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.

Highlights

Tested biomass showed different behaviours depending on the algae/bacteria ratio.

Enzymatic hydrolysis of DWB yielded high glucose and low xylose extraction.

Sugars from PMWB were completely released by enzymatic hydrolysis but oxidized.

Acetic, formic and succinic acids were the main byproducts from released sugars.

Pretreatment enhanced enzymatic hydrolysis performance for almost all biomass tested.

[†] 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.

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, biofertilizers, 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 optimization of processes 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 feedstock 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 maximize 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 optimized (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 sterilization process (autoclaving) before using wastewater microalgae biomass for butanol production.

A biomass sterilization 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) optimized 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 sterilization 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 a 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 a 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 a 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^{-1} (Celluclast 1.5L - Cellulase from *Trichoderma reesei*) and 20 CBU g^{-1} (*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 deionized 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 fertilizers (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 $g \cdot L^{-1} \cdot d^{-1}$ for A1 and A2, 1.5 $g \cdot L^{-1} \cdot d^{-1}$ for C, and 2.5 $g \cdot L^{-1} \cdot d^{-1}$ for B1 and B2.

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 sugars 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 abundance in these samples of bacteria able to oxidize 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 oxidized, 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.

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 solubilized 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 solubilized 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. 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 solubilized 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

utilization 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 others 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. $\approx$ 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 solubilization. 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.

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.

6. References

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Figure Captions

Figure 1: Total carbohydrates (g/100g raw material) obtained from three consecutive acid hydrolysis.

Figure 1
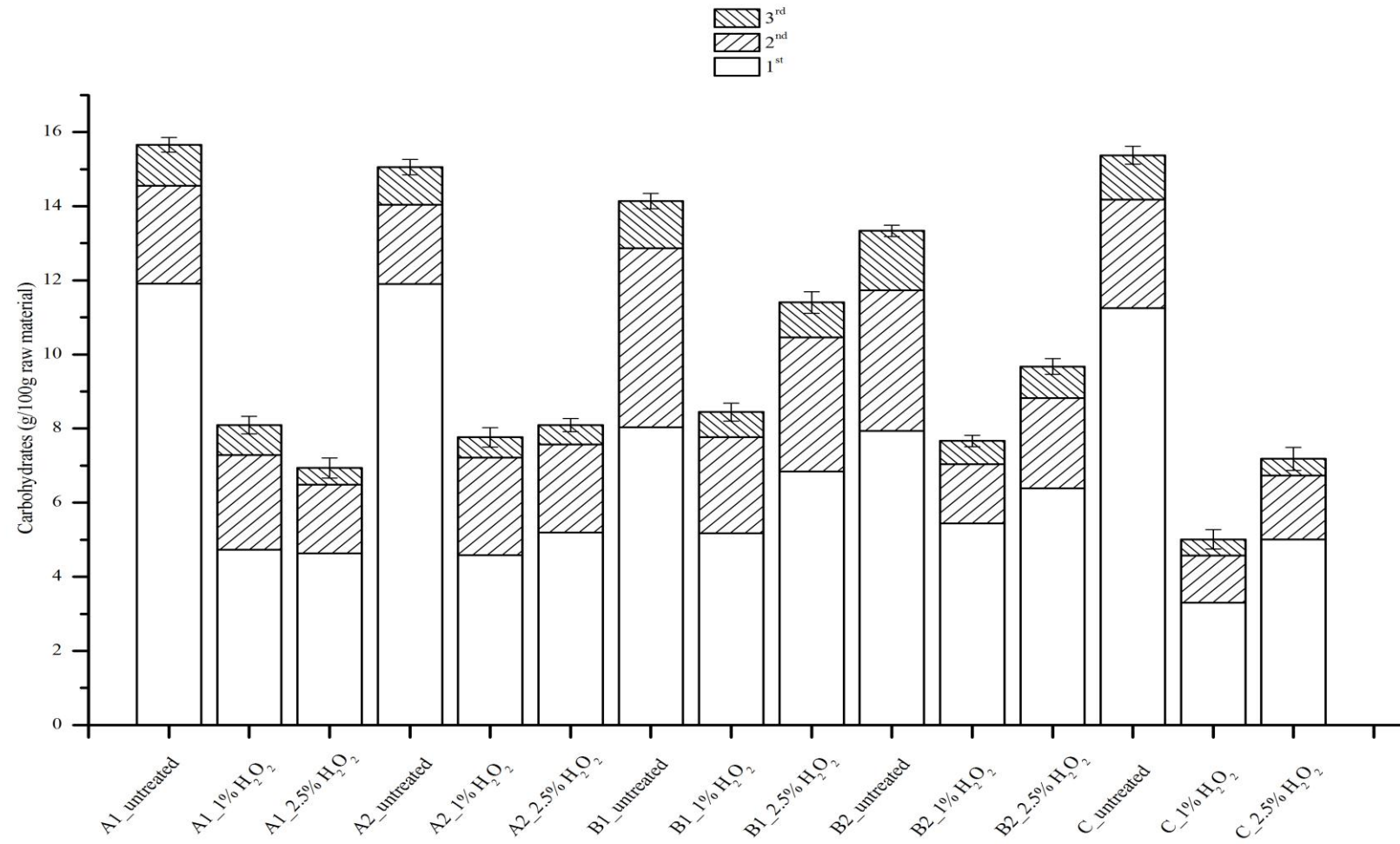
Tables

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

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

Table 3: Sugars composition in the solid fractions (%), solubilized 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