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Anaerobic digestion of microalgae biomass for volatile fatty acids production

Digestión anaerobia de microalgas para la producción de ácidos grasos volátiles

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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ANAEROBIC DIGESTION OF MICROALGAE BIOMASS FOR VOLATILE FATTY ACIDS PRODUCTION

DIGESTIÓN ANAEROBIA DE MICROALGAS PARA LA PRODUCCIÓN DE ÁCIDOS GRASOS VOLÁTILES

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ABSTRACT

The carboxylate platform arises as a potential technology to produce bio-based chemicals from renewable resources. Anaerobic digestion (AD), traditionally employed for biogas production, can be shortened to the fermentative stages (anaerobic fermentation AF, acidogenesis and acetogenesis) to produce volatile fatty acids (VFAs) or carboxylates. In this manner organic acids namely acetic, propionic, (iso)butyric, (iso)valeric and caproic acids are produced. The main interest of these compounds, currently obtained petro-chemically, lies in their added value with respect to biogas and the wide range of applications in the chemical industry.

Concurrently, the use of microalgae to treat wastewater can decrease the energy cost associated to aeration in traditional systems. The biomass grown constitutes a potential residue to valorize via AF. Notwithstanding, this biomass requires pretreatments prior digestion due to the hard cell wall of certain strains **(Publication I)**. Even though this biomass has been lately studied for biogas production, the operational conditions and anaerobic microbiome remains unknown when it comes to VFAs production. The novelty of this work, including the use of microalgae biomass as feedstock for VFAs production, is the operational conditions optimization focusing on methanogenic inhibition to avoid VFAs consumption **(Publication II** and **III)**. Archaea inhibition is of outmost importance since these species contribute to VFAs degradation.

The main objective was to evaluate microalgae biomass (*Chlorella* sp.) as feedstock for VFAs production via AF. The employed proteolytic pretreatment responded to the high protein content of the microalgae as well as to avoid any hydrolytic barrier and fully focus on the acidogenic stage. Two approaches were followed, namely shaping the inoculum via pretreatments and tuning operational parameters in order to maximize VFAs production by decreasing archaea activity.

Firstly, batch fermentations were run in order to identify the best conditions that should be further tested in semicontinuous mode. The first approach entailed the use of pretreatments applied to the anaerobic inoculum. Both, chemical (40-50% COD-VFAs/COD_{in}) and thermal pretreatments (up to 70% COD-VFAs/COD_{in}) resulted in high VFAs conversion yields in BCPs **(Publication IV)**. Nevertheless, VFAs yields were not enhanced compared to the control in semicontinuous fermentation mode. This fact was most probably due to the short-term effect of these pretreatments observed in the scarce

variations in process performance combined with the low HRT (8 days) directly imposed to the non-adapted sludge.

The second approach focused on understanding the effect of operational conditions: temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT) and reactor configuration (continuous stirred tank reactor (CSTR) and upflow anaerobic sludge blanket reactor (UASB)). Additionally, the anaerobic microbiome was analyzed by bioinformatics tools to gain insights regarding the key species involved in organic acids production.

BCPs were set at 25ºC, 35ºC and 50ºC and different initial pH values (5.5 and 7.5) (**Publication V)**. Results showed conversion yields ranging 40-48% COD-VFAs/COD_{in} at 25ºC for both pH values (5.5 and 7.5) and at 35ºC for pH 5.5. This study highlighted the need of working at neutral and low pH values and mesophilic or psychrophilic conditions to maximize VFA yields. These results were further assessed in semicontinuous mode in which 25ºC resulted in better conversions (OLR 1.5 g COD/Ld, 35% COD-VFAs/COD_{in}) than mesophilic digestion (OLR 1.5 and 3 g COD/Ld, 25% COD-VFAs/COD_{in}) **(Publication VI)**.

Aiming at increasing the VFAs conversion yields, the use of different HRTs (8 and 12 days) was tested with adapted sludge (OLR 1.5 g COD/Ld). HRT 8 days resulted in similar conversion yields and acidogenic efficiency (39% COD-VFAs/COD_{in} and 0.8 COD-VFAs/**s**COD_{out}). Taking into account that increasing OLR in the mesophilic range did not affect conversion yields, a stepwise increase was applied (OLR 3 g COD/Ld). Digester operation resulted in 39% COD-VFAs/COD_{in} demonstrating the possibility of working at higher OLRs whilst maintaining conversions into VFAs. For this reason, in order to assess the robustness of the system performance, a lack of feeding for two weeks was applied **(Publication VII)**. Results showed a drop in VFAs conversion (from 39 to 30% COD-VFAs/COD_{in}) due to the hydrogenotrophic archaea adaptation, which outcompeted the acidogens activity in this stage. Thus, aiming at recovering and maximizing VFAs production yields, stepwise OLR increases from 3 to 15 g COD/Ld were applied **(Publication VIII)**. COD-VFAs/COD_{in} was maximized at 9 and 12 g COD/Ld, recovering yields showed before starvation (37-39% COD-VFAs/COD_{in}; 0.7 COD-VFAs/**s**COD_{out}). Nevertheless, further OLR increases did not report enhanced VFAs productions yields. The high OLR provided hampered acidogens (0.5 COD-

VFAs/**s**COD_{out}) due to the combined effect of high ammonium (4.4 NH_4^+ g/L NH_4^+), sodium (4.9 Na^+ g/L) and VFAs concentrations (36 g COD/L). Butyric acid led VFAs profile at high OLRs (from 3 COD/Ld, 32%) whereas acetic acid was the most abundant 1.5 g COD/Ld (20%).

Given that UASB configuration could be operated for decoupling HRT and SRT, this reactor was operated at 25ºC for comparison purposes with CSTRs **(Publication IX)**. Low OLRs (2 and 4 g COD/Ld) values provided high methane production (around 50% biodegradability) at lower HRTs (6 days) than those commonly employed in CSTRs. On the contrary, high OLRs (9 g COD/Ld) contributed to VFAs accumulation (37% COD-VFAs/COD_{in}). Propionic acid outstood as the most abundant product (34%).

The microbial analysis showed low diversity (low Shannon index) when compared to reactors employed for biogas production. Firmicutes phylum outstood as the most abundant community (CSTR and UASB) followed by Bacteroidetes or Actinobacteria, which presented functional redundancy. Additionally, the same inoculum gave as a result different microbial structures in both reactors reflecting the importance of reactor configuration. Methanogenesis was mainly related to the hydrogenotrophic pathway in CSTRs whereas acetoclastic dominated at low OLRs (2-4 g COD/Ld) in the UASB.

To conclude, microalgae biomass was confirmed as an attractive feedstock to obtain VFAs. The use of low HRTs (6-8 days), temperatures (25ºC) and high OLRs (9 g COD/Ld) in CSTR and UASB reactors achieved competitive conversion yields (37% COD-VFAs/COD_{in}) with respect to other substrates. Firmicutes presence was highlighted regardless of the reactor configuration employed, which influenced the microbiome in the reactor. Nevertheless, both reactors were able to obtain similar conversions showing the functional redundancy of the microbiome.

RESUMEN

La plataforma de los carboxilatos es una alternativa prometedora para la obtención de bioproductos a partir de recursos renovables. La digestión anaerobia (DA), tradicionalmente empleada para la producción de biogás, puede reducirse tras las etapas fermentativas (fermentación anaerobia (FA), acidogénesis y acetogénesis) para producir ácidos grasos volátiles (AGVs). De este modo, se obtienen ácido acético, propiónico, (iso)butírico, (iso)valérico y caproico. El principal interés de estos compuestos, actualmente obtenidos petroquímicamente, radica en su valor añadido con respecto al biogás y la amplia gama de aplicaciones en la industria química.

Las microalgas para el tratamiento de aguas residuales suponen una alternativa para disminuir los costes energéticos asociados a los sistemas de tratamiento más tradicionales. Esta biomasa, revalorizable mediante FA, a menudo requiere pretratamientos debido a la pared celular presente en ciertas cepas (**Publicación I**). Aunque las microalgas han sido ampliamente estudiadas para producir biogás, las condiciones operacionales y el microbioma anaerobio para obtener AGVs son aún desconocidos. La novedad de este trabajo, que incluye el uso de biomasa de microalgas para la producción de AGVs, es la optimización de las condiciones operacionales centrándose en la inhibición metanogénica (arqueas) debido a que estas especies contribuyen a la degradación de los AGVs durante la metanogénesis (**Publicación II y III**).

El objetivo principal fue la evaluación de biomasa de la microalga *Chlorella* sp. como sustrato para producir AGVs por FA. El pretratamiento proteolítico empleado respondió al alto contenido de proteínas de las microalgas, evitando cualquier barrera hidrolítica y centrando así la investigación en la acidogénesis. Se siguieron dos estrategias; acondicionar el fango anaerobio mediante pretratamientos y optimizar las condiciones operacionales. Ambas tuvieron como fin maximizar la producción de AGVs e inhibir las arqueas. Primero se realizaron fermentaciones en modo discontinuo (BCPs) para identificar las mejores condiciones, y posteriormente se evaluaron en modo semicontinuo.

En primer lugar, los pretratamientos aplicados al inóculo anaerobio en BCPs dieron lugar a altos rendimientos (**Publicación IV**): 40-50% DQO-AGVs/DQO_{in} con pretratamiento

químico, y hasta 70% DQO-AGVs/DQO_{in} con pretratamiento térmico. Sin embargo, no hubo mejora en modo semicontinuo debido al efecto a corto plazo de estos pretratamientos combinados con el bajo TRH (8 días).

La segunda estrategia para maximizar la producción de AGVs se centró en la optimización de las condiciones operacionales: temperatura, pH, carga orgánica (CO), tiempo de retención hidráulica (TRH) y configuración del reactor (de mezcla completa (CSTR) y de flujo ascendente (UASB)). Además, se analizó el microbioma anaerobio para obtener información sobre las especies involucradas en la producción de AGVs.

En primer lugar, los BCPs se establecieron a 25, 35 y 50ºC y diferentes valores iniciales de pH (5,5 y 7,5) (**Publicación V**). Los resultados mostraron rendimientos en el rango de 40-48% DQO-AGVs/DQO_{in} a 25ºC a pH 5,5 y 7,5 y a 35ºC y pH 5,5. Al llevar a cabo un análisis del efecto de la temperatura en condiciones semicontinuas, los resultados obtenidos demostraron que a 25ºC (1,5 g DQO/Ld, 35% DQO-AGVs/DQO_{in}) se alcanzó una mayor conversión que a 35ºC (1,5 y 3 g DQO/Ld, 25% DQO-AGVs/DQO_{in}) (**Publicación VI**).

Con el objetivo de incrementar el rendimiento obtenido previamente, se evaluó el efecto del TRH (8 y 12 días) a 1,5 g DQO/Ld empleando un inóculo adaptado a condiciones psicrófilas. El TRH de 8 días mantuvo los rendimientos de conversión total y acidogénicos (39% DQO-AGVs/DQO_{in} y 0,8 DQO-AGVs/sDQO_{out}). El aumento de carga a 3 g DQO/Ld no afectó a la conversión (39% DQO-AGVs/DQO_{in}), demostrando la posibilidad de trabajar a cargas más altas.

Para evaluar la robustez del sistema, se simuló una falta de sustrato para alimentar el reactor durante dos semanas (**Publicación VII**). Durante este periodo, las arqueas hidrogenotróficas recuperaron su actividad y la conversión alcanzada fue 30% DQO-AGVs/DQO_{in}. Para recuperar los valores de conversión de AGVs, se aplicaron incrementos graduales de CO de 3 a 15 g de DQO/Ld (**Publicación VIII**). La conversión total fue maximizada a 9-12 g DQO/Ld, recuperando los rendimientos mostrados antes de la ausencia de alimentación (37-39% DQO-AGVs/DQO_{in}; 0,7 DQO-AGVs/sDQO_{out}). Sin embargo, la CO de 15 g DQO/Ld resultó en una bajada de la eficiencia acidogénica (0,5 DQO-AGVs/sDQO_{out}) debido al efecto combinado de alta concentración de amonio

(4,4 g NH_4^+/L), sodio (4,9 g Na^+/L) y AGVs (36 g COD/L). El ácido butírico fue el producto más abundante a partir de 3 g DQO/Ld (32%) mientras que el ácido acético fue el más abundante a baja carga (1,5 g DQO/Ld, 20%).

Dado que el uso de un reactor tipo UASB puede desacoplar el TRH y el TRC, se usó esta configuración para realizar una comparación con el CSTR a 25ºC (**Publicación IX**). A baja carga (2 y 4 g DQO/Ld) se obtuvo una alta biometanización (alrededor del 50% de biodegradabilidad) trabajando a HRTs más bajos (6 días) que los empleados comúnmente en CSTRs. Por el contrario, la acumulación de AGVs (37% DQO-AGVs/DQO_{in}) tuvo lugar al aplicar mayor CO (9 g DQO/Ld). En este caso, el ácido propiónico destacó como el producto más abundante (34%).

El análisis microbiano mostró baja diversidad (bajo índice Shannon) en comparación con los reactores empleados para la producción de biogás. El filo Firmicutes fue el más abundante (CSTR y UASB), seguido de Bacteroidetes/Actinobacteria, que presentaron redundancia funcional. Además, el mismo inóculo resultó en un desarrollo de diferentes poblaciones microbianas, lo que refleja la importancia de la configuración del reactor. La vía hidrogenotrófica destacó en los CSTRs y UASB, mientras que la via acetoclástica dominó a bajas cargas orgánicas (2-4 g DQO/Ld) en el UASB.

Para concluir, la biomasa de microalgas se confirmó como un sustrato competitivo para obtener AGVs. Se lograron alcanzar rendimientos de conversión (37% DQO-AGVs/DQO_{in}) similares a otros sustratos. Dicha optimización se alcanzó mediante el uso de TRHs bajos (6-8 días), temperaturas (25ºC) y OLRs altas (9 g DQO/Ld) en reactores CSTR y UASB. La presencia de Firmicutes destacó en ambas configuraciones de reactores. La configuración del reactor afectó a la comunidad microbiana obtenida pero no tuvo efecto en la conversión a AGVs, lo que demostró la redundancia funcional del microbioma anaerobio desarollado en ambas configuraciones.

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ABBREVIATIONS

AD: Anaerobic digestion

AF: Anaerobic fermentation

AAS: Adapted anaerobic sludge

CE: Chain elongation

COD: Chemical oxygen demand

CSTR: Continuous stirred tank reactor

GA3P: Glycerlaldehyde-3-phosphate

HRT: Hydraulic retention time

MCFA: Medium chain fatty acids

MFC: Microbial fuel cells

OLR: Organic loading rate

OTUs: Operational taxonomic units

PHA: Polyhydroxyalkanoate

SAOB: Syntrophic acetate oxidizing bacteria

SCOD: Soluble chemical oxygen demand

SRT: Solid retention time

STP: Standard temperature and pressure

T: Temperature

TAN: Total ammonia nitrogen

TCOD: Total chemical oxygen demand

TS: Total solids

VFAs: Volatile fatty acids

VS: Volatile solids

WWTP: Wastewater treatment plant

INTRODUCTION

1. INTRODUCTION

1.1. Importance of volatile fatty acids (VFAs)

European countries are nowadays joining forces under Europe 2020 growth strategy. The main target of the European Union (EU) in environmental matter has been to gradually update its legislation to promote a shift to a circular economy sustainable model [1]. One of the main concerns is to reduce the current carbon footprint of the state members. Recently, the European Commission has launched a "Green Deal" in order to transform the EU into a fair and prosperous society with no net emissions of greenhouse gases in 2050 [2]. Achieving the EU´s climate and environmental goal require of a new industrial approach based on the circular economy. According to this, the European Green Deal is committed for a green transformation of the industrial sector. Among the sectors that should be tackled for achieved a carbon neutral Europe by 2050, energy and chemical industries are key players. Within these two sectors, anaerobic digestion for revalorizing residual waste streams can be of high interest. As an alternative to the traditional fossil fuels, waste streams can be used as feedstocks for the production of chemicals and energy, Focusing on bioproducts employed in the industrial sector, one of the investigation lines gaining importance nowadays is the use of mixed cultures to produce high added value products such is the case of carboxylates (volatile fatty acids, VFAs) through anaerobic fermentation (AF). Production of VFAs through this via is known as the carboxylate platform [3,4]. The traditional anaerobic digestion (AD) process converts complex substrates into a gas stream (biogas, containing methane) and a liquid stream (digestate). However, AF entails the conversion of organic substrates to bulk chemicals (VFAs) instead of biogas, increasing process profitability [5,6]. As a matter of fact, acetic acid, which is the shortest VFA has a market price of 400-800 €/Ton [7] whereas propionic and butyric acids increase their value up to 2,500 and 1,650 €/Ton, respectively [5,8]. Nonetheless, the economic revenues obtained from VFAs are considerably higher than those resulting from biogas production (around 200€/Ton, [9]). Hence, the use of AF from wastes can be regarded as an interesting option once some technical barriers, such as methanogenic inhibition or VFAs purification steps, are overcome. Acetic, propionic, (iso)butyric, (iso)valeric and caproic acids are VFAs traditionally obtained through the petrochemical pathway. These compounds can be further used as building blocks in

different fields of the industry including food additives, pharmaceuticals, adhesives, solvents or chemical intermediates [10–12]. For instance, acetic acid has an important role in food industry [13], propionic acid is mainly used as acidifier for animal feed and grain [14], and butyric acid can be utilized as a precursor on biofuels production [15]. As a summary, industrial applications of VFAs are gathered in Figure 1.

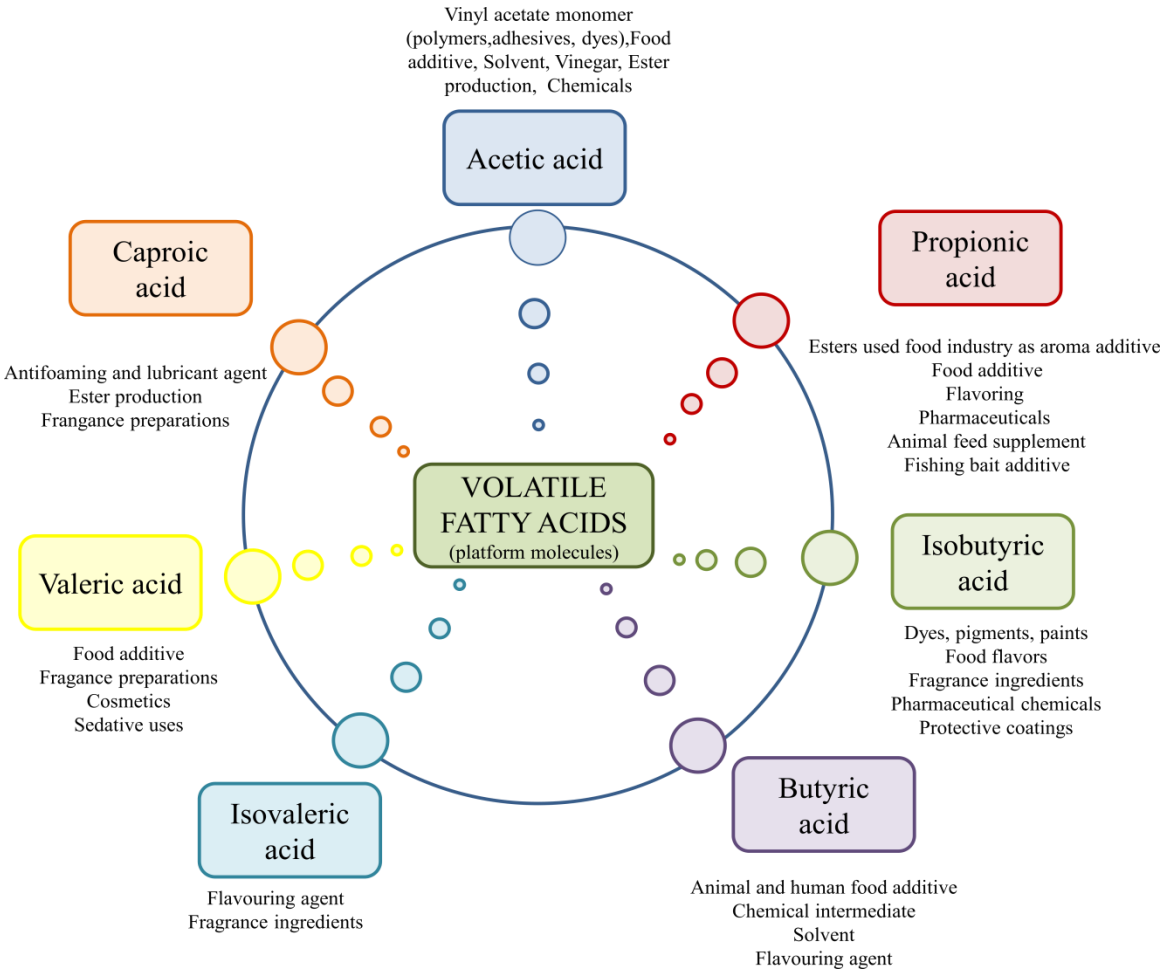

Figure 1. Different applications of VFAs obtained via AF [10–15].

1.2. VFAs production by means of anaerobic fermentation

AD is a well-known and robust process. A wide variety of substrates can be subjected to this technology regardless of their macromolecular composition. The chosen substrate undergoes four different steps which include hydrolysis, acidogenesis, acetogenesis and methanogenesis. Firstly, exo-enzymes belonging to hydrolytic bacteria are in charge of degrading the complex organic matter composed of carbohydrates, proteins and lipids into its respective monomers namely, sugars, amino acids and long chain fatty acids. The efficiency of this stage often rules the overall process yields, as it determines the organic matter availability. Secondly, the acidogenic bacteria oxidize the soluble monomers originating the VFAs, CO_2, alcohols, H_2, and lactic acid (Figure 2).

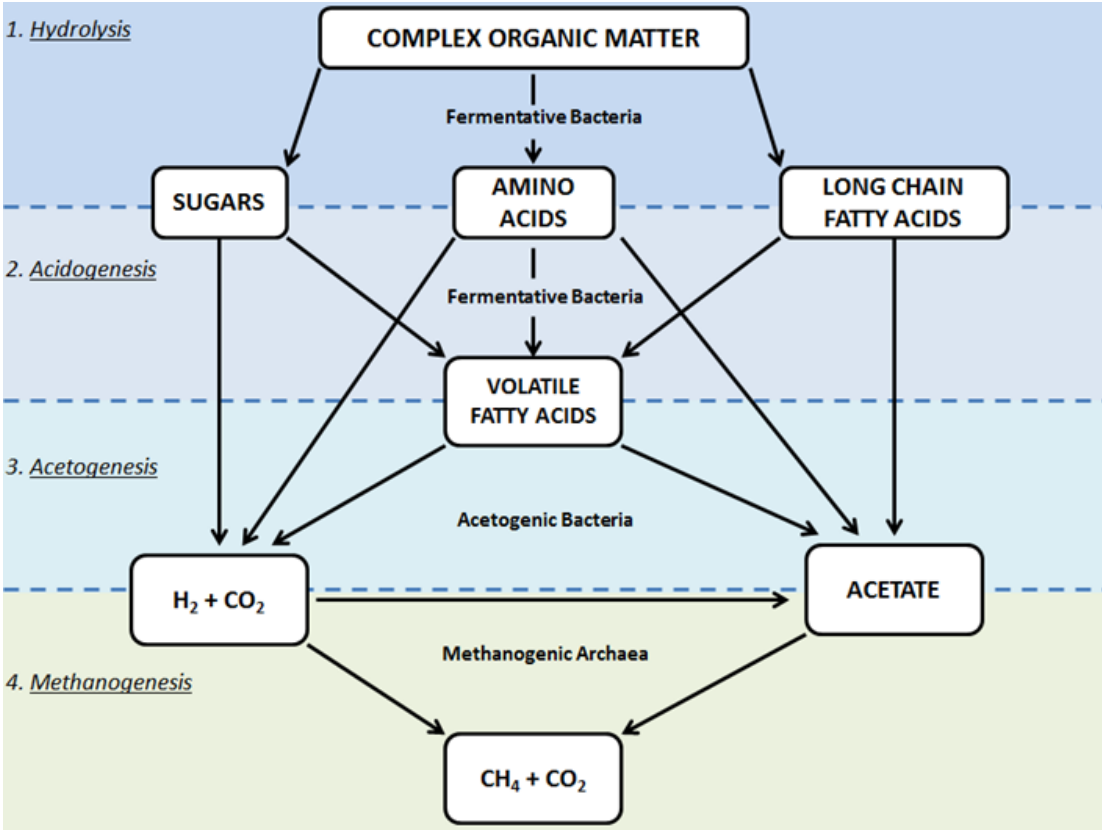

Figure 2. General scheme of the anaerobic digestion process.

Volatile fatty acids (VFAs) are formed during the fermentative stages (acidogenesis and acetogenesis) of the AD process through intricate metabolic pathways (Figure 3). VFAs production through AF occurs at milder temperature and pressure conditions than

petrochemical pathways, resulting beneficial in terms of process energy consumption [16].

During acetogenesis, acetic acid, CO_2 and H_2 are produced by acetogenic bacteria. These products are the main substrates for the methanogenic archaea, which are in charge of the methanogenic stage. These microorganisms can be classified in two different groups depending on the substrate metabolized for biogas production. Acetoclastic archaea use acetic acid to produce methane whereas hydrogenotrophic microorganisms use H_2 and CO_2 as main substrates to obtain methane. The inhibition of this latter step in AD is considered crucial in the context of carboxylates production since otherwise VFAs would be degraded and finally transformed into biogas instead of accumulated (which is the purpose of the present thesis).

VFAs production via AF requires a revisit of the AD process traditionally used for biogas production. In this sense, there are different variables that deserve further study such as (i) potential substrates to be employed, (ii) optimum operational conditions to be implemented for maximum VFA yield production, and (iii) the microbial communities developed within the bioreactor.

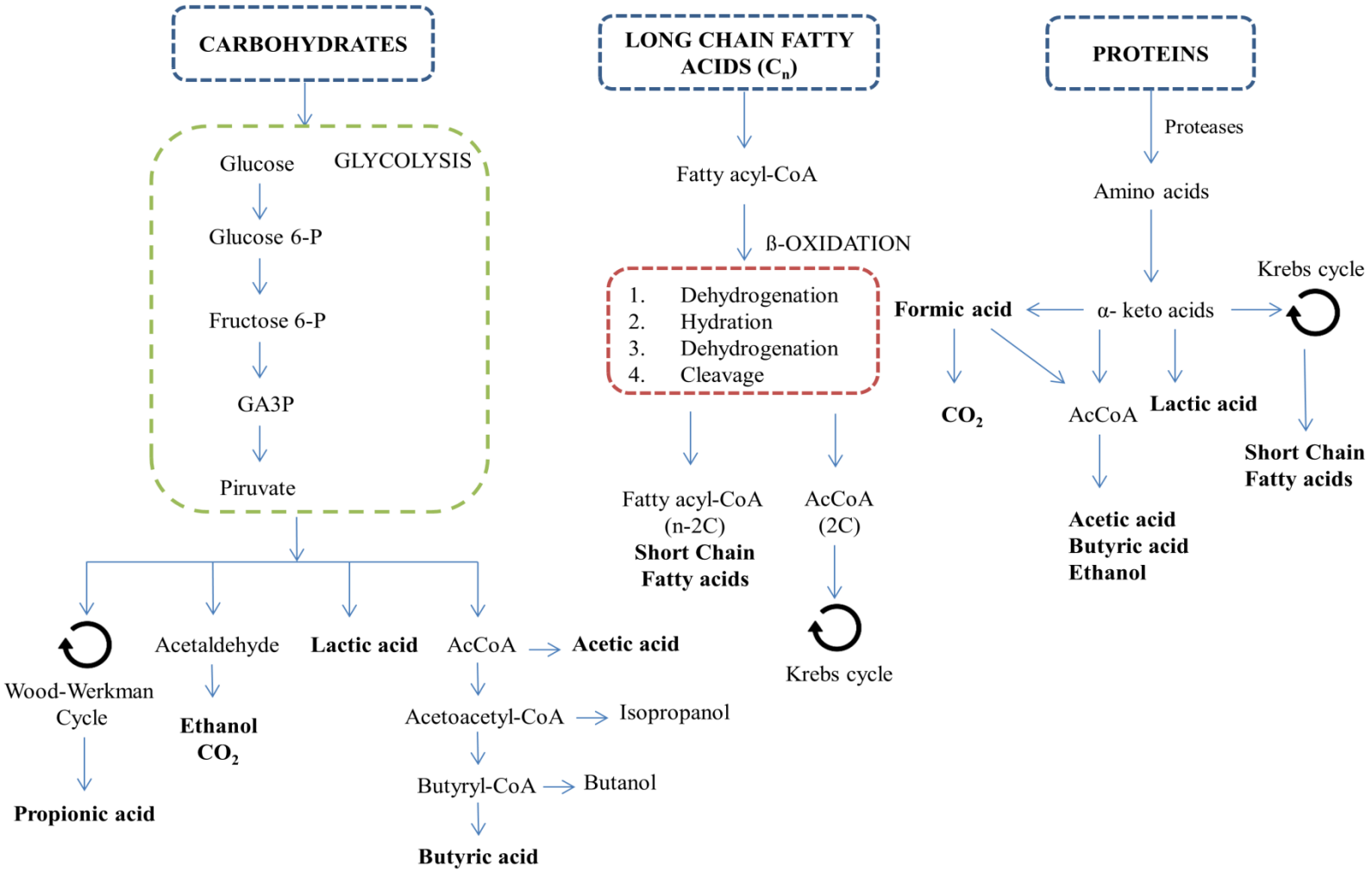

Figure 3. A simplified overview of metabolic pathways involved in VFAs synthesis from the main macromolecules [17–19].

1.2.1. Microalgae biomass as substrate for VFAs production

The selection of cost-effective substrates for VFAs production is of paramount importance to decrease production costs. Up to date, different sugar-based carbon sources have been tested for VFAs production [20,21]. However, cheaper feedstocks, such as waste streams, could be ideally employed maintaining production efficiencies. In particular, food wastes, agriculture residues, and sewage sludge are some of the residual streams that have been employed for VFAs production [22–24]. These residues gather high amounts of organic matter, nitrogen, and phosphorous, which are indispensable nutrients for the metabolic activities of the microorganisms. However, the varying macromolecular composition of food and agricultural wastes responds to seasonal and geographical location changes [25,26], which might represent a drawback to achieve constant VFAs production [27].

Microalgae biomass can be considered a residual stream when grown in wastewater. Microalgae culture systems for wastewater treatment have been shown to be a promising technology for biofuel production [28,29]. Once the biomass is produced, a straightforward process would be to obtain methane via AD, however the generated biomass might be also converted into more valuable compounds than biogas (Figure 4).

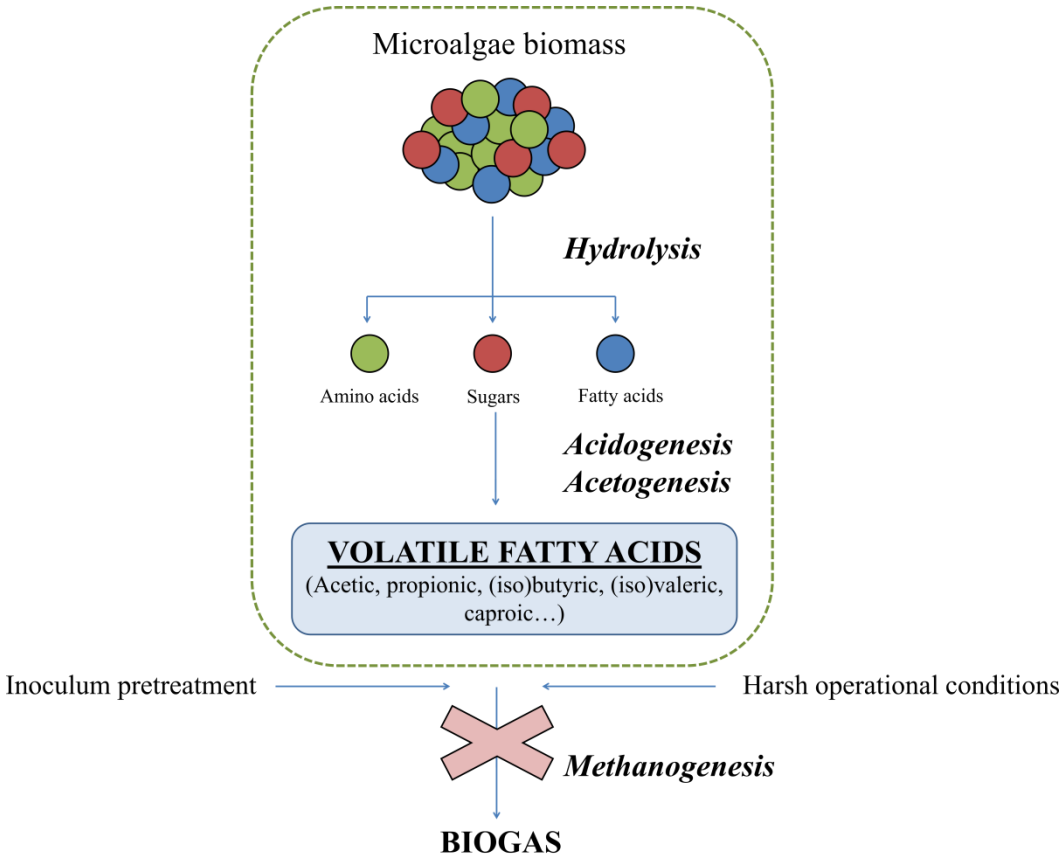

Figure 4. The use of microalgae biomass as feedstock for the carboxylate platform.

Among the feedstocks that can be subjected to AF, microalgae biomass arises as a potential alternative for VFAs production. This biomass does not need arable lands to grow, and it is able to thrive in residual effluents [30,31]. Wastewater bioremediation by means of photosynthetic microorganisms can overcome the well-known limitation of nutrients costs for their cultivation associated to any bioproduct generation from microalgae biomass [29]. When microalgae biomass is cultivated in residual effluents, its use for nutraceutical, feed or food purposes is restricted. Moreover, the harvested biomass is normally poor in fermentable sugar or transesterificable lipids and thus, the most straightforward use of algal biomass is AD. Hence, microalgae biomass cultivated in residual effluents can be considered a renewable resource that can be further valorized for VFAs generation. The physical state and macromolecular composition of a substrate directly affect the hydrolysis efficiency during AF. Low hydrolytic rates due to substrate complexity results in less organic matter available for the subsequent stages and hence, VFAs conversion yields are low. In this sense, as robust strains grown in wastewater

often exhibit a sturdy cell wall, microalgae biomass is considered a complex substrate [32,33]. Aiming at enhancing the hydrolytic step when using microalgae as substrate in AD, different pretreatment methods (e.g., thermal, mechanical, chemical or biological) have been proved to increase biomass solubilization by means of cell wall disruption/hydrolysis. These pretreatment techniques have been widely studied for biogas production using microalgae biomass [16,34]. Since AF includes the 3 first stages of AD, it can be inferred that hydrolysis is also a crucial stage for an efficient VFA productions. In this manner, it can be assumed that the studied pretreatments to increase biogas production from microalgae biomass can also be applied to improve VFAs production.

Besides of the wall protecting microalgae cells, another important aspect is the macromolecular distribution of the substrate that can be classified regarding its content in carbohydrates, proteins and lipids. Fractions of each macromolecule are very variable depending on growth conditions and assessed strain [35,36]. However, in general terms, proteins are the most abundant macromolecule of green microalgae accounting from 30 to 60% of their dry weight when grown in high strength wastewater [37,38]. Digesting protein-rich substrates might constitute a drawback in AD. Proteins are characterized by a tertiary and quaternary structure, which makes them less susceptible to proteolytic enzymes [39]. Hence, the hydrolytic step is often identified as the slowest stage [40]. During organic matter hydrolysis, the protein fraction (most abundant fraction in microalgae biomass) is cleaved into simple amino acids releasing the nitrogen contained in form of ammonium (NH_4^+) and free ammonia (NH_3). The ratio NH_4^+/NH_3 relies on pH and temperature in the system. When targeting biogas production, high amounts of these chemical compounds are associated with digestion failure [41] since they cause methanogenic step inhibition [42–45]. Therefore, this methanogenic weakness towards NH_4^+ and NH_3 that affects biogas production might actually represent an advantage for VFAs generation, as the inhibition of this microbial community would contribute to VFAs accumulation. Thus, this protein prevalence in their macromolecular distribution renders microalgae biomass as an attractive feedstock when AF is targeted for the production of VFAs.

Proteins, carbohydrates and lipids entail different hydrolysis rates affecting process productivity [46]. However, the relative amount of each macromolecule has an influence on total product concentrations and VFA profiles. For instance, proteins-rich substrates have shown enhancement of odd-numbered and longer carboxylates such as valeric and

caproic acids [47,48]. With regard to microalgae biomass, results collected in Table 1 shows that acetic and propionic acids are the most abundant products. Nevertheless, besides the macromolecular composition, there are other factors affecting VFAs production and profiles, such as the established operational conditions and the microorganisms carrying out the biological process.

Table 1. Overview microalgae strains employed in batch mode for VFAs productions: VFAs profiles and bioconversion yields.

Strain	Temperature (°C)	pH	Pretreatment	Substrate Composition (% VS)			VFAs profile (%)							COD-VFAs/COD_{in} (%)	Reference
				Carb.	Prot.	Lipids	Ac	Pr	But	IBut	Val	IVal	Cap		
Chlorella vulgaris	15	6.4	-	26	62	12	70	30	0	-	-	-	-	17.4	[49]
	35						70	20	10	-	-	-	-	38.2	
	55						70	10	20	-	-	-	-	40.5	
Chlorella vulgaris	15	6.4	After lipids extraction	33	66	1	70	30	0	-	-	-	-	9.4	[49]
	35						50	40	10	-	-	-	-	33.4	
	55						60	10	30	-	-	-	-	42.1	
Chlorella sp.	25	5.5	Enzymatic (proteases)	25	65	10	41	28	7	9	7	8	-	47.7	[50]
	35						26	35	9	12	8	9	-	39.1	
	50						33	11	15	14	-	27	-	34.5	
Chlorella sp.	25	7.5	Enzymatic (proteases)	25	64	10	54	21	6	6	6	7	-	45.1	[50]
	35						57	21	6	8	1	7	-	48.3	
	50						46	17	12	15	-	9	-	37.1	
Microcystis	25	10	Control	6	63	4	38	14		36		12	-	13.1	[51]
			0.5 g/L Act. carbon				50	13		21.1		15.9	-	31.5	

Chlorella Pyrenoidosa	35	6.5	-	34	48	18	52	11	35	-	-	-	3	4.3	[52]
			140ºC, 10 min 1% H_2SO_4				41	14	37	-	-	-	8	9.1	
Arthrospira Platensis	37	6	2.5% dilute H_2SO_4 at 135°C for 15 min	19	76	5	40	5	28	5	5	9	8	NA	[53]
Desmodesmus Scenedesmus and *Chlamydomonas*	35	6.9	-	NA	NA	NA	86	2	2	0	0	10	0	20.0	[54]
	45						74	8	5	2	0	10	0	33.0	
	55						66	15	5	1	2	11	0	50.0	
Ettlia	35	7.2	1% NaOH + ultrasound	53	41	6	64	25	11	-	-	-	-	25.3	[55]

1.2.2. Reactor operational conditions for VFAs production

The manipulation of process variables such as the inoculum, pH, temperature, HRT and OLR have a great influence not only in the VFAs accumulation, but also in the obtained VFAs profile [56,57]. The manipulation of operational conditions affects the relations among the microbial populations and determines the fate of organic matter either for biogas production or to VFAs accumulation. Therefore, in the context of VFAs production, the ultimate goal is to inhibit the methanogenic step of the AD and strengthen hydrolytic and acidogenic bacteria activity by tuning operational parameters. Methanogenic inhibition might take place when manipulating process parameters because archaea species are more sensitive to operational changes than organic acid producers [58]. As it was aforementioned (Section 1.2), archaea are the main responsible of VFAs consumption, and thus, their inhibition is considered of paramount importance to attain competitive VFAs yields.

Inoculum

Species present in the anaerobic sludge are very diverse and thus, playing different roles in the AD process. Hydrolytic, acidogenic and acetogenic bacteria, together with methanogenic archaea, gather the biodiversity present in the process. When selecting a substrate for AD, it is necessary to consider the different interactions that might occur with the anaerobic populations. For instance, marine microalgae species, such as *Isochrysis galbana*, *Dunaliella salina* or *Nannochloropsis salina*, hindered biogas production due to their associated high salinity [59–61]. In this sense, as shown by the high external osmotic pressure, high salt concentrations might cause plasmolysis of the anaerobic populations (both bacteria and archaea). Possible solutions to overcome this issue are a long acclimation period for the inoculum, the use of compatible solutes and the employment of halophilic anaerobic populations [62–64].

Even though during the whole process many different species take part, each stage of the AD process is characterized by a group of microorganisms. These species use different molecules as substrates and release different products, resulting in a complex scheme of reactions and products. To achieve a high VFAs accumulation, one possible approach is to reduce the methanogenic population by means of inoculum pretreatments.

Acid/alkali treatments consist on keeping the inoculum for a certain period of time under basic (pH 9–11) or acidic (pH 2–4) conditions. Extreme pH values are able to inhibit methanogenic activity. For instance, acid/alkali pretreatments were conducted by adjusting the pH of the inoculum to 3 and 11, respectively, with 1 N HCl and 1 N NaOH, and maintained for 24 h firstly, and restoring the pH to 6.8 before the fermentation starts [65]. The latter study showed the complete suppression of methanogenic activity at acidic conditions but also showed the influence of the inoculum pretreatment on the VFAs spectrum. Acetate and n-butyrate were dominated in alkali pretreated sludge while propionate was observed when the sludge was pretreated with acid. This outcome was also supported by other study in which acetic acid was the most abundant product (60-70%) after alkali pretreatment, whereas acidic pretreatment showed acetic, propionic and butyric acids [66]. Most likely, those pretreatments affected more communities than just the methanogenic archaea. Therefore, these pretreatments need to be optimized (exposure time and pH conditions) because they do not only inhibit the methanogenic step, but they also might alter the VFAs profile obtained.

Thermal pretreatments imply subjecting the inoculum to high temperatures for a determined period of time to eliminate non-spore forming microorganisms (mainly acetoclastic archaea). For instance, a mixture of *Desmodesmus* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. was digested with an anaerobic inoculum subjected to a thermal pretreatment (100ºC for 2 h) to inactivate methanogens and the results showed organic matter conversions into VFAs of 50% COD-VFAs/COD_{in} at 55°C [54]. Unfortunately, no control was run by these authors for comparison of fresh or pretreated inoculum. Another example includes the pretreatment of an anaerobic inoculum at 120ºC for 10 and 30 min using *C. vulgaris* as substrate which increased the organic matter conversion from 48% COD-VFAs /COD_{in} (maximum conversion of the control) to 71% COD-VFAs/COD_{in} [67]. On the contrary, low temperature pretreatments (80ºC for 10 min, respectively) promoted biogas production (275 mL CH_4/gCOD_{in}) with respect to the control (198 mL

$CH_4/gCOD_{in}$), resulting in low organic matter conversion into VFAs (maximum value of 40% COD-VFAs /COD_{in}) [67]. In that case, 80ºC and 10 min pretreatment contributed to a better hydrolysis and acidogenesis activity linked to a promoted bacterial activity. Apart from microalgae biomass, this type of pretreatment has been applied in literature to other residual streams such as food waste or model substrates such as sucrose [68,69]. However, thermal pre-treatments can be sometimes counterproductive since too harsh conditions can not only eliminate methanogens but also organic acid producers [70].

An alternative to that is the use of more specific pretreatments such is the case of the addition of chemicals to block the enzymatic systems of methanogens. Different compounds (2-bromoethanesulfonate (BES), iodoform or chloroform) have been investigated for this goal [71,72]. In this context, BES (50 mM) prevented methanogenesis when microalgae biomass (*S. quadricauda* and *C. vulgaris*) was used for VFAs production [73]. This trend was maintained when treating an inoculum with lower concentrations of BES (10 and 30 mM) in which no methane was detected and VFAs were accumulated by 50% COD-VFAs /COD_{in} [67]. Likewise, when *Laminaria japonica* was employed as substrate (AF conducted at 35°C and pH 6.5-7), iodoform (0.07, 0.12 and 0.17 mM) inhibited methanogens [74]. This latter study demonstrated that VFAs concentration (8 g/L VFAs) was maximized when using 0.12 mM of iodoform whereas further increases negatively affected VFAs productions (0.17 mM reported values similar to those found in the control, 6 g/L), suggesting the negative effect of iodoform in the rest of the microbiome. This inhibition is in agreement with other studies in which iodoform and chloroform resulted in a reduction of acetic acid production by inhibiting acetogens activity as well [71,75]. In this manner, it can be concluded that even though some chemicals might be archaea specific, some others can affect the whole microbial system and thus, a careful selection of the most appropriated chemical should be conducted.

In general terms, chemical and thermal pretreatments applied to the inoculum are able to inactivate methanogens. At this point, it is worth to highlight that organic matter conversions into VFAs attained in the investigations reviewed were higher when using a thermal pretreatment than employing chemicals. Nevertheless, the high prices, the high energy input requirements in the case of thermal pretreatments and the environmental concerns associated to certain chemicals are the main drawbacks for further implementations. In addition, these strategies often show short term-effect in continuous

operation towards methanogens and thus, other methods (manipulation of operational conditions) are rather recommended for VFAs accumulation.

pH

The pH value of the process has a direct effect on the growth rate of the fermentative microorganisms as well as the optimum enzymatic activities. Each group of microorganisms has an optimum pH working value. Whereas methanogenic archaea grow better at pH close to neutrality, acidogenic and hydrolytic bacteria have a wider pH growth range. Previous studies have estimated the optimum range for the acidogenic bacteria around 5 and 7 [21,76]. However, investigations regarding the effect of pH on VFAs production from microalgae biomass did not show a clear trend, most likely due to the wide range of microalgae macromolecular compositions and operational conditions employed.

The use of pH values in the basic range (pH 10 and 25ºC) using *Microcystis* as substrate retrieved an organic matter conversion into VFAs of 31.5% COD-VFAs /COD_{in} [51]. Agreeing with the fact that basic pH favor VFAs production, another study focusing on VFAs accumulation from excess sludge found that maximum accumulation was achieved at pH 10 (3 g COD/L) over those VFAs attained in the acidic pH range (1 g COD/L) [77]. Likewise, alkaline fermentation (pH of 8 and 10) of primary sludge for VFAs production caused higher VFAs accumulation (1.8-fold) when compared to digestions conducted at pH 3.0–7.0 [78]. In the present thesis, digestion of *Chlorella* sp. was carried out under alkaline conditions (pH of 9, data not published) resulting in maximum organic matter conversion into VFAs of 33% COD-VFAs/COD_{in}.

As it can be seen in Table 1, microalgae biomass has been also evaluated for VFAs production in the neutral and acidic pH range For instance, digestion of *Chlorella* sp. at acidic pH values (5.5) and 25ºC resulted in 48% COD-VFAs /COD_{in}, similarly to the values attained in the same experiment at neutral pH values (45% COD-VFAs /COD_{in}) [50]. The higher conversions obtained at neutral and acidic pH values were supported by investigations carried out by Kim et al., and Cho et al., ([49,54]) who obtained similar conversions (ranging 42-50% COD-VFAs /COD_{in}) when using microalgae biomass as substrate at slightly acidic pH (6.4-6.9).

Temperature

Temperature is a parameter closely related to pH. 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. This correlation was demonstrated when waste activated sludge was employed as substrate for VFAs production at 4, 14 and 24ºC [79]. Results showed an increase in the hydrolysis constant at 24ºC (0.17 $days^{-1}$) in comparison with values attained at 4ºC (0.04 $days^{-1}$). The increase in organic matter availability at 24ºC resulted in the highest VFAs productions (2154 mg COD-VFAs/L vs 782 mg COD-VFAs /L at 4ºC). With regard to microalgae biomass, a recent investigation using pretreated *Chlorella* sp. as substrate obtained similar conversion yields (45-48% COD-VFAs /COD_{in}) at temperature of 25ºC and 35ºC when compared to 50ºC (37% COD-VFAs /COD_{in}) [50]. On the contrary, high fermentation temperatures (50ºC) resulted in high conversion yields (40% COD-VFAs/COD_{in}) when non-pretreated *Chlorella* sp. was digested at pH 6.4, while the use of 25ºC mediated lower conversions (17% COD-VFAs /COD_{in}) [49]. Whereas Magdalena et al., [50] used a proteolytic pretreatment, Kim et al., [49] did not carry out any pretreatment prior AD. Hence, the high temperatures at which this latter investigation was conducted most likely increased biomass solubilization and thus, VFAs yields were higher at the highest temperature. In this manner, temperature increases are associated with higher solubilization rates but not necessarily with a raise in VFAs production. For instance, when maize silage and cow manure were fermented for VFAs production at 37ºC and 55ºC, the highest VFA yield was achieved at 37ºC (18.3 % COD-VFAs/kg VS [80]). Even though organic matter solubilization was more efficient at 55ºC, this study showed higher acidification at 37ºC. Authors suggested that the lower acidification yields reached at 55ºC could be related to a slow adaptation of the thermophilic culture. Similarly, 30ºC was found to be the optimum temperature for VFAs production (3,400 mg/L VFAs) in experiments carried out at 25, 30 and 40ºC when cassava water was used as substrate [81].

Temperature not only affects the physical state of the biomass, but it also has an impact on the anaerobic microbiome. In this manner, selecting a temperature promotes

specific species over others resulting in varying populations, which might impact final VFAs productions and profiles.

Organic Loading Rate (OLR)

The organic loading rate (OLR) is the amount of organic matter present in the substrate (in terms of COD or solids) applied to a certain volume of media per unit of time. OLR selection is process specific. With regard to studies devoted to VFAs production, the general trend is an increasing VFAs production at increasing OLR [82–85]. This may be due to the fact that VFAs accumulation leads to a pH drop, which results in a final decay of methanogens. Nevertheless, it is also true that experimental studies showed a maximum OLR threshold where no further improvements in VFAs production can be obtained [83–85]. These latter studies found that the bottleneck was the hydrolytic stage of the AD process. For instance, OLRs of 3.2, 5.6, 7.4, 9.6, 11.0, 12.9, 14.0 and 15.1 g COD/Ld were evaluated to produce VFAs from olive mill solid residue [84]. The optimum value was 12.9 g COD/Ld whereas further increases resulted in a significant decrease in the acetic acid concentration in the effluent. Following the same trend, acidogenesis of food waste resulted in a decrease in the yields from 0.34–0.37 to 0.29–0.30 (g VFA/g VS_{in}) when the OLR was increased from 5 g VS/Ld to 13 g VS/Ld [85]. Authors of the two latter investigations stated that reactors fed at increasing OLR values might exceed the hydrolytic capacity of the system, and thus, no further improvement in VFAs production is reached. However, the limiting step might be also encountered in other stages of the AF. As a matter of fact, the two previous studies did not employ any substrate pretreatment prior digestion and thus, hydrolytic deficiencies were likely to occur. Notwithstanding, a recent study analyzing the effect of stepwise OLR increases (3, 6, 9, 12, 15 g COD/Ld) for VFAs production using a pretreated *C. vulgaris* as substrate revealed an optimum VFAs production at 12 g COD/Ld (0.37±0.02 COD-VFAs/COD_{in}) with respect to the highest OLR employed (15 g COD/Ld, 0.29±0.01 COD-VFAs/COD_{in}) [86]. In this case, authors used a proteolytic pretreatment to overcome hydrolytic deficiencies and highlighted that the acidogenic stage was the bottleneck for a further increase in VFAs. Authors point out that this was due to the combined effect of high NH_4^+, VFAs and sodium concentrations. Overall, it could be

stated that depending on the organic matter bioavailability, increasing OLRs might affect different AD stages.

Retention time: Hydraulic retention time (HRT) and Solid retention time (SRT)

The hydraulic retention time (HRT) is a design parameter that stablishes the time that the substrate employed to obtain VFAs remains in the reactor. It is closely related with the OLR selected for the process and the substrate employed. At the same time, the solid retention time (SRT) describes the time that the biomass (anaerobic microorganisms) stay within the reactor. HRT and SRT are given by the reactor configuration. A continuous stirred tank reactor (CSTR) configuration provides complete contact between the phases (HRT=SRT) while other reactors such as the up-flow anaerobic sludge blanket (UASB) reactor or the anaerobic membrane bioreactor (AnMBR) can decouple HRT and SRT, which allows setting a low HRT while working at high OLRs. The type of substrate is an important factor that might affect the selected retention time. Substrates can be grouped into three categories according to their polymer composition: rich in carbohydrate, protein, or lipids, each of them with different hydrolysis rates [87]. In this sense, Pavlostathis et al., [88] reported hydrolysis constants of 0.18, 0.43 and 3.2 days for carbohydrates, proteins and lipids, respectively. In general, it can be said that the higher the hydrolysis rate, the lower HRT/SRT can be employed. Nevertheless, it should be kept in mind, that HRT/SRT need to be set up not only based on the hydrolysis stage since a too fast hydrolysis together with a too short RT can be leading to a inhibition of the acidogenesis stage. More specifically, RT needs to be set up to avoid organic overloading of acidogens and allow their proper activity for conversion of organic matter into VFAs.

Given a CSTR operating at low HRT values (and hence, same SRT), microorganisms exhibiting low growth rates can possibly be washed out from the reactor since they do not have time enough to grow. Since methanogenic archaea have been reported to exhibit lower growth rates than acidogenic bacteria [89], employing low HRT values may provoke a drop in the species diversity [90]. Therefore, low HRTs could be used as a tool to select the most suitable populations in charge of organic acids accumulation favoring

the wash out of methanogens. However, HRT values must be high enough to allow the proper activity of anaerobic microorganisms conducting the hydrolysis and acidogenesis of the substrate. While HRT values for biogas production using complex substrates ranges 15-30 [43,91], HRTs for VFAs production can be considerably decreased. More specifically, values reported in literature varied from 0.125 days to 12 days for semi-continuous fermentations [56]. For instance, acidogenesis of food waste was studied at different HRT values (4, 8 and 12 days) [85]. Results indicated that 8 days was optimal to obtain VFAs achieving yields of 0.34-0.37 g VFAs/g VS_{in}. Further decrease of the HRT did not result in an improvement in organic matter conversion into VFAs (0.26-0.32 g VFAs/g VS_{in}) because the HRT was too low for the proper activity of hydrolytic and acidogenic bacteria. Following this trend, the use of low HRTs favored VFAs production (around 39% COD-VFAs/COD_{in}) in a semi-continuous bioreactor fed with *C. vulgaris* in which the use of HRT 8 days reported higher productivities (0.73 g COD-VFAs/Ld) than conversions attained when operating at 10 and 12 days (0.46-0.50 g COD-VFAs/Ld). This fact was attributed to a better activity of methanogens at higher HRTs [90]. Hence, processes devoted for VFAs production would need shorter periods of time than the ones established for biogas production, having a direct impact in a reduction of the total economic process costs. In the particular case of microalgae biomass as substrate in AD, HRT of 15 and 20 days have been used with *C. vulgaris* biomass for biogas production [43] while the HRT can be reduced to 8 days for VFAs accumulation purposes. Nevertheless, other reactor configurations are worth to study since effluents produced in CSTRs present high amounts of solids, which is detrimental for VFAs separation.

The UASB reactor has been claimed to be an optimum choice for the anaerobic degradation of wastewater [92,93] but its use for complex organic substrates (such is the case of microalgae biomass) remain limited. Some investigations employing microalgae biomass as substrate under this reactor configuration are devoted for biogas production. For instance, Soboh and co-workers [94] digested microalgae biomass and attained COD removal values of 79% in a UASB reactor (OLR 5.4 g COD/Ld and HRT of 7.2 days). Following this trend, Tartakovsky et al., [95] employed a UASB reactor (OLR of 2.25 g VS/Ld and HRT of 3.8 days) to digest *Scenedesmus* biomass resulting in a methane yield of 0.22 L CH_4 g VS_{in} (COD removals of 47% considering COD/VS ratio of 1.3). It is worth to mention at this point that high-rate reactor systems, such as UASB, are interesting to decrease the HRTs normally employed in CSTRs (15-30 days, [96]). As a

matter of fact, HRTs employed for biogas production from microalgae biomass in a UASB reactor ranged 2-7 days [94,95]. With regard to VFAs production, UASB reactors produce effluents with high quality (low amount of solids), which can be seen as an advantage for a subsequent VFAs separation and purification step. However, literature is scarce when it comes to produce VFAs under this reactor configuration. When using other substrates different to microalgae, a recent study using a mixture of methanol, ethanol and acetone as substrate at HRT of 3.1 days and OLR 8.6 g COD/Ld resulted in a conversion ranging 52–70% [97]. Another important feature of the UASB reactor is that its configuration mode allows to work at higher OLR values than those normally employed in CSTRs (1-5 g COD/Ld [91]).

The AnMBR configuration has been extensively employed for biogas production using wastewater [98,99] and more complex substrates as well. For instance, the AnMBR configuration was employed in semi-continuous mode for biogas production (OLR 0.52 g COD/Ld; HRT 30 days; SRT 100 days) for the co-digestion of primary sludge and microalgae biomass (*Scenedesmus* sp. and *Chlorella* sp.) resulting in 73% COD removal [100]. In the same way as UASB reactor, the AnMBR configuration was also employed for VFAs production by reducing the HRTs. A recent investigation elucidated the optimum HRT and OLR to obtain VFAs from low strength wastewater and the maximum VFAs yields (48.2 % COD-VFAs/g COD_{in}) were attained at HRT 8 h and OLR 1.65 g COD/Ld [101].

As a summary, literature evidences that most of the experiments to obtain VFAs using microalgae biomass as substrate have been carried out in CSTRs. However, UASB/AnMBR might contribute to VFAs production by decoupling HRT and SRT, as well as mediating high quality effluents due to their low solid content. This characteristic facilitates further VFAs separation and purification steps. Decoupling retention time of the microbial population from the hydraulic time also allows higher anaerobic microbiome diversity [102]. In this manner, depending on the microalgae biomass employed or the use of biomass pretreatments, the use of different reactor configuration should be carefully evaluated.

1.3. Microbial populations involved in VFAs production

Microbial populations present in an anaerobic inoculum have a determining influence in the AD performance. The relative abundance of each group of species during AD/AF might affect the fate of the organic matter. In this sense, a recent study regarding the metagenome for biogas generation highlighted the high flexibility, diversity and adaptability of the anaerobic community to different operational conditions and substrates [103]. Opposite to that, reactors involved in VFAs production are often less diverse and exhibit different species than those devoted to biogas production [27,104]. Some of the main genera identified in AD processes using microalgae biomass as substrate have been collected in Figure 5.

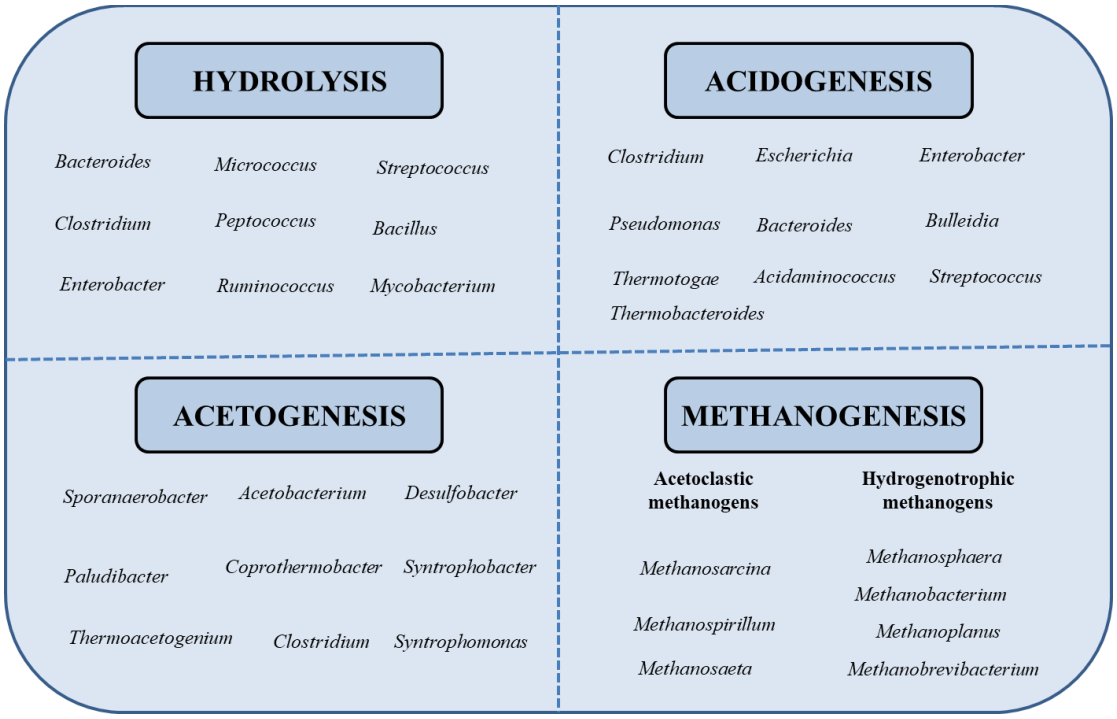

Figure 5. Main genus encountered in AD of microalgae biomass [86,102,105].

In general, Bacteroidetes, Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Spirochaetes, Thermotogae, and Synergistetes are commonly found at different relative abundances depending on the operational parameters and substrates employed when targeting biogas production [103,105]. For instance, batch assays digesting *Chlorella*

sorokiniana and *Scenedesmus*, in the mesophilic range (35ºC), showed that the bacterial distribution was mainly dominated by Proteobacteria (46–51%) followed by Firmicutes (20%) and Bacteroidetes (2-6%) [106]. Continuous operation in a CSTR set in the thermophilic temperature range (55ºC) revealed a remarkable presence of microorganisms that exhibit high hydrolytic capabilities such as Thermotogae (44%) and Firmicutes (17%) when digesting *Scenedesmus* biomass [107]. In this latter study the high presence of Clostridiales (up to 65%) within Firmicutes was highlighted. Using *Scenedesmus* as substrate, microbial communities characterized in an AnMBR (35ºC) showed the dominance of Chloroflexi (27.9%) and Proteobacteria (15.4%) when compared to a CSTR (55ºC) in which Firmicutes led the profile with a relative abundance of 34.6% [104]. In that case, it is not really clear if the different microbiome developed in both reactors was due to the different reactor configuration (high SRTs in the AnMBR) or digestion temperature. When *Spirulina* was digested at extreme alkaline conditions to produce biogas, the anaerobic microbiome analysis showed that Bacteroidetes led relative abundance (27%) followed by Halanaerobiales (15%) and the family Clostridiales (11%) [108]. Therefore, the anaerobic microbiome for biogas production was demonstrated to be very variable. The leading phylum relies on the operational conditions of the digestion, the reactor configuration and the substrate employed.

With respect to the bacterial community in anaerobic fermenters devoted to VFAs productions, Firmicutes, Proteobacteria and Bacteroidetes have been identified as the major contributors phyla (in terms of relative abundance). These phyla have been claimed to produce VFAs as well as actively degrade proteins and polysaccharides, that in fact represent a high percentage in the macromolecular distribution of microalgae biomass (Table 1) [109]. The PCR-DGGE analysis carried out when microalgae biomass was digested at different temperatures (35, 45 and 55ºC) for VFAs production displayed a clear dominance of microbial species belonging to Firmicutes, Proteobacteria and Bacteroidetes [54]. Besides, this investigation also concluded that diversity decreased at the highest temperature, in which VFAs production achieved the highest conversion (50% COD-VFAs/COD_{in}). In the same line, Proteobacteria (65.7%) and Firmicutes (29.0%) were dominant when *Microcystis* was used as substrate for VFAs production [51]. Species belonging to Firmicutes were the most abundant (45-70%) followed by Bacteroidetes (10-35%) when cyanobacterial biomass was digested for VFAs production [110]. All these investigations were carried out in batch mode. Nevertheless, it should be

highlighted that the dominance of Firmicutes (up to 80%) in the bacterial community has been also reported in semi-continuous fermenters fed with *C. vulgaris* [86,90].

Methanogenic species represent a small percentage (in terms of relative abundance) of the microbiome in anaerobic reactors devoted to VFAs production. These species can thrive in extreme conditions of temperature and salinity. Archaea are classified in three orders: (i) Methanobacteriales, (ii) Methanococcales and (iii) Methanomicrobiales [105]. These species, which belong to the Euryarchaeota phylum, are detrimental for VFAs accumulation because their metabolic activity is linked to syntrophic carboxylate-oxidation reactions of propionic and butyric acids to form acetate and hydrogen, which reduces the amount of VFAs in the digestate [4]. Hence, their inactivation is of high importance to achieve competitive VFAs production yields. According to their metabolism, archaea species can be divided into acetoclastic or hydrogenotrophic depending on the substrates employed to generate methane. Hydrogenotrophic archaea are often more robust than the acetoclastic ones [111]. With regard to the archaeal community, contrary to acidogenic reactors, acetoclastic methanogens usually dominate biogas reactors. For instance, an investigation for biogas production in an anaerobic membrane bioreactor (AnMBR) fed with *Chlorella sp.* and *Scenedesmus* highlighted the importance of *Methanosaeta* (acetoclastic methanogen) [112]. This genus was also found in a similar investigation in the same type of reactor using *Scenedesmus* as substrate [104]. On the contrary, the hydrogenotrophic pathway usually gains importance in acidogenic reactors. In this sense, hydrogenotrophic species, such as *Methanobacterium*, have been reported in studies targeting VFAs production [27,86,113].

Overall, reactors devoted for biogas production are expected to be more diverse in terms of bacteria and archaea communities. On the contrary, digesters devoted for VFAs production are often composed by a less diverse microbiome as the imposed operational conditions (i.e. OLR, HRT) might result in a sludge specialization where methanogenic activity is outcompeted by fermentative bacteria. This imbalance is intended to hamper biogas production and in turn, boost VFAs accumulation.

1.4. Separation and purification

Once VFAs are produced, an appropriate technology for its subsequent separation and purification should be selected. Based on techno-economic analysis, this separation process entails technical challenges and is responsible of the main production costs [114]. Several separation techniques have been proposed to recover VFAs from aqueous solution (Table 2). The choice of a specific recovery method relies on the future application of the recovered VFA stream. For instance, methods such as reverse osmosis or high voltage electrodialysis render VFAs with high purity, but can be costly due to high energy costs associated to the recovery process. As mentioned in Section 1.2.2., reactor configurations such as UASB or AnMBR might be employed when the future VFA application require high purity levels, as the subsequent purification is facilitated by the low solid content in the digestate. VFAs at high purities could be employed for instance for drug or cosmetics preparation. On the contrary, if recovered VFAs are suitable for a particular application at low purities, there will be no need to use a high-cost recovery method.

Table 2. Advantages and disadvantages of methods employed for VFAs separation from aqueous solution [115]**.**

METHODS	DESCRIPTION	ADVANTAGES	DISADVANTAGES	LITERATURE
Precipitation	Calcium salts neutralize the acids. Resulting calcium carboxylate solutions, can be concentrated crystallized and separated from the mother liquor	- Well established - Higher product yields - Low capital costs - Products of high purities	- Generating solid wastes	[116–118]
Distillation	Ammonia neutralizes the acids forming ammonia carboxylate. This compound mixed with alcohol to form esters, which are separated by distillation	- Well established - Highly pure products - Byproducts can be used as fertilizer	- High energy and capital costs related to distillation	[119]
Adsorption	Ion Exchange resins used to adsorb carboxylate ions	- Well established. - Easily operable	- High resins costs - High energy demand due to resin regeneration - Low adsorption capacities - Separation is not highly selective	[120–124]

Electrodialysis	Negatively charged carboxylate ions move through an anion exchange membrane towards the anode by using an electric current	- Carboxylate is concentrated in an aqueous solution - No acid treatment to adjust pH is required	- High impurities - Difficulties in scaling up - High energy demand - Prone to fouling	[125–128]
Solvent Extraction	Organic acids are used to extract carboxylic acids from the stream	- High product yields - Suitable for carboxylate salt production	- The feed needs to be acidified for efficient extraction - Extractants need to be regenerated by distillation or back extraction	[129–132]
Membrane Separations	Use of membrane filters of various pore sizes to treat the mixed effluents for solids removal and fractionate the desired substances for recovery	- Developing technology - High product yields - Low energy required - Easy to scale up	- Membrane fouling and clogging - Largely untried in complex waste systems	[133,134]

Among them, forward osmosis, electrodialysis/electrocoagulation and pervaporation are the techniques most used for VFAs separation.

Forward osmosis is based on separation of feed and draws solution via osmotic pressure. More specifically, high osmotic pressure (compared to the feed solution) induces the water to flow through the membrane. A rejection of 100% of the feed solution indicates that only water passes through the membrane. This would mean a high VFAs concentration on the other side of the membrane. This membrane-based technology is very dependent on pH since this parameter affects the rejection rate. For instance, Jung et al., [133] highlighted that the rejection rate was higher at pH 8 in which 97% rejection was attained for a synthetic solution of 35 g/L solution (6:3:1 ratio acetic, propionic and butyric acid). On the contrary, lower pH (4) resulted in a rejection of VFAs of 40%.

Electrodialysis can be applied to recover charged components from liquid effluents obtaining high quality products. There are two types of ion exchange membranes: anion-fixed; permeable to anions and cation-fixed membranes; permeable to cations [135]. The only requirement for a proper performance of these membranes is that the compound of interest remains in the ionized form so it can be transported by an electrical current (driving force). In electrodialysis, the VFA (anions) and the cations would migrate toward the electrodes with opposite charges when an electrical voltage is applied between the electrodes. The anionic exchange membrane only allows the VFA anions to pass and retains the cations, whereas the cationic exchange membrane does the opposite. For instance, removal of VFAs via ED was tested from hydrogen production fermenters with efficiencies higher than 90% [136,137]. Authors pointed out to the importance of the chain length, as longer molecules might negatively affect process efficiency. Electrocoagulation is an alternative to electrodialysis. In this case, the process uses sacrificial electrodes, which produce metal ions that can be used to coagulate the organic matter and nutrients whilst VFAs remain in the liquid phase [138].

Pervaporation is a membrane-based separation process relying on the difference in solubility and diffusivity of components through a dense membrane. Pervaporation has been used for organic solvent separation [139]. The separation is mainly governed by the hydrophobicity. Therefore, in the case of VFAs, this technology can be suitable since

hydrophobicity increases as carbon chain length increases. While some other separation techniques are able to separate all the VFAs in a global manner, this technology is able to discriminate among particular VFAs. When this technology has been applied for VFAs recovery, valeric and caproic acids were preferentially separated over acetic, propionic and butyric acids [140].

1.5. VFAs as building blocks for the industry

VFAs produced from microalgae biomass fermentation might be a product by itself (after separation and purification, Section 1.4) or serve as platform molecules for different applications within several fields in the industry. Some of the promising applications that these molecules might encounter include the production of biodegradable plastics such as polyhydroxyalkanoates (PHAs), energy generation from microbial fuel cells (MFCs), medium chain carboxylates via chain elongation (CE) and their use as building blocks for oil-based chemistry via oleaginous microbial fermentation (Figure 6).

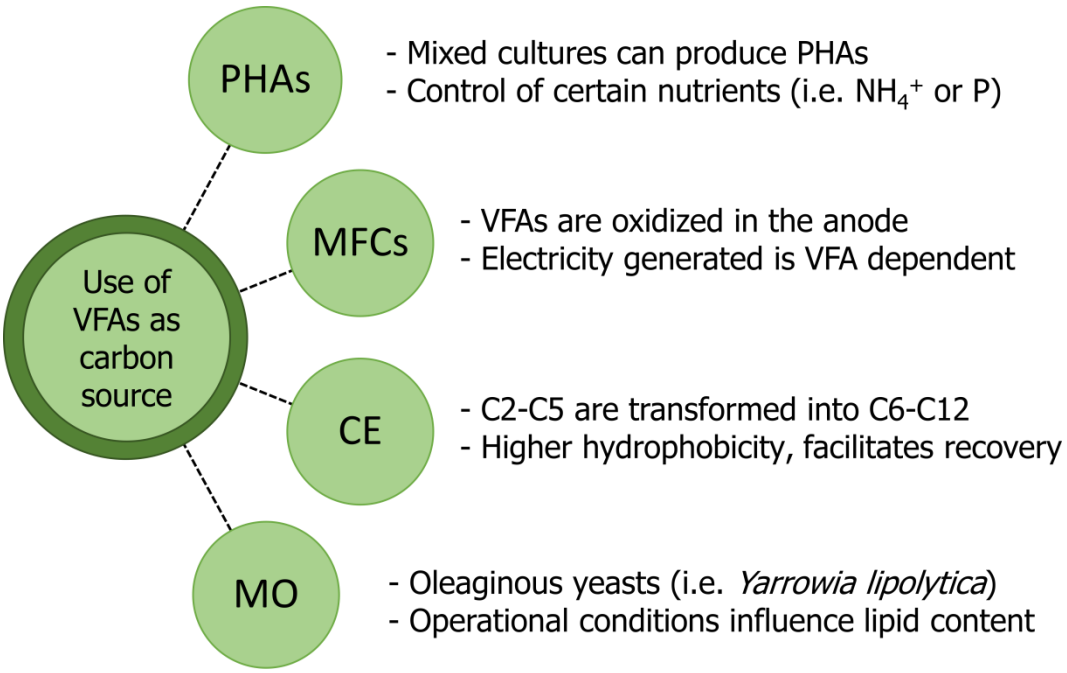

Figure 6. Main industrial applications of VFAs as carbon sources.

PHAs are currently produced using microbial isolates and well defined substrates, which increase overall production costs [141]. However, VFAs produced from waste streams appear as a promising alternative to reduce process costs [142]. In this sense, PHAs might be produced from the VFAs present in the digestate obtained after microalgae fermentation. This broth should be filtered to remove microorganisms and the NH_4^+ and phosphorous controlled to allow PHAs production [143]. Results using different fermented wastes as substrates in mixed cultures have resulted in microbial systems exhibiting PHAs contents ranging 40-77% (DW) [11]. Likewise, it should be highlighted that some authors have addressed the importance of VFAs distribution on final PHAs composition [144,145].

Another application might be the electricity generation in MFCs [146]. MFCs are made up of two electrodes: a bioanode and a cathode. In the bioanode, a biofilm oxidizes the soluble VFAs producing electrons. These electrons flow towards the cathode through an external electric circuit generating an electric current. In the cathode, those electrons react with an electron acceptor, which is thereby reduced. This technology has attracted lately the attention of the scientific community since VFAs can be used as alternative carbon sources. This is the case of Rabaey et al., [147] who used glucose medium as carbon source to obtain electricity in a MFC resulting in a power output of 49 W/m^3. Alternatively to glucose, some VFAs have been tested as carbon sources. Besides, the contribution to electricity generation is VFA dependent. For instance, the electricity generated in a MFC using a mixture of VFAs was mainly attributed to the presence of acetic and propionic acids whereas butyric acid exerted a negative impact [148].

CE process transforms short VFAs (C2-C5) into medium chain carboxylates (C6-C12) [82]. These compounds have more value than biogas or VFAs and can be further used in several fields of the industry (aviation fuels, solvents, lubricants or feed additives) [149]. In addition, C6-C12 organic acids are more hydrophobic than shorter VFAs. This feature makes them more attractive as a product because it facilitates the subsequent recovery step. The CE is catalyzed by an anaerobic microbiome via a metabolic route called reverse β-oxidation. In this pathway, an acetyl CoA molecule is added to a carboxylate (acetate) finally elongating two carbons at a time. The oxidation of an electron donor such as ethanol, methanol, hydrogen or lactic acid is necessary for this process to take place. The impact of different operational conditions (selected electron

donor, methane inhibitor or the substrate used) on medium chain carboxylate productions is nowadays the focus of intensive investigation [150]. In general, low productivities are attained due to the use of mixed culture fermentations, and thus, the study of the microbiome may serve to enhance process yields.

VFAs are also regarded as low-cost carbon sources for lipid biosynthesis to produce oil-based products [151]. The similar characteristics of plant and microbial oils (similar fatty acids profile) make microbial oil production a promising biotechnological tool for biofuel and bioproducts generation. Among the oleaginous microbial systems, oleaginous yeasts exhibit high cell densities and fast growth rates [151]. In addition, they can be cultivated in a wide range of wastes [152] and exhibit higher lipid content than bacteria [153]. For instance, oleaginous yeasts, such as *Yarrowia lipolytica* or *Cryptococcus curvatus,* can accumulate up to 60% of their dry weight in form of lipid bodies [154]. In fact, different approaches have been attempted to increase microbial lipid content such as changes in temperature, pH, C/N ratio, culture mode or fermentation time [155]. Within this research field, the yields obtained range 0.1-0.2 g lipid/ g VFAs [156,157]. While the stoichiometry is very well-known in the case of glucose as a substrate, the use of VFAs in microbial oils is still in its infancy and yet, it is not even clear the metabolic route that yeast use to convert them into oils. In this sense, efforts are also being directed to research new strains and process configurations to maximize oil content using VFAs as carbon source [158,159].

OBJECTIVES

2. OBJECTIVES

The use of the carboxylate platform from microalgae biomass might be useful to produce bulk chemicals as well as for proper waste management. Microalgae biomass arises as a potential feedstock for bio-based VFAs production. This biomass has been lately studied for biogas production in the context of wastewater treatment plants. Nevertheless, this new approach presents the additional benefit of recovering all the carbon contained in the biomass instead of losing a side stream as CO_2 when producing biogas. For this reason, the general objective of the present PhD thesis was to evaluate the potential of microalgae biomass for VFAs production. To achieve this objective, VFAs production yields and product profile were assessed using microalgae biomass as substrate for AF. In order to fulfill this general objective, three specific objectives were established:

a. Understanding how operational parameters can be tuned for maximizing VFAs yield. For such a goal, the impact of operational parameters, temperature, pH, HRT, OLR and reactor configuration on VFAs productions and profiles was studied.

b. Inhibiting the methanogenic step in order to cause VFAs accumulation by applying pretreatments to the anaerobic inoculum. More specifically, the influence of thermal and chemical pretreatments was assessed in terms of organic matter conversion into VFAs and VFAs productions and profiles.

c. Identifying microbial communities as related to fermentation with desirable performance outcome. With the aim of identifying key species involved in VFAs production from microalgae biomass, the anaerobic microbiome developed in each scenario was analyzed.

Finally, for a better comprehension of the overall PhD thesis, the different publications used in the results and discussion section can be found in Figure 7.

