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FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Valorisation of lignocellulosic residues for lactic acid and bioethanol production in a biorefinery context

Aprovechamiento de residuos lignocelulósicos para la producción de ácido láctico y bioetanol en un contexto de biorrefinería

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PRESENTADA POR

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ABBREVIATIONS

ADP: Adenosine Diphosphate

ALE: Adaptive Laboratory Evolution

ANOVA: Analysis of Variance

ATB: Leibniz Institute of Agricultural Engineering and Bio-economy

ATP: Adenosine Triphosphate

β-Glu: Novozym® 188

β-Xyl: β-Xylosidase Megazyme

BM: Basal Medium

BMI: Basal Medium with low Inhibitors concentration

CBA: Carboxylic acid

CBP: Consolidated Bioprocessing

CcpA: Carbon catabolite control protein A

CCR: Carbon Catabolite Repression

CECT: from Spanish, “Colección Española de Cultivos Tipo”

Cel: Celluclast® 1.5 L

CIEMAT: from Spanish, “Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas”

DSMZ: German Collection of Microorganisms and Cell Cultures GmbH

DNA: Deoxyribonucleic acid

E-GH: Ethanol-rich gardening hydrolysate

E-WSH: Ethanol-rich wheat straw hydrolysate

ED: Entner-Doudoroff pathway

EMP: Embden-Meyerhof-Parnas

EXA: Ethanol and Xylose Adapted

FPU: Filter Paper Units

GH: Gardening Hydrolysate

GP: Gardening Prehydrolysate

GRAS: Generally Recognised As Safe

5-HMF: 5-hydroxymethyl furfural

HPLC: High Performance Liquid Chromatrography

IU: International Units

LA: Lactic Acid

LAB: Lactic Acid Bacteria

LDH: Lactate Dehydrogenase

MAX2: Mutant Acid Xylose 2

MRS: Man Rogosa Sharpe

M-GH3: Modified GH3

NAD(P): Nicotinamide Adenine Dinucleotide (Phosphate)

NREL: National Renewable Energy Laboratory

OD: Optical Density

PEP-PTS: Phosphoenolpyruvate-dependent phosphotransferase system

PLA: Poly-lactic acid

PK: Phoskphoketolase

PP: Pentoses-Phosphate

ROS: Reactive Oxygen Species

RNA: Ribonucleic acid

R-XL: Reactor with X liters

rpm: Revolutions per minute

SHF: Separated Hydrolysis and Fermentation

SSF: Simultaneous Saccharification and Fermentation

v v^{-1}: volume/volume relation

WIS: Water Insoluble Solids

WSH: Wheat Straw Hydrolysate

WSP: Wheat Straw Prehydrolysate

WT: wild-type

w w^{-1}: weight/weight relation

w v^{-1}: weight/volume relation

YPD: Yeast Peptone Dextrose

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ABSTRACT

Lignocellulosic biomass is considered an interesting substrate for bioproduction of fuels and chemicals due to its sugars content, high availability and low price. Interestingly, different products can be obtained from the carbon sources contained in lignocellulose through fermentative processes. Among the bioproducts, lactic acid is a building block to obtain other chemicals and biopolymers (Article I) and a versatile chemical with plenty of applications in food and pharma. On the other hand, bioethanol is a biofuel used as a substitute for gasoline in a complete or partial way. The co-generation of lactic acid and bioethanol from different lignocellulosic materials, including wheat straw and gardening residues, is proposed in this Thesis as a biorefinery-oriented approach.

When utilising lignocellulosic substrates, a pretreatment is always needed to increase the sugars accessibility of the materials. In this way, steam explosion promotes lignocellulose deconstruction by recovering cellulose in the water insoluble solids fraction, while hemicellulose is solubilised and recovered in the liquid fraction or prehydrolysate. These two fractions can be separated and valorised in different streams or, alternatively, the whole slurry can be valorised in an integrated strategy.

Glucose obtained from the cellulose-rich solid fraction has been widely used for bioethanol production. Nevertheless, the use of the hemicellulose-rich liquid fractions needs to be optimised due to its high content in five-carbon sugars, which cannot be metabolised by most of wild-type microorganisms, and inhibitory degradation compounds formed during pretreatment.

In the first part of this Thesis, a separated bioethanol and lactic acid production approach was carried out: hemicellulose-rich prehydrolysates were used for lactic acid production with pentoses-utilising bacteria, while the cellulose-rich water insoluble solids fractions were used for bioethanol production by yeasts.

Different pretreatment conditions were tested and the highest sugars recovery in the prehydrolysates were achieved when biomass was subjected to aqueous extraction followed by H_2SO_4-catalysed steam explosion. When required, steam-exploded wheat straw and gardening prehydrolysates were subjected to hydrolytic steps. Enzymatic hydrolysis resulted in the most adequate method to increase the amount of monosaccharides from these liquid fractions. In fact,

the addition of cellulases together with hemicellulases increased the release of monomeric sugars from these materials (Articles II and III).

The heterolactic bacteria *Lactobacillus pentosus* CECT 4023T demonstrated its ability to co-metabolise different sugars from wheat straw and gardening liquid fractions with low carbon catabolite repression effect. While only lactic acid was produced from six-carbon sugars, both lactic acid and acetic acid were produced from five-carbon sugars by the phosphoketolase pathway. The inhibitory effect produced by the presence of degradation compounds together with oxidative and acid stress was alleviated by reducing the oxygen presence and controlling the culture pH (Articles II and III). In fact, under strictly anaerobic and controlled pH conditions by base automatic addition, lactic acid yields of 0.52 ± 0.00 g g^{-1} and 0.70 g g^{-1} were obtained from wheat straw and gardening hydrolysates, respectively, implying 81 % and 95 % of the theoretical maximum.

Higher lactic acid yields, between 0.90 ± 0.01 g g^{-1} and 1.00 ± 0.03 g g^{-1} (90 % and 100 % of the theoretical maximum), were obtained from gardening hydrolysates by homolactic *Bacillus coagulans* strains because five-carbon sugars were metabolised by the pentoses phosphate pathway without by-products generation. Furthermore, *B. coagulans* produced optically pure L-lactic acid, with higher added value than the racemic mixture produced by *L. pentosus*. The inhibitory effect of different degradation compounds mixtures was screened at increasing inhibitors concentrations on different *B. coagulans* strains. In this case, attained lactic acid productivities were higher in case of the isolated A166 and A162 strains than the collection strain DSM 2314. In fact, A162 was able to remove part of these inhibitors, producing a biodetoxification of defined and gardening media (Article IV).

Although higher sugars consumption was obtained from wheat straw and gardening hydrolysates under controlled pH conditions, the addition of neutralisers implies the need to separate the free lactic acid from the lactate salts. For this reason, a novel downstream processing method for free lactic acid recovery and purification from fermented gardening hydrolysate was assayed. This method included different membrane-based steps, like bipolar electrodialysis. Most of impurities were removed, including the ones derived from the material, pretreatment, fermentation and neutralisation, showing different advantages in comparison with the calcium lactate precipitation technology commonly used in industrial LA production.

In the same context, an evolved *L. pentosus* strain with increased xylose consumption rate at low pH was obtained with the aim to reduce the need of neutralising agents and to produce free

lactic acid, facilitating the purification step (Article V). *L. pentosus* was subjected to adaptive laboratory evolution by serial batch cultivation at increasing xylose concentrations in order to increase xylose uptake despite the pH drop. As a result, the evolved strain presented between 1.5 and 2-fold more xylose consumption and lactic acid production than the wild-type strain from 20 g L^{-1} xylose, independently of the initial pH value.

Concerning bioethanol production, the cellulose-rich water insoluble solids fraction from gardening residues was used for separated hydrolysis and fermentation by *Saccharomyces cerevisiae* Ethanol Red. As a result, the whole glucose content was consumed, reaching 0.43 ± 0.00 g g^{-1} of bioethanol yield, corresponding with 84 % of the theoretical maximum. In this way, it was demonstrated that both fractions obtained from gardening residues (prehydrolysates and water insoluble solids) could be separately valorised for the production of lactic acid and bioethanol.

In the case of wheat straw, the whole slurry was used for simultaneous saccharification and ethanol fermentation experiments. The highest bioethanol concentrations, 32.00 ± 0.22 g L^{-1}, and yields, 0.49 ± 0.00 g g^{-1} (96 % of the theoretical maximum) were obtained with 15 % (w v^{-1}) of total solids in fed-batch mode when the substrate feed was added at the earliest time (8 h), demonstrating the high solids and inhibitors tolerance of Ethanol Red. Since *S. cerevisiae* is not able to ferment pentoses, the resulting medium rich in ethanol and C5 sugars, was used for lactic acid fermentation following a sequential yeast-bacteria cultivation and allowing an integrated bioethanol and lactic acid production.

With this in mind, an evolved *B. coagulans* was obtained by adaptive laboratory evolution in chemostat, making it more resistant to ethanol (Article VI). Remarkably, L-lactic acid yields obtained by the evolves strain (EXA) were enhanced by around 2.5-fold in comparison with the wild-type *B. coagulans*, reaching 0.89 ± 0.02 g g^{-1} and 0.89 ± 0.03 g g^{-1} (89 % of the theoretical maximum) from xylose in presence of 6 % (v v^{-1}) ethanol from defined media and 5 % (v v^{-1}) bioethanol from fermented gardening media, respectively. Finally, the evolved bacterium was used for fermentation of five-carbon sugars from a 50 % (v v^{-1}) bioethanol-rich wheat straw hydrolysate previously obtained by simultaneous saccharification and ethanol fermentation of the whole slurry. As a result, the evolved *B. coagulans* reached the maximum lactic acid theoretical yield (1 g g^{-1}), tolerating the combined inhibitory effect of 2 % (v v^{-1}) bioethanol and the presence of lignocellulosic inhibitors.

In this Thesis, the complete valorisation of the whole sugars content, including six- and five-carbon sugars, from different lignocellulosic materials to bioethanol and lactic acid has been demonstrated. Most of the work has been focused on improving the conversion of lignocellulosic sugars into lactic acid, which has provided new knowledge about the optimum fermentation conditions and the stress tolerance of different hetero- and homolactic lactic acid producers. Furthermore, many efforts have been invested in the development of new lactic acid-producing strains fitted to industrial fermentation processes using adaptive laboratory evolution. Different process configurations for *S. cerevisiae* Ethanol Red cultivation have also been tested to improve the bioethanol production from glucose derived from different lignocellulosic materials. Both separated and integrated lactic acid and bioethanol production strategies offer different particular strengths and can be considered to be applied and up-scaled in future commercial lignocellulosic biorefineries.

RESUMEN

La biomasa lignocelulósica está ganando mucho interés como sustrato para la producción de biocombustibles y otros bioproductos debido a su contenido en azúcares, gran disponibilidad y bajo precio. De hecho, numerosos productos pueden ser obtenidos a partir de los azúcares contenidos en la lignocelulosa mediante procesos fermentativos. Entre ellos, el ácido láctico es un compuesto químico muy versátil dada su gran variedad de aplicaciones en la industria alimentaria, farmacéutica y química, especialmente como molécula plataforma para producir otros compuestos y biopolímeros (Artículo I). Por otra parte, el bioetanol es un biocombustible apto para utilizarse como sustituto de la gasolina de forma parcial o completa. Teniendo en consideración el inminente desarrollo de las biorefinerías, en esta Tesis se plantea una estrategia basada en la producción de ácido láctico y bioetanol a partir de diferentes materiales lignocelulósicos, como la paja de trigo y los residuos de poda de jardines.

La aplicación de un pretratamiento es necesario para aumentar la disponibilidad de los azúcares contenidos en la biomasa lignocelulósica. La explosión de vapor permite la fragmentación y deconstrucción de la lignocelulosa. Como resultado, la celulosa se recupera en la fracción de residuo sólido insoluble, mientras que la hemicelulosa se solubiliza en la fracción líquida o prehidrolizado. Estas fracciones pueden ser separadas por filtración y valorizadas en diferentes corrientes o, por el contrario, el material pretratado completo puede ser valorizado siguiendo un enfoque integrado. La producción de bioetanol lignocelulósico se ha centrado principalmente en la utilización de glucosa obtenida del residuo sólido insoluble. Sin embargo, la utilización de la fracción líquida, rica en hemicelulosa, presenta diferentes limitaciones, como la presencia de azúcares de cinco carbonos (no metabolizables por la mayoría de los microorganismos) y productos inhibidores formados durante el pretratamiento.

En la primera parte de la Tesis se abordó la producción separada de ácido láctico y bioetanol. Los prehidrolizados ricos en azúcares hemicelulósicos fueron utilizados por bacterias capaces de metabolizar azúcares de cinco carbonos para producir ácido láctico. Por su parte, los residuos sólidos insolubles, ricos en celulosa, fueron utilizados para la producción de bioetanol con levaduras.

Tras estudiar diferentes condiciones de pretratamiento, la mayor recuperación de azúcares en los prehidrolizados tuvo lugar cuando la biomasa fue sometida a extracción acuosa, seguida de explosión de vapor catalizada con H_2SO_4. En aquellos casos en los que fue necesario, se aplicó

un tratamiento de hidrólisis enzimática mediante la adición conjunta de celulasas y hemicelulasas, mejorando la liberación de azúcares monoméricos, tanto de seis como de cinco carbonos (Artículos II y III).

La bacteria heteroláctica *Lactobacillus pentosus* CECT 4023T fue capaz de co-metabolizar diferentes fuentes de carbono provenientes de hidrolizados de paja de trigo y de poda, sin observarse un marcado efecto de represión por catabolito. Mientras que esta bacteria produce ácido láctico a partir de azúcares de seis carbonos, el metabolismo de azúcares de cinco carbonos a partir de la ruta de la fosfocetolasa produce ácido láctico y ácido acético. El efecto inhibidor producido por la presencia de productos de degradación y ácidos, así como por el estrés oxidativo fue aliviado mediante la reducción de la presencia de oxígeno y el control del pH del medio (Artículos II y III). De hecho, los rendimientos de ácido láctico más altos a partir de hidrolizados de paja de trigo y poda de jardines, 0.52 ± 0.00 g g^{-1} y 0.70 g g^{-1} (81 % y 95 % del máximo teórico), respetivamente, fueron obtenidos en condiciones de anaerobiosis estricta y control de pH mediante adición automática de sosa.

Por otra parte, diferentes cepas homolácticas de *Bacillus coagulans* fueron capaces de producir mayores rendimientos de ácido láctico a partir de hidrolizados de poda, entre 0.90 ± 0.01 g g^{-1} y 1.00 ± 0.03 g g^{-1} (90 % - 100 % del máximo teórico), dada su habilidad de fermentar azúcares de cinco carbonos mediante la ruta de las pentosas fosfato, sin producir ácido acético como subproducto. Además, *B. coagulans* produce el isómero puro L-ácido láctico, el cual presenta mayor valor añadido que la mezcla racémica, pudiéndose utilizar como monómero para la producción de ácido poliláctico. Se llevaron a cabo diferentes experimentos con concentraciones crecientes de compuestos de degradación para comprobar la tolerancia de varias cepas de *B. coagulans* a estos inhibidores. En este caso, las productividades de ácido láctico obtenidas por A166 y A162, cepas aisladas de materiales reales, fueron significativamente mayores que las obtenidas por la cepa de colección DSM 2314. Además, la cepa A162 fue capaz de reducir las concentraciones de inhibidores, produciendo la biodetoxificación parcial de diferentes medios sintéticos e hidrolizados de poda (Artículo IV).

Aunque la aplicación de métodos de control de pH mejoró el consumo de azúcares y la producción de ácido láctico, el uso de neutralizantes implica añadir pasos adicionales para separar el ácido láctico libre de las sales de lactato. Por este motivo, se aplicó un método para la recuperación y purificación de ácido láctico libre a partir del hidrolizado de poda ya fermentado. Este método, compuesto por diferentes pasos de membranas (incluyendo la electrodiálisis bipolar), permitió que la mayor parte de impurezas fueran eliminadas, incluyendo

aquellas provenientes del material y formadas durante el pretratamiento, fermentación y neutralización.

En ese contexto, una nueva cepa de *L. pentosus* con mayor velocidad de consumo de xilosa a pH bajo fue obtenida con el objetivo de reducir la necesidad de agentes neutralizantes durante la fermentación. *L. pentosus* fue sometido a evolución adaptativa mediante cultivo seriado en lotes, aumentando progresivamente la concentración de xilosa. La nueva cepa presentaba entre 1.5 y 2 veces mayor consumo de xilosa y producción de ácido láctico en comparación con la cepa parental, con independencia del pH inicial del medio.

Con respecto a la producción de bioetanol, el residuo sólido insoluble obtenido de la biomasa de poda de jardines fue sometido a una hidrólisis y fermentación separada con *Saccharomyces cerevisiae* Ethanol Red. Como resultado, se obtuvo un alto rendimiento de etanol, 0.43 ± 0.00 g g^{-1}, equivalente a un 84 % del máximo teórico. De esta forma, se comprobó cómo ambas fracciones obtenidas a partir de residuos de poda de jardines podían ser valorizadas de forma separada para la producción de bioetanol y ácido láctico.

En cuanto a la paja de trigo, el material pretratado completo se utilizó en experimentos de sacarificación y fermentación simultánea para la producción de bioetanol. El mayor rendimiento de bioetanol, 0.49 ± 0.00 g g^{-1} (96 % del máximo teórico), fue obtenido con 15 % (p v^{-1}) de sólidos totales en semicontinuo por lotes alimentados (*fed-batch)* con adición temprana de sustrato (8 h), demostrando la gran tolerancia de Ethanol Red a altas concentraciones de sólidos e inhibidores. El medio resultante presentaba altas concentraciones de bioetanol y azúcares de cinco carbonos no metabolizables por *S. cerevisiae*. Este medio fue destinado a la producción de ácido láctico, siguiendo un método de fermentación secuencial levadura-bacteria para la producción integrada de bioetanol y ácido láctico.

Con este fin, *B. coagulans* fue sometido a evolución adaptativa en quimiostato, haciéndola más resistente a etanol (Artículo VI). Cabe destacar que los rendimientos de L-ácido láctico obtenidos a partir de xilosa por la cepa evolucionada (EXA) fueron 2.5 veces mayores que los de la cepa parental, alcanzando 0.89 ± 0.02 g g^{-1} y 0.89 ± 0.03 g g^{-1} (89 % del máximo teórico), en presencia de 6 % (v v^{-1}) de etanol en medio sintético y de 5 % (v v^{-1}) de bioetanol en medio fermentado de poda de jardines, respectivamente. Finalmente, la bacteria evolucionada fue utilizada para la fermentación de azúcares de cinco carbonos de un medio de paja de trigo rico en bioetanol previamente obtenido mediante sacarificación y fermentación alcohólica simultáneas y diluido al 50 % (v v^{-1}). Como resultado, la bacteria evolucionada fue capaz de

resistir la inhibición producida por la presencia 2 % (v v^{-1}) de bioetanol y de productos de degradación, alcanzando el máximo teórico de ácido láctico (1.00 g g^{-1}).

En esta Tesis se ha conseguido una valorización completa de todos los azúcares, tanto de seis como de cinco carbonos, procedentes de diferentes materiales lignocelulósicos, para la producción de ácido láctico y bioetanol. Gran parte del trabajo se ha centrado en la mejora de la conversión de azúcares lignocelulósicos en ácido láctico, proporcionando un mayor conocimiento sobre las condiciones óptimas de fermentación y la tolerancia a estrés de diferentes bacterias hetero- y homofermentativas productoras de ácido láctico. Asimismo, se han dedicado muchos esfuerzos al desarrollo de nuevas bacterias productoras de ácido láctico evolucionadas con características mejoradas que las hacen idóneas para diferentes procesos fermentativos industriales. Además, se han optimizado las condiciones de cultivo de *S. cerevisiae* Ethanol Red para la producción de bioetanol a partir de glucosa proveniente de diferentes materiales lignocelulósicos. Tanto el enfoque de producción separada de bioetanol y ácido láctico como el enfoque integrado ofrecen diferentes ventajas y pueden ser considerados para su aplicación y escalado en futuras biorefinerías lignocelulósicas comerciales.

1. INTRODUCTION

1. INTRODUCTION

1.1. Lignocellulosic biorefineries

Current global economy is based on the exploitation of fossil resources, like petroleum oil, coal and natural gas to cover energetic, transport and industrial necessities for human development. As a result, high amounts of greenhouse gases are released during extraction and combustion of these sources. In fact, a 39 % increase in the atmospheric concentration of these gases has been measured in comparison with preindustrial levels resulting in what is known as greenhouse effect (Owusu and Asumadu-Sarkodie, 2016). As a result of the greenhouse effect, global average surface temperature was increased by 1 °C between 1880 and 2017 (Allen et al., 2018).

In spite of their continued depletion, geopolitical and military conflicts, price fluctuations and the mentioned environmental impacts, fossil resources demand has been still increasing during the last years. Remarkably, due to the population and economic growth, especially in emerging market economies, global oil demand increased from 2500 million tonnes in the early seventies to almost 4500 million tonnes in 2018 (IEA, 2019a).

In this context, renewable energy sources provide opportunities in energy security, social and economic development, and reduction of environmental and health impacts, being less polluting, both in local emissions and greenhouse gases, than fossil resources (Owusu and Asumadu-Sarkodie, 2016). Within this group, different sources can be highlighted, including hydropower, geothermal, solar, wind, ocean and biomass. Since 1990, these renewable sources have considerably grown, reaching 26 % of global electricity generation (REN21, 2019) and 13.6 % of fuels share (IEA, 2019b).

Biomass is defined as "the biodegradable fraction of products, waste and residues with biological origin derived from agriculture, forestry and related industries, as well as from industrial and municipal waste" (European Comission, 2017). Biomass is a very appropriate source for the production of chemicals, materials and energy due to its availability and versatile chemical composition (European Comission, 2017). Furthermore, most of the biomass-derived chemicals and materials present biodegradable properties, which ensures a sustainable disposal and feedstock regeneration through different techniques, like composting or anaerobic digestion (European Comission, 2017). When used as energy source, it is carbon neutral, since the

greenhouse gas emission rate is balanced to the fixation rate, closing the loop of CO_2 within ecological cycles (Fabbri and Torri, 2016).

Biorefineries, in contrast to conventional petrochemical refineries, have emerged as sustainable alternatives for the processing of biomass into a spectrum of marketable bio-based products (chemicals and materials) and bioenergy (fuels, power, and/or heat) (Motola et al., 2018). Co-production of biofuels and bioproducts from the same feedstock in biorefineries reduces materials and energy consumption assigned to each individual product and promotes technology advances, which could, ultimately, increase the profitability of these economical activities (Dunn, 2019).

High efforts are being undertaken to make biorefineries a reality. Indeed, different measures and policies have been implemented in the EU, including the allocation of part of the 2014-2020 European Structural Investment Funds and Horizon 2020 funds for the development of new technologies and markets related to bioeconomy in which biorefineries are enclosed (BBI, 2014). The public and private investments in the bioeconomy sector have been translated in a huge increase in published research works and the expansion of commercial and pilot biorefinery plants (Motola et al., 2018; Wenger and Stern, 2019).

Among different types of biomass, lignocellulosic biomass is usually inexpensive, available throughout the year and worldwide distributed as well as readily and locally available, which can bring high benefits to the development of rural economies (Bolivar-Telleria et al., 2018). Furthermore, lignocellulose corresponds with non-edible parts of the plant and its valorisation does not interfere with food supplies (Isikgor and Becer, 2015).

Lignocellulosic biomass can be valorised through chemical, thermochemical and biotechnological processes or combinations of these approaches. For instance, lignocellulose can be decomposed by pyrolysis, gasification or liquefaction, generating new compounds like syngas, biochar and bio-oil (Fabbri and Torri, 2016).

Different examples of biochemical processes for lignocellulose valorisation can be found in **Table 1.1**. As it is shown, a great variety of compounds can be obtained from lignocellulose by biological reactions using microorganisms or enzymes (Gall et al., 2017).

In this sense, the different sugars contained in lignocellulose can be used as platform molecules for microbial fermentation, producing an array of biofuels, like bioethanol and butanol; as well as bio-based chemicals, like lactic acid (LA), succinic acid, xylitol, butanol and 1,3-propanediol

(Chandel et al., 2018). The transformation of lignocellulosic sugars via fermentation will be addressed in depth in the following sections of this Thesis.

Table 1.1. Main biofuels and bio-based products obtained from lignocellulose by biochemical processes

Product	Production process	Applications	Ref[1]
Alcohols			
Bioethanol	Fermentation	Biofuel	(1)
Butanol	Fermentation	Biofuel, adhesives, plasticiser and cleaning products	(2)
Xylitol	Fermentation / xylose bioreduction	Sweetener, preservative, tooth care and pharma	(1, 2)
Sorbitol	Fermentation	Sweetener, cosmetic, tooth care and ascorbic acid	(2)
Glycerol	By-product of biodiesel	Building block, pharma, food, cosmetics	(2)
1,3-propanediol	Fermentation	Polyesters, polyethers, and polyurethanes	(2)
2,3-butanediol	Fermentation	Inks, perfumes, food and pharma	(2)
Aldehydes			
Furfural	Biodehydration of xylose	Building block, solvents, fungicides and food	(1,2)
Acetaldehyde	Ethanol biodehydrogenation	Acetic acid, perfumes, dyes, pharma, plasticiser	(2)
Carboxylic acids and esters			
Lactic acid	Fermentation	Building block, pharma, food, biopolymers precursor	(1,2,3,4)
Acrylic acid	Lactic acid biodehydration	Building block, acrylate esters, detergents	(3,4)
Citric acid	Fermentation	Food additive and pharma	(4)
Succinic acid	Fermentation	Polyesters, paints, fuel, herbicides and pharma	(2,3)
Itaconic acid	Fermentation	Lubricant, dyes, plastics, rubber, resins, and fibers	(2,4)
Short-chain fatty acids	Fermentation /anaerobic digestion	Animal feed, biofuels, textiles, etc.	(4,5)
Hydroxy-alcanoates	Microbial accumulation	Biopolymers	(4)
Biodiesel	Esterification of microbial lipids	Biofuel	(6)
Others			
Biogas	Anaerobic fermentation	Biofuel	(7)

[1] Ref: (1) Naidu et al., 2018; (2) Rosales-Calderón and Arantes., 2019; (3) Pleissner et al., 2017a; (4) Sauer et al., 2008; (5) Llamas et al., 2020; (6) Cho et al., 2018; (7) Kainthola et al., 2019.

1.2. Lignocellulosic biomass

Lignocellulosic biomass is the most abundant renewable organic source present in plants, with a global annual production of 182 billion tonnes (Dahmen et al., 2019), including: softwood, hardwood, herbaceous biomass, agricultural residues and energy crops.

Among different lignocellulosic substrates, valorisation of agricultural and herbaceous residues is the most attractive approach, coupling the elimination of residual biomass with the generation of added-value products. In this sense, 1.2 billion tonnes of lignocellulosic residues are currently valorised for different purposes, representing 25 % of the lignocellulosic residues annual production (Dahmen et al., 2019).

The utilisation of these residues is expected to increase in the following years with the implementation of multi-products biorefineries, playing an important role in the transition from a linear towards a circular economy.

1.2.1. Structure and composition of lignocellulose

Lignocellulosic biomass is mainly composed of two carbohydrate polymers rich in fermentable sugars (cellulose and hemicellulose), an aromatic polymer -lignin- and small amounts of pectin, proteins, and extractives.

Cellulose

Cellulose is the main constituent of lignocellulose, corresponding with 30 % - 50 % (w w^{-1}) of its dry weight (Agbor et al., 2011). This linear homopolysaccharide is formed by the polymerisation of cellobiose units, a disaccharide of two β-D-glucoses linked with a β-1,4-glucosidic bond (**Fig. 1.1**). The polymerisation degree of cellulose ranges from 10,000 to 15,000 glucose moieties (Agbor et al., 2011).

Elongation of the polymer is carried out from the reducing end of glucose units. Hydrogen and van der Waals bonds are formed between different cellulose chains, grouping them together in microfibrils (**Fig. 1.1**). These microfibrils can be organised in macrofibrils and fibers (crystalline regions), but they can also be disposed as amorphous regions (Bhatia et al., 2020).

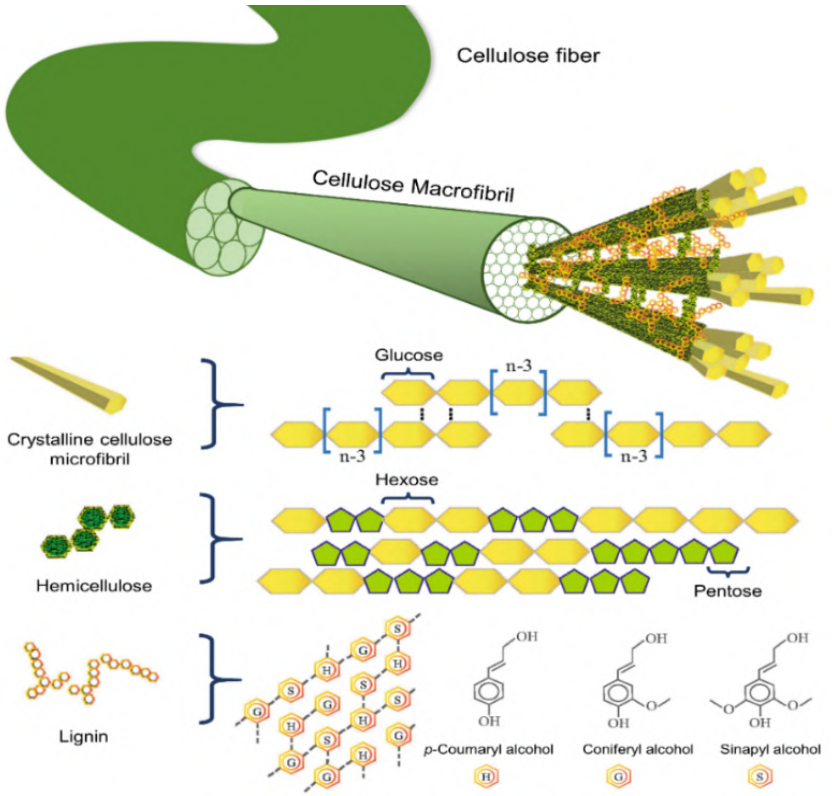

Fig. 1.1. Structure and composition of the different components from lignocellulose. Modified from: Biswas et al. (2015).

Hemicellulose

Hemicellulose is the second most abundant component of lignocellulose, corresponding with 20 % - 50 % (w w^{-1}) of its dry weight. This heteropolysaccharide acts as a cross-linker between cellulose and lignin, reinforcing the rigidity of the plant structure (Agbor et al., 2011). The polymerisation degree of hemicellulose is considerably lower than cellulose's, with a backbone containing from 70 to 200 units of hexoses, pentoses and uronic acids. However, they also present single-sugars side chains and acetyl groups (**Fig. 1.1**) (Bhatia et al., 2020).

Hemicellulose chains can be classified as xyloglucan, xylans, mannans and glucomannans, which present a β-(1,4)-linked backbone structure. Galactans, arabinans, and arabinogalactans are sometimes included in the hemicellulose group, but they do not share the same backbone structure (Scheller and Ulvskov, 2010). Moreover, there is a high heterogeneity in the hemicellulose among different types of biomass. Softwood hemicellulose components are galactoglucomannan and arabinoglucuronoxylan, while xylan and glucuronoxylan are the main components of hemicellulose in hardwood and herbaceous materials (Bhatia et al., 2020).

Lignin

Lignin constitutes 15 % - 30 % (w w^{-1}) of the dry weight of lignocellulose, being the third most abundant component. This amorphous heteropolymer is formed by the linkage of the hydroxyphenylpropane units *p*-hydroxyphenyl, guaiacyl and syringyl that are derived from the phenyl propionic alcohols precursors *p*-coumaryl, coniferyl and sinapyl alcohol, respectively (**Fig. 1.1**) (Agbor et al., 2011). As it is the case for hemicellulose, the proportion of lignin moieties significantly varies among different lignocellulosic materials. Lignin from softwoods is mainly composed by guaiacyl units, while hardwoods are rich in both guaiacyl and syringyl units. Herbaceous materials contain the three moieties, with high presence of *p*-hydroxyphenyl groups. Lignin binds the different components of lignocellulosic biomass together, providing structural support, impermeability and mechanical forces resistance (Bhatia et al., 2020).

The composition and relative amount of each component in lignocellulose (*i.e.* cellulose, hemicellulose and lignin) are dependent on the material, age, climate, harvesting and storage aspects, with high differences between softwood, hardwood and herbaceous and agricultural residues. While the total content of cellulose and hemicellulose is higher in hardwoods and herbaceous/agricultural residues than in softwoods, lignin content is normally higher in softwoods (Bhatia et al., 2020). An overview of the composition of different lignocellulosic materials can be found in **Table 1.2**.

Table 1.2. Composition (% w w^{-1}) of different lignocellulosic materials.

Material	Glucan	Xylan	Arabinan	Galactan	Mannan	Lignin	Ref [1]
Softwood							
Spruce	49.9	5.3	1.7	2.3	12.3	28.7	(1)
	42.4	5.6	4.3	1.3	3.4	33.8	(2)
Pine	41.9	5,5	1.7	2.9	11.7	28.6	(3)
	53.3	1.1	-	-	0.9	37.3	(4)
Hardwood							
Willow	43.0	14.9	1.2	2.0	3.2	26.6	(5)
Poplar	43.5	15.5	1.5	2.3	2.5	26.2	(6)
Agricultural and herbaceous residues							
Wheat straw	40.5	22.7	2.1	1.3	-	18.1	Articles II, V
	40.7	23.7	1.3	2.6	-	17.0	(7)
	38.8	22.2	1.4	2.7	1.7	18.5	(8)
	32.6	20.1	3.3	0.8	-	26.5	(9)
	40.5	22.7	2.1	1.3	-	18.1	(10)
Gardening residues	24.8	10.1	2.6	2.0	1.0	21.6	Articles III, IV, VI
Barley straw	33.1	20.2	3.8	0.9	-	16.1	(11)
	36.8	17.2	5.3	2.2	-	14.3	(12)
Corn stover	37.2	21.1	1.7	1.2	-	26.0	(13)
	36.8	22.2	5.5	2.9	-	23.1	(14)
	36.8	21.7	2.6	0.7	0.3	17.2	(15)

[1] Ref: (1) Soderstrom et al., 2003; (2) Frankó et al., 2015; (3) Tian et al., 2010; (4) Zhou et al., 2014; (5) Sassner et al., 2006; (6) Negro et al., 2003; (7) Tomás-Pejó et al., 2009; (8) Erdei et al., 2013; (9) Linde et al., 2008; (10) Oliva-Taravilla et al., 2015; (11) García-Aparicio et al., 2006; (12) Linde et al., 2007; (13) Öhgren et al., 2006; (14) Ohgren et al., 2005; (15) Zhu et al., 2007.

Due to the complex and recalcitrant structure of lignocellulose, different steps need to be performed to allow its valorisation through biochemical processes, including pretreatment, saccharification (enzymatic hydrolysis) and fermentative processes.

1.2.2. Pretreatment step

Lignocellulose has evolved to resist degradation. As mentioned, the recalcitrant properties of lignocellulose are due to its rigid structure and compositional complexity, being dependant of the cellulose degree of polymerisation and crystallinity, pore size, accessible surface area and hemicellulose and lignin shielding on cellulose (Agbor et al., 2011). For this reason, when lignocellulose is used for the production of sugars as platform molecules via enzymatic hydrolysis, pretreatment step is needed to facilitate the accessibility of the material to enzymes (Agbor et al., 2011; Alvira et al., 2010; Tomás-Pejó et al., 2011). In this sense, pretreatment should produce different changes in the raw material, including:

- Fragmentation and size reduction to increase the biomass surface area.
- Disruption of the crystalline structure of cellulose by separation of cellulose fibers.
- Separation of different lignocellulosic components in different fractions (*e.g.,* hemicellulose solubilisation).
- Hemicellulosic sugars recovery with high yields in a separate fraction.
- Cellulosic sugars recovery and partial hydrolysis.
- Recovery of lignin to simplify downstream processes.

Due to the different changes produced on biomass, the pretreatment type has a large impact on the following hydrolysis and fermentation steps (Tomás-Pejó et al., 2011). The desired properties of lignocellulose pretreatment have been deeply reviewed (Agbor et al., 2011; Alvira et al., 2010; Tomás-Pejó et al., 2011) and are listed as follows:

- Avoid carbohydrate loss and degradation into inhibitory compounds.
- Effectiveness on a wide range of lignocellulosic material.
- Non-production of solid wastes that present processing or disposal challenges.
- Cost effective process (low water, energy and chemicals demand; and low capital and operational costs). Effectiveness at low moisture content to reduce energy and water consumption and avoid dilution of the material.

Different pretreatment technologies have been studied for lignocellulose valorisation, including physical (mechanical comminution, extrusion, microwave, ultrasound), chemical (acid/alkali pretreatment, organosolv, ozonolysis, ionic liquid extraction, etc.), physicochemical (steam explosion, liquid hot water, ammonia fiber explosion, supercritical fluids and wet oxidation) and biological (fungal pretreatment) (Agbor et al., 2011; Jönsson and Martín, 2016; Tomás-Pejó et al., 2011).

1.2.2.1. Steam explosion pretreatment

Steam explosion is a hydrothermal physicochemical pretreatment in which biomass is subjected to pressurised steam for a period of time and then suddenly depressurised, combining mechanical forces (explosive decompression) and chemical effects. Chemical autohydrolysis can be produced at high temperatures by water and acetic acid derived from hemicellulose acetyl groups. External catalysts, like H_2SO_4, SO_2 or $FeCl_3$, can also be added. By modifying the severity of the process conditions (residence time, temperature, and/or catalyst addition), pretreated materials with different characteristics can be obtained from the same biomass.

Among the main advantages of steam explosion, it is worth to mention the possibility of using large chip size, good hydrolysis yields in enzymatic hydrolysis, and its feasibility at industrial scale (Tomás-Pejó et al., 2011). It also presents a low environmental impact and capital investment, high energy efficiency and less hazardous process chemicals and conditions than other pretreatment technologies (Tomás-Pejó et al., 2011).

This pretreatment produces certain separation of cellulose fibers, solubilisation and partial hydrolysis of hemicellulose and redistribution of lignin. As a result, the generated slurry can be filtrated into two fractions: (1) A liquid fraction rich in monomeric and oligomeric sugars from hemicelluloses solubilisation together with different degradation compounds, and; (2) a water insoluble solid (WIS) fraction rich in cellulose and lignin.

Although steam explosion works well on hardwoods, it is not suitable for softwood if acid catalyst is not added due to the low amount of acetyl groups in their hemicellulosic fraction (Agbor et al., 2011). Nevertheless, it is very appropriate for agricultural and herbaceous residues and it has been widely utilised for pretreatment of wheat straw, rapeseed and barley straw, as well as for pruning residues like grasses and branches (García-Aparicio et al., 2006; López-Linares et al., 2015; Montipo et al., 2018; Oliva-Taravilla et al., 2015; Sasaki et al., 2014).

For this reason, steam explosion was selected and used as pretreatment method for the lignocellulosic biomass used as in the present Thesis, including agricultural residues such as wheat straw and herbaceous materials like gardening residues.

1.2.2.2. Inhibitory degradation compounds

Harsh conditions during stem explosion pretreatment lead to a partial hemicellulose and lignin degradation and generation of inhibitory compounds. These degradation compounds can be divided in three groups: furan derivatives, carboxylic acids (CBA) and phenolic compounds (**Fig. 1.2**).

Main furans are furfural and 5-hydroxymethyl furfural (HMF) derived from C5 and C6 sugars degradation, respectively (Jönsson and Martín, 2016) (**Fig. 1.2**). CBA are formed due to the release of acetyl groups from hemicellulose scaffold and also after degradation of furfural into formic acid and of HMF into levulinic and formic acid (Tomás-Pejó et al., 2011) (**Fig. 1.2**). Low amounts of uronic acids derived from sugars oxidation can also be found, especially in pectine-rich materials (Jönsson et al., 2013).

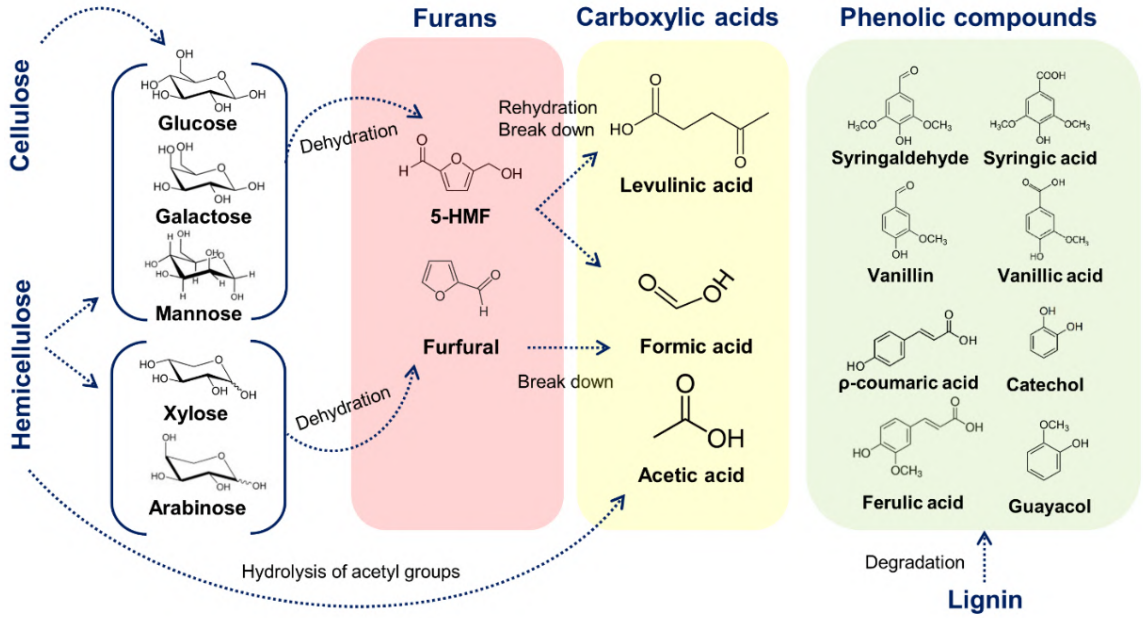

Fig. 1.2. Inhibitory compounds formed by degradation of lignocellulose components during biomass pretreatment.

Phenolic compounds are originated during degradation of lignin (**Fig. 1.2**), mainly because of the splitting of the β-O-4 ether and other acid–labile linkages (Jönsson and Martín, 2016). Although low phenols concentrations are solubilised during pretreatment, a high variety of

these compounds can be found. According to their functional groups, they can be divided into aldehydes (4-hydroxybenzaldehyde, vanillin, coniferyl aldehyde, syringaldehyde, etc.), acids (ferulic acid, *p*-coumaric acid, hydroxybenzoic acid, cinammic acid, syringic acid, vanillic acid, etc.), benzoquinones (catechol) and ketons (Hibbert's ketones) (Jönsson and Martín, 2016; Larsson et al., 1999; Tomás-Pejó et al., 2011).

All these inhibitors are able to produce a strong inhibition on enzymatic hydrolysis (Jung and Kim, 2017; Panagiotou and Olsson, 2007) and fermentative microorganisms (Jung and Kim, 2017; van der Pol et al., 2016b).

Furans are involved in the formation of ROS, which, as explained, can produce oxidative damage to different molecules and structures like enzymes, organelles and chromatin (Allen et al., 2010; van der Pol et al., 2016b), thus hindering enzymatic hydrolysis and fermentation. Indeed, high concentrations of furans produce a prolonged lag phase during batch fermentation by damaging the membrane and interfering with the glycolytic enzymes (Tomás-Pejó et al., 2011; van der Pol et al., 2016).

At low pH values, CBA are able to denaturate hydrolytic enzymes and cross the microbial cell membranes. Once inside the cell, CBAs dissociate due to the higher pH and the excess of protons is pumped out at the expenses of ATP. When the capacity of the microorganism to pump protons out of the cell is overpassed, it leads to cytosol acidification and cell death. The low molecular weight and the stronger polar groups of formic acid makes it the most inhibitory compound within this group for enzymatic hydrolysis and fermentative microorganisms, followed by levulinic acid and acetic acid (Almeida et al., 2007; Jung and Kim, 2017; Panagiotou and Olsson, 2007).

Interestingly, smaller molecular size and greater carbonyl content of the phenolic compounds have associated a higher inhibitory effect to enzymatic cellulose hydrolysis (Zhai et al., 2018). Although inhibitory mechanisms of phenolic compounds on microorganisms have not yet been completely elucidated (Bai et al., 2015), they are known to affect cell membrane permeability and functionality, hindering the performance of respiration and the maintenance of the pH homeostasis because of the dissipation of ionic gradients (Almeida et al., 2007). Indeed, phenolic aldehydes, like vanillin and syringaldehyde, have been reported to produce a high damage on membrane and glycolytic and fermentative enzymes (Bai et al., 2015).

The inhibition mechanism of the degradation compounds is not only based on the effect caused by each compound individually but also on their interaction and synergy (Oliva et al., 2004; van der Pol et al., 2016b).

Different detoxification methods can be applied to reduce the inhibitors content of the pretreated materials, including physical (evaporation, membrane filtration), chemical (solvent extraction, resin ion exchange, active carbon adsorption, overliming, zeolites) and biological (enzymes and microorganisms) (Bhatia et al., 2020). In this sense, biodetoxification methods are gaining ground as the greenest solutions to this problem. Laccase and peroxidase enzymes obtained from different microorganisms like *Tinea versicolor* and *Trametes villosa* allow the direct oxidation of phenolic and non-phenolic lignin compounds (Moreno et al., 2015). Furthermore, different biodetoxifying microorganisms, like *Aspergillus nidulans* or *Khurtia huakuii* can transform phenolic and furan aldehydes into less toxic compounds (Xie et al., 2018; Yu et al., 2011). These approaches can decrease the toxicity of the material, improving hydrolysis and fermentation.

However, additional detoxification steps for lignocellulose processing could increase the overall production costs. To avoid this, new fermentative microorganisms with the ability to tolerate (Gao et al., 2017; Moreno et al., 2019a) or even biodetoxify (Jiang et al., 2016; Liu et al., 2005) high inhibitors concentrations can be isolated or developed by different methodologies, including evolutionary approaches, as will be explained hereafter.

1.2.3. Enzymatic hydrolysis

After pretreatment, a hydrolysis step is generally needed to release the potentially fermentable monosaccharides from the material. In this case, enzymatic hydrolysis offers many advantages in comparison with acid hydrolysis, like higher conversion yields, no inhibitors formation and lower energy requirements. However, hydrolysis rate decreases rapidly along time while monosaccharides are being released, leading to decreased yields and the need of high enzymes dosages (Ballesteros, 2010).

Cellulolytic and hemicellulolytic enzymes used during lignocellulose hydrolysis are mainly produced by *Trichoderma* and *Aspergillus* fungi and they have a catalytic domain and a carbohydrate-binding module.

The different types of cellulolytic enzymes, *i.e.* exoglucanases, endoglucanases and *β*-glucosidases, catalyse the hydrolysis of crystalline cellulose by a synergistic action. Exoglucanases facilitate the release of glucose and cellobiose units from the end of cellulose chains, while endoglucanases catalyse the hydrolysis of *β*-1,4-glycosidic bonds from different parts of amorphous cellulose. On the other hand, *β*-glucosidases promote the split of cellobiose disaccharides into two D-glucose molecules.

In the same way as cellulolytic enzymes, the hemicellulases endo-1,4-*β*-D-xylanases catalyse the release of xylo- and arabino- oligomers from hemicellulose, while *β*-D-xylosidases catalyse the release of monomeric xylose and arabinose. Arabinofuranosidases, *α*-galactosidases, *α*-glucuronidases, mannases and pectinases can also be added for hydrolysis hemicellulose from softwood and grass feedstocks rich in arabinoxylan, (galacto)glucomannan and poly-*α*-(rhamno)-galacturonic acids (Sweeney and Xu, 2012; Xin et al., 2019).

Enzymatic hydrolysis yields on lignocellulosic materials are highly limited by different facts (Ballesteros, 2010; Dondelinger et al., 2016; Jönsson and Martín, 2016):

- Cellulose with high crystallinity and degree of polymerisation is very resistant to hydrolytic enzymes.
- Hemicellulose and lignin can limit cellulose enzymatic hydrolysis by acting as physical barriers or by producing non-productive binding to enzymes.
- Soluble xylooligosaccharides can produce competitive inhibition on cellulases.
- High monosaccharides concentrations can produce end-product inhibition on cellulases and hemicellulases.
- Suboptimal process conditions, including temperature, pH, agitation and presence of inhibitory degradation compounds produce low stability and inactivation of enzymes.

In order to overcome the mentioned obstacles during lignocellulosic biomass enzymatic hydrolysis, intensive research is being carried out for the application of (1) pretreatment techniques that allow cellulose digestion, hemicellulose solubilisation and lignin removal before the hydrolytic step (López-Linares et al., 2015), (2) new synergetic enzymatic cocktails with higher stability and catalytic activity and lower costs (Dondelinger et al., 2016; Lopes et al., 2018), and (3) different hydrolysis and fermentation process configurations that tackle end-product inhibition (Neves et al., 2016).

1.2.4. Fermentation

There is a great spectrum of microorganisms able to convert different lignocellulosic sugars into biofuels and bio-based products through fermentative processes, and a few examples can be seen in **Table 1.3**.

In order to choose the proper fermentative microorganisms for a certain bioprocess from lignocellulose, different questions must be taken into account, including the raw material, product yields and productivities, nutritional requirements, growth and fermentation conditions, (temperature, oxygen, pH, agitation, etc.) and resistance to inhibitors (Idler et al., 2015).

Regarding sugars utilisation, most of fermentative microorganisms are able to metabolise hexoses, like D-glucose, but not pentoses like D-xylose and L-arabinose (**Table 1.3**). For instance, in the case of bioethanol production, natural C5-fermenting yeasts are very unusual, they require microaerophilic conditions, and present low tolerance to high ethanol concentrations, inhibitors and pH changes (Tomás-Pejó, 2011).

Table 1.3. Culture conditions for bioproduction of biofuels and bio-based chemicals by different fermentative microorganisms.

Fermentative microorganism		Temperature	pH	Sugars		Product	Ref [1]
				C6	C5		
Yeasts	*Saccharomyces cerevisiae*	30-35 °C	5.5	+	-	Bioethanol	(1)
	Scheffersomyces stipitis	26-35 °C	5.5	+	+	Bioethanol	(2)
	Candida tropicalis	30 °C	5.5	+	+	Xylitol	(3)
Gram (+) bacteria	*Lactobacillus rhamnosus*	30 - 40 °C	5.5-7	+	-	Lactic acid	(4)
	Lactobacillus pentosus	30 - 40 °C	5.5-7	+	+	Lactic acid	
	Bacillus coagulans	30 - 55 °C	5.5-7	+	+	Lactic acid	
	Clostridium acetobutylicum	37 °C	5	+	-	Butanol	(5)
Filamentous fungi	*Aspergillus* strains	30-35 °C	3-3.5	+	-	Itaconic acid	(6)

[1] Ref: (1) Dickinson and Schweizer, 1999; (2) Slininger et al., 1990; (3) El Baz, 2011; (4) Idler et al., 2015; (5) Al-Shorgani et al., 2018; (6) Hajian et al., 2017.

Utilisation of cellulosic glucose from steam-exploded WIS fractions is well established, especially for bioethanol production. However, since the prehydrolysates contain mixtures of C6 and C5 sugars, the valorisation of these fraction is still a big challenge. Hence, alternative methods are being studied to solve this, including: (1) generation of a unique fermentation product from lignocellulose by robust C6 and C5 co-fermenting microorganisms or using (2) different C6- and C5-utilising microorganisms, producing different products from lignocellulosic biomass in a biorefinery context.

1.2.5. Process configurations for hydrolysis and fermentation

Different process configurations can be applied when using lignocellulosic substrates to produce biofuels and bio-based products (**Fig. 1.3**). The proper configuration should be chosen taking into account the properties of the lignocellulosic material and the fermenting microorganism.

Separated hydrolysis and fermentation (SHF). Sugars from the material are firstly enzymatically hydrolysed and the resulting media rich in monosaccharides is subsequently fermented. SHF has been commonly used in bioproduction because optimum conditions for enzymatic hydrolysis and fermentation can be independently set. Moreover, fermentation may be carried out in liquid medium since insoluble solids can be removed after enzymatic hydrolysis (Kawaguchi et al., 2016).

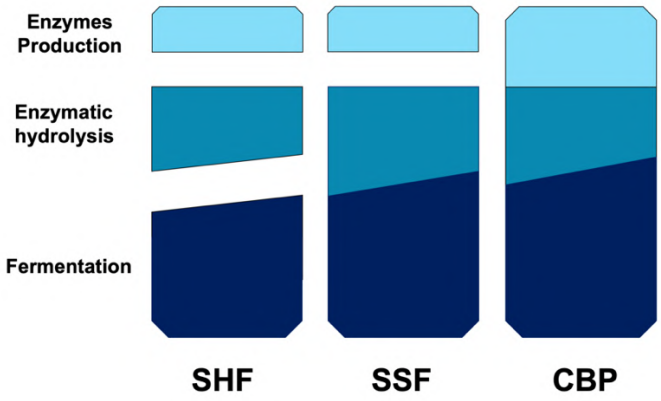

Fig. 1.3. Process configurations for enzymatic hydrolysis and fermentation of lignocellulosic biomass.

Simultaneous saccharification and fermentation (SSF) is an integrated process in which both hydrolysis and fermentation steps are simultaneously produced in the same vessel. In this case, sugars are consumed as they are hydrolysed and enzymes are not subjected to end-product inhibition (Olofsson et al., 2008). As a consequence, higher hydrolysis yields and shorter reaction times are achieved. Furthermore, the need of high enzyme loadings is reduced, which is translated in a significant cost reduction (Tomás-Pejó et al., 2008).

However, these processes have to be carried out at compromised pH and temperature conditions for both enzymes and fermenting microorganisms (Olofsson et al., 2008).

Consolidated bioprocessing (CBP) is the ultimate and most integrated process configuration and it is based on the use of cellulolytic microorganisms with the ability to (1) release monosaccharides from lignocellulose and (2) convert the released sugars into biofuels or bio-products through fermentative pathways (Kawaguchi et al., 2016). CBP has less operational steps than SHF or SSF. Furthermore, since no exogenous enzymes are required, the process costs could be reduced. However, low hydrolysis yields and/or product productivities are obtained by these CBP-oriented microorganisms (Kawaguchi et al., 2016). Genetic modification and metabolic engineering are being investigated to obtain new strains that present efficient hydrolytic enzymes production and fermentation of the released sugars to be applied in CBP (Amoah et al., 2017; Oh and Jin, 2020).

Depending on the substrate addition method, fermentation can be operated in different ways:

Batch culture. The microorganism is inoculated in a fixed volume of medium and the desired product is recovered at the end of the process. Hence, the culture conditions change as the microorganism passes through the different growth passes, *i.e.* lag, exponential, stationary and death phases (Sauer, 2011).

When the culture is started at high substrate loading, high sugar concentrations are present, which can produce substrate inhibition together with mass and heat transfer limitations. In this case, microbial growth can be repressed due to osmotic stress, reducing water activity and provoking cell lysis (Abdel-Rahman and Sonomoto, 2016; Chang et al., 2018). Furthermore, the expression of hexose transporters have demonstrated to be downregulated at high glucose concentrations (Chang et al., 2018).

In the same way, some fermentation products, like alcohols and organic acids can become toxic for the microorganisms at high concentrations, producing product inhibition and disrupting the integrity of the cell membrane (Klotz et al., 2016; Zhang et al., 2015).

Fed-batch culture. Inoculation is performed in batch and after a period of time, more substrate (and, if required, more inoculum) is fed in one or more stages. The feeding times are set to maintain an adequate substrate concentration. If the process is carried out in SSF, enzymes can also be added in different stages.

In this case, higher productivities are obtained because (1) sugar concentrations are always on subcritical levels for substrate inhibition (Abdel-Rahman et al., 2011) and (2) inhibitory degradation compounds are gradually added to the culture (Tomás-Pejó et al., 2009).

Continuous fermentations. Fresh medium is introduced in the culture vessel at a steady flow rate, while fermented medium and cells are harvested at the same flow rate. This method allows to maintain constant concentrations of growth-limiting nutrients, fixing the microorganism growth rate and cell density (Kuenen and Johnson, 2009). As a result, production and consumption rates also remain in a steady state (Mears et al., 2017). To achieve this, a proper dilution rate has to be settled to avoid the efflux of non-fermented sugars and the cell wash-out.

In contrast with fed-batch mode, a constant working volume is maintained, which facilitates the scale-up of the process. The high risk of contamination and the high capital costs are the main drawbacks of these processes. Both substrate and product inhibition can be overcome by continuous operation coupled with other techniques:

On one hand, high cell densities can be obtained by coupling a continuous culture with cell immobilisation or cell recycling. In this case, high flow rates can be operated without producing cell wash-out and the substrate concentration can be kept low, avoiding substrate and product inhibition, which results in higher productivities than batch culture (Tashiro et al., 2011).

On the other hand, when using continuous extractive fermentation, the desired product is continuously removed as it is formed, keeping it at low concentration in the culture vessel and avoiding product inhibition (Mears et al., 2017, Boontawan et al., 2011).

1.3. Production of lactic acid and bioethanol from lignocellulosic biomass

1.3.1. Lactic acid production from lignocellulose

Most of chemicals are still produced from petrochemical sources. However, bio-based chemicals and polymers production are starting to gain ground, being estimated at 50 million tons per year, with an annual growth of 3 % - 4 % (Chandel et al., 2018). In this sense, lignocellulosic biomass has received great attention as raw material, and more than 200 value-added chemicals can be produced in a lignocellulosic biorefinery (Isikgor and Becer, 2015). As a matter of fact, LA was identified among the most promising bio-based products from lignocellulosic biorefineries, by US Department of Energy (USDOE, Washington) (Bozell and Petersen, 2010) and the European Comission (Fabbri et al., 2018).

LA, or 2-hydroxypropanoic acid, is an organic acid with a hydroxyl group adjacent to the carboxyl group (**Fig 1.4**). Due to its chirality, two optical isomers can be found: L-(+)-lactic acid (L-LA) and D-(-)-lactic acid (D-LA) (**Fig. 1.4**).

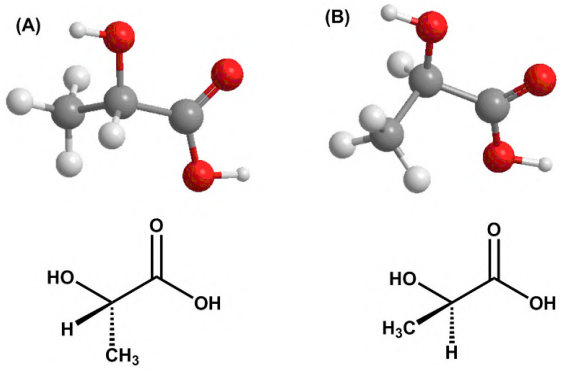

Fig. 1.4. Three-dimensional structure of L-LA (A) and D-LA (B) (Article I).

LA has been commercially used in food industry as an emulsifying, acidulant and preserving agent (Abdel-Rahman et al., 2011; Wang et al., 2015). Due to its antibacterial and ink erasure properties, it has also been used in textile, detergent and paper industry (Secchi et al., 2012). Currently, LA is starting to conquer new market niches related to added-value products, such as pharma, cosmetic and chemical industry (**Fig. 1.5**) (Article I).

The racemic mixture can be used for the production of green solvents like ethyl lactate, acetaldehyde, propylene glycol, 2,3-pentadione and other CBA like pyruvic, oxalic, acrylic and propanoic acid (Isikgor and Becer, 2015; López-Gómez et al., 2019) (**Fig. 1.5**). However, pure isomers are more valuable than the racemic mixture. For instance, L-LA is the biologically active form in humans due to the presence of L-lactate dehydrogenase (LDH), being used in food and pharma industries (Wang et al., 2015). In the field of biopolymers, pure enantiomers are needed for the production of poly-lactic acid (PLA) (**Fig. 1.5**).

PLA has emerged as an alternative to petrochemical-based plastics. It exhibits high chemical and mechanical resistance, being very appropriate for the manufacture of fibers, non-woven fabrics, and films for packaging (Zhang and Vadlani, 2013). Furthermore, fabrication of PLA-based prosthetic devices, surgical sutures and controlled drug delivery systems is being investigated given its biocompatible features (Ulery et al., 2011) (**Fig. 1.5**). When mixtures of both isomers are used in the polymerisation of PLA, the resulting polymer is amorphous and unstable. Nevertheless, using optically pure D-LA or L-LA, highly crystalline PD-LA or PL-LA is formed, with higher thermal stability and easier biodegradation (Klotz et al., 2016).

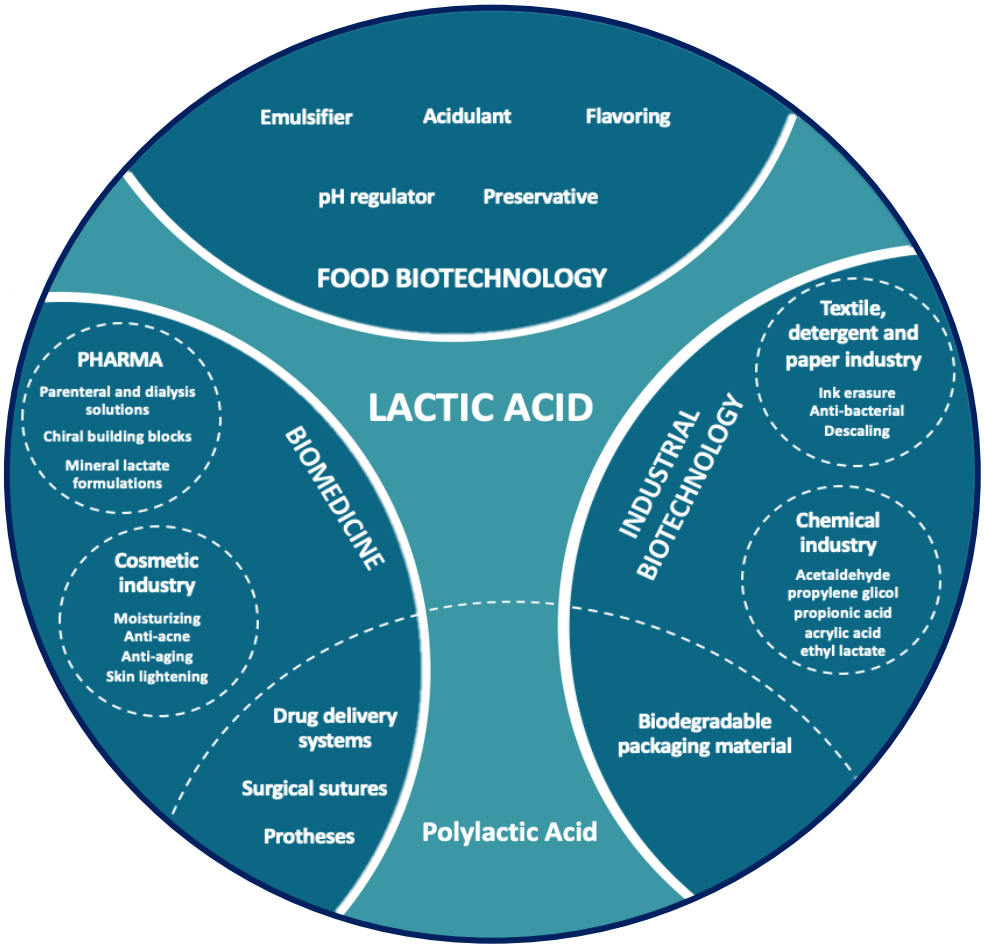

Fig. 1.5. Industrial application of LA and PLA (Article I).

LA can be synthetised by chemical synthesis and microbial fermentation. The first one involves the production of lactonitrile by the reaction of acetaldehyde and hydrogen cyanide, which derives from petrochemical sources (Eiteman and Ramalingam, 2015). Furthermore, chemical synthesis yields a racemic mixture of LA. On the other hand, LA can be produced by sugars fermentation with the possibility of producing optically-pure LA.

The microbial LA production process from starchy materials is commercially mature, with a global LA market size of $2.64 billion in 2018 (GVR, 2019). The major manufacturers include NatureWorks LLC (USA), Archer Daniels Midland Company (USA), Corbion Purac (The Netherlands) and Galactic S.A. (Belgium).

While LA production from these starchy substrates is well-established, different particular limitations need to be faced when using steam-exploded pretreated prehydrolysates. As a result, lignocellulosic LA production remains at demonstration scale stage.

For instance, pretreated lignocellulosic materials contain different impurities, like inhibitory degradation compounds, that can hamper both bacterial growth and LA purification. In addition, the presence of C6 and C5 sugar mixtures, mainly from the hemicellulosic fraction, requires the use of versatile LA-producing microorganisms with the ability to convert different carbon sources into LA.

The microorganisms and metabolic pathways involved in fermentation of different sugars into LA are detailed hereafter.

1.3.1.1. Lactic acid fermentation metabolism

Different microbial groups are able to produce LA from the sugars platform, including, fungi, like *Rhizopus* and *Candida* strains, algae and, especially, bacteria (Idler et al., 2015). The bacterial group includes lactic acid bacteria (LAB), *Bifidobacterium* genus, some *Escherichia coli*, and different *Corynebacterium* and *Bacillus* strains (Abdel-Rahman et al., 2013).

- *Lactobacillales* order or LAB can be described as gram-positive, mesophilic, non-sporeforming and microaerophilic or facultative anaerobic bacteria that produce LA as predominant or unique fermentation product (Juturu and Wu, 2015). LAB need plenty of nitrogen-rich sources because they natively lack various biosynthetic pathways (Abdel-Rahman et al., 2013). Different species of *Lactobacillus, Pediococcus, Leuconostoc, Oenococcus, Weissella, Streptococcus and Lactococcus* genera are included in this group (Juturu and Wu, 2015).

- *Bacillales* order. Although LA fermentation has traditionally been studied in LAB, some Bacilli are able to convert sugars into LA with high yields. These bacteria are described as gram positive and facultative anaerobic bacteria, but in contrast with LAB, they are spore-forming and have low nutritional requirements. The sporulation ability increases their tolerance to harsh conditions and some of them are described as facultative thermophilic. In this group different *Sporolactobacillus* and *Bacillus* strains can be found. One of the microorganisms that has become most popular in the last years is *B. coagulans*, being a pentose-utilising bacterium that produces optically pure L-LA (Idler et al., 2015).

The metabolic pathways involved in LA production are the Embden-Meyerhof-Parnas (EMP) pathway, the Pentose Phosphate (PP) pathway and the Phosphoketolase (PK) pathway

(**Table 1.4**). Many LA-producing bacteria have one or more of these metabolic pathways and the conversion yield and energy generated will depend on the particular strain and growth conditions (Eiteman and Ramalingam, 2015).

Table 1.4. LA and ATP yields of different metabolic pathways involved in fermentation of C6 and C5 sugars (Article I).

	C6 SUGARS		C5 SUGARS	
Metabolic pathway	LA yield (mol mol^{-1})	ATP yield (mol mol^{-1})	LA yield (mol mol^{-1})	ATP yield (mol mol^{-1})
EMP	2	2	-	-
PP	1.67	1.67	1.67	0.67
PK	1	2	1	2

Homolactic fermentation of C6 sugars

Lactobacillus helveticus, L. rhamnosus, Lactobacillus delbrueckii, *Lactobacillus acidophilus*, *Lactococcus lactis* and *Enterococcus* strains are LAB with the ability to convert glucose into pyruvate, through EMP pathway, and then to LA using the enzyme lactate dehydrogenase (LDH) (**Fig. 1.6**) with a theoretical yield of 2 mol mol^{-1} (**Table 1.4**). By this route, one molecule of glucose yields two molecules of LA, generating two ATP (1).

(1) $C_6H_{12}O_6 + 2\ ADP + 2\ Pi \rightarrow 2\ C_3H_6O_3 + 2\ ATP + 2\ H_2O$

Nonetheless, pentoses cannot be fermented by these strains because of lack of xylulose PK enzyme (Zhang et al. 2016; Idler et al., 2015).

Homolactic fermentation of C6 and C5 sugars

Although homolactic LAB are not able to metabolise pentoses, some Bacilli are able to ferment both C6 and C5 sugars homofermentatively (Ma et al., 2016). Glucose can be metabolised by EMP pathway (1) or by PP pathway (2). In both cases, fructose-6-P is formed from glucose, which is converted to glyceraldehyde 3-phosphate, and consequently, to LA (**Fig. 1.6**). However, the theoretical yield from PP pathway is lower, 1.67 mol mol^{-1} (**Table 1.4**), because of the formation of CO_2 (2).

(2) $3\ C_6H_{12}O_6 + 6\ NAD(P) + 5\ Pi + 5\ ADP \rightarrow 5\ C_3H_6O_3 + 3\ CO_2 + 6\ NAD(P)H + 5\ ATP + 2\ H_2O$

B. coagulans can convert three molecules of xylose to five molecules of LA (3), with higher yields than the obtained with heterolactic LAB from C5 (**Table 1.4**) (Juturu and Wu, 2015).

(3) $3\ C_5H_{10}O_5 + 2\ ADP + 2\ Pi \rightarrow 5\ C_3H_6O_3 + 2\ ATP$

Heterolactic fermentation of C6 and C5 sugars

Some LAB metabolise both C6 and C5 sugars via the PK pathway because of the lack of enzyme aldolase. One molecule of CO_2 is cleaved from a C6 sugar, forming ribulose-5-P. This ribulose-5-P is cleaved again, catalysed by PK enzyme, resulting in one molecule of glyceraldehyde 3-phosphate, which is converted to LA, and another of acetyl-phosphate, that is directed to acetic acid (4) or ethanol (5) to close the electron balance (**Fig. 1.6**).

(4) $C_6H_{12}O_6 + 2\ ADP + Pi \rightarrow C_3H_6O_3 + C_2H_4O_2 + CO_2 + 2\ ATP + H_2O$

(5) $C_6H_{12}O_6 + 2\ NAD(P) + 2\ ADP + Pi \rightarrow C_3H_6O_3 + CO_2 + C_2H_5OH + 2\ NAD(P)H + ATP$

When pentoses are used as carbon source, there is not NAD(P)H, CO_2 and ethanol production and the acetyl-phosphate is converted directly to acetic acid (6). The maximum theoretical yield is 1 mol LA per mol C6/C5 sugar (**Table 1.4**).

(6) $C_5H_{10}O_5 + 2\ ADP + 2\ Pi \rightarrow C_3H_6O_3 + C_2H_4O_2 + 2\ ATP + 2\ H_2O$

Lactobacillus bifermentans, *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Lactobacillus sanfranciscensis* and *Lactobacillus brevis*, as well as *Leuconostoc* and *Oenococcus* strains are assigned to this group (Idler et al., 2015; Prückler et al., 2015).

Facultative heterolactic fermentation

L. pentosus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei and *Pediococcus acidilactici* are assigned to this group (Idler et al., 2015; Zhang et al., 2016; Zhu et al., 2007). These bacteria metabolise hexoses via EMP pathway (1) and pentoses via PK pathway (5), with a maximum yield of 1 and 2 mol LA per mol sugar, respectively.

In general terms, the targeted bacteria for LA production from hemicellulosic fraction of lignocellulosic biomass should be a C6 and C5-utilising strain with high resistance to inhibitors released during the pretreatment.

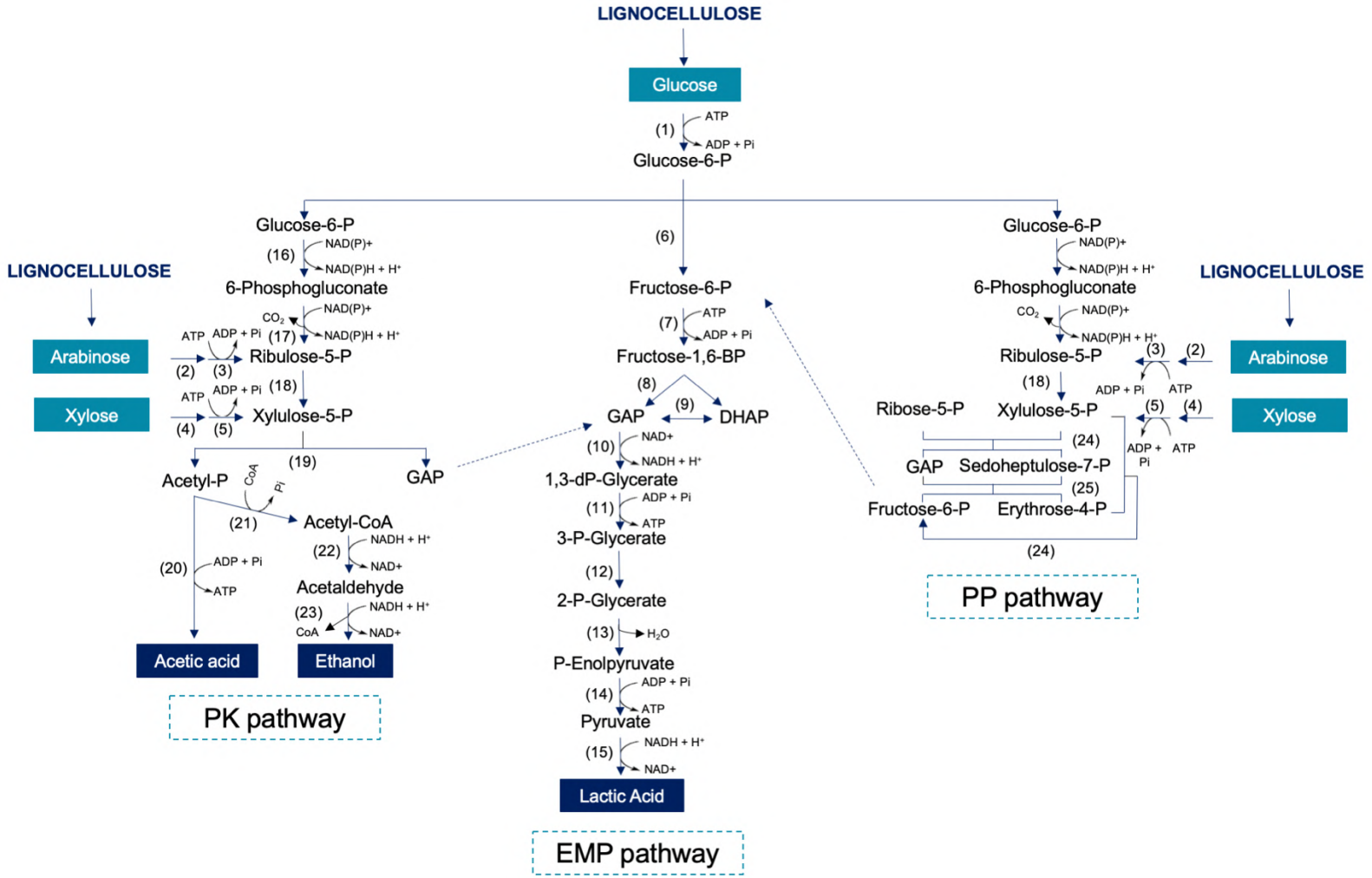

Fig. 1.6. Metabolic pathways involved in LA production from different carbon sources. (1) hexokinase, (2) arabinose isomerase, (3) ribulokinase, (4) xylose isomerase, (5) xylulokinase, (6) glucose-6-P isomerase, (7) 6-phosphofructokinase, (8) fructose-bisphosphate aldolase, (9) triose-phosphate isomerase, (10) glyceraldehyde-3-P dehydrogenase, (11) phosphoglycerate kinase, (12) phosphoyglycerate mutase, (13) enolase, (14) pyruvate kinase, (15) lactate dehydrogenase, (16) glucose-6-phosphate dehydrogenase, (17) 6-phosphogluconate dehydrogenase, (18) ribulose-5-phosphate-3-epimerase, (19) phosphoketolase, (20) acetate kinase, (21) phosphotransacetylase, (22) aldehyde dehydrogenase, (23) alcohol dehydrogenase, (24) transketolase, (25) transaldolase (Article I).

1.3.1.2. Downstream processing for lactic acid recovery and purification

Bearing in mind that the production of high added value products needs a highly pure LA as building block, recovery and purification are crucial steps for commercialisation. Markedly, separation and purification can account for 30 % - 40 % of total production costs, being steps that determine the economic viability of the process (López-Garzón and Straathof, 2014).

Currently, the calcium lactate method is the most widely used process for both LA neutralisation and separation, being commercially applied by NatureWorks and Purac in LA production from starch (López-Garzón and Straathof, 2014). The addition of $CaCO_3$ provides an effective calcium lactate precipitation that facilitates LA recovery. However, accumulation of $CaCO_3$ and calcium lactate crystals can inhibit the growth of LA producers (Ye et al., 2013b). Furthermore, an additional hydrolytic step is needed to dissociate free LA from calcium lactate, consuming significant volumes of H_2SO_4 and releasing high amounts of gypsum ($CaSO_4$) as solid waste, which generates environmental and economic concerns (Singhvi et al., 2018).

In the context of green chemistry, the calcium lactate method should be replaced by new approaches like the use of acid-resistant bacteria and novel purification methods. Alternative purification methods, such as solvent extraction, crystallisation, membrane separation and reactive distillation, are being tested for removal of impurities from lignocellulosic materials and recovery of free LA from sodium/calcium lactate, lowering the cost and energy consumption (Khunnonkwao et al., 2012; López-Garzón and Straathof, 2014).

Chromatographic techniques are well-recognised by their high selectivity and separation yields, low waste generation, short operation times and moderate operational conditions (Bishai et al., 2015). In electrodialysis, the application of an electric field in the junction of two anion and cation exchanges membranes produce water splitting, thus generating protons and hydroxyl ions that force the separation of free LA (Lech and Trusek, 2018; Neu et al., 2016).

Reactive distillation is a separation process in which the desired product is subjected to a chemical reaction to favour the subsequent distillation (Coker, 2010). This technique has been shown to be economically feasible in many catalysed reactions, like LA esterification with ethanol (Komesu et al., 2015; Vudata Venkata et al., 2014).

Although some of these techniques are very advantageous in terms of sustainability, environment preservation and energy savings, they are still in pilot/demonstration scale and many efforts are needed for their implementation.

1.3.1.3. Challenges of lignocellulosic lactic acid production

LA production can be seriously affected by several factors during the whole process. As it is the case of other products produced from lignocellulose, the need of pretreatment, a multi-enzymatic hydrolysis steps, some fermentation constraints and purification processes can harshly increase the production costs (Pleissner et al., 2017a). Regarding the fermentation step, the most important challenges that need to be tackled in the future years for an efficient lignocellulosic LA production, focused on the hemicellulosic fraction, are mentioned below.

Use of C5 sugars and carbon catabolite repression effect

In LA-producing bacteria, glucose and xylose can be transported by facilitated diffusion through phosphoenolpyruvate-dependent phosphotransferase systems (PEP-PTS) (Abriouel et al., 2017; Chaillou et al., 1999). Normally, when both C5 and C6 sugars are present, different carbon catabolic repression (CCR) effects are activated (**Fig. 1.7**), which leads to a sequential sugar uptake that can become a handicap during fermentation of hemicellulose-rich materials (Abdel-Rahman and Sonomoto, 2016).

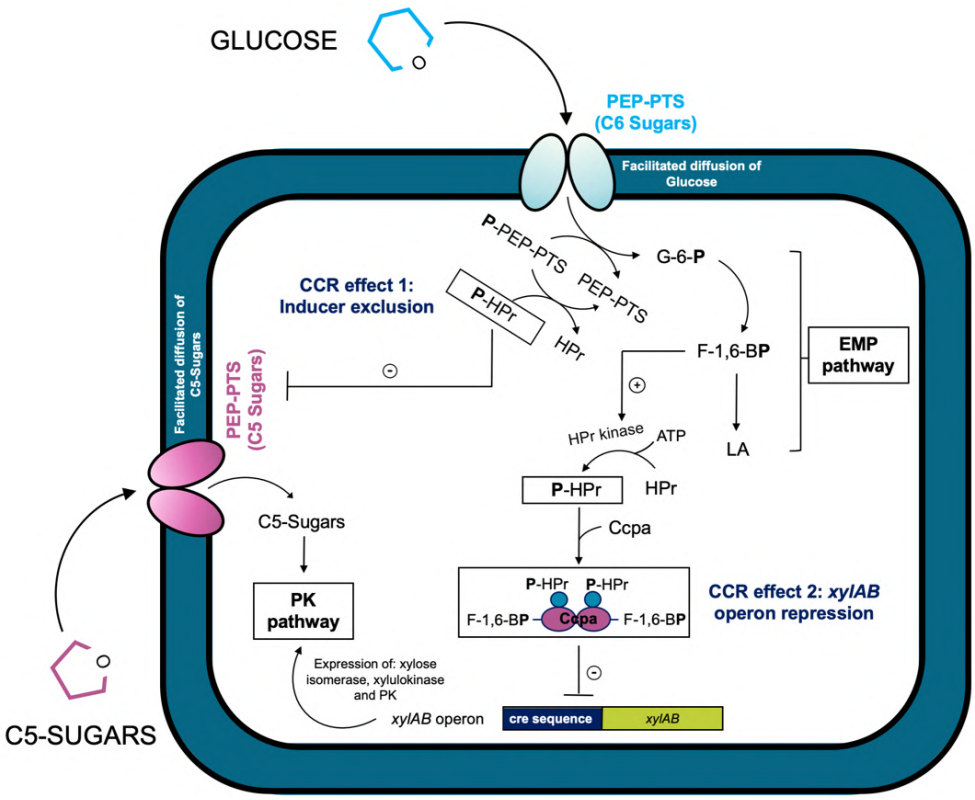

Fig. 1.7. C5 sugars transport systems and CCR effect on heterolactic LAB. G-6-P (glucose-6-phosphate), F-1,6-BP (fructose-1,6-bisphosphate).

In LAB, CCR mechanism is performed by a heat-stable phosphocarrier protein (HPr) (Kim et al., 2009). In the presence of glucose, phosphorylated forms of HPr, can: (1) provide phosphate groups for PEP-PTS transport of glucose, (2) prevent the translocation of C5 sugars by interacting with their corresponding PEP-PTS systems and (3) inhibit the expression of genes involved C5 sugars metabolism (*xylAB* operon) (**Fig. 1.7**) (Chen et al., 2018; Kim et al., 2010).

In the latter case, the complex formed between HPr, a carbon catabolite protein (CcpA) and glycolytic intermediates (fructose-1,6-bisphosphate) binds to an upstream catabolite repression element of these genes (**Fig. 1.7**) (Chen et al., 2018; Kim et al., 2010). However, when glucose is depleted, there is a reduction of glycolytic intermediates that forces LA-producing bacteria to use other carbon sources (Papadimitriou et al., 2016).

By-product formation during heterolactic fermentation

By-products like acetic acid or ethanol are produced by several LAB using PK pathway (**Fig. 1.6**). This is the case for C6 and C5 sugars fermentation with obligate heterolactic strains like *L. brevis,* and C5 sugars fermentation by facultative heterolactic strains like *L. pentosus* (Idler et al., 2015). By-product formation implies a significant decrease in LA yield.

Inhibitory degradation compounds

The presence of lignocellulosic degradation compounds is one of the main drawbacks that affect the LA production. For instance, LA production on different bacteria has reported to be inhibited at concentrations of around 10 g L^{-1}, 2.5 g L^{-1} and 1 g L^{-1} of CBA, furans and phenols, respectively (Boguta et al., 2014; van der Pol et al., 2016b).

Optical purity of lactic acid

As explained above, production of optically pure L or D-LA is preferred against the racemate. It is important to notice that optical purity of LA is not guaranteed unless the proper LA-producing bacteria is chosen. For example, *L. delbrueckii*, *Lactobacillus coryniformis*, *Lactobacillus jensenii*, and *Lactobacillus vitulinus* produce D-LA; *L. casei*, *L. paracasei*, *L. rhamnosus* and *B. coagulans* produce L-LA; and *L. pentosus*, *L. plantarum*, *L. brevis* and *L. acidophilus* produce DL-LA (Kuo et al., 2015).

Oxidative stress

Different oxygen requirements for metabolism can be found in different LA-producing bacteria. On one hand, LAB lack a functional respiratory chain and catalases, being considered aerotolerant or microaerophilic bacteria (Idler et al., 2015). On the other hand, *Bacillus* strains are catalase positive facultative anaerobic bacteria, being able to use oxygen as electron acceptor (Idler et al., 2015).

Oxidative stress can cause different effects on microorganisms like metabolic pathway disruptions, and damage on DNA, cell membrane and proteins. This situation is produced when oxygen is partially reduced in cell metabolism by the expression of NADH oxidases, leading to the formation of ROS like H_2O_2 and O^{2-} (Papadimitriou et al., 2016). Under these conditions, ROS detoxification responses are activated, like the expression of NADH peroxidase to remove the excess of H_2O_2 (Papadimitriou et al., 2016). Both NADH oxidase and peroxidase enzymes compete with LDH for NADH molecules. Consequently, LA production is reduced, and pyruvate is redirected to mixed fermentations (Qin et al., 2010).

Inhibition at low pH

Most of the LA-producing bacteria are acid tolerant, being able to grow when moderate amounts of LA are present during fermentation. Nonetheless, when using lignocellulosic materials with high sugar concentrations, high amounts of LA are accumulated in the media, which produce a severe pH drop that can inhibit bacterial growth and metabolism (**Fig. 1.8 A**).

The situation becomes harder when (1) using LAB that produce both LA and acetic acid by PK pathway (**Fig. 1.8**), and when (2) fermenting materials with high concentrations of CBA formed during pretreatment (**Fig. 1.2**).

As a result, the culture pH drops to values lower than the pKa of LA (3.8), acetic acid (4.8), formic acid (3.75) and levulinic acid (4.6), and they become able to cross the cell membrane in their undissociated forms (Trcek et al., 2015). Due to the higher intracellular pH, these acids dissociate back inside the cell and energetic supplies are arrested to pump protons out of the cell. As a result, there is a reduction of the ATP available for the cell (growth inhibition) and, when this is overcome, the cytosol acidification leads to the denaturation of proteins and the change of membrane potential, ending with membrane disruption and cell death (**Fig. 1.8 B**) (Trcek et al., 2015). Hence, the presence of organic acids exerts an inhibitory effect on bacterial growth and fermentation when the pH is not controlled.

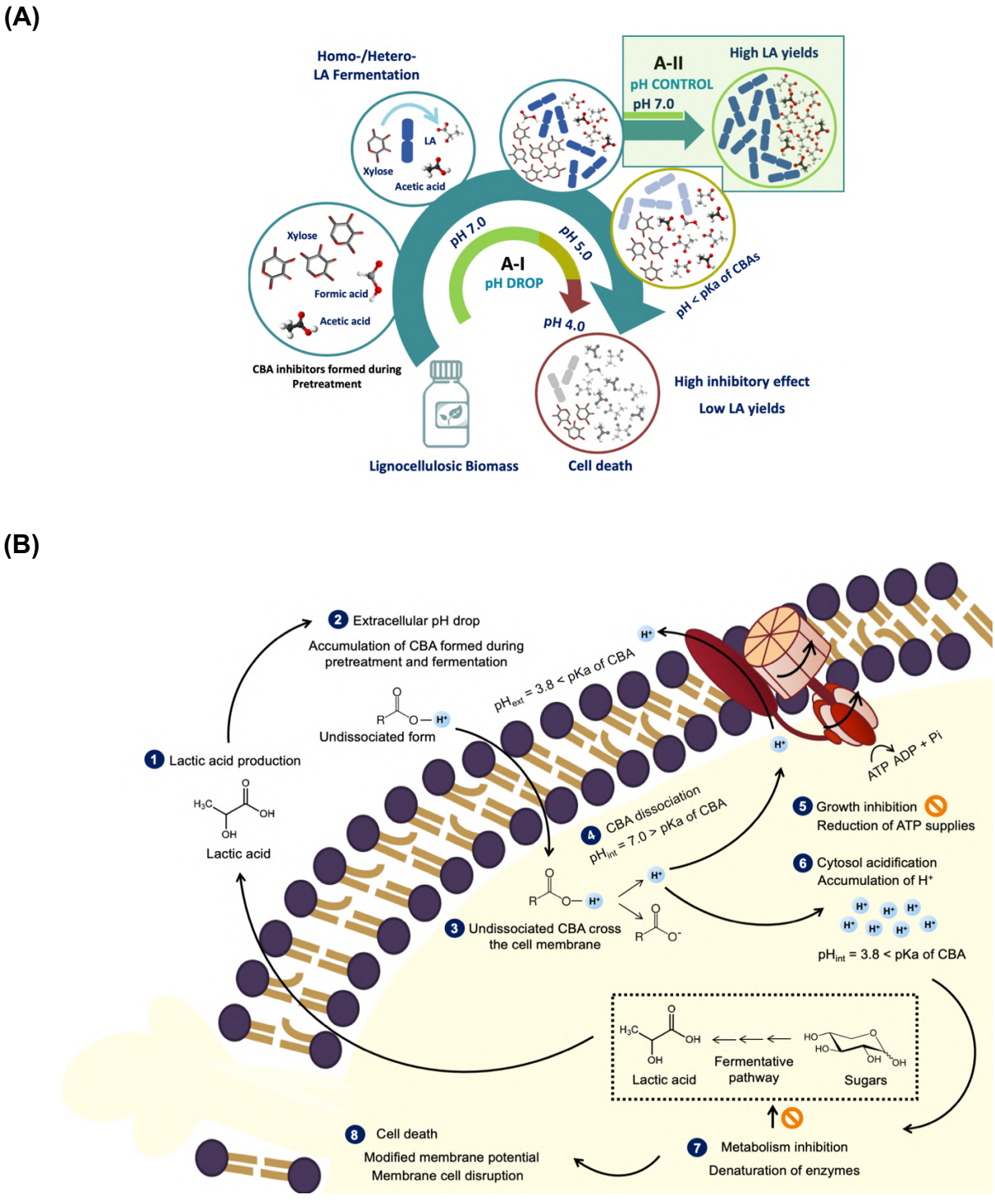

Fig. 1.8. Evolution of bacterial population in LA fermentation (A) under non-controlled (A-I) and controlled (A-II) pH conditions; and intracellular inhibitory effect of CBA on bacterial growth and LA production at low pH (B).

1.3.2. Bioethanol production from lignocellulose

Transport accounts for about a third of the world's energy use, half of global oil consumption and a fifth of global greenhouse gases emissions (IRENA, 2016). Different alternatives can be found to replace oil in transport sector, including electricity, natural gas and biofuels. Biofuels are defined by the European Comission as liquid fuels for transport produced from biomass (European Comission, 2019). These compounds should be considered interesting options to replace conventional fossil fuels for transport in the medium term due to their non-toxic, biodegradable, and carbon neutral features (Toor et al., 2020).

Although biofuels are still playing a limited role, being less than 4 % of the total transport energy, the global production of these alternative energy sources for transport is sharply increasing (IEA, 2019a). Indeed, 154 billion L of biofuels were produced in 2018, with a 7 % year-on year increase (IEA, 2019a). Bioethanol is the biofuel with the most established production technology and with the highest global production (Toor et al., 2020). According to bioethanol production as biofuel, the two larger producers in 2018 were Brazil and the US, reaching 33 billion L and 61 billion L from sugarcane and corn-starch, respectively (RFA, 2018), while almost 9 billion L were produced in the EU (EPURE, 2019).

Bioethanol is a potential substitute for gasoline in transport and its use in gasoline mixtures is broadly employed and even mandatory in some countries (USDA, 2019). Ethanol and gasoline can be blended at different ratios from 10 % to 85 % (v v^{-1}) ethanol. Flexible fuel vehicles are able to use any ethanol and gasoline blend, including ethanol 85 % (E85) and 100 % (v v^{-1}) (E100) (Delavarrafiee and Frey, 2018). Bioethanol can also be used for power generation, fuel cells, co-generation systems and as a building block for the production of other chemicals, polymers and green solvents (Choi et al., 2015).

Among its main characteristics, bioethanol has a higher octane number than gasoline, which results in a better combustion and fuel efficiency. Due to its higher oxygen content, there is a reduction in emissions (particulate solids, hydrocarbons and NO_x) during combustion and the toxicity and risk of explosion in case of spill is also much lower. However, it has less energy density (33 % lower) and higher water miscibility than gasoline, so higher temperatures are needed to evaporate the fuel (Toor et al., 2020). On the other hand, it can produce corrosion and degradation of the engines at high concentrations due to presence of water and oxygen (Thangavelu et al., 2016).

Although ethanol can be obtained by thermochemical routes, nowadays it is mostly produced by fermentation processes because of the high selectivity, conversion efficiency and low energy consumption (Robak and Balcerek, 2018). The industrial production relies on sugar crops (sugarcane, cane juice or molasses) and starchy materials (corn, wheat, cassava or barley).

However, the use of renewable sugar sources, avoiding the competition of water and land-use with food, is an interesting alternative. Advanced bioethanol can be produced from lignocellulosic biomass, municipal solid wastes, industrial by-products or crude glycerol. Lignocellulosic bioethanol is considered a feasible option for fossil fuels replacement. Although only 4 % of bioethanol production in the EU derives from lignocellulosic raw materials (**Fig. 1.9**) (EPURE, 2019), its market size is expected to increase (Choi et al., 2015).

Indeed, various commercial and demonstration ethanol biorefineries have settled in the last years in Europe, like, St1 Biofuels Oy (Finland), Avantium (Netherlands), Borregaard Industries AB (Norway), Procethol2G (France) and Perseo IMECAL (Spain) (Passoth and Sandgren, 2019; Perseo, 2020; Procethol2G, 2020).

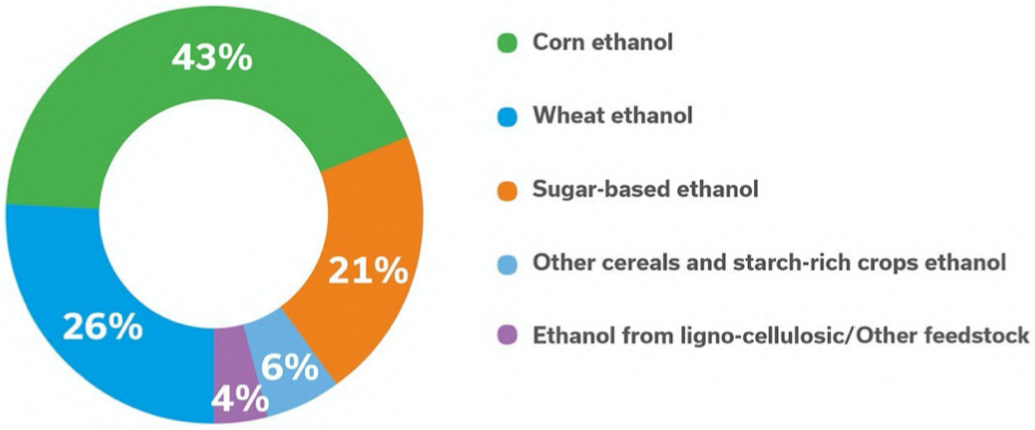

Fig. 1.9. Share of European renewable bioethanol produced from each feedstock type. Modified from: EPURE, 2019.

1.3.2.1. Alcoholic fermentation metabolism

Different microorganisms are able to convert sugars into ethanol by fermentative pathways. Among them, the yeast *S. cerevisiae* is the most utilised microorganism for bioethanol production due the high ethanol yields obtained from glucose by the EMP pathway (**Fig. 2.11**), its high tolerance to inhibitors and ethanol, and because it is generally recognised as safe (GRAS) (Tomás-Pejó, 2011). *Kluveromyces marxianus* is also a widely used GRAS ethanologenic yeast, being characterised by its thermotolerant features (Tomás-Pejó, 2011).

Among bacterial groups, *Zymomonas mobilis* is able to ferment glucose through the Entner-Doudoroff (ED) pathway, which yields only half as much ATP per mole glucose as the EMP pathway (**Fig. 1.10**). As a result, it produces less biomass than yeasts, leading more carbon to ethanol. This GRAS ethanologenic bacterium presents high ethanol and inhibitors tolerance, but it is more sensitive to inorganic ions (Tomás-Pejó, 2011).

In both EMP and ED pathways, one molecule of glucose yields two molecules of pyruvate, which are subsequently converted into two molecules of acetaldehyde, catalysed by the pyruvate decarboxylase, releasing two molecules of CO_2. Lastly, the alcohol dehydrogenase catalyses the conversion of acetaldehyde molecules into ethanol, with a theoretical yield of 0.51 g g^{-1} (7) (**Fig. 1.10**).

(7) $C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$

The main disadvantage of the mentioned microorganisms is that they are not able to ferment C5 sugars released from hemicellulose. However, some yeast strains, like *Candida shehatae*, *S. stipitis* and *Pachysolen tannophilus* are able to convert these sugars into ethanol. In these cases, three molecules of xylose are converted into 5 molecules of each ethanol and CO_2 (8), following the PP pathway coupled with EMP or ED pathways (**Fig. 1.10**).

(8) $3\ C_5H_{10}O_5 \rightarrow 5\ CH_3CH_2OH + 5\ CO_2$

Although native *E. coli* strains are able to produce ethanol from C6 and C5 sugars, they lack the gene encoding pyruvate decarboxylase, expressing pyruvate formate lyase instead. The catalysed reactions produce ethanol at the expenses of NADH, which is compensated by the production of different acids to regenerate NADH, resulting in low ethanol yields (Tomás-Pejó, 2011).

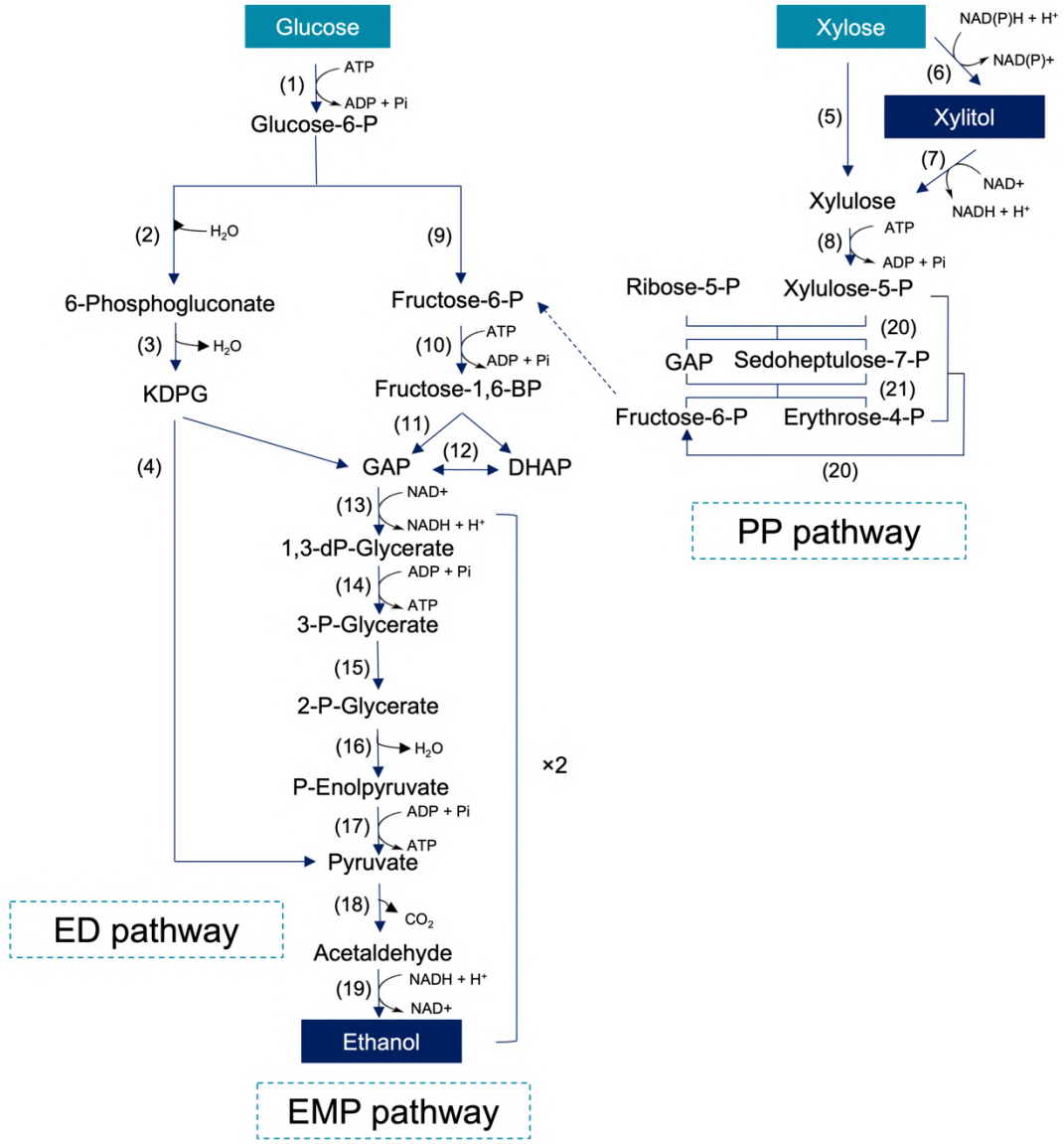

Fig. 1.10. Metabolic pathways for bioethanol production. (1) hexokinase, (2) glucose-6-phosphate dehydrogenase, (3) 6-phosphogluconate dehydratase, (4) KDPG aldolase, (5) xylose isomerase, (6) xylose reductase, (7) xylitol dehydrogenase, (8) xylulokinase, (9) glucose-6-P isomerase, (10) 6-phosphofructokinase, (11) fructose-bisphosphate aldolase, (12) triose-phosphate isomerase, (13) glyceraldehyde-3-P dehydrogenase, (14) phosphoglycerate kinase, (15) phosphoyglycerate mutase, (16) enolase, (17) pyruvate kinase, (18) pyruvate decarboxylase, (19) alcohol dehydrogenase, (20) transketolase, (21) transaldolase.

1.3.2.2. Downstream processing for ethanol recovery and purification

Ethanol purification requires a distillation and a subsequent dehydration step. The distillation step is carried out in two different columns. In the stripper columns, ethanol is concentrated and separated from solid and non-volatile compounds, being further concentrated to a value close to the azeotropic point in rectification columns (Galbe et al., 2011). For enrichment beyond the azeotropic mixture, other methods need to be employed to dehydrate ethanol, like azeotropic distillation, pervaporation and molecular sieve adsorption (Baeyens et al., 2015). Several methods are recommended to substitute the whole distillation process, such as membrane distillations, being more efficient, easier to operate, and with a lower energy requirement (Muhammad and Rosentrater, 2020).

Distillation step implies 40 % of total energy demand in bioethanol production process (Muhammad and Rosentrater, 2020). It is very important to maximise ethanol concentration in order to facilitate the subsequent purification step.

In comparison with sugar crops and starchy materials, higher substrate loading is needed when using lignocellulose to achieve high sugar concentrations due to the lower sugars content of the material. For this reason, high initial solids loading (15-20 %) (w w^{-1}) is required in most of lignocellulosic biomass to reach the concentration benchmark for a cost-effective distillation: 4 % (w w^{-1}) ethanol (32 g L^{-1}) (Zacchi and Axelsson, 1989). The high solids content, together with the low density and high hygroscopicity of lignocellulosic materials, results in a medium with high viscosity, which produces different limitations during bioconversion, as will be explained below (Koppram et al., 2014).

1.3.2.3. Challenges of lignocellulosic bioethanol production

Different limitations can affect lignocellulosic bioethanol production. Some of them are common in biochemical processes from lignocellulose, like the ones mentioned in LA production, but others are intrinsic to ethanol fermentation.

As mentioned, bioethanol production at high substrate concentrations is considered a cost-effective process that allows to reach the ethanol concentration benchmark while lowering energy and water input (Koppram et al., 2014). Nonetheless, the high substrate loading (> 20 % w w^{-1}) also implies high amount of sugars, inhibitors, solids and ethanol accumulation that can hinder both enzymes and microorganisms (**Fig. 1.11**):

Use of C5 sugars

The main disadvantage of native industrial ethanologenic microorganisms is the lack of the enzymes needed to ferment C5 sugars into ethanol. In the case of WT C5-utilising microorganisms, low bioethanol yields are obtained because of their low tolerance to high ethanol concentrations, inhibitors and pH changes (Toor et al., 2020). Furthermore, xylitol accumulation can take place due to a cofactor imbalance produced because xylose reductase prefers NADPH over NADH, while xylitol dehydrogenase exclusively uses NAD^+ (**Fig 1.10**) (Runquist et al., 2010).

