Process Safety and Environmental Protection 174 (2023) 276–285

Contents lists available at ScienceDirect

Process Safety and Environmental Protection

journal homepage: www.journals.elsevier.com/process-safety-and-environmental-protection

Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass

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ARTICLE INFO

Keywords:
Ultrasounds
Microwaves
Proteins
Peptides
Monosaccharides
Piggery wastewater

ABSTRACT

Despite the interest in the utilization of photobioreactors as an alternative wastewater treatment, the research about posterior recovery and valorization of nutrients accumulated in the biomass is still limited. This work compared several hydrolysis methods for the recovery of proteins and carbohydrates from the biomass grown in a photobioreactor treating swine wastewater. Ultrasound-assisted and microwave-assisted enzymatic hydrolysis at mild conditions and chemical methods at different temperatures (40, 60, 120ºC) were applied to the microalgae and bacteria biomass. Alkaline hydrolysis provided the greatest peptide recoveries, increasing with temperature up to a maximum of 81%, but with very small peptide sizes in all temperature range. Acid hydrolysis provided the highest carbohydrate recoveries (60.7% at 120ºC) but degraded proteins, even at mild temperatures. Protein degradation did not vary with temperature in each chemical hydrolysis, obtaining similar peptide sizes in all temperatures, while carbohydrate losses were higher at lower temperatures. Ultrasound-assisted enzymatic extraction recovered 43.6% of the initial proteins as large peptides (up to 135 kDa) with the highest peptide purity (46.7%). Microwave-assistance increased the carbohydrate solubilization of enzymatic hydrolysis, achieving yields of 73% of xylose, but with significant losses.

1. Introduction

Microalgae are photosynthetic organisms able to produce proteins, carbohydrates, lipids, and other organic substances by using solar energy, CO_2, and nutrients from an aquatic media (Matos, 2019). These nutrients can be obtained from different sources, such as wastewater streams (Nagarajan et al., 2020), and, therefore, photobioreactors are a promising approach for the purification of wastewater, and the accumulation of nutrients in the generated biomass (Zhang et al., 2020). Algal biomass grown in wastewater treatment photobioreactors is composed of a consortium of microalgae and bacteria working symbiotically. In wastewater treatment process, bacterial cells oxidize organic matter to inorganic compounds (CO_2), consuming O_2, while microalgae cells use light to carry out the photosynthesis and capture these inorganic compounds and thus, producing biomass and O_2 (Acién et al., 2016).

Microalgae biomass grown in nitrogen rich wastewater is mostly composed of proteins (Grossmann et al., 2020) and could be an important source of peptides which are used in animal feed or in agriculture as bio-stimulants and have a broad range of industrial uses (Rojo et al., 2021). The peptide characteristics will determine their applications, since the peptide sizes influence their techno-functional properties (e.g. foaming or emulsifying) for industrial uses and the profile of amino acids is important to evaluate their potential for feed applications. The amino acid profiles of hydrolysates from different treatments can provide, also, interesting information for further studies about the mechanisms of the hydrolysis methods. Peptides are very sensitive to harsh conditions, so the method used for protein solubilization should provide significant yields while trying to conserve their properties (Lorenzo-Hernando et al., 2019). Also, biofuel or other bioproducts could be produced from the fermentable monosaccharides recovered from the carbohydrate fraction, and so, it is also important to know the type of monosaccharide recovered as it influences the fermentation yields (Ezeji et al., 2014).

The first step of component extraction is the disruption of the microorganisms' cell walls. Several methods have been assayed, including

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https://doi.org/10.1016/j.psep.2023.03.067
Received 13 June 2022; Received in revised form 26 March 2023; Accepted 27 March 2023
Available online 30 March 2023
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chemical, biological, and physical methods (Nitsos et al., 2020). Chemical treatments, usually applied to microalgae biomass, combine moderate to high temperatures with HCl, H_2SO_4, NaOH, acetic acid, or nitric acid (Timira et al., 2021). In biological methods, enzymes are used to catalyze hydrolysis of cell wall components and/or to bind to the target compounds (Wen et al., 2020). (Martin Juárez et al., 2021) treated algal biomass cultivated in pig manure, obtaining high carbohydrate solubilizations (98.2% with 2 M HCl) and protein solubilizations (85.5% with 2 M NaOH) after only 1 h of chemical hydrolysis at 121ºC. However, these chemical treatments significantly degraded the solubilized carbohydrates (specially xylose over glucose in the alkaline hydrolysis), releasing a high concentration of byproducts and resulting in low carbohydrate recoveries. No data about peptide recovery were provided in this work, but it would be expected that the severe treatment conditions also affect the extracted peptides, being very small in size or even degraded to simple nitrogenous compounds. The application of mild treatments would result in low degradation of the solubilized peptides and monosaccharides, allowing the fractional recovery of the algal biomass components. Therefore, (Rojo et al., 2021) carried out enzymatic hydrolysis of a microalgal-bacteria biomass grown in piggery wastewater with different enzymes and found very low protein degradation. The hydrolysis with Protamex enzyme resulted in low peptide recoveries (<20%), but large sizes (up to 135 kDa). Then, although the peptide size was interesting, it is necessary to increase the yields to achieve a more efficient process. The recovery of the carbohydrate fraction is also important and the further process of valorization of this component depends on the type of recovered monosaccharides (López-Linares et al., 2020; Travaini et al., 2016).

The coupling of physical and biological methods could improve the extraction results, increasing the solubilization yields with minimal impact on the targeted molecule (Khadhraoui et al., 2021; Wen et al., 2020). (Hildebrand et al., 2020) investigated the enzymatic extraction of protein from pure *Chlorella vulgaris* coupled with ultrasounds, treating the microalgae with UAEE during 10 min followed by 1 h of incubation with protease AP30L at 50ºC. The combination of pretreatments increased the protein solubilization up to 80% compared to only enzymatic hydrolysis (60%). However, they did not provide data about degradation or peptide recovery yields nor the peptide sizes nor the co-solubilization and recovery of carbohydrates.

Ultrasounds and microwave enzymatic-assisted extraction have been used as powerful tools to achieve high extraction yields, to obtain large size peptides for different industrial applications and high-quality products from other raw materials, such as sesame bran, seaweed, flaxseed, and peanut shells (Wen et al., 2020). From the results with other materials, it is expected that the use of these combined technologies would allow for a greater solubilization and recovery of microalgae biomass compounds at mild conditions.

This work is a pioneer in the study of protein and carbohydrate extraction from a microalgal-bacterial consortium grown in wastewater (formed mainly by *Scenedesmus almeriensis*) comparing the ultrasound- and microwave-assisted enzymatic extraction methods to conventional acid and alkaline hydrolysis. The effect of temperature of chemical hydrolysis on the solubilization, compound degradation, and size of the recovered peptides was analyzed, working between 40 and 120ºC. Likewise, the results of enzymatic hydrolysis, UAEE, and MAEE with protease alone and with a cocktail of cellulase and protease were studied and compared. The solubilization and recovery yields of peptides and monosaccharides were determined, and the purity, size, and amino acid profile of the released peptides were analyzed. The solid fractions after hydrolysis were analyzed by scanning electron microscopy (SEM).