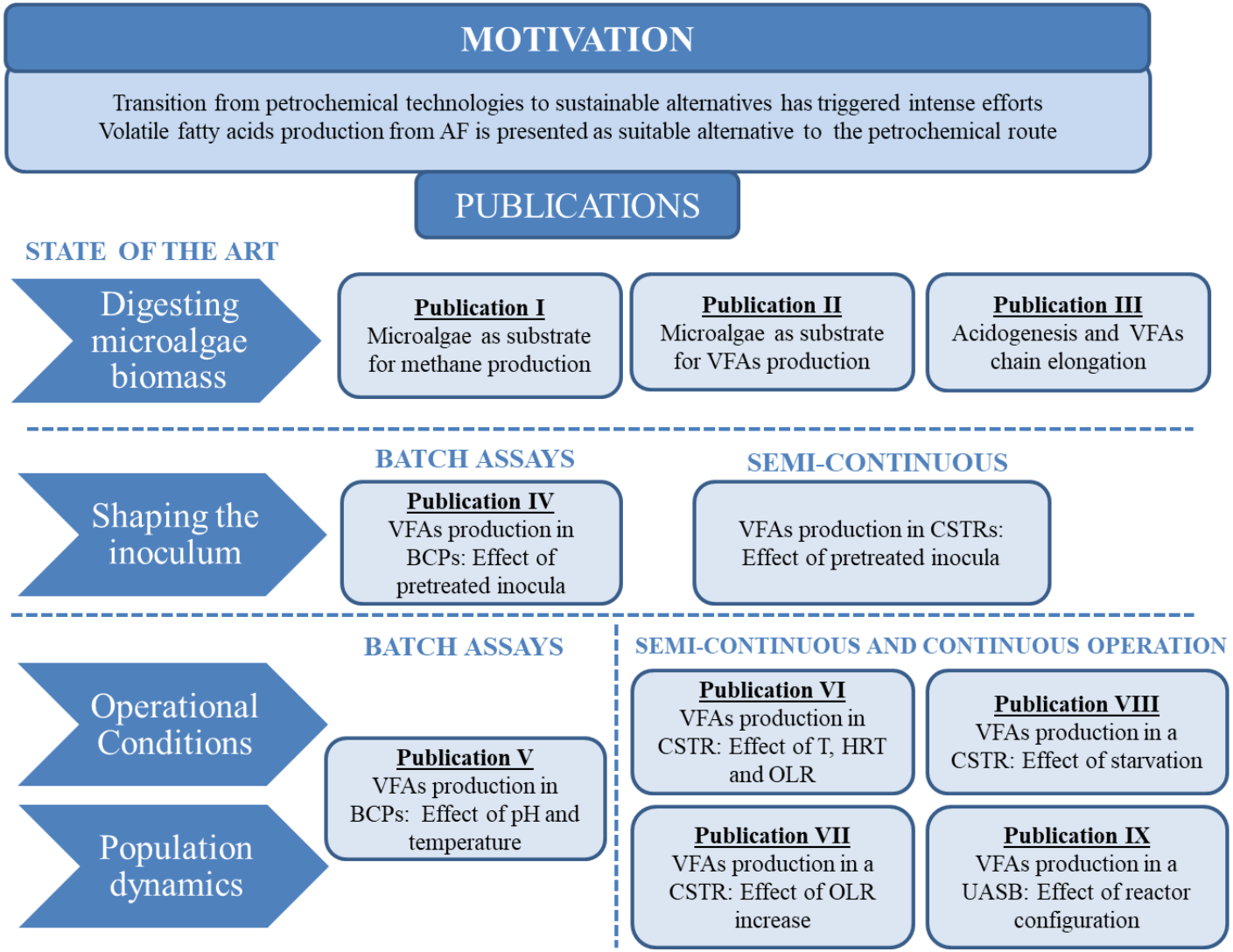

Figure 7. Schematic summary of the performed research to achieve the global objective of this thesis.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Microalgae biomass and sludge used as seed inoculum

Microalgae biomass used in the present PhD thesis belonged to the specie *Chlorella* sp. The sCOD/tCOD ratio of this raw microalgal biomass was low (0.1). For this reason, a pretreatment was carried out in order to increase the organic matter availability. The pretreated microalgae biomass was characterized in each investigation. Average values, taking into account all experiments, are shown in Table 3.

Table 3 . Characterization of *Chlorella* sp. used as substrate (after pretreatment) for VFAs production**.**

Chemical Parameter	Value
COD/TS	1.5 ± 0.5
COD/VS	1.8 ± 0.5
sCOD/tCOD	0.59 ± 0.03
pH	8 ± 0.3
Proteins (% VS)	59 ± 5
Carbohydrates (% VS)	26 ± 3
Lipids (% VS)	9 ± 5

The high amount of proteins (Table 3) exhibited by this microalgae strain might entail an added-advantage for VFAs production. During AD, proteins are degraded into NH_4^+ and free NH_3. High concentrations of these compounds inhibit methanogenic archaea, and thus, contributing to VFAs accumulation [41].

The anaerobic and aerobic sludge's used along the different experiments in the present PhD thesis were kindly provided by the wastewater treatment plant of Valladolid (Spain). The sludge was periodically collected and thus, chemical and microbiological characterization was carried out in each experiment. In general, the anaerobic sludge employed in the present PhD thesis presented the following chemical characterization: VS/TS=0.7±0.1, sCOD/tCOD=0.15±0.1, NH_4^+=0.4±0.1 g/L and pH 7.5±0.2.

3.2. Enzymatic pretreatment applied to microalgae biomass

As mentioned in Section 3.1., a pretreatment was used to solubilize the particulate organic matter present in the microalgae biomass. This solubilization rendered the organic matter more bioavailable for the microbiome present in the anaerobic sludge, thus favoring the hydrolytic step. Since the goal of the thesis is to evaluate the potential of this biomass, it was decided that all microalgae biomass used herein would be pretreated according to the previous data attained for this strain in the context of biogas production purposes [91]. In this manner, the most common stage hampering the fermentation, hydrolysis, was facilitated by using a proteolytic pretreatment. The use of this pretreatment responded to previous works that highlighted the importance of the protein fraction in terms of methane production during AD [45,91].

First of all, microalgae biomass was concentrated by centrifugation in a Thermo Scientific Heraeus Megafuge 16 R at 5000 rpm. Afterwards, the commercial proteolytic cocktail Alcalase 2.5L (Novozymes, Denmark) was added taking into account the total solids of the non-pretreated biomass. The dose to pretreat microalgae biomass was established at 0.585 UA/ g TS [91]. During biomass pretreatment, operational parameters such as pH and temperature were adjusted periodically to 8 and 50ºC, respectively (according to the supplier specifications). Pretreatment lasted for 3 hours at 130 rpm. After this time, the temperature was raised to 75ºC for 30 min in order to deactivate the enzymes.

3.3. Inoculum pretreatment

Aiming at decreasing the methanogenic community activity, the present investigation used thermal and chemical pretreatments:

3.3.1. Thermal pretreatment

Thermal pretreatment was applied to the anaerobic inoculum to suppress methanogenic activity. Temperature can be easily controlled and its effect is microbial specific. In this sense, high temperatures might inactivate non-spore-forming microorganisms (like methanogens) while maintaining spore-forming acid producers (See Section 1.2.2). The present study was designed to cover temperatures 80, 100 and 120ºC in periods ranging from 10 to 30 min. Temperature and time were selected according to previous studies [56,160]. More specifically, the experimental design combined these values to cover the whole range of both parameters and thus, the anaerobic sludge was pretreated at (i) 80ºC for 15 and 30 min, (ii) 100ºC for 20 min and (iii) 120ºC for 15 and 30 min.

3.3.2. Chemical pretreatment

Sodium 2-bromoethanesulfonate (BES, purchased from Sigma Aldrich) affects methanogenic activity by inhibiting archaea enzymes [161]. This chemical was tested at two different concentrations, namely 10 mM and 30 mM. These concentrations were selected based on the investigation of Webster and co-workers [162]. Moreover, BES, both at 10 mM and 30 mM, was also combined with a thermal pretreatment (80ºC and 120ºC for 10 min) in order to study any possible synergistic effect between both pretreatment methods.

3.4. Anaerobic biodegradability of microalgae

3.4.1. Biochemical carboxylate potential assays (BCPs): Batch mode

The biochemical methanogenic potential (BMP) of microalgae biomass gives information regarding the biodegradability of this biomass under a standardized process methodology [163]. This method, however, might also be applied to determine the carboxylate potential of a certain substrate in batch mode (BCPs). For this task, BCPs were run in triplicate at different conditions depending on the experiment (Table 4).

Table 4. Operational conditions and characteristic of the inoculum used in the different BCP assays.

Article	Temperature (ºC)	pH	Pretreated inoculum
V	25	5.5	No
	25	7.5	
	35	5.5	
	35	7.5	
	50	5.5	
	50	7.5	
IV	35	7.5	Yes*

* In this investigation, the inoculum was subjected to different pretreatments but operational conditions (T and pH) were maintained.

120 mL serum glass bottles with 70 mL of working volume were set at 3 g COD substrate/ g VS inoculum. This ratio causes AD imbalance, resulting beneficial for VFAs production [109]. pH was adjusted to a certain value depending on the experiment (Table 4) at the beginning of the assay but not further controlled. 1.5 g $CaCO_3$/L was supplied to batches to buffer the system and prevent pH changes. Bottles were flushed with helium to remove the oxygen and ensure anaerobic conditions. Additionally, blank measurements were conducted to estimate the endogenous methane and VFAs production. The overall methane and VFAs production was calculated by subtracting the blank productions measured in each sample. 0.5 mL digestate were extracted periodically and filtered

through 0.2 micrometers to analyze VFAs through liquid chromatography. Figure 8 shows a schematic approach of the BCPs experiment. The substrate was expressed as in terms of biodegradability by dividing the cumulative methane volume by the theoretical cumulative methane volume, which is obtained from the chemical ratio of 1 g COD = 350 mL CH_4 at standard temperature and pressure conditions (STP).

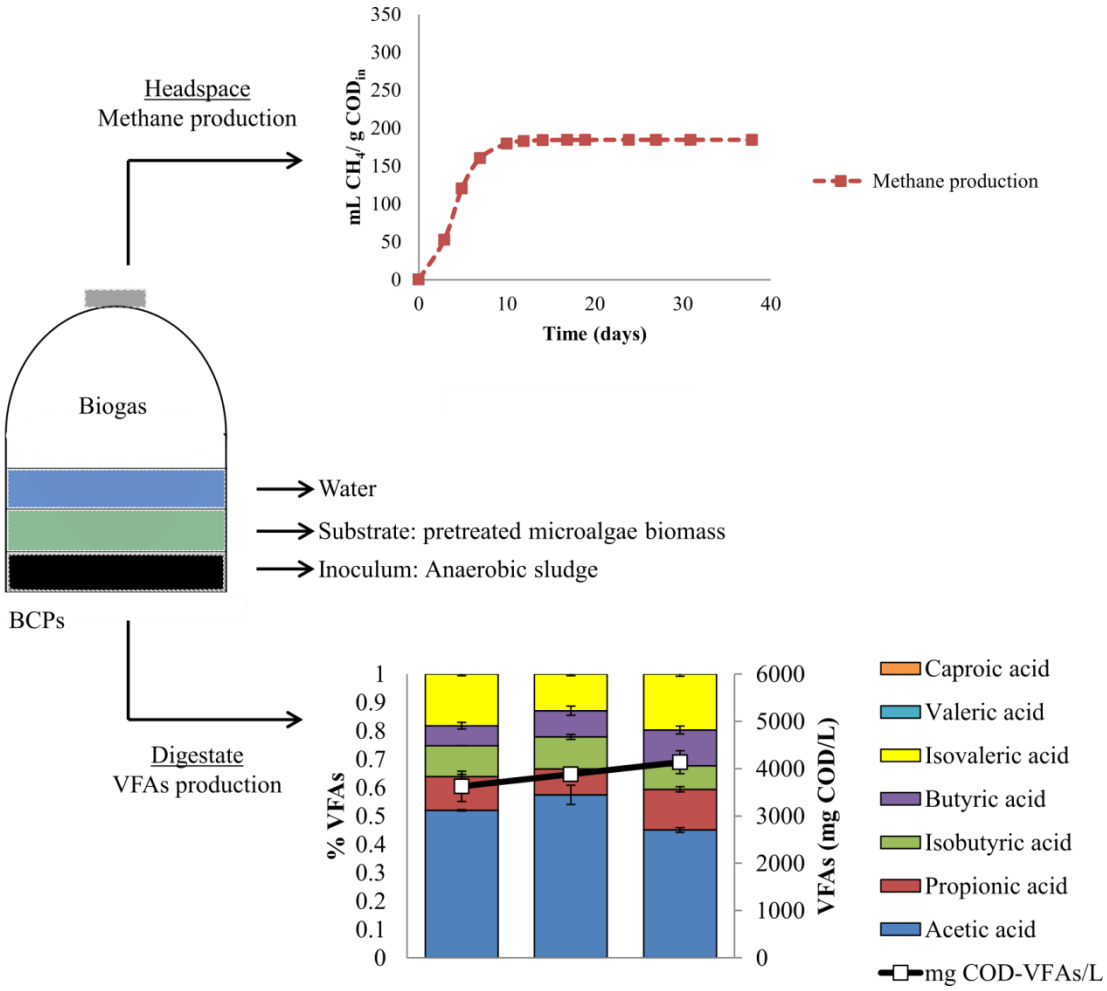

Figure 8. General scheme of BCPs: analysis of the biogas production provides the biodegradability of the substrate whereas the produced VFAs are present in the digestate.

The biogas volume produced was calculated by measuring the pressure of the bottle's headspace. The gas productions were expressed in standard temperature (0ºC) and pressure (1 atm) conditions (STP conditions) according to the following equations:

$$P.V = n.R.T \qquad \text{(Eq.1)}$$

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

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

$$P^o.V^o=n.R.T^o \qquad \text{(Eq.2)}$$

Where: P^o is reference pressure (1 atm = 1.013 bar), V^o is biogas production at 0ºC and 1 atm, during the test T^o is standard temperature (0ºC, 273 Kelvin)

By substituting n (as it is fixed) in two former equations:

$$PV/T = P^oV^o/T^o \qquad \text{(Eq.3)}$$

Therefore,

$$V^o = P.V.T^o/P^o.T \qquad \text{(Eq.4)}$$

Additionally, experimental data obtained from methane productions can be fitted to mathematical models. One of the models employed in the present thesis was the modified Gompertz model. Experimental data from BMP (biochemical methane potential) were fitted with the modified Gompertz equation (Eq.5) [164].

$$P(t)=P_\infty \exp\left[-\exp\left(\frac{R_m^e}{P_\infty}(\lambda-t)+1\right)\right] \qquad \text{(Eq. 5)}$$

P(t) is the accumulated methane production at Standard Temperature and Pressure (mL CH_4 STP/g COD_{in}), P_∞ is the potential methane production (mL CH_4 STP/ g COD_{in}), R_m

the maximum methane production rate (mL CH_4/day), λ the lag phase (days) and t the elapsed time (days).

3.4.2. Semi-continuous anaerobic digestion in a continuous stirred tank reactor (CSTR)

AF was carried out in continuous stirred tank reactors (CSTRs) of 1 L working volume under semi-continuous feeding mode (Figure 9). Reactors were stirred magnetically at 250 rpm and temperature was maintained with a water bath. Depending on the experiment, digester operational conditions (temperature, OLR and HRT) are presented in Table 5. Steady state was considered after 3 HRTs and stable effluent COD and VFAs concentrations had been achieved. pH was monitored but not controlled during the experiments.

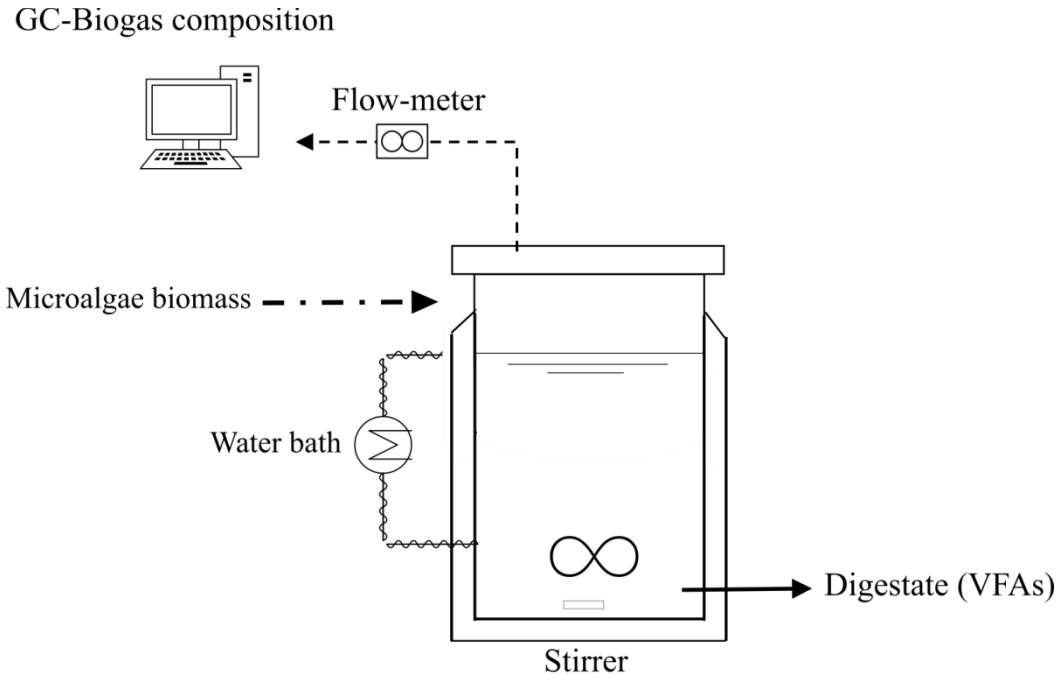

Figure 9. Schematic diagram of a CSTR fed with microalgae biomass for VFAs production.

A controlled disturbance namely starvation for a period of two weeks was investigated to analyze AF performance and microbial community dynamics. Temperature, OLR and HRT conditions selected were based on the investigation carried out in Section 3.4.1.

Table 5. Operational conditions of the CSTRs employed in the present investigation

Article	Temperature (ºC)	OLR (g COD/Ld)	HRT (days)
VI	35	1.5	10
	35	3	10
	25	1.5	10
	25	1.5	12
	25	1.5	8
VII	25	3	8
	25	starvation	-
	25	3	8
VIII	25	6	8
	25	9	8
	25	12	8
	25	15	8

3.4.3. Continuous anaerobic digestion in an up-flow sludge anaerobic blanket reactor (UASB)

AF of *C. vulgaris* was carried out in a UASB reactor of 4.41 L working volume (Figure 10, Article IX). The up-flow velocity supplied (±0.3 cm/h) from the base of the reactor was used to help the sludge coalesce back to the bottom of the reactor. Operation temperature was set at 25ºC (psychrophilic range). The reactor was fed in continuous mode by using a peristaltic pump at stepwise OLR increases (Table 6). pH was monitored but not controlled during the experiment.

Table 6. Operational conditions imposed during UASB operation.

		STAGE		
		I	**II**	**III**
UASB operational conditions	Upflow velocity (cm/h)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Q (L/d)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
	HRT (d)	6.4 ± 1.1	7.2 ± 1.2	6.3 ± 0.7
	OLR (g COD/Ld)	2.3 ± 0.2	3.6 ± 0.9	8.7 ± 1.2

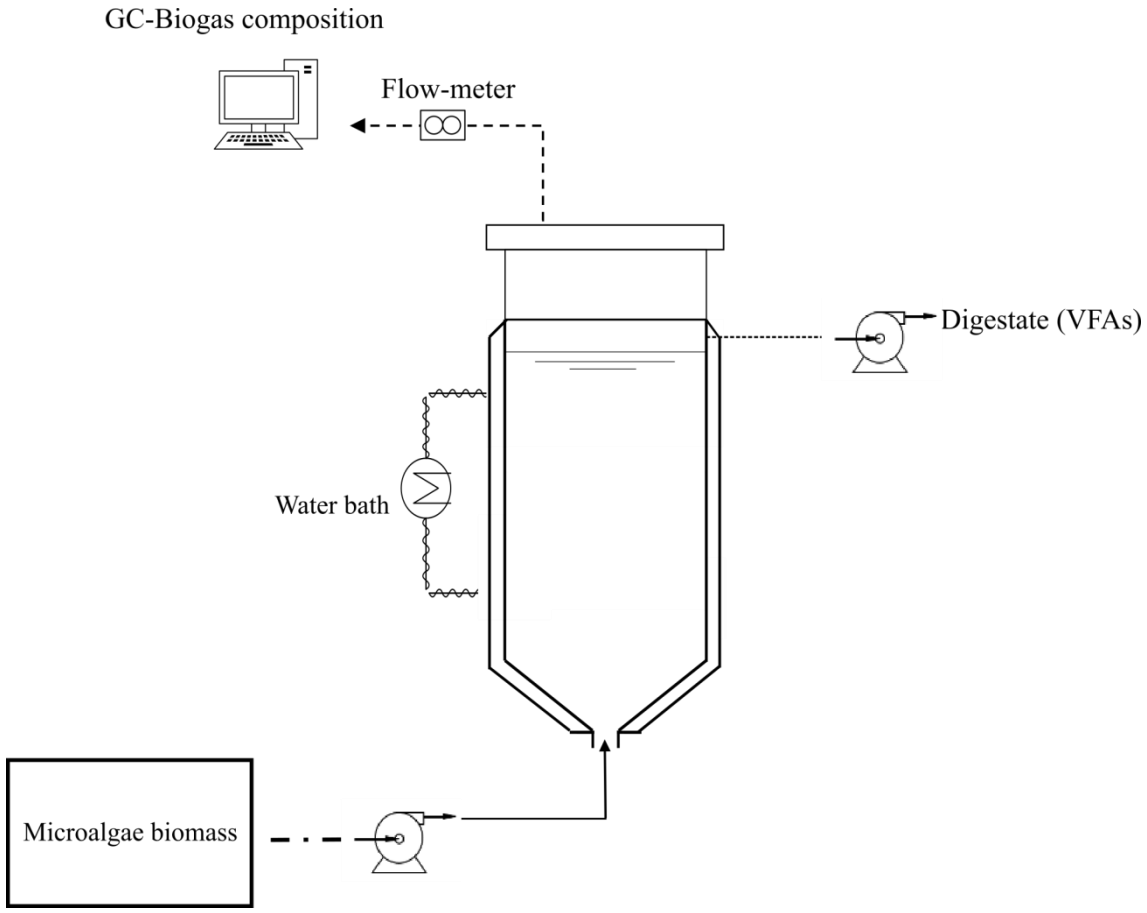

Figure 10. Schematic diagram of an UASB reaction during continuous operation to obtain VFAs from microalgae biomass.

3.5. Process performance

The process performance was evaluated 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. COD balance gives information regarding the methanogenic efficiency and bioconversion into VFAs of the evaluated processes. More specifically, the COD removal account for the organic matter that has been totally reduced to methane out of the total organic matter fed into the system. The COD removal can be calculated as follows (Equation 6):

$$\%\text{COD removal} = \frac{(\text{COD}_{in} - \text{COD}_{out})}{\text{COD}_{in}} \cdot 100 \qquad \text{(Eq. 6)}$$

Additionally, two parameters were considered to evaluate organic matter bioconversion efficiency, namely COD-VFA/COD_{in} and COD-VFA/$sCOD_{out}$. COD-VFAs/COD_{in} was employed in BCPs and semicontinuous and continuous mode to measure the total process efficiency and select the appropriate operational conditions. COD-VFAs/$sCOD_{out}$ provides information regarding the acidogenic stage of the AF.

3.6. Analytical procedures

3.6.1. Total and volatile solids (TS and VS)

Following the standard methods, total and volatile solids (TS/VS) determination was carried out by using a gravimetric balance (Sartorius TE64) [165]. This method consists on the evaporation of the water contained in the sample. For this task, the sample is placed in a crucible, previously dried and weighted (P1), and placed in an oven (Binder) for 24 h. Afterwards, the sample is allowed to cool down at room temperature in a desiccator and subsequently weighted (P2). The difference between the two weights, and taking into account the sample volume, is the result of TS (Eq.7, i.e. g/L). To determine

VS content, the sample obtained after following the TS procedure is incinerated in a muffle (Carbolite 2000 W) at 550ºC for 3 h. Thereupon, the sample is introduced firstly in an oven (Binder) and secondly in a desiccator to cool down and is subsequently weighted (P3). The decrease in crucible weight represents the VS contained in the sample (Eq. 8).

$$TS\ \left(\frac{g}{L}\right)=\frac{\left(P_2\ (g)\text{-}P_1(g)\right)\cdot 1000}{Vsample\ (mL)} \qquad (Eq.7)$$

$$VS\ \left(\frac{g}{L}\right)=\frac{(P_3\ (g)\text{-}P_2(g))\cdot 1000}{Vsample\ (mL)} \qquad (Eq.8)$$

3.6.2. Chemical oxygen demand (COD)

The main goal of this analysis was to follow up organic matter conversion into VFAs along the experimental time. Chemical oxygen demand (COD) was determined through a colorimetric method corresponding to DIN ISO 15705. This parameter indicated the amount of oxygen coming from potassium dichromate that reacts with the oxidable compounds contained in an aqueous sample. The same method was employed to determine the soluble COD. However, in this case, the sample was first filtered through 0.45 μm.

The analytical method consists on the use of commercial kit Merck ISO15705, that oxidizes 3 mL of sample adequately diluted with potassium dichromate at 148ºC for 2 h in a thermoreactor (Spectroquant TR420 M) using silver sulphate as catalyst. Subsequently, COD content was determined in a Spectroquant Pharo 100M.

3.6.3. Carbohydrates content determination

Carbohydrate content in liquid samples was determined by the phenol sulphate method [166]. Briefly, 200 μl of liquid sample was diluted up to sugars concentration range between 0.05 and 0.5 mg/mL. Deionized water, as blank sample, and glucose standards solution to prepare a calibration curve (0-0.5 mg/mL) were also employed. Then, 50 μl of phenol solution (at concentration of 5% v/v) and 5 mL of sulphuric acid

(98% v/v) were added to each sample and mixed. After 30 minutes, the amount of total soluble sugars was determined by using a Spectrophotometer (wavelength of 485 nm) (Spectrostar Omega S/N 415-1414, Germany).

3.6.4. Total Kjeldahl Nitrogen (TKN): Proteins determination

TKN involves digestion, distillation and titration. A fixed volume of microalgae sample was digested with 12 mL sulphuric acid (95%) and a catalyst mixture (K_2SO_4 and $CuSO_4$) at 420ºC for 1 h. The digestion step was conducted in FOSS Tecator TM scrubber. After digestion, the sample was distilled using Kjeltce TM 8200 autodistillation unit. The digestion fraction was made alkaline with 50 mL of sodium hydroxide solution (40% v/v), and the released ammonia was steam distilled into a receiver filled with 25 mL of 4% boric acid with Kjeldahl indicator. Lastly, the contents were titrated with HCl 0.01N. Nitrogen content was calculated according to the equation below (Eq.9):

$$\text{nitrogen (\%)} = \frac{(T\text{-}B)\cdot N\cdot 14.007\cdot 100}{W} \qquad \text{(Eq. 9)}$$

where: T: titrated volume of HCl for sample (mL); B : titrated volume of HCl for blank, (mL); N : normality of hydrochloric acid; 14.007 - molar mass of N (mg/mmol); W : sample weight (mg).

Proteins content was estimated by multiplying the total Kjeldahl nitrogen by a correction factor of 5.95 [167].

3.6.5. Lipids content determination

Lipids were estimated as the remaining fraction of TS after the determination of proteins, carbohydrates and ash content.

3.6.6. Ash content determination

The ash content (inorganic matter) was calculated as follows: %Ash = 100 - %VS/TS.

3.6.7. pH measurement

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

3.6.8. Sodium determination

Na^{+} was measured by ion chromatography (ICS 3000, Dionex) equipped with pre-columns and separation columns CG 16 and CS16 (3 mm ø) for cations. The column temperature was set at 35ºC.

3.6.9. Ammonium (NH_4^{+}) and ammonia (NH_3)

NH_4^{+} determination was carried out by using a colorimetric method corresponding to DIN 38406-5 and a commercial kit (Merck, ISO 000683). This kit has 2 main reagents: liquid (R1) and solid (R2). For the analysis, 5 mL of R1 and 200 µL of sample were conveniently diluted and mixed with tablespoon of R2. The sample is strenuously mixed and set aside for 15 min. Finally, concentration of nitrogen in form of ammonium (N-NH_4^{+}) is determined by using a Spectroquant Pharo 100M).

Ammonia concentrations (NH_3) rely on total ammonia nitrogen (TAN, Eq (10)), pH and temperature. Ammonia can be calculated by taking into account the equilibrium NH_4^{+}/NH_3.

$$TAN = NH_4^{+} + NH_3 \qquad \text{(Eq. 10)}$$

The resulting equation from which ammonia was calculated was Equation 11.

$$NH_3 = \frac{TAN}{1 + \frac{10^{-pH}}{10^{-(0.09018+2729.92/T)}}} \qquad \text{(Eq. 11)}$$

3.6.10. Biogas composition

Biogas is a mixture of gases mainly composed of CO_2 and CH_4 and other gases in a minor extent (H_2, NH_3 or H_2S). The main goal of these measurements was to verify the methanogenic inhibition. Methane content was determined by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (70 × 1/8" O.D.) and a MS13X4-09SF2 40/60 P packed column (9' × 1/8" O.D.) (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 200ºC, respectively. The injected sample volume was 100 μL.

3.6.11. Volatile fatty acids (VFAs) composition

Volatile fatty acids were extracted from the digestate of the reactor by filtering samples through 0.2 μm. VFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids) were analyzed by liquid chromatography using an Agilent 1260 HPLC-RID (Agilent) 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 composition was 5 mM H_2SO_4, and elution was conducted in isocratic mode at a flow rate of 0.35 mL/min. The injected sample volume was 20 μL, and the oven and detector temperatures were 25 and 35ºC, respectively.

Organic matter conversion into VFAs (COD-VFAs/CODin) was calculated based on the equivalence of each VFA (mg/L) in terms of COD (mg COD/L) as follows: acetic acid (1.07), propionic acid (1.51), isobutyric acid (1.82), butyric acid (1.82), isovaleric acid (2.04), valeric acid (2.04) [168].

$CH_3COOH + 2O_2 \rightarrow 2\,CO_2 + 2\,H_2O$ COD Acetic acid $= \frac{64 \frac{g}{mol}\, O_2}{60 \frac{g}{mol}\, CH_3COOH} = 1.07 \frac{g\ COD}{g\ VFA}$

$C_3H_6O_2 + \frac{7}{2} O_2 \rightarrow 3\,CO_2 + 3\,H_2O$ COD Propionic acid $= \frac{112 \frac{g}{mol}\, O_2}{74 \frac{g}{mol}\, C_3H_6O_2} = 1.51 \frac{g\ COD}{g\ VFA}$

$C_4H_8O_2 + 5\,O_2 \rightarrow 4\,CO_2 + 4\,H_2O$ COD (Iso)Butyric acid $= \frac{160 \frac{g}{mol}\, O_2}{88 \frac{g}{mol}\, C_4H_8O_2} = 1.82 \frac{g\ COD}{g\ VFA}$

$C_5H_{10}O_2 + \frac{13}{2} O_2 \rightarrow 5\,CO_2 + 5\,H_2O$ COD (Iso)Valeric acid $= \frac{208 \frac{g}{mol}\, O_2}{102 \frac{g}{mol}\, C_5H_{10}O_2} = 2.04 \frac{g\ COD}{g\ VFA}$

$C_6H_{12}O_2 + 8\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$ COD Caproic acid $= \frac{256 \frac{g}{mol}\, O_2}{116 \frac{g}{mol}\, CH_3COOH} = 2.2 \frac{g\ COD}{g\ VFA}$

3.7. Anaerobic microbiome analysis

The analysis of the microbial population allows identifying the microorganisms involved in the anaerobic fermentation of microalgae and thus, they should be promoted in the reactors in order to maximize the efficiency of VFAs production.

Microbial communities were studied in the sludge employed as inoculum in all experiments as well as in the reactors (CSTRs and UASB), which allow evaluating the microbial switch provoked by the imposed operational conditions. Regarding the reactors, the samples were taken during the stationary state, which are considered biologically representative since there were no variations of the bioprocess parameters. All samples were straightforward (immediately) frozen (-20ºC). DNA was extracted and subsequently sequenced by using next-generation sequencing technique (Illumina MiSeq) (Figure 11).

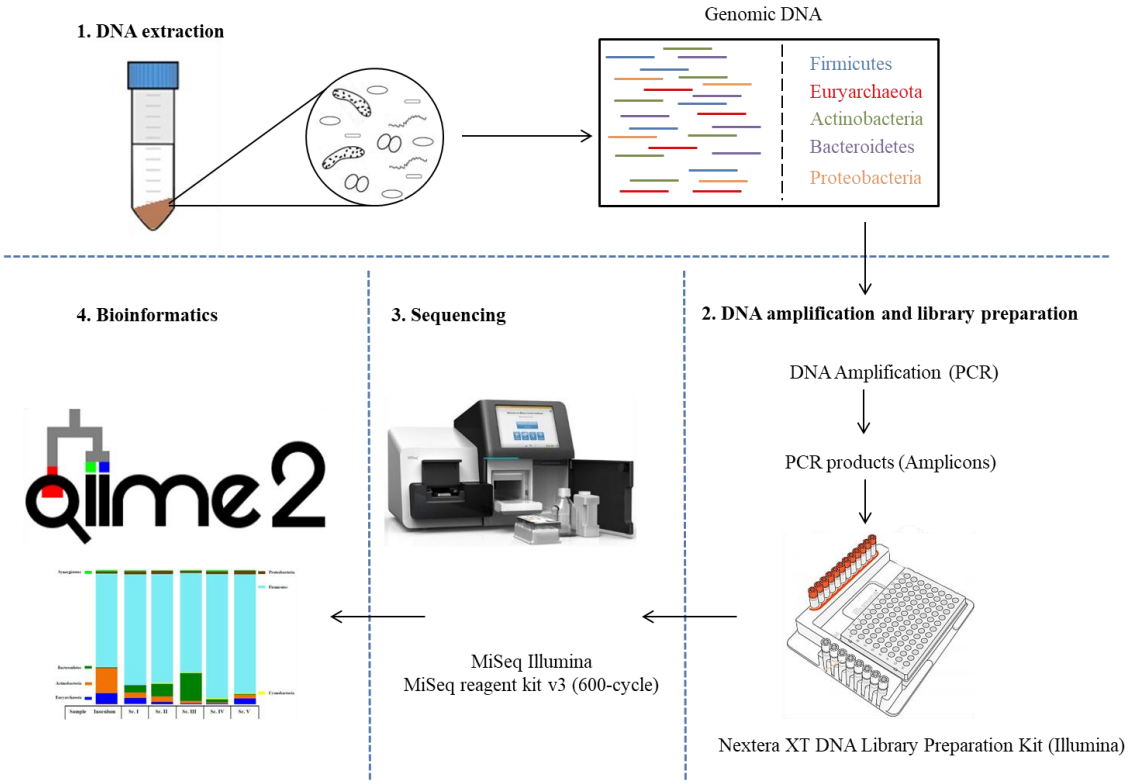

Figure 11. Work flow to determine the anaerobic populations developed in the reactors.

In general terms, this analysis is carried out by amplification of the hypervariable regions of gene ARNr 16S. This gen is widely known to be much conserved among prokaryotic species. Normally, out of the nine variable regions, most of the bacteria and archaea are detected in region V4 [169]. The extracted DNA must be firstly amplified through a polymerase chain reaction (PCR). PCR replicates the strands of DNA through the enzyme DNA polymerase. This reaction is first initiated by a primer. The primer consists of a series of complementary nucleotides to the 16S rRNA gene region to amplify. Two primers are commonly needed to define the region. The primers act as a starting point for the DNA polymerase to begin the nucleotide addition replicating the DNA. Once the amplicons (PCR products) are obtained, they are sequenced using a sequencer, in this case Illumina MiSeq. This sequencer uses a synthesis sequencing technology that employs terminator nucleotides labeled with fluorescence. The fluorescence emitted by these nucleotides when excited is detected by the equipment, thus determining the composition of the DNA strand.

Sequences obtained were further processed by using bioinformatics tools. During this process, sequence quality is tested. Sequences are aligned and put together forming operational taxonomic units (OTUs). OTU is defined as sequences presenting high similarity percentage (97%). Afterwards, species are determined by comparison with database. Concretely, the genomic analysis performed in the presented work is explained in detail below.

3.7.1. DNA extraction, amplification and sequencing

Samples were defrosted and heavy metals chelated by using 0.5 % w/v EDTA solution. Afterwards, DNA was extracted from 1 mL of sample by using the kit "FastDNA SPIN Kit for Soil" (MP Biomedicals, LCC), according to the protocol provided by the manufacturer. Quality of the DNA extracted was checked using a Nanodrop by measuring 260/280 and 260/230 ratios along with the amount of DNA extracted (ng/mL). The primers used for the amplification of the 16S rRNA gene were 341F and 805R (F – CCTACGGGNGGCWGCAG and R – GACTACHVGGGTATCTAATCC), which targeted the hypervariable regions V3 and V4 of both bacteria and archaea. Amplicons resulting from PCR were sequenced on a MiSeq Sequencer (Illumina) by Life Sequencing (University of Valencia, Spain) with MiSeq reagent kit v3 (600-cycle), according to the manufacturer's protocol.

3.7.2. Bioinformatic analysis

The resulting sequence data were processed by using bioinformatics tools. First of all, paired-end reads were merged using the program PEAR [170]. Afterwards, sequence quality was filtered using PRINSEQ and only sequences with a quality score of 30 and minimum lengths of 350 bp were taken into account for further analysis [171]. Primer sequences were removed using Mothur [172] while chimeric sequences were removed and the resulted sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity (OTU 0.97). The microbial identification step was performed by

USEARCH using the Greengenes database gg_13_8 [173], which is implemented in the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software package [172,174,175]

3.7.3. Biodiversity and statistical analysis

Diversity was evaluated by taking into account the species richness in a specific sample and also by evaluating the species replacement between samples. This latter approach was evaluated by comparing samples between reactors set at different operational conditions (Table 7). For this task, diversity indices (such as observed OTUs, Shannon and Simpson), the number of observed species, rarefaction curves, principal coordinate analysis (PCoA), principal component analysis (PCA) and statistical test ANOSIM were carried out.

Diversity indices

These parameters are employed to express the species richness in terms of number of species, but also in terms of evenness, which refers to the equal distribution of species in a sample. In this manner, a sample is more diverse when the number of microorganisms and distribution uniformity is higher.

Observed OTUs ($OTUs_{obs}$)

This parameter represents the number of microorganisms detected in a sample.

Simpson Index (D)

This parameter expresses the probability of randomly extracting two individuals belonging to different species from a microbial community. This parameter is calculated through the following equations (Eq. 12 and 13):

$$D = \sum_{i=1}^{S} P_i^2 \qquad \text{(Eq. 12)}$$

$$P_i = \frac{n_i}{N}$$

(Eq. 13)

Where n_i is the number of sequences of the specie *i*, *N* is the number of total sequences and *S* is the number of species. The value of this index goes from zero, representing maximum diversity, to 1 in samples that are dominated by only one species.

Shannon index (H')

This index expresses both the richness in species as well as their abundance in a specific sample by using the following expression (Eq. 14):

$$H' = -\sum_{i=1}^{S} P_i \cdot \ln P_i \qquad \text{(Eq. 14)}$$

Where *S* is the number of species and P_i is defined by Equation 6. The value of this index goes from zero when only one species exists in the sample, to *lnS*. In this manner, the higher this parameter is, the higher the diversity present in the sample.

Methods for data comparison and interpretation

Rarefaction curves

These curves are employed to compare number of species when the size of samples are different, estimating the richness of species based on the sample with the lowest number of sequences. This method allows the graphical representation of number of OTUs (axis x) against the total number of sequences (axis y), showing a curve indicating the sample's diversity. The higher the slope in this curve is, the higher the diversity. Likewise, if the curve reaches a plateau, it indicates that most of the microorganisms present in the sample have been identified. This analysis was carried out by using QIIME [175].

PCA

This method allows to summarize and to visualize the information described by multiple inter-correlated quantitative variables. PCA is used to extract the important information from a multivariate data table and to express this information as a set of few new variables called principal components. The points in the graphs of the present study represent the samples of the reactors, and distances correspond with dissimilitude among samples composition based on the physicochemical characterization. Axis X (PC1)

explains the highest differences between samples whilst axis Y (PC2) has the same function but in this case differences explained are lower. In the present thesis this analysis was carried out by using PAST software [176].

PCoA

It is a method to explore and to visualize similarities or dissimilarities of multivariable samples. In this manner each sample is assigned a location in a graph whose geometrical dimensions are reduced to 2 or 3. In the present thesis this analysis has been employed to identify similarities and differences between samples based on microbial communities' distribution. These analyses were carried out by using QIIME [175].

ANOSIM (Analysis of similarities)

This analysis allows evaluating the statistical significance of differences between certain groups of samples. This analysis was performed with a p-value of 0.05 in order to test differences in microbial community composition between scenarios [176]. This statistical test results in the R-values matrix where values close to 1 indicate strong dissimilarity between samples.

To sum up, results of VFAs productions were subsequently analyzed in different investigations (Table 7). Experiments were firstly carried out in batch mode and best conditions were further implemented in semicontinuous/continuous mode. Figure 12 collects the operational conditions employed for the investigations conducted during the development of this thesis.

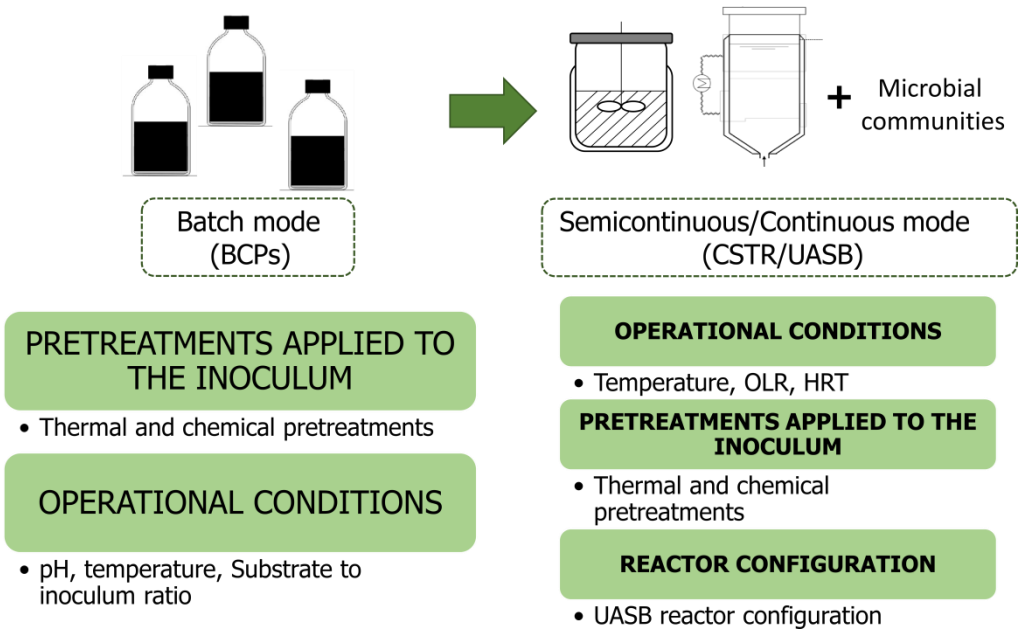

Figure 12. Investigations were first carried out in batch mode (Section 4.1, left) and results were confirmed in semicontinuous/continuous mode (Section 4.2, right)

Table 7. Summary of the operational conditions imposed in the different experimental designs of the present PhD thesis

Section	Mode	Reactor configuration	pH	T (ºC)	HRT (days)	OLR (g COD/Ld)	$\frac{COD_{in}}{VS_{in}}$	Inoculum	Substrate (*Chlorella* sp.)
4.1.1.	Batch	BCP	7.5	35	-	-	0.5 0.5 3	Anaerobic sludge	Non-pretreated Protease pretreated Protease pretreated
4.1.2.	Batch	BCP	7.5	35	-	-	3	Aerobic sludge Anaerobic sludge	Protease pretreated
4.1.2.	Batch	BCP	7.5	35	-	-	3	Anaerobic sludge	80ºC - 10 min 80ºC - 30 min 100ºC - 20 min 120ºC - 10 min 120ºC - 30 min BES 10 mM and BES 30 mM BES 10 mM + 80ºC - 10 min BES 10 mM + 80ºC - 10 min BES 30 mM + 120ºC - 10 min BES 30 mM + 120ºC - 10 min
4.1.3.	Batch	BCP	5.5	25 35 50	-	-	3	Anaerobic sludge	Protease pretreated
			7.5	25 35 50					
			9	25 35 50					

4.2.1	Semi-continuous	CSTR	7.5	25 35	10 10	1.5 1.5	-	Anaerobic sludge	Non-pretreated
4.2.1.	Semi-continuous	CSTR R1 R2	7.5 7.5	35 35	10 10	1.5 3	-	Anaerobic sludge	Protease pretreated
4.2.2.	Semi-continuous	CSTR R1 R3	7.5 7.5	35 25	10 10	1.5 1.5	-	Anaerobic sludge	Protease pretreated
4.2.3.	Semi-continuous	CSTR R4 R5	7.5 7.5	25 25	8 12	1.5 1.5	-	R3	Protease pretreated
4.2.4.	Semi-continuous	CSTR	7.5	25	8	1.5	-	Control: Anaerobic sludge BES 10 mM 120ºC - 10 min 120ºC - 30 min	Protease pretreated
4.2.6. 4.2.7.	Semi-continuous	CSTR	7.5	25	8	3 starvation (2 weeks) 3 6 9 12 15	-	R4	Protease pretreated
4.3	Continuous	UASB	7.5	25	6.4 7.2 6.3	2.3 3.6 8.7	-	After starvation period	Protease pretreated

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Results obtained during the development of this PhD thesis were discussed in two different sections:

- VFAs production optimization in BCPs (batch mode fermentations): These experiments were carried out in batch mode in order to identify appropriate conditions (COD/VS ratio, pH and temperature) for volatile fatty acids production. Additionally, the effect of pretreating the anaerobic sludge was also tested for inhibiting methanogens. These parameters were the basis to decide the conditions to be implemented in semicontinuous fermentations.
- VFAs production in CSTR and UASB reactors (semicontinuous fermentations): Operational conditions (temperature, HRT, OLR, inoculum pretreatment) were assessed to understand their impact on VFAs production yields and profiles. Furthermore, microbial populations were evaluated to identify key species involved in VFAs production.

4.1. VFAS PRODUCTION OPTIMIZATION IN BCPs

4.1.1. Effect of COD/VS ratio in VFAs production

AD was carried out under standard conditions (0.5 g COD/g VS, T=35ºC, pH=7.5 and 150 rpm) to evaluate the biodegradability potential of microalgae. Methane yield was 94±2 mL CH_4 STP (Standard Temperature and Pressure)/ g COD_{in} for the raw biomass while it was enhanced to 175±1 mL CH_4 STP/g COD_{in} (50% biodegradability) in experiments with protease pretreated biomass (Figure 13). This increase was in agreement with previous results using proteases as pretreatment method prior to AD. For instance, methane production in batch assays using pretreated *C. vulgaris* and *Chlamydomonas reinhardtii* biomass enhanced methane production by 51% and 17%, respectively, when compared to raw biomass [32].

Since experiments at 0.5 g COD/g VS were conducted under optimal conditions for biogas production, VFAs were not accumulated. Higher organic overload is reported to cause a destabilization in the AD by exceeding the methanogenic capacity of the archaea community, resulting in a reduction of methane production [177]. To cause organic overloading and thereby inhibiting methanogenic activity, a substrate to inoculum ratio of 3 g COD/g VS was tested. In fact, maximum organic matter conversion into VFAs rose up to 48.3% COD-VFAs/COD_{in} when using COD/VS=3 against negligible concentrations detected at COD/VS=0.5 ratios. The presence of high amounts of VFAs involves a pH drop, which normally results in methanogenic inhibition [57]. In this sense, one promising strategy to accumulate VFAs in BCPs is to increase the substrate to inoculum ratio.

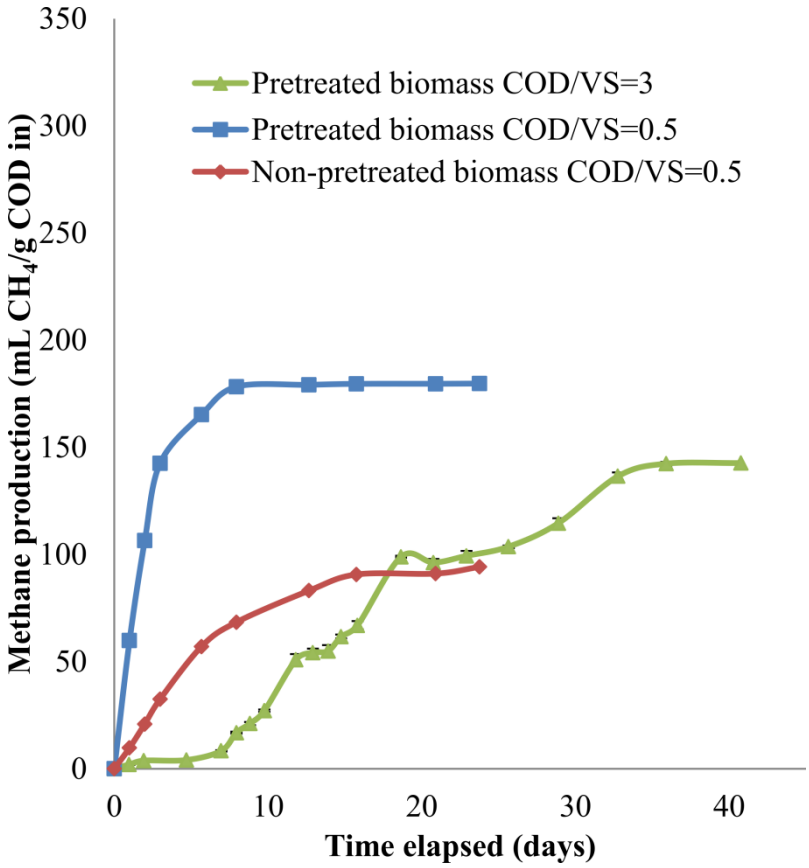

Figure 13. Methane production at different substrate to inoculum ratio (standard deviation < 5%).

As it can be seen in Figure 13, the methane yield was slightly lower (142±1 mL CH_4 STP/g COD_{in}) than the obtained at 0.5 g COD/g VS. These results showed that organic

overloading affected methanogenesis negatively. González and co-workers concluded that high COD/VS ratio (3 g COD/g VS) were negative for methane production due to the accumulation of VFAs [179]. Remarkably, this reduction was also observed in the production rate. While the maximum production was achieved in 8 days at low substrate to inoculum ratio (0.5 g COD/g VS), almost 30 days were needed to obtain maximum production at 3 g COD/g VS. Since VFAs accumulation is the target of the present PhD thesis, organic loadings of 3 g COD / g VS were selected to test the influence of pH and temperature on VFAs production in BCP mode.

4.1.2. Effect of inoculum in VFAs production

Aerobic sludge vs anaerobic sludge

As methanogens are obligate anaerobes, selection of aerobic sludge was expected to avoid the presence of methanogenic species favouring acidogenic population (facultative microorganisms). Aerobic sludge has been previously tested for VFAs production. Values obtained by an aerobic sludge digesting hardwood spent liquor achieved competitive organic matter conversions into VFAs in continuous operation (36% COD-VFAs/COD_{in},) [180]. The comparison conducted herein mediated higher methane potential and VFAs productions (198.2±1.7 mL CH_4/g COD_{in} and 48.6% COD-VFAs/COD_{in}) for the anaerobic sludge than the aerobic sludge (155.2±2.8 mL CH_4/g COD_{in} and 35.5% COD-VFAs/COD_{in}) in batch mode. Thus, in order to enhance VFAs production yields by means of operational parameters, the anaerobic inoculum was selected.

Anaerobic sludge subjected to thermal pretreatment

Besides methane producers, the anaerobic microbiome is also rich in acid-producing species. To avoid methanogenic activity, inoculum pretreatments might be of importance when operational parameters are selected in a range where methanogens naturally grow. These pretreatments have been efficiently employed in anaerobic sludge devoted to methane production inhibition [66,68,181]. To analyze the effectiveness of the pretreatment, methane production was monitored and data were fitted to Gompertz model (Figure 14). In the present investigation, according to Gompertz modelling results, methane productions were very different depending on the pretreatment applied to the inoculum (Table 8). Low temperature pretreatments (80ºC-10 min) promoted methane productions and shortened the lag phase in the assays (2.1 d^{-1} with respect to 4.1 d^{-1} in the non-pretreated anaerobic sludge). Results showed a 39% enhancement compared to the non-pretreated inoculum, contributing to methane generation instead of VFAs accumulation. Investigations in literature showed similar yield increase (30%) with other inoculum pretreated at low temperature (70ºC) and different heat exposure times (9, 24, 48 and 72 h) [182]. A possible explanation might be a better hydrolysis and acidogenesis activity linked to an increase in the sCOD from 2.7% in the non-pretreated inoculum to 8.1% when pretreated at 80ºC for 10 and 30 min. The increase in temperature pretreatment (100ºC for 20 min) showed similar methane production yields in comparison with the non-pretreated inoculum (207.7 ± 1.8 mL CH_4/g COD_{in} and 198.2 ± 1.7 mL CH_4/g COD_{in}, respectively) and a sCOD increase of 12%. In this case, the lag phase attained (4.6±0.2) was similar to the one obtained in the non-pretreated anaerobic inoculum (4.1±0.5). Despite of the solubility enhancement, most likely pretreatment conditions established were too harsh to promote acidogenesis in the anaerobic microbiome. At the highest temperature sCOD values increased 3.9 and 4.5-fold (120ºC for 10 and 30 min, respectively) but these assays did not show any methane production. This might be because methanogenic archaea are more sensitive to temperature than bacteria. This latter group of microorganims have the ability to form spores under stress conditions (such as heat or chemicals addition) and resume their activity when proper conditions are given [72].

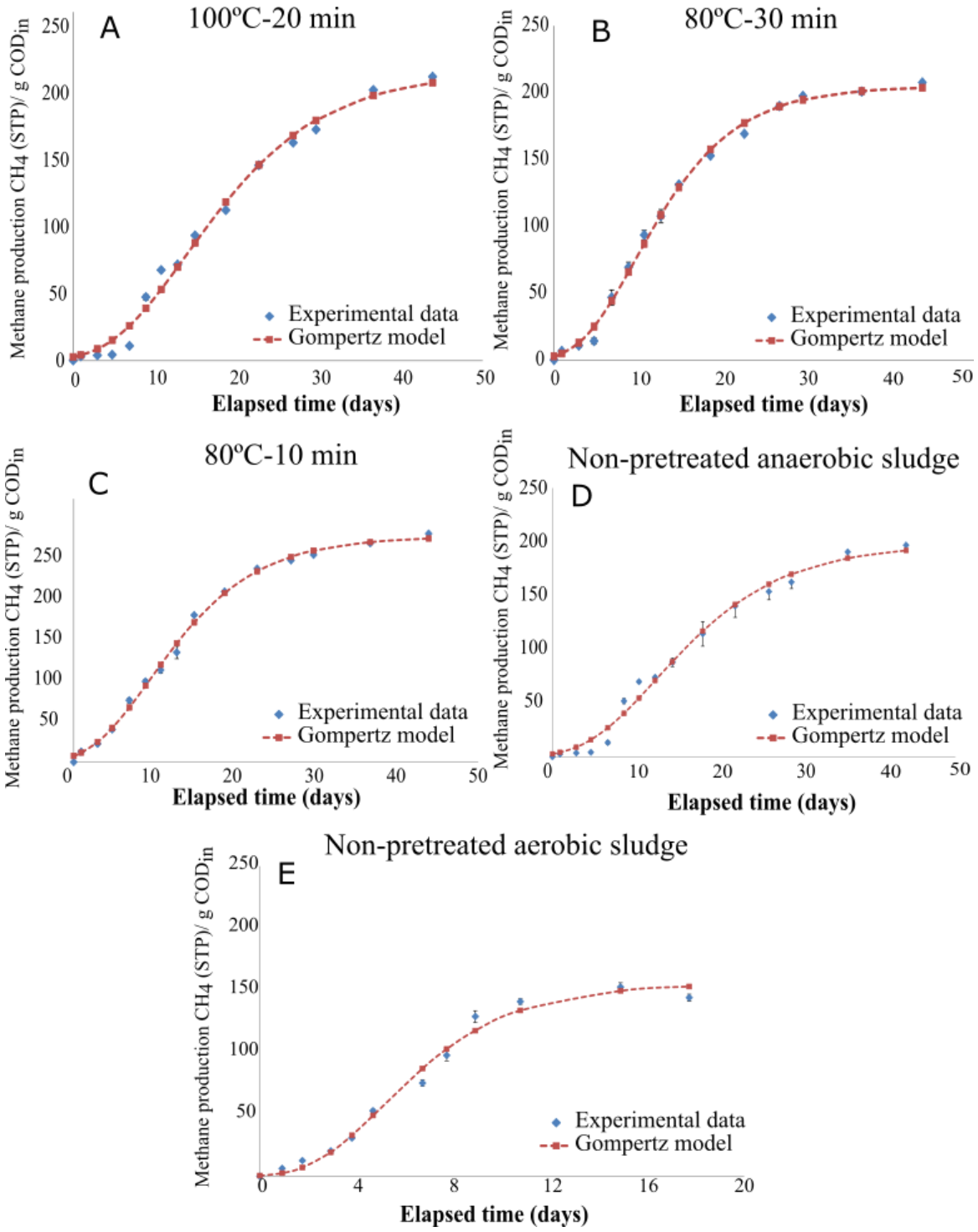

Figure 14. Methane production yields using thermal pretreated anaerobic sludge (A, B and C), non-pretreated anaerobic sludge (D) and non-pretreated aerobic sludge (E).

Table 8. BMP results according to Gompertz model.

	100°C 20 min	80°C 30 min	80°C 10 min	Non-pretrated anaerobic sludge	Non-pretreated aerobic sludge
mL CH_4/g COD_{in}	207.7 ± 1.8	204.8 ± 19.2	275.9 ± 0.2	198.2 ± 1.7	155.2±2.8
mL CH_4/g COD_{in}·d	3.1 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	3.2 ± 0.1	4.3±0.1
Lag phase (d^{-1})	4.6 ± 0.2	3.1 ± 0.1	2.1 ± 0.1	4.1 ± 0.5	2.1±0.1

The non-pretreated anaerobic sludge achieved higher methane production 198.2±1.7 mL CH_4/g COD_{in} than the BCP set at the same conditions in Section 4.1.1. (142±3.0 mL CH_4/g COD_{in}). The varying biodegradability might be linked to the anaerobic sludge employed in each investigation. As described in Section 3.1., anaerobic sludge was regularly provided by the WWTP of Valladolid, and hence, the sludge might present different species depending on the collection season (See Microbiology Section 4.2.1. and 4.2.4.). This difference in microbial population, and hence in metabolic activities, might be the responsible for the difference in the obtained results. Nevertheless, since controls were included in all BCPs, the inherent activity of the sludge used in each of them has been taken into consideration for comparison purposes.

With respect to VFAs production, the highest concentrations were detected in batches were methane was totally inhibited, namely when the sludge was pretreated at 120ºC for 10 and 30 min. In those BCPs, organic matter conversion into VFAs yielded 71.6% and 63.8% COD-VFAs/COD_{in}, respectively (Figure 15). The lower conversion (63.8%) obtained when the sludge was subjected at 120ºC for 30 min was attributed to the longer exposure time that might had damaged acidogenic microorganisms. In this sense, thermal pretreatments applied to sludge for hydrogen production also showed that an increase in the exposure time of the pretreatment was detrimental for hydrogen production [183]. These conversions were comparatively higher than the control (48.6% COD-VFAs/COD_{in}) and the other thermal pretreatments (Figure 15).

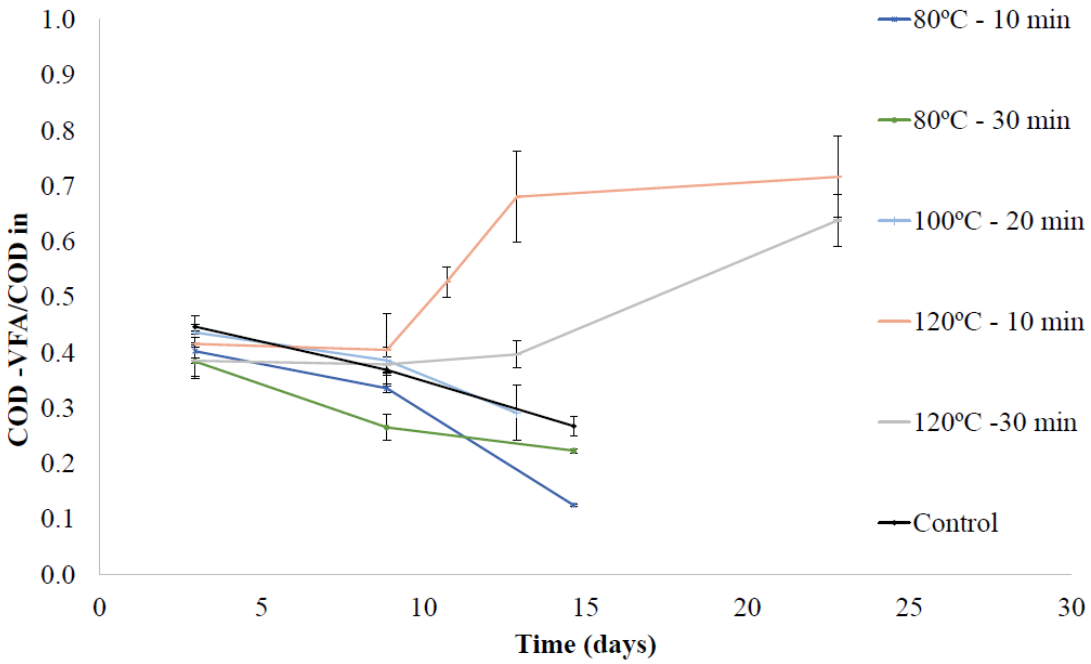

Figure 15. Organic matter conversion yields when anaerobic sludge was subjected at thermal pretreatments.

VFAs profile also depended on the pretreatment employed. Acetic acid was accumulated in the experiments at 120ºC (Figure 16, D-E) whereas it was quickly consumed in the rest of assays along the fermentation time (Figure 16, A-B-C). This feature is in agreement with the methane production observed in assays conducted with the anaerobic sludge pretreated at lower temperatures. In fact, acetic acid is the main substrate for methane production via the acetoclastic pathway and thus, it was accumulated in the assays where the methanogenic activity was inhibited (assays conducted at 120ºC).

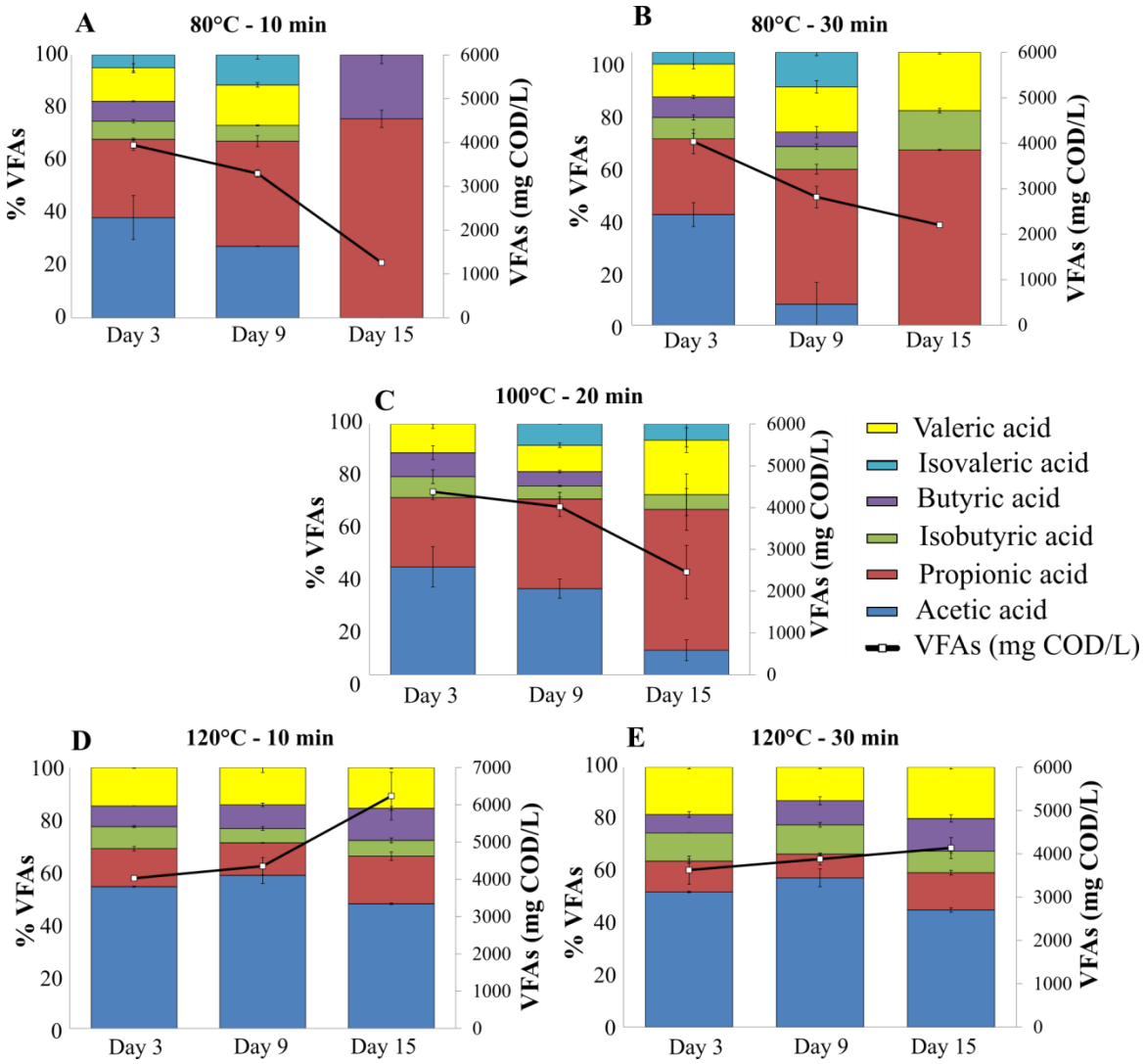

Figure 16. VFAs productions and profiles for thermally pretreated sludge.

Propionic acid accumulated at 80ºC (82% and 70% for 10 and 30 min, respectively out of the total VFAs production expressed as g COD/L), 100ºC (63%) and the untreated sludge (65%). It should be highlighted that propionic degradation is the most thermodynamically unfavorable (ΔG=+76.1 KJ/mol) [89]. The low propionic acid accumulation at 120ºC was in agreement with other studies, which stated that pretreatments can suppress the activity of propionic acid producers (Figure 16, A-B-C vs D-E) [56]. Along the digestion time of the assays conducted with sludge pretreated at low temperature, it seems likely that longer VFAs were converted to shorter VFAs. This can be seen in the propionic acid increase with regard to the rest of the VFAs. Opposite to that, highest temperatures employed in the sludge pretreatment showed a more drastic effect on the microbial systems since

much less changes were registered along the fermentation time (Figure 16, D-E). VFAs spectrum also remained unaltered along time in another study employing a pretreated sludge at high temperature (112ºC) and exposure time (5 h) for hydrogen production from sucrose in which a similar VFAs distribution was highlighted regardless of the initial sucrose concentrations (6, 12, 18 and 24 g/L) [69]. In this latter case, among the detected VFAs, butyrate was the most abundant (75.4–91.9%), followed by acetate (19.6-6.3%).

The experimental design helped to elucidate the conditions that maximized VFAs production and minimized methane production. High temperature pretreatments to the anaerobic inoculum were selected as a potential strategy to tailor the microbial system used as inoculum for VFAs production. In this manner, this sludge pretreatment was further tested in semi-continuous fermentation mode.

Anaerobic sludge subjected to chemical pretreatment

The chemical BES suppresses methane production by blocking Methyl-Coenzyme M formation pathway [184]. BES is a structural analogue of coenzyme M (2-mercaptoethanesulfonic acid), the methyl carrier in the final reductive step of methanogenesis. Coenzyme M accepts methyl groups generated from methanol or CO_2 to form methylcoenzyme. This cofactor is found in all methanogens but not in bacteria [185,186]. Aiming at methanogenic inhibition, BES has been previously used for VFAs production [71,187]. Despite of inhibiting methanogenic activity, the use of chemicals also entails disadvantages such as the high prices and the toxicity for the environment [56]. For this reason, their use to accumulate VFAs must be supported by high process conversions efficiencies.

VFAs accumulated at both tested concentrations (10 mM and 30 mM of BES) and remained stable after 15 days of digestion (Figure 18, A-B). BES addition impeded methanogens to carry out their metabolic functions. Despite of that, organic matter conversion into VFAs after chemically pretreating the anaerobic sludge (47±2% COD-VFA/COD_{in}, Figure 17) was considerably lower than the obtained after thermal pretreatment. In this sense, the use of this chemical might have damaged not only

methanogens, but also VFAs producers. Despite of the specificity towards methanogens, there are studies addressing altered the bacterial community structure when low BES concentrations (3 mM) are added [111,188]. Additionally, organic matter conversion into VFAs did not increase with BES concentration and hence, the use of a lower dose to suppress methanogenic activity was recommended in case of using this inhibitor. The use of this chemical was previously tested (BES 50 mM) for VFAs production from *C. vulgaris* and *Scenedesmus quadricauda,* reaching 40% g VFAs/g sCOD at neutral pH values [73]. These authors detected hydrogen production during the first days of experiments but also highlighted the absence of methane as well. When compared to literature, results obtained during this PhD thesis were comparatively higher since in the above reference the ratio was calculated based on the soluble COD while the efficiency of BES in BCPs conducted in this thesis were based in total COD used as substrate.

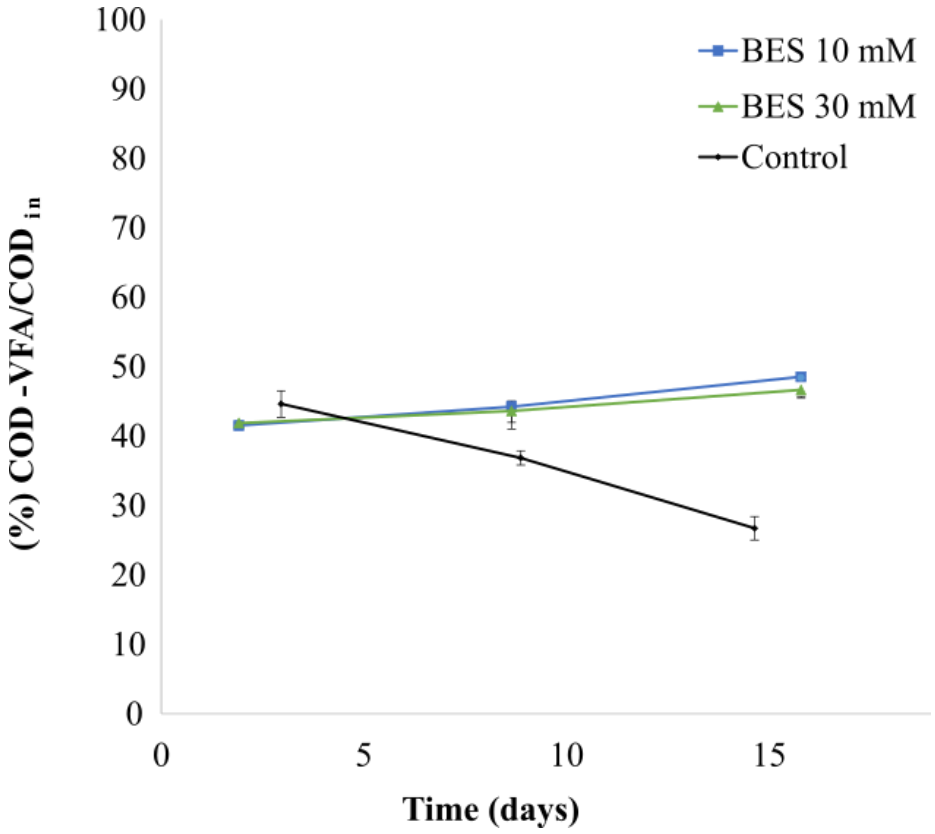

Figure 17. Organic matter conversion yields of chemical pretreatments applied to anaerobic sludge.

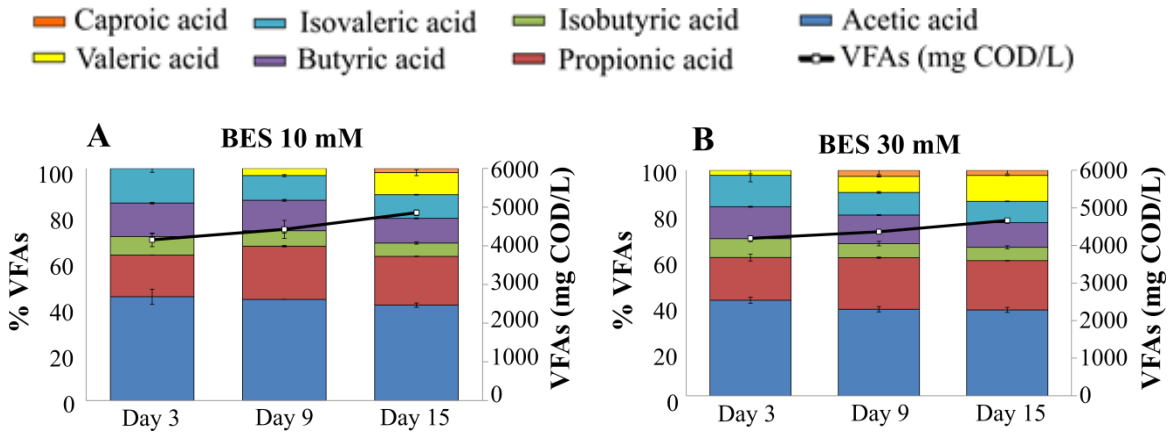

Figure 18. VFAs productions and profiles under chemical pretreatment at different BES concentrations.

Regardless of BES concentration, VFAs profiles and production yields were similar. Regarding VFAs profiles, acetic acid was the most abundant acid (40±3% out of the total VFAs production (g COD/L)) in all BCPs assays. Propionic and butyric acids represented around 20±2% and 13±2% out of the total VFAs production (g COD/L) (Figure 18 A-B). This trend was in agreement with previous studies stating that the higher hydrogen production, when BES was employed, was a result of the stimulation of microorganisms involved in propionic and butyric acids productions [189].

Anaerobic sludge subjected to a combined chemical and thermal pretreatment

A combination of both thermal and chemical pretreatment was assessed to test possible synergistic effects. Pretreatments employed inhibited methane formation in all cases. When thermal and chemical pretreatments were combined, conversion yields were in the range of 40±2% COD-VFA/COD_{in} in all cases (Figure 19). Hence, the pretreatment combination did not provide any additional positive effects on VFAs accumulation with respect to the use of both pretreatments separately. Since BES and the combination of pretreatments obtained similar values but lower than when only applying thermal treatment, it could be concluded that the use of BES might have damaged VFAs producers as well as methanogenic microorganisms. It is therefore important to find a balance on the pretreatments conditions employed to affect only methanogenic species.

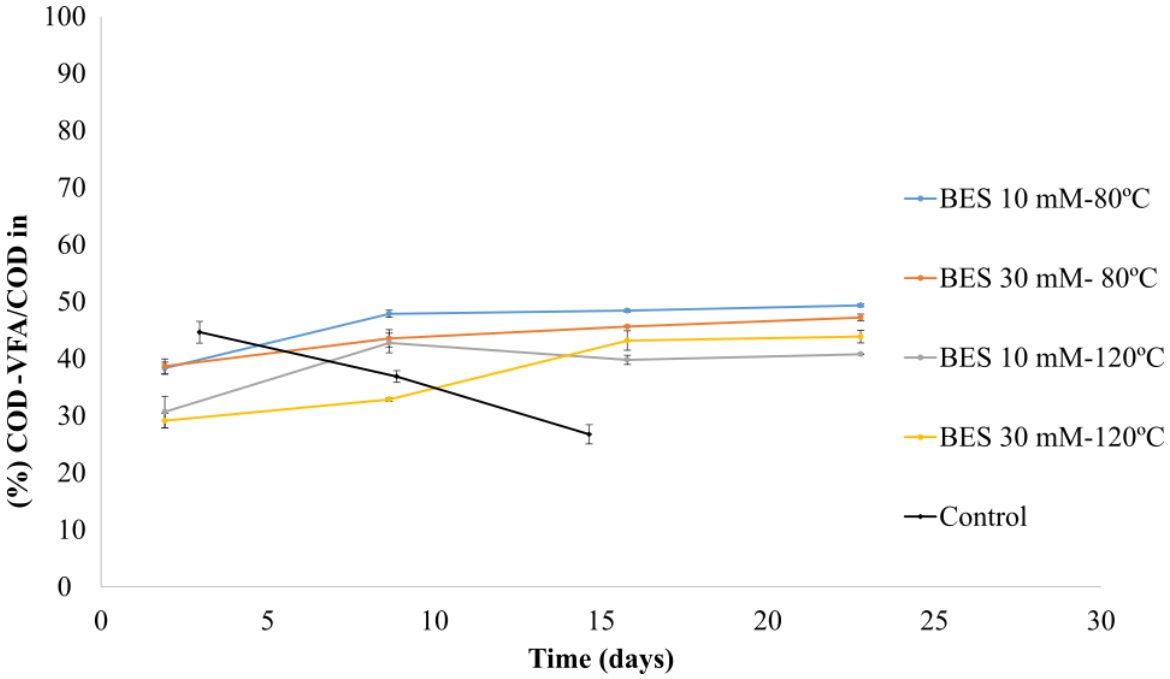

Figure 19. Organic matter conversion yields of the combination of chemical and thermal pretreatments applied to the anaerobic sludge.

With regard to VFAs profile distribution, propionic percentage was higher when using 80ºC, while the combination of BES with 120ºC favored butyric acid (Figure 20). It was also remarkable the presence of caproic and valeric (linear and iso-form) in the sludge subjected to BES and 80ºC (Figure 20 A-B). This trend was also observed when only BES was used. Opposite to that, the combination of BES with 120ºC provided a slightly different VFAs profile when compared with only BES addition. However, no important changes were registered at different BES concentrations and same temperature. This fact indicated that BES concentrations were not relevant in terms of VFAs profile distribution within the range tested herein. The effect of combining BES at 1 mM with thermal pretreatment (100ºC for 1 h) was analyzed to pretreat the anaerobic sludge for hydrogen production using dairy wastewater as substrate [190]. In their study, the chemical pretreatment outstood as the best pretreatment when compared to thermal and the combination of pretreatments. Most probably, exposure time to thermal pretreatment was too high causing the decay of methanogens and other bacteria. In this sense, little is still known about the combination of pretreatments applied to the sludge to enhance VFAs accumulation which highlighted the interest of the present study.