High sugars content

The use of high substrate loading results in high concentrations of the hydrolysis end-products cellobiose and glucose, which can produce end-product inhibition on cellulases, being a limiting factor for lignocellulose hydrolysis (Kristensen et al., 2009). Furthermore, high initial glucose concentrations (120-180 g L^{-1}) may cause osmotic stress on microorganisms and thus lead to a decrease growth rate and ethanol production (Chang et al., 2018). Under these conditions it is known that the expression of enzymes from hexoses monophosphate and glycolytic pathways are downregulated (Tesfaw and Assefa, 2014). However, these high glucose concentrations are not commonly achieved from lignocellulosic materials.

Inhibitory degradation compounds

In the same way as in LA-producing bacteria, degradation compounds formed during pretreatment can hinder ethanologenic microorganisms growth and ethanol production. Furans and phenols exhibit high inhibitory effects at a concentration of 1 g L^{-1}, while up to 2 g L^{-1} of CBA can produce a significant impact (Palmqvist and Hahn-Hägerdal, 2000). Severe inhibitory effects on *S. cerevisiae*, *S. stipitis* and *C. sheatae* were shown at furans concentrations between 0.5 g L^{-1} and 4 g L^{-1}, while phenolic acids (ferulic and coumaric acid) and aldehydes (vanillin and syringaldehyde) inhibited *S. cerevisiae* metabolism at concentrations as low as 0.20 g L^{-1} (Jönsson and Martín, 2016; Larsson et al., 1999; Palmqvist and Hahn-Hägerdal, 2000).

Insoluble solids and viscosity

At high solids content, adequate mixing is often not achieved, hindering the convective heat transfer, the uniform distribution of chemical components and the interactions between enzymes and microorganisms with the substrate, which could ultimately decrease the biomass

conversion (Koppram et al., 2014). The presence of high concentrations of insoluble solids during SSF and CBP processes may also represent an important stress factor for ethanologenic microorganisms. On one hand, friction and collision mechanisms between these solids and cells can produce an alteration of cell morphology and even the disruption of cell membranes and cell death. On the other hand, under these circumstances there is an alteration of gene expression pattern, which could derive in ROS formation and the alteration of the sugars conversion into bioethanol (Moreno et al., 2019b).

Ethanol inhibition and oxidative damage

Ethanol accumulation in the culture broth can become a significant stress factor during fermentation. Ethanol is able to increase the fluidity and permeability of cell membrane at concentrations higher than 4 % (v v^{-1}). It can also produce the denaturation enzymes involved in glycolysis, respiration and fermentation, lowering ATP levels and decreasing specific growth rate (Stanley et al., 2010). It is also involved in the formation of ROS, which causes oxidative damage on DNA and other structures (Navarro-Tapia et al., 2018).

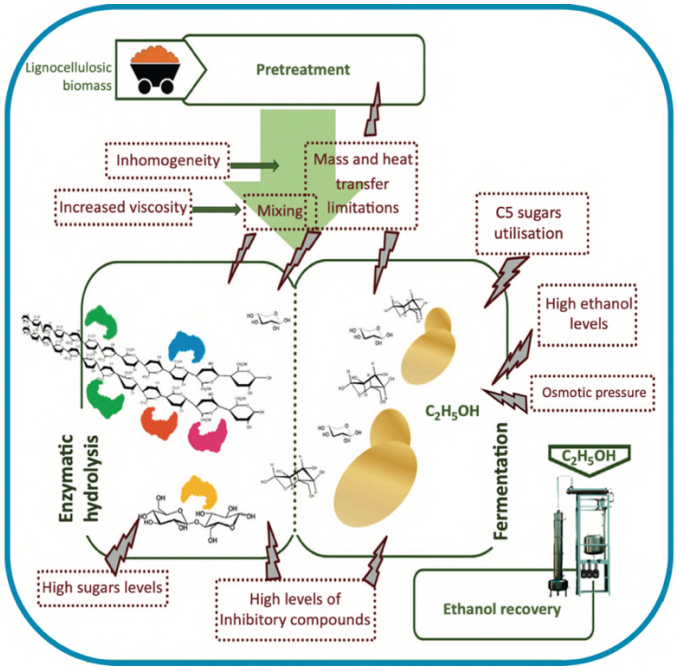

Fig. 1.11. Challenges on lignocellulosic bioethanol production. Modified from: Koppram et al. (2014).

The mentioned stressful conditions are particularly damaging for ethanologenic bacteria. Although ethanologenic yeasts are known to be more robust than bacteria, synergy between the mentioned inhibiting conditions could be expected, especially in processes with high substrate

loading, where the concentrations of insoluble solids, inhibitory degradation compounds, sugars and ethanol could be elevated (Koppram et al., 2014) (**Fig. 1.11**). For this reason, different strategies should be applied to overcome the mentioned limitations and maximise ethanol production from lignocellulosic materials.

1.3.3. Novel approaches to improve fermentation processes for lignocellulose valorisation

Considering the different challenges that need to be faced when using lignocellulosic materials, the effectiveness of LA and bioethanol production can be improved using different process- and microorganism-oriented approaches (**Table 1.5**).

Process configurations and culture conditions

Different process configurations can be applied to solve the challenges associated to high substrate loading, including the inhibitory effect produced by high concentrations of sugars, degradation compounds, solids and fermentation products (Koppram et al., 2014).

When coupling SSF process with fed-batch substrate addition, solids are gradually added to the culture, which increases LA or ethanol productivities (Klotz et al., 2016; Toor et al., 2020). Furthermore, if the slurry is used, inhibitors are kept at low concentration during fed-batch processes (Tomás-Pejó et al., 2010). In this sense, multi-feed SSF has shown to improve high-gravity ethanol production, reducing the negative effects of inhibitors on ethanologenic yeasts (Westman et al., 2017). As previously explained, continuous cultivation coupled with extractive mitigation could reduce the end-product inhibition since the product is continuously separated from the media as it is produced (Boontawan et al., 2011). Moreover, a high cell concentration of microbes is achieved in continuous cultivation coupled with cell immobilization/recycling, which gives higher production and short processing time (**Table 1.5**) (Toor et al., 2020).

Apart from the process configuration and the feeding strategies, some other culture conditions must be taken into account, including temperature, agitation, oxygen presence and culture pH. In both LA and ethanol fermentation, the oxygen presence should be adjusted to the metabolism and oxidative tolerance of the microorganism (**Table 1.5**) (Article II). The addition of neutraliser for pH control becomes imperative in LA fermentation since an acidic product is obtained (**Table 1.5**) (Article III).

Table 1.5. Challenges of lactic acid and ethanol fermentation from lignocellulosic materials and novel approaches applied to overcome them.

Challenges	Approaches	
	Lactic acid production	Bioethanol production
Lignocellulose structure and composition		
Necessity of hydrolysis step	Use of thermotolerant fermentative microorganisms suitable for SSF processes (1), (2) that favour higher hydrolysis yields	
	Isolating or engineering cellulolytic microorganism that can both hydrolyse and ferment sugars in CBP processes (3), (4)	
High viscosity	Use of fed-batch strategies (5), (6)	
Substrate inhibition and osmotic pressure	Use of fed-batch strategies	
	Isolating or engineering osmotic-tolerant fermentative microorganisms (7)	
High concentrations of inhibitory degradation compounds	Use of fed-batch strategies	
	Adding a biodetoxification step prior hydrolysis and fermentation (8), (9)	
	Isolating or engineering new fermentative microorganisms with increased inhibitors tolerance or with biodetoxification abilities (10), (11)	
C5 sugars utilisation and carbon catabolite repression effect	Use of hetero- or homo-lactic C5-utilising bacteria (12)	Heterologous expression of PP pathway genes in industrial ethanologenic microorganisms (13)
	Isolation or engineering fermentative microorganisms with low carbon catabolite repression effect (14), (15)	
	Co-cultivation of different C6- and C5-utilising microorganisms to produce a unique fermentation product (lactic acid or bioethanol) or different products in a biorefinery (16), (17)	
Fermentation conditions		
Oxidative stress	Controlling the culture conditions to minimise the ROS formation due to the presence of oxygen and other compounds like furans and ethanol (18)	
	Isolation or engineering ROS-resistant fermentative microorganisms (19)	
Inhibition at low pH by lactic acid accumulation	Use of effective neutralisers (20)	-
	Continuous culture coupled with extractive fermentation (21)	
	Isolation or engineering acid-resistant bacteria (22)	
Ethanol inhibition	-	Isolation or engineering ethanol-resistant microorganisms (23)
Product characteristics		
Optical purity of lactic acid	Isolating or engineering new bacteria encoding a LDH with high selectivity (24)	-
By-products formation	Isolating or engineering new homofermentative bacteria (25)	-

Ref: (1) Ou et al., 2011; (2) Choudhary et al., 2017; (3) Hu et al., 2011; (4) Vianna et al., 2019; (5) Hu et al., 2016; (6) Jiang et al., 2013; (7) Ge et al., 2011; (8) Xie et al., 2018; (9) Yu et al., 2011; (10) Gao et al., 2017; (11) Smith et al., 2014; (12) Glaser and Venus, 2018; (13) Vilela et al., 2015; (14) Reddy et al., 2015; (15) Shin et al., 2015; (16) Zhang and Vadlani, 2015; (17) Wang et al., 2018; (18) Qin et al., 2010; (19) Yuan et al., 2019; (20) Nakano et al., 2012; (21) Boontawan et al., 2011; (22) Singhvi et al., 2015; (23) Moreno et al., 2019a; (24) Yi et al., 2016; (25) Zhang et al., 2016.

Co-cultures and sequential cultures for complete sugars utilisation

In principle, the use of pure cultures is extended for the production of bioproducts, allowing a predictable fermentation, reducing the risks of contamination and ensuring a constant quality of the desired product. However, using co-cultures to valorise complex substrates is desirable in some cases.

Interestingly, C6 and C5 sugars co-utilisation can be achieved when adding different C6 and C5-utilising microorganisms for the production of a unique fermentation product (Cui et al., 2011; Sahoo and Jayaraman, 2019; Zhang and Vadlani, 2015) or different biofuels and bio-based products (Mandegari et al., 2017; Wang et al., 2018) (**Table 1.5**), as it may be the case for sequential bioethanol and LA co-generation (Article VI).

Isolation and/or selection of appropriated fermentative microorganisms

Isolation of new robust strains from stressful environments is a feasible approach to reach improved resistance to different inhibitory effects commonly found in lignocellulosic biomass.

In the case of LA-producing bacteria, Lactobacilli such as *L. brevis*, *L. plantarum, L. paracasei* and *Pediococcus*, and different *Bacillus* strains have been isolated from cabbage, soil, corn stover slurry, and food waste (Aulitto et al., 2017; Bischoff et al., 2010; Guo et al., 2010; Juodeikiene et al., 2016b; Kuo et al., 2015; Meng et al., 2012; Nguyen et al., 2012; Peng et al., 2013; Zhao et al., 2013; Zhou et al., 2013). In the same way, ethanologenic yeasts from different genera (*Saccharomyces*, *Candida*, *Pichia*, *Wickerhamomyces*, *Kluyveromyces, Debaryomyces, Cryptococcus*, etc.) have been isolated from different media like rotten fruits and barks of trees (Choudhary et al., 2017; Rao et al., 2008).

The selection of thermotolerant strains is very useful in SSF processes. These strains are able to ferment at temperatures close to the optimal for hydrolytic enzymes, with lower risk of contamination (**Table 1.5**). Indeed, *B. coagulans* strains can grow and produce LA at temperatures close to 50 ºC (Idler et al., 2015), while different *Saccharomyces*, *Candida*, *Pichia*, *Wickerhamomyces* and *Kluyveromyces* strains are able to grow and produce ethanol at 40 °C (Tomás-Pejó et al., 2009).

The inhibitory effect of degradation compounds, both in defined and lignocellulosic media, was deeply studied in different works by screening different LA-producing bacteria (Alexandri et al., 2019; Boguta et al., 2014; López-Gómez et al., 2019; van der Pol et al., 2016; Article IV) and ethanologenic microorganisms (Almeida et al., 2007; Gu et al., 2015; Jung and Kim, 2017;

Lujan-Rhenals et al., 2014). The isolation of robust fermenting microorganisms that can cope with the inhibitors or even remove their presence through biodetoxification processes is crucial for achieving a cost-competitive production process (Moreno et al., 2015).

Furthermore, microbial strains can also be selected according to their capacity to ferment pentoses with low CCR and without by-products formation (**Table 1.5**). *B. coagulans* strains are able to convert both C6 and C5 sugars into LA via homolactic fermentation, producing just small amounts of 2.3-butanediol, acetoin, acetic acid and ethanol (Idler et al., 2015).

Directed genetic modification of microorganisms

Heterologous expression is an interesting methodology for improving different microorganisms' genotypes by modifying fermentative routes and enzymes, switching metabolic pathways fluxes.

Regarding LA-producing bacteria, the production of each LA stereoisomer is a consequence of the LDH selectivity. For this reason, deletion of D-LDH or L-LDH would lead to an optically pure L-LA and D-LA, respectively (**Table 1.5**) (Aso et al., 2019; Yi et al., 2016). Furthermore, LA fermentation can be switched from hetero- to homolactic by substituting endogenous PK genes by heterologous genes from the PP pathway, avoiding by-products formation and increasing LA yields (Guo et al., 2014; Okano et al., 2009; Qiu et al., 2018).

In the same way, heterologous expression of different PP pathway enzymes (xylose reductase, xylulokinase and xylitol dehydrogenase) and xylose transporters can be carried out on industrial robust yeasts. The resulting recombinant strains are fitted for utilisation of both C6 and C5 sugars from lignocellulosic materials (Tomás-Pejó, 2011; Vilela et al., 2015) (**Table 1.5**). The deletion and insertion of specific genes involved in ethanol resistance and biosynthesis has also resulted in new ethanol tolerant yeast strains with great potential to be used for bioethanol production (**Table 1.5**) (Abreu-Cavalheiro and Monteiro, 2014; Martínez-Alcántar et al., 2019).

CBP-oriented strains can be obtained by introducing genes encoding for hydrolytic enzymes in fermenting microorganisms. Following this approach, heterologous expression of different cellulases, xylanases and α-amylases genes in LA- and ethanol-producing microorganisms resulted in recombinant strains with the ability to directly convert lignocellulosic materials into LA and ethanol, respectively (**Table 1.5**) (Amoah et al., 2017; Aso et al., 2019; Chang et al., 2013; Hu et al., 2011; Treebupachatsakul et al., 2016; Vianna-Bernardi et al., 2019).

Non-directed genetic modification of microorganisms. Adaptive Laboratory Evolution

In addition to isolation or directed genetic modification, other techniques can be applied for obtaining appropriate microorganisms for fermentation, including non-directed genetic modification approaches. The application of these methodologies can result in new microbial strains with improved features in comparison with the wild-type (WT) strains.

Cell growth and fermentation in stressful conditions are generally controlled by multiple loci. In this sense, non-directed techniques like random mutagenesis, genome shuffling and adaptive laboratory evolution (ALE) are more appropriated than rational genetic modification to alter different loci at the same time (Ye et al., 2013a).

ALE is a strain-improvement method based on the implementation of cultivation conditions to: (1) confer a selective advantage to mutants with an industrially relevant trait and (2) allow the identification of the molecular mechanisms involved (Mans et al., 2018). By means of gradually increasing the selective pressure, the selection of different mutations leads to the establishment of clones with desired phenotypes (Sauer, 2001). These methods are becoming very popular to improve stress tolerance and multiple sugar utilisation in fermentative processes (Chen and Dou, 2016). ALE is mostly applied following two different set-ups:

- **Serial batch cultivation**. Adaptation is performed by serially transferring aliquots of cells from one shake flask to a new shake flask with fresh medium under selective conditions (**Fig. 1.12**) (Dragosits and Mattanovich, 2013). Having in mind the simplicity of this method, numerous parallel cultures can be run simultaneously (Dragosits and Mattanovich, 2013).

  In this case, fluctuations in the population density, nutrient supply and pH conditions are produced as the population passes through the lag, exponential and stationary phases during the batch culture. The exposure of cells to non-optimal pH values and/or nutrients starvation can result in an unpredictable evolved phenotype because different unintended traits can be selected together with the desired one (Bachmann et al., 2017).

- **Chemostat cultivation**. A chemostat is a flow-controlled continuous culture which is maintained by the continuous addition of nutrients-rich fresh medium coupled with the outflow of fermented medium for prolonged periods of time (**Fig. 1.12**) (Kuenen and Johnson, 2009; Sauer, 2001).

In this ALE method strict environmental conditions can be settled, allowing the application of an independent selective pressure. Indeed, selection of particular constant growth rates, named auxostat, is very useful when the desired product is formed at the expenses of growth rate or under limitation of nutrients (Sauer, 2001). However, the set-up and operational conditions of a chemostat are laborious, since complex bioreactors are needed, with considerably higher costs than serial batch cultivation in shake flasks.

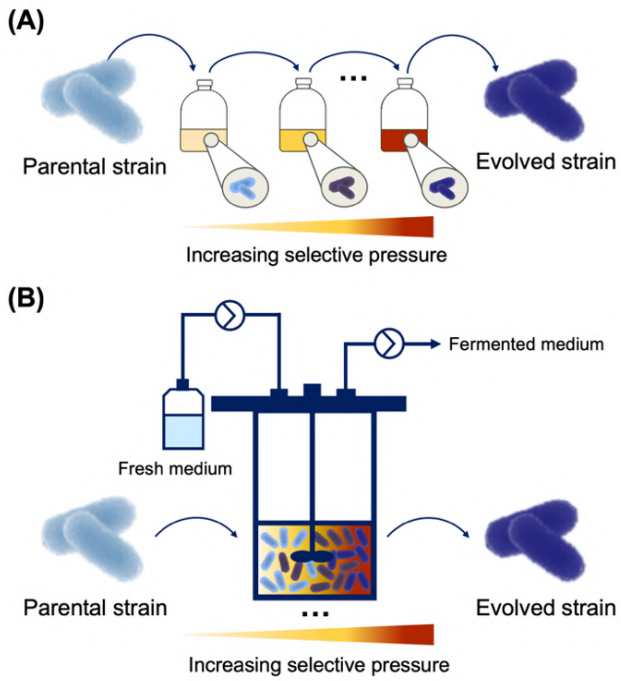

Fig. 1.12. ALE experiments by serial batch cultivation (A) and chemostat cultivation (B).

Acid resistant bacteria are very convenient in LA production since lower concentration of neutralising agent is required during fermentation. For these reason, different ALE experiments have been performed to increase the acid tolerance of LA-producing bacteria (**Table 1.5**) (Article V, Singhvi et al., 2015; Ye et al., 2013a; Zhang et al., 2012). By means of ALE, the inhibitors tolerance has also been increased both in LA-producing bacteria (Jiang et al., 2016) and ethanologenic microorganisms (Wallace-Salinas and Gorwa-Grauslund, 2013) (**Table 1.5**).

In order to favour the use of alternative carbon sources, like C5 sugars, prolonged cultivation in presence of these sugars can increase their consumption rates, even in stressful conditions. ALE has been applied to improve ethanol production from C5 sugars in presence of inhibitors (Moreno et al., 2019a; Smith et al., 2014; Tomás-Pejó et al., 2010), but few works have been performed with LA-producing bacteria (Qiu et al., 2018) (**Table 1.5**).

1.3.4. Co-generation of lactic acid and bioethanol for a complete valorisation of lignocellulosic sugars in a biorefinery

Biorefineries have received plenty of attention during the last years. Governments and companies are directing many efforts for the development of new technologies for utilisation of the different components of lignocellulosic biomass, with particular emphasis on lignocellulosic sugars.

As previously explained, two different fractions are obtained after steam explosion of lignocellulosic materials: a WIS fraction (containing C6 cellulose oligomers and lignin) and a liquid fraction or prehydrolysate (containing C6 and C5 hemicellulose oligomers) (Tomás-Pejó et al., 2011).

The use of the WIS fraction for bioethanol production has been broadly studied. Nonetheless, valorisation of the liquid fraction is still challenging due to the high abundance of C5 sugars, that cannot be fermented by most WT yeast, and the presence of inhibitory degradation compounds formed during pretreatment (Martín et al., 2018).

C5-utilising and inhibitor-tolerant yeasts strains have been obtained to address these issues, but scalability is not feasible yet (Robak and Balcerek, 2018). Therefore, C5 sugars contained in steam-exploded materials are usually wasted (Ji et al., 2012**)**. As explained, different native LA-producing bacteria are able to convert C5 sugars into LA (Idler et al., 2015), showing a promising opportunity for valorisation of these sugars.

The main objective of lignocellulosic biorefineries is the multi-production of a wide range of bioproducts, including biofuels and bio-based chemicals and polymers, from lignocellulosic materials (Chandel et al., 2018).

From this point of view, valorisation of the whole sugars content from lignocellulose can be achieved by applying different fermentative steps, taking advantage of the different sugar preference of microorganisms.

Having this in mind, different process configurations can be applied for LA and bioethanol co-generation:

- **Separated LA and bioethanol production.** This method takes advantage of the fractionating capacity of steam explosion to separately valorise the resulting fractions of lignocellulosic biomass. After filtration of the pretreated slurry, WIS and

prehydrolysates are individually saccharified for the subsequent production and purification of bioethanol and LA, respectively, in different streams (**Fig. 1.13 A**).

- **Integrated LA and bioethanol production.** The whole slurry is directly used in a serial process consisting of: saccharification and C6 fermentation to ethanol, C5 fermentation to LA and downstream processing of both products in just one stream (**Fig. 1.13 B**).

  This approach has different advantages: (1) the slurry is not filtered reducing the costs, (2) both fermentation stages could be performed in the same vessel and (3) downstream costs are reduced because ethanol and LA could be recovered in the same stream by a reactive distillation process.

Different works have recently simulated the feasibility of LA and bioethanol co-generation systems to valorise the different fractions obtained from lignocellulosic materials (Farzad et al., 2017; Mandegari et al., 2017). However, few experimental studies have addressed this topic. Montipó and co-workers have recently studied the separated LA and bioethanol production approach from grass, using the WIS and prehydrolysate fractions to produce bioethanol and LA, respectively (Montipó et al., 2018). Other studies have tested integrated approaches for bioethanol and LA co-generation from corn stalk and poplar wood (Wang et al., 2018; Zhang et al., 2014). Nothwithstanding, different specific strategies were carried out in these works that contrast with the integrated approach described above. Wang and co-workers performed a gas stripping step after bioethanol fermentation to remove this compound before LA fermentation of C5 sugars. Another system involved the addition of hemicellulosic hydrolysate to the medium after ethanol fermentation instead of using the whole slurry in the first place (Zhang et al., 2014).

Having in mind the high interest of these co-generation approaches, greater knowledge and new insights in this field are needed for the development of future biorefineries with complete valorisation of lignocellulosic sugars.

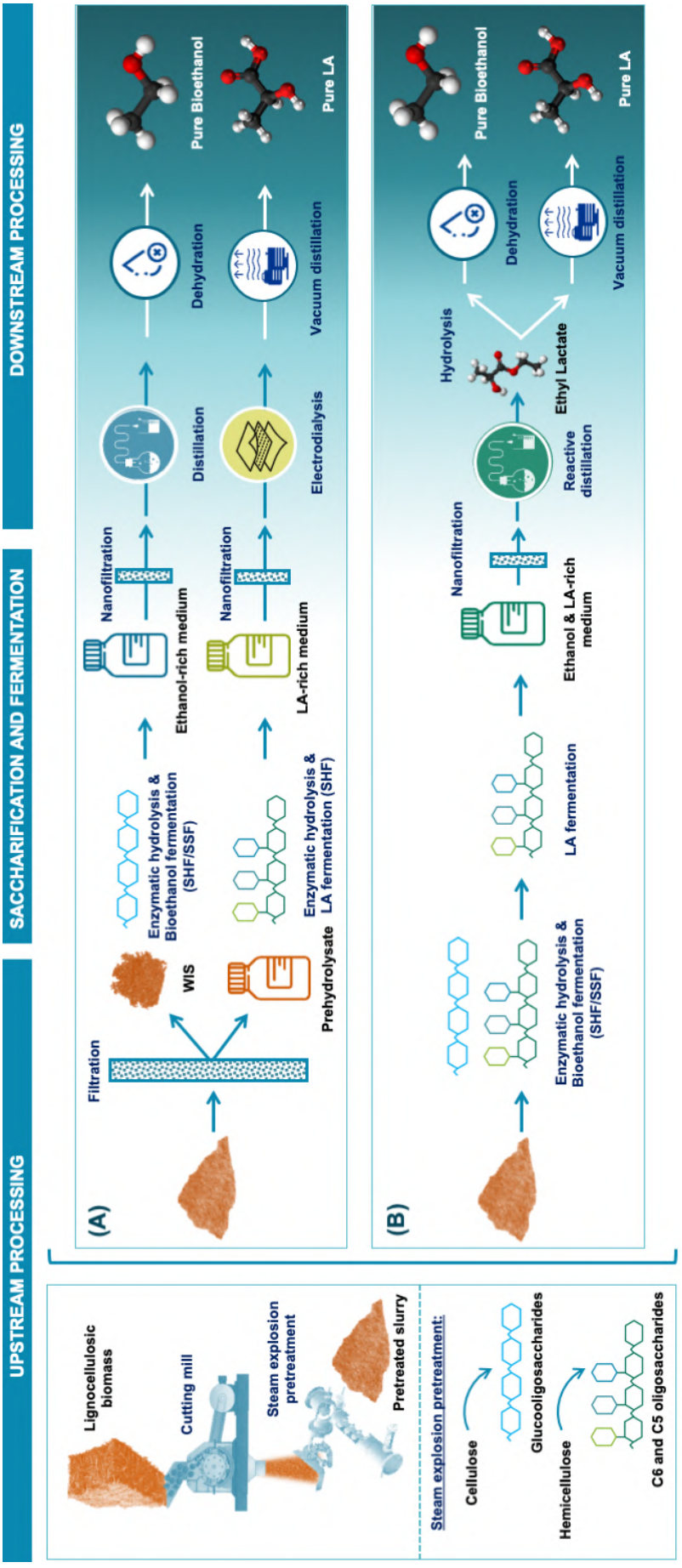

Fig. 1.13. Valorisation of steam exploded lignocellulosic materials into bioethanol and LA by different processes: separated bioethanol and LA production from WIS and liquid fraction (A) and integrated bioethanol and LA production from the whole slurry (B).

2. OBJECTIVES & HYPOTHESIS

2. OBJECTIVES AND HYPOTHESIS

The different studies described in this Thesis have been performed in the Biotechnological Processes Unit of IMDEA Energy. This Unit focuses its research lines in the microbial production of biofuels and bio-based products from organic residues, such as lignocellulose, food residues and municipal solid wastes. Part of the experiments with *B. coagulans*, as well as the application of purification techniques, were performed in the Bioengineering department from the Leibniz Institute of Agricultural Engineering and Bio-economy (ATB) in Potsdam (Germany).

The main focus of lignocellulosic biorefineries is the use of a broad spectrum of biomass for the generation of different products and energy. In this regard, two residues with high differences in terms of composition and origin were used in this Thesis. Specifically, wheat straw was chosen due to the worldwide abundance of this agricultural residue in rural areas, while gardening residues were used considering the expansion of green spaces in urban areas in the context of smart sustainable cities. These lignocellulosic materials were subjected to steam explosion due to its wide utilisation for pretreatment of both agricultural and herbaceous residues.

The different sugars released in the pretreated materials, including the whole slurry, the WIS and prehydrolysates fractions, were used for LA and bioethanol production following a separated and an integrated approach. In the first case, the C6 sugars from the WIS fraction were used for bioethanol production; while the C6 and C5 sugars mixtures from the prehydrolysates were directed to LA production. By contrast, the whole slurry was used in the integrated approach for sequential ethanol and LA co-generation.

All things considered, the main objective of this Thesis is the total valorisation of C6 and C5 sugars from lignocellulosic materials into bioethanol and LA, respectively.

One of the most relevant challenges of the described processes is the need of C5-utilising robust LA-producing bacteria able to resist stressful conditions like high inhibitors content, fermentation products (ethanol and LA) and oxidative and acid stress. The heterolactic LAB *L. pentosus* was chosen owing its widely reported C5 consumption with low CCR, through the PK pathway. *B. coagulans* was also selected considering its growing interest as homolactic producer from C5 through the PP pathway. The first produces the racemate L,D-LA, a building

block for the production of chemicals like ethyl lactate and 2,3-pentadione, while the Bacilli produce optically pure L-LA, with higher commercial value as monomer for PLA production.

In order to attain the main objective, different specific objectives were accomplished:

- To review the different characteristics of LA-producing bacteria and the limitations commonly found when using lignocellulosic materials (Article I).
- To evaluate different methods for hydrolysis optimisation of the prehydrolysates and to study the effect of inhibitors, oxygen presence and different pH control methods on LA fermentation (Articles II and III).
- To screen the capacity of different *B. coagulans* strains to tolerate and biodetoxify inhibitors mixtures, both in defined media and in prehydrolysates (Article IV).
- To perform an ALE of *L. pentosus* to improve xylose consumption rate at low pH (Article V).
- To maximise the conversion of glucose released from WIS and whole slurries into bioethanol by *S. cerevisiae* Ethanol Red using different process configurations (Article VI).
- To obtain an ethanol-tolerant *B. coagulans* strain by ALE to be used in a sequential yeast-bacteria culture (Article VI).
- To perform a successful yeast-bacteria cultivation from different media in an integrated ethanol and LA production process (Article VI).

3. MATERIALS & METHODS

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3.1. Raw materials

In this Thesis, different lignocellulosic materials were used as raw material for LA and bioethanol production, being selected according to their origin, availability and potential sugars content (**Table 3.1**). Wheat straw was obtained from CEDER-CIEMAT (Soria, Spain), while gardening residues were provided by "BECSA" (Castellón, Spain).

Lignocellulosic biomass was milled in a laboratory cutting mill (SM 2000, Retsch, Germany) to obtain milled chips with a particle size between 2 and 10 mm.

Table 3.1. Composition (% w w^{-1}) of the raw materials used in this Thesis.

Material	Cellulose	Hemicellulose				Lignin	Ashes	Extractives	Proteins	Acetyl groups	Articles
		Xylan	Arabinan	Galactan	Mannan						
Wheat straw	40.5	22.7	2.1	1.3	-	18.1	5.1	14.6	-	-	II, V
Gardening residues	24.8	10.1	2.6	2.0	1.0	21.6	7.2	18.2	7.2	2.1	III, IV, VI

3.2. Steam explosion pretreatment

The milled lignocellulosic biomass was subjected to different steam explosion conditions (**Table 3.2**).

Table 3.2. Steam explosion conditions used with different lignocellulosic materials.

Pretreatment condition	Wheat straw		Gardening Residues		
Aqueous extraction	No	No	No	No	Yes
Temperature	200 °C	180 °C	180 °C	180 °C	180 °C
Time	7 min	4 min	20 min	5 min	10 min
Catalyst	No addition	H_2SO_4 0.1M	$FeCl_3$ 0.1 M	H_2SO_4 0.25 M	H_2SO_4 0.33 M
Articles	II	V	III	III	III, IV, VI

After steam explosion, the resulting slurries contained around 20 % (w w^{-1}) of total solids and they were filtered to separate the WIS fraction and the liquid fraction or prehydrolysates (**Fig. 3.1**). As will be seen in the results section on this Thesis, both wheat straw prehydrolysates (WSP) and gardening prehydrolysates (GP) were rich in different C6 and C5 oligomers and in

inhibitory degradation compounds formed during pretreatment. Their corresponding WIS fractions contained mainly cellulose (**Fig. 3.1**). WSP and GP were used for LA production (Section 4.1, Articles II-V), while the gardening WIS fraction was used as substrate for bioethanol production and sequential LA production (Section 4.2, Article VI). Wheat straw WIS fraction was not used for bioethanol production in this Thesis. Nonetheless, the wheat straw whole slurry was used for integrated bioethanol and LA production, without filtration (Section 4.2 and 4.3).

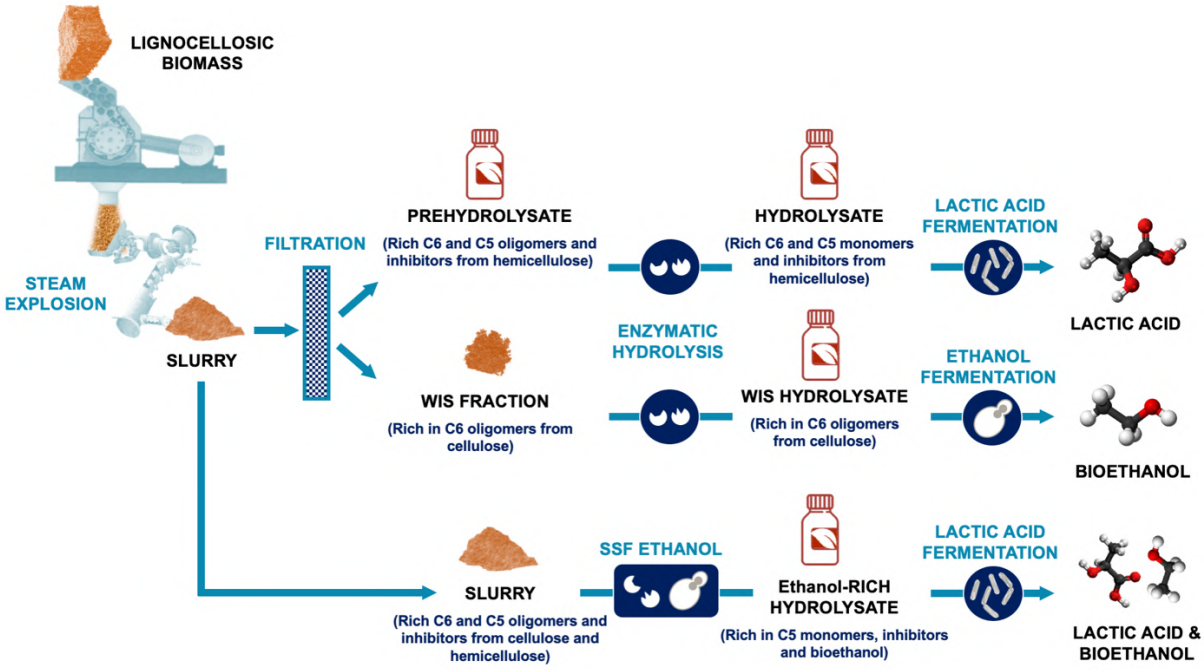


Fig. 3.1. Utilisation of the different fractions obtained after steam explosion pretreatment of lignocellulosic materials.

3.3. Microorganisms and preculture conditions

3.3.1. Lactic acid-producing bacteria

The heterolactic *L. pentosus* CECT 4023T was purchased from the CECT (Valencia, Spain) and it was used in Articles II, III and V. For preculture, an aliquot of 20 µL of cells was taken from glycerol stocks and inoculated in 50-mL Falcon tubes with 15 mL of Man, Rogosa and Sharpe medium (MRS), containing (g L^{-1}): glucose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.2, $MnSO_4 \cdot H_2O$, 0.05 and $CaCO_3$, 10 g L^{-1} as buffer. The preculture was grown in a rotatory shaker at 32 °C and 150 rpm for 16 h (until the culture reached the late exponential growth phase).

Different homolactic *B. coagulans* strains were used for LA production. *B. coagulans* DSM 2314 was obtained from the DSMZ (Braunschweig, Germany), while A166 and A162 strains were isolated in ATB (Potsdam, Germany). All strains were L-LA producers (more than 99 % of L-LA purity). MRS medium was used for preculture, with glucose 20 g L^{-1} (Article IV) or xylose 20 g L^{-1} (Article VI) as carbon source, for fermentation of mixed sugars and xylose media, respectively. Cells were inoculated from slants in 60 mL of MRS (Article IV) or glycerol stocks (200 µL in 25 mL of MRS) (Article VI). Precultures were incubated in rotatory shakers at 40 °C-52 °C and 100 rpm for 16 h.

3.3.2. Ethanologenic yeasts

The industrial strain *S. cerevisiae* Ethanol Red® was provided by Fermentis S.L., Lessafre (France). The yeast was maintained in YPD medium in agar plates, containing (g L^{-1}): glucose, 20; yeast extract, 10; peptone, 20; agar, 20. For preculture, one colony was inoculated in 250-mL flasks with 100 mL of YPD medium and incubated at 32 ºC and 150 rpm for 16 h. Precultures were centrifuged at 4000 rpm for 4 min at 4 ºC, supernatant was discarded and cells were diluted to the corresponding inoculum size.

3.4. Separated hydrolysis and lactic acid fermentation of prehydrolysates

3.4.1. Hydrolysis of hemicellulosic prehydrolysates

After pretreatment, hemicellulosic prehydrolysates were mainly rich in oligosaccharides. A hydrolysis step was applied to wheat straw and gardening prehydrolysates (namely WSP1, WSP2, GP1 and GP2) to increase their monomeric sugars content, producing wheat straw hydrolysates (WSH1 and WSH2, Articles II and V) and gardening hydrolysates (GH1, GH2, Article III). GH3 and modified GH3 (M-GH3) were directly derived from GP3 without the need of hydrolysis since most of their sugars were already in monomeric form after pretreatment (Articles III and IV).

The enzymatic hydrolysis of WSPs and GPs were run at different volumes. 50-mL Falcon centrifuge tubes with 10 mL of medium were used in preliminary studies for enzymatic hydrolysis optimisation. On the other hand, 25 mL and 200 mL of media were used for

enzymatic hydrolysis to produce high volumes of hydrolysates for further fermentation step. The enzymatic hydrolysis experiments were run in triplicates at 40 °C, 150 rpm and pH 5.5 for 24 h-72h. In the case of WSP1, acid hydrolysis was also carried out. Acid hydrolysis was performed by adding 4 % (w w^{-1}) H_2SO_4 to the WSP1 and subjecting the mixture to 120 °C for 30 min.

Different cellulolytic and hemicellulolytic commercial enzymes were added as catalysts. Specifically, the cellulases Celluclast® 1.5 L (60 FPU mL^{-1}) (Cel) and the *β*-D-glucosidases Novozym® 188 (600 UI mL^{-1}) (*β*-Glu) (Novozymes, Denmark) were used for depolymerisation of glucose oligomers. The enzymatic cocktail of xylanases Cellic-HTec2® (300 U mg^{-1}, Novozymes, Denmark) and the *β*-D-xylosidase EC 3.2.1.37 (118 U mg^{-1}, Megazyme®, USA) (*β*-Xyl) were used to release the monomeric sugars from xylooligosaccharides. Different dosages of Cel, *β*-Glu, Cellic-HTec2 and *β*-Xyl were screened to obtain the highest glucose, galactose, mannose, xylose and arabinose hydrolysis yields. Cel and *β*-Glu were added as Filter Paper Units (FPU) and International Units (IU), respectively, per g of glucooligosaccharides. Cellic-HTec2 and *β*-Xyl were added as mg g^{-1} of xylooligosaccharides.

Addition of cellulases and hemicellulases cocktails was carried out following two strategies: i) hemicellulases were added together with the cellulases and ii) hemicellulases were added 24 h prior cellulases addition. Furthermore, different enzymes dosages were tested, as can be seen in **Table 3.3**.

Table 3.3. Enzymes dosages used for enzymatic hydrolysis of hemicellulosic prehydrolysates: Wheat straw prehydrolysate 1 (WSP1), wheat straw prehydrolysate 2 (WSP2), gardening prehydrolysate 1 (GP1) and gardening prehydrolysate 2 (GP2).

Material	Test	Cel (FPU g^{-1})[1]	*β*-Glu (IU g^{-1})[2]	Cellic-HTec2 (mg g^{-1})	*β*-Xyl (mg g^{-1})[3]	Article
WSP1	-	10	10	50	0.1	II
WSP2	-	0	0	30	0.1	V
GP1	A	16	16	0	0	III
	B	16	16	15	0.1	
	C	16[4]	16[4]	15	0.1	
GP2	D	8	8	15	0.1	
	E	12	12	15	0.1	
	F	16	16	15	0.1	
	G	16	16	15	0.05	
	H	16	16	15	0.5	

[1] Cel (Celluclast® 1.5L), [2] *β*-Glu (Novozym® 188) and [3] *β*-Xyl (*β*-D-xylosidase EC 3.2.1.37).
[4] Cel and *β-Glu* were added 24 h after the addition of Cellic-HTec2 and *β*-Xyl.

3.4.2. Lactic acid fermentation of defined media and hemicellulosic hydrolysates

Before fermentation tests, the pH of the WSHs and the GHs was adjusted to 6-7 and the MRS compounds, except glucose, were added. Afterwards, the hydrolysates were filtrated using membrane filters (Thermo Scientific® Nylon 0.2 µm) and stored at 4 °C until use.

Lactic acid fermentation tests in shake flask:

Fermentation tests with *L. pentosus* (Articles II, III and V) were performed under aerobic, anaerobic and strictly anaerobic conditions. To favour oxygen transfer (aerobic conditions), cultivation was performed in 150-mL shake flasks covered with cellulose caps. In order to reduce the oxygen transfer, shake flasks were covered with rubber caps (anaerobic conditions). Finally, to get strict anaerobic conditions, 120 mL-clamped flasks were flushed with filtered nitrogen or helium using a needle (gas inlet) at 0.5 bar for 2 min. The contained air -including oxygen- was ejected through another needle (gas outlet). In the latter case, the effect of adding $CaCO_3$ (half of the sugars concentration) to alleviate the pH drop during fermentation was also tested.

L. pentosus was inoculated from the preculture at an initial OD_{600} of 0.1 in 25 mL of medium and incubated at 32 °C, pH 7 and 150 rpm. The following tests were performed:

- Fermentation of MRS with glucose 20 g L^{-1} and different concentrations of WSP1 (25 %, 50 % and 75 % v v^{-1}) under non-controlled pH and aerobic, anaerobic or strictly anaerobic conditions in duplicate (Article II).
- Fermentation of MRS with sugars mixtures (5 g L^{-1} glucose and 15 g L^{-1} xylose) and xylose 20 g L^{-1} at different concentrations of WSP1 (25 %, 50 % and 75 % v v^{-1}) under strictly anaerobic and non-controlled pH conditions in duplicate (Article II).
- Fermentation of acid and enzymatically hydrolysed WSH1 under strictly anaerobic and non-controlled pH conditions in triplicate (Article II).
- Fermentation of GH2 and GH3 under strictly anaerobic conditions in triplicate. In the case of GH3, fermentation was carried out comparing with and without $CaCO_3$ addition (Article III).

- Fermentation of MRS with xylose:glucose mixtures (g L^{-1}): 15:5, 17.5:2.5, 19:1 and 20:0, at different initial pH values (7, 6, 5 and 4) under strictly anaerobic and non-controlled pH conditions in triplicate. WT and evolved strains were inoculated (Article V).

Lactic acid fermentation tests in bioreactor:

Fermentation tests in bioreactor were performed in batch, maintaining the culture pH at pH 6-7 by automatic addition of NaOH 5 M. Depending on the bacteria used the fermentation conditions varied as follow:

Fermentation tests with *L. pentosus* were performed under strictly anaerobic conditions by sparging with 0.5 L min^{-1} of nitrogen gas until the pO2 sensor (HAMILTON; USA) detected less than 5 % (v v^{-1}) oxygen. The bacteria were incubated at 32 °C and 150 rpm. Different configurations were used: a 1.5-L bioreactor (Minifors2; Infors-HT; Switzerland) with a working volume of 0.5 L (R-0.5L) and a 42-L bioreactor (BIOSTAT Cplus 30-3 MO; Sartorius; Germany) with a working volume of 10 L (R-10L).

Different synthetic and lignocellulosic media were fermented:

- Fermentation of GH3 in bioreactor (R-0.5L and R-10L) under strictly anaerobic and controlled pH conditions (NaOH automatic addition) (Article III).
- Fermentation of MRS with xylose 20 g L^{-1} in bioreactor (R-0.5L) in strict anaerobiosis under non-controlled and controlled pH conditions (NaOH automatic addition) in duplicate, comparing WT and evolved strains (Article V).
- Fermentation of WSH2 in bioreactor (R-0.5L) under strictly anaerobic and controlled pH conditions (NaOH automatic addition) in duplicate, comparing WT and evolved strains (Article V).

Fermentation tests with *B. coagulans* were performed at 52 °C and 400 rpm without controlling the oxygen presence in duplicate. A 0.5-L Eloferm multifermentation system (Biotronix GmbH, Germany) with 0.25 L of working volume (R-0.25L) was used for fermentation of M-GH3 and a basal medium (BM) containing (g L^{-1}): glucose, 5.4; xylose, 14.1, arabinose, 3.7 and yeast extract, 5. Different compounds were added to BM to obtain a series of basal media with inhibitors (BMI) (**Table 3.4**) (Article IV).

Table 3.4. Composition of the basal media with inhibitors (BMI) used for fermentation of different *B. coagulans* strains

Concentration (g L^{-1})	BMI	BMI A 10	BMI A 20	BMI F 2.5	BMI F 5	BMI P 2.5	BMI P 5
Acetic acid	1.0	**5.0**	**10.0**	1.0	1.0	1.0	1.0
Formic acid	1.0	**5.0**	**10.0**	1.0	1.0	1.0	1.0
Furfural	0.25	0.25	0.25	**1.25**	**2.5**	0.25	0.25
5-HMF	0.25	0.25	0.25	**1.25**	**2.5**	0.25	0.25
Vanillin	0.25	0.25	0.25	0.25	0.25	**1.25**	**2.5**
Syringaldehyde	0.25	0.25	0.25	0.25	0.25	**1.25**	**2.5**

Sampling times of the different LA fermentation tests varied depending on each experiment, including 0, 2, 4, 6, 8, 10, 24, 32, 48, 72, 96, 120 and 144 h. The sample volume ranged from 1 to 5 mL, depending on the total culture volume and required analysis and measurements needed, *i.e.*, bacterial growth, culture pH, residual sugars, fermentation products and other compounds.

3.5. Adaptive laboratory evolution experiments

3.5.1. ALE in serial batch cultivation

Serial batch cultivation of *L. pentosus* was carried out in 50-mL clamped flasks with 10 mL of MRS medium. Cells were incubated at 150 rpm, 32 °C and pH 7 with an initial OD_{600} of 0.01 under strictly anaerobic conditions. Different xylose:glucose mixtures (g L^{-1}: 15:5, 17.5:2.5, 19:1 and 20:0) were used as carbon source. To improve LA production from xylose, the xylose:glucose ratio increased gradually as evolution proceeded, when an improvement in the bacterial growth and xylose consumption was detected. Each round of ALE started by inoculating an aliquot of cells from the previous culture and lasted 24 h. Apart from improved xylose consumption rate, a concomitant improved tolerance to low pH was expected since no pH control was applied during ALE (Article V). ALE was run for 100 rounds and different populations (rounds 25, 50, 75 and 100) were chosen for fermentation of xylose:glucose mixtures (g L^{-1}: 15:5, 17.5:2.5, 19:1 and 20:0) to check the improvements achieved along ALE.

After ALE, clone isolation was performed at acid pH in order to ensure the isolation of those clones with improved xylose fermentation under acid stress. Clones were isolated from the final population of cells in MRS plates at different conditions: (1) 20 g L^{-1} xylose at pH 5 and (2) 15

g L^{-1} xylose with 5 g L^{-1} glucose at pH 5. Selected clones were subsequently tested in MRS media with 20 g L^{-1} of xylose al pH 7 and 32 ºC to analyse their growth, xylose consumption and LA production. The clone that showed the highest LA yields was selected for further experiments mentioned in Section 3.4.2.

3.5.2. ALE in chemostat cultivation

An ALE in chemostat was carried out with *B. coagulans* DSM 2314 to increase its ethanol tolerance. Chemostat cultivation was performed in bioreactor with 0.5 L of MRS medium (R-0.5L) and 40 g L^{-1} of xylose at 52 °C and 150 rpm. Input and output flow were set at 21 mL h^{-1} to maximise xylose consumption without washing the cells out of the reactor. The ethanol concentration in the MRS medium of the input flow was gradually increased as evolution proceeded, being: 0, 2, 3, 4, 5 and 6 % (v v^{-1}).

Samples were taken daily and, when improvements in bacterial growth, xylose consumption and L-LA production were detected, ethanol concentration in the evolution medium was increased. Strict anaerobic conditions were obtained by sparging the medium with 0.5 L min^{-1} of nitrogen gas until the pO_2 sensor (HAMILTON; USA) detected less than 5 % (v v^{-1}) oxygen. pH was automatically controlled at 7 with NaOH 5 M. ALE started by inoculating an aliquot of cells (5 % (v v^{-1})) from the preculture. Due to difficutiles on preparation of selection agar plates with ethanol as selective pressure, clone isolation was not viable. The evolved population was used in sequential yeast-bacteria cultures for integrated bioethanol-LA production from gardening (Article VI) and wheat straw residues, as will be explained in Section 3.8.

3.6. Separated hydrolysis and bioethanol fermentation of WIS

3.6.1. Enzymatic hydrolysis of WIS

Enzymatic hydrolysis of gardening WIS fraction was performed with 25 % (w v^{-1}) of total solids in 250-mL shake flasks at 50 °C and pH 5 for 72 h. The following enzymes dosages were added: Cellic-CTec2®, 32 FPU g^{-1}; Cellic-HTec2®, 0.9 mg g^{-1} and Viscozyme L® (Merck, Germany), 4.5 mg g^{-1} of glucan. After enzymatic hydrolysis, a WIS gardening hydrolysate was obtained (WIS-GH).

3.6.2. Bioethanol fermentation of WIS hydrolysate

The WIS-GH was supplemented with (g L^{-1}): yeast extract, 5; NH_4Cl, 2; KH_2PO_4, 1 and $MgSO_4 \cdot 7H_2O$, 0.3; and the pH was adjusted to 5.5. Ethanol fermentation was performed in 250-mL flasks with a working volume of 100 mL in triplicate. Fermentation with *S. cerevisiae* Ethanol Red was carried out at 32 °C and 150 rpm for 24 h and flasks were covered with rubber caps to favour anaerobic conditions. An inoculum size of 1 g L^{-1} cells (dry weight) was used. Samples were withdrawn at 1, 2, 3, 4, 5, 6, 7, 8 and 14 h of fermentation. As a result, and ethanol-rich gardening hydrolysate (E-GH) was obtained.

3.7. Simultaneous saccharification and bioethanol fermentation of slurry

The wheat straw slurry was used in batch and fed-batch SSF processes for bioethanol production under non-sterile conditions. SSF experiments were run in triplicate at 37 °C and 150 rpm in 250-mL flasks covered with rubber caps to favour anaerobic conditions. A working volume of 50 mL was used. An inoculum size of 2 g L^{-1} of *S. cerevisiae* Ethanol Red cells (dry weight) was used.

Enzyme loading was 15 FPU g^{-1} glucan of Celluclast® 1.5L (Novozymes, Denmark), 15 UI g^{-1} glucan of Novozym® 188 (Novozymes, Denmark), 0.5 % xylan of Cellic-HTec2® (Novozymes, Denmark) and 0.1 % xylan of β-xylosidase EC 3.2.1.37 (Megazyme®, USA).

Batch SSF was performed with a 10 % (w v^{-1}) of total solids. Thus, 25 g of slurry (containing 5 g dry weight) were suspended in 25 mL of SSF medium, containing (g L^{-1}): yeast extract, 5; NH_4Cl, 2; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.3.

Fed-batch SSF were carried out with 15 % of total solids (w v^{-1}). In this case, 18.75 g of slurry (containing 3.75 g dry weight) were added to 12.5 mL of SSF medium. Another 18.75 g of slurry were subsequently fed into the medium at 8 h (t_8) or 24 h (t_{24}) of SSF. Inoculation was carried out at the initial time (t_0) with 2 g L^{-1} (dry weight) of inoculum. The pH was adjusted to 5.5 with NaOH 5 M. Enzyme dosage was divided in two identical addition points, the first at t_0 and the second at the corresponding feeding time (t_8 or t_{24}).

In all cases, samples were taken at 0, 2, 4, 6, 8, 24, 48 and 72 h. As a result, and ethanol-rich wheat straw hydrolysate (E-WSH) was obtained.

3.8. Integrated bioethanol and lactic acid production

B. coagulans DSM 2314 WT and evolved strains were grown on C5 sugars in different defined ethanol-rich media and bioethanol-rich hydrolysates obtained after SHF of gardening WIS and SSF of wheat straw slurry with *S. cerevisiae*. The main purpose of these experiments was to check the feasibility of these strains for an integrated ethanol and LA production by sequential yeast-bacteria cultivation.

Shake flask fermentation tests were carried out in nitrogen-flushed 120 mL-clamped flasks. An aliquot of cells from the preculture was inoculated in 25 mL of medium in batch and incubated at 52 °C, pH 7 and 150 rpm. Different experiments were performed:

- Fermentation of MRS with xylose 20 g L^{-1} and 0 %, 4 % and 6 % (v v^{-1}) ethanol. LA fermentation with WT and evolved strains was performed under strictly anaerobic and controlled pH ($CaCO_3$) conditions in triplicate. An aliquot of cells (5 % (v v^{-1})) from the preculture was inoculated. LA fermentation was carried out comparing WT and evolved strains (Article VI).
- Fermentation of E-GH. This medium was rich in bioethanol previously produced from a WIS-GH by *S. cerevisiae*. Since this material derived from the hydrolysis of the WIS fraction and did not contain C5 sugars, 25 g L^{-1} of xylose were added, together with the MRS compounds. An aliquot of cells (5 % (v v^{-1})) from the preculture was inoculated. LA fermentation with WT and evolved strains was performed under strictly anaerobic and controlled pH ($CaCO_3$) conditions in triplicate (Article VI).
- Fermentation of E-WSH. This medium was rich in bioethanol and C5 sugars previously obtained by SSF of wheat straw slurry. Different diluted media were obtained to check the effect of different inhibitors and bioethanol concentrations on LA production, *i.e*, 100 %, 75 % and 50 % (v v^{-1}). The MRS compounds, except glucose, were added and the pH was adjusted at 7. An aliquot of cells (10 % (v v^{-1})) from the preculture was inoculated: 5 % (v v^{-1}) at t_0 and 5 % (v v^{-1}) at t_{24}. LA fermentation was performed with the evolved strain under strictly anaerobic and controlled pH ($CaCO_3$) conditions in triplicate.

Sampling times varied depending on fermentation experiment, including 0, 2, 4, 6, 8, 10, 24, 32, 48, 72, 96, 120 and 144 h of culture. Samples (1 mL) were taken to measure residual sugars, fermentation products, ethanol and other compounds.

3.9. Summary of fermentation and SSF experiments

Table 3.5 summarises the different fermentation and SSF tests performed for the production of LA and bioethanol mentioned in Sections 3.5-3.8:

Table 3.5 Summary of the fermentation experiments performed in the framework of this Thesis (I).

Lactic acid production from hemicellulosic liquid fractions									
Medium	**Mode**	**Sugars (g L^{-1})**	**Inhibitors**	**pH control**	**O_2 conditions**	**Microorganism**	**Objectives**	**Section**	**Article**
MRS	Flask Batch	Glc[1] 20		No	Aerobic, anaerobic and S. anaerobic[2]	*L. pentosus* CECT 4023T	Effect of inhibitors and oxygen presence on LA production from different sugars	4.1.2.1	II
		Glc 5 and Xyl[1] 15	WSP 25, 50 and 75 % (v v^{-1})		Anaerobic				
		Xyl 20			S. anaerobic				
WSH1	Flask Batch	From WSH1		No	S. anaerobic	*L. pentosus* CECT 4023T	Effect of inhibitors and pH control on LA production with different LA-producing bacteria	4.1.2.2	V
WSH2 50 % (v v^{-1})	R-0.5L Batch	From WSH2 50 % (v v^{-1})		NaOH	S. anaerobic				-
GH2	Flask Batch	From GH2		No	S. anaerobic				
GH3	Flask Batch	From GH3		No	S. anaerobic				III
				$CaCO_3$					
	R-0.5L Batch			NaOH					
	R-10L Batch								
M-GH3	R-0.25L Batch	From M-GH3		NaOH	Not controlled	*B. coagulans* DSM 2314, A166 and A162		4.1.2.3	IV
BMI	R-0.25L Batch	Same as M-GH3	Inhibitors mixtures						
MRS	Flask Serial Batch	Glc and Xyl mixtures	No	No	S. anaerobic	*L. pentosus* CECT 4023T	ALE to improve xylose consumption at low pH	4.1.3.2	V
		Glc and Xyl mixtures	No	No	S. anaerobic	*L. pentosus* CECT 4023T	Check improvements in rounds 25, 50, 75 and 100 of ALE		
MRS	Flask Batch	Glc and Xyl 15:5	No	No	S. anaerobic	*L. pentosus* CECT 4023T and evolved strains	Comparison of WT and evolved strains performance		
		Glc and Xyl 17.5:2.5							
		Glc and Xyl 19:1							
		Xyl 20							
MRS	R-0.5L Batch	Xyl 20	No	No	S. anaerobic				
				NaOH	S. anaerobic				
WSH2 50 % (v v^{-1})	R-0.5L Batch	From WSH2 50 % (v v^{-1})		NaOH	S. anaerobic				

Table 3.5 Summary of the fermentation experiments performed in the framework of this Thesis (II).

Bioethanol production from cellulosic fractions									
Medium	**Mode**	**Sugars (g L^{-1})**	**Inhibitors**	**pH control**	**O_2 conditions**	**Microorganism**	**Objectives**	**Section**	**Article**
WIS-GH	Flask Batch	From WIS-GH	No	No	Anaerobic	*S. cerevisiae* Ethanol Red	Feasibility of ethanol production from WIS fraction	**4.2.1**	VI
Wheat straw slurry	Flask SSF 10 % (w v^{-1}) batch Flask SSF 15 % (w v^{-1}) fed-batch t_{24} Flask SSF 15 % (w v^{-1}) fed-batch t_8	From wheat straw slurry	From wheat straw slurry	No	Anaerobic	*S. cerevisiae* Ethanol Red	Feasibility of ethanol production from whole slurry	**4.2.2**	-
Integrated lactic acid and bioethanol production									
Medium	**Mode**	**Sugars (g L^{-1})**	**Inhibitors**	**pH control**	**O_2 conditions**	**Microorganism**	**Objectives**	**Section**	**Article**
MRS-ethanol 0- 6 % (v v^{-1})	R-0.5L Continuous	Xyl 40	No	NaOH	S. anaerobic	*B. coagulans* DSM 2314 WT and evolved bacteria	ALE to improve xylose fermentation in ethanol stress conditions	**4.3.1**	VI
MRS-ethanol 0, 4 and 6 % (v v^{-1})	Flask Batch	Xyl 20	No	$CaCO_3$	S. anaerobic	*B. coagulans* DSM 2314 WT and evolved bacteria	Comparison of WT and evolved strains performance. Feasibility of sequential ethanol-LA co-generation	**4.3.1**	VI
E-GH	Flask Batch							**4.3.2.1**	VI
E-WSH 100 %, 75 % and 50 % (v v^{-1})	Flask Batch	From E-WSH	From E-WSH	$CaCO_3$	S. anaerobic	*B. coagulans* DSM 2314 evolved bacteria	Effect of inhibitors and bioethanol on LA production. Feasibility of sequential ethanol-LA co-generation	**4.3.2.2**	-

[1] Glc (Glucose), Xyl (Xylose).
[2] S. anaerobic (Strict Anaerobic).

3.10. Downstream processing for lactic acid purification

A multi-step downstream processing method was carried out on lignocellulosic LA-rich fermented media, as described in Neu et al. (2016).

The fermentation LA-rich broth was microfiltrated at 1.5 bar and 15 °C using an UFI-TEC cross-flow microfiltration system (UFI-TEC, Germany) equipped with 4 TAMI membranes (TAMI Industries, France) with a pore size of 0.2 μm for 1.78 h. Permeate obtained by microfiltration was nanofiltrated at 30 bar using an UFI-TEC cross-flow nanofiltration system (UFI-TEC, Germany) equipped with OS-UO-201-CO membranes (Osmota Membrantechnik GmbH, Germany) with a cut-off of 150–300 Da for 20 min.

Softening was performed to remove cations (calcium and magnesium) from permeate obtained by nanofiltration. Separation of cations was carried out using PUROLITE S950 acid chelating resin (Purolite, Germany). The pH-value of the permeate was increased to 10 by adding NaOH 5 M. Permeate was supplied to the column from below in an expanded bed setting and it was subjected to the softening step for 1.33 h.

Bipolar electrodialysis was carried out at 30 V and 5 A with a sheet flow stack consisting of 11 cation exchange membranes Type II (Fujifilm, the Netherlands) and 10 anion exchange membranes Type II (Fujifilm, The Netherlands) at 20 V and 3 A. By applying of an electric potential, anions in the salt cycle move through the membrane into the acid cycle, while cations move into the base cycle. As a result, the lactate solution was splitted in into free LA and base (caustic). Bipolar electrodialysis was stopped when the conductivity of the retentate was below 25 mS cm^{-1}. The acid stream was used in the following steps.

Decolourisation was performed to remove colorants from the LA acid stream using PUROLITE MN-502 (Purolite, Germany), supplied from below in an expanded bed setting. The flow was set at 6 bed volumes h^{-1}. Decolourisation was finished after rinsing the column with purified water until conductivity was below 1 mS cm^{-1}.

Cation exchange chromatography with RELITE EXC 08 (Resindion S. R. L., Italy) resin was performed in order to separate LA from salt-ions. Column volume was 1 L and the loading was carried out from below at 6 bed volumes h^{-1}. Afterward, the column was washed with purified water until conductivity reached 1 mS cm^{-1}. An outdoor power meter 3/32 A Type sinus 85 SO (TIP, USA) was used to measure the power consumption of each downstream step.

3.11. Analytical methods

The raw and steam exploded materials were analysed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, LAP-004, LAP-017, and LAP-019). Dry weight was determined by drying samples at 105 °C for 24 h (LAP-001).

The gas composition in clamped flasks used for strict anaerobic cultures was analysed by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (70 × 1/8" O.D.) and a MS13X4-09SF2 40/60 P packed column (9' × 1/8" O.D.) (PerkinElmer).

Cell growth was assessed by two methods: (1) measuring optical density in a spectrophotometer (SPECTROstar® Omega) at 600 nm and (2) by drying pre-weighted samples with known culture volume until constant weight at 105 °C.

For the quantification of sugars and fermentation products, samples were taken from the fermentation broth and centrifuged at 14.000 g for 4 min. When fermentations in flasks were performed with $CaCO_3$, H_2SO_4 0.2 M was added to the samples to release the free LA from the calcium lactate (100 µL of H_2SO_4 2 M were added to 900 µL of sample). Then, samples were filtered using membrane filters (Thermo Scientific® Nylon 0.2 µm) and stored at -20 °C until analysed. pH was measured using a benchtop pH meter (Sension+ PH31).

In experiments in articles II, III, V and VI, glucose, xylose, ethanol, acetic acid and LA were quantified by HPLC (DIONEX, USA) equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) was employed at the following conditions: column temperature, 50 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.5 mL min^{-1} and injection volume, 20 µL. Arabinose, galactose and mannose were quantified with a CARBOSep CHO-682 column (Transgenomic, Omana, NE) at the following conditions: column temperature, 80 °C: mobile phase, ultrapure water; flow rate, 0.35 mL min^{-1} and injection volume, 20 µL. Vanillin, *p*-coumaric acid, furfural, 5-HMF, syringaldehyde, ferulic acid and formic acid were also quantified by HPLC (Agilent, Waldbronn, Germany). The system was equipped with a Coregel 87H3 column (Transgenomic, San Jose, CA, USA). The operating temperature was 65 °C, and the mobile phase was 89 % (v v^{-1}) 5 mM H_2SO_4 and 11 % (v v^{-1}) acetonitrile, with a flow rate of 0.7 mL min^{-1}. All these compounds were identified by a 1050 photodiode-array detector (Agilent, Waldbronn, Germany).

In experiments included in article IV, LA, acetic acid, formic acid, glucose, xylose and arabinose concentrations were measured by HPLC (DIONEX, USA) with a Eurokat H column (300 mm × 8 mm × 10 μm) at the following conditions: column temperature, 50 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.8 mL min^{-1} and injection volume, 10 μL. Detection was carried out by a refractive index detector (RI-71, SHODEX, Japan). Phenols, furfural and 5-HMF were determined by HPLC (DIONEX, USA) with a UV detector (280 nm). Ultrapure water (A) and acetonitrile 50 % (B) in a multistep-gradient were used as eluents with a flow rate of 1 mL min^{-1}. The enantiomeric purity of LA was also determined via HPLC (Dionex, USA), using a Phenomenex Chirex 3126 (150 9 4.6 mm ID, Phenomenex, USA) column, operating at 30 °C, with 1 mM Cu_2SO_4 as eluent at a flow rate of 1 mL min^{-1}. Detection was carried out with an ultraviolet detector.

Cations and anion concentrations were analysed by ion chromatography (DIONEX, USA). For quantification of cations, 25 μL of sample volume was added on an IonPac CS 16 column (250 mm × 4 μm, DIONEX, USA) and eluted isocratically with 1.0 mL min^{-1} of 30 mM CH_3SO_3H at 40 °C. For quantification of anions, 25 μL of sample volume was added on an IonPac AS9-HC column (250 mm × 4 μm, DIONEX, USA) and eluted isocratically with 1.2 mL min^{-1} of 9 mM Na_2CO_3 at room temperature. Detection of cations and anions was carried out by a conductivity cell. Each analysis was carried in duplicate and peak areas were compared to analyses of known concentrations of salt-solutions consisting of cations and anions of interest. The mean concentration of two analyses is presented.

Kjeldahl-nitrogen content was determined according to the DIN-EN-25663 standard method. Protein content was calculated by multiplying the Kjeldahl-N content by 5.7 (Leung et al., 2012). Free amino nitrogen (NH_4-N)) concentration was measured using the ninhydrin reaction method (Lie, 1973). Glycine was used as standard. The total phosphorus content was measured by flow injection analysis (FIA), according to the international standard ISO 15681-1, 2003.

3.12. Calculations

The enzymatic hydrolysis yields (Y_{EH}) were calculated considering the final concentration of monomeric sugars (FS) at 72 h of hydrolysis, and the potential monomeric sugars (PS) in WIS and liquid fractions, as indicated hereafter:

(9) $Y_{EH} = [(FS) \div (PS)] \times 100$

The PS are the total amount of monosaccharides (glucose, xylose, arabinose, galactose and mannose) that could be released from the mentioned materials. These values were determined by an acid hydrolysis (4 % (v v^{-1}) H_2SO_4, 120 °C and 30 min).

The specific growth rate (μ) was calculated as the increase in OD_{600}/biomass per unit of time:

(10) $\mu\ (h^{-1}) = \ln (x_2 \div x_1) \div (t_2 - t_1)$,

where x is biomass (g L^{-1}) or OD_{600} obtained at each sample time, t (h).

LA and ethanol yields (Y_{LA} and Y_{EtOH}, g g^{-1}) were defined as the concentrations (g L^{-1}) of these compounds at the indicated fermentation time per initial sugars (g L^{-1}). The percentage of the theoretical maximum ($\%Y_{MaxLA}$ and $\%Y_{MaxEtOH}$) was calculated taking into account the maximum theoretical yield in each case. Volumetric productivities (Q_{LA} and Q_{EtOH}, g L^{-1} h^{-1}) were calculated as the concentrations produced divided by the h at the indicated fermentation times.

Statistical analyses were carried out using the software Microsoft Excel. Results are given as the average ± standard deviation for descriptive statistics. In order to determine the statically significant differences between different fermentations, analysis of variance (ANOVA) was carried out. The level of significance was set at $p < 0.05$.

4. RESULTS & DISCUSSION

4. RESULTS AND DISCUSSION

The benefits of using lignocellulosic materials for the generation of biofuels and bio-based products lie, among other, in their wide availability, high carbohydrates content, low cost, renewability, and no competition of water and land-use with food crops (Article I). Indeed, high amounts of fermentable sugars, potentially used as platform molecules, can be released from these materials due to their cellulose and hemicellulose content.

A high variety of biomass with different origins can be used as suitable substrate for the production of biofuels and biochemicals. Wheat straw is one of the most abundant agriculture residues in rural areas, with a total global production of 529 million tons per year (Zhao et al., 2017). This residual biomass contains, approximately, 35 % - 45 % (w w^{-1}) cellulose, 20-30 % (w w^{-1}) hemicelluloses and 15 % (w w^{-1}) lignin (del Río et al., 2012). On the other hand, high amounts of lignocellulosic residues are generated in gardening and pruning activities due to the expansion of green spaces in urban areas, reaching even 1.5 kg per m^2 (Tetma, 2018). The chemical composition of these residues, including tree branches, hedge cuttings, leaves and grass clippings, is about 40 % (w w^{-1}) cellulose, 20 % - 30 % (w w^{-1}) hemicelluloses and 25-30 (% w w^{-1}) lignin (Boldrin and Christensen, 2010; Shi et al., 2013).

A complete valorisation of the different fractions obtained from wheat straw and gardening residues is addressed in this Thesis for the production of LA and bioethanol (**Fig. 4.1**), being an attractive option in terms of sustainability and environment preservation and promoting the implementation of circular economy principles. Nevertheless, different bottlenecks have to be faced when using lignocellulosic materials like the need of pretreatment, multi-enzymatic hydrolysis steps and purification processes that significantly increase the production costs (Pleissner et al., 2017a).

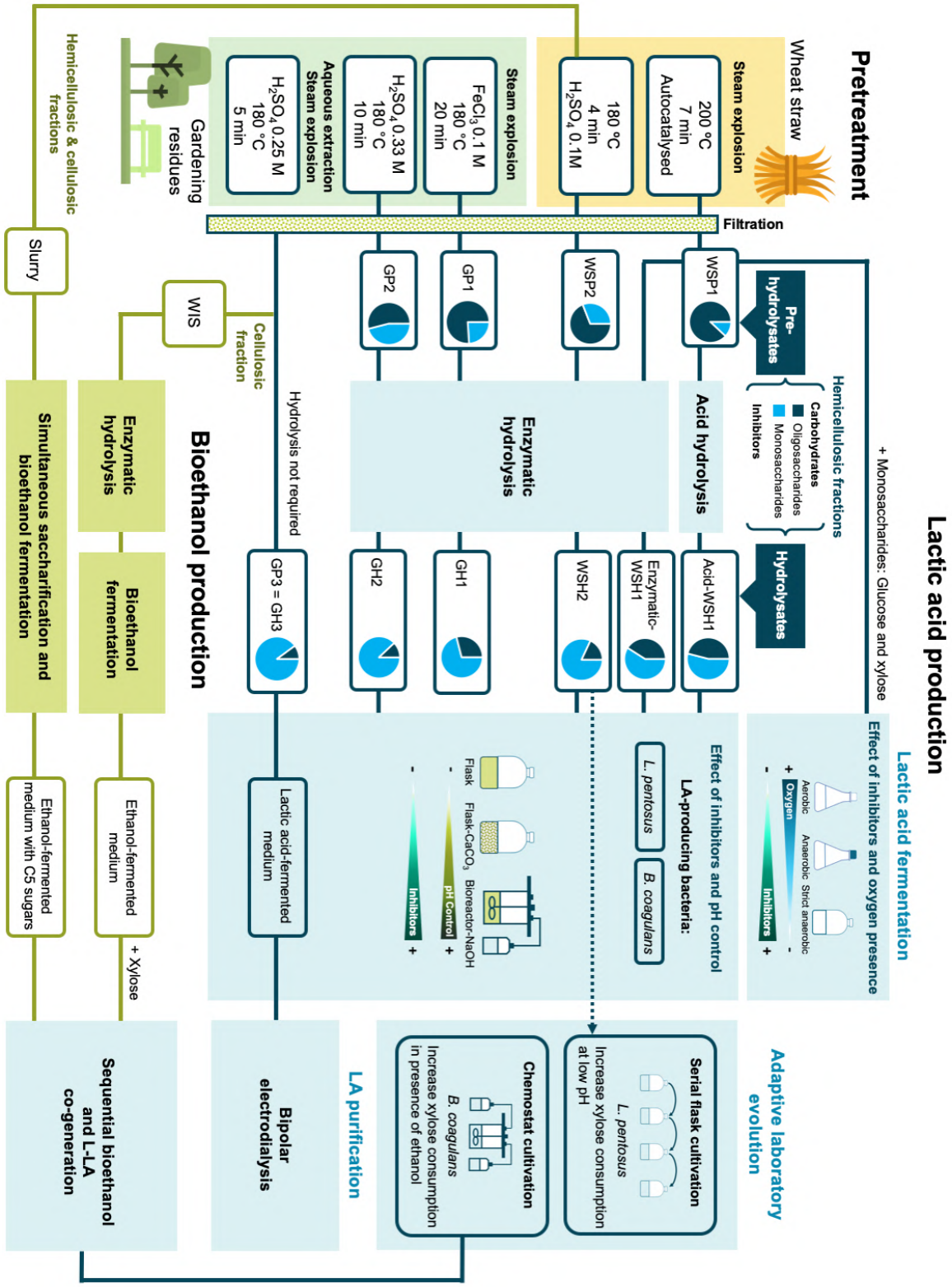


Fig. 4.1. Schematic representation of the main experiments performed to reach a complete valorisation of lignocellulosic materials in the framework of this Thesis

4.1. Lactic acid production from liquid fractions of different steam-exploded lignocellulosic materials

The use of cellulosic glucose has been extensively studied for bioethanol production by yeasts (Robak and Balcerek, 2018). However, the use of the liquid fractions rich in hemicellulosic sugars to produce biofuels or bio-based products is still very challenging because of the presence of inhibitory compounds produced during pretreatment and mixed C6 and C5 sugars, that cannot be metabolised by most of wild-type microorganisms. In this context, different bacteria are able to metabolise pentoses and sugar mixtures into LA, exhibiting an opportunity for the valorisation of liquid fractions from pretreated lignocellulosic materials.

4.1.1. Obtaining different liquid fractions by pretreatment and hydrolysis

4.1.1.1. Production of steam-exploded liquid fractions

Steam explosion has been commonly used for pretreatment of herbaceous agricultural residues, like wheat straw (Aulitto et al., 2017; Ballesteros et al., 2006; Linde et al., 2008; Tomás-Pejó et al., 2017), but few works have addressed the pretreatment of gardening residues. Among these, thermochemical pretreatments like steam explosion and wet explosion (steam explosion combined with oxygen addition) have been used to pretreat cocksfoot grass and pruned branches from pear trees (Njoku et al., 2013; Sasaki et al., 2014).

As indicated in Articles II-V, during steam explosion of wheat straw and gardening residues performed in this Thesis, hemicellulose was solubilised and the released hemicellulosic sugars, mostly oligomers, were recovered in the liquid fractions or prehydrolysates (**Table 4.1**).

Different pretreatment conditions were tested, evaluating the effect of different catalysers, catalyser concentrations, temperature and reaction times (**Table 3.1**), which determined the composition of the resulting wheat straw and gardening prehydrolysates (WSPs and GPs) (**Table 4.1**). After steam explosion pretreatment of wheat straw at 200 °C for 7 min without external catalyst addition, most of sugars were recovered in oligomeric form in the resulting WSP1 (Article II**, Table 4.1**). In fact, only 1.03 g L^{-1} and 2.70 g L^{-1} of glucose and xylose, respectively, were detected in monomeric form that imply a 20 % and 10 % of the potential glucose and xylose in the prehydrolysate, respectively (**Table 4.1**).

Table 4.1. Composition of different steam-exploded wheat straw and gardening hemicellulosic liquid fractions (prehydrolysates) and derived media obtained through hydrolytic treatments (hydrolysates).

Concentration (g L^{-1})	Wheat straw hemicellulosic media					Gardening hemicellulosic media				
	WSP1	Acid-WSH1	Enzymatic-WSH1	WSP2	WSH2	GP1	GH1	GP2	GH2	GP3 = GH3[2]
Sugars: Monosaccharides (Total)[1]										
Glucose	1.03 (5.06)	2.41	2.28	3.00 (5.80)	4.50	2.37 (6.80)	5.41 ± 0.08	4.25 (8.09)	7.51 ± 0.08	5.50 ± 0.03 (6.40)
Xylose	2.70 (26.50)	18.06	19.86	33.30 (47.50)	40.00	0.12 (4.60)	3.60 ± 0.06	0.94 (4.83)	4.40 ± 0.05	17.00 ± 0.23 (17.00)
Arabinose	-	-	-	4.29 (5.55)	4.00	1.05 (2.80)	1.97 ± 0.01	2.86 (3.94)	3.89 ± 0.31	3.89 ± 0.08 (4.90)
Galactose	-	-	-	-	-	0.36 (2.30)	0.60 ± 0.05	0.84 (2.61)	1.26 ± 0.09	3.66 ± 0.13 (4.70)
Mannose	-	-	-	-	-	0.55 (0.55)	0.55 ± 0.00	0.55 (0.55)	0.55 ± 0.00	0.90 ± 0.00 (1.40)
Inhibitors										
Furfural	1.26	0.36	0.52	0.41		0.02		0.09		0.20
5-HMF	0.07	0.15	0.23	0.09		0.08		0.13		0.21
Acetic acid	7.19	8.86	6.67	2.36		0.65		0.42		1.15
Formic acid	-	-	-	0.58		0.61		0.17		0.23
Syringaldehyde	0.01	-	-	0.02		-		-		-
Vanillin	0.04	-	-	0.03		-		-		-
Ferulic acid	-	-	-	0.02		-		-		-
Coumaric acid	0.02	-	-	0.05		-		-		-
Articles	II			V		III		III		III, IV

[1] Total sugars concentrations (including monomers and oligomers), determined by acid treatment, are indicated in brackets.

[2] GH3 was directly derived from GP3 because enzymatic hydrolysis was not required.

In different works, biomass impregnation with H_2SO_4 and $FeCl_3$ has shown to improve sugars release during pretreatment, facilitating further hydrolytic steps (Ballesteros et al., 2006; López-Linares et al., 2013). For this reason, wheat straw pretreatment was also performed with H_2SO_4 0.1 M as catalyser at 180 °C for 7 min (Article V). As a result, the total sugars in the liquid fraction, considering both monomers and oligomers, were notably higher in WSP2, with 2-fold increase in total C5 content in comparison with WSP1 (**Table 4.1**). Furthermore, 52 % of the glucose, 70 % of the xylose, and 77 % of the arabinose were in monomeric form (**Table 4.1**). The addition of H_2SO_4 at high temperatures has previously resulted in high sugar solubilisation in wheat straw steam explosion, reaching even 85 % of sugars recovery in the liquid fraction (Ballesteros et al., 2006).

Considering the higher sugar concentrations obtained with wheat straw under catalysed-steam explosion, gardening residues were impregnated with two different catalysts ($FeCl_3$ and H_2SO_4) (Article III). When $FeCl_3$ 0.1 M was used in steam explosion at 180 °C for 20 min, only 24 % of monomeric sugars were detected in the resulting GP1 (**Table 4.1**). On the other hand, when 0.25 M H_2SO_4 was added as catalyst in steam explosion at 180 °C for 5 min, 46 % of the sugars were detected in monomeric form (**Table 4.1**), showing almost 2-fold increase in comparison with pretreatment with $FeCl_3$.

Previous studies have demonstrated the ability of aqueous extraction to reduce the amounts of extractives that could hinder pretreatment and enzymatic hydrolysis (Ballesteros et al., 2011). In fact, when applying an aqueous extraction step prior steam explosion (180 °C with 0.33 M H_2SO_4 for 10 min), 90 % of sugars were present in monomeric form in GP3 (**Table 4.1**, Article III), corresponding with almost 2-fold increase in comparison with employing H_2SO_4 without water extraction (GP2). GP3 contained the highest amount of total (34.40 g L^{-1}) and monomeric (31.00 g L^{-1}) sugars of the obtained GPs (**Table 4.1**). However, these results were considerably lower than the ones obtained from H_2SO_4-catalysed wheat straw pretreatment. In this case, 59.0 g L^{-1} and 40.60 g L^{-1} of total and monomeric sugars were obtained in WSP2, respectively (**Table 4.1**), owing to the higher hemicellulose content of wheat straw (26 %) in comparison with gardening residues (16 %) (**Table 3.1**).

Acetic acid and furfural are formed through the release of acetyl groups and the degradation of pentoses from the hemicellulosic fraction, respectively, being solubilised in the prehydrolysates at higher concentrations than other inhibitors. Thus, a higher hemicellulose content has associated a higher amount of acetyl groups and pentoses that can be released and degraded

into acetic acid and furfural, respectively, which could explain the higher concentrations of these inhibitors in WSPs in comparison with GPs (**Table 4.1**). Low 5-HMF concentrations were detected in WSPs and GPs because this inhibitor is formed by degradation of hexoses, which are lower in hemicellulose of herbaceous biomass and mostly remain in the cellulosic fraction (WIS) after steam explosion (Tomás-Pejó et al., 2008) (**Table 4.1**).

Phenolic compounds, formed by lignin degradation, are normally present at low concentrations due to their lower solubilisation (Tomás-Pejó et al., 2011). While these compounds were not detected in GPs, between 0.02 and 0.05 g L^{-1} of phenols were detected in WSPs, including acids (ferulic and coumaric acid derived from cinnamic acid) and aldehydes (vanillin and syringaldehyde derived from guaiacyl- and syringylpropane units, respectively) (**Table 4.1**).

Although the addition of H_2SO_4 is known to increase the production of inhibitors (Alvira et al., 2010), steam explosion of wheat straw without external catalyst addition produced 3-fold more acetic acid and furfural concentrations than H_2SO_4-catalysed (**Table 4.1**). The higher temperature (200 °C) and longer reaction time (7 min) used for the production of WSP1 compared to WSP2 could explain the higher amounts of these compounds. Formic acid is formed by furfural degradation, which can also explain the lower furfural and higher formic acid content of WSP2 in comparison with WSP1.

Regarding inhibitors formation in GPs, lower CBA and higher furans concentrations were formed in GP2 (H_2SO_4-catalysed pretreatment) than in GP1 ($FeCl_3$-catalysed pretreatment) (**Table 4.1) (**Article III). The addition of inorganic salts in steam explosion, especially in the case of $FeCl_3$, was reported to promote xylose degradation to furfural (Liu and Wyman, 2006). At last, furfural can be converted into formic acid, which could explain the higher formic acid and the lower xylose and furfural content in GP1 (0.61 g L^{-1}, 0.12 g L^{-1} and 0.02 g L^{-1} for formic acid, xylose and furfural, respectively) when compared with GP2 (0.17 g L^{-1}, 0.94 g L^{-1} and 0.09 g L^{-1} for formic acid, xylose and furfural, respectively) (**Table 4.1**). The highest inhibitors content was obtained in GP3, including both CBA (1.38 g L^{-1}) and furans (0.41 g L^{-1}) (**Table 4.1**), presumably because of the water extraction process applied and the highest H_2SO_4 concentration and longest pretreatment time.

The above-mentioned inhibitory degradation compounds were previously identified in a similar range when using steam-exploded herbaceous materials (Ballesteros et al., 2006; Moreno et al., 2019a; Njoku et al., 2013; Tomás-Pejó et al., 2009, 2008).

Considering that most of sugars in WSP1, WSP2, GP1 and GP2 (Articles II-V) were in oligomeric form, different hydrolysis tests were carried out to favour the monomeric sugar release from these prehydrolysates before the fermentation step. Since 90 % of sugars from GP3 were fermentable monosaccharides, this material did not require a hydrolytic step and was directly used for LA production.

4.1.1.2. Production of acid- and enzymatically-hydrolysed liquid fractions

As previously mentioned, to increase the monomeric sugars content of the liquid fractions to proceed with fermentation step, different hydrolysis methods were applied to WSPs and GPs, consequently obtaining wheat straw and gardening hydrolysates (WSHs and GHs).

Acid and enzymatic hydrolysis of wheat straw prehydrolysates

Firstly, an acid and an enzymatic hydrolysis were applied on WSP1 achieving similar hydrolysis yields (Article II). The acid-WSH1 contained 2.41 g L^{-1} glucose and 18.06 g L^{-1} xylose while the enzymatic-WSH1 contained 2.28 g L^{-1} glucose and 19.86 g L^{-1} xylose (**Table 4.1**). These values represented a Y_{EH} of 34 % and 31 % for glucose and 65 % and 72 % for xylose in acid and enzymatic hydrolysis, respectively, showing that the addition of cellulases (Cel 10 FPU g^{-1} glucan and *β*-Glu 10 IU g^{-1} glucan) and hemicellulases (Cellic-HTec2 50 mg g^{-1} xylan and *β*-Xyl 0.1 mg g^{-1} xylan) (**Table 3.2**) produced similar monosaccharides' release to the acid hydrolysis.

For enzymatic hydrolysis, the cellulases preparation from *T. reesei* (Celluclast® 1.5) and the *β*-glucosidase from *A. niger* (Novozym®188) were used to release glucose and glucooligosaccharides from cellulose and cleave cellobiose units to glucose, respectively, reducing the end-product inhibition on the cellulases. The combination of these two commercial enzymes has been extensively used for glucan hydrolysis from different lignocellulosic materials (Arantes & Saddler, 2011; Liang et al., 2014; Mosier et al., 2005; Rodrigues et al., 2015). Due to the high xylan and arabinan content of the hemicellulosic fraction, supplementation with hemicellulases is crucial for an efficient enzymatic hydrolysis (Dondelinger et al., 2016). While Cellic-HTec2 releases xylo- and arabino-oligosaccharides from soluble hemicellulose (Novozymes, 2010), the *β*-xyl from *Selenomonas ruminantium* is able to cleave the monosaccharides residues from these oligomers (Jordan et al., 2007), alleviating their end-product inhibition on Cellic-HTec2.

In light of the results obtained after enzymatic hydrolysis of WSP1, WSP2 was also subjected to enzymatic hydrolysis with Cellic-HTec2 and *β*-xyl. Cellulases were not added in this case due to the low glucooligomers content in WSP2 (**Table 4.1**). In this case, a xylose Y_{EH} of 47 % was attained, being lower than the one obtained after enzymatic hydrolysis of WSP1. A possible explanation is that the higher amount of xylose and arabinose monomers in WSP2 than in WSP1 (**Table 4.1)** could have produced end-product inhibition on hemicellulases. In fact, it has been demonstrated that the presence of high xylose and arabinose monosaccharides content inhibits the enzymatic activity of the *S. ruminantium β*-xyl used in this work (Jordan and Braker, 2007). In addition, the lower Cellic-HTec2 loading used can also explain the lower pentoses Y_{EH} obtained in WSH2 in comparison with enzymatic-WSH1.

As previously mentioned, the presence of degradation compounds formed during pretreatment can negatively affect the activity and stability of cellulolytic enzymes, mainly through hydrophobic interactions, leading to their deactivation (Jung and Kim, 2017). Although WSP1 contained around 3-fold more furfural and acetic acid concentrations than WSP2, formic acid was only present in WSP2, reaching almost 0.60 g L^{-1} (**Table 4.1**). Some works have reported that the inhibitory effect of degradation compounds on cellulolytic enzymes follows the order: Phenolic compounds > 5-HMF > formic acid > furfural > acetic acid (Cao et al., 2013; Jung and Kim, 2017). For this reason, since similar concentrations of 5-HMF and phenolic compounds were present in both WSPs, the presence of formic acid in WSP2 could explain the lower enzymatic yields attained (**Table 4.1**). Indeed, formic acid was concluded to present the highest cellulolytic and hemicellulolytic inactivation effect on enzymes among different lignocellulosic inhibitors from pretreated wheat straw (Panagiotou and Olsson, 2007). Thus, the combination of the higher formic acid and C5 monosaccharides content in WSP2 could explain the inhibitory effect occurred on hemicellulases.

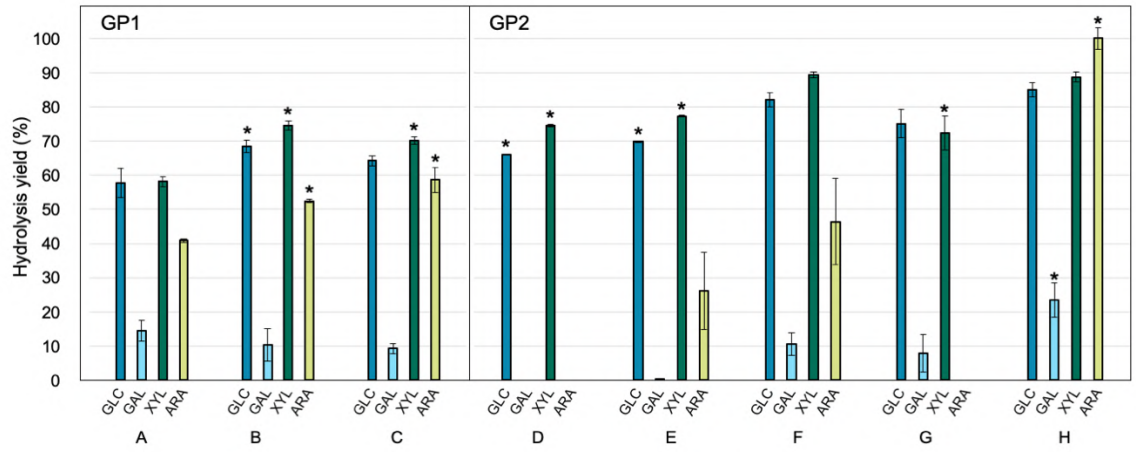
After increasing the monomeric sugars content of these materials, the resulting hydrolysates: acid-WSH1, enzymatic-WSH1 and enzymatic-WSH2 were used as substrate for LA production.

Enzymatic hydrolysis of gardening prehydrolysates

The effect of adding only cellulases or cellulases together with hemicellulases was tested in enzymatic hydrolysis of GP1. In this sense, addition of cellulase and hemicellulase cocktails in GP1 was carried out following three strategies (Article III): (1) only cellulases (**Fig. 4.2 A**), (2)

hemicellulases together with cellulases (**Fig. 4.2 B**) and (3) hemicellulases 24 h prior cellulases (**Fig. 4.2 C**).

The addition of cellulases and hemicellulases to GP1 (**Fig. 4.2 B)** resulted in a significant 19 % increase in glucose $Y_{EH,}$ as well as a 28 % increase in xylose and arabinose Y_{EH} (**Fig. 4.2 B**) when compared with only cellulases (**Fig. 4.2 A**). In fact, while only 4.93 ± 0.19 g L^{-1} and 2.73 ± 0.07 g L^{-1} of glucose and xylose, respectively, were detected in GH1 after 72 h with only cellulases (**Fig. 4.3 A**), 5.41 ± 0.08 g L^{-1} and 3.60 ± 0.06 g L^{-1} of these sugars were detected when adding both hemi- and cellulases (**Fig. 4.3 B**).



	A	B	C	D	E	F	G	H
Cel (FPU g^{-1})	16	16	16[1]	8	12	16	16	16
β-Glu (IU g^{-1})	16	16	16[1]	8	12	16	16	16
Cellic-HTec2 (mg g^{-1})	0	15	15	15	15	15	15	15
β-Xyl (mg g^{-1})	0	0.1	0.1	0.1	0.1	0.1	0.05	0.5

Fig. 4.2. Hydrolysis yields obtained in the hydrolysis of GP1 using different enzyme dosages and addition strategies. The mean difference with the control for GP1 (A) and GP2 (F) is significant at p-value < 0.05 (*). GLC (glucose), GAL (galactose), XYL (xylose) and ARA (arabinose).

[1] Cel and β-Glu were added 24 h after the addition of Cellic-HTec2 and β-Xyl.

The improved glucose Y_{EH} when adding both Cellic-HTec2 and *β*-Xyl when compared with only cellulases could be due to the cellulolytic activity present in Cellic-HTec2 as indicated by the supplier (Novozymes, 2010). Moreover, high concentrations of xylooligosaccharides have been reported to competitively inhibit cellulose hydrolysis by mimicking its structure and binding to the catalytic site of cellulases (Dondelinger et al., 2016; Qing and Wyman, 2011). In this context, xylose may exert lower end-product inhibitory effect on cellulases than xylooligosaccharides and thus the addition of the hemicellulases Cellic-HTec2 and *β*-Xyl could increase glucose yields. Indeed, 27 % improvement in glucan conversion was reported when supplementing cellulases with xylanase and *β*-xyl in the enzymatic hydrolysis of pretreated corn

stover (Qing and Wyman, 2011). This phenomenon was also reported with steam-exploded corn stover, in which case cellulose and xylan Y_{EH} were improved by a partial replacement of cellulases with xylanases without increasing enzyme loading (Hu et al., 2011).

When GP1 was hydrolysed by adding cellulases 24 h after xylanases, 64, 70, 9 and 59 % of total glucose, xylose, galactose and arabinose were released, respectively (**Fig. 4.2 C**). In this case, hydrolysis yields were similar to the simultaneous addition of cellulases and hemicellulases (**Fig. 4.2 B**). Indeed, no significant differences were shown in xylose Y_{EH}.

Figure 4.3 shows the enzymatic hydrolysis time courses of GP1, including addition of only cellulases (**Fig. 4.3 A**), simultaneous addition of cellulases and hemicellulases (**Fig. 4.3 B**) and addition of cellulases 24 h after hemicellulases (**Fig. 4.3 C**).

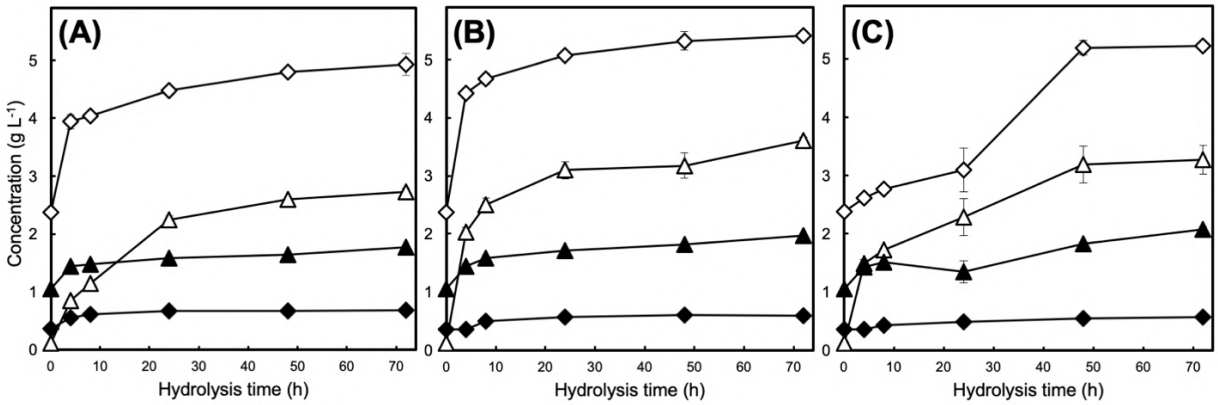


Fig. 4.3. Hydrolysis time courses of GP1 with only cellulases (A), cellulases and hemicellulases (B) and hemicellulases added 24 h before cellulases (C). Glucose (◇), xylose (△), galactose (◆) and arabinose (▲).

As can be seen, a low xylose release along time was attained when adding only cellulases, releasing only 1.14 ± 0.03 g L^{-1}, 2.25 ± 0.07 g L^{-1} and 2.73 ± 0.07 g L^{-1} of xylose at 8 h, 24 h and 72 h of hydrolysis, respectively (**Fig. 4.3 A**). When both cellulases and hemicellulases were added, different results were attained regarding the addition method. Xylose release was significantly higher in the first h of hydrolysis when adding hemicellulases and cellulases simultaneously (**Fig. 4.3 B**) in comparison with adding hemicellulases 24 h before cellulases (**Fig. 4.3 C**). Indeed, 3.10 ± 0.15 g L^{-1} of xylose were measured at 24 h when cellulases and xylanases were added simultaneously (**Fig. 4.3 B**), while only 2.28 ± 0.32 g L^{-1} were present at the same time point when cellulases were added 24 h after xylanases (**Fig. 4.3 C**). However, similar concentrations of xylose, 3.26 ± 0.25 g L^{-1} - 3.60 ± 0.06 g L^{-1} (**Fig. 4.3 B and C**), were achieved in both cases at 72 h of enzymatic hydrolysis which explains that no significant

differences were found in final xylose Y_{EH} (**Fig. 4.2 B and C**). No differences in arabinose hydrolysis along time were found in these cases either.

Even though Y_{EH} was not affected by the sequential addition of the enzymes, higher hydrolysis rates were shown when cellulases and hemicellulases were added simultaneously. For this reason, the simultaneous addition of both cellulases and hemicellulases was selected as the optimum for the enzymatic hydrolysis of GP1 into GH1.

The effect of increasing the cellulases dosages on enzymatic hydrolysis yields was studied in GP2 (Article III). As expected, higher Y_{EH} of hexoses was obtained when increasing the cellulases dosage. Indeed, glucose Y_{EH} increased from 66 % **(Fig. 4.2 D)** to 70 % **(Fig. 4.2 E)** and even to 82 % **(Fig. 4.2 F)**, when the cellulases dosages were increased from 8 FPU g^{-1} of Cel and 8 IU g^{-1} of *β*-Glu to 16 FPU g^{-1} of Cel and 16 IU g^{-1} of *β*-Glu. Xylose Y_{EH} was also enhanced when increasing the dosage of Cel and *β*-Glu from 8 FPU/IU g^{-1} to 16 FPU/IU g^{-1} (**Table 3.3**), switching from around 75 % (**Fig. 4.2 D**, **E)** to almost 90 % (**Fig. 4.2 F)**.

The effect of increasing *β*-Xyl loading at the highest cellulases dosages (16 FPU g^{-1} of Cel and 16 IU g^{-1} of *β*-Glu) in GP2 was also studied (**Fig. 4.2 G**, **F** and **H**). A positive effect on glucose Y_{EH} was observed when increasing *β*-Xyl loading. Glucose Y_{EH} increased from 75 % (**Fig. 4.2 G**) to 82 % (**Fig. 4.2 F**) and to 85 % (**Fig. 4.2 H**) when *β*-Xyl loading was 0.05, 0.10 and 0.50 mg g^{-1}, respectively. In **Fig. 4.2 G, F** and **H,** 8 %, 10 % and 23 % of galactose was released, following the same trend as glucose when increasing the *β*-Xyl dosage.

Significantly higher xylose and arabinose hydrolysis was produced when increasing the concentration of *β*-Xyl. Xylose Y_{EH} was 72 % with 0.05 mg g^{-1} of *β*-Xyl (**Fig. 4.2 G**), while 90 % of xylose Y_{EH} was reached with 0.1 mg g^{-1} and 0.5 mg g^{-1} (**Fig. 4.2 F, H)**. Arabinose hydrolysis was not detected with 0.05 mg g^{-1} of *β*-Xyl (**Fig. 4.2 G**). However, when increasing the enzyme content to 0.1 mg g^{-1}, arabinose Y_{EH} was 46 % (**Fig. 4.2 F**) and even reached 100 % when adding 0.5 mg g^{-1} of *β*-Xyl (**Fig. 4.2 H**). Since similar results were obtained in **Fig. 4.2 F and H** in terms of glucose, galactose and xylose hydrolysis, the enzymes dosages used in **Fig. 4.2 H** (**Table 3.3**) were chosen as the optimum for the enzymatic hydrolysis of GP2 due to the higher arabinose Y_{EH}.

The capacity of *β*-Xyl to release both arabinose and xylose has been previously reported, demonstrating the bifunctionality of this kind of enzymes, which not only presents *β*-xyl activity, but also α-arabinosidase. In fact, the *β*-xyl from *Paenibacillus woosongensis* was able to hydrolyse xylose and arabinose from paranitrophenyl-*β*-xylopyranoside and

paranitrophenyl-*α*-arabinofuranoside, respectively, with high specific activity in both cases (Kim and Yoon, 2010). The *β*-xyl from *S. ruminantium* used in this Thesis was shown to catalyse the hydrolysis of 4-nitrophenyl-*β*-D-xylopyranoside with 10-fold preference than the 4-nitrophenyl-*α*-L-arabinofuranoside, confirming the prevalence of *β*-Xyl over α-arabinosidase activity (Jordan et al., 2007).

Higher Y_{EH} were reached with GP2 than with GP1 at the same enzyme loadings (**Fig. 4.2 B** and **F**). Considering the whole sugars content, 61 % of overall Y_{EH} was obtained in GH1, while 78 % was obtained in GH2. As mentioned before, inhibitors formed during steam explosion are known to affect hydrolytic enzymes (García-Aparicio et al., 2006; Zhai, et al., 2018). For this reason, the difference in Y_{EH} could be related with the higher formic acid concentration in GP1 (0.61 g L^{-1}) in comparison with GP2 (0.17 g L^{-1}), as it was the case for WSP2 (**Table 4.1**). Even though acetic acid concentrations, 0.65 g L^{-1} and 0.42 g L^{-1} in GP1 and GP2, respectively, were higher than formic concentrations in both prehydrolysates (**Table 4.1**), the higher inhibitory effect of formic acid has been previously demonstrated (Jung and Kim, 2017).

4.1.2. Effect of lignocellulosic inhibitors on lactic acid fermentation of different carbon sources

The following sections aim to take advantage of the sugars mixtures present in the wheat straw and gardening hydrolysates, checking their suitability as substrate for LA production. In this way, different fermentation conditions were tested in terms of microorganisms (including WT and evolved hetero- and homolactic bacterial strains), inhibitors content, oxygen presence and pH control, studying the effect on bacterial growth, sugars consumption and LA production.

4.1.2.1. Oxygen as key factor for inhibitors sensitivity in *Lactobacillus pentosus*

LAB are anaerobic or aerotolerant microorganisms that lack a functional respiratory chain and catalases (Idler et al., 2015; Article I). Therefore, as long as respiration is not induced, energy is produced via fermentation (Guidone et al., 2013; Papadimitriou et al., 2016). In presence of oxygen, the formation of ROS is promoted, which can produce oxidative damage on DNA, cell membrane and proteins. Furthermore, metabolism can be disrupted if the NADH supplies are arrested to detoxify these ROS (by NADH peroxidases) instead of being used for energy production (by pyruvate conversion into LA through LDH). As a result, cell integrity, energy production and LA production can be severely hindered by oxygen, especially in combination

with other inhibitory effects produced by degradation compounds from lignocellulosic pretreated materials.

The use of *L. pentosus* CECT 4023T to produce LA from lignocellulosic streams has been proposed by several authors due to its ability to metabolise C6 sugars via the EMP pathway and C5 sugars via the PK pathway, even in the presence of inhibitors produced during pretreatment (Bustos et al., 2005; Buyondo and Liu, 2011; Costa-Trigo et al., 2019; Garde et al., 2002; Rivera et al., 2009; Wischral et al., 2019).

In this Thesis, the effect of oxygen presence on the susceptibility of *L. pentosus* CECT 4023T to the inhibitors formed during pretreatment was evaluated (Article II). For such a purpose, different experimental configurations (aerobic, anaerobic and strict anaerobic) were tested in fermentation media with increasing concentration of WSP1, *i.e*, 25 %, 50 % and 75 % (v v^{-1}), from now on WSP25, WSP50 and WSP75, respectively, which means increasing concentration of inhibitors. The ability of this LAB to metabolise different synthetic sugars in presence of the inhibitors in these prehydrolysates was also investigated. Different carbon sources were added in this sense, including glucose (20 g L^{-1}), glucose and xylose (5 g L^{-1} and 15 g L^{-1}, respectively) and xylose (20 g L^{-1}).

The composition of the WSP25, WSP50 and WSP75 obtained after dilution of WSP1 and sugars supplementation of can be found in **Table 4.2**.

Table 4.2. Composition of Wheat Straw Prehydrolysates (WSP) used in this study.

Concentration (g L^{-1})	Wheat straw prehydrolysate 1 diluted and supplemented media		
	WSP25 (WSP1 25 % v v^{-1})	WSP50 (WSP1 50 % v v^{-1})	WSP75 (WSP1 75 % v v^{-1})
Monosaccharides[1]			
Glucose	**20/5/0**	**20/5/0**	**20/5/0**
Xylose	**0/15/20**	**0/15/20**	**0/15/20**
Inhibitors			
Furfural	**0.32**	**0.63**	**0.95**
5-HMF	**0.02**	**0.04**	**0.06**
Acetic acid	**4.19**	**5.63**	**6.19**
Syringaldehyde, vanillin, coumaric acid	**< 0.01**	**< 0.01**	**< 0.01**

[1] Sugar concentrations in these cases depend on the carbon source added in each assay: Glucose 20 g L^{-1}; Glucose 5 g L^{-1} and Xylose 15 g L^{-1} and Xylose 20 g L^{-1}.