2. Materials and methods

2.1. Raw material and reagents

A biomass formed by a microalgae and bacteria consortium was used. This biomass was harvested from a 1200 L thin-layer photobioreactor working with a dilution rate of 0.33 d^{-1} and fed with 10% diluted swine wastewater in Almería, Spain (Morales-Amaral et al., 2015), centrifuged, freeze-dried, and well mixed to ensure identical composition and properties of the initial biomass used in all the experiments. Biomass lyophilization was used in laboratory experiments to provide the identical and stable initial biomass necessary to compare different hydrolysis methods. Previous works of this research group found that there were no differences between results of hydrolysis experiments working with fresh and freeze-dried biomass (Martín Juárez et al., 2016). Also, SEM images of fresh and freeze-dried biomass confirmed there is no cell rupture preserving cell structure and constituents (Chen et al., 2015). The most abundant microalgae species in the consortium was *Scenedesmus almeriensis* (96%), which was identified and quantified by optical microscopy (Rojo et al., 2021). Previous analysis of biomasses grown in similar conditions have provided percentages of microalgae in the range of 65%− 82% (Sánchez-zurano et al., 2021), being the dominant bacterial species from the phyla *Proteobacteria, Firmicutes and Cyanobacteria* (Collao et al., 2022; García et al., 2019). The protein, carbohydrate, and lipid content of this biomass was 41.3%, 22.7%, and 6.7%, respectively, while carbohydrates were composed of 11.1% glucose, 10.6% xylose, < 1% cellobiose, and 1.6% starch (all percentages on a dry-weight ash-free basis).

All of the reagents used in this work (HCl, NaOH, glucose, xylose, cellobiose, arabinose, bovine serum albumin, aspartic acid, methanol, and chloroform), with a purity > 95% or reagent grade, were purchased from Sigma Aldrich (Spain) and Panreac Applichem (Spain). Protamex as the protease and Celluclast 1.5 L as the cellulase were kindly supplied by Novozymes A/S (Bagsværd, Denmark).

2.2. Extraction methods

Several methods were compared for the solubilization and recovery of the microalgal compounds, including acid hydrolysis with HCl, alkaline hydrolysis with NaOH, enzymatic hydrolysis, UAEE, and MAEE (Fig. 1). For this research, all the assays were performed in duplicate with a biomass concentration of 5% ($w_{dry\ biomass}/w$).

After each treatment, the suspensions were centrifuged at 10,000 rpm and 4 ºC for 10 min to separate the liquid and solid fractions. Mass balances were checked by analyzing weights, volatile solid (VS), and total nitrogen concentrations in both fractions. Scanning electron microscopy (SEM) was used to analyze the initial biomass and some solid fractions after extraction treatments to evaluate the cell wall rupture. The presence of microorganisms and their growth in some hydrolysate samples was also observed by microscopy.

The protein, carbohydrate, and lipid contents were analyzed in the exhausted solid fractions. Peptide and monosaccharide concentrations and peptide sizes and free amino acids were determined in the liquid fractions. The amino acid profile was determined in the liquid and solid fractions. All analyses were carried out in replicate.

2.2.1. Acid and alkaline hydrolysis

Acid (2 M HCl) and alkaline (2 M NaOH) hydrolysis experiments were carried out with a working volume of 250 mL in 500 mL Erlenmeyer flasks and were preheated before the addition of the chemical reagents. Three temperatures (120ºC, 60ºC, and 40ºC) were tested for both chemical methods to analyze the effect of temperature on protein solubilization and recovery and peptide sizes. The 40 and 60ºC experiments were performed in a rotatory shaker at 300 rpm with a hydrolysis time of 1 h. The temperature was measured inside the conical flasks during all the experimentation. For the highest temperature, an autoclave at a pressure of 1 bar during 1 h was used, preheating the suspension up to 80ºC before the reagents addition. The highest temperature (120ºC) during 1 h was used because it provided high solubilization yields in previous works with similar biomasses (Lorenzo-Hernando et al., 2019; Martin Juárez et al., 2021). The lower

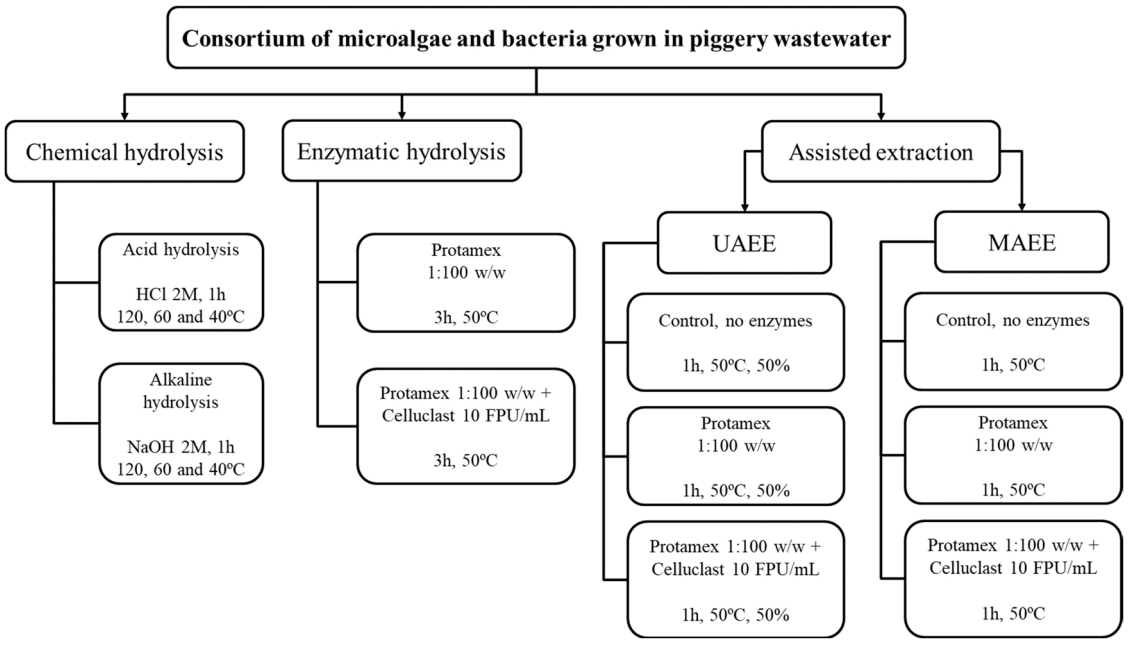

Fig. 1. Protein and carbohydrate extraction methods.

temperatures (60ºC and 40ºC) were chosen in an attempt to minimize degradation during the chemical hydrolysis. (Lorenzo-Hernando et al., 2019) also analyzed the effect of hydrolysis time during alkaline hydrolysis, without finding significant differences, so 1 h was chosen for our assays.

2.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis control assays were carried out using Protamex in some experiments and a cocktail of Protamex and Celluclast 1.5 L in others at 50ºC and pH of 6.5 according to the manufacturer's instructions and internal enzymatic analysis protocols (Marcos et al., 2013). No buffer addition was necessary. The Protamex enzyme is an endo-protease, consisting of a mixture of Alcalase and Neutrase, with 1.5 AU-A/g of enzymatic activity, while Celluclast 1.5 L is a cellulase enzyme which catalyzes the cellulose hydrolysis by attacking the glycosidic linkages. In previous studies, working with a similar algal biomass, Protamex enzyme provided moderate protein solubilization yields but promising peptide sizes, while Celluclast enzyme, used individually, provided very low protein solubilization yields (Martin Juárez et al., 2021; Rojo et al., 2021). As the cell wall of the microalgae is formed mostly of polysaccharides and glycoproteins, the combination of both enzymes could increase the solubilization yield.

