Recovery of proteins from biomass grown in pig manure microalgae-based treatment plants by alkaline hydrolysis and acidic precipitation.

Ana Lorenzo Hernando[a, c], Javier Ruiz Vegas[a], Marisol Vega Alegre[b, c], Silvia Bolado Rodríguez[a, c *]

[a] Department of Chemical Engineering and Environmental Technology, University of Valladolid, Calle Doctor Mergelina s/n, 47011, Valladolid, Spain

[b] Department of Analytical Chemistry. Campus Miguel Delibes, 8. Paseo Belén 7, 47011, Valladolid, Spain.

[c] Institute of Sustainable Processes. University of Valladolid, 47011, Valladolid, Spain

[*] **Corresponding author**: silvia@iq.uva.es

Abstract

The influence of three variables on key parameters of the protein extraction process (an alkaline hydrolysis followed by an acidic precipitation) for biomass from innovative photo-bioreactors for pig manure treatment was evaluated. Alkaline hydrolysis provided high solubilisation values (up to 66.5% of the biomass), augmenting with increasing values of the three studied variables (NaOH concentration, temperature and time). Nevertheless, moderate total (13.2%) and protein extraction yields (16.9%) were obtained, which was attributable to protein denaturation or to the low effectivity of the precipitation method. Extracts rich in proteins (53.5% – 77.9%) with suitable amino acid profiles were obtained, but significant amounts of the initial lipids (up to 44.6%) were co-extracted probably due to fatty acids saponification. These results establish the first step for future studies in enhancing cell wall disruption and protein recovery by coupling alkaline hydrolysis with other mechanical pre-treatments, while considering alternative separation and purification methods.

Highlights

- Protein recovery by pH-shift from a consortium microalgae-bacteria biomass.
- ANOVA of NaOH concentration, temperature and time effect on process parameters.
- Intermediate alkaline concentration (0.5M NaOH) provided maxima extraction yields.
- High solubilisation by alkaline hydrolysis but low acidic precipitation efficiency.
- Co-extraction of lipids increased with process intensity.

Keywords

Extraction, Lipids, pH-shift, *Scenedesmus almeriensis*, Solubilisation, Wastewater

1. Introduction

Consortia of microalgae and bacteria have been recently used in innovative wastewater and pig manure treatment plants to enhance removal efficiency and reduce energy requirements compared to conventional processes. Since microorganisms use the pollutants contained in the wastewaters to grow, these processes generate high amounts of biomass. Specifically, microalgae metabolise nutrients present in the wastewaters into proteins, carbohydrates and lipids through photosynthesis. This process provides a mixed microalgae-bacteria biomass rich in microalgae, with a macromolecular composition similar to that of any other microalgae biomass obtained in sterilized media (Acién et al., 2016). This biomass produced by wastewater remediation could be a very cheap and valuable source of a multitude of products by applying the current concepts of circular economy or biorefinery (Chew et al., 2017; Molinuevo-Salces et al., 2015; Vanthoor-Koopmans et al., 2013). From the three main macromolecular components (proteins, lipids and carbohydrates) of the biomass, protein fraction is usually the richest (up to 70% of the dry weight of the biomass (Becker, 2007)), but also the most sensitive to harsh operation conditions such as low pH or high temperatures. Thus, to preserve their properties as much as possible, protein extraction would be one of the first steps to be considered in a biorefinery approach. Since the culture media used for biomass production is wastewater, proteins obtained through these processes cannot be used for human consumption, but they are perfectly suitable for other commercial applications such as animal feed, bio-fertilizers, as pigments source like phycobiliproteins, and foaming or emulsifying agents (Benelhadj et al., 2016; Bleakley and Hayes, 2017; Suganya et al., 2016).

An important factor when using microalgae-rich biomass as raw material is the

presence of a rigid cell wall protecting the intracellular content and acting as a barrier against pathogens and severe environmental conditions (Phong et al., 2018b). This cell wall could be especially recalcitrant in microalgae species able to grow in stressing environments and used for wastewater remediation, such as *Scenedesmus sp.* or other coccoid green algae (Dunker and Wilhelm, 2018; Scholz et al., 2014; Voigt et al., 2014), given that it provides tolerance to high organic loads and physiological stresses (Gupta et al., 2016). In this regard, several mechanical treatments, most of them under alkaline conditions, have been tested. In the great majority of cases, tests were done for particular microalgae species grown in sterilized synthetic media, and for pre-defined "optimal" conditions, which does not allow for an understanding of the influence of the operation parameters on the protein extraction process. One of the few published studies that includes the effect of operation parameters was performed by (Gerde et al., 2013), who studied the effect of temperature, time, pH and defatting pre-treatment when evaluating a protein extraction method from *Nannochloropsis* sp. biomass combining ultrasonication and pH-shift. They found a maximum recovery of around 15% from a 1% suspension of sonicated non-defatted biomass after extraction at 60º C, pH 11 for 5h. (Ursu et al., 2014) used a high-pressure cell disrupter instead of ultrasonication for enhancing alkaline protein extraction from *Chlorella vulgaris* biomass, achieving 98% of protein solubilisation after 2 x 2.7 kbar at pH 12. They also reported a minimum solubilization when using only chemical treatment (2.3% at pH 12). In contrast, the work by (Safi et al., 2014) showed remarkable protein release (around 25%) from *C. vulgaris* and *N. oculata* biomass when using only alkaline treatment (pH 12, 2h and 40º C, followed by acid precipitation). (Cavonius et al., 2015) applied bead milling and pH-shift processing for *N. oculata* biomass, obtaining a maximum protein yield of 86%, and

extracts containing 23%, 12% and 42% of protein, lipid and carbohydrate content, respectively. (Teuling et al., 2017) systematically compared composition (proximate, amino acid and fatty acid compositions) and solubility of protein extracts from four pure microalgae species, being one of them *Scenedesmus dimorphus*, using a delicate isolation process based on bead milling as in the previous example but using a potassium phosphate buffer to bring the solution under alkaline conditions (pH 8.0). They obtained the lowest protein yield (17%) for *S. dimorphus* biomass which was attributed to its highly recalcitrant cell wall. Recently, (Ansari et al., 2018) used in their work biomass obtained from the cultivation of an isolated strain of *Scenedesmus obliquus* in municipal wastewater as culture medium, but they mainly focused on lipid extraction.

Alkaline hydrolysis conditions are crucial to obtain high yields and quality of extracts because they are responsible for an efficient protein solubilisation. Cell wall configuration, composition and proteins nature could show a great variability depending on the environmental growth conditions of the biomass (Phong et al., 2018b). Therefore, deductions for microalgae-bacteria mixtures cultured in wastewaters cannot be directly inferred from results for pure species, and a preliminary study should be done to optimize this process in terms of chemicals, energy and time prior to any coupling with other mechanical pre-treatments. In this work, a pH-shift process for biomass grown in photo-bioreactors for pig manure treatment has been studied. The protein extraction process was adapted from (Gerde et al., 2013).

The influence of three operational parameters (alkali concentration, temperature and time) on the total extraction yield and the protein extraction yield, solubilisation and protein-to-lipid ratio has been evaluated through a multi-factorial ANOVA. Extracts

quality was evaluated considering the amino acid profile of extracts as well as their proximate composition. The distribution of the organic nitrogen among the different final phases has also been studied.

2. Materials and methods

2.1. Raw Biomass

Freeze-dried biomass used in this work was kindly provided by University of Almería (Spain) and stored at 4º C until use. This biomass was recovered from a photo-bioreactor used for pig manure wastewater remediation during September-October 2016, freeze-dried and stored at 4º C until further use. It was mainly composed of the microalgae *Scenedesmus almeriensis.* The macromolecular composition of this biomass was determined as 46.7 ± 0.8% proteins, 43.5 ± 4.3% carbohydrates, and 9.4% ± 0.3% lipids in dry-weight ash-free basis. Ash content of the original raw material was 15.6 ± 0.28% in dry-weight basis.