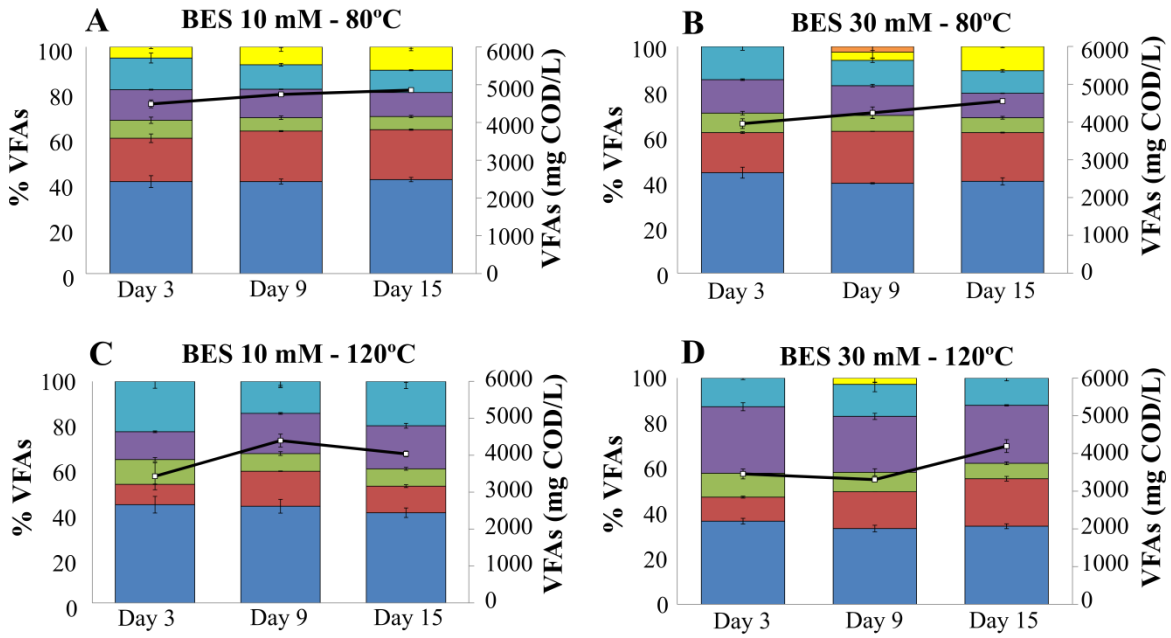

Figure 20. VFAs productions and profiles for the combination of chemical and thermal pretreatments.

In general terms, thermally-pretreated anaerobic sludge displayed higher organic matter conversion values into VFAs than the rest of the pretreatments assessed, and thus, this pretreatment was selected to be tested in semi-continuous mode.

4.1.3. Optimization of pH and temperature in BCPs to maximize VFAs production yields

Effect of initial pH and digestion temperature in methane yield

The acidogenic and hydrolysis steps of acid fermentation are significantly influenced by pH. This parameter has an effect on growth conditions and enzymatic activities. As a matter of fact, pH changes might be used to favour the acidogenic stage [76] and counteract methanogens activity [191]. Methane yields obtained over digestion time (30-35 days) at different initial pH and temperatures are shown in Table 9 and Figure 21. All batches showed VFAs consumption after a lag phase, and thus methane production

started after 5-12 days in all assays. This fact is in accordance with the previous lag phases registered in the other BCPs in which high COD/VS ratio was employed. The lag phase was especially marked in batches set at psychrophilic conditions and pH 5.5 and 9 (Figure 21 A-C). Lower methanogenic activity was registered at these pH values when compared to pH 7.5 (Figure 21 B) and no methane was produced at all at 50ºC and pH 5.5 (Figure 21 A).

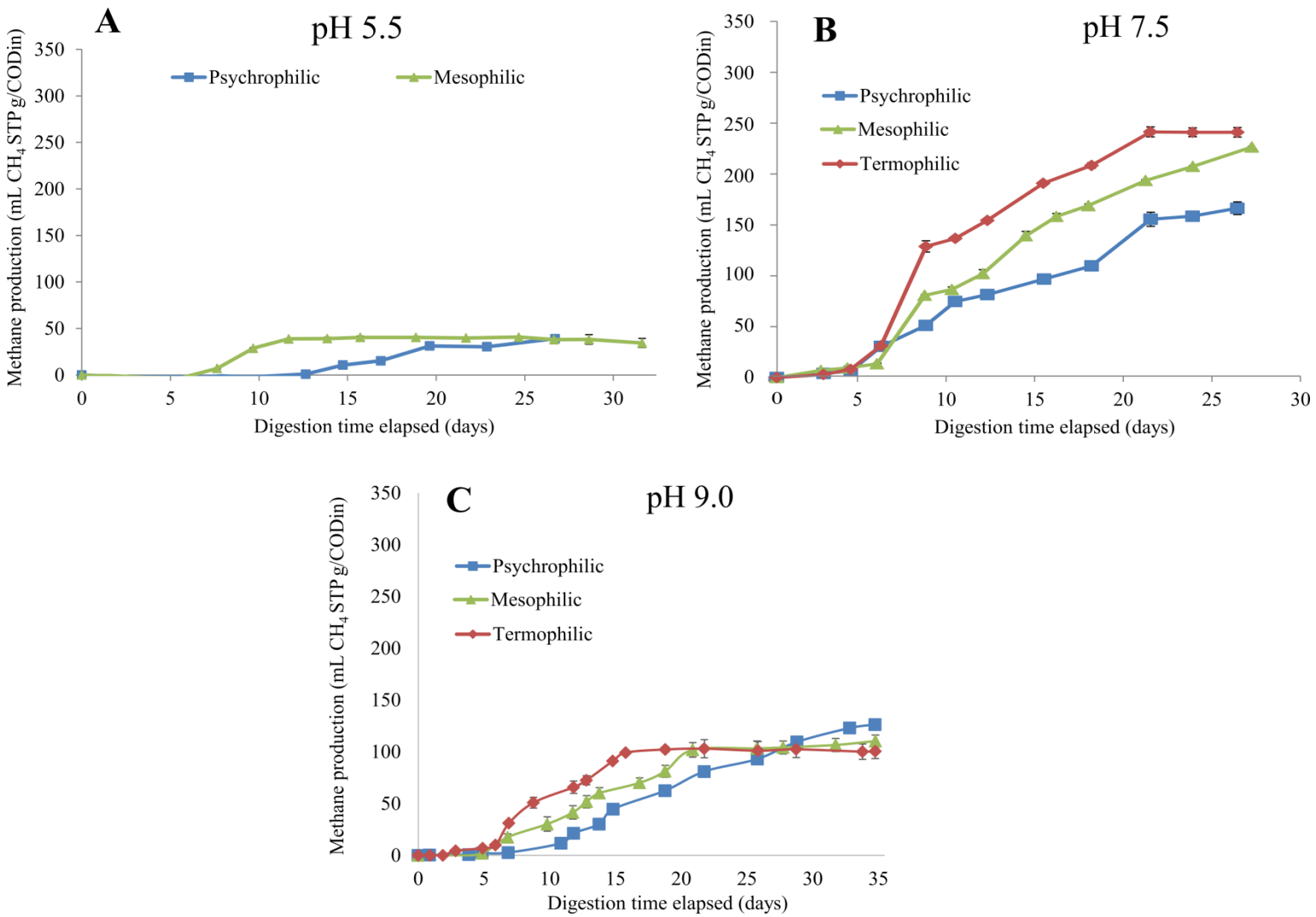

Figure 21. Methane production at initial pH=5.5 (A), 7.5 (B) and 9 (C) at different temperature ranges (25ºC - 35ºC – 50ºC).

Table 9. Methane and VFAs production yields obtained at different pH and temperature values.

Parameters	pH = 5.5			pH = 7.5			pH = 9		
Temperature	25ºC	35ºC	50ºC	25ºC	35ºC	50ºC	25ºC	35ºC	50ºC
mL CH_4 STP g COD_{in}^{-1}	39±0.4	40.4±1.3	0	166.4±6.3	235.3±1.0	235.6±4.1	126.3±2.2	110.3±5.9	100.6±7.0
%CH_4-COD_{max}/COD_{in}*	11.1±2.7	11.5±0.6	0	47.7±2.5	67.2±1.3	67.3±1.4	36.1±0.6	31.5±1.7	28.7±2.0
%VFA-COD_{max}/COD_{in}	47.7±0.1	39.1±0.2	34.5±0.5	45.1±1.4	48.3±0.9	37.1±1.5	33.4±1.2	28.1±2.1	31.6±1.5

* 1 g COD= 350 mL CH_4

In general, pH 7.5 favored the methanogenic step with respect to 5.5 and 9. The initial pH of 5.5 and 9 could have caused an early acidification/basification of the media resulting in methanogenic inhibition [192]. At psychrophilic conditions, methane yield increased from 38.9±2.7 and 126.3±2.2 mL CH_4/g COD_{in} at pH 5.5 and 9, respectively to 166.4±6.3 mL CH_4/g COD_{in} at pH 7.5. A similar trend was observed at mesophilic conditions where methane yield increased from 38.2±5.1 and 110.3±5.9 at pH 5.5 and 9, respectively to 235.4±1.0 mL CH_4/g COD_{in} at pH 7.5. It is worth to mention that pH 7.5 and 35ºC resulted in 142 mL CH_4/g COD_{in} in Section 4.1.1. and 198 mL CH_4/g COD_{in} in Section 4.1.2. As pointed out previously in 4.1.2., the use of anaerobic sludge with different compositions might be the responsible of different microbial activities resulting in different methane potentials [193] (See Microbiology Section 4.2.1. and 4.2.4.). At thermophilic conditions, methane yield was the highest again at pH 7.5 (235.6±4.1 mL CH_4/g COD_{in}). In principle, thermophilic conditions enhance enzymatic hydrolysis efficiency and the growth rate of methanogens, thereby methane productivity can be improved but the methane yield remains the same (in this case, 235 mL CH_4/g COD_{in}) [194]. However, as observed herein, other authors concluded that the mesophilic temperature range supported higher anaerobic biodegradability than thermophilic when using lipid-extracted *Nannochloropsis gaditana* as substrate for methane production [195]. In fact, the use of mesophilic conditions over thermophilic with protein rich substrates (such is the case of microalgae biomass) has been associated with more chances of suffering microbial inhibition associated to higher NH_4^+ toxicity in the thermophilic temperature range [106].

Effect of initial pH and temperature on VFAs production yield

When pH 5.5 was evaluated, a COD-VFAs/COD_{in} of 47.7±0.1% was obtained at 25ºC (Table 9). Organic matter conversions decreased concomitantly at 35ºC and 50ºC (39.1±0.2 and 34.5±0.5 COD-VFAs/COD_{in}, respectively).

With regard to pH 7.5, psychrophilic (25ºC) and mesophilic (35ºC) digestions ranged similar COD conversion efficiency (COD-VFAs/COD_{in}= 45-48% at pH 7.5, Table 9). These values were higher when compared to the yields obtained at 50ºC (37.1±1.5 COD-

VFAs/COD_{in}). Cho and co-workers digested microalgae biomass at neutral pH (6.9) and different temperatures (35ºC, 45ºC, 55ºC) and their results showed a concomitant increase of VFAs yields (20.0, 33.0 and 50.0 COD-VFAs/COD_{in}, respectively) [54]. Nevertheless, these latter authors did not employ any biomass pretreatment and hence, the increase in temperature was correlated to an increase in organic matter availability explaining the better yields at the highest temperature. Another study showed conversions of 10% COD-VFAs/COD_{in} when investigating the digestion of a non-treated microalgae mixture at 35ºC and pH 7 [54]. The use of non-pretreated microalgae as substrate most probably resulted in low hydrolytic rates, explaining the low organic matter conversions into VFAs.

When pH 9 was assessed, COD-VFAs/COD_{in} was similar regardless of digestion temperature (28-33% COD-VFAs/COD_{in}). Nevertheless, conversions into VFAs were low compared to 5.5 and 7.5 pH values which indicated that alkaline initial pH values were not suitable to produce VFAs from microalgae biomass. COD-VFAs/COD_{in} of 31.5% was reached when *Microcystis* was used as substrate at pH 10 [51]. Another study carried out by Yuan and co-workers to produce VFAs from waste activated sludge at pH 11 achieved an organic matter conversion of 20.2% COD-VFAs/COD_{in} [196]. In this sense, similar values were attained when using microalgae biomass under alkaline conditions but still far below the values attained when conducting BCPs at neutral pH values.

During AF, pH changes affect hydrolytic and acidogenic bacteria. In this sense, non-optimum pHs might affect negatively the overall process conversion of organic matter into VFAs. Hydrolysis has been found optimal in the pH range of 5-7, and thus these conditions seem appropriate to obtain the highest hydrolysis and acidification yields simultaneously [197]. In this particular case, the nature of the anaerobic sludge used as inoculum, which was adapted to work at pH close to neutrality, might also have contributed to organic matter conversions into VFAs achieved at initial pH of 7.5.

When evaluating the assessed temperatures ranges, psychrophilic conditions (25ºC) supported similar organic matter conversions into VFAs at 5.5 and 7.5 pH values (45-47% COD-VFAs/COD_{in}). When comparing AF at mesophilic conditions, only the assays conducted at pH 7.5 mediated a similar organic matter conversion into VFAs (48.3% COD-VFAs/COD_{in}). Regardless of the tested pH, the lowest conversions were attained in thermophilic digestions (Table 9). As determined herein, other studies employing alternative substrates (such as olive mill wastewater) have shown lower COD-VFAs/COD_{in} conversion at thermophilic temperatures [198].

Hence, pH 5.5 and 7.5 resulted in the highest organic matter conversion into VFAs. As the effluent of AF is better to be in neutral conditions to avoid any reagent addition, pH 7.5 was selected to carry out the rest of the experiments in this PhD thesis. With respect to temperature, 25ºC and 35ºC were found to be the most appropriate for VFAs production and both of them were assessed in the following investigations conducted in semi-continuous scale to determine the best operating temperature.

Effect of initial pH and temperature on VFAs profile

Regardless of temperature, acetic acid was the most abundant VFA representing maximum concentrations between 40-57% (in terms of COD, Figure 22). The slight decrease of acetic acid along digestion can be explained by its use for methane generation. As it can be seen in Figure 21, there is a lag phase (days 1-5) where concentration of VFAs remained stable (Figure 22. After those days, VFAs concentrations dropped (data not shown) and methane started to be produced.

Regardless of the tested pH, propionic acid showed its lowest relative abundances at 50ºC whilst it was the second most abundant product at 25ºC and 35ºC (15-28%). Muller et al., [199] pointed out that the oxidation of propionate is energetically unfavorable and thus, its accumulation is common in unbalanced digestions. Moreover, this oxidation is influenced by factors such as pH, temperature, hydrogen partial pressure or VFAs present

in the process [200]. Thus, the alteration of parameters such as the substrate to inoculum ratio or pH could have affected this oxidation and caused the accumulation.

The abundance of acetic and propionic acids as main products has been already reported in literature for microalgae biomass. For instance, as in the present study, VFAs production using *Scenedesmus quadricauda* and *C. vulgaris*, resulted in acetic acid as the main product of the digestion at low initial pH values (5.5) and mesophilic conditions [73]. This trend was maintained in other studies employing microalgae biomass as substrate (See Table 1, Section 1.2.1.). Additionally, acetic acid has also led VFAs profiles (up to 57%) when using other protein rich substrates (municipal solid waste) [48].

Butyric and isobutyric acids remained in the same range regardless of temperature and pH (around 10% each) whereas valeric acid production seemed to be linked to temperature as it was not detected in thermophilic digestion whilst representing 7-10% at 25ºC and 35ºC regardless of the pH value. Opposite, the maximum isovaleric percentage was obtained during the digestion conducted at thermophilic conditions. At this temperature, isovaleric acid represented up to 20% of the VFAs profile, and remained stable throughout the experiment.

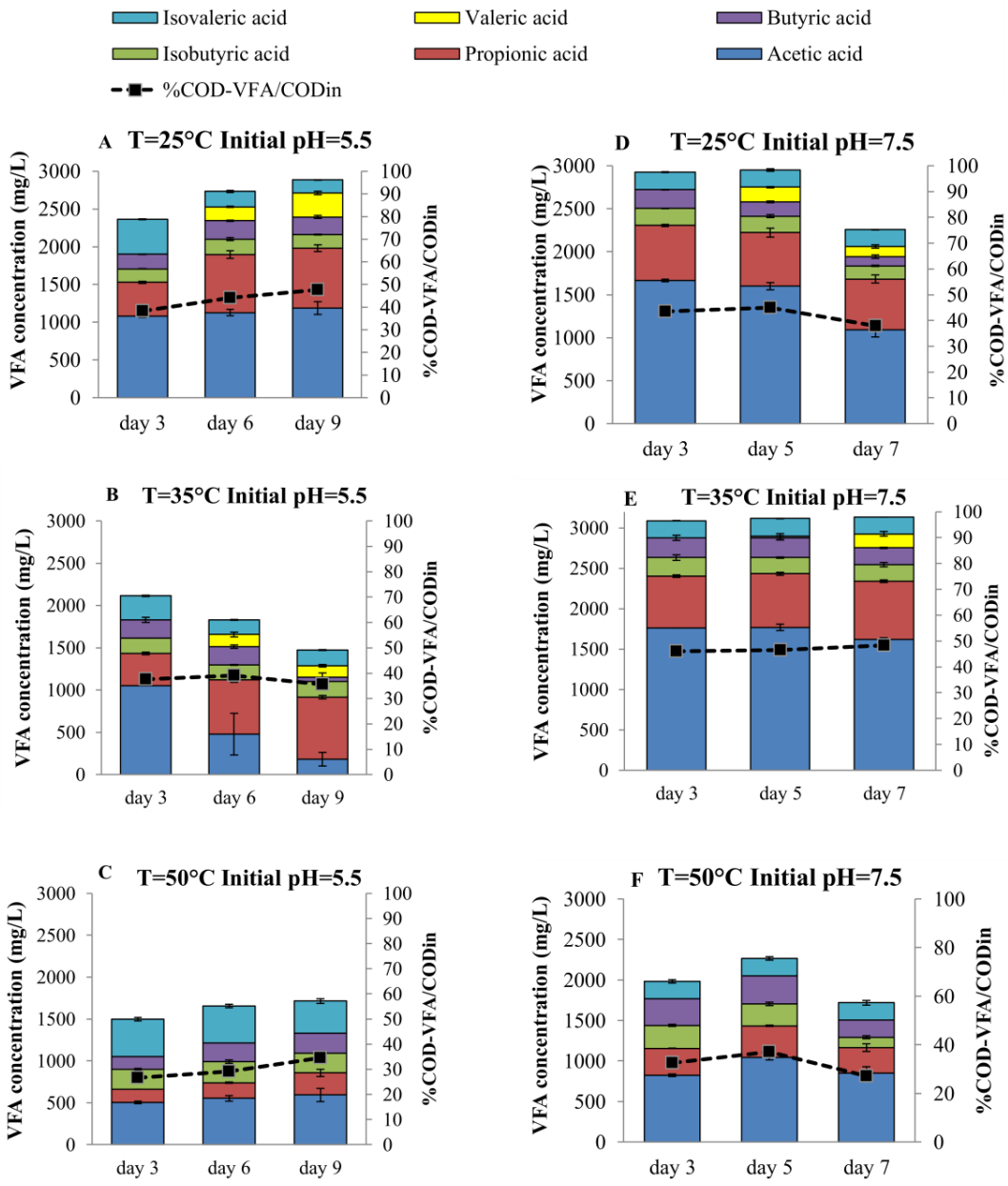

Figure 22. VFAs productions and profiles in BCPs at initial pH of 5.5 (A, B, C) and 7.5 (D, E, F).

Overall, results showed that best conditions for VFAs production were mesophilic temperature ranges (35°C) at neutral initial pH values (7.5), and psychrophilic temperature ranges (25°C) at low initial pH values (5.5), which resulted in a conversion of organic matter into VFAs of 48% COD-VFAs/COD_{in}, respectively. This value is in good agreement with the values of organic matter conversion into VFAs reported for microalgae biomass (Table 1).

4.2. OPTIMIZATION OF VFAS PRODUCTION IN CSTR

4.2.1. Organic loading rate effect in semi-continuous mode at mesophilic conditions

AF performance of non-pretreated microalgae biomass at 35ºC

The control experiment consisted on the use of anaerobic sludge at 35ºC using non-pretreated microalgae biomass as substrate (HRT 10 days and OLR 1.5 g COD/Ld). As it is the most conventional range of temperatures applied in AD [35,201], reactors were firstly run at 35ºC. In order to promote hydrolytic and acidogenic stages, the HRT selected was lower than those normally employed in methane production. For instance, Ras et al., [202] achieved better process performance using *C. vulgaris* at longer residence times when targeting methane production. More specifically, those authors reported an increase in organic matter removal from 33% to 51% by increasing the HRT in a CSTR from 16 to 28 days. This increase in HRT resulted in a methane yield enhancement of 1.6-fold. Since the objective herein was to produce VFAs, HRT was set to 10 days in a first attempt. Results showed a low $sCOD_{effluent}/tCOD_{effluent}$ ratio (around 0.15), which reflected the low hydrolytic capacity of the microbiome. Due to the low hydrolysis achieved in the reactor, VFAs production in semi-continuous mode with non-pretreated microalgae was very low (below 5% COD-VFAs/COD_{in}). This fact was consistent with former studies in which non-pretreated microalgae biomass used as substrate for methane production in batch mode and semicontinuous operation (CSTRs) showed very low biodegradability [91]. Therefore, it can be inferred from this result that a pretreatment is of outmost importance to achieve competitive VFA yields from microalgae biomass. As a matter of fact, protease pretreated microalgae rendered a high organic matter conversion into VFAs (up to 48%, see Section 4.1.3). In this sense, operation in BCPs can provide information regarding the biodegradation of a certain feedstock. However, as carbon and nutrients availability decline and are not replenished, microbial growth rates and community structure shift over time are not considered in

BCPs. BCPs methodology is useful for discriminating among ranges of different operational parameters, but results should be always confirmed in semicontinuous feeding mode.

Diverse types of pretreatments are currently used to disrupt microalgae biomass for promoting organic matter solubilization and increase organic matter availability [45,201]. This thesis was designed to assess the potential of microalgae biomass for VFAs production and hence, the hydrolysis stage was facilitated to fully focus on the acidogenic stage. Proteins were recently pointed out as the macromolecules responsible of hindering the AD process for methane production [45]. Additionally, the microalgae used as substrate presented high protein content (59±5% DW, see section 3.1). For all these reasons, the hydrolysis was facilitated by employing a proteolytic pretreatment (Alcalase 2.5L, Novozymes). This pretreatment was applied for the rest of the experiments in order to promote VFAs production.

AF performance of protease pretreated microalgae biomass: Effect of organic loading rate at mesophilic temperature

As presented in Section 4.1., the effect of substrate to inoculum ratio was analyzed in batch mode. Results showed that overloading the system might contribute to VFAs accumulation. As more organic matter is available for the anaerobic microbiome and given the slow activity of archaea compared to bacteria, VFAs might accumulate. For this reason, the effect of OLR was assessed in semicontinuous mode by comparing reactors R1 and R2 set at 1.5 and 3 g COD/Ld, 35ºC and HRT 10 days, respectively. The inoculum employed for this investigation was anaerobic sludge provided by the WWTP of Valladolid.

Results showed similar COD removals for the mesophilic reactors R1 and R2 (23.6±2.3% and 26.3±2.6%, respectively, Table 10). COD removals were low when compared to a process devoted for biogas production. For instance, microalgae biomass digested in a semicontinuous CSTR (35ºC, 1.5 g COD/Ld and HRT of 20 days) showed a methane production of 128.4±15.3 mL CH_4 (STP)/g COD_{in} (56% COD removal) [43]. Indeed, those authors pointed out that increasing HRT could be used as a tool to increase COD

removal. This conclusion was also supported by previous results obtained in a continuous anaerobic digestion fed with microwave pretreated microalgae biomass [203]. In that case, methane yield improved (from 36% to 42% COD removal) when HRT was increased from 15 to 20 days. Therefore, the use of low HRTs is an important parameter in order to decrease COD removal and hence, accumulate VFAs. With regard to the biogas composition, methane content was 52.1±2.4% (v/v) for R1 and 48.9±5.5% for R2. The similar methane content in the biogas, together with the obtained COD removals, showed that methanogenesis was equally affected regardless of the OLR employed. Hence, the low COD removals were mainly attributed to the short HRT imposed.

Table 10. Main process parameters measured in the effluents during R1 (OLR 1.5 g COD/Ld) and R2 (3 g COD/Ld) operation.

	R1 (1.5 g COD/Ld)	R2 (3 g COD/Ld)
% CH_4 in biogas (v/v)	52.1±2.4	48.9±5.5
% COD removal	23.6±2.3	26.3±2.6
% COD-VFAs/COD_{in}	25.6±3.0	25.8±3.9
COD-VFAs/$sCOD_{out}$	0.7±0.1	0.7±0.1
pH	6.9±0.1	7.1±0.1
NH_4^+ (g/L)	0.7±0.1	1.2±0.1

NH_4^+ and NH_3 accumulation might occur when proteins are degraded during AD/AF [204]. NH_4^+ and NH_3 concentrations are important parameters since high concentrations of these compounds may result inhibitory for methanogenic archaea, resulting in methanogenesis inhibition. In this particular case, NH_4^+concentration was high, especially for R2, but not yet above the inhibitory threshold considered for un-acclimated inoculum (1.7–1.8 g/L [41]). Hence, even though the microalgae biomass employed was rich in proteins, the OLR employed was still not high enough to cause methanogenic inhibition due to NH_4^+ toxicity. NH_4^+/NH_3 equilibrium relies mainly on pH and temperature. In fact, NH_3 concentrations are minimized at neutral and acidic pH values and low temperatures. According to the values of pH and process temperature (35ºC, Table 10), NH_3 concentration was very low (6.2 and 16.9 mg/L NH_3 for R1 and R2, respectively). More specifically, those values were below the inhibitory threshold (150 mg/L [205]). The pH values obtained, close to neutrality, were appropriate for methanogenic activity (Section

1.2.2.). At this point, it should be highlighted that protein-rich substrates, such as microalgae biomass, might drive the process to methanogenic inhibition due to NH_4^+ toxicity [43]. However, carbon and nitrogen mineralization can differ during the fermentative process. For instance, a similar study for organic acid production from crop silaging led to a pH decrease and inhibition of methanogenic activity [206]. Transformation of organic carbon (partial hydrolysis and acidogenesis) occurred, but no high nitrogen mineralization was reached. This latter example can be associated with the present process in which organic acids were formed.

VFAs production: conversion yields and profiles

VFAs concentration was 2-fold higher in R2 than R1, reaching nearly 10,000 mg COD-VFAs/L. However, organic matter conversion into VFAs (25% COD-VFAs/COD_{in}, Table 10) was not affected by OLR and similar conversion yields were reached by both reactors (Figure 23). In the same manner, the efficiency of the acidogenic stage was similar for both reactors analyzed (0.7±0.1 COD-VFAs/$sCOD_{out}$). Opposite to that trend, a similar study was carried out in semi-continuous mode to evaluate VFAs production from tuna waste (37ºC, HRT of 10 days) at pH ranging from 5 to 9. These authors highlighted an increase in organic matter conversion into VFAs when the OLR was increased from 2 to 4 g COD/Ld at pH 9 (from 25 to 30% COD-VFAs/COD_{in}, respectively) [207]. In the present study, pH was monitored but not controlled. As explained in Section 1.2.2., the working pH is dependent on the residue used as feedstock. Those authors found that the most appropriate pH was the alkaline range whereas in the present study, alkaline values did not enhance organic matter conversions into VFAs (see Section 4.1.3.). Despite of the differences in the OLR effect, it should be pointed out that conversion yields of COD into VFAs are in the range of those obtained herein.

Regarding the effect of the OLR used to feed the reactors on VFAs production profile (% COD-each VFA/total COD-VFAs), propionic acid concentration prevailed in both reactors (36.0±2.0% for R1 and 31.8±4.9% for R2, Table 11).

Table 11. VFAs spectrum of mesophilic reactors (R1 and R2).

	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid
R1	14.1±2.3	36.0±2.0	10.7±0.9	9.8±1.4	11.3±1.2	18.2±1.8	0
R2	18.1±6.5	31.8±4.9	10.2±0.8	11.6±1.8	11.3±1.3	16.9±2.2	0

Accumulation of propionic acid is related to the Gibbs energy associated to its degradation reactions and has been previously reported in unbalanced AD [208]. Degradation of propionic acid is the less favorable reaction ($\Delta G = +76.1$ kJ mol^{-1}) when compared to other VFAs such as acetic acid, which is a spontaneous process ($\Delta G = -30$ kJ/mol) [209]. Hydrogen is one of the products released upon propionic acid degradation [210]. Thus, as hydrogen is removed from the media by anaerobic microorganisms, propionic acid is degraded avoiding its accumulation. According to propionic acid accumulation registered herein, it could be assumed that the harsh operational conditions imposed to the system caused a drop of syntrophic microorganism's activity. Syntrophy is defined as the closely associated relationship between two or more species. In the present case, the syntrophic relation would be established between syntrophic acetogens and methanogenic archaea [211]. In fact, methanogenic archaea can only metabolize a few substrates to produce methane. Hence, hydrolytic and acidogenic products, such as propionic acid (or longer VFAs), need to be transformed by syntrophic acetogens to form acetate, H_2 or CO_2, which are the products that archaea can metabolize.

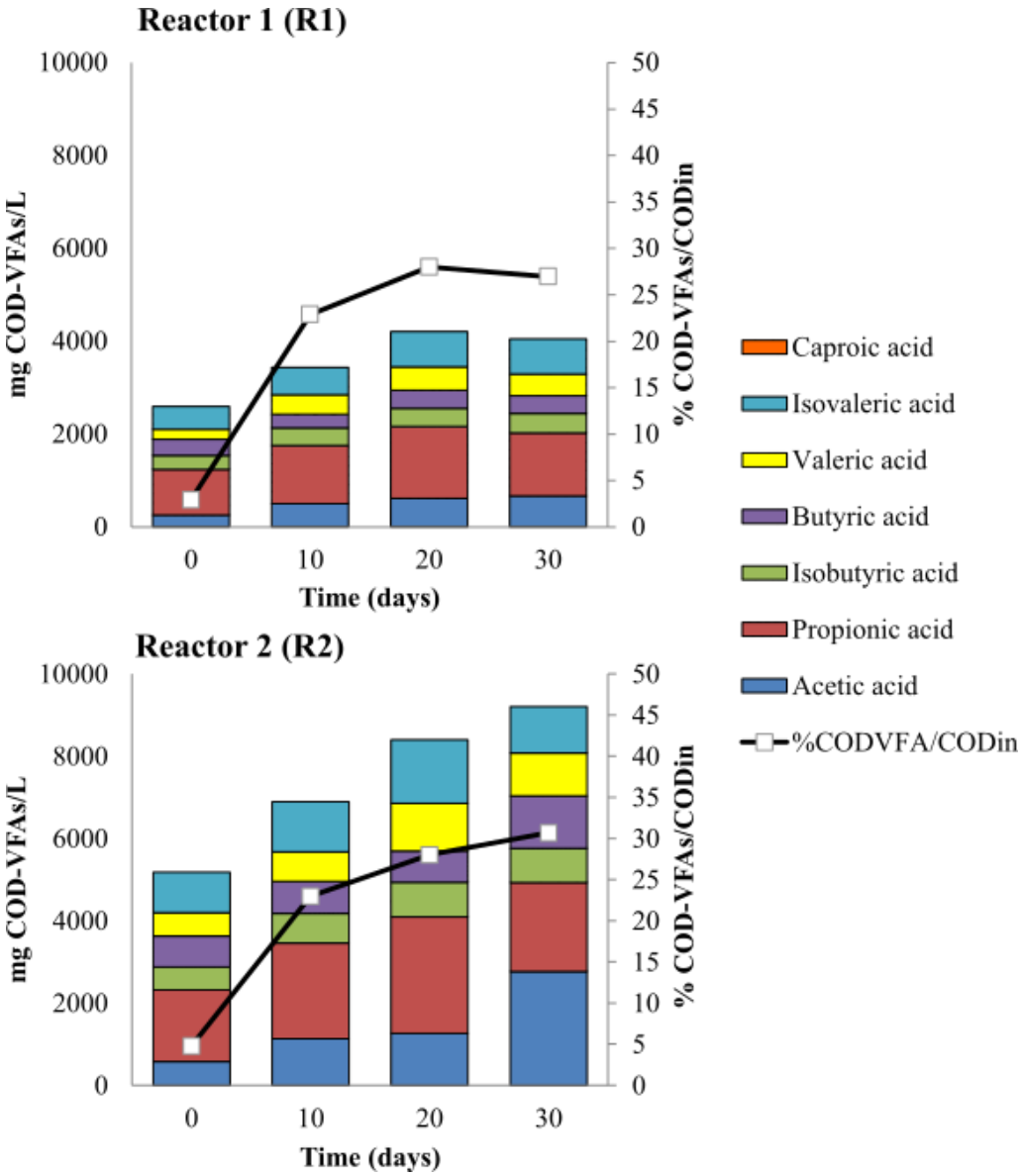

Figure 23. VFAs production and conversion for R1 (1.5 g COD/Ld) and R2 (3 g COD/Ld). Representative samples from the initial days, HRT, 2HRT and 3HRT to follow up VFAs productions were included.

Acetic acid was the second most abundant VFA in these reactors (14.1±2.3% and 18.1±6.5%, R1 and R2, respectively). The abundance of acetic acid might result from VFAs degradation. In this sense, longer VFAs chains are converted into acetate and hydrogen through the ß-oxidation pathway (see Section 1.2). This VFA was also found to be among the most abundant products in other studies using different substrates such as waste activated sludge, maize silage and whey [47,73]. These two VFAs (acetic and

propionic acids) accounted for up to 50% of the VFAs obtained in R1 and R2. The rest of them (C4-C5) ranged 10-18% each and were not influenced by the different OLRs applied (Table 11).

These results are in accordance with other study where microalgae biomass was employed as substrate [73]. Jankowska and co-workers carried out mixed culture fermentations with *Scenedesmus quadricauda* and *C. vulgaris*. They found that acetic acid was the most abundant product (42%) during the first days, followed by propionic and butyric acids (19% each), and isovaleric acid (12%) while the rest of the VFAs percentages were even lower. With regard to other substrates, the prevalence of acetic acid is a common feature (Table 1, Section 1.2.1.). This might be caused by the degradation of longer VFAs via β-oxidation, which gives acetic acid as the main degradation product. Therefore, the fact that the VFAs pool was prevalent on short chain VFAs remains within a conventional AF performance.

Microbial communities

The main objective of analyzing the anaerobic microbiome is to link microbial structure with differences in process parameters. Aiming at further explaining the VFAs productions and digestion performance in semicontinuous digestion mode, DNA was extracted to analyze the microbial populations involved in the processes.

The Shannon index was employed to characterize the diversity in a microbial community. Lower values (4.37-4.03) were determined for reactors fed with microalgae biomass (R1 and R2) when compared to the inoculum (6.83). These results evidenced a lower microbial diversity in reactor operated for VFAs production (R1 and R2). The differences in diversity were attributed to two main reasons: i) the substrate fed into the reactors and ii) the operational conditions. The inoculum came from a WWTP fed with mixed primary and secondary sludge while the substrate fed herein was microalgae biomass. For this reason, once the inoculum was subjected to the AF process at different OLRs (R1 and

R2), a drastic population change was observed (Figure 24). Therefore, changing the substrate can be associated to a change of microbial population [27]. This is a normal feature inherent to the adaptation of the microbial community to a new substrate. Additionally, the operational conditions imposed (HRT, OLR, and temperature) for methanogenesis inhibition could have caused the death and wash out of other species resulting in a specialization of the inoculum. This specialization would be thus the responsible for the lowered Shannon value during reactors operation.

The anaerobic sludge employed as inoculum presented bacteria population belonging to Bacteroidetes (15%), Proteobacteria (11%), Chloroflexi (11%), Firmicutes (9%), Actinobacteria (7%), Synergistetes (6.1%) and Spirochaetes (5.2%), as the most abundant phyla. Euryarchaeota population in charge of the methanogenic step presented a relative abundance of 4%. This phylum was mainly constituted by species such as *Methanobacterium* and *Methanosaeta*. The low presence of archaea when compared to other anaerobic sludge in AD (8% [106]) might be an advantage when the objective is to produce VFAs.

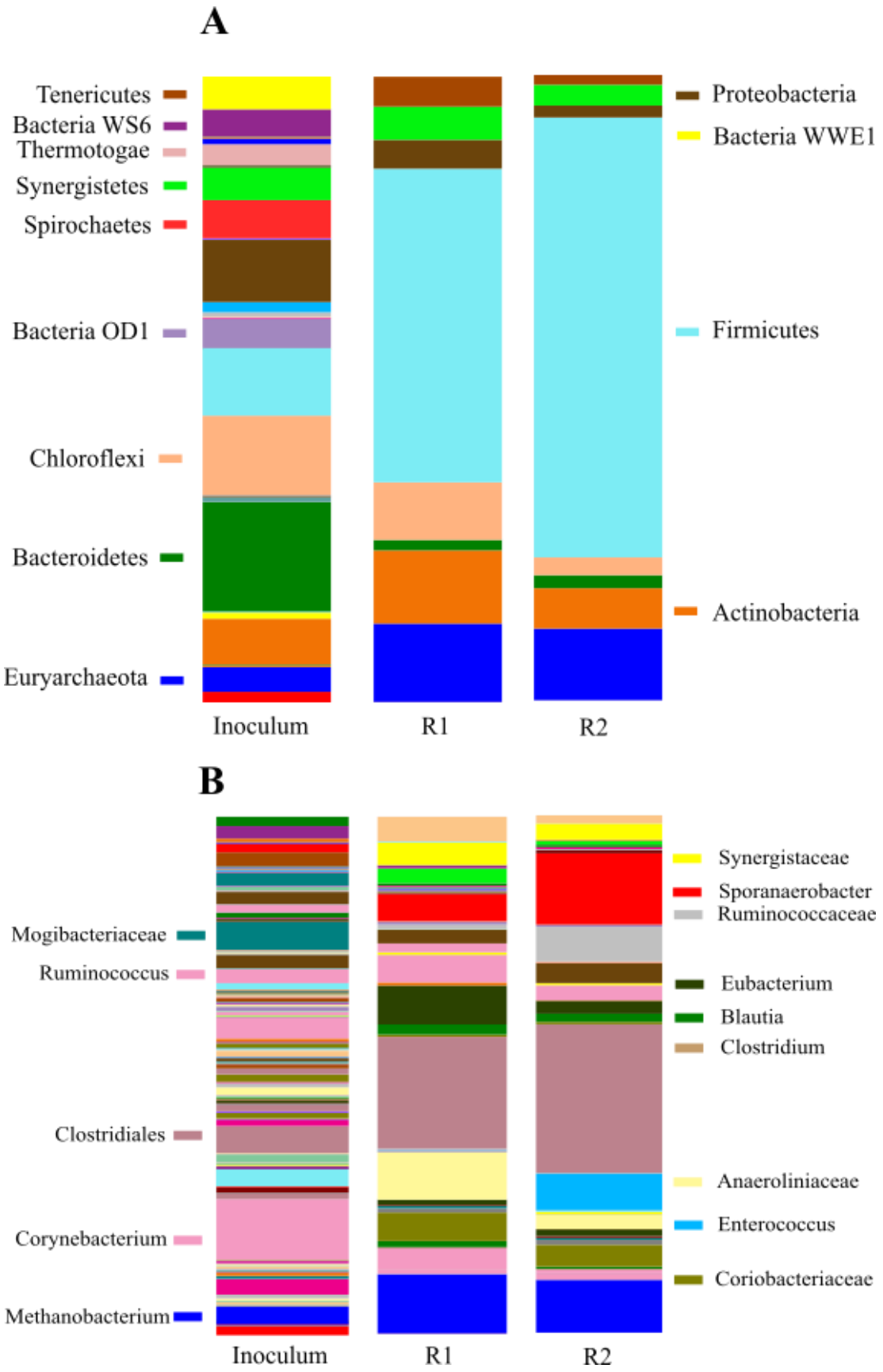

Figure 24. Main phyla (A) and genera (B) encountered in the anaerobic inoculum employed and the mesophilic reactors R1 (1.5 g COD/Ld) and R2 (3 g COD/Ld).

At phylum level, bacterial distribution was highly represented by Firmicutes accounting for 51% and 73%, respectively, in both mesophilic reactors R1 and R2 (Figure 24A). Firmicutes phylum, include species associated with the anaerobic environment and mesophilic temperature and contains most known acidogenic bacteria responsible for VFAs production [212]. Species distribution in both reactors within this phylum was similar favoring the growth of Clostridiales family (21.5 and 28.7, respectively, Figure 24B) that usually release different products such as VFAs (acetate and butyrate), formate, CO_2 or hydrogen [213]. In fact, acetic acid and butyric acid represented nearly 25% of total VFAs production in R1 and R2. The other encountered genera were present in both reactors at different relative abundances most likely because process parameters (i.e. NH_4^+ or VFAs concentrations) associated to the increase of OLR had an impact on growth rates. For instance, *Sporanaerobacter* gained importance in R2 with respect to R1 (13.8 vs 5.3%). This genus has been identified in acidogenic reactors fed with microalgae biomass and has been pointed out to be responsible of metabolizing sugars, peptides and single amino acids into acetate [214].

Opposite to these results, other studies in literature using microalgae biomass as substrate in anaerobic digesters showed a different microbial structure. For instance, anaerobic digestion at mesophilic conditions of *Scenedesmus* for biogas production resulted in the abundance of Chloroflexi (27.9%) whilst Firmicutes only represented 3.6% [104]. Chlorofexi phylum are commonly found in activated sludge systems [215]. However, it has been confirmed the low tolerance of Chloroflexi species to operational conditions, explaining their absence in the digesters of the present investigation [104]. Proteobacteria is another important phylum in AD [106], as these species are represented in high proportion in anaerobic sludge [216]. Members of this phylum share a syntrophic relation with methanogens but herein their presence was negligible in R1 and R2, which might be another reason to explain VFAs accumulation. On the contrary, the relative abundance of Firmicutes phylum was found to be considerably lower in reactors operated for biogas production purposes [106,217]. Gonzalez-Fernandez et al. [106] used *Chlorella sorokiniana* and *Scenedesmus* sp. for methane generation and obtained a diverse community characterized by the presence of Proteobacteria (46-51%) whilst Firmicutes only accounted for 20%. At this point, differences in terms of phylum when the digestion is devoted to biogas or VFAs production should be highlighted. According to the

available literature, it seems likely that anaerobic microbiome devoted to biogas production is mainly represented by Proteobacteria or Chloroflexi.

The Euryarchaeota phylum responsible of carrying out the methanogenic step presented a relative abundance of around 12.5% in R1 and 11.5% in R2. These values were higher with respect to the ones obtained in the inoculum (4%). Even though COD removals were lower than studies devoted for biogas production, the methane content analyzed in the biogas indicated that archaea were active. Nevertheless, the low HRT imposed might have hampered their full development. Archaea presence is in accordance with similar studies devoted for biomethane production. For instance, the archaeal population represented 7-8% in the case of digesting sewage sludge with species such as *Methanosaeta, Methanomicrobiales, Methanomassiliicocus, Methanosarcina* or *Methanothermobacter* [106]. However, population found at genus level in the present study was less diverse and only *Methanobacterium* genus was identified. This fact might be associated with the low COD removals obtained in R1 and R2, as methane was only produced via the hydrogenotrophic metabolic pathway.

Overall, the OLR had an effect on the relative abundances of the developed species. The low COD removal percentages achieved in both reactors might be explained by the only presence of the hydrogenotrophic archaea and the low HRT imposed. Both reactors were dominated by members belonging to Firmicutes phylum. This first analysis of the anaerobic microbiome revealed strong differences with the bacterial population of reactors devoted to biogas production.

4.2.2. Temperature optimization in semi-continuous mode

AF performance of non-pretreated microalgae biomass at 25ºC

In Section 4.1.3, the most appropriate temperatures in BCPs for VFAs production were 35ºC and 25ºC, which presented the highest organic matter conversions into VFAs (up to 48% COD-VFAs/COD_{in}). The present investigation was intended to confirm the most appropriate temperature for VFAs production in semi-continuous digestion mode. Similar to the control digester at 35ºC described in Section 4.2.1, AF was set at 25ºC using non-pretreated microalgae biomass as substrate (HRT 10 days and OLR 1.5 g COD/Ld). Again, a low $sCOD_{effluent}/tCOD_{effluent}$ ratio (around 0.15) was obtained with an organic matter conversion into VFAs of 7±3% COD-VFAs/COD_{in}. These values were similar to the results obtained by the non-pretreated microalgae in the mesophilic temperature range (Section 4.2.1.). Therefore, the proteolytic pretreatment was again applied for the CSTRs in this temperature range.

AF performance of protease pretreated microalgae biomass: Effect of temperature

A new CSTR (R3) was set in the psychrophilic temperature range (HRT=10 days and OLR 1.5 g COD/Ld). This digester was compared with R1 (Section 4.2.1) set at 35 ºC (HRT=10 days and OLR 1.5 g COD/Ld). Additionally, the inoculum employed was the same as Section 4.2.1.

Results showed higher COD removal for the mesophilic reactor R1 (23.6±2.3%) than for the psychrophilic reactor R3 (11.9±3.0%, Table 12). Besides, temperature had an effect in biogas composition. In this case, methane content was lower in R3 (20.8±2.6% v/v) when compared to R1 (52.1±2.4% v/v). The lower methane content and COD removals obtained in R3 compared to R1 confirmed a higher inhibition of the methanogenic step in R3. It should be highlighted that R1 and R2 already achieved lower COD removal values

than those obtained for biogas production (Section 2.4.4). Hence, the combined effect of low HRT and temperature was even more detrimental for methanogenesis.

Table 12. Main process parameters measured in the digesters effluents during reactor operation.

	R1 (35ºC)	R3 (25ºC)
% CH_4 in biogas (v/v)	52.1±2.4	20.8±2.6
% COD removal	23.6±2.3	11.9±3.0
% COD-VFAs/COD_{in}	25.6±3.0	35.5± 3.0
COD-VFAs/$sCOD_{out}$	0.7±0.1	0.8±0.1
pH	6.9±0.1	6.3±0.1
NH_4^+ (g/L)	0.7±0.1	0.7±0.1

As observed in the case of mesophilic operation conducted in R1 and R2, NH_4^+ concentrations remained at low levels (0.7±0.1 g/L NH_4^+, Table 12). Those values were not considered inhibitory, however it should be highlighted that pH values slightly dropped in R3 (6.3±0.1) with respect to R1 (6.9±0.1). This small change might be a result of the higher organic matter conversion into VFAs at 25ºC (35.5±3.0% COD-VFAs/COD_{in}) when compared to the one obtained at 35ºC. Additionally, the slightly acidic pH in the medium could have contributed to hinder COD removal in R3 because methanogenesis is favored at values close to neutrality (see pH in Section 1.2.2.).

This investigation confirmed that digestion temperature affected the methanogenic step. Since bacterial growth and conversion processes are slower, lower methane productions are normally associated to lower temperature digestions [218]. A low COD removal value, which might be beneficial for VFAs productions, was especially observed in R3. This fact confirmed that low temperature digestion (25ºC) was more appropriate than 35ºC to inhibit methanogenesis.

VFAs production: conversion yields and profiles

VFAs production (mg COD/L) and organic matter conversion into VFAs are collected in Figure 25. Average conversion yields in the stationary state were 25.6±3.0% and 35.5± 3.0% COD-VFAs/COD_{in} for R1 and R3, respectively. Additionally, acidogenic efficiency slightly increased (0.8±0.1 COD-VFAs/$sCOD_{out}$) when compared to the values obtained by mesophilic reactors (Section 4.2.1.).

VFAs production (mg COD-VFAs/L) was higher when the experiment was performed at 25ºC in R3 (5,056±348 mg COD-VFAs/L) than at 35ºC (4,057±512 mg COD-VFAs/L in R1). Temperature has been regarded as a tunable parameter for VFAs production in AF. For instance, Zhuo et al., [219] tested a range of temperatures (10, 20, 37, 55°C) on the hydrolysis and acidification stages of AD using waste activated sludge as substrate. Those authors reported a concomitant organic matter conversion into VFAs with temperature; 10ºC (COD-VFAs/COD_{in} = 10.4%), 20ºC (COD-VFAs/COD_{in} = 29.8%) and 37ºC (COD-VFAs/COD_{in} = 41.5%). The authors attributed the progressive VFAs increase to the better hydrolysis of proteins and carbohydrates at higher temperatures. However, at 55ºC, the conversion dropped to 25.0% COD-VFAs/COD_{in}. Consumption of soluble proteins and carbohydrates by the anaerobic microbiome decreased at the highest temperature, suggesting the inhibition of certain species involved in the acidogenic stage. With regard to the present study, the temperature effect on the hydrolysis stage was negligible since a proteolytic pretreatment was carried out prior to AF to discard hydrolytic problems.

Average conversion values attained in R3 at 25ºC (COD-VFAs/COD_{in} = 35.5%) are in the range of the conversions obtained by Zhuo et al., [219] and Oktem et al., [220] in semi-continuous operation mode using waste activated sludge and pharmaceutical wastewater as substrate. These results showed that organic matter conversions into VFAs obtained herein from microalgae biomass are in the range shown by other investigations using different substrates. Therefore, it can be suggested at this point that microalgae biomass would be as good as any other substrate employed in AF.

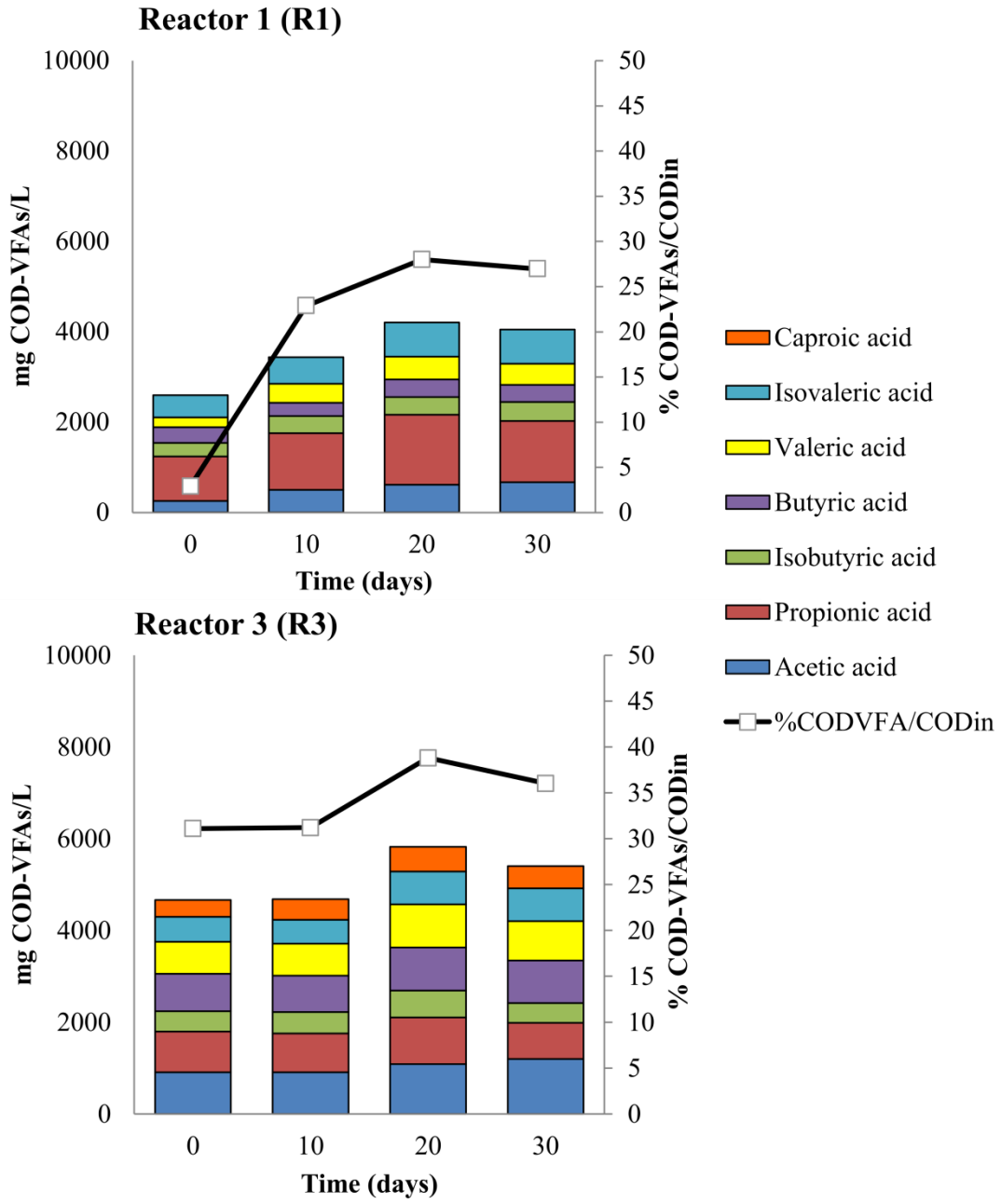

Figure 25. VFAs production and conversion for R1 (35ºC) and R3 (25ºC). Representative samples from the initial days, HRT, 2HRT and 3HRT to follow up VFAs productions were included.

Regarding the effect of digestion temperature on VFAs profile, out of the total COD represented by VFAs, acetic acid (19.9±1.5%) together with propionic (17.3±1.9%), and butyric acids (16.9±0.6%) were the most abundant products in R3 whereas propionic acid stood out as the most abundant product in R1 (36.0±2.0%) followed by acetic acid (14.1%±2.3%). The higher acetic acid accumulation recorded in R3 in comparison to R1,

indicated an imbalance on the AD process since this compound is normally transformed into methane by acetoclastic archaea [221]. Thus, acetic accumulation confirmed the inhibition of the acetoclastic pathway of methanogenesis in R3, supporting the low COD removal (11.9±2.9%). With regard to propionic acid, digestion temperature seemed to affect its production and degradation rates. The low propionic acid presence in R3 compared to R1 might be related with the process temperature and the degradation pathways of longer VFAs. As a matter of fact, no C6-VFA was detected at 35ºC whereas this acid could accumulate at 25ºC (R3, 8.9±0.7%, R3, Figure 25). Likewise, the presence of C4-C5 in R1 (35ºC) was 50% whereas in R3 was 63% (25ºC). This is a high relative abundance of C4-C5-C6 in comparison with other studies using microalgae biomass as substrate [52,54] since the VFAs spectrum is often dominated by acetic and propionic acids (Section 1.2.1., Table 1).

Table 13. VFAs spectrum of mesophilic reactors (R1 and R3).

	Acetic acid	**Propionic acid**	**Isobutyric acid**	**Butyric acid**	**Isovaleric acid**	**Valeric acid**	**Caproic acid**
R1	14.1±2.3	36.0±2.0	10.7±0.9	9.8±1.4	11.3±1.2	18.2±1.8	0
R3	19.9±1.5	17.3±1.9	9.4±0.9	16.9±0.6	15.4±0.6	12.1±1.0	8.9±0.7

Overall, 25ºC and 35ºC were the most appropriate temperatures in BCPs for VFAs production (Section 4.1.3.). Nevertheless, investigation carried out in semi-continuous mode evidenced 25ºC as the most promising temperature to achieve such a goal. Selection of this temperature for future experiments was based on the lower COD removals attained when compared to mesophilic operation and the higher organic matter conversions into VFAs (COD-VFAs/COD_{in}).

Microbial communities

The influence of temperature on microbial communities was assessed through the comparison of the populations obtained in R1 (35ºC) and R3 (25ºC). The psychrophilic reactor R3 showed a sludge specialization similar (4.07) to that exhibited in R1 and R2 (Shannon index 4.37 and 4.03, Section 4.2.2.) when compared to the anaerobic inoculum (6.83).

At phylum level, Firmicutes stood out again as the most abundant phylum in both reactors and increased its relative abundance in R3 (83%) when compared to R1 (51%) (Figure 26A). Genera found in the psychrophilic digester (R3) were similar to those found in R1. However, relative abundances of the different species belonging to Firmicutes increased in R3. For instance, microorganisms belonging to order Clostridiales (32%), *Ruminococcaceae* (11%), *Enterococcus* (11%), or *Eubacterium* (5%) had a major prevalence in the psychrophilic reactor. Since the abundance of this phylum is related with the acidification phase of anaerobic digestion [222], the higher *Firmicutes* relative abundance in R3 with respect to R1 was in accordance to the increase in VFAs production registered at psychrophilic conditions.

The relative abundance of the Euryarchaeota phylum in R3 averaged 4.6%. More specifically, the *Methanobacterium* population percentage decrease registered in R3 evidenced the lower removal capacity of R3 (COD removal 11.9±2.9%) in comparison to R1 (COD removal 23.6±2.3%) for methane production. As reported in the previous section (Section 4.2.1.), acetic acid accumulation detected may be related with the lack of archaeal species belonging to *Methanosarcinales*, order known to perform acetoclastic methanogenesis [223].

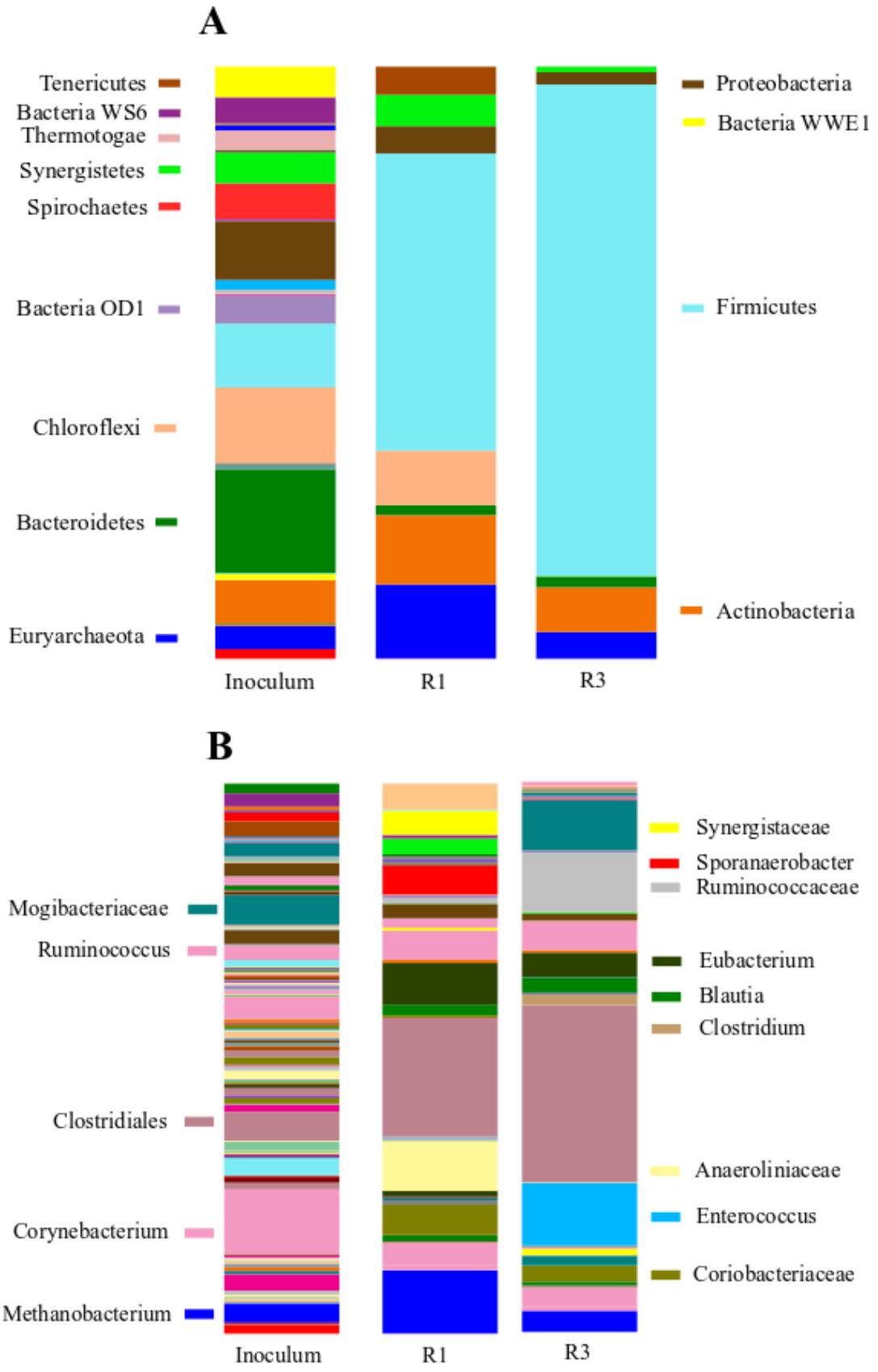

Figure 26. Main phyla (A) and genera (B) encountered in the anaerobic inoculum employed and the mesophilic reactors R1 (35ºC) and R3 (25ºC) at OLR of 1.5 g COD/Ld.

Overall, it could be concluded that operational temperature had an impact on the developed microbial population when compared to the inoculum source. Reactor set at 25ºC exhibited a less diverse microbial community characterized by a high Firmicutes presence. Archaea species were more developed at mesophilic conditions (R1), which most likely caused the increase in COD removal in that reactor.

4.2.3. HRT optimization in semi-continuous mode

AF performance: Effect of HRT

Since low fermentation temperature (R3, Section 4.2.2) provided the highest VFA production yields, the AF process was further studied at psychrophilic conditions. Aiming at increasing the VFAs production and conversion yields, the use of different HRTs (R4; 8 days and R5; 12 days) was tested at 25ºC. Given the good results obtained in terms of VFAs yields in R3 (Section 4.2.2.), the specialized sludge obtained after fermentation of this reactor was employed as seed inoculum for the present investigation. Hence, the sludge is considered to be adapted to the substrate and operational conditions employed.

COD removals obtained for R4 and R5 (8.9±3.5% and 10.4±2.7%, respectively) were similar to the ones obtained in R3 (11.9±2.9%). Once again, these values were comparatively lower than those obtained in reactors devoted for methane production [43,203]. Reactors showed lower methane content (13.2±1.7% and 10.5±4.2% (v/v) for R4 and R5, respectively) in the biogas to the previously shown by R3 (20.8±2.6% v/v) (Table 14). The similar COD removals and methane compositions suggested that methanogenesis was not affected by changes in the HRT. NH_4^+ and pH values remained in the same range than those obtained previously in R3 (Section 4.2.3). Thereby, the AF performance was very similar regardless of the HRT employed.

Table 14. Main results of different parameters assessed in R4 and R5 at 1.5 g COD/Ld.

	R4 (HRT 8 days)	R3 (HRT 10 days)	R5 (HRT 12 days)
% CH_4 in biogas (v/v)	13.2±1.7	20.8±2.6	10.5±4.2
% COD removal	8.9±3.5%	11.9±3.0	10.4±2.7
% COD-VFAs/COD_{in}	39.8±1.8	35.5± 3.0	38.0±1.0
COD-VFAs/$sCOD_{out}$	0.8±0.1	0.8±0.1	0.8±0.1
pH	6.4±0.1	6.3±0.1	6.4±0.1
NH_4^+ (g/L)	0.7±0.1	0.7±0.1	0.7±0.1

VFAs production: conversion yields and profiles

The use of different HRTs showed similar organic matter conversion yields into VFAs in R4 and R5 (38.0±1.0% and 39.8±1.8%, respectively for R4 and R5) than those exhibited in R3 (35.4±3.8%). Additionally, acidogenic efficiency also remained similar to that obtained in R3 (0.8±0.1 COD-VFAs/$sCOD_{out}$). In reactors with adapted inoculum (R4 and R5), even though final conversion values were similar, both digesters reached the stability in a different period of time. Whereas R5 (HRT=12 days) required 16 days to achieve the maximum conversion yield, R4 (HRT=8 days) achieved the same yield after one week of operation. VFAs production registered a more stable trend when the adapted sludge was used than in those operated with non-adapted sludge (R1, R2 and R3). In this sense, total VFAs concentration remained similar in R4 and R5 (5,696±161 and 5,803±223 mg COD-VFAs/L, respectively, Figure 27). However, the VFA daily production rate increased from R5 (466 mg COD-VFAs/Ld) to R4 (734 mg COD-VFAs/Ld). Thus, production rate values in R4 were considerably higher than those obtained with the non-adapted sludge (R3, around 489.5 mg COD-VFAs/Ld). This fact suggested that the anaerobic microbiome was underestimated since their volumetric productivity could be higher as evidenced during the operation of R4. Jankowska et al., [224] analyzed the retention time impact in VFAs productivity using primary sludge and waste activated sludge as substrate. They concluded that short retention time at acid pH were the best conditions to promote VFAs productivity. This conclusion was in accordance to the results attained herein.

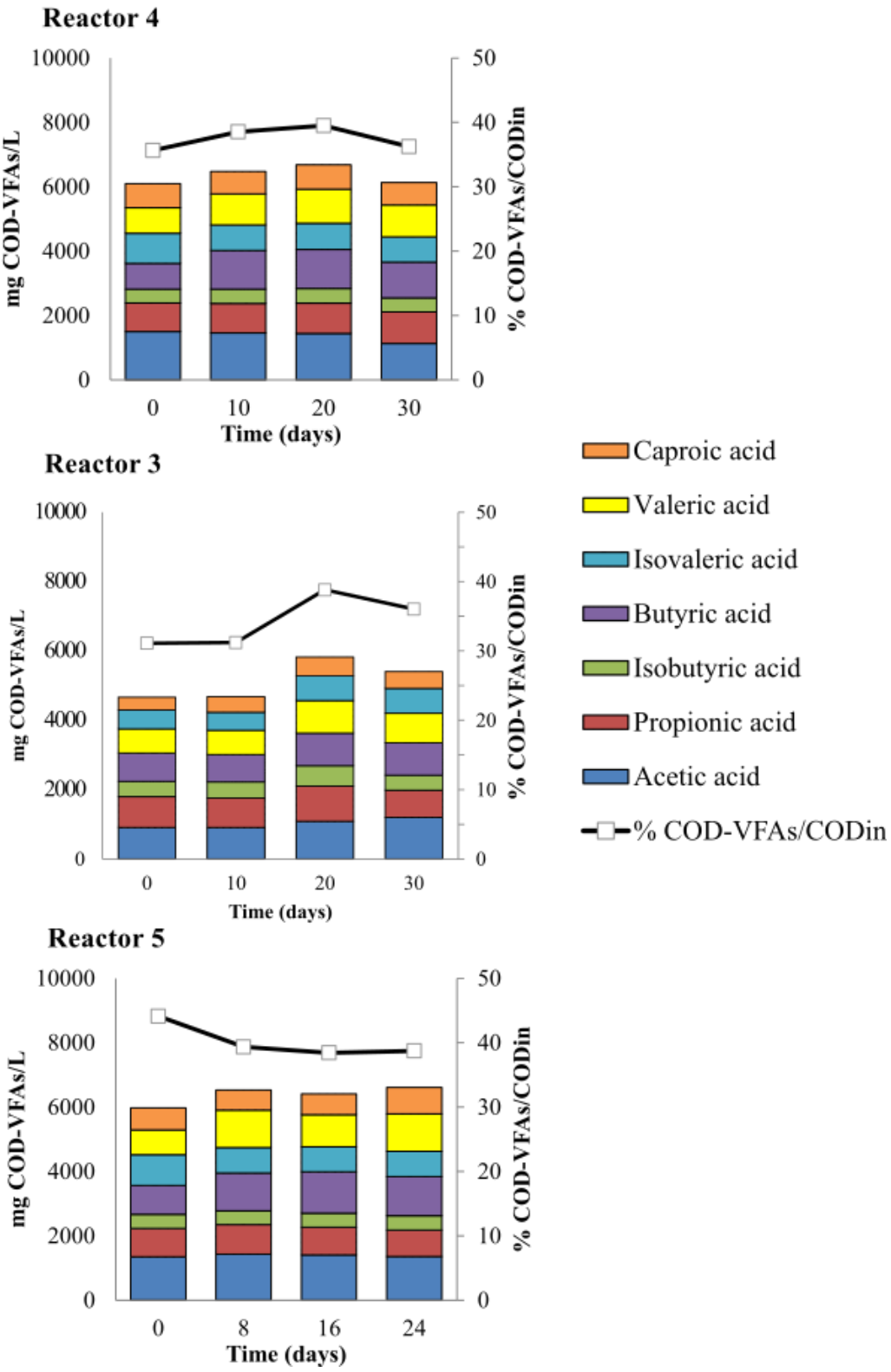

Figure 27. VFAs production at 1HRT, 2HRT and 3HRTs for R4 (HRT 8 days), R3 (HRT 10 days) and R5 (HRT 12 days).

With regard to VFAs profile, R4 and R5 exhibited a similar VFAs profile to that obtained in R3. Partial acetic acid concentrations (COD of each VFA out of the total VFAs COD) reached average values of 24.5±2.1% and 24.3±0.6% of the total concentration in R4 and R5 and remained similar throughout the experiment. Acetic acid was followed by butyric acid (18.1%). No great differences were appreciated in terms of VFAs profile between R3 and reactors with adapted sludge, in which long chain VFAs maintained the dominance showed previously in R3 (63% C4-C5-C6, Table 15). Therefore, the use of adapted sludge supported a quite stable production of VFAs while maintaining the inhibitory conditions for the methanogenic step.

Table 15. VFAs profile exhibited by mesophilic digesters and psychrophilic digesters (R1-R5).

	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Caproic acid
R3	19.9±1.5	17.3±1.9	9.4±0.9	16.9±0.6	15.4±0.6	12.1±1.0	8.9±0.7
R4	20.1±1.3	14.7±0.9	7.0±0.1	17.8±0.3	12.6±0.3	16.4±0.6	11.5±0.2
R5	21.7±0.6	13.6±0.7	6.8±0.3	18.1±1.4	12.5±1.4	16.4±1.6	11.0±0.9

To sum up, the use of low HRTs (8 days) reported similar conversion yields than R3 (10 days) and R5 (12 days). Moreover, digesters with adapted sludge did not show differences in their VFAs profile. For this reason, an HRT of 8 days was selected as the most suitable of the HRTs assessed for VFAs production.

Microbial communities

The HRT effect was assessed by analyzing the microbiome in the stationary state of R4, R3 and R5 (HRT 8, 10 and 12 days, respectively) set at T=25ºC and OLR=1.5 g COD/Ld. Shannon index showed that the anaerobic sludge maintained the specialization for both reactors R5 and R4 (4.19 and 3.7, respectively) with respect to R3 (4.07) (Section 4.2.2.). Biodiversity agreed with the HRT values employed in the digesters. The highest diversity

was attributed to R5 (HRT 12 days) whereas the lowest values was observed in R8 (HRT 8 days).

With respect to bacteria populations, Firmicutes phylum was the most abundant in all reactors. Nevertheless, a decrease of Firmicutes phylum in R4 and R5 with respect to R3 was observed (68%, 81% and 65%, respectively for R4, R3 and R5, Figure 28A) with a concomitant increase of Actinobacteria (18% in R4 and R5 vs 8% in R3). Additionally, Bacteroidetes phylum slightly increased in R4 and R5 (3.1% and 6.5%, respectively) when compared to R3 (1.4%). However, these values are far below from the ones reported in studies targeting biogas production from casein, starch and cream, where Bacteroidetes presented higher relative abundance (58.9%) [225]. This new balance in R4 and R5 did not alter VFAs conversion yields suggesting microbial redundancy.

With regard to the bacterial community (Figure 28B), the similar results exhibited by R4 and R5 in terms of microbial genera explained the similar VFAs productions, profiles and COD removals reported by both of them. Major contributors identified were species related with Clostridiales order (32-39%), other microorganism's belonging to Coriobacteriaceae family (17%) as well as genera such as *Ruminococcus* (13%), *Sporanaerobacter* (7%) and *Methanobacterium* (6%).

With respect to archaea, results obtained in terms of their relative abundance (3.3 and 4.2%, respectively) supported the low methane productions registered in reactors with adapted sludge (R4 and R5) regardless of the HRT (Table 14). As observed in the previous section (Section 4.2.2.), the hydrogenotrophic pathway dominated the scarce methane production.

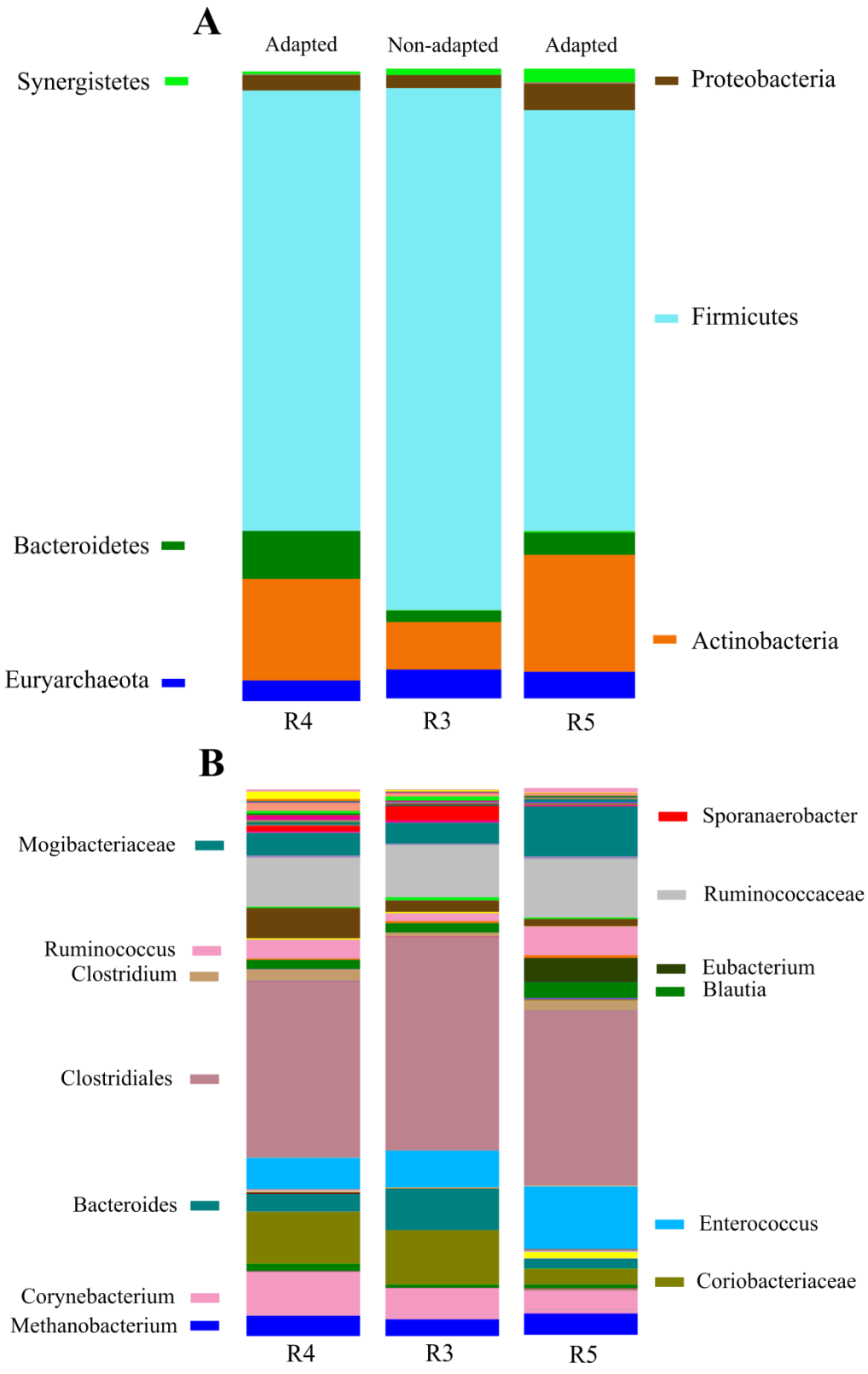

Figure 28. Main phyla (A) and genera (B) found in semicontinuous operation: CSTRs R4 (HRT 8 days), R3 (HRT 10 days) and R5 (HRT 12 days).

In general, adapted sludge exhibited a less diverse microbial community characterized by: i) a high Firmicutes presence and ii) low archaea presence than that obtained in Section 4.2.1. The results obtained in terms of populations dynamics strongly contrast when compared to those aiming at biogas production bringing to the forefront the necessity of studying the microorganisms present in the digester for the better understanding of the process. Likewise, the control of the operational parameters could be used as a tool to select desired microorganism populations to achieve targeted VFAs or the inhibition of the methanogenic step to accumulate VFAs.

4.2.4. Effect of anaerobic inoculum pretreatment evaluated in semi-continuous mode

The promising results obtained in BCPs when the inoculum was subjected to chemical and thermal pretreatments required further confirmation in semicontinuous fermentation mode (CSTR). In the attempt of further increasing the VFAs accumulation, this investigation was designed to elucidate whether the use of inoculum pretreatment could be an advantage for VFAs production. According to the previous results (Sections 4.2.2., 4.2.3. and 4.2.4), fermenters were operated at 25ºC and HRT 8 days. A reactor with protease pretreated biomass and non-pretreated anaerobic sludge coming from the WWTP of Valladolid was set as control. Additionally, anaerobic sludge was pretreated chemically (BES 10 mM) and thermally (120ºC for 10 min and 120ºC for 30 min). Non-adapted anaerobic sludge was selected for pretreatments due to the potential presence of archaea species. In this sense, pretreatments applied to an adapted inoculum would not be effective since archaea species presence is already very low.

AF performance: methane yields

COD removals and methane composition obtained for the digesters using pretreated inocula and the non-pretreated control were low (6-13% COD removal, Table 16) and in the range of those obtained previously in Section 4.2.3. In the same manner, pH values

and NH_4^+/NH_3 concentrations were also similar. Hence, no additional benefits were observed when using chemical or thermal pretreatments for the inoculum. These results suggested that the methanogenic step can be inhibited by imposing appropriate operational conditions rather than using additional chemicals or energy for pretreating the anaerobic sludge.

Table 16. Main process parameters measured in the digesters effluents during reactor operation of the non-pretreated control and the chemical and thermal pretreated inocula.