These assays were carried out with a working volume of 250 mL in 500 mL Erlenmeyer flasks, which were preheated before the addition of the different enzymes. According to previous studies about the effect of process parameters on enzymatic extraction (Rojo et al., 2021), the experiments were performed in a rotatory shaker at 50 ºC and 300 rpm with a hydrolysis time of 1 h (higher hydrolysis time did not resulted in higher solubilization yields) with enzyme concentrations of 1:100 $w/w_{dry\ biomass}$ of Protamex in single protease experiments and 1:100 $w/w_{dry\ biomass}$ of Protamex and 10 $FPU/g_{carbohydrate}$ of Celluclast 1.5 L in protease-cellulase cocktail experiments. Temperature of the suspension was measured during the entire hydrolysis process.

2.2.3. Ultrasound-assisted enzymatic extraction (UAEE)

UAEE was carried out by applying the operational conditions previously described for the enzymatic hydrolysis experiments (except the hydrolysis time), and sonication with the ultrasonic probe UIP1000hd (1000 W, 20 kHz, Hielscher Ultrasound Technology, Germany) at 50% of amplitude for 1 h. Experiments were carried out with 1:100 $w/w_{dry\ biomass}$ of Protamex and combining 1:100 $w/w_{dry\ biomass}$ of Protamex and 10 $FPU/g_{carbohydrate}$ of Celluclast 1.5 L in a temperature-controlled jacketed vessel to maintain the working temperature (50ºC) and pH 6.5. A control experiment without enzymes was also carried out under the same conditions.

2.2.4. Microwave-assisted enzymatic extraction (MAEE)

MAEE was performed using a closed microwave-assisted system (Multiwave PRO SOLV 16HF100, Anton Paar GmbH, Austria) for 1 h at the working temperature (50ºC) and pH 6.5 using an enzyme concentrations of 1:100 $w/w_{dry\ biomass}$ of Protamex in single protease experiments and 1:100 $w/w_{dry\ biomass}$ of Protamex and 10 $FPU/g_{carbohydrate}$ of Celluclast 1.5 L in protease-cellulase cocktail experiments. The equipment included temperature and pressure probes to control the reaction conditions (López-Linares et al., 2020). A control experiment without enzymes was also carried out under the same conditions.

2.3. Calculations of extraction yields

To calculate the solubilization yields, Eq. 1 was used:

$$Compound\ solubilization\ yield\ (\%) = \left(1 - \frac{g\ compound\ in\ exhausted\ solid\ phase}{g\ compound\ in\ initial\ biomass}\right) \times 100 \quad (1)$$

where compounds were proteins or carbohydrates.

To calculate the recovery yields in the liquid fraction along with the peptide purity, the following equations (Eqs. 2 and 3) were used:

$$Compound\ recovery\ yield\ (\%) = \left(\frac{g\ compound\ in\ liquid\ phase}{g\ compound\ in\ initial\ biomass}\right) \times 100 \quad (2)$$

$$Peptide\ purity\ (\%) = \left(\frac{g\ peptides\ in\ liquid\ phase}{g\ VS\ in\ liquid\ phase}\right) \times 100 \quad (3)$$

where compounds were peptides or monosaccharides, and VS was the

volatile solids content. The purity of the peptides was determined based on the VS (content on organic matter of the hydrolysate) as this fraction includes peptides, monosaccharides and other organic by-products present in this product. Ash could be efficiently removed in a further electrodialysis step. During the hydrolysis process, losses of protein and carbohydrates occurred and were determined with the next equation (Eq. 4):

$$Compound\ losses(\%) = \left(1 - \frac{g\ compound\ in\ liquid\ phase + g\ compound\ in\ exhausted\ solid\ phase}{g\ compound\ in\ initial\ biomass}\right) \times 100 \quad (4)$$

where compounds were proteins or carbohydrates in the solid phase and peptides or monosaccharides in the liquid phase.

2.4. Analytical methods

The microalgal species in the initial biomass were identified and quantified by optical microscopy according to (Martín-Juárez et al., 2019). Volatile solids and the nitrogen content in the initial biomass and both fractions after the microalgal biomass treatment were analyzed by gravimetry using a NREL protocol (Van Wychen and Laurens, 2016) and the Total Kjeldahl Nitrogen method (TKN) by digestion, distillation, and titration, respectively. Electronic micrographs were taken using a Jeol JSM-820 scanning electronic microscope (SEM). The presence of microorganisms was observed by microscopy (LEICA DM4000 B) after 72 h of growth in hydrolysates at room temperature.

The carbohydrate content of the initial biomass and the solid fractions was determined as monosaccharides by using a NREL modified protocol based on an acid hydrolysis and liquid chromatography (HPLC) with an IR detector (Martín-Juárez et al., 2019). The monosaccharide content of the liquid fractions was analyzed by HPLC with an IR detector. External standards of glucose, xylose, arabinose, and cellobiose were used as multi-standard calibration solutions. To determine the starch content of the initial biomass, a polarimetric methodology was employed according to (Martín-Juárez et al., 2019). The lipid content of the initial biomass was analyzed using a modified Kochert extraction method (Kochert, 1978) using as a solvent chloroform-methanol. As usual for algal biomass grown in photobioreactors treating piggery wastewater with high nitrogen and microalgal species able to grow in stressing media (Hernández et al., 2018; Villaró et al., 2022; Zittelli et al., 2022), the initial composition of lipids resulted too low (6.7%) to consider its valorization (Rojo et al., 2021), and this component was not monitored through the studied processes. The protein content of the initial biomass was determined by applying a nitrogen-protein factor of 4.3, calculated from the aminoacid profile of the initial biomass obtained by HPLC (Rojo et al., 2021). The free amino acids in the liquid fractions were analyzed using the ninhydrin method according to a Sigma Aldrich protocol (Trigueros et al., 2021) and an aspartic acid solution was used a standard. The total amino acid profile of the initial biomass and of liquid and solid fractions after hydrolysis were analyzed by HPLC and UV detector according to internal analytical standards from the Instrumental Techniques Laboratory (LTI – UVa) described in (Rojo et al., 2021). A sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page) described in (Rojo et al., 2021) was employed to determine the peptide sizes in the liquid fractions in all the experiments.

2.5. Statistical analysis

Two-factor ANOVA was applied to chemical hydrolysis experiments, to study the effect of the temperature (3 levels: 120ºC, 60ºC and 40ºC) and the type of chemical (2 levels: NaOH and HCl). On the other hand, a second two-factor ANOVA was applied to the assisted enzymatic methods to study the effect of the treatment (non-assisted, ultrasound-assisted and microwave-assisted) and the enzymes (Protamex and Protamex with Celluclast). A LSD test was used to evaluate the statistically significant differences among the mean yields with a significance level of $\alpha = 0.05$. The results are expressed as means ± standard deviations of 4 analytical determinations (duplicated experiments analyzed in replicate). All the data were analyzed using Statgraphics Centurion XVIII software.