2.2. pH-shift process: Alkaline hydrolysis and acidic precipitation

Figure 1 shows a diagram of the process. Biomass suspensions were prepared at 5% w/w by adding 5M NaOH and distilled water up to the required alkali concentration. Alkaline hydrolysis assays were conducted in an orbital shaker at 200 rpm at the corresponding temperature for each run. Once the reaction time was completed, hydrolysates were centrifuged at 18500xg for 15 minutes at 4º C. Solids recovered from this first centrifugation, denoted as “spent biomass”, were dried at 40º C, its weigh recorded and stored at 4º C for further analysis (evaluated properties were total solids content (TS, %), ash content (%) and – for some selected experiments – the organic nitrogen content). Supernatants from this centrifugation step – containing solubilised proteins – were pooled and proteins were recovered by pH adjustment, inducing

precipitation by adding 2M HCl until reaching pH 2.5. Acidified suspension was then centrifuged (18500xg for 15 minutes at 4º C). The liquid phase obtained after this second centrifugation step (called acid supernatant) was recovered for some selected runs and kept separately for further analysis of organic nitrogen content. The solid phase (the so-called extract) was dried at 40º C, its weigh recorded and stored at 4º C for further analysis: TS content, ash content and proximate composition (protein, carbohydrate and lipid content). The amino acid profile was also evaluated for some selected extracts (Cavonius et al., 2015; Gerde et al., 2013).

The total extraction yield, as well as the protein, lipid and carbohydrate extraction yields, the solubilisation yield and the protein-to-lipid ratio of the extracts, were calculated as expressed in the following equations. Since the ash content was expected to be considerably high due to the combined use of NaOH and HCl during the different steps of the extraction process, all the calculations were made in a dry-weight ash-free basis.

$$Y_T\ (\%) = m_{extract}/m^0 \times 100 \quad \text{(Eq. 1)}$$

$$Y_P\ (\%) = ((m_{extract} \times P_{extract}(\%))/(m^0 \times P^0\ (\%))) \times 100 \quad \text{(Eq. 2)}$$

$$Y_L\ (\%) = ((m_{extract} \times L_{extract}\ (\%))/(m^0 \times L^0\ (\%))) \times 100 \quad \text{(Eq. 3)}$$

$$Y_{CH}\ (\%) = ((m_{extract} \times CH_{extract}\ (\%))/(m^0 \times CH^0\ (\%))) \times 100 \quad \text{(Eq. 4)}$$

$$S\ (\%) = 100 - (m_{spent}/m^0 \times 100) \quad \text{(Eq. 5)}$$

$$P\text{-to-}L_{ratio}\ (\text{dim.-less}) = P_{extract}/L_{extract} \quad \text{(Eq. 6)}$$

Where Y_T (%) is the total extraction yield, $m_{extract}$ is the dry-basis ash-free mass of extract, m^0 is the dry-basis ash-free initial biomass, Y_P (%) is the protein extraction yield, $P_{extract}$ (%) is the protein content of the extract, P^0 (%) is the protein content of the raw biomass, Y_L (%) is the lipid extraction yield, $L_{extract}$ (%) is the lipid content of the

extract, L^0 (%) is the lipid content of the raw biomass, Y_{CH} (%) is the carbohydrate extraction yield, $CH_{extract}$ (%) is the carbohydrate content of the extract, CH^0 (%) is the carbohydrate content of the raw biomass, S (%) is the solubilisation yield, m_{spent} is the dry-basis ash-free amount of the spent biomass recovered after alkaline hydrolysis, and P-to-L_{ratio} is the mass protein-to-lipid ratio of the extracts.

The effect of the three process parameters (alkali concentration – B (mol L^{-1}) –, temperature – T (º C) –, and time – t (h) –) and the interactions between them were evaluated at three levels (low, medium and high which were numbered as 0, 1, 2). Parameters levels were selected according to previous works (Gerde et al., 2013; Safi et al., 2014), introducing the most common values for each parameter as the intermediate level magnitude and adding rational minimum and maximum values according to them. It has been considered as well operational restrictions; e.g. a maximum temperature of 55º C had to be defined to avoid protein denaturation (Bischof and He, 2005), or 5h as hydrolysis time was selected since longer times could not be industrially suitable.

Parameter levels were hence defined as follows: the NaOH concentration selected values were 0.1, 0.5 and 2M; the temperature selected values were 25, 40 and 55 º C, and the hydrolysis time selected values were 0.5, 2 and 5h. Experimental runs have been coded according to the corresponding levels applied, in the same order as described previously, e.g. experimental Run 210 would correspond with the hydrolysis conditions of an alkali concentration of 2M NaOH (Level 2), a temperature of 40º C (Level 1) and a hydrolysis time of 0.5h (Level 0), and thus coded as 2 1 0.

All parameters were evaluated at all levels (3^3) in duplicate and non-consecutive experiments, which permitted the researchers to study the influence of the three factors along with the interactions between them up to second and third degree (B – T, B – t

and T – t; and B – T – t) for four outputs: total extraction yield, protein extraction yield, lipid extraction yield, and solubilisation yield. For this, a multi-factor analysis of variance (ANOVA) was applied to determine the significant differences (considering a confidence interval of 95%) among the levels of operational parameters in terms of the previously mentioned outputs. The data were analysed using Statgraphics Centurion XVII software.

2.3. Analytical methods

The total solids content and ash content of the raw biomass, the extracts and spent biomass were determined by the gravimetric method according to internal analytical standards from the Instrumental Techniques Laboratory (LTI – UVa). The identification, quantification, and biometry measurements of raw biomass (fixed with lugol solution at 5% and stored at 4 °C prior to analysis) were carried out by microscopic examination (OLYMPUS IX70) according to (Sournia, 1978).

The carbohydrates content in the raw biomass and extracts was determined by HPLC-RI using a modified NREL (National Renewable Energy Laboratory – USA) procedure (Sluiter et al., 2008). First, 300 mg dry-weight basis samples were subjected to a concentrated acid hydrolysis for 1 h by adding 3 mL of H_2SO_4 (72% w/w) at 30º C. Then, 84 mL of deionized water was added to dilute the acid concentration to 4% w/w prior to hydrolysis at 121º C for 1h. Then, liquid samples were obtained by filtration and analysed. 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 on the mentioned liquid samples. A mobile phase of 0.025M H_2SO_4 was eluted at a flow ratio of 0.6 mL/min and 50º C. External standards were used for quantification.

The organic nitrogen content of the raw biomass, extracts and some selected samples of spent biomass and acid supernatants were determined using the Total Kjeldahl nitrogen method. The protein content of the extracts was calculated by applying an N-to-P ratio of 5.95 (González-López et al., 2010). The total amino acid profile of the raw biomass and the selected extracts was determined, in order to evaluate the protein extracts quality, according to internal analytical standards from the Instrumental Techniques Laboratory (LTI – UVa) based on (Moore and Stein, 1948). Samples were hydrolysed with 6 N hydrochloric acid. Then, the acid was removed using N_2 until dry, the samples were re-suspended in 0.1M HCl and the final solution was filtered with 0.22 μm nylon membrane filters. Samples underwent pre-column derivatization with ortho-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC). The analysis was performed by HPLC using a Zorbax Eclipse AAA 4.6 x 150 mm 3.5-micron column, with mobile phases A (Buffer $NaH_2PO_4 \cdot H_2O$ pH 7.8) and B (AcN:MetOH:ddi water 45:45:10), and UV detector. The lipid content of the raw material and the extracts was determined using a modified protocol based on the Kochert method (Kochert, 1978).

All values were determined in replicate for each duplicated experiment. The results are presented as the mean of the replicated duplicates and the corresponding standard deviation.

