

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS



TESIS DOCTORAL

Valorización de microalgas para la producción de ácidos grasos volátiles, aceites microbianos y biogás,
en un contexto de biorrefinería

Microalgae biomass valorization for volatile fatty acids, microbial oils and biogas production in a biorefinery
context

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Memoria para optar al grado de Doctor presentada por:

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PhD THESIS

Microalgae biomass valorization for volatile fatty acids, microbial
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INFORMAN

Que el trabajo de investigación titulado “Valorización de microalgas para la producción de ácidos grasos volátiles, aceites microbianos y biogás, en un contexto de biorrefinería / Microalgae biomass valorization for volatile fatty acids, microbial oils and biogas production in a biorefinery context” ha sido realizado bajo su dirección en la Unidad de Procesos Biotecnológicos de IMDEA Energía, y constituye la memoria que presenta Dña. Mercedes Llamas Redondo para optar al Grado de Doctor.

Y para que conste a los efectos oportunos, firman la presente, en Madrid a 17 de Marzo de 2021.

Dra. Cristina González Fernández

Dra. Elia Tomás Pejó

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ABBREVIATIONS

A: Alcalase
AD: Anaerobic digestion
AF: Anaerobic fermentation
AnMBR: Anaerobic membrane bioreactor
BMP: Biomethane potential
COD: Chemical oxygen demand
CSTR: Continuous stirred tank reactor
FA: Fatty acid
HRT: Hydraulic retention time
LB: Lipid bodies
MO: Microbial oils
NP: Non pretreated
OD: Optical density
OY: Oleaginous yeasts
OLR: Organic loading rate
SRT: Solid retention time
sCH: Soluble carbohydrates
sCOD: Soluble chemical oxygen demand
sPr: Soluble proteins
STP: Standard temperature and pressure
T: Thermal
tCH: total carbohydrates
tCOD: Total chemical oxygen demand
tPr: Total proteins
TS: Total solid
UASB: Up-flow anaerobic sludge blanket reactor
V: Viscozyme
VFAs: Volatile fatty acids
VS: Volatile solids

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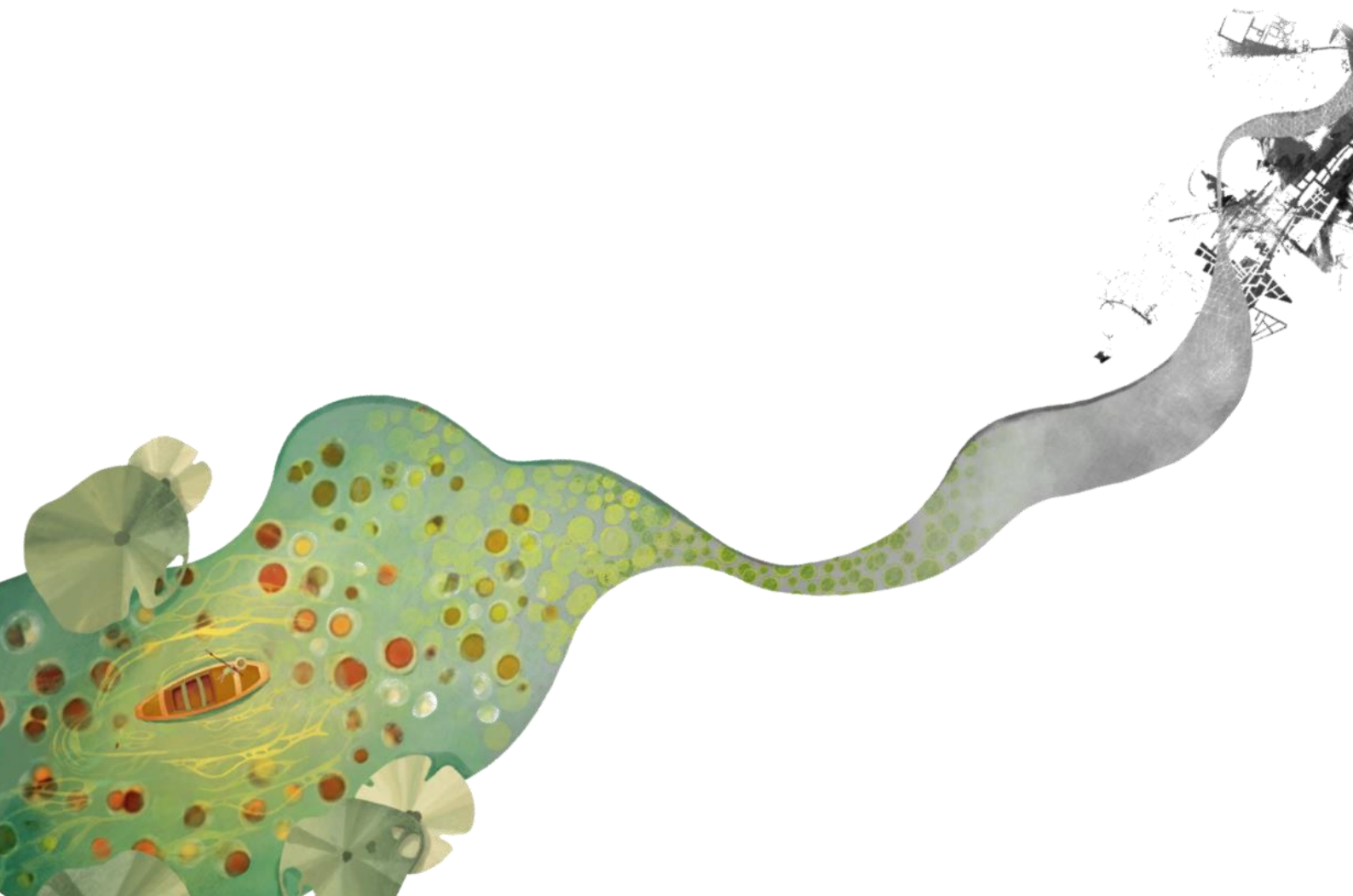
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ABSTRACT

RESUMEN



ABSTRACT

Residual microalgae biomass, generated in the context of wastewater treatment, has been traditionally devoted to biogas production via anaerobic digestion (AD). Following an innovative biorefinery approach, value-added products can be firstly produced, generating concomitantly low-cost products, such as energy carriers, from the spent biomass (Publication II).

Among the bioproducts, volatile fatty acids (VFAs) are regarded as building blocks with a wide range of industrial applications. VFAs can be obtained by an unbalanced AD, known as anaerobic fermentation (AF). After VFAs generation, obtained AF effluent can be further used with a two-fold purpose. The VFAs-rich liquid fraction arises as low-cost carbon source for microbial oils (MO) production by means of oleaginous yeasts (OY) fermentation, while the remaining solid fraction can be harnessed via conventional AD for biogas production.

For this purpose, the main objective of this Thesis was to evaluate the parameters that allow the optimization of multi-product generation (VFAs, MO and biogas) from microalgae biomass by integrating three fermentative bioprocesses (namely AF, oleaginous fermentation and AD).

In the first part of this Thesis, some challenges hindering VFAs production from microalgae via AF were tackled. Due to the hard cell wall of certain strains, microalgae biomass requires pretreatments to render the organic matter available for AF. One thermal and two enzymatic pretreatments (protease and carbohydrase) were tested on *Chlorella vulgaris* biomass prior to AF (Publication I). Protease pretreatment resulted the most efficient in terms of microalgae solubilization (38.6 % hydrolytic efficiency) and bioconversions into VFAs (33.4 % $\text{VFA}_{\text{COD}}/\text{tCOD}_{\text{in}}$), displaying a 4-fold enhancement compared with raw biomass. These results confirmed that macromolecule-specific pretreatments were essential and highlighted the key role of proteins during AF of microalgae biomass.

AF operational parameters were tuned to avoid VFAs consumption by methanogenic archaea during methane production. The effect of temperature was assessed by using adapted sludge to psychrophilic (25 °C) and thermophilic (55 °C) temperatures. Despite using temperature-adapted inocula, 25 °C promoted higher bioconversions into VFAs (34.2 % $\text{VFA}_{\text{COD}}/\text{tCOD}_{\text{in}}$) than 55 °C (14.1 % $\text{VFA}_{\text{COD}}/\text{tCOD}_{\text{in}}$). This Thesis evidenced the need of working at low temperatures during AF to slow down methanogenic activity while maximizing VFAs yields. The effect of hydraulic retention time (HRT) reduction in VFAs production during AF was also evaluated. HRT values below 10 days prevented methanogenic activity. However, reducing HRT below a critical value resulted in harsh conditions for acidogenic bacteria as well, which ultimately caused low VFAs bioconversions. HRT reduction in a stepwise mode favored a microbial adaptation to operational conditions. This fact allowed to work at lower HRT values (HRT 4 days) than when low HRT was directly imposed (HRT 6 days) without affecting the bioconversions efficiencies (32 % $\text{VFA}_{\text{COD}}/\text{tCOD}_{\text{in}}$).

A major challenge associated to the use of VFAs is the need of extraction or even purification. To overcome this drawback, the direct use of VFAs-rich digestates as low-cost carbon sources for OY to produce MO arises as an outstanding alternative to the traditional sugar-based substrates (Publication IV). These MO are expected to have a great impact in the oleochemical sector in the near future.

To gain insights on yeast's VFAs tolerance, uptake preference and lipid accumulation capacity, individual and mixtures of synthetic VFAs as well as real digestate were tested. The ability of *Yarrowia lipolytica* to grow on VFAs as unique carbon source was demonstrated (Publication V), exhibiting biomass concentrations and yields close to those achieved from glucose. Long-chain VFAs exerted higher inhibitory effect on OY than short-chain VFAs. Besides, *Y. lipolytica* sequentially utilized different types of VFAs, showing preference for acetic acid coupled to the consumption of long-chain VFAs. When using real VFAs-rich digestate as culture medium, biomass production was 2.4-fold higher than the obtained in synthetic medium despite having the same VFA concentration and profile. This positive effect was attributed to the presence of nutrients (such as nitrogen, phosphorous, etc.). In fact, the inhibitory effect of VFAs at

high concentrations (above 10 g VFAs/L) on *Y. lipolytica* was overcome when using real digestate as substrate.

In the search of efficient OY to produce MO from VFAs-rich digestates, five OY were screened at three VFAs concentrations (5, 10 and 15 g VFAs/L) (Publication VI). The highest biomass yields were exhibited by *Cryptococcus curvatus*, *Lipomyces lipofer* and *Y. lipolytica* (values higher than 0.3 g/g). *Williopsis saturnus* and *C. curvatus* showed lipid contents up to 36.9 % (w/w) and 33.9 % (w/w) respectively, corresponding to the highest lipids yields (0.11-0.13 g/g). Moreover, the fatty acid profile produced by all strains was similar to that of vegetable oils commonly used in the industry. This part of the Thesis confirmed the feasibility of using VFAs-rich effluents as innovative culture medium for OY.

Lastly, aiming at a full valorization of AF streams, the residual solid fraction recovered after the VFAs-rich liquid separation was used as substrate for biogas production. This Thesis assessed methane potential of this fraction via conventional AD in batch assays (Publication II). The high methane yield (187.3 mL CH₄/g tCOD_{in}) and biodegradability (57 % COD removal) obtained in batch assays proved the suitability of the solid spent as substrate for biogas production. This bioprocess was validated in CSTR (Publication III), resulting in 168.9 mL CH₄ /g tCOD_{in} and 48.3 % biodegradability. These values were competitive with respect to the reported when microalgae biomass was digested for biogas production. This Thesis demonstrated that the solid residue biodegradability was enhanced by the previous AF process, which in turns boosted biogas production in AD.

Overall, the entire microalgae biomass valorization by the implementation of three bioprocesses was proved. The integration of AF, AD and OY fermentation supports not only wastes treatment but also carbon recovery maximization through an efficient multi-product generation (VFAs, MO and biogas).

RESUMEN

Las microalgas generadas durante el tratamiento de aguas residuales han sido usadas tradicionalmente para producir biogás mediante digestión anaerobia (DA). Bajo el concepto innovador de biorrefinería, pueden obtenerse bioproductos de alto valor añadido y posteriormente, otros de menor valor a partir de los subproductos generados (Artículo II).

Entre ellos, los ácidos grasos volátiles (AGV) son considerados moléculas de interés con multitud de aplicaciones industriales. Estos compuestos pueden producirse mediante una DA desbalanceada, conocida como fermentación anaerobia (FA). El efluente de la FA puede ser usado con una doble finalidad. La fracción líquida, rica en AGV, se presenta como una fuente de carbono de bajo coste para la producción de aceites microbianos (AM) mediante fermentación con levaduras oleaginosas (LO). Por otro lado, el residuo sólido puede ser aprovechado como sustrato de la DA generando biogás.

Siguiendo este enfoque, el objetivo principal de esta Tesis fue evaluar los parámetros que permiten optimizar la generación de múltiples productos (AGV, AM y biogás) a partir de microalgas integrando tres bioprocesos (FA, fermentación oleaginosa y DA).

En una primera parte de esta Tesis, se abordaron algunos de los retos que dificultan la producción eficiente de AGV a partir de microalgas. La robusta pared celular de ciertas cepas de microalgas, hace necesaria la aplicación de pretratamientos que mejoren la disponibilidad de la materia orgánica. Por ello, se evaluaron tres pretratamientos en *Chlorella vulgaris* previos a la FA: uno térmico y dos enzimáticos (proteasa y carbohidrasa) (Artículo I). El tratamiento proteolítico resultó ser el más eficiente en términos de solubilización (38.6 % eficiencia hidrolítica) y de bioconversión a AGV (33.4 % $\text{AGV}_{\text{DQO}}/\text{tDQO}_{\text{in}}$) siendo 4 veces superior a la obtenida usando microalgas sin pretratar. Esto demostró la importancia de aplicar pretratamientos específicos, destacando el papel fundamental de las proteínas durante la FA de microalgas.

Los parámetros operacionales de la FA fueron ajustados para evitar la competencia por AGV de las arqueas durante la producción de metano. El efecto de la temperatura durante la FA se evaluó usando fangos adaptados a temperaturas psicrófilas (25 °C) y

termófilas (55 °C). Como resultado, se obtuvo una mayor bioconversión a AGV durante la FA a 25 °C (34.2 % AGV_{DQO}/tDQO_{in}) que a 55 °C (14.1 % AGV_{DQO}/tDQO_{in}). Esto evidenció la necesidad de trabajar a bajas temperaturas, reduciendo así la actividad metanogénica y maximizando la producción de AGV. Por otro lado, también se evaluó el efecto del tiempo hidráulico de retención (THR) en la producción de AGV. Aunque la aplicación de THR inferiores a 10 días previene la actividad metanogénica, un valor excesivamente bajo puede resultar perjudicial para las bacterias acidogénicas. La reducción progresiva del THR propició la adaptación de la población microbiana a las condiciones operacionales. Esto permitió disminuir el THR hasta valores inferiores a los que se puede trabajar cuando un THR bajo es inicialmente aplicado (THR 4 días vs 6 días, respectivamente), sin comprometer la bioconversión a AGV.

Una de las principales limitaciones en la aplicación de los AGV es su extracción o purificación. Para evitarla, el uso de digestatos ricos en AGV como fuente de carbono para producir AM mediante LO se presenta como una alternativa prometedora a los azúcares tradicionalmente empleados para este fin (Artículo IV). Se prevé que estos AM tengan un gran impacto en el sector de la oleoquímica en un futuro próximo.

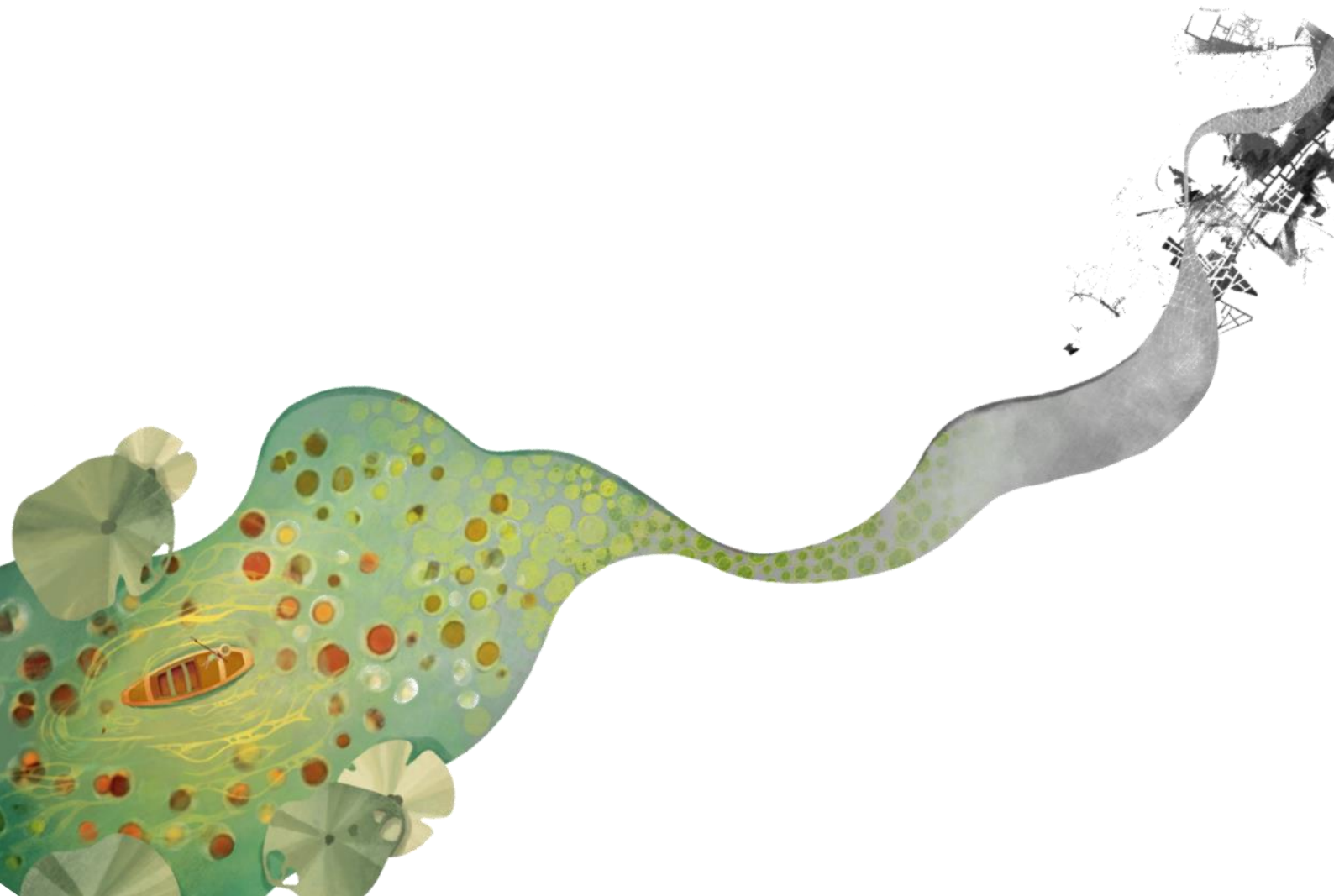
Para conocer el efecto de los AGV en *Yarrowia lipolytica* y las preferencias de consumo, se evaluaron diversos medios sintéticos (AGV individuales y mezclas) y digestato real como medio de cultivo. *Y. lipolytica* demostró ser capaz de crecer usando AGV como única fuente de carbono (Artículo V) alcanzando rendimientos de biomasa cercanos a los obtenidos a partir de azúcares. Los AGV de cadena larga resultaron tener un efecto inhibitorio en la LO superior a los obtenidos con AGV de cadena corta. Además, el consumo de AGV por parte de *Y. lipolytica* se realizó de un modo secuencial, mostrando preferencia por el ácido acético en presencia de AGV de cadena larga. El uso de digestato como medio de cultivo, dio lugar a una producción de biomasa 2,4 veces superior a la obtenida empleando un medio sintético con similar concentración y perfil de AGV. Este efecto positivo fue atribuido a la presencia de nutrientes en el digestato (nitrógeno, fósforo, etc.). De hecho, al usar digestato real como medio de cultivo, disminuyó el efecto inhibitorio provocado por concentraciones superiores a 10 g AGV/L en *Y. lipolytica*.

Posteriormente, se realizó un cribado de cinco LO a tres concentraciones de AGV (5, 10 y 15 g/L) con el objetivo de encontrar las cepas más eficientes en producción de AM a partir de digestatos ricos en AGV (Artículo VI). Los mejores resultados en términos de crecimiento fueron obtenidos por *Cryptococcus curvatus*, *Lipomyces lipofer* y *Y. lipolytica* (0,3 g biomasa/g AGV). *Williopsis saturnus* y *C. curvatus* mostraron una acumulación de lípidos de 36,9 % p/p y 33,9 % p/p, respectivamente, resultando en los mayores rendimientos de lípidos (0,11-0,13 g/g). Además, el perfil de ácidos grasos generado por todas las cepas fue similar a la composición de los aceites vegetales usados industrialmente. Esta Tesis, por tanto, confirmó la viabilidad de usar efluentes ricos en AGV como medio de cultivo prometedor para las LO.

Por último, para aprovechar completamente el efluente de la FA, la fracción sólida obtenida tras la separación de la fase líquida rica en AGV, se usó para producir biogás mediante DA. En primer lugar, se evaluó el potencial de la fracción sólida como sustrato de la DA en discontinuo (Artículo II). El alto rendimiento de producción de metano (187,3 mL CH₄/g tDQO_{in}) y la biodegradabilidad (57 % eliminación de DQO) obtenidos demostraron su idoneidad para ser usado en la producción de biogás. Este proceso fue validado en continuo (Artículo III), resultando en 168,9 mL CH₄ /g tDQO_{in} y una biodegradabilidad del 48,3 %. Estos valores resultaron competitivos con los obtenidos usando microalgas para producir biogás. Por tanto, se demostró que la aplicación del proceso de FA previo a la DA aumentó la biodegradabilidad del residuo sólido.

En esta Tesis se demostró la valorización completa de microalgas integrando tres bioprocesos (FA, DA y fermentación oleaginoso). Esto no solo permitió el tratamiento de residuos orgánicos, sino maximizar la recuperación de carbono a través de una generación eficiente de múltiples productos (AGV, MO y biogás).

1. INTRODUCTION



1. Introduction

Growing population, resources limitation, climate change and ecosystem degradation force us to seek for new production pathways that comply with the ecological boundaries of our planet. Among them, replacing fossil-derived compounds is considered a top priority in today's society. In fact, the European Commission has launched the "Green Deal" with the target to convert Europe in a greenhouse gases emissions neutral continent by 2050 (European Commission, 2019). To achieve such a goal, energy and chemical industries are key players to be taken into consideration. In this context, the implementation of sustainable conversion processes of renewable feedstock into products and fuels is of utmost importance.

Oposite to the conventional refineries that produce fuels and chemicals from fossil resources, biorefineries convert biomass into a range of marketable bio-based products and bioenergy (Motola et al., 2018). Biomass is defined as "the biodegradable fraction of products, wastes and residues with biological origin derived from agriculture, forestry and related industries, as well as from industrial and municipal wastes" (European Commission, 2019). The wide availability and versatile chemical composition of biomass stand out as their main advantages.

Biomass has appeared in the last years as a promising raw material for the bio-based industries to produce chemicals, plastics, pharmaceuticals, biofuels and bioenergy (Figure 1.1). The analysis of the Eurostat data shows that the total bioeconomy turnover ranged 2.5 billion € in the EU-28 (Piotrowski et al., 2020).

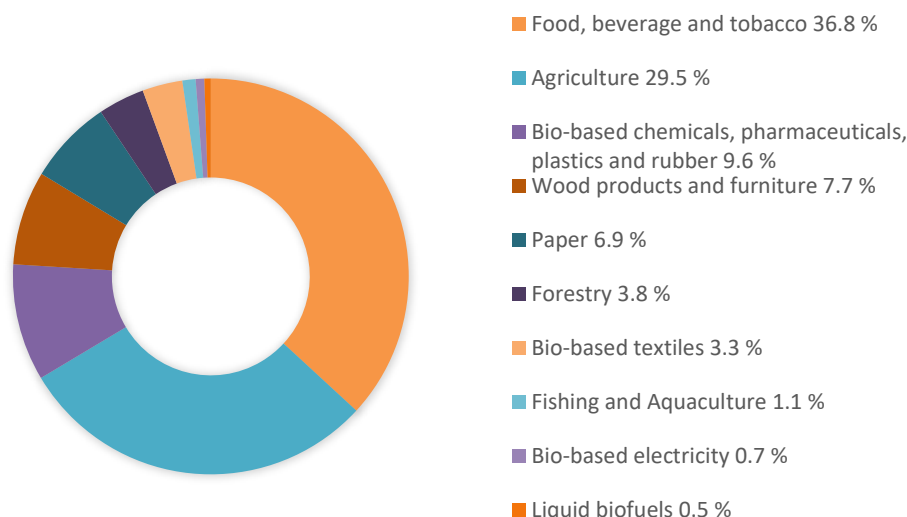


Figure 1.1. Turnover of the bioeconomy by sectors in EUR-28 (2017). Source: Based on DataM – Bioeconomics, database elaborated by the European Commission / Joint Research Centre IPTS and nova Institut.

One of the main bottlenecks for an appropriate development of a biobased society is the economic feasibility of the bioproducts. Indeed, the raw material used as substrate has been claimed to be a key issue for attaining economically-competitive bioprocesses (Ng et al., 2020; Sharma et al., 2020). The utilization of waste streams, as low-cost substrates for bioproducts, is currently presented as an attractive option to decrease production costs. This strategy exhibits a two-fold benefit, namely the production of different bioproducts from widely available residues and the valorization of wastes that otherwise should be treated before disposal. In this manner, the use of organic wastes can definitively contribute to the transition from a linear to a renewable circular economy.

Given the great heterogeneity of organic wastes, anaerobic digestion (AD) arises as the most advantageous technology to valorize them since it can be applied to a wide variety of substrates regardless of their macromolecular composition (Raposo et al., 2012). Among different types of residual biomass, microalgae generated in the context of wastewater treatment is an interesting option. This biomass can be produced from residual effluents as cultivation media and once harvested, it can be utilized for the production of different fuels (biodiesel, bioethanol or biogas). Nevertheless, the

economic revenue of biofuels is most of the times not enough to cover the production expenses. For this reason, the biorefinery scheme seems to be the most interesting approach to take full advantage of the available substrates. In this manner, biorefineries are designed to convert biomass into value-added products, generating subsequently low-cost products such as energy carriers from the spent biomass.

Following a biorefinery scheme, this Thesis presents an innovative approach in which the whole microalgae biomass is used for the production of volatile fatty acids (VFAs), microbial oils (MO) and biogas.

VFAs are intermediate compounds of the AD that can be used as precursors for bio-based products such as biofuels, bioplastics and biotextiles. VFAs accumulation can be promoted by shortening the traditional AD process to an anaerobic fermentation (AF) process. The obtained digestates during AF process can be further used with a two-fold purpose. On one hand, the VFAs-rich liquid fraction is regarded as low-cost carbon sources for MO by means of oleaginous yeast (OY) fermentation (Llamas et al., 2019). On the other hand, the remaining solid fraction can be harnessed via conventional AD for biogas production which could be energetically revalorized.

Henceforth, the alternative use of microalgae biomass by integrating these three fermentative bioprocesses (namely AF, oleaginous fermentation and AD) (Figure 1.2) allows the valorisation of residues by reducing associated feedstock costs, while increasing the revenues coming from the production of high-value chemicals (VFAs and MO) and energy precursors (biogas).

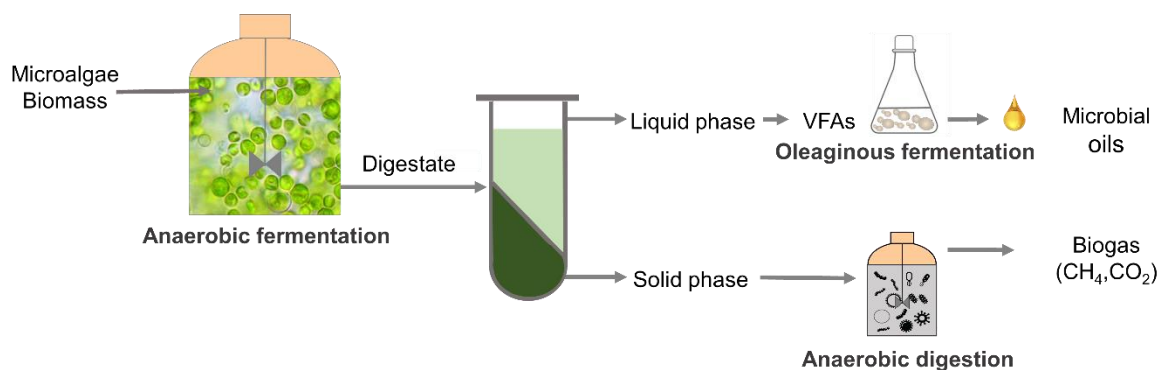


Figure 1.2. Integration of VFAs, MO and biogas production processes proposed in this Thesis.

This section includes an overview of the three bioprocesses (AF, AD and oleaginous fermentation) involved in microalgae valorization, covering process description, substrates employed, microorganisms and metabolic pathways as well as key parameters to be controlled for an optimum fermentation. Additionally, the main challenges and recent approaches for enhancing their performances are also discussed.

1.1. Anaerobic digestion

AD is a robust technology widely employed for wastes treatment, in which both pollution control and energy recovery can be accomplished. This is a well-known degradative process where an organic substrate is converted into a gas stream (biogas). This biogas can be further used for electricity and heat production in cogeneration engines. AD is composed of sequential and simultaneous steps where the products of one step are used as substrate for the following ones (Figure 1.3).

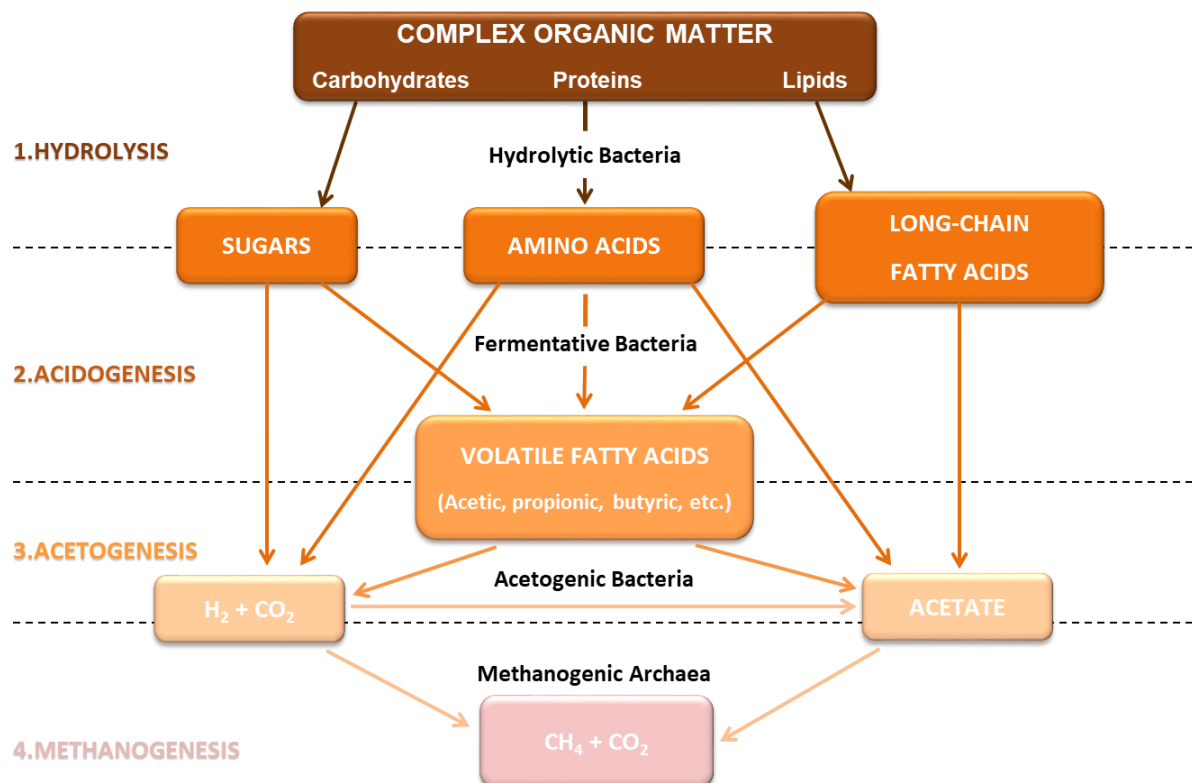


Figure 1.3. Scheme of AD process stages and involved microbial population.

AD can be divided in four different stages including hydrolysis, acidogenesis, acetogenesis and methanogenesis (González-Fernández et al., 2015). This complex bioprocess is based on the metabolic interaction of different anaerobic microorganisms, namely archaea and bacteria.

During the hydrolysis stage, exo-enzymes belonging to hydrolytic bacteria are in charge of degrading the complex organic matter composed of carbohydrates, proteins and lipids into their monomers (sugars, amino acids and long chain fatty acids, respectively). The efficiency of this stage often determines the overall process yields, as it establishes the organic matter availability (Méndez et al., 2013; Córdova et al., 2019). In the acidogenesis, acidogenic bacteria oxidize the soluble monomers and produce VFAs, CO₂, alcohols, H₂, and lactic acid. During acetogenesis, acetic acid, CO₂ and H₂ are produced by acetogenic bacteria. These last products are the main substrates for the methanogenic archaea, which are in charge of producing biogas (CH₄ and CO₂) in the methanogenesis step.

1.1.1. Biogas production via traditional anaerobic digestion

The biogas produced through the AD of organic matter is a mixture of CO₂ and CH₄. Compared to bioethanol and biodiesel, biogas production via AD is a more flexible process since the entire organic matter, not only carbohydrates or lipids, is used to produce the desired product. In fact, each macromolecule exhibits specific methane yields, being 1.014, 0.496 and 0.415 L CH₄ g/ VS_{in} for lipids, proteins and carbohydrates, respectively (Angelidaki and Sanders, 2004). The fact that AD technology can be performed regardless the substrate composition is quite relevant, since it allows the use of a wide variety of feedstocks.

An stable AD process requires a dynamic equilibrium among the involved microbial communities (bacteria and archaea). Methanogenic population (archaea) is the most sensitive group towards operational conditions or inhibitors (Hwang et al., 2010; Madigan, 2012). Archaea have lower growth rates than the bacteria performing the three first steps of AD, which might render AD into a extended bioprocess

(Vanwonterghem et al., 2016). Generally, methanogenic microorganisms can be classified in two different groups, namely acetoclastic and hydrogenotrophic. The main difference lies in the substrate metabolized for biogas production. While acetoclastic archaea use acetic acid to produce methane, hydrogenotrophic microorganisms use H_2 and CO_2 as main substrates. Hydrogenotrophic archaea play an important role in the low H_2 partial pressure required to obtain high biogas production (Bajpai, 2017). Nonetheless, biogas is mainly formed through the acetoclastic pathway (Zamorano-López et al., 2020).

1.1.2. Volatile fatty acids production via anaerobic fermentation

The traditional AD process (including the 4 reaction steps) ends up in biogas production. However, AF technology is drawing considerably attention in the scientific community for VFAs production (Strazzera et al., 2018; Greses et al., 2020b). VFAs, also known as carboxylates, are intermediate compounds produced after the hydrolytic and the acidogenic stages of AD (Figure 1.3). Acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids are the VFAs normally encountered in AD. VFAs concentration in conventional AD is normally low since they are used as substrate to ultimately produce biogas. Nevertheless, the traditional AD bioprocess can be shortened, through the inhibition of the methanogenesis step, thus resulting in VFAs accumulation in the liquid stream. AD shortening (so-called AF) increases process robustness since the methanogenesis, which is the most sensitive microbiological step, is avoided (Tamis et al., 2015).

VFAs might be a product by themselves after separation or purification (Holtzapfel and Granda, 2009; Agler et al., 2011) or they can serve as building blocks for several bioproducts (Table 1.1). VFAs applications include food additives, pharmaceuticals, adhesives, solvents or chemical intermediates (Lee et al., 2014; Kim et al., 2018; Gao et al., 2020). For instance, acetic acid has an important role in food industry (Sengun and Karabiyikli, 2011), propionic acid is mainly used as acidifier for animal feed and grain

(Ahmadi et al., 2017), and butyric acid can be utilized as a precursor for biofuels production (Dwidar et al., 2012). Despite their wide range of applications, their production feasibility is hampered by the high costs and technological constraints associated to their purification (Magdalena et al., 2019a). Therefore, the search of applications where VFAs can be used without the need of extraction and purification is a crucial step forward in the implementation of the VFAs platform. To this end, some of the most promising uses of these molecules include the production of biodegradable plastics such as polyhydroxyalkanoates (Kumar et al., 2019), the generation of energy in microbial fuel cells (Nosek and Cydzik-Kwiatkowska, 2020), the production of medium chain carboxylates via chain elongation (Magdalena et al., 2019a), and their use as building blocks for oil-based chemistry via oleaginous microbial fermentation (Llamas et al., 2019).

Table 1.1. Application of VFAs.

	Acid	Application
Direct application	Acetic	Vinyl acetate monomer (polymer, adhesives, dyes), food additives, solvent, ester production
	Propionic	Food additive, flavouring, pharmaceuticals, animal feed supplement, fishing bait additive
	Iso-butyric	Dyes, pigments, paints, food flavour, fragrance ingredient, pharmaceutical, protective coating
	Butyric	Animal and human food additive, chemical intermediate, solvents, flavouring
	Isovaleric	Flavouring agent, fragrance ingredient
	Valeric	Food additive, fragrance ingredient, cosmetics, sedative uses
	Caproic	Antifoaming agent, lubricant, ester production, fragrance preparations
Building blocks	VFAs mixture	Biopolymers: PHAs production via fermentation
		Electricity production (MFCs): VFAs are oxidized in the anode
		Medium chain carboxylates via chain elongation by fermentation
		MO via oleaginous fermentation

(Agler et al., 2011)

1.1.2.1. Parameters affecting VFAs production via anaerobic fermentation

To favor VFAs accumulation and increase process efficiency, attention must be directed to the substrate, the inoculum and the operational conditions. These factors strongly affect the microbial population development which ultimately determines the organic matter's fate and therefore, the VFAs production yields and profile (Wainaina et al., 2019).

1.1.2.1.1. Anaerobic inoculum

Culture conditions that favour microbial growth and activity of specific microorganisms must be set to develop a suitable microbial population for VFAs production. Most commonly, the seed inoculum is anaerobic sludge. Bacteria species present in the anaerobic sludge are very diverse and play different roles in the AD process. Hydrolytic, acidogenic and acetogenic bacteria, together with methanogenic archaea, gather the biodiversity required for such a complete and complex bioprocess (Figure 1.4). Even though many different species take part, each stage of the process is characterized by a group of microorganisms.

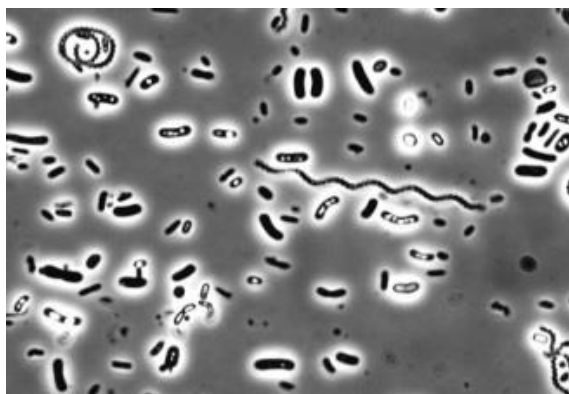


Figure 1.4. Optic microscopic image of the microbial diversity in the anaerobic inoculum used in this Thesis.

The use of an anaerobic inoculum with low archaea population could be beneficial for VFAs accumulation. Methanogenic archaea are involved in the last step of AD where

biogas is produced by means of VFAs consumption. Considering methanogenic archaea sensitivity, inoculum pretreatments could be one possible approach to reduce this population and boost VFAs accumulation. Among the pretreatments, thermal ones are the most widely employed. Thermal pretreatment implies subjecting the anaerobic inoculum to high temperatures for a determined period of time and it has been used for eliminating non-spore forming microorganisms. This pretreatment has been shown to increase organic matter bioconversion into VFAs due to the inactivation of the most sensitive methanogenic microorganisms (mainly acetoclastic archaea) (Cho et al., 2015a; Magdalena and González-Fernández, 2020).

Previous works have also reported the complete inhibition of methanogenic population (acetoclastic and hydrogenotrophic archaea) when keeping the inoculum under extreme pH conditions (basic (9-11) or acid (2-4)) (Wang and Wan, 2008; Ren et al., 2008). These pretreatments might not only eliminate methanogenic archaea, but also alter the organic acid producers (Luo et al., 2010). More particularly, chemicals that specifically block the enzymatic systems of methanogens have been also applied when targeting VFAs accumulation. For instance, specific reagents such as 2-bromoethanesulfonate or iodoform demonstrated to favour VFAs accumulation by preventing methanogens population development (Pham et al., 2012; Magdalena and González-Fernández, 2020). The overload of these chemicals can lead to a negative effect on the rest of the microbiome, resulting in a reduction of VFAs production by inhibiting acetogens activity as well. Therefore, all these pretreatments applied to anaerobic sludge must be carefully controlled to affect only the desired microbial population.

Economic costs, environmental concerns and the high energy demand of some of the pretreatments are the main drawbacks identified when using these technologies for archaea inactivation. In addition, these strategies often show short-term effect in continuous operation (Arslan et al., 2016). As an alternative to inoculum pretreatments, the selection of appropriate substrates and operational parameters that decrease archaea activity while promoting the activity of the microorganisms involved in AF stages could be a more economic and environmentally friendly approach to favor VFAs accumulation.

1.1.2.1.2. Microalgae biomass as a suitable substrate for VFAs production

As previously mentioned, the selection of cost-effective substrates is of paramount importance to decrease VFAs production costs. Cheap feedstocks, such as waste streams (*e.g.* food wastes, agriculture residues, and sewage sludge) have been proposed to be ideal candidates for VFAs production (Strazzera et al., 2018; Luo et al., 2019; Uludag-Demirer et al., 2019). These residues exhibit high amounts of organic matter, nitrogen and phosphorous, which are indispensable nutrients for the metabolic activities of the anaerobic microorganisms. Out of the potential organic wastes that can be used in AF, microalgae biomass arises as a promising feedstock.

“Microalgae” term describes microscopic photosynthetic algae able to transform light energy into organic matter using inorganic carbon sources and nutrients. Microalgae are organisms of 2-200 μm of size and they can be unicellular or exhibiting simple multicellular structures (Figure 1.5). Although about 35000 species of microalgae have been identified, it has been estimated that between 200000 and 800000 species are still undiscovered (Ratha and Prasanna, 2012).

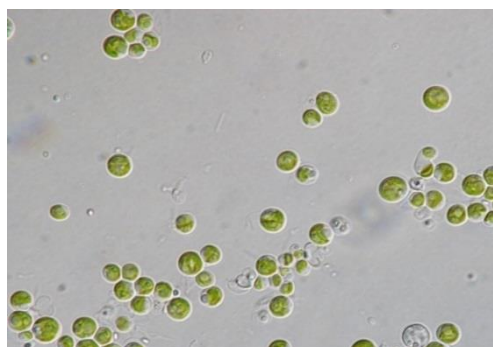


Figure 1.5. Optic microscopic image of *Chlorella vulgaris* used in this Thesis.

The interest in microalgae lies in their potential utilization to produce biomass for biofuels and high-value chemicals. One of their main advantages is that microalgae thrive in wastewater and hence, do not require expensive nutrients or arable lands (Davis et al., 2011; Xin et al., 2016; Paches et al., 2020; Shahid et al., 2020). Microalgae

biomass harvested from wastewater is normally poor in fermentable sugars or transesterificable lipids and thus, the most straightforward use is AD.

Moreover, this biomass can be considered an appropriated renewable resource for VFAs production given its macromolecular distribution. Although the macromolecular distribution is very variable and highly depends on growth conditions and strain, proteins generally are the most abundant macromolecules (Sialve et al., 2009; Milledge et al., 2019). Proteins usually account for 30 % to 60 % of their dry weight (DW) in green microalgae (such as *Chlorella* and *Scenedesmus*) (Mahdy et al., 2014c; Mata et al., 2010). Additionally, these strains present around 20-35 % (w/w) carbohydrates and 15-20 % (w/w) lipids (de Carvalho et al., 2020).

The use of protein-rich substrates might constitute an important advantage in VFAs production processes where methanogenic community inhibition is sought. When proteins are cleaved into simple aminoacids, nitrogen is released in the form of ammonium (NH_4^+) and free ammonia (NH_3). High concentrations of these compounds can inhibit methanogenic population (Mahdy et al., 2015, 2016a; Magdalena et al., 2018a; Tian et al., 2018) and thus, favour VFAs accumulation.

In spite of all the positive aspects mentioned above, there are many challenges to be overcome when using microalgae as a feedstock for AF. Their complex nature and structure can result in low hydrolytic rates, resulting in less organic matter available for the subsequent stages, which eventually leads to a low VFAs production (Passos et al., 2014). Tertiary and quaternary structures of some proteins might actually hamper the hydrolytic step of this bioprocess (Battista and Bolzonella, 2018). Additionally, microalgae present a membrane usually surrounded by a cell wall, which constitutes a selective barrier that protects internal components (Andersen and Koeppe, 2007; Manrique et al., 2014). These facts are particularly relevant in the case of microalgae species grown in wastewater. *Chlorella* and *Scenedesmus*, two of the most common microalgae, often exhibit a robust cell wall hampering anaerobic degradation (Mahdy et al., 2016b). According to de Carvalho and co-workers (2020), *Chlorella vulgaris* cell wall is composed by carbohydrate-based fibrillar structure covered by an algenan-based layer (Figure 1.6). In order to expose internal macromolecular components to anaerobic

microorganisms, cell wall disruption/solubilisation and membrane weakening are essential to favour AD/AF processes.

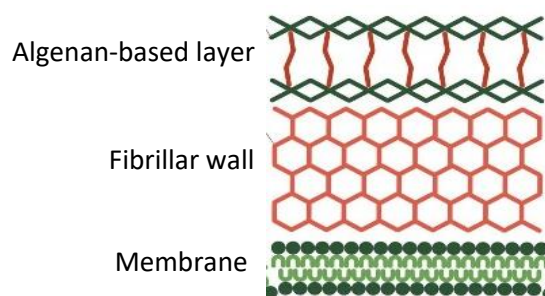


Figure 1.6. Cell wall structure of *C. vulgaris* illustrating the main components.
(Adapted from Carvalho et al., 2020)

Pretreatments to increase microalgae solubilization

Different pretreatments have been deeply studied in terms of microalgae disruption and solubilization for different purposes. This up-stream process seems to be essential to extract high-value products and to increase the access to the organic matter available within microalgae biomass (González-Fernández et al., 2012; Silva et al., 2014). The knowledge of microalgae composition and cell wall structure allows the selection of the most suitable pretreatment for specific targeted molecules. Microalgae pretreatment methods can be divided in four categories: thermal, mechanical, chemical and biological processes (Figure 1.7). This section describes these pretreatments and outlines their effect on microalgae organic matter solubilization. In the field of AD from microalgae biomass, it should be highlighted that most of the available literature deals with microalgae pretreatments devoted to increase methane production. Since AF entails the first stages of AD, pretreatment effects are expected to be very similar in both AF and AD in terms of increasing the organic matter bioavailability.

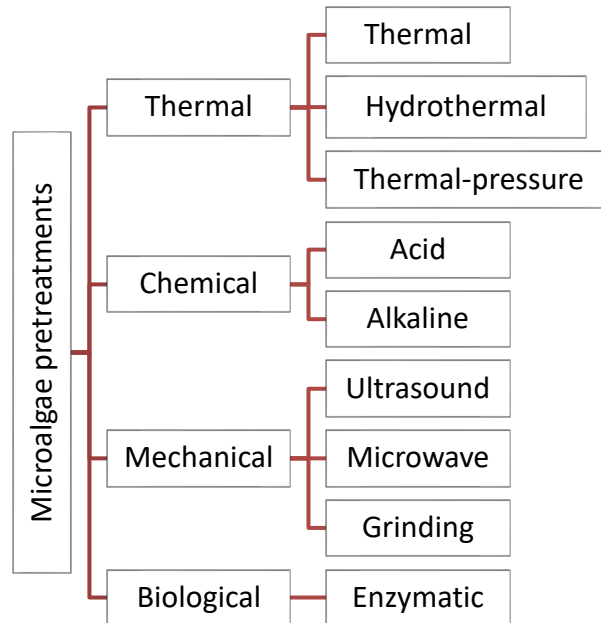


Figure 1.7. Different pretreatment technologies employed to improve microalgae solubilization. (Adapted from Passos and Ferrer, 2014)

Thermal pretreatments

The application of specific temperature has been the most widely studied pretreatment prior to AD (Schwede et al., 2013; Passos and Ferrer, 2014). Low thermal pretreatment consists on applying temperatures below 100 °C under atmospheric pressure. Some works that evaluated thermal pretreatment effect on *C. vulgaris* and *Scenedesmus* sp. reported that pretreatment efficiency is specie-dependent and stated that high temperatures cause higher organic matter solubilisation than low temperatures regardless the microalgae employed (González-Fernández et al., 2012b; Passos et al., 2013a; Alzate et al., 2014; Mahdy et al., 2014b). These studies also evidenced that high organic matter solubilisation is not necessarily correlated with high AD process efficiencies. This could be explained by the fact that pretreatments might entail not only the release of degradable biomass components but also process inhibitors (Cho et al., 2013; Méndez et al., 2013).

On the other hand, high temperatures pretreatments (above 100 °C) result in a pressure increase. After the thermal application, pressure can be released gradually or rapidly. Hydrothermal pretreatment implies gradual pressure release while the sudden pressure

drop to ambient conditions is defined as thermal pretreatment with steam explosion (Passos et al., 2014). One of the advantages of these methods is the shorter reaction times (15–30 min) in comparison with low temperature pretreatments (3–24 h). Hydrothermal pretreatment has been shown to produce high biodegradability of microalgae biomass (Cho et al., 2013; Mendez et al., 2013; Ometto et al., 2014). Although pretreatment efficiency is influenced by both temperature and exposure time, it has been reported that temperature is the most crucial factor on biomass solubilization (Passos et al., 2014).

The main drawback of this pretreatment is that high economic cost is associated (Passos et al., 2014). Therefore, pretreatment optimization or the employment of low energy demanding pretreatments must be considered.

Chemical pretreatments

Acid and alkali reagents are commonly used to solubilize polymers. Although several works did not find a significant enhancement in terms of organic matter solubilization after microalgae pretreatment with NaOH or H₂SO₄, the combination of these chemicals with thermal pretreatments increased the solubilized organic matter (Cho et al., 2013; Bohutskyi et al., 2014). As an example, when aiming at biogas, the increase in organic matter solubilisation from thermochemical-pretreated microalgae did not correspond with the slight improvement registered in biogas production (Cho et al., 2013; Méndez et al., 2013). Additionally, residual acid/alkali compounds remaining in the pretreated biomass may affect the downstream processes. For these reasons, it has been concluded that chemical pretreatments are less appropriated than other pretreatments (de Carvalho et al., 2020).

Mechanical pretreatments

Mechanical pretreatments (*e.g.* grinding, ultrasounds, microwave, etc.) act by directly breaking microalgae cells through a physical force. The aim of grinding is to reduce the biomass size by breaking all cell structures. Ultrasounds consist in rapid compression

and decompression cycles of sonic waves while the application of microwaves entails short waves of electromagnetic energy varying in frequency from 300 MHz to 300 GHz (Passos et al., 2014). Mechanical pretreatments are less dependent on microalgae species, but require higher energy input if compared with chemical, thermal and biological methods (Lee et al., 2012).

Both ultrasounds and microwave pretreatments have already shown enhancements in organic matter solubilization of microalgae biomass (Kumar et al., 2017; Feng et al., 2019). Energy and exposure time are the main controllable parameters influencing these pretreatments. The effect of these pretreatment on biomass solubilisation (as well as on methane yield when this is targeted) increases concomitantly with the applied energy (González-Fernández et al., 2012; Alzate et al., 2014; Ometto et al., 2014). Comparing similar energy input, microalgae biodegradability is increased at higher extent when using microwave treatment than with ultrasounds pretreatment (Passos et al., 2013b).

In conclusion, enhancing organic matter solubilization in microalgae biomass by mechanical pretreatments requires high energy input and may not be economically feasible when a low-cost product is targeted. Therefore, alternative pretreatments where biogas production offsets the reagents or energy demand must be implemented.

Enzymatic pretreatments

Enzymes convert biopolymers of microalgae cells to low molecular weight compounds that are more readily available for anaerobic bacteria. The most important parameters affecting this pretreatment efficiency are enzyme type and dose, temperature, pH and exposure time. Proteases, carbohydrases (celluloses, xylanases, etc.) and several enzymatic mixtures have been demonstrated to enhance organic matter solubility of different microalgae species (*C. vulgaris*, *Chlamydomonas reinhardtii*, or *Rhizoclonium* sp.), leading as well to methane yields increases (Ehimen et al., 2013; Mahdy et al., 2014a; Passos et al., 2016). These results suggest the use of enzymes as a promising pretreatment to increase bioprocess yields given the lower energy requirements and

the presumably low formation of inhibitory compounds. However, substrate-specificity and enzymes production costs are among the major drawbacks of this technology (de Carvalho et al., 2020).

All pretreatments described in this section have been widely applied on microalgae biomass devoted to methane production, however, there is a lack of information about the effect of such pretreatments in AF for VFAs production. Since pretreatments aim to favour organic matter release, it could be assumed that the positive effect of pretreatments on organic matter solubility would most probably result in an increase in VFAs production yields.

1.1.2.1.3. Operational parameters affecting VFAs production

As previously mentioned, the inhibition of the methanogenic step and the enhancement of hydrolytic and acidogenic bacterial activity is sought in AF. For such a purpose, different operational strategies can be applied to promote VFAs production in AF. Taking advantage of the higher sensibility of archaea species to operational changes when compared to organic acid producers, fine-tuning of process parameters can be used to inhibit methanogenic population (Tamis et al., 2015). Process parameters such as the type of inoculum, pH or temperature have a great influence not only in the VFAs production yield but also in the obtained VFAs profile (Arslan et al., 2016; Khan et al., 2016a).

pH

Each group of microorganisms and enzymes has an optimum pH working value, and therefore, this parameter has a direct effect on AF. Whereas methanogenic archaea grow better at pH close to neutrality (Mukhuba et al., 2020), acidogenic and hydrolytic bacteria have a wider pH growth range (Bengtsson et al., 2008). Considering that the optimum pH range for acidogenic bacteria is around 5 and 7, acidic and neutral pH ranges have been widely employed for VFAs production (Cho et al., 2015a; Magdalena

et al., 2018b; Kim et al., 2019). Nevertheless, some attempts have been also accomplished at alkaline pH. Previous studies devoted to VFAs production reported that alkaline fermentation favored VFAs production when microalgae biomass was employed as substrate. This fact was attributed to a hydrolytic enhancement of microalgae biomass and an inhibited methanogenic population at alkaline pH (Wu et al., 2010; Jie et al., 2014; Xie et al., 2019).

Temperature

Temperature affects not only the metabolism of the microorganisms and their enzymatic activities, but also the physical state of the organic matter. In this manner, temperature is positively correlated with organic matter solubilization (Yuan et al., 2011). Kim and co-workers (2019) reported higher organic matter conversion from non-pretreated *Chlorella* sp. into VFAs at thermophilic range than at psychrophilic range. They concluded that high fermentation temperature enhanced hydrolysis rates, promoting the enzymatic activity. Remarkably, when using pretreated microalgae biomass, the positive effect of using high temperatures was not observed (Cavinato et al., 2017; Magdalena et al., 2018b). Biomass pretreatment results in organic matter solubilisation, which ultimately results in a decrease of the positive effect assigned to high fermentation temperatures.

An increase in AF temperature could be associated with higher organic matter solubilization rates but not necessarily with an increase in VFAs production. This was the case observed when using maize silage and cow manure or cassava water as substrate (Hasan et al., 2015; Cavinato et al., 2017). These authors showed higher VFAs production at psychrophilic and mesophilic conditions than at thermophilic temperatures but organic matter solubilization was more efficient at thermophilic ones. It could be inferred, thus, that thermophilic fermentation enhances biomass hydrolysis while psychrophilic and mesophilic range seems to favour acidogenic step. Moreover, temperature does not only affect the physical state of biomass, but it also has an impact on the anaerobic microbiome. In this manner, temperature promotes the growth and metabolism of

specific species that might impact final VFAs productions and profiles (Greses et al., 2017).

Organic loading rate

The organic loading rate (OLR) is the concentration of organic matter present in the substrate fed in terms of organic matter or volatile solids per unit of time. Although the OLR selection is process specific, studies devoted to VFAs production stated that increasing OLRs resulted in an enhancement in VFAs production (Lim et al., 2008; Rincón et al., 2008; Tang et al., 2016; De Groof et al., 2019). These previous works found an optimum range of OLR of 12-15 g COD/L d when targeting VFAs production. Nevertheless, a maximum OLR threshold with no further improvement in VFAs production has been identified due to product inhibition (Lim et al., 2008; Rincón et al., 2008; Tang et al., 2016). Too high OLR values might exceed the hydrolytic capacity of the system, whereby no further improvement in VFAs production is attained.

A recent study assessed the effect of stepwise OLR increase (3, 6, 9, 12, 15 g COD/L d) for VFAs production using pretreated *C. vulgaris* as substrate. This investigation revealed an optimum VFAs production at 12 g COD/L d while the highest OLR (15 g COD/L d) resulted in lower bioconversion efficiencies (Magdalena et al., 2019b). In this case, authors pointed out that the combined effect of high NH_4^+ , VFAs and sodium concentrations inhibited the acidogenic population activity. Therefore, OLR should be carefully tuned according to the organic matter bioavailability and its effect on the microbial population during the different AF stages.

Hydraulic retention time

Hydraulic retention time (HRT) establishes the time that the substrate remains in the reactor. The substrate employed might affect the selected HRT since the lower hydrolysis rate, the higher should be the HRT. When too short HRT is applied, a fast hydrolysis can cause inhibition of the acidogenesis stage because HRT must be high enough to allow the proper activity of acidogenic population. Thus, HRT may not be set

up only based on the hydrolytic stage but also according to microorganisms growth rates.

While HRT values for biogas production might range 15-30 days (Mahdy et al., 2015; Liu et al., 2019), HRTs for VFAs production can be considerably lower since archaeal activity is not required. The reason behind these values relies in the fact that methanogenic archaea exhibit lower growth rates than acidogenic bacteria (Henze et al., 2008). HRT values in literature employed for VFAs production varied from 0.125 to 10 days for semi-continuous fermentations (Arslan et al., 2016; Strazzera et al., 2018; Magdalena et al., 2019b; Battista et al., 2020). Therefore, the use of low HRTs could be a tool to select the most suitable populations in charge of VFAs accumulation while favoring the wash out of methanogens. On the other hand, it should be also highlighted that HRT must be high enough to allow the proper activity of anaerobic microorganisms conducting the hydrolysis and acidogenesis steps.

Operational mode

Reactor configuration is crucial in AF since it determines the mixing of all the fermentation elements affecting the microbial growth and activity. Proper process configuration should be chosen taking into account the properties of the substrate, microorganisms and targeted products. Some examples of the most common reactor configuration are explained below.

Continuous Stirred Tank Reactor (CSTR)

CSTR configuration enables the full contact between phases providing a complete mixture of substrate, microorganisms and products (Figure 1.8). This configuration has been the most commonly employed in AF due to its simple design and operation.

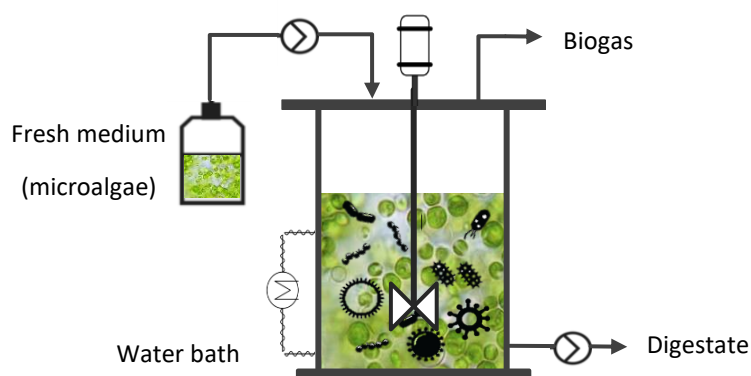


Figure 1.8. Schematic diagram of a CSTR fed with microalgae biomass.

In CSTRs, the substrate flow rate (marked by HRT) and solids flow rate (marked by solids retention time (SRT)) are the same. Because of that, the use of low HRT or high OLR is hampered since this might cause microorganisms wash-out or substrate overloading. As a matter of fact, HRTs employed for biogas production from microalgae biomass in a CSTR reactor range 15-30 days (Zabed et al., 2020) while OLR values range 1-5 g COD/L d (Mahdy, 2016).

In the context of VFAs, one limitation of CSTRs is associated to the high amount of solids present in the effluents. This, as mentioned before, can entail more complex and expensive methods for VFAs extraction and purification for determined application. Decoupling HRT and SRT could reduce solids content in effluents, decreasing the costs of VFAs downstream processing.

Upflow Anaerobic Sludge Blanket (UASB)

UASB is a tubular bioreactor working in continuous feeding mode and upflow. In UASB reactors, the substrate is added at the lower part of the reactor and the output exits in the upper part. In this manner, a longitudinal concentration gradient takes place that allows decoupling the HRT and SRT (Figure 1.9).

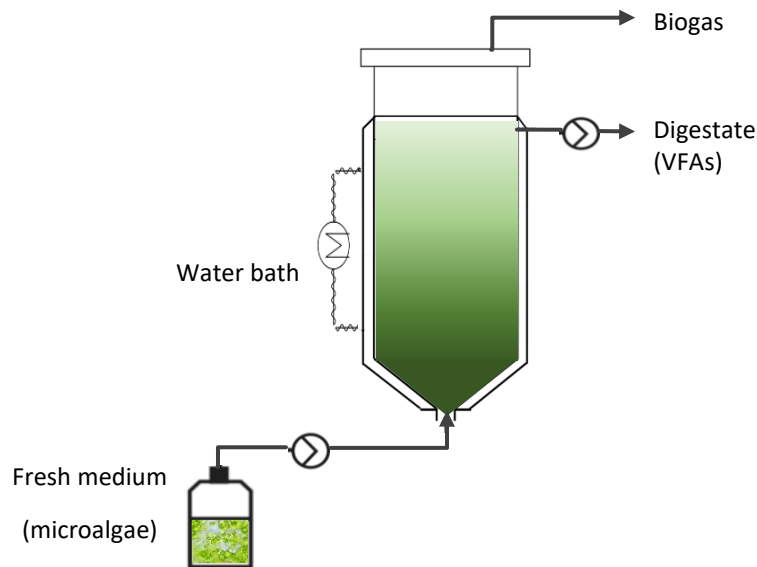


Figure 1.9. Schematic diagram of UASB reactor configuration fed with microalgae biomass.

This configuration arises as the optimum choice for simple substrates (Bakraoui et al., 2020; Chen et al., 2020) since it allows to work at high OLR and low HRT while offering high treatment efficiencies. One of the main advantages of UASB reactor configuration is the low HRTs at which it can be operated. In the context of biogas production, previous works have used HRTs from 2 to 7 days (Tartakovsky et al., 2015; Soboh et al., 2016). Additionally, this reactor configuration enables to work at OLR values higher than 1-5 g COD/L normally employed in CSTRs (Abdelmohsen and Mohamed, 2016).

The use of UASB in the case of complex organic substrates like microalgae biomass is limited. Only few research studies have dealt with this type of reactor for biogas production from microalgae biomass (Tartakovsky et al., 2015; Soboh et al., 2016). Nevertheless, it should be stressed that this type of reactor configuration might be an interesting option for VFAs production in which the activity of slow archaea is not required. In spite of the promising features of this configuration, only one research investigation has used UASB reactor configuration for VFAs production (Magdalena et al., 2020).

Anaerobic Membrane Bioreactor (AnMBR)

AnMBR configuration consists on a CSTR reactor where the effluent is filtered giving rise to two flows: a clean permeate and a solid concentrate fraction (retentate) (Figure 1.10). Under this configuration, the retentate (composed by the most complex organic fraction) is returned to the reactor to be further degraded.

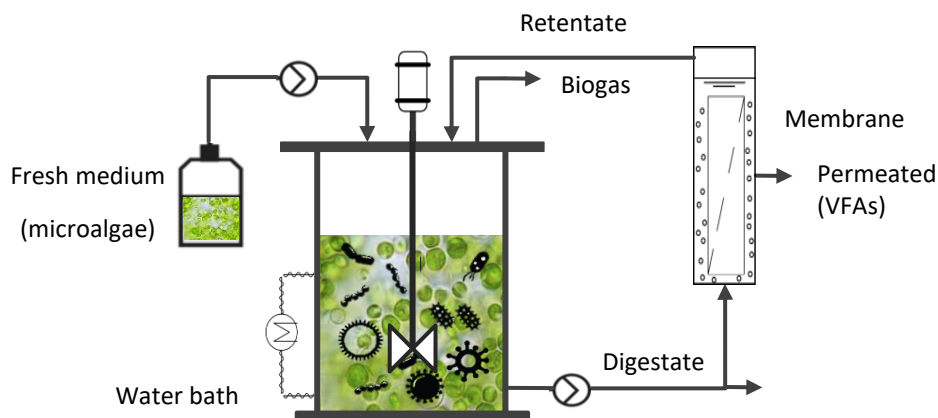


Figure 1.10. Schematic diagram of AnMBR configuration fed with microalgae biomass.

AnMBR has been extensively employed for biogas production using simple substrates, such as wastewater (Khan et al., 2016b; Charfi et al., 2017), and complex ones, like the primary sludge and microalgae biomass (*Scenedesmus* sp. and *Chlorella* sp.) (Magdalena et al., 2020; Serna-García et al., 2020). Similarly to the UASB reactor, the goal of AnMBR configuration is decoupling SRT and HRT while enriching the reactor with the desired microbial population. Recent investigations have demonstrated the viability of VFAs production using AnMBR reactor from sewage sludge, reaching 38 % bioconversion (g VFAs_{COD}/g VS) (Liu et al., 2019). Similarly, 48 % bioconversion (g VFAs_{COD}/g COD_{in}) was reported by Khan and co-workers (2019) when using low strength wastewater in AnMBR.

As a summary, although CSTR configuration has been mostly employed to obtain VFAs using microalgae biomass as substrate, UASB/AnMBR might contribute to favor VFAs production because both configurations allow to decouple HRT and SRT. Besides, both UASB/AnMBR configurations produce high quality effluents in the AF, facilitating or even

avoiding VFAs separation and purification (Li et al., 2020). For these reasons, depending on the microalgae biomass employed or the use of biomass pretreatments, different reactor configuration should be carefully evaluated.

1.1.2.2. Microbial populations involved in VFAs production

Microbial populations present in an anaerobic inoculum have a strong effect in the AD/AF performance. The relative abundance of different microbial species might affect the fate of the organic matter and thus, the final product.

A recent study highlighted the high flexibility, diversity and adaptability of the anaerobic community upon different operational conditions, reactor configurations and substrates when the process is devoted to biogas production (Campanaro et al., 2019) (Table 1.2).

Table 1.2. Main genus encountered in AD of microalgae biomass.

AD step	Microorganisms involved in each step
Hydrolysis	<i>Bacteroides</i> , <i>Micrococcus</i> , <i>Streptococcus</i> , <i>Clostridium</i> , <i>Peptococcus</i> , <i>Bacillus</i> , <i>Enterobacter</i> , <i>Ruminococcus</i> , <i>Mycobacterium</i>
Acidogenesis	<i>Clostridium</i> , <i>Escherichia</i> , <i>Enterobacter</i> , <i>Pseudomonas</i> , <i>Bacteroides</i> , <i>Bulleidia</i> , <i>Thermotogae</i> , <i>Acidaminococcus</i> , <i>Streptococcus</i> , <i>Thermobacteroides</i>
Acetogenesis	<i>Sporanoaerobacter</i> , <i>Acetobacterium</i> , <i>Desulfobacter</i> , <i>Paludibacter</i> , <i>Coprothermobacter</i> , <i>Syntrophobacter</i> , <i>Thermoacetogenium</i> , <i>Clostridium</i> , <i>Syntrophomonas</i>
	Acetoclastic: <i>Methanosarcina</i> , <i>Methanospirillum</i> , <i>Methanosaeta</i>
Methanogenesis	Hydrogenotrophic: <i>Methanospaera</i> , <i>Methanobacterium</i> , <i>Methanoplanus</i> , <i>Methanovrevibacterium</i>

(Greses et al., 2018; Magdalena et al., 2019b; Córdova and Chamy, 2020)

Bacteroidetes, Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Spirochaetes, Thermotogae, and Synergistetes are commonly present in anaerobic communities (Campanaro et al., 2019; Córdova and Chamy, 2020). However, their relative abundance depends on the operational parameters and substrates employed. For instance, some authors reported the dominance of Proteobacteria phylum under mesophilic conditions (35 °C) when digesting *Chlorella sorokiniana* and *Scenedesmus* sp. (González-Fernández et al., 2018), while Firmicutes and Thermotogae were found as the main phyla when

reactors were operated at thermophilic temperature (55 °C) (Greses et al., 2017; Zamorano-López et al., 2020). With regard to the substrate employed, the microbial analysis in batch assays fed with *Dunaliella* sp. at 37 °C, showed prevalence of Proteobacteria phylum, while Bacteroidetes and Proteobacteria were found the most abundant phyla when *C. vulgaris* was fermented under the same conditions (Lakaniemi et al., 2011).

Regarding the effect of reactor configuration on microbial population, important differences were also found when comparing CSTRs with AnMBRs employing *Scenedesmus* sp. as substrate. While Proteobacteria phylum was prevailing in CSTRs, the most abundant phyla in AnMBR reactor were Chloroflexi and Proteobacteria (Greses et al., 2017). This suggests that reactor configuration plays a key role in selecting the prevailing communities.

In reactors operated for VFAs production purposes, bacterial population changes with regard to that normally encountered in conventional digesters devoted to biogas. In fact, AF reactors presented different species and exhibited also lower biodiversities (Greses et al., 2017; Greses et al., 2020b). Firmicutes, Proteobacteria and Bacteroidetes have been identified as the main phyla in batch AF reactors regardless of the employed microalgae strain or the operational parameters (temperature or OLR) (Cho et al., 2018, 2015a; Magdalena et al., 2019b; Xie et al., 2019). These phyla have been claimed to produce VFAs as well as actively degrade proteins and polysaccharides (Sanz et al., 2016; Hartati et al., 2018; Gao et al., 2019), that represent a high percentage of the macromolecular composition of microalgae biomass.

When compared to digesters devoted to biogas production, fermenters operated for VFAs production show an archaea population decrease (Magdalena and González-Fernández, 2019). In anaerobic reactors devoted to VFAs production, methanogenic species represent a small percentage of the microbiome in terms of relative abundance. In this sense, applied operational conditions might result in a sludge specialization where methanogenic activity is outcompeted by fermentative bacteria (Magdalena et al., 2019b; Peces et al., 2021). This imbalance is intended to hamper biogas production and in turn, boost VFAs accumulation.

Methanogenic species are within the Euryarchaeota phylum (Córdova and Chamy, 2020). The activity of these species is detrimental for VFAs accumulation because their metabolic activity is linked to syntrophic carboxylate-oxidation reactions of propionic and butyric acids to form acetate and H_2 , which reduces the amount of VFAs in the digestate (Agler et al., 2011). It can be thus claimed that methanogenic archaea inactivation is essential to achieve high VFAs production yields. Previous studies targeting VFAs production have reported the presence of the hydrogenotrophic archaea *Methanobacterium* (Magdalena et al., 2019b; Greses et al., 2020b). By opposite, *Methanosaeta* genus (acetoclastic methanogen) prevailed in reactors devoted to biogas production from acetate when digesting *Chlorella* sp. and *Scenedesmus* (Greses et al., 2017; Zamorano-López et al., 2020). Contrary to biogas reactors where acetoclastic methanogens usually dominate, hydrogenotrophic species gain importance in acidogenic reactors. This is supported by the fact that hydrogenotrophic archaea are more robust than the acetoclastic ones (Xu et al., 2010) and thus, they can handle better the harsh conditions (high OLR and short HRT) implemented in reactors operated for VFAs production.

1.2. Microbial oils production using VFAs as carbon source

Among the bio-based industries, oil-based chemistry arises as a promising alternative to petroleum. In the transport sector, biodiesel produced from oleaginous feedstocks might replace diesel fuel (Bharathiraja et al., 2017; Madani et al., 2017; Diwan et al., 2018b). Additionally, fatty acids (FA) and derivatives can find wide range of applications: i) precursors for agricultural chemicals like biocides (Avis et al., 2000; Clausen et al., 2010); ii) nutritional (Beopoulos et al., 2009a; Bellou et al., 2016) and pharmaceutical purposes (Jenkins et al., 2015; Pfeuffer and Jaudszus, 2018); and iii) industrial chemicals such as flavour and plasticizers (Ochsenreither et al., 2016) (Table 1.3).

Table 1.3. Oil-based chemistry applications

Application	Acid
Energy: biodiesel	C14-C18 mixtures
Agriculture: biocide	C17, C10
Nutrition: Food additive, animal feed supplement, flavours	PUFAs ω -3/ ω -6 (C18-C16)
Cosmetics and pharmaceutical: antiinflammatory, biomarker, antibiotic	C15, C17, 20:4 ω -6, 22:6 ω -3
Industrial chemicals: plasticizers, solvent, fragrances, detergents	Fatty alcohols, methyl ester, wax ester

(Madani et al., 2017)

The conventional raw materials employed for oleochemicals include vegetable and animal oils. The competition with food necessities makes them unsustainable, leading to the need of finding non-edible sources. In order to solve supply issues, MO have emerged as promising alternatives to vegetable oils because of their higher lipid and biomass productivities, shorter cultivation period and lower surface requirements than plants (Cho and Park, 2018). Furthermore, MO share similar characteristics and FA profile than the plants oils most commonly used in industry (Madani et al., 2017). Due to all these interesting features, MO are expected to have a great impact in the oleochemical sector.

Microorganisms are able to consume the carbon source from the medium and convert it into lipid storage material. Although all microorganisms are able to synthesize lipids, only some of them are able to accumulate more than 20 % DW. These type of microorganisms are classified as oleaginous and belong to different species of bacteria (Meng et al., 2009; Goswami et al., 2017), microalgae (Fei et al., 2015; Shin et al., 2015) and yeasts (Athenaki et al., 2018).

During the last decades, microalgae have been pointed out as a suitable alternative to vegetable crops for oil production due to their fast growth, high lipid yields per unit of cultivation area, and reduced CO₂ emissions (Spolaore et al., 2006; González-Fernández and Muñoz, 2017). To obtain an economically viable lipid production from microalgae, research efforts have been conducted to favor microalgal growth on digestates coming from AD of wastewater, food waste or manure (Cho et al., 2015a; Fei et al., 2015; Shin et al., 2015). However, the high ammonium concentration in AD effluents is, in some cases, inhibitory for microalgae growth (González-Fernández and Muñoz, 2017). Besides

this, microalgae require favorable light and temperature conditions, and exhibit slower growth rates than bacteria and yeasts (Kempes et al., 2012). For all these reasons, alternative microorganisms are being proposed to produce MO via biotechnological routes. Bacteria are easily cultured, achieving high cell density in short period of times, but their lipid content is on average lower than those exhibited by microalgae or yeasts (Meng et al., 2009).

Yeasts exhibit several advantages over other microorganisms for MO production such their fast growth rates, ability to thrive at high cell densities and a robust physiology. All these features render yeasts well suited for bioreactor cultivations (Meng et al., 2009). OY can accumulate lipids in significant quantities (up to 60 % DW) in separate structures called lipid bodies (LB), which facilitates the oil extraction during downstream processing (Papanikolaou and Aggelis, 2011a; Sitepu et al., 2014). Additionally, yeasts have the outstanding capability to be cultivated on a wide range of wastes such as sugar-based materials (lignocellulosic residues), glycerol and hydrophobic substrates (crude oil, industrial FA, etc.) (Papanikolaou and Aggelis, 2011b; Diwan et al., 2018b; Tomás-Pejó et al., 2021). Sugar-based feedstocks have been widely employed as substrates for MO production. However, the use of these substrates as carbon source, apart from their restricted availability, contributes to increase the bioprocess cost. In fact, during MO production, the cost of the substrate can account for 60 % of the overall production cost (Fei et al., 2011a). This renders the process to be economically unfeasible when using sugar-rich media as substrate. One way to reduce MO production costs is to use alternative low-cost carbon sources, such is the case of VFAs derived from the AF of organic wastes.

VFAs as carbon source

Limited information is available related to OY cultivation on VFAs as sole carbon source mostly focused on synthetic medium (Llamas et al., 2019). When VFAs are used as carbon source, concentrations higher than 5 g/L have been reported to be inhibitory for yeast growth (Fei et al., 2011a; Fontanille et al., 2012; Huang et al., 2016; Liu et al.,

2017a). Besides, several studies have concluded that the inhibitory effect of VFAs on yeast increases concomitantly with the VFAs chain length (Gao et al., 2017; Liu et al., 2017a). Despite the fact that longer lag phases and fermentation periods are required when using long-chain VFAs (C4-C6), some works have reported higher biomass and lipid yields in comparison to the obtained when using short-chain VFAs (C2-C3) (Liu et al., 2017a)

The VFAs profile in the media may not only affect yeast growth but also VFAs consumption rates. In this sense, the preferential utilization of acetic acid in the case of *Cryptococcus curvatus* was reported (Vajpeyi and Chandran, 2015), while other authors demonstrated the simultaneous utilization of different acids with the same OY (Xu et al., 2015). Higher lipid content and yield in *Cryptococcus albidus* was also promoted by predominance of acetic acid in VFAs mixture when compared to mixtures where propionic or butyric acids were the most abundant (Fei et al., 2011a). Remarkably, not only the lipid concentration but also lipid distribution is affected by different VFAs. It has been demonstrated that increasing propionic and butyric acids could enhance odd-chain FA production (Zheng et al., 2012; Kolouchová et al., 2015; 2017a; Park et al., 2018). At this point, it is worth highlighting that most of these studies have been performed using synthetic media and the information about lipid production from VFAs-rich digestates produced via AF is still scarce.