Fermentation of glucose-rich media under aerobic, anaerobic and strictly anaerobic conditions at increasing inhibitors concentrations

The effect of oxygen on *L. pentosus* growth and LA production from 20 g L^{-1} of glucose in presence of inhibitory degradation compounds of WSP1 was analysed (Article II). For such purpose, fermentation experiments were performed under aerobic, anaerobic and strictly anaerobic conditions. Xylose fermentation was not taken into account since it was mostly solubilised as xylooligomers in WSP1 and negligible concentrations of monomeric xylose were measured in the resulting diluted media (**Table 4.1**).

As shown in **Table 4.3**, the highest μ_{max}, ranging between 0.27 ± 0.01 h^{-1} and 0.41 ± 0.03 h^{-1}, were obtained under strict anaerobiosis in spite of the presence of different concentrations of WSP1. In anaerobic conditions, there was a slight reduction in μ_{max} (0.20 ± 0.01 h^{-1}- 0.38 ± 0.02 h^{-1}) when compared with strict anaerobiosis and the lowest growth rates (< 0.10 h^{-1}) were attained in aerobic conditions.

Table 4.3. Effect of oxygen presence on growth rate and lactic acid yields of *L. pentosus* from MRS with glucose 20 g L^{-1} (control) and from glucose-rich media with increasing concentrations of wheat straw prehydrolysate 1 (WPS1) in flask.

Fermentation conditions	Carbon source (g L^{-1})	Liquid fraction[1]	μ_{max} (h^{-1})	Y_{LA} 48h (g g^{-1})[2]	%Y_{MaxLA} 48h
Aerobic	Glucose 20	Control	**0.22** ± 0.01	**0.53** ± 0.00	**53**
		WSP25	**0.10** ± 0.02	**0.35** ± 0.01	**35**
		WSP50	**0.06** ± 0.02	**0.13** ± 0.00	**13**
		WSP75	**0.08** ± 0.01	**0.05** ± 0.00	**5**
Anaerobic	Glucose 20	Control	**0.46** ± 0.02	**0.72** ± 0.00	**72**
		WSP25	**0.31** ± 0.00	**0.69** ± 0.01	**70**
		WSP50	**0.38** ± 0.02	**0.62** ± 0.03	**62**
		WSP75	**0.20** ± 0.01	**0.47** ± 0.05	**47**
Strictly anaerobic	Glucose 20	Control	**0.37** ± 0.01	**0.90** ± 0.01	**90**
		WSP25	**0.41** ± 0.03	**0.84** ± 0.00	**84**
		WSP50	**0.27** ± 0.01	**0.82** ± 0.00	**82**
		WSP75	**0.27** ± 0.01	**0.81** ± 0.02	**81**

[1] 25, 50 and 75 % (v v^{-1}) of WSPI are WSP25, WSP50 and WSP75, respectively.
[2] Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

The lowest growth rates obtained in aerobiosis, confirmed that *L. pentosus* preferably grows under anaerobic conditions. Some authors have previously pointed out to the toxicity of oxygen for LAB (Papadimitriou et al., 2016). Among the different fermentation parameters, temperature and oxygen level were determinant on survival phenotypes and gene expression

level of *L. lactis* (Dijkstra et al., 2014). Indeed, aerated growth of *L. lactis* was reported to cause damage on proteins, cell membrane and chromosomal DNA due to the oxidative stress produced by ROS (Rezaiki et al., 2004).

Following the same trend as growth rates, the highest Y_{LA} were achieved in strict anaerobiosis, reaching values between 0.81 ± 0.02 g g^{-1} and 0.84 ± 0.00 g g^{-1} (**Table 4.3**), without showing significant differences among WSP25, 50 and 75. Furthermore, only 7-10 % of reduction in LA yields were observed from WSP25, 50 and 75 when compared with the control (MRS with glucose 20 g L^{-1}) (**Table 4.3**).

Y_{LA} was more affected when increasing the concentration WSP1 (meaning increasing inhibitors content) in the media in aerobiosis and anaerobiosis than in strict anaerobiosis**.** As a matter of fact, while no significant differences were shown in anaerobiosis in WSP25 (0.69 ± 0.01 g g^{-1}) in comparison with the control (0.72 ± 0.00 g g^{-1}), LA yields were reduced by 14 % and 35 % in WSP50 (0.62 ± 0.03 g g^{-1}) and WSP75 (0.47 ± 0.05 g g^{-1}), respectively, when compared to the control (**Table 4.3**).

In aerobic conditions, *L. pentosus* was more susceptible to the inhibitors present in the WSP1, which resulted in a reduction of LA production, reaching Y_{LA} of 0.35 ± 0.01 g g^{-1} (WSP25), 0.13 ± 0.00 g g^{-1} (WSP50) and 0.05 ± 0.00 g g^{-1}(WSP75) (**Table 4.3**). As a result, the Y_{LA} were reduced by 34 % (WSP25), 75 % (WSP50) and 91 % (WSP75), in comparison with the control.

The reduction in LA production due to oxygen presence has been previously reported. As mentioned, under oxidative stress conditions, ROS detoxification mechanisms are activated, like the expression of NADH peroxidase to reduce the presence of these compounds (Papadimitriou et al., 2016) or the accumulation of poly-phosphate (Gray and Jakob, 2015). NADH peroxidases compete with LDH for NADH molecules. For instance, LA production is reduced and pyruvate is redirected to mixed fermentations in *L. lactis* (Miyoshi et al., 2003).

According to sugars consumption, while only 46 % of glucose was consumed in aerobiosis (**Fig. 4.4 A**), this sugar was totally consumed in anaerobic (**Fig. 4.4 B**) and strictly anaerobic conditions (**Fig. 4.4 C**) in WSP25 fermentation. In case of WSP50, only 17 % of glucose was consumed in aerobiosis (**Fig. 4.4 D**), increasing to 85 % and 100 % in anaerobiosis and strictly anaerobiosis, respectively (**Fig. 4.4 E, F**). Focusing on the most challenging conditions tested in terms of inhibitors concentrations (WSP75), less than 5 % of glucose was consumed in aerobic conditions and only 0.79 ± 0.07 g L^{-1} of LA were produced (**Fig. 4.4 G**). In anaerobic conditions, more than 75 % of glucose was consumed, producing 7.90 ± 0.89 g L^{-1} of LA

(**Fig. 4.4 H**). Furthermore, in strict anaerobiosis, all the glucose was consumed at 48 h, with a LA concentration of nearly 17.63 ± 0.38 g L^{-1} (**Fig. 4.4 I**) demonstrating again that this condition was the most appropriated for LA production.

As will be discussed later, the ability of *L. pentosus* to grow in the presence of different inhibitors commonly found in steam-exploded prehydrolysates has been addressed in several works (Boguta et al., 2014). However, the novelty of the present study relied on elucidating the correlation between the tolerance to lignocellulosic inhibitors and the oxygen presence in order to minimise the combined inhibitory effect of these degradation compounds and the oxidative damage (Article II).

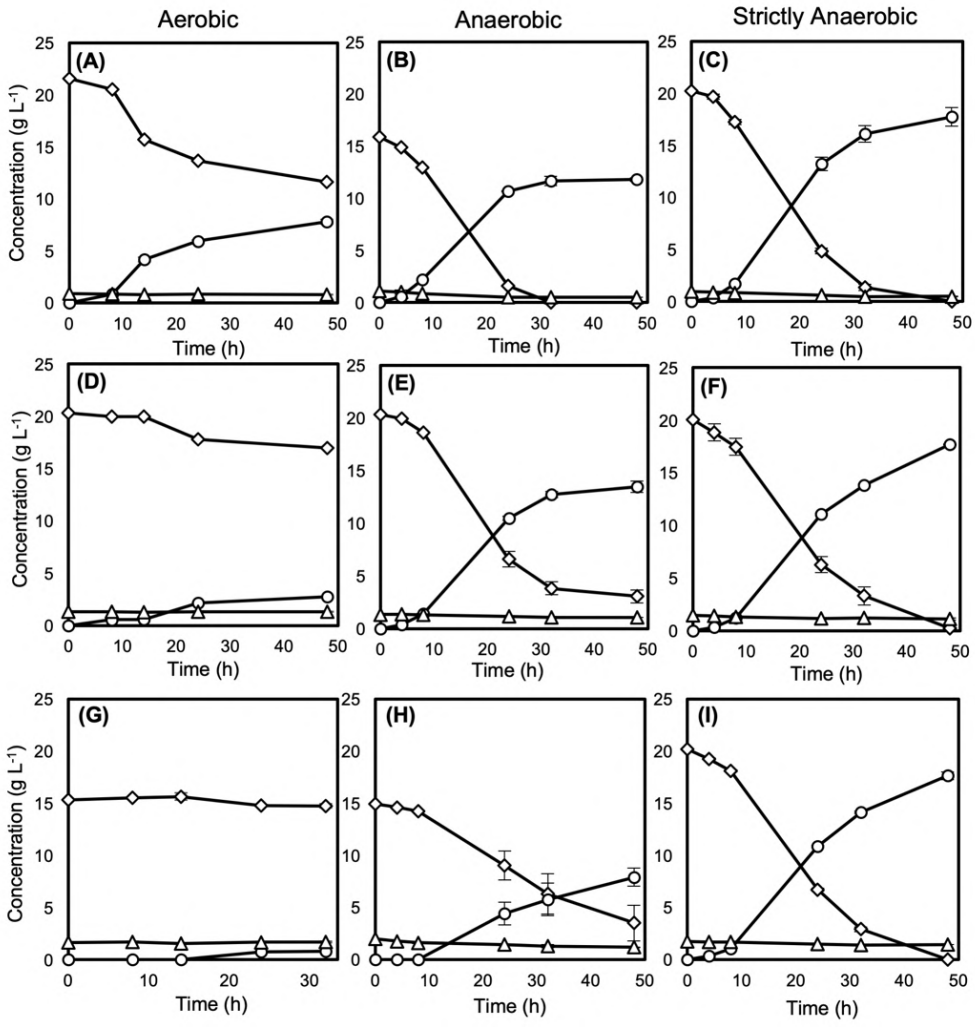


Fig. 4.4. Effect of oxygen presence on fermentation time courses of *L. pentosus* CECT 4023T from 20 g L^{-1} glucose with 25 % (A-C), 50 % (D-F) and 75 % (G-I) (v v^{-1}) of WSP1. Glucose (◇), xylose (△) and LA (○).

Fermentation of xylose-rich media under strictly anaerobic conditions at increasing concentrations of lignocellulosic inhibitors

In order to check the ability of *L. pentosus* to metabolise both C6 and C5 sugars in presence of inhibitors from WSP1, several experiments were carried out using: 1) a 1:3 (glucose:xylose) mixture and 2) xylose 20 g L^{-1} as carbon sources (Article II). Experiments were performed in strict anaerobiosis because these conditions showed the highest LA production in glucose media, as stated in the previous section.

According to the bacterial growth, μ_{max} between 0.19 ± 0.01 h^{-1} and 0.45 ± 0.00 h^{-1} were obtained in the different experiments with sugar mixtures and with only xylose (**Table 4.4**). As it was the case for glucose fermentation, lower μ_{max} were obtained when increasing the concentration of WSP1 and, consequently, the inhibitors concentrations (**Table 4.4**). Boguta et al. (2014) reported that several *L. pentosus* isolates could utilise xylose and reach μ_{max} values around 0.20 h^{-1} in the presence of different inhibitors commonly found in pretreated wheat straw (*i.e.* furfural 0.15 g L^{-1} and acetic acid 2.70 g L^{-1}). Remarkably, the μ_{max} obtained in the present Thesis with real WSP75 containing higher concentration of inhibitors were significantly higher.

Table 4.4. Growth rate and lactic acid yields attained by *L. pentosus* from MRS media with glucose 5 g L^{-1} and xylose 15 g L^{-1} or with only xylose 20 g L^{-1} (controls) and from xylose-rich media with increasing concentrations of wheat straw prehydrolysate 1 (WPS1) in flask.

Fermentation conditions	Carbon source (g L^{-1})	Liquid fraction[1]	μ_{max} (h^{-1})	Y_{LA} 48h (g g^{-1})[2]	$\%Y_{MaxLA}$ 48h
Strictly anaerobic	Glucose 5 Xylose 15	Control	**0.47** ± 0.00	**0.41** ± 0.00	**60**
		WSP25	**0.45** ± 0.00	**0.47** ± 0.00	**67**
		WSP50	**0.35** ± 0.00	**0.46** ± 0.00	**67**
		WSP75	**0.29** ± 0.01	**0.34** ± 0.00	**49**
Strictly anaerobic	Xylose 20	Control	**0.39** ± 0.01	**0.34** ± 0.00	**56**
		WSP25	**0.36** ± 0.01	**0.32** ± 0.02	**53**
		WSP50	**0.29** ± 0.01	**0.34** ± 0.01	**56**
		WSP75	**0.19** ± 0.01	**0.28** ± 0.00	**46**

[1] 25, 50 and 75 % (v v^{-1}) of WSP1 are WSP25, WSP50 and WSP75, respectively.
[2] Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

The Y_{LA} obtained from glucose in WSP1 under strictly anaerobic conditions were more than 2-fold higher than those from the sugars mixture and from only xylose (**Table 4.3** and **4.4**). Since *L. pentosus* employs the PK pathway to metabolise C5, lower LA yields were obtained with this pathway due to the production of acetic acid as a by-product. For this reason, the percentage of the theoretical yield, $\%Y_{MaxLA}$, was calculated to normalise the results between different experiments.

During fermentation of glucose:xylose mixture (5 g L^{-1}:15 g L^{-1}), no differences in LA yields were shown between in WSP25 and WSP50, reaching 67 $\%Y_{MaxLA}$ in both cases, being even higher than the one obtained with the control without inhibitors (60 $\%Y_{LA}$) (**Table 4.4**). Nevertheless, 49 $\%Y_{MaxLA}$ was attained when WSP1 concentration was increased to WSP75, meaning an 18 % reduction in comparison with the control (**Table 4.4**). These LA yields were similar to others reported in previous studies using *L. pentosus* (69 $\%Y_{LA}$) cultivated in glucose:xylose defined mixtures (Garde et al., 2002).

At 48 h of fermentation, around 50 % of xylose was consumed with the mixed sugars fermentation, producing between 8.35 ± 0.00 g L^{-1} and 9.14 ± 0.04 g L^{-1} of LA and between 1.38 ± 0.40 g L^{-1} and 2.61 ± 0.18 g L^{-1} of acetic acid independently of the percentage of WSP1 present (**Fig. 4.5 A-C**). At 24 h of process the glucose was completely consumed in all cases and xylose became the unique sugar present in the broth. From this point, *L. pentosus* could only use the PK pathway and LA production rate decreased at the expense of the acetic acid production (**Fig. 4.5 A-C**).

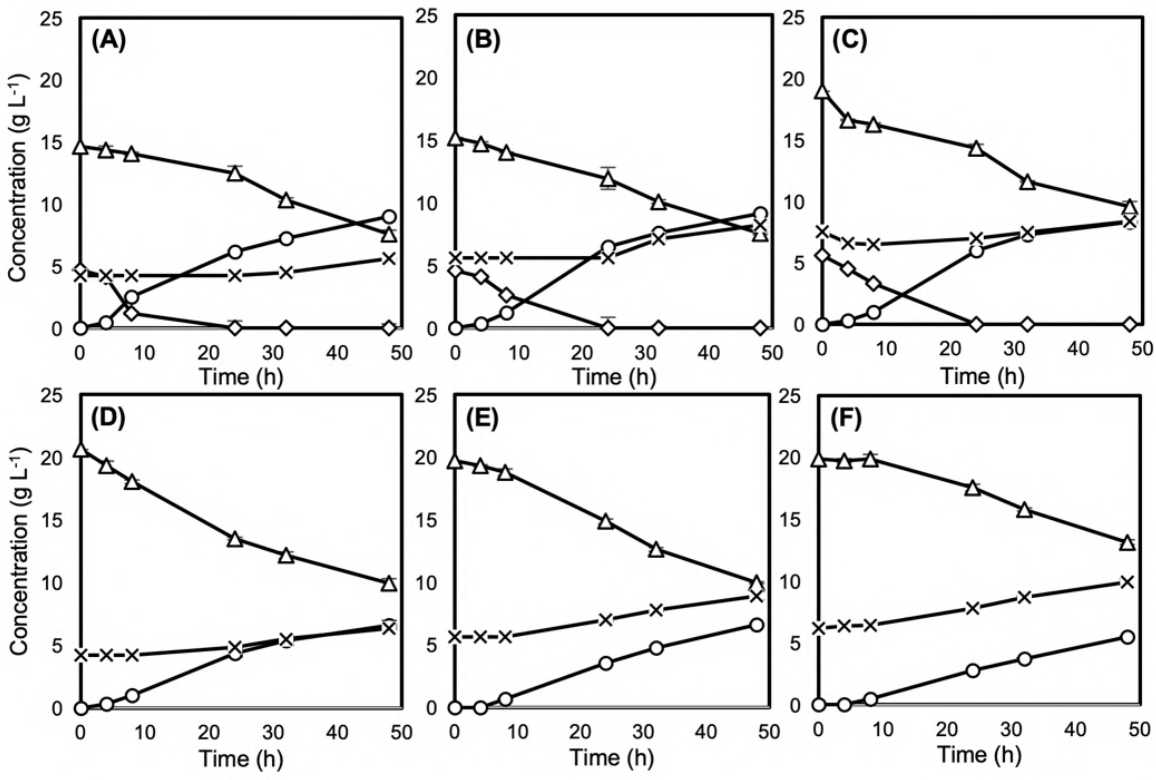


Fig. 4.5. Fermentation time courses of *L. pentosus* 4023T from mixtures of sugars: xylose 15 g L^{-1} and glucose 5 g L^{-1} with 25 % (A), 50 % (B) and 75 % (C) (v v^{-1}) of WSP1; and xylose 20 g L^{-1} with 25 % (D), 50 % (E) and 75 % (F) (v v^{-1}) of WSP1. Glucose (◇), xylose (△), LA (○) and acetic acid (×).

Due to its high abundance, bacterial metabolic pathways have evolved for glucose utilisation, promoting a hierarchical control of sugar utilisation (Kim et al., 2010). In *L. pentosus*, the genes encoding for the main enzymes of the PK pathway (operon *xylAB*) are induced by the presence of pentoses and are repressed by glucose, mediated by CcpA and by the PEP-PTS for sugars transport (Kim et al., 2010; Posthuma et al., 2002). It is worth highlighting that, in this Thesis, a co-consumption of both glucose and xylose when *L. pentosus* was grown with sugars mixtures was observed (**Fig. 4.5 A-C**). Interestingly, these results suggested that CCR did not have a marked effect in this case, since the presence of glucose did not hinder xylose consumption. Having in mind that most of xylose-utilising LAB are affected by this phenomenon, the fact that *L. pentosus* CECT 4023T was able to perform a simultaneous sugar consumption with relaxed CCR arises as an interesting approach to take advantage of the heterogeneous sugars composition of lignocellulosic biomass (Article II).

The glucose and xylose co-consumption ability of *L. pentosus* has been previously confirmed during fermentation of sugarcane bagasse (Wischral et al., 2019). *L. brevis* has also demonstrated to present a soft CCR and it can perform a simultaneous fermentation of a range of carbon sources (Grewal and Khare, 2018; Kim et al., 2009). This co-consumption was also observed in other LA-producing microorganisms like *Enterococcus mundtii* QU 25 from initial concentrations of 25 g L^{-1} of glucose and 50 g L^{-1} of xylose (Abdel-Rahman et al., 2015).

With 20 g L^{-1} of xylose as sole carbon source, between 46 % and 56 % Y_{MaxLA} at 48 h were attained (**Table 4.4**). In the same way as with the sugars mixture, no significant differences in LA yields were found when the WSP1 concentration was increased from 0 % to 25 % (v v^{-1}) (WSP25) and 50 % (v v^{-1}) (WSP50) (**Table 4.4**). However, Y_{LA} was reduced by 18 % in fermentation of WSP75 in comparison with the control (**Table 4.4**). In these cases, between 34 % (WSP75) and 50 % (WSP25 and WSP50) of initial xylose was consumed at 48 h of fermentation (**Fig. 4.5 D-F**).

The low xylose consumption observed in all the xylose-containing media used in these experiments can explain the lower %Y_{MaxLA} (**Table 4.4**). This fact might be due to an inhibitory effect on xylose consumption caused by the decrease in pH during fermentation. When the culture pH drops to values lower than the pKa of the CBA formed during pretreatment (acetic and formic acid) and fermentation (LA and acetic acid), the acids become able to cross the cell membrane in their undissociated form (Trcek et al., 2015). Once inside, CBA dissociate back due to the higher intracellular pH, producing an accumulation of protons. The extra need of ATP to pump protons out of the cell reduces the energetic supply of the cell which causes

growth inhibition and, when this is overcome, the cytosol acidification leads to the denaturation of proteins, membrane disruption and cell death (Trcek et al., 2015).

Therefore, the absence of pH control could have influenced the detrimental effect of CBAs present in the media, including end-products and degradation compounds. The damaging effect produced by CBA, combined with the presence of other compounds like furans and phenols could explain the high inhibitory effect of WSP1 on xylose fermentation. In fact, the highest inhibitory effect on xylose-containing media was produced in presence in WSP75, in which case, the concentration of inhibitors at the end of fermentation was (g L^{-1}): furfural, 1.00; 5.HMF, 0.06; acetic acid, 10.00 and LA, 5.50-6.60 (**Table 4.2, Fig. 4.5**).

It seems likely that the combination of acids produced during fermentation and lignocellulosic inhibitors hindered the xylose conversion to LA when the pH dropped. By contrast, *L. pentosus* was able to completely consume 20 g L^{-1} of glucose in spite of the pH drop and the inhibitors from WSP1 (**Fig. 5.4**) (Article II).

As previously introduced, it is known that, in presence of glucose, the PEP-PTS involved in the transport of alternative sugars, like xylose, are inhibited by phosphorylated forms of a phosphocarrier protein named HPr, preventing the translocation of alternative carbon sources and repressing the genes involved in their catabolism as CCR methods (Chaillou et al., 1999; Kim et al., 2009).

Once the monosaccharides have accessed to the cytosol, glucose enters the EMP pathway, while xylose is metabolised by the enzymes of the PK pathway. Previous works have demonstrated that the xylose transport mechanisms of *L. pentosus* present low velocity and low affinity, being the rate-controlling step in the metabolism and growth on this compound (Chaillou et al., 1999). This fact would explain why xylose consumption could be more affected than glucose by the pH drop and the inhibitors present in WSP1 since the low-speed transport of this sugar could be further slowed down under these stress conditions.

4.1.2.2. Importance of pH control for inhibitor tolerance in *Lactobacillus pentosus* and *Bacillus coagulans*

Although the application of strictly anaerobic conditions improved LA yields in *L. pentosus*, a low xylose consumption was attained due to the pH drop produced by LA and acetic acid accumulation along fermentation combined with the presence of inhibitors produced during pretreatment.

For this reason, different pH control methods were applied in the LA fermentations from wheat straw and gardening hydrolysates, following an SHF approach (Articles II-V). In case of gardening hydrolysates, fermentations were carried out comparing two different LA-producing bacteria: the heterolactic *L. pentosus* strain used in previous fermentations, and different homolactic *B. coagulans* strains.

Heterolactic fermentation of lignocellulosic hydrolysates by *Lactobacillus pentosus* under non-controlled pH conditions

L. pentosus was cultivated in acid- and enzymatically-hydrolysed WSH1 obtained in section 4.1.1.2 to check the suitability of these two hydrolysis methods for SHF processes. Flask fermentations were carried out under strictly anaerobic and non-controlled pH conditions to compare the results with the ones obtained from WSP25, WSP50 and WSP75. The monomeric sugars released from acid and enzymatic hydrolysis of WSP1 were used as carbon sources for LA production in this case (Article II).

Unexpectedly, *L. pentosus* was not able to grow on acid-WSH1 and, therefore, no LA production was obtained **(Fig 4.6 A)**. By contrast, high growth was attained from enzymatically hydrolysed WSH1, reaching a $\mu_{max} = 0.38 \pm 0.01$ h^{-1} (Article II).

The inhibitory effect that the acid-WSH1 had on the LAB could be explained by the increased amount of inhibitors produced during acid-hydrolysis, which has been previously reported (Tomás-Pejó et al., 2011). In fact, a significant acetic acid increase was observed in acid-WSH after hydrolysis, reaching 8.86 g L^{-1} (**Table 4.1**). By contrast, during enzymatic hydrolysis, the acetic acid concentration was maintained around 7.00 g L^{-1} (WSH1) (**Table 4.1**) supporting the adequacy of enzymatic methods.

In fermentation of enzymatically-hydrolysed WSH1, 38 % of the xylose was consumed in 48 h, resulting in 67 $\%Y_{MaxLA}$ (**Table 4.5**) and a Q_{LA48h} of 0.20 ± 0.00 g L^{-1} h^{-1}. When extending

the fermentation time to 120 h, the xylose uptake increased to 55 % producing 12.58 ± 0.22 g L^{-1} of LA (86 $\%Y_{LA}$) (**Fig. 4.6 B,** Article II). In addition, more than 15 g L^{-1} of acetic acid (**Fig. 4.6 B**) were accumulated in the medium, similarly to the experiments with synthetic xylose (**Fig. 4.5**).

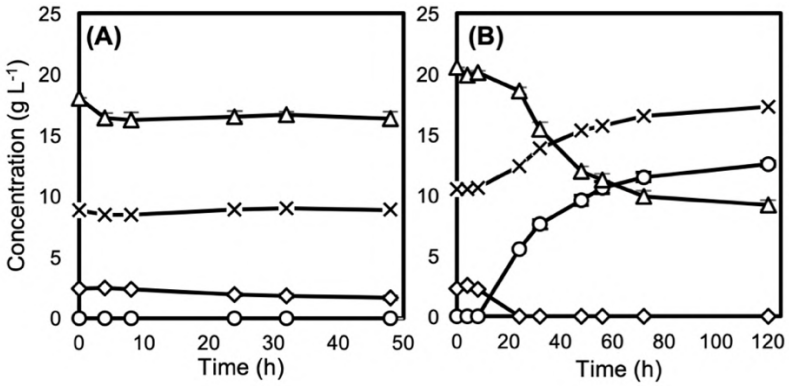


Fig. 4.6. Fermentation time courses of *L. pentosus* CECT 4023T from acid-hydrolysed WSH1 (A) and enzymatically-hydrolysed WSH1 (B) in flask under non-controlled pH conditions. Glucose (◇), xylose (△), LA (○), acetic acid (×).

L. pentosus was also cultivated in flask without pH control for LA fermentation of GH2 and GH3 (Article III). While GH2 contained the same amount of pentoses as hexoses, GH3 presented a ratio 2:1 (pentoses:hexoses) (**Table 4.1**). GH1 was not fermented due to its lower sugars content (**Table 4.1**).

While glucose, galactose, mannose and arabinose were totally consumed, less than 30 % of total xylose was consumed in both GH2 and GH3 after 48 h of fermentation (**Fig. 4.7 A**). As a result, around 12.35 ± 0.09 g L^{-1}-13.80 ± 0.03 g L^{-1} of LA (**Fig. 4.7 C)** were produced from GH2 and GH3, meaning 86 % and 66 $\%Y_{MaxLA}$, respectively. Although xylose consumption was increased when the fermentation time was extended to 72 h, more than 60 % of xylose was not consumed in GH2 and GH3 (**Fig. 4.7 A**, Article III). The lower $\%Y_{MaxLA}$ obtained from GH3 in comparison with WSH1 and GH2 (**Table 4.5**) in non-controlled pH fermentation can be explained due to the higher initial xylose and inhibitors concentrations (**Table 4.1**), which resulted in higher residual sugars after fermentation. Indeed, 10.98 ± 0.05 g L^{-1} of acetic acid were accumulated in GH3 after 48 h of fermentation, while only 5.80 ± 0.42 g L^{-1} were present in GH2 (**Fig. 4.7 C**). Due to the LA and acetic acid accumulation, a marked pH drop took place during GH2 and GH3 culture, switching from 7 to 4.5 in 24 h (**Fig. 4.7 D**).

When fermentation of enzymatically-hydrolysed WSH1, GH2 and GH3 was performed without pH control, xylose was not completely consumed in any case, not even when fermentation times

were extended from 48 h to 72 h or 120 h (**Fig. 4.6 and 4.7**). Similarly, xylose from chestnut residues hydrolysate was not completely consumed by *L. pentosus* in a 36-h long fermentation under non-controlled pH conditions, producing low LA yields (52 $\%Y_{LA}$) (Costa-Trigo et al., 2019) (**Table 4.5**).

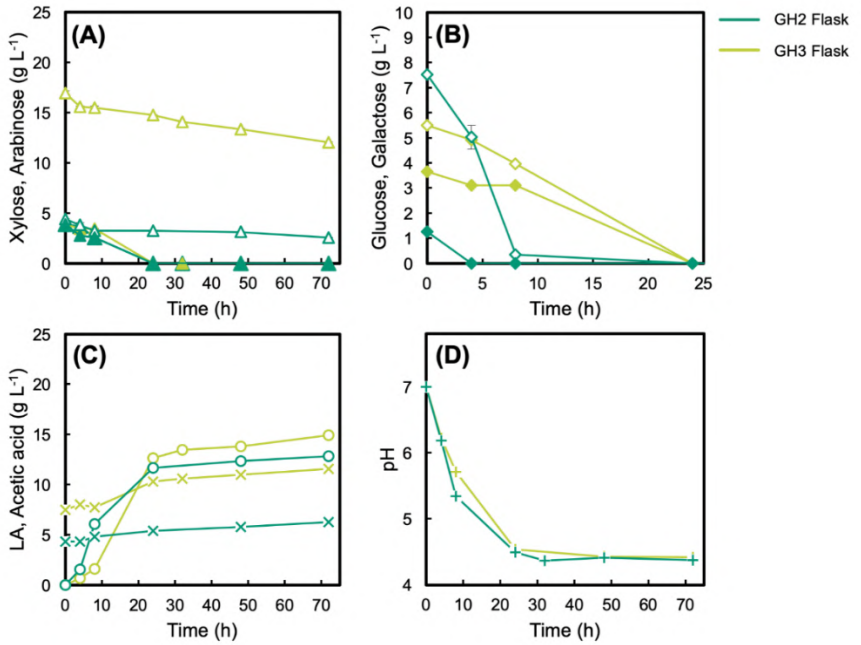


Fig. 4.7. Fermentation time courses of *L. pentosus* CECT 4023T from GH2 and GH3: sugars consumption (A-B) acids production (C) and culture pH (D) in flask under non-controlled pH conditions. Glucose (◇), xylose (△), galactose (◆), arabinose (▲), LA (○), acetic acid (×) and culture pH (+).

Heterolactic fermentation of lignocellulosic hydrolysates by *Lactobacillus pentosus* under controlled pH conditions

With the aim to avoid the incomplete xylose consumption produced by the pH drop, different pH control methods were used for fermentation of hydrolysates, including the addition of $CaCO_3$ at the starting of the culture and the automatic addition of NaOH along fermentation (Articles II and V):

For instance, 0.5 L of WSH2 obtained by enzymatic hydrolysis of WSP2 were fermented in bioreactor (R-0.5L) with automatic addition of 5M NaOH (Article V). The WSH2 was diluted 1:2 in order to achieve a similar sugars concentration than enzymatic-WSH1 (**Table 4.1**). In this case, a growth rate near 0.40 h^{-1} was obtained, being similar than the one from WSH1 fermentation (Article V). By contrast, after 48 h of fermentation, total sugars consumption was achieved, producing 12.54 ± 0.00 g L^{-1} of LA (**Fig. 4.8)**, reaching 81 $\%Y_{MaxLA}$ (**Table 4.5**) and a Q_{LA48h} of 0.26 ± 0.00 g L^{-1} h^{-1} (Article V). Thus, the pH control applied in bioreactor resulted

in 1.2- and 1.3-fold improvement in Y_{LA} and Q_{LA48h}, respectively, in comparison with enzymatic-WSH1 fermentation in flask.

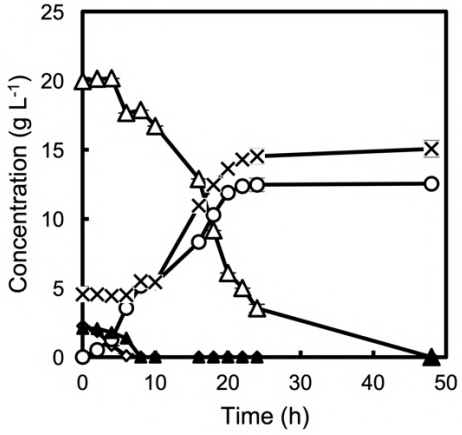


Fig. 4.8. Fermentation time courses of *L. pentosus* CECT 4023T from enzymatically-hydrolysed WSH2 in bioreactor under controlled pH conditions by automatic NaOH 5M addition. Glucose (◇), xylose (△), arabinose (▲), LA (○), acetic acid (×).

Different fermentation modes and controlled pH conditions were applied for GH3 fermentation, *i.e.,* (1) $CaCO_3$ addition in flask, and (2) automatic addition of 5 M NaOH in bioreactor at different working volumes (R-0.5L and R-10L) (Article III).

In the first case, 70 % of xylose was consumed at 48 h of fermentation (**Fig. 4.9 A**), while arabinose, glucose and galactose were totally consumed at the same fermentation time (**Fig. 4.9 A-B**). Considering acids production, 17.84 ± 0.24 g L^{-1} of LA and 13.23 ± 0.05 g L^{-1} of acetic acid were accumulated in the medium (**Fig. 4.8 C**). As a result, 82 $\%Y_{MaxLA}$ was achieved, meaning 1.3-fold increase in comparison with GH3 flask fermentation without pH control (**Table 4.5**, Article III). The higher xylose consumption and LA yield obtained in comparison with the non-controlled pH fermentation was produced due to the more progressive pH drop. In this case, culture pH dropped from 7 to 5 and 4.8 in 24 h and 48 h, respectively (**Fig. 4.9 D**).

Lower $\%Y_{LA}$ (62 % and 69 %) were obtained by *L. pentosus* in fermentation of inhibitors-rich trimming vine shoots and grape marc hydrolysates when using $CaCO_3$ to control the pH (Bustos et al., 2005; Rivera et al., 2009) (**Table 4.5**). The use of $CaCO_3$, as an insoluble neutraliser, is limited by the mass transfer because the reaction with LA takes place in solid:liquid heterogeneous phases. Furthermore, when using $CaCO_3$, high amounts of solid waste (gypsum) are formed after releasing the LA from the calcium lactate (Singhvi et al., 2015).

During GH3 fermentation with automatic NaOH addition in bioreactor, all the carbon sources, including xylose, were exhausted in 48 h (**Fig. 4.9 A-B**), reaching 19.95 g L^{-1}-21.38 g L^{-1} of LA and 14.86 g L^{-1}-15.49 g L^{-1} of acetic acid in R-0.5L and R-10L (**Fig. 4.9 C**). As a result of maintaining the culture pH at 7 (**Fig. 4.9 D**), between 89 % and 95 $\%Y_{MaxLA}$ were achieved (**Table 4.5**), meaning around 1.5-fold improvement in comparison with flask fermentation without pH control (Article III).

These yields were consistent with the ones obtained by *L. pentosus* from 50 % (v v^{-1}) WSH2 (81 $\%Y_{MaxLA}$) and from other inhibitors-rich materials like sugarcane bagasse, corncob and wood-extract hydrolysates in bioreactor with NaOH automatic addition (88 % - 97 $\%Y_{MaxLA}$) (**Table 4.5**) (Buyondo and Liu, 2011; Moldes et al., 2006; Wischral et al., 2019).

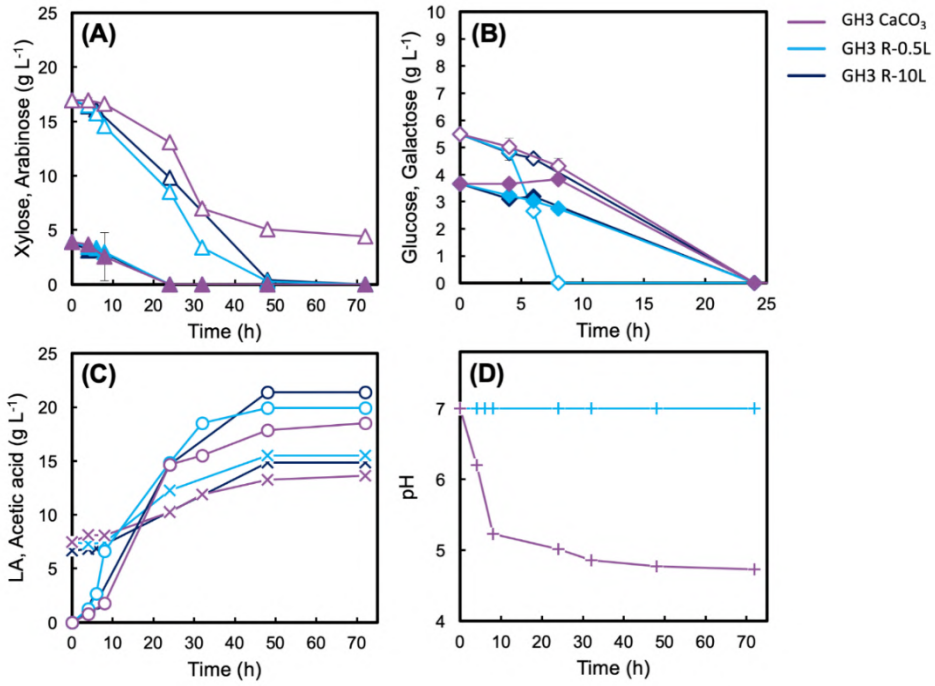


Fig. 4.9. Fermentation time courses of *L. pentosus* CECT 4023T from GH3: sugars consumption (A-B) acids production (C) and culture pH (D) under different controlled pH conditions. Glucose (◇), xylose (△), galactose (◆), arabinose (▲), LA (○), acetic acid (×) and culture pH (+).

It is worth to mention that all the carbon sources present in WSHs and GHs, including C6 (glucose) and C5 sugars (xylose and arabinose) were simultaneously co-consumed (**Fig. 4.6 B, 4.7 A-B, 4.8** and **4.9 A-B**). This fact confirmed the low CCR effect of this LAB, not only with synthetic mixed sugars (**Fig. 4.5 A-C**), but also from lignocellulosic sugars. For this reason, *L. pentosus* CECT 4023T is proposed as a great candidate for valorisation of mixtures of sugars from different heterogeneous lignocellulosic materials in biorefineries.

Table 4.5. Effect of pH control on lactic acid production from inhibitors-rich lignocellulosic hydrolysates by *L. pentosus* CECT 4023T.

Material	Inhibitors	pH control	Y_{LA} 48h (g g^{-1})[1]	Y_{MaxLA} (%)	Ref[2]
Enzymatic-WSH1	Table 5.2	No	**0.43** ± 0.01	**67**	Article II
50 % (v v^{-1}) WSH2	Table 5.2	NaOH (R-0.5L)	**0.52** ± 0.00	**81**	Article V
GH2	Table 5.3	No	**0.70** ± 0.01	**86**	This work
GH3	Table 5.3	No	**0.46** ± 0.01	**61**	Article III
		$CaCO_3$	**0.59** ± 0.02	**81**	
		NaOH (R-0.5L)	**0.65**	**89**	
		NaOH (R-10L)	**0.70**	**95**	
Chestnut residues hydrolysate	5-HMF 0.02 Acetic acid 4.14 Phenols 0.02	No	-	**52**	(1)
Trimming vine shoots hydrolysate	Furfural 1.34 5-HMF 0.11 Acetic acid 19.40	$CaCO_3$	**0.46**	**62**	(2)
Grape marc hydrolysate	Acetic acid 2.20	$CaCO_3$	**0.53**	**69**	(3)
Sugarcane bagasse hydrolysate	Furfural 0.19 5-HMF 0.06 Acetic acid 12.10	NaOH	**0.55**	**88**	(4)
Corncob hydrolysate	Not indicated	NaOH	**0.56**	**90**	(5)
Wood-extract hydrolysate	Acetic acid 2.17	NaOH	-	**97**	(6)

[1]Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).
[2]Ref: (1) Costa-Trigo et al., 2019; (2) Bustos et al., 2005; (3) Rivera et al., 2009; (4) Wischral et al., 2019; (5) Moldes et al., 2006; (6) Buyondo and Liu, 2011.

Different works have addressed the effect of lignocellulosic inhibitors on Lactobacilli. For instance, the presence of acetic acid and formic acid at concentrations ranging from 5.00 g L^{-1}-12.00 g L^{-1} and 1.00 g L^{-1}-7.70 g L^{-1}, respectively, were able to hinder bacterial growth of *L. casei*, *L. delbureckii*, *L. lactis*, *L. pentosus*, *L. brevis* and *L. plantarum* (Boguta et al., 2014; van der Pol et al., 2016b). These LAB were also inhibited by high furfural (1.00 g L^{-1}-3.50 g L^{-1}) and 5-HMF (1.00 g L^{-1}-5.90 g L^{-1}) concentrations. Besides, it was also concluded that phenolic acids can significantly inhibit the growth of most LAB at concentrations above 1.00 g L^{-1} (van der Pol et al., 2016b). Nevertheless, mentioned research works did not show the effects on LA production.

Altogether, it must be pointed out that the inhibitor concentrations present in wheat straw and gardening hydrolysates did not considerably hamper *L. pentosus* CECT 4023T growth and fermentation performance when reducing the oxygen presence and avoiding the pH drop during fermentation. In fact, this robust LAB was able to produce high LA yields in spite of the possible synergistic inhibitory effects between different inhibitors that has been previously reported (van der Pol et al., 2016b).

The results obtained in this Thesis with lignocellulosic hydrolysates highlighted the importance of having strict anaerobic and pH control conditions to avoid a marked inhibitory effect of lignocellulosic degradation compounds (CBA, furans and phenols) and fermentation products (LA and acetic acid) on LAB metabolism.

Homolactic fermentation of lignocellulosic hydrolysates by *Bacillus coagulans* under controlled pH conditions

Having in mind the higher bacterial growth, sugars consumption and LA yields obtained with *L. pentosus* when the pH was controlled in comparison with flask fermentation, the following experiments with *B. coagulans* were performed under controlled pH conditions (Article IV).

LA-producing Bacilli (*Bacillales* order) present different advantages in comparison with LAB in terms of fermentation features. In the LAB group (*Lactobacillales* order), C5 sugars like D-xylose and L-arabinose can only be metabolised by heterolactic strains by the PK pathway, producing LA together with other by-products (Idler et al., 2015). Such is the case for the *L. pentosus* strain used in this Thesis, which converted pentoses from wheat straw and gardening hydrolysates into LA and acetic acid. By contrast, *B. coagulans* strains are able to perform a homolactic fermentation of pentoses through the PP pathway, reaching higher LA yields than heterolactic LAB (Idler et al., 2015). Furthermore, *B. coagulans* strains produce optically pure L-LA (Juturu and Wu, 2015). Pure enantiomers are required for the polymerisation of LA into a crystalline and stable PLA, suitable to be used as a compostable packaging material and as a biocompatible material for biomedicine (Article I).

Having this in mind, three *B. coagulans* strains were screened for fermentation of gardening hydrolysates. *B. coagulans* strains included collection strains and isolates obtained in ATB (Potsdam, Germany) facilities: (1) DSM 2314, from DSMZ; (2) A166, isolated from hemp biomass fiber processing and (3) A162, isolated from washing water of a starch factory.

The composition of GH3 was slightly modified in comparison with previous experiments, and the available sugars for fermentation were reduced probably because of the dilution of the media for pH adjustment. The modified GH3 medium (M-GH3) composition can be found in **Table 4.6**. Fermentation of a basal medium (BM) with the same sugar concentrations as M-GH3 but without inhibitors was also utilised as a control.

Unlike Lactobacilli, LA-producing *Bacillus* strains, as catalase positive bacteria, have the ability to use oxygen as electron acceptor. Thus, *B. coagulans* strains are able to consume

oxygen, producing (1) the natural removal of this gas, reducing the risk of ROS formation; and (2) an increased cell biomass formation by respiration, which provides higher amounts of ATP than L-LA fermentation. In fact, *B. coagulans* is usually cultured in presence of oxygen at the first h of fermentation, reducing the lag phase and, eventually, increasing LA yields (Wang et al., 2018). Thus, the effect of oxygen presence on LA fermentation was not addressed in this study with *B. coagulans* and the reactors were run aerobically without sparging. In this case, part of oxygen would be consumed by the Bacilli and the formation of ROS would be less significant than for *Lactobacillus* (Vassilyadi and Archibald, 1985).

In the case of bacterial growth, A166 and A162 reached μ_{max} between 0.33 ± 0.00 h^{-1} and 0.34 ± 0.00 h^{-1} from M-GH3, while DSM 2314 showed a significantly lower μ_{max}, 0.24 ± 0.01 h^{-1}, being reduced by 14 % in comparison with the one attained from the control with only sugars (**Table 4.6**). A162 strain presented the highest cell biomass production, reaching almost 3.68 ± 0.026 g L^{-1}, while less than 3 g L^{-1} were achieved by A166 and DSM 2314 strains (**Fig. 4.10**).

Table 4.6. Growth rates, lactic acid yields and productivities obtained from the inhibitors-rich modified gardening hydrolysate 3 (M-GH3) and a basal medium (BM) without inhibitors as a control by different *B. coagulans* strains under controlled pH conditions (NaOH addition).

Medium	Initial sugars (g L^{-1})[1]	Inhibitors (g L^{-1})		*B. coagulans*	μ_{max} (h^{-1})	Y_{LA} 24h (g g^{-1})[2]	Q_{LA} 10h (g L^{-1} h^{-1})
BM	Glc 5.42 ± 0.29 Xyl 14.18 ± 0.40 Ara 3.74 ± 0.18	-		DSM 2314	**0.24** ± 0.01	**0.79** ± 0.07	**1.94** ± 0.34
				A166	**0.33** ± 0.00	**0.78** ± 0.05	**1.89** ± 0.11
				A162	**0.34** ± 0.03	**0.84** ± 0.00	**1.96** ± 0.08
M-GH3	Glc 4.94 ± 0.29 Xyl 15.12 ± 0.24 Ara 3.39 ± 0.19	Furfural 5-HMF Acetic acid Formic acid	0.20 0.21 1.15 0.23	DSM 2314	**0.28** ± 0.00	**1.00** ± 0.03	**1.63** ± 0.22
				A166	**0.29** ± 0.02	**0.99** ± 0.08	**1.96** ± 0.04
				A162	**0.34** ± 0.02	**0.90** ± 0.01	**2.36** ± 0.04

[1] Initial sugar concentrations were the mean values in the media tested with the three *B. coagulans* strains. Glc (glucose), Xyl (xylose) and Ara (arabinose).
[2] Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

Strikingly, *B. coagulans* DSM 2314, A166 and A162 reached between 90 and 100 % of the theoretical LA yield after 24 h of fermentation, being even higher than the ones obtained in the BM with only sugars (around 0.80 g g^{-1}) (**Table 4.6**). The main differences between the three strains were found in their fermentation kinetics. While xylose was totally consumed in 24 h by DSM 2314 and A166 (**Fig. 4.10 A and B**), A162 needed only 8 h to consume all xylose (**Fig. 4.10 C**). As a result, *B. coagulans* A162 presented the highest Q_{LA10h} (2.36 ± 0.04 g L h^{-1}), followed by A166 (1.96 ± 0.04 g L h^{-1}) and by DSM 2314 (1.63 ± 0.22 g L h^{-1}) (**Table 4.6**, Article IV). Thus, LA yields and productivities were not affected by the presence of lignocellulosic inhibitors in M-GH3 in any of the tested strains when compared to BM.

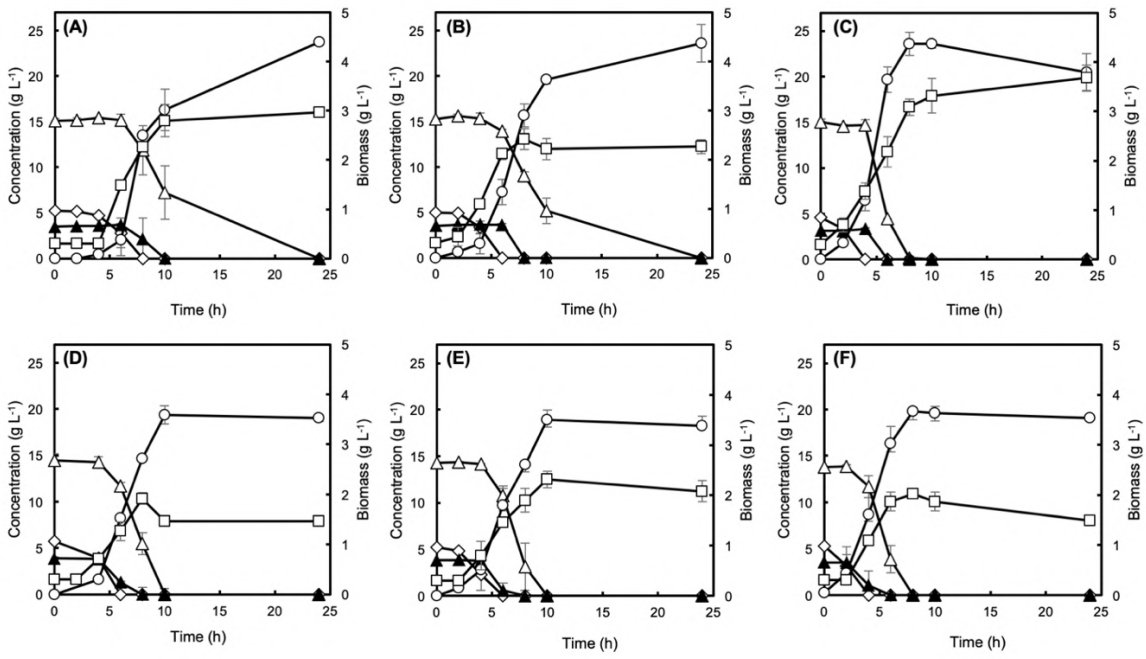


Fig. 4.10. Fermentation time courses of *B. coagulans* from M-GH3 by DSM 2314 (A), A166 (B) and A162 (C); and from BM by DSM 2314 (D), A166 (E) and A162 (F). Glucose (◇), xylose (△), arabinose (▲), LA (○) and biomass (□).

B. coagulans DSM 2314, as a collection strain, presented lower growth and fermentation rates than A166 and A162 isolates when fermenting a medium with inhibitors. Different screening experiments have previously showed that yeast or bacterial strains isolated from selective media presented more fitted characteristics than reference collection strains (Bautista-Gallego et al., 2013; Demeke et al., 2013; Shaghaghi-Moghaddam et al., 2018).

As it was the case for *L. pentosus*, when *B. coagulans* strains were cultured under controlled pH conditions by automatic NaOH addition, they were able to tackle the inhibitory effect produced by acid compounds (acetic acid, formic acid, L-LA, etc.). Since the pH was maintained at 6 along fermentation, CBA were in dissociated form, being unable to cross the cell membrane. As a result, sugars were completely consumed by the three strains in 24 h (**Fig. 4.9**), reaching almost the theoretical maximum (1 g g^{-1}) (**Table 4.6**).

Contrary to *L. pentosus*, *B. coagulans* was severely affected by the CCR effect when fermenting hexoses:pentoses mixtures from M-GH3. In fact, none of the three strains tested was able to consume C5 sugars until glucose was exhausted after 4 – 8 h of culture (**Fig. 4.10**).

Both *L. pentosus* and *B. coagulans* reached almost the LA theoretical yield from GH3 and M-GH3, respectively, showing their ability to successfully consume C6 and C5 sugars in presence of the inhibitors contained in these materials (**Table 4.5** and **4.6**). It should be noted that, when using C5 sugars, *B. coagulans* is able to produce 1 mol mol^{-1} of LA by the PP pathway, while

L. pentosus can only produce 0.67 mol mol^{-1} via the PK pathway (Article I). Consequently, higher LA yields, in terms of g of LA per g of initial sugars were obtained by *B. coagulans* (0.90 ± 0.01 g g^{-1} - 1.00 ± 0.03 g g^{-1}) (**Table 4.6**), while around 0.70 g g^{-1} of LA were produced by the *Lactobacillus* (**Table 4.5**). For this reason, *B. coagulans* strains have become very popular for LA production from lignocellulosic hydrolysates rich in C5 sugars and inhibitory degradation compounds.

Some works have recently studied the effect of inhibitors mixtures from other lignocellulosic hydrolysates in *B. coagulans* strains under controlled pH conditions, showing similar LA yields than the ones obtained in this Thesis (Article IV). When different *B. coagulans* strains were cultivated in wheat straw, sugarcane bagasse and oil palm empty fruit bunch hydrolysates containing different degradation compounds (mostly acetic acid and furfural), LA yields between 0.87 g g^{-1} than 0.97 g g^{-1} were obtained (Aulitto et al., 2017; van der Pol et al., 2016a; Ye et al., 2014) (**Table 4.7**). In the same way, 0.83 g g^{-1} of LA were obtained by an engineered inhibitor tolerant *B. coagulans* GKN316 strain from an inhibitors-rich corn stover hydrolysate, showing 3-fold increase in comparison with the WT strain (Jiang et al., 2016) (**Table 4.7**).

Table 4.7. Lactic acid production from inhibitors-rich agricultural hemicellulosic hydrolysates by *B. coagulans* under controlled pH conditions.

Material	Inhibitors (g L^{-1})		pH control	*B. coagulans*	Y_{LA} (g g^{-1})[1]	Ref[2]
Wheat straw hydrolysate	Furfural 5-HMF Acetic acid	4.00 1.40 3.80	NaOH	MA-13	**0.92**	(1)
Sugarcane bagasse hydrolysate	Furfural Acetic acid Formic acid Glycolyc acid Coumaric acid Vanillin	1.00 2.00 0.05 0.17 0.06 0.02	$Ca(OH)_2$	DSM 2314	**0.87**	(2)
Oil palm empty fruit bunch hydrolysate	Furfural 5-HMF Acetic acid	0.41 0.16 18.39	$Ca(OH)_2$	JI12	**0.97**	(3)
Corn stover hydrolysate	5-HMF Vanillin Acetic acid Formic acid Levulinic acid Syringaldehyde ρ-Hydroxy-benzaldehyde	0.39 0.04 2.00 0.72 0.28 0.02 0.02	$CaCO_3$	GKN316	**0.86**	(4)

[1] Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).
[2] Ref: (1) Aulitto et al., 2017; (2) van der Pol et al., 2016a; (3) Ye et al., 2014; (4) Jiang et al., 2016.

4.1.2.3. Assessment of different *Bacillus coagulans* strains for inhibitors tolerance and biodetoxification

Homolactic fermentation of defined media at increasing concentrations of inhibitors by *B. coagulans*

Different synthetic inhibitors were added to BM to screen the tolerance of the *B. coagulans* strains to the degradation compounds (Article IV). The resulting medium, named BMI, contained 1 g L^{-1} of each CBA and 0.25 g L^{-1} of each furan and phenolic aldehyde (**Table 3.4**).

According to the bacterial growth, a μ_{max} of 0.25 ± 0.02 h^{-1} was obtained in BMI by *B. coagulans* DSM 2314, while significantly higher growth rates, around 0.32 h^{-1}, were reached by A166 and A162 (**Fig. 4.11 A**). Since sugars were totally consumed in all cases at 24 h, similar Y_{LA} were obtained, ranging from 0.81 ± 0.08 g g^{-1} to 0.87 ± 0.01 g g^{-1} (**Fig. 4.11 B**).

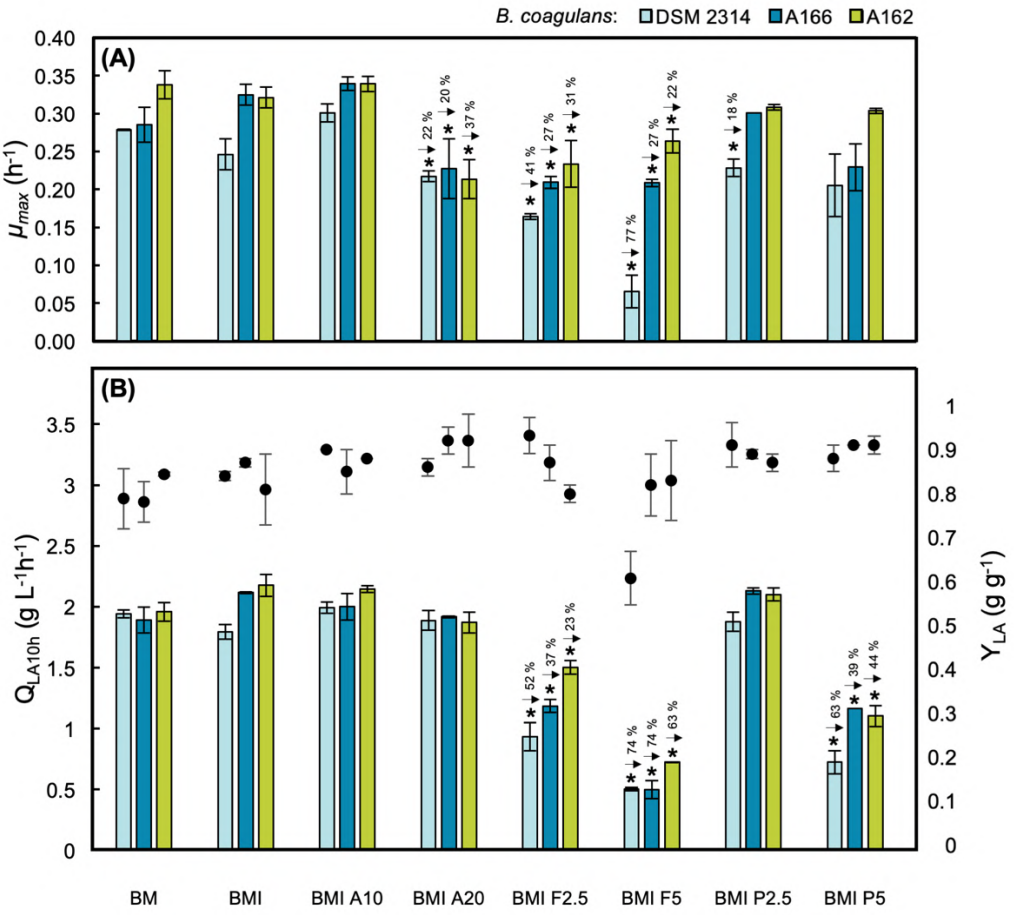


Fig. 4.11. Effect of different inhibitors-rich defined media on growth rate (A), LA yield per initial sugars (B, dots) and LA productivity at 10 h (B, bars) of three *B. coagulans* strains. When the differences between the results obtained with each medium and the control (BM) are significant at p-value < 0.05 (*), the % of reduction in growth rate and LA productivity are indicated.

Important differences in terms of LA productivities were found between the three strains. The highest Q_{LA10h} in BMI was obtained by both A166 and A162 strains (2.12 ± 0.01 g L h^{-1} and 2.18 ± 0.09 g L h^{-1}, respectively) while DSM 2314 only produced 1.79 ± 0.06 g L h^{-1} of LA (**Fig. 4.11 B**, Article IV). However, no differences were observed among the screened strains in terms of μ_{max} and Q_{LA10h} when comparing BMI and the control (BM). Based on the robustness showed by these *B. coagulans* strains in BMI, the concentration of each inhibitors group was increased 5- or 10-fold to ascertain the maximum concentration of these compounds that could be tolerated in a lignocellulosic hydrolysate (**Table 3.4)**.

When increasing the CBA concentrations to 10 g L^{-1} (BMI-A10), μ_{max} and Q_{LA10h} around 0.3 h^{-1} and 2 g L h^{-1}, respectively, were obtained in all cases (**Fig. 4.11 A**). However, when adding 20 g L^{-1} of acids (BMI-A20), lower μ_{max} (around 0.22 h^{-1}) were attained with the three strains, being reduced by 20 % – 37 % in comparison with BM (**Fig. 4.11 A**). Although growth rates were reduced at high CBA concentrations (20 g L^{-1}), no effect on Q_{LA10h} and Y_{LA} were shown in any of the screened strains when compared to BM (**Fig. 4.11 B**). Van der Pol and co-workers (2016) previously demonstrated that 10 g L^{-1} of acetic and formic acids were able to reduce *B. coagulans* DSM 2314 growth by 26 % and 51 %, respectively, in comparison with a reference culture without inhibitors in 48-well plates without pH control (van der Pol et al., 2016b). Although the concentration of CBA was higher in the present work (20 g L^{-1}), lower inhibitory effect on DSM 2314 growth was shown in comparison with the results of van der Pol et al. (2016b), which could be explained because the pH was maintained at 6 along fermentation.

Presumably, at pH 6 most of acetic acid (pKa = 4.8), formic acid (pKa = 3.75) and LA (pKa = 3.8) were not able to cross the cell membrane. For this reason, only growth inhibition took place (**Fig. 4.11 A, Fig. 4.12 A-C**), but LA metabolism was not affected, confirming that pH control is crucial in this kind of processes. During fermentation of xylose-rich defined and lignocellulosic media with *L. pentosus* CECT 4023T (Articles II, III and V), the inhibitory effect of CBA was considerably alleviated when switching from flask fermentation without pH control to automatic NaOH addition in bioreactor. As a result, 43 % and 44 % increase in LA production was attained with defined and lignocellulosic medium, respectively (Articles II, III and V).

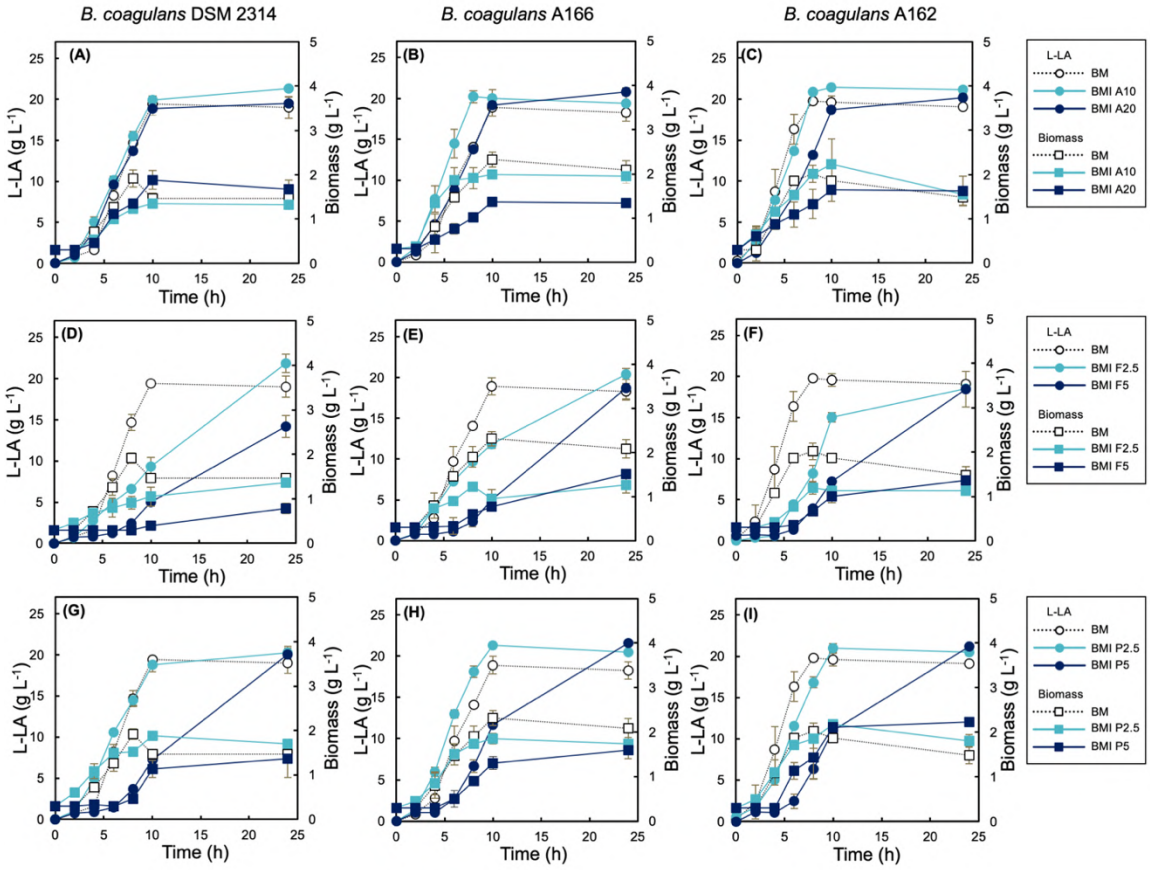


Fig. 4.12. Growth and fermentation time courses of three *B. coagulans* strains from different inhibitors-rich defined media.

Marked differences between the three strains were observed comparing to BM when raising the furans concentrations to 2.5 g L^{-1} and 5 g L^{-1}. According to bacterial growth, μ_{max} was 0.23 ± 0.03 h^{-1} and 0.26 ± 0.02 h^{-1} in BMI-F2.5 and BMI-F5, respectively with A162, while μ_{max} of A166 strain was around 0.21 h^{-1} in both media (**Fig. 4.11 A**). DSM 2314 strain attained the lowest μ_{max}, especially in BMI-F5, reaching 0.07 ± 0.02 h^{-1}, which was significantly lower than the obtained with the A166 and A162 strains (**Fig. 4.11 A**). In fact, when compared to BM, 77 % of reduction in μ_{max} was shown in DSM 2314 from BMI-F5, while μ_{max} in A162 and A166 were only reduced by 22 % and 27 %, respectively (**Fig. 4.11 A**).

As it was the case for fermentation of BMI, A166 and A162 strains appeared to be more robust than the collection strain DSM 2314, presenting higher tolerance to furans, probably owing to the fact that they were isolated from real materials.

Focusing on Q_{LA10h}, A162 reached 1.50 ± 0.05 g L^{-1} h^{-1} and 0.72 ± 0.00 g L^{-1} h^{-1} in BMI-F2.5 and BMI-F5, respectively (**Fig. 4.11 B**). This means that Q_{LA10h} was reduced by 23 % and 63 % in those media in comparison with the control (BM) (**Fig. 4.11 B**). Significantly lower Q_{LA10h} were attained by A166 and DSM2314 strains, especially in BMI-F5 (**Fig. 4.11 B**). In fact, 74 %

of reduction in Q_{LA10h} was shown for both A166 and DSM 2314 strains (**Fig. 4.11 B**), highlighting their lower tolerance to the high furans concentrations in BMI-F5 (5 g L^{-1}) in comparison with A162. BMI-F5 produced the highest inhibitory effect, with a clear influence in growth and LA production (**Fig. 4.12 D-F**). In contrast with the isolated strains, DSM 2314 was unable to completely consume the sugars in BMI-F5 in 24 h (**Fig. 4.11 B**), which resulted in lower Y_{LA} (0.61± 0.06 g g^{-1}) than A166 (0.82 ± 0.07 g g^{-1}) and A162 (0.83 ± 0.09 g g^{-1}).

The addition of 2.5 g L^{-1} of furfural or 5-HMF has been previously reported to produce around 70 % of growth inhibition in *B. coagulans* DSM 2314 (van der Pol et al., 2016b). Similarly, in this Thesis, 77 % of growth rate inhibition was shown in DSM 2314 when grown in BMI-F5 (**Fig. 4.11 A**). Notwithstanding, it is worth mentioning that this medium contained double furans content (5 g L^{-1}). Furthermore, while the fermentation experiments of this Thesis were performed in stirred bioreactors with NaOH 5M automatic addition (Article IV), van der Pol and co-workers performed the screening experiment in 48-well plates. Multi-well cultivation method has a simple set-up, reducing process times and costs, but it presents several limitations like uneven agitation, inefficient oxygen and mass transfer, evaporation effects, non-controlled pH or increased wall growth due to the larger surface:volume ratio (Tajsoleiman et al., 2019). These effects could be the reason for the different tolerance to furans showed in these studies.

Higher furans tolerance was shown by *Sporolactobacillus inulinus*, being able to grow with 3 g L^{-1} of furfural and 5-HMF with only 29 % and 24 % inhibition in growth and LA production, respectively (Bai et al., 2015), showing similar results than A162 in this Thesis.

Pre-cultivation in stressful conditions is a feasible strategy to pre-adapt the bacteria to a certain inhibition effect. Indeed, *B. coagulans* MA-13 showed 50 % improved Q_{LA} from wheat straw hydrolysates, containing acetic acid (3.8 g L^{-1}), furfural (4.0 g L^{-1}) and 5-HMF (1.4 g L^{-1}), when it was pre-cultivated with 30 % hydrolysate (Aulitto et al., 2019). Thus, preculture in presence of inhibitors could be a promising approach to further increase the productivities obtained by A162 strain.

In case of phenolic aldehydes, few differences in μ_{max} (**Fig. 4.11 A**) and biomass (**Fig. 4.12 G-I**) were found among the 3 strains when comparing BMI-P2.5 with the control (BM). Only 18 % of reduction of μ_{max} was shown in DSM 2314 (**Fig. 4.11 A**). Neither LA yields nor productivities (**Fig. 4.11 B**) were affected by the presence of 2.5 g L^{-1} of phenols and the lowest concentrations of other inhibitors (**Table 3.4**). However, a significant inhibition was shown in BMI-P5 in terms of LA productivity, especially for DSM 2314, showing 63 % of reduction in Q_{LA10h} in comparison

with BM (**Fig. 4.11 B**). In the case of A166 and A162, no significant differences were shown between them, and their Q_{LA10h} were reduced by 39 % and 44 %, respectively (**Fig. 4.11 B**). As previously seen with the media with high furans concentrations (BMI-F2.5 and BMI-F5), the isolated strains presented higher LA productivities than the collection strain in presence of high concentrations of phenolic compounds.

Low-molecular weight phenols have been previously reported to be more toxic than aliphatic acids or furans for different bacteria (Gu et al., 2015; Mills et al., 2009). However, the reduction in Q_{LA10h} when compared to BM was more evident in BMI-F5 than in BMI-P5 (**Fig. 4.11 B**).

Indeed, LA fermentation by *Rhizopus oryzae* and *S. inulinus* showed more than 90 % decrease in growth and LA concentration when being exposed to only 1 g L^{-1} of syringaldehyde (Bai et al., 2015; L. Zhang et al., 2016). A different study reported 43 % and 73 % of growth inhibition in *B. coagulans* DSM 2314 in presence of 2.5 g L^{-1} of syringaldehyde and vanillin, respectively (van der Pol et al., 2016b). As it was the case for CBA and furans, the *B. coagulans* strains tested in this Thesis were less inhibited by the presence of high phenols concentrations (5 g L^{-1}) than in the mentioned works. The higher phenols resistance showed in this Thesis could also be explained by the differences in the cultivation method since fermentation in stirred bioreactors offers a higher control of different parameters affecting growth (pH, agitation, mass transfer) than cultivation in 48-well plates (Tajsoleiman et al., 2019).

Altogether, in spite of the reduction in μ_{max} and Q_{LA10h} in different media with the highest inhibitors concentrations (BMI-A20, BMI-F2.5, BMI-F5 and BMI-P5) when compared to the control (BM), it should be highlighted that LA yields close to the theoretical maximum have been reached by A166 and A162 at 24 h in all cases (**Fig. 4.11 B**). Remarkably, the inhibitors concentrations tested in this Thesis (Article IV) were considerably higher than the normally attained during lignocellulose pretreatment (Jönsson et al., 2013). However, higher inhibitors content could be found in lignocellulosic hydrolysates obtained through harsh pretreatment conditions (Roque et al., 2019) or even at high gravity processes (Koppram et al., 2014).

Overall, as it was the case for fermentation of M-GH3, A162 presented the highest LA productivities in defined media with high furans concentrations (BMI-F2.5 and BMI-F5) in comparison with the other strains tested. A162 strain could be efficiently used in future works for fermentation of highly inhibitory lignocellulosic hydrolysates, without needing a previous detoxification step, being able to resist the presence of considerably high levels of inhibitors and able to valorise both C6 and C5 sugars.

Biodetoxification capacity of inhibitors in *Bacillus coagulans*

Since the highest Q_{LA10h} were obtained by A162 strain, furans and phenolic aldehydes concentrations at the end of fermentations were measured to assess the possible biodetoxification responses (Article IV). As explained, no inhibitory effect was produced on L-LA production by CBAs due to the pH control applied. For this reason, the concentrations of these compounds at the end of fermentation were not taken into account.

As can be seen in **Table 4.8**, furans concentrations were reduced during fermentation. Less than 0.02 ± 0.00 g L^{-1} of furfural were detected in all cases at 24 h, removing even 2.5 g L^{-1} of this compound in BMI F5 (**Table 4.8**). In the case of 5-HMF, the final concentration ranged from 0.02 ± 0.00 g L^{-1} to 0.06 ± 0.03 g L^{-1}. As an exception, 1.04 ± 0.02 g L^{-1} of 5-HMF were detected in BMI F5, showing a 58 % of removal (**Table 4.8**). It seems that, under these conditions, up to 1.5 g L^{-1} of 5-HMF could have been removed in 24 h. Considering BMI A20 and BMI P5 media, in which cases 0.25 g L^{-1} of 5-HMF were initially added (**Table 3.4**), 0.15 ± 0.06 g L^{-1} and 0.10 ± 0.03 g L^{-1} of 5-HMF were detected after 24 h (40 % and 60 % of removal) (**Table 4.8**). This fact suggested that the removal of 5-HMF could be hampered by high CBA or phenolic aldehydes content.

Some works have studied microbial mechanisms for furans biodetoxification. Furfural and 5-HMF concentrations from steam-exploded corn stover were considerably reduced after inoculation of *A. nidulans* (Yu et al., 2011). It is also known that these compounds can be reduced into furfuryl and 5-hydroxymethylfurfuryl alcohol, respectively, in *S. cerevisiae* (Liu et al., 2005; Moreno et al., 2015). In fact, Sárvári and co-workers concluded that the cultivation mode of *S. cerevisiae* (aerobic or anaerobic) determined the type of conversion products obtained from furfural (Sárvári et al., 2003). While furfural was exclusively oxidised to furoic acid during respiratory growth, furfural could be converted into furfuryl alcohol in anaerobic conditions to regenerate NAD^+, at the expenses of glycerol formation (Sárvári et al., 2003).

Nevertheless, the involved mechanisms for furans biodetoxification in *B. coagulan*s have not been exhaustively determined (Jiang et al., 2016; Yan et al., 2018; Ye et al., 2014). In the case of *B. coagulans* strains, furfural content completely disappeared after 36 h of fermentation of a xylose-rich medium with 3 g L^{-1} of each furfural and 5-HMF by *B. coagulans* GKN316, while furfuryl and 5-hydroxymethylfurfuryl alcohols were detected in the resulting media (Jiang et al., 2016). Similarly, it was shown that *B. coagulans* JI12 was able to convert 1.08 g L^{-1} of furfural into 0.97 g L^{-1} of furoic acid within 9 h in LB media (Ye et al., 2014).

Table 4.8. Inhibitors removal on different Basal Media with Inhibitors (BMI) and Modified Gardening Hydrolysate 3 (M-GH3) at 24 h of fermentation with *B. coagulans* A162.

Inhibitors		BMI	BMI A 10	BMI A 20	BMI F 2.5	BMI F 5	BMI P 2.5	BMI P 5	M-GH3
Furfural	Concentration (g L^{-1})	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	% Removal	96	96	96	99	99	96	96	95
5-HMF	Concentration (g L^{-1})	0.00 ± 0.00	0.02 ± 0.01	0.15 ± 0.06	0.06 ± 0.03	1.04 ± 0.02	0.02 ± 0.00	0.1 ± 0.00	0.00 ± 0.00
	% Removal	100	92	40	95	58	92	60	100
Vanillin	Concentration (g L^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	% Removal	100	100	100	100	100	100	100	100
Syring-aldehyde	Concentration (g L^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	% Removal	100	100	100	100	100	100	100	100

In the case of phenolic aldehydes, vanillin and syringaldehyde were not detected after 24 h of culture, even in the media with 2.5 g L^{-1} of each compound (**Table 4.8**). Different fungi like *K. huakuii* and *Trichoderma viride* were reported to produce dioxygenase enzymes able to convert different phenolic aldehydes, including syringaldehyde, vanillin and *ρ*-hydroxybenzaldehyde, into less toxic acids (syringic acid, vanillic acid and hydroxybenzoic acid, respectively) (Liu et al., 2020; Xie et al., 2018). In this way, LA yields obtained by *B. coagulans* from pretreated materials (corn stovers and corncobs) were significantly improved by previously inoculating *K. huakuii* or adding the enzyme preparation from *T. viride* (Liu et al., 2020; Xie et al., 2018).

Nevertheless, it should also be highlighted that the addition of these additional steps would increase the production costs associated with enzymes production (enzymatic biodetoxification) or would reduce the sugar concentrations available for fermentation (microbial biodetoxification). For this reason, the use of LA-producing microorganisms with phenolic aldehydes biodetoxification capacities is a preferred option. In this sense, *B. coagulans* GKN316 was able to convert syringaldehyde, vanillin and ρ-hydroxybenzaldehyde into syringyl alcohol, vanillyl alcohol and ρ-hydroxybenzyl alcohol, without the need of using additional microorganisms or enzymes (Jiang et al., 2016).

All these facts, together with the reduction and removal of inhibitors in the current work, suggest a biodetoxification response of *B. coagulans* A162. In short, *B. coagulans* A162 was able to completely remove furfural, vanillin and syringaldehyde and make a considerable reduction of 5-HMF concentration (**Table 4.8**). From these results it should be highlighted that the use of this bacterium can produce a double effect on lignocellulosic media: (1) the efficient conversion of the present sugars into optically pure L-LA and (2) the removal of toxic compounds (Article IV). This system would avoid the need of adding additional enzymatic or microbial detoxification steps before fermentation and could also facilitate the following purification steps.

4.1.3. Novel approaches to facilitate lactic acid production and purification from lignocellulosic liquid fractions

Although the different pH control methods applied during fermentation considerably improved xylose consumption and LA production in WSHs and GHs, their use could imply different limitations in purification steps.

When $CaCO_3$ is added for LA neutralisation and recovery at industrial scale, additional operational steps are always required to dissociate free LA from calcium lactate, consuming high amounts of H_2SO_4, increasing the purification costs, and releasing high amounts of gypsum ($CaSO_4$) as solid waste to the environment (Singhvi et al., 2018). For this reason, the calcium lactate method is not desirable for an environmentally-friendly LA large-scale production.

Automatic pH control in bioreactor is a feasible way to avoid the pH drop and maximise xylose consumption, but it requires high amounts of NaOH, increasing the fermentation costs. Furthermore, base addition for pH adjustment results in high salt (sodium lactate) and ions concentrations in the resulting fermentation broth (Singhvi et al., 2018). Free LA is released from sodium lactate by different downstream processing methods like electrodialysis, which present lower drawbacks than the H_2SO_4-based dissociation of calcium lactate. Even so, it results in increased LA recovery and purification costs (Pleissner et al., 2017b).

In this regard, different strategies were performed in this Thesis to address the different limitations of neutralisation on LA production and purification:

1. **Use of downstream processing methods** for the separation and recovery of free LA from lactate salts and the removal of ions and impurities from the fermented media, derived both from the material and from pH neutralisation (Alexandri et al., 2018). As will be explained in section 4.1.3.1, the LA-rich fermentation broth obtained by *L. pentosus* from GH3 was subjected to a multi-step membrane-based process in order to increase the final LA chemical purity.

2. **Increasing the acid tolerance of the LA producer**. This approach could minimise the need of neutralisation during fermentation, producing free LA instead of calcium/sodium lactate, which could facilitate the downstream processing (Sauer et al., 2008). As will be addressed in section 4.1.3.2, an evolutionary engineering approach was performed on *L. pentosus* to improve the capacity of this strain to ferment xylose at low pH.

4.1.3.1. Membrane-based downstream processing methods for a selective lactic acid recovery and purification

Membrane techniques have emerged as high selective separation methods, which makes them very attractive for the recovery of LA from lignocellulosic materials with high impurities (Pal et al., 2009). In this sense, they are also more environmentally-friendly than other LA purification techniques like calcium lactate precipitation method, avoiding the formation of solid wastes.

For this reason, 10 L of fermentation broth derived from GH3 fermentation with *L. pentosus* in bioreactor were processed using different steps (**Fig. 4.13**). This medium contained around 19 g L^{-1} (189 g) of LA, and other CBA like acetic acid (15.30 g L^{-1}) and formic acid (0.81 g L^{-1}). Residual monosaccharides were not detected since they were totally consumed during fermentation. Around 25 g L^{-1} of ions were measured, being the most abundant: Na^+ (41 %), introduced by NaOH addition during fermentation, SO_4^{2-} (28 %) and K^+ (25 %) (**Table 4.9**).

Bacterial cells and other solid components were removed from the hydrolysate by microfiltration (**Fig. 4.13**). As a result, 13.60 L of permeate with 12.79 g L^{-1} (172.72 g) of LA were recovered (**Table 4.9**), accounting for 91 % of LA recovery. Nanofiltration was applied to further remove biomass and different macromolecules and multivalent anions (**Fig. 4.13**). Indeed, the dry weight of the media was reduced from 9.2 g to 0 after the filtration steps and the resulting 13.50 L of permeate contained 3.04 g L^{-1} (41.04 g) of LA (**Table 4.9**). While more than 80 % of LA is normally recovered after filtration (Alexandri et al., 2018; Sikder et al., 2012), only 22 % of LA recovery was attained in this Thesis because most of LA was retained in nanofiltration retentate due to the low initial volume and LA concentration of the medium. However, nanofiltration was necessary due to the sensitivity of electrodialysis to residual sugars and proteins. Cations were also removed from permeate through a softening step (**Fig. 4.13**).

During the bipolar electrodialysis step, water splitting was produced in the junction of two anion and cation exchanges membranes, thus generating protons and hydroxyl ions that forced the conversion of sodium lactate into the corresponding acid (free LA) and base (NaOH) (Lech and Trusek, 2018) (**Fig. 4.13**). Three streams were produced after bipolar electrodialysis (acid, base and salt). The acid stream, which contained 6.02 g L^{-1} of LA (**Table 4.9**), was used for decolourisation and cation-exchange chromatography to further reduce the salt-ions concentration (Neu et al., 2016).

In fact, 98 % of ions from the fermentation broth were removed and negligible amounts were detected after the whole purification process, switching from 25 g L^{-1} to 0.45 g L^{-1} (**Table 4.9**). Furthermore, almost 100 % of total nitrogen was removed, as well as 92 % of total phosphorous (**Table 4.9**). Although formic acid was not detected in the purified stream, 4.4 g L^{-1} of acetic acid was measured, meaning 71 % of removal (**Table 4.9**). The LA concentration after the downstream processing was 4.61 g L^{-1} (54.86 g) in 11.90 L (**Table 4.9**). The LA recovery increased from 22 % after the filtration steps to 29 % because few mL of pure L-LA were added to the electrodialysis acid stream to ensure the flow of current (**Fig. 4.13**). The LA concentration and purity could be further increased once reducing the water and removing the remaining acetic acid of the medium by vacuum distillation.

Although micro- and nanofiltration and electrodialysis are cost-intensive processes in terms of operational costs, especially for acquisition and maintenance, low energy consumption is produced. Indeed, only 0.82 kW h^{-1}, 0.39 kW h^{-1} and 2.70 kW h^{-1} were consumed in micro-, nanofiltration and electrodialysis, respectively, while the input for power consumption was almost negligible for softening, decolourisation and cation exchanging (**Table 4.9**).

Previous works have demonstrated the suitability of micro-, nanofiltration, electrodialysis and chromatographic techniques for LA recovery. Coffee mucilage and coffee pulp hydrolysates were used for LA production with *B. coagulans* and a downstream processing similar to the one used in this Thesis resulted in 38 % and 23 % of LA recovery, respectively (Neu et al., 2016; Pleissner et al., 2016). While these studies aimed to obtain LA from lignocellulosic materials with high sugars content, the present work was focused in valorisation of the lignocellulosic liquid fraction. For this reason, the main limitation was the low sugars concentration present in these hydrolysates. Although high LA yields were obtained from the present sugars (**Table 4.5**), the LA concentration of GH3 and the medium volume were not sufficiently high to allow a higher LA recovery since most of LA was lost in retentates (**Table 4.9**). In any case, it has been demonstrated that gardening hydrolysates can be used for LA production and purification through membrane-based downstream processing. This method presented different advantages in comparison with the calcium lactate precipitation, especially regarding environmental concerns, allowing an effective removal of impurities from the lignocellulosic material and ions added for pH control during fermentation.

This approach was carried out as a proof of concept and further studies should be performed to optimise the process and reach higher LA recovery and purity. In this sense, different questions need to be considered to reduce the LA loss in the retentates, like (1) collection of higher

volumes of gardening residues for pretreatment, LA fermentation and downstream processing; (2) increasing the sugar concentrations in hydrolysates to reach higher LA concentrations; and (3) recycling of retentates in order to recover part of the rejected LA.

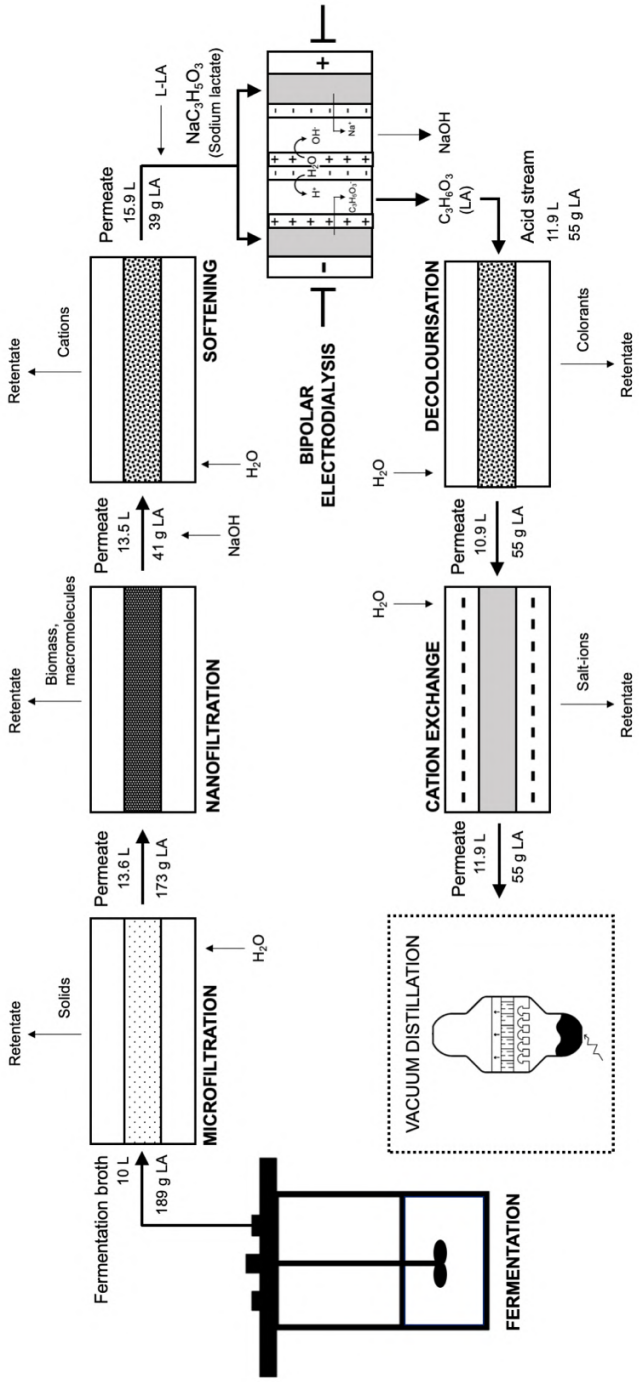


Fig. 4.13. Scheme of the different downstream processing steps performed on fermented GH3 for LA recovery. Vacuum distillation is proposed as an additional step to increase final LA purity and concentration.

Table 4.9. Composition of the different fractions obtained after each downstream processing step.

Step	Power consumption (kW h^{1})	Fractions	Volume (L)	Water addition (L)	Dry weight (%)	pH	N_{kjel} (g L^{-1})	P_{total} (g L^{-1})	Acetic acid (g L^{-1})	Formic acid (g L^{-1})	LA (g L^{-1})
Starting medium	-	-	10.00	-	9.17	6.36	3.54	0.53	15.3	0.81	18.90
Microfiltration	0.82	Permeate	13.60	5.00	5.79	6.39	2.04	0.28	10.2	1.04	12.70
		Retentate	1.70		5.89	6.40	3.56	0.69	6.30	n.n.	7.10
Nanofiltration	0.39	Permeate	13.50	-	1.41	6.33	0.21	0.03	4.88	0.37	3.04
		Retentate	4.20	5.00	8.03	6.42	3.26	0.48	8.42	0.26	16.2
Softening	0.01	-	15.90	2.40	0.00	9.32	0.11	0.03	4.13	0.29	2.46
Bipolar Electrodialysis	2.70	Acid	9.40	8.00	0.00	2.14	0.26	0.05	6.35	0.42	6.02
		Base	10.50	11.00	0.00	12.6	0.17	0.00	0.58	n.n.	n.n.
		Salt	15.30	14.90	0.00	5.25	0.09	0.01	0.20	n.n.	0.21
Decolourisation	0.02	-	10.90	1.60	0.00	1.93	0.02	0.04	5.80	n.n.	5.09
Cation exchanger	0.02	-	11.90	1.00	0.00	2.03	0.01	0.04	4.41	n.n.	4.61

Step	Fractions	PO_4^{3-} (mg L^{-1})	Cl^- (mg L^{-1})	SO_4^{2-} (mg L^{-1})	NO_2 (mg L^{-1})	NO_3 (mg L^{-1})	Na^+ (mg L^{-1})	K^+ (mg L^{-1})	Mg^{2+} (mg L^{-1})	Ca^{2+} (mg L^{-1})	NH_4^+ (mg L^{-1})	Total ions (g L^{-1})
Starting medium	-	366	518	7163	<0.2	<0.2	10305	6261	132	354	306	25.41
Microfiltration	Permeate	242	337	4744	<0.2	<0.2	6836	4106	84.30	220	199	16.77
	Retentate	193	190	2820	<0.2	<0.2	4509	2969	73.80	234	167	11.16
Nanofiltration	Permeate	28.40	249	268	<0.2	<0.2	2080	1274	6.79	21.40	56.20	3.98
	Retentate	385	60.60	8300	<0.2	<0.2	8656	5264	156	400	253	23.47
Softening	-	24.20	209	229	<0.2	<0.2	2491	315	<0.1	7.88	1.80	3.28
Bipolar Electrodialysis	Acid	20.60	346	335	<0.2	0.54	185	8.12	0.33	3.91	1.87	0.90
	Base	<1	10.80	5.50	<0.2	<0.2	6199	410	<0.1	<0.1	1.69	6.62
	Salt	1.92	2.26	14.80	<0.2	<0.2	117	5.20	0.12	9.14	0.56	0.15
Decolourisation	-	30.10	274	269	<0.02	0.40	0.62	0.18	0.02	<0,01	0.14	0.57
Cation exchanger	-	21.60	231	215	<0.2	0.40	0.33	0.11	<0.01	0.11	0.01	0.47

4.1.3.2. Adaptive laboratory evolution to improve xylose consumption at low culture pH

The addition of NaOH and $CaCO_3$ has demonstrated in this Thesis to avoid the pH drop and the accumulation of inhibiting undissociated forms of CBA during LA fermentation, improving LA yields by means of increasing xylose consumption. However, controlled pH fermentations need high amounts of neutralisers (increasing fermentation costs) and additional downstream operations to dissociate LA from its salts and to remove the ions added to the medium during neutralisation (increasing purification costs). For this reason, acid-tolerant LAB are being investigated to reduce or even avoid the need of neutralisers, producing free LA and easing downstream processing (Singhvi et al., 2018).

The ability of *L. pentosus* CECT 4023T to co-metabolise mixtures of sugars from different hemicellulosic media has been demonstrated (Articles II and III). The goal of this section is to subject this LAB to an evolutionary approach to increase the xylose consumption capacity without the need of adding neutralising agents, concomitantly improving tolerance to low pH produced by LA and acetic acid accumulation (Article V). For this purpose, an ALE strategy in serial batch cultivation at increasing xylose concentrations was carried out (**Fig. 4.14**).

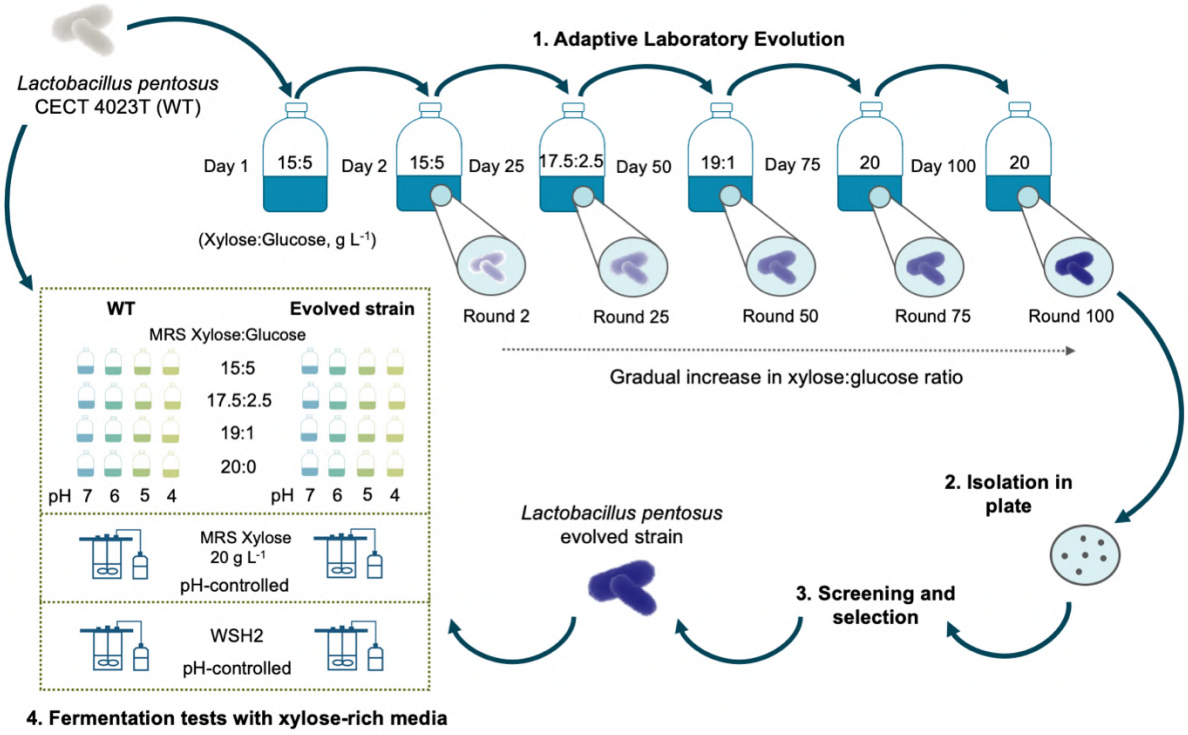


Fig. 4.14. Schematic representation of the ALE experiment applied to increase xylose consumption at low pH, comparing the fermentation performance of the WT *L. pentosus* CECT 4023T with the evolved strain.

ALE approach in batch mode is commonly used in biotechnology as an operational simple method to obtain microorganisms better adapted to certain conditions (Sauer, 2001). When microorganisms are cultivated in defined selective conditions for prolonged periods of time, selection of different mutations leads to the establishment of clones with desired phenotypes (Sauer, 2001). As a result of maintaining a constant or continually increasing selective pressure, serial-transfer experiments have yielded microorganisms with improved stress tolerance and improved rates of substrate consumption (Mans et al., 2018). In this Thesis, an ALE was carried out in flask without pH control, using synthetic medium with different sugars mixtures at increasing concentrations of xylose, *i.e.* MRS (xylose:glucose, g L^{-1}): 15:5, 17.5:2.5, 19:1 and 20:0. The xylose ratio of the mixture was increased when a change in bacterial growth (OD_{600}) and LA production was seen.

Furthermore, in order to check the cumulative changes in xylose consumption and LA production, different batch LA fermentation experiments in flask were carried out with the *L. pentosus* populations obtained as evolution proceeded. Considering that the ALE was run for 100 rounds (around 850 generations), the following populations were chosen for the fermentation experiments: rounds 25, 50, 75 and 100. As can be seen in **Fig. 4.15**, both xylose consumption and $\%Y_{MaxLA}$ at 72 h of fermentation gradually increased from bacterial population of round 0 to round 100 in MRS medium at pH 7 with the different sugar mixtures used during ALE. Remarkable improvements were achieved in all the media tested, especially in those with higher xylose ratio (MRS 19:1 and 20:0). The main changes in xylose consumption and $\%Y_{MaxLA}$ in all media tested were achieved in rounds 75 and 100 (**Fig. 4.15**).

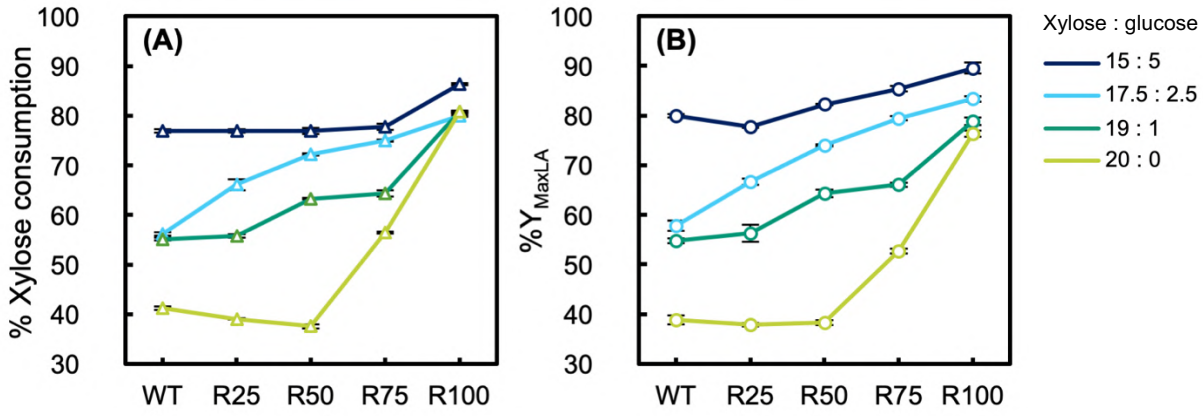


Fig. 4.15. Gradually increase in xylose consumption at 72 h (A) and $\%Y_{MaxLA}$ (B) in batch fermentation of different sugars mixtures by the bacterial populations obtained throughout ALE: round 25 (R25), round 50 (R50), round 75 (R75) and round 100 (R100). Xylose (△), Y_{MaxLA} (○).

After 100 rounds of ALE, a heterogeneous population of cells with stable phenotypes and improved fitness was obtained. Therefore, isolation of different clones was required to find the proper strain for xylose fermentation at low pH values. 4 clones were isolated from the plate with 20 g L^{-1} xylose at pH 5 (clones 1-4) and 2 clones from the plate with 15 g L^{-1} xylose and 5 g L^{-1} glucose at pH 5 (clones 5 and 6) (**Fig. 4.16**), being subsequently utilised for LA fermentation of MRS with xylose 20 g L^{-1} (Article V).

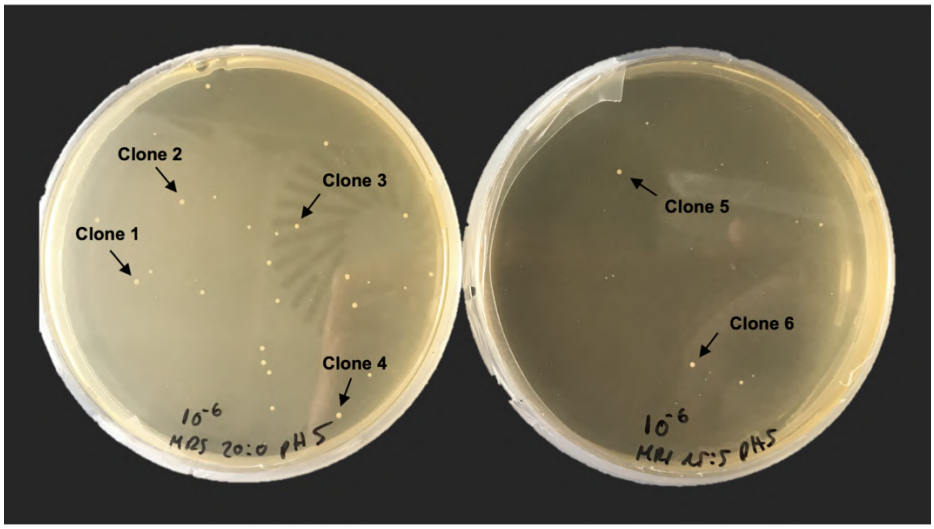


Fig. 4.16. Isolation of clones from the bacterial population with 100 rounds of ALE in MRS at pH 5 with: xylose 20 g L^{-1} (left) and with 15 g L^{-1} of xylose and 5 g L^{-1} of glucose (right). The selected clones are indicated with arrows.

The highest xylose consumption and LA production were obtained with clone 2 (from now on named Mutant Acid Xylose 2 strain, MAX2 strain), reaching 8.01 ± 0.08 g L^{-1} of LA and consuming around 15 g L^{-1} of xylose, followed by clones 4, 3 and 1 (**Fig. 4.17**). As expected, mutants 5 and 6 showed the lowest xylose uptake and LA production since both clones were isolated from a plate containing glucose:xylose mixture as carbon source instead of only xylose. This fact illustrates the importance of maintaining the selection pressure applied during the ALE in the clone isolation, to ensure the selection of the most fitted variants (Tomás-Pejó et al., 2014).

The improvements of the evolved MAX2 strain were subsequently checked in fermentation tests with different sugars mixtures in defined media and in 50 % (v v^{-1}) WSH2, checking the effect of different initial culture pH (7, 6, 5 and 4) and pH control conditions (Article V).

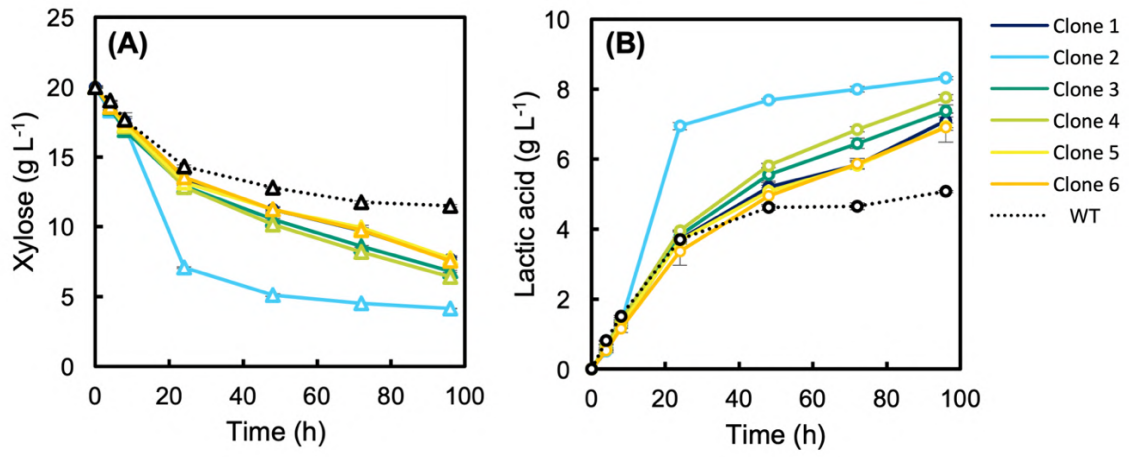


Fig. 4.17. Xylose consumption (A) and LA production (B) with WT strain and clones isolated after ALE. Xylose (△), LA (○).

Comparison of MAX2 and WT strains: Lactic acid fermentation from xylose-rich MRS media in flask at different initial pH values

Figure 4.18 A and **B** show the xylose consumption and the $\%Y_{MaxLA}$ at 72 h of fermentation of MRS media with several xylose:glucose mixtures. It is clearly shown that there was an increase in both xylose consumption and $\%Y_{MaxLA}$ in MAX2 strain when compared with the WT strain in most of the tested conditions. As expected, differences between both strains were more marked when the xylose concentration increased since the selection pressure of the ALE was the gradual increase in xylose ratio.

