Universidad de Valladolid

TESIS DOCTORAL:

Recovery of the carbohydrate fraction from microalgal biomass grown in wastewater treatment photobioreactors: a biorefinery approach

Presentada por Judit Martín Juárez para optar
al grado de
Doctora por la Universidad de Valladolid

Dirigida por:
Silvia Bolado Rodríguez

**PROGRAMA DE DOCTORADO EN INGENIERÍA
QUÍMICA Y AMBIENTAL**

TESIS DOCTORAL:

**Recuperación de la fracción de carbohidratos
procedentes de biomasa microalgal cultivada en
fotobioreactores de tratamiento de aguas residuales:
un enfoque de biorefinería**

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Memoria para optar al grado de Doctor,

con **Mención Doctor Internacional**,

presentada por la Ingeniería Química:

Judit Martín Juárez

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Catedrática

Departamento de Ingeniería Química y
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Certifican que:

JUDIT MARTÍN JUÁREZ ha realizado bajo su dirección el
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Considerando que dicho trabajo reúne los requisitos
para ser presentado como Tesis Doctoral expresan su
conformidad con dicha presentación.

Valladolid, a _____de_____ de 2019

Fdo. Silvia Bolado Rodríguez

Reunido el tribunal que ha juzgado la tesis doctoral "Recovery of the carbohydrate fraction from microalgal biomass grown in wastewater treatment photobioreactors: a biorefinery approach", presentada por Judit Martín Juárez y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por _____ la calificación de _____.

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PRESIDENTE   SECRETARIO

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Resumen

En los últimos años, la búsqueda de nuevas materias primas renovables ha aumentado exponencialmente debido al gran consumo de materiales y los limitados recursos fósiles. Al mismo tiempo, un importante esfuerzo de investigación se centra en la gestión de las grandes cantidades de desechos generadas por las actividades humanas. Combinando ambos objetivos de investigación, la recuperación y la valorización de los componentes procedentes de desechos parecen ser la única solución sostenible. Las aguas residuales, que contienen gran cantidad de materia orgánica y nutrientes, son uno de los mayores residuos generados en nuestra sociedad. Se están estudiando procesos biotecnológicos con consorcios de microalgas y bacterias para el tratamiento de aguas residuales con el objetivo de mejorar los rendimientos de recuperación de los nutrientes (C, N, P, S ...), así como con el fin de obtener agua limpia para otras aplicaciones. Por lo tanto, esta tesis tiene como objetivo abordar la valorización de la biomasa de microalgas-bacterias cultivadas en fotobiorreactores de tratamiento de aguas residuales. Este trabajo se centra en la valorización de la fracción de carbohidratos de esta biomasa para producir biogás y monosacáridos fermentables, pero considerando el efecto de este proceso en otras fracciones valiosas de la biomasa, como proteínas y lípidos, y la generación de otros subproductos. El estudio de la recuperación de carbohidratos se aborda como una primera etapa de un proceso secuencial para la valorización integral de la biomasa, aplicando un concepto de biorrefinería.

Se aplicaron diferentes métodos de ruptura de la pared celular (molino de bolas, ultrasonidos, explosión de vapor, alcalino peróxido, alcalino y ácido) en diferentes condiciones de operación, como un primer paso para la producción de biogás o para la recuperación de azúcares fermentables mediante hidrólisis enzimática.

Los tests BMP proporcionaron la producción máxima de metano (377 ml de CH_4 / g VS) a partir de muestras pretratadas con NaOH 2M (120ºC, 60 min), mientras que los pretratamientos ácidos provocaron una inhibición severa. El pretratamiento con alcalino peróxido mejoró la producción de metano en un 73% con respecto a la biomasa no tratada, mientras que el molino de bolas y la explosión de vapor aumentaron la tasa de producción

de metano en un factor de 5 y 3, respectivamente. La composición de los residuos tras la digestión anaeróbica fue adecuada para su uso como fertilizante.

Los mayores rendimientos de solubilización de carbohidratos se obtuvieron mediante hidrólisis enzimática de muestras pretratadas con ácido y base. La hidrólisis de muestras pretratadas con HCl 2M, 120ºC, 60 minutos proporcionó el 98% de solubilización de carbohidratos y el 81% de recuperación de monosacáridos con una baja degradación, pero también solubilizó el 76% de las proteínas y el 56% de los lípidos. El pretratamiento alcalino peróxido alcanzó una mejora significativa durante la hidrólisis enzimática con rendimientos solubilización del 70% para carbohidratos y 55% para lípidos, mientras que solo un 35% para proteínas. La hidrólisis enzimática de muestras pretratadas del molino de bolas proporcionó resultados altamente selectivos con altos rendimientos de solubilización de carbohidratos (84%) pero con alta generación de subproductos, principalmente metanol y etanol (4.5 g / L).

La optimización de los parámetros operacionales de los pretratamientos ácidos y alcalinos junto con la etapa de hidrólisis enzimática confirmó como los parámetros más significativos la temperatura del pretratamiento, el tipo de agente químico y la concentración del agente químico. La etapa de hidrólisis enzimática no resultó necesaria, logrando rendimientos de solubilización de carbohidratos superiores al 84% para diferentes biomasas de microalgas, para el pretratamiento ácido (120ºC, HCl 2M). Sin embargo, los medios de crecimiento de la biomasa tuvieron un impacto relevante en la generación del subproducto, con rendimientos de recuperación de monosacáridos que van desde el 80% para la biomasa cultivada en medio sintético hasta el 53% para la biomasa cultivada en aguas residuales de cerdos.

Finalmente, se estudió el posible uso de la biomasa de microalgas como sustrato para la producción de enzimas por *Trichoderma reesei* y se optimizaron los parámetros de operación. Las producciones máximas de celulasas y xilanasas (28.35 FPU / g para FPasa, 16.76 U / g para β-glucosidasa, 1113.45 U / g para xilanasa y 3.81U / g para β-xilosidasa) se lograron utilizando una relación 50:50 de biomasa: bagazo, 5 días, 28ºC, pH 4, y

extracción de fosfato a 22ºC durante 1 h, trabajando con diferentes contenidos de humedad de la biomasa.

Los resultados obtenidos en la tesis actual presentan información importante y herramientas valiosas para comprender las diferentes vías para valorizar la biomasa de microalgas y bacterias del tratamiento de aguas residuales de purines de cerdo. Además, se evalúa una comparación de los resultados generales con otro tipo de biomasas de microalgas para proporcionar soluciones únicas y viables en un contexto general.

Abstract

In the recent years, the search of new renewable raw materials has exponentially increased due to the huge consumption of materials and the limited fossil resources. At the same time, an important research effort is addressed to the management of the increasing amounts of wastes generated by human activities. Combining both research goals, the recovery and valorisation of the components of wastes seem to be the only sustainable solution. Wastewaters, containing great amount of organic matter and nutrients, are one of the largest residues generated in our society. Biotechnological processes with consortia of microalgae and bacteria are being studied for wastewater treatment with the aim to enhance the yields of recovery of the nutrients (C, N, P, S…) as well as to obtain clean water for other applications. Thus, this thesis aims at tackling the valorisation of the microalgae-bacteria biomass grown in wastewater treatment photobioreactors. This work is focused on the valorisation of the carbohydrate fraction of this biomass to produce biogas and fermentable monosaccharides but considering the effect of this process stages on other valuable fractions of the biomass, as proteins and lipids and the generation of by-products. The study of carbohydrates recovery is addressed as a first stage of a sequential process for the integral valorisation of the biomass, applying a biorefinery concept.

Different cell wall disruption methods (bead mill, ultrasound, steam explosion, alkali-peroxide, alkaline and acid) were applied at different operation conditions, as a first step for biogas production or for recovery of fermentable sugars by enzymatic hydrolysis.

BMP tests provided the maximum methane production (377mL CH_4/g VS) from alkali pretreated samples (NaOH 2M, 120ºC, 60 min), whereas acid pretreatments provoked a severe inhibition. Alkaline peroxide pretreatment enhanced the methane production 73% respect to untreated biomass, while bead mill and steam explosion increased the methane production rate by a factor of 5 and 3, respectively. The composition of the residues after the anaerobic digestion was adequate for their use as fertiliser.

The higher carbohydrate solubilisation yields were obtained by enzymatic hydrolysis of acid and alkaline pretreated samples. Hydrolysis of samples pretreated with HCl 2M,

120ºC, 60 min provided 98% of carbohydrates solubilisation and 81% of monosaccharides recovery with low degradation, but also solubilised 76% of the proteins and 56% of the lipids. Alkaline-peroxide pretreatment reached significant improvement during the enzymatic hydrolysis with solubilised yields of 70% for carbohydrates and 55% for lipids while only 35% for proteins. Enzymatic hydrolysis of bead mill pretreated samples provided highly selective results with high carbohydrate solubilisation yields (84%) but high by-products generation mainly methanol and ethanol (4.5g/L).

The optimisation of operational parameters of acid and alkaline pretreatments coupled with enzymatic hydrolysis confirmed temperature of pretreatment, kind of chemical agent and concentration of chemical agent as the most significant parameters. Enzymatic hydrolysis step did not result necessary, achieving carbohydrate solubilisation yields higher than 84% from different microalgal biomasses, applying only acid pretreatment (120ºC, HCl 2M). However, the growth media of biomass had a relevant impact on the by-product's generation, with monosaccharides recovery yields ranging from 80% for biomass cultivated in synthetic medium to 53% for biomass grown in piggery wastewater.

Finally, the possible use of microalgal biomass as substrate for enzymes production by *Trichoderma reesei* was studied and the operation parameters were optimised. The maximum cellulases and xylanases productions (28.35 FPU/g for FPase, 16.76 U/g for β-glucosidase, 1113.45 U/g for xylanase and 3.81U/g for β-xylosidase) were achieved using a 50:50 ratio biomass:bagasse, 5 days, 28ºC, pH 4, and phosphate extraction at 22ºC for 1 h, working with different moisture contents of the biomass.

The results fulfilled in the current thesis present important information and valuable tools to understand different pathways to valorise the microalgae-bacteria biomass from pig manure wastewater treatment. Furthermore, a comparative of the overall results with other kind of microalgal biomasses is evaluated to provide unique and feasible solutions in general context.

List of publications

The publications cited below are presented as a part of the current thesis. All papers were published in international journals indexed in ISI web of Knowledge (Papers I, III, and IV). Paper II have been submitted for publication in Algal Research and Paper V is being prepared.

Manuscript I. Martín-Juárez, J., Riol-Pastor, E., Muñoz-Torre, R., Fernández-Sevilla, J.M., García-Encina, P.A., Bolado-Rodríguez, S. (2018). Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants, Biores. Technol. 257: 30–38.

Manuscript II. Martín-Juárez, J., Martínez-Páramo, S., García-Encina, P.A., Muñoz-Torre, R., Bolado-Rodríguez, S. Evaluation of pretreatments for production of fermentable monosaccharides from microalgae biomass grown in piggery wastewater. Submitted for publication in Algal Research.

Manuscript III. Martín-Juárez, J., Lorenzo-Hernando, A., Muñoz-Torre, R., Blanco-Lanza, S., Bolado-Rodríguez, S. (2016). Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment, Biores. Technol. 218: 265–271.

Manuscript IV. Martín-Juárez, J., Vega-Alegre, M., Riol-Pastor, E., Muñoz-Torre, R., Bolado-Rodríguez, S. (2019). Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater, Biores. Technol. 281: 239–249.

Manuscript V. Martín-Juárez, J., Collantes-Doyague, M., Vega-Alegre, M., Bolado-Rodríguez, S. Valorisation of microalgae biomass grown in piggery wastewater treatment by cellulases and xylanases production from *Trichoderma reesei* QM9414. In preparation.

Contribution to the papers included in the thesis

Manuscript I. During the execution of this work, I was in charge of the design, start-up and operation of the experimental set-up, with the collaboration of Miss Elena Riol Pastor. I was the responsible of results evaluation and the preparation of the manuscript under the supervision of Dr. Pedro García Encina, Dr. Raúl Muñoz Torre and Dra. Silvia Bolado Rodríguez.

Manuscript II. In this work, I was the responsible for the design, start-up and operation of the experimental set-up. Dra. Sonia Martínez was the responsible of the molecular biology analysis. I prepared the manuscript under the supervision of Dr. Pedro García Encina, Dr. Raúl Muñoz Torre and Dra. Silvia Bolado Rodríguez.

Manuscript III. In this research, I was in charge of the design, start-up and operation of the experimental set-up. Dr. Saul Blanco Lanza was the responsible of the microalgae specie analysis. I prepared the manuscript under the supervision of Miss Ana Lorenzo Hernando, Dr. Raúl Muñoz Torre and Dra. Silvia Bolado Rodríguez.

Manuscript IV. In this research, I was the responsible for the design, start-up and operation of the experimental set-up, with the collaboration of Miss Elena Riol Pastor. Dra. Marisol Vega Alegre was the responsible of the statistical methods and analysis, where I actively collaborated. I prepared the manuscript under the supervision of Dra. Marisol Vega Alegre, Dr. Raúl Muñoz Torre and Dra. Silvia Bolado Rodríguez.

Manuscript V. In this work, I was in charge of the design, start-up and operation of the experimental set-up, with the collaboration of Miss Maria Collantes Doyague. Dra. Marisol Vega Alegre was the responsible of the statistical methods and analysis, where I actively collaborated. I prepared the manuscript under the supervision of Dra. Marisol Vega Alegre and Dra. Silvia Bolado Rodríguez.

Introduction

1. Algal biomass for wastewater treatment

World population has exponentially increased in the last years from 3 (1959) to 7.6 (2019) billion people as shown the Fig. 1. This expansion coupled with the improvement of life standards leads to an exponential increase in raw materials consumption. Additionally, the available and arable lands are mainly destined by food cultivation to supply at the same trend as growth population. In this context, the search of new renewable resources as raw materials is crucial to solve this enormous requirement and to reduce the harmful impacts on the environment by the use of fossil sources (El-Dalatony et al., 2019).
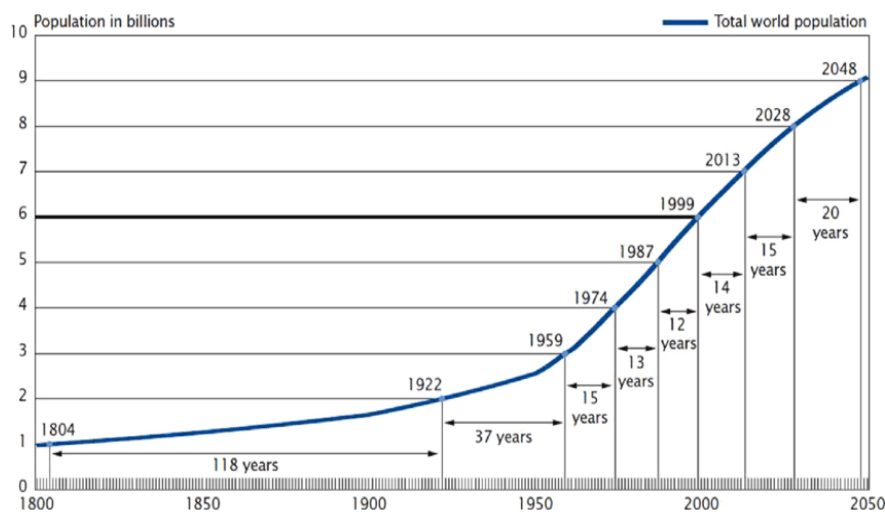
Figure 1. World Population 1800-2050 (Source: U.S. Census Bureau, 2017)

Simultaneously, the change in the way of life and consumerism have led to the continuous generation of a large amount of wastes from industries to houses. Thus, the use of these wastes as new renewable materials is the fundamental point and resolution for these two global problems, applying a bio-refinery concept (Lam et al., 2017). One of the main wastes with highlighted attention is the wastewaters due to the contamination of natural water bodies, environmental pollution and mal odours. The treatment of these wastewaters also achieves clean water useful, for example, for agriculture purposes, contributing to solve the problem of water demand (Yen et al., 2013).

Conventionally, common biological processes (such as active sludge) have been used for wastewaters treatment in the past years. However, the low recovery yields of nitrogen and phosphorous and the huge energy demand to reach the specific aeration for this treatment promote the search of new environmentally friendly techniques (Nghiem et al., 2017). Last years, the photosynthetic bioreactors (based on microalgae-bacteria

consortium) are being developed as a favourable platform for wastewater treatment. Algae and bacteria reduce the levels of organic matter and nutrients in wastewater via aerobic carbon oxidation and nutrient assimilation into biomass (García et al., 2017a). The symbiosis interaction between microalgae and the bacteria present on the biomass of photobioreactor plays a fundamental role in wastewater treatment. Microalgae generate oxygen that bacteria need to consume organic pollution faster than anaerobic bacteria. Nitrogen and phosphorus are removed from the wastewater by microalgae and used for their growth (Fuentes et al., 2016). By degrading organic matter, bacteria produce carbon dioxide - a nutrient necessary for the efficient growing of microalgae (Figure 2).
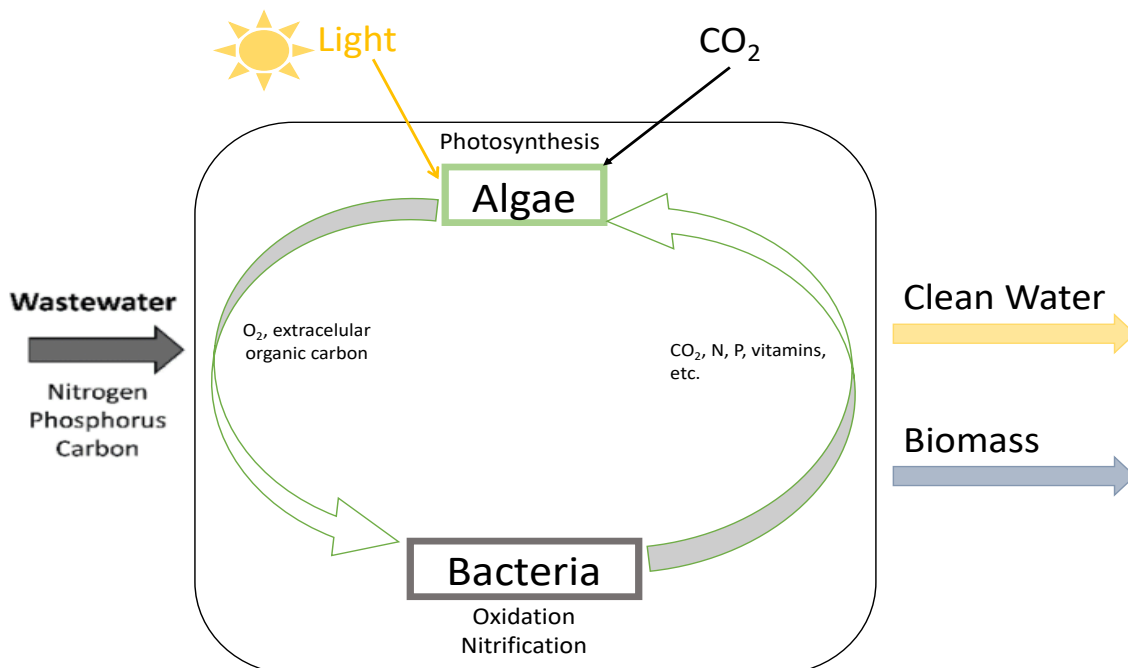
Figure 2: Symbiosis between microalgae and bacteria on wastewater treatment process

The processes of microalgae and bacteria provide important advantages supporting a low-cost photosynthetic oxygenation (by solar energy), a greater assimilation of nutrients compared with the conventional processes of active sludge (as a result of the combination of autotrophic and heterotrophic metabolisms) and an effective elimination of pathogens and emerging contaminants (due to the high pHs and oxygen concentrations induced by microalgal photosynthesis, and the action of UV radiation) (Salama et al., 2017).

Among all the wastewaters, domestic and agro-industrial activities generate large quantities of wastes containing high amounts of organic and nutrients (N and P), with a great potential to recovery (García et al., 2017b). Several works have demonstrated the efficiency of systems based on microalgae-bacteria consortium to treat the domestic and

agro-industrial wastewaters (Cheng et al., 2019). Special attention is paid to piggery wastewater. Global statistics demonstrated that Europe Union (EU) is currently one of the largest pig producers, with an average of $154 \cdot 10^6$ pig heads over the last 10 years (Statista, 2018). It generates $215 - 430 \cdot 10^6$ m^3/year (4-8 L/day/pig) of piggery wastewater. Their estimated average organic matter and nutrients load in 2018 were 8.923.000 t chemical oxygen demand (COD)/year, 890.000 t nitrogen (N)/year and 223.000 t phosphorous (P)/year (Statista, 2018). Table 1 shows several examples of these biotechnological processes with the main results. However, despite of the proved efficiency of the treatment for the elimination of organic matter and nutrients, studies about the valorisation and recovery of these nutrients from the obtained biomass are limited (Acién et al., 2017).

Table 1: Overview of studies of pollutants removal (%) from different wastewaters using microalgae-bacteria consortium

Microalgae species	Wastewater	Conditions	Pollutant removal			Reference
			COD (%)	Nitrogen (%)	Phosphorus (%)	
Chlorella and *Chlamydomonas*	Anaerobically digested swine manure	Photo-sequencing batch reactor (PSBR); using organic carbon source		90		Wang et al., (2015)
Scenedesmus sp.	Municipal wastewater	Bacteria: *Flavobacteria and Sphingobacteria*	92.3	95.7	98.1	Lee et al., (2016)
Chlorella. sorokiniana	Swine wastewaters	Nitrification efficiency: 75.7 % denitrification efficiency: 53.8%	62.3	82.7	58	Hernández et al., (2013)
Scenedesmus obliquus	Primary domestic	TSS-RE ≈ 82% Biomass productivity ≈ 2.5 g m^{-2} d^{-1}	74	67	96	Zamalloa et al., (2013)
Phormidium, Oocystis and *Microspora*	Primary domestic	Max. Biomass productivity ≈ 3.6 g m^{-2} d^{-1}	89	92	96	Posadas et al., (2014)
Microspora willeana	Dairy manure	Biomass composition: N (4.9– 7.1%); P (1.5– 2.1%) Max. Biomass productivity ≈ 5.5 g m^{-2} d^{-1}	95	62	93	Wilkie and Mulbry, (2002)
Unknown	Diluted centrates and primary domestic		80	70	85	Posadas et al., (2013)
Unkown	Diluted centrates	Low lipid content (2.9-11.2%)	99	100	82	Posadas et al., (2016)
Scenedesmus quadricauda	Dairy wastewater	No dilution Airlift photobioreactor (12L)	64	86	90	Daneshvar et al., (2018)
Acutodesmus dimorphus	Dairy wastewater	No dilution Flask (1L)	90	100	100	Chokshi et al., (2016)

Scenedesmus, Nitzschia and *Chlamydomonas*	Swine manure	Max. biomass concentration:HRAP A (CO_2 flue gas) ≈ 500 mg VSS/L HRAP B (no CO_2 flue gas) ≈ 400 mg VSS/L	56	98	15	De Godos et al., (2010)
Chlorella sorokiniana	Swine manure	Biomass productivity ≈ 21-28 g m^{-2} d^{-1} Higher microalgae biodiversity in summer than in winter	76	88	10	De Godos et al., (2009)
Chlorella vulgaris	Piggery wastewater	Open photobioreactors outdoors conditions TSS concentration ≈ 680 mg/L	91	72	81	García et al., (2017a)
Chlamydomonas sp., Chlorella kessieri, Chlorella vulgaris and *Scenedesmus acutus,*	Piggery wastewater	3L open photobioreactors Dilution of wastewater: 5% TSS concentration ≈ 275 mg/L	84	87	91	García et al., (2019)

2. Algal biomass composition

The macromolecular composition of microalgae is extremely variable depending on the specie, environmental conditions for their growth and operational conditions during their cultivation. Thus, the percentages of their principal components can vary in a wide range: carbohydrates (4%– 64%), proteins (6%– 61%), and lipids (2%– 40%). Microalgae can contain also other value-added components as phycobiliproteins, carotenoids, vitamins, toxins or sterols in small quantities (Bastiaens et al., 2017). Table 2 displays the range of principal components composition of pure species of microalgae.

Table 2. Chemical composition (carbohydrates, proteins and lipids in percentage) of several species of pure microalgae (Lam and Lee, 2015; Sudhakar et al., 2019; Kadir et al., 2018)

Microalgae species	Carbohydrates	Proteins	Lipids
Chlamydomonas rheinhardii	17	48	35
Chlorella pyrenoidosa	26	57	17
Chlorella sp.	19	55	26
Chlorella vulgaris	12-17	51-58	25-37
Chlorococcum sp.	33	50	19
Dunaliella bioculata	4	49	47
Dunaliella salina	32	57	11
Euglena gracilis	14-18	39-61	14-20
Isochrysis galbana	8-14	45-85	7-40
Isochrysis sp.	5-16	40-80	7-33
Mychonaster afer	28	50	25
Nannochloropsis oculata	8	60	23-30
Porphyridium cruentum	40	28-39	18-30
Prymnesium parvum	25-33	20-45	20-60
Scenedesmus abundans	41	45	18
Scenedesmus dimorphus	21-52	8-18	30-65
Scenedesmus obliquus	15-28	50-56	11-55
Spirogya sp.	33-64	5-18	18-62
Spirulina platensis	8-14	46-63	4-17
Spirulina maxima	13-16	60-71	4-15
Synechoccus sp.	15	63	20
Tetraselmis maculate	15	52	30
Tetraselmis sp.	24	60	15
Tetraselmis suecica	15-50	23-76	8-23

2.1 Carbohydrate content of algal biomass

The carbohydrate content of microalgae-bacteria biomass can be found in the outer cell wall (e.g., pectin, agar, alginate), the inner cell wall (e.g., cellulose, and other materials such as hemicellulose and glycoprotein) and inside the cell as storage products (e.g., starch in microalgae and glycogen in cyanobacteria) (Phwan et al., 2018).

During photosynthesis, microalgae produce the monosaccharide glucose. This glucose is used as an energy and carbon source to produce proteins, lipids and other carbohydrates. When irradiance is too high or when the inorganic nutrients supply is limited (e.g.

nitrogen stress), the rate of glucose production during photosynthesis can exceed the rate of glucose consumption by the cell. This excess cannot be stored due to the disturbance of the cell's osmotic balance (de Farias Silva and Bertucco, 2016). Therefore, the overproduced glucose is converted either into polysaccharides or into lipids, which will act as carbon and energy storage for future use. Because glucose conversion into polysaccharides is much faster than into lipids, microalgae will often first accumulate carbohydrates and afterwards lipids. Microalgae-bacteria biomasses from the wastewater treatment support a high stress and, hence, the production of lipids and the storage as starch are limited, being carbohydrates and proteins the main fractions (Chen et al., 2013).

Carbohydrates can also be found in the microalgae cell wall, containing cellulose (β-(1-4) glucan). Multiple cellulose chains are linked by hydrogen bonds to form a complex and crystalline structure that is resistant to enzymatic degradation (Popper and Tuohy, 2010). Most microalgae also contain hemicellulose, a polysaccharide composed of several types of monosaccharides connected by β-(1-4) and occasionally β-(1-3) glycosidic bonds (Cheng et al., 2015).

2.2. Operational factors influencing on biomass composition

The composition of biomass can vary widely depending on environmental and operational factors such as nutrients availability, light intensity, temperature and pH. It is possible to follow strategies in order to facilitate the accumulation of one specific fraction. In fact, this approach is attractive from the point of view of the valorisation (Salama et al., 2018).
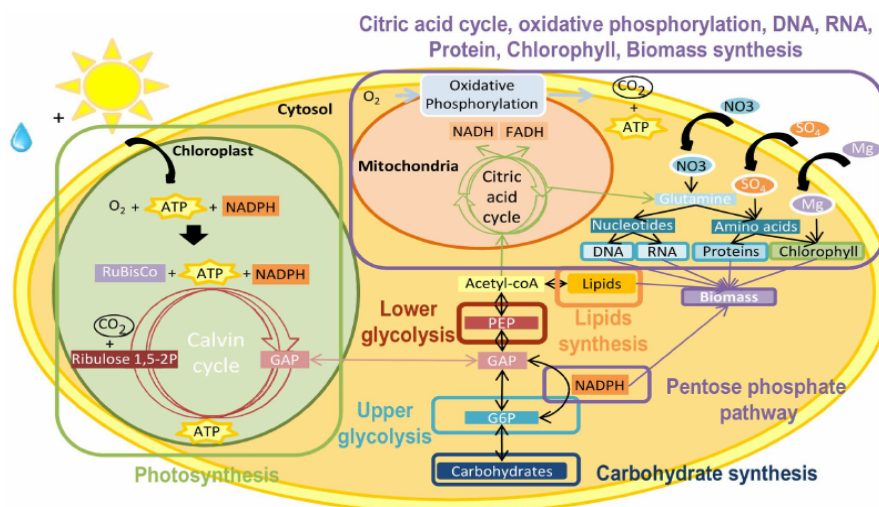
Figure 3: Outline of components pathway in microalgae biomass and their valorisation (Baroukh et al., 2013)

Chapter 1: Introduction

The limitation of a concrete nutrient such as nitrogen, phosphorus, potassium or sulphur affects the microalgae cells growth and, hence, could have a significant impact on the biochemical composition (Kamalanathan et al., 2015). Microalgae need the nitrogen to synthetise various essential biomolecules (proteins, DNA or pigments). Pathway of photosynthetically fixed carbon is modified by the nitrogen starvation provoking a change of the metabolism from the protein synthesis to the accumulation of lipids or carbohydrates. This tendency is totally different depending on the type of microalgae, while oleaginous eukaryotic microalgae tend to store energy in form of lipids, the rest of algae and cyanobacteria tend to produce carbohydrates (González-Fernández and Ballesteros, 2012). For example, Brányiková et al., (2011) accounted an accumulation of carbohydrates up to 41% in *Chlorella vulgaris* under nitrogen limitation, Ji et al., (2011) around 35% for *Tetraselmis* and Sassano et al., (2010) about 65% for *Spirulina platensis*.

Phosphorus is also essential for metabolic process and its limitation entails an accumulation of carbohydrates (Markou, 2012). It is carried out due to the non-consumption of phosphorus in the carbohydrates synthesis and begins when the intracellular phosphorus drops below a threshold limitation level (Cade-Menun and Paytan, 2010). The phosphorus limitation increased the content of carbohydrates from 10% to 55% in *Chlorella* sp (Brányiková et al., 2011) and from 20% to 63% in *Spirulina platensis* (Markou, 2012). Limitation of minority nutrients as sulphur, potassium or manganese also implies an accumulation of carbohydrates. For example, Melis, (2007) reported an increment of 10-fold in the carbohydrate content under sulphur limitation of *Chlamydomonas reindhardtii*. Sulphur limitation could be the most appropriate strategy for the production of carbohydrate-rich microalgae because cells contain around 60% of carbohydrates for longer time compared to other nutrient starvation methods (nitrogen and phosphorus) before the cell-death phase (Torzillo et al., 2014).

Microalgae biomass use the light to fix carbon through photosynthesis, affecting the light quantity and quality on the biomass growth and composition (Khajepour et al., 2015). An increase of the light involves an increment on the biomass growth (normally an accumulation of carbohydrates) until a maximum level of light (typical saturation intensity is 200 - 400 $\mu mol_{photons}$ m^{-2} s^{-1}) whereas a further increase may inhibit photosynthesis (Lu and Vonshak, 1999). Under high salinity, microalgae typically respond by accumulating intracellular carbohydrates of low molecular weight to adjust

the intracellular pressure and protect themselves from osmotic lysis (Rao et al., 2007)..
The manipulation of salinity along with nutrient limitation has been proposed as an
effective strategy for carbohydrate accumulation (Yao et al., 2013).

3. Cell disruption technologies

The type of cell wall of algae biomass has significant influence on the kind of
pretreatment for disrupt the wall. For example, microalgae biomass from the wastewater
treatment, has a rigid and resistant cell wall due to their capability to support severe and
stressful conditions. So, the application of severe pretreatments is required to disrupt the
cell wall facilitating the access to the components inside the biomass (Günerken et al.,
2015). The effect of pretreatments has been only studied on pure microalgae and in some
particular cases on the biogas production from microalgae-bacteria biomass. For this
reason, it is necessary to study the effect of the applied pretreatments on bacteria present
on the biomass for different alternatives.

3.1. Algal biomass cell wall

As previously explained above, the cell wall of the microalgae biomass is hugely variable
depending on the species of microalgae, the cultivation factors, and, hence, on the
chemical composition (Yoo et al., 2014). Different cell wall structures could be
distinguished from tiny membranes to multi-layered complex structures (Fig. 4):
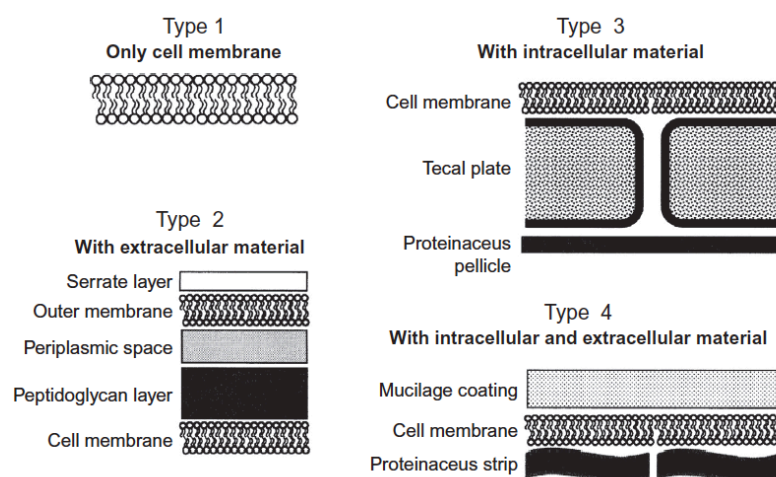
Figure 4: Types of algae biomass cell wall (D'Hondt et al., 2017)

Type 1 consists of a simple cell membrane with a bilayer of lipid and peripheral proteins.
Algae in short-lived stages (when the algae are growing as gametes), chrysophytes,

raphidophytes, green algae *Dunaliella* or haptophytes *Isochrysis* present this kind of cell wall. A glycolipids and glycoproteins layer occasionally envelopes the outer surface.

Type 2 includes extracellular material on the external layer of the wall (mucilage and sheaths, scales, frustules, lorica, skeleton), and it is the typical cell wall of cyanobacteria (*Aphanizomenon, Arthrospira*) and many groups of common algae (*Haematococcus, Scenedesmus/Desmodesmus, Chlorella, Tetraselmis, Porphyridium, Nannochloropsis...*). This cell wall is rigid, homogenous, and multi-layered. The peptidoglycan layer overlaps the inner cell membrane and strongly connected with the outer membrane of the wall. Mucilages and sheaths protect the cell and support the movement while scales envelope the surface with organic and inorganic scattered structures. Frustules are only ornaments made of amorphous hydrated silica. Lorica is presented as a specific structure from cellulose or chitin. Finally, skeletons are situated outside the plasma membrane with three-dimensional structure.

Most of the microalgae used for commercial and biotechnological applications have a cell wall type 2. Figure 5 shows a schematic overview of the cell wall of the main microalgae species.
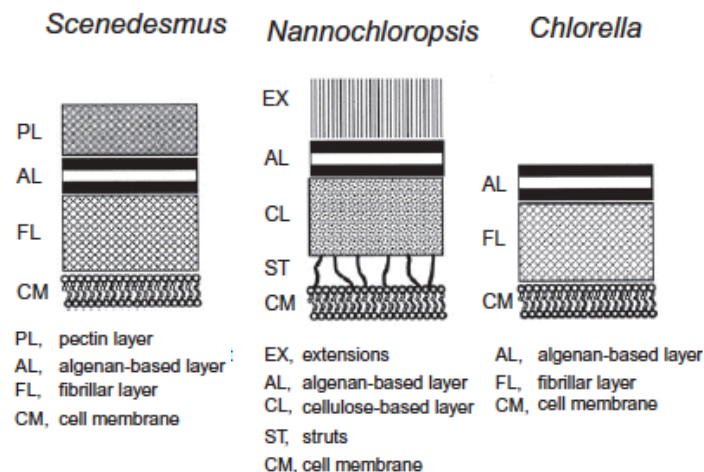
Figure 5: Cell wall of the main and renowned microalgae species (modified D'Hondt et al., 2017)

Scenedesmus is composed of three layers: an inner cellulosic layer delimiting individual cells, a thin middle algaenan-based layer and an outer pectic layer joining the cells into coenobium (Voigt et al., 2014). *Nannochloropsis* consists of four multi-layers where extensions of unknown composition protrude from the outer surface layer. Algaenan

layers comprise a thin trilaminar sheath in the cell periphery. The inner layer is primarily composed of cellulose and glucose; and amino acids represent an integral cell wall constituent. This layer is connected to the plasma membrane by the struts (Alhattab et al., 2018). Growth conditions have a relevant impact on the cell wall of the strains of *Chlorella*. Mostly, the inner cell wall layer is composed a rigid microfibrillar structure fixed into a constant matrix of cellulose and chitin-like glycan. Moreover, the outer cell wall of different species may include a trilaminar algaenan or form a thin homogeneous monolayer (Abdul Razack et al., 2016).

Type 3 is commonly presented in dinoflagellates with additional intracellular material in vesicles known as amphiesma. The amphiesma consists of a continuous plasma membrane, outer plate membrane and a single membrane bounded thecal vesicle. There are a number of cellulosic plates inside this thecal vesicle subtended by a pellicular layer.

Type 4 includes cell membranes with intracellular and extracellular material, and they are characteristic for euglenophytes and cryptophytes. The inner layer contains proteins and may consist of fibril material, a single sheet or multiple plates. The outer component could have plates, heptagonal scales, mucilage, or combinations.

3.2. Physical-mechanical pretreatments

Physical-mechanical pretreatments apply pressure, temperature or shear forces to disrupt the cell wall, facilitating the further release of cellular components. They are effective breakthrough methods, entailing low degradation compounds but sometimes with high energy consumption. This type of pretreatments are non-specific, releasing carbohydrates, proteins and lipids simultaneously, and decreasing the economic feasibility of the sequential valorisation process and the quality of obtained products (Hu et al., 2019). The main physical pretreatments, commonly used with other biological materials are: bead mill, ultrasounds, microwave and thermal pretreatment (<120ºC). These methods have been essayed for lipid extraction or for improvement of the biogas production from microalgae (Lara and Graciano, 2019).

During the bead milling, the recalcitrant cell walls of microalgae biomass are disrupted by the collision or friction and shear stress provoked when beads are in movement. The

principal parameters with a relevant effect in this method are: bead type (loading, size and material), feed rate, biomass properties (species and humidity) and time (Chandra et al., 2019).

Ultrasounds pretreatment consists on the application of low and high-pressure cycles in the biomass medium provoking cavitation through the waves. This cavitation implies microalgae cell wall disruption and organic matter solubilisation. The power and exposure time, defining the specific energy, temperature and number of cycles are the main factors on the ultrasound method. Different range of frequencies have been tested from 20kHz to 1 MHz; even the efficiency of the process also depends on microalgae specie and its concentration (Kim et al., 2016). Ultrasonic method is significantly more intense at low frequency (<100kHz) than at high frequency (>100kHz). A low temperature is favourable for an effective sonolysis, to continuously cool the medium and prevent the temperature from increasing due to heat loss. However, the energy consumption is increased due to the cooling and the high power of the ultrasound. Moreover, the scaling-up is difficult because the cavitation occurs in regions near the ultrasonic probes (Onumaegbu et al., 2018).

Microwave pretreatment provokes similar effects as ultrasounds, exciting the polar water molecules of the suspension by the short electromagnetic waves causing local heating and pressure increase. It leads the damage of the cell wall and the release of intracellular compounds. In this case, the temperature increment is more homogenous, and the process is higher effectiveness, robustness and easy scaled-up due to the simplicity. The parameters with more impact during this method are the same as reported by ultrasound pretreatment (Günerken et al., 2015) (Lee et al., 2010).

The thermal pretreatments with mild temperatures lower than 120ºC are commonly considered in this section. The method harnesses the temperature to break the cell wall thanks to the activity of thermophilic and hyper-thermophilic bacteria as biological pre-treatment. The main operational factors for this pretreatment are temperature, time, pressure and type of microalgae. In addition, Table 3 shows some sugar release results applying these pretreatments to microalgae biomass.

Table 3. Examples of sugar release from microalgae biomass by applying physico-mechanical pretreatments

Microalgae species/ (%carbohydrates)	Method	Conditions	Sugar release yield (g/g algae)	References
Scenedesmus obliquus (31.8%)	Bead mill	0.4–0.6 mm glass beads	0.03	Miranda et al., (2012)
Neochloris oleoabundans (17%)	Bead mill	0.4–0.6 mm zirconia beads, 2000 rpm, 45 min	0.12	Günerken et al., (2016)
Scenedesmus obliquus	Ultrasounds	2200W, 15 min	0.450	Choi et al., (2011)
Scenedesmus obliquus (37%)	Ultrasounds	2200 W, 15 min	0.120	Jeon et al., (2013)
Chlorella sp.	Ultrasounds	800 W, 80 min, 1.52 L/min	0.370 (glucose)	Zhao et al., (2013)
Scenedesmus obliquus (31.8%)	Ultrasounds	200 W, 30 s, 5 cycles	0.020	Miranda et al., (2012)
Nannochloropsis spp. (30%)	Ultrasounds	200W, 600s, pH 8.5	0.030	Parniakov et al., (2015)
Chlorella sorokiniana	Microwave	150W, 40 s	0.021	Hernández et al., (2015)
Scenedesmus obliquus (31.8%)	Thermal	120ºC, 30 min	0.04	Miranda et al., (2012)

3.3. Chemical pretreatments

The application of chemicals has been widely studied since the start of the use of microalgae biomass due to the previous experience of breakthrough of biomass structure by applying these methods to other residues as lignocellulosic materials. The reported results working with microalgae varied widely, depending on the microalgae specie and composition (Rizwan et al., 2018).

The principal parameters on these methods are the type of chemical and its concentration, temperature, time, biomass concentration and microalgae species. Moreover, each chemical reagent acts discordantly on the different fractions of the microalgae biomass, such as acids tend to liberate more carbohydrates whereas basics usually release proteins and lipids (Velazquez-Lucio et al., 2018). These pretreatments are generally fast and appreciably inexpensive due to non-requirement of electricity to break. The increase of reagent concentration rinses the component release yields but also the degradation compounds generation, the equipment corrosion and the operational costs. Moreover, a pH readjustment prior to the subsequent valorisation steps can be required, especially in biological processes. Degradation compounds generated by these pretreatments can also inhibit further biological steps, and an intermediate detoxification step can be required. The temperature and time are other relevant factors, being usually applied inversely:

elevated temperatures (>100°C) with short times (<15min), or low temperatures (<100°C) with longer times (30-90 min) (Onumaegbu et al., 2018).

Considering acid methods, H_2SO_4 is the most applied acid but HCl, H_3PO_4 or HNO_3 are also used. However, NaOH is the most studied chemical for alkali pretreatment. Table 4 summarises some results of sugar release by applying acid and alkali pretreatments to microalgae biomass in the last years.

Table 4. Sugar release by chemical pretreatments of microalgae biomass

Microalgae species	Conditions	Total carbohydrates (%)	Sugar release yield (g/g algae)	References
Chlorella vulgaris	1% (v/v) H_2SO_4, 121°C, 120 min	50.4	0.472	Ho et al., (2013)
Scenedesmus bijugatus (Post-lipid extraction)	2% (v/v) H_2SO_4, 130°C, 45 min	26.0	0.218	Ashokkumar et al., (2015)
Chlamydomonas reinhardtii	3% (v/v) H_2SO_4, 110°C, 30 min	60.0	0.580	Nguyen et al., (2009)
Scenedesmus obliquus	2 N H_2SO_4, 120°C, 30 min	31.8	0.286	Miranda et al., (2012)
Spirulina platensis	0.5 N HNO_3,100°C, 180 min	58.0	0.522	Markou et al., (2013)
Scenedesmus obliquus	3N NaOH, 120°C, 30 min	31.8	0.025	Miranda et al., (2012)

Other chemical method as alkaline-peroxide has been applied in the recent years. This pretreatment combined the use of H_2O_2 and NaOH as chemicals, providing high yield of release working at moderate temperatures for lignocellulosic biomass (de Araújo Padilha et al., 2017). Besides, Li et al., (2016) optimised the hydrogen peroxide treatment on seaweed *Ulva prolifera* biomass (49.1% of carbohydrates), achieving promising results with a maximum sugar released yield of 0.42 g/g algae at 0.2% H_2O_2, 50ºC, pH 4.0 and 12h.

Other innovative methods as ozonolysis, ionic liquids and supercritical fluids are emerging to soften the harsh conditions that are commonly required by acid/alkali. Ozonolysis consists on the oxidative reaction between ozone and the cell wall protective components. The principal advantages are the absence of liquid phase, mild conditions, and in-site ozone production. However, some hurdles appear such as the high toxicity, flammability, corrosivity, reactivity, and hence special materials for the equipment are required, increasing its costs (Travaini et al., 2016). The main process parameters are reactor design, moisture, ozone concentration, ozone/air flow rate and time. This method

has been widely applied for lignocellulosic materials but also has been used for macroalgae pretreatment. Schultz-Jensen et al., (2013) reported low formation of degradation compounds from ozonated *Chaetomorpha linum*, with complete glucan and arabinan recovery and 75% xylan recovery in the solid fraction. Cardeña et al., (2017) applied the ozone pretreatment to improve the anaerobic digestion of mixed microalgae biomass (*Scenedesmus*, *Keratococcus* and *Oscillatoria*). They achieved 432.7 mL CH_4/g VS using 382 mg O_3/ g VS; respect to 260 mL CH_4/g VS from untreated biomass.

The ionic liquids are promising solvents for disrupting cells and extracting components from algal biomass, because of their interesting properties as low volatility, high dissolving power, and easy and complete recovery from water. However, its application to large-scale processes is still a challenge due to their elevated cost. This method has been exclusively studied for lipid extraction and ethanol production process from microalgae (Kim et al., 2012). For instance, Zhou et al., (2012) obtained 0.65 g_{sugar} /g_{algae} dw applying [Emim]Cl and 7 % w/w HCl at 105°C for 3 h to *Chlorella* sp. (73.58% of initial carbohydrates).

3.4. Combined pretreatments

Some pretreatments combine different physical and/or chemical effect, being difficult of classifying in some of the above explained methods. Thermal pretreatment at temperatures higher than 120ºC is the most studied combined methods (Phwan et al., 2018).

Thermal pretreatments at temperatures higher than 120°C are commonly associated to this combination method due to the acid behaviour of water in these operation conditions. Their classification (hydrothermal or steam explosion) depends on how the pressure is relieved. Hydrothermal processes consist on a change of the water physicochemical characteristics caused by an increase of temperature and pressure, and a smooth relief of pressure when the reaction finishes (Carrere et al., 2016). For example, Mendez et al., (2014) studied the effect of this pretreatment (140, 160, and 180°C; 3, 6, and 10bars; 10 and 20min) on the solubilisation of different fractions of *Chlorella vulgaris* (36.6% of carbohydrates), achieving 69% of carbohydrates solubilisations at 180°C, 10 bar and 20 min. The steam explosion differs from hydrothermal pretreatment in the sudden release

of the pressure and the quick shift of the biomass to a flash vessel with the resulting cooling effect. It involves great cell wall disruption and high biomass components solubilisation (Carrere et al., 2016). Most of the studies of this pretreatement have been addressed to enhance biogas production in batch test experiments (BMP) Nonetheless, Lorente et al., (2015) applied steam explosion to *Nannochloropsis gaditana* (initial carbohydrates of 13.5%) at 120 and 150°C for 5 minutes. Both temperatures led to a 0.06 g_{sugar} /g_{algae} dw (44.4% of sugar solubilisation yield).

To sum up, Table 5 reviews the advantages and drawbacks of all the mentioned pretreatments.

Table 5. Advantages and disadvantages of pretreatments applied for the cell wall disruption of the microalgae biomass (Phong et al., 2018)

Pretreatment	Advantages	Disadvantages
Bead milling	Simple equipment Rapid process High disruption efficiency Easy scale-up Can be applied on algal slurry	High energy consumption Requires extensive cooling for thermolabile compounds Formation of very fine cell debris
Microwave	Rapid process Effective for robust species Easy to scale up Low operating costs Not require dewatering of algal biomass	High energy consumption and maintenance costs High temperature Recovery of thermolabile compounds may require cooling Lipid degradation and protein aggregation, denaturation Formation of free radicals
Ultrasonication	Simple Short extraction time High reproducibility Operated continuously Environmentally friendly	Moderate energetic costs Temperature rise Hinders product release Production of reactive hydroxyl radicals Not applicable to large-scale Energy effective in small volume
Mild temperature	Low energy consumption Simple Can be applied on algal slurry Cost effectiveness	Time-consuming Low effectiveness for algae with complex cell wall Algae species sensitive
Chemicals	Fast High reaction rate	High temperature High pressure Degradation of some compounds Corrosion of equipment Difficult separation from algae
Ozonolysis	Low inhibitory compounds No chemical requirements Liquid phase absence Mild conditions In-site and direct ozone production	High operational costs High toxicity High flammability High corrosivity Special materials for the equipment

Ionic liquids	Short reaction times	High cost
	Versatile	High toxicity
	Less hazardous	
	Excellent recyclability	
Steam explosion	Easy to scale up	High energy consumption
	Can be applied on algal slurry	Species-specific effectiveness
	Short time	Degradation of some compounds

4. Sugar release by enzymatic hydrolysis

The step of enzymatic hydrolysis can be used as a biological pretreatment or as a further process after the physic-chemical pretreatments. Enzymatic hydrolysis has numerous advantages over chemical hydrolysis: mild operational conditions (with subsequent lower energy requirements), higher selectivity and biological specificity (leading to higher conversion yields and lower by-products formation), and easier scale-up. Nevertheless, it has also remarkable weaknesses, such as enzymes cost and problematic recovery, which could make the process economically unfeasible. The main operational factors are: type and concentration of microalgae; temperature; pH; time and enzymes type and concentration and, hence, an optimisation of the diverse parameters must be done for achieving maximum yields and reducing costs. The application of a previous pretreatment step permits to reduce the quantity of enzymes and, hence, the cost of the global process (Brasil et al., 2017).

The election of enzyme type is influenced by the composition of the desired macromolecular fraction. Carbohydrates can be in form of cellulose, hemicellulose, or starch depending on the microalgae biomass and its cultivation conditions, requiring the use of cellulases, hemicellulases, and amylases enzymes (Carrillo-Reyes et al., 2016).

Cellulases encompass a group of enzymes that hydrolyse the crystalline structure of cellulose into small oligosaccharides and subsequently to glucose. They consist of at least three major enzymatic components: 1) endoglucanases (EC 3.2.1.4 - endo-β-1,4-glucanases), which randomly hydrolyse glycosides bonds in amorphous regions of the cellulose, leading to a diminution in chain length and generation of reducing ends; 2) exoglucanases or cellobiohydrolases (EC 3.2.1.74 - exo-β-1,4-glucanases), which act on both reducing and non-reducing ends, releasing glucose or cellobiose; and 3) β-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose or oligosaccharides to glucose (Lam and Lee, 2015).

Xylanases are hydrolytic enzymes which cleave the β-1,4 backbone of the cell wall of polysaccharide xylan. The structure of xylan is composed of a linear polymer of xylopyranosyl groups substituting various carbon positions with different sugars and/or acidic compounds. Thus, sequence processes have to be carried out as explained above

for cellulase: 1) endo-1,4-β D-xylanase (E.C. 3.2.1.8), randomly cleaves the xylan backbone; 2) β-D-xylosidases (E.C. 3.2.1.37) cleaves xylose monomers; and 3) α-L-arabinofuranosidases (E.C. 3.2.1.55), α-D glucuronidases (E.C. 3.2.1.139) and acetylxylan esterases (E.C. 3.1.1.72), which eliminate acetyl and phenolic side subdivisions and act synergistically on the complex polymer (Walia et al., 2017).

For starch, their α-(1-4) D-glucosidic linkages are hydrolysed by α-amylase (EC 3.2.1.1), in a process known as liquefaction. Maltodextrin is the obtained product, which is composed of oligosaccharides with three or more α-(1-4)-linked D-glucose units. After this, the saccharification takes place when maltodextrin is converted into simple reducing sugars by amyloglucosidase (β-amylase (EC 3.2.1.2) and γ-amylase (EC 3.2.1.3)). These enzymes act on both α- (1-4) and α-(1-6) D-glucosidic linkages (van der Maarel et al., 2002).

Besides, there are other specific enzymes for the hydrolysis of the rest of fractions (proteins and lipids). Proteases constitute a wide group of enzymes that catalyse peptide-bond cleavage in proteins and peptides. Lipases (E.C. 3.1.1.3) are enzymes that naturally hydrolyse triglyceride into fatty acids and glycerol (Singh et al., 2016).

To end up, Table 6 reviews the main results of enzymatic hydrolysis from different microalgae biomass.

Table 6: Summary of enzymatic hydrolysis results on different microalgae biomass

Microalgae	Enzyme	Conditions	Product	Reference
Chlamydomonas reinhardtii	Alcalase 2.5 L	0.2 mL/g dw at pH 8 for 2 h, 50 °C	289 mL CH_4/g COD; 10% increase the methane production	Mahdy et al., (2014)
Chlorella vulgaris	Alcalase 2.5 L	0.2 mL/g dw at pH 8 for 2 h, 50 °C	287 mL CH_4/g COD; 51% increase the methane production	Mahdy et al., (2014)
Chlorella pyrenoidosa	Cellulase	2% enzyme/ g dw, 24 h, pH 4.6, 50 °C	62% of glucose yield 75% of lipid yield	Fu et al., (2010)
Chlorella vulgaris	Alcalase 2.5 L	0.585 U/g dw, 130 rpm, 3 h, 50 °C	49% of protein yield; 256 mL $CH_{4/g}$ COD, increasing methane production 1.59-fold	Mahdy et al., (2014b)
Chlorella vulgaris	Cellulase	5 mg/L, 10 h, pH 4.8, 55 °C	8.1-fold lipid yield more than untreated cell	Zheng et al., (2011)
Chlorella vulgaris	Lysozyme	5 mg/L, 10 h, 55 °C	7.6-fold lipid yield more than untreated cell	Zheng et al., (2011)
Chlorella vulgaris	Pectinase (Pectinex SP-L)	240 U/mg protein, pH 4.8, 200 rpm, 72h	79% of glucose yield	Kim et al., (2014)
Chloroccum sp.	Cellulase from *T. reesei* ATCC 26921	0.02g enzyme/g algae, 40 °C, pH 4.8, 72 h. 100 mL	64% of glucose yield	Harun and Danquah, (2011)
Nannochloropsis oculata	Cellulase	5 mg/L, 37 °C, pH 5.5, 12 h.	33% of lipids yield in nitrogen rich cultures; 52% of lipids yield under nitrogen starvation condition	Surendhiran and Vijay, (2014)
Chlorella vulgaris	Enzyme mixture	Endoglucanase (0.65 U/mL), β-glucosidase (1.50 U/mL) and amylase (0.09 U/mL), 45ºC, 48h	97% of glucose yield	Ho et al., (2013)
Chlamydomonas reinhardtii	Amylase	0.005% v/w α-amylase, 0.2% v/w amyloglucosidase, 90ºC, 30 min	94% of glucose yield	Choi et al., (2010)
Chlorella vulgaris	Alcalase 2.5 L Viscozyme	3.2% w/v, 50ºC, 3h 5.5% w/v, 50ºC, 3h	54.7% of protein yield 28.4% of carbohydrate yield	Mahdy et al., (2016)

Despite the great advantages on enzymatic hydrolysis, in order to achieve an economically viable process, the efficiency of enzymes production has to be improved, (Farinas, 2018). Currently, most of cellulase enzymes are produced by solid state fermentation (SSF) to avoid environmental pollution or wastes and simulate the natural habitats of fungi. Moreover, the SSF is cheaper than the fermentation in liquid phase because of its low investment and operational cost, simple equipment and high productivity per reactor volume (Hansen et al., 2015).

Among several factors as moisture content, temperature, pH, time, oxygen levels, concentrations of nutrients and particle size of substrate affecting the enzyme production, the substrate selection results of special relevance. The ideal substrate should not only provide the nutrients to the fungi growth but also should serve as anchorage for the cells (Guoweia et al., 2011). Besides, the production of enzymes using as substrate the same material that will be subsequently hydrolysed results in more complex and specific enzymes (Ray and Behera, 2017). Commonly, agricultural wastes – wheat bran, banana peel, rice straw, wheat straw, cassava peel, peanut shell, sorghum stover, soybean meal - have been studied as substrates for enzyme production with a variety of fungi and bacteria (Ahmed Simair et al., (2018), Xu et al., (2018), Hu et al., (2018), Khanahmadi et al., (2018), Leite da Silva et al., (2018)).

Among the numerous applications of algae, the use as a substrate for the production of high-added products as enzymes is an emerging alternative. In this field, the green seaweed *Ulva fasciata* was also used as substrate in the solid-state fermentation of *C. sphaerospermum* for cellulase enzyme production (Trivedi et al., 2015), studying the effect of moisture content (40-100%), temperature (25-40ºC), pH (2-6) and incubation time (2-6days). The optimum was achieved at 60% of moisture content, 25ºC, pH 4 and 4 days, reporting 10.2 U/g for CMCase and 9.6 U/g for FPase, but they added saline solutions to supplement the substrate. In order to reduce the costs of enzymes production, the research should be addressed to find new cheap and efficient substrates, no requiring supplementation of nutrients (Ray and Behera, 2017).

5. Bioproducts from carbohydrate fraction

Once the microalgae biomass has been treated to break the cell wall or/and hydrolysed to obtain simple components, different process can be applied to achieve commercial products Most of the research on carbohydrate fraction valorisation addresses to fermentation processes for biogas, bioalcohols, or even polyesters production.

5.1. Biogas production

Anaerobic digestion consists on the conversion of the whole microalgae biomass into biogas through biochemical reactions, but the carbohydrate fraction is the most easily biodegradable. Biogas is commonly composed of methane from 50 to 70%; carbon dioxide, and traces of other gases as N_2, H_2S, etc. Anaerobic digestion process leads four distinct stages (Fig. 6): hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Gonzalez-Fernandez et al., 2015).
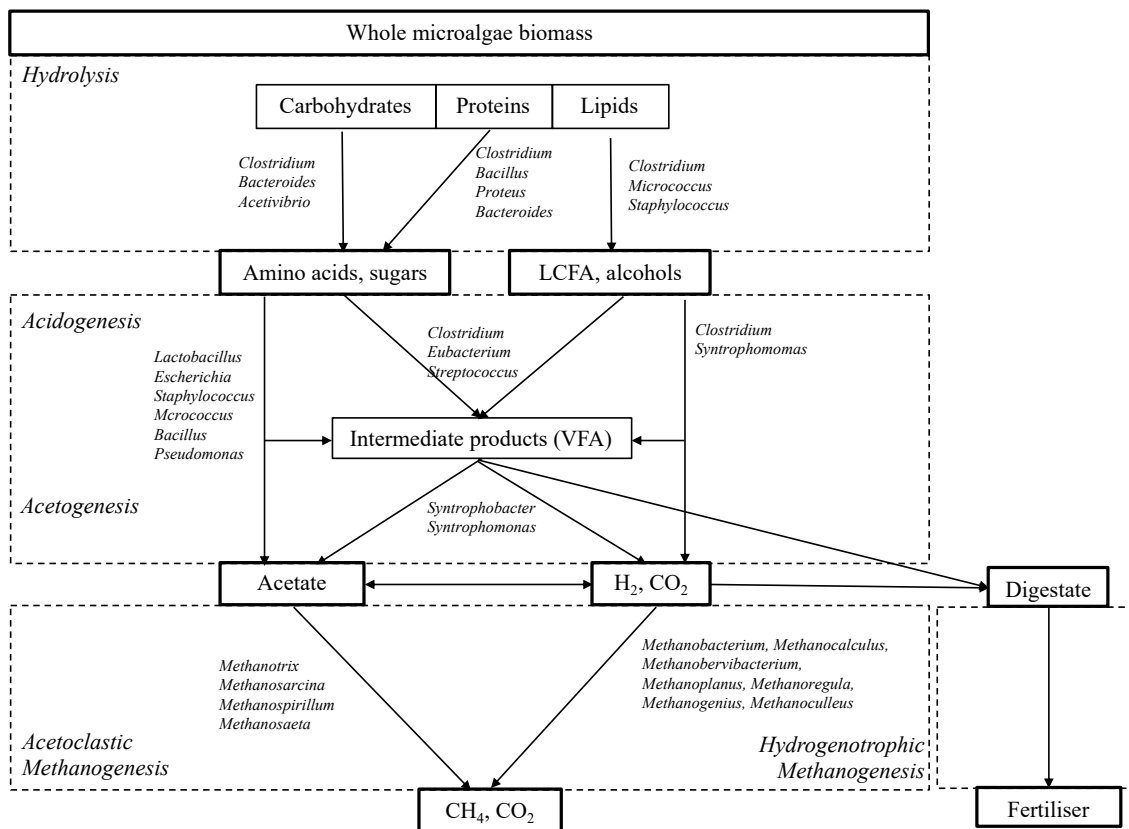
Figure 6: Overview of the four stages on the anaerobic digestion for the biogas production from complete biomass (modified Cavinato et al., 2017)

During the first step (hydrolysis), insoluble and high molecular weight organic compounds are degraded into soluble organic substances. In the next step (acidogenesis), the disruption continues and acidogenic bacteria produces volatile fatty acids. The third

stage is the acetogenesis, where the by-products of acidogenesis are further degraded by acetogens to acetic acid, CO_2, and H_2. Finally, the methanogenesis produces methane by two pathways: acetoclastic methanogens convert acetate into methane and carbon dioxide while hydrogenotrophic methanogens use hydrogen as the electron donor and carbon dioxide as the electron acceptor to produce methane (Habouzit et al., 2014).