1.2.1. Oleaginous yeasts

Some well-known OY strains can accumulate intracellular lipids up to 60 % DW (*i.e.* *Cryptococcus* sp., *Lipomyces* sp., *Rhodosporidium* sp., *Rhodotorula* sp., *Yarrowia* sp. and *Trichosporon* sp.) (Papanikolaou and Aggelis, 2011a). Lipids from OY present similar compositions to common edible vegetable oils, such as rapeseed, sunflower, palm and soybean (Sitepu et al., 2013). OY lipids are mainly composed of triacylglycerols (TAGs) rich in FA (myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Amara et al., 2016). Other lipidic components can be present in important quantities such as free fatty acids (FFAs), neutral lipids, sterols, and polar fractions (*e.g.* phospholipids, sphingolipids, glycolipids)

(Beopoulos et al., 2009a). While the stoichiometry and operational conditions are well-established when employing glucose as a substrate (Papanikolaou and Aggelis, 2011a), the use of VFAs in MO is still in its infancy and it is not even clear the metabolic route that yeasts use to convert VFAs into oils.

1.2.2. Lipids metabolic pathways in oleaginous yeasts

1.2.2.1. Lipids synthesis and accumulation

In yeasts, lipids may be synthesized and accumulated via two different strategies depending on the substrate (Figure 1.11, Publication IV): 1) *de novo* synthesis, producing FA biosynthesis precursors in presence of sugar substrates and induced by the limitation of a primary nutrients, and 2) *ex novo* synthesis, involving the uptake of FA from the culture independently of nutrients concentration.

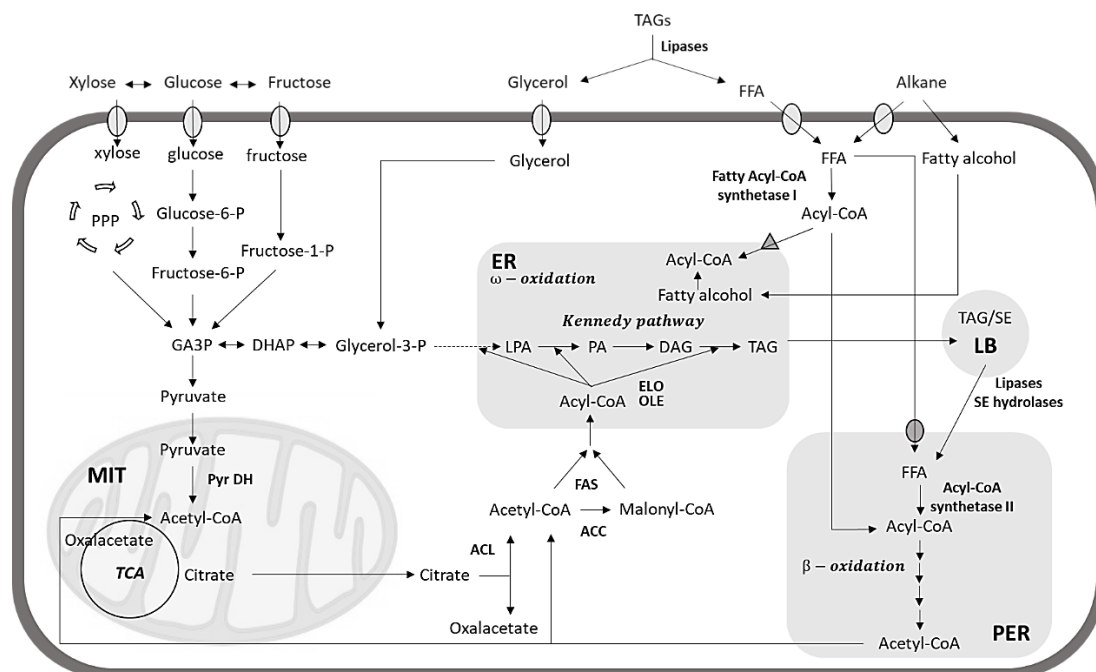


Figure 1.11. A simplified overview of metabolic pathways involved in oleaginous yeast synthesis of FA from various substrates.

Abbreviations: ACC, Acetyl-CoA carboxylase; ACL, ATP-citrate lyase; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; ELO, Elongase ER, endoplasmic reticulum; FAS, fatty acid synthase; FFA, free fatty acids; GA3P, glyceraldehyde-3-phosphate; LBs, lipid bodies; LPA, lysophosphatidic acid; MIT, mitochondria; OLE, desaturase PA, phosphatidic acid; PER, peroxisome; PPP, pentose phosphate pathway; Pyr DH, pyruvate dehydrogenase; SE, steryl ester; TAC, tricarboxylic acid cycle; TAG, triacylglycerol. (Llamas et al., 2019)

1.2.2.1.1. *De novo* pathway

This anabolic biochemical process involves the production of lipids precursors such as acetyl- and malonyl-CoA and their integration into lipid synthesis pathway (Beopoulos et al., 2009a; Xie, 2017). Oleaginous microorganisms do not possess a hyperactive system of FA biosynthesis, but they are capable of producing significant quantities of acetyl-CoA, which is the basic unit for *de novo* FA biosynthesis (Figure 1.11, Publication IV) (Papanikolaou and Aggelis, 2011a).

The key factor for lipid accumulation from sugar-based substrates in yeasts is the limitation of essential nutrients (Papanikolaou and Aggelis, 2011a). Under these circumstances, yeast continues assimilating the carbon source from the medium, which is channelled towards lipid synthesis instead of proteins and nucleic acids (Economou et al., 2010).

1.2.2.1.2. *Ex novo* pathway

Some OY are able to grow and develop adaptation mechanisms for the efficient use of hydrophobic substrates (*i.e.* n-alkanes, FAs and TAGs) (Vasiliadou et al., 2018). As it can be seen in Figure 1.11, the *ex novo* pathway involves the uptake of FAs from the culture medium (Beopoulos and Nicaud, 2012). This pathway requires hydrolysis of complex hydrophobic substrates and the subsequent transport of the released FA within the cell (Beopoulos et al., 2009a). In general, FFAs uptake depends on the carbon chain length and saturation degree of the available FAs (Papanikolaou and Aggelis, 2003). Once inside the cell, the FFAs can be stored in LB or can be degraded for growth needs through the β -oxidation pathway (Section 1.2.2.2). In both cases, FFAs should be converted in Acyl-CoA. Afterwards, structural and reserve lipids are produced from fatty Acyl-CoA (regardless of *de novo* or *ex novo* synthesis). LBs are primarily composed of neutral lipids, particularly TAGs (85 %) and some steryl ester (SE) (8 %) (Xie, 2017).

Although lipid accumulation in yeasts when hydrophobic substrates are utilized as sole carbon source is not clear, it seems to be a growth-coupled process. Contrary to what occurs in *de novo* lipid biosynthesis that takes place only after nitrogen exhaustion, lipid

accumulation from VFAs in the presence of assimilable nitrogen takes place along with cell growth (Athenaki et al., 2018). There are some examples related to microorganism's growth with hydrophobic and hydrophilic materials as co-substrates, indicating a possible simultaneous *ex novo* and *de novo* lipid biosynthesis pathways (Beopoulos et al., 2009a; Fontanille et al., 2012; Gao et al., 2017).

1.2.2.2. Lipids turnover

As a general rule, microorganisms consume their own lipid reserves when their metabolic requirements cannot be fulfilled by extracellular carbon sources. Lipids turnover has been observed regardless of the lipid biosynthesis route (Papanikolaou and Aggelis, 2011a). Lipid degradation is a complex process that can be generally divided in three sequential steps: remobilization of the LBs, transport and activation, and β -oxidation process (Dulermo et al., 2015) (Figure 1.11, Publication IV).

In order to carry out LB breakdown, microorganisms should obligatory possess intracellular lipases and steryl ester hydrolases (Dulermo et al., 2013). Subsequently, the released FFAs should be activated and transported to the peroxisome where lipid degradation takes place via the β -oxidation process (Ledesma-Amaro and Nicaud, 2016).

1.2.3. Approaches for increasing lipids accumulation

Lipid accumulation and composition is genetically determined (Meng et al., 2009). Apart from genetic factors, the lipid content can be also altered by tuning culture conditions (Beopoulos et al., 2009a). Several approaches to improve the fermentation process for an efficient VFAs conversion to lipids are discussed below.

1.2.3.1. Microorganisms-oriented approaches

1.2.3.1.1. Isolation and screening of new oleaginous yeast

Some OY such as *Candida tropicalis*, *Pichia kudriavzevii*, *Lipomyces lipoferrus*, *Kodamaea ohmeri*, *Trichosporonoides spathulata* and *Rhodotorula babjevae* have been isolated and identified from rotten fruits, soil and glycerol, among others (Duarte et al., 2013; Ayadi et al., 2018; Diwan et al., 2018a; Guerfali et al., 2019).

There is only limited information on OY cultivation on VFAs as the sole carbon source and thus, only a narrow range of microorganisms with this capacity have been studied (mostly *C. curvatus* and *Y. lipolytica*). The search of new yeasts with the ability to grow on these novel substrates and to accumulate high lipid quantities becomes essential. For instance, *Naganishia liquefaciens* NITTS2, isolated from a wastewater treatment plant, was selected due to its ability to use VFAs-rich digestate of waste sludge attaining high lipid content (50 % DW) (Selvakumar and Sivashanmugam, 2018). Kolouchová and co-workers (2015) highlighted the ability of *Trichosporon cutaneum* as lipid-accumulating microorganisms (43 % DW) when performing an screening of OY for lipid production using VFAs as carbon source.

Due to a general stress response mechanism, OY isolated from extreme environments, such as wastes of oil mills or biodiesel plants, are able to grow in adverse conditions. In this sense, isolation, selection, and characterization of microorganisms with appropriate features for lipid production can significantly improve fermentation yields.

1.2.3.1.2. Co-cultures and mixed cultures

Although pure cultures are widely extended in biotechnological applications, research on co-cultivation of microorganisms has mainly been conducted to improve the limitation of single strains. For instance, some yeasts (*Rhodotorula glutinis*, *Trichosporonoides spathulata*) have been co-cultured with microalgae (*C. vulgaris*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus*) for lipid production (Wang et al., 2016; Liu et al., 2018). The co-cultivation of yeasts and microalgae appears to be

advantageous since these two microorganisms can form a symbiotic relationship due to the balance between oxygen-CO₂ and pH adjustment during the cultivation (Zuccaro et al., 2019). Within this synergic activity, microalgae provide (by photosynthetic means) the oxygen necessary for aerobic yeast, consuming in turn inorganic carbon (CO₂) released from yeast respiration. As a matter of fact, higher final lipid production from VFAs was determined when co-culturing *Rhodospiridium toruloides* and *C. pyrenoidosa* than in separated cultivation (Ling et al., 2014). A co-culture of *C. vulgaris* and *R. glutinis*/*R. toruloides* in industrial food wastes resulted in higher lipid concentration and shorter cultivation times than pure cultures (Cheirsilp et al., 2011; Zeng et al., 2018). These results point out at mix-cultures as a promising strategy for lipid production from waste streams.

1.2.3.1.3. Genetic modifications of oleaginous yeasts

Mutation and adaptive evolution are interesting tools for diverting yeast metabolism towards desired pathways for improving OY features. Yeasts can be genetically modified for lipid overproduction by optimization of FA synthesis (Ledesma-Amaro and Nicaud, 2016). In this sense, current strategies include the overexpression of genes involved in lipid synthesis and storage (Ferreira et al., 2018; Niehus et al., 2018) or those involved in the supply of lipid precursors (Qiao et al., 2017), the blocking of different pathways competing with lipid biosynthesis (Dourou et al., 2018; Sagnak et al., 2018) and the avoidance or reduction of lipid degradation pathways (Ledesma-Amaro and Nicaud, 2016; Marella et al., 2018). It is worth mentioning that most of the studies focused on improving FA synthesis through genetic engineering have been performed using sugars-based substrates (following *de novo* pathway) and there are only a few studies about genetic modification of genes encoding enzymes involved in the *ex novo* pathway (Sabirova et al., 2011; Park et al., 2018; Lee et al., 2019) .

On the other hand, adaptive evolution consists on microorganisms culture under selective pressure conditions for many generations in order to enrich favorable genetic or phenotypic changes (Dourou et al., 2018). Since lipid production routes from VFAs

are not completely understood, adaptive evolution is considered as a powerful tool to improve microorganism's properties due to its simplicity and effectiveness. Successful evolution strategies have been conducted to improve yeasts lipid accumulation using VFAs while overcoming other environmental stress factors such as the presence of inhibitors, unfavourable pH or temperature (Liu et al., 2015; Díaz et al., 2018).

1.2.3.2. Process-oriented approaches

To optimize the lipid production process not only the use of new or developed strains is needed but also the optimization of specific culture conditions. Henceforth, the optimization of the fermentation process is also crucial to overcome challenges associated to lipid production. Substrate type and concentration, agitation, temperature, pH and nutrients or oxygen concentrations are culture conditions that can strongly affect microbial lipid accumulation as discussed below.

1.2.3.2.1. Culture conditions

C/N ratio

It is generally accepted that C/N ratio plays an important role in MO production when sugar-based substrates are employed. Generally, MO are produced with an excessive carbon source but limited nutrient concentration. The lack of some nutrients such as nitrogen, iron or magnesium can channel carbon flux promoting lipid accumulation as well as suppress lipid degradation pathways (Papanikolaou and Aggelis, 2011a).

However, results on C/N effect on lipid production from VFAs are sometimes contradictory. For instance, an improvement in lipid content from 16.3 % DW to 48.2 % DW was obtained in *R. toruloides* when increasing the C/N from 5 to 200 using acetic acid as substrate (Huang et al., 2016). However, an excessively high C/N ratio was found to limit growth and lipid production. For instance, no significant increase of lipids content was obtained by Béligon and co-workers (2015) when *C. curvatus* was grown in acetate with C/N ratio above 300. Gong and co-workers (2015) reported higher lipid

yields when growing *C. curvatus* on acetate under nitrogen-rich conditions than under nitrogen-limited conditions. Liu and co-workers (2017a) identified different effects of C/N ratio on lipid accumulation in *C. curvatus* depending on VFAs concentration. Opposite to what is well-known for sugar-based substrates, where the C/N ratio only changes carbon flow within the cells, it seems likely that the C/N ratio also affects substrate utilization when using VFAs as carbon source (Huang et al., 2016). Therefore, the suitable C/N ratio should be carefully determined depending on the substrate used to optimize lipid accumulation in OY.

Temperature, pH and aeration

Temperatures ranging from 25-28 °C are commonly used in lipid production by OY since temperatures higher than 30 °C have been reported to hamper microbial growth and lipid accumulation (Fei et al., 2011a; Gao et al., 2017).

pH also has a crucial effect on OY lipid accumulation when VFAs are used as carbon source because it can influence the acid form (protonated/deprotonated) in solution and the acid transport inside the cell (Fei et al., 2011a; Béligon et al., 2015; Liu et al., 2017a). OY have been found to be strongly inhibited at pH lower than 4, while the degree of inhibition is lower at neutral or basic pH (Fei et al., 2011a; Liu et al., 2017a). Furthermore, the highest biomass production and lipid accumulation has been proposed to take place at pH around 6-7 (Chi et al., 2011).

Aeration can also be an important requirement when aerobic yeasts are used (Blazeck et al., 2014; Qiao et al., 2017). Fei and co-workers (2011b) found that the amount of oxygen in the air was enough for cell growth but the supply of an oxygen-enriched air (40 %) enhanced lipid accumulation. Thus, it can be speculated that providing an oxygen supply could be an effective method to increase lipid accumulation using VFAs as substrate during aerobic fermentations.

1.2.3.2.2. Operational mode

Depending on the substrate addition method, fermentation can be operated in different modes. Culture mode has a great impact on lipid accumulation and culture optimization can be used as a tool to enhance microbial lipid production. A compilation of biomass and lipid yields from VFAs using different operational modes is presented in Table 1.4 (Publication IV).

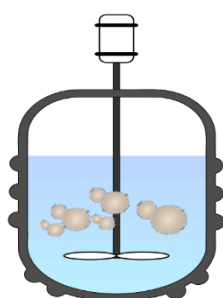
Table 1.4. MO production by OY cultured on VFAs using different culture strategies.

Yeast strain	Substrate	Operational mode	Biomass (g/L)	Lipid content (% DW)	Lipid yield (g/g)	Ref (*)
<i>C. albidus</i>	VFAs (5 g/L)	Batch	2.6	25.1	0.13	(1)
	Ac (6 g/L)	Batch	2.8	25.8	0.12	
	G (30 g/L)+Ac	Two-stage batch	8.1	38.3	0.25	
	G (30 g/L)+VFAs	Two-stage batch	7.9	43.8	0.27	
	G (30 g/L)+Ac	Two-stage fed-batch	27.0	55.0	0.17	
<i>R. toruloides</i>	Ac (20 g/L)	Batch	4.3	48.2	0.12	(2)
	G (40 g/L)+Ac (20 g/L)	Two-stage batch	6.8	50.1	0.10	
	Ac (4 g/L)	Sequential batch	4.2	38.6	0.08	
<i>C. curvatus</i>	VFAs (3.35 g/L)	Sequential batch		39.6	0.61	(3)
<i>C. curvatus</i>	VFAs (9.27 g/L)	Batch	3.7	33.1	0.13	(4)
	VFAs (9,27 g/L)	Fed-batch repeated	2.4	61.0	0.15	
<i>C. curvatus</i>	Ac (4 g/L)	Fed-batch		30.5		(5)
<i>C. curvatus</i>	Ac (5 g/L)	Fed-batch		53.0	0.23	(6)
<i>C. curvatus</i>	VFAs (12+4 g/L)	Fed batch	6.8	42.0	0.13	(7)
<i>Y. lipolytica</i>	Ac (12 g/L)	Fed-batch	5.9	30.8	0.15	(8)
	P (8 g/L)	Fed-batch	3.4	25.7	0.11	
	B (12 g/L)	Fed-batch	3.4	25.1	0.07	
	G+Ac	Two-stage fed-batch	30.8	40.7	0.13	
	G+P	Two-stage fed-batch	39.0	38.1	0.20	
	G+B	Two-stage fed-batch	31.8	24.0	0.11	
	G+VFAs (3:1:1)	Two-stage fed-batch	41.1	40.2	0.20	
	Gl+Ac	Two-stage fed-batch	40.9	38.4	0.17	
	Gl+P	Two-stage fed-batch	36.8	38.8	0.17	
	Gl+B	Two-stage fed-batch	30.8	37.6	0.19	
	Gl+ VFAs (3:1:1)	Two-stage fed-batch	41.0	34.6	0.16	
<i>C. curvatus</i>	G (15 g/L)+Ac (5 g/L)	Two-stage fedbatch		50.9	0.15	(9)
	G (5 g/L)+Ac (5 g/L)	Two-stage batch		47.3	0.19	
<i>C. curvatus</i>	Ac (5 g/L)	Continuous		56.7	0.18	(10)
	Ac (30 g/L)	Continuous		66.4	0.12	
<i>C. albidus</i>	VFAs (6.5 gCOD /L)	Continuous	1.1	28.3	0.04	(11)

Abbreviations: Ac, acetic acid; B, butyric acid; G, glucose; Gl, glycerol; P, propionic acid; Ref*: (1) Fei et al., (2011b); (2) Huang et al., (2016); (3) Liu et al., (2016); (4) Xu et al., (2015); (5) Chi et al., (2011); (6) Bélignon et al., (2015); (7) Bélignon et al., (2016); (8) Fontanille et al., (2012); (9) Christophe et al., (2012); (10) Gong et al., (2015); (11) Vajpeyi and Chandran, (2015).

Batch culture

When dealing with batch cultures, the microorganism is inoculated in a fixed volume of medium and the desired product is recovered at the end of the process (Figure 1.12). In this cultivation mode, culture parameters change with the microorganism activity which passes through the different growth phases, *i.e.* lag, exponential, stationary and death phases (Sauer, 2011).



VFAs

Figure 1.12. Schematic diagram of batch configuration during oleaginous fermentation.

Batch fermentation has several limitations considering substrate and by-product inhibition. More specifically, when using VFAs for MO production, initial inhibition of microorganisms at certain VFAs concentration must be considered (Zheng et al., 2012; Gao et al., 2017).

In the same way, nutrient and carbon source depletion during batch cultivation can lead to a shift into secondary microbial metabolism, inducing by-product synthesis with a concomitant decrease of microbial growth and growth-associated products. This is the case, for instance, of MO produced using sugar based substrates. In this case, substrate exhaustion in the medium gives rise to a citric acid production instead of lipid formation (Beopoulos et al., 2009a).

Therefore, it is essential to develop culture strategies that allow C/N ratio control and the reduction of substrate inhibition (*i.e.* two-stage batch, sequential batch, fed-batch, two-stage fed-batch and continuous mode) (Table 1.4).

Two-stage batch

This cultivation mode is a common practice to focus each stage on a specific target (*i.e.* microbial growth or lipid accumulation). Normally, the first step is designed to attain an optimum biomass growth. After a batch period in suitable conditions for yeast growth, cells are transferred into a medium with VFAs as sole carbon source to enhance lipid production (Figure 1.13). This two-stage batch mode has been successfully applied for obtaining higher biomass and lipid yields than the obtained in single batch cultures using only VFAs as carbon source (Fei et al., 2011b; Christophe et al., 2012; Huang et al., 2016).

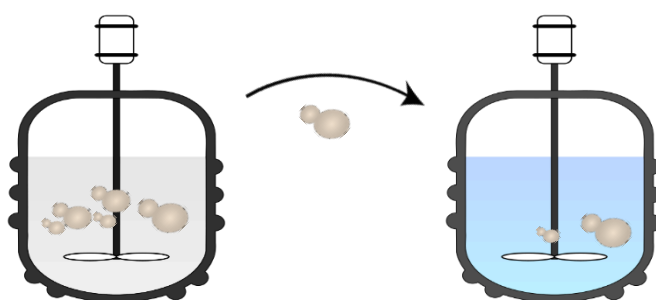


Figure 1.13. Schematic diagram two-stage batch strategy for MO production from VFAs.

Sequential batch

Another strategy to enhance VFAs conversion efficiencies into MO is the use of sequential batch, where the medium is replaced when substrate is finished. Due to the microbial adaptation to the medium, this approach has been reported to be successful to increase biomass and lipid content (Xu et al., 2015; Huang et al., 2016; Liu et al., 2016) (Figure 1.14).

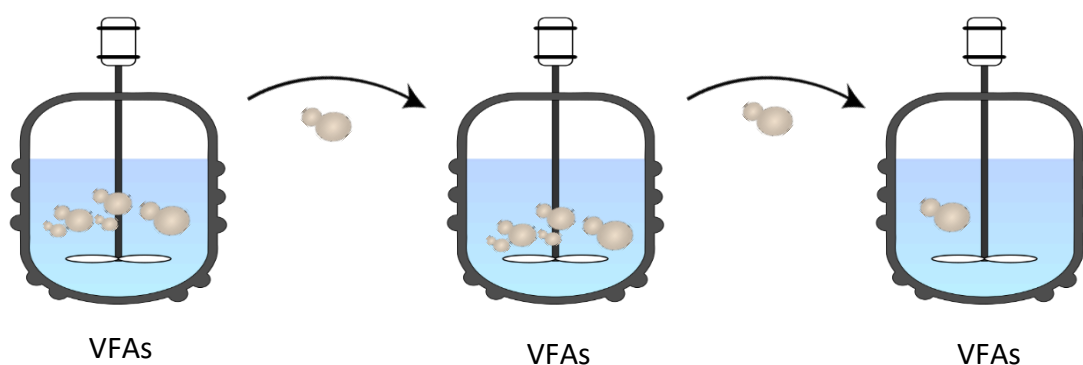


Figure 1.14. Schematic diagram of sequential batch strategy for MO production from VFAs.

When compared to batch culture, an increase in lipid content in *C. curvatus* from 50 % to 61 % DW was obtained after 2 cycles of culture (Xu et al., 2015). Huang and co-workers (2016) compared sequential batch cultivation with a two-stage batch strategy for *R. toruloides* using VFAs as a substrate and they concluded that sequential batch was a better option in terms of lipid production given the carbon-excess condition provided periodically along with the yeast adaptation to VFAs.

Due to the aforementioned inhibitory effect caused by high concentration of VFAs, acid concentrations in the fermentation media must be maintained at low levels. This fact is considered a drawback since low carbon source concentration hampers biomass and lipid production using batch, two stage batch and sequential batch cultivation.

Fed-batch culture

Yeast inoculation is performed similarly to batch culture, and after a period of time, a new substrate batch (and, if required, more inoculum) is fed in one or more stages (Figure 1.15), resulting in an increase in working volume. In this case, feeding addition must be adequate to maintain the correct substrate concentration. Fed-batch could be problematic when substrate concentration is low thus introducing a high dilution rate. Usually feed-batch are performed with high concentrated substrates.

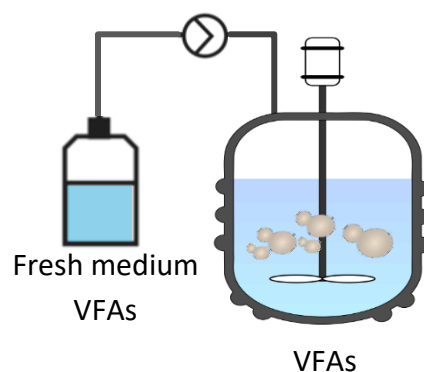


Figure 1.15. Schematic diagram of fed-batch strategy for MO production from VFAs.

Fed-batch mode results in higher stability than batch mode because fresh nutrients are added before microorganisms enter in the stationary phase (Hewitt and Nienow, 2007).

Furthermore, higher biomass and lipids productions are obtained because VFAs concentrations are always below the inhibitory threshold and other inhibitory compounds are gradually added to the culture (Kitcha and Cheirsilp, 2013).

As shown in Table 1.4, when using two-stage fed-batch mode (similar strategy than two-stage batch but working both bioreactor on fed-batch mode), lipid content and yields are much higher than those obtained in fed-batch mode. Using fed-batch (Chi et al., 2011; Fontanille et al., 2012; Zheng et al., 2012; Béligon et al., 2016, 2015) and two-stage fed-batch mode (Christophe et al., 2012; Fontanille et al., 2012), significant improvements in the lipid content could be accomplished since extra carbon source can be provided while avoiding high VFAs concentrations. Fontanille and co-workers (2012) evaluated these two culture modes for *Y. lipolytica* grown on acetic, propionic and butyric acid after glucose or glycerol-rich medium.

Continuous fermentations

When continuous mode is employed, fresh medium is continuously added after microbial inoculation (Figure 1.16). Simultaneously, fermented medium, cells and products are harvested at the same flow rate. Therefore, a constant working volume is maintained. To achieve stability in process parameters during steady state, a proper dilution rate and operational conditions must be settled allowing cell growth while avoiding the wash out of cells (Mears et al., 2017).

Employing continuous mode, substrate and product inhibition can be overcome since the continuous input/output allows to keep low VFAs concentration and remove products from the reactor. However, contamination of pure cultures is one of the main drawbacks of this operational mode (Roukas, 1996).

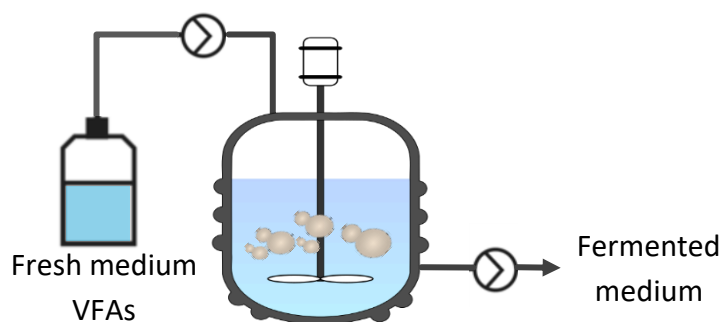
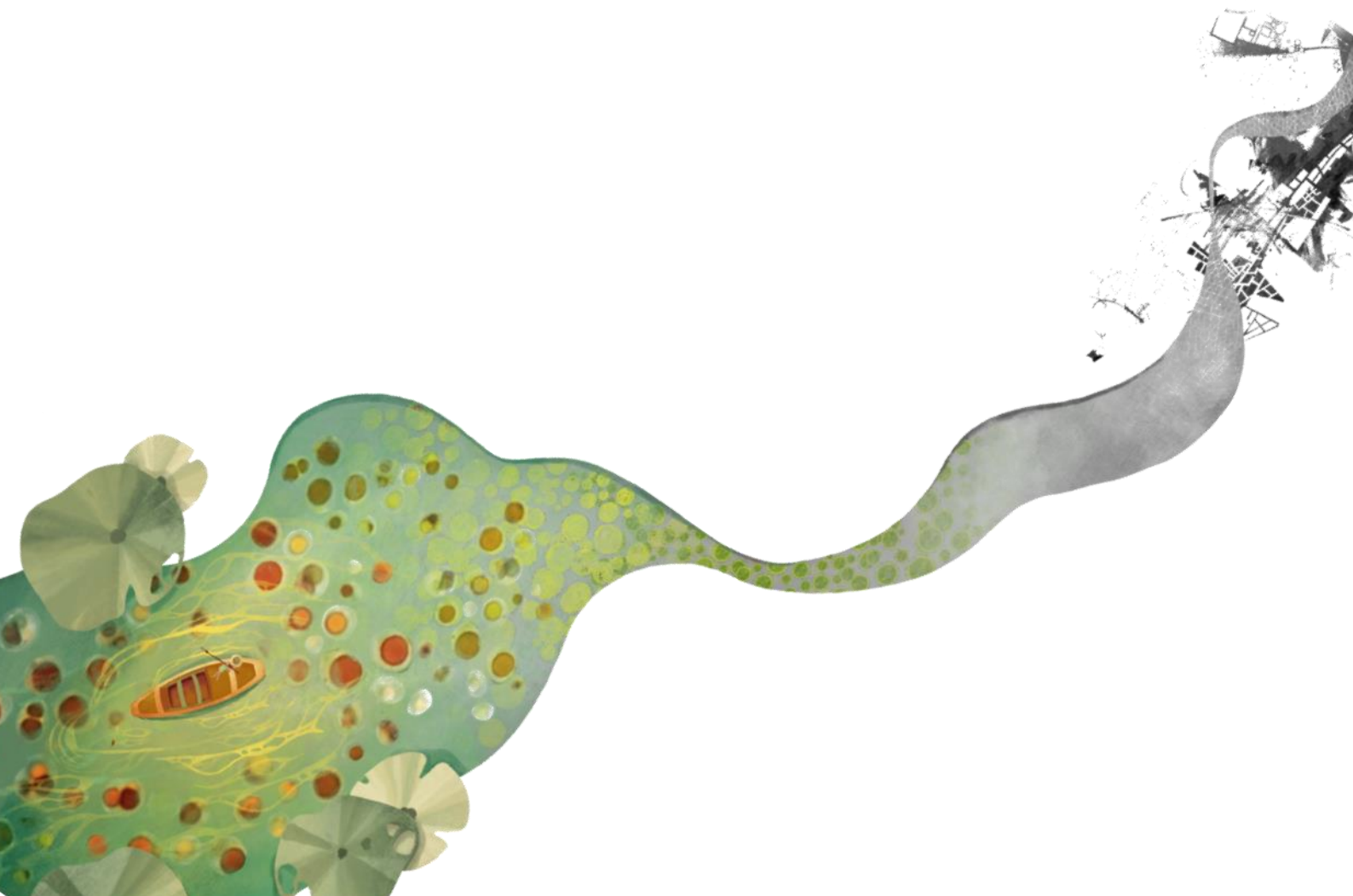


Figure 1.16. Schematic diagram of continuous operation for MO production from VFAs.

Only few reports about continuous MO production from VFAs have been published (Gong et al., 2015; Vajpeyi and Chandran, 2015). Gong and co-workers (2015) found that lipid yields dropped at increasing dilution rate from 0.04 h^{-1} to 0.14 h^{-1} , confirming that low dilution rates give rise to higher lipid and biomass concentration. Thus, the optimization of the process when using continuous mode must involve optimal operational conditions (temperature, pH and O_2) as well as optimal dilution rates.

2. OBJECTIVES



2. Objectives

The different studies described in this Thesis have been performed in the Biotechnological Processes Unit of IMDEA Energy. The research of this Unit focuses on the production of biofuels and bio-based products from organic residues, such as lignocellulose, microalgae, food and municipal solid wastes. Additionally, the study related to the screening of oleaginous yeasts was performed in the Department of Microbiology at University of Patras (Greece).

Microalgae biomass generated in the context of wastewater treatment plants has been mainly devoted to biodiesel, bioethanol or biogas production. Nevertheless, this biomass can be used to produce not only biofuels but also high value-added products. In this context, microalgae arises as a potential feedstock for bio-based VFAs production via AF. After VFAs generation, other products can be produced from the spent biomass maximizing carbon recovery. Within this perspective, digestates obtained from the AF can be further used within a two-fold approach to obtain bioproducts and bioenergy.

On one hand, VFAs in the liquid fraction can be exploited as marketable chemicals or can be assessed as carbon sources for MO production. These MO are expected to have a great impact in the oleochemical sector as promising alternatives to petroleum for the production of chemicals, polymers, plasticizers and lubricants. Due to the high production cost of MO produced from traditional carbon sources (*i.e.* glucose), considerable efforts have been addressed to find alternative substrates for yeasts growth. Some OY are able to utilize the VFAs from the culture medium and convert them into lipid storage material. Therefore, VFAs are regarded as low-cost carbon sources for lipid production via oleaginous fermentation.

Together with the liquid fraction rich in VFAs, the more recalcitrant organic matter that is not converted in the AF still remains in a solid fraction. This remaining solid fraction can be further used for biogas production via conventional AD. In this manner, biogas produced can serve as an energy source in a biorefinery plant, closing the loop of the circular economy approach.

Objectives

All things considered, the general objective of the present Thesis was to evaluate the parameters that allow the optimization of multi-product generation from microalgae biomass in consecutive bioprocesses. In this manner, the proposed strategy entailed the production of VFAs, MO and biogas.

Pursuing this main objective, the following specific objectives were established:

Ob.i. To maximize VFAs yield production via AF of *C. vulgaris* by tuning operational parameters. More specifically, the effect of temperature and HRT were studied in terms of organic matter conversion into VFAs and profile.

Ob.ii. To assess the benefits of *C. vulgaris* biomass pretreatments (one thermal and two enzymatic: protease and carbohydrase) on the AF in terms of VFAs production and profile.

Ob.iii. To evaluate the feasibility of using the VFAs present in digestates as carbon source for *Y. lipolytica*. For this purpose, individual and mixtures of synthetic VFAs as well as real digestate were tested to determine uptake rates and potential inhibitory effects.

Ob.iv. To find new suitable OY strains to produce MO from VFAs-rich digestates by comparing yeast ability to metabolize VFAs and produce lipid. To this end, the most promising yeasts were screened.

Ob.v. To revalorize the digestate solid fraction as substrate for biogas production purposes. For that purpose, the methane potential of this fraction was assessed via conventional AD in batch and continuous mode.

For a better comprehension of the overall PhD Thesis, an scheme of the performed research to achieve the general and specific objectives together with the different publications used in the results and discussion section can be found in Figure 2.1.

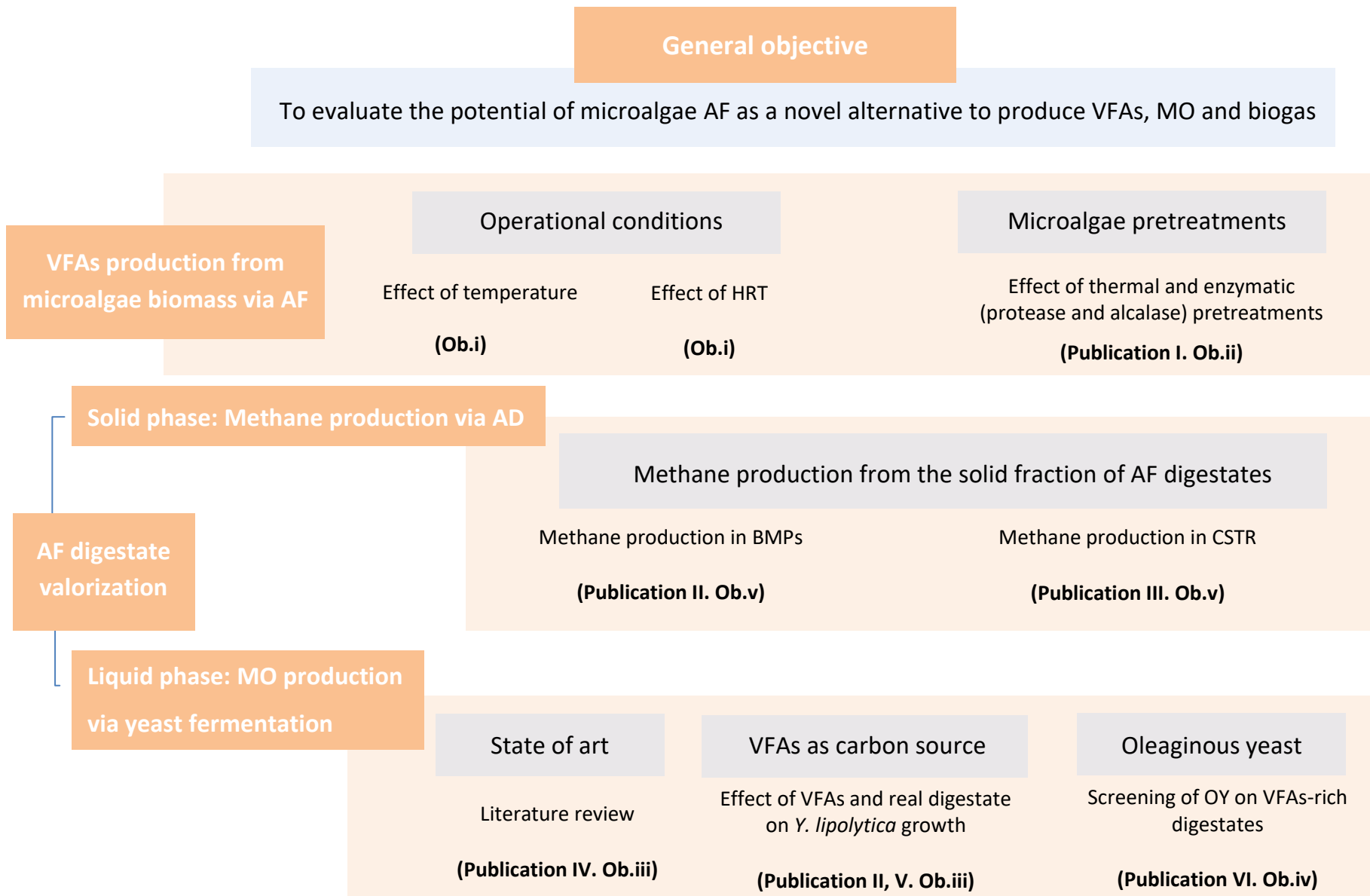
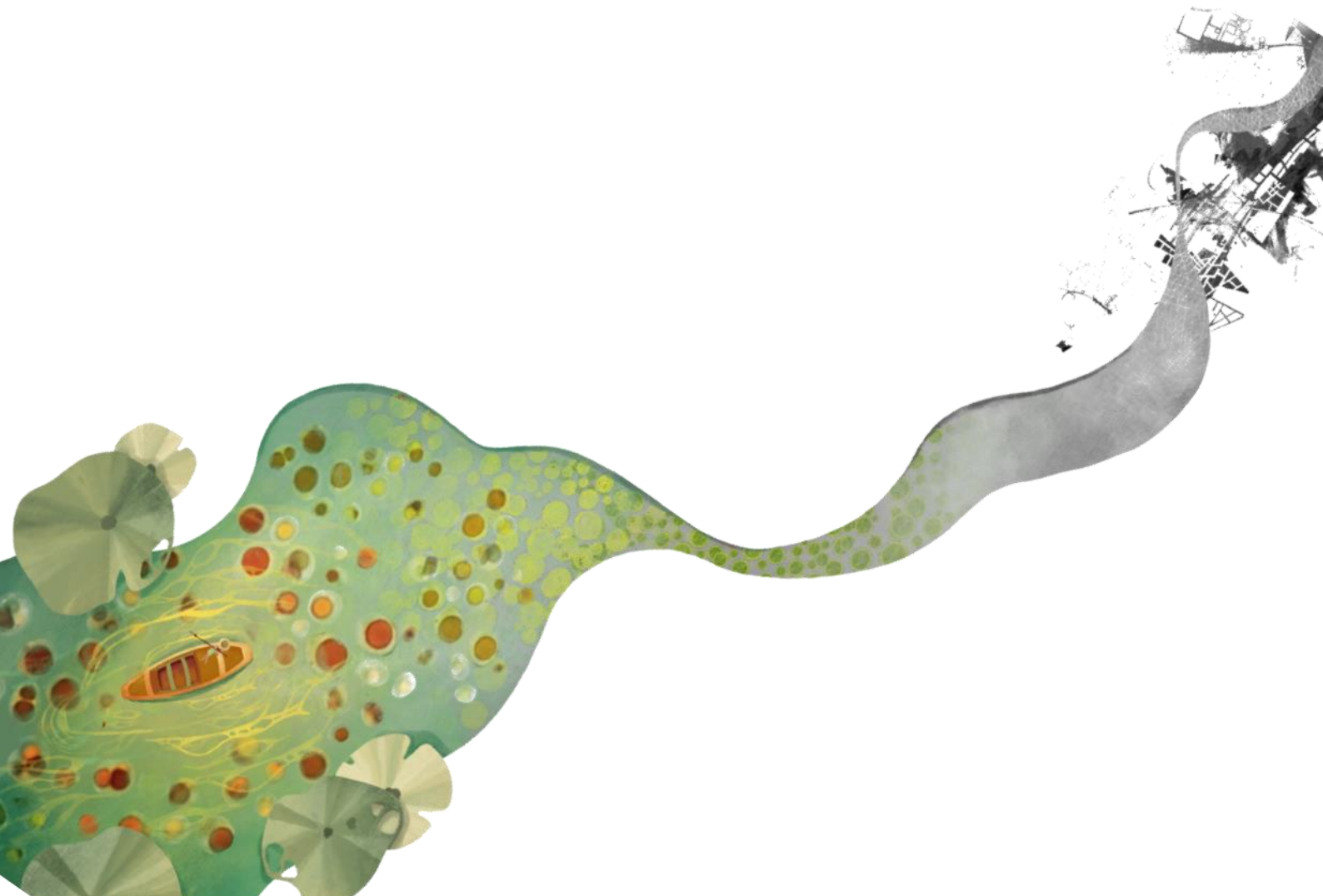


Figure 2.1. Schematic summary of the performed research to achieve the general objective of this Thesis.

3. MATERIALS AND METHODS



3. Materials and methods

3.1. Anaerobic fermentation for VFAs production

3.1.1. Substrate for VFAs production

Microalgae biomass used in this Thesis as AF substrate belonged to the specie *C. vulgaris*. This biomass was purchased from Allmicroalgae (Lisbon, Portugal) and it was characterized in each experiment. Average characterization values can be found in Table 3.1.

Table 3.1. Characterization of *C. vulgaris* used as substrate for VFAs production in this Thesis.

Chemical parameters	Average $\pm$ Standard deviation
TS (g/L)	70.3 $\pm$ 4.2
VS/TS (% DW)	91.3 $\pm$ 0.9
sCOD (g/L)	10.3 $\pm$ 4.5
tCOD (g/L)	153.6 $\pm$ 2.2
sCOD/tCOD (%)	6.9 $\pm$ 0.4
pH	6.5 $\pm$ 0.5
N-NH ₄ ⁺ (g/L)	0.1 $\pm$ 0.0
Carbohydrates (% DW)	21.5 $\pm$ 2.6
Proteins (% DW)	63.8 $\pm$ 5.2
Lipids (% DW)	6.0 $\pm$ 0.8
Ash (% DW)	8.7 $\pm$ 0.9

The low sCOD/tCOD ratio exhibited by the raw microalgae (about 7 %) evidenced the need of a pretreatment to enhance the organic matter availability. As previously mentioned, the selection of this biomass was based on its high protein content. This fact could help inhibiting archaea activity and thus, seemed one of the most appropriated biomass to produce VFAs.

C. vulgaris biomass was subjected to three different pretreatments prior to AF: one thermal and two enzymatic (*i.e.* protease and carbohydrase). Pretreatments performance in terms of organic matter solubilization and their influence on AF process was investigated in the present Thesis in Section 4.1.1 (Publication I).

Thermal pretreatment

When applying high temperatures, thermal pretreatment was previously found to produce cell wall damage, improving biomass anaerobic biodegradability (González-Fernández et al., 2012b). For this reason, the selected temperature (120 °C) was in the range of values optimized by other authors to enhance microalgae biomass biodegradability (Méndez et al., 2013). Approximately 200 mL of wet *C. vulgaris* biomass contained in 500-mL glass bottle were subjected to a thermal pretreatment in an autoclave (Selecta, Spain). To identify optimal hydrolysis conditions, samples were withdrawn and cooled down to room temperature every 10 min for 40 min.

Carbohydrase pretreatment

Carbohydrase enzymatic hydrolysis was performed using the commercial cocktail Viscozyme supplied by Novozymes (Denmark). This cocktail is a multienzyme complex containing a wide range of β -glucanase, arabinase, cellulase, β -glucanase, hemicellulase and xylanase activities. To select the most appropriated enzyme dosage, the enzymatic cocktail was applied at 0.3 mL enzyme/g TS and 0.5 mL enzyme/g TS based on Viscozyme dosage ranges previously used (Greses, 2017). The pretreatment was performed in a glass bottle containing 200 mL of wet *C. vulgaris* in a water bath for 4 h where temperature and agitation were maintained at 50 °C and 150 rpm, respectively. Initial pH was 5.5 and it was periodically adjusted with HCl (5 M), according to supplier recommendations, to maintain the enzymatic cocktail at the optimum activity. Samples were withdrawn every 30 min to evaluate organic matter solubilization along the pretreatment time. Finally, temperature was raised to 75 °C for 30 min for enzyme inactivation.

Protease pretreatment

The proteolytic pretreatment was performed with endopeptidase enzymes using the commercial cocktail Alcalase (Novozymes). Microalgae biomass was subjected to an enzymatic dose of 0.2 mL enzyme/g TS and 0.3 mL/g TS based on Alcalase dosage ranges

previously used (Mahdy, 2016). The procedure was carried out according to Mahdy and co-workers (2014a). Briefly, 200 mL of wet *C. vulgaris* biomass contained in 500-mL glass bottle was placed in a shaking water bath at 50 °C for 4 h. pH value was periodically adjusted to 8 with NaOH (3 M). For the assessment of pretreatment efficiencies along the experimental time, samples were taken every 30 min. After the enzymatic treatment, temperature was raised to 75 °C for 30 min for enzyme deactivation.

Hydrolysis efficiency

Samples were taken out periodically in order to evaluate the effect of temperature and enzymes on organic matter, protein and carbohydrate solubilisation along the pretreatments time. The efficiency of each pretreatment applied on microalgae biomass was calculated according to equation 1, where COD refers to the organic matter measured as chemical oxygen demand, in the soluble (sCOD) and total (tCOD) fractions.

$$\text{Hydrolysis efficiency (\%)} = \frac{\text{sCOD after pretreatment} - \text{sCOD raw biomass}}{\text{tCOD raw biomass} - \text{sCOD raw biomass}} \cdot 100 \quad (\text{Eq. 1})$$

Likewise, proteins and carbohydrates solubilization efficiencies were also calculated by using the same formula, but considering the soluble and total fraction of protein (sPr and tPr, respectively) and carbohydrate (sCh and tCh) (equations 2 and 3, respectively). These parameters were chosen because solubilized COD is directly linked to total organic matter released while both carbohydrates and proteins are the main components of the total microalgae dry weight (Table 3.1).

$$\text{Pr solubilization (\%)} = \frac{\text{sPr after pretreatment} - \text{sPr raw biomass}}{\text{tPr raw biomass} - \text{sPr raw biomass}} \cdot 100 \quad (\text{Eq. 2})$$

$$\text{Ch solubilization (\%)} = \frac{\text{sCh after pretreatment} - \text{sCh raw biomass}}{\text{tCh raw biomass} - \text{sCh raw biomass}} \cdot 100 \quad (\text{Eq. 3})$$

3.1.2. Inoculum for VFAs production

Along the different AF experiments presented in this Thesis (Section 4.1), two different anaerobic inocula were employed. Depending on the working temperature of the inocula, conditions were classified as mesophilic (35 °C) and thermophilic (55 °C).

Mesophilic anaerobic sludge was collected at the wastewater treatment plant of Valladolid (Spain) and it was kept at room temperature until use. The sludge was periodically collected and thus, chemical and microbiological characterizations were carried out in each experiment. The anaerobic sludge presented the following chemical characterization: $VS/TS=0.6\pm0.1$, $sCOD/tCOD=0.15\pm0.1$, $NH_4^+=0.4\pm0.1$ g/L and pH 7.5 ± 0.2 .

Thermophilic anaerobic sludge was collected at a mechanical-biological treatment plant for municipal solid wastes conducting AD at thermophilic range located in Tarragona (Spain). Chemical characterization of this anaerobic sludge was $VS/TS=0.46\pm0.1$, $sCOD/tCOD=0.11\pm0.1$, $NH_4^+=0.4\pm0.1$ g/L and pH 7.5 ± 0.3 .

3.1.3. Semi-continuous anaerobic fermentation

Microalgae AF was evaluated in 1-L CSTRs under semi-continuous feeding mode (Figure 3.1, Section 4.1, Publication I). Mesophilic anaerobic sludge described in the previous section was employed as inoculum. Reactors were magnetically stirred at 250 rpm and temperature (Table 3.2) was maintained by using a water bath. Same volume of digestate was daily collected (effluent) and added (influent) to the digesters using plastic syringes.



Figure 3.1. Picture of a CSTR fed with microalgae biomass for VFAs production employed in this Thesis.

The specific microalgae employed in each fermentation for VFAs production, as well as imposed operational conditions (temperature, OLR and HRT) are summarized in Table 3.2.

Table 3.2. Operational conditions of the CSTRs devoted to VFAs production employed in this Thesis.

Publication	Substrate	Temperature (°C)	OLR (g COD/L d)	HRT (d)
I (Section 4.1.1)	a. Non-pretreated <i>C. vulgaris</i>	25	1.5	8
	b. Thermal pretreated <i>C. vulgaris</i>	25	1.5	8
	c. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	8
	d. Viscozyme pretreated <i>C. vulgaris</i>	25	1.5	8
(Section 4.1.2)	a. Alcalase pretreated <i>C. vulgaris</i>	55	1.5	10
	b. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	10
(Section 4.1.3)	a. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	10
	b. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	8
	c. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	6
	d. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	4
	e. Alcalase pretreated <i>C. vulgaris</i>	25	1.5	2

The evaluation of the process was carried out according to the COD balance and a stable VFAs production. These parameters were used to identify the stationary state of the process in each experiment. Steady state was considered after 3 HRTs and stable effluent in terms of COD and VFAs concentrations. OLR applied to all fermenters was 1.5 g COD/L d. According to the pH value of the non-pretreated biomass, pH of the

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feeding was adjusted to neutral values (6.5 ± 0.2). During the experiments, pH in the reactor was monitored but not controlled.

AF efficiency was evaluated in terms of organic matter bioconversion into VFAs, which was calculated as detailed in equation 4:

$$\text{Bioconversion (\%)} = \frac{(\text{VFAs-COD}_{\text{out}})}{\text{tCOD}_{\text{in}}} \cdot 100 \quad (\text{Eq. 4})$$

Where VFAs-COD_{out} refers to the organic matter related to VFAs in the effluent, while tCOD_{in} stands for the total organic matter fed in the reactor. The equivalence of each acid (mg/L) in terms of COD (mg COD/L) was 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for isobutyric and butyric acids, 2.04 for isovaleric and valeric acids and 2.2 for caproic acid. These values were calculated as follow:

$$\text{CH}_3\text{COOH} + 2\text{O}_2 \rightarrow 2\text{CO}_2 + 2\text{H}_2\text{O} \quad \text{COD-Acetic acid} = \frac{64 \frac{\text{g}}{\text{mol}} \text{O}_2}{60 \frac{\text{g}}{\text{mol}} \text{CH}_3\text{COOH}} = 1.07 \frac{\text{g COD}}{\text{g VFA}}$$

$$\text{C}_3\text{H}_6\text{O}_2 + \frac{7}{2}\text{O}_2 \rightarrow 3\text{CO}_2 + 3\text{H}_2\text{O} \quad \text{COD-Propionic acid} = \frac{112 \frac{\text{g}}{\text{mol}} \text{O}_2}{74 \frac{\text{g}}{\text{mol}} \text{C}_3\text{H}_6\text{O}_2} = 1.51 \frac{\text{g COD}}{\text{g VFA}}$$

$$\text{C}_4\text{H}_8\text{O}_2 + 5\text{O}_2 \rightarrow 4\text{CO}_2 + 4\text{H}_2\text{O} \quad \text{COD-(Iso) Butyric acid} = \frac{160 \frac{\text{g}}{\text{mol}} \text{O}_2}{88 \frac{\text{g}}{\text{mol}} \text{C}_4\text{H}_8\text{O}_2} = 1.82 \frac{\text{g COD}}{\text{g VFA}}$$

$$\text{C}_5\text{H}_{10}\text{O}_2 + \frac{13}{2}\text{O}_2 \rightarrow 5\text{CO}_2 + 5\text{H}_2\text{O} \quad \text{COD-(Iso) Valeric acid} = \frac{208 \frac{\text{g}}{\text{mol}} \text{O}_2}{102 \frac{\text{g}}{\text{mol}} \text{C}_5\text{H}_{10}\text{O}_2} = 2.04 \frac{\text{g COD}}{\text{g VFA}}$$

$$\text{C}_6\text{H}_{12}\text{O}_2 + 8\text{O}_2 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O} \quad \text{COD-Caproic acid} = \frac{256 \frac{\text{g}}{\text{mol}} \text{O}_2}{116 \frac{\text{g}}{\text{mol}} \text{CH}_3\text{COOH}} = 2.2 \frac{\text{g COD}}{\text{g VFA}}$$

The COD balance was used for determining the methanogenic efficiency and bioconversion into VFAs. More specifically, the COD removal shows the organic matter that has been reduced to methane out of the total organic matter fed into the system. The COD removal was calculated as follows (equation 5):

$$\text{COD removal (\%)} = \frac{(t\text{COD}_{\text{in}} - t\text{COD}_{\text{out}})}{t\text{COD}_{\text{in}}} \cdot 100 \quad (\text{Eq. 5})$$

The reactor effluents obtained after AF of microalgae biomass were centrifuged at 14000 rpms for 10 min in a Heraeus Megafuge 16R centrifuge, and separated into two different fractions for further revalorization (Figure 3.2). The liquid fraction was used as culture media for lipid production by OY (Section 3.3) whereas the solid fraction was subjected to AD to obtain bioenergy in the form of methane (Section 3.2).

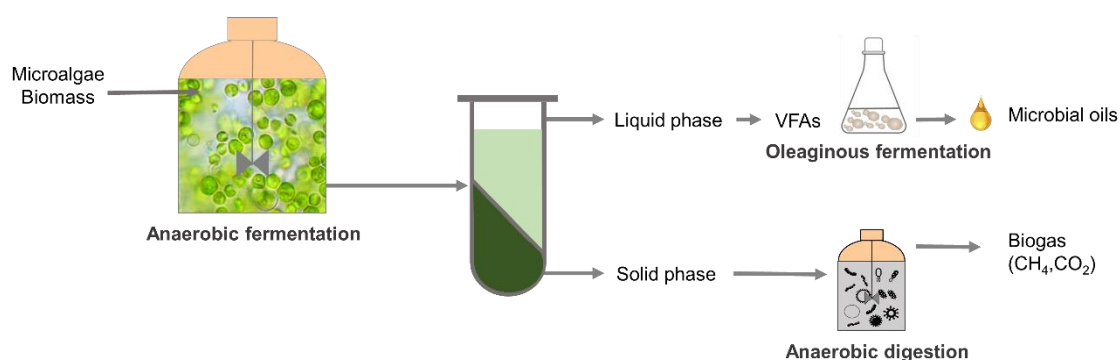


Figure 3.2. Schematic diagram of microalgae AF effluent valorization: liquid phase (rich in VFAs) employed as substrate of OY and solid phase devoted to biogas production via AD.

3.2. Anaerobic digestion for biogas production

3.2.1. Substrate for biogas production

The performance of batch tests and CSTRs (Publications II and III, respectively) were evaluated to determine the methane yield of the remaining solid fraction contained in the AF effluents. The substrates employed in all batch experiments were obtained after semi-continuous AF of *C. vulgaris*, as detailed in a previous work from (Magdalena et al., 2019b). Briefly, these authors performed AF in 1-L CSTR working at 25 °C, HRT of 8 days and OLRs of 6, 9 and 12 g COD/L d. To further confirm the potential of the solid fraction for methane production purposes, a CSTR was fed with the residual solid fraction from the AF of *C. vulgaris* working at 25 °C, HRT of 8 days and OLR of 1.5 g COD/L d (Table 3.2). Average characterization values of the AF solid fraction can be found in Table 3.3.

Table 3.3. Characterization of AF solid fraction used as substrate for biogas production in this Thesis.

Chemical parameters	Average $\pm$ Standard deviation
TS (g/L)	14.1 $\pm$ 1.3
VS/TS (%DW)	90.7 $\pm$ 0.9
sCOD (g/L)	8.2 $\pm$ 2.8
tCOD (g/L)	30.9 $\pm$ 0.9
sCOD/tCOD (%)	26.5 $\pm$ 0.8
pH	5.7 $\pm$ 0.5
N-NH ₄ ⁺ (g/L)	0.3 $\pm$ 0.1
Carbohydrates (% DW)	28.7 $\pm$ 2.6
Proteins (% DW)	57.9 $\pm$ 3.6
Lipids (% DW)	4.9 $\pm$ 0.9
Ash (% DW)	9.5 $\pm$ 0.2

3.2.2. Inoculum for biogas production

AD of the solid fraction obtained after microalgae AF was carried out using mesophilic anaerobic sludge described in Section 3.1.2.

3.2.3. Biochemical methane potential

Biochemical methane potential assays (BMPs) were carried out to evaluate the methane production yields of the solid residues obtained after AF performed at three different OLRs: 6, 9 and 12 g COD/L d (Publication II) under the operational conditions detailed in Table 3.4 using a standardized process methodology (Angelidaki et al., 2009).

Table 3.4. Operational conditions of AD experiments devoted to biogas production from the solid fraction of AF developed in this Thesis.

Publication	Substrate	Temperature (°C)	Feeding mode
II (Section 4.2)	Solid residue from OLR 6 g COD/L d [*]	35	Batch
	Solid residue from OLR 9 g COD/L d [*]	35	Batch
	Solid residue from OLR 12 g COD/L d [*]	35	Batch
III (Section 4.2)	Solid residue from OLR 1.5 g COD/L d HRT 8 d [^]	35	CSTR (OLR 1.5 g COD/L d; HRT 20 d)

[*] Residues obtained from a previous work (Magdalena et al., 2019b)

[^] Residue obtained from experiment described in Section 4.1.3.

According to González-Fernández and García-Encina (2009), substrate and anaerobic sludge were mixed to attain a ratio of 0.5 g COD substrate/VS anaerobic sludge (as inoculum). This ratio ensures an optimal organic matter conversion into methane, avoiding organic matter overloading or underestimation of anaerobes activity.

AD was carried out in triplicate in 120-mL bottles, containing 70 mL working volume at mesophilic temperature (35 °C). This temperature was previously reported as optimal for methane production (Heaven et al., 2011). Figure 3.3 shows a scheme of the BMPs experiments. Blank experiments containing only anaerobic sludge were set in order to estimate the endogenous methane potential. Initial pH was adjusted to 7.5 and oxygen was removed by flushing the headspaces of the bottles with helium (ensuring anaerobic conditions). Batch reactors were incubated until the biogas production reached steady state, being this period characterized by a stable methane production (fluctuations lower than 5 %, approximately 3-5 weeks).

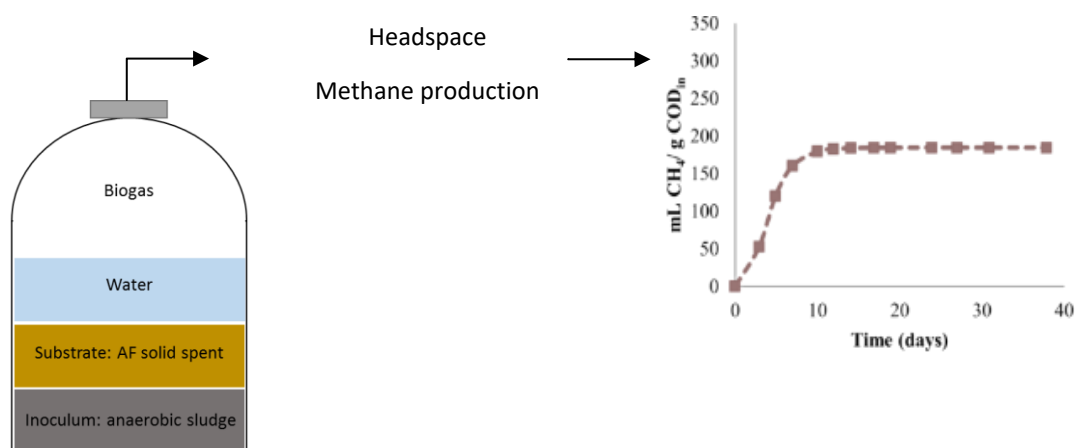


Figure 3.3. General scheme of BMP assays to determine the substrate biodegradability: reactor composition and biogas analysis.

The produced biogas volume was measured via bottle's headspace pressure. Methane production was calculated at standard temperature (0 °C) and pressure (1 atm) conditions (STP) by subtracting the methane production in the blank to the amount of methane measured in each sample (as detailed in the following equations):

$$P.V = n.R.T \quad (\text{Eq. 6})$$

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Where P is pressure variation during the process (bar), V is reactor volume (L), n is amount of generated gas (mole), R is gas constant (bar·L/K·mole) and T is the process temperature (Kelvin).

The produced biogas was recalculated to normal pressure and temperature (0 °C and 1 atm) by using the following equation:

$$P^{\circ} \cdot V^{\circ} = n \cdot R \cdot T^{\circ} \quad (\text{Eq. 7})$$

Where P° is reference pressure (1 atm = 1.013 bar), V° is biogas production at 0 °C and 1 atm, during the test, T° is standard temperature (0 °C = 273 Kelvin).

By substituting n in equations 6 and 7:

$$PV/T = P^{\circ}V^{\circ}/T^{\circ} \quad (\text{Eq. 8})$$

Therefore,

$$V^{\circ} = P \cdot V \cdot T^{\circ} / P^{\circ} \cdot T \quad (\text{Eq. 9})$$

Additionally, experimental methane productions from the different BMPs were fitted with the modified Gompertz equation (Equation 10) (Diaz et al., 2011).

$$P(t) = P_{\infty} \exp \left[-\exp \left(\frac{R_m^e}{P_{\infty}} (\lambda - t) + 1 \right) \right] \quad (\text{Eq. 10})$$

Where $P(t)$ is the accumulated methane production at STP (mL CH₄ STP g/COD_{in}), P_{∞} is the potential methane production (mL CH₄ STP g/COD_{in}), R_m the maximum methane production rate (mL CH₄/day), λ the lag phase (days) and t the elapsed time (days).

3.2.4. Semi-continuous anaerobic digestion

After batch experiments, CSTRs were employed for the AD of the solid phase obtained in the AF of *C. vulgaris* (Publication III, Table 3.4). A digester with a working volume of

1- L (Figure 3.4) was maintained at mesophilic temperature (35 °C) by circulating water through a water jacket. Constant mixing was provided by a magnetic stirrer. pH was not controlled but it was monitored every day immediately after effluent withdrawal. The OLR for the whole experimental period was 1.5 g COD/L d and the HRT was set at 20 days (Table 3.4). These conditions were selected because the traditional AD process devoted to biogas production usually works at HRT of 15-30 days to ensure methanogenic population development (Zabed et al., 2020).



Figure 3.4. Picture of a CSTR fed with the solid residue of AF for biogas production employed in this Thesis.

Daily biogas volume was measured by using a flowmeter (Bioprocess®). Biogas composition was determined as detailed in Section 3.4.3. The methane content was analyzed three times per week. Steady state was characterized by a stable gas production and COD concentration of the effluents digesters.

3.3. Oleaginous fermentation for lipid production

3.3.1. Yeast strains

OY employed in this Thesis were *Cryptococcus curvatus* NRRL-Y-1511, *Lipomyces lipofer* NRRL-Y-11555, *Rhodospiridium toruloides* NRRL-Y-27012, *Williopsis saturnus* NRRL-Y-17396 and *Yarrowia lipolytica* ACA DC 50109 (Figure 3.5). These yeasts were selected according to their intrinsic ability for lipid accumulation (Meng et al., 2009; Sitepu et al.,

2014). Strains were maintained at -80 °C in 30 % v/v glycerol. Strains were regularly sub-cultured on yeast extract-peptone-dextrose (YPD) agar plates, containing yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) and agar (20 g/L) and maintained at 4 ± 1 °C.

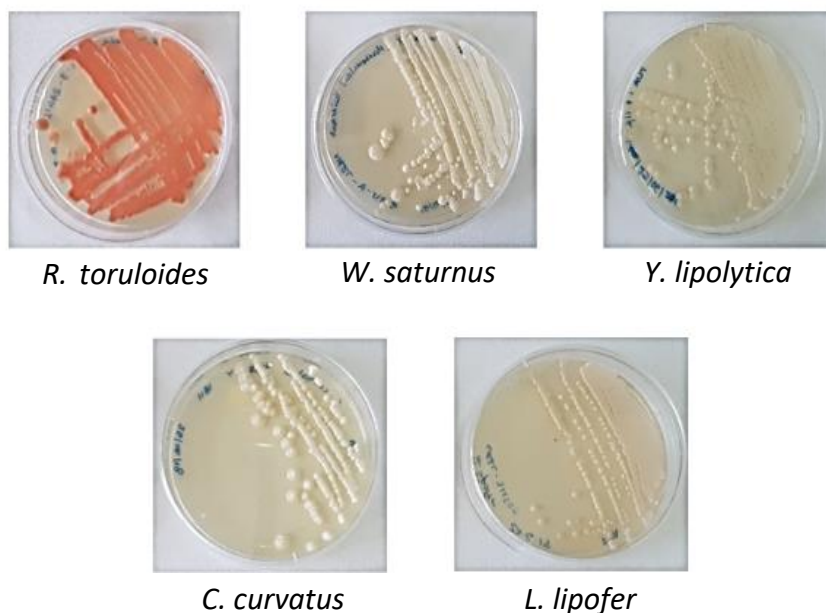


Figure 3.5. Oleaginous yeast strains used in this Thesis.

3.3.2. Substrates employed for oleaginous fermentation

Synthetic media

The seven predominant VFAs in AF digestate (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acid) were evaluated independently and in mixtures (Publication V, Table 3.5).

In all synthetic media, VFAs were added to yeast nitrogen base medium (YNB) (Conda, Cat.1553.00) supplemented with 7.5 g/L of $(\text{NH}_4)_2\text{SO}_4$. In order to identify the effect of each individual VFA on yeast growth, VFAs were evaluated as sole carbon source at 0.8 g C/L and 4 g C/L. These concentrations were selected for comparison purposes with a model substrate (glucose). In this manner, 0.8 g C/L and 4 g C/L of glucose (corresponding with 2 and 10 g glucose/L, respectively) were also tested as a model substrates.

VFAs mixtures were assessed as carbon source to evaluate the effect of VFAs interactions on yeast growth and VFAs usage preference. For this purpose, five synthetic VFAs mixtures were also tested comprising for each acid: I) 0.11 g C/L, II) 0.8 g C/L, III) 2 g C/L, IV) 4 g C/L and V) 1.51 g/L. Since carbon content of each acid is different due to their different chain lengths, corresponding concentrations in g/L are also indicated in Table 3.5.

Real digestate

Real digestate was obtained through semi-continuous AF of *C. vulgaris* biomass (as detailed in Section 3.1.3). Once AF processes reached the steady-state, the reactor effluent was centrifuged at 14000 rpms for 10 min. Na₂HPO₄ and KH₂PO₄ (Fluka, Steinheim, Germany) were added at 12 g/L to buffer the fermentation medium and maintain pH at 6.5 ± 0.5. The culture medium was sterilized by filtration through 0.22 µm pore (VWR international).

VFAs-rich digestate (without additional nutrients) was also used as substrate for oleaginous fermentation (Table 3.5). In Publication V, real effluent from microalgae AF was tested for comparison purposes with a synthetic medium containing the same VFAs concentration and profile. Real digestate was also used to screen the growth of different OY to find the most promising strain for lipid accumulation (Publication VI).

The inhibitory effect of VFAs on yeast was evaluated by using real digestate. For such a purpose, real digestate (containing 4.34 g VFAs/L) was supplemented with synthetic VFAs, up to 25 g/L and 50 g/L, to compare with the synthetic mixtures III and IV (Table 3.5, Publication V).

Table 3.5. Composition of VFAs mixtures and real AF effluent employed as substrates for OY in this Thesis.

Publication	Substrate	VFAs concentration (g/L)							Total
		Acetic	Propionic	Butyric	Isobutyric	Valeric	Isovaleric	Caproic	
V (Section 4.3)	I. Mixture 0.11 g C/L	0.28	0.23	0.20	0.20	0.19	0.19	0.18	1.46
	II. Mixture 0.8 g C/L	2.00	1.65	1.47	1.47	1.36	1.36	1.29	10.60
	III. Mixture 2 g C/L	5.00	4.12	3.67	3.67	3.40	3.40	3.23	26.49
	IV. Mixture 4 g C/L	10.00	8.23	7.34	7.34	6.81	6.81	6.45	52.99
	V. Mixture 1.51 g/L	1.51	1.51	1.51	1.51	1.51	1.51	1.51	10.60
	Real liquid phase of AF [[^]]	1.42	0.78	0.24	0.68	0.47	0.54	0.21	4.34
	Synthetic digestate	1.32	0.85	0.22	0.70	0.47	0.43	0.19	4.18
	Supplemented 2 g C/L	5.00	4.12	3.67	3.67	3.40	3.40	3.23	26.49
	Supplemented 4 g C/L	10.00	8.23	7.34	7.34	6.81	6.81	6.45	52.99
V, VI (Section 4.3)	Real liquid phase of AF OLR 3 [[*]]	1.62	0.64	0.23	1.22	0.33	0.6	0.32	4.96
	Real liquid phase of AF OLR 6 [[*]]	3.23	1.27	0.47	2.44	0.66	1.20	0.64	9.91
	Real liquid phase of AF OLR 9 [[*]]	4.87	1.92	0.7	3.67	0.99	1.81	0.96	14.92

[[^]] Real liquid phase rich in VFAs obtained in the previous work (Magdalena et al., 2019c)

[^{*}] Real liquid phase rich in VFAs obtained in the previous work (Magdalena et al., 2019b)

3.3.3. Batch fermentation

For pre-culture, one colony was inoculated into YPD liquid medium (composition described in Section 3.3.1, without agar). The inoculum was grown in a rotary shaker at 150 rpm and 25 °C overnight, until the culture reached the late exponential growth phase.

Batch fermentation experiments were inoculated at initial optical density at 600 nm (OD_{600}) of 0.3 based on previous experiments not included in the present Thesis where this inoculum size was found suitable to evaluate yeast behavior. Experiments were performed in triplicate under aseptic conditions in 250 mL Erlenmeyer with baffles on the bottom, containing 50 mL medium and cellulose caps. Initial pH was adjusted to 6.0 ± 0.5 (without any further pH control during the process). All cultures were incubated in a rotary shaker (Figure 3.6) at 150 rpm and 25 °C until the available substrate was exhausted (exception made for inhibited cultures). To evaluate yeasts performance, dry yeast biomass, VFAs consumption and lipid accumulation were determined.



Figure 3.6. Oleaginous fermentation in batch assays.

Specific growth rate (μ) was calculated as the increase in biomass OD_{600} /biomass per unit of time (Equation 11):

$$\mu \text{ (h}^{-1}\text{)} = \frac{\ln \frac{x_2}{x_1}}{(t_2 - t_1)} \quad (\text{Eq. 11})$$

Where x is biomass (g/L) or OD (OD_{600}) obtained at each sample of time (h).

Specific substrate consumption rate (q_s) was calculated as the decrease in VFAs in the culture medium per unit of time (Equation 12):

$$q_s(\text{g/Lh}) = \frac{(S_{t2} - S_{t1})}{(t_2 - t_1)} \quad (\text{Eq. 12})$$

Where S is the VFAs concentration (g/L) obtained at each sample of time (t).

3.4. Analytical methods

3.4.1. Microbiological techniques

Biomass determination

Yeast growth was estimated by measuring optical density in a spectrophotometer (Spectroquant® Pharo 100) at 600 nm. For cell dry weight, 5 mL of culture were filtered through a 0.45 μm pre-weighted glass fiber membrane (Merck Millipore). Collected cells were washed several times with distilled water and subsequently, samples were dried at 105 °C until constant weight (Rice et al., 1980).

Cell monitoring

Optical microscopy

Yeast cell morphology was observed using a 40x and 100x magnification in an optical microscope Carl Zeiss (GmbH, Göttingen, Germany), equipped with a video camera (Exwave HAD, Sony, Tokyo, Japan).

Fluorescence microscopy

LB were stained with Nile red fluorescence dye as follows: 0.1 g of wet biomass was suspended in water and mixed (1:10 v/v) with a solution of Nile red (Sigma) in ethanol (1 mg/mL). After incubation with the dye for 1 h at room temperature, cells were washed twice with distilled water and viewed under an Axiostar 40 (Zeiss, Cambridge,

United Kingdom) fluorescence microscope equipped with an excitation filter of 470/40 nm and a ProgRes camera (Jenoptik CF cool, Jena, Germany).

3.4.2. Macromolecular composition determination

Carbohydrates content

Carbohydrate content of the different substrates used in the fermentations and digestions was determined by the colorimetric method relying on sugars reduction with sulphuric acid and phenol (Dubois et al., 1956). Deionized water was used as blank sample, and glucose standards were employed to prepare the calibration curve (0-0.5 mg/mL). Then, 50 µL of phenol solution (5 % v/v) and 5 mL of sulphuric acid (98 % v/v) were added to each sample and mixed. After 30 min at room temperature, soluble sugars were determined by measuring the absorbance at 485 nm wavelength using a spectrophotometer (Spectrostar Omega S/N 415-1414, Germany).

Proteins content: Total Kjeldahl Nitrogen (TKN)

Proteins content was estimated by multiplying the total Kjeldahl nitrogen (TKN) by a correction factor of 5.95 (Lopez et al., 2010). TKN analysis involves three steps: digestion, distillation and titration. Firstly, total organic nitrogen was converted in ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) via acid digestion. A fixed volume of biomass employed as substrate was digested with 12 mL H_2SO_4 (95 %) at 420 °C for 1 h in presence of a catalyst mixture (K_2SO_4 and CuSO_4 mixture). This reaction was performed in FOSS Tecator TM scrubber. After digestion, the sample was distilled using Kjeltce TM 8200 autodistillation unit. The digested fraction was made alkaline with 50 mL of NaOH solution (40 % v/v) and the released ammonia was steam distilled into a receiver filled with 25 mL of 4 % H_3BO_3 with indicator. Lastly, the contents were titrated with HCl (0.01 N).

Nitrogen content was calculated according to the equation 13:

$$\text{Nitrogen (\%)} = \frac{(T-B) \cdot N \cdot 14.007}{W} \cdot 100 \quad (\text{Eq. 13})$$

Where T is the titrated volume of HCl for sample (mL); B, titrated volume of HCl for blank (mL); N, normality of HCl; 14.007 - molar mass of N (mg/mmol) and W, sample weight (mg).

Lipid content

Lipid quantification was performed when maximum yeast growth was reached and the substrate was completely exhausted. For lipid analysis, yeast biomass was harvested at the end of the fermentation process by centrifugation at 14000 rpm for 10 min at 4 °C (Heraeus, Megafuge16, Thermo Scientific). Total cellular lipids extraction was performed according to the method described by Folch (Folch and Lees, 1957) with modifications (Dourou et al., 2017). Briefly, yeast biomass was washed three times with 0.9 % NaCl for medium cleaning and subsequently dried overnight at 100 °C. Intracellular lipids were extracted with chloroform:methanol (2:1 v/v) under reflux for 4 h. The extract was filtered through Watman N.1 paper and the organic phase was washed twice with a 0.88 % w/v KCl solution (Sigma). Samples were dried over anhydrous Na₂SO₄ (Sigma). Finally, the solvent was evaporated at 40 °C under vacuum using a Rotavapor R-215 device (BUCHI), and the total cellular lipids were gravimetrically determined. The lipid content was expressed as gram lipid per gram of dry biomass (% DW).

3.4.3. Analytical determinations

Total and volatile solids

Total solids (TS) and volatile solids (VS) content were measured gravimetrically according to the Analytical Standard Methods (APHA/AWWA/WEF, 2017). TS was determined as the residue after water evaporation of the sample dried in an oven (Binder) at 105 °C for 24 h. The difference between the wet and dry weights divided by the sample volume was the result of TS. To determine VS content, after determining TS, the sample was incinerated in a muffle (Carbolite 2000 W) at 550 °C for 3 h. The weight loss, measured in a balance (Sartorius TE64), represented the VS of the sample.

Ash content

The ash content (inorganic matter) was calculated as follows:

$$\text{Ash (\%)} = 100 - \% \text{ VS/TS} \quad (\text{Eq. 14})$$

Chemical oxygen demand

Chemical oxygen demand (COD) describes the oxygen required to completely oxidize the organic matter content in the sample under aerobic conditions. This parameter was measured by a colorimetric method using a commercial test kit (Merck, ISO 15705) that oxidizes 3 mL of sample with potassium dichromate at 148 °C for 2 h in a thermoreactor (Spectroquant TR420 M) using silver sulphate as catalyst. Subsequently, COD content was determined in a spectrophotometer (Spectroquant Pharo 100M). The same method was employed to determine total and soluble COD. However, soluble phase was obtained after sample centrifugation for 5 min (Mini-spin Eppendorf 5424) and filtering through 0.45 µm.