3. Results and discussion

3.1. Extraction and solubilisation yields.

The total extraction yields (Y_T) at the different operation conditions are shown in Figure 2. Maximum Y_T was found to be 13.2 ± 0.4%. Interestingly, this maximum yield was not obtained when applying the maximum process intensity, but for Run 122,

corresponding to the hydrolysis conditions of 0.5M, 55º C and 5h. On the other hand, minimum Y_T (4.3 ± 0.8%) was found for Run 001, which corresponded to 0.1M, 25º C and 2h. These results were in agreement with those values reported by (Safi et al., 2014), who found slightly higher extraction yields for pure *C. vulgaris* biomass (around 15%) when applying chemical alkaline treatment at pH 12 and 40º C for 2h. This higher value could be attributed to the fact that *C. vulgaris* cell walls is less resistant than those of *S. almeriensis* (Baudelet et al., 2017). Moreover, the biomass used in our work underwent more stress due to the culture media and that had more stressful environmental conditions, which could have caused the development of a stronger and more resistant strain. As expected, larger extraction yields were observed when both temperature and time were increased. Regarding the alkali concentration, larger yields were observed for experiments where this variable was increased from 0.1M to 0.5M, but little improving effect or even a decrease in the extraction yields were detected when increasing the NaOH concentration from 0.5M to 2M. Since maximum yield values were obtained for milder conditions rather than the stronger conditions, it could be expected that the combination of these individual effects might eventually lead to some degradation of the extracted bioproducts influencing the extract quantity (yields) and quality (composition). The individual and combined effect of the operation parameters was evaluated by ANOVA and is shown in Section 3.3. The conditions that yielded the maximum total extraction yield (Run 122) also provided the highest protein extraction yield (Y_P = 16.9 ± 1.1%). In general terms, the Y_P values also showed the same trend as the total yields with respect to the variation of the experimental conditions – the values increased with higher temperature and time with maximum alkali concentrations values of 0.5M NaOH of alkali concentration. The minimum Y_P

was found for Run 001, with a yield of just 4.9 ± 1.1%.

The lipid extraction yields (Y_L) steadily increased with process severity, presumably influenced by all three variables equally. Y_L values oscillated between 3.7 ± 0.1% for Run 000 and 44.6 ± 0.5% for run 222, i.e. almost half of the initial lipid content was retrieved within the final extract. These results showed a co-extraction that could be relevant for the quality of extracts (whether those extracted lipids could provide valuable properties to the extract or not), and it could also be important when considering a sequential valorisation of the components, according to the biorefinery concept. Under alkaline conditions, lipids contained within the cells would be transformed by saponification into water-soluble sodic salts, favouring protein solubilisation (Sari et al., 2013). In addition, the consecutive acidification of the solution not only caused protein agglomeration but also the precipitation of these sodic salts, and thus were recovered along with the proteins in the extract. On the contrary, carbohydrate extraction yields reported very low values, and only between 0.6 and 3.6% of the initial carbohydrate content was found in the extracts. Complex carbohydrate chains might have been hydrolysed and transformed into simple sugars, which are water-soluble (Sun and Cheng, 2002) and probably would have remained in the acid supernatant (Figure 1).

Although the extraction yields were not found to be high, it should be noted that those values were the global efficiency of the process, as the combination of two steps: solubilisation and precipitation. The previously defined Solubilisation yield (S (%)) would therefore help to describe the specific efficiency of the alkaline hydrolysis step (Figure 1), where solubilisation took place, and complete the understanding of the pH-shift method for this particular case. Solubilisation yield values could also help

understand how much of the initial raw material was lost due to either degradation of the extracted compounds or a low efficiency of the acidification step. As a matter of fact, S% ranged from 38.0 ± 4.1% (for Run 011) to 66.5 ± 1.4% (for Run 222), which were considerably higher values than total extraction yields (6.0 ± 0.2% and 12.3 1.2± % respectively). Solubilisation yield values increased with stronger extraction conditions, even with higher alkali concentration, in a manner contrary to what was observed for the total extraction yields. For example, Y_{TS} for Runs 022, 122 and 222 were 10.9 ± 1.1%, 13.2 ± 0.4% and 12.3 ± 1.2%, whereas solubilisation yield values for these runs were found to be 43.9 ± 2.6%, 52.1 ± 5.1% and 66.5 ± 1.4% respectively. Solubilisation yield values for 0.1M experiments oscillated between 38.0 ± 4.1% and 50.1 ± 1.0%, for 0.5M experiments varied from 41.2 ± 1.2% and 52.1 ± 5.1%, and from 54.3 ± 0.2% and 66.5 ± 1.4% for 2M experiments, suggesting a strong correlation of solubilisation with alkali concentration. These will be further analysed when applying ANOVA method in section 3.3. These values were in agreement with (Gerde et al., 2013), who found that the soluble fraction remaining in the acid supernatant accounted for 52% of the initial biomass when extracting proteins from pure *Nannochloropsis* sp. These values were higher than those reported by (Phong et al., 2018a), who obtained 25% of solubilization yield when extracting proteins from *Chlorella sorokiniana* working at 0.5M KOH, but using a very short extraction time (60s).

Since carbohydrates and proteins are especially sensitive to alkaline and high-temperature conditions (Bischof and He, 2005; Clark and Pazdernik, 2013), these compounds might have been denaturalized and transformed into other smaller compounds (such as simple sugars – glucose, xylose – and peptides, respectively, or even suffer chemical transformations (Schwass and Finley, 1984)). On one hand, this

could lead a loss of some carbohydrates (reducing valuable carbohydrate content of the spent biomass used for successive steps in the biorefinery) and, on the other hand, it could affect proteins re-folding ability and eventually reduce their recovery rate during the acidic precipitation step.

3.2. Extracts composition

Regarding the composition of extracts, ash content required a first insight since all contents are reported in dry-weight ash-free basis. The ash content of the precipitated extracts was directly related to the NaOH concentration used for each run: the ash content oscillated between 5.4 ± 0.3% and 8.2 ± 2.0% for experiments with an alkali concentration of 0.1M NaOH; for 0.5M NaOH experiments, the ash content went from 10.3 ± 2.0% to 16.0 ± 0.9%; and finally for 2M NaOH alkali, the ash content was between 24.1 ± 0.7% and 31.7 ± 3.7%. The higher the amount of NaOH added for the experiments, the higher the amount of HCl was needed for acidification and so the final ash content in the extracts. These results are in agreement with the data reported by (Gerde et al., 2013), who worked at pH 11 and whose protein extracts had around 8% ash content.

Figure 3 displayed the proximate composition (protein, lipid and carbohydrate content) of extracts in dry-weight ash-free basis. The protein content showed some variation, with values between 53.5 ± 0.3% and 77.9 ± 3.4%, (corresponding to Runs 022 and 201 respectively), but was scarcely relatable to operation conditions or yields (Fig. 3). The application of identical conditions for the acidification step can likely be related to the similarity obtained in the extract proximate composition. Slightly higher composition values (between 63% and 77%) were found for the algae soluble protein isolates obtained by (Teuling et al., 2017) from pure *Scenedesmus dimorphus* biomass.

They also observed that, despite the differences found in the initial protein content and between protein solubilisation yields, the protein content of the different extracts were similar at the end of the process. The proximate composition of the extract obtained for Run 122, which was found to have the highest total extraction yield as well as the highest protein extraction yield, was 60.0 ± 1.8% (w/w dry basis ash-free) proteins, 20.6 ± 1.8% (w/w dry basis ash-free) lipids and 11.8 ± 0.6% (w/w dry basis ash-free) carbohydrates (Fig. 3), with an ash content of 10.3 ± 3.7%.

Unlike the protein content, the carbohydrate and lipid contents showed a trend which was clearly dependent on the severity of the extraction process. The lipid content values rose as the levels of the operation conditions increased, from 7.5 ± 0.2% to 34.9 ± 3.8% for the Runs 000 and 222 respectively, while the carbohydrate content of the extracts decreased from an average value of 13% when using 0.1M and 0.5M NaOH to values around 4% for 2M NaOH experimental runs (Fig. 3). This should be considered especially relevant since an enrichment in lipid content and a steep diminution in carbohydrate content for the extracts was observed, compared to the raw biomass that had a lipid content of 9.4 ± 0.3% and a carbohydrate content of 43.5 ± 4.3%.

In light of the fact that a large amount of lipids was apparently being co-extracted along with proteins (and hence transferred to the extracts), and considering the low carbohydrate content of the extracts, an additional property was defined: a protein-to-lipid ratio. This additional property is considered important for the evaluation of extracts quality. The two extreme experiments showed the highest (7.21 ± 0.3 for Run 000) and the lowest (1.66 ± 0.2 for Run 222) protein-to-lipid ratio, which consequently followed the opposite trend that lipids extraction yield, and decayed with process intensity. (Cavonius et al., 2015) suggested in their work that the presence of lipids

(fatty acids) in these extracts can improve the nutritional profile as well as the organoleptic properties of proteinaceous products. In our case, due to the source of the biomass an exhaustive preliminary study of toxicity and pathogenesis of the extracts would be compulsory before considering their applications e.g. as an enriched feed ingredient.