For this reason, small differences between WT and MAX2 strains were found in MRS 15:5 (**Fig. 4.18**). Notwithstanding, 1.4-fold more xylose consumption and $\%Y_{MaxLA}$ were obtained with MAX2 in MRS 17.5:2.5 at pH 7 and pH 6 in comparison with the WT strain. In this case, around 80 $\%Y_{MaxLA}$ of LA was produced (**Fig. 4.18**). Similarly, with MRS 19:1 at pH 7, 6 and 5, 1.5-fold more xylose consumption and $\%Y_{MaxLA}$ were obtained, reaching 75 %, 70 % and 55 % of the theoretical yield, respectively (**Fig. 4.18**).

A marked increase in xylose uptake and $\%Y_{MaxLA}$ was observed in MRS 20 g L^{-1} xylose medium with MAX2 strain. The evolved strain consumed and produced around 2-fold more xylose and LA, respectively, at pH 7, 6 and 5 than the WT (**Fig. 4.18**). Remarkably, 1.5-fold more xylose consumption and $\%Y_{MaxLA}$ were attained at the lowest pH (pH 4) (**Fig. 4.18**, Article V).

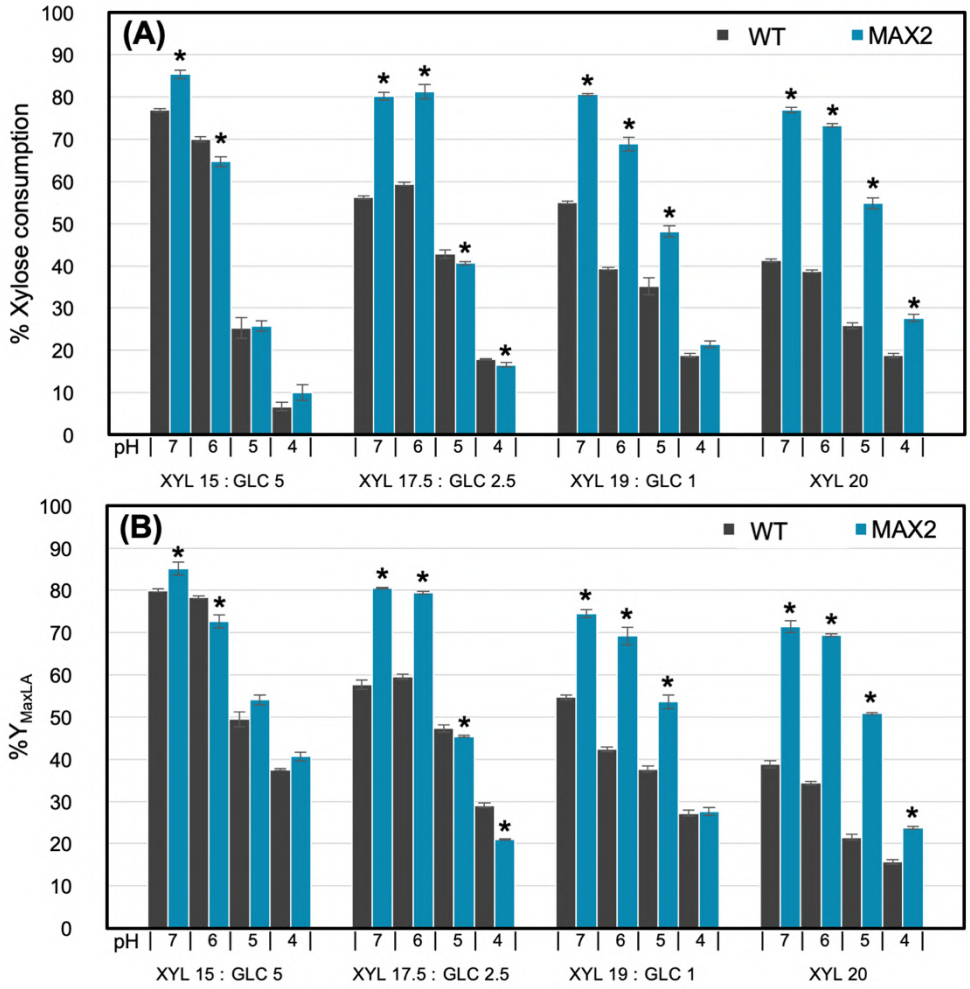


Fig. 4.18. Comparison of xylose consumption (A) and $\%Y_{MaxLA}$ (B) between MAX2 and WT strain from MRS medium with several xylose:glucose mixtures at different pH values. The mean difference between both strains is significant at p-value < 0.05 (*). GLC (glucose), XYL (xylose).

In this same line, other studies also reported enhanced xylose fermentation with different microorganisms after applying ALE. For instance, LA production from C5 sugars was improved in a LA-producing *E. coli* strain by genetic engineering followed by 75 generations of ALE at increasing xylose concentration. As a result, the engineered strains reached even a 95 $\%Y_{MaxLA}$ (Utrilla et al., 2012). ALE also resulted in a 2.5-fold increase of xylose consumption in *S. cerevisiae* when the evolved strain BSPX013 was cultured in a mixture 1:1 of glucose and xylose (Shen et al., 2012). Moreover, xylose consumption in *S. cerevisiae* was increased from 17.70 g L^{-1} to 29.70 g L^{-1}, thus attaining 1.1-fold increase in ethanol production (Li et al., 2017).

As in previous sections, both xylose and glucose were co-consumed during the first hours of fermentation. In fact, the MAX2 strain presented higher xylose uptake than the WT strain in the first 8 h of fermentation of MRS with the following xylose:glucose ratios (g L^{-1}): 15:5,

17.5:2.5 and 19:1 at pH 7. Specifically, consumed xylose at 8 h switched from 0.60 g L^{-1}, 0.50 g L^{-1} and 2.30 g L^{-1} in the WT strain to 3.90 g L^{-1}, 2.20 g L^{-1} and 3.40 g L^{-1} in MAX2, in MRS 15:5, 17.5:2.5 and 19:1, respectively (**Fig. 4.19 A-D**). The increased xylose consumption in presence of glucose was also shown at lower pH values, *i.e*, pH 6 (MRS 17.5:2.5 and 19:1) (**Fig. 4.19 F-G**) and pH 5 (MRS 19:1) (**Fig. 4.19 K**). For this reason, it can be stated that this new strain presented a reduced CCR effect in comparison with the WT since it was able to consume higher amounts of xylose in the presence of glucose. Nevertheless, in the case of pH 4, no differences in sugar consumption were found between WT and evolved strain in the first hours of fermentation of sugar mixtures (**Fig. 4.19 M-O**).

According to MRS xylose 20 g L^{-1} fermentation, the WT strain consumed 8.30 g L^{-1} of xylose and produced 4.66 ± 0.10 g L^{-1} of LA (39 $\%Y_{MaxLA}$) at pH 7 (**Fig. 4.19 D, Fig. 4.18 B**). In contrast, MAX2 strain was able to consume 15.50 g L^{-1} of xylose. As a result of the increase in sugars uptake, MAX2 produced more LA (8.55 ± 0.43 g L^{-1}) (**Fig 4.19 D**), reaching a 71 $\%Y_{MaxLA}$ at the end of fermentation (**Fig. 4.18 B**). 7.19 ± 0.08 g L^{-1} and 9.58 ± 0.05 g L^{-1} of acetic acid were accumulated in the medium by the WT strain and MAX2 strain, respectively (**Fig. 4.19 D**). In this case, a final pH of 4.6 was reached by the WT strain while pH dropped to 4 with MAX2 strain due to its capacity to keep growing and producing LA at lower pH values. Between the 8 h and the 24 h of fermentation (exponential growth phase), the higher xylose uptake of MAX2 led to higher biomass production from xylose, with an OD_{600} of 4.85 ± 0.11, while the WT reached only an OD_{600} = 1.36 ± 0.04 (Article V). The increase in biomass production allowed MAX2 to resist the high acid accumulation along fermentation. When the initial culture pH was adjusted to pH 6 (**Fig. 4.19 H**), similar results to those observed at pH 7 were obtained.

Interestingly, there were also remarkable improvements in xylose fermentation at pH 5. As previously mentioned, 2-fold increase in LA final concentration was attained, switching from 2.58 ± 0.09 g L^{-1} (21 % Y_{MaxLA}) to 6.10 ± 0.04 g L^{-1} (51 % Y_{MaxLA}) of LA, as a result of the higher xylose consumption (**Fig. 4.18 B**, **Fig. 4.19 L**). In the same way than at initial pH 7 and pH 6, acetic acid production increased from 4.83 ± 0.04 g L^{-1} with the WT strain to 7.34 ± 0.19 g L^{-1} with MAX2 strain (**Fig. 4.19 L**).

Xylose consumption and LA production were severely hampered in both strains when the initial pH of the media was 4 (**Fig. 4.19 P**). It is worth to mention that LAB optimal pH for growth ranges from 5.5 to 6.5, being strongly inhibited below pH 4.5 (Juodeikiene et al., 2016a). As explained, culture pH below the acids pKa (3.86 and 4.75 for LA and acetic acid, respectively)

produces the accumulation of their undissociated forms able to cross the cell membrane and produce the cell death. Even so, the MAX2 strain presented an increased xylose consumption and higher LA final concentration in comparison with the WT strain in xylose fermentation with initial pH 4 (**Fig. 4.19 P**). Considering that pH below 6 is certainly stressful for *L. pentosus*, the capacity of MAX2 to grow and produce more LA than the WT strain at pH values around 5 evidenced its improved acidity tolerance. The higher resistance towards low pH of MAX2 strain when compared with the WT strain could be due to a cross-resistance effect produced after prolonged exposure to high LA and acetic acid concentrations during ALE in flask without pH control.

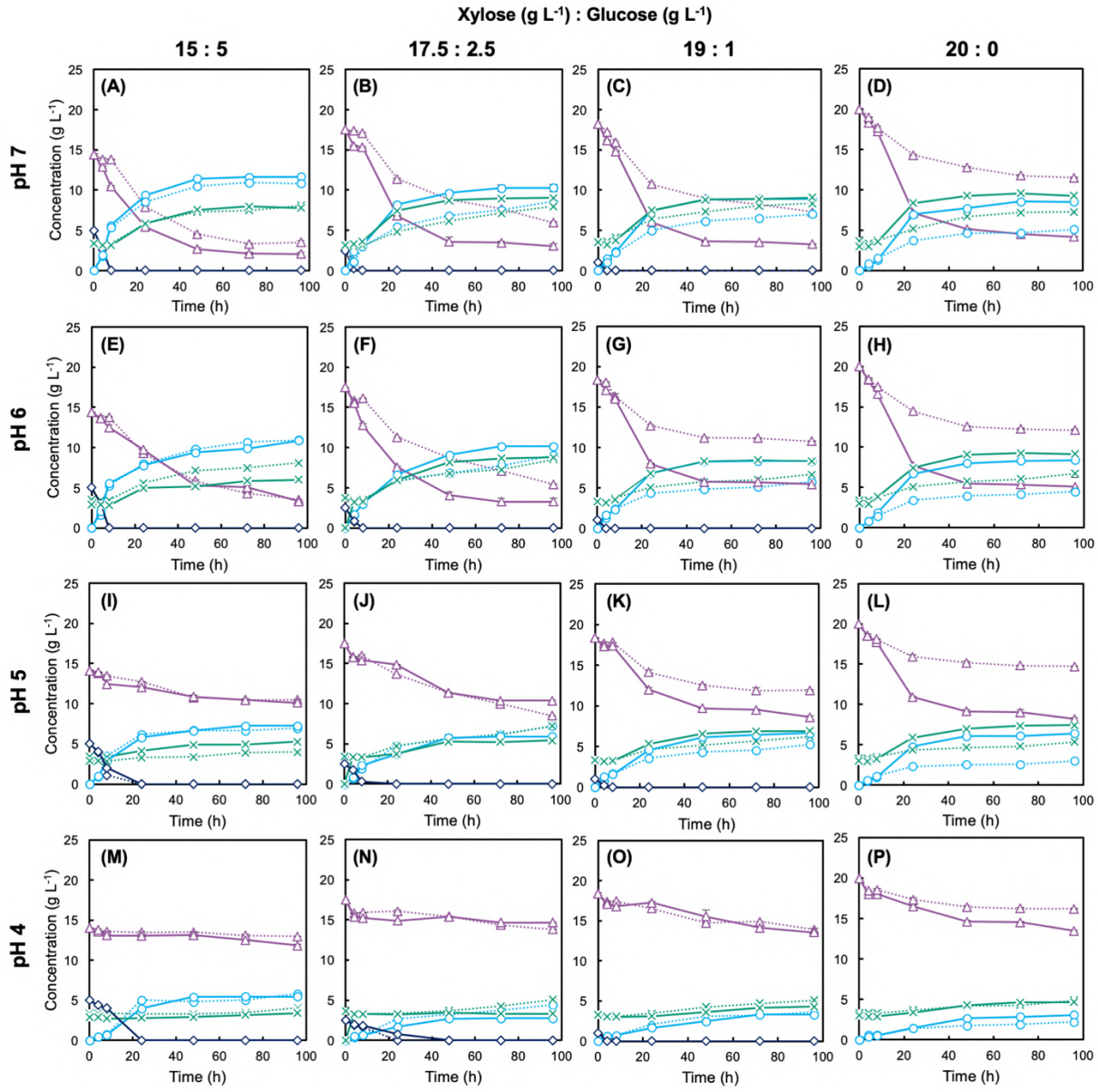


Fig. 4.19. Fermentation time courses of *L. pentosus* CECT 4023T MAX2 (continuous lines) and WT strains (dotted lines) from MRS medium at pH 7, 6, 5 and 4 with different xylose:glucose mixtures. Glucose (◇), xylose (△), LA (○) and acetic acid (×).

Several studies have addressed the improvement of LAB in terms of acid tolerance by different experimental methods. For instance, four *L. rhamnosus* ATCC 11443 strains that could grow at pH 3.6, a *L. plantarum* mutant with 64 % more LA production than the WT at pH 4 and a *Lactobacillus* strain with 3-fold more LA production at pH 4 were obtained after several rounds of genome shuffling (Patnaik et al., 2002; Triratna et al., 2011; Wang et al., 2007). After 70 days of serial exposure to low pH conditions, a *L. casei* strain able to grow at pH 4.3 was obtained, with 14 % increase in LA concentration (Zhang et al., 2012). Error-prone whole-genome amplification method was also used in *L. pentosus*, obtaining a mutant able to ferment glucose at pH 3.8 (Ye et al., 2013a).

The improvements obtained by Patnaik an co-workers and Triratna and co-workers when comparing WT with evolved or shuffled strains were higher than the ones attained in this Thesis at pH 4. However, it is worth to highlight that these studies were carried out with glucose as carbon source, focusing on reaching a remarkable increase in LA production. In this Thesis, the objective was to improve the xylose consumption in spite of the pH drop produced during fermentation. Thus, low pH adaptation has been concomitantly achieved in MAX2 strain.

Fermentation at the LA *pKa* (3.8) or below could reduce the production cost and facilitate LA purification because a higher ratio of the acid is in the free acid form instead of in calcium/sodium lactate. When neutralisers are used to maintain high pH value, LA extensive purification is required (Singhvi et al., 2018). For this reason, the improved xylose fermentation at pH 5 and 4 in MAX2 strain makes the resulting strain very attractive for the valorisation of this sugar from lignocellulosic materials.

Comparison of MAX2 and WT strains: Lactic acid fermentation from xylose-rich MRS media in bioreactor

L. pentosus MAX2 and WT strains were cultivated in MRS medium with 20 g L^{-1} of xylose at initial pH 7 in bioreactor with and without pH adjustment (Article V). Without pH adjustment, the WT strain consumed less than 10 g L^{-1} of xylose (42 %), while MAX2 strain was able to consume more than 15 g L^{-1} (76 %) (**Fig. 4.20 A**, **Table 4.10**). In spite of the rapid decrease in culture pH during fermentation with both strains, dropping from 7 to 4.5 in 24 h (**Fig. 4.20 B**), high growth was produced with MAX2 strain. In fact, 4-fold higher OD_{600} was attained by MAX2 strain, reaching 5.79 ± 0.13, while no more than OD_{600} = 1.49 ± 0.09 was obtained by the WT strain was (**Fig. 4.20 B**). As a result, 41 % and 70 %Y_{MaxLA} were produced by WT and MAX2 strains, respectively (**Table 4.10**). These results were practically identical to those

obtained with 20 g L^{-1} of xylose at pH 7 in flask (**Fig. 4.18**, **Fig 4.19 D**), which clearly confirmed the lower requirements of pH adjustment in the evolved strain.

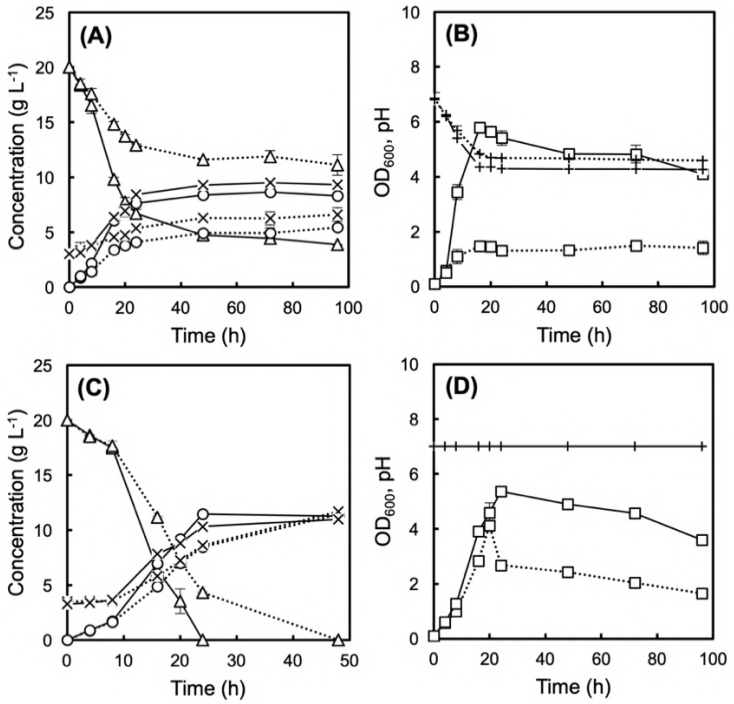


Fig. 4.20. Growth and fermentation time courses of *L. pentosus* CECT 4023T MAX2 (continuous lines) and WT strains (dotted lines) in bioreactor from MRS medium with xylose 20 g L^{-1} under non-controlled (A-B) and controlled (C-D) pH conditions. Glucose (◇), xylose (△), LA (○), acetic acid (×), OD_{600} (□) and culture pH (+).

LA yields from xylose of both *L. pentosus* strains were considerably higher under controlled pH conditions when compared with non-controlled pH conditions in bioreactor and in flask. In fact, both WT and MAX2 strains were able to convert most of the xylose into LA (**Fig. 4.20 C**) when maintaining the pH at 7 (**Fig. 4.20 D**) and no differences in LA yields at 48 h were found since xylose was exhausted in both cases, reaching 94 %Y_{MaxLA} (**Table 4.10**).

Table 4.10. Xylose consumption and lactic acid yields and productivities obtained from MRS medium in bioreactor under non-controlled and controlled pH conditions by *L. pentosus* CECT 4023T wild-type (WT) and MAX2 strains.

Medium	pH conditions	Strain	% Xylose consumption (48h)	Y_{LA} 48h (g g^{-1})[1]	Y_{MaxLA} 48h (%)	Q_{LA}16h (g L^{-1} h^{-1})
MRS Xylose 20 g L^{-1}	Non-controlled	WT	**42**	**0.24** ± 0.01	**41**	**0.21** ± 0.00
		MAX2	**76**	**0.42** ± 0.02	**70**	**0.38** ± 0.00
	Controlled (pH 7)	WT	**100**	**0.57** ± 0.01	**94**	**0.31** ± 0.00
		MAX2	**100**	**0.57** ± 0.01	**94**	**0.44** ± 0.02

[1]Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

Nonetheless, significant differences were found during the first 16 h of fermentation (**Fig. 4.20, Table 4.10**). In the same way than in flask fermentation, as a result of the ALE, MAX2 strain presented higher xylose consumption rate, which implied a 1.4-fold increase in LA productivity at 16 h, *i.e*. Q_{LA16h} = 0.44 ± 0.02 g L^{-1} h^{-1} with MAX2 while the WT only produced 0.31 ± 0.00 g L^{-1} h^{-1} (**Table 4.10**, Article V). The higher xylose consumption of MAX2 is shown in **Fig. 4.20 C**. 68 %, 82 % and 100 % of total xylose was consumed at 16 h, 20 h and 24 h, while the WT only consumed 44 %, 64 % and 78 % at the same fermentation time points. Concerning acetic acid production, 7.84 ± 0.03 g L^{-1} were accumulated in the medium by MAX2 strain at 16 h while the WT strain only reached 5.83 ± 0.08 g L^{-1}, verifying the acceleration of xylose uptake coupled with LA and acetic acid production (**Fig. 4.20 C**).

The WT strain produced 8.48 ± 0.00 g L^{-1} of LA in 24 h, with a maximum OD_{600} of 4.12 ± 0.02 in 20 h and required 48 h of fermentation for xylose exhaustion and reaching the maximum LA concentration (11.41 ± 0.12 g L^{-1}) (**Fig. 4.20 C-D**). By contrast, MAX2 strain was able to reach an OD_{600} of 5.37 ± 0.00, producing the maximum LA concentration, 11.45 ± 0.14 g L^{-1} of LA, at 24 h, (**Fig. 4.20 C-D**).

In this case, it can be inferred that the increase in xylose consumption rate in MAX2 strain was not only due to its increased pH tolerance because culture pH was maintained at 7. One possible mechanism to explain this increased xylose consumption rate may be that the prolonged exposure to this sugar has produced an alteration in the expression of enzymes involved in xylose assimilation, catabolism and fermentation, including xylose transporters, PK enzymes and glycolytic enzymes (Abriouel et al., 2017; Chaillou et al., 1999; Utrilla et al., 2012).

As previously explained, the xylose PEP-PTS in natural *L. pentosus* presents lower sugar affinity than glucose PEP-PTS, which explains the low xylose transport rate, being the limiting factor on xylose metabolism (Chaillou et al., 1999). Considering this, the improved xylose consumption of MAX2 strain could be due to an increased expression of those genes involved in this facilitated diffusion system for xylose transport. For instance, when *E. coli* was engineered and adapted by serial transfers increasing xylose concentration, different changes were found in glycolytic enzymes concentration, but xylose transporter GatC was concluded to be the limiting step in xylose fermentative metabolism (Utrilla et al., 2012).

All things considered, the resulting phenotype of MAX2 strain could be a combination of an increased acid resistance, together with an accelerated xylose transport and metabolism.

Comparison of MAX2 and WT strains: Lactic acid fermentation from xylose-rich wheat straw hydrolysate

In order to determine the feasibility of the new MAX2 strain to ferment lignocellulosic substrates, 50 % (v v^{-1}) WSH2 was used as fermentation media for LA production (Article V). This substrate contained both C6 (glucose) and C5 sugars (xylose and arabinose) and it was previously used for LA fermentation with *L. pentosus* CECT 4023T WT in bioreactor with pH control (section 4.1.1). Although many works have reported the improvements in terms of LA production of new strains obtained by ALE in synthetic media (Triratna et al., 2011; Utrilla et al., 2012; Zhang et al., 2012), few reports have addressed the fermentation of a real lignocellulosic hydrolysate.

In this case, both glucose and arabinose present in 50 % (v v^{-1}) WSH2 were consumed in 8 h of fermentation by the WT and the MAX2 strains (**Fig. 4.21 A**). As in MRS medium, MAX2 strain consumed xylose at higher rates than the WT strain. Specifically, 2.10 g L^{-1}, 7.10 g L^{-1} and 16.40 g L^{-1} of xylose were consumed by the WT strain at 8 h, 16 h and 24 h of fermentation, respectively, while MAX2 strain was able to consume 3.40 g L^{-1}, 10.40 g L^{-1} and 19.80 g L^{-1} of xylose at the same fermentation times (**Fig. 4.21 A**). In the same way as in fermentation of MRS with 20 g L^{-1} of xylose under controlled pH conditions, the productivity at 16 h was 1.4-fold higher with MAX2 strain than with the WT strain, switching from 0.52 ± 0.00 g L h^{-1} to 0.74 ± 0.00 g L h^{-1} of LA (**Table 4.11**, Article V). The reduction in fermentation length is a relevant fact since LA productivity is a key parameter in determining the final production cost. *P. acidilactici* was engineered and evolved to accelerate xylose assimilation by 66 successive transfers of ALE with 40 g L^{-1} of xylose. The resulting strain was used for SSF of wheat straw. As a result, it produced 35 % more LA than the WT strain (Qiu et al., 2018), being similar to the 1.4-fold increase in LA productivity attained at in the present Thesis from 50 % (v v^{-1}) WSH2 (**Table 4.11**).

In this Thesis, high growth was obtained by MAX2 in wheat straw hydrolysate, reaching a maximum OD_{600} = 8.46 ± 0.00 (**Fig. 4.21 B**). This value was even higher than the one reached from MRS with xylose 20 g L^{-1} under controlled pH conditions (**Fig. 4.20 D**) because of the increased sugar content, including glucose and arabinose. By contrast, only OD_{600} = 5.83 ± 0.27 was obtained by the WT strain from 50 % (v v^{-1}) WSH2 (**Fig. 4.21 B**).

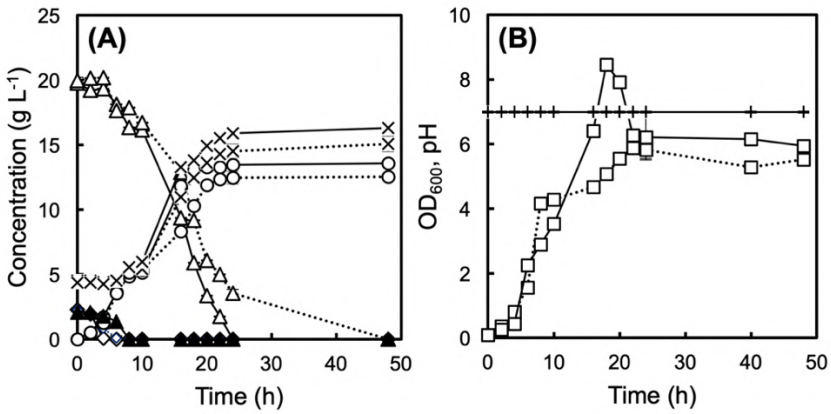


Fig. 4.21. Growth and fermentation time courses of *L. pentosus* CECT 4023T MAX2 (continuous lines) and WT strains (dotted lines) in bioreactor from WSH2 50 % (v v^{-1}) under controlled pH conditions (A-B). Glucose (◇), xylose (△), arabinose (▲), LA (○), acetic acid (×), OD_{600} (□) and culture pH (+).

Nevertheless, few changes could be found at 48 h of fermentation, when xylose was exhausted for both strains. 15.07 ± 0.60 g L^{-1}-16.29 ± 0.00 g L^{-1} of acetic acid were accumulated in the medium taking into account the one produced by pretreatment, added as sodium acetate of MRS, and produced by *L. pentosus* from xylose (**Fig. 4.21 A**). LA concentrations between 12.54 ± 0.00 g L^{-1} and 13.59 ± 0.10 g L^{-1} were produced by WT and MAX2 strains (**Fig. 4.21 A**), reaching similar % Y_{MaxLA} at the end of fermentation 81 % - 86 %Y_{MaxLA} (**Table 4.11**). Although these %Y_{MaxLA} were slightly lower than the ones from MRS fermentation (**Table 4.11**), both WT and MAX2 strains demonstrated a high robustness against the CBA, furans and phenols (**Table 4.5**) under controlled pH conditions as in previous sections.

Table 4.11. Xylose consumption and lactic acid yields and productivities obtained from 50 % (v v^{-1}) wheat straw hydrolysate 2 (WSH2) in bioreactor under controlled pH conditions by *L. pentosus* CECT 4023T wild-type (WT) and MAX2 strains.

Medium	Strain	% Xylose consumption (48h)	Y_{LA} 48h (g g^{-1})[1]	Y_{MaxLA} 48h (%)	Q_{LA}16h (g L^{-1} h^{-1})
WSH2 50 % (v v^{-1})	WT	**100**	**0.52** ± 0.00	**81**	**0.52** ± 0.00
	MAX2	**100**	**0.56** ± 0.01	**86**	**0.74** ± 0.00

[1]Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

The results presented in the previous sections not only demonstrated the ability of *L. pentosus* and *B. coagulans* to metabolise different sugars in presence of inhibitors, but also evidenced their suitability to be applied for valorisation of C5-rich liquid fractions from wheat straw and gardening residues into LA. This approach could be successfully combined with the use of the C6-rich WIS fraction for ethanol fermentation, allowing a complete valorisation of the different fractions obtained after steam explosion. In this sense, the whole sugars content of lignocellulose could be converted into different biofuels and bio-based products.

4.2. Bioethanol production from cellulosic fractions of different lignocellulosic materials

During the last decades, many research efforts have been made to reduce bioethanol production costs, making the cellulosic glucose conversion to ethanol a well-known technology. The advances achieved in this sense have been recently materialised by the starting-up of the first industrial cellulosic ethanol plants in the world (Motola et al., 2018). *S. cerevisiae* is the yeast mainly used for fermentation in these plants because of its high ethanol yields from glucose and high ethanol tolerance. However, its main drawback is that it cannot ferment pentoses (Toor et al., 2020).

Having this in mind, an interesting approach is addressed in this Thesis, directing the cellulosic and hemicellulosic sugars to ethanol and LA fermentation, respectively. Thus, the industrial strain *S. cerevisiae* Ethanol Red was used in this section for fermentation of glucose released from cellulose-rich materials. In this way, different substrates were used in terms of feedstock origin (wheat straw and gardening residues) and pretreated material (whole slurry and WIS fraction). Furthermore, different process configurations were studied to check their effect on bioethanol yields (**Fig. 4.1**).

4.2.1. Separated hydrolysis and ethanol fermentation of gardening WIS

After aqueous extraction of gardening residues, biomass was impregnated with H_2SO_4 0.25 M and subjected to steam explosion pretreatment at 180 °C during 5 min (**Table 4.2**). The resulting slurry was filtered and two different fractions were obtained: 1) A liquid fraction rich in hemicellulosic sugars and degradation compounds (GH3), which was used in previous section 4.1 for LA production (Articles III and IV); and 2) a WIS fraction containing mainly (% w w^{-1}): digestible cellulose, 40.6; lignin, 49.2 and a minor part of hemicellulose, 4.6.

This WIS fraction was used for SHF (Article VI). Firstly, an enzymatic hydrolysis step with 25 % (w v^{-1}) of gardening WIS was carried out. For this purpose, different enzymes, including Cellic-CTec2 and HTec2 as well as Viscozyme L, were simultaneously added at t_0. Cellic-CTec2 and HTec2 are cellulases and endoxylanases preparations, respectively, that in combination, have proven an effective enzymatic hydrolysis on a wide variety of pretreated lignocellulosic materials for bioethanol production (Novozymes, 2010; Rodrigues et al., 2015).

The Viscozyme L preparation from *Aspergillus aculeatus* contains both cellulase and hemicellulase activities and it can act on pectin-like substances found in plant cell walls, reducing the viscosity of the medium. Although most of xylan and arabinan (and their corresponding oligomers) were solubilised in the liquid fraction, hemicellulases were added to the enzymatic cocktail to tackle the possible inhibition that these compounds could produce on cellulases.

After 72 h of enzymatic hydrolysis, a WIS gardening hydrolysate (WIS-GH) was obtained, which contained 102.58 ± 0.51 g L^{-1} of glucose (taking into account monomers and oligomers), meaning 84 % Y_{EH} (**Table 4.12**). This yield was calculated considering the maximum amount of glucose that could be released from the material under these conditions, determined by a mild acid treatment.

When WIS-GH was used as fermentation media, *S. cerevisiae* Ethanol Red showed a fast grow, with a μ_{max} = 0.47 ± 0.03, reaching a maximum OD_{600} of 15.20 ± 0.87 at 14 h of fermentation (**Fig. 4.22**). In fact, the whole glucose content of WIS-GH was consumed in 14 h (**Fig. 4.22**), producing 43.92 ± 0.41 g L^{-1} of ethanol, meaning a Y_{EtOH} = 0.43 ± 0.00 g g^{-1} (83 %$Y_{MaxEtOH}$) (**Table 4.13**). As a result, a bioethanol-rich gardening hydrolysate (E-GH) was obtained.

Table 4.12. Composition of different media used for bioethanol fermentation: water insoluble solids gardening hydrolysate (WIS-GH) and wheat straw slurry.

Concentration (g L^{-1})[1]	**WIS-GH**	
Glucose	102.58 ± 0.51	
Concentration (g L^{-1})[2]	**Wheat straw slurry 10 % (w v^{-1})**	**Wheat straw slurry 15 % (w v^{-1})**
Glucose	41.54[3]; 2.32[4]	62.00[3]; 3.48[4]
Xylose	5.49[3]; 19.00[4]	8.20[3]; 28.50[4]
Arabinose	2.22[4]	3.33[4]
Furfural	0.16	0.25
5-HMF	0.04	0.05
Acetic acid	0.94	1.42
Formic acid	0.23	0.35
Syringaldehyde	<0.01	0.01
Vanillin	0.01	0.02
Ferulic acid	<0.01	0.01
Coumaric acid	0.02	0.03

[1] Measured values.
[2] Calculated values.
[3] Sugars (monomers or oligomers) from WIS fraction.
[4] Sugars (monomers or oligomers) from liquid fraction.

The results obtained for ethanol production from WIS-GH were in good agreement with literature using similar substrates and fermentation approaches. Like in the present Thesis, 0.45 g g^{-1} of ethanol were produced by *S. cerevisiae* from sugarcane bagasse (Neves et al., 2016). Furthermore, between 0.43 g g^{-1} and 0.51 g g^{-1} of ethanol were yielded after SHF of pretreated eucalyptus and spruce wood chips, respectively (Chiarello et al., 2020; Frankó et al., 2015).

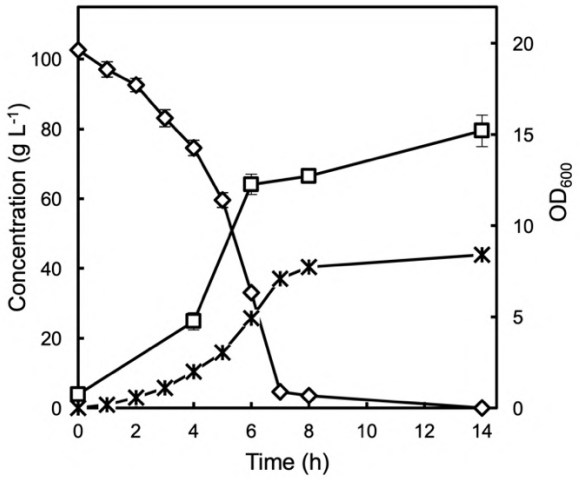


Fig. 4.22. Growth and fermentation time courses of *S. cerevisiae* Ethanol Red from WIS-GH (A). Glucose (◇), ethanol (∗) and OD_{600} (□).

4.2.2. Simultaneous saccharification and ethanol fermentation of wheat straw slurry: effect of different feeding strategies

When using the whole slurry, the filtration step is avoided, reducing operational costs, freshwater consumption and wastewater generation (Tomás-Pejó et al., 2008). However, the effect of different stressful conditions on enzymes and yeasts, including inhibitors presence and high insoluble solids content, should be taken into account when whole slurry is used. In this Thesis, the slurry obtained after H_2SO_4 0.1 M-catalysed steam explosion of wheat straw at 180 °C for 4 min (**Table 3.2**), was used for SSF. This material contained 62 % of glucose and 8.2 % of pentoses. Since *S. cerevisiae* is not capable of fermenting pentoses, ethanol was not produced from this sugar and was not taken into account to calculate the ethanol yields. However, C5 sugars could be further converted into LA through a sequential yeast-bacteria cultivation, following an integrated ethanol and LA production approach.

Different SSF experiments were carried out in order to optimise: (1) glucose release and conversion to ethanol and (2) pentoses release.

When using slurry at 10 % (w v^{-1}) total solids as substrate loading, 18.47 ± 0.66 g L^{-1} of ethanol was produced in batch mode at 72 h of SSF. As a result, a Y_{EtOH} of 0.42 ± 0.02 g g^{-1} was obtained, meaning 83 % of the theoretical yield (**Table 4.13**). While glucose was exhausted, 23.79 ± 0.82 g L^{-1} of pentoses were detected at 72 h (**Fig. 4.23 A**). Taking into account the monomeric C5 present in the slurry at t_0, 8.76 g L^{-1} of xylose were released (**Fig. 4.23 A**), meaning 75 %Y_{EH}.

The ethanol yield obtained after batch SSF fermentation of wheat straw slurry was very similar than the one obtained after batch SHF of WIS-GH. From these results, it can be inferred that the industrial strain Ethanol Red was not significantly hindered by the presence of the solids and inhibitors content at 10 % (w v^{-1}) of total solids in wheat straw slurry. However, the attained ethanol concentration was not high enough to reach the ethanol benchmark (32 g L^{-1}) for an economically viable distillation step. In fact, to reach the minimum ethanol concentration recommended to make distillation costs affordable, at least 14 % – 15 % (w v^{-1}) of substrate loading is normally required (Koppram et al., 2014).

For this reason, substrate loading was increased from 10 % to 15 % (w v^{-1}), which increased the available glucose (monomeric and oligomeric) to 65.48 g L^{-1} (**Table 4.12**). However, the higher presence of solids can promote an uneven distribution of yeast, enzyme, substrate, pH and temperature (Moreno et al., 2019b; Tomás-Pejó et al., 2010). Higher concentrations of inhibitors (**Table 4.12**) could also produce a marked inhibition on enzymes and yeast. To solve this, SSF experiments with wheat straw slurry at 15 % (w v^{-1}) of total solids were performed in fed-batch configuration, adding 7.5 % (w v^{-1}) of substrate at t_0 and 7.5 % (w v^{-1}) at different feeding time (t_8 and t_{24}).

The gradual addition of fresh substrate and enzymes in fed-batch SSF is very appropriate when using the whole slurry because, in this case, enzymes and microorganisms have to face lower concentration of degradation compounds and solids when the culture is started.

When the feed was at t_{24}, ethanol concentration increased from 18.47 ± 0.66 g L^{-1} in case of batch SSF at 10 % (w v^{-1}) to 25.58 ± 0.87 g L^{-1} as a result of the increased substrate content, but Y_{EtOH} was reduced from 0.42 ± 0.02 g g^{-1} to 0.38 ± 0.01 g g^{-1} (**Table 4.13**). Furthermore, around 11.0 g L^{-1} of pentoses were released (63 % of xylose Y_{EH}), reaching a final C5 sugars concentration (xylose + arabinose) of 33.59 ± 0.31 g L^{-1} (**Fig. 4.23 B**). Both xylose hydrolysis and ethanol production were clearly slowed down from 24 h to 72 h (**Fig. 4.23 C**). Presumably,

enzymatic activities and yeast metabolism were already compromised at 24 h of culture, when the feeding time was set. For this reason, an earlier feeding time was tested.

Table 4.13. Ethanol yields obtained from fermentation of different water insoluble solid fractions and whole slurries by ethanologenic yeasts.

Material	Culture mode	Substrate loading % (w v^{-1})	Ethanol (g L^{-1})	Y_{EtOH} (g g^{-1})[1]	Yeast	Ref[5]
WIS fraction						
Gardening	SHF Batch	25	**43.92** ± 0.41	**0.43** ± 0.00	*S. cerevisiae* Ethanol Red	Article VI
Spruce wood	SHF Batch	10	**41.50**	**0.51**	*S. cerevisiae* Ethanol Red	(1)
Eucalyptus wood	SHF Batch	4	**21.40**	**0.43**	*S. cerevisiae* CAT-1	(2)
Sugarcane bagasse	SHF Batch	12	**24.90**	**0.45**	*S. cerevisiae* Thermossac Dry	(3)
Wheat straw	SSF Fed-batch t_{24}[2]	14	**30.70**	**0.28**	*K. marxianus* CECT 10875	(4)
	SSF Fed-batch t_{12}[2]	14	**36.20**	**0.33**		
Whole slurry						
Wheat straw	SSF Batch	10	**18.47** ± 0.66	**0.42** ± 0.02	*S. cerevisiae* Ethanol Red	This Thesis
	SSF Fed-batch t_{24}[2]	15	**25.58** ± 0.87	**0.38** ± 0.01		
	SSF Fed-batch t_8[2]	15	**32.00** ± 0.22	**0.49** ± 0.00		
	SSF Fed-batch $t_{12,36}$[2]	11.25	**21.80**	**0.23**	*S. cerevisiae* F12	(5)
	SSF Fed-batch $t_{6,24}$[2]	11.25	**24.80**	**0.36**		
	SSF Batch	10	**12.70**	**0.30**	*K. marxianus* CECT 10875	(6)
Corncob	SSF Fed-batch	10	**47.0**	**0.35**	*S. cerevisiae* KE6-12	(7)
Lodgepol pine wood	SHF Batch	15	**28.14**	**0.46**	*S. cerevisiae* D5A	(8)
			22.96	**0.38**	*S. cerevisiae* YRH400	

[1] Y_{EtOH} was defined as the bioethanol concentrations (g L^{-1}) at 14 h (gardening WIS fraction) and 72 h (wheat straw slurry) per initial sugars (g L^{-1}).
[2] Feeding times.
[5] Ref: (1) Frankó et al., 2015; (2) Chiarello et al., 2020; (3) Neves et al., 2016; (4) Tomás-Pejó et al., 2009; (5) Tomás-Pejó et al., 2010: (6) Moreno et al., 2016, (7) Koppram et al., 2013; (8) Zhou et al., 2014.

When the feeding time was set at t_8, 32.00 ± 0.22 g L^{-1} of ethanol were produced. In this case, Y_{EtOH} was significantly improved to 0.49 ± 0.00 g g^{-1}, meaning 96 %$Y_{MaxEtOH}$ (**Table 4.13**). As a result, the ethanol benchmark was reached. In addition, 13.17 g L^{-1} of pentoses were released, reaching the same hydrolysis yield as that from 10 % (w v^{-1}) of substrate in batch (75 % of xylose Y_{EH}), meaning 1.2-fold improvement in comparison with fed-batch at t_{24}. In fact, at 72 h of culture, nearly 35.72 ± 2.47 g L^{-1} of xylose were present in the media (**Fig. 4.23 C**).

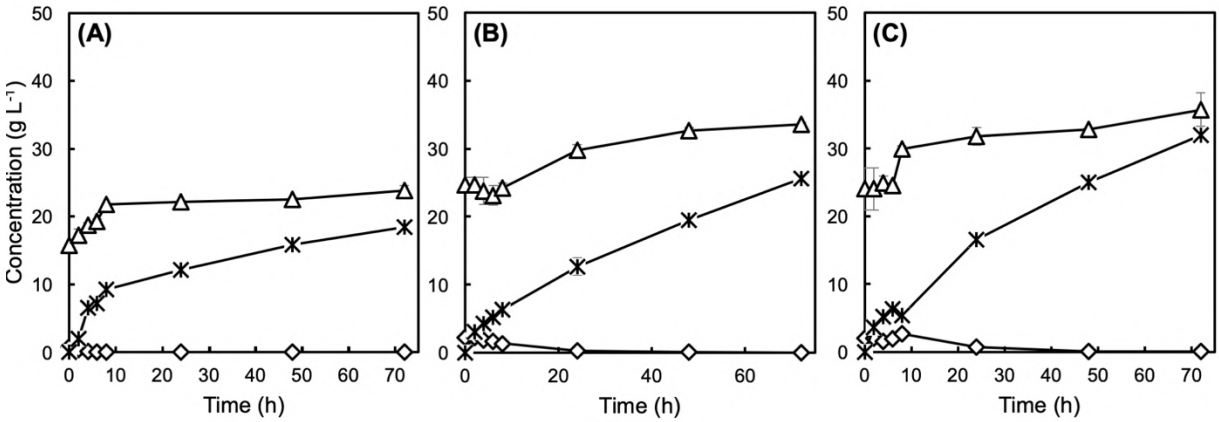


Fig. 4.23. SSF time course for *S. cerevisiae* Ethanol Red from wheat straw slurry 10 % (w v^{-1}) in batch mode (A) and from wheat straw slurry 15 % (w v^{-1}) in fed-batch mode with feeding time at 24 h (B) and 8 h (C) of culture. Glucose (◇), C5-sugars (xylose and arabinose) (△), ethanol (∗).

In the same way as in enzymatic hydrolysis of prehydrolysates for LA production, high xylose and arabinose release was produced by the addition of hemicellulases (Cellic-HTec2 and *β*-xyl) together with cellulases (Cel and *β*-glu) during SSF of the whole slurry. By contrast, lower xylose hydrolysis yields (20 % Y_{EH}) were obtained in previous works with wheat straw slurry when only Cel and *β*-glu were added (Tomás-Pejó et al., 2008).

Thus, the benefits of fed-batch, in comparison with batch SSF, for ethanol production from glucose and for C5 sugars release were apparent when using an early substrate feeding time. In previous works, after screening different fed-batch profiles in SSF of a wheat straw slurry with the C5 and C6-utilising *S. cerevisiae* F12 strain, the highest Y_{EtOH} (0.26 g g^{-1}) was also achieved when feeding at earlier times (Tomás-Pejó et al., 2010) (**Table 4.13**). An earlier substrate feeding results in higher ethanol yields because cell viability and cellular metabolism could be compromised after long culture times (Tomás-Pejó et al., 2010, 2009).

As in previous LA fermentation studies from wheat straw hydrolysates, different degradation compounds, like CBA (acetic and formic acid), furan derivatives (furfural and 5-HMF) and phenolic compounds, were present in the SSF from slurry. In fact, a higher substrate loading had associated a higher concentrations of degradation compounds. As can be seen in **Table 4.12**, the most abundant compounds were CBA, with 1.42 g L^{-1} and 0.35 g L^{-1} of acetic acid and formic acid, respectively, followed by furfural (0.25 g L^{-1}) and finally, by 5-HMF and phenolic compounds (≤ 0.05 g L^{-1}).

Although, yeasts are known to be more acid tolerant than bacteria, the pH drop produced during ethanol fermentation due to CO_2 solubilisation and carbonic acid accumulation can increase the

inhibitory effect of acetic and formic acid, being able to permeate the cell wall. Once the levels of protons inside the cytosol increase by the dissociation of these acids, the excess of protons is pumped out at the expenses of ATP, hampering *S. cerevisiae* growth (Caspeta et al., 2015; Robak and Balcerek, 2018).

Furthermore, lower biomass yield and ethanol productivity have also been shown in the presence of furan derivatives by inactivation of glycolytic enzymes (Caspeta et al., 2015). Furfural induces ROS accumulation in *S. cerevisiae*, producing oxidative damage to different organelles and nuclear chromatin (Allen et al., 2010). In most of yeasts, furans can exert severe inhibitory effects at a concentration of 1 g L^{-1}, whereas up to 2 g L^{-1} are needed to show a significant inhibition with CBA (Caspeta et al., 2015).

Phenolic acids (ferulic and coumaric acid) and aldehydes (vanillin and syringaldehyde) can inhibit yeast metabolism at concentrations as low as 0.2 g L^{-1} (Jönsson and Martín, 2016). Although the individual concentration of each mentioned inhibitor in wheat straw slurry is not supposed to cause great damage in *S. cerevisiae* growth, a synergistic effect between furans, phenolic compounds and acetic, formic and levulinic acid has been found (Mussatto and Roberto, 2004).

The results obtained in batch with slurry at 10 % (w v^{-1}) of total solids (**Fig. 4.23 A**) and in fed-batch t_8 with 15 % (w v^{-1}) of slurry as substrate loading (**Fig. 4.23 C**), evidenced the high solids and inhibitors tolerance of *S. cerevisiae* Ethanol Red. While some ethanologenic yeasts have shown to be incapable of growing from inhibitors-rich lignocellulosic slurries, different inhibitors-tolerant *S. cerevisiae* and *K. marxianus* strains have been isolated or engineered through evolutionary approaches (Tomás-Pejó et al., 2010, 2009).

For instance, high level of toxicity was shown when using the slurry of pretreated lodgepole pine containing (g L^{-1}): 5-HMF, 1.21; acetic acid, 4.90 and formic acid, 0.28. With this substrate, only two *S. cerevisiae* strains out of five were able to grow, producing Y_{EtOH} between 0.38 g g^{-1} and 0.46 g g^{-1} (Zhou et al., 2014) (**Table 4.13**). An SSF of corncob slurry containing around 1.00 g L^{-1}, 2.24 g L^{-1} and 4.65 g L^{-1} of 5-HMF, furfural and acetic acid, respectively, was performed with *S. cerevisiae* KE6-12, yielding 0.35 g g^{-1} (Koppram et al., 2013) (**Table 4.13)**. Similarly, 0.30 g g^{-1} of ethanol was obtained by *K. marxianus* after SSF of laccase-threated wheat straw slurry containing (g L^{-1}): furfural, 0.3; 5-HMF, 0.1; acetic acid, 3.3; formic acid, 2.1 and phenols, 1.4 (Moreno et al., 2016) (**Table 4.13**).

Wheat straw slurry SSF was more challenging than WIS-GH fermentation due to the presence of different inhibitory compounds and the higher viscosity of the media. Even though, after optimising the culture mode and feeding strategy, an ethanol yield as high as 0.49 ± 0.00 g g^{-1} was obtained (**Table 4.13**).

These results verified that fed-batch SSF processes are well-suited approaches to tackle the different limitations found in bioethanol production from the whole slurry. When using slurry, the production costs can be reduced by avoiding the filtration step after steam explosion and the necessity of separated hydrolysis steps for each fraction (*i.e.* WIS and prehydrolysates). At the end of the fed-batch SSF (t_8) experiment, a hydrolysate containing 32.00 ± 0.22 g L^{-1} of ethanol was obtained, named ethanol-rich wheat straw hydrolysate (E-WSH). Furthermore, 35.72 ± 2.47 g L^{-1} of xylose that could not be fermented by Ethanol Red were present in the medium. This medium could be further used for LA fermentation to allow a complete sugars valorisation from pretreated wheat straw, as will be addressed in the next section.

4.3. Integrated bioethanol and lactic acid production

The use of steam-exploded WIS and liquid fractions for the production of bioethanol and LA, respectively, has been demonstrated in this Thesis to be a feasible way to valorise different lignocellulosic materials (Articles II-V). Despite this, valorisation of both cellulosic and hemicellulosic fractions in an integrated way (Article VI) could avoid the need of expensive operations, such as (1) the filtration step for the slurry fractionation and (2) the duplication of different steps like enzymatic hydrolysis and purification that takes place when bioproduction is segregated in two different streams.

Considering these advantages, different works have recently addressed the integrated sequential co-generation of biofuels and biochemicals in one stream, such as bioethanol, biogas, fatty acids, xylitol and LA (Bondesson et al., 2015; Cheng et al., 2010; Demichelis et al., 2017; Kim et al., 2016; Pleissner et al., 2015; Wang et al., 2018; Zhang et al., 2014).

4.3.1. Adaptive laboratory evolution to increase ethanol tolerance of *Bacillus coagulans*

The development of an ethanol tolerant *B. coagulans* DSM 2314 by ALE is proposed as an innovative approach that can be applied in yeast-bacterial sequential fermentation for bioethanol and L-LA co-generation (**Fig. 4.24**) (Article VI). This method can favour the valorisation of different sugars from heterogeneous lignocellulosic materials, making a great contribution to the implementation of integrated biorefinery-based processes.

In order to increase the ethanol tolerance, *B. coagulans* was cultivated in a chemostat with MRS-xylose 40 g L^{-1} at increasing concentrations of ethanol. During chemostat cultivation, a constant environment is maintained by the continuous addition of nutrients coupled with the outflow of cells and fermented medium. Bacterial population does not undergo the different batch cultivation phases, being maintained in the exponential phase (Sauer, 2001). By contrast, as it is the case in serial batch cultivation of LA-producing strains, the exposure of cells to low pH values and/or nutrients-starvation makes the evolved phenotype more unpredictable (Bachmann et al., 2017). In this sense, two different traits were selected during ALE in batch mode of *L. pentosus* in previous section, resulting in both increased xylose consumption rate and increased tolerance to acid culture pH in Section 4.1.3.2 (Article V).

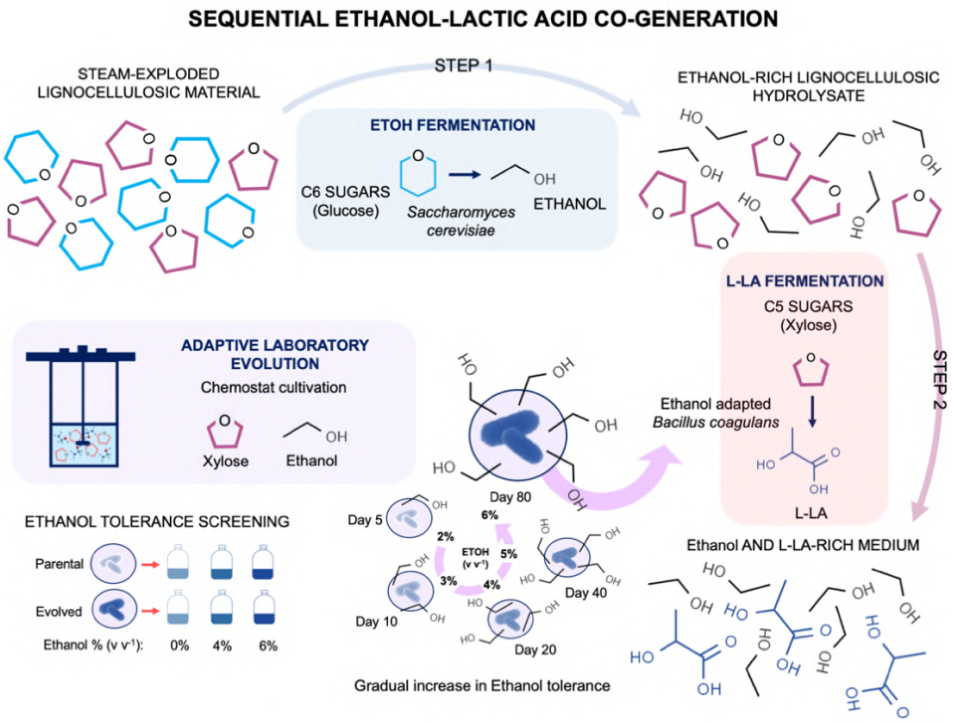


Fig. 4.24. Schematic representation of the ALE experiment to increase ethanol tolerance in *B. coagulans* DSM 2314 for sequential yeast-bacteria cultivation.

Although *B. coagulans* A166 and A162 showed higher L-LA yields from inhibitors-rich lignocellulosic materials in previous studies, DSM 2314 strain was chosen for ALE experiment because the whole genome of this strain is annotated. For this reason, the study of the possible genotypic changes accumulated in this collection strain after the evolutionary engineering would be much simpler. The bacterium was cultivated for 80 days (831 generations) in a bioreactor with xylose as carbon source (40 g L^{-1}) at increasing ethanol concentrations, *i.e.,* 0, 2, 4, 5 and 6 % (v v^{-1}). Higher concentrations were not tested during ALE because 5 % – 6 % (v v^{-1}) of ethanol production is considered a feasible concentration to make distillation economically feasible (Zacchi and Axelsson, 1989). Chemostats with at least 20 generations are normally used for evolution studies, in which cases, changes in fermentation fitness are already expected (Sauer, 2001).

Fig. 4.25 shows changes in bacterial growth (OD_{600}) and L-LA production throughout ALE. When ethanol concentration was firstly increased to 2, 3, 4, 5 and 6 % (v v^{-1}), a decrease in growth and L-LA production was observed for some generations. Nonetheless, once the bacteria became adapted to the environment, growth and L-LA values considerably increased. This phenomenon was more pronounced on day 20 when ethanol concentration was firstly set at 5 % (v v^{-1}) (40 g L^{-1}). In this case, OD_{600} abruptly decreased from 4.7 to 1.2 and the L-LA content in the bioreactor dropped from 33.90 g L^{-1} to 2.60 g L^{-1} (**Fig. 4.25**). Between days 25

and 30 (after 103 generations), a sharp increase in OD_{600} and LA concentration was observed, becoming stable at OD_{600} = 3.2 and 32.00 g L^{-1} of LA (**Fig. 4.25**). This fact indicated that the population had become adapted to the presence of 5 % (v v^{-1}) ethanol.

By the end of ALE, with 6 % (v v^{-1}) of ethanol (47 g L^{-1}) in the medium, an OD_{600} of 5.0 and a L-LA concentration of 35.60 g L^{-1} were reached, almost identical results than the ones obtained at day 2, with 2 % (v v^{-1}) of ethanol (**Fig. 4.25**).

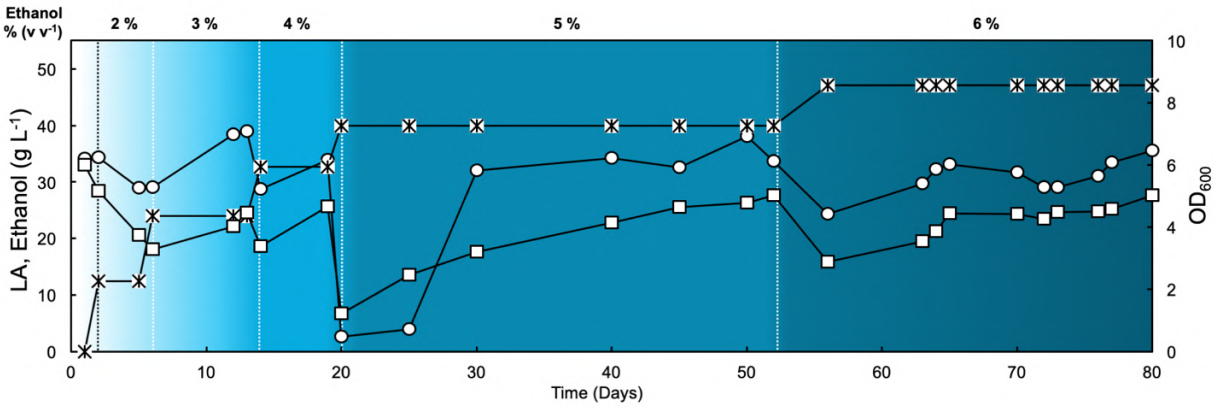


Fig. 4.25. ALE of *B. coagulans* DSM 2314 in chemostat with MRS-xylose 40 g L^{-1} at increasing ethanol concentrations. LA (○), ethanol (⁎) and OD_{600} (□).

Ethanol is known to increase membrane fluidisation and to hinder the performance of membrane-embedded proteins (van Bokhorst-van de Veen et al., 2011). As a result, membrane permeability is increased, facilitating intracellular acidification (Olguin et al., 2009).

Different protection responses to face the ethanol challenge have been reported in LAB. *L. plantarum*, *Lactobacillus acetotolerans* and *Oenococcus oeni* are known to reduce their cell membrane fluidity, by modifying its fatty acids composition after the prolonged exposure to different ethanol concentrations (8 % - 12 % (v v^{-1})), and even changing their cell morphology (Bravo-Ferrada et al., 2015; Grandvalet et al., 2008; Silveira et al., 2004; van Bokhorst-van de Veen et al., 2011; Yang et al., 2017). For instance, a rougher appearance of *L. plantarum* cells (van Bokhorst-van de Veen et al., 2011) and an increased cell length in *L. acetotolerans*, in comparison with the controls (Yang et al., 2017) have been reported. An aggregation of *O. oeni* cells was also observed when increasing the level of ethanol increased in the medium (Elahwany, 2012).

Apart from that, ethanol presence is also involved in the production ROS, causing oxidative damage (Silveira et al., 2004).

Successful ethanol adaptation by ALE has also been reported for the LAB *O. oeni* and the yeasts *Candida intermedia* and *K. marxianus* (Betteridge et al., 2018; Mo et al., 2019; Moreno et al., 2019a). These microorganisms are commonly found in ethanol-rich media since they are used for malolactic fermentation in wine production and ethanol fermentation, respectively. After 330 generations of continuous cultivation of *O. oeni* at increasing ethanol concentrations, a strain with 56 % improvement in the presence of 15 % (v v^{-1}) ethanol was obtained (Betteridge et al., 2018). Xylose consumption was enhanced in *C. intermedia* when fermenting a medium supplemented with 35.50 g L^{-1} ethanol after evolutionary engineering at increasing ethanol and lignocellulosic inhibitors concentration (Moreno et al., 2019a). In the same context, after 100 days of ALE in 6 % (v v^{-1}) ethanol, a population of *K. marxianus* with increased ethanol tolerance up to 10 % (v v^{-1}) was obtained (**Table 4.11**) (Mo et al., 2019).

However, no previous ALE-based ethanol adaptation has been performed on LA-producing bacteria for the co-generation of ethanol and L-LA from lignocellulosic biomass. Results presented in this Thesis indicated that the new LA-producing bacteria obtained by ALE, named as Ethanol and Xylose Adapted (EXA), presented higher ethanol tolerance than the WT strain and could be used as a robust L-LA producer from xylose and ethanol-riches lignocellulosic materials. In order to check the improvements of the evolved strain, L-LA fermentation in MRS with xylose as carbon source (20 g L^{-1}) and different ethanol concentrations, *i.e.* 0 %, 4 % and 6 % (v v^{-1}), was carried out with *B. coagulans* DSM 2314 WT and EXA.

As expected, no changes between both EXA and WT were found in fermentations with defined medium without ethanol, with practically identical fermentation profiles (**Fig. 4.26 A**). The same LA concentration (around 20 g L^{-1}) was obtained, reaching, the maximum theoretical yield from this sugar by the PP pathway (**Table 4.14**). When adding 4 % (v v^{-1}) ethanol, high differences were observed during fermentation time courses. Xylose consumption at 24 h increased from 29 % to 47 % when comparing WT with EXA (**Fig. 4.26 B**). Although the theoretical maximum LA from xylose was reached in both cases by the end of fermentation (120 h), 1.7-fold improvement in L-LA productivity at 24 h was achieved by EXA (**Table 4.14**).

The highest improvements in comparison with the WT strain were attained in fermentation of MRS with xylose 20 g L^{-1} and 6 % (v v^{-1}) ethanol. In this case, only 41 % of xylose was consumed by WT at the end of fermentation, reaching a $Y_{LA} = 0.37 \pm 0.03$ g g^{-1} (**Table 4.14**). On the other hand, EXA was able to consume 93 % of the xylose (**Fig. 4.26 C**). As a result, a LA yield of 0.89 ± 0.02 g g^{-1} was obtained, meaning 2.4-fold increase in comparison with the WT (**Table 4.14**).

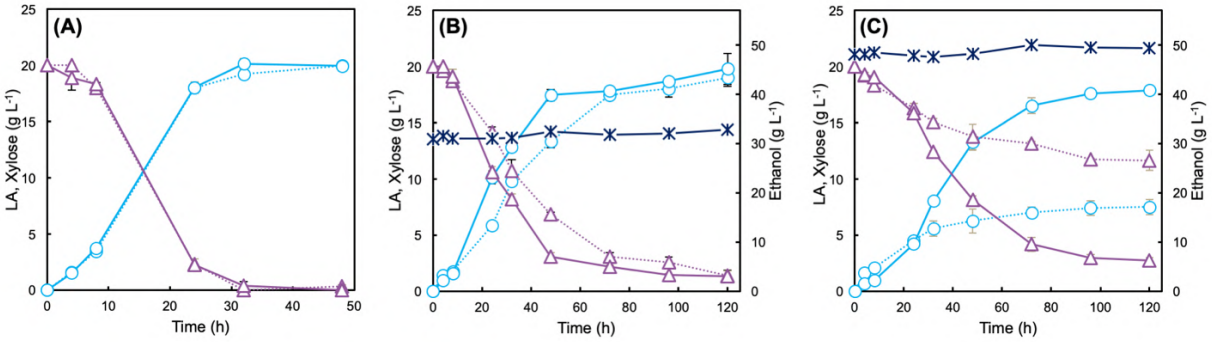


Fig. 4.26. Screening for LA production from xylose with *B. coagulans* DSM 2314 EXA (continuous lines) and WT (dotted lines) in MRS media with 0 % (A), 4 % (B) and 6 % (C) (v v^{-1}) ethanol. Xylose (△), LA (○) and ethanol (∗).

As previously mentioned, malolactic bacteria and yeasts have been adapted to ethanol in other works using ALE (Betteridge et al., 2018; Moreno et al., 2019a). After 330 generations of continuous cultivation of *O. oeni* at increasing ethanol concentrations, a strain with 56 % improvement in fermentation time in the presence of 15 % (v v^{-1}) ethanol was obtained (Betteridge et al., 2018). Xylose consumption was enhanced in *C. intermedia* when fermenting a medium supplemented with 35.50 g L^{-1} ethanol after evolutionary engineering at increasing ethanol and lignocellulosic inhibitors concentration (Moreno et al., 2019a). In the same context, after 100 days of ALE in 6 % (v v^{-1}) ethanol, a population of *K. marxianus* with increased ethanol tolerance up to 10 % (v v^{-1}) was obtained (**Table 5.11**) (Mo et al., 2019)

In bioethanol production processes, the final concentration must be above 40 g L^{-1} in order to achieve an economically-viable bioprocess (Koppram et al., 2014). Since *B. coagulans* EXA was able to produce high L-LA yields even in the presence of nearly 50 g L^{-1} of ethanol, this new LA-producing bacterium was subsequently used for sequential LA-bioethanol production from lignocellulosic materials.

Table 4.14. L-lactic acid production from xylose in MRS medium with increasing ethanol concentrations comparing *B. coagulans* DSM 2314 wild-type (WT) and EXA.

Medium	Ethanol (g L^{-1})	Ethanol % (v v^{-1})	Bacteria	L-Lactic acid (g L^{-1})	Y_{LA} (g g^{-1})[1]	Q_{LA} 24h (g L^{-1} h^{-1})
MRS Xylose 20 g L^{-1}	**0.00** ± 0.00	**0**	WT	**19.97**[2] ± 0.29	**1.00**[2] ± 0.01	**0.75** ± 0.02
			EXA	**19.94**[2] ± 0.16	**1.00**[2] ± 0.01	**0.75** ± 0.02
	32.00 ± 0.01	**4**	WT	**19.02**[3] ± 0.76	**0.95**[3] ± 0.04	**0.25** ± 0.00
			EXA	**19.80**[3] ± 1.38	**0.99**[3] ± 0.08	**0.42** ± 0.02
	47.00 ± 0.00	**6**	WT	**7.49**[3] ± 0.66	**0.37**[3] ± 0.03	**0.19** ± 0.02
			EXA	**17.88**[3] ± 0.42	**0.89**[3] ± 0.02	**0.18** ± 0.01

[1] Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).
[2] Results obtained at 48 h of fermentation.
[3] Results obtained at 120 h of fermentation.

4.3.2. L-Lactic acid production from bioethanol-rich lignocellulosic hydrolysates

The ethanol tolerance of *B. coagulans* WT and evolved populations in MRS media was studied in Section 4.3.1. Following studies will address the LA fermentation of lignocellulosic media in presence of bioethanol previously obtained by yeast fermentation. As explained, two different lignocellulosic materials were used in this Thesis, gardening residues and wheat straw, which were subjected to a pretreatment step, producing different steam exploded slurries.

Two fractions were obtained by filtration of the steam-exploded gardening slurry: a cellulose-rich WIS fraction, and prehydrolysates containing hemicellulosic C6 and C5 oligomers, together with degradation compounds (**Fig. 4.27**). These different fractions were used for separated bioethanol and LA production following SHF methods, resulting in different fermented media rich in LA and bioethanol (E-GH, Section 4.2.1), respectively.

The whole slurry from wheat straw was not subjected to a filtration step and, therefore, it contained all the sugars derived from cellulose and hemicellulose, as well as the inhibitors from sugars and lignin degradation (**Fig. 4.27**). This material was ideal for integrated bioethanol and LA production considering its high sugars heterogeneity. Following this approach, an E-WSH with C5-sugars and bioethanol was obtained by SSF with *S. cerevisiae* (Section 4.2.2), which could be directed to LA fermentation.

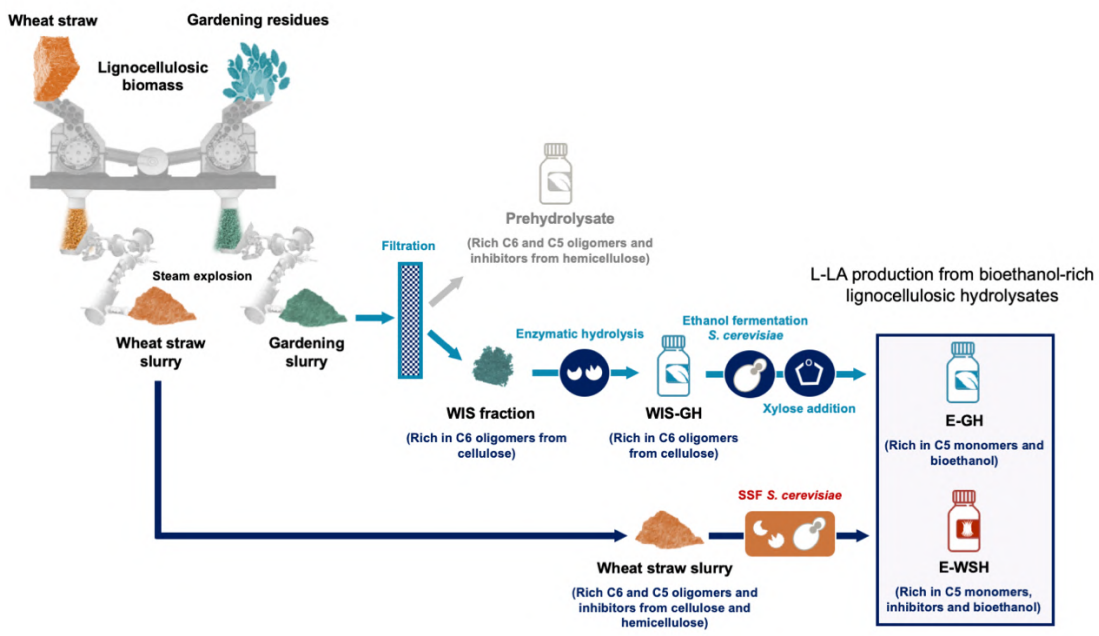


Fig. 4.27. Bioethanol-rich lignocellulosic hydrolysates used for L-LA production with *B. coagulans* DSM 2314 EXA and WT.

Taking into consideration the composition of the different bioethanol-rich media obtained in previous sections, two different experiments were carried out:

1. The E-GH enriched with synthetic xylose was used for fermentation with *B. coagulans* WT and EXA, as a first approximation for integrated bioethanol and LA production (Section 4.3.2.1, Article VI). Since E-GH did not contain degradation compounds, the aim of this study was to check the individual effect of bioethanol on xylose fermentation (**Fig. 4.27**).
2. The E-WSH was used for L-LA production with *B. coagulans* EXA. This medium contained C5 sugars released during SSF of wheat straw slurry, together with bioethanol and degradation compounds. The aim of this experiment was to verify the applicability of integrated bioethanol and LA production approach when using the whole slurry. In this context, the possible synergistic inhibitory effect of degradation compounds and bioethanol on L-LA production is addressed (**Fig. 4.27**).

4.3.2.1. Lactic acid production from xylose in bioethanol-rich gardening hydrolysate

As introduced, an E-GH was firstly used for LA production with *B. coagulans* DSM 2314 WT and EXA. This medium contained around 5 % (v v^{-1}) (40.50 ± 1.65 g L^{-1}) ethanol and 25 g L^{-1} of synthetic xylose (**Table 4.15**). This ethanol concentration was slightly lower than the one obtained after *S. cerevisiae* fermentation due to the processing of the media for L-LA fermentation, including pH adjustment, that produced a dilution effect.

High differences were shown between WT and EXA in E-GH fermentation. While only 40 % of xylose was consumed by WT, the 25 g L^{-1} of xylose were completely consumed by EXA (**Fig 4.28**). As a result, a Y_{LA} of 0.89 ± 0.03 g g^{-1} was reached after 120 h with EXA, meaning 2.6-fold increase in comparison with the WT (**Table 4.15**). It is worth to mention that the Y_{LA} attained in this case from a lignocellulosic medium with 5 % (v v^{-1}) ethanol was identical to the one obtained from MRS medium with 6 % (v v^{-1}) ethanol, in which case 2.4-fold increase was reached. Therefore, these results were consistent with previous experiments in defined medium.

Table 4.15. L-lactic acid production from xylose in bioethanol-rich gardening hydrolysate (E-GH) comparing *B. coagulans* DSM 2314 wild-type (WT) and EXA

Medium	Bioethanol (g L^{-1})	Bioethanol % (v v^{-1})	Bacteria	Y_{LA} 120 h (g g^{-1})[1]	Q_{LA24h} (g L^{-1} h^{-1})
E-GH Xylose 25 g L^{-1}	**40.50** ± 1.65	**5.13**	WT	**0.34** ± 0.01	**0.19** ± 0.00
			EXA	**0.89** ± 0.03	**0.14** ± 0.01

[1]Y_{LA} was defined as the LA concentrations (g L^{-1}) at the indicated fermentation time per initial sugars (g L^{-1}).

L-LA production and sugar consumption in WT and EXA followed a slow rate in the first 48 h of fermentation (**Fig. 4.28**). Most probably, cell energetic supplies were mainly being used to induce and run protection responses against the high ethanol content, which explains the large lag phase and the low L-LA productivity at 24 h of both *B. coagulans* WT and EXA (**Table 4.15**). After that, an increased xylose consumption and L-LA production rate was shown by EXA until the end of fermentation (**Fig. 4.28**).

As mentioned, considerably higher L-LA yields were obtained in fermentation of xylose in E-GH by *B. coagulans* EXA in comparison with the WT. These results verified the increased bioethanol tolerance of the evolved population during C5 sugars conversion into L-LA. The resulting bacteria could be used together with *S. cerevisiae* on integrated bioethanol and LA production, avoiding the need of ethanol removal before the inoculation of the bacteria.

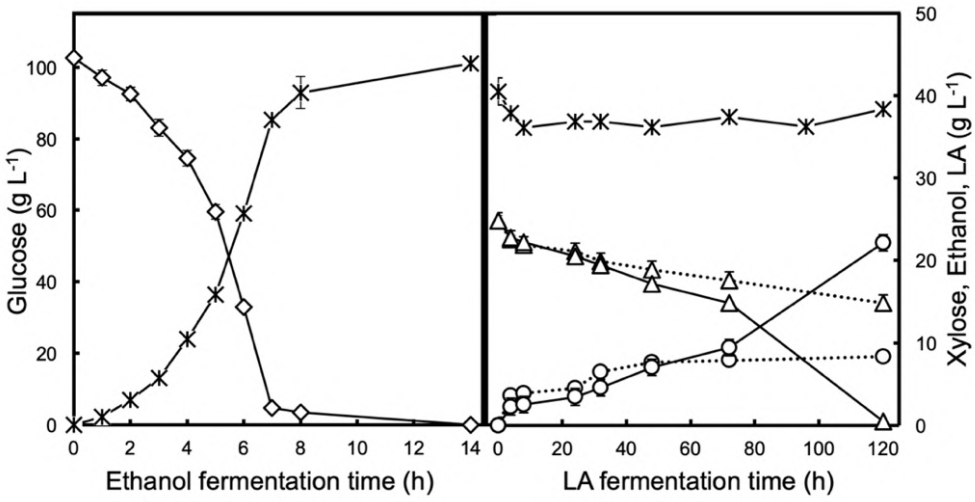


Fig. 4.28. Integrated bioethanol and LA production via sequential cultivation: Ethanol fermentation of WIS-GH with *S. cerevisiae* Ethanol Red and LA fermentation of E-GH with *B. coagulans* DSM 2314 EXA (continuous lines) and WT (dotted lines). Glucose (◇), xylose (△), LA (○) and ethanol (∗).

In this case, glucose released from the WIS fraction of gardening residues was previously used for bioethanol production, while synthetic xylose was subsequently added for L-LA production, as a first approximation (**Fig. 4.28**). C5 sugars contained in a lignocellulosic whole slurry were not used in this study to avoid the combined inhibitory effect of the degradation compounds contained in the material and the ethanol produced on the LA-producing bacteria. Since this proof of concept was successfully validated, following section will address the conversion of both C6 and C5 sugars from a lignocellulosic slurry into bioethanol and L-LA, respectively, via sequential cultivation of yeast and bacteria.

4.3.2.2. Lactic acid production from lignocellulosic C5-sugars in bioethanol-rich wheat straw hydrolysate

Having in mind the high L-LA yields obtained by *B. coagulans* EXA from synthetic xylose in ethanol-rich MRS and bioethanol-rich gardening media, this population was used for fermentation of lignocellulosic C5 sugars from E-WSH. This medium, previously obtained by SSF of wheat straw slurry with *S. cerevisiae* (Section 4.2.2), was used to corroborate the feasibility of integrated bioethanol and LA production.

After SSF, E-WSH contained, approximately, 32 g L^{-1} of bioethanol (**Table 4.13**) and 36 g L^{-1} of C5 sugars (**Fig. 4.23**). However, as it was the case for E-GH, the concentration of these compounds was reduced due to the processing of the media for L-LA fermentation, which produced a dilution effect. After medium processing, 20.70 ± 0.00 g L^{-1} and 26.31 ± 0.40 g L^{-1} of C5 sugars and bioethanol, respectively, were measured in E-WSH 100 % (v v^{-1}) (**Table 4.16**).

Two main differences can be found in LA fermentation of E-WSH in comparison with E-GH: (1) the use of lignocellulosic C5 sugars instead of adding defined xylose, and (2) the presence of inhibitory degradation compounds formed during biomass pretreatment (**Table 4.16**). Although *B. coagulans* DSM 2314 has demonstrated a remarkable tolerance to much higher inhibitors concentrations than the ones present in E-WSH (Article IV), no previous works have reported the possible combined damaging effect of ethanol and lignocellulosic inhibitors on LA-producing bacteria. For this reason, an array of diluted media with different bioethanol and inhibitors concentrations were obtained, resulting in: 100 %, 75 % and 50 % (v v^{-1}) of E-WSH (**Table 4.16**).

Table 4.16. Composition of the bioethanol-rich wheat straw hydrolysates (E-WSH) used in this study.

E-WSH	100 % (v v^{-1})	75 % (v v^{-1})	50 % (v v^{-1})
Sugars (g L^{-1})[1]			
C5 sugars	**26.31** ± 0.40	**19.73** ± 0.30	**13.15** ± 0.20
Bioethanol[1]			
(g L^{-1})	**20.70** ± 0.00	**16.00** ± 0.00	**11.00** ± 0.00
(% v v^{-1})	**2.62**	**2.03**	**1.39**
Inhibitors (g L^{-1})[2]			
Furfural	**0.25**	**0.19**	**0.13**
5-HMF	**0.05**	**0.04**	**0.03**
Acetic acid	**1.42**	**1.07**	**0.71**
Formic acid	**0.35**	**0.26**	**0.18**
Syringaldehyde	**0.01**	**< 0.01**	**< 0.01**
Vanillin	**0.02**	**0.02**	**0.01**
Ferulic acid	**< 0.01**	**< 0.01**	**< 0.01**
Coumaric acid	**0.02**	**0.02**	**0.01**

[1] Measured values.
[2] Calculated values.

In the first case, when using the non-diluted E-WSH 100 % (v v^{-1}), low sugars consumption was attained (48 %) (**Fig. 4.29**). Indeed, just 12.50 g L^{-1} of C5 sugars were consumed at 120 h of culture (**Fig. 4.30 A**). As a result, 9.49 ± 0.05 g L^{-1} of L-LA were produced (**Fig. 4.30 A**) and a low Y_{LA} was obtained, 0.36 ± 0.02 g g^{-1} (**Fig. 4.29**). Surprisingly, it seems that a bioethanol concentration of 20.70 ± 0.00 g L^{-1} coupled with around 0.30 g L^{-1}, 1.77 g L^{-1} and 0.05 g L^{-1} of furans, CBA and phenolic compounds, respectively, (**Table 4.16**) in E-WSH 100 % (v v^{-1}) can preclude C5 sugars consumption.

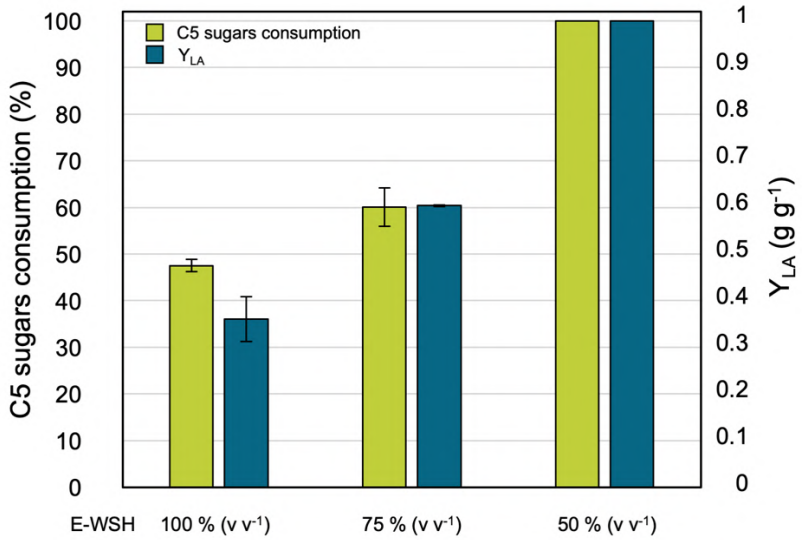


Fig. 4.29. Effect of different concentrations of E-WSH in C5 sugars consumption and LA yields obtained by *B. coagulans* DSM 2314 EXA.

After diluting E-WSH to 75 % (v v^{-1}), the resulting medium contained 19.73 ± 0.30 g L^{-1} of C5-sugars, together with 16.00 ± 0.00, 0.23, 1.33 and 0.04 g L^{-1} of bioethanol, furans, CBA and phenolic compounds, respectively (**Table 4.16**). Under these conditions, *B. coagulans* EXA reached higher sugars consumption, around 60 %, producing 11.91 g L^{-1} of L-LA, which resulted in a Y_{LA} = 0.60 ± 0.00 g g^{-1} (**Fig. 4.29, Fig. 4.30 B**).

It seems that the bioethanol content in E-WSH 100 % and 75 % (v v^{-1}), combined with the mentioned inhibitors concentrations in these media, were able to inhibit the C5 sugars consumption in *B. coagulans* EXA, resulting in low Y_{LA} (**Fig. 4.29**). In fact, this evolved bacterium was able to tolerate between 2- and 2.5-fold higher bioethanol concentrations in E-GH (40.50 g L^{-1}) (**Table 4.15**), in which case lignocellulosic inhibitors were not present.

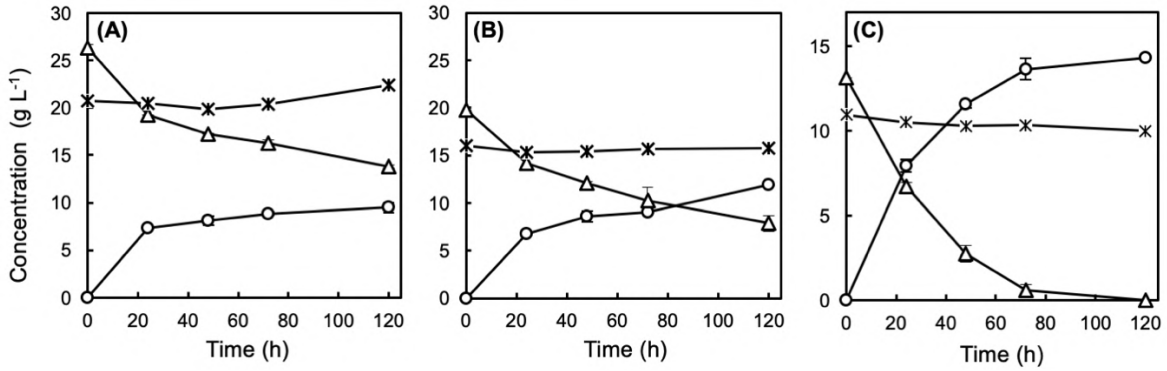


Fig. 4.30. Integrated bioethanol and LA production via sequential cultivation: LA fermentation time courses of E-WSH 100 % (A), 75 % and 50 % (v v^{-1}) with *B. coagulans* DSM 2314 EXA. C5 sugars (△), LA (○) and ethanol (∗).

The combined inhibitory effect of bioethanol and degradation compounds has been previously observed in other fermentative microorganisms. For instance, 21 % of growth inhibition was shown in *S. cerevisiae* NGY10 when cultivated in a medium containing (g L^{-1}): ethanol, 39.45; furfural, 0.62; 5-HMF, 0.75; and acetic acid, 1.89 (Pandey et al., 2019).

When mixtures of inhibitors are present during fermentation, a synergistic effect can take place. For instance, the alteration of cell membrane permeability produced by CBA allows other inhibitors to enter the cell, increasing their damaging effect on enzymes and structures (Caspeta et al., 2015). However, the culture pH was maintained at 5 by $CaCO_3$ addition in the current work, so a significant contribution of undissociated CBA to the whole inhibitory effect was not expected (Caspeta et al., 2015).

In a similar way, bioethanol has been shown to produce an alteration of cell membrane, increasing its fluidisation and permeability by means of altering the phospholipids composition and the membrane-embedded proteins performance (van Bokhorst-van de Veen et al., 2011). Thus, the accessibility of furans and phenols to the cytosol could have been facilitated by the presence of bioethanol in *B. coagulans*. Although the amounts of furans and phenols tested in previous works were considerably higher (Article IV), higher concentrations of these compounds could have entered the cell due to the ethanol presence.

In addition to that, both furans and bioethanol are involved in the formation of ROS (Allen et al., 2010; Pérez-Gallardo 2013), producing a massive oxidative damage on glycolytic enzymes, organelles and chromatin. As a result, energy and NADH molecules would be required for inhibitors and ROS biodetoxification, at the expenses of C5 sugars conversion into LA via PP pathway (Papadimitriou et al., 2016; Qin et al., 2010). Oxidative damage has also shown to produce translation repression, increasing the levels of non-translating messenger RNAs (Iwaki et al., 2013).

In this sense, addition of different concentrations of ethanol and furans have shown to produce messenger RNA accumulation in the form of granules in *S. cerevisiae* (Iwaki et al., 2013; Kato et al., 2011). While messenger RNA granules formation has not been reported in prokaryotes, the accumulation of polyphosphate granules has been shown both in yeasts and LA-producing bacteria (Gray and Jakob, 2015). Polyphosphate granules formation can be induced by the presence of oxidative stress (Gray and Jakob, 2015), which can be produced by both ethanol and furans presence. In this way, these granules have demonstrated to be involved in different activities like inhibiting RNA degradation and acting as an ATP reservoir (Alcántara et al., 2014). Considering that microorganisms induce common stress responses to the damaging effects produced by ethanol and lignocellulosic degradation compounds, it is conceivable that these compounds can produce a synergistic inhibition. As mentioned, this fact would explain the low sugars consumption of *B. coagulans* EXA in E-WSH 100 % and 75 % (v v^{-1}).

A different trend was shown during fermentation of E-WSH 50 % (v v^{-1}). In this case, total sugars consumption was achieved at 120 h of fermentation, producing 14.33 ± 0.00 g L^{-1} of LA (**Fig. 4.30 C**), reaching the LA theoretical maximum from initial sugars (Y_{LA} = 1.00 ± 0.00) (**Fig. 4.29**). In this case, the LA yield obtained was 2.8-fold higher than with the non-diluted medium. Apparently, a bioethanol concentration of 11.00 ± 0.00 g L^{-1} was not high enough to produce a significant inhibitory effect in combination with the inhibitors contained, *i.e.*, 0.15, 0.89 and 0.03 g L^{-1} of furans, CBA and phenols, respectively (**Table 4.16**). In essence, *B.*

coagulans DSM 2314 EXA was able to completely consume lignocellulosic C5 sugars in E-WSH 50 % (v v^{-1}) in spite of the presence of inhibitors and bioethanol previously formed during pretreatment step and SSF for bioethanol production step, respectively.

The evolved *B. coagulans* obtained in the present study was able to tolerate even 40.50 ± 1.65 g L^{-1} of bioethanol in E-GH (**Table 4.15**) and 11.00 ± 0.00 g L^{-1} of bioethanol in E-WSH 50 % (v v^{-1}) (**Table 4.16**), without the need of removing this compound before LA fermentation. The integrated bioethanol and LA production approach have been validated, producing both biofuels and a bio-based product from different types of lignocellulosic sugars in one stream.

In a higher-scale fermentation, it would be possible to simultaneously purify LA and ethanol at the end of the process. Reactive distillation can be used in this sense, taking advantage of esterification of both L-LA and ethanol to allow a direct purification of ethyl lactate, which can be distillated in one step (Komesu et al., 2015; Vudata et al., 2014). Ethyl lactate can be used as an industrial relevant product, but it can also be subjected to hydrolysis with water in presence of an ion-exchange resin in order to separate both products and yield pure ethanol and PLA-grade L-LA (Komesu et al., 2015).

As mentioned, when using the whole slurry for integrated bioethanol and LA production, different production costs could be reduced in comparison with the separated approach, avoiding the slurry filtration and the duplication of enzymatic hydrolysis needed for both WIS and prehydrolysate fractions. Furthermore, after optimising different conditions, the two fermentation stages could be sequentially performed in the same vessel, without the need of media processing in between them. Finally, downstream processing costs could also be reduced by using reactive distillation.

4.4. Comparison of different approaches for bioethanol and lactic acid co-production

Co-generation and multi-generation of different products from residual substrates is being recently studied as a promising approach for future biorefineries. In this sense, two different approaches were addressed in this Thesis to achieve a total valorisation of lignocellulosic sugars: separated and integrated bioethanol and LA production.

Both wheat straw and gardening residues were used for separated bioethanol and LA production. In this sense, the different fractions obtained after filtration of steam exploded slurries were subjected to SHF in separated streams.

According to LA fermentation of C6 and C5 sugars from hemicellulosic prehydrolysates, L,D-LA yields of 0.52 ± 0.00 g g^{-1} (81 $\%Y_{MaxLA}$) (**Table 4.5**) and 0.56 ± 0.01 g g^{-1} (86 $\%Y_{MaxLA}$) (**Table 4.11**) were obtained by *L. pentosus* WT and MAX2 strains, respectively, from WSH2 50 % (v v^{-1}). On the other hand, while *L. pentosus* yielded around 0.70 g g^{-1} of L,D-LA (95 $\%Y_{MaxLA}$) (**Table 4.5**) from GH3, between 0.90 ± 0.01 g g^{-1} and 1.00 ± 0.03 g g^{-1} (90 % - 100 $\%Y_{MaxLA}$) of L-LA were obtained from M-GH3 by different *B. coagulans* strains (**Table 4.6**).

On the other hand, *S. cerevisiae* Ethanol Red produced 0.43 ± 0.00 g g^{-1} of bioethanol (83 $\%Y_{MaxEtOH}$) from cellulosic glucose through SHF of WIS gardening fraction. However, the corresponding WIS fraction obtained from wheat straw biomass was not used in the framework of this Thesis.

Results obtained by *L. pentosus* from WSHs and GHs (**Table 4.5**) in this Thesis were similar to the ones obtained by Montipó and co-workers (2018) (0.50 g g^{-1} of L,D-LA and 68 % Y_{MaxLA}) with *Lactobacillus buchnery* to valorise the different fractions obtained by steam explosion of elephant grass because both LAB perform a heterolactic fermentation of C5 sugars by the PK pathway. However, considerably higher LA yields were obtained by *B. coagulans* from hemicellulosic prehydrolysates in this Thesis through the PP pathway (**Table 4.6**). On the other hand, 0.38 g g^{-1} of bioethanol (75 $\%Y_{MaxEtOH}$) were produced by *S. cerevisiae* CAT-I from glucose in the WIS fraction (Montipó et al., 2018), which were also lower than the ones obtained in this work by Ethanol Red from gardening WIS fraction.

High bioethanol and LA yields were also obtained in this Thesis by integrated bioethanol and LA production approach. Indeed, 0.89 ± 0.02 g g^{-1} of LA were yielded by the ethanol-adapted *B. coagulans* EXA from added xylose in E-GH (**Table 4.15**), which was previously obtained

by ethanol fermentation of WIS fraction from gardening residues. In the case of wheat straw slurry, 0.49 ± 0.00 g g^{-1} of bioethanol were obtained after SSF with *S. cerevisiae* Ethanol Red (**Table 4.13**). However, a 1:2 dilution of the resulting E-WSH media was required in before LA fermentation to avoid the combined inhibitory effect of bioethanol and lignocellulosic inhibitors on the evolved LA-producing bacterium. As a result, 1.00 ± 0.00 g g^{-1} of LA was yielded (**Table 4.16**).

Similar bioethanol and LA yields than in this Thesis were obtained by sequential cultivation of *S. cerevisiae* M3013 and *B. coagulans* LA1507 from corn stalk, yielding 0.46 g g^{-1} and 0.86 g g^{-1} of bioethanol and LA, respectively (Wang et al., 2018). Nevertheless, different aeration stages were applied in this case for the gas stripping of ethanol, reducing its concentration from 50.50 g L^{-1} to less than 6.00 g L^{-1}, so an ethanol tolerant LA producer was not required (Wang et al., 2018). The interest of the integrated approach in this Thesis is the engineering of an ethanol tolerant LA producer to obtain a fermentation broth rich in both bioethanol and LA available for reactive distillation.

All in all, complete C6 and C5 sugars utilisation has been achieved and high yields have been obtained by the different bioethanol and LA producers, independently of the lignocellulosic substrate used, both in separated and integrated production approaches. Different advantages and drawbacks of each method must be highlighted and evaluated. While different production costs could be reduced following the integrated approach, microorganisms are subjected to softer stress factors when biomass is fractionated after pretreatment in the separated method.

In this regard, future research could be aimed at subjecting the evolved bacteria obtained in this Thesis to further ALE methods to develop brand-new LA-producing strains able to resist multiple stresses like low pH, bioethanol and lignocellulosic inhibitors. These strains would be very versatile for valorisation of hemicellulosic liquid fractions and ethanol-rich hydrolysates previously fermented by yeast. Moreover, genomic and transcriptomic analysis could be performed with the evolved LA-producing bacteria obtained, *L. pentosus* MAX2 and *B. coagulans* EXA, in order to figure out the possible gene expression changes that are responsible for the desired traits of these microorganisms for industrial processes.

5. CONCLUSIONS

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1. Steam explosion is an effective method for pretreatment of both wheat straw and gardening residues, producing different fractions (slurries, WIS and prehydrolysates). Among the different pretreatment conditions, the highest sugars release in WSPs and GPs were obtained when H_2SO_4 was added as catalyst, especially when water extraction was applied to gardening residues before steam explosion. The application of high temperatures, long reaction times and the water extraction process appears to be decisive in the formation of degradation compounds.

2. When WSPs and GPs are subjected to enzymatic hydrolysis, the addition of hemicellulases increases both C5 and C6 monomeric sugars release. In this sense, the hydrolysis of xylo- and arabino-oligosaccharides by hemicellulases can alleviate the competitive inhibition of these compounds on cellulases. The presence of high concentrations of C5 monomers in WSPs and formic acid in WSPs and GPs could explain the inhibitory effects occurred in the hydrolytic enzymes.

3. When *L. pentosus* CECT 4023T was used for fermentation of glucose in presence of different WSPs concentrations under aerobic conditions, low LA yields were obtained. However, the inhibitory effect produced by the lignocellulosic inhibitors present in WSP and oxygen presence on *L. pentosus* was alleviated when cultivation method changed to strictly anaerobic conditions, producing higher LA yields than in aerobiosis.

4. *L. pentosus* CECT 4023T was able to co-utilise hexoses and pentoses from defined and lignocellulosic media, showing low CCR effect. In this case, L,D-LA was produced from C6 sugars, like glucose (EMP pathway), while both L,D-LA and acetic acid were obtained from C5 sugars, like xylose (PK pathway). When cultivated in flask without pH control, xylose consumption in *L. pentosus* was severely affected by the pH drop produced along fermentation. It can be inferred that the combined effect of inhibitors and acid stress could especially hinder xylose consumption since the transport of this C5 sugars in *L. pentosus* is produced at lower rates than glucose.

5. The cultivation of *L. pentosus* in bioreactor with automatic NaOH 5M for pH control allowed the xylose exhaustion in 48 h, resulting in marked increased in LA yields from

WSHs and GHs in comparison with flask fermentation without pH control. Thus, having strictly anaerobic and controlled pH conditions is very important to avoid a marked inhibitory effect of lignocellulosic degradation compounds and fermentation products on *L. pentosus* metabolism.

6. The *B. coagulans* strains used in this Thesis were able to perform a homolactic fermentation of both C6 (EMP pathway) and C5 sugars (PP pathway). Indeed, when cultivated under controlled pH conditions in bioreactor, high yields of optically pure L-LA were obtained, without being affected by the inhibitors contained in GHs.

7. When analysing the inhibitors tolerance of different *B. coagulans* strains, higher biomass and L-LA yields were attained by those strains isolated from real materials (A166 and A162) than by the collection strain DSM 2314. Therefore, isolated strain could be a great candidate to ferment lignocellulosic hydrolysates containing high sugars and inhibitors concentrations.

8. A downstream processing method including different filtration, bipolar electrodialysis and chromatographic steps was a succesful approach to allow the separation of free LA from lactate salts formed during pH neutralisation and to remove most of ions and impurities from fermented gardening hydrolysate. However, further optimisation is needed to increase the final LA concentration and purity.

9. ALE by serial batch cultivation was a feasible approach to increase the xylose consumption rate of *L. pentosus* at low pH, reducing the need of neutralisation during fermentation of mixed sugars from lignocellulosic hydrolysates. Indeed, the evolved MAX2 strain showed higher xylose consumption and LA production than the WT strain from xylose in defined media independently of the initial pH. Furthermore, MAX2 presented an increased LA productivity from WSH under controlled pH conditions.

10. *S. cerevisiae* Ethanol Red was capable of producing high Y_{EtOH} from cellulose independently of the type of lignocellulosic feedstock (gardening residues and wheat straw) and substrate fraction (WIS fraction and whole slurry). The high bioethanol yields obtained after SHF of the gardening WIS fraction, coupled with the utilisation of GHs by LA-producing bacteria demonstrated the suitability of biomass fractionation for a

separated bioethanol and LA production, reaching a complete valorisation of lignocellulosic sugars.

11. Fed-batch strategy allowed to increase the substrate loading in SSF of wheat straw slurry up to 15 % (w v^{-1}). An earlier substrate feeding (8 h) had associated an increased yeast tolerance to solids and inhibitors, which resulted in higher Y_{EtOH} than feeding at 24 h. Under these conditions, the ethanol concentration benchmark for a cost-effective distillation could be obtained, with a concomitant release of C5 monosaccharides that cannot be fermented by the yeast. The resulting media was suitable to be used in a subsequent LA fermentation step.

12. ALE by chemostat cultivation at increasing ethanol concentrations was a feasible method to improve ethanol tolerance of *B. coagulans* DSM 2314. Indeed, the evolved EXA population showed a remarkable increase in LA yield from xylose in presence of 6 % (v v^{-1}) ethanol and in presence of 5 % (v v^{-1}) bioethanol from gardening media previously fermented by *S. cerevisiae*. The combination of bioethanol and lignocellulosic inhibitors produced a significant inhibition on DSM 2314 EXA growth and LA production. The evolved bacterium was able to completely valorise lignocellulosic C5 sugars from E-WSH 50 % (v v^{-1}), previously obtained by SSF of wheat straw slurry with *S. cerevisiae*. This fact demonstrated that sequential yeast-bacteria cultivation with *B. coagulans* and *S. cerevisiae* is a successful method for integrated bioethanol and LA production from lignocellulosic slurries.