Ammonium

Ammonium (N-NH_4^+) concentration was determined using a colorimetric method based on the ammonia transformation into a blue indophenol derivative. A commercial test kit (Merck, ISO 000683) composed by two main reagents (R1 liquid and R2 solid) was employed. For the analysis, 5 mL of R1 and 200 µL of sample were mixed, a tablespoon of R2 was added and left for 15 min at room temperature. Two subsequently reactions take place in this analysis. In the first one, ammonium nitrogen in the form of ammonia medium reacts with hypochlorite ions to form monochloramine. In the second one, this product reacts with a substituted phenol to form a blue indophenol derivative, which is determined photometrically in a spectrophotometer (Spectroquant® pharo 100, EU).

pH measurement

The pH was measured using a pH meter (Crison Basic 20+, EU). The pH meter was daily calibrated with pH 4.01, 7.0 and 9.21 buffers (HANNA, HI).

VFAs

For VFAs determination, the collected samples were filtered through 0.2 μm after centrifugation at 14000 rpm to facilitate the filtering step. VFAs were analyzed from C2 to C6 (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acid) by high performance liquid chromatography (HPLC) equipped with a refractive index detector (Agilent 1260 HPLC-RID). A Cation H Refill Cartridge Microguard column (Biorad) and an Aminex HPX-87H ion exclusion column (300 x 7.8 mm I.D.) (Biorad) were used. Mobile phase was 5 mM H_2SO_4 (VWR) and elution was conducted in isocratic mode at a flow rate of 0.6 mL/min. The injected sample volume was 20 μL , and the oven and detector temperatures were 50 $^{\circ}\text{C}$ and 35 $^{\circ}\text{C}$, respectively. VFAs were identified by using a standards calibration curve using pure VFAs mixtures (Sigma-Aldrich) from 0.05 g/L to 1 g/L of each acid.

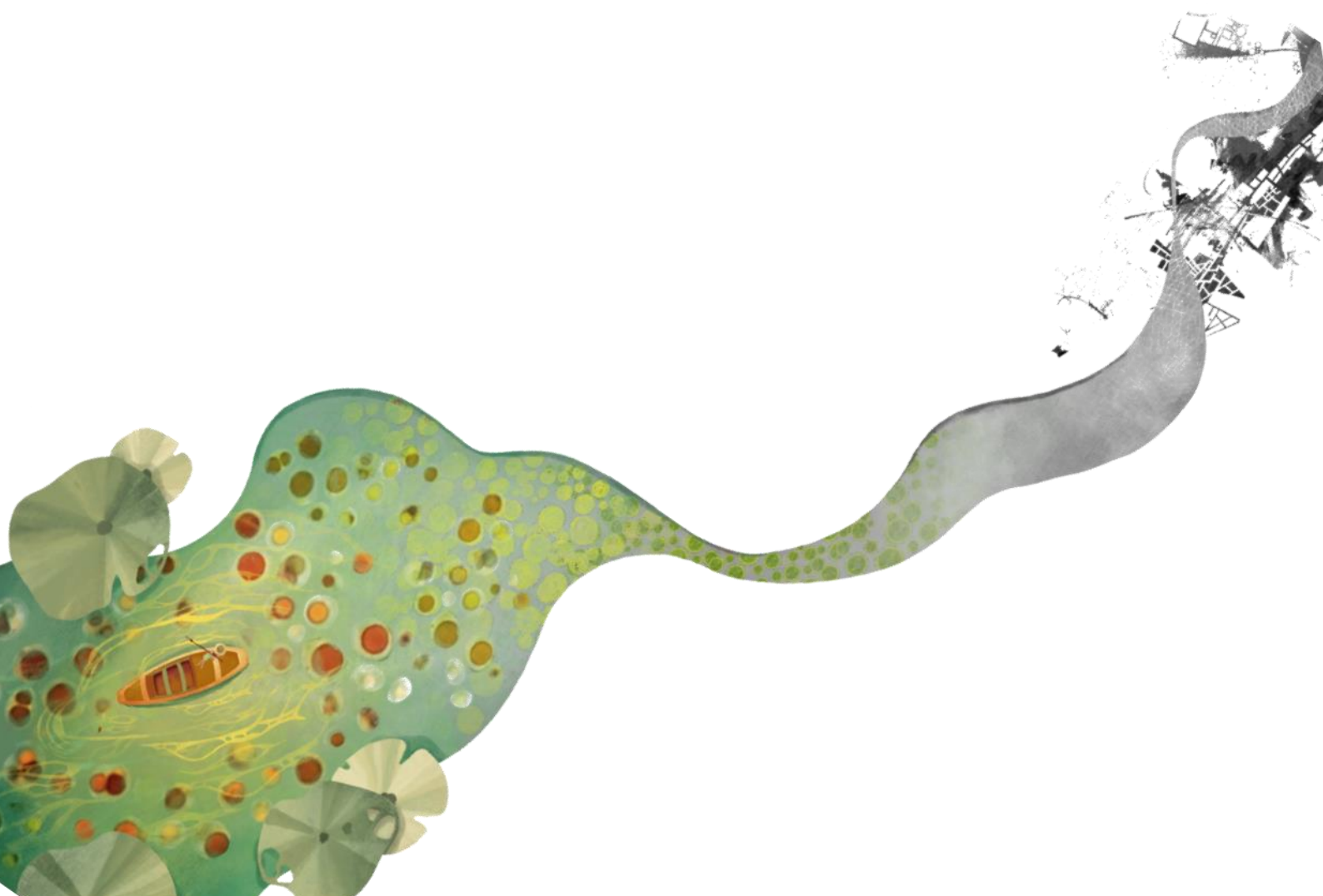
FA composition of yeast lipids

FA composition of cellular lipids was determined by gas chromatography (GC) after FA trans-methylation (AFNOR, 1984). FA moieties were converted into their fatty acid methyl esters (FAMES) in a two-stage reaction. Samples were analysed in an Agilent 7890 A gas chromatograph equipped with a HP-88 column (60 m $\times$ 0.32 mm) and a FID detector. GC conditions were as follows: helium was used as carrier gas at a flow rate of 1 mL/min, injection temperature was 250 $^{\circ}\text{C}$, oven temperature 200 $^{\circ}\text{C}$ and FID temperature 280 $^{\circ}\text{C}$. FAMES (from C10 to C22 and unsaturated) were identified by using a standards calibration curve (Sigma Aldrich).

Biogas composition

To determine the biogas composition (mainly composed of CO_2 and CH_4), a GC coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) was employed. The GC was equipped with an HSN6–60/80 Sulfinert P packed column (70 $\times$ 1/8" O.D.) and a MS13X4-09SF2 40/60 P packed column (9' $\times$ 1/8" O.D.) (Perkin Elmer). Helium was used as carrier gas at a flow rate of 30 mL/min. The injector, oven and detector temperatures were 80, 62, and 200 $^{\circ}\text{C}$, respectively. The injected sample volume was 100 μL .

4. RESULTS AND DISCUSSION



4. Results and Discussion

Microalgae have been the focus of many studies mainly devoted to biodiesel, bioethanol or biogas production. Nevertheless, little information exists on producing two or more bioproducts out of this biomass.

AD has emerged as one of the most promising options to improve the economic and environmental sustainability of microalgae-based wastewater treatment (Colzi-Lopes et al., 2018; Al-jabri et al., 2021). Opposite to this conventional approach, a shortened AD known as AF, can also be used as an alternative bioprocess to produce VFAs instead of biogas. However, there are still few barriers that prevent VFAs production from microalgae biomass, namely the substrate low biodegradability, the persistence of methanogens competing for VFAs and the limitations in VFAs recovery. To circumvent the two first barriers, the present Thesis focuses on the evaluation of suitable substrate pretreatments and operational conditions. To overcome the third barrier, the avoidance of VFAs recovery and purification steps, will imply a step forward in the use of the VFAs platform.

To this end, the use of VFAs as a carbon source for OY, instead of the traditional sugar-rich substrates for oil production, is a very innovative approach. In spite of that, the knowledge on VFAs potential and limitations as low-cost carbon source is really scarce and the effect of real digestates on OY has been scarcely studied. Thus, experiments in this Thesis were designed to gain insights on yeast's VFAs tolerance, uptake preference and lipid accumulation capacity from these carbon sources.

Lastly, and to propose a full valorization of all the available streams, the solid spent recovered from the AF after the separation of the VFAs-rich liquid fraction, can still be employed for methane production. In this sense, this Thesis also evaluated the potential of using the solid spent as substrate in AD process for biogas production.

Within this integration perspective, microalgae biomass employed in this Thesis has been considered a potential resource to be used in a biorefinery facility, as shown in Figure 4.1.

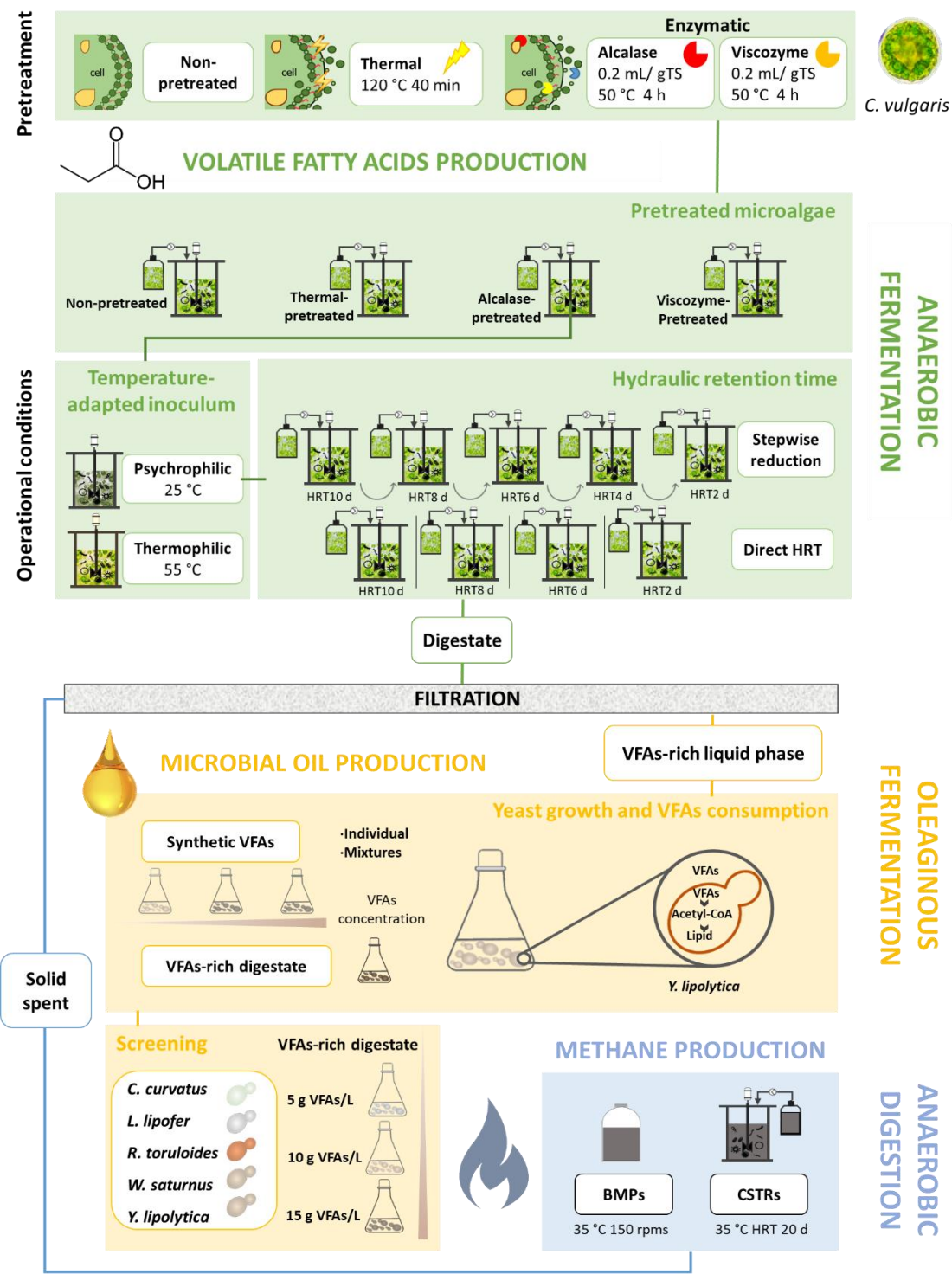


Figure 4.1. Schematic representation of the main experiments performed to reach a full valorisation of microalgae biomass in this Thesis.

The results obtained in this Thesis are discussed in three different sections:

1. VFAs production via AF of *C. vulgaris*

Different microalgae pretreatments were tested to evaluate the improvement in substrate biodegradability and to understand their impact on VFAs production yields and profile. The effect of different inocula and applied operational conditions (temperature and HRT) in the reactors were also assessed in semi-continuous mode.

2. AF solid spent valorisation into biogas via traditional AD

Batch experiments were firstly carried out in order to evaluate the suitability of this substrate for biogas production. Subsequently, this bioprocess was validated in semi-continuous operation mode.

3. VFAs-rich liquid phase valorisation into MO via OY fermentation

The feasibility of using VFAs-rich AF effluent as carbon source for OY was evaluated in terms of yeast growth and lipid accumulation. Synthetic individual VFAs and mixtures were firstly tested to determine uptake rates and potential inhibitory effect. Real AF effluent was also evaluated to elucidate whether some other digestate products could negatively affect yeast performance. Additionally, a screening of OY for lipid production using VFAs-rich AF effluent as carbon source was conducted to identify the most suitable candidate for MO production.

4.1. VFAs production via AF of microalgae biomass

To optimize and maximize VFAs production, attention must be directed to the substrate, the inoculum employed and the operational conditions applied in the fermenters (mentioned in Section 1.1.2.1). These factors strongly affect the microbial population development, which ultimately determines the organic matter's fate and therefore, the VFAs production yields and profiles.

Although microalgae biomass presents potential advantages for VFAs production via AF (see Section 1.1.2.1.2), the bioconversion efficiency of this bioprocess can be hampered by the hard cell wall of some species. Publication I addressed the effect of *C. vulgaris* pretreatments on the AF. Considering the wide number of microorganisms involved in this process, another challenge is to drive the process towards a specific product. This can be achieved by tuning operational conditions. In this sense, the effect of using temperature-adapted inoculum was evaluated. More specifically, AF process performance was studied by seeding the reactors with sludge adapted to psychrophilic and thermophilic temperatures (Section 4.1.2). Lastly, how the HRT reduction affects VFAs production in terms of bioconversion efficiencies and acid profiles was evaluated in Section 4.1.3.

4.1.1. Effect of microalgae pretreatments on VFAs production via AF

Microalgae grown in wastewater effluents, such as *C. vulgaris*, present a rigid cell wall that could hamper the hydrolysis step of AF (Ometto et al., 2014). This hydrolytic stage determines the organic matter availability and ultimately could affect AF process yields and productivities. Therefore, application of pretreatment methods for cell wall degradation/disruption to favor organic matter solubilization seems to be essential for an efficient bioconversion of microalgae biomass into VFAs via AF.

Microalgae pretreatments have been widely studied for methane production purposes (Passos et al., 2014). Nevertheless, information about the effect of pretreatments in VFAs production during AF is scarce. For this reason, in this Thesis, one thermal and two

enzymatic pretreatments (carbohydrases and proteases) were employed to enhance *C. vulgaris* biomass solubilization prior to AF (Publication I).

Previous investigations evidenced that the cell wall was disrupted by applying thermal pretreatment at high temperatures (55-170 °C), improving anaerobic biodegradability of microalgae biomass (Córdova et al., 2019). Different enzymatic cocktails were also used to target specific microalgae cell macromolecules (*i.e.* carbohydrates or proteins) to enhance organic matter availability. According to de Carvalho and co-workers (2020), microalgae cell wall is composed by a carbohydrate-based fibrillar structure covered by an algenan-based layer susceptible to be broken down by thermal and carbohydrase pretreatments (Méndez et al., 2013; Mahdy et al., 2014a). Likewise, proteases were a rather logical biocatalyst to be tested owing to the high protein content of microalgae biomass (Table 3.1, Section 3.1.1). For these reasons, this Thesis includes the evaluation of thermal (T), carbohydrolytic (Viscozyme, V) and proteolytic (Alcalase, A) pretreatments. For comparison purposes, non-pretreated (NP) microalgae was also assessed in CSTR configuration for VFAs production.

4.1.1.1. Biomass solubilization during pretreatments

Organic matter solubilization

COD of the soluble phase is directly linked to organic matter availability for the anaerobic microbiome. Raw microalgae are characterized by exhibit low biodegradable potential ($sCOD/tCOD = 6.9 \pm 0.4 \%$, Table 3.1). Figure 4.2 shows sCOD along the experimental time for the thermal, protease and carbohydrase pretreatments of *C. vulgaris*.

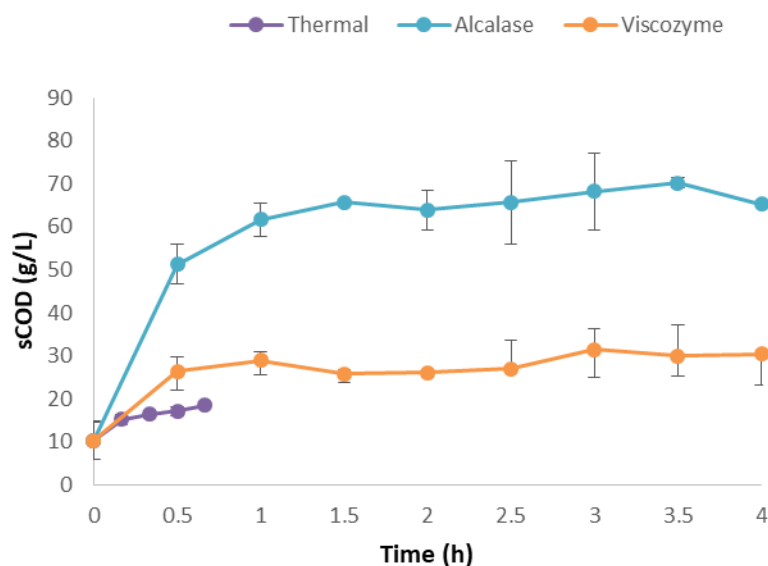


Figure 4.2. sCOD for the thermal, protease and carbohydrase pretreatments along the experimental time.

As it can be seen, pretreatments notably increased the sCOD/tCOD ratios from 6.9 % in the NP microalgae to 12.1 ± 0.2 , 19.9 ± 0.4 and 42.8 ± 0.7 % for T, V and A treated biomass, respectively (Table 4.1).

Table 4.1. Pretreatments efficiencies in terms of COD, proteins and carbohydrates solubilization.

	Thermal	Viscozyme	Alcalase
sCOD/tCOD (%)	12.1 ± 0.2	19.9 ± 0.4	42.8 ± 0.7
COD Hydrolysis efficiency (%)	5.8 ± 0.2	14.1 ± 0.5	38.6 ± 0.8
Proteins solubilisation (%)	3.4 ± 0.9	9.4 ± 0.8	81.6 ± 1.6
Carbohydrates solubilisation (%)	24.8 ± 1.2	61.9 ± 1.1	34.1 ± 1.4

T pretreatment resulted in a linear organic matter solubilization along the experimental time, exhibiting the lowest sCOD (1.8-fold higher than the NP sCOD). This value corresponded with a hydrolytic efficiency of 5.8 ± 0.2 %. Longer exposure times in thermal pretreatment were not tested since side reactions at high temperatures can convert soluble organic matter into complex inhibitory molecules for the AF process (Méndez et al., 2013).

On the other hand, enzymatic pretreatments (V and A) showed a similar organic matter solubilization trend. Maximum hydrolysis was reached after 1 h of pretreatment and longer hydrolysis times did not provide any solubilization enhancement. The highest COD was attained by biomass subjected to A pretreatment (6.5-fold increase compared to NP), while the V pretreatment only reached 3-fold increase with respect to the NP biomass. The use of A and V cocktails resulted in final hydrolytic efficiencies of $38.6 \pm 0.8 \%$ and $14.1 \pm 0.5 \%$, respectively (Table 4.1). This could be explained by the high amount of proteins, in comparison to carbohydrates, present in *C. vulgaris*. These results were in agreement with Mahdy and co-workers (2014a) who found the highest organic matter solubilization when using A pretreatment.

In order to fully understand the effect of the pretreatment on organic matter solubilization, an in-depth analysis of carbohydrates and proteins released upon pretreatments was conducted.

Carbohydrates and proteins solubilisation

Microalgae biomass was composed by $21.5 \pm 2.6 \%$ DW of carbohydrates (Table 3.1, Section 3.1.1), out of which 21 % were soluble (3.3 g/L, Figure 4.3.a).

Carbohydrates solubilization profile varied depending on the pretreatment employed. As it can be seen in Table 4.1, V pretreatment gave rise to the highest carbohydrates solubilization ($61.9 \pm 1.1 \%$ of total carbohydrates), followed by A and T pretreatments with $34.1 \pm 1.4 \%$ and $24.8 \pm 1.2 \%$, respectively. The cellulolytic activity of the V enzymatic cocktail was able to release 10.6 g/L of soluble carbohydrates. On the contrary, during T and A pretreatments considerable lower carbohydrates solubilization was reached probably due to the non-specific affinity of these pretreatments towards carbohydrates.

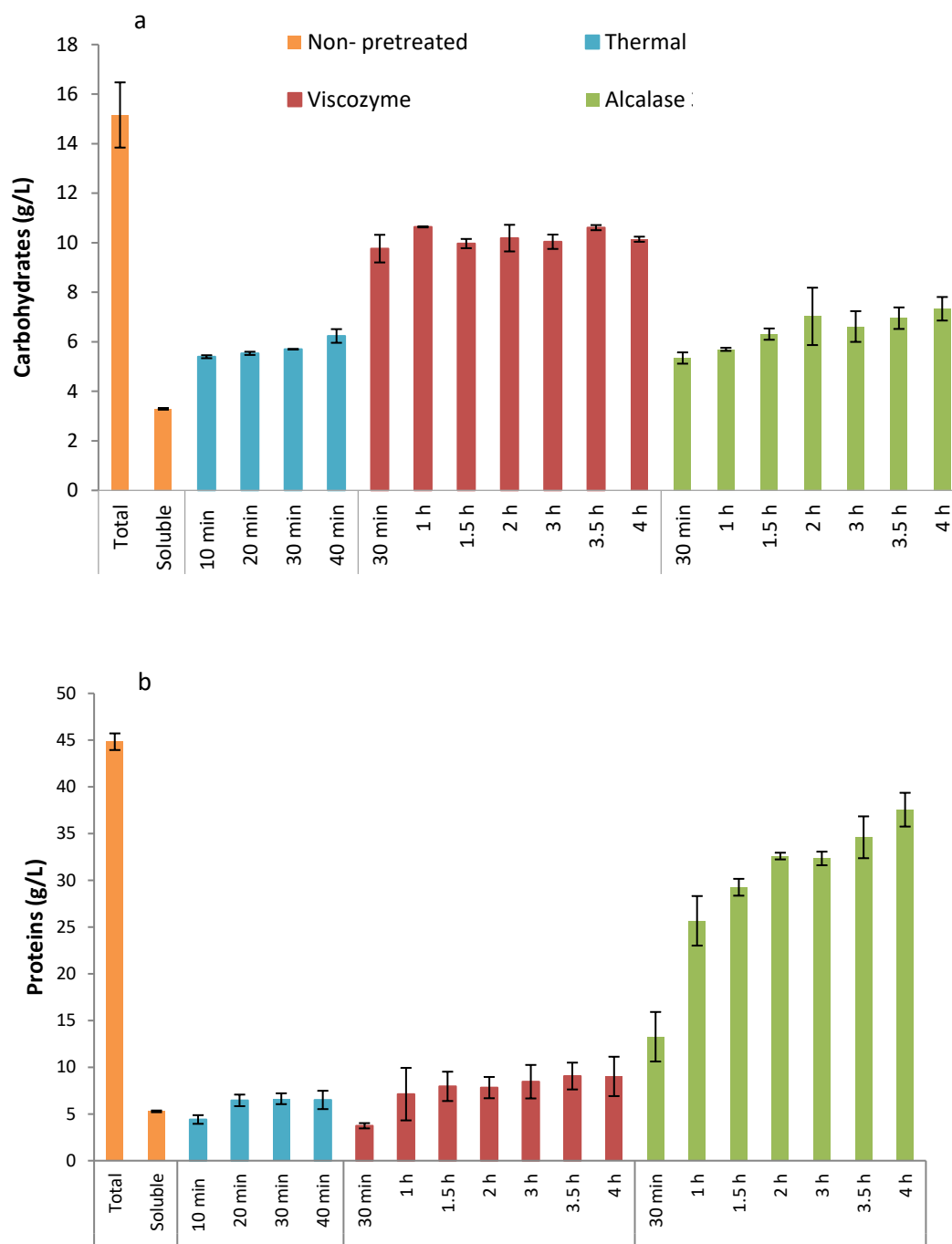


Figure 4.3. Carbohydrates (a) and proteins (b) solubilization during thermal, Viscozyme and Alcalase pretreatments compared with non-pretreated microalgae.

Even though all pretreatments markedly affected the carbohydrates fraction, the same trend was not observed for the proteins fraction. As shown in Figure 4.3.b, the highest proteins solubilization resulted when using A pretreatment (81.6 % of the total proteins

content). This result demonstrated the suitability of the proteolytic cocktail to degrade protein-rich microalgae biomass. However, it should be noted that proteins solubilization when using A increased along the hydrolysis time, reaching the maximum after 3.5-4 h. The same cannot be stated for T and V pretreatments in which the solubilized proteins fraction remained stable after 1 h. More importantly, T and V pretreatments displayed lower proteins solubilization values ($3.4 \pm 0.9 \%$ and $9.4 \pm 0.8 \%$, respectively) than the obtained with A (Table 4.1). These results are in agreement with Mendez and co-workers (2013) who also observed that thermal pretreatments of microalgae were more effective on carbohydrates solubilization than proteins solubilization.

4.1.1.2. Effect of microalgae pretreatment on AF

In order to elucidate the pretreatment effect on the VFAs production process, pretreated and NP *C. vulgaris* biomass were subjected to AF. AF was conducted in CSTRs working at 25 °C, HRT 8 days and OLR 1.5 gCOD/Ld based on previous works (Magdalena et al., 2019c)

4.1.1.2.1. Effect of microalgae pretreatment on organic matter removal

Although VFAs are targeted in this Thesis, methanogenic archaea could consume and metabolize VFAs into biogas, thereby reducing VFAs bioconversion yields. To demonstrate that biogas production is not being produced during AF process, COD removal was evaluated (Table 4.2).

In this sense, COD removal (3.5-11.8 %) and methane yields ($12.3\text{-}41.9 \text{ mL CH}_4/\text{tCOD}_{\text{in}}$) in all CSTR evaluated, were considerably lower than the values usually exhibited in reactors where biogas is desired. For instance, Mahdy and co-workers (2015) obtained $129 \text{ mL CH}_4/\text{tCOD}_{\text{in}}$ in CSTR when using A-pretreated *C. vulgaris* biomass at the same OLR (1.5 g COD/L d) and HRT of 15 days when biogas production was targeted. It can be thus concluded that given the COD removal values obtained in this Thesis, the bioprocess was not suitably working for biogas production.

Table 4.2. Main parameters and efficiencies evaluated in CSTRs fed with non-pretreated and thermal-, Viscozyme- and Alcalase-pretreated microalgae.

	Non-pretreated	Thermal	Viscozyme	Alcalase
pH	6.0 ± 0.1	6.2 ± 0.1	5.7 ± 0.1	6.3 ± 0.1
TS (g/L)	9.9 ± 0.4	5.0 ± 0.5	6.6 ± 0.8	4.8 ± 0.7
VS (g/L)	8.8 ± 0.5	4.5 ± 0.5	5.5 ± 0.9	4.1 ± 0.8
N-NH₄⁺(g/L)	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
tCOD (g/L)	10.2 ± 0.5	8.3 ± 0.8	10.2 ± 0.7	9.1 ± 0.4
sCOD (g/L)	1.8 ± 0.2	2.1 ± 0.9	2.3 ± 0.2	4.7 ± 0.1
sCOD/ tCOD (%)	15.3 ± 0.9	22.7 ± 2.9	27.5 ± 1.6	51.5 ± 3.5
COD removal (%)	3.5 ± 3.1	11.8 ± 1.8	3.5 ± 1.5	7.6 ± 2.2
Methane yield (mL CH₄/g COD_{in})	12.3 ± 15.1	41.9 ± 9.0	12.2 ± 7.3	26.5 ± 10.7

When protein rich-substrates are employed, methanogenic population is usually inhibited by high ammonium concentration and therefore methane production may be lowered or suppressed (Hansen et al., 1998). The high proteins solubilization observed during A pretreatment gave rise to the highest ammonium concentration in the CSTRs (0.4 ± 0.1 g N-NH₄⁺/L, Table 4.2). The ammonium values obtained in this study were below the methanogens inhibitory threshold (above 3 g N-NH₄⁺/L) (Yenigün and Demirel, 2013) and thus, the low methanogenic activity observed was likely associated to the operational conditions imposed instead of the ammonium presence (Cavinato et al., 2017). In this manner, it could be thus concluded that the initial operational conditions were well selected for VFAs accumulation.

Efficiencies of the hydrolytic step were evaluated in terms of sCOD/tCOD (calculated as detailed in Section 3.1.3). Significant differences among CSTRs were obtained in terms of sCOD/tCOD (Table 4.2). The high hydrolysis efficiency obtained in the CSTR fed with A-pretreated biomass (51.5 ± 3.5 % sCOD/tCOD) confirmed the high hydrolytic activity of the process since it involved high production of soluble organic matter to be transformed into VFAs by acidogenic bacteria. T and V pretreatments implied lower hydrolysis efficiencies (22.7 ± 2.9 % and 27.5 ± 1.6 % sCOD/tCOD, respectively) than the obtained in A (51.5 ± 3.5 %). These results agreed with the organic matter solubilization achieved after pretreatments (Table 4.1), confirming that hydrolysis rate is the bottleneck in AF. This fact supports the need of applying pretreatments prior to AF to attain suitable organic matter conversion into VFAs.

4.1.1.2.2. Effect of microalgae pretreatment on VFAs production: conversion yields and profiles

AF bioconversion efficiencies

AF efficiencies were evaluated in terms of organic matter conversion into VFAs once the bioprocess reached the steady state (Figure 4.4, Publication I). The low VFAs production attained from NP microalgae (1.0 ± 0.1 g VFAs_{COD}/L), corresponding with 8.1 ± 0.7 % VFAs_{COD}/tCOD_{in}, demonstrated that the use of a pretreatment step was crucial to enhance microalgae biomass bioconversion into VFAs. As it was previously mentioned (Section 1.1.2.1.2), microalgae present a high resistant cell wall that hampers bacterial hydrolysis reducing the efficiency of the AD. In this case, low percentage of easily biodegradable organic matter (sCOD/tCOD = 6.9 %) was exhibited by the NP *C. vulgaris* (Table 3.1).

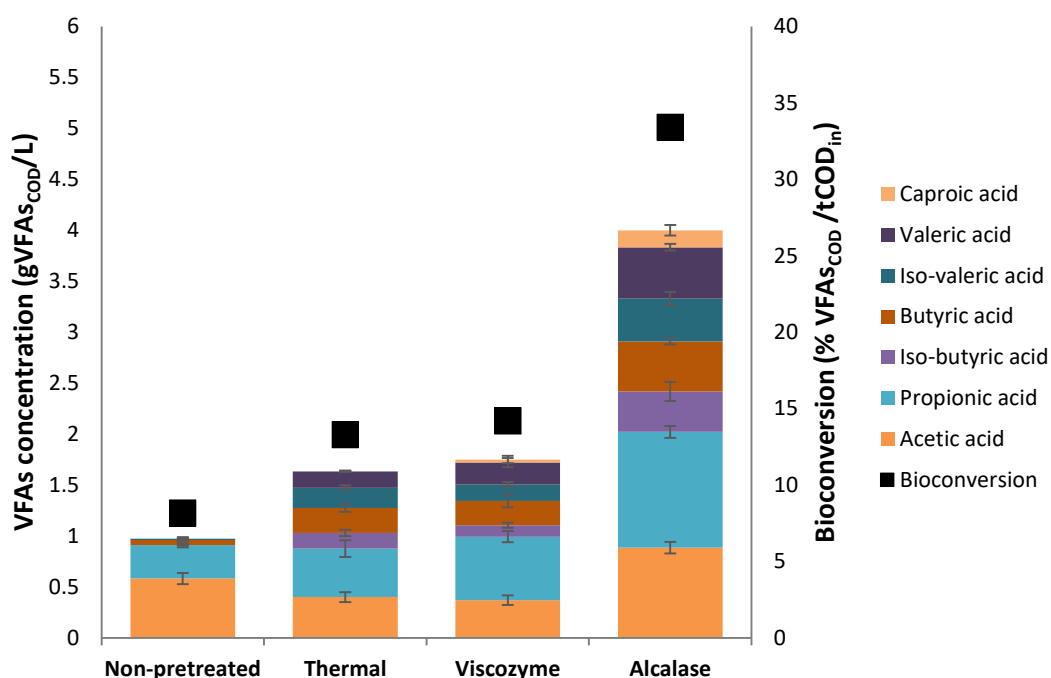


Figure 4.4. VFAs production and bioconversion in the AF of non-pretreated, thermal-, Viscozyme- and Alcalase-pretreated *C. vulgaris*.

The CSTRs fed with pretreated microalgae biomass gave rise to a VFAs concentration increase of 1.6 ± 0.2 , 1.7 ± 0.3 and 4.0 ± 0.4 g VFAs_{COD}/L for T, V and A, respectively (Figure 4.4). These values corresponded with bioconversion efficiencies of 13.6 ± 1.6 ,

14.2 ± 1.4 and 33.4 ± 2.2 % VFAs_{COD}/tCOD_{in}, respectively. Acidogenesis efficiencies were evaluated in terms of COD acidified (VFAs_{COD}/sCOD, calculated as detailed in Section 3.1.3). The similar acidification efficiency achieved independently of the pretreatment (69.3-72.4 % VFAs_{COD}/sCOD, Table 4.3) demonstrated that acidogenesis was not the limiting step in the studied AF. This fact supported the previous statement about the hydrolysis limitations found during anaerobic bioprocess.

Table 4.3. VFAs production and efficiencies evaluated in CSTRs fed with non-pretreated and thermal-, Viscozyme- and Alcalase-pretreated microalgae.

	Non-pretreated	Thermal	Viscozyme	Alcalase
Acetic acid (% w/w)	58.0 ± 6.1	24.4 ± 3.8	21.4 ± 3.3	22.6 ± 2.8
Propionic acid (% w/w)	28.3 ± 3.5	29.1 ± 0.2	35.8 ± 0.4	29.0 ± 3.9
Iso-butyric acid (% w/w)	0.0 ± 0.0	9.5 ± 0.1	6.5 ± 0.0	10.2 ± 2.1
Butyric acid (% w/w)	5.1 ± 0.7	14.9 ± 1.9	13.7 ± 1.5	12.5 ± 1.7
Iso-valeric acid (% w/w)	2.1 ± 0.5	12.1 ± 1.4	9.2 ± 1.0	10.7 ± 2.1
Valeric acid (% w/w)	6.5 ± 2.1	9.9 ± 0.8	12.5 ± 2.6	12.8 ± 3.6
Caproic acid (% w/w)	0.0 ± 0.5	0.1 ± 0.1	0.8 ± 0.6	2.1 ± 0.6
Total VFAs (gCOD/L)	1.0 ± 0.1	1.6 ± 0.2	1.7 ± 0.3	4.0 ± 0.4
% Bioconversion (gVFAs _{COD} /gCOD _{in})	8.1 ± 0.7	13.6 ± 1.6	14.2 ± 1.4	33.4 ± 2.2
% COD acidified (VFAs _{COD} /sCOD)	55.6 ± 1.8	69.3 ± 3.5	71.6 ± 2.9	72.4 ± 3.7

Although, organic matter conversion into VFAs is variable depending on substrate and operational conditions (Cho et al., 2015a; Magdalena and González-Fernández, 2019), bioconversion into VFAs attained from biomass subjected to proteolytic pretreatment (A) (33.4 % VFAs_{COD}/tCOD_{in}) was similar or higher than that reported in literature from different waste streams. For instance, Jiang and co-workers (2021) found that AF of sewage sludge achieved a bioconversion of 19 % VFAs_{COD}/tCOD_{in}, while Dahiya and co-workers (2015) reported a maximum bioconversion of 25 % VFAs_{COD}/tCOD_{in} when food wastes were used for AF. Both investigations were performed in CSTRs at mesophilic temperature and alkaline pH conditions (pH 10). In comparison with the result showed herein, their lower bioconversions into VFAs may be caused by the harsh operational conditions applied in these studies to suppress methanogenesis (extreme pH), which concomitantly affected the acidogenic activity.

In case of using microalgae biomass for VFAs production, Kim and co-workers (2019) determined 35 % bioconversion ($\text{VFAS}_{\text{COD}}/\text{tCOD}_{\text{in}}$) when *C. vulgaris* AF was conducted in batch mode at 35 °C. Despite the similar bioconversion into VFAs achieved by these authors in comparison to the present study, it is important to highlight that batch reactors commonly result in higher process efficiency. Moreover, the applied temperature (35 °C) would imply higher energy requirements reducing the cost-effectiveness of the process. The use of the same proteolytic cocktail (A) resulted in 35.5 % $\text{VFAS}_{\text{COD}}/\text{tCOD}_{\text{in}}$ in a CSTR set at 25 °C and HRT 10 days (Magdalena et al., 2019b). The highest bioconversion into VFAs attained by the proteolytic pretreatment (A) in this Thesis agreed with the high organic matter solubilization obtained by means of this enzymatic cocktail (Table 4.1, Section 4.1.1.1). It is worth highlighting that, T and V pretreatments have been used for the first time in CSTR with *C. vulgaris* to obtain VFAs.

When T pretreatment was applied, AF bioconversion efficiency was 13.6 ± 1.6 % $\text{VFAS}_{\text{COD}}/\text{tCOD}_{\text{in}}$ compared to approximately 8 % $\text{VFAS}_{\text{COD}}/\text{tCOD}_{\text{in}}$ when employing NP biomass as substrate (Figure 4.4, Table 4.3). This slight increase would not justify the use of this pretreatment. Despite the higher carbohydrates solubilization during V pretreatment in comparison with T (61.9 % vs 24.8 %), similar VFAs bioconversion values were obtained in both reactors (Figure 4.4, Table 4.3). These results evidenced that high organic matter solubilization during pretreatment does not necessarily correlate with high AF process efficiencies. Previous studies concluded that the harsh conditions during thermo-chemical pretreatments of microalgae cause the formation of recalcitrant compounds in Maillard side-reactions hampering biogas production (González-Fernández et al., 2012; Méndez et al., 2014, 2013). Thus, the use of pretreatments might entail not only the release of degradable organic matter from biomass but also process inhibitors or more recalcitrant compounds.

The low VFAs bioconversion and low biogas production (% COD removal), obtained when using T- and V-pretreated microalgae revealed that solubilization of the carbohydrates fraction was less important than the solubilization of the proteins fraction for VFAs production from protein-rich microalgae biomass. This fact indicated that the use of a macromolecule-specific pretreatment prior to AF is essential to

enhance organic matter bioconversion into VFAs. Although carbohydrates have been claimed to be the bottleneck for microalgae degradation (de Carvalho et al., 2020), it has been demonstrated that the proteins fraction should be hydrolyzed prior to AF to attain a proper bioconversion into VFAs.

VFAs profile

The study of the VFAs distribution in the CSTRs showed similar profiles among the assessed pretreatments (Figure 4.5, Table 4.3). When using pretreated microalgae, acetic, propionic, iso-butyric and butyric acids outstood as the most abundant organic acids, ranging from 74 % (w/w) to 78 % (w/w) of the total VFAs in terms of COD. Longer-chain VFAs (C5-C6) accounted for around 20 % (w/w) of the total VFAs.

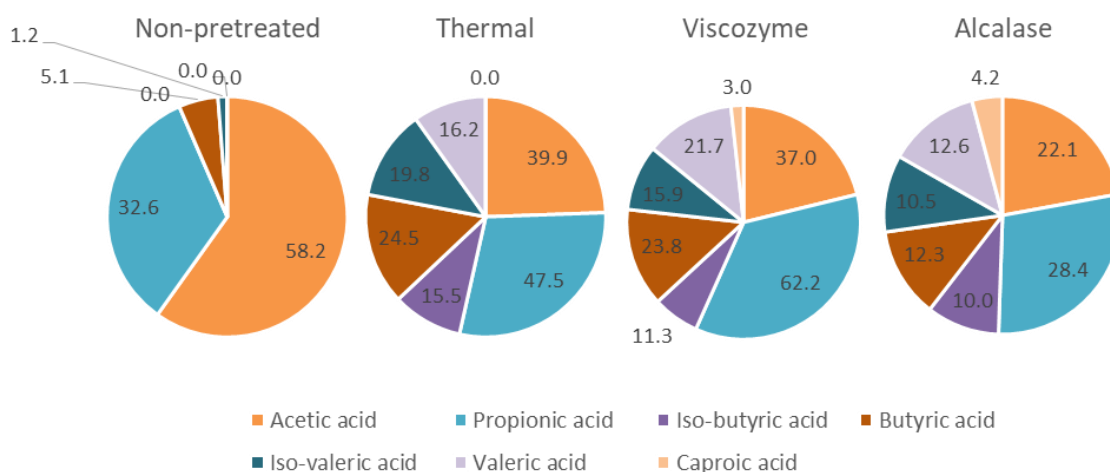


Figure 4.5. VFAs distribution profile in the AF of non-pretreated and thermal-, Viscozyme- and Alcalase-pretreated *C. vulgaris*.

This phenomenon could be explained by the fact that AF is a degradative process where longer FA are degraded, giving rise to short chain VFAs accumulation (Moretto et al., 2019). Opposite to that, when using NP biomass only acetic and propionic acid accounted for more than 90 % (w/w) of the total VFAs. Acetic acid is quickly consumed by methanogenic archaea in conventional AD. Thus, acetic acid accumulation revealed an unbalanced process with low acetoclastic methanogenesis. These facts bolster the

aforementioned results in Table 4.2, where negligible COD removal and methane production yields were shown.

Regueira and co-workers (2020a) stated that odd-chain acids, especially propionic acid, are mainly associated to protein-rich substrates. Thus, the presence of high concentration of propionic acid was mainly consequence of the high protein content of the microalgae biomass (Table 3.1, Section 3.1.1). In addition, propionic acid prevalence has been previously reported in unbalanced AD since its oxidation is energetically unfavorable and strongly influenced by process conditions (Moretto et al., 2019). By contrast, even carbon number VFAs have been reported to prevail during AF of carbohydrate-rich substrates (Greses et al., 2020b). This fact agreed with the low content of butyric and caproic acids in the present Thesis since the carbohydrates fraction in microalgae biomass only accounted for 26.2 % DW.

Valeric acid accumulation has been correlated with methanogenic activity inhibition (Strazzera et al., 2018). Higher concentration of long chain VFAs supported the higher bioconversions of organic matter into VFAs reached in fermenters fed with pretreated biomass in comparison with that using NP microalgae. This fact was more relevant when *C. vulgaris* was pretreated with A, where C5 and C6 acids accounted for the remarkably 25 % (w/w) of the VFAs content in terms of COD. The obtained profiles agreed with other studies in which VFAs were targeted using microalgae biomass as substrate (Jankowska et al., 2017; Magdalena et al., 2019b; Moretto et al., 2019).

All this considered, it was evidenced that the use of a pretreatment targeting a specific macromolecule has a strong effect on VFAs conversion yields from microalgae biomass. Out of the three assayed pretreatments, protease pretreatment (A) resulted in both, the highest organic matter solubilization and bioconversion into VFAs.

After selecting the protease pretreatment as the most promising method to be applied prior to AF of microalgae biomass, the importance of tuning operational parameters such as temperature or HRT to optimize VFAs production via AF will be discussed in Sections 4.1.2 and 4.1.3.

4.1.2. Effect of temperature on VFAs production via AF using temperature adapted inoculum

The impact of temperature on VFAs production via AF of A-pretreated microalgae biomass was evaluated by comparing two CSTRs operated at 25 °C and 55 °C using temperature adapted anaerobic sludge as inoculum (see Section 3.1.2).

4.1.2.1. Effect of temperature on organic matter removal

The results attained during the steady state of each CSTR are summarized in Table 4.4.

Table 4.4. Main parameters and efficiencies evaluated in the AF of *C. vulgaris* at psychrophilic and thermophilic conditions.

	Psychrophilic CSTR 25 °C	Thermophilic CSTR 55 °C
pH	6.2 ± 0.1	7.2 ± 0.1
TS (g/L)	5.7 ± 0.3	4.1 ± 0.7
VS (g/L)	4.1 ± 0.4	3.2 ± 0.4
N-NH ₄ ⁺ (g/L)	0.5 ± 0.1	0.6 ± 0.1
tCOD (g/L)	15.1 ± 0.7	11.2 ± 0.6
sCOD (g/L)	7.5 ± 0.4	4.2 ± 0.4
sCOD/ tCOD (%)	49.4 ± 2.1	38.4 ± 2.1
COD removal (%)	3.4 ± 2.9	20.9 ± 7.4
Methane yield (mL CH ₄ /g tCOD _{in})	11.9 ± 10.1	73.1 ± 25.9

As previously stated, it is important to corroborate that applied operational conditions are suitable for VFAs production by avoiding methanogenic activity. In this manner, biogas production in terms of COD removal was evaluated. Higher COD removal was exhibited by the CSTR operated under thermophilic conditions (20.9 ± 7.4 %) than at psychrophilic conditions (3.4 ± 2.9 %). According to these results, process temperature affected the methanogenic step. Low digestion temperatures are usually associated with lower CH₄ production since bacterial growth and conversion processes are slower (Magdalena et al., 2019c). Hence, the low COD removal under psychrophilic conditions evidenced a reduced methanogenic activity, which was beneficial for VFAs accumulation.

Although the thermophilic CSTR exhibited higher biogas production than the psychrophilic reactor, both COD removals were remarkably low when compared with a

process devoted to biogas production (Méndez et al., 2014; Prajapati et al., 2014; Mahdy et al., 2016a). Mahdy and co-workers (2015) reported 128.4 mL CH₄/g tCOD_{in} (56 % COD removal) when using A-pretreated microalgae biomass in a CSTR under mesophilic temperature (35 °C) and working at OLR 1.5 g COD/L d and HRT 20 days. These authors, used longer HRT values (20 days) than in present study (10 days) because CH₄ production was targeted. The low CH₄ obtained in this Thesis, regardless of the operation temperature, suggested that methanogenic activity was limited by the short HRT imposed. Therefore, taking advantage of the slower methanogens growth compared to hydrolytic bacteria, biogas production could be reduced favouring VFAs accumulation (as discussed in the following Section 4.1.3).

Apart from methanogenic activity inhibition, elucidating the effect of temperature on hydrolytic and acidogenic population is essential when VFAs are desired. To assess the organic matter availability to be transformed into VFAs, hydrolytic efficiencies were evaluated. Differences in terms of sCOD/tCOD between psychrophilic and thermophilic CSTRs were found. Even though some authors reported an enhanced hydrolytic step at higher temperatures (Zhuo et al., 2012), lower sCOD/tCOD were exhibited by the CSTR at 55 °C in comparison with 25 °C (38.4 ± 2.1 % vs 49.4 ± 2.1 %, Table 4.4). This hydrolytic improvement by temperature was negligible in the present Thesis because a proteolytic pretreatment was carried out to discard hydrolytic problems of microalgae biomass (focusing on the acidogenesis stage). Despite the fact that A-pretreated microalgae was employed as substrate, the non-solubilized fraction after the pretreatment must be hydrolyzed during AF. Therefore, it could be stated that operational temperature at 55 °C negatively affected hydrolytic microorganisms activities as well.

4.1.2.2. Effect of temperature on VFAs production: conversion yields and profiles

AF bioconversion efficiencies

VFAs concentration (5.1 ± 0.3 g VFAs_{COD}/L) at psychrophilic temperature was 2-fold higher than the obtained at thermophilic temperature (2.4 ± 0.1 g VFAs_{COD}/L),

corresponding with average conversion yields of $34.2 \pm 0.9 \%$ and $14.1 \pm 0.3 \%$ VFAs_{COD}/tCOD_{in}, respectively (Figure 4.6, Table 4.5).

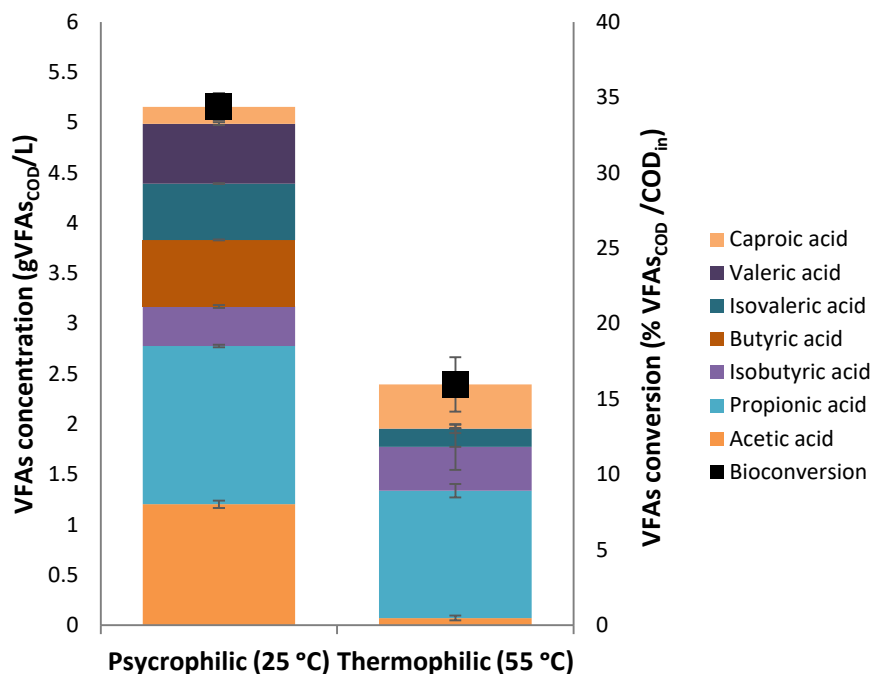


Figure 4.6. VFAs production and bioconversion in the AF of *C. vulgaris* at psychrophilic and thermophilic conditions.

Similarly to the hydrolytic efficiencies showed above, lower acidogenic efficiency was exhibited at 55 °C ($56.9 \pm 2.9 \%$) in comparison with the fermentation conducted at 25 °C ($68.9 \pm 3.4 \%$). In this manner, it should be highlighted that even though temperature-adapted inocula were employed to ensure the proper activities at each fermentation temperature, a negative effect on hydrolytic and acidogenic population by high temperature was detected.

Table 4.5. VFAs production and efficiencies evaluated in the AF of *C. vulgaris* at psychrophilic and thermophilic conditions.

	Psychrophilic CSTR 25 °C	Thermophilic CSTR 55 °C
Acetic acid (% w/w)	23.3 ± 4.2	2.9 ± 0.0
Propionic acid (% w/w)	30.5 ± 5.1	52.8 ± 7.6
Iso-butyric acid (% w/w)	7.6 ± 2.3	18.1 ± 5.4
Butyric acid (% w/w)	12.8 ± 3.9	0.1 ± 0.0
Iso-valeric acid (% w/w)	10.8 ± 1.7	7.4 ± 1.6
Valeric acid (% w/w)	11.5 ± 1.0	0.2 ± 0.0
Caproic acid (% w/w)	3.2 ± 0.0	18.3 ± 2.5
Total VFAs (g COD/L)	5.1 ± 0.3	2.3 ± 0.1
% Bioconversion (g VFAs _{cod} /g tCOD _{in})	34.2 ± 0.9	14.1 ± 0.3
% COD acidified (g VFAs _{cod} /g sCOD)	68.9 ± 3.4	56.9 ± 2.9

Temperature is a well-known tuneable parameter that can affect VFAs production (Cho et al., 2015a; Zamanzadeh et al., 2016; Magdalena et al., 2019c). For instance, Zhuo and co-workers (2012) tested the effect of temperature (10, 20, 37 °C) on the hydrolysis and acidification stages of AD using waste-activated sludge as substrate. Opposite to the trend observed herein, these authors reported an increase on VFAs production when increasing operation temperature, reaching the maximum bioconversion (41 % VFAs_{cod}/tCOD_{in}) at 37 °C. As aforementioned, they attributed the progressive VFAs increase to the efficient hydrolysis at higher temperatures when NP microalgae was employed as substrate. In the present Thesis, the difference in AF bioconversion efficiencies was related to the methanogenic stage rather than the hydrolysis-acidogenesis stages. The increase in COD accumulated as VFAs in the reactor working at 25 °C in comparison with the one at 55 °C was attributed to the low methanogenic activity, resulting in low organic matter removal (3.4 ± 2.9 % at 25 °C and 20.9 ± 7.4 % at 55 °C).

Maximum COD conversion values into VFAs at psychrophilic conditions 25 °C were in the range of other studies found in literature. For instance, Zhuo and co-workers (2012), reported 30 % bioconversion (VFAs_{cod}/tCOD_{in}) at 20 °C from waste activated sludge. However, when other residues were employed as substrates higher bioconversions into VFAs than the obtained herein have been shown. For instance, Oktem and co-workers (2006) achieved 44 % bioconversion into VFAs with an acidogenic reactor fed with

pharmaceutical wastewater at 35 °C while Greses and co-workers (2020b) reported up to 52 % bioconversion into VFAs when sugar-rich food wastes were used. It should be noted that those substrates contain easily biodegradable organic matter. Thus, the bioconversion into VFAs around 32 % attained at psychrophilic temperatures from microalgae could be considered an efficient process taking into account that 50 % bioconversion was attained when full microalgae biomass was devoted to biogas production at mesophilic temperatures (Zabed et al., 2020). Additionally, after VFAs extraction, the remaining solid residue can be further digested for biogas production, maximizing products output generation from a single substrate.

VFAs profile

Regarding the effect of AF temperature on VFAs profile, great differences were obtained (Figure 4.7). In the psychrophilic AF, acetic and propionic acids were the most abundant products, accounting for the 54 % (w/w) of the total VFAs, whereas in the thermophilic AF, propionic acid stood out as the main product (52 % w/w of total VFAs).

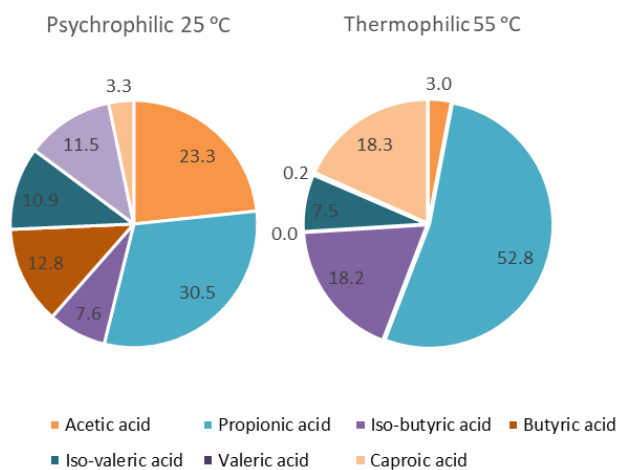


Figure 4.7. VFAs distribution profile in the AF of *C. vulgaris* at psychrophilic and thermophilic conditions.

As previously explained in Section 4.1.1, acetic acid accumulation is considered a process performance indicator. High concentration of this acid evidences an unbalanced AD process since this acid is immediately transformed into CH₄ by methanogenic archaea

via the acetoclastic pathway in well balanced digesters. Thus, acetic accumulation confirmed the inhibition of methanogenesis in the psychrophilic reactor, which indeed was supported by the low COD removal observed (3.4 %). The abundance of acetic acid might result from longer chain VFAs degradation. Acetic acid has been frequently reported to be among the most abundant products in other studies devoted to VFAs accumulation when using different substrates such as food waste, maize silage and whey (Cavinato et al., 2017; Jankowska et al., 2017; Greses et al., 2020b). Indeed, the VFAs distribution profile achieved in this Thesis at 25 °C was in accordance with another investigation employing microalgae biomass as feedstock. Jankowska and co-workers (2017) carried out a mixed culture fermentation using *Scenedesmus quadricauda* and *C. vulgaris* as substrate. In that study, acetic acid was the most abundant product (42 % w/w) during the first days of fermentation, followed by propionic and butyric acids (19 % w/w each). At this point, it is worth to highlight the low acetic acid presence in the fermenter running at thermophilic conditions. This fact suggested that acetic acid was consumed by methanogenic population, agreeing with the 20.9 % COD removal attained in thermophilic reactor.

The high production of propionic acid exhibited at both temperatures was probably caused by the high protein content of microalgae biomass used as substrate (Table 3.1). As previously mentioned, some works have demonstrated that odd-chain VFAs presence (specially propionic acid) is linked to protein-rich substrate composition (Regueira et al., 2020; Gálvez-Martos et al., 2021). Besides, oxidation of propionic acid is an energetically unfavourable reaction when compared to other VFAs such as acetic acid, which is a spontaneous process (van Lier et al., 2008; Müller et al., 2010). Thus, propionic acid accumulation could be related to the high energy required for its degradation.

AF temperature affected the production and degradation of long chain VFAs C4-C5-C6 as well (Table 4.5). Under psychrophilic temperature, C4 and C5 acids (butyric, iso-butyric, valeric and iso-valeric acids) accounted for around 10 % (w/w) each while caproic acid (C6) was not observed. Under thermophilic conditions, caproic and iso-butyric acid prevailed in the VFAs pool, accounting each of them for almost 20 % (w/w) of total VFAs. These differences found in VFAs profiles could be related not only to the temperature effect on the AF process, but also to the fact that two different

temperature-adapted inocula were used for the psychrophilic and thermophilic reactors. In this manner, the different metabolic activities present in the initial microbial population along with the operational conditions could cause the differences in VFAs profiles.

Overall, this study confirmed that psychrophilic fermentation temperatures (25 °C) promoted higher VFAs accumulation when compared to thermophilic temperatures (55 °C). The selected fermentation temperature also had a direct impact on VFAs profile. This change in the VFAs distribution was associated to the production of biogas at thermophilic temperature, which concomitantly resulted in a consumption of acetic acid.

4.1.3. Effect of HRT reduction on VFAs production via AF

Anaerobic inocula contain a wide diversity of bacteria and archaea with different activities and kinetics, nutritional requirements and growth rates (Vanwonterghem et al., 2016). Operational conditions can inhibit a certain microbial community while favouring the development of others, thereby promoting specific fermentation pathways. In the case of CSTRs, the HRT is a key parameter that establishes the flow rate daily fed into the reactor and thus it determines available time for organic matter degradation, microbial growth and intermediate compounds accumulation in the reactor. The HRT control is essential not only for adjusting microbial activity but also in a full-scale perspective.

Considering the lower growth rate of methanogenic archaea in comparison with acidogenic bacteria (Henze et al., 2008), the HRT control can be used as a strategical tool to force the wash out methanogens from the reactor. Nevertheless, it should be also highlighted, as explained in Section 1.1.2.1.3, that HRT should be long enough to allow complex organic matter solubilization. In fact, reducing the HRT below a critical value (shorter than hydrolytic and acidogenic bacteria growth rate) can cause the inhibition of the acidogenic step as well. In this sense, a proper HRT trade-off should be found to optimize the overall process performance.

In this section of the Thesis, the shorter HRT that can be applied without compromising the bioconversion yields into VFAs was determined. For this purpose, the effect of HRT as individual parameter on AF performance of A-pretreated *C. vulgaris* for VFAs production was evaluated. Considering that HRT values below 10 days have been demonstrated to prevent methanogenic activity (avoiding VFAs consumption), a stepwise decrease of HRT from 10 to 8, 6, 4 and 2 days was conducted. CSTRs were operated at 25 °C while keeping constant the rest of the variables. The same inoculum was sequentially used for the following HRT decrease (*i.e.* the inoculum used for HRT 8 days was collected from the reactor operated at HRT 10 days, the inoculum used for 6 days HRT was the one collected from the reactor operated at 8 days HRT, and so on) (Figure 4.8.a). For comparison purposes, the HRT effect was also evaluated by implementing the same HRTs above mentioned to the initial inoculum. This means that the inoculum used for 10 days of HRT was directly operated at 8, 6 and 4 days without any adaptation period (Figure 4.8.b).

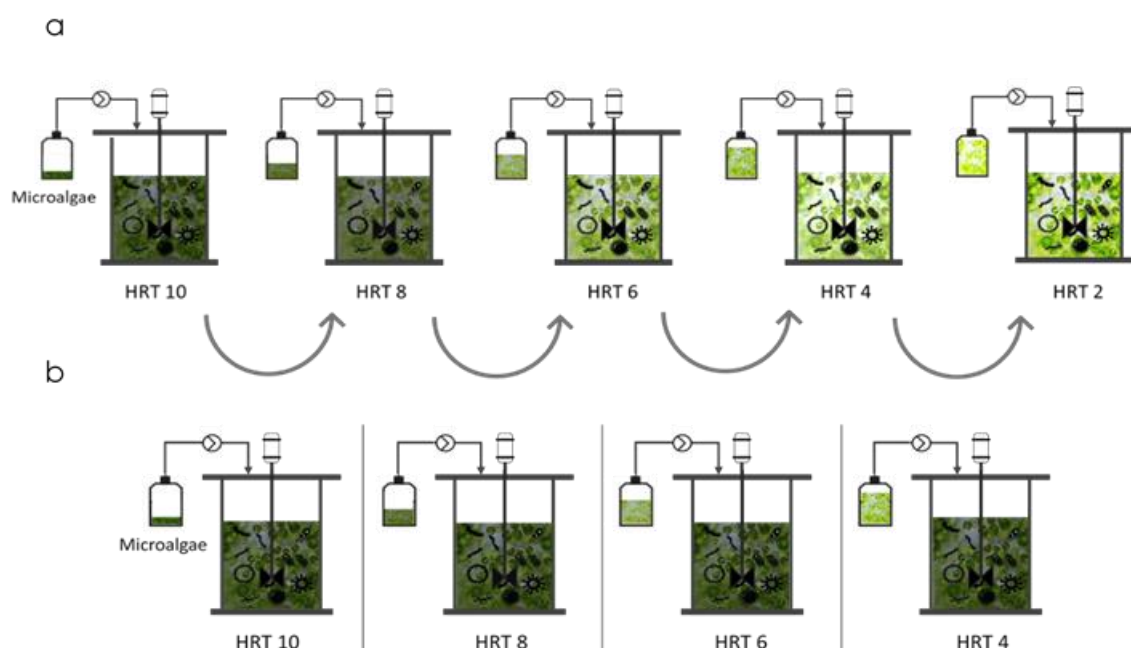


Figure 4.8. Schematic representation of the experiments performed to evaluate the HRT effect on AF of *C. vulgaris* biomass: a) stepwise HRT decrease from 10 to 2 days; b) direct HRT evaluation of HRT at 10, 8, 6 and 4 days.

4.1.3.1. Effect of stepwise HRT decrease on AF

4.1.3.1.1. Effect of stepwise HRT reduction on organic matter removal

Reactor operation was divided into five stages, each of them corresponding to a stepwise decrease in HRT (see details in Table 3.2). Figure 4.9 shows the organic matter evolution in the AF over time. Each scenario achieved a new steady state when VFAs production as well as TS/VS, COD and NH_4^+ were stable (3-4 HRT).

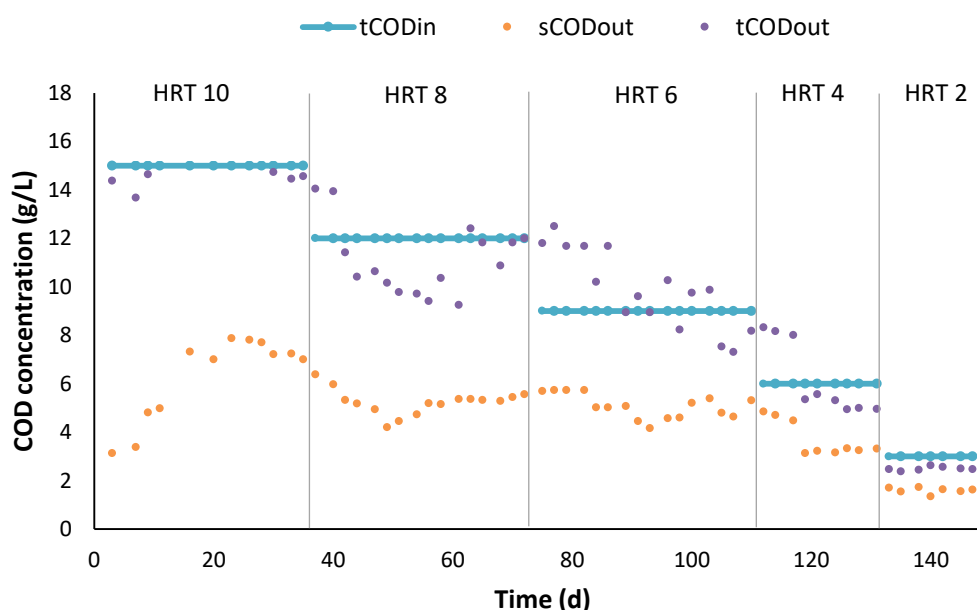


Figure 4.9. Time course of the sCOD and tCOD in the influent and effluent obtained during stepwise HRT decrease.

As it can be seen in Figure 4.9, tCOD_{out} values determined in the effluents and tCOD_{in} fed in each scenario were similar. All the HRT gave rise to COD removals in the narrow range of 7- 12 % (Table 4.6). It can be stated that regardless of the HRT, low biogas production was attained. These low values indicated that HRT below 10 days did not mediate a significant biogas production, which evidenced a hampered AD process favouring VFAs accumulation. HRT values usually employed in literature for biogas production via conventional AD of microalgae biomass (20-30 days) are longer than the ones employed herein and thereby, mediating higher COD removals. For instance, COD removals higher than 50 % were reported when *C. vulgaris* was digested for biogas production purposes

when working at 20-28 days of HRT (Ras et al., 2011; Mahdy et al., 2016a). Considering that methanogenic inhibition is desired for VFAs production, the use of HRTs lower than 10 days was confirmed to be appropriate for such a goal.

Table 4.6. Main parameters and efficiencies evaluated during stepwise HRT reduction from 10 to 2 days in the AF of *C. vulgaris* biomass.

	HRT 10	HRT 8	HRT 6	HRT 4	HRT 2
pH	6.0 ± 0.1	6.1 ± 0.1	6.2 ± 0.1	6.1 ± 0.1	6.3 ± 0.1
TS (g/L)	5.6 ± 0.4	3.9 ± 0.2	3.1 ± 0.3	1.9 ± 0.1	1.1 ± 0.0
VS (g/L)	4.1 ± 0.4	2.9 ± 0.3	1.9 ± 0.3	1.4 ± 0.1	0.8 ± 0.0
VS/TS	69.9 ± 3.2	72.7 ± 1.8	68.5 ± 2.2	70.9 ± 4.6	69.4 ± 3.1
N-NH ₄ ⁺ (g/L)	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
tCOD (g/L)	15.0 ± 0.4	11.8 ± 0.3	8.5 ± 0.8	5.2 ± 0.1	2.5 ± 0.0
sCOD (g/L)	7.4 ± 0.2	5.6 ± 0.1	4.9 ± 0.2	3.3 ± 0.1	1.6 ± 0.0
sCOD/ tCOD (%)	49.4 ± 2.1	47.9 ± 1.9	56.9 ± 4.6	61.1 ± 4.7	59.2 ± 1.8
COD removal (%)	8.7 ± 0.9	7.6 ± 1.8	11.7 ± 3.6	9.8 ± 3.2	6.8 ± 1.5
Methane yield (mL CH ₄ /g COD _{in})	30.4 ± 15.1	26.6 ± 9.0	40.9 ± 7.3	34.9 ± 6.7	23.8 ± 2.7

The ratio sCOD/tCOD at different HRT showed quite stable values ranging 48-61 %. These values confirmed the hydrolytic activity of the process since it involved high production of soluble organic matter to be transformed into VFAs by acidogenic bacteria. These results are in good agreement with the values (around 50 % sCOD/tCOD) obtained in the experiments previously showed in Sections 4.1.1 and 4.1.2. The similar ratios attained regardless of the HRT reduction evidenced that the hydrolysis stage was not limited in any of the evaluated HRT scenarios.

Ammonium concentration showed a decrease trend from 0.6 ± 0.1 to 0.1 ± 0.0 g/L (Table 4.6). The decrease of ammonium concentration was related with the concomitant reduction in the fed organic matter concentration required for the stepwise HRT decrease (at constant OLR). As previously explained, ammonium released from proteins degradation could be accumulated and should be carefully controlled given their influence on methanogenic population. Similarly to reactors described in Sections 4.1.1 and 4.1.2, ammonium concentrations were far below the methanogenic inhibition threshold values (Yenigün and Demirel, 2013). Hence, this factor was not the

responsible for inhibiting methanogenic archaea, but the imposed operational conditions were mainly the ones fostering VFAs accumulation.

4.1.3.1.2. Effect of stepwise HRT reduction on VFAs production: conversion yields and profiles

AF bioconversion efficiencies

VFAs production and corresponding bioconversion efficiencies obtained when stepwise HRT reduction was conducted from HRT 10 to 2 days, are depicted in Figure 4.10.

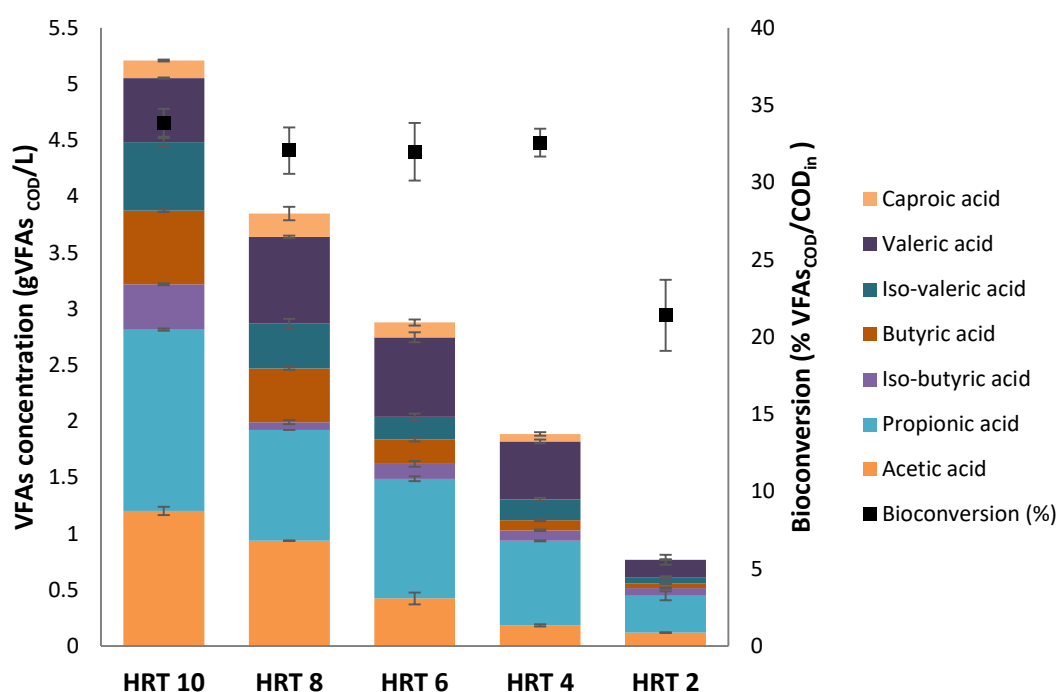


Figure 4.10. VFAs production and bioconversions in AF of *C. vulgaris* biomass during stepwise HRT reduction from 10 to 2 days.

Although a decrease in VFAs production was noticed (from 5.2 ± 0.2 g COD/L to 1.9 ± 0.1 g COD/L), similar organic matter bioconversion into VFAs (values around 32 % $\text{VFAs}_{\text{COD}}/\text{COD}_{\text{in}}$) were obtained when decreasing HRT from HRT 10 to 4 days. This fact could be explained by the reduction in the fed organic matter content to keep the OLR constant at 1.5 g COD/L d (see Table 3.2, Section 3.1.3). The acidification efficiency achieved in the AF reactors ranged 57-70 % $\text{VFAs}_{\text{COD}}/\text{sCOD}$, (Table 4.7). These values were in the range of previous studies (54-69 % $\text{VFAs}_{\text{COD}}/\text{sCOD}$), where vegetable wastes

were fermented under similar operational conditions (25 °C and pH 6) (Kumar and Mohan, 2018; Greses et al., 2020a).

Table 4.7. VFAs production and efficiencies evaluated during stepwise HRT reduction from 10 to 2 days in the AF of *C. vulgaris* biomass

	HRT 10	HRT 8	HRT 6	HRT 4	HRT 2
Acetic acid (% w/w)	23.8 ± 3.8	24.3 ± 0.3	14.7 ± 5.3	9.3 ± 1.1	15.5 ± 0.2
Propionic acid (% w/w)	31.0 ± 1.1	25.6 ± 0.1	37.0 ± 2.1	39.9 ± 0.4	42.5 ± 3.0
Iso-butyric acid (% w/w)	7.7 ± 0.8	1.8 ± 1.9	4.6 ± 2.6	4.9 ± 0.6	8.9 ± 0.6
Butyric acid (% w/w)	12.6 ± 1.2	12.4 ± 1.3	7.5 ± 1.8	4.8 ± 0.9	5.2 ± 0.2
Iso-valeric acid (% w/w)	11.7 ± 3.7	10.4 ± 3.1	7.0 ± 3.0	9.8 ± 1.5	7.6 ± 0.9
Valeric acid (% w/w)	10.9 ± 0.7	20.1 ± 1.1	24.6 ± 4.3	27.4 ± 1.8	19.9 ± 1.4
Caproic acid (% w/w)	3.0 ± 0.8	5.4 ± 5.0	4.6 ± 1.6	3.5 ± 1.6	0.3 ± 0.4
Total VFAs (g COD/L)	5.2 ± 0.2	3.9 ± 0.2	2.9 ± 0.1	1.9 ± 0.1	0.8 ± 0.1
% Bioconversion (gVFAs _{cod} /gCOD _{in})	33.9 ± 0.9	32.1 ± 0.6	32.0 ± 1.4	32.6 ± 0.9	21.4 ± 2.3
% COD acidified (VFAs _{cod} /sCOD)	70.4 ± 3.5	68.7 ± 2.2	58.8 ± 2.1	57.2 ± 3.6	48.1 ± 1.9

It could be stated that the imposed operational conditions in the reactors operating at 25 °C and HRT of 10, 8, 6 and 4 days promoted the hydrolysis and acidogenesis stages while preventing methanogenic activity. Despite the low HRTs, the organic matter conversion efficiencies into VFAs were not affected, evidencing the robustness of the process until HRT of 4 days. A further decrease to 2 days caused a considerable decrease in organic matter bioconversion. In the last scenario working at HRT of 2 days, 0.8 ± 0.1 g VFAs_{cod}/L was achieved corresponding to 21.4 ± 2.3 % VFAs_{cod}/tCOD_{in}. Considering that the hydrolytic step was not a bottleneck for VFAs production along the stepwise HRT reduction and that the methanogenic activity was negligible, the diminishment in the bioconversion efficiency into VFAs could be attributed to the acidogenic stage inhibition. The harsh operational conditions applied to suppress methanogenesis also affected the acidogenic activity in the case of HRT at 2 days.

The HRT effect has been explored using different substrates in anaerobic bioprocesses. For instance, Zhang and co-workers (2017) evaluated the HRT reduction from 52 to 5 days working at 38 °C using chicken manure and they found that VFAs accumulation occurred when HRT was below 10 days. Similarly, Peces and co-workers (2021) determined the impact of sequential HRT reduction from 15 to 2 days on AD and

microbial population evolution using sugar-based wastes as substrate. These studies evaluated how decreasing HRT could drive the anaerobic process from biogas towards VFAs production. However, information about the lowest HRT that can be applied to attain maximum VFAs yields is scarce. Cavinato and co-workers (2017) reported an optimum HRT of 4 days to attain a maximum 18.3 % VFAs_{COD}/tCOD_{in} when cow manure and maize silage were fermented at 37 °C and 55 °C. It is important to stress that this value is considerable lower than the one obtained here from microalgae biomass at the same HRT but at even lower temperature. Strikingly, in this Thesis, the effect of the HRT has been separated from the other variables. It is worth mentioning that most commonly studies investigating the effect of decreasing HRT are associated with a concomitant increase in OLR (Romero Aguilar et al., 2013; Cavinato et al., 2017).

VFAs profile

The stepwise HRT decrease was also evaluated in terms of the produced VFAs profile (Figure 4.11).

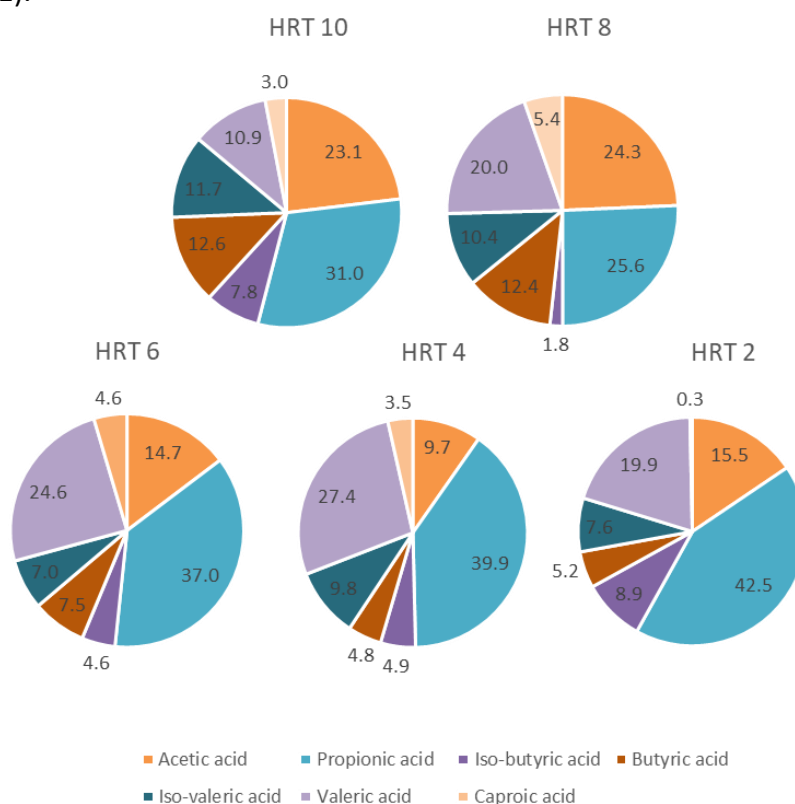


Figure 4.11. VFAs distribution profile in the AF of *C. vulgaris* during stepwise HRT reduction from 10 to 2 days.