Despite the easy and straightforward way of biogas production, there are some constrains due to variable characteristics of microalgal biomass with determining factors such as C:N ratio, chemical composition, and the kind of cell wall. Common ratio C:N for microalgae biomass is below 10, due to the high content of proteins of this biomass. Besides, pretreatments promote the disrupt the rigid cell wall facilitating the first step of hydrolysis (Murphy et al., 2015).

Two distinct range of temperatures are used for anaerobic digestion: mesophilic (30–42°C) and thermophilic (43–55°C). Mesophilic conditions are the most prevalent selection for anaerobic digestion of any kind of biomass due to the stability and economy of the process. Nevertheless, thermophilic conditions provide faster reaction times and higher elimination of volatile solids. Furthermore, the elimination of pathogens is fulfilled at temperature of 50ºC, which is endorsed for biomass grown in wastewater treatment (Kim et al., 2013).

Table 7 summarises the recent results of biogas production from microalgae biomass.

Table 7. Biogas production from several microalgae biomass (Córdova et al., 2018)

Microalgae species	Medium microalgae growth	Conditions	Pretreatment	Methane/biogas yield		References
				Untreated	Pretreated	
Scenedesmus obliquus	Synthetic medium	35ºC, 33 days, batch reactor	Thermal, 70ºC, 15 min	0.076 L CH_4/g COD	0.085 L CH_4/g COD	González-Fernández et al., (2012)
Nannochloropsis sp	Marine	35ºC, 30 days, batch reactor		0.357 L CH_4/g VS		Zhao et al., (2014)
Stigeoclonium sp., Monorraphidium sp., Nitzchia sp., Amphora sp.	Urban Wastewater	37ºC, continuous test	Thermal, 95ºC, 10h	0.10 L CH_4/g VS	0.12 L CH_4/g VS	Passos and Ferrer, (2014)
Scenedesmus obliquus	Fresh water	33ºC, 30 days		0.13 L CH_4/g VS		Zamalloa et al., (2012)
Chlorella vulgaris	Swine manure	37ºC, 24 days, batch reactor		0.228 L CH_4/g VS		Mendez et al., (2014)
H. reticulatum	Urban Secondary wastewater	35ºC, 25 days, batch reactor		0.110 L CH_4/g VS		Lee et al., (2014)
Microalgal biomass	Urban Primary wastewater	35ºC, continuous test	Microwave, 900W, 3 min	0.17 L CH_4/g VS	0.27 L CH_4/g VS	Passos et al., (2014)

Chlorella vulgaris	Swine wastewater	35ºC, 25 days, batch reactor		0.229 L CH$_4$/g VS		Park et al., (2013)
Algal biomass: Scenedesmus obliquus, Chlorella vulgaris	Primary urban wastewater	35ºC, 46 days, batch reactor		0.117 L CH$_4$/g VS		Passos et al., (2013)
Nannochloropsis salina		38ºC, 40 days, batch test	Thermal, 100ºC, 8h	0.347 L biogas/g VS	0.549 L biogas/g VS	Schwede et al., (2011)
			Microwave, 600W, 2450 MHz	0.347 L biogas/g VS	0.487 L biogas/g VS	Schwede et al., (2011)
			Ultrasound, 200W, 30 kHz	0.347 L biogas/g VS	0.274 L biogas/g VS	Schwede et al., (2011)
Chlorella vulgaris		35ºC, 30 days, batch test	Thermochemical, 120ºC, 20 min	0.139 L CH$_4$/g VS	0.180 L CH$_4$/g VS	Mendez et al., (2013)
			Thermochemical, 120ºC, 40 min	0.139 L CH$_4$/g VS	0.268 L CH$_4$/g VS	Mendez et al., (2013)
Isochrysis galbana		30ºC, 15 days, batch test	H$_2$SO$_4$, 40ºC, 0.2% v/v, 16h	0.017 L CH$_4$/g VS	0.017 L CH$_4$/g VS	Santos et al., (2014)

In addition, digestate is obtained after the anaerobic digestion as a liquid fraction rich in mineralised nutrients, which can be used as fertiliser. Several considerations are required by the strict legislation on this area. The content of NPK must be superior to the minimum legal threshold value of 7% (w/w) and the ratio C/N should be lower than maximum allowed value of 15. In smaller components, the limits are totally diverse, being the strictest for the content of As with a maximum limit of 50 mg/kg. The limit allowed for other components depends on the fertiliser use: extensive and grazing cultivation, fertirrigation or horticultural use, and foliar (European Parliament and of the Council, 2003).

5.2. Bioalcohols

Bio-alcohols production is performed by a fermentation process, converting the monomeric sugars released from the biomass into alcohols. Many authors are studied the production of ethanol from the carbohydrate fraction of pure microalgae (Table 8). For bio-ethanol production, the most used yeasts are *Saccharomyces* and *Zymomonas*; and the maximum possible stoichiometric production from glucose is 0.511 $g_{ethanol}/g_{glucose}$ (de Farias Silva and Bertucco, 2016). The genus Clostridia is the most common microorganism for bio-butanol production, carrying out the conversion of the sugars into a mixture of acetone, butanol and ethanol (ABE) with typical ratio of 3:6:1 (Bellido et al., 2014). Table 8 shows a brief of literature about the bioethanol production.

Table 8. Principal results of bio-ethanol production by fermentation of released sugars from microalgae biomass

Microalgae species	Hydrolysis	Yeast/Bacteria	Initial biomass concentration (g/L)	Total carbohydrates (%)	Ethanol yield (g/g algae)	References
Chlorella vulgaris	Acid	*Zymomonas mobilis*	50	51	0.233	Ho et al., (2013)
Chlorella vulgaris	Enzymatic	*Zymomonas mobilis*	20	51	0.178	Ho et al., (2013)
Chlamydomonas reinhardtii UTEX 90	Enzymatic	*Saccharomyces cerevisiae*	50	60	0.235	Choi et al., (2010)
Chlorella vulgaris	Enzymatic	*Saccharomyces cerevisiae*	10	22	0.070	Kim et al., (2014)
Chlorella vulgaris	Enzymatic	*Zymomonas mobilis*	20	51	0.214	Ho et al., (2013)
Chlamydomonas fasciata	Enzymatic	*Saccharomyces cerevisiae*	100	-	0.194	Asada et al., (2012)
Scenedesmus abundans	Chemical (H_2SO_4) and enzymatic	*Saccharomyces cerevisiae*	50	36	0.103	Guo et al., (2013)
Scenedesmus bijugatus	Thermochemical (H_2SO_4)	*Saccharomyces cerevisiae*	20	31	0.158	Ashokkumar et al., (2015)

The possible inhibition by the degradation compounds generated or released by the pretreatment or the enzymatic hydrolysis results a critical issue during the fermentation for alcohol production. For example, 5-hydroxymethylfurfural and furfural (furanic compounds) usually found in acid hydrolysates can inhibit the cell growth damaging the DNA while phenolic compounds modify the membrane permeability provoking the loss of intracellular components, whenever affecting the enzymatic pathways. Currently, most of the utilised yeasts or bacteria are genetically modified to avoid or reduce some of these problems (Monlau et al., 2014).

The limited research works on bio-butanol production from microalgae are caused by this inhibitory problem, involving low efficient process and most of the reported studies are only confined to the laboratory stage (Lin et al., 2018).

6. Bio-refinery of algal biomass

Most of the published research about valorisation of microalgae biomass is focused on only one specific product with low-medium value-added and from only pure species of microalgae. Particularly for microalgae-bacteria biomass, scarce works has been published and mainly for biogas production. Thus, the development of economic and environmentally sustainable processes requires an integral valorisation of all the microalgae components, applying a bio-refinery concept (Zhu, 2015). This well-known concept leads a complete extraction of the compounds and its conversion into a spectrum of bio-based products and bio-energy (Chandra et al., 2019). The research on this field is still in its infancy stage due to the lack of studies on extraction processes integration and reduction of the wastes maximising profitability and benefits (Demirbas, 2009).

As explained in the cell disruption section, extraction of valuable compounds from microalgae from wastewaters treatment is difficult in comparison with other biomass, as their cell wall is composed of several layers with a rigid structure and the coexistence with bacteria. Therefore, an intensive pretreatment of the algal biomass must be required to extract the intracellular compounds of interest in an efficient way (Menegazzo and Fonseca, 2019). The sequential valorisation of all the fractions requires not only the highest yield of one compound optimised but also the analysis of its impact on the other

compounds (Chew et al., 2017). For example, extraction of proteins requires mild methods to avoid degradation or denaturation.
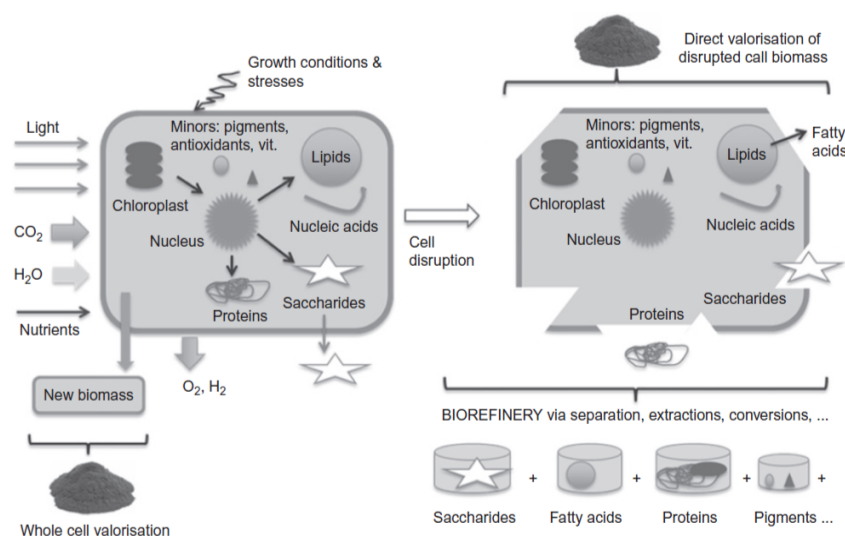
Figure 7: Overview of the biorefinery concept for valorisation of microalgae biomass (Bastiaens et al., 2017)

Numerous cascading biorefinery theoretical approaches have been elaborated to multiple marketable fractions but only few projects have been carried out al laboratory scale and they have always addressed to pure microalgae, classifying in various categories (Gouveia et al., 2014):

- Valorisation of microalgae biomass to produce low-value compounds as energy and the next use of the residual biomass.
- Valorisation of microalgae biomass to produce medium-value bulk products and the next use of the residual biomass.
- Valorisation of microalgae biomass to produce high-value products coupled with residual biomass use.

Table 9 summarises the laboratory scale studies applying a bio-refinery concept in the last years.

Table 9. Principal species of microalgae used for an integration process and the main products obtained

Microalgae	Targeted compounds	References
Chlorella protothecoids	Carotenoids, lipids	Campenni' et al., (2013)
Chlorella reindhardtii	Biogas, biohydrogen	Mussgnug et al., (2010)
Chlorella vulgaris	Biodiesel, methane	Ehimen et al., (2011)

Dunaliella salina	Biodiesel, methane	Sialve et al., (2009)
Dunaliella tertiolecta	Pyrolysis products bio-oil and char, lipids (beta-carotene, phytosterol, fatty acids)	Francavilla et al., (2015)
Nannochloropsis sp.	Fatty acids, carotenoids, biohydrogen	Nobre et al., (2013)
Isochrysis galbana	Fucoxanthin-carotenoids, polar lipids	Gilbert-López et al., (2015)
Scenedesmus acutus	Bioethanol (from hydrolysed sugars), oil	Dong et al., (2016)
Scenedesmus sp.	Biogas, amino acids	Ramos-Suárez et al., (2014)
Haematococcus pluvialis	Biodiesel, asthaxanthin, PHB	Prieto et al., (2017)
Chlamydomonas sp.	Methyl ester and e-polylysine	Sivaramakrishnan et al., (2019)

Despite the renowned issue to apply the approach of bio-refinery concept, the research in sequential valorisation of complete microalgae is really scarce. So far, no studies have been done using microalgae-bacteria biomass grown in wastewater treatment plant.

7. References

[1] Abdul Razack, S., Duraiarasan, S., Mani, V., 2016. Biosynthesis of silver nanoparticle and its application in cell wall disruption to release carbohydrate and lipid from *C. vulgaris* for biofuel production. Biotechnol. Reports 11, 70–76. doi:10.1016/j.btre.2016.07.001

[2] Acién, F.G., Molina, E., Reis, A., Torzillo, G., Zittelli, G.C., Sepúlveda, C., Masojídek, J., 2017. 1 - Photobioreactors for the production of microalgae. In: Microalgae-Based biofuels and Bioproducts. 1–44. https://doi.org/10.1016/B978-0-08-101023-5.00001-7

[3] Ahmed, A., Sattar, A., Pirah, S., Khushk, I., Klykov, S.P., Haider, C., Lu, C., 2018. An integrated bioprocess for xylanase production from agriculture waste under open non-sterilized conditions : Biofabrication as fermentation tool 193, 194–205.

[4] Alhattab, M., Kermanshahi pour, A., Su-Ling Brooks, M., 2018. Microalgae disruption techniques for product recovery: influence of cell wall composition, Journal of Applied Phycology. doi:10.1007/s10811-018-1560-9

[5] Asada, C., Doi, K., Sasaki, C., Nakamura, Y., 2012. Efficient Extraction of Starch from Microalgae Using Ultrasonic Homogenizer and Its Conversion into Ethanol by Simultaneous Saccharification and Fermentation. Nat. Resour. 03, 175–179. doi:10.4236/nr.2012.34023

[6] Ashokkumar, V., Salam, Z., Tiwari, O.N., Chinnasamy, S., Mohammed, S., Ani, F.N., 2015. An integrated approach for biodiesel and bioethanol production from *Scenedesmus bijugatus* cultivated in a vertical tubular photobioreactor. Energy Convers. Manag. 101, 778–786. doi:10.1016/j.enconman.2015.06.006

[7] Baroukh, C., Muñoz-Tamayo, R., Steyer, J.P., Bernard, O., 2013. A new framework for metabolic modeling under non-balanced growth. Application to carbon metabolism of unicellular microalgae. IFAC Proc. Vol. 12, 107–112. doi:10.3182/20131216-3-IN-2044.00006

[8] Bastiaens, L., Van Roy, S., Thomassen, G., Elst, K., 2017. Biorefinery of algae: Technical and economic considerations. Microalgae-Based Biofuels Bioprod. From Feed. Cultiv. to End-Products. 327 – 345. doi:10.1016/B978-0-08-101023-5.00014-5

[9] Bellido, C., Loureiro Pinto, M., Coca, M., González-Benito, G., García-Cubero, M.T., 2014. Acetone-butanol-ethanol (ABE) production by *Clostridium beijerinckii* from wheat straw hydrolysates: Efficient use of penta and hexa carbohydrates. Bioresour. Technol. 167, 198–205. doi:10.1016/j.biortech.2014.06.020

[10] Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae-novel highly efficient starch producers. Biotechnol. Bioeng. 108, 766–776. doi:10.1002/bit.23016

[11] Brasil, B.S.A.F, Siquerira, F.G., Salum, T.F.C., Zanette, C.M., Spier, M.R. (2017) Microalgae and cyanobacteria as enzyme biofactories. Algal Reser. 25, 76–89.

[12] Bureau, U.S.C., 2012. The Emergency and Transitional Shelter Population: 2010 .1–22.

[13] C'ordova, O., Santis, J., Ruiz-Fillipi, G., Zuñiga, M.E., Fermoso, F.G., Chamy, R. 2018. Microalgae digestive pretretament for increasing biogas production. Renew. and Sust. Energ. Review. 82, 2806–2813.

[14] Cade-Menun, B.J., Paytan, A., 2010. Nutrient temperature and light stress alter phosphorus and carbon forms in culture-grown algae. Mar. Chem. 121, 27–36. doi:10.1016/j.marchem.2010.03.002

[15] Campenni', L., Nobre, B.P., Santos, C.A., Oliveira, A.C., Aires-Barros, M.R., Palavra, A.M.F., Gouveia, L., 2013. Carotenoid and lipid production by the autotrophic microalga *Chlorella protothecoides* under nutritional, salinity, and luminosity stress conditions. Appl. Microbiol. Biotechnol. 97, 1383–1393. doi:10.1007/s00253-012-4570-6

[16] Cardeña, R., Moreno, G., Bakonyi, P., Buitrón, G., 2017. Enhancement of methane production from various microalgae cultures via novel ozonation pretreatment . Chem. Eng. J. 307, 948–954.

[17] Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., 2016. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. Bioresource Technology. 199, 386–397.

[18] Carrillo-Reyes, J., Barragán-Trinidad, M., Buitrón, G., 2016. Biological pretreatments of microalgal biomass for gaseous biofuel production and the potential use of rumen microorganisms: A review. Algal Res. 18, 341–351. doi:10.1016/j.aspen.2018.01.017

[19] Cavinato, C., Ugurlu, A., de Godos, I., Kendir, E., Gonzalez-Fernandez, C., 2017. Biogas production from microalgae. Microalgae-Based Biofuels Bioprod. From Feed. Cultiv. to End-Products 155–182. doi:10.1016/B978-0-08-101023-5.00007-8

[20] Chandra, R., Iqbal, H.M.N., Vishal, G., Lee, H.S., Nagra, S., 2019. Algal biorefinery: A sustainable approach to valorize algal-based biomass towards multiple product recovery. Bioresour. Technol. 278, 346–359. doi:10.1016/j.biortech.2019.01.104

[21] Chen, C.Y., Zhao, X.Q., Yen, H.W., Ho, S.H., Cheng, C.L., Lee, D.J., Bai, F.W., Chang, J.S., 2013. Microalgae-based carbohydrates for biofuel production. Biochem. Eng. J. 78, 1–10. doi:10.1016/j.bej.2013.03.006

[22] Cheng, D.L., Ngo, H.H., Guo, W.S., Chang, S.W., Nguyen, D.D., Kumar, S.M., 2019. Microalgae biomass from swine wastewater and its conversion to bioenergy. Bioresour. Technol. 275, 109–122. doi:10.1016/j.biortech.2018.12.019

[23] Cheng, Y.S., Labavitch, J.M., Vandergheynst, J.S., 2015. Elevated CO_2 concentration impacts cell wall polysaccharide composition of green microalgae of the genus *Chlorella*. Lett. Appl. Microbiol. 60, 1–7. doi:10.1111/lam.12320

[24] Chew, K.W., Yap, J.Y., Show, P.L., Suan, N.H., Juan, J.C., Ling, T.C., Lee, D.J., Chang, J.S., 2017. Microalgae biorefinery: High value products perspectives. Bioresour. Technol. 229, 53–62. doi:10.1016/j.biortech.2017.01.006

[25] Choi, J.-A., Hwang, J.-H., Dempsey, B. a., Abou-Shanab, R. a. I., Min, B., Song, H., Lee, D.S., Kim, J.R., Cho, Y., Hong, S., Jeon, B.-H., 2011. Enhancement of fermentative bioenergy (ethanol/hydrogen) production using ultrasonication of *Scenedesmus obliquus* YSW15 cultivated in swine wastewater effluent. Energy Environ. Sci. 4, 3513. doi:10.1039/c1ee01068a

[26] Choi, S.P., Nguyen, M.T., Sim, S.J., 2010. Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. Bioresour. Technol. 101, 5330–5336. doi:10.1016/j.biortech.2010.02.026

[27] Chokshi, K., Pancha, I., Maurya, R., Paliwal, C., Ghosh, T., Ghosh, A., Mishra, S., 2016. Growth medium standardization and thermotolerance study of freshwater microalgae

Acutodesmus dimorphus - A potential strain for biofuel production, Journal of Applied Phycology. 28. doi:10.1007/s10811-016-0826-3

[28] D'Hondt, E., Martín-Juárez, J., Bolado, S., Kasperoviciene, J., Koreiviene, J., Sulcius, S., Elst, K., Bastiaens, L., 2017. Cell disruption technologies. Microalgae-Based Biofuels Bioprod. From Feed. Cultiv. to End-Products 133–154. doi:10.1016/B978-0-08-101023-5.00006-6

[29] Daneshvar, E., Zarrinmehr, M.J., Hashtjin, A.M., Farhadian, O., Bhatnagar, A., 2018. Versatile applications of freshwater and marine water microalgae in dairy wastewater treatment, lipid extraction and tetracycline biosorption. Bioresour. Technol. 268, 523 – 530. doi:10.1016/j.biortech.2018.08.032

[30] de Araújo Padilha, C.E., de Oliveira Campos, A., de Macedo, G.R., dos Santos, E.S., dos Santos, D.A., da Silva, F.L., de Sousa Junior, F.C., de Oliveira Júnior, S.D., 2017. Pretreatments of *Carnauba (Copernicia prunifera)* straw residue for production of cellulolytic enzymes by *Trichoderma reesei* CCT-2768 by solid state fermentation. Renew. Energy 116, 299–308. doi:10.1016/j.renene.2017.09.064

[31] de Farias Silva, C.E., Bertucco, A., 2016. Bioethanol from microalgae and cyanobacteria: A review and technological outlook. Process Biochem. 51, 1833–1842. doi:10.1016/j.procbio.2016.02.016

[32] de Godos, I., Blanco, S., García-Encina, P.A., Becares, E., Muñoz, R., 2010. Influence of flue gas sparging on the performance of high rate algae ponds treating agro-industrial wastewaters. J. Hazard. Mater. 179, 1049–1054. doi:10.1016/j.jhazmat.2010.03.112

[33] De Godos, I., González, C., Becares, E., García-Encina, P.A., Muñoz, R., 2009. Simultaneous nutrients and carbon removal during pretreated swine slurry degradation in a tubular biofilm photobioreactor. Appl. Microbiol. Biotechnol. 82, 187–194. doi:10.1007/s00253-008-1825-3

[34] Demirbas, A. 2009. Biorefineries: Current activities and future developments. Energy Convers. Manag. 50, 2782 –2801. doi:10.1016/j.enconman.2009.06.035

[35] Dong, T., Knoshaug, E.P., Davis, R., Laurens, L.M.L., Van Wychen, S., Pienkos, P.T., Nagle, N., 2016. Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts. Algal Res. 316 – 323. doi:10.1016/j.algal.2015.12.021

[36] Ehimen, E.A., Sun, Z.F., Carrington, C.G., Birch, E.J., Eaton-Rye, J.J., 2011. Anaerobic digestion of microalgae residues resulting from the biodiesel production process. Appl. Energy. 88, 3454 – 3463. doi:10.1016/j.apenergy.2010.10.020

[37] El-Dalatony, M.M., Salama, E.S., Kurade, M.B., Kim, K.Y., Govindwar, S.P., Kim, J.R., Kwon, E.E., Min, B., Jang, M., Oh, S.E., Chang, S.W., Jeon, B.H., 2019. Whole conversion of microalgal biomass into biofuels through successive high-throughput fermentation. Chem. Eng. J. 360, 797–805. doi:10.1016/j.cej.2018.12.042

[38] European Parliament and of the Council, 2003. Regulation (EC) No 2003/2003 of the wEuropean Parliament and of the Council of 13 October 2003 relating to fertilisers. Official Journal L304, 21/11/2003, 0001–0194

[39] Farinas, C.S., 2018. Production of Cellulolytic Enzymes and Their Use in the Saccharification of Lignocellulosic Biomass. In: Current Developments in Biotechnology and Bioengineering, 169–183.

[40] Francavilla, M., Kamaterou, P., Intini, S., Monteleone, M., Zabaniotou, A. (2015) Cascading microalgae biorefinery: Fast pyrolysis of *Dunaliella tertiolecta* lipid extracted-residue. Algal Reser. 11, 184–193.

[41] Fu, C., Hung, T., Chen, J., Su, C., Wu W. 2010. Hydrolysis of microalgae cell walls for production of reducing sugar and lipid extraction. Bioresour. Technol. 101, 8750 – 8754.

[42] Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., Vilchez, C., 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Mar. Drugs 14. doi:10.3390/md14050100

[43] García, D., de Godos, I., Domínguez, C., Turiel, S., Bolado, S., Muñoz, R., 2019. A systematic comparison of the potential of microalgae-bacteria and purple phototrophic bacteria consortia for the treatment of piggery wastewater. Bioresour. Technol. 276, 18–27. doi:10.1016/j.biortech.2018.12.095

[44] García, D., Posadas, E., Blanco, S., Acién, G., García-encina, P., Bolado, S., Muñoz, R., 2017a. Evaluation of the dynamics of microalgae population structure and process performance during piggery wastewater treatment in algal- bacterial photobioreactors. Bioresour. Technol. 248, 120–126. doi:10.1016/j.biortech.2017.06.079

[45] García, D., Posadas, E., Grajeda, C., Blanco, S., Martínez-Páramo, S., Acién, G., García-Encina, P., Bolado, S., Muñoz, R., Martínez-, S., 2017b. Comparative evaluation of piggery wastewater treatment in algal-bacterial pho-tobioreactors under indoor and outdoor conditions. Bioresour. Technol. 245, 483–490. doi:10.1016/j.biortech.2017.08.135

[46] Gilbert-López, B., Mendiola, J.A., Fontecha, J., Van Den Broek, L.A.M., Sijtsma, L., Cifuentes, A., Herrero, M., Ibáñez, E., 2015. Downstream processing of *Isochrysis galbana*: a step towards microalgal biorefinery. Green Chem. 17, 4599–4609. doi:10.1039/c5gc01256b

[47]   González-Fernández, C., Ballesteros, M., 2012. Linking microalgae and cyanobacteria culture conditions and key-enzymes for carbohydrate accumulation. Biotechnol. Adv. 30, 1655–1661. doi:10.1016/j.biotechadv.2012.07.003

[48]   González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.P., 2012. Thermal pretreatment to improve methane production of Scenedesmus biomass. Biomass and Bioenergy. 40, 105 –111. doi:10.1016/j.biombioe.2012.02.008

[49]   Gonzalez-Fernandez, C., Sialve, B., Molinuevo-Salces, B., 2015. Anaerobic digestion of microalgal biomass: Challenges, opportunities and research needs. Bioresour. Technol. 30, 1655 – 1661. doi:10.1016/j.biortech.2015.09.095

[50]   Gouveia, L., Neves, C., Sebastião, D., Nobre, B.P., Matos, C.T., 2014. Effect of light on the production of bioelectricity and added-value microalgae biomass in a Photosynthetic Alga Microbial Fuel Cell. Bioresour. Technol. 8, 357 – 361. doi:10.1016/j.biortech.2013.12.049

[51]   Günerken, E. D´Hondt, E., Eppink, M., Els, K., Wijffels, R. 2016. Influence of nitrogen depletion in the growth of N. oleoabundans on the release of cellular components after bead milling. Bioresour. Technol. 214, 89–95. doi:10.1016/j.biortech.2016.04.072

[52]   Günerken, E., Hondt, E.D., Eppink, M.H.M., Garcia-gonzalez, L., Elst, K., Wijffels, R.H., 2015. Cell disruption for microalgae biorefineries 33, 243–260. doi:10.1016/j.biotechadv.2015.01.008

[53]   Guo, H., Daroch, M., Liu, L., Qiu, G., Geng, S., Wang, G., 2013. Biochemical features and bioethanol production of microalgae from coastal waters of Pearl River Delta. Bioresour. Technol. doi:10.1016/j.biortech.2012.10.006

[54]   Guoweia, S., Man, H., Shikai, W., He, C., 2011. Effect of some factors on Production of cellulase by *Trichoderma reesei* HY07. Procedia Environ. Sci. 8, 357–361. doi:10.1016/j.proenv.2011.10.056

[55]   Habouzit, F., Hamelin, J., Santa-Catalina, G., Steyer, J.P., Bernet, N., 2014. Biofilm development during the start-up period of anaerobic biofilm reactors: The biofilm Archaea community is highly dependent on the support material. Microb. Biotechnol. 7, 257–264. doi:10.1111/1751-7915.12115

[56]   Hansen, G.H., Lübeck, M., Frisvad, J.C., Lübeck, P.S., 2015. Production of cellulolytic enzymes from ascomycetes : Comparison of solid state and submerged fermentation. Process Biochem. 50, 1327–1341.

[57]   Harun, R., Danquah, M.K., 2011. Enzymatic hydrolysis of microalgal biomass for bioethanol production. Chem. Eng. J. 168, 1079–1084. doi:10.1016/j.cej.2011.01.088

[58] Hernández, D., Riaño, B., Coca, M., García-González, M.C., 2015. Saccharification of carbohydrates in microalgal biomass by physical , chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chem. Eng. J. 262, 939–945. doi:10.1016/j.cej.2014.10.049

[59] Hernández, D., Riaño, B., Coca, M., García-González, M.C., 2013. Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass. Bioresour. Technol. 135, 598–603. doi:10.1016/j.biortech.2012.09.029

[60] Ho, S.H., Huang, S.W., Chen, C.Y., Hasunuma, T., Kondo, A., Chang, J.S., 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresour. Technol. 135, 191–198. doi:10.1016/j.biortech.2012.10.015

[61] Hu, Y., Du, C., Leu, S., Jing, H., Li, X., Sze, C., Lin, K., 2018. Valorisation of textile waste by fungal solid state fermentation: An example of circular waste-based biorefinery. Resources , Conservation & Recycling. 129, 27–35.

[62] Hu, Y., Gong, M., Feng, S., Xu, C., Bassi, A., 2019. A review of recent developments of pre-treatment technologies and hydrothermal liquefaction of microalgae for bio-crude oil production. Renew. Sustain. Energy Rev. 101, 476–492. doi:10.1016/j.rser.2018.11.037

[63] Jeon, B.-H., Choi, J.-A., Kim, H.-C., Hwang, J.-H., Abou-Shanab, R.A.I., Dempsey, B.A., Regan, J.M., Kim, J.R., 2013. Ultrasonic disintegration of microalgal biomass and consequent improvement of bioaccessibility/bioavailability in microbial fermentation. Biotechnol. Biofuels 6, 37. doi:10.1186/1754-6834-6-37

[64] Ji, C.F., Yu, X.J., Chen, Z.A., Xue, S., Legrand, J., Zhang, W., 2011. Effects of nutrient deprivation on biochemical compositions and photo-hydrogen production of *Tetraselmis subcordiformis*. Int. J. Hydrogen Energy 36, 5817–5821. doi:10.1016/j.ijhydene.2010.12.138

[65] Kadir, W.N.A., Lam, M.K., Uemura, Y., Lim, J.W., Lee, K.T., 2018. Harvesting and pre-treatment of microalgae cultivated in wastewater for biodiesel production: A review. Energy Convers. Manag. 171, 1416–1429. doi:10.1016/j.enconman.2018.06.074

[66] Kamalanathan, M., Gleadow, R., Beardall, J., 2015. Impacts of phosphorus availability on lipid production by *Chlamydomonas reinhardtii*. Algal Res. 12, 191–196. doi:10.1016/j.algal.2015.08.021

[67] Khajepour, F., Hosseini, S.A., Ghorbani Nasrabadi, R., Markou, G., 2015. Effect of Light Intensity and Photoperiod on Growth and Biochemical Composition of a Local Isolate of Nostoc calcicola. Appl. Biochem. Biotechnol. 176, 2279–2289. doi:10.1007/s12010-015-1717-9

[68]   Khanahmadi, M., Arezi, I., Amiri, M., Miranzadeh, M., 2018. Bioprocessing of agro-industrial residues for optimisation of xylanase production by solid- state fermentation in flask and tray bioreactor. Biocatalysis and Agricultural Biotechnology. 13, 272–282.

[69]   Kim, D., Vijayan, D., Praveenkumar, R., Han, J., Lee, K., Park, J., Chang, W., Lee, J., Oh, Y., 2016. Bioresource Technology Cell-wall disruption and lipid / astaxanthin extraction from microalgae : *Chlorella* and *Haematococcus* Biores. Techn. 199, 300–310.

[70]   Kim, K.H., Choi, I.S., Kim, H.M., Wi, S.G., Bae, H.J., 2014. Bioethanol production from the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation. Bioresour. Technol. 153, 47–54. doi:10.1016/j.biortech.2013.11.059

[71]   Kim, C.W., Yoo, G., Lee, H., Lim, J., Kim, J., Kim, K., Park, M.S., Yang, J.-W.,2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol. Adv. 31, 862 – 876, doi:10.1016/j.biotechadv.2013.04.006

[72]   Kim, Y.H., Choi, Y.K., Park, J., Lee, S., Yang, Y.H., Kim, H.J., Park, T.J., Hwan Kim, Y., Lee, S.H., 2012. Ionic liquid-mediated extraction of lipids from algal biomass. Bioresour. Technol. 109, 312–315. doi:10.1016/j.biortech.2011.04.064

[73]   Lam, G.P., Vermuë, M.H., Eppink, M.H.M., Wijffels, R.H., Berg, C. Van Den, 2017. Multi-Product Microalgae Biore fi neries : From Concept Towards Reality. Trends Biotechnol. 36, 1–12. doi:10.1016/j.tibtech.2017.10.011

[74]   Lam, M.K., Lee, K.T., 2015. Bioethanol Production from Microalgae. Handb. Mar. Microalgae 197–208. doi:10.1016/B978-0-12-800776-1.00012-1

[75]   Lara, M., Graciano, G., 2019. Biomass recovery and lipid extraction processes for microalgae biofuels production : A review. Renew. Sustain. Energy Rev. 107, 87–107. doi:10.1016/j.rser.2019.01.064

[76]   Lee, J.Y., Yoo, C., Jun, S.Y., Ahn, C.Y., Oh, H.M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101, S75–S77. doi:10.1016/j.biortech.2009.03.058

[77]   Lee, K., Chantrasakdakul, P., Kim, D., Kong, M., Park, K.Y., 2014. Ultrasound pretreatment of filamentous algal biomass for enhanced biogas production. Waste Manag. 34, 1035 – 1040. doi:10.1016/j.wasman.2013.10.012

[78]   Leite, F., Oliveira, A. De, Alves, D., Júnior, D.O., Eduardo, C., Padilha, D.A., 2018. Pretreatments of Carnauba (Copernicia prunifera) straw residue for production of cellulolytic enzymes by *Trichorderma reesei* CCT-2768 by solid state fermentation. Renew. Ener. 116, 299–308.

[79] Li, Y., Cui, J., Zhang, G., Liu, Z., guan, H., Hwang, H., Aker, W.G., Wang, P. 2016. Optimisation study on the hydrogen peroxide pretreatment and production of bioethanol from seaweed *Ulva* prolifera biomass. Bioresour. Technol. 144 – 149. doi:10.1016/j.biortech.2016.04.090.

[80] Lin, L., Danquah, M.K., Yeong, T.K., Jiao, K., Pan, S., Zeng, X., 2018. Microalgae for biobutanol production – Technology evaluation and value proposition. Algal Res.31, 367–376. doi:10.1016/j.algal.2018.02.029

[81] Lorente, E., Farriol, X., Salvadó, J., 2015. Steam explosion as a fractionation step in biofuel production from microalgae. Fuel Process. Technol. 131, 93–98. doi:10.1016/j.fuproc.2014.11.009

[82] Lu, C., Vonshak, A., 1999. Photoinhibition in outdoor *Spirulina platensis* cultures assessed by polyphasic chlorophyll fluorescence transients. J. Appl. Phycol. 11, 355–359. doi:10.1023/A:1008195927725

[83] Mahdy, A., Ballesteros, M., González-Fernández, C., 2016. Enzymatic pretreatment of *Chlorella vulgaris* for biogas production: Influence of urban wastewater as a sole nutrient source on macromolecular profile and biocatalyst efficiency. Bioresour. Technol. 199, 319–325. doi:10.1016/j.biortech.2015.08.080

[84] Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C., 2014a. Enhanced methane production of Chlorella vulgaris and *Chlamydomonas reinhardtii* by hydrolytic enzymes addition. Energy Convers. Manag. 85, 551 – 557. doi:10.1016/j.enconman.2014.04.097

[85] Mahdy, A., Mendez, L., Blanco, S., Ballesteros, M., González-Fernández, C., 2014b. Protease cell wall degradation of *Chlorella vulgaris*: Effect on methane production. Bioresour. Technol. 171, 421–427. doi:10.1016/j.biortech.2014.08.091

[86] Markou, G., 2012. Alteration of the biomass composition of *Arthrospira (Spirulina) platensis* under various amounts of limited phosphorus. Bioresour. Technol. 116, 533–535. doi:10.1016/j.biortech.2012.04.022

[87] Markou, G., Angelidaki, I., Nerantzis, E., Georgakakis, D., 2013. Bioethanol production by carbohydrate-enriched biomass of *Arthrospira (Spirulina) platensis*. Energies 6, 3937–3950. doi:10.3390/en6083937

[88] Melis, A., 2007. Photosynthetic H_2 metabolism in *Chlamydomonas reinhardtii* (unicellular green algae). Planta 226, 1075–1086. doi:10.1007/s00425-007-0609-9

[89] Mendez, L., Mahdy, A., Demuez, M., Ballesteros, M., González-Fernández, C., 2014. Effect of high pressure thermal pretreatment on *Chlorella vulgaris* biomass: Organic matter

solubilisation and biochemical methane potential. Fuel. 117, 674 – 679. doi:10.1016/j.fuel.2013.09.032

[90] Mendez, L., Mahdy, A., Timmers, R.A., Ballesteros, M., González-Fernández, C., 2013. Enhancing methane production of *Chlorella vulgaris* via thermochemical pretreatments. Bioresour. Technol. 149, 136–141. doi:10.1016/j.biortech.2013.08.136

[91] Miranda, J. R., Passarinho, P.C., Gouveia, L., 2012. Pre-treatment optimisation of Scenedesmus obliquus microalga for bioethanol production. Bioresour. Technol. 104, 342–348. doi:10.1016/j.biortech.2011.10.059

[92] Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.P., Carrère, H., 2014. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolysate inhibit anaerobic mixed cultures? A comprehensive review. Biotechnol. Adv. 32, 934–951. doi:10.1016/j.biotechadv.2014.04.007

[93] Murphy, J.D., Drosg, B., Allen, E., Jerney, J., Xia, A., Herrmann, C., 2015. A Perspective on algal biomass. 1–40. doi:10.13140/RG.2.1.4268.7127

[94] Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56. doi:10.1016/j.jbiotec.2010.07.030

[95] Nghiem, L., Koch, K., Bolzonella, D., Drewes. 2017. Full scale co-digestion of wastewater sludge and food waste Bottlenecks and possibilities. Renew. and Sust. Ener. Rev. 72, 354 – 362.

[96] Nguyen, M.T., Choi, S.P., Lee, J., Lee, J.H., Sim, S.J., 2009. Hydrothermal acid pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. J. Microbiol. Biotechnol. 19, 161–166. doi:10.4014/jmb.0810.578

[97] Nobre, B.P., Villalobos, F., Barragán, B.E., oliveira, A.C., Batista, A.P., Marques, P.A.S.S., Mendes, R.L., Sovová, H., Palavra, A.F., Gouveia, L. 2013. Biorefinery from *Nannochloropsis* sp. microalga - Extraction of microalgal oils and pigments. Production of biohydrogen from biomass leftover biomass. Bioresour. Technol. 135, 128–136. doi:10.1016/j.biortech.2012.11.084.

[98] Oh, H.-S., Kim, H.-S., Ahn, C.-Y., Lee, C.S., Oh, H.-M., 2015. Two-phase photoperiodic cultivation of algal–bacterial consortia for high biomass production and efficient nutrient removal from municipal wastewater. Bioresour. Technol. 200, 867–875. doi:10.1016/j.biortech.2015.11.007

[99] Onumaegbu, C., Mooney, J., Alaswad, A., Olabi, A.G., 2018. Pre-treatment methods for production of biofuel from microalgae biomass. Renew. Sustain. Energy Rev. 93, 16–26. doi:10.1016/J.RSER.2018.04.015

[100] Park, K.Y., Kweon, J., Chantrasakdakul, P., Lee, K., Cha, H.Y., 2013. Anaerobic digestion of microalgal biomass with ultrasonic disintegration. Int. Biodeterior. Biodegrad. 85, 598 –602. doi:10.1016/j.ibiod.2013.03.035

[101] Parniakov, O., Barba, F.J., Grimi, N., Marchal, L., Jubeau, S., Lebovka, N., Vorobiev, E., 2015. Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae nannochloropsis. Algal Res. 8, 128–134. doi:10.1016/j.algal.2015.01.014

[102] Passos, F., Ferrer, I., 2014. Microalgae Conversion to Biogas: Thermal Pretreatment Contribution on Net Energy Production. Environ. Sci. Technol. 48, 7171–7178. doi:10.1021/es500982v

[103] Passos, F., Hernández-Mariné, M., García, J., Ferrer, I., 2014. Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment. Water Res. 49, 351 – 359. doi:10.1016/j.watres.2013.10.013

[104] Passos, F., Solé, M., García, J., Ferrer, I., 2013. Biogas production from microalgae grown in wastewater: Effect of microwave pretreatment. Appl. Energy. 108, 168 – 175. doi:10.1016/j.apenergy.2013.02.042

[105] Phong, W.N., Show, P.L., Ling, T.C., Juan, J.C., Ng, E.P., Chang, J.S., 2018. Mild cell disruption methods for bio-functional proteins recovery from microalgae—Recent developments and future perspectives. Algal Res. 31, 506–516. doi:10.1016/j.algal.2017.04.005

[106] Phwan, C.K., Ong, H.C., Chen, W.H., Ling, T.C., Ng, E.P., Show, P.L., 2018. Overview: Comparison of pretreatment technologies and fermentation processes of bioethanol from microalgae. Energy Convers. Manag. 173, 81–94. doi:10.1016/j.enconman.2018.07.054

[107] Popper, Z.A., Tuohy, M.G., 2010. Beyond the Green: Understanding the Evolutionary Puzzle of Plant and Algal Cell Walls. Plant Physiol. 153, 373–383. doi:10.1104/pp.110.158055

[108] Posadas, E., García-Encina, P.-A., Soltau, A., Domínguez, A., Díaz, I., Muñoz, R., 2013. Carbon and nutrient removal from centrates and domestic wastewater using algal–bacterial biofilm bioreactors. Bioresour. Technol. 139, 50–58. doi:10.1016/j.biortech.2013.04.008

[109] Posadas, E., Bochon, S., Coca, M., García-González, M.C., García-Encina, P.A., Muñoz, R., 2014. Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. J. Appl. Phycol. 26, 2335–2345. doi:10.1007/s10811-014-0263-0

[110] Posadas, E., Szpak, D., Lombó, F., Domínguez, A., Díaz, I., Blanco, S., García-Encina, P.A., Muñoz, R. Feasibility study of biogas upgrading coupled with nutrient removal from anaerobic effluents using microalgae-based processes. Applied Microbiology and Biotechnology. 1–21.

[111] Prieto, C.V.G., Ramos, F.D., Estrada, V., Villar, M.A., Diaz, M.S., 2017. Optimisation of an integrated algae-based biore fi nery for the production of biodiesel astaxanthin and PHB. Energy, 139, 1159–1172.

[112] Ramos-Suárez, J.L., Cuadra, F.G., Acién, F.G., Carreras, N., 2014. Benefits of combining anaerobic digestion and amino acid extraction from microalgae. Chem. Eng. J. 258, 1–9. doi:10.1016/j.cej.2014.07.086

[113] Rao, A.R., Dayananda, C., Sarada, R., Shamala, T.R., Ravishankar, G.A., 2007. Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. Bioresour. Technol. 98, 560–564. doi:10.1016/j.biortech.2006.02.007

[114] Ray, R.C., Behera, S.S., 2017. Solid State Fermentation for Production of Microbial Cellulases. In: biotechnology of Microbial Enzymes, 43–79.

[115] Rizwan, M., Mujtaba, G., Memon, S.A., Lee, K., Rashid, N., 2018. Exploring the potential of microalgae for new biotechnology applications and beyond: A review. Renew. Sustain. Energy Rev. 92, 394–404. doi:10.1016/j.rser.2018.04.034

[116] Salama, E.S., Hwang, J.H., El-Dalatony, M.M., Kurade, M.B., Kabra, A.N., Abou-Shanab, R.A.I., Kim, K.H., Yang, I.S., Govindwar, S.P., Kim, S., Jeon, B.H., 2018. Enhancement of microalgal growth and biocomponent-based transformations for improved biofuel recovery: A review. Bioresour. Technol. 258, 365–375. doi:10.1016/j.biortech.2018.02.006

[117] Salama, E.S., Kurade, M.B., Abou-Shanab, R.A.I., El-Dalatony, M.M., Yang, I.S., Min, B., Jeon, B.H., 2017. Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation. Renew. Sustain. Energy Rev. 79, 1189–1211. doi:10.1016/j.rser.2017.05.091

[118] Santos, N.O., Oliveira, S.M., Alves, L.C., Cammarota, M.C., 2014. Methane production from marine microalgae *Isochrysis galbana*. Bioresour. Technol. 157, 60 – 67. doi:10.1016/j.biortech.2014.01.091

[119] Sassano, C.E.N., Gioielli, L.A., Ferreira, L.S., Rodrigues, M.S., Sato, S., Converti, A., Carvalho, J.C.M., 2010. Evaluation of the composition of continuously-cultivated *Arthrospira (Spirulina) platensis* using ammonium chloride as nitrogen source. Biomass and Bioenergy 34, 1732–1738. doi:10.1016/j.biombioe.2010.07.002

[120] Schultz-Jensen, N., Thygesen, A., Leipold, F., Thomsen, S.T., Roslander, C., Lilholt, H., Bjerre, A.B., 2013. Pretreatment of the macroalgae *Chaetomorpha linum* for the production of bioethanol–Comparison of five pretreatment technologies. Bioresour. Technol. 140, 36–42.

[121] Schwede, S., Kowalczyk, A., Gerber, M., Span, R., 2011. Influence of Different Cell Disruption Techniques on Mono Digestion of Algal Biomass. Proc. World Renew. Energy Congr. – Sweden, 8–13 May, 2011, Linköping, Sweden 57, 41–47. doi:10.3384/ecp1105741

[122] Sialve, B., Bernet, N., Bernard, O., Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal Biotechnol. Adv. 27, 409–416. doi:10.1016/j.biotechadv.2009.03.001

[123] Singh, R., Kumar, M., Mittal, A., Mehta, P.K., 2016. Microbial enzymes: industrial progress in 21st century. 3 Biotech 6 –174. doi:10.1007/s13205-016-0485-8

[124] Sivaramakrishnan, R., Suresh, S., Incharoensakdi, A., 2019. *Chlamydomonas* sp. as dynamic biorefinery feedstock for the production of methyl ester and ε-polylysine. Bioresour. Technol. 272, 281–287. doi:10.1016/j.biortech.2018.10.026

[125] Statista, 2018. Global pork production in 2018, by country [WWW Document]. URL https://www.statista.com (accessed 7.07.19).

[126] Sudhakar, M.P., Kumar, B.R., Mathimani, T., Arunkumar, K., 2019. A review on bioenergy and bioactive compounds from microalgae and macroalgae-sustainable energy perspective. J. Clean. Prod. 228, 1320–1333. doi:10.1016/j.jclepro.2019.04.287

[127] Surendhiran, D., Vijay, M., 2014. Effect of Various Pretreatment for Extracting Intracellular Lipid from *Nannochloropsis oculata* under Nitrogen Replete and Depleted Conditions. Chem. Eng. 2014, 1–9. doi:10.1155/2014/536310

[128] Torzillo, G., Scoma, A., Faraloni, C., Giannelli, L., 2014. Advances in the biotechnology of hydrogen production with the microalga *Chlamydomonas reinhardtii.* Crit. Rev. Biotechnol. 8551, 1–12. doi:10.3109/07388551.2014.900734

[129] Travaini, R., Martín-Juáre, J., Loreno-Hernando, A., Bolado-Rodrígue, S., 2016. Oonolysis: An advantageous pretreatment for lignocellulosic biomass revisited. Bioresour. Technol. 199, 2–12. doi:10.1016/j.biortech.2015.08.143

[130] Trivedi, N., Reddy, C.R.K., Radulovich, R., Jha, B., 2015. Solid state fermentation (SSF)-derived cellulase for saccharification of the green seaweed *Ulva* for bioethanol production. Algal Res. 9, 48–54. doi:10.1016/j.algal.2015.02.025

[131] van der Maarel, M.J.E.C., van der Veen, B., Uitdehaag, J.C.M., Leemhuis, H., Dijkhuien L., 2002. Properties and applications of starch-converting enymes of the α-amylase family. J. Biotech. 94, 137 – 155.

[132] Velaque-Lucio, J., Rodríguez-Jasso, R.M., Colla, L.M., Sáenz-Galindo, A., Cervantes-Cisneros, D.E., Aguilar, C.N., Fernandes, B.D., Ruiz, H.A., 2018. Microalgal biomass

pretreatment for bioethanol production: a review. Biofuel Res. J. 5, 780–791. doi:10.18331/BRJ2018.5.1.5

[133] Voigt, J., Stolarczyk, A., Zych, M., Malec, P., Burczyk, J., 2014. The cell-wall glycoproteins of the green alga Scenedesmus obliquus. The predominant cell-wall polypeptide of *Scenedesmus obliquus* is related to the cell-wall glycoprotein gp3 of *Chlamydomonas reinhardtii.* Plant Sci. 215, 39 – 47. doi:10.1016/j.plantsci.2013.10.011

[134] Walia, A., Guleria, S., Mehta, P., Chauhan, A., Parkash, J., 2017. Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7 – 11. doi:10.1007/s13205-016-0584-6

[135] Wang, M., Yang, H., Ergas, S.J., van der Steen, P., 2015. A novel shortcut nitrogen removal process using an algal-bacterial consortium in a photo-sequencing batch reactor (PSBR). Water Res. 87, 38–48. doi:10.1016/j.watres.2015.09.016

[136] Wilkie, A.C., Mulbry, W., 2015. Recovery of dairy manure nutrients by benthic freshwater algae. Bioresour. Technol. 8524, 81–91. doi:10.1016/S0960-8524(02)00003-2

[137] Xu, X., Lin, M., Zang, Q., Shi, S., 2018. Solid state bioconversion of lignocellulosic residues by *Inonotus obliquus* for production of cellulolytic enzymes and saccharification. Bioresour. Technol. 247, 88–95. doi:10.1016/j.biortech.2017.08.192

[138] Yao, C.H., Ai, J.N., Cao, X.P., Xue, S., 2013. Salinity manipulation as an effective method for enhanced starch production in the marine microalga *Tetraselmis subcordiformis*. Bioresour. Technol. 146, 663–671. doi:10.1016/j.biortech.2013.07.134

[139] Yen, H.W., Hu, I.C., Chen, C.Y., Ho, S.H., Lee, D.J., Chang, J.S., 2013. Microalgae-based biorefinery - From biofuels to natural products. Bioresour. Technol. 135, 166–174. doi:10.1016/j.biortech.2012.10.099

[140] Yoo, G., Park, M.S., Yang, J.W., 2014. Chemical Pretreatment of Algal Biomass, Pretreatment of Biomass: Processes and Technologies. 227 – 258. doi:10.1016/B978-0-12-800080-9.00012-8

[141] Zamalloa, C., Boon, N., Verstraete, W., 2013. Decentralized two-stage sewage treatment by chemical-biological flocculation combined with microalgae biofilm for nutrient immobilization in a roof installed parallel plate reactor. Bioresour. Technol. 130, 152–160. doi:10.1016/j.biortech.2012.11.128

[142] Zamalloa, C., Boon, N., Verstraete, W., 2012. Anaerobic digestibility of *Scenedesmus obliquus* and *Phaeodactylum tricornutum* under mesophilic and thermophilic conditions. Appl. Energy. 733 – 738. doi:10.1016/j.apenergy.2011.08.017

[143] Zhao, B., Ma, J., Zhao, Q., Laurens, L., Jarvis, E., Chen, S., Frear, C., 2014. Efficient anaerobic digestion of whole microalgae and lipid-extracted microalgae residues for

methane energy production. Bioresour. Technol. 423 – 430. doi:10.1016/j.biortech.2014.03.079

[144] Zhao, G., Chen, X., Wang, L., Zhou, S., Feng, H., Chen, W.N., Lau, R., 2013. Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation. Bioresour. Technol. 128, 337–344. doi:10.1016/j.biortech.2012.10.038

[145] Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., Dou, C., 2011. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl. Biochem. Biotechnol. 164, 1215–24. doi:10.1007/s12010-011-9207-1

[146] Zhou, N., Zhang, Y., Gong, X., Wang, Q., Ma, Y., 2012. Ionic liquids-based hydrolysis of *Chlorella biomass* for fermentable sugars. Bioresour. Technol. 118, 512–517. doi:10.1016/j.biortech.2012.05.074

[147] Zhu, L., 2015. Biorefinery as a promising approach to promote microalgae industry: An innovative framework. Renew. Sustain. Energy Rev. 1376 – 1384. doi:10.1016/j.rser.2014.09.040

Aims and scope of the thesis

2.1. Justification of the thesis

During the last decades, the consumption of all raw materials has exponentially increased by the growth of the human population, involving an urgent transition from fossil raw materials into renewable sources. Besides, the available lands are required to cultivate food for this increasing population. In this regard, the recovery of the abundant and problematic wastes, and their use as raw materials for new processes seem to be the most feasible solution. Wastewaters are promising raw materials, renewable and alternative to fossil fuels, and to produce bio-energy and other bio-products due to their high content of organic matter and nutrients. Microalgae-bacteria biomass grown in photobioreactors have a remarkable potential to accumulate these organic matter and nutrients from wastewaters, resulting a promising raw material and providing clean water useful for recycle in other applications.

However, the valorisation of this microalgal biomass grown in wastewater treatment photobioreactors is still on an early stage. Therefore, further research is required to optimise the implementation of both steps, the treatment and the biomass valorisation, to ensure cost-effective recovery processes and to support the development of microalgae-based bio-refineries.

This thesis aims to address the principal problems hindering the valorisation of microalgal biomass - cultivated in wastewater treatment photobioreactors - into bioenergy and bioproducts: i) the effect of pretreatments (chemical, physic-mechanical, combined) on solubilisation of macromolecular components of the biomass, ii) the degradation of solubilised components by the action of the pretreatments or the microorganisms present on the biomass, iii) the effect of pretreatments on the yields obtained in further valorisation steps, iv) the optimisation of operational conditions during pretreatments and further valorisation steps as anaerobic digestion or enzymatic hydrolysis, and v) the search of new alternatives for integral valorisation of this microalgae biomass.

2.2. Main objectives

The overall purpose of this thesis is the valorisation of microalgae biomass grown in pig manure treatment photobioreactors to produce bio-energy and bio-products. The work is focused on the valorisation of the carbohydrate fraction of this biomass but considering the effect of the processes in the other fractions (proteins and lipids), applying a bio-refinery concept. Biogas production and recovery of fermentable sugars are the two principal alternatives evaluated in this thesis. Different pretreatments are studied for both alternatives followed by anaerobic digestion and enzymatic hydrolysis, respectively. The solid residues after anaerobic digestion from microalgae biomass are assessed as a fertiliser.

The generation of by-products are determined for the pretreatment and enzymatic hydrolysis steps, as well as the solubilisation of proteins and lipids. Further, for the valorisation of carbohydrate fraction, the use of microalgal biomass as substrate for the production of cellulases and xylanases is evaluated, using solid-state fermentation.

More explicitly, the following specific objectives are pursued:

Objective 1. To evaluate the effect of different pretreatments on the limiting step, yield and kinetic of biogas production from microalgae-bacteria biomass grown in piggery treatment photobioreactors.

Objective 2. To analyse the potential use as bio-fertiliser of digestates of biogas production from microalgae-bacteria biomass grown in piggery treatment photobioreactors, in order to achieve an integral valorisation of the biomass.

Objective 3. To select the pretreatment more adequate for the valorisation of microalgae-bacteria biomass grown in piggery treatment photobioreactors by producing biogas and fertilisers.

Objective 4. To evaluate the effect of different pretreatments on the yields of carbohydrates solubilisation and monosaccharides recovery from microalgae-bacteria biomass grown in piggery treatment photobioreactors.

Objective 5. To evaluate the effect of coupling different pretreatments and enzymatic hydrolysis on the yields of carbohydrates solubilisation, and monosaccharides recovery from microalgae-bacteria biomass grown in piggery treatment photobioreactors.

Objective 6. To analyse the formation of by-products and the solubilisation of proteins and lipids by the application of different pretreatments and coupled pretreatment and enzymatic hydrolysis processes to biomass grown in piggery treatment photobioreactors, in order to obtain information for a further bio-refinery advance.

Objective 7. To select the most reasonable process for the valorisation of the fraction carbohydrate of microalgae-bacteria biomass grown in piggery treatment photobioreactors, considering its effect on the further valorisation of other fractions.

Objective 8. To analyse the effect of the main operational parameters of acid and alkaline pretreatments and enzymatic hydrolysis on the carbohydrates, proteins and lipids solubilised yields, and on the generation of by-products from microalgae-based biomass.

Objective 9. To optimise the operation conditions of chemical pretreatment coupled with enzymatic hydrolysis for carbohydrates solubilisation and monosaccharides recovery from microalgae biomass, considering a high variability in the biomass growth media.

Objective 10. To evaluate the use of microalgae biomass from pig manure wastewater treatment photobioreactors as substrate for production of enzymes - cellulases and xylanases - and optimise the main operational parameters of the solid-state fermentation and the enzymes extraction

2.3. Development of the thesis

Five series of experiments are conducted to fulfill the particular aims aforementioned:

Valorisation of microalgae biomass from pig manure treatment photobioreactors as substrate for biogas and biofertiliser production (**Chapter 3**).

- Biogas productions by anaerobic digestion of untreated and different pretreated microalgae biomass are compared to determine the feasibility of different techniques for cell wall disruption.

- Biogas productions by anaerobic digestion of only solid fraction and whole suspension from pretreatments are compared to determine the possible inhibitory effect of by-products present in the liquid fractions.

- Cumulative biogas production results are fitted to identify the limiting step of the anaerobic digestion of each pretreated biomass, and to quantify the pretreatment effects on the potential and the kinetic of biogas production.

- The possible use of solid residues after anaerobic digestion as fertiliser is evaluated, applying a bio-refinery concept.

The performance of several pretreatments at different conditions and coupled pretreatment and enzymatic hydrolysis processes is evaluated (**Chapter 4 and 5**).

- Acid, alkaline, alkaline-peroxide, steam explosion, bead mill and ultrasound are applied to microalgae-based biomasses grown in wastewater treatment photobioreactors, at different operation conditions.

- The yields of solubilisation of carbohydrates, proteins and lipids, and recovery of fermentable sugars are quantified for each pretreatment experiment.

- The main possible by-products are analysed in all the liquid phases from pretreatments.

- Enzymatic hydrolysis of only solid fraction and of whole suspensions from pretreatments were carried out, evaluating the yields of solubilisation of carbohydrates, proteins and lipids, and the recovery of fermentable sugars in this step.

- The main possible by-products are analysed in all the liquid phases from enzymatic hydrolysis.

- The different pretreatments are compared in terms of overall yields of the coupled pretreatment and enzymatic hydrolysis processes.

- Biological analysis of bacteria is conducted to evaluate the effect of each pretreatment and enzymatic hydrolysis on viability of these microorganisms.

Optimisation of operational conditions for selected pretreatments and enzymatic hydrolysis is conducted using a Taguchi design, and three distinct biomass grown in pig manure, domestic wastewater and synthetic medium (**Chapter 6**).

- The more significant parameters of pretreatments and enzymatic hydrolysis are selected, defining their ranges and the Taguchi Orthogonal Array design.
- The effect of operational parameters on carbohydrates, proteins and lipids solubilization; recovery of fermentable sugars; and generation of by-products is analysed.
- The optimal conditions for the maximisation of the carbohydrates solubilisation and the recovery of fermentable sugars are selected, considering also the solubilisation of proteins and lipids, and the generation of by-products, in a bio-refinery approach.
- The robustness of the processes faced with variations on the microalgae growth media is studied.

Valorisation of microalgae biomass from pig manure treatment photobioreactors as substrate for enzymes production (**Chapter 7**).

- The main operational parameters of solid-state fermentation and enzymes extraction are selected, defining their studied ranges and the Taguchi Orthogonal Array design.
- Enzymatic activities of FPase, xylanase, β-glucosidase and β-xylosidase are analysed in the extracts of solid-state fermentations using *Trichoderma reesei.*
- The effect of operational parameters on the enzymatic activities is analysed, and the optimal conditions of enzymes production are selected.
- The robustness of the processes faced with changes on the substrate moisture content is studied.

Chapter 3

Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants

Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants

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ABSTRACT

Methane production from pretreated and raw mixed microalgae biomass grown in pig manure was evaluated. Acid and basic pretreatments provided the highest volatile solids solubilisation (up to 81%) followed by alkaline-peroxide and ultrasounds (23%). Bead milling and steam explosion remarkably increased the methane production rate, although the highest yield (377 mL CH_4/g SV) was achieved by alkali pretreatment. Nevertheless, some pretreatments inhibited biogas production and resulted in lag phases of 7-9 days. Hence, experiments using only the pretreated solid phase were performed, which resulted in a decrease in the lag phase to 2-3 days for the alkali pretreatment and slightly increased biomass biodegradability of few samples. The limiting step during the BMP test (hydrolysis or microbial inhibition) for each pretreatment was elucidated using the goodness of fitting to a first order or a Gompertz model. Finally, the use of digestate as biofertiliser was evaluated applying a biorefinery concept.