3.3. Influence of the operation parameters: ANOVA analysis

ANOVA analysis showed that the total extraction yield was individually affected by all the three operation parameters, with the alkali concentration being especially important since it represented 31.6% of the contribution, followed by time (24.6%) and temperature (19.3%). To the best of our knowledge, only (Gerde et al., 2013) briefly studied the effect of temperature, alkali concentration and time on protein extraction despite the extended use of the pH-shift process with microalgae biomass. These authors described no significant differences between using 0.5 or 1M NaOH concentrations in protein solubilisation from a 1% suspension of defatted biomass since the pH of these solutions was around 13, which provided the highest solubilisation yields according to the protein solubility curve. Temperature increased protein solubilisation at 45º C and 60º C compared to 30º C, and the hydrolysis time was found to be reduced with higher temperatures, obtaining a 12% yield after only 2 – 5h at 60º C and after only 8 – 16h at 45º C. For the individual multi-range tests, differences between alkali concentrations were all statistically significant, with the most important being within the range of 0.1M – 0.5M. An increase in the total yield values was achieved when increasing the concentrations from 0.1M to 0.5M and from 0.1M to 2M, but a decrease in the total yield values occurred in the 0.5M – 2M range. This means that the optimum alkali concentration for obtaining the highest yields is 0.5M. The total yields

increased with temperature and time. In both cases, the most relevant difference between the extreme values was 25º C – 55 º C and 0.5h – 5h. By analysing interactions between parameters, it was found that neither B – T nor B – t interactions were statistically significant even though the individual alkali concentration (B) was the parameter primarily affecting the total extraction yield. By contrast, the other two-parameter interaction T – t was statistically significant with an influence of 10.4%. The T – t interaction graph showed no maxima but instead showed a steady increase in average yield values and the important effect that temperature had on the total extraction yields when hydrolysis time was increased (Fig 4a and b). That is, extraction yields were almost independent of temperature when hydrolysing for 0.5h (6.3 ± 0.4% at 25 and 40º C and 6.9 ± 0.4% at 55º C). However, a more significant difference was found on the average total extraction yields when applying a temperature of 25º C, 40º C or 55º C (7.3 ± 0.4%, 8.8 ± 0.4% and 12.2 ± 0.4% respectively) after a hydrolysis time of 5h. The three-parameter interaction was not found to be statistically significant.

Comparable conclusions were obtained from the ANOVA analysis of the protein extraction yield. This variable was statistically affected by the three operational parameters evaluated~~,~~ especially by the NaOH concentration, whose influence accounted for 49.1%, almost 20 percentage points higher than the NaOH effect on the total yields. The influence of time was again more important than temperature; however, the influence of time was lower for the total yields, with values of 18.0 and 14.9%, respectively. Similar multi-range tests figures to the ones acquired for the total extraction yield were obtained for the protein extraction yields. Alkali concentration levels were individually contrasted and only intervals 0.1 – 0.5 and 0.1 – 2 presented statistically significant differences. However, (Sari et al., 2013) found the highest

protein extraction yields (between 15-35%) at alkaline pH (9.5, 10 and 11) after 3h of incubation at 60º C of *Chlorella fusca* biomass cultured in agricultural wastewaters, but small differences were reported when working at pH 9.5, 10 or 11. On the contrary, the multi-range tests of temperature and time levels were all found to be statistically significant, with the most important being the contrast of average protein extraction yield values between levels 0 – 2 for both parameters. In contrast with total extraction yields, no relevant interactions between the parameters were found.

The lipid extraction yield was found to be homogeneously affected by the three studied parameters B, T and t, with a contribution of 24.1% for the alkali concentration, 24.2% for temperature, and a 30.4% for time. This similar influence on the lipid extraction yield from all parameters tested was also observed in the multi-range test, which showed statistically significant differences for every parameter on all the ranges tested, with the most relevant being the difference between levels 0 and 2: 0.1 – 2M, 25 – 55 º C and 0.5 – 5h. Regarding the interactions between parameters, p-values were all found to be below 0.05 but T – t was the only one with a relevant contribution (7.0%), higher than that of the residues. Interactions proportionally increased in the lipid extraction yield either by increasing the alkali concentration, temperature or time. Differences between average Y_L values of each level were found to be steeper when increasing the level of the other studied variables. The carbohydrate extraction yields were not studied by ANOVA since differences were low and not significant.

Solubilisation, on the contrary, strongly – and only – depended on the alkali concentration (80.2% of the share). Although the p-value of temperature was below 0.05, its contribution (4.8%) could be considered merely nominal. The hydrolysis time was also found to be relevant as its p-value was below 0.05, but its contribution was

lower than that of the residues. The multi-range test of the alkali concentration for solubilisation average values revealed that the ranges 0 – 2 and 1 – 2, corresponding to 0.1M – 2M and 0.5M – 2M were the most relevant, in opposition to what was found for total yield and protein extraction yield. The solubilisation values were statistically similar for range 0.1M – 0.5M and increased for range 0.1M – 2M whereas the extraction yields increased for interval 0.1M – 0.5M but decreased for 0.5M – 2M. That is, protein recovery worsened even though solubilisation was being favoured by the extraction conditions. The temperature multi-range test showed significant differences for intervals 40º C – 55º C and 25º C – 55º C, whereas the significant differences were found for the hydrolysis time for intervals 0.5h – 2h and 0.5h – 5h. Analysis of the interactions determined that B – T and B – t interplays were influencing solubilisation, with a contribution of 3.1 and 4.5% respectively, which again was barely relevant compared to the influence of the alkaline concentration alone. Interaction graphs (Figure 4c and d) display the average behaviour expected from the results previously described, with increasing solubilisation values for increasing alkali concentrations, and overlapped lines and similar trends for temperature and time variables.

For the extract composition, the protein-to-lipid ratio was influenced by the extraction time (35.9% of the share), followed by the temperature (25.4% of the share) and scarcely affected by the alkali concentration, with just a 7.7% of the share. Regarding parameter interaction, only the three-parameter interaction was statistically relevant with a substantial 16.2% of the share. Any strengthening in variable levels caused a reduction in the average P-to-L ratio values, although this reduction was much less significant for variations of the alkali concentration when the hydrolysis temperature was 40º C, and for the temperature range 1 – 2 (40º C – 55º C) at Level 0 of

hydrolysis time (0.5h). Multi-range tests of all the variables showed that the most significant differences between average P-to-L ratio values were found for range 0 – 2.

In view of these results, a compromise between the protein extraction yield, extract protein content and protein-to-lipid ratio should be achieved, to obtain a good protein extraction yield but at the same time a proper protein-to-lipid ratio. To select the best point, the protein content of extracts and the protein-to-lipid ratio values were evaluated, and the highest values checked against the total extraction yields and the protein extraction yields. The extract from Run 122, which provided the highest extraction yields (13.2 ± 0.4% and 17.0 ± 1.1% for the total yields and the protein extraction yields respectively), presented a poor protein-to-lipid ratio (only 2.9 ± 0.2 $g_{proteins}/g_{lipids}$). On the one hand, the maximum protein-to-lipid ratio was obtained for Run 000, with 7.76 ± 0.3 $g_{proteins}/g_{lipids}$ and a 58.41 ± 0.8% of protein content, but with the lowest extraction yields (4.6 ± 0.0% of the total yield and 5.4 ± 0.1% of the protein extraction yield). On the other hand, maximum protein content (77.8 ± 3.4%) was found for the extract from Run 201, but only 5.0 ± 0.2 of the protein-to-lipid ratio and 11.1 ± 1.8% of the protein extraction yield. The extract with the second highest protein content (74.0 ± 0.1%) was obtained from Run 210, with a Y_T of 7.1 ± 0.3%, Y_P of 11.3 ± 0.6% and a P-to-L ratio of 5.8 ± 0.2. Since a compromise between all the parameters was needed, the experimental conditions for this run (2M NaOH, 40º C and 0.5h) were chosen as the best ones to obtain a high-quality protein extract from biomass by pH-shift, because it presented the second highest protein content and an appreciable protein-to-lipid ratio, with an intermediate protein extraction yield value. The lipid and carbohydrate content of these extracts were 12.7 ± 0.4% and 4.1 ± 0.1% respectively. Nevertheless, the considerable ash content (29.9 ± 1.0%) might require additional processing to further

improve its quality, like membrane ultrafiltration techniques.