REFERENCES

REFERENCES

Abdel-Rahman, M.A., Sonomoto, K., 2016. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. J. Biotechnol. 236, 176–192. https://doi.org/10.1016/j.jbiotec.2016.08.008

Abdel-Rahman, M.A., Tashiro, Y., Sonomoto, K., 2013. Recent advances in lactic acid production by microbial fermentation processes. Biotechnol. Adv. 31, 877–902. https://doi.org/10.1016/j.biotechadv.2013.04.002

Abdel-Rahman, M.A., Tashiro, Y., Sonomoto, K., 2011. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. J. Biotechnol. 156, 286–301. https://doi.org/10.1016/j.jbiotec.2011.06.017

Abdel-Rahman, M.A., Xiao, Y., Tashiro, Y., Wang, Y., Zendo, T., Sakai, K., Sonomoto, K., 2015. Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. J. Biosci. Bioeng. 119, 153–158. https://doi.org/10.1016/j.jbiosc.2014.07.007

Abreu-Cavalheiro, A., Monteiro, G., 2014. Solving ethanol production problems with genetically modified yeast strains. Braz. J. Microbiol. 44, 665–671. https://doi.org/10.1590/s1517-83822013000300001

Abriouel, H., Pérez Montoro, B., Casimiro-Soriguer, C.S., Pérez Pulido, A.J., Knapp, C.W., Caballero Gómez, N., Castillo-Gutiérrez, S., Estudillo-Martínez, M.D., Gálvez, A., Benomar, N., 2017. Insight into Potential Probiotic Markers Predicted in *Lactobacillus pentosus* MP-10 Genome Sequence. Front. Microbiol. 8, 891. https://doi.org/10.3389/fmicb.2017.00891

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B., 2011. Biomass pretreatment: Fundamentals toward application. Biotechnol. Adv. 29, 675–685. https://doi.org/10.1016/J.BIOTECHADV.2011.05.005

Al-Shorgani, N.K.N., Shukor, H., Abdeshahian, P., Kalil, M.S., Yusoff, W.M.W., Hamid, A.A., 2018. Enhanced butanol production by optimization of medium parameters using *Clostridium acetobutylicum* YM1. Saudi J. Biol. Sci. 25, 1308–1321. https://doi.org/10.1016/j.sjbs.2016.02.017

Alcántara, C., Blasco, A., Zúñiga, M., Monedero, V., 2014. Accumulation of polyphosphate in *Lactobacillus* spp. and its involvement in stress resistance. Appl. Environ. Microbiol. 80, 1650–1659. https://doi.org/10.1128/AEM.03997-13

Alexandri, M., Neu, A.K., Schneider, R., López-Gómez, J.P., Venus, J., 2019. Evaluation of various *Bacillus coagulans* isolates for the production of high purity L-lactic acid using defatted rice bran hydrolysates. Int. J. Food Sci. Technol. 54, 1321–1329. https://doi.org/10.1111/ijfs.14086

Alexandri, M., Schneider, R., Venus, J., 2018. Membrane Technologies for Lactic Acid Separation from Fermentation Broths Derived from Renewable Resources. Membranes (Basel). 8. https://doi.org/10.3390/membranes8040094

Allen, M.R., Dube, O.P., Solecki. W., Aragón-Durand, F., Cramer, W., Humphreys, S., Kainuma, M., Kala, J., Mahowald, N., Mulugetta, Y., Perez, R., Wairiu, M., Zickfeld, K., 2018. Framing and Context, in: Global Warming of 1.5°C. An IPCC Special Report on the Impacts of Global Warming of 1.5°C above Pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat of Climate Change. https://www.ipcc.ch/sr15/download/

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 2. https://doi.org/10.1186/1754-6834-3-2

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82, 340–349. https://doi.org/10.1002/jctb.1676

Alonso, S., Herrero, M., Rendueles, M., Díaz, M., 2014. Physiological heterogeneity in *Lactobacillus casei* fermentations on residual yoghurt whey. Process Biochem. 49, 732–739. https://doi.org/10.1016/j.procbio.2014.01.033

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour. Technol. 101, 4851–4861. https://doi.org/10.1016/j.biortech.2009.11.093

Amoah, J., Ishizue, N., Ishizaki, M., Yasuda, M., Takahashi, K., Ninomiya, K., Yamada, R., Kondo, A., Ogino, C., 2017. Development and evaluation of consolidated bioprocessing yeast for ethanol production from ionic liquid-pretreated bagasse. Bioresour. Technol. 245, 1413–1420. https://doi.org/10.1016/j.biortech.2017.05.171

Arantes, V., Saddler, J.N., 2011. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. Biotechnol. Biofuels 4, 3. https://doi.org/10.1186/1754-6834-4-3

Aso, Y., Hashimoto, A., Ohara, H., 2019. Engineering *Lactococcus lactis* for D-Lactic Acid Production from Starch. Curr. Microbiol. 76, 1186–1192. https://doi.org/10.1007/s00284-019-01742-4

Aulitto, M., Fusco, S., Bartolucci, S., Franzen, C.J., Contursi, P., 2017. *Bacillus coagulans* MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. Biotechnol. Biofuels 10, 210. https://doi.org/10.1186/s13068-017-0896-8

Aulitto, M., Fusco, S., Nickel, D.B., Bartolucci, S., Contursi, P., Franzén, C.J., 2019. Seed culture pre-adaptation of *Bacillus coagulans* MA-13 improves lactic acid production in simultaneous saccharification and fermentation. Biotechnol. Biofuels 12, 45. https://doi.org/10.1186/s13068-019-1382-2

Bachmann, H., Molenaar, D., Branco dos Santos, F., Teusink, B., 2017. Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria. FEMS Microbiol. Rev. 41, S201–S219. https://doi.org/10.1093/femsre/fux024

Baeyens, J., Kang, Q., Appels, L., Dewil, R., Lv, Y., Tan, T., 2015. Challenges and opportunities in improving the production of bio-ethanol. Prog. Energy Combust. Sci. 47, 60–88. https://doi.org/10.1016/j.pecs.2014.10.003

Bai, Z., Gao, Z., He, B., Wu, B., 2015. Effect of lignocellulose-derived inhibitors on the growth and D-lactic acid production of *Sporolactobacillus inulinus* YBS1-5. Bioprocess Biosyst. Eng. 38, 1993–2001. https://doi.org/10.1007/s00449-015-1440-5

Ballesteros, I., Ballesteros, M., Cara, C., Saez, F., Castro, E., Manzanares, P., Negro, M.J., Oliva, J.M., 2011. Effect of water extraction on sugars recovery from steam exploded olive tree pruning. Bioresour. Technol. 102, 6611–6616. https://doi.org/10.1016/j.biortech.2011.03.077

Ballesteros, I., Negro, M.J., Oliva, J.M., Cabañas, A., Manzanares, P., Ballesteros, M., 2006. Ethanol production from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 130, 496–508. https://doi.org/10.1385/ABAB:130:1:496

Ballesteros, M., 2010. 6 - Enzymatic hydrolysis of lignocellulosic biomass A2 - Waldron, Keith, in: Bioalcohol Production. Woodhead Publishing, pp. 159–177. http://doi.org/10.1533/9781845699611.2.159

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50, 135–142. https://doi.org/10.1016/j.foodres.2012.10.004

BBI, 2014. Combining BBI (H2020) and European Structural and Investment Funds (ESIF) to deploy the European bioeconomy. https://www.bbi-europe.eu/sites/default/files/documents/Guidelines_BBI-ESIF-Final.pdf

Betteridge, A.L., Sumby, K.M., Sundstrom, J.F., Grbin, P.R., Jiranek, V., 2018. Application of directed evolution to develop ethanol tolerant *Oenococcus oeni* for more efficient malolactic fermentation. Appl. Microbiol. Biotechnol. 102, 921–932. https://doi.org/10.1007/s00253-017-8593-x

Bhatia, S.K., Jagtap, S.S., Bedekar, A.A., Bhatia, R.K., Patel, A.K., Pant, D., Rajesh Banu, J., Rao, C. V, Kim, Y.-G., Yang, Y.-H., 2020. Recent developments in pretreatment technologies on lignocellulosic biomass: Effect of key parameters, technological improvements, and challenges. Bioresour. Technol. 300, 122724. https://doi.org/10.1016/j.biortech.2019.122724

Bischoff, K.M., Liu, S., Hughes, S.R., Rich, J.O., 2010. Fermentation of corn fiber hydrolysate to lactic acid by the moderate thermophile *Bacillus coagulans*. Biotechnol. Lett. 32, 823–828. https://doi.org/10.1007/s10529-010-0222-z

Bishai, M., De, S., Adhikari, B., Banerjee, R., 2015. A platform technology of recovery of lactic acid from a fermentation broth of novel substrate *Zizyphus oenophlia*. 3 Biotech. 5, 455–463. https://doi.org/10.1007/s13205-014-0240-y

Biswas, R., Uellendahl, H., Ahring, B.K., 2015. Wet Explosion: a Universal and Efficient Pretreatment Process for Lignocellulosic Biorefineries. BioEnergy Res. 8, 1101–1116. https://doi.org/10.1007/s12155-015-9590-5

Boguta, A.M., Bringel, F., Martinussen, J., Jensen, P.R., 2014. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. Microb. Cell Fact. 13. https://doi.org/10.1186/s12934-014-0097-0

Boldrin, A., Christensen, T.H., 2010. Seasonal generation and composition of garden waste in Aarhus (Denmark). Waste Manag. 30, 551–557. https://doi.org/10.1016/j.wasman.2009.11.031

Bolivar-Telleria, M., Turbay, C., Favarato, L., Carneiro, T., de Biasi, R.S., Fernandes, A.A.R., Santos, A.M.C., Fernandes, P.M.B., 2018. Second-Generation Bioethanol from Coconut Husk. Biomed Res. Int. 2018, 4916497. https://doi.org/10.1155/2018/4916497

Bondesson, P.-M., Dupuy, A., Galbe, M., Zacchi, G., 2015. Optimizing Ethanol and Methane Production from Steam-pretreated, Phosphoric Acid-impregnated Corn Stover. Appl. Biochem. Biotechnol. 175, 1371–1388. https://doi.org/10.1007/s12010-014-1358-4

Boontawan, P., Kanchanathawee, S., Boontawan, A., 2011. Extractive fermentation of L-(+)-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. Biochem. Eng. J. 54, 192–199. https://doi.org/10.1016/j.bej.2011.02.021

Bozell, J.J., Petersen, G.R., 2010. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chem. 12, 539–554. https://doi.org/10.1039/B922014C

Bravo-Ferrada, B.M., Gomez-Zavaglia, A., Semorile, L., Tymczyszyn, E.E., 2015. Effect of the fatty acid composition of acclimated oenological *Lactobacillus plantarum* on the resistance to ethanol. Lett. Appl. Microbiol. 60, 155–161. https://doi.org/10.1111/lam.12350

Bustos, G., Moldes, A.B., Cruz, J.M., Domínguez, J.M., 2005. Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. Biotechnol. Prog. 21, 793–798. https://doi.org/10.1021/bp049603v

Buyondo, J.P., Liu, S., 2011. Lactic acid production by *Lactobacillus pentosus* from wood extract hydrolysates. J. Sci. Technol. For. Prod. Process. 1.

Cao, G., Ximenes, E., Nichols, N.N., Zhang, L., Ladisch, M., 2013. Biological abatement of cellulase inhibitors. Bioresour. Technol. 146, 604–610. https://doi.org/10.1016/j.biortech.2013.07.112

Caspeta, L., Castillo, T., Nielsen, J., 2015. Modifying Yeast Tolerance to Inhibitory Conditions of Ethanol Production Processes. Front. Bioeng. Biotechnol. 3, 184. https://doi.org/10.3389/fbioe.2015.00184

Chaillou, S., Pouwels, P.H., Postma, P.W., 1999. Transport of D-xylose in *Lactobacillus pentosus*, Lactobacillus casei, and Lactobacillus plantarum: evidence for a mechanism of facilitated diffusion via the phosphoenolpyruvate:mannose phosphotransferase system. J. Bacteriol. 181, 4768–4773. https://doi.org/10.1128/JB.181.16.4768-4773.1999

Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A.F., da Silva, S.S., 2018. The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. Bioresour. Technol. 264, 370–381. https://doi.org/10.1016/j.biortech.2018.06.004

Chang, J.J., Ho, F.J., Ho, C.Y., Wu, Y.C., Hou, Y.H., Huang, C.C., Shih, M.C., Li, W.H., 2013. Assembling a cellulase cocktail and a cellodextrin transporter into a yeast host for CBP ethanol production. Biotechnol. Biofuels 6, 19. https://doi.org/10.1186/1754-6834-6-19

Chang, Y.-H., Chang, K.S., Chen, C.Y., Hsu, C.L., Chang, T.C., Jang, H.D., 2018. Enhancement of the Efficiency of Bioethanol Production by *Saccharomyces cerevisiae* via Gradually Batch-Wise and Fed-Batch Increasing the Glucose Concentration. Fermentation 4, 45. https://doi.org/10.3390/fermentation4020045

Chen, C., Lu, Y., Wang, L., Yu, H., Tian, H., 2018. CcpA-Dependent Carbon Catabolite Repression Regulates Fructooligosaccharides Metabolism in *Lactobacillus plantarum*. Front. Microbiol. 9, 1114. https://doi.org/10.3389/fmicb.2018.01114

Chen, R., Dou, J., 2016. Biofuels and bio-based chemicals from lignocellulose: metabolic engineering strategies in strain development. Biotechnol. Lett. 38, 213–221. https://doi.org/10.1007/s10529-015-1976-0

Cheng, K.K., Zhang, J.A., Chavez, E., Li, J.P., 2010. Integrated production of xylitol and ethanol using corncob. Appl. Microbiol. Biotechnol. 87, 411–417. https://doi.org/10.1007/s00253-010-2612-5

Chiarello, L.M., A. Ramos, C.E., dos Santos, L.F.F., Silveira, M.H.L., Zaccaron, S., Schiehser, S., Santo, M.E., Guimarães, F.E.G., de Azevêdo, E.R., Polikarpov, I., Potthast, A., Ramos, L.P., 2020. Characterization of Pretreated Fractions and Cellulosic Ethanol Production from Steam-Exploded *Eucalyptus urograndis*. Energy & Fuels 34, 535–545. https://doi.org/10.1021/acs.energyfuels.9b03405

Cho, H.U., Park, J.M., 2018. Biodiesel production by various oleaginous microorganisms from organic wastes. Bioresour. Technol. 256, 502–508. https://doi.org/https://doi.org/10.1016/j.biortech.2018.02.010

Choi, S., Song, C.W., Shin, J.H., Lee, S.Y., 2015. Biorefineries for the production of top building block chemicals and their derivatives. Metab. Eng. 28, 223–239. https://doi.org/10.1016/j.ymben.2014.12.007

Choudhary, J., Singh, S., Nain, L., 2017. Bioprospecting thermotolerant ethanologenic yeasts for simultaneous saccharification and fermentation from diverse environments. J. Biosci. Bioeng. 123, 342–346. https://doi.org/10.1016/j.jbiosc.2016.10.007

Coker, A.K., 2010. Chapter 12 - Enhanced distillation types, in: Coker, A.K.B.T.-L.A.P.D. for C. and P.P. (Fourth E. (Ed.), . Gulf Professional Publishing, Boston, pp. 345–371. https://doi.org/10.1016/B978-0-7506-8366-1.10012-X

Costa-Trigo, I., Otero-Penedo, P., Outeiriño, D., Paz, A., Domínguez, J.M., 2019. Valorization of chestnut (*Castanea sativa*) residues: Characterization of different materials and optimization of the acid-hydrolysis of chestnut burrs for the elaboration of culture broths. Waste Manag. 87, 472–484. https://doi.org/10.1016/j.wasman.2019.02.028

Cui, F., Li, Y., Wan, C., 2011. Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. Bioresour. Technol. 102, 1831–1836. https://doi.org/10.1016/j.biortech.2010.09.063

Dahmen, N., Lewandowski, I., Zibek, S., Weidtmann, A., 2019. Integrated lignocellulosic value chains in a growing bioeconomy: Status quo and perspectives. GCB Bioenergy 11, 107–117. https://doi.org/10.1111/gcbb.12586

del Río, J., Rencoret, J., Prinsen, P., Martinez, A.T., Ralph, J., Gutiérrez, A., 2012. Structural Characterization of Wheat Straw Lignin as Revealed by Analytical Pyrolysis, 2D-NMR, and Reductive Cleavage Methods. J. Agric. Food Chem. 60. https://doi.org/10.1021/jf301002n

Delavarrafiee, M., Frey, H.C., 2018. Real-world fuel use and gaseous emission rates for flex fuel vehicles operated on E85 versus gasoline. J. Air Waste Manage. Assoc. 68, 235–254. https://doi.org/10.1080/10962247.2017.1405097

Demeke, M.M., Dumortier, F., Li, Y., Broeckx, T., Foulquie-Moreno, M.R., Thevelein, J.M., 2013. Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. Biotechnol. Biofuels 6, 120. https://doi.org/10.1186/1754-6834-6-120

Demichelis, F., Pleissner, D., Fiore, S., Mariano, S., Navarro Gutierrez, I.M., Schneider, R., Venus, J., 2017. Investigation of food waste valorization through sequential lactic acid fermentative production and anaerobic digestion of fermentation residues. Bioresour. Technol. 241, 508–516. https://doi.org/10.1016/j.biortech.2017.05.174

Dijkstra, A., Alkema, W., Starrenburg, M., Hugenholtz, J., Hijum, S., Bron, P., 2014. Fermentation-induced variation in heat and oxidative stress phenotypes of *Lactococcus lactis* MG1363 reveals transcriptome signatures for robustness. Microb. Cell Fact. 13, 148. https://doi.org/10.1186/s12934-014-0148-6

Dondelinger, E., Aubry, N., Ben Chaabane, F., Cohen, C., Tayeb, J., Rémond, C., 2016. Contrasted enzymatic cocktails reveal the importance of cellulases and hemicellulases activity ratios for the hydrolysis of cellulose in presence of xylans. AMB Express 6, 24. https://doi.org/10.1186/s13568-016-0196-x

Dragosits, M., Mattanovich, D., 2013. Adaptive laboratory evolution – principles and applications for biotechnology. Microb. Cell Fact. 12, 64. https://doi.org/10.1186/1475-2859-12-64

Dunn, J.B., 2019. Biofuel and bioproduct environmental sustainability analysis. Curr. Opin. Biotechnol. 57, 88–93. https://doi.org/10.1016/j.copbio.2019.02.008

Eiteman, M.A., Ramalingam, S., 2015. Microbial production of lactic acid. Biotechnol. Lett. 37, 955–972. https://doi.org/10.1007/s10529-015-1769-5

El Baz, A., 2011. Xylitol production by *Candida tropicalis* under different statistically optimized growth conditions. AFRICAN J. Biotechnol. 10. https://doi.org/10.5897/AJB10.1575

Elahwany, A.M.D., 2012. Genetic and physiological studies on *Oenococcus oeni* PsuI in response to ethanol stress. Acta Biol. Hung. 63, 128–137. https://doi.org/10.1556/ABiol.63.2012.1.10

EPURE, 2019. Share of European renewable ethanol produced from each feedstock type 2018. https://www.epure.org/resources/statistics-and-infographics/

Erdei, B., Galbe, M., Zacchi, G., 2013. Simultaneous saccharification and co-fermentation of whole wheat in integrated ethanol production. Biomass and Bioenergy 56, 506–514. https://doi.org/10.1016/j.biombioe.2013.05.032

European Comission, 2017. Commission expert group on bio-based products. Final Report. https://ec.europa.eu/growth/content/commission-expert-group-bio-based-products-calls-alignment-bioeconomy-strategy-eu-policy_en

European Comission, 2019. Sustainability criteria for biofuels specified. https://ec.europa.eu/commission/presscorner/detail/en/MEMO_19_1656

Fabbri, D., Torri, C., 2016. Linking pyrolysis and anaerobic digestion (Py-AD) for the conversion of lignocellulosic biomass. Curr. Opin. Biotechnol. 38, 167–173. https://doi.org/10.1016/j.copbio.2016.02.004

Fabbri, P., Viaggi, D., Cavani, F., Bertin, L., Michetti, M., Carnevale, E., Velasquez-Ochoa, J., Martinez G.A., Esposti, M.D., Fischer, K.P, Wydra, S., Schwarz, A., Marscheider, F., 2018. Top emerging bio-based products, their properties and industrial applications. https://www.ecologic.eu/15776

Farzad, S., Mandegari, M.A., Guo, M., Haigh, K.F., Shah, N., Görgens, J.F., 2017. Multi-product biorefineries from lignocelluloses: a pathway to revitalisation of the sugar industry? Biotechnol. Biofuels 10, 87. https://doi.org/10.1186/s13068-017-0761-9

Frankó, B., Galbe, M., Wallberg, O., 2015. Influence of bark on fuel ethanol production from steam-pretreated spruce. Biotechnol. Biofuels 8, 15. https://doi.org/10.1186/s13068-015-0199-x

Galbe, M., Wallberg, O., Zacchi, G., 2011. Techno-Economic Aspects of Ethanol Production from Lignocellulosic Agricultural Crops and Residues, in: Comprehensive Biotechnology. pp. 615–628. https://doi.org/10.1016/B978-0-08-088504-9.00298-1

Gall, D.L., Ralph, J., Donohue, T.J., Noguera, D.R., 2017. Biochemical transformation of lignin for deriving valued commodities from lignocellulose. Curr. Opin. Biotechnol. 45, 120–126. https://doi.org/10.1016/j.copbio.2017.02.015

Gao, J., Feng, H., Yuan, W., Li, Y., Hou, S., Zhong, S., Bai, F., 2017. Enhanced fermentative performance under stresses of multiple lignocellulose-derived inhibitors by overexpression of a typical 2-Cys peroxiredoxin from *Kluyveromyces marxianus*. Biotechnol. Biofuels 10, 79. https://doi.org/10.1186/s13068-017-0766-4

García-Aparicio, M.P., Ballesteros, I., González, A., Oliva, J.M., Ballesteros, M., Negro, M.J., 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl. Biochem. Biotechnol. 129, 278–288. https://doi.org/10.1385/ABAB:129:1:278

Garde, A., Jonsson, G., Schmidt, A.S., Ahring, B.K., 2002. Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*. Bioresour. Technol. 81, 217–223. https://doi.org/10.1016/s0960-8524(01)00135-3.

Ge, X.Y., Yuan, J., Qin, H., Zhang, W.G., 2011. Improvement of L-lactic acid production by osmotic-tolerant mutant of *Lactobacillus casei* at high temperature. Appl Microbiol Biotechnol 89, 73–78. https://doi.org/10.1007/s00253-010-2868-9

Glaser, R., Venus, J., 2018. Co-fermentation of the main sugar types from a beechwood organosolv hydrolysate by several strains of *Bacillus coagulans* results in effective lactic acid production. Biotechnol. Rep. 18, e00245. https://doi.org/10.1016/j.btre.2018.e00245

Grandvalet, C., Assad-Garcia, J.S., Chu-Ky, S., Tollot, M., Guzzo, J., Gresti, J., Tourdot-Marechal, R., 2008. Changes in membrane lipid composition in ethanol- and acid-adapted *Oenococcus oeni* cells: characterization of the cfa gene by heterologous complementation. Microbiology 154, 2611–2619. https://doi.org/10.1099/mic.0.2007/016238-0

Gray, M.J., Jakob, U., 2015. Oxidative stress protection by polyphosphate—new roles for an old player. Curr. Opin. Microbiol. 24, 1–6. https://doi.org/10.1016/j.mib.2014.12.004

Grewal, J., Khare, S.K., 2018. One-pot bioprocess for lactic acid production from lignocellulosic agro-wastes by using ionic liquid stable *Lactobacillus brevis*. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2017.12.056

Gu, H., Zhang, J., Bao, J., 2015. High tolerance and physiological mechanism of *Zymomonas mobilis* to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnol. Bioeng. 112, 1770–1782. https://doi.org/10.1002/bit.25603

Guidone, A., Ianniello, R.G., Ricciardi, A., Zotta, T., Parente, E., 2013. Aerobic metabolism and oxidative stress tolerance in the *Lactobacillus plantarum* group. World J. Microbiol. Biotechnol. 29, 1713–1722. https://doi.org/10.1007/s11274-013-1334-0

Guo, W., He, R., Ma, L., Jia, W., Li, D., Chen, S., 2014. Construction of a constitutively expressed homo-fermentative pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. 98, 6641–6650. https://doi.org/10.1007/s00253-014-5703-x

Guo, W., Jia, W., Li, Y., Chen, S., 2010. Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics. Appl. Biochem. Biotechnol. 161, 124–136. https://doi.org/10.1007/s12010-009-8857-8

GVR, 2019. Lactic Acid Market. https://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market

Hajian, H., Al-Shorgani, N., Abdullah, S.R.S., Yusoff, W.M.W., 2017. Optimization of itaconic acid production by *Aspergillus terreus* SUK-1 using Responce Surface Methodology. Asian J. Microbiol. Biotechnol. Environ. Sci. 19, 210–217.

Hu, C.-Y., Chi, D.-J., Chen, S.-S., Chen, Y.-C., 2011. The direct conversion of xylan to lactic acid by *Lactobacillus brevis* transformed with a xylanase gene. Green Chem. 13, 1729–1734. https://doi.org/10.1039/c1gc15169j

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels 4, 36. https://doi.org/10.1186/1754-6834-4-36

Hu, J., Lin, Y., Zhang, Z., Xiang, T., Mei, Y., Zhao, S., Liang, Y., Peng, N., 2016. High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. Bioresour. Technol. 214, 74–80. https://doi.org/10.1016/j.biortech.2016.04.034

Idler, C., Venus, J., Kamm, B., 2015. Microorganisms for the Production of Lactic Acid and Organic Lactates BT - Microorganisms in Biorefineries, in: Kamm, B. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 225–273. https://doi.org/10.1007/978-3-662-45209-7_9

IEA, 2019a. Oil Information 2019. https://www.iea.org/reports/oil-information-2019

IEA, 2019b. Renewables Information 2019. https://www.iea.org/reports/renewables-information-2019

Intan Shafinas Muhammad, N., A Rosentrater, K., 2020. Economic Assessment of Bioethanol Recovery Using Membrane Distillation for Food Waste Fermentation. Bioeng. (Basel, Switzerland) 7. https://doi.org/10.3390/bioengineering7010015

IRENA, 2016. Innovation Outlook Advanced Liquid Biofuels. https://www.irena.org/publications/2016/Oct/Innovation-Outlook-Advanced-Liquid-Biofuels

Isikgor, F.H., Becer, C.R., 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym. Chem. 6, 4497–4559. https://doi.org/10.1039/C5PY00263J

Iwaki, A., Kawai, T., Yamamoto, Y., Izawa, S., 2013. Biomass conversion inhibitors furfural and 5-hydroxymethylfurfural induce formation of messenger RNP granules and attenuate translation activity in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 79, 1661–1667. https://doi.org/10.1128/AEM.02797-12

Ji, X.J., Huang, H., Nie, Z.K., Qu, L., Xu, Q., Tsao, G.T., 2012. Fuels and chemicals from hemicellulose sugars. Adv. Biochem. Eng. Biotechnol. 128, 199–224. https://doi.org/10.1007/10_2011_124

Jiang, H.-L., He, Q., He, Z., Hemme, C.L., Wu, L., Zhou, J., 2013. Continuous cellulosic bioethanol fermentation by cyclic fed-batch cocultivation. Appl. Environ. Microbiol. 79, 1580–1589. https://doi.org/10.1128/AEM.02617-12

Jiang, T., Qiao, H., Zheng, Z., Chu, Q., Li, X., Yong, Q., Ouyang, J., 2016. Lactic Acid Production from Pretreated Hydrolysates of Corn Stover by a Newly Developed *Bacillus coagulans* Strain. PLoS One 11. https://doi.org/10.1371/journal.pone.0149101

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels 6, 16. https://doi.org/10.1186/1754-6834-6-16

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Jordan, D.B., Braker, J.D., 2007. Inhibition of the two-subsite beta-d-xylosidase from *Selenomonas ruminantium* by sugars: competitive, noncompetitive, double binding, and slow binding modes. Arch. Biochem. Biophys. 465, 231–246. https://doi.org/10.1016/j.abb.2007.05.016

Jordan, D.B., Li, X.L., Dunlap, C.A., Whitehead, T.R., Cotta, M.A., 2007. Beta-D-xylosidase from *Selenomonas ruminantium* of glycoside hydrolase family 43. Appl. Biochem. Biotechnol. 137–140, 93–104. https://doi.org/10.1007/s12010-007-9042-6

Jung, Y.H., Kim, K.H., 2017. Evaluation of the Main Inhibitors from Lignocellulose Pretreatment for Enzymatic Hydrolysis and Yeast Fermentation. Bioresour. 12, 4. https://doi.org/10.15376/biores.12.4.9348-9356

Juodeikiene, G., Zadeike, D., Bartkiene, E., Bartkevics, V., Dikiy, A., Shumilina, E., 2016a. Potential of an Exploitation of Acid-Tolerant Antimicrobial Microorganisms Evolving Enzyme Systems for the Utilization of Dairy By-products and Lignocellulosic Biomass to Lactic Acid. Front. Bioeng. Biotechnol. 4, 92. https://doi.org/10.3389/fbioe.2016.00092

Juodeikiene, G., Zadeike, D., Bartkiene, E., Klupsaite, D., 2016b. Application of acid tolerant *Pedioccocus* strains for increasing the sustainability of lactic acid production from cheese whey. LWT - Food Sci. Technol. 72, 399–406. https://doi.org/10.1016/j.lwt.2016.05.023

Juturu, V., Wu, J.C., 2015. Microbial production of lactic acid: the latest development. Crit. Rev. Biotechnol. 1–11. https://doi.org/10.3109/07388551.2015.1066305.

Kainthola, J., Kalamdhad, A.S., Goud, V. V, 2019. A review on enhanced biogas production from anaerobic digestion of lignocellulosic biomass by different enhancement techniques. Process Biochem. 84, 81–90. https://doi.org/https://doi.org/10.1016/j.procbio.2019.05.023

Kato, K., Yamamoto, Y., Izawa, S., 2011. Severe ethanol stress induces assembly of stress granules in *Saccharomyces cerevisiae*. Yeast 28, 339–347. https://doi.org/10.1002/yea.1842

Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Curr. Opin. Biotechnol. 42, 30–39. https://doi.org/10.1016/j.copbio.2016.02.031

Khunnonkwao, P., Ariyawong, C., Lertsiriyothin, W., Boontawan, A., 2012. Purification of D-(-)-Lactic Acid from Fermentation Broth Using Nanofiltration, Esterification, Distillation, and Hydrolysis Technique. Adv. Mater. Res. 550–553, 2945–2952. https://doi.org/10.4028/www.scientific.net/AMR.550-553.2945

Kim, J.-H., Block, D.E., Mills, D.A., 2010. Simultaneous consumption of pentose and hexose sugars: an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass. Appl. Microbiol. Biotechnol. 88, 1077–1085. https://doi.org/10.1007/s00253-010-2839-1

Kim, J.H., Shoemaker, S.P., Mills, D.A., 2009. Relaxed control of sugar utilization in *Lactobacillus brevis*. Microbiology 155, 1351–1359. https://doi.org/10.1099/mic.0.024653-0

Kim, M.-S., Na, J.-G., Lee, M.-K., Ryu, H., Chang, Y.-K., Triolo, J.M., Yun, Y.-M., Kim, D.-H., 2016. More value from food waste: Lactic acid and biogas recovery. Water Res. 96, 208–216. https://doi.org/10.1016/j.watres.2016.03.064

Klotz, S., Kaufmann, N., Kuenz, A., Prusse, U., 2016. Biotechnological production of enantiomerically pure d-lactic acid. Appl. Microbiol. Biotechnol. 100, 9423–9437. https://doi.org/10.1007/s00253-016-7843-7

Komesu, A., Martinez, P.F.M., Lunelli, B.H., Filho, R.M., Maciel, M.R.W., 2015. Lactic acid purification by reactive distillation system using design of experiments. Chem. Eng. Process. Process Intensif. 95, 26–30. https://doi.org/10.1016/j.cep.2015.05.005

Komesu, A., Oliveira, J.A.R. de, Martins, L.H. da S., Wolf Maciel, M.R., Maciel Filho, R., 2017. Lactic Acid Production to Purification: A Review. Bioresour. 12, 2. https://doi.org/10.15376/biores.12.2.Komesu

Koppram, R., Nielsen, F., Albers, E., Lambert, A., Wännström, S., Welin, L., Zacchi, G., Olsson, L., 2013. Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales. Biotechnol. Biofuels 6, 2. https://doi.org/10.1186/1754-6834-6-2

Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol. 32, 46–53. https://doi.org/10.1016/j.tibtech.2013.10.003

Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol. Biofuels 2, 11. https://doi.org/10.1186/1754-6834-2-11

Kuenen, J.G., Johnson, O.J., 2009. Continuous Cultures (Chemostats), in: Schaechter, M.B.T.-E. of M. (Third Ed.). Academic Press, Oxford, pp. 130–147. https://doi.org/https://doi.org/10.1016/B978-012373944-5.00112-7

Kuo, Y.C., Yuan, S.F., Wang, C.A., Huang, Y.J., Guo, G.L., Hwang, W.S., 2015. Production of optically pure L-lactic acid from lignocellulosic hydrolysate by using a newly isolated and D-lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain. Bioresour. Technol. 198, 651–657. https://doi.org/10.1016/j.biortech.2015.09.071

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.O., 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb. Technol. 24, 151–159. https://doi.org/10.1016/S0141-0229(98)00101-X

Lech, M., Trusek, A., 2018. Batch Electrodialysis of Lactic Acid Obtained from Lab Fermentation. Polish J. Chem. Technol. 20, 81–86. https://doi.org/10.2478/pjct-2018-0042

Leung, C.C.J., Cheung, A.S.Y., Zhang, A.Y.-Z., Lam, K.F., Lin, C.S.K., 2012. Utilisation of waste bread for fermentative succinic acid production. Biochem. Eng. J. 65, 10–15. https://doi.org/10.1016/j.bej.2012.03.010

Li, Y.C., Zeng, W.Y., Gou, M., Sun, Z.Y., Xia, Z.Y., Tang, Y.Q., 2017. Transcriptome changes in adaptive evolution of xylose-fermenting industrial *Saccharomyces cerevisiae* strains with delta-integration of different xylA genes. Appl. Microbiol. Biotechnol. 101, 7741–7753. https://doi.org/10.1007/s00253-017-8494-z

Liang, Y., Jarosz, K., Wardlow, A.T., Zhang, J., Cui, Y., 2014. Lipid production by *Cryptococcus curvatus* on hydrolysates derived from corn fiber and sweet sorghum bagasse following dilute acid pretreatment. Appl. Biochem. Biotechnol. 173, 2086–2098. https://doi.org/10.1007/s12010-014-1007-y

Lie, S., 1973. The EBC-Ninhydrin method for determination of free alpha amino nitrogen. J. Inst. Brew. 79, 37–41. https://doi.org/10.1002/j.2050-0416.1973.tb03495.x

Linde, M., Galbe, M., Zacchi, G., 2007. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. Enzyme Microb. Technol. 40, 1100–1107. https://doi.org/10.1016/j.enzmictec.2006.08.014

Linde, M., Jakobsson, E.-L., Galbe, M., Zacchi, G., 2008. Steam pretreatment of dilute H_2SO_4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. Biomass and Bioenergy 32, 326–332. https://doi.org/10.1016/j.biombioe.2007.09.013

Liu, C., Wyman, C.E., 2006. The enhancement of xylose monomer and xylotriose degradation by inorganic salts in aqueous solutions at 180°C. Carbohydr. Res. 341, 2550–2556. https://doi.org/https://doi.org/10.1016/j.carres.2006.07.017

Liu, J., Cai, Y., Liu, L., Zhan, R., Zhao, S., Liang, Y., Peng, N., Zhu, J., Li, H., Xiao, N., Hu, J., He, M., Hu, G., 2020. Enhanced lactic acid production by *Bacillus coagulans* through simultaneous saccharification, biodetoxification, and fermentation. Biofuels, Bioprod. Biorefining 14, 533–543. https://doi.org/10.1002/bbb.2086

Liu, Z.L., Slininger, P.J., Gorsich, S.W., 2005. Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. Appl. Biochem. Biotechnol. 121–124, 451–460. https://doi.org/10.1385/ABAB:121:1-3:0451

Llamas, M., Magdalena, J.A., González-Fernández, C., Tomás-Pejó, E., 2020. Volatile fatty acids as novel building blocks for oil-based chemistry via oleaginous yeast fermentation. Biotechnol. Bioeng. 117, 238–250. https://doi.org/10.1002/bit.27180

Lopes, A.M., Ferreira Filho, E.X., Moreira, L.R.S., 2018. An update on enzymatic cocktails for lignocellulose breakdown. J. Appl. Microbiol. 125, 632–645. https://doi.org/10.1111/jam.13923

López-Garzón, C.S., Straathof, A.J.J., 2014. Recovery of carboxylic acids produced by fermentation. Biotechnol. Adv. 32, 873–904. https://doi.org/10.1016/j.biotechadv.2014.04.002

López-Gómez, J.P., Latorre-Sánchez, M., Unger, P., Schneider, R., Lozano, C.C., Venus, J., 2019. Assessing the organic fraction of municipal solid wastes for the production of lactic acid. Biochem. Eng. J. 107251. https://doi.org/10.1016/j.bej.2019.107251

López-Linares, J.C., Ballesteros, I., Touran, J., Cara, C., Castro, E., Ballesteros, M., Romero, I., 2015. Optimization of uncatalyzed steam explosion pretreatment of rapeseed straw for biofuel production. Bioresour. Technol. 190, 97–105. https://doi.org/10.1016/j.biortech.2015.04.066

López-Linares, J.C., Romero, I., Moya, M., Cara, C., Ruiz, E., Castro, E., 2013. Pretreatment of olive tree biomass with $FeCl_3$ prior enzymatic hydrolysis. Bioresour. Technol. 128, 180–187. https://doi.org/10.1016/J.BIORTECH.2012.10.076

Lujan-Rhenals, D.E., Morawicki, R.O., Ricke, S.C., 2014. Tolerance of *S. cerevisiae* and *Z. mobilis* to inhibitors produced during dilute acid hydrolysis of soybean meal. J. Environ. Sci. Health. B. 49, 305–311. https://doi.org/10.1080/03601234.2014.868683

Ma, K., Hu, G., Pan, L., Wang, Z., Zhou, Y., Wang, Y., Ruan, Z., He, M., 2016. Highly efficient production of optically pure l-lactic acid from corn stover hydrolysate by thermophilic *Bacillus coagulans*. Bioresour. Technol. 219, 114–122. https://doi.org/10.1016/j.biortech.2016.07.100

Mandegari, M.A., Farzad, S., van Rensburg, E., Görgens, J.F., 2017. Multi-criteria analysis of a biorefinery for co-production of lactic acid and ethanol from sugarcane lignocellulose. Biofuels, Bioprod. Biorefining 11, 971–990. https://doi.org/10.1002/bbb.1801

Mans, R., Daran, J.-M.G., Pronk, J.T., 2018. Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. Curr. Opin. Biotechnol. 50, 47–56. https://doi.org/10.1016/j.copbio.2017.10.011

Martín, C., Wu, G., Wang, Z., Stagge, S., Jönsson, L.J., 2018. Formation of microbial inhibitors in steam-explosion pretreatment of softwood impregnated with sulfuric acid and sulfur dioxide. Bioresour. Technol. 262, 242–250. https://doi.org/10.1016/j.biortech.2018.04.074

Martínez-Alcántar, L., Madrigal, A., Sánchez-Briones, L., Díaz-Pérez, A.L., López-Bucio, J.S., Campos-García, J., 2019. Over-expression of Isu1p and Jac1p increases the ethanol tolerance and yield by superoxide and iron homeostasis mechanism in an engineered *Saccharomyces cerevisiae* yeast. J. Ind. Microbiol. Biotechnol. 46, 925–936. https://doi.org/10.1007/s10295-019-02175-5

Mears, L., Feldman, H., Falco, F.C., Bach, C., Wu, M., Nørregaard, A., Gernaey, K. V, 2017. Continuous Fermentation for Biopharmaceuticals? Contin. Manuf. Pharm., Wiley Online Books. https://doi.org/doi:10.1002/9781119001348.ch6

Meng, Y., Xue, Y.F., Yu, B., Gao, C.H., Ma, Y., 2012. Efficient production of L-lactic acid with high optical purity by alkaliphilic Bacillus sp WL-S20. Bioresour. Technol. 116, 334–339. https://doi.org/10.1016/j.biortech.2012.03.103

Michael, D.J.R.; S., 1999. The metabolism and molecular physiology of *Saccharomyces cerevisiae*. Taylor & Francis, Philadelphia.

Mills, T.Y., Sandoval, N.R., Gill, R.T., 2009. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol. Biofuels 2, 26. https://doi.org/10.1186/1754-6834-2-26

Miyoshi, A., Rochat, T., Gratadoux, J.-J., Le Loir, Y., Oliveira, S., Langella, P., Azevedo, V., 2003. Oxidative stress in *Lactococcus lactis*. Genet. Mol. Res. 2, 348–359.

Mo, W., Wang, M., Zhan, R., Yu, Y., He, Y., Lu, H., 2019. *Kluyveromyces marxianus* developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways. Biotechnol. Biofuels 12, 63. https://doi.org/10.1186/s13068-019-1393-z

Moldes, A.B., Torrado, A., Converti, A., Domínguez, J.M., 2006. Complete bioconversion of hemicellulosic sugars from agricultural residues into lactic acid by *Lactobacillus pentosus*. Appl. Biochem. Biotechnol. 135, 219–228. https://doi.org/10.1385/ABAB:135:3:219

Montipó, S., Ballesteros, I., Fontana, R.C., Liu, S., Martins, A.F., Ballesteros, M., Camassola, M., 2018. Integrated production of second generation ethanol and lactic acid from steam-exploded elephant grass. Bioresour. Technol. 249, 1017–1024. https://doi.org/10.1016/j.biortech.2017.11.001

Moreno, A.D., Carbone, A., Pavone, R., Olsson, L., Geijer, C., 2019a. Evolutionary engineered *Candida intermedia* exhibits improved xylose utilization and robustness to lignocellulose-derived inhibitors and ethanol. Appl. Microbiol. Biotechnol. 103, 1405–1416. https://doi.org/10.1007/s00253-018-9528-x

Moreno, A.D., Gonzalez-Fernandez, C., Ballesteros, M., Tomas-Pejo, E., 2019b. Insoluble solids at high concentrations repress yeast's response against stress and increase intracellular ROS levels. Sci. Rep. 9, 12236. https://doi.org/10.1038/s41598-019-48733-w

Moreno, A.D., Ibarra, D., Alvira, P., Tomas-Pejo, E., Ballesteros, M., 2015. A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit. Rev. Biotechnol. 35, 342–354. https://doi.org/10.3109/07388551.2013.878896

Moreno, D.A., Ibarra, D., Mialon, A., Ballesteros, M., 2016. A Bacterial Laccase for Enhancing Saccharification and Ethanol Fermentation of Steam-Pretreated Biomass. Fermentation 2. https://doi.org/10.3390/fermentation2020011

Mosier, N., Hendrickson, R., Brewer, M., Ho, N., Sedlak, M., Dreshel, R., Welch, G., Dien, B., Aden, A., Ladisch, M., 2005. Industrial Scale-Up of pH-Controlled Liquid Hot Water Pretreatment of Corn Fiber for Fuel Ethanol Production. Appl. Biochem. Biotechnol. 125, 77–97. https://doi.org/10.1385/ABAB:125:2:077

Motola, V., De Bari, I., Pierro, N., Giocoli, A., 2018. Bioeconomy and biorefining strategies in the EU Member States and beyond. https://www.ieabioenergy.com/wp-content/uploads/2018/12/Bioeconomy-and-Biorefining-Strategies_Final-Report_DEC2018.pdf

Mussatto, S.I., Roberto, I.C., 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresour. Technol. 93, 1–10. https://doi.org/10.1016/j.biortech.2003.10.005

Naidu, D.S., Hlangothi, S.P., John, M.J., 2018. Bio-based products from xylan: A review. Carbohydr. Polym. 179, 28–41. https://doi.org/10.1016/j.carbpol.2017.09.064

Nakano, S., Ugwu, C.U., Tokiwa, Y., 2012. Efficient production of D-(-)-lactic acid from broken rice by *Lactobacillus delbrueckii* using $Ca(OH)_2$ as a neutralizing agent. Bioresour. Technol. 104, 791–794. https://doi.org/10.1016/j.biortech.2011.10.017

Navarro-Tapia, E., Querol, A., Pérez-Torrado, R., 2018. Membrane fluidification by ethanol stress activates unfolded protein response in yeasts. Microb. Biotechnol. 11, 465–475. https://doi.org/10.1111/1751-7915.13032

Negro, M.J., Manzanares, P., Ballesteros, I., Oliva, J.M., Cabanas, A., Ballesteros, M., 2003. Hydrothermal pretreatment conditions to enhance ethanol production from poplar biomass. Appl. Biochem. Biotechnol. 105–108, 87–100. https://doi.org/10.1385/abab:105:1-3:87

Neu, A.-K., Pleissner, D., Mehlmann, K., Schneider, R., Puerta-Quintero, G.I., Venus, J., 2016. Fermentative utilization of coffee mucilage using *Bacillus coagulans* and investigation of down-stream processing of fermentation broth for optically pure l(+)-lactic acid production. Bioresour. Technol. 211, 398–405. https://doi.org10.1016/j.biortech.2016.03.122

Neves, P. V, Pitarelo, A.P., Ramos, L.P., 2016. Production of cellulosic ethanol from sugarcane bagasse by steam explosion: Effect of extractives content, acid catalysis and different fermentation technologies. Bioresour. Technol. 208, 184–194. https://doi.org/10.1016/j.biortech.2016.02.085

Nguyen, C.M., Kim, J.S., Hwang, H.J., Park, M.S., Choi, G.J., Choi, Y.H., Jang, K.S., Kim, J.C., 2012. Production of L-lactic acid from a green microalga, *Hydrodictyon reticulum*, by *Lactobacillus paracasei* LA104 isolated from the traditional Korean food, makgeolli. Bioresour. Technol. 110, 552–559. https://doi.org/10.1016/j.biortech.2012.01.079

Njoku, S.I., Iversen, J.A., Uellendahl, H., Ahring, B.K., 2013. Production of ethanol from hemicellulose fraction of cocksfoot grass using *Pichia stipitis*. Sustain. Chem. Process. 1, 13. https://doi.org/10.1186/2043-7129-1-13

Novozymes, 2010. CELLIC Ctec and Htec2—Enzymes for Hydrolysis of Lignocellulosic Materials, Sheet, Fuel Ethanol Application. http://www.shinshu-u.ac.jp/faculty/engineering/chair/chem010/manual/Ctec2.pdf

Oh, E.J., Jin, Y.S., 2020. Engineering of *Saccharomyces cerevisiae* for efficient fermentation of cellulose. FEMS Yeast Res. 20. https://doi.org/10.1093/femsyr/foz089

Ohgren, K., Galbe, M., Zacchi, G., 2005. Optimization of steam pretreatment of SO_2-impregnated corn stover for fuel ethanol production. Appl. Biochem. Biotechnol. 121–124, 1055–1067. https://doi.org/10.1385/abab:124:1-3:1055

Öhgren, K., Rudolf, A., Galbe, M., Zacchi, G., 2006. Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content. Biomass Bioenergy – Biomass Bioenerg 30, 863–869. https://doi.org/10.1016/j.biombioe.2006.02.002

Okano, K., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2009. Homo-d-Lactic Acid Fermentation from Arabinose by Redirection of the Phosphoketolase Pathway to the Pentose Phosphate Pathway in l-Lactate Dehydrogenase Gene-Deficient *Lactobacillus plantarum*. Appl. Environ. Microbiol. 75, 5175–5178. https://doi.org/10.1128/AEM.00573-09

Olguin, N., Bordons, A., Reguant, C., 2009. Influence of ethanol and pH on the gene expression of the citrate pathway in *Oenococcus oeni*. Food Microbiol. 26, 197–203. https://doi.org/10.1016/j.fm.2008.09.004

Oliva-Taravilla, A., Moreno, A.D., Demuez, M., Ibarra, D., Tomás-Pejó, E., González-Fernández, C., Ballesteros, M., 2015. Unraveling the effects of laccase treatment on enzymatic hydrolysis of steam-exploded wheat straw. Bioresour. Technol. 175, 209–215. https://doi.org/10.1016/j.biortech.2014.10.086

Oliva, J.M., Ballesteros, I., Negro, M.J., Manzanares, P., Cabañas, A., Ballesteros, M., 2004. Effect of Binary Combinations of Selected Toxic Compounds on Growth and Fermentation of *Kluyveromyces marxianus*. Biotechnol. Prog. 20, 715–720. https://doi.org/10.1021/bp034317p

Olofsson, K., Bertilsson, M., Liden, G., 2008. A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1, 7. https://doi.org/10.1186/1754-6834-1-7

Ou, M.S., Ingram, L.O., Shanmugam, K.T., 2011. l(+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*. J. Ind. Microbiol. Biotechnol. 38, 599–605. https://doi.org/10.1007/s10295-010-0796-4

Owusu, P.A., Asumadu-Sarkodie, S., 2016. A review of renewable energy sources, sustainability issues and climate change mitigation. Cogent Eng. 3, 1167990. https://doi.org/10.1080/23311916.2016.1167990

Pal, P., Sikder, J., Roy, S., Giorno, L., 2009. Process intensification in lactic acid production: A review of membrane based processes. Chem. Eng. Process. Process Intensif. 48, 1549–1559. https://doi.org/10.1016/j.cep.2009.09.003

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33. https://doi.org/10.1016/S0960-8524(99)00161-3

Panagiotou, G., Olsson, L., 2007. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol. Bioeng. 96, 250–258. https://doi.org/10.1002/bit.21100

Pandey, A.K., Kumar, M., Kumari, S., Kumari, P., Yusuf, F., Jakeer, S., Naz, S., Chandna, P., Bhatnagar, I., Gaur, N.A., 2019. Evaluation of divergent yeast genera for fermentation-associated stresses and identification of a robust sugarcane distillery waste isolate *Saccharomyces cerevisiae* NGY10 for lignocellulosic ethanol production in SHF and SSF. Biotechnol. Biofuels 12, 40. https://doi.org/10.1186/s13068-019-1379-x

Papadimitriou, K., Alegría, Á., Bron, P.A., de Angelis, M., Gobbetti, M., Kleerebezem, M., Lemos, J.A., Linares, D.M., Ross, P., Stanton, C., Turroni, F., van Sinderen, D., Varmanen, P., Ventura, M., Zúñiga, M., Tsakalidou, E., Kok, J., 2016. Stress Physiology of Lactic Acid Bacteria. Microbiol. Mol. Biol. Rev. 80, 837–890. https://doi.org/10.1128/MMBR.00076-15

Passoth, V., Sandgren, M., 2019. Biofuel production from straw hydrolysates: current achievements and perspectives. Appl. Microbiol. Biotechnol. 103, 5105–5116. https://doi.org/10.1007/s00253-019-09863-3

Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W.P., Ryan, C.M., del Cardayre, S., 2002. Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat. Biotechnol. 20, 707–712. https://doi.org/10.1038/nbt0702-707

Peng, L.L., Wang, L.M., Che, C.C., Yang, G., Yu, B., Ma, Y.H., 2013. *Bacillus* sp strain P38: An efficient producer of L-lactate from cellulosic hydrolysate, with high tolerance for 2-furfural. Bioresour. Technol. 149, 169–176. https://doi.org/10.1016/j.biortech.2013.09.047

Pérez-Gallardo, R. V, Briones, L.S., Díaz-Pérez, A.L., Gutiérrez, S., Rodríguez-Zavala, J.S., Campos-García, J., 2013. Reactive oxygen species production induced by ethanol in *Saccharomyces cerevisiae* increases because of a dysfunctional mitochondrial iron–sulfur cluster assembly system. FEMS Yeast Res. 13, 804–819. https://doi.org/10.1111/1567-1364.12090

Perseo, 2020. Proceso PERSEO Bioethanol. http://www.imecal.com/perseo/proceso-perseo-bioethanol/

Pleissner, D., Dietz, D., van Duuren, J.B.J.H., Wittmann, C., Yang, X., Lin, C.S.K., Venus, J., 2017a. Biotechnological Production of Organic Acids from Renewable Resources. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–38. https://doi.org/10.1007/10_2016_73

Pleissner, D., Lau, K.Y., Schneider, R., Venus, J., Lin, C.S.K., 2015. Fatty acid feedstock preparation and lactic acid production as integrated processes in mixed restaurant food and bakery wastes treatment. Food Res. Int. 73, 52–61. https://doi.org/10.1016/j.foodres.2014.11.048

Pleissner, D., Neu, A.K., Mehlmann, K., Schneider, R., Puerta-Quintero, G.I., Venus, J., 2016. Fermentative lactic acid production from coffee pulp hydrolysate using *Bacillus coagulans* at laboratory and pilot scales. Bioresour. Technol. 218, 167–173. https://doi.org/10.1016/j.biortech.2016.06.078

Pleissner, D., Schneider, R., Venus, J., Koch, T., 2017b. Separation of lactic acid and recovery of salt-ions from fermentation broth. J. Chem. Technol. Biotechnol. 92, 504–511. https://doi.org/10.1002/jctb.5023

Posthuma, C.C., Bader, R., Engelmann, R., Postma, P.W., Hengstenberg, W., Pouwels, P.H., 2002. Expression of the xylulose 5-phosphate phosphoketolase gene, xpkA, from *Lactobacillus pentosus* MD363 is induced by sugars that are fermented via the phosphoketolase pathway and is repressed by glucose mediated by CcpA and the mannose phosphoenolpyruvate phosphotransferase. Appl. Environ. Microbiol. 68, 831–837. https://doi.org/10.1128/aem.68.2.831-837.2002

Procethol2G, 2020. 2nd generation biofuels: an industrial first for french futurol™ technology. https://www.ifpenergiesnouvelles.com/article/2nd-generation-biofuels-industrial-first-french-futuroltm-technology

Prückler, M., Lorenz, C., Endo, A., Kraler, M., Dürrschmid, K., Hendriks, K., Soares da Silva, F., Auterith, E., Kneifel, W., Michlmayr, H., 2015. Comparison of homo- and heterofermentative lactic acid bacteria for implementation of fermented wheat bran in bread. Food Microbiol. 49, 211–219. https://doi.org/10.1016/j.fm.2015.02.014

Qin, J., Wang, X., Zheng, Z., Ma, C., Tang, H., Xu, P., 2010. Production of l-lactic acid by a thermophilic *Bacillus* mutant using sodium hydroxide as neutralizing agent. Bioresour. Technol. 101, 7570–7576. https://doi.org/10.1016/j.biortech.2010.04.037

Qing, Q., Wyman, C.E., 2011. Supplementation with xylanase and *β*-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. Biotechnol. Biofuels 4, 18. https://doi.org/10.1186/1754-6834-4-18

Qiu, Z., Gao, Q., Bao, J., 2018. Engineering *Pediococcus acidilactici* with xylose assimilation pathway for high titer cellulosic l-lactic acid fermentation. Bioresour. Technol. 249, 9–15. https://doi.org/10.1016/j.biortech.2017.09.117

Rao, R.S., Bhadra, B., Shivaji, S., 2008. Isolation and characterization of ethanol-producing yeasts from fruits and tree barks. Lett. Appl. Microbiol. 47, 19–24. https://doi.org/10.1111/j.1472-765X.2008.02380.x

Reddy, L. V, Park, J.H., Wee, Y.J., 2015. Homofermentative production of optically pure L-lactic acid from sucrose and mixed sugars by batch fermentation of *Enterococcus faecalis* RKY1. Biotechnol. Bioprocess Eng. 20, 1099–1105. https://doi.org/10.1007/s12257-015-0379-3

REN21, 2019. Renewables 2019 Global Status Report. https://www.ren21.net/gsr-2019/

Rezaiki, L., Cesselin, B., Yamamoto, Y., Vido, K., van West, E., Gaudu, P., Gruss, A., 2004. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. Mol. Microbiol. 53, 1331–1342. https://doi.org/10.1111/j.1365-2958.2004.04217.x

RFA, 2018. Pocket Guide to Ethanol. https://ethanolrfa.org/wp-content/uploads/2019/02/RFA2019PocketGuide.pdf

Rivera, O.M.P., Torrado, A.M., Moldes, A.B., Dominguez, J.M., 2009. Minerals and organic nitrogen present in grape marc hydrolyzates enhance xylose consumption by *Lactobacillus pentosus*. Appl. Biochem. Biotechnol. 152, 262–274. https://doi.org/10.1007/s12010-008-8252-x

Robak, K., Balcerek, M., 2018. Review of Second Generation Bioethanol Production from Residual Biomass. Food Technol. Biotechnol. 56, 174–187. https://doi.org/10.17113/ftb.56.02.18.5428

Rodrigues, A.C., Haven, M.Ø., Lindedam, J., Felby, C., Gama, M., 2015. Celluclast and Cellic® CTec2: Saccharification/fermentation of wheat straw, solid–liquid partition and potential of enzyme recycling by alkaline washing. Enzyme Microb. Technol. 79–80, 70–77. https://doi.org/10.1016/j.enzmictec.2015.06.019

Roque, L.R., Morgado, G.P., Nascimento, V.M., Ienczak, J.L., Rabelo, S.C., 2019. Liquid-liquid extraction: A promising alternative for inhibitors removing of pentoses fermentation. Fuel 242, 775–787. https://doi.org/10.1016/j.fuel.2018.12.130

Rosales-Calderon, O., Arantes, V., 2019. A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol. Biotechnol. Biofuels 12, 240. https://doi.org/10.1186/s13068-019-1529-1

Rosgaard, L., Andric, P., Dam-Johansen, K., Pedersen, S., Meyer, A.S., 2007. Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. Appl. Biochem. Biotechnol. 143, 27–40. https://doi.org/10.1007/s12010-007-0028-1

Runquist, D., Hahn-Hägerdal, B., Bettiga, M., 2010. Increased Ethanol Productivity in Xylose-Utilizing *Saccharomyces cerevisiae* via a Randomly Mutagenized Xylose Reductase. Appl. Environ. Microbiol. 76, 7796 – 7802. https://doi.org/10.1128/AEM.01505-10

Sahoo, T.K., Jayaraman, G., 2019. Co-culture of Lactobacillus delbrueckii and engineered *Lactococcus lactis* enhances stoichiometric yield of D-lactic acid from whey permeate. Appl. Microbiol. Biotechnol. 103, 5653–5662. https://doi.org/10.1007/s00253-019-09819-7

Sárvári Horváth, I., Franzén, C.J., Taherzadeh, M.J., Niklasson, C., Lidén, G., 2003. Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats. Appl. Environ. Microbiol. 69, 4076–4086. https://doi.org/10.1128/aem.69.7.4076-4086.2003

Sasaki, C., Okumura, R., Asada, C., Nakamura, Y., 2014. Steam explosion treatment for ethanol production from branches pruned from pear trees by simultaneous saccharification and fermentation. Biosci. Biotechnol. Biochem. 78, 160–166. https://doi.org/10.1080/09168451.2014.877818

Sassner, P., Galbe, M., Zacchi, G., 2006. Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated Salix at high dry-matter content. Enzyme Microb. Technol. 39, 756–762. https://doi.org/10.1016/j.enzmictec.2005.12.010

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26, 100–108. https://doi.org/10.1016/J.TIBTECH.2007.11.006

Sauer, U., 2001. Evolutionary engineering of industrially important microbial phenotypes. Adv. Biochem. Eng. Biotechnol. 73, 129–169. https://doi.org/10.1007/3-540-45300-8_7

Scheller, H.V., Ulvskov, P., 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61, 263–289. https://doi.org/10.1146/annurev-arplant-042809-112315

Secchi, N., Giunta, D., Pretti, L., Garcia, M.R., Roggio, T., Mannazzu, I., Catzeddu, P., 2012. Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. J. Ind. Microbiol. Biotechnol. 39, 175–181. https://doi.org/10.1007/s10295-011-1013-9

Shaghaghi-Moghaddam, R., Jafarizadeh-Malmiri, H., Mehdikhani, P., Jalalian, S., Alijanianzadeh, R., 2018. Screening of the five different wild, traditional and industrial *Saccharomyces cerevisiae* strains to overproduce bioethanol in the batch submerged fermentation. Z. Naturforsch. C. 73, 361–366. https://doi.org/10.1515/znc-2017-0180

Shen, Y., Chen, X., Peng, B., Chen, L., Hou, J., Bao, X., 2012. An efficient xylose-fermenting recombinant *Saccharomyces cerevisiae* strain obtained through adaptive evolution and its global transcription profile. Appl. Microbiol. Biotechnol. 96, 1079–1091. https://doi.org/10.1007/s00253-012-4418-0

Shi, Y., Ge, Y., Chang, J., Shao, H., Tang, Y., 2013. Garden waste biomass for renewable and sustainable energy production in China: Potential, challenges and development. Renew. Sustain. Energy Rev. 22, 432–437. https://doi.org/10.1016/j.rser.2013.02.003

Shin, H.Y., Nijland, J.G., de Waal, P.P., de Jong, R.M., Klaassen, P., Driessen, A.J.M., 2015. An engineered cryptic Hxt11 sugar transporter facilitates glucose-xylose co-consumption in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 8, 176. https://doi.org/10.1186/s13068-015-0360-6

Sikder, J., Chakraborty, S., Pal, P., Drioli, E., Bhattacharjee, C., 2012. Purification of lactic acid from microfiltrate fermentation broth by cross-flow nanofiltration. Biochem. Eng. J. 69, 130–137. https://doi.org/10.1016/j.bej.2012.09.003

Silveira, M.G., Baumgärtner, M., Rombouts, F.M., Abee, T., 2004. Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. Appl. Environ. Microbiol. 70, 2748–2755. https://doi.org/10.1128/aem.70.5.2748-2755.2004

Singhvi, M., Gurjar, G., Gupta, V., Gokhale, D., 2015. Biocatalyst development for lactic acid production at acidic pH using inter-generic protoplast fusion. Rsc Adv. 5, 2024–2031. https://doi.org/10.1039/c4ra11104d

Singhvi, M., Zendo, T., Sonomoto, K., 2018. Free lactic acid production under acidic conditions by lactic acid bacteria strains: challenges and future prospects. Appl. Microbiol. Biotechnol. 102, 5911–5924. https://doi.org/10.1007/s00253-018-9092-4

Slininger, P.J., Bothast, R.J., Ladisch, M.R., Okos, M.R., 1990. Optimum pH and temperature conditions for xylose fermentation by *Pichia stipitis*. Biotechnol. Bioeng. 35, 727–731. https://doi.org/10.1002/bit.260350710

Smith, J., van Rensburg, E., Görgens, J.F., 2014. Simultaneously improving xylose fermentation and tolerance to lignocellulosic inhibitors through evolutionary engineering of recombinant *Saccharomyces cerevisiae* harbouring xylose isomerase. BMC Biotechnol. 14, 41. https://doi.org/10.1186/1472-6750-14-41

Soderstrom, J., Pilcher, L., Galbe, M., Zacchi, G., 2003. Combined use of H_2SO_4 and SO_2 impregnation for steam pretreatment of spruce in ethanol production. Appl. Biochem. Biotechnol. 105–108, 127–140. https://doi.org/10.1385/abab:105:1-3:127

Stanley, D., Bandara, A., Fraser, S., Chambers, P.J., Stanley, G.A., 2010. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J. Appl. Microbiol. 109, 13–24. https://doi.org/10.1111/j.1365-2672.2009.04657.x

Sweeney, M.D., Xu, F., 2012. Biomass Converting Enzymes as Industrial Biocatalysts for Fuels and Chemicals: Recent Developments. Catalysts 2. https://doi.org/10.3390/catal2020244

Tajsoleiman, T., Mears, L., Kruhne, U., Gernaey, K. V, Cornelissen, S., 2019. An Industrial Perspective on Scale-Down Challenges Using Miniaturized Bioreactors. Trends Biotechnol. 37, 697–706. https://doi.org/10.1016/j.tibtech.2019.01.002

Tashiro, Y., Kaneko, W., Sun, Y., Shibata, K., Inokuma, K., Zendo, T., Sonomoto, K., 2011. Continuous d-lactic acid production by a novelthermotolerant *Lactobacillus delbrueckii* subsp. lactis QU 41. Appl. Microbiol. Biotechnol. 89, 1741–1750. https://doi.org/10.1007/s00253-010-3011-7

Tesfaw, A., Assefa, F., 2014. Current Trends in Bioethanol Production by *Saccharomyces cerevisiae*: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. Int. Sch. Res. Not. 2014, 532852. https://doi.org/10.1155/2014/532852

Tetma, 2018. TETma y CENTREVerd siguen con su compromiso por mejorar el medioambiente con el proyecto BIO_LIGWASTE. https://tetma.com/2018/03/28/tetma-y-centreverd-siguen-con-su-compromiso-por-mejorar-el-medioambiente-con-el-proyecto-bio-ligwaste/

Thangavelu, S.K., Ahmed, A., Ani, F., 2016. Corrosive characteristics of bioethanol and gasoline blends for metals. Int. J. Energy Res. 40. https://doi.org/10.1002/er.3555

Tian, S., Luo, X.L., Yang, X.S., Zhu, J.Y., 2010. Robust cellulosic ethanol production from SPORL-pretreated lodgepole pine using an adapted strain *Saccharomyces cerevisiae* without detoxification. Bioresour. Technol. 101, 8678–8685. https://doi.org/10.1016/j.biortech.2010.06.069

Tomás-Pejó, E., 2011. Fermenting microorganisms for 2nd generation bioethanol production. Bioprocess Sci. Technol. 187–208.

Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Chapter 7 - Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion A2 - Pandey, Ashok, in: Larroche, C., Ricke, S.C., Dussap, C.-G., Gnansounou, E. (Eds.), Biofuels. Academic Press, Amsterdam, pp. 149–176. https://doi.org/10.1016/B978-0-12-385099-7.00007-3

Tomás-Pejó, E., Ballesteros, M., Oliva, J.M., Olsson, L., 2010. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. J.Ind. Microbiol. Biotechnol. 37, 1211–1220. https://doi.org/10.1007/s10295-010-0768-8

Tomás-Pejó, E., Bonander, N., Olsson, L., 2014. Industrial yeasts strains for biorefinery solutions: Constructing and selecting efficient barcoded xylose fermenting strains for ethanol. Biofuels, Bioprod. Biorefining 8, 626–634. https://doi.org/10.1002/bbb.1472

Tomás-Pejó, E., Oliva, J.M., Ballesteros, M., Olsson, L., 2008. Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting *Saccharomyces cerevisiae* strains. Biotechnol. Bioeng. 100, 1122–1131. https://doi.org/10.1002/bit.21849

Tomás-Pejó, E., Oliva, J.M., González, A., Ballesteros, I., Ballesteros, M., 2009. Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous saccharification and fermentation fed-batch process. Fuel 88, 2142–2147. https://doi.org/10.1016/j.fuel.2009.01.014

Toor, M., Kumar, S.S., Malyan, S.K., Bishnoi, N.R., Mathimani, T., Rajendran, K., Pugazhendhi, A., 2020. An overview on bioethanol production from lignocellulosic feedstocks. Chemosphere 242, 125080. https://doi.org/10.1016/j.chemosphere.2019.125080

Trcek, J., Mira, N.P., Jarboe, L.R., 2015. Adaptation and tolerance of bacteria against acetic acid. Appl Microbiol. Biotechnol. 99, 6215–6229. https://doi.org/10.1007/s00253-015-6762-3

Treebupachatsakul, T., Nakazawa, H., Shinbo, H., Fujikawa, H., Nagaiwa, A., Ochiai, N., Kawaguchi, T., Nikaido, M., Totani, K., Shioya, K., Shida, Y., Morikawa, Y., Ogasawara, W., Okada, H., 2016. Heterologously expressed Aspergillus aculeatus beta-glucosidase in *Saccharomyces cerevisiae* is a cost-effective alternative to commercial supplementation of beta-glucosidase in industrial ethanol production using Trichoderma reesei cellulases. J. Biosci. Bioeng. 121, 27–35. https://doi.org/10.1016/j.jbiosc.2015.05.002

Triratna, L., Saksono, B., Sukmarini, L., Suparman, A., 2011. Genome-Shuffling-Improved Acid Tolerance and Lactic Acid Production in *Lactobacillus plantarum* for Commercialization. Microb. Indonesia 5, 10. https://doi.org/10.5454/mi.5.1.4

Ulery, B.D., Nair, L.S., Laurencin, C.T., 2011. Biomedical Applications of Biodegradable Polymers. J. Polym. Sci. B. Polym. Phys. 49, 832–864. https://doi.org/10.1002/polb.22259

USDA, 2019. Brazil Biofuels Annual 2019.
https://apps.fas.usda.gov/newgainapi/api/report/downloadreportbyfilename?filename=Biofuels%20Annual_Sao%20Paulo%20ATO_Brazil_8-9-2019.pdf

Utrilla, J., Licona-Cassani, C., Marcellin, E., Gosset, G., Nielsen, L.K., Martinez, A., 2012. Engineering and adaptive evolution of *Escherichia coli* for D-lactate fermentation reveals GatC as a xylose transporter. Metab. Eng. 14, 469–476. https://doi.org/10.1016/j.ymben.2012.07.007

van Bokhorst-van de Veen, H., Abee, T., Tempelaars, M., Bron, P.A., Kleerebezem, M., Marco, M.L., 2011. Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 77, 5247–5256. https://doi.org/10.1128/AEM.00515-11

van der Pol, E.C., Eggink, G., Weusthuis, R.A., 2016a. Production of l(+)-lactic acid from acid pretreated sugarcane bagasse using *Bacillus coagulans* DSM2314 in a simultaneous saccharification and fermentation strategy. Biotechnol. Biofuels. 15, 248. https://doi.org/10.1186/s13068-016-0646-3

van der Pol, E.C., Vaessen, E., Weusthuis, R.A., Eggink, G., 2016b. Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small-scale screening method. Bioresour. Technol. 209, 297–304. https://doi.org/10.1016/j.biortech.2016.03.037

Vassilyadi, M., Archibald, F., 1985. Catalase, superoxide dismutase, and the production of O_2-sensitive mutants of *Bacillus coagulans*. Can. J. Microbiol. 31, 994–999. https://doi.org/10.1139/m85-188

Vianna Bernardi, A., Kimie Yonamine, D., Akira Uyemura, S., Magnani Dinamarco, T., 2019. A Thermostable *Aspergillus fumigatus* GH7 Endoglucanase Over-Expressed in *Pichia pastoris* Stimulates Lignocellulosic Biomass Hydrolysis. Int. J. Mol. Sci. 20. https://doi.org/10.3390/ijms20092261

Vilela, L. de F., de Araujo, V.P.G., Paredes, R. de S., Bon, E.P. da S., Torres, F.A.G., Neves, B.C., Eleutherio, E.C.A., 2015. Enhanced xylose fermentation and ethanol production by engineered *Saccharomyces cerevisiae* strain. AMB Express 5, 16. https://doi.org/10.1186/s13568-015-0102-y

Vudata Venkata, P.B.R., Kumar, P., Sailu, C., Rao, S., 2014. Recovery of Lactic Acid by Reactive Distillation. J. Appl. Sci. 14, 1289–1293. https://doi.org/10.3923/jas.2014.1289.1293

Wallace-Salinas, V., Gorwa-Grauslund, M.F., 2013. Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature. Biotechnol. Biofuels 6, 151. https://doi.org/10.1186/1754-6834-6-151

Wang, C., Chang, T., Yang, H., Cui, M., 2015. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella Enteritidis*, *Escherichia coli* and *Listeria monocytogenes*. Food Control 47, 231–236. https://doi.org/10.1016/j.foodcont.2014.06.034

Wang, Y., Li, Y., Pei, X., Yu, L., Feng, Y., 2007. Genome-shuffling improved acid tolerance and l-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. J. Biotechnol. 129, 510–515. https://doi.org/10.1016/j.jbiotec.2007.01.011

Wang, Y., Liu, J., Cai, D., Zhao, G., 2018. Co-generation of ethanol and l-lactic acid from corn stalk under a hybrid process. Biotechnol. Biofuels 11, 331. https://doi.org/10.1186/s13068-018-1330-6

Wenger, J., Stern, T., 2019. Reflection on the research on and implementation of biorefinery systems – a systematic literature review with a focus on feedstock. Biofuels, Bioprod. Biorefining 13, 1347–1364. https://doi.org/10.1002/bbb.2021

Westman, J.O., Wang, R., Novy, V., Franzén, C.J., 2017. Sustaining fermentation in high-gravity ethanol production by feeding yeast to a temperature-profiled multifeed simultaneous saccharification and co-fermentation of wheat straw. Biotechnol. Biofuels 10, 213. https://doi.org/10.1186/s13068-017-0893-y

Wischral, D., Arias, J.M., Modesto, L.F., de Franca Passos, D., Pereira Jr., N., 2019. Lactic acid production from sugarcane bagasse hydrolysates by *Lactobacillus pentosus*: Integrating xylose and glucose fermentation. Biotechnol. Prog. 35, e2718. https://doi.org/10.1002/btpr.2718

Xie, Y., Hu, Q., Feng, G., Jiang, X., Hu, J., He, M., Hu, G., Zhao, S., Liang, Y., Ruan, Z., Peng, N., 2018. Biodetoxification of Phenolic Inhibitors from Lignocellulose Pretreatment using *Kurthia huakuii* LAM0618(T) and Subsequent Lactic Acid Fermentation. Molecules 23. https://doi.org/10.3390/molecules23102626

Xin, D., Chen, X., Wen, P., Zhang, J., 2019. Insight into the role of α-arabinofuranosidase in biomass hydrolysis: cellulose digestibility and inhibition by xylooligomers. Biotechnol. Biofuels 12, 64. https://doi.org/10.1186/s13068-019-1412-0

Yan, Y., Bu, C., He, Q., Zheng, Z., Ouyang, J., 2018. Efficient bioconversion of furfural to furfuryl alcohol by *Bacillus coagulans* NL01. RSC Adv. 8, 26720–26727. https://doi.org/10.1039/C8RA05098H

Yang, X., Teng, K., Zhang, J., Wang, F., Zhang, T., Ai, G., Han, P., Bai, F., Zhong, J., 2017. Transcriptome responses of *Lactobacillus acetotolerans* F28 to a short and long term ethanol stress. Sci. Rep. 7, 2650. https://doi.org/10.1038/s41598-017-02975-8

Ye, L., Zhao, H., Li, Z., Wu, J.C., 2013a. Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. Bioresour. Technol. 135, 459–463. https://doi.org/10.1016/j.biortech.2012.10.042

Ye, L., Zhou, X., Hudari, M.S. Bin, Li, Z., Wu, J.C., 2013b. Highly efficient production of l-lactic acid from xylose by newly isolated *Bacillus coagulans* C106. Bioresour. Technol. 132, 38–44. https://doi.org/10.1016/j.biortech.2013.01.011

Ye, L.D., Bin Hudari, M.S., Li, Z., Wu, J.C., 2014. Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid production by *Bacillus coagulans* JI12. Biochem. Eng. J. 83, 16–21. https://doi.org/10.1016/j.bej.2013.12.005

Yi, X., Zhang, P., Sun, J.E., Tu, Y., Gao, Q.Q., Zhang, J., Bao, J., 2016. Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock. J. Biotechnol. 217, 112–121. https://doi.org/10.1016/j.jbiotec.2015.11.014

Yu, Y., Feng, Y., Xu, C., Liu, J., Li, D., 2011. Onsite bio-detoxification of steam-exploded corn stover for cellulosic ethanol production. Bioresour. Technol. 102, 5123–5128. https://doi.org/10.1016/j.biortech.2011.01.067

Yuan, L., Zhao, H., Liu, L., Peng, S., Li, H., Wang, H., 2019. Heterologous expression of the puuE from *Oenococcus oeni* SD-2a in *Lactobacillus plantarum* WCFS1 improves ethanol tolerance. J. Basic Microbiol. 59, 1134–1142. https://doi.org/10.1002/jobm.201900339

Zacchi, G., Axelsson, A., 1989. Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions. Biotechnol. Bioeng. 34, 223–233. https://doi.org/10.1002/bit.260340211

Zhai, R., Hu, J., Saddler, J.N., 2018. Extent of Enzyme Inhibition by Phenolics Derived from Pretreated Biomass Is Significantly Influenced by the Size and Carbonyl Group Content of the Phenolics. ACS Sustain. Chem. Eng. 6, 3823–3829. https://doi.org/10.1021/acssuschemeng.7b04178

Zhang, J., Wu, C., Du, G., Chen, J., 2012. Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. Biotechnol. Bioprocess Eng. 17, 283–289. https://doi.org/10.1007/s12257-011-0346-6

Zhang, L., Li, X., Yong, Q., Yang, S.-T., Ouyang, J., Yu, S., 2016. Impacts of lignocellulose-derived inhibitors on L-lactic acid fermentation by *Rhizopus oryzae*. Bioresour. Technol. 203, 173–180. https://doi.org/10.1016/j.biortech.2015.12.014

Zhang, Liming, You, T., Zhang, Lu, Li, M., Xu, F., 2014. Comprehensive utilization of waste hemicelluloses during ethanol production to increase lactic acid yield: from pretreatment to fermentation. Biotechnol. Biofuels 7, 494. https://doi.org/10.1186/s13068-014-0189-4

Zhang, Q., Wu, D., Lin, Y., Wang, X., Kong, H., Tanaka, S., 2015. Substrate and Product Inhibition on Yeast Performance in Ethanol Fermentation. Energy & Fuels 29, 1019–1027. https://doi.org/10.1021/ef502349v

Zhang, Y., Vadlani, P. V, 2013. D-Lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation. Bioprocess Biosyst. Eng. 36, 1897–1904. https://doi.org/10.1007/s00449-013-0965-8

Zhang, Y.X., Vadlani, P. V, 2015. Lactic acid production from biomass-derived sugars via co-fermentation of *Lactobacillus brevis* and *Lactobacillus plantarum*. J. Biosci. Bioeng. 119, 694–699. https://doi.org/10.1016/j.jbiosc.2014.10.027

Zhang, Y.X., Vadlani, P. V, Kumar, A., Hardwidge, P.R., Govind, R., Tanaka, T., Kondo, A., 2016. Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 100, 279–288. https://doi.org/10.1007/s00253-015-7016-0

Zhao, K., Qiao, Q.G., Chu, D.Q., Gu, H.Q., Dao, T.H., Zhang, J., Bao, J., 2013. Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2. Bioresour. Technol. 135, 481–489. https://doi.org/10.1016/j.biortech.2012.09.063

Zhao, Z., Yang, Y., Abdeltawab, A.A., Yakout, S.M., Chen, X., Yu, G., 2017. Cholinium amino acids-glycerol mixtures: New class of solvents for pretreating wheat straw to facilitate enzymatic hydrolysis. Bioresour. Technol. 245, 625–632. https://doi.org/10.1016/j.biortech.2017.08.209

Zhou, H., Lan, T., Dien, B.S., Hector, R.E., Zhu, J.Y., 2014. Comparisons of five *Saccharomyces cerevisiae* strains for ethanol production from SPORL-pretreated lodgepole pine. Biotechnol. Prog. 30, 1076–1083. https://doi.org/10.1002/btpr.1937

Zhou, X.D., Ye, L.D., Wu, J.C., 2013. Efficient production of l-lactic acid by newly isolated thermophilic *Bacillus coagulans* WCP10-4 with high glucose tolerance. Appl. Microbiol. Biotechnol. 97, 4309–4314. https://doi.org/10.1007/s00253-013-4710-7

Zhu, Y., Lee, Y.Y., Elander, R.T., 2007. Conversion of aqueous ammonia-treated corn stover to lactic acid by simultaneous saccharification and cofermentation. Appl. Biochem. Biotechnol. 137–140, 721–738. https://doi.org/10.1007/s12010-007-9092-9

RESEARCH ACTIVITIES

RESEARCH ACTIVITIES DURING PhD

List of publications

Article I. Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2018. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate. Biofuels, Bioprod. Biorefining. https://doi.org/10.1002/bbb.1852.

Article II. Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2019. *Lactobacillus pentosus* CECT 4023 T co-utilizes glucose and xylose to produce lactic acid from wheat straw hydrolysate: Anaerobiosis as a key factor. Biotechnol. Prog. 35, e2739. https://doi.org/10.1002/btpr.2739.

Article III. Cubas-Cano, E., González-Fernández, C., Ballesteros, I., Tomás-Pejó, E., 2020. Efficient utilization of hydrolysates from steam-exploded gardening residues for lactic acid production by optimization of enzyme addition and pH control. Waste Manag. 107, 235–243. https://doi.org/https://doi.org/10.1016/j.wasman.2020.04.003.

Article IV. Cubas-Cano, E., Venus, J., González-Fernández, C., Tomás-Pejó, E., 2020. Assessment of different *Bacillus coagulans* strains for L-lactic acid production from defined media and gardening hydrolysates: effect of lignocellulosic inhibitors. J. Biotechnol. 323, 9-16. https://doi.org/10.1016/j.jbiotec.2020.07.017

Article V. Cubas-Cano, E., González-Fernández, C., Tomás-Pejó, E., 2019. Evolutionary engineering of *Lactobacillus pentosus* improves lactic acid productivity from xylose-rich media at low pH. Bioresour. Technol. 288, 121540. https://doi.org/https://doi.org/10.1016/j.biortech.2019.121540.

Article VI. Cubas-Cano, E., López-Gómez, J.P., González-Fernández, C., Ballesteros, I., Tomás-Pejó, E., 2020. Towards sequential bioethanol and l-lactic acid co-generation: Improving xylose conversion to l-lactic acid in presence of lignocellulosic ethanol with an evolved *Bacillus coagulans*. Renew. Energy 153, 759–765. https://doi.org/https://doi.org/10.1016/j.renene.2020.02.066.

Conferences

"Producción de ácido láctico a partir de hidrolizado de paja de trigo: estudio de las condiciones de proceso". Enrique Cubas Cano, Mercedes Ballesteros, Cristina González Fernández, Elia Tomás Pejó. Oral presentation. **XIX Workshop Lignocel.** Madrid (Spain), 9th October 2017.

"Lactic acid production by *Lactobacillus pentosus* CECT4023 from wheat straw prehydrolysate: Study of the process conditions". Enrique Cubas Cano, Mercedes Ballesteros,

Cristina González Fernández, Elia Tomás Pejó. Poster presentation. **6th Annual Workshop of Young Researchers of IMDEA Energy.** Móstoles (Spain), 15th December 2017.

"Lactic acid production from hemicellulosic hydrolysates by *Lactobacillus pentosus* CECT 4023T in a biorefinery context". Enrique Cubas Cano, Mercedes Ballesteros, Cristina González Fernández, Elia Tomás Pejó. Oral presentation. **4th Iberoamerican Congress on Biorefineries (4-CIAB).** Jaén (Spain), 24th – 26th October 2018.

"Evolutionary engineering to improve lactic acid production from xylose. Obtaining an acid pH tolerant *Lactobacillus pentosus* strain". Enrique Cubas Cano, Cristina González Fernández, Elia Tomás Pejó. Oral presentation. **7th Annual Workshop of Young Researchers of IMDEA Energy.** Móstoles (Spain), 13th – 14th December 2018.

"Evolutionary engineering to improve lactic acid production from xylose-rich hemicellulosic hydrolysates: Obtaining an acid pH tolerant *Lactobacillus pentosus* strain". Enrique Cubas Cano, Cristina González Fernández, Elia Tomás Pejó. Oral presentation. **27th European Biomass Conference and Exhibition (EUBCE 2019).** Lisbon (Portugal), 27th – 29th May 2019.

"Ingeniería evolutiva para mejorar la producción de ácido láctico a partir de hidrolizados hemicelulósicos ricos en xilosa: obtención de una cepa de *Lactobacillus pentosus* resistente a pH ácido". Enrique Cubas Cano, Cristina González Fernández, Elia Tomás Pejó. Oral presentation. **XX Workshop Lignocel**. Miraflores de la Sierra (Spain), 4th – 5th June 2019.

"Efficient production of lactic acid from gardening residues importance of pH control for the resistance of inhibitory compounds". Enrique Cubas Cano, Cristina González-Fernández, Elia Tomás Pejó. Poster presentation. **EuroMicropH COST Action Open Meeting**. Lisbon (Portugal), 12th – 14th February 2020.

International short-term stay

"Production of highly pure L-lactic acid from lignocellulosic materials by optimisation of fermentation and downstream processing". Supervisor: Dr. Joachim Venus. **Bioengineering Department. Leibniz Institute of Agricultural Engineering and Bio-economy** e.V. (ATB). Potsdam (Germany), 16th September – 16th December 2020.

ARTICLE I

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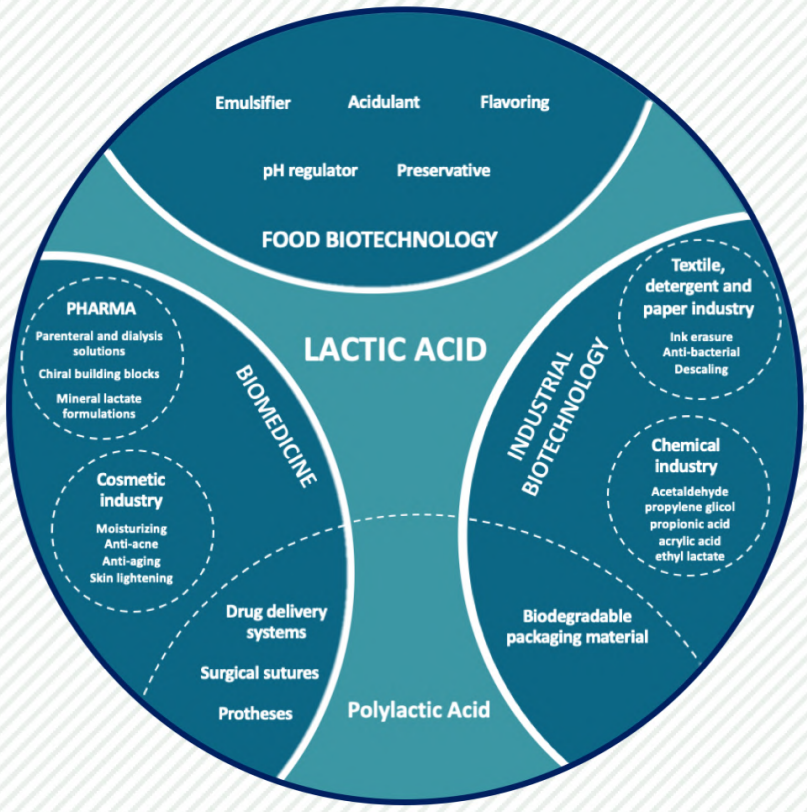

REVIEW

Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate

Enrique Cubas-Cano, Cristina González-Fernández, Mercedes Ballesteros, Elia Tomás-Pejó

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Review

Biofpr Biofuels Bioproducts & Biorefining

Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate

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Abstract: The production of high added-value products from lignocellulose is proposed as a suitable alternative to petroleum-based resources in terms of environmental preservation, sustainability, and circular economy. Lactic acid is a versatile building block that can be produced via fermentative routes by several groups of microorganisms, including yeasts and microalgae, which are bacteria recognized to achieve the highest concentrations. Lactic acid, among other substances, can be used as a starting point in the production of poly-lactic acid, which is a biopolymer with many applications due to its resistance, durability, biodegradability, and biocompatibility. Lactic acid production can be performed from lignocellulosic biomass. However, lactic acid production from lignocellulose faces several hurdles such as carbohydrate hydrolysis to release sugars, the co-utilization of sugar mixtures by the fermenting microorganism, and the presence of degradation compounds released during pretreatment. In this review, a general overview of lactic-acid bacterial fermentation from lignocellulose is provided, starting from the potential substrates and their composition, the different metabolic pathways involved, and the purification steps. The main challenges are discussed and the newest approaches to solve the limitations of the process are proposed. © 2018 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: lactic acid; lignocellulose; lactic acid bacteria; polylactic acid; fermentation

Introduction

The utilization of microorganisms for the production of bioproducts from renewable resources offers many advantages in terms of environmental sustainability and it is expected to play an important role in the bioeconomy in the near future. In this context, one of the most promising resources is lignocellulose. Lignocellulosic biomass, such as wood, agricultural wastes, and food-processing by-products, is rich in carbohydrates that can be transformed into different products via the sugar platform. Identifying the key benefits and development needs to use the sugar platform fully could lead to the production of biofuels and high-value products in an environmentally friendly manner through biochemical processes.

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Lactic acid (LA) is a natural organic acid that has been commercially used in the food industry as an emulsifying, acidulant, buffering, and preserving agent.[1,2] Due to its antibacterial and ink-erasure properties, it has also been used in the textile, detergent, and paper industries.[3] Currently, LA is starting to conquer new market niches related to added value products, such as pharmaceutical, cosmetic and chemical industry (Fig. 1).

The global LA market was 714 kt in 2013 and it is expected to reach 1960 kt by 2020. The largest market for LA is based in North America and was valued at USD 444 million in 2013; this is followed by Europe and Asia Pacific (http://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market). The major manufacturers include NatureWorks LLC (United States), Archer Daniels Midland Company (United States), Purac (The Netherlands) and Galactic S.A. (Belgium) (http://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market).

Lactic acid presents a hydroxyl group adjacent to the carboxyl group (2-hydroxypropanoic acid, CH3–CH(OH)–COOH). Due to the chirality of LA, there are two optical isomers, L-(+)-lactic acid (L-LA) and D-(-)-lactic acid (D-LA) (Fig. 2). Pure isomers are more valuable than the racemic mixture because each independent isomer has a specific industrial role. There is a preference in the use of L-LA in food and pharmaceutical industries because it can

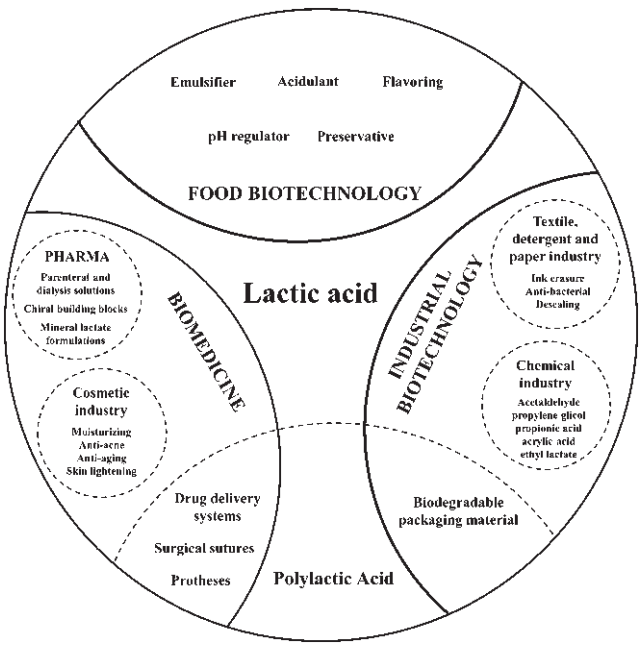

Figure 1. Lactic acid applications in different fields of biotechnology. Polylactic acid is included as the most promising and demanded LA-derived product.

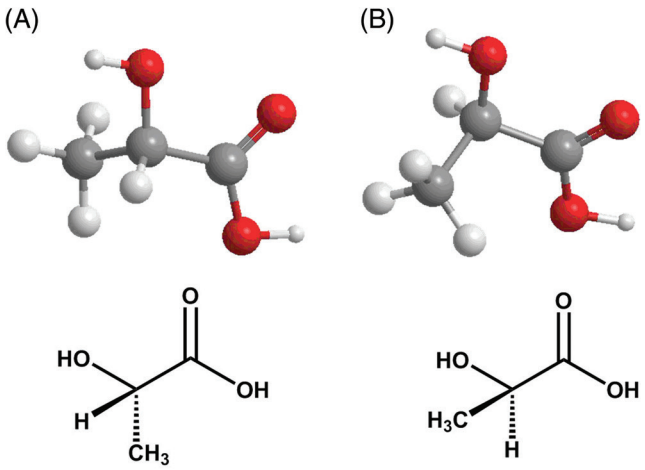

Figure 2. Three-dimensional structure of L(+)-lactic acid (A) and D(-)-lactic acid (B).

be completely metabolized by the human body due to the presence of L-lactate dehydrogenase.[2] In the field of green chemistry, both L-LA and D-LA can be used in the production of acetaldehyde, propylene glycol, propionic acid, acrylic acid, 2,3-pentadione and, mainly, as monomers in the production of polylactic acid (PLA) (Fig. 1).[4] Polylactic acid is a biodegradable alternative to petrochemical-based plastics. It exhibits high chemical resistance, rendering this polymer as an appropriate material for the manufacture of fibers, nonwoven fabrics, and films.[5] The use of this polymer in packaging is also starting to gain ground. Furthermore, numerous studies about fabrication of PLA-based prosthetic devices, surgical sutures, and controlled drug delivery systems in humans are being carried out given the biocompatible features of PLA.[6] When mixtures of both isomers (L-LA and D-LA) are used in the polymerization of PLA, the resulting polymer is amorphous and unstable. Nevertheless, using optically pure D-LA or L-LA, highly crystalline poly-D-(-)-lactic acid (PDLA) or poly-L-(+)-lactic acid (PLLA) is formed. This bioplastic has a lower melting point than conventional plastics, so several companies are working to enhance the heat resistance of PLA by co-polymerization with other monomers. Mixing and blending PLLA and PDLA results in an even more crystalline complex with high thermal stability, increasing the melting point.[7]

Lactic acid can be synthetized by chemical synthesis and microbial fermentation. The first involves the production of lactonitrile by the reaction of acetaldehyde with hydrogen cyanide, which derives from petrochemical sources, resulting in environmental negative consequences.[8] Furthermore, chemical synthesis yields a racemic mixture of LA. On the other hand, LA can also be produced by

fermentative processes and interestingly, some microorganisms can produce optically pure LA. Most worldwide LA production via fermentation derives from lactic fermentation using starchy substrates.[9] However, starchy raw materials compete with food-crops and alternative renewable non-food substrates are required. In this context, the use of renewable sugar sources such as lignocellulosic biomass, avoiding the competition of land-use with food is an interesting alternative.[10] Nonetheless, utilization of lignocellulosic biomass has some drawbacks. Only few microorganisms can metabolize pentoses from hemicellulose to LA, and the ones that possess this ability produce LA together with some other by-products via heterolactic fermentation.[11] As a matter of fact, there is a hierarchical sugar utilization in which glucose is consumed before pentoses.[12] The feasibility of this microbial bioprocess is limited by the presence of degradation compounds, end-product inhibition, and the need for accurate pH control, among others, being the LA purification another critical step.

This review describes the most up-to-date approaches for LA production from lignocellulosic biomass with lactic acid bacteria (LAB) and proposes alternatives to circumvent the main limitations of this process.

Novel substrates for lactic acid production: lignocellulosic biomass

The viability of LA production by fermentation depends on many different factors, the cost of the raw materials being one of the most significant. Lignocellulosic materials appear as attractive options due to their wide availability, low cost, renewability, and no competition with food crops. The annual production of lignocellulosic biomass has been estimated worldwide as more than 10^{10} MT.[13] Thus, lignocellulose is likely to be an important substrate for the bioeconomy in the near future.

The most commonly used lignocellulosic sources include agricultural residues (cereal straw, sugarcane bagasse, corn stover, etc.) and forestry woody feedstocks (spruce, eucalyptus, birch, etc.). However, energy crops (poplar, switch grass, giant reed, elephantgrass, *Miscanthus giganteus*, etc.), industrial residues (brews' spent grains, paper mill residues, cheese whey, etc.) and the organic fraction of municipal solid wastes are considered attractive alternatives with high potential for the production of biofuels and bioproducts.

Alongside other less abundant components such as extractives and ash, lignocellulosic materials are mainly composed of: (1) cellulose, a linear polymer of D-glucose, (2) hemicellulose, a branched polymer with more than one kind of sugar (D-xylose, L-arabinose, D-mannose, D-glucose and D-galactose), and (3) lignin, an amorphous monocrystalline polymer built up by ρ-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

Lignocellulosic materials need to be subjected to a hydrolysis step in order to convert the polysaccharides into potentially fermentable sugars (Fig. 3). Hydrolysis can be performed by means of acid catalysts or through enzymatic catalysts. Acid hydrolysis requires relatively high temperatures and generates toxic compounds, but enzymatic hydrolysis requires lower energy input and shows higher conversion efficiencies.[14] Enzymatic hydrolysis is, however, hindered by the high recalcitrant structure of lignocellulosic biomass. In this sense, a pretreatment is necessary to facilitate an efficient and rapid enzymatic hydrolysis of carbohydrates. Harsh conditions applied during pretreatment lead to a partial hemicellulose and lignin degradation and generation of toxic compounds that can act as inhibitors of the hydrolytic enzymes and fermentative microorganisms.[15] Despite this, lignocellulosic LA production has received a lot of attention in the last decade, as is shown by examples depicted below.

Lactic acid-producing bacteria

Lactic acid bacteria can be described as gram-positive, non-sporeforming, and aerotolerant bacteria that produce LA as a predominant or unique fermentation product.[16] Lactic acid bacteria are included in the order Lactobacillales by 16S rRNA sequencing. It contains six families with 38 genera, such as *Lactobacillus, Pediococcus, Enterococcus, Leuconostoc, Streptococcus,* and *Lactococcus*.[16] Lactic acid bacteria need plenty of rich nitrogen sources as, natively, they lack various biosynthetic pathways. Most of them are able to tolerate acidic conditions and a high temperature range.[17] There are also some non-LAB that produce LA with significant yields,

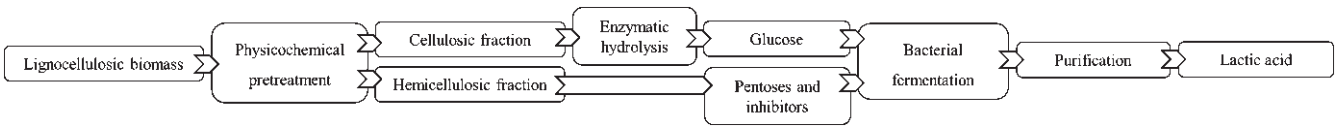

Figure 3. Sequential steps in LA production using C5 and C6 sugars from lignocellulosic biomass.

such as *Escherichia coli*, *Corynebacterium glutamicum* and, among them, several *Bacillus* strains.[17]

The metabolic pathways involved in LA production are the Embden-Meyerhof-Parnas (EMP) pathway, the pentose phosphate (PP) pathway and the phosphoketolase (PK) pathway. Many LAB have one or more of these metabolic pathways and the conversion yield and energy generated will rely on the particular strain and growth conditions. [18] Based on this, LAB can be classified as follows.

Homolactic LAB

Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus delbrueckii and *Lactobacillus acidophilus* are included in this group.[17,19] They convert hexoses to pyruvate via the EMP pathway –encoding aldolase enzyme – and then to lactate using the enzyme lactate dehydrogenase (LDH) (Fig. 4). One molecule of glucose yields two molecules of LA, generating two ATP (1).

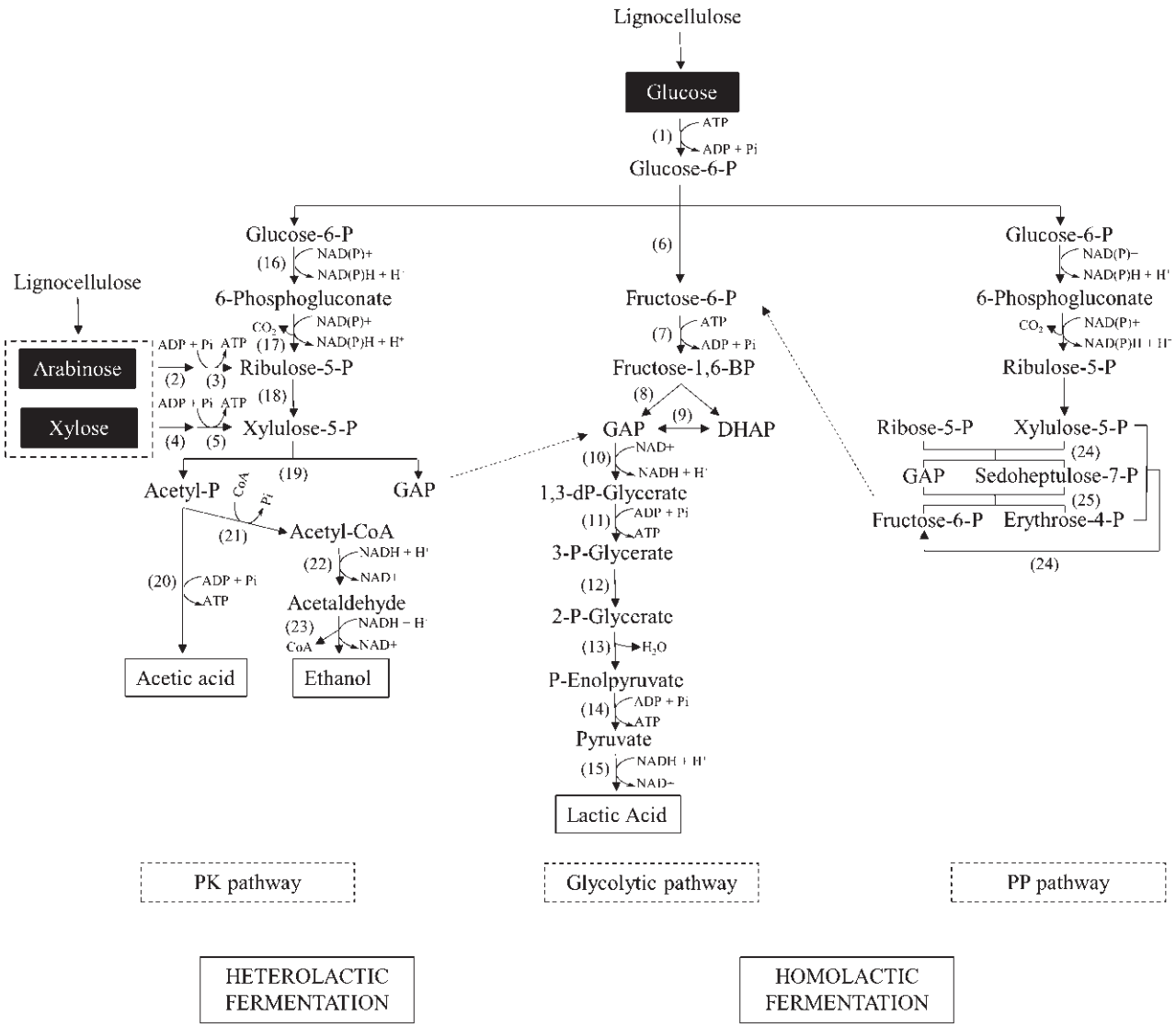

Figure 4. Metabolic pathways involved in LA production from different lignocellulosic carbon sources. (1) hexokinase, (2) arabinose isomerase, (3) ribulokinase, (4) xylose isomerase, (5) xylulokinase, (6) glucose-6-P isomerase, (7) 6-phosphofructokinase, (8) fructose-bisphosphate aldolase, (9) triose-phosphate isomerase, (10) glyceraldehyde-3-P dehydrogenase, (11) phosphoglycerate kinase, (12) phosphoyglycerate mutase, (13) enolase, (14) pyruvate kinase, (15) lactate dehydrogenase, (16) glucose-6-phosphate dehydrogenase, (17) 6-phosphogluconate dehydrogenase, (18) ribulose-5-phosphate-3-epimerase, (19) phosphoketolase, (20) acetate kinase, (21) phosphotransacetylase, (22) aldehyde dehydrogenase, (23) alcohol dehydrogenase, (24) transketolase, (25) transaldolase.

$$\text{Glucose} + 2\ \text{Pi} + 2\ \text{ADP} \rightarrow 2\ \text{LA} + 2\ \text{ATP} + 2\ H_2O \quad (1)$$

This metabolic pathway leads to a maximum theoretical yield of 2 mol LA per mol glucose (Table 1). Most of them have the ability to use hexoses as carbon source for LA production, but cannot use pentoses derived from hemicellulose.[20]

In some strains, glucose is metabolized by the PP pathway (2), reaching glyceraldehyde-3-P (GAP) and fructose-6-P (Fig. 4). The later compound is finally converted to GAP via EM pathway. In this case, the maximum theoretical yield is 1.67 mol LA per mol glucose (Table 1).

$$3\ \text{Glucose} + 6\ \text{NAD(P)} + 5\ \text{Pi} + 5\ \text{ADP} \rightarrow 5\ \text{LA} + 3\ CO_2 + 6\ \text{NAD(P)H} + 5\ \text{ATP} + 2\ H_2O \quad (2)$$

Obligately heterolactic LAB

These LAB metabolize hexoses and pentoses exclusively via the PK pathway. One molecule of CO_2 is cleaved from a six-carbon sugar, forming a ribulose-5-P. This ribulose-5-P is cleaved again, catalyzed by phosphoketolase, resulting in one molecule of GAP and another of acetyl-phosphate. Then, GAP is converted to LA via EMP pathway and the acetyl-phosphate is hydrolyzed to acetic acid (3) or converted into ethanol (4) and closing the electron balance (Fig. 4.). However, when pentoses are used as carbon sources, there is no NAD(P)H production, so ethanol is not produced and the acetyl-phosphate is converted directly to acetic acid (5). The maximum theoretical yield is 1 mol LA per mol hexose or pentose (Table 1). *Lactobacillus bifermentans*, *Leuconostoc lactis*, *Lactobacillus sanfranciscensis* and *Lactobacillus brevis* are assigned to this group.[21]

$$\text{Glucose} + 2\ \text{ADP} + \text{Pi} \rightarrow \text{LA} + \text{Acetic acid} + CO_2 + 2\ \text{ATP} + H_2O \quad (3)$$

$$\text{Glucose} + 2\ \text{NAD(P)} + 2\ \text{ADP} + \text{Pi} \rightarrow \text{LA} + CO_2 + \text{Ethanol} + 2\ \text{NAD(P)H} + \text{ATP} \quad (4)$$

$$\text{Xylose} + 2\ \text{ADP} + 2\ \text{Pi} \rightarrow \text{LA} + \text{Acetic acid} + 2\ \text{ATP} + 2\ H_2O \quad (5)$$

Facultative heterolactic LAB

Lactobacillus pentosus and *Lactobacillus plantarum* are recognized as facultative heterolactic bacteria.[20,22] These bacteria present both pathways in their metabolism. They metabolize hexoses via the EM pathway (1) and pentoses via the PK pathway (5). Because of this, acetic acid is not produced until all the glucose is completely depleted, shifting from homo- to heterofermentative.[23] The maximum yield is 1 and 2 mol LA per mol sugar from pentoses and hexoses, respectively.

Table 1. Lactic acid and energetic yields comparison between different metabolic pathways involved in LA fermentation of different sugars.

	Hexose		Pentose	
Metabolic pathway	LA yield (mol LA/mol hexose)	ATP yield (mol ATP/mol hexose)	LA yield (mol LA/mol pentose)	ATP yield (mol ATP/mol pentose)
EMP	2	2	—	—
PP	1.67	1.67	1.67	0.67
PK	1	2	1	2

In general terms, the targeted bacteria for LA production from lignocellulose would be a homofermentative strain that produces optically pure LA and tolerates inhibitors released during the pretreatment, high temperatures, and low pH with the ability to metabolize different sugars.

Lactic acid purification

The production of high added-value products, such as PLA and other chemicals, needs a highly pure LA as building block, so purification of LA from the fermentation broth is a crucial step for its commercialization. Some applications need optically pure D-LA or L-LA, so the purification step can be even more complicated when both isomers are produced during fermentation.[24] Separation and purification steps can account for 30–40% of total production costs.[25] Because of this, along with the feedstock price, purification is one of the steps that determine the economic viability of the process. In fact several synergic downstream steps are often needed for an efficient purification, increasing the LA price.

Since the accumulation of LA in the fermentation broth implies product inhibition, product removal from the culture as soon as it is formed is envisaged as a proper approach to increase the products' yields, requiring appropriate separation techniques.[26]

Currently, the calcium lactate process is the most used for separation purposes. This method is based on calcium hydroxide precipitation and it is already commercially applied by NatureWorks and Purac in LA production processes from starch.[25] After the addition of calcium hydroxide to the fermentation broth, calcium lactate crystals, which tend to precipitate, are formed. However, large amounts of these crystals inhibit the growth of LA producers such as *L. delbrueckii* and *B. coagulans*.[9] In addition, the purification of LA from calcium lactate produces

large amounts of gypsum ($CaSO_4$), which generates environmental and economic problems.[27,28]

Lactic acid can be recovered by other alternative methods such as extraction, crystallization, adsorption, ion exchange, membrane separation, electrodialysis, and reactive distillation.[29] High selectivity has been reported in crystallization, but its scalability is difficult.[30] Moreover, it has to be combined with other separation methods.[31] Solvent extraction is limited by the high toxicity of extractants and membrane separation by the high cost of membranes, together with the high energy consumption and fouling.[29]

Main challenges in lactic acid production using lignocellulosic substrates

Lactic acid yields can be seriously affected by several phenomena taking place during the production process. Most of these hurdles are common to any LA microbial production process but they are strengthened when lignocellulosic materials are used as substrate.