Keywords: anaerobic digestion, fertiliser, inhibition, kinetic model, methane

1. Introduction

Over the past decades, the concurrent developments in society, science, and technology have resulted in a higher demand for energy. One of the principal challenges in today's society is to provide a reliable energy supply for the future, which is hindered by the increasing prices of oil and gas (Kavitha et al., 2017a). Multiple eco-friendly alternatives, such as the production of bioethanol, biodiesel or biogas from wastes, have been considered and developed to make processes more environmentally friendly and feasible. The conversion of residual biomass into biogas via anaerobic digestion is considered the simplest and most straightforward way, since it requires mild pretreatments and low-cost equipment (Kavitha et al., 2017b).

Biomass grown in wastewater treatment plants is a suitable substrate for biogas production. Among the possible biological wastewater treatment alternatives, the use of microalgae is an emerging challenge, especially for effluents such as pig manure with a high nutrient concentration. Microalgae are able to grow in these wastewaters assimilating organic matter, N and P. Although wastewater treatment coupled to the anaerobic digestion of the microalgae biomass produced is a sustainable and interesting

alternative, most studies on biogas production from microalgae have focused on single species (Mussgnug et al., 2010).

The type of microalgae and the cultivation conditions are essential parameters affecting its macromolecular composition and the cell wall resistance, and hence its potential biogas production (Klassen et al., 2016). Murphy et al., (2015) reported different theoretical methane yields from each organic fractions of the biomass (1.390 L/g VS from lipids, 0.851 L/g VS from proteins, and 0.746 L/g VS from carbohydrates). Additionally, biomass grown in microalgae-based treatment plants contains resistant microalgae species and a huge number of bacteria. To evaluate the feasibility of the combined process of wastewater treatment and biomass valorisation, the study of biogas production from this type of mixed microalgae biomass is required (Jankowska et al., 2017).

The application of pretreatments to disrupt the cell wall represents a promising alternative to increase the biodegradability of mixed microalgae biomass composed of recalcitrant microalgae species. Most of the information reported in literature refers to microalgae grown in domestic wastewater. Passos et al., (2015) carried out different pretreatments such as ultrasound and hydrothermal pretreatments in a mixed microalgae biomass cultivated in domestic wastewater (*Stigeoclonium sp.* and *Monoraphidium sp.* and diatoms *Nitzschia sp.* and *Navicula sp.*). Hydrothermal pretreatment (130ºC) increased the methane yield (135 mL CH_4/g VS) compared to the untreated control (106 mL CH_4/g VS). However, in this case, ultrasound pretreatment (26700 J/g TS) did not significantly improve methane production. In another study, Passos et al., (2016a) studied the effect of two thermochemical pretreatments (KOH and HCl) on biogas production from microalgal biomass. They reported an increase in methane production up to 82% and 86% compared to the untreated biomass (78 mL CH_4/g VS) for alkaline and acid pretreatments, respectively.

Nevertheless, Passos et al., (2016a) also observed an inhibitory effect under severe pretreatment conditions. Most of the reported degradation compounds generated by pretreatments in algae (Martín Juárez et al., 2016) or other types of biomasses were soluble and released to the liquid phase (Toquero and Bolado, 2014, Bolado-Rodríguez et al., 2016). Therefore, the systematic comparison of biogas production using both

fractions (solid and liquid fractions) or only the solid fraction of pretreated samples will provide a valuable information about the effect of the pretreatment technology on the biodegradability of biomass and generation of inhibitory compounds.

Following the valorisation as biogas of the organic matter present in microalgae, a significant load of nutrient is expected in the digestates, especially from biomass grown in wastewater with high N and P content. The use of the residual effluent from microalgae anaerobic digestion as fertiliser would lead the integral valorisation of the mixed microalgae biomass (Acién et al, 2014).

This study aimed at investigating the production of biogas by anaerobic digestion of mixed algal biomass grown in pig manure treatment plants. This work evaluated first the efficiency of different pretreatments (bead mill, alkaline, steam explosion, alkali-peroxide, ultrasound, and acid pretreatments) under two extreme operating conditions on CH_4 productivity. Furthermore, the methane productions from the whole suspension and the only solid fraction from pretreatment were compared in terms of the methane production yield to evaluate the generation of any potential inhibition induced by the pretreatments, kinetic modelling being used to identify the limiting step of the anaerobic digestion of the pretreated biomass. Finally, the composition of the digestates was analysed and their potential use as bio-fertilisers was evaluated to recover the high nutrients load of pig manure using a bio-refinery approach.

2. Materials and methods

2.1. Microalgae biomass

Fresh mixed microalgae biomasses were cultivated in a thin-layer photobioreactor with a volume of 1200L fed with pig manure diluted at 10% at two different times of the year: February and March. The composition during February was 23.67% carbohydrates, 43.31% proteins, 16.74% lipids, 83.17% volatile solids, and 987 mg O_2/ kg of COD, all of them in a dry basis. The microalgae species were *Tetradesmus obliquus* (29%), *Tetradesmus lagerheimii* (26%), *Desmodesmus opoliensis* (16%), *Aphanothece saxicola* (11%), *Chlorella vulgaris* (5%), *Scenedesmus magnus* (4%), *Parachlorella kessleri* (3%), and others in lesser amounts. The composition during March was 38.11% carbohydrates, 24.83% proteins, 12.51% lipids, 74.5% % volatile solids and 1150 mg O_2/ kg in a dry

basis. The microalgae species were *Desmosdesmus opoliensis* (47%), *Navicula reichardtiana* (27%), *Tetradesmus obliquus* (12%), *Scenedesmus sp.* (9%), and *Scenedesmus acuminatus* (5%). The biomass was supplied by the Cajamar Foundation (Almeria, Spain) and centrifuged at 78.75% (February) and 77.91% (March) of moisture and refrigerated at 4ºC prior to use.

2.2. Pretreatments

The pretreatments performed for the biomass from February were bead mill, alkaline (NaOH), steam explosion, and alkaline-peroxide (H_2O_2) pretreatments, all of them at 5% (w/w) dry weight. Two levels of bead mill pretreatments (Postma et al., 2017) were carried out: A (small beads 1.25 mm and 5 minutes) and B (big beads 2.50 mm and 60 min), using distilled water in the mill until 200 mL of total volume (Pascal Engineering Co. Ltd). The alkaline pretreatment was carried out in 1 L borosilicate bottles with NaOH 0.5M (C) and 2M (D). Adequate volumes of NaOH solutions (of the selected concentrations) were added to the known mass of microalgae to obtain 200 mL volume, and, then, suspensions were autoclaved at 121ºC for 60 minutes (Bolado-Rodríguez et al., 2016). The steam explosion pretreatment was carried out using saturated steam at 130ºC during 5 minutes (E) and at 170ºC during 20 minutes (F) in a 5L stainless steel reactor filled with 800 mL of suspension (Alzate et al., 2012). After the selected operation time, the steam was flashed and the biomass was cooled down in another vessel (Marcos et al., 2013). For the alkaline-peroxide pretreatment, known mass of microalgae were placed in 1 L bottles and adequate volumes of H_2O_2 solutions of the selected concentrations 0.5% (G) and 7.5% (H) were added to obtain 200 mL of total volume (Martín Juárez et al., 2016). Then, the pH was adjusted to 11.5 with 2 M NaOH, a few drops of antifoam were added, and the systems were incubated in a rotatory shaker at 50ºC and 120 rpm for 60 minutes.

Ultrasound and acid (HCl) pretreatments at 5% (w/w) dry weight were performed on the biomass from March. The ultrasound pretreatment was carried to a total volume of 400 mL of microalgae biomass diluted with distilled water in Ultrasound Technology (Hielscher UIP1000hd), during 5 (I) and 21 minutes (J), (Alzate et al., 2012). Power was calculated to expend identical amount of energy (7186 J/g TS) for the two operation conditions, according to Equation (1). This consumption of energy, considered a limit

value, was calculated as the difference between energy from the maximum theoretical potential of biogas production and the experimental biogas production from the raw biomass.

$$.. \qquad\qquad (Eq.\ 1)$$

where P is the average ultrasonic power (Watts), t is the ultrasonic time (seconds), V is the sample volume (liters), and TS is the initial total solid concentration (g TS/L).

The acid pretreatment was carried out in borosilicate bottles with HCl 0.5 (K) and 2M (L) (Bolado-Rodríguez et al., 2016). The known mass values of microalgae were placed in 1 L bottles, adequate volumes of HCl solutions (of the selected concentrations) were added to obtain a volume of 200 mL of, and suspensions were autoclaved at 121ºC for 60 minutes. All the pretreatments were conducted in duplicate.

After the pretreatments, the resulting suspensions were centrifuged at 10000 rpm, for 10 minutes. The solid and liquid fractions were weighed. Next, the total and the volatile solids were analysed both in the solid and liquid fractions and in the pretreated whole. Samples of whole pretreated suspensions (named 1) and only solid fractions (named 2) were stored at 4ºC for biogas production experiments. The following parameter was defined to calculate the percentage of volatile solids retained:

Eq. (2)

2.3. Biogas production

Biochemical methane potential (BMP) tests were carried out to study the biodegradability of the microalgae biomass in triplicate following the protocol of Angelidaki et al., (2009). Batch mode assays were performed under mesophilic conditions in 300 mL borosilicate glass bottles with a working volume of 100mL. The effluent from a pilot scale mesophilic anaerobic digester processed mixed sludge from a municipal wastewater treatment plant, with a volatile solids (VS) concentration of 9.1 ± 0.08 g VS/kg was used as inoculum for the tests. Two series of experiments were performed to determine the influence of the pretreatment and the inhibitory effect of the compounds present in the liquid phase: (1) using the whole pretreated suspension; and (2) using only the solid fractions from

pretreatments. A control test without a substrate was also conducted which aimed to check the methanogenic activity of the inoculum (Bolado-Rodríguez et al., 2016).

NaOH or HCl were added, if necessary, to pre-neutralise the samples to pH values 8 for alkaline samples or 5.5 for acid samples. Identical mass of inoculum was used in all the BMPs tests of untreated microalgae biomass, whole suspensions, and solid fractions from pretreatments. Based on previous studies, weighed amounts of pre-neutralised algal biomass were added to obtain an identical ratio of substrate/inoculum of 0.5 g VS/g VS in all the experiments (Alzate et al., 2012). Distilled water was used to fill the 100 mL working volume, when it was required. The pH of the initial mixture was always between 6.5 and 7. Before starting the tests, the bottles were closed with rubber septa and aluminum crimps. Helium gas was circulated inside the gas chamber for 5 minutes and the test started after releasing the pressure. The bottles were placed horizontally on a rotary desk with constant mixing under mesophilic conditions in a thermostatic room (37 ± 0.5 ºC) (Bolado-Rodríguez et al., 2016).

Biogas production in the headspace of each bottle was measured periodically by a manual pressure transmitter (PN5007, range 0–1 bar, IFM Electronics) over a period of 30-45 days. The biogas composition was determined by gas chromatography. Specific methane yields are expressed as the volume of methane under standard conditions, i.e. 0ºC and 1 atm for gases, as defined by the International Union of Pure Applied Chemistry (IUPAC), per gram of VS in the substrate fed into the assay (N mL CH_4/g VS). Theoretical methane yields, calculated from the ratio of COD/VS performed for every substrate, were 415 mL and 540 mL CH_4/g VS for February and March, respectively.

After the anaerobic digestion, the possible use of selected digestates as fertiliser was evaluated, analysing TS, VS, elements (C, H, N, S, P), heavy metals (Al, As, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Zn, Hg), and pathogens (*Salmonella spp.* and *E. Coli*).

2.4. Kinetic models

First order model (Eq. (3)) and the modified Gompertz equation (Eq. (4)) were applied to fit the cumulative methane production data from the experiments (Lay et al., 1996). The first order model fits successfully results of anaerobic biodegradability tests when the

hydrolysis reaction is the rate-limiting step. The modified Gompertz model fits better the cumulative methane production in batch assays when occurs inhibition, assuming that the methane production is function of bacterial growth (Bolado-Rodríguez et al., 2016). Moreover, the model parameters were calculated by minimising the least square difference between observed and predicted values.

$$\text{(Eq. 3)}$$

$$\text{(Eq. 4)}$$

In these equations, B represents the cumulative methane production (mL CH_4/g VS) and t is the time of the assay (d). These models estimate the methane production potential B_0 (mL CH_4/ g VS, related to the substrate biodegradability), the hydrolysis coefficient k_H (d^{-1}), the maximum biogas production rate Rm (mL CH_4/g VS d), and the lag time λ (d).

2.5. Analytical methods

The identification, quantification, and biometry measurements of microalgae were carried out by microscopic examination (OLYMPUS IX70) of microalgae samples (fixed with lugol acid at 5% and stored at 4°C prior to analysis) according to Sournia, (1978). The COD concentration was determined according to APHA Standard Methods (2005). The total and volatile solids were measured following the NREL (Van Wychen and Laurens, 2015a). The carbohydrate content was determined by acid hydrolysis and HPLC-RI using an NREL procedure (Van Wychen and Laurens, 2015b). The protein content in the raw materials was correlated with the Total Nitrogen Kjeldahl, multiplied by a factor of 5.95, and the lipid content was determined by the Kochert method (González Lopez et al., 2010). The determination of the carbon, nitrogen, and hydrogen content of the biomass was performed using a LECO CHNS-932 analyzer, while phosphorus, sulphur, and all the heavy metals analyses were carried out spectrophotometrically after acid digestion in a microwave according to the internal protocol of the Laboratory of Instrumental Analysis of The University of Valladolid.

The CO_2, H_2S, CH_4, O_2, and N_2 concentrations in the gas phase of biogas samples were determined using a Varian CP-3800 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 mm × 15 µm) and a CP-Pora BOND Q (25 m × 0.53 mm × 15 µm) columns (Posadas et al., 2015). The analysis of *Salmonella spp.* and *Escherichia Coli*

were measured following the UNE-EN ISO 6579:2003/A1:2007 and UNE-EN ISO 9308-2:2014, respectively.

3. Results and discussion

3.1. The pretreatments effect in terms of volatile solids solubilisation

Mixed biomasses were used in this study with different macromolecular compositions as shown in Section 2.1. These differences, mainly in carbohydrate and protein compositions, had an influence on the biogas production and kinetic. So, the comparison between pretreatments applied to the different biomasses was only studied in terms of general results. Molinuevo-Salces et al., (2016), who treated swine slurry at different temperatures, illumination periods, and NH_4^+ concentrations, also observed the influence of operational conditions in the biomass composition. Carbohydrate content increased from 35%-40% under non-favorable conditions and up to 50%-60% in the summer experiments.

Mass balances were made for all the experiments considering retained volatile solids in the solid fraction and released volatile solids in the liquid fraction. Additionally, the total mass of the both fraction from pretreatment were considered. The differences found between the initial VS and the total VS after pretreatment were always lower than ±10%. All the performed pretreatments solubilised volatile solids, but in different amounts, as shown in Figure 1 as the percentage of volatile solids retained. The alkaline and acid methods involved a high solubilisation of volatile solids while the bead milling or ultrasound methods solubilised only a small fraction of these solids. Contrary to what was expected, the retained volatile solid yield of alkaline-peroxide pretreatment was high, much like the results of the mechanical ultrasound method. This high solid recovery compared to the results of the basic pretreatment could be related to the low concentration of NaOH in these experiments. The most intense condition only increased remarkably volatile solids solubilisation for acid pretreatment with yields of retained volatile solids decreasing from 40% to 19%. A light increase was found for alkaline-peroxide (from 81% to 73%) and ultrasound pretreatments (from 86 to 76%). As previously reported for alkaline-peroxide pretreatment of mixed microalgae biomass composed mainly by *Scenedesmus* (Martín Juárez et al., 2016), no clear effect of severity in the studied range was observed for other pretreatments apart from the acid one.
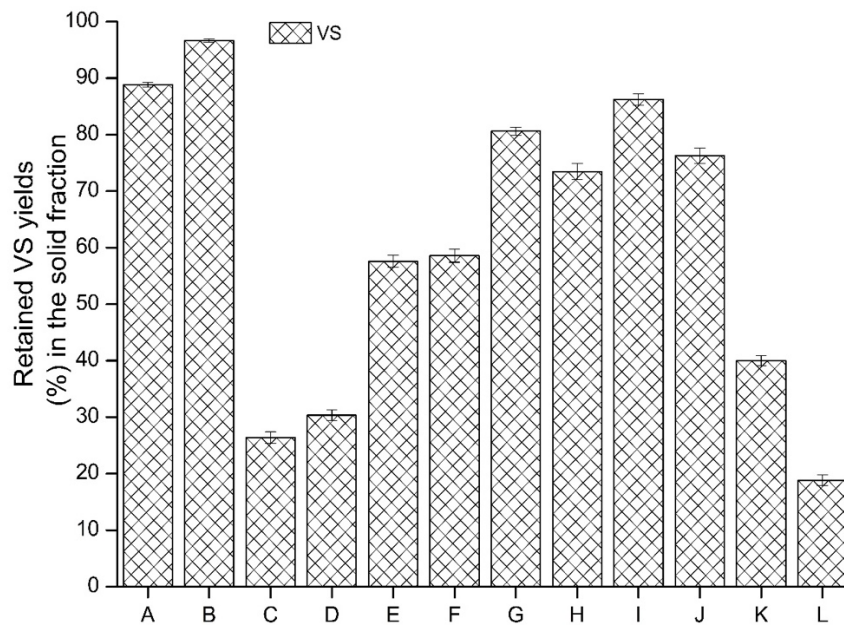
Figure 1. Percentage of volatile solids retained in the solid fractions with respect to the initial content of volatile solids.

Passos et al., (2016a) applied KOH and HCl at different concentrations (0.5, 1.25, and 2% w/w) at 80ºC for 2 hours to the biogas production from microalgal biomass grown in urban wastewater treatment. They reported around 50% of TOC solubilisation for the acid pretreatment and up to 200% for the alkaline pretreatment with respect to the thermal pretreatment (80ºC, 2 hours) as their control.

3.2. Biogas production

3.2.1. Test 1: BMP of untreated raw materials and of pretreated whole suspensions

The anaerobic digestion of whole suspensions after the pretreatments was carried out to harness volatile solids released in the liquid phase and to avoid a separation step. Figures 2 and 3 present the cumulative methane production curves from Test 1 in terms of methane production (the volume of methane gas produced per gram of volatile solid in the substrate). This test worked with untreated and pretreated whole suspensions from the microalgae biomass from February. Figure 4 presents the results of the microalgae biomass from March. Other terms such as biodegradability – defined as the percentage of the theoretical methane yield determined for raw substrates – and normalised production of methane (NP) – defined as the ratio between the production of methane per gram of VS from treated and untreated microalgae biomass – are used in this discussion.

Figure 2. Experimental results and fitting curves of cumulative methane production: untreated and whole pretreated fraction of microalgae biomass (Test 1). A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M.

Figure 3. Experimental results and fitting curves of cumulative methane production: untreated and whole pretreated fraction of microalgae biomass (Test 1). E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%.

Figure 4. Experimental results and fitting curves of cumulative methane production: untreated and whole pretreated fraction of microalgae biomass (Test 1). I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M.

For both biomasses, the biodegradability of the untreated microalgae was 39% with respect to the theoretical methane yield (415 mL CH_4/g VS for February and 540 mL CH_4/g VS for March algae). These values of biogas production from untreated biomass are comparable to a range of 106 mL to 146 mL CH_4 g/COD as reported by Molinuevo-Salces et al., (2016) who worked with different microalgae biomasses grown in pig manure. Contrary to our experiment, Passos et al., (2016b) reported lower methane yields in the biomass from March than in the biomass from February, with values of 72 mL and 128 mL CH_4/g COD, respectively.

The highest methane production of all the assays was achieved by alkaline pretreatment at the high NaOH concentration (D1) after overcoming an initial delay, with 377 mL CH_4/g VS; 91% of biodegradability and an NP value of 2.34. Although C pretreatment reported a slightly higher volatile solids release than D, the biogas production was remarkably lower and very similar to the untreated biomass (C1: 173 mLCH_4/g VS; 42% biodegradability and NP 1.08) and also contained a considerable lag phase. Passos et al., (2016a) reported increases on methane production of 82% with respect to the untreated biomass for alkaline pretreatment at low NaOH concentrations (0.5%, 80ºC, 2 hours), but

the methane production from the untreated biomass was very low in this study (78 mL CH_4/g VS).

The second-best result was achieved by the alkaline-peroxide pretreatment but working with a low peroxide concentration (G1: 279 mL CH_4/g VS; 67% of biodegradability and NP 1.73). In this case, the increase in the severity of the condition caused methane production to be slightly lower than the methane production of the untreated material (H1: 148 mL CH_4/g VS; 36% of biodegradability and NP 0.92), probably due to an inhibition that could not be coped with.

Despite the low effect on biodegradability, some pretreatments such as bead milling, and steam explosion had an advance of methane production. Biomass pretreated with both pretreatments achieved 90% of its total methane production at day 4. This advance was also reported by Gruber-brunhumer et al., (2015) but they reached an increase of 51% (289 mL CH_4/g VS) using milling (100 g of biomass mixed with 40 g of glass beads for 20 minutes, cooling to 20ºC) with respect to the untreated biomass (191 mL CH_4/g VS). No enhancement of methane production was observed at severe conditions of both pretreatments, reporting NP values of 1.00 and 0.91 for B1 and F1, respectively. For the mildest conditions, methane production increased slightly, reaching NP values of 1.06 and 1.11 for A1 and E1, respectively. Passos et al., (2015) reported a significant increase of 28% on the methane yield by hydrothermal pretreatment at 130ºC for 15 minutes (135 mL CH_4/g VS) with respect to the untreated mixed microalgae biomass from urban wastewater treatment.

The other pretreatment assays recorded no improvement with respect to the untreated biomass in terms of methane production and biodegradability. Acid pretreatments provided even lower methane production than untreated material with an NP of 0.95 for K1 and 0.90 for L1. However, Passos et al., (2016a) reported an increase of methane production of 86% with respect to the untreated biomass for acid pretreatment at 0.5%, 80ºC for 2 hours. However, as mentioned previously, the methane production in this study was very low.

Surprisingly, the biogas production was remarkably reduced by ultrasound pretreatment and further for the higher time conditions (J1: 137 mL CH$_4$/g VS; 25% of biodegradability and NP 0.66). The lag phase detected in biogas production from ultrasound pretreated biomass confirmed the possible inhibitory effect of this method. The decrease on biogas production with pretreatment time, even expending identical energy amount, could be related with the higher impact of time in inhibition. Similar behavior was observed by Passos et al., (2015) with no increase in methane production by ultrasound pretreatment. Gruber-brunhumer et al., (2015) reported an increase of 52% (292 mL/g VS) with respect to the untreated biomass by ultrasound pretreatment but they expended 20000 J/g TS, working with pure microalgae (*Acutodesmus obliquus*).

3.2.2. Test 2: BMP of solid fraction from pretreatments

Cumulative methane production curves from Test 2 are presented in Figures 5 and 6 (for February) and in Figure 7 (for March). These figures show the results from the solid fractions after the pretreatments and the results from the untreated microalgae biomasses.
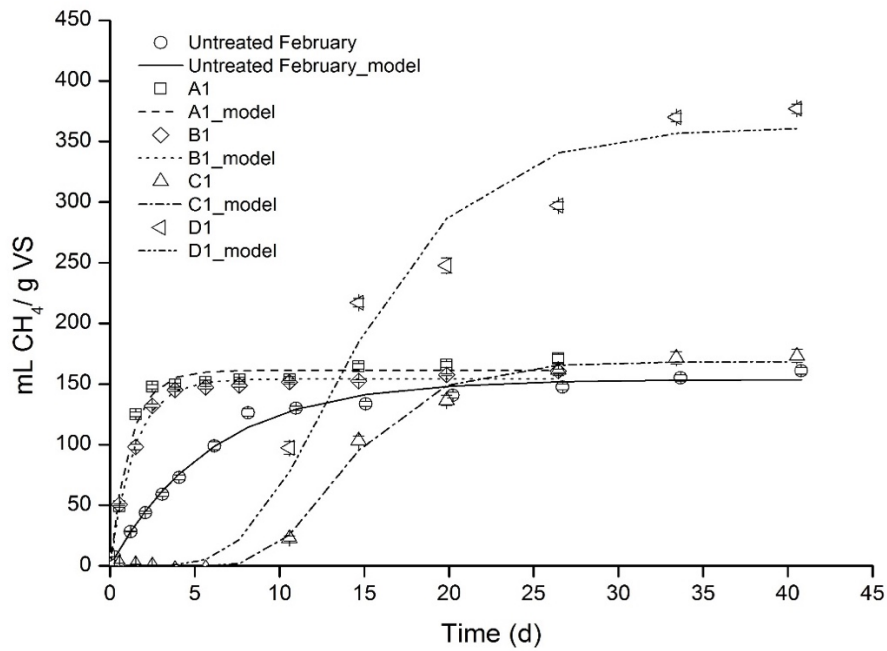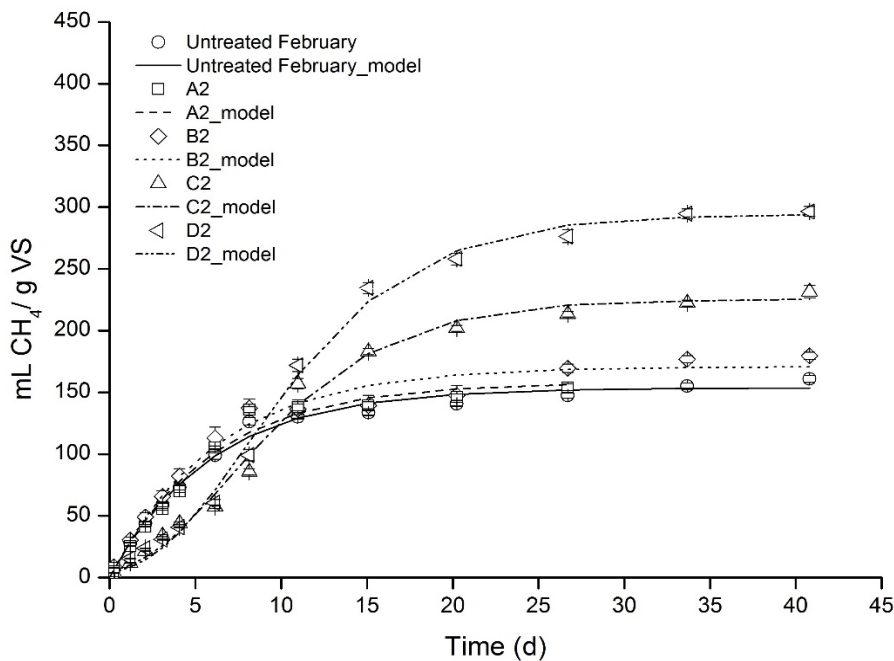

Figure 5. Experimental results and fitting curves of cumulative methane production: untreated and solid pretreated fraction of microalgae biomass (Test 2). A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M.
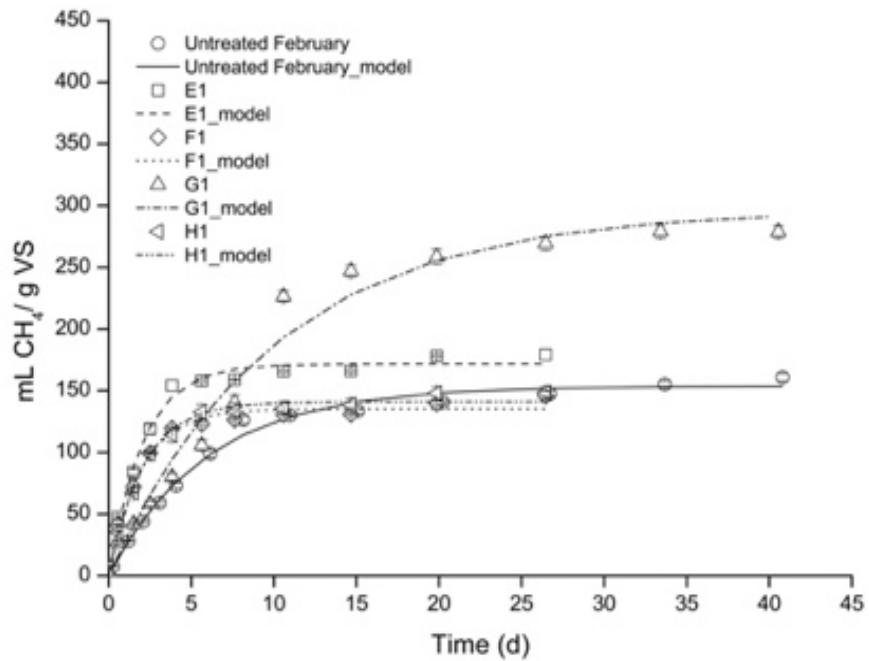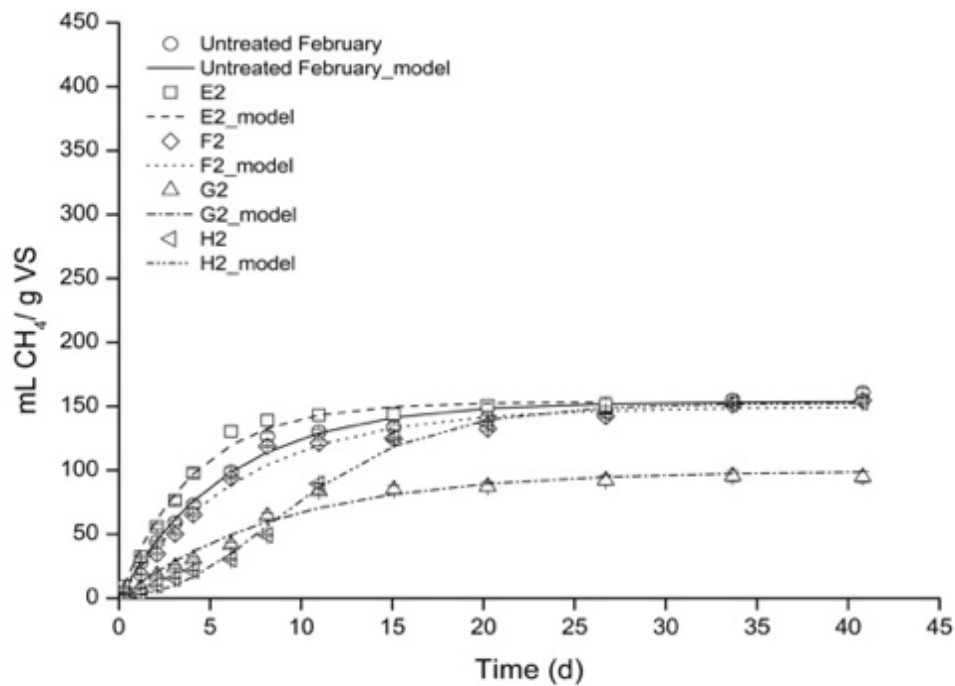
Figure 6. Experimental results and fitting curves of cumulative methane production: untreated and solid pretreated fraction of microalgae biomass (Test 2). E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%.
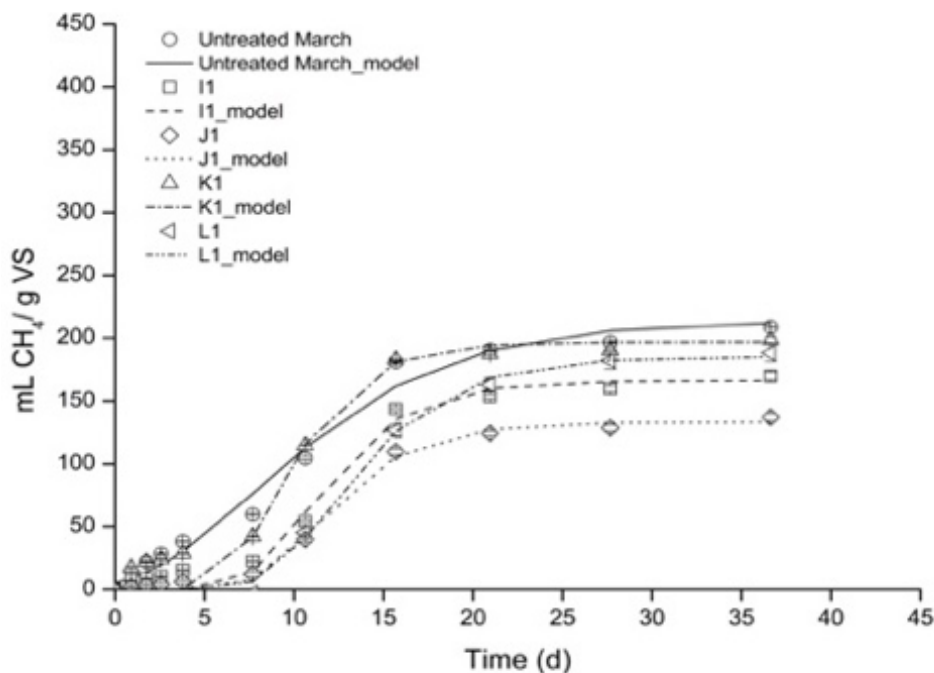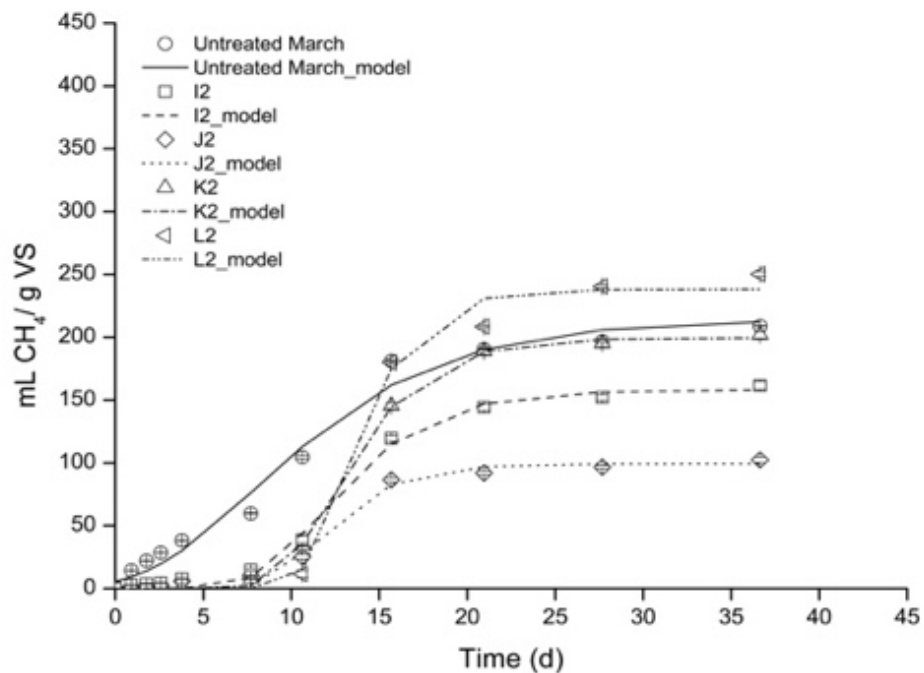

Figure 7. Experimental results and fitting curves of cumulative methane production: untreated and solid pretreated fraction of microalgae biomass (Test 2). I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M.

In this test, the solid fractions from alkaline pretreatment again provided the highest increase in methane production. Material pretreated with NaOH 2M (D2) achieved

methane production values of 296 mL/g VS, 71% of biodegradability and 1.84 of NP. Despite the fact that these values were the highest for Test 2, they were lower than the results achieved from whole suspension, demonstrating that the VS of liquid fractions were more biodegradable than the VS of solids. However, this behavior was not detected with the solid fraction of NaOH 0.5M which reached a higher methane production (232mL CH_4/g of VS) than whole fraction, with 56% biodegradability and an NP 1.44. In this case, the inhibition was reduced or avoided by removing the liquid phase since most of the possible inhibitory compounds were soluble. This low inhibition was confirmed with the shortening of lag phase with respect to experiments with whole suspensions.

Apart from the alkaline pretreatment, only acid pretreatment with HCl 2M increased the methane production (L2: 250 mL CH_4/g of VS; 46% biodegradability and NP 1.20) with respect to the untreated biomass and to the whole suspension. The inhibition played a key role in this pretreatment and decreased when the liquid fraction was removed.

The biodegradability of VS on the solid fraction from the alkaline-peroxide pretreatment at mild conditions was very low (G2: 95 mL CH_4/g of VS; 23% biodegradability and NP 0.59), showing a drastic reduction with respect to whole suspension but also to the untreated material. The VS retained in the solid fraction was high in this experiment (81%), and the possible high biodegradability of VS solubilised into the liquid fraction cannot justify this huge difference.

In the same way, bead milling pretreatment did not advance the anaerobic digestion of the solid fraction. However, a slight increase in methane production was only observed for B2 (180 mL CH_4/g of VS; 41% biodegradability and NP 1.12). Results of the other applied pretreatments were similar to those obtained from the whole suspension experiments.

In order to calculate the global methane production balance, the losses of volatile solids solubilised to the liquid phase during the pretreatment and removed in these experiments must be considered (Figure 1). Referring the methane production from the pretreated solid to the initial VS in the raw biomass before the pretreatment, only the bead mill

pretreatment for 60 minutes (B2) slightly enhanced the methane production with respect to the untreated biomass, with an NP of 1.08. For the other pretreatments, the increase in methane production by gram of volatile solid did not counteract the volatile solids' losses in the removed liquid fraction. If VS removal is considered, even the pretreatments with the highest biodegradability provided global NP values lower than 1, such as 0.38 (C2) and 0.56 (D2) for alkaline pretreatment or 0.39 (K2) and 0.23 (L2) for acid pretreatment.

3.3. Kinetics

Two different models were tested to fit the experimental results of cumulative methane production and to calculate the kinetic parameters. The first order model considers the hydrolysis reaction as the limiting step while the modified Gompertz equation considers bacterial growth and, hence, the inhibition of the process as the limiting step. Table 1 shows the model kinetic parameters that provided the best fit of methane production for each pretreatment and operational condition, working with the whole suspension and with only the solid fraction.

Table 1. Kinetic model and parameters of fitting equations of cumulative methane production from untreated and pretreated microalgae biomass using whole suspension and solid fractions from pretreatment.

Sample[a]	Kinetic Model[b]	B_0[c]	k_H[d]	λ[e]	R_m[f]	R^2[g]
Untreated_February	First order	154	0.167			0.9914
A1	First order	161	0.852			0.9805
A2	First order	158	0.168			0.9821
B1	First order	154	0.711			0.9951
B2	First order	172	0.166			0.9933
C1	Gompertz Model	168		9.34	18.18	0.9943
C2	Gompertz Model	226		1.89	15.90	0.9921
D1	Gompertz Model	362		7.80	27.09	0.9710
D2	Gompertz Model	295		2.63	19.97	0.9960
E1	First order	172	0.487			0.9890
E2	First order	153	0.246			0.9912
F1	First order	135	0.528			0.9868
F2	First order	150	0.147			0.9865
G1	First order	297	0.100			0.9788
G2	First order	100	0.112			0.9775
H1	First order	141	0.491			0.9913
H2	Gompertz Model	153		3.04	11.10	0.9957
Untreated_March	First order	214	0.055			0.9901
I1	Gompertz Model	167		7.39	19.11	0.9870
I2	Gompertz Model	158		7.91	16.10	0.9957
J1	Gompertz Model	133		7.79	15.34	0.9954
J2	Gompertz Model	99		8.60	14.00	0.9918

K1	Gompertz Model	197	6.18	26.63	0.9733
K2	Gompertz Model	200	9.25	24.35	0.9994
L1	Gompertz Model	185	8.44	18.36	0.9965
L2	Gompertz Model	238	10.67	37.96	0.9930

[a] Codes: Pretreatment: A: bead mill 5min; B: bead mill 60 min; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 min; J: ultrasound 21 min; K: HCl 0.5M; L: HCl 2M. Fractions used: 1, whole slurry and 2, solid fraction.
[b] B_0: methane production potential (mL CH_4/g VS). (Equations 3 and 4).
[c] k_H: hydrolysis coefficient in the first order kinetic model (d^{-1}). (Equation 3).
[d] λ: lag time (d). (Equation 4).
[e] R_m: maximum biogas production rate in the Gompertz model (mL CH_4/ g VS·d). (Equation 4).
[f] R^2: coefficient of determination.

In the case of the biomass from February, methane production from untreated and bead mill pretreatment (A and B) were fit using the first order kinetic. Bead mill pretreatment is a mild method, which gently opens the cell wall, generating scarce amounts of degradation compounds. Thus, the hydrolysis reaction was the limiting step in these cases. The methane potentials obtained for all the bead mill experiments were similar to that of the untreated microalgae biomass. The rapid increase of methane production previously mentioned for experiments with whole suspensions was reflected in the hydrolysis coefficient, which remarkably increased even more at the mildest conditions (A1).

Gompertz model was required for fitting the whole suspensions and solid fractions from alkaline conditions. This pretreatment was the most effective, increasing the methane potential up to 234% for NaOH 2M when working with the whole suspension. As expected, the lag period (inhibition) was longer for experiments with whole suspensions due to the presence of degradation compounds in the liquid fraction. However, the inhibition effect decreased with the NaOH concentration while also increasing the maximum biogas production rate. Pretreatment with NaOH 0.5 M caused a high lag phase but the mild conditions did not open the structure and enhance the methane production potential. The lag phase using only solid fractions was shorter, and pretreatment increased the methane production potential by nearly 150% for NaOH 0.5M and 200% for NaOH 2M. Nevertheless, they did not achieve the results that were obtained by using the whole fractions at a high NaOH concentration. Moreover, the high mass losses by solubilisation in these experiments should be still considered. Passos et al., (2016a) also used the Gompertz model to fit the methane production from microalgae grown in urban wastewater and pretreated with KOH, even while working with lower concentrations.

They reported lag phases that increased with the alkaline concentration from 1.20 days with KOH 0.5% up to 6 days with KOH 2.0%.

The results of steam explosion pretreatment were fit with first order model as the untreated biomass, with hydrolysis as the limiting step. The pretreatment increased the kinetic coefficients of whole suspensions three times (E1 and F1), but the methane production only increased 11% for E1. The results of methane production were similar to the untreated material. As detected in the bead milling pretreatment, the steam explosion pretreatment reduced the reaction time when working with whole suspensions but maintained or slightly increased the biogas production.

Regarding alkaline-peroxide pretreatment, all the conditions were fit with the first order model except for H2 which required the use of the Gompertz model. This behaviour was the opposite of that noticed in other chemical pretreatments because the inhibition appeared using only the solid fractions. Nevertheless, the methane production potential of H2 achieved the values of the untreated material with a lag period of 3 days while B_0 decreased remarkably for G2 (with milder conditions and no apparent inhibition). Regarding the whole suspensions, G1 practically doubled the methane production potential but decreased the kinetic. This effect was exactly the opposite when increasing the pretreatment severity.

The untreated biomass from March was fit with a first order model with higher methane production potential but a lower kinetic coefficient than the untreated biomass from February. The experimental results from all the assayed pretreatments were fit using the Gompertz model with a long lag phase from 6.2 to 10.7 days, showing a remarkable inhibitory effect. The only pretreatment providing a certain increase of methane production potential (20%) was the acid pretreatment at severe conditions (L2), when using only the solid fraction but with the longest lag phase (10.7). Passos et al., (2016a), working with HCl, reported lag phases that increased with the acid concentration (0.43 days for 0.5%, 3 days for 1.25%, and 5 days for 2%), but all the experiments required the Gompertz model to fit the results.

Additional research is necessary in order to identify the inhibitory compounds generated

by some of the pretreatments, which were unexpectedly retained in the solid phase. Further continuous anaerobic digestion tests would provide relevant information about acclimation of microorganisms to the pretreated substrates, which would enhance both methane production yields and microbial kinetic.

3.4. Fertilisers analysis

Table 2 shows the composition of some residues after anaerobic digestion in order to evaluate their possible application as fertilisers. Digestates from tests that achieved higher methane production than untreated biomass were selected (alkaline, alkaline-peroxide, and acid pretreatments). The content of nitrogen was clearly reduced in the samples from alkali media due to the effect of basic pH on protein release and ammonia stripping. The NPK content of digestate from pretreated samples was always lower than from untreated biomasses, but higher than the minimum legal threshold value of 7% (w/w). This excess was very low for samples from the biomass from March. The ratio C/N increased in basic pretreatments, because of N removal, but remained lower than the maximum allowed value of 15. The content of As was much lower than the maximum limit of 50 mg/kg. The minimum legal content of the other analysed elements depends of the fertilisers use: extensive and grasing cultivation, fertirrigation or horticultural use, and foliar; but Cu and Mn supplementation would likely be necessary (Reglamento CE 2003/2003, 2003).

Regarding microbiology, the digestate from the untreated biomass from February did not contain pathogens and the results did not provide information about a possible sterilisation effect of these pretreatments. However, a clear sterilising effect of acid pretreatment was observed, remarkably reducing the *E.coli* content of the final digestate.

In summary, the digestates from anaerobic digestion of algal biomass grown in pig manure have a potential application as fertilisers. The initial microalgae biomass composition should be considered, mainly for the variability of nitrogen content throughout the year and the cultivation conditions.

Table 2. Main parameters analysed for the characterisation as a fertiliser of anaerobic digestate of untreated and selected pretreated algal biomasses

	Untreated_February	C2	D1	D2	G1	Untreated_March	K1	K2	L1	L2
ST	1.090	1.232	1.453	2.504	1.423	1.824	1.931	1.843	2.048	2.366
SV[a]	51.656	46.541	36.619	23.695	45.566	40.185	34.147	33.900	35.800	23.732
C[a]	34.260	21.270	17.670	10.180	19.260	21.100	21.890	17.220	19.100	14.720
N[a]	7.500	2.640	1.870	1.360	2.470	2.420	2.120	1.800	1.710	1.520
P[a]	4.105	4.091	3.765	2.155	3.470	2.177	1.749	2.167	1.669	1.772
S[a]	1.705	1.383	1.348	0.801	1.284	1.073	0.958	1.060	0.935	0.841
Hg[a]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Al[a]	1.041	0.817	0.762	0.375	0.691	0.547	0.512	0.623	0.519	0.517
As[a]	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Ca[a]	5.018	5.252	6.036	2.229	4.830	10.079	9.754	9.677	9.457	9.854
Cr[a]	0.003	0.003	0.003	0.001	0.002	0.002	0.002	0.002	0.002	0.002
Cu[a]	0.020	0.024	0.032	0.014	0.020	0.013	0.017	0.013	0.011	0.010
Fe[a]	2.399	1.783	1.626	0.824	1.532	1.219	1.057	1.280	1.065	1.090
K[a]	3.013	2.308	1.643	1.387	2.489	1.267	0.912	1.139	0.836	0.936
Mg[a]	1.156	0.987	0.939	0.433	0.923	0.461	0.367	0.642	0.384	0.495
Mn[a]	0.023	0.049	0.064	0.022	0.032	0.000	0.000	0.000	0.000	0.000
Ni[a]	0.006	0.005	0.005	0.002	0.004	0.003	0.003	0.003	0.003	0.003
Pb[a]	0.005	0.004	0.004	0.002	0.003	0.003	0.003	0.003	0.003	0.003
Zn[a]	0.248	0.246	0.362	0.144	0.237	0.169	0.104	0.179	0.118	0.141
Salmonella[b]	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence	Absence
E.coli[c]	<1	<1	<1	<1	<1	1.10E+05	1.00E+05	9.10E+04	<1	1.30E+03

[a]: percentage in dry weight (g*100/g dried)
[b]: 25g. Limit: absence
[c]: NMP/g. Limit: <1.0E3

4. Conclusions

Acid and alkaline pretreatments solubilised high percentage of VS but induced a remarkable inhibition. The highest methane production enhancement was achieved with whole broth of alkaline (234%) and alkaline-peroxide (173%) pretreatments, while bead mill and steam explosion increased the methane production rate by a factor of 5 and 3, respectively. The methane yield was not improved by removing the liquid phase. The fitting to kinetic models revealed the impact of each pretreatment in terms of hydrolysis or inhibition. Finally, the composition of the digestates, with NPK higher than 7% (w/w) and C/N lower than 15, allows their use as fertilisers.

5. Acknowledgements

6. References

[1] Acién F.G., Fernández, J.M., Molina-Grima E., 2014. Economics of Microalgae Biomass Production, in: Pandey A., Lee D.J., Chisti Y., Soccol C.R. (Eds) Biofuels from Algae, Burlington, pp. 313–325

[2] Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I., 2012. Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment. Bioresour. Technol. 123, 488–494.

[3] Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J.B., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays. Water Sci. Technol. 59, 927–934.

[4] APHA, Water Environment, APHA, 2005. Standard Methods for the Examination of Water and Wastewater Part 1000 Standard Methods for the Examination of Water and Wastewater.

[5] Bolado-Rodríguez, S., Toquero, C., Martín-Juárez, J., Travaini, R., García-Encina, P.A., 2016. Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. Bioresour. Technol. 201, 182–190.

[6]   Reglamento CE 2003/2003. Diario Oficial de la Unión Europea, Luxemburgo, 13 Octubre 2003.

[7]   González López, C.V., Cerón García M.C., Acién Fernández F.G., Segovia Bustos C., Chisti Y., Fernández Sevilla J.M., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591.

[8]   Gruber-brunhumer, M.R., Jerney, J., Zohar, E., Nussbaumer, M., Hieger, C., Bochmann, G., 2015. Acutodesmus obliquus as a benchmark strain for evaluating methane production from microalgae: Influence of different storage and pretreatment methods on biogas yield. Algal Research, 12, 230–238.

[9]   Jankowska, E., Sahu, A.K., Oleskowicz-Popiel, P., 2017. Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. Renew. Sustain. Energy Rev. 75, 692–709.

[10] Kavitha, S., Subbulakshmi, P., Rajesh Banu, J., Gobi, M., Tae Yeom, I., 2017a. Enhancement of biogas production from microalgal biomass through cellulolytic bacterial pretreatment. Bioresour. Technol. 233, 34–43.

[11] Kavitha, S., Yukesh Kannah, R., Rajesh Banu, J., Kaliappan, S., Johnson, M., 2017b. Biological disintegration of microalgae for biomethane recovery-prediction of biodegradability and computation of energy balance. Bioresour. Technol. 244, 1367–1375.

[12] Klassen, V., Blifernez-Klassen, O., Wobbe, L., Schlüter, A., Kruse, O., Mussgnug, J.H., 2016. Efficiency and biotechnological aspects of biogas production from microalgal substrates. J. Biotechnol. 234, 7–26.

[13] Lay, J.J., Li, Y.Y., Noike, T., 1996. Effect of Moisture Content and Chemical Nature on Methane Fermentation Characteristics of Municipal Solid Wastes. J. Environ. Syst. and Eng. 552, 101–108.

[14] Marcos, M., García-Cubero, M.T., González-Benito, G., Coca, M., Bolado, S., Lucas, S., 2013. Optimisation of the enzymatic hydrolysis conditions of steam-exploded wheat straw for maximum glucose and xylose recovery. J. Chem. Technol. Biotechnol. 88, 237–246.

[15] Martín Juárez, J., Lorenzo Hernando, A., Muñoz Torre, R., Blanco Lanza, S., Bolado Rodríguez, S., 2016. Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment. Bioresour. Technol. 218, 265–271.

[16] Molinuevo-Salces, B., Mahdy, A., Ballesteros, M., González-Fernández, C., 2016. From piggery wastewater nutrients to biogas: Microalgae biomass revalorisation through anaerobic digestion. Renew. Energy 96, 1103–1110.

[17] Murphy, J.D., Drosg, B., Allen, E., Jerney, J., Xia, A., Herrmann, C., 2015. A Perspective on algal biomass.

[18] Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J. Biotechnol. 150, 51–56.

[19] Passos, F., Carretero, J., Ferrer, I., 2015. Comparing pretreatment methods for improving microalgae anaerobic digestion: Thermal, hydrothermal, microwave and ultrasound. Chem. Eng. J. 279, 667–672.

[20] Passos, F., Felix, L., Rocha, H., Pereira, J. de O., de Aquino, S., 2016a. Reuse of microalgae

grown in full-scale wastewater treatment ponds: Thermochemical pretreatment and biogas production. Bioresour. Technol. 209, 305–312.

[21] Passos, F., Federal, U., Preto, O., Brockmann, D., Transfert, I., 2016b. Microalgae production in wastewater treatment systems , anaerobic digestion and modelling using ADM1. Algal Research, 10, 55–63.

[22] Posadas, E., Serejo, M.L., Blanco, S., Pérez, R., García-Encina, P.A., Muñoz, R., 2015. Minimisation of biomethane oxygen concentration during biogas upgrading in algal – bacterial photobioreactors. Algal Research, 12, 221–229.

[23] Postma, P.R., Suarez-Garcia, E., Safi, C., Olivieri, G., Olivieri, G., Wijffels, R.H., Wijffels, R.H., 2017. Energy efficient bead milling of microalgae: Effect of bead size on disintegration and release of proteins and carbohydrates. Bioresour. Technol. 224, 670–679.

[24] Sournia, A., 1978. Phytoplankton Manual, Unesco, United Kingdom.

[25] Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.

[26] Van Wychen, S., Laurens, L.M.L., 2015a. Determination of Total Solids and Ash in Algal Biomas. NREL/TP-5100-60956.

[27] Van Wychen, S., Laurens, L.M.L., 2015b. Determination of Total Carbohydrates in Algal Biomass. NREL/TP-5100-60957.

Chapter 4

Evaluation of pretreatments for production of fermentable monosaccharides from microalgae biomass grown in piggery wastewater

Evaluation of pretreatments for production of fermentable monosaccharides from microalgae biomass grown in piggery wastewater

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ABSTRACT

Microalgae-bacteria biomass cultured in wastewater is an interesting renewable material capable of metabolising nutrients into proteins, carbohydrates and lipids through photosynthesis. The recovery and valorisation of these components would improve the economic viability of microalgae-based processes. This work evaluates several alternatives for monosaccharides production from the carbohydrate fraction of this biomass through different mechanical and chemical disruption methods, coupling these pretreatments with enzymatic hydrolysis processes. Six different pretreatments (bead milling, alkaline hydrolysis, steam explosion, alkali-peroxide treatment, ultrasounds, and acid hydrolysis) were evaluated at two extreme (low and high) conditions to study not only the carbohydrate solubilisation, but also the degradation and the recovery of monosaccharides. The co-solubilisation of proteins and lipids was also analysed as a result of applying the bio-refinery concept. The acid pretreatment with 2M HCl provided the highest carbohydrate solubilisation yield (98%) and monosaccharide recovery (81%), with low degradation. This pretreatment also solubilised 76% of the proteins and 56% of the lipids. Chemical pretreatments generated high concentrations of degradation byproducts and completely degraded the bacterial DNA of the biomass, as shown by agarose gel electrophoresis of genomic DNA analysis. The effect of the subsequent enzymatic hydrolysis step of pretreated samples was evaluated. The hydrolysis of the solid phase from pretreatment and of the whole pretreated suspension were compared to determine the inhibitory effect of the degradation compounds generated in the pretreatment step and present in the liquid phase. Enymatic hydrolysis enhanced carbohydrate solubilisation, reaching yields of 92% for 2M NaOH or 85% for bead mill pretreated biomass using the whole suspension from pretreatment. Removal of the liquid phase from pretreated samples did not improve the enzymatic hydrolysis efficiency.

Keywords: Bacteria; Degradation; Enzymatic hydrolysis; Lipids; Proteins;Valorisation.

1.   Introduction

The cultivation of microalgae has been boosted in recent decades due to their high potential for CO_2 mitigation and wastewater treatment and their use as feed for animals or as feedstock for fertilisers or biofuel production. A cost-competitive process could be achieved by coupling wastewater treatment in algal-bacterial photo-bioreactors with the

valorisation of the biomass produced [1][2]. Bacteria and microalgae grow symbiotically in wastewater treatment photo-bioreactors, using the oxygen generated by microalgal photosynthesis for the oxidation of organic matter and the assimilation of nutrients. Indeed, these algal-bacterial photo-bioreactors for wastewater treatment are nowadays under research in order to enhance the recovery of nutrients and reduce the cost of microalgae cultivation [3][4]. This research is particularly relevant in the valorisation of livestock wastewaters as a result of their high content of N and P [5].

The composition of the microalgae biomass produced during wastewater treatment depends on the wastewater composition and the cultivation conditions [6]. Typically, the main application of microalgae biomass grown in wastewater has been to produce biogas [7][8]. However, the economic viability of microalgae-based processes could be significantly improved through the fractional recovery of the main components of the biomass (carbohydrates, proteins and lipids) using a bio-refinery approach [9]. The development of sequential valorisation schemes for microalgae-bacteria biomass is economically and technically important. In this context, the recovery of carbohydrates as fermentable monosaccharides, which are useful for the production of sustainable biofuels, is of special interest [10]. However, most microalgae species able to grow in wastewaters are very recalcitrant and the application of pretreatments to disrupt the cell wall and release the carbohydrates is required [11][12]. Thus, microalgae biomass pretreatment plays a critical role in the downstream process and affects both the product recovery and the quality of the extracted products [13]. Additionally, when working with consortia of microalgae and bacteria, the possible sterilising effect of chemical pretreatment is of great interest in order to avoid the metabolic degradation of released components or further products by alive microorganisms [14].

Biomass pretreatments result in the solubilisation of carbohydrates, but also of other components like proteins and lipids. Understanding of the effect of the microalgae biomass pretreatment on the solubilisation of each component is essential to design sequential biomass valorisation processes [15]. Moreover, solubilised carbohydrates could be degraded either by severe pretreatment conditions or by the active metabolism of microrganisms present in the raw biomass. This degradation ultimately decreases the recovery of monosaccharides and can produce inhibitory byproducts that reduce both the

quantity and the quality of the final products [16]. For example, Hernandez et al. [17] reported the production of formic and acetic acids applying a combination of acid hydrolysis with autoclave to cultures of pure microalgae. Likewise, Miranda et al. [18] detected the presence of acetic, formic, lactic, butyric and propionic acids in the acid hydrolysates of pure cultures of *Scenedesmus obliquus*. Therefore, the determination of these inhibitory compounds is essential for the optimisation of the global process, although scarce information about this topic is found in literature.

The aim of this work is to evaluate alternative processes for the production of fermentable monosaccharides from microalgae biomass grown in pig manure treatment photo-bioreactors. The effect of different pretreatments (bead milling, alkaline hydrolysis, steam explosion, alkali-peroxide, ultrasounds, and acid hydrolysis) at different operational conditions on carbohydrate solubilisation and degradation was analysed. Besides the fermentable monosaccharides production, solubilisation of proteins and lipids was also determined. Enzymatic hydrolysis of the whole pretreated suspension and of the solid pretreated fraction after pretreatment was performed, analysing carbohydrate, protein and lipid solubilisation and monosaccharide recovery and carbohydrate degradation in this step. The concentration of degradation byproducts after the pretreatments and the enzymatic hydrolysis was quantified in order to evaluate the possible inhibition of further processes. Finally, aiming to assess the possible competition between microorganisms to be used in subsequent fermentation steps and bacteria and the risk of metabolic degradation of products, the effect of the pretreatments and the enzymatic hydrolysis on bacteria viability was estimated.

2. Materials and methods

4.1. Microalgae biomass

The biomass used in this work was the same that was used in our previous study about biogas production [8]. Fresh algal-bacterial biomass was cultivated in a thin-layer 1200 L photo-bioreactor [19] treating pig manure and collected at two different times of the year. The biomass composition from the first batch (on a dry weight basis) was 23.67% carbohydrates, 42.55% proteins, 16.74% lipids, and 16.83% ash, and the main microalgae families in the biomass were Scenedesmaceae (71%), Aphanothecaceae (11%) and Chlorellaceae (12%). The biomass composition from the second batch was 38.11%

carbohydrates, 24.83% proteins, 12.51% lipids, and 24.50% ash; and the families of microalgae identified within were Scenedesmaceae (73%) and Naviculaceae (27%) [8]. The biomass, concentrated by centrifugation at ~ 21%, was kindly supplied by the Cajamar Foundation (Almeria, Spain) and refrigerated at 4 °C for a maximum of 48 h prior to use.

4.2. Pretreatments

Bead mill, alkaline (NaOH), steam explosion, and alkali-peroxide (H_2O_2) pretreatments were applied to the biomass from Batch 1, and ultrasound and acid (HCl) pretreatments were applied to the biomass from Batch 2. Each pretreatment was performed at two different levels (Table 1), in identical operational conditions as those previously described in [8]. All the experiments were performed in duplicate for this research, working with biomass suspensions of 5% (w/w dw) concentration.

Table 1. Experimental conditions of the pretreatment tests

Code	Type of pretreatment	Conditions	Time (minutes)	Temperature
A	Bead mill	1.25 mm beads	5	Room temperature
B	Bead mill	2.50 mm beads	60	Room temperature
C	Alkaline	NaOH 0.5M	60	121ºC
D	Alkaline	NaOH 2M	60	121ºC
E	Steam explosion	Saturated steam + flash	5	130ºC
F	Steam explosion	Saturated steam + flash	20	170ºC
G	Alkali-peroxide	H_2O_2 0.5% (w/w) pH 11.5	60	50ºC
H	Alkali-peroxide	H_2O_2 7.5% (w/w) pH 11.5	60	50ºC
I	Ultrasound	479 W, 7186 J/g TS	5	Room temperature
J	Ultrasound	115W, 7186 J/g TS	21	Room temperature
K	Acid	HCl 0.5M	60	121ºC
L	Acid	HCl 2M	60	121ºC

Two types of enzymatic hydrolysis experiments were carried out after each pretreatment, one using the whole pretreated suspensions (both the solid and liquid fractions (hereby denoted as W)), and other using only the solid pretreated fraction (hereby denoted as S). The solid fractions were obtained as follows: a portion of each pretreated suspension was centrifuged at 10000 rpm for 10 min, the solid and liquid fractions were weighed and a sample of the solid fraction was used for enzymatic hydrolysis experiments. Total solids

and volatile solids were analysed in both the solid and the liquid fractions; carbohydrate, protein and lipid contents were analysed in the solid fractions; and monosaccharides and degradation byproducts (oxalic, formic, acetic, lactic, butyric, succinic and levulinic acids, as well as methanol, xylitol, glycerol, ethanol, acetone, furfural and HMF) were analysed in the liquid fractions [18]. Bacteria viability and DNA integrity were analysed in the solid fractions.