3.4. Organic nitrogen balance and amino acid profile of selected extracts

The organic nitrogen content in the different phases (solid extract, solid spent biomass and liquid acid supernatant) was evaluated on some runs (001, 011, 101, 120, 122, 201, 211, 220, 221 and 222) in order to have a clearer perspective of the influence of the alkaline solubilisation and pH-shift recovery steps on the overall protein extraction process. A parameter called "Process intensity" was computed by summing up the numerical values of the levels applied for each run and used as classification criterion, i.e. Run 011 would present a process intensity of 2 (0 + 1 + 1) and Run 122 would represent a process intensity of 5 (1 + 2 + 2). Two experimental runs, corresponding to the maximum and minimum total extraction yields, were selected for process intensity values 2, 3, 4 and 5; for values 1 and 6 only the minimum and the maximum values were respectively selected. Organic nitrogen content (using the Kjeldahl method) is a reliable indicator of the actual protein (González-López et al., 2010; Mæhre et al., 2018) .

As explained before, higher extraction yields were not necessarily obtained from runs where higher solubilisation yields were detected, probably because of a poor efficiency in the acidification step. Consequently, the organic nitrogen content on each final phase has been analysed to check the organic nitrogen distribution in the different phases depending on the intensity of the operation parameters (Figure 5). The graph showed that the organic nitrogen content in the spent biomass decreased with increasing intensity, which is in line with the solubilisation values and proved that the alkaline hydrolysis process was solubilising the protein fraction into the liquid phase. Retained organic nitrogen (defined as the amount of organic nitrogen not solubilised and

expressed as $g_{\text{organic nitrogen in spent}}/100g_{\text{initial organic nitrogen in raw biomass}}$) in the spent biomass went from 69.6 ± 3.28% for Run 001 down to 27.0 ± 5.9% for Run 222. As observed from the low values of the protein extraction yields, a small amount of the solubilised protein – and hence of the organic nitrogen – was recovered in the extracts (with values ranging from 5.4 ± 0.24% for Run 001 to 20.5 ± 1.0% for Run 122). Meanwhile, the amount of organic nitrogen not recovered by the acidification step – and hence remaining in the acid supernatant – progressively increased with the intensity of the parameters from 17.3 ± 0.8% for Run 001 to a value as high as 54.9 ± 13.5% for Run 222, suggesting that the extracted proteins might have undergone one or several different chemical and physical changes (such as irreversible denaturation, break of covalent bonds, etc. (Bischof and He, 2005)) that made them unable to refold and/or aggregate during the acidification step, and thus remaining in the liquid phase (Gerde et al., 2013). In terms of the total recovery, all of the initial organic nitrogen content was recovered for all the runs tested, distributed throughout those different phases, excluding a possible loss of nitrogen due to degradation, e. g. into ammonia form. (Gerde et al., 2013) also evaluated nitrogen mass balance, but on a moisture-free basis (since the reported content on sodium chloride was low and constant) and in terms of total nitrogen instead of organic nitrogen. They obtained a 100% nitrogen recovery, and an approximate distribution of 40%, 30% and 30% of total nitrogen in the pH 11 insoluble fraction (equivalent to the spent biomass defined for this work), the extract and the pH 11&3.2 soluble fraction (called acid supernatant in our work) when extracting proteins from pure *Nannochloropsis* sp. at pH 11, 60º C and 5h. Comparing to the equivalent run in our work (Run 022), our recovery values (33.3% within the spent biomass, 20.5% in the extract and 42.7% in the acid supernatant) were similarly

distributed and within the range of those reported by (Gerde et al., 2013). Since it was observed that the acidic precipitation was not as selective as expected, other separation methods should be considered for future work, like three-phase partitioning or membrane filtration ((Safi et al., 2017; Waghmare et al., 2016))

The amino acid profile of the raw biomass (Table 1) presented a suitable essential-to-total amino acid ratio (Eaa/Taa), with high values of essential amino acids such as tyrosine and isoleucine, but slightly lower values of lysine or phenylalanine, compared to WHO/FAO recommendations (Becker, 2007). The amino acid profile of the extracts was found to be standardly constant for all of them, and hence independent of the intensity of the process. In general, a good Eaa/Taa amino acid ratio was found for all the extracts, slightly higher than that of the raw biomass, presenting values from 28.3 to 37.3% which are within the range of the average Eaa/Taa of an egg (34.7%) (Becker, 2007). In fact, the extraction process caused an enrichment of the content of several amino acids, including those that were in scarcity in the raw biomass. The extraction process caused the enrichment of some essential amino acids, namely Phe, Leu and Lys, and the non-essential amino acids Ala, Val and Asp. On the contrary, a substantial decay in Arg content was observed, along with Tyr, Gly and Ile content. Despite these variations and even though some of the lower limit values defined by the WHO/FAO were not attained in the raw biomass used in this work (e.g. for Phe or Lys), the amount of these amino acids (Tyr, Val, Met, Phe, Ile, Leu and Lys) was above these lower limits in almost all of the analysed extracts (Becker, 2007). (Safi et al., 2013) analysed the amino acid profile of several species, including *Chlorella* sp., as well as that of the obtained extracts. In general terms, the microalgae-bacteria consortium studied in this work originally had a lower Eaa/Taa, although Met and Ile content was more favourable

compared to raw pure *Chlorella* sp. However, the extracts obtained from the microalgae biomass grown in piggery wastewater in this work had a higher ratio and a better amino acid distribution, with higher contents of essential amino acids such as Met, Phe or Leu, and lesser amounts of Ala or Glu, than those obtained from the *Chlorella* sp. biomass. (Teuling et al., 2017) compared protein extracts from different pure microalgae species, including *Scenedesmus dimorphus*. The amino acid profile of *S. dimorphus* raw biomass partially differed in some of the amino acid content (such as Arg, Phe, Leu and Lys) and presented an Eaa/Taa 10 percentage points higher than the raw biomass used in this work. However, the amino acid profile of the extract obtained from *S. dimorphus* biomass (with an Eaa/Taa ratio of 34.2%) did not differ very much from the average obtained in this work and despite the different initial distribution.

4. Conclusions

ANOVA showed that all variables (alkali concentration, temperature and time) influenced the total (31.6%, 19.3% and 24.6% of influence), protein (49.1%, 14.9% and 18.0%) and lipid (24.1%, 24.2% and 30.4%) yields. Protein-to-lipid ratio decreased due to time and temperature (35.9% and 25.4%) while protein solubilisation solely increased because of the alkali concentration (80.2%). Acidic precipitation provided low efficiency and selectivity, thus a different separation method should be considered. The most suitable conditions, pondering yields and selectivity, were 2M - 40º C - 0.5h, which resulted in 11.3% of protein yield, providing an extract comprised of 73.9% proteins and 12.7% lipids.

5. Acknowledgements

This work was supported by the regional government of Castilla y León (UIC 071, CLU 2017-09 and VA080G18). The authors also thank “Ministerio de Ciencia,

Innovación y Universidades" (CTQ2017-84006-C3-1-R) and the EU-FEDER (CLU 2017-09 and CTQ2017-84006-C3-1-R) for the financial support of this work. Ana Lorenzo would like to thank the University of Valladolid for her doctorate scholarship.