Carbon catabolite repression effect

The preference in the utilization of glucose by LAB leads to a sequential sugar uptake. For this reason, when a hydrolysate with a mixture of C5 and C6 sugars is used, the pentoses will not be fermented until the glucose level drops considerably.[27] In the presence of a mixture of sugars, activation of the catabolic repression element by glycolytic intermediates in the cells occurs. The repressor inhibits the expression of genes involved in the metabolism of alternative carbon sources. As the glucose is depleted and pH decreases, there is a reduction of glycolytic intermediates, which forces LAB to use carbon sources by heterofermentation.[32] Given that the non-simultaneous utilization of pentoses and hexoses decreases fermentation efficiency, new strategies must be developed in order to overcome this problem. As discussed later, the use of isolated or engineered strains capable of metabolizing mixtures of sugars or co-culturing pentose-utilizing LAB with homofermenters LAB could be a potential alternative to circumvent the carbon catabolite repression effect (Table 2).[11]

By-product formation during lactic acid heterofermentation

As a result of the use of pentoses for LA production, by-products like acetic acid or ethanol and CO_2 are produced by several LAB using the PK pathway (Fig. 4). This fact implies a decrease in LA yield. However, the production of these by-products can be avoided using novel or genetic modified strains that metabolize pentoses using a different metabolic pathway (Table 2).[33–37]

Table 2. Challenges of LA production and novel approaches to overcome them.

Challenges	Main approaches to circumvent the limitations
Recalcitrant lignocellulosic biomass	• SSF: co-culturing a hydrolytic enzyme producer microorganism with a LA producer[62]
	• SSF: engineering a LA producer to produce hydrolytic enzymes[22]
Carbon catabolite repression effect	• Bacteria that can use mixed sugars with relaxed carbon catabolite repression
	• Isolated or engineered xylose utilizing bacteria[20,67]
	• Co-cultures of hexose and pentose-utilizing bacteria[63]
By-product formation	• Isolated or engineered homofermentative bacteria[33–36]
Optical purity	• Isolated or engineered bacteria encoding a LDH with high selectivity[35,36,66]
	• Efficient purification of the desired isomer
Low cell densities	• Cell recirculation: repeated fed-batch and membrane-integrated continuous culture [17,52]
	• Cell immobilization[51,53,54]
Substrate inhibition	• Fed-batch and continuous cultures[7,22,50,51]
	• Osmotic-resistant bacteria[28,38]
	• Osmoprotectants[39]
LA and pH growth inhibition	• Isolated, engineered or adapted bacteria to low pH[19,71,72]
	• Using an effective neutralizing agent[40]
	• LA recovery using membrane extractive fermentation[26,32]
Inhibitors released during pretreatment	• Isolated, adapted or engineered bacteria with increased tolerance to inhibitors[24,42,56–61]
	• Optimization of pretreatment and hydrolysis methods to minimize inhibitors' formation[46]

Optical purity of lactic acid

As explained above, production of optically pure L or D-LA is preferred against the mixture of both isomers because each one has specific applications. It is important to notice that the optical purity of LA is not guaranteed unless the proper LAB is chosen. For example, *L. delbrueckii*, *Lactobacillus coryniformis*, *Lactobacillus jensenii*,

and *Lactobacillus vitulinus* produce D-LA; *Lactobacillus casei*, *Lactobacillus paracasei*, and *L. rhamnosus* produce L-LA; and *L. pentosus*, *L. plantarum*, *L. brevis*, *Lactobacillus sake*, and *L. acidophilus* produce DL-LA.[24] For this reason, new isolated or engineered bacteria with the capacity of producing the desired isomer with purity higher than 95% must be developed (Table 2).

Furthermore, as previously stated, when optical pure LA is needed, the purification from lignocellulosic hydrolysates is even more challenging due to the complexity of the fermentation media.

Substrate inhibition

At high sugar concentration in the broth, substrate inhibition may take part, repressing the growth of LA producers due to osmotic stress, reducing water activity and provoking cell lysis.[27] Glucose concentration higher than 20 g/L has been inhibitory for *L. casei,* reducing cell growth and LA production.[28] In this sense, as mentioned in the next section, substrate feeding strategies and continuous cultures are the best options to reduce substrate inhibition, avoiding the accumulation of large amount of sugars in the media.[7] In addition, the obtaining of osmotic-resistant microorganisms and the supplementation of the media with osmoprotectants such as betaine have also been reported as appropriate methods to address this limitation (Table 2).[28,38,39]

End-product inhibition: lactic acid accumulation and pH drop

Most of the LAB are acid tolerant and are able to grow when LA is accumulated in the medium. Nonetheless, the prolonged exposure to the protonated form of LA modifies their physiology and metabolism. Once LA crosses the cell membrane, the intracellular LA increases and cell membrane is disrupted due to the change in membrane potential, resulting in cell death.[27,40] An optimum pH can also be maintained using neutralizing agents or reducing the soluble LA. The application of fed-batch, LA continuous removal, or the use of acidic and osmotic-resistant bacteria, would be suitable ways to tackle this obstacle (Table 2).

Inhibitors released during pretreatment

The presence of biomass-degradation products is one of the main drawbacks that affect the fermentation step. The nature and concentration of the inhibitory compounds depend on the raw material, the pretreatment type, and conditions employed during pretreatment as well as the use of catalysts.[41]

The degradation products produced in the lignocellulosic biomass pretreatment can be divided into three groups: furan derivatives, weak acids, and phenolic compounds. The main furans are furfural and 5-hydroxymethyl furfural (HMF) derived from degradation of pentoses and hexoses, respectively, which cause a prolonged lag phase during batch fermentation.[42,43] Acetic and formic acid are released due to the solubilization of acetyl groups and from furfural and HMF degradation, respectively. Both acetic and formic acid reduce the microorganism growth, formic acid being one of the most inhibitory compounds.[44] A recent study has concluded that the phenolic compounds such as coumaric and ferulic acid significantly inhibit the growth of several LA-producing microorganisms at concentrations above 1 g/L.[43]

As a matter of fact, the inhibition mechanism of the degradation compounds is not only based on the inhibitory effect caused by each compound individually but also on their interaction and synergy.[43,45] In this sense, the use of robust fermenting microorganisms that can cope with the inhibitors is crucial for achieving a cost-competitive production process (Table 2). Over these years, several strategies to overcome the problems associated with the release of inhibitors after biomass pretreatment have been identified such as the removal of inhibitors before fermentation or the use of the biodetoxification capability of fermenting microorganisms.[46]

Novel approaches to improve lactic acid production from lignocellulose

Several approaches to overcome the challenges faced in LA production will be discussed and compared in this section (Table 2). Besides, approaches to further improve the LA production processes from lignocellulose, such as the utilization of high initial cell concentration, are also mentioned. A compilation of LA yields and productivities from different lignocellulosic feedstock using pioneering approaches for LA production are summarized in Table 3.

Process-oriented approaches

The effectiveness of LA fermentation can be improved using different process configurations, fermenter designs, product-recovery techniques, or increasing cell concentration.

SS(C)F processes

The main process configurations when producing bioethanol or bioproducts from lignocellulosic substrates are separated hydrolysis and fermentation (SHF) and

Table 3. LA yields, titers and productivities obtained by LAB using different lignocellulosic feedstock.

Substrate	LA yield (g/g)	LA titer (g/L)	LA productivity (g/L·h)	Bacterial strain	Approach
Corn stover	0.65	74.80	0.70	*L. pentosus*	Fed-batch[22]
Corn stover	0.78	31.20	0.43	*L. brevis and L. plantarum*	Co-culture[63]
Corn stover	0.65	77.66	1.06	*P. acidilactici* DQ2	D-LDH gene disruption[66]
Corn stover	0.58	76.76	1.02	*P. acidilactici* DQ2	L-LDH gene disruption[66]
Corn stover and soybean meal extract	0.77	61.40	0.32	*L. plantarum*	Expression of *xylAB* operon[20]
Hardwood pulp	0.88	102.30	2.29	*L. plantarum*	Expression of *xylAB* operon and L-LDH gene disruption[67]
Lactose from whey	—	106.20	1.77	*L. rhamnosus*	Fed-batch[7]
Lactose from whey	—	57.00	1.18	*L. rhamnosus*	Batch[7]
Raw sugar cane	0.96	67.00	12.20	*S. laevolacticus*	Continuous culture[50]
Raw sugar cane	0.88	77.00	0.86	*S. laevolacticus*	Batch[50]
Artichoke flour	0.95	120.50	3.35	*Lactobacillus* sp. G-02 and *A. niger* SL-09	Co-culture with SSF[62]
Cassava bagasse starch	0.97	—	—	*L. casei* NCIMB 3254	Polyurethane Immobilization [53]
Cassava bagasse starch	—	38.00	1.02	*Lactobacillus sp.* co-culture	Polyurethane Immobilization with alginate entrapment[54]

simultaneous saccharification and fermentation (SSF). The latter is usually stated as simultaneous saccharification and co-fermentation (SSCF) when co-utilization of different kind of sugars takes place. Opposite to SHF, in SS(C)F, enzymatic hydrolysis (saccharification) and fermentation are performed simultaneously in the same vessel which implies an integrated process translated in cost reduction. Another advantage of SS(C)F in comparison to SHF is the reduction of end-product inhibition during the enzymatic hydrolysis step because sugars are simultaneously consumed by fermenting microorganisms. As a consequence, higher hydrolysis yields, shorter reaction times and reduced enzyme loadings are achieved.[47] Furthermore, since glucose is consumed as soon as it is released, there is less glucose available for opportunistic microorganisms and the risk of contamination is markedly diminished. Furthermore, as is the case for yeast in bioethanol production, the lower accumulation of glucose could favor xylose utilization.

One of the SS(C)F shortcomings in cellulosic ethanol production is the different optimum temperature that hydrolytic enzymes and yeast might require.[48] However, this drawback is not significant when using LA-producing bacteria as most of them can cope with wide range of temperatures. Some successful examples of SS(C)F processes in LA from lignocellulosic materials are displayed in Table 3.

Batch, fed-batch and continuous fermentation

As mentioned before, the conventional use of batch fermentation in LA production has several limitations derived from substrate and by-product inhibition. For this reason, the potential of different fermentation configurations as alternatives to batch cultures has been investigated, feeding strategies (i.e. fed-batch) and continuous fermentation being the most outstanding.[7,49,50]

Compared to batch, fed-batch mode exhibits a reduced lag phase of the microorganism, thus improving LA production.[51] As a matter of fact, LA concentration increased from 40 g/L with batch SSF to 74.8 g/L with fed-batch SSF of corn stover using *L. pentosus*.[22] Moreover, in a fed-batch fermentation of lactose from whey, 106.2 g/L of LA were produced by *L. rhamnosus*. This concentration was clearly higher than that which resulted from the corresponding batch culture, 57.0 g/L (Table 3).[7]

A few reports about continuous LA fermentation are appearing. Growing microorganisms in continuous culture implies the need to maintain the pH, concentration of nutrients, metabolic products, oxygen, etc. As a result, microbial growth occurs at a constant rate and the cells remain in a particular growth phase, without entering the stationary phase. This technique increases LA productivity due to the mitigation of end-product inhibition.

Productivity values 11-fold higher than with batch fermentation have been reported in continuous fermentation of raw sugar cane with *Sporolactobacillus laevolacticus*.[50]

Cell recycle: biomass recirculation

Membrane cell recycling systems are a good way to enhance the conversion rate of the substrate by achieving high cell biomass.

Re-inoculation of used biomass in repeated cycles of fresh-medium (repeated batch fermentation) is a feasible way to increase the initial cell concentration in the reactor and to overcome the carbon catabolite repression effect. As proof, using *Enterococcus faecalis* RKY1 in a 10 repeated-batch fermentation with different carbon and nitrogen sources, LA productivity increased from 3.2 g/L·h to 6.37 g/L·h.[52]

Cell-recirculation can also be coupled with continuous fermentation. In this way, it is possible to operate at higher dilution rates without cell washout, achieving high LA productivity.[17]

Cell immobilization

Cell immobilization, which is based on microorganism attachment to an inert support, is also a useful strategy that enables the repeated use of microorganisms and the maintenance of high cell density. For instance, *L. casei* NCIMB 3254 was adhered to polyurethane foam cubes and used for the production of LA from cassava bagasse starch hydrolysate, achieving about 0.95–0.97 g LA/g glucose.[53] Polyurethane foam immobilization was also combined with cross-linking alginate entrapment. In this case, using cassava bagasse starch as substrate and an immobilized biofilm composed of a co-culture of lactobacilli, LA productivity increased from 0.32 g/L·h to 1.6 g/L·h.[54] When fibrous bed bioreactors were utilized with Jerusalem artichoke hydrolysate, cell immobilization in the fibrous matrix allowed the formation of 35–42 g/L of biomass, while only 7–10 g/L were reached in free fermentation broth, implying an improvement of 28% in LA yield.[51]

New lactic acid recovery techniques

Alternatives to traditional LA purification methods are being tested to simplify the process and lower the cost and energy consumption.[25]

Chromatographic techniques are well recognized by their low cost, high selectivity, and moderate operational conditions. For LA purification, ion exchange chromatography has shown low waste generation, high separation yields and shorter operation times than other downstream processes.[30]

Using an anion exchange extraction method in glucose batch fermentations with *L. lactis* ATCC 11454, a 1.2-fold increase in total LA in comparison with the resin-free culture was obtained.[26] This method was also combined with reactive extraction using organic solvents, yielding up to 70% recovery of LA.[55] A two-step separation process including both anion and cation exchange chromatography was carried out with the fermentation broth of a novel substrate *Zizyphus oenophlia*, obtaining LA with 99% purity.[30]

The combination of both electrodialysis and ion exchange results in a hybrid method called electrodeionization, allowing continuous membrane regeneration by an electric field. Glucose fermentation of *Pediococcus pentosaceus* coupled with electrodeionization extraction led to an alleviation of product inhibitory effect and, therefore, improving the half-life of the biocatalyst and achieving 185 g LA /L.[26]

Microorganism oriented approaches

Although improvement of fermentation operation is needed to optimize the production process, the use of new isolated or developed strains tailored to LA production is also a promising approach to circumvent challenges associated with lignocellulosic LA production.[56]

Isolation and screening of new lactic-acid-producing bacteria strains

Lactic acid bacteria isolated from extreme environments are able to grow in extreme conditions due to general stress response mechanisms, and to produce LA at high productivities.[32] Thus, isolation, selection, and characterization of microorganisms with the appropriate features for LA production can significantly improve fermentation yields.

Lactobacilli such as *L. brevis*, *L. plantarum, L. paracasei;* some species of *Pediococcus* or even different *Bacillus* strains have been isolated from cabbage, soil, corn stover slurry, composted dairy manure.[24,42,56–61] These newly isolated microorganisms showed high tolerance to inhibitors, LA and salts. Likewise, they also showed their ability to grow at high temperature, and low pH. In this context, *L. casei* decreases the flux of carbohydrate import system at low-pH conditions.[32] Another example involves the redirection of the sugar metabolism from LA, acetic acid or ethanol production to the formation of acetyl-CoA for the biosynthesis of fatty acids and the successive strengthening of the cell membrane observed in *L. delbrueckii* and *L. rhamnosus*.[32]

Some strains have also been selected due to their ability to use pentoses homofermentatively with high yields and to avoid by-product formation. For instance, *Enterococcus mundtii* QU25 and *Enterococcus faecium* QU50, isolated from ovine fecal samples and soil, respectively, metabolized xylose and produced exclusively LA with high optical purity and yield.[35,36]

Co-cultures and mixed cultures

In principle, the use of pure cultures may be extended and recommended in biotechnological processes because it allows a predictable fermentation, reducing the risks of contamination and ensuring a constant quality of the desired product. However, the use of co-cultures appears to be advantageous over a single microorganism, especially with complex substrates. There are few examples of a co-culture of hydrolytic enzyme producing microorganisms with LAB in SSF experiments. A co-culture of *Lactobacillus* sp. G-02 and *Aspergillus niger* SL-09, which produces inulinase and invertase, resulted in 120.5 g/L LA.[62] Interestingly, using co-cultures of heterofermentative and homofermentative LAB strains, co-utilization of both hexoses and pentoses can be achieved. In case of corn stover, glucose was metabolized by *L. rhamnosus* via EM pathway, while the resulting xylose was converted to LA and acetic acid via PK pathway by *L. brevis*. The final yields were about 19% and 30% higher than those obtained by single cultures of *L. rhamnosus* and *L. brevis*, respectively.[4] Glucose and xylose present in poplar and corn stover hydrolysates were also utilized by a co-culture of *L. brevis* and *L. plantarum*, producing LA yields of 0.8 g/g and 0.78 g/g, respectively, with very low by-product formation.[63]

Indigenous mixed culture obtained from microflora of the substrate can also be used as a seed for fermentation instead of specific LAB.[64] For example, 0.46 g/g of LA was produced using the indigenous bacteria of food wastes, *Lactobacillus* being the dominant species.[65]

Genetic modification of lactic acid-producing bacteria

Mutation, heterologous expression and directed evolution are interesting options for improving the phenotype and genotype of LA-producing microorganisms, fitting the metabolisms to LA production, and increasing stress tolerance.

Genetic engineering

As mentioned above, LA homofermentation is preferred to heterofermentation in terms of yield. In this context, a homofermentative pathway for LA production was constructed in *L. brevis* by heterologous expression of two EMP/PP pathways genes from *L. rhamnosus*, encoding for fructose-6-phosfate kinase and fructose-1,6-biphosfate aldolase.[34] With this strategy, 34% more LA in comparison to native strain was produced. In case of *L. plantarum*, LA fermentation was switched from heterofermentative to homofermentative by substituting an endogenous PK gene with the heterologous transketolase gene of *L. lactis* IL 1403, replacing the PK pathway by the PP pathway.[33]

Since the production of each stereoisomer is a consequence of the lactate dehydrogenase (LDH) selectivity, deletion of D-LDH or L-LDH by gene disruption would lead to optically pure production of L-LA and D-LA, respectively. Following this strategy, two engineered L-LA- and D-LA-producing bacteria were obtained from the wild-type *Pediococcus acidilactici* DQ2 that were able to produce 77 g/L of L-LA and 76 g/L of D-LA, respectively, using pretreated corn stover as substrate.[66]

An appropriate strategy to maximize sugar utilization of lignocellulosic substrates is to engineer xylose-utilizing bacteria. To this end, xylose assimilating *xylAB* operon from *L. pentosus* –encoding xylose isomerase and xylulokinase – was introduced in *L. plantarum*, yielding 0.88 g/g of LA from delignified hardwood pulp.[67] In the same way, the *xylAB* operon from *L. brevis* was introduced in *L. plantarum*, giving a recombinant strain able to produce LA from corn stover and soybean meal extract and yielding 0.77 g/g.[20]

Simultaneous-saccharification-and-fermentation oriented LAB strains can be obtained by introducing genes encoding for hydrolytic enzymes. With this aim, a xylanase gene was introduced in *L. brevis*. The genetically modified strain was able to produce 1.7 g/L of LA from xylan with a xylanase activity of 0.412 U/mL.[68]

Genetic modification approaches have also been applied to improve LAB thermotolerance and osmotolerance, which are interesting traits when producing LA from lignocellulose. Heterologous expression of a heat-shock protein known as DnaK from *E. coli* in *L. lactis* resulted in a LAB strain more tolerant to NaCl, ethanol, and LA, and able to grow and ferment at 40 °C.[69] Likewise, overproduction of RecO protein from *L. casei* Zhang in *L. lactis* NZ9000 resulted in higher growth rates and LA yields than the control strain under heat stress and high concentrations of LA, salts (3% NaCl), oxygen (0.1 mM H_2O_2), and ethanol (5%).[70]

Non-directed mutagenesis

When the genetic pathways related with a phenotype are clearly well known, directed mutagenesis and heterologous

expression of specific genes are very useful techniques for strain improvement. Nonetheless, cell growth and LA production in stressful conditions are generally controlled by multiple loci.[71] For this reason and due to the hurdles when transferring multiple genes, random mutagenesis (e.g. error-prone PCR, DNA shuffling, use of mutagens, etc.) or protoplast fusion are very appropriate techniques to alter different loci and metabolic pathways at the same time.

Acid-resistant LAB are very convenient in LA production because a lower concentration of neutralizing agent is required during fermentation. In this context, using UV irradiation and nitrous acid as mutagens, followed by recursive protoplast fusion, a mutant of *L. rhamnosus* was obtained with a 3.1-fold increase in LA production at pH 3.8.[19] Similarly, a mutant that produced fivefold more LA at acidic pH was generated using protoplast fusion between *L. delbrueckii* Mut Uc-3 and the acid-tolerant *Acetobacter pasteurianus*.[72] Error-prone amplification of *L. pentosus* genomic DNA allowed the isolation of a mutant-producing 17-fold LA at pH 3.8.[71]

Concluding remarks

Lactic acid production from renewable sugar sources has drawn a lot of attention in recent years, searching for improved fermentation conditions and fitted microbial strains. This report gathers the novel approaches addressed to improve LA production from lignocellulose, from process- to microorganism-oriented strategies.

The cost of the raw material is one of the most significant factors affecting the viability of LA production. So, selecting an appropriate raw material is a key factor for the profitability of the process. The implementation of efficient separation and purification techniques is also crucial for obtaining high-quality LA required for industrial applications.

From the process point of view, efforts have been made to increase yields and productivity by applying new process configurations (SSF, fed-batch, continuous culture, etc.) and increasing cell density by recirculation or immobilization of microorganisms.

From the microorganism point of view, the development of highly acid tolerant strains and the ability to produce optically pure LA remain major obstacles to reaching cost-effective LA production. In this sense, research has mainly been directed to genetically modify lactic acid producing bacteria to acquire the desired traits.

Considering the recent advances in LA production, much attention should be given to implement new process strategies and develop new metabolic engineering approaches, to reach efficient LA production processes from lignocellulose. The increasing number of publications in this field and the current research efforts by the scientific community, point to significant progress in the field in the near future.

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References

1. Das D and Goyal A, *Microorganisms in Sustainable Agriculture and Biotechnology.* Springer Netherlands, Dordrecht, pp. 757–772 (2012).
2. Wang C, Chang T, Yang H and Cui M, Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella Enteritidis*, *Escherichia coli* and *Listeria monocytogenes*. *Food Control* **47**:231–236 (2015).
3. Secchi N, Giunta D, Pretti L, Garcia MR, Roggio T, Mannazzu I *et al.*, Bioconversion of ovine scotta into lactic acid with pure and mixed cultures of lactic acid bacteria. *J Ind Microbiol Biotechnol* **39**:175–181 (2012).
4. Cui F, Li Y and Wan C, Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*. *Bioresour Technol* **102**:1831–1836 (2011).
5. Zhang Y and Vadlani PVD, Lactic acid biosynthesis from biomass-derived sugars via *Lactobacillus delbrueckii* fermentation. *Bioprocess Biosyst Eng* **36**:1897–1904 (2013).
6. Ulery BD, Nair LS and Laurencin CT, Biomedical Applications of Biodegradable Polymers. *J Polym Sci, Part B: Polym Phys* **49**:832–864 (2011).
7. Bernardo MP, Coelho LF, Sass DC and Contiero J, l-(+)-Lactic acid production by *Lactobacillus rhamnosus* B103 from dairy industry waste. *Braz J Microbiol* **47**:640–646 (2016).
8. Guha A, Production of Lactic Acid from Sweet Meat Industry Waste by *Lactobacillus delbruki*. *Int J Res Eng Technol* **2:**630–634 (2013).
9. Ye L, Zhou X, Hudari MSB, Li Z and Wu JC, Highly efficient production of l-lactic acid from xylose by newly isolated *Bacillus coagulans* C106. *Bioresour Technol* **132**:38–44 (2013).
10. Ahring BK, Traverso JJ, Murali N and Srinivas K, Continuous fermentation of clarified corn stover hydrolysate for the production of lactic acid at high yield and productivity. *Biochem Eng J* **109**:162–169 (2016).
11. Ma K, Hu G, Pan L, Wang Z, Zhou Y, Wang Y *et al.*, Highly efficient production of optically pure l-lactic acid from corn stover hydrolysate by thermophilic *Bacillus coagulans*. *Bioresour Technol* **219**:114–122 (2016).
12. Abdel-Rahman MA, Xiao Y, Tashiro Y, Wang Y, Zendo T, Sakai K *et al.*, Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. *J Biosci Bioeng* **119**:153–158 (2015).
13. Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EW, Sijtsma L, Stams AJM *et al.*, Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology* **52**:741–755 (1999).

14. Ballesteros M, Enzymatic hydrolysis of lignocellulosic biomass in *Bioalcohol Production,* ed by Waldron K. Woodhead Publishing, UK, pp. 159–177 (2010).
15. Hu J, Lin Y, Zhang Z, Xiang T, Mei Y, Zhao S *et al*., High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. *Bioresour Technol* **214**:74–80 (2016).
16. Juturu V and Wu JC, Microbial production of lactic acid: the latest development. *Crit Rev Biotechnol* **36**:967–977 (2015).
17. Abdel-Rahman MA, Tashiro Y and Sonomoto K, Recent advances in lactic acid production by microbial fermentation processes. *Biotechnol Adv* **31**:877–902 (2013).
18. Eiteman MA and Ramalingam S, Microbial production of lactic acid. *Biotechnol Lett* **37**:955–972 (2015).
19. Wang Y, Li Y, Pei X, Yu L and Feng Y, Genome-shuffling improved acid tolerance and l-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. *J Biotechnol* **129**:510–515 (2007).
20. Zhang YX, Vadlani PV, Kumar A, Hardwidge PR, Govind R, Tanaka T *et al*., Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. *Appl Microbiol Biotechnol* **100**:279–288 (2016).
21. Prückler M, Lorenz C, Endo A, Kraler M, Dürrschmid K, Hendriks K *et al*., Comparison of homo- and heterofermentative lactic acid bacteria for implementation of fermented wheat bran in bread. *Food Microbiol* **49**:211–219 (2015).
22. Zhu Y, Lee YY and Elander RT, Conversion of aqueous ammonia-treated corn stover to lactic acid by simultaneous saccharification and cofermentation. *Appl Biochem Biotechnol* **137–140**:721–738 (2007).
23. Bustos G, Moldes AB, Cruz JM and Domínguez JM, Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. *Biotechnology Prog* **21**:793–798 (2005).
24. Kuo YC, Yuan SF, Wang CA, Huang YJ, Guo GL and Hwang WS, Production of optically pure L-lactic acid from lignocellulosic hydrolysate by using a newly isolated and D-lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain. *Bioresour Technol* **198**:651–657 (2015).
25. López-Garzón CS and Straathof AJJ, Recovery of carboxylic acids produced by fermentation. *Biotechnol Adv* **32**:873–904 (2014).
26. Boontawan P, Kanchanathawee S and Boontawan A, Extractive fermentation of L-(+)-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. *Biochem Eng J* **54**:192–199 (2011).
27. Abdel-Rahman MA and Sonomoto K, Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. *J Biotechnol* **236**:176–192 (2016).
28. Ge XY, Yuan J, Qin H and Zhang WG, Improvement of L-lactic acid production by osmotic-tolerant mutant of *Lactobacillus casei* at high temperature. *Appl Microbiol Biotechnol* **89**:73–78 (2011).
29. Abdel-Rahman MA, Tashiro Y and Sonomoto K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. *J Biotechnol* **156**:286–301 (2011).
30. Bishai M, De S, Adhikari B and Banerjee R, A platform technology of recovery of lactic acid from a fermentation broth of novel substrate *Zizyphus oenophlia*. *3 Biotech* **5**:455–463 (2015).
31. Komesu A, Oliveira JARdM, Martins LHdS, Wolf Maciel MR and Maciel Filho R, Lactic acid production to purification: a review. *BioResources* **12**:4364–4383 (2017).
32. Papadimitriou K, Alegría Á, Bron PA, de Angelis M, Gobbetti M *et al*., Stress physiology of lactic acid bacteria. *Microbiol Mol Biol Rev* **80**:837–890 (2016).
33. Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H and Kondo A, Homo-d-Lactic Acid Fermentation from Arabinose by Redirection of the Phosphoketolase Pathway to the Pentose Phosphate Pathway in l-Lactate Dehydrogenase Gene-Deficient *Lactobacillus plantarum*. *Appl Environ Microbiol* **75**:5175–5178 (2009).
34. Guo W, He R, Ma L, Jia W, Li D and Chen S, Construction of a constitutively expressed homo-fermentative pathway in *Lactobacillus brevis*. *Appl Microbiol Biotechnol* **98**:6641–6650 (2014).
35. Abdel-Rahman MA, Tashiro Y, Zendo T, Hanada K, Shibata K and Sonomoto K, Efficient Homofermentative l-(+)-Lactic Acid Production from Xylose by a Novel Lactic Acid Bacterium, *Enterococcus mundtii* QU 25. *Appl Environ Microbiol* **77**:1892–1895 (2011).
36. Abdel-Rahman, MA, Tashiro Y, Zendo T, Sakai K, and Sonomoto K, *Enterococcus faecium* QU 50: a novel thermophilic lactic acid bacterium for high-yield l-lactic acid production from xylose. *FEMS Microbiol Lett* **362**:1–7 (2015).
37. Boguta AM, Bringel F, Martinussen J and Jensen PR, Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. *Microb Cell Fact* **13** (2014).
38. Yu L, Pei X, Lei T, Wang Y and Feng Y, Genome shuffling enhanced l-lactic acid production by improving glucose tolerance of *Lactobacillus rhamnosus*. *J Biotechnol* **134**:154–159 (2008).
39. Zou H, Wu Z, Xian M, Liu H, Cheng T and Cao Y. Not only osmoprotectant: Betaine increased lactate dehydrogenase activity and l-lactate production in lactobacilli. *Bioresour Technol* **148**:591–595 (2013).
40. Nakano S, Ugwu CU and Tokiwa Y, Efficient production of D-(-)-lactic acid from broken rice by *Lactobacillus delbrueckii* using $Ca(OH)_2$ as a neutralizing agent. *Bioresour Technol* **104**:791–794 (2012).
41. Tomás-Pejó E, Alvira P, Ballesteros M and Negro MJ, Chapter 7 – Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion in *Biofuels*, ed by Ashok P, Christian L, Steven CR, Claude-Gilles D and Edgard G. Academic Press, Amsterdam, pp. 149–176 (2011).
42. Peng LL, Wang LM, Che CC, Yang G, Yu B and Ma YH. *Bacillus* sp strain P38: An efficient producer of L-lactate from cellulosic hydrolysate, with high tolerance for 2-furfural. *Bioresour Technol* **149**:169–176 (2013).
43. van der Pol EC, Vaessen E, Weusthuis RA and Eggink G, Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small-scale screening method. *Bioresour Technol* **209**:297–304 (2016).
44. Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G and Gorwa-Grauslund MF, Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J Chem Technol Biotechnol* **82**:340–349 (2007).
45. Oliva JM, Ballesteros I, Negro MJ, Manzanares P, Cabañas A and Ballesteros M, Effect of Binary Combinations of Selected Toxic Compounds on Growth and Fermentation of *Kluyveromyces marxianus*. *Biotechnol Prog* **20**:715–720 (2004).
46. Moreno AD, Ibarra D, Alvira P, Tomas-Pejo E and Ballesteros M. A review of biological delignification and detoxification

methods for lignocellulosic bioethanol production. *Crit Rev Biotechnol* **35**:342–354 (2015).
47. Tomás-Pejó E, Oliva JM, Ballesteros M and Olsson L, Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting *Saccharomyces cerevisiae* strains. *Biotechnol Bioeng* **100**:1122–1131 (2008).
48. Olofsson K, Bertilsson M and Liden G, A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol Biofuels* **1**:1–14 (2008).
49. Ou MS, Ingram LO and Shanmugam KT, l(+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*. *J Ind Microbiol Biotechnol* **38**:599–605 (2011).
50. Sawai H, Na K, Sasaki N, Mimitsuka T, Minegishi S, Henmi M *et al.*, Membrane-Integrated Fermentation System for Improving the Optical Purity of D-Lactic Acid Produced during Continuous Fermentation. *Biosci Biotechnol Biochem* **75**:2326 (2011).
51. Shi ZM, Wei PL, Zhu XC, Cai J, Huang L and Xu ZN, Efficient production of L-lactic acid from hydrolysate of Jerusalem artichoke with immobilized cells of *Lactococcus lactis* in fibrous bed bioreactors. *Enzyme Microb Technol* **51**:263–268 (2012).
52. Reddy LV, Kim YM, Yun JS, Ryu HW and Wee YJ, L-Lactic acid production by combined utilization of agricultural bioresources as renewable and economical substrates through batch and repeated-batch fermentation of *Enterococcus faecalis* RKY1. *Bioresour Technol* **209**:187–194 (2016).
53. John RP, Nampoothiri KM and Pandey A, Polyurethane foam as an inert carrier for the production of L(+)-lactic acid by *Lactobacillus casei* under solid-state fermentation. *Lett Appl Microbiol* **44**:582–587 (2007).
54. John RP and Nampoothiri KM, Co-culturing of *Lactobacillus paracasei* subsp paracasei with a *Lactobacillus delbrueckii* subsp *delbrueckii* mutant to make high cell density for increased lactate productivity from cassava bagasse hydrolysate. *Curr Microbiol* **62**:790–794 (2011).
55. Boonmee M, Cotano O, Amnuaypanich S and Grisadanurak N, Improved lactic acid production by in situ removal of lactic acid during fermentation and a proposed scheme for its recovery. *Arab J Sci Engineering* **41**:2067–2075 (2016).
56. Guo W, Jia W, Li Y and Chen S, Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics. *Appl Biochem Biotechnol* **161**:124–136 (2010).
57. Nguyen CM, Kim JS, Hwang HJ, Park MS, Choi GJ, Choi YH *et al.*, Production of L-lactic acid from a green microalga, *Hydrodictyon reticulum*, by *Lactobacillus paracasei* LA104 isolated from the traditional Korean food, makgeolli. *Bioresour Technology* **110**:552–559 (2012).
58. Zhao K, Qiao QG, Chu DQ, Gu HQ, Dao TH, Zhang J *et al.*, Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2. *Bioresour Technol* **135**:481–489 (2013).
59. Juodeikiene G, Zadeike D, Bartkiene E and Klupsaite D, Application of acid tolerant *Pedioccocus* strains for increasing the sustainability of lactic acid production from cheese whey. *LWT – Food Science and Technology* **72**:399–406 (2016).
60. Bischoff KM, Liu S, Hughes SR and Rich JO, Fermentation of corn fiber hydrolysate to lactic acid by the moderate thermophile *Bacillus coagulans*. *Biotechnol Lett* **32**:823–828 (2010).
61. Meng Y, Xue YF, Yu B, Gao CH and Ma Y, Efficient production of L-lactic acid with high optical purity by alkaliphilic *Bacillus* sp WL-S20. *Bioresour Technol* **116**:334–339 (2012).
62. Ge XY, Qian H and Zhang WG, Improvement of L-lactic acid production from Jerusalem artichoke tubers by mixed culture of *Aspergillus niger* and *Lactobacillus* sp. *Bioresour Technol* **100**:1872–1874 (2009).
63. Zhang YX and Vadlani PV, Lactic acid production from biomass-derived sugars via co-fermentation of *Lactobacillus brevis* and *Lactobacillus plantarum*. *J Biosci Bioeng* **119**:694–699 (2015).
64. Bader J, Mast-Gerlach E, Popovic MK, Bajpai R and Stahl U, Relevance of microbial coculture fermentations in biotechnology. *J Appl Microbiol* **109**:371–387 (2010).
65. Tang JL, Wang XC, Hu YS, Zhang YM and Li YY, Lactic acid fermentation from food waste with indigenous microbiota: Effects of pH, temperature and high OLR. *Waste Manag* **52**:278–285 (2016).
66. Yi X, Zhang P, Sun JE, Tu Y, Gao QQ, Zhang J *et al.*, Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock. *J Biotechnol* **217**:112–121 (2016).
67. Hama S, Mizuno S, Kihara M, Tanaka T, Ogino C, Noda H *et al.*, Production of d-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. *Bioresour Technol* **187**:167–172 (2015).
68. Hu CY, Chi DJ, Chen SS and Chen YC, The direct conversion of xylan to lactic acid by Lactobacillus brevis transformed with a xylanase gene. *Green Chem* **13**:1729–1734 (2011).
69. Abdullah-Al-Mahin, Sugimoto S, Higashi C, Matsumoto S and Sonomoto K, Improvement of multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli* DnaK. *Appl Environ Microbiol* **76**:4277–4285 (2010).
70. Wu C, Zhang J, Du G and Chen J, Heterologous expression of *Lactobacillus casei* RecO improved the multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 during salt stress. *Bioresour Technol* **143**:238–241 (2013).
71. Ye L, Zhao H, Li Z and Wu JC, Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. *Bioresour Technology* **135**:459–463 (2013).
72. Singhvi M, Gurjar G, Gupta V and Gokhale D, Biocatalyst development for lactic acid production at acidic pH using inter-generic protoplast fusion. *RSC Adv* **5**:2024–2031 (2015).

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ARTICLE II


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Lactobacillus pentosus CECT 4023 T Coutilizes Glucose and Xylose to Produce Lactic Acid from Wheat Straw Hydrolysate: Anaerobiosis as a Key Factor

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Lactic acid is a versatile chemical that can be produced via *fermentation of lignocellulosic materials. The heterolactic strain* Lactobacillus pentosus *CECT 4023 T, that can consume glucose and xylose, was studied to produce lactic acid from steam exploded wheat straw prehydrolysate. The effect of temperature and pH on bacterial growth was analyzed. Besides, the effect of oxygen on lactic acid production was tested and fermentation yields were compared in different scenarios. This strain showed very high tolerance to the inhibitors contained in the wheat straw prehydrolysate. The highest lactic acid yields based on present sugar, around 0.80 g* g^{-1}*, were obtained from glucose in presence of 25%, 50%, and 75% v* v^{-1} *of prehydrolysate in strict anaerobiosis. Lactic fermentation of wheat straw hydrolysate obtained after enzymatic hydrolysis of the prehydrolysate yielded 0.39 g of lactic acid per gram of released sugars, which demonstrated the high potential of* L. pentosus *to produce lactic acid from hemicellulosic hydrolysates. Results presented herein not only corroborated the ability of* L. pentosus *to grow using mixtures of sugars, but also demonstrated the suitability of this strain to be applied as an efficient lactic acid producer in a lignocellulosic biorefinery approach.* © 2018 American Institute of Chemical Engineers
Keywords: lactic acid, lactic acid bacteria, Lactobacillus pentosus, wheat straw hydrolysate, anaerobiosis

Introduction

Lactic acid (LA) is an industrially relevant organic acid that has become very popular in recent years. Besides its implemented utilization in food, textile, detergent, and paper industry, it is recently being used for the formulation of new high value products, particularly pharmaceuticals, cosmetics, chemicals and biopolymers like poly-lactic acid (PLA).[1,2] Because of the high biodegradability and the biocompatible features of PLA,[3] it is considered as a suitable alternative to petrochemical-based plastics for packaging, as well as in fabrication of prosthetic devices and drug delivery systems.[4,5] Furthermore, the industrial applications of this acid are dependent on the LA isomer produced. While L-LA is preferred in food and pharmaceutical industries, both optically pure D-LA and L-LA are used for the production of different chemicals and PLA polymers.[12] LA can be produced by chemical or by microbial processes. For industrial production, the fermentative process presents several advantages when compared to lactonitrile-based chemical synthesis as the latter uses petrochemical sources which implies environmental concerns.[6] LA can be produced *via* microbial fermentation using several microorganisms, such as lactic acid bacteria (LAB), yeasts, microalgae, and some *Bacillus* strains.[6]

Most of LA manufacturers use glucose or starchy materials as carbon source supplemented with yeast extract in LA fermentation.[7] The use of these substrates is often considered economically unfavorable since they can represent up to 68% of the total production cost.[8] Furthermore, starchy materials are also used for food or animal feed.[9] To circumvent these challenges, the use of lignocellulosic materials, that is, forest wastes, crop residues, industrial residues, food-processing by-products, and so forth, has emerged as an attractive alternative to sugars and starchy crops for the microbial production of biofuels and high added value bioproducts such as LA.[7,10]

Lignocellulosic biomass is composed of two carbohydrate polymers rich in fermentable sugars—cellulose and hemicellulose—and an aromatic polymer (lignin). One of the most abundant lignocellulosic agriculture residues is wheat

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straw, with a total global production of, approximately, 529 million tons per year.[11] In addition to its availability and worldwide distribution, it does not compete with food crops. Therefore, the use of this or similar residues is an attractive option for the production of LA based chemicals and biopolymers in terms of renewability, sustainability and environment preservation, promoting the implementation of circular economy principles.[12] Nonetheless, purification and neutralization of LA should also be taken into account as they both represent a high percentage of the production costs and, in many cases, neutralization leads to the production of gypsum, which triggers environmental concerns.[12]

To improve sugar release from cellulose and hemicellulose by increasing the accessibility of the recalcitrant lignocellulose structure to enzymes, a pretreatment step is needed. Besides facilitating enzymatic hydrolysis, pretreatment also leads to partial sugar and lignin degradation and, thus, the generation of degradation compounds that can inhibit enzymes and microbial growth.[13,14]

Among pretreatments, steam explosion is the one of the most widely used technology for lignocellulosic biomass.[15] One of the main characteristics of steam explosion is the solubilization of hemicellulosic sugars, mainly xylose, and the remaining water insoluble solids (WIS) is mainly composed of cellulose, and lignin (Figure 1).[14] The production of bioethanol from the cellulosic fraction (i.e., WIS) has been successfully implemented.[16,17] However, the use of the liquid fraction or prehydrolysate for bioethanol production is still very challenging because xylose is not fermented by the most common fermenting yeast *S. cerevisiae* and the content of inhibitory compounds produced during pretreatment. In this context, the use of heterofermentative LAB that can metabolize xylose and sugar mixtures is an appealing option to valorize lignocellulosic prehydrolysates for LA production. In the recent years, the complete utilization of lignocellulosic sugars has been addressed with the isolation of new evolved and engineered yeasts and bacterial strains with the ability to metabolize both hexoses and pentoses. For instance, in the case of LA production, the isolated strain *Enterococcus mundtii* QU 25 was engineered to produce LA from glucose: xylose mixtures.[18] An efficient LA-producing *S. cerevisiae* strain was also developed when the LA pathway was introduced in a strain able to metabolize xylose.[19] Furthermore, by the introduction of a xylose-metabolism pathway and further adaptive evolution, an efficient LA producer *Pediococcus* strain was obtained.[20]

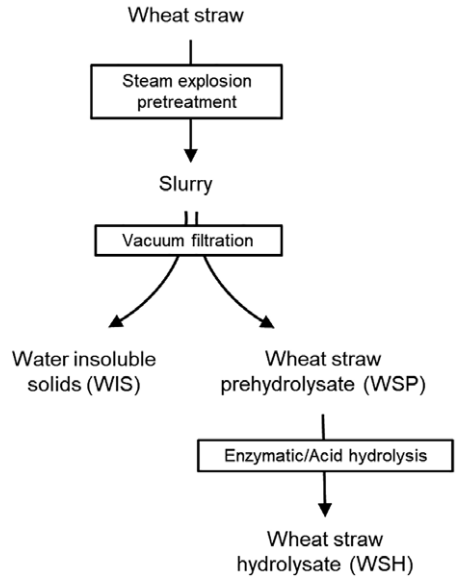

Figure 1. Schematic representation of wheat straw prehydrolysate (WSP) and wheat straw hydrolysate (WSH) production from lignocellulosic wheat straw.

Lactobacillus pentosus is considered a facultative heterolactic bacteria due to its ability to metabolize glucose *via* the Embden-Meyerhof-Parnas (EMP) pathway and xylose and other pentoses *via* the phosphoketolase (PK) pathway.[21] In this sense, higher LA yields are obtained from glucose than from xylose because acetic acid is produced as a by-product in the PK pathway. As a result, the maximum yield obtained by this LAB is 1 and 2 mol LA per mol of pentoses and hexoses, respectively (i.e., 1 and 0.6 g g^{-1} of glucose and xylose, respectively).[22]

The use of *L. pentosus* for producing LA from lignocellulosic streams has been proposed by several authors due to its ability to metabolize pentoses and grow in the presence of degradation compounds produced during pretreatment.[21,23,24] However, few studies have been published using wheat straw hydrolysates (WSH) for LA production (Figure 1). For instance, an isolated *Bacillus coagulans* strain was able to grow and produce LA in presence of high concentrations of pretreated wheat straw.[25] After a screening of several LAB, some *Lactococcus*, *Leuconostoc* and *Lactobacillus* strains were found to ferment WSH combined with several concentrations of tomato juice medium.[26]

Having in mind that most efforts are focused on the valorization of the cellulosic fraction, this work aims to take advantage of the hemicellulosic sugars present in the liquid fraction obtained after steam explosion. In this study, the ability of *L. pentosus* CECT 4023 T to grow and produce LA from WSH was tested. For this reason, to check the capacity of the LAB to grow in presence of these materials, LA fermentation of glucose was performed adding different concentrations of wheat straw prehydrolysates (WSP). Additionally, the effect of oxygen on LA fermentation was tested and compared in terms of bacterial growth and LA production in the presence of WSP. Furthermore, the ability of this LAB to metabolize hexoses and pentoses was investigated by: (i) adding xylose and glucose: xylose mixtures as carbon sources with WSP and (ii) using monomeric sugars released from the WSP by different hydrolysis methods.

Materials and Methods

Wheat straw prehydrolysate/hydrolysate

Wheat straw used in this study presented the following composition (% dry weight [DW]): cellulose, 40.5; hemicellulose, 26.1 (xylan, 22.7; arabinan, 2.1; and galactan, 1.3); Klason lignin, 18.1; ash, 5.1; and extractives, 14.6.[27] The wheat straw was firstly milled to obtain a chip size between 2 and 10 mm using a laboratory hammer mill. The chips were then pretreated by autocatalyzed (no acid addition) steam explosion in a 2 L reactor at 200°C for 7 min.[27] To determine the maximum amount of glucose and xylose that could be released from its oligomeric form, a strong acid hydrolysis treatment was performed. As a result, 5.06 g L^{-1} of glucose and 26.50 g L^{-1} of xylose were released. This data was used in the calculation of the hydrolysis yields in section lactic acid production from WSH obtained with different hydrolysis methods.

Table 1. Composition of Wheat Straw Prehydrolysates and Hydrolysates used in this Study

Composition (g L^{-1})	WSP	WSP[a] 25% v v^{-1}	WSP[a] 50% v v^{-1}	WSP[a] 75% v v^{-1}	Acid WSH	Enzymatic WSH
Glucose	1.03	20/5/0	20/5/0	20/5/0	2.41	2.28
Xylose	2.70	0/15/20	0/15/20	0/15/20	18.06	19.86
Acetic acid	7.20	4.19	5.63	6.19	8.86	6.67
Furfural	1.26	0.32[b]	0.63[b]	0.95[b]	0.36	0.52
Hydroxymethylfurfural	0.08	0.02[b]	0.04[b]	0.06[b]	0.15	0.23

[a]Glucose and xylose concentrations in these cases depend on the carbon source added in each assay: Glucose 20 g L^{-1}; Glucose 5 g L^{-1} and Xylose 15 g L^{-1}; Xylose 20 g L^{-1}.
[b]Calculated values using the corresponding dilution factors.

After the pretreatment, the slurry was recovered. The slurry was vacuum filtered to obtain two fractions: (i) water insoluble solid (WIS) fraction and (ii) wheat straw prehydrolysate (WSP) or liquid fraction (Figure 1). Most of the sugars present in the liquid fraction were oligomers. Because of that, a fraction of WSP was hydrolyzed enzymatically or chemically, as detailed in section lactic acid production from WSH obtained with different hydrolysis methods, to obtain the wheat straw hydrolysate (WSH). The composition of both WSP and WSH, including monomeric sugars and potential growth inhibitors, are showed in Table 1.

Acid and enzymatic hydrolysis of WSP

To obtain monomeric sugars from the WSP, two hydrolysis methods were compared. The acid hydrolysis was carried out by adding 4% (w w^{-1}) H_2SO_4 to the WSP and subjecting the mixture to 120°C for 30 min. After hydrolysis, the hydrolysate was adjusted to pH 7.0 with NaOH.

The enzymatic hydrolysis was performed using commercial enzymes. Celluclast® 1.5 L (60 FPU mL^{-1}), Novozym® 188 (600 UI mL^{-1}) and Cellic-HTec2® (300 U mg^{-1}) were provided by Novozymes (Denmark), while β-D-xylosidase (EC 3.2.1.37; 118 U mg^{-1} dry xylooligosaccharides) was purchased from Megazyme® (USA). All the enzymes were added simultaneously and the enzyme loading was 10 FPU and 10 UI of cellulase and β-D-glucosidase per gram of glucooligosaccharides, respectively. 5 g of xylanase per 100 g of xylooligosaccharides and 0.1 mg of β-D-xylosidase per gram of xylooligosaccharides were added. The enzymatic hydrolysis was run at 40°C, 150 rpm and pH 5.5 for 24 h. As required for the following fermentation tests, the pH was subsequently adjusted to 7.0.

Both hydrolysates were filtered using membrane filters (Thermo Scientific® Nylon 0.2 μm) and stored at 4°C until use.

Micro-organisms and preinoculum growth

Lactobacillus pentosus CECT 4023 T (ATCC-8041) was obtained from the Spanish Type Culture Collection (Valencia, Spain). For preinoculum growth, an aliquot of 20 μL of cells was taken from glycerol stocks with an optical density (OD_{600}) of 0.4 at 600 nm. This aliquot was inoculated in 50-mL Falcon tubes with 15 mL of Man, Rogosa and Sharpe medium (MRS), containing (g L^{-1}): glucose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; $MgSO_4{\cdot}7\ H_2O$, 0.2 and $MnSO_4{\cdot}H_2O$, 0.05 (pH 6.2). The preculture was grown in a rotatory shaker at 150 rpm and 32 °C overnight (until the culture reached the late exponential growth phase).

Effect of temperature and pH on bacterial growth

To determine the effect of temperature and pH on *L. pentosus* growth, three different temperatures (32°C, 35 °C and 37 °C) and pH values (6.0, 6.5, and 7.0) were tested in 150 mL shake flasks with 25 mL of MRS medium and 20 g L^{-1} of glucose at 150 rpm with inoculum size of OD_{600} 0.01 in aerobic conditions.

Lactic acid fermentation tests

LA fermentation tests were performed in MRS media at 150 rpm, 32°C, pH 7.0 and with an initial OD_{600} of 0.1. To study the effect of the oxygen—especially in terms of LA production—fermentation experiments were performed in different scenarios, comparing aerobic, anaerobic, and strict anaerobic conditions. In all cases, different concentrations of WSP were tested: 25%, 50%, and 75% v v^{-1}. The amounts of glucose and xylose varied as indicated hereafter. All fermentation tests were carried out in duplicate.

To favor oxygen transfer (aerobic conditions), *L. pentosus* was cultured in 150 mL shake flasks with 25 mL MRS medium with diluted WSP and 20 g L^{-1} of glucose covered with cellulose caps. Conversely, to reduce the oxygen transfer, lactic fermentations were also performed at the same conditions (20 g L^{-1} glucose) in shake flasks covered with oxygen-impermeable rubber caps (anaerobic conditions). Finally, to get strict anaerobic conditions, 120 mL clamped flasks with 25 mL of medium were flushed with filtered helium using a needle (gas inlet) at 0.5 bar for 2 min. The contained air—including oxygen—was ejected through another needle (gas outlet). As a result, the sealed flask was filled with helium. In the latter case, both glucose (20 g L^{-1}) and xylose (20 g L^{-1}), were added separately as carbon sources. Furthermore, both sugars were also combined at 1:3 ratio (5 g L^{-1} glucose and 15 g L^{-1} xylose), thus simulating the sugar composition of hemicellulosic hydrolysates. Samples were taken at 4, 8, 24, 32, and 48 h of fermentation for measuring the bacterial growth, residual sugars, and fermentation products.

Fermentation tests with acid and enzymatically obtained WSH were performed under the strict anaerobic conditions mentioned before. In this case, fermentation tests were performed in triplicate and samples were additionally taken at 56, 72, and 120 h for a prolonged fermentation time.

Calculations

The LA yield (Y, g g^{-1}) was defined as the LA produced (g L^{-1}) per initial sugar (glucose and/or xylose) (g L^{-1}). The percentage of the theoretical maximum LA was calculated taking into account the maximum stoichiometric theoretical yield in each case. LA volumetric productivity (Q_{48h}, g L^{-1} h^{-1})

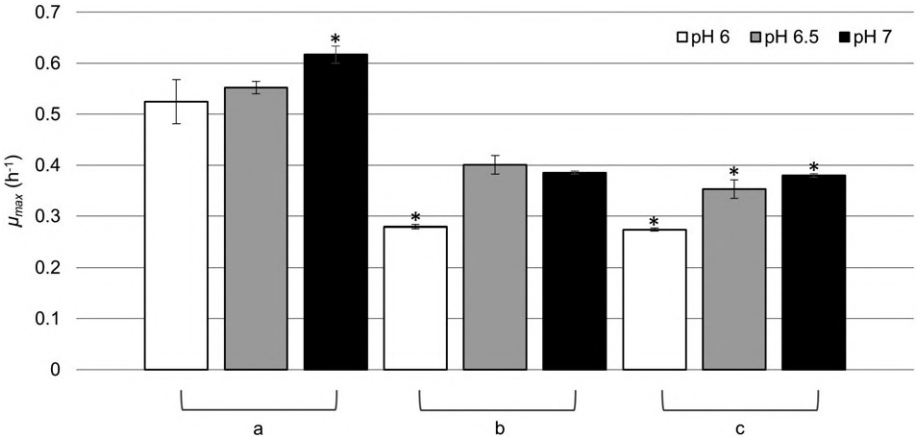

Figure 2. Maximum growth rates for *L. pentosus* CECT 4023 T in MRS medium at different temperature and pH. (a) (32°C), (b) (35°C), and (c) (37°C). The mean difference is significant at (*) 0.05.

was calculated as the LA concentration produced (g L^{-1}) at 48 h.

The software Microsoft Excel was used for the statistical analysis of the data. The results are given as the average ± standard deviation for descriptive statistics. To determine the statically significant differences in terms of growth and LA production in fermentations experiments at different conditions (assays at different pH, temperature, carbon sources, oxygen presence, % WSP, etc.), analysis of variance (ANOVA) was performed. The level of significance was set at $P < 0.05$.

Analytical methods

Cell growth was estimated by measuring optical density in a spectrophotometer (Spectroquant® Pharo 100) at 600 nm and 650 nm for MRS cultures and WSP/WSH containing media, respectively. For determining sugars and fermentation products, samples (1 mL) were taken from the fermentation broth and centrifuged at 14.000 g for 4 min. Then, supernatants were filtered using membrane filters (Thermo Scientific® Nylon 0.2 μm) and stored at −20°C until analysis. Glucose, xylose, LA and acetic acid were quantified by HPLC equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) was used at the following conditions: column temperature, 50°C; mobile phase, 5 mM H_2SO_4; flow rate, 0.5 mL min^{-1} and injection volume, 20 μL. The concentrations of residual sugars and fermentation products were calculated using calibration curves obtained with standard solutions.

Vanillin, p-coumaric acid, furfural and 5-hydroxymethylfurfural (5-HMF) were quantified by HPLC (Agilent, Waldbronn, Germany). The system was equipped with a Coregel 87H3 column (Transgenomic, San Jose, CA). The operating temperature was 65 °C, and the mobile phase was 89% v v^{-1} 5 mM H_2SO_4 and 11% v v^{-1} acetonitrile, with a flow rate of 0.7 mL min^{-1}. All these compounds were identified by a 1050 photodiode-array detector (Agilent, Waldbronn, Germany).

Results and Discussion

Effect of temperature and pH on bacterial growth

Both temperature and pH have significant effect on *L. pentosus* growth. Maximum specific growth rates were obtained at 32°C for all pH tested (Figure 2). With the increase of pH from 6 to 7, increase in the specific growth rate was observed. The highest *L. pentosus* growth rate (0.60 h^{-1}) was attained at 32°C and pH 7.0. This rate was significantly higher than that obtained at the same temperature with pH 6.0 and 6.5. However, at pH 7.0, growth rates decreased at 35°C and 37°C, being below 0.40 h^{-1} in both cases.

Lactic acid production from glucose in WSP: Effect of oxygen presence

To determine the combined effect of oxygen and the inhibitors present in the WSP, different experimental configurations were tested with 20 g L^{-1} of glucose as carbon source in the presence of increasing concentrations of WSP, which means increasing concentration of toxic compounds. In this experiment, glucose was the predominant sugar and very low amount of xylose was detected because it was mostly solubilized as xylooligomers instead of as monomeric xylose during steam explosion pretreatment.

LABs are considered aerotolerant or microaerophilic microorganisms, lacking a functional respiratory chain and catalases. Therefore, as long as respiration or other aerobic metabolisms are not induced, energy is produced *via* fermentation.[28,29] In this study, the lowest growth rates were obtained in aerobiosis, confirming that *L. pentosus* preferably grows under anaerobic conditions (Table 2). Indeed, the highest μ_{max} (0.41 h^{-1}) from WSP 75% v v^{-1}, was obtained under strict anaerobiosis. As a matter of fact, some authors have even pointed out to the toxicity of oxygen for LAB.[30] Aerated growth of *Lactococcus lactis* was reported to cause cellular protein, membrane and chromosomal DNA damage due to the oxidative stress produced by reactive oxygen species.[30]

In terms of LA production, the highest LA yields, around 0.81 and 0.84 g g^{-1}, were achieved in strict anaerobiosis, independently of the WSP concentration (Table 2). As there were not significant differences in LA production from glucose among cultures with 25%, 50% and 75% v v^{-1} of WSP, it may be inferred that a low concentration of oxygen inside the flasks had associated a higher tolerance to the inhibitors present in the WSP. By contrast, it must be highlighted that LA production (titer and yield) was more affected by the different concentration of WSP in aerobiosis and anaerobiosis than in strict anaerobiosis. LA yields varied significantly from

Table 2. Effect of Oxygen Presence and Carbon Source in *L. pentosus* Growth Rate and LA Production from Different Concentrations of WSP[a] and Enzymatically Hydrolysed WSH[b]

Fermentation Condition	Carbon Source	(v v^{-1})	LA Concentration (g L^{-1})	Y (g g^{-1})	%[c]	Q_{48h} (g L^{-1} h^{-1})	μ_{max} (h^{-1})
Aerobic	Glucose 20 g L^{-1}	25% WSP	7.82 ± 0.24*	0.35 ± 0.01*	35	0.24 ± 0.01	0.10 ± 0.02
		50% WSP	2.73 ± 0.01*	0.13 ± 0.00*	13	0.09 ± 0.00	0.06 ± 0.02
		75% WSP	0.79 ± 0.07*	0.05 ± 0.00*	5	0.03 ± 0.00	0.08 ± 0.01
Anaerobic	Glucose 20 g L^{-1}	25% WSP	11.80 ± 0.17	0.69 ± 0.01	70	0.25 ± 0.00	0.31 ± 0.00
		50% WSP	13.47 ± 0.55	0.62 ± 0.03	62	0.28 ± 0.01	0.38 ± 0.02
		75% WSP	7.90 ± 0.89*	0.47 ± 0.05*	47	0.17 ± 0.02	0.20 ± 0.01
Strictly anaerobic	Glucose 20 g L^{-1}	25% WSP	17.76 ± 0.09	0.84 ± 0.00	84	0.37 ± 0.00	0.41 ± 0.03
		50% WSP	17.70 ± 0.05	0.82 ± 0.00	82	0.37 ± 0.00	0.27 ± 0.01
		75% WSP	17.63 ± 0.38	0.81 ± 0.02	81	0.37 ± 0.01	0.27 ± 0.01
Strictly anaerobic	Glucose 5 g L^{-1} Xylose 15 g L^{-1}	25% WSP	8.99 ± 0.03	0.47 ± 0.00	67	0.19 ± 0.00	0.45 ± 0.00
		50% WSP	9.14 ± 0.46	0.46 ± 0.00	67	0.19 ± 0.00	0.35 ± 0.00
		75% WSP	8.35 ± 0.00*	0.34 ± 0.00*	49	0.17 ± 0.00	0.29 ± 0.01
Strictly anaerobic	Xylose 20 g L^{-1}	25% WSP	6.61 ± 0.36	0.32 ± 0.02	53	0.14 ± 0.01	0.36 ± 0.01
		50% WSP	6.62 ± 0.10	0.34 ± 0.01	56	0.13 ± 0.00	0.29 ± 0.01
		75% WSP	5.46 ± 0.07*	0.28 ± 0.00*	46	0.11 ± 0.00	0.19 ± 0.01
Strictly anaerobic	Glucose 2.3 g L^{-1} Xylose 19.3 g L^{-1}	100% WSH	12.58[d] ± 0.22	0.55 ± 0.01	86	0.22 ± 0.00	0.38 ± 0.01

[a]Wheat straw prehydrolysate obtained after the filtration of steam pretreated wheat straw.
[b]Wheat straw hydrolysate obtained after the enzymatic hydrolysis of the WSP.
[c]Percentage of the theoretical maximum.
[d]LA concentration obtained after 120 h.
*mean values are different at 0.05 level of significance.

0.47 to 0.69 g g^{-1} in anaerobiosis and from 0.05 to 0.35 g g^{-1} in aerobiosis (Table 2). In the presence of oxygen, *L. pentosus* presented lower μ_{max} and cells were more susceptible to the toxicity of the inhibitors present in the WSP (weak acids, furan derivatives, and phenolic compounds), which resulted in a reduction of LA production (Table 2).

Previous works reported the ability of several *L. pentosus* strains to grow in the presence of 0.15 and 2.70 g L^{-1} of furfural and acetic acid, respectively.[31] However, in the present study, the concentrations of the mentioned inhibitors (Table 1), were much higher. For instance, acetic acid concentrations ranged from 4.2 to 6.2 g L^{-1} and furfural concentrations ranged from 0.32 to 0.95 g L^{-1} in the different assays with 25%, 50%, and 75% v v^{-1} of WSP (Table 1). In addition, instead of adding the toxic compounds to a synthetic medium, a real lignocellulosic prehydrolysate, composed of a wide range of inhibitory compounds was used to check the inhibitors tolerance of *L. pentosus.*

Focusing on the most challenging conditions tested for *L. pentosus* (i.e., WSP 75% v v^{-1}), a general overview of the sugars consumption and the LA production over time is shown in Figure 3. In aerobic conditions, less than 5% of glucose was consumed (Figure 3a). As a result, the final LA titer was less than 1 g L^{-1}. In anaerobic conditions, more than 75% of glucose was consumed, producing around 8 g L^{-1} of LA (Figure 3b). As mentioned before, the most appropriated condition for LA production was strict anaerobiosis. In this case, all the glucose was consumed at 48 h, with a LA titer of nearly 18 g L^{-1} (Figure 3c). Under these conditions, acetic acid concentration did not increase along fermentation since *L. pentosus* only produces acetic acid when it metabolizes pentoses by the PK pathway.[7]

Lactic acid production from xylose in WSP

In order to check the ability of *L. pentosus* to metabolize both hexoses and pentoses, several experiments were carried out: (i) with a mixture 1:3 of glucose and xylose and (ii) with xylose 20 g L^{-1} as carbon source. Experiments were performed in strict anaerobiosis with WSP because these conditions showed the highest LA production in glucose media.

According to the growth rates, slight differences could be observed between the fermentation with glucose, with the mixture of sugars and with only xylose, reaching μ_{max} between 0.30 and 0.40 h^{-1} in all cases (Table 2). Boguta et al. reported that several *L. pentosus* isolates could utilize xylose and reach μ_{max} values around 0.20 h^{-1} in the presence of different inhibitors commonly found in pretreated wheat straw (i.e., furfural 0.15 g L^{-1} and acetic acid 2.70 g L^{-1}).[31] Remarkably, the μ_{max} obtained in this study with real WSP containing higher concentration of inhibitors were significantly higher (Table 1).

As *L. pentosus* uses the PK pathway to metabolize pentoses, lower LA yields were obtained in this case, due to the production of acetic acid as a by-product. LA yields between 0.34 and 0.47 g g^{-1} were obtained from the glucose-xylose mixture (1:3) in the presence of different concentrations of WSP (Table 2), which represented between 49% and 67% of the theoretical maximum. These results were similar to other reported in previous studies (69%) using *L. pentosus* in MRS medium with a mixture of glucose and xylose.[23] An engineered *E. mundtii* strain was grown in batch fermentations with different ratios of glucose: xylose mixtures reaching LA

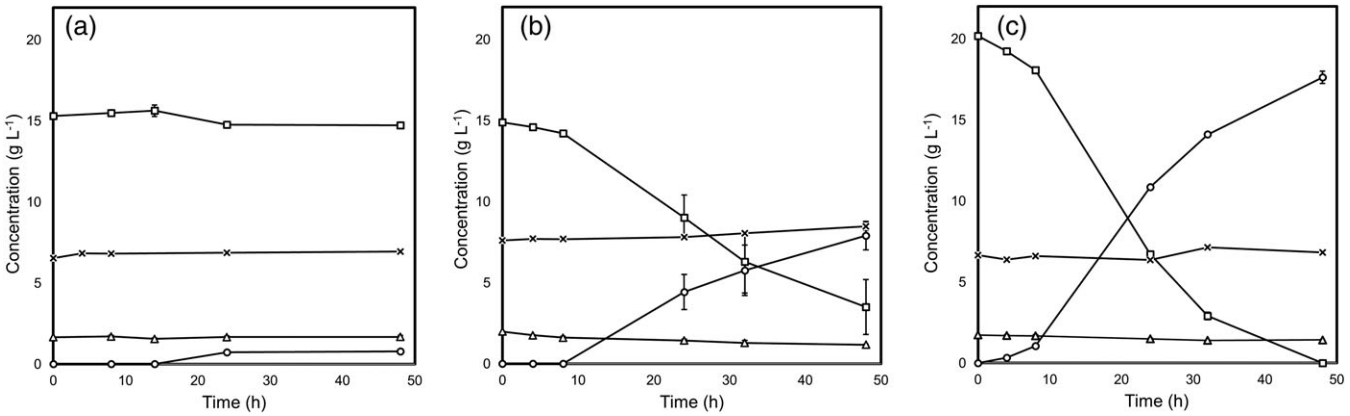

Figure 3. Sugar consumption and acid production profiles during *L. pentosus* CECT 4023 T fermentation of 20 g L^{-1} glucose with 75% v v^{-1} of WSP in aerobiosis (a), anaerobiosis (b) and strict anaerobiosis (c). Lactic acid (circle), acetic acid (cross), glucose (square), xylose (triangle).

yields around 0.80 g g^{-1}. The high yields obtained in this case could be explained because xylose is metabolized homofermentatively by the pentose phosphate pathway, without acetic acid formation.[18] Conversely, 0.58 g g^{-1} of LA were obtained with *Lactobacillus brevis* from a mixture 1:1 of glucose and xylose.[32] Remarkably, in this study, these high yields were obtained in presence of lignocellulosic inhibitors. As expected, the yields reached with the sugars mixture were higher than the ones obtained with xylose alone. In the latter case, LA yields around 0.30 g g^{-1} were attained (Table 2). As it can be seen in Table 2, no significant differences in LA production from xylose were found when the WSP concentration was increased from 25% v v^{-1} to 50% v v^{-1}. However, LA yield decreased when 75% v v^{-1} WSP was used. In this case, the concentration of acetic acid, furfural and hydroxymethilfurfural was approximately 6, 1, and 0.06 g L^{-1}, respectively (Table 1). It seems that these concentrations of inhibitors in 75% v v^{-1} WSP could affect the xylose conversion to LA, while this was not the case with glucose as carbon source in strict anaerobiosis (Table 2).

The sugar consumption, as well as LA and acetic acid production in the fermentation test with WSP 75% v v^{-1} is shown in Figure 4. When using the sugars mixture as carbon source (Figure 4a), 50% of the xylose was consumed, producing 8.35 g L^{-1} and 2 g L^{-1} of LA and acetic acid, respectively. As expected, LA production was lower than the obtained with only glucose as carbon source with WSP 75% v v^{-1} (Table 2). As can be seen in Figure 4a, there was a coconsumption of both glucose and xylose during the first hours of fermentation. Interestingly, these results suggested that the carbon catabolite repression did not have a marked effect in this case, as the presence of glucose did not hinder the xylose consumption. Having in mind that most of xylose-utilizing LAB are affected by this phenomenon, the fact that *L. pentosus* CECT 4023 T was able to perform a simultaneous mixed sugar consumption in the presence of WSP was very remarkable. Indeed, the use of LAB that can metabolize mixed sugars to LA with high efficiency and relaxed carbon catabolite repression arises as an interesting approach to take advantage of the heterogeneous sugar composition of lignocellulosic biomass.[12] This co-consumption was also observed in LA production by *E. mundtii* QU 25 from initial concentrations of 25 g L^{-1} glucose and 50 g L^{-1} xylose.[18] *L. brevis* has demonstrated to present a lax control of carbohydrate utilization and can perform a simultaneous fermentation of a range of carbon sources.[32] Indeed, *L. brevis* was able to consume simultaneously both glucose and xylose in a mixture with a ratio 1:1.[33]

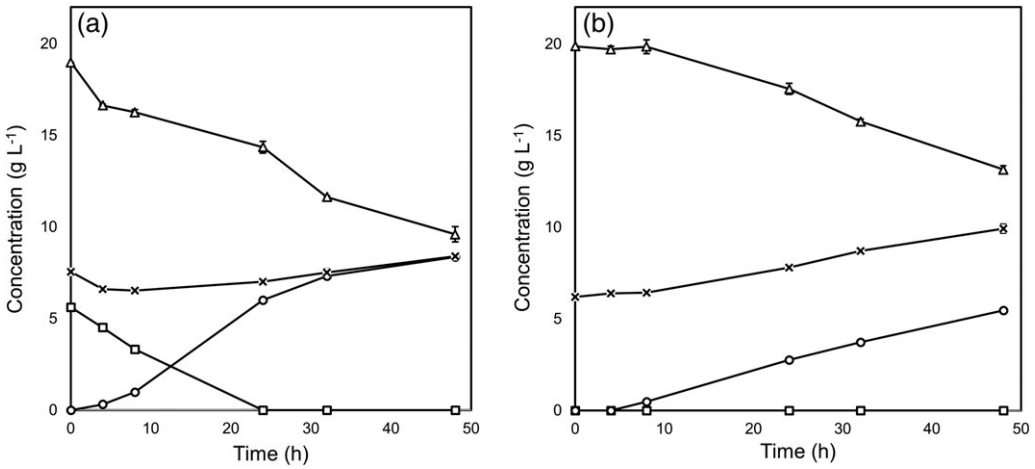

Figure 4. Sugar consumption and acid production profiles during *L. pentosus* CECT 4023 T lactic fermentation with 75% v v^{-1} of WSP in strict anaerobiosis from (a) glucose: xylose (1:3) and (b) xylose 20 g L^{-1}. Lactic acid (circle), acetic acid (cross), glucose (square), xylose (triangle).

When there was only xylose as carbon source in the fermentation broth with WSP 75% v v^{-1}, only 34% of the xylose was consumed at 48 h of fermentation, producing 5.4 g L^{-1} of LA (Figure 4b). By taking into account the acetic acid originated as a by-product during the xylose metabolism by the PK pathway and the amount previously originated from deacetylation of hemicellulose during steam explosion pretreatment, around 10 g L^{-1} of acetic acid were accumulated in the medium. In this sense, the low sugar consumption in this case might be a consequence of the inhibition produced by the pH fall and the acetic acid accumulation. The acetic acid could permeate the cell membrane and dissociate inside the cytoplasm, thus decreasing intracellular pH and injuring the bacterial growth by means of cell membrane disruption.[12] Indeed, the lack of control of essential conditions such as pH could have aggravated the detrimental effect of the inhibitors present in WSP. This drawback could be alleviated by changing the process conditions from uncontrolled to pH-controlled batch fermentation.[34] Furthermore, the pH fall could also be prevented by combining different separation methods in order to avoid the accumulation of LA or acetic acid in the culture. In this sense, the combined effect of anion exchange and organic solvent extraction presented a high LA recovery yield in *Lactococcus lactis* fermentation.[35] High LA yields were also obtained from glucose fermentation of *Pediococcus pentosaceus* coupled with the combination of electrodialysis and ion exchange extraction.[36]

Lactic acid production from WSH obtained with different hydrolysis methods

To determine the proper hydrolysis technique for releasing fermentable sugar from its oligomeric form present in WSP, an acid and an enzymatic approach were carried out. Concerning the hydrolysis yields, similar results were obtained with both methods. The acid-WSH contained 2.4 g L^{-1} glucose and 18.0 g L^{-1} xylose while the enzymatic-WSH contained 2.2 g L^{-1} glucose and 19.0 g L^{-1} xylose (Table 1). These values represented a hydrolytic efficiency of 48% and 43% for glucose and 68% and 72% for xylose in acid and enzymatic hydrolysis, respectively. In terms of inhibitory compounds, a significant acetic acid increase was observed in acid-WSH, reaching 8.86 g L^{-1}. Nevertheless, with enzymatic hydrolysis, the acetic acid concentration was maintained around 7.0 g L^{-1} (Table 1). In the case of furfural, there was a decrease in the concentration of this degradation compound after the hydrolysis step. In fact, furfural concentration decreased from 1.26 to 0.36 g L^{-1} and 0.52 g L^{-1} after acid and enzymatic hydrolysis, respectively (Table 1). Similar final concentrations of 5-hydroxymethilfurfural were reached after acid (0.15 g L^{-1}) and enzymatic hydrolysis (0.23 g L^{-1}) (Table 1).

According to the fermentation of these hydrolysates, very slow growth was obtained with acid-WSH and, therefore, no LA production was obtained (data not shown). By contrast, high growth of *L. pentosus* was obtained in the enzymatic-WSH, reaching a μ_{max} near to 0.40 h^{-1}, which was similar to that obtained in previous experiments where glucose and xylose were added to the WSP (Table 2). These results contrasted with those reported by Garde et al., who concluded that both enzymatic and acidic WSH could be used for LA fermentation with similar yields.[23] Nevertheless, the temperature and reaction time tested in the acid hydrolysis were softer in the mentioned work, so the bacterial inhibition effect of the resulting hydrolysate could be less significant. The inhibitory effect that the acid-WSH had on the LAB could be explained by the increased amount of inhibitors like acetic acid produced during acid-hydrolysis, which has been previously reported, suggesting advantageous the enzymatic hydrolysis methods.[37]

Acetic acid begins to affect cell growth even at concentrations as low as 5 g L^{-1} both in Gram-positive and Gram-negative bacteria.[38] As mentioned, when acetic acid crosses the membrane and decreases intracellular pH, transmembrane gradient is affected and energy is used to maintain that pH gradient instead of growing.[39] However, it has been reported that some *Lactobacillus plantarum* and *L. pentosus* strains were able to grow at high concentrations of this acid, even at 12.14 g L^{-1}.[31] Furthermore, these isolates also tolerated 7.9 and 5.9 g L^{-1} of furfural and 5-hydroxymethilfurfural, respectively, which were higher than the concentrations present in the WSH. Nevertheless, these assays were carried out with glucose as carbon source and without taking into account that the interaction effects among the different toxic compounds would also imply synergistic effects between different kind of inhibitors.

In terms of LA production, LA yield of 0.55 g g^{-1} and LA productivity of 0.22 g L^{-1} h^{-1} were reached using enzymatic WSH (Table 2). In this case, all the glucose was consumed, as well as 55% of total xylose at 120 h of fermentation (Figure 5). As there was a low xylose uptake at 48 h of fermentation in previous experiments with xylose as carbon source (30% of present xylose) it became necessary to extend the fermentation time to 120 h to maximize the sugar consumption. For this reason, as a result of the higher sugar utilization and longer fermentation time, the LA yields were significantly greater and 12.58 g L^{-1} of LA were produced (Figure 5). As already discussed, the low xylose consumption rate in these cases can be explained by the pH fall produced as a result of LA and acetic acid accumulation. In fact, more than 10 g L^{-1} of acetic acid were accumulated in the medium similarly to the experiment with 20 g L^{-1} xylose. It is worth

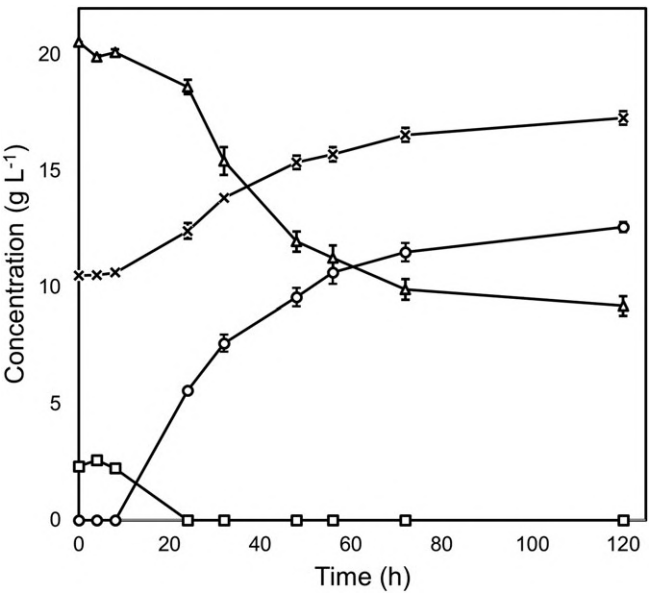

Figure 5. Sugar consumption and acid production profiles during *L. pentosus* CECT 4023 T lactic fermentation of WSH 100% v v^{-1} obtained by enzymatic hydrolysis of WSP. Lactic acid (circle), acetic acid (cross), glucose (square), xylose (triangle).

pointing out that since enzymes were not deactivated before fermentation, some enzymatic hydrolysis might be taking place during fermentation and thus, available sugars would be higher than the detected concentrations after 24 h-long enzymatic hydrolysis. LA yields obtained from WSH were comparable to results attained in this study in strict anaerobiosis (Table 2) and with previous reports with *L. pentosus* strains using lignocellulosic hydrolysates. For example, around 0.50 g g^{-1} of LA were produced from barley bran husks and corncobs hydrolysates using *L. pentosus.*[40] Moreover, using trimming vine shoots hydrolysates (with xylose and glucose in a relation of 2:1), LA yields around 0.70 g g^{-1} were obtained.[21,40] Between 66% and 89% of LA were obtained at 48 h of fermentation of ammonia-treated corn stover by *L. pentosus*,[41] while 0.83 g g^{-1} of LA were produced from wood-extract hydrolysate.[42] 0.92 g g^{-1} of LA were produced by an isolated homofermentative *B. coagulans* strain from WSH without acetic acid production.[25] After a screening of heterofermentative LAB, two *Lactobacillus hilgardii* and *Leuconostoc mesenteroides* strains were found to produce more than 10 g L^{-1} of LA from WSH supplemented with tomato juice medium. Nonetheless, most of the mentioned studies, fermentations were carried out maintaining the optimal pH for fermentation by dosing with NaOH, $CaCO_3$ and other neutralizing solution, avoiding the inhibition produced by the pH fall. To reduce the amounts of these neutralizers, new engineered strains could be obtained with enhanced tolerance to LA and acetic acid. For instance, a heat shock protein from *Escherichia coli* was expressed in *Lactococcus lactis*, improving its tolerance to NaCl, ethanol and LA at 40 °C.[43] The combination of selection in chemostat at low pH and genome shuffling resulted in an improved *Lactobacillus* LA tolerant strain with threefold increase in LA production.[44]

Overall, taking into account released sugar after enzymatic hydrolysis of WSP, fermentation yields reached 86% of the theoretical maximum without pH control (Table 2), which was the highest yield obtained in this work.

Conclusions

The heterolactic bacteria *L. pentosus* CECT4023T was able to grow on different concentrations of WSP, tolerating the presence of inhibiting compounds and producing LA as fermentation product. LA production was enhanced when the oxygen presence was reduced by changing the fermentation conditions from aerobic, to anaerobic and, especially, strictly anaerobic. Therefore, in the presence of WSP, less oxygen concentration in the culture exhibited higher *L. pentosus* growth and LA production.

The use of different sugars as carbon source highlighted the efficient coutilization of hexoses and pentoses of this LAB. When xylose was used as carbon source–mixed with glucose or alone-, lower LA yields than with glucose were obtained because of the production of acetic acid as by-product of the PK pathway. Nonetheless, most of the consumed sugars were being used for the production of LA.

When WSP was hydrolyzed, the oligomeric sugars were properly released, especially xylose, which was the predominant sugar of this liquid fraction. Although similar hydrolysis yields were obtained with enzymatic and acid treatment, the high inhibitors increase produced in the latter affected the bacterial growth and hampered the LA production. By contrast, high LA yield (0.55 g g^{-1}) was obtained from enzymatically hydrolysed WSH at 120 h of fermentation without pH control. Both glucose and xylose were also coutilized when using this hemicellulosic hydrolysate as substrate for LA production. Having in mind the high yields obtained without controlling the pH of the culture, further studies may be performed in the future to improve the resistance of this LAB to the low pH reached during heterolactic fermentation, avoiding the use of neutralizing agents. With this in mind, adaptive evolution and genetic engineering experiments could be carried out in order to improve the resistance of this strain to acid pH, thus improving LA yields from xylose and other pentoses present in hemicellulosic hydrolysates. Although purification steps can increase sharply the production costs, different separation methods can also be applied to remove the acidic fermentation products from the culture as soon as they are formed, preventing the acidification of the medium and the product inhibition.

The results presented in this study not only corroborated the ability of *L. pentosus* to grow using mixtures of sugars, but also demonstrated the suitability of this strain to be applied as an efficient LA producer, using lignocellulosic hydrolysates in a biorefinery approach.

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Literature Cited

1. Abdel-Rahman MA, Sonomoto K. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. *J Biotechnol.* 2016;236:176–192.
2. Das D, Goyal A. Lactic acid bacteria in food industry. In: Satyanarayana T, Johri BN, editors. *Microorganisms in Sustainable Agriculture and Biotechnology*, Vol 2012. Netherlands: Springer:757–772.
3. Reddy LV, Kim YM, Yun JS, Ryu HW, Wee YJ. L-lactic acid production by combined utilization of agricultural bioresources as renewable and economical substrates through batch and repeated-batch fermentation of *Enterococcus faecalis* RKY1. *Bioresour Technol.* 2016;209:187–194.
4. Ahring BK, Traverso JJ, Murali N, Srinivas K. Continuous fermentation of clarified corn Stover hydrolysate for the production of lactic acid at high yield and productivity. *Biochem Eng J.* 2016;109:162–169.
5. Ulery BD, Nair LS, Laurencin CT. Biomedical applications of biodegradable polymers. *J Polym Sci B Polym Phys.* 2011;49:832–864.
6. Juturu V, Wu JC. Microbial production of lactic acid: the latest development. *Crit Rev Biotechnol.* 2015;1–11.
7. Abdel-Rahman MA, Tashiro Y, Sonomoto K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. *J Biotechnol.* 2011;156:286–301.
8. Bai Z, Gao Z, Sun J, Wu B, He B. D-lactic acid production by *Sporolactobacillus inulinus* YBS1-5 with simultaneous utilization of cottonseed meal and corncob residue. *Bioresour Technol.* 2016;207:346–352.
9. Ye L, Zhou X, Hudari MSB, Li Z, Wu JC. Highly efficient production of L-lactic acid from xylose by newly isolated *Bacillus coagulans* C106. *Bioresour Technol.* 2013;132:38–44.
10. Moreno AD, Alvira P, Ibarra D, Tomás-Pejó E. Production of ethanol from Lignocellulosic biomass. In: Fang Z, Smith JR, Qi X, editors. *Production of Platform Chemicals from Sustainable Resources*, Vol 2017. Singapore: Springer:375–410.
11. Zhao Z, Yang Y, Abdeltawab AA, Yakout SM, Chen X, Yu G. Cholinium amino acids-glycerol mixtures: new class of solvents

for pretreating wheat straw to facilitate enzymatic hydrolysis. *Bioresour Technol.* 2017;245(Part A:625–632.
12. Cubas-Cano E, González-Fernández C, Ballesteros M, Tomás-Pejó E. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate. *Biofuels Bioprod Biorefin.* 2018;12:290–300.
13. Jönsson LJ, Martín C. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. *Bioresour Technol.* 2016;199:103–112.
14. Tomás-Pejó E, Alvira P, Ballesteros M, Negro MJ. Pretreatment technologies for lignocellulose-to-bioethanol conversion. *Biofuels*; 2011b:149–176.
15. Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresour Technol.* 2010;101: 4851–4861.
16. Alvira P, Moreno AD, Ibarra D, Sáez F, Ballesteros M. Improving the fermentation performance of *Saccharomyces cerevisiae* by laccase during ethanol production from steam-exploded wheat straw at high-substrate loadings. *Biotechnol Prog.* 2013;29:74–82.
17. Tomás-Pejó E, Fermoso J, Herrador E, Hernando H, Jiménez-Sánchez S, Ballesteros M, González-Fernández C, Serrano DP. Valorization of steam-exploded wheat straw through a biorefinery approach: bioethanol and bio-oil co-production. *Fuel.* 2017;199: 403–412.
18. Abdel-Rahman MA, Xiao Y, Tashiro Y, Wang Y, Zendo T, Sakai K, Sonomoto K. Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. *J Biosci Bioeng.* 2015;119:153–158.
19. Turner TL, Zhang GC, Kim SR, Subramaniam V, Steffen D, Skory CD, Jin YS. Lactic acid production from xylose by engineered *Saccharomyces cerevisiae* without PDC or ADH deletion. *Appl Microbiol Biotechnol.* 2015;99:8023–8033.
20. Qiu Z, Gao Q, Bao J. Engineering Pediococcus acidilactici with xylose assimilation pathway for high titer cellulosic l-lactic acid fermentation. *Bioresour Technol.* 2018;249:9–15.
21. Bustos G, Moldes AB, Cruz JM, Domínguez JM. Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. *Biotechnol Prog.* 2005;21:793–798.
22. Eiteman MA, Ramalingam S. Microbial production of lactic acid. *Biotechnol Lett.* 2015;37:955–972.
23. Garde A, Jonsson G, Schmidt AS, Ahring BK. Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*. *Bioresour Technol.* 2002;81:217–223.
24. Hu J, Lin Y, Zhang Z, Xiang T, Mei Y, Zhao S, Liang Y, Peng N. High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn Stover using fed-batch simultaneous saccharification and fermentation. *Bioresour Technol.* 2016;214:74–80.
25. Aulitto M, Fusco S, Bartolucci S, Franzen CJ, Contursi P. *Bacillus coagulans* MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. *Biotechnol Biofuels.* 2017;10:210.
26. Gasser E, Ballmann P, Droge S, Bohn J, Konig H. Microbial production of biopolymers from the renewable resource wheat straw. *J Appl Microbiol.* 2014;117:1035–1044.
27. Moreno AD, Ibarra D, Mialon A, Ballesteros M. A bacterial Laccase for enhancing Saccharification and ethanol fermentation of steam-pretreated biomass. *Fermentation.* 2016;2.
28. Guidone A, Ianniello RG, Ricciardi A, Zotta T, Parente E. Aerobic metabolism and oxidative stress tolerance in the *Lactobacillus plantarum* group. *World J Microbiol Biotechnol.* 2013;29:1713–1722.
29. Papadimitriou K, Alegría Á, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, Turroni F, van Sinderen D, Varmanen P, Ventura M, Zúñiga M, Tsakalidou E, Kok J. Stress physiology of lactic acid bacteria. *Microbiol Mol Biol Rev.* 2016;80:837–890.
30. Rezaiki L, Cesselin B, Yamamoto Y, Vido K, van West E, Gaudu P, Gruss A. Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. *Mol Microbiol.* 2004;53:1331–1342.
31. Boguta AM, Bringel F, Martinussen J, Jensen PR. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. *Microb Cell Fact.* 2014;13:97.
32. Kim JH, Shoemaker SP, Mills DA. Relaxed control of sugar utilization in lactobacillus brevis. *Microbiology.* 2009;155: 1351–1359.
33. Grewal J, Khare SK. One-pot bioprocess for lactic acid production from lignocellulosic agro-wastes by using ionic liquid stable *Lactobacillus brevis*. *Bioresour Technol.* 2017;251:268–273.
34. Alonso S, Herrero M, Rendueles M, Díaz M. Physiological heterogeneity *in Lactobacillus casei* fermentations on residual yoghurt whey. *Process Biochem.* 2014;49:732–739.
35. Boonmee M, Cotano O, Amnuaypanich S, Grisadanurak N. Improved lactic acid production by in situ removal of lactic acid during fermentation and a proposed scheme for its recovery. *Arabian Journal for Science and Engineering.* 2016;41:2067–2075.
36. Boontawan P, Kanchanathawee S, Boontawan A. Extractive fermentation of L-(+)-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. *Biochem Eng J.* 2011;54: 192–199.
37. Tomás-Pejó E, Alvira P, Ballesteros M, Negro MJ. Chapter 7 - pretreatment technologies for lignocellulose-to-bioethanol conversion A2 - Pandey, Ashok. In: Larroche C, Ricke SC, Dussap CG, Gnansounou E, editors. *Biofuels*. Amsterdam: Academic Press; 2011:149–176.
38. Trcek J, Mira NP, Jarboe LR. Adaptation and tolerance of bacteria against acetic acid. *Appl Microbiol Biotechnol.* 2015;99: 6215–6229.
39. Othman M, Ariff AB, Rios-Solis L, Halim M. Extractive fermentation of lactic acid in lactic acid bacteria cultivation: a review. *Front Microbiol.* 2017;8:2285.
40. Moldes AB, Torrado A, Converti A, Domínguez JM. Complete bioconversion of hemicellulosic sugars from agricultural residues into lactic acid by *Lactobacillus pentosus*. *Appl Biochem Biotechnol.* 2006;135:219–228.
41. Zhu Y, Lee YY, Elander RT. Conversion of aqueous ammonia-treated corn Stover to lactic acid by simultaneous saccharification and cofermentation. *Appl Biochem Biotechnol.* 2007;137-140: 721–738.
42. Buyondo JP, Liu S. Lactic acid production by *Lactobacillus pentosus* from wood extract hydrolysates. *J Sci Technol Forest Products Processes.* 2011;1:38–47.
43. Abdullah-Al-Mahin SS, Higashi C, Matsumoto S, Sonomoto K. Improvement of multiple-stress tolerance and lactic acid production in *Lactococcus lactis* NZ9000 under conditions of thermal stress by heterologous expression of *Escherichia coli* DnaK. *Appl Environ Microbiol.* 2010;76:4277–4285.
44. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WP, Ryan CM, del Cardayre S. Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nat Biotechnol.* 2002;20:707–712.

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ARTICLE III


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Efficient utilization of hydrolysates from steam-exploded gardening residues for lactic acid production by optimization of enzyme addition and pH control
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Efficient utilization of hydrolysates from steam-exploded gardening residues for lactic acid production by optimization of enzyme addition and pH control

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ABSTRACT

The expansion of urban green areas has boosted the accumulation of gardening lignocellulosic residues that could be potentially used to produce platform chemicals like lactic acid. However, when using lignocelluloses, pretreatment step, such as steam explosion, is often needed to favour sugar release. Considering that the conversion of glucose from cellulose has been widely addressed, this work is focused on the valorisation of the steam-exploded gardening liquid fraction rich in hemicellulosic sugars. Since oligomeric sugars are usually solubilized during steam explosion, an enzymatic hydrolysis step was required in some cases to increase the monosaccharides content. Although the presence of inhibitors released during pretreatment (e.g. formic acid) hindered hydrolysis yields, the addition of hemicellulases and the enzyme dosage optimization resulted in 85%, 89% and 95% of glucose, xylose and arabinose release from soluble oligomers, respectively. *Lactobacillus pentosus* CECT4023T was used for lactic acid fermentation of C6 and C5 sugars from the hydrolysate with the highest sugars concentration, that did not require enzymatic hydrolysis. Xylose consumption was hampered due to the inhibitory effect of acids that produced pH drop. Different pH control systems were applied and automatic NaOH addition in bioreactor resulted in 21 g L^{-1} of lactic acid (95% of the maximum theoretical yield) that implied 44% increase in lactic acid production when compared with flask fermentation. These results provide new insights for the valorisation of emerging lignocellulosic materials like gardening residues into high added-value products.

1. Introduction

The continued depletion of petrochemical sources as well as their environmental impacts make necessary the use of alternative resources for the increasing demands of energy and chemicals. Lignocellulosic materials are considered a suitable option for the production of bio-based products because they are available throughout the year at low prices, avoiding the competition of water and land-use with food (Bolivar-Telleria et al., 2018).

The rise and expansion of open green spaces bring to the forefront the opportunity to valorise the residual lignocellulosic biomass produced in the gardening of these areas. Large volumes of gardening residues, like tree branches from pruning, hedge cuttings, leaves and grass clippings are generated annually (Boldrin and Christensen, 2010). In fact, even 1.5 kg per m^2 of residues are generated during the gardening and pruning of these areas (Tetma, 2018). Gardening residues have already been used for energy production by advanced wood combustion, gasification, pyrolysis, anaerobic digestion and ethanol production (Míguez et al., 2012; Robak and Balcerek, 2018). However, these residues are still an undervalued resource and their valorisation is hindered by its heterogeneity, being harder to deal with and more complex than forest, plantation or crop residues.

The chemical composition of gardening residues is about 40% cellulose, 20–30% hemicelluloses and 25–30% (w w^{-1}) lignin (Shi et al., 2013). A pretreatment step is required to improve sugars release from the feedstock by increasing the accessibility of enzymes to the recalcitrant lignocellulose structure. Few works have addressed the pretreatment of gardening residues, like cocksfoot grass and branches pruned from pear trees, using wet and steam explosion (Njoku et al., 2013; Sasaki et al., 2014). In this context, steam explosion is one of the most widely applied technologies, producing the separation of cellulose fibers, solubilisation

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and partial hydrolysis of hemicellulose and redistribution of lignin (Tomás-Pejó et al., 2011). As a result, two fractions are generated: (i) water insoluble solids (WIS) fraction rich in cellulose and lignin and (ii) liquid fraction or prehydrolysate rich in monomeric and oligomeric sugars from hemicelluloses solubilisation together with degradation compounds.

Valorisation of the cellulose present in the WIS fraction has been traditionally addressed, while the use of the liquid fraction is still challenging because of the high abundance of C5 sugars that cannot be fermented by most wild-type microorganisms (Cubas-Cano et al., 2018). Furthermore, the heterogeneous structure of hemicellulose, including different constituents and chemical bonds, makes its enzymatic hydrolysis very complex, requiring several groups of enzymes and complex enzymatic systems (Ji et al., 2012). In this sense, optimizing an enzymatic cocktail to accelerate the release of monomeric sugars from soluble hemicellulosic oligosaccharides could be crucial, especially for grass-based materials enriched in xylan and arabinoxylan (Sweeney and Xu, 2012).

Some bacterial groups are able to metabolise pentoses from hemicellulosic liquid fractions into lactic acid (LA). This building block present novel high-value applications in the production of chemicals, pharmaceuticals and bioplastics (Cubas-Cano et al., 2018). Although, woody lignocellulosic materials have received great attention for the sustainable production of organics acids (Pleissner et al., 2017), gardening residues have not been previously used as substrate to produce LA. For this reason, the present study is focused on the use of steam exploded gardening liquid fractions as novel substrates for the bioproduction of LA.

In this context, a multi-stage approach for producing biofuels, like bioethanol, and bio-based products, like LA, could be performed from each fraction obtained after steam explosion of lignocellulose, coupling the reduction of organic residues with the production of an array of commodities. This topic was recently studied in a previous work from Cubas-Cano et al. (2020), focused on the total valorisation of the different sugars from lignocellulose following a sequential cultivation approach.

In this work, different steam explosion conditions that determined the composition of the resulting Gardening Prehydrolysates (GP) were tested for pretreatment of gardening residues. When necessary, GP were subjected to enzymatic hydrolysis to release the monomeric sugars from oligomers, subsequently obtaining Gardening Hydrolysates (GH). The GP containing the highest concentrations of fermentable monosaccharides did not require an hydrolytic step and was directly used for LA fermentation with *Lactobacillus pentosus* CECT4023T. This bacterial strain is able to metabolise both C6- and C5-sugars through the Embden-Meyerhof-Parnas (EMP) and the phosphoketolase (PK) pathways, respectively, even in presence of different inhibitors formed during pretreatment (Cubas-Cano et al., 2019). The weak acids produced during pretreatment (i.e., acetic acid, formic acid) and fermentation (LA, acetic acid) can severely inhibit sugars consumption at low pH values. For this reason, different pH control methods were applied to maximize xylose consumption. Results presented herein will provide new insights for process optimization to valorise the increasing volumes of gardening residues and produce high-added value commodities like LA.

2. Materials and methods

2.1. Pretreatment and gardening prehydrolysates

The gardening residues were firstly milled in a laboratory cutting mill (SM 2000, Retsch, Germany) to a particle size of 10 mm. The chips were then pretreated by steam explosion in a 2-L reactor. Three different pretreatment conditions were used according to previous works with other lignocellulosic materials: (1) $FeCl_3$-catalyzed (0.1 M, 30 mg g^{-1} biomass) pretreatment at 180 °C for 20 min, (2) H_2SO_4-catalyzed (0.25 M, 45 mg g^{-1} biomass) pretreatment at 180 °C for 5 min and (3) aqueous extraction followed by H_2SO_4-catalyzed (0.33 M, 60 mg g^{-1} biomass) pretreatment at 180 °C for 10 min (Ballesteros et al., 2006; Ballesteros et al., 2011; López-Linares et al., 2013). Aqueous extraction was performed at 130 °C for 20 min with a 1:10 solid:liquid (v v^{-1}) relation. After pretreatment, the obtained slurries were vacuum filtered in order to obtain the hemicellulosic liquid fractions. GPs from pretreatment (1) and (2) (GP1 and GP2) were subjected to enzymatic hydrolysis to obtain gardening hydrolysates GH1 and GH2, respectively. GH3 was directly derived from GP3 since it did not require enzymatic hydrolysis because most of the sugars were in monomeric form. A scheme of the whole process is shown in Fig. 1. The compositions of the GPs are shown in Table 1. Potential monomeric sugars were the monosaccharides (glucose, xylose, arabinose, galactose and mannose) that could be released from oligomers present in the GP. The potential monomeric sugars were used to calculate the enzymatic hydrolysis yields, as explained hereunder, and were determined by an acid hydrolysis (4% (v v^{-1}) H_2SO_4, 120 °C and 30 min) (Table 1).

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of GP1 and GP2 to obtain monomeric sugars were carried out by adding different cellulolytic and hemicellulolytic commercial enzymes. Specifically, the cellulose Celluclast® 1.5 L (*Cel*) and the β-D-glucosidase Novozym® 188 (*β-Glu*) (Novozymes, Denmark) were used for depolymerization of glucose oligomers, while the enzymatic cocktail of xylanases Cellic-HTec2® (*C-HTec2*) (Novozymes, Denmark) and the β-D-xylosidase EC 3.2.1.37 (*β-Xyl*) (Megazyme®, USA) were used to release the monomeric sugars from xylooligosaccharides.

Different dosages of *Cel*, *β-Glu, C-HTec2* and *β-Xyl* were screened to obtain the highest glucose, galactose, mannose, xylose and arabinose hydrolysis yields. *Cel* and *β-Glu* were added as Filter Paper Units (FPU) g^{-1} and International Units (IU) g^{-1} of glucooligosaccharides, respectively. *C-HTec2* was added as % of xylooligosaccharydes and *β-Xyl* as mg g^{-1} of xylooligosaccharides. Detailed information of the enzymes dosages used are stated in Table 2. The highest cellulases dosages were set at 16 FPU g^{-1} and 16 IU g^{-1} because, at higher enzyme loadings, enzyme could be adsorbed on the substrate, restricting the diffusion process (Martín et al., 2012). The hemicellulases loadings were selected according to the results obtained in previous works with hemicellulosic hydrolysates (Cubas-Cano et al., 2019).

Addition of cellulase and hemicellulase cocktails in GP1 was carried out following two strategies: i) hemicellulases were added together with the cellulases and ii) hemicellulases were added 24 h prior to cellulases addition. Furthermore, the effect of increasing the cellulases dosages was studied in GP2, as well as the effect of increasing the *β-Xyl* loading at the highest cellulases dosages.

The enzymatic hydrolysis tests were run in 50-mL Falcon centrifuge tubes with 10 mL of medium at 40 °C, 150 rpm and pH 5.5 for 72 h. Enzymatic hydrolysis assays were carried out in triplicate. Samples were taken at 0, 24, 48 and 72 h of hydrolysis. After hydrolysis of GP1 and GP2, two GHs were obtained (GH1 and GH2, respectively).

2.3. Microorganisms and culture conditions

Lactobacillus pentosus CECT 4023 T (ATCC-8041) was obtained from the Spanish Type Culture Collection (Valencia, Spain). For preculture, an aliquot of 20 μL of cells was taken from glycerol

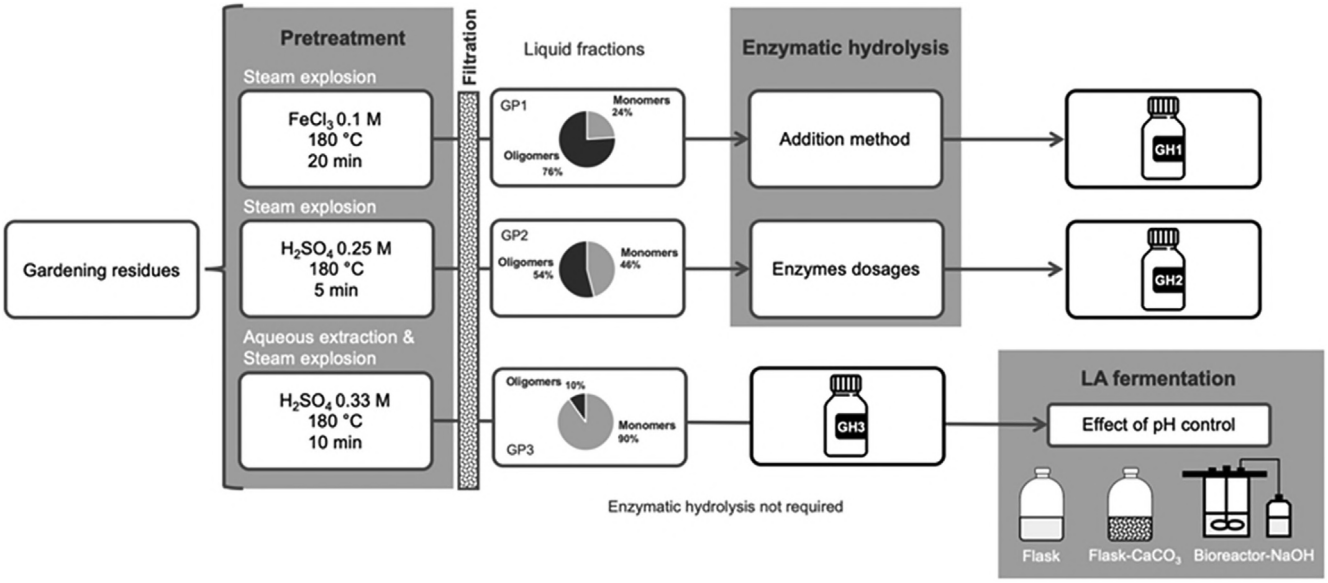

Fig. 1. Experiments carried out with different gardening lignocellulosic (pre)hydrolysates in the current work.

stocks with an optical density (OD_{600}) of 0.4. This aliquot was inoculated in 50-mL Falcon tubes with 15 mL of Man, Rogosa and Sharpe medium (MRS), containing (g L^{-1}): glucose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; $MgSO_4 \cdot 7\ H_2O$, 0.2 and $MnSO_4 \cdot H_2O$, 0.05 (pH 6.2). The preculture was grown in a rotatory shaker at 150 rpm and 32 °C overnight (until the culture reached the late exponential growth phase).

2.4. Lactic acid fermentation of gardening hydrolysate in flask

GH3 was selected for LA fermentation due to its higher monomeric sugars content in comparison with GH1 and GH2. LA fermentation was performed in 120-mL nitrogen-purged clamped flasks with 25 mL of working volume at 32 °C and 150 rpm in strict anaerobiosis as detailed in Cubas-Cano et al. (2019). The gas composition was analysed by gas chromatography to corroborate that flasks contained <5% (v v^{-1}) of oxygen. The pH of GH3 was adjusted at 7.0 with NaOH 5 M and the compounds from MRS medium, except glucose, were added. *L. pentosus* was inoculated at an initial OD_{600} of 0.1. The effect of the pH drop during LA fermentation was studied by comparing the addition of 10 g L^{-1} of $CaCO_3$ with non-controlled pH (Fig. 1).