Propionic, acetic and valeric acids were the most abundant acids in the AF reactors. As aforementioned in Sections 4.1.1 and 4.1.2, apart from acetic acid, the high presence of propionic and valeric acids may be related to the high protein content of the microalgae biomass used as feedstock (Table 3.1, Section 3.1.1) because protein-rich substrates lead to odd-chain VFAs production (Regueira et al., 2020)

Important differences in VFAs profiles were found when the HRT was decreased. Propionic and valeric acids showed an increasing trend from 31.0 % (w/w) to 42.5 % (w/w) and from 10.9 % (w/w) to 27.3 % (w/w), respectively (Table 4.7) along the HRT decrease. The accumulation of longer chain VFAs, such as valeric acid, has been correlated with methanogenic activity inhibition (Strazzera et al., 2018). Therefore, the high concentration of this acid evidenced the high efficiencies of organic matter conversion into VFAs at HRT 10, 8, 6 and 4 days. By opposite, iso-butyric and caproic acids presented lower abundance, accounting for less than 10 % (w/w) of total VFAs produced regardless of the HRT applied.

As explained in Sections 4.1.1 and 4.1.2, it is common feature of AF that longer chain VFAs are shortened to smaller molecules, giving rise to C2-C3 VFAs accumulation. Changes in relative VFAs distribution have been previously observed during HRT reduction (Strazzera et al., 2018). In general, acetic acid predominance was observed when short HRTs were applied, whereas propionic and butyric acids were the main VFAs when working at long HRTs (Lim et al., 2008). In reactors targeting VFAs production, working at short HRT, acetic acid presence is considered an indicator of a successful AF performance since acetoclastic archaea inactivity can be assumed when acetic acid is not consumed. This fact supported the low COD removal values attained in this work (6.8-11.7 % COD removal, Table 4.6).

However, as it can be seen in Figure 4.11, the presence of acetic acid strongly decreased along the HRT reduction from 23.0 % (w/w) to 9.7 % (w/w), which could be attributed to a process inhibition probably mediated by acidogenic population wash-out. Previous studies reported that microbial population become less diverse when HRT decreases (Zhang et al., 2006; Peces et al., 2021). Microbial population shifts during HRT reduction have been determined in works focused on biogas production where methanogenic

population disappeared during biogas production failure. Nevertheless, information about microbial population in AF working at HRT lower than 10 days is scarce. Some authors have reported the correlation between VFAs production and hydrolytic/acidogenic population when working at short HRT employing other substrates (Bi et al., 2020; Peces et al., 2021). In addition to the changes in hydrolytic, acidogenic and acidogenic microbial populations, shifts in fermentative pathways were also observed when reducing the HRT. For instance, similarly to the results obtained herein, Peces and co-workers (2021) observed a transition from butyrate and propionate degradation to acetic and ethanol degradation when working at HRT lower than 4-2 days.

4.1.3.2. Effect of direct HRT decrease on AF

In order to optimize the HRT avoiding a stepwise reduction, which in turns requires higher resources, time and costs, the effect of directly operating AF at initial HRT 8, 6 and 4 days (without any adaptation period) is addressed in this section. An AF reactor set at initial HRT 2 days was not evaluated since it caused process failure during stepwise HRT reduction (Section 4.1.3.1).

4.1.3.2.1. Effect of HRT on organic matter removal

When comparing the organic matter removal obtained during the stepwise HRT reduction (Table 4.6) and the obtained directly starting at the same HRT (Table 4.8), no important differences were shown.

Table 4.8. Main parameters and efficiencies evaluated in the AF of *C. vulgaris* directly operated at initial HRT 10, 8, 6 and 4 days.

	i-HRT 10	i-HRT 8	i-HRT 6	i-HRT 4
pH	6.0 ± 0.1	6.1 ± 0.1	6.0 ± 0.0	5.9 ± 0.1
TS (g/L)	5.6 ± 0.4	4.8 ± 0.7	2.8 ± 0.1	2.0 ± 0.1
VS (g/L)	4.1 ± 0.4	3.4 ± 0.8	2.0 ± 0.1	1.6 ± 0.1
VS/TS	69.9 ± 3.2	68.7 ± 2.0	72.5 ± 1.6	73.7 ± 5.4
N-NH ₄ ⁺ (g/L)	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
tCOD (g/L)	15.0 ± 0.4	9.1 ± 0.4	7.3 ± 0.3	5.9 ± 0.5
sCOD (g/L)	7.4 ± 0.2	4.7 ± 0.1	3.8 ± 0.2	3.0 ± 0.2
sCOD/ tCOD (%)	49.4 ± 2.1	51.5 ± 3.5	53.8 ± 2.4	51.2 ± 7.8
COD removal (%)	8.7 ± 0.9	7.6 ± 2.2	7.1 ± 3.5	3.6 ± 1.9
Methane yield (mL CH ₄ /g COD _{in})	30.4 ± 15.1	26.5 ± 10.7	24.8 ± 6.2	12.6 ± 2.8

Abbreviation: i-HRT: initial HRT

When working at HRT of 8, 6 and 4 days, COD removals were considerably low (4-7 % COD removal). Similarly to the COD removal obtained in the stepwise HRT reduction, these values pointed to an inhibition of biogas production. Independently of the HRT evaluated, the ratio sCOD/tCOD close to 50 % and the VS/TS ratio about 70 % confirmed that a high hydrolytic activity was maintained despite the direct application of low HRT. These results agreed with the ratios aforementioned in Sections 4.1.1 and 4.1.2.

Ammonium concentration and pH values in the reactors directly set at HRT 8, 6 and 4 days were similar than those achieved in the stepwise HRT reduction, being both parameters in the suitable range for VFAs production.

4.1.3.2.2. Effect of HRT on VFAs production: conversion yields and profiles

AF bioconversion efficiencies

Figure 4.12 shows obtained VFAs when the reactors were directly operated at initial HRT 10, 8, 6 and 4 days. As it can be seen, VFAs productions were 5.2 ± 0.2 g COD/L at HRT 10 days; 4.0 ± 0.4 g COD/L at HRT 8 days; 2.8 ± 0.6 g COD/L at HRT 6 days and 1.3 ± 1.1 g COD/L at HRT 4 days. These values gave rise to bioconversion efficiencies of 33.8 ± 0.9, 33.4 ± 2.2, 31.5 ± 0.7 and 19.7 ± 2.6 % g VFAs_{cod}/COD_{in} (Table 4.9).

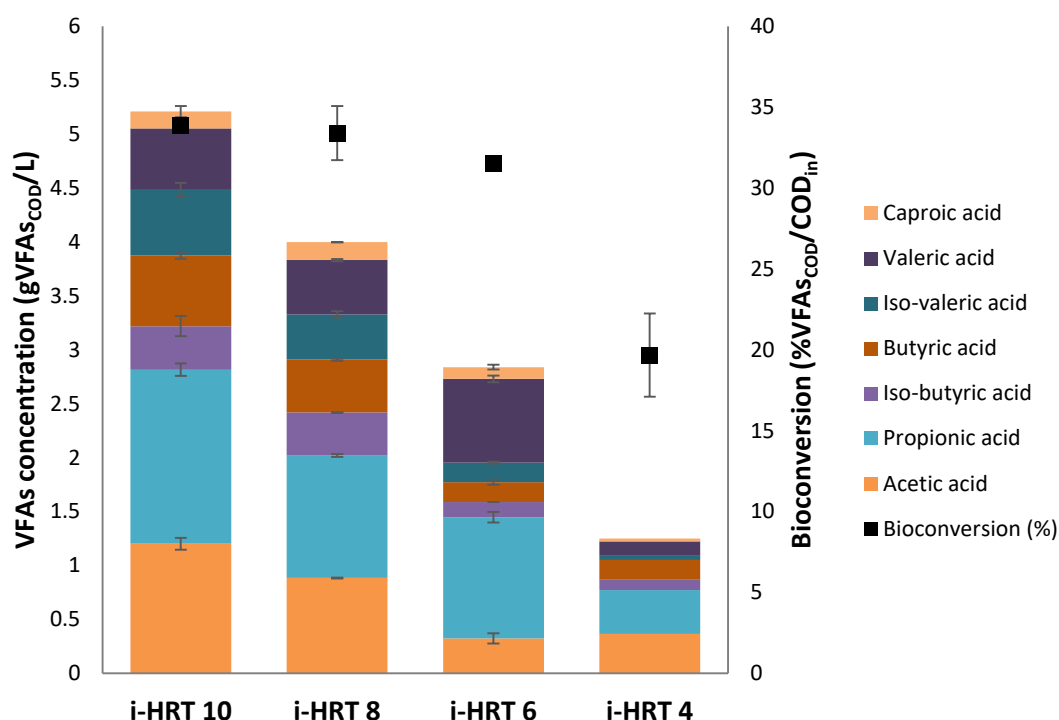


Figure 4.12. VFAs production and bioconversions in the AF of *C. vulgaris* directly operated at initial HRT 10, 8, 6 and 4 days. Abbreviation: *i-HRT*: initial HRT

Although organic matter removal was similar when comparing stepwise reduction and direct application of low HTR, differences in VFAs bioconversion efficiencies were evidenced. The reactors operated at initial HRT of 10, 8 and 6 days maintained a similar bioconversion of around 32 % VFAs_{COD}/tCOD_{in}, which was comparable to the stepwise operation mode. However, when the HRT was directly set at 4 days, a decrease in VFAs bioconversion from 32.6 % VFAs_{COD}/tCOD_{in} to 19.7 % VFAs_{COD}/tCOD_{in} was observed. This trend can also be observed in the acidification efficiencies obtained in the reactors (Table 4.9). Similarly to the stepwise HRT reduction, values close to 70 % VFAs_{COD}/sCOD were calculated when working directly at initial HRT of 10, 8 and 6 days. The acidogenic efficiency was decreased from 70 % to 43 % when the reactors were operated directly at initial HRT of 4 days.

Table 4.9. VFAs production and efficiencies evaluated in the AF of *C. vulgaris* directly operated at initial HRT 10, 8, 6 and 4 days.

	i-HRT 10	i-HRT 8	i-HRT 6	i-HRT 4
Acetic acid (% w/w)	23.8 ± 3.8	22.6 ± 3.1	11.4 ± 2.7	29.1 ± 3.3
Propionic acid (% w/w)	31.0 ± 1.1	29.0 ± 3.3	39.5 ± 4.3	32.6 ± 4.0
Iso-butyric acid (% w/w)	7.7 ± 0.8	10.2 ± 1.5	4.9 ± 0.5	8.0 ± 2.2
Butyric acid (% w/w)	12.6 ± 1.2	12.5 ± 1.9	6.5 ± 1.0	14.4 ± 1.5
Iso-valeric acid (% w/w)	11.7 ± 3.7	10.7 ± 1.1	6.4 ± 1.1	3.1 ± 0.7
Valeric acid (% w/w)	10.9 ± 0.7	12.8 ± 3.9	27.3 ± 2.5	10.5 ± 1.4
Caproic acid (% w/w)	3.0 ± 0.8	2.1 ± 0.4	3.8 ± 0.3	0.0 ± 0.0
Total VFAs (gCOD/L)	5.2 ± 0.2	4.0 ± 0.4	2.8 ± 0.6	1.3 ± 1.1
% Bioconversion (gVFAs _{cod} /gCOD _{in})	33.9 ± 0.9	33.4 ± 2.2	31.5 ± 0.7	19.7 ± 2.6
%COD acidified (VFAs _{cod} /sCOD)	70.4 ± 3.5	72.4 ± 3.7	73.6 ± 4.1	43.3 ± 1.1

These results indicated that the reactors could be set up to work directly at HRT of 10, 8 and 6 days without compromising the hydrolysis and acidogenesis stages. However, when the reactors were directly started at HRT of 4 days, the acidogenic activity was hindered. This fact evidenced that the stepwise decrease of HRT allowed the microbial system to be adapted to a stressful situation, such is the low HRT imposed. The adaptation of the anaerobic population to operational conditions as temperature, OLR increase, salinity or ammonium also resulted in an enhanced performance during AD in previous studies where methane was desired (Ghasimi et al., 2015; Muñoz Sierra et al., 2018; Tian et al., 2018). Tian and co-workers (2018) demonstrated that the acclimation of the anaerobic population to extreme ammonia levels overcame the toxicity problems associated to biogas reactors when microalgae was employed as substrate. Similarly, Magdalena and co-workers (2019c) reported an improvement from 35 % VFAs_{cod}/tCOD_{in} to 39 % VFAs_{cod}/tCOD_{in} by the use of a temperature and substrate-adapted inoculum when *C. vulgaris* was devoted to VFAs production in semi-continuous mode.

VFAs profile

In terms of VFAs profile, no important differences were achieved among the reactors working at different initial HRT (Figure 4.13).

Chapter 4

Regardless of the HRT established, propionic and acetic acids were the main products in the AF effluents, accounting for 50-60 % (w/w) of total VFAs. Unlike the VFAs profile obtained in the stepwise HRT reduction experiment, valeric acid presence was lower than 10 % (w/w) of total VFAs (Table 4.9). It is worth to highlight that despite the process failure in terms of VFAs yields when the reactor was directly operated at 4 days of HRT, VFAs distribution in the effluent was not affected.

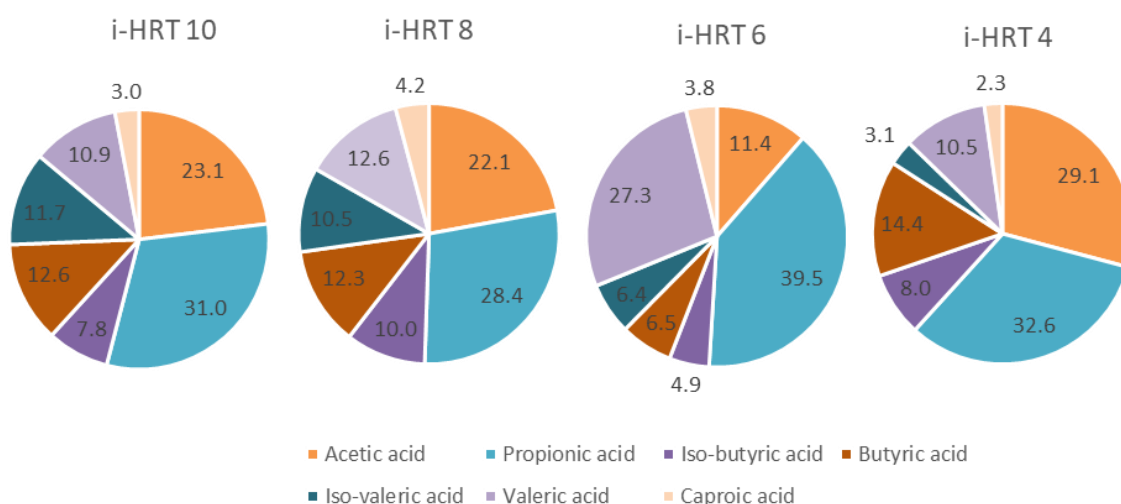


Figure 4.13. VFAs distribution profile in the AF of *C. vulgaris* directly operated at initial HRT 10, 8, 6 and 4 days.

The comparison of stepwise HRT reduction with the direct operation at low HRT evidenced a microbial community adaptation to the harsh operational conditions (low HRT). This adaptation allowed a reduction of the HRT until 4 days, treating higher flow rates while keeping up the high AF efficiency. When the reactors were directly operated at HRT values below 6 days, the harsh operational conditions did not only affect the methanogenic archaea but were also detrimental for acidogenic bacteria activity resulting in low bioconversions into VFAs. At this point, it should be stressed that HRT of 4 days was a very low value for the digestion of complex organic matter (such as microalgae biomass). HRT normally found in literature for VFAs production from microalgae biomass was not lower than 8 days (Magdalena et al., 2019b, 2019c; Llamas et al., 2021).

In conclusion, the high bioconversion around 32 % achieved during the stepwise HRT reduction to 4 days and in the reactor directly set at HRT 6 days, confirmed that high volumetric production of VFAs could be attained by appropriate HRT adjustment. HRT below 10 days was demonstrated to have a positive influence on AF performance by preventing methanogenic activity. However, decreasing the HRT below a critical value also affected acidogenic bacteria, ultimately leading to low VFAs bioconversion yields. Stepwise HRT reduction strategy implied microbial adaptation to operational conditions which in turn allowed HRT reduction to lower values.

4.2. Valorization of the AF solid fraction for biogas production via conventional AD

The effluent of the AF consists in VFAs-rich liquid phase and a solid spent containing unfermented organic matter and anaerobic biomass. In the present Thesis, the residual solid fraction obtained after liquid fraction separation is proposed as substrate to produce methane in AD reactors (Section 1.1). By using this approach, multiproduct generation from microalgae biomass can be maximized via this innovative two-stage sequential anaerobic bioprocesses.

Most commonly, when employing a two-stage configuration in AD, two bioreactors are implemented with different operational conditions to enable a decoupled digestion. More specifically, a first reactor is set to conduct the hydrolytic-acidogenic stages and a second one is set for methanogenesis. This digestion strategy allows implementing optimum environmental conditions for each microbial group, favouring bioprocess control and maximizing production yields. In the conventional two-stage AD, the entire VFAs-rich effluent obtained in the acidogenic reactor is fed in a second reactor for methane production (Figure 4.14.a). However, the novel approach presented in this Thesis aims to increase products output (by recovering VFAs in the first reactor) instead of just maximizing biogas production (Figure 4.14.b). This integration supports not only wastes treatment but also carbon recovery maximization through multiproduct generation.

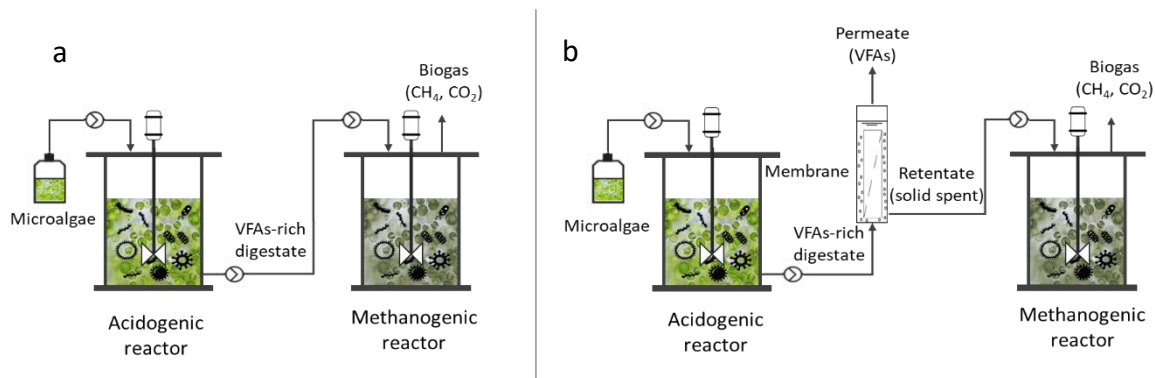


Figure 4.14. Schematic diagram of (a) conventional two-stage AD strategy and (b) novel two-stage AD strategy presented in this Thesis.

To address this alternative, this section is focused on the use of the solid spent biomass recovered from AF in batch (Publication II) and semi-continuous digestion mode (Publication III) for biogas production.

4.2.1. Biogas production via AD from the AF solid spent in batch mode

To evaluate the potential of the AF solid spent for methane production, BMPs of three different solid fractions were carried out (Publication II). Standard conditions for biogas production (0.5 g COD/g VS, $T=35^{\circ}\text{C}$, $\text{pH}=7.5$ and 150 rpm) were selected to conduct the BMPs, as described in Section 3.2.3 (Table 3.4). The three solid spents employed as substrate were obtained from the work previously performed by Magdalena and co-workers (2019b), where the impact of OLR increase in the AF performance of *C. vulgaris* biomass was studied. Regardless of the OLRs applied (6, 9 and 12 g COD/L d), these authors reported similar bioconversion efficiencies into VFAs (around 0.30 g VFAs_{COD/g} tCOD_{in}). In fact, not only VFAs yields but also similar VFAs profiles were attained for the three AF effluents.

The solid spent of the AF conducted at OLR 6, 9 and 12 g COD/L d were collected and subjected to BMP assays. Figure 4.15 illustrates the specific methane yields (mL CH₄/g tCOD_{in}) against time (Publication II). Independently of the OLR from which the solid spent was collected, an average methane yield of 187.3 ± 3.7 mL CH₄/g tCOD_{in} (around

57 % biodegradability) was reached after 38 days of AD. The similar biodegradability exhibited by the three solid fractions used as AD substrates was in agreement with the trend reported by Magdalena and co-workers (2019b) during the AF. Considering that VFAs conversion yields were similar upon the different OLRs, the remaining organic matter biodegradability was expected to be similar as well. Nevertheless, this should be confirmed since different OLR in AF could affect not only VFAs production but also the formation of others by-products that could in turn hamper biogas production from AF solid spent.

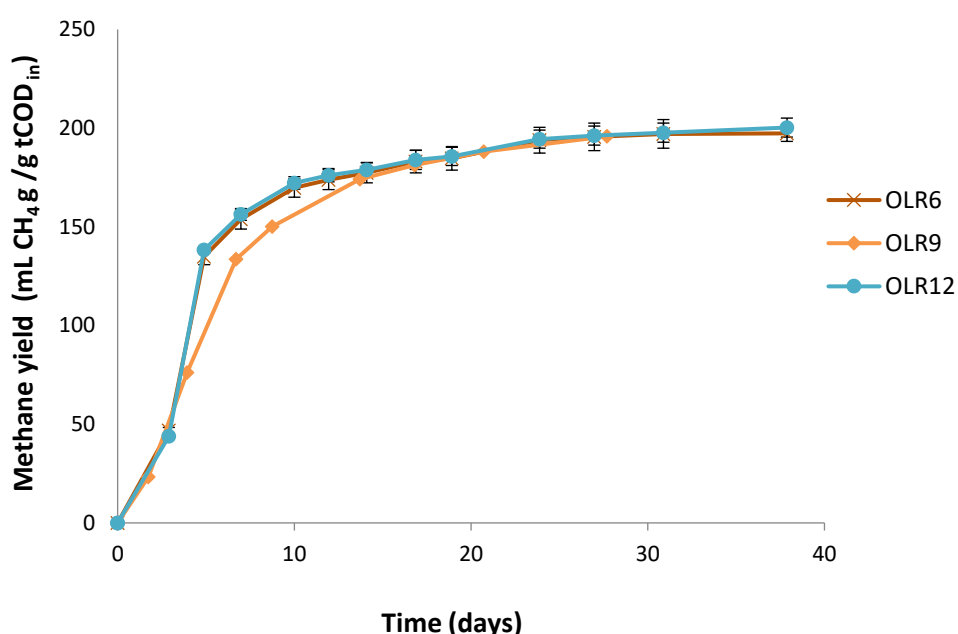


Figure 4.15. Cumulative methane yields achieved in BMPs using different AF solid spent solid as substrate.

In terms of methane content in the biogas, no differences were observed regardless the substrate employed in AD, ranging from 61 % (v/v) CH₄ to 67 % (v/v) CH₄ in all cases. Methane yield achieved was in the range of other studies where microalgae biomass was particularly devoted to biogas production. Mahdy and co-workers (2014b) reported a maximum 255.6 mL CH₄/g tCOD_{in} (73 % anaerobic biodegradability) in batch assays when using the same A-pretreated microalgae strain. This fact evidenced the high biodegradability of the organic matter present in the solid fractions obtained from the AF effluents conducted at different OLRs.

The BMPs results were modelled according to Gompertz model. As it can be seen in Table 4.10 (Publication II), the model presented a good fit (R^2 values). This model also allowed to confirm that kinetic parameters, including methane productivity and lag phase, were similar for the three AF spent solid fractions obtained at different OLRs.

Table 4.10. BMP results according to Gompertz model using AF solid spent obtained in AF at OLR 6, 9 and 12 g COD/L d as substrate.

	OLR 6	OLR 9	OLR 12
P (mL CH₄/g tCOD_{in})	184.6 ± 5.7	188.6 ± 2.2	188.7 ± 3.4
Rm (mL CH₄/g tCOD_{in} d)	5.4 ± 0.1	4.6 ± 0.1	5.7 ± 0.1
Lambda (d⁻¹)	1.5 ± 0.1	0.8 ± 0.1	1.7 ± 0.1
R²	0.98 ± 0.01	0.99 ± 0.00	0.98 ± 0.01

Few studies have evaluated the potential energy contribution of AD to other microalgae-based processes and these are mainly devoted to recover energy after microalgae lipid extraction. Different authors indicated that a considerable part of the total energy contained within the biomass could be recovered if AD of the lipid-extracted microalgae is implemented (Batista et al., 2017; Choi et al., 2017). However, this process has not been applied to recover energy out of the solid fraction generated after AF, highlighting the novelty of the results and the interesting findings presented in this Thesis.

Despite the fact that the substrate was a residue collected from AF, the high methane yields achieved in BMPs evidenced the high biodegradability of the organic matter in the remaining solid fraction. In this sense, the suitability of this side-product as a substrate for biogas production was proved. Coupling the two bioprocesses (acidogenesis for VFAs production and entire AD for methane production) did not affect the methane production potential that can be attained from the solid fraction generated in AF.

Once confirmed the potential of AF solid fractions as substrate for biogas production, this bioprocess was validated in semi-continuous mode (as described in the following Section 4.2.2 (Publication III)).

4.2.2. Biogas production via AD from the AF solid spent in semi-continuous mode

Batch assays are an efficient tool to perform a preliminary determination of substrates potential for biogas production. However, conversions into methane obtained in batch mode are often higher than those observed in semi-continuous mode (Mahdy et al., 2015; Méndez et al., 2015). For this reason, in the present Thesis, the performance of a CSTR fed with the solid spent recovered from AF was evaluated.

The residual solid spent obtained from AF of microalgae at HRT 8 days (Section 4.1) was anaerobically digested in a CSTR (Publication III). As summarized in Table 3.4, Section 3.2.3, the CSTR was operated under similar conditions than the ones implemented for BMP assays.

The AD reactor reached values of 1.1 ± 0.3 g COD/L and 16.5 ± 1.9 g COD/L for sCOD and tCOD, respectively, corresponding to a COD removal of 48.5 ± 1.5 % (Table 4.11, Publication III).

Table 4.11. Main parameters and efficiencies evaluated during the stable period of AD-CSTR fed with AF solid spent.

	AD-CSTR
pH	7.3 ± 0.1
tCOD (g/L)	16.5 ± 1.9
sCOD/tCOD (%)	6.8 ± 1.9
TS (g/L)	10.0 ± 0.4
VS (g/L)	8.7 ± 0.4
N-NH ₄ ⁺ (g/L)	0.4 ± 0.1
Total VFAs (g COD/L)	0.2 ± 0.0
COD removal (%)	48.5 ± 1.5
VS removal (%)	32.5 ± 2.9
N mineralization (%)	15.5 ± 0.9
CH ₄ (%)	75.2 ± 3.7
Methane yield (mL CH ₄ /g tCOD _{in})	168.9 ± 25.7
% Biodegradability	48.3 ± 9.2

The COD removal obtained under these conditions was in the range of previous studies devoted to biogas production where more than 50 % COD removal are usually reported (Ramos-Suárez and Carreras, 2014; Greses et al., 2017). These values were remarkably

higher than the obtained when targeting at VFAs production. This is due to the need of restricting methanogenic activity for maximizing VFAs accumulation. For instance, COD removals lower than 12 % were attained in Section 4.1 when VFAs were desired. Therefore, the low VFAs concentrations detected in the effluent of the AD reactor fed with the solid spent ($0.02 \text{ g VFAs}_{\text{COD}}/\text{L}$) pointed out to a well-balanced AD process where VFAs are consumed during biogas production by the methanogenic population. Environmental conditions in the AD reactor (pH of 7.3 and $0.4 \text{ g N-NH}_4^+/\text{L}$, Table 4.11) were suitable for methanogenic activity. All these results suggested that the imposed operational conditions in the CSTR fed with the solid spent (HRT 20 days, 35°C and OLR $1.5 \text{ g COD}/\text{L d}$, Table 3.5) promoted the hydrolysis, acidogenesis and methanogenesis of this particular feedstock.

Considering the low soluble organic matter available in the employed feedstock (27.7 % sCOD/tCOD, Table 3.4), a high hydrolytic activity was determined in the AD reactor. The solid concentration reduction (32.5 % VS removal) confirmed the solubilization of organic matter for the subsequent stages. This fact is very important considering that the substrate is the residual fraction obtained after AF. The high hydrolytic activity detected may be related not only to the operational conditions applied but also to the hydrolytic and acidogenic population enrichment, as a consequence of the microorganisms dragged from the AF to the AD reactor.

The specific methane yield ($\text{mL CH}_4/\text{g tCOD}_{\text{in}}$) obtained in the AD reactor is illustrated in Figure 4.16 (Publication III). Methane content in the biogas was 75.2 % v/v and an average methane yield of $168.9 \pm 25.7 \text{ mL CH}_4/\text{g tCOD}_{\text{in}}$ was obtained in semi-continuous mode during the steady-state. Based on a maximum conversion of $350 \text{ mL CH}_4 \text{ produced}/\text{g tCOD}_{\text{in}}$, the methane yield corresponded to a biodegradability of $48.3 \pm 9.2 \%$ (Table 4.11).

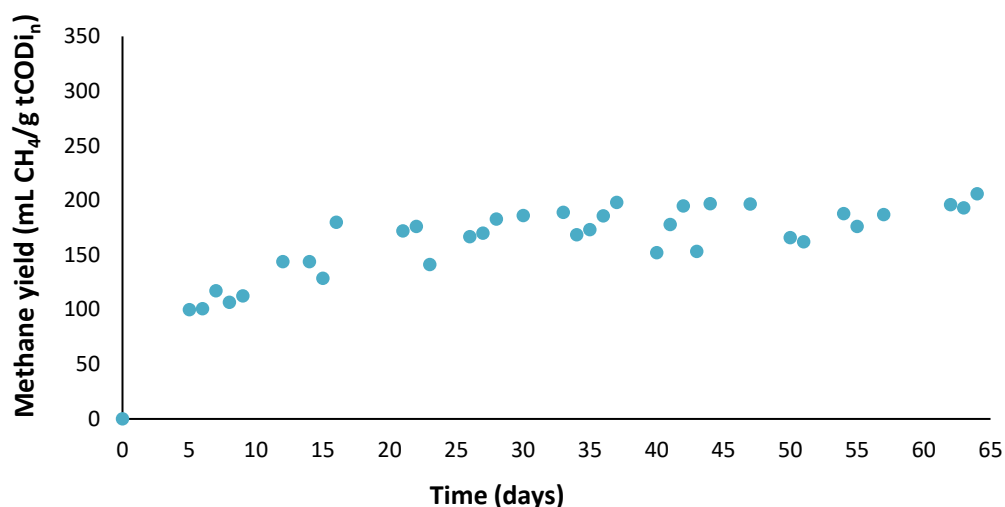


Figure 4.16. Time course of methane yield achieved in AD reactor fed with AF solid spent.

The methane yield obtained in the CSTR fed with solid spent was slightly lower than the values found in BMPs under same conditions (188 mL CH₄/g tCOD_{in} and 57 % biodegradability, previously shown in Section 4.2.1 (Publication II). However, the methane yield achieved herein was similar to the values reported in other studies evaluating the potential of microalgae AD for energy recovery under semi-continuous digestion mode. For instance, methane yields of 110-150 mL CH₄ g /tCOD_{in} have been reported when semi-continuous AD of raw microalgae was conducted (Mendez et al., 2014; Prajapati et al., 2014) while 136-220 mL CH₄ g /tCOD_{in} were attained when microalgae was pretreated with thermal, mechanical or enzymatic pretreatments (Passos et al., 2013b; Passos and Ferrer, 2014; Mahdy et al., 2016a).

As mentioned above, two-stage AD of microalgae biomass has been also studied by feeding the entire VFAs-rich effluent from the first AF reactor to a second AD reactor (Vergara-Fernández et al., 2008; Varol and Ugurlu, 2016; Markphan et al., 2020). When implementing a two-stage AD strategy, a methane yield increase between 10 % and 30 % has been reported when compared with the traditional single-stage AD (Rajendran et al., 2020). Splitting AD stages provides optimum conditions for each microbial community, thereby enhancing the methane yield and productivity (Vergara-Fernández

et al., 2008). Considering the methane yield ($136 \text{ mL CH}_4 / \text{g tCOD}_{\text{in}}$) reported by Mahdy and co-workers (2016) when A-pretreated *C. vulgaris* was digested under similar conditions, the methane yield reached in this Thesis showed a 24 % improvement.

This study confirmed that the results previously obtained in batch assays could be extrapolated to CSTR digestion mode. It seems likely that the AF conducted previously to the AD process favoured solid residue biodegradability. Furthermore, the hydrolytic and acidogenic population enrichment as a consequence of the microorganisms dragged from the AF to the AD reactor, could have contributed to promote the high biogas production achieved as well.

4.3. Microbial oils production from VFAs-rich liquid fraction via oleaginous fermentation

VFAs can be a product by themselves after their separation or purification or, they can serve as building blocks for several bioproducts (see Section 1.1.2, Table 1.1). In this Thesis, the VFAs present in the liquid fraction obtained from AF have been investigated as novel carbon sources for MO production (explained in Section 1.2).

Despite the interest in MO as a petroleum alternative and the necessity to find low-cost lipid sources, most of the studies on lipid production with OY have been carried out using sugars as carbon source. The use of VFAs, instead of sugars, arises as a promising strategy since these acids can be produced from a wide range of wastes. However, scarce information about the use of VFAs as substrate for OY is available, and the full potential of VFAs-rich liquid streams as cultivation media is still unknown. To cover this gap of knowledge, the most recent approaches dealing with MO production from VFAs were reviewed in Publication IV.

Gaining insight on how VFAs are metabolized by OY is a fundamental issue to fully understand this bioprocess. In this Thesis, experiments with synthetic VFAs were performed (Publication V) to elucidate VFAs utilization by OY and their bioconversion into lipids. When VFAs suitability as carbon source was proven, VFAs-rich effluents

collected from real AF of microalgae biomass were tested for yeast fermentation in Publication V. Lastly, in Publication VI, a screening of the most promising OY was accomplished by investigating their lipid production capacity using VFAs-rich effluent recovered from the AF of *C. vulgaris*.

4.3.1. Assessment of VFAs as carbon source for OY

Y. lipolytica growth and substrate consumption were evaluated using the VFAs present in anaerobic effluents (Publication V). *Y. lipolytica* was selected to perform these experiments given its capacity to use hydrophilic and hydrophobic compounds as substrates (Fukuda and Ohta, 2013; Papanikolaou and Aggelis, 2011a). Additionally, *Y. lipolytica* is the most widely studied yeast for MO production and its full sequenced genome is available, which would facilitate the understanding of the VFAs routes for lipid biosynthesis.

Synthetic individual VFAs (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids), as well as mixtures, were tested at different concentrations and ratios to determine uptake rates and potential inhibitory effect (see Table 3.5, Section 3.3.2). Additionally, real AF effluent rich in VFAs was compared with synthetic medium to evaluate the potential of this substrate for practical application.

4.3.1.1. Effect of individual synthetic VFAs as carbon source on *Y. lipolytica* growth

As described in Section 3.3.2, the seven VFAs were tested independently as carbon source for *Y. lipolytica* at a low concentration (*i.e.* 0.8 g C/L) and high concentration (*i.e.* 4 g C/L) to evaluate the individual inhibitory effect of each acid. For comparison purposes, 0.8 g C/L and 4 g C/L of glucose (corresponding with 2 and 10 g glucose/L, respectively) were also tested.

When each acid was independently used at 0.8 g C/L, *Y. lipolytica* was able to grow regardless of the tested VFAs. The maximum biomass growth, however, increased

concomitantly with VFAs chain length. As it can be seen in Table 4.12 (Publication V), the highest biomass concentration was obtained when using caproic acid (0.93 ± 0.12 g/L) as a substrate while the lowest biomass was reached from acetic acid (0.52 ± 0.12 g/L). The same trend was attained for the biomass yields (g of biomass/g of substrate), showing higher values when longer VFAs chain lengths were used (0.72 ± 0.19 g/g from caproic acid and 0.25 ± 0.13 g/g from acetic acid). This tendency was similar to the pattern described by Liu and co-workers (2017a) who pointed out that longer chain VFAs had higher theoretical growth yield coefficient.

Table 4.12. *Y. lipolytica* growth parameters on individual VFAs at different concentrations.

	Carbon source	VFAs conc. (g /L)	Exhaustion time (h)	Lag phase (h)	Biomass conc. (g/L)	$Y_{x/s}$ (g/g)
0.8 g C/L	Glucose	2	24	2	1.50 ± 0.12	0.75 ± 0.15
	Acetic acid	2	32	4.5	0.52 ± 0.14	0.25 ± 0.13
	Propionic acid	1.64	24	4.5	0.63 ± 0.15	0.42 ± 0.20
	Butyric acid	1.47	24	7	0.76 ± 0.05	0.48 ± 0.06
	Iso-butyric acid	1.47	32	9	0.80 ± 0.08	0.55 ± 0.11
	Valeric acid	1.36	24	7	0.81 ± 0.14	0.63 ± 0.22
	Iso-valeric acid	1.36	24	9	0.84 ± 0.07	0.70 ± 0.12
	Caproic acid	1.29	24	7	0.93 ± 0.12	0.72 ± 0.19
4 g C/L	Glucose	10	48	2	5.82 ± 0.59	0.58 ± 0.03
	Acetic acid	10	120	4.5	1.38 ± 0.03	0.15 ± 0.01
	Propionic acid	8.23	150	7	2.48 ± 0.04	0.33 ± 0.01
	Butyric acid	7.34	72	9.5	3.90 ± 0.21	0.44 ± 0.05
	Iso-butyric acid	7.34	120	9	3.21 ± 0.21	0.50 ± 0.06
	Valeric acid	6.81	100	9	3.41 ± 0.24	0.54 ± 0.08
	Iso-valeric acid	6.81	120	9	3.37 ± 0.36	0.54 ± 0.05
	Caproic acid	6.45	240	120	3.11 ± 0.21	0.51 ± 0.07

Y_{x/s}: growth yield

When using 0.8 g C/L of VFAs, acid consumption was always associated with cell growth. Most of the VFAs were exhausted in 24 h of cultivation and only acetic and iso-butyric acids were consumed after 32 h (Table 4.12). These consumption rates were considerably faster than the reported in previous studies where more than 84 h of fermentation were required for exhaustion of 2.5 g/L acetic, propionic and butyric acids (Gao et al., 2017). In the present Thesis, the lag phases with acetic and propionic acids

were shorter (4.5 h) than for the other evaluated acids (7-9 h), which suggested lower inhibitory effect of short-chain VFAs. Despite having the shortest lag-phase, acetic acid exhaustion required the longest fermentation time (32 h). This fact could be explained by the higher concentration of this acid when compared with the longer ones (*i.e.* 2 g/L of acetic vs 1.3 g/L of caproic acid).

When acids were added at 4 g C/L, an increase in biomass production from 1.38 ± 0.03 g/L to 3.21 ± 0.21 g/L (corresponding to biomass yields of 0.15 ± 0.01 g/g and 0.50 ± 0.06 g/g, respectively) was observed when carbon chain length increased from C2 (acetic) to C4 (iso-butyric acid) (Table 4.12). Nevertheless, when the chain length exceeded C4 (*i.e.* valeric, iso-valeric and caproic acids), no significant differences in yeast growth were observed and biomass yields ranged from 0.51 ± 0.07 g/g to 0.54 ± 0.08 g/g.

Remarkably, *Y. lipolytica* grown on caproic acid showed a prolonged lag phase (120 h), requiring more than 240 h for substrate exhaustion. However, no significant differences in biomass production were found when VFAs with chain length from C4 to C6 were used at 4 g C/L. Thus, it may be inferred that long-chain VFAs at high concentration have associated an inhibitory effect on yeast. These results are in good agreement with previous studies related to VFAs inhibition. Gao and co-workers (2017) stated VFAs inhibitory effect on *Y. lipolytica* in the following order: butyric>propionic>acetic acid. The VFAs inhibitory effect could be explained by the pH increase during VFAs consumption, reaching basic values that hamper microbial growth. Yeast cells have limited pH tolerance since pH changes can interfere with membrane transporters and ATP expenses, consequently inhibiting biomass and lipid production (Rodrigues and Pais, 2000).

When comparing high and low VFAs concentrations, biomass yields were considerably lower at high VFAs concentrations (4 g C/L) independently of the acid used as carbon source. For instance, from acetic acid at 0.8 g C/L, 0.25 ± 0.13 g/g of biomass yield was attained whereas 0.15 ± 0.01 g/g was obtained at 4 g C/L (Table 4.12). Therefore, inhibitory effects of caproic acid and acetic acid could be suggested when reaching 6.5 g/L and 10 g/L, respectively. Fontanille and co-workers (2012) and Fei and co-workers (2011) also showed inhibition of *Y. lipolytica* and *C. albidus* growth when using

mixtures of acetic, propionic and butyric acids at higher concentrations than 5 g total VFAs/L. At this point, it should be highlighted that in the present Thesis when 4 g C/L of VFAs were used, all VFAs were above that threshold.

Biomass concentrations and yields were very close to those achieved with glucose as carbon source (Table 4.12). In this case, 0.75 ± 0.09 g/g and 0.58 ± 0.03 g/g of biomass were produced from 0.8 g C glucose/L and 4 g C glucose/L, respectively. This fact proved that carboxylate-based substrates can be an interesting alternative to sugar-based substrates for MO production with *Y. lipolytica*.

Since AF effluents contain VFAs mixtures from C2 to C6, it is necessary to unravel VFAs effects on yeast growth when present in mixtures.

4.3.1.2. Effect of synthetic VFAs mixtures as carbon source on *Y. lipolytica* growth

Once evaluated the individual effect of VFAs on *Y. lipolytica* growth, the impact of synthetic mixtures was also studied. The ability of several OY to grow in the presence of mixtures of acetic, propionic and butyric acid has been previously reported (Fei et al., 2011a; Fontanille et al., 2012; Gong et al., 2015; Kolouchová et al., 2015; Gao et al., 2017; Liu et al., 2017a). However, iso-butyric, valeric, iso-valeric and caproic acids can account for more than 30 % (w/w) of the VFAs present in the digestate (Magdalena et al., 2019b) and their presence should not be neglected. In this Thesis, *Y. lipolytica* performance was assessed on five synthetic VFAs mixtures, containing of each acid: I) 0.11 g C/L, II) 0.8 g C/L, III) 2 g C/L, IV) 4 g C/L and V) 1.51 g/L (Table 3.5).

Figure 4.17 (Publication V) shows *Y. lipolytica* growth on mixtures I-V during fermentation time. *Y. lipolytica* was able to grow on 0.11 g C/L, 0.8 g C/L and 2 g C/L of each acid. However, 2 g C/L of each acid had a strong inhibitory effect that hampered yeast growth during the first hours of fermentation (lag phase of 144 h, Figure 4.17).

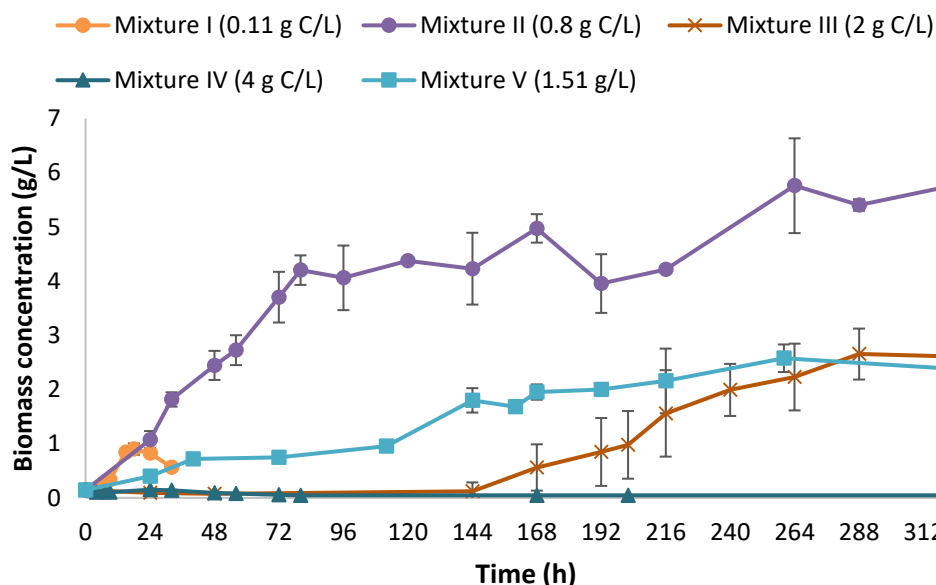


Figure 4.17. Cell dry weight during *Y. lipolytica* cultivation in mixtures of VFAs.

As shown in Table 4.13 (Publication V), a total VFAs concentration of approximately 10.6 g/L (mixture II) gave rise to the highest biomass growth concentration and similar biomass yield than 1.46 g VFAs /L (mixture I).

Table 4.13. *Y. lipolytica* growth parameters on VFAs mixtures.

Carbon source	Total VFAs conc. (g /L)	Fermentation time (h)	Lag phase (h)	Biomass conc. (g/L)	$Y_{x/s}$ (g/g)
I. Mix 0.11 g C/L each	1.45	15	4	0.89 ± 0.10	0.61 ± 0.13
II. Mix 0.8 g C/L each	10.6	240	9	5.75 ± 0.07	0.58 ± 0.07
III. Mix 2 g C/L each	26.5	380	144	3.15 ± 0.24	0.12 ± 0.2
IV. Mix 4 g C/L each	52.9	-	∞	n.d.	n.d.
V. Mix 1.51 g/L each	10.6	380	9	2.57 ± 0.25	0.25 ± 0.12

$Y_{x/s}$: growth yield; n.d.: not detected.

As mentioned before, these results differed from those reported by Fontanille and co-workers (2012) who concluded that VFAs concentration higher than 5 g VFAs/L implies inhibitory effects on *Y. lipolytica* growth. Biomass production obtained in mixtures I and II were 0.89 ± 0.10 g/L and 5.75 ± 0.07 g/L, respectively (Figure 4.17), corresponding to a biomass yields close to 0.6 g/g (Table 4.13).

The biomass yields were slightly higher than those obtained when individual VFAs were used (section 4.3.1.1). For instance, biomass yield (0.58 ± 0.07 g/g) attained in mixture II with 10.6 g/L of VFAs was higher than the obtained when acids were tested individually

at 4 g C/L that accounted for 10 g/L of VFAs or lower (0.54 g/g, Table 4.12). The results indicated that VFAs mixtures do not inhibit yeast growth but they could even increase biomass yield.

When using mixture III with 2 g C/L of each acid (26.5 g total VFAs/L), maximum biomass was 3.15 ± 0.24 g/L. This value was 45 % lower than the one obtained with mixture II (10.5 g total VFAs/L). The 5-fold lower biomass yield attained in mixture III evidenced the potential inhibitory effect of 26.5 g VFAs/L (Table 4.13). In fact, barely 7.6 g/L of the 26.5 g VFAs/L available were consumed after 380 h of culture (data not shown). Moreover, no yeast growth was observed when 4 g C/L of each VFA (mixture IV, Table 3.5) were used as carbon source. It is worth to mention that this mixture made up a total VFAs concentration of 52.9 g/L.

Considering the inhibitor potential of longer-chain VFAs reported in Section 4.3.1.1, not only VFAs mixture concentration must be considered since VFAs distribution in the mixture was also evidenced to be a crucial factor to be taken into account.

4.3.1.3. Effect of VFAs profile in VFAs consumption by *Y. lipolytica*

In order to determine the influence of the VFAs profile on *Y. lipolytica* growth, a mixture containing 1.51 g/L of each VFAs (mixture V, Table 3.5) was performed for comparison purposes with mixture II. Both mixtures (II and V) had the same total VFAs concentration of 10.6 g/L (corresponding to 5.6 g C/L), but different individual VFAs profiles (Table 3.5). In this manner, mixture V presented higher concentrations of long-chain VFAs than mixture II and consequently, lower short-chain VFAs concentration. Time courses of VFAs mixtures utilization (II and V) by *Y. lipolytica* are depicted in Figure 4.18 (Publication V).

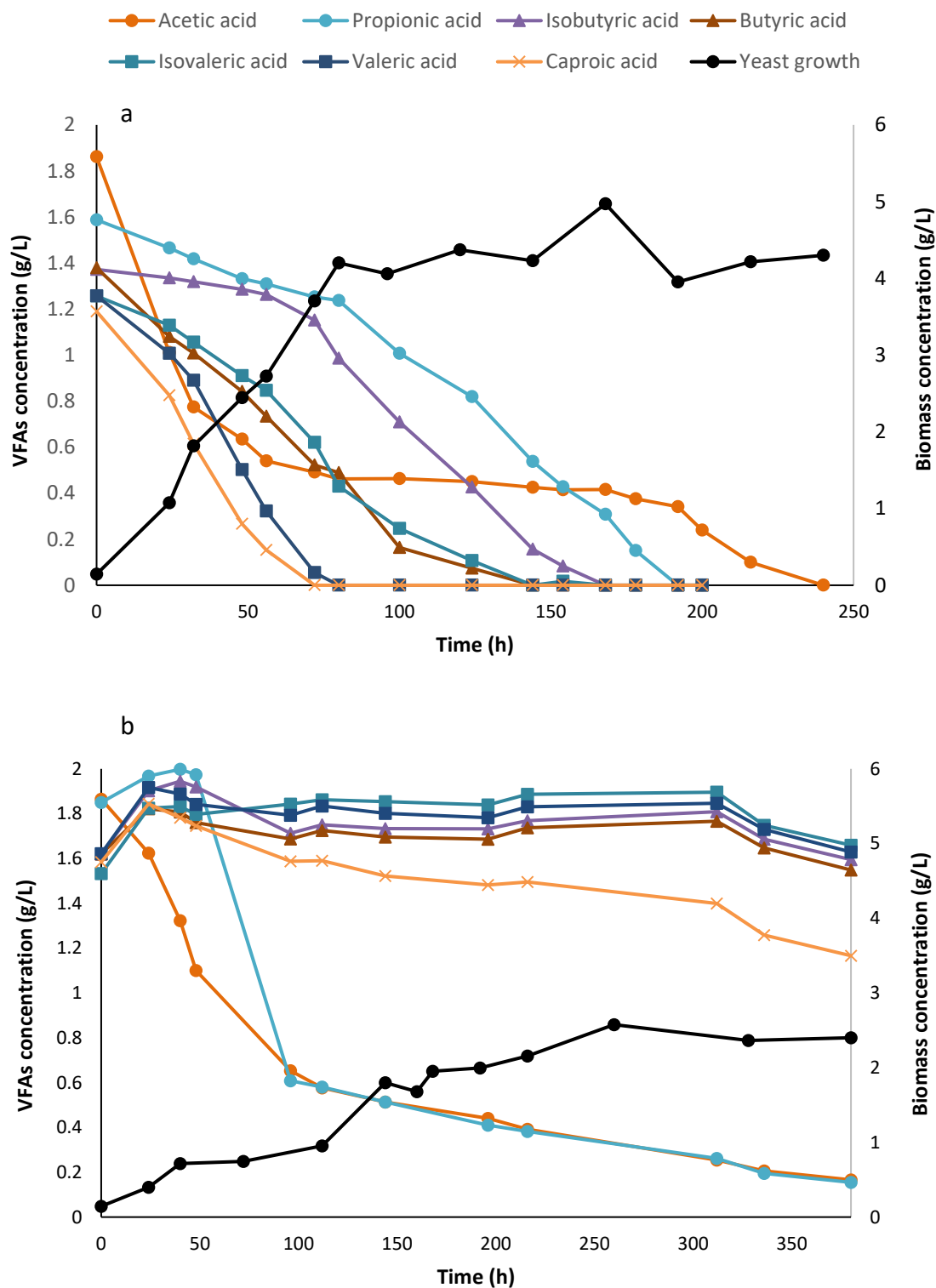


Figure 4.18. Time courses of VFAs utilization by *Y. lipolytica* on (a) mix II (0.8 g C/L each acid) and (b) mix V (1.51 g/L each acid).

Y. lipolytica was able to grow on mixture V, reaching 2.57 ± 0.25 g/L of biomass, which corresponded to 0.25 ± 0.12 g/g biomass yield (Table 4.13). These results were 2-fold lower than the obtained in mixture II, and thereby pointing at a preference for short-chain VFAs. This was in agreement with some studies that reported preferential utilization of short-chain VFAs instead of long-chain VFAs by OY (Fei et al., 2011a; Vajpeyi and Chandran, 2015).

When 0.8 g C/L of each acid were added (mixture II), 50 % of the available carbon sources were consumed after 56 h of fermentation and VFAs were totally exhausted after 250 h (Figure 4.18.a). In mixture V, however, barely 3.77 g/L of the 10.6 g/L available VFAs were consumed (75 % (w/w) of VFAs remained after 380 h).

These results confirmed that VFAs profile in the media had crucial effects on yeast growth and thus, not only total VFAs concentration should be evaluated. In this sense, it would be necessary to elucidate the particular effect of different VFAs profiles. According to previous experiments, predominance of short-chain VFAs in the medium triggered yeast growth (as it was the case of mixture II with 0.8 g C/L). Opposite, high proportion of long-chain VFAs (e.g. mixture V with 1.51 g/L of each acid) led to a decrease in biomass production, hampering yeast growth. These results agreed with previous studies using VFAs mixtures at different ratios where the highest biomass productions and yields were obtained when acetic acid was the predominant acid in the mixture (Fei et al., 2011a; Liu et al., 2017a). For instance, Fei and co-workers (2011a) reported biomass yields of 0.6 g/g with a mixture 8:1:1 of acetic, propionic and butyric, while 0.32 g/g was attained when the ratio was 4:3:3. The lack of reports dealing with the use of long-chain VFAs as carbon source for OY, makes necessary to progress in this field to clarify the chain-length effect.

Regarding the VFAs consumption in mixture II (Figure 4.18.b), *Y. lipolytica* showed preference for acetic acid over the other VFAs in the first 24 h of fermentation. During this period, acetic acid consumption rate (0.035 g/Lh) was significantly higher than the exhibited for the other acids (0.001-0.015 g/Lh). This was in agreement with Gao and co-workers (2017) who found that *Y. lipolytica* exhibited faster utilization rates of acetic over propionic and butyric acid. These authors attributed this fact to the different

metabolic routes after VFAs intake. More specifically, inside the cell acetic acid is cleaved directly into acetyl-CoA, unlike other acids which consumption is controlled by the rate of other biochemical transformations towards acetyl-CoA. In mixture II, more than 50 % of acetic, valeric and caproic acids were consumed in 32 h. It is worth noting that propionic and iso-butyric acids were moderately consumed until valeric and caproic acids were exhausted (72 h of fermentation), after which their utilization was accelerated. The fact that acetic acid consumption was practically stopped after valeric and caproic exhaustion may indicate that acetic acid consumption was coupled to long-chain VFAs uptake. The lack of literature in this topic renders the comparison with other studies very limited, highlighting the novelty of this investigation.

Results in this Thesis showed that *Y. lipolytica* sequentially utilized different types of VFAs. In the presence of long-chain VFAs, the metabolism of short-chain VFAs (propionic and iso-butyric acids) might be halted. Gao and co-workers (2017) reported a similar behavior when using C2 to C4 VFAs. Nevertheless, when long-chain VFAs were present at inhibitory concentrations (as it was the case for mixture V), these acids were practically unconsumed (consumption rates 0-0.002 g/Lh) while acetic and propionic acid showed the fastest consumption rates (0.013 g/Lh at 96 h of fermentation) (Figure 4.18.b). As previously explained in Section 4.3.1.1, it could be concluded that long-chain VFAs at high concentration have associated an inhibitory effect on yeast growth.

In this Thesis, *Y. lipolytica* sequentially utilized different types of VFAs when present in mixtures, showing preference for short-chain VFAs. Considering these results, the digestate obtained from AF of microalgae biomass (Section 4.1), where short-chain VFAs accounted for 60-70 % (w/w) of total VFAs, arises as an appropriated substrate for OY.

4.3.1.4. Real anaerobic digestate vs synthetic medium as substrate for *Y. lipolytica*

Several studies have investigated the conversion of synthetic VFAs into storage lipid by OY. However, only few studies have addressed the use of VFAs produced during AF. To

further evaluate the potential of VFA-rich digestate as substrate for OY, a real digestate from AF of microalgae biomass (without additional nutrients) was used as culture media for *Y. lipolytica*. A comparative assay between real AF digestate and synthetic medium with the same initial VFAs concentration and profile was also carried out to evaluate the effect that other components present in real digestate might exert on yeast growth.

Short-chain (C2-C4) VFAs were the predominant VFAs in real digestate (more than 75 % (w/w) of total carbon source) and small amounts of long-chain (C5-C6) were detected (around 25 % (w/w)) (Table 3.5). Despite the fact that VFAs distribution during AF might be affected by the feedstock characteristics and the fermentation conditions, this VFAs profile was considered conventional when using organic residues as feedstock (Magdalena et al., 2018a). As reported in Section 4.1, acetic acid was the most abundant VFA (32.7 % (w/w)), followed by propionic acid (18 % (w/w)). This profile is in agreement with values previously reported in literature where microalgae biomass or wastewater were fermented for VFAs production purposes (Magdalena et al., 2018b; Moretto et al., 2019).

Time courses for biomass growth and VFAs consumption on synthetic and real digestates are shown in Figure 4.19 (Publication V). Strikingly, biomass production in real digestate (3.17 ± 0.35 g/L) was 2.4-fold higher than in synthetic medium (1.34 ± 0.30 g/L) (Table 4.14).

Table 4.14. *Y. lipolytica* growth parameters on real and synthetic digestate

Carbon source	Total VFAs conc. (g/L)	Fermentation time (h)	Lag phase (h)	Biomass conc. (g/L)	$Y_{x/s}$ (g/g)
Real digestate	4.3	32	4	3.17 ± 0.35	0.74 ± 0.16
Synthetic digestate	4.3	168	9	1.34 ± 0.30	0.32 ± 0.14

Y_{x/s}: growth yield; n.d.: not detected

Despite having the same VFAs concentration and profile, the highest biomass yield was reached on real digestate (0.74 ± 0.16 g/g). These results pointed out to the presence of additional carbon sources, nutrient compounds (such as nitrogen, phosphorous, etc.) or by-products in the digestate that could enhance biomass production.

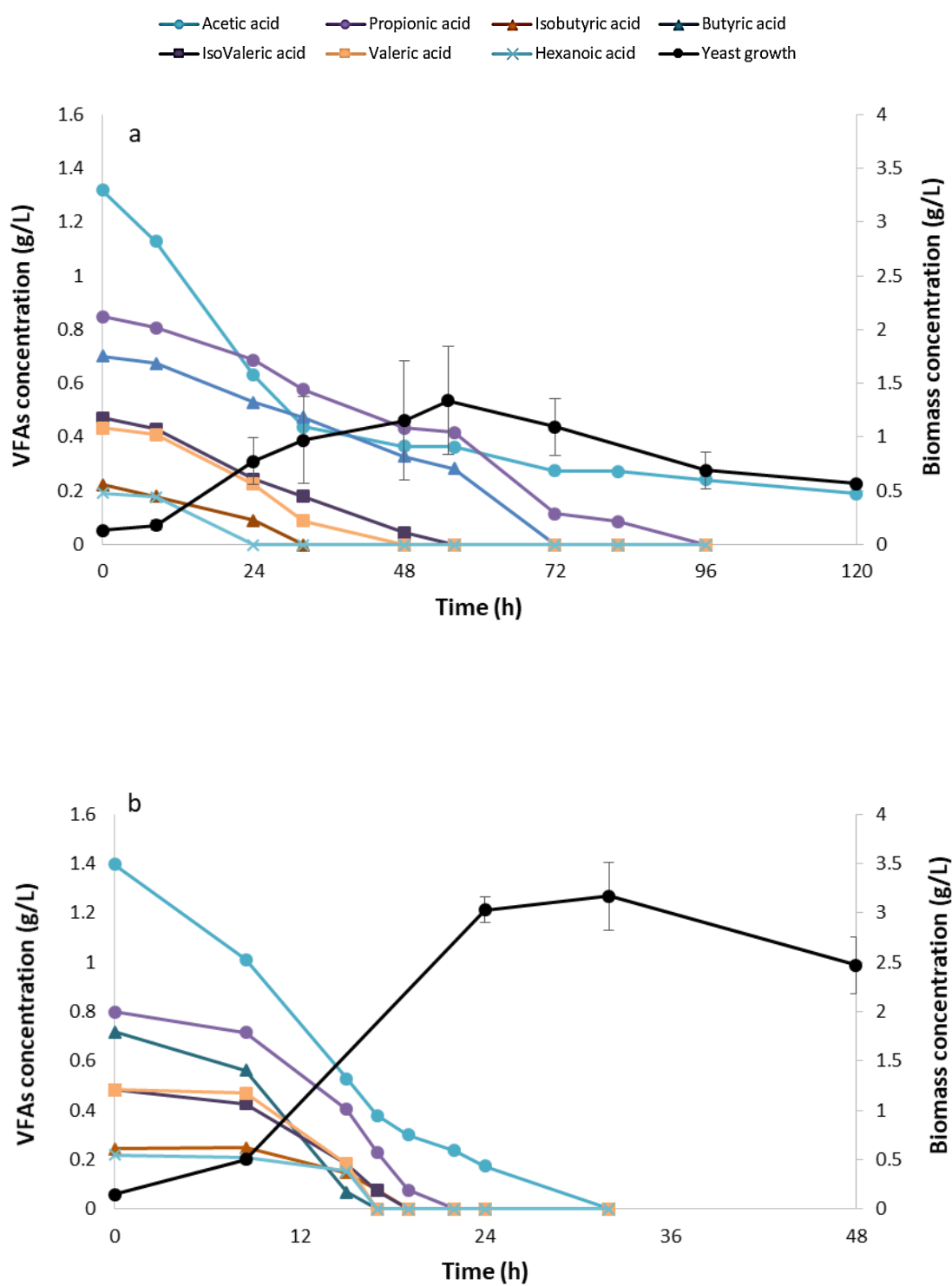


Figure 4.19. Time courses of *Y. lipolytica* growth and VFAs utilization on synthetic digestate (a) and real digestate (b).

Aiming to find additional carbon sources in the real digestate that were not present in synthetic media, initial and final organic matter in terms of COD was evaluated. At the initial fermentation time, 8.8 g COD/L and 7.0 g COD/L were determined for real and synthetic media, respectively. According to the stoichiometric calculation based on the composition of the real and synthetic media (Table 3.5), the organic matter due to the VFAs was 6.9 g COD/L in both media. These results demonstrated that VFAs accounted for 78.4 % (w/w) of the overall COD obtained in the real digestate and consequently, 21.6 % (w/w) of COD was due to other compounds. In this sense, 1.8 g COD/L (equivalent to the initial COD not given by VFAs) were detected at the end of fermentation when real digestate was employed as substrate. This demonstrated that the additional organic matter available in real digestate was not consumed by *Y. lipolytica*. Consequently, the biomass improvement in real digestate could not be simply attributed to the presence of additional carbon sources but to the presence of other nutrients, which may favor *Y. lipolytica* growth.

Other studies reported similar biomass production and yields when comparing *Y. lipolytica* growth on VFAs-rich real digestates with synthetic VFAs mixtures (around 0.85 g/g) (Gao et al., 2017). Notably, Xu and co-workers (2015) reported lower biomass yield (0.28 g/g) than the result obtained in the present Thesis, when using digestate from macroalgae fermentation. One possible explanation could be the higher nitrogen content (1.4 g NH_4^+ /L) in the digestate from microalgae in comparison with the digestate coming from macroalgae biomass (0.11 g NH_4^+ /L). During AF of protein-rich substrates (such is the case of microalgae), ammonium is released, as previously detailed in Section 1.1.2.1. In this particular case, the high nitrogen coming from the AF of protein-rich microalgae might have been beneficial for OY growth. Nitrogen constitutes an essential nutrient necessary for cell material synthesis (Papanikolaou and Aggelis, 2011a). It could be hypothesised that the high ammonium concentration in the broth could have boosted biomass growth.

While all VFAs were utilized simultaneously in both, real and synthetic digestates (Figure 4.19), different VFAs consumption rates were found. As a matter of fact, VFAs contained in real digestate were consumed faster than in synthetic medium. All VFAs in real

digestate were completely exhausted after 32 h of culture while 144 h were required for the consumption of 96 % (w/w) of VFAs in synthetic medium. In accordance with previous results, acetic acid showed the fastest utilization rates at early stages in both cases. Despite its high consumption rates, 11.5 % (w/w) of acetic acid remained after 120 h of process on synthetic medium (Figure 4.19.a) and it was exhausted on real digestate after 32 h of fermentation (Figure 4.19.b). The fact that acetic acid was not exhausted in synthetic medium could be explained by coupled consumption of acetic acid and long-chain VFAs (valeric and caproic). As previously discussed in Section 4.3.1.3, caproic and valeric acid exhaustion led to slower acetic acid consumption rate. These two acids were totally consumed before the short-chain VFAs probably due to the lower concentration of long-chain VFAs in the fermentation broth (far below inhibitory values).

Considering the potential that the real digestate showed over yeast growth when compared to the synthetic media, the positive effect of digestate was further evaluated at high VFAs concentration.

4.3.1.5. Supplemented digestate as substrate for *Y. lipolytica*

Real digestates were supplemented with VFAs to corroborate the positive effect of digestate on yeast growth. For comparison purposes with mixtures III and IV, the digestate was supplemented with synthetic acids but maintaining the same VFAs profile (Table 3.5). Real digestate containing 4.34 g VFAs/L was supplemented with synthetic VFAs up to 26.5 g VFAs/L (2 g C/L of each acid) and 52.9 g VFAs/L (4 g C/L of each acid). As depicted in Figure 4.20 (Publication V), *Y. lipolytica* grew in the medium supplemented at 2 g C/L of each acid but not on the digestate supplemented at 4 g C/L.

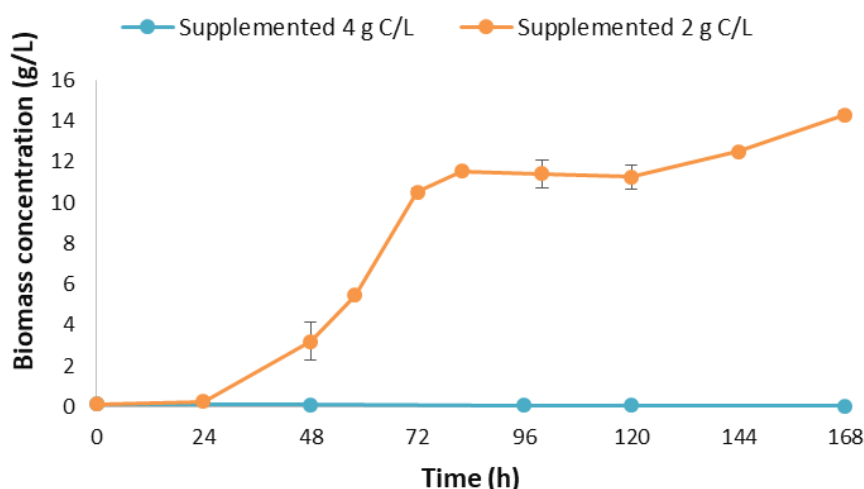


Figure 4.20. *Y. lipolytica* growth on supplemented digestates at 26.5 g/L VFAs (2 g C/L each acid) and 52.9 g/L VFAs (4 g C/L each acid).

Synthetic media containing VFAs at 26.5 g VFAs/L (mixture III) was shown to be a harsh substrate since *Y. lipolytica* growth was hampered during the first 144 h of fermentation (Figure 4.17). However, real digestate supplemented with VFAs up to 26.5 g VFAs/L was fermentable by *Y. lipolytica* after a short lag phase, indicating that the inhibitory effect of the acids was counterbalanced favouring yeast growth.

Biomass growth and yield in presence of 26.5 g VFAs/L varied significantly from 3.15 ± 0.24 g/L to 14.13 ± 0.21 g/L and from 0.12 ± 0.2 g/g to 0.56 ± 0.01 g/g, with mixture III and supplemented digestate up to 2 g C/L, respectively (Table 4.15, Section 4.3.1.3).

Table 4.15. *Y. lipolytica* growth parameters on supplemented digestate.

Carbon source	Total VFAs conc. (g/L)	Fermentation time (h)	Lag phase (h)	Biomass conc. (g/L)	$Y_{x/s}$ (g/g)
Supplemented 2 g C/L	26.5	100	24	14.13 ± 0.21	0.56 ± 0.01
Supplemented 4 g C/L	52.9	-	∞	n.d.	n.d.

$Y_{x/s}$: growth yield; n.d.: not detected

VFAs were exhausted after 100 h of fermentation in supplemented digestate containing 26.5 g VFAs/L. By contrast, 380 h were needed to completely consume all VFAs in mixture III (data not shown). Attained biomass yield with real digestate supplemented

to 26.5 g VFAs/L (0.56 ± 0.01 g/g) was comparable to those obtained in mixtures I and II containing same profile but 1.45 g total VFAs/L and 10.6 g total VFAs/L (around 0.6 g/g, Table 4.13). This fact confirmed that the inhibitory effect caused by high concentrations of VFAs was overcome.

These results pointed out to the presence of some compounds (*e.g.* nutrients) in the real digestate that could enhance yeast growth. The strong inhibitory effect of VFAs at high concentrations was diminished when using real digestate as substrate. This fact revealed that real digestates can be not only successfully used as possible fermentation media at high VFAs concentrations but also contain some other compounds beneficial for yeast growth.

The importance of several trace nutrients (such as nitrogen, phosphorus, zinc, magnesium, iron, etc.) during yeast fermentation have been widely reported (Madani et al., 2017). Lack of essential nutrients can repress cell growth and the availability of trace elements also has a strong influence in yeast metabolism driving pathways towards different products (Madani et al., 2017). In this context, besides being a carbon-rich medium, AF effluent has been reported to be a useful nutrients source (including nitrogen, phosphorous, magnesium, iron, sulfur, etc.) for microbial cultivation (Bjornsson et al., 2013; Flores-Alsina et al., 2016). For instance, Xu and co-workers (2015) reported that macroalgae-AF effluent was an appropriate source of micro- and macronutrients for *C. curvatus* growth during oleaginous fermentation. Despite the positive effect of nutrient limitation on lipid accumulation reported when using sugar-based substrates, when VFAs are employed as carbon source this effect is unclear. For instance, Gong and co-workers (2015) demonstrated not only a more efficient growth but a higher lipid accumulation when *C. curvatus* was cultured in acetic acid under nitrogen-rich conditions when compared with nitrogen-limited conditions. Therefore, the suitable nutrients ratio to optimize lipid accumulation in OY should be determined taking into account the substrate used.

Considering that most OY are unable to metabolize VFAs, the ability of *Y. lipolytica* to grow on digestate using VFAs as unique carbon source was very remarkable. Besides, the positive effect of certain compounds in the real digestate to overcome VFAs

inhibitory effect was also a very positive asset. All these results evidenced the suitability of the AF digestate as a substrate for OY, allowing the integration of two bioprocesses (AF and oleaginous fermentation) for the production of MO. As mentioned in Section 1.2.3.1, limited information is available about the conversion of VFAs-rich digestates into lipids by OY and just a narrow range of microorganisms with this capacity have been studied (mostly *Cryptococcus* and *Y. lipolytica*). Therefore, to optimize MO production, it is essential the search of new OY strains able to efficiently metabolize VFAs and channel these acids to lipid accumulation.

4.3.2. Screening of OY

In this Thesis, different OY, often utilized in the literature for MO production using sugar carbon sources (*i.e.* *C. curvatus*, *L. lipofer*, *R. toruloides*, *W. saturnus* and *Y. lipolytica*), were explored for their ability to metabolize VFAs-rich digestates in Publication VI. Specifically, yeasts growth and lipid accumulation capacity were evaluated when cultivated on VFAs from AF of *C. vulgaris* biomass (produced as detailed in Section 4.1) at three different concentrations (5, 10 and 15 g/L). The FA composition of the produced lipids was determined and compared to that produced on glucose (model substrate).

4.3.2.1. Yeasts growth on VFAs containing digestate

Similar to the AF effluent from microalgae employed in Section 4.3.1, the predominant acids in the VFAs-rich effluent utilized for yeast screening were short-chain VFAs (C2 to C4), accounting for 75 % (w/w) of the total VFAs (Table 3.5). The VFAs digestate was composed by 32.6, 24.5 and 12.9 % (w/w) for acetic, propionic and butyric acid, respectively. This is a common VFAs profile given that during AF the long-chain fatty acids are predominantly degraded to short-chain VFAs (Llamas et al., 2019; Moretto et al., 2019). According to the results previously showed in Section 4.3.1, this VFAs profile was considered suitable for yeast growth.

Time courses of cell growth of OY cultivated on digestate at different VFAs initial concentrations are displayed in Figure 4.21 (Publication VI).

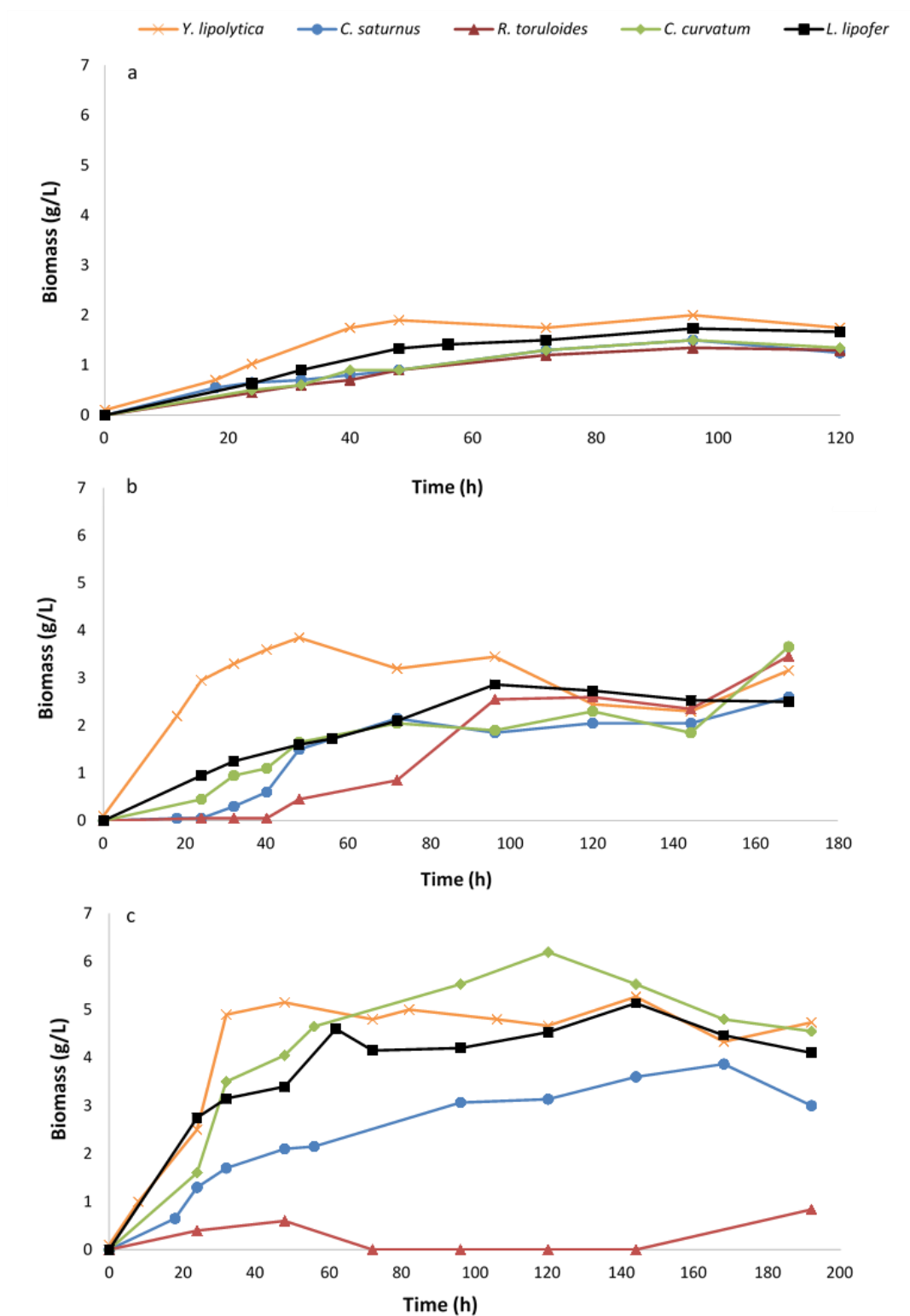


Figure 4.21. Time courses of five OY growth on digestates containing VFAs at: 5 (a), 10 (b) and 15 (c) g/L. Standard deviations were not included since their values were below 5 %.

C. curvatus and *Y. lipolytica* are already well known for their ability to metabolize VFAs (Fontanille et al., 2012; Kolouchová et al., 2015; Huang et al., 2016; Liu et al., 2017a) and results presented herein confirmed these previous findings. In addition, *W. saturnus* and *L. lipofer* were able to grow on VFAs in all cases, while *R. toruloides* was completely inhibited in the medium with 15 g VFAs/L.

Biomass production and growth rates are shown in Table 4.16 (Publication VI). In all cases, apart from *R. toruloides*, an increase in VFAs concentration resulted in higher biomass production.

Table 4.16. Yeast growth parameter attained using AF digestates at 5, 10 and 15 g VFAs/L.