	Control	BES 10 mM	120ºC-10 min	120ºC-30 min
% CH_4 in biogas (v/v)	20.3±2.6	22.5±3.7	19.8±9.8	15.2±6.3
% COD removal	13.5±3.6	12.3±2.1	7.8±4.0	6.4±3.0
% COD-VFAs/COD_{in}	24.9±2.3	26.8±2.1	27.5±2.8	29.2±3.9
COD-VFAs/$sCOD_{out}$	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
pH	6.3±0.1	6.3±0.1	6.3±0.1	6.3±0.1
NH_4^+ (g/L)	0.7±0.1	0.7±0.1	0.7±0.1	0.6±0.1

AF performance: VFAs conversion yields and profiles

Results showed that the pretreated inocula assessed (chemical or thermal) and the control (non-pretreated anaerobic sludge) ranged 25-30% COD-VFAs/COD_{in} (Figure 29). Therefore, the implementation of pretreatments applied to the inoculum in semi-continuous feeding mode did not enhance VFAs yields with respect to the use of non-pretreated sludge. Conversion yields obtained in semicontinuous mode were substantially lower than those found in batch mode (70% COD-VFAs/COD_{in}, Section 4.1.2). This outcome was supported by other investigation that pointed out that pretreatments applied to the inoculum might have a short-term effect [226]. Additionally, it is important to mention that conversion values retrieved by the non-pretreated inoculum (HRT 8 days 1.5 g COD/Ld and 25ºC) were lower than those obtained in R4 at the same conditions (Section 4.2.3.). This difference might be explained by the different inoculum employed in both investigations. Whereas herein the inoculum employed was an anaerobic sludge coming from a WWTP, in Section 4.2.3. adapted sludge was employed (R4 was operated

using sludge coming from R3 set at HRT 10 days OLR 1.5 g COD/Ld and 25ºC). Thus, imposing HRT 8 days directly in the present experiment was detrimental for acidogenesis. In fact, COD-VFAs/sCOD$_{out}$ in reactors dropped to 0.5±0.1 compared to 0.7±0.1 showed by the previous reactors (Section 4.2.1.-4.2.3.).

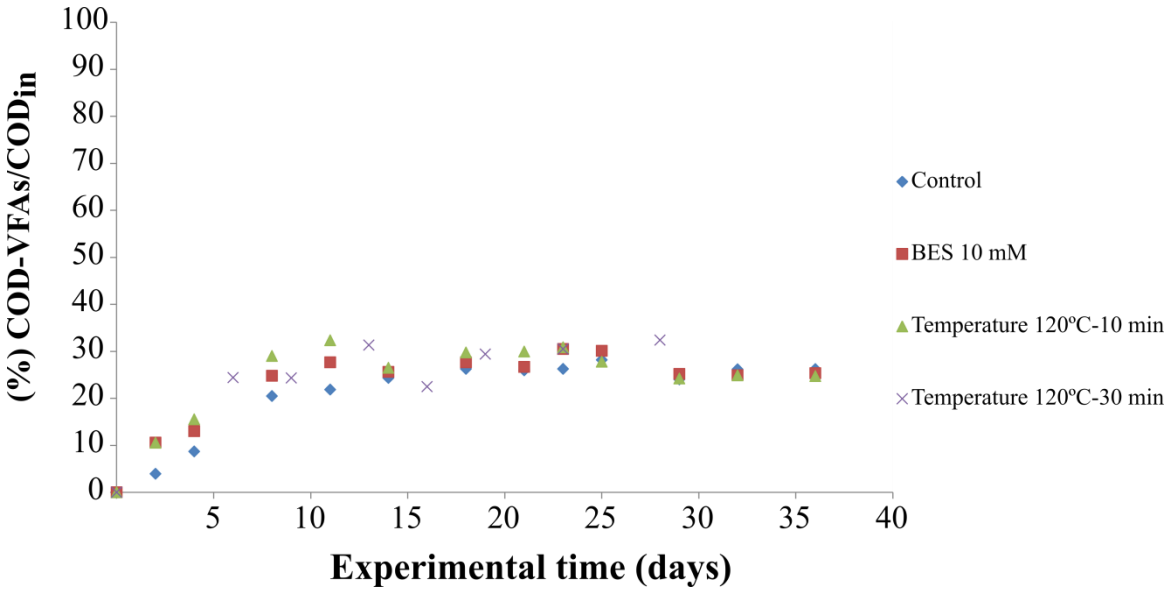

Figure 29. Organic matter conversion into VFAs achieved in semi-continuous mode when the inoculum was subjected to thermal and chemical pretreatments.

With regard to the VFAs profile (Figure 30), results showed that acetic and propionic acids were again the most abundant products after digesters operation. Another study pointed out that thermal pretreatments might reduce the activity of propionic acid producers [56], explaining the lower production in this reactor (120ºC-30 min).

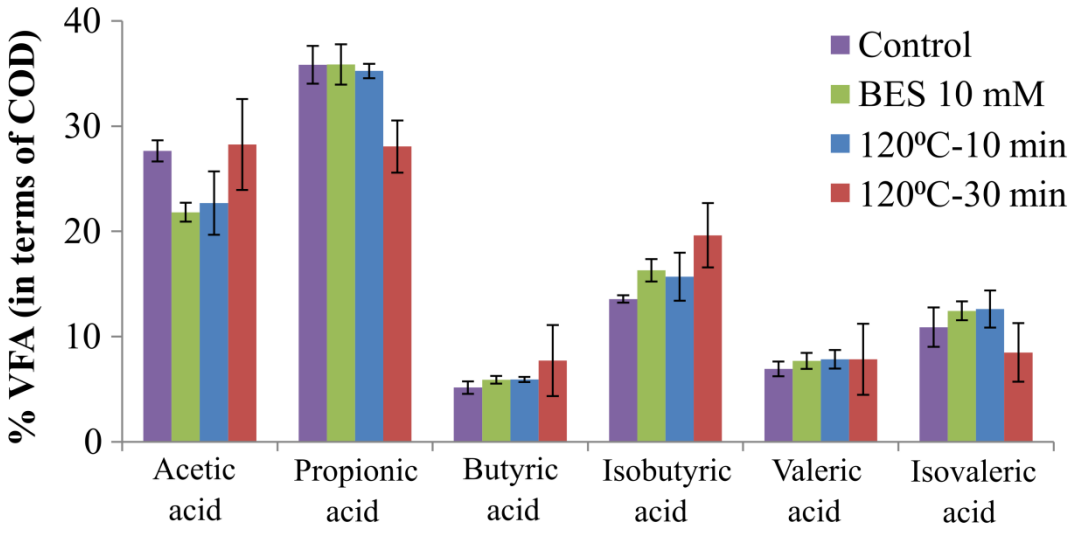

Figure 30. VFAs profile for the non-pretreated anaerobic sludge and the sludge subjected to chemical (BES 10 mM) and thermal pretreatments (120ºC for 10 and 30 min).

Overall, the application of pretreatments to the inoculum did not enhance VFAs yields and thus, it was concluded that some other alternatives such as the manipulation of the operational conditions would be better strategies to enhance VFAs production rather than inoculum pretreatments. In this sense, the high energy input required for thermal pretreatments and the environmental concerns associated with the use of chemical could be dismissed by tuning the operational conditions in fermenters devoted for VFAs accumulation.

Microbial communities

The anaerobic sludge employed in the present investigation showed higher initial diversity (Shannon index 7.571) than the one employed in Section 4.2.1. The rest of samples analyzed at the end of the investigation for the non-pretreated and pretreated anaerobic inocula showed a slight decrease in diversity with regard to the initial anaerobic sludge (Control: 5.471; BES: 5.541; 120ºC-10 min: 4.72 and 120ºC-10 min: 4.71). The chemically pretreated anaerobic sludge did not show differences in terms of diversity with

respect to the control confirming the short term effect of this pretreatment. Anaerobic sludge exposed to thermal pretreatments was less diverse than the control digester.

The anaerobic sludge employed as inoculum was very diverse presenting bacteria phyla belonging to Chloroflexi (16%), Bacteroidetes (15%), Actinobacteria (14%), Proteobacteria (11%) and Firmicutes (9%). This anaerobic sludge presented some differences with respect to the one obtained from the WWTP employed in Section 4.2.1. As a matter of fact, while a similar presence of the Euryarchaeota phylum (3-4%) was determined, the relative abundance of Chloroflexi (11%) and Actinobacteria (7%) was lower in the former one.

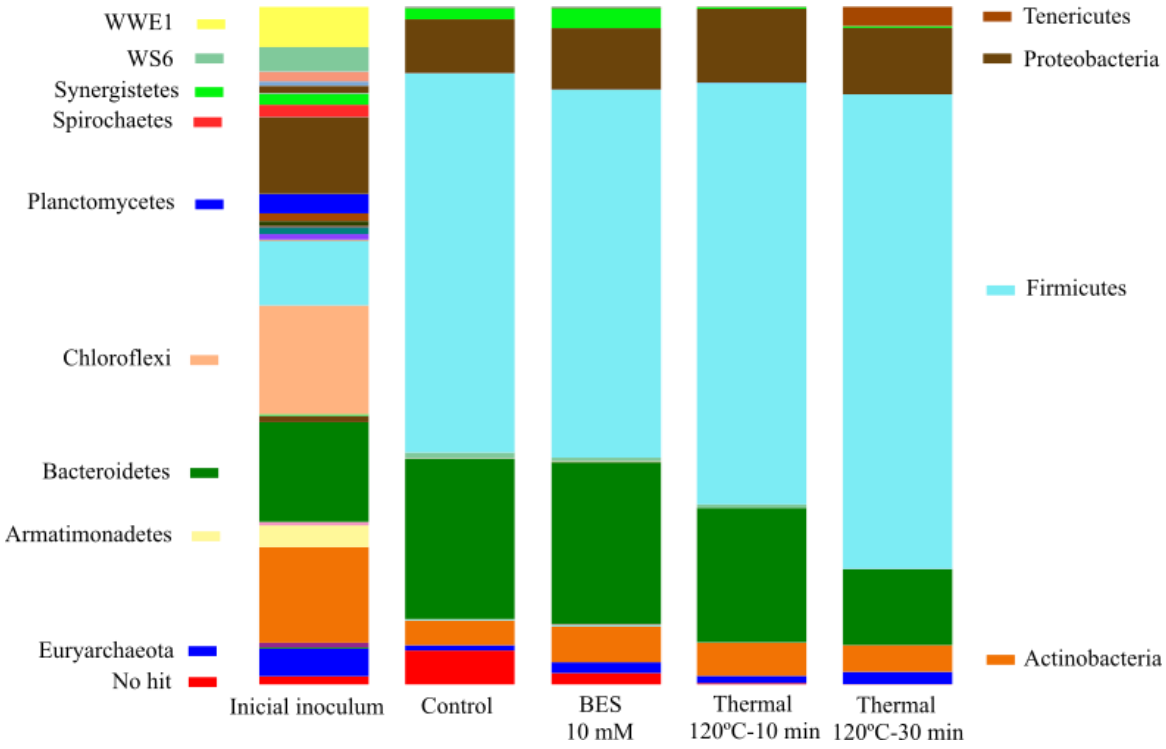

Figure 31. Main phyla found in the control and CSTRs with pretreated anaerobic inoculum: chemical (BES 10 mM) and thermal pretreatments (120ºC-10 min and 120ºC-30 min).

With regard to the non-pretreated anaerobic sludge used as control, Firmicutes phylum stood out as the most abundat phylum (56%), followed by Bacteroidetes (24%) and

Proteobacteria (8%) (Figure 31). The high abundance of Bacteroidetes and Proteobacteria herein attained in this control contrasted with the values obtained in R3 (Section 4.2.2.). The non-pretreated anaerobic sludge was established directly at HRT 8 days whereas HRT in R3 was 10 days. This difference in HRT might be responsible for the change in population observed for Bacteroidetes, Proteobacteria and Actinobacteria. Even though Firmicutes was the most abundant phylum, organic matter conversions into VFAs were slower than those obtained previously in Section 4.2.3. The high relative abundance of Proteobacteria species herein when compared to the former study (Section 4.2.3.), might have contributed to the lower organic matter conversions into VFAs obtained herein (24.9±2.3% COD-VFAs/COD_{in}).

Chemically pretreated sludge and the control digester resulted in a similar bacterial structure. This fact, together with the process parameters analyzed in the effluent of both reactors (Table 16) indicated that BES did not affect process performance nor anerobic microbiome thereby operational conditions influenced process performance in a greater extent than the chemical pretreatment. Thermally pretreated anaerobic sludge showed a concomitant increase of Firmicutes with the exposure time of the pretreatment (62 and 70%, for 120ºC-10 min and 120ºC-30 min, respectively) outcompeting Bacteroidetes abundance (20 and 11%, respectively). However, the shift in microbial structure did not report any changes in VFAs production yields. With respect to the Euryarchaeota community, regardless of the assessed digester, archaea species displayed low abundance values, supporting the low COD removals (Table 16).

As control digester exhibited similar COD removals than reactors with the pretreated inocula, inhibition of methanogenesis in the present study was attributed to the operational conditions applied rather than to the evaluated pretreatments. In this sense, pretreatments were applied Bacterial profile attained in the different reactors exhibited differences in Firmicutes and Bacteroidetes abundance, but organic matter conversions into VFAs remain unaltered. To this point, the low organic matter conversions into VFAs can be explained in two manners: i) microbial structure or ii) imposed operational conditions. According to the microbial systems evaluated until now, it could be stated that not only a high presence of Firmicutes would be of importance, this investigation showed that attention should be also directed to other phyla. Whereas Actinobacteria and Bacteroidetes might have redundancy in functions with respect to Firmicutes (Section

4.2.3.), the presence of Proteobacteria could be detrimental for VFAs production. With respect to operational conditions, digesters were set directly at HRT 8 days, which was very low considering that the sludge was not adapted to these operational conditions. The use of low HRTs in non-adapted anaerobic sludge resulted in archaea inhibition. This fact could have caused as well the low acidogenic efficiency ratio, suggesting that the anaerobic microbiome would require an adaptation step at higher HRTs to achieve good organic matter conversion into VFAs and after that point HRTs could be decreased to lower values.

4.2.5. Organic loading rate effect in semicontinuous mode at psychrophilic conditions

Since the effect of pretreating the anaerobic sludge was negligible when operating the reactors in semicontinuous feeding mode, the alternative to keep on increasing VFAs production yield was to find the most appropriate operational conditions. According to Section 4.2.1, increasing the OLR resulted in similar conversion yields at 35ºC (R1,R2=25% COD-VFAs/COD_{in}), which were below the ones obtained when the fermentation was conducted at 25ºC (R4=38% COD-VFAs/COD_{in}, Section 4.2.3.). Hence, a new fermenter (R6) was set at 25ºC, HRT 8 days and OLR 3 g COD/Ld to study the effect of increasing the OLR using R4 as seed inoculum.

AF performance: methane yield

Results showed similar COD removals for both psychrophilic reactors R4 and R6 (10.4±2.7 and 5.1±2.2, respectively, Table 17). These COD removals agreed with the low values obtained in Section (4.2.3.). With regard to the biogas composition, methane content was 23.8±6.7% (v/v) for R4 and 28.8±4.2% for R6. The similar methane content in the biogas, together with the obtained COD removals, showed that methanogenesis was equally affected regardless of the OLR employed as in mesophilic reactors R1 and R2 (Section 4.2.1.). NH_4^+/NH_3 and pH values remained in the same range in both reactors (R4 and R6) as well as in R3 (Section 4.2.3), thereby the AF performance was very similar regardless of the OLR employed.

Table 17. Main process parameters measured in the effluents during R4 (OLR 1.5 g COD/Ld) and R6 (3 g COD/Ld) operation at 25ºC.

	R4 (HRT 8 days)	R6 (HRT 8 days)
% CH_4 in biogas (v/v)	23.8±6.7	28.8±4.2
% COD removal	10.4±2.7	5.1±2.2
% COD-VFAs/COD_{in}	38.0±1.0	39.0±1.0
COD-VFAs/$sCOD_{out}$	0.8±0.1	0.7±0.1
pH	6.4±0.1	6.3±0.1
NH_4^+ (g/L)	0.7±0.1	1.3±0.1

This investigation demonstrated the possibility of working at higher OLRs whilst maintaining the organic matter conversions into VFAs. For this reason, in order to put a strain on the system performance and assess its robustness, a disturbance was applied. This perturbation consisted on a starvation period of two weeks (Section 4.2.6.) were VFAs conversion yields and profiles, as well as microbial communities, were analysed before and after the starvation period.

4.2.6. Effect of a disturbance in semi-continuous mode: starvation

Controlled perturbation experiments can provide useful information in terms of AF performance and microbial community dynamics. This investigation was designed to cover the gap of knowledge related to the effect that potential disturbances can cause in fermentative processes for VFAs production. With this objective, fermentation performance was evaluated in terms of VFAs yields and bacterial and archaea response after a starvation period of two weeks. The selection for this starvation period was based on the fact that this would be the time to recover an algal based system operating at hydraulic retention time of 4 days (typical value for urban wastewater treatment by means of algae consortium [227,228]). In this manner, this study attempted to simulate a lack of feeding for 14 days due to a crash in the microalgae production system.

AF performance: methane yields

In the previous section (4.2.5.), R6 mediated the highest organic matter conversions into VFAs (39% COD-VFAs/COD_{in}) at 3 g COD/Ld, HRT 8 days and 25ºC. The negligible COD removal attained in scenario 3-Before (±5%, Table 18) indicated that imposed operational conditions inhibited methanogenesis. Main parameters during reactor operation and starvation period are shown in Figure 32.

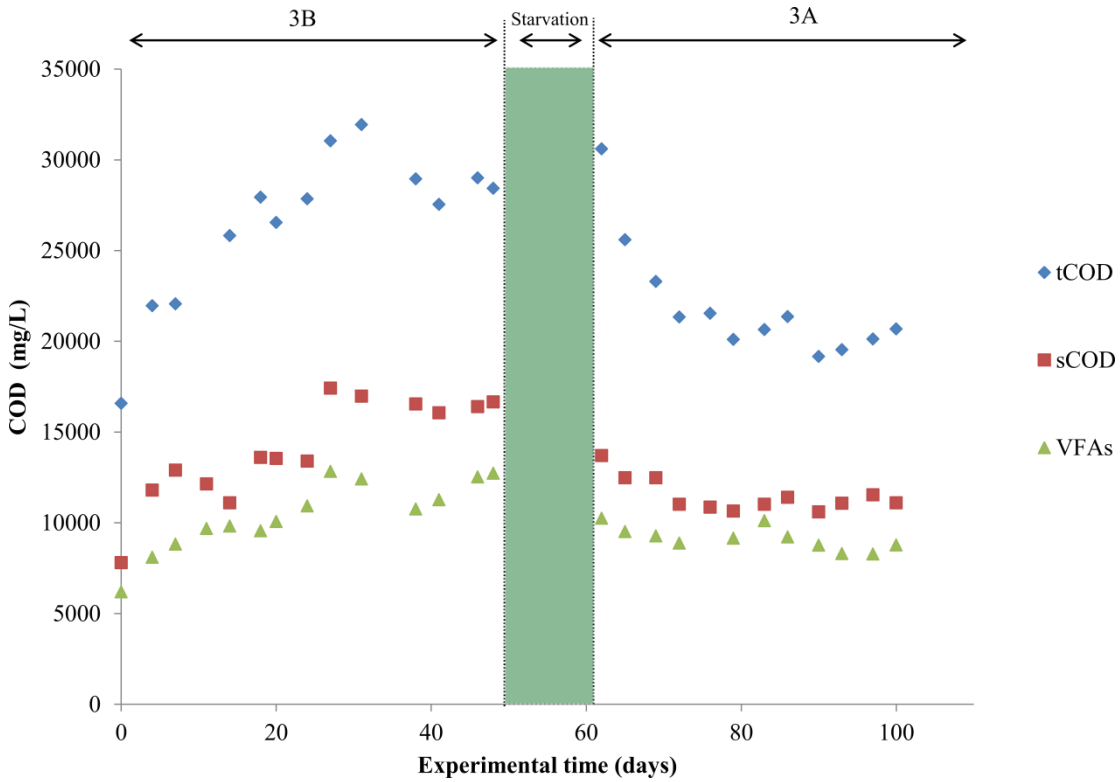

Figure 32. Main operational parameters assessed during reactor operation: tCOD, sCOD and VFAs.

Once stationary state was achieved, the system was subjected to a starvation period of two weeks. Starvation length is quite arbitrary in scientific literature. While some studies employ long-term starvation [229], others evidenced modest changes with just one day of starvation [230]. The effect of the lack of feeding can affect microbial activities [231], impacting ultimately the bioprocess efficiency. In the present investigation, after

the starvation period (Scenario 3-After), the reactor was operated at the same initial conditions (HRT 8 days, OLR 3 g COD/Ld and T=25ºC). Starvation period affected COD removal capacity of the system, which increased to 32.5±2.7% (Table 18).

Table 18. Effluent results of the different parameters assessed before (3-Before) and after starvation (3-After) at 3 g COD/Ld.

	3-Before	3-After
% CH_4 in biogas (v/v)	28.8±4.2	31.7±1.5
% COD removal	5.1±2.2	32.5±2.7
% COD-VFAs/COD_{in}	39.0±1.0	30.1±2.2
COD-VFAs/$sCOD_{out}$	0.7±0.1	0.8±0.1
pH	6.3±0.1	6.1±0.1
NH_4^+ (g/L)	1.3±0.1	0.9±0.1

Total ammonia nitrogen was mainly present in form of NH_4^+ due to the slightly acidic pH (6.3±0.1) and the low process temperature (25ºC), but values were not considered inhibitory for methanogens (1.3±0.1 g NH_4^+/L, Table 18). Despite of the slightly lower acidic pH values observed after starvation, methanogenic species were able to adapt during the starvation period to these conditions as COD removal increased to 32.5±2.7%.

AF performance: VFAs conversion yields and profiles

VFAs conversion yield at steady state (39.0±1.0% COD-VFAs/COD_{in}) was in agreement with previously reported values from microalgae biomass in Section 4.2.4. However, after the starvation period, conversion yields dropped to 30.1±2.2% COD-VFAs/COD_{in}. The different stages of AD were analyzed in order to find which stage was affected by the disturbance. With regard to the hydrolytic stage, the comparison of $sCOD_{out}$/$tCOD_{in}$ of both scenarios revealed a similar ratio (0.58±0.1 in 3-Before and 0.55±0.1 in 3-After). Thereby, hydrolytic differences were discarded. Analysis of the fermentative stages (acidogenesis and acetogenesis) showed a value of 0.8 COD-VFAs/sCODout in scenario 3-After, similar to that obtained in 3-Before, evidencing that VFAs production was not affected by the starvation period. This value was in agreement

with the acidogenic efficiency obtained in previous sections (Section 4.2.3.). However, results in terms of COD removal were quite different and thus, it could be concluded that the starvation period affected the methanogenic stage.

In terms of VFAs profile distribution, butyric acid was the VFA exhibiting the highest percentage before starvation (23±2% of total VFA in terms of COD), followed by acetic acid (20±1%) and the odd chain VFAs (15±1% of propionic and valeric acids). As previously mentioned in Section 4.2.3., butyric and acetic acids were the main fermentation products of R4, thereby the VFAs profile was not affected by the increase in OLR. As it can be seen in Figure 33, this trend was maintained after starvation (scenario 3A). The only remarkable difference was attained for caproic acid that decreased from 10±3 to 4±2% of total VFA as COD. However, the differences before and after starvation were minimal and thus, it can be pointed out that the implemented disturbance did not greatly affect VFAs distribution.

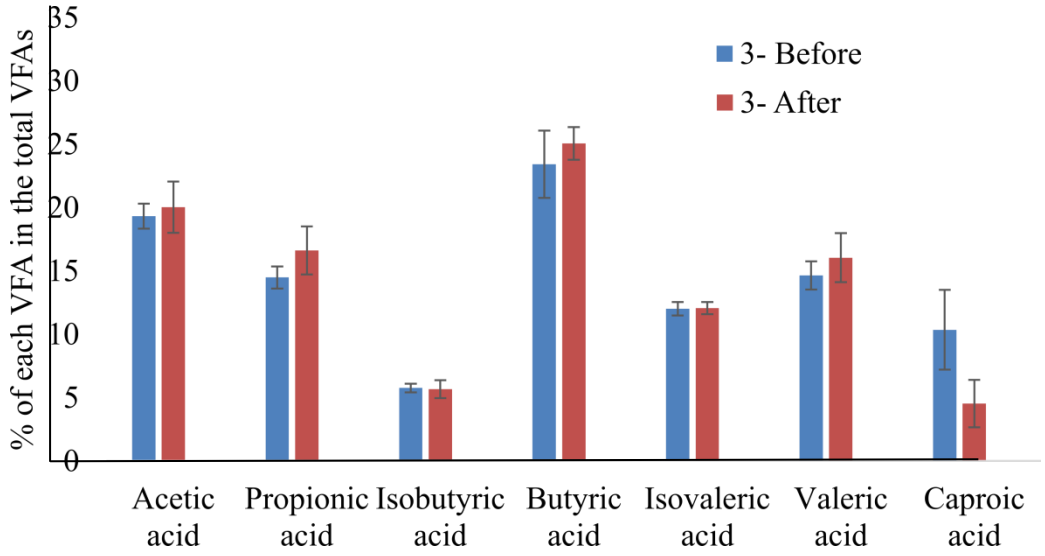

Figure 33. VFAs profiles exhibited at the stationary state of the different scenarios in terms of COD of each VFA out of the total COD-VFAs.

The starvation period did not affect the fermentative stages (hydrolytic and acidogenic), but it had an influence in the methanogenic stage (COD removal increased). The lack of feeding most probably contributed to the development of the archaea community because no effluent was extracted from the acidogenic reactor in 14 days. A

similar study assessing a starvation stage concluded that this period favored hydrogenotrophic methanogens [232]. Overall, the starvation period was shown to be detrimental for VFAs accumulation since this period resulted to be crucial for archaea recovery and thus, VFAs were consumed for methane production purposes.

Microbial communities

In order to link digestion results with the existing microbiome, microbial populations were analyzed before and after the starvation period (3-Before and 3-After). As indicated in Section 4.2.3., Shannon index for R4 employed as inoculum was 3.7. The increase of OLR to 3 g COD/Ld resulted in similar Shannon index values (3.8). This indicated that the increase in OLR did not affect the microbiome in terms of diversity. After the starvation period of two weeks, the Shannon index decreased (3.4) most probably due to the fact that some species decayed because of the lack of feeding. Once the reactor was fed again, diversity increased to 4.1. These latter values were very similar to the ones exhibited by the reactor before the starvation period.

The bacterial community in R4 before starvation consisted of Firmicutes (68%) as the major phylum, followed by Bacteroidetes (18%) and Actinobacteria (10%). Samples taken immediately after starvation, before restarting the feeding, showed no differences in terms of Firmicutes, while in the case of Bacteroidetes, the population drastically decreased to 0.5% (Figure 34). In addition, even though the relative abundance of Firmicutes was not affected, a decrease in Bacilli and an increase in Tissierellia class were observed (Figure 35 A-B). Reactor operation after the starvation period returned Tissierellia and Bacilli values to those showed initially and gave rise to a sensitive increase in Firmicutes.

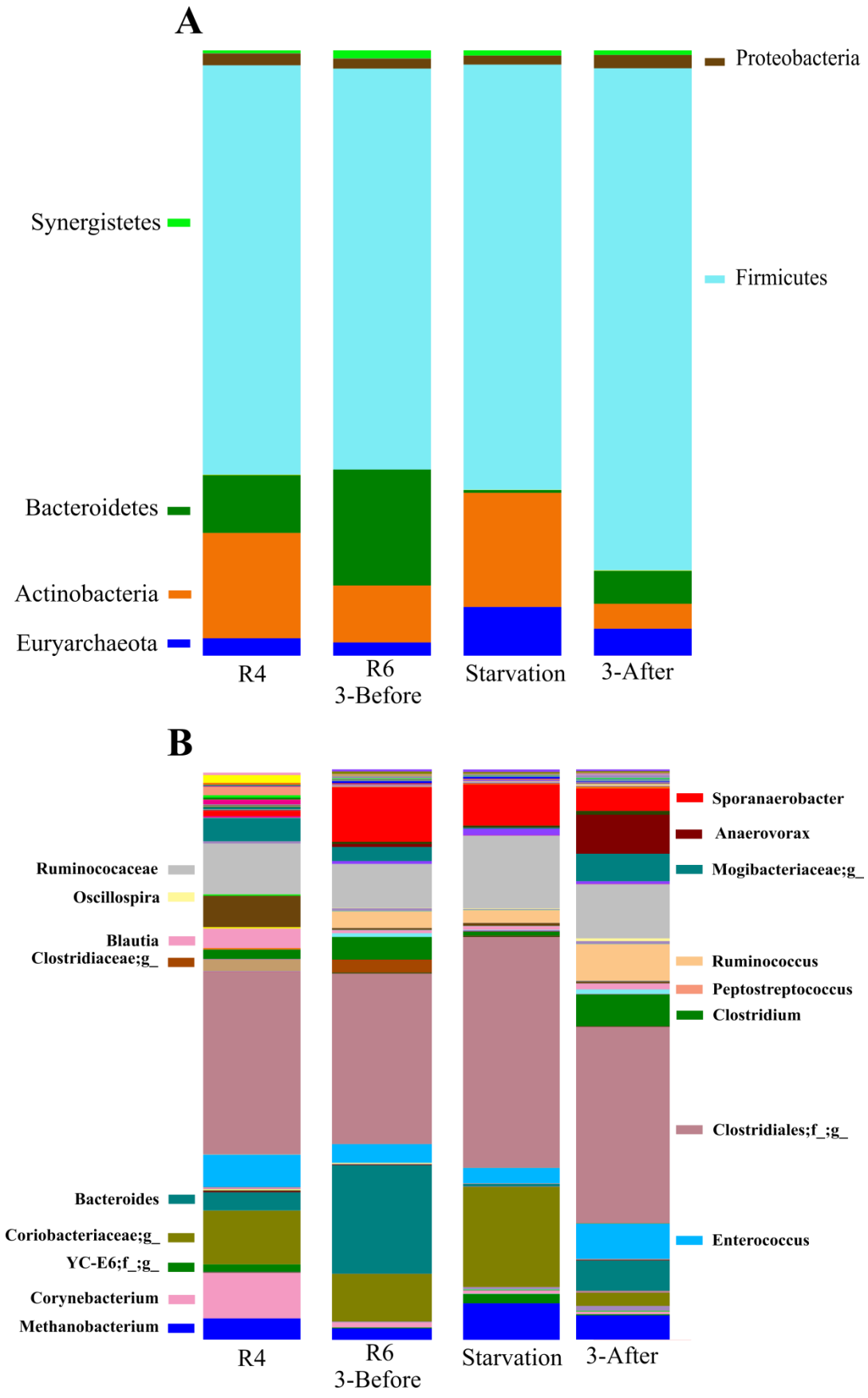

Figure 34. Phylum (A) and genera (B) distribution in the different scenarios: R4, R6 (3-Before starvation); After the starvation period of two weeks and 3-After.

In general terms, the predominance of Firmicutes agreed with the previous sections of the present investigation. This phylum prevails in environments devoted for VFAs production [90,233,234]. However, as seen in Section 4.2.4., with pretreated inocula in semicontinuous mode, Firmicutes presence do not necessarily mean competitive organic matter conversions into VFAs. In the present case, Firmicutes was followed by Bacteroidetes and Actinobacteria, which seem to contribute to VFAs accumulation.

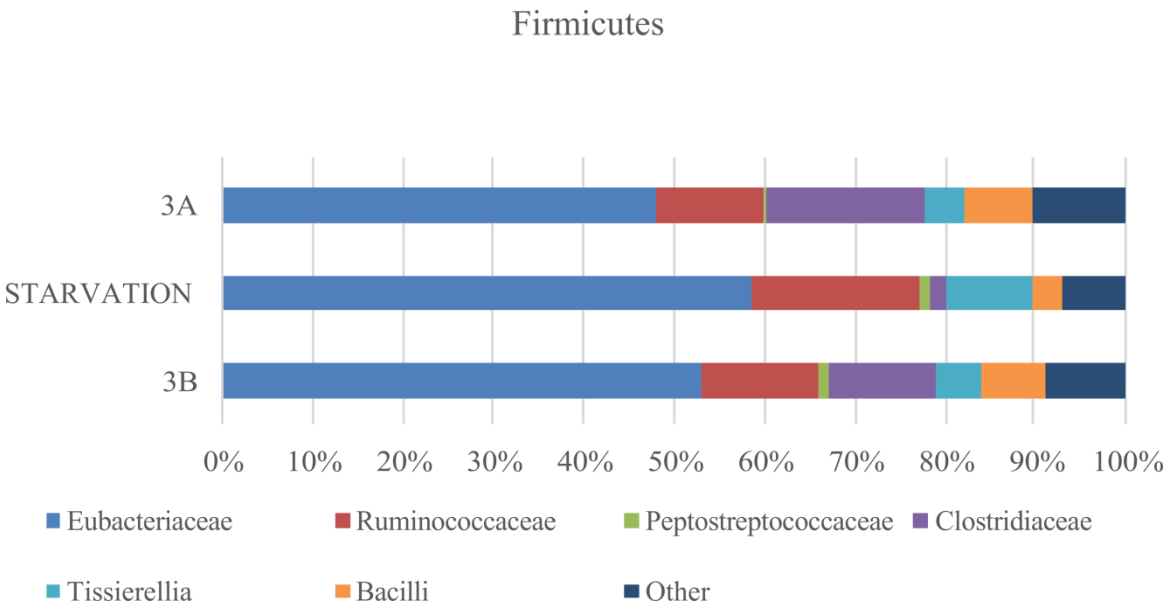

Figure 35. Distribution of Firmicutes phylum in the different scenarios: 3B (3-Before starvation); After the starvation period of two weeks; 3A (3-After).

More importantly, the percentage of Euryarchaeota community (archaea) displayed a significant increase during starvation confirming the recovery of this community (Figure 34A) and agreeing with the values attained in terms of COD removal. Note worth to mention that the main strain determined among this population was the hydrogenotrophic *Methanobacterium* [235].

In this context, there are two major methanogenic pathways as mentioned in Section 1.2.: a) acetoclastic pathway and b) hydrogenotrophic pathway. Additionally, syntrophic acetate oxidizing bacteria (SAOB) might occur. These species oxidize acetate and produce H_2 and CO_2 or formate. This H_2 generated might be used as well for hydrogenotrophic methanogenesis. Acetoclastic pathway is mediated by families related

with Methanosarcinaceae spp. and Methanosaetaceae spp., while species belonging to order Methanomicrobiales spp., Methanobacteriales spp. (such as *Methanobacterium*), and Methanococcales spp., are responsible for the hydrogenotrophic pathway [236]. It should be highlighted that this latter methanogenic route is preferred over the acetoclastic pathway when difficult methanogenesis environments are imposed, as seen in Section 4.2.3. As a matter of fact, the acetoclastic archaea are more sensitive than hydrogenotrophic species [111]. For instance, digesters operating at high NH_4^+ or VFAs concentrations, which can be potentially toxic, have shown hydrogenotrophic pathway preference for methanogenesis [237,238]. These adverse conditions for methanogenesis were also evidenced in scenario 3-Before while immediately after the starvation period, methanogens activity resumed as it could be seen by archaea population increase after starvation in Figure 34.

This feature is in agreement with Kim and co-workers who pointed out that under starvation conditions methanogens are able to enter a quiescent state until favorable conditions for growth are attained again [239]. The lower conversion yield in terms of COD-VFAs/COD_{in} attributed to the consumption of VFAs was also related to the presence of syntrophic acetate oxidizing bacteria (SAOB). SAOB are normally working together with their hydrogenotrophic counterpartners to keep an optimum hydrogen trade off in the anaerobic system. Acetate oxidation only proceeds when the hydrogen level is kept low by hydrogenotrophic methanogens consumption [240]. SAOB are affiliated with Firmicutes phylum, more particularly to Clostridia class (*Thermacetogenium phaeum*, *Tepidanaerobacter acetatoxydans* or *Syntrophaceticus schinkii*), Tissierellia class (*Clostridium ultunense*) and Thermotogae phylum (*Pseudothermotoga lettingae*) [241,242]. However, other members of Firmicutes have been attributed to perform SAO activities. In fact, species belonging to Clostridia class have been previously related with the SAO pathway [243]. In this sense, the highest COD removals and lowest COD-VFAs/COD_{in} conversions were attained after starvation (3-After), which showed the highest Clostridia population (72%). Moreover, the methanogens recovery during starvation might also be linked to the lower NH_4^+ concentration of the digestates after starvation (0.89 ± 0.02 g NH_4^+/L, Table 18). Indeed, the nitrogen mineralization percentage was not recovered since NH_4^+ levels in the effluents after starvation did not reach the same concentration as in scenario 3-Before. This could be explained by the

different fate of carbon and nitrogen during AD [244]. In this case, it seems likely that nitrogen mineralization did not recover its initial efficiency after the starvation period.

Overall, the starvation period was detrimental for organic matter conversion into VFAs. The lack of feeding allowed methanogenic species to adapt and promote methanogenesis. Additionally, SAO coupled with hydrogenotrophic methanogenesis might have taken place during that period.

4.2.7. Effect of stepwise OLR increases

Conventional AD processes used for maximizing methane production must have a balanced HRT and OLR, since these are key parameters in process optimization [245]. Low and high OLR values can drive the process either to starvation or to incomplete organic matter degradation due to inhibition by overloading. Since methanogenic inhibition is desired for VFAs production, the selection of low HRT and high OLR values were considered appropriate for such a goal. As pointed out in Section 4.2.6., OLR disturbance affected process yields. The present study aimed at recovering and maximizing VFAs production yields by stepwise OLR increases. The process was evaluated from the Scenario after starvation. Afterwards reactor was fed at 3 g COD/Ld (as showed in Section 4.2.6., Scenario I), 6 (Scenario II), 9 (Scenario III), 12 (Scenario IV) and 15 g COD/Ld (Scenario V).

AF performance: organic matter removal

Stepwise OLR increases resulted in concomitantly decreasing COD removals (Figure 36). Methanogenic instability was evidenced until OLR of 12 g COD/Ld (Table 19). However, when the system was operated at OLR 15 g COD/Ld (Sc. V), the COD removal percentage seemed to increase slightly when compared to Sc. IV (14.1±2.7% against < 5%). Nevertheless, the recorded values for total COD removal were too low within the carbon balance. As a matter of fact, fermentation of organic compounds by acidogenic bacteria and methanogenic archaea is also devoted for the growth of new cells (0.15 kg

VSS/kg COD for acidogenic bacteria and 0.03 kg VSS/kg COD in the case of methane producers) [246]. Overall, the COD removal from Sc. II onwards was considered too low in the carbon flow directed to biogas and thus, removal percentages were rather attributed to anaerobic microorganism's growth.

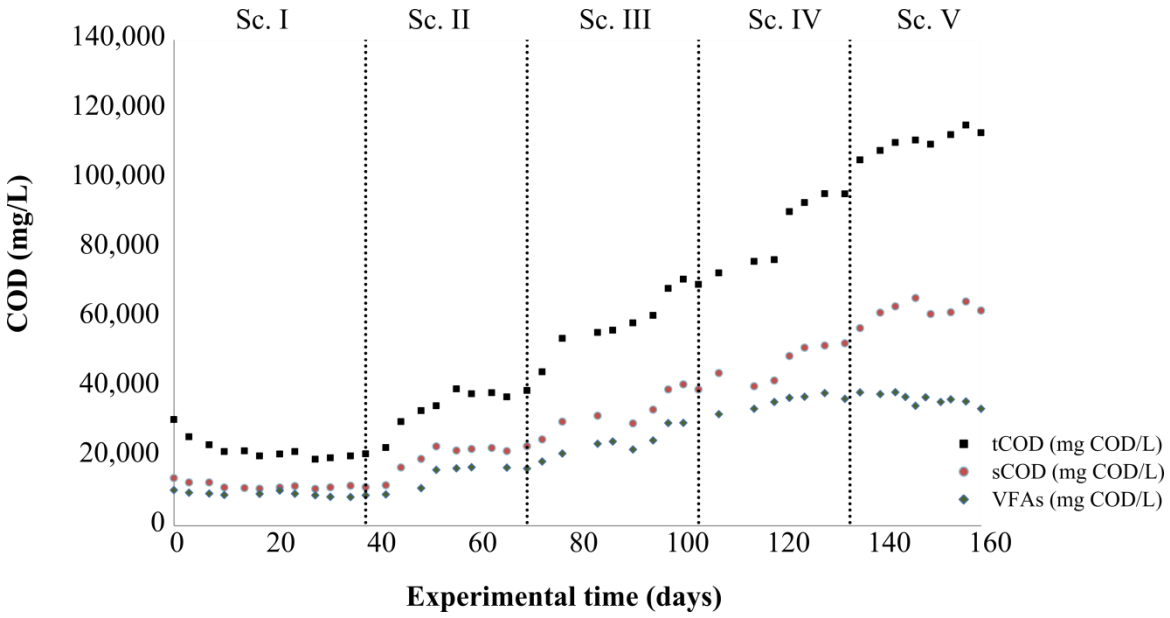

Figure 36. Time course of tCOD, sCOD and VFAs along the different scenarios I-V corresponding to the stepwise OLR at values 3, 6, 9, 12 and 15 g COD/Ld.

Table 19. Average values achieved during the CSTR operation at the different stepwise OLR increases.

	OLR (g COD/Ld)	**tCOD (g/L)**	**% COD removal**	**sCOD (g/L)**	**TS (g/L)**	**VS (g/L)**	**pH**	**NH_4^+ (g/L)**	**VFAs (g COD/L)**	**COD-VFAs/COD_{in}**
Sc. I	3	21.9±3.2	29.3±6.1	11.4±0.9	8.3±0.5	6.3±0.5	6.3±0.3	0.9±0.1	9.1±0.6	0.30±0.02
Sc. II	6	38.3±0.8	20.1±1.9	20.1±3.7	14.2±1.4	10.2±0.4	6.3±0.1	1.4±0.2	16.5±3.2	0.34±0.01
Sc. III	9	69.6±1.3	3.3±1.8	29.9±3.2	24.9±0.5	19.6±0.5	6.3±0.1	2.4±0.3	28.0±2.3	0.39±0.04
Sc. IV	12	91.1±6.2	2.2±3.1	47.2±5.1	33.1±2.5	27±2.0	6.5±0.1	3.8±0.2	36.8±2.1	0.37±0.02
Sc. V	15	109.9±3.4	14.1±2.7	62.2±2.9	45.4±0.1	35.5±0.6	6.5±0.1	4.4±0.1	36.4±1.5	0.29±0.01

NH_4^+ concentrations showed a growing trend up to 4,410 mg NH_4^+/L at 15 g COD/Ld (Table 19). The last scenarios (Sc. III, IV and V 2.4±0.3, 3.8±0.2 and 4.4±0.1 g/L NH_4^+, respectively, Figure 37) resulted in NH_4^+ values above the threshold indicated in literature (above 1.5 g/L of total ammonia nitrogen) to provoke methanogenesis inhibition [247,248]. With regard to free ammonia (NH_3), the concentrations attained during the experiment were very low as indicated in the previous sections due to the low process temperature (25ºC) and pH values (6.4±0.1) (Table 19). According to literature, inhibition due to this compound occurs at 80 mg/L NH_3 [41]. This value was far above from the ones attained in the present study (below 10 mg/L NH_3). Thus, this compound was presumably not the responsible for inhibiting methanogenic archaea but it cannot be neglected that total ammonia (NH_4^+ and NH_3) were in the inhibition level for methanogenic archaea. pH remained stable along the experimental time. In this sense, the pretreated microalgae fed at pH 8 might have buffered the system, avoiding the pH drop associated normally to high VFAs concentration.

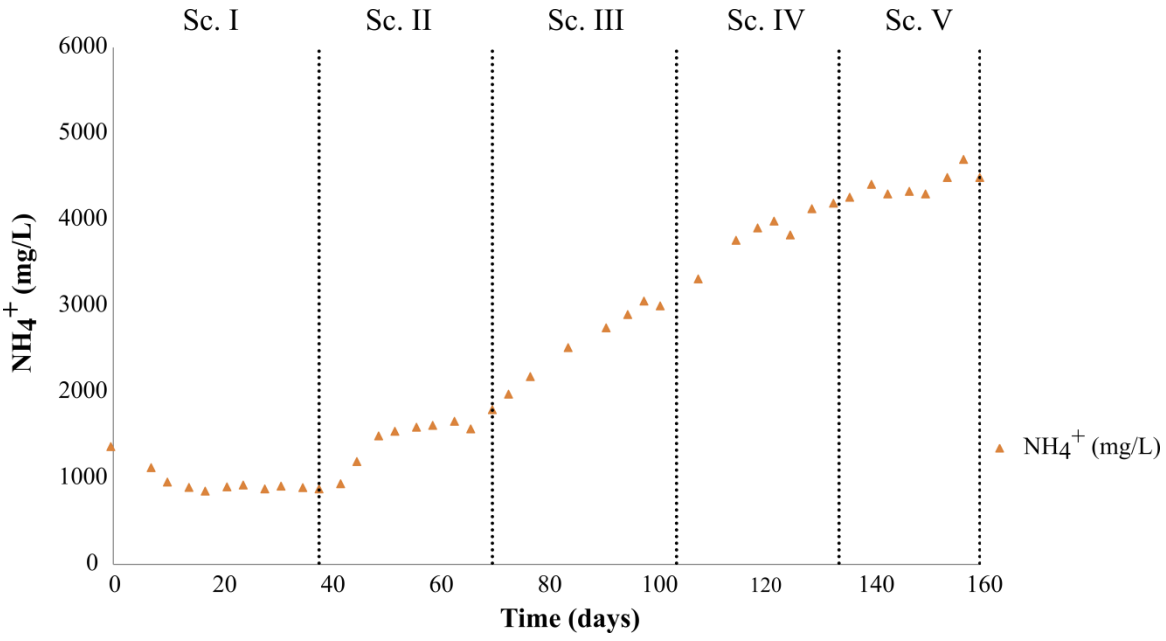

Figure 37. Time course of the NH_4^+ concentrations along the experimental time.

VFAs production: concentration, yields and profiles

The efficiency of the different scenarios was assessed by calculating the organic matter conversion yields into VFAs (COD-VFAs/COD_{in}). Sc. I exhibited the lowest value (0.30±0.02, after the starvation period) concomitantly with the highest % COD removal (Table 19). From that point onwards, the system increased organic matter conversion into VFAs in the following scenarios (Sc. II, 0.34±0.01; Sc. III 0.39±0.04; Sc. IV 0.37±0.02 COD-VFAs/COD_{in}) until Sc. V, in which the conversion dropped (0.29±0.01 COD-VFAs/COD_{in}).

An increase in VFAs production (mg COD-VFAs/L) was noticed throughout the experimental time at increasing OLR values from Sc. I-V (Figure 38). However, the last scenario fed at 15 g COD/Ld resulted in a decrease in VFAs concentration. Similar experiments available in literature conclude on the existence of an optimum OLR value from which VFAs production does not increase. These studies attribute this point of inflection to the hydrolytic capacity of the system. When this point is exceeded, the first step of the AD becomes limiting. For instance, AD of olive mill solid residue was carried out under different OLR values from 3.2 to 15.1 g COD/Ld equivalent to HRT from 50 to 10.7 days at continuous feeding mode [84]. Those researchers pointed out that the optimum value was 12.9 g COD/Ld (HRT 12.4 days) resulting in VFAs production of 15-20 g COD-VFAs/L whilst 15.1 g COD/Ld did not report higher VFAs productions yields. The inhibition of the process was characterized by a strong decrease of the most abundant product acetic acid. VFAs productions were as well monitored in a similar study at OLR 5; 6.6; 10 and 13.3 g COD/Ld and decreasing HRT values 4; 3; 2; 1.5 days at mesophilic conditions (37ºC) in a process devoted for biohydrogen production from a waste stream of palm oil [249]. Results showed a maximum VFAs production of 1.5 g VFAs/L at high OLR values and low HRT (10 g COD/Ld and 2 days). Final VFAs productions concentrations in those studies were below the ones attained herein, probably due to the use of substrates with different macromolecular composition and operational conditions. Both former studies attributed the drop in VFAs production to a deficient hydrolytic step. At this point, and as mentioned in Section 3.2., the present study subjected microalgae biomass to a proteolytic pretreatment to avoid any hydrolysis limitation with the focus put on the acidogenesis stage of AD. In fact, the ratio $sCOD_{out}/tCOD_{out}$ in the effluent of the

different scenarios showed quite stable values ranging 0.52-0.58. This fact suggested that the hydrolytic step was not a bottleneck for VFAs production along the increasing OLRs applied since similar ratios were attained (Table 19).

Analysis of the acidogenic stage (COD-VFAs/$sCOD_{out}$) resulted in ratios in the range of 0.8-0.9 from Scenarios I-IV. However, in the last stage (Sc. V) this ratio dropped to 0.6. Thus, it was inferred that an inhibition of the acidogenic step took place at the highest OLR assessed. In this sense, the acidogenic inhibition step has been previously studied and different compounds were pointed out as responsible for the acidogenic inhibition. K^+, Na^+, clorophenols and heavy metals (Cu>Zn>Cr>Cd>Ni>Pb) are toxic for acidogenesis [250]. Out of these compounds, sodium may have affected acidogenic activity in the present study, as NaOH was used to control pH during the enzymatic pretreatment of the microalgal biomass. The analysis revealed increasing Na^+ concentrations from Scenario I to V. This concentration concomitantly increased from 1.0 g/L determined in Scenario I, 1.8 g/L, 2.8 g/L, 3.7 g/L and 4.9 g/L Na^+ in Scenario V. This compound affects the specific growth rate of microorganisms because it plays a role in the formation of adenosine triphosphate and NADH oxidation. Although it is beneficial at minor concentrations (<1 g/L Na^+), higher amounts might alter anaerobic species growth [250]. Since AD has been devoted traditionally for biogas production, the influence of sodium in methanogens has been more studied [251,252]. In this sense, moderate methanogenic inhibition at Na^+ values ranging 3.5-5.5 g/L has been pointed out [191]. However, hydrolytic, acidogenic and acetogenic species are known to be more sensitive to Na^+ [253]. Hence, taking into account the acidogenic sensitivity aforementioned, it could be inferred that Na^+ affected process yields in terms of VFAs production.

Likewise, high NH_4^+ concentrations have been also found to affect the acidogenic step. As a matter of fact, the high NH_4^+ concentrations attained at the highest OLR (4.4 g/L) were above the level (3.1 g/L) identified for acidogenic bacteria inhibition [254]. Finally, high VFAs concentrations have been studied as well as possible inhibitors of the acidogenesis. Investigations found a slight inhibitory effect at 4 g VFAs/L during the fermentation of glucose [255]. Since these values are far below the VFAs productions

obtained in the present study, high VFAs concentrations determined herein could have also hampered the acidogenic stage in the last scenario.

VFAs profiles were assessed to evaluate the influence of increasing OLR values (Figure 38). Similarly to what was described in previous sections (Section 4.2.3.), butyric acid was the most abundant product obtained in the digesters. Butyric acid accounted for 11.7±0.8 g COD/L at 15 g COD/Ld, which corresponded to 32.2% of total VFAs production. This VFA registered an increasing trend from Sc. I to Sc V (25.8% to 32.2% out of the total VFAs in terms of COD). Accumulation of this acid can occur due to different reasons. For instance, it is regarded as a signal of higher hydrogen partial pressure than when the process is devoted to biogas production. In this sense, when hydrogen-utilising methanogens are exposed to hydrogen partial pressures above 10^{-4} atm, VFAs such as butyric acid accumulate in the system [256]. Another possible reason might be related with the slightly acidic pH observed in the digesters, which is often related to higher butyric acid biosynthesis [257]. The second most abundant product in each stage was acetic acid (26% in Sc. V out of total VFAs production vs 19-20% in the rest of the stages). This fact might be explained by the degradation of the longest VFAs (such as isovaleric valeric and caproic acids) into butyric and acetic acids (from 8.6%, 15.4%, and 8.4% isovaleric, valeric and caproic acids, respectively in Sc. IV to 7.5%, 10.4% and 6.9% in Sc. V). As mentioned in Section 1.2. the underlying abundance or shortage of a concrete VFA is due not only to the substrate employed, but also to the operational conditions and substrates employed in the system [73,258]. In the present study, high OLRs and low HRTs promoted butyric acid accumulation over the rest of the VFAs spectrum.

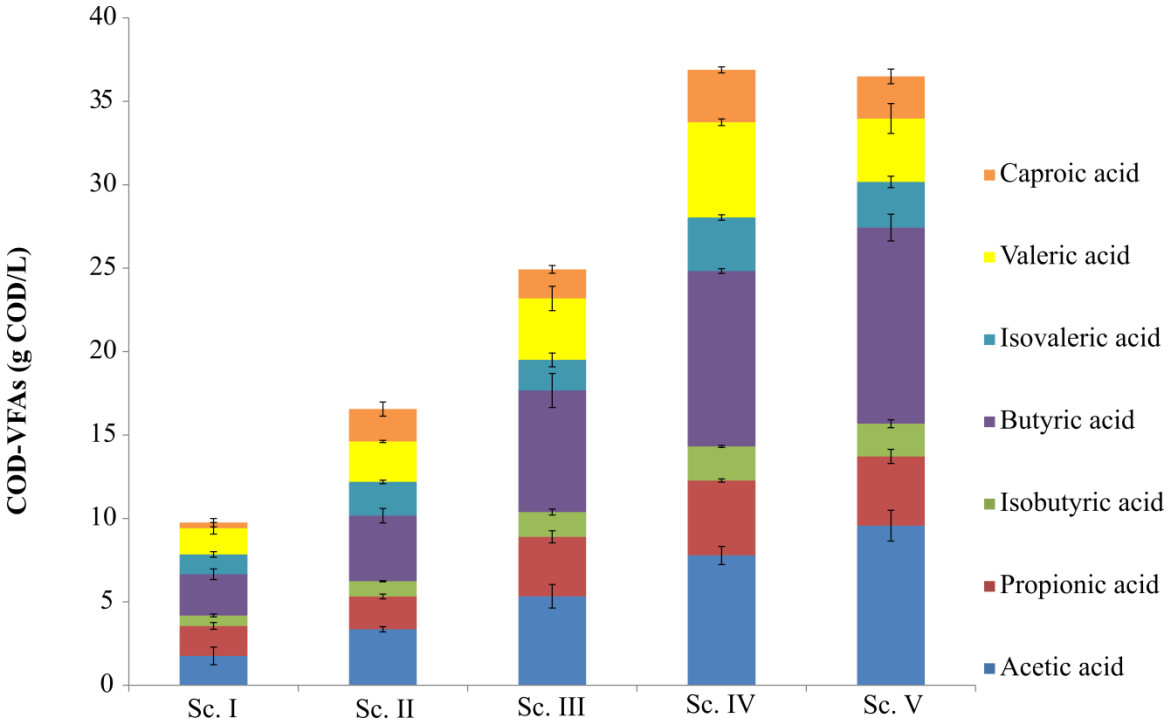

Figure 38. VFAs production along the stepwise OLR increases for the different scenarios (Sc. I: 3 g COD/Ld; Sc. II: 6 g COD/Ld; Sc. III: 9 g COD/Ld; Sc. IV: 12 g COD/Ld; Sc. V: 15 g COD/Ld).

Overall, the lower organic matter conversion efficiency into VFA determined at the highest OLR tested (15 g COD/Ld) was attributed to a drop in acidogenic stage efficiency. Combination of NH_4^+, VFAs and sodium concentrations might have damaged this step. In addition, this decrease in organic matter conversion into VFAs agreed with the higher COD removal registered in Sc. V (Table 19).

Microbial communities

Microbial community analysis

Since promoting specific acidogenic bacteria population is a key factor for maximizing VFA production, microbial communities were analysed during the steady-state of each scenario in order to evaluate the effect of increasing OLR on the relative abundance of the dominant microorganisms. In fact, there was a clear microbial trend along the experimental scenarios in terms of diversity, statistics and microbial distribution analyses. As it can be seen in Table 20, Shannon index reflected a slight diversity increase from the period after starvation (3.357) to Sc. I (4.110) fed at 3 g COD/Ld. During this first scenario, operation of the reactor likely promoted the growth of microorganisms. Likewise, once OLR was increased in the following scenarios (II and III), an increase in Shannon index was detected (4.417 and 4.469, respectively) suggesting an adaptation of the anaerobic biomass present in the reactor to the conditions imposed in the system. However, the subsequent OLR increase in Sc. IV and Sc. V resulted in lower diversity than the previous scenarios (3.870 and 3.802, respectively, Table 20.

Table 20. OTUs and Shannon/Simpson indexes calculated for the samples in each scenario.

Scenario	Shannon
Inoculum	3.357
I	4.110
II	4.417
III	4.469
IV	3.870
V	3.802

It should be taken into account that diversity is not only represented by richness but also by evenness and thus, the higher the microorganisms detected as well as their homogeneity (in terms of relative abundance), the higher the diversity in the system [259]. The influence of OLR was displayed in the PCoA statistical analysis (Figure 39A), which reflected that microbial samples were clustered distinctly according to the different OLR ranges: (i) inoculum and Sc. I, (ii) Sc. II-III and (iii) Sc. IV-V. Thus, physico-chemical parameters values changed (NH_4^+/NH_3, VFAs) due to the progressive OLR

increase, definitely affecting microbial populations. As it can be seen in PCA analysis, the VFA concentration registered at the highest OLR was mainly related to the high NH_4^+ concentrations released to the medium (Figure 39B). Both, VFAs and NH_4^+, are compounds that might be toxic for the microbiome, explaining the specialization at increasing OLRs [250,254]. An ANOSIM (Figure 40) test confirmed the strong dissimilarity between the clusters detected through PCoA, as well as a high similarity between the scenarios that constituted each cluster. In addition, microorganism's population changed throughout the different scenarios with a concomitant increase in organic matter conversion into VFAs. However, population changes was not reflected in VFA profiles obtained, which remained stable throughout the different scenarios (Figure 38).

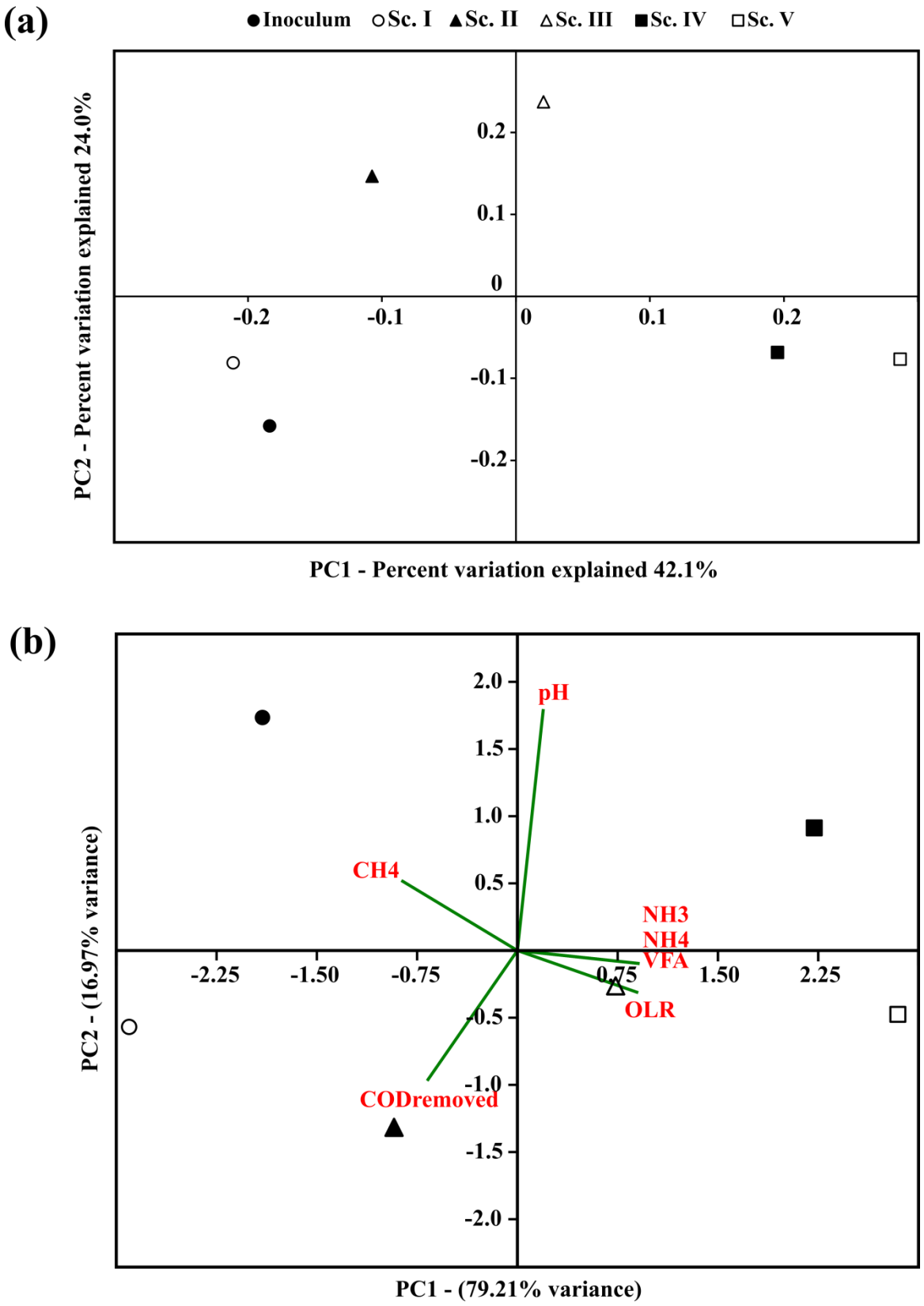

Figure 39. Principal coordinate analysis (PCoA) (A) and principal components analysis (PCA) (B).

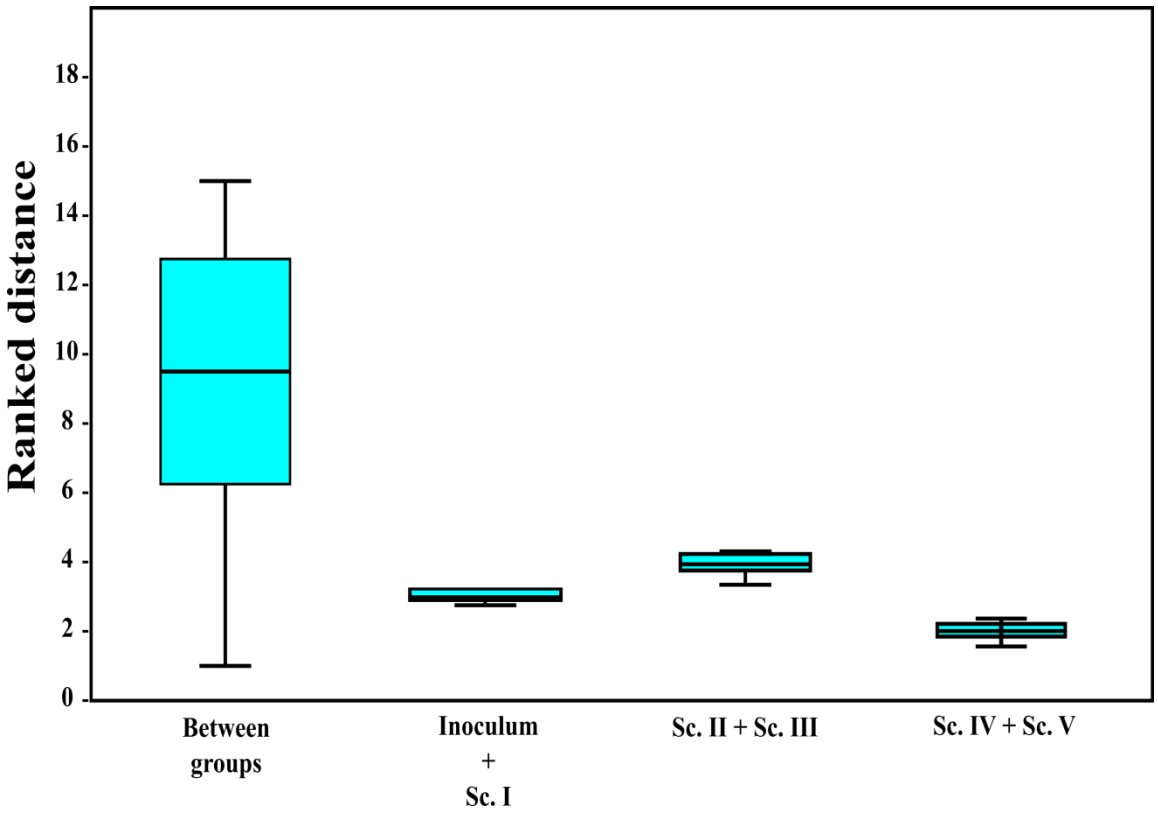

Figure 40. ANOSIM test carried out for the different scenarios assessed.

Microbial community composition

The 16S rRNA gene analysis revealed that Firmicutes, Bacteroidetes and Actinobacteria were the most abundant phyla in the whole experimental period, further followed by Proteobacteria, Synergistetes and Euryarchaeota (Figure 41). As mentioned in Section 4.2.6., after starvation, the sludge was mainly composed by Firmicutes phylum (68%), Actinobacteria (18%) and Euryarchaeota (8%). The high presence of bacteria belonging to Firmicutes phylum can be explained by the anaerobic sludge origin, which was an acidogenic anaerobic reactor (R4, Section 4.2.3.). The community structure in the sludge was composed by microorganisms exhibiting hydrolytic and acidogenic activities [260].

At phylum level, the progressive OLR increase influenced the microbial population dynamics. Sc. II and Sc. III (6 and 9 g COD/Ld) were characterized by the progressive disappearance of Actinobacteria and Euryarchaeota and the slight increase of

Bacteroidetes (up to 20.7% in Sc. III, Figure 41). At this point, it is important to highlight that Sc. III coincided with the highest organic matter conversions into VFAs (COD-VFAs/COD_{in}) obtained and acidification ratio (COD-VFAs/$sCOD_{out}$) (Table 19). Thus, the balance established between Bacteroidetes and Firmicutes relative abundance as well as the reduction of the methanogenic activity (Euryarchaeota phylum) might play a key role in maximizing VFAs production. DNA analysis from Sc. IV, and especially Sc. V, showed a gradual increase of Firmicutes and Euryarchaeota together with the disappearance of Bacteroidetes (Figure 41). These factors likely caused the drop of organic matter into VFAs conversion registered at the end of the experimental time (Sc. V, Table 19). The dominance of Bacteroidetes and Firmicutes in acidogenic fermentation from grass biomass acidification was previously reported at 37ºC and 55ºC, respectively [261]. This latter study and the one carried out by Greses and co-workers showed the low presence of Bacteroidetes in a process devoted for biogas production [104]. Moreover, the relative abundances (%) in those studies between Bacteroidetes and Euryarchaeota were very different to those reported in the present investigation where Bacteroidetes stood out when methanogenic species were suppressed. This combination resulted in high VFAs productions. At this point, it could be stated that the existing differences in terms of microbial population between an anaerobic community devoted to biogas production and the acidogenic inoculum presented herein indicated that the adapted inoculum chosen was appropriate for VFAs maximization.

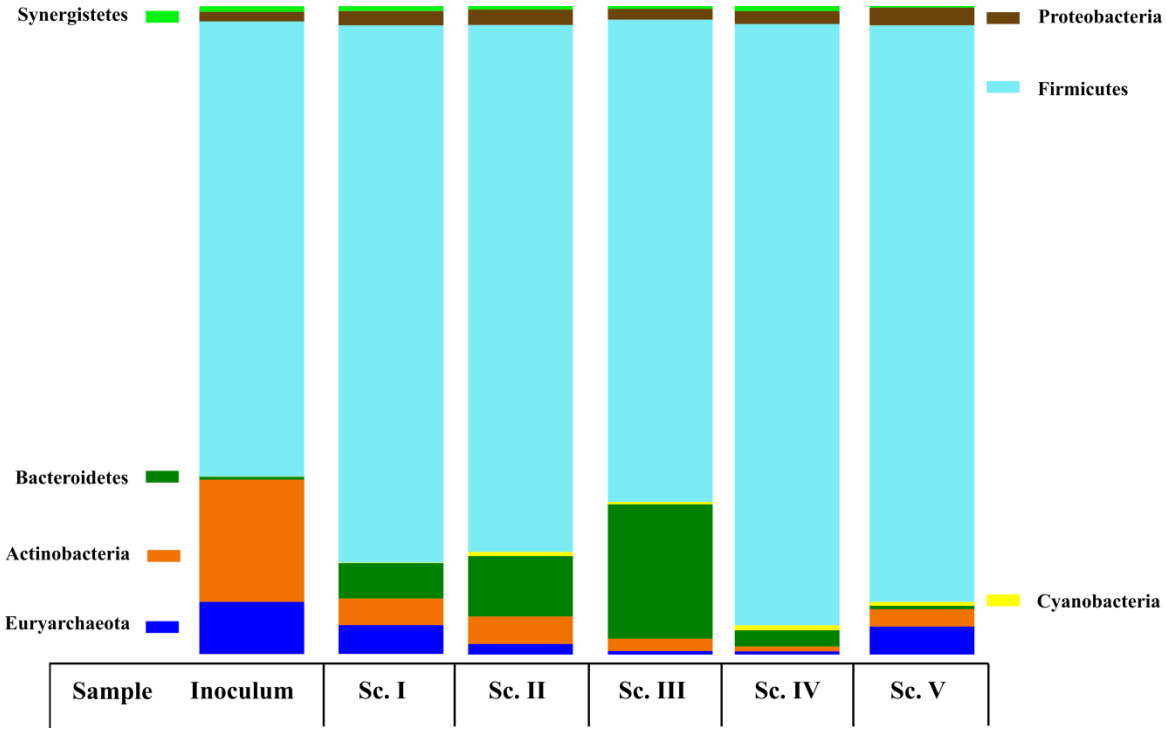

Figure 41. Main phyla detected during reactor operation.

At genera level, operational conditions affected species differently. Whereas some of them gradually disappeared such as *Ruminococcus*, *Anaerovorax*, or microorganisms related with the Coriobacteriaceae family, others increased its relative abundance. In this sense, *Sporanaerobacter*, *Clostridium*, *Peptostreptococcus* and *Enterococcus* belonging to Firmicutes phylum gained importance along the experiment (Figure 42).

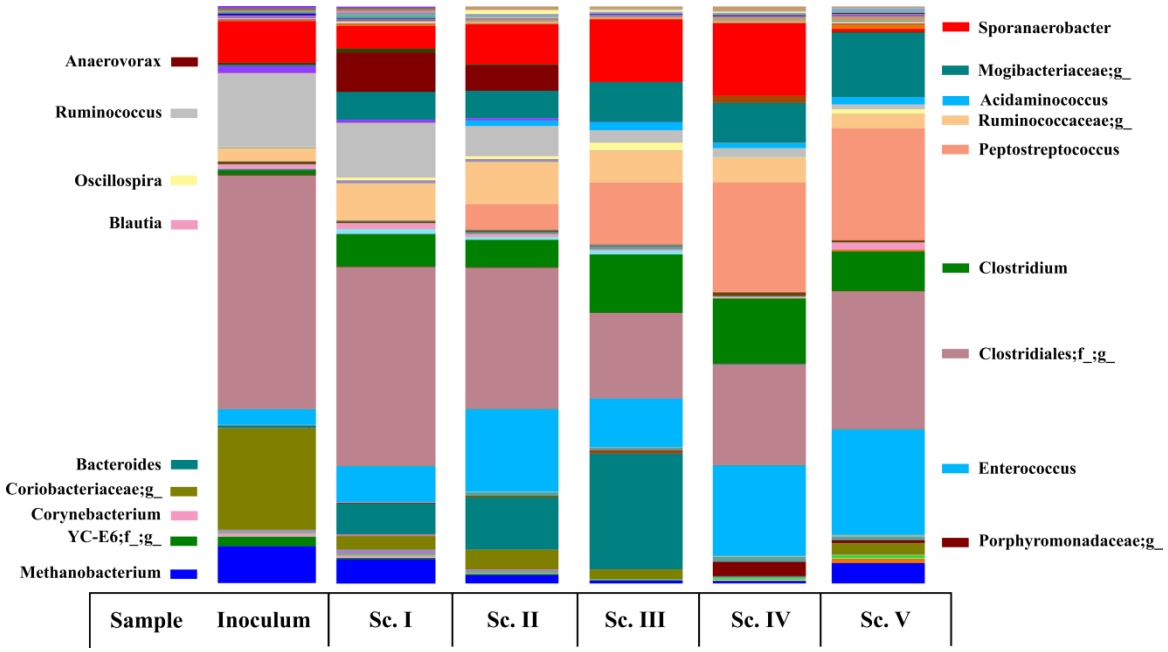

Figure 42. Main genera detected during reactor operation.

Clostridium genus is involved in butyrate, acetic acid, lactic acid and ethanol production due to their ability to carry out mixed acid and alcohol fermentations [262], explaining the butyric acid dominance (from 25.8% in Sc. I to 32.2% in Sc. V) in the VFAs profile as well as the high acetic acid productions (Figure 38). *Peptostreptococcus* is associated with the presence of propionic and succinic acids in anaerobic digesters [263]. All of these species decreased their relative abundance during the last scenario contributing to the lower VFAs production attained.

With regard to archaea species, the dominant genus found was again *Methanobacterium.* The gradual decrease in terms of relative abundance of these genera (6.4% vs 0.5%) agreed with the concomitant drop of COD removal percentages encountered throughout the process (Table 19). Exception made for the last scenario, in which abundance levels raised once again (3.5%). This fact suggested that the hydrogenotrophic genus *Methanobacterium* was able to get adapted at the end of the experimental time. Hydrogenotrophic species are reported to be more resistant than acetoclastic methanogens to high VFAs and NH_4^+ concentrations [264,265]. Additionally, the adaptive capacity of methanogenic archaea to specific process conditions has been widely proven in literature [204,266].

Thus, the high NH_4^+, VFAs levels and presence of certain ions (Na^+) might have caused the efficiency drop in the acidogenic stage. The high tolerance of hydrogenotrophic methanogens was demonstrated during Sc. V, in which no VFAs enhancement was reported and COD removal increased with respect to the previous scenarios.

4.3. EFFECT OF REACTOR CONFIGURATION

Opposite to the CSTR configuration employed in the previous sections, the UASB reactor offers the possibility of working at low HRT and high SRT. UASB reactors have been claimed to be an optimum choice for the anaerobic degradation of wastewater but its use for complex organic substrates (such is the case of microalgae biomass) remain limited. This configuration might be of interest to decrease the HRTs normally employed in CSTRs. Additionally, the high quality effluents produced (low amount of solids) might facilitate further VFAs purification steps.

The UASB configuration was tested in order to assess VFAs conversion and profiles. Stepwise OLR increases (Section 3.4.3.) at three Stages (I, II and III) were carried out to evaluate the influence of the OLR in this configuration. For this investigation, the adapted anaerobic sludge employed as inoculum was the same used for the stepwise OLR increase in CSTR. This sludge corresponded to the reactor after the starvation period.

AF performance: organic matter removal

Stages I and II (OLR 2.3±0.2 g COD/Ld and 3.6±0.9 g COD/Ld) mediated COD removals percentages of 39.4±11.2% and 48.8±10.4%, respectively (Table 21). These COD removals corresponded to methane production yields of 138±39 and 170±36 mL CH_4 (STP)/g COD_{in}. During Stage III, only 24.6±8.2% COD removal was achieved. The stability exhibited by the reactor in Stages I and II was characterized by a conventional methane content in the biogas composition (65.4±1.8 and 61.2±1.9 % v/v CH_4), stable pH

values (7.8±0.2 and 8.2±0.3) and moderate NH_4^+ concentrations (0.6±0.1 and 1.2±0.1 g NH_4^+/L). On the contrary, Stage III showed a marked drop of the methanogenic step. In this sense, methane composition in the biogas attained was significantly below Stages I and II (45.0±4.5% v/v CH_4). The latter stage fed at the highest OLR reported a COD removal in the range of those exhibited in the previous section in the CSTR configuration (Section 4.2.3., 4.2.7.). Thus, methanogenic partial inhibition was achieved only at the highest OLR.

Table 21. Average results of the parameters assessed in the effluent along the different stages of UASB operation.

Parameter	Stage I	Stage II	Stage III
TS (g/L)	4.1 ± 0.4	7.5 ± 1.6	20.7 ± 0.7
VS (g/L)	3.1 ± 0.4	5.6 ± 1.4	16.1 ± 0.5
tCOD (g/L)	8.0 ± 1.4	12.3 ± 2.6	42.9 ± 1.2
sCOD (g/L)	3.8 ± 0.9	5.0 ± 0.9	27.6 ± 0.7
sCOD/tCOD	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
% COD removal	39.4 ± 11.2	48.8 ± 10.4	24.6 ± 8.2
%VS removal	36.9 ± 8.1	30.4 ± 9.0	23.5 ± 7.4
(%) COD-VFAs/COD_{in}	14 ± 8	10 ± 4	37 ± 4
pH	7.8 ± 0.2	8.2 ± 0.3	7.2 ± 0.3
Total VFAs (g COD-VFAs/L)	1.8 ± 0.9	2.5 ± 1.2	20.8 ± 0.8
NH_4^+ (g/L)	0.6 ± 0.1	1.2 ± 0.1	2.7 ± 0.1

Literature regarding the digestibility of microalgae biomass in UASB reactors is scarce and devoted for methane production. For instance, Soboh et al., [94] reported microalgal high biomethanization values (79% of COD removal) in an UASB reactor under similar operational conditions (gradual OLR increase from 0.9 to 5.4 g COD/Ld and HRT from

7.2 to 5.5 days). The higher biodegradability obtained by those authors was mainly related to the microalgal biomass composition since *Chlamydomonas* and *Synedra* were used as substrate. Anaerobic biodegradability values of microalgae biomass is well-known to be strain dependent [40,267]. Whereas the microalgae biomass employed by Soboh and co-workers does not possess a hard cell wall, one of the main problems of *Chlorella* used in the present study is the rigid cell wall [268]. Indeed, *Chlorella* sp. and *Scenedesmus* sp. are probably among the most robust microalgae biomass and that is why they are most of the times able to thrive in wastewater. This cell hardness does not only protect them, but in the context of anaerobically degrading this biomass, their cell walls hamper the AD process and considerably reduce the hydrolysis step efficiency. In this sense, the use of *Scenedesmus* resulted in a degradation efficiency close to 50% in terms of COD when using a UASB reactor at HRT 2-4 days and OLR 2.25-3.23 g VS/Ld [95]. This value was in good agreement with the ones obtained herein for Stages I and II when using *Chlorella* biomass as substrate. Moreover, these values were also in line with those reported for digestions conducted in CSTRs. A COD removal of 56% was attained when digesting pretreated microalgae biomass *C. vulgaris* in a CSTR [43]. However, it should be noted that the HRT employed in this latter study was higher (HRT 20 days) than that applied in the present study (HRT≈6.5 days, Table 6). Agreeing with this latter research, other authors achieved a similar COD removal (51%) in a CSTR when digesting *C. vulgaris* under HRT 28 days and OLR 1 g COD/Ld [202]. The results confirmed the inherent potential of the strain *C. vulgaris* for methane production using an UASB configuration. Additionally, the solid content obtained (TS/VS) in the UASB effluent at the highest OLR was reduced by 20% when compared to the one obtained at a similar OLR in a CSTR (Section 4.2.7.). As pointed out in Section 1.4., separation and purification steps from a high quality effluent facilitates this step. Thereby, the UASB reactor should be envisaged as a reactor configuration able to reduce the operational economic investment required using *Chlorella* biomass as feedstock for VFAs production.