Mass balances were checked using the total and volatile solids. The following parameters were defined in order to understand the process and to determine the solubilisation of the components, the degradation of solubilised carbohydrates and, hence, the recovery of monosaccharides from the pretreatment step:

$$Eq. (1)$$

$$Eq. (2)$$

$$Eq. (3)$$

where PR is the initial raw biomass; component refers to carbohydrates, proteins and lipids; PRS is the solid fraction after pretreatment, and PRL is the liquid fraction after pretreatment.

4.3. Enzymatic hydrolysis

Enzymatic hydrolysis assays were carried out with the whole suspension (solid and liquid fractions) after biomass pretreatment and with the solid fractions after pretreatment resuspended in distilled water at 5% w/w dry biomass in order to determine the influence of the pretreatment and the potential inhibitory compounds on the solubilisation of biomass and the monosaccharide recovery. The tests were performed in 100 mL Erlenmeyer flasks containing 25 mL of pretreated biomass suspension (pH was adjusted at 4.9 ± 0.1), 1M citrate buffer and the volume of enzymes required for each pretreated sample to obtain 10 FPU/g of cellulose (Celluclast 1.5L - Cellulase) and 20 CBU/g of cellulose (Novozyme 188 – β-glucosidase) [20]. The assays were incubated in a rotatory shaker at 50 ºC and 300 rpm for 12h. The experiments were performed in duplicate for each sample obtained from replicated pretreatment experiments.

After the enzymatic hydrolysis, the solid and liquid fractions were separated by centrifugation (10 min, 10000 rpm), weighed and analysed. Total and volatile solids were analysed in both fractions which was used to estimate mass balances. Carbohydrates, proteins, and lipids were analysed in the solid fractions; and monosaccharides and probable degradation byproducts were analysed in the liquid fractions [8]. Bacteria viability and DNA integrity was analysed in the solid fractions after enzymatic hydrolysis experiments.

To quantify the effect of the enzymatic hydrolysis and the global yields of the combined processes (pretreatment plus enzymatic hydrolysis), the following parameters were defined:

$$\text{Eq. (4)}$$

$$\text{Eq. (5)}$$

$$\text{Eq. (6)}$$

$$\text{Eq. (7)}$$

$$\text{Eq. (8)}$$

where EH is the biomass subjected to the enzymatic hydrolysis, component refers to carbohydrates, proteins and lipids, PRS is the solid fraction after the pretreatment (PRS = initial biomass for enzymatic hydrolysis of raw biomass samples), EHS is the solid fraction after the enzymatic hydrolysis, EHL is the liquid fraction after the enzymatic hydrolysis, PRL is the liquid fraction after the pretreatment (which is only applicable on the tests where the whole pretreated suspension was used (W) (PRL= 0 for raw biomass and the experiments with solid fracions from pretreatments)) and PR is the initial biomass before pretreatment.

4.4. Analytical methods

Following the NREL protocols, the total and volatile solids content (TS, %; VS, %) were measured in the raw biomass, solid fractions, liquid fractions, and whole suspension after the biomass pretreatment and the enzymatic hydrolysis experiments [21]. The lipid content was determined using a protocol based on a chloroform-methanol 2:1 extraction applying the Kochert method [22]. The protein content was determined using the Total Nitrogen Kjeldahl method and applying an N-to-P ratio of 5.95 [23].

Monosaccharides and degradation byproducts were quantified by HPLC using a Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module. A refractive index detector (Waters 2414) was used to quantify the concentration of monosaccharides and degradation byproducts, such as methanol, xylitol, glycerol, ethanol and acetone [8]. Other degradation byproducts (oxalic, formic, acetic, lactic, butyric, succinic and levulinic acids, furfural and HMF) were measured with a photodiode detector (Waters 2998) at 210nm. External standards of monosaccharides and degradation byproducts with a purity > 95% were used for quantification (Sigma Aldrich, Spain). The carbohydrate content in the solid fractions was determined as monosaccharides, after concentrated acid hydrolysis, using an NREL procedure [24] and the HPLC-IR method previously described. All the analyses were carried out in duplicate for each experiment.

4.5. Bacteria viability and DNA integrity

DNA integrity was visualised in an 1.6% agarose gel to estimate the effect of each pretreatment on the bacterial viability. To quantify viable bacteria in the biomass samples, the genes of the bacterial 16S ribosomal ribonucleic acid (rRNA) were amplified using a quantitative polymerase chain reaction (qPCR), using a standard curve to estimate the copy number concentration of standard DNA molecules (copies/µL). To differentiate live and dead bacteria, the biomass was incubated with propidium monoazide (PMA), a nucleic acid intercalating dye that selectively penetrates bacteria with compromised membranes, which can be considered dead [25][26]. Once inside the bacteria, and after exposure to strong visible light, PMA covalently crosslinks DNA, interfering with DNA amplification by qPCR. Thus, after incubation with PMA, only genomes of bacteria with the entire lipid membrane barrier, not affected by PMA, can be amplified with qPCR and quantified as viable bacteria.

Briefly, 2 mL aliquots of biomass were incubated with PMA (20μM; Biotium, USA) on darkness for 20 min. Then, the tubes were set on ice at 20 cm under a Floodlight LED 100W 4000K lamp (Ledvance Projector LED, 2017) for 30 min. The samples were centrifuged (10,000 g, 10 min, 4ºC), and the pellets were used for DNA extraction.

DNA was extracted, before and after the incubation with PMA, from the raw biomass, and from the solid fractions after each pretreatment and after each enzymatic hydrolysis assays, using the Fast® DNA Spin Kit for Soil (MP Biomedicals, LLC) according to the manufacturer's instructions. The integrity of the extracted DNA was visualised in an 1.6% agarose gel. The genes of the bacterial 16S ribosomal ribonucleic acid (rRNA) were amplified by Quantitative Polymerase Chain Reaction (PCR) using the universal Eubacteria primers E1052f (5'TGCATGGYTGTCGTCAGCTCG) and E1193r (5'CGTCRTCCCCRCCTTCC) (Wang and Qian, 2009). For each sample, three 20-μL PCR reactions, each containing 10 μL SYBR® Green Supermix (BioRad, USA), 0.8 μl of each primer (10nM), and 1μL DNA template, were conducted in the iCycler IQTM Real-Time PCR Detection System (Bio-Rad, USA), set as 95 °C 5 min, 25 x (15 s 95 °C; 20 s 63.5 °C; 20 s 72 °C; 15 s 81 ºC) and 81 x (81 °C 30 s) and hold at 4 ºC.

5. Results and discussion

5.1. *Influence of biomass pretreatments on carbohydrate, protein and lipid solubilisation*

The analysis of the total and volatile solids of the different fractions before and after biomass pretreatment are in agreement with the mass balances. The alkaline and acid methods solubilised the highest amounts of carbohydrates, which resulted in PR yields of 56% when using 0.5M NaOH for the biomass in Batch 1 (Fig. 1) and a yield of 98% when using 2M HCl for the biomass in Batch 2 (Fig. 2). Mahdy et al. [27] and Kassim and Bhattacharya [28] reported slightly lower carbohydrate solubilisation yields of 43.5% (5% NaOH (w/v), 50ºC, 48h) for pure *Scenedesmus* sp, and 36% (2% KOH (w/v), 120ºC, 2h) for pure *Tetraselmis suecica* microalgae. However, Shokrkar et al. [29] achieved higher carbohydrate solubilisation (80%) using alkali pretreatment, but similar values

(94%) using acid pretreatment, working with a microalgae-bacteria biomass cultured in domestic wastewater, under comparable conditions.
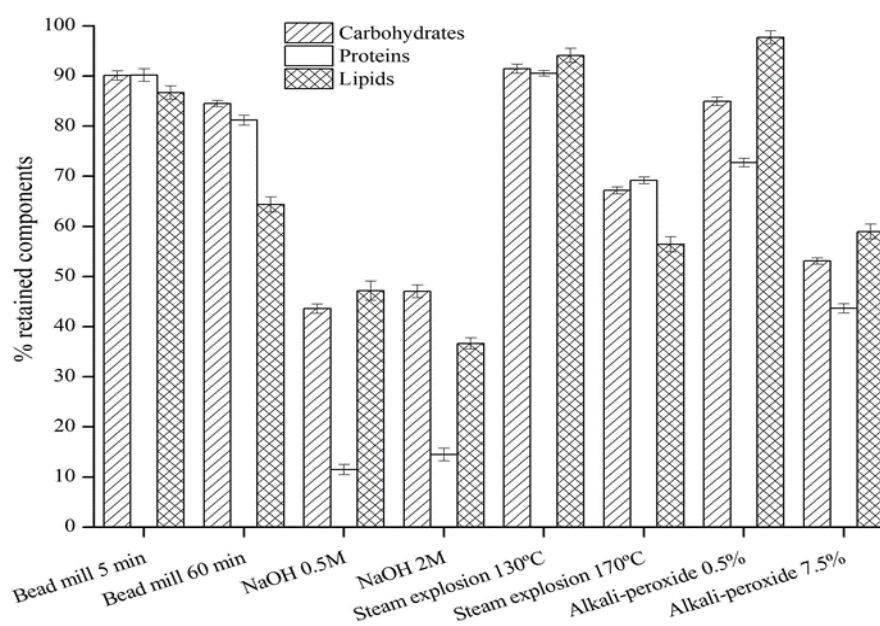
Figure 1. Retained components (%) in the solid fraction after biomass pretreatments based on their initial content for Batch 1 biomass. The results are expressed as means ± standard deviations of 4 analytical determinations (duplicated treatments analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Alkaline pretreatment at 0.5 and 2 M NaOH also resulted in a high solubilisation of proteins (88% and 85%, respectively) and lipids (53% and 63%, respectively) (Fig. 1). In this context, biomass pretreatment at 0.5M NaOH solubilised carbohydrates, proteins and lipids in a mass ratio of 1.51/4.26/1.00 compared to the 1.41/2.54/1.00 ratio determined in the raw biomass. Therefore, alkaline pretreatment released proteins preferentially by cleaving intermolecular linkages between complex polysaccharydes and fibers and other polymeric compounds while acid reagents are able to break complex carbohydrates into monosaccharides, as explained by Solé-Bundó et al. [30]. Previous studies about protein recovery from pure microalgae using alkaline hydrolysis always applied low temperatures and short contact times to preserve the structure of the desired product. These milder pretreatment conditions typically resulted in lower protein solubilisation yields than those obtained in this work, but probably maintained a higher preservation proteins [31].
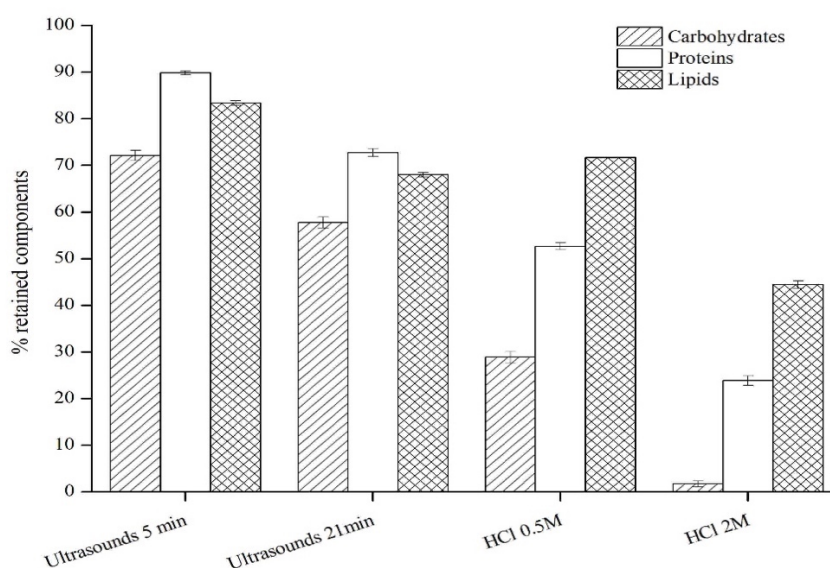
Figure 2. Retained components (%) in the solid fraction after biomass pretreatments based on their initial content for Batch 2 biomass. The results are expressed as means ± standard deviations of 4 analytical determinations (duplicated treatments analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Acid pretreatment also provided high solubilisation yields for all the biomass components, and a remarkable effect of the HCl concentration (Fig. 2) was observed. This significant impact of chemical concentration of dilte acid pretreatment was also reported by Dong et al. [32] working with different microalgae strains (*Chlorella*, *Nannochloropsis* and *Scenedesmus*). An increase in HCl concentration from 0.5 to 2M enhanced the PR carbohydrate, protein and lipid solubilisation yields from 71%, 47% and 28% to 98%, 76% and 56%, respectively. The solubilised carbohydrate/protein/lipid mass ratio after 0.5M HCl method was 7.67/3.33/1.00, respectively, compared to the ratio of 3.05/1.99/1.00 in the raw biomass of Batch 2. Markou et al. [33] also reported an increase on carbohydrate solubilisation with the acid concentration, solubilising 90% of carbohydrates for pure *Spirulina platensis* using 0.5N HNO_3 or 2.5N HCl (100°C and 180min).

Alkali-peroxide and steam explosion pretreatments remarkably solubilised all the components only when using the most severe operational conditions. Alkali-peroxide pretreatment using 7.5% H_2O_2 resulted in PR carbohydrate, protein and lipid solubilisation yields of 47%, 56% and 41%, respectively. Martín Juárez et al. [34] studied the effect of alkali-peroxide pretreatment on *Scenedesmus* biomass cultured in domestic

wastewater and obtained carbohydrate solubilisation yields similar to this work (51.3%), also using 7.5% H_2O_2.

Steam explosion at 170ºC resulted in PR carbohydrate, protein and lipid solubilisation yields of 33%, 31% and 44%, respectively. Lorente et al. [35] and Mendez et al. [36] reached higher carbohydrate solubilisation under both milder and similar conditions (44% for pure *Nannochloropsis gaditana* at 150ºC, 20 min, and 69% for pure *Chlorella vulgaris* at 180ºC, 20 min). This difference could be attributed to the high resistance of the cell wall of the genus *Scenedesmus* [37].

Ultrasound pretreatment exhibited a remarkable effect of the application time under identical energy inputs. Thus, PR carbohydrate, protein and lipid solubilisation yields increased from 28%, 10% and 17%, respectively, after 5 minutes of ultrasound pretreatment (479 W, I) to 42%, 27% and 32% after 21 minutes of ultrasound pretreatment (115W, J). The key role of the ultrasound time on carbohydrate solubilisation was previously reported by Zhao et al. [38], but in experiments using different amounts of energy. On the contrary, Passos et al. [39] obtained a negligible release of macromolecular components when applying an ultrasound treatment (70W, 30 min, 26700 J/g TS) to microalgae cultured in urban wastewater. Likewise, Souza Silva et al. [40] only obtained 13.3% of lipid solubilisation using this treatment (80W, 40 min) on biomass cultivated in sewage, despite the long application times and the high energy consumption. The lowest solubilisation yields were obtained for the bead mill pretreatment, with most of the PR solubilisation yields lower than 19% (excluding lipids, who had a yield of 36% for 60 min). Low solubilisation yields were also reported by Miranda et al. [18] when pretreating pure *Scenedesmus obliquus* with bead milling, and by Günerken et al. [41] when working with *Nannochloropsis,* likely due to the presence of resistant algaenan layers on the cell wall of both species [42][43].

5.2. *Influence of the pretreatments on the recovery and degradation of monosaccharides*

The evaluation of alternatives for carbohydrate valorisation requires quantifying the monosaccharide recovery in the liquid fraction after pretreatment and considering the

degradation of the solubilised carbohydrates as a result of the pretreatment conditions or the metabolism of the biomass [44].

The acid pretreatments supported the highest PR monosaccharide recovery yields, 54 and 81% for 0.5M HCl (K) and 2M HCl (L), respectively (Fig. 3). Dong et al. [45] also achieved 80% of monosaccharide recovery from *Scenedesmus acutus* applying H_2SO_4 dilute acid pretreatment. PR carbohydrate degradation factors were around 20%, regardless of the acid concentration; and the PR monosaccharide recovery yields for the other pretreatments were below 20% (Fig. 3). The high PR carbohydrate degradation factors of 77% and 66% for 0.5 and 2 M NaOH, and 71% for 7.5% H_2O_2 pretreatments, jeopardised the remarkably high carbohydrate solubilisation supported by these pretreatments. Very low PR monosaccharides recovery yields were obtained from steam explosion (4.4%) and bead mill (0.4%), as a result of a low carbohydrate solubilisation and a high carbohydrate degradation.
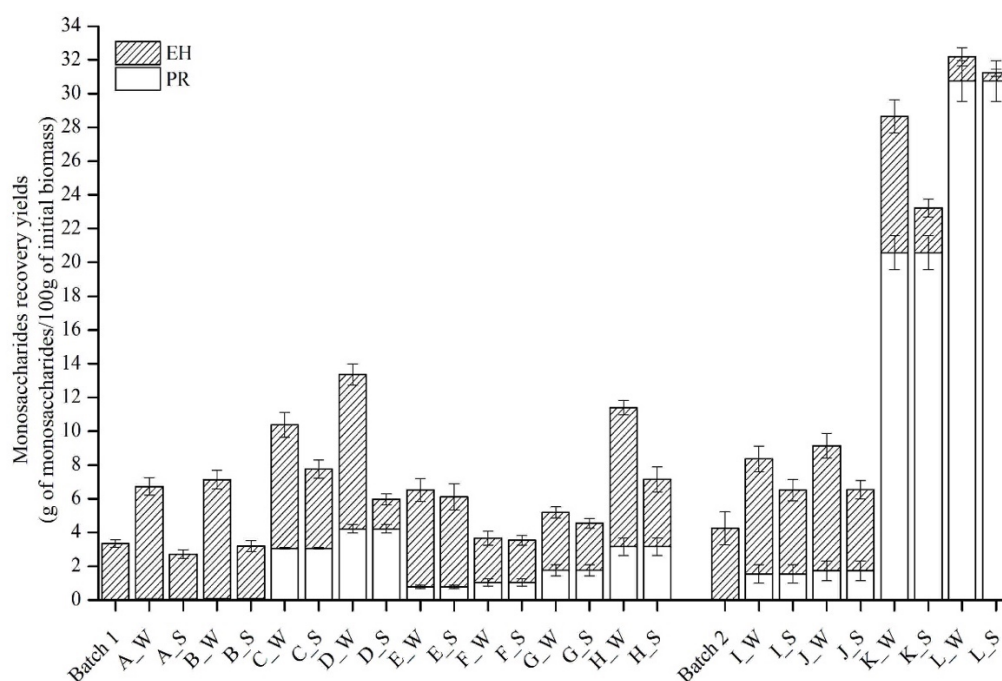
Figure 3. Monosaccharide recovery yields referred to the initial dried biomass from biomass pretreatment (PR) and enzymatic hydrolysis (EH). A: bead mill 5 min.; B: bead mill 60 min.; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 min.; J: ultrasound 21 min.; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations for each stage (pretreatment and enzymatic hydrolysis) of 4 analytical determinations (duplicated stage analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Figure 4. Total concentrations (g/L) of degradation byproducts from the biomass pretreatment (PR) and enzymatic hydrolysis (EH). A: bead mill 5 min.; B: bead mill 60 min.; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 min.; J: ultrasound 21 min.; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations for each stage (pretreatment and enzymatic hydrolysis) of 4 analytical determinations (duplicated stage analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Additionally, the most typical degradation byproducts from biomass pretreatments were analysed in the liquid fractions to estimate the degradation of the different solubilised components and to elucidate any potential inhibitory effects of these byproducts on further valorisation steps. The concentration of the degradation byproducts increased with the harshness of the operational conditions for each pretreatment, except for bead mill (Fig. 4). Chemical pretreatments entailed the highest concentrations of degradation byproducts, with values of 5.2, 5.2 and 5.5 g/L for 2M NaOH, 2M HCl and 7.5% H_2O_2, respectively. Conversely, physical-mechanical pretreatments generated low concentrations of degradation byproducts (Fig. 4), with the minimum concentrations recorded for bead milling (1.09 and 1.04 g/L for conditions A and B, respectively). The nature of the degradation compounds seemed to be related to the biomass origin. Despite their same provenance, Batches 1 and 2 presented some differences in terms of the microalgae species and macromolecular composition. Lactic, acetic and formic acids

were detected to a greater extent in pretreatments for the biomass in Batch 1, while oxalic was dominant in the biomass in Batch 2. No furfural or HMF were detected in any sample.

5.3. Enzymatic hydrolysis

5.3.1. Enzymatic hydrolysis of raw biomass

Enzymatic hydrolysis provided very similar solubilisation yields for the biomass in both Batch 1 and Batch 2, regardless of their different compositions. EH carbohydrate, protein and lipid solubilisation yields accounted for 69%, 6% and 40% in the biomass of Batch 1 and 68%, 12% and 36% in the biomass of Batch 2, respectively (Fig. 5 and 6). The type of enzymes used in these hydrolysis experiments selectively solubilised carbohydrates, therefore the mass ratios of solubilised carbohydrates/proteins/lipids after enzymatic hydrolysis were 5.93/1.00/2.40 for the biomass of Batch 1 and 8.92/1.00/1.56 for the biomass of Batch 2 [42]. In this context, Al-Zuhair et al. [46] obtained EH protein solubilisation yields of 28% for pure *Scenedesmus* and an almost complete protein release for *Chlorella*, under identical hydrolysis conditions.
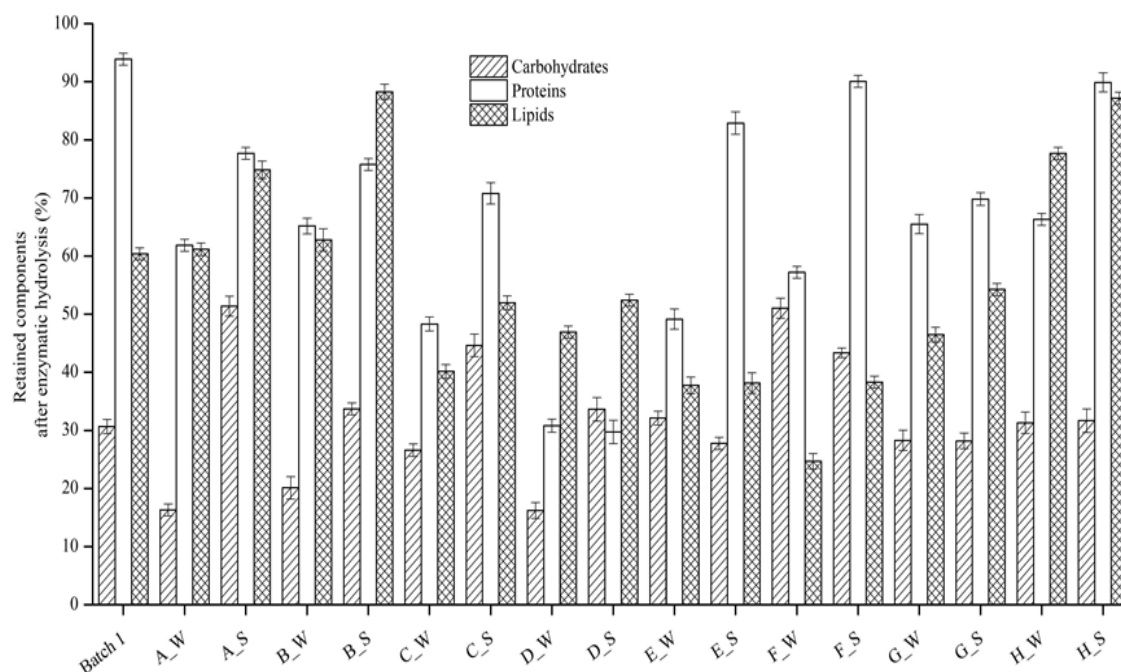
Figure 5. Retained carbohydrate, protein and lipid yields (%) in the solid fraction after enzymatic hydrolysis based on the content of each component in the solid fraction before enzymatic hydrolysis for Batch 1 biomass. A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations of 4 analytical determinations (duplicated hydrolysis analysed in duplicate). The vertical interval lines represent standard deviation of the means.

EH monosaccharide recovery yields for the raw biomass were lower than 14% for both batches, and the high carbohydrate degradation factors (~80%) were attributed to the active metabolism of bacteria present in the raw biomass [14] (Fig. 3). Martín Juárez et al. [34] reported high EH carbohydrate solubilisation yield (81.7%) with an EH monosaccharide recovery as low as 1%, working with non-pretreated biomass grown in piggery wastewater with a high bacterial content.

Methanol, ethanol, acetic acid and succinic acid were found in significant concentrations in the hydrolysates of the untreated biomasses of both Batch 1 and 2 (Fig. 4).
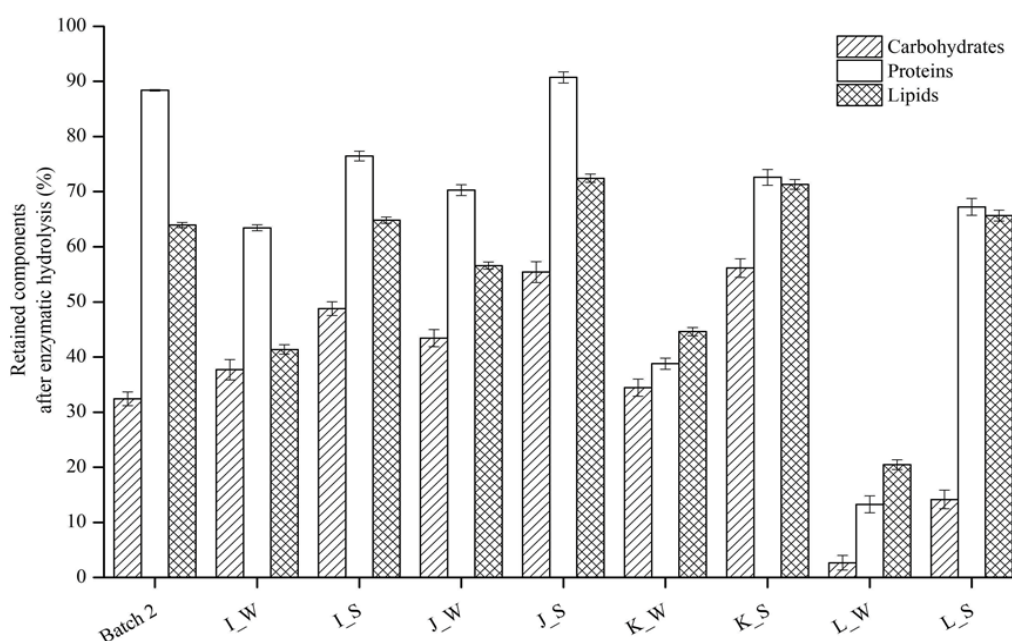
Figure 6. Retained carbohydrate, protein and lipid yields (%) in the solid fraction after enzymatic hydrolysis based on the content of each component in the solid fraction before enzymatic hydrolysis for Batch 2 biomass. I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations of 4 analytical determinations (duplicated hydrolysis analysed in duplicate). The vertical interval lines represent standard deviation of the means.

5.3.2. *Enzymatic hydrolysis of the whole suspension of pretreated biomass*

Enzymatic hydrolysis of the biomass pretreated with 2M HCl (L_W) produced the maximum carbohydrate, protein and lipid solubilisation yields (97%, 87% and 80%, respectively). However, the total amount of carbohydrates yielded from the enzymatic hydrolisis was very low since carbohydrates were already solubilised during the pretreatment step. The enzymatic hydrolysis of 0.5M HCl pretreated samples (K_W)

resulted in lower solubilisation yields (66%, 61% and 55% for carbohydrates, proteins, and lipids, respectively) due to less damage to the cell wall, making the solubilisation difficult (Fig. 6). The enzymatic hydrolysis of alkaline pretreated samples also supported high EH carbohydrate, protein and lipid solubilisation yields (73%, 52% and 60% for C_W and 84%, 69% and 53% for D_W, respectively). Moreover, the concentration of NaOH on the carbohydrate and protein solubilisation yields had a remarkable effect (Fig. 5). The high concentration of degradation byproducts in the liquid fraction of the acid and alkaline pretreated samples did not cause a significant inhibitory effect on the enzymatic hydrolysis step.

The enzymatic hydrolysis of bead mill pretreated biomass induced high EH carbohydrate solubilisation yields (84% for A_W and 80% for B_W), and moderate protein and lipid solubilisation yields (38 and 39% for A_W, and 35 and 37% for B_W, respectively), which made this pretreatment the most selective during the enzymatic hydrolysis step, likely due to its mild effect in disrupting the cell wall and enhancing the action of the specific enzymes [47]. The enzymatic hydrolysis of the biomass subjected to the pretreatments not mentioned above did not provide promising results – the carbohydrate solubilisation yields were similar to or even lower than those obtained for the raw biomass, while increasing the solubilisation of proteins and lipids.

Regarding specific recovery and degradation indicators, the 2M HCl pretreated samples provided the highest EH monosaccharide recovery yields (92%) after enzymatic hydrolysis, concomitantly with a very low carbohydrate degradation factor (5%), whereas the 0.5M HCl pretreated samples presented a monosaccharide recovery yield of only 48% with a carbohydrate degradation factor of 27% after enzymatic hydrolysis. The enzymatic hydrolysis of alkaline and alkali-peroxide pretreatments also resulted in high EH monosaccharide recovery yields (71% for C_W, 82% for D_W and 65% for H_W). In comparison, Kassim and Battacharya [28] recovered 55% of the monosaccharides for pure *Tetraselmis suecica* pretreated with 2% (w/v) KOH at 120ºC for 120 min and 63% for pure *Chlorella* sp. pretreated with 2% (w/v) NaOH at 120ºC for 30 min, when applying enzymatic hydrolysis for 48h. Unfortunately, no data about carbohydrate degradation was reported in this work. The remarkable carbohydrate solubilisation achieved during enzymatic hydrolysis of bead mill pretreated samples was counteracted

by the high carbohydrate degradation factor occurring in these experiments (62% in A_W and 56% in B_W).

Low concentrations of degradation byproducts were generated in the enzymatic hydrolysis step of chemically pretreated samples due to the high concentrations already generated during these pretreatments (Fig. 4). By contrast, the enzymatic hydrolysis of the bead mill pretreated samples generated remarkable concentrations of degradation byproducts, in concordance with the high EH carbohydrate solubilisation and degradation factors found in these samples. This degradation was likely mediated by the metabolic activity of the microorganisms in the biomass, which used the intracellular compounds as substrates and even facilitated the hydrolysis [14].

Overall, the type of degradation byproducts generated during enzymatic hydrolysis depended more on the pretreatment than on the origin of the biomass. Methanol and ethanol were produced from the physically-pretreated biomass (bead-milling and ultrasounds), oxalic acid was produced from the biomass pretreated using steam explosion and alkaline and lactic acid was produced from the HCl pretreated biomass.

5.3.3. *Enzymatic hydrolysis of the solid fractions from pretreated biomass*

As shown in the previous section, high concentrations of degradation byproducts were found in hydrolysates of the whole pretreated samples. In an attempt to reduce the concentration of byproducts which can further inhibit valorisation processes [47], enzymatic hydrolysis of the solid fraction of the pretreated biomass was tested.

The enzymatic hydrolysis of the solid fractions from pretreatment supported similar or lower solubilisation yields than the enzymatic hydrolysis of the whole pretreated biomass suspension, regardless of the pretreatment or target component. As an average of all pretreatments, EH carbohydrate, protein and lipid solubilisation yields decreased from 72%, 47% and 53%, respectively, for whole pretreated biomass suspensions from pretreatment to 61%, 26% and 37% for pretreated solid fractions, which resulted in a higher selectivity of carbohydrate solubilisation (Fig. 5 and 6).

The enzymatic hydrolysis of the 2M HCl pretreated solid fraction supported the highest EH carbohydrate solubilisation yield (86%). However, the total amount of carbohydrates was low in these samples. Thus, the authors do not find these results to be particularly relevant. On the contrary, a low EH protein and lipid yield solubilisation was obtained in this test (33% and 34%, respectively). Unexpectedly, the EH carbohydrate degradation factor (15%) increased compared to factor obtained for the enzymatic hydrolysis of the whole suspension, but was still lower than that of the untreated biomass, which supported the highest EH monosaccharide recovery yield in this series of experiments (73%). A very low carbohydrate degradation factor was found in the hydrolysates from the 0.5M NaOH pretreated biomass (18%), which supported a 45% monosaccharide recovery even with the moderate carbohydrate solubilisation achieved in this test (55%).

In most of the solid fraction from pretreated samples, the enzymatic hydrolysis resulted in lower carbohydrate solubilisation yields than those obtained in the untreated biomasses. The enzymatic hydrolysis of solid fraction from pretreatment also resulted in higher carbohydrate degradation factors which ultimately entailed low monosaccharide recovery yields. Only the enzymatic hydrolysis of the solid fraction from steam explosion (E) supported higher monosaccharide recovery (25%) and lower carbohydrate degradation factors (66%) than the enzymatic hydrolysis of the untreated biomass. This monosaccharide recovery yield was lower than the value obtained with pure *Tetraselmis suecica* pretreated with 2% KOH (w/v) at 120ºC for 120 min (55%) or the value obtained with pure *Chlorella sp.* pretreated with 2% NaOH (w/v) at 120ºC for 30 min (63%) [28].

Finally, it should be stressed that the generation of degradation byproducts during enzymatic hydrolysis was higher when using only the solid fraction when the biomass was pretreated using bead milling (A), alkaline solution (D), steam explosion (F), ultrasounds (I, J) and acid treatment (L) (Fig. 4). The concentrations of the generated degradation byproduct were very similar to those produced during the enzymatic hydrolysis of whole pretreated samples, except for the lactic and acetic acids generated from the solid fractions of the steam explosion and alkali-peroxide pretreated samples.

5.4. *Assessment of the global process efficiency*

The highest biomass carbohydrate solubilisation and monosaccharide recovery yields were supported by acid pretreatment (Fig. 3 and 7).
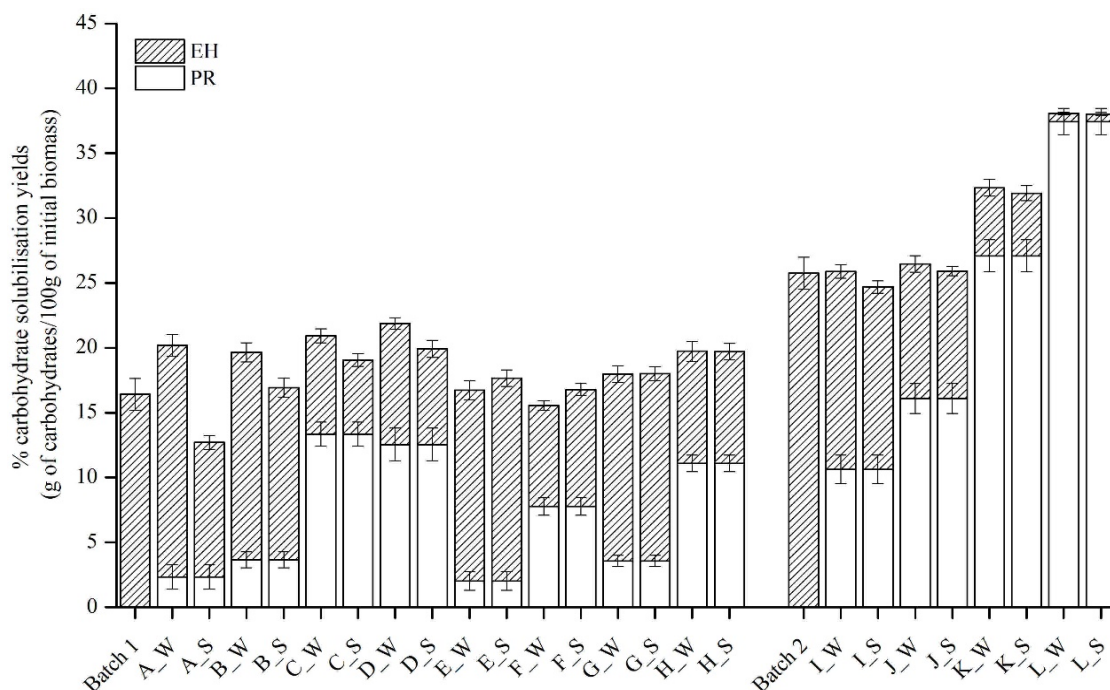
Figure 7. Carbohydrate solubilisation yields referred to the initial dried biomass from biomass pretreatment (PR) and enzymatic hydrolysis (EH). A: bead mill 5 min.; B: bead mill 60 min.; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 min.; J: ultrasound 21 min.; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations for each stage (pretreatment and enzymatic hydrolysis) of 4 analytical determinations (duplicated stage analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Chemical pretreatment with 2M HCl resulted in a complete carbohydrate solubilisation and a monosaccharide recovery of the initial carbohydrates of 84.49%, after applying enzymatic hydrolysis to the whole suspension from pretreatment. As a result of the high carbohydrate solubilisation yield of the 2M HCl pretreatment, the subsequent enzymatic hydrolysis step only solubilised proteins and lipids, decreasing the selectivity of the process (Fig. 8 and 9).
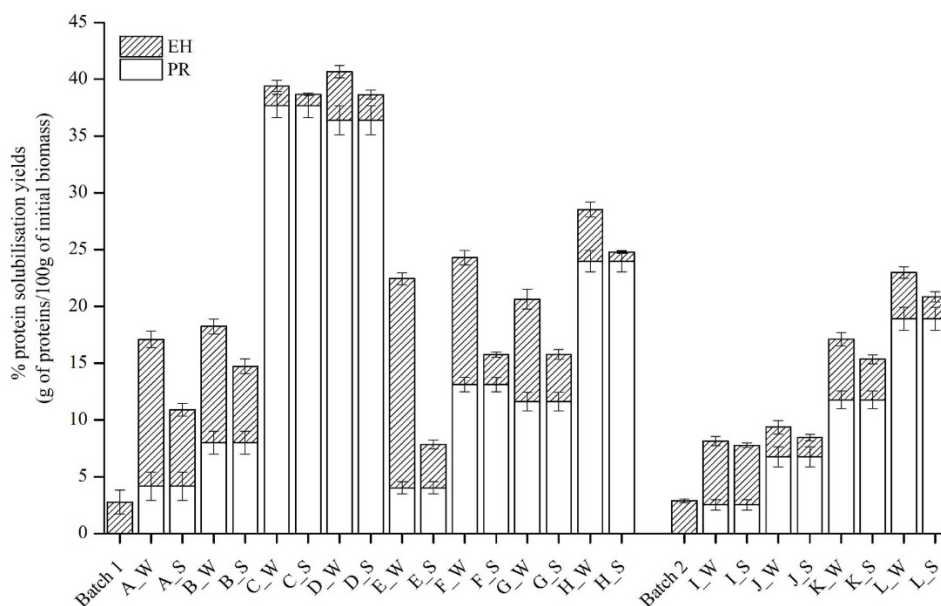
Figure 8. Biomass protein solubilisation yields based on to the initial dried microalgae biomasses. A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations for each stage (pretreatment and enzymatic hydrolysis) of 4 analytical determinations (duplicated stage analysed in duplicate). The vertical interval lines represent standard deviation of the means.

Therefore, the amount of carbohydrates, proteins and lipids that were solubilised increased from 37.44, 18.90 and 6.90 g per 100 g of the initial biomass in the pretreatment step, respectively, to 38.07, 22.97 and 9.81 g per 100 g of the initial biomass in the global process. The acid concentration constituted a key operational parameter for the solubilisation of the biomass components, but it caused a limited increase in the biomass monosaccharide recovery yields. Sequential recovery of components after dilute acid pretreatment would require separation of solubilised fractions or the application of configurations as the Combined Algal Processing developed by Dong et al. [15]. In this approach, the whole algal slurry after acid pretreatment is directly used for ethanol fermentation, and lipids are recovered from the fermentation broth, improving the energy yield.
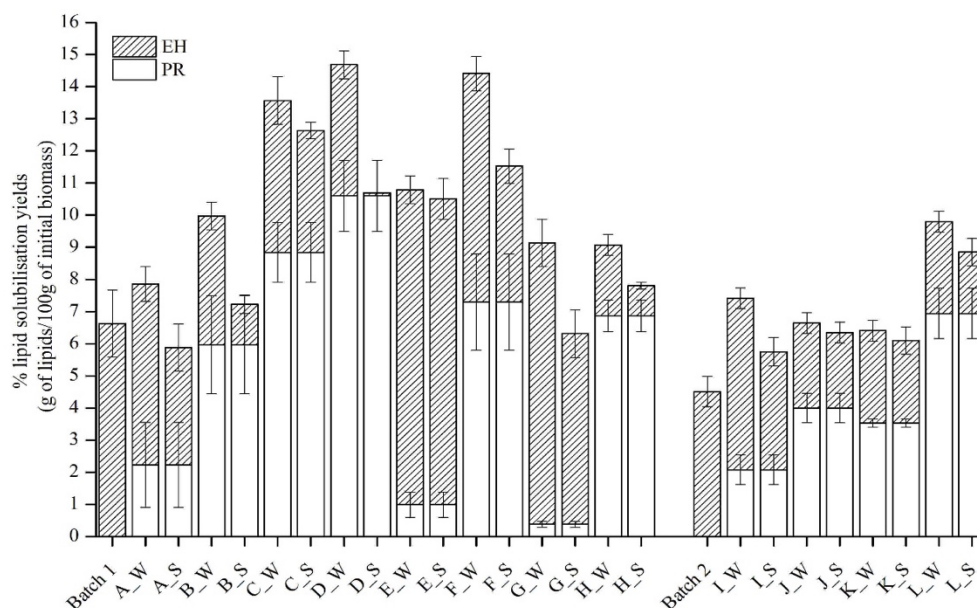
Figure 9. Biomass lipid solubilisation yields based on to the initial dried microalgae biomasses. A: bead mill 5 minutes; B: bead mill 60 minutes; C: NaOH 0.5M; D: NaOH 2M; E: steam explosion 130ºC; F: steam explosion 170ºC; G: H_2O_2 0.5%; H: H_2O_2 7.5%; I: ultrasound 5 minutes; J: ultrasound 21 minutes; K: HCl 0.5M; L: HCl 2M. W: hydrolysis of the whole suspension of the pretreated biomass; S: hydrolysis of the solid fraction of the pretreated biomass. The results are expressed as means ± standard deviations for each stage (pretreatment and enzymatic hydrolysis) of 4 analytical determinations (duplicated stage analysed in duplicate). The vertical interval lines represent standard deviation of the means.

High global solubilisation and monosaccharide recovery yields were also obtained using the alkaline pretreatment (NaOH) followed by enzymatic hydrolysis of the whole pretreated suspensions. Thus, biomass pretreatment with 2M NaOH combined with enzymatic incubation solubilised 92% of the initial carbohydrates, 96% of the initial proteins and 88% of the initial lipids contained in the biomass, corresponding to 22 g of carbohydrates, 41 g of proteins and 15 g of solubilised lipids per 100 g of biomass. In this particular case, the enzymatic hydrolysis contributed remarkably to the carbohydrate solubilisation (compensating the high protein solubilisation during pretreatment step) and the monosaccharide recovery. The increase in biomass monosaccharide recovery yields with the NaOH concentration of the pretreatment step (from 10.4% in C_W to 13.4 % in D_W) was attributed to a decrease in carbohydrate degradation due to high sterilisation of the biomass. Enzymatic hydrolysis of the whole biomass suspension resulted in higher carbohydrate and lipid solubilisation and monosaccharide recovery yields. However, the protein solubilisation yields were similar to those of the enzymatic hydrolysis of the solid fraction of the pretreated biomass (Fig. 7, 8 and 9).

A moderate monosaccharide recovery (48% of the initial carbohydrates) was obtained using alkali-peroxide pretreatment (7.5% H_2O_2) coupled with enzymatic hydrolysis of the whole suspension of pretreated biomass. The alkali-peroxide pretreatment preferentially solubilised proteins, while enzymatic hydrolysis contributed remarkably to the solubilisation of carbohydrates (although to a lesser extent than alkaline pretreatment). Similarly, the hydrogen peroxide concentration was the main factor that determined carbohydrate degradation and monosaccharide recovery.

Bead mill was the physical pretreatment that supported the highest biomass solubilisation yields (85% of the initial carbohydrates, 40% of the initial proteins, 47% of the initial lipids), when combining 5 min bead milling with the enzymatic hydrolysis of the whole suspension of pretreated biomass. Nevertheless, the high degradation that occured during the enzymatic hydrolysis resulted in a recovery of only 28% of the carbohydrates in the initial biomass.

5.5. *Bacterial viability and DNA integrity*

The integrity of the bacterial DNA was analysed in order to estimate the effect of each pretreatment on the bacterial viability. Viable bacteria on the biomass would degrade the solubilised components and further products and compete with fermentation microorganisms in subsequent valorisation stages. The results of the agarose gel electrophoresis of the genomic DNA isolated from the raw biomass, pretreated samples, and enzymatically hydrolysed samples, suggest the bacterial DNA degraded during the enzymatic hydrolysis. Indeed, while the electrophoresis of the raw biomass provided compact, narrow and well-defined bands, longer and diffuse traces along the gel were obtained after enzymatic hydrolysis of the untreated samples. Zhang et al. [49] demonstrated that enzymatic hydrolysis caused significant alterations in the structure of the cell wall of microalgae, and it is expected that the peptidoglycan wall surrounding the cytoplasmic membrane of bacterial cells could be also compromised, causing the bacteria to be more susceptible to damage.

No bacterial DNA was found in the samples after the acid or alkaline pretreatments or in the samples after the alkali-peroxide pretreatment with 7.5% H_2O_2. The absence of bands in the gel could indicate a complete sterilisation, suggesting that the generation of

degradation byproducts could be attributed to chemical reactions during these pretreatments. Since no viable bacteria were found independently of the NaOH and HCl concentrations, the increase in the amount of degradation byproducts with the chemical reagent concentration supports the chemical origin of these compounds. For the alkali-peroxide samples, the concentration of H_2O_2 had a relevant impact on the bacterial DNA integrity. The effect of the H_2O_2 concentration increased after enzymatic hydrolysis, showing more diffuse bands at higher concentrations of H_2O_2, as a result of the oxidative damage prompted by this reactive species that poses a significant threat to cellular integrity in terms of damage to DNA, lipids, proteins and other macromolecules [50]. Bacterial activity was likely the cause of the higher carbohydrate degradation and lower recovery of monosaccharides found during enzymatic hydrolysis of the biomass pretreated at mild alkali-peroxide conditions, even when similar carbohydrate solubilisation yields were obtained with H_2O_2 0.5% and H_2O_2 7.5%. Ultrasound decreased the bacteria viability, mainly when a higher power was used, but to a lesser extent than the chemical pretreatments. A very limited bacterial DNA degradation was observed in the solid biomass fraction after bead mill and steam explosion pretreatments as suggested by the clear and defined bands.

qPCR analysis was performed to quantify the number of viable bacteria in the samples with acceptable DNA integrity in regard to the results obtained in the agarose gel. Thus, only DNA obtained from the raw biomass samples and after bead mill pretreatment were used for this analysis. The live bacteria fraction in the raw biomass samples (66.7% of the population) decreased to 0.6% and 0.4% after bead mill pretreatments A and B, respectively, despite the DNA integrity observed in the agarose gel. Therefore, it can be hypothesised that bead milling broke bacterial membranes, thus reducing the total number of viable cells, without affecting the integrity of the DNA in a significant way. Finally, the enzymatic hydrolysis of the pretreated samples degraded the bacteria damaged during pretreatment, increasing by 14% the percentage of viable bacteria, in spite of the the constant number of live bacteria, as a result of the decrease of the total number of bacteria.

6. Conclusions

This work successfully demonstrated the efficiency of acid and basic diluted pretreatments for carbohydrate solubilisation from microalgae-bacteria biomass cultured in piggery wastewater. The acid and alkaline pretreatments resulted in high solubilisation and low carbohydrate degradation, but also in low selectivity due to the co-solubilisation of high percentages of the protein and lipid fractions. High monosaccharide recoveries were achieved by using 2M HCl pretreatment (81%). Enzymatic hydrolysis was a necessary step after alkaline pretreatment, achieving 56.4% of monosaccharide recovery yield from suspensions of samples pretreated with 2M NaOH. No viable bacteria were found in samples pretreated with HCl and NaOH, according with their low carbohydrate's degradation.

The enzymatic hydrolysis of the biomass pretreated mechanically with bead mill reached 85% of carbohydrate solubilisation but an 80% of degradation, related with the high percentage of viable bacteria found in these samples. Coupled bead mill and enzymatic hydrolysis resulted an efficient and selective process for carbohydrate solubilisation, but it would also require a previous sterilisation step in order to enhance the monosaccharide recovery, allow subsequent fermentation steps and preserve products.

The removal of the liquid phase of pretreated samples did not enhance the solubilisation yields of the enzymatic hydrolysis step, making the separation step unnecessary and resulting in a more advantageous process.

References

[1]   E.S. Salama, M.B. Kurade, R.A.I. Abou-Shanab, M.M. El-Dalatony, I.S. Yang, B. Min, B.H. Jeon, Recent progress in microalgal biomass production coupled with wastewater treatment for biofuel generation, Renew. Sustain. Energy Rev. 79 (2017) 1189–1211. doi:10.1016/j.rser.2017.05.091.

[2]   T.M. Aida, T. Nonaka, S. Fukuda, H. Kujiraoka, Y. Kumagai, R. Maruta, M. Ota, I. Suzuki, M.M. Watanabe, H. Inomata, R.L. Smith, Nutrient recovery from municipal sludge for microalgae cultivation with two-step hydrothermal liquefaction, Algal Res. 18 (2016) 61–68. doi:10.1016/j.algal.2016.06.009.

[3]   G. Quijano, J.S. Arcila, G. Buitrón, Microalgal-bacterial aggregates: Applications and perspectives for wastewater treatment, Biotechnol. Adv. 35 (2017) 772–781. doi:10.1016/j.biotechadv.2017.07.003.

[4]   A.L. Gonçalves, J.C.M. Pires, M. Simões, A review on the use of microalgal consortia for wastewater treatment, Algal Res. 24 (2017) 403–415. doi:10.1016/j.algal.2016.11.008.

[5]   D. García, E. Posadas, C. Grajeda, S. Blanco, S. Martínez-Páramo, G. Acién, P. García-Encina, S. Bolado, R. Muñoz, S. Martínez, Comparative evaluation of piggery wastewater treatment in algal-bacterial pho-tobioreactors under indoor and outdoor conditions. Bioresour. Technol. 245 (2017) 483–490. doi:10.1016/j.biortech.2017.08.135.

[6]   B. Colling Klein, A. Bonomi, R. Maciel Filho, Integration of microalgae production with industrial biofuel facilities: A critical review, Renew. Sustain. Energy Rev. 82 (2018) 1376–1392. doi:10.1016/j.rser.2017.04.063.

[7]   E. Jankowska, A.K. Sahu, P. Oleskowicz-Popiel, Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion, Renew. Sustain. Energy Rev. 75 (2017) 692–709. doi:10.1016/j.rser.2016.11.045.

[8]   J. Martín Juárez, E. Riol Pastor, J.M. Fernández Sevilla, R. Muñoz Torre, P.A. García-Encina, S. Bolado Rodríguez, Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants, Bioresour. Technol. 257 (2018) 30–38. doi:10.1016/j.biortech.2018.02.063.

[9]   M. Rizwan, G. Mujtaba, S.A. Memon, K. Lee, N. Rashid, Exploring the potential of microalgae for new biotechnology applications and beyond: A review, Renew. Sustain. Energy Rev. 92 (2018) 394–404. doi:10.1016/j.rser.2018.04.034.

[10]   K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.J. Lee, J.S. Chang, Microalgae biorefinery: High value products perspectives, Bioresour. Technol. 229 (2017) 53–62. doi:10.1016/j.biortech.2017.01.006.

[11]   E. D'Hondt, J. Martín-Juárez, S. Bolado, J. Kasperoviciene, J. Koreiviene, S. Sulcius, K. Elst, L. Bastiaens, Cell disruption technologies, Microalgae-Based Biofuels Bioprod. From Feed. Cultiv. to End-Products. (2017) 133–154. doi:10.1016/B978-0-08-101023-5.00006-6.

[12]   B. B. Makut, D. Das, G. Goswami, Production of microbial biomass feedstock via co-cultivation of microalgae-bacteria consortium coupled with effective wastewater treatment: A sustainable approach, 37 (2019) 228–239. doi:10.1016/j.algal.2018.11.020

[13]   E. Günerken, E.D. Hondt, M.H.M. Eppink, L. Garcia-gonzalez, K. Elst, R.H. Wijffels, Cell disruption for microalgae biorefineries, 33 (2015) 243–260. doi:10.1016/j.biotechadv.2015.01.008.

[14]   J.L. Fuentes, I. Garbayo, M. Cuaresma, Z. Montero, M. González-Del-Valle, C. Vílchez, Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds, Mar. Drugs. 14 (2016). doi:10.3390/md14050100.

[15]   T. Dong, E, P.Knoshaug, R. Davis. L.M.L. Laurens, S. Van Wychen, P. T. Pienkos, N. Nagle, Combined algal processing: A novel integrated biorefinery process to produce algal biofuels and bioproducts, Algal Res. 19 (2016) 316-323. doi: 10.1016/j.algal.2015.12.021

[16]   C.K. Phwan, H.C. Ong, W.H. Chen, T.C. Ling, E.P. Ng, P.L. Show, Overview: Comparison of pretreatment technologies and fermentation processes of bioethanol from microalgae, Energy Convers. Manag. 173 (2018) 81–94. doi:10.1016/j.enconman.2018.07.054.

[17]   D. Hernández, B. Riaño, M. Coca, M.C. García-González, Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production, Chem. Eng. J. 262 (2015) 939–945. doi:10.1016/j.cej.2014.10.049.

[18]   J.R. Miranda, P.C. Passarinho, L. Gouveia, Pre-treatment optimisation of *Scenedesmus obliquus* microalga for bioethanol production, Bioresour. Technol. 104 (2012) 342–348. doi:10.1016/j.biortech.2011.10.059.

[19]   M.M. Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Outdoor production of *Scenedesmus* sp. in thin layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source, Algal Res. (2015) 297–305.

[20]   R. Travaini, E. Barrado, S. Bolado-Rodríguez, Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse, Bioresour. Technol. 214 (2016) 150–158. doi:10.1016/j.biortech.2016.04.102.

[21]   S. Van Wychen, L.M.L. Laurens, Determination of Total Solids and Ash in Algal Biomas, (2015) NREL/TP-5100-60956. doi:10.2172/1118077.

[22]   G. Kochert, Quantification of the macromolecular components of microalgae. Hanbook of Phycological Methods. Physiological and Biochemical Methods. University Press. (1978) 189-195.

[23]   C.V. González lopez, M.C. Cerón García, F.G. Acién Fernández, C. Segovia Bustos, Y. Chisti, J.M. Fernández Sevilla, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591. doi:10.1016/j.biortech.2010.04.077.

[24]   S. Van Wychen, L.M.L. Laurens, Determination of Total Carbohydrates in Algal Biomass, (2015) NREL/TP-5100-60957. doi:10.2172/1118077.

[25]   G.A. Cangelosi, J.S. Meschke, Dead or alive: Molecular assessment of microbial viability, Appl. Environ. Microbiol. 80 (2014) 5884–5891. doi:10.1128/AEM.01763-14.

[26]   J. Desneux, M. Chemaly, A.M. Pourcher, Experimental design for the optimisation of propidium monoazide treatment to quantify viable and non-viable bacteria in piggery effluents, BMC Microbiol. 15 (2015) 1–9. doi:10.1186/s12866-015-0505-6.

[27]   A. Mahdy, L. Mendez, M. Ballesteros, C. González-Fernández, Autohydrolysis and alkaline pretreatment effect on *Chlorella vulgaris* and *Scenedesmus* sp. methane production, Energy. 78 (2014) 48–52. doi:10.1016/j.energy.2014.05.052.

[28]   M. A. Kassim, S. Bhattacharya, Dilute alkaline pretreatment for reducing sugar production from *Tetraselmis suecica* and *Chlorella* sp. biomass, Process Biochemistry, 51 (2015) 1757–1766. doi: 0.1016/j.procbio.2015.11.027.

[29]   H. Shokrkar, S. Ebrahimi, M. Zamani, Bioethanol production from acidic and enzymatic hydrolysates of mixed microalgae culture, Fuel. 200 (2017) 380–386. doi:10.1016/j.fuel.2017.03.090.

[30]   M. Solé-Bundó, H. Carrère, M. Garfí, I. Ferrer, Enhancement of microalgae anaerobic digestion by thermo-alkaline pretreatment with lime (CaO), Algal Res. 24 (2017) 199–206. doi:10.1016/j.algal.2017.03.025.

[31]   W.N. Phong, P.L. Show, T.C. Ling, J.C. Juan, E.P. Ng, J.S. Chang, Mild cell disruption methods for bio-functional proteins recovery from microalgae—Recent developments and future perspectives, Algal Res. 31 (2018) 506–516. doi:10.1016/j.algal.2017.04.005.

[32]   T. Dong, S. Van Wychen, N. Nagle, P.T. Pienkos, L.M.L. Laurens, Impact of biochemical composition on susceptibility of algal biomass to acid-catalysed pretreatment for sugar and lipid recovery, Algal Res. 18 (2016) 69–77. doi:10.1016/j.algal.2016.06.004.

[33]   G. Markou, I. Angelidaki, E. Nerantzis, D. Georgakakis, Bioethanol production by carbohydrate-enriched biomass of *Arthrospira (Spirulina) platensis*, Energies. 6 (2013) 3937–3950. doi:10.3390/en6083937.

[34]   J. Martín Juárez, A. Lorenzo Hernando, R. Muñoz Torre, S. Blanco Lanza, S. Bolado Rodríguez, Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment, Bioresour. Technol. 218 (2016) 265–271. doi:10.1016/j.biortech.2016.06.087.

[35]   E. Lorente, X. Farriol, J. Salvadó, Steam explosion as a fractionation step in biofuel production from microalgae, Fuel Process. Technol. 131 (2015) 93–98. doi:10.1016/j.fuproc.2014.11.009.

[36]   L. Mendez, A. Mahdy, R. a Timmers, M. Ballesteros, C. González-Fernández, Enhancing methane production of *Chlorella vulgaris* via thermochemical pretreatments, Bioresour. Technol. 149 (2013) 136–41. doi:10.1016/j.biortech.2013.08.136.

[37]   M.E. Alzate, R. Muñoz, F. Rogalla, F. Fdz-Polanco, S.I. Pérez-Elvira, Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment, Bioresour. Technol. 123 (2012) 488–494. doi:10.1016/j.biortech.2012.06.113.

[38]   G. Zhao, X. Chen, L. Wang, S. Zhou, H. Feng, W.N. Chen, R. Lau, Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation, Bioresour. Technol. 128 (2013) 337–344. doi:10.1016/j.biortech.2012.10.038.

[39]   F. Passos, J. Carretero, I. Ferrer, Comparing pretreatment methods for improving microalgae anaerobic digestion: Thermal, hydrothermal, microwave and ultrasound, Chem. Eng. J. 279 (2015) 667–672. doi:10.1016/j.cej.2015.05.065.

[40]   A. P. F. De Souza Silva, M. Carantino Costa, A. Colzi Lopes, E. F. Abdala Neto, R. Carrhá Leitao, C. Rossas Mota, A. Bezerra dos Santos, Comparison of pretreatment methods for total lipids extraction from mixed microalgae, Renew. Ener. 63 (2014) 762–766.

[41]   E. Günerken, E. D'Hondt, M. Eppink, K. Elst, R. Wijffels, Influence of nitrogen depletion in the growth of *N. oleoabundans* on the release of cellular components after beadmilling, Bioresour. Technol. 214 (2016) 89–95. doi:10.1016/j.biortech.2016.04.072.

[42]   S. Dunker, C. Wilhelm, Cell wall structure of coccoid green algae as an important trade-off between biotic interference mechanisms and multidimensional cell growth, Front. Microbiol. 9 (2018). doi:10.3389/fmicb.2018.00719.

[43]   M.J. Scholz, T.L. Weiss, R.E. Jinkerson, J. Jing, R. Roth, U. Goodenough, M.C. Posewitz, H.G. Gerken, Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall, Eukaryot. Cell. 13 (2014) 1450–1464. doi:10.1128/EC.00183-14.

[44]   C.Y. Chen, X.Q. Zhao, H.W. Yen, S.H. Ho, C.L. Cheng, D.J. Lee, F.W. Bai, J.S. Chang, Microalgae-based carbohydrates for biofuel production, Biochem. Eng. J. 78 (2013) 1–10. doi:10.1016/j.bej.2013.03.006.

[45]   T. Dong, E.P. Knoshaug, R. Davis, L.M.L. Laurens, S. Van Wychen, P.T. Pienkos, N. Nagle, Combined algal processing : A novel integrated biorefinery process to produce algal biofuels and bioproducts, Algal Res. 19 (2016) 316–323.

[46]   S. Al-Zuhair, S. Ashraf, S. Hisaindee, N. Al Darmaki, S. Battah, D. Svistunenko, B. Reeder, G. Stanway, A. Chaudhary, Enzymatic pre-treatment of microalgae cells for enhanced extraction of proteins, Eng. Life Sci. 17 (2017) 175–185. doi:10.1002/elsc.201600127.