Yeast strain	5 g VFAs/L		10 g VFAs/L		15 g VFAs/L	
	X_m (g/L)	μ (h ⁻¹)	X_m (g/L)	μ (h ⁻¹)	X_m (g/L)	μ (h ⁻¹)
<i>Y. lipolytica</i>	1.9 ± 0.7	0.04	3.5 ± 0.6	0.11	4.9 ± 0.3	0.14
<i>W. saturnus</i>	1.6 ± 0.1	0.02	2.2 ± 0.2	0.04	3.4 ± 0.4	0.05
<i>R. toruloides</i>	1.2 ± 0.2	0.01	2.5 ± 0.3	0.01	0.8 ± 0.0	0.01
<i>C. curvatus</i>	1.7 ± 0.3	0.01	2.4 ± 0.6	0.02	5.2 ± 0.3	0.09
<i>L. lipofer</i>	1.6 ± 0.2	0.03	2.7 ± 0.2	0.02	4.6 ± 0.4	0.07

Abbreviations: X_m : maximum biomass concentration; μ : growth rate.

When VFAs content was 5 g/L, no important differences were observed among the strains tested in terms of cell growth (Figure 4.21.a). The maximum biomass production ranged from 1.2 ± 0.2 g/L to 1.9 ± 0.7 g/L. *Y. lipolytica* exhibited the highest specific growth rate (0.04 h⁻¹) while *R. toruloides* showed the lowest (0.01 h⁻¹). These results indicated that there was no inhibitory effect on yeast growth when digestate was diluted to 5 g VFAs/L, which agreed with the values reported in Section 4.3.1. This fact was also supported by previous studies that concluded that keeping VFAs concentration below 5 g/L, acid inhibitory effect was avoided (Fei et al., 2011a; Fontanille et al., 2012; Liu et al., 2017a).

When VFAs concentration in the medium was 10 g/L, significant differences among strains were found (Figure 4.21.b). More specifically, in terms of biomass production, *Y. lipolytica* reached 3.5 ± 0.6 g/L, followed by *L. lipofer* and *C. curvatus* that attained 2.7 ± 0.2 g/L and 2.5 ± 0.3 g/L, respectively. The lowest biomass production was reached by

W. saturnus (2.2 ± 0.2 g/L). As shown in Table 4.16, and following the same trend that at 5 g VFAs/L, *Y. lipolytica* exhibited the highest specific growth rate (0.11 h^{-1}) and practically no lag phase in presence of 10 g VFAs/L, reaching the maximum biomass production after 48 h. Oppositely, *R. toruloides* displayed the lowest specific growth rate (0.02 h^{-1}) and a 40 h-long lag phase, requiring 96 h to reach the maximum biomass production. These features indicated that an increase in VFAs concentration resulted in an inhibitory effect of VFAs on this strain.

When VFAs concentration was 15 g/L, 5.2 ± 0.3 , 4.9 ± 0.3 and 4.6 ± 0.4 g/L of biomass were observed in *C. curvatus*, *L. lipofer* and *Y. lipolytica*, respectively (Figure 4.21.c, Table 4.16). In terms of specific growth rate, *Y. lipolytica* exhibited the fastest growth, achieving the maximum biomass production in 24 h. *C. curvatus* and *L. lipofer* specific growth rates (0.07 h^{-1} and 0.09 h^{-1} , respectively) were lower than that achieved by *Y. lipolytica* (0.14 h^{-1}). While the inhibitory effect of high VFAs concentrations (>5 g/L) on *Y. lipolytica* and *C. curvatus* growth has been widely reported (Fei et al., 2011a; Fontanille et al., 2012; Huang et al., 2016; Liu et al., 2017a), these strains together with *L. lipofer* were not affected the VFAs concentration used in this Thesis. This feature evidenced a high tolerance of these strains to VFAs. Increasing VFAs concentration from 5 g/L to 15 g/L resulted in an important decrease in *R. toruloides* and *W. saturnus* biomass yields, indicating an inhibitory effect of VFAs on these strains. Specifically, substrate exhaustion (data not shown) and maximum biomass production occurred after 144 h in *W. saturnus* when VFAs content in the medium was 15 g/L. In the same way, at this VFAs concentration, *R. toruloides* was totally inhibited and no growth was detected even after 144 h of incubation.

Biomass yields attained during this Thesis (Publication VI) are summarized in Table 4.17.

Table 4.17. Biomass yields (g/g) attained using AF digestates at 5, 10 and 15 g VFAs/L as carbon source.

Yeast strain	5 g VFAs/L	10 g VFAs /L	15 g VFAs /L
<i>Y. lipolytica</i>	0.37	0.35	0.33
<i>W. saturnus</i>	0.32	0.22	0.22
<i>R. toruloides</i>	0.24	0.25	0.06
<i>C. curvatus</i>	0.34	0.30	0.35
<i>L. lipofer</i>	0.32	0.30	0.30

Standard deviations were not included since their values were below 5 %.

Y. lipolytica, *C. curvatus* and *L. lipofer* biomass yields were not affected, remaining above 0.3 g/g regardless of the VFAs concentration in the medium. *Y. lipolytica* and *C. curvatus* have been previously investigated using VFAs produced from waste streams and their robustness facing inhibitors during fermentation have been also described (Atasoy et al., 2018). For instance, VFAs produced via AF of rice straw hydrolysates or macroalgae biomass were used as a carbon source for *C. curvatus* (Xu et al., 2015; Park et al., 2017). These investigations reported similar biomass yields than the ones determined in this Thesis (0.3 g/g). Likewise, *Y. lipolytica* exhibited biomass yields greater than 0.3 g/g when VFAs-rich digestate produced from food-waste was employed as carbon source (Gao et al., 2020). Interestingly, biomass production and yields obtained by *C. curvatus*, *L. lipofer* and *Y. lipolytica* in this Thesis were very close to those attained when glucose or glycerol are used as carbon source (Papanikolaou and Aggelis, 2011a). Thus, it could be confirmed the suitability of using VFAs-based substrates as alternative to sugar-based substrates for OY growth with *Y. lipolytica*, *C. curvatus* and *L. lipofer*.

On the contrary, in the case of *W. saturnus*, the biomass yields decreased from 0.32 g/g to 0.22 g/g when VFAs concentration increased from 5 g/L to 15 g/L. The low tolerance of this strain to increasing VFAs concentration was not expected since *W. saturnus* has been identified as promising candidate due its ability to utilize many sugar-based carbon sources withstanding the presence of inhibitors (Sitepu et al., 2014). These authors also demonstrated *W. saturnus* tolerance to acetic acid at low concentration (2.5 g/L). However, the effects of longer-chain VFAs and higher concentrations were not evaluated.

In this Thesis, *R. toruloides* was not able to grow when the VFAs content was 15 g/L, but no differences were found when grown at 5 g/L and 10 g/L. Although similar biomass yields were obtained at these VFAs concentrations (0.24 g/g and 0.25 g/g, Table 4.17), significant differences in specific growth rates and lag phases were observed. *R. toruloides* sensitivity to inhibitors has been previously reported. For instance, Huang and co-workers (2016) showed that acetic acid as sole carbon source at concentration higher than 10 g/L caused the complete inhibition of this OY growth. Similarly, even when xylose or glucose were employed as carbon sources, acetic acid was determined to be a

strong lignocellulosic-derived inhibitor on *R. toruloides* (Chen et al., 2009; Hu et al., 2009).

All these results proved that the VFAs-rich digestate was inhibitory for the growth of *W. saturnus* and *R. toruloides*, but not for *Y. lipolytica*, *C. curvatus* and *L. lipofer*. Therefore, the inhibitory effect of VFAs was demonstrated to be specie and probably strain specific.

4.3.2.2. Microbial oil production

Lipid accumulation

In order to assess the oleaginous capacity of the studied strains on VFAs-rich digestate, yeast lipid content was quantified at the end of the fermentation time when carbon source was completely exhausted and maximum yeasts biomass was achieved.

From a viewpoint of biotechnological applications, the most important feature of OY is the high lipid accumulation that ranged from 7.4 % DW to 36.9 % DW in the studied strains (Figure 4.22, Publication VI).

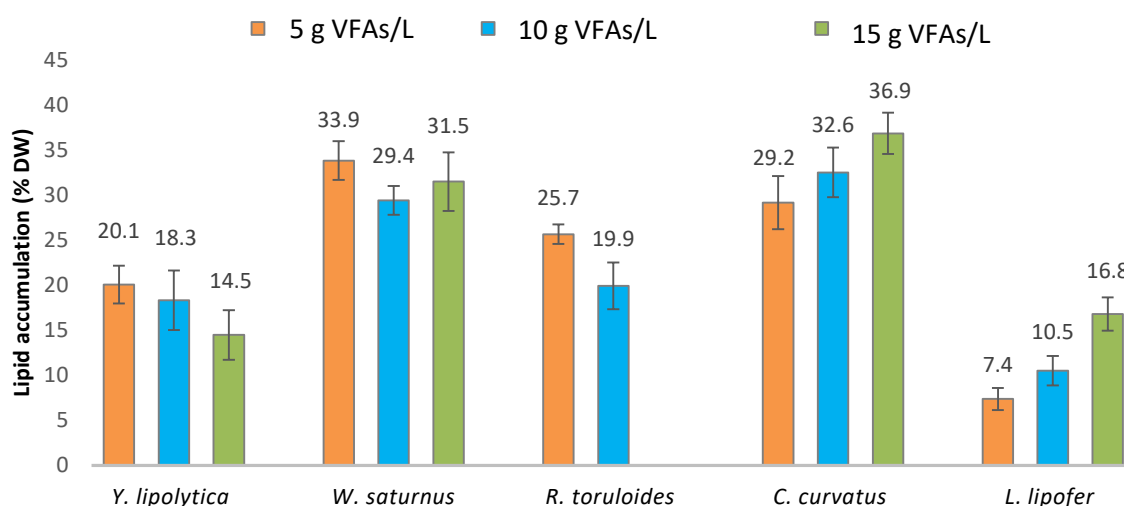


Figure 4.22. Lipid content (% DW) of the five selected strains cultivated on digestate at different concentrations of VFAs.

These results confirm that lipid content in yeast varies among strains (Papanikolaou and Aggelis, 2011a). As presented in Section 1.2, oleaginous microorganisms are known to accumulate storage lipids above 20 % DW. In this Thesis, *W. saturnus* and *C. curvatus* displayed the highest lipid accumulation, reaching values of around 29.2-36.9 % DW regardless of the VFAs concentration. These lipidic contents confirmed the oleaginous character of these two strains.

On the other hand, *L. lipofer* exhibited the lowest lipid production (7.4-16.8 % DW). Lipid accumulation in *R. toruloides* and *Y. lipolytica* was negatively affected by the increase in VFAs concentration. For instance, lipid accumulation in *Y. lipolytica* decreased from 20.1 % DW to 14.5 % DW when VFAs concentration increased from 5 g/L to 15 g/L. In the case of *R. toruloides*, a decrease in lipid accumulation from 25.7 % DW to 19.9 % DW was observed when VFAs concentration increased from 5 to 10 g/L.

This indicated that apart from cell growth, lipid accumulation pathway seemed to be also affected in *R. toruloides* and *Y. lipolytica* in presence of high VFAs concentration. The slight decrease in biomass yields along the increasing VFAs concentration gave rise to a lipid yield decrease in *Y. lipolytica* (Table 4.18). The drop in lipid content exhibited by *Y. lipolytica* evidenced that the carbon source was channeled towards other products instead of lipids (Papanikolaou et al., 2002; Fickers et al., 2005; Sagnak et al., 2018). The decrease in lipid accumulation together with the growth inhibition suggested that lipid production takes place during primary metabolic growth, according to the *ex-novo* lipid accumulation mode (Llamas et al., 2019). In *W. saturnus*, *C. curvatus* and *L. lipofer*, lipids content either remained constant or increased when VFAs concentration of the medium increased. For instance, the lipid content of *W. saturnus* slightly varied from 29.4 % DW to 33.9 % DW. On the other hand, *C. curvatus* and *L. lipofer* exhibited a significant increase in lipid content from 29.2 % DW to 36.9 % DW and from 7.4 % DW to 16.8 % DW when VFAs concentration increased from 5 g/L to 15 g/L.

Considering the high quantities of ammonium released during microalgae AF (explained in Section 1.1.2.1), the C/N ratio in the digestate used in the current Thesis was very low (*i.e.* C/N=3.8) (Table 3.5). Although the metabolic pathways involved in VFAs assimilation by OY and factors involved in lipid accumulation are still unclear, the results obtained

herein supported the hypothesis that lipid biosynthesis occurred via *ex-novo* pathway. In this case, lipid accumulation takes place during the primary metabolic growth, regardless of nutrient limitations (Vasiliadou et al., 2018; Llamas et al., 2019). Previous works have also reported high lipid yields in *C. curvatus* and *R. toruloides* growing on nitrogen-rich media having acetic acid as carbon source (Gong et al., 2015; Huang et al., 2016).

As summarized in Table 4.18, high lipid yields per consumed VFAs were obtained by *C. curvatus*, *W. saturnus* and *Y. lipolytica* (0.07-0.13 g/g), with *C. curvatus* showing the highest conversion yield when cultivated on 15 g VFAs/L (0.13 g/g). These lipid yields were in good agreement with the results obtained by Xu and co-workers (2015) that reported 0.15 g/g when *C. curvatus* was grown in VFAs-rich effluent from macroalgae AF at concentrations of 10 g VFAs/L using sequential-batch mode cultivation.

Table 4.18. Lipid yields (g/g) attained using AF digestates with 5, 10 and 15 g VFAs/L.

Yeast strains	5 g VFAs/L	10 g VFAs/L	15 g VFAs/L
<i>Y. lipolytica</i>	0.08	0.06	0.05
<i>W. saturnus</i>	0.11	0.07	0.07
<i>R. toruloides</i>	0.06	0.05	n.d
<i>C. curvatus</i>	0.10	0.10	0.13
<i>L. lipofer</i>	0.02	0.03	0.05

Standard deviations were not included since their values were below 5 %.

Abbreviations: n.d. Non detected

Y. lipolytica presented a lipid yield of 0.08 g/g when cultivated on 5 g VFAs/L but this value decreased with the increase of VFAs concentration in the medium. The rest of the yeasts exhibited low lipid yield from VFAs ranging from 0.02 g/g to 0.06 g/g. Gao and co-workers (2020) showed lipid yields of 0.14 g/g when *Y. lipolytica* was grown on VFAs obtained from AF of fruits and vegetables after optimization of the culture conditions. Thus, process optimization could be essential to increase the lipid yields obtained herein.

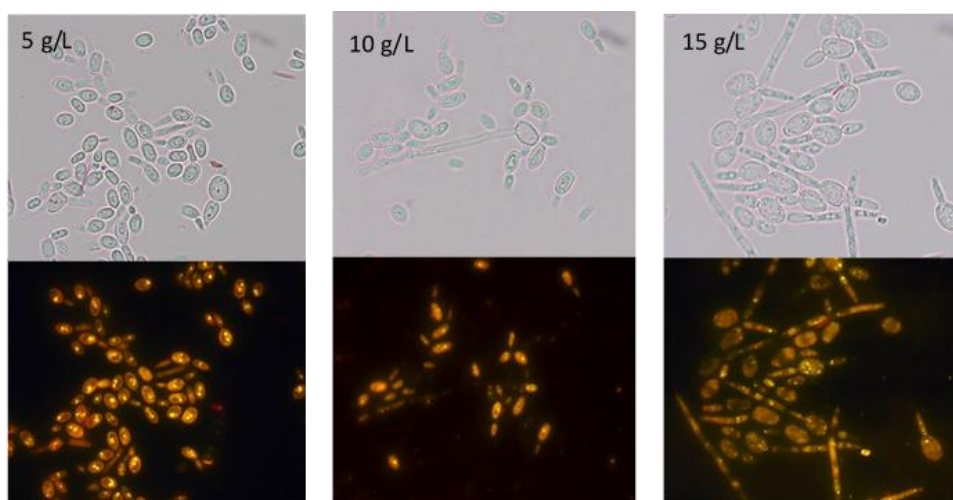
The maximum theoretical lipid yield when using glucose or glycerol as carbon sources are 0.32 g/g and 0.30 g/g, respectively (Papanikolaou and Aggelis, 2011a). In this Thesis,

the experimentally obtained lipid yields were rarely higher than 0.20 g/g. Therefore, the improvement of the fermentation process is of utmost importance to maximize lipid production in yeasts. As deeply described in Publication IV, optimization of process configurations and culture conditions, genetic engineering as well as adaptive evolution are interesting strategies to overcome challenges associated to lipid production in OY. For instance, some studies summarized in Table 1.4 (Section 1.2.3.2) have reported lipid yield improvement by applying operational mode strategies such as two-stage batch or two-stage feed-batch feeding mode. Likewise, adaptive evolution could be an efficient tool to overcome VFAs toxic effect on yeasts. This method has been used not only to improve the environmental stress resistance but also to increase lipid accumulation of strains obtained through synthetic biology (Ledesma-Amaro et al., 2016; Díaz et al., 2018). An evolved *Y. lipolytica* strain obtained after random mutagenesis exhibited 87 % DW lipid content compared with 71 % DW and 55 % DW obtained when only the evolved strain and wild type were used (Liu et al., 2015).

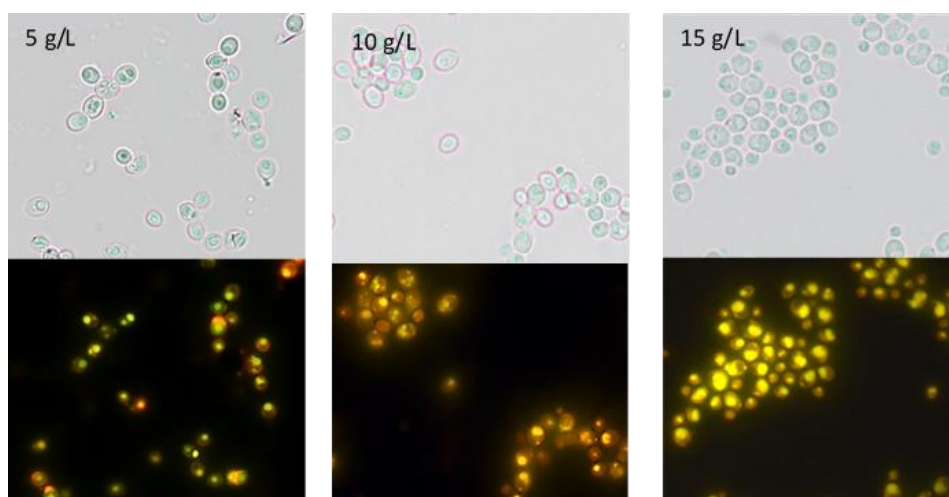
Morphology of lipid bodies

Fluorescence microscopy pictures were taken at different fermentation times to evaluate the LB within the yeast cells (Figure 4.23, Publication VI).

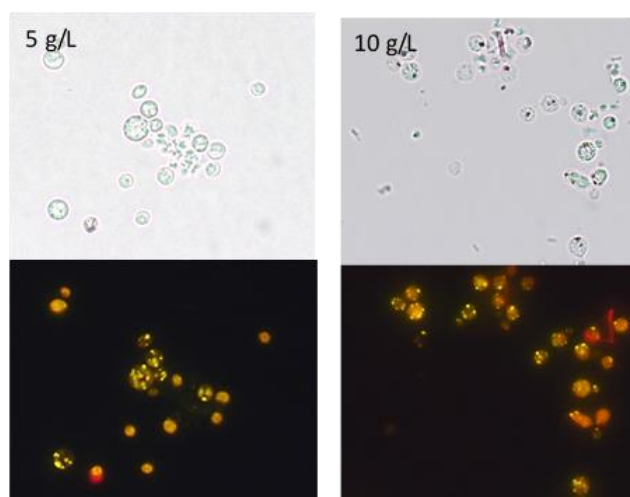
Y. lipolytica (a)



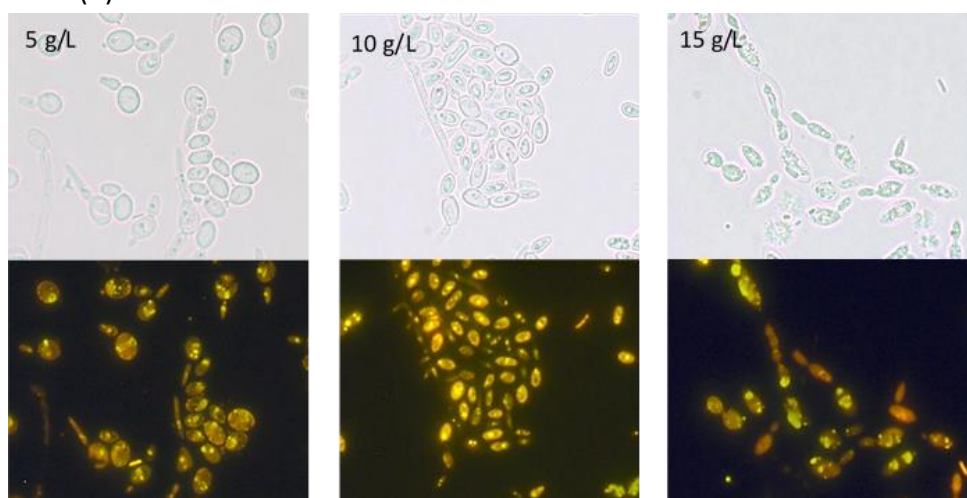
W. saturnus (b)



R. toruloides (c)



C. curvatus (d)



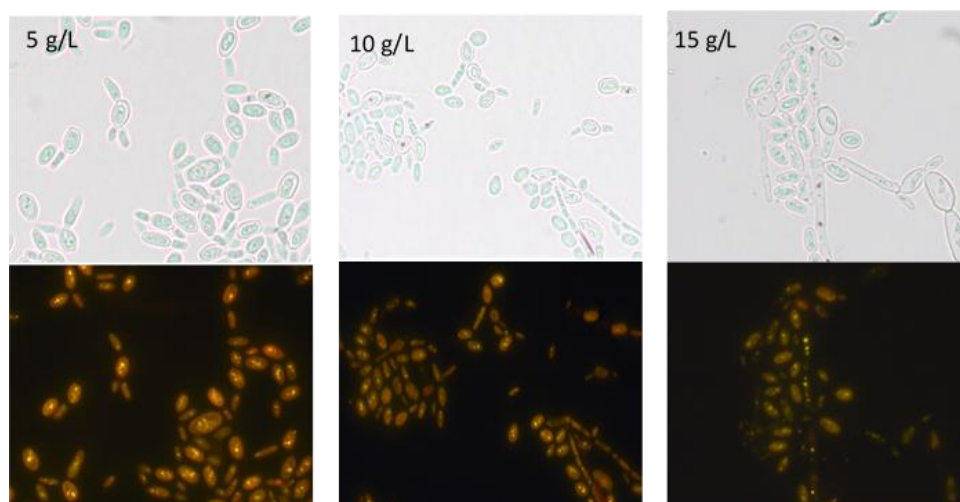
L. lipofer (e)

Figure 4.23. Lipid bodies morphology in *Y. lipolytica* (a), *W. saturnus* (b), *R. toruloides* (c), *C. curvatus* (d) and *L. lipofer* (e) cultivated in 5, 10 and 15 g VFAs/L, after staining with the Nile Red. Upper: optical microscope, bottom: fluorescence microscope ($\times 1000$)

As it can be seen in Figure 4.23, *L. lipofer* did not practically show lipids inside the cell, while *Y. lipolytica*, *W. saturnus*, *R. toruloides* and *C. curvatus* presented lipid droplets of diverse sizes indicating that the amount, localization and morphology of LB widely varied even among closely related species (Arous et al., 2017; Ayadi et al., 2018). *W. saturnus* cells had spherical and voluminous LB in the middle of the cell, whereas *Y. lipolytica* presented one or two small and central droplets per cell. *C. curvatus*, *R. toruloides* and *L. lipofer* also presented several small LB along the cell. The biggest LB, shaping more than 30 % of the cells (Figure 4.23) were found in *W. saturnus* grown on VFAs at 10 g/L and 15 g/L, and in *C. curvatus* grown on 15 g VFAs/L.

Morphological changes were observed in *Y. lipolytica*, *C. curvatus* and *L. lipofer* when the VFAs concentration increased up to 10 g/L or 15 g/L. Usually, pseudomycelia formation takes places when environmental conditions stress yeast cells (Rakicka et al., 2015). Important factors regulating the dimorphic transitions are pH, carbon and nitrogen exhaustion, citrate formation and anaerobic stress (Barth and Gaillardin, 1997; Bellou et al., 2014). The formation of pseudomycelia, that has been determined antagonist to lipogenic stage (Rakicka et al., 2015), could explain the low lipid content in *Y. lipolytica* and *L. lipofer* in presence of high VFAs concentration.

Fatty acid composition

The FA composition of total lipid was quantitatively determined for each strain cultivated on the three VFAs concentrations. The obtained FA profiles were compared to the composition produced on synthetic medium containing 15 g/L glucose (Table 4.19, Publication VI).

Similar FA composition was observed in all the strains. This confirmed that consumed VFAs were mainly devoted to biosynthesis of reserve lipid. The main FA were oleic acid (C18:1) (30-67 % w/w), palmitic acid (C16:0) (7-19 % w/w) and linoleic acid (C18:2) (9-29 % (w/w)). The fact that all the strains contained C16 and C18 FA at high ratio (up to 87 % (w/w)) revealed the high similarity of these lipids with vegetable oils traditionally used in industry (see application summarized in Table 1.3, Section 1.2). Similar results were shown by other authors when comparing the composition of vegetable oils and MO in terms of FA (Kraisintu et al., 2010; Viñarta et al., 2016).

Comparing the FA profile obtained with glucose with that observed when VFAs were used as carbon source, a decrease in the content of C16:0 and C16:1 was shown in the latter case. It is worth highlighting the presence of odd-chain FA (C17:0), being unusually produced by OY grown in conventional substrates (Park et al., 2018). C17:0 accounted for the 6.4 %-6.8 % (w/w) of total FA when *Y. lipolytica*, *C. curvatus* and *L. lipofer* were grown on VFAs-rich digestates at 5, 15 and 5 g VFAs/L, respectively. Nevertheless, no C17:0 was detected in any of the yeast strains when grown on glucose (Table 4.19). Odd-chain FA, rarely found in the nature, have been considered precursors for exceptional lipids (Ledesma-Amaro and Nicaud, 2016). The remarkable percentage of this FA, could be explained by the VFAs composition in the substrate since propionic acid utilization by yeast may lead to a high content of odd-numbered FAs (Park et al., 2018). This is in agreement with the fact that propionic acid accounted for 24.5 % (w/w) of the total VFAs in the used digestate.

Table 4.19. FA composition of the lipid produced by the different yeast strains grown on VFAs-rich digestates and glucose.

Strain	Substrate	Relative amount of FA (% w/w)										
		C14:0	C14:1	C16:0	C16:1	C16:2	C17:0	C18:0	C18:1 n-9	C18:2	C18:3 α	others*
<i>Y. lipolytica</i>	15 g Glu/L	0.3	nd	13.3	17.0	0.5	nd	2.7	58.5	7.8	nd	nd
	5 g VFAs/L	2.3	1.7	13.6	4.8	1.4	6.4	2.7	35.6	14.5	2.4	14.5
	10 g VFAs/L	1.7	2.2	14.2	2.9	2.6	4.0	3.7	42.7	16.3	5.4	4.3
	15 g VFAs/L	0.6	0.7	7.1	6.1	1.9	3.9	2.0	52.6	21.6	2.0	1.4
<i>W. saturnus</i>	15 g Glu/L	1.5	nd	25.6	7.0	Nd	nd	1.9	49.4	12.5	2.2	0.3
	5 g VFAs/L	0.3	0.5	15.8	1.2	0.9	3.0	4.0	68.8	1.9	1.4	3.6
	10 g VFAs/L	1.5	2.6	13.5	6.9	7.8	nd	25.2	32.4	8.8	1.3	0.5
	15 g VFAs/L	0.5	4.0	15.4	5.4	7.3		23.1	30.1	9.4	3.7	1.1
<i>R. toruloides</i>	15 g Glu/L	1.7	0.2	30.4	3.8	0.1	nd	2.5	50.7	9.4	1.2	0.2
	5 g VFAs/L	2.3	2.2	14.7	6.2	2.4	3.3	3.2	39.3	14.9	5.8	5.6
	10 g VFAs/L	1.3	0.7	9.7	4.6	5.1	0.5	5.8	33.9	29.3	6.4	2.3
	15 g VFAs/L	-	-	-	-	-	-	-	-	-	-	-
<i>C. curvatus</i>	15 g Glu/L	1.3	1.0	10.9	7.6	0.6	nd	3.0	52.9	22.0	nd	0.7
	5 g VFAs/L	3.6	3.3	18.6	8.4	5.3	nd	3.6	32.6	18.5	nd	5.9
	10 g VFAs/L	1.4	0.8	16.4	3.8	1.4	nd	4.5	66.7	3.9	nd	2.1
	15 g VFAs/L	1.5	2.0	12.9	4.5	2.1	6.8	6.6	52.6	9.7	nd	1.4
<i>L. lipofer</i>	15 g Glu/L	0.1	0.1	12.8	21.4	0.3	nd	1.8	55.0	8.3	nd	nd
	5 g VFAs/L	2.7	7.0	14.8	4.7	2.0	6.4	3.9	46.7	13.3	nd	6.4
	10 g VFAs/L	0.6	0.7	9.1	8.7	2.3	5.7	1.6	55.6	13.2	4.3	0.5
	15 g VFAs/L	1.7	0.7	12.5	4.8	1.5	4.2	5.3	52.8	10.0	3.2	3.2

Abbreviations: nd:non detected

5. CONCLUSIONS



5. Conclusions

1. When using microalgae biomass as feedstock, a pretreatment step prior to AF was essential for the substrate bioavailability enhancement. Among the different evaluated pretreatments (thermal, carbohydrase and protease), proteolytic pretreatment resulted the most efficient in terms of both organic matter solubilization and bioconversion into VFAs. This fact evidenced that protein fraction should be hydrolyzed prior to AF, confirming the key role of proteins in this bioprocess. The use of a macromolecule-specific pretreatment on microalgae biomass appeared to be decisive in VFAs production yields during AF.
2. Temperature had a strong effect on AF of alcalase-pretreated *C. vulgaris* in CSTRs. Despite employing temperature-adapted inocula, psychrophilic fermentation temperatures (25 °C) promoted higher VFAs accumulation than thermophilic temperatures (55 °C). The enhancement in the hydrolytic step usually associated to high temperatures was found negligible in this thesis because pretreated microalgae was used as substrate. Low temperature AF slowed down methanogenic activity, causing higher COD accumulation as VFAs in the reactor at 25 °C than at 55 °C.
3. HRT was demonstrated to have a strong influence on AF performance. HRT values below 10 days prevented slow-growing archaea involved in methanogenic activity, resulting in low methane production. Reducing HRT below a critical value resulted in harsh operational conditions not only for methanogenic archaea but also for acidogenic bacteria, giving rise to low VFAs bioconversions. In addition, stepwise HRT reduction implied microbial adaptation to operational conditions which in turn allowed HRT reduction to lower values, treating higher flow rates and maximizing AF efficiency.
4. VFAs production yields and profiles were linked to the macromolecular composition of the employed substrate, to the pretreatment applied and to the imposed operational conditions.

5. High methane yields could be achieved when using AF solid spent for biogas production in BMPs and continuous mode. In spite of being a residue from AF, the obtained biogas evidenced the high biodegradability of the organic matter contained in this fraction. The previous AF did not negatively affect the methane potential but indeed improved biogas production via AD, since the solid residue biodegradability was enhanced during AF. These results supported the suitability for this residual stream as substrate for biogas production.

6. Despite the fact that the same anaerobic inoculum was employed for biogas production via AD and for VFAs production via AF, microbial community specialization of each bioprocess was promoted by applying proper operational conditions. Therefore, operational conditions ruled the fate of organic matter and drive the process towards a specific product.

7. The ability of *Y. lipolytica* to grow on digestate using VFAs as unique carbon source was demonstrated. Biomass concentrations and yields were very close to those achieved with glucose as carbon source. This fact indicated that carboxylate-based substrates are an interesting alternative to sugar-based substrates for OY.

8. The assessment of VFAs uptake rates and potential toxicity revealed that VFAs chain length had a crucial effect on yeast growth. Despite the fact that longer chain VFAs had higher growth yield, they exerted higher inhibitory effect on yeasts than short-chain VFAs.

9. When VFAs mixtures were evaluated as carbon source for OY, not only total VFAs concentration should be considered but also VFAs distribution in mixtures. *Y. lipolytica* sequentially utilized different types of VFA, showing preference for acetic acid coupled to the consumption of long-chain VFAs (valeric and caproic). When long-chain VFAs were available as carbon source, the metabolism of short-chain VFAs (propionic and iso-

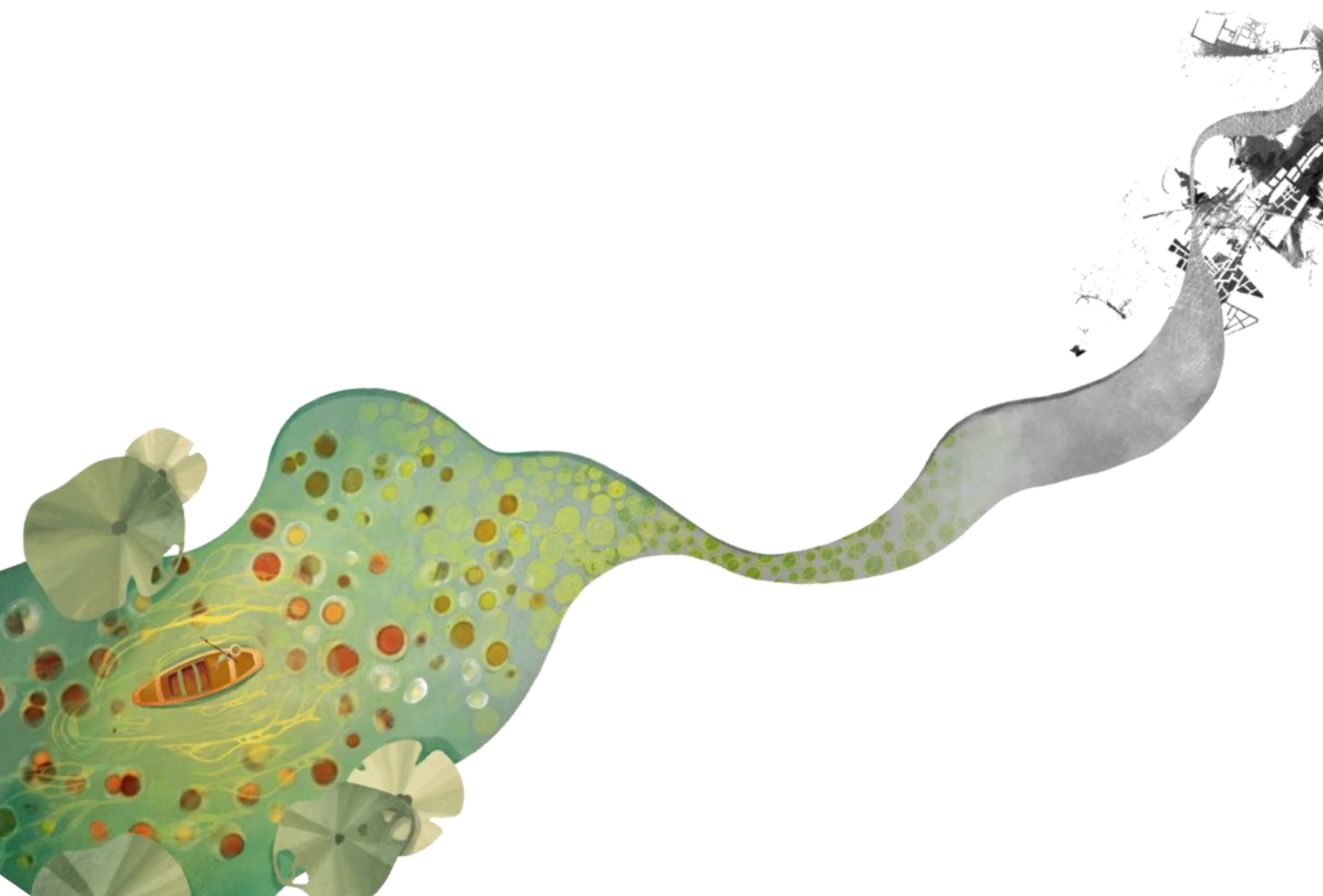
butyric acids) might be halted and these acids were only moderately consumed until valeric and caproic acids were exhausted.

10. Despite having the same VFAs profile, the biomass yield using real digestate was higher than the obtained in synthetic medium. This positive effect could not be simply attributed to additional carbon sources but to the presence of other nutrients (such as nitrogen, phosphorous, etc.) or by-products in the digestate that could enhance yeast growth. Additionally, the strong inhibitory effect of VFAs at high concentrations on *Y. lipolytica* was overcome when using real digestate as substrate. The suitability of using VFAs-rich liquid fraction obtained from AF as a novel carbon source for MO production was confirmed.

11. In the search of new efficient OY strains able to metabolize VFAs-rich digestates, the highest biomass growth was exhibited by *C. curvatus*, *L. lipofer* and *Y. lipolytica*. In these cases, biomass yields very close to the obtained when glucose or glycerol are used as carbon sources. *W. saturnus* and *C. curvatus* showed the highest lipid accumulation regardless of the substrate concentration in the medium. Moreover, the FA profile produced by all strains, mainly consisting of oleic, palmitic and linoleic acid, was similar to that of vegetable oils commonly used in the industry. Remarkably, significant amounts of the odd-chain FA C17:0 were produced by *Y. lipolytica*, *L. lipofer* and *C. curvatus*. Although process performance optimization would require further studies, *C. curvatus* was found to be the most promising strain during this Thesis in terms of both, biomass and lipid production.

12. Instead of the conventional use of microalgae for biogas production via AD, the present Thesis proved the efficient multi-product generation (VFAs, MO and biogas) from a single waste by the integration of three bioprocesses.

6. FUTURE PERSPECTIVES



6. Future perspectives

The research presented in this Thesis has demonstrated the successful production of bioenergy and bioproducts from an organic residue. These are preliminary results of the direct interconnection of different processes. Nevertheless, further studies could be performed to gain insights on each bioprocess and optimize process performance.

Proteomic-based studies would aid to understand the substrate-microbiome-products nexus, which could be employed as a tool to optimize VFAs production in AF. Indeed, a better knowledge of the AF bioprocess could also allow driving the AF towards a specific VFA production. Additionally, the generation of other AF bioproducts of interest, such as ethanol or lactic acid, during AF could be studied for other applications.

To cover the wide gap of knowledge about MO production from VFAs is crucial to elucidate the metabolic pathways involved in VFAs utilization by yeast. Complementary studies, including operational parameters optimization (oxygen concentration, micro- and macro-nutrients, etc.) or reactors configuration during oleaginous yeast fermentation, could help increasing yeasts growth and lipid production. Besides, to evaluate how pH affects the inhibitory effect of VFAs on yeasts, could allow working at high VFAs concentration.

The integration of three bioprocesses presented in this Thesis (*i.e.* AF, AD and oleaginous fermentation) has demonstrated the efficient multi-product generation from a single organic waste. However, to meet the circular economy needs, the conversion of different renewable substrates into marketable bioproducts is of utmost importance (Figure 6.1). Other low-cost organic wastes such as food wastes, agricultural residues or lignocellulosic materials could be tested as potential feedstocks. Targeting to broaden the biorefinery approach, additional studies could be performed to completely valorize all the available streams. In this sense, the utilization of the residual AD effluent obtained after methane generation as a potential fertilizer with agronomic value could be a challenging approach allowing nutrients recovery maximization.

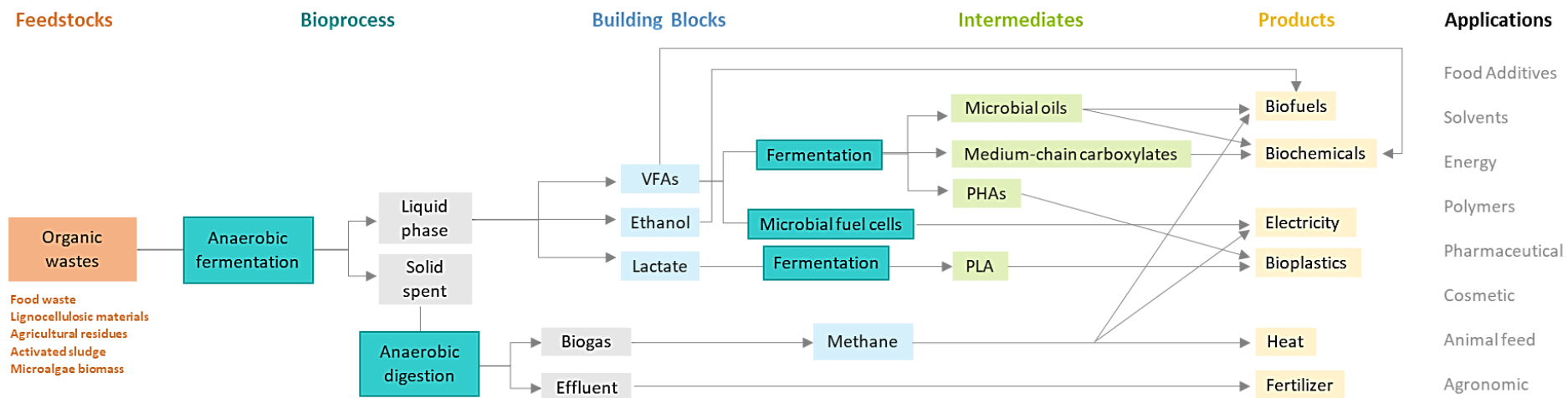
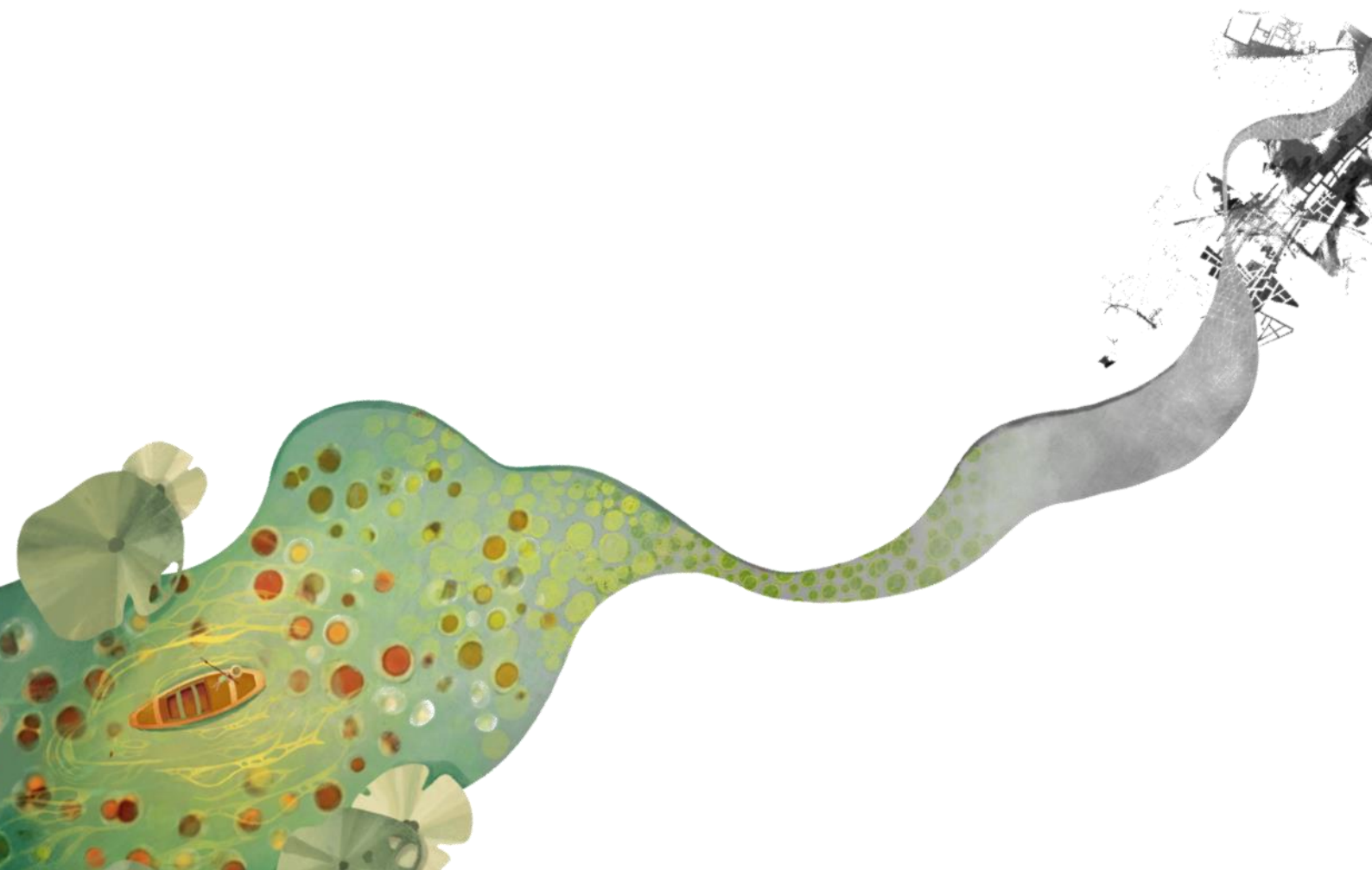


Figure 6.1. General biorefinery overview from organic wastes to biofuel and value-added products recovery.

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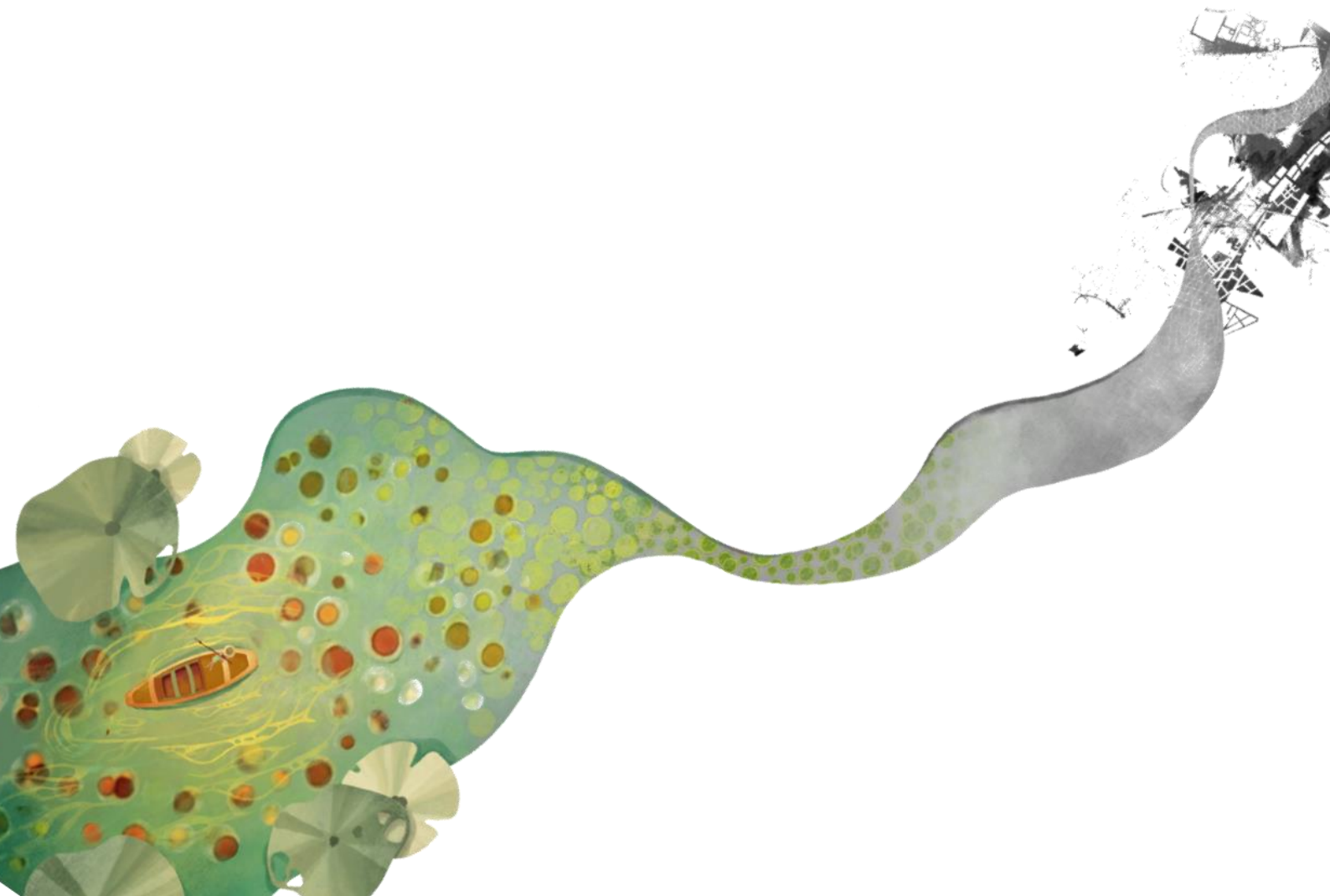
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ORIGINAL PUBLICATIONS



Publication I

Insights on the microbial communities developed during the anaerobic fermentation of raw and pretreated microalgae biomass

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Insights on the microbial communities developed during the anaerobic fermentation of raw and pretreated microalgae biomass



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HIGHLIGHTS

- Microalgae pretreatments effect on AF microbiome development was elucidated.
- Protease-pretreated *Chlorella vulgaris* enhanced SCFAs production.
- Biodiversity decreased when pretreated microalgae was used as substrate.
- Protease pretreated substrate promoted Firmicutes phylum development.
- Short HRT imposed (8 days) hindered methane production by archaea.

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ABSTRACT

Short-chain fatty acids (SCFAs) are considered building blocks for bioproducts in the so-called carboxylate platform. These compounds can be sustainably produced via anaerobic fermentation (AF) of organic substrates, such as microalgae. However, SCFAs bioconversion efficiency is hampered by the hard cell wall of some microalgae. In this study, one thermal and two enzymatic pretreatments (carbohydrases and proteases) were employed to enhance *Chlorella vulgaris* biomass solubilization prior to AF. Pretreated and non-pretreated microalgae were assessed in continuous stirred tank reactors (CSTRs) for SCFAs production. Aiming to understand microorganisms' roles in AF depending on the employed substrate, not only bioconversion yields into SCFAs were evaluated but microbial communities were thoroughly characterized. Proteins were responsible for the inherent limitation of raw biomass conversion into SCFAs. Indeed, the proteolytic pretreatment resulted in the highest bioconversion (33.4% SCFAs-COD/COD_{in}), displaying a 4-fold enhancement compared with raw biomass. Population dynamics revealed a microbial biodiversity loss along the AF regardless of the applied pretreatment, evidencing that the imposed operational conditions specialized the microbial community. In fact, a reduced abundance in Euryarchaeota phylum explained the low methanogenic activity, implying SCFAs accumulation. The bacterial community developed in the reactors fed with pretreated microalgae exhibited high acidogenic activities, being dominated by Firmicutes and Bacteroidetes. Firmicutes was by far the dominant phylum when using protease (65% relative abundance) while Bacteroidetes was prevailing in the reactor fed with carbohydrase-pretreated microalgae biomass (40% relative abundance). This fact indicated that the applied pretreatment and macromolecule solubilization have a strong effect on microbial distribution and therefore in SCFAs bioconversion yields.

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1. Introduction

Short-chain fatty acids (SCFAs) are drawing considerable

attention in the chemical industry. SCFAs have by themselves market prices of 400–2500€/ton (Atasoy et al., 2018; Bhatia and Yang, 2017) and they can even serve as precursors of a wide range of products such as biopolymers, chemicals and derivatives (Agler et al., 2011; Lee et al., 2014). SCFAs are formed during the middle stages of anaerobic digestion (AD) and subsequently degraded to produce methane. For this reason, anaerobic

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fermentation (AF), a shortened version of the traditional AD, was used to avoid methanogenesis and thus resulting in SCFAs accumulation (Agler et al., 2011; Holtzaple and Granda, 2009). Therefore, although SCFAs have been traditionally obtained through petroleum processing, they can be sustainably produced from renewable sources via AF (Dahiya et al., 2015). Among the substrates that can be subjected to AF for SCFAs production, microalgae biomass presents potential advantages such as the non-competition for arable lands, the contribution to CO₂ mitigation or the ability to be cultivated in wastewater (Passos et al., 2014). Another advantage is the high protein content exhibited by *Chlorella vulgaris*, accounting from 30 to 60% of their dry weight (Mata et al., 2010). When microalgae proteins are broken down during AD, ammonium and free ammonia are released to the medium. In this manner, the use of protein-rich substrates might lead to high total ammonia concentrations contributing to methanogens inhibition (Tian et al., 2018) and thus favoring SCFAs accumulation.

However, microalgae species usually grown in wastewater, such as *C. vulgaris*, present a rigid cell wall that hampers the hydrolysis step of AF. It should be highlighted that the hydrolytic stage determines the organic matter availability and ultimately could affect AF process yields. Therefore, application of pretreatment methods for cell wall degradation and organic matter solubilization is essential for an efficient bioconversion into SCFAs via AF. In the present work, thermal pretreatment was chosen since previous investigations found cell wall damage (disruption) by applying high temperatures (55–170 °C), improving microalgae biomass anaerobic biodegradability (Córdova et al., 2019). Different enzymatic cocktails were also used to target specific microalgae cell macromolecules (i.e. carbohydrates or proteins) to enhance biomass solubility. Owing to the high protein content of microalgae biomass, a protease cocktail was tested. Besides, the knowledge of microalgae cell wall composition is essential to select the most suitable pretreatment to disrupt/solubilize this structure and increase microbial bioaccessibility to organic matter. According to de Carvalho et al. (2020), microalgae cell wall is composed by a carbohydrate-based fibrillar walls covered by an algenan-based layer (de Carvalho et al., 2020). For this reason, carbohydrase enzymatic cocktail was employed as well.

Microalgae pretreatments have been widely studied for methane production purposes (Alzate et al., 2014; Feng et al., 2019; Kumar et al., 2017; Mahdy et al., 2016, 2014a; Mendez et al., 2013; Passos et al., 2013). Nevertheless, information about the effect of pretreatments in SCFAs production and microbial communities developed during AF is scarce. This latter point has been recently highlighted to be crucial when aiming at maximizing SCFAs conversion efficiency (Atasoy et al., 2018). Microbial communities' trend during AF process is uncertain since these populations are strongly affected by factors like the inoculum source, the substrate employed and implemented operational conditions (Arslan et al., 2016). In this context, AF of microalgae biomass requires further research to understand how by-products released during pretreatments can affect the anaerobic microbiome and thus, the conversion into SCFAs.

To cover this gap of knowledge, the present investigation addressed the effect of three pretreatments (one thermal and two enzymatic: protease and carbohydrase) on *C. vulgaris* biomass and their impact on the AF process by linking microbial communities to SCFAs production (yields and profile). Moreover, a canonical correspondence analysis was performed to further elucidate the effect of the environmental parameters on the microorganism communities' development and performance.

2. Material and methods

2.1. Microalgae biomass used as feedstock

The microalgae *C. vulgaris* was selected as substrate due to its ability to grow in wastewater. This latter characteristic is important when it comes to bioprocesses costs. It should be highlighted that nutrients might account for 30% of the operational costs (Xin et al., 2016). Harvested wet microalgae biomass employed in this work was purchased from Allmicroalgae (Lisbon, Portugal). Characterization of the raw microalgae biomass in terms of total and volatile solids (TS and VS), total and soluble Chemical Oxygen Demand (COD), ammonium (N–NH₄⁺), total carbohydrates, proteins, lipids and ashes, can be found in Table 1.

To avoid hydrolytic limitations during AF, raw microalgae biomass was subjected to different pretreatments.

2.2. Biomass pretreatments

Selected temperature and application time length during thermal pretreatment (abbreviated as T) were 120 °C and 100 KPa for 40 min. These values were in the range of values previously confirmed beneficial to enhance microalgae biomass solubility, causing cell wall disruption (Mendez et al., 2013). Wet biomass was pretreated in an autoclave (Selecta, Spain).

Carbohydrase enzymatic hydrolysis was performed using the commercial cocktail Viscozyme (abbreviated as V) kindly supplied by Novozymes (Denmark). This cocktail was composed by a variety of cellulolytic enzymes with β -glucanase, arabinase, cellulase, hemicellulose and xylanase activities. According to the supplier recommendations, the enzymatic cocktail was applied at 0.3 mL/g TS dose. The proteolytic pretreatment was performed with endopeptidase enzymes using the commercial cocktail Alcalase 2.5 L (from now abbreviated as A), also provided by Novozymes. The enzyme dosage applied was 0.2 mL/g TS and the pretreatment procedure was carried out according to previously optimized parameters (Mahdy et al., 2014b).

According to manufacturer's recommendations, both enzymatic pretreatments were performed at 50 °C and continuously stirred by using an orbital water bath shaker for 4 h. Initial pH was 5.5 and 8.0 for V and A cocktails, respectively. In order to maintain the enzymes at their optimum activity, pH was periodically adjusted with a pH-meter (Crison, Spain) using 5 M HCl or 6 M NaOH. After hydrolysis, temperature was raised to 75 °C for 30 min for enzymes deactivation.

Samples were taken and cooled down at room temperature (every 10 min during T pretreatment and every 30 min during A and V pretreatments) to assess organic matter solubilization along

Table 1
Chemical characterization of raw microalgae *C. vulgaris*.

Chemical parameters	Average $\pm$ Standard deviation
TS (g/L)	70.3 $\pm$ 4.2
VS (g/L)	64.2 $\pm$ 4.5
VS/TS (%DW)	91.3 $\pm$ 0.9
Soluble COD (g/L)	10.3 $\pm$ 4.5
Total COD (g/L)	153.6 $\pm$ 2.2
Soluble COD/Total COD (%)	6.9 $\pm$ 0.4
pH	6.5 $\pm$ 0.5
N–NH ₄ ⁺ (mg/L)	69.9 $\pm$ 1.5
Carbohydrates (%DW)	21.5 $\pm$ 2.6
Proteins (%DW)	63.8 $\pm$ 5.2
Lipids (%DW)	6.0 $\pm$ 0.8
Ash (%DW)	8.7 $\pm$ 0.9

DW: dry weight.

the experimental time. Pretreatments were considered finished when organic matter solubilization in terms of COD was stable.

The efficiency of the pretreatment employed was calculated according to equation (1):

$$\text{Hydrolysis efficiency (\%)} = \frac{\text{soluble COD after pretreatment} - \text{soluble COD raw biomass}}{\text{total COD raw biomass} - \text{soluble COD raw biomass}} \cdot 100 \quad (1)$$

where COD refers to the organic matter measured as chemical oxygen demand.

Likewise, proteins and carbohydrates solubilization efficiencies were measured by using the same formula, but considering carbohydrates (Ch) and proteins (Pr) instead of COD

$$\text{Ch solubilization (\%)} = \frac{\text{soluble Ch after pretreatment} - \text{soluble Ch raw biomass}}{\text{total Ch raw biomass} - \text{soluble Ch raw biomass}} \cdot 100 \quad (2)$$

$$\text{Pr solubilization (\%)} = \frac{\text{soluble Pr after pretreatment} - \text{soluble Pr raw biomass}}{\text{total Pr raw biomass} - \text{soluble Pr raw biomass}} \cdot 100 \quad (3)$$

2.3. Semi-continuous anaerobic fermentation in CSTRs

Microalgae AF was evaluated in four CSTRs of 1-L working volume. Fermenters were inoculated with anaerobic sludge collected from the anaerobic digester of the wastewater treatment plant of Valladolid (Spain). Anaerobic sludge presented total solids (TS) concentration of 15.8 ± 0.2 g/L and Volatile solids (VS)/TS of $60.4 \pm 0.1\%$. Reactors were maintained at 25°C by using a water bath. This low temperature was selected since previous studies reported that 25°C promoted organic matter conversions into SCFAs by avoiding methanogenesis (Magdalena et al., 2019a). In the reactors, pH was monitored but not controlled. Similarly to non-pretreated biomass, pH of the feeding was adjusted to neutral values (6.5 ± 0.2) in all pretreated substrates. Organic loading rate (OLR) applied was 1.5 g COD/(L d) and Hydraulic retention time (HRT) was set at 8 d to prevent methanogenic activity by washing out the archaea population (Leitão et al., 2006; Magdalena et al., 2019a).

2.4. Analytical methods

Total Kjeldahl nitrogen (TKN), TS and VS were measured according to Standard Methods (APHA/AWWA/WEF, 2017). Colorimetric commercial methods were used for the quantification of total and soluble chemical oxygen demand (COD) and ammonium (Merck, ISO 15705 and ISO 7150–1, respectively). Protein content was determined multiplying total organic nitrogen by 5.95 (López et al., 2010). Total organic nitrogen was determined by Kjeldahl method which consists on an acid digestion followed by distillation and titration (Owusu-Apenten, 2002). Carbohydrates content was analyzed by phenol–sulphuric acid method (Dubois et al., 1956). To

determine SCFAs concentration, collected samples were filtered through $0.2\ \mu\text{m}$ nylon filters (Labbox, Spain) and analyzed by liquid chromatography using an Agilent 1260 HPLC-RID (Agilent, USA) equipped with a Cation H Refill Cartridge Microguard column

(Biorad) and an Aminex HPX-87H ion exclusion column (300×7.8 mm I.D.) (Biorad). Mobile phase was 5 mM H_2SO_4 , and elution was conducted in isocratic mode at a flow rate of 0.6 mL/min. The injected sample volume was 20 μL , and the oven and detector temperatures were 50 and 35°C , respectively.

Once the bioprocesses reached the pseudo-steady state, which

was characterized by a stable SCFAs production as well as TS, VS, COD and NH_4^+ values after 3 HRTs, the process efficiency was evaluated in terms of bioconversion of organic matter into SCFAs and calculated as follows (equation (4)):

$$\text{Bioconversion (\%)} = \frac{\text{SCFAs} - \text{COD out}}{\text{total COD in}} \cdot 100 \quad (4)$$

where SCFAs-COD_{out} refers to the organic matter related to SCFAs measured in the effluent as g COD/L, while total COD_{in} represents the total organic matter fed in the reactor measured as g COD/L. SCFAs stands for acetic acid (HAc), propionic acid (HPro), isobutyric acid (isoHBu), butyric acid (HBu), isovaleric acid (isoHVal), valeric acid (HVal) and caproic acid (HCa). The equivalence of each SCFA in terms of COD was 1.07 for HAc, 1.51 for HPro, 1.82 for HBu/isoHBu, 2.04 for HVal/isoHVal and 2.2 (mg COD/mg SCFAs) for HCa (Eastman and Ferguson, 1981).

2.5. DNA extraction and sequencing

Microbial communities were analyzed in the inoculum and in samples collected in CSTRs once bioprocesses reached the pseudo-steady state. For that purpose, DNA was extracted from 1 mL of sample using the kit 'FastDNA SPIN Kit for Soil' (MP Biomedicals, LCC). DNA extraction was conducted according to manufacturer's protocol. Concentration and quality of the extracted DNA was checked by using a nanodrop spectrophotometer (LVis Plate, BMG Labtech, DE). Amplification of the 16S rRNA hypervariable regions V3–V4 of archaea and bacteria was performed using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) and subsequently sequenced on a MiSeq Sequencer (Illumina) by FISABIO (Valencia, Spain).

Table 2
OTUs and Shannon indices calculated for the samples.

	Observed OTUs	Shannon
Inoculum	823	7.378
Non-pretreated	381	5.767
Thermal pretreatment	336	5.001
Viscozyme pretreatment	309	4.811
Alcalase pretreatment	313	4.999

OTUs: Operational taxonomic units.

2.6. Diversity analysis and statistical method

Raw sequence were computationally processed by using bioinformatics tools as follows: in the first step, the paired-end reads from each sample were merged using the program PEAR (Zhang et al., 2014). Afterwards, sequences were quality sieved with a mean score of 30 using PRINSEQ (Schmieder and Edwards, 2011). Primers sequences were removed using Mothur program (Schloss et al., 2009) and then, chimeric sequences were also deleted while the resulting sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity (OTU0.97) by USEARCH (Edgar, 2010; Edgar et al., 2011), using Greengenes database gg_13_8 (McDonald et al., 2012) which is implemented in the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software package (Caporaso et al., 2010). QIIME was used to evaluate the biodiversity in terms of richness and evenness by calculating the observed OTUs and Shannon index. Canonical Correspondence Analysis (CCA) was performed using PAST (Hammer et al., 2001) to evaluate the relationship between community composition and environmental variables such as organic matter, macromolecular solubilization and SCFAs produced.

3. Results and discussion

3.1. Assessment of the microbial biodiversity in the fermenters

Microbial communities of the inoculum and the microbial systems developed at the pseudo-steady state of each fermenter were compared. The 16S rRNA analysis revealed remarkable changes in microbial diversity (Table 2). The inoculum exhibited the highest biodiversity in terms of observed OTUs (823). Similar trend was

also confirmed by the Shannon index (7.378), which evaluates both richness and evenness of the sample. The high biodiversity detected in the inoculum was likely associated with the sludge origin since it was collected from a mesophilic anaerobic digester devoted to biogas production. Conventional anaerobic digesters are normally operated at high HRT (20–30 days) in order to promote slow-growing microorganisms' development, such as methanogens. Therefore, high biodiversities are commonly detected in these conventional systems (Gonzalez-Fernandez et al., 2018). Opposite, samples taken during the pseudo-steady state of the CSTRs showed a biodiversity decrease in terms of observed OTUs (309–381) and Shannon indexes (5.767–4.811) when compared with the inoculum. This decrease was likely related to the operational conditions. Operational conditions have a direct effect on microbial population dynamics since imposing a low HRT (8 days) along with low temperature (25 °C) likely provoked the wash out of methanogens (Cavinato et al., 2017), suggesting a specialization of the microbiome for SCFAs production.

Comparing different CSTRs, it should be noted that the biodiversity when using non-pretreated microalgae as substrate was higher than the ones obtained when using pretreated biomass. This fact could be related to the amount and type of soluble organic matter available since raw microalgae presented lower soluble organic matter than the pretreated biomass (Fig. 1). Thus, the higher particulate fraction of raw microalgae probably promoted the adhesion of microorganisms on the particles surface (Tsapekos et al., 2017), increasing the microbial biodiversity.

3.2. Microbial communities present in the inoculum

The inoculum was mainly constituted by six phyla, covering 77% of the total sequences in terms of relative abundance (Fig. 2a). These variety of phyla included Chloroflexi (17.1%), Proteobacteria (15.8%), Bacteroidetes (13.0%), Actinobacteria (12.6%), Firmicutes (11.5%) and Euryarchaeota (7.2%). Despite the fact that bacterial population is strongly variable due to the operational conditions, similar phyla were detected in the anaerobic inoculum used in previous studies focused on biogas production (Gonzalez-Fernandez et al., 2018; Greses et al., 2017; Sundberg et al., 2013). It could be stated that the employed inoculum exhibited a quite conventional microbial phyla.

More specifically, Chloroflexi was mainly comprised by

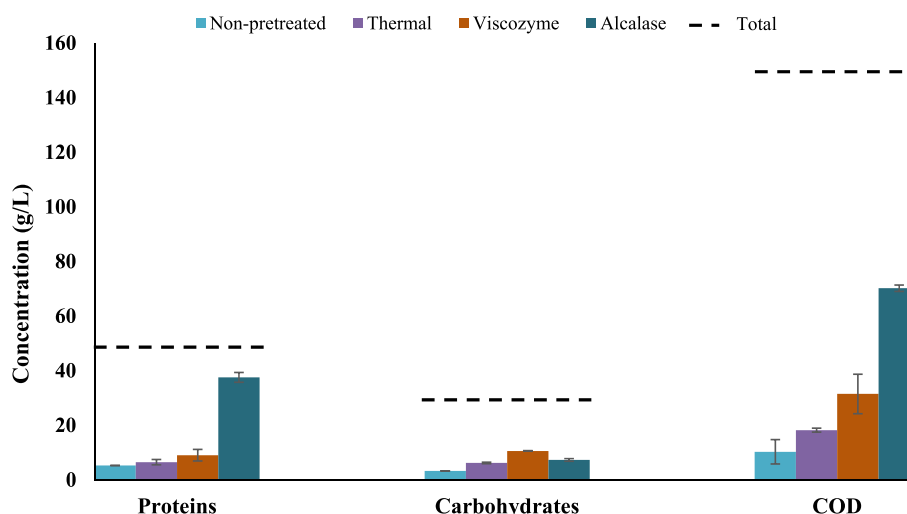


Fig. 1. Organic matter (COD), protein and carbohydrate fraction solubilization comparing the non-pretreated biomass with the different pretreatments assessed: Thermal, Viscozyme and Alcalase. Dashed lines correspond to the total protein, carbohydrates and COD concentration.

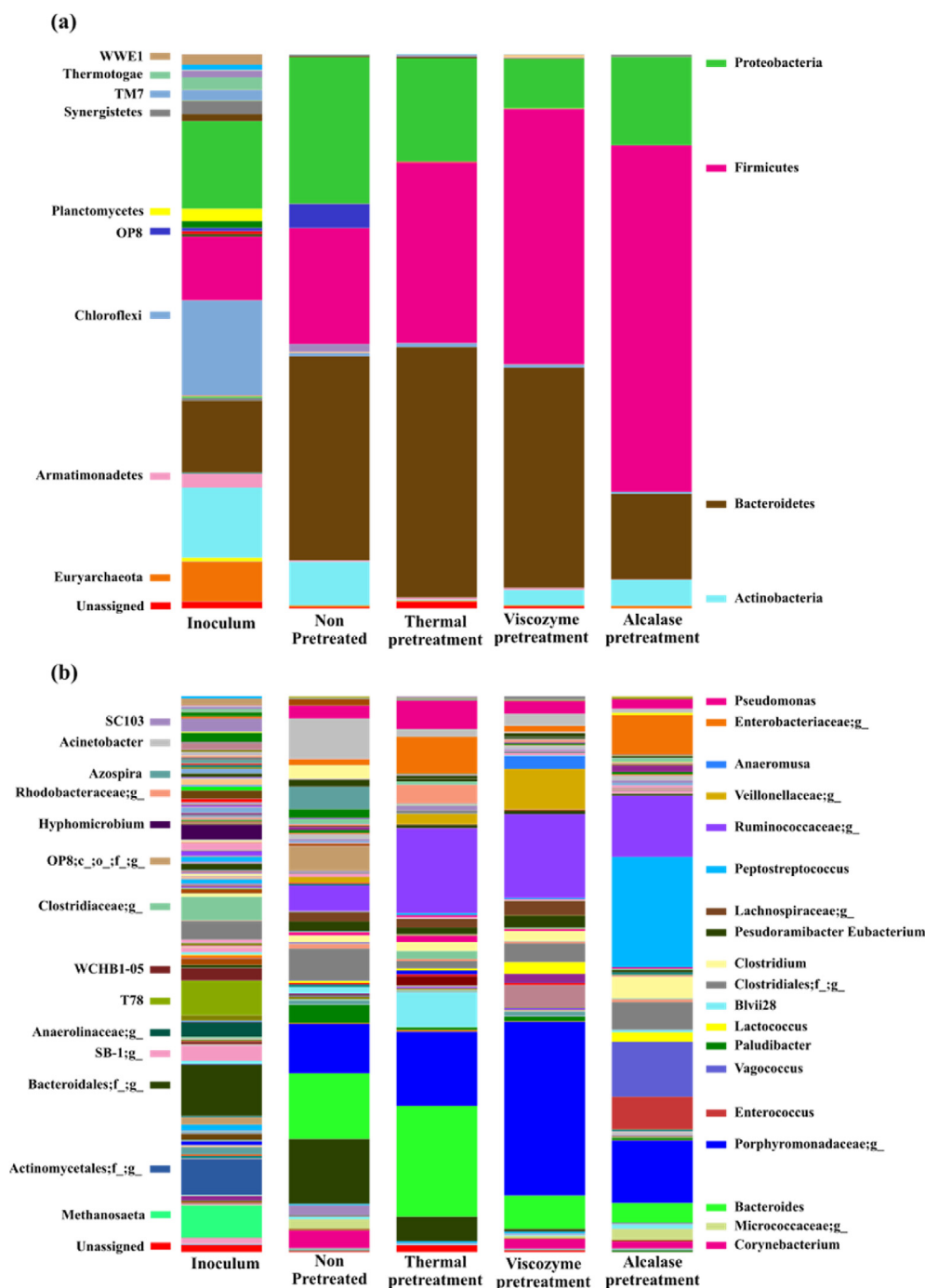


Fig. 2. Relative abundance of Bacterial and Archaeal OTUs identified in the inoculum as well as in the pseudo-steady state of the CSTRs at phyla (a) and genera levels (b). Taxonomic groups with relative abundance lower than 1% were excluded from the legend.

Caldilineaceae (1.6%) and Anaerolineaceae (12.4%) families where T78 and WCHB1-05 were the most abundant genera (Fig. 2b). These microorganisms played important roles in carbohydrates hydrolysis and acidogenesis (Braz et al., 2019; Liao et al., 2016; Wang et al., 2016). *Hyphomicrobium* (2.6%) was the most abundant genus in Proteobacteria phylum, which has been identified as a facultative bacteria able to degrade different carbon sources to facilitate the methanogenic step (Hartati et al., 2018). Bacteroidetes, included members of bacteroidales class (9.2%), SB-1 (2.7%) families and *Blvii28* genus (0.6%), while Actinobacteria phylum was composed by members of the order Actinomycetales (6.5%). Both phyla are

characterized by high cellulolytic activities (Cirne et al., 2007; Gao et al., 2019; Granada et al., 2018; Su et al., 2014; Ziganshin et al., 2011). In the case of Firmicutes, this phylum was mainly constituted by Clostridia class (10.8%), being also related to anaerobic degradation of protein and carbohydrates (Sanz et al., 2016). Regarding Euryarchaeota phylum, *Methanosaeta* (5.7%) was the most abundant genus. *Methanosaeta* has been commonly reported to be in charge of HAC bioconversion into methane via acetoclastic metabolism (Lim et al., 2020). Therefore, the sludge selected as inoculum presented a wide range of hydrolytic activities to degrade a great variety of organic components. Additionally, the inoculum

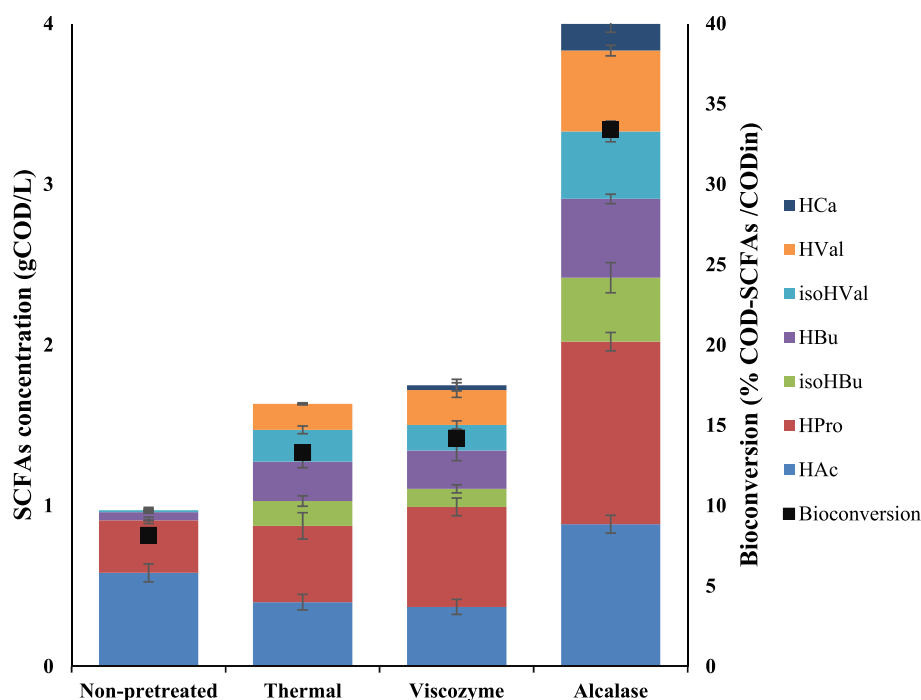


Fig. 3. SCFAs production, distribution profile and bioconversion during AF.

Table 3
Main parameters assessed during CSTR operation.

	Non-pretreated	Thermal pretreatment	Viscozyme	Alcalase
COD removal (%)	3.5 ± 3.1	11.8 ± 1.8	3.5 ± 1.5	7.6 ± 2.2
mL CH ₄ /g COD _{in}	12.3 ± 15.1	41.9 ± 9.0	12.2 ± 7.3	26.5 ± 10.7
N-NH ₄ ⁺ (mg/L)	110.1 ± 24.8	168.3 ± 37.9	96.0 ± 27.3	410.7 ± 37.0
Bioconversion (%)	8.1 ± 0.7	13.6 ± 1.6	14.2 ± 1.4	33.4 ± 2.2
pH	6.0 ± 0.1	6.2 ± 0.1	5.7 ± 0.1	6.3 ± 0.1

sludge displayed also a high relative abundance of methanogens (7.2%), which supported its capability for biogas production.

3.3. Fermenter fed with non-pretreated microalgae biomass: performance and microbial communities developed

Raw *C. vulgaris* presented a low percentage of easily degradable organic matter (soluble COD/total COD = $6.9 \pm 0.4\%$, Table 1). Likewise, low SCFAs production was attained when non-pretreated microalgae was employed as substrate (1.0 ± 0.1 g SCFAs-COD/L), which resulted in a bioconversion efficiency of $8.1 \pm 1.4\%$ SCFAs-COD/COD_{in} (Fig. 3).

As previously described in conventional anaerobic processes, microalgae present a highly resistant cell wall that hampers bacterial hydrolysis resulting in low bioprocess efficiencies (Ometto et al., 2014). With regard to the SCFAs profile shown in Fig. 3, only HAc and HPro accounted for more than 90% of the total SCFAs. This phenomenon could be explained by β -oxidation pathway during AF (Moretto et al., 2019). The high presence of HPro was mainly associated to the protein-rich substrate employed as feedstock. This agreed with Regueira and co-workers who stated that protein-rich substrates lead to high odd-chain production, specially HPro (Regueira et al., 2020). In a conventional anaerobic processes, HAc is quickly consumed by acetoclastic methanogenic archaea and therefore, its accumulation confirmed a low methanogenic activity. In fact, a concomitantly drastic decrease in *Methanosaeta*

community, also supported the low methane production yields determined in the fermenter (COD removal was about 4%, Table 3). Therefore, it should be highlighted that the imposed conditions (HRT, fermentation temperature and OLR) were appropriated for SCFAs accumulation since methanogenic activity was negligible, avoiding SCFAs conversion into methane.