6. References

Acién, F.G., Gómez-Serrano, C., Morales-Amaral, M.M., Fernández-Sevilla, J.M., Molina-Grima, E., 2016. Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment? Appl. Microbiol. Biotechnol. 100, 9013–9022. doi:10.1007/s00253-016-7835-7

Ansari, F.A., Gupta, S.K., Nasr, M., Rawat, I., Bux, F., 2018. Evaluation of various cell drying and disruption techniques for sustainable metabolite extractions from microalgae grown in wastewater: A multivariate approach. J. Clean. Prod. 182, 634–643. doi:10.1016/j.jclepro.2018.02.098

Baudelet, P.H., Ricochon, G., Linder, M., Muniglia, L., 2017. A new insight into cell walls of Chlorophyta. Algal Res. 25, 333–371. doi:10.1016/j.algal.2017.04.008

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–10. doi:10.1016/j.biotechadv.2006.11.002

Benelhadj, S., Gharsallaoui, A., Degraeve, P., Attia, H., Ghorbel, D., 2016. Effect of pH on the functional properties of *Arthrospira* (*Spirulina*) platensis protein isolate. Food Chem. 194, 1056–1063. doi:10.1016/j.foodchem.2015.08.133

Bischof, J.C., He, X., 2005. Thermal stability of proteins. Ann. N. Y. Acad. Sci. 1066, 12–33. doi:10.1196/annals.1363.003

Bleakley, S., Hayes, M., 2017. Algal Proteins: Extraction, Application, and Challenges Concerning Production. Foods 6, 33. doi:10.3390/foods6050033

Cavonius, L.R., Albers, E., Undeland, I., 2015. pH-shift processing of *Nannochloropsis*

oculata microalgal biomass to obtain a protein-enriched food or feed ingredient. Algal Res. 11, 95–102. doi:10.1016/j.algal.2015.05.022

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

Clark, D.P., Pazdernik, N.J., 2013. Molecular biology. Elsevier.

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

Gerde, J.A., Wang, T., Yao, L., Jung, S., Johnson, L.A., Lamsal, B., 2013. Optimizing protein isolation from defatted and non-defatted *Nannochloropsis* microalgae biomass. Algal Res. 2, 145–153. doi:10.1016/j.algal.2013.02.001

González-López, C. V., Cerón-García, M.C., Acién, F.G., Bustos, C.S., Chisti, Y., Fernandez-Sevilla, J.M., López, C.V.G., del Carmen Cerón García, M., Fernández, F.G.A., Bustos, C.S., Chisti, Y., Sevilla, J.M.F., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591. doi:10.1016/j.biortech.2010.04.077

Gupta, S.K., Ansari, F.A., Shriwastav, A., Sahoo, N.K., Rawat, I., Bux, F., 2016. Dual role of *Chlorella sorokiniana* and *Scenedesmus obliquus* for comprehensive wastewater treatment and biomass production for bio-fuels. J. Clean. Prod. 115, 255–264. doi:10.1016/j.jclepro.2015.12.040

Kochert, G., 1978. Quantitation of the macromolecular components of microalgae. Handb. Phycol. methods. Physiol. Biochem. methods 189–195.

Mæhre, H.K., Dalheim, L., Edvinsen, G.K., Elvevoll, E.O., Jensen, I., 2018. Protein

Determination—Method Matters. Foods 7, 5. doi:10.3390/foods7010005

Molinuevo-Salces, B., Mahdy, A., Ballesteros, M., González-Fernández, C., 2015. From piggery wastewater nutrients to biogas: Microalgae biomass revalorization through anaerobic digestion. Renew. Energy 1–8. doi:10.1016/j.renene.2016.01.090

Moore, S., Stein, W.H., 1948. Partition chromatography of amino acids on starch. Ann. N. Y. Acad. Sci. 49, 265–278.

Phong, W.N., Show, P.L., Le, C.F., Tao, Y., Chang, J.S., Ling, T.C., 2018a. Improving cell disruption efficiency to facilitate protein release from microalgae using chemical and mechanical integrated method. Biochem. Eng. J. 135, 83–90. doi:10.1016/j.bej.2018.04.002

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

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

Safi, C., Charton, M., Pignolet, O., Silvestre, F., Vaca-Garcia, C., Pontalier, P.Y., 2013. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. J. Appl. Phycol. 25, 523–529. doi:10.1007/s10811-012-9886-1

Safi, C., Ursu, A.V., Laroche, C., Zebib, B., Merah, O., Pontalier, P.Y., Vaca-Garcia,

C., 2014. Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. Algal Res. 3, 61–65. doi:10.1016/j.algal.2013.12.004

Sari, Y.W., Bruins, M.E., Sanders, J.P.M., 2013. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Ind. Crops Prod. 43, 78–83. doi:10.1016/j.indcrop.2012.07.014

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

Schwass, D.E., Finley, J.W., 1984. Heat and alkaline damage to proteins: Racemization and lysinoalanine formation. J. Agric. Food Chem. 32, 1377–1382. doi:10.1021/jf00126a040

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of ash in biomass: Laboratory Analytical Procedure (LAP), NREL/TP-510-42622.

Sournia, A., 1978. Phytoplankton: manual. UNESCO, United Kingdom.

Suganya, T., Varman, M., Masjuki, H.H., Renganathan, S., 2016. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. Renew. Sustain. Energy Rev. 55, 909–941. doi:10.1016/j.rser.2015.11.026

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production : a review q. Bioresour. Technol. 83, 1–11. doi:10.1016/S0960-8524(01)00212-7

Teuling, E., Wierenga, P.A., Schrama, J.W., Gruppen, H., 2017. Comparison of Protein

Extracts from Various Unicellular Green Sources. J. Agric. Food Chem. 65, 7989–8002. doi:10.1021/acs.jafc.7b01788

Ursu, A.V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., Michaud, P., 2014. Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*. Bioresour. Technol. 157, 134–139. doi:10.1016/j.biortech.2014.01.071

Vanthoor-Koopmans, M., Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2013. Biorefinery of microalgae for food and fuel. Bioresour. Technol. 135, 142–149. doi:10.1016/j.biortech.2012.10.135

Voigt, J., Stolarczyk, A., Zych, M., Malec, P., Burczyk, J., 2014. The cell-wall glycoproteins of the green alga *Scenedesmus obliquus*. The predominant cell-wall polypeptide of Scenedesmus obliquus is related to the cell-wall glycoprotein gp3 of Chlamydomonas reinhardtii. Plant Sci. 215–216, 39–47. doi:10.1016/j.plantsci.2013.10.011

Waghmare, A.G., Salve, M.K., LeBlanc, J.G., Arya, S.S., 2016. Concentration and characterization of microalgae proteins from *Chlorella pyrenoidosa.* Bioresour. Bioprocess. 3. doi:10.1186/s40643-016-0094-8

Figure Captions

Figure 1. Scheme of the process followed for the extraction of the protein fraction of biomass from pig manure treatment photo-bioreactors. The three process parameters defined and abbreviated in this figure are alkali (NaOH) concentration – B (mol L^{-1}) –, temperature – T (º C) –, and time – t (h) –.

Figure 2. Total ($g_{extract}/100g_{raw\ biomass}$) and protein ($g_{proteins}/100g_{proteins\ in\ raw\ biomass}$) extraction yields. Values shown are the mean of duplicate values, and the standard deviation is shown as lines. Experimental runs codification stands for the three levels (0, 1 and 2) of the three evaluated parameters, i.e. alkali concentration (0.1, 0.5 and 2M NaOH), temperature (25, 40 and 55 ºC), and hydrolysis time (0.5, 2 and 5h).

Figure 3. Proximate composition of extracts ($g_{macromol.\ component}/100g_{extract\ dry\text{-}weight\ ash\text{-}free}$). Values shown are the mean of duplicate values, and the standard deviation is shown as lines. Experimental runs codification stands for the three levels (0, 1 and 2) of the three evaluated parameters, i.e. alkali concentration (0.1, 0.5 and 2M NaOH), temperature (25, 40 and 55 ºC), and hydrolysis time (0.5, 2 and 5h).

Figure 4. Interaction graphs obtained from ANOVA. (a) Total extraction yield (%): NaOH concentration vs. Temperature; (b) Total extraction yield (%): Temperature vs. time; (c) Solubilisation (%): NaOH concentration vs. Temperature; (d) Solubilisation (%): NaOH vs. time.

Figure 5. Organic nitrogen balance ($g_{OrgN\ in\ each\ phase}/100g_{\ initial\ OrgN}$) in each phase obtained after alkaline hydrolysis and acidic precipitation of the biomass.

Table 1. Amino acid composition (%) and essential-to-total amino acid ratio (Eaa/Taa) of selected extracts from the protein extraction process.