3. Results and discussion

3.1. Protein solubilization

All the mass balances calculated for the liquid and solid fractions showed losses of volatile solids lower than 5%. Comparing the N solubility obtained from the solid phase and its recovery in the liquid phase (all determined using the Total Kjeldahl Nitrogen Method), very low losses were calculated (between 0% and 3.3%) with almost complete N recovery after the process in all experiments. The amount of N supplied by the enzyme was considered negligible for these calculations, since the enzyme/substrate ratio is very low.

The protein solubilization yields achieved by the different extraction methods are shown in Fig. 2. As previously reported by (Martin Juárez et al., 2021) comparing different pretreatments (including alkaline and acid hydrolysis), alkaline hydrolysis at 120ºC for 1 h provided the highest protein solubilization from biomass grown in wastewater (up to 90%) while acid hydrolysis achieved the highest carbohydrate solubilization (up to 98%). The high protein extraction of alkaline hydrolysis has been related to disulfide cross-linking breakage (Du et al., 2020) and to disintegration of the cells by saponification of lipids in the membrane/cell wall (Nagarajan et al., 2020). On the other hand, the carbohydrate solubilization by acid treatment has been related to the hydrolysis of heterocyclic ether bonds between sugar monomers of the polymers presents in the cell wall by the protons released by the acid (Bonifácio-Lopes et al., 2019). Different to what happened in alkaline and other treatments, SEM images did not shown holes in the cell wall by acid treatment. In the alkaline hydrolysis, the increase in temperature improved protein solubilization from 36.1% at 40ºC to 54.1% at 60ºC and the maximum yield at 120ºC. The acid hydrolysis at 60ºC and 40ºC resulted in the lowest protein solubilizations, with values around 19%. This remarkable effect of temperature on protein solubilization has been previously reported by (Martín-Juárez et al., 2019) when they applied chemical hydrolysis with HCl and NaOH in a temperature range from 80ºC to 120ºC to microalgal biomasses cultivated in different media. (Lorenzo-Hernando et al., 2019) also noted the remarkable effect of temperature on protein solubilization from microalgal biomass composed mainly of *Scenedesmus almeriensis* and grown in piggery wastewater by alkaline hydrolysis.

The ANOVA analysis confirmed that the two operational parameters of chemical hydrolysis studied in this work – temperature and chemical reagent type – had a significant effect on the protein solubilization yield ($p < 0.05$). In agreement with the results reported by (Martín-Juárez et al., 2019), the effect of temperature (with a contribution of 80.5%) was higher than that of the chemical reagent (with a contribution of 15.8%). There were significant differences between the three temperatures (120, 60, and 40ºC) in the alkaline hydrolysis, and between 120ºC

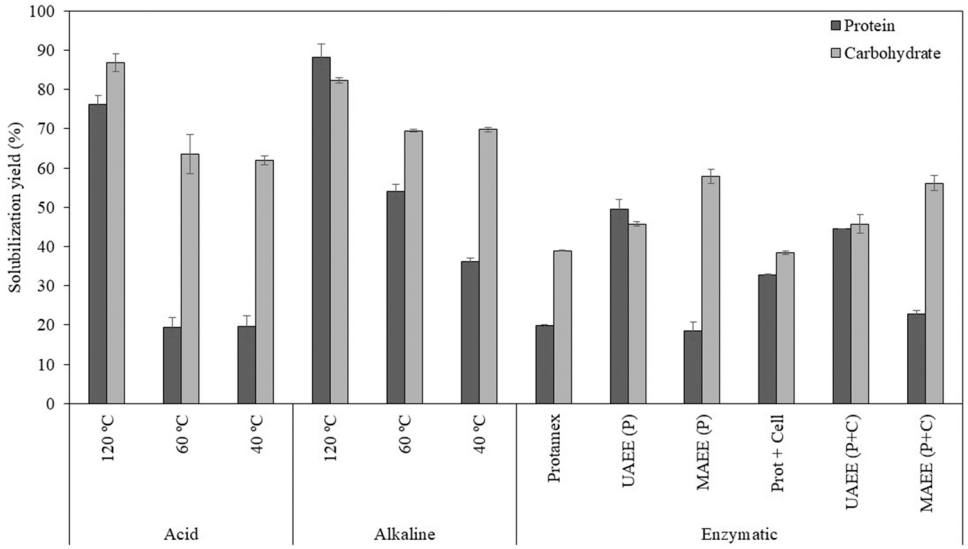

Fig. 2. Protein solubilization yield (%) in reference to the protein content in the initial microalgal biomass and carbohydrate solubilization yield (%) in reference to the carbohydrate content in the initial microalgal biomass. The data are provided as means ± standard deviations of 4 analytical determinations (duplicated assays analyzed in replicate). The standard deviation of the means is represented by vertical interval lines.

and the two lowest temperatures in the acid hydrolysis.

The enzymatic experiments provided low protein solubilizations, reaching a yield of 19.8% after 1 h with the Protamex enzyme alone and of 32.8% using the combination of Protamex and Celluclast 1.5 L. This demonstrated that the use of cellulase along with the protease enzyme improved the protein solubilization yield. The low protein solubilization yields from algal biomass by enzymatic hydrolysis have been previously reported. (Martin Juárez et al., 2021) carried out an enzymatic hydrolysis with Celluclast 1.5 L and Novozyme 188 to algal-bacterial biomass (*Scenedesmaceae*) grown in pig manure obtaining a protein solubilization of around 10%, while (Rojo et al., 2021) performed an enzymatic hydrolysis with Protamex to a similar biomass to our work, achieving 25% of protein solubilization. Also, (Sedighi et al., 2019) obtained low protein solubilization yields (20%) after treating pure *Spirulina platensis* cultures in synthetic media with serine proteases. These results with only enzymes were low and therefore, this work proposes the combination of physical and enzymatic methods. These low yields were enhanced using ultrasounds which provided protein solubilization yields of 35.1% in the control experiment without enzymes and 49.6% in the assisted enzymatic hydrolysis (UAEE) using the Protamex enzyme alone. SEM microscopy analysis also evidenced the effect of ultrasounds on microalgae, even showing some cell wall holes, which could facilitate the diffusion from the cell, explaining the increase on protein extraction. However, the effect of the ultrasounds assistance resulted lower than expected when working with the cocktail of enzymes, obtaining a protein solubilization yield of 44.5%. Comparing UAEE experiments, the cocktail of proteins resulted in slightly lower protein solubilization yield that the use of Protamex alone. It was possible that ultrasounds and cellulase enzyme promoted similar changes in microalgae cell wall structure and their effects were overlapping. In addition, the presence of Celluclast may have favored different degradation routes, generating by-products that can influence the hydrolysis process. To our knowledge, there are no previously published results about the combination of enzymatic hydrolysis with ultrasounds for the extraction of proteins from a consortium of bacteria and microalgae, although it has been employed for other types of biomasses like sludge (Yan et al., 2022), pure microalgae (Hildebrand et al., 2020) and seaweed (Le Guillard et al., 2016). (Le Guillard et al., 2016) applied UAEE to the red seaweed *Grateloupia turuturu* at 40ºC for 6 h with an enzymatic cocktail of four commercial carbohydrases (Sumizyme MC, Sumizyme TG, Ultraflo XL, and Multifect CX), obtaining a protein solubilization yield of 91% which was higher than the solubilization with only the cocktail enzyme (71%). The results of these works confirm that the application of ultrasounds during the enzymatic hydrolysis improves the efficiency of the process, being a promising method. Ultrasounds favored enzyme aided reactions through the increase in the mass transfer and the cell wall disruption (appreciated in the SEM images) attributed to the implosion of cavitation bubbles (Singla and Sit, 2021; Wang et al., 2021). Additional research is necessary to evaluate the effect of the different UAEE operational parameters (amplitude, time, enzyme and /or biomass concentration…) in the recovery yields, the properties of the recovered compounds and the economy of the process.