In all cases, samples were taken at 0, 4, 8, 24, 32, 48 and 72 h of fermentation for measuring the concentration of sugars, pH values and fermentation products. All fermentation tests were carried out in triplicate.

2.5. Lactic acid fermentation of gardening hydrolysate in bioreactor

Fermentation of GH3 was also conducted in bioreactor in order to apply strict pH control (Fig. 1). Two different equipment were used: a 1.5-L bioreactor (Minifors2; Infors-HT; Switzerland) with a working volume of 0.5 L (Reactor 0.5, R-0.5L) and a 42-L bioreactor (BIOSTAT Cplus 30–3 MO; Sartorius; Germany) with a working volume of 10 L (Reactor 10, R-10*L*). Fermentation was operated in batch mode at 32 °C and 150 rpm. *L. pentosus* was inoculated at an initial OD_{600} of 0.1. Strict anaerobic conditions were obtained by sparging with 0.5 L min^{-1} of nitrogen gas until the pO_2 sensor (HAMILTON; USA) detected<5% (v v^{-1}) of oxygen. The pH of GH3 was initially adjusted at 7.0 with NaOH 5 M and the compounds from MRS medium, except glucose, were added. pH was automatically maintained at 7 with NaOH 5 M along culture. Samples were taken at 0, 4, 6, 8, 24, 32, 48 and 72 h from R-0.5L while they were collected at 0, 4, 6, 24, 48 and 72 h from R-10L.

2.6. Analytical methods

Cell growth during preculture was estimated by measuring OD in a spectrophotometer (Spectroquant® Pharo 100) at 600 nm to determine inoculum size for fermentation. Cell growth was not measured in fermentation experiments because the addition of $CaCO_3$ for pH control impeded the correct cell growth determination by means of OD_{600} measurement.

The gas composition in clamped flasks was analysed by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (70 × 1/8″ O.D.) and a MS13X4-09SF2 40/60P packed column (9′ × 1/8″ O.D.) (PerkinElmer). pH was measured using a benchtop pH meter (Sension + PH31).

For determining sugars and fermentation products (LA and acetic acid), samples (1 mL) were taken from the fermentation broth and centrifuged at 14.000 g for 4 min. When fermentations in flasks were performed with $CaCO_3$, H_2SO_4 0.2 M was added to the samples to release the free LA from the calcium lactate (100 μL of H_2SO_4 2 M were added to 900 μL of sample). Then, samples were filtered using membrane filters (Thermo Scientific® Nylon 0.2 μm) and stored at −20 °C until analysis.

LA, acetic acid, glucose and xylose were quantified by HPLC (Agilent, Waldbronn, Germany) equipped with a refractive index detector with an Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) at 50 °C. Mobile phase was H_2SO_4 5 mM; flow rate was 0.5 mL min^{-1} and injection volume was 20 μL. Galactose, mannose and arabinose were quantified with a CARBOSep CHO-682 column (Transgenomic, Omana, NE) with the following conditions: column temperature, 80 °C; mobile phase, ultrapure water; flow rate, 0.35 mL min^{-1} and injection volume, 20 μL.

Furfural, 5-hydroxymethylfurfural (5-HMF) and formic acid were quantified with a Coregel 87H3 column (Transgenomic, San Jose, CA, USA). The operating temperature was 65 °C, and the

Table 1
Composition of the hemicellulosic mediums used in this study.

Composition (g L^{-1})	GP1	GP2	GP3
Glucose (potential)[1]	2.37 (6.80)	4.25 (8.09)	5.50 (6.40)
Xylose (potential)[1]	0.12 (4.60)	0.94 (4.83)	17.00 (17.00)
Galactose (potential)[1]	0.36 (2.30)	0.84 (2.61)	3.66 (4.70)
Arabinose (potential)[1]	1.05 (2.80)	2.86 (3.94)	3.89 (4.90)
Mannose (potential)[1]	0.55 (0.55)	0.55 (0.55)	0.90 (1.40)
Furfural	0.02	0.09	0.20
5-HMF	0.08	0.13	0.21
Acetic acid	0.65	0.42	1.15
Formic acid	0.61	0.17	0.23

[1] Potential sugars concentrations, determined by acid treatment, are indicated in brackets.

mobile phase was 89% (v v^{-1}) 5 mM H_2SO_4 and 11% (v v^{-1}) acetonitrile, with a flow rate of 0.7 mL min^{-1}. All these compounds were identified by a 1050 photodiode-array detector (Agilent, Waldbronn, Germany).

2.7. Calculations

The enzymatic hydrolysis yields (Y_{EH}) were calculated considering the initial sugars (IS) in monomeric form before hydrolysis, the concentration of final monomeric sugars (FS) at 72 h of hydrolysis, and the potential monomeric sugars (PS), as indicated hereafter:

$$Y_{EH} = [(FS - IS) \div (PS - IS)] \times 100$$

LA yield (Y, g g^{-1}) was defined as the LA produced (g L^{-1}) at 72 h of fermentation per initial sugars (g L^{-1}). The percentage yield of LA (% Y_{LA}) was calculated considering the stoichiometric theoretical yield in each case according to the initial sugars' composition of the medium. In this sense, 2 mol of LA can be produced per mol of hexoses via the EMP pathway, while 1 mol mol^{-1} is produced from pentoses by the PK pathway.

Microsoft Excel was used for the statistics analysis. The results are given as the average ± standard deviation for descriptive statistics. Analysis of variance (ANOVA) was carried out for comparisons of hydrolysis and fermentation data obtained in all the experiments. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Production of different gardening prehydrolysates by steam explosion pretreatment of gardening residues

Among the different steam explosion conditions tested to favour the release of hemicellulosic sugars from GRs, three conditions were selected to evaluate the effect of different catalysers (including $FeCl_3$ and H_2SO_4), catalysers concentrations and reaction times. The three resulting prehydrolysates presented different characteristics in terms of sugars and inhibitors concentrations (Table 1) and their susceptibility for enzymatic hydrolysis and LA production was subsequently evaluated for GP1/GP2, and for GP3, respectively.

Biomass impregnation with $FeCl_3$ and H_2SO_4 has previously demonstrated to improve pretreatment of different lignocellulosic materials, like olive tree biomass and wheat straw (Ballesteros et al., 2006; López-Linares et al., 2013). However, when $FeCl_3$ 0.1 M was added to catalyse gardening residues pretreatment, only 24% of monomeric sugars were detected in the resulting GP1 after 20 min of pretreatment (Fig. 1). On the other hand, when 0.25 M H_2SO_4 was used as catalyst in steam explosion at 180 °C for 5 min, 46% of the sugars were detected in monomeric form (Fig. 1), showing almost 2-fold increase in comparison with pretreatment with $FeCl_3$. The addition of H_2SO_4 as catalyst at high temperatures is known to produce a high solubilisation and hydrolysis of hemicellulose. As a matter of fact, almost complete solubilization of hemicellulose from wheat straw was attained with H_2SO_4-impregnated biomass at 180 °C for 10 min, reaching 85% of sugars recovery in the liquid fraction (Ballesteros et al., 2006). Aqueous extraction has been previously used before steam explosion pretreatment to reduce the amounts of extractives that could hinder pretreatment and enzymatic hydrolysis (Ballesteros et al., 2011). As expected, the efficiency of the process was further increased by applying an aqueous extraction, followed by steam explosion at 180 °C with 0.33 M H_2SO_4 for 10 min. As a result, 90% of sugars were present in monomeric form in GP3 (Fig. 1), corresponding with almost 2-fold increase in comparison with employing H_2SO_4 without water extraction.

Although the water extraction process prior to steam explosion and the higher H_2SO_4 concentration and pretreatment time resulted in the highest amount of monomeric sugars, these harsher conditions also promoted the release of furan derivatives like furfural and 5-HMF, which explains their higher concentrations in GP3 in comparison with GP2 (Table 1). Degradation compounds, including furans, weak acids and phenolic compounds, are able to preclude both enzymatic hydrolysis and fermentation by inhibiting the catalytic reactions and the growth of enzymes and microorganisms, respectively (Cubas-Cano et al., 2018). The addition of inorganic salts in steam explosion, especially in the case of $FeCl_3$, was reported to promote a huge xylose degradation to furfural (Liu and Wyman, 2006). At last furfural can be converted into formic acid (Tomás-Pejó et al., 2011), which could explain the higher formic acid concentration and the lower xylose and furfural content in GP1 when compared with H_2SO_4-catalysed pretreatments (Table 1).

In essence, among the inhibitors concentrations found in the three different prehydrolysates, it must be highlighted that GP1 contained higher concentration of carboxylic acids (1.26 g L^{-1}) than furans derivatives (0.1 g L^{-1}). On the other hand, GP2 contained lower acids concentration (0.65 g L^{-1}) and higher furans concentrations (0.22 g L^{-1}) than GP1. Furthermore, the highest inhibitors content was obtained in GP3, including both acids

Table 2
Enzymes dosages used in different tests for GP1 and GP2 enzymatic hydrolysis.

Material	Test	*Cel* (FPU g^{-1})	*β-Glu* (IU g^{-1})	*C-HTec2* (%)	*β-Xyl* (*mg* g^{-1})
GP1	A	16	16	0	0
	B	16	16	1.5	0.1
	C	16[1]	16[1]	1.5	0.1
GP2	A	8	8	1.5	0.1
	B	12	12	1.5	0.1
	C	16	16	1.5	0.1
	D	16	16	1.5	0.05
	E	16	16	1.5	0.5

[1] Cel and *β-Glu* were added 24 h after the addition of *C-HTec2* and *β-Xyl*.

(1.38 g L^{-1}) and furans (0.41 g L^{-1}) (Table 1). The different concentrations of degradation compounds in these prehydrolysates could be decisive for the following enzymatic hydrolysis and LA fermentation processes, showing significant differences between the materials.

Considering that GP3 contained more than 30 g L^{-1} of monomeric sugars ready for fermentation (90% of total sugars) (Table 1), this material did not require further hydrolytic steps and was directly used for LA production as GH3. As a matter of fact, high concentrations of monosaccharides were released in the resulting hemicellulosic prehydrolysate after wet explosion of pear trees branches, reaching even 35 g L^{-1} of pentoses and 1.83 g L^{-1} of hexoses, which were similar results than the ones obtained in GP3 in the present work. However higher inhibitors concentrations were produced during wet oxidation, both in terms of furans (0.63 g L^{-1}) and carboxylic acids (1.72 g L^{-1}) (Njoku et al., 2013).

Conversely, since most of sugars in GP1 and GP2 were in oligomeric form, different experiments were carried out to optimize the enzymatic hydrolysis conditions for these prehydrolysates

3.2. Optimization of enzymatic hydrolysis of gardening prehydrolysates for lactic fermentation

3.2.1. Combined effect of cellulases and hemicellulases: Simultaneous and sequential addition of enzymes

The addition of cellulases and hemicellulases (Fig. 2B) to GP1 resulted in a significant 19% increase in glucose Y_{EH} when compared with only cellulases (p-value < 0.05) (Fig. 2**A**). Furthermore, it also implied a significant 28% increase in xylose and arabinose Y_{EH}. (p-values < 0.05) (Fig. 2**B**). In fact, 5.4 and 3.6 g L^{-1} of glucose and xylose, respectively, were detected in GH1 after 72 h, when adding both hemi- and cellulases (Fig. 3**B**), while only 4.9 and 2.7 g L^{-1} of these monosaccharides were detected in the medium with just cellulases (Fig. 3**A**). The improved glucose Y_{EH} when adding *C-HTec2* and *β-Xyl* to the enzyme mixture could be due to the cellulolytic activity present in *C-HTec2* as indicated by the supplier. Moreover, high concentrations of xylooligosaccharides have been reported to competitively inhibit cellulose hydrolysis by mimicking its structure and binding to the catalytic site of cellulases (Dondelinger et al., 2016; Qing and Wyman, 2011). In this context, xylose may exert lower inhibitory effect on cellulases than xylooligosaccharides and thus the addition of the hemicellulases *C-HTec2* and *β-Xyl* could increase glucose yields. Indeed, 27% improvement in glucan conversion was reported when supplementing cellulases with xylanase and β-xylosidase in the enzymatic hydrolysis of pretreated corn stover (Qing and Wyman, 2011). This phenomenon was also reported with steam-exploded corn stover in which case cellulose and xylan Y_{EH} were improved by a partial replacement of cellulases with xylanase without increasing enzyme loading (Hu et al., 2011)

When GP1 was hydrolysed by adding cellulases 24 h after xylanases 64%, 70%, 9% and 59% of total glucose, xylose, galactose and arabinose were released, respectively (Fig. 2**C**). In this case, hydrolysis yields at 72 h were similar to the simultaneous addition of all enzymes (Fig. 2B). Indeed, no significant differences were shown in xylose Y_{EH} (p-value > 0.05). However, release of xylose and arabinose were significantly higher in the first hours of hydrolysis when adding hemicellulases and cellulases simultaneously (Fig. 3**B**). Indeed, 3 g L^{-1} of xylose were measured at 24 h when cellulases and xylanases were added simultaneously (Fig. 3**B**), while only 2 g L^{-1} were present at the same time point when cellulases were added 24 h after xylanases (Fig. 3**C**). Therefore, even though Y_{EH} was not affected by addition sequence of the enzymes, higher hydrolysis rates were shown when cellulases and hemicellulases were added simultaneously.

3.2.2. Screening of different cellulolytic and hemicellulolytic enzymes dosages

In order to determine the optimum conditions to release the monomeric sugars, different enzymes dosages were tested for the hydrolysis of GP2 (Table 2**)**.

The effect of increasing the cellulases dosages was studied in GP2 (tests A, B and C). As expected, higher Y_{EH} of hexoses was obtained when increasing the dosage of cellulases. Indeed, glucose Y_{EH} increased from 66% **(**Fig. 4**A)** to 70% **(**Fig. 4**B)** and even to 82% **(**Fig. 4**C)**, when the cellulases dosages were increased from 8 FPU g^{-1} of *Cel* and 8 IU g^{-1} of *β-Glu* up to 16 FPU g^{-1} of *Cel* and 16 IU g^{-1} of *β-Glu*. Xylose Y_{EH} was also enhanced when increasing the dosage of *Cel* and *β-Glu,* switching from around 75% (Fig. 4**A, B)** to almost 90% (Fig. 4**C)**.

The effect of increasing *β-Xyl* loading at the highest cellulases dosages (16 FPU g^{-1} of *Cel* and 16 IU g^{-1} of *β-Glu*) in GP2 was also studied (Fig. 4 **D**, **E** and **C**). A positive effect on glucose Y_{EH} was observed when increasing *β-Xyl* loading. Glucose Y_{EH} increased from 75% (Fig. 4**D**) to 82% (Fig. 4**C**) and to 85% (Fig. 4**E**) when *β-Xyl* loading was 0.05, 0.1 and 0.5 mg g^{-1}, respectively. In Fig. 2 **C, D** and **E**, 8%, 10% and 23% of galactose was released, following the same trend than glucose when increasing the *β-Xyl* dosage. Significantly, higher xylose and arabinose hydrolysis was clearly produced when increasing the concentration of *β-Xyl*. Xylose Y_{EH} was 72% with 0.05 mg g^{-1} of *β-Xyl* (Fig. 4**D**), while 90% of xylose Y_{EH} was reached with 0.1 and 0.5 mg g^{-1} of this enzyme (Fig. 4**C, E)**. In the case of arabinose, no hydrolysis was shown with 0.05 mg g^{-1} of *β-Xyl* (Fig. 4**D**). However, when increasing the enzyme content to 0.1 mg g^{-1}, arabinose Y_{EH} was 46% (Fig. 4**C**) and even reached 100% when adding 0.5 mg g^{-1} of *β-Xyl* (Fig. 4**E**).

The capacity of *β-Xyl* in releasing both arabinose and xylose has been previously reported, demonstrating the bifunctionality of this kind of enzymes, which not only presents β-xylosidase activity, but also α-arabinosidase. In fact, the β-xylosidase from *Paenibacillus woosongensis* was able to hydrolyse the monomeric xylose and arabinose from paranitrophenyl-β-xylopyranoside and paranitrophenyl-α-arabinofuranoside, respectively, reaching high specific activity in both cases (Kim and Yoon, 2010). In the case of the enzyme used in this study, the β-xylosidase from *Selenomonas ruminantium*, it was shown to catalyse the hydrolysis of 4-nitrophenyl-β-D-xylopyranoside with 10-fold preference than the 4-nitrophenyl-α-L-arabinofuranoside (Jordan et al., 2007).

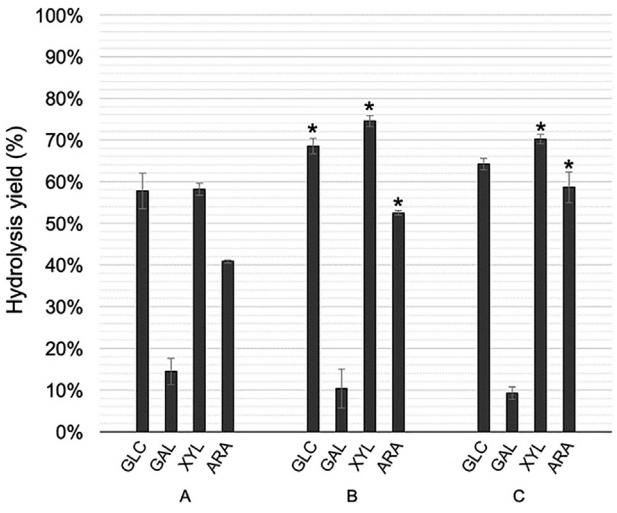

Fig. 2. Hydrolysis yields obtained in the hydrolysis of GP1 using different enzyme dosages and addition strategies. The mean difference with the reference (Fig. 2A) is significant at p-value < 0.05 (*).

As mentioned before, degradation inhibitors formed during steam explosion are known to affect hydrolytic enzymes (García-Aparicio et al., 2006; Zhai, et al., 2018). In this sense, different pretreatment conditions for GP1 ($FeCl_3$-catalyzed) and GP2 (H_2SO_4-catalyzed) led to different concentration of degradation compounds (Table 1). As it can be seen, higher Y_{EH} were reached with GP2 than with GP1 at the same enzymes dosages (Figs. 2**B** and 4**C**). In this case, considering the whole sugars content, 61% of overall enzymatic hydrolysis yield was obtained from GP1 (Fig. 2**B**), while 78% was obtained from GP2 (Fig. 4**C**). This difference in Y_{EH} could be related with the higher formic acid concentration in GP1. Even though acetic acid concentration was higher than formic acid in both GP1 and GP2, the stronger polar groups and the smaller molecular weight of the formic acid could explain its stronger hydrolysis inhibition (Jung and Kim, 2017). In fact, among different potential inhibiting compounds commonly found in wheat straw hydrolysates, formic acid presented the higher inactivation of cellulolytic and hemicellulolytic enzymes (Panagiotou and Olsson, 2007).

3.3. Effect of pH control and inhibitory compounds on LA fermentation

GP3 presented higher sugars concentration (31 g L^{-1}) than GP1 and GP2, especially xylose. Since most of the sugars in GP3 were in monomeric form, it did not require enzymatic hydrolysis and directly corresponded to GH3 (Table 1). As discussed before, this material also contained higher amount of furfural, 5-HMF and acetic acid than GP1 and GP2.

As mentioned, the toxicity of the acids (present in the hydrolysate or produced during fermentation) increased at low pH values. For this reason, fermentation tests were carried out with different pH control methods and working volumes, as shown in Fig. 5. Glucose, mannose, galactose and arabinose were totally consumed in the first 24 h (Sup. Table 1) in all cases. When fermentation was carried out in flask without $CaCO_3$, only 29% of total xylose was consumed in 72 h. Indeed, 11.5 g L^{-1} of acetic acid and 15 g L^{-1} of LA were produced (Fig. 5), corresponding to 66% Y_{LA} (Table 3). In contrast, 74% of xylose was consumed in flask-$CaCO_3$ at 72 h, reaching 18.5 and 13.6 g L^{-1} of LA and acetic acid, respectively (Fig. 5). In this case, 82% Y_{LA} was achieved (Table 3), meaning 24% increase in LA yield. The highest LA concentrations and the maximum xylose consumption rates of this study were obtained in fermentation tests in bioreactor (R-0.5L and R-10*L*) with automatic pH adjustment, in which cases, xylose was exhausted in 48 h, reaching 18 and 21 g L^{-1} of LA and 14–15 g L^{-1} of acetic acid (Fig. 5). As a result, LA yields between 88% and 95% of the theoretical yield were achieved. Thus, compared to flask fermentation, automatic NaOH addition offered a fitter pH control, with 33–44% improvement in Y_{LA}.

As mentioned, carboxylic acids are produced both as pretreatment-derived degradation compounds (acetic and formic acid) and as fermentation products of the PK pathway (acetic acid and LA) (Cubas-Cano et al., 2018). As a result, when the culture pH drops to values lower than the pKa of these acids, they become able to cross the cell membrane in their undissociated form. When these acids dissociate back inside the cell, energetic supplies are arrested to pump protons out, which culminate in cytosol acidification, denaturation of proteins, membrane disruption and, finally, cell death (Tomás-Pejó et al., 2011; Trcek et al., 2015). Acetic acid has been reported to produce a slight growth inhibition on LAB, while formic acid has demonstrated a high toxic effect on these bacteria at lower concentrations than other organic acids (van der Pol et al., 2016b). Thus, the presence of organic acids, including LA, acetic and formic acid, exert an inhibitory effect on *L. pentosus* growth and fermentation when the pH is not controlled.

In this study, the most appropriated pH control method was the automatic addition of NaOH 5 M in R-0.5L and R-10L, in which cases, pH was maintained at 7.0 along fermentation (Fig. 5**D**). As expected, a marked pH drop (from 7.0 to 4.5 in 24 h) took place when the pH was not controlled in flask fermentation (Fig. 5**D**). In the case of fermentation in flask with $CaCO_3$, pH dropped to 5.0 and 4.7 in 24 h and 72 h, respectively. Although addition of $CaCO_3$ improved LA fermentation in flask, LA yields and productivities were considerably lower than in bioreactor because xylose depletion took longer. Furthermore, $CaCO_3$ addition for LA neutralization and recovery at industrial scale has several drawbacks. In fact, additional operations are always required to dissociate LA from calcium lactate, consuming high amounts of H_2SO_4 in the process and releasing to the environment high amounts of gypsum ($CaSO_4$) as solid waste (Singhvi et al., 2018). For this reason, the calcium lactate method is not desirable for an environmentally friendly LA large-scale production and other novel methods are being developed to avoid its use, like obtaining new acid-resistant LAB (Cubas-Cano et al., 2018; Singhvi et al., 2018).

As it can be seen in Table 3, the highest LA yields from hemicellulosic hydrolysates were always attained in bioreactor, with pH controlled conditions by NaOH or $Ca(OH)_2$ automatic addition. For instance, fermentation of inhibitors-rich wheat straw, sugarcane bagasse and oil palm empty fruit bunch hydrolysate yielded 92%, 87% and 97% of the theoretical yield, respectively, with different *Bacillus coagulans* strains (Aulitto et al., 2017; van der Pol, et al., 2016a; Ye et al., 2014) (Table 3). These results were very similar to

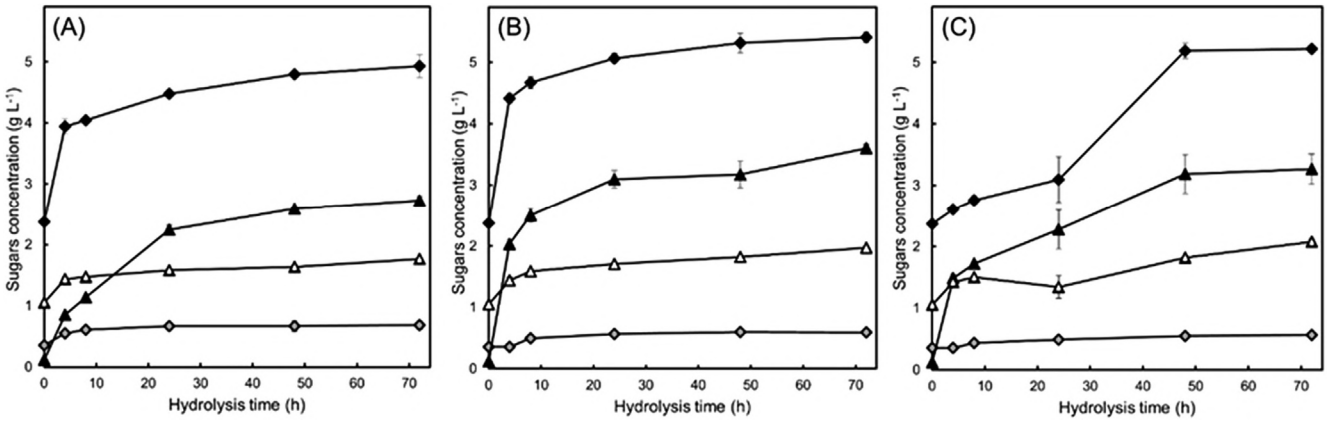

Fig. 3. Hydrolysis time courses of GP1. Glucose (◆), galactose (◈), xylose (▲), arabinose (△). A) only cellulases, B) cellulases and hemicellulases, C) hemicellulases added 24 h before cellulases.

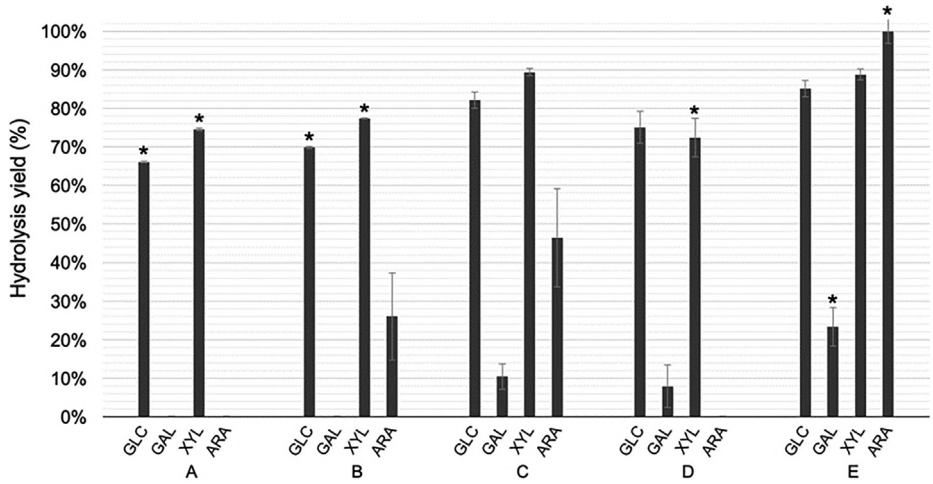

Fig. 4. Hydrolysis yields obtained in the hydrolysis of GP2 using different enzyme dosages. The mean difference with the reference (Fig. 4C) is significant at p-value < 0.05 (*).

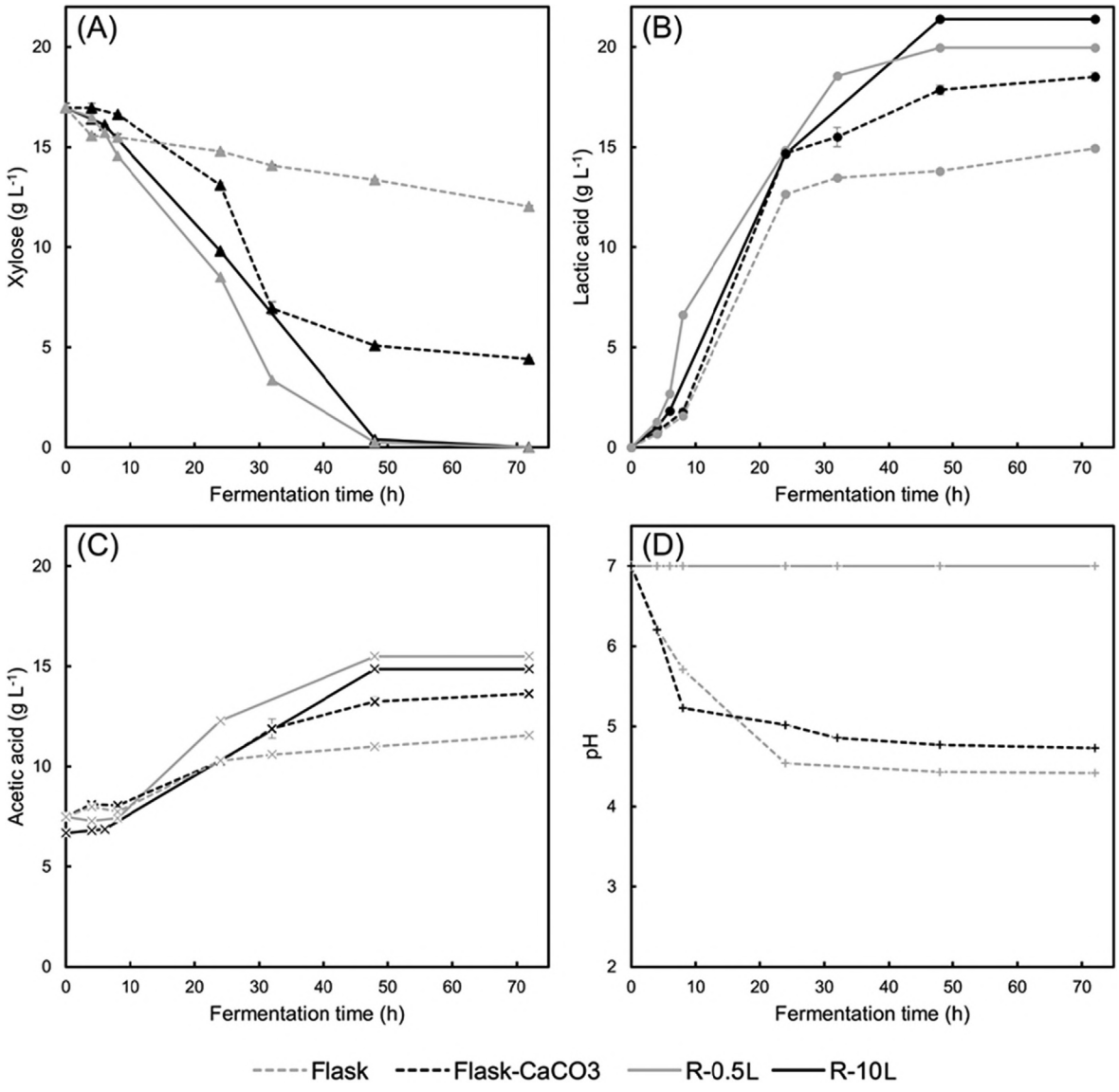

Fig. 5. Fermentation time courses for GH3 with different pH control. Xylose consumption (A), LA production (B), acetic acid production (C) and culture pH (D) of different conditions regarding pH control. Flask fermentation with no pH control (Grey dashed line), Flask fermentation with $CaCO_3$ (black dashed line), 0.5L-reactor with automatic pH control (continuous grey line), 10*L*-reactor with automatic pH control (continuous black line).

Table 3
Effect of pretreatment-derived inhibitors and pH control on LA production from agricultural hemicellulosic hydrolysates by LAB in literature.

Material	Inhibitors ($g\ L^{-1}$)		pH control	Y_{LA} ($g\ g^{-1}$)	$\%Y_{LA}$	Strain	Reference
Gardening hydrolysate (GH3)	Furfural	0.20	No	0.48	66	*L. pentosus* CECT4023T	This study
	5-HMF	0.21	$CaCO_3$	0.60	82		
	Acetic acid	1.15	NaOH (R-0.5)	0.65	88		
	Formic acid	0.23	NaOH (R-10)	0.70	95		
Trimming vine shoots hydrolysate	Furfural	1.34	$CaCO_3$	0.46	67	*L. pentosus* CECT4023T	Bustos et al. (2005)
	5-HMF	0.11					
	Acetic acid	19.40					
Softwood prehydrolysate	Furfural	1.41	$CaCO_3$	–	60	*L. rhamnosus* ATCC-10863	Shi et al. (2015)
	5-HMF	1.02					
	Acetic acid	2.61					
	Levulinic acid	0.20					
Wheat straw hydrolysate	Furfural	4.0	NaOH	0.92	92	*B. coagulans* MA-13	Aulitto et al. (2017)
	5-HMF	1.4					
	Acetic acid	3.8					
Sugarcane bagasse hydrolysate	Furfural	1.00	$Ca(OH)_2$	0.87	87	*B. coagulans* DSM2314	van der Pol et al. (2016b)
	Acetic acid	2.00					
	Formic acid	0.05					
	Glycolyc acid	0.17					
	Coumaric acid	0.06					
	Vanillin	0.02					
Oil palm empty fruit bunch hydrolysate	Furfural	0.41	$Ca(OH)_2$	0.97	97	*B. coagulans* JI12	Ye et al. (2014)
	5-HMF	0.16					
	Acetic acid	18.39					

the ones obtained in the present work (88–95% Y_{LA}) from GH3 in bioreactor (R-0.5L and R-10*L*) (Table 3). However, lower yields (60 and 67% Y_{LA}) were obtained in fermentation of inhibitors-rich trimming vine shoots and softwood hydrolysates with different *Lactobacillus* strains when using $CaCO_3$ to control the pH (Bustos et al., 2005; Shi et al., 2015) (Table 3). Indeed, it should be noticed that, in spite of the presence of inhibitors, when controlling the culture pH, complete sugars consumption was achieved, reaching almost the theoretical maximum of LA (Fig. 5). This fact confirmed the importance of having an accurate pH control method in LA fermentation to avoid the toxic effect of organic acids, including the ones formed during both pretreatment and fermentation.

From a biorefinery point of view, valorisation of the whole sugar content from lignocellulose can be achieved by using the different fractions produced after steam explosion for the production of different biofuels and bio-based products, without wasting any carbon source contained in the materials. Cellulose-rich WIS fraction can be used for the production of a high variety of products because almost all the industrial relevant microorganisms are able to ferment glucose, but the use of the hemicellulose-rich liquid fractions needs to be optimised. Considering the high LA yields obtained by *L. pentosus* CECT 4023 T in the present work, the suitability of this strain to be used for fermentation of different C5-sugars rich hemicellulosic liquid fractions derived from renewable sources has been demonstrated.

4. Conclusions

Gardening of urban green areas offer high amounts of underutilized polysaccharides-rich organic wastes that can be directed to LA production instead of their traditional and low added-value applications. In this work, the application of different pretreatment conditions and the use of different dosages of cellulases and hemicellulases resulted in an efficient release of both C6 and C5 sugars from gardening prehydrolysates. The detrimental effect caused by the pH drop during LA and acetic acid production appeared to be critical, increasing the damaging effect of organic acids and precluding total xylose consumption. Nevertheless, improved LA yields were obtained in bioreactor when fermenting gardening hydrolysates with proper pH control by NaOH addition in comparison with flask and flask-$CaCO_3$ fermentation. The tolerance of the bacteria to low pH could be increased in future works in order to reduce fermentation costs associated with the addition of neutralizers. *L. pentosus* CECT4023T has showed its suitability to be used on fermentation of gardening hemicellulosic hydrolysates, without being affected by lignocellulosic degradation compounds. This approach could be successfully combined with WIS fraction utilisation, producing different biofuels and bio-based products from different carbon sources in a lignocellulosic biorefinery. Having in mind the abundance of gardening residues and the increasing demand of bio-based chemicals, future efforts may be focused in this research line, taking advantage of these wastes and promoting their complete valorisation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Aulitto, M., Fusco, S., Bartolucci, S., Franzen, C.J., Contursi, P., 2017. *Bacillus coagulans* MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. Biotechnol. Biofuels 10, 210. https://doi.org/10.1186/s13068-017-0896-8.

Ballesteros, I., Negro, M.J., Oliva, J.M., Cabañas, A., Manzanares, P., Ballesteros, M., 2006. Ethanol production from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 129–132, 496–508. https://doi.org/10.1385/abab:130:1:496.

Ballesteros, I., Ballesteros, M., Cara, C., Sáez, F., Castro, E., Manzanares, P., Negro, M.J., Oliva, J.M., 2011. Effect of water extraction on sugars recovery from steam exploded olive tree pruning. Bioresour. Technol. 102 (11), 6611–6616. https://doi.org/10.1016/j.biortech.2011.03.077.

Boldrin, A., Christensen, T.H., 2010. Seasonal generation and composition of garden waste in Aarhus (Denmark). Waste Manage. 30 (4), 551–557. https://doi.org/10.1016/j.wasman.2009.11.031.

Bolivar-Telleria, M., Turbay, C.L.F., Tarcio, C., de Ronaldo, S.B., Fernandes, A.A.R., Santos, A.M.C., Fernandes, P.M.B., 2018. Second-generation bioethanol from coconut husk. Biomed. Res. Int. 4916497. https://doi.org/10.1155/2018/4916497.

Bustos, Guadalupe, Moldes, Ana Belén, Cruz, José Manuel, Domínguez, José Manuel, 2005. Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. Biotechnol. Prog. 21 (3), 793–798. https://doi.org/10.1021/(ISSN)1520-603310.1021/bp049603v.

Cubas-Cano, Enrique, González-Fernández, Cristina, Ballesteros, Mercedes, Tomás-Pejó, Elia, 2019. *Lactobacillus pentosus* 4023 T co-utilizes glucose and xylose to produce lactic acid from wheat straw hydrolysate: Anaerobiosis as a key factor. Biotechnol. Prog. 35 (1), e2739. https://doi.org/10.1002/btpr.2739.

Cubas-Cano, Enrique, González-Fernández, Cristina, Ballesteros, Mercedes, Tomás-Pejó, Elia, 2018. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate: Lactic acid production from lignocellulose: Biotechnological advances. Biofuels Bioprod. Bioref. 12 (2), 290–303. https://doi.org/10.1002/bbb.2018.12.issue-210.1002/bbb.1852.

Cubas-Cano, E., López-Gómez, J.P., González-Fernández, C., Ballesteros, I., Tomás-Pejó, E., 2020. Towards sequential bioethanol and l-lactic acid co-generation: Improving xylose conversion to l-lactic acid in presence of lignocellulosic ethanol with an evolved *Bacillus coagulans*. Renew. Energy 153, 759–765. https://doi.org/10.1016/j.renene.2020.02.066.

Dondelinger, E., Aubry, N., Chaabane, F.B., Cohen, C., Tayeb, J., Rémond, C., 2016. Contrasted enzymatic cocktails reveal the importance of cellulases and hemicellulases activity ratios for the hydrolysis of cellulose in presence of xylans. AMB Express 6 (1), 24. https://doi.org/10.1186/s13568-016-0196-x.

García-Aparicio, M.P., Ballesteros, I., González, A., Oliva, J.M., Ballesteros, M., Negro, M.J., 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl. Biochem. Biotechnol. 129 (1), 278–288. https://doi.org/10.1385/ABAB:129:1:278.

Hu, C., Chi, D., Chen, S., Chia Chen, Y., 2011. The direct conversion of xylan to lactic acid by *Lactobacillus brevis* transformed with a xylanase gene. Green Chem. 13 (7), 1729–1734. https://doi.org/10.1039/c1gc15169j.

Ji, X.J., Huang, H., Nie, Z.K., Qu, L., Xu, Q., Tsao, G.T., 2012. Fuels and chemicals from hemicellulose sugars. Adv. Biochem. Eng. Biotechnol. 128, 199–224. https://doi.org/10.1007/10_2011_124.

Jordan, D.B., Li, X.L., Dunlap, C.A., Whitehead, T.R., Cotta, M.A., 2007. Beta-D-xylosidase from *Selenomonas ruminantium* of glycoside hydrolase family 43. Appl. Biochem. Biotechnol. 137–140 (1–12), 93–104. https://doi.org/10.1007/s12010-007-9042-6.

Jung, Y.H., Kim, K.H., 2017. Evaluation of the main inhibitors from lignocellulose pretreatment for enzymatic hydrolysis and yeast fermentation. Bioresearch 12 (4), 9348–9356.

Kim, Y.A., Yoon, K.H., 2010. Characterization of a *Paenibacillus woosongensis* beta-Xylosidase/alpha-Arabinofuranosidase produced by recombinant *Escherichia coli*. J. Microbiol. Biotechnol. 20 (12), 1711–1716.

Liu, C., Wyman, C.E., 2006. The enhancement of xylose monomer and xylotriose degradation by inorganic salts in aqueous solutions at 180 °C. Carbohydr. Res. 341 (15), 2550–2556. https://doi.org/10.1016/j.carres.2006.07.017.

López-Linares, J.C., Romero, I., Moya, M., Cara, C., Ruiz, E., Castro, E., 2013. Pretreatment of olive tree biomass with $FeCl_3$ prior enzymatic hydrolysis. Bioresour. Technol. 128, 180–187. https://doi.org/10.1016/j.biortech.2012.10.076.

Martín, C., de Moraes Rocha, G.J., Alves dos Santans, J.B., de Albuquerque Wanderley, M.C., Gowela, E.R., 2012. Enzyme loading dependence of cellulose hydrolysis of sugarcane bagasse. Quim. Nova 35 (10), 1927–1930.

Míguez, J.L., Moran, J.C., Granada, E., Porteiro, J., 2012. Review of technology in small-scale biomass combustion systems in the European market. Renew. Sustain. Energy Rev. 16 (6), 3867–3875. https://doi.org/10.1016/j.rser.2012.03.044.

Njoku, S.I., Iversen, J.A., Uellendahl, H., Ahring, B.K., 2013. Production of ethanol from hemicellulose fraction of cocksfoot grass using *Pichia stipitis*. Sustain. Chem. Process. 1, 13. https://doi.org/10.1186/2043-7129-1-13.

Panagiotou, G., Olsson, L., 2007. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol. Bioeng. 96 (2), 250–258. https://doi.org/10.1002/bit.21100.

Pleissner, D., Dietz, D., van Duuren, J.B.J.H., Wittmann, C., Yang, X., Lin, C.S.K., Venus, J., 2017. Biotechnological production of organic acids from renewable resources. Adv. Biochem. Eng. Biotechnol. 166, 373–410. https://doi.org/10.1007/10_2016_73.

Qing, Q., Wyman, C.E., 2011. Supplementation with xylanase and β-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. Biotechnol. Biofuels 4 (1), 18. https://doi.org/10.1186/1754-6834-4-18.

Robak, K., Balcerek, M., 2018. Review of second generation bioethanol production from residual biomass. Food Technol. Biotechnol. 56 (2), 174–187 https://doi.org/10.17113/ftb.56.02.18.5428.

Sasaki, C., Okumura, R., Asada, C., Nakamura, Y., 2014. Steam explosion treatment for ethanol production from branches pruned from pear trees by simultaneous saccharification and fermentation. Biosci. Biotechnol. Biochem. 78 (1), 160–166. https://doi.org/10.1080/09168451.2014.877818.

Shi, S., Kang, L., Lee, Y.Y., 2015. Production of lactic acid from the mixture of softwood pre-hydrolysate and paper mill sludge by simultaneous saccharification and fermentation. Appl. Biochem. Biotechnol. 175 (5), 2741–2754. https://doi.org/10.1007/s12010-014-1451-8.

Shi, Y., Ge, Y., Chang, J., Shao, H., Tang, Y., 2013. Garden waste biomass for renewable and sustainable energy production in China: potential, challenges and development. Renew. Sustain. Energy Rev. 22, 432–437. https://doi.org/10.1016/j.rser.2013.02.003.

Singhvi, M., Zendo, T., Sonomoto, K., 2018. Free lactic acid production under acidic conditions by lactic acid bacteria strains: challenges and future prospects. Appl. Microbiol. Biotechnol. 102 (14), 5911–5924. https://doi.org/10.1007/s00253-018-9092-4.

Sweeney, M.D., Xu, F., 2012. Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2 (2). https://doi.org/10.3390/catal2020244.

Tetma, 2018. http://tetma.com/2018/03/28/tetma-y-centreverd-siguen-con-su-compromiso- (accessed 22 March 2020).

Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion. In Biofuels, Alternative Feedstocks and Conversion Processes. Pandey, A., Larroche, C., Ricke, S.C., Dussap, C., Gnansounou, E. (Eds). pp., 149-176.

Trcek, J., Mira, N.P., Jarboe, L.R., 2015. Adaptation and tolerance of bacteria against acetic acid. Appl. Microbiol. Biotechnol. 99 (15), 6215–6229. https://doi.org/10.1007/s00253-015-6762-3.

van der Pol, E.C., Eggink, G., Weusthuis, R.A., 2016a. Production of l(+)-lactic acid from acid pretreated sugarcane bagasse using *Bacillus coagulans* DSM2314 in a simultaneous saccharification and fermentation strategy. Biotechnol. Biofuels 15 (9), 248. https://doi.org/10.1186/s13068-016-0646-3.

van der Pol, E.C., Vaessen, E., Weusthuis, R.A., Eggink, G., 2016b. Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small-scale screening method. Bioresour. Technol. 209, 297–304. https://doi.org/10.1016/j.biortech.2016.03.037.

Ye, L.D., Bin Hudari, M.S., Li, Z., Wu, J.C., 2014. Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid production by *Bacillus coagulans* JI12. Biochem. Eng. J. 83, 16–21. https://doi.org/10.1016/j.bej.2013.12.005.

Zhai, R., Hu, J., Saddler, J.N., 2018. Extent of enzyme inhibition by phenolics derived from pretreated biomass is significantly influenced by the size and carbonyl group content of the phenolics. ACS Sustain. Chem. Eng. 6 (3), 3823–3829. https://doi.org/10.1021/acssuschemeng.7b04178.

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Assessment of different *Bacillus coagulans* strains for L-lactic acid production from defined media and gardening hydrolysates: Effect of lignocellulosic inhibitors

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ABSTRACT

Cellulose valorisation has been successfully addressed for years. However, the use of hemicellulosic hydrolysates is limited due to the presence of C5-sugars and inhibitors formed during pretreatment. *Bacillus coagulans* is one of the few bacteria able to utilize both C6- and C5-sugars to produce L-lactic acid, but its susceptibility to the lignocellulosic inhibitors needs further investigation. For such a purpose, the tolerance of different *B. coagulans* strains to increasing concentrations of inhibitors is studied. The isolated A162 strain reached the highest L-lactic acid productivity in all cases (up to 2.4 g L^{-1} h^{-1}), even in presence of 5 g L^{-1} of furans and phenols. Remarkably, most of furans and phenolic aldehydes were removed from defined media and hemicellulosic gardening hydrolysate after fermentation with A162. Considering the high productivities and the biodetoxifying effect attained, A162 could be pointed out as a great candidate for valorisation of mixed sugars from hemicellulosic hydrolysates with high inhibitors concentration, promoting the implementation of lignocellulosic biorefineries.

1. Introduction

Lactic acid (LA) is a chemical compound with a wide number of industrial applications (Cubas-Cano et al., 2018). LA can be produced by chemical synthesis, using fossil resources and producing a racemic mixture (Pleissner et al., 2017). Conversely, throught the biotechnological route, some bacteria are able to use sugar-rich lignocellulosic substrates to produce optically pure isomers, which present higher added-value. L-LA isomer can be used as a monomer for poly-lactic acid (PLA), an attractive biopolymer for the production of petrochemical alternative plastics (Cubas-Cano et al., 2018). Furthermore, it can be used for food industry applications (Juturu and Wu, 2015; Klotz et al., 2016), which make it the preferable isomer in bioproduction.

Lignocellulosic biomass is widely distributed and does not compete with food crops, which makes it an attractive source of sugars for the biotechnological production of LA by promoting the implementation of circular economy principles. Lignocellulosic biomass is formed by two carbohydrate polymers rich in fermentable sugars—cellulose and hemicellulose—and lignin. Cellulose is composed of glucose molecules and hemicellulose mainly consists of non-cellulosic sugars such as xylose, mannose, galactose, arabinose, etc. (Tomás-Pejó et al., 2011). Due to the structural characteristics of lignocellulose, a pretreatment is necessary to favour the release of sugars (Tomás-Pejó et al., 2011). However, when the raw material is subjected to harsh conditions inherent to pretreatment, a partial cellulose, hemicellulose and lignin degradation may take place. This fact could imply the formation of different inhibitory compounds that can affect bacterial growth and LA production (Alves de Oliveira et al., 2018) These soluble degradation compounds are usually classified in three groups: furans, carboxylic acids and phenols.

Furans, like furfural and 5-hydroxymethilfurfural (5-HMF), are formed after dehydration of xylose and glucose, respectively (Jönsson and Martín, 2016). These compounds are able to cause membrane damage and interfere with the glycolytic enzymes and the synthesis of macromolecules, provoking an extended lag phase (Tomás-Pejó et al., 2011). Different studies have also verified their correlation with reactive oxygen species fomation, which can produce oxidative damage to enzymes, organelles and chromatine (Allen et al., 2010; van der Pol et al., 2016). The formation of carboxylic acids is produced due to the release of acetyl groups (acetic acid) from hemicellulose scaffold and also after degradation of furans (levulinic and formic acid) (Tomás-Pejó et al., 2011). These acids are able to cross the cell membrane and

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produce cytosol acidification, leading to protein denaturation, membrane lysis and cell death (Trcek et al., 2015). Phenolic compounds are originated during depolymerisation and degradation of lignin. For instance, vanillin and syringadelhyde are formed by degradation of the guaiacylpropane and syringyl propane units, respectively (Palmqvist and Hahn-Hägerdal, 2000). Phenols can affect membrane permeability and functionality, affecting the maintenance of the pH homeostasis due to the dissipation of ionic gradients (Almeida et al., 2007). Having in mind that different inhibitors can act synergistically (Oliva et al., 2004), it is of utmost importance to assess the tolerance of LA-producing strains to different mixtures of these compounds when using lignocellulosic sugars for LA.

The valorisation of the cellulosic fraction of lignocellulose has been widely studied since glucose can be metabolised by most microorganisms. However, the utilisation of hemicellulosic fraction, is more difficult because of the presence of the mentioned inhibitory compounds and sugars mixtures (both C6 and C5) (Cubas-Cano et al., 2019). As a matter of fact, *Bacillus coagulans* has become one of the most popular bacterium used in research for LA production due to its capacity to metabolize C5 sugars via the pentoses phosphate (PP) pathway and to produce optically pure L-LA with high yields (Alexandri et al., 2019; Aulitto et al., 2017; Pleissner et al., 2016) These traits make *B. coagulans* an interesting candidate for the production of L-LA at industrial level in future biorefineries (Su and Xu, 2014).

The novelty of this work relies on the use of new isolated *B. coagulans* strains for fermentation of (i) defined media with inhibitors mixtures at high concentrations (ii) hemicellulosic gardening hydrolysate. This substrate has been used for bioethanol production (Njoku et al., 2013; Sasaki et al., 2014) and in LA production in a previous work (Cubas-Cano et al., 2020). The assessment of high inhibitors concentration in this study, could be crucial when high gravity processes from lignocellulose are targeted. Furthermore, most of the studies screening different LA-producing bacteria for their inhibitors tolerance are focused on analysing the effect on bacterial growth in microplates, without showing the outcomes in LA production and inhibitors biodetoxification (Boguta et al., 2014; van der Pol et al., 2016). The results presented hereunder imply a significant contribution to the knowledge about the tolerance and the biodetoxifying capacity of *B. coagulans* against the inhibitors formed during lignocellulose pretreatment, which highlights the suitability of these strains to efficiently convert hemicellulosic sugars into L-LA.

2. Materials & methods

2.1. Microorganisms and preculture

Three *B. coagulans* strains were tested for LA production. *B. coagulans* DSM 2314 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), while A166 and A162 strains were isolated from the Leibniz-Institute of Agricultural Engineering and Bioeconomy (Potsdam, Germany) (Sup. Table 1). These strains were characterised as Gram positive using Bactident Aminopeptidase 1.13301.0001 (Merck®, Germany) (Sup. Table 1). Catalase activity was measured using a Catalase Assay Procedure kit (Megazyme®, US) (Sup. Table 1). Spores formation, cell mobility and cell/colony morphologies were determined using an optical microscope (Olympus®, Japan) (Sup. Table 1).

The strains were identified as *B. coagulans* species using API 50 CHL method (BioMérieux®, US) and MALDI TOF. API 50 CHL allows the identification of the genus *Lactobacillus* and related genera by the fermentation of 49 carbohydrates. The method was performed at 52 °C for 24 h and 48 h in 10 mL of 50 CHL medium, containing (g L^{-1}): polypeptone, 10; yeast extract, 5; dipotassium phosphate, 2; sodium acetate, 5; diammonium citrate, 2; magnesium sulfate heptahydrate, 0.2; manganese sulfate monohydrate, 0.05; bromcresol purple, 0.17; with Tween 80, 0.1 % (v v^{-1}) (Sup. Table 2).

Table 1
Inhibitory composition of the defined and hemicellulosic media used in the screening for inhibitors tolerance assay.

Inhibitor (g L^{-1})	BM	BMI	BMI A 10	BMI A 20	BMI F 2.5	BMI F 5	BMI P 2.5	BMI P 5	GH
Acetic acid	0.00	1.00	**5.00**	**10.00**	1.00	1.00	1.00	1.00	1.15
Formic acid	0.00	1.00	**5.00**	**10.00**	1.00	1.00	1.00	1.00	0.23
Furfural	0.00	0.25	0.25	0.25	**1.25**	**2.50**	0.25	0.25	0.20
5-HMF	0.00	0.25	0.25	0.25	**1.25**	**2.50**	0.25	0.25	0.23
Vanillin	0.00	0.25	0.25	0.25	0.25	0.25	**1.25**	**2.50**	0.00
Syringaldehyde	0.00	0.25	0.25	0.25	0.25	0.25	**1.25**	**2.50**	0.00

For preculture, cells were inoculated from slants in 250-mL conic flasks with 60 mL of Man, Rogosa & Sharpe medium (MRS) (Merck®, Germany), containing (g L^{-1}): glucose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; dipotassium phosphate, 2; magnesium sulfate heptahydrate, 0.2; manganese sulfate monohydrate, 0.05; and 0.67 Everzit Dol (Evers®, Germany) as buffer. Precultures were incubated at 40 °C and 100 rpm for 16 h.

2.2. Hemicellulosic gardening hydrolysate

The gardening residues were firstly milled in a laboratory cutting mill (SM 2000, Retsch, Germany) to a size of 10 mm. Afterwards, an aqueous extraction was carried out at 130 °C for 20 min with a 1:10 solid:liquid (v v^{-1}) relation. The chips were then pretreated by steam explosion at 180 °C for 10 min in a 2-L reactor with H_2SO_4 (0.33 M, 60 mg g^{-1} biomass) impregnation (Cubas-Cano et al., 2020). After pretreatment, the hemicellulosic-gardening hydrolysate (GH) containing sugars and inhibitors was obtained after vacuum filtering the resulting pretreated material. The GH presented the following composition (g L^{-1}): glucose, 5; xylose, 15; arabinose, 3.4; furfural, 0.20; 5-HMF, 0.23; acetic acid 1.15; formic acid, 0.23 (Table 1).

2.3. Fermentation

LA fermentations of defined media and GH were carried out in Eloferm multifermentation system (Biotronix GmbH, Germany) with 250 mL of working volume. Culture conditions were chosen according to previous works (Alexandri et al., 2019). Fermentations tests were performed at 52 °C and 400 rpm for 24 h in duplicates. The culture pH was maintained at pH 6 by automatic addition of NaOH (5 M). 5 % (v v^{-1}) of inoculum volume was used. Samples were withdrawn at 0, 2 4, 6, 8, 10 and 24 h of culture.

For Basal Media (BM) fermentation, C5 and C6 sugars were added to reach the same sugar concentration of GH. 5 g L^{-1} of yeast extract were also added. Different concentrations of inhibitors, (*i.e.* acetic acid, formic acid, furfural, 5-HMF, vanillin and syringaldehyde (Merck, Germany)) were tested as indicated in Table 1. BM without inhibitors was used as control. BM with Inhibtiors (BMI) contained 1 g L^{-1} of each carboxylic acid and 0.25 g L^{-1} of each furan and phenolic aldehyde (Table 1). The concentration of inhibitors added in BMI were chosen taking into consideration the carboxylic acids and furans content in GH. This material did not contain phenolic aldehydes in spite of their abundance in other steam-exploded-lignocellulosic materials such as wheat straw (Cubas-Cano et al., 2019). Thus, due to their importance in lignocellulosic hydrolysates, syringaldehyde and vanillin were added at the same concentrations than furans. In order to check the effect on microbial growth and fermentation of increasing each of the inhibitors groups, different media were obtained by increasing 5- and 10-fold the concentrations of carboxylic acids, furans and phenolic aldehydes of BMI (Table 1). For GH fermentation, the same salts present in MRS used for the preinoculum growth were added to the material.

2.4. Analytical methods

Bacterial growth was determined by drying pre-weighted samples at 105 °C until constant weight.

LA, acetic acid, formic acid and sugars (glucose, xylose and arabinose) concentrations were measured by HPLC (DIONEX, USA). 10 μL of sample were added to the Eurokat H column (300 mm × 8 mm × 10 μm). H_2SO_4 (5 mM) was used as mobile phase, at a flow rate of 0.8 mL min^{-1}. Detection was carried out by a refractive index detector (RI-71, SHODEX, Japan). Phenols, furfural and 5-HMF were measured at the end of fermentation experiments with A162 to check possible biodetoxification effects. These compounds were determined by HPLC (DIONEX, USA) with a UV detector (280 nm). Ultrapure water (A) and acetonitrile 50 % (B) were used as eluents with a flow rate of 1 mL min^{-1}. The solvent composition was changed in several stages during the measurement (multistep-gradient).

The enantiomeric purity of the produced LA was also determined via HPLC (Dionex, USA), using a Phenomenex Chirex 3126 (150 9 4.6 mm ID, Phenomenex, USA) column, operating at 30 °C, with 1 mM Cu_2SO_4 as eluent at a flow rate of 1 mL min^{-1}. Detection was carried out with an ultraviolet detector. More than 99 % of L-LA purity was measured in all the samples.

2.5. Calculations

The specific growth rate (μ) was calculated as the increase in biomass per unit of time:

$\mu\ (h^{-1}) = \ln (x_2 / x_1)/(t_2 - t_1)$, where x is biomass (g L^{-1}) obtained at each sample time, t (h).

LA yield (Y_{LA}, g g^{-1}) was defined as the LA (g L^{-1}) produced at 24 h of culture per initial sugars (g L^{-1}). LA productivity (Q_{LA10h}) was calculated by taking into consideration the LA (g L^{-1}) produced at 10 h of fermentation.

Microsoft Excel was used for the statistics analysis. The results are given as the average ± standard deviation for descriptive statistics. Analysis of variance (ANOVA) was carried out for comparisons of hydrolysis and fermentation data obtained in all the experiments. The level of significance was set at $p < 0.05$.

3. Results & discussion

3.1. LA production in defined basal media with different concentrations of inhibitors

3.1.1. LA production in presence of inhibitors mixture

BMI (Table 1) was used to preliminary screen the tolerance of the *B. coagulans* strains to the tested degradation compounds.

According to the bacterial growth, a μ_{max} of 0.25 h^{-1} was obtained in BMI by *B. coagulans* DSM 2314, while significantly higher growth rates, around 0.32 h^{-1}, were reached by A166 and A162 (p-values < 0.05) (Fig. 1A). Since sugars were totally consumed in all cases at 24 h, the Y_{LA} were similar with the three strains, ranging from 0.81 to 0.87 g g^{-1} (Fig. 1B). Thus, the inhibitors concentrations tested in BMI did not cause a detrimental effect in any of the screened strains in terms of final LA concentration (Fig. 2).

However, high differences were found in LA productivities. The highest Q_{LA10h} in BMI were obtained by both A166 and A162 strains, between 2.1 and 2.2 g L h^{-1}, while DSM 2314 only produced 1.8 g L h^{-1} of LA from BMI (Fig. 1B) (p-values < 0.05). It is worth mentioning that, A166 and A162 strains were isolated from lignocellulosic media, i.e., hemp biomass during the fibre processing and washing water from a starch factory, respectively, which could be the reason for the higher growth and fermentation rates. By contrast, DSM 2314, as a collection strain, presented lower growth and fermentation rates when fermenting a medium with inhibitors. Different screening experiments have previously showed that yeast or bacterial strains isolated from selective media presented more fitted characteristcs than the reference collection strains (Bautista-Gallego et al., 2013; Demeke et al., 2013). For instance, different *Lactobacillus pentosus* strains isolated from table olives showed more promising probiotic and differentiated characteristics in comparison with the probiotic reference strains (Bautista-Gallego et al., 2013).

Based on the high robustness showed by these *B. coagulans* strains in BMI, without showing a significant reduction in growth rates and LA yields and productivities in comparision with BM, the concentration of each inhibitors groups was increased 5- or 10-fold to ascertain the maximum concentration of these compounds that could be tolerated in a hemicellulosic hydrolysate.

3.1.2. Effect of increasing the concentration of carboxylic acids

When increasing the carboxylic acids concentrations to 10 g L^{-1} (BMI-A10), μ_{max} and Q_{LA10h} around 0.3 h^{-1} and 2 g L h^{-1}, respectively, were obtained in all cases (Fig. 1). However, when 20 g L^{-1} acids were added (BMI-A20), lower μ_{max} (0.22 h^{-1}) were obtained with the three strains, being reduced by 20–37 % in comparison with BM (Fig. 1A). Although growth rates were reduced at high carboxylic acids concentrations (20 g L^{-1}), no effect on Q_{LA10h} and Y_{LA} (Fig. 1B) were shown in any of the screened strains when compared to BM. Van der Pol and co-workers previously demonstrated that 10 g L^{-1} of acetic and formic acid were able to reduce *B. coagulans* DSM 2314 growth by 26 and 51 %, respectively, in comparison with the reference culture without inhibitors, in 48-well plates without pH control (van der Pol et al., 2016). Although the concentration of carboxylic acids was higher in the present work (20 g L^{-1}), lower inhibitory effect on DSM 2314 growth rate was shown in comparison with the results of van der Pol and co-workers, which could be explained due to the pH control applied, maintaining the culture pH at 6 along fermentation.

pH is a quite relevant parameter in fermentative processes since it is well known that carboxylic acids are able to pass through the cell membrane in their undissociated form (Trcek et al., 2015). However, these compounds are in their undissociated form when the culture pH is below their pKa. Once inside, they dissociate back due to the higher pH, producing an accumulation of protons. The extra need of ATP to pump protons out of the cell reduces the energetic supply of the cell (growth inhibition) and, when this is overcome, the cytosol acidification lead to the denaturation of proteins (metabolism inhibition) and the cell death (Trcek et al., 2015).

In the current work, the pH was maintained at 6 along the fermentation, and, presumably, most of acetic acid (pKa = 4.8), formic acid (pKa = 3.75) and LA (pKa = 3.8) were not able to cross the membrane. For this reason, only growth inhibition took place (Figs. 1A, 2 A–C), but LA metabolism was not affected, indicating that pH control is crucial in these kind of processes. During fermentation of xylose-rich defined and lignocellulosic media with *L. pentosus* CECT4023 T, the inhibitory effect of carboxylic acids (LA, acetic acid and formic acid) considerably decreased when switching from flask fermentation without pH control to automatic NaOH addition in bioreactor. As a result, 43 % and 44 % increase in LA production was attained with defined and lignocellulosic medium, respectively (Cubas-Cano et al., 2020, 2019).

3.1.3. Effect of increasing the concentration of furans

Marked differences between the three strains were observed when rising the furans concentrations to 2.5 and 5 g $L^{-1}$when compared to BM. According to bacterial growth, μ_{max} between 0.23 and 0.26 h^{-1} were reached by A162 strain in BMI-F2.5 and BMI-F5, respectively, while μ_{max} of A166 strain was around 0.21 h^{-1} in both media (Fig. 1A). DSM 2314 strain attained the lowest μ_{max}, especially in BMI-F5, reaching just 0.07 h^{-1}, which was significantly lower than the obtained with the isolates A166 and A162 (p-values < 0.05) (Fig. 1A). In this case, when compared to BM, 77 % of reduction in μ_{max} was shown in DSM 2314, while μ_{max} on A162 and A166 were only reduced by 22 and

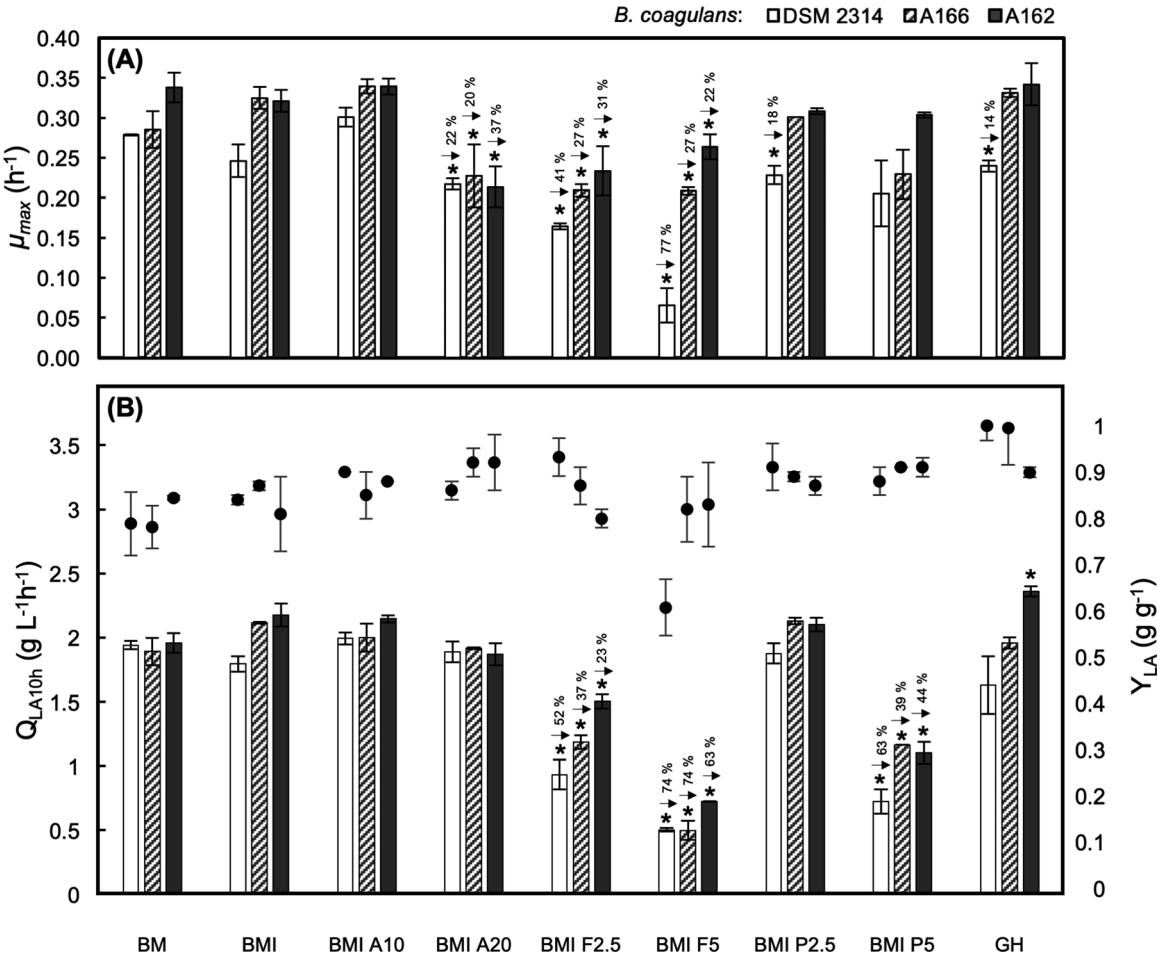

Fig. 1. Effect of different inhibitors-rich defined media and hemicellulosic gardening hydrolysate on growth rate (A), LA yield (B, dots) and LA productivity at 10 h (B, bars) of three *B. coagulans* strains. The mean differences between the results obtained with each medium and the control (BM) is significant at p-value < 0.05 (*). When significant, the % of reduction in growth rate and LA productivity are indicated.

27 %, respectively (Fig. 1A). As it was the case for fermentation of BMI, A166 and A162 strains appeared to be more robust than the collection strain DSM 2314, presenting higher tolerance to the inhibitors presence, presumably owing to the fact that they were isolated from real materials, as previously discussed.

Focusing on LA productivity at 10 h, A162 reached 1.50 and 0.72 g L^{-1} h^{-1} in BMI-F2.5 and BMI-F5, respectively (Fig. 1B). This means that Q_{LA10h} was reduced by 23 and 63 % in those media in comparison with the control (BM) (Fig. 1B). Significant lower Q_{LA10h} were attained by A166 and DSM 2314 strains, especially in BMI-F5 (p-values < 0.05) (Fig. 1B). In fact, 74 % of reduction in Q_{LA10h} was shown in this medium both for A166 and DSM 2314 strains (Fig. 1B), highlighting their lower tolerance to the high furans concentrations in BMI-F5 (5 g L^{-1}) in comparsion with A162. BMI-F5 produced the highest inhibitory effect of this study, with a clear influence in growth and LA production (Fig. 2D–F). In fact, in contrast with the isolated strains, DSM 2314 was unable to completely consume the sugars in BMI-F5 in 24 h, which resulted in lower Y_{LA} (0.61 g g^{-1}) than A166 (0.82 g g^{-1}) and A162 (0.83 g g^{-1}) (Fig. 1B).

The addition of 2.5 g L^{-1} of furfural or 5-HMF has been previously reported to produce around 70 % of growth inhibition in *B. coagulans* DSM 2314 (van der Pol et al., 2016). Similarly, 75 % of growth rate inhibition was shown in the current work with DSM 2314 in BMI-F5 (Fig. 1A), but this medium contained 2-fold more furans content (5 g L^{-1}). A possible explanation could be these results of van der Pol and co-workers were obtained from a screening experiment in 48-well plates. This cultivation method has a simple set-up, reducing process times and costs, but it presents several limitations like uneven agitation, uneficcient oxygen and mass transfer, evaporation effects, non-pH control or increased wall growth due to the larger surface:volume ratio (Tajsoleiman et al., 2019). These effects could be the reason for the different tolerance to furans showed in these studies. By contrast, *Sporolactobacillus inulinus* was able to grow with 3 g L^{-1} of furfural and 5-HMF with only 29 % and 24 % inhibition in growth and LA production, respectively (Bai et al., 2015), showing similar results than A162 in the current work. Moreover, pre-cultivation in stressful conditions is a feasible strategy to adapt the bacteria to a certain inhibition effect. Indeed, DSM 2314 showed 50 % improved L-LA productivity from wheat straw hydrolysates, containing acetic acid (3.8 g L^{-1}), furfural (4.0 g L^{-1}) and HMF (1.4 g L^{-1}), when it was pre-cultivated in presence of 30 % hydrolysate (Aulitto et al., 2019). Thus, preculture in presence of inhibitors could be a promising approach to further increase the productivity values obtained by A162 strain.

3.1.4. Effect of increasing the concentration of phenolic aldehydes

According to the fermentation of BMI-P2.5, few differences in μ_{max} (Fig. 1A) and biomass were found in comparison with the control (Fig. 2G–I). Only 18 % of reduction of μ_{max} was shown in DSM 2314 (Fig. 1A). Neither LA yields nor productivities were affected by these inhibitors concentrations (Fig. 1B). However, a significant inhibition in BMI-P5 in terms LA productivity was observed, especially for DSM 2314, showing 63 % of reduction in Q_{LA10h} in comparison with BM (Fig. 1B) (p-values < 0.05). In the case of A166 and A162, no significant differences were shown between them, and their Q_{LA10h} were

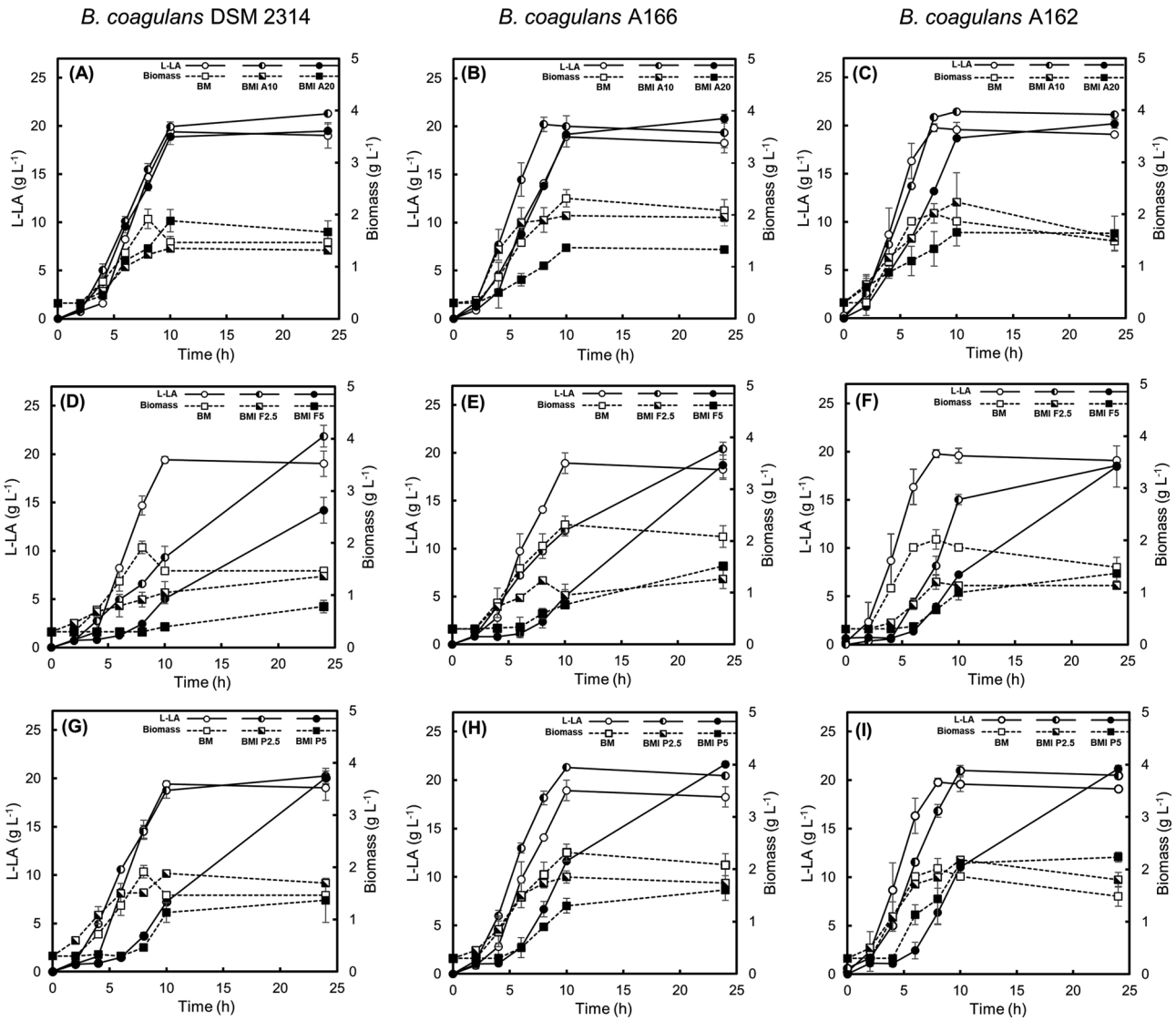

Fig. 2. L-LA and biomass production along time from inhibitors-rich defined media with different *B. coagulans* strains.

reduced by 39 % and 44 %, respectively (p-value < 0.05) (Fig. 1B). As previously seen with the media with high furans concentrations (BMI-F2.5 and BMI-F5), the isolated strains presented higher LA productivities than the collection strain. Surprisingly, higher inhibitory effect was shown with BMI-F5 than with BMI-P5 in the current work (Fig. 1B), while low-molecular weight phenols have been previously reported to be more toxic than aliphatic acids or furans for different bacteria (Gu et al., 2015; Mills et al., 2009).

Indeed, LA fermentation by *Rhizopus oryzae* and *S. inulinus* showed more than 90 % decrease in growth and LA concentration when being exposed to only 1 g L^{-1} of syringaldehyde (Bai et al., 2015; Zhang et al., 2016). Other works have reported 43 % and 73 % of growth inhibition of *B. coagulans* DSM 2314 in presence of 2.5 g L^{-1} of syringaldehyde and vanillin, respectively (van der Pol et al., 2016). As it was the case for carboxylic acids and furans, the *B. coagulans* strains tested in this work were less inhibited by the presence of high phenols concentrations (5 g L^{-1}). In fact, no significant differences in μ_{max} in comparison with the control (BM) were shown. The higher phenols resistance showed in this work coud also be explained by the differences in the cultivation method, as previously stated.

Altogether, in spite of the inhibition that took place in μ_{max} and Q_{LA10h} in different media with high inhibitors concentrations (BMI-F2.5, BMI-F5, BMI-P2.5 and BMI-P5), it should be reminded that LA yields close to the theoretical maximum have been reached by A166 and A162 in 24 h (Fig. 1B). To the best of the authors knowledge, no previous works have studied the inhibitors tolerance of *Bacillus coagulans* strains at concentrations as high as 5 g L^{-1} of furans or phenols and as 20 g L^{-1} of carboxylic acids. In fact, the inhibitors concentrations tested in these media were considerably higher than the ones normally attained during lignocellulose pretreatment (Jönsson et al., 2013). However, higher inhibitors content could be found in lignocellulosic hydrolysates obtained through harsh pretreatment conditions in which case, a detoxification method might be needed (Roque et al., 2019). With this in mind, *B. coagulans* A166 and A162 isolated strains would be good candidates to valorise hemicellulosic hydrolysates, without needing a previous detoxification step, being able to resist the presence of considerably high levels of inhibitors and able to valorise both C6 and C5 sugars.

3.2. LA production from hemicellulosic gardening hydrolysate

A GH containing inhibitors formed during steam explosion pretreatment was also used for L-LA production by *B. coagulans* DSM 2314, A166 and A162. *B. coagulans* isolates A166 and A162 used in this work have been previously used for fermentation of different hydrolysates obtained from municipal organic waste (López-Gómez et al., 2019) and

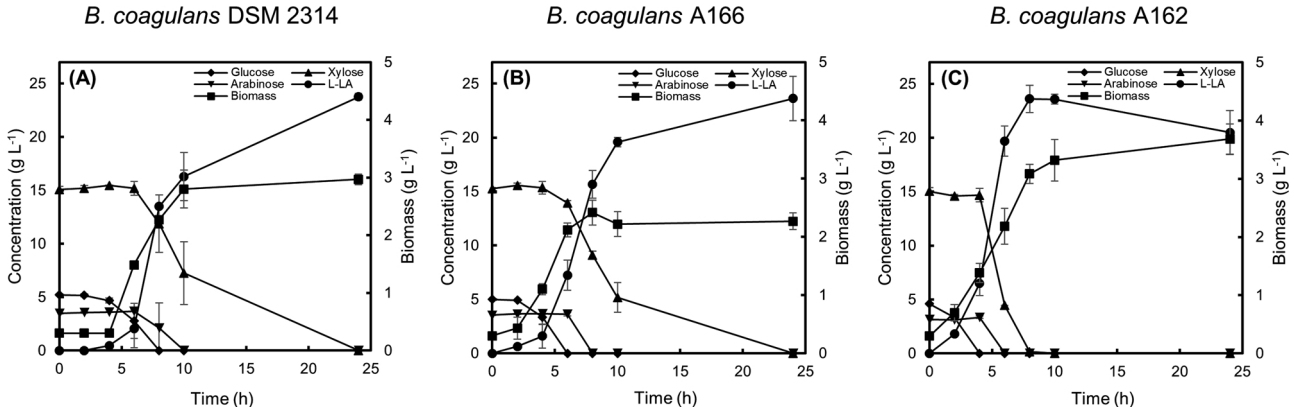

Fig. 3. Fermentation kinetics of hemicellulosic gardening hydrolysates with different *B. coagulans* strains.

defatted rice bran (Alexandri et al., 2019). However, no previous works have addressed the fermentation of hemicellulosic hydrolysates obtained from gardening residues with the mentioned strains. In fact, this novel substrate has only been used for LA production in a previous work, using a different LA-producing bacteria, *Lactobacillus pentosus* (Cubas-Cano et al., 2020).

This GH contained similar concentrations of carboxylic acids and furans than BMI previously tested, but vanillin and syringaldehyde were not detected (Table 1).

According to the bacterial growth, A166 and A162 reached μ_{max} between 0.33 and 0.34 h^{-1}, while DSM 2314 showed a significantly lower μ_{max}, 0.24 h^{-1} (p-values < 0.05), being reduced by 14 % in comparison with the one attained from the control with only sugars (BM) (Fig. 1B). A162 strain presented the highest biomass production, reaching almost 4 g L^{-1}, while no more than 3 g L^{-1} were achieved by A166 and DSM 2314 strains (Fig. 3).

In this case, *B. coagulans* DSM 2314, A166 and A162 showed high Y_{LA} at the end of fermentation, reaching almost the theoretical maximum (1 g g^{-1}) (Fig. 1B). The main differences were found in the fermentation kinetics. Xylose was totally consumed by A162 in just 8 h (Fig. 3C), while the other strains needed 24 h (Fig. 3A, B). As a result, *B. coagulans* A162 presented the highest Q_{LA10h} (2.36 g L h^{-1}), followed by A166 (1.96 g L^{-1}) and, finally, by DSM 2314 (1.63 g L h^{-1}) (Fig. 1B). Thus, no significant inhibition on LA yields and LA productivities was observed in any of the tested strains when cultivated in the GH in comparison with BM.

Recently, *B. coagulans* has become very popular for efficient LA production from lignocellulosic hydrolysates. A screening of different *B. coagulans* isolates was carried out with defatted rice bran hydrolysate as substrate. In this case, A107 strain high LA yield (0.90 g g^{-1}) and productivity (2.7 g L^{-1}) (Alexandri et al., 2019). When *B. coagulans* MA-13 was cultivated in wheat straw hydrolysate containing (g L^{-1}): acetic acid, 3.80; furfural, 4.00 and 5-HMF, 1.40, LA yields higher than 0.90 g g^{-1} were also obtained (Aulitto et al., 2017). An evolved inhibitor tolerant *B. coagulans* GKN316 strain was used for fermentation of a hemicellulosic corn stover hydrolysate which was concentrated by rotatory evaporation. As a result, not only sugars concentrations were increased, but also the degradation compounds, i.e. (g L^{-1}): acetic acid, 2.00; formic acid, 0.72; levulinic acid, 0.28; 5-HMF, 0.39; vanillin, 0.04; syringaldehyde, 0.02 and ρ-hydroxybenzaldehyde, 0.02 (Jiang et al., 2016). This evolved *B. coagulans* strain reached a LA yield of 0.83 g g^{-1}, showing 3-fold increase in comparison with the parental strain (Jiang et al., 2016). These results were very similar to the LA yields and productivities obtained in the present work from an inhibitors-rich gardening hydrolysate by A162 strain (Fig. 1B).

Overall, A162 was the strain which presented the highest LA productivities in defined media, especially with high furans concentrations (BMI-F2.5 and BMI-F5), and in hemicellulosic GH. A162 strain could be efficiently used in future works for fermentation of highly toxic hemicellulosic hydrolysates since it has demonstrated its tolerance to the presence of high inhibitors concentrations.

3.3. Biodetoxification capacity of inhibitors in Bacillus coagulans

Since the highest Q_{LA10h} were obtained by A162 strain, furans and phenolic aldehydes concentrations at the end of A162 fermentations were measured to check for possible biodetoxification responses. As explained, no inhibitory effect was produced on L-LA production by carboxylic acids due to the pH control applied. For this reason, the concentrations of these compounds at the end of fermentation were not taken into account.

As can be seen in Table 2, furans concentrations were reduced during fermentation. Less than 0.02 g L^{-1} of furfural were detected in all cases, removing even 2.5 g L^{-1} of this compound in BMI F5 (Table 2). In the case of 5-HMF, its final concentration ranged from 0.02 to 0.06 g L^{-1} in most of the cases. As an exception, 1.04 g L^{-1} of 5-HMF were detected in BMI F5, showing just a 58 % of removal (Table 2). For this reason, it seemed likely that, in these conditions, up to 1.5 g L^{-1} of 5-HMF could have been removed in 24 h. Considering BMI A20 and BMI P5 media, in which cases 0.25 g L^{-1} of 5-HMF were initially added, 0.15 and 0.10 g L^{-1} were detected after 24 h (40 % and 60 % of removal) (Table 2). This fact suggested that the removal of 5-HMF could

Table 2
Concentration of inhibitors at 24 h of fermentation and inhibitors removal (%) on defined media and gardening hydrolysate fermented by *B. coagulans* A162.

Inhibitors		BMI	BMI A 10	BMI A 20	BMI F 2.5	BMI F 5	BMI P 2.5	BMI P 5	GH
Furfural	Concentration (g L^{-1})	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	% Removal	96	96	96	99	99	96	96	95
5-HMF	Concentration (g L^{-1})	0.00 ± 0.00	0.02 ± 0.01	0.15 ± 0.06	0.06 ± 0.03	1.04 ± 0.02	0.02 ± 0.00	0.1 ± 0.00	0.00 ± 0.00
	% Removal	100	92	40	95	58	92	60	100
Vanillin	Concentration (g L^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	% Removal	100	100	100	100	100	100	100	100
Syring-aldehyde	Concentration (g L^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	% Removal	100	100	100	100	100	100	100	100

be hampered by high carboxylic acids or phenolic aldehydes concentrations.

Some works have studied the removal of furans with different microoganisms like fungi and yeast. Furfural and 5-HMF concentrations from steam exploded corn stover were considerably reduced after inoculation of *Aspergillus nidulans* (Yu et al., 2011). It is also known that furfural and 5-HMF can be reduced into furfuryl and 5-hydroxymethylfurfuryl alcohol, respectively, by the expression of NADPH-dependant reductases in *S. cerevisiae* (Moreno et al., 2015).

Nevertheless, the involved mechanisms for furans biodetoxification in *B. coagulans* have not been exhaustively determined (Jiang et al., 2016; Yan et al., 2018; Ye et al., 2014). In the case of *B. coagulans* strains, furfural content completely disappeared after 36 h of fermentation of a xylose-rich medium with 3 g L^{-1} of each furfural and 5-HMF by *B. coagulans* GKN316, while furfuryl and 5-hydroxymethylfurfuryl alcohols were detected in the resulting media (Jiang et al., 2016). Similarly, it was shown that *B. coagulans* JI12 was able to convert 1.08 g L^{-1} of furfural into 0.97 g L^{-1} of furoic acid within 9 h in LB media (Ye et al., 2014).

In the case of phenolic aldehydes, vanillin and syringaldehyde were not detected after 24 h of culture, even in the media with 2.5 g L^{-1} of each compound (Table 2). Different fungi like *Khurtia huakuii* and *Trichoderma viride* were reported to produce dioxygenase enzymes able to convert different phenolic aldehydes, including syringaldehyde, vanillin and ρ-hydroxybenzaldehyde into less toxic acids (Liu et al., 2020; Xie et al., 2018). In this way, LA yields obtained by *B. coagulans* from pretreated materials (corn stovers and corncobs) were significantly improved by previously inoculating *K. huakuii* or adding the enzyme preparation from *T. viride* (Liu et al., 2020; Xie et al., 2018). Nevertheless, it should also be highlighted that the addition of these additional steps would increase the production costs associated with enzymes production (enzymatic biodetoxification) or with the reduction in the sugars concentrations available for fermentation (microbial biodetoxification). For this reason, the use of LA-producing microorganisms with phenol biodetoxification capacities is a preferred option. In this sense, *B. coagulans* GKN316 was able to convert syringaldehyde, vanillin and ρ-hydroxybenzaldehyde into syringyl alcohol, vanillyl alcohol and ρ-hydroxybenzyl alcohol, without the need of using additional microorganisms or enzymes (Jiang et al., 2016).

All these facts, together with the reduction and removal of inhibitors in the current work, suggest a biodetoxification response of *B. coagulans* A162.

In short, furfural, vanillin and syringaldehyde were completely removed after fermentation with *B. coagulans* A162, while there was a considerable reduction of 5-HMF concentration, ranging from 40 % to 100 % (Table 2). For this reason, inoculation of this bacterium could produce a double effect on the media: (i) the efficient conversion of the present sugars into optically pure L-LA and (ii) the removal of toxic compounds. This system would avoid the need of adding additional enzymatic or microbial detoxification operations before fermentation and could also facilitate the following purification steps.

4. Conclusions

Different *B. coagulans* strains have demonstrated their ability to produce LA from sugars mixtures in presence of inhibitory compounds. The highest LA productivities (2.4 g L^{-1} h^{-1}) were attained by A162 strain, both in defined and gardening lignocellulosic media, tolerating the presence of inhibitors at concentrations as high as 20 g L^{-1} of carboxylic acids, 5 g L^{-1} of furans or 5 g L^{-1} of phenolic aldehydes. Furthermore, a reduction in the concentration of these compounds at the end of fermentation was shown, which suggests the presence of biodetoxification responses in A162 strain. Therefore, this strain could be a great candidate to be up-scaled to ferment lignocellulosic hydrolysates containing high sugars and inhibitors concentrations, producing L-LA at high yields.

CRediT authorship contribution statement

Enrique Cubas-Cano: Conceptualization, Investigation, Writing - original draft. **Joachim Venus:** Conceptualization, Supervision, Writing - review & editing. **Cristina González-Fernández:** Writing - review & editing. **Elia Tomás-Pejó:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.07.017.

References

Alexandri, M., Neu, A.-K., Schneider, R., López-Gómez, J.P., Venus, J., 2019. Evaluation of various *Bacillus coagulans* isolates for the production of high purity L-lactic acid using defatted rice bran hydrolysates. Int. J. Food Sci. Technol. 54, 1321–1329. https://doi.org/10.1111/ijfs.14086.

Allen, S.A., Clark, W., McCaffery, J.M., Cai, Z., Lanctot, A., Slininger, P.J., Liu, Z.L., Gorsich, S.W., 2010. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 3, 2. https://doi.org/10.1186/1754-6834-3-2.

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol. 82, 340–349. https://doi.org/10.1002/jctb.1676.

Alves de Oliveira, R., Vaz Rossell, C.E., Venus, J., Cândida Rabelo, S., Maciel Filho, R., 2018. Detoxification of sugarcane-derived hemicellulosic hydrolysate using a lactic acid producing strain. J. Biotechnol. 278, 56–63. https://doi.org/10.1016/j.jbiotec.2018.05.006.

Aulitto, M., Fusco, S., Bartolucci, S., Franzen, C.J., Contursi, P., 2017. *Bacillus coagulans* MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. Biotechnol. Biofuels 10, 210. https://doi.org/10.1186/s13068-017-0896-8.

Aulitto, M., Fusco, S., Nickel, D.B., Bartolucci, S., Contursi, P., Franzén, C.J., 2019. Seed culture pre-adaptation of *Bacillus coagulans* MA-13 improves lactic acid production in simultaneous saccharification and fermentation. Biotechnol. Biofuels 12, 45. https://doi.org/10.1186/s13068-019-1382-2.

Bai, Z., Gao, Z., He, B., Wu, B., 2015. Effect of lignocellulose-derived inhibitors on the growth and D-lactic acid production of *Sporolactobacillus inulinus* YBS1-5. Bioprocess Biosyst. Eng. 38, 1993–2001. https://doi.org/10.1007/s00449-015-1440-5.

Bautista-Gallego, J., Arroyo-López, F.N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., Cocolin, L., 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50, 135–142. https://doi.org/10.1016/j.foodres.2012.10.004.

Boguta, A.M., Bringel, F., Martinussen, J., Jensen, P.R., 2014. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. Microb. Cell Fact. 13. https://doi.org/10.1186/s12934-014-0097-0.

Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2018. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate. Biofuels Bioprod. Biorefin. 12 (2). https://doi.org/10.1002/bbb.1852.

Cubas-Cano, E., González-Fernández, C., Tomás-Pejó, E., 2019. Evolutionary engineering of *Lactobacillus pentosus* improves lactic acid productivity from xylose-rich media at low pH. Bioresour. Technol. 288, 121540. https://doi.org/10.1016/j.biortech.2019.121540.

Cubas-Cano, E., González-Fernández, C., Ballesteros, I., Tomás-Pejó, E., 2020. Efficient utilization of hydrolysates from steam-exploded gardening residues for lactic acid production by optimization of enzyme addition and pH control. Waste Manag. 107, 235–243. https://doi.org/10.1016/j.wasman.2020.04.003.

Demeke, M.M., Dumortier, F., Li, Y., Broeckx, T., Foulquie-Moreno, M.R., Thevelein, J.M., 2013. Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. Biotechnol. Biofuels 6, 120. https://doi.org/10.1186/1754-6834-6-120.

Gu, H., Zhang, J., Bao, J., 2015. High tolerance and physiological mechanism of *Zymomonas mobilis* to phenolic inhibitors in ethanol fermentation of corncob residue. Biotechnol. Bioeng. 112, 1770–1782. https://doi.org/10.1002/bit.25603.

Jiang, T., Qiao, H., Zheng, Z., Chu, Q., Li, X., Yong, Q., Ouyang, J., 2016. Lactic acid production from pretreated hydrolysates of corn stover by a newly developed *Bacillus coagulans* strain. PLoS One 11. https://doi.org/10.1371/journal.pone.0149101.

Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009.

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol. Biofuels 6, 16. https://doi.org/10.1186/1754-6834-6-16.

Juturu, V., Wu, J.C., 2015. Microbial production of lactic acid: the latest development. Crit. Rev. Biotechnol. 1–11.

Klotz, S., Kaufmann, N., Kuenz, A., Prusse, U., 2016. Biotechnological production of enantiomerically pure d-lactic acid. Appl. Microbiol. Biotechnol. 100, 9423–9437. https://doi.org/10.1007/s00253-016-7843-7.

Liu, J., Cai, Y., Liu, L., Zhan, R., Zhao, S., Liang, Y., Peng, N., Zhu, J., Li, H., Xiao, N., Hu, J., He, M., Hu, G., 2020. Enhanced lactic acid production by *Bacillus coagulans* through simultaneous saccharification, biodetoxification, and fermentation. Biofuels Bioprod. Biorefin. 14, 533–543. https://doi.org/10.1002/bbb.2086.

López-Gómez, J.P., Latorre-Sánchez, M., Unger, P., Schneider, R., Lozano, C.C., Venus, J., 2019. Assessing the organic fraction of municipal solid wastes for the production of lactic acid. Biochem. Eng. J., 107251. https://doi.org/10.1016/j.bej.2019.107251.

Mills, T.Y., Sandoval, N.R., Gill, R.T., 2009. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol. Biofuels 2, 26. https://doi.org/10.1186/1754-6834-2-26.

Moreno, A.D., Ibarra, D., Alvira, P., Tomas-Pejo, E., Ballesteros, M., 2015. A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit. Rev. Biotechnol. 35, 342–354. https://doi.org/10.3109/07388551.2013.878896.

Njoku, S.I., Iversen, J.A., Uellendahl, H., Ahring, B.K., 2013. Production of ethanol from hemicellulose fraction of cocksfoot grass using *Pichia stipitis*. Sustain. Chem. Process. 1, 13. https://doi.org/10.1186/2043-7129-1-13.

Oliva, J.M., Ballesteros, I., Negro, M.J., Manzanares, P., Cabañas, A., Ballesteros, M., 2004. Effect of binary combinations of selected toxic compounds on growth and fermentation of *Kluyveromyces marxianus*. Biotechnol. Prog. 20, 715–720. https://doi.org/10.1021/bp034317p.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33. https://doi.org/10.1016/S0960-8524(99)00161-3.

Pleissner, D., Neu, A.K., Mehlmann, K., Schneider, R., Puerta-Quintero, G.I., Venus, J., 2016. Fermentative lactic acid production from coffee pulp hydrolysate using *Bacillus coagulans* at laboratory and pilot scales. Bioresour. Technol. 218, 167–173. https://doi.org/10.1016/j.biortech.2016.06.078.

Pleissner, D., Dietz, D., van Duuren, J.B.J.H., Wittmann, C., Yang, X., Lin, C.S.K., Venus, J., 2017. Biotechnological Production of Organic Acids From Renewable Resources. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–38. https://doi.org/10.1007/10_2016_73.

Roque, L.R., Morgado, G.P., Nascimento, V.M., Ienczak, J.L., Rabelo, S.C., 2019. Liquid-liquid extraction: a promising alternative for inhibitors removing of pentoses fermentation. Fuel 242, 775–787. https://doi.org/10.1016/j.fuel.2018.12.130.

Sasaki, C., Okumura, R., Asada, C., Nakamura, Y., 2014. Steam explosion treatment for ethanol production from branches pruned from pear trees by simultaneous saccharification and fermentation. Biosci. Biotechnol. Biochem. 78, 160–166. https://doi.org/10.1080/09168451.2014.877818.

Su, F., Xu, P., 2014. Genomic analysis of thermophilic *Bacillus coagulans* strains: efficient producers for platform bio-chemicals. Sci. Rep. 4, 3926. https://doi.org/10.1038/srep03926.

Tajsoleiman, T., Mears, L., Kruhne, U., Gernaey, K.V., Cornelissen, S., 2019. An industrial perspective on scale-down challenges using miniaturized bioreactors. Trends Biotechnol. 37, 697–706. https://doi.org/10.1016/j.tibtech.2019.01.002.

Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Chapter 7 – pretreatment technologies for lignocellulose-to-bioethanol conversion A2 – Pandey, Ashok. In: Larroche, C., Ricke, S.C., Dussap, C.-G., Gnansounou, E. (Eds.), Biofuels. Academic Press, Amsterdam, pp. 149–176. https://doi.org/10.1016/B978-0-12-385099-7.00007-3.

Trcek, J., Mira, N.P., Jarboe, L.R., 2015. Adaptation and tolerance of bacteria against acetic acid. Appl. Microbiol. Biotechnol. 99, 6215–6229. https://doi.org/10.1007/s00253-015-6762-3.

van der Pol, E.C., Vaessen, E., Weusthuis, R.A., Eggink, G., 2016. Identifying inhibitory effects of lignocellulosic by-products on growth of lactic acid producing micro-organisms using a rapid small-scale screening method. Bioresour. Technol. 209, 297–304. https://doi.org/10.1016/j.biortech.2016.03.037.

Xie, Y., Hu, Q., Feng, G., Jiang, X., Hu, J., He, M., Hu, G., Zhao, S., Liang, Y., Ruan, Z., Peng, N., 2018. Biodetoxification of phenolic inhibitors from lignocellulose pretreatment using *Kurthia huakuii* LAM0618(T) and subsequent lactic acid fermentation. Molecules 23. https://doi.org/10.3390/molecules23102626.

Yan, Y., Bu, C., He, Q., Zheng, Z., Ouyang, J., 2018. Efficient bioconversion of furfural to furfuryl alcohol by *Bacillus coagulans* NL01. RSC Adv. 8, 26720–26727. https://doi.org/10.1039/C8RA05098H.

Ye, L.D., Bin Hudari, M.S., Li, Z., Wu, J.C., 2014. Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid production by *Bacillus coagulans* JI12. Biochem. Eng. J. 83, 16–21. https://doi.org/10.1016/j.bej.2013.12.005.

Yu, Y., Feng, Y., Xu, C., Liu, J., Li, D., 2011. Onsite bio-detoxification of steam-exploded corn stover for cellulosic ethanol production. Bioresour. Technol. 102, 5123–5128. https://doi.org/10.1016/j.biortech.2011.01.067.

Zhang, L., Li, X., Yong, Q., Yang, S.-T., Ouyang, J., Yu, S., 2016. Impacts of lignocellulose-derived inhibitors on L-lactic acid fermentation by *Rhizopus oryzae*. Bioresour. Technol. 203, 173–180. https://doi.org/10.1016/j.biortech.2015.12.014.

ARTICLE V

Evolutionary engineering of *Lactobacillus pentosus* improves lactic acid productivity from xylose-rich media at low pH

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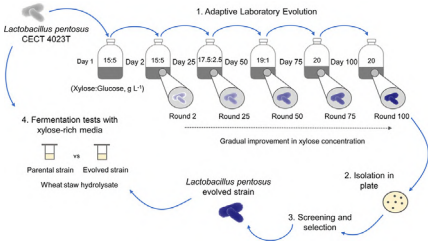
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Lactic acid
Xylose
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Lactobacillus pentosus
Low-pH tolerance

ABSTRACT

Since xylose is the second most abundant sugar in lignocellulose, using microorganisms able to metabolize it into bio-based chemicals like lactic acid is an attractive approach. In this study, *Lactobacillus pentosus* CECT4023T was evolved to improve its xylose fermentation capacity even at acid pH by adaptive laboratory evolution in repeated anaerobic batch cultures at increasing xylose concentration. The resulting strain (named MAX2) presented between 1.5 and 2-fold more xylose consumption and lactic acid production than the parental strain in 20 g L^{-1} xylose defined media independently of the initial pH value. When the pH was controlled in bioreactor, lactic acid productivity at 16 h increased 1.4-fold when MAX2 was grown both in xylose defined media and in wheat straw hydrolysate. These results demonstrated the potential of this new strain to produce lactic acid from hemicellulosic substrates at low pH, reducing the need of using neutralizing agents in the process.

1. Introduction

Lactic acid bacteria (LAB) are gram-positive, aerotolerant and non-spore forming bacteria with the ability to produce lactic acid (LA) as fermentation product. Organic acids like LA present several novel applications as building blocks for chemicals and polymers. Indeed, the biodegradable plastic poly-lactic acid (PLA) synthetized from LA is a very promising compound to replace conventional petroleum-based plastics (Pleissner et al., 2017).

LA can be produced by chemical and fermentative methods, the latter being the most commonly used at industrial scale since lactic fermentative microorganisms perform at higher yields. Furthermore, chemical processes are based on the reaction of acetaldehyde with hydrogen cyanide, which derives from petrochemical sources (Cubas-Cano et al., 2018). Among LA-producing microorganisms, some LAB are able to use different carbon sources apart from glucose (i.e. xylose, arabinose, etc.) (Yoshida et al., 2011). This is very interesting when using lignocellulose that contains different types of polysaccharides. The high abundance of lignocellulose (200×10^9 tons produced per year), its low price and renewability make this feedstock an ideal raw material in terms of substrate availability and sustainability (Zhang, 2008).

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The use of glucose obtained from the hydrolysis of cellulose has been extensively studied for bioethanol production by yeasts (Bolivar-Telleria et al., 2018; Das et al., 2013; Erdei et al., 2012; Jiang et al., 2013; Tomás-Pejó et al., 2008; Tomás-Pejó et al., 2017; Yuan et al., 2017). However, although xylose is the predominant sugar released from the hemicellulosic fraction and the second most abundant sugar in lignocellulosic biomass, most of wild type bioethanol-producing yeasts cannot metabolize this pentose. Furthermore, fermentation of xylose-containing sugars mixtures by recombinant yeasts leads to an incomplete sugar uptake or suboptimal bioethanol yields (Tomás-Pejó et al., 2010). For this reason, improving the conversion of xylose to targeted bio-based chemicals is crucial to ensure a cost-competitive full utilization of lignocellulose.

Facultative heterolactic bacteria like *Lactobacillus pentosus* are able to ferment glucose and xylose. This LAB produces LA from glucose homofermentatively via the Embden-Meyerhof-Parnas pathway, while xylose is metabolized by the phosphoketolase pathway, producing acetic acid as a by-product (Cubas-Cano et al., 2018). As a result of carbon catabolite repression (CCR), xylose is not consumed in most LAB until the preferred carbon source, i.e. glucose, is depleted (Abdel-Rahman et al., 2015).

Despite of being moderately acid tolerant, the high accumulation of fermentation products, i.e. LA and acetic acid, leads to the continuous acidification of the culture, inhibiting the metabolic activity of the LAB (Singhvi et al., 2018). Among other inhibition mechanisms, undissociated forms of LA and acetic acid at low pH are able to cross the cell membrane by simple diffusion, thus dissociating in the cytoplasm and becoming insoluble (Thomas & Ingledew, 2001; Trcek et al., 2015). The accumulation of these acids decreases intracellular pH, which affects the transmembrane pH gradient. Bacterial growth is then arrested since cellular functions are disabled and energy is being used to maintain the pH gradient instead of growing (Othman et al., 2017; Trcek et al., 2015). As a result, LA production slows down until cell membrane is finally disrupted (Abdel-Rahman & Sonomoto, 2016; Qin et al., 2010). To avoid pH-drop, neutralizing agents ($CaCO_3$, CaOH, NaOH and $NH_3{\cdot}H_2O$) are frequently utilized in LA fermentation (Qin et al., 2010). Having in mind the downstream processing costs of LA purification (50% of total production cost), acid-tolerant LAB are being investigated to reduce the amounts of neutralizers (Singhvi et al., 2018).