Tables and Figures

Figure 1

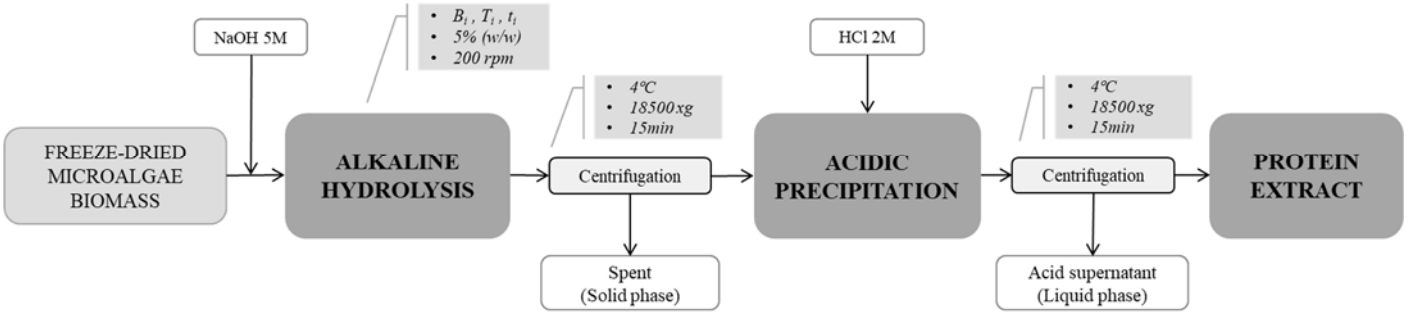

Figure 2

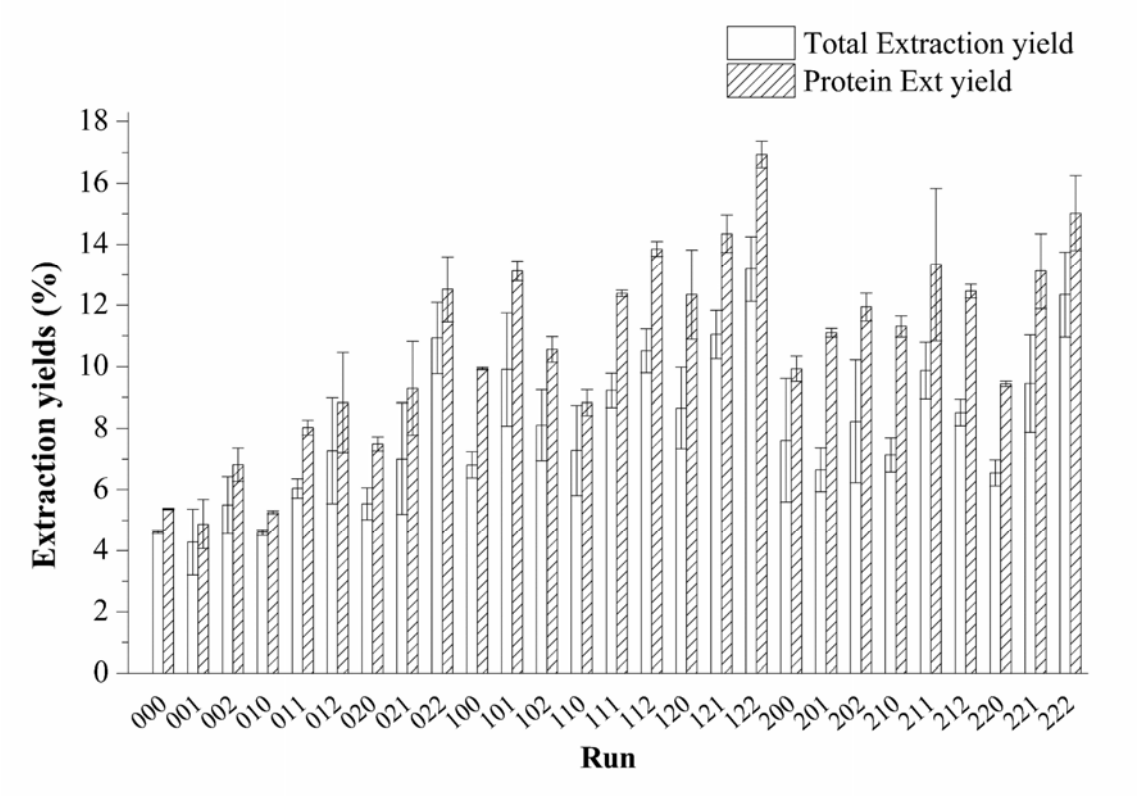
Total Extraction yield
Protein Ext yield
Extraction yields (%)
0
2
4
6
8
10
12
14
16
18
000
001
002
010
011
012
020
021
022
100
101
102
110
111
112
120
121
122
200
201
202
210
211
212
220
221
222
Run

Figure 3

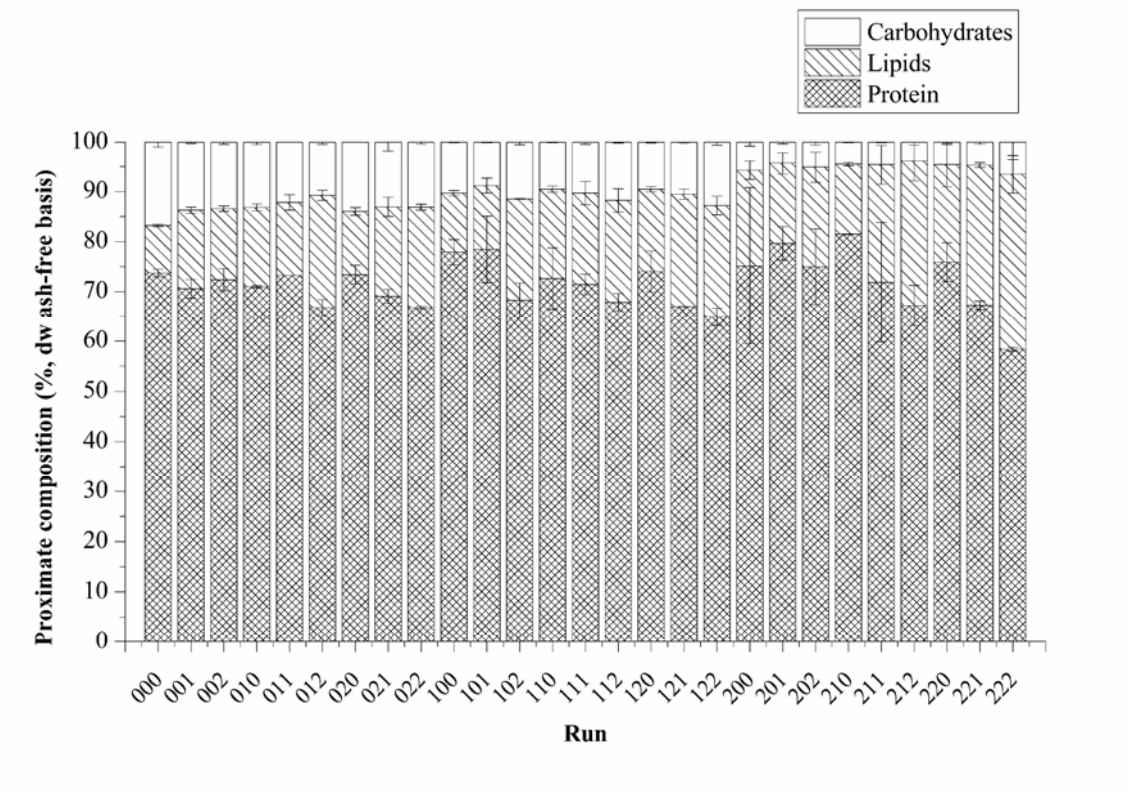
Carbohydrates
Lipids
Protein
Proximate composition (%, dw ash-free basis)
100
90
80
70
60
50
40
30
20
10
0
000
001
002
010
011
012
020
021
022
100
101
102
110
111
112
120
121
122
200
201
202
210
211
212
220
221
222
Run