On the other hand, microwaves surprisingly did not improve the efficiency of the protein solubilization, being not statistically significant the differences between MAEE with Protamex and the enzymatic control assay (≈ 19%) according to the LSD test, although the enzyme-free control with microwaves alone solubilized even less protein (15.3%). SEM analysis showed loss of turgor in the microalga cell wall after applying MAEE, but no rupture was observed. The lower solubilization yield by MAEE with Protamex + Celluclast 1.5 L (1 h) is noteworthy, since only 22.9% of the initial proteins were solubilized when microwaves and enzymatic hydrolysis were combined, while 32.8% were solubilized in the enzymatic control assay with the enzyme cocktail (3 h). The microwave assistance was not able to compensate the effect of the shorter enzymatic hydrolysis time in the case of protein solubilization. As shown in Fig. 2, microwaves favored carbohydrate solubilization but not proteins, may be due to the type of changes in the cell wall caused by microwaves, since this physical method withdraw the acetyl groups of hemicellulose and thus, the reaction of the substrate with the enzymes was enhanced for carbohydrates. Likewise, experiments with only microwaves achieved a protein solubilization of only 15.3%, lower than the yield obtained with ultrasounds alone (35.1%), which indicated that ultrasounds were more adequate to extract proteins from cells. As previously stated, to the best of our knowledge, no research has been published about the use of enzymes in combination with microwaves for the extraction of proteins from microalgae biomass, although it has been employed in other types of biomasses. (Görgüç et al., 2020) carried out MAEE experiments with sesame bran, and they obtained a maximum protein solubilization yield of 91.7% by combining microwaves with the Alcalase enzyme (49ºC, 98 min, and 1.94 AU/100 g). This yield was higher than for the experiment with only microwaves, where they achieved a protein solubilization yield of 62.3%. Thus, they achieved

very high solubilization yields by applying microwave-assisted enzymatic hydrolysis, contrary to the results of our work, possibly due to using a different biomass and enzyme.

The ANOVA analysis showed that both studied individual operational parameters of assisted enzymatic hydrolysis had a significant effect on the protein solubilization ($p < 0.05$), with the type of treatment being the most important with a contribution of 87%. The LSD Test confirmed that there were significant differences between the three types of enzymatic extraction methods (only enzymatic hydrolysis, UAEE, and MAEE).

Despite the increase of protein solubilization yields by applying ultrasounds during enzymatic hydrolysis, these results were lower than those obtained with chemical treatments, so further research and optimization of UAEE processes is still needed to improve solubilization.

3.2. Carbohydrate solubilization

The highest carbohydrate solubilizations were achieved in both chemical treatments at 120ºC, obtaining similar yields (86.8% for acid hydrolysis and 82.3% for alkaline hydrolysis) as shown in Fig. 2. These yields decreased to 63.6% (acid hydrolysis) and 69.5% (alkaline hydrolysis) as the temperature was reduced to 60ºC, although as the temperature dropped further to 40 °C, the solubilization yields did not vary significantly. In contrast to the proteins, the effect of temperature is lower on the solubilization of carbohydrates since moderate yields are achieved at both low temperatures. The increase on carbohydrate solubilization when the temperature is raised above 100ºC has been reported for different biomasses. Hydrogen bonds are more stable and harder to break at low than at high temperatures (Salakkam et al., 2021). The solubilization of the glucose and xylose (the most abundant monosaccharides) were determined individually, as shown in Fig. 3, to analyze the results in more detail.

The glucose solubilization yields were higher in acid hydrolysis assays (on average 69.9% vs 55.8% obtained in alkaline hydrolysis). Furthermore, in these experiments, the same amount of xylose was solubilized as glucose. This result is similar to those obtained by (Martin Juárez et al., 2021) who treated a fresh algal-bacterial biomass grown in diluted pig manure at 120ºC. On the other hand, alkaline hydrolysis preferentially solubilized xylose (on average 90.6% vs 70.1% in acid hydrolysis). Probably, the higher xylose solubilization by NaOH was due to the saponification of intermolecular ester bonds linking the xylan of the hemicellulose (Amezcua-Allieri et al., 2017; Kucharska et al., 2018). The behavior with temperature was similar for both monosaccharides since glucose and xylose solubilization increased with temperature, obtaining the highest value at 120ºC (83.9% and 69.3% of glucose solubilization yields with acid and alkaline hydrolysis and 88.7% and 94.4% of xylose solubility with acid and alkaline hydrolysis, respectively). Also, between 60 and 40ºC there were negligible differences in glucose and xylose solubilization yields for both chemical treatments.

Enzymatic control treatments achieved lower carbohydrate solubilizations than by chemical methods, reaching final carbohydrate solubilization yields after 1 h of 38.9% with Protamex enzyme alone and 38.4% using the combination of Protamex and Celluclast 1.5 L. These results are surprising, since cellulases catalyze the hydrolysis of cellulose (Rojo et al., 2021) and the increase of carbohydrates, and especially glucose solubilization by adding cellulases was expected. (Rojo et al., 2021) achieved a carbohydrate solubilization yields of 33% after 1 h of enzymatic hydrolysis with only Celluclast 1.5 L. Probably, the addition of Celluclast in this work did not increase carbohydrate solubilization since the protease enzyme already provided a higher carbohydrate extraction (38.9%) than that reported previously with cellulase alone. The presence of Celluclast enzyme also could promote other types of reactions (denoted by the appearance of a new peak in hydrolysates) that would have reduced the cellulase activity for carbohydrate hydrolysis and extraction. Similar carbohydrate solubilization yields (≈39%) were obtained in control microwave experiment without enzymes, being higher than the carbohydrate solubilization yield achieved with only ultrasounds (33.7%).