Rational approaches like metabolic and genetic engineering to improve acid tolerance are limited due to the incomplete knowledge of acid stress responses in LAB. For this reason, evolutionary engineering approaches are becoming very popular to obtain desirable phenotypes like inhibitor or stress tolerance and multiple sugar utilization (Chen & Dou, 2016). Xylose consumption rates have been successfully improved in *Saccharomyces cerevisiae* and *Escherichia coli* using adaptive laboratory evolution (ALE) (Li et al., 2017; Reider et al., 2016; Sandberg et al., 2017; Wisselink, et al., 2009), but few studies have been carried out using LAB. Qiu and co-workers increased xylose consumption in *Pediococcus acidilactici* by combining genetic engineering with ALE (Qiu et al., 2018). ALE has also been applied to improve temperature, H_2O_2 or salt tolerance in LAB but the improvement of xylose fermentation is still limited (Bachmann et al., 2017; Patnaik et al., 2002).

The ability of *L. pentosus* CECT 4023 T to co-metabolize mixtures of sugars in synthetic and lignocellulosic media with a slight CCR effect has been previously reported (Cubas-Cano et al., 2019). The goal of the present work is to subject this LAB to an evolutionary engineering approach to maximize the LA production from xylose, concomitantly improving tolerance to low pH. For this reason, a sequential batch cultivation was carried out by increasing gradually the xylose:glucose ratio of the medium. As a result, MAX2 strain was obtained. This newly developed strain showed higher growth rates, LA production and acid-pH tolerance than the parental strain.

2. Materials and methods

2.1. Microorganisms and preculture media

Lactobacillus pentosus CECT 4023 T (ATCC-8041) was obtained from the Spanish Type Culture Collection (Valencia, Spain) since its ability to consume xylose has been previously reported, with high tolerance to inhibitory compounds present in hemicellulosic hydrolysates (Cubas-Cano et al., 2019). For preculture, an aliquot of 20 μL of cells was taken from glycerol stocks. Cells were inoculated in 50-mL Falcon tubes with 15 mL of Man, Rogosa and Sharpe medium (MRS), containing ($g\,L^{-1}$): glucose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; $MgSO_4{\cdot}7\ H_2O$, 0.2, $MnSO_4{\cdot}H_2O$, 0.05 (pH 7). The preculture was grown in a rotatory shaker at 150 rpm and 32 °C overnight (until the culture reached the late exponential growth phase).

2.2. Wheat straw hydrolysate

Wheat straw prehydrolysate was obtained after vacuum filtering the slurry produced by steam explosion of wheat straw chips at 180 °C for 4 min with H_2SO_4 (0.5%) impregnation. Prehydrolysate was enzymatically hydrolysed to release the hemicellulosic monomeric sugars from their oligomeric forms and obtain the wheat stray hydrolysate that was used for fermentation. The enzymatic hydrolysis was performed at 40 °C, 150 rpm and pH 5.5 for 72 h using the commercial enzymes Cellic-HTec2® (300 U mg^{-1}, Novozymes) and β-D-xylosidase (EC 3.2.1.37; 118 U mg^{-1}, Megazyme®), respectively. Enzymes loading were 0.03 g Cellic-HTec2®/g xylooligosaccharides and 0.1 mg β-D-xylosidase/g xylooligosaccharides. The composition of the resulting wheat straw hydrolysate (WSH) was ($g\,L^{-1}$): glucose, 4.5; xylose, 40; arabinose, 4; 5-hydroxymethylfurfural, 0.09; furfural, 0.41; vanillin, 0.028; syringaldehyde, 0.024; coumaric acid, 0.046; ferulic acid, 0.02; formic acid, 0.57 and acetic acid, 2.36. After the enzymatic hydrolysis, the pH was adjusted to 7 and hydrolysate was supplemented with MRS compounds except glucose.

For fermentation experiments, the hydrolysate was diluted 1:2 in order to achieve a xylose concentration near 20 $g\,L^{-1}$.

2.3. Adaptive laboratory evolution in sequential batch cultivation

ALE was performed in 50 mL clamped flasks with 10 mL of MRS medium. Cells were incubated at 150 rpm, 32 °C and pH 7 with an initial OD_{600} of 0.01 in strict anaerobic conditions. To get strict anaerobic conditions, clamped flasks were flushed with filtered nitrogen using a needle (gas inlet) at 0.5 bar for 2 min. The contained air, including oxygen, was ejected through another needle (gas outlet) (Cubas-Cano et al., 2019). The gas composition in clamped flasks was analysed by gas chromatography to corroborate that flasks contained less than 5% of oxygen.

The xylose:glucose ratio increased gradually as evolution proceeded, being (xylose:glucose, $g\,L^{-1}$): 15:5, 17.5:2.5, 19:1 and 20:0. When an improvement in the bacterial growth (measured as OD_{600}) and xylose consumption was detected, xylose:glucose ratio in the evolution media was increased. Each round of ALE started by inoculating an aliquot of cells from the previous culture and lasted 24 h. Clones were isolated from the final population of cells in MRS plates at different conditions: i) 20 $g\,L^{-1}$ xylose at pH 5 and ii) 15 $g\,L^{-1}$ xylose with 5 $g\,L^{-1}$ glucose at pH 5. This process was carried out at acid pH in order to ensure the isolation of those clones with improved xylose fermentation even under acid stress. Clones with prominent growth on plates were selected and confirmed to be *L. pentosus* via 16S sequencing. Selected clones were subsequently tested in MRS media with 20 $g\,L^{-1}$ xylose al pH 7 and 32 °C to analyse their growth, xylose consumption and LA production. The clone that showed the best fermentation performance and cell growth was selected for further experiments.

2.4. Lactic fermentation tests in flask without pH control

LA fermentation tests were carried out in 25 mL of MRS medium using 120 mL nitrogen-purged clamped flasks at 32 °C and 150 rpm in strict anaerobic conditions (Cubas-Cano et al., 2019). Xylose and glucose were used as carbon source at the same ratios specified for ALE (i.e., 15:5, 17.5:2.5, 19:1 and 20:0). Cells were inoculated at OD_{600} of 0.1 and at different initial pH values (pH 7, 6, 5 and 4). All fermentation tests were carried out in triplicate. Samples were taken at 4, 8, 24, 48, 72 and 96 h of fermentation for measuring the bacterial growth, pH value, residual sugars and fermentation products.

2.5. Lactic fermentation tests in bioreactor with and without pH control

A 1.5-L bioreactor (Minifors2; Infors-HT; Switzerland) with a working volume of 0.5 L was used for lactic fermentation. *L. pentosus* parental and evolved strains were cultured at the same conditions (temperature, agitation, inoculation and oxygen presence) than in flask fermentations. Strict anaerobic conditions were obtained by sparging with 0.5 L min^{-1} of nitrogen gas until the pO_2 sensor (HAMILTON; USA) detected a 0% of oxygen.

MRS with 20 g L^{-1} of xylose was used as medium for fermentation with the parental and the evolved strains. Experiments were performed in duplicate with and without pH control. When controlled, pH was automatically maintained at 7 with NaOH 5 M. WSH was also fermented in pH-controlled conditions to prove the improved ability of the evolved strain to ferment a xylose-rich lignocellulosic hydrolysate. Samples were taken at 4, 8, 16, 20, 24, 48, 72 and 96 h of fermentation for measuring the bacterial growth, residual sugars and fermentation products. In the case of fermentation of WSH, samples were taken at 2, 4, 6, 8, 16, 18, 20, 22, 24 and 48 h of fermentation.

2.6. Calculations

LA yield (Y, g g^{-1}) was defined as the LA produced (g L^{-1}) at 72 h of fermentation per initial sugar (glucose and/or xylose) (g L^{-1}). The percentage of the theoretical maximum LA (%Y_{maxLA}) was calculated taking into account the maximum stoichiometric theoretical yield in each case, being 2 mol of LA per mol of glucose and 1 mol of LA per mol of xylose, respectively. LA volumetric productivity (Q, g L^{-1} h^{-1}) was calculated as the LA concentration produced (g L^{-1}) at 72 h. In the case of fermentation in bioreactor with pH neutralization, LA yields and productivities were calculated taking into account the results at 16 h and 48 h of fermentation since, when neutralizing, less time was needed to achieve the maximum LA titer.

Statistical analyses were carried out using the software Microsoft Excel. The results are given as the average ± standard deviation for descriptive statistics. In order to determine the statically significant differences between fermentations with the parental and the evolved strains, analysis of variance (ANOVA) was carried out. The level of significance was set at p less than 0.05.

2.7. Analytical methods

The gas composition in clamped flasks used for ALE was analysed by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (70 × 1/8″ O.D.) and a MS13X4-09SF2 40/60P packed column (9′ × 1/8″ O.D.) (PerkinElmer).

Cell growth was assessed by measuring optical density in a spectrophotometer (SPECTROstar® Omega) at 600 nm. Samples (2 mL) were taken from the fermentation broth and centrifuged at 14.000 g for 4 min to determine pH values, sugars, acetic and LA concentration. Supernatants were filtered using membrane filters (Thermo Scientific® Nylon 0.2 µm) and stored at −20 °C until analysis. pH was measured using a benchtop pH meter (Sension + PH31). LA and acetic acid were quantified by HPLC equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) was employed at the following conditions: column temperature, 50 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.5 mL min^{-1} and injection volume, 20 µL. Glucose, xylose and arabinose were quantified with a CARBOSep CHO-682 column (Transgenomic, Omana, NE) at the following conditions: column temperature, 80 °C: mobile phase, ultrapure water; flow rate, 0.35 mL min^{-1} and injection volume, 20 µL. Vanillin, *p*-coumaric acid, furfural, 5-hydroxymethylfurfural, syringaldehyde, ferulic acid and formic acid were quantified by HPLC (Agilent, Waldbronn, Germany). The system was equipped with a Coregel 87H3 column (Transgenomic, San Jose, CA, USA). The operating temperature was 65 °C, and the mobile phase was 89% v v^{-1} 5 mM H_2SO_4 and 11% v v^{-1} acetonitrile, with a flow rate of 0.7 mL min^{-1}. All these compounds were identified by a 1050 photodiode-array detector (Agilent, Waldbronn, Germany).

3. Results and discussion

3.1. Obtaining an improved xylose-fermenting L. Pentosus strain via adaptive laboratory evolution

ALE approach in batch mode is commonly used in biotechnology to obtain microorganisms better adapted to certain conditions (Tomás-Pejó et al., 2010). When microorganisms are cultivated in defined condition for prolonged periods of time, selection of different mutations leads to the establishment of clones with desired phenotypes (Sauer, 2001). As a result of maintaining a constant or continually increasing selective pressure, serial-transfer experiments have yielded microorganisms with improved stress tolerance and improved rates of substrate consumption (Mans et al., 2018).

During ALE, a heterogeneous population of cells with stable phenotypes and improved fitness is obtained, and therefore isolation of the desired clone is required (Tomás-Pejó et al., 2014). In this study, clone isolation to find the proper strain for xylose fermentation was performed from the population that resulted after 100 rounds of ALE (around 850 generations). 4 clones were isolated from the plate with 20 g L^{-1} xylose at pH 5 (clone 1–4) and 2 clones from the plate with 15 g L^{-1} xylose and 5 g L^{-1} glucose at pH 5 (clone 5 and 6). The highest xylose consumption and LA production were obtained with clone 2 (from now on named Mutant Acid Xylose 2 strain, MAX2 strain), reaching 8 g L^{-1} of LA and consuming around 15 g L^{-1} of xylose, followed by clones 4, 3 and 1 (Fig. 1). As expected, mutants 5 and 6 showed the lowest xylose consumption and LA production since both clones were isolated from a plate containing glucose:xylose mixture as carbon source instead of only xylose. This fact illustrates the importance of maintaining the selection pressure applied during the ALE in the isolation of clones, to ensure the selection of the most fitted and desired variants.

3.2. Lactic acid fermentation from xylose-rich sugar mixtures at different pH values comparison of MAX2 and parental strains.

Fig. 2A and B show the xylose consumption percentage (%XYL_{cons}) and the LA percentage of the theoretical yield from available sugars (%Y_{maxLA}) at 72 h of fermentation. There was an increase in both %XYL_{cons} and %Y_{maxLA} in MAX2 strain when compared with the parental strain in most of the tested conditions. As expected, differences between both strains were more marked as the xylose concentration increased since the selection pressure of the ALE was the gradual increase in xylose ratio. For this reason, few differences between parental and MAX2 strains were found in MRS 15:5. In this case, a slight increase in %XYL_{cons} and %Y_{maxLA} was obtained with MAX2 strain at pH 7, 5 and 4 (around 1.1-fold), while no significant differences were found at pH 6 (Fig. 2). Notwithstanding, 1.4-fold more xylose consumption and LA production were obtained with MAX2 in MRS 17.5:2.5 at pH 7 and pH 6 in comparison with the parental strain. In this case, around 80 %Y_{maxLA}

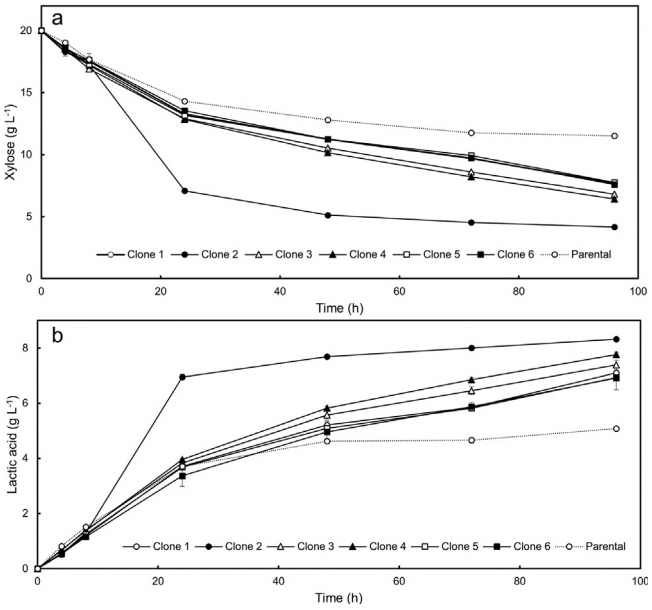

Fig. 1. Xylose consumption (a) and LA production (b) with parental strain and clones isolated after ALE.

of LA was produced (Fig. 2). Similarly, 1.5-fold more %XYL_{cons} and %Y_{maxLA} were obtained with MRS 19:1 at pH 7, 6 and 5, reaching 75%, 70% and 55% of the Y_{maxLA}, respectively (Fig. 2). A marked increase in %XYL_{cons} and %Y_{maxLA} was observed in 20 g L^{-1} xylose medium with MAX2. The evolved strain consumed and produced around 2-fold more xylose and LA, respectively, at pH 7, 6 and 5. Remarkably, 1.5-fold more xylose consumption and LA production were attained at the lowest pH (pH 4) (Fig. 2). In this same line, other studies also reported enhanced xylose fermentation after applying ALE. For instance, LA production from C5-sugars was improved in a LA-producing *E. coli* strain by genetic engineering followed by 75 generations of ALE at increasing xylose concentration. As a result, the engineered strains reached even a 95 %Y_{maxLA} (Utrilla et al., 2012). ALE also resulted in a 2.5-fold increase of xylose consumption in *S. cerevisiae* when the evolved strain BSPX013 was cultured in a mixture 1:1 of glucose and xylose (Shen et al., 2012). Moreover, xylose consumption in *S. cerevisiae* was increased from 17.7 g L^{-1} to 29.7 g L^{-1}, thus attaining 1.11-fold increase in ethanol production (Li et al., 2017).

The hierarchical utilization of carbon sources ruled by CCR is a common problem in lignocellulosic biomass fermentation because glucose presence represses the consumption of other sugars in mixtures. In LAB, this regulation mechanism is performed by the heat-stable phosphocarrier protein CcpA (Kim et al., 2009). As it can be seen in Fig. 3, the strain MAX2 presented higher xylose uptake than the parental strain in the first 8 h of fermentation of MRS with the following xylose:glucose ratios (g L^{-1}): 15:5, 17.5:2.5 and 19:1 at pH 7. Remarkably, both xylose and glucose were co-consumed during this period. Specifically, xylose consumption switched from 0.6 g L^{-1}, 0.5 g L^{-1} and 2.3 g L^{-1} in the parental strain to 3.9 g L^{-1}, 2.2 g L^{-1} and 3.4 g L^{-1} in MAX2, in each sugar mixture, respectively. For this reason, it can be inferred that this strain presented a reduced CCR effect at pH 7 since it was able to consume higher amounts of xylose than the parental strain in the presence of glucose. In the case of the fermentations tests at pH 6, similar results to those at pH 7 were obtained in terms of reduced CCR effect. Nevertheless, in the case of pH 5 and 4, no changes in sugar consumption were found between parental and evolved strains in the first hours of fermentation of sugar mixtures (data not shown).

The parental strain consumed 8.3 g L^{-1} of xylose and produced 4.7 g L^{-1} of LA (39 %Y_{maxLA}) in fermentation of MRS 20:0 at pH 7 (Fig. 4). In contrast, MAX2 strain was able to consume 15.5 g L^{-1} of xylose. As a result of the increase in sugars uptake, MAX2 presented higher growth and produced more LA (8.5 g L^{-1}), reaching a %Y_{maxLA} of 71% at the end of fermentation (Fig. 2). The increase in the LA production was concomitant with acetic acid generation because both acids are co-produced when xylose is metabolized via the phosphoketolase pathway (Abdel-Rahman & Sonomoto, 2016). In this case, higher and faster acidification of the medium also occurred. Indeed, 7.2 g L^{-1} and 9.6 g L^{-1} of acetic acid were accumulated in the medium by the parental strain and MAX2 strain, respectively (Fig. 4a). In this case, a final pH of 4.6 was reached by the parental strain while it dropped until pH 4 with MAX2 strain. Between the 8 h and the 24 h of culture (exponential growth phase), the accelerated xylose uptake of MAX2 led to higher biomass production from xylose, with an OD_{600} near 5, while the parental was not able to exceed an OD_{600} of 1 (Fig. 4a). The increase in biomass production allowed MAX2 to resist the high acid accumulation along fermentation. When the initial culture pH was adjusted to pH 6 (Fig. 4b), similar results to those observed at pH 7 were obtained.

Interestingly, there were also remarkable improvements in xylose fermentation at pH 5. As previously mentioned, 2-fold increase in LA final titer was attained, switching from 2.6 (21% Y_{LA}) to 6.1 g L^{-1} (51% Y_{LA}) of LA, as a result of the higher xylose consumption and bacterial growth (Fig. 2, Fig. 4c). In the same way than at initial pH 7 and pH 6, acetic acid production increased from 4.8 g L^{-1} with the parental strain to 7.3 g L^{-1} with MAX2 strain.

Xylose consumption and LA production were severely hampered in both strains when the initial pH of the media was 4. It is worth to mention that LAB optimal pH for growth ranges from 5.5 to 6.5, being strongly inhibited below pH 4.5 (Juodeikiene et al., 2016). Acid pH produces an alteration of cell wall and cell membrane as well as a cytosol acidification, leading to protein denaturation, affecting the metabolism and ending with the cell death (Papadimitriou et al., 2016). Even so, as it can be seen in Fig. 4d, the MAX2 strain presented an increased xylose consumption and LA final concentration in a medium with initial pH 4 in comparison with the parental strain. Considering that pH 5 or 4 are certainly stressful for *L. pentosus*, the fact that the MAX2 strain was able to keep growing and producing more LA than the parental strain at these pH values evidenced its improved acidity tolerance. Hypothetically, the higher resistance towards low pH of MAX2 strain when compared with the parental strain could be due to a cross-resistance effect produced after prolonged exposure to high LA and acetic acid concentrations during ALE.

Several studies have addressed the improvement of LAB in terms of acid tolerance by different experimental methods. For instance, four *Lactobacillus rhamnosus* ATCC 11443 strains that could grow at pH 3.6, a *L. plantarum* mutant with 64% more LA production than the wild type at pH 4 and a *Lactobacillus* strain with 3-fold more LA production at pH 4 were obtained after several rounds of genome shuffling (Patnaik et al., 2002; Triratna et al., 2011; Wang et al., 2007). After 70 days of serial exposure to low pH conditions, a *L. casei* strain able to grow at pH 4.3 was obtained, with 13.6% increase in LA concentration (Zhang et al., 2012). Error-prone whole-genome amplification method was also used in *L. pentosus*, obtaining a mutant able to ferment glucose at pH 3.8 (Ye et al., 2013). The improvements obtained by Patnaik et al. and Triratna et al. when comparing parental with evolved or shuffled strains were higher than the ones attained in this study at pH 4 in which 1.5-fold improvement in LA production was reached. However, it is worth to highlight that these studies were carried out with glucose as carbon source, focusing on reaching a remarkable increase in LA production at pH close to 4. In the present work, the objective was to improve the consumption of xylose and low pH adaptation has been concomitantly achieved in MAX2 strain. Fermentation at the LA *pKa* (3.8) or below can reduce the production cost and facilitate LA organic extraction because a higher ratio of the acid is in the free acid form. When neutralizers like calcium carbonate are used to maintain higher pH value, LA extensive purification is required, generating considerable waste after releasing LA from calcium lactate (Juturu et al., 2015). For this reason, the

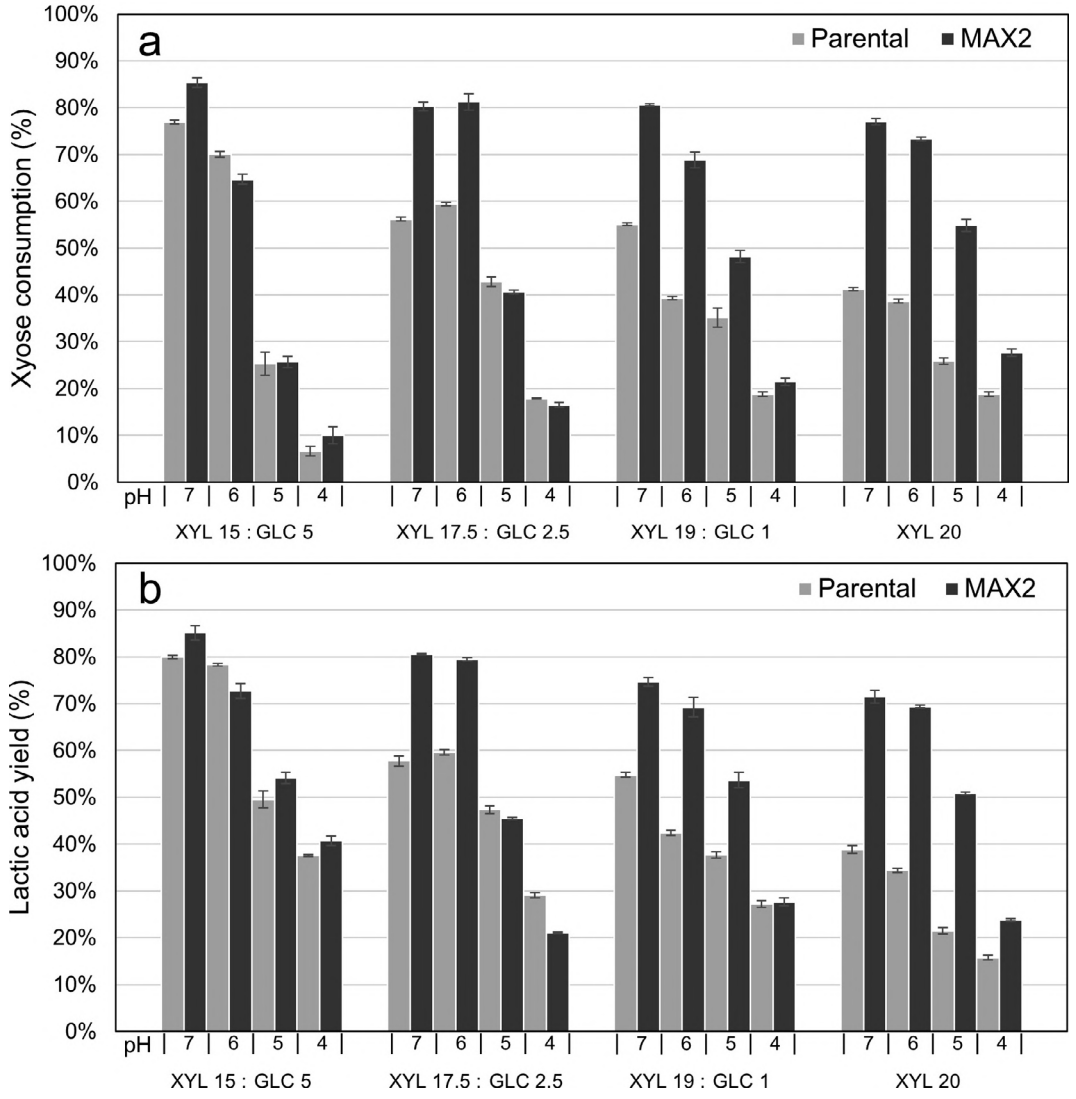

Fig. 2. Comparison of percentage of xylose consumption (a) and LA percentage of the theoretical yield (b) between MAX2 and parental strain from MRS medium with xylose:glucose mixtures at different pH values.

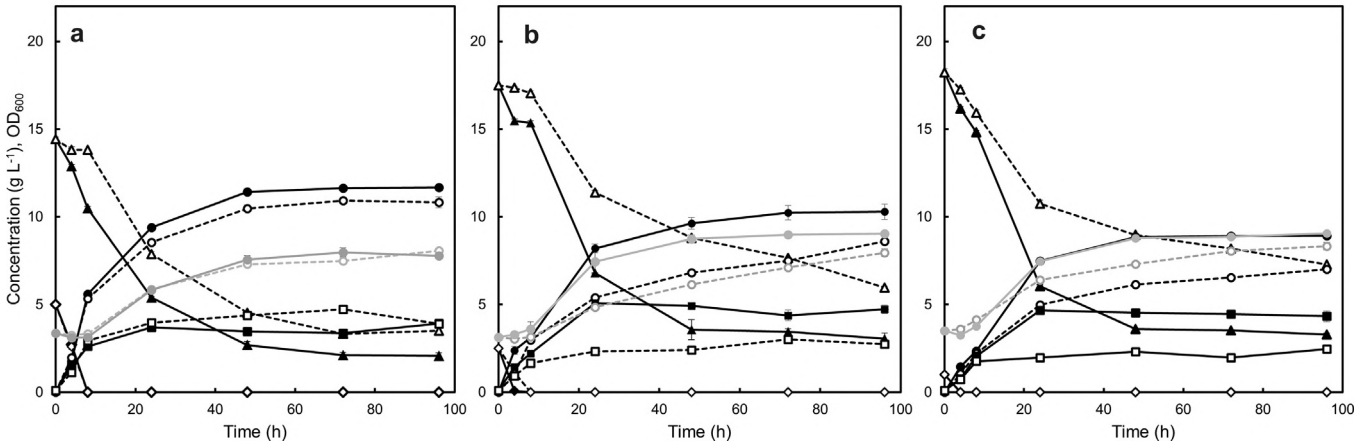

Fig. 3. Fermentation and growth time courses of MAX2 and parental strains in MRS medium at pH 7 with different mixtures xylose:glucose (g L^{-1}): 15:5 (a), 17.5:2.5 (b) and 19:1 (c). Xylose (▲, △), glucose (◆, ◊), LA (●, ○), acetic acid (●, ○), OD_{600} (■, □). Data of MAX2 and parental strains are represented with filled and empty symbols, respectively.

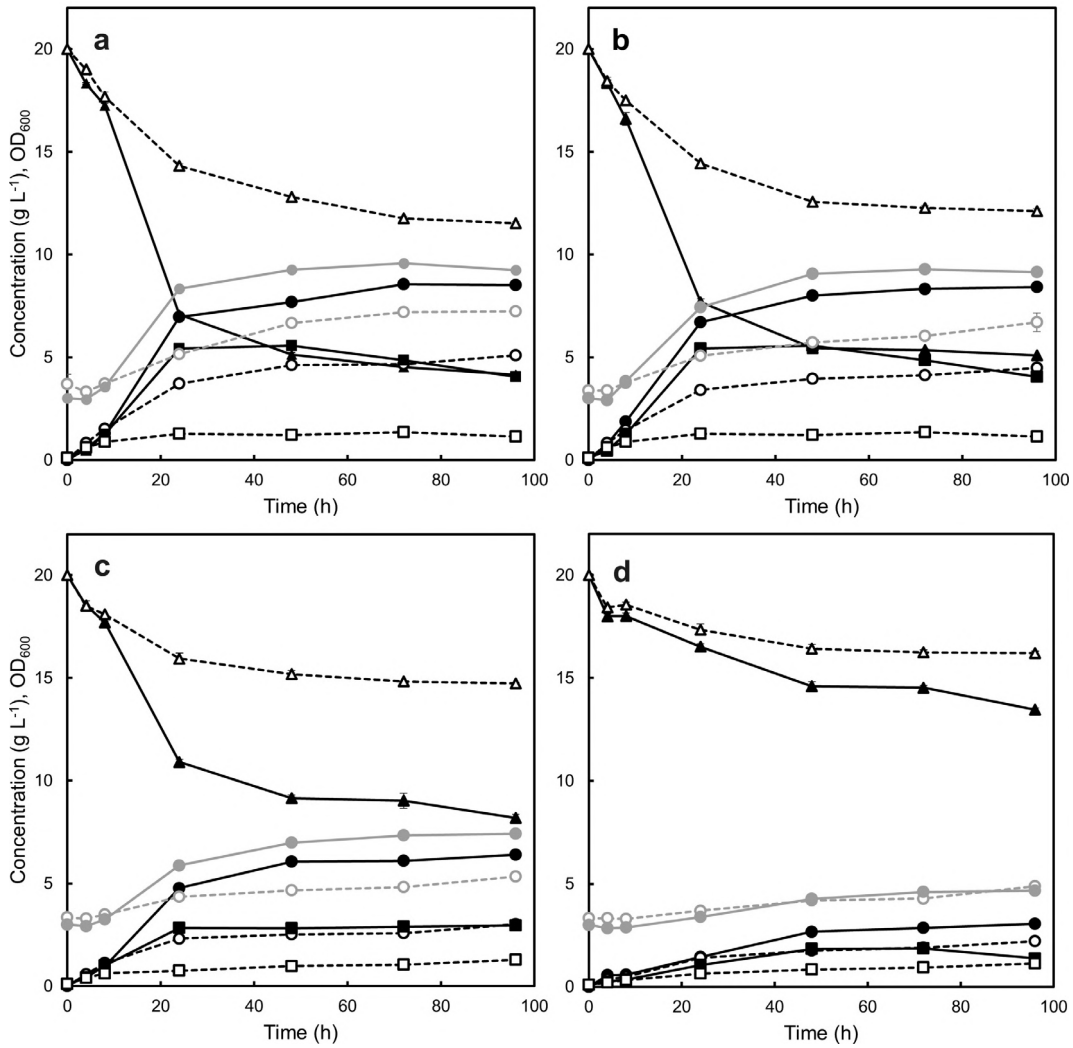

Fig. 4. Fermentation and growth time courses of MAX2 and parental strains in MRS medium with 20 g L^{-1} of xylose at pH 7 (a), pH 6 (b), pH 5 (c) and pH 4 (d). Xylose (▲, △), LA (●, ○), acetic acid (●, ○), OD_{600} (■, □). Data of MAX2 and parental strains are represented with filled and empty symbols, respectively.

improved xylose fermentation at pH 5 and 4 reached by MAX2 strain makes the resulting strain very attractive when it comes to valorization of this sugar from lignocellulosic materials. Even so, MAX2 fermentation performance at pH 4 could be further improved by ALE in terms of gradually reducing the initial pH of the culture. With this in mind, the next challenges could be focused on obtaining a new strain with higher acid pH tolerance in xylose fermentation, even below LA *pKa*.

3.3. LA production from xylose-rich synthetic media in bioreactor

L. pentosus strains were cultured in bioreactor in MRS medium with 20 g L^{-1} of xylose at initial pH 7 with and without pH adjustment (Fig. 5a). Without pH adjustment, 41 $\%Y_{maxLA}$ and 70% Y_{maxLA} were produced by parental and MAX2 strains, respectively (Table 1). These results, which were practically identical to those obtained with 20 g L^{-1} of xylose at pH 7 in flask (Section 3.2) clearly confirmed the lower requirements of pH adjustment in the evolved strain.

LA yields from xylose of both *L. pentosus* strains were considerably higher when maintaining the pH at 7 in the bioreactor when compared with non-controlled pH conditions both in bioreactor and in flask. When producing LA under pH-controlled conditions, both parental and evolved strains were able to convert most of the xylose in LA, reaching almost the maximum theoretical yield, with 94–95$\%Y_{maxLA}$ (Table 1).

Although no differences can be found between parental and MAX2 strains at the end of fermentation when controlling the pH, since xylose was exhausted in both cases, significant differences could be found during the first 16 h of fermentation (Fig. 5b, Table 1). In the same way than in flask fermentation, as a result of the ALE experiment, MAX2 strain presented higher xylose consumption rate, which concluded in a 1.4-fold increase in LA productivity at 16 h of culture, i.e. Q_{16h} = 0.44 g L^{-1} h^{-1}, while the parental strain only produced 0.31 g L^{-1} h^{-1} (Table 1). The accelerated xylose consumption of MAX2 is shown in Fig. 5b. 68%, 82% and 100% of total xylose was consumed at 16, 20 and 24 h, while the parental strain just consumed 44%, 64% and 78% at the same fermentation times. Concerning acetic acid production, 7.8 g L^{-1} were accumulated in the medium by MAX2 strain at 16 h while the parental strain only reached 5.8 g L^{-1}, verifying the acceleration of xylose uptake coupled with LA and acetic acid production. As a result, MAX2 strain reached the maximum LA titer and bacterial growth at 24 h, i.e. 11.5 g L^{-1} of LA and an OD_{600} of 5.4. In contrast, the parental strain produced only 8.5 g L^{-1} of LA in 24 h (with an OD_{600} of 2.7) and required 48 h of fermentation for xylose exhaustion and reaching the maximum LA concentration (Fig. 5b).

It can be inferred that the increase of xylose consumption rate in MAX2 strain was not only due to its increased pH tolerance because culture pH was controlled at 7 in this case. Among the different possibilities that can explain this increased xylose consumption rate, one possible mechanism may be that the prolonged exposure to this sugar

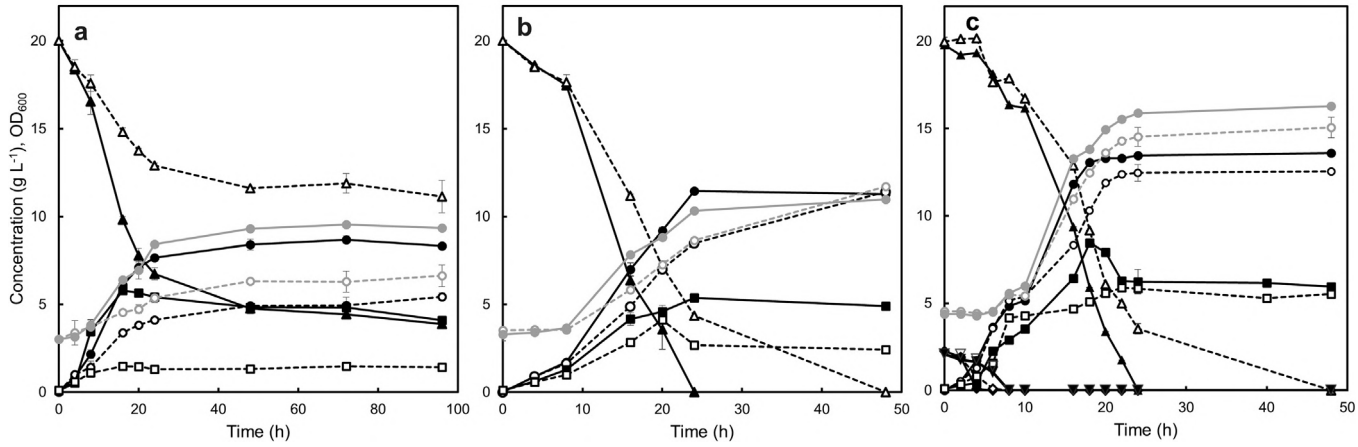

Fig 5. Fermentation and growth time courses of MAX2 and parental strains in bioreactor from MRS medium without pH control (a), MRS medium with pH control (b) and WSH with pH control (c). Xylose (▲, △), glucose (◆, ◊), arabinose (▼, ▽), LA (●, ○), acetic acid (●, ○), OD_{600} (■, □). Data of MAX2 and parental strains are represented with filled and empty symbols, respectively.

have produced an alteration in terms of glycolytic fluxes or in the expression of enzymes involved in xylose assimilation, catabolism and fermentation (Abriouel et al., 2017; Chaillou et al., 1999; Utrilla et al., 2012). For instance, when *E. coli* was engineered and adapted by serial transfers increasing xylose concentration, major changes were found in glycolytic enzymes concentration, as well as in the expression of the xylose transporter GatC (Utrilla et al., 2012).

3.4. Improved fermentation of a xylose-rich wheat straw hydrolysate by MAX2 strain

In order to determine the feasibility of the new strain to ferment lignocellulosic substrates, WSH was used as raw material for LA production. This substrate contained both C6 (glucose) and C5 sugars (xylose and arabinose) and it was diluted 1:2 in order to adjust the xylose content to a similar concentration as in the previous experiments with MRS medium. Although many works have reported the improvements in terms of LA production of new strains obtained by ALE in synthetic media (Triratna et al., 2011; Utrilla et al., 2012; Zhang et al., 2012), few reports have addressed the fermentation of a real lignocellulosic hydrolysate, checking the applicability of the evolved strains in a biorefinery.

In the same way than in fermentation tests with mixtures of sugars (Section 3.2), *L. pentosus* showed a slight CCR effect because both parental and MAX2 strains were able to co-utilize glucose and xylose, as previously pointed by Cubas-Cano et al. (2019). In the present work, it can also be noticed that arabinose was consumed together with the rest of sugars at the same time. In fact, with both strains, the whole content of glucose and arabinose present in the hydrolysate, i.e. 2 g L^{-1} of each sugar, were consumed in 8 h of culture (Fig. 5c). In the case of xylose, as in MRS medium, MAX2 strain consumed this sugar at higher rates than the parental strain. Specifically, 2.1, 7.1 and 16.4 g L^{-1} of xylose were consumed by the parental strain at 8, 16 and 24 h of fermentation, respectively, while MAX2 strain was able to consume 3.4, 10.4 and 19.8 g L^{-1} of xylose at the same fermentation times. As a result, MAX2 strain required less time than the parental strain to consume all the carbon sources of the medium (Fig. 5c). In the same way than in xylose fermentation with controlled pH, the productivity at 16 h of fermentation was 1.4-fold higher with MAX2 strain in comparison with the parental strain, switching from 0.52 to 0.74 g L^{-1} h^{-1} of LA. Nevertheless, few changes could be found in LA production at 48 h of fermentation, when all xylose was exhausted for both strains, reaching between 12.5 g L^{-1} and 13.5 g L^{-1} of LA (Table 1).

In the case of acetic acid production, 13.3 g L^{-1} and 11 g L^{-1} were accumulated in the medium at 16 h in fermentation of MAX2 and parental strain, respectively. At the end of fermentation, between 15 and 16 g L^{-1} were accumulated in both cases taking into account the acetic acid produced after steam explosion pretreatment, the sodium acetate of MRS and the acetic acid produced by *L. pentosus* from xylose. The reduction in fermentation times is a relevant fact since LA productivity is a key parameter in determining the final production cost. In the same way, *Pediococcus acidilactici* was engineered and evolved to accelerate xylose assimilation by 66 successive transfers of ALE with 40 g L^{-1} of xylose. The resulting strain was applied to the simultaneous saccharification and co-fermentation under high solids loading of wheat straw. As a result, it produced 35% more LA than the parental strain (Qiu et al., 2018). This result was similar to the 1.4-fold increase in LA from WSH attained at 16 h by MAX2 when compared with the parental strain (Fig. 5c)

At the end of fermentation, when all the sugars were depleted, Y_{maxLA} of 81 and 86% were obtained by the parental and MAX2 strains, respectively (Table 1). The slight decrease in LA yield when compared with values obtained in MRS with 20 g L^{-1} xylose fermentation in bioreactor with neutralization could be explained by the presence of pretreatment-derived inhibitors in the hydrolysate. Inhibitors like weak acids (formic and acetic acid) and furan derivatives (furfural and 5-hydroxymethylfurfural) have been reported to hinder bacterial growth by inducing reactive oxygen species (ROS) generation (Gao et al.,

Table 1
Effect of controlled pH conditions in xylose consumption and LA yields in fermentation of MRS medium with xylose and fermentation of WSH in bioreactor.

pH control	Medium	Strain	$\%XYL_{cons}$ (48 h)	$\%Y_{maxLA\ (48h)}$	Q_{16h} (g L^{-1} h^{-1})
Non-controlled	MRS 20:0	Parental	42	41	0.21 ± 0.00
		MAX2	76	70	0.38 ± 0.00
Controlled (pH 7.0)	MRS 20:0	Parental	100	94	0.31 ± 0.00
		MAX2	100	95	0.44 ± 0.02
	WSH	Parental	100	81	0.52 ± 0.00
		MAX2	100	86	0.74 ± 0.00

2017). The presence of inhibitors found in sugarcane bagasse and wheat straw was reported to cause an inhibition of *E. coli* growth by 36% and 21%, respectively. The same inhibitor concentration caused up to 4% growth inhibition of *L. pentosus* LMG 17,672 (Boguta et al., 2014). Indeed, *L. pentosus* CECT 4023 T demonstrated high tolerance to inhibitory compounds in previous works with WSH, which contained 0.52, 0.23 and 6.67 $g\,L^{-1}$ of furfural, 5-hydroxymethylfurfural and acetic acid, respectively. However, in the previous study, only 55% of total xylose was consumed due to the higher inhibitor concentration, the lack of pH control and the absence of evolutionary engineering (Cubas-Cano et al., 2019). In contrast, complete xylose consumption was attained in the present study at 24 h and 48 h by MAX2 and parental strain, respectively. Preadaptation of a *Bacillus coagulans* strain to 30% hydrolysate has recently reported to produce 2.4 $g\,L^{-1}\,h^{-1}$ of LA in simultaneous saccharification and fermentation of steam exploded wheat straw containing 0.61, 0.21 and 0.58 $g\,L^{-1}$ of furfural and 5-hydroxymethylfurfural, respectively (Aulitto et al., 2019).

When sugar-rich substrates are used for lactic fermentation, LA is produced with higher yield and purity than from lignocellulosic raw materials. However, the benefit of using these residual substrates lies in their wide availability, low cost, renewability, and no competition with food (Cubas-Cano et al., 2018). The bottlenecks when using lignocellulosic materials for LA production are the need of physicochemical pretreatment, multi-enzymatic hydrolysis steps and purification processes that harshly increase the production costs. For this reason, it would be necessary to optimize the entire value chain, including feedstock, pretreatment, fermentation and downstream processing steps (Pleissner et al., 2017). Having this in mind, a new *L. pentosus* strain has been obtained. Its improved fermentation capacity from xylose might play a key role in a lignocellulosic biorefinery.

4. Conclusions

The suitability of adaptive laboratory evolution of *Lactobacillus pentosus* to xylose-rich media has been demonstrated. Remarkable improvements in xylose uptake were found when culturing the evolved strain with and without pH control, even at acid initial pH. The higher tolerance to acidic conditions would imply low requirements of neutralizing agents reducing the final cost of the process. The great features of MAX2 strain were also shown when xylose was co-consumed with other sugars in WSH, demonstrating its potential to ferment hemicellulosic hydrolysates with improved xylose consumption rate and LA productivity.

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References

Abdel-Rahman, M.A., Sonomoto, K., 2016. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. J. Biotechnol. 236, 176–192.

Abdel-Rahman, M.A., Xiao, Y., Tashiro, Y., Wang, Y., Zendo, T., Sakai, K., Sonomoto, K., 2015. Fed-batch fermentation for enhanced lactic acid production from glucose/xylose mixture without carbon catabolite repression. J. Biosci. Bioeng. 119 (2), 153–158.

Abriouel, H., Pérez Montoro, B., Casimiro-Soriguer, C.S., Pérez Pulido, A.J., Knapp, C.W., Caballero Gómez, N., Castillo-Gutiérrez, S., Estudillo-Martínez, M.D., Gálvez, A., Benomar, N., 2017. Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence. Front. Microbiol 8 891–891.

Aulitto, M., Fusco, S., Nickel, D.B., Bartolucci, S., Contursi, P., Franzén, C.J., 2019. Seed culture pre-adaptation of *Bacillus coagulans* MA-13 improves lactic acid production in simultaneous saccharification and fermentation. Biotechnol. Biofuels 12, 45.

Bachmann, H., Molenaar, D., Branco dos Santos, F., Teusink, B., 2017. Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria. FEMS Microbiol. Rev. 41, 201–219.

Boguta, A.M., Bringel, F., Martinussen, J., Jensen, P.R., 2014. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. Microb Cell Fact. 13 (1), 97.

Bolivar-Telleria, M., Turbay, C., Favarato, L., Carneiro, T., de Biasi, R.S., Fernandes, A.A.R., Santos, A.M.C., Fernandes, P.M.B., 2018. Second-generation bioethanol from coconut husk. BioMed Res Int.

Chaillou, S., Pouwels, P.H., Postma, P.W., 1999. Transport of D-xylose in *Lactobacillus pentosus*, *Lactobacillus casei*, and *Lactobacillus plantarum*: evidence for a mechanism of facilitated diffusion via the phosphoenolpyruvate:mannose phosphotransferase system. J Bacteriol. 181 (16), 4768–4773.

Chen, R., Dou, J., 2016. Biofuels and bio-based chemicals from lignocellulose: metabolic engineering strategies in strain development. Biotechnol. Lett. 38 (2), 213–221.

Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2018. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate. Biofpr 12 (2), 290–303.

Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2019. *Lactobacillus pentosus* CECT 4023 T co-utilizes glucose and xylose to produce lactic acid from wheat straw hydrolysate: anaerobiosis as a key factor. Biotechnol. Prog. 35 (1), e2739.

Das, S.P., Ghosh, A., Gupta, A., Goyal, A., Das, D., 2013. Lignocellulosic fermentation of wild grass employing recombinant hydrolytic enzymes and fermentative microbes with effective bioethanol recovery. BioMed Res. Int.

Erdei, B., Frankó, B., Galbe, M., Zacchi, G., 2012. Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw. Biotechnol. Biofuels 5 (1), 12.

Gao, J., Feng, H., Yuan, W., Li, Y., Hou, S., Zhong, S., Bai, F., 2017. Enhanced fermentative performance under stresses of multiple lignocellulose-derived inhibitors by overexpression of a typical 2-Cys peroxiredoxin from *Kluyveromyces marxianus*. Biotechnol. Biofuels 10, 79.

Jiang, H.L., He, Q., He, Z., Hemme, C.L., Wu, L., Zhou, J., 2013. Continuous cellulosic bioethanol fermentation by cyclic fed-batch cocultivation. Appl. Environ. Microbiol. 79 (5), 1580–1589.

Juodeikiene, G., Zadeike, D., Bartkiene, E., Klupsaite, D., 2016. Application of acid tolerant Pediococcus strains for increasing the sustainability of lactic acid production from cheese whey. LWT – Food Sci. Technol. 72, 399–406.

Juturu, V., Wu, J.C., 2015. Microbial production of lactic acid: the latest development. Crit. Rev. Biotechnol. 1–11.

Kim, J.H., Shoemaker, S.P., Mills, D.A., 2009. Relaxed control of sugar utilization in *Lactobacillus brevis*. Microbiology 155 (4), 1351–1359.

Li, Y.C., Zeng, W.Y., Gou, M., Sun, Z.Y., Xia, Z.Y., Tang, Y.Q., 2017. Transcriptome changes in adaptive evolution of xylose-fermenting industrial *Saccharomyces cerevisiae* strains with delta-integration of different *xylA* genes. Appl. Microbiol. Biotechnol. 101 (20), 7741–7753.

Mans, R., Daran, J.G., Pronk, J.T., 2018. Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production. Curr. Opin. Biotechnol. 50, 47–56.

Othman, M., Ariff, A.B., Rios-Solis, L., Halim, M., 2017. Extractive fermentation of lactic acid in lactic acid bacteria cultivation: a review. Front Microbiol. 8, 2285.

Papadimitriou, K., Alegría, Á., Bron, P.A., de Angelis, M., Gobbetti, M., Kleerebezem, M., Lemos, J.A., Linares, D.M., Ross, P., Stanton, C., Turroni, F., van Sinderen, D., Varmanen, P., Ventura, M., Zúñiga, M., Tsakalidou, E., Kok, J., 2016. Stress physiology of lactic acid bacteria. Microbiol. Mol. Biol. Rev. 80 (3), 837–890.

Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W.P., Ryan, C.M., del Cardayre, S., 2002. Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat. Biotechnol. 20 (7), 707–712.

Pleissner, D., Dietz, D., van Duuren, J.B.J.H., Wittmann, C., Yang, X., Lin, C.S.K., Venus, J., 2017. Biotechnological Production of Organic Acids from Renewable Resources. In Springer, Berlin Heidelberg, pp. 1–38.

Qin, J., Wang, X., Zheng, Z., Ma, C., Tang, H., Xu, P., 2010. Production of l-lactic acid by a thermophilic *Bacillus* mutant using sodium hydroxide as neutralizing agent. Bioresour. Technol. 101 (19), 7570–7576.

Qiu, Z., Gao, Q., Bao, J., 2018. Engineering *Pediococcus acidilactici* with xylose assimilation pathway for high titer cellulosic l-lactic acid fermentation. Bioresour. Technol. 249, 9–15.

Reider Apel, A., Ouellet, M., Szmidt-Middleton, H., Keasling, J.D., Mukhopadhyay, A., 2016. Evolved hexose transporter enhances xylose uptake and glucose/xylose co-utilization in *Saccharomyces cerevisiae*. Sci. Rep. 6, 19512.

Sandberg, T.E., Lloyd, C.J., Palsson, B.O., Feist, A.M., 2017. Laboratory evolution to alternating substrate environments yields distinct phenotypic and genetic adaptive strategies. Appl. Environ. Microbiol. 83 (13).

Sauer, U., 2001. Evolutionary engineering of industrially important microbial phenotypes. Adv. Biochem. Eng. Biotechnol. 73, 129–169.

Shen, Y., Chen, X., Peng, B., Chen, L., Hou, J., Bao, X., 2012. An efficient xylose-fermenting recombinant *Saccharomyces cerevisiae* strain obtained through adaptive evolution and its global transcription profile. Appl. Microbiol. Biotechnol. 96 (4), 1079–1091.

Singhvi, M., Zendo, T., Sonomoto, K., 2018. Free lactic acid production under acidic conditions by lactic acid bacteria strains: challenges and future prospects. Appl. Microbiol. Biotechnol. 102 (14), 5911–5924.

Thomas, K.C., Ingledew, W.M., 2001. Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms AU – Narendranath, Neelakantam V. J. Am. Soc. Brew Chem. 59 (4), 187–194.

Tomás-Pejó, E., Ballesteros, M., Oliva, J.M., Olsson, L., 2010. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in

different fed-batch SSF processes. J. Ind. Microbiol. Biotechnol. 37 (11), 1211–1220.
Tomás-Pejó, E., Bonander, N., Olsson, L., 2014. Industrial yeasts strains for biorefinery solutions: constructing and selecting efficient barcoded xylose fermenting strains for ethanol. Biofpr 8 (5), 626–634.
Tomás-Pejó, E., Fermoso, J., Herrador, E., Hernando, H., Jiménez-Sánchez, S., Ballesteros, M., González-Fernández, C., Serrano, D.P., 2017. Valorization of steam-exploded wheat straw through a biorefinery approach: bioethanol and bio-oil co-production. Fuel 199, 403–412.
Tomás-Pejó, E., Oliva, J.M., Ballesteros, M., Olsson, L., 2008. Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting Saccharomyces cerevisiae strains. Biotechnol. Bioeng. 100 (6), 1122–1131.
Trcek, J., Mira, N.P., Jarboe, L.R., 2015. Adaptation and tolerance of bacteria against acetic acid. Appl. Microbiol. Biotechnol. 99 (15), 6215–6229.
Triratna, L., Saksono, B., Sukmarini, L., Suparman, A., 2011. Genome-Shuffling-Improved Acid Tolerance and Lactic Acid Production in *Lactobacillus plantarum* for Commercialization. Microb Indo 5, 10.
Utrilla, J., Licona-Cassani, C., Marcellin, E., Gosset, G., Nielsen, L.K., Martinez, A., 2012. Engineering and adaptive evolution of *Escherichia coli* for D-lactate fermentation reveals GatC as a xylose transporter. Metab Eng. 14 (5), 469–476.
Wang, Y., Li, Y., Pei, X., Yu, L., Feng, Y., 2007. Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. J. Biotechnol. 129 (3), 510–515.
Wisselink, H.W., Toirkens, M.J., Wu, Q., Pronk, J.T., van Maris, A.J., 2009. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl. Environ. Microbiol. 75 (4), 907–914.
Ye, L., Zhao, H., Li, Z., Wu, J.C., 2013. Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. Bioresour. Technol. 135, 459–463.
Yoshida, S., Okano, K., Tanaka, T., Ogino, C., Kondo, A., 2011. Homo-d-lactic acid production from mixed sugars using xylose-assimilating operon-integrated *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 92 (1), 67.
Yuan, S.F., Guo, G.L., Hwang, W.S., 2017. Ethanol production from dilute-acid steam exploded lignocellulosic feedstocks using an isolated multistress-tolerant *Pichia kudriavzevii* strain. Microb. biotechnol. 10 (6), 1581–1590.
Zhang, Y.P., 2008. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J. Ind. Microbiol. Biotechnol. 35 (5), 367–375.
Zhang, J., Wu, C., Du, G., Chen, J., 2012. Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. Biotechnol. Bioprocess. Eng. 17 (2), 283–289.

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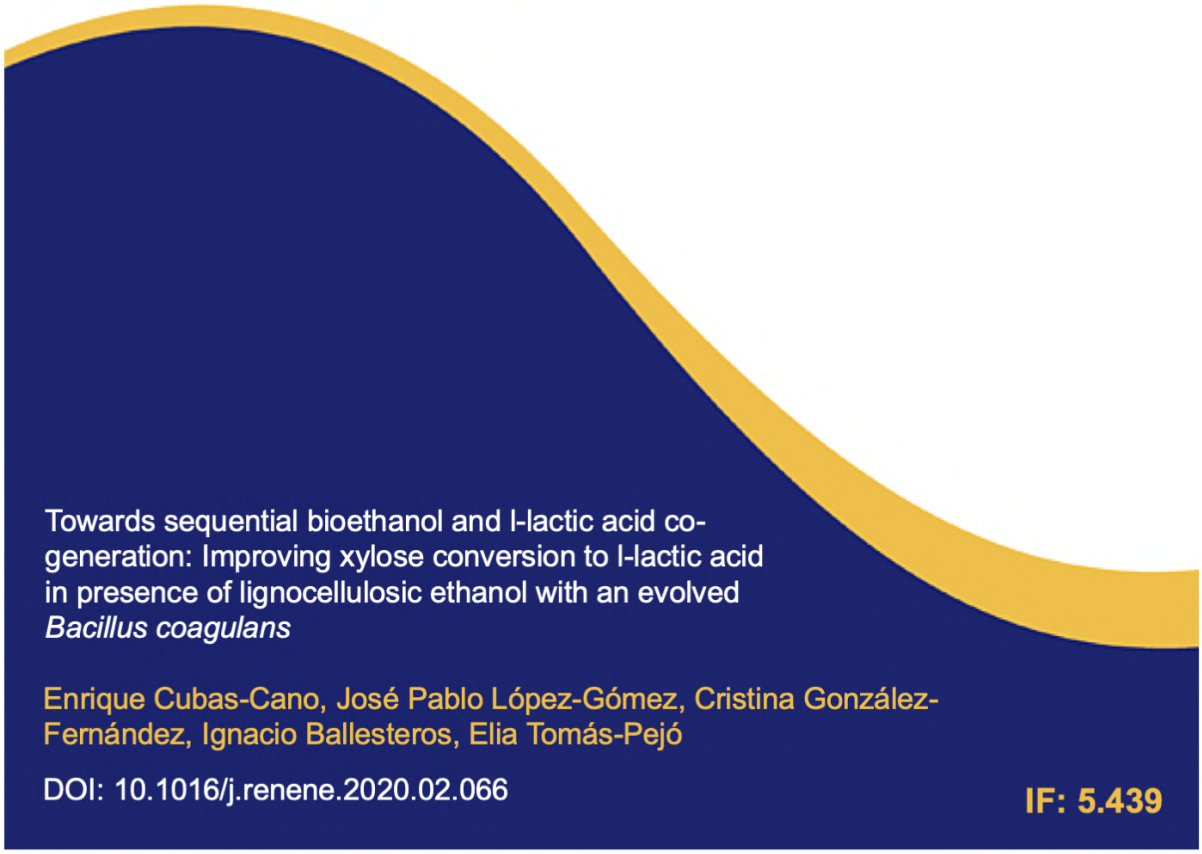

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Towards sequential bioethanol and L-lactic acid co-generation: Improving xylose conversion to L-lactic acid in presence of lignocellulosic ethanol with an evolved *Bacillus coagulans*

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ABSTRACT

A wide range of biofuels and bio-based products can be produced from lignocellulose considering its high compositional diversity. Ethanol production by yeasts from cellulosic glucose is well-known, while hemicellulosic xylose utilization is still challenging. This work proposes the use the xylose for L-lactic acid fermentation. In this context, a sequential cultivation of *Saccharomyces cerevisiae* and the C5-utilizing *Bacillus coagulans* is studied. High ethanol yields, around 0.44 g g^{-1}, were obtained from a cellulosic-gardening hydrolysate. The high ethanol concentrations did not affect the evolved *B. coagulans* A20-EXA obtained by adaptive evolution to ethanol. As a result, 2.6-fold increase in lactic acid yield was achieved when compared with parental *B. coagulans* strain in presence of 5% (v v^{-1}) ethanol. These results demonstrated the suitability of *B. coagulans* A20-EXA to be used together with *S. cerevisiae* for the sequential co-generation of ethanol and lactic acid from lignocellulosic biomass in a biorefinery approach.

1. Introduction

One of the main goals to be achieved for the coming decades is to gradually reduce the dependency on petrochemical resources. Biofuels, like bioethanol, and bio-based products, such as lactic acid (LA), can be produced by fermentation of renewable sources. In this sense, lignocellulosic biomass is the cheapest, widest distributed and most abundant renewable carbon source in the world, with an annual production of 10^{10} MT, including different agricultural, forestry and industrial residues [1,2]. Although higher fermentation yields are always obtained when pure sugars or starch-rich feedstock are fermented, the use of lignocellulosic biomass avoids any impact on the food chain [2]. However, due to the close association of the three components of lignocellulose (cellulose, hemicellulose and lignin), a pretreatment and an enzymatic hydrolysis are needed to allow the release of the sugars contained in the cellulose and hemicellulose fractions before fermentation [1].

Bioethanol from lignocellulose is considered a feasible option for fossil fuels replacement and its market size is expected to increase owing its wide application as a fuel additive [3,4]. Bioethanol can also be converted into other chemicals such as carboxylic acids and ethylene, that can be used as starting point for the production of a variety of polymers and green solvents [3]. However, one of the current technical limitations of ethanol production from lignocellulose is that most of the industrial *Saccharomyces cerevisiae* strains can metabolize hexoses from the cellulosic fraction but not pentoses, such as xylose, derived from the hemicellulosic fraction [5].

Apart from bioethanol, many other bio-based products can be produced from lignocellulosic biomass due to their high compositional diversity [6]. For instance, LA is an organic acid with a wide variety of applications. Several lactic acid-producing bacteria can produce LA not only from C6 but also from C5 sugars of lignocellulose. Indeed, *Bacillus coagulans* strains are able to ferment pentoses such as D-xylose and L-arabinose, through the pentoses phosphate (PP) pathway, producing pure L-LA enantiomer [7]. Pure enantiomers, not produced in LA chemical synthesis, are required for the polymerization of LA into a crystalline and stable poly-lactic

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acid (PLA), suitable to be used as a sustainable packaging material [2,8].

Many works have highlighted the interest in reaching a complete valorisation of the different lignocellulosic sugars, producing an array of products such as bioethanol, biogas, fatty acids, xylitol and LA [9–15]. In this sense, co-production of biochemicals along with biofuels is an attractive approach that deserves further research and development. With this in mind, implementation of multi-product lignocellulosic biorefineries might be achieved in future decades, coupling the reduction of organic wastes with the production of essential commodities that nowadays are produced from petrochemical sources.

In this work, an integrated sequential ethanol and L-LA fermentation process has been implemented to valorise gardening residues. These organics resides (e.g. tree branches from pruning, hedge cuttings, leaves, grass clippings, etc.) are produced in large quantities due to expansion of urban green areas and, therefore, they offer an interesting source of lignocellulosic biomass. In the first stage, glucose fermentation of a gardening hydrolysate was carried out by *S. cerevisiae* and, in the second stage, the resulting ethanol-rich medium was consecutively fermented by *B. coagulans* to convert xylose into L-LA. The resulting ethanol and LA-rich medium could be purified by reactive distillation, including: i) esterification of both compounds into ethyl lactate, without the need of adding external ethanol, ii) distillation of ethyl lactate and iii) hydrolysis into ethanol and L-LA [16].

Since high ethanol concentrations may produce a loss of membrane integrity and secondary effects on bacterial metabolism [17], an Adaptive Laboratory Evolution (ALE) experiment in chemostat with increasing ethanol concentrations was carried out in this study. The improved ethanol tolerance of the resulting bacteria was evaluated in defined and gardening media, checking its suitability to be used together with yeasts for the sequential production of bioethanol and PLA-grade L-LA in an integrated biorefinery-based process.

2. Materials and methods

2.1. Microorganisms and preculture media

The industrial strain *S. cerevisiae* Ethanol Red® was provided by Fermentis S.L., Lessafre (France). The microorganism was maintained in YPD (yeast extract, peptone and dextrose) medium in agar plates, containing (g L^{-1}): glucose, 20; yeast extract, 10; peptone, 20; agar, 20. For preculture, one colony was inoculated in 250-mL flasks with 100 mL of YPD medium. After 16 h of incubation at 32 °C and 150 rpm, precultures were centrifuged at 4000 rpm for 4 min at 4 °C. Supernatant was discarded and cells were diluted to obtain the desired inoculum size.

Bacillus coagulans A20 was kindly provided by the Bioengineering Department of Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB Potsdam, Germany). For preculture, an aliquot of 200 μL of cells from glycerol stocks was inoculated in 25 mL of modified Man, Rogosa and Sharpe medium (mMRS), containing (g L^{-1}): xylose, 20; yeast extract, 5; beef extract, 10; peptone, 10; sodium acetate, 5; ammonium citrate, 2; K_2HPO_4, 2; $MgSO_4 \bullet 7\ H_2O$, 0.2, $MnSO_4 \bullet H_2O$, 0.05 and $CaCO_3$, 10. The preculture was grown for 16 h (until the culture reached the late exponential growth phase) at 52 °C and 150 rpm in strict anaerobic conditions.

2.2. Adaptive Laboratory Evolution of Bacillus coagulans A20 in chemostat cultivation

A 1.5-L bioreactor (Minifors2; Infors-HT; Switzerland) with 0.5 L of mMRS (40 g L^{-1} of xylose) at 52 °C and 150 rpm was used for the adaptive evolution experiment. Strict anaerobic conditions were obtained by sparging the medium with 0.5 L min^{-1} of nitrogen gas until the pO_2 sensor (HAMILTON; USA) detected a 0% (v v^{-1}) oxygen. pH was automatically controlled at 7 with NaOH 5 M. ALE started by inoculating an aliquot of cells (5% v v^{-1}) from the preculture. Different input and output flow values were screened before starting ALE (including, 42, 32 and 21 mL h^{-1}) to determine the conditions that maximized xylose consumption without washing the cells out of the reactor. Consequently, input/output flow was set at 21 mL h^{-1}. Samples were taken daily and when an improvement in bacterial growth (measured as OD_{600}), xylose consumption and L-LA production was detected, ethanol concentration in the evolution media was increased. Ethanol concentration in the mMRS medium of the input flow was gradually increased as evolution proceeded, being: 0, 2, 3, 4, 5 and 6% (v v^{-1}).

2.3. Comparison experiments with parental and evolved B. coagulans

Ethanol tolerance assays with *B. coagulans* A20 parental and evolved strains were performed at 52 °C and 150 rpm. Fermentation was carried out in 120-mL anaerobic clamped flasks with 25 mL of mMRS medium containing 20 g L^{-1} of xylose as carbon source. Fermentations in the presence of 0, 4 and 6% (v v^{-1}) ethanol were performed. The pH was initially adjusted to 7.0 by adding NaOH 5 M. Furthermore, to maintain the pH along fermentation, 10 g L^{-1} of $CaCO_3$ was added. The ratio $CaCO_3$:sugars of 1:2 was optimized in previous experiments (data not shown). Clamped flasks were flushed with filtered nitrogen using a needle (gas inlet) at 0.5 bar for 2 min to achieve strict anaerobic conditions. The contained air, including oxygen, was ejected through another needle (gas outlet). The gas composition in clamped flasks was analysed by gas chromatography to corroborate that flasks contained less than 5% of oxygen (v v^{-1}).

All fermentation tests were carried out in triplicate. Samples were taken at 0, 4, 8, 24, 32, 48, 72, 96 and 120 h of fermentation for the quantification of residual sugars and fermentation products.

2.4. Lignocellulosic biomass: cellulosic gardening hydrolysate

The gardening residues contained (% w w^{-1}): cellulose, 24.8; hemicellulose, 15.7; lignin, 21.6; extractives, 18.2; proteins, 7.2; acetyl groups, 2.1; ashes, 7.2; other compounds, 3.2. The biomass was milled in a laboratory cutting mill (SM 2000, Retsch, Germany) with sieve of 10 mm to obtain milled gardening chips. After aqueous extraction and steam explosion at 180 °C for 10 min and H_2SO_4 0.33 M impregnation (60 mg of H_2SO_4 per gram of biomass), the resulting slurry was filtered to obtain two different fractions, i.e., water insoluble solids (WIS) and liquid fraction. The WIS fraction represented 60% of the initial insoluble weight after aqueous extraction and pretreatment. In this fraction, 85.5% of glucose and 21.5% of xylose was recovered, while 9% of glucose 63% of xylose was recovered in the liquid fraction, in relation with the starting material. WIS fraction was used in a Separate Hydrolysis and Fermentation (SHF) process. Since this work was focused in the inhibition caused by high ethanol concentrations, the liquid fraction obtained from the slurry was not used to avoid the negative effect that inhibitory degradation compounds cause on yeast and bacteria. The WIS fraction contained (% w w^{-1}): cellulose, 40.6; hemicellulose, 4.6; lignin, 49.2; acetyl groups, 1.1 and ashes, 4.3. Enzymatic hydrolysis of pretreated gardening residues was performed with 25% of total solids (w w^{-1}) at 50 °C and pH 5.0 for 72 h. The following enzymes dosages were added: Cellic-CTec2® (Novozymes, Denmark), 32 FPU g^{-1} of glucan; Cellic-HTec2® (Novozymes, Denmark), 0.9 mg of protein g^{-1} glucan and

Viscozyme L (Merck, Germany), 4.5 mg protein g^{-1} of glucan. The resulting cellulosic gardening hydrolysate (GH) was supplemented with (g L^{-1}): yeast extract, 5; NH_4Cl, 2; KH_2PO_4, 1 and $MgSO_4 \bullet 7H_2O$, 0.3 and the pH was adjusted to 5.5. The GH was used as fermentation medium for ethanol production with *S. cerevisiae*.

2.5. Batch ethanol fermentation of gardening hydrolysate by Saccharomyces cerevisiae

Ethanol fermentation of cellulosic GH was performed in triplicate in 250-mL flasks with a working volume of 100 mL. Incubation was carried out at 32 °C and 150 rpm for 24 h. 1 g L^{-1} cells (dry weight) were used as inoculum. Samples were withdrawn at 1, 2, 3, 4, 5, 6, 7, 8, 14, 16, 18, 20, 22 and 24 h of fermentation. The pH was set at 7.0 by NaOH 5 M addition. The resulting ethanol-rich gardening hydrolysate (E-GH) was used for LA fermentation.

2.6. Lactic acid production from xylose in ethanol-rich gardening hydrolysate by Bacillus coagulans A20 parental and evolved strains

LA fermentations of the E-GH were carried out at 52 °C and 150 rpm in anaerobic conditions using 120-mL clamped flasks with a working volume of 25 mL. Since this material derived from the hydrolysis of the WIS fraction and did not contain C5 sugars, around 25 g L^{-1} of xylose were added, together with the previously mentioned mMRS compounds. The pH was also maintained at 5.0 by adding 12.5 g L^{-1} of $CaCO_3$ (half of sugars concentration) to the E-GH.

Lactic fermentations started by inoculating an aliquot of cells (5% v v^{-1}) from the preculture. All fermentation tests were carried out in triplicate. Samples were taken at 0, 4, 8, 24, 48, 72 and 120 h of fermentation.

2.7. Calculations

The enzymatic hydrolysis yields (Y_{EH}) were calculated considering the final concentration of monomeric sugars (FS) at 72 h of hydrolysis, and the potential monomeric sugars (PS) in WIS, as indicated hereafter:

(1) $Y_{EH} = [(FS) \div (PS)] \times 100$

LA yield (Y, g g^{-1}) was defined as the LA produced (g L^{-1}) at the end of fermentation per initial sugar (glucose and/or xylose) (g L^{-1}). LA volumetric productivity (Q, g L^{-1} h^{-1}) was calculated as the LA concentration produced (g L^{-1}) at 24 h.

Statistical analyses were carried out using the software Microsoft Excel. Results are given as the average ± standard deviation for descriptive statistics. In order to determine the statically significant differences between fermentations with the parental and the evolved strains, analysis of variance (ANOVA) was carried out. The level of significance was set at $p < 0.05$.

2.8. Analytical methods

The raw material and WIS fraction were analysed using the National Renewable Energy Laboratory (NREL) standard methods for determination of structural carbohydrates and lignin in biomass (LAP-002, LAP-003, LAP-004, LAP-017, and LAP-019). Dry weight of WIS and slurry was determined by drying samples at 105 °C for 24 h (LAP-001).

The gas composition in clamped flasks used for ALE was analysed by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (70 × 1/8″ O.D.) and a MS13X4-09SF2 40/60 P packed column (9’ × 1/8” O.D.) (PerkinElmer).

Cell growth was assessed by measuring optical density in a spectrophotometer (SPECTROstar® Omega) at 600 nm.

For the quantification of sugars and fermentation products, samples were taken from the fermentation broth and centrifuged at 14.000 g for 4 min. When fermentations in flasks were performed with $CaCO_3$, H_2SO_4 0.2 M was added to the samples to release the free LA from the calcium lactate (100 μL of H_2SO_4 2 M were added to 900 μL of sample). Then, samples were filtered using membrane filters (Thermo Scientific® Nylon 0.2 μm) and stored at −20 °C until analysed. pH was measured using a benchtop pH meter (Sension + PH31). Glucose, xylose, ethanol and LA were quantified by HPLC equipped with a refractive index detector. An Aminex HPX-87H column (Bio-Rad Labs, Hercules, CA) was employed at the following conditions: column temperature, 50 °C; mobile phase, 5 mM H_2SO_4; flow rate, 0.5 mL min^{-1} and injection volume, 20 μL.

3. Results and discussion

3.1. Adaptive Laboratory Evolution to increase ethanol tolerance in B. coagulans

In a chemostat cultivation, a constant environment is maintained by the continuous addition of nutrients coupled with the outflow of cells and fermented medium. Bacterial population does not undergo the different batch cultivation phases, being maintained in the exponential phase [18]. The exposure of cells to low pH values and/or nutrients-starvation, as it is the case in batch cultivation of LA-producing strains, makes the evolved phenotype more unpredictable [19]. In this sense, ALE in batch mode also resulted in an increased tolerance to acid culture pH in previous work [5].

In this work, *B. coagulans* A20 was cultured for 80 days (831 generations) in a bioreactor with xylose as the carbon source (40 g L^{-1}) at increasing ethanol concentrations, i.e., 0, 2, 4, 5 and 6% (v v^{-1}). Higher concentrations were not tested during ALE since 5–6% (v v^{-1}) of ethanol production is considered a feasible titer to make distillation economically feasible [20]. Chemostats with at least 20 generations are normally used for evolution studies, in which cases, changes in fermentation fitness are already expected [18]. Fig. 1 shows changes in bacterial growth (OD_{600}) and LA production throughout ALE. When ethanol concentration was firstly increased, a decrease in growth and LA production was observed for some generations. Nonetheless, once the bacteria became adapted to the environment, growth and LA values considerably increased. This phenomenon was more pronounced on day 20 when ethanol concentration was firstly set at 5% (v v^{-1}) (40 g L^{-1}). OD_{600} abruptly decreased from 4.7 to 1.2 and the LA content in the bioreactor dropped from 33.9 to 2.6 g L^{-1} (Fig. 1). After some generations under these stressful conditions, bacterial growth rose again, becoming stable and reaching an OD_{600} of 3.2 on day 30. By the end of ALE, with 6% (v v^{-1}) of ethanol (47 g L^{-1}) in the medium, an OD_{600} of 5.0 and a LA titer of 35.6 g L^{-1} were reached, almost identical results than the ones obtained at day 2, with 2% (v v^{-1}) of ethanol (Fig. 1).

Ethanol is known to increase membrane fluidization and to hinder the performance of membrane-embedded proteins [17]. As a result, membrane permeability is increased, facilitating intracellular acidification [21]. Different protection responses to face the ethanol challenge have been reported in LAB. *Lactobacillus plantarum*, *Lactobacillus acetotolerans* and *Oenococcus oeni* are known to reduce their cell membrane fluidity, by modifying its fatty acids composition, after the prolonged exposure to different ethanol concentrations, ranging from 8 to 12% (v v^{-1}) [17,22–25]. As a result, different cell morphology changes have been reported by scanning electron microscopy analysis, like a rougher appearance of

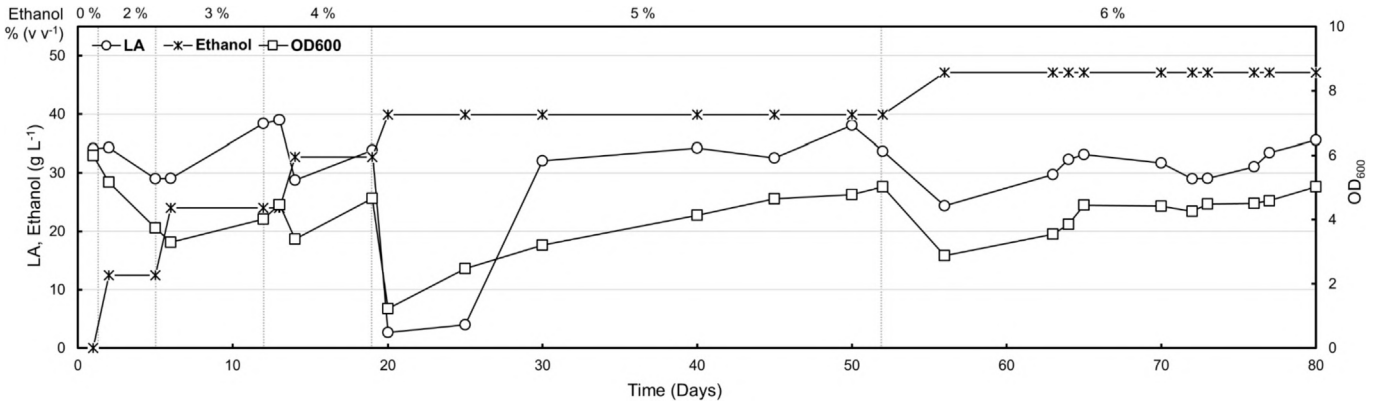

Fig. 1. Adaptive Laboratory Evolution to increased ethanol concentrations with *Bacillus coagulans* A20.

L. plantarum cells [17] and an increased cell length in *L. acetotolerans* [22] after 24 h of exposure to synthetic media with 8 and 12% (v v^{-1}) ethanol, respectively, in comparison with the controls. As well, an aggregation of *O. oeni* cells was seen as the level of ethanol increased in the medium [26].

Apart from that, ethanol presence is also involved in the production of reactive oxygen species (ROS), causing oxidative damage [23]. Induced expression of different stress regulators has also been reported as a cross-protection to heat and oxidative stresses [17,23]. In addition, overexpression of citrate metabolism was observed in some LAB, in order to generate membrane potential and produce more ATP to balance the energy loss produced by ethanol stress [17,21].

Successful ethanol adaptation by ALE has also been reported for the LAB *O. oeni* and the yeasts *Candida intermedia* and *Kluyveromyces marxianus* [27–29] (Table 1). These microorganisms are commonly found in ethanol-rich media since they are used for malolactic fermentation in wine production and ethanol fermentation, respectively. To the best of our knowledge, no previous ALE-based ethanol adaptation has been performed on lactic acid-producing bacteria for the co-production of ethanol and L-LA from lignocellulosic biomass. Results presented herein indicated that the new lactic acid-producing bacteria obtained by ALE, named as A20 Ethanol and Xylose Adapted (A20-EXA), could have higher ethanol tolerance than the parental strain and could be used as a robust L-LA producer from xylose and ethanol-riches lignocellulosic materials.

3.2. Screening ethanol tolerance of Bacillus coagulans A20 and A20-EXA in defined medium

LA fermentation in defined mMRS with xylose as carbon source (20 g L^{-1}) and different ethanol concentrations, i.e. 0, 4 and 6% (v v^{-1}), was carried out with *B. coagulans* A20 and A20-EXA. As expected, no changes between both bacteria were found in fermentations with defined medium without ethanol, with practically identical fermentation profiles (Fig. 2a). The same LA titer was obtained, reaching a Y_{PS} of 1 g g^{-1} of xylose (Table 2), the theoretical yield from this sugar by the PP pathway [7].

When adding 4% (v v^{-1}) ethanol, high differences were observed during fermentation time courses. Xylose consumption at 24 h increased from 29% to 47% when comparing A20 with A20-EXA (Fig. 2b). Although the theoretical maximum LA from xylose was reached in both cases by the end of fermentation (120 h), 1.7-fold improvement in LA productivity at 24 h was achieved by A20-EXA (Table 2).

The highest A20-EXA improvements, in comparison with the parental strain, were attained in fermentation of mMRS with 6% (v v^{-1}) ethanol. In this case, only 41% of xylose was consumed by *B. coagulans* A20, reaching a Y_{PS} of 0.37 g g^{-1} at the end of the fermentation (Table 2). Conversely, A20-EXA was able to consume 93% of the xylose (Fig. 2c). As a result, 0.89 g of LA per gram of xylose were produced, meaning 2.4-fold increase in Y_{LA} (Table 2).

As previously mentioned, malolactic bacteria and yeasts have been adapted to ethanol in other works using ALE. After 330 generations of continuous cultivation of *O. oeni* at increasing ethanol

Table 1
Compilation of different experiments with wild type and evolved microorganisms subjected to ethanol stress in biotechnological processes.

Medium	Ethanol % (v v^{-1})	Microorganisms	Approach	Results	Reference
Defined (modified MRS)	15	*O. oeni*	Directed evolution to withstand high ethanol concentrations	The new strain A90 completed malolactic fermentation in 40% of the time required by the parental	[26]
Defined (YPX)	4.5	*C. intermedia*	ALE to improve xylose consumption at increased concentrations of inhibitors and ethanol	The evolved strain was able to ferment xylose in presence of 4.5% (v v^{-1}) ethanol, while the parental strain was not able to grow	[27]
Defined (YPX)	6	*K. marxianus*	ALE in 6% (v v^{-1}) ethanol	2-fold increase in biomass in comparison with the parental strain	[28]
Furfural residues	0.5	*S. cerevisiae* and different LAB	SSF with a simultaneous mixed culture	Higher cellulose conversion rate in comparison with separate ethanol and lactic SSF. When using high substrate concentration at 38 °C, an acid/ethanol ratio close to 1 was obtained.	[32]
Corn stalk	6.4 (0.7)[a]	*S. cerevisiae* M3013 and *B. coagulans* LA1507	Sequential cultivation	0.46 g g^{-1} and 0.86 g g^{-1} of ethanol and LA, respectively, were yielded	[13]

[a] Ethanol concentration inside the fermentor was drastically reduced by different aeration stages before LA fermentation.

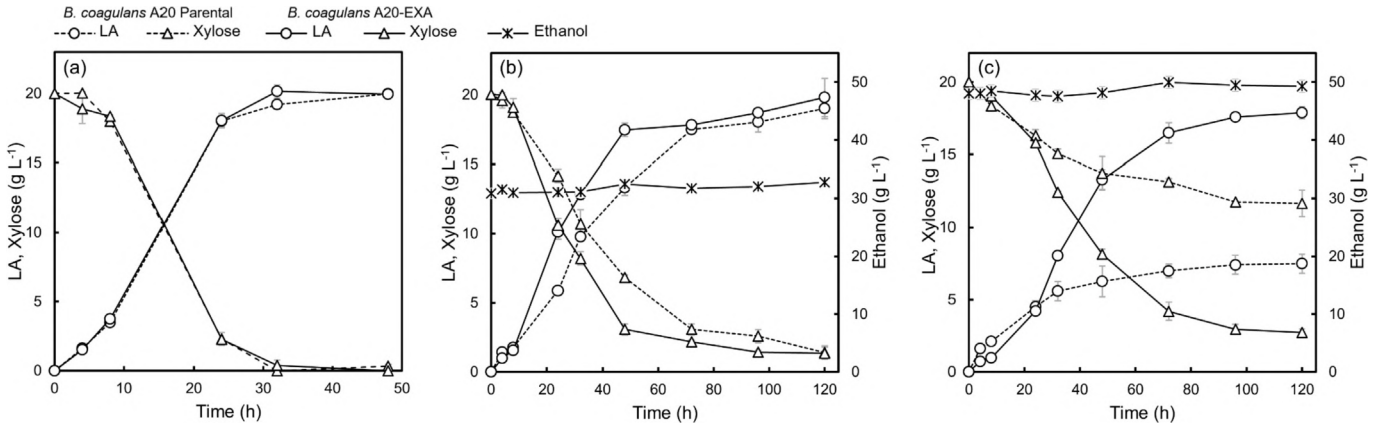

Fig. 2. Screening for LA fermentation with parental and evolved strains in mMRS media with 0 (a), 4 (b) and 6 (c) % (v v^{-1}) ethanol.

Table 2
Lactic acid fermentation of ethanol-rich defined and gardening media comparing *B. coagulans* A20 and A20-EXA.

Medium	Ethanol (g L^{-1})	Ethanol % (v v^{-1})	Strain	LA (g L^{-1})	Y_{LA} (g g^{-1})	Q_{LA24h} (g L^{-1} h^{-1})
mMRS	0.00	0	A20	19.97	1.00[a]	0.75
			A20-EXA	19.94	1.00[a]	0.75
	32.00	4	A20	19.02	0.95[b]	0.25
			A20-EXA	19.80	0.99[b]	0.42
	47.00	6	A20	7.49	0.37[b]	0.19
			A20-EXA	17.88	0.89[b]	0.18
E-GH	40.45	5	A20	8.35	0.34[b]	0.19
			A20-EXA	22.15	0.89[b]	0.14

[a] Y_{LA} at 48 h of fermentation.
[b] Y_{LA} at 120 h of fermentation.

concentrations, a strain with 56% improvement in fermentation time in the presence of 15% (v v^{-1}) ethanol was obtained (Table 1) [27]. Xylose consumption was enhanced in *C. intermedia* when fermenting a medium supplemented with 35.5 g L^{-1} ethanol after evolutionary engineering at increasing ethanol and lignocellulosic inhibitors concentration (Table 1) [28]. After 100 days of ALE in 6% (v v^{-1}) ethanol, a population of *K. marxianus* with increased ethanol tolerance up to 10% (v v^{-1}) was obtained (Table 1) [29]. However, the mentioned strains were not used in LA production and, therefore, direct comparison could not be done.

In bioethanol production processes, the final concentration must be above 40 g L^{-1} in order to achieve an economically-viable bioprocess [20]. Since *B. coagulans* A20-EXA was able to produce high L-LA yields even in the presence of nearly 50 g L^{-1} of ethanol, this new lactic acid-producing bacteria was subsequently used for sequential yeast-LAB cultivation in lignocellulosic materials.

3.3. Ethanol fermentation of glucose in cellulosic gardening hydrolysate by Saccharomyces cerevisiae

In order to study the feasibility of sequential ethanol-LA co-generation, a WIS fraction derived from steam-exploded gardening residues was subjected to a SHF process. 103 g L^{-1} of glucose were measured in the GH after hydrolysis, meaning that 86% of glucose was released.

After *S. cerevisiae* fermentation, almost 44 g L^{-1} of ethanol were produced (Fig. 3a), meaning a Y_{EtOH} of 0.44 (86% of the theoretical yield). Similar results to the ones attained in this study were reported in fermentation of sulfite pretreated lodgepole pine whole hydrolysate with *S. cerevisiae* D5A, reaching 90.1% of the theoretical yield [30]. 90% of ethanol was also yielded after SHF of a hydrolysate obtained after pretreatment of mixtures of spruce bark and wood chips [31]. SSF of steam exploded branched pruned from pear trees with *K. marxianus* NBRC resulted in 76% of the theoretical yield [32]. Overall, results obtained for ethanol production were in good agreement with literature using similar substrates and fermentation approaches. The resulting Ethanol-rich Gardening Hydrolysate (E-GH) was used for L-LA production.

3.4. Lactic acid production from xylose in ethanol-rich gardening hydrolysate by Bacillus coagulans parental and evolved bacteria

B. coagulans A20 and A20-EXA were cultured in E-GH, which contained around 5% (v v^{-1}) (40.5 g L^{-1}) ethanol and 25 g L^{-1} of added xylose (Table 2). These ethanol concentrations were slightly lower than the one obtained after *S. cerevisiae* fermentation due to the processing of the media for LA fermentation, including pH adjustment, that produced a dilution effect. Moreover, part of the ethanol content could have been evaporated during the process.

High differences were shown between A20 and A20-EXA in E-GH fermentation. While only 40% of xylose was consumed by the former, the 25 g L^{-1} of xylose were completely depleted by the evolved population (Fig. 3b). As a result, a Y_{LA} of 0.89 g g^{-1} was reached, meaning 2.6-fold increase in comparison with the parental strain (Table 2). It is worth to mention that the Y_{LA} attained in this case from a lignocellulosic medium with 5% (v v^{-1}) ethanol was identical to the one obtained from mMRS medium with 6% (v v^{-1}) ethanol, in which case 2.4-fold increase was reached. Therefore, the results were consistent with previous experiments in defined medium. LA production and sugar consumption in A20 and A20-EXA followed a slow rate in the first 48 h of culture (Fig. 3b). Most probably, cell energetic supplies were mainly being used to induce and run protection responses against the high ethanol content, which explains the large lag phase and the low LA productivity at 24 h of A20-EXA (Table 2). After that, an increased xylose consumption and LA production rate was shown by the

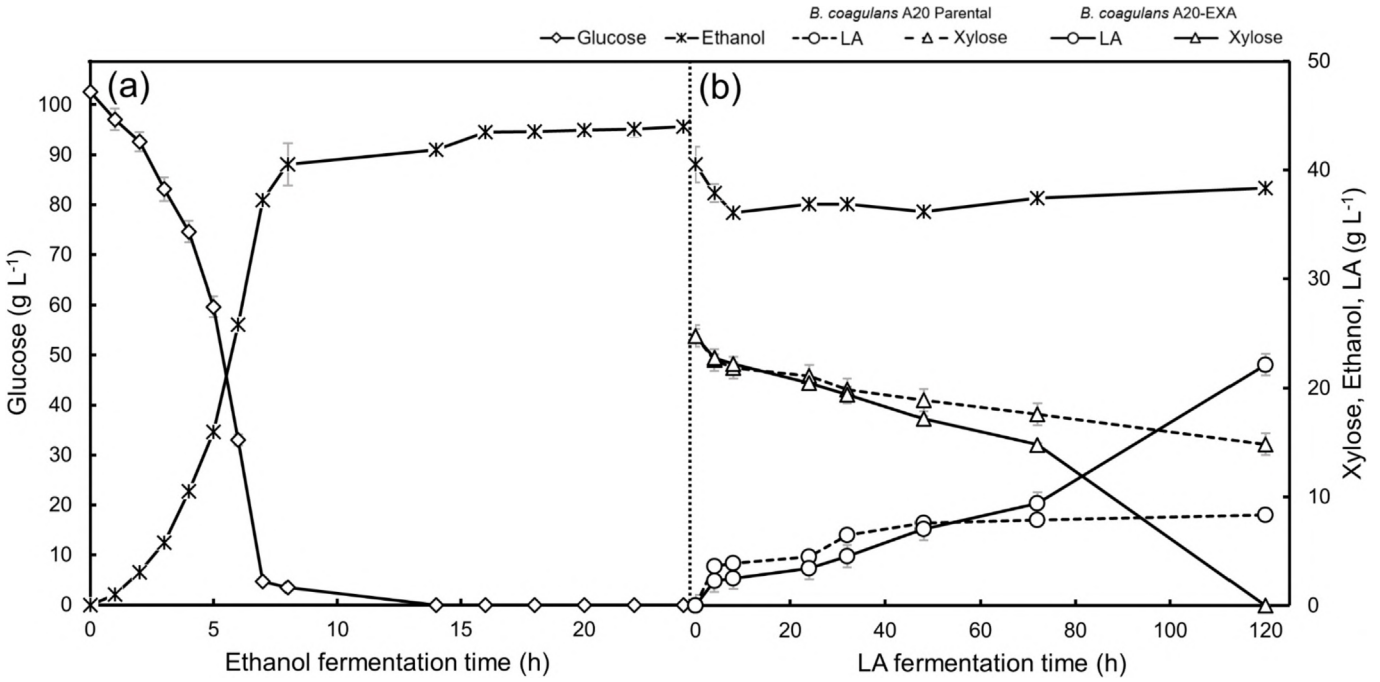

Fig. 3. Sequential ethanol-LA fermentation with *S. cerevisiae* Ethanol Red and *B. coagulans* A20 or A20-EXA. Ethanol fermentation of cellulosic GH (a), LA fermentation of E-GH (b).

evolved population until the end of fermentation (Fig. 3b).

As mentioned, higher results were obtained in fermentation of xylose added to ethanol-rich GH by the new engineered *B. coagulans* A20-EXA in comparison with the parental strain. The novelty of the present work lies, therefore, in the evolutionary approach carried out to increase ethanol tolerance, focusing on the use of the resulting strain together with *S. cerevisiae* on bioethanol and L-LA co-generation. In this work, glucose released from gardening residues was previously used for ethanol production, while synthetic xylose was subsequently added for L-LA production, as a first approximation. Future studies will address the conversion of both lignocellulosic glucose and xylose into ethanol and L-LA, respectively, via sequential cultivation of yeast and bacteria.

Co-generation and multi-generation of different products from residual substrates is being studied as a promising approach for future biorefineries. Few studies have been focused on ethanol and LA co-generation via simultaneous or sequential cultivation of microorganisms. SSF of furfural residues with a simultaneous mixed culture of LAB and *S. cerevisiae* showed high cellulose conversion rate and LA concentration by fluctuating temperature along culture (Table 1) [33]. Co-generation of ethanol and L-LA from corn stalk by sequential cultivation of *S. cerevisiae* M3013 and *B. coagulans* LA1507 yielded 0.46 g g^{-1} and 0.86 g g^{-1} of ethanol and LA, respectively (Table 1) [13]. These results were similar to the ones obtained in this study with gardening residues, 0.44 g g^{-1} and 0.89 g g^{-1} of ethanol and LA, respectively. However, different aeration stages were applied by Wang and co-workers for the gas stripping of ethanol, reducing its concentration from 50.50 to less than 6 g L^{-1}, in which case an ethanol-tolerant lactic acid-producing bacteria was not required [13]. The evolved *B. coagulans* obtained in the present study was able to tolerate even 40 g L^{-1} of ethanol in gardening hydrolysate, so ethanol removal by gas stripping was not required, avoiding the corresponding operational costs at industrial scale. In fact, it would be possible to purify LA and ethanol at the end of the process, without the need of removing ethanol before LA fermentation. Since the boiling point of water is higher than ethanol and lower than LA, ethanol could be separated firstly by distillation (78.4 °C), while L-LA could be subsequently separated by electrodialysis followed by ion exchange chromatography and distillation [34]. Reactive distillation can also be used in this process, taking advantage of esterification of both L-LA and ethanol to allow a direct purification of ethyl lactate, which can be distillated in one step. Ethyl lactate can be used as an industrial relevant product, but it can also be subjected to hydrolysis with water in presence of an ion-exchange resin in order to separate both products and yield pure ethanol and PLA-grade L-LA [17].

4. Conclusions

The suitability of yeast-bacteria sequential cultivation for the co-generation of ethanol and L-LA was assessed. High ethanol yields (0.46 g g^{-1}) were obtained by *S. cerevisiae* from glucose after SHF of gardening residues, producing an ethanol-rich gardening hydrolysate. An evolutionary engineering approach was carried out to increase the tolerance of *B. coagulans* A20 to high ethanol concentrations. As a result, 0.89 g g^{-1} of LA were obtained by the evolved A20-EXA from xylose added to the ethanol-rich gardening hydrolysate, meaning 2.6-fold improvement in comparison with the parental strain. Although, the liquid fraction from the slurry was not used in this work to avoid the combined effect of inhibitory degradation compounds and ethanol, it would be interesting to address the use of lignocellulosic xylose instead of synthetic in future works. The above results verified the increased ethanol tolerance of the evolved population, which would avoid the need of ethanol removal before the inoculation of the bacteria. All in all, the valorisation of both cellulosic and hemicellulosic fractions may circumvent the duplication of expensive operations, such as enzymatic hydrolysis and purification. In a nutshell, development of an ethanol tolerant *B. coagulans* by ALE is proposed as an innovative approach that can be applied in yeast-bacterial sequential fermentation for bioethanol and L-LA co-generation. This method can increase the valorisation of different sugars from heterogeneous lignocellulosic materials, making a great contribution to the implementation of biorefineries.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Enrique Cubas-Cano: Conceptualization, Investigation, Writing - original draft. **José Pablo López-Gómez:** Resources, Writing - original draft. **Cristina González-Fernández:** Writing - review & editing. **Ignacio Ballesteros:** Resources, Methodology. **Elia Tomás-Pejó:** Conceptualization, Writing - review & editing, Supervision.

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References

[1] P. Alvira, E. Tomas-Pejo, M. Ballesteros, M.J. Negro, Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review, Bioresour. Technol. 101 (2010) 4851–4861, https://doi.org/10.1016/j.biortech.2009.11.093.

[2] E. Cubas-Cano, C. González-Fernández, M. Ballesteros, E. Tomás-Pejó, Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate, Biofuels, Bioprod. Biorefin. (2018) n/a, https://doi.org/10.1002/bbb.1852.

[3] S. Choi, C.W. Song, J.H. Shin, S.Y. Lee, Biorefineries for the production of top building block chemicals and their derivatives, Metab. Eng. 28 (2015) 223–239, https://doi.org/10.1016/j.ymben.2014.12.007.

[4] A.D. Moreno, D. Ibarra, P. Alvira, E. Tomas-Pejo, M. Ballesteros, A review of biological delignification and detoxification methods for lignocellulosic bioethanol production, Crit. Rev. Biotechnol. 35 (2015) 342–354, https://doi.org/10.3109/07388551.2013.878896.

[5] E. Cubas-Cano, C. González-Fernández, E. Tomás-Pejó, Evolutionary engineering of Lactobacillus pentosus improves lactic acid productivity from xylose-rich media at low pH, Bioresour. Technol. 288 (2019) 121540, https://doi.org/10.1016/j.biortech.2019.121540.

[6] M. Bilal, M. Asgher, H.M.N. Iqbal, H. Hu, X. Zhang, Biotransformation of lignocellulosic materials into value-added products—a review, Int. J. Biol. Macromol. 98 (2017) 447–458, https://doi.org/10.1016/j.ijbiomac.2017.01.133.

[7] V. Juturu, J.C. Wu, Microbial production of lactic acid: the latest development, Crit. Rev. Biotechnol. (2015) 1–11. https://www.ncbi.nlm.nih.gov/pubmed/26260005.

[8] J.P. López-Gómez, M. Latorre-Sánchez, P. Unger, R. Schneider, C.C. Lozano, J. Venus, Assessing the organic fraction of municipal solid wastes for the production of lactic acid, Biochem. Eng. J. (2019) 107251, https://doi.org/10.1016/j.bej.2019.107251.

[9] K.K. Cheng, J.A. Zhang, E. Chavez, J.P. Li, Integrated production of xylitol and ethanol using corncob, Appl. Microbiol. Biotechnol. 87 (2010) 411–417, https://doi.org/10.1007/s00253-010-2612-5.

[10] D. Pleissner, K.Y. Lau, R. Schneider, J. Venus, C.S.K. Lin, Fatty acid feedstock preparation and lactic acid production as integrated processes in mixed restaurant food and bakery wastes treatment, Food Res. Int. 73 (2015) 52–61, https://doi.org/10.1016/j.foodres.2014.11.048.

[11] M.-S. Kim, J.-G. Na, M.-K. Lee, H. Ryu, Y.-K. Chang, J.M. Triolo, Y.-M. Yun, D.-H. Kim, More value from food waste: lactic acid and biogas recovery, Water Res. 96 (2016) 208–216, https://doi.org/10.1016/j.watres.2016.03.064.

[12] F. Demichelis, D. Pleissner, S. Fiore, S. Mariano, I.M. Navarro Gutierrez, R. Schneider, J. Venus, Investigation of food waste valorization through sequential lactic acid fermentative production and anaerobic digestion of fermentation residues, Bioresour. Technol. 241 (2017) 508–516, https://doi.org/10.1016/j.biortech.2017.05.174.

[13] Y. Wang, J. Liu, D. Cai, G. Zhao, Co-generation of ethanol and l-lactic acid from corn stalk under a hybrid process, Biotechnol. Biofuels 11 (2018) 331, https://doi.org/10.1186/s13068-018-1330-6.

[14] L. Zhang, T. You, L. Zhang, M. Li, F. Xu, Comprehensive utilization of waste hemicelluloses during ethanol production to increase lactic acid yield: from pretreatment to fermentation, Biotechnol. Biofuels 7 (2014) 494, https://doi.org/10.1186/s13068-014-0189-4.

[15] P.-M. Bondesson, A. Dupuy, M. Galbe, G. Zacchi, Optimizing ethanol and methane production from steam-pretreated, phosphoric acid-impregnated corn stover, Appl. Biochem. Biotechnol. 175 (2015) 1371–1388, https://doi.org/10.1007/s12010-014-1358-4.

[16] P. Khunnonkwao, C. Ariyawong, W. Lertsiriyothin, A. Boontawan, Purification of D-(-)-Lactic acid from fermentation broth using nanofiltration, esterification, distillation, and hydrolysis technique, Adv. Mater. Res. 550–553 (2012) 2945–2952. https://doi.org/10.4028/www.scientific.net/AMR.550-553.2945.

[17] H. van Bokhorst-van de Veen, T. Abee, M. Tempelaars, P.A. Bron, M. Kleerebezem, M.L. Marco, Short- and long-term adaptation to ethanol stress and its cross-protective consequences in Lactobacillus plantarum, Appl. Environ. Microbiol. 77 (2011) 5247–5256, https://doi.org/10.1128/AEM.00515-11.

[18] U. Sauer, Evolutionary engineering of industrially important microbial phenotypes, Adv. Biochem. Eng. Biotechnol. 73 (2001) 129–169.

[19] H. Bachmann, D. Molenaar, F. Branco dos Santos, B. Teusink, Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria, FEMS Microbiol. Rev. 41 (2017) S201–S219, https://doi.org/10.1093/femsre/fux024.

[20] G. Zacchi, A. Axelsson, Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions, Biotechnol. Bioeng. 34 (1989) 223–233, https://doi.org/10.1002/bit.260340211.

[21] N. Olguin, A. Bordons, C. Reguant, Influence of ethanol and pH on the gene expression of the citrate pathway in Oenococcus oeni, Food Microbiol. 26 (2009) 197–203, https://doi.org/10.1016/j.fm.2008.09.004.

[22] X. Yang, K. Teng, J. Zhang, F. Wang, T. Zhang, G. Ai, P. Han, F. Bai, J. Zhong, Transcriptome responses of Lactobacillus acetotolerans F28 to a short and long term ethanol stress, Sci. Rep. 7 (2017) 2650, https://doi.org/10.1038/s41598-017-02975-8.

[23] M.G. Silveira, M. Baumgärtner, F.M. Rombouts, T. Abee, Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of Oenococcus oeni, Appl. Environ. Microbiol. 70 (2004) 2748–2755, https://doi.org/10.1128/aem.70.5.2748-2755.2004.

[24] B.M. Bravo-Ferrada, A. Gomez-Zavaglia, L. Semorile, E.E. Tymczyszyn, Effect of the fatty acid composition of acclimated oenological Lactobacillus plantarum on the resistance to ethanol, Lett. Appl. Microbiol. 60 (2015) 155–161, https://doi.org/10.1111/lam.12350.

[25] C. Grandvalet, J.S. Assad-Garcia, S. Chu-Ky, M. Tollot, J. Guzzo, J. Gresti, R. Tourdot-Marechal, Changes in membrane lipid composition in ethanol- and acid-adapted Oenococcus oeni cells: characterization of the cfa gene by heterologous complementation, Microbiology 154 (2008) 2611–2619, https://doi.org/10.1099/mic.0.2007/016238-0.

[26] A.M.D. Elahwany, Genetic and physiological studies on Oenococcus oeni PsuI in response to ethanol stress, Acta Biol. Hung. 63 (2012) 128–137, https://doi.org/10.1556/ABiol.63.2012.1.10.

[27] A.L. Betteridge, K.M. Sumby, J.F. Sundstrom, P.R. Grbin, V. Jiranek, Application of directed evolution to develop ethanol tolerant Oenococcus oeni for more efficient malolactic fermentation, Appl. Microbiol. Biotechnol. 102 (2018) 921–932, https://doi.org/10.1007/s00253-017-8593-x.

[28] A.D. Moreno, A. Carbone, R. Pavone, L. Olsson, C. Geijer, Evolutionary engineered Candida intermedia exhibits improved xylose utilization and robustness to lignocellulose-derived inhibitors and ethanol, Appl. Microbiol. Biotechnol. 103 (2019) 1405–1416, https://doi.org/10.1007/s00253-018-9528-x.

[29] W. Mo, M. Wang, R. Zhan, Y. Yu, Y. He, H. Lu, Kluyveromyces marxianus developing ethanol tolerance during adaptive evolution with significant improvements of multiple pathways, Biotechnol. Biofuels 12 (2019) 63, https://doi.org/10.1186/s13068-019-1393-z.

[30] H. Zhou, T. Lan, B.S. Dien, R.E. Hector, J.Y. Zhu, Comparisons of five Saccharomyces cerevisiae strains for ethanol production from SPORL-pretreated lodgepole pine, Biotechnol. Prog. 30 (2014) 1076–1083, https://doi.org/10.1002/btpr.1937.

[31] B. Frankó, M. Galbe, O. Wallberg, Influence of bark on fuel ethanol production from steam-pretreated spruce, Biotechnol. Biofuels 8 (2015) 15, https://doi.org/10.1186/s13068-015-0199-x.

[32] C. Sasaki, R. Okumura, C. Asada, Y. Nakamura, Steam explosion treatment for ethanol production from branches pruned from pear trees by simultaneous saccharification and fermentation, Biosci. Biotechnol. Biochem. 78 (2014) 160–166, https://doi.org/10.1080/09168451.2014.877818.

[33] Yong Tang, Danqing Zhao, Liwei Zhu, Jianxin Jiang, Simultaneous saccharification and fermentation of furfural residues by mixed cultures of lactic acid bacteria and yeast to produce lactic acid and ethanol, Eur. Food Res. Technol. 233 (2011), https://doi.org/10.1007/s00217-011-1542-5, 489-495–2011 v.233 no.3.

[34] A.-K. Neu, D. Pleissner, K. Mehlmann, R. Schneider, G.I. Puerta-Quintero, J. Venus, Fermentative utilization of coffee mucilage using Bacillus coagulans and investigation of down-stream processing of fermentation broth for optically pure l(+)-lactic acid production, Bioresour. Technol. 211 (2016) 398–405, https://doi.org/10.1016/j.biortech.2016.03.122.