The gradual increase in NH_4^+ concentrations during reactor operation (Table 21) pointed out at this compound as a possible inhibitor of the methanogenic activity. The limiting NH_4^+ threshold starts around 1.5 g NH_4^+/L [41]. Hence, NH_4^+ values determined in Stage III (Table 21) most likely contributed to the methanogenic inhibition.

VFAs conversion yields and profiles

VFAs production was highly dependent on the OLR applied. Stages I and II resulted in 1.8±0.9 and 2.5±1.2 g COD-VFAs/L, respectively (Table 21). In general, a threshold concentration of 1.5 g/L VFAs has been stated for an optimum AD process for methane production [269,270]. Thus, concentrations obtained herein at low OLR values were comparable to the aforementioned limit (1.2±0.5 and 1.7±0.8 g/L VFAs). These productions corresponded to conversion values in the range of 10-14% COD-VFA/COD_{in} Despite of the VFA levels registered, AD performance was not affected since COD removals attained in Stage II increased with respect to those obtained in Stage I. In this line, other authors have reported a correct AD performance at high VFAs concentration making clear that each system must be evaluated independently. Such is the case of Wang and co-workers who studied different VFAs at higher concentration (acetic and butyric acids, 2.4 g/L VFAs) and did not encounter significant inhibitions [271]. Finally, the highest OLR (Stage III) reported a notable VFAs production increase (20.8±0.8 g COD-VFA/L) which evidenced the effect of OLR with regard to Stages I and II. These VFAs values corresponded to organic matter conversions of 37±4% COD-VFA/COD_{in} (Figure 43). To the best of authors' knowledge, microalgae biomass is an innovative substrate to be fed in UASB reactors and hence, literature is scarce regarding VFAs accumulation. As a matter of fact, authors using microalgae as substrate did not report accumulation of these chemicals [94,95,272]. Nevertheless, it should be highlighted that the 37±4% COD-VFA/COD_{in} achieved by the UASB reactor fed continuously are in accordance with values registered in CSTR throughout the present investigation work (See Sections 4.2.7 and 4.2.3.) whilst working at lower HRTs (6 days).

When analyzing the acidogenic stage, COD-VFAs/$sCOD_{out}$ ratio was 0.5±0.1 for Stages I and II, and 0.8±0.1 for Stage III. The lower ratio attained for Stages I and II agreed with the higher COD removals. However, Stage III retrieved similar acidogenic efficiency as previously showed in the CSTR at similar OLR (Section 4.2.7.). This fact indicated that high OLRs can be used as a tool in the UASB reactor to promote acidogenic species and decrease methanogenic activity.

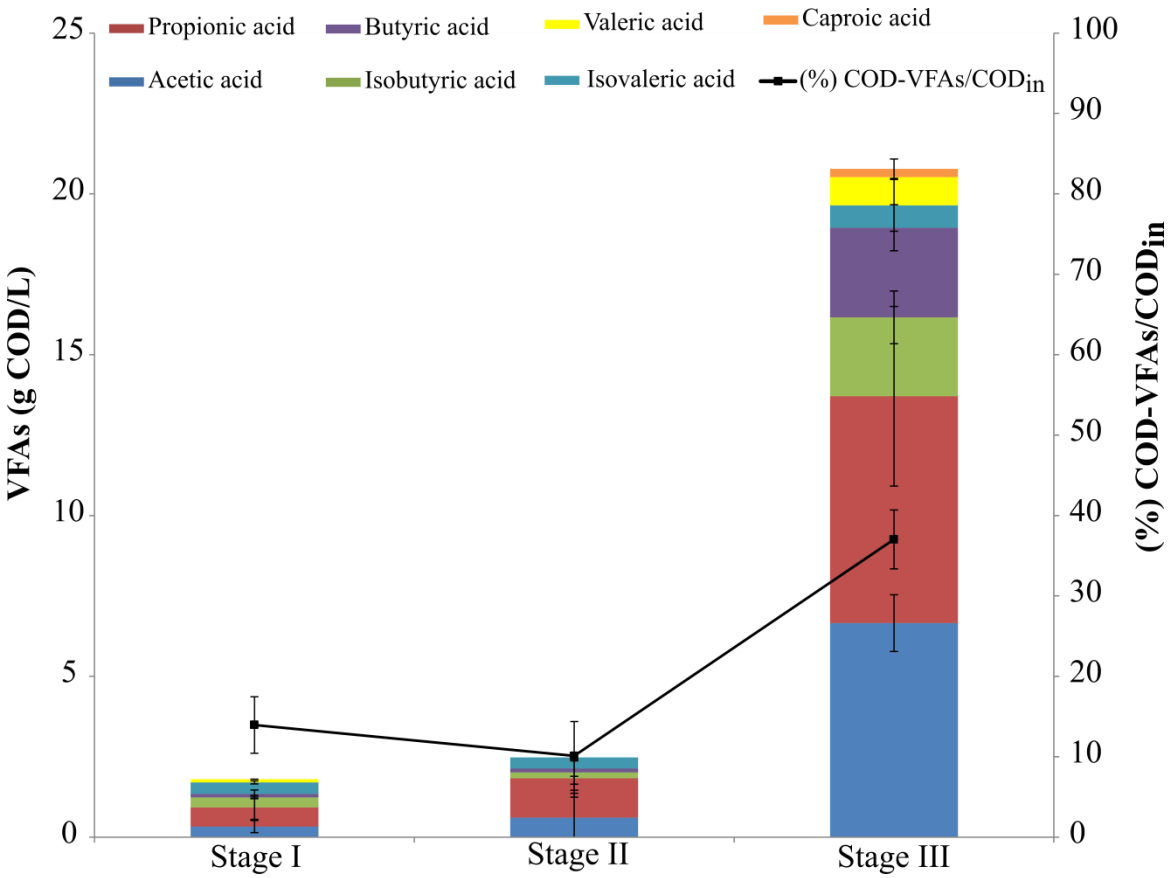

Figure 43. VFAs productions at stepwise OLR increases using a UASB configuration.

With regard to the VFAs profile, propionic and acetic acids were found to be the most abundant products (Figure 43), contrasting with the results obtained in the CSTR in which VFAs profile was led by butyric acid (Section 4.2.7). Acetic and propionic acids production notably increased during Stage III achieving final concentrations of 6.7 and 7.1 g COD-VFAs/L, respectively. This accumulation may be related to the aforementioned methanogenic inhibition. Acetic acid serves as substrate for acetoclastic methanogens species [273] whilst propionic acid accumulation has been previously reported in unbalanced digestions [208]. Moreover, isobutyric, butyric, valeric, isovaleric and caproic acids were also measured although their presence was comparably lower. As a matter of fact, total production of C4 (butyric and isobutyric acids) and C5 (valeric and isovaleric acids) during Stages I and II was 0.9 g COD-VFAs/L and 0.7 g COD-VFAs/L, respectively, whereas Stage III raised C4 concentrations up to 5.2 g COD-VFAs/L. C5 remained comparatively lower (1.6 g COD-VFAs/L) and for the first time C6 concentrations were detected in a minor extent (0.3 g COD-VFAs/L). In this case C4-C5-

C6 VFAs represented 34% of the total VFAs detected with respect to 63% in CSTRs (Section 4.2.2.)

Microbial communities

The use of DNA sequencing with bacterial and archaeal primers revealed a microbial shift in the bacterial community structure along the OLR imposed. The population diversity increased according to the Shannon index from the inoculum (3.406) up to 7.359, 6.759 and 5.234 in Stages I, II and III, respectively (Table 22).

Table 22. Shannon indices showed in Stages I, II and III during UASB operation.

	Shannon
Inoculum	3.4
Stage I	7.4
Stage II	6.8
Stage III	5.2

The highest diversity obtained during Stage I might be related with the high SRT established, which most likely promoted low growth rate microorganisms. In fact as introduced in Section 1.2.2., UASB reactor are able to decouple HRT from SRT allowing slow growing species to thrive. However, these values decreased from Stages I to III with the concomitant increase of the OLR. This fact evidenced the influence of this parameter in the microbial system. Bacterial composition of the adapted anaerobic inoculum was described in Sections 4.2.5. and 4.2.6. This sludge was taken after the starvation period and was mainly represented by Firmicutes accounting for 70% of the encountered bacteria (Figure 44).

Despite of their predominance in acidogenic microorganisms and its conversion yields in the stepwise OLR increase in CSTR (Section 4.2.7.), the inoculum showed its efficiency in terms of biogas production. This fact highlighted the importance of reactor configuration over the anaerobic population of the inoculum used. In this sense,

developed population using the same adapted inoculum were different in CSTR (Section 4.2.7) and UASB configurations.

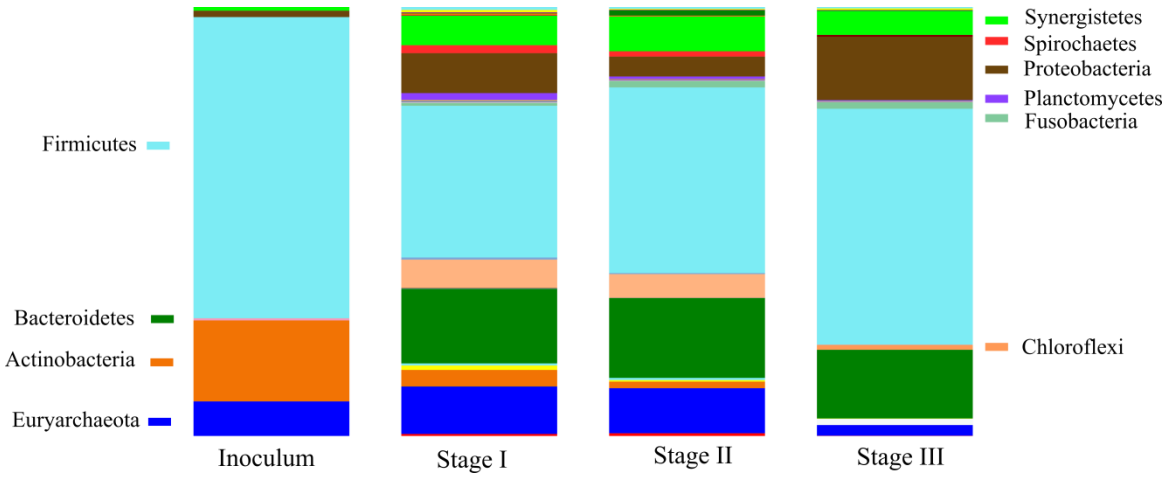

Figure 44. Main phyla identified during UASB reactor operation.

UASB reactor operation in Stages I, II and III confirmed the diversity increase observed in the Shannon index and showed a drastic shift in the microbial composition when compared to the inoculum. In fact, Firmicutes and Actinobacteria phyla were reduced in Stage I to 35% and 3.8% (Figure 44), respectively, whereas species belonging to Bacteroidetes, Synergistetes, Proteobacteria, Chloroflexi and Euryarchaeota gained importance. All these phyla have been previously found in AD environments for biogas production [274]. Bacteroidetes increased its relative abundance during operation (Stages I, II and III; 17-18%) when compared to the inoculum (<1%). This phylum is in charge of protein degradation for propionic and acetic productions [275]. Synergistetes phylum increased up to values ranging 5.5-8.1% (Figure 44) during reactor operation. This phylum gather microorganisms with amino acid degrading activity, which is consistent with the high protein content exhibited by the microalgae biomass used as substrate [276]. The low presence detected of this phylum in CSTRs (Section 4.2.7.) might be associated with the low HRTs employed in those reactors which could have caused the washed out of these species. Chloroflexi (5-6% in Stages I and II, Figure 44) has been claimed to develop hydrolytic functions and the main genus encountered, T78 (4.4% Stages I and II), was previously identified in a CSTR and anaerobic membrane bioreactors (AnMBR) for

methane production from microalgae biomass at low NH_4^+ concentrations (below 1 g/L NH_4^+) [102]. The increasing NH_4^+ concentrations registered herein (Table 21) might have played a role in species belonging to Chloroflexi phylum disappearance. As a matter of fact, Stage III showed NH_4^+ concentrations in the range of those reporting growth inhibition [277]. In addition, Proteobacteria species, that closely work with genera belonging to Euryarchaeota in the syntrophic degradation of organic acids and proteins, were present in a minor extent when compared to other studies (46-51%, [106], explaining the slight VFAs accumulation occurred in Stages I and II (Figure 43). However, Proteobacteria gained importance with respect to the CSTRs employed in the present experiment (Section 4.2.7.). The latter stepwise OLR increase (Stage III) showed an increase in the relative abundance of Firmicutes (55%) followed by Proteobacteria (14%). The dominance of Firmicutes was previously reported along the present investigation and in other studies [90,278].

The bacteria community encountered in the UASB at genus level was more diverse when compared to the one obtained in the CSTR. In this sense, only a few species were present in both reactors. For instance, *Enterococcus* (14%) and *Peptostreptococcus* (21%) belonging to Firmicutes phylum, were present in UASB, but also in CSTR (see Section 4.2.7.). However, even though species were different, organic matter conversion into VFAs was in the same range, which indicated the redundant functionality of the microbial system.

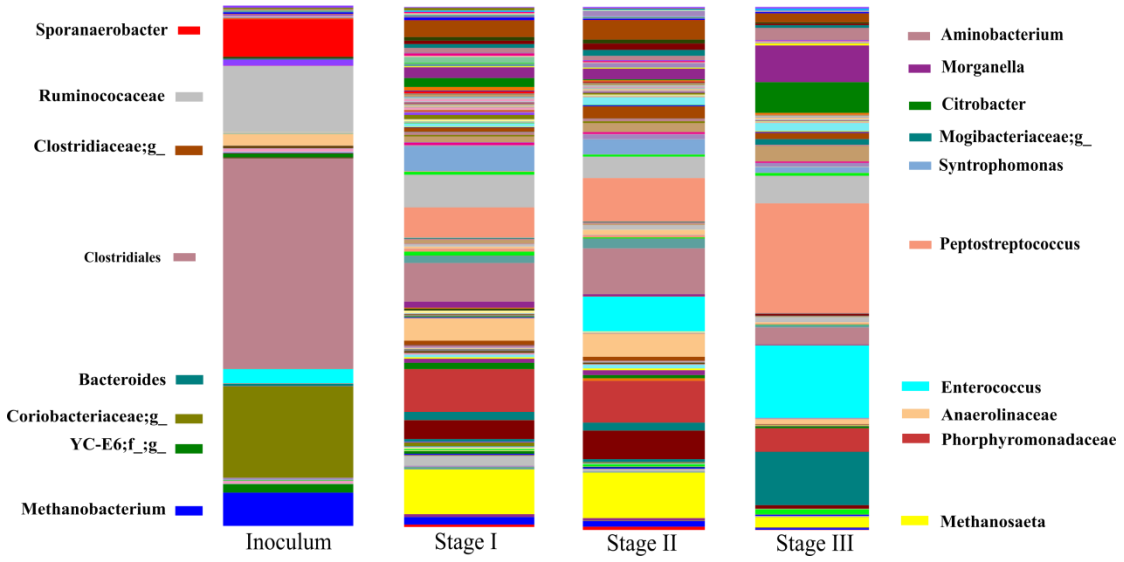

Figure 45. Main phyla identified in Stages I, II and III during UASB reactor operation.

Archaea genera in Stages I and II were detected in a major extent (11%), explaining the high COD removals attained. Although hydrogenotrophic species were mainly present in the inoculum (*Methanobacterium*), the acetoclastic pathway dominated UASB reactor operation as *Methanosaeta* was the main genus identified (8.6 and 8.7%, respectively, Figure 45). The wide bacterial community developed, most likely due to the high SRT of the UASB reactor together with the presence of archaea species, supported the high methane yields during Stages I and II. However, similarly to what happened to the bacteria community, the last OLR increase also retrieved a microbial shift on the archaea structure. As a matter of fact, there was a sharp drop in the relative abundance of Euryarchaeota phylum to values of 2.5%. The significant reduction of *Methanosaeta* in Stage III, when compared to Stages I and II, may be explained by the increasing acetic acid concentration attained in the reactor during the last stage, which is claimed to be inhibitory for this genus [279]. Consequently, the COD removal obtained during Stage III was lower than in Stages I and II. In addition, the high NH_4^+ and VFAs concentrations might have also contributed to the inhibition of the acetoclastic route, as these species are known to be more sensitive than the hydrogenotrophic archaea [111].

Overall, the relative abundance of each genus was dependent on the OLR stablished in the reactor and the reactor configuration. The high SRT allowed in the UASB reactor

promoted the growth of species, increasing reactor diversity. Low OLR values positively correlated with species diversity promoting microorganisms in charge of microalgae biomass complete degradation to methane. On the contrary, the highest OLR resulted in a more specialized sludge in which hydrolytic and fermentative bacteria gained importance outcompeting the methanogenic activity. This fact decreased the relative abundance of Euryarchaeota and Chloroflexi species whilst promoting hydrolytic and fermentative microorganisms (Firmicutes), hampering biogas production and in turn boosting VFAs accumulation.

CONCLUSIONS

5. CONCLUSIONS

Conclusions drawn from this work focused on VFAs production from microalgae biomass have been clustered in five different blocks.

Biomass pretreatment

- A pretreatment step prior digestion was found crucial to promote VFAs production from microalgae biomass. The proteolytic pretreatment used in the present investigation enhanced the organic matter bioavailability of the substrate, which enabled higher VFAs conversion yields compared to non-pretreated biomass.

Operational conditions

- The assessment of temperature in BCPs showed that the best temperature conditions were 35ºC and 25ºC. Nevertheless, low temperature (psychrophilic, 25ºC) operation in CSTRs gave the best results in terms of organic matter conversion into VFAs (35±3% COD-VFAs/COD_{in}) with respect to mesophilic (25±3% COD-VFAs/COD_{in}).

- Initial neutral and slight acidic pH values promoted VFAs accumulation in comparison to initial basic pH values in BCPs. Therefore, neutral pH is recommended to avoid chemicals addition.

- HRTs employed for VFAs production (8 days) in CSTRs when using adapted sludge were substantially lower than those found optimal for methane production using microalgae as feedstock (15-20 days). Organic matter conversion into VFAs was maintained at values close to 37±2% COD-VFAs/COD_{in}. When the same HRT (8 days) was assessed in a CSTR inoculated with non-adapted sludge the organic matter conversion dropped to 25% COD-VFAs/COD_{in}.

- Opposite to the reactors devoted for biogas production, high OLRs could be used without decreasing organic matter conversion into VFAs. OLR was stepwise increased from 1.5 to 12 g COD/Ld in CSTRs while preserving organic matter conversion efficiency at maximum levels (37±2% COD-VFAs/COD_{in}). Nevertheless, an OLR threshold was encountered at 15 g COD/Ld that inhibited the acidogenic stage reflected in the acidogenic efficiency ratio (0.6 against 0.8-0.9 COD-VFAs/$sCOD_{out}$). Different relevant factors including NH_4^+, VFAs and other ions concentrations (i.e. Na^+) were identified to decrease organic matter conversion into VFAs at OLR 15 g COD/Ld.

- NH_4^+ was inhibitory for methanogenic archaea when working at high OLRs. This parameter, together with the imposed operational condition, contributed to methanogenic inhibition. On the contrary, NH_3 concentrations were low due to the acidic process pH and temperature and did not represent a threat for methanogens activity.

Reactor configuration

- CSTR in semicontinuous mode and UASB configuration fed continuously resulted in similar organic matter conversions into VFAs (37±2% COD-VFAs/COD_{in}) at high loading rates (12 and 9 g COD/Ld, respectively).

- The OLR effect was dependent on reactor configuration. Low OLR values (2-4 g COD/Ld) promoted methanogenesis in the UASB configuration (50% biodegradability) at lower HRT values than those established in conventional CSTRs whereas high values (9 g COD/Ld) favored organic matter conversions into VFAs. With regard to the CSTR, the stepwise OLR recovered and maintained VFAs conversions at 37±2% COD-VFAs/COD_{in}. Exception made for the highest OLR applied (15 g COD/Ld) in which the acidogenic stage was inhibited.

- UASB configuration resulted in a reduction of 20% of TS than those obtained in the CSTR. This fact might be of importance when VFAs are produced as

marketable products or as chemical platforms. VFAs purification and separation steps would be technically easier in low solids content effluents.

- VFAs profile in terms of COD was affected by the different temperatures, OLR and reactor configurations assessed (CSTR and UASB). In this manner, butyric acid led VFAs profile in CSTR configuration at high OLRs (from 3 COD/Ld, 32%) and 25ºC, whereas acetic acid was the most abundant product at low OLR (1.5 g COD/Ld, 20%). Propionic acid outstood as the most abundant product when employing the UASB reactor configuration at 25ºC and high OLRs (34%).

Inoculum

- The use of adapted inoculum contributed to maintain organic matter conversion yields into VFAs. In this sense, adapted inoculum was able to cope with harsh operational conditions (HRT 8 days) whereas the non-adapted inoculum exhibited a decrease in VFAs conversion yields.

- Despite of using the same anaerobic inoculum, the use of different reactor configurations resulted in different process development. Therefore, reactor configuration ruled the fate of organic matter.

- Out of the pretreatments applied to the inoculum and evaluated in BCPs (thermal, chemical and a combination of both of them), thermal pretreatments were found to be the most effective to convert organic matter into VFAs (70% COD-VFAs/COD_{in}). Chemical pretreatments with BES allowed VFAs to remain in the digestate instead of being converted to methane but conversion values were lower (48% COD-VFAs/COD_{in}) than the ones attained after thermal pretreatment. Nevertheless, inoculum pretreatment was ineffective when tested in semicontinuous conditions, which evidenced a short-term effect of the pretreatment with no further process improvement with respect to the non-pretreated inoculum.

Anaerobic microbiome

- Regardless of the reactor employed (CSTR or UASB), microbial communities and diversity found in digesters targeting VFAs production were rather different to the ones often encountered when biogas production is desired. In this manner, according to Shannon index, CSTR displayed the lowest diversity whereas UASB configuration allowed higher diversity due to the high SRT of this configuration.

- With regard to the bacterial community, Firmicutes was the most abundant phylum regardless of the employed reactor (CSTR or UASB). Additionally, Bacteroidetes and Actinobacteria were present in CSTR configuration in different extent depending on the operational conditions, developing redundant functions as VFAs conversion was maintained. On the other hand, Bacteroidetes, Proteobacteria and Synergistetes were highlighted in the UASB reactor. Hence, association of Firmicutes and Bacteroidetes was found essential to achieve competitive organic matter conversions into VFAs.

- With regard to the Euryarchaeota phylum, the species encountered in the present investigation produced methane via the hydrogenotrophic pathway in CSTRs. The operational conditions and the harsh cultivation broth (high VFAs and NH_4^+ concentrations) rendered hydrogenotrophic archaea prevailing over the acetoclastic pathway. This latter route only was highlighted during UASB operation at low OLR values. Acetoclastic archaea were able to thrive due to the high SRT in the UASB.

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6. REFERENCES

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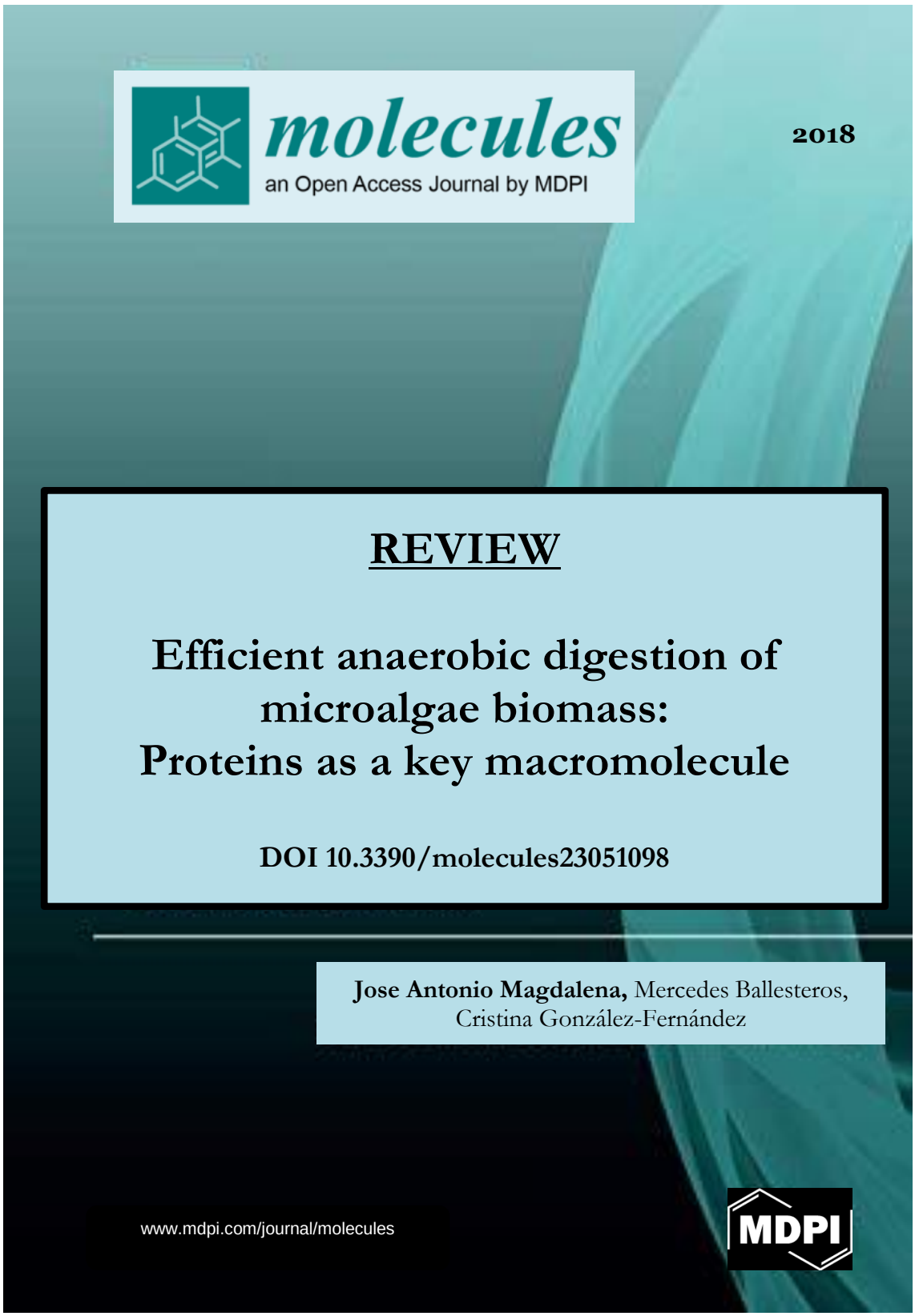
ORIGINAL PUBLICATIONS

PUBLICATION I



molecules

MDPI

Review

Efficient Anaerobic Digestion of Microalgae Biomass: Proteins as a Key Macromolecule

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Abstract: Biogas generation is the least complex technology to transform microalgae biomass into bioenergy. Since hydrolysis has been pointed out as the rate limiting stage of anaerobic digestion, the main challenge for an efficient biogas production is the optimization of cell wall disruption/hydrolysis. Among all tested pretreatments, enzymatic treatments were demonstrated not only very effective in disruption/hydrolysis but they also revealed the impact of microalgae macromolecular composition in the anaerobic process. Although carbohydrates have been traditionally recognized as the polymers responsible for the low microalgae digestibility, protease addition resulted in the highest organic matter solubilization and the highest methane production. However, protein solubilization during the pretreatment can result in anaerobic digestion inhibition due to the release of large amounts of ammonium nitrogen. The possible solutions to overcome these negative effects include the reduction of protein biomass levels by culturing the microalgae in low nitrogen media and the use of ammonia tolerant anaerobic inocula. Overall, this review is intended to evidence the relevance of microalgae proteins in different stages of anaerobic digestion, namely hydrolysis and methanogenesis.

Keywords: microalgae; anaerobic digestion; proteins; biogas; inhibition

1. Introduction

Environmental issues and energy self-sufficiency concerns related to fossil fuels have led to research on new approaches to improve renewable energy production to substitute them. Anaerobic digestion is one of those technologies devoted to the production of biofuels, which involves the degradation of organic matter through the action of different microorganisms. Anaerobic digestion exhibits many advantages such as its efficiency for organic matter removal, its applicability at any scale and the wide variety of substrates that can be used as feedstock. Likewise, the multiproduct generation attained during digestion is also a major benefit of this technology. Those end-products, including biogas and digestate, are easy to separate and can be a source of energy and fertilizers, respectively [1].

Among the different substrates that can be employed, microalgae are being recently studied since this biomass can be grown in residual effluents, do not need arable land to be cultivated while contributing to CO_2 mitigation and wastewater bioremediation [2]. Previous studies have demonstrated the technoeconomic and environmental benefits of microalgae biomass for bioenergy purposes when considered as by-product in other technologies [3–8]. In the same manner, out of the bioenergy producing technologies where microalgae can be used as feedstocks, anaerobic digestion is probably the most economically feasible since it does not require highly concentrated biomass [9] and anaerobes can use proteins, carbohydrates and lipids for methane production purposes [10]. Microalgae biomass has

a wide range of compositions, depending on growth conditions and species [11,12]. In general terms, biochemical profile of chlorophytes range 30–60% of proteins, 20–40% of carbohydrates, and 4–57% of lipids [13,14]. Each macromolecule has different achievable methane yields [10]. Thus, in principle, different microalgae compositions produce different methane yields [12]. At the same time, microalgae composition varies depending not only among strains but also on the growth conditions (nutrients availability and operational conditions) [15,16]. In addition to the different macromolecular composition that microalgae might exhibit, this biomass also differs in structural features. Most of the microalgae able to thrive in wastewater effluents have a chemically complex and structurally robust cell wall composed of low biodegradable substances that hinder the anaerobic digestion [17,18]. Some of these compounds are sporopollenin, algaenan, and polymeric carbohydrates that offer a barrier towards anaerobes [19,20]. During anaerobic digestion, cell walls are degraded by extracellular enzymes of hydrolytic bacteria. Nevertheless, this process might be too slow and thus, a limited hydrolysis rate renders the anaerobic digestion as a lengthy and inefficient bioprocess. Pretreatments are used in order to facilitate the accessibility of these extracellular enzymes whereby improving hydrolysis stage. Different microalgae pretreatments have been studied such as thermal, chemical, mechanical or biological. Methane yields improvements achieved with those different pretreatments can be found elsewhere [21–24]. Out of the different pretreatments, biological approach is the most environmentally friendly [25]. Opposite to other pretreatments, the additional benefits of biological pretreatments are the absence of inhibiting by-products [26] and the high selectivity of the reactions [27]. This approach might not only be used for biomass hydrolysis but also to provide crucial information related to the macromolecule that reduces the anaerobic biodegradability of microalgae biomass. In this manner, this review summarizes the main results attained during the last years of research devoted to microalgae pretreatments in the biogas production context. Moreover, this period of research highlighted the importance of proteins on different stages of the digestion. This review attempts to provide comprehensive evidences of the key role of microalgae proteins.

2. Pretreatment of Microalgae Biomass to Improve Biogas Production

Since low biodegradability is a common issue in anaerobic digestion of different substrates (such as activated sludge, lignocellulose and photosynthetic microorganisms), a wide range of pretreatments are available to enhance the hydrolysis step [28]. Cell wall rupture or hydrolysis is needed to make available microalgae organic matter to anaerobic microorganisms [29]. Pretreatments are classified in four main groups, namely thermal, mechanical (ultrasound and microwave), chemical (acidic, alkaline, solvents and ozonation) and thermo-chemical (acid or alkali reagents addition combined with high temperatures) and biological (enzymes and microorganisms). Those pretreatments have been intensively studied during the last decade to improve biogas production of microalgae biomass (Table 1). Most of them have been assessed in Biochemical Methane Potential (BMP) assays (batch digestion mode).

Table 1. Studied pretreatments to improve biogas production using microalgae as substrates.

High Demanding Energy Pretreatments	Operation Mode	Biomass	Conditions	Methane Yield Increase	References
Thermal	Batch	*Scenedesmus* sp.	75 °C for 10 h 95 °C for 10 h	58% 69%	[29–31]
	Batch	*Scenedesmus* sp.	80 °C for 15 min	60%	[32]
	Batch	*Chlorella* sp.	70 °C for 30 min 90 °C for 30 min	37%48%	[33]
	Batch	*Stigeoclonium* sp. *Monoraphidium* sp and *Nitzschia*	130 °C for 15–30 min	28%	[31]
	Semi-continuous	*Chlorella* sp.	120 °C40 min	1.5-fold	[34]
Mechanical	Batch	*Scenedesmus* sp.	128.9 KJ/g TS for 30 min	87%	[32]
	Batch	*Monoraphidium* sp. and *Stigeoclonium* sp.	26.7 KJ/g TS for 30 min	85%	[31]
	Batch	*Mixture of microalgae biomass*	10; 27; 40; 57 KJ/g TS	6-24%	[35]
Chemical	Batch	*Chlorella* sp. and *Scenedesmus* sp.	CaO (4 and 10% w/w) at 25, 55 and 72 °C	25%	[36]
	Batch	*Chlorella* sp.	4 M H_2SO_4 at 120 °C for 20–40 min	72.5%	[37]
Low Demanding Energy Pretreatments		**Biomass**	**Solubilization**	**Methane Yield**	**References**
Proteases	Batch	*C. reinhardtii* *C. vulgaris*	86-96% for both biomasses	51% in *Chlorella* biomass 7% C. *reindhartii*	[38]
	Batch	*Scenedesmus* sp.	30%	1.53-fold	[39]
	Semi-continuous	*C.vulgaris*	47%	2.6-fold	[39]
	Semi-continuous	*C. vulgaris*	54%	5 and 6.3-fold (OLR= 1.5 g/L d and OLR= 3 g/L d)	[40]
Carbohydrases	Batch	*C. vulgaris* and *Scenedesmus* sp.	84% 36%	1.2-fold	[41]

2.1. High Energy Demanding Pretreatments

Thermal, thermo-chemical and mechanical pretreatments are considered as high energy demanding processes and, in order to evaluate its efficiency, the final energy balance of the pretreatment process has to be addressed. Given that thermal energy is available in biogas production facilities, the most used pretreatment is thermal application. Thermal pretreatments involve biomass heat up in a wide range of temperatures (50–270 °C) and reaction time (from minutes to hours). With regard to thermal application, the effect on the biomass depends on the microalgae strain and applied temperature [30]. Passos et al. [31] and Passos and Ferrer [42] applied thermal pretreatment to *Scenedesmus* sp. biomass at 75 °C and 95 °C for 10 h resulting in methane yield enhancement of 58% and 69%, respectively . Similar values were attained by González-Fernández et al. [43] when treating *Scenedesmus* at 80 °C for only 15 min, highlighting the impact of temperature rather than the heating time as the most relevant parameter in thermal pretreatment. Similar temperatures were tested in *Chlorella* biomass (70 and 90 °C) for 30 min resulting in an enhanced methane yield of 37% and 48% compared to the raw biomass (322 mL CH_4/g VS_{in}) [32]. These results evidenced that thermal pretreatments are strain specific and thus, at the same temperature applied, different methane yields enhancement can be attained among the different biomass used. Higher temperatures (130 °C for 15–30 min) were also tested, resulting in 28% methane yield increase when compared to a raw biomass composed by a mixture of green algae (*Stigeoclonium* sp. and *Monoraphidium* sp.) and diatoms (*Nitzschia*) (105 mL CH_4/g VS_{in}) [31]. Due to the potential formation of Maillard compounds at higher temperatures, moderate temperatures in the range of 80–120 °C are most widely tested. Moreover, thermal pretreatments have been tested not only in batch mode, but also in semicontinuous mode. Méndez et al. [33] reported a methane yield enhancement of 1.5-fold compared to raw *Chlorella* biomass (84 mL CH_4/g COD_{in}) when using 120 °C for 40 min for feeding a Completely Stirred Tank Reactor (CSTR). Although no common inhibitors were identified, the results obtained in the CSTR were considerably lower (50% less) than the ones obtained in batch mode digestion. This experimentation corroborated the need to test each pretreatment in different feeding modes. Although thermal pretreatments normally present positive results in terms of methane yield, the values attained are very diverse depending on different variables such as the pretreated biomass, temperature, pretreatment time employed and operation mode during the digestion. Moreover, as mentioned above, these methods involved some drawbacks such as the formation of recalcitrant compounds that could potentially decrease the performance of the process [34,35].

Mechanical pretreatments are commonly employed to disrupt different kind of organic substrates in industrial processes [44,45]. Ultrasound treatment has been applied to disrupt microalgae cell wall in different bioprocess devoted to biofuel production, such as ethanol production from *Chlorella* biomass [46] and biodiesel generation from *Spirulina* biomass [47]. In the case of anaerobic digestion, ultrasound pretreatment has also shown positive results in terms of methane yield enhancement. González-Fernández et al. [43] applied 128.9 kJ/g TS at 85 °C and 30 min to enhance methane yield of *Scenedesmus* biomass from 81 mL CH_4/g COD_{in} to 153 mL CH_4/g COD_{in} (87% enhancement). Nevertheless, those authors also pointed out the fact that ultrasound application is having associated an increase in temperature which also acts as a pretreatment. As a matter of fact, when it comes to the pretreatment of *Scenedesmus* sp., the benefits of ultrasound application were rather questionable compared to the enhancement in methane yield attained only with the application of temperature. Ultrasound pretreatment (26.7 KJ/g TS for 30 min) was also applied to *Monoraphidium* sp. and *Stigeoclonium* sp. biomass and their methane yields were enhanced from 105 mL CH_4/g COD_{in} to 196 mL CH_4/g COD_{in} [42]. When testing different energy inputs (10; 27; 40; 57 KJ/g TS), applied to different mixtures of microalgae biomass (mixture A: 40% *Chlamydomonas*, 20% *Scenedesmus* and 40% *Nannocloropsis*; mixture B: 58% *Acutodesmus obliquus*, 36% *Oocystis* sp., 1% *Phormidium* and 5% *Nitzschia* sp; Mixture C: *Microspora* ≈ 100%), an increase in methane yield ranging from 6 to 24% at 10 MJ/kg TS was determined, while higher energy inputs did not report any significant increase [34]. Despite all those positive results in terms of methane yields enhancement, the main limitation of ultrasound

pretreatment is the high energy input required when compared to thermal, chemical or biological methods [21].

Chemical methods are often combined with heat pretreatment. Thermochemical pretreatments have been less employed than thermal and mechanical pretreatments due to its potential toxicity for the anaerobes. Cell wall disruption with alkali and acid pretreatments has been tested with positive results for the production of ethanol, butanol and biomethane when using microalgae biomass as a feedstock [48,49]. Studies related to microalgae biomass solubilization using thermo-alkaline methods include for instance the use of reagents such as NaOH or CaO. Different doses of CaO (4 and 10% *w*/*w*) and different temperatures (25, 55 and 72 °C) resulted in maximum proteins and carbohydrates solubilization of 32.4% and 31.4%, respectively, and methane yield enhancement of 25% compared to the raw biomass (260 mL CH_4/g VS_{in}) at the highest temperature and lime dose tested (72 °C and 10% *w*/*w*) [50]. When using NaOH (0.5, 2 and 5% *v*/*v*) in *Chlorella* and *Scenedesmus* biomass, the conducted experiments revealed that despite of the biomass solubilisation, the methane yield enhancement was really low (10%, [36]). Thermo-acid pretreatments have been less employed than thermo-alkali. For instance, *Chlorella* biomass was heated at 120 °C either for 20 min and 40 min. Sulphuric acid addition combined with 120 °C for 40 min enhanced carbohydrates solubilization by 7-fold, although no solubilization of the protein fraction was reported. In terms of methane production, this thermo-acid pretreatment improved the methane yield from the untreated biomass from 139 mL CH_4 g/COD_{in} to 230 mL CH_4 g/COD_{in} [51]. Since anaerobic digestion is taking place at around pH 7, one of the main limitations of chemical pretreatments is the need to readjust the pH previously to the digestion. In this manner, chemical costs limit the use of these pretreatments. Moreover, some of the chemicals need to be removed previously to the anaerobic digestion as they can be toxic for anaerobes [27].

In conclusion, high energy demanding pretreatment methods report high values in terms of methane yield. However, they are energetically unbalanced. This means that the energy required to carry out the pretreatment is higher than the one obtained in form of biogas. This is why research has been directed towards the use of low energy demanding pretreatments

2.2. Low Energy Demanding Pretreatments

Compared to other pretreatments, the biological approach presents some advantages such as lower energy demand and high specificity [37]. These pretreatments include the use of suitable enzymes or microorganisms to hydrolyze microalgae biomass. Information about the cell wall composition is scarce, but necessary in order to select the most suitable enzyme for the pretreatment. For that reason, a wide range of biocatalysts have been tested. In principle, given the similarities between higher plants and microalgae, the most studied catalysts are carbohydrases. Among them, cellulases, hemicellulases, amylases and pectinases are the most tested ones [37,52]. Some other enzymatic cocktails employed for microalgae biomass hydrolysis include lysozyme (catalyzing the hydrolysis of 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycan [53]), proteases (hydrolyzing peptide bonds [39]) and laccases [25]. Overall, the best results have been evidenced by adding commercial proteases cocktails. For instance, carbohydrases and proteases were compared hydrolyzing *Chlamydomonas reinhardtii* and *Chlorella vulgaris* [38]. Enzyme doses applied for carbohydrases and proteases were 0.3 mL/g DW and 0.2 mL/g DW, respectively. The enzymatic pretreatment lasted for 5 h and results obtained after carbohydrases addition were 86% and 96% carbohydrate solubilization for *C. vulgaris* and *C. reindhartii* while in the case of protease addition both biomass resulted in 96% protein solubilization. However, the authors pointed out that despite of the high carbohydrate solubilization, only a 14% enhancement methane yield was observed in *Chlorella* biomass, whereas no improvement was observed in *Chlamydomonas*. In the case of protease pretreated biomass, methane yield was enhanced by 51% in the *C. vulgaris* and 7% for *C. reindhartii*. The reason for the low methane yield enhancement recorded for *C. reindhartii* was due to the inherent high anaerobic biodegradability of this strain (75%, 263 mL CH_4 g/COD_{in}). Methane yield is limited

by the inherent methane yield that the biomass can attain. However, the kinetics might be enhanced by the use of pretreatments. More specifically, methane yield might be enhanced by protease pretreatment in the range of 1.07 to 6.3 fold depending on the targeted microalgae biomass within 10–15 days of digestion [38,40].

An alternative to improve economically the enzymatic pretreatment and avoid the addition of high cost cocktails is the addition of hydrolytic secretomes released by other microorganisms. For instance, 0.7 g/L of cellulase-secreting bacteria was added to *Chlorella vulgaris* for 48 h resulting in an increase of 18% organic matter solubilization and 2-fold methane yield compared to the raw biomass [54]. Non-specific extracellular enzymes of *Anthracophyllum discolor* were employed to disrupt the cell wall of *Botryococcus braunii*, resulting in an improvement of 60% methane yield, when enzymatic concentration of 1000 U/mL was applied [55]. Likewise, cellulolytic marine bacteria were applied to *Botryococcus braunii* and *Nannochloropsis gaditana* biomass 1:1 ratio DW resulting in a methane enhancement of 140% and 150%, respectively compared to the raw biomass [56].

As it is observed in Table 1, almost all tested pretreatments improved methane production yields although a direct linkage between solubilization and methane enhancement still requires in-depth research in continuous systems to determine the energy balance and costs of the overall process [57]. Even though this pretreatment is economically unfeasible yet, enzymatic pretreatments, targeting at specific molecules, could provide important information in order to identify which is the microalgae macromolecule hampering biogas production when using this biomass [23].

3. Biological Approach to Enhance Biogas Production: Enzymatic Pretreatment

Opposite to other pretreatments, biological reactions show high selectivity and absence of inhibitory compounds. Biocatalysts do not only disrupt the cell wall, but they also hydrolyze the macromolecules during biological pretreatment. As it was indicated above, these methods are energetically competitive since they require soft temperatures and smooth shaking. Different parameters must be taken into account such as pH, temperature, enzyme dose, and exposure time [21]. Given the different macromolecular composition, structural features and cell wall composition among microalgae strains, a wide range of biocatalysts can be used. Despite of the high economic cost of the enzymatic cocktails, the use of biocatalysts can provide crucial information to identify the macromolecule hampering anaerobic digestion of microalgae biomass. Moreover, the costs could be reduced either by in situ enzymes production [54,58] or by particular enzymes secreted by bacteria and fungi via sludge bioaugmentation [23,59,60].

3.1. Carbohydrases

Carbohydrases are in charge of hydrolysing carbohydrates polymers present within the cell wall and inside the cells into simple sugars. Since it is believed that carbohydrates are the responsible of cell wall toughness, cellulaseshave been tested in microalgae biomass to enhance the hydrolysis. Cellulases from *Trichoderma reseei* were mixed with metal oxides to treat *Chlorella* biomass resulting in glucose yield of 91% of the theoretical maximum [61]. Furthermore, enzymatic cocktails aimed at degrading the compartmentalized cell material such as amylases and amyloglucosidases have been tested to promote the efficiency of the hydrolysis step. As a matter of fact, a combination of amylases and cellulases was tested to degrade the cell wall and the cell material with acid hydrolysis in *Chlorella sorokiniana*, *Nannochloropsis gaditana*, and *Scenedesmus*. This treatment produced a sugar release of 128 mg/g DW, 129 mg/g DW and 60 mg/g DW, respectively against control values for the different biomass (70 mg/g DW, 20 mg/g DW and 25 mg/g DW) [62]. Carbohydrases have also been tested to facilitate lipid extraction by using exoglucanase, endoglucanase, xylanase and laccase produced by different biomass-degrading bacteria, improving lipid extraction up to 40% [63]. All those studies are mainly focused on carbohydrates solubilisation while, only recently, the biomass subjected to carbohydrases has been investigated for biogas production purposes. Ometto et al. [9] tested different enzymatic cocktails on three different biomass, namely *Scenedesmus obliquus*, *Chlorella sorokiniana* and *Arthrospira*

maxima [5]. Out of the tested enzymatic cocktails, mixtures of cellulase plus pectinase and esterase plus protease were the most effective catalysts for organic matter hydrolysis of all three biomass. In the same manner, commercial cocktails hydrolyzing the carbohydrate fraction such as Viscozyme, Celluclast and Pectinase (from Novozymes, Bagsværd, Denmark) have been employed in *C. vulgaris* and *Scenedesmus*. The use of Viscozyme provided carbohydrate fraction solubilization of 84% and 36% for *C. vulgaris* and *Scenedesmus* respectively, while the methane yield enhancement was 1.2-fold for both of them, despite of the different biomass composition and strain [41]. This experimentation suggested that the carbohydrate fraction cannot be understood as a whole to elucidate the relation between solubilization efficiency and the methane yield achievable. Instead of this, an in-depth research must be done concerning the carbohydrates composition of microalgae cell wall.

3.2. Lipases

When compared to other macromolecular constituents, lipids could be very useful substrates for anaerobic digestion due to its high potential methane yield. More specifically, theoretical methane yield for lipids is 1.014 L CH_4/g VS compared to 0.496 and 0.415 L CH_4/g VS for proteins and carbohydrates, respectively [10]. However, instability of the system can easily occur due to the formation of long chain fatty acids when lipids are hydrolyzed [64]. As a matter of fact, studies are mainly focused on the optimal concentration of lipids that makes possible to carry out anaerobic digestion without inhibition. In this way, it has been highlighted that lipid fraction should not be over 30% to avoid process inhibition [65]. To overcome such an inhibition, different strategies have been developed. For instance, Palatsi et al. [66] tested different recovery strategies to reduce the negative effect of long chain fatty acids by using different feeding patterns and adsorbents addition. Despite of the high lipid potential to enhance methane yield, microalgae biomass grown in wastewater does not present high lipid content [67,68]. At this point, it should be stressed that microalgae grown in residual effluents is the only feasible way to produce biofuel using this feedstock. In this manner, really limited information on lipases treatment of microalgae biomass for biogas production can be found in literature. For instance, an enzymatic mixture containing protease, α-amylase, xylanase, lipase and cellulase employed for *Rhizoclonium* biomass (filamentous green algae) hydrolysis resulted in 40% yield enhancement [69]. In this case, the mixture of enzymes made difficult the identification of the enzymatic activity responsible for such an enhancement. Ometto et al. [9] also tested esterases in different lipid rich microalgae biomass. Moreover, this investigation reported the use of esterases alone and the mixture of esterases and proteases. No biogas production was attempted for the biomass pretreated with esterases alone and thus, no conclusion could be withdrawn. Nevertheless, their work revealed that this later enzymatic mixture supported much higher organic matter solubilization than the values attained for esterases application alone, highlighting the importance of microalgae proteins.

3.3. Proteases

Microalgae biomass is normally prevailing in protein content. As a matter of fact, this polymer might represent approximately 40–60% of the microalgae dry weight [24,70]. Protein fraction might be degraded by proteases since they hydrolyze peptides into amino acids. The use of proteases is receiving particular interest in last years, especially in combination with other pretreatments or other commercial enzymatic cocktails [71,72]. Some examples on the use of proteases in different microalgae biomass were evaluated in terms of organic matter solubilization and methane yields [38–40]. In the context of anaerobic digestion, methane yields of *C. vulgaris* and *Scenedesmus* sp. were enhanced by 2.6-fold and 1.53-fold, respectively, when pretreated with protease [39]. It is important to note that those results were attained with proteins rich biomass. More specifically, *Chlorella vulgaris* exhibited 64% protein and 22% carbohydrate content. When dealing with carbohydrate rich *C. vulgaris* biomass (39.6%), protease hydrolysis efficiency (54%) displayed higher organic matter values than carbohydrolase hydrolysis (approx. 26%). The different effect of both enzymatic cocktails was also observed in the methane yields attained by both pretreated biomass. In that case, methane yield achieved with the biomass

pretreated with proteases was 137 mL CH_4 g/COD_{in} while 65 mL CH_4 g/COD_{in} was obtained for the biomass pretreated with carbohydrases [40]. This fact showed that even working with carbohydrate rich *C. vulgaris*, the proteolytic cocktail supported high organic matter hydrolysis and methane yields.

Comparison of different studies regarding enzymatic pretreatments suggested that proteins are the molecules that hindered the access of anaerobic bacteria to microalgae organic matter in the anaerobic digestion process to a greater extent than carbohydrates or lipids. Therefore, the protein fraction has been carefully analyzed during the anaerobic digestion process of microalgae biomass in the subsequent section

4. Biomass Proteins in Anaerobic Digestion of Microalgae

Anaerobic digestion is divided in four different stages including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1). When protein rich microalgae are subjected to anaerobic digestion, the bioprocess can be affected at different stages.

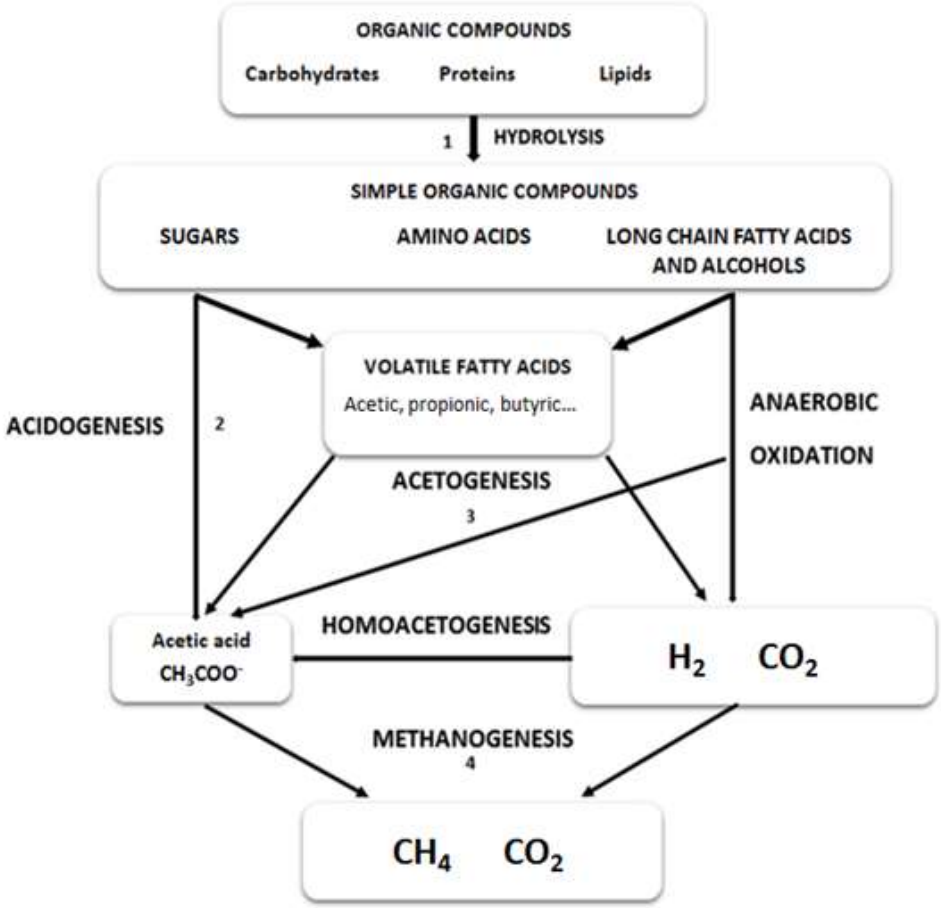

Figure 1. Reactive scheme for the anaerobic digestion of polymeric microalgal biomass.

Anaerobic degradation of proteins and lipids has not been investigated in depth compared to that of carbohydrates. Proteins are hydrolyzed to aminoacids by extracellular enzymes. Anaerobic and facultatively anaerobic bacteria, mainly *Clostridium*, are responsible of aminoacids fermentation. Clostridia obtain energy by coupled oxidation-reduction reaction between aminoacids via the so-called Stickland reaction. This reaction entails the oxidation (dehydrogetation) of one aminoacid and the reduction of a second aminoacids (hydrogenation) (Figure 2).

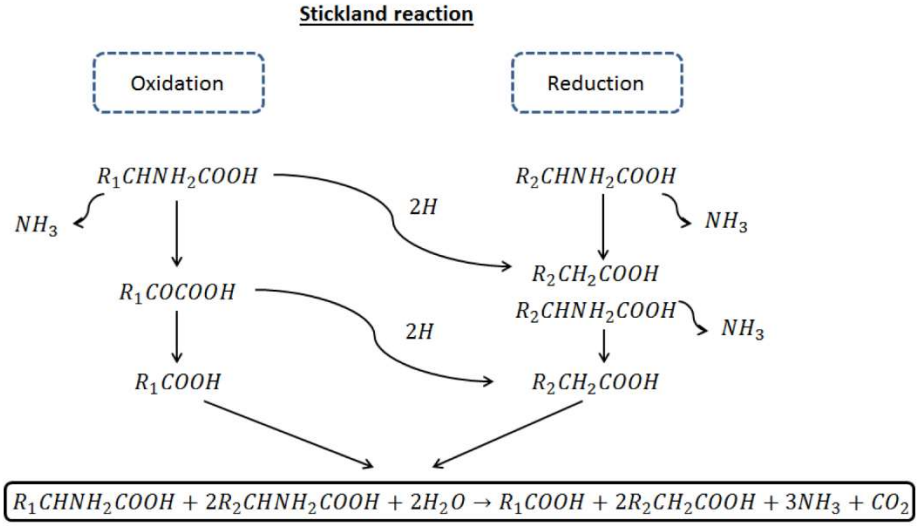

Figure 2. Stickland reactions scheme.

Aminoacids can act as electron acceptors or donors. In the first case, the aminoacid form a carboxylic acid with one carbon shorter than the original acid (e.g alanine to acetate) while when acting and electron acceptor, it retains the carbon to form a carboxylic acid with the same chain length as the original aminoacid (e.g., glycine to acetate). The aminoacid is de-ammonified by anaerobic oxidation, yielding volatile fatty acids and hydrogen, as shown in Table 2 [73].

Table 2. Aminoacid products based on Stickland reaction (modified from [73]).

Amino Acid	Formula	HAc	HProp	HBu	HVa	IN	IC	Other	H_2	ATP
Arginine	$C_6H_{14}O_2N_4$	0.5	0.5	0	0.5	4	1	0	−1	1
Histidine	$C_6H_9O_2N_3$	1	0	0.5	0	3	1	1	0	2
Lysine	$C_6H_{14}O_2N_2$	1	0	1	0	2	0	0	0	1
Tyrosine	$C_9H_{11}O_3N$	1	0	0	0	1	1	0.882	1	1
Tryptophan	$C_{11}H_{12}O_3N$	0	0	0	0	1	1	1.471	2	1
Phenylalanine	$C_9H_{11}O_2N$	0	0	0	0	1	1	1.176	2	1
Cysteine	$C_3H_6O_2NS$	1	0	0	0	1	1	0	1	1
Methionine	$C_5H_{11}O_2NS$	0	1	0	0	1	1	0	1	1
Threonine	$C_4H_9O_3N$	1	0	0.5	0	1	0	0	−1	1
Serine	$C_3H_7O_3N$	1	0	0	0	1	1	0	1	1
Leucine/Isoleucine	$C_6H_{13}O_2N$	0	0	0	1	1	1	0	2	1
Valine	$C_5H_{11}O_2N$	0	0	1	0	1	1	0	2	1
Glutamine	$C_5H_9O_4N$	1	0	0.5	0	1	1	0	0	2
Aspartate	$C_4H_7O_4N$	1	0	0	0	1	2	0	2	2
Glycine	$C_2H_5O_2N$	1	0	0	0	1	0	0	−1	0
Alanine	$C_3H_7O_2N$	1	0	0	0	1	1	0	2	1
Proline	$C_5H_9O_2N$	0.5	0.5	0	0.5	1	0	0	−1	0

4.1. The Relevance of Microalgae Proteins in the Hydrolysis Stage of Anaerobic Digestion

The first biological process involved in anaerobic digestion is hydrolysis, which is the limiting step and its effectiveness is crucial for the overall process [9,74]. Focusing on proteins, they are hydrolyzed into amino acids by extracellular enzymes secreted by different bacteria such as *Clostridium*, *Vibrio*, *Peptococcus*, *Bacillus*, *Proteus*, or *Bacteroides* [23]. As reviewed above, research devoted to microalgae digestion conducted over last years showed higher methane production in protease pretreated biomass compared to raw biomass and biomass treated with carbohydrases [40]. Methane production of protease pretreated *C. vulgaris* was enhanced by 51% compared to the raw biomass, showing the benefits of having proteins in the soluble phase. Similarly, methane yield enhancement (37%) of

cyanobacteria was also attributed to the proteolytic activity developed upon biomass storage [74]. Even though protease addition has revealed the importance of microalgae proteins in microalgae digestion, it is clear that the use of commercial cocktails would not make biogas production profitable. In this manner, the use of commercial proteases helped in the identification of the macromolecule opposing more resistance to an optimal anaerobic digestion but cheaper alternatives should be investigated for avoiding the addition of commercial enzymes. Two main strategies can be applied for such a purpose. The first one entails the use of in-situ released enzymes by fungi or bacteria. Through the so-called bioaugmentation, microorganisms can be added to the anaerobic sludge used as degradation consortium. In this manner, once identified the microorganisms producing the enzymatic cocktail required for the targeted microalgae biomass, it can be added to the anaerobic sludge. Obviously, the appropriate microbial species should be carefully selected to be effective, not only for microalgae hydrolysis, but also to be viable and present good activity within the anaerobic microbiome. The potential of bioaugmentation, including the main benefits and limitations, has been recently reviewed [75]. This approach has been applied in more conventional substrates while literature available on bioaugmentation strategies devoted to microalgae anaerobic digestion is scarce. Nevertheless, this strategy was successfully applied to improve methane production of *C. vulgaris* biomass [60]. Those researchers showed an enhanced methane yield (18–38%) after adding *Clostridium thermocellum* at various inoculum ratios to degrade the carbohydrate fraction of microalgae biomass. Likewise, the same bacteria, *C. thermocellum*, was reported to enhance methane yield (18–38%) when degrading *Haematococcus pluvialis*. Therefore, this acidogenic phase bacteria is nowadays considered as a promising biotechnological tool to improve anaerobic digestion of microalgae through bioaugmentation.

The second alternative to increase the hydrolytic activity of anaerobic sludge is the use of metals. The addition of trace metals as micronutrients have been proven to stimulate methane production. The dosing needs to be well balanced to support the desired microbial activity or growth rate above which the trace metals become inhibitory or toxic. These metals are essential in the anaerobic reactions, since most of them are part of the active site of enzymes. The effect on different trace metal on anaerobic digestion can be found elsewhere [76]. Even though the use of trace elements is beneficial in most cases, the response of the system is uncertain due to the complexity of the anaerobic digestion process. It is recommended for substrates which initially have low trace element content. For instance, Kim et al. [77] evaluated the effect of trace elements at different range temperatures highlighting the benefits of using Fe, Co. or Ni for the hydrolysis step due to the increase of COD solubilization and organic acids production.

4.2. The Relevance of Microalgae Proteins in the Methanogenesis Stage of Anaerobic Digestion

Out of the subsequent stages involved in anaerobic digestion, hydrogen and acetic acid are converted to methane gas and carbon dioxide during methanogenesis. This last stage is performed by archaea. When compared to anaerobic bacteria involved in anaerobic digestion, archaea are more sensitive to toxic compounds and also exhibit lower growth rates. Acidifiers present ten to twenty-fold higher growth rates and five-fold conversion rates than methanogens [1,69]. With regard to their sensibility toward toxic compounds, methanogens exhibit low tolerance against ammonium nitrogen. Depending on digester pH and operation temperature, the ammonium/ammonia equilibrium might shift. This latter component has been claimed to be highly toxic for methanogens. Ammonia diffuses freely through the permeable membrane of methanogens cells causing changes in intracellular pH and resulting in potassium deficiency and/or proton imbalance [78]. Moreover, ammonium can also inhibit enzymes that are involved in methane production [79]. Yenigün and Demirel [80] reported inhibition of the methanogenesis stage at total ammonia nitrogen (TAN) and ammonia concentrations of 1700–1800 mg/L and 150 mg/L, respectively. As a result, the high concentration of TAN (NH_3 and NH_4^+) can lead to volatile fatty acids accumulation. This last process involves acidification of the anaerobic broth, which in turns inhibits methanogens activity. Therefore, the main drawback of

protein rich biomass, such as microalgae, during digestion is the high amount of nitrogen released in form of ammonium that can inhibit methane formation. In fact, this inhibition has been already evidenced by Mahdy et al. [38] during the digestion of protein rich *Chlorella vulgaris*. Those authors attributed the stepwise methane production decrease to the high nitrogen mineralization (77%) taking place during the digestion of protease pretreated microalgae biomass. In this manner, microalgae proteins are not only limiting the hydrolysis stage of the anaerobic digestion but they might also be detrimental in methanogenesis stage. Similar to the developed strategies to overcome the negative effect of microalgae proteins in hydrolysis, some solutions have been proposed to overcome the issues that proteins might cause in methanogenesis during those last years of research.

To avoid inhibition by ammonium, different strategies can be implemented. One of them entails the use of nitrogen poor media for microalgae cultivation. Due to the low nitrogen availability in the medium, proteins accumulation is restricted while lipids and carbohydrates fractions become more abundant in the grown biomass [81,82]. Biogas production was modified using this method in different studies [80,83]. This strategy can be easily applied by using urban wastewater as culture media, which normally contains considerable lower nitrogen concentrations than synthetic salt media (≈60 vs. 300–600 mg N/L). The benefit of this strategy has been evidenced recently using *Spirulina* biomass for biogas production [12]. Similar results were obtained with *C. vulgaris*, where a higher accumulation of carbohydrates (40%) was observed when microalgae was grown in urban wastewater while only 22% was obtained in biomass grown in synthetic medium. Concomitantly with the increase in carbohydrates, protein biomass content was reduced (from 64 to 33%) and thus, methane production was enhanced [40].

A second approach to avoid ammonium inhibition is through sludge bioaugmentation. This approach consists in introducing or enriching specific anaerobic microorganisms with special features. Thus, anaerobic microorganisms that are tolerant to high NH_4^+ concentrations should be used within the anaerobic sludge to accomplish this goal. Although it is generally believed that total ammonia levels above 3 g/L have toxic effect on the methanogens, the resistance of methanogens can be increased by exposing the microorganisms to high nitrogen concentrations [83]. The use ammonia tolerant inocula has been recently demonstrated as an efficient option for digestion of *C. vulgaris* and cattle manure [84]. In this study, the effectiveness of adapted methanogens resulted in a 33% methane yield increase. This approach allowed operating the digester at 3.7–4.2 g NH_4^+-N/L. Tian et al. [85] operated an acclimation experiment in continuous anaerobic reactors fed with substrate rich in the protein fraction such as microalgae and cattle slurry manure. Results showed a stable biomethanization process despite of the high ammonium concentration (10 g NH_4^+-N/L). Authors stressed the changes on the anaerobic population taking place as the responsible feature to handle high ammonium concentration. Even though this biological strategy is very promising, it is necessary to do further research due to the challenges that might arise such as the different behavior that the bioaugmented inocula under different operational conditions imposed in the reactors. Attention must be directed to microorganism's population since they might fail to thrive or be washed out from the reactors.

5. Conclusions

Anaerobic digestion of microalgae has been presented as a promising alternative for generation of bioenergy. The implementation of this process requires pretreatment of the rigid algae cell wall in order to make available the organic matter to anaerobes. Enzymatic pretreatment with proteases showed the best performance in terms of organic matter solubilization and methane production. This feature already highlighted the importance of proteins in the hydrolysis stage of anaerobic digestion. Solving this problem with protease addition could result in methanogens inhibition mediated by high ammonium concentrations reached during nitrogen mineralization. Two solutions are proposed to overcome potential inhibition, namely the reduction of nitrogen levels of microalgae biomass using a low nitrogen concentration culture media and the use of ammonium tolerant anaerobic inocula. This fact showed that protein embedded in microalgae cell wall might be responsible for their

inherent low biodegradability. Microalgae proteins might be crucial not only in the hydrolytic phase but also during methanogenesis.

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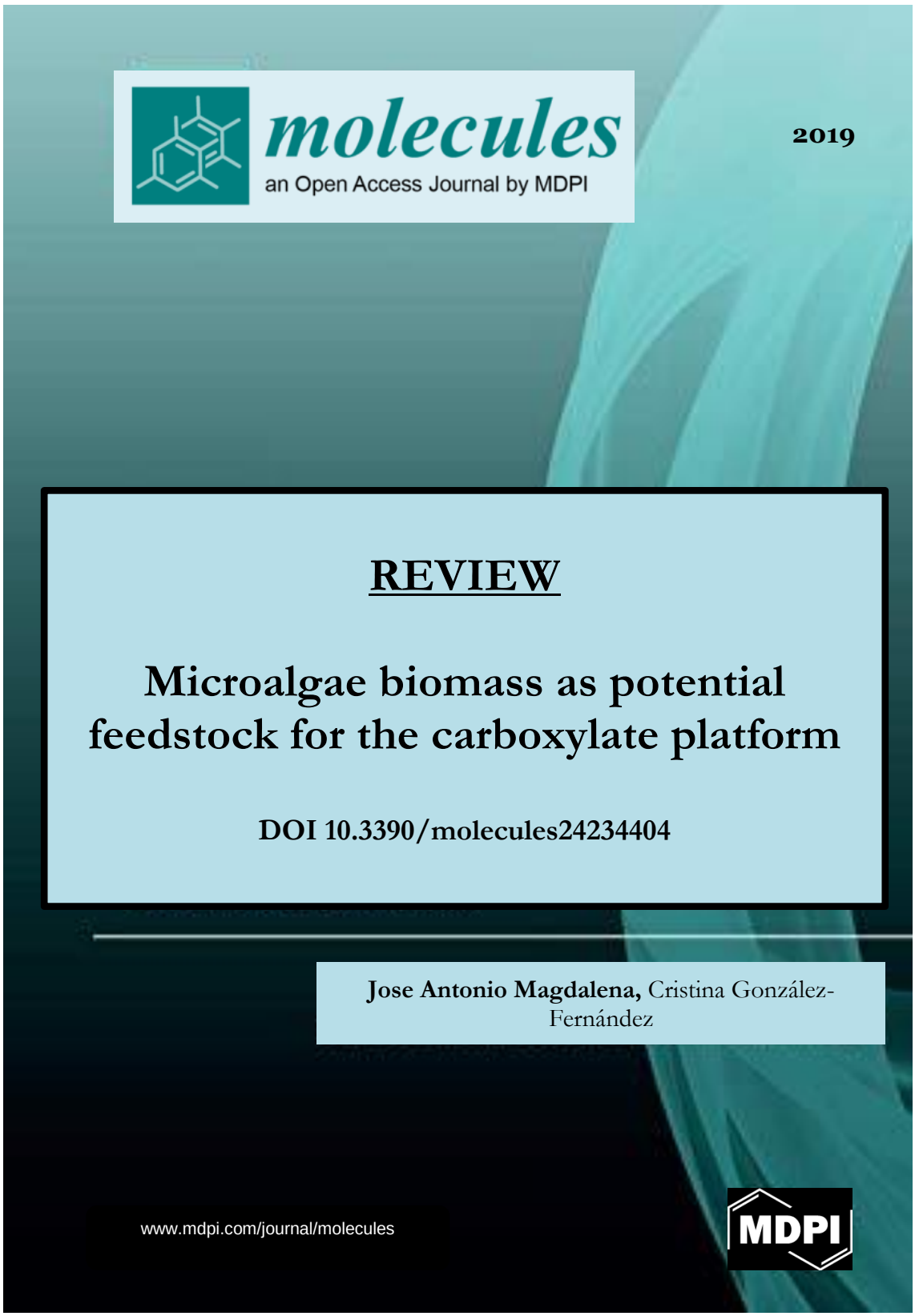
PUBLICATION II



molecules

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Review

Microalgae Biomass as a Potential Feedstock for the Carboxylate Platform

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Abstract: Volatile fatty acids (VFAs) are chemical building blocks for industries, and are mainly produced via the petrochemical pathway. However, the anaerobic fermentation (AF) process gives a potential alternative to produce these organic acids using renewable resources. For this purpose, waste streams, such as microalgae biomass, might constitute a cost-effective feedstock to obtain VFAs. The present review is intended to summarize the inherent potential of microalgae biomass for VFA production. Different strategies, such as the use of pretreatments to the inoculum and the manipulation of operational conditions (pH, temperature, organic loading rate or hydraulic retention time) to promote VFA production from different microalgae strains, are discussed. Microbial structure analysis using microalgae biomass as a substrate is pointed out in order to further comprehend the roles of bacteria and archaea in the AF process. Finally, VFA applications in different industry fields are reviewed.

Keywords: anaerobic fermentation; carboxylate platform; microalgae; microbial communities; operational conditions; volatile fatty acids

1. Introduction

Under the Europe 2020 growth strategy, the European Union (EU) is currently updating its legislation to promote a shift to a more sustainable model known as a circular economy [1]. The use of waste streams and renewable resources appear as a core priority to reduce the actual carbon footprint of the state members. These directives prioritize the development of efficient alternatives to the traditional fossil fuels employed for energy and commodity generation. Nowadays, one of the investigation lines gaining importance is the use of microbial consortia to produce high value added products such as carboxylates (volatile fatty acids, VFAs) through anaerobic fermentation (AF), mostly known as the carboxylate platform [2,3]. Traditionally, anaerobic digestion (AD) converts complex substrates into biogas, containing methane (bioenergy) and a digestate. However, this new approach involves the conversion of biomass to bulk chemicals (bioproducts), which is economically more profitable than biogas production [4,5]. Acetic, propionic, (iso)butyric, (iso)valeric and caproic acids are VFAs traditionally obtained through the petrochemical pathway. These compounds can be further used as building blocks in different fields of the industry including food additives, pharmaceuticals, adhesives, solvents or chemical intermediates [6,7].

Among the feedstocks that can be subjected to AF, microalgae biomass arises as a potential alternative for VFA production. It should be highlighted that this biomass can be valorized for high value bioproducts instead of being produced specifically for VFA generation. More specifically, one of the weaknesses of microalgae-based bioproduct production is the nutrients required. However, when this technology is combined, for instance, with wastewater bioremediation by photosynthetic means, the overall balance becomes positive [8]. When cultivated in this manner, biomass cannot be

used for nutraceutical, feed or food purposes. Moreover, the harvested biomass is normally poor in fermentable sugar or transesterificable lipids, and thus, the most straightforward use of algal biomass is AD.

VFA production requires a revisit of the AD process for better comprehension of the overall process as this technology has been traditionally used for biogas production. In this sense, there are different variables that deserve further study, such as (i) the substrate employed, (ii) the operational conditions imposed, and (iii) the developed microbiome within the bioreactor. The use of waste streams is cost-effective, and also helps decrease the overall process costs as it contributes to residues management. However, microorganisms in AD are often not able to directly utilize complex organic matter. Hence, a pretreatment step is needed prior to digestion by using physical, chemical, or enzymatic methods to increase the soluble organic matter availability [9,10]. Additionally, there is a need to consider factors related to the operational conditions in the system. Temperature, inoculum used, retention times (solid and hydraulic), organic loading rate (OLR) and pH directly affect VFA production and profiles [11,12]. Finally, the existing bacterial populations must assure a good conversion of organic matter into VFAs. For such a goal, it is considered crucial to inhibit the methanogenic population to accumulate VFAs. As a matter of fact, archaea activity is linked to secondary syntrophic carboxylate-oxidation reactions of propionic and butyric acids to acetate and hydrogen, reducing the amount of VFAs in the digestate [3].

The aim of this work is to review the potential advantages of waste streams as feedstock for VFA production, paying specific attention to microalgae biomass. In addition, the operational conditions and the microbiome related to the acidogenic stage of the AD will be reviewed.

2. Volatile Fatty Acid Production by Means of Anaerobic Fermentation

Volatile fatty acids (VFAs) are formed during the fermentative stages (acidogenesis and acetogenesis) of the AD process (Figure 1). These chemicals are very versatile as building blocks and are commonly used in different industrial processes. The range of applications is quite broad [7,13]. For instance, acetic acid has an important role in the food industry [14], propionic acid is mainly used as an acidifier for animal feed and grain [15], and butyric acid can be utilized as a precursor for biofuel production [16]. Therefore, VFA production through biological catalysis in the AF process is a very promising technology due to its wide range of applications. VFA production through AF occurs at milder temperature and pressure conditions than petrochemical pathways, which is beneficial in terms of energy consumption [17].

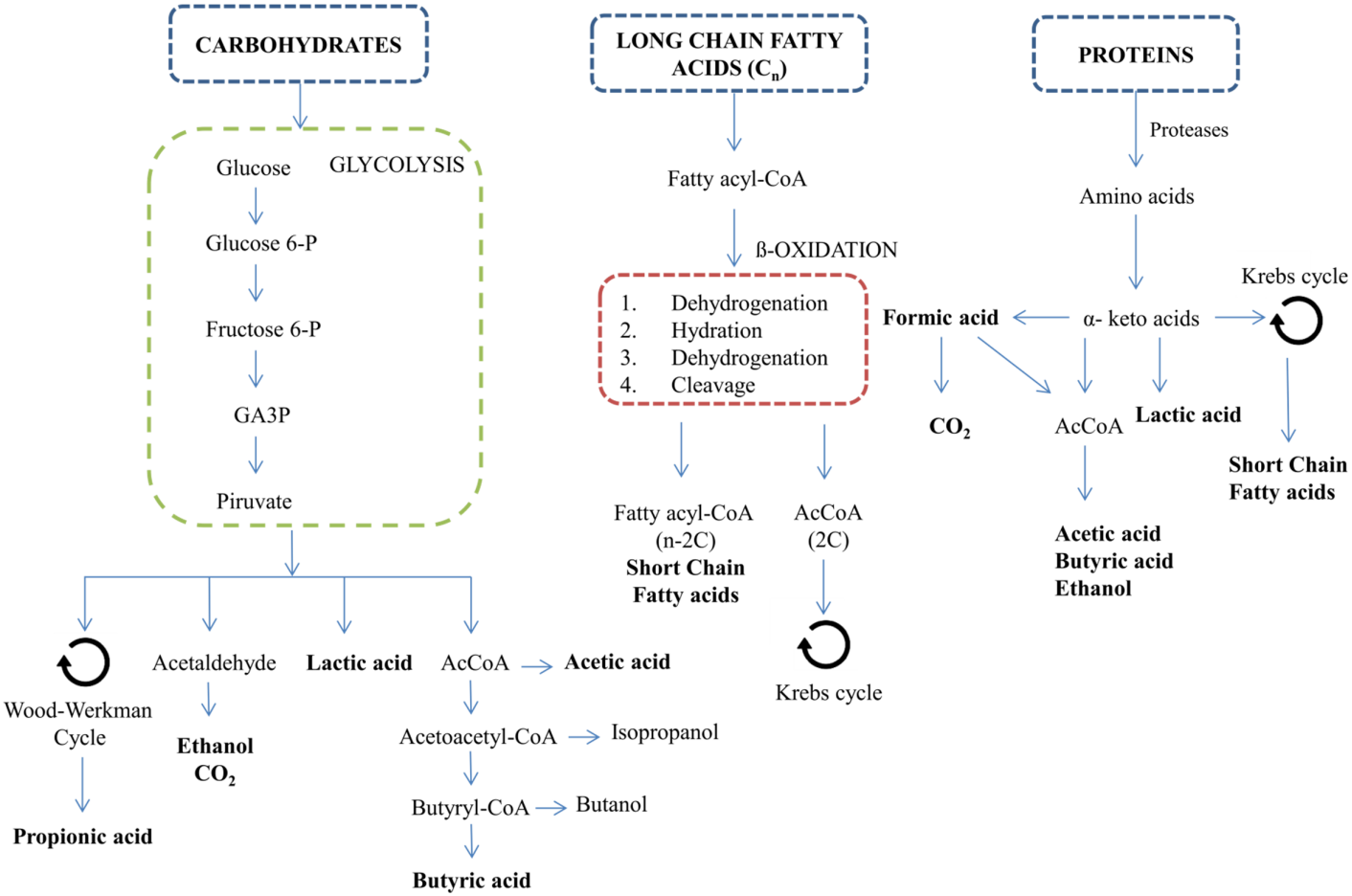

Figure 1. A simplified overview of metabolic pathways involved in VFA synthesis from the main macromolecules of microalgae biomass. GA3P, glyceraldehyde-3-phosphate; AcCoA, Acetyl-CoA.