These low yields were improved by combining enzymatic hydrolysis and microwaves (MAEE), obtaining a final solubilization yield of 57.9% with Protamex and 56.2% with the Protamex and Celluclast 1.5 L cocktail. Xylose was preferentially solubilized by MAEE with yields around 73% for the MAEE experiments with both types of enzymes, a higher value than in the control enzymatic experiments (≈43%). Previous works with lignocellulosic materials have reported that microwaves degrade hemicellulose which is composed mainly of xylose (Norazlina et al., 2022; Ríos-González et al., 2021), an important component in the cell wall of microalgae. Therefore, the higher carbohydrate extraction would be related to the ability of microwave to promote the withdraw of the acetyl groups of hemicellulose (xylose solubilization) (López-Linares et al., 2020). This microwave effect is also observed comparing the carbohydrate solubilization of individual

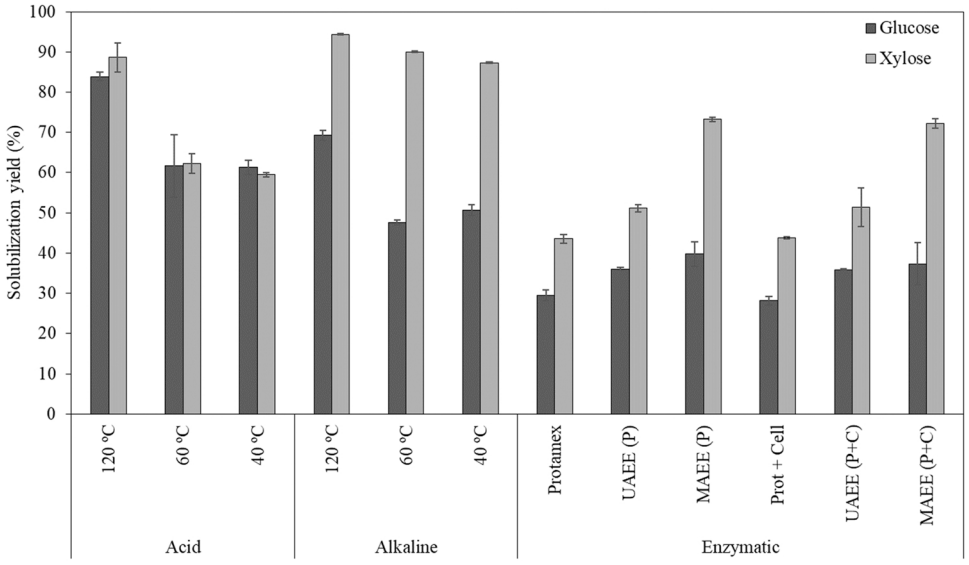

Fig. 3. Glucose solubilization yield (%) in reference to the glucose content in the initial microalgal biomass and xylose solubilization yield (%) in reference to the xylose content in the initial microalgal biomass. The data are provided as means ± standard deviations of 4 analytical determinations (duplicated assays analyzed in replicate). The standard deviation of the means is represented by vertical interval lines.

microwave and ultrasounds control treatments. On the other hand, ultrasounds (UAEE) also enhanced carbohydrate solubilization, although not as much as the microwaves. The ANOVA analysis clearly indicated that the type of method used for the assisted enzymatic hydrolysis affected carbohydrate solubilization ($p < 0.05$). The LSD Test confirmed that there were significant differences between the three types of extraction methods (only enzymatic hydrolysis, UAEE, and MAEE). (Ríos-González et al., 2021) also obtained better xylose yields by applying microwaves (77.5%) instead of ultrasounds (28.2%) to enhance enzymatic hydrolysis of agave with the enzymatic complex Cellic CTec3.

The efficiency improvement by microwaves and ultrasounds could be due to actions of physical forces by irradiation and sonication, which produced the disruptions of cell walls components and led to a greater contact area between the solid and liquid phase (Cheng et al., 2015).

3.3. Peptide and monosaccharide recovery

The amino acid profiles for initial biomass and hydrolysates are displayed in Table 1. Regarding the profile of the initial microalgal biomass, the major amino acid was glutamic acid (13.9%), followed by aspartic acid (10.6%) and alanine (9.7%). According to (Trigueros et al., 2021), the main amino acids for many algae are aspartic and glutamic acids, which is in accordance with the results obtained for our biomass. After hydrolysis treatments, all amino acids were found in the liquid fraction, highlighting the high percentage of essential amino acids in the enzymatic hydrolysates (39.5% with Protamex and 38.4% with Protamex and Celluclast), which could make them suitable for animal feed. In the acid hydrolysis assays, there were notable percentages of glutamic acid and alanine which were preferably recovered at low temperature in this solvent, while the recovery of aspartic acid and leucine increased significantly with temperature. In the alkaline hydrolysis assays, there were remarkable solubilizations of cysteine, glycine, and alanine, recovering arginine and serine at low temperatures. For both chemical treatments, the percentage of essential amino acids increased with temperature achieving a value around 31% at 120ºC. The decrease of the nitrogen-protein factor from 4.3 in the raw biomass to 1.6 in the hydrolysates of the acid treatments at low temperatures (40 and 60ºC) stands out, which indicates losses and/or degradation of proteins into other compounds like ammonium, imides, or amides (Planyavsky et al., 2015) while in the rest of experiments, it did not decrease as much, always remaining above 3.2. In the UAEE experiments, the high amount of aspartic acid, alanine, and glutamic acid found was noteworthy, while MAEE recovered alanine and glutamic acid. There were no significant differences between the use of Protamex alone and the enzyme cocktail. The best results for essential amino acids content were provided by the enzymatic control treatments (~39%) and ultrasonic-assisted assays (33–35%), being these hydrolysates useful for feed.

The mass of recovered peptides and the protein losses were calculated from the amino acid profiles (Fig. 4). The protein losses were dependent on the type of hydrolysis performed. Alkaline experiments, with the highest protein solubilization yields, resulted in protein losses of ~8% of the proteins in the initial biomass, with no significant decrease with temperature. However, the losses of the acid experiments were a bit higher than in the alkaline (~14% of proteins in the initial biomass) and again without significant influences of temperature. Although a high percentage of the solubilized protein were degraded in acid hydrolysis at mild temperature (71% and 66% at 40ºC and 60ºC respectively), the low recovery of peptides in this type of treatment is due to the low solubilization of proteins. These results were different from those expected, since as the hydrolysis temperature decreased, degradation should also have decreased while recovery have increased, but that did not actually occur. Thus, the highest peptide recovery was obtained with alkaline hydrolysis at 120ºC as shown in Fig. 4, where a protein recovery yield of 81% was achieved. This was a much higher value than at other temperatures (46.1% for 60ºC and 26.8% for 40ºC). By contrast, acid hydrolysis provided very low peptide recoveries at 40ºC and 60ºC (about 6%), while at 120ºC a high recovery of 61.5% was achieved. Therefore, the recovery of peptides showed the same behavior as protein solubilization which increased with temperature.

In the enzymatic control experiments, higher protein losses were obtained with the Protamex and Celluclast 1.5 L cocktail (13.8%) than for Protamex alone (0.2%), indicating that the use of both enzymes led to higher protein losses. The UAEE and MAEE experiments provided average protein losses of around 4.5%. However, remarkable differences were found for UAEE depending on the enzymes. UAEE protein losses achieved 10.4% when used with Protamex alone (where microorganisms were observed by microscopy, and so metabolic activity was expected), while these protein losses were only 0.9% with the enzymes cocktail. Therefore, the higher peptide recovery yields of enzymatic experiments were obtained with UAEE (39.2% using Protamex alone and 43.6% using Protamex with Celluclast 1.5 L), confirming that the combination of ultrasounds and enzymes improved the extraction of peptides. No published research studying the effect of ultrasounds or microwaves with enzymes on the recovery of peptides from algal biomass has been found. However, some authors, such as (Ardiles et al., 2020), concluded that ultrasounds were effective in the recovery of phycoerythrin, a pigment present in red microalgae *Porphyridium,* with water or phosphate buffer as solvent, while microwaves did not improve the pigment recovery yield.