 [47]   C. Safi, L. Cabas Rodriguez, W.J. Mulder, N. Engelen-Smit, W. Spekking, L.A.M. van den Broek, G. Olivieri, L. Sijtsma, Energy consumption and water-soluble protein release by cell wall disruption of *Nannochloropsis gaditana*, Bioresour. Technol. 239 (2017) 204–210. doi:10.1016/j.biortech.2017.05.012.

[48]   L. Bastiaens, S. Van Roy, G. Thomassen, K. Elst, Biorefinery of algae: Technical and economic considerations, Elsevier Ltd., 2017. doi:10.1016/B978-0-08-101023-5.00014-5.

[49]   Y. Zhang, X. Kong, Z. Wang, Y. Sun, S. Zhu, L. Li, P. Lv, Optimisation of enzymatic hydrolysis for effective lipid extraction from microalgae *Scenedesmus* sp., Renew. Energy. 125 (2018) 1049–1057. doi:10.1016/j.renene.2018.01.078.

[50]   J. Cadet, K.J.A. Davies, Oxidative DNA damage & repair: An introduction, Free Radic. Biol. Med. 106 (2017) 100–110. doi:10.1016/j.freeradbiomed.2017.03.030.

Supplementary Material

Figure 1. Agarose gel electrophoresis of genomic DNA **(a)** from the solid samples. 1: weight marker, 2: untreated raw biomass from batch 1, 3: enzymatic hydrolysis of untreated raw biomass from batch 1 at 12h, 4: bead mill 5 min, 5: enzymatic hydrolysis (12h) of whole pretreated suspension from bead mill 5 min, 6: enzymatic hydrolysis (12h) of solid pretreated fraction from bead mill 5 min, 7: bead mill 60 min, 8: enzymatic hydrolysis (12h) of whole pretreated suspension from bead mill 60 min, 9: enzymatic hydrolysis (12h) of solid pretreated fraction from bead mill 60 min, 10: NaOH 0.5M, 11: enzymatic hydrolysis (12h) of whole pretreated suspension from NaOH 0.5M, 12: enzymatic hydrolysis (12h) of solid pretreated fraction from NaOH 0.5M, 13: NaOH 2M, 14: enzymatic hydrolysis (12h) of whole pretreated suspension from NaOH 2M, 15: enzymatic hydrolysis (12h) of solid pretreated fraction from NaOH 2M, 16: steam explosion 130ºC, 17: enzymatic hydrolysis (12h) of whole pretreated suspension from steam explosion 130ºC, 18: enzymatic hydrolysis (12h) of solid pretreated fraction from steam explosion 130ºC, 19: steam explosion 170ºC, 20: enzymatic hydrolysis (12h) of whole pretreated suspension from steam explosion 170ºC, 21: enzymatic hydrolysis (12h) of solid pretreated fraction from steam explosion 170ºC, 22: H_2O_2 0.5%, 23: enzymatic hydrolysis (12h) of whole pretreated suspension from H_2O_2 0.5%, 24: enzymatic hydrolysis (12h) of solid pretreated fraction from H_2O_2 0.5%, 25: H_2O_2 7.5%, 26: enzymatic hydrolysis (12h) of whole pretreated suspension from H_2O_2 7.5%, and 27: enzymatic hydrolysis (12h) of solid pretreated fraction from H_2O_2 7.5%.

b) 1: weight marker, 2: untreated raw biomass from batch 2, 3: enzymatic hydrolysis of untreated raw biomass from batch 2 at 12h, 4: ultrasound 5 min, 5: enzymatic hydrolysis (12h) of whole pretreated suspension from ultrasound 5 min, 6: enzymatic hydrolysis (12h) of solid pretreated fraction from ultrasound 5 min, 7: ultrasound 21 min, 8: enzymatic hydrolysis (12h) of whole pretreated suspension from ultrasound 21 min, 9: enzymatic hydrolysis (12h) of solid pretreated fraction from ultrasound 21 min, 10: HCl 0.5M, 11: enzymatic hydrolysis (12h) of whole pretreated suspension from HCl 0.5M, 12: enzymatic hydrolysis (12h) of solid pretreated fraction from HCl 0.5M, 13: HCl 2M, 14: enzymatic hydrolysis (12h) of whole pretreated suspension from HCl 2M and 15: enzymatic hydrolysis (12h) of solid pretreated fraction from HCl 2M.

Figure 1

(a)
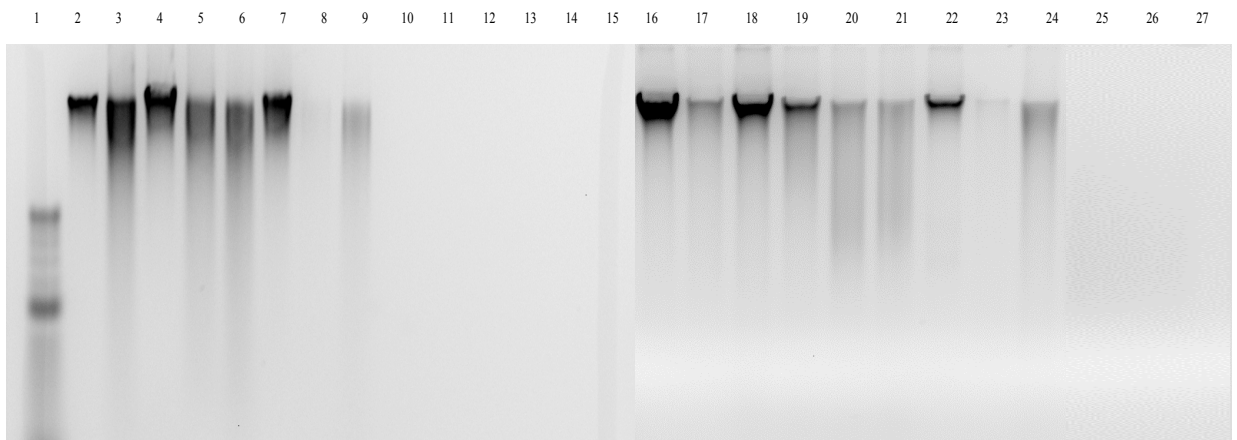
(b)
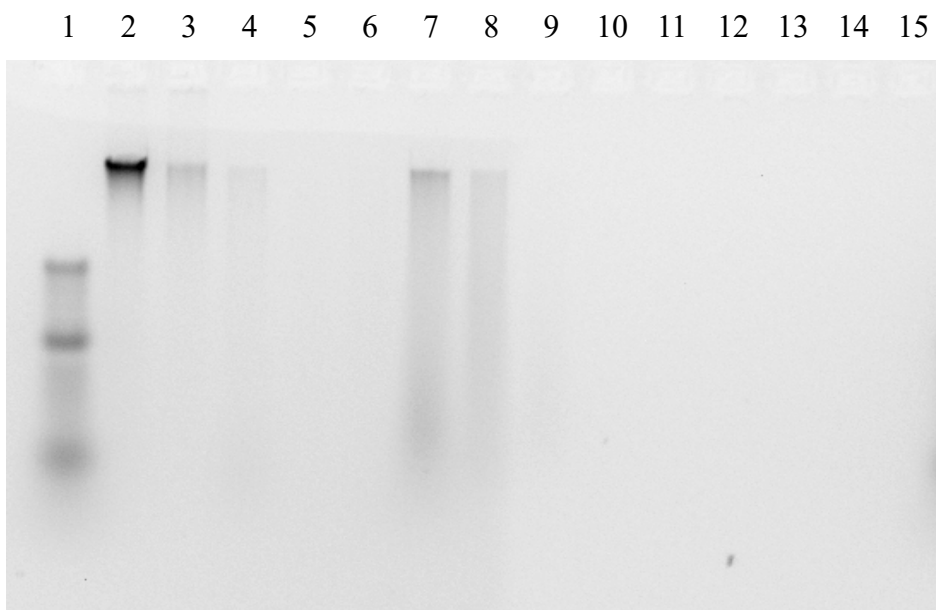
Chapter 5

Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment.

Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment.

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ABSTRACT

An enzymatic method for the carbohydrate hydrolysis of different microalgae biomass cultivated in domestic (DWB)[*] and pig manure (PMWB) wastewaters, at different storage conditions (fresh, freeze-dried and reconstituted), was evaluated. The DWB provided sugars yields between 40 and 63%, although low xylose yields ($< 23.5\%$). Approximately 2% of this biomass was converted to byproducts as succinic, acetic and formic acids. For PMWB, a high fraction of the sugars (up to 87%) was extracted, but mainly converted into acetic, butyric and formic acids, which was attributed to the bacterial action. In addition, the performance of an alkaline-peroxide pretreatment, conducted for 1 hour, 50ºC and H_2O_2 concentrations from 1 to 7.5% (w/w), was essayed. The hydrolysis of pretreated microalgae supported a wide range of sugars extraction for DWB (55-90%), and 100% for PMWB. Nevertheless, a large fraction of these sugars (~30% for DWB and 100% for PMWB) was transformed to byproducts.

Keywords: Enzymatic hydrolysis; Glucose; Xylose; Wastewater; Alkaline-peroxide pretreatment

1. Introduction

World human population and industrial activity have exponentially increased during last decades, with a concomitant raise in global energy demand. This growth has been traditionally based on fossil fuels, whose side effects have turned this dependence environmentally unsustainable (Chisti, 2007). New renewable fuel sources and biorefinery approaches for designing cost-effective and "green" processes are expected to create more efficient and sustainable economies (Daroch et al., 2013). During the past decade, microalgae have experimented a continuous and positive development due to their wide range of practical applications: wastewater treatment, nitrogen and phosphorous recovery, biogas upgrading, production of biofuels, biofertilisers, animal and fish feed, etc. Despite Oswald and co-workers were pioneers in introducing the microalgae biorefinery concept in the 60's, the combination and optimisation of processes

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[*] Abbreviations: DWB, domestic wastewater biomass; PMWB, pig manure microalgae biomass; HRT, hydraulic retention time; SRT, sludge retention time; CO_2, carbon dioxide; CH_4, methane.

for the valorisation of microalgae biomass obtained from wastewaters treatment remains a challenge nowadays (Acién et al, 2014).

Microalgae biomass is mainly composed of proteins (6% - 52%), lipids (5% - 23%) and carbohydrates (7% - 23%) (Tijani et al., 2015). This content may vary within microalgae strains and is highly dependent on cultivation conditions, especially under nutrients-deprivation scenarios. Among them, carbohydrates are one of the preferred feedstocks for obtaining a variety of biofuels. Carbohydrates are mainly present in microalgae cell wall as cellulose and hemicellulose, and/or inside the cell as starch. Cell walls are mainly composed of biopolymers such as sporopollenin or algaenan, which confer the cell a high rigidity and resistance to chemical attack (González-Hernández et al., 2012) and are characteristic of microalgae strains like *Scenedesmus* (Miranda et al., 2012).

In order to make available the valuable compounds present inside microalgae cells; pretreatments are often needed in order to disrupt cell walls. Microalgae pretreatment allows for an efficient release of the carbohydrate content, enhancing saccharification and sugars bioavailability to maximise biofuels production (Hernández et al., 2015). Due to the lack of lignin, microalgae-based biofuels are expected to be cheaper compared to second-generation biofuels (Chen et al., 2013), but most of the literature references use pure cultures of microalgae grown on synthetic media, which would turn microalgae biofuel production prohibitive from an economic point of view (Lam and Lee, 2015). For instance, Miranda et al., (2012) evaluated the performance of several chemical and mechanical pretreatments for cell disruption and sugar extraction of wet and dried *Scenedesmus obliquus* biomass. H_2SO_4 hydrolysis was selected and optimised (120ºC, 2N sulfuric acid, 50 g biomass/L, one single step), and a synergistic effect between microalgae drying and sugar extraction for the acid pretreatment was reported. This study also confirmed the key role of cell disruption on the efficiency of sugar extraction from *Scenedesmus*. Harun and Danquah, (2011a) and (2011b) assessed the efficiency of pretreatments such as acid hydrolysis and ultrasound followed by enzymatic hydrolysis with cellulose on *Chlorococcum humicola* for bioethanol production. Despite no values of released sugars or byproducts were provided after acid hydrolysis, the authors obtained a maximum released glucose yield of 68.2% with 10g/L of biomass concentration after enzymatic hydrolysis at 40ºC and pH 4.5. Furthermore, it is also desirable to develop

pretreatment methods with chemicals and effluents streams that have a lower impact on the environment. Some works have been published studying the use of green solvents, as supercritical fluids and ionic liquids, (Silveira et al., 2015) for pure culture algae pretreatment. For example, Schultz-Jensen et al., (2013) applied ozonolysis to increase the digestibility of *Chaetomorpha linum* macroalgae, reporting 75% of xylan recovery; and Zhou et al., (2012) obtained 0.65g of released sugars/g algae applying [Emim]Cl and 7 wt% HCl at 105°C for 3 h to *Chlorella sp.* biomass (73.58% of initial sugars). Similarly, Ometto et al., (2014) evaluated the energy consumption and impact of four pretreatments (enzymatic treatment, thermal, thermal hydrolysis and ultrasound) on the preferential release of the biochemical fractions of axenic *Scenedesmus obliquus, Chlorella sorokiniana and Arthrospira maxima* strains. The authors concluded that enzymatic hydrolysis was the best method for carbohydrate release and the only one with a positive energy balance due to the mild operational conditions needed.

Based on the benefits and popularization of microalgae-based wastewater treatment, there is a recent interest on developing strategies for the valorisation of this residual microalgae biomass. This biomass often contains significant concentrations of heterotrophic and nitrifying bacterial due to the high concentration of organic matter and ammonium present in domestic or livestock wastewaters, which could have some effect on the pretreatments results. Nevertheless, only some authors mentioned this bacteria contribution, like Alzate et al., (2012) working in biogas production or Castro et al., (2015) who considered necessary to apply sterilisation process (autoclaving) before using wastewater microalgae biomass for butanol production.

A biomass sterilisation effect could be expected from the application of alkaline peroxide pretreatment, which has also shown high sugars release yields when used for lignocellulosic materials (Monlau et al., (2012); Toquero and Bolado, (2014)). Compared with other chemical pretreatments, alkaline-peroxide pretreatment is carried out at mild temperatures, and it leads to a lesser formation of inhibitors than in other processes (Bolado-Rodríguez et al., 2016). Tijani et al., (2015) suggested this pretreatment as a suitable process for microalgae biomass rich in hemicellulose, thanks to its moderate operating conditions and its high efficiency releasing xylose. For macroalgae, its viability has just started to be tested. Li et al., (2016) optimised hydrogen peroxide as pretreatment

for *Ulva prolifera* waste biomass, in order to improve ulterior enzymatic hydrolysis process. When applying optimum conditions (0.2% H_2O_2, 50ºC, 12h and pH 4.0) they obtained 420 mg/g biomass of reducing sugars. Nevertheless, to the authors' knowledge, the potential of this pretreatment to enhance sugar release from microalgae biomass has never been explored.

The aim of this work was the elucidation of the performance of enzymatic hydrolysis for saccharification of microalgae biomass cultivated in different types of wastewaters. An analysis of the influence of biomass composition and storage conditions, such as freeze-drying or cooling, on the released sugars yields and their transformation on other byproducts was conducted. Finally, the potential of alkaline-peroxide pretreatment for hemicellulose solubilisation and biomass sterilisation was herein assessed for the first time.

2. Materials and methods

2.1. Microalgae

Freeze-dried microalgae biomass (A1) and the same biomass reconstituted with distilled water at a concentration of 150g/L (A2) were obtained from a thin-layer photobioreactor fed with domestic wastewater at HRT (hydraulic residence time) of 3.3 days. Microalgae biomass was composed of *Scenedesmus obliquus* (95%), *Scenedesmus quadricauda* (4%) and *Nitzschia sp.* (1%). Freeze-dried (B1) and fresh (B2) microalgae biomass were also cultivated in a thin-layer photobioreactor at HRT 3.3 days fed with pig manure wastewater diluted at 10%. The composition of B1 and B2 was *Aphanothece sp.* (61%) and *Scenedesmus obliquus* (39%). Biomass A1, A2, B1 and B2 were kindly supplied by Cajamar Foundation (Almeria, Spain). Finally, fresh microalgae biomass (C) was cultivated at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid (Spain) in an anoxic–aerobic algal–bacterial photobioreactor with biomass recirculation (Alcántara et al., 2015). The photobioreactor was operated at HRT 2 days and a sludge retention time (SRT) of 10 days using fresh domestic wastewater. Biomass C was composed of *Scenedesmus obliquus* (48%), *Desmodesmus spinosus* (45%) and *Nitzschia palea* (7%) and it was centrifuged for 10 min at 10000 rpm and maintained at 4ºC prior to use.

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis assays of untreated and pretreated microalgae were performed in 100 mL Erlenmeyer flasks containing 6% w/w dry solid and a mixture of 10 FPU/g (Celluclast 1.5L - Cellulase from *Trichoderma reesei*) and 20 CBU/g (*Novozyme 188 –* β-glucosidase from *Aspergillus niger*) of cellulose (dry basis) (Travaini et al., 2013). The pH was adjusted at 4.9 ± 0.1. The hydrolysis assays were carried out in a rotary shaker at 50 ºC and 300 rpm for 48 h. Samples were drawn after hydrolysis and stored at 4ºC prior to the determination of the concentration of sugars (glucose, xylose, cellobiose and arabinose) and potential byproducts (oxalic, formic, acetic, butyric, succinic and levulinic acids, methanol and xylitol).

2.3. Alkaline-peroxide microalgae pretreatment

Based on previously published experiments conducted with lignocellulosic materials (Toquero and Bolado, (2014); Karagöz et al., (2012)), H_2O_2 concentrations ranging from 1% to 7.5% were initially selected for the pretreatment of microalgae biomass A1 and A2. The high H_2O_2 concentrations used in A1 and A2 assays involved harsh reactions, which resulted in gas generation, biomass losses by splashing and even break of some bottles. Therefore, only H_2O_2 concentrations of 1% and 2.5% were later on applied to B1, B2 and C. Known mass of microalgae were placed in 1 L bottles and adequate volumes of H_2O_2 solutions (of the selected concentrations), were added to obtain 5% w/w suspensions. Then, the pH was adjusted to 11.5 with 2 M NaOH and the systems incubated in a rotatory shaker at 50ºC and 120 rpm for 60 min. The slurry was cooled down to room temperature, and the residual solid was separated by centrifugation (10 min, 10000 rpm). The experiments were conducted in duplicate. The liquid and solid fractions were stored at 4 ºC for further composition analysis of sugars (glucose, xylose, cellobiose and arabinose). In addition, the potential byproducts formed during biomass pretreatment (oxalic, formic, acetic, butyric, succinic, and levulinic acids, methanol and xylitol) were analysed in the liquid fraction. The solid fractions were used as a substrate in a subsequent enzymatic hydrolysis assay carried out as described above (Toquero and Bolado, 2014).

2.4. Analytical methods

The identification, quantification and biometry measurements of microalgae were carried out by microscopic examination (OLYMPUS IX70) of microalgae samples (fixed with lugol acid at 5% and stored at 4 °C prior to analysis) according to Sournia, (1978). The absorbance ratio [(ABS at 680nm - ABS at750nm)/ ABS at 680nm)], measured in a GENESYS 20 visible spectrophotometer, was used as a qualitative estimation of the microalgae to bacteria ratio (Fairchild et al., 2005).

The determination of the carbon and nitrogen content of the biomass was performed using a LECO CHNS-932 analyzer, while phosphorus and sulphur content analyses were carried out spectrophotometrically after acid digestion in a microwave according to the internal protocol of the Laboratory of Instrumental Analysis of Valladolid University. The starch content was measured following the 996.11 AOAC method. The protein and lipid content were determined using the Lowry method and Kochert method, respectively (Serejo et al., 2015).

The content of moisture, extractives, ash and insoluble residue in raw biomass samples was analysed following NREL (*National Renewable Energy Laboratory* – USA) analytical procedures. The carbohydrate content in the raw and pretreated microalgae was determined by HPLC-RI using a modified NREL procedure. First, biomass was subjected to a concentrated acid hydrolysis for 1 h by adding 3 mL of H_2SO_4 (72% w/w) at 30ºC to a 300 mg dry biomass sample. Then, 84 mL of deionised water was added to dilute the acid concentration to 4% w/w prior to autoclaving at 121ºC for 1h. Then, solid and liquid fractions were separated by centrifugation (10 min, 10000 rpm). The liquid fraction was stored at 4ºC for the determination of sugars, whereas the solid fraction was used for successive acid hydrolysis. This procedure was repeated three consecutive times in order to ensure a complete release and quantification of the sugars present in the biomass. A Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module equipped with Waters 2414 refractive index detector was used to quantify the concentration of sugars (glucose, xylose, cellobiose and arabinose) and byproducts (oxalic, formic, acetic, butyric, succinic and levulinic acids, methanol and xylitol) in the liquid fractions from the pretreatment and hydrolysis assays (hydrolysates). A mobile

phase of 0.025M H_2SO_4 was eluted at a flow ratio of 0.6 mL/min and 50ºC. External standards were used for quantification.

3. Results and discussion

3.1. Algae biomass composition

Table 1 shows the elemental and macromolecular composition of the microalgae biomass evaluated. The most abundant sugars identified were cellulose (as glucose) and hemicellulose (as xylose), although other sugars such as cellobiose and arabinose were also detected in small quantities. On the other hand, starch content was low in all microalgae tested, which determined the nature of the enzymes used during the enzymatic hydrolysis (targeting cellulose and hemicellulose). The C, N and P content of the microalgae biomass grown in domestic wastewater (A1, A2 and C) was in agreement with values typically reported in literature (Posadas et al., 2014), and confirmed the balanced microalgae growth in domestic wastewater. The high ash content recorded in A1 and A2 (~ 40 %) was likely due to the high evaporation losses in the thin layer outdoor photobioreactor, compared to the low ash content measured in the biomass obtained from the enclosed anoxic-aerobic photobioreactor. Unexpectedly, the C, N and P content in the biomass grown in diluted manure (B1 and B2) was lower despite the moderate ash content recorded (~23 %), which suggest a higher oxygen and hydrogen content in this biomass. The results of the elemental composition of the microalgae evaluated correlated with the high lipid content in B1 and B2 (~ 24 %) and the high protein content in C. Microalgae grown in wastewater in excess of nutrients typically exhibit low lipids and carbohydrates contents (Posadas et al., 2015). In this context, a similar carbohydrate content was recorded in all tested biomass (13-16 %). Despite the low content of carbohydrates, a sequential valorisation of the different fractions of these biomass is intended to perform in order to use the whole and have an economically feasible balance, for example using the fraction of proteins to produce fertilisers (Acién et al, 2014). Finally, the qualitative estimation of the microalgae/bacteria ratio revealed a higher abundance of microalgae in all DWB compared to PMWB. The absorbance ratios measured were ~36 for A1 ≈ A2, ~30 for C, and ~10 for B1 ≈ B2. This ratio is related to the biomass growth, decreasing the biomass productivity with the increase of

microalgae/bacteria ratio, with values of 1 g/(L·d) for A1 and A2, 1.5 g/(L·d) for C, and 2.5 g/(L·d) for B1 and B2.

Table 1: Chemical composition in mass percentage of the evaluated microalgae biomass

Parameters	A1	A2	B1	B2	C
Elemental analysis[a]	C (45.03), N (7.80), P (1.99), S (0.52)	C (45.03), N (7.80), P (1.99), S (0.52)	C (37.86), N (4.99), P (1.03), S (0.79)	C (37.86), N (4.99), P (1.03), S (0.79)	C (46.73), N (8.31), P (1.35), S (0.84)
Moisture	4.36 ± 0.81	87.53 ± 0.80	9.10 ± 0.81	80.52 ± 0.85	86.87 ± 0.85
Ash	41.26 ± 1.25	40.20 ± 1.02	23.93 ± 1.24	22.02 ± 1.12	7.68 ± 0.21
Total carbohydrates[a]	15.66 ± 0.20	15.05 ± 0.21	14.18 ± 0.21	13.34 ± 0.15	15.37 ± 0.24
Cellulose[a]	8.09 ± 0.21	7.93 ± 0.23	7.01 ± 0.20	6.52 ± 0.18	7.46 ± 0.17
Hemicellulose[a]	7.25 ± 0.23	6.98 ± 0.18	6.34 ± 0.24	5.74 ± 0.17	7.00 ± 0.31
Proteins[a]	33.35 ± 1.26	33.04 ± 1.26	37.34 ± 1.54	37.04 ± 1.54	63.00 ± 2.74
Lipids[a]	4.47 ± 0.34	4.25 ± 0.34	23.96 ± 0.57	23.56 ± 0.57	16.00 ± 0.50
Insoluble compounds[a]	5.55 ± 0.51	5.60 ± 0.47	2.14 ± 0.24	3.52 ± 0.24	1.52 ± 0.21
Extractives[a]	3.80 ± 0.15	3.80 ± 0.15	4.01 ± 0.20	4.01 ± 0.20	2.42 ± 0.18
Starch[a]	0.77 ± 0.05	0.80 ± 0.05	0.21 ± 0.05	0.61 ± 0.05	0.76 ± 0.05

[a]mass percentage in dry basis

3.2. Enzymatic hydrolysis of raw materials

Table 2 shows the sugars and byproducts concentrations in the liquid fraction from the enzymatic hydrolysis of the microalgae biomass. High released glucose yields were obtained for biomass from domestic wastewater: 93.6% for A1, 87.1% for A2 and 65.1% for C. Nevertheless, remarkably low released xylose yields of 23.5%, 21.2% and 12.6% were recorded for A1, A2 and C, respectively. The different microalgae species may explain this lower sugar release from sample C compared to that from samples A1 and A2. Contrary to A cultures, C biomass was composed of a large fraction of *Desmodesmus* cells. *Desmodesmus* contains four sporopolleninic wall layers along with certain submicroscopic structures on the outermost layer, which do not appear in species of *Scenedesmus*, and could have conferred an especially high resistance to hydrolysis (An et al., 1999). Succinic, acetic and formic acid were the main byproducts obtained in the hydrolysate of DWB. Methanol was also detected in A1 and A2 hydrolysates. Very low concentrations of glucose, no xylose and high concentrations of byproducts were detected in the hydrolysates of samples B1 and B2. These results could be attributed to the high

Table 2: Released sugars (g /100g untreated and pretreated material) and byproducts concentration (g/L) in the liquid fraction after enzymatic hydrolysis

Sample	Released sugars			Byproducts					
	Glucose	Xylose	Total sugars	Acetic acid	Formic acid	Methanol	Succinic acid	Butyric acid	Total byproducts
Untreated A1	7.57 ± 0.18	1.70 ± 0.04	9.84 ± 0.20	0.15 ± 0.00	0.08 ± 0.00	0.10 ± 0.00	0.76 ± 0.06	ND [a]	1.09 ± 0.10
A1_1% H_2O_2	5.12 ± 0.07	0.04 ± 0.00	5.33 ± 0.14	0.05 ± 0.00	0.09 ± 0.00	0.20 ± 0.00	0.04 ± 0.00	ND [a]	0.51 ± 0.04
A1_2.5% H_2O_2	6.18 ± 0.09	0.03 ± 0.00	6.37 ± 0.11	0.06 ± 0.00	0.14 ± 0.00	0.23 ± 0.01	0.04 ± 0.02	ND [a]	0.63 ± 0.06
A1_5% H_2O_2	5.42 ± 0.12	0.03 ± 0.00	5.93 ± 0.13	0.08 ± 0.00	0.27 ± 0.00	0.26 ± 0.02	0.02 ± 0.00	ND [a]	0.83 ± 0.06
A1_7.5% H_2O_2	4.08 ± 0.04	0.00 ± 0.00	5.53 ± 0.11	0.06 ± 0.00	0.23 ± 0.00	0.52 ± 0.02	0.03 ± 0.01	ND [a]	0.97 ± 0.08
Untreated A2	6.91 ± 0.13	1.48 ± 0.04	8.50 ± 0.19	0.13 ± 0.03	0.07 ± 0.01	0.08 ± 0.00	0.61 ± 0.05	ND [a]	0.89 ± 0.12
A2_1% H_2O_2	4.71 ± 0.11	0.05 ± 0.00	4.87 ± 0.11	0.04 ± 0.00	0.09 ± 0.00	0.17 ± 0.01	0.04 ± 0.00	ND [a]	0.50 ± 0.05
A2_2.5% H_2O_2	4.96 ± 0.14	0.07 ± 0.00	5.16 ± 0.21	0.06 ± 0.00	0.14 ± 0.00	0.19 ± 0.01	0.04 ± 0.00	ND [a]	0.63 ± 0.06
A2_5% H_2O_2	4.90 ± 0.04	0.04 ± 0.00	5.38 ± 0.10	0.06 ± 0.00	0.19 ± 0.00	0.24 ± 0.02	0.03 ± 0.01	ND [a]	0.71 ± 0.06
A2_7.5% H_2O_2	3.51 ± 0.05	0.00 ± 0.00	4.05 ± 0.09	0.05 ± 0.00	0.23 ± 0.00	0.44 ± 0.02	0.01 ± 0.01	ND [a]	0.86 ± 0.07
Untreated B1	0.02 ± 0.00	ND [a]	0.02 ± 0.00	5.92 ± 0.26	0.55 ± 0.01	ND [a]	ND [a]	0.91 ± 0.08	7.38 ± 0.24
B1_1% H_2O_2	ND [a]	0.00 ± 0.00	0.01 ± 0.00	5.98 ± 0.24	0.37 ± 0.00	ND [a]	ND [a]	0.86 ± 0.07	7.21 ± 0.39
B1_2.5% H_2O_2	ND [a]	0.03 ± 0.00	0.03 ± 0.00	6.05 ± 0.31	0.60 ± 0.02	ND [a]	ND [a]	1.04 ± 0.10	7.69 ± 0.54
Untreated B2	0.11 ± 0.00	ND [a]	0.11 ± 0.00	5.33 ± 0.25	0.23 ± 0.01	ND [a]	ND [a]	0.91 ± 0.14	6.47 ± 0.42
B2_1% H_2O_2	0.04 ± 0.00	ND [a]	0.04 ± 0.00	5.04 ± 0.25	0.18 ± 0.00	ND [a]	ND [a]	0.72 ± 0.10	5.94 ± 0.28
B2_2.5% H_2O_2	ND [a]	ND [a]	0.01 ± 0.00	5.41 ± 0.28	0.24 ± 0.01	ND [a]	ND [a]	0.99 ± 0.11	6.63 ± 0.34
Untreated C	4.86 ± 0.13	0.88 ± 0.03	6.16 ± 0.14	0.57 ± 0.01	0.16 ± 0.00	ND [a]	0.75 ± 0.03	ND [a]	1.55 ± 0.14
C_1% H_2O_2	3.38 ± 0.06	0.61 ± 0.02	4.16 ± 0.16	0.19 ± 0.03	0.03 ± 0.00	ND [a]	0.19 ± 0.09	0.03 ± 0.01	0.53 ± 0.09
C_2.5% H_2O_2	3.36 ± 0.06	0.78 ± 0.02	4.19 ± 0.12	0.35 ± 0.11	0.06 ± 0.00	ND [a]	0.11 ± 0.05	0.04 ± 0.01	0.99 ± 0.14

[a] ND: not detected

abundance in these samples of bacteria able to oxidise the released sugar to organic acids, mainly acetic acid (6g/L). Butyric and formic acids were the other byproducts found in these hydrolysates. The glucose release yields of A1, A2 and C were in agreement with previous literature studies using pure algae cultures. Thus, Noraini et al., (2014) reported high saccharification yields of 90% during the enzymatic hydrolysis of macroalgae species such as *Ulva fasciata, Sargassum sp and Gracialaria verrucosa* using cellulase and β-glucosidase. Likewise, Ho et al., (2013) obtained 90.4% glucose release from *Chlorella vulgaris* using endoglucanase, β-glucosidase and amylase. Choi et al., (2010) recorded a 94% glucose release from *Chlamydomonas reinhardtii* with a high starch content using a α-amylase-amyloglucosidase pretreatment at 90ºC for 30 min.

In terms of total sugar release, the yields accounted for 62.8%, 56.5% and 40.1% for A1, A2 and C, respectively. A lower reducing sugar yield of 232 mg/g was reported by Li et al., (2016) from *Ulva prolifera* residue during a similar enzymatic hydrolysis at 50ºC and pH 4 for 48 hours. This difference could be attributed to the stronger cell wall of *Ulva prolifera* compared to the species in this study. Considering both the released sugars and the byproducts generated from sugar bioconversion, the percentage of total sugars that were not released and therefore remained in the biomass after the enzymatic hydrolysis were 25.6% for A1, 33.7% for A2, and 43.1% for C. In the particular case of the enzymatic hydrolysis of PMWB, most sugars were released but rapidly oxidised, the fractions of sugars retained in the biomass accounting for 13.1% in B1 and 18.3% in B2.

No remarkable effect of freeze-drying in the release and oxidation of sugars was observed. In fact, the freeze-dried samples A1 and B1 retained a slightly lower percentage of sugars than the reconstituted A2 and the fresh B2, respectively, and even with a small increase on sugar conversion by the bacterial action. However, Gruber-brunhumer et al., (2015) concluded that freeze-drying could be considered as a preliminary pretreatment capable of increasing biomethane production during the anaerobic digestion of *Scenedesmus obliquus*.

The results of sugar extraction with three successive acid hydrolysis, used as analytical method to determine the total sugar content of microalgae (Fig.1), were systematically compared to the results of the released sugars by enzymatic hydrolysis. Extracted sugars

by acid hydrolysis for A1, A2 and C accounted for 76% of the total carbohydrate content in the first cycle, 16.5% in the second cycle and 7.5% in the last one. Unexpectedly, B1 and B2 were more resistant to acid hydrolysis than DWB, with released sugar yields of ~60%, ~30% and 10% in the first, second and third cycle, respectively. The action of bacteria may explain this mismatch between enzymatic and acid hydrolysis. Thus, bacteria could have enhanced sugar release during enzymatic hydrolysis, but were inhibited by the low pH present during acid hydrolysis. In addition, only a slight improvement mediated by freeze-drying was found during acid hydrolysis. In this context, Miranda et al., (2012) observed a significant increase of 55% in sugars solubilisation from *Scenedesmus obliquus* by acid hydrolysis when comparing the potential of wet and dried biomass for bioethanol production. No additional sugar extraction cycles were required by these authors when acid hydrolysis was conducted at 2N sulphuric acid, 50ºC and 2 min. Nevertheless, three consecutive cycles were always necessary to completely extract the sugars present in the different biomass tested in our study, regardless of the storage procedure.
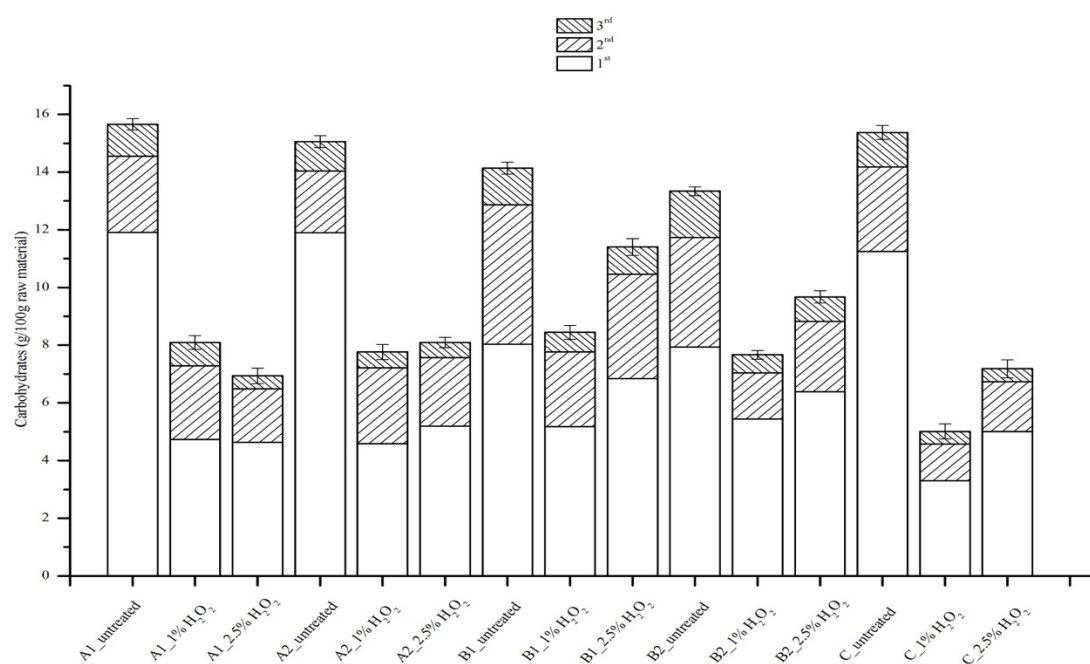
Figure 1. Total carbohydrates (g/100g raw material) obtained from three consecutive acid hydrolysis.

The results here obtained represent a great opportunity for the application of the biorefinery concept to residual microalgae biomass generated from wastewater treatment with moderate to high bacteria/microalgae ratios. Nevertheless, low xylose release

efficiencies and high transformation were observed in the hydrolysates of untreated raw materials. In this regard, alkaline peroxide seems to be a suitable pretreatment to increase the xylose release and reduce the sugar transformation into byproducts.

3.3 Alkaline-peroxide pretreatment

3.3.1 Sugars in solid and liquid fractions and byproducts generation

The cellulose (as glucose) and hemicellulose (as xylose) content of the pretreated solid fractions of the biomass and the concentrations of solubilised sugars and total byproducts are shown in the Table 3. Large differences on sugar solubilisation during pretreatment were observed among the different microalgae evaluated. Similarly, to the acid hydrolysis assays, B1 and B2 were the most resistant biomass and thus supported the lowest values of sugar solubilisation and transformation. In terms of sugars in the liquid fractions, a solubilisation higher for xylose than for glucose was detected in most cases for A1 and A2. Total byproducts concentration was approximately 1g/L for A1, A2 and C, and 0.15g/L for B1 and B2. The solubilised glucose increased with increasing H_2O_2 concentration and represented 9.4, 15.8, 17.5 and 41.8% of the cellulose present in the untreated biomass for A1, and 9.8, 14.1, 18.4 and 30.0% for A2 at 1, 2.5, 5 and 7.5% H_2O_2, respectively. These results were in agreement with the observations of Karagöz et al., (2012), who reported increases in glucose solubilisation from 10.5 to 12.0% in rapeseed straw when increasing hydrogen peroxide concentration from 1.25 to 5% H_2O_2. Similar glucose solubilisations of 13.7 and 15.3% of the total cellulose present in C were recorded at 1 and 2.5% H_2O_2. However, low glucose solubilisations were measured for samples B1 and B2 (0.6 and 2.0% for B1; and 0.9 and 1.2% for B2 at 1 and 2.5 % H_2O_2, respectively).

Surprisingly, the solubilised xylose was not correlated to H_2O_2 concentration, with extraction yield of 30% of the hemicellulose initially present in the raw material for A1 and A2. The xylose solubilisation values were remarkably low for B1, B2 and C (contrary to the common behavior of hemicellulose, being much easier hydrolysed than cellulose), and were inversely correlated to H_2O_2 concentration.

Table 3: Sugars composition in the solid fractions (%), solubilised sugars (g/L) and total byproducts (g/L) in the liquid fractions

Sample	Solid fraction (%)			Liquid fraction (g/L)			
	Cellulose (as glucose)	Hemicellulose (as xylose)	Total sugars	Glucose	Xylose	Total sugars	Total byproducts
A1_1% H_2O_2	7.36 ± 0.20	5.45 ± 0.14	12.98 ± 0.24	0.38 ± 0.03	1.22 ± 0.01	1.76 ± 0.05	1.01 ± 0.02
A1_2.5% H_2O_2	7.82 ± 0.20	5.75 ± 0.14	13.71 ± 0.27	0.64 ± 0.05	1.14 ± 0.03	1.97 ± 0.02	1.46 ± 0.09
A1_5% H_2O_2	7.06 ± 0.19	5.30 ± 0.19	12.51 ± 0.18	0.71 ± 0.02	1.11 ± 0.02	2.02 ± 0.04	1.53 ± 0.07
A1_7.5% H_2O_2	6.06 ± 0.17	3.45 ± 0.11	9.59 ± 0.24	1.69 ± 0.05	1.11 ± 0.02	3.00 ± 0.05	1.78 ± 0.07
A2_1% H_2O_2	7.35 ± 0.20	5.07 ± 0.15	12.55 ± 0.26	0.35 ± 0.02	1.12 ± 0.03	1.62 ± 0.05	0.97 ± 0.04
A2_2.5% H_2O_2	7.79 ± 0.19	5.09 ± 0.14	13.05 ± 0.18	0.56 ± 0.01	1.12 ± 0.02	1.78 ± 0.05	1.28 ± 0.08
A2_5% H_2O_2	6.93 ± 0.17	5.23 ± 0.14	12.30 ± 0.21	0.73 ± 0.02	1.16 ± 0.01	2.08 ± 0.06	1.45 ± 0.06
A2_7.5% H_2O_2	5.38 ± 0.17	2.59 ± 0.09	8.05 ± 0.23	1.19 ± 0.04	0.98 ± 0.07	2.32 ± 0.08	1.54 ± 0.07
B1_1% H_2O_2	5.92 ± 0.24	4.32 ± 0.17	11.48 ± 0.24	0.02 ± 0.00	0.10 ± 0.01	0.12 ± 0.02	0.15 ± 0.01
B1_2.5% H_2O_2	6.70 ± 0.24	5.50 ± 0.17	13.28 ± 0.29	0.07 ± 0.00	0.01 ± 0.00	0.08 ± 0.01	0.18 ± 0.01
B2_1% H_2O_2	4.37 ± 0.17	4.01 ± 0.16	9.61 ± 0.15	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
B2_2.5% H_2O_2	4.77 ± 0.17	5.10 ± 0.22	10.92 ± 0.21	0.04 ± 0.00	0.00 ± 0.00	0.04 ± 0.01	0.14 ± 0.01
C_1% H_2O_2	4.62 ± 0.19	5.25 ± 0.15	10.71 ± 0.26	0.51 ± 0.02	0.28 ± 0.01	0.79 ± 0.02	0.89 ± 0.08
C_2.5% H_2O_2	5.19 ± 0.23	5.48 ± 0.12	11.54 ± 0.31	0.57 ± 0.08	0.02 ± 0.00	0.59 ± 0.01	1.18 ± 0.11

In this context, Yu et al., (2015) also observed a slight decrease of glucose and xylose solubilisation from sugarcane bagasse when increasing the H_2O_2 concentration, which was attributed to monomers degradation under high dosage of H_2O_2. Unfortunately, the authors did not report the concentrations of byproducts formed during pretreatment.

The concentration of total byproducts in the liquid fraction was correlated to the concentration of solubilised glucose after pretreatment. Hence, higher byproduct concentrations were observed at increasing H_2O_2 concentration in A1, A2 and C. The main byproducts found in the liquid fraction after pretreatment of A1 and A2 biomass were formic acid (~60%) and acetic acid (20%), with methanol and succinic acid detected at very low concentrations. On the other hand, acetic acid represented 50% of the total byproducts after pretreatment in the liquid fraction of B1, B2 and C, while formic, butyric, succinic and levulinic acids and xylitol were produced at trace levels. Methanol was only detected in the liquid fraction of sample C after pretreatment. Finally, and in agreement with the results reported by other authors when applying alkaline peroxide pretreatment for lignocellulosic materials (Karagöz et al., 2012), neither furfural nor HMF (inhibitory compounds) were detected in this work.

Sugars solubilisation and transformation during the pretreatment of DWB represented a noteworthy loss of total sugar potential. The losses increased with H_2O_2 concentration, accounting for 35.4, 43.8, 45.3 and 61.0% in A1, 34.4, 40.7, 46.9 and 51.3% in A2, and 25.2 and 26.5% in C. These high sugar losses during pretreatment allowed foreseeing a final low sugar release yield during enzymatic hydrolysis in A1 and A2. At this point, it should be remarked that the final sugar content of the microalgae hydrolysate is critical for the economic sustainability of microalgae biorefineries devoted to ferment the released sugars. In our particular study, the low sugars concentration, along with the high concentration of byproducts and potentially inhibitory residues from alkaline-peroxide pretreatment would hinder the fermentation of the hydrolysates by a diauxic microorganism such as *Pichia stipitis*. On the other hand, these losses were barely noticeable in PMWB (3.8 and 3.7% in B1, and 2.1 and 2.7% in B2 at 1 and 2.5% H_2O_2, respectively). Again, the biomass from pig manure wastewater was more resistant in a chemical inhibitory medium. This finding highlighted the beneficial effect of alkaline-peroxide pretreatment on the further utilisation of biomass with high bacteria/algae ratios. On the other hand, the freeze-drying and initial moisture content of the biomass exhibited

a scanty effect on the sugar release and further bioconversion during H_2O_2 pretreatment. Thus, only slightly higher solubilisation yields and byproducts generation were obtained for freeze-dry biomass (A1 and B1) and reconstituted (A2) or fresh biomass (B2).

Significant biomass losses during pretreatment of ~30% of the initial microalgae mass were estimated for samples A1 and A2 from the results in Table 3 (data not shown). These high values suggested a solubilisation of other components than sugars during pretreatment, whose determination was out of the scope of this study. In fact, alkaline-peroxide pretreatment is capable of supporting high lignin solubilisations in wheat straw at operating conditions compared to those used in this work (5% H_2O_2, pH 11.5, 1h, 50ºC) (Toquero and Bolado, 2014). In addition, a decrease in cellulose and hemicellulose content compared to the raw biomass was observed for all solid fractions of pretreated material.

3.3.2 Enzymatic hydrolysis of pretreated samples

Table 2 shows the concentration of released sugars and byproducts resulting from the enzymatic hydrolysis of pretreated samples. No clear correlation between hydrogen peroxide concentration and the yields of glucose and xylose release was found, considering the different sugars concentrations in the pretreated materials before enzymatic hydrolysis (Table 3). These results were in agreement with Li et al., (2016), who reported an increase in the reducing sugar yield when increasing H_2O_2 concentration up to 0.5%, followed by a reduction of sugars yield when increasing H_2O_2 concentration to 2 %.

The concentration of released glucose from all pretreated samples was lower than that from untreated samples. The released glucose yield for A1 varied from 67.3 to 78.8% in pretreated samples, which was significantly lower than the 93.6% for untreated A1 biomass. Similar released glucose yields ranging from 63.7 to 70.7% were obtained for A2. However, comparable glucose yields (~65%) were found during the enzymatic hydrolysis of untreated and pretreated samples of biomass C. These glucose release yields recorded in pretreated biomass were very similar to the value of 64% reported by Harun and Danquah, (2011a) during the cellulose-based hydrolysis of *Chlorococum sp.* pretreated by ultrasounds. On the other hand, very low xylose release yields were obtained for all pretreated microalgae samples, despite most studies investigating the enzymatic

hydrolysis of lignocellulosic materials pretreated with H_2O_2 under alkaline conditions reported an increase on released xylose yield. For example, this yield increased from 6.4% to 28.9% when sugarcane bagasse was pretreated (Yu et al., 2015) and from 9.3% to 48% when pretreating wheat straw (Toquero and Bolado, 2014).

In general terms, the concentration of byproducts was similar in hydrolysates from pretreated samples and in those from raw materials, which suggests that H_2O_2 pretreatment did not exerted a significant disinfectant effect. In fact, the concentration of byproducts increased with H_2O_2 concentration likely due to a chemical mediated sugars oxidation. Enzymatic hydrolysis released almost the entire sugar content of pretreated B1 and B2 samples, which was transformed to byproducts at concentrations similar to those recorded in untreated biomass samples (e.g. ≈ 6 g/L acetic acid). Freeze-drying resulted in a higher concentration of byproducts in the hydrolysate compared to the hydrolysate of the pretreated fresh sample B2. In addition to acetic, formic and butyric acid, succinic acids were obtained in the hydrolysate of microalgae C, although at lower concentrations than those recorded for A1 and A2. Biomass pretreatment promoted the generation of oxalic acid and increased methanol production in samples A1 and A2, along with the formation of acetic, formic and succinic acids.

The concentration of sugars released from pretreated samples by successive acid hydrolysis is shown in Fig. 1. The pretreatment of biomass grown in domestic wastewater (A1, A2 and C) decreased the release of sugars in the first acid hydrolysis compared to untreated biomass. Extraction efficiencies of 58-69%, 24-33% and 6-10% were measured in the first, second and last cycle. Nevertheless, the sugar released in the first acid hydrolysis cycle increased with H_2O_2 concentration in the three DWB samples. These experimental observations could be attributed to the antagonistic effects of the pretreatment. Indeed, while H_2O_2 pretreatment disrupts biomass structure, it promotes the loss of easily releasable sugars by solubilisation. On the other hand, the pretreatment of samples B1 and B2 increased the released sugar during the first acid hydrolysis compared to untreated PMWB samples, which resulted in yields of 60-70%. Sugar solubilisation during the pretreatment of PMWB was low and the disruption of the cell wall structure was dominant. Surprisingly, the first acid hydrolysis after pretreatment did not achieve the high values of sugar solubilisation obtained by enzymatic hydrolysis of untreated samples. It was hypothesis the disruption effect of bacteria is higher than that of the tested

pretreatment, but the low pH values during acid hydrolysis inhibited the hydrolytic mechanisms of bacteria.

In order to evaluate the overall performance of the process, solubilisation of glucose and xylose and their further oxidation during both pretreatment and enzymatic hydrolysis must be considered. The alkaline-peroxide pretreatment increased sugar solubilisation from biomass by enzymatic hydrolysis, but at decreasing or similar sugar recovery yields due to the generation of byproducts. At the highest H_2O_2 concentration tested, only 10 % of the initial sugars present in A1 and A2 remained in the pretreated and hydrolysed biomass residues. Sugar extraction in samples B1 and B2 was however complete. No influence of H_2O_2 concentration on sugar solubilisation was found in sample C.

4. Conclusions

Enzymatic hydrolysis supported high efficiencies of glucose release from DWB but a low xylose release. Despite the efficient sugar solubilisation from PMWB mediated by the enzymatic method tested, the high bacterial content of this biomass promoted a rapid oxidation of the released sugars to organic acids and methanol. No significant influence of the biomass storage conditions was observed during enzymatic hydrolysis. Finally, alkaline-peroxide pretreatment increased the global sugar solubilisation, considering both, pretreated liquid fractions and hydrolysates from enzymatic hydrolysis. Overall, the evaluated alkaline-peroxide pretreatment increased sugar oxidation to organic acids and methanol regardless of the biomass type and storage conditions.

5. Acknowledgements

6. References

[1] Acién F.G., Fernández, J.M., Molina-Grima E., 2014. Economics of Microalgae Biomass Production, in: Pandey A., Lee D.J., Chisti Y., Soccol C.R. (Eds) Biofuels from Algae, Burlington, pp. 313–325.

[2] Alcántara., C., Domínguez, J.M., García, D., Blanco, S., Pérez, R., García-Encina, P.A., Muñoz, R., 2015. Evaluation of wastewater treatment in a novel anoxic – aerobic algal – bacterial photobioreactor with biomass recycling through carbon and nitrogen mass balances. Bioresour. Technol. 191, 173–186.

[3] Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I., 2012. Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment. Bioresour. Technol. 123, 488–494.

[4] An, S.S., Friedel, T., Hegewald, E., 1999. Phylogenetic relationships of Scenedesmus and Scenedesmus-like coccoid green algae as inferred from ITS-2 rDNA sequence comparison. Plant Biol. 1, 418–428.

[5] Bolado-Rodríguez, S., Toquero, C., Martín-Juárez, J., Travaini, R., García-Encina, P.A., 2016. Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. Bioresour. Technol. 201, 182–190.

[6] Castro, Y.A., Ellis, J.T., Miller, C.D., Sims, R.C., 2015. Optimisation of wastewater microalgae saccharification using dilute acid hydrolysis for acetone, butanol, and ethanol fermentation. Appl. Energy 140, 14–19.

[7] Chen, C.Y., Zhao, X.Q., Yen, H.W., Ho, S.H., Cheng, C.L., Lee, D.J., Bai, F.W., Chang, J.S., 2013. Microalgae-based carbohydrates for biofuel production. Biochem. Eng. J. 78, 1–10.

[8] Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

[9] Choi, S.P., Nguyen, M.T., Sim, S.J., 2010. Enzymatic pretreatment of Chlamydomonas reinhardtii biomass for ethanol production. Bioresour. Technol. 101, 5330–5336.

[10] Daroch, M., Geng, S., Wang, G., 2013. Recent advances in liquid biofuel production from algal feedstocks. Appli. Energy 102, 1371–1381.

[11] Fairchild, G.W., Anderson, J.N., Velinsky, D.J., 2005. The trophic state "chain of relationships" in ponds: Does size matter? Hydrobiologia 539, 35–46.

[12] González-Fernández C., Sialve B., Bernet N., Steyer J.P., 2012. Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. Review, biofuels, Bioprod. Biorefining 6, 246–256.

[13] Gruber-brunhumer, M.R., Jerney, J., Zohar, E., Nussbaumer, M., Hieger, C., Bochmann, G., 2015. Acutodesmus obliquus as a benchmark strain for evaluating methane production from microalgae: Influence of different storage and pretreatment methods on biogas yield. Algal Research 12, 230–238.

[14] Harun, R., Danquah, M.K., 2011a. Enzymatic hydrolysis of microalgal biomass for bioethanol production. Chem. Eng. J. 168, 1079–1084.

[15] Harun, R., Danquah, M.K., 2011b. Influence of acid pre-treatment on microalgal biomass for bioethanol production. Process Biochem. 46, 304–309.

[16] Hernández, D., Riaño, B., Coca, M., García-González, M.C., 2015. Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chem. Eng. J. 262, 939–945.

[17] Ho, S., Huang, S., Chen, C., Hasunuma, T., Kondo, A., 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresour. Technol. 135, 191–198.

[18] Karagöz, P., Rocha, I. V, Özkan, M., Angelidaki, I., 2012. Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by Same Vessel Saccharification and Co-Fermentation. Bioresour. Technol. 104, 349–357.

[19] Lam, M.K., Lee, K.T., 2015. Bioethanol Production from Microalgae, in: Kim S.K. (Ed.), Handb. Mar. Microalgae, pp. 197–208.

[20] Li, Y., Cui, J., Zhang, G., Liu, Z., Guan, H., Hwang, H., Aker, W.G., Wang, P., 2016. Optimisation study on the hydrogen peroxide pretreatment and production of bioethanol from seaweed Ulva prolifera biomass. Bioresour. Technol. 214, 144–149.

[21] Miranda, J.R., Passarinho, P.C., Gouveia, L., 2012. Pre-treatment optimisation of Scenedesmus obliquus microalga for bioethanol production. Bioresour. Technol. 104, 342–348.

[22] Monlau, F., Barakat, A., Steyer, J.P., Carrere, H., 2012. Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks. Bioresour. Technol. 120, 241–247.

[23] Noraini, M.Y., Ong, H.C., Badrul, M.J., Chong, W.T., 2014. A review on potential enzymatic reaction for biofuel production from algae. Renew. Sustain. Energy Rev. 39, 24–34.

[24] Ometto, F., Quiroga, G., Pšenička, P., Whitton, R., Jefferson, B., Villa, R., 2014. Impacts of microalgae pre-treatments for improved anaerobic digestion: Thermal treatment, thermal hydrolysis, ultrasound and enzymatic hydrolysis. Water Res. 65, 350–361.

[25] Posadas, E., García-Encina, P.A., Domínguez, A., Díaz, I., Becares, E., Blanco, S., Muñoz, R., 2014. Enclosed tubular and open algal-bacterial biofilm photobioreactors for carbon and nutrient removal from domestic wastewater. Ecol. Eng. 67, 156–164.

[26] Posadas, E., Szpak, D., Lombó, F., Domínguez, A., Díaz, I., Blanco, S., 2015. Feasibility study of biogas upgrading coupled with nutrient removal from anaerobic effluents using microalgae-based processes.J. Appl. Phycol. doi:10.1007/s10811-015-0758-3

[27] Schultz-Jensen, N., Thygesen, A., Leipold, F., Thomsen, S.T., Roslander, C., Lilholt, H., Bjerre, A.B., 2013. Pretreatment of the macroalgae Chaetomorpha linum for the production of bioethanol – Comparison of five pretreatment technologies. Bioresour. Technol. 140, 36–42.

[28] Serejo, M.L., Posadas, E., Boncz, M.A., Blanco, S., García-Encina, P., Muñoz, R., 2015. Influence of biogas flow rate on biomass composition during the optimisation of biogas upgrading in microalgal-bacterial processes. Environ. Sci. Technol. 49, 3228–3236.

[29] Silveira, M.H.L., Morais, A.R.C., Da Costa Lopes, A.M., Olekszyszen, D.N., Bogel-Lukasik, R., Andreaus, J., Pereira Ramos, L., 2015. Current Pretreatment Technologies for the Development of Cellulosic Ethanol and Biorefineries. ChemSusChem 8, 3366–3390.

[30] Sournia, A., 1978. Phytoplankton Manual, Unesco, United Kingdom.

[31] Tijani, H., Abdullah, N., Yuzir, A., 2015. Integration of microalgae biomass in biomethanation systems. Energy Rev. 52, 1610–1622.

[32] Toquero, C., Bolado, S., 2014. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. Bioresour. Technol. 157, 68–76.

[33] Travaini, R., Derly, M., Otero, M., Coca, M., Da-silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. Bioresour. Technol. 133, 332–339.

[34] Yu, H., You, Y., Lei, F., Liu, Z., Zhang, W., Jiang, J., 2015. Comparative study of alkaline hydrogen peroxide and organosolv pretreatments of sugarcane bagasse to improve the overall sugar yield. Bioresour. Technol. 187, 161–166.

[35] Zhou, N., Zhang, Y., Gong, X., Wang, Q., Ma, Y., 2012. Ionic liquids-based hydrolysis of Chlorella biomass for fermentable sugars. Bioresour. Technol. 118, 512–517.

Chapter 6

Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater

Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater

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Chapter 6

ABSTRACT

Biomass grown in wastewater treatment photobioreactors is a cheap raw material with high contents of carbohydrates, proteins and lipids. This work studies the production of fermentable monosaccharides from three biomasses grown in piggery wastewater (P), domestic wastewater (W) and synthetic medium (S) by applying chemical pretreatment and enzymatic hydrolysis, using a Taguchi design.

ANOVA identified temperature, chemical reagent type and chemical reagent concentration as significant operational parameters. However, the biomass concentration, pretreatment time, enzyme dosage and enzymatic hydrolysis time had no remarkable effect. The bacterial content of the biomass had no relevant impact on carbohydrate and protein solubilisation but had a remarkable effect on the degradation of the released carbohydrates (57, 60 and 37% for P, W and S), while also affecting lipid solubilisation. Pretreatment with HCl 2M at 120ºC resulted the optimal conditions, achieving a monosaccharide recovery of 53, 59 and 80% for P, W and S biomasses, respectively.

Keywords: Enzymatic hydrolysis; Lipids; Pig manure; Pretreatment; Proteins; Taguchi method

1. Introduction

Microalgae are considered a promising bio-based feedstock and a great source of carbohydrates, proteins and lipids, which has increased their use in the recent years. Microalgae photosynthetically consume CO_2 as a carbon source, use sunlight as an energy source, can treat different types of wastewaters and exhibit high areal productivities in non-arable land (Jankowska et al., 2017; Su et al., 2017). Nowadays, the cultivation of axenic microalgae is costly (Zhuang et al., 2018), but the integration of microalgae cultivation and wastewater treatment significantly reduces the production costs of microalgae biomass. By contrast, complex mixtures of different microalgae species and bacteria grow symbiotically in these treatment photobioreactors hinder the valorisation of the biomass (Kadir et al., 2018; Chen et al., 2013).

At an industrial scale, microalgae are currently used to produce extracts of specific high added value products, such as astaxanthin or pigments, but the rest of components are typically not valorised, which jeopardises the economic sustainability of these processes

(Koutra et al., 2018). Thereby, one of the main challenges of microalgae cultivation is the valorisation of every fraction of the microalgae biomass. Among the different components, the carbohydrate fraction could be used as a carbon source for fermentation processes for the production of biofuels like bioethanol, biohydrogen, biobutanol (Sankaran et al., 2018) and even for the production of polyhydroxyalkanoates (Rahman and Miller, 2017).

Cell wall disruption is typically the main bottleneck to valorise the components of algal biomass. This step becomes even more critical for algal-bacterial biomass grown in wastewater treatment photobioreactors, due to the resistant and recalcitrant cell wall of microalgae species able to growth in these media (Onumaegbu et al., 2018). Among the possible alternatives, chemical pretreatments have been successfully tested to support microalgae cell wall disruption, resulting in a fast and relatively inexpensive cell breakdown while providing high carbohydrate solubilisation. As examples of effective chemical pretreatments, Shokrkar et al., (2017) achieved a monosaccharide recovery of 94% from a mixture of pure microalgae species using 2M HCl at 120ºC for 30 min. Markou et al., (2013) obtained a carbohydrate solubilisation of 90% from *Spirulina platensis* using 0.5N HNO_3 at 100ºC for 3h. Likewise, Harun et al., (2011) pretreated *Chlorococcum infusionum* biomass with alkali, achieving a maximum yield of 0.350 $g_{glucose}/g_{dw}$ at 0.75% (w/v) NaOH, 120ºC for 30 min. In addition, the potential sterilisation effect of chemical pretreatment is of great interest when pretreating microalgae-bacteria consortia, due to the prevention of the microbial degradation of the released components by microorganisms present in the cultivation broth (Fuentes et al., 2016).