In addition to the decrease in archaea population registered in the fermentation conducted with raw microalgae biomass, bacterial community also changed from that observed in the inoculum. At phylum level, Chloroflexi presence was drastically decreased at the expenses of an increased population of Bacteroidetes (36.8%), Proteobacteria (26.4%) and Firmicutes (21%) (Fig. 2a). Firmicutes detected in the reactor fed with raw microalgae presented bacteria belonging to Clostridiales order (5.8%) as well as uncultured Ruminococcaceae (4.6%), supporting the high HAc content 67% of total SCFAs, Fig. 3). Members of Bacteroidetes and Proteobacteria phyla are hydrolytic bacteria involved in the degradation of cellulose and protein (Bi et al., 2020; Cho et al., 2015; Greses et al., 2017). The high relative abundance of these phyla found when using non-pretreated microalgae could be related to the high content of particulate COD. In Proteobacteria phylum, *Acinetobacter* accounted for the highest relative abundance in the CSTR fed with non-pretreated microalgae (7.2%), probably due to the hydrolytic activity of this genus to degrade cellulose. Actinobacteria phyla (7.9%) was mainly detected in the fermentation conducted with raw microalgae. This phylum is involved in complex carbohydrates degradation

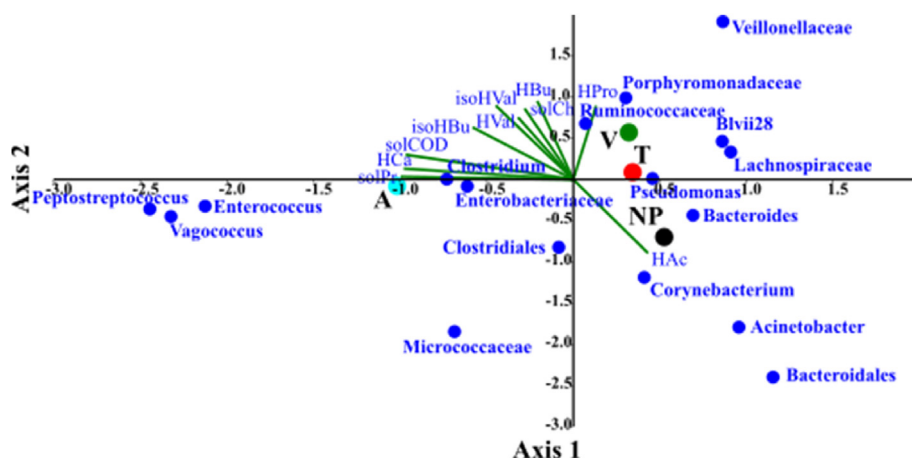


Fig. 4. Canonical correspondence analysis ordination triplot performed for the samples retrieved during the pseudo-steady state of the CSTRs fed with non-pretreated (NP) microalgae as well as Thermal (T), Viscozyme (V) and Alcalase (A) pretreated microalgae biomass.

Table 4

Pretreatments efficiencies in terms of COD, proteins and carbohydrates solubilization.

	Thermal	Viscozyme	Alcalase
Soluble COD/Total COD (%)	12.1 ± 0.2	19.9 ± 0.4	42.8 ± 0.7
COD Hydrolysis efficiency (%)	5.8 ± 0.2	14.1 ± 0.5	38.6 ± 0.8
Proteins solubilization (%)	3.4 ± 0.9	9.4 ± 0.8	81.6 ± 1.6
Carbohydrates solubilization (%)	24.8 ± 1.2	61.9 ± 1.1	34.1 ± 1.4

(Ziganshin et al., 2011) and thus, its abundance was likely related to the complex carbohydrates in *C. vulgaris* cell wall. Additionally, Fig. 4 showed that *Acinetobacter* was linked with HAC production during the degradation of particulate biomass. This is in agreement with previous works where the correlation of this genus with HAC production was reported (Fitamo et al., 2017).

The decrease of Actinobacteria phylum with regard to the inoculum has been previously reported in studies dealing with the use of raw microalgae as feedstock for AD process (Cirne et al., 2007; Sanz et al., 2016). Moreover, the abundance of this phylum observed in the different reactors seems to be associated to the carbohydrate state since pretreated microalgae presented a higher fraction of carbohydrates with lower complexity than the raw biomass (Fig. 1). In this manner, when the substrate is more complex, the microbial systems need a wider range of hydrolytic activities and hence, hydrolytic phyla become more abundant. At genera level (Fig. 2b), *Corynebacterium* accounted for 3.2%. This genus converts carbohydrates into HAC and HPro [67]. This result was supported by the correlation found in Fig. 4, where *Corynebacterium* distribution displayed an interconnection with HAC when using raw biomass as substrate.

3.4. Fermenters fed with thermally-pretreated microalgae biomass: performance and microbial communities developed

In order to elucidate pretreatments effect on the bioprocess performance, AF efficiencies were evaluated in terms of organic matter bioconversion into SCFAs and developed microbial communities compared to the raw microalgae. As it can be seen in Fig. 1, the use of T pretreatment roughly doubled the soluble COD/total COD ratios from $6.9 \pm 0.4\%$ in the raw microalgae biomass to $12.1 \pm 0.2\%$ in the raw microalgae biomass to $12.1 \pm 0.2\%$, corresponding with a hydrolytic efficiency of $5.8 \pm 0.2\%$ (Table 4). After T pretreatment, 24.8% carbohydrates and 3.4% proteins solubilization was attained.

These results were in agreement with Mendez and co-workers (Mendez et al., 2013), who also observed that carbohydrates solubilization prevailed over protein during microalgae T pretreatments. Despite of the carbohydrate release after the pretreatment, AF bioconversion efficiency was only enhanced to $13.6 \pm 1.6\%$ compared to approximately 8% when employing raw biomass as substrate (Fig. 3, Table 3). Therefore, a slight increase was achieved but not enough to justify the use of this pretreatment. SCFAs profile slightly changed in comparison to that obtained for raw biomass fermentation. When using T-pretreated biomass, longer SCFAs (C4 and C5) accounted for 37% of the SCFAs-COD (Fig. 3).

When comparing with the microbial communities of the inoculum, a significant shift was observed (Fig. 2). Phyla diversity were further decreased from that registered in reactors fed with raw microalgae. In this case, three main phyla composed the microbial community: Bacteroidetes (45.1%), Firmicutes (35.2%) and Proteobacteria (18.6%). Bacteroidetes and Firmicutes increased their population when compared to the microbiome determined for the raw biomass while Proteobacteria decreased. Within Bacteroidetes, it must be highlighted the presence of *Blvii28* genus exclusively in CSTR fed with T-pretreated microalgae (6.3%). This genus is involved in longer carboxylic acids fermentation into HAC and HBU (Shen et al., 2017). Hence, the presence of *Blvii28* was correlated with the absence of HCa in this CSTRs due to its degradation into shorter carboxylic acids, such HAC (Fig. 4).

3.5. Fermenters fed with enzymatically-pretreated microalgae biomass: performance and microbial communities developed

As it can be seen in Fig. 1, the use of enzymatic pretreatments notably increased the soluble COD/total COD ratios from $6.9 \pm 0.4\%$ in the raw microalgae biomass to $19.9 \pm 0.4\%$ and $42.8 \pm 0.8\%$ for V and A treated biomass, respectively. These values corresponded to hydrolytic efficiencies of $14.1 \pm 0.5\%$ and $38.6 \pm 0.8\%$ (Table 4). With regard to the carbohydrates solubilization, the highest value was reached by V-pretreated biomass ($61.9 \pm 1.1\%$ of total carbohydrates) while A pretreatment reached $24.8 \pm 1.2\%$ (Table 4, Fig. 1). Even though all pretreatments affected the carbohydrate fraction, the same trend was not observed for proteins. Higher COD solubilization values were obtained after A pretreatment when compared with V or T pretreatments. This fact could be explained by the high protein content in *C. vulgaris*. In fact, A resulted in the highest protein solubilization ($81.6 \pm 1.6\%$ of total protein), whereas V and T

only reached 9.4 ± 0.8 and $3.4 \pm 0.9\%$, respectively (Table 4). The highest hydrolytic efficiency attained with A evidenced the suitability of the proteolytic pretreatment for releasing readily biodegradable organic matter.

When microalgae pretreated with V and A were fed in AF reactors, 1.7 ± 0.3 and 4.0 ± 0.4 g SCFAs-COD/L were obtained, respectively (Fig. 3). These values corresponded to bioconversion efficiencies of $14.2 \pm 1.6\%$ and $33.4 \pm 2.2\%$ (Table 3). The highest organic matter bioconversion into SCFAs attained by the proteolytic pretreatment agreed with the highest organic matter solubilization obtained by means of this enzymatic cocktail (Table 3, Fig. 1). However, despite of the higher solubilization during V pretreatment in comparison with T, similar SCFAs conversion were obtained in both reactors (Fig. 3). This fact evidenced that high organic matter solubilization did not necessarily correlate with high AF process efficiencies. This could be explained by the fact that not all soluble organic matter is ready to be degraded since the use of pretreatments might entail not only the release of degradable biomass components but also process inhibitors (Mendez et al., 2013).

Therefore, although carbohydrates fraction have been claimed to be the bottleneck for microalgae degradation (de Carvalho et al., 2020; Passos et al., 2014), it has been demonstrated that the use of a proteolytic pretreatment step is crucial to enhance organic matter bioconversion into SCFAs. The results obtained from the proteolytic pretreatment were similar or higher of those reported in literature from different waste streams. For instance, Iglesias-iglesias et al. (2019) found that sewage sludge only reached 22% bioconversion, Huang and co-workers (2016) achieved 25% bioconversion when swine manure was fermented while Valentino et al. (2018) reported 31% bioconversion from municipal solid wastes (Huang et al., 2015; Iglesias-iglesias et al., 2019; Valentino et al., 2018). Additionally, similar results were reported by other authors employing microalgae biomass as substrate. Magdalena and co-workers found that *C. vulgaris* biomass pretreated with A resulted in 39.5% SCFAs-COD/CODin in a CSTR operated under similar operational conditions using adapted sludge (Magdalena et al., 2019b). Therefore, these results evidenced the potential of pretreated microalgae as feedstock for SCFAs production.

Regarding the SCFAs distribution profile, slight variations were found among the assessed enzymatic pretreatments (Fig. 3). HAc, HPro, isoHBU and HBU acids outstood as the most abundant organic acids ranging from 79 to 83% of the total SCFAs production in terms of COD while around 20% was distributed in longer-chain fatty acids C5–C6. HVal and HCa (C5–C6) accumulation has been correlated with methanogenic activity inhibition (Strazzer et al., 2018). Higher concentration of these acids supported the higher efficiencies of organic matter conversion into SCFAs reached in fermenters fed with pretreated biomass in comparison with that using raw microalgae. The SCFAs profiles were in agreement with other studies aiming at producing SCFAs from microalgae biomass (Jankowska et al., 2017; Magdalena et al., 2019a; Moretto et al., 2019).

Regarding the microbial communities (Fig. 2), Firmicutes was the most abundant phylum in fermenters fed with A-pretreated biomass (65.2% of relative abundance). This value was comparably higher to fermenters fed with V (46.1%) and T (32.5%) pretreated microalgae and raw microalgae (21.0%). Previous research studies have reported Firmicutes as dominant phylum in AF aiming at SCFAs production (Greses et al., 2020; Magdalena et al., 2019a). Firmicutes play an important role in both, the hydrolytic and acidogenic steps of polysaccharides degradation (Greses et al., 2017). Therefore, the high bioconversion efficiency (33.4% SCFAs-COD/CODin) attained when microalgae biomass was pretreated with A was likely related with the prevalence of Firmicutes. The

different soluble components released after microalgae pretreatment can indeed have an effect on prevailing microbial communities. In this manner, the high proteins solubilization obtained after microalgae pretreatment with A might prevent the proteolytic population development, promoting the bacteria involved in the hydrolysis and acidogenesis of carbohydrates, such as Firmicutes.

The high soluble COD and proteins content in the sample collected from CSTR fed with A-pretreated microalgae were greatly correlated to the presence of genus *Peptostreptococcus* (19.8%), *Vagococcus* (9.8%), *Enterococcus* (5.8%) and *Clostridium* (3.9%). These genera were only detected in CSTR fed with A-pretreated microalgae. *Peptostreptococcus* was found as one of the main genus involved in the fermentation of proteins to produce HAc, HBU and HVal (Shen et al., 2017). On the other hand, *Vagococcus* is a fermentative bacteria able to metabolize carbohydrates into HAc and lactic acid in environments with high amino acids content since this genus is limited by its low capacity to synthesise amino acids (Jin et al., 2019). The presence of both genera supported the correlation found with the content of HAc, HBU and HVal (Fig. 4) and hence, it could be inferred that the high content of soluble proteins in A-pretreated microalgae promoted their co-existence. Moreover, *Enterococcus* is also involved carbohydrates fermentation to release lactic acid and HPro as main products (Lim et al., 2020). The correlation found with the high soluble COD content was likely due to carbohydrates solubilization occurring during A pretreatment (34.1% solubilized carbohydrates, Table 4). The growth of lactic acid producers (*Vagococcus* and *Enterococcus*) might be responsible of the strong correlation found with *Clostridium* and HCa in CSTR fed with A-pretreated microalgae. Moreover, some bacteria included in Clostridiales order are also known as HAc producers from the carbohydrates hydrolysis (Hung et al., 2011). Therefore, these bacteria could be the responsible microorganisms of the HAc accumulation in this reactor along with the methanogenesis inhibition.

Similar results were encountered in V-pretreated and T-pretreated fermenters. Bacteria belonging to Ruminococcaceae family were positively correlated with T and V samples, (accounting for 15.3% and 15.0%, respectively). Ruminococcaceae are involved in the hydrolysis and acidogenesis of polysaccharides, releasing HAc as main product (Greses et al., 2020). Similar trend was observed with member of Veillonellaceae family (1.8 and 7.3% in T and V samples, respectively), which showed a correlation with HPro content (Fig. 4). Veillonellaceae family exhibits high hydrolytic activity for carbohydrate degradation, producing HPro as main metabolite (Braz et al., 2019) and thus, supporting the correlation observed in Fig. 4. Therefore, the presence of both families could be related to the hydrolysis of carbohydrates still contained in the solid fraction.

Members of Lachnospiraceae family are cellulolytic microorganisms that produce HBU and lactic acid during the metabolism (Moestedt et al., 2020) and thus, not only contributed to the HBU accumulated in the CSTRs but also to the synergic metabolism with *Clostridium* and member belonging to Clostridiales. As it was described above, these bacteria utilize lactic acid and HAc to produce HCa, which was detected in CSTR fed with V-pretreated microalgae. However, HCa was not found in T samples probably due to the notable reduction of both *Clostridium* genus and Clostridiales order (Fig. 2b).

Bacteroidetes phylum, commonly involved in cellulose and protein degradation (Bi et al., 2020; Cho et al., 2015), was detected in low relative abundance when using A-pretreated microalgae (15.5%), while the abundance of this community was markedly higher in the other fermenters (36.8–45.1%). The lower presence of this phylum in the fermentation conducted with A-pretreated biomass could be explained by the highest hydrolytic efficiency of

this pretreatment, avoiding the need for the microbial community to develop high hydrolytic capabilities. Concerning genera distribution, Bacteroidetes phylum was mainly composed by *Bacteroides*, uncultured Bacteroidales order, and Porphyromonadaceae family. These microorganisms are producers of HAc and HPro during cellulose fermentation (Greses et al., 2020) respectively, which could be related with the high content of HPro and HAc in CSTRs (60–65% of the total SCFAs). *Bacteroides* population abundance, when using enzymatically-pretreated biomass, accounted for 6% in V and 3.5% in A, whereas members of Bacteroidales order, including *Bacteroides*, accounted for 23.5% and 24.3% in CSTRs fed with non-pretreated and T-pretreated microalgae, respectively. Since *Bacteroides* are involved in the hydrolysis of carbohydrates, lipids and proteins, the high organic matter solubilization resulting from the V and A pretreatments probably prevented these species to grow (Lim et al., 2020). On the contrary, the highest abundance of Porphyromonadaceae family (31.2%) was found in the microbiome corresponding to V-pretreated substrate. This family is composed by acidogenic bacteria of carbohydrates (Braz et al., 2019; Wang et al., 2016) and therefore, the high relative abundance might be related to the high percentage of carbohydrates solubilized during V pretreatment (61.9%).

3.6. Canonical correspondence analysis: linking fermenter output with microbial communities

The pretreatments applied to microalgae and the soluble components released definitely affected the microbial population distribution in the AF reactor and thus, the bioprocess efficiency. Some trends could be drawn by analysing the correlation between the abiotic and biotic results retrieved from the CSTRs (Fig. 4).

Microbial community developed in the CSTRs fed with raw, V and T-pretreated microalgae presented a higher hydrolytic activity in comparison with the CSTR fed with A-pretreated microalgae. As it can be seen in Fig. 4, CCA revealed a strong correlation between non-pretreated as well as V and T samples with bacteria involved in the hydrolytic stage (Bacteroidales, Bacteroidetes, *Acinetobacter*, *Corynebacterium*). The significance of this correlation lies in the fact that these samples were situated opposite to soluble COD (Fig. 4). In this regard, the absence of pretreatment and the low efficiency of T and V pretreatments likely provoked the highest presence of strong hydrolytic communities needed to degrade a sturdy feedstock, such as microalgae biomass. In fact, the relative positions of hydrolytic microorganisms near to HAc indicated the linkage of HAc production during degradation of particulate biomass in these samples. Additionally, the shorter distance between samples taken from V and T-pretreated microalgae indicated a close relationship, supported by the similar results exhibited by these fermenters. These samples exhibited a positive correlation with bacteria belonging to Ruminococcaceae, Veillonellaceae, Lachnospiraceae, Porphyromonadaceae families and *Blvii28* genus (Fig. 4). The distribution of these microorganisms evidenced a correspondence with HAc, HPro and HBU production during acidogenesis of carbohydrates. In this sense, the high relative abundance of these microorganisms might be correlated with the carbohydrates solubilized during these pretreatments (V and T).

During A pretreatment, an opposite trend to macromolecular solubilization was found since statistical analysis showed A sample location in the line of soluble protein and COD maximization. The high percentage of organic matter solubilization promoted by this pretreatment supported the result observed in Fig. 4. Moreover, the high COD and proteins content in the soluble phases were greatly correlated to the presence of genus *Peptostreptococcus*, *Vagococcus*, *Enterococcus* and *Clostridium* (Fig. 4). The presence of these genera supported the interrelation found with the content of HAc, HBU and

HVal.

Overall, the linkage found between microorganisms, feedstock composition and SCFAs production (yield and profile) provided a valuable knowledge for further process optimization. These results revealed the microorganisms that should be promoted to maximize SCFAs production, based on the macromolecular components condition (particulate and soluble fractions) in the feedstock.

4. Conclusions

When using microalgae biomass as feedstock, the use of a pretreatment and specific macromolecule solubilization have a strong effect on microbial distribution and therefore, in SCFAs bioconversion yields. Out of the three tested pretreatments, protease pretreatment resulted in both highest solubilization and organic matter conversions into SCFAs. Additionally, operational conditions also affected the bioconversion efficiency. The low HRT (8 days) and 25 °C promoted bacterial community specialization and prevented slow-growing microorganisms involved in the methanogenic step. The outcome of this microbial specialization was a SCFAs accumulation. The use of a pretreated microalgae, whereby high organic matter was solubilized, mediated the development of acidogenic population instead of hydrolytic communities needed to degrade sturdy substrates such as microalgae. While the use of proteases as a pretreatment promoted the abundance of bacteria involved in the fermentation of carbohydrates (Firmicutes), the use of carbohydrases promoted Bacteroidetes phylum development due to its proteolytic capacity. In this sense, the relative abundance of the different determined phyla was influenced by the macromolecular solubilization obtained after each pretreatment. Therefore, the knowledge about substrate-microbiome-products nexus can be employed as a tool to optimize AF for SCFAs.

Credit authors statement

An accurate and detailed description of authors contributions to the published work is summarized herein:

Mercedes Llamas: Conceptualization, Formal analysis and Writing - original draft. Jose Antonio Magdalena: Conceptualization, Formal analysis and Writing - original draft. Silvia Greses: Software, Formal analysis, Data curation and Writing - original draft. Elia Tomás-Pejó: Validation, Writing - review & editing, Supervision and Funding acquisition. Cristina González-Fernández: Conceptualization, Validation, Writing - review & editing, supervision and Funding acquisition.

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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Publication II

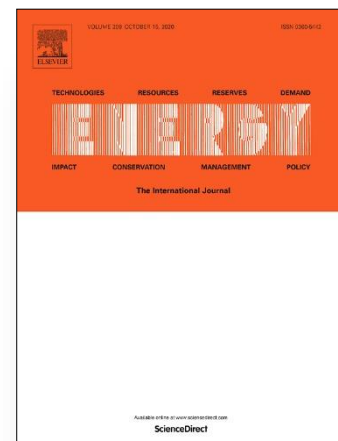
Microalgae-based anaerobic fermentation as a promising technology for producing biogas and microbial oils

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Microalgae-based anaerobic fermentation as a promising technology for producing biogas and microbial oils

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ABSTRACT

This study assessed the potential of using microalgae anaerobic fermentation as a novel choice for producing microbial oils and biogas. Microalgae biomass (*Chlorella vulgaris*) was used for volatile fatty acids (VFAs) production at three organic loading rates (OLR = 6, 9, 12 g COD/Ld). Regardless of the OLR, anaerobic systems displayed similar organic matter conversion yields into VFAs (VFAs-COD/COD_{in} = 38%) and VFAs distribution profiling. Obtained digestates were further used with a two-fold purpose, producing microbial oils from the VFA rich liquid fraction, and generating methane from the remaining solid spent. The yeast *Yarrowia lipolytica* was able to grow on VFAs concentration up to 17.6 g/L while the highest VFAs concentration (24.2 g/L, digestate obtained at 12 g COD/Ld) resulted inhibitory. Despite of that, *Y. lipolytica* exhibited remarkably high lipid content (15–23%) when grown in digestates. The three spent solid fractions (corresponding to the 3 OLRs) reached the same methane potential.

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1. Introduction

Microalgae have been the focus of a large amount of research studies due to their capacity of producing not only biofuels but also high value-added products. Within this perspective, microalgae have been considered a potential resource to be used in a biorefinery facility [1–3]. In this context, value-added products can be firstly produced, generating concomitantly low cost products such as energy carriers from the spent biomass. When dealing with added-value products for food or feed purposes, microalgae biomass should be cultivated in expensive synthetic media and axenic conditions. However, when targeting at low-cost downstream processing, their cultivation in wastewater streams has been highly studied [4,5]. When microalgae thrive in such residual effluents, this biomass cannot be used for edible purposes but another type of low cost product biorefinery can be envisaged.

In principle, the microalgae biomass generated in wastewater is mainly devoted to biodiesel, bioethanol or biogas production. Nevertheless, little information exists on producing two or more bioproducts out of this biomass cultivated under non-axenic conditions. Indeed, most of the research has been focused on producing

biogas since anaerobic microorganisms, in charge of conducting this bioprocess, are robust and can use the three microalgae macromolecules (proteins, carbohydrates and lipids) [6]. Anaerobic digestion of the microalgae biomass has emerged as one of the most promising options for improving the economic and environmental sustainability of microalgae-based wastewater treatment [7,8]. Furthermore, anaerobic digestion could also be used as an alternative bioprocess to produce volatile fatty acids (VFAs) instead of biogas. VFAs are valuable chemicals produced during the middle stages (acidogenesis and acetogenesis) of anaerobic digestion. The interest in VFAs relies on their use as building blocks within the renewable-based biorefinery concept [9,10]. These short-chain carboxylates have been mainly investigated as marketable chemicals [9], converted to their corresponding alcohols [11] or elongated to medium chain carboxylates (C7–C9) [12]. However, there are still barriers that prevent VFAs production from waste streams, namely the persistence of methanogens competing for VFAs and VFAs recovery limitations. The use of protein rich microalgae can be extremely beneficial to circumvent the first barrier since degradation of protein-rich substrates leads to high ammonium concentration, which in turn inhibits archaea activity hampering methane production [13]. Likewise, in order to overcome the second barrier, avoiding VFAs recovery and purification requirements will be a step forward in the use of the VFAs platform. Under this approach, the use of oleaginous yeasts utilizing VFAs as a carbon source instead of

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the traditional sugar-based substrates for oil production is nowadays an innovative approach of high interest.

Stoichiometric yields that can be attained by oleaginous yeasts using xylose or glucose are well known [14], while the knowledge on VFAs potential and limitations as low cost carbon source is really limited. Moreover, the effect of real digestates containing VFAs mixtures together with other chemicals that digestates might contain has been scarcely studied since normally synthetic mixtures have been used [15,16]. Within this research field, the current investigation was designed to gain insights on yeast's VFAs tolerance, uptake preference and lipid accumulation capacity.

The aim of the current investigation was to assess the potential of microalgae anaerobic fermentation as a novel choice to produce microbial oils and biogas. For such a goal, anaerobic digestion of microalgae biomass was conducted at three increasing organic loading rate (OLR) to investigate the microalgae biomass bioconversion into VFAs and evaluating the use of this microalgae digestate within a two-fold approach. On one hand, the liquid fraction (rich in VFAs) was assessed for microbial oil production via oleaginous yeast fermentation while the solid fraction was further used for biogas production via conventional anaerobic digestion. This alternative use of digestate allows energy recovery in the form of biogas and enables the use of VFAs-rich effluents for oil production (fuel precursor).

2. Material and methods

2.1. Substrate and microorganisms employed

2.1.1. Microalgae

Chlorella vulgaris, purchased from Allmicroalgae (Lisbon, Portugal), was used as substrate for anaerobic digestion. Macromolecular composition of microalgae biomass was 57.9% proteins, 21.6% carbohydrates, 13.4% lipids and 7.1% ashes (w/w, dry weight). A proteolytic pretreatment "Alcalase 2.5 L" was applied to avoid hydrolysis limitation and enhance VFAs production. The enzyme dosage ($0.585 \text{ UA g}^{-1} \text{ TS}^{-1}$) and pretreatment procedure was based on previous results [13,17].

2.1.2. Anaerobic sludge

Continuous anaerobic digestion of the pretreated microalgae biomass was carried out using adapted anaerobic sludge in a completely stirred tank reactor (CSTR). This inoculum was taken from an acidogenic reactor (25°C and fed with *C. vulgaris* as substrate) in order to obtain high VFAs concentrations. Total solids (TS) and volatile solids (VS) were 8.3 and 6.3 g/L, respectively.

Batch anaerobic digestion assays (BMPs) of the solid residue were carried out using mesophilic anaerobic sludge provided by the wastewater treatment plant (WWTP) of Valladolid (Spain). In this case, TS and VS were 23.7 g/L and 17.1 g/L, respectively.

2.1.3. Yeast

Yarrowia lipolytica (CECT1240) was obtained from the Spanish Type Culture Collection. The strain was maintained at $4 \pm 1^\circ\text{C}$ and regularly sub-cultured on yeast extract-peptone-dextrose (YPD) agar plates, containing (g/L): yeast extract, 10; peptone, 20; glucose, 20; and agar, 20. For preinoculum growth, one colony was inoculated into 100 mL YPD liquid medium (composition described above except for the agar). The preculture was grown in a rotary shaker at 150 rpm and 25°C overnight, until the culture reached the late exponential growth phase.

2.2. Digesters set up for the different tested OLRs

Continuous acidogenic anaerobic digestion to achieve high VFAs

production was carried out in 1 L CSTR (250 rpm, 25°C , HRT 8 days $^{-1}$). pH was monitored but not controlled during the experiment. Increasing OLRs (6, 9 and 12 g COD/Ld) were applied to test the influence of this parameter on VFAs production. The effluent of this reactor composed of three different VFAs concentrations depending on the OLR were centrifuged and separated into two different fractions for its revalorization. The liquid fraction was used as culture media for lipid production by oleaginous yeasts whereas the solid fraction was subjected to anaerobic digestion to obtain bioenergy in form of methane.

2.3. Oleaginous fermentation of the liquid digestate fractions

The liquid fraction of anaerobic digestates was sterilized by filtration ($0.22 \mu\text{m}$ pore) and subsequently used as substrate for microbial oil production by *Y. lipolytica*. Experiments were performed in batch mode in 250 mL Erlenmeyer with baffles containing 50 mL digestate without any additional nutrient. All the cultures were inoculated at initial OD_{600} of 0.3. Temperature and agitation were maintained at 25°C and 150 rpm in a rotary shaker. pH was adjusted at 6.0 ± 0.5 and not controlled during the process. Biomass production and VFAs consumption were monitored daily. Once fermentation was finished (substrate entirely exhausted and maximum biomass growth), yeast lipid production was evaluated. All fermentation tests were performed in triplicate.

2.4. Biochemical methane potential (BMPs) of the solid digestate fractions

Biochemical methane potential assays (BMPs) were run in triplicate to evaluate methane potential of the solid residues obtained at 3 different OLR (6, 9 and 12 g COD/Ld, Fig. 1). Mesophilic temperature (35°C) and organic load of $0.5 \text{ g COD g VS}^{-1}$ were selected to conduct the BMPs. Anaerobic digestion was carried out in 120 mL bottles containing 70 mL working volume. Blank experiments were set in order to estimate the inherent methane potential of the sludge. Initial pH was adjusted to 7.5 at the beginning of the experiment and bottles were flushed with helium to ensure anaerobic conditions. Methane production at standard temperature and pressure (STP) was calculated by subtracting the blank methane production to the amount of methane measured in each sample. Experimental methane productions from the different BMPs were fitted with the modified Gompertz equation (Eq. (1)) [18].

$$P(t) = P_{\infty} \exp \left[- \exp \left(\frac{R_m}{P_{\infty}} (\lambda - t) + 1 \right) \right] \quad (1)$$

$P(t)$ is the accumulated methane production at Standard Temperature and Pressure ($\text{mL CH}_4 \text{ STP g COD}_{\text{in}}^{-1}$), P_{∞} is the potential methane production ($\text{mL CH}_4 \text{ STP g COD}_{\text{in}}^{-1}$), R_m the maximum methane production rate ($\text{mL CH}_4 \text{ day}^{-1}$), λ the lag phase (days) and t the elapsed time (days).

2.5. Analytical methods

Test kits (Merck, ISO 15705, ISO 000683) were employed for quantification of total and soluble COD and N-NH_4^+ , respectively.

VFAs (acetic, propionic, iso-butyric/butyric, iso-valeric/valeric and caproic acid) were measured by liquid chromatography (HPLC) analysed through an Agilent 1260 HPLC-RID. A Cation H Refill Cartridge Microguard column (Biorad) and an Aminex HPX-87H ion exclusion column ($300 \times 7.8 \text{ mm I.D.}$) (Biorad) were used. Mobile phase was 5 mM H_2SO_4 , and elution was conducted in isocratic mode at a flow rate of 0.35 mL min^{-1} . The injected sample volume

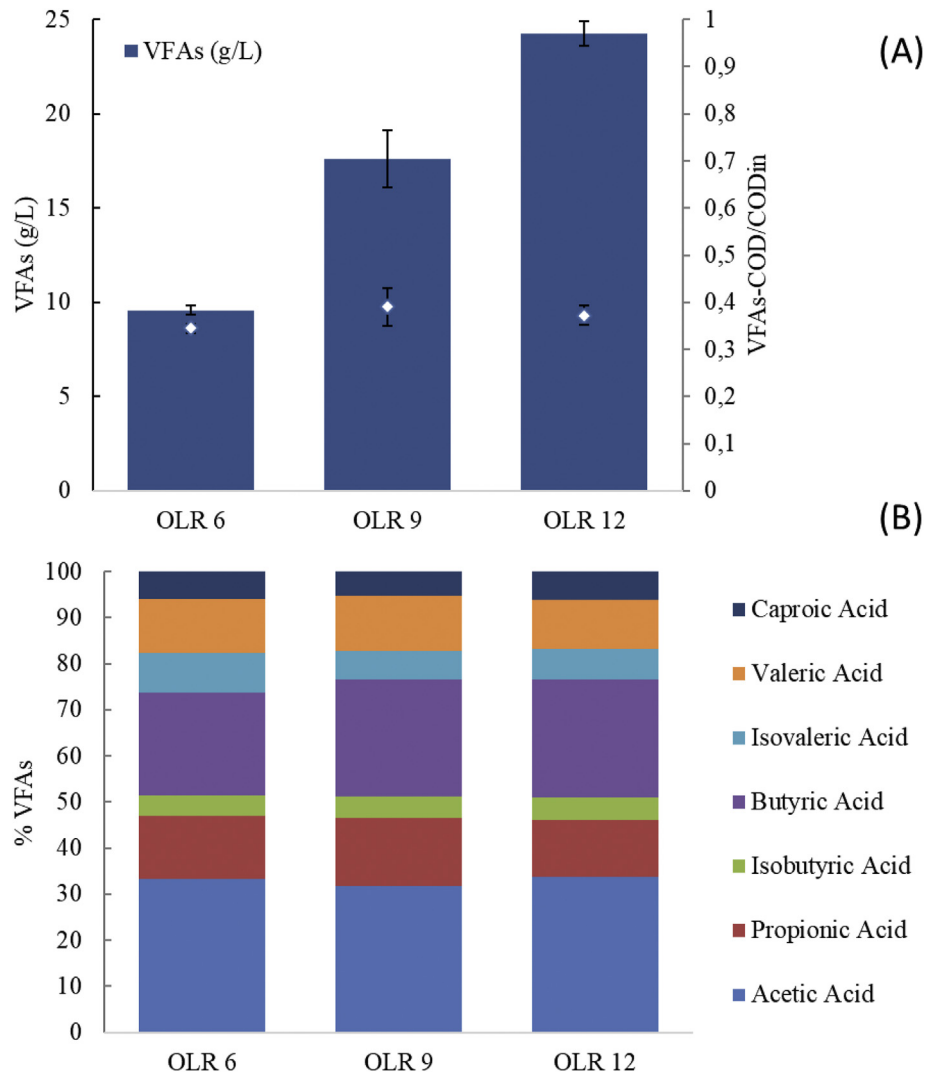


Fig. 1. VFAs concentration and organic matter conversion yields (empty dots) (A) and VFAs profile (B) achieved at the steady state during the anaerobic fermentation of microalgae biomass at increasing OLR.

was 20 μ L, and the oven and detector temperatures were 25 and 35 $^{\circ}$ C, respectively. VFAs (acetic, propionic, butyric and iso-butyric, valeric and iso-valeric and caproic) mixtures from 0.01 to 1 g/L of each acid (Sigma-Aldrich, Spain) were used as external standards. Gas chromatography was used to analyse biogas composition. The equipment was coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6-60/80 Sulfinert P packed column (7' x 1/8" O.D.) and a MS13X4-09SF2 40/60 P packed column (9' x 1/8" O.D.) (PerkinElmer). Several gas mixtures were employed as calibration standards in the GC-TCD measurements. Specifically, pure H₂; 3% H₂ and mixtures 50% CO₂/CH₄, 25% CO₂-75% CH₄ supplied by Praxair-Nippon (Spain) were employed.

Yeast growth was estimated by measuring optical density in a spectrophotometer (Spectroquant® Pharo 100) at 600 nm. Dry cell weight was determined gravimetrically by filtering 2 mL of culture through a 0.45 μ m glass fiber membrane (Merck Millipore). Filters were washed with MilliQ water and dried to a constant weight at 105 $^{\circ}$ C. For lipid analysis, yeast biomass was harvested at the end of the fermentation process by centrifugation at 14,000 rpm for 10 min at 4 $^{\circ}$ C (Heraeus, Megafuge16, Thermo Scientific). The supernatant was collected and stored at -20 $^{\circ}$ C for further analysis.

Total cellular lipids extraction was performed according to the method described by Folch [19]. Briefly, intracellular lipids were extracted in chloroform:methanol (2:1 v/v) at 100 $^{\circ}$ C under reflux for 4 h. The extract was filtered and the organic phase was washed with a 0.88% (w/v) KCl solution. Finally, the solvent was evaporated at 40 $^{\circ}$ C under vacuum using a Rotavapor R-215 device (BUCHI, Flawil, Switzerland), and the total cellular lipids were gravimetrically determined.

3. Results and discussion

3.1. Anaerobic digestion of microalgae biomass: VFAs production

Microalgae biomass was digested in a CSTR at 3 increasing OLRs. VFAs concentration at the stationary state increased concomitantly with the OLR applied. Total VFAs concentration was 9.6, 17.6, 24.2 g/L for the OLR of 6, 9 and 12 g COD/Ld, respectively (Fig. 1A). Nevertheless, the organic matter conversion efficiency (VFAs-COD/COD_{in}) was similar at the 3 assessed OLRs. More specifically, the values ranged 0.30 VFAs-COD/COD_{in} (i.e. 30-36%) regardless the OLRs (Fig. 2A). This value was in good agreement with values attained for microalgae biomass (38%) [20] or waste activated

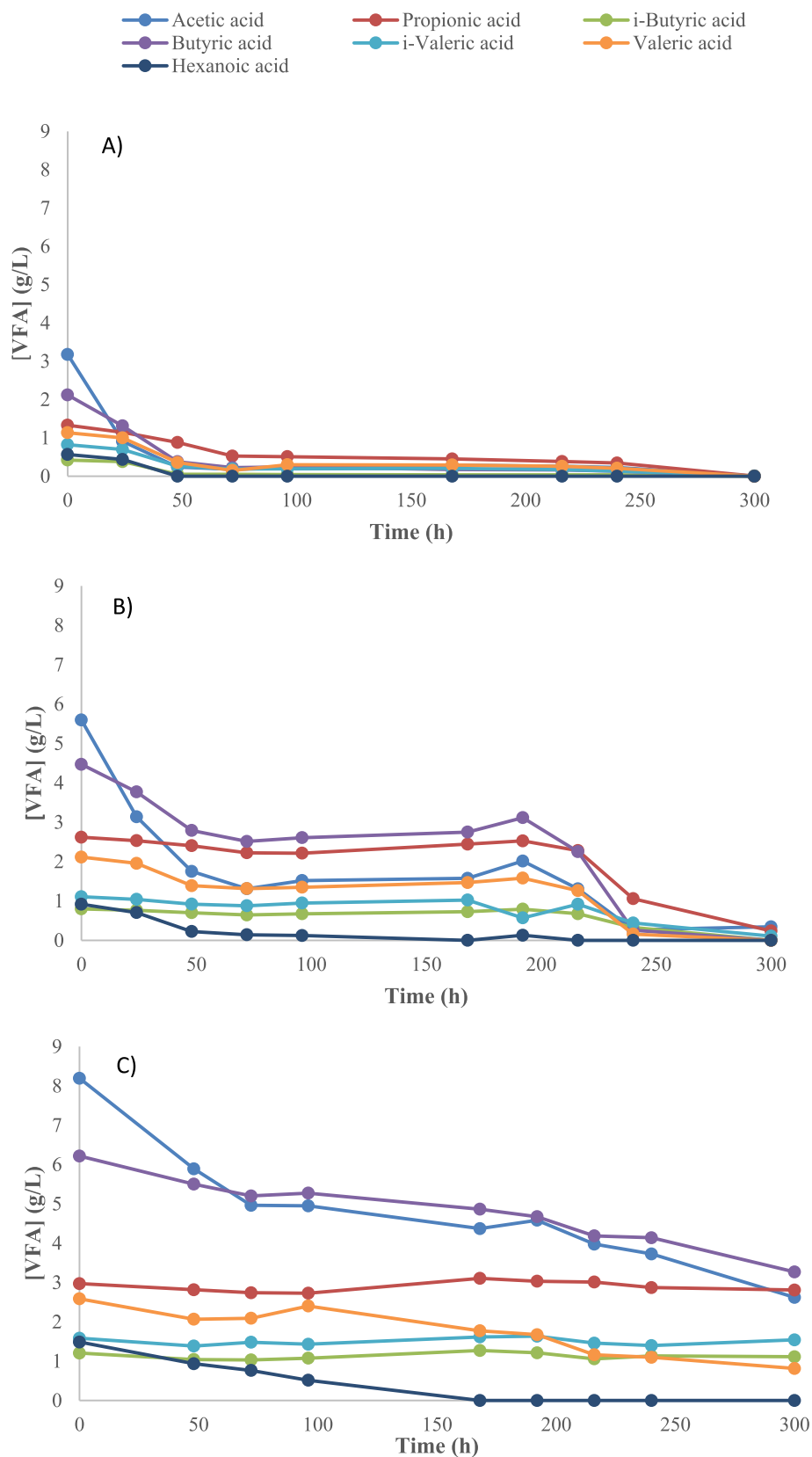


Fig. 2. Time course of VFAs consumption via yeast fermentation for the different digestates (A) OLR 6, (B) OLR 9 and (C) OLR 12 g COD/Ld.

sludge (30%) [21] operating CSTRs under psychrophilic temperature. The maintenance of the organic matter conversion efficiencies into VFAs independently of the OLR increase clearly showed the robustness of the process. This lack of OLR effect on VFAs conversion yields could be explained by the rates at which each phase of the anaerobic digestion proceeds. OLR and hydraulic retention time are interconnected digestion parameters. In this sense, an optimum trade-off between both parameters should be set to avoid organic overloading or underestimated feeding requirements. Out of the four stages involved in anaerobic digestion, methanogenesis and hydrolysis are the slowest phases while acidogenesis is the fastest [22]. In this particular case, in which VFAs were produced from pretreated microalgae biomass, it can be concluded that high OLR could be used without diminishing the conversion yield while producing high VFAs concentration (approximately 25 g/L, Fig. 1A). It should be highlighted that in the present study only the first stages of anaerobic digestion were targeted for VFAs production (hydrolysis and acidogenesis). More specifically, hydrolysis rate limitation was considered negligible since a proteolytic pretreatment of microalgae biomass was used to fully focus on the acidogenesis stage. At the same time, operational conditions imposed to the system such as the low HRT (8 days⁻¹) was intended to reduce the methanogens activity by provoking their wash out from the system. In this way, methane production was avoided and organic matter remained in VFAs-form instead of being transformed into biogas. Therefore, the HRT used was enough to provide similar VFAs conversion yields regardless the OLRs applied.

Regarding the effect of the OLR used to feed the reactors on VFAs production profile, no changes were evidenced (Fig. 1B). Acetic acid represented approximately 33% of the total VFAs. The abundance of this acid might result from acetoclastic methanogenesis inhibition and/or VFAs degradation. In this sense, long-chain VFAs are converted into acetate and hydrogen through the β -oxidation pathway. Acetic acid was also found to be among the most abundant products in other studies using different substrates such as wasted activated sludge, maize silage and whey [23,24]. After acetic acid, the second main group of VFAs were C4–VFAs (butyric and isobutyric acids) ranging 27–30% and C5–VFAs (valeric and isovaleric acids) ranging 17–20% of the total VFAs. This profile was unexpected since normally propionic acid prevails in this type of microalgae biomass fermentation. According to previous works [20], propionic acid was the most prominent acid (31–36% of the total) when digesting *C. vulgaris* at 35 °C with HRT of 10 days, and OLR = 1.5–3 g COD/Ld. Most probably, the different digestion temperature combined with the high OLR used herein were responsible of the VFAs profile shift. With regard to the VFAs profile, the anaerobic microbial community showed again its robustness not only in terms of conversion yields but also in VFAs distribution upon the different OLR applied.

3.2. Oleaginous yeast cultivation in VFAs rich digestate: lipid production

3.2.1. VFAs consumption

Although all microorganisms are able to synthesize lipids, only some of them (called oleaginous) are able to accumulate more than 20% w/w of their dry weight. Microorganisms with high capacity to accumulate lipids can be found among bacteria, microalgae and fungi. Out of these microorganisms, yeasts present some advantages since they exhibit short life cycle, do not need of arable land, are easy to scale-up, can grow at high density and are not subjected to seasonal weather conditions. In this manner, the yeast *Y. lipolytica* was selected for VFAs consumption based on its metabolic flexibility in terms of carbon source usage. Opposite to what was observed during the anaerobic fermentation, the different VFAs

concentrations produced at different OLRs affected the oleaginous fermentation. In this manner, higher VFAs concentration implied lower consumption rates. Time course of VFAs consumption on the three digestates are shown in Fig. 2. 86% of the total VFAs present in the digestate coming from OLR 6 were consumed within the first 72 h of fermentation, while in the digestate coming from OLR 9, 240 h were needed to consume the same amount of VFAs. This difference in consumption rates was even more marked in the digestate coming from OLR 12, in which case only 50% of the total VFAs were consumed after 300 h. The final point of the oleaginous fermentation was set at 300 h, since at this point; VFAs were totally exhausted at OLR 6. In all cases, VFAs consumption seemed to take place in two stages (Fig. 2). The first stage would entail the first 72 h of cultivation followed by a lag phase. After 170 h, VFAs consumption resumed and was nearly exhausted in OLR 9 (Fig. 2B). In the case of OLR12, VFAs were consumed (as indicated in Fig. 2C) but total exhaustion was not achieved in 300 h of process. The limitation encountered at the highest VFAs concentration might be related to the chemical interference with the membrane transport of phosphate. In this sense, according to Rodrigues and Pais [25], high VFAs concentration would require high ATP expenditures and thus lipid accumulation and yeast growth might be hampered. As a matter of fact, it should be highlighted that *Y. lipolytica* was able to consume almost 18 g VFAs/L (OLR 9) after 300 h of fermentation. This VFAs concentration is far above the substrate used for *Cryptococcus curvatus* [26] or activated sludge [27] which could not grow at VFAs concentration higher than 10 g/L.

Acetic acid was by far the organic molecule that *Y. lipolytica* consumed the fastest. The ability for acetic acid consumption seems to be yeast and concentration specific. For instance, Yahara et al. [28], observed that yeast growth was disturbed when exceeding 2 g/L of acetic acid with *Brettanomyces bruxellensis* while Fei et al. [26], encountered no growth problems when using 3 g/L with *C. albidus*. In agreement with this later finding, *Y. lipolytica* was able to handle 5.6 g/L of acetic acid in the digestate OLR 9 or even 8.2 g/L of OLR 12 that could be presumably consumed with longer fermentation time. Previously to the lag phase (initiated at 72 h), the second fastest consumed VFA was butyric acid. Each VFA requires different metabolic steps to be used. While acetic acid is directly converted to acetyl-CoA and used in the tricarboxylic acid cycle, the rest of organic acids must be degraded to be further used. In the case of butyric acid, it is converted to butyryl-CoA and possibly degraded to 2 mol acetyl CoA via β -oxidation [29]. It can be assumed that hexanoic acid is used in a similar manner. As a matter of fact, hexanoic and valeric acids showed slightly lower consumption rates than butyric acid. It is also important to point out that it seems likely that each VFA might be used for different purposes. While acetic acid is used for growth requirement, butyric acid promotes lipid production [30]. In this manner, the digestates used in this study were suitable to foster not only yeast growth but also lipid accumulation. Propionic acid consumption mechanism is not clear but it is supposed to be similar to microalgae uptake [29]. In this case, propionic acid consumption was lower than that of acetic acid. Indeed, odd-VFAs (propionic and valeric acids) were only slightly consumed before the lag phase while their consumption rate increased during the second stage of the fermentation. Lastly, iso-VFAs forms also exhibited a much lower consumption rate than their linear counterparts indicating that isomeric forms could hamper their usage.

Despite of the different VFAs utilization by *Y. lipolytica* observed upon OLR, the carbon recycling ratio was similar for all of them. Carbon recycling ratios were calculated by multiplying the carbon conversion yield from VFAs to biomass and taking into account yeast carbon content of 49% [31]. The value of carbon recycling ratio ranged 30.4–34.5%. This means that although 50% of the VFAs

remained unconsumed in the case of OLR 12, the carbon contained in the VFAs was converted in biomass as efficiently as OLR 6. Moreover, it should be highlighted that this value was considerably higher than the values attained with microalgae consuming VFAs. More specifically, Kim et al. [32], calculated a carbon recycling ratio from 6.3 to 23.8% using *Chlorella vulgaris*. This fact evidenced the higher carbon recycling capacity of yeasts when compared to other microbial systems.

3.2.2. Yeast growth and lipid accumulation

As it can be seen in Fig. 3, yeast growth patterns were different for each OLR. In the case of OLR 6, steady biomass production of 3.38 ± 0.33 g/L was attained after 50 h of fermentation. This fermentation time corresponded as well to the moment at which most VFAs were consumed (Fig. 2A.). In the digestate coming from OLR 9, a diauxic growth was observed. More particularly, the first growth stage also reached stationary phase with 2.51 ± 0.30 g/L biomass at 50 h. After this point, a lag phase until 192 h of fermentation took place. After this time point, the second growth stage resulted in a total biomass concentration of 5.56 ± 0.51 g/L. This diauxic growth is a response of the microorganism making an adaptation to new conditions, i.e. a second substrate or carbon source. In fact, digestates are a clear example of a multi-nutrient environment. As it was mentioned above, VFAs were consumed differently depending on the carbon length and usage purposes (growth or lipid accumulation). With regard to the carbon length, the second growth stage was attributed to the consumption of odd carbon VFAs (C3 and C5) and iso-forms. During the 140 h of lag phase, the cell metabolisms changed to be able to consume those VFAs. In this manner, after this adaptive period, *Y. lipolytica* reached the highest biomass concentration. Opposite to that, digestate from OLR12 was inhibitory for this yeast that showed a biomass concentration of 2.88 ± 0.36 g/L after 150 h of fermentation. In this case, as it can be seen in Fig. 2C, the fermentation time set up was not enough to consume the odd carbon VFAs (C3 and C5) and iso-forms, as they remained in similar levels from the beginning of the fermentation.

Soluble CODs were measured at the end of the cultivation time to discard yeasts being fed on other carbon sources present in the digestate. By subtracting the contribution of VFAs from the initial soluble CODs, the values of soluble COD at the beginning of the fermentation were 6.9, 13.2 and 22.4 g/L for OLR 6, 9 and 12, respectively. At the end of the fermentation, the values remained constant pointing out to the fact that only VFAs were used as carbon source for growth purposes. Accordingly, biomass yields were

0.35 g/g VFAs for OLR 6 and OLR 9 in which cases all VFAs were consumed (Table 1). Likewise, since only 50% of VFAs were consumed in OLR 12, biomass yield attained in the case of OLR 12 was half of that obtained when using OLR 6 and OLR 9 digestates.

Biomass lipid content values were quite high regardless of the digestate used (Table 1). Lipid content was about 20% (w/w) of the biomass for the three tested digestates and thus lipids yields accounted for 0.07 g lipid/g VFAs for OLR 6 and 9. Even though the lipid yield was diminished in OLR 12 (0.02 g lipid/g VFAs) due to the decrease in biomass yield, it should be highlighted that the ability to accumulate lipids was not affected by the high VFAs concentration of this digestate. Likewise, ammonium concentration of the digestates were considerably high. This fact was of relevance since it has been claimed that nitrogen can hamper lipid accumulation when the substrates follow *de novo* lipids synthesis pathway [14]. Ammonium concentrations at the beginning of the fermentation were 1.9, 2.9 and 4.2 g/L for OLR 6, 9 and 12, respectively. Despite of the different ammonium concentration, COD/NH₄⁺ (C/N ratio) remained in the narrow range of 14.0–14.7 for the three digestates. Remarkably, the lipids synthesis pathway that the VFAs follow is still not clear. Hydrophilic substrates follow *de novo* pathway and are thus, affected by nitrogen content in the medium, while hydrophobic substrate follow *ex novo* pathway and are not affected by nitrogen [14]. Regardless of their water solubility characteristics, VFAs are sometimes classified as hydrophobic substrates [33] or hydrophilic [34]. It is then worth to mention that only a restricted number of yeast strains are able to grow using both hydrophobic and hydrophilic substrates, such is the case of *Y. lipolytica* [35]. Most probably, the use of such flexible metabolic strain helped in the consumption of different carbon length VFAs without nitrogen affecting the biomass lipid content.

3.3. Revalorization of the digestate solid fraction: methane production

Fig. 4 illustrates the variation of the specific methane yield (mL CH₄/g COD_{in}) against time for the BMP tests carried out on the solid samples recovered after digestate solid-liquid separation. The methane yields achieved after 38 days of digestion were approximately 200 mLCH₄/g COD_{in} (57% biodegradability) regardless the OLR of which the solid fraction was obtained. Unexpectedly, this value was considerable high when compared to the methane yield of microalgae biomass devoted to biogas production. As a matter of fact, the organic matter exhibited slightly lower biodegradability as pretreated microalgae biomass. For instance, Mahdy and co-

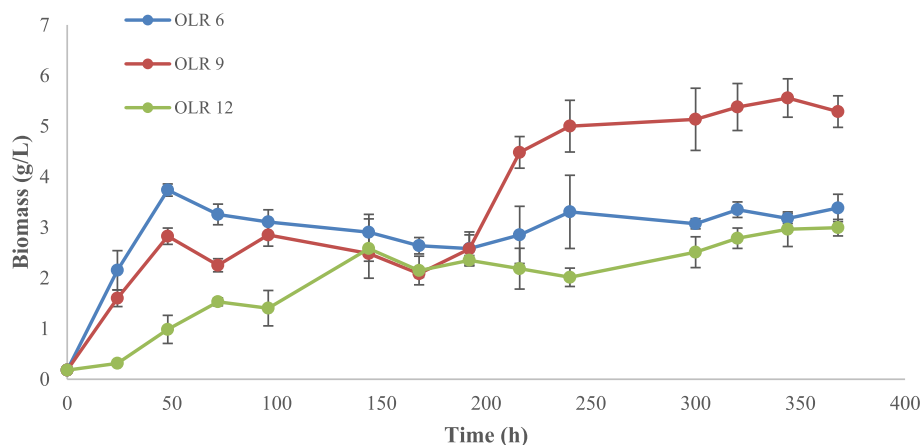
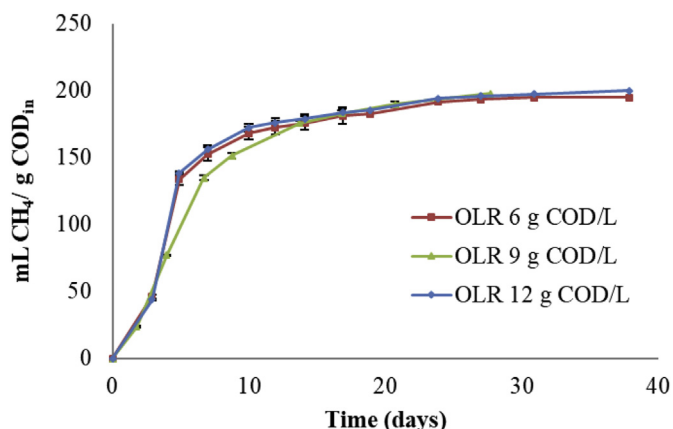


Fig. 3. Yeast growth patterns when cultured in the different digestates.

Table 1*Y. lipolytica* growth and lipid parameters on the different digestates used as culture medium.

Substrate Digestate	OLR6	OLR9	OLR12
VFAs concentration (g/L)	9.56 ± 0.24	17.62 ± 1.52	24.24 ± 0.66
Maximum biomass concentration (g/L)	3.38 ± 0.27	5.56 ± 0.51	2.88 ± 0.41
Biomass Yield (g/g)	0.35	0.32	0.12
Lipid content (%)	15.2 ± 2.84	19.6 ± 1.71	23.2 ± 3.15
Lipid concentration (g/L)	0.51	1.1	0.38
Lipid yield (g/g)	0.06	0.07	0.02

**Fig. 4.** Cumulative methane yield achieved by the spent solid fraction of the different digestates.

workers reported 73% anaerobic biodegradability of the same microalgae strain pretreated with the same proteolytic cocktail [17]. This fact evidenced the high biodegradability of the organic matter in the solid fractions obtained at the different OLRs. The similar biodegradability was in agreement with the trends observed during the fermentation conducted for VFAs production (Section 3.1.). In this manner, as VFAs conversion yields were similar upon the different OLRs, the remaining organic matter was expected to be similar as well. The BMPs results were modelled according to Gompertz model. As it can be seen in Table 2, the model presented a good fit (R^2 values). This modelization also allowed to confirm that kinetic parameters, including methane productivity and lag phase, were similar for the three solid fractions obtained from the different OLRs digestions. In terms of methane content in the biogas, no differences were recorded neither ranging from 61 to 67% v/v CH_4 .

Few studies have evaluated the potential energy contribution of anaerobic digestion to other microalgae based processes. The few reports available in the literature are mainly devoted to recover energy after microalgae lipid extraction. Those studies indicated that a considerable part of the total energy contained within the biomass can be recovered if anaerobic digestion of lipid-extracted microalgae is implemented [36,37]. However, this process has not been applied to recover energy out of the solid fraction generated after anaerobic fermentation conducted at different OLRs and thus,

Table 2

BMP results according to Gompertz model.

	OLR 6	OLR 9	OLR 12
P (mL CH_4 /g CODin)	184,64 ± 5,7	188,6 ± 2,2	187,2 ± 3,4
Rm (mL CH_4 /g CODin · d)	5,4 ± 0,1	4,6 ± 0,	1 5,7 ± 0,1
Lambda (d-1)	1,5 ± 0,1	0,8 ± 0,1	1,7 ± 0,1
R2	0,98 ± 0,01	0,99 ± 0,00 0,	98 ± 0,01

no comparison with literature could be accomplished. Overall, the BMPs showed that coupling the two bioprocesses, acidogenesis for VFAs production and full digestion for methane production did not affect the methane potential that can be attained from the solid fraction generated upon increasing OLRs.

4. Conclusion

Instead of the conventional use of microalgae biomass for biogas production purposes, this investigation has proven the possibility of producing microbial oils and biogas from microalgae anaerobic digestates. The microalgae biomass bioconversion into VFAs remained constant ($\pm 38\%$) regardless of the OLRs applied (6, 9 and 12 g COD/Ld). Not only VFAs yields but also VFAs profile were similar for the three digestates. The digestates liquid fraction were a good carbon source for lipid accumulation, as yeast lipid content reached 23% DW. Nevertheless, yeast growth was dependent on VFAs concentration. Despite of that, carbon-recycling ratio was considerably high and independent of the VFAs concentration used. The anaerobic biodegradability of the solid fraction was similar for the three digestates probably mediated by the similar behavior attained during the acidogenesis stage. This alternative use of digestate allows energy recovery in the form of biogas and enables the use of VFAs rich effluents for oil production.

CRediT authorship contribution statement

Mercedes Llamas: Investigation, Writing - original draft. **Jose Antonio Magdalena:** Investigation, Writing - review & editing. **Elia Tomás-Pejó:** Validation, Supervision, Writing - review & editing. **Cristina González-Fernández:** Conceptualization, Writing - original draft, 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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Publication III

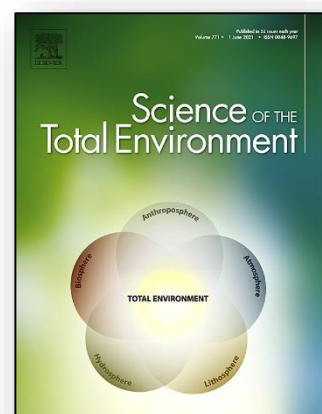
Driving microbial community towards microalgae biomass harnessing: short-chain fatty acids and biogas production

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DRIVING MICROBIAL COMMUNITY TOWARDS MICROALGAE BIOMASS HARNESSING: SHORT-CHAIN FATTY ACIDS AND BIOGAS PRODUCTION

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KEYWORDS: Anaerobic fermentation; anaerobic digestion; *Chlorella vulgaris*; short-chain fatty acids; methane; microbial community.

ABSTRACT

Microalgae have been extensively studied as feedstock for biogas production. As an alternative to this approach, this work evaluates the potential of microalgae to produce not only biogas but also high value-added products via an innovative two-stage sequential anaerobic bioprocesses. For this purpose, *Chlorella vulgaris* was subjected to anaerobic fermentation (AF) for short-chain fatty acids (SCFAs) production. Subsequently, the solid spent of AF effluent was valorized for biogas production via anaerobic digestion (AD). Aiming at understanding microorganisms' roles in both bioprocess, microbial communities were thoroughly characterized.

Microbial community in AF exhibited a notable specialization to convert microalgae biomass into SCFAs. Firmicutes was the prevailing phylum, accounting for the 64 % of the population, followed by Proteobacteria (11.7 %) and Actinobacteria (11.4 %) phyla. The high hydrolytic and acidogenic activities of these phyla mediated the accumulation of propionic, acetic and valeric acids (25.6, 24.3 and 20 % of total SCFAs, respectively),

26 resulting in a SCFAs bioconversion efficiency of 32.1 % g SCFAs-COD/g COD_{in}. The
27 AD of the solid spent exhibited wider microbial biodiversity than the AF reactor. During
28 this AD, the microbiome was mainly composed by Firmicutes (28.6 %), Euryarchaeota
29 (17.7 %), Proteobacteria (15.3 %), and Bacteroidetes (10.9 %). This microbial population
30 gave rise to a methane yield of 168.9 mL CH₄ /g COD_{in}, similar to the values obtained
31 when subjecting the whole microalgae biomass to AD.

32 This study proved the efficient generation of two bioproducts, SCFAs and biogas, from
33 *C. vulgaris* biomass by applying two sequential bioprocesses. Despite of the fact that both
34 bioprocesses were seeded with the same anaerobic inoculum, microbial community
35 specialization of each bioprocess was promoted by applying proper operational
36 conditions. This fact reveals the tremendous flexibility of the anaerobic microbial systems
37 to adapt and give rise to different bioproducts. AF performed prior AD enhanced the solid
38 residue biodegradability. Additionally, hydrolytic and acidogenic microorganisms
39 dragging from AF reactor to AD reactor boosted biogas production from the solid residue.
40 Overall, this unconventional two-stage anaerobic bioprocess allows the valorization of
41 microalgae biomass into biogas after SCFAs extraction.

1. BACKGROUND

In the transition to a sustainable bio-based society, implementing processes where renewable feedstock are converted into bioproducts and biofuels is of utmost importance. The economic feasibility of the bioproducts is essential for a suitable development of the bio-based industry. In this sense, the use of low-cost renewable sources, such as waste streams, arises as an attractive option to decrease production costs while meeting the goals of the circular economy [1].

Anaerobic digestion (AD) is the most exploited bioprocesses for wastes treatment since it can be applied to a wide variety of substrates regardless of their macromolecular composition. During AD, complex organic matter is degraded and converted into biogas, which can be energetically revalorized. AD is performed by a synergic microbial consortium in four sequential steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) where the products of one step are used as substrate for the following stage. This bioprocess can be performed in the traditional way where the four steps end up in biogas production, or it can be applied to produce short-chain fatty acids (SCFAs) in a shortened AD [2,3]. SCFAs are carboxylic acids from C2 to C6 acid generated after hydrolysis and acidogenesis in the so-called anaerobic fermentation (AF). These carboxylates, considered valuable building blocks for the chemical industry, might be a product by themselves or can serve as precursors for several bioproducts (alcohols aldehydes, ketones, elongated to medium chain carboxylates, etc.), entailing applications in different fields including solvents, adhesives, food additives, cosmetics and pharmaceutical industries [4]. SCFAs production through AF is proposed as a suitable and environmentally-friendly alternative to their conventional petrochemical production. Microalgae biomass that can be generated during wastewater bioremediation may be considered an attractive low-cost feedstock for SCFAs production [5]. Its protein-rich

composition (30-60 % dry weight) has been reported to be an advantageous feature when SCFAs are targeted [6]. During protein degradation, nitrogen is released in the form of ammonium (NH_4^+) and free ammonia (NH_3). High concentrations of these compounds can inhibit methanogenic population that consume SCFAs for biogas production, thereby fostering SCFAs accumulation [7].

Microalgae has been extensively studied for biogas production under different digestion configuration [8,9]. One option to increase biogas production might be the use of two-stage AD strategy [10–12]. This configuration decouples the digestion in two bioreactors, namely hydrolytic-acidogenic reactor and acetogenic-methanogenic reactor. This two-stage digestion allows implementing optimum environmental conditions for each microbial group, favouring bioprocess control and maximizing production yields. An alternative to this approach is to use the two-stage strategy to increase products output of this biomass instead of just maximizing biogas production. This integration supports not only wastes treatment but also carbon recovery maximization through multiproduct generation. To address this alternative, the current work assessed the use of microalgae biomass for the production of SCFAs-rich liquid fraction and the further use of the solid spent biomass after AF for biogas production. Aiming at establishing a link between microbial activity and process performance to ensure high product yields, the sequential two-step anaerobic bioprocess was evaluated in terms of SCFAs production and profile, methane yields and microbial communities developed in each reactor to identify key microorganisms.

2. RESULTS AND DISCUSION

2.1. Microbial diversity determined in the AD and AF reactors

Both AF and AD reactors were started up with the same inoculum but fed with different substrate (pretreated microalgae in AF or solid spent of fermented microalgae in AD). The microbial community in the inoculum and reactors was analyzed to elucidate the microbial shift associated to the reactors producing SCFAs or CH₄.

The effect of substrate composition and operational parameters on the microbial community can be observed in the calculated biodiversity indexes (**Table 1**). 1141 OTUs were identified in the inoculum corresponding with 7.506 Shannon index, which was the highest microbial diversity of all samples. This is a common feature in conventional anaerobic digesters devoted to biogas production since these processes are normally conducted at high hydraulic retention time (HRT) (15-30 days) to promote hydrolytic activity and slow-growing microorganisms such as methanogenic archaea. High biodiversity is a key requirement for inocula since the presence of a wide variety of microorganisms and metabolic pathways able to transform the organic matter into the desirable product favors the microbial adaptation to different processes.

Table 1. Biodiversity indexes calculated for the microbial community of inoculum, anaerobic fermenter and anaerobic digester

Biodiversity index	Inoculum	AF reactor	AD reactor
OTUs	1141	363	747
Shannon	7.506	5.545	6.818

Opposite to the inoculum, microbial community in AF reactor exhibited a notable diversity decrease to 363 OTUs (5.545 Shannon index), reflecting a possible microbial community adaptation and specialization to transform microalgae biomass into SCFAs. In the case of the microbial community developed in the AD reactor, the biodiversity also decreased when compared to the inoculum according to calculated Shannon index (6.818) but not as drastically as observed for the AF. Inoculum and AD reactor were aimed at

biogas production while AF reactor was set to avoid the methanogenic stage and promote SCFAs accumulation. This fact could imply the highest decrease in biodiversity observed in the AF reactor and it could be verified by evaluating the shared OTUs within the samples (**Figure 1**). As a matter of fact, the 3 samples shared 324 OTUs, while inoculum and AD reactor exclusively shared 291 OTUs. These values revealed the adaptation of the microorganisms in the inoculum to the AD of the solid spent. By contrast, inoculum and AF reactor microbiomes uniquely shared 1 OTU, and no OTUs were shared with the AD reactor. These values suggested that the 324 OTUs shared between all samples (inoculum, AF and AD) corresponded to hydrolytic and fermentation stages while microorganisms involved in biogas production were probably eliminated from the system in the AF reactor due to the imposed operational conditions.

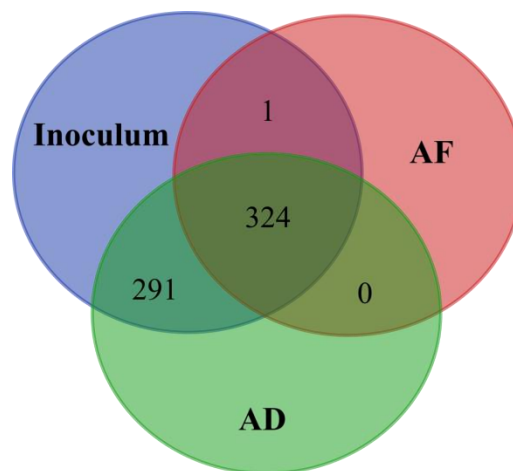


Figure 1. Venn diagram showing the OTUs shared among the inoculum, AF and AD reactors.

2.2. Microbial community present in the inoculum

As it can be seen in **Figure 2a**, the inoculum was represented by a balanced variety of bacterial phyla that comprised Chloroflexi (20.9 %), Actinobacteria (19.9 %),

Proteobacteria (11.8 %), Bacteroidetes (11.2 %), Firmicutes (10.0 %), Amatinomonadetes (2.9 %) and Synergistetes (2.6 %), among others. These phyla and their even profile are common in sewage anaerobic digesters operated at conventional HRT (15-30 days) for producing biogas [13,14]. Chloroflexi was mainly characterized by *Caldilinea* and T78 genera (Figure 2b, Table 2).

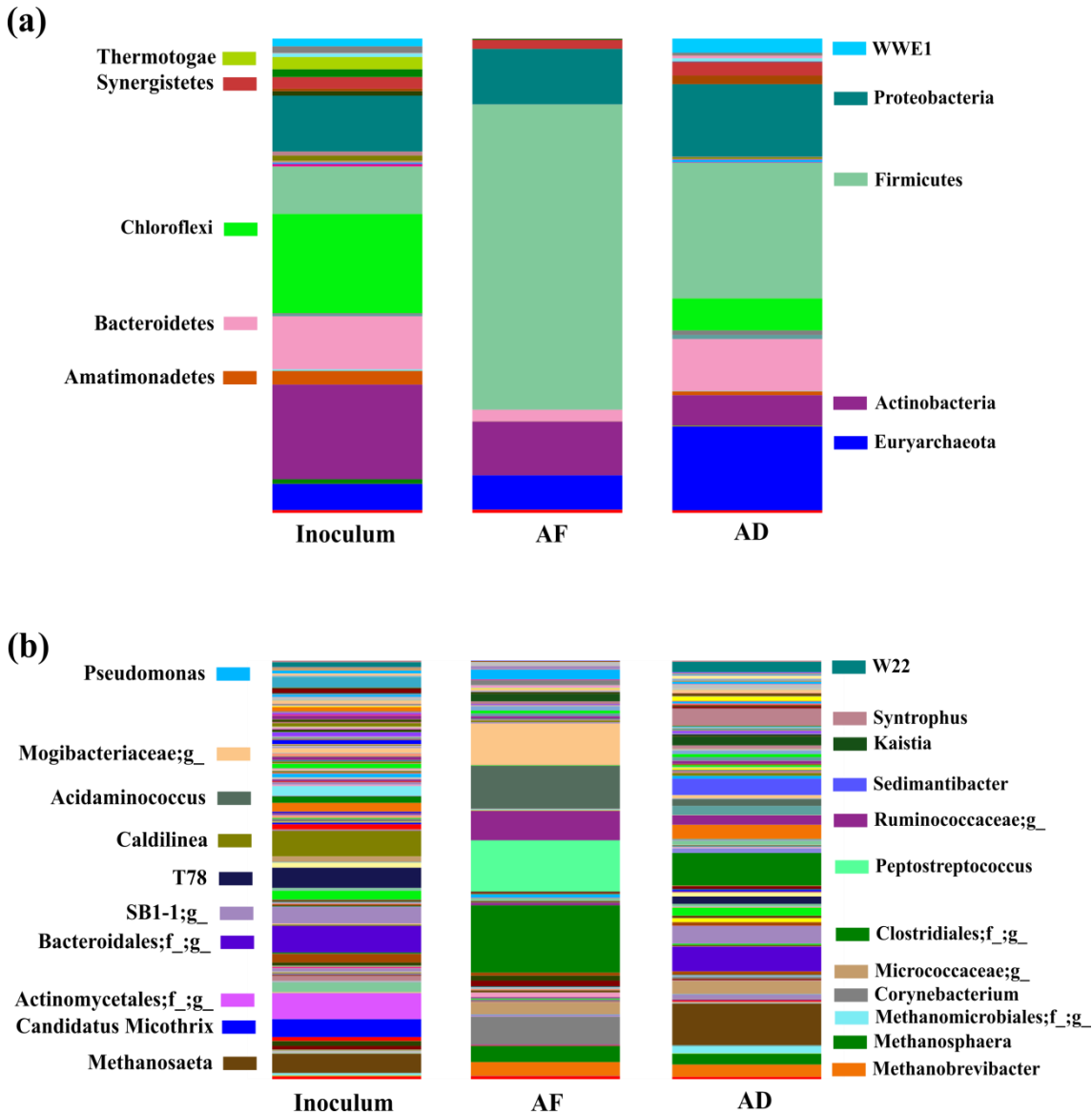


Figure 2. Bacteria and archaea composition in the inoculum, AF and AD reactors in terms of relative abundance at phyla (a) and genera levels (b). The legend displays taxonomic groups with relative abundance higher than 1 %.

Table 2. Most abundant bacteria and archaea identified in the inoculum, AF and AD.

Phylum	Class	Order	Family	Genus	Relative abundance (%)
Inoculum					
Bacteria domain (93.9 %)					
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthroxaceae	<i>Candidatus Microthrix</i>	4.3
Actinobacteria	Actinobacteria	Actinomycetales	-	-	6.2
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	-	2.3
Bacteroidetes	Bacteroidia	Bacteroidales	-	-	6.3
Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	-	3.9
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	-	2.1
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78	6.1
Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	<i>Caldilinea</i>	6.1
Firmicutes	Clostridia	Clostridiales	-	-	3.8
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	-	2.3
Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	SC103	2.5
WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22	1.2
Archaea domain (5.5 %)					
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	<i>Methanosaeta</i>	4.6
Euryarchaeota	Methanomicrobia	Methanomicrobiales	-	-	0.5
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobrevibacter</i>	0.2
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanosphaera</i>	0.1
AF					
Bacteria domain (92.1 %)					
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	<i>Corynebacterium</i>	6.6
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	-	2.9
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	<i>Bacteroides</i>	1.1
Firmicutes	Clostridia	Clostridiales	-	-	16.1
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	<i>Peptostreptococcus</i>	12.2
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-	7.1
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	<i>Acidaminococcus</i>	10.3
Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	-	9.8
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Kaistia</i>	1.8
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	2.3
Archaea domain (7.2 %)					
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobrevibacter</i>	3.3
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanosphaera</i>	3.8
AD					
Bacteria domain (81.7 %)					
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	-	3.1
Bacteroidetes	Bacteroidia	Bacteroidales	-	-	5.9
Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	-	4.2
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	-	1.9
Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78	1.8
Firmicutes	Clostridia	Clostridiales	-	-	7.9
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	-	3.4
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-	2.3
Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	<i>Syntrophomonas</i>	2.2
Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	<i>Sedimentibacter</i>	3.9
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Kaistia</i>	2.1
Proteobacteria	Deltaproteobacteria	Syntrophobacteriales	Syntrophaceae	<i>Syntrophus</i>	4.1
WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22	2.5
Archaea domain (17.7 %)					
Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	<i>Methanosaeta</i>	9.9
Euryarchaeota	Methanomicrobia	Methanomicrobiales	-	-	1.7
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobrevibacter</i>	2.9
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanosphaera</i>	2.6

These bacteria are known as hydrolytic and acidogenic microorganisms that exhibit low growth rates and a negative response against process inhibitors [15]. Consequently, the presence of bacteria belonging to Chloroflexi phylum indicated that the inoculum came from a stable AD operated at an elevated HRT. Actinobacteria, mainly constituted by Acidomicrobiales and Actinomycetales orders (**Table 2**), are involved in the degradation of complex carbohydrates and proteins, as well as in the acetic acid (HAc) production during the fermentation stage [16]. Similarly, bacteria belonging to Bacteroidetes phylum also play a key role in HAc and propionic acid (HPro) production [17]. Both phyla, Actinobacteria and Bacteroidetes, have been reported as growth promoters of syntrophic bacteria and acetoclastic archaea [17]. Proteobacteria phylum comprises carbon-degrading bacteria including syntrophic acetogens [18], while Firmicutes phylum is characterized by hydrolytic and acidogenic bacteria involved in SCFAs production (HAc and butyric acid (HBu)) and in syntrophic acetate oxidation such as some members of Clostridiales order (**Table 2**) [17,19]. This bacterial distribution indicated that the inoculum exhibited high hydrolytic activity for degrading feedstock with different compositions in terms of carbohydrates and proteins. Moreover, the high abundance of acetate-producers and syntrophic microorganisms suggested acetoclastic methanogenesis as main pathway for methane production. This was confirmed by looking into the Archaea domain, since *Methanosaeta* (4.6 %) dominated the Euryarchaeota phylum (5.5 %). *Methanosaeta* is a strictly acetoclastic archaea that exclusively consume HAc and release methane as end metabolic product [20], evidencing the high methanogenic activity of the inoculum.

The high hydrolytic, acidogenic and methanogenic metabolic activities present in the inoculum confirmed the suitability of the microbial community to perform both processes (AF and AD).

2.3. Microbial community involved in SCFAs production via AF

2.3.1. SCFA production yield

As a first stage of microalgae valorization, *C. vulgaris* was subjected to AF in a continuous stirred tank reactor (CSTR) to assess process performance and the microbial community that determined SCFAs as end metabolites.

Figure 3a shows the time course of SCFAs production, profile and bioconversion efficiency obtained in the AF. An average SCFAs concentration of 3.8 ± 0.2 g SCFAs-Carbon Oxygen Demand (COD)/L was reached once AF exhibited stability, giving rise to a bioconversion efficiency of 32.1 ± 1.5 % (g SCFAs-COD/g COD_{in}) (**Figure 3a, Table 3**).

Table 3. Process parameters and calculated efficiency percentages during the stable period for AF and AD reactors.