Table 1
Amino acid profile (%) of initial microalgal biomass and liquid fractions after extraction treatment (referred to the mass of total amino acids).

	Initial biomass	HCl 120ºC	HCl 60ºC	HCl 40ºC	NaOH 120ºC	NaOH 60ºC	NaOH 40ºC	Protamex	UAEE (P)	MAEE (P)	Protamex + Celluclast	UAEE (P + C)	MAEE (P + C)
Aspartic acid	10.57	13.74	8.21	8.26	13.56	14.18	13.73	11.94	13.29	11.45	12.43	12.78	12.34
Glutamic acid	13.95	14.67	28.36	30.58	16.22	16.34	17.29	13.26	14.53	17.17	12.71	13.11	18.37
Serine	5.67	5.97	4.48	4.13	1.76	4.98	5.42	4.77	5.59	5.12	5.08	5.56	5.77
Histidine*	2.15	0.19	0.75	0.83	0.54	0.65	0.34	1.86	1.74	1.20	5.93	2.00	1.05
Glycine	6.60	7.83	5.22	4.95	10.23	8.98	9.15	8.22	7.33	6.63	7.91	7.33	6.56
Threonine*	4.70	0.50	3.73	3.31	0.24	2.59	3.22	6.37	5.84	5.12	5.93	3.67	5.25
Arginine	6.45	1.06	8.96	9.09	0.24	2.59	1.69	3.71	4.84	6.33	3.67	5.56	6.56
Alanine	9.70	10.81	17.91	19.83	12.83	11.80	13.73	7.43	10.93	12.95	8.19	9.56	13.12
Tyrosine	3.71	4.79	1.49	0.83	4.96	4.22	3.73	4.24	4.10	3.01	3.95	4.11	3.15
Cysteine	0.88	4.23	4.48	3.31	5.15	5.84	5.59	0.80	0.50	0.60	0.85	0.67	0.52
Valine*	4.10	5.09	2.24	1.65	5.27	4.55	4.41	5.31	3.85	3.31	4.52	4.11	3.41
Methionine*	2.22	2.49	0.00	0.00	2.42	2.16	2.20	2.39	2.11	1.51	1.98	2.44	1.31
Phenylalanine*	5.22	5.28	1.49	1.65	5.75	4.76	4.58	4.77	4.22	3.31	4.24	4.89	3.41
Isoleucine*	3.10	3.05	1.49	1.65	1.27	2.81	2.71	2.65	2.36	5.12	1.41	2.78	1.84
Leucine*	8.92	9.14	2.99	2.48	9.93	8.11	7.12	8.22	5.71	6.33	7.91	8.89	6.56
Lysine*	6.39	6.09	4.48	4.13	5.33	3.89	3.56	7.96	7.45	6.02	6.50	6.56	6.04
Proline	5.66	5.09	3.73	3.31	4.29	1.62	1.53	6.10	5.59	4.82	6.78	6.00	4.72
Essential AA*	32.70	31.82	17.16	15.70	30.75	29.44	28.14	39.52	33.29	31.93	38.42	35.33	28.87

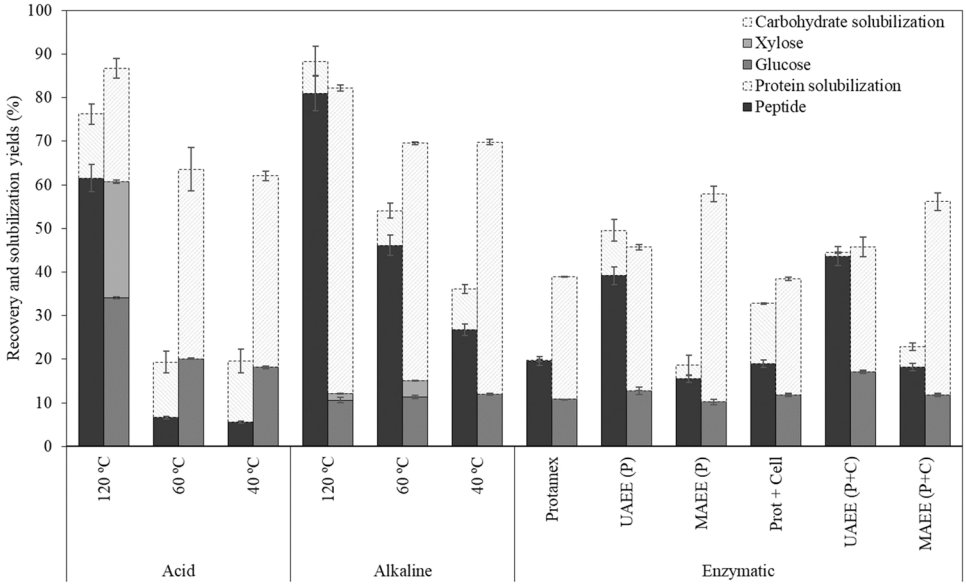

Fig. 4. Peptide recovery yield (%) in reference to the protein content in the initial microalgal biomass, glucose recovery yield (%) and xylose recovery yield (%) in reference to the carbohydrate content in the initial microalgal biomass. Discontinuous bars show solubilization yields (%) of proteins and carbohydrates in reference to the initial content. The data are provided as means ± standard deviations of 4 analytical determinations (duplicated assays analyzed in replicate). The standard deviation of the means is represented by vertical interval lines.

Regarding the monosaccharide recovery, higher monosaccharide recovery yields were obtained by acid hydrolysis than by alkaline hydrolysis at the three temperatures studied. Acid hydrolysis at 120ºC could be used to recover monosaccharides useful to produce bioproducts such as polyhydroxyalkanoates (PHA) by fermentative processes (recently the research group has demonstrated the PHA production capacity of *Paraccocus denitrificans* in this type of hydrolysate). Glucose losses were moderate in both chemical treatments (Fig. 4), ranging from 14.6% at 120ºC to 24.4% at 40ºC for acid hydrolysis and from 24.4% at 40ºC to 33.7% at 120ºC for alkaline hydrolysis. The low losses in the acid hydrolysis at 120ºC, despite the severity of the operation conditions could be attributed to the sterilization effect of this pretreatment. No presence of microorganisms in this hydrolysate was observed under the microscope, which can be related to low metabolic degradation. (Martin Juárez et al., 2021) analyzed the bacterial viability in hydrolysates of algal biomass grown in piggery wastewater after HCl treatment at 120ºC by DNA extraction. They found a massive bacterial disintegration (no bacterial DNA detected), resulting in complete sterilization. Although it was expected that losses would be low at mild temperature, favoring the recovery of macrocompounds, the application of high temperature achieved better recoveries, decreasing the losses which could be attributed to the sterilization effect of this pretreatment. In the two types of treatment, the highest glucose recovery yield of 69.3% for acid hydrolysis and 35.6% for alkaline hydrolysis was achieved at the highest temperature of 120ºC. On the other hand, xylose losses were high and decreased with temperature. The xylose losses ranged from 31.7% at 120ºC to around 60% at 60ºC and 40ºC in acid hydrolysis, and from 61.3% at 120ºC to 87.3% at 40ºC in alkaline hydrolysis. As a result, the xylose recovery was very low or negligible in both chemical methods at 40ºC and 60ºC and the highest xylose recovery was achieved with acid hydrolysis at 120ºC (56.9%). This remarkable transformation of xylose into other organic forms and degradation by-products, mainly organic acids (Martin Juárez et al., 2021), was observed as small peaks in the chromatograms. The LSD Test confirmed that there were significant differences between acid and alkaline hydrolysis, as well as between 120ºC and the other two lower temperatures.