AD is a robust and well-known process, and a wide variety of substrates can be subjected to this technology regardless of their macromolecular composition. The substrate chosen undergoes four different steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis. Firstly, exo-enzymes belonging to hydrolytic bacteria degrade the complex organic matter composed of carbohydrates, proteins and lipids into their respective monomers namely, sugars, amino acids and long chain fatty acids. The efficiency of this stage often conditions the overall process yields, as it determines the total soluble organic matter availability. Secondly, the acidogenic bacteria anaerobically oxidize the soluble monomers originating from the VFAs, CO_2, alcohols, H_2, and lactic acid. Afterwards, acetic acid, CO_2 and H_2 are produced by acetogenic bacteria. These products are the main substrates for the methanogenic archaea, which are in charge of the methanogenic stage. These microorganisms can be classified into two different groups depending on the substrate metabolized for biogas production. Acetoclastic archaea use acetic acid to produce methane, whereas hydrogenotrophic microorganisms use H_2 and CO_2 as the main substrates to obtain methane. The inhibition of this latter step of the AD is considered crucial as, otherwise, VFAs would be degraded and finally transformed into biogas (Figure 2).

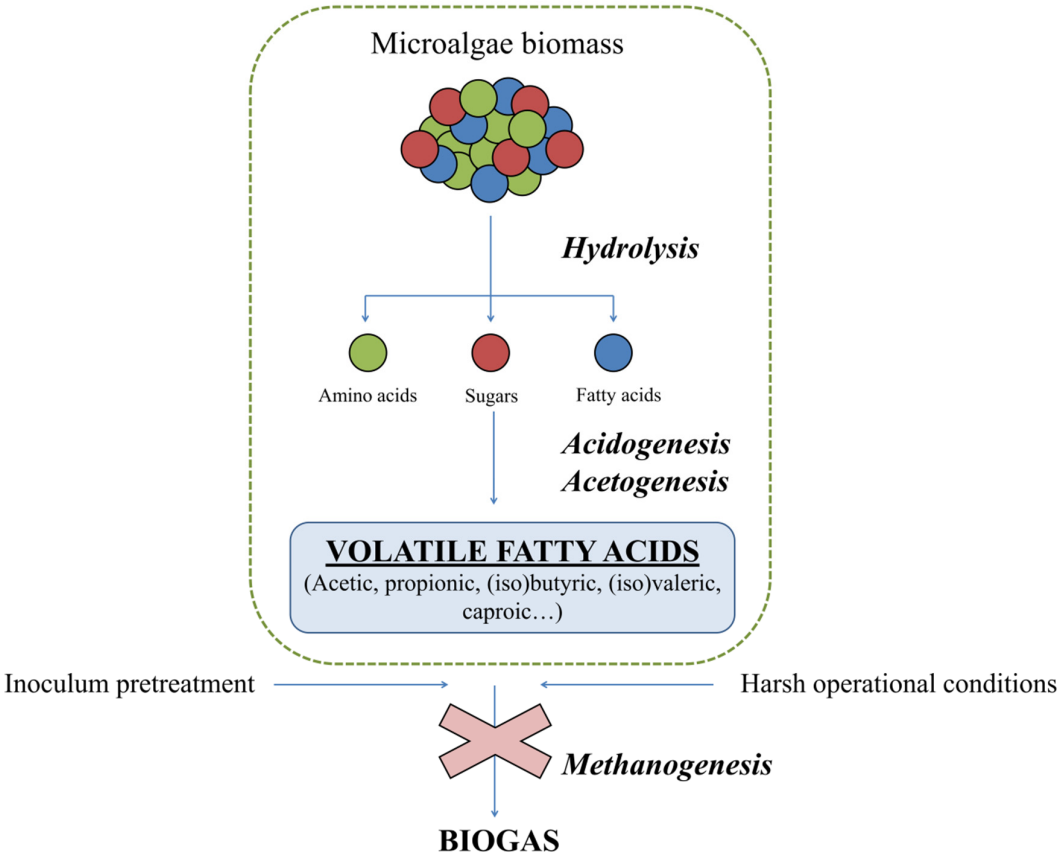

Figure 2. The use of microalgae biomass as feedstock for the carboxylate platform.

2.1. Microalgae Biomass as a Substrate for VFA Production

The selection of a cost-effective substrate for VFA production is of paramount importance to decrease the overall production cost. Up to date, different sugar-based carbon sources have been tested for VFA production [18,19]. However, cheaper substrates such as waste streams could be ideally used to reduce the costs of the process. Microalgae, for instance, can be considered a residual stream when grown in wastewater. Microalgae-based systems for wastewater treatment have been

shown to be a promising technology [8,20]; however, the biomass generated can be further processed into something more valuable than biogas.

The physical state and composition of a substrate directly affect the hydrolysis efficiency and hence, the VFA conversion yields. In this sense, microalgae biomass is considered a complex substrate and that is why recent research efforts have been conducted to improve the hydrolysis step [9]. Different pretreatment methods (e.g., thermal, mechanical, chemical or biological) have been proven to increase the solubilization of the organic matter by means of cell wall disruption/hydrolysis. These pretreatment techniques have been widely studied for biogas production using microalgae biomass [17,21]. These methods could be an interesting option to improve VFA production from microalgae biomass.

In addition to the cell wall protecting microalgae cells, another important aspect is the macromolecular distribution of the substrate that can be classified regarding its content in carbohydrates, proteins and lipids. With regard to microalgae biomass, the amount of each macromolecule appears to be very variable depending on the growth conditions and the strain assessed [22]. However, in general terms, proteins are the most abundant macromolecule of green microalgae, accounting from 30 to 60% of their dry weight [23]. The AD of a protein-rich substrate might constitute a drawback for biogas production. Indeed, this macromolecule has been shown to hamper biogas production when using protein-rich microalgae biomass [9,24]. Nevertheless, it may turn out as an attractive feedstock characteristic when AF is directed towards VFA production. The first stage of the AD entails the hydrolysis of the complex macromolecules composing the microalgae biomass. During this phase, the protein fraction is cleaved into simple amino acids and releases the nitrogen contained in the form of ammonium (NH_4^+), and free ammonia (NH_3). The amount of each species relies on pH and temperature conditions. High amounts of these compounds [25] are often associated with digestion failure when targeting biogas production due to the inhibition of the methanogenic step [26,27]. Therefore, this methanogenic weakness towards NH_4^+ and NH_3 seen in biogas production might in fact represent an advantage for VFA generation, as the inhibition of this microbial community would contribute to VFA accumulation.

Proteins, carbohydrates and lipids present different hydrolysis rates [28]. As a result, different VFA conversion yields and profiles can be obtained depending on the substrate composition (Table 1). Results collected in Table 1 suggest that besides the macromolecular composition of the microalgae biomass, there are other factors affecting VFA production and profiles, such as the operational conditions established and the microorganisms carrying out the biological process.

Table 1. Overview of microalgae strains employed in batch and semi-continuous mode for VFA productions: VFA profiles and conversions.

BATCH MODE														
Strain	**Temperature (°C)**	**pH**	**Pretreatment**	**Composition**	**% DW**	**Ac**	**Pr**	**But**	**IBut**	**Val**	**IVal**	**Cap**	**COD-VFAs/CODin (%)**	**Reference**
Chlorella vulgaris	15	6.4	-	Carbohydrates	47.5	70	30	0	-	-	-	-	17.37	[29]
	35			Proteins	20.4	70	20	10	-	-	-	-	38.17	
	55			Fatty acids	0.9	70	10	20	-	-	-	-	40.47	
C. vulgaris	15	6.4	-	Carbohydrates	28.6	70	30	0	-	-	-	-	9.44	[29]
	35			Proteins	56.8	50	40	10	-	-	-	-	33.40	
	55			Fatty acids	0.004	60	10	30	-	-	-	-	42.03	
Chlorella sp.	25	5.5	Enzymatic (proteases)	Carbohydrates	25	41	28	7	9	7	8	-	47.7	[30]
	35			Proteins	64	26	35	9	12	8	9	-	39.1	
	50			Lipids	10	33	11	15	14	-	27	-	34.5	
Chlorella sp.	25	7.5	Enzymatic (proteases)	Carbohydrates	25	54	21	6	6	6	7	-	45.1	[30]
	35			Proteins	64	57	21	6	8	1	7	-	48.3	
	50			Lipids	10	46	17	12	15	-	9	-	37.1	
Microcystis	25	10	Control	Carbohydrates	6	38	14		36		12	-	13.09	[31]
			0.5 Activated carbon (g/L)	Proteins	63	50	13		21.1		15.9	-	31.50	
				Lipids	4									
Chlorella Pyrenoidosa	35	6.5	-	Carbohydrates	34									[32]
				Proteins	48	52	11	35	-	-	-	3	4.28	
			140 °C, 10 min 1% H_2SO_4	Lipids	18	41	14	37	-	-	-	8	9.14	
Scenedesmus quadricadua and *C.vulgaris*	35	5	Non pretreated	NA		40	20	5	20	15	-	-	0.25	[33]
		7.4				50	20	5	10	10	5	-	5.42	
				Carbohydrates	19									[34]

Arthrospira Platensis	37	6	2.5% dilute H_2SO_4 at 135 °C for 15 min	Proteins Lipids	76 5	40	5	28	5	5	9	8	NA	
Desmodesmus sp.,*Scenedesmus* sp., and *Chlamydomonas* sp	35	6.9	-	Carbohydrates	NA	86	2	2	0	0	10	0	20.0	[35]
	45			Proteins	NA	74	8	5	2	0	10	0	33.0	
	55			Lipids	NA	66	15	5	1	2	11	0	50.0	
Ettlia sp	35	7.2	1% NaOH + ultrasound	Carbohydrates Proteins Lipids	45.5 35 5.5	64	25	11	-	-	-	-	25.25	[36]
SEMI-CONTINUOUS MODE														
Strain	**Temperature (º C)**	**Operational conditions**		**Composition (%) DW**		**Ac**	**Pr**	**But**	**Ibut**	**Val**	**Ival**	**Cap**	**COD-VFAs/CODin**	**Reference**
Scenedesmus sp. Frozen	35	HRT 15 days		Carbohydrates	45	11	3	57	18	3	3	4	0.171 g COD-VFAs/g VSin	[37]
	55	OLR 2.5 VS/Ld		Proteins Lipids	44 4	14	1	48	2	0	0	0	0.088 g COD-VFAs/g VSin	
C. vulgaris Enzymatic pretreatment	35	HRT 10 days OLR 1.5 g COD/Ld				14	36	10	11	11	18	-	25.6	[38]
	35	HRT 10 days OLR 3 g COD/Ld		Carbohydrates	21.6	18	32	12	10	11	17	-	25.8	
	25	HRT 10 days OLR 1.5 g COD/Ld		Proteins	57.9	20	17	9	17	15	12	9	35.4	
	25	HRT 12 days OLR 1.5 g COD/Ld		Lipids	13.4	24	16	8	20	14	18	13	38.0	
	25	HRT 8 days OLR 1.5 g COD/Ld				24	15	8	20	14	18	12	39.8	

* All the investigations collected in Table 1 were carried out at the lab scale; COD: Chemical oxygen demand.

2.2. Operational Conditions for VFA Production

The manipulation of process variables such as inoculum, pH, temperature, HRT and OLR have a great influence not only on VFA accumulation, but also on the obtained VFA profiles [11,12]. This is because these operational conditions ultimately affect the delicate relations among microbial populations. Methanogenic archaea are more sensitive to operational changes than organic acid producers [39]. As it was aforementioned, archaea are the main organisms responsible for VFA consumption, and thus, their inhibition is considered of paramount importance for attaining competitive VFA yields.

2.2.1. Inoculum

Microorganisms present in the anaerobic sludge are very diverse as many species are involved in the AD process. When selecting microalgae biomass for AD, it is important to take into account the interactions with the anaerobic microbiome. For instance, marine microalgae species, such as *Isochrysis galbana*, *Dunaliella salina* or *Nannochloropsis salina,* have been proposed for AD for biogas production [40–42]. These strains hinder the AD process due to high salinity causing plasmolysis in the anaerobic populations (VFA producers and archaea) due to high external osmotic pressure. A long acclimation period for the inoculum, the use of compatible solutes and the employment of halophilic populations are regarded as strategies to be applied to the inoculum to overcome these issues in order to be able to use these species as substrates.

Each stage of the AD process is characterized by different groups of microorganisms. Among others, organic acid-producing bacteria are distinguished during the fermentative stages (hydrolysis and acidogenesis), and methanogenic archaea during methanogenesis. The species involved use different molecules as substrates, and release different products according to their metabolism, resulting in a complex scheme of reactions and products. Therefore, when VFA production is desired, reduction of methanogenic archaea in the inoculum is appropriate to avoid VFA consumption. Strategies applied to the inoculum, such as thermal pretreatments and the addition of chemicals, have been tested. Thermal pretreatment implies subjecting the inoculum to high temperatures during determinate periods of time with the aim of eliminating non spore forming microorganisms. This type of pretreatment has been applied in the literature to substrates other than microalgae [43,44]. A mixture of *Desmodesmus* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. was digested with an anaerobic inoculum subjected to a thermal pretreatment (100 °C for 2 h) to inactivate methanogens and the results showed organic matter conversion into VFAs up to 50% VFAs-COD/CODin at 55 °C [35]. Additionally, pretreatment of inoculum at 120 °C for 10 and 30 min using *C. vulgaris* as the substrate rendered organic matter conversion into VFAs up to 71% [45]. On the contrary, low temperature pretreatments in this study promoted biogas production. However, thermal pre-treatments should be conducted in such a way that only methanogens are affected, as conditions that are too harsh can not only eliminate methanogens but also organic acid producers [46].

The addition of chemicals is used to block methanogen enzymes. Different compounds have been used for this goal, such as 2-bromoethanesulfonate (BES), iodoform or chloroform. In this context, BES (50 μmol/mL) prevented methanogenesis when microalgae biomass composed of *S. quadricadua* and *C. vulgaris* was used for VFA production [33]. This trend was maintained when treating an inoculum with BES (10 and 30 mM) [45]. No methane was detected and VFAs accumulated up to 50% VFAs-COD/CODin. In addition, iodoform (30, 50 and 70 ppm *v*/*v*) inhibited methanogens, causing VFA accumulation, when *Laminaria japonica* was employed as a substrate in an AD process (35 °C and pH 6.5–7) [47]. VFA concentration (8 g/L VFAs) was maximized when using 50 ppm of iodoform, whereas further increases negatively affected VFA productions (70 ppm, reported values similar to those found in the control, 6 g/L), suggesting the negative effect of iodoform on the rest of the microbiome.

In general, the use of chemicals and thermal pretreatments applied to the inoculum are able to inactivate methanogens. Nevertheless, the high prices, the environmental concerns, and the high energy input requirements are the main drawbacks. In addition, these strategies often show short-

term effects on the continuous operation towards methanogens and thus, other methods (manipulation of operational conditions) are recommended for VFA accumulation.

2.2.2. pH

pH influences the growth rate of the fermentative microorganisms in charge of VFA production and the optimum enzymatic activities during the hydrolytic step. Moreover, each group of microorganisms has an optimum pH working value. Whereas methanogenic archaea grow better at a pH close to neutrality, acidogenic and hydrolytic bacteria have a wider pH growth range. Previous studies have estimated the optimum range for the acidogenic bacteria at around 5 to 7 [19,48]. Investigations regarding the effect of pH on VFA productions from microalgae biomass showed different results, most likely due to the wide range of microalgae strains and operational conditions employed. For instance, digestion of *Chlorella* sp. at acidic pH values (5.5) and 25 °C showed up to 47% VFAs-COD/CODin, similar to what was attained in the same experiment at neutral pH values and the same temperature (7.5, 45.1% VFAs-COD/CODin) [30]. However, the use of pH values in the basic range has also resulted in good organic matter conversions into VFAs. The digestion of *Microcystis* at pH 10 and 25 °C retrieved an organic matter conversion into VFAs of 31.5% VFAs-COD/CODin. In this sense, some authors pointed to the higher hydrolysis rates achieved at basic pH values as the reason for the conversion yield attained [49]. This feature was also observed when using other protein-rich substrates [50]. Both studies gave acetic and propionic acids as the main fermentation products. Other investigations using microalgae biomass as a substrate for VFA production followed this trend (Table 1).

2.2.3. Temperature

Similar to pH, 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 and determines the development of certain microbial communities impacting VFA production and profiles. High fermentation temperatures (50 °C) resulted in high conversion yields (40% VFAs-COD/CODin) when non-pretreated *Chlorella* sp. was digested at pH 6.4, while the use of 25 °C and 35 °C mediated lower conversions (17 and 38% VFAs-COD, respectively) [29]. On the contrary, other investigations obtained similar conversion yields (45–48% VFAs-COD/CODin) at temperatures of 25 °C and 35 °C when compared to 50 °C (37% VFAs-COD/CODin) when using protease pretreated *Chlorella* sp. as a substrate [30]. These differences might rely on the state of the biomass (raw or pretreated). Whereas Magdalena et al. (2018) used a proteolytic pretreatment, Kim et al. (2019) did not hydrolyze the biomass prior to AD. Hence, the high temperatures at which this latter investigation was conducted most likely increased biomass solubilization, and thus, VFA yields, at the highest temperature. With regard to VFA profiles, acetic and propionic acids were the most abundant products regardless of the temperature employed and the butyric acid fraction gained importance at higher temperatures in these experiments [29,30].

2.2.4. Organic Loading Rate (OLR)

The organic loading rate (OLR) is the amount of organic matter present in the substrate applied to a certain volume of media per unit of time. OLR selection is process specific and has been studied previously for biogas production [51]. The general trend observed with other substrates is an increasing VFA production at stepwise OLR increases [52]. VFA accumulation leads to a drop in pH and a final decay of methanogens. Nevertheless, it is also true that there is a maximum OLR threshold where no further improvements are obtained. This fact might be explained by taking into account the hydrolytic stage of the AD process. When reactors are fed at high OLR (values), the hydrolytic capacity of the system is exceeded, and thus, the process becomes unstable and no further improvement is noticed. A recent study analyzing the effect of stepwise OLR increases (3, 6, 9, 12, 15 g COD/Ld) for VFA production using *C. vulgaris* as a substrate revealed an optimum VFA production at 12 g COD/Ld (0.37 ± 0.02 COD-VFAs/CODin) with respect to the highest OLR (0.29 ± 0.01 COD-

VFAs/CODin) [53]. Authors used a proteolytic pretreatment to overcome hydrolytic deficiencies and highlighted that the bottleneck of the process was found to be the acidogenic stage, most probably due to the combined effect of high ammonium, VFAs and Na^+ concentrations.

2.2.5. Hydraulic Retention Time (HRT)

The hydraulic retention time (HRT) is a design parameter that establishes the time that the organic matter remains in the reactor. It is closely related to the OLR selected for the process. At low HRT values, microorganisms exhibiting low growth rates are possibly washed out from the reactor as they do not have enough time to grow and adapt to the harsh environmental conditions. These conditions may provoke a drop in the species diversity present in the system as certain species are not able to grow [38]. On the contrary, when HRT values are high, more populations are likely to grow and take part in the AD process. In this context, methanogenic archaea have been reported to exhibit lower growth rates than acidogenic bacteria [54]. Therefore, this parameter could be used as a tool to select the most suitable populations in charge of organic acid accumulation, as the use of low HTR could favor the wash out of methanogens. However, values for HRT must be high enough to allow the anaerobic microorganisms to carry out the hydrolysis and acidogenesis of the substrate. For instance, the use of low HRT favored VFA production in a semi-continuous bioreactor fed with *C. vulgaris* in which the use of HRT for 8 days showed higher productivities than 10 and 12 days, most probably because of a better activity of methanogens at higher HRTs [38]. This fact means that maximum conversion yield can be achieved in a shorter period of time than that needed for biogas production with a direct impact on the reduction of the total economic process costs. For instance, HRT of 15 and 20 days has been used for the AD of *C. vulgaris* biomass for biogas production [27], while the HRT can be reduced to 8 days for VFA accumulation purposes.

Therefore, considering the high heterogeneity of microalgae strains and macromolecular composition, even among the same species, as well as the different operational conditions imposed on the system, it is only possible to set approximate guidelines for maximum conversion of organic matter into VFAs.

3. Microbial Populations Involved in VFAs Productions

Microbial populations present in an anaerobic inoculum have a determining influence on the AD performance. Each AD stage presents different microorganisms (hydrolytic and fermentative bacteria) during hydrolysis, acidogenesis, acetogenesis and (methanogenic archaea) during methanogenesis. The relative abundance of species during AD might affect the fate of the organic matter. In this sense, manipulation of operational conditions could promote organic acid producers and inhibit those microorganisms in charge of methanogenesis to enhance VFA accumulation. In addition, the study of microbial populations can shed light on AD assigning roles to microbial populations.

Microbial structure is widely dependent on the AD final goal, either biogas or VFA production. This difference is caused by the operational conditions imposed on the system (Section 2.2) resulting in a sludge specialization. In this sense, a recent study regarding the metagenome for biogas generation highlighted the high flexibility, diversity and adaptability to operational conditions and substrates of the anaerobic community [55]. Opposite to that, reactors involved in VFA production are often less diverse and exhibit different species than those devoted to biogas production.

With respect to the bacterial community, Firmicutes, Proteobacteria and Bacteroidetes have been identified as the major contributing phyla in anaerobic fermenters devoted to VFA productions. These phyla have been claimed to produce VFAs as well as actively degrade proteins and polysaccharides, which in fact represent a high percentage of the macromolecular distribution of microalgae biomass (Table 1) [56]. The PCR-DGGE analysis carried out at different temperatures (35, 45 and 55 °C) when microalgae biomass was digested for VFA production displayed a clear dominance of microbial species belonging to Firmicutes, Proteobacteria and Bacteroidetes [35]. Furthermore, this investigation also concluded that diversity decreased at the highest temperature, in which VFA production achieved the highest conversion (COD-VFAs/CODin). Following this

trend, Proteobacteria (65.7%) and Firmicutes (29.0%) were dominant when activated carbon was used to enhance VFA production from *Microcystis* [31]. Likewise, species belonging to Firmicutes were the most abundant (45–70% in terms of relative abundance) followed by Bacteroidetes (10–35%) when cyanobacterial biomass was digested for VFA production [57]. A similar investigation also remarked on the presence of the Firmicutes phylum with species such as *Sporanaerobacter acetigenes* (13%) or *Soehngenia* (12%), identified as the major VFA producers when the microalgae strain *Ettlia* sp. was subjected to AD [58]. All of these investigations were carried out at batch scale; however, when operating semi-continuous fermenters fed with *C. vulgaris*, Fimicutes dominated in the bacterial community [38].

Concerning the Euryarchaeota phylum, these species are detrimental for VFA accumulation [3]. Hence, their inactivation is of high importance in achieving competitive VFA production. According to their metabolism, archaea species can be divided into acetoclastic or hydrogenotrophic. The latter ones are often more robust than the acetoclastic ones [59]. Thus, it is expected that in the case that biogas is produced, reactors devoted to VFA production remove organic matter through the hydrogenotrophic pathway. In fact, hydrogenotropic species such as *Methanobacterium* were reported by Magdalena et al. (2019), while other investigations did not find a significant archaea, acetoclastic or hydrogenotrophic presence [58]. In this particular case, those authors applied daily iodoform (8 mg/L) to suppress any methanogenic activity and this would explain the lack of archaea.

Overall, the reviewed investigations related to the microbial structure of bioprocesses aiming at VFA production are clearly different to the ones using microalgae biomass as a substrate for biogas generation. The relative abundance of each phylum is dependent on the operational conditions established in the reactor. Hence, the use of harsh operational conditions to inactivate methanogens and promote VFA producers results in a sludge specialization, where methanogenic activity is outcompeted by fermentative bacteria. This fact might hamper biogas production and in turn boost VFA accumulation.

4. VFAs As Building Blocks for the Industry

VFAs produced from microalgae biomass fermentation might be a product by itself (after separation and purification) or serve as platform molecules for different applications within several fields in the industry. Some of the promising applications that these molecules might encounter include the production of biodegradable plastics such as polyhydroxyalkanoates (PHAs), energy generation from microbial fuel cells (MFCs), VFA elongation into longer fatty acids via reverse ß oxidation and their use as building blocks for oil-based chemistry via oleaginous yeast fermentation.

PHAs are currently produced using microbial isolates and well-defined substrates, which increase overall production costs [60]. However, VFAs produced from waste streams appear as a promising alternative to reduce the price of the process [61]. In this sense, PHAs might be produced from the VFAs present in the digestate obtained after microalgae fermentation. Filtering this broth is advised to remove microorganisms and to control the amount of ammonium and phosphorous to allow PHA production [62]. Results using different fermented wastes as substrates in mixed cultures have resulted in microbial systems exhibiting PHA contents in the range of 40–77% (DW %) [7], whereas other authors have addressed the importance of VFA distribution on final PHA composition [63,64].

Another application might be the electricity generation in MFCs [65]. MFCs are made up of an anode where the biofilm oxidizes the soluble VFAs, producing electrons. This current flows towards the cathode where an electron acceptor is reduced. Recently, this technology has attracted the attention of the scientific community [66,67], but operational conditions still need to be optimized as process yields significantly vary depending on the VFA profiles [68]. As a matter of fact, the investigation conducted by Teng et al. (2010) found a different contribution of acetic, propionic and butyric acids to electricity generation. Those authors attributed electricity generation mainly to the presence of acetic and propionic acids, whereas butyric acid exerted a negative impact [69].

The chain elongation (CE) process transforms short VFAs (C2–C5) into medium carboxylates (C6–C12) [52]. These compounds have more value than biogas or VFAs and can be further used in

several fields of the industry (aviation fuels, solvents, lubricants or feed additives) [70]. In addition, C6–C12 organic acids are more hydrophobic than shorter VFAs. This feature makes them more attractive as a product because it facilitates the subsequent recovery step. The CE is catalyzed by an anaerobic microbiome in strict anaerobic conditions in a metabolic pathway called reverse β-oxidation. In this pathway, an acetyl CoA molecule is added to a carboxylate (acetate), finally elongating two carbons at a time. The oxidation of an electron donor such as ethanol, methanol, hydrogen or lactic acid is necessary for this process to take place. The impact of different operational conditions, such as the selected electron donor, methane inhibitor or the substrate used, on medium carboxylate production has been studied [71]. In general, low productivities are attained due to the use of mixed culture fermentations, and thus, the study of the microbiome may serve to enhance process yields.

Finally, VFAs are regarded as low-cost carbon sources for lipid biosynthesis to produce oil-based products [72]. Oleaginous yeasts such as *Yarrowia Lipolytica* or *Cryptococcus Curvatus* can accumulate up to 60% of their dry weight in the form of lipid bodies [73]. The use of VFAs obtained from waste such as microalgae can help decrease the overall process production costs mainly impacted by the high price of current substrates [74]. The similar characteristics of plant and microbial oils (similar fatty acids profile) make microbial oil production a promising biotechnological tool for biofuel and bioproduct generation.

5. Conclusions

Overall, the use of the carboxylate platform from microalgae biomass might be useful for added-value product generation as well as a feasible technology for proper waste management. Microalgae biomass arises as a potential feedstock for bio-based VFA productions. The effect of operational conditions on VFA production was reviewed. There are yet no optimum operational conditions for VFA production considering the amount of microalgae strains and conditions employed and thus, further investigation is still needed. To fully understand how these variables influence VFA production and profiles, a possible approach might be to direct attention towards the microbial communities developed during the reactor operation. As a matter of fact, operational conditions are interconnected with the microbiome and hence, the study of the combined effect might result in valuable information for VFA production from microalgae biomass.

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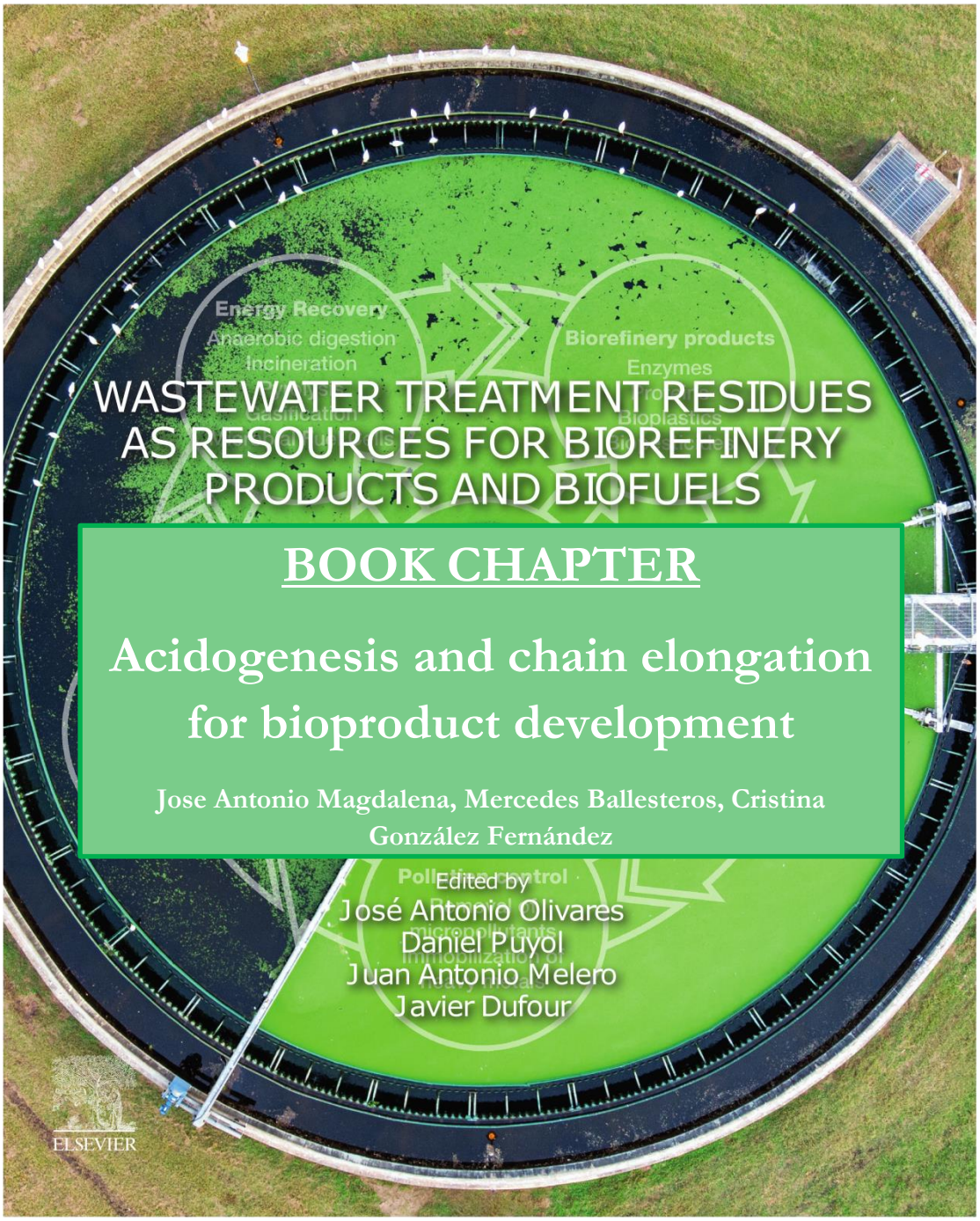
PUBLICATION III



CHAPTER

17

Acidogenesis and chain elongation for bioproduct development

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Introduction

Nowadays, energy and commodities utilized worldwide are mainly produced by the petrochemical industry. Petroleum is essential in fields such as transportation, manufacturing, household products, agriculture, and forestry. However, the increase in the oil demand motivated by population growth and environmental concerns, such as climate change, has led to look for sustainable alternatives to reduce the dependence of oil-based energy sources. Within this challenging context, bioenergy and bioproducts produced from residues are feasible alternatives for progressive fossil fuel reduction. The use of waste streams as feedstock is considered a critical factor that can help reduce greenhouse gas emissions, as well as decoupling oil prices from energy costs. Various technologies have been devoted to the conversion of biomass to biofuels. Out of the variety of fuels that can be produced from waste resources, gaseous fuels, such as hydrogen and methane, are probably the most commonly produced. The biogas platform is aimed at producing methane from biomass and other residues through anaerobic digestion (AD) processes. Biogas is a mixture of gases mainly composed of methane and carbon dioxide, along with hydrogen, sulfur hydrogen, and ammonia. However, the AD process also mediates the bio-based production of chemical building blocks of high importance for the chemical industry, namely, volatile fatty acids (VFAs). VFAs are organic acids, traditionally produced through the petrochemical route, which account from two to six carbon chains (i.e., acetic acid [C2], propionic acid [C3], butyric acid [C4], valeric acid [C5], and caproic acid [C6], as well as their isoforms, isobutyric and isovaleric acids). In this sense, the AD process using organic waste as a substrate, along with undefined microbial communities, is regarded as a tool to handle the complexity and variability of this feedstock to produce VFAs in the so-called carboxylate platform [1, 2].

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Moreover, the use of the AD process for VFA production also presents the added advantage of recovering most carbon contained in the substrate as VFAs instead of being lost as CO_2 in the biogas stream. These molecules are employed as building blocks in different industries, such as pharmaceutical, food, and textile [3]. As a matter of fact, their price in the global market fluctuates depending on the carbon length [3]. Nevertheless, a sustainable VFA production is constrained by technical barriers, such as low product selectivity and production rates, and the separation and purification of VFAs from the digestate, which ultimately increase the costs to implement the carboxylate platform.

The present chapter is aimed at providing knowledge related to the biology and operational parameters for VFA production via AD. This process is described as an interesting tool to produce VFAs from low-cost and residual feedstocks. However, the microbiota and the biochemical pathways that take part in the AD process remain poorly understood. Additionally, VFA separation and purification, as well as the chain elongation process, will be discussed, providing valuable information related to the carboxylate platform.

Anaerobic digestion

AD is a complex organic matter degradation process where numerous reactions and microorganisms interact to transform the organic matter into products, such as methane, carbon dioxide, hydrogen sulfide, water, and ammonia. It is composed of four different phases including hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 17.1).

Different microbial groups participate in each phase of the AD process, exhibiting different metabolic behaviors. Firstly, the complex biomass constituted by carbohydrates, proteins, and lipids is hydrolyzed. The efficiency of the microbial consortia in solubilizing the substrate has a determining influence in the final production yields of the system, since microorganisms cannot assimilate particulate organic matter. Soluble products from the hydrolytic step are able to pass through the cell membranes of the fermentative bacteria. These microorganisms convert the soluble monomers into simpler compounds, including VFAs, carbon dioxide, lactic acid, hydrogen, ammonia, and hydrogen sulfide, which are further excreted by the bacterial cells in the acidogenesis phase. Afterward, during the acetogenesis step, the acetogenic bacteria oxidize the products originated by the acidogenic bacteria by transforming them into suitable substrates for methanogenic archaea. The main products originated in this step are acetic acid, carbon dioxide, and hydrogen. The hydrogen can be used directly by methanogenic archaea for methane formation or by acidogenic bacteria to produce VFAs. Among all products originated during the process, methanogenic archaea can only use a few substrates to produce methane (i.e., acetic acid, hydrogen, carbon dioxide, formic acid, or methanol, among others). In this last step of the AD process, methanogenic microorganisms are divided into two different groups depending on the molecules from which they produce methane. The first pathway of methane production is the acetoclastic methanogenesis, which employs acetic acid to produce methane. The second pathway is led by the hydrogenotrophic methanogens. Those methanogens use hydrogen and carbon dioxide for methane production. When a population of methanogenic microorganisms is present in a sufficient amount, they readily consume VFAs for methane production. Under these conditions, chemical parameters, such as the pH, remain in a range favorable for their activity and development. However, a low presence of methanogenic archaea or unfavorable environmental or operational conditions might cause accumulation of those chemicals resulting in a pH drop in the system.

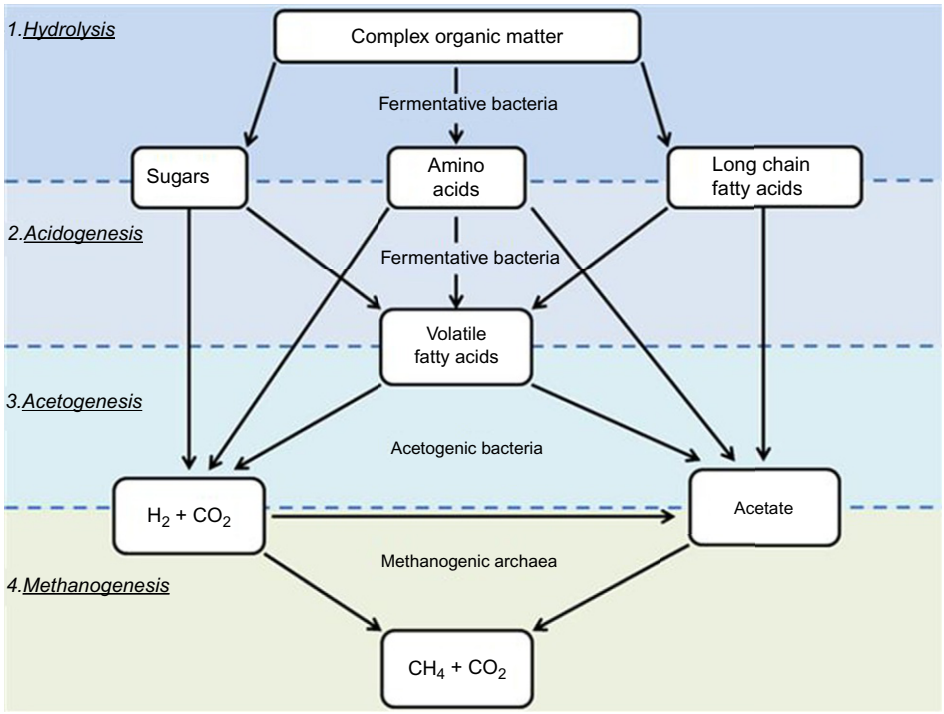

FIG. 17.1 Diagram of the anaerobic digestion process.

Therefore, since the main challenge for VFA accumulation is avoiding their conversion into methane, detailed research must be focused on the operational parameters and microbiology favoring VFA production during the acidogenesis stage. This knowledge is crucial to fully comprehend VFA formation and methanogenesis inhibition.

Impact of parameters in the anaerobic digestion process

The impact of pH, hydraulic retention time (HRT), organic loading rate (OLR), temperature, and inoculum pretreatment are the operational parameters most commonly studied to understand their effect on VFA compositions and yields.

The pH is considered a critical factor, because microorganism populations and enzymatic activities vary, according to its value. More specifically, the threshold of tolerance against pH for methanogenic archaea is lower than for acidogenic bacteria [4, 5]. The latter microorganisms are able to stand more acidic or alkaline pH values than methanogenic archaea. In this sense, methanogenic archaea decreased from 58% to 2% with increasing pH values (from 7 to 10), whereas the relative abundance of *Proteobacteria* and *Actinobacteria* phyla increased [6]. Thus, the selection of an appropriate pH value can affect the development of those acidogenic microorganisms, favoring substrate hydrolysis that ultimately results in higher VFA production. Different studies have been conducted to assess the optimal pH for VFA production. For instance, pH values from acidic to alkaline (from

4 to 11) were tested for VFA production when waste activated sludge was used as substrate at two different digestion temperatures (35°C and 55°C) [7]. In unit terms of chemical oxygen demand for volatile fatty acid (COD-VFA's) formation to volatile suspended solids (VSS), the authors achieved a maximum conversion of 368 mg COD-VFAs g^{-1} VSS, at pH = 8 and $T = 55$°C and stated that VFA accumulation was favored at this pH value due to a decrease in the methanogenic activity. Some other studies also reinforced this conclusion in which alkaline conditions were found to be best for VFA production [8, 9]. However, acidic conditions have been identified as more suitable for certain materials. As a matter of fact, when pharmaceutical wastewater was used as substrate, the use of pH 5.5 evidenced a maximum acidification level of 44% COD-VFAs/COD_{in} when carrying out a continuous AD (at: 35°C; HRT of 0.5 days; OLR of 13 kg COD m^{-3} d^{-1}) [10]. Therefore, it is necessary to take into account additional parameters, such as the composition of the substrate and the HRT in the reactor. As a matter of fact, this interlinkage among operational parameters has been reported in a study by Jankowska et al. [11]. In this study, acidic conditions (pH 4) were identified as the best conditions for VFA production with respect to added volatile solids (VS_{added}) (i.e., 0.24 g VFAs g^{-1} VS_{added}) at low HRT (5 days), whereas basic conditions (pH 10) produced higher VFA concentrations (0.62 g VFAs g^{-1} VS_{added}) at longer HRT (15 days).

The **retention time** includes hydraulic (HRT) and solid retention time (SRT). HRT is the time that the substrate is within the reactor, and thus, it is connected to the time that microorganisms have to metabolize it, whereas SRT defines the time that microorganisms are in the reactor. The geometry of the reactor (configuration) and retention time can affect population dynamics and VFA production. A continuous stirred tank reactor (CSTR) configuration provides good contact between the phases (HRT = SRT), whereas the effluents produced present high amounts of solids, which are detrimental to the feasibility for separation of VFAs. Other reactors can decouple HRT and SRT, which allows setting a lower HRT while working at high charges. The decrease in HRT can lead to methane inhibition due to the methanogenic archaea washing out. This results in the VFAs accumulating because the growth rates of these microorganisms are lower in comparison with acidogenic bacteria [4]. As a matter of fact, the study of growth kinetics of methanogenic archaea species, such as *Methanosarcina* and *Methanosaeta*, showed growth rates of 0.43 d^{-1} and 0.12 d^{-1}, respectively. In contrast, the acidogenic bacteria *Acetobacterium*, showed growth rates of 1.104 d^{-1} [12, 13]. However, there is no agreement in the literature over the influence of this operational parameter in VFA production. On one hand, Mahdy et al. [14] reported VFA accumulation by ammonia inhibition when carrying out a semicontinuous AD of protease pretreated *Chlorella vulgaris* biomass at a HRT of 15 days. The authors pointed out that increasing HRT could be used to degrade the VFAs due to a better balance of bacteria and archaea, supported by an increase in methane production. Opposite to this trend, Fang et al. [15] reported an increase in VFA yield in the AD of dairy wastewater when increasing the HRT from 4 h (723 mg COD-VFAs L^{-1}) to 24 h (1447 mg COD-VFAs L^{-1}). These authors attributed the increase in VFA yields to a better hydrolysis of the substrate at increasing HRT values. In this sense, too low HRT does not allow the hydrolytic bacteria to degrade the substrate, resulting in a decrease in VFA production yields due to less availability of soluble organic matter in the system. While this latter investigation dealt with an easily degradable substrate (dairy wastewater), the study of Mahdy et al. [14] dealt with a more complex organic matter (microalgae biomass). Most probably, the different biodegradability and easiness for hydrolysis affected the HRT required to maximize acidogenesis. In this sense, the importance not only of the

operational parameters but also of the feedstock used for VFA production can be highlighted. Moreover, retention time has been pointed out to influence the VFA composition. For instance, caproate was only detected at 20 and 30 days when vegetable and salad waste was used as substrate at various SRT (10, 20, and 30 days) [16].

The **organic loading rate** (OLR) is the amount of organic matter fed into the system per unit of volume and time. This value establishes the amount of organic matter subjected to the AD process and, thus, also plays a key role in the amount of VFAs ($g L^{-1}$) that may be produced. The OLR fed into a system is strongly dependent on different factors, such as the substrate composition and the reactor volume and geometry. Values found in the literature for large-scale processes are focused on biogas production [17]. However, there are bench-scale experiments in which the OLR influence was assessed for VFA production. OLR values tend to be lower (1–14 g COD $L^{-1} d^{-1}$) [10] when experiments are carried out in continuous stirred tank reactors than those set in upflow anaerobic sludge blanket reactors (up to 60 g COD $L^{-1} d^{-1}$) [18, 19]. The latter geometry allows higher loading values to be employed, due to the decoupling of HRT and SRT, resulting in better settling properties. For instance, OLR values from 3.2 to 15.1 g COD $L^{-1} d^{-1}$ were evaluated in an acidogenic fermentation of two-phase olive mill solid residue [20]. The best VFA production was obtained at 12.9 g COD $L^{-1} d^{-1}$ (14 g L^{-1} VFAs), whereas higher values (14 and 15.1 g COD $L^{-1} d^{-1}$) mediated an inhibition of the AD process. The authors attributed this inhibition to a low hydrolysis efficiency, which caused a reduction of the acetic acid and hydrogen concentration, resulting in lower VFA production (8 g L^{-1} VFAs). Similarly, another study investigated the influence of the OLR in the digestion of pretreated olive mill wastewater in batch reactors, covering an OLR from 5 to 40 g COD $L^{-1} d^{-1}$ [21]. The optimum value was found to be 20 g COD $L^{-1} d^{-1}$ (producing 27 g L^{-1} VFAs). At higher OLR values, a sharp decrease in VFA productions to 15 g L^{-1} was observed, which was attributed to the stress caused to the acidogenic bacteria when the OLR was increased. Therefore, it can be concluded that VFA production increases with increasing initial OLR values. However, a decrease in VFA yields has been observed when further charges are employed [20, 21]. Thus, an in-depth characterization of the effects of OLR is necessary to balance this parameter and optimize VFA production, taking into account related parameters, such as the type of substrate, and the HRT.

Temperature is an easily adjustable parameter that influences the microbiota present in the reactor. It favors the development of certain microorganisms and affects their growth rate and metabolic reactions, due to its significant influence on enzymatic activities. The influence of temperature in the psychrophilic range was tested for VFA production when waste activated sludge was used as substrate at 4, 14, and 24°C [22]. Results showed an increase in the hydrolysis constant at 24°C (0.17 $days^{-1}$) in comparison with values attained at 4°C (0.04 $days^{-1}$). The increase in organic matter availability at 24°C resulted in the highest VFA production (2154 mg COD-VFAs L^{-1} vs 782 mg COD-VFAs L^{-1} at 4°C). Higher temperatures have been also tested to enhance VFA productions. Another study assessed different temperatures (37°C and 55°C) and HRT (2, 4, and 6 days) when digesting maize silage and cow manure for VFA production [23]. The highest VFA yield was achieved at 37°C and HRT 4 days (183.2 g COD-VFAs kg^{-1} VS). This study showed higher acidification at 37°C, even though organic matter solubilization was more efficient at 55°C. The authors suggested that the lower acidification yields reached at 55°C could be related to a slow adaptation of the thermophilic culture. Similarly, 30°C was found to be the optimum temperature for VFA production (34 g L^{-1} VFAs) in experiments carried out at 25, 30, and 40°C when cassava

wastewater was used as substrate [24]. Following this trend, a similar study focused on the effect of temperature (35, 45, and 55°C) on VFA production from food waste [25]. The highest VFA yields were reported at 45°C and 35°C (47.8 and 41.3 $g L^{-1}$ VFAs vs 14.9 $g L^{-1}$ VFAs at 55°C). Therefore, based on the presented data, it can be concluded that a mesophilic range of temperatures seems to provide higher acidification yields resulting in higher VFA production.

Different **pretreatments applied to the inocula** have been regarded as possible ways to inhibit the methanogenic activity and, thereby, enhance the growth of organic acid-producing microorganisms. In this sense, thermal and chemical pretreatments applied to anaerobic inocula or addition of chemical reagents in the digestion medium has been studied. Thermal treatments are based on spore formation of the acidogenic bacteria, which can resist high temperatures. It is generally performed by boiling the sludge at different temperatures and time [26]. In chemical pretreatments, the sludge is maintained at acidic or alkali pH values [27, 28]. Another possible approach is the use of chemical inhibitors, such as 2-bromoethanesulfonate (BES), chloroform, or iodoform. Those chemicals interact with specific enzymes of methanogens, resulting in an inhibition of their activity [29]. However, all these approaches present drawbacks involving economic costs or environmental toxicity.

Apart from the operational conditions previously exposed, the microorganisms involved in the AD process also play a key role in the final VFA yield. As a matter of fact, different groups of microorganisms, namely, bacteria (syntrophic, acidogenic, and acetogenic) and methanogenic archaea, are interconnected with the ultimate goal of degrading the organic matter during the AD process. The study of the biology implied in each stage can shed light on the most important species to favor VFA production.

Biology

The behavior of the different microorganisms present in AD plays a key role in the decomposition and assimilation of complex organic matter. The presence of different species is linked to the different AD phases (hydrolytic and acidogenic bacteria or methanogenic archaea). However, the microorganisms involved in the hydrolytic step (belonging to the phyla *Bacteroidetes* and *Firmicutes*) are often active during the acidogenic stage [30]. In this sense, these microorganisms are referred to as fermentative bacteria, being facultative anaerobes or strict anaerobes. Moreover, the molecules targeted as substrates by each of the microorganisms are very variable. During the fermentative phases (hydrolysis and acidogenesis), fermentative bacteria transform the products from the hydrolysis stage (sugars, amino acids, and proteins) into VFAs, alcohols, aldehydes, carbon dioxide, and hydrogen. During the acidogenic step, >50 bacterial groups are involved, but the main ones include *Proteobacteria*, *Acinetobacter*, or *Clostridium* belonging to *Bacteroidetes*, *Formicutes*, *Chloroflexi*, and *Proteobacteria* [30, 31]. These bacteria must be in balance with the archaea in charge of the methanogenic step to achieve an efficient biogas production. Nevertheless, if the AD is devoted to VFA production, an unbalance of the mentioned populations is desired. More specifically, an increase in organic acid bacteria producers is targeted at the expense of methanogenic microorganisms. Hence, VFAs can accumulate in the digestate and remain in the liquid phase instead of being transformed into biogas. Macromolecules composing the organic matter (carbohydrates, proteins, and lipids) have different degradation pathways during the acidogenesis step.

The polymeric nature of carbohydrates makes them too big to enter the bacterial cell. Thus, complex carbohydrates are degraded outside the cells using exoenzymes to form simple sugars, such as galactose, fructose, ribose, or

TABLE 17.1 Main degradation products of glucose as a substrate in acidogenesis.

Products	Reaction	ATP/mol glucose
Acetate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	4
Propionate	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	Low
Butyrate	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	4/3
Lactate	$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$	3
Ethanol	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$	2

glucose, which are subsequently able to go through the cell membrane. Modeling studies, taking glucose as a model substrate, revealed the main degradation products of this substrate (Table 17.1). As it can be seen in Table 17.1, VFAs are not the only acidogenesis products, as lactate and ethanol are also normally encountered in the digestion broth. Some of the species in charge of carbohydrate degradation are *Enterobacter*, *Lactobacillus*, *Clostridium*, *Streptococcus*, and *Propionibacterium* [32, 33].

The breakdown of proteins gives amino acids through the use of proteases and peptidases in the anaerobic digester. Amino acids, such as arginine, glutamate, glycine, or lysine are transported inside the cell and are used in the production of organic acids. The principal genera that metabolize these compounds are *Clostridium* [34]. The acidogenesis step of amino acids resulting from protein hydrolysis occurs mainly through two different pathways: the Stickland oxidation-reduction reactions or the oxidation of single amino acid using an external electron acceptor requiring a hydrogen-utilizing bacteria (hydrogen or carbon dioxide) [35]. In Stickland reactions, one amino acid acts as an electron donor, whereas another one acts as an electron acceptor (some of them can play both roles).

Lipids are hydrolyzed by extracellular lipases into long-chain fatty acids (LCFA) (e.g., palmitate and oleate) and glycerol, during the AD process. Further conversion of these molecules takes place inside the cell. Glycerol is converted into acetate by acidogenesis, and the LCFA, which vary in degree of saturation and chain length, are converted into acetate and hydrogen through the ß-oxidation pathway [36]. This latter step is rather slow in comparison with the hydrolytic stage [37]. Identified bacteria species that can anaerobically degrade LCFA belong to the *Syntrophomonadaceae* and *Syntrophaceae* families [38].

The intermediate compounds formed in acidogenesis, VFAs, such as propionate, butyrate, valerate, and their isoforms, must be further degraded before methanogens can metabolize them for biogas generation. The process in which this transformation occurs is named syntrophic acetogenesis giving acetate, H_2, and CO_2 as final products. The association of proton-reducing acetogens with methanogens (either hydrogenotrophic or aceticlastic) takes place when the partial pressure of hydrogen is kept low (10^{-4}atm) [39]. *Pelotomaculum*, *Smithella*, and *Syntrophobacter* are some of the genus in charge of the propionic syntrophic degradation [40–42]. Butyrate and some other VFAs are degraded by species, such as *Syntrophothermus* and *Syntrophomonas* [43, 44].

Separation and purification

For some of the applications in which VFAs might be useful, these chemicals need to be separated and purified from the anaerobic broth.

Based on technoeconomic analysis, this separation process entails technical challenges and is responsible of the main production costs [45]. The separation is challenging due to the low VFA concentrations generally found in anaerobic effluents. As reviewed in the following paragraphs, several separation techniques have been proposed to recover VFAs from aqueous solution (see Table 17.2).

(i) Precipitation is a well-established technique that involves low capital costs and is highly selective, and thus, it renders high product yields and purities. The most studied precipitation agents in the literature are $Ca(OH)_2$ and $CaCO_3$ [47, 48]. Precipitation involves four steps: First, the sample is filtered to remove undesirable solids. Then, the sample is treated with

TABLE 17.2 Advantages and disadvantages of the most common methods employed for the separation volatile fatty acids from aqueous solution [46].

Methods	Description	Advantages	Disadvantages
Precipitation	Calcium salts are added in the medium, to neutralize the acids. The resulting calcium carboxylate solutions can be concentrated by evaporation, crystallized, and separated of the mother liquor	Well established. Higher product yields, low capital costs, products of high purities	Generating solid wastes as sulfuric acid is used to release carboxylic acids from the calcium carboxylates
Distillation	Ammonia is used to neutralize the acids reacting to form ammonia carboxylate, which is then mixed with alcohol to form esters, to be separated by distillation	Well established. Highly pure products, byproducts can be used as fertilizer	High energy and capital costs related to distillation that is used to separate the alcohol from carboxylic acids after formed esters are hydrolyzed
Adsorption	Ion exchange resins used to exchange to adsorb carboxylate ions of the feeds	Well established. Easily operable	High resins costs, high energy demand due to resin regeneration, low adsorption capacities; separation is not highly selective
Electrodialysis	Negatively charged carboxylate ions move through an anion exchange membrane toward the anode in the electrodialyzer through electric current	Carboxylate is concentrated in aqueous solution, does not require acid treatment to adjust pH	The products have high impurities; further purification might be required; difficulties in scaling up, high energy demand. Prone to fouling
Solvent extraction	Organic acids use to extract carboxylic acids from the stream	Higher product yields, suitable for carboxylate salt production, lower costs	The feed needs to be acidified for efficient extraction; extractants need to be regenerated by distillation or back extraction
Membrane separations	Use of membrane filters of various pore sizes to treat the mixed effluents for solid removal and fractionate the desired substances for recovery	Developing technology, high product yields, suitable for a wide range of applications, low energy, economic, easy to scale up	Membrane fouling, clogging, largely untried in complex waste systems

sulfuric acid. This step allows the precipitation of $CaSO_4$ and the release of free organic acids, which are further purified via active carbon adsorption or ion exchange. In this manner, traditional calcium precipitation coupled with ion exchange adsorption offered 92% recovery of succinic acid [49]. The main drawbacks are the solid wastes generated during the use of sulfuric acid for the carboxylate release. Currently, investigations dealing with this technique are devoted to the chemicals used for precipitation. More specifically, a reusable chemical agent is a hot topic in this field [50].

(ii) **Distillation** is a straightforward process separation method based on the different volatilities of the components involved. Even though it is efficient at separating organic acids at low concentrations, the yield decreases at higher concentrations. Moreover, these processes entail high energy and capital costs. As a matter of fact, separation techniques attempting to recover VFAs by removing water are not economically viable. An attempt to make this process viable was tested by Rakesh et al. [51], when a reactive distillation process was carried out to recover lactic acid from an aqueous solution. Their strategy consisted on the formation of methyl lactate to overcome the azeotrope barrier. Results showed an efficient lactic acid recovery achieving 99.95% conversion of methyl lactate and 81 g kg^{-1} concentration of lactic acid in the solution.

(iii) **Adsorption** is an affinity method that allows separation of certain compounds from dilute solutions with several compounds involved. This methodology can be applied for VFA separation, but finding the most appropriate affinity agent is challenging. VFA adsorption or ion exchange (IE) relies on the interaction between the carboxylate groups and the active sites of a solid matrix. Adsorption techniques require a protonated VFA, which is physically linked to the adsorbent, whereas IE occurs through ionic bond formation between ionized acids and cations (charged resins are commonly used for such a purpose). Functional groups that are normally used as reactive sites include amines (primary, secondary, and tertiary) and quaternary ammonium. Amines adsorb VFAs only when their charge is neutral by creating bonds with hydrogen or transferring protons, whereas quaternary ammonium adsorbents can be employed in anion exchange techniques. For instance, a study carried out by Rebecchi et al. [52] found tertiary amino resins to be the most suitable among four amino IE resins. Better results were attained with longer-chain VFAs suggesting that molecular weight played a key role in VFA physical adsorption.

With regard to the adsorbents, recent studies have evaluated them for VFA adsorption [53–55]. Recently, Reyhanitash et al. [56] studied polystyrene divinylbenzene nonfunctionalized resins and primary, secondary, and tertiary amines for their functionalization. Results differed depending on the resin employed. On one hand, high VFA selectivity was reported by the nonfunctionalized resins, but on the other hand, amine-functionalized resins adsorbed mineral acids preferentially. As for the regeneration step, nonfunctionalized resins permitted VFA fractionation by using two different stages of washing with water and evaporation at different temperatures. It is noteworthy to mention that butyric acid (initially 0.25 wt%) finally achieved purities of 91 wt%. In the case of the amine-functionalized adsorbents, chloride, sulfate, and phosphate salts resulted in coadsorption of their acidic forms, which severely reduced the VFA adsorption capacity.

It should be also highlighted that, after the complete adsorption process, a desorption step is needed in which the molecules of interest (adsorbate) need to be desorbed from the adsorbent to finish the VFA recovery process and regenerate the adsorbent [52]. Water and basified ethanol have been recently tested for such a goal [52, 56]. In this work, all VFAs were desorbed when using basified ethanol, while evaporation resulted in a VFA concentrated solution [52].

(iv) **Liquid-liquid extraction** is the oldest and better-established chemical operation methodology for VFA separation. It is based on the relative solubility of the compounds in different immiscible liquids. Its efficiency depends on the organic acid targeted for extraction and on the extractant and its concentration. Generally, alcohols, ketones, esters, and aliphatic hydrocarbons are used as extractants. Ijmker et al. [57] used medium-chain fatty acids (MCFA) diluted in an organic solvent as extractants. The advantage of such extractant is the double hydrogen bond formation. According to this investigation, MCFA have excellent capability for VFA separation due to their ability for dimerization and low water solubility. Acetic, propionic, and butyric acids were extracted with medium-chain fatty acids diluted in n-hexane and toluene. Their results showed that VFA chain length increase caused a significant increase in the extraction efficiency (i.e., acetic acid < propionic acid < butyric acid). This effect was less relevant for the medium-chain fatty acids employed (i.e., hexanoic, octanoic, and decanoic acid). Other extractants that are widely used in liquid-liquid extraction are amines. These compounds are derivatives of ammonia, where one or more hydrogen atoms have been replaced by a substituent, such as an alkyl or aryl group. Strong amine interaction with the acid allows the formation of acid-amine complexes, which increases their distribution coefficients. While primary amines are too water-soluble and secondary amines are susceptible to amide formation, tertiary and quaternary amines were also studied for carboxylic acid extraction [58]. Their results confirmed that amine extraction efficiency was pH dependent. In this manner, when no pH adjustment of the anaerobic effluent is desired, the quaternary amine is more suitable, while, if the bioprocess can tolerate a pH of around 4.0, the tertiary amine might also be an option. More recently, ionic liquids have gained interest for extraction of carboxylic acids, as they are considered "green" solvents. For instance, the ionic liquid [P666,14][Phos] and trioctylamine (TOA) dissolved in n-octanol were applied as solvents to extract acetic acid from fermented wastewater model solutions [59]. These researchers concluded that [P666,14][Phos] is superior to TOA in terms of extraction capacity and selectivity. They started with a solution containing 1 wt% HAc and different salts and finally obtained a 30 X more concentrated solution when water and HAc were completely recovered (34% HAc). However, the authors recognized that the process could be improved by presurizing CO_2 because large amounts of water were still involved. Carboxylic hydrophobicity is an essential parameter in liquid-liquid extraction that takes into account that the longer the carboxylic acids are, the more hydrophobic. Hence, it is not surprising that C5-VFAs are extracted at higher efficiencies than C2-VFAs. For instance, acetic acid is recovered at 50% (using 10% trioctylphosphine oxide at pH2.5), while almost 100% is recovered in the case of valeric acid [60].

pH control is an important parameter for VFA extraction due to the similar pKa

values that these chemicals exhibit. Indeed, at pH values lower than the pKa, VFAs are extracted more efficiently as they are present in undissociated forms. This was observed in previous investigations where, for example, the extraction efficiency was higher at pH 2.5 than at 5.5 [60]. At this latter pH value (5.5), the extraction efficiency remained comparably low (26%–32%) when compared with that achieved (53%–67%) at pH value 2.5, despite an increase in the concentration of trioctylphosphine oxide. Nevertheless, it should also be pointed out that acidogenic anaerobic microorganisms thrive at pH 5–7, and thus, the challenge remains in extracting VFAs at those pHs.

(v) **Membrane-based solvent extraction**. This technology is an alternative to the more traditional liquid-liquid extraction. The main difference is that, in this case, immiscible liquids are replaced by an immobilized interface.

Membrane separation involves the use of a semipermeable barrier (membrane) through which chemicals move with varying rates. This technology is interesting due to the high selectivity observed in membranes. Membrane-based technologies entail some drawbacks, such as the high pressure required and high electrical input. Likewise, membrane fouling is a common issue that limits their efficiency given the complex nature of acidogenic digestates. Nevertheless, membranes have been widely reported to be efficient for VFA separation. The most common membrane separation technologies are included in Table 17.3.

Of the preceding text, forward osmosis, electrocoagulation, and pervaporation are the techniques most used for VFA separation. Forward osmosis is based on separation of feed and draws solution via osmotic pressure. More specifically, high osmotic pressure (compared with the feed solution) induces the water to flow through the membrane. A rejection of 100% of the feed solution indicates that only water passes through the membrane. This would mean a high VFA concentration on the other side of the membrane. This membrane-based technology has been tested and described in the literature [61]. The results showed that this technology was pH dependent. The rejection rate was higher at pH 8, while the flux was greater at lower pH values. 97% Rejection was attained for a synthetic solution of 35 g L^{-1} solution (6:3:1 ratio acetic, propionic, and butyric acid). Nevertheless, the water flux could be enhanced by changing the operational temperature and draw solution concentration (sodium chloride). Moreover, these parameters did not affect the high VFA rejection rate.

On the other hand, it should be pointed out that the optimum performance was obtained with synthetic medium, whereas when using real digestate the osmotic pressure increased due to the complex organic matter contained in the fermentation broth. 33% Lower forward osmosis efficiency was recorded in real digestates. Once again, the authors highlighted that varying operational conditions could improve those rejection efficiencies. For instance, the effect of pH was evaluated for the fouling properties on the forward osmosis membrane surface [62]. Fouling at pH 9.0 was greater than at 5 due to carboxylic acid protonation (pKa values of VFAs). These mechanisms resulted in electrostatic repulsion between the carboxylates and the membrane surface, and hence, membrane fouling decreased.

Electrodialysis has been widely applied for the recovery of small-chain organic acids from aqueous solutions, using synthetic solutions or fermentation broths. This technology has been tested for many purposes. In the context of VFAs, namely, to increase VFA concentration [63], to convert sodium salts of VFAs into the corresponding acids [64], and for fractionation of VFA mixtures [65]. A recent investigation was conducted by Scoma et al. [66]. Their study

TABLE 17.3 The most common membrane separation technologies.

Process	Principle	Type of membrane	Driving force
Microfiltration	Separation of organic and polymeric compounds	Micropore range of 0.1–10 μm	Pressure difference 35–350 KPa
Ultrafiltration	Separation of water and microsolutes from macromolecules and colloids	Micropore range of 1–100 μm	Pressure difference 140–700 KPa
Reverse osmosis	Passage of solvent through a dense membrane that is permeable to solvent but not solutes	Dense solution-diffusion	Pressure difference 700–7000 KPa
Electrodialysis	Ions are transported through a membrane from one solution to another under the influence of an electrical potential	Electrically charged films	Voltage difference 1–2 V
Pervaporation	Component of a mixture diffuses through, evaporates under low pressure, and is removed by a vacuum	Dense solution-diffusion	Vapor pressure 7–70 KPa

(Modified from R.R. Singhania, A.K. Patel, G. Christophe, P. Fontanille, C. Larroche, Biological upgrading of volatile fatty acids, key intermediates for the valorization of biowaste through dark anaerobic fermentation, Bioresour. Technol. 145 (2013) 166–174.)

showed the removal of VFAs from real olive mill wastewater at room temperature and pH values close to 6–6.5, with no membrane damage. The electric current was kept constant at 500 mA (31 A m^{-2}). VFA removal was about 30%–35% and resulted into a concentration factor between 1.2 and 1.5 with respect to the initial solution (14 g L^{-1}). The major drawback identified during this research was a competition between chloride and acidic anions that occurred as long as the chloride concentration was high, whereas acidic anions transport across the membrane increased after 50% NaCl removal. Moreover, these authors also highlighted the effect of the stearic hindrance associated with each VFA. In this sense, transport of each acidic anion across the membrane was affected by the concomitant role of concentration and diffusivity, which can shift the natural order imposed by the steric hindrance of the species (caproic and acetic acids exhibiting highest and lowest hindrance, respectively).

Electrocoagulation is an alternative to electrodialysis. In this case, the process uses sacrificial electrodes, which produce metal ions that can be used to coagulate the organic matter and nutrients. This technology has been widely used in wastewater treatment. For the particular application of VFA separation, a recent investigation demonstrated its efficiency [67]. Within this approach, solids and nutrients were removed, while VFAs remained entirely in the liquid phase. Out of the parameters evaluated during electrocoagulation, namely, employed time, electrode material, current density, initial pH, or interelectrode distance, none of them affected the VFA concentration in the effluent. The VFAs were not removed nor oxidized nor adsorbed on flocs, due most probably to the small molecular weight compared with floc pores and similar electrostatic properties.

Pervaporation is defined as the separation of a mixture in which a component is diffused through a semipermeable membrane and evaporated. This technology is an energy-efficient

alternative to distillation for removing volatile organic compounds from water. Pervaporation has been used for organic solvent separation [68]. The separation is mainly governed by the hydrophobicity of the acids, since hydrophobicity increases as carbon chain length increases. Only recently has this technology been applied for the recovery of VFAs. More specifically, butyric acid was separated by a pervaporation process using polyether block amide composite membranes [69]. The mechanical and thermal properties of the membrane were strengthened by adding nanomaterials. The best results (separation factor of 21, starting with a concentration of butyric acid in the feed of 0.6%) were attained when the membrane was loaded with graphene, since the pervaporation process was taking place at 70°C.

Biochemical chain elongation

The chain elongation (CE) process consists in converting the products of anaerobic fermentation (i.e., short-chain carboxylic acids [SCCAs]) into more valuable medium-chain carboxylic acids (MCCAs), under anaerobic conditions. While SCCAs include acetate, propionate, butyrate, and valerate, the MCCA group involves hexanoic acid (caproic), heptanoic acid (enanthic), octanoic acid (caprylic), and nonanoic acid (pelargonic). MCCAs are saturated fatty acids with relatively low solubility in water due to their hydrophobic carbon chain. At low pH (below their pKa), MCCAs form an oil liquid easy to separate from the water phase.

CE takes place via reverse β-oxidation. This process adds an acetyl-CoA molecule to a carboxylate, elongating its carbon chain length with two carbons at a time (Fig. 17.2). This cyclic process entails the oxidation of an electron donor, such as ethanol to acetyl-CoA by NAD^+, and the reduction of ferredoxin by NADH. This redox reaction includes an electron acceptor (SSCA) and an electron donor (i.e., ethanol). In principle, acetate is the initial feedstock for reverse β-oxidation. However, MCCA formation (caproate and caprylate) has been reported without acetate and using CO_2 and H_2 [71]. As most studies available in literature work with acetate (electron acceptor) and ethanol (electron donor), mainly, even-carbon number carboxylates have been reported. In fact, most of the literature is focused on caproate production. Odd-carbon number carboxylates have also been detected but at lower production yields [72]. Likewise, long-chain fatty acid elongation rate is lower than for shorter fatty acids [73]. CE is a complex process in which different parameters, subsequently discussed, affect final MCCA yields and productions.

Factors affecting the chain elongation process

The electron donor, hydrogen partial pressure, substrate complexity, pH and the microorganisms involved are the parameters most commonly studied in CE process.

Ethanol is preferred as an **electron donor** for high-rate CE, but other chemicals can be used. In fact, ethanol and acetate have been reported to provide the highest growth rate for *Clostridium kluyveri*, which suggests a preference for short molecules in CE [74]. Nevertheless, the need for ethanol (as an electron donor) is reduced for elongation of butyrate compared with acetate [75]. Besides, it is suspected that a concentration of 10–20 $g\,L^{-1}$ ethanol may be inhibitory to the CE microbiomes [76]. Apart from ethanol, some other molecules are used as electron donors, such as methanol, propanol, lactate, or glucose. Following ethanol, lactate is most probably the second most investigated electron donor in CE. In fact, during the CE process (Fig. 17.3), part of the lactate is converted to propionate via the acrylate pathway using acrylyl coenzyme A as intermediate with the consumption of NADH, and another part is released as CO_2 to oxidize

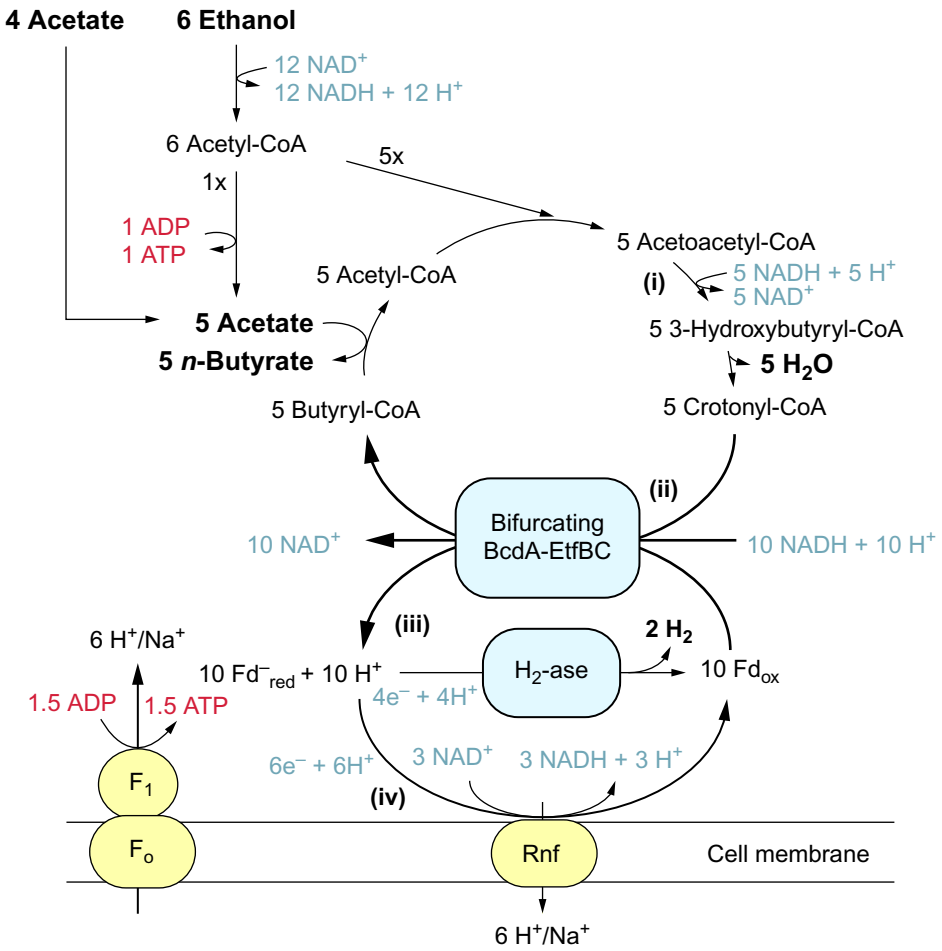

FIG. 17.2 Chain elongation pathway (reverse ß-oxidation) with ethanol and acetate to n-butyrate for *C. kluyveri* at high substrate concentration [70]. Ethanol is oxidized to Acetyl-CoA by NAD^+. Abbreviations: *BcdA-EtfBC*, butyryl-CoA dehydrogenase-electron transferring flavoprotein complex; *CoA*, Coenzyme A; *F0F1*, H^+/Na^+ pumping ATP synthase complex; Fd_{ox}, oxidized ferredoxin; Fd^-_{red}, reduced ferredoxin; H_2*-ase*, hydrogenase; *Rnf*, ferredoxin-NAD reductase complex.

lactate into acetyl-CoA [78]. This pathway has been found in different bacteria, such as *C. propionicum*, *Megasphaera elsdenii*, and *Prevotella ruminicola*, which use other substrates apart from lactate, such as serine, alanine, or ethanol [79]. In this manner, not all the lactate is used for CE but consumed in other carbon pathways. The use of lactate as an electron donor has been previously reported in the literature. Liang and Wan [80] pointed out an increase of SCCA production when using lactate as the electron donor, while caproate production was enhanced when using ethanol. Likewise, the cooperative relationship between electron donors, such as ethanol and lactate, and electron acceptors in CE has been recently investigated. A recent investigation showed the results of combining different electron acceptors and donors (ethanol and lactate)

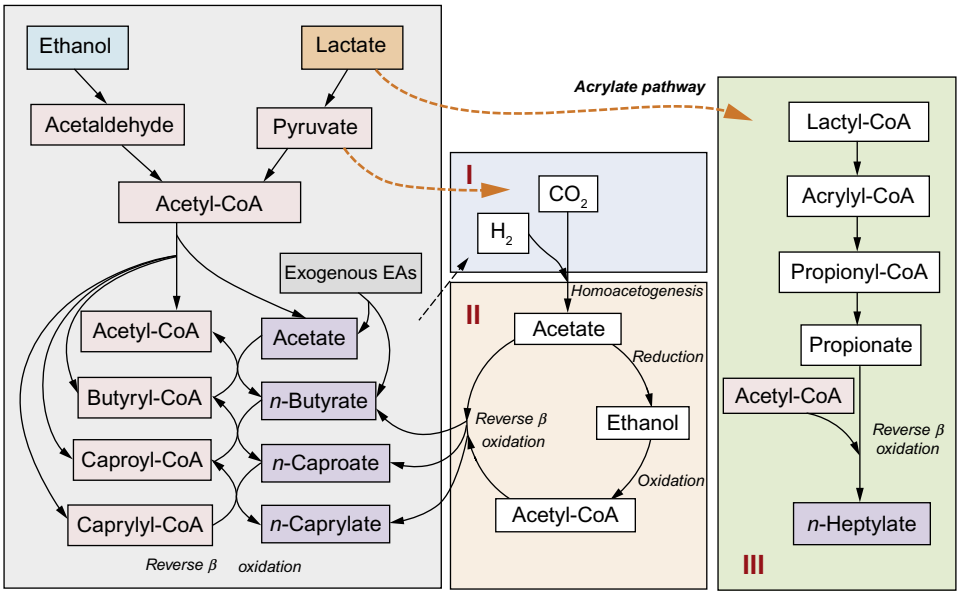

FIG. 17.3 Simplified reaction pathways for the cooperative use of ethanol and lactate in the production of medium-chain carboxylic acids [77]. I: CO_2 use from lactate oxidation for homoacetogenesis. II: Acetate reduction to ethanol and further oxidation to acetyl-CoA for n-butyrate, n-caproate, and n-caprylate production through reverse β-oxidation. III: Acrylate pathway for propionate production from lactate oxidation and further elongation to n-heptylate through reverse β-oxidation.

in CE [77]. This study was based on taking advantage of the CO_2 released during lactate oxidation to compensate for the CO_2 shortage in the ethanol-based CE. Following the trend reported by Liang and Wan [80], results showed a higher caproate production using ethanol. However, the use of lactate contributed to a higher production of caprylate. Combination of both electron donors gave as a result higher heptylate and caprylate production than when used independently. Carbon flow was directed mainly to caproate production (61%), while heptylate and caprylate were produced in similar amounts (around 10% each).

CO is another molecule that has been recently investigated as an electron donor and carbon source [72]. In this latter study, CO partial pressure was increased from 0.15 to 0.60 atm. This upper limit stopped the production of caproate and caprylate, and thus, it was pointed out as an inhibitory CO concentration. Another compound studied was CO_2, which was determined essential not only for growth purposes but also for stimulating the syntrophic ethanol oxidation pathway. The experimentation by Roghair et al. [81] used ethanol for upgrading into caproate and propionate and further upgrading to heptanoate, by supplying different CO_2 loading rates (0–2.5 L CO_2 $L^{-1}d^{-1}$). Results showed that caproate production increased 3.6-fold at high loading rates (2.5 L CO_2 $L^{-1}d^{-1}$), compared with values attained at 0.5 L CO_2 $L^{-1}d^{-1}$ (3 g caproate $L^{-1}d^{-1}$). The combination of substrates (ethanol and propionate) provided evidence that enzymes catalyzing chain elongation have a preference for acetate, as the electron acceptor for ethanol upgrading. This higher catalytic preference is the reason why odd-numbered fatty acid production is challenging. In this manner, reducing the ethanol concentration would limit even-carbon

number fatty acid production with a gain in odd-numbered fatty acids. Since CO_2 loading rate controls syntrophic ethanol oxidation, the supply of CO_2 could be used as a tool to produce even- or odd-numbered fatty acids. Grootscholten et al. [82] tested even higher CO_2 flow rates (from 2.4 to 4.8 L CO_2 $L^{-1}d^{-1}$) and their results showed acetate accumulation instead of chain elongation, due presumably to the low hydrogen partial pressure, which ended up in anaerobic SCCA oxidation. When using ethanol as the electron donor, CO_2 is required by CE bacteria. This nutrient is crucial since it is used for microbial protein synthesis [83]. Indeed, Steinbusch et al. [84] observed a long lag phase due to the absence of CO_2 in the elongation medium.

Hydrogen partial pressure. This parameter should be high enough to avoid oxidation of the electron donor and carboxylates. According to Angenet et al. [70], low hydrogen partial pressure ($\leq$0.1 KPa) is imperative for fast elongation rates of n-caproate. The importance of hydrogen is related to the presence of hydrogenotrophic methanogens in the anaerobic culture. Indeed, Ding et al. [85] stated that caproate formation is hydrogenogenic rather than hydrogenotrophic. Ethanol concentration might affect this microbial group's activity, and thus, elevated hydrogen partial pressure occurs. In this sense, when a reduced substrate, such as ethanol, is added as the electron donor, hydrogen partial pressure is high enough since the limiting substrate for hydrogenotrophic methanogenesis is carbon dioxide [82]. This parameter can also be important in the carboxylates used for CE. In this manner, high hydrogen partial pressure can drive anaerobic fermentation toward butyrate [86].