The high variability and the bacterial content of the biomass grown in wastewater treatment photobioreactors are also major challenges to be considered (Oh et al., 2018). Biomass grown in open photobioreactors is strongly dependent on uncontrollable factors, such as climatic and environmental conditions (Kumar et al., 2019), as well as on the characteristics of the wastewater (García et al., 2017; Iasimone et al., 2018; Lv et al., 2018; Ganeshkumar et al., 2018). A robust optimisation of the process that would be able to provide high extraction yields independently of the intrinsic variability of biomass grown in wastewater treatment photobioreactors is a requirement to successfully implement the process at both pilot and industrial scales (El-Dalatony et al., 2019).

This work aims at optimising the production of fermentable monosaccharides from the carbohydrate fraction of algal-bacterial biomass grown in photobioreactors. Based on previous results (Martín Juárez et al., 2018), a two-step process with a chemical pretreatment followed by an enzymatic hydrolysis was selected. A Taguchi $L_{27}(3^{13})$ design was used to evaluate the influence of the main experimental parameters and their interaction effects on carbohydrate solubilisation and monosaccharide recovery, and to analyse the loss of released sugars via chemical or metabolic degradation. The effect of the pretreatment and the enzymatic hydrolysis on proteins and lipids was also evaluated by applying the concept of bio-refinery. In order to achieve a robust optimisation, independent of the substrate characteristics, the complete experimental design was applied to three types of biomass grown in piggery wastewater, domestic wastewater and a synthetic medium. These particular wastewater streams were selected in order to obtain a wide variation of bacterial content in the microalgae biomass, which is a main objective of this study. The microalgae grown in synthetic medium, without bacteria, is an extreme condition and is comparable to most of the previously published research in this field which worked with pure microalgae.

2. Materials and methods

2.1. Raw materials

The biomass used in this work was cultivated in a 1.2 m^3 outdoor thin-layer photobioreactor operating under steady-state at the facilities of the Cajamar Foundation (Almería, Spain) (Morales-Amaral et al., 2015). Three experiments were performed feeding the photobioreactor with different media: piggery wastewater (P), domestic wastewater (W) and synthetic culture medium (S). The different types of biomass cultivated were concentrated through centrifugation up to a concentration of 20% (P), 24% (W) and 18% (S). The biomass was refrigerated at 4 °C prior to use for a maximum of 48 h. The chemical composition of these fresh biomasses was as follows: 22.3% of carbohydrates (including 1.7% of starch), 51.7% of proteins and 13.4% of lipids for P grown biomass; 24.2% of carbohydrates (including 1.4% of starch), 45.4% of proteins and 14.0% of lipids for W grown biomass; and 21.9% (including 1.9% of starch) of carbohydrates, 58.0% of proteins and 13.7% of lipids for S grown biomass (percentages refer to dry mass).

The main microalgae species present in the three biomasses were as follows: *Scenedesmus acutus* (32%), *Chlorella kessieri* (23%), *Scenedesmus obliquus* (17%), *Scenedesmus sp.* (12%) and *Aphanothece saxicola* (12%) in biomass P; *Scenedesmus acutus* (65%), *Scenedesmus acuminatus* (27%) and *Chlorella kessieri* (7%) in biomass W; and *Scenedesmus acutus* (98%) in biomass S.

The identification and quantification measurements of the microalgae species were performed by microscopic examination (OLYMPUS IX70) using at least three different samples using a counting chamber according to Sournia, (1978). Biomass samples were fixed with lugol acid at 5% and stored at 4 °C prior to analysis.

2.2. Pretreatments

Weighted amounts of biomass and the corresponding volumes of 5 M HCl or NaOH and distilled water – to achieve a total volume of 300 mL of suspension – were introduced in 1 L borosilicate bottles. The bottles were introduced in a thermostatic bath or in an autoclave at the pre-established temperature during the time selected for each experiment. The pretreated suspensions were stored at 4 ºC for a maximum period of 24 h for further enzymatic hydrolysis experiments. Additional aliquots were centrifuged at 10,000 rpm for 6 min to separate the solid and liquid fractions, which were then weighted. The content of carbohydrates, proteins and lipids was analysed in the solid fractions and the monosaccharide concentration was measured in the liquid fractions. In order to check the mass balances, total and volatile solids were determined in the solid and liquid fractions, as well as in the whole suspensions.

2.3. Enzymatic hydrolysis

Assays to study the enzymatic hydrolysis conditions in the pretreated biomass were carried out at a biomass concentration of 5 % w/w and adjusting the final concentration with distilled water when necessary. The pH was adjusted to 4.9 ± 0.1. The tests were performed in 100 mL Erlenmeyer flasks with a working volume of 25 mL by adding the required enzyme dosage (Celluclast 1.5L - Cellulase from *Trichoderma reesei*) and a 1 M citrate buffer (Travaini et al., 2016). The assays were carried out in a rotatory shaker at 50 ºC and 300 rpm at the tested incubation times. The experiments were performed in duplicate.

The solid and liquid fractions were separated by centrifugation (10 min, 10,000 rpm) and weighted after the enzymatic hydrolysis. The carbohydrate, protein and lipid concentrations were determined in the solid fractions and the monosaccharide concentration was determined in the liquid fractions (Martín Juárez et al., 2016). Total and volatile solids were determined in the solid and liquid fractions as well as in the whole suspensions to check the mass balances. All analyses were carried out in duplicate.

2.4. Calculation of yields

The following parameters were defined to understand the process and to determine the solubilisation of carbohydrates, proteins and lipids, the loss of carbohydrates via degradation and the recovery of monosaccharides in the liquid fractions during the pretreatment step and the global process (pretreatment + enzymatic hydrolysis):

$$\text{Eq. (1)}$$
$$\text{Eq. (2)}$$
$$\text{Eq. (3)}$$

where "components" are carbohydrates, proteins and lipids and "PR" is the initial biomass. The solid and liquid fractions were from the pretreatment for the pretreatment step yields and from the enzymatic hydrolysis for the global yields.

2.5. Optimisation of operational conditions by Taguchi's robust parameter design

Seven operational parameters (control factors) were selected in this study based on previous works on monosaccharide production from solid wastes by applying chemical pretreatments and enzymatic hydrolysis: biomass concentration (C_A), chemical reagent (H), chemical reagent concentration (C_Q), temperature (T) and pretreatment time (t) on the pretreatment step and enzyme dosage (E) and time (t_H) for the enzymatic hydrolysis. Interaction effect of some control factors ($C_Q \times T$, $C_Q \times t$ and $T \times t$) were also considered. The optimisation was carried out using the Taguchi's orthogonal arrays (OA) $L_{27}(3^{13})$ design. This experimental design, with 27 freedom degrees, permits three levels for each control factor in order to detect quadratic or non-linear effects of the parameters and to obtain information over a wide range of the factors. Additionally, this design provides information about the interaction effect of 3 combinations of control factors (Taguchi et al., 2007).

Table 1. Levels of the studied control factors[a] for the optimisation by Taguchi design compared with other published works.

References	Statistical design	Microalgae biomass	C_Q	T	T min	C_A g/L	H	E FPU/g	t_H hours	Remarks
This study	Taguchi design, three levels (1, 2, 3)	P, W: Mixed microalgae-bacteria S: *Scenedesmus Almeriense*	1: 0.5M 2: 1M 3: 2M	1: 80 ºC 2: 100 ºC 3: 120 ºC	1: 10 2: 30 3: 60	1: 50 g/L 2: 75 g/L 3: 100 g/L	1: HCl 2: NaOH 3[b]: HCl	1: 10 2: 30 3: 60	1: 3 2: 6 3: 12	
Asyraf Kassim and Bhattacharya, (2016)	Response surface method	*Chlorella sp.*	0.1 to 0.5 M	60 to 120ºC	30 to 120		NaOH	-	-	Sugar yield: 88mg/g at 120ºC, 2% NaOH, 30 min
Harun et al., (2011)	Central composite design	*Chlorococcum humicola*	0.2 to 0.75 M	60 to 140ªC	15 to 60		NaOH			Glucose yield: 350 mg/g at 0.75%, 120ºC, 30 min
Hernández et al., (2015)		*C.sorokiniana N.gaditana S. almeriensis*	0 to 2.5M	121ºC	30		H_2SO_4	15 Celluclast 1.5L		Maximum sugar release *C.sorokiniana*: 100mg/g *N.gaditana:* 125mg/g *S. almeriensis:* 50mg/g
Pancha et al., (2016)		*Scenedesmus sp.* CCNM 1077	0.1 to 3M	121ºC	15 to 60	20 to 100	HCl, H_2SO_4, NaOH, KOH	Cellulase	6, 24, 48, 72	HCl, 60 min, 0.5M, 6% of biomass, 72h.
Shokrkar et al., (2017)		Mixed microalgae-bacteria biomass	0.5, 1 and 2M	121ºC	10 to 40		HCl, H_2SO_4, NaOH			Sugar yield: 95% at HCl, 2M, 30 min
Sivaramakrishnan and Incharoensakdi, (2018)		*Scenedesmus sp.*	0.1, 0.2 and 0.3N	60 to 120ºC	10 to 40		HCl, H_2SO_4, NaOH, KOH			Sugar yield: 80% at 0.3N, 120ºC, 20min, NaOH

[a]C_Q: Concentration of chemical reagent; T: Temperature; t: time; C_A: concentration of microalgae biomass; H: reagent; E: dosage of enzyme; t_H: time during the enzymatic hydrolysis.
[b]Level 3 for chemical reagent corresponds again to HCl (dummy effect).

Chapter 6

The range as well as the specific values of each operational parameter were selected based on previous results and unpublished research (Table 1). Individual control factors and interactions of control factors were assigned to the columns of the OA according to the adequate triangular table and linear graph (Taguchi and Konishi, 1987). The chemical reagent type (H) was tested at only two levels, using HCl and NaOH solutions. The dummy treatment allowed for the accommodation of the factor H at only two levels into a column with three levels while orthogonality was maintained by repeating one of the two levels (Ross, 1995). The experimental design matrix is shown in Table 2. The execution order of each set of 27 experiments was randomised.

The variability of the microalgae biomass, inherent and uncontrollable in a real wastewater treatment process, was introduced in the experimental design as a noise factor by using three microalgae biomass grown in rather different media to achieve a robust response. Each of the 27 combinations of factor levels defined by the OA were run at the three levels of the noise factor.

The effect of the individual control factors and the interactions of control factors on the different target responses was studied by analysis of variance (ANOVA). No replicate of experiments was performed, and hence residual error was estimated from the results of the unassigned degree of freedom of the design (dummy error in factor H, e_H). Sums of squares and degrees of freedom of dummy error and of its interaction with the noise factor, $e_H \times N$, were pooled for a first estimation of the residual variance. Non-significant factors/interactions were then iteratively pooled into the residual error until only significant effects arose. To estimate the experimental conditions less affected by the variability of microalgal biomass, the ANOVA of the signal-to-noise ratio (S/N) of the 27 combinations was analysed (Taguchi et al., 2007).

For those factors that contributed considerably to the target responses, the Duncan multiple range test was used. This test allowed for the evaluation of the statistically significant differences between the tested factor values for the identification of the factor level that yielded the optimum response (Ross, 1988). A significance level p=0.05 was used in all statistical calculations.

Table 2: Taguchi's $L_{27}(3)^{13}$ orthogonal array and experimental results of carbohydrate, protein and lipid solubilisation yields, and monosaccharide recovery yields during the pretreatment step.

Orthogonal array matrix														Experimental results, in %											
														Carbohydrates			Monosaccharides			Proteins			Lipids		
	1	2	3	4	5	6	7	8	9	10	11	12	13												
Exp. No.	C_Q[a]	T[b]	$C_Q{\times}T$	$C_Q{\times}T$	t[c]	$C_Q{\times}t$	$C_Q{\times}t$	$T{\times}t$	C_A[d]	E[e]	Txt	t_H[f]	H[g]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]
1	1	1	1	1	1	1	1	1	1	1	1	1	1	28	37	13	4	10	4	18	13	18	1	62	44
2	1	1	1	1	2	2	2	2	2	2	2	2	2	45	33	48	9	8	17	37	34	48	2	40	78
3	1	1	1	1	3	3	3	3	3	3	3	3	1'	40	54	20	5	9	7	26	23	33	11	67	26
4	1	2	2	2	1	1	1	2	2	2	3	3	1'	75	44	57	10	15	31	34	46	29	30	69	45
5	1	2	2	2	2	2	2	3	3	3	1	1	1	73	67	69	16	15	30	45	26	38	20	29	14
6	1	2	2	2	3	3	3	1	1	1	2	2	2	40	45	76	12	17	32	67	73	88	63	71	88
7	1	3	3	3	1	1	1	3	3	3	2	2	2	55	54	40	4	12	28	62	56	51	59	65	77
8	1	3	3	3	2	2	2	1	1	1	3	3	1'	85	85	78	76	56	70	67	57	68	12	32	16
9	1	3	3	3	3	3	3	2	2	2	1	1	1	85	75	75	56	52	51	58	49	35	7	19	23
10	2	1	2	3	1	2	3	1	2	3	1	2	1'	52	34	22	3	9	8	13	17	20	7	66	46
11	2	1	2	3	2	3	1	2	3	1	2	3	1	57	64	51	4	8	16	13	21	22	13	44	44
12	2	1	2	3	3	1	2	3	1	2	3	1	2	25	53	67	14	10	27	67	53	81	5	50	89
13	2	2	3	1	1	2	3	2	3	1	3	1	2	61	61	45	19	9	30	64	54	64	9	7	77
14	2	2	3	1	2	3	1	3	1	2	1	2	1'	82	84	67	44	47	57	56	58	58	16	44	49
15	2	2	3	1	3	1	2	1	2	3	2	3	1	74	80	74	73	51	64	54	61	55	2	39	28
16	2	3	1	2	1	2	3	3	1	2	2	3	1	87	88	78	54	62	72	52	63	62	12	34	41
17	2	3	1	2	2	3	1	1	2	3	3	1	2	58	45	65	22	15	37	86	75	86	14	64	78
18	2	3	1	2	3	1	2	2	3	1	1	2	1'	85	82	67	55	58	52	71	63	43	22	30	22
19	3	1	3	2	1	3	2	1	3	2	1	3	2	52	28	28	8	8	21	56	61	34	3	53	92
20	3	1	3	2	2	1	3	2	1	3	2	1	1'	60	67	67	24	15	31	28	50	35	10	20	51
21	3	1	3	2	3	2	1	3	2	1	3	2	1	84	74	64	60	30	55	54	24	41	17	41	43
22	3	2	1	3	1	3	2	2	1	3	3	2	1	86	94	85	49	44	77	51	92	75	10	78	59
23	3	2	1	3	2	1	3	3	2	1	1	3	2	55	76	79	13	14	32	82	67	89	37	93	96
24	3	2	1	3	3	2	1	1	3	2	2	1	1'	75	84	71	68	52	59	42	71	51	5	48	18
25	3	3	2	1	1	3	2	3	2	1	2	1	1'	85	85	84	53	59	80	60	67	75	16	1	59
26	3	3	2	1	2	1	3	1	3	2	3	2	1	88	83	78	48	50	67	67	72	51	26	40	33
27	3	3	2	1	3	2	1	2	1	3	1	3	2	67	77	87	33	21	40	86	78	96	44	93	96

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1'=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

2.6. Analytical methods

The total and volatile solid contents were measured according to the NREL protocols in the raw material, solid and liquid fractions, and whole suspensions to check the mass balance in all the experiments (Van Wychen and Laurens, 2015a). The lipid content was determined using a modified protocol based on a chloroform-methanol 2:1 extraction by applying the Kochert method (Kochert, 1978) and the protein content was calculated by multiplying the Kjeldahl Total Nitrogen by a factor of 5.95 (González Lopez et al., 2010).

The carbohydrate content was determined as total monosaccharides in the raw materials and solid fractions by using an NREL procedure (Van Wychen and Laurens, 2015b). The biomass samples (300 mg dry biomass) were subjected to a concentrated acid hydrolysis for 1 h by adding 3 mL of 72% w/w H_2SO_4 at 30 ºC. Then, 84 mL of deionised water was added to dilute the acid concentration to 4% w/w and the samples were autoclaved at 121 ºC for 1 h. Then, solid and liquid fractions were separated by filtration and the resulting liquid fraction was stored at 4 ºC for in order to determine the total carbohydrate content by HPLC-RI.

A Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module was used for the quantification of the monosaccharide content. A refractive index detector (Waters 2414) was used to quantify the monosaccharide concentration obtained in the liquid fractions. An aqueous solution of 0.025 M H_2SO_4 was eluted at a flow rate of 0.6 mL/min and 50ºC (Martín-Juárez et al., 2016). The external calibration method was used for quantification. Multi-standard calibration solutions were prepared by adequate dilution of individual standards commercially available with a purity >95% (Sigma Aldrich, Spain). The starch content was determined using the polarimetric methodology using an internal procedure of the Laboratory of Animal Nutrition (Serida, Spain).

3. Results and discussion

3.1. Effect of the experimental parameters on the performance of the pretreatment step

High solubilisation yields of the different macromolecular components of biomass were achieved in the pretreatment step for some of the combinations of the operational parameters (Table 2). Specifically, an average carbohydrate solubilisation yield of 64%

was obtained, with similar values ranging from 25% to 94% for biomasses grown in piggery and domestic wastewaters and slightly lower (from 13% to 85%) for microalgae grown in synthetic medium. A high protein solubilisation yield was also achieved, with average yields of 53% (identical for the three biomass) and experimental values ranging from 13% to 96%. These similar carbohydrate and protein solubilisation yields concurred with the analogous composition and predominant microalgae species determined in the three biomasses used in this study. Therefore, these results could indicate the insignificant effect of the bacteria present in the biomass in the release of these components during acid or basic diluted pretreatment. Lipid solubilisation resulted in the largest differences with average yields of only 18% for biomass grown in piggery wastewater, while 48% and 52% of the lipid fraction was solubilised from biomass W and S, respectively.

The experimental design applied allowed for the elucidation of the individual effects that each operational parameter, interaction of selected factors and noise factor had on carbohydrate, protein and lipid solubilisation, as well as on the monosaccharide recovery.

3.1.1. Carbohydrate solubilisation and monosaccharide recovery

The effect of each factor level on the mean values of carbohydrate solubilisation yields during the pretreatment step is shown in Figure 1. The mean results at the different noise factor levels have been represented separately to highlight the variability of the type of biomass.
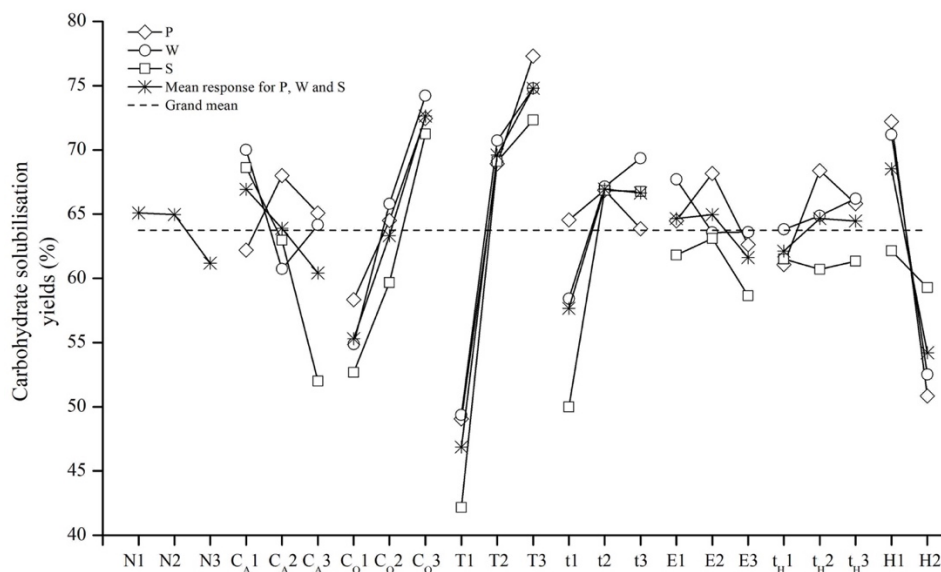
Figure 1. Main effect plots on the carbohydrate solubilisation yields (in %) for the chemical pretreatment step. Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

The ANOVA analysis revealed that temperature, chemical reagent concentration and chemical reagent type were the most influential parameters with the respective percentages of contributions of 38, 13 and 12%, being higher than the residual error (8%). Similarly, the ANOVA S/N disclosed the most influential factors in the robustness of the carbohydrate solubilisation during the pretreatment step against the variability of microalgae biomass used as a substrate. The main parameters identified by ANOVA were confirmed by the ANOVA S/N, with a contribution of 48% for temperature and 15% for the chemical reagent concentration and a residual contribution of 9%. It was also determined that the effect of the reagent type depended on the biomass.

The effect of temperature was very similar for the three types of biomass, with a rapid increase in the yields between 80 and 100°C and slight differences between 100 and 120 ºC. For instance, the carbohydrate solubilisation yield in experiments with microalgae grown in synthetic medium pretreated with HCl 0.5 M increased from 13% at 80ºC to 69% at 100ºC and to 75% at 120ºC. HCl provided higher carbohydrate solubilisation yields than NaOH, increasing the significance of the type of chemical reagent with the concentration of chemical reagent (Figure 1). The biomass type exhibited a significant influence on the effect of the chemical reagent factor, with significant differences for algal-bacteria biomass grown in wastewater, but minor variances for microalgae grown in synthetic medium.

Despite the insignificant effect of the pretreatment time in the mean responses of the three biomasses, this control factor had a significant impact on the results from microalgae grown in synthetic medium. Indeed, carbohydrate solubilisation yields increased remarkably from Level 1(10 minutes) to Level 2 (20 minutes) in the S biomass. The bacteria present in the biomasses grown in wastewater jeopardised the effect of pretreatment time.

Monosaccharide recovery yields varied from 3% to 76% for biomass grown in piggery wastewater, from 8% to 62% for biomass grown in domestic wastewater and from 4% to 80% for microalgae grown in synthetic medium (Table 2). These values were low compared with the high monosaccharide recovery yields reported by Shokrkar et al., (2017), who achieved a maximum yield of 94% from mixed microalgae grown in synthetic medium by applying acid pretreatment with 2M HCl at 121ºC for 30 min. This

difference could be attributed to the previous drying and grinding applied to the biomass or to the microalgae species composition (data not provided).

Despite the fact that comparable average carbohydrate solubilisation yields were obtained for the three types of biomass, the average monosaccharide recovery yields were significantly higher for the S microalgae (41%) than for biomasses grown in wastewaters (31% for P and 28% for W). These differences revealed average carbohydrate degradation factors of 37% for the S microalgae and ~ 60% for the P and W biomasses. The presence of bacteria in the biomass exerted a relevant and negative influence on monosaccharide recovery by increasing the microbial degradation of the monosaccharides released (Fuentes et al., 2016).

The impact of the control factor levels on the mean monosaccharide recovery yields during the pretreatment step is shown in Figure 2. According to the ANOVA analysis, the effects of temperature (33% of the share) and the reagent concentration (9% of the share) in the monosaccharide recovery were very similar to those obtained for carbohydrate solubilisation. However, a higher contribution of the chemical reagent type was calculated for monosaccharide recovery (20% of the share) than for carbohydrate solubilisation. Chemical degradation of the solubilised carbohydrates could also increase with the severity of the pretreatment conditions, resulting in lower recovery yields (Anburajan et al., 2018). No significant contributions were found for the rest of individual and combined operational parameters in the pretreatment step. Some authors have reported the significant influence of the microalgae concentration (Shokrkar et al., 2017) and the pretreatment time (Sivaramakrishnan and Incharoensakdi, 2018) on monosaccharide recovery, but these studies only used microalgae species grown in synthetic media and conducted non-statistical analysis.

The ANOVA S/N confirmed that temperature was the most influential factor (with a share of 42%). The effect of the other factors was rather variable dependent on the different biomass and, hence, common conclusions cannot be drawn (23% of residual). Higher impact of temperature on monosaccharide recovery was recorded from Level 1 (80ºC) to 2 (100ºC) than from Level 2 to 3 (120ºC). Sivaramakrishnan and Incharoensakdi, (2018) observed a similar effect of temperature during the chemical pretreatment of

Scenedesmus sp. with 0.3M NaOH, with an increase in the monosaccharide recovery yield, from 45% at 60ºC to 78% at 100ºC, but with no further improvement at 120ºC.

Despite the differences among biomasses, the mean values of monosaccharide recovery were higher using HCl instead of NaOH (Figure 2). Therefore, a monosaccharide recovery of 80% was achieved with HCl, while the maximum monosaccharide recovery using NaOH was only 40%. The superior performance of acid reagents was also reported by Shokrkar et al., (2017) when comparing the hydrolysis of microalgae mixtures with different acid reagents (H_2SO_4, HCl, H_3PO_3) and NaOH. However, Sivaramakrishnan and Incharoensakdi, (2018) achieved higher monosaccharide recovery yields with NaOH (45%) instead of HCl (28%) under mild pretreatment conditions (0.2M, 80ºC).

Figure 2. Main effect plots on the monosaccharide recovery yields (in %) for (a) the pretreatment step. Plotted values represent the mean yields for each factor level considering individual noise levels P ($\Diamond$), W ($\bigcirc$) and S ($\square$) and the mean response of the three noise levels ($*$).

Monosaccharide recovery increased with the chemical reagent concentration in the three types of biomass tested in this study. Only a slight difference was observed in monosaccharide recovery from the W biomass, where the recovery yield increased slightly when the reagent concentration increased from 1M to 2M. In this context, the carbohydrate solubilisation from the W biomass using acid pretreatment at 80ºC increased from 28% at HCl 0.5M to 84% at HCl 2M. Similarly, Sivaramakrishnan and Incharoensakdi, (2018) also reported an increment on the monosaccharide recovery yields with a chemical reagent concentration from 35% at 0.1M NaOH to 60% at 0.3M NaOH.

According with the carbohydrate solubilisation results, the contribution of pretreatment time on monosaccharide recovery was particularly relevant in microalgae grown in synthetic medium, but it was not significant for the mean values of the three biomasses.

3.1.2. Protein and lipid solubilisation

The application of chemical pretreatments resulted in the solubilisation of other macromolecular components of the biomass (proteins and lipids) (Lorenzo Hernando et al., 2018). Thus, similar protein solubilisation yields were obtained for the three types of biomass, ranging from 13% to 96% (Table 2). Figure 3 displays the effect of the control factors on the mean protein solubilisation yields for the three noise levels. No divergence on protein solubilisation for the three microalgae was detected and, hence, a great robustness of this result against the variations of microalgae biomass in the process was determined.
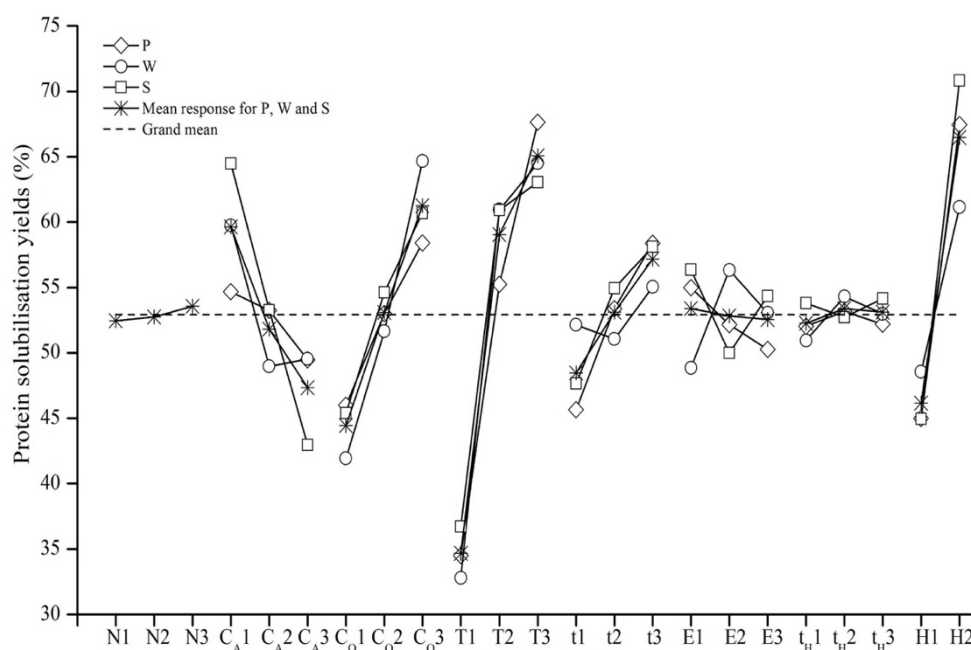
Figure 3. Main effect plots on the protein solubilisation yields (in %) for (a) the pretreatment step. Plotted values represent the mean yields for each factor level considering individual noise levels P ($\diamond$), W ($\bigcirc$) and S ($\square$) and the mean response of the three noise levels ($\ast$).

The ANOVA analysis provided the contributions of the most influential parameters to protein solubilisation: temperature (39%), chemical reagent type (21%), and the chemical reagent concentration (11%), with residual of 8%. These results, analogous to those obtained for the carbohydrate solubilisation yields, were confirmed by ANOVA of S/N.

Protein solubilisation increased with temperature and chemical reagent concentration, reaching the maximum at 2M and 120ºC, which confirmed the simultaneous solubilisation of carbohydrates and proteins. However, the best chemical reagent for protein solubilisation was NaOH. It is well known that alkaline pHs promote protein solubilisation, whereas carbohydrates are better solubilised under acidic conditions (Phong et al., 2018). The highest protein solubilisation yield was obtained for the S microalgae with NaOH 2M and 120ºC (96%), while only a maximum yield of 75 % was achieved for this biomass with HCl 2M at 120ºC.

The noise effect exerted a significant impact on lipid solubilisation yields along with chemical reagent type used according to the ANOVA. The impact of the type of biomass is shown in Figure 4. The lipid solubilisation yields from the P biomass were remarkably lower than those obtained from the W and S biomasses. HCl solubilised lower amounts of lipids than NaOH under all experimental conditions tested. This effect was especially notable for the S microalgae. The chemical reagent was also the only significant factor in ANOVA signal to noise, with 55% of the share (residual: 45%). Therefore, the use of acid reagents was selected as the best option to minimise lipid release.
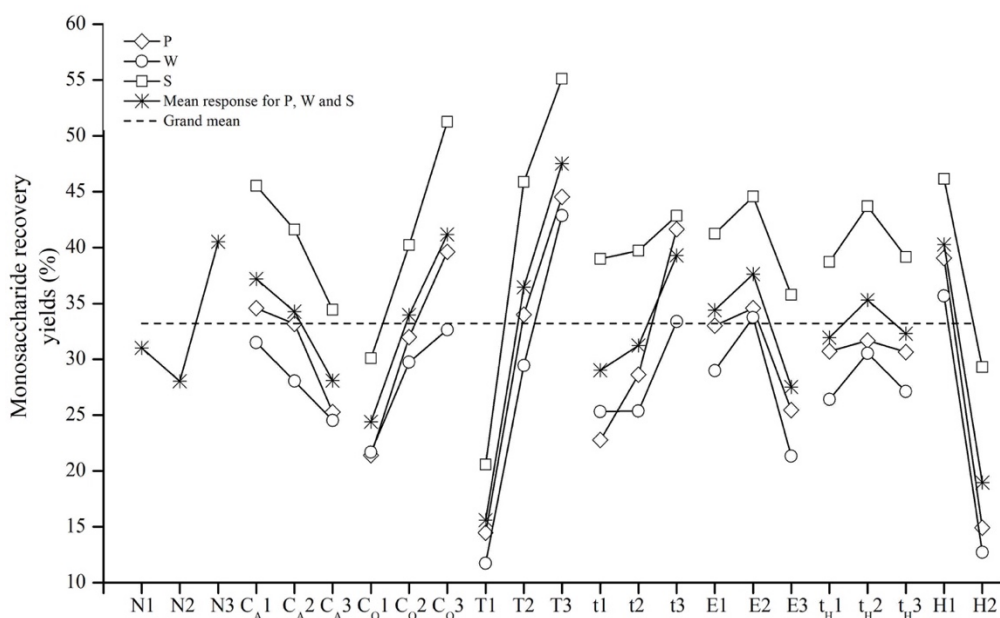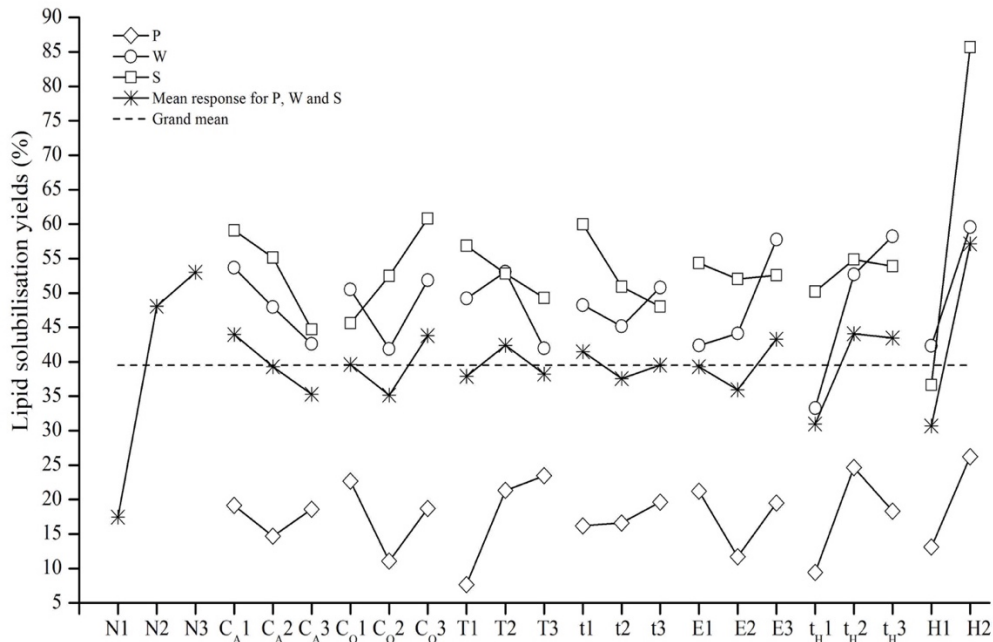

Figure 4. Main effect plots on the lipid solubilisation yields (in %) for (a) the pretreatment step. Plotted values represent the mean yields for each factor level considering individual noise levels P ($\Diamond$), W ($\bigcirc$) and S ($\square$) and the mean response of the three noise levels ($\ast$)

3.2. Effect of the operational parameters on the global process yields

The application of enzymatic hydrolysis after chemical pretreatment was also evaluated using the same experimental design. Two additional factors of the enzymatic process were also included (enzyme dosage, E, and time, t_H). Considering the low concentration of starch in the microalgae biomasses used in this work, a commercial cocktail containing cellulases and -glucosidases was selected for the enzymatic hydrolysis in order to obtain fermentable monosaccharides, as previously reported by other authors (González-Fernández et al., 2012; Hernández et al., 2015; Passos et al., 2014; Yin et al., 2010). The assessment of global yields (pretreatment followed by enzymatic hydrolysis) was investigated in this section in order to determine the feasibility of an additional enzymatic hydrolysis step compared to a single chemical pretreatment stage. Despite the use of specific enzymes for carbohydrates, enzymatic hydrolysis increased the average global solubilisation values of all the macromolecular components to 83% for carbohydrates, 77% for proteins and 59% for lipids. This simultaneous solubilisation of intracellular content (carbohydrates, proteins and lipids) could be attributed to the cell wall breakthrough by the enzymatic hydrolysis. The multilayer cell wall of microalgae present in these biomasses contain structural polysaccharides (cellulose and hemicellulose) which were degraded by the enzymes actions (Cordova et al., 2018). Proteins are also an integral cell wall constituent, covalently linked to algaenan or carbohydrates (Zhang et al., 2018). Thus, it could be expected that these proteins release in the media after polysaccharides hydrolysis.

The effect of enzymatic hydrolysis was different depending on the type of biomass. Therefore, enzymatic hydrolysis resulted in a lower impact on the global carbohydrate solubilisation of the P biomass (average of 78%) than in the W biomass (average of 89%) and the S microalgae (average of 81%). The opposite effect was found in the global protein solubilisation, with the highest yields recorded in the P biomass (average of 83%) compared to the W and S biomass (76% and 70%, respectively).

The enzymatic hydrolysis also boosted the global monosaccharide recovery yields, but to a lower extent than the global carbohydrate solubilisation yields, with average yields of 39% in the P biomass, 44% in the W biomass and 53% in the S microalgae. The maximum global monosaccharide recovery yields were 86% for the P biomass, 72% for the W

biomass and 91% for the S biomass. The biomass cultivated in the synthetic medium also provided the highest global monosaccharide recoveries. Differences between the global carbohydrate solubilisation yields and the global monosaccharide recovery yields allowed for an estimation of the global carbohydrate degradation factors – 57% for the P biomass, 60% for the W biomass and 37% for the S microalgae. These factors, very similar to those previously estimated for the chemical pretreatment step highlighted the metabolic degradation of solubilised carbohydrates by the bacteria present in biomasses grown in wastewater.

3.2.1. Global carbohydrate solubilisation and monosaccharide recovery

The effect of the operational parameters on the global carbohydrate solubilisation yields is shown in Figure 5. The ANOVA showed that temperature was the only factor with an important contribution on the global yields (37%). The enzymatic hydrolysis stage counteracted the differences found in the pretreatment step for the rest of the operational parameters. No influence of the analysed operational factors of the enzymatic hydrolysis was identified. Rehman and Anal, (2019) also detected no impact of enzyme concentration on sugar yields from *Chlorococcum* sp. using cellulase enzyme at 45ºC, 72h.

Figure 5. Main effect plots on the carbohydrate solubilisation yields (in %) for the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P ($\diamond$), W ($\bigcirc$) and S ($\square$) and the mean response of the three noise levels ($\ast$).

Regarding the noise effect, the W biomass provided higher global carbohydrate solubilisation yields than the P and S biomass. The ANOVA S/N confirmed that temperature was the most influential factor with a 58% of the share, where an increase in the carbohydrate solubilisations yields was observed at increasing temperatures.

Temperature was also the most influential parameter on the mean values of global monosaccharide recovery (Figure 6), with a 41% of the share. The ANOVA S/N of the global monosaccharide recovery yields confirmed this major contribution of temperature (51%, with a residual of 30%).

Figure 6. Main effect plots on the monosaccharide recovery yields (in %) for the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P ($\diamond$), W ($\circ$) and S ($\square$) and the mean response of the three noise levels ($\ast$).

Regarding the results for each biomass, temperature, chemical reagent type and chemical reagent concentration exhibited a noteworthy impact on the global monosaccharide recovery yields in the P biomass. Average global monosaccharide recoveries of 45% were obtained using HCl, whereas a recovery of 26% was reached with NaOH. Moreover, an increase in chemical reagent concentrations greatly improved the yields (24% at 0.5M and 58% at 2M).

However, only temperature and chemical reagent type exerted a significant effect on global monosaccharide recovery yields in the W biomass. In this case, the average values

were 49% using HCl and 34% using NaOH. Finally, only temperature exhibited a relevant impact on the global monosaccharide recovery yields in the S biomass. Therefore, the effect of the chemical reagent type and concentration on monosaccharide recovery yields seems to be related to the sterilising effect of the pretreatment, and with the metabolic degradation of solubilised carbohydrates by the viable bacteria remaining after pretreatment.

3.2.2. Global protein and lipid solubilisation

Figure 7 shows the effect of the control factors on the mean values of global protein solubilisation yields. The trend was similar to the results obtained in the protein solubilisation tests conducted with a single pretreatment step. However, the significant operational parameters had a lower influence on these yields. Temperature and chemical reagent type were the most influential factors with 29% and 18% of the share, respectively (residual 13%).
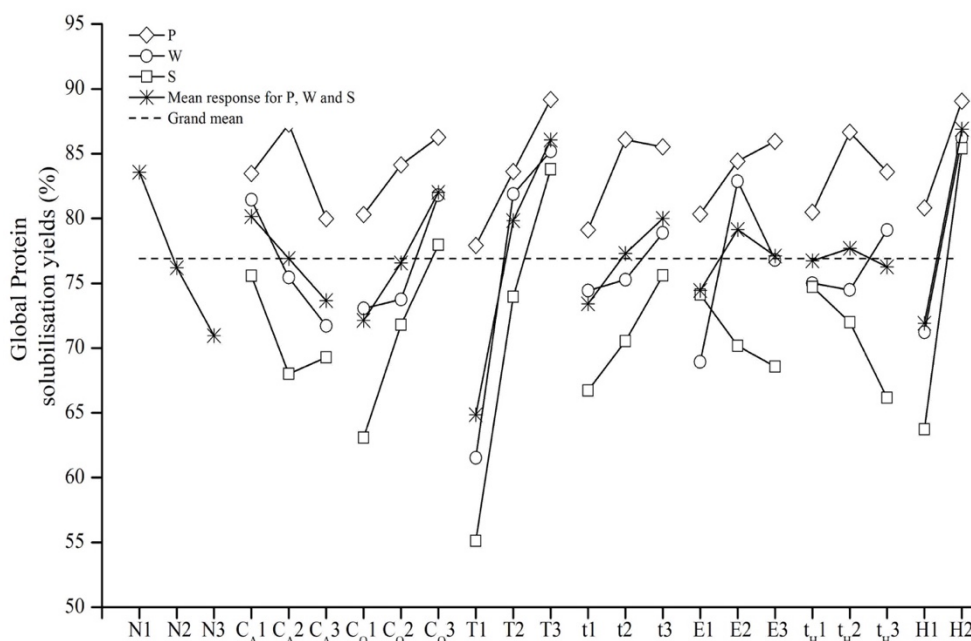
Figure 7. Main effect plots on the protein solubilisation yields (in %) for the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✶).

Unlike of the results obtained in the chemical pretreatment step, the noise factor exerted a significant impact on this global yield, with remarkably different results among the three types of biomass tested. The enzymatic hydrolysis step increased the average protein solubilisation yield by 30% in the P biomass, 31% in the W biomass and 17% in the S

biomass. The bacteria present in the biomass could contribute to the proteins release during the enzymatic hydrolysis step. It could be corroborated with the fact that Maffei et al., (2018) obtained constant protein content after the application of cellulase on pure *Nannochloropsis* at 53ªC and pH 4.4.

The ANOVA S/N confirmed the key role of temperature (39% of the share) and the chemical reagent type (23% of the share) on the global protein solubilisation, but to a lesser extent than the ANOVA analysis, because of the differences between the biomasses (38% of residual). The global protein solubilisation yields increased with temperature and NaOH as the chemical reagent. These results were consistent with those previously recorded for the pretreatment step.

On the other hand, the effect of the individual parameters on the global lipid solubilisation yields was identical to that found in the chemical pretreatment tests (Figure 8). The only difference was the increase in the yields after enzymatic hydrolysis in all the experiments. The chemical reagent and biomass type were identified as the only influential control factors on the global lipid solubilisation yields.
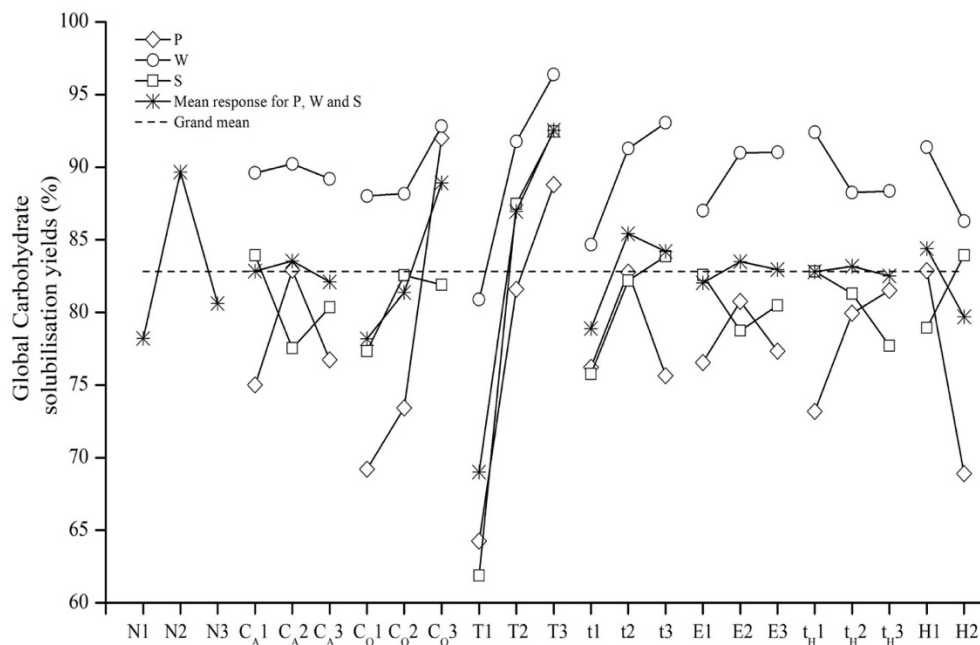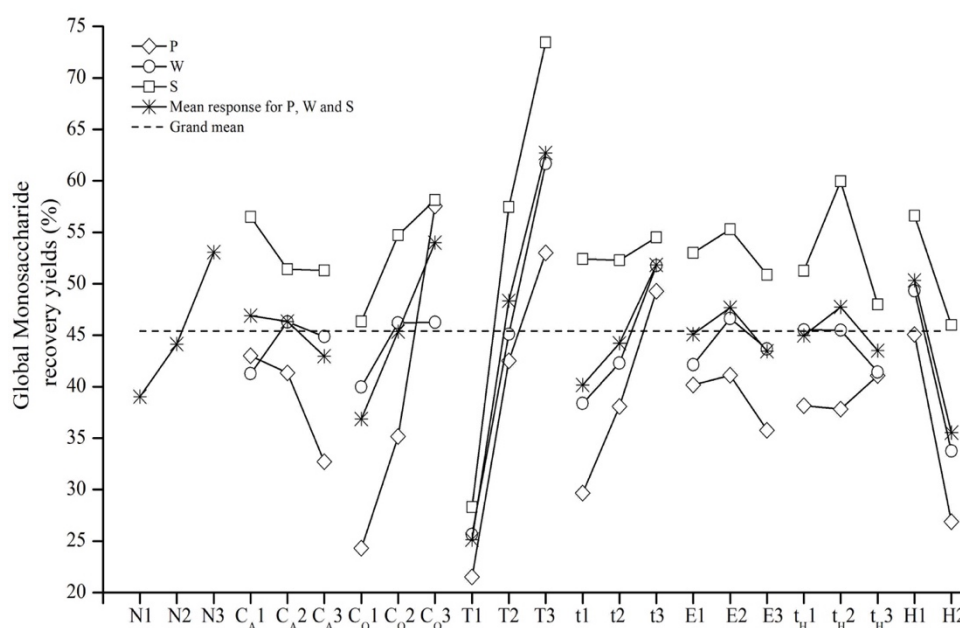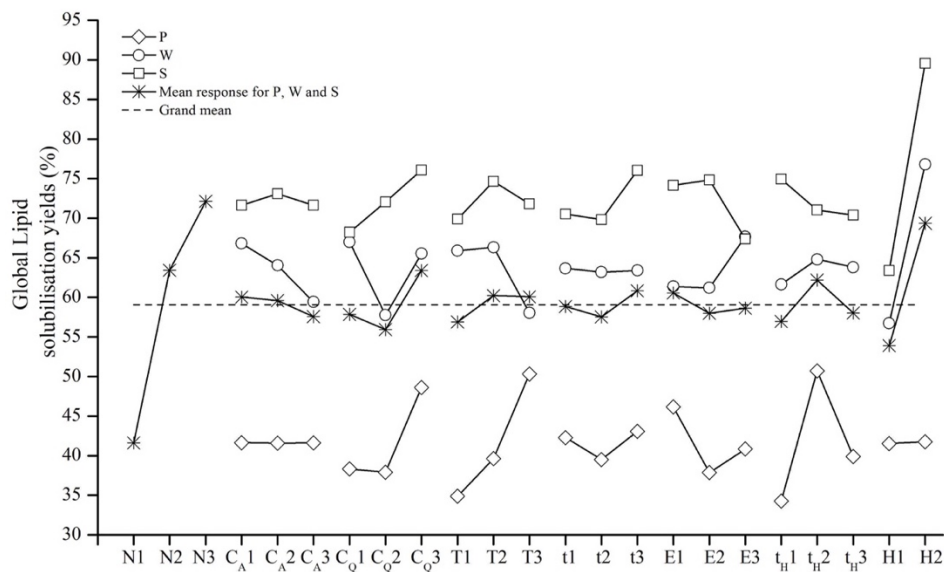

Figure 8. Main effect plots on the lipid solubilisation yields (in %) for the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

The highest global lipid solubilisation yields were recorded in microalgae grown in the synthetic medium and the lowest yields were recorded in microalgae grown in piggery wastewater. The ANOVA established the global lipid solubilisation dependence of only these two parameters, with contributions of 40% for the type of biomass and 13% for the chemical reagent type (residual of 24%). In this regard, Zhang et al., (2018) identified temperature, enzyme dosage and enzymatic hydrolysis time as the key variables in the optimisation of lipid solubilisation in Scenedesmus sp. using enzymatic hydrolysis, although these tests were conducted with an initial chemical pretreatment step.

Finally, the ANOVA S/N demonstrated that the chemical reagent type was significant in every biomass, with a 61% of the share. HCl was the chemical reagent that caused minimal global lipid solubilisation and was less sensitive to noise.

3.3. Process optimisation

In order to optimise a robust process capable of coping with a variable biomass composition, the typical effects of the main significant control factors should be used. A Duncan multiple range test of the most influential parameters was performed to elucidate the factor levels providing the highest improvement of the target variables. The analysis of the protein solubilisation yields showed an inevitable co-solubilisation of carbohydrates and proteins. Most of the operational conditions mediating a carbohydrate release also caused a solubilisation of proteins. Therefore, the protein solubilisation yields cannot be used as a target response and process optimisation should target maximising carbohydrate solubilisation and/or monosaccharide recovery and minimising lipid solubilisation. Thus, a fractional valorisation of macromolecular components of microalgae-based biomass using HCl or NaOH pretreatment would require a further step to separate monosaccharides and proteins (Suarez Garcia et al., 2018).

The temperature of the pretreatment was identified as the most important factor, with higher temperature increasing carbohydrate and protein solubilisation and monosaccharide recoveries in both the chemical pretreatment tests and the global process. Interestingly, no significant influence of temperature on lipid solubilisation yields was recorded. Differences between temperature levels were all significant for carbohydrate solubilisation and monosaccharide recovery, with Level T3 (120°C) being selected as the

optimal temperature. The reagent type exerted a higher influence on the pretreatment step than on the global process. The use of HCl favored carbohydrate solubilisation and monosaccharide recovery, mainly in the pretreatment step, while the NaOH pretreatment favored protein and lipid solubilisation. Therefore, HCl was selected as the optimal chemical reagent. The increase in the chemical reagent concentration induced higher carbohydrate and protein solubilisation and monosaccharide recovery in both the chemical pretreatment tests and the global process but exhibited no impact on lipid solubilisation. The Duncan Test conducted revealed that the only significant difference was between Level 1 (0.5M) and Level 3 (2M), and between Level 2 (1M) and Level 3 (2M) during carbohydrate solubilisation and monosaccharide recovery. Therefore, Level 3 was selected as the optimal concentration.

Carbohydrate solubilisation increased with the pretreatment time from Level 1 (10 minutes) to Level 2 (30 minutes), but no significant differences were found from Level 2 to Level 3 (60 minutes). Nevertheless, the effect of the pretreatment time on the monosaccharide recovery was highly dependent on the type of biomass, with the degradation factor increasing remarkably in biomass grown in wastewater. An optimal pretreatment time of 10 minutes was selected based on economic considerations. Finally, economic or technical criteria should be applied for the values selection of the rest of the operational parameters since no significant impact was recorded (Lam et al., 2017).

The results obtained in experiment number 25, which involved all the selected levels of the influential parameters, provided carbohydrate solubilisations of 85%, 85% and 84% in the pretreatment step, and monosaccharide recoveries of 53%, 59% and 80% in the P, W and S biomasses, respectively. Likewise, protein solubilisation yields of 85%, 85% and 84% and lipid solubilisation yield of 16%, 1% and 59% were obtained in the chemical pretreatment tests in the P, W and S biomasses, respectively, under optimal operational conditions.

In the particular case of the P biomass, experimental conditions numbers 8 and 15 provided high monosaccharide recovery yields (76 and 73%, respectively). Carbohydrate solubilisation was similar or lower in these experiments than in experiment number 25. The high monosaccharide recovery recorded in experiments 8 and 15 was likely due to

the low degradation of the solubilised carbohydrates under these particular combinations of operational parameters.

The enzymatic hydrolysis of pretreated samples obtained under the selected optimal conditions supported global carbohydrate solubilisation values of 97%, 98% and 95% and, therefore, global monosaccharide yields of 64%, 68% and 91% in the P, W and S biomasses, respectively. This slight improvement in the yield was not likely sufficient to counterbalance the additional cost of the enzymatic step. The economic viability of applying an enzymatic hydrolysis step could be considered only in the case that a relevant enhancement of the monosaccharide recovery is achieved. Interestingly, enzymatic hydrolysis did not solubilise additional proteins under these conditions, but lipid solubilisation yields increased up to 49%, 46% and 66% in the P, W and S biomass, respectively.

4. Conclusions

This study optimised the operational conditions of the chemical pretreatment and the enzymatic hydrolysis for the fermentable monosaccharide production from microalgae biomass. The experimental design provided the optimal conditions for the significant control factors (120ºC, 2M HCl) independently of the kind of microalgae biomass. The other parameters (10 min, 75g/L) were selected applying economic considerations. At these conditions, the carbohydrate solubilisations were 84% for all biomasses with a degradation of 37, 31 and 5% for biomass grown in piggery wastewater, domestic wastewater and synthetic medium, respectively. The global process improved the solubilisation up to 97% while the degradation remained constant.

5. Acknowledgements

6. References

[1] Anburajan, P., Yoon, J.J., Kumar, G., Park, J.H., Kim, S.H., 2018. Evaluation of process performance on biohydrogen production in continuous fixed bed reactor (C-FBR) using acid algae hydrolysate (AAH) as feedstock. Int. J. Hydrogen Energy. doi:10.1016/j.ijhydene.2018.09.098

[2] Asyraf, Kassim, M., Bhattacharya, S., 2016. Dilute alkaline pretreatment for reducing sugar production from Tetraselmis suecica and Chlorella sp. biomass. Process Biochem. 51, 1757–1766.

[3] Córdova, O., Santis, J., Ruiz-Fillipi, G., Zuñiga, M.E., Fermoso, F.G., Chamy, R., 2018. Microalgae digestive pretretament for increasing biogas production. Renew.Sust. Ener. Review. 82, 2806–2813.
[4] Chen, C.Y., Zhao, X.Q., Yen, H.W., Ho, S.H., Cheng, C.L., Lee, D.J., Bai, F.W., Chang, J.S., 2013. Microalgae-based carbohydrates for biofuel production. Biochem. Eng. J. 78, 1–10.

[5] El-Dalatony, M.M., Salama, E.S., Kurade, M.B., Kim, K.Y., Govindwar, S.P., Kim, J.R., Kwon, E.E., Min, B., Jang, M., Oh, S.E., Chang, S.W., Jeon, B.H., 2019. Whole conversion of microalgal biomass into biofuels through successive high-throughput fermentation. Chem. Eng. J. 360, 797–805.

[6] Fuentes, J.L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., Vílchez, C., 2016. Impact of microalgae-bacteria interactions on the production of algal biomass and associated compounds. Mar. Drugs 14.

[7] Ganeshkumar, V., Subashchandrabose, S.R., Dharmarajan, R., Venkateswarlu, K., Naidu, R., Megharaj, M., 2018. Use of mixed wastewaters from piggery and winery for nutrient removal and lipid production by Chlorella sp. MM3. Bioresour. Technol. 256, 254–258.

[8] García, D., Posadas, E., Blanco, S., Acién, G., García-encina, P., Bolado, S., Muñoz, R., 2017. Evaluation of the dynamics of microalgae population structure and process performance during piggery wastewater treatment in algal-bacterial photobioreactors. Bioresour. Technol. 248, 120–126.

[9] González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.-P., 2012. Impact of microalgae characteristics on their conversion to biofuel. Part I: Focus on cultivation and biofuel production, Biofuels, Bioproducts and Biorefining. 6, 105-113.

[10] González Lopez, C.V., Cerón García, M.C., Acién Fernández, F.G., Segovia Bustos, C., Chisti, Y., Fernández Sevilla, J.M.., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591.

[11] Harun, R., Jason, W.S.Y., Cherrington, T., Danquah, M.K., 2011. Exploring alkaline pretreatment of microalgal biomass for bioethanol production. Appl. Energy 88, 3464–3467.

[12] Hernández, D., 2015. Riaño, B., Coca. M., García-González, M.C., 2015. Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production. Chem. Eng. J. 262, 939–945.

[13] Iasimone, F., Panico, A., De Felice, V., Fantasma, F., Iorizzi, M., Pirozzi, F., 2018. Effect of light intensity and nutrients supply on microalgae cultivated in urban wastewater: Biomass production, lipids accumulation and settleability characteristics. J. Environ. Manage. 223, 1078–1085.

[14] Jankowska, E., Sahu, A.K., Oleskowicz-Popiel, P., 2017. Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. Renew. Sustain. Energy Rev. 75, 692–709.

[15] Kadir, W.N.A., Lam, M.K., Uemura, Y., Lim, J.W., Lee, K.T., 2018. Harvesting and pretreatment of microalgae cultivated in wastewater for biodiesel production: A review. Energy Convers. Manag. 171, 1416–1429.

[16] Kochert, G., 1978. Quantification of the macrolecular components of microalgae. Hand. Phycol. Methods. Physicol. Biochem., 189-175.

[17] Koutra, E., Economou, C.N., Tsafrakidou, P., Kornaros, M., 2018. Bio-Based Products from Microalgae Cultivated in Digestates. Trends Biotechnol. 36, 819–833.

[18] Kumar, G., Nguyen, D.D., Huy, M., Sivagurunathan, P., Bakonyi, P., Zhen, G., Kobayashi, T., Xu, K.Q., Nemestóthy, N., Chang, S.W., 2019. Effects of light intensity on biomass, carbohydrate and fatty acid compositions of three different mixed consortia from natural ecological water bodies. J. Environ. Manage. 230, 293–300.

[19] Lam, G.P., Vermuë, M.H., Eppink, M.H.M., Wijffels, R.H., Berg, C. Van Den, 2017. Multi-Product Microalgae Biore fi neries : From Concept Towards Reality. Trends Biotechnol. 36, 1–12.

[20] Lorenzo Hernando, A., Ruiz Vegas, J., Vega Alegre, M., Bolado Rodríguez, S., 2018. Recovery of proteins from biomass grown in pig manure microalgae-based treatment plants by alkaline hydrolysis and acidic precipitation. Bioresour. Technol. 273, 599–607.

[21] Lv, J., Liu, Y., Feng, J., Liu, Q., Nan, F., Xie, S., 2018. Nutrients removal from undiluted cattle farm wastewater by the two-stage process of microalgae-based wastewater treatment. Bioresour. Technol. 264, 311–318.

[22]Maffei, G., Bracciale, M.P., Broggi, A., Zuorro, A., Santarelli, M.L., Lavecchia, R., 2018. Effect of an enzymatic treatment with cellulase and mannanase on the structural properties of Nannochloropsis microalgae. Bioresour. Technol. 249, 592–598.

[23] Markou, G., Angelidaki, I., Nerantzis, E., Georgakakis, D., 2013. Bioethanol production by carbohydrate-enriched biomass of Arthrospira (Spirulina) platensis. Energies 6, 3937–3950.

[24] Martín Juárez, J., Lorenzo Hernando, A., Muñoz Torre, R., Blanco Lanza, S., Bolado Rodríguez, S., 2016. Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment. Bioresour. Technol. 218, 265–271.
[25] Martín Juárez, J., Riol Pastor, E., Fernández Sevilla, J.M., Muñoz Torre, R., García-Encina, P.A., Bolado Rodríguez, S., 2018. Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants. Bioresour. Technol. 257, 30–38.

[26] Morales-Amaral, M.M., Gómez-Serrano, C., Acién, F.G., Fernández-Sevilla, J.M., Molina-Grima, E., 2015. Outdoor production of Scenedesmus sp. in thin layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source. Algal Res. 12, 99–108.

[27] Oh, Y.K., Hwang, K.R., Kim, C., Kim, J.R., Lee, J.S., 2018. Recent developments and key barriers to advanced biofuels: A short review. Bioresour. Technol. 257, 320–333.

[28] Onumaegbu, C., Mooney, J., Alaswad, A., Olabi, A.G., 2018. Pre-treatment methods for production of biofuel from microalgae biomass. Renew. Sustain. Energy Rev. 93, 16–26.

[29] Pancha, I., Chokshi, K., Maurya, R., Bhattacharya, S., Bachani, P., Mishra, S., 2016. Comparative evaluation of chemical and enzymatic saccharification of mixotrophically grown de-oiled microalgal biomass for reducing sugar production. Bioresour. Technol. 204, 9–16.

[30] Passos, F., Uggetti, E., Carrère, H., Ferrer, I., 2014. Pretreatment of microalgae to improve biogas production: A review. Bioresour. Technol. 172, 403–412.

[31] Phong, W.N., Show, P.L., Ling, T.C., Juan, J.C., Ng, E.P., Chang, J.S., 2018. Mild cell disruption methods for bio-functional proteins recovery from microalgae—Recent developments and future perspectives. Algal Res. 31, 506–516.

[32] Rahman. A., Miller, C.D., 2017. Microalgae as a Source of Bioplastics. Algal Green Chemistry, 121–138.

[33]Rehman, Z.U., Anal, A.K., 2019. Enhanced lipid and starch productivity of microalga (Chlorococcum sp. TISTR 8583) with nitrogen limitation following effective pretreatments for biofuel production. Biotechnol. Reports 21. doi:10.1016/j.btre.2018.e00298.