Figure 4

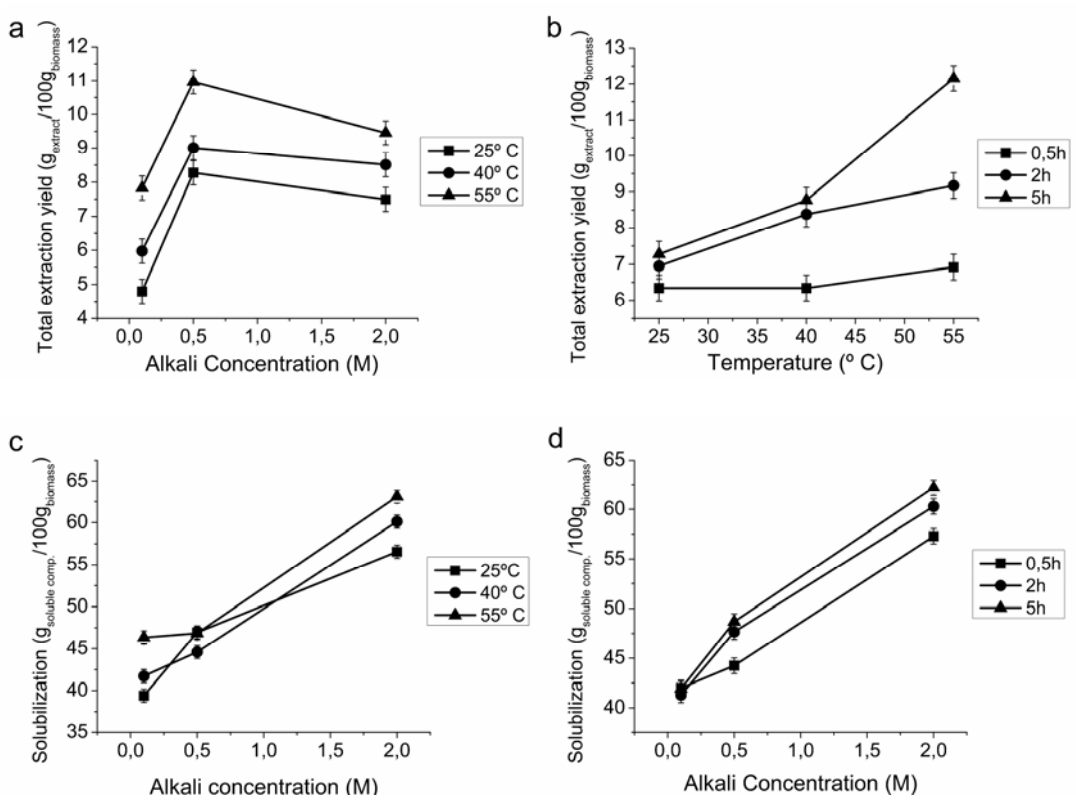
a
Total extraction yield ($g_{extract}$/100$g_{biomass}$)
Alkali Concentration (M)
25º C
40º C
55º C
b
Total extraction yield ($g_{extract}$/100$g_{biomass}$)
Temperature (º C)
0,5h
2h
5h
c
Solubilization ($g_{soluble\ comp.}$/100$g_{biomass}$)
Alkali concentration (M)
25ºC
40º C
55º C
d
Solubilization ($g_{soluble\ comp.}$/100$g_{biomass}$)
Alkali Concentration (M)
0,5h
2h
5h

Figure 5

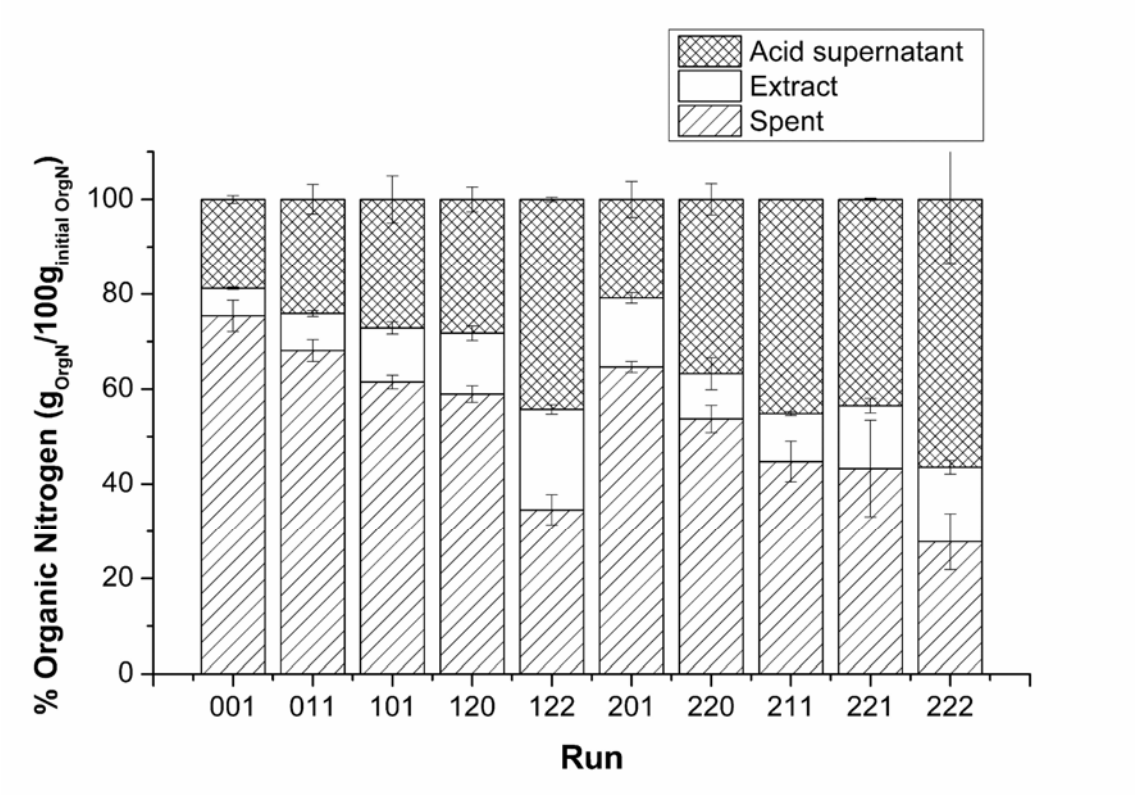
Acid supernatant
Extract
Spent
% Organic Nitrogen (g_{OrgN}/100$g_{initial\ OrgN}$)
0
20
40
60
80
100
001
011
101
120
122
201
220
211
221
222
Run

Table 1

$g/100g_{prot}$	**Initial**	**001**	**011**	**101**	**120**	**122**	**201**	**211**	**220**	**221**	**222**
Asp	10.52	11.41	11.76	12.34	12.47	12.71	10.98	11.38	10.65	11.22	11.29
Glu	15.48	15.80	13.16	13.66	13.54	14.43	13.50	13.38	12.69	13.09	13.37
Ser	4.64	4.72	4.92	4.36	4.26	3.49	3.38	3.45	2.94	2.59	2.08
His	2.12	1.74	1.77	1.97	2.02	2.02	2.10	2.11	2.02	2.10	2.10
Gly	9.47	6.48	6.61	6.18	6.38	6.53	4.69	5.01	4.80	4.87	4.94
Thr	4.38	5.75	5.99	4.19	4.03	2.87	3.24	3.29	2.76	2.23	1.84
Arg	16.80	8.05	7.83	6.68	6.42	6.65	6.72	6.63	6.10	5.91	5.55
Ala	1.54	9.62	8.73	8.49	8.59	8.95	8.37	8.45	8.62	8.96	8.94
Tyr	8.67	3.60	3.69	4.32	4.51	4.54	4.38	4.40	4.26	4.57	4.55
Cys	0.00	0.64	0.50	0.10	0.10	0.04	0.32	0.09	0.29	0.22	0.65
Val	2.44	5.53	5.82	5.87	5.96	5.25	6.21	6.22	6.53	5.98	5.79
Met	3.69	1.62	1.63	2.14	2.22	2.57	2.63	2.53	2.73	3.06	3.00
Phe	0.18	4.70	5.56	5.89	5.94	6.03	6.77	6.70	7.55	7.05	7.01
Ile	7.40	3.90	4.35	4.64	4.71	4.05	5.20	5.23	5.66	4.86	4.68
Leu	7.65	7.76	8.79	9.90	9.99	10.55	11.55	11.36	12.39	12.42	12.26
Lys	0.00	4.56	4.51	5.04	5.07	5.05	6.09	5.86	6.20	6.19	7.70
Pro	5.03	4.11	4.38	4.22	3.80	4.27	3.89	3.88	3.81	4.68	4.27
Eaa/Taa	**29.3**	**28.3**	**30.9**	**31.8**	**32.0**	**31.1**	**35.5**	**35.0**	**37.3**	**35.8**	**36.5**