In the enzymatic control treatments, low glucose losses were found, resulting in losses of 1.9% with the enzyme cocktail and 4.1% with Protamex. The glucose losses were higher in assisted enzymatic hydrolysis experiments, especially in MAEE, where losses reached up to 19.1% with Protamex alone and 13.3% with the enzymatic cocktail. As a result, glucose recoveries were low, ranging from 20.7% to 25% in all the enzymatic treatments, except in the UAEE with the cocktail where the highest value was reached (34.9%). No recovery of xylose was found in any enzymatic treatment (control assays, UAEE, and MAEE) which implied total degradation or transformation of this monosaccharide. In this sense, a new peak was found in hydrolysates of the enzyme cocktail experiments (enzyme control, UAEE and MAEE), which could be attributed to xylulose from xylose isomerization (Kim and Zhang, 2016; Lindén and Hahn-Hägerdal, 1989). Therefore, it is possible that a xylose isomerase enzyme was present in our experiments with Protamex and Celluclast that resulted in different reactions and the production of xylulose from xylose during hydrolysis.

To author's knowledge, no research was published about monosaccharide recovery from microalgal biomass using assisted enzymatic hydrolysis, although it was applied to other types of biomasses to obtain bioactive carbohydrates. (Chen et al., 2014) optimized the extraction of polysaccharides from corn silk by UAEE, obtaining a maximum monosaccharide recovery of only 7.1% (cellulase concentration of 7.5%, 150 min at 55 °C and a liquid–solid ratio of 31.8).

3.4. Peptide purity and sizes

The peptide purities were calculated as the ratio between the peptide concentration (determined from the amino acid profile) and the volatile solids concentration in the liquid phases. The highest peptide purities were provided by the UAEE assays (46.7% with Protamex alone and 46.0% with the cocktail of Protamex and Celluclast 1.5 L). Lower values were obtained from the MAEE assays (31.3% with Protamex alone and 33.9% with Protamex and Celluclast 1.5 L) and the control experiments with only enzymes (32.6% for Protamex and 30.2% for the Protamex and Celluclast 1.5 L cocktail). On the other hand, for the chemical treatments, the purity of the recovered peptides increased with temperature, from 15.7% at 40ºC to 37.2% at 120ºC for alkaline hydrolysis and from 12.4% at 40ºC to 42.9% at 120ºC for acid hydrolysis, which means that temperature favored the recovery of peptides over other organic compounds of the microalgal biomass.

The sizes of the recovered peptides in the hydrolysates were obtained using SDS-page, which revealed remarkable differences between assays. The control experiments of enzymatic hydrolysis resulted in peptides with molecular sizes of around 11 kDa, 63 kDa, 75 kDa, and 135 kDa, with Protamex alone or the Protamex and Celluclast 1.5 L cocktail. The enzymatic hydrolysis as a single process did not completely hydrolyze the proteins and they remained as long chain peptides in the liquid

phase. UAEE also provided large peptides of various sizes (≈11 kDa, 63 kDa, and 135 kDa for assays with Protamex, and ≈11 kDa,48 kDa, 63 kDa, 100 kDa, and 135 kDa for assays with Protamex and Celluclast 1.5 L), so ultrasounds did not break the proteins and allowed for similar sizes to be obtained like in enzymatic control experiments. In the case of MAEE and alkaline hydrolysis, both processes degraded the solubilized proteins into small peptides or even amino acids with a size smaller than 11 kDa. No bands were detected in the gel electrophoresis of acid hydrolysates.

A ninhydrin analysis was used to determine the presence of free amino acids in the liquid fractions of all the experiments. From this, positive results were obtained for the control enzymatic hydrolysis, UAEE, and MAEE experiments. However, negative results were obtained for acid and alkaline hydrolysates, despite the amino acid profiles provided by the HPLC analysis, which indicated the presence of amino acids in these hydrolysates, and the electrophoresis protein profile of alkaline hydrolysates, where small-sized peptide bands (<11 kDa) were found. The ninhydrin method could be affected by interferences, including cations like Fe^{3+}, Cu^{2+} (Moore et al., 2010), which are present in piggery wastewater. Haven and Jørgensen (2014) also reported interferences by monosaccharides and dark color of complex samples from chemical hydrolysis (like our acid and alkaline hydrolysates) in this analysis. Therefore, this method resulted inefficient for the detection of free amino acids in these types of samples from a chemical hydrolysis treatment.

4. Conclusions

The study compares new extraction methods applied for the first time to biomass grown in wastewater treatment plants with chemical pre-treatments at different temperatures, with the aim of recover peptides and monosaccharides from a consortium of microalgae and bacteria grown in a piggery wastewater treatment photobioreactor. The highest protein recovery yield (81%) was achieved by alkaline hydrolysis at 120ºC (with remarkable effect of temperature). Chemical hydrolysis produced small peptide sizes in all the temperature range (40–120ºC) but percentages of essential amino acids ≈ 31% at 120ºC. Decreasing the temperature of chemical hydrolysis did not reduce the losses of solubilized components, resulting in lower recovery yields. The ultrasound-assisted enzymatic hydrolysis provided moderated peptide recoveries (39–44%), but the best results in terms of purity (46–47%) and size (up to 135 kDa) of the peptides. Cavitation produced by ultrasounds enhanced cellular disruption, improving the solubilization yield of the enzymatic hydrolysis with low protein losses. Regarding carbohydrate fraction, acid hydrolysis at 120ºC achieved the highest recoveries of monosaccharides, 69% of glucose and 57% of xylose, with low losses at this temperature that could be related to the sterilizing effect of this treatment. Microwaves assistance enhanced carbohydrate solubilization by the enzymatic hydrolysis, especially in the case of xylose (>70%), which could be attributed to the previously reported ability of microwaves to disrupt the acetyl groups of hemicellulose. Therefore, it is possible to recover proteins and carbohydrates from biomass grown in wastewater treatment photobioreactors and the selection of the extraction treatment will depend on the application and characteristics of the final product desired. Further research is necessary to optimize operational parameters of the UAEE.

Authorship contribution

Elena M. Rojo: Experimental work, Results analysis, drafting and Writing – original draft. **Angel A. Filipigh**: Experimental work, Results analysis, and Investigation. **Silvia Bolado**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the "Ministerio de Ciencia, Innovación y Universidades" of Spain (PID2020–113544RB-I00 y PDC2021–121861-C22). The authors also thank the regional Government of Castilla y León (UIC 338, CLU 2017–09, CL-EI-2021-07) for the financial support of this work. E. Rojo and A. Filipigh would like to thank the "Ministerio de Ciencia, Innovación y Universidades" for their doctorate scholarship (PRE2018-083845 and PRE2021-100176).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psep.2023.03.067.

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