[34] Ross, P.J., 1995. Taguchi Techniques for Quality Engineering: Loss Function, Orthogonal Experiments, Parameter and Tolerance Design, ISBN-10: 0070539588

[35] Sankaran, R., Show, P.L., Nagarajan, D., Chang, J.-S., 2018. Exploitation and Biorefinery of Microalgae, Waste Biorefinery. 571-601.

[36] Shokrkar, H., Ebrahimi, S., Zamani, M., 2017. Bioethanol production from acidic and enzymatic hydrolysates of mixed microalgae culture. Fuel 200, 380–386.

[37] Sivaramakrishnan, R., Incharoensakdi, A., 2018. Utilisation of microalgae feedstock for concomitant production of bioethanol and biodiesel. Fuel 217, 458–466.

[38] Sournia, A., 1978. Phytoplankton Manual, Unesco, United Kingdom.

[39] Su, Y., Song, K., Zhang, P., Su, Y., Cheng, J., Chen, X., 2017. Progress of microalgae biofuel's commercialization. Renew. Sustain. Energy Rev. 74, 402–411.

[40] Suarez Garcia, E., Suarez Ruiz, C.A., Tilaye, T., Eppink, M.H.M., Wijffels, R.H., van den Berg, C., 2018. Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system. Sep. Purif. Technol. 204, 56–65.

[41] Taguchi, G., Chowdhury, S., Wu, Y., 2007. Taguchi's Quality Engineering Handbook.

[42] Taguchi, G., Konishi, S., 1987. Taguchi methods, orthogonal arrays and linear graphs, tools for quality engineering. Dearborn.

[43] Travaini, R., Barrado, E., Bolado-Rodríguez, S., 2016. Effect of ozonolysis pretreatment parameters on the sugar release, ozone consumption and ethanol production from sugarcane bagasse. Bioresour. Technol. 214, 150–158.

[44] Van Wychen, S., Laurens, L.M.L., 2015a. Determination of Total Solids and Ash in Algal Biomas. NREL/TP-5100-60956.

[45] Van Wychen, S., Laurens, L.M.L., 2015b. Determination of Total Carbohydrates in Algal Biomass. NREL/TP-5100-60957.

[46] Yin, L.-J., Jiang, S.-T., Pon, S.-H., Lin, H.-H., 2010. Hydrolysis of Chlorella by Cellulomonas sp YJ5 Cellulases and Its Biofunctional Properties, Journal of food science. 75, 317–323.

[47] Zhang, Y., Kong, X., Wang, Z., Sun, Y., Zhu, S., Li, L., Lv, P., 2018. Optimisation of enzymatic hydrolysis for effective lipid extraction from microalgae Scenedesmus sp. Renew. Energy 125, 1049–1057.

[48] Zhuang, L.L., Yu, D., Zhang, J., Liu, F. fei, Wu, Y.H., Zhang, T.Y., Dao, G.H., Hu, H.Y., 2018. The characteristics and influencing factors of the attached microalgae cultivation: A review. Renew. Sustain. Energy Rev. 94, 1110–1119.

SUPPLEMENTARY MATERIALS

Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater

CONTENT

Table S1: Volatile solids solubilisation yields of the pretreatment and the global process (pretreatment and enzymatic hydrolysis)

Orthogonal array matrix													**Experimental results, in %**					
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**P[h]**		**W[i]**		**S[j]**	
Exp. No. $C_Q{}^a$	**T[b]**	$C_Q{\times}T$	$C_Q{\times}T$	**t[c]**	$C_Q{\times}t$	$C_Q{\times}t$	**T×t**	$C_A{}^d$	**E[e]**	**Txt**	$t_H{}^f$	**H[g]**	**PR**	**Global**	**PR**	**Global**	**PR**	**Global**
1	1	1	1	1	1	1	1	1	1	1	1	1	22	47	7	51	8	28
2	1	1	1	2	2	2	2	2	2	2	2	2	24	67	10	80	39	64
3	1	1	1	3	3	3	3	3	3	3	3	1'	16	30	17	29	18	44
4	2	2	2	1	1	1	2	2	2	3	3	1'	26	51	26	69	32	54
5	2	2	2	2	2	2	3	3	3	1	1	1	30	55	24	49	26	55
6	2	2	2	3	3	3	1	1	1	2	2	2	62	85	32	69	77	94
7	3	3	3	1	1	1	3	3	3	2	2	2	46	71	34	62	41	79
8	3	3	3	2	2	2	1	1	1	3	3	1'	51	83	50	80	48	86
9	3	3	3	3	3	3	2	2	2	1	1	1	53	86	45	69	28	85
10	2	1	2	3	1	2	3	1	2	3	1	2	1'	15	38	18	38	12	22
11	2	1	2	3	2	3	1	2	3	1	2	3	1	15	43	12	38	19	51
12	2	1	2	3	3	1	2	3	1	2	3	1	2	54	65	33	87	68	87
13	2	2	3	1	1	2	3	2	3	1	3	1	2	25	52	40	65	44	82
14	2	2	3	1	2	3	1	3	1	2	1	2	1'	49	76	52	79	38	75
15	2	2	3	1	3	1	2	1	2	3	2	3	1	44	73	50	74	43	73
16	2	3	1	2	1	2	3	3	1	2	2	3	1	43	74	53	79	58	83
17	2	3	1	2	2	3	1	1	2	3	3	1	2	51	77	38	78	66	92
18	2	3	1	2	3	1	2	2	3	1	1	2	1'	53	77	47	75	28	85
19	3	1	3	2	1	3	2	1	3	2	1	3	2	16	35	41	61	13	35
20	3	1	3	2	2	1	3	2	1	3	2	1	1'	27	64	19	58	26	62
21	3	1	3	2	3	2	1	3	2	1	3	2	1	51	82	26	53	35	73
22	3	2	1	3	1	3	2	2	1	3	3	2	1	38	77	54	78	70	85
23	3	2	1	3	2	1	3	3	2	1	1	3	2	55	79	75	92	72	92
24	3	2	1	3	3	2	1	1	3	2	2	1	1'	39	79	51	81	38	84
25	3	3	2	1	1	3	2	3	2	1	2	1	1'	54	87	52	83	66	86
26	3	3	2	1	2	1	3	1	3	2	3	2	1	66	94	56	85	53	79
27	3	3	2	1	3	2	1	2	1	3	1	3	2	58	83	80	94	91	97

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1'=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

Table S2: ANOVA tables of the results from the pretreatment step showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at three noise levels. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	4063	0.000	13	2	3829	0.000	9	2	3819	0.000	11	*2*	*1009*		
T	2	11913	0.000	38	2	141809	0.000	34	2	13985	0.000	39	*2*	*342*		
C_Q x T	*4*	*349*			4	2022	0.000	5	*4*	*92*			*4*	*1826*		
t	2	1508	0.000	5	2	1579	0.000	4	2	1023	0.001	3	*2*	*206*		
C_Q x t	4	831	0.010	3	4	2787	0.000	7	4	1034	0.004	3	*4*	*2796*		
T x t	4	1523	0.000	5	4	1306	0.004	3	4	886	0.008	2	*4*	*301*		
C_A	2	576	0.009	2	2	1173	0.001	3	2	2096	0.000	6	*2*	*1015*		
H	1	3685	0.000	12	1	8179	0.000	20	1	7430	0.000	21	1	12607	0.000	21
N	*2*	*265*			2	2303	0.000	6	*2*	*18*			2	19993	0.000	33
C_QxN	*4*	*109*			*4*	*277*			*4*	*289*			*4*	*1183*		
TxN	*4*	*164*			*4*	*156*			*4*	*343*			*4*	*1820*		
$(C_Q$xT)xN	8	1265	0.011	4	*8*	*430*			*8*	*681*			*8*	*2365*		
txN	4	834	0.010	3	*4*	*568*			*4*	*313*			*4*	*696*		
$(C_Q$xt)xN	*8*	*270*			*8*	*355*			*8*	*390*			*8*	*919*		
(Txt)xN	*8*	*418*			*8*	*474*			8	1370	0.008	4	*8*	*1537*		
C_AxN	4	1254	0.001	4	*4*	*70*			4	781	0.016	2	*4*	*636*		
HxN	2	1197	0.000	4	*2*	*183*			2	571	0.011	2	2	4641	0.001	8
Residual	45	2477		8	57	4365		10	49	2818		8	75	22698		38
Total	80	31126			80	41722			80	35814			80	59939		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, H: Chemical reagent, and N: microalgae biomass harvested from different wastewater treatments (noise).

Table S3: ANOVA tables for the signal to noise values of the 27 experiments for pretreatment results, showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and factor interactions for the experimental design at three noise levels.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	47	0.001	15	2	208	0.014	13	2	41	0.023	9	2	12		
T	2	153	0.000	48	2	676	0.000	42	2	231	0.000	50	2	16		
C_Q x T	4	13			4	77			4	7			4	25		
t	2	28	0.007	9	2	142	0.045	9	2	17			2	20		
C_Q x t	4	10			4	105			4	23			4	32		
T x t	4	32	0.020	10	4	31			4	21			4	3		
C_A	2	2			2	76			2	19			2	10		
H	1	26	0.003	8	1	212	0.004	13	1	94	0.000	21	1	185	0.000	55
Residual	15	30		10	19	368		23	21	93		20	25	150		45
Total	26	315			26	1606			26	459			26	335		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, H: Chemical reagent, and N: noise.

Table S4: Taguchi's $L_{27}(3)^{13}$ orthogonal array and experimental results for carbohydrates, proteins and lipids solubilisation, and monosaccharides recovery in the global process (pretreatment followed by enzymatic hydrolysis).

	Orthogonal array matrix													Experimental results, in %											
	1	2	3	4	5	6	7	8	9	10	11	12	13	Carbohydrates			Monosaccharides			Proteins			Lipids		
Exp. No.	$C_Q{}^a$	T^b	C_QxT	C_QxT	t^c	$C_Q$xt	$C_Q$xt	Txt	$C_A{}^d$	E^e	Txt	$t_H{}^f$	H^g	P^h	W^i	S^j	P^h	W^i	S^j	P^h	W^i	S^j	P^h	W^i	S^j
1	1	1	1	1	1	1	1	1	1	1	1	1	1	38	75	39	5	14	9	54	41	40	11	77	65
2	1	1	1	1	2	2	2	2	2	2	2	2	2	67	87	58	11	20	24	89	78	68	14	87	83
3	1	1	1	1	3	3	3	3	3	3	3	3	1'	47	99	54	6	53	15	67	66	50	26	68	46
4	1	2	2	2	1	1	1	2	2	2	3	3	1'	86	87	69	12	25	34	77	72	35	40	71	60
5	1	2	2	2	2	2	2	3	3	3	1	1	1	82	98	91	19	46	47	85	61	50	31	57	53
6	1	2	2	2	3	3	3	1	1	1	2	2	2	50	69	100	15	34	55	86	85	91	68	79	89
7	1	3	3	3	1	1	1	3	3	3	2	2	2	69	87	97	15	35	83	86	77	79	60	69	78
8	1	3	3	3	2	2	2	1	1	1	3	3	1'	91	92	97	78	61	87	88	91	81	37	53	58
9	1	3	3	3	3	3	3	2	2	2	1	1	1	94	97	92	57	72	64	91	87	74	57	41	82
10	2	1	2	3	1	2	3	1	2	3	1	2	1'	65	61	49	7	23	18	80	45	42	56	76	61
11	2	1	2	3	2	3	1	2	3	1	2	3	1	72	70	67	7	11	25	71	32	38	47	53	61
12	2	1	2	3	3	1	2	3	1	2	3	1	2	34	89	84	16	18	38	91	84	87	23	80	89
13	2	2	3	1	1	2	3	2	3	1	3	1	2	66	91	94	20	38	41	77	79	95	40	55	94
14	2	2	3	1	2	3	1	3	1	2	1	2	1'	89	98	86	48	59	76	83	82	72	36	48	71
15	2	2	3	1	3	1	2	1	2	3	2	3	1	81	91	86	76	57	75	91	84	64	16	41	65
16	2	3	1	2	1	2	3	3	1	2	2	3	1	93	100	93	56	74	84	81	88	70	48	42	51
17	2	3	1	2	2	3	1	1	2	3	3	1	2	71	98	90	30	67	61	97	95	93	27	76	82
18	2	3	1	2	3	1	2	2	3	1	1	2	1'	91	96	94	57	70	74	87	75	85	48	51	75
19	3	1	3	2	1	3	2	1	3	2	1	3	2	76	63	57	27	19	32	85	91	64	30	58	98
20	3	1	3	2	2	1	3	2	1	3	2	1	1'	86	85	77	46	21	38	78	68	58	44	50	64
21	3	1	3	2	3	2	1	3	2	1	3	2	1	95	98	71	69	53	56	86	50	49	62	44	62
22	3	2	1	3	1	3	2	2	1	3	3	2	1	98	99	91	61	49	80	91	99	82	46	79	62
23	3	2	1	3	2	1	3	3	2	1	1	3	2	91	94	87	46	31	39	92	91	94	53	94	98
24	3	2	1	3	3	2	1	1	3	2	2	1	1'	92	99	84	86	67	71	70	84	81	26	73	80
25	3	3	2	1	1	3	2	3	2	1	2	1	1'	97	98	95	64	68	91	82	77	93	49	46	66
26	3	3	2	1	2	1	3	1	3	2	3	2	1	97	99	86	57	66	74	92	80	80	65	51	59
27	3	3	2	1	3	2	1	2	1	3	1	3	2	97	99	89	62	42	42	99	96	99	61	94	96

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1'=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

Table S5: ANOVA tables for the global process (pretreatment and enzymatic hydrolysis) responses showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at three levels of noise. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	1639	0.000	7	2	3953	0.000	8	2	1319	0.000	6	2	819	0.048	2
T	2	8158	0.000	37	2	19387	0.000	41	2	6409	0.000	29	*2*	*190*		
C_Q x T	*4*	*359*			4	2874	0.001	6	*4*	*317*			*4*	*1098*		
t	2	651	0.032	3	2	1903	0.001	4	2	591	0.009	3	*2*	*151*		
C_Q x t	*4*	*349*			4	1640	0.016	3	4	1442	0.000	6	*4*	*549*		
T x t	4	1274	0.011	6	4	1710	0.013	4	*4*	*181*			*4*	*603*		
C_A	*2*	*29*			*2*	*249*			2	571	0.011	3	*2*	*94*		
E	*2*	*29*			*2*	*247*			*2*	*299*			*2*	*98*		
t_H	*2*	*6*			*2*	*251*			*2*	*29*			*2*	*412*		
H	1	395	0.040	2	1	3940	0.000	8	1	4032	0.000	18	1	4311	0.000	13
e_H	*1*	*16*			*1*	*1*			*1*	*66*			*1*	*47*		
N	2	1973	0.000	9	2	2734	0.000	6	2	2171	0.000	10	2	13341	0.000	40
C_QxN	4	1292	0.011	6	4	2121	0.004	5	*4*	*278*			*4*	*567*		
TxN	*4*	*683*			*4*	*522*			4	938	0.006	4	4	1432	0.034	4
(C_QxT)xN	*8*	*1045*			*8*	*1097*			*8*	*736*			8	3322	0.004	10
txN	*4*	*312*			*4*	*718*			*4*	*137*			*4*	*120*		
($C_Q$xt)xN	*8*	*358*			*8*	*1159*			*8*	*175*			*8*	*1267*		
(Txt)xN	*8*	*1017*			*8*	*1026*			*8*	*132*			*8*	*578*		
C_AxN	*4*	*472*			*4*	*581*			*4*	*399*			*4*	*170*		
ExN	*4*	*223*			*4*	*81*			4	881	0.009	4	*4*	*769*		
t_HxN	*4*	*572*			*4*	*592*			4	603	0.046	3	*4*	*1000*		
HxN	2	1083	0.004	5	*2*	*177*			2	543	0.013	2	2	2203	0.001	7
e_HxN	*2*	*2*			*2*	*5*			*2*	*198*			*2*	*129*		
Residual	61	5472		25	55	6704		14	51	2946		13	61	7842		24
Total	80	21938			80	46966			80	22447			80	33269		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, E: dosage of enzyme, t_H: time of enzymatic hydrolysis, H: Chemical reagent, e_H: dummy effect, and N: noise.

Table S6: ANOVA tables for the signal to noise values of the 27 experiments for global (pretreatment and enzymatic hydrolysis) results, showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contribution (C) of factors and factor interactions for the experimental design at three noise levels.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	12	0.014	13	2	203	0.004	20	2	9			2	4		
T	2	53	0.000	58	2	527	0.000	51	2	45	0.000	39	2	0		
C_Q x T	4	5			4	97			4	5			4	6		
t	2	5			2	55			2	5			2	1		
C_Q x t	4	3			4	63			4	14			4	2		
T x t	4	7			4	11			4	1			4	5		
C_A	2	1			2	18			2	4			2	0		
E	2	0			2	22			2	3			2	0		
t_H	2	1			2	6			2	1			2	2		
H	1	4			1	35			1	27	0.001	23	1	34	0.000	61
e_H	1	0			1	1			1	1			1	0		
Residual	22	26		29	22	310		30	23	43		38	25	21		39
Total	26	91			26	1040			26	114			26	55		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, E: dosage of enzyme, t_H: time of enzymatic hydrolysis, H: Chemical reagent, and e_H: dummy effect.

Chapter 7

Valorisation of microalgae biomass grown in piggery wastewater treatment by celullases and xylanases production from *Trichoderma reesei* QM9414

Valorisation of microalgae biomass grown in piggery wastewater treatment by celullases and xylanases production from *Trichoderma reesei* QM9414

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ABSTRACT

Eco-friendly applications of microalgae biomass from wastewaters has grown up in the last years to implement a circular bio-economy. This work evaluated the valorisation of microalgae biomass from piggery as a substrate to produce enzymes by solid-state fermentation using *Trichoderma reesei* QM9414. Taguchi Orthogonal Array design $L_{27}(3^{13})$ was used for the optimisation of the main operational parameters during cellulase and xylanases productions with the supplementation of sugarcane bagasse.

ANOVA provided that temperature of fermentation was the main significant parameters for β-glucosidase and β-xilosidase activities. While the buffer and temperature of enzyme extraction were the most relevant values for xylanase production. Besides, the ratio of susbtrate had a great influence on FPase enzyme. The optimum conditions were ratio biomass:sugarcane bagasse 50:50, 5 days of fermentation time, pH of 4, temperature of 28ºC, phosphate buffer, 22ºC of temperature of extraction and 1 hour of extraction time.

Keywords: Enzyme; Fungi; Optimisation; Solid-state fermentation; Sugarcane Bagasse

1. Introduction

The conversion of different biomasses as raw material in chemical products, energy and high-value products through environmentally sustainable processes and encompassing the demanded energy problem, is being considered one of the main challenges in our society, trying to apply a concept of bio-refinery (Yamakawa et al., 2018). In this context, the enzymatic hydrolysis is a key step where diverse enzymes facilitate the cleavage of bonds in molecules such as cellulolytic enzymes breaking the cellulose into monomers. However, the efficiency of enzymes production has to be improved, since their cost have a relevant economical influence on the overall process (Farinas, 2018).

The common process to obtained enzymes cocktails is a fermentation conducted in a liquid phase (SmF) or using solid state processes (SSF). Despite 90% of industrial-scale enzyme production processes are carried out by SmF, most cellulase enzymes are produced by SSF to avoid environmental pollution or wastes and simulate the natural habitats of fungi (Manan and Webb, 2017). Moreover, the SSF is an economical process for its low investment and operational cost, simple equipment and high productivity per reactor volume (Hansen et al., 2015; Thomas et al., 2013).

Several factors have an important impact on the enzymes production and even quite difficult to control depend on the substrate. The main parameters are moisture content, temperature, pH, time, oxygen levels, concentrations of nutrients and particle size of substrate (Farinas, 2015). Besides, the use of enzymes produced in the same material that is subsequently hydrolysed is a beneficial starting point due to the creation of more complex and specific enzymes. Different substrates have been used since the beginning of the production of enzymes from lignocellulose biomass to agricultural residues, such as: wheat bran, wheat straw, rice bran, rice husk, sugarcane bagasse, oil palm, wood chips, etc… (Ray and Behera, 2017) (Bala and Singh, 2019). The head works have focused on the use of agricultural waste as substrate with a variety of fungi or bacteria. Ahmed Simair et al., (2018) compared the xylanase production by solid state fermentation from *B. cereus* TH-050 using wheat bran, millet waste, banana peel and sugarcane bagasse as substrates. Xu et al., (2018) made a screening with different substrates (birch branch, beech branch, rice straw, wheat straw, wheat bran, sugarcane bagasse, cassava peel and peanut shell) under SSF from *Inonotus obliquus*.

Although, the most studied were *Trichoderma* and *Aspergillus* due to the wide range of produced enzymes and high protein secretion (Behera and Ray, 2016). Leite et al., (2018) performed the SSF (30ºC, 168h, 60% of moisture, with saline solution) using carnauba straw as substrate and *Trichoderma reesei* CCT2768. Xie et al., (2015) studied the SSF using a mixture of rice straw, wheat bran and corncob (ratio 4:4:2, respectively, from *Trichoderma reesei* strains at 28ºC for 144h. Hu et al., (2018) investigated the feasibility of textile waste as feedstock for enzymes production in SSF doing a screening of the fungi strain, moisture and substrate ratio. Khanahmadi et al., (2018) researched the optimisation of xylanase production by SSF of *Aspergillus niger* CCUG33991 using different substrates (wheat bran, sorghum stover, corn cob and soybean meal).

The global cost of enzymes production is related to the selection of substrate and its availability. The ideal substrate should not only provide the nutrients to the fungi growth but also serves as anchorage for the cells. However, the majority of the substrates have not enough or available nutrients, being necessary to supplement (Guoweia et al., 2011). In this context, *Ulva fasciata* (green seaweed) was used as emerging substrate in the solid-state fermentation for cellulase enzyme production with *C. sphaerospermum* (Trivedi et al., 2015). However, they also continued adding saline solutions to supplement the

necessary nutrients. Therefore, the utilisation of new sources as substrate is necessary to investigate in order to achieve the ideal substrate without supplementation of nutrients (Ray and Behera, 2017; Marín et al., 2019).

This work aims the production of cellulases and xylanases enzymes in solid-state fermentation from *Trichoderma reesei QM9414* using microalgae biomass grown in piggery wastewater treatment as alternative substrate. Taguchi Orthogonal Array design $L_{27}(3^{13})$ was applied in the enzymes production to optimise the main operational parameters of the fermentation (ratio microalgae biomass: sugarcane bagasse, pH, temperature and time) and the extraction (type of buffer, temperature and time). The moisture content, which can vary among tests of microalgae biomass and may be difficult to control, was assayed as a noise factor at two levels - 85% moisture content (common percentage for microalgae biomass after centrifugation) and no moisture adjustment - to find an optimum enzyme yield robust against variable moisture degree.

2. Materials and methods

2.1. Raw materials

Fresh mixed microalgae biomass was cultivated in a thin-layer photobioreactor with a volume of 1200L fed with pig manure wastewater diluted at 10% (Morales-Amaral et al., 2015). The microalgae biomass composition (22.50% of total solids) was 23.31% of carbohydrates, 51.73% of proteins and 13.41% of lipids and 88.56% of volatile solids, all of them in a dry basis. The biomass was kindly supplied by Cajamar Foundation (Almeria, Spain) and refrigerated at 4ºC prior to use.

Sugarcane bagasse was donated by Usina Vale, city of Onda Verde, São Paulo State, Brazil. It was washed with distilled water to remove sugar residues and particulate material, dried in a ventilated oven at 37ºC and ground in an agricultural crusher (Trapp Model TRF400) to a size of 3–5 mm. The chemical composition was 46.21% of cellulose, 20.86% of xylan and 22.67% of total lignin (Travaini et al., 2013).

The wheat bran used for the control was commercial with a composition of 44.10% of cellulose.

2.2. Microorganism

The fungus *Trichoderma reesei* QM9414 was used in this study as the most common one to produce cellulolytic enzymes. Stock cultures are maintained in cryo tubes, in 20% glycerol solution at 80ºC. The fungus was grown in petri dishes (25 mL of autoclaved Potato Dextrose Agar medium) for 7 days and 28ºC. These plates were used to prepare the inoculum for the liquid cultures. This inoculum was performed in Erlenmeyer 250mL with 50mL of autoclaved Potato Dextrose Agar and one-loop from the petri dish for 7 days and 28ºC. After this period, 50mL of autoclaved distilled water was added, and the mycelium were broken with a sterile inoculating loop.

2.3. Solid state fermentation

The required amount of substrate with and without adjustment of moisture content was introduced in 250 mL Erlenmeyer flask and the pH was adjusted up to the established values. After this, the flasks were autoclaved at 121ºC during 20 min before inoculation. Erlenmeyer flasks were inoculated with 1ml of inoculum and incubated at certain temperature and time. Control assay was performed with 2.5g of wheat straw and 2.5g of sugarcane bagasse adding 10 ml of saline solution, at 28ºC, for 5 days.

2.4. Enzymes extraction

After this period, 50 mL of the buffer were added to each flask, the mixture was homogenised with a glass bar, stirred in an orbital shaker (150rpm) for a determined time. The extraction of control experiment with distilled water was carried out for 1h. Then, it was filtered through nylon cloth disks and centrifuged at 10000g, for 20 min, at 5ºC. The supernatants obtained were stored to analyse the enzymes activities.

2.5. Design of experiments using orthogonal array for enzymes production optimisation

The effect of several parameters was considered based on literature as the major factors in solid-state fermentation and extraction enzyme: microalgae biomass: sugarcane bagasse ratio, pH, temperature and time of fermentation; and type of buffer, temperature and time during the extraction. Moreover, the moisture content has a relevant impact on the enzymes production, but it may be an uncontrollable factor due to the variable degree of hydration of the microalgae biomass. Therefore, the moisture content was introduced in the experimental design as a noise factor.

Orthogonal array experimental design $L_{27}(3^{13})$ with seven control factors at three levels was used to select the optimum combination of these operational parameters involved in the solid-state fermentation and enzymes extraction. Three levels for each assayed factor were chosen to detect quadratic or non-linear effects of the parameters, to obtain information over wide ranges of the factors, and to find experimental conditions providing a maximum signal-to-noise ratio (Taguchi et al., 2007). The noise factor was assayed at two extreme values (adjustment of moisture content to 85% with water and without water addition) to obtain an optimum combination of factor levels yielding a robust response. Control factors at their selected levels and the $L_{27}(3^{13})$ experimental array involving 27 experiments are shown in Table 1.

The remaining six columns of the OA were used to investigate three potential factor interactions: microalgae biomass: sugarcane bagasse ratio with fermentation time, pH with fermentation time and extraction temperature with extraction time. The assignment of factors and interactions to the columns of the OA was made according to the linear graphs and triangular tables devised by Taguchi (Taguchi et al., 2007).

Each of the 27 experiments of the OA were run at two levels of the noise factor, without and with adjustment of moisture content to 85%. The realisation order of the 54 experiments was randomised.

The orthogonality of the experimental array allows to separate the effect of each factor and interaction, and their interactions with the noise factor, enabling optimisation of control factors and reduction of process variability. The effect of factors and interactions on the enzymes activities was resolved by analysis of variance (ANOVA). The least significant factors/interactions were pooled to calculate the residual error, and factors affecting significantly the responses were identified. Statistically significant differences between the levels of those factors and level values producing the optimum response were assessed by the Duncan multiple range test (Ross, 1995). A significance level p=0.05 was used in all statistical calculations.

Table 1: Taguchi's $L_{27}(3)^{13}$ orthogonal array and experimental results of FPase, β-glucosidase, xylanase and β-xylosidase productions.

Exp. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	FPase (FPU/g) NWʰ	WWⁱ	β-glucosidase (U/g) NWʰ	WWⁱ	Xylanase (U/g) NWʰ	WWⁱ	β-xylosidase (U/g) NWʰ	WWⁱ
	Rᵃ	tᵇ	R x t	R x t	pHᶜ	R x pH	R x pH	t x pH	Tᵈ	Bᵉ	t x pH	Teᶠ	teᵍ								
1	1	1	1	1	1	1	1	1	1	1	1	1	1	11.37	14.99	10.50	3.86	427.49	662.20	3.05	1.44
2	1	1	1	1	2	2	2	2	2	2	2	2	2	1.03	2.88	0.34	10.18	24.39	504.27	2.76	2.19
3	1	1	1	1	3	3	3	3	3	3	3	3	3	0.05	3.70	4.28	1.22	471.08	590.11	3.46	1.49
4	1	2	2	2	1	1	1	2	2	2	3	3	3	1.64	5.87	3.73	10.78	25.98	70.91	1.00	2.81
5	1	2	2	2	2	2	2	3	3	3	1	1	1	0.48	2.98	2.37	0.98	50.29	707.46	0.91	2.10
6	1	2	2	2	3	3	3	1	1	1	2	2	2	0.40	1.39	9.57	0.64	55.74	320.37	0.69	2.19
7	1	3	3	3	1	1	1	3	3	3	2	2	2	0.84	5.01	2.68	1.63	98.91	478.54	0.97	1.62
8	1	3	3	3	2	2	2	1	1	1	3	3	3	0.16	5.19	4.60	2.90	69.82	270.24	0.73	2.30
9	1	3	3	3	3	3	3	2	2	2	1	1	1	0.70	5.47	6.63	9.55	722.21	673.09	4.21	1.54
10	2	1	2	3	1	2	3	1	2	3	1	2	3	13.14	7.04	10.51	8.89	680.64	544.01	4.29	2.34
11	2	1	2	3	2	3	1	2	3	1	2	3	1	5.85	8.13	3.00	2.86	69.15	466.72	0.91	1.68
12	2	1	2	3	3	1	2	3	1	2	3	1	2	2.74	4.87	3.91	2.75	254.82	156.92	1.01	1.51
13	2	2	3	1	1	2	3	2	3	1	3	1	2	6.46	9.79	3.42	2.41	74.52	554.48	1.66	2.22
14	2	2	3	1	2	3	1	3	1	2	1	2	3	6.73	6.06	0.90	1.54	30.18	324.48	1.87	1.56
15	2	2	3	1	3	1	2	1	2	3	2	3	1	2.82	3.54	11.13	11.12	437.13	261.02	1.52	2.74
16	2	3	1	2	1	2	3	3	1	2	2	3	1	4.63	9.49	3.45	3.02	52.22	611.90	1.05	1.18
17	2	3	1	2	2	3	1	1	2	3	3	1	2	4.13	6.38	2.38	10.04	74.60	620.28	1.55	1.98
18	2	3	1	2	3	1	2	2	3	1	1	2	3	7.09	2.80	2.37	1.27	76.70	114.42	1.40	1.22
19	3	1	3	2	1	3	2	1	3	2	1	3	2	9.23	10.00	7.11	2.48	699.92	196.56	1.67	1.71
20	3	1	3	2	2	1	3	2	1	3	2	1	3	10.28	12.69	1.64	2.85	653.81	628.67	1.45	2.27
21	3	1	3	2	3	2	1	3	2	1	3	2	1	6.74	2.90	3.36	9.14	375.52	415.42	1.66	1.93
22	3	2	1	3	1	3	2	2	1	3	3	2	1	8.48	8.45	10.39	1.85	570.83	543.84	1.69	1.26
23	3	2	1	3	2	1	3	3	2	1	1	3	2	6.30	5.95	9.45	11.13	577.54	459.35	1.74	2.29
24	3	2	1	3	3	2	1	1	3	2	2	1	3	6.76	10.22	3.30	1.56	178.79	528.08	0.90	2.72
25	3	3	2	1	1	3	2	3	2	1	2	1	3	8.40	15.80	4.48	11.35	66.22	252.81	1.62	1.30
26	3	3	2	1	2	1	3	1	3	2	3	2	1	4.35	11.37	2.97	2.13	70.83	640.40	1.53	1.61
27	3	3	2	1	3	2	1	2	1	3	1	3	2	6.20	10.66	10.08	2.96	71.58	542.50	1.76	1.13

ᵃRatio microalgae biomass:sugarcane bagasse. 1=100-0, 2=75-25, 3=50-50.
ᵇFermentation time. 1=3 days, 2=5 days, 3=7 days
ᶜpH in the raw material. 1=2, 2= 4, 3=6.
ᵈFermentation temperature. 1=22ºC, 2=28ºC, 3=35ºC.
ᵉKind of buffer during the extraction. 1=distilled water, 2=acetate buffer, 3=phosphate buffer.
ᶠExtraction temperature. 1=22ºC, 2=28ºC, 3=35ºC.
ᵍExtraction time. 1=1 hour, 2=2 hours, 3=3 hours.
ʰNW: non-water adjustment of the moisture content tests.
ⁱWW: adjustment of the moisture content (at 85%) with distilled water.

The aim of introducing the noise factor is to minimise the variation of the enzymes production when moisture contents vary. To estimate the experimental conditions less affected by moisture of the raw material the signal-to-noise ratio, S/N, was calculated for each of the 27 trials of the experimental design as (Taguchi):

where n is the number of repetitions of each trial (2 in this work) and y_i is the response (enzyme activity).

The evaluation by ANOVA of the 27 S/N ratio values allows to obtain experimental conditions less prone to be affected by variability in moisture content.

2.6. Analytical methods

The total and volatile solid contents were measured according to the NREL protocols in the raw material (Van Wychen and Laurens, 2015a). The lipid content was determined using a modified protocol based on a chloroform-methanol 2:1 extraction by applying the Kochert method (Kochert, 1978) and the protein content was calculated by multiplying the Kjeldahl Total Nitrogen by a factor of 5.95 (González Lopez et al., 2010).

The carbohydrate content was determined as total monosaccharides in the raw materials by using an NREL procedure (Van Wychen and Laurens, 2015b). The biomass samples (300 mg dry biomass) were subjected to a concentrated acid hydrolysis for 1 h by adding 3 mL of 72% w/w H_2SO_4 at 30 ºC. Then, 84 mL of deionised water was added to dilute the acid concentration to 4% w/w and the samples were autoclaved at 121 ºC for 1 h. Then, solid and liquid fractions were separated by filtration and the resulting liquid fraction was stored at 4 ºC for in order to determine the total carbohydrate content by HPLC-RI. A Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module with a refractive index detector (Waters 2414) was used for the quantification of the monosaccharide content. An aqueous solution of 0.025 M H_2SO_4 was eluted at a flow rate of 0.6 mL/min and 50ºC. The external calibration method was used for quantification. Multi-standard calibration solutions were prepared by adequate dilution of individual standards commercially available with a purity >95% (Sigma Aldrich, Spain) (Martín-Juárez et al., 2019).

FPase activity was determined according the standarised NREL method (Adney and Nrel, 2008). The activity was carried out with 0.5mL of produced enzymes mixing with 1ml of sodium citrate buffer (0.05M, pH 4.8) and a Whatman No.1 paper filter. It was incubated at 50°C for 60min. Xylanase activity was evaluated as reported by Ahmed Simair et al., (2018) with certain modifications. 0.450 mL of xylan (2%) was mixed with 0.450 mL of buffer citrate 0.1M and 0.100 mL of produced enzyme. The mixture was incubated at 50°C for 10 min. After the incubated time of both enzymes, dinitro-salicylic acid (DNS) was use to quantify the reducing sugar release (Miller, 1959). For FPase and xylanase, one unit of enzyme activity was defined as the amount of enzyme required to release the equivalent to 1 µmol of reducing sugars under assay conditions.

β-glucosidase activity was assayed with 0.250 mL of pNPG (4mM), 0.250 mL of sodium buffer citrate (50mM) and 0.050 mL of produced enzymes. This mixture was incubated at 50°C for 10 min. Then, 2ml of sodium carbonate (2M) were added, and the amount of p-nitrophenol was determined by UV spectrophotometer at 410nm. The measurement of β-xylosidase activity was evaluated as the same protocol for β-glucosidase, but using pNPX instead of pNPG (Hu et al., 2018).

3.   Results and discussion

3.1. Assessment of influencing experimental parameters on enzymes activities

Enzymes productions (FPase, β-glucosidase, xylanase and β-xylosidase) from microalgae biomass at the two noise levels - without (NW) and with adjustment with water at 85% of moisture content (WW) - are shown in Table 1 for the design experiments. Average FPase production was 6.12 and the activities ranged from 0.05 to 15.80 FPU/g for both noise levels but resulting in higher values for WW than NW in the most cases. The values reported for β-glucosidase varied from 0.64 to 11.35 U/g for both two tests. High xylanase productions were achieved independently of the moisture adjustment with an average of 353.70U/g, reaching activities from 24.39 to 722.21U/g. The lowest productions were obtained for β-xylosidase, accounting values from 0.69 to 4.29 U/g for NW and from 1.13 to 2.81U/g for WW. The enzymes productions were higher for WW than NW in most all the cases, showing the same tendency for all the activities. As a control assay using sugarcane bagasse and wheat straw, the activities reached were 1.37 FPU/g, 6.24, 348.25 and 1.07U/g for FPase, β-glucosidase, xylanase and β-xylosidase, respectively. These values were in agreement with the average productions for all the enzymes apart from

FPase with lower result. By analogy with the control test, Paganini et al., (2018) achieved close value of xylanase activity (351.74U/g) but slight low enzymes productions (0.26 for FPase, 2.97 for β-glucosidase and 0.53 for β-xylosidase) using a mixture of sugarcane bagasse and wheat straw in a SSF of *Trichoderma viridae* PAJ 03 at 28°C for 7 days.

3.1.1. FPase enzyme

Figure 1 displays the effect of operational parameters on the mean values of FPase production for the two noise levels, representing separately to disclose the variability of moisture content.
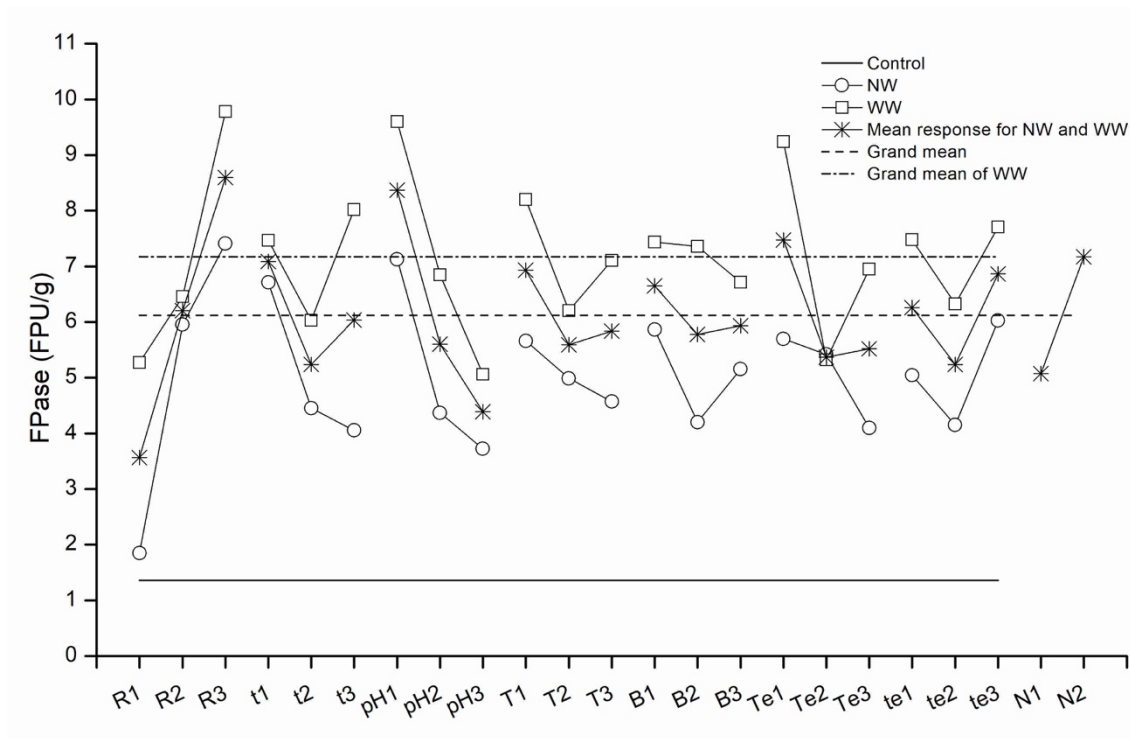

Figure 1. Main effect plots on FPase (FPU/g) production. Plotted values represent the mean productions for each factor level considering the individual noise levels NW (○), and WW (□), and the mean response of the two noise levels (✳).

The main parameters with a relevant influence were ratio biomass:sugarcane bagasse (R) and pH, with a 29 and 19% contribution to total variance, respectively (Table 2). Subsequently, the interaction of time with pH, noise factor (N), and temperature of extraction (T_e) were the next with a less considerable impact (7, 7 and 6% of the share, respectively). All contributions of factors were lower than the residual error (32%). However, ANOVA S/N detected the most influential parameters in the robustness of the FPase activity against the variations of moisture content in the substrate for enzyme production (Table 3). This analysis confirmed the same main parameters with contributions of 57% for ratio microalgae biomass: sugarcane bagasse, 14% of pH, 10%

of interaction of time with pH, and 5% of temperature of extraction (6% of the share for residual error). Besides, the interaction of ratio with pH had significant impact on FPase activity (8% of the share).

Despite similar tendency for both noise levels, values reported for WW were higher than for NW. The increment of the moisture content provided lower productions at 100 microalgae biomass: 0 sugarcane bagasse (1.6FPU/g) than 6.8FPU/g using a ratio of 50:50 at NW both experiments. However, the enzyme production from WW test had same tendency adjusting the moisture content at the same percentage. However, Trivedi et al., (2015) only increased the enzyme activity (9.2 FPU/g) up to 60% of moisture much lower than studied content in this study but thereafter their activity declined (7.6 FPU/g) at 80% of moisture. Their optimum condition was 60% of moisture, achieving 9.20 FPU/g using seaweed *Ulv*a in SSF with *Cladosporium sphaerospermum*. Xu et al., (2018) also detected the same tendency studying the effect of substrate:moisture ratio from 1:1 (50%) to 1:4 (80%), achieving the maximum production (3.30IU/g) at 1:2.5 (71%) for 7 days at 28ºC using sugarcane bagasse (pH 6) from *Inonotus obliquus*. Consequently, they observed that the influence of moisture content had different effect depending on the kind of used substrate but always achieving a maximum at one moisture point content and afterwards with a declined fact. Other factor with a determining effect on the moisture content was the type of fungi and even the strains from the same fungi as reported by Hu et al., (2018). They provided scant enhancement of FPase activity from 0.8 (80%) to 1.3 (85%) using *Trichoderma reesei* with textile waste after 7 days.

Figure 1 confirmed the attraction of FPase enzyme to the acid pH, obtaining higher results at pH1 (2) than the other two levels (4 and 6). The same tendency of enzyme production with the pH was detected by Trivedi et al., (2015), reaching 9.6U/g at pH 4 and 6.3U/g at pH 6. The non-effect of temperature for cellulase enzyme was also reported by Lopez-Ramirez et al., (2018) reaching values around 9U/g from 26 to 36ªC after 2 days in the fermentation of pine sawdust and saline solution with *Trichoderma harzianum.*

Table 2: ANOVA tables of the enzymes production responses showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at two noise levels. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	FPase (FPU/g)				β-glucosidase (U/g)				Xylanase (U/g)				β-xylosidase (U/g)			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
R	2	228.24	0.000	29	*2*	*5.76*			*2*	*120567.37*			*2*	*0.80*		
t	*2*	*30.96*			*2*	*6.52*			*2*	*178360.93*			2	1.84	0.090	6
R x t	*4*	*25.24*			*4*	*29.62*			4	226794.09	0.225	7	*4*	*1.54*		
pH	2	149.44	0.000	19	*2*	*11.63*			*2*	*5015.41*			*2*	*0.09*		
R x pH	*4*	*17.92*			*4*	*9.21*			4	352117.65	0.077	12	*4*	*2.10*		
t x pH	4	56.52	0.074	7	*4*	*34.34*			*4*	*135786.18*			*4*	*2.20*		
T	*2*	*18.23*			2	334.15	0.000	47	*2*	*15518.89*			2	4.18	0.006	13
B	*2*	*7.76*			*2*	*3.96*			2	235067.07	0.058	8	*2*	*0.57*		
Te	2	49.40	0.025	6	*2*	*13.30*			*2*	*70700.25*			*2*	*0.15*		
te	*2*	*24.45*			*2*	*19.54*			*2*	*162245.65*			*2*	*0.49*		
N	1	59.39	0.003	7	*1*	*5.64*			1	496536.14	0.000	16	*1*	*0.20*		
R x N	*2*	*19.70*			*2*	*7.11*			*2*	*56215.15*			*2*	*0.15*		
t x N	*2*	*25.05*			*2*	*10.24*			*2*	*159227.40*			2	4.09	0.007	13
(Rxt) x N	*4*	*17.00*			*4*	*16.17*			4	186098.82	0.317	6	4	3.26	0.079	10
pH x N	*2*	*3.87*			*2*	*14.18*			*2*	*137395.21*			*2*	*1.01*		
(RxpH) x N	*4*	*1.67*			*4*	*11.06*			4	272818.96	0.151	9	*4*	*2.80*		
(txpH) x N	*4*	*17.39*			*4*	*32.26*			*4*	*79864.42*			4	4.35	0.029	14
T x N	*2*	*5.23*			2	116.69	0.000	16	*2*	*79276.76*			*2*	*0.50*		
B x N	*2*	*7.62*			*2*	*6.77*			*2*	*714.12*			*2*	*0.41*		
Te x N	*2*	*33.69*			*2*	*14.66*			*2*	*48543.34*			*2*	*0.55*		
te x N	*2*	*1.32*			*2*	*13.59*			*2*	*38397.69*			*2*	*0.46*		
Residual	42	257.11		32	49	266.57		37	28	787994.78		42	39	14.01		44
Total	53	800.10			53	716.40			53	3057261.47			53	31.74		

[a]R: ratio microalgae biomass: sugarcane bagasse; t: fermentation time; pH: pH in the raw material; T: fermentation temperature; B: kind of buffer during the extraction; T_e: extraction temperature; t_e: extraction time; and N: noise.

3.1.2. β-glucosidase enzyme

For β-glucosidase, the average productions for both tests (NW and WW) had the same tendency and analogous values, comparable to the control assay (Figure 2). In this context, ANOVA (Table 2) revealed the temperature of fermentation process as the most significant parameter on this activity. Similarly, ANOVA S/N (Table 3) confirmed the same results with a contribution of 59% (residual error: 29% of the share). None of the studied interactions had a significant effect to be considered in the production of this enzyme. T2 (28ºC) was the best condition considering the both noise levels.
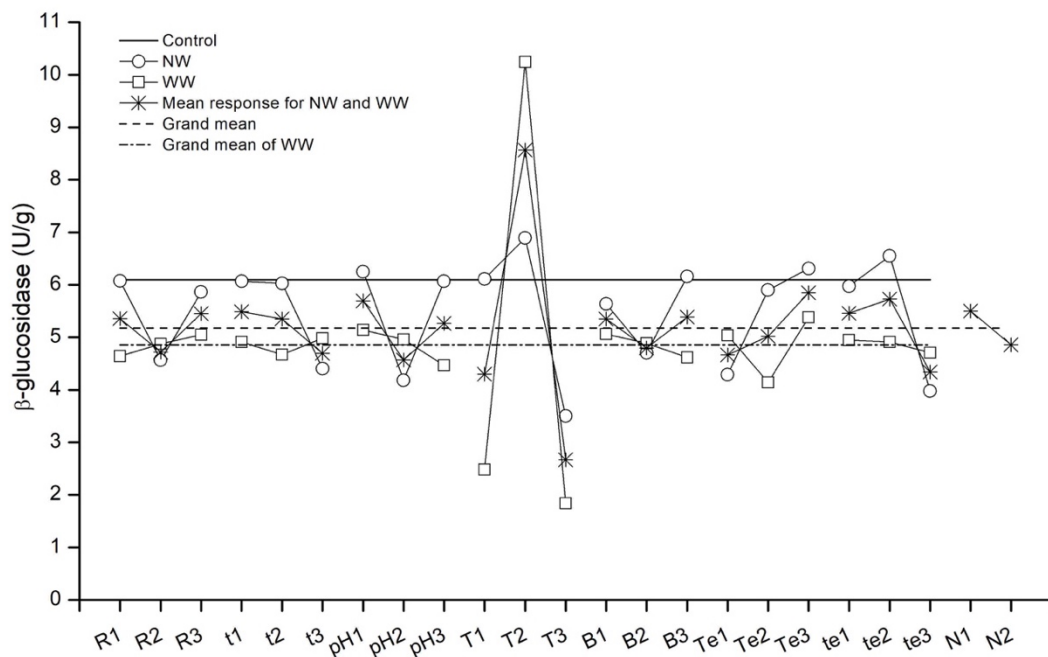

Figure 2. Main effect plots on β-glucosidase (U/g) production. Plotted values represent the mean productions for each factor level considering the individual noise levels NW (○), and WW (□), and the mean response of the two noise levels (✳).

Xu et al., (2018) did not observe a significant effect varying the susbtrate:moisture ratio, the same behaviour reported in this study with or without adjustment of moisture content. However, they reached much lower β-glucosidase activity (1.85IU/g), at substrate: moisture ratio of 1:1.5 (60%) for 7 days at 28ºC using sugarcane bagasse (pH 6) from *Inonotus obliquus*, than achieved in this study (11.35 CBU/g) at ratio 50:50 for 7 days at pH 2 and 28ºC. In this study, pH had no significant impact on this enzyme activity but other authors (Xu et al., 2018; Hirasawa et al., 2019) highlighted the optimal range from 4.0 to 6.0 and as a determinative parameter affecting the charge of cell membrane and the secretion of enzymes.

Table 3: ANOVA tables for the signal to noise values of the 27 experiments, showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and factor interactions for the experimental design at two noise levels.

Source of variation[a]	FPase (FPU/g)				β-glucosidase (U/g)				Xylanase (U/g)				β-xylosidase (U/g)			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
R	2	1816.86	0.000	57	2	6.28			2	186.28	0.120	11	2	2.14		
t	2	19.78			2	40.27			2	241.48	0.079	14	2	23.87	0.009	16
R x t	4	20.31			4	106.54	0.144	11	4	425.21	0.083	24	4	31.89	0.015	21
pH	2	459.24	0.001	14	2	27.50			2	158.78	0.151	9	2	2.09		
R x pH	4	252.66	0.029	8	4	70.48			4	251.16	0.202	14	4	11.76		
t x pH	4	333.14	0.012	10	4	34.33			4	112.95	0.503	6	4	17.90	0.074	12
T	2	56.86			2	559.32	0.000	59	2	22.75			2	48.73	0.001	33
B	2	50.79			2	3.25			2	210.20	0.099	12	2	6.85		
Te	2	160.10	0.026	5	2	63.72			2	59.77			2	3.88		
te	2	44.32			2	29.57			2	98.81			2	0.11		
Residual	12	192.06		6	20	275.39		29	6	181.32		10	10	15.06		10
Total	26	3214.06			26	941.25			26	1767.38			26	149.21		

[a]R: ratio microalgae biomass: sugarcane bagasse; t: fermentation time; pH: pH in the raw material; T: fermentation temperature; B: kind of buffer during the extraction; T_e: extraction temperature; and t_e: extraction time.

3.1.3. Xylanase enzyme

Regarding the average reported in Figure 3 for each factor level, the adjustment of the moisture content provided the most diverse results in this enzyme activity with average productions of 258 and 450 U/g for NW and WW, respectively. In accordance with the results, other parameters had a relevant influence on xylanase production as ratio microalgae:sugarcane bagasse (R), time of fermentation (t), type of buffer (B), and the last one the time of extraction (te).
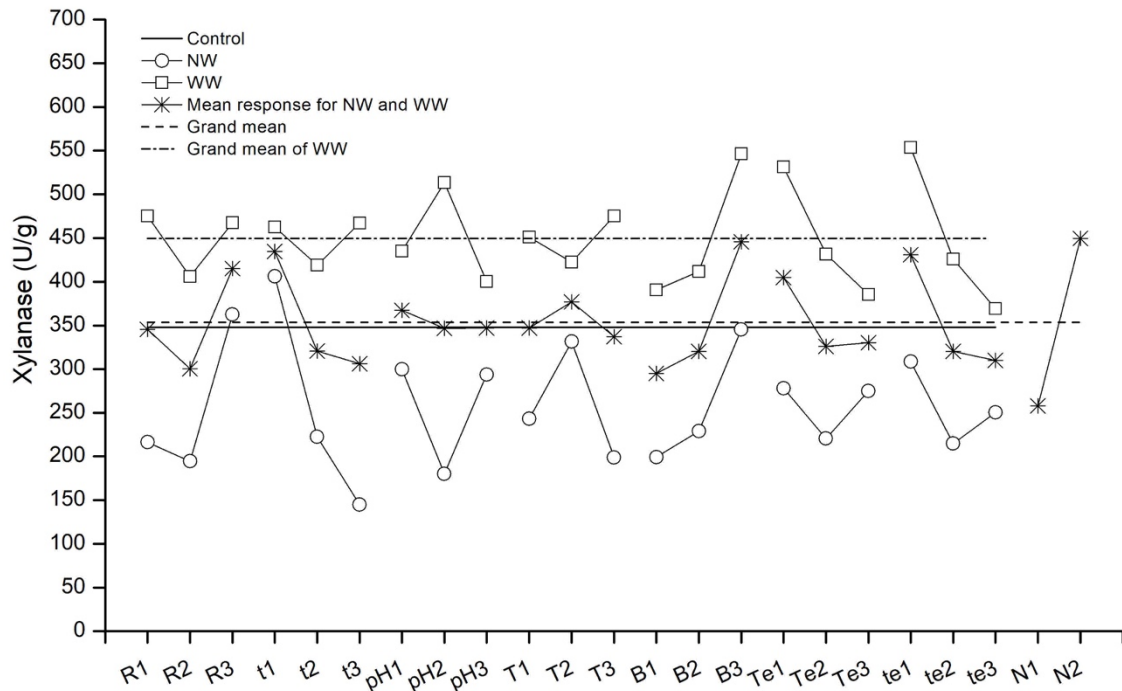

Figure 3. Main effect plots on xylanase (U/g) production. Plotted values represent the mean productions for each factor level considering the individual noise levels NW (○), and WW (□), and the mean response of the two noise levels (✳).

In this context, ANOVA (Table 2) demonstrated the same influential factors but with a subtle distinction. All the factors had lower contributions than the residual error (42%) and the unique parameter with p-value <0.05 was the adjustment of moisture content (noise-N). It explained the high-relevance of the study of the experimental conditions less affected by moisture content (ANOVA S/N). Therefore, several factors and interactions had a significant influence with contributions (24% of Rxt, 14% for time -t-, 14% for R x pH, 12% for type of buffer -B-, and 11% for ratio -R-) higher than the residual error (10% of the share).

However, the trends in activity response when the factor levels change were similar, in general, for the two noise levels. In other works (Khanahmadi et al., 2018), the effect of

moisture content had also a relevant influence on xylanase activity. They provided the highest results (2000U/g) at 70% of moisture content of wheat bran using *Aspergillus niger* CCUG33991 at 2 days, and a declination at 75%. Recurrently, the importance of moisture content determination comes to the fore on the solid-state fermentation for each specific fungi with unique substrate. High content of moisture prevents the oxygen penetration but low amount of water inhibits microbial growth with poor accessibility to the nutrients and decrement of enzymes activities (Maurya et al., 2012; Libardi et al., 2017).

3.1.4. β-xylosidase enzyme

β-xylosidase activity exhibited alike trend as β-glucosidase production resulting in an extreme influence of the temperature of fermentation but with further significant deviation between other factors levels as time of fermentation (t) (Figure 4).
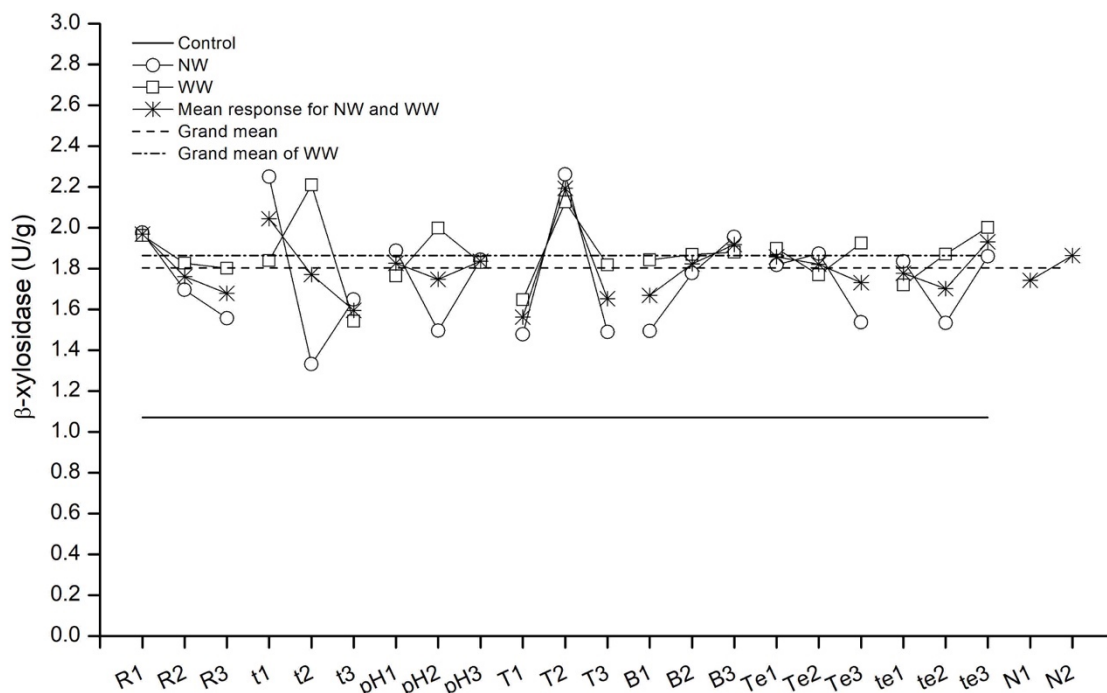

Figure 4. Main effect plots on β-xylosidase (U/g) production. Plotted values represent the mean productions for each factor level considering the individual noise levels NW (○), and WW (□), and the mean response of the two noise levels (✳).

ANOVA supported this data with p-values close or lower than 0.05 for these two factors (time and temperature of fermentation) and, as well as, for some parameters and interactions with the noise effect (Table 2). The contributions of the main factors accounted for 13% for temperature, and 6% for time. Significant effect of time but with

contrary trend was observed by Hu et al., (2018), involving high enzyme activity with a longer time up to (1600U/g) after 15 days with *Aspergillus niger* CKB and textile waste.

Regarding the ANOVA of the S/N ratio, the factors that give maximum response with minimum variation when moisture content varies were T, t, Rxt with 33, 16 and 21% of the share, respectively. The rest of interactions had no a substantial impact on the robustness of β-xylosidase activity against water content in the support media. According to the average responses between noises in Figure 4, it was specially observed differences in ratio microalgae biomass:sugarcane bagasse (R), but it did not report an effect on this activity.

Forasmuch as the results accounted for all the enzymes activities, the noise had a noteworthy consequence achieving higher productions for WW in majority of cases. In a practise work, this factor is easily controlled. Therefore, it was highlighted the necessity to investigate the effect of the studied experimental parameters and interactions when moisture content was adjusted to 85% (WW) since it is likely that distinctive effects of the experimental variables could be observed, and others may appear.

3.2. Assessment of influencing experimental parameters on enzymes activities in controlled moisture media

As explained above, Table 1 presents the results of the experimental design used to study the effect of seven parameters and three interactions explained on the FPase, β-glucosidase, xylanase and β-xylosidase activities for the tests using water to adjust (WW) the moisture content (85%). The activity values ranged from 1.39 to 15.80 FPU/g, 0.64 to 11.35 U/g, 70.91 to 707.46 U/g and 1.13 to 2.81 U/g for FPase, β-glucosidase, xylanase and β-xylosidase, respectively. The limits were analogous as summarised in the previous section, but the bottom limit was slightly higher for this case. The average values from this test were also elevated compared to the control. The enzymes activities (1.37FPU/g and 348U/g of FPase and xylanase, respectively) from the control were in agreement to the productions achieved by Taherzadeh-Ghahfarokhi et al., (2019). They reported 2.2 FPU/g and 300U/g, respectively; using *Trichoderma reesei* ATCC 13631 in the SSF of wheat straw.

3.2.1. FPase enzyme

Seemingly, the results obtained for the FPase average of both tests were analogous that achieved by WW (Figure 1). ANOVA of the results from only WW test indicated as significant factors ratio biomass:sugarcane bagasse (R), pH and temperature of extraction (T_e) contributing to the total variance with 26, 25 and 18%, respectively. In this case, none of interactions had a relevant impact on FPase enzyme (Table 4). In this study, no impact of the time was detected, but Xu et al., (2018) disclosed a significant time effect on FPase activity, showing higher results when the time increases. Furthermore, Trivedi et al., (2015) also detected the influence of the fermentation time achieving the maximum enzyme production at 4 days and, then, having a declined effect. Despite de non-statistically significance in this work, it was observed that shortest time (3 days) reached higher results than longer.

For each significant factor, the level responsible of the significant effect yielding the maximum enzyme activity was identified by using the Duncan's multiple range test for comparison of mean responses at the different factor levels assayed. For R, there was no significant difference between R1 and R2 but both differed from R3, being ratio R3 (50 of microalgae – 50 of sugarcane bagasse) the optimum condition for this enzyme production. Accordingly, Li et al., (2019) also found a great effect of the concentration of duckweed on cellulase production in SSF by *Trichoderma reesei* Rut C-30.