Substrate complexity. Depending on the fermented feedstock, the reactor configuration to be employed will be different. In fact, complex substrates normally require long residence times ($\geq$10 days), while easily digested substrates require residence times of a few hours. The need for long retention times is associated with the growth of acetoclastic methanogens. Since they might compete for the carboxylate substrate, this group of microorganisms needs to be inhibited (see "Impact of parameters in the anaerobic digestion process" section). In the case of low retention times, this time needs to be enough to avoid microorganisms washout. For instance, *C. kluyveri* has a growth rate of 0.1 h^{-1} [87], while the acetoclastic methanogen *Methanobacterium*'s growth rate is 0.01 h^{-1} [37].

pH. This operational parameter is important not only as an outcompeting strategy but also to ensure availability of nutrients. In principle, anaerobic fermentation takes place around neutral pH, but studies dealing with CE have revealed high conversion rates at acidic pH (e.g., 5.5) [76, 88]. At this point, it is important to highlight the need for MCCA extraction as they are produced, due to the potential toxicity in their undissociated form [89]. Besides, pH also affects the carbon equilibrium. In this sense, a pH around 6.4 has been determined as optimum for *C. kluyveri* growth [87]. This pH ensures the availability of CO_2 for growth requirements (as the pKa CO_2 (H_2CO_3)/$HCO_3^- = 6.3$).

Presence of other microorganisms. The anaerobic microbiome is obviously not an axenic culture, and thus, competitive processes might take place. The need for limiting methanogenesis is of paramount importance when SCCAs are the substrate for CE. Out of the two microbial groups that could compete for SCCAs in methanogenesis, it seems likely that hydrogenotrophic methanogens are less harmful than acetotrophic methanogens [90]. With the aim of washing out these competitive microorganisms, this later investigation used a reactor filled with polyurethane cubes as carrier material and applied an upflow velocity of 1.2 $m\,s^{-1}$ to ensure detachment of methanogens. In this case, since growth rates for hydrogenotrophic methanogens (i.e., *Methanobrevibacter*) are $\geq$0.1 h^{-1}, their wash out

is harder than for acetotrophic methanogens that exhibit lower growth rates ($0.013\,h^{-1}$, *Methanosarcina* [91]). Another alternative to get rid of competing microorganisms is the addition of chemicals that inhibit methanogenesis (as discussed previously) or the use of heat shocks, which would not affect spore-forming bacteria like *C. kluyveri* while affecting methanogens.

Production yields and production rates

As mentioned at the beginning of this section, the current investigation is mainly focused on caproate production. Nevertheless, most of the time, caproate and caprylate productions are reported together, most probably due to the formation of even MCCAs. Grootscholten et al. [90] achieved a maximum volumetric production of $15.7\,g\,L^{-1}d^{-1}$ with a concentration of $11.1\,g\,L^{-1}$ for caproate, which was 30 times higher than that of Steinbusch et al. [84]. This later investigation produced MCCAs from acetate with hydrogen and/or ethanol as the electron donors. A stable microbial population dominated by *C. kluyveri* was able to produce $8.17\,g\,L^{-1}$ caproate and $0.32\,g\,L^{-1}$ caprylate in a stable reactor run, under methanogenesis suppressed conditions. An enhancement by Grootscholten et al. [90] was mediated by the higher ethanol load use. Not only higher production rates but also product selectivity was achieved. Caprylate production rate, being a longer MCCA, was lower than caproate production rate. Using this approach, Grootscholten et al. [90] also increased the production rate of caprylate to $0.9\,g\,L^{-1}\,d^{-1}$ with a concentration of $0.6\,g\,L^{-1}$, which was 16 times higher than by Steinbusch et al. [84]. Roghair et al. [81] reached lower caproate production rates ($10.8\,g\,L^{-1}\,d^{-1}$) despite of the high CO_2 flow rate employed. Much lower values were attained by He et al. [72]. In this study, concentrations of 0.22 and $0.15\,g\,L^{-1}$ and production rates of 0.032 and $0.016\,g\,L^{-1}\,d^{-1}$ were obtained for caproic and caprylate production. Nevertheless, it should be highlighted that in that investigation MCCAs were produced by using exclusively CO. In principle, all research studies evidenced a lower production rate and yield of caprylate compared with that of caproic acid; however, Wu et al. [77] demonstrated caprylate selectivity when using butyrate as electron acceptor instead of acetate, while when using caproate the production of caprylate was negligible. Even though most of the research is conducted with *C. kluyveri*, the constant search for alternative microorganisms also showed the effectiveness of other microbial groups for CE. This is the case for *Megasphaera* sp. that metabolizes fructose and produces MCCAs. Jeon et al. [92] evaluated different electron acceptors and obtained maximum caproic concentration ($9.7\,g\,L^{-1}$) when using acetate combined with butyrate and maximum caprylic acid ($1.2\,g\,L^{-1}$) when using caproic and acetate. Despite the normally higher values for *C. kluyveri* in terms of caproic production, it should be highlighted that *Megasphaera* presented higher production rates ($0.41\,g\,L^{-1}\,d^{-1}$).

With regard to odd-carbon number fatty acids, investigations are scarce. The production of heptanoate reported in literature is in the range of that of caprylate. Roghair et al. [81] determined a production rate of $1.8\,g\,L^{-1}\,d^{-1}$ when using propionate and ethanol, together with a CO_2 loading rate of 1 L CO_2 $L^{-1}\,d^{-1}$. According to Grootscholten et al. [93], heptanoate selectivity is reduced due to the formation of valerate, which can be produced as an intermediate from propionate elongation. Nevertheless, those authors were able to increase heptanoate production rate to $4.5\,g\,L^{-1}\,d^{-1}$ and achieve a concentration of $3.2\,g\,L^{-1}$ by increasing ethanol loading and decreasing acetate load in the CE reaction. Moreover, for the first time, this later investigation also reported the production of nonanoic acid at low concentrations. Slightly higher concentration values were recorded by Jeon et al. [92] when using *Megasphaera* with

acetate and pentanoate as electron acceptors. This approach resulted in 3.6 g L^{-1} heptanoate, while this concentration was slightly lower (2.7 g L^{-1}) when using acetate and propionate.

Biology in chain elongation

Clostridium kluyveri is the most studied microorganism in the literature, and it is by far the most reported microorganism in CE processes. This species uses the reverse β-oxidation pathway to elongate the carbon chain of SCCAs. As a matter of fact, *C. kluyveri* dominated the cultures at 50% in recent investigations [71, 84, 89]. Similar to *C. kluyveri*, *Ruminococcaceae bacterium*, *Eubacterium pyruvativorans*, and *Megasphaera elsdenii* all share analogous metabolic properties when it comes to SCCA elongation. While *C. kluyveri* is mostly used for caproate production using ethanol as the electron donor, *M. elsdenii* is known by its ability for caproate production from lactate [78]. Both *C. kluyveri* and *M. elsdenii* are mostly used in pure systems, since most probably they would have problems to adapt to a real anaerobic effluent. Some other *Clostridia* have been used with the purpose of CE. This is the case by Dams et al. [94], who bioaugmented the anaerobic sludge used for CE with these microorganisms. Their results showed an enhanced production of caproic and caprylic acids using *C. acetobutylicum* ATCC 824 in the bioaugmented sludge.

Eubacterium pyruvativorans grows on amino acids or peptides. In this case, two carbon atoms from amino acids are used for elongation. Indeed, their growth is increased when adding SCCAs [95]. *Megasphaera elsdenii* produced a mixture of carboxylates (C2–C6) using glucose and lactate [96] and sucrose and butyrate [97]. More recently, this species was used on the production of heptanoic and octanoic acid using a pure anaerobic culture [92]. Indeed, *Megasphaera* sp. MH demonstrated the fastest productivity of hexanoic acid.

Some other microbial groups supporting CE include *Firmicutes* [89] and *Acinetobacter* [78]. *Acinetobacter* was dominant (49.1%) in a study dealing with CO as the start-up molecule for chain elongation [72].This bacterium plays an important role in the reverse β-oxidation pathway, since it possess the gene encoding fatty acyl-CoA reductases. Together with *Acinetobacter* and *Clostridium*, *Alcaligenes* and *Rhodobacteraceae* were pointed out as microorganisms that could use CO for chain elongation. Indeed, *Rhodocyclaceae* was identified as the dominating microorganism family in the production of caprylate from ethanol and acetate [98].

In addition to the reverse β-oxidation, the fatty acid biosynthesis route was also highlighted as a potential pathway for CE. Unlike acetyl-CoA, in reverse β-oxidation, malonyl-CoA plays the role of two-carbon donor in fatty acid biosynthesis (Fig. 17.4). Both pathways add two carbon atoms to the starting molecule per cycle. Nevertheless, this latter pathway is longer and requires more energy (ATP). Besides, it was more active in MCCA production compared with reverse β-oxidation, in the experiment conducted by Han et al. [99], which focused on chain elongation using ethanol in an upflow blanket filter reactor and a suppressed methanogenic sludge. In this study, *Bordetella avium* and *Plactomycetaceae* were determined as active participants in CE.

Conclusion and perspective

Renewable chemical production via the so-called carboxylate platform is nowadays considered as an attractive biotechnological approach to convert complex organic residues into valuable chemicals. This bioprocess does not require sterilization, and hence, lower capital and operating costs compared with axenic cultures are involved.

Acidogenesis, the primary fermentation stage for MCCAs, is the anaerobic process by which

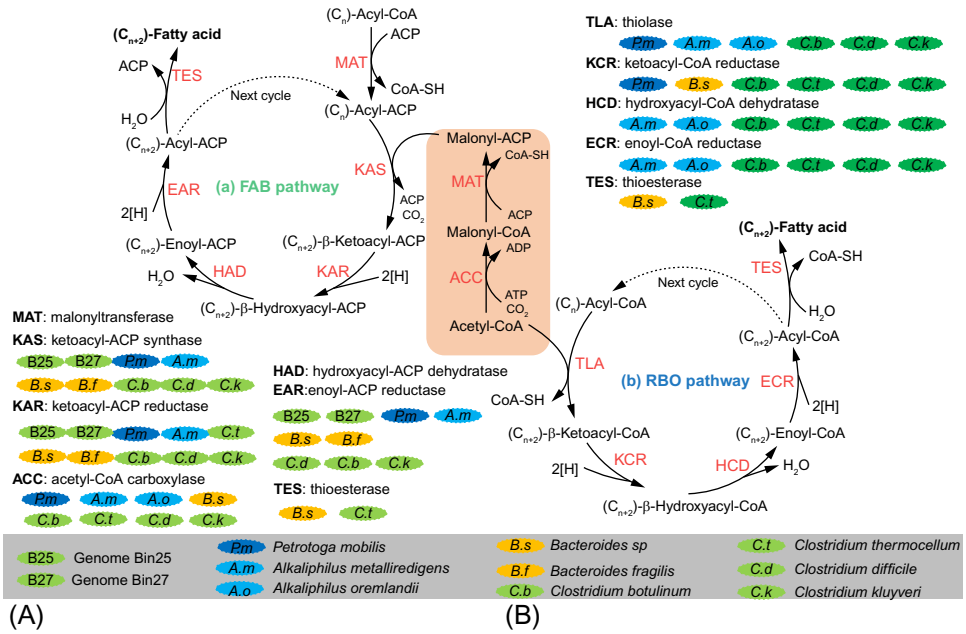

FIG. 17.4 Metabolic pathways and possible participants in chain elongation via (A) the fatty acid biosynthesis pathway and (B) reverse β-oxidation pathway [99].

sustainable biochemicals might be produced. Until now, AD has been mainly devoted to biogas production, and thus, the presence of carboxylates was mostly associated with process failure. Nevertheless, within this new biochemical production platform, carboxylate production requires further research. Production of specific targeted SCCAs, anaerobic microbiome responses toward operational parameter changes, and outcompeting methanogens efficiently are some of the challenges that deserve further investigation.

Between primary and secondary fermentation stages, a separation and/or purification step, often identified as a bottleneck due to the high economic costs, is required. The sensitivity of chain-elongating microorganisms rules the separation efficiency, as well as the purity needed for subsequent carboxylate application. At this moment, there are different available technologies for VFA separation, which entail different purities. This should be addressed as a key parameter to decide the appropriate separation technology.

CE, the secondary fermentation stage for MCCA production, has been named as an important bioprocess for study in the coming years. Nowadays, limited data are available. Most studies have involved ethanol as the electron donor, while the use of alternative ones may broaden the products and, hence, the bioproduct applications. Likewise, a large part of the investigations have been conducted in synthetic medium, while the use of real digestate remains to be addressed. Alternative microorganisms with higher conversion rates and yields might also be of interest in this fermentation stage, as well as identifying by-product inhibition thresholds.

Overall, this technology may become an important biotechnological pillar in the production of value-added products from wastes, such

as wastewater sludges, at industrial scale. However, as reviewed in this chapter, some technological and microbiological drawbacks still require further research before successful implementations at scale will be seen.

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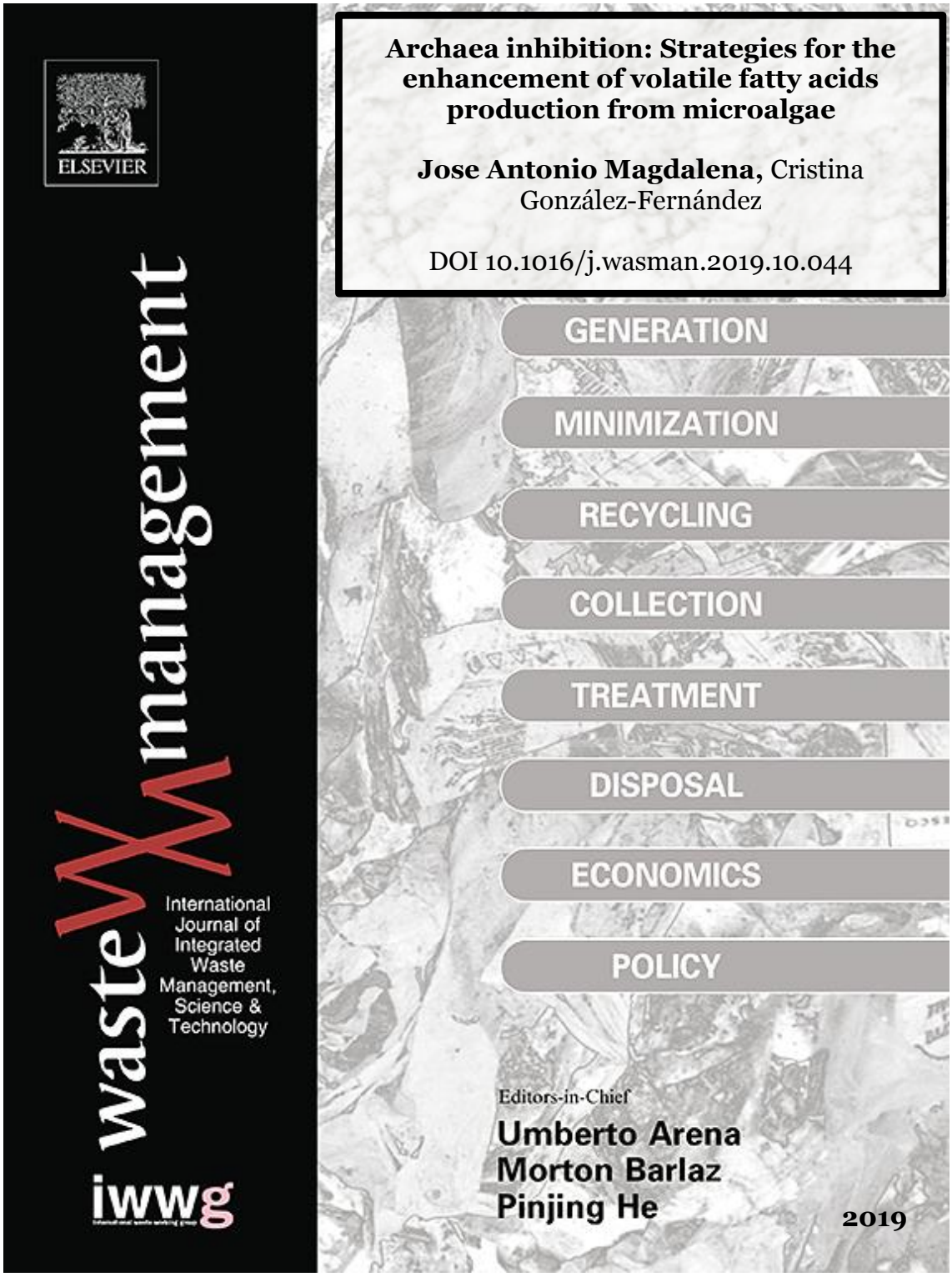
PUBLICATION IV



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Archaea inhibition: Strategies for the enhancement of volatile fatty acids production from microalgae

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ABSTRACT

In the present study, anaerobic sludge was subjected to thermal and chemical pretreatments to favour VFAs production from a protein-rich waste (i.e. microalgae biomass). Sludge pretreatments have been previously used in hydrogen production; however, information about how they can affect VFAs production from microalgae is still lacking. Thermal pretreatment was studied at: (i) 80 °C for 10 and 30 min; (ii) 120 °C for 10 and 30 min; and (iii) 100 °C for 20 min. 2-bromoethanesulfonate (BES) at 10 mM and 30 mM was used as chemical pretreatment. Besides, a combination of both pretreatment methods (80 °C and 120 °C at 10 mM and 30 mM BES) was also tested. Thermal pretreatment increased organic matter conversions into VFAs (up to 71% COD-VFAs/COD_{in}) when compared to control values (40% in the untreated anaerobic sludge). Acetic acid was the most abundant VFAs at high temperatures (120 °C) and when BES was employed (up to 60% and 40%, respectively, in terms of COD). On the other hand, propionic acid was the most abundant product at low temperatures and in the untreated anaerobic sludge (up to 60% in terms of COD). This research work might set guidelines in order to choose a suitable sludge pretreatment for VFAs production from microalgae.

1. Introduction

Anaerobic digestion (AD) is a mature technology employed worldwide for the treatment of organic wastes. AD is a complex process that can be divided into four individual stages, namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Given the complexity of this bioprocess, AD does not only produce methane as ultimate product but also volatile fatty acids (VFAs). These acids are considered chemical building blocks of high importance for the chemical industry. VFAs, traditionally produced through petrochemical routes, are carboxylates that account from two to six carbons (acetic, propionic, butyric, valeric, and caproic acid). The interest of these compounds lies in their wide applications and their increasing market demand (Calt, 2015). In this sense, acetic acid reached a market size of 14,000–17,000 kton/year and an average price of 400–800 €/ton, while propionic acid increased its price up to 2,500 €/ton (Atasoy et al., 2018).

Since AD has been traditionally used for biogas production, the VFAs production in the carboxylate platform requires a revisit of the AD process. Indeed, VFAs accumulation has been always pointed out as a failure in the biogas production process. Nevertheless, within this new approach of carboxylates production via AD, VFAs accumulation becomes the main goal of the process. During AD, VFAs might be degraded into acetate, hydrogen and carbon dioxide by bacteria and subsequently metabolized by methanogenic archaea for biogas production. However, in the context of the carboxylate platform, VFA consumption should be avoided. In this sense, microalgae biomass arises as a promising feedstock due to their macromolecular composition (containing fermentable carbohydrates, proteins and lipids). The high protein content exhibited by some microalgae strains (50–70% w/w) can cause AD destabilization driven by high ammonium concentration (Magdalena et al., 2018; Yenigün and Demirel, 2013). This imbalance results in methanogenesis inhibition causing VFAs accumulation (Mahdy et al., 2015). Furthermore, microalgae offer the advantage of treating wastewater at lower cost than activated sludge systems due to their *in situ* oxygen production via photosynthetic activity (Acién et al., 2016). In this manner, similarly to activated sludge, microalgae biomass can be regarded as a waste to be potentially revalorized as substrate for VFAs production

Abbreviations: VFAs, Volatile fatty acids; AD, Anaerobic digestion; BES, 2-bromoethanesulfonate; BMP, Biochemical methane potential; BCP, Biochemical carboxylate potential; TS, Total solids; VS, Volatile solids; HRT, Hydraulic retention time; OLR, Organic loading rate.

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PUBLICATION V

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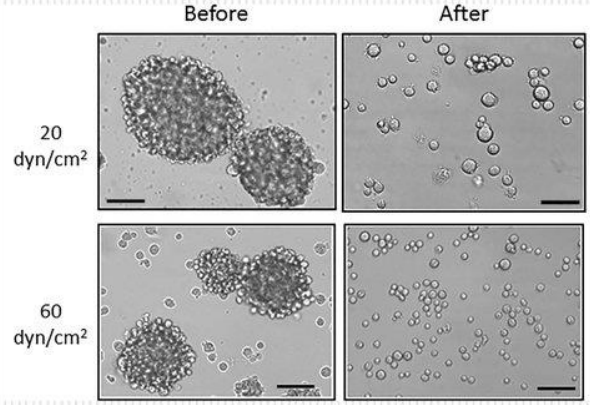
2018

BIOTECHNOLOGY PROGRESS



Volatile fatty acids production from protease pretreated *Chlorella* biomass via anaerobic digestion

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Volatile Fatty Acids Production from Protease Pretreated *Chlorella* Biomass via Anaerobic Digestion

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Volatile fatty acids (VFAs) produced via anaerobic digestion (AD) are regarded as a low cost production process of building blocks of interest for the chemical industry. In this study, VFAs and methane production were assessed in batch reactors at different temperature ranges (psychrophilic 25°C, mesophilic 35°C, thermophilic 50°C) and different pH values (5.5 and 7.5) using protease pretreated *Chlorella* sp. biomass as substrate. Acetic acid and propionic acid were the most abundant products (up to 73% of the total VFAs) during the first days independently of the conditions. VFAs concentration decreased over time as methane was produced after a lag phase of 7–10 days. Results showed that best conditions for VFAs production were mesophilic temperature ranges (35°C) at neutral initial pH values (7.5), and psychrophilic temperature ranges (25°C) at low initial pH values (5.5) which resulted in a conversion of the initial COD into VFAs of 48%, respectively.
Keywords: volatile fatty acids, biogas, microalgae, protease, anaerobic digestion

Introduction

Volatile fatty acids (VFAs) are widely applied in the chemical industry as building blocks, for the production of food preservatives, polymers, inks, and paints, as well as solvents and fuels. Different soluble organic acids are included under the name of VFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids) and their market price is highly dependent on the number of carbons.[1] Until now, VFAs-based industry relies on petro-chemistry, in this context, approximately 4% of the global oil consumption is devoted to chemical and plastics production.[2] Petrochemical processes entail high pressure and temperature and thus, high energy requirements are associated with this technology. Besides, oil reserves are finite and prices instability is inherent to the petrochemical route. As an alternative, VFAs might be produced by means of microorganisms under milder conditions during anaerobic digestion (AD). Four biological processes are involved in the AD of organic matter, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. VFAs are produced during the acidogenic phase of AD and are the precursors for the subsequent methanogenesis. Therefore, the inhibition of the final step (methanogensis) of the AD could provide a very interesting bioprocess to produce VFAs from inexpensive and residual organic streams.

AD has been extensively employed for biogas production. This bioprocess is very effective for biodegradable organic matter removal, can be developed at any scale and can be operated using a wide variety of low-priced feedstocks. In this sense, the use of the AD technology to produce VFAs has attracted increasing interest as a promising alternative to petrochemical production.

Among the substrates that can be used for AD, microalgae arise as a promising feedstock due to their fast growth, their ability to thrive in residual effluents or the non-necessity of arable lands. AD of microalgae for biogas production has been intensively investigated over the last decade and the main bottlenecks have been identified.[3,4] One of the main drawback of using microalgae as feedstock for AD is the robust cell wall exhibited by some strains, preventing organic matter accessibility to bacteria attack.[3] The composition and structure of microalgae cell walls is highly specie- and growth conditions-dependent, ranging from simple and thin membranes to complex and rigid structures. Most frequently, strains growing in outdoors conditions, present more complex and robust cell walls. To overcome this drawback, a great number of pretreatments have been investigated over the last decade to facilitate cell wall disruption and promote the first hydrolysis stage of AD. Enzymatic pretreatment with proteases is a very effective method for hydrolysing microalgae cells.[5] However, the high

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Semi-continuous anaerobic digestion of protease pretreated *Chlorella* biomass for volatile fatty acids production

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Semicontinuous anaerobic digestion of protease pretreated *Chlorella* biomass for volatile fatty acids production

Jose Antonio Magdalena, Mercedes Llamas, Elia Tomás-Pejó and Cristina González-Fernández*

Abstract

BACKGROUND: Anaerobic digestion (AD) could be designed as a source of volatile fatty acids (VFAs). However, acidogenesis optimization for novel substrates such as *Chlorella vulgaris* biomass needs to be investigated considering parameters such as temperature (T), organic loading rate (OLR), hydraulic retention time (HRT) and the adaptation of the sludge to temperature and substrate.

RESULTS: The best organic matter conversion into VFAs (COD_{VFA}/COD_{in}; COD, chemical oxygen demand) was achieved with HRT = 8 d, adapted anaerobic sludge (AAS) and OLR = 1.5 gCOD L^{-1} d^{-1} (COD_{VFA}/COD_{in} = 39.8 ± 1.0% and productivity VFA = 0.5 ± 0.1 g L^{-1} d^{-1}). Acetic and butyric acids represented 50% of the total VFAs. The microbiota related to acidogenesis and acetogenesis (Firmicutes 55% of the operational taxonomic units in R5) and the low archaeal population resulted in VFA accumulation at 25 °C.

CONCLUSIONS: The use of low HRT and temperatures promoted VFAs production especially when AAS was employed. Microbial communities were strikingly different to the ones often found in AD targeted at biogas production. The relevance of the Firmicutes phylum (≤55% in R3 and R5) and *euryarchaeota* absence at 25 °C contributed to VFA accumulation. The use of AAS reported an increase in *Actinobacteria* species.

Supporting information may be found in the online version of this article.

Keywords: volatile fatty acids (VFAs); carboxylate platform; microalgae; microbial population; *Chlorella vulgaris*

INTRODUCTION

Volatile fatty acids (VFAs) are valuable building blocks for the chemical industry due to their wide applications in fields including food preservation, cosmetics, textiles and pharmaceuticals. VFAs have attracted the attention of the scientific community in the so-called carboxylate platform.[1–3] These compounds have been traditionally obtained by petrochemical means. Nevertheless, the increasing search for renewable sources together with environmental concerns is boosting the need of developing new production models. Biological VFAs synthesis through anaerobic digestion (AD) represents a low-cost and environmentally friendly alternative to fossil petroleum-based technology due to the milder conditions established in bioprocesses (lower temperature and pressure if compared to petrochemical pathways).

The four-stage complex organic matter degradation process of AD involves different microbial consortia connected in an intricate reaction scheme. During the hydrolysis step, a complex organic substrate composed of carbohydrates, proteins and lipids is depolymerized by exoenzymes. Subsequently, the produced compounds are metabolized by acidogenic bacteria giving rise to VFAs during the fermentative stages (acido- and acetogenesis). In order to avoid the transformation of VFAs into biogas, the inhibition of the following AD stage (methanogenesis) is critical. However, achieving VFA accumulation is one of the most significant challenges due to impediments to by-product toxicity and microbial competition for VFAs substrate.[4] In this sense, microalgae arise as a promising feedstock due to their macromolecular composition. In fact, the high protein content that some microalgae strains exhibit (50–70%[5,6]), could cause AD destabilization driven by high concentration of ammonium (NH_4^+). This imbalance results in methanogenesis inhibition and accumulation of VFAs. To avoid methanogen toxicity, the ammonium concentration threshold has been set around 1700–1800 mgN-NH_4^+ L^{-1} (150 mgNH_3 L^{-1} [7]). When this range is overcome, VFAs accumulate instead of being transformed into biogas.[8] Another possible approach to inhibit the methanogenesis step might be to take into account the metabolic features of the different species involved in the AD process. In this sense, the slow growth and sensitivity of methanogenic archaea with regard to those of anaerobic bacteria can be used as a tool to mediate methanogenesis inhibition.[3]

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Operational conditions such as temperature (T), organic loading rate (OLR) and hydraulic retention time (HRT) can be manipulated to affect population dynamics within the bioreactor to favor the activity and prevalence of those species in charge of VFA production.[9]

Biogas production has traditionally used AD, and thus, the carboxylate platform requires revisiting the AD process. Indeed, VFA accumulation has been always pointed out as a process failure. Nevertheless, within this new approach of biochemical production via AD, the goal is not to use VFAs for methanogenesis but, rather, to accumulate them for further use. The aim of the present study was to gain insights into the effect that operational parameters (T, HRT, OLR) have on VFA production using microalgae biomass as substrate. The effect of different temperature ranges (psychrophilic 25 °C and mesophilic 35 °C), OLR (1.5 gCOD L^{-1} d^{-1} and 3 gCOD L^{-1} d^{-1}; COD, chemical oxygen demand), HRT (8, 10 and 12 d) and inocula (adapted or not) were assessed in terms of VFA production yields and profile. Furthermore, microbial communities (bacteria and archaea) were identified in the semicontinuous anaerobic digesters and correlated with the reactor performance.

MATERIAL AND METHODS

Inoculum and substrate

Mesophilic anaerobic sludge was supplied by the wastewater treatment plant (WWTP) of Valladolid (Spain). Total solids (TS) and volatile solids (VS) were 17.9 g L^{-1} and 11.8 g L^{-1}, respectively. This mesophilic sludge (AS) was used during the operation of Reactor 1 (R1), Reactor 2 (R2) and Reactor 3 (R3) (Table 1). Once stabilized, inoculum adapted to psychrophilic temperature range was taken from R3 (AAS) operated at 25 °C and used as inoculum for Reactor 4 (R4) and Reactor 5 (R5).

Digesters were fed with protease-pretreated *C. vulgaris*. Raw microalgae biomass was purchased from Allmicroalgae (Lisbon, Portugal) and kept frozen at −20 °C. The biomass exhibited 57.9% (w/w) proteins, 21.6% carbohydrates, 13.4% lipids and 7.1% ashes. Because the goal of this study was to investigate the acidogenesis stage, biomass pretreatment was applied to avoid hydrolysis limitation. The commercial enzymatic cocktail 'Alcalase 2.5 L' (Novozyme, Denmark) was employed to pretreat the biomass (TS = 46.8 ± 0.1 g L^{-1} and VS = 43.6 ± 0.2 g L^{-1}) and make available the organic matter to anaerobic microorganisms. The dosage (0.585 UA gTS $^{-1}$) and procedure was based on results obtained for *C. vulgaris*.[8,10]

Experimental set-up

The AD was carried out in continuous stirred tank reactors (CSTRs) of 1-L working volume under semicontinuous feeding mode. Reactors were stirred magnetically at 250 rpm. Digester operational conditions are presented in Table 1. Steady state was considered to have occurred after 3 HRT when a stable effluent COD concentration had been achieved. Parameters such as total COD, soluble COD, NH_4^+ concentration, biogas composition, VFAs and pH were measured twice per week. Total COD and soluble COD (filtered through 0.45-µm mesh) were assessed using commercial test kits (Merck ISO, 15705). The NH_4^+ concentration of digesters effluent was measured with a commercial kit (Merck, indophenol blue method 000683). The % COD removal was calculated according to Eqn (1):

$$\%\text{COD removal} = \frac{\left(COD_{in} - COD_{out}\right)}{COD_{in}} \cdot 100 \qquad (1)$$

Table 1. Conditions applied for the anaerobic reactors

	Temperature (°C)	OLR (g $L^{-1}d^{-1}$)	HRT (d)	Sludge
R1	35	1.5	10	AS[a]
R2	35	3	10	AS
R3	25	1.5	10	AS
R4	25	1.5	12	AAS[a]
R5	25	1.5	8	AAS

[a] AS, anaerobic sludge; AAS, adapted anaerobic sludge.

The methane (CH_4) content in the biogas was determined by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6-60/80 Sulfinert P packed column [7′ × 1/8″ outer diameter (o.d.)] and a MS13X4-09SF2 40/60 P packed column (9′× 1/8″ o.d.) (PerkinElmer). To determine VFA concentration, the sample was filtered through 0.2-µm mesh and analyzed by liquid chromatography using an Agilent 1260 HPLC-RID (Agilent) equipped with a Cation H Refill Cartridge Microguard column (Bio-Rad) and an Aminex HPX-87H ion exclusion column [300 × 7.8 mm inner diameter (i.d.)] (Bio-Rad). The pH was monitored but not controlled during CSTR operation.

DNA extraction

Initial inoculum and samples coming from the five digesters were collected for DNA extraction. The kit 'FastDNA SPIN Kit for Soil' was used (MP Biomedicals, LCC) for this purpose. The quality of the extracted DNA was checked by measuring the absorbance ratio 260/280 nm, which was between 1.8 and 1.9. Subsequently the samples were sent to Life Sequencing (University of Valencia, Spain) where a capture of the 16 s rRNA hypervariable regions V3–V4 was performed.[11] The database BLAST was used in order to associate results obtained to a taxonomical group. Sequence abundance <2% was considered negligible and thus deleted from the analysis.

Data analysis

Statgraphics CENTURION XV computer software was used for the statistical analysis of the data. A parametric one way ANOVA was used for assessing the CSTR performance (confidence interval 95%). Differences were considered significant at *P*-value ≤0.05.

RESULTS AND DISCUSSION

Effect of OLR and temperature on the methanogenic step and VFA production using nonadapted mesophilic inoculum

COD removals

The slow growth of methanogens compared to hydrolytic bacteria[12] can be used as a tool to decrease biogas production favoring VFAs accumulation. Hence, to study the effect of temperature and OLR on VFA production anaerobic bioreactors were operated at HRT = 10 d with the aim of washing out methanogenic archaea to decrease biogas production. Results showed similar % COD removals for the mesophilic reactors R1 and R2 (23.6 ± 2.3% and 26.3 ± 2.6%, respectively), which were higher than for the psychrophilic reactor R3 (11.9 ± 2.9%) (Table 2). With regard to the biogas composition, CH_4 content was 52.1 ± 2.4% (v/v) for R1, 48.9 ± 5.5% for R2 and 20.8 ± 2.6% for R3. COD removals were low when compared to a process devoted to biogas production.

Table 2. Results of different parameters assessed in the anaerobic reactors

	R1	R2	R3	R4	R5
% CH_4 in biogas (v/v)	52.1 ± 2.4	48.9 ± 5.5	20.8 ± 2.6	10.5 ± 4.2	13.2 ± 1.7
% COD removal	23.6 ± 2.3	26.3 ± 2.6	11.9 ± 3.0	10.4 ± 2.7	8.9 ± 3.5
pH	6.9 ± 0.1	7.1 ± 0.1	6.3 ± 0.1	6.4 ± 0.1	6.4 ± 0.1
NH_4^+ (g L^{-1})	0.7 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1

For instance, Mahdy et al. (2015) used similar biomass in a semi-continuous anaerobic digestion at mesophilic conditions (35 °C) and their results showed a CH_4 production of 128.4 ± 15.3 mL CH_4 $gCOD_{in}^{-1}$ (56% COD removal) when the reactor was fed with OLR = 1.5 gCOD L^{-1} d^{-1} and HRT = 20 d. Because CH_4 production was targeted, authors used longer HRT values (15 and 20 d) than in the present study (10 d). Indeed, Mahdy et al. (2015) reported VFA accumulation (1 gVFA L^{-1}) caused by NH_4^+ inhibition and they pointed out that increasing HRT could be used as a tool to decrease VFAs overloading to ensure a better balance between bacteria and methanogenic archaea, whereas the opposite was intended in the present study. This conclusion also was supported by previous results obtained in a continuous anaerobic digestion fed with microwave-pretreated microalgae biomass.[13] In that case, CH_4 yield was improved by 30% when HRT was increased from 15 to 20 d (from 36% to 42% COD removal). With regard to the present study, the low CH_4 productions observed in the reactors ($P = 0.13$), regardless of temperature or applied OLR, suggested that methanogenic activity was limited due to the short HRT imposed.

Digestion temperature, the other tested operational condition, affected the methanogenic step according to the COD removals attained in the mesophilic (R1, R2) and the psychrophilic reactors (R3). In this sense, lower CH_4 production is normally associated with lower temperature digestion because bacterial growth and conversion processes are slower.[14] This association, beneficial for VFA production, was observed especially in R3. The % COD removal was lower in the psychrophilic range (11.9%) compared to the attained values for mesophilic digestions (23.6 and 26.3%). Therefore, to ensure sufficient bacterial mass was retained in the reactor, psychrophilic digestion (R3) might require longer retention times than for mesophilic reactors (R1 and R2). Hence, the COD removal and biogas composition in R3 were evidence of the higher methanogenic inhibition than in R2 and R1, confirming that low-temperature digestion achieved better VFA accumulation.

VFAs production: Conversion yields and profiles

The effects of OLR and temperature were evaluated in terms of COD conversion into VFAs (% COD_{VFA}/COD_{in}) and VFA profile in R1, R2 and R3. R1 and R2 achieved the maximum conversion during the third HRT (days 20–30, 26.7 ± 0.1% and 30.0 ± 0.1% COD_{VFA}/COD_{in}), whereas R3 achieved the maximum conversion in an earlier stage of the process, which was during the second HRT (days 10–20, 38.8 ± 3.4% COD_{VFA}/COD_{in}). Average conversion yields were 25.6 ± 3.0%, 25.8 ± 3.9% and 35.5 ± 3.0% COD_{VFA}/COD_{in} for R1, R2 and R3, respectively (Fig. 1).

The effect of OLR was assessed by comparing reactors R1 and R2 set at 1.5 and 3 gCOD L^{-1} d^{-1}, respectively. Results showed that VFA production was two-fold higher in R2 ($P = 1.7 \times 10^{-5}$) than R1, reaching nearly 10 000 $mgCOD_{VFA}$ L^{-1}. However, organic matter conversion into VFAs was not affected by OLR and similar conversion yields (25%) were reached by both of them (Fig. 1). Opposite to that trend, Bermúdez-Penabad et al. (2017)[15] fed a semicontinuous reactor with tuna waste to evaluate VFA production and highlighted the increase in organic matter conversion into VFAs when higher OLR values were employed. More specifically, those authors reported 5000 mg COD_{VFA} L^{-1} in a reactor fed with OLR 2 gCOD L^{-1} d^{-1} and HRT = 10 d (25% COD_{in} converted into VFAs), whereas when OLR of 4 gCOD L^{-1} d^{-1}, 30% COD_{in} was converted into VFAs. The increasing pH control set by those authors (pH 5–9) may have caused this improvement in VFA conversion, whereas in the present study the pH was monitored but not controlled. Despite the differences attained with regard to the OLR effect, conversion yields of COD into VFAs are in the range of those obtained herein.

The effect of temperature was evaluated by comparing R1 and R3, which were operated at 35 °C and 25 °C, respectively. The total maximum VFA concentration ($mgCOD_{VFAs}$ L^{-1}) was higher when the experiment was performed within the psychrophilic range temperature (5056 $mgCOD_{VFAs}$ L^{-1} in R3 *versus* 4057 $mgCOD_{VFAs}$ L^{-1} in R1). The enhancement in VFA production was reflected also on the maximum conversion yield obtained at Day 20 of digestion (COD_{VFA}/COD_{in} = 28.0% for R1 and COD_{VFA}/COD_{in} = 38.8% for R3). Temperature has been regarded as a tunable parameter to affect VFA production in the literature. As a matter of fact, Zhuo et al. (2012)[16] tested the effect of temperature (10, 20, 37, 55 °C) on the hydrolysis and acidification stages of AD using waste-activated sludge as substrate. Opposite to the trend observed in this study, they reported an increase of VFA conversion from 10 °C (COD_{VFA}/COD_{in} = 10%) to 37 °C (COD_{VFA}/COD_{in} = 41%), whereas at 20 °C they obtained a conversion of COD_{VFA}/COD_{in} = 30%. The authors attributed the progressive VFA increase to the better hydrolysis of proteins and carbohydrates at higher temperatures. However, in this study, the hydrolysis enhancement associated to the increasing temperature digestion was negligible because a proteolytic pretreatment was carried out to discard hydrolytic problems of microalgae biomass. In this sense, the low methanogenic activity, resulting in scarce organic matter removal, could have caused the increase in COD accumulated as VFAs in R3 in comparison with R1. At this point, it should be highlighted that maximum COD conversion values attained in R3 at 25 °C (COD_{VFA}/COD_{in} = 38.8%) are in the range of other studies found in literature. In this manner, Zhuo et al., (2012) reported COD_{VFA}/COD_{in} = 30% at 20 °C, whereas Oktem et al. (2006)[17] achieved COD_{VFA}/COD_{in} = 44% when working with an acidogenic reactor fed with pharmaceutical wastewater (35 °C, HRT = 8–24 h).

Regarding the effect of OLR used to feed the reactors on VFA production profile (% COD_{VFA}/ total COD_{VFAs}), propionic acid concentration in mesophilic reactors R1 and R2 increased with digestion time, reaching average values of 36.0 ± 2.0% for R1 and 31.8 ± 4.9% for R2. Accumulation of propionic acid is related to the Gibbs energy from the degradation reactions. Degradation of propionic acid is the less favorable reaction ($\Delta G = +76.1$ kJ mol^{-1}) when compared to other VFAs such as acetic acid, which is a spontaneous process ($\Delta G = -30$ kJ mol^{-1}).[18] Hydrogen is one of the products released upon propionic acid degradation;[19] thus, as hydrogen is removed from the media by anaerobic microorganisms, propionic acid is degraded as well. Results suggest that the harsh environment imposed to the system could have caused

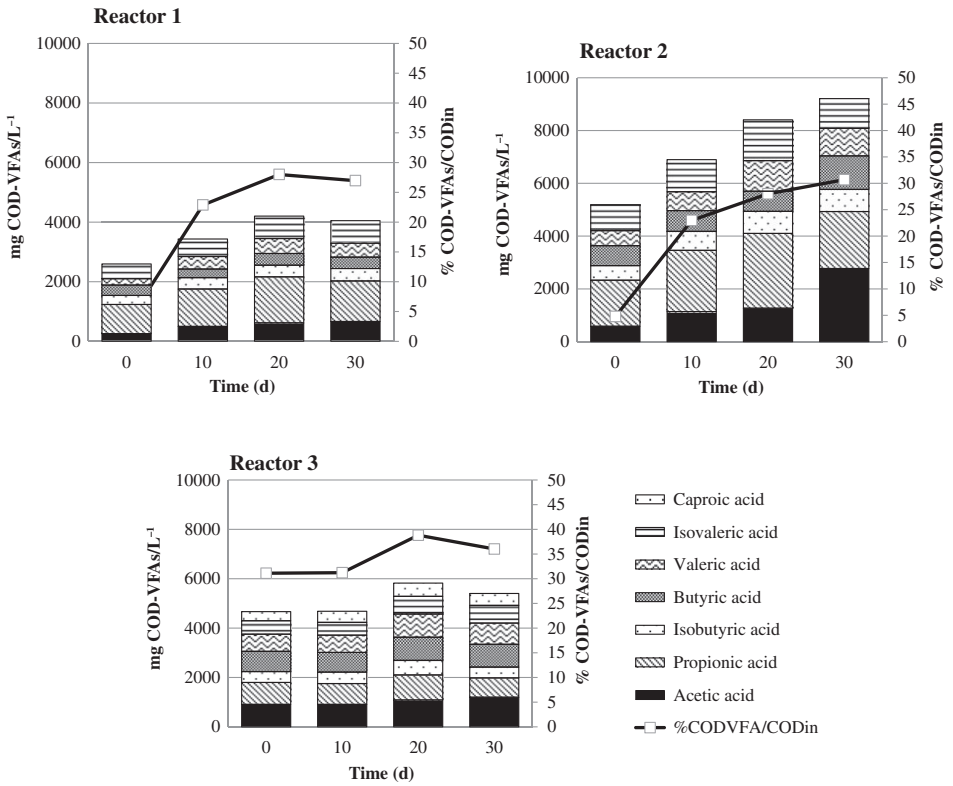

Figure 1. Representative samples from the initial days, HRT, 2HRT and 3HRT providing evidence for VFA production and conversion.

a drop of syntrophic microorganism activity, resulting in propionic acid accumulation. Following propionic acid, acetic acid was the second most abundant acid in these reactors (14.1 ± 2.3% and 18.1 ± 6.5% in R1 and R2, respectively). The abundance of acetic acid might result from VFA degradation. In this sense, longer VFAs chains are converted into acetate and hydrogen through the ß-oxidation pathway. This VFA also was found to be among the most abundant products in other studies using different substrates such as wasted activated sludge, maize silage and whey.[20,21] These two VFAs accounted for ≤50% of the VFAs obtained in R1 and R2. The rest of them ranged from 10 to 20% each and were not influenced by the different OLR applied. These results are in accordance with another study where microalgae biomass was employed. Jankowska et al. (2017) carried out a mixed culture fermentation with *Scenedesmus quadricadua* and *C. vulgaris*. They found that acetic acid was the most abundant product (42%) during the first days, followed by propionic and butyric acids (19% each), and isovaleric acid (12%) whereas the rest of the VFA percentages were even lower.

Regarding the effect of digestion temperature on VFA profile, acetic acid was the most abundant product in R3 (19.9 ± 1.5%) together with 17.3 ± 1.9% propionic and 16.95 ± 0.6% butyric acids, whereas propionic acid stood out as the most abundant product in R1 (36.0 ± 2.0%) followed by acetic (14.1% ± 2.3%) and isovaleric (18.2 ± 1.8%) acids. The accumulation of acetic acid as the most abundant VFA in R3 indicated an imbalance in the AD process because this compound is immediately transformed into CH_4 by methanogenic archaea.[22] Moreover, other compounds formed by the subsequent VFA degradation such as other VFAs, lactate or ethanol are converted into acetic acid in the acetogenesis step which seems likely to have increased the percentage of acetic acid. Thus, acetic accumulation confirmed inhibition of methanogenesis in R3 supporting the low COD removal observed (26.3 ± 2.6%) in comparison to R1 (11.9 ± 2.9%). Digestion temperature also seemed to affect the production rate of propionic acid. Propionic acid was consumed during the first days of the experiment in R3, and its concentration dropped continually through the digestion time until 15.9% of the total VFAs when the steady state was achieved. Digestion temperature affected the production and degradation of C4-C5-C6 VFAs differently. The average amount of C4-C5-C6 in R1 was 50%, whereas it was 62% in R3. This difference is attributed to the fact that caproic acid was not observed in mesophilic reactors, possibly because the raise in the digestion temperature caused its degradation to smaller VFAs. In this sense, caproic acid contributed ≤8.9 ± 0.1% in R3. In the same way, butyric acid percentage reached higher values in R3 (18.0 ± 0.1%) compared to 9.7 ± 1.4% determined in R1.

Effect of HRT on VFA production using adapted inoculum under psychrophilic conditions

Because low digestion temperature provided the highest COD conversion yields into VFAs, the AD process was further studied at psychrophilic digestion. Aiming at increasing VFA production and conversion yields, the use of adapted sludge under different HRTs was tested at psychrophilic temperatures. Adapted sludge from R3 was collected to inoculate R4 and R5 which were operated at different HRTs (Table 1).

COD removals

COD removals obtained with the adapted sludge for R4 and R5 were similar to those obtained previously in R3 (11.9 ± 2.9% for R3 against 10.4 ± 2.7% and 8.9 ± 3.5% for R4 and R5, respectively). Reactors showed similar CH_4 content [23.8 ± 6.7% and 25.9 ± 1.6%

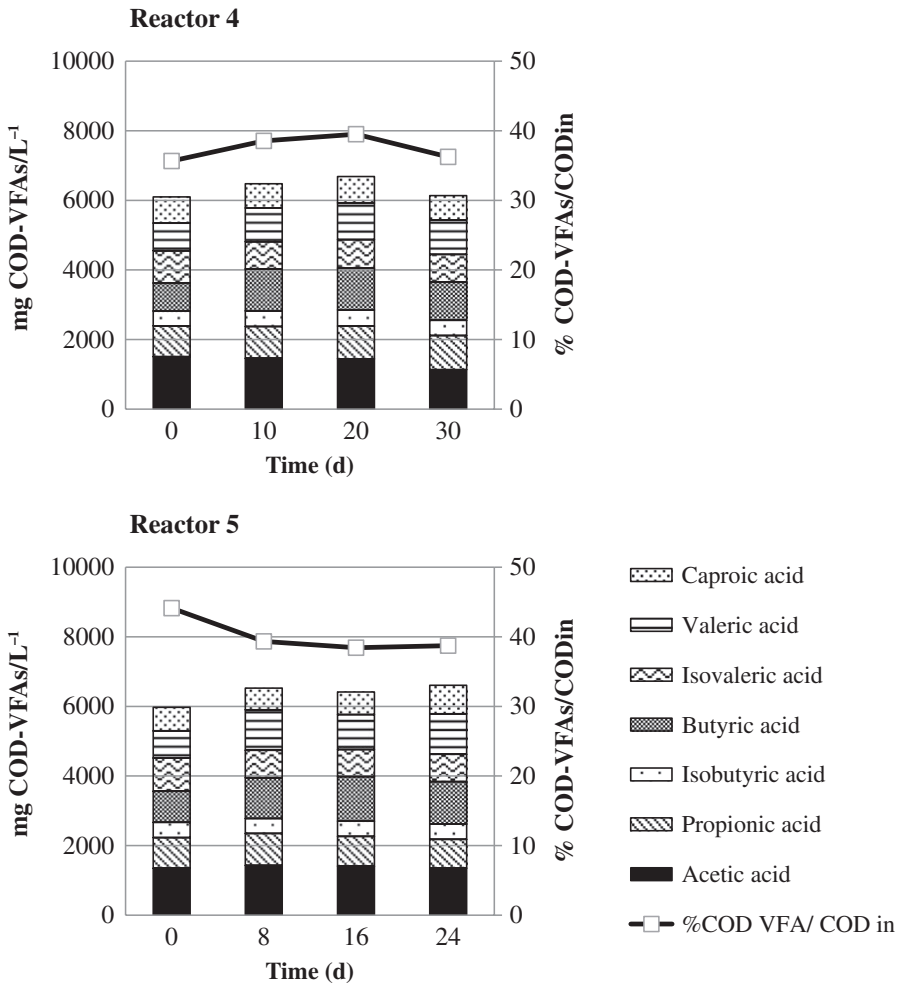

Figure 2. Representative samples from the initial days, HRT, 2HRT and 3HRT providing evidence for VFA production and conversion.

(v/v)] in the biogas to R3 (20.8 ± 2.6% v/v). Psychrophilic conditions and the low HRT values applied in R4 and R5 maintained the inhibition of the methanogenesis step already achieved in R3. Regardless of the HRT, the methanogenic population performance remained unaltered.

VFA production: conversion yields and profiles

The use of the adapted inoculum maintained conversion yields (% COD_{VFA}/COD_{in}) in R4 and R5. Both digesters presented values of 38.0 ± 1.0% and 39.8 ± 1.8%, respectively, against 35.4 ± 3.8% determined for R3 when nonadapted sludge was used (Fig. 2). In reactors with adapted inoculum (R4 and R5), even though final conversion values were similar, both digesters reached the stability in a different period of time. Although R4 (HRT = 12 d) required 16 d to achieve the maximum conversion yield, R5 (HRT = 8 d) achieved the same yield after one week of operation. VFA production (mg L^{-1}) demonstrated a more stable (Fig. 2; RSD < 3.8%) trend when the adapted sludge was used compared with operation using nonadapted sludge (R1, R2 and R3). In this sense, total VFA concentration remained similar in R4 and R5 (5696 ± 161 and 5803 ± 223 mgCOD_{VFA} L^{-1}, respectively). However, the VFA daily production rate increased from R4 (466 mgCOD_{VFA} L^{-1} d^{-1}) to R5 (734 mgCOD_{VFA} L^{-1} d^{-1}). Thus, production rate values in R5 were considerably higher than those obtained with the nonadapted sludge (R3, *c.* 489.5 mgCOD_{VFA} L^{-1} d^{-1}). This fact suggested that the anaerobic microbiome was underestimated because its volumetric productivity could be higher, as evidenced during the operation of R5 (734 against 466 mgCOD_{VFA} L^{-1} d^{-1} for R5 and R4, respectively). Jankowska et al. (2015)[23] analyzed the retention time impact in VFA productivity when primary sludge and waste-activated sludge were employed as substrate. They concluded that short retention time at acid pH were the best conditions to promote VFAs productivity. This conclusion was in accordance to the results attained herein.

With regard to VFA profile, as described previously for the nonadapted sludge reactor at psychrophilic conditions (R3), acetic acid was the most abundant VFA in both digesters (R4 and R5). Partial acetic acid concentrations reached average values of 24.5 ± 2.1% and 24.3 ± 0.6% of the total concentrations in R4 and R5 (respectively) and remained similar throughout the experiment. Propionic acid concentration was similar in R3, R4 and R5 (15 – 17%). The main difference was found with regard to C4-C5-C6 VFAs. For instance, butyric acid increased in R4 and R5 reaching maximum values of 20.3 ± 1.6% in R5. In general, R4 and R5 enhanced the formation of C4-C5-C6 VFAs, representing around 73% of the total amount of VFAs when compared to R3 (63%). Therefore, the use of adapted sludge (R4, R5) supported a quite stable production of long-chain VFAs while maintaining the inhibitory conditions necessary for the methanogenic step.

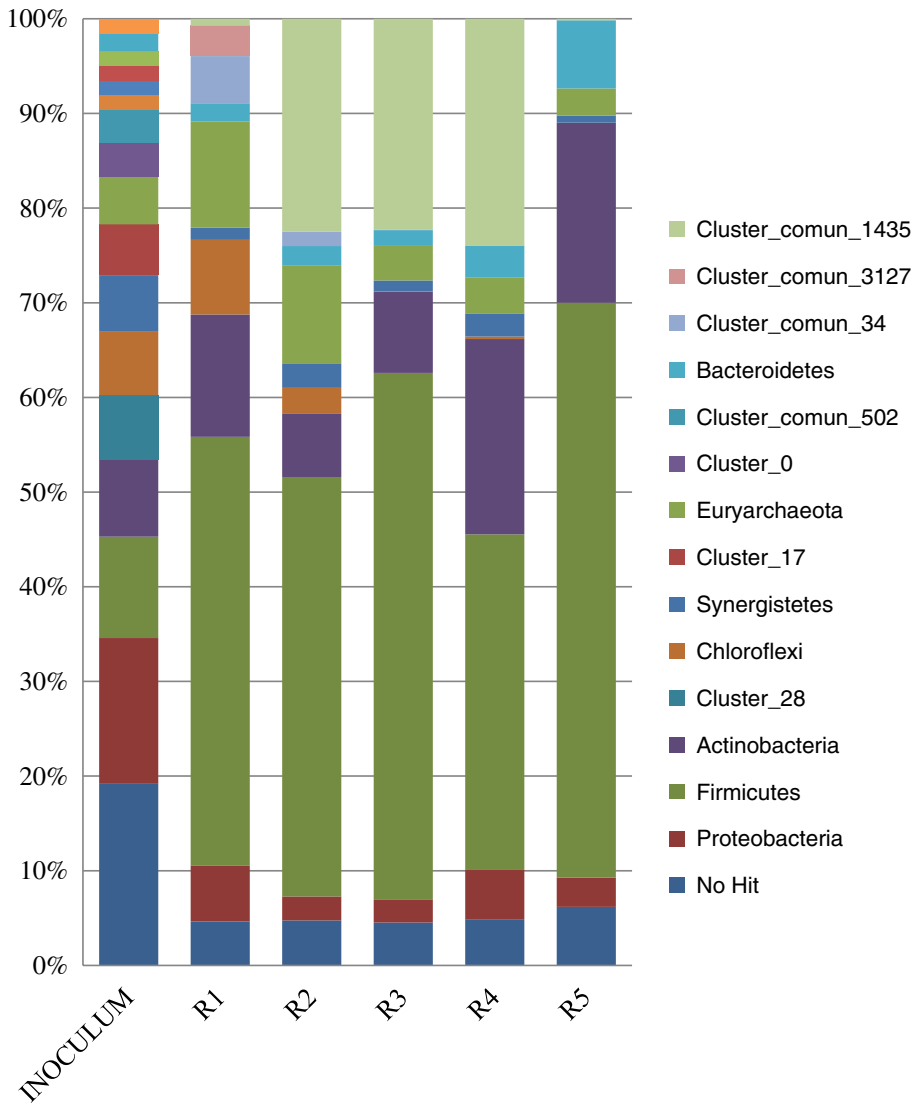

Figure 3. Taxonomic profiles at phylum level for the bacteria and archaea communities found in the inoculum and reactors (R1 – R5).

Dynamics of the microbial communities

The importance of linking VFA production to microbial communities has been reviewed recently.[24] Aiming at further explaining the results obtained in the digesters in terms of COD removals and VFA production, DNA was extracted from the anaerobic sludge (inoculum) and the biomass developed in the reactors (R1 – R5) to analyze the microbial population involved in the processes.

The rarefaction curves (Supporting Information, Fig. S1) obtained at genus level showed a plateau in R1 to R5, indicating that the diversity achieved in the sample was correctly represented. This also was noticeable taking into account the Shannon index, a parameter commonly used to characterize microbial community diversity. Lower values indicate low diversity (R1 – R5 = 0.2 – 0.3), whereas higher values indicate higher diversity (inoculum = 2.66). The different diversity was attributed to the substrate fed into the reactors. The inoculum came from a WWTP fed with mixed primary and secondary sludge, whereas the substrate fed herein was microalgae biomass. Moreover, the operational conditions imposed (HRT, OLR and temperature) for methanogenesis inhibition could have caused the death and wash-out of other species, resulting in a specialization of the inoculum. This specialization would be thus the responsible for the lowered Shannon value. This trend also has been reported by Gonzalez-Fernandez et al. (2018).[25]

Once the inoculum was subjected to the AD process at different operational conditions, population changes were observed. The OLR effect was evaluated comparing the samples from R1 and R2 (1.5 and 3 gCOD L^{-1} d^{-1}, respectively, and T = 35 °C). Profiles of these samples changed drastically compared to the anaerobic sludge used as inoculum. At the phylum level, bacterial distribution was highly represented by Firmicutes accounting for ≤40% in both mesophilic reactors R1 and R2 (Fig. 3). However, the genera distribution in both reactors within this phylum was different. The lowest applied OLRs (R1) favored the growth of genus *Acetobacterium* (15%) and *Eubacterium* (8%) (Fig. 4) that usually release different products such as VFAs (acetate and butyrate), formate, CO_2 or hydrogen.[26] In fact, acetic acid and butyric acid represented *c.* 25% of total VFA production in R1 and R2. The use of the highest OLR (3 gCOD L^{-1} d^{-1}) in R2 promoted the presence of *Sporanaerobacter* (12%) and a cluster that was associated with the Ruminococcaceae family (21%, identified with a similarity of 97%). Found genera, belonging to the Firmicutes phylum include species

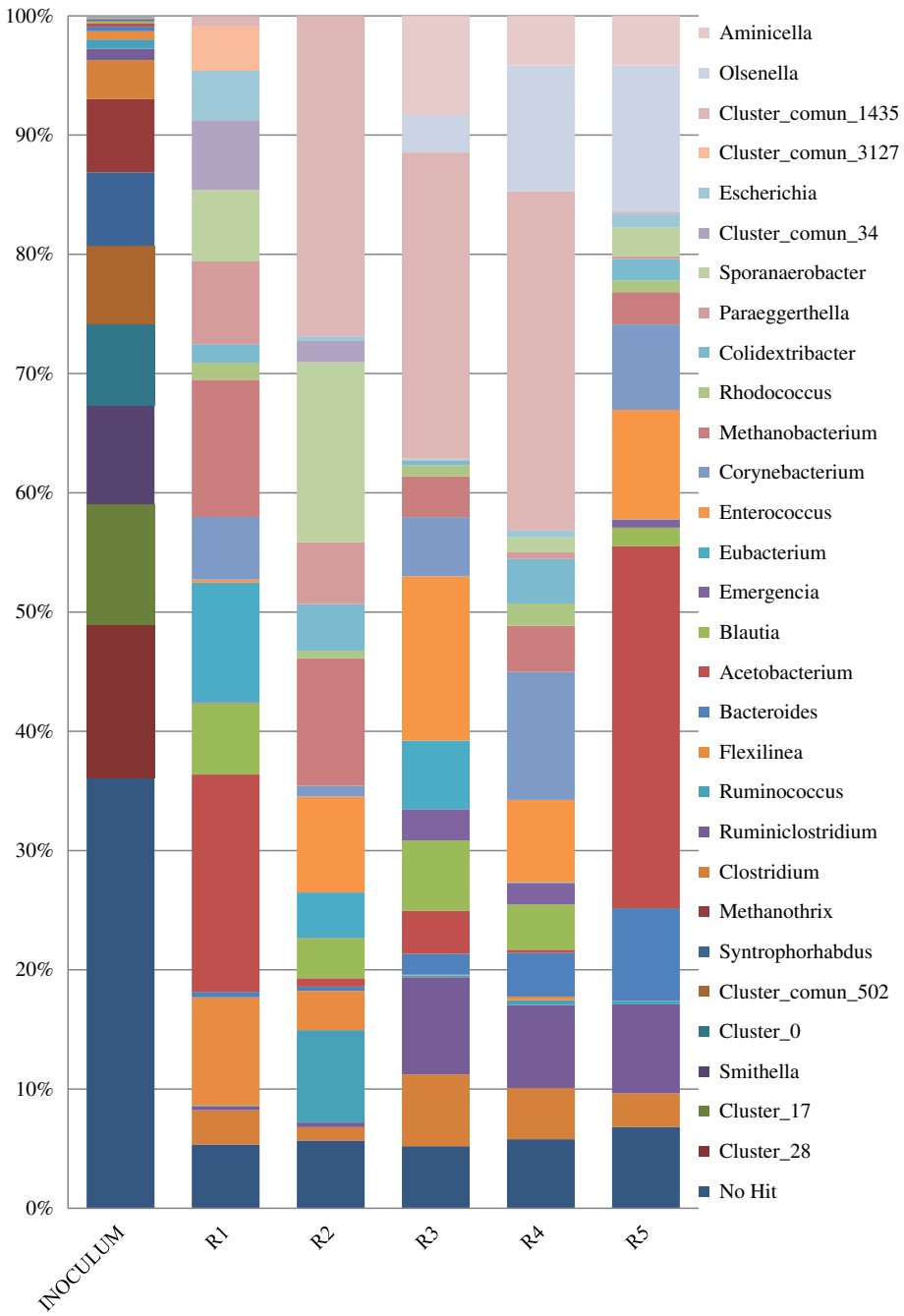

Figure 4. Taxonomic profiles at genera level for the bacteria and archaea communities found in the inoculum and reactors (R1 – R5).

associated with the anaerobic environment and mesophilic temperature, and contains most known acidogenic bacteria responsible for VFA production.[27] In fact, *Sporanaerobacter* was found in anaerobic digesters at the same temperature range used in the present study when food waste was used as substrate.[28] Opposite to these results, the phylum Firmicutes represented only 3.6% of the total when *Scenedesmus* biomass was subjected to anaerobic digestion at mesophilic conditions.[29] In this latter study, the most abundant phylum was Chloroflexi (27.9%). However, the authors reported the low tolerance of these species to operational conditions, which might have caused the absence of Firmicutes in the reactors assessed. It is important to note the differences in terms of phyla when the digestion is devoted to biogas or VFA production. In this sense, it seems likely that Proteobacteria and Chloroflexi are the phyla most present when biogas is produced, and Firmicutes the dominant phylum in the case of VFA production.[25,29]

The second main phylum found in R1 and R2 was Euryarchaeota (10%). The relative abundance of this phylum was in accordance to similar studies focusing on biomethane production. For instance, the archaeal population represented 7 – 8% in the case of digesting sewage sludge with species belonging to *Methanosaeta, Methanomicrobiales, Methanomassiliicocus, Methanosarcina* or *Methanothermobacter*.[25] However, populations in the present study were less diverse at the genus level and only genera *Methanothrix* and *Methanobacterium* were identified, possibly due to the imposed operational conditions, explaining the low

CH_4 production reflected by the low COD removals achieved by these reactors (R1 and R2).

With the aim of explaining the high propionic acid accumulation in R1 and R2 (>30% in both), attention was directed towards the Proteobacteria phylum (comprising 5% and 2% in R1 and R2, respectively). In this sense, *Deltaproteobacteria* is the main genus in charge of propionic acid oxidation, which requires compulsory syntrophic consortia between acetogenic bacteria and methanogens.[30] The low bacterial presence belonging to this genus (<1% in both reactors) may be related to the accumulation of propionic acid. Moreover, values found in both reactors contrast strongly with the relative abundance of this phylum (42.1%) in anaerobic digestion processes targeting CH_4 production.[31]

The influence of temperature was assessed through the comparison of the populations obtained in R1 (T = 35 °C) and R3 (T = 25 °C), both set at 1.5 gCOD L^{-1} d^{-1} and HRT = 10 d. Once again, Firmicutes stood out as the most abundant phylum in both reactors and slightly increased its relative abundance in R3 (52%) when compared to R1 (42%). In this case, the most abundant family and genera found in the psychrophilic digester (R3), belonging to Firmicutes phylum, were the *Ruminococcaceae* cluster (21%), *Enterococcus* (11%), *Ruminiclostridium* (7%) and *Clostridium* (5%). Because the abundance of this phylum is related to the acidification phase of anaerobic digestion, the higher Firmicutes relative abundance in R3 with respect to R1 was in accordance with the increase in VFA production registered at psychrophilic conditions.[32] In addition, phylum Euryarchaeota was better represented in R1 (10%) than in R3 (3.5%). More specifically, the abundance of *Methanobacterium* decreased in R3 to 2.8% (9.2% in R1), attesting to the lower capacity of R3 (COD removal 11.9 ± 2.9%) in comparison to R1 (COD removal 23.6 ± 2.3%) for CH_4 production. Acetic acid accumulation detected in R3 may be related to the lack of archaeal species belonging to the Methanosarcinales order, which are known to perform aceticlastic methanogenesis.[33]

The effect of HRT was assessed by taking into account the results provided by R3, R4 and R5 (HRT = 10, 12 and 8 d, respectively) set at T = 25 °C and OLR = 1.5 gCOD L^{-1} d^{-1}. The Firmicutes phylum was the most abundant phylum in the three reactors (52%, 32% and 55% for R3, R4 and R5, respectively). The most abundant genus for R4 and R3, was the cluster associated with Ruminococcaceae (21% with 97% similarities), even though some other genera also were dominant, namely *Ruminiclostridium* (5%) and *Enterococcus* (5%). However, the cluster associated with Ruminococcaceae had less importance in R5 than in the other reactors and the most abundant genus was *Acetobacterium* (25%). This fact is attributed to the lower HRT set in R5, which could have caused the wash-out of thosee species involved in the cluster mediating *Acetobacterium* predominance. A decrease of phylum Firmicutes was noted in R4 and the disappearance of the cluster associated with Ruminococcaceae in R5 concomitantly with an increase of Actinobacteria. This latter phylum was highlighted in R4 and R5 (≈18%) in comparison with R3 (8%). The increase of bacteria belonging to the Firmicutes phylum, such as *Olsenella* (10% in R5 *versus* 2.5% in R3) and *Corynebacterium* (8% in R4 vs 4% in R3), might be related to the use of AAS, which favored the growth of these bacteria regardless of the HRT imposed. Finally, the Bacteroidetes phylum slightly increased in R4 and R5 (3.1% and 6.5% for R4 and R5, respectively) when compared to R3 (1.4%). However, these values are far from the ones reported in other study targeting biogas production where this phylum's relative abundance was higher (58.9%).[34] Moreover, the similar results exhibited by R4 and R5 in terms of microbial genera explained their similar VFA production, profiles and COD removals. In fact, the low abundance of archaea was related with the scarce CH_4 production potential of the psychrophilic reactors (2% for R3, R4 and R5) which contributed to VFAs accumulation in comparison with the mesophilic reactors (10% for R1 and R2).

Overall, it could be concluded that operational temperature had a greater impact on the developed microbial population when compared to the inoculum source. Adapted sludge exhibited a less diverse microbial community characterized by a high Firmicutes presence and the absence of archaea. The results obtained strongly contrast in terms of population dynamics with reactors aimed at biogas production, highlighting the necessity of studying the microorganisms present in any digester for the better understanding of the process. Likewise, the control of the operational parameters could be used as a tool to select the desired microorganism populations to achieve targeted VFAs or the inhibition of the methanogenic step to accumulate VFAs.

CONCLUSIONS

Imposed conditions affected VFA production and COD removals as well as the microbial communities developed in each reactor. The highest conversions into VFAs and lowest COD removals were achieved under psychrophilic conditions (R5, $COD_{VFA}/COD_{in} = 39.8 \pm 1.0\%$). Hence, the use of psychrophilic conditions was considered the most suitable to inhibit the methanogenic step. Propionic acid in R1 and R2, and acetic acid in R3-R4-R5 led VFA profiles. Adapted sludge promoted C4-C5-C6 VFAs (72% R4 – R5), highlighting the use of psychrophilic conditions and low HRT to promote VFA production. Microbial communities were rather different to the ones often found when biogas production is targeted. It is important to highlight the high microbial specialization determined in the sludge, wherein species specializing in organic acid production gained importance (phylum Firmicutes up to 55% in R3 and R5). This fact, together with the low activity and the scarcity of species belonging to the Euryarchaeota phylum at 25 °C contributed to VFA accumulation. Likewise, the use of adapted sludge led to an increase in Actinobacteria species.

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Supporting Information

Supporting information may be found in the online version of this article.

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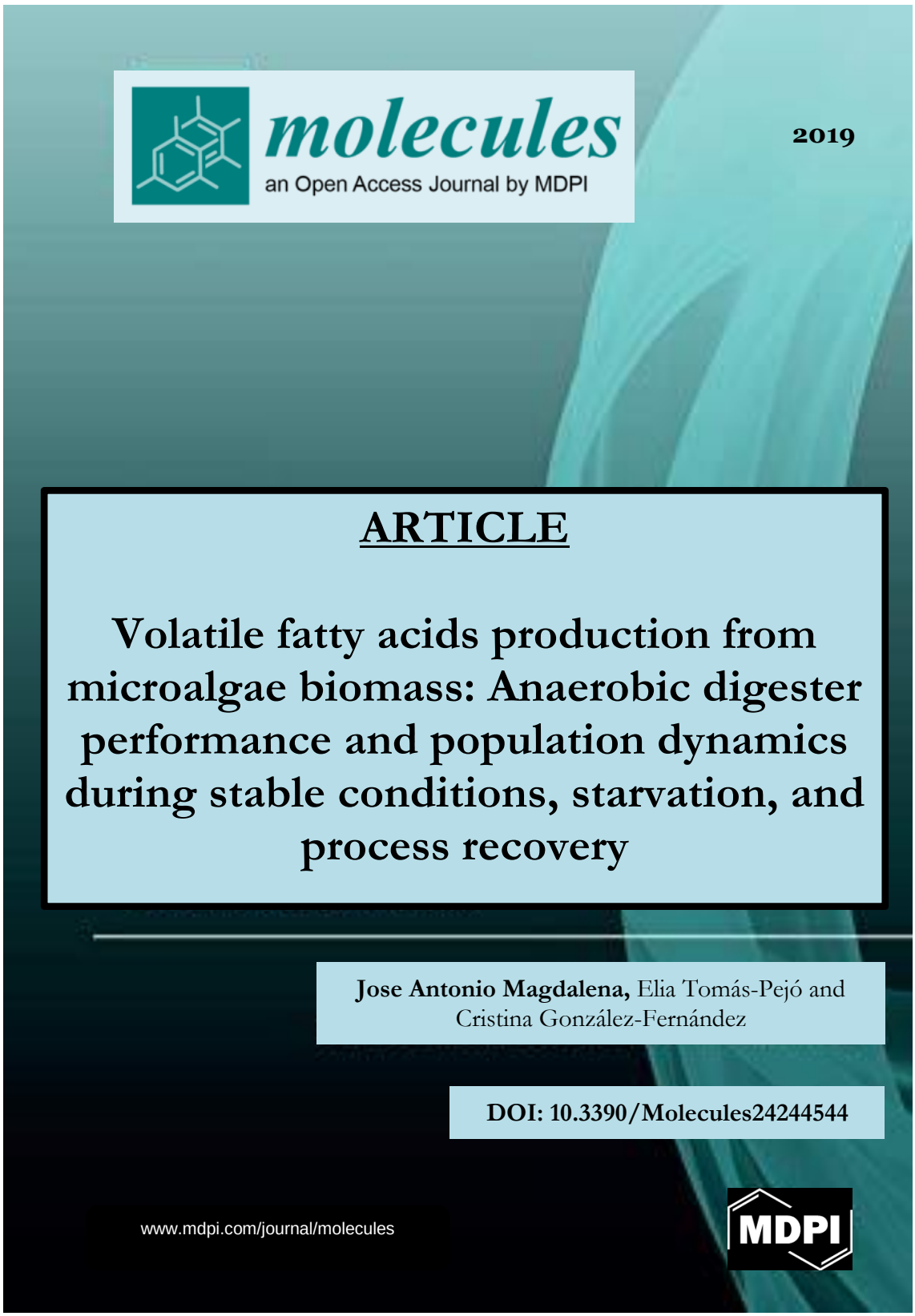

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Article

Volatile Fatty Acids Production from Microalgae Biomass: Anaerobic Digester Performance and Population Dynamics during Stable Conditions, Starvation, and Process Recovery

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Abstract: Disturbances in anaerobic digestion (AD) negatively impact the overall reactor performance. These adverse effects have been widely investigated for methane generation. However, AD recently appeared as a potential technology to obtain volatile fatty acids (VFAs) and thus, the impact of process disturbances must be evaluated. In this sense, microbial response towards a starvation period of two weeks was investigated resulting in a conversion of organic matter into VFAs of 0.39 ± 0.03 COD-VFAs/CODin. However, the lack of feeding reduced the yield to 0.30 ± 0.02 COD-VFAs/CODin. Microbial analysis revealed that the starvation period favored the syntrophic acetate-oxidizing bacteria coupled with hydrogenotrophic methanogens. Finally, the system was fed at 9 g COD/Ld resulting in process recovery (0.39 ± 0.04 COD-VFAs/CODin). The different microbiome obtained at the end of the process was proved to be functionally redundant, highlighting the AD robustness for VFAs production.

Keywords: anaerobic digestion; disturbance; microalgae; population dynamics; volatile fatty acids

1. Introduction

Replacing products obtained from fossil fuels with others obtained from renewable resources is becoming a worldwide issue. A shift to bio-based chemicals seems crucial to circumvent the negative effects of petrochemicals in the environment and overcome supply limitations. As a matter of fact, the so-called "bioeconomy" aims at the gradual use of renewable feedstocks. Among those marketed fossil fuels derivatives, carboxylates, also known as volatile fatty acids (VFAs), could be produced using an alternative route [1]. Acetic, propionic, (iso) butyric, (iso) valeric, and caproic acid are VFAs that account from two to six carbons. The interest in these compounds as platform molecules lies in their wide chemical industry applications [2].

VFAs are produced during the middle stages (acidogenesis and acetogenesis) of anaerobic digestion (AD). Typically, VFAs are anaerobically oxidized to acetate, which is the main substrate that methanogens use to produce biogas. However, VFAs production from AD requires digestion shortening to avoid the methanogenic step favoring VFAs accumulation. The biochemical process for VFAs production pretends to exploit what should be normally prevented in digesters devoted for biogas production. AD for biogas production is a robust technology applied to a wide range of organic substrates. Several research studies regarding microbial response to disturbances during biogas production were mainly targeted at evaluating this latter AD stage [3,4]. These investigations pointed out to methanogenesis as the most sensitive step due to the slow growth rates and susceptibility to inhibitory substances of methanogens [5,6]. However, the new interest in VFAs makes of utmost

importance the study of the bacterial response to disturbances as well as the crucial inhibition of the archaea community to achieve competitive process yields.

With regard to the substrate, the use of microalgae biomass presents potential advantages for the process because of the high protein content exhibited by some strains. During AD, proteins degradation results in the release of ammonium and free ammonia to the medium, which could cause the destabilization of the AD process. As a matter of fact, high concentration of these compounds is toxic for methanogenic archaea, which in turn promotes VFAs accumulation [6].

Common process disturbances studied in the context of biogas production include temperature changes, salinity stress, or feeding alterations [7–9]. As feedstock availability can fluctuate along the year, it is important to assess the effects and to propose proper management strategies to overcome this event. Starvation and organic overloading are frequent perturbations in full scale AD, which might affect the microbiome behavior [10]. A microbial ecosystem will be considered resistant when no changes are observed upon a perturbation. Alternatively, if the microbial population is sensitive and does change, it could be resilient and quickly recover to its initial composition. Finally, if the perturbed population is sensitive and displaced by other microorganisms with similar function, the microbial system can be considered functionally redundant.

In this sense, controlled perturbation experiments can provide useful information in terms of fermenters performance and microbial community dynamics. Suitable VFA yields associated with certain bacterial species can contribute to further proposing recovery strategies and quickly anticipate process failure as well as identifying key organic acid producers. This investigation was designed to cover the gap of knowledge related to the effect that potential perturbations can cause in fermentative processes for VFAs production. With this objective, this investigation evaluated VFA yields and the bacterial and archaea response towards starvation. Population dynamics analysis throughout the different scenarios (stable operation, starvation, feeding re-start, recovery) was assessed to find out the involved microorganisms developing key roles in VFAs production.

2. Material and Methods

2.1. Inoculum and Substrate Pretreatment

Temperature and substrate adapted anaerobic sludge was collected from a previous anaerobic reactor set at psychrophilic range temperature (25 °C) and fed with enzymatic pretreated *Chlorella vulgaris*. This acidogenic reactor was previously established for VFAs production. More details regarding characterization of the effluent in the stationary state of this reactor, which was used as anaerobic inoculum in the present study, can be found elsewhere [11]. The substrate *C. vulgaris* was purchased from Allmicroalgae (Lisbon, Portugal) revealing a composition (% *w*/*w* dry weight) of 57.9 proteins, 21.6 carbohydrates, 13.4 lipids and 7.1 ash. Since the goal of this study was to investigate the acidogenic stage, biomass pretreatment was applied to avoid hydrolysis limitation. Commercial enzymatic cocktail "Alcalase 2.5 L" (Novozyme, Denmark) was employed to pretreat the biomass and make available the organic matter to anaerobic microorganisms. The dosage (0.585 UA g^{-1} TS^{-1}) and procedure was based on results obtained for *C. vulgaris* [6].