	AF reactor	AD reactor
pH	6.1 ± 0.1	7.3 ± 0.1
tCOD (g/L)	11.4 ± 1.1	16.5 ± 1.9
sCOD (g/L)	5.5 ± 0.2	1.1 ± 0.1
sCOD/tCOD (%)	48.7 ± 0.4	6.8 ± 1.9
TS (g/L)	3.8 ± 0.2	10.0 ± 0.4
VS (g/L)	3.0 ± 0.4	8.7 ± 0.4
N-NH ₄ ⁺ (mg/L)	433.9 ± 23.8	452.0 ± 0.4
HAc (%w/w)	24.4 ± 0.6	56.2 ± 0.5
HPro (%w/w)	25.7 ± 0.7	4.7 ± 0.1
Iso-HBu (%w/w)	1.8 ± 0.3	1.1 ± 0.1
HBu (%w/w)	12.5 ± 0.1	37.9 ± 0.3
Iso-HVal (%w/w)	10.4 ± 0.5	0.0 ± 0.0
HVal (%w/w)	20.1 ± 0.3	0.0 ± 0.0
HCa (%w/w)	5.4 ± 0.9	0.0 ± 0.3
Total SCFAs (g COD/L)	3.84 ± 0.1	0.2 ± 0.0
% Bioconversion (g SCFAs-COD/ g COD in)	32.1 ± 0.7	0.1 ± 1.6
COD removal (%)	4.6 ± 1.1	48.5 ± 1.5
VS removal (%)	49.5 ± 5.9	32.5 ± 2.9
% COD acidified (SCFAs-COD/sCOD)	71.7 ± 4.1	0.0 ± 0.0
N mineralization (%)	58.2 ± 2.3	15.54 ± 0.9
CH ₄ (%)	57.7 ± 6.0	75.2 ± 3.7
Methane yield (mL CH ₄ /g COD _{in})	16.2 ± 1.7	168.9 ± 25.7

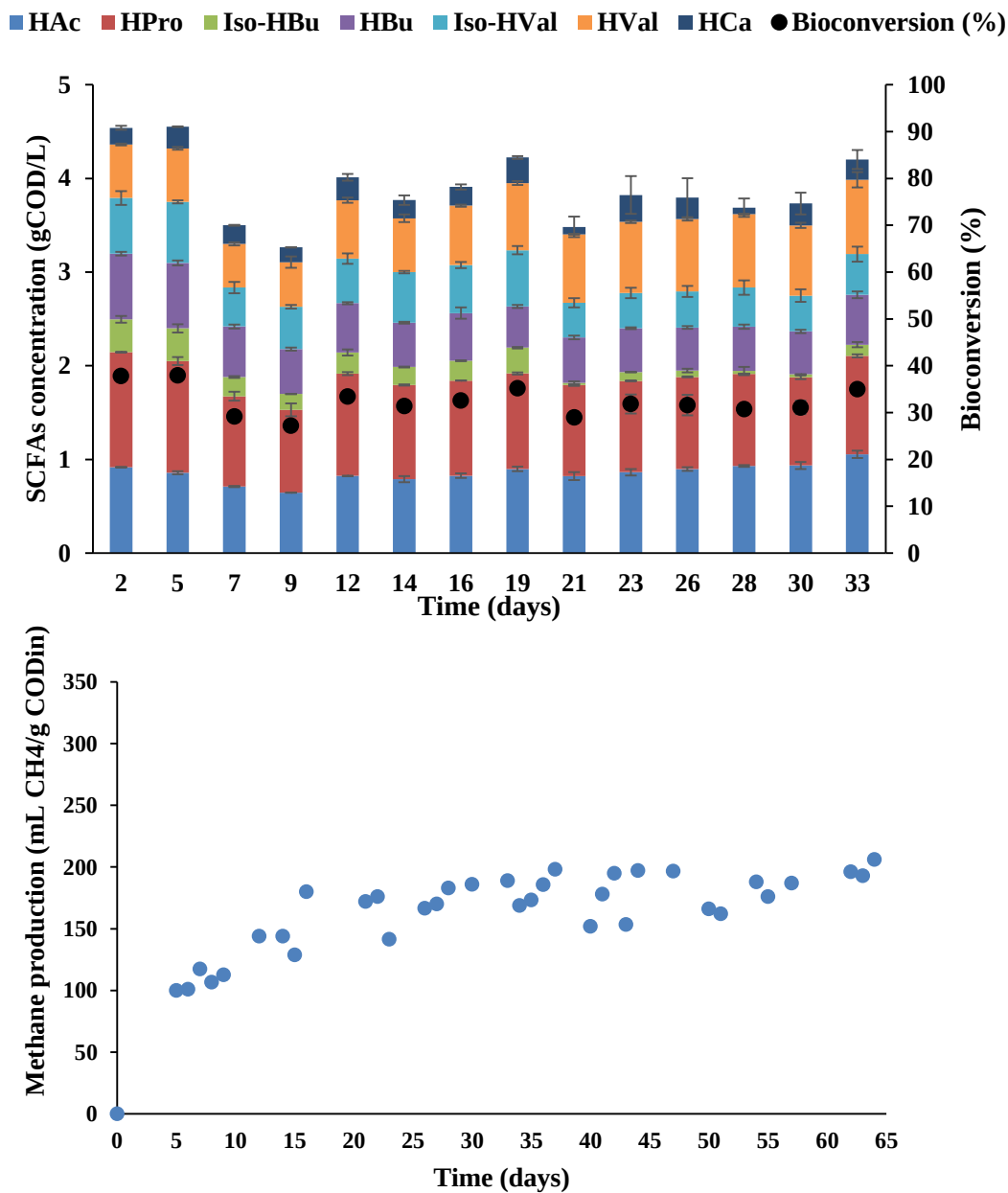


Figure 3. Time course of (a) SCFAs production, distribution and bioconversion achieved in AF reactor and (b) methane yield reached by AD reactor.

These results evidenced a higher process performance than those reported in previous studies employing different waste streams as fermentation feedstock. Jiang et al. [21] found that sewage sludge fermentation achieved 18.6 % bioconversion (g SCFAs-COD/g COD_{in}) while Dahiya et al. [22] reported a maximum bioconversion of 25 % g SCFAs-COD/g COD_{in} when food wastes were fermented. Both investigations were performed in CSTRs at mesophilic temperature and alkaline pH conditions (pH 10). Their low bioconversions in comparison with the result showed herein may be caused by the harsh operational conditions applied to suppress methanogenesis, which concomitantly affected the acidogenic activity. Kim et al. [4] reported 35 % bioconversion (g SCFAs-COD/g COD_{in}) when *C. vulgaris* AF was conducted in batch mode at 35 °C. In spite of the similar bioconversion achieved by these authors in comparison to the present study, it is important to highlight that batch reactors commonly result in higher process efficiency. Moreover, 35 °C would imply higher energy requirements reducing the cost-effectiveness of the process.

Imposed operational conditions in AF reactor promoted hydrolysis and acidogenesis while preventing methanogenic activity. Furthermore, the selected fermentation temperature (25 °C) along with a slight acidic pH (6.1) mediated higher bioconversions into SCFAs than alkaline conditions [23].

The high volatile solid (VS) removal (49.5 ± 5.9 %) confirmed the hydrolytic activity of the process since it involved high production of soluble organic matter to be transformed into SCFAs by acidogenic bacteria. Although microalgae were subjected to a proteolytic pretreatment to avoid hydrolysis limitation, further hydrolysis might be taking place for the remaining proteins and carbohydrates fractions. The acidification efficiency achieved in the AF reactor (71.7 ± 4.1 % g SCFAs-COD/g sCOD, **Table 3**) was in the range of previous studies (54-69 % g SCFAs-COD/g sCOD), where vegetable wastes were

fermented under similar operational conditions (25 °C and pH 6) [19,24]. This result proved that operational conditions established in the AF reactor promoted both the hydrolysis and acidogenic activities.

The microbial community developed during the AF was particularly specialized in SCFAs production. The anticipated microbial shift based on the biodiversity analysis (section 2.1) was supported by the identified microorganisms. 16S rRNA analysis revealed an important change from the inoculum to the AF reactor microbiome (**Figure 2**). Once AF reactor reached the steady state, the bacterial population was mainly represented by Firmicutes phylum (64.2 %), followed by Proteobacteria (11.7 %) and Actinobacteria (11.4 %) phyla. These phyla exhibit high hydrolytic and acidogenic activities (as discussed in section 2.2) and thus, their dominance supported the high VS degradation and SCFAs accumulation achieved in the AF reactor.

One of the most important microbial shifts was related to the disappearance of Chloroflexi, evidencing a process unbalance for biogas production while favoring SCFAs accumulation. Given the fact that Chloroflexi grow in stable AD processes devoted to biogas production and conducted at high HRT [15], the low HRT imposed (8 d) and the presence of disturbances such as a low pH (6.1) and temperature (25 °C) likely caused Chloroflexi wash-out. The decrease in Bacteroidetes (from 11.2 % in the inoculum to 2.4 % in AF) also supported the methanogenic activity reduction due to the fact that this phylum establishes a synergic metabolism with acetoclastic methanogens for biogas production. Bacteroidetes decrease could be explained by the feedstock employed. Considering the important role of Bacteroidetes in complex protein degradation [25], the proteolytic pretreatment employed in the present investigation might have prevented the proteolytic population development. As a result, Bacteroidetes relative abundance was reduced. The uneven microbial phyla distribution and the enrichment in hydrolytic and

fermentative bacteria evidenced a microbial shift from the inoculum (coming from a stable AD process) to an AF for SCFAs as main metabolite.

Regarding the Archaea domain, the relative abundance of Euryarchaeota phylum surprisingly increased from the inoculum (5.5 %) to the AF reactor (7.2 %). According to Zhang et al. [26] this could be related to the shift in the methanogenic systems that takes place when decreasing the pH to 6. This decrease in pH from the inoculum to the imposed conditions for AF, likely promoted hydrogenotrophic methanogens more able to tolerate acidic conditions. Actually, *Methanosphaera* (3.8 %) and *Methanobrevibacter* (3.3 %), which are known to be hydrogenotrophic archaea, dominated Euryarchaeota phylum [3]. *Methanosphaera* is an acid tolerant methanogen able to metabolize H₂ and CO₂ into CH₄ at pHs lower than 6 [27], while *Methanobrevibacter* has been identified as one of the most resilient archaea against process inhibitors [28]. It is important to highlight that methanogenic activity is significantly limited at pHs lower than 7. This could explain the low methane yield and COD removal reached in AF reactor (**Table 3**).

2.3.2. SCFAs production profile

Regarding the SCFAs profile, the most abundant acids were HPro, HAc and valeric acid (HVal), accounting for the 25.6, 24.3 and 20.0 % of the total SCFAs, respectively (**Table 3**). By opposite, iso-Butyric (iso-HBu) and caproic acid (HCa) acids presented lower abundance, accounting both for less than 10 % of total SCFAs produced. In AF, longer chain fatty acids are usually shortened giving rise to C2-C3 fatty acids accumulation [29].

In reactors targeting SCFAs production, HAc accumulation is considered a success indicator of AF process performance. During AD, HAc is consumed by acetoclastic archaea and thus, methanogenic population inactivity can be assumed when HAc is

accumulated. Indeed, the methane production attained in the AF was considerable low in comparison with anaerobic processes particularly devoted to methane production. According to the COD removal value (4.7 ± 1.1 %, **Table 3**), methane yield obtained was 16.2 ± 1.7 mL CH₄ /g COD_{in}. This low value agreed with the SCFAs accumulation in the AF reactor. Given that methanogenic microorganisms have slower growth rates than hydrolytic and acidogenic bacteria, the combination of low HRTs (8 d), 25 °C and slight acidic pH can be used as tool to decrease methanogenic activity when SCFAs are targeted.

Apart from HAc, the AF reactor generated an effluent dominated by odd carbon number (mainly HPro and HVal). The high HPro presence obtained in the present study may be related to the high protein content of the microalgae biomass used as feedstock (**Table 3**). Regueira et al. [30] stated that odd-chain acids, specially HPro, are mainly associated to protein-rich substrates, regardless of the reactor pH. Even carbon number SCFAs have been reported to prevail during carbohydrate-rich substrate fermentation [3]. This fact agreed with the low content of HBu and HCa acids in the present study since the carbohydrates fraction in microalgae biomass only accounted for 26.2 ± 0.8 % (**Table 4**). With regard to the longest carboxylic acids, such as HVal, their accumulation has been correlated with methanogenic activity inhibition [31]. In this sense, the high concentration of these acids evidenced the high efficiencies of organic matter conversion into SCFAs.

Table 4. Chemical characterization of the pretreated *C. vulgaris* used as substrate for SCFAs production and the solid spent from AF employed as substrate for biogas production.

Chemical parameters	Average $\pm$ Standard deviation	
	<i>C. vulgaris</i>	AF solid spent
TS (g/L)	6.8 $\pm$ 0.1	14.1 $\pm$ 1.3
VS (g/L)	5.7 $\pm$ 0.3	12.8 $\pm$ 1.2
VS/TS (%DW)	83.6 $\pm$ 0.1	90.5 $\pm$ 0.2
sCOD (g/L)	5.8 $\pm$ 0.3	8.2 $\pm$ 2.8
tCOD (g/L)	11.8 $\pm$ 1.2	30.9 $\pm$ 0.3
sCOD/tCOD (%)	49.4 $\pm$ 0.4	27.7 $\pm$ 4.4
pH	6.5 $\pm$ 0.5	5.7 $\pm$ 0.5
N-NH ₄ ⁺ (mg/L)	203.2 $\pm$ 9.6	304 $\pm$ 8.0
Carbohydrates (%DW)	26.2 $\pm$ 0.8	28.7 $\pm$ 2.6
Proteins (%DW)	56.1 $\pm$ 01.6	57.9 $\pm$ 3.6
Lipids (%DW)	6.3 $\pm$ 0.8	4.9 $\pm$ 0.9
Ash (%DW)	11.4 $\pm$ 0.1	9.53 $\pm$ 0.2

In the attempt to further link the SCFAs profile to the microbial population developed in the AF, a closer investigation to the bacterial genera was conducted. The sequences retrieved from Firmicutes phylum were assigned to the Clostridiales order, which increased from 8.2 % in the inoculum to 59.8 % in the AF reactor. Clostridiales was mainly composed by *Peptostreptococcus* (12.2 %), *Acidaminococcus* (10.3 %), uncultured Mogibacteriaceae (9.8 %) and uncultured Ruminococcaceae (7.1 %). *Peptostreptococcus* is commonly detected in AF of protein-rich feedstock since these bacteria are involved in the fermentation of peptides and amino acids, releasing HPro as main metabolite [32] and producing HAc via homoacetogenesis [33]. Therefore, the high HPro and HAc concentrations in the AF (0.99 g COD/L and 0.94 g COD/L respectively, **Figure 3a**) were likely related to these microorganisms. The activity related to amino acids and peptides degradation can be proved by the high nitrogen mineralization

achieved in AF reactor (58.2 ± 0.9 %, **Table 3**). Moreover, HPro concentration higher than 0.9 g COD/L has been reported to be inhibitory for acetoclastic methanogenesis [34]. This fact suggested that *Peptostreptococcus* had a key role in SCFAs accumulation by producing HPro and HAc at levels that provoked methanogenesis inhibition. *Acidaminococcus* has been identified in anaerobic processes conducted at pH lower than neutral [26]. According to Zhang et al.[26], these bacteria genus entails monosaccharides degradation to produce H₂ and SCFAs such as HAc and H₂u. This would be in agreement with the H₂u and the HAc found in the AF (**Figure 3a**). Mogibacteriaceae and Ruminococcaceae families include bacteria identified in systems devoted to H₂ production [27]. Whereas Mogibacteriaceae are able to produce H₂ and HAc [27], Ruminococcaceae release H₂, isoH₂u and H₂a when AF is conducted at slightly acidic conditions [19], which justified the presence of these SCFAs in the AF reactor. This Likewise, *Pseudomonas*, from the Proteobacteria phylum, also increased its abundance in the AF reactor when compared to the inoculum. This trend also agreed with the accumulation of HAc since this genus exhibit a high fermentative activity that breaks down monosaccharides and amino acids into HAc [33].

Given the fact that there was a high relative abundance of H₂-producer microorganisms (*Acidaminococcus*, Mogibacteriaceae, Ruminococcaceae, and *Pseudomonas*), it could be concluded that AF balance entailed the presence of H₂-consumers since not H₂ was detected in the gas phase.

Overall, the low HRT did not cause archaea population wash-out but the imposed operational conditions strongly affected the pH in the reactor, which in turn prevented archaea metabolic activity, resulting in low methane production. In fact, the imposed operational conditions and the feedstock characteristics (protease pretreated protein rich substrate) promoted a robust microbial population involved in high hydrolytic (mainly

related to the carbohydrate fraction of microalgae biomass) and fermentative activities, which resulted in high organic matter bioconversion into SCFA.

2.4. Microbial community involved in the solid residue valorization via AD

Once microalgae biomass was firstly valorized as SCFAs, the residual solid fraction obtained from the AF reactor was anaerobically digested in a CSTR. An average methane yield of 168.9 ± 23.7 mL CH₄ /g COD_{in} was obtained in continuous mode (**Figure 3b**). Based on a maximum conversion of 350 mL CH₄ produced/g COD_{in}, the methane yield corresponded to a biodegradability of 48.3 ± 9.2 % (**Table 3**). Although the result obtained herein was slightly lower than the values found in batch mode (200 mL CH₄/g COD_{in} [35]), the methane yield was comparably higher to the values reported in other studies. Methane yields of 110-180 mL CH₄ g /COD_{in} have been reported when AD of raw microalgae was conducted in continuous mode [36–38].

Two-stage AD of microalgae biomass has been also studied by feeding the entire SCFAs-rich effluent from the first AF reactor to a second AD reactor [10–12]. Recent publications stated a methane yield increase between 10 % and 30 % when implementing a two-stage AD strategy over the traditional AD [39]. Considering the methane yield (136 CH₄ /g COD_{in}) reported by Mahdy et al. [38] when alcalase pretreated *C. vulgaris* was digested under similar conditions, the methane yield reached herein showed a 24 % improvement. Opposite to the strategy performed in the present work, during the traditional two-stage AD, the high-value SCFAs are not extracted but consumed by methanogenic population and thus, the carbon recovery is not maximized as it is in the present investigation.

Overall, it could be concluded that despite of the fact that the substrate employed in this AD was a residue from AF, the high methane yield achieved proved its suitability as substrate for biogas production.

Similarly to SCFAs production, biogas production yields is determined by the microbial community developed in the AD reactor. In this case, bacteria domain was mainly represented by 6 phyla: Firmicutes (28.6 %), Proteobacteria (15.3 %), Bacteroidetes (10.9 %), Chloroflexi (6.8 %), Actinobacteria (6.4 %) and WWE1 (3.0 %). The high hydrolytic activity of those phyla can be proved by the reduction of solids concentration (32.5 ± 2.9 % VS removal, **Table 3**), which allows increasing the soluble organic matter available for the subsequent stages. This fact may be related not only to the increase in feedstock bioaccessibility along the AF process, but also to the hydrolytic and acidogenic population enrichment, as a consequence of the microorganisms dragged from the AF to the AD reactor.

Firmicutes relative abundance was higher in the AD reactor (28.6 %) than in the inoculum (10.0 %). This fact could be explained by the feedstock used for feeding this reactor. The solid spent of the previous AF reactor used as AD substrate, was composed by fermented microalgae and fermentative bacteria mainly dominated by Firmicutes (**Figure 2a**). Moreover, Firmicutes exhibits high resistance to process variation and a metabolic growth rate increase with operational temperature increases [40]. Consequently, the high abundance of Firmicutes in the feedstock along with the temperature increase from 25 °C (solid fraction from AF) to 35 °C (AD reactor) likely promoted the presence of this phylum. Sequences belonging to phylum Firmicutes were mainly assigned to order Clostridiales (25.7 %), comprised by members of uncultured Clostridiales (7.9 %) order, Peptostreptococcaceae (3.4 %) and Ruminococcaceae (2.3 %) families. Considering the high cellulolytic activity of these bacteria [27], their development in AD reactor might be

due to both, the temperature increase and the affinity for sturdy polysaccharides that compose microalgae cell walls. Other OTUs identified in the microbiome from AD included *Sedimentibacter* (3.9 %) and *Syntrophomonas* (2.2 %). These genera typically grow in syntrophic acetate oxidation communities co-occurring with hydrogenotrophic methanogens [41], suggesting methane production via the hydrogenotrophic pathway.

The relative abundance of Proteobacteria remained similar in all the samples but in the AD microbiome, *Syntrophus* (4.1 %) and *Kaistia* (2.1 %) displayed higher relative abundances. *Syntrophus* and *Kaistia* are involved in fermentative processes, releasing HAc as end metabolite. However, low SCFAs concentration was detected in AD (0.02 ± 0.01 g SCFAs-COD/L, **Table 3**). The negligible SCFAs concentration pointed out to a well-balanced AD process where acetate-degraders played a major role.

Regarding Bacteroidetes, this phylum persisted in AD reactor in the same relative abundance and bacterial composition as in the inoculum (**Figure 2a**). Bacteroidetes comprised members of the order Bacteroidales (10.8 %) that are common in anaerobic processes with low SCFAs concentration [42]. This was in agreement with the low SCFAs levels registered in the AD reactor (**Table 3**). According to Gulhane et al.[17], Bacteroidales promotes syntrophic bacteria growth along with acetoclastic archaea. The functional role of Proteobacteria and Bacteroidetes detected in the AD reactor confirmed the development of an active acetotrophic pathway as one of the main metabolic process for methane production. Indeed, acetoclastic methanogenesis has been previously reported when microalgae biomass is digested for biogas production [15].

Chloroflexi showed a relative abundance decrease in comparison with the inoculum. Bacteria of Chloroflexi and Firmicutes exhibit high hydrolytic activities and share some metabolic pathways [43]. As previously stated, the high relative abundance of Firmicutes in AF reactor likely provoked Firmicutes enrichment in AD microbiome. Chloroflexi relative abundance in the AD microbiome was lower than the inocula commonly employed for AD purposes, such is the case of the inoculum employed herein (20.9 %). Considering that Chloroflexi presence declined, it can be assumed that Firmicutes covered part of the metabolic functions of Chloroflexi.

The bacterial community detected in AD reactor evidenced a flexible metabolic population able to produce different methane precursors (HAc or H₂ and CO₂). These results agreed with the developed archaea population. Euryarchaeota phylum increased to 17.7 %, comprising acetoclastic (*Methanosaeta*, 9.9 %) and hydrogenotrophic methanogens (*Methanobrevibacter*, *Methanosphaera* and uncultured Methanomicrobiales with relative abundance of 2.9, 2.6 and 1.7 %, respectively). Their relative abundance can be supported by the process parameters determined in the AD reactor (**Table 3**). The NH₄⁺ concentration was below inhibitor levels for methanogenic population (452 mg NH₄⁺ /L), pH around 7.3 and temperature of 35 °C have been reported to be suitable for methanogenic activity [44]. As it can be seen in Table 3, no SCFAs were detected in the AD effluent while high methane yields and COD removal were reached. Therefore, the increase of this archaeal community confirmed SCFAs consumption for methane production purposes.

Considering the microbial population development, it could be concluded that biomass recirculation from AF to AD reactor promoted a microbial enrichment of the anaerobic

microbiome, able to optimally degrade the residual carbohydrates and proteins present in the substrate and maximizing the valorization into biogas through both methanogenic pathways.

3. CONCLUSIONS

The anaerobic sludge used as seed inoculum was able to shape its microbial population to efficiently produce SCFAs and biogas via a non-conventional two-stage sequential bioprocess.

The high hydrolytic and acidogenic microbial populations (mainly Firmicutes phylum) developed in the AF reactor resulted in high bioconversion of *C. vulgaris* biomass into SCFAs. This microbial specialization, along with the lack of methanogenic archaea activity, was promoted by the imposed operational conditions.

The wide phyla diversity determined in the AD reactor allowed a high methane production yield. The microorganisms dragged from the AF into the AD reactor mediated Firmicutes enrichment of the anaerobic microbiome. Even though SCFAs-rich effluent was not fed in the AD reactor, the organic matter bioaccessibility of solid residue increased during AF, which ultimately supported the suitability of this solid spent as substrate for biogas production.

This study proved the efficient generation of SCFAs and biogas from *C. vulgaris* biomass by applying two sequential bioprocesses, increasing multiproduct recovery from a single waste.

4. MATERIAL AND METHODS

4.1 Anaerobic sludge employed as inoculum

The inoculum employed for AF and AD reactors was mesophilic anaerobic sludge collected from the anaerobic digester of the wastewater treatment plant of Valladolid (Spain). Anaerobic sludge presented 12.6 ± 0.2 g/L Total solids (TS) and VS/TS of $61.8 \pm 0.1\%$.

4.2. Substrates

C. vulgaris was employed for SCFAs production via AF and the solid spent of AF effluent was used as substrate for methane production via conventional AD. **Table 4** shows both substrates composition in terms of tCOD and sCOD, TS and VS, $\text{NH}_4^+\text{-N}$, pH, total carbohydrates, proteins, and lipids. These values corresponded to the mean and standard deviation calculated during the whole experimental time.

4.2.1. Microalgae employed for SCFAs production via AF

Wet *C. vulgaris* employed in this work was purchased from Allmicroalgae (Lisbon, Portugal) and characterized before feeding the reactors. This study aimed at investigating the acidogenic stage, and thus, to avoid hydrolytic limitations during AF, microalgae biomass was subjected pretreatment. Taking into account that proteins are the main barrier hampering hydrolysis step [45], a proteolytic pretreatment was applied employing a commercial enzymatic cocktail with endopeptidase enzymes “Alcalase 2.5 L” (Novozyme, Denmark). The enzyme dosage applied was 0.2 mL/g TS and the pretreatment procedure was carried out according to previously optimized parameters

[46]. Pretreated *C. vulgaris* was diluted with tap water to finally obtain the concentration required to feed the AF reactor.

4.2.2. AF solid spent employed for methane production via AD

After microalgae AF, the effluent of this reactor was centrifuged at 14000 rpms and separated into liquid and solid fraction. The solid fraction of the effluent was diluted with tap water to finally obtain the desired concentration to feed the AD reactor.

4.3. Experimental set up

AF of microalgae biomass was evaluated in a CSTR with 1-L working volume. Agitation was performed by mechanical stirring. Operational conditions included organic loading rate (OLR) of 1.5 g COD/Ld, HRT of 8 days and 25 °C digestion temperature. This low temperature was selected based on previous studies that reported that psychrophilic conditions and short HRT (lower than 10 days) promoted organic matter conversions into SCFAs by avoiding methanogenesis [15,31]. The pH of the substrate was adjusted to 6.5 $\pm$ 0.2. In the reactors, pH was daily monitored but not controlled.

AD of solid spent obtained from AF was also performed in a 1L-CSTR provided with mechanical stirring. The operational conditions were OLR 1.5 g COD/Ld, HRT 20 days and 35 °C digestion temperature. Mesophilic conditions and high HRT (15-20 days) were set to allow methanogenic population development. In this case, the substrate pH was 5.7 $\pm$ 0.3 and no pH control was conducted in the reactor.

Both CSTRs were monitored 2–3 times per week, until parameters stability was reached along the experimental period. The biological process was considered at steady state when

the reactor was operated at least during 3 HRT and the effluent was characterized by stable parameters concentration. Once, the AF and AD reached the steady state, bioprocesses efficiencies were evaluated.

AF efficiency was evaluated in terms of bioconversion of organic matter into SCFAs as described in equation 1.

$$\% SCFAs \text{ bioconversion} = \frac{SCFAs(COD)_{out}}{tCOD_{in}} \cdot 100 \quad (Eq. 1)$$

Where SCFAs_{out} (COD) indicated the organic matter related to SCFAs measured in the effluent as g COD/L, while total COD_{in} represents the total organic matter fed in the reactor measured as g COD/L. SCFAs measured were HAc, HPro, iso-HBu, HBu, iso-HVal, HVal and HCa.

On the other hand, AD efficiency was assessed as the percentage of biomethanization and COD removal calculated according to equations 2 and 3, respectively:

$$\% \text{ biomethanization} = \frac{mL \text{ methane}_{out}}{tCOD_{in}} \cdot 100 \quad (Eq. 2)$$

$$\% COD \text{ removal} = \frac{tCOD_{out} - tCOD_{in}}{tCOD_{in}} \cdot 100 \quad (Eq. 3)$$

Where mL methane referred to the volume of methane produced by the AD reactor, and tCOD indicated the total organic matter fed in the bioreactor in terms of g COD/L.

To evaluate hydrolysis and acidogenesis steps efficiencies, the percentage of VS removal and COD acidified were calculated as indicated in equations 4 and 5, respectively. VS_{in/out} makes reference to the VS measured in the feeding and the effluent of the bioreactor while sCOD_{out} represents the soluble organic matter in the bioreactor effluent measured as g COD/L.

$$\% VS\ removal = \frac{VS_{out} - VS_{in}}{VS_{in}} \cdot 100 \quad (Eq. 4)$$

$$\% COD\ acidified = \frac{SCFAs(COD)_{out}}{sCOD_{out}} \cdot 100 \quad (Eq. 5)$$

Taking into consideration the biomass protein content, the initial $N-NH_4^+$ contained in the biomass used as substrate and the $N-NH_4^+$ measured during the steady state ($N-NH_4^+_{in}$ and $N-NH_4^+_{out}$ respectively), the organic nitrogen converted to NH_4^+ (nitrogen mineralization) can be calculated as follows (equation 6):

$$\% Nitrogen\ mineralization = \frac{N-NH_4^+_{out} - N-NH_4^+_{in}}{tN_{in} - N-NH_4^+_{in}} \cdot 100 \quad (Eq. 6)$$

4.4. Analytical methods

For substrates characterization, protein content was determined multiplying total nitrogen by 5.95. Total Kjeldahl nitrogen (TKN) was measured according to Standard Methods. Carbohydrates content in the biomass was analysed by phenol–sulphuric acid method described by Dubois et al. [47].

CSTR effluents were analysed in terms of TS, VS, tCOD, sCOD and $N-NH_4^+$. All these parameters were performed in accordance with APHA standard methods [48]. The soluble fraction of AF effluent was obtained after centrifugation at 14.000 rpm for 3 min and 0.45 μm pore filtration. Commercial kits were used for colorimetric quantification of tCOD, sCOD and $N-NH_4^+$ (Merck, ISO 15705 y ISO 7150-1, respectively).

To determine SCFAs concentration, collected samples were filtered through 0.2 μm and analyzed by liquid chromatography using an Agilent 1260 HPLC-RID (Agilent, USA) equipped with a Cation H Refill Cartridge Microguard column (Biorad) and an Aminex HPX-87H ion exclusion column (300 x 7.8 mm I.D.) (Biorad). Mobile phase was 5 mM H_2SO_4 , and elution was conducted in isocratic mode at a flow rate of 0.6 mL min⁻¹. The

injected sample volume was 20 μ L, and the oven and detector temperatures were 50 and 35 $^{\circ}$ C, respectively.

Gas chromatography was used to analyse biogas composition. The equipment was coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) equipped with an HSN6-60/80 Sulfinert P packed column (70 x 1/800 O.D.) and a MS13X4-09SF2 40/60 P packed column (90x 1/800 O.D.) (PerkinElmer). Several gas mixtures (3% H₂ and mixtures 50% CO₂/CH₄, 25% CO₂-75 CH₄% from Praxair-Nippon, Spain) were employed as calibration standards in the GC-TCD measurements. Helium was used as carrier gas (30 mL/min). The temperatures of the injector, column and detector were 80, 62 and 200 $^{\circ}$ C, respectively.

4.5. Microbial community analysis

Microbial communities were analyzed in the inoculum and in samples collected from CSTRs when the AF and the AD reached the steady state, as detailed by Greses et al [3]. For this purpose, DNA extraction was conducted according to manufacturer's protocol using the kit 'FastDNA SPIN Kit for Soil' (MP Biomedicals, LCC). Extracted DNA concentration and quality were checked by using a nanodrop spectrophotometer (LVis Plate, BMG Labtech, DE).

The primers used for the amplification of the hypervariable regions V3–V4 of 16S rRNA of archaea and bacteria were 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). Amplicons obtained were sequenced on a MiSeq Sequencer (Illumina) by FISABIO (Valencia, Spain).

Raw sequences were computationally processed by using bioinformatics tools as follows: firstly, the paired-end reads from each sample were merged using the program PEAR.

Afterwards, sequences were quality sieved with a mean score of 30 using PRINSEQ. Primers sequences were removed using Mothur program and then, chimeric sequences were also deleted while the resulting sequences were clustered into operational taxonomic units (OTUs) at 97 % sequence identity (OTU0.97) by USEARCH, using Greengenes database gg_13_8 which is implemented in the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software package. QIIME was used to evaluate the biodiversity in terms of richness and evenness by calculating the observed OTUs and Shannon index. The Venn diagram calculates the intersection of list of elements. In this work, Venn diagram of shared OTUs was used to depict the similarity on species abundance between the three communities.

ABBREVIATIONS

AD: Anaerobic digestion

AF: Anaerobic fermentation

CSTR: Continuous stirred tank reactor

HAc: Acetic acid

HBu: Butyric acid

HCa: Caproic acid

HPro: Propionic acid

HRT: Hydraulic retention time

HVal: Valeric acid

Iso-HBut: Isobutyric acid

Iso-HVal: Isovaleric acid

OLR: Organic loading rate

OTUs: Operational taxonomic units
SCFAs: Short-chain fatty acids
sCOD: Soluble chemical oxygen demand
tCOD: Total chemical oxygen demand
TS: Total solids
TKN: Total Kjeldahl nitrogen
VS: Volatile solids

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Publication IV

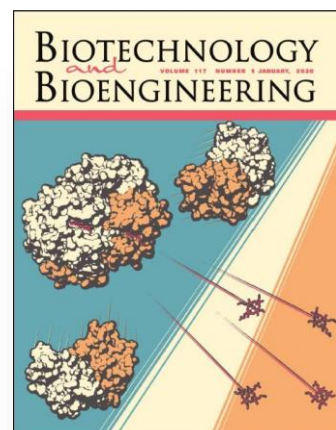
Volatile fatty acids as novel building blocks for oil-based chemistry via oleaginous yeast fermentation (Review)

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REVIEW

Volatile fatty acids as novel building blocks for oil-based chemistry via oleaginous yeast fermentation

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Abstract

Microbial oils are proposed as a suitable alternative to petroleum-based chemistry in terms of environmental preservation. These oils have traditionally been studied using sugar-based feedstock, which implies high costs, substrate limitation, and high contamination risks. In this sense, low-cost carbon sources such as volatile fatty acids (VFAs) are envisaged as promising building blocks for lipid biosynthesis to produce oil-based bioproducts. VFAs can be generated from a wide variety of organic wastes through anaerobic digestion and further converted into lipids by oleaginous yeasts (OYs) in a fermentation process. These microorganisms can accumulate in the form of lipid bodies, lipids of up to 60% wt/wt of their biomass. In this context, OY is a promising biotechnological tool for biofuel and bioproduct generation using low-cost VFA media as substrates. This review covers recent advances in microbial oil production from VFAs. Production of VFAs via anaerobic digestion processes and the involved metabolic pathways are reviewed. The main challenges as well as recent approaches for lipid overproduction are also discussed.

KEYWORDS

anaerobic digestion, lipids, oleaginous yeasts, volatile fatty acids, waste valorization

1 | INTRODUCTION: MICROBIAL OILS FOR THE PRODUCTION OF BIOCHEMICALS

The implementation of a sustainable bio-based economy is considered a top priority in today's society. To achieve such a goal, there is no doubt about the need for producing renewable bio-products to replace fossil-derived compounds. As a matter of fact, the European Union (EU) through the challenge "Secure, efficient and clean energy" (European Commission, 2017) is committed to a transition from a fossil fuel-based industry to a more energetically sustainable industry based on renewable raw materials. In this context, the implementation of economically viable conversion processes of different types of feedstock into bioproducts and biofuels is of utmost importance.

The raw material for oleochemicals include vegetable and animal oils. Nevertheless, to solve supply issues, microbial oils have emerged as promising alternatives to vegetable oils because of their higher

lipid and biomass productivities, shorter cultivation period, and lower surface requirements than plants (Cho & Park, 2018). Furthermore, microbial oils share similar characteristics and fatty acids (FAs) profile with the plants oils most commonly used in industry (Madani, Enshaeieh, & Abdoli, 2017), and therefore, are expected to have a great impact in the oleochemical sector.

The economic feasibility of microbial oils is, however, uncertain due to the high cost of the substrate, which may account for up to 60% of overall production cost in a typical fermentation process (Fei, et al., 2011b). In this sense, it is of utmost importance to use low-cost renewable resources as substrates. Among others, food waste, agricultural residues or lignocellulosic substrates are proposed as low-cost renewable feedstock to be used as carbon sources for microbial oil production (Chang, Kim, Kang, & Jeong, 2010; Cho & Park, 2018; Diwan, Parkhey, & Gupta, 2018b). These low-cost substrates may undergo different transformation routes for their

conversion into potentially usable carbon sources for oleaginous microorganisms.

The production of sugars as platform molecules has conventionally been proposed as one of the most attractive biotechnological ways to valorize carbohydrate-rich organic wastes or lignocellulosic substrates via enzymatic hydrolysis (Tomás-Pejó, Bonander, & Olsson, 2014). Anaerobic digestion (AD), a counterpart of enzymatic hydrolysis, entails a biochemical route by which organic matter can be converted into volatile fatty acids (VFAs; also known as carboxylates). In this manner, there are nowadays two platforms, namely sugar and carboxylates, that could be potentially used for the partial replacement of the petrochemical industry. Given the great waste heterogeneity, AD may be considered a very favorable technology as all the organic matter present in the substrate (carbohydrates, proteins and lipids) is potentially used for VFA production instead of solely the carbohydrate fraction, such as is the case for the sugar platform.

As the use of the carboxylate platform, by means of VFAs, is nowadays considered as a cutting-edge technology for the development of future biotechnology industries, this review focuses on recent advances in microbial oil production using these carbon sources.

2 | VFAS AS A NOVEL SUBSTRATE FOR OIL PRODUCTION

VFAs, that can be obtained through AD, are considered as building blocks in the so-called carboxylate platform (Aglar, Wrenn, Zinder, & Angenent, 2011; Holtzapple & Granda, 2009). The AD is a complex organic matter degradation process where an organic substrate is converted into bioenergy in form of biogas in four different steps, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. More specifically, VFAs are intermediate compounds produced after the hydrolytic and the acidogenic stage of AD. Acetic, propionic, isobutyric, butyric, iso-valeric, valeric and caproic acids are the most commonly found VFAs (Magdalena, Tomás-pejó, Ballesteros, & González-fernandez, 2018). These chemicals can be further used as

carbon sources for oleaginous yeasts, which accumulate lipids in lipid bodies (LBs; Figure 1). Nevertheless, to enhance VFA production, attention must be directed to different aspects regarding the substrate used and the operational conditions in the AD process (Magdalena, Llamas, Tomás-Pejó, & González-Fernández, 2019; Strazzera, Battista, Garcia, Frison, & Bolzonella, 2018).

Microbial populations in AD are affected by substrate composition and selected operational conditions, which ultimately determines the final VFA productions and profiles (Arslan et al., 2016; Khan et al., 2016). Macromolecular composition of a given substrate would entail different VFA conversion yields due to the different hydrolysis rates of carbohydrates, proteins and lipids (Angelidaki & Sanders, 2004). As a result, different VFAs profiles can be attained depending on substrate composition (Arslan et al., 2012). Additionally, different operational strategies can be applied to the AD process to enhance VFA production (Magdalena et al., 2019). As a matter of fact, working with an anaerobic inoculum particularly prepared for VFA accumulation might help in reducing archaea population, which uses VFAs as a carbon source in the AD process. For such a purpose, thermal application, alkali or the addition of chemicals have been tested (Jankowska, Chwialkowska, Stodolny, & Oleskowicz-Popiel, 2017; Tao, Chen, Wu, He, & Zhou, 2007; Yang, Guo, Xu, Fan, & Luo, 2011). However, economic costs, environmental concerns as well as the high energy demand of these techniques are the main drawbacks identified for archaea inactivation to inhibit methanogenesis. Thus, the most appropriate approach to favor VFAs accumulation relies on finding the optimum balance in the operational parameters applied to the anaerobic system. In this sense, studies on key parameters such as pH (Chen, Jiang, Yuan, Zhou, & Gu, 2007; Lee et al., 2008; Neyens, 2004), organic loading rate (Piyarat, Black, & Holtzapple, 2003; Rincón et al., 2008), hydraulic retention time (Bengtsson, Hallquist, Werker, & Welander, 2008; Kim, Shin, Lim, & Hwang, 2013; Lim et al., 2008; Beopoulos et al., 2009a; Xie, 2017) and temperature (Komemoto et al., 2009; Yuan, Sparling, & Oleszkiewicz, 2011) have recently been reviewed. However, when targeting for VFA production, operational conditions should be set-up according to the substrate characteristics and desired product.

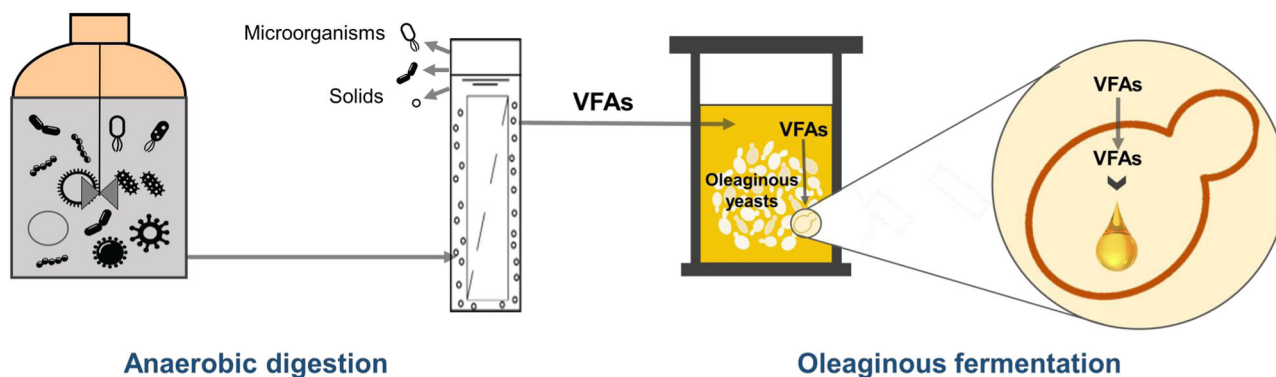


FIGURE 1 Schematic diagram of volatile fatty acid (VFA) production and lipid production process integration

3 | OLEAGINOUS MICROORGANISMS FOR OIL PRODUCTION

Although all microorganisms are able to synthesize lipids, only some of them are able to accumulate more than 20% (wt/wt) dry weight. These microorganisms, belonging to different species of bacteria, fungi, microalgae and yeasts, are called oleaginous microorganisms and are considered the most suitable chassis for bio-oil production (Madani et al., 2017). Lipid accumulation and composition is genetically determined (Meng et al., 2009). However, apart from genetic factors, the lipid content can be also manipulated by culture conditions (such as temperature, pH, C/N ratio, culture mode, fermentation time, and so forth; Beopoulos et al., 2009a). The economic viability of microbial oil production strongly depends not only on the substrate cost but also on the microorganism's performance.

During the last decade, microalgae have been pointed out as a suitable alternative to vegetable crops for bio-oil production due to their fast growth, high lipid yields per unit of cultivation area, and reduced CO₂ emissions (González-Fernández & Muñoz, 2017). Furthermore, microalgae showed extremely high lipid accumulation ability, reaching up to 90% (wt/wt) of the dry weight of lipids under certain conditions (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). To obtain an economically viable lipid production from microalgae, current research efforts have been conducted to favor microalgal growth on digestates proceeding from AD of wastewater, food waste or manure as a low-cost carbon source (Cho et al., 2015; Fei, Fu, Shang, Brigham, & Chang, 2015; Shin et al., 2015). However, the high ammonium concentration in AD effluents might be inhibitory for microalgae growth (González-Fernández & Muñoz, 2017). Besides this, microalgae require favorable light and temperature conditions, and they exhibit slower growth rates than bacteria and yeast. For all these reasons, alternative microorganisms are being proposed to produce microbial oils via biotechnological means.

Bacteria are easily cultured achieving high cell density in short times, but their lipid content is on average lower than those exhibited by microalgae or yeasts (Meng et al., 2009). Recently, *Rhodococcus opacus* grown on gasification wastewater as carbon source resulted in a remarkable 55% (wt/wt) lipid accumulation while only 26% (wt/wt) was achieved when grown on glucose (Goswami, Tejas Nambodiri, Vinoth Kumar, Pakshirajan, & Pugazhenth, 2017). Notwithstanding, yeasts present various advantages including fast growth rates and high cell densities. Due to their robust physiology, yeasts are well suitable for bioreactor cultivations, and, depending on the species, they can even tolerate elevated temperatures, low pHs and organic solvents (Meng et al., 2009). Moreover, yeast can accumulate lipids in significant quantities in separate LB, which facilitate downstream processes (Sitepu et al., 2014).

Yeasts have the outstanding capability to be cultivated on a wide range of wastes, such as sugar-based materials (lignocellulosic residues), glycerol, and hydrophobic substrates (HS; crude oil, industrial fatty acids, and so forth; Diwan et al., 2018b; Papanikolaou & Aggelis, 2011a). Therefore, the utilization of organic wastes for

lipid production by oleaginous yeasts (OYs) can be considered as a cost-effective technology to partially replace petroleum in oil-based chemistry within the so-called circular bioeconomy.

3.1 | Oleaginous yeasts

Some well-known OY strains can accumulate intracellular lipids up to 60% (wt/wt) of their dry weight (i.e. *Cryptococcus* sp., *Lipomyces* sp., *Rhodospiridium* sp., *Rhodotorula* sp., *Yarrowia* sp., and *Trichosporon* sp.). Microbial lipids from OY present similar compositions to common edible vegetable oils, such as rapeseed, sunflower, palm and soybean (Sitepu et al., 2013). They are mainly composed of triacylglycerols (TAGs) rich in FAs (myristic acid [C14:0], palmitic acid [C16:0], stearic acid [C18:0], oleic acid [C18:1], linoleic acid [C18:2], and linolenic acid [C18:3]; Amara et al., 2016). Other lipidic components can be present in important quantities, such as free fatty acids (FFAs), neutral lipids, sterols, and polar fractions (e.g. phospholipids, sphingolipids, glycolipids; Beopoulos et al., 2009a).

3.2 | Lipid metabolism in OY

3.2.1 | Metabolic pathways for lipid synthesis and accumulation

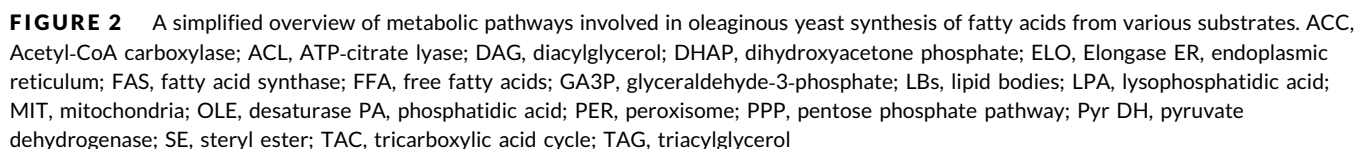
Lipids may be synthesized and accumulated via two different strategies depending on the substrate (depicted in Figure 2): (a) de novo synthesis, producing FA biosynthesis precursors in the presence of sugar substrates and induced by the limitation of a primary nutrients, and (b) ex novo synthesis, involving the uptake of FA from the culture independently of nutrient concentration.

De novo pathway

A very high number of sugar-based substrates have been considered in de novo synthesis. This anabolic biochemical process involves the production of lipids precursors, such as acetyl- and malonyl-CoA, and their integration into the lipid synthesis pathway. Oleaginous microorganisms do not possess a hyperactive system of FA biosynthesis, but they are capable of producing significant quantities of acetyl-CoA, which are the basic unit for de novo FA biosynthesis (Figure 2).

After sugar uptake, glycolysis proceeds. Thus, pyruvic acid enters into mitochondria where pyruvate-dehydrogenase (Pyr DH) catalyzes the formation of acetyl-CoA, which enters the Krebs cycle and is converted to citrate. In the cytosol, ATP-citrate lyase (ACL), which is present only in OY, is responsible for the production of acetyl-CoA through the cleavage of citrate (Papanikolaou & Aggelis, 2011b). Acetyl-coA carboxylase (ACC) can transform acetyl-CoA into malonyl-CoA, these being the building blocks of lipid synthesis. Later, the fatty acid synthase (FAS) complex produces acyl-CoA using both acetyl-CoA and malonyl-CoA molecules (Beopoulos et al., 2009a; Xie, 2017).

The key factor for lipid accumulation in yeasts from sugar-based substrates is the limitation of essential nutrients. Cell proliferation slows down when nitrogen is limited as it is an essential nutrient for



Ex novo pathway

depends on the carbon chain length and saturation degree of the available FAs (Papanikolaou & Aggelis, 2003).

Once inside the cell, the FFAs can be stored in LB or can be degraded for growth needs in β -oxidation (Section 3.2.2). In both cases, FFAs should be converted in Acyl-CoA by Acyl-CoA synthetase (Figure 2). A FAs transport and activation model in *Yarrowia lipolytica* has recently been proposed by Dulermo et al. (2015). These authors suggested two alternative pathways regarding chain-length preferences (Figure 2). More specifically, short chain FAs would enter the peroxisome directly in a nonactivated form by unknown transporters followed by an activation inside of the peroxisome by virtue of Acyl-CoA synthetase II. On the other hand, long-chain FAs may turn into an activated form in the cytosol through Acyl-CoA. Then, Acyl-CoA would be stored in LB or enter in the peroxisome by specific transporters.

Afterwards, the fatty Acyl-CoA (regardless of de novo or ex novo synthesis) is esterified with glycerol generating structural (phospholipids, sphingolipids, and so forth) and reserve lipids (Papanikolaou & Aggelis, 2011b; Ratledge & Cohen, 2008). Non-polar lipid synthesis in yeasts is performed from activated FAs through acylation of the glycerol backbone to synthesize TAG (the so-called Kennedy pathway) or by the esterification of sterols to produce steryl ester (SE; Beopoulos et al., 2009a). LBs are primarily composed of neutral lipids, particularly TAGs (85%) and

some SE (8%; Xie, 2017) and incorporated via the endoplasmic reticulum (ER) into FA droplets. The FFAs, envisaged to be stored as TAGs, are accumulated directly or after being subjected to enzyme-catalyzed modifications such as desaturation or elongation reactions (Athenaki et al., 2018).

When HSs are utilized as the sole carbon source, lipid accumulation is a growth-coupled process in the presence of assimilable nitrogen, in contrast to what occurs in de novo lipid biosynthesis that takes place only after nitrogen exhaustion when cell growth ceases (Athenaki et al., 2018). Besides this, in the ex novo pathway, the microbial lipids produced contain lower quantities of TAGs and somehow higher quantities of FFAs as compared with lipid produced through de novo process (Papanikolaou & Aggelis, 2011b). There are some examples related to the microorganism's growth with hydrophobic and hydrophilic materials as cosubstrates (Beopoulos et al., 2009a; Fontanille, Kumar, Christophe, Nouaille, & Larroche, 2012; Gao, Li, Zhou, Cheng, & Zheng, 2017) indicating possible simultaneous ex novo and de novo lipid biosynthesis pathways.

3.2.2 | Lipid turnover

As a general rule, oleaginous microorganisms consume their own lipid reserves when their metabolic requirements cannot be fulfilled by an extracellular carbon source. Lipid turnover has been observed regardless of the lipid biosynthesis route (Papanikolaou & Aggelis, 2011b). Lipid degradation is a complex process that can be generally divided into three sequential steps: remobilization of the LBs, transport and activation, and the β -oxidation process (Dulermo et al., 2015; Figure 2).

LBs are dynamic structures that represent an additional carbon source once the nutrients of the media have been depleted. To carry out LB breakdown, microorganisms should obligatorily possess intracellular lipases and steryl ester hydrolases (Dulermo et al., 2013). Both TAGs and SE are substrates of these intracellular enzymes, which release FFAs at the surface of the LBs. Subsequently, the released FFAs should be activated and transported to the peroxisome, where lipid degradation takes place via the β -oxidation process (Ledesma-Amaro & Nicaud, 2016). The β -oxidation process is a four reaction cycle, resulting in two carbon shortenings of the FAs backbone, accompanied by the release of one molecule of acetyl-CoA. The acetyl-CoA will be further converted via the Krebs cycle and the anaplerotic by-pass of glyoxylic acid. It is also important to point out that an alternative degradation route to β -oxidation does exist. The so-called ω -oxidation process takes place in the ER and give rise to a dicarboxylic acid from a FA. This is a minor process, but it can be important when the β -oxidation process is altered (Xie et al., 2017).

3.3 | Approaches for increasing lipid accumulation

Several approaches to improve the fermentation process for an efficient VFA conversion to lipids are discussed in this section.

3.3.1 | Microorganism-oriented approaches

Isolation and screening of new oleaginous yeast

Due to a general stress response mechanism, OY isolated from extreme environments, such as wastes of oil mills or biodiesel plants, are able to grow in adverse conditions. In this sense, isolation, selection, and characterization of microorganisms with appropriate features for lipid production can significantly improve fermentation yields.

Some new OY such as *Candida tropicalis*, *Pichia kudriavzevii*, *Lipomyces lipoferrus*, *Kodamaea ohmeri*, *Trichosporonoides spathulata*, and *Rhodotorula babjevae* have been isolated and identified from rotten fruits, soil and glycerol, among others (Ayadi, Belghith, Gargouri, & Guerfali, 2018; Diwan & Gupta, 2018a; Duarte, de Andrade, Ghiselli, & Maugeri, 2013; Guerfali et al., 2019; Kitcha & Cheirsilp, 2011; Kraisintu, Yongmanitchai, & Limtong, 2010; Pan et al., 2009).

There is only limited published information on OY cultivation on VFA as the sole carbon source, and thus, only a narrow range of microorganisms with this capacity have been studied (mostly *C. curvatus* and *Y. lipolytica*). In this context, the search for new yeast with the ability to grow on new substrates and to accumulate high lipid quantities becomes essential. For instance, *Naganishia liquefaciens* NITTS2, isolated from a wastewater treatment plant, was selected due to their ability to use a digestate of waste sludge attaining high lipid content (50% wt/wt; Selvakumar & Sivashanmugam, 2018). Kolouchová, Schreiberová, Sigler, Masák, and Řezanka (2015) highlighted the ability of *Trichosporon cutaneum* as lipid accumulating microorganisms (43% wt/wt) when evaluating yeast lipid production using VFAs as the carbon source.

Cocultures and mixed cultures

Pure cultures are widely extended in biotechnological applications because they allow predictable fermentation, ensuring a constant quality of the desired product. However, research on cocultivation of microorganisms has mainly been conducted to improve the limitation of single strains.

There are a few examples of yeasts and microalgae coculture for lipid production from sugar or glycerol using yeast species such as *Rhodotorula glutinis*, *T. spathulata*, and microalgae *C. vulgaris*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Cheirsilp, Kitcha, & Torpee, 2012; Kitcha & Cheirsilp, 2014; L. Liu, Chen, Lim, & Wei, 2018; Wang, Wu, & Wang, 2016; Yen, Chen, & Chen, 2015). Yeast and microalgae cocultivation appears to be advantageous as these two microorganisms can form a symbiotic relationship due to the balance between oxygen-CO₂ and pH adjustment during the cultivation (Zuccaro, Steyer, & van Lis, 2019). Interestingly, the improvement in lipid production from low-cost residual substrates by using these cocultures has also been confirmed. For instance, digestate obtained from wastewater AD was used as substrate for microalgae-yeast fermentation (Ling, Nip, Cheok, de Toledo, & Shim, 2014; Qin, Liu, Wang, Chen, & Wei, 2018). Ling et al. (2014) reported a higher final lipid production (4.6 g/L) when coculturing *Rhodospiridium toruloides*

and *C. pyrenoidosa* than when using the yeast (3.02 g/L) and the microalgae (2.53 g/L), separately. Industrial food wastes have also been utilized by a coculture of *C. vulgaris* and *R. glutinis*/*R. toruloides* for lipid production (Cheirsilp et al., 2011; Y. Zeng, Xie, et al., 2018). In these cases, cocultures obtained higher lipid concentration and shorter cultivation times than pure cultures. These results point at mix-cultures as a good strategy for waste management and lipid production.

Genetic modifications of oleaginous yeasts

The correct use of metabolic tools to increase lipid production capacity in OY requires the knowledge of the lipid metabolic process (described in Section 3.2). As lipid production routes are not completely understood, mutation and adaptive evolution are interesting options for diverting yeast metabolism towards the desired pathways for improving OY features.

Genetic engineering

Yeasts have often been used as tools for the understanding of lipid biochemical mechanisms (Mlícková et al., 2004) and can even be genetically modified for lipid overproduction or enrichment in a specific oil (Ji, 2018; Ledesma-Amaro & Nicaud, 2016). Improving lipid biosynthesis capacity requires an overall optimization of FA synthesis and assimilation. In this sense, central carbon metabolism, cofactor balance, regulation, and metabolite transport should be optimized. Current strategies to improve lipid production include increasing the supply of lipid precursors and/or avoiding lipid degradation together with the overcoming of limiting factors (Marella, Holkenbrink, Siewers, & Borodina, 2018; González-Fernández and Muñoz, 2017).

For instance, the overexpression of genes encoding ACC, ACL, and FAS in *Y. lipolytica*, *R. toruloides*, and *S. cerevisiae*, successfully improved lipid production (Blazeck et al., 2014; Runguphan & Keasling, 2014; Tai & Stephanopoulos, 2013; Zhang et al., 2014, 2016).

The overexpression of genes encoding enzymes (phospholipid: diacylglycerol acyltransferase or diacylglycerol acyltransferases) that catalyze lipid storage in TAG, has also generated high-lipid producing strains (Ferreira et al., 2018). Furthermore, blocking different pathways competing with lipid biosynthesis, such as those involved in polysaccharide storage or organic acid excretion, could also increase yeast ability to accumulate lipids (Dourou, Aggeli, Papanikolaou, & Aggelis, 2018; Kavšček, Bhutada, Madl, & Natter, 2015; Sagnak, Cochot, Molina-Jouve, Nicaud, & Guillouet, 2018).

An important point in lipid synthesis is the presence of NADPH, which is mainly generated via the pentose phosphate pathway and through decarboxylation of malate to pyruvate catalyzed by malic enzyme. Although no improvement was reported by Zhang et al. (2013) overexpressing malic enzyme in *Y. lipolytica*, when this approach was followed in *R. glutinis*, lipid concentration was increased two-fold (Li et al., 2013). In the same context, an engineered *Y. lipolytica* with less oxygen requirements to regenerate NADH by respiration, reached a lipid productivity of 1.2 g/Lh (close

to fulfilling a competitive productivity of 1.3 g/Lh; Qiao, Wasylenko, Zhou, Xu, & Stephanopoulos, 2017).

Another strategy to enhance lipid accumulation capacity is to avoid or diminish lipid degradation pathways. In this sense, some researchers have inhibited FA transport to the peroxisome or have reduced intracellular lipases activities. Some others have altered or even completely blocked β -oxidation flux (Beopoulos, Chardot, & Nicaud, 2009b; Dulermo et al., 2013; Ledesma-Amaro & Nicaud, 2016).

It is worth mentioning that most of the studies focused on improving FA synthesis through genetic engineering have been performed using sugar-based substrates, and there are only a few studies about genetic modification of genes encoding enzymes involved in the ex novo pathway (Fickers et al., 2005; Mauersberger, Wang, Gaillardin, Barth, & Nicaud, 2001; Sabirova et al., 2011).

Despite the big progress in increasing microbial lipid production by genetic modification, attained yields are still insufficient for commercial production. To make this process economically viable, apart from obtaining higher productivities and yields, the downstream process costs should be minimized. In this sense, research efforts are diverted to engineer strains able to secrete FA (Ledesma-Amaro, Dulermo, Niehus, & Nicaud, 2016) or even to achieve a strain producing biodiesel directly through fermentation (Nawabi, Bauer, Kyrpides, & Lykidis, 2011).

Evolutionary engineering

Due to its simplicity and effectiveness, adaptive evolution has been introduced as a powerful tool to improve a microorganism's properties. During adaptive evolution, microorganisms are cultured under selective pressure conditions for many generations to enrich favorable genetic or phenotypic changes (Dourou et al., 2018). In recent years, many successful evolution strategies have been conducted to improve the production capacity of the desired compounds and to overcome other environmental stress factors such as the presence of inhibitors, pH, temperature, and so forth (Mans, Daran, & Pronk, 2018; Tomás-Pejó, Ballesteros, Oliva, & Olsson, 2010). This method has widely been used not only to develop novel biological and phenotypic functions but also for the improvement of strains obtained through synthetic biology. For instance, an evolved *R. toruloides* strain growing on straw hydrolysate containing inhibitors produced during pretreatment was able to accumulate 61% (wt/wt) of lipids compared with the 55% (wt/wt) obtained by the parental strain (Díaz et al., 2018). Similarly, a *Y. lipolytica* evolved strain obtained after random mutagenesis exhibited 87% (wt/wt) lipid content compared with 71% and 55% (wt/wt) obtained when only the evolved strain and wild type were used (Liu, Pan, Spofford, Zhou, & Alper, 2015; Ledesma-Amaro and Nicaud, 2016).

3.3.2 | Process-oriented approaches

To optimize the lipid production process, not only the use of new or developed strains is needed but also the optimization of specific culture conditions. Hence, the optimization of fermentation process

is also crucial to overcome challenges associated to lipid production. Different process configurations or culture conditions to improve the efficiency of microbial lipid production are discussed below.

Culture condition

Substrate type and concentration, agitation, temperature, pH and nutrients or oxygen concentrations are culture conditions that can strongly influence microbial lipid accumulation.

When VFAs are used as a carbon source, the inhibitory effect of high acid concentration (up to 5 g/L) on yeast growth is well known (Fei et al., 2011b; Fontanille et al., 2012; Huang et al., 2016; Liu, Yuan, Liu, & Huang, 2017a). In this sense, several studies have concluded that the toxic effect on yeast increases with increasing VFA chain length (Gao et al., 2017; Liu, Yuan, Liu, & Huang, 2017b). However, J. Liu et al. (2017b) reported higher biomass and lipid yields (1.6 and 0.4 g/g C, respectively) from 5 g/L of butyric acid than from acetic or propionic acids in *C. curvatus*. Regarding VFA preferences of yeasts, researchers reported the preferential utilization of acetic acid in *C. curvatus* (Vajpeyi & Chandran, 2015), while some others demonstrated the simultaneous utilization of different acids (Xu, Kim, Cho, Park, & Park, 2015). On the other hand, a higher lipid content and yield in *C. albidus* was promoted by predominance of acetic acid (Fei et al., 2011b). In addition, not only the lipid concentration but also lipid distribution was affected by different VFAs, as it has been demonstrated that increasing propionic and butyric acid could enhance odd-chain FA production in several OY (Kolouchová et al., 2015; J. Liu et al., 2017b; Park, Dulermo, Ledesma-Amaro, & Nicaud, 2018).

It is generally accepted that the C/N ratio plays an important role in microbial oil production. Generally, microbial oils are produced with an excessive carbon source but limited nutrient concentration. The lack of some nutrients such nitrogen, iron or magnesium can channel carbon flux promoting lipid accumulation as well as suppress lipid degradation pathways (Papanikolaou & Aggelis, 2011b). Some researchers obtained an improvement in lipid content when increasing the C/N ratio (Huang et al., 2016). However, an excessively high C/N ratio leads would limit growth and lipid production (Bélignon et al., 2015). For instance, Huang et al. (2016) improved lipid content from 16.3% to 48.2% (wt/wt) in *R. toruloides* when the C/N of the media increased from 5 to 200 using acetic acid (20 g/L) as substrate. Nevertheless, other authors obtained higher lipid yields when growing *C. curvatus* on acetate under nitrogen-rich conditions (0.18 g/g at C/N 1.76) than under nitrogen-limited conditions (0.12 g/g at C/N ratio 35.5; Gong et al., 2015). J. Liu et al. (2017a) identified different effects of C/N on lipid accumulation in *C. curvatus* regarding substrate concentration. Therefore, the suitable C/N ratio should be determined depending of the substrate used to optimize lipid accumulation in OY. Unlike sugar-based substrates, where C/N only changes carbon flow within the cells, when using VFAs as carbon source, the C/N ratio also affects substrate utilization (Huang et al., 2016).

In terms of fermentation conditions, temperatures ranging from 25°C to 28°C are commonly used in lipid production by

OY as temperatures higher than 30 °C hamper microbial growth and lipid accumulation (Fei et al., 2011b; Gao et al., 2017). pH also has a crucial effect on OY lipid accumulation when VFAs are used as carbon source because it can influence the acid form (protonated/desprotonated) in solution and acid transport inside the cell (Bélignon et al., 2015; Fei et al., 2011b; J. Liu et al., 2017a). Several authors have found that OYs are strongly inhibited at pH lower than 4.0, but at neutral or basic pH, the degree of inhibition is lower (Fei et al., 2011b; J. Liu et al., 2017a). Besides this, the highest biomass production and lipid accumulation were reached when the pH was around 6–7 (Chi, Zheng, Ma, & Chen, 2011). Aeration can also be an important requirement when aerobic yeast are used (Blazek et al., 2014; Qiao et al., 2017). Fei and co-workers (2011a) concluded that providing an oxygen-enriched (40%) air supply could be an effective method to increase lipid productivity using VFAs as substrate.

Operational mode

A compilation of lipid yields and productivities from VFAs using different culture modes are summarized in Table 1.

Batch fermentation in microbial lipid production has several limitations considering VFAs toxicity and by-product inhibition. Nitrogen exhaustion during the process led to a shift into secondary microbial metabolism, inducing by-product synthesis (e.g. citric acid) and decreasing lipid yields (Beopoulos et al., 2009a). For these reasons, it is essential to develop culture strategies, which allow C/N ratio control as well as the reduction of substrate inhibition (i.e. two-stage batch, sequencing batch, fed-batch, two-stage fed-batch and continuous mode; Table 1).

When using two-stage batch cultivation, cells are cultured in glucose or glycerol rich medium and low C/N ratio to boost biomass accumulation during the first step. Then, cells are transferred into a nitrogen limited medium with VFAs as the sole carbon source to enhance lipid content. Some authors have applied this two-stage batch mode obtaining a higher biomass and lipid yields than those obtained in single batch cultures (Christophe et al., 2012; Fei et al., 2011a; Huang et al., 2016). Moreover, reducing the VFAs inhibitory effect in the second stage led to more efficient VFA use in comparison with flask cultures (Fei et al., 2011a). These authors showed an increase in lipid yields from 0.125 to 0.27 g/g when using *C. albidus* in two stage batch culture with 9 g VFAs/L.

Another strategy to enhance VFA conversion efficiencies is the use of a sequential batch, where the medium is replaced when the substrate is finished resulting in an increase in biomass and lipid content (Huang et al., 2016; J. Liu et al., 2016; Xu et al., 2015). For instance, compared with batch cultures, an increase in lipid content in *C. curvatus* from 50.4% to 61% (wt/wt) was obtained after 2 cycles of culture (Xu et al., 2015). Huang et al. (2016) compared a sequencing batch with two-stage batch strategies for *R. toruloides* on a VFA substrate concluding that the sequencing batch was the optimal strategy in terms of lipid productivity (0.57 vs. 0.26 g·L⁻¹·d⁻¹).

TABLE 1 Microbial oil production by oleaginous yeast cultured on VFAs using different culture strategies

Yeast strain	Substrate	Operational mode	Biomass (g/L)	Lipid content (%)	Lipid yield (g/g)	Reference
<i>C. albidus</i>	VFAs (5)	Batch	2.57	25.1	0.125	Fei et al. (2011a)
	Ac (6)	Batch	2.85	25.8	0.123	
	G (30) + Ac	Two-stage batch	8.14	38.3	0.25	
	G (30) + VFAs	Two-stage batch	7.96	43.8	0.27	
	G (30) + Ac	Two-stage fed-batch	27	55	0.172	
<i>R. toruloides</i>	Ac (20)	Batch	4.35	48.2	0.125	Huang et al. (2016)
	G (40) + Ac (20)	Two-stage batch	6.75	50.1	0.104	
	Ac (4)	Sequencing batch	4.21	38.6	0.084	
<i>C. curvatus</i>	VFAs (3.35)	Sequencing batch		39.6	0.61	J. Liu et al. (2016)
<i>C. curvatus</i>	VFAs (9,27)	Batch	3.7	33.1	0.13	Xu et al. (2015)
	VFAs (9,27)	Fed-batch repeated	2.4	61	0.15	
<i>C. curvatus</i>	Ac (4)	Fed-batch		30.46		Chi et al. (2011)
<i>C. curvatus</i>	Ac (5)	Fed-batch		53	0.23	Béligon et al. (2015)
<i>C. curvatus</i>	VFAs (12 + 4)	Fed-batch	6.8	42	0.13	Béligon et al. (2016)
<i>Y. lipolytica</i>	Ac (12)	Fed-batch	5.89	30.76	0.15	Fontanille et al. (2012)
	P (8)	Fed-batch	3.38	25.73	0.11	
	B (12)	Fed-batch	3.39	25.07	0.07	
	G + Ac	Two-stage fed-batch	30.83	40.69	0.13	
	G + P	Two-stage fed-batch	39	38.06	0.2	
	G + B	Two-stage fed-batch	31.76	24	0.11	
	G + VFAs (3:1:1)	Two-stage fed-batch	41.02	40.22	0.2	
	Gl + Ac	Two-stage fed-batch	40.93	38.43	0.17	
	Gl + P	Two-stage fed-batch	36.82	38.81	0.17	
	Gl + B	Two-stage fed-batch	30.83	37.59	0.19	
<i>C. curvatus</i>	Gl + VFAs (3:1:1)	Two-stage fed-batch	41.02	34.59	0.16	Christophe et al. (2012)
	G (15) + Ac (5)	Two-stage fed batch		50.9	0.15	
<i>C. curvatus</i>	G (5) + Ac (5)	Two-stage batch		47.3	0.19	Gong et al. (2015)
	Ac (5)	Continuous		56.7	0.18	
<i>C. curvatus</i>	Ac (30)	Continuous		66.4	0.12	Vajpeyi and Chandran (2015)
	VFAs*	Continuous	1.1	28.3	0.04	

Abbreviations: Ac, acetic acid; B, butyric acid; G, glucose; Gl, glycerol; P, propionic acid; VFA, volatile fatty acid.

*VFAs mixture of 6.5 g COD/L.

As mentioned before, considering substrate inhibition on yeast growth, low VFA concentration are widely used. However, low carbon source concentration resulted in relatively low biomass concentration and lipid production using a batch, two stage batch and sequential batch cultivation. Using the fed-batch (Béligon et al., 2016; Béligon et al., 2015; Chi et al., 2011; Fontanille et al., 2012; Zheng et al., 2012) and two-stage fed-batch mode (Christophe et al., 2012; Fontanille et al., 2012), significant improvements in the lipid content and productivity could be accomplished as an extra carbon source can be provided avoiding high VFA concentrations. Fontanille et al. (2012) evaluated these two culture modes for *Y. lipolytica* grown on acetic, propionic and butyric acid after glucose or glycerol rich medium. When using the two-stage fed-batch mode, lipid content and yields (40.22% (wt/wt) and 0.2 g/g when using glucose and mixture VFAs) were much higher than those obtained in the fed-batch mode (30.76% (wt/wt) and 0.15 g/g when using acetic acid).

Some studies have compared the two-stage batch with the two-stage fed-batch (Christophe et al., 2012; Fei et al., 2011a). Although higher lipid concentrations were obtained applying the

two-stage fed-batch, the lipid yield decreased. In this context, Christophe et al. (2012) obtained 47.3% (wt/wt) lipid content in *C. curvatus* and 0.19 g/g lipid yield when using acetate in the two-stage batch mode. On the other hand, 50.9% (wt/wt) of lipid content and 0.15 g/g lipid yield were obtained in two-stage fed-batch mode.

Only few reports about continuous microbial oil production have been published (Gong et al., 2015; Vajpeyi & Chandran, 2015). A continuous culture implies the maintenance of the pH and C/N ratio for a given dilution rate. Gong et al. (2015) obtained a maximum lipid content and lipid yield of 56.7% (wt/wt) and 0.18 g/g, respectively when using *C. curvatus* on acetate medium at a dilution rate of 0.04 hr⁻¹. This study confirmed that low dilution rates give rise to a higher lipid and biomass concentration than those obtained for higher dilutions rates. Thus, the optimization of the process involves determining the optimal dilution rate with optimal C/N ratio.

These results suggested that culture modes had a great impact on lipid accumulation and culture optimization can be used as a tool to enhance microbial lipid production.

Cell recycle: biomass recirculation and immobilization

High specific growth rate and high cell density considerably affect lipid production cost. In this sense, the recirculation of microorganisms is an interesting strategy to improve lipid yields and productivities. Cell recycling based on repeated cycling of fresh-medium or microorganism immobilization on an inert support, are techniques used for the maintenance of high cell density. As a matter of proof, the immobilization of *Y. lipolytica* in a bioreactor resulted in 29% higher biomass concentration than in the case of using non-immobilised cells (Vandermies et al., 2018). The co-encapsulation of *T. spathulata* with microalgae *C. vulgaris* in alginate gel beads achieved the highest biomass of 12.2 g/L with a high lipid content of 47% (wt/wt; Kitcha & Cheirsilp, 2014). Moreover, the use of enzymes immobilized with yeasts can improve lipid yields or simplify downstream process steps (Gen, Wang, & Chi, 2014; Zhao et al., 2011). For instance, the combination of a immobilized lipases able to synthesize biodiesel with the yeast *Candida* sp. resulted in 40.6% FAME yields (Duarte et al., 2015).

4 | CONCLUDING REMARKS AND PERSPECTIVES

Microbial oils are interesting precursors for the oleochemical industry, which is envisaged as a promising technology for sustainable production of chemicals and fuels. For this reason, these oils have drawn significant attention in recent years, and the search for new low-cost carbon sources, improved fermentation conditions and fitted microbial strains has been addressed here.

This review gathers together recent novel approaches (from microorganisms to process oriented strategies) for microbial oil overproduction using VFAs as substrate.

As the substrate cost is one of the challenges for microbial oil production, the optimization of VFA production from organic wastes is a key factor for process profitability. So far, most of the studies on lipid production with oleaginous yeasts have been carried out using sugars as the carbon source. Thus, elucidating metabolic pathways involved in VFAs utilization by yeast is also crucial for obtaining efficient bioconversions into lipids.

One of the main obstacles for lipid production from VFAs is their toxicity. In this sense, efforts have been made to research new strains and to apply new process configurations (fed batch, two-stage batch, sequencing batch, and so forth) to overcome the inhibitory effect. Besides this, apart from the optimization of the culture condition to maximize lipid yields and productivities, research efforts have mainly been directed to genetically modify yeast for lipid overproduction and avoidance of lipid degradation.

Considering the recent advances in microbial oil production and the challenges associated with the use of these novel carbon sources, much attention should be given to implement new process strategies

and develop new metabolic engineering approaches. The expansion in the number of research publications in this field are indicative of significant progress in the near future to attain efficient lipid production processes from VFAs.

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Publication V

Volatile fatty acids from organic wastes as novel low-cost carbon source for *Yarrowia lipolytica*

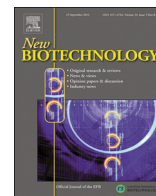
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Full length Article

VOLATILE FATTY ACIDS FROM ORGANIC WASTES AS NOVEL LOW-COST CARBON SOURCE FOR *Yarrowia lipolytica*

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ABSTRACT

Microbial oil biosynthesis is envisaged as a promising technology for sustainable production of chemicals and fuels. Sugar-based substrates are the most typical carbon sources used for this purpose where metabolic pathways and stoichiometry are well known. However, the use of low-cost substrates is crucial for the economic viability of the process. Volatile fatty acids (VFAs) are considered to be a novel low-cost carbon source for microbial lipid production. They can be utilized by oleaginous yeasts to produce and store fatty acids in form of intracellular lipid bodies. In this work, *Yarrowia lipolytica* growth and substrate consumption were evaluated using the major VFAs present in anaerobic effluents. Individual VFAs as well as synthetic mixtures were tested at different concentrations to determine uptake rates and potential toxicity. Increasing VFA chain length resulted in greater biomass yield although, when added individually, 4 g Carbon/L VFA (e.g. 6.45 g/L of caproic and 10 g/L of acetic acid) caused inhibitory effects. Remarkably, biomass growth increased by 2.5-fold on real anaerobic fermentation effluent compared with synthetic mixtures. When real digestate was supplemented with synthetic VFAs up to 26.5 g/L, the inhibitory effect of the acids was counterbalanced. The results provided evidence of robustness of *Y. lipolytica* towards low-cost fermentation effluents and present this yeast as a promising candidate for the sustainable production of microbial oil using real digestates.

1. Introduction

Bio-oil based chemistry is a promising alternative to petroleum for the production of chemical precursors in synthesis of polymers, plasticizers and lubricants [1]. Currently, the lipid industry is based mainly on agricultural sources, but their limited supply as well as competition with food necessities make the identification of alternative low-cost lipid sources a priority. Microbial oils (MO) are receiving considerable attention because of their higher lipid and biomass productivities, shorter cultivation period and lower volume surface requirements than plants [2]. MOs mostly consist of triacylglycerols (composed of C14 to C18 fatty acids (FA)) similar in composition to vegetable oils [3]. Among the microorganisms able to accumulate lipids, oleaginous yeasts (OY) have the capacity to consume carbon sources converting them into acetyl-CoA, a central intermediate in lipid biosynthesis. Under certain conditions, OY can accumulate lipids up to 60 % of their dry cell weight [4–6]. *Y. lipolytica* is the most widely studied OY due to its ability to use hydrophilic and hydrophobic compounds efficiently as substrates [7,8]. Most studies related to lipid production via OY have been carried out with glycerol and sugar-based media as carbon sources [9–14].

However, owing to the high cost of such substrates, considerable efforts have been directed to finding alternative low-cost carbon sources [2,6,15]. VFAs are organic acids that could be used as carbon source by OY. The acids require a shorter transformation pathway and have higher theoretical lipid conversion efficiencies than their counter-partner sugar-based substrates [16]. VFAs can also be sustainably produced through anaerobic fermentation (AF) of organic wastes.

Anaerobic digestion is a complex process whereby organic matter is first degraded to intermediate products (VFAs) which will be finally transformed into biogas. The VFAs are regarded as building blocks in the so-called carboxylate platform [17–19]. Anaerobic digestion may be considered a favorable technology by which all organic matter (carbohydrates, proteins and lipids) is converted into VFAs, instead of only the carbohydrate fraction, as is the case for the sugar platform. Within the carboxylate platform, VFAs should accumulate and not be consumed during anaerobic digestion. Using controversial substrates for biogas production could help to hamper VFA consumption, e.g. protein rich substrates can result in unbalanced anaerobic digestion systems due to the high nitrogen concentration, in which case VFAs are not consumed but accumulated [20]. Due to their high protein content and

Abbreviation: AF, Anaerobic fermentation; COD, Carbon oxygen demand; MO, Microbial oil; OY, Oleaginous yeast; VFA, Volatile fatty acid

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ability to thrive in residual effluents, microalgae have emerged as a promising feedstock for VFA production. Despite the interest in the use of low-cost substrates, only a few studies have reported the use of VFAs as raw materials for lipid production. Those available have investigated the effect of one specific acid (commercial acetic, propionic or butyric acids) [5,21–23] and limited reports have assessed the use of real VFAs produced during AF [24–27].