However, Libardi et al., (2017) did not account for the influence of the concentration of domestic sanitary wastewater on cellulase production by *Trichoderma harzianum* HBA03. But they detected an increase of enzyme activity respect to the control (using only distilled water) due to the presence of micronutrients and nitrogen, carbon and phosphorous content in the wastewater. They mentioned the great capability of *Trichoderma* to harsh environment and the necessity of search of substrates without adding nutrients and convenient for fungi growth (Drani et al., 2011).

Table 4: ANOVA tables of the enzymes production responses showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at level WW tests. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	FPase (FPU/g)				β-glucosidase (U/g)				Xylanase (U/g)				β-xylosidase (U/g)			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
R	2	98.27	0.002	26	*2*	*0.77*			*2*	*25895.35*			*2*	*0.14*		
t	*2*	*18.99*			*2*	*0.49*			*2*	*12815.20*			*2*	*2.01*		
R x t	*4*	*38.79*			*4*	*1.81*			4	82330.29	0.050	9	4	0.45	0.002	31
pH	2	94.30	0.003	25	*2*	*2.17*			2	60658.75	0.029	7	*2*	*0.26*		
R x pH	*4*	*13.48*			*4*	*3.05*			4	307546.28	0.001	34	*4*	*0.77*		
t x pH	*4*	*17.33*			*4*	*0.61*			*4*	*7842.73*			4	1.32	0.056	20
T	*2*	*17.98*			2	393.98	0.000	96	*2*	*12603.09*			2	1.05	0.026	16
B	*2*	*2.81*			*2*	*0.91*			2	128190.37	0.003	14	*2*	*0.01*		
Te	2	69.88	0.010	18	2	7.40	0.002	2	2	100318.60	0.007	11	*2*	*0.12*		
te	*2*	*9.87*			*2*	*0.30*			2	160349.84	0.001	18	*2*	*0.36*		
Residual	20	119.25		31	22	10.11		2	10	59156.37		7	18	2.11		32
Total	26	381.70			26	411.49			26	898550.50			26	6.50		

[a]R: ratio microalgae biomass: sugarcane bagasse; t: fermentation time; pH: pH in the raw material; T: fermentation temperature; B: kind of buffer during the extraction; T_e: extraction temperature; and t_e: extraction time.

The optimum value of pH was 2 (pH1), showing a significant difference with pH2 and pH3, but no significant difference between pH2 and pH3 was observed. However, Xu et al., (2018) obtained the maximum production of FPase (3.21 IU/g) at pH 4 with sugarcane bagasse and *Inonotus obliquus*, the lowest value proved in their work. While for T_e, there was only a relevant difference between T_e1 with T_e2, being the best condition T_e1 (22ºC).

3.2.2. β-glucosidase enzyme

The effect of the temperature on the β-glucosidase production was certainly clear studying both NW and WW conditions simultaneously, as described in the previous section. However, it was fundamentally caused by WW tests as exhibited in Figure 2. Additionally, a slight significant influence of T_e was identified in the case of WW (Table 4). Nevertheless, other authors highlighted the increment of β-glucosidase enzyme throughout the time. Teles et al., (2018) provided a wide and high range from 15 (at 1 day) to 90U/g (at 3 days) using a mixture of grape pomace and wheat straw, at 37ºC, 60% of moisture in a SSF by *Aspergillus niger* 3T5B8. The effect of pH was statistically insignificant for this enzyme, contrary to the behaviour observed for FPase and xylanase. Other authors (Karray et al., 2016) showed a variability of β-glucosidase activity at different pH from 2 to 8, being the optimum at 4 using *Aspergillus niger* with *Ulva rigida*.

Their optimum conditions for the relevant parameters were T2 (28ºC) and T_e3 (35ºC). Duncan's test showed no statistical distinction between T1 (22ºC) and T3 (35ºC) but T2 (28ºC) reported considerable differences with the other two levels. For T_e, there was no relevant deviation between T_e1 (22ºC) and T_e3 (35ºC) while their differences with T_e2 (28ºC) were significant.

3.2.3. Xylanase enzyme

The effect of the main parameters on xylanase production was not really detected in the ANOVA considering the two noises (Table 2), due to the great influence of the noise as previously explained in the above section. Therefore, ANOVA of the xylanase activities obtained in WW support media (Table 4) provided the predominant sources of variation with p-values less than 0.05. These parameters were pH, type of buffer (B), temperature (T_e) and time (t_e) of extraction with the following percentages of the share – 7, 14, 11 and

18%, respectively. Besides, the main contribution was the interaction of ratio microalgae biomass with pH (34%), demonstrating the facilitation of this kind of enzyme when combination substrate with pH is fitted. Leite et al., (2018) accounted a significance time influence on the xylanase production from 35 to 50U/g at 3 and 7 days, respectively. Despite the use of same strain of fungi (*Trichoderma reesei*), they utilised Carnauba straw with a 60% of moisture adding saline solution. However, Kogo et al., (2017) reached a noticeable 50 unit/mL (corresponding to 2500U/g) of xylanase activity using rice straw in SSF with *Trichoderma reesei* (ATCC 66589) at 30ºC for 7 days and 200 rpm. Consequently, the kind of substrate for this enzyme is more significant factor than the mixture of substrate used.

The optimum levels for xylanase activity were t_e1 (1 hour), B3 (phosphate buffer), T_e1 (22ºC) and pH2 (4). The parameters during the extraction (t_e and T_e) had the same tendency, statistically distinguishing level 1 with levels 2 and 3, but without difference between level 2 and 3. In the case of pH, the unique difference was found between level 2 and level 3; contrary to this result Ahmed Simair et al., (2018) reported a higher xylanase activity at higher pH (8) at 37ºC for 2 days using *Bacillus Cereus* TH-050.

The higher extraction with phosphate buffer (B3) and the significant divergence with B1 (water) and B2 (acetate buffer) were also corroborated by Ahmed Simair et al., (2018), obtaining high xylanase concentration (3566U/g dry matter) using wheat bran at 50ºC for 48h but with *Bacillus cereus* TH-050.

3.2.4. β-xylosidase enzyme

Concerning β-xylosidase activities, time and temperature of fermentation, and interaction of txpH were the main influential parameters with p-values<0.05 (Table 4). Entirely, the significant effect of t and T were also detected in the previous ANOVA (Table 2) but with lower contribution, and other interactions with the noise had an influence on this enzyme activity. Contrarily, their contributions varied, and the time had further influence than temperature with corresponding percentages of the share – 31 for time and 16% for temperature. The test of Duncan confirmed that the optimum conditions were t2 (5 days) and T2 (28ºC). Level t2 (5 days) had a significant difference with levels t1 (3 days) and t3 (7 days), while T2 (28ºC) had only with level 1.

In summary, the tested parameters had diverse influence on the enzyme's activities, provoking higher impact or without effect. Therefore, the optimum conditions for the highest productions based on the results reported were: ratio biomass:sugarcane bagasse 50:50 (R3), 5 days of fermentation time (t2), pH of 4 (pH2), temperature of 28°C (T2), phosphate buffer (B3), 22°C of temperature of extraction (T_e1) and 1 hour of extraction time (t_e1).

3.3. Optimum conditions: confirmatory experiments

Apart from the general optimum condition in the above section, the experiment number 11 (ratio 75:25, 3 days of fermentation, pH 4, 35°C; extraction of: water, 35°C, 1 hour) was selected considering the lowest deviation of the enzymes production respect to the average as shown in Figures 1, 2, 3 and 4. It was used as an internal control to corroborate the experimental design when the microalgae biomass changes notably its composition throughout the year for the environmental conditions.

The confirming experiments were carried out at the optimum and internal control conditions adjusting the moisture content (85%) using microalgae biomass at the ratio stablished at these conditions, uniquely sugarcane bagasse, and again as control the mixture sugarcane bagasse with wheat bran (50:50). New fresh mixed microalgae biomass was cultivated in the same condition as the previous reported in the materials and methods section but collected in July. Therefore, the microalgae composition (23.50% of total solids) appreciably varied and was composed by 27.32% of carbohydrates, 40.54% of proteins and 11.59% of lipids and 82.76% of volatile solids, all of them in a dry basis. The sugarcane bagasse and wheat straw were always the same for all the assays.

Firstly, the control using a mixture of sugarcane bagasse and wheat straw obtained close productions (1.30 ± 0.49 FPU/g for FPAse, 5.83 ± 0.58 U/g for β-glucosidase, 300.32 ± 8.29 U/g for xylanase, and 1.00 ± 0.34 U/g for β-xylosidase) as the orthogonal design in the previous section. Newly to confirm the correct experimental set up, the internal control experiment (number 11) obtained using microalgae with sugarcane bagasse reported 7.83 ± 0.67 FPU/g for FPAse, 2.83 ± 0.44 U/g for β-glucosidase, 500.32 ± 10.09

U/g for xylanase, and 1.80 ± 0.56 U/g for β-xylosidase. These values undoubtedly corroborated the precise internal working process, reporting similar enzymes production independently of the kind of microalgae biomass and its composition. At the same conditions of internal experiment (number 11), new experiment but using only sugarcane bagasse was evaluated to identify the contribution of this substrate (sugarcane bagasse) into the solid-state fermentation. The enzymes productions were 1.00 ± 0.27 FPU/g for FPAse, 2.31 ± 0.35 U/g for β-glucosidase, 100.45 ± 12.29 U/g for xylanase, and 0.87 ± 0.17 U/g for β-xylosidase. Therefore, sugarcane uniquely involved a higher contribution for β-glucosidase and β-xylosidase than FPAse and xylanase.

The enzymes activities obtained using a mixture of microalgae biomass and sugarcane bagasse at the optimum conditions were 28.35 ± 0.43 FPU/g for FPAse, 19.76 ± 0.44 U/g for β-glucosidase, 1113.45 ± 68.65 U/g for xylanase, and 3.81 ± 0.26 U/g for β-xylosidase. These values were much higher than reported in all the previous tests from the experimental design and the other confirming experiments. Using the same fungi (*Trichoderma reesei* CCT2768), Leite da Silva et al., (2018) obtained simply 0.2 and 50U/g of FPase and xylanase enzymes under SSF using carnauba straw as substrate (30ºC, 60% of moisture, 7 days and adding saline solution).

However, Xie et al., (2015) investigated the effect of several strains of Trichoderma, being G26 (known as *Trichoderma longibrachiatum*), the one that achieved the highest productions (40.9 and 71.8 IU/g for FPase and β-glucosidase, respectively) using a mixture of rice straw, wheat bran and corncob (ratio 4:4:2) at 28ºC for 144h (6 days), even achieving higher enzymes activities at 72 days (3 days) than reported in this study. In this context, Hu et al., (2018) achieved higher β-glucosidase activity (1773 U/g) but with other fungi (*Aspergillus niger* CKB) for longer time (14 days) at 28ºC with 75% of moisture content in a mixture of cotton/PET (80/20). Similar value (1137U/g) was reported by Khanahmadi et al., (2018) using other strain of *Aspergillus niger* CCUG33991 but only in 72h (3 days). Therefore, this comparison determined that despite the excellent results at the optimal conditions, the use of *Aspergillus* fungi commonly promoted the production of β-glucosidase enzyme (Raveendran et al., 2018).

Repeatedly, the same experiment at optimal conditions but only using sugarcane bagasse was assessed to determine the involvement of this substrate. The enzymes activities values were 2.30 ± 0.28 FPU/g for FPAse, 2.40 ± 0.31 U/g for β-glucosidase, 200.50 ± 13.60 U/g for xylanase, and 1.99 ± 0.13 U/g for β-xylosidase. Consequently, the unique use of sugarcane bagasse did not enhance the enzymes production, and hence the microalgae biomass was the most contributing substrate and wheat straw in the control assays. Most recently, Fernandes et al., (2019) achieved lower enzymes activities (350 of xylanase and 8U/g of β-glucosidase) than reached in this study at the optimum conditions even using a macroalgae (*Ulva rigida*) without nutrient supplementation using *Aspergillus ibericus*.

4. Conclusions

The microalgae biomass grown in pig manure was a profitable substrate for cellulases and xylanases productions by SSF. The study of the moisture content determined the enhancement of enzymes activities with an easily controllable adjustment. Statistically significant parameters for each enzyme were ratio substrate, pH and extraction temperature for FPase; fermentation and extraction temperature for β-glucosidase; pH, buffer, temperature and time of extraction for xylanase; and temperature and time of fermentation for β-xylosidase. The highest values obtained at the optimum conditions were 28.35 FPU/g for FPAse, 19.76 U/g for β-glucosidase, 1113.45 U/g for xylanase, and 3.81 U/g for β-xylosidase.

5. Acknowledgements

6. References

[1] Adney, B., Nrel, J.B., 2008. Measurement of Cellulase Activities. NREL/TP-510-42628.

[2] Ahmed Simair, A., Sattar Qureshi, A., Pirah Simair, S., Khushk, I., Klykov, S.P., Haider Ali, C., Lu, C., 2018. An integrated bioprocess for xylanase production from agriculture waste under open non-sterilised conditions: Biofabrication as fermentation tool. J. Cleaner Product. 193, 194–205.

[3]   Bala, A., Singh, B., 2018. Cellulolytic and xylanolytic enzymes of thermophiles for the production of renewable biofuels. Renew. Energy 136, 1231–1244.

[4]   Behera, S.S., Ray, R.C., 2016. Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int. J. Biol. Macromol. 86, 656–669.

[5]   Drani, A., Zahangir Alam, Md., Muyibi, S.A., 2011. Screening of potencial strains and co-substrate for production of cellulase enzyme using sewage treatment plant sludge as major substrate. Biotechnology, 1–6.

[6]   Farinas, C.S., 2018. Chapter 9. Solid-State Fermentation for the on-site production of Cellulolytic Enzymes and Their Use in the Saccharification of Lignocellulosic Biomass. In: Current Developments in Biotechnology and Bioengineering, 169–183.

[7]   Farinas, C.S., 2015. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. Renew. Sustain. Energy Rev. 52, 179–188.

[8]   Fernandes, H., Manuel, J., Martins, N., Peres, H., Oliva-teles, A., Belo, I., 2019. Sequential bioprocessing of *Ulva rigida* to produce lignocellulolytic enzymes and to improve its nutritional value as aquaculture feed. Bioresour. Technol. 281, 277–285.

[9]   González Lopez, C.V., Cerón García, M.C., Acién Fernández, F.G., Segovia Bustos, C., Chisti, Y., Fernández Sevilla, J.M., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591.

[10] Guoweia, S., Man, H., Shikai, W., He, C., 2011. Effect of some factors on Production of cellulase by *Trichoderma reesei* HY07. Procedia Environ. Sci. 8, 357–361.

[11] Hansen, G.H., Lübeck, M., Frisvad, J.C., Lübeck, P.S., 2015. Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. Proc. Biochem. 50, 1327–1341.

[12] Hirasawa, H., Shioya, K., Mori, K., Tashiro, K., Aburatani, S., Shida, Y., Kuhara, S., Ogasawara, W., 2019. Cellulase productivity of *Trichoderma reesei* mutants developed in Japan varies with varying pH conditions. J. Biosc. Bioeng. doi.org/10.1016/j.jbiosc.2019.03.005

[13] Hu, Y., Du, C., Leu, S., Jing, H., Li, X., Sze, C., Lin, K., 2018. Valorisation of textile waste by fungal solid state fermentation: An example of circular waste-based biorefinery. Resources, Conservation & Recycling 129, 27–35.

[14] Karray, R., Hamza, M., Sayadi, S., 2016. Production and characterisation of enzymatic cocktail produced by *Aspergillus niger* using green macroalgae as nitrogen source and its application in the pre-treatment for biogas production from *Ulva rigida*. Bioresour. Technol. 216, 622–628.

[15] Khanahmadi, M., Arezi, I., Amiri, M., Miranzadeh, M., 2018. Bioprocessing of agro-industrial residues for optimisation of xylanase production by solid- state fermentation in flask and tray bioreactor. Biocatalysis and Agricultural Biotechnology. 13, 272–282.

[16] Kochert, G., 1978. Quantification of the macrolecular components of microalgae. Hand. Phycol. Methods. Physicol. Biochem. 189–1175.

[17] Kogo, T., Yoshida, Y., Koganei, K., Matsumoto, H., Watanabe, T., Ogihara, J., Kasumi, T., 2017. Production of rice straw hydrolysis enzymes by the fungi *Trichoderma reesei* and *Humicola insolens* using rice straw as a carbon source. Bioresource Technology. 233, 67–73.

[18] Leite, F., Oliveira, A. De, Alves, D., Júnior, D.O., Eduardo, C., Padilha, D.A., 2018. Pretreatments of Carnauba (*Copernicia prunifera*) straw residue for production of cellulolytic enzymes by *Trichorderma reesei* CCT-2768 by solid state fermentation. Renewable Energy. 116, 299–308.

[19] Li, C., Li, D., Feng, J., Fan, X., Chen, S., Zhang, D., He, R., 2019. Duckweed (*Lemna minor*) is a novel natural inducer of cellulase production in *Trichoderma reesei*. J. Biosc. Bioeng. 127, 486–491.

[20] Libardi, N., Soccol, C.R., Góes-Neto, A., Oliveira, J. de, Vandenberghe, L.P. de S., 2017. Domestic wastewater as substrate for cellulase production by *Trichoderma harzianum*. Process Biochem. 57, 190–199.

[21] Lopez-Ramirez, N., Volke-Sepulveda, T., Gaime-Perraud, I., Saucedo-Castañeda, G., Favela-Torres, E., 2018. Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor. Bioresour. Technol. 265, 291–298.

[22] Manan, M.A., Webb, C., 2017. Modern microbial solid state fermentation technology for future biorefineries for the production of added-value products. Biofuel Research J. 16, 730–740.
[23] Marín, M., Sánchez, A., Artola, A., 2019. Production and recovery of cellulases through solid-state fermentation of selected lignocellulosic wastes. J. Clean. Prod. 209, 937–946.

[24] Martín-Juárez, J., Vega-Alegre, M., Riol-Pastor, E., Muñoz-Torre, R., Bolado-Rodríguez, S., 2019. Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater. Bioresour. Technol. 281, 239–249.

[25] Maurya, D., Singh, D., Pratap, D., Maurya, J., 2012. Optimisation of solid state fermentation conditions for the production of cellulase by *Trichoderma reesei*. J. Environ.Biol. 33, 5–8.

[26] Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

[27] Morales-Amaral, M.M., Gómez-Serrano, C., Acién, F.G., Fernández-Sevilla, J.M., Molina-Grima, E., 2015. Outdoor production of Scenedesmus sp. in thin layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source. Algal Res. 12, 99–108.

[28] Paganini, N., Cassia, J. De, Gomes, E., Regina, A., Ferreira, H., Rodrigues, A., Johana, K., Alonso, D., 2018. Cellulases and xylanases production by endophytic fungi by solid state fermentation using lignocellulosic substrates and enzymatic sacchari fi cation of pretreated sugarcane bagasse. Industrial Crops & Products 122, 66–75.

[29] Raveendran, S., Parameswaran, B., Ummalyma, S.B., Abraham, A., Kuruvilla, A., Madhavan, A., Rebello, S., Pandey, A., 2018. Applications of Microbial Enzymes in Food Industry 56, 16–30.

[30] Ray, R.C., Behera, S.S., 2017. Chapter 3. Solid State Fermentation for Production of Microbial Cellulases. In: Biotechnology of Microbial Enzymes, 43–79.

[31] Ross, P.J., 1995. Taguchi Techniques for Quality Engineering: Loss Function, Orthogonal Experiments, Parameter and Tolerance Design. ISBN-10: 0070539588

[32] Taguchi, G., Chowdhury, S., Wu, Y., 2007. Taguchi's Quality Engineering Handbook.

[33] Taherzadeh-Ghahfarokhi, M., Panahi, R., Mokhtarani, B., 2019. Optimising the combination of conventional carbonaceous additives of culture media to produce lignocellulose-degrading enzymes by *Trichoderma reesei* in solid state fermentation of agricultural residues. Renew. Energy 131, 946–955.

[34] Teles, A.S.C., Chávez, D.W.H., Oliveira, R.A., Bon, E.P.S., Terzi, S.C., Souza, E.F., Gottschalk, L.M.F., Tonon, R. V., 2018. Use of grape pomace for the production of hydrolytic enzymes by solid-state fermentation and recovery of its bioactive compounds. Food Res. Int. doi:10.1016/j.foodres.2018.10.083

[35] Thomas, L., Larroche, C., Ashok, P., 2008. Current Developments in Solid-State Fermentation. Bioch. Eng. J. 81, 146–161.

[36] Travaini, R., Derly, M., Otero, M., Coca, M., Da-silva, R., Bolado, S., 2013. Sugarcane bagasse ozonolysis pretreatment : Effect on enzymatic digestibility and inhibitory compound formation. Bioresource Technology 133, 332–339.

[37] Trivedi, N., Reddy, C.R.K., Radulovich, R., Jha, B., 2015. Solid state fermentation (SSF)-derived cellulase for saccharification of the green seaweed *Ulva* for bioethanol production. Algal Res. 9, 48–54.

[38] Van Wychen, S., Laurens, L.M.L., 2015a. Determination of Total Solids and Ash in Algal Biomass. NREL/TP-5100-60956.

[39] Van Wychen, S., Laurens, L.M.L., 2015b. Determination of Total Carbohydrates in Algal Biomass. NREL/TP-5100-60957.

[40] Xie, L., Zhao, J., Wu, J., Gao, M., Zhao, Z., Lei, X., Zhao, Y., Yang, W., Gao, X., Ma, C., Liu, H., Wu, F., Wang, X., Zhang, F., Guo, P., Dai, G., 2015. Efficient hydrolysis of corncob residue through cellulolytic enzymes from *Trichoderma* strain G26 and L -lactic acid preparation with the hydrolysate. Bioresource Technology 193, 331–336.

[41] Xu, X., Lin, M., Zang, Q., Shi, S., 2018. Solid state bioconversion of lignocellulosic residues by *Inonotus obliquus* for production of cellulolytic enzymes and saccharification Bioresource Technology 247, 88–95.

[42] Yamakawa, C.K., Qin, F., Mussatto, S.I., 2018. Advances and opportunities in biomass conversion technologies and biorefineries for the development of a bio-based economy. Biomass and Bioenergy 119, 54–60.

Conclusiones y trabajo futuro

Conclusiones y trabajo futuro

Diversas alternativas de valorización de biomasas microalgales crecidas en fotobioreactores para el tratamiento de aguas residuales fueron evaluados, centrándose en la recuperación de la fracción carbohidrato como primer paso para la valorización integral de la biomasa.

Los pretratamientos comparados para la ruptura de la pared celular de la biomasa de consorcios de microalgas con bacterias antes de la degradación anaerobia y la hidrólisis enzimática fueron molino de bolas, ultrasonidos, explosión de vapor, peróxido de hidrógeno, alcalino y ácido en dos condiciones extremas.

Los métodos mecánicos (molino de bolas) proporcionaron una baja solubilización de sólidos volátiles (<20%) y, por lo tanto, bajos rendimientos de liberación (<19%) para todas las fracciones en ambas condiciones, excepto los lípidos con un rendimiento del 36% durante 60 min. La degradación anaerobia de las muestras pretratadas en el molino de bolas dio como resultado la misma producción de metano que la biomasa no tratada (154 ml de CH_4 / g VS) en 20 días. Sin embargo, esta producción se logró en solo 4 días con una cinética notablemente mayor de producción de biogás (0.8 d^{-1}) que la biomasa no tratada (0.17d^{-1}). Estas producciones fueron controladas por la etapa de hidrólisis debido a la baja concentración de subproductos (alrededor de 1 g/L). Además, el pretratamiento de ultrasonido también proporcionó baja solubilización de sólidos volátiles, pero aumentó con la duración del pretratamiento (15 y 25% a los 5 y 21 min, respectivamente). La misma tendencia se observó en liberación de carbohidratos, proteínas y lípidos con valores de 28%, 10% y 17%, respectivamente, después de 5 minutos a 42%, 27% y 32% después de 21 minutos. Sin embargo, el pretratamiento con ultrasonido no aumentó el potencial de producción de biogás (<167 ml de CH_4/ g VS) para todas las condiciones analizadas y la cinética se vio afectada por la inhibición, con una larga fase de retraso (> 7 días) calculada utilizando el modelo Gompertz. Ambos métodos mecánicos mostraron la presencia de ADN bacteriano y, por lo tanto, ningún efecto de esterilización de las bacterias presentes en la biomasa de consorcios de microalgas y bacterias.

El pretratamiento combinado (explosión de vapor) presentó resultados intermedios en la solubilización (alrededor del 40% de los sólidos volátiles solubilizados). La liberación de todas las fracciones aumentó con la temperatura, logrando 33%, 31% y 44% de carbohidratos, proteínas y lípidos, respectivamente. Sin embargo, se obtuvieron resultados diferentes en las producciones de biogás en las dos condiciones operacionales, con una mejora del 11% en la producción de biogás a 130ºC en un tiempo más corto (<5 días) y ninguna mejora a 170ºC. Ambos experimentos de degradación anaerobia fueron controlados por la etapa de hidrólisis con un coeficiente cinético de 0.48 d^{-1} a 130ºC.

Los pretratamientos ácidos alcanzaron la mayor solubilización de carbohidratos (98%) y recuperación de monosacáridos (81%), pero también una baja selectividad debido a la co-solubilización con altos porcentajes de las fracciones de proteínas (76%) y lípidos (56%). No obstante, el pretratamiento ácido con los valores más altos para la solubilización de sólidos volátiles (80%) inhibió la digestión anaerobia, ajustándose los resultados al modelo de Gompertz con fases de retraso de hasta 10 días y producciones de biogás inferiores a la biomasa no tratada (214 ml de CH_4/ g VS).

Los métodos alcalinos proporcionaron una alta liberación de sólidos volátiles (75%) y principalmente proteínas (88%) pero también carbohidratos (60%) y lípidos (63%). El pretratamiento mejoró la producción de metano 2.34 veces en comparación con la biomasa no tratada. A pesar de las grandes mejoras, la producción de biogás se vio afectada por la inhibición, con resultados ajustados al modelo Gompertz con una fase de retardo larga (> 7.8 días). Esto está relacionado con la gran degradación de carbohidratos (77%) detectada en estas muestras.

Los pretratamientos alcalino peróxidos proporcionaron una solubilización del 30% para sólidos volátiles. En condiciones severas, la liberación de las fracciones (47%, 56% y 41% para carbohidratos, proteínas y lípidos, respectivamente) fue mayor que en condiciones leves (0.5% H_2O_2); pero con un 71% de degradación de carbohidratos. Este método mejoró la producción de metano 1.73 veces al 0.5% de H_2O_2 en comparación con la biomasa no tratada y sin aumento al 7.5% de H_2O_2. A pesar de las diferencias entre las condiciones, ambos modelos cinéticos fueron controlados por etapa de hidrólisis.

No se encontraron bacterias viables en muestras pretratadas con HCl, NaOH y 7.5% de H_2O_2, de acuerdo con su baja degradación de carbohidratos con alrededor de 5 g/L de subproductos para todas las muestras. Los residuos después de la digestión anaerobia de las muestras pretratadas químicamente (alcalinos, alcalino peróxido y ácido) demostraron una buena calidad para su uso como bio-fertilizante para los campos con NPK superior al 7% (p /p) y C/N inferior a 15.

Además, estos pretratamientos se combinaron con una etapa posterior de hidrólisis enzimática para obtener azúcares fermentables. La hidrólisis enzimática no mejoró significativamente la solubilización para muestras de pretratamientos con alta solubilización durante la etapa de pretratamiento. En el caso de muestras pretratadas con ácido, la hidrólisis enzimática produjo nuevamente los rendimientos máximos de liberación de carbohidratos, proteínas y lípidos (97%, 87% y 80%, respectivamente) y la recuperación máxima de monosacáridos (92%). Las muestras pretratadas con NaOH también proporcionaron grandes solubilizaciones para todas las fracciones (84, 69 y 53%, respectivamente). La hidrólisis enzimática de los pretratamientos alcalinos y alcalino peróxido también dio como resultado altos rendimientos de recuperación de monosacáridos (82% y 65%, respectivamente). Sin embargo, la aplicación de hidrólisis enzimática si mejoró la solubilización para muestras de pretratamientos mecánicos. Los rendimientos de solubilización de carbohidratos alcanzaron el 84% por hidrólisis enzimática de muestras pretratadas con molino de bolas, mientras las solubilizaciones moderadas de proteínas y lípidos se alcanzaron (39 y 37%, respectivamente). Esta notable y selectiva solubilización de carbohidratos fue contrarrestada por el alto factor de degradación de carbohidratos que es detectado en estos experimentos (62%).

Además, se han realizado pruebas de digestión anaerobia e hidrólisis enzimática eliminando la fase líquida de los pretratamientos, para evitar el posible efecto inhibidor de los subproductos solubles. Sin embargo, el uso de solo fracciones sólidas de estos pretratamientos no mejoró los resultados e incluso proporcionó producciones más bajas que el uso de suspensiones completas. Esto explica la dificultad de acceder a los compuestos biodegradables en las fracciones sólidas únicamente.

En resumen, el pretratamiento de molino de bolas en condiciones severas (60 min) mejoró altamente la producción de biogás cinética; y el alcalino y el alcalino-peróxido mejoraron el potencial de las producciones de biogás y la cinética. Aunque se detectó una fase de retardo significativa en las muestras alcalinas destacando el interés de estudiar procesos continuos con lodos aclimatados. Para el pretratamiento con la hidrólisis enzimática, el molino de bolas conlleva una solubilización completa y selectiva de carbohidratos, pero con una degradación significativa que requiere una etapa de esterilización adicional. Inesperadamente, los pretratamientos alcalino-peróxido al 0.5% de H_2O_2 y la explosión de vapor no tuvieron efecto de esterilización. Las grandes recuperaciones y los rendimientos de solubilización de los pretratamientos alcalinos y ácidos hacen que estos pretratamientos sean los más prometedores para la recuperación de azúcares fermentables. Finalmente, los principales subproductos detectados durante los pretratamientos y la hidrólisis enzimática fueron ácidos orgánicos y metanol.

Basados en los resultados anteriores, se evaluó el estudio de los parámetros operacionales de los pretratamientos químicos para encontrar condiciones óptimas y únicas para la recuperación de azúcares fermentables de biomasas de microalgas cultivadas en diferentes medios: purines de cerdo, aguas residuales domésticas y medio sintético esterilizados. La temperatura, el tipo y la concentración del agente químico se identificaron como los parámetros estadísticamente más significativos para la recuperación de monosacáridos. Sin embargo, la concentración de biomasa, el tiempo de pretratamiento, la concentración de las enzimas y el tiempo de hidrólisis enzimática no tuvieron un efecto notable. El medio de crecimiento de la biomasa no tuvo un impacto relevante en la liberación de carbohidratos y proteínas, pero tuvo un efecto considerable en la degradación de los carbohidratos liberados, así como en la solubilización de lípidos.

El diseño experimental proporcionó las condiciones óptimas para los factores de control significativos (120ºC, 2M HCl). Se seleccionaron otros parámetros como el tiempo de pretratamiento (10 min) y la concentración de biomasa (75 g/L) en base a consideraciones económicas. En estas condiciones, la solubilización de carbohidratos fue 84% para todas las biomasas con degradaciones de 37, 31 y 5% para biomasa cultivada en purines de cerdo, aguas residuales domésticas y medios sintéticos, respectivamente.

El proceso adicional de hidrólisis enzimática tuvo el mayor impacto en la solubilización de carbohidratos y la recuperación de monosacáridos de la biomasa de microalgas cultivadas en aguas residuales domésticas, con incrementos de 15 a 25% respecto a los valores obtenidos solo durante la etapa de pretratamiento. A pesar de esta mejora, se encontró una alta degradación de carbohidratos (35-55%) para esta biomasa. La hidrólisis enzimática de la biomasa de microalgas cultivada en medio sintético solo consiguió una solubilización adicional entre 5 y 18%. En términos de solubilización de proteínas, la hidrólisis enzimática proporcionó un mayor efecto sobre la biomasa de microalgas de purines de cerdo con incrementos de hasta el 40%, mientras que la biomasa crecida en medio sintético reportó el aumento más bajo (10%). Para los lípidos, el 30% de la mejora se atribuyó para las microalgas de purines de cerdo y solo el 10-15% para la biomasa cultivada en medio sintético.

En general, después de la hidrólisis enzimática, el rendimiento máximo de carbohidratos liberados fue del 99.90% para la biomasa de microalgas cultivadas en aguas residuales domésticas pretratadas con HCl 1M o NaOH 2M, a 120ºC durante 10 minutos y además con una liberación de proteínas (88%) y lípidos (42%). Mientras que el máximo para la recuperación de monosacáridos fue del 91.20% para la biomasa cultivada en medio sintético pretratado con HCl 2M, 120ºC, 10 minutos con una gran solubilidad simultanea de carbohidratos (95%) y proteínas (93%). La solubilización casi completa de las proteínas se alcanzó para la biomasa de microalgas cultivadas en purines de cerdo pretratado con NaOH 2M, 120ºC, 60 min y, por lo tanto, una alta solubilización de carbohidratos (97%) y lípidos (61%).

La biomasa de microalgas del tratamiento de purines de cerdos resultó un sustrato adecuado para la producción de celulasas y xilanasas por fermentación en estado sólido usando *Trichoderma reesei*. La principal ventaja de la biomasa de microalgas con respecto a los sustratos tradicionales (paja de trigo, salvado de trigo ...) es la gran cantidad de nutrientes dentro de la biomasa sin la necesidad de una suplementación adicional de solución salina. El efecto de los parámetros principales como la relación biomasa: bagazo de caña de azúcar, temperatura, tiempo, pH; y tipo de tampón, temperatura y tiempo de extracción fueron analizados, y las condiciones operacionales óptimas seleccionadas. El

diseño experimental de Taguchi $L_{27}(3^{13})$ permitió determinar la robustez de este estudio frente a la variabilidad del contenido de humedad en la biomasa.

Los parámetros de operación tenían diferentes tendencias frente a cada actividad enzimática. Para FPasa, los parámetros más influyentes fueron la relación biomasa: bagazo de caña de azúcar, pH y temperatura de extracción con valores óptimos de 50:50, pH de 2, 22°C, respectivamente. La actividad de la β-glucosidasa solo se vio afectada por la temperatura de fermentación y extracción. Su óptimo se logró a 28°C para la fermentación y 35°C para la extracción. La producción de la enzima xilanasa fue influenciada por el tipo de buffer, la temperatura y el tiempo de extracción. Sus condiciones óptimas fueron a pH 4, buffer de fosfato, 22°C y 1 hora, respectivamente. El tiempo y la temperatura de extracción fueron los únicos dos parámetros con un impacto significativo en la actividad de la β-xilosidasa. La condición óptima fue a los 5 días y 28°C.

Combinando el efecto de los parámetros sobre las diferentes actividades enzimáticas, se seleccionaron las condiciones óptimas como: 50:50, 5 días de tiempo de fermentación, pH de 4, temperatura de 28°C, buffer de fosfato, 22°C de temperatura de extracción y 1 hora de tiempo de extracción. Las actividades obtenidas para estas condiciones fueron 28.35 FPU/g para FPAsa, 19.76 U/g para β-glucosidasa, 1113.45 U/g para xilanasa y 3.81 U/g para β-xilosidasa. Estos altos valores de actividades en comparación con las producciones comunes de paja de trigo corroboraron la posible valorización de la biomasa de microalgas como sustrato para la producción de enzimas.

En base a los resultados y las limitaciones encontradas en esta tesis, investigación adicional sobre alternativas de valorización debería centrarse en:
1. El estudio de la producción continua de biogás utilizando lodos aclimatados y la co-digestión con otros sustratos.
2. El estudio de los subproductos generados y el posible efecto inhibidor de estos compuestos en etapas adicionales de valorización.
3. La evaluación de nuevas alternativas de valorización capaces de transformar los subproductos generados por pretratamientos y microorganismos presentes en la biomasa en productos de valor añadido.

4. La búsqueda de nuevas técnicas para separar las bacterias y las microalgas presentes en la biomasa, o para esterilizar la biomasa, minimizando la generación de subproductos.

5. El desarrollo de procesos de separación para aprovechar los componentes macromoleculares de la biomasa de consorcios de microalgas y bacterias para la valorización secuencial.

6. La búsqueda de nuevos posibles productos de valor añadido de la biomasa de consorcios de microalgas y bacterias.

7. El estudio de metales pesados y contaminantes emergentes para determinar su influencia en la calidad de los productos obtenidos.

8. La evaluación de la hidrólisis enzimática para la liberación de azúcar de la biomasa de consorcios de microalgas y bacterias con las enzimas producidas utilizando como sustrato la misma biomasa.

9. La capacidad de la biomasa de consorcios de microalgas y bacterias como sustrato para la producción de otro tipo de enzimas, como proteasas o lipasas.

Chapter 9

Conclusions and future work

Conclusions and future work

Several alternatives for the valorisation of microalgae biomass grown in wastewater treatment photobioreactors were evaluated, focused on recovery of the carbohydrate fraction, as a first step of the biomass integral valorisation.

The pretreatments compared for the cell disruption of the microalgae-bacteria biomass before anaerobic degradation and enzymatic hydrolysis were bead mill, ultrasound, steam explosion, alkali-peroxide, alkaline and acid under two extreme operational conditions.

Mechanical methods (bead mill) provided low solubilisation of volatile solids (<20%) and, hence, low solubilisation yields (<19%) for all the fractions at both operational conditions apart from lipids with a yield of 36% for 60 min. Anaerobic degradation of bead mill pretreated samples resulted in the same methane production as untreated biomass (154 mL CH_4/g VS) at 20 days. Nevertheless, this production was achieved in only 4 days with remarkably higher kinetic of biogas production (0.8 d^{-1}) than untreated biomass (0.17d^{-1}). These productions were controlled by hydrolysis step due the low concentration of by-products (around 1g/L). Besides, ultrasound method also reported low solubilisation of volatile solids but increasing with the duration of pretreatment (15 and 25% at 5 and 21 min, respectively). The same tendency was observed in solubilisations of carbohydrates, proteins and lipids with values from 28%, 10% and 17%, respectively, after 5 minutes to 42%, 27% and 32% after 21min. However, ultrasound pretreatment did not increase the biogas production potential (<167 mL CH_4/g VS) for all assayed conditions and the kinetic was affected by inhibition, with a long lag phase (>7days) calculated using Gompertz model. Both mechanical methods showed the presence of bacterial DNA and, hence, the no effect of sterilisation for the bacteria present in the microalgae-bacteria biomass.

Combined method (steam explosion) accounted intermediate results on solubilisation (around 40% of solubilised volatile solids). The solubilisation of all fractions incremented with the temperature, achieving 33%, 31% and 44% of carbohydrates, proteins and lipids, respectively. However, different results on biogas productions at the two operational conditions were obtained, with an improvement of 11% on biogas production at 130ºC in

a shorter time (<5days) and no enhancement at 170ºC. Both anaerobic degradation experiments were controlled by the hydrolysis step with a kinetic coefficient of 0.48 d^{-1} at 130ºC.

The acid pretreatments resulted in the highest solubilisation (98%) and monosaccharides recovery (81%), but also in low selectivity due to the co-solubilisation of high percentages of the protein (76%) and lipid (56%) fractions. Nonetheless, acid pretreatment with the highest values for the solubilisation of volatile solids (80%) inhibited the anaerobic digestion, fitting the results to Gompertz model with lag phases up to 10 days and biogas productions lower than untreated biomass (214 mL CH_4/g VS).

Alkali methods provided high solubilisation of volatile solids (75%) and mainly solubilised protein fraction (88%) but also carbohydrates (60%) and lipids (63%). Alkali pretreatment improved the methane production 2.34-fold compared to the untreated biomass. Despite the great improvements, the biogas production was affected by the inhibition, with results fitted by Gompertz model with a long phase (>7.8 days). It was related to the great carbohydrate degradation (77%) detected in these samples.

Alkaline-peroxide pretreatments provided a solubilisation of 30% for volatile solids. At severe condition, the release of the fractions (47%, 56% and 41% for carbohydrates, proteins and lipids, respectively) was higher than at mild condition (0.5% H_2O_2); but with a 71% of carbohydrate degradation. This method improved the methane production 1.73-fold at 0.5% H_2O_2 compared to the untreated biomass and with no increase at 7.5% H_2O_2. Even the difference between conditions, both kinetic models were controlled by hydrolysis step.

No viable bacteria were found in samples pretreated with HCl, NaOH and 7.5% of H_2O_2, according with their low carbohydrate's degradation with around 5g/L of by-products for all the samples. The residues after the anaerobic digestion of the chemically pretreated (alkaline, alkaline-peroxide and acid pretreatments) samples demonstrated a good-quality for use as bio-fertiliser for the fields with NPK higher than 7% (w/w) and C/N lower than 15.

Besides, these pretreatments were coupled with a following enzymatic hydrolysis step to obtain fermentable sugars. Enzymatic hydrolysis did not enhance significantly the solubilisation for samples from pretreatments with high solubilisation during the pretreatment step. In the case of acid pretreated samples, the enzymatic hydrolysis newly produced the maximum carbohydrate, protein and lipid solubilisation yields (97%, 87% and 80%, respectively) and the maximum monosaccharides recovery (92%). Alkali samples also provided great solubilisations for all the fractions (84, 69 and 53%, respectively). The enzymatic hydrolysis of alkaline and alkali-peroxide pretreatments also resulted in high monosaccharide recovery yields (82% and 65%, respectively). Nevertheless, the application of enzymatic hydrolysis improved the solubilisation for mechanical samples. Carbohydrate solubilisation yields achieved 84% by enzymatic hydrolysis of bead mill pretreated samples, while moderate solubilisations were accounted for proteins and lipids (39 and 37%, respectively). This remarkable and selective carbohydrate solubilisation was counteracted by the high carbohydrate degradation factor occurring in these experiments (62%).

Additionally, tests of anaerobic digestion and enzymatic hydrolysis have been carried out by removing the liquid phase from pretreatments, to avoid the possible inhibitory effect of soluble by-products. However, the use of only solid fractions from these pretreatments did not improve the results and even provided lower productions than using whole suspensions. It meant that the difficulty to access to the biodegradable compounds in the only solid fractions.

Summarising, bead mill method at severe condition (60 min) enhanced highly the biogas production kinetic; and alkali and alkaline peroxide improved the potential of biogas productions and kinetics. Although significant lag phase was detected in alkali samples, pointing the interest of studying continuous processes with acclimatised sludge. For pretreatment coupled to enzymatic hydrolysis, bead mill fulfilled solubilise selectively carbohydrates but with significant degradation which require an additional sterilisation stage. Unexpectedly, alkaline peroxide at 0.5% H_2O_2 and steam explosion did not have the sterilising effect. The great recoveries and solubilisation yields from alkali and acid methods, make these pretreatments the most promising for the recovery of fermentable

sugars. Finally, the principal by-products detected during the pretreatments and enzymatic hydrolysis were organic acids and methanol.

Based on the previous results, the study of the operational parameters of chemical pretreatments was assessed to find unique optimal operational conditions for recovery of fermentable sugars from microalgae biomasses grown in different media: pig manure, domestic wastewater and sterile synthetic medium. Temperature, chemical reagent type and chemical reagent concentration were identified as the most statistically significant operational parameters for recovery of monosaccharides. However, the biomass concentration, pretreatment time, enzyme dosage and enzymatic hydrolysis time had no remarkable effect. The growth medium of the biomass had no relevant impact on carbohydrate and protein solubilisation but had a remarkable effect on the degradation of the released carbohydrates as well as lipid solubilisation.

The experimental design provided the optimal conditions for the significant control factors (120ºC, 2M HCl). Other parameters as pretreatment time (10 min) and biomass concentration (75g/L) were selected applying economic considerations. At these conditions, the carbohydrate solubilisations were 84% for all the biomasses with degradations of 37, 31 and 5% for biomass grown in piggery wastewater, domestic wastewater and synthetic medium, respectively.

The further enzymatic hydrolysis process had the most relevant impact on solubilisation of carbohydrates and recovery of monosaccharides from microalgae biomass grown in domestic wastewater, with increments from 15 to 25% respect to the values obtained only during the pretreatment step. Despite this enhancement, high carbohydrates degradation (35-55%) was found for this biomass. Enzymatic hydrolysis of pretreated microalgae biomass cultivated in synthetic medium resulted only in additional carbohydrate solubilisations between 5 to 18%. In terms of proteins solubilisation, enzymatic hydrolysis supported higher effect on microalgal biomass from pig manure with increases up to 40% while biomass grown in synthetic medium reported the lowest augmentation (10%). For lipids, a 30% of implementation was accounted for microalgae from pig manure and only 10-15% for biomass cultivated in synthetic medium.

In general, after enzymatic hydrolysis, the maximum carbohydrates solubilised yield was 99.90% for microalgae biomass grown in domestic wastewater pretreated with HCl 1M or NaOH 2M, at 120ºC for 10 min with also release of proteins (88%) and lipids (42%).

While the maximum for monosaccharides recovery was 91.20% for biomass cultivated at synthetic medium pretreated with HCl 2M, 120ºC, 10 min with a great co-solubilisation of carbohydrates (95%) and proteins (93%). Almost complete solubilisation of proteins was accounted for microalgae biomass grown in pig manure pretreated with NaOH 2M, 120ºC, 60 min and, hence, a high solubilisation of carbohydrates (97%) and lipids (61%).

Microalgae biomass from piggery wastewater treatment resulted an adequate substrate for cellulases and xylanases production by solid fermentation using *Trichoderma reesei*. The main advantage of microalgal biomass respect to the traditional substrates (wheat straw, wheat bran…) is the high amount of nutrients inside the biomass without supplementation of saline solution. The effect of the principal parameters such as ratio biomass: sugarcane bagasse, temperature, time, pH; and type of buffer, temperature and time of extraction were analysed, and the optimal operational conditions selected. Taguchi $L_{27}(3^{13})$ experimental design permitted to determine the robustness of this study faced to the variability of moisture content in the biomass.

The operational parameters had different tendencies faced to each enzyme activity. For FPase, the most influential parameters were ratio biomass:sugarcane bagasse, pH and temperature of extraction with optimum values at 50:50, pH of 2, 22ºC, respectively. β-glucosidase activity was only affected by temperature of fermentation and extraction. Their optimum was achieved at 28ºC for fermentation and 35ºC for extraction. The production of xylanase enzyme had been influenced by pH, buffer, temperature and time of extraction. Its optimal point was at pH of 4, phosphate buffer, 22ºC and 1 hour, respectively. The time and temperature of extraction were the only two operational parameters with a significant impact on the activity of β-xylosidase. The optimum condition was at 5 days and 28ºC.

Combining the effect of parameters on the different enzymatic activities, optimal conditions were selected as: 50:50, 5 days of fermentation time, pH of 4, temperature of

28ºC, phosphate buffer, 22ºC of temperature of extraction and 1 hour of extraction time. The highest values obtained at this condition were 28.35 FPU/g for FPAse, 19.76 U/g for β-glucosidase, 1113.45 U/g for xylanase, and 3.81 U/g for β-xylosidase. These great activities values compared to common productions from wheat straw corroborated the possible valorisation of microalgae biomass as substrate for enzymes production.

Based on the outcomes and limitations found in this study, further research on valorisation alternatives should focus on:

1. The study of continuous biogas production using acclimated sludge and the co-digestion with other substrates.
2. The study of the by-products generated and the possible inhibitory effect of these compounds in further valorisation steps.
3. The evaluation of new alternatives of valorisation able to transform the by-products generated by pretreatments and microorganisms present in the biomass into valuable products.
4. The search of new techniques to separate bacteria and microalgae present in the biomass, or to sterilise the biomass, minimising the generation of by-products.
5. The development of separation processes to harness of the macromolecular components from microalgae-bacteria biomass for sequential valorisation.
6. The search of new possible added-value products from microalgae-bacteria biomass.
7. The study of heavy metals and emergent contaminants to determine their influence on the quality of the obtained products.
8. The evaluation of enzymatic hydrolysis for the sugar release from microalgae-bacteria biomass with the enzymes produced using as substrate the same microalgae biomass.
9. The capability of microalgae-bacteria biomass as substrate for the production of other kind of enzymes, as proteases or lipases.

Chapter 10

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En la vida ni se gana ni se pierde, ni se fracasa ni se triunfa. En la vida se aprende, se crece, se descubre; se escribe, borra y reescribe; se hila, se deshila y se vuelve a hilar.

Chapter 11

Biography

Biography

Judit Martín Juárez (Valladolid, 1990) started Chemical Engineering in 2008 at the University of Valladolid. She did her Final Project in Technische Universität Hamburg-Harburg from September 2013 to April 2014. Afterwards, Judit joined to the Environmental Technology Research Group under the supervision of Dra. Silvia Bolado (Department of Chemical Engineering and Environmental Technology – University of Valladolid). Judit was awarded in November 2015 with a PhD contract by 'Junta de Castilla y León'.

Her PhD study was focused on the valorisation of microalgae biomass from piggery wastewater treatment. This thesis was belonged to complementary projects, one funded by INIA titled "Aprovechamiento de nutrientes de efluentes agroalimentarios mediante crecimiento y valorización de biomasa algal" and other funded by 'Junta de Castilla y León' with a title of "Valorización de residuos agroalimentarios generando bioenergía y bioproductos en procesos con microalgas". Fundación Cajamar and University of Almeria collaborated in both Projects.

Initially, the application of different pretreatments was studied to disrupt the cell wall of microalgae biomass from wastewaters. Besides, statistical methods were used to optimise the best conditions of carbohydrate solubilisation and valorisation as substrate for enzyme production. During her PhD, the candidate carried out two research stay for three month each one in University of Wageningen (2016, The Netherlands) and University of Novi Sad (2017, Serbia).

Publications in international journals

I. Martín-Juárez, J., Lorenzo-Hernando, A., Muñoz-Torre, R., Blanco-Lanza, S., Bolado-Rodríguez, S. (2016). Saccharification of microalgae biomass obtained from wastewater treatment by enzymatic hydrolysis. Effect of alkaline-peroxide pretreatment, Biores. Technol. 218: 265–271.

II. Bolado-Rodríguez, S., Toquero, C., Martín-Juárez, J., Travaini, R., García-Encina, P.A. (2016). Effect of termal, acid, alkaline and alkaline-peroxide

pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. Biores. Technol. 201: 182–190.

III. <u>Martín-Juárez, J.</u>, Riol-Pastor, E., Muñoz-Torre, R., Fernández-Sevilla, J.M., García-Encina, P.A., Bolado-Rodríguez, S. (2018). Effect of pretreatments on biogas production from microalgae biomass grown in pig manure treatment plants, Biores. Technol. 257: 30–38.

IV. Lorenzo-Hernando, A., <u>Martín-Juárez, J.</u>, Bolado-Rodríguez, S. (2018). Study of steam explosion pretreatment and preservation methods of commercial cellulose. Carbohydrate Polymers. 191: 234–241.

V. García-Cubero, R., Wang, W., <u>Martín, J.</u>, Bermejo, E., Sijstma, L., Togtema, A., Barbosa, M., Kleinegris, Dorinde. (2018). Milking exopolysaccharides from *Botryococcus braunii* CCALA778 by membrane filtration. Algal Res. 34: 175–181.

VI. <u>Martín-Juárez, J.</u>, Vega-Alegre, M., Riol-Pastor, E., Muñoz-Torre, R., Bolado-Rodríguez, S. (2019). Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater, Biores. Technol. 281: 239–249.

VII. <u>Martín-Juárez, J.</u>, Martínez-Páramo, S., García-Encina, P.A., Muñoz-Torre, R., Bolado-Rodríguez, S. Evaluation of pretreatments for production of fermentable monosaccharides from microalgae biomass grown in piggery wastewater. Submitted for publication in Algal Research.

VIII. <u>Martín-Juárez, J.</u>, Collantes-Doyague, M., Vega-Alegre, M., Bolado-Rodríguez, S. Valorisation of microalgae biomass grown in piggery wastewater treatment by cellulases and xylanases production from *Trichoderma reesei* QM9414. In preparation.

IX. <u>Martín-Juárez, J.</u>, Vladic, J., Bolado-Rodríguez, S., Vidovic, S. Sequential valorisation of microalgae biomass grown in pig manure wastewater treatment plants. In preparation.

Review

I. Travaini, R., <u>Martín-Juárez, J.</u>, Lorenzo-Hernando, A., Bolado-Rodríguez, S. (2016). Ozonolysis: An advantages pretreatment for lignocellulosic biomass revisited. Biores. Technol. 199: 2–12.

Book chapters

I. D'Hondt, E., <u>Martín-Juárez, J.</u>, Bolado, S., Kasperoviciene, J., Koreiviene, J., Sulcius, S., Elst, K., Bastiaens, L. (2017). Chapter 6. Cell disruption technologies. In: Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products. Edited by González-Fernández, C. and Muñoz, R. 133–154.

II. <u>Martín-Juárez, J.</u>, Markou, G., Muylaert, K., Lorenzo-Hernando, A., Bolado, S. (2017). Chapter 8. Breakthroughs in bioalcohol production from microalgae: Solving the hurdles. In: Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products. Edited by González-Fernández, C. and Muñoz, R. 183–208.

Contributions to conferences

I. Bolado-Rodríguez, S., Travaini, R., García-Encina, P., <u>Martín, J.</u> Effect of chemical pretreatments on digestibility of lignocellulosic materials and bioenergy production. In: International Conference on Emerging Trends in Biotecnology, 6-9 November 2014, New Delhi, India (Invited Oral Lecture).

II. Lorenzo, A., <u>Martín, J.</u>, García, D., Bolado, S. Influencia de las condiciones de producción de biomasa microalgal procedente de aguas residuales en la liberación de azúcares mediante hidrólisis enzimática. Bio.Iberoamérica 2016: Biotecnología integrando continentes. 5-8 June 2016. Salamanca, Spain (Poster).

III. Martín Juárez, J., Travaini, R., Carvajal Guevara, A., Bolado Rodriguez, S., Production of Cellulases and Xylanases from *Trichoderma reesei* QM9414 using microalgae biomass as substrate. 24th European Biomass Conference & Exhibition (EUBCE), 6 - 9 June 2016 Amsterdam, The Netherlands (Poster).

IV. Bolado, S., Martín, J., Lebrero, R., Pérez, S., García, D., Marín, D., Lorenzo, A., García, P.A., Muñoz, R. Tratamiento y valorización de aguas residuales mediante microalgas. XII reunión de la Mesa Española de Tratamiento de Aguas Residuales. 20-22 June 2016, Madrid, Spain (Oral Presentation).

V. Martín-Juárez, J., Lorenzo-Hernando, A., Saavedra, R., Bolado-Rodriguez, S. Valorización de aguas residuales a través de bioenergía y bioproductos usando microalgas. Investigadoras de la Uva en La Aventura de la Ciencia y la Tecnología, 10 March 2017, Valladolid, Spain. (Oral Presentation).

VI. Martín-Juárez, J., Lorenzo-Hernando, A., Bolado-Rodriguez, S. Revalorisation of solid waste microalgae biomass from pig manure water treatment towards biogas and fertilisers production. 5th International Conference on Sustainable Solid Waste Management. 21-24 June 2017, Athens, Greek. (Oral Presentation).

VII. Martin Juárez, J., Bolado-Rodríguez, S. Valorisation of wastewaters via bioenergy and bioproducts using carbohydrates from microalgae. Training School Microalgae processes: from fundamentals to industrial scale. 13-15 September 2017. Almería, Spain. (Poster).

VIII. Martín-Juárez, J., Riol-Pastor, E., Stanimirov-Stoychev, P., Muñoz-Torre, R., García-Encina, P., Bolado-Rodríguez, S. Biogas production from microalgae biomass grown in pig manure wastewater treatment plants. International Conference on Alternative Fuels: Future and Challenges. 23-24 October 2017, Daegu, South Korea. (Poster).

IX. Martín-Juárez, J., Vladic, J., Bolado, S., Vidovic, S. Sequential extraction of microalgae biomass grown in pig manure wastewater treatment: a step further towards a bio-refinery into added-value products. International Conference on

Renewable Energy ICREN 2018. 25-27 April 2018, Barcelona, Spain. (Oral presentation).

X. <u>Martín Juárez, J.</u>, Pastor Riol, E., Vega, M., Muñoz Torre, R., Bolado Rodríguez, S. Valorisation of carbohydrate fraction from microalgae-bacteria biomass grown in wastewater treatment plants: Optimisation of chemical pretreatment and enzymatic hydrolysis.1st International Conference on Water Resources and Sustainability & 3rd International Conference on Alternative Fuels, Energy and Environment. 28-31 October 2018, Nanjing/Yixing, China. (Poster).

Research stays

I. Technische Universität Hamburg-Harburg, (Germany) September 2013 – April 2014. Supervisor: Gabriela Juárez. Scope: Study of the bioremediation of the oil spill in the Gul f of Mexico. (Final Project Stay).

II. Institute of Food and Bio-based Research of Wageningen, University of Wageningen, (The Netherlands) January 2016 – April 2016. Supervisor: Dra. Maria Barbosa, Dra. Dorinde M.M. Kleinegris and Dr. Rafael García-Cubero. Scope: Development in situ continuous technologies to recover and separate the products (hydrocarbons and polysaccharides) of *Botryococcus braunii* and to further convert these to renewable polymers. (PhD stay).

III. Department of Biotechnology and Pharmaceutical Engineering, Faculty of Technology, University of Novi Sad, (Serbia) September 2017 – December 2017. Supervisor: Dra Senka Vidovic. Scope: Supercritical carbon dioxide extraction, subcritical water extraction, ultrasound-assisted and microwave-assisted extraction of various bioactive compounds from distinct microalgae. (PhD stay).

Awards

I. Special prize for the best Poster Presentation in the Training School Microalgae processes: from fundamentals to industrial scale. 13-15 September 2017. Almería, Spain.

II. Grant for research stay in University of Novi Sad (2017). University of Valladolid.

III. Grant for attending to 24th European Biomass Conference & Exhibition (EUBCE) in The Netherlands. University of Valladolid.

IV. Short Term Scientific Mission Grant for research stay in University of Wageningen (2016). EUAlgae COST ACTION (ES1408-120116-067087).

Co-supervision

I. Master Thesis: Lucia Castaño Ojero (February 2015 – July 2015). 'Estudio del Efecto del Pretratamiento Básico con Peróxido de Hidrógeno en la Liberación de Azúcares de Microalgas'. University of Valladolid, Spain.

II. Research Project: Elena Riol Pastor (February 2017 – July 2017). ''University of Valladolid, Spain.

III. Research Project: María Collantes Doyague (February 2018 – July 2018). 'Optimisación de la producción de celulasas y xilanasas mediante Trichoderma reesei QM9414 y usando biomasa microalgal como sustrato' University of Valladolid, Spain.

IV. Final year Project: Elena Riol Pastor (February 2018 – July 2018). 'Comparativa de valorización de la fracción carbohidrato de diferentes biomasas microalgales aplicando técnicas de diseño de experimentos' University of Valladolid, Spain.

Teaching

I. 2016-2017: Lecturer of 'Cálculo y Diseño de Operaciones de Separación' in Chemical Engineering Degree. University of Valladolid. 1.2 ECTS.

II. 2017-2018: Lecturer of 'Cálculo y Diseño de Operaciones de Separación' in Chemical Engineering Degree. University of Valladolid. 1.2 ECTS.

Attended short-course and seminars

I.   Course of Microalgae Cultivation. University of Valladolid. 24/11/2014 – 27/11/2014. 10 hours.

II.   Course of Scientific Communication in Engineering. University of Valladolid. 12/01/2015. 4 hours.

III. Complementary subject 'Introduction to the Research'. University of Valladolid. 10/2014 – 06/2015. 6ECTS.

IV.   Complementary subject 'Environmental Biotechnology'. University of Valladolid. 02/2015 – 06/2015. 3ECTS.

V.   Complementary subject 'Technologies of Production and Preservation of Food Industry'. University of Valladolid. 02/2015 – 06/2015. 3ECTS.

VI.   Course 'Microbiology and Microbial Ecology of Biofilms in Environmental Biotechnology'. University of Valladolid. 07/09/2015 – 14/09/2015. 12 hours.

VII. Technical Seminar 'Characterisation and management of odours and greenhouse gases in WWTPs'. University of Valladolid. 05/10/2015 – 05/10/2015. 8 hours.

VIII. Seminar on anaerobic Biochemical Methane Potential and activity tests. University of Valladolid. 14/10/2015 – 14/10/2015. 1 hour.

IX.   Course 'Vegetal biotechnology. Vegetal cellular cultures as biorefineries for the production of high-added value products'. University of Valladolid. 30/11/2015 – 04/12/2015. 12 hours.

X.   Course 'Bioproducts Engineering and Biorefineries'. University of Valladolid. 14-17/06/2016. 10 hours.

XI. Speaking Workshop for 3 Minutes Thesis Competition. University of Valladolid. 19 – 21/09/2016. 8 hours.

XII. 3 Minutes Thesis Competition. University of Valladolid. 07/10/2016. 3 hours.

XIII. Workshop 'Valorización de biomasa algal obtenida de efluentes agroalimentarios para su uso en acuicultura'. Centro de Investigación en Acuicultura, Zamarramala (Segovia). 24/11/2016. 5 hours.

XIV. 4th Workshop of PhD students in Chemical and Environmental Engineering. University of Valladolid. 01/12/2016. 4 hours.

XV. Course:'Coaching. El arte de ser profesional'. University of Valladolid. 29/02/2017 and 07/03/2017. 8 hours.

XVI. Conference 'Investigadoras de la Uva en la Aventura de la Ciencia y la Tecnología. University of Valladolid. 10/03/2017. 8 hours.

XVII. 5th Workshop of PhD students in Chemical and Environmental Engineering. University of Valladolid. 29/11/2017. 4 hours.

XVIII. English Course. Level C1. University of Valladolid. 22/01/2018 – 19/03/2018. 51 hours.