2.2. Experimental Set up

Anaerobic fermentation was carried out under semi-continuous feeding mode in 1 L-CSTR. Agitation was performed by magnetic stirring. The operational temperature and hydraulic retention time (HRT) were 25 °C and 8 days, respectively. The low temperature and HRT were selected according to previous investigations in which these conditions were found appropriate for VFAs production [11,12]. Three scenarios were evaluated in terms of organic loading rate (OLR), namely: scenario 3B (48 days, OLR = 3 g COD/Ld before starvation), scenario 3A (38 days, OLR = 3 g COD/Ld after starvation (feeding re-start)) and scenario 9R (32 days, OLR = 9 g COD/Ld, recovery stage). The biological process was considered at steady state condition when VFAs resulted in a constant

value and the reactor was operated during 3-HRTs. As samples were taken consecutively once the process had achieved the steady-state, multiple time point did not show variation. For this reason, samples collected and analyzed along the steady state offered a constant trend. pH was monitored but not controlled during the experiment. The starvation period lasted 14 days (2 weeks). The selection for this starvation period was based on the fact that this would be the time to recover an algal based system operating at hydraulic retention time of 4 days (typical value for urban wastewater treatment by means of algae consortium). In this manner, this study attempted to simulate a lack of feeding for 14 days due to a crash in the microalgae production system.

2.3. Analytical Methods

Total and soluble COD and NH_4^+ were measured twice per week using test kits (Merck, ISO 15705, ISO 000683, Darmstadt, Germany). Similarly, VFAs were measured by liquid chromatography (HPLC) an analyzed through an Agilent 1260 HPLC-RID (Agilent, Santa Clara, CA, USA) equipped with a Cation H Refill Cartridge Microguard column (Bio-Rad, Hercules, CA, USA) and an Aminex HPX-87H ion exclusion column (300 × 7.8 mm I.D.) (Bio-Rad). The biological process was considered at steady state condition when VFAs resulted in a constant value over three sampling points and the reactor was operated during three HRTs.

2.4. DNA Extraction

At the steady-state, samples (15 mL) were collected and frozen at −20 °C. The kit "FastDNA SPIN Kit for Soil" (MP Biomedicals, LCC, Illkrich, France) was used to extract DNA according to the protocol provided by the manufacturer. A nanodrop (LVis Plate, BMG LABTECH) was used to quantify the amount of extracted DNA (ng/mL) and analyze its quality by measuring 260/280 and 260/230 ratios. The primers used for the amplification of the 16S rRNA gene were 341F and 805R (F – CCTACGGGNGGCWGCAG and R – GACTACHVGGGTATCTAATCC), which targeted the hypervariable regions V3 and V4 of both bacteria and archaea [13]. Amplicons resulting from PCR were sequenced on a MiSeq Sequencer (Illumina, San Diego, CA, USA) and by Life Sequencing (University of Valencia, Spain) with MiSeq reagent kit v3 (600-cycle). 50 ng were amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina). In brief, the first amplification step, primers were designed containing: 1) a universal linker sequence allowing amplicons for incorporation indexes and sequencing primers by Nextera XT Index kit (Illumina); and 16S rRNA gene universal primers [13] and in the second and last amplification indexes were included. Libraries were quantified by fluorimetry using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher, Waltham, MA, USA) and pooled prior to sequencing on the MiSeq platform (Illumina), configuration 300 cycles paired reads. The size and quantity of the pool were assessed on the Bioanalyzer 2100 (Agilent) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. PhiX Control library (v3) (Illumina) was combined with the amplicon library (expected at 20%). Sequencing data were available within approximately 56 h. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument. The resulting sequences were split taking into account the barcode introduced during the PCR reaction, while R1 and R2 reads were overlapped using PEAR program version 0.9.1 [14] providing a single FASTQ file for each of the samples. Quality control of the sequences was performed in different steps, (i) quality filtering (with a minimum threshold of Q20) was performed using fastx tool kit version 0.013, (ii) primer (16s rRNA primers) trimming and length selection (reads over 300 nts) was done with cutadapt version 1.4.1 [15]. These FASTQ files were converted to FASTA files and UCHIME program version 7.0.1001 was used in order to remove chimeras that could arise during the amplification and sequencing step. Those clean FASTA files were BLAST [16] against NCBI 16s rRNA database using blastn version 2.2.29+. The resulting XML files were processed using a python script developed by Lifesequencing S.L.-ADM (Paterna, Valencia, Spain) in order to annotate each sequence at different phylogenetic levels.

2.5. Statistical Analysis

Data were presented as mean values ± standard deviation of the mean and statistical significances was assessed by analysis of variance (ANOVA). Values were considered statistically significant when p value was lower than 0.05.

3. Results and Discussion

3.1. Effect of Starvation on Reactor Performance

3.1.1. Reactors Performance: VFAs Yields and Profiles

The negligible COD removal attained in scenario 3B (±5%, Table 1) indicated that operational conditions imposed were suitable for VFAs production. Low COD removals were the result of a poor methanogenesis and VFAs remained unconsumed. Most probably the use of an acidogenic inoculum with scarce methanogenic activity together with the low HRT imposed (8 days) contributed to VFAs accumulation by inhibiting the methanogenic stage. It should be highlighted that decreasing the HRT is considered a strategy to wash out methanogenic archaea as their growth rates are lower than those exhibited by acidogenic bacteria [17,18]. VFAs conversion yields attained at steady state (0.39 ± 0.03 COD-VFAs/COD in) were in agreement with previously reported values from microalgae biomass [11]. The measured soluble COD was about 16.41 ± 0.26 g/L. By taking into consideration the VFAs contribution in terms of COD (11.82 ± 0.96 g/L), it could be stated that the acidification stage (acidogenesis and acetogenesis) was efficient since the COD-VFA/sol COD ratio was 0.72.

Table 1. Effluent results of the different parameters assessed at the different scenarios.

Scenario	OLR (g COD/Ld)	%COD Removal	Soluble COD (g COD/L)	VFAs (g COD/L)	COD-VFAs/CODin	NH_4^+ (g/L)	pH
3B	3	5.1 ± 2.2	16.42 ± 0.26	11.82 ± 0.96	0.39 ± 0.03	1.28 ± 0.02	6.3 ± 0.1
3A	3	32.5 ± 2.7	11.12 ± 0.33	8.90 ± 0.69	0.30 ± 0.02	0.89 ± 0.02	6.1 ± 0.1
9R	9	3.3 ± 1.8	38.16 ± 0.32	27.92 ± 2.90	0.39 ± 0.04	2.83 ± 0.02	6.3 ± 0.1

OLR: Organic loading rate; %COD removal: chemical oxygen demand removal; soluble COD: soluble chemical oxygen demand; VFAs: Volatile fatty acids; COD-VFAs/CODin: Volatile fatty acids in terms of chemical oxygen demand out of the total chemical oxygen demand fed in the system.

After stable operation, the system was subjected to a starvation period of two weeks. Starvation length is quite arbitrary in scientific literature. While some studies employ long-term starvation [19], others evidenced modest changes with just one day of starvation [20]. The effect of the lack of feeding can affect microbial activities [21], impacting ultimately the bioprocess efficiency. In the present investigation, after the starvation period, the reactor was operated at the same initial conditions. However, results in terms of COD elimination were quite different. In scenario 3A, COD removal increased up to approximately 33% (Table 1), and thus the organic matter conversion yield into VFAs decreased to 0.30 ± 0.02 COD-VFAs/CODin. With regard to the acidification stage, similarly to scenario 3B, high values were recorded when taking into consideration the VFAs contribution to the soluble COD (0.8 COD-VFA/sol COD ratio). This feature evidenced that the formation of VFAs was not affected by the starvation period. Lastly, when comparing effluent sol COD/tot CODin of both scenarios, a similar ratio was observed (0.58 ± 0.1 in 3B and 0.55 ± 0.1 in 3A). Therefore, it could be stated that starvation did not affect the fermentative stages (hydrolytic and acidogenic), but it had an influence in the methanogenic stage, as COD removal increased. The starvation period most probably contributed to the development of the archaea community because no effluent was extracted from the acidogenic reactor in 14 days. Additionally, archaea species might have recovered due to the lower NH_4^+ concentration detected after starvation. The trade-off of the main parameters evaluated is shown in Figure 1.

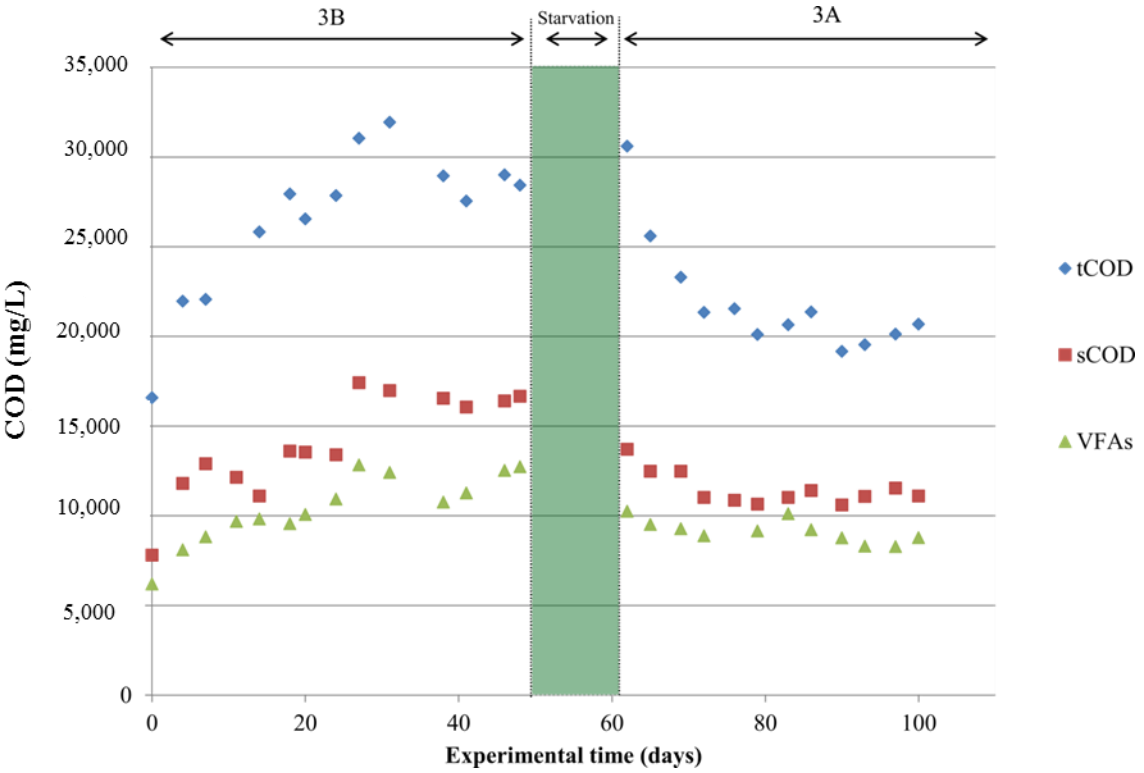

Figure 1. Main operational parameters assessed during reactor operation: total/soluble chemical oxygen demand (tCOD and sCOD) and volatile fatty acids (VFAs).

In terms of VFAs profile distribution, butyric acid was the VFA exhibiting the highest percentage (23% ± 2% of total VFA as COD), followed by acetic acid (20% ± 1% of total VFA as COD) and the odd chain VFAs (15% ± 1% of total VFA as COD of propionic and valeric acids). Generally, acetic, propionic, and butyric acids are the main products when microalgae biomass is subjected to AD [22,23]. As seen in Figure 2, this trend was maintained after starvation (scenario 3A). The only remarkable difference was attained for caproic acid that decreased from 10% ± 3% to 4% ± 2% of total VFA as COD ($p < 0.05$). However, the differences before and after starvation were minimal and thus, it can be pointed out that implemented disturbances did not greatly affect VFAs distribution.

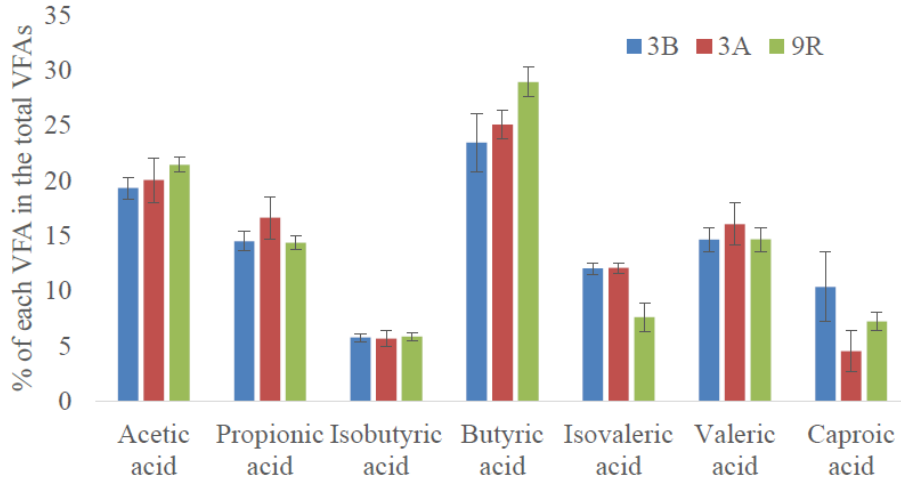

Figure 2. VFAs profiles exhibited at the stationary state of the different scenarios (3A, 3B, and 9R).

3.1.2. Microbial Population Dynamics

In order to link fermenters chemical outputs with the existing microbiome, microbial populations were analyzed before and after the starvation period (scenarios 3B and 3A, Figure 3). Anaerobic populations were evaluated in terms of relative abundance (%). The bacterial community before starvation consisted of Firmicutes (68%) as the major phylum, followed by Bacteroidetes (10%), and Actinobacteria (18%). Samples taken immediately after starvation, before restarting the feeding, showed no differences in terms of Firmicutes and Actinobacteria, while in the case of Bacteroidetes, the population drastically decreased to 0.5%. In addition, even though the relative abundance of Firmicutes was not affected, a decrease in Bacilli and an increase in Tissierellia class were observed (Figure 3A,B). Reactor operation after the starvation period returned Tissierellia and Bacilli values to those showed initially and gave rise to a sensitive increase in Firmicutes (Figure 3C). This might be attributed to Clostridia class, which increased from 58% to 72% (before and after starvation) with respect to the total sequences analyzed (Figure 3A–C).

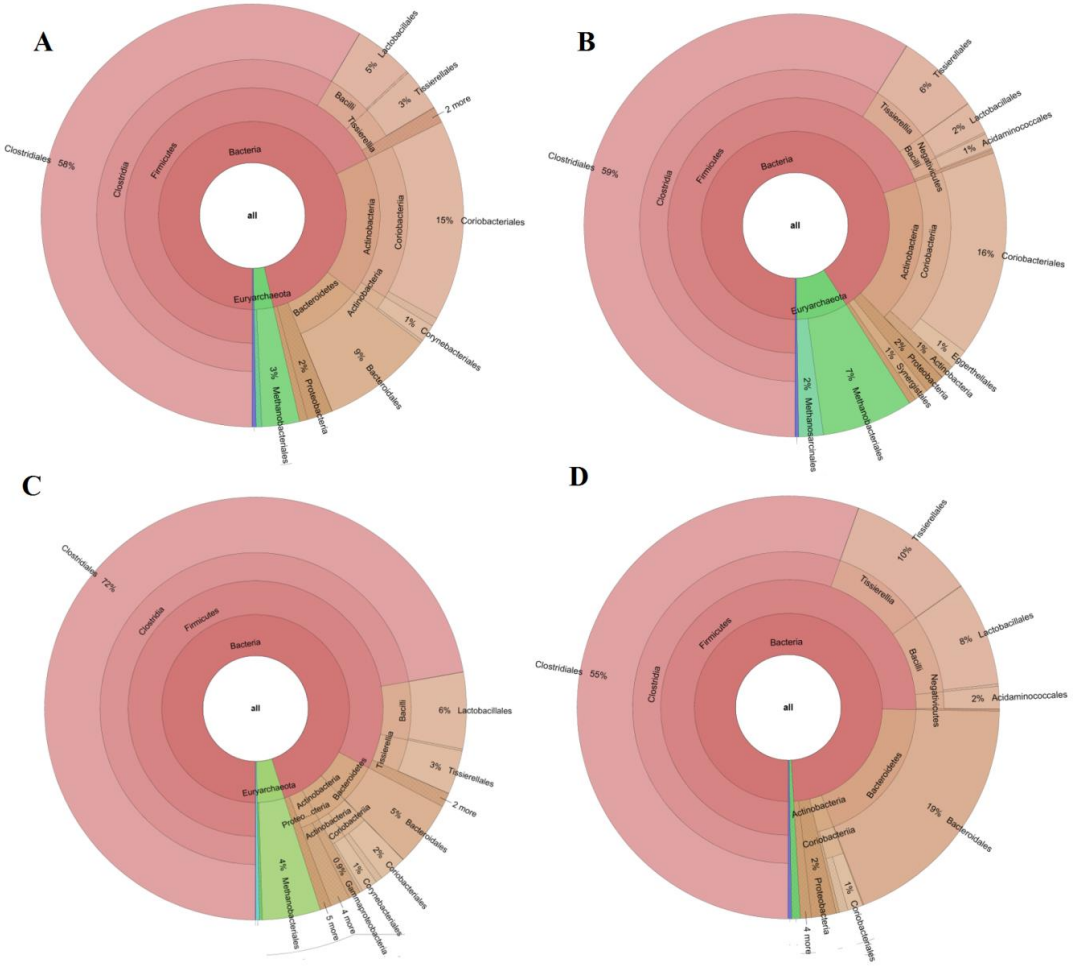

Figure 3. Krona graphics extracted from each scenario: 3B(**A**); starvation (**B**); 3A(**C**); 9R(**D**).

In general terms, the predominance of Firmicutes agreed with previous research studies dealing with the production of VFAs from microalgae biomass [11]. In fact, the bacterial population is markedly different to the obtained herein when the digestion is targeted for biogas production. Bacterial community when biogas is the end-product is mainly represented by Cloroflexi (under low ammonium levels [24]) or by Proteobacteria [25] while the relative abundance of Firmicutes is considerably lower [25,26]. Other studies stated that a high presence of Firmicutes is related to poor biogas production performance, which is in fact the scenario sought herein [7]. Ammonium and

ammonia might be toxic for anaerobes and therefore it should be carefully analyzed. With regard to ammonium, the concentration determined during scenario 3B was 1.28 ± 0.02 g NH_4^+/L (Table 1). In this particular case, ammonium concentration was high but not yet in the inhibitory concentration range considered for un-acclimated inoculum (1.7–1.8 g/L, [27]).

More importantly, the percentage of Euryarchaeota community (archaea) displayed a significant increase during starvation confirming the recovery of this community (Figure 3). Note worth to mention that the main strain determined among this population was *Methanobacterium*. This strain has been claimed to be a hydrogenotrophic methanogen [28]. In this context, there are two major methanogenic pathways: a) the acetoclastic pathway and b) the hydrogenotrophic pathway. Additionally, syntrophic acetate oxidizing bacteria (SAOB) might occur. These species oxidize acetate and produce H_2 and CO_2 or formate. This H_2 generated might be used as well for hydrogenotrophic methanogenesis.

Acetoclastic pathway is mediated by families related with Methanosarcinaceae spp. and Methanosaetaceae spp., while species belonging to order Methanomicrobiales spp., Methanobacteriales spp. (such as *Methanobacterium*), and Methanococcales spp., are responsible for the hydrogenotrophic pathway [29]. It should be highlighted that this latter methanogenic route prevails over the acetoclastic pathway when difficult methanogenesis environments are imposed. As a matter of fact, the acetoclastic archaea are more sensitive than hydrogenotrophic species [30]. For instance, digesters operating at high ammonium or VFAs concentrations, which can be potentially toxic, have shown hydrogenotrophic pathway preference for methanogenesis [4,31]. These adverse conditions for methanogenesis were also evidenced in scenario 3B while immediately after the starvation period, methanogens activity resumed as it could be seen by archaea population increase after starvation in Figure 3. This feature is in agreement with Kim et al., (2015) who pointed out that under starvation conditions methanogens are able to enter a quiescent state until favorable conditions for growth are attained again. The lower conversion yield in terms of COD-VFAs/CODin attributed to the consumption of VFAs was also related to the presence of syntrophic acetate oxidizing bacteria (SAOB). SAOB are normally working together with their hydrogenotrophic counter partners to keep an optimum hydrogen trade off in the anaerobic system. Acetate oxidation only proceeds when the hydrogen level is kept low by hydrogenotrophic methanogens consumption [32]. Whereas, the presence of Chloroflexi has been negatively correlated with VFAs production, other phylum such as Firmicutes prevails in environments devoted for VFAs production [11,33]. SAOB are affiliated with Firmicutes phylum, more particularly to Clostridia class (*Thermacetogenium phaeum, Tepidanaerobacter acetatoxydans*, or *Syntrophaceticus schinkii*), Tissierellia class (*Clostridium ultunense*) and Thermotogae phylum (*Pseudothermotoga lettingae*) [34,35]. However, other members of Firmicutes have been attributed to perform SAO activities. In fact, species belonging to Clostridia class have been previously related with the SAO pathway [36]. In this sense, the highest COD removals and lowest COD-VFAs/CODin conversions were attained under scenario 3A, which showed the highest Clostridia population (72%). Moreover, the methanogens recovery during starvation might also be linked to the lower ammonium concentration of the digestates after starvation (0.89 ± 0.02 g NH_4^+/L, Table 1). Indeed, the nitrogen mineralization percentage was not recovered since ammonium levels in the effluents after starvation did not reach the same concentration as in scenario 3B.This could be explained by the different fate of carbon and nitrogen during AD [37]. In this case, it seems likely that nitrogen mineralization did not recover its initial efficiency.

3.2. Recovery Strategy: OLR Increase

3.2.1. Reactors Performance VFAs Yields and Profiles during Fermenter Recovery

The OLR has been reported as a bioengineering management tool to shape anaerobic digesters performance [7]. Aiming at recovering the organic matter conversion into VFAs, OLR was stepwise increased to reach 9 g COD/Ld (scenario 9R).This strategy resulted in the same conversion yield (0.39 ± 0.04 COD-VFAs/CODin) attained before starvation (3B). Likewise, COD removal decreased to values similar to scenario 3B (Table 1). Moreover, it should be highlighted that ammonium concentration

in scenario 9R increased to 2.89 ± 0.23 g NH_4^+/L (Table 1) and VFAs concentration was 18.3 ± 0.3 g/L. Both parameters, ammonium and VFAs, were above the threshold limits identified for proper biogas production [27,31]. These two facts likely contributed to methanogenesis inhibition resulting in similar COD removals values and organic matter conversion into VFAs previously showed in 3B. The acidogenesis stage also remained stable when compared with the two previous scenarios (0.73 COD-VFA/sol COD ratio) indicating that acidogenesis was not affected by starvation, ammonium concentration or the OLR increase. With regard to the hydrolysis stage, the increase in OLR (scenario 9R) also supported an increase in the ratio effluent sol COD/tot CODin (0.59), which was similar to the values attained in scenario 3B. Based on the effluent ammonium concentration attained during scenario 9R, it could be stated that nitrogen mineralization efficiency was similar to scenario 3A. In this manner, the diminished activity in nitrogen mineralization registered after the starvation still remained after the OLR increase. Thus, organic matter conversion yield into VFAs was recovered but not the nitrogen mineralization. It should be highlighted that the goal of this recovery strategy was to obtain the same VFA conversion yield as before of the starvation period.

In terms of VFA distribution, slight differences in VFAs content were determined even though a similar profile trend to the one obtained in scenario 3B was observed. In general terms, butyric (29% ± 1% of total VFA as COD) and acetic acids (21% ± 1% of total VFA as COD) were the dominant VFAs. As it was aforementioned, this is a normal trend in microalgae biomass AD. These values represented slightly higher values than the ones obtained in scenario 3B. At the expenses of the increased percentage of those two acids, lower percentages of the longest VFAs (C5 and C6) were attained (Figure 2).

3.2.2. Microbial Population Dynamics during Fermenter Recovery

As seen in Figure 3, microbial population changed when increasing OLR to 9 g COD/Ld (scenario 9R). One of the main differences in scenario 9R was associated to the stepwise decrease of the Euryarchaeota population with respect to the starvation period and 3A. In this case, archaea accounted for 1% of the microbial population. This fact combined as aforementioned with higher VFAs productions and NH_4^+ concentration, which might entail toxicity for the anaerobic populations, weakened the organic matter removal in the system. This fact underpinned the low COD removal values determined in this scenario. When compared to scenario 3A, not only archaea community decreased but also a marked increase in Bacteroidetes phylum was observed (20%, Figure 3D). Opposite to that, the increase in OLR did not affect the Actinobacteria population percentage that stayed at low values (2%). In spite of the similar VFAs conversion values in scenarios 3B and 9R, their microbial populations were slightly different. More specially, Bacteroidetes and Euryarchaeota were present at similar percentages while Firmicutes increased to 75% and Actinobacteria decreased to 2% at 9R. Despite the increased percentage of Firmicutes, the prevalence of Clostridiales within this phylum attained its initial value (58% and 55% in scenarios 3B and 9R, respectively). Some other remarkable changes within the anaerobic microbiome at OLR 9 g COD/Ld included the drastic decrease of Ruminococcaceae and Eubacteriaceae and the increase of Peptrostreptococcaceae (Figure 4). Therefore, even though Firmicutes phylum remained similarly high to the previous scenarios, the relative abundance of the bacterial class was quite different. Overall, it could be concluded that despite of the conversion yield recovery in terms of VFAs production, microbial community did not return to the initial structure (scenario 3B) after recovery (scenario 9R). In this sense, the microbial systems developed under scenario 9R and 3B were functionally redundant indicating that the new microbial community could maintain similar performance efficiency supporting similar VFAs yields. This behavior was previously reported in literature when targeting biogas production [8,38]. However, to the best of the knowledge authors, AD robustness was not proven previously for VFAs production.

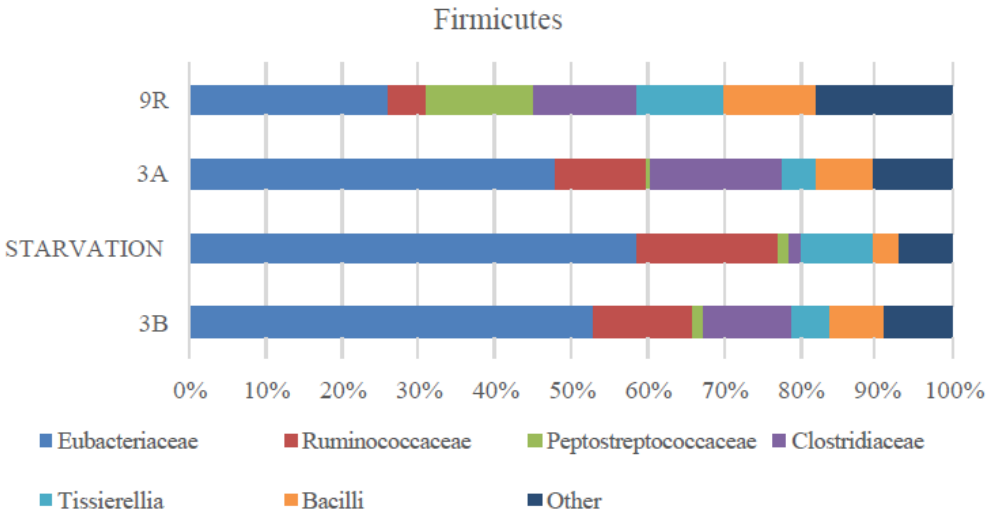

Figure 4. Firmicutes phylum distribution at the different scenarios assessed (3B, starvation, 3A, and 9R).

4. Conclusions

The starvation period affected the overall process performance (VFAs yields and nitrogen mineralization) as well as the microbiome involved. More specifically, methanogenic archaea were able to thrive after the lack of feeding resulting in an increase in COD removal via the hydrogenotrophic pathway. The recovery strategy of applying an OLR increase recovered conversion values showed initially (0.39 ± 0.04 COD-VFAs/CODin). This approach weakened methanogenesis and contributed to maintain archaea and Clostridia levels similar to those showed initially. Remarkably, microbial systems developed (represented by Firmicutes) were functionally redundant since the new community could maintain similar performance efficiency highlighting the robustness of anaerobic fermentation for VFAs production.

Author Contributions: J.A.M. was responsible for the manuscript preparation and data interpretation. E.T.-P. was responsible for revising the manuscript. C.G.-F. was responsible of the experimental design and manuscript revision. The final publication was prepared with contribution from all authors.

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Conflicts of Interest: The authors declare no conflict of interest.

Data accessibility: These sequence data have been submitted to GenBank database in a project (accession number PRJNA529178).

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PUBLICATION VIII

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ARTICLE

Impact of Organic Loading Rate in Volatile Fatty Acids Production and Population Dynamics Using Microalgae Biomass as Substrate

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Impact of Organic Loading Rate in Volatile Fatty Acids Production and Population Dynamics Using Microalgae Biomass as Substrate

Jose Antonio Magdalena, Silvia Greses & Cristina González-Fernández*

Volatile fatty acids (VFAs) are regarded as building blocks with a wide range of applications, including biofuel production. The traditional anaerobic digestion used for biogas production can be alternatively employed for VFAs production. The present study aimed at maximizing VFAs productions from *Chlorella vulgaris* through anaerobic digestion by assessing the effect of stepwise organic loading rates (OLR) increases (3, 6, 9, 12 and 15 g COD L^{-1} d^{-1}). The biological system was proven to be robust as organic matter conversion efficiency into VFAs increased from 0.30 ± 0.02 COD-VFAs/COD_{in} at 3 g COD L^{-1} d^{-1} to 0.37 ± 0.02 COD-VFAs/COD_{in} at 12 g COD $L^{-1}d^{-1}$. Even though, the hydrolytic step was similar for all studied scenario sCOD/tCOD = 0.52–0.58), the highest OLR (15 g COD L^{-1} d^{-1}) did not show any further increase in VFAs conversion (0.29 ± 0.01 COD-VFAs/COD_{in}). This fact suggested acidogenesis inhibition at 15 g COD $L^{-1}d^{-1}$. Butyric (23–32%), acetic (19–26%) and propionic acids (11–17%) were the most abundant bioproducts. Population dynamics analysis revealed microbial specialization, with a high presence of Firmicutes followed by Bacteroidetes. In addition, this investigation showed the microbial adaptation of Euryarchaeota species at the highest OLR (15 g COD $L^{-1}d^{-1}$), evidencing one of the main challenges in VFAs production (out-competition of archaea community to avoid product consumption). Stepwise OLR increase can be regarded as a tool to promote VFAs productions. However, acidogenic inhibition was reported at the highest OLR instead of the traditional hydrolytic barriers. The operational conditions imposed together with the high VFAs and ammonium concentrations might have affected the system yields. The relative abundance of Firmicutes (74%) and Bacteroidetes (20%), as main phyla, together with the reduction of Euryarchaeota phylum (0.5%) were found the best combination to promote organic matter conversion into VFAs.

Volatile fatty acids (VFAs) are valuable chemicals produced during the middle stages (acidogenesis and acetogenesis) of anaerobic digestion (AD)[1]. The interest in VFAs relies on their use as building blocks within the renewable-based biorefinery concept[2–4]. AD is a complex organic matter degradation process composed of four different phases (hydrolysis, acidogenesis, acetogenesis and methanogenesis). Numerous reactions and microorganisms interact to transform the organic matter firstly into intermediate products (VFAs) and finally into biogas. AD optimization towards VFAs accumulation need to circumvent VFAs consumption in the methanogenic stage[5]. In this sense, different strategies have been adopted to drive AD to VFAs production such as the use of specific substrates, manipulation of operational conditions or the use of microbial biomass rich in organic acids producers[6,7].

With regard to the substrate, the use of microalgae biomass presents potential advantages for the process because of the high protein content exhibited by some strains. During AD, proteins degradation results in the release of ammonium and free ammonia to the medium, which could cause the destabilization of the AD process. As a matter of fact, high concentration of these compounds is toxic for methanogenic archaea, which in turn promotes VFAs accumulation[8,9].

Manipulation of operational conditions such as pH, temperature, hydraulic retention time (HRT) and organic loading rate (OLR) must be taken into account when targeting VFAs production[10–12]. OLR expresses the amount of organic matter fed into a system in terms of Chemical Oxygen Demand (COD). High OLR values can lead to

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	OLR (g COD·d^{-1}·L^{-1})	tCOD (g L^{-1})	% COD removal	sCOD (g L^{-1})	TS (g L^{-1})	VS (g L^{-1})	pH	NH_4^+ (g L^{-1})	$\%CH_4$	$\%CO_2$	VFAs (g COD L^{-1})	VFAs-COD/ COD_{in}
Sc. I	3	21.9 ± 3.2	29.3 ± 6.1	11.4 ± 0.9	8.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.3	0.9 ± 0.1	39.5 ± 11.9	60.5 ± 11.9	9.1 ± 0.6	0.30 ± 0.02
Sc. II	6	38.3 ± 0.8	20.1 ± 1.9	20.1 ± 3.7	14.2 ± 1.4	10.2 ± 0.4	6.3 ± 0.1	1.4 ± 0.2	25.7 ± 5.5	68.9 ± 5.6	16.5 ± 3.2	0.34 ± 0.01
Sc. III	9	69.6 ± 1.3	3.3 ± 1.8	29.9 ± 3.2	24.9 ± 0.5	19.6 ± 0.5	6.3 ± 0.1	2.4 ± 0.3	15.4 ± 1.9	79.5 ± 2.4	28.0 ± 2.3	0.39 ± 0.04
Sc. IV	12	91.1 ± 6.2	2.2 ± 3.1	47.2 ± 5.1	33.1 ± 2.5	27 ± 2.0	6.5 ± 0.1	3.8 ± 0.2	14.2 ± 1.4	80.0 ± 2.1	36.8 ± 2.1	0.37 ± 0.02
Sc. V	15	109.9 ± 3.4	14.1 ± 2.7	62.2 ± 2.9	45.4 ± 0.1	35.5 ± 0.6	6.5 ± 0.1	4.4 ± 0.1	11.7 ± 0.9	83.8 ± 2.1	36.4 ± 1.5	0.29 ± 0.01

Table 1. Average values achieved throughout the different scenarios of the CSTR operation. *tCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand; TS: Total solids; VS: Volatile solids;VFAs: Volatile fatty acids.

pH drop due to the fast generation of VFAs. Indeed, changes in OLR affect AD process in terms of population dynamics and organic matter availability and thus, final VFAs productions yields and profile might also vary. VFAs can be obtained from a wide amount of substrates[7]. Nevertheless, valorisation of microalgae biomass is an important hotspot due to the key role of this biomass in studies related to wastewater treatment[13]. The novelty of this investigation lies on the use of this feedstock, since the surplus of biomass generated during wastewater bioremediation might constitute an attractive substrate to obtain added-value bio-based compounds. In this context, the study of different OLRs to assess VFAs production from microalgae is relevant to identify system boundaries that should not be overcome to ensure maximum conversion efficiency. In this manner, this study aimed at elucidating the effect of increasing OLR values (3, 6, 9, 12 and 15 g COD L^{-1} d^{-1}) on VFAs production yields and profiles in a continuous stirred tank reactor (CSTR) using *Chlorella vulgaris* (protein rich substrate). Moreover, population dynamics analysis throughout the different scenarios was assessed to find out the involved microorganisms to identify those who develop a key role in VFAs production.

Methods

Inoculum and substrate pretreatment. Adapted anaerobic sludge to temperature and substrate was collected from a previous anaerobic reactor set at psychrophilic range temperature (25 °C) and fed with enzymatic pretreated *C. vulgaris*. In this sense, the anaerobic inoculum was adapted to low temperature operation and to the substrate as well. The substrate *C. vulgaris* was purchased from Allmicroalgae (Portugal) revealing a composition (dry weight) of 57.9% proteins (w/w), 21.6% carbohydrates, 13.4% lipids and 7.1% ashes. Since the goal of this study was to investigate the acidogenesis stage, biomass pretreatment was applied to avoid hydrolysis limitation. Commercial enzymatic cocktail "Alcalase 2.5 L" (Novozyme, Denmark) was employed to pretreat the biomass and make available the organic matter to anaerobic microorganisms. The dosage (0.585 UA g^{-1} TS^{-1}) and procedure was based on results obtained for *C. vulgaris*[9,14].

Experimental set up. AD was carried out under semi-continuous feeding mode in 1 L CSTR. Agitation was performed by mechanical stirring at 250 rpm. The operational temperature was maintained at 25 °C using a water bath and the HRT was set at 8 $days^{-1}$. OLRs applied to test the influence of this parameter on VFAs production allowed dividing the experimental period into five different scenarios (Sc. I-V), as it can be seen in Table 1. pH was monitored but not controlled. Total and soluble COD and N-NH_4^+ were measured using test kits (Merck, ISO 15705, ISO 000683). Total and soluble COD together with ammonium, VFAs and pH were measured twice per week. VFAs were measured by liquid chromatography (HPLC) and analysed through an Agilent 1260 HPLC-RID (Agilent) 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). Na^+ was measured by ion chromatography (ICS 3000, Dionex) equipped with pre-columns and separation columns CG 16 and CS16 (3 mm ø) for cations. The column temperature was set at 35 °C. Biogas composition was analysed by gas chromatography coupled with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and equipped with an HSN6–60/80 Sulfinert P packed column (7′ × 1/8″ O.D.) and a MS13X4-09SF2 40/60 P packed column (9′ × 1/8″ O.D.) (PerkinElmer). The biological process was considered at steady state condition when VFAs resulted in a constant value and the reactor was operated during 3-HRTs. COD removal was calculated according to Eq. 1, where CODin is the total organic matter fed into the system and COD_{out} is the total organic matter recovered in the effluent:

$$\%\text{COD removal} = \frac{(COD_{in} - COD_{out})}{COD_{in}} \cdot 100 \quad (1)$$

DNA extraction. Once each scenario achieved the steady-state, samples were collected and immediately frozen at −20 °C. DNA was extracted using the kit "FastDNA SPIN Kit for Soil" (MP Biomedicals, LCC), according to the protocol provided by the manufacturer. Quality of the DNA extracted was checked using a nanodrop by measuring 260/280 and 260/230 ratios and the amount of DNA extracted (ng/mL). The primers used for the amplification of the 16 S rRNA gene were 341 F and 805 R (F–CCTACGGGNGGCWGCAG and R–GACTACHVGGGTATCTAATCC), which targeted the hypervariable regions V3 and V4 of both bacteria and archaea. Amplicons resulting from PCR were sequenced on a MiSeq Sequencer (Illumina) by Life Sequencing (University of Valencia, Spain) with MiSeq reagent kit v3 (600-cycle), according to the manufacturer's protocol. Sequence data were processed by using bioinformatics tools. First of all, paired-end reads were merged using the program PEAR[15]. Afterwards, sequence quality was filtered using PRINSEQ and only sequences with a quality

score of 30 and minimum lengths of 350 bp were taken into account for further analysis[16]. Primer sequences were removed using Mothur[17] while chimeric sequences were removed and the resulted sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity (OTU 0.97). The latter step was performed by USEARCH using the Greengenes database gg_13_8[18], which is implemented in the Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software package[17,19,20]. These sequence data have been submitted to GenBank database in a project (accession number PRJNA529178).

Regarding microbial statistics, diversity was analysed with estimation of Shannon index as well as the number of observed OTUs, providing the microbial evenness and richness of the samples. To determine changes in the microbiome population due to the OLR increase in the system, weighted UniFrac distance matrix was used to elaborate Principal Coordinate Analysis (PCoA). Besides, the effect of the physicochemical parameters on reactor performance was evaluated by Principal Components Analysis (PCA) using PAST[21], which takes into account the experimental measurements of each stage of the reactor. Additionally, variances in microbial composition between scenarios were evaluated through the ANOSIM statistical analysis with a p-value of 0.05, also using PAST[21]. The ANOSIM statistical analysis was performed with a p-value of 0.05 in order to test for differences in microbial community composition between the scenarios. This statistical test resulted in the R-values matrix shown in Table S1, where values close to 1 indicated a strong dissimilarity between samples and 0 indicated no difference.

Results and Discussion

Reactor performance. The present study aimed at maximizing VFAs productions by assessing the effect of stepwise OLR increases. The initial scenario (Sc. I, 3 COD $L^{-1}d^{-1}$) evidenced average tCOD and sCOD values of 21.9 ± 3.2 g COD L^{-1} and 11.4 ± 0.9 g COD L^{-1}, respectively, during the steady-state, which corresponded to a COD removal of 29.3 ± 6.1% (Table 1). The COD removal attained during this scenario showed that AD process was not working well for biogas production probably due to the low HRT (8 days) and high OLR (3 g COD·L^{-1}·d^{-1}) values imposed. Conventional AD processes used for maximizing methane production must have a balanced HRT and OLR, since these are key parameters in process optimization[22]. Too short HRT might cause incomplete substrate degradation or microbial population death by starvation whereas low and high OLR values can drive the process either to starvation or to incomplete organic matter degradation due to inhibition by overloading. As a matter of fact, HRT and OLR values usually employed in literature for microalgal biomass degradation via AD for biogas production are very different to the ones employed in the present study and showed higher COD removals. For instance, 51% COD removal took place in an anaerobic digester fed with *C. vulgaris* (1 g COD L^{-1} d^{-1}) at 28 days HRT[23]. Since methanogenic inhibition is desired for VFAs production, the selection of low HRT and high OLR values were appropriated for such a goal. Nevertheless, the COD removal was still high and thus, an important carbon fraction was still lost in the biogas stream. For this reason, further OLR increases were applied.

Stepwise OLR increases resulted in concomitantly increasing organic matter conversion into VFAs (Fig. 1). Accordingly, Sc. II (6 g COD L^{-1} d^{-1}) reached values of 38.3 ± 0.8 and 20.1 ± 3.7 g COD L^{-1} (tCOD and sCOD, respectively, Table 1), reducing the COD removal from 29.3% attained in Sc. I to 20.1%, as a consequence of methanogenic instability. Considerably lower values were achieved at the end of Sc. III-IV (% COD removals less than 5%, tCOD of 69.6 ± 1.3 and 91.1 ± 6.2 g COD L^{-1} and sCOD of 29.9 ± 3.221 and 47.2 ± 5.1 for 9 and 12 g COD L^{-1} d^{-1}, respectively). However, when the system was operated at OLR 15 g COD L^{-1} d^{-1} (Sc. V), the COD removal percentage seemed to increase slightly when compared to Sc. IV (14.1 ± 2.7% against <5%). In this latter scenario, an acclimation of the anaerobic archaea community to consecutive OLR increasing values might have taken place, improving the organic matter removal efficiency (Table 1). The adaptive capacity of methanogenic archaea to specific process conditions has been already proven in literature[24,25]. Nevertheless, the recorded values for total COD removal were too low within the carbon balance. As a matter of fact, fermentation of organic compounds by acidogenic bacteria and methanogenic archaea is also devoted for the growth of new cells (0.15 kg VSS/kg COD for acidogenic bacteria and 0.03 kg VSS/kg COD in the case of methane producers)[26]. Overall, the COD removal from Sc. II onwards was considered too low in the carbon flow directed to biogas but percentages were rather attributed to anaerobic microorganism's growth.

Ammonium (NH_4^+) and free ammonia (NH_3) concentrations are important parameters since high concentrations of these compounds may result inhibitory for methanogenic archaea resulting in methanogenesis inhibition. NH_4^+ and NH_3 accumulation might occur when proteins are degraded during AD[24]. Achieved NH_4^+ concentrations showed a growing trend up to 4,410 mg NH_4^+-N L^{-1} at 15 g COD L^{-1} d^{-1} (Table 1). Even though NH_4^+-N was registered throughout the experimental time, only the two last scenarios (Sc. IV and V 3.8 ± 0.2 and 4.4 ± 0.1 g L^{-1} NH_4^+-N, respectively) resulted in values close to the threshold indicated in literature (above 3 g L^{-1} of total ammonia nitrogen) to provoke methanogenesis inhibition[27,28]. With regard to free ammonia (NH_3), the concentrations attained during the experiment were very low since temperature was set at the psychrophilic range temperature (25 °C) and pH values were always between 6 and 7 (Table 1). According to literature, inhibition due to this compound occurs at 80 mg L^{-1} N-NH_3[29]. This value was far from the ones attained in the present study (below 10 mg L^{-1} NH_3-N). Thus, this compound was presumably not the responsible for inhibiting methanogenic archaea but it cannot be neglected that total ammonia (ammonium + ammonia) were in the inhibition level for methanogenic archaea. In this sense, the operational conditions imposed in the system were considered suitable for VFA accumulation rather than consumed by archaea.

VFAs production: concentration, yields and profiles. An increase in VFAs production (mg COD-VFA L^{-1}) was noticed throughout the experimental time at increasing OLR values from Sc. I-V (Fig. 1 and Fig. 2). However, the last scenario fed at 15 g COD L^{-1} d^{-1} resulted in a decrease in VFAs concentration. Similar experiments available in literature conclude on the existence of an optimum OLR value from which VFAs production

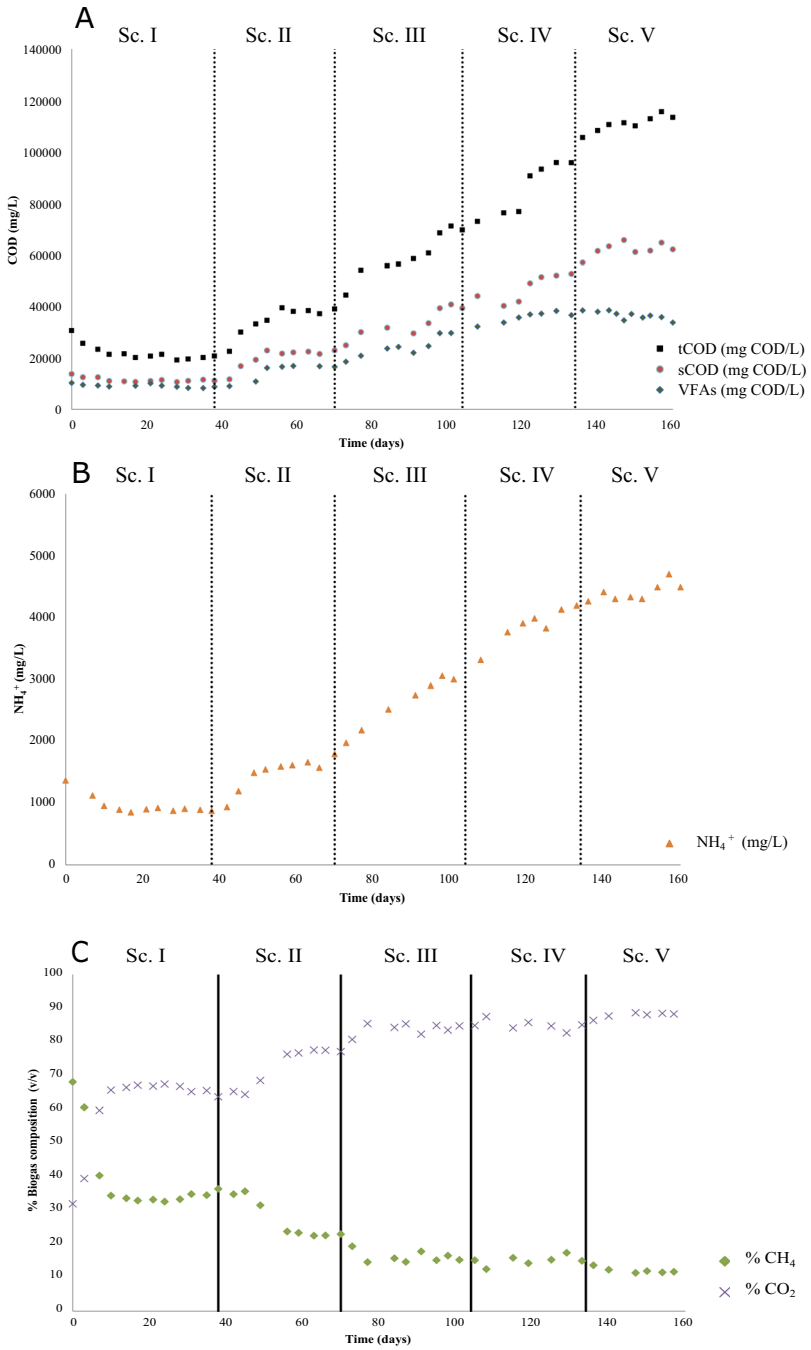

Figure 1. Time course of the main experimental parameters during reactor operation: (**A**) Total/soluble chemical oxygen demand and volatile fatty acids; (**B**) ammonium and (**C**) biogas composition.

does not increase. These studies attribute this point of inflection to the hydrolytic capacity of the system. When this point is exceeded, the first step of the AD becomes limiting. For instance, AD of olive mill solid residue was carried out under different OLR values from 3.2 to 15.1 g COD L^{-1} d^{-1} equivalent to HRT from 50 to 10.7 days at continuous feeding mode[30]. Those researchers pointed out that the optimum value was 12.9 g COD L^{-1} d^{-1} (HRT 12.4 days) resulting in VFAs production of 15–20 g COD-VFA L^{-1}. A subsequent OLR increase did not report higher VFAs productions. The inhibition of the process was characterized by a strong decrease of the most abundant product acetic acid. VFAs productions were as well monitored in a similar study at OLR 5; 6.6; 10 and 13.3 g COD L^{-1} d^{-1} and decreasing HRT values 4; 3; 2; 1.5 days at mesophilic conditions (37 °C) in a process devoted for biohydrogen production from a waste stream of palm oil[31]. Results showed a maximum VFAs production of 1.5 g VFAs L^{-1} at high OLR values and low HRT (10 g COD L^{-1} d^{-1} and 2 days). Final VFAs productions in these studies were below the ones attained herein, probably due to the use of substrates with different macromolecular composition and operational conditions. Both former studies attributed the drop in VFAs production to a deficient hydrolytic step. At this point, and as mentioned in Section 2.1, the present study subjected microalgae

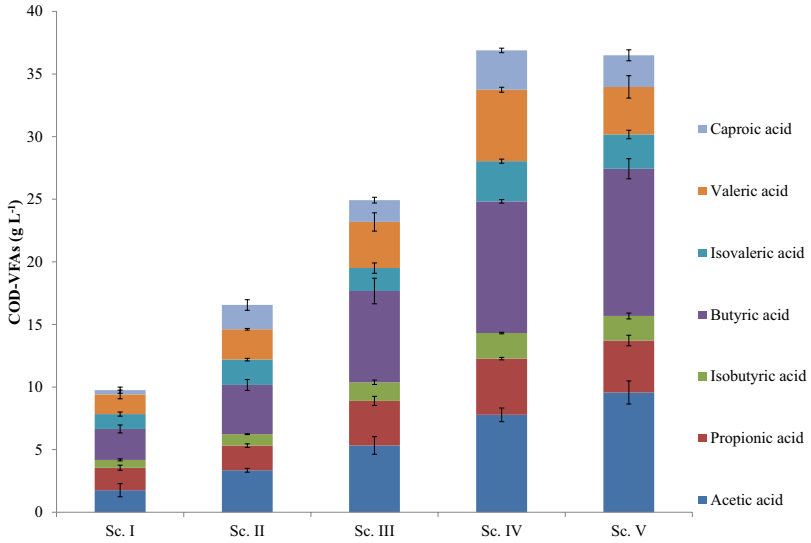

Figure 2. Volatile fatty acids (VFAs) production from scenarios I to V.

biomass to a proteolytic pretreatment to avoid any hydrolysis limitation with the focus put on the acidogenesis stage of AD. In fact, the ratio sCOD/tCOD comparison of the different scenarios showed quite stable values ranging 0.52–0.58. This fact suggested that the hydrolytic step was not a bottleneck for VFAs production along the increasing OLRs applied since similar ratios were attained (Table 1). Thus, it was inferred that an inhibition of the acidogenic step took place. In this sense, the acidogenic inhibition step has been previously studied and different compounds were pointed out as responsible for the acidogenic inhibition. K^+, Na^+, clorophenols and heavy metals (Cu > Zn > Cr > Cd > Ni > Pb) are toxic for acidogenesis[32]. Out of these compounds, sodium may have affected acidogenic activity in the present study, as NaOH was used to control pH during the enzymatic pretreatment of the microalgal biomass. The analysis revealed increasing Na^+ concentrations from Scenario I to V. This concentration concomitantly increased from 1.02 g L^{-1} determined in Scenario I, 1.8 g L^{-1}, 2.8 g L^{-1}, 3.7 g L^{-1} and 4.9 g L^{-1} Na^+ in Scenario V. This compound affects the specific growth rate of microorganisms because it plays a role in the formation of adenosine triphosphate and NADH oxidation. Although it is beneficial at minor concentrations (<1 g L^{-1} Na^+), higher amounts might alter anaerobic species growth[32]. Since AD has been devoted traditionally for biogas production, the influence of sodium in methanogens has been more studied[33,34]. However, hydrolytic, acidogenic and acetogenic species are known to be more sensitive to Na^+ [35]. In this sense, there are studies showing moderate methanogenic inhibition at Na^+ values ranging 3.5–5.5 g L^{-1} [36]. Hence, taking into account the acidogenic sensitivity aforementioned, it could be inferred that Na^+ affected process yields in terms of VFAs production.

Likewise, high ammonium concentrations have been also found to affect the acidogenic step. As a matter of fact, the high ammonium concentrations attained at the highest OLR (4.4 g L^{-1}) were above the level (3.1 g L^{-1}) identified for acidogenic bacteria inhibition[37]. Finally, high VFAs concentrations have been studied as well as possible inhibitors of the acidogenesis. Investigations found a slight inhibitory effect at 4 g VFAs L^{-1} during the fermentation of glucose[38]. Since these values are far below the VFAs productions obtained in the present study, high VFAs concentrations determined herein could have also hampered the acidogenic stage.

The efficiency of the different scenarios was assessed by calculating the organic matter conversion yields into VFAs (COD-VFAs/COD_{in}). Sc. I exhibited the lowest value (0.30 ± 0.02) concomitantly with the highest % COD removal (Table 1). From that point onwards, the system increased organic matter conversion into VFAs in the following scenarios (Sc. II, 0.34 ± 0.01; Sc. III 0.39 ± 0.04; Sc. IV 0.37 ± 0.02 COD-VFAs/COD_{in}) until Sc. V, in which conversion dropped (0.29 ± 0.01 COD-VFAs/COD_{in}). The lower organic matter conversion efficiency into VFA determined at the highest OLR tested (15 g COD $L^{-1}d^{-1}$) was attributed to a combination of high ammonium, VFAs and sodium concentrations. In addition, this decrease in organic matter conversion into VFAs agreed with the higher COD removal registered in Sc. V (Table 1).

VFAs profiles were assessed to evaluate the influence of increasing OLR values (Fig. 2). Butyric acid was the most abundant product obtained in the digesters accounting up to 11.7 ± 0.8 g COD L^{-1} at 15 g COD L^{-1} d^{-1}, which corresponded to 32.2% of total VFAs production. This VFA registered an increasing trend from Sc. I to Sc V (25.8% to 32.2%). Accumulation of butyric acid is regarded as a signal of higher hydrogen partial pressure than when the process is devoted to biogas production. In this sense, when hydrogen-utilising methanogens are exposed to hydrogen partial pressures above 10^{-4} atm, VFAs such as butyric acid accumulate in the system[39]. The second most abundant product in each stage was acetic acid (26% in Sc. V out of total VFAs production vs 19–20% in the rest of the stages). This fact might be explained because of the degradation of the longest VFAs (such as isovaleric valeric and caproic acids) into butyric and acetic acids (from 8.6%, 15.4%, and 8.4% isovaleric, valeric and caproic acids, respectively in Sc. IV to 7.5%, 10.4% and 6.9% in Sc. V). Similarly to the present study, another investigation using a mixture of *C. vulgaris* and *Scenedesmus quadricula* as substrate was carried out for

Scenario	Observed OTUs	Shannon
Inoculum	123,000	3.357
I	148,700	4.110
II	213,200	4.447
III	178,600	4.469
IV	166,600	3.809
V	144,400	3.889

Table 2. OTUs and Shannon and Simpson indices calculated for the samples. OTUs: Operational taxonomic units.

VFAs production. Acetic, propionic and butyric acids were also the most abundant products although their distribution was slightly different (46% acetic acid; 19% propionic acid and 19% butyric acid).The latter experiment was carried out in batch mode, which makes difficult the comparison. The underlying abundance or shortage of a concrete VFA is due not only to the substrate employed, but also to the operational conditions set in the system[40]. In addition, different substrates also result in varying VFAs productions. For instance, VFAs production from sucrose for hydrogen production from AD reported a different VFAs profile consisting of 52% butyric acid, 9% propionic acid and 32% acetic acid at 40 g COD L^{-1} d^{-1} and HRT 12 h[41]. Overall, comparison with literature is hard since substrate, feeding mode or operational conditions affect VFA production yields and profiles.

Microbial population dynamics. *Microbial community analysis.* Since promoting specific acidogenic bacteria population is a key factor for maximizing VFA production, microbial communities were analysed during the steady-state of each scenario in order to evaluate the effect of increasing OLR on the relative abundance of the dominant microorganisms. In fact, there was a clear microbial trend along the experimental scenarios in terms of diversity, statistics and microbial distribution analyses. As it can be seen in Table 2, Shannon index reflected a slight diversity increase from the inoculum (3.357) to Sc. I (4.110). During this first scenario, operation of the reactor likely promoted the growth of microorganisms. Likewise, once OLR was increased in the following scenarios (II and III), an increase in Shannon index was detected (4.417 and 4.469, respectively) suggesting an adaptation of the anaerobic biomass present in the reactor to the conditions imposed in the system. However, the subsequent OLR increase in Sc. IV and Sc. V resulted in lower diversity than the previous scenarios (3.870 and 3.802, respectively). These values indicated the specialization of the microorganisms present in the reactor at high OLR values and displayed the crucial influence that this parameter wields over the process. Regarding the number of OTUs observed per sample, these values did not exhibit the same trend that Shannon index (Table 2). It should be taken into account that diversity is not only represented by richness but also by evenness and thus, the higher the microorganisms detected as well as their homogeneity (in terms of relative abundance), the higher the diversity in the system[42]. However, it can be seen that the observed OTUs decreased substantially from Sc. II (153, 200) to Sc. IV (100,400). This fact confirmed the microbial adaptation to the operational conditions and the microbial consortia specialization. The influence of OLR was displayed in the PCoA statistical analysis (Fig. 3a), which reflected that microbial samples were clustered distinctly according to the different OLR ranges: (i) inoculum and Sc. I, (ii) Sc. II-III and (iii) Sc. IV-V. Thus, physico-chemical parameters values changed ($N\text{-}NH_4^+$/$N\text{-}NH_3$, VFAs) due to the progressive OLR increase, definitely affecting microbial populations. pH remained stable along the experimental time. In this sense, the pretreated microalgae fed at pH 8 might have buffered the system, avoiding the pH drop associated normally to high VFAs concentration. As it can be seen in PCA analysis, the VFA concentration registered at the highest OLR was mainly related to the high NH_4^+ concentrations released to the medium (Fig. 3b). Both, VFAs and NH_4^+, are compounds that might be toxic for the microbiome, explaining the specialization at increasing OLRs[32,37]. An ANOSIM (Fig. S1) test confirmed the strong dissimilarity between the clusters detected through PCoA as well as a high similarity between the scenarios that constituted each cluster. In addition, microorganism's population changed throughout the different scenarios with a concomitant increase in organic matter conversion into VFAs. However, population changes was not reflected in VFA profiles obtained, which remained stable throughout the different scenarios (Fig. 2).

Microbial community composition. The 16S rRNA gene analysis revealed that Firmicutes, Bacteroidetes and Actinobacteria were the most abundant phyla in the whole experimental period, further followed by Proteobacteria, Synergistetes and Euryarchaeota (Fig. 4A). The inoculum was dominated by Firmicutes phylum (70.2%), Actinobacteria (18.9%) and Euryarchaeota (8.1%). The high presence of bacteria belonging to Firmicutes phylum can be explained by the anaerobic sludge origin, which was an acidogenic anaerobic reactor (Section 2.1). Major contributors identified were species related with Clostridiales order (40.5%), other microorganism's belonging to Coriobacteriaceae family (17.6%) as well as genera such as *Ruminococcus* (12.8%), *Sporanaerobacter* (7.2%) and *Methanobacterium* (6.4%). Overall, the community structure in the sludge was composed by microorganisms exhibiting hydrolytic and acidogenic activities[43].

At phylum level, the progressive OLR increase influenced the microbial population dynamics. During Sc. I, the first applied OLR provoked an increase in relevance of Firmicutes phylum (82.9%) and Bacteroidetes (5.4%) with a concomitant decrease of Actinobacteria (4.1%) and Euryarchaeota (4.4%) (Fig. 3a). Sc. II and Sc. III were characterized by the progressive disappearance of Actinobacteria and Euryarchaeota and the slight increase of Bacteroidetes (up to 20.7% in Sc. III). At this point it is important to highlight that Sc. III coincided with the highest organic matter conversions into VFAs obtained (Table 1). Thus, the balance established between Bacteroidetes

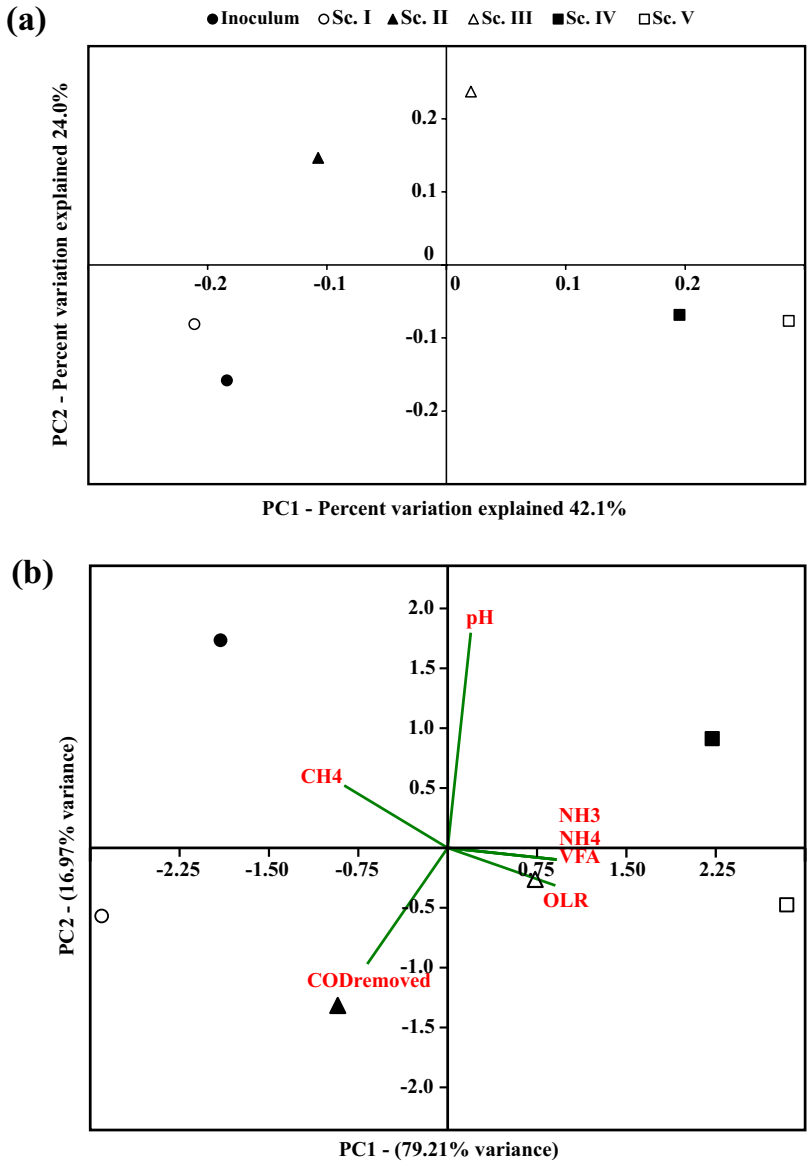

Figure 3. Principal coordinate analysis (PCoA) (**a**) and principal components analysis (PCA) (**b**).

and Firmicutes relative abundance as well as the reduction of the methanogenic activity (Euryarchaeota phylum) might play a key role in maximizing VFAs production. DNA analysis from Sc. IV and especially Sc. V showed a gradual increase of Firmicutes and Euryarchaeota together with the disappearance of Bacteroidetes (Fig. 4A). These factors likely caused the drop of organic matter into VFAs conversion registered at the end of the experimental time (Sc. V, Table 1). The dominance of Bacteroidetes and Firmicutes in acidogenic fermentation from grass biomass acidification was previously reported at 37 °C and 55 °C, respectively[44]. In addition, low diversity is encountered in acidogenic reactors when compared to populations observed in AD processes devoted to biogas production. In fact, similar studies using microalgae as feedstock for methane generation revealed higher phylum diversity than in the study presented herein. Gonzalez-Fernandez *et al.* (2018) used *Chlorella sorokiniana* and *Scenedesmus* sp. for methane generation and obtained a diverse community characterized by the presence of different phyla such as Proteobacteria (46–51%), Firmicutes (20%), Bacteroidetes (2–6%) and Euryarchaeota (7–8%)[45]. This latter study and the one carried out by Greses and co-workers[46] showed the low presence of Bacteroidetes in a process devoted for biogas production. Moreover, the relative abundances (%) in those studies between Bacteroidetes and Euryarchaeota were very different to those reported in the present investigation where Bacteroidetes stood out when methanogenic species were suppressed. This combination resulted in high VFAs productions. Following the same trend, Proteobacteria is another phylum which showed variation between acidogenic fermentation and AD for biogas production. Whereas in the present study this phylum showed values below 3% other studies devoted to biogas production reported values drastically different (46–51%)[45]. Moreover, the low COD removals percentages achieved along the experiment (Table 1) suggested that the syntrophic association among Proteobacteria and Euryarchaeota was weakened. Overall, it could be

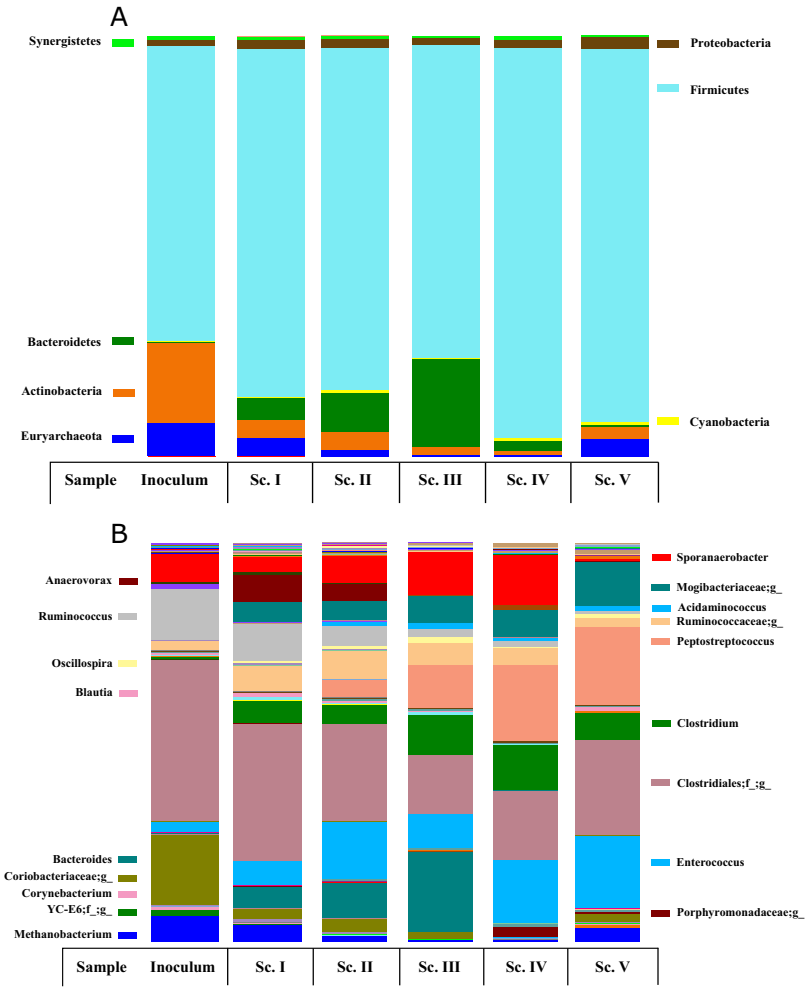

Figure 4. Main phyla (**A**) and genera (**B**) found during reactor operation.

stated that the existing differences in terms of microbial population between an anaerobic community devoted to biogas production and the acidogenic inoculum presented herein indicated that the inoculum chosen was appropriate for VFAs maximization.

At genera level, operational conditions affected species differently. Whereas some of them gradually disappeared such as *Ruminococcus*, *Anaerovorax*, or microorganisms related with the Coriobacteriaceae family, others increased its relative abundance. In this sense, *Sporanaerobacter, Clostridium, Peptostreptococcus* and *Enterococcus* belonging to Firmicutes phylum gained importance along the experiment. More specifically, *Sporanaerobacter* has been identified in acidogenic reactors fed with microalgae biomass and has been pointed out to be responsible of metabolizing sugars, peptides and single amino acids into acetate[47]. *Clostridium* genus is involved in butyrate, acetic acid, lactic acid and ethanol production due to their ability to carry out mixed acid and alcohol fermentations[48], explaining the butyric acid dominance (from 25.8% in Sc. I to 32.2% in Sc. V) in the VFAs profile as well as the high acetic acid productions (Fig. 2). *Peptostreptococcus* is associated with the presence of propionic and succinic acids in anaerobic digesters[49]. All of these species decreased their relative abundance during the last scenario contributing to the lower VFAs production attained (Fig. 1). In addition, the dominant genus found from Euryarchaeota phylum was *Methanobacterium*. The gradual decrease in terms of relative abundance of these genera (6.4% vs 0.5%) agreed with the concomitant drop of COD removals percentages encountered throughout the process (Table 1). Exception made for the last scenario, in which abundance levels raised once again (3.5%). This fact suggested that the hydrogenotrophic genus *Methanobacterium* was able to get adapted at the end of the experimental time. Hydrogenotrophic species are reported to be more resistant than acetoclastic methanogens to high VFAs and ammonium concentrations[50,51]. In fact acetic acid accumulated (Fig. 2) but no acetoclastic species were found along the evaluated scenarios. Thus, harsh operational conditions (low HRT and high OLR), causing the wash out of archaea species, as well as the high ammonium and VFAs levels detected most likely explained the acetoclastic inhibition and the low hydrogenotrophic methanogens presence[32]. The high tolerance of this latter species was demonstrated during Sc. IV and V, in which no VFAs enhancement was reported.

VFAs applications, future perspective and challenges. VFAs are currently produced via the petro-chemical pathway. Nevertheless, their production through AD via the carboxylate platform is an attractive biotechnological technology to valorize organic residues in an environmentally friendly manner. Transition into

a circular economy based on sustainable use of resources is, nowadays, a must. For this purpose, waste streams constitute a cost-effective raw material from which volatile fatty acids (VFAs) can be obtained through AD. AD has been mainly devoted to biogas production. Nevertheless, carboxylate production through this via requires further research.

This bioprocess does not require sterilization and hence, lower capital and operating costs compared to axenic cultures are involved. The economic value of the VFAs generally relies on the chain length. The market price increases from acetic (600$/ton) to butyric acids (2163$/ton)[52]. VFAs have been used for a wide variety of applications such as ester, alcohols production, food preservatives[2]. The possibility of elongating VFAs through the reverse ß oxidation pathway or single cell oils is being currently studied to give additional value to the products obtained[53,54]. In addition, these chemicals have gained importance in the biofuels field, namely biodiesel[55] and biohydrogen[56] production or electricity generation via microbial fuel cells[57]. Lastly, they can also be used for biopolymers production. However, distribution of these chemicals (VFAs profile) must be considered for some applications such as their use for polyhydroxyalkanoates production (PHAs). In this sense, a specific VFAs profile implies predictable PHAs characteristics. The prevalence of acids with even number of carbons promotes 3-hydroxybutyrate synthesis whereas 3-hydroxyvalerate is favoured by odd number VFAs[58].

Despite of their wide potential, there are still barriers that require to be overcome in order to implement the carboxylate technology at industrial scale. For instance, the methanogenic step of the AD must be suppressed to favour VFAs accumulation in the digestate by using chemicals[59] or operational strategies (low HRTs and high OLRs, as presented herein). Likewise, more research should be conducted to avoid acidogenic inhibition that may result from the high VFAs and ammonium concentration[37]. In this sense, efforts need to be directed to identify the key acidogens and to apply different process configurations and operational conditions to overcome inhibitory effects. For instance, when conversion yields are decreased at high OLR, increasing the HRT or bioaugment the anaerobic microbiome might be beneficial.

Some other technological key aspects rely on the VFA application. Whereas biogas is easily separated from the digestate, separation and purification steps might be required depending on the VFAs application. The product quality needed for a specific application will determine the separation method employed[60]. Hence, the proper choice of the separation method is of paramount importance for the process to be cost effective. In terms of the biology of the system, there are more topics deserving further investigation such as the production of targeted VFAs or the anaerobic microbiome response towards operational changes or potential perturbation that the system might suffer.

Overall, the carboxylate platform might become an efficient technology to produce value added-products from microalgae biomass. The flexibility of anaerobic digestion towards different organic feedstocks and the alternative products that can be attained further than biogas, makes this technology an important producer of valuable chemicals. Nevertheless, as aforementioned, more research is required to move from the most conventional product (biogas) to the new bio-based materials required in the chemical industry.

Conclusions

VFAs production yields and microbial populations were affected by increasing OLRs. Butyric, acetic and propionic acids were the most abundant products (23–32%, 19–26%, 11–17%, respectively, out of the total VFAs). Organic matter conversion into VFAs was maximized at 9 and 12 g COD L^{-1} d^{-1} reaching VFAs productions up to 36.8 ± 2.1 g COD-VFA L^{-1} (conversion yields of 0.37 ± 0.02 COD-VFAs/COD_{in}). During these stages a good balance between microbial populations (Bacteroidetes and Firmicutes) as well as low methanogenic presence was observed. However, the last scenario (OLR 15 g COD L^{-1} d^{-1}) did not report an enhancement on VFAs productions. VFAs production was hampered at the acidogenic stage due to the combined effect of high ammonium, sodium and VFAs concentrations. The microbial follow up in this latter scenario revealed a reduction of Bacteroidetes phylum as well as the increase of methanogenic population. This microbial shift was found crucial in the lower organic matter conversions into VFAs obtained in Sc. V.

Data availability

All data generated or analyzed during this study are included in this published article and Additional file 1.

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Author contributions

J.A.M. executed experiments, performed analyses, interpreted data, and drafted the manuscript. S.G. contributed to data interpretation, performed the microbial analysis and revised the manuscript. C.G.F. is principal investigator and contributed to experimental design, data interpretation, and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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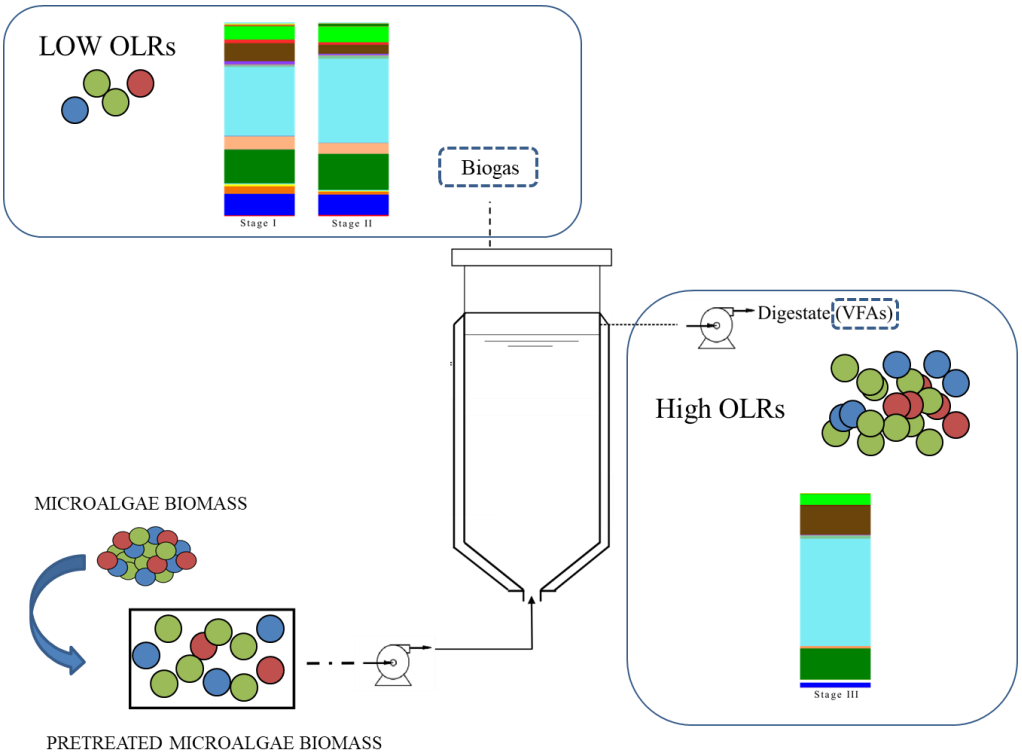
PUBLICATION IX

ARTICLE

ANAEROBIC FERMENTATION OF MICROALGAE BIOMASS IN A UASB REACTOR: ORGANIC LOADING RATE EFFECT ON PRODUCT OUTPUT AND MICROBIOLOGY



Jose Antonio Magdalena, Silvia Greses, Cristina González-Fernández

ANAEROBIC FERMENTATION OF MICROALGAE BIOMASS IN A UASB REACTOR: ORGANIC LOADING RATE EFFECT ON PRODUCT OUTPUT AND MICROBIOLOGY

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ABSTRACT

Efficient biomethane production from the microalgae *Chlorella vulgaris* was aimed in an upflow anaerobic sludge bed (UASB) reactor at 25ºC and stepwise organic loading rate (OLR) increases (OLR; 2.3±0.2, 3.6±0.9 and 8.7±1.2 g COD $L^{-1}d^{-1}$). Low OLRs retrieved a COD removal of around 48%, showing a good digestibility of the substrate at lower HRTs (around 6 days) than the normally employed in CSTRs. On the contrary, the highest OLR employed resulted in an organic matter conversion into volatile fatty acids (VFAs) of 37% VFAs-COD/COD_{in} (COD removal of 24%). Microbial analysis revealed at low OLR values, a high relative abundance of Firmicutes (35-43%) together with Bacteroidetes (17-18%) and Euryarchaeota (11%). However, high OLRs resulted in a sludge specialization promoting Firmicutes (55%) and Proteobacteria (14%) while Euryarchaeota (2.5%) population decreased, which most likely mediated VFAs accumulation. Results showed the effectiveness of the UASB reactor to produce biogas or VFAs (valuable platform molecules).

KEYWORDS: anaerobic digestion; microalgae; population dynamics; UASB; volatile fatty acids