The aim of the present study was to investigate the feasibility of an AF effluent (real digestate) from microalgal biomass to be used as carbon source for *Y. lipolytica*. Yeast growth from the main VFAs present in digestates was investigated. Synthetic individual VFAs (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids), as well as mixtures, were tested at different concentrations and ratios to determine uptake rates and potential toxicity. Real digestate was compared with synthetic medium to evaluate the potential of this substrate for practical application.

2. Materials and methods

2.1. Microorganism, inoculum preparation and culture conditions

Yarrowia lipolytica (CECT1240) was obtained from the Spanish Type Culture Collection. The strain was maintained at 4 ± 1 °C and regularly sub-cultured on yeast extract-peptone-dextrose (YPD) agar plates, containing yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) and agar (20 g/L). For pre-culture, one colony was inoculated into YPD liquid medium (composition as above except for the agar). The inoculum was grown in a rotary shaker at 150 rpm and 25 °C overnight, until the culture reached the late exponential growth phase. Batch culture experiments were inoculated at initial optical density at 600 nm (OD_{600}) of 0.3. Experiments were performed in triplicate in 250 mL Erlenmeyer with baffles on the bottom containing 50 mL medium at initial pH 6.0 ± 0.5 without pH control during the process. All cultures were incubated in a rotary shaker at 150 rpm and 25 °C until the available substrate was exhausted (exception made for inhibited cultures).

2.2. Substrates used

The seven main VFAs present in AF digestate (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acid) were evaluated independently at 0.8 g C(carbon)/L and 4 g C/L as carbon source for *Y. lipolytica*. For comparison purposes, 0.8 g C/L and 4 g C/L of glucose (2 and 10 g glucose/L, respectively) were also tested as an ideal substrate.

Y. lipolytica performance was also assessed on five synthetic VFA mixtures, comprising for each acid: I) 0.11 g C/L, II) 0.8 g C/L, III) 2 g C/L, IV) 4 g C/L and V) 1.51 g/L. Since carbon (C) content of each acid is different due to their lengths, corresponding concentrations in g/L and mol/L are indicated in Table 1. In all synthetic media, VFAs were added to yeast nitrogen base medium (YNB) (Conda, Cat.1553.00) supplemented with 7.5 g/L of $(NH_4)_2SO_4$.

Lastly, real digestate without additional nutrient was used as substrate for *Y. lipolytica*. It was extracted from AF of microalgal biomass carried out in 1 L continuous stirred tank reactors under semi-continuous feeding mode as detailed elsewhere [28]. Selected digestate was obtained at 35 °C, hydraulic retention time of 10 days and organic loading rate in terms of Carbon Oxygen Demand (COD) of 3 g/Ld.

Once AF processes reached the steady-state, digestate was centrifuged at 14,000 rpms for 10 min. in a Heraeus Megafuge 16R centrifuge and subsequently sterilized by membrane filtration (0.22 µm pore). Batch cultures on synthetic media with the same VFAs concentration were also performed for comparative purposes. Furthermore, in order to compare with mixtures III and IV, real digestate (containing 4.34 g VFAs/L) was supplemented with synthetic VFAs up to 25 and 50 g/L VFAs.

Table 1
Composition of substrates.

	Acid concentration (g/L)							Acid concentration (mM/L)								
	Acetic	Propionic	i-Butyric	Butyric	i-Valeric	Valeric	Caproic	Total	Acetic	Propionic	i-Butyric	Butyric	i-Valeric	Valeric	Caproic	Total
I. Mixture 0.11 g C/L	0.28	0.23	0.20	0.20	0.19	0.19	0.18	1.46	4.67	3.10	2.27	2.27	1.86	1.86	1.55	17.58
II. Mixture 0.8 g C/L	2.00	1.65	1.47	1.47	1.36	1.36	1.29	10.60	33.32	22.27	16.68	16.68	13.32	13.32	11.11	126.7
III. Mixture 2 g C/L	5.00	4.12	3.67	3.67	3.40	3.40	3.23	26.49	83.30	55.62	41.65	41.65	33.29	33.29	27.81	316.61
IV. Mixture 4 g C/L	10.00	8.23	7.34	7.34	6.81	6.81	6.45	52.99	166.61	111.10	83.30	83.30	66.68	66.68	55.53	633.20
V. Mixture 1.51 g/L	1.51	1.51	1.51	1.51	1.51	1.51	1.51	10.60	25.16	20.38	17.14	17.14	14.79	14.79	13.00	122.39
Real digestate	1.42	0.78	0.24	0.68	0.47	0.54	0.21	4.34	23.66	10.53	2.72	7.72	4.60	5.29	1.81	56.33
Synthetic digestate	1.32	0.85	0.22	0.70	0.47	0.43	0.19	4.18	21.99	11.47	2.50	7.94	4.60	4.21	1.64	54.36
Supplemented 2 g C/L	5.00	4.12	3.67	3.67	3.40	3.40	3.23	26.49	83.30	55.62	41.65	41.65	33.29	33.29	27.81	316.61
Supplemented 4 g C/L	10.00	8.23	7.34	7.34	6.81	6.81	6.45	52.99	166.61	111.10	83.30	83.30	66.68	66.68	55.53	633.20

2.3. Analytical methods

Cell growth was estimated by measuring OD₆₀₀ (Spectroquant® Pharo 100 spectrophotometer). For dry biomass, 5 mL of culture media was filtered using a pre-dried and weighed nitrocellulose filter (0.45 µm, Millipore, MA, USA) using a vacuum pump. Dry cell weight was determined gravimetrically after drying the samples to a constant weight at 105 °C. The supernatant was collected and stored at -20 °C for further analysis. Colorimetric commercial methods were used for quantification of COD and ammonium (Merck Darmstadt, Germany, ISO 15,705 y ISO 7150-1, respectively). VFAs were analyzed by liquid chromatography using an Agilent 1260 HPLC-RID (Agilent, Santa Clara, CA, USA) equipped with a Cation H Refill Cartridge Microguard column (Biorad, Hercules, CA, USA) and an Aminex HPX- 87H ion exclusion column (300 × 7.8 mm I.D.) (Biorad). The mobile phase was 5 mM H₂SO₄, and elution was conducted in isocratic mode at a flow rate of 0.35 mL min⁻¹. The injected sample volume was 20 µL, and the oven and detector temperatures were 25 and 35 °C, respectively.

3. Results and discussion

3.1. Individual VFAs as carbon source

When individual VFAs at low carbon concentrations (i.e. 0.8 g C/L) were used as substrate, *Y. lipolytica* was able to grow regardless of VFA chain length. The maximum biomass, however, increased commensurately with increasing chain length. As shown in Table 2, the highest biomass concentration was obtained from caproic acid (0.93 g/L) while the lowest biomass level was reached from acetic acid (0.52 g/L). The same trend was attained for the biomass yields showing higher values when longer chain lengths were used (0.72 g/g from caproic acid and 0.25 g/g from acetic acid). This tendency was similar to the pattern described by others [21] who pointed out that longer chain VFAs had a higher theoretical growth yield coefficient. VFA consumption is always associated with cell growth. Most of the VFAs were exhausted in 24 h of cultivation and only acetic and isobutyric acids were consumed after 32 h (Table 2). These rates were considerably faster than reported in previous studies where more than 84 h of fermentation was required for exhaustion of 2.5 g/L acetic, propionic and butyric acids [27]. Here, the lag phase obtained with acetic and propionic acids was shorter (4.5 h) than for the other acids evaluated (7–9 h), which suggested lower toxicity of short-chain VFAs. Despite having the shortest lag-phase, acetic acid exhaustion required the longest fermentation time (32 h). This could be explained by the higher concentration of this acid over others (i.e. 2 g/L of acetic vs 1.3 g/L of caproic acid).

Similar behavior was found for short chain VFAs (C2–C4) evaluated

at 4 g C/L. In this case, an increase in biomass production from 1.38 to 3.21 g/L (corresponding to yields of 0.15 and 0.50 g/g, respectively) was observed when carbon chain length increased from C2 (acetic) to C4 (isobutyric acid) (Table 2). When chain length exceeded C4 (valeric, isovaleric and caproic acids), no significant differences in yeast growth were obtained and biomass yields ranged from 0.51 to 0.54 g/g. Remarkably, *Y. lipolytica* grown on caproic acid showed a prolonged lag phase (120 h), requiring more than 240 h for substrate exhaustion. As there were no significant differences in biomass production when VFAs with chain length from C4 to C6 were used at 4 g C/L, it may be inferred that long-chain VFAs at high concentration had an associated inhibitory effect on yeast. During VFA consumption, pH can increase reaching basic values that will also inhibit microbial growth (Table 2). Yeast cells have limited pH tolerance, since pH changes can interfere with membrane transporters and ATP expenditure, consequently inhibiting biomass and lipid production [29]. These results are in good agreement with previous studies related to VFAs toxicity. Reports by others [27] concluded that high concentrations of different VFAs (> 5, 10 and 20 g/L of butyric, propionic and acetic acid, respectively) exerted inhibitory effects on *Y. lipolytica*. Moreover, these authors found that the VFAs toxicity order was butyric > propionic > acetic acid.

When comparing high and low VFAs concentration, biomass yields were considerably lower at high VFA concentrations (4 g C/L) independent of the acid used as carbon source. From acetic acid at low concentration (0.8 g C/L), 0.25 g/g of biomass yield was attained whereas 0.15 g/g was obtained at 4 g C/L (Table 2). Thus, inhibitory effects may occur when using VFA concentrations of 6.5 g/L (caproic acid) and 10 g/L (acetic acid). The inhibitory effect was also reported using different OYs. For instance, it was shown [5,23] that *Y. lipolytica* and *Cryptococcus albidus* exhibited growth inhibition when mixtures of acetic, propionic and butyric acids were used at concentrations > 5 g/L. It should be noted that in the test conducted at 4 g C/L, all VFAs were above that threshold.

Biomass concentrations and yields were very close to those achieved with glucose as carbon source. The comparison performed here showed 0.75 g/g biomass from 0.8 g C glucose/L and 0.58 g/g from 4 g C glucose/L. This indicates that carboxylate-based substrates may be an interesting alternative to sugar-based substrates for *Y. lipolytica* CECT1240.

3.2. VFA mixtures as carbon source

The ability of several OYs to grow in the presence of mixtures of acetic, propionic and butyric acids has been reported [5,21–23,27,30]. However, isobutyric, valeric, isovaleric and caproic acids can account for more than 30 % of the VFAs present in the digestate [28] and their

Table 2
Y. lipolytica growth parameters on individual VFAs at different concentrations.

	Carbon source	Exhaustion time (h)	Lag phase (h)	Biomass conc. (g/L)	Y _{x/s} (g/g) ¹	Y _{x/s} (g/gC) ²	Final pH
0.8 g C/L (0.067 molC/L)	Acetic acid (2 g/L)	32	4.5	0.52 ± 0.14	0.25 ± 0.13	0.65 ± 0.13	7.9 ± 0.02
	Propionic Acid (1.64 g/L)	24	4.5	0.63 ± 0.15	0.42 ± 0.20	0.78 ± 0.19	7.44 ± 0.05
	Butyric Acid (1.47 g/L)	24	7	0.76 ± 0.05	0.48 ± 0.06	0.95 ± 0.07	7.17 ± 0.09
	Isobutyric Acid (1.47 g/L)	32	9	0.80 ± 0.08	0.55 ± 0.11	1 ± 0.11	7.01 ± 0.10
	Valeric Acid (1.36 g/L)	24	7	0.81 ± 0.14	0.63 ± 0.22	1.02 ± 0.23	7.11 ± 0.05
	Isovaleric Acid (1.36 g/L)	24	9	0.84 ± 0.07	0.70 ± 0.12	1.05 ± 0.13	6.98 ± 0.09
	Caproic acid (1.29 g/L)	24	7	0.93 ± 0.12	0.72 ± 0.19	1.16 ± 0.19	7.07 ± 0.09
	Acetic acid (10 g/L)	120	4.5	1.38 ± 0.03	0.15 ± 0.01	0.35 ± 0.02	8.65 ± 0.02
4 g C/L (0.33 molC/L)	Propionic Acid (8.23 g/L)	150	7	2.48 ± 0.04	0.33 ± 0.01	0.62 ± 0.02	8.7 ± 0.06
	Butyric Acid (7.34 g/L)	72	9.5	3.90 ± 0.21	0.44 ± 0.05	0.97 ± 0.07	8.42 ± 0.06
	Isobutyric Acid (7.34 g/L)	120	9	3.21 ± 0.21	0.50 ± 0.06	0.80 ± 0.06	8.67 ± 0.03
	Valeric Acid (6.81 g/L)	100	9	3.41 ± 0.24	0.54 ± 0.08	0.85 ± 0.09	8.4 ± 0.05
	Isovaleric Acid (6.81 g/L)	120	9	3.37 ± 0.16	0.54 ± 0.05	0.84 ± 0.05	8.93 ± 0.08
	Caproic acid (6.45 g/L)	240	120	3.11 ± 0.21	0.51 ± 0.07	0.77 ± 0.06	8.17 ± 0.07

1: Y_{x/s}: growth yield (grams biomass/grams total VFA).

2: Y_{x/s}: growth yield (grams biomass/grams of total C in VFA).

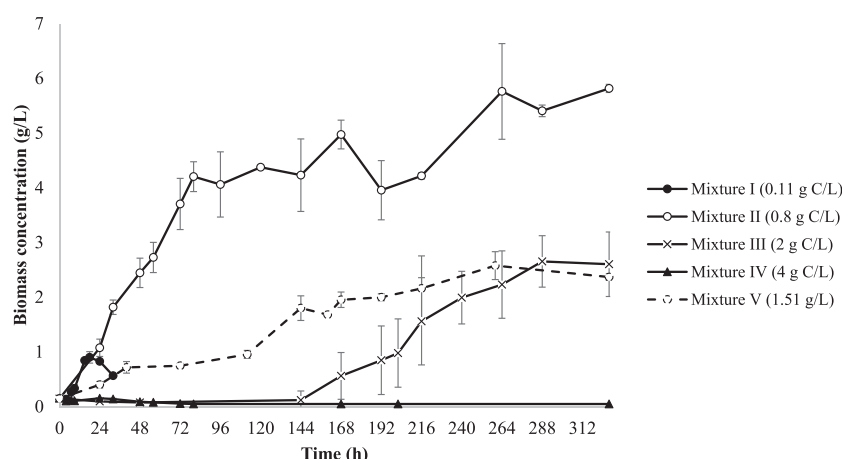


Fig. 1. Dry cell weight during *Y. lipolytica* cultivation in mixtures of VFAs.

presence should therefore not be ignored. Due to their high toxicity potential, it is important to unravel their effects on yeast growth when present in VFA mixtures.

Y. lipolytica CECT1240 was able to grow on 0.11 g C/L (I), 0.8 g C/L (II) and 2 g C/L (III) of each acid, although the latter had a strong inhibitory effect hampering yeast growth during the first period of the culture (lag phase of 144 h) (Fig. 1). A total VFA concentration of approximately 10.6 g/L (mixture II) displayed the highest biomass growth concentration and similar biomass yield as I with 1.46 g/L VFA. As stated, these results differ from those reported by others [5] who concluded that VFA concentrations > 5 g/L imply inhibitory effects on *Y. lipolytica* growth. Biomass yields obtained in mixtures I and II were close to 0.6 g/g (Table 3), corresponding to a biomass production of 0.89 and 5.75 g/L, respectively (Fig. 1). The biomass yield values were slightly greater than those obtained above with individual VFAs, e.g. biomass yield obtained in substrate II (0.58 g/g) was higher than that obtained on 4 g C/L of each acid (Table 2). The results indicated that mixtures of VFAs do not inhibit yeast growth and could even boost biomass yield.

When using mixture III with 2 g C/L of each acid (26.5 g/L total VFAs), the maximum biomass was 3.15 g/L, 45 % lower than with mixture II (10.5 g/L total VFAs). The 5-fold lower biomass yield attained in mixture III indicated the potential toxicity of 26.5 g/L VFA (Table 3). In fact, barely 7.6 of the 26.5 g/L available VFA was consumed after 380 h of culture (data not shown). In contrast, no yeast growth was observed when 4 g C/L of each VFA (mixture IV) was used as carbon source. This mixture made up a total VFA concentration of 52.9 g/L.

3.3. Effect of VFAs profile

In terms of VFAs utilization, some studies have reported preferential utilization of short-chain rather than long-chain VFAs by OY [23,25]. In order to determine the influence of VFAs profile on *Y. lipolytica* growth, a mixture of VFAs at 1.51 g/L (mixture V) was performed for comparison with mixture II. Both substrates (II and V) had the same total VFAs concentration of 10.6 g/L corresponding to 5.6 g C/L, but different individual VFA concentration profile (Table 1). Substrate V presented higher concentrations of long-chain VFAs compared with substrate II and consequently, lower short-chain VFA concentrations. *Y. lipolytica* was able to grow on substrate V, reaching 2.57 g/L of biomass, corresponding to 0.25 g/g biomass yield (Table 3). These results were half those obtained on substrate II, thereby indicating a preference for short-chain VFAs. The time course of VFA mixtures utilization (substrates II and V) by *Y. lipolytica* is shown in Fig. 2. When VFAs were added at 0.8 g C/L each (substrate II), 50 % of the available substrate was consumed after 56 h fermentation and substrates were totally exhausted after 250 h (Fig. 2A). With substrate V, however, only 3.77 out of the 10.6 g/L of available VFAs was consumed (75 % of VFAs remained after 380 h). These results confirmed that the VFA profile in the media had crucial effects on yeast growth and thus, not only total VFA concentration should be evaluated but also systematic studies are required to elucidate their particular effects. According to previous work, a predominance of short-chain VFAs in the medium triggered yeast growth as was the case for mixture II, 0.8 g C/L. In contrast, a high proportion of long-chain VFAs (present in a mixture with 1.51 g/L each) led to a decrease in biomass production, by hampering yeast growth. These results agree with previous studies using VFA mixtures at different ratios, where the highest biomass production and yields were obtained when acetic acid was the predominant acid in the mixture [21,23]. For instance, others [23] reported yields of 0.6 g/g with a mixture 8:1:1 of acetic, propionic and butyric, while 0.32 g/g was attained when the ratio was 4:3:3. It should be noted again that there are few reports dealing with long-chain VFAs and in depth studies should be performed to clarify the chain-length effect.

Regarding VFAs consumption in mixture II (Fig. 2A), *Y. lipolytica* showed a preference for acetic acid over other VFAs substrates during the first 24 h fermentation. Although acetic acid was the last acid to be exhausted, its consumption rate during the first fermentation hours was significantly higher (0.035 g/Lh) than that for the other acids (0.001–0.015 g/Lh). This agrees with other reports [27] where it was found that *Y. lipolytica* exhibited faster utilization rates of acetic over propionic and butyric acid. The authors attributed this to the different metabolic routes after intake: acetic acid is cleaved directly into acetyl-CoA, unlike other acids for which uptake rates are controlled by the rate of other biochemical transformations towards acetyl-CoA. After 32 h of

Table 3

Y. lipolytica growth parameters on VFA mixtures and real digestate.

Carbon source	$Y_{x/s}$ (g/g) ¹	$Y_{x/s}$ (g/gC) ²	Final pH
I. Mix 0.11 g C/L (1.45 g/L)	0.61 ± 0.07	1.16 ± 0.13	7.61 ± 0.04
II. Mix 0.8 g C/L (10.6 g/L)	0.58 ± 0.07	1.10 ± 0.15	8.92 ± 0.07
III. Mix 2 g C/L (26.5 g/L)	0.12 ± 0.2	0.23 ± 0.2	8.86 ± 0.06
IV. Mix 4 g C/L (52.9 g/L)	N.D.	N.D.	6.0 ± 0.01
V. Mix 1.51 g/L (10.6 g/L)	0.25 ± 0.12	0.46 ± 0.11	7.99 ± 0.03
Real digestate (4.3 g/L)	0.74 ± 0.16	1.49 ± 0.15	8.69 ± 0.03
Synthetic digestate (4.3 g/L)	0.32 ± 0.14	0.63 ± 0.13	8.35 ± 0.08
Supplemented 2 g C/L (26.5 g/L)	0.56 ± 0.01	0.98 ± 0.03	9.37 ± 0.05
Supplemented 4 g C/L (52.9 g/L)	N.D.	N.D.	6.0 ± 0.01

1: $Y_{x/s}$: growth yield (grams biomass/grams total VFA).

2: $Y_{x/s}$: growth yield (grams biomass/grams of total C in VFA).

N.D: not detected.

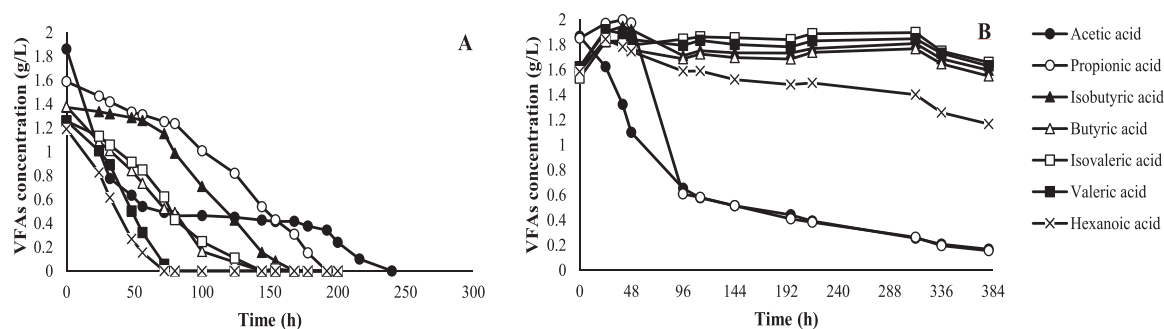


Fig. 2. Time course of utilization of VFAs by *Y. lipolytica* on (A) equal carbon concentration (0.8 g C/L VFAs) and (B) equal concentration (1.51 g/L VFAs).

fermentation, more than 50 % of acetic, valeric and caproic acids were consumed. Propionic and isobutyric acid were only moderately consumed until valeric and caproic acids were exhausted (72 h), after which their utilization was accelerated. However, the fact that acetic acid consumption practically stopped after valeric and caproic exhaustion may indicate that acetic acid consumption was coupled to long-chain VFAs. On the other hand, when those long-chain VFAs are available as carbon source, the metabolism of short-chain VFAs (propionic and isobutyric acids) would be halted. These results showed that in VFA mixtures, *Y. lipolytica* sequentially utilized different VFA types, as also reported when using C2 to C4 VFAs [27]. When long-chain VFAs were present at inhibitory concentrations, as was the case for mixture V, acetic and propionic acid showed the highest consumption rates (0.013 g/Lh at 96 h). In this case, long-chain VFAs were practically unconsumed (0–0.002 g/Lh) (Fig. 2B). This observation may be explained by consideration of VFAs toxicity results (above).

3.4. Real anaerobic digestate vs synthetic substrate

To further evaluate the potential of VFA-rich digestate as substrate for OY, a comparative assay between real AF digestate and synthetic medium with the same initial VFAs concentration was carried out. In the digestate used, short-chain (C2–C3) were the predominant VFAs (> 50 % of total substrate) and small amounts of long-chain (C5–C6) were detected (~25 %) (Table 1). Although VFA distribution during AF appeared to be influenced by feedstock characteristics as well as fermentation conditions, this VFA profile has been conventionally obtained since the fatty acids present in the broth are mainly degraded via β -oxidation, giving rise to short-chain VFA accumulation [31]. Acetic acid was the most abundant VFA (32.7 %) followed by propionic acid (18 %). This is in agreement with values reported in literature irrespective of the substrate in AF [17,32]. This may be explained by the fact that acetate formation reactions involved in AF are thermodynamically favorable [33]. Propionic acid accumulation is also common in unbalanced digestions since its oxidation is energetically unfavorable and strongly influenced by the process parameters [17,34,35].

Time courses for biomass growth and VFAs consumption on real and synthetic digestates are shown in Fig. 3. Biomass production obtained using real digestate was 2.4-fold higher than on synthetic medium (3.14 g/L and 1.34 g/L, respectively). In this way, biomass yield obtained in synthetic medium (0.32 g/g) was lower than expected based on results previously obtained (around 0.60 g/g on substrates I and II) (Table 3). While having the same VFA profile, the highest biomass yield was found on real digestate (0.74 g/g). These results indicated the presence of additional carbon sources, nutrient compounds (such as nitrogen, phosphorous, etc) or by-products in the digestate that could enhance biomass production. According to the stoichiometric calculation based on the composition of the real and synthetic media (Table 1), the COD due to the VFAs was 6.9 g/L; 8.8 and 7.0 g COD/L were determined for real and synthetic media respectively. Thus, the VFAs constitute 78.4 %

of the overall COD obtained in the real digestate, with 21.6 % due to other compounds. Hence, biomass improvement in real digestate could not be simply attributed to the organic matter content but to the presence of other nutrients, which may enhance *Y. lipolytica* growth.

Other studies have reported similar biomass production and yields when comparing *Y. lipolytica* growth on VFAs from an anaerobic fermenter with a synthetic VFAs mixture (around 0.85 g/g) [27]. Notably, a lower biomass yield (0.28 g/g) than the result obtained in the present study was reported when using macroalgal fermentation digestate [24]. A possible explanation for this could be the high nitrogen content (1.4 g/L NH_4^+) present in the digestate from microalgae compared with that from macroalgal biomass (0.11 g NH_4^+ /L). Proteins can account up to 70 % of the macromolecular composition of microalgae. During AF, they are degraded to total ammonia (ammonium ion + free ammonia) [36]. As nitrogen is an essential nutrient for cell synthesis, its high concentration in the broth may have boosted biomass growth.

While all VFAs were utilized simultaneously in both, real and synthetic digestates (Fig. 3), different VFA consumption rates were found. VFAs contained in real digestate were consumed faster than in synthetic medium (Fig. 3). All VFAs in real digestate were completely exhausted after 32 h culture while 144 h was required for consumption of 96 % of VFAs in synthetic medium. In agreement with previous results, acetic acid showed the fastest utilization rate at early stages in both cases. In spite of its high consumption rate, 11.5 % of acetic acid remained after 120 h of process on synthetic medium (Fig. 2A) and total exhaustion on real digestate was achieved after 32 h fermentation (Fig. 2B). As previously noted, *Y. lipolytica* showed acetic acid preference in the presence of long-chain VFAs and caproic and valeric acid exhaustion led to slower acetic acid consumption. In addition, and probably due to their lower concentration in the fermentation broth (far from inhibitory values), long-chain VFAs were totally consumed before short-chain.

3.5. Supplemented digestate

Real digestates were supplemented with VFAs to confirm the positive effect of the digestates on yeast growth and evaluate their suitability. In order to compare with substrates III and IV, digestate was supplemented maintaining the same VFAs profile (Table 1). Real digestate containing 4.34 g/L VFA was supplemented with synthetic VFAs up to 26.5 g/L (2 g C/L of each) and 52.9 g/L VFAs (4 g C/L of each). As shown in Fig. 4, *Y. lipolytica* grew on the medium supplemented up to 2 g C/L of each acid although no biomass growth was reported on that supplemented up to 4 g C/L. Synthetic VFAs at 26.5 g VFAs/L were shown to be a harsh substrate (Fig. 1) while real digestate supplemented with the same VFA concentration was fermentable by *Y. lipolytica* CECT1240 after a short lag phase.

Biomass growth and yield varied significantly from 3.15 to 14.15 g/L and from 0.12 to 0.56 g/g with mixture III and supplemented digestate respectively, both containing 26.5 g/L VFAs (Table 3). In addition, VFAs were totally exhausted after 100 h fermentation in digestate supplemented with up to 26.5 g VFAs/L. In contrast, 380 h were

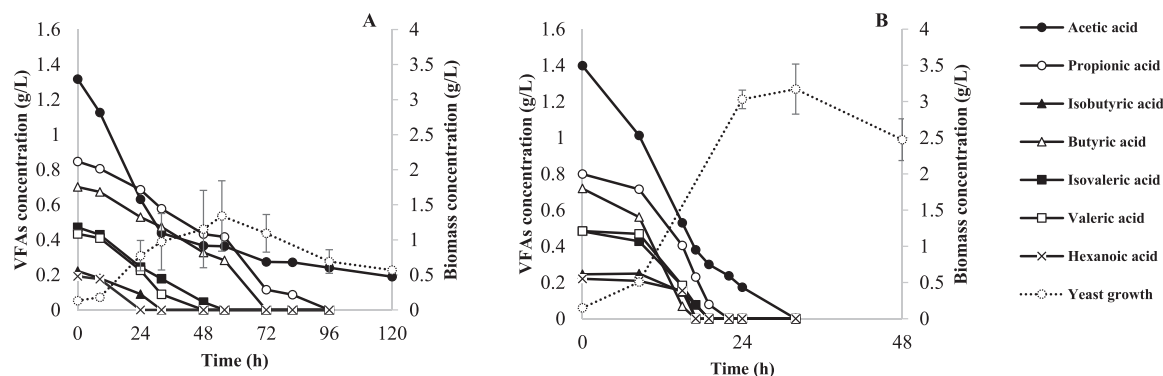


Fig. 3. Time course of *Y. lipolytica* growth and VFA utilization on synthetic digestate (A) and real digestate (B).

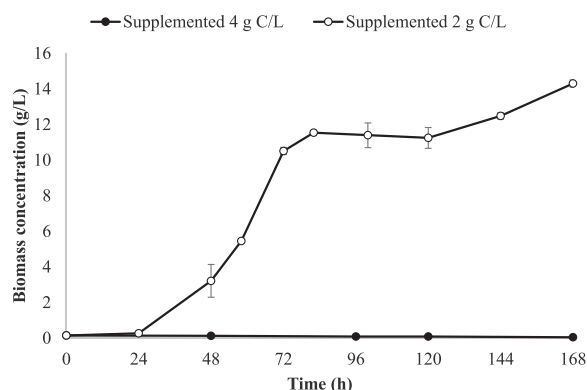


Fig. 4. *Y. lipolytica* growth on supplemented digest.

required to consume all VFAs completely on substrate III (data not shown). This result also indicated the presence of compounds (e.g. nutrients) in the digestate that could boost yeast growth and suggested that the strong inhibitory effect of VFAs at high concentrations was diminished when using real digestate as substrate. Biomass yields attained with real digestate supplemented up to 26.5 g/L were comparable to those obtained in mixtures I and II (0.6 g/g), confirming the feasibility of working with high VFA concentrations in real digestates.

4. Conclusions

Since most OYs are unable to metabolize VFAs, the ability of *Y. lipolytica* CECT 1240 to grow on digestate using VFAs as unique carbon source was very remarkable. Long-chain VFAs were evaluated as substrates for yeast for the first time. VFA chain length had a crucial effect on yeast growth since predominance of long-chain in VFA mixtures exerted an inhibitory effect on the yeast strain. At concentrations below the inhibitory level (4 g C/L), the biomass yields increased commensurately with increasing chain length. *Y. lipolytica* sequentially utilized different types of VFA when present in mixtures showing preference for acetic acid coupled to the consumption of long-chain VFAs. Biomass yield using real digestate was 2.5-fold higher than in synthetic digestate. This positive effect can be attributed to by-products present in the medium which enhanced yeast growth. Although VFA mixtures at concentrations > 10.6 g/L were found to be inhibitory for the strain in synthetic media, this could be overcome by using real digestate (up to 26.5 g/L VFAs) as culture media. This fact confirmed the suitability of AF digestate as a substrate for *Y. lipolytica*, allowing the integration of two bioprocesses (AF and VFAs fermentation) for the production of microbial oils.

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Publication VI

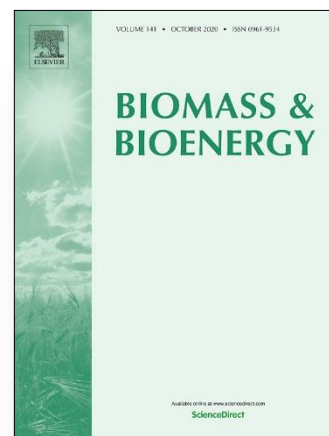
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Research paper

Screening of oleaginous yeasts for lipid production using volatile fatty acids as substrate

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ABSTRACT

Using residual material instead of sugars as substrate for oleaginous microorganisms is a promising approach that may reduce the production costs of microbial lipid. In this study, five oleaginous yeasts were screened for their ability to grow and produce lipid utilizing volatile fatty acids (VFAs), generated from anaerobic fermentation of microalgal biomass, as the only carbon and energy source. Yeasts growth and lipid accumulation capacity at three VFAs concentrations (i.e. 5, 10 and 15 g L⁻¹) were evaluated. Regardless of VFAs concentration four of the five strains were able to grow in digestates reaching biomass yields from VFAs between 0.22 and 0.37 g g⁻¹. The highest lipid content in dry biomass was observed in *Cutaneotrichosporon curvatum* and *Cyberlindnera saturnus* (36.9 and 33.9% on dry biomass, respectively) corresponding to lipid yields from VFAs of 0.11 and 0.13 g g⁻¹, respectively. Oleic, palmitic and linoleic acids were the major fatty acids, accounting for more than 70% of the fatty acids contained in total yeast lipids, profile similar to that of common vegetable oils. The above findings suggest that microalgal biomass derived VFAs could be converted into yeast lipid suitable as feedstock in the chemical (including biofuel) industry.

1. Introduction

Significant environmental and technical motivations encourage the replacement of petroleum-based compounds with renewable bio-products. Microbial oil (single cell oil - SCO) is regarded as a promising raw material for sustainable production of chemicals and biofuels, due to their similar fatty acid composition to the vegetable oils that are commonly used in industry [1]. The capability of some oleaginous yeasts to accumulate high quantities of lipids offers the potential for commercial SCO production, eventually combined with the production of fine chemicals, such as surfactants and carotenoids. In this context, SCO may play a critical role in the transition to a renewable based economy in the near future. At present, due to the high production cost of SCOs only the high-value SCOs, such as those containing polyunsaturated fatty acids, are produced on industrial scale, while those having a common fatty acid composition lag behind [2,3]. Sugar-based feedstock such as simple sugars (e.g. glucose and fructose), lactose, sucrose, whey, glucose enriched wastes or molasses, are easy assimilable by most

oleaginous microorganisms and have been traditionally considered as substrates for SCO production [4]. However, the use of these substrates as carbon source, apart from their restricted availability, contribute to the high SCO production cost. In this sense, the utilization of alternative low-cost renewable feedstocks will boost the economic viability of the SCO production process.

Volatile fatty acids (VFAs) produced during the anaerobic fermentation (AF) of wastes have emerged as novel substrates for oleaginous yeasts. AF is a simple technology which can be applied to a large range of organic wastes (industrial food waste, agricultural residues, etc.) with different macromolecular composition. VFAs are short-chain organic acids (C2 to C6) regarded in the chemical industry as building blocks in the so-called carboxylate platform [5,6]. VFAs can be sustainably produced after the hydrolytic and the acidogenic stages of anaerobic digestion and they can be utilized as a carbon source by oleaginous yeasts to produce lipid reserves that are stored in form of intracellular lipid bodies [7]. Compared to the sugar-based substrates, VFAs have shorter metabolic pathways which could theoretically lead to higher

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Table 1
Anaerobic digestion effluents characterization.

	Concentration			Mass fraction profile (% of total VFAs)
	5 g L ⁻¹	10 g L ⁻¹	15 g L ⁻¹	
Acetic acid	1.6	3.2	4.9	32.6
Propionic acid	0.6	1.3	1.9	12.9
Isobutyric acid	0.2	0.5	0.7	4.5
Butyric acid	1.2	2.5	3.7	24.5
Isovaleric acid	0.3	0.7	1.0	6.5
Valeric acid	0.6	1.2	1.8	12.0
Caproic acid	0.3	0.6	1.0	6.7
Total VFAs	5.0	10.0	14.9	
Ammonium	0.8	1.6	2.9	

lipid conversion efficiencies [8,9]. Metabolic pathways and key factors involved in lipid accumulation when using sugar-based substrates are well known and detailed [2]. Nevertheless, information about VFAs as substrate for oleaginous yeasts is scarce. Therefore, gaining insight on VFAs metabolism in oleaginous yeasts is a fundamental issue to fully understand this bioprocess. Several studies have investigated the conversion of pure VFAs into storage lipid by oleaginous yeasts [8,10,11]. However, only few studies have addressed the use of unpurified VFAs produced during AF [12–16], leaving unanswered questions concerning the ability of the oleaginous yeasts to overcome the inhibitory effect of fermented broth.

To cover this gap of knowledge, the current work explored the efficiency of some oleaginous yeasts, often utilized in the literature for SCO production (i.e. *Cutaneotrichosporon curvatum*, *Lipomyces lipofer*, *Rhodotorula toruloides*, *Cyberlindnera saturnus* and *Yarrowia lipolytica*), to metabolize VFAs arisen from AF of microalgal (*Chlorella vulgaris*) biomass. Specifically, yeasts growth and lipid accumulation capacity were evaluated on different VFAs concentration (5, 10 and 15 g L⁻¹). The fatty acid composition of the lipid produced was determined and compared to that produced on glucose. We concluded that anaerobically fermented waste material rich in VFAs can be converted into SCO with a high coefficient of efficiency. The fatty acid composition of the lipids produced differed from that observed when glucose was used as a carbon source, which is probably interesting in terms of lipid production having an exceptional fatty acid composition.

2. Material and methods

2.1. Microorganisms and substrate used

2.1.1. Yeast strains

The oleaginous yeasts screened in this study were *Cutaneotrichosporon curvatum* (previously known as *Cryptococcus curvatus*) NRRL-Y-1511, *Lipomyces lipofer* NRRL-Y-11555, *Rhodotorula (Rhodosporidium) toruloides* NRRL-Y-27012, *Cyberlindnera (Williopsis) saturnus* NRRL-Y-17396 and *Yarrowia lipolytica* ACA DC 50109. These yeast strains were selected according to their intrinsic ability of lipid accumulation reported previously in literature [17,18]. The strains were maintained for long term conservation at -80 °C in glycerol 30%. They were grown out of the frozen stock on potato dextrose agar (PDA, Himedia Laboratories, Mumbai, India) and maintained at 8 ± 1 °C and regularly sub-cultured on agar plates.

2.1.2. Substrate preparation

Digestate rich in VFAs was obtained through semi-continuous AF of *Chlorella vulgaris* biomass as detailed in the work performed by Magdalena and co-workers [19]. Briefly, the substrate *C. vulgaris* was purchased from Allmicroalgae (Portugal) and preserved at -20 °C until the enzymatic pretreatment. Microalgae macromolecular composition in dry weight was 57.9% proteins, 21.6% carbohydrates, 13.4% lipids and 7.1% ash. In order to avoid hydrolysis limitation, a protease

pretreatment with a commercial enzymatic cocktail “Alcalase” (Novozyme, Denmark), was applied to the microalgal biomass. AF process was carried out in 1 L reactor working at 25 °C, hydraulic retention time of 8 d and organic loading rate in terms of COD of 9 g L⁻¹ d⁻¹. Once the AF process reached the steady-state, reactor effluent rich in VFAs was centrifuged at 21.496 RCF (Heraeus, Megafuge16, Thermo Scientific) for 15 min to obtain the soluble fraction (digestate). Three different digestate preparations containing VFAs at 5, 10 and 15 g L⁻¹ were obtained by diluting the original centrifuged digestate with sterilized water. The above preparations (composition described in Table 1) were directly used as culture media without any additional nutrient, except for Na₂HPO₄ and KH₂PO₄ (Fluka, Steinheim, Germany) that were added at 12 g L⁻¹ each in order to buffer the growth medium. Finally, the culture medium was sterilized by filtration through 0.22 µm pore (VWR international). pH of all media was 6.5 ± 0.5.

2.2. SCO fermentation

Batch cultures were performed under aseptic conditions in triplicate in 250 mL Erlenmeyer containing 50 mL AF effluent as growth medium. Flasks were sterilized at 121 °C for 20 min, aseptically filled with sterilized AF effluent suitably diluted and inoculated with a pre-culture found at the end of exponential growth phase (OD₆₀₀ = 0.3). Pre-cultures were performed in 50 mL potato dextrose broth (PDB, Himedia Laboratories, Mumbai, India) medium incubated at 27 ± 1 °C at 2 RCF. All cultures were performed in a rotary shaker at 27 ± 1 °C and 2 RCF and pH was daily adjusted with 5 mM HCl (VWR international) to 6.5 ± 0.5.

2.3. Microscopy

Cell morphology was observed using an optical microscope Carl Zeiss (GmbH, Göttingen, Germany), equipped with a video camera (Exwave HAD, Sony, Tokio, Japan). Lipid bodies were stained with Nile red fluorescence dye as follows: 0.1 g of wet biomass was suspended in water and mixed (1:10 v v⁻¹) with a solution of Nile red (Sigma) in ethanol (1 mg mL⁻¹). After incubation with the dye for 1 h at room temperature, cells were washed twice with distilled water and viewed under an Axiostar 40 (Zeiss, Cambridge, United Kingdom) fluorescence microscope equipped with an excitation filter of 470/40 nm and a ProgRes camera (Jenoptik CF cool, Jena, Germany).

2.4. Analytical methods

2.4.1. Biomass determination

Yeast growth was estimated by measuring optical density in a spectrophotometer (Spectroquant® Pharo 100) at 600 nm. For cell dry weight, 5 mL of culture were filtered through a 0.45 µm glass fiber membrane (Merck Millipore). Collected cells were washed several times with distilled water and subsequently, samples were dried at 105 °C until constant weight.

2.4.2. VFAs determination

VFAs were analysed by liquid chromatography (HPLC) equipped with a refractive index detector (Agilent 1260 HPLC-RID). A Cation H Refill Cartridge Microguard column (Biorad) and an Aminex HPX-87H ion exclusion column (300 × 7.8 mm I.D.) (Biorad) were used. Mobile phase was 5 mM H₂SO₄ (VWR, international) and elution was conducted in isocratic mode at a flow rate of 0.35 mL min⁻¹. The injected sample volume was 20 µL, and the oven and detector temperatures were 25 and 35 °C, respectively.

2.4.3. Lipid extraction and purification

Lipid quantification was performed when maximum growth was reached and substrate was completely exhausted. For lipid analysis, yeast biomass was harvested at the end of the fermentation process by

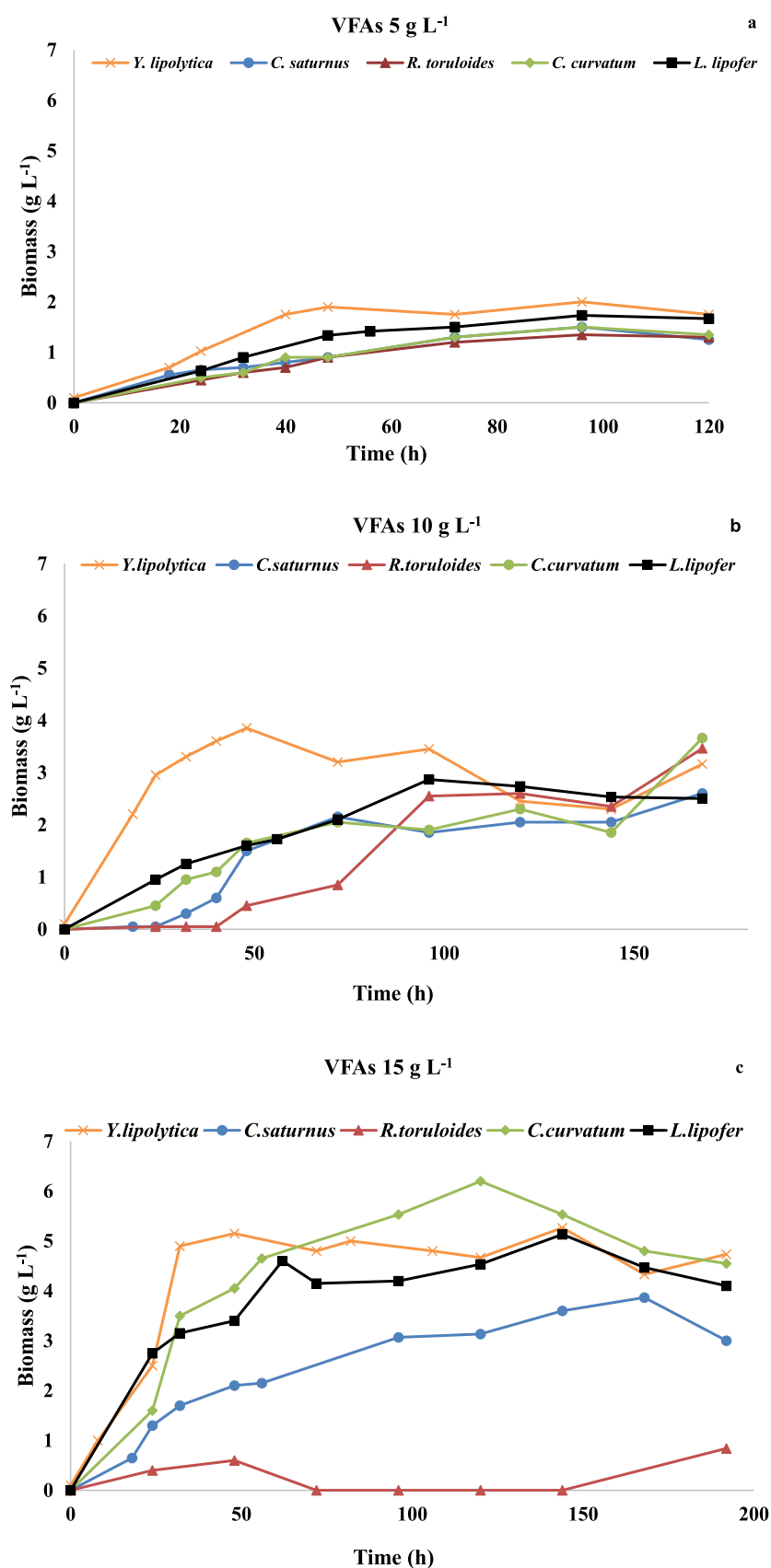


Fig. 1. Time course of the five yeast growth in anaerobic digestion effluent at VFAs 5 (a), 10 (b) and 15 (c) g L⁻¹. Standard deviations were below 5%.

centrifugation at 21,496 RCF for 10 min at 4 °C (Heraeus, Megafuge16, Thermo Scientific). Total cellular lipids extraction was performed according to the method described by Folch [20] with modifications [21]. Briefly, intracellular lipids were extracted in chloroform:methanol (2:1 v v⁻¹) under reflux for 4 h. The extract was filtered through Watman N.1 paper and the organic phase was washed twice with a 8.8 g L⁻¹ KCl solution (Sigma). Samples were dried over anhydrous Na₂SO₄ (Sigma). Finally, the solvent was evaporated at 40 °C under vacuum using a Rotavapor R-215 device (BUCHI), and the total cellular lipids were gravimetrically determined. The lipid content was expressed as gram lipid per gram of dry biomass (%).

2.4.4. Fatty acid composition of yeast lipids

The fatty acid composition of cellular lipids was determined by gas chromatography (GC) after fatty acid *trans*-methylation, according to the AFNOR method [22]. Fatty acid moieties were converted into their fatty acid methyl esters (FAMES) in a two stage reaction. Samples were analysed in an Agilent 7890 A gas, equipped with a HP-88 column (60 m × 0.32 mm) and a FID detector. GC conditions were as follows: Helium was used as carrier gas at a flow rate of 1 mL min⁻¹, injection temperature was 250 °C, oven temperature 200 °C and FID temperature 280 °C. Peaks of FAMES were identified by reference to standards.

2.4.5. Ammonium determination

A colorimetric commercial method, based on the ammonia transformation into a blue indophenol derivative, was used for quantification of ammonium concentration (N-NH₄⁺) using a test kit (Merck, ISO 000683). Briefly, two subsequently reactions took place. In the first one, ammonium nitrogen found in the form of ammonia due to a strongly alkaline medium reacts with hypochlorite ions to form monochloramine. In the second one, this turn reacts with a substituted phenol to form a blue indophenol derivative which will be determined photometrically in a spectrophotometer (Spectroquant® pharo 100, EU).

2.4.6. Statistic methods

A parametric one way ANOVA was used for the assessment of the yeasts growth and lipid accumulation (confidence interval 90%). Differences were considered significant at p-value ≤ 0.1.

3. Results and discussion

3.1. Yeasts growth on VFA containing digestate

The predominant acids in the anaerobic digestate used as substrate for oleaginous yeasts were short-chain VFAs (from C2 to C4) (Table 1), accounting for 75% of the total VFAs. This is a common VFAs profile obtained during AF in which the long chain fatty acids are predominantly degraded through β-oxidation giving rise to short-chain VFAs [6, 23]. Besides, acetic, propionic and butyric oxidation reactions are energetically unfavorable, and therefore, their accumulation in the AF reactor is boosted [24]. These facts agreed with the highest percentage of these acids in the digestate accounting for 32.6%, 12.9% and 24.5%, for acetic, propionic and butyric acid, respectively.

This VFAs profile seems to be suitable for yeast growth according to

previous results were the VFAs toxicity on yeast was evaluated. For instance, Gao and co-workers concluded that the inhibitory effect of VFAs on yeast increased when increasing VFAs chain length [13]. Thus, the low percentage of long-chain VFAs, such as valeric and caproic acid, supports the use of this digestate as a potential low-cost substrate suitable for SCO production.

Time courses of cell growth of the studied yeast strains cultivated on digestate containing VFAs at different initial concentrations are shown in Fig. 1. Some of the strains used in the current work, such as *C. curvatum*, *R. toruloides* and *Y. lipolytica*, are already known for their ability to metabolize VFAs [12,25–28] and this paper confirms previous findings. In addition, *C. saturnus* and *L. lipofer*, were able to grow on VFAs, while *R. toruloides* was completely inhibited in the medium with a VFAs content of 15 g L⁻¹.

Yeast growth parameters (biomass production and growth rates) obtained by the strains under consideration are shown in Table 2. When VFAs concentration in the medium was 5 g L⁻¹, no significant differences were observed among the different strains (p < 0.1) behavior in terms of cell growth (Fig. 1a). The maximum biomass production attained by all yeast strains ranged from 1.2 to 1.9 g L⁻¹. *Y. lipolytica* exhibited the highest specific growth rate (0.04 h⁻¹) while *R. toruloides* was the slowest yeast, presenting a specific growth rate equal to 0.01 h⁻¹. These results indicated that there was no inhibitory effect on yeast growth when digestate was diluted to obtain a VFAs concentration of 5 g L⁻¹. This fact is in good agreement with previous studies which concluded that keeping VFAs concentration below 5 g L⁻¹, acid inhibitory effect was avoided [10,11,26].

When VFAs concentration in the medium increased to 10 g L⁻¹, significant differences among strains were found at p < 0.1 (Fig. 1 b.). Specifically, in terms of biomass production, *Y. lipolytica* reached 3.5 g L⁻¹ followed by *L. lipofer* and *C. curvatum* that attained 2.7 and 2.5 g L⁻¹, respectively. The lowest biomass production was reached by *C. saturnus* (2.2 g L⁻¹). As shown in Table 2, *Y. lipolytica* exhibited the highest specific growth rate (i.e. 0.11 h⁻¹) and practically no lag phase, reaching the maximum biomass production in 48 h. Oppositely, *R. toruloides* exhibited the lowest specific growth rate (i.e. 0.02 h⁻¹) and a 40 h lag phase, requiring 96 h to reach the maximum biomass production. Therefore, although similar biomass yields were observed in *R. toruloides* growing on 5 or 10 g VFAs L⁻¹ (Table 3) (p > 0.1), significant differences were observed in specific growth rates (p < 0.1) and lag phases indicated

Table 3

Biomass and lipid yields. Abbreviations: *Y_{X/S}*, biomass growth yield on VFAs (g g⁻¹); *Y_{L/S}*, lipid yield as lipid on VFAs (g g⁻¹); nd, non detected. Standard deviations were below 5%.

Yeast Strain	VFAs 5 g L ⁻¹		VFAs 10 g L ⁻¹		VFAs 15 g L ⁻¹	
	<i>Y_{X/S}</i>	<i>Y_{L/S}</i>	<i>Y_{X/S}</i>	<i>Y_{L/S}</i>	<i>Y_{X/S}</i>	<i>Y_{L/S}</i>
<i>Yarrowia lipolytica</i>	0.37	0.08	0.35	0.06	0.33	0.05
<i>Cyberlindnera saturnus</i>	0.32	0.11	0.22	0.07	0.22	0.07
<i>Rhodotorula toruloides</i>	0.24	0.06	0.25	0.05	0.06	nd
<i>Cutaneotrichosporon curvatum</i>	0.34	0.10	0.30	0.10	0.35	0.13
<i>Lipomyces lipofer</i>	0.32	0.02	0.30	0.03	0.30	0.05

Table 2

Yeasts growth parameters. Abbreviations: *X_m*, Maximum biomass (g L⁻¹); *μ*, growth rate (h⁻¹). Standard deviations lower than 5% were omitted.

Yeast Strain	5 g VFAs L ⁻¹		10 g VFAs L ⁻¹		15 g VFAs L ⁻¹	
	<i>X_m</i>	<i>μ</i>	<i>X_m</i>	<i>μ</i>	<i>X_m</i>	<i>μ</i>
<i>Yarrowia lipolytica</i>	1.9 ± 0.5	0.04	3.5 ± 0.4	0.11	4.9 ± 0.3	0.14
<i>Cyberlindnera saturnus</i>	1.6 ± 0.1	0.02	2.2 ± 0.2	0.04	3.4 ± 0.4	0.05
<i>Rhodotorula toruloides</i>	1.2 ± 0.2	0.02	2.5 ± 0.3	0.01	0.8 ± 0.1	0.01
<i>Cutaneotrichosporon curvatum</i>	1.7 ± 0.3	0.02	2.4 ± 0.4	0.02	5.2 ± 0.3	0.09
<i>Lipomyces lipofer</i>	1.6 ± 0.2	0.03	2.7 ± 0.2	0.02	4.6 ± 0.4	0.07

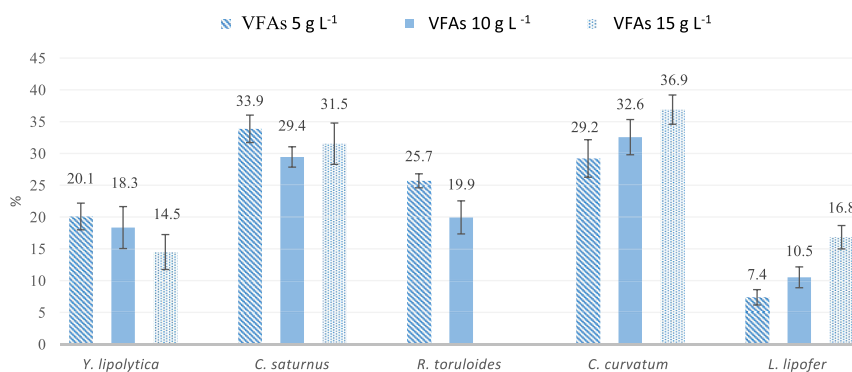


Fig. 2. Lipid content (% ww⁻¹) of the five selected strains cultivated on digestate.

that an increase in VFAs concentration resulted in a partially at least inhibitory effect of VFAs on this strain.

When VFAs concentration increased to 15 g L⁻¹, important biomass production was observed in *C. curvatum*, *L. lipofer* and *Y. lipolytica*, reaching the values of 5.2, 4.9 and 4.6 g L⁻¹, respectively (Fig. 1c). In terms of specific growth rate, *Y. lipolytica* exhibited the fastest growth achieving the maximum biomass production in 24 h. *C. curvatum* and *L. lipofer* specific growth rates (i.e. 0.07 and 0.09 h⁻¹, respectively) were lower than that achieved by *Y. lipolytica* (i.e. 0.14 h⁻¹).

Increasing VFAs concentration from 5 to 15 g L⁻¹, an important decrease in *R. toruloides* and *C. saturnus* yields ($p < 0.05$) observed indicating an inhibitory effect of VFAs on these strains. Specifically, *C. saturnus* was partially inhibited when VFAs content in the medium was 15 g L⁻¹, since both, the substrate exhaustion (data not shown) and maximum biomass production occurred after a long incubation period (i.e. 144 h). In the same way, at this VFA concentration *R. toruloides* was totally inhibited, as no growth was detected even after 144 h of incubation. While the inhibitory effect of high VFAs concentrations (>5 g L⁻¹) on *Y. lipolytica* and *C. curvatum* growth has been reported in many studies [10,11,25,26], in the present study, these strains together with *L. lipofer* seem to be unaffected by VFAs used even at 15 g L⁻¹ concentration. This feature indicated high tolerance of these strains to VFAs (Table 2).

Scenarios comparison indicated that, excepting for *R. toruloides*, an increase in VFAs concentration resulted in higher biomass production (Table 2). In addition, *Y. lipolytica*, *C. curvatum* and *L. lipofer* biomass yields were not negatively affected by the VFAs concentration in the medium (Table 3), remaining over 0.3 g g⁻¹ regardless of the substrate concentration (comparisons performed at $p < 0.1$). On the contrary, in the case of *C. saturnus*, the biomass yields strongly decreased from 0.32 to 0.22 (statistically significant at $p < 0.1$) when VFAs concentration increased from 5 to 15 g L⁻¹. Although *R. toruloides* was not able to grow when the VFAs content was 15 g L⁻¹, no difference was found between its performance at 5 and 10 g L⁻¹ since similar biomass yields were obtained at these VFA concentrations (0.24 and 0.25 g g⁻¹) ($p > 0.1$). These results indicate substrate inhibition on the growth of *C. saturnus* and *R. toruloides*, but not on *Y. lipolytica*, *C. curvatum* and *L. lipofer*. Therefore, we conclude that VFAs inhibitory effect is species and probably strain specific.

Interestingly, biomass production and yields obtained by *C. curvatum*, *L. lipofer* and *Y. lipolytica*, were very close to those achieved with glucose or glycerol used as carbon sources [4]. This fact proves the suitability of using carboxylate-based substrates as alternative to sugar-based substrates for oleaginous yeasts.

3.2. Single cell oil production

3.2.1. Lipid accumulation

In order to assess the oleaginous character of the strains under consideration growing on VFAs rich digestate, yeast lipid was quantified at the end of the fermentation when carbon source was completely exhausted and maximum yeasts biomass was achieved. High lipid productivity is considered as the most important feature of oleaginous yeasts from the viewpoint of biotechnological applications [29]. Lipid accumulation in the tested strains cultivated on medium containing VFAs at 5, 10 and 15 g L⁻¹ ranged from around 7 to 37%, ww⁻¹ of dry biomass (Fig. 2). Oleaginous microorganisms are known to accumulate storage lipids at above 20% on the basis of dry cell weight, reaching even 60% under certain circumstances [4]. Lipid content in yeast is varying even among strains belonging to same species. Apart from their physiological ability to accumulate lipids, many other factors such as substrate type or environmental conditions can strongly affect lipid accumulation [30]. Depending on the substrate used, storage lipid may be synthesized and accumulated via two different pathways. When sugars and similarly metabolized substrates are employed as carbon sources, lipid synthesis follows the so called *de novo* pathway. Prerequisite for lipid accumulation in this pathway, occurring during secondary metabolic growth, is the disturbance of the TCA cycle due to nutrient (usually nitrogen) limitation. Under nutrient limitation, the carbon source found in excess in the growth medium is converted into storage lipid, mostly triacylglycerols. It is generally accepted that the C/N ratio plays a key role in the *de novo* lipid biosynthesis [4]. On the other hand, *ex novo* lipid biosynthesis pathway occurs when hydrophobic substrates are employed as carbon sources. In this case, lipid accumulation takes place during the primary metabolic growth, regardless of nutrient limitations [6,31]. Nevertheless, the metabolic pathways involved in VFAs assimilation by oleaginous yeasts, as well as the key factors involved in lipid accumulation are unidentified. Owing to the high quantities of ammonium released during microalgae AF, the C/N ratio in the digestate used in the current study was very low (i.e. 3.8 ± 0.2) (Table 1), suggesting that lipid accumulation occurred during primary metabolic growth, as in the case of long chain fatty acids used as substrates in the growth medium. High lipid yields were also obtained by *C. curvatum* and *R. toruloides* growing on nitrogen-rich media having acetic acid as carbon source [25,32].

C. saturnus and *C. curvatum* showed the highest lipid accumulation reaching values of around 30–37% on dry biomass regardless the substrate concentration, which confirms the oleaginous character of these two strains. On the other hand, *L. lipofer* exhibited the lowest lipid-producing ability (7.4–16.8% dry biomass). Lipid accumulation in *R. toruloides* and *Y. lipolytica* was negatively affected by the increase in VFAs concentration. For instance, in *Y. lipolytica* lipid accumulation decreased from 20.1 to 14.5% ($p < 0.1$) of its biomass when VFAs

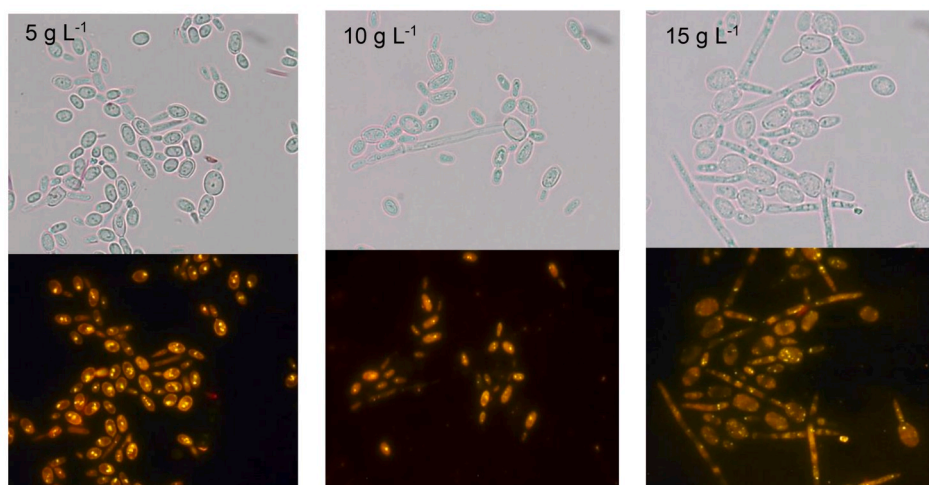
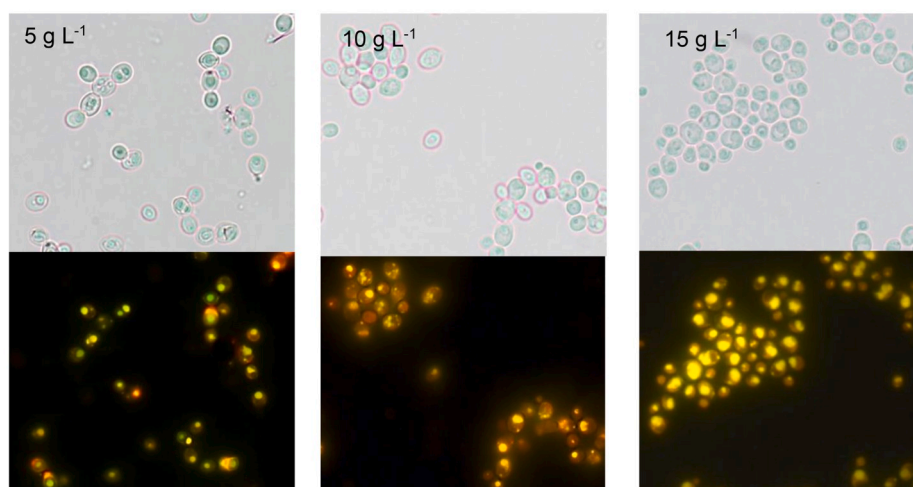
Y. lipolytica (a)*C. saturnus* (b)

Fig. 3. Lipid droplets morphology in *Y. lipolytica*, *C. saturnus*, *R. toruloides*, *C. curvatum* and *L. lipofer* cultured on VFAs 5, 10 and 15 g L⁻¹.

concentration increased from 5 to 15 g L⁻¹, while in the case of *R. toruloides*, a decrease in lipid accumulation from 25.7 to 19.9% ($p < 0.1$) of its biomass was observed when VFAs concentration increased from 5 to 10 g L⁻¹. The data of *R. toruloides* growth show that this strain was strongly inhibited when VFAs concentration increased (Fig. 1), which could explain the decrease in lipid accumulation which takes place in parallel with cell growth according to the *ex novo* lipid accumulation mode. *Y. lipolytica* showed a slight decrease in biomass yields ($p < 0.1$) which resulted in a lipid content decrease with the increase of VFAs concentration (Table 3). These results could be explained by the substrate channelling towards other products instead of lipids [33–35].

In the other yeasts, lipids content either remained constant or increased when VFAs concentration increased. For instance, the lipid content of *C. saturnus* ranged between 29.4 and 33.9% of the dry biomass. On the other hand, *C. curvatum* and *L. lipofer* exhibited an increase in lipid content (statistically significant at $p < 0.1$) from 29.2 to 36.9% and from 7.4 to 16.8% when VFAs concentration increased from 5 to 15 g L⁻¹.

High lipid yield per unit of VFAs consumed were obtained by *C. curvatum*, *C. saturnus* and *Y. lipolytica* (i.e. 0.07–0.13 g g⁻¹), with *C. curvatum* showing the highest conversion yield when cultivated on 15

g VFAs L⁻¹ (0.13 g g⁻¹). *Y. lipolytica* presented a satisfying lipid yield when cultivated on 5 g VFAs L⁻¹ (i.e. 0.08 g g⁻¹) but this decreased with the increase of VFAs concentration in the medium. The remaining of the yeasts showed low lipid yield on consumed VFAs, ranging from 0.02 to 0.06 g g⁻¹.

In spite of the maximum theoretical lipid yield from other substrates such as glucose or glycerol are 0.32 and 0.30 g g⁻¹ respectively, the actual lipid yields obtained by oleaginous yeasts are rarely higher than 0.20 g g⁻¹ [4]. Therefore, the values obtained in this work should be considered relatively high. At this point, it should be highlighted that improvement of the fermentation process is of high importance in order to maximize lipid productivity in yeasts. In this sense, beside the important contribution of the genetic engineering in the field [2] different process configurations [25,26,36–38], culture conditions [32, 37,39,40] and adaptive evolution [41–43], are interesting strategies to overcome challenges associated to lipid accumulation. For instance, some studies have reported lipid yield improvement up to 0.19–0.23 g g⁻¹ in *C. curvatum* and *Y. lipolytica* on synthetic VFAs applying operational mode strategies such as two-stage batch or two-stage feed-batch feeding mode [26,32,36,40].

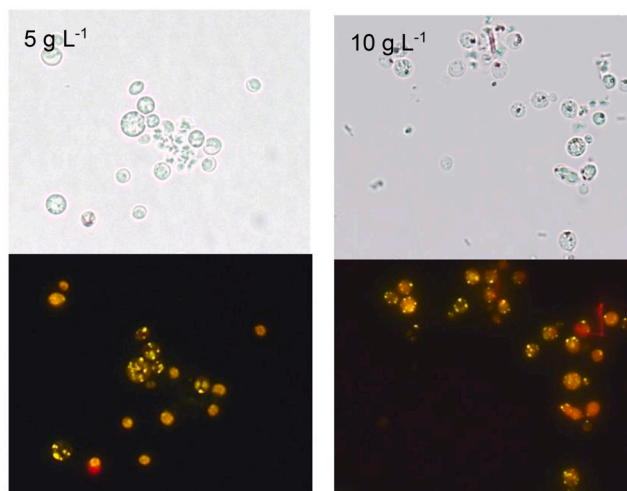
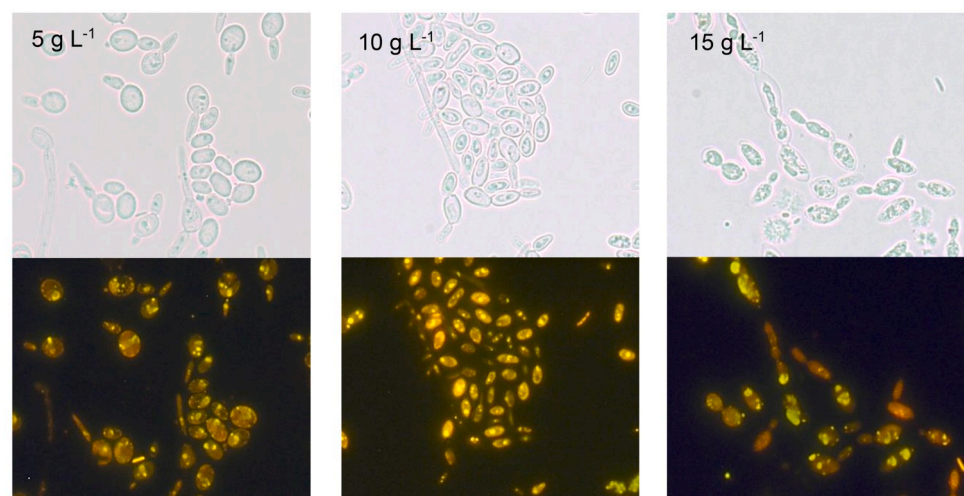
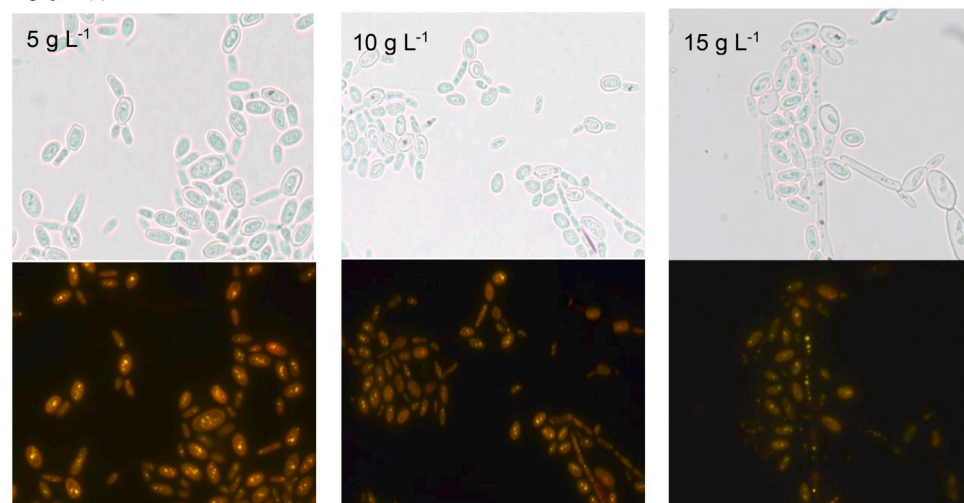
R. toruloides (c)*C. curvatum* (d)*L. lipofer* (e)

Fig. 3. (continued).

3.2.2. Morphology of lipid bodies

Fluorescence microscopy pictures were taken at different fermentation times to evaluate the lipid bodies within the yeast cells (Fig. 3). As it

can be seen in Fig. 3, *L. lipofer* (d) did not practically show lipids inside the cell, while *Y. lipolytica* (a), *C. saturnus* (b), *R. toruloides* (c) and *C. curvatum* (a) presented lipid droplets of varying sizes. It has been

Table 4
Fatty acid composition of the lipid produced by tested yeast strains. *Tr:traces*.

Strain	Substrate	Relative amount of fatty acids (% w w ⁻¹)										
		C14:0	C14:1	C16:0	C16:1	C16:2	C17:0	C18:0	C18:1 n-9	C18:2	C18:3 α	others*
<i>Yarrowia lipolytica</i>	Glu 15 g L ⁻¹	0.3	tr	13.3	17.0	0.5	tr	2.7	58.5	7.8	tr	tr
	VFAs 5 g L ⁻¹	2.3	1.7	13.6	4.8	1.4	6.4	2.7	35.6	14.5	2.4	14.5
	VFAs 10 g L ⁻¹	1.7	2.2	14.2	2.9	2.6	4.0	3.7	42.7	16.3	5.4	4.3
	VFAs 15 g L ⁻¹	0.6	0.7	7.1	6.1	1.9	3.9	2.0	52.6	21.6	2.0	1.4
<i>Cyberlindnera saturnus</i>	Glu 15 g L ⁻¹	1.5	tr	25.6	7.0	tr	tr	1.9	49.4	12.5	2.2	0.3
	VFAs 5 g L ⁻¹	0.3	0.5	15.8	1.2	0.9	3.0	4.0	68.8	1.9	1.4	3.6
	VFAs 10 g L ⁻¹	1.5	2.6	13.5	6.9	7.8	tr	25.2	32.4	8.8	1.3	0.5
	VFAs 15 g L ⁻¹	0.5	4.0	15.4	5.4	7.3		23.1	30.1	9.4	3.7	1.1
<i>Rhodotorula toruloides</i>	Glu 15 g L ⁻¹	1.7	0.2	30.4	3.8	0.1	tr	2.5	50.7	9.4	1.2	0.2
	VFAs 5 g L ⁻¹	2.3	2.2	14.7	6.2	2.4	3.3	3.2	39.3	14.9	5.8	5.6
	VFAs 10 g L ⁻¹	1.3	0.7	9.7	4.6	5.1	0.5	5.8	33.9	29.3	6.4	2.3
	VFAs 15 g L ⁻¹	–	–	–	–	–	–	–	–	–	–	–
<i>Cutaneotrichosporon curvatum</i>	Glu 15 g L ⁻¹	1.3	1.0	10.9	7.6	0.6	tr	3.0	52.9	22.0	tr	0.7
	VFAs 5 g L ⁻¹	3.6	3.3	18.6	8.4	5.3	tr	3.6	32.6	18.5	tr	5.9
	VFAs 10 g L ⁻¹	1.4	0.8	16.4	3.8	1.4	tr	4.5	66.7	3.9	tr	2.1
	VFAs 15 g L ⁻¹	1.5	2.0	12.9	4.5	2.1	6.8	6.6	52.6	9.7	tr	1.4
<i>Lipomyces lipofer</i>	Glu 15 g L ⁻¹	0.1	0.1	12.8	21.4	0.3	tr	1.8	55.0	8.3	tr	tr
	VFAs 5 g L ⁻¹	2.7	7.0	14.8	4.7	2.0	6.4	3.9	46.7	13.3	tr	6.4
	VFAs 10 g L ⁻¹	0.6	0.7	9.1	8.7	2.3	5.7	1.6	55.6	13.2	4.3	0.5
	VFAs 15 g L ⁻¹	1.7	0.7	12.5	4.8	1.5	4.2	5.3	52.8	10.0	3.2	3.2

demonstrated that the amount, localization and morphology of lipid bodies varied widely even among closely related species [30,44]. *C. saturnus* cells have a spherical and voluminous lipid bodies in the middle of the cell, while *Y. lipolytica* presented one or two small and central droplets per cell. *C. curvatum*, *R. toruloides* and *L. lipofer* also presented several small lipid bodies along the cell. It was found that *C. saturnus* (b) on VFAs at 10 and 15 g L⁻¹ and *C. curvatum* (d) on 15 g L⁻¹ exhibited the biggest lipid bodies shaping more than 30% of the cells.

In addition, morphological changes in *Y. lipolytica* (a), *C. curvatum* (d) and *L. lipofer* (e) were observed when the VFAs concentration increase up to 10 or 15 g L⁻¹. Usually, pseudomycelia formation takes places when environmental conditions change stressing yeast cells [45]. Some authors reported that important factors regulating the dimorphic transitions are pH, carbon and nitrogen exhaustion, citrate formation and anaerobic stress [46,47]. The formation of pseudomycelia, that has been determined antagonist to lipogenesis [45], could explain the low lipid content in *Y. lipolytica* and *L. lipofer* when high VFAs concentration in the medium was used.

3.2.3. Fatty acid composition

The fatty acid composition of total lipid was quantitatively determined for each strain cultivated on the three VFA concentrations and compared to the composition of lipid produced on a conventional medium containing 15 g L⁻¹ glucose (Table 4).

Similar fatty acid composition of lipids produced in all the strains was observed, indicated that VFAs are vastly utilized as building blocks for biosynthesis of reserve lipid. The main fatty acids were oleic acid (C18:1) (30–67%), palmitic acid (C16:0) (7–19%) and linoleic acid (C18:2) (9–29%). The fact that all the strains contained C16 and C18 fatty acids at high ratio (up to 87% of the total) revealed the high similarity of these lipids with vegetable oils traditionally used in industry. Similar results were shown by other authors when comparing the composition of vegetable oils and yeasts oils in terms of fatty acids [48, 49].

Comparing the fatty acid profile obtained with glucose as substrate with that observed when VFAs were used as substrate, we note that in the case of VFAs a decrease in the content of C16:0 and C16:1 was registered. Moreover, it is noteworthy the presence of heptadecanoic acid (C17:0), which is an odd carbon chain fatty acid, being unusually produced by oleaginous yeasts grown in conventional substrates. For

instance, *Y. lipolytica*, *C. curvatum* and *L. lipofer* synthesized C17:0 at high percentages (i.e. 6.8%) when grown on VFAs, but this fatty acid was absent when growth performed on glucose (Table 4). Currently, odd carbon chain fatty acids, rarely found in the nature, have attracted significant attention being considered precursors for exceptional lipids [50]. The remarkable percentage of this fatty acid, could be explained by the VFAs composition in the substrate. Previous studies have reported that propionic acid abundance leads to the high content of odd-numbered fatty acids, especially C17:0 [8]. This is in agreement with the fact that propionic acid conformed the 12.9% of the total VFAs in the digestate tested.

4. Conclusions

This work demonstrated the feasibility of utilizing AF effluents as an innovative substrate for oleaginous yeasts. Four of the strains screened in this study (*Y. lipolytica*, *C. saturnus*, *C. curvatum* and *L. lipofer*) grew well in VFAs-rich digestate at three different VFAs concentrations. The highest lipid accumulation was observed in *C. curvatum* (36.9% dry biomass) and *C. saturnus* (33.9% dry biomass) corresponding with lipid yields from VFAs of 0.11 and 0.13 g g⁻¹, respectively. These results were comparable to those achieved with sugar-based media. Moreover, the fatty acid profile of lipids produced by all strains, consisted of oleic, palmitic and linoleic acid, was similar to that of vegetable oils commonly used in the industry. Remarkably, significant amounts of the odd-chain fatty acid C17:0 were produced by *Y. lipolytica*, *L. lipofer* and *C. curvatum*. This study opens new perspectives in organic wastes bioconversion to SCO which ultimately implies a wider number of substrates that can be used for lipid production via AF.

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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GLOSSARY

AF: Anaerobic fermentation

SCO: single-cell oil

VFAs: Volatile fatty acids