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Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors

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Abstract Additional evidence about the influence of the cell wall physical and chemical characteristics on protein extractability was determined by calculating the conversion factors of five different microalgae known to have different cell wall composition, and their protein extracts. The conversion factors obtained for crude rigid cell walled *Chlorella vulgaris*, *Nannochloropsis oculata* and *Haematococcus pluvialis* were 6.35, 6.28 and 6.25, respectively, but for their protein extracts the values were lower with 5.96, 5.86 and 5.63. On the other hand, conversion factor obtained for fragile cell walled microalgae *Porphyridium cruentum* and *Athrospira platensis* was 6.35 for the former and 6.27 for the latter, with no significant difference for their protein extract with 6.34 for the former and 6.21 for the latter. In addition, the highest hydro-soluble protein percentage recovered from total protein was for *P. cruentum* 80.3 % and *A. platensis* 69.5 % but lower for *C. vulgaris* with 43.3 %, *N. oculata* with 33.3 % and *H. pluvialis* with 27.5 %. The study spotted the light on the influence of the cell wall on evaluating the conversion factor and protein extractability. In addition, it showed the necessity of finding the conversion factor every time accurate protein quantification is required, and proved that there is not a universal conversion factor that can be recommended.

Introduction

Microalgae have been consumed long time ago by the Aztecs and other Mesoamericans who used this biomass as an important food source (Vonshak 1997). Nowadays, in Japan for instance, *Chlorella vulgaris* is added to food such as noodles and pasta (Fradique et al. 2010) to improve the nutritional quality of the meal.

Microalgae are gaining interest due to their capacity to accumulate important amounts of multiple components (proteins, lipids, carbohydrates and pigments) compared to any other sources, and therefore protein content is considered as one of the cardinal components determining their nutritional value. For instance, *Arthrospira* (*Spirulina*) *maxima* can accumulate proteins up to 71 % dry weight (Becker 1994). Thus, analysing and quantifying the protein content are key factors that should be thoroughly investigated. A capital point is to calculate precisely the nitrogen-to-protein conversion factor (NTP). While the standard value of 6.25 is used, Kjeldahl or elemental analysis may lead to an overestimation or underestimation of the protein quantity. Moreover, these two methods take into account the totality of the nitrogen present in the biomass from which 59–98 % (Lourenço et al. 1998; Fujihara et al. 2001; Lourenço et al. 2004; Gonzalez

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Lopez et al. 2010) of total nitrogen belongs to protein and the rest comes from pigments, nucleic acids and other inorganic components. It is true that the colorimetric method of Lowry (Lowry et al. 1951) is an accurate method for protein quantification (Peterson 1979; Gonzalez Lopez et al. 2010) and it does not require a conversion factor. Nevertheless, this method determines only the hydro-soluble proteins (Crossman et al. 2000; Diniz et al. 2011) and not the total protein content.

In addition, the extraction of proteins can be diminished by the cell wall barrier, which can prevent the solubilisation of all the intracellular proteins affecting thus the value of the nitrogen-to-protein conversion factor. Therefore, the impact of the cell wall characteristics on protein extractability should be taken into account and analysed in order to prevent an incorrect estimation of the protein content.

Multiple studies have focused on finding a method to recommend the right conversion factor; for instance, Gonzalez Lopez et al. (2010) focused on obtaining the conversion factor of five microalgae after breaking the cell wall, and then finding a correlation between protein content and total nitrogen content (elemental analysis or Kjeldahl). As a result, among five microalgae a new mean conversion factor was estimated to 4.44 (elemental analysis) and 5.95 (Kjeldahl). Another study (Lourenço et al. 2002) determined the conversion factor for 19 tropical seaweeds harvested directly from the beach; and in a second study (Lourenço et al. 1998), 12 marine microalgae were analysed under different growth phases and a mean value of 4.58 was found.

The following study assesses the impact of the cell wall on the protein extractability and the evaluation of the NTP for five microalgae intensively grown worldwide and having wide taxonomic diversity.

Materials and methods

The microalgae used are: the cyanobacterium *Arthrospira platensis* (strain PCC 8005), two Chlorophyceae *C. vulgaris* (strain SAG 211-19), *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium cruentum* (strain UTEX 161) and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain).

Each alga was cultivated in a different culture medium: Hemerick medium for *P. cruentum*, Sueoka medium for *C. vulgaris*, Basal medium for *H. pluvialis*, Conway medium for *N. oculata*, and Zarrouk medium for *A. platensis*. All were grown in batch mode in an indoor tubular air-lift photobioreactor (PBR, 10 L) at 25 °C (Loubiere et al. 2011) after inoculation from a prior culture in a flat panel air-lift PBR (1 L). Culture mixing was by sterile air injection at the bottom of the PBR. The pH and temperature were recorded by a pH/temperature probe and pH was regulated at 7.5 with CO_2. The algae were harvested during the exponential growth phase and concentrated by centrifugation, and then supplied as a frozen paste from Alpha Biotech (Asserac, France). The biomass contained 20 % dry weight. The frozen paste of crude microalgae was freeze-dried in a Fisher Bioblock Scientific Alpha 2–4 LD Plus device (Illkirch, France).

Protein extraction

Stock solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust the solution to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of stock solution. The mixture was heated to 40 °C with stirring for 1 h followed by centrifugation at $5,000 \times g$ for 10 min. Samples were taken for analysis by the colorimetric method of Lowry et al. (1951), elemental analysis and amino acid analysis.

Lowry method

A calibration curve was prepared using a concentration range of bovine serum albumin from 0 to 1,500 μg mL^{-1}. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were withdrawn and then 1 mL of modified Lowry reagent was added to each sample. Each sample was then vortexed and incubated for exactly 10 min. After incubation, 100 μL of Folin–Ciocalteu reagent (1 N) were added and again vortexed and incubated for exactly 30 min. The absorbance was then measured at 750 nm.

Elemental analysis

Total nitrogen of the freeze-dried biomass was evaluated using a PerkinElmer 2400 series II elemental analyser. Samples of 2 mg were placed in tin capsules and then heated at 925 °C using pure oxygen as the combustion gas and pure helium as the carrier gas, then evaluating the nitrogen percentage and converting it into protein percentage by using the conversion factors calculated for each microalga in this study.

Amino acid analysis and NTP calculation

The determination of the amino acid composition of the biomass was performed according to a widely used standard method (Moore and Stein 1948). The samples were hydrolysed with 6 N hydrochloric acid at 103 °C for 24 h. Then, the hydrolysed material was adjusted to pH 2.2 with 6 N NaOH and stabilised with a pH 2.2 citrate buffer solution. The final solution was then filtered with 0.45 μm PTFE

membrane to remove any residual solids remaining in the solution. The analysis was performed by using an amino acid analyser Biochrom Ltd 32+ (Cambridge, UK) equipped with a high pressure PEEK "column+pre-column" (size, 200×4.6 mm) packed with Ultropac cation exchange resin containing sodium. The separation of amino acids is carried

out by elution with loading buffers (flow rate 25 mL h^{-1}) at different pH. After reaction with ninhydrin (flow rate 35 mL h^{-1}), amino acids are detected with a UV detector at a wavelength of 570 nm, with the exception of proline, for which detection occurs at 440 nm. Calculation of NTP was carried out according to the method (k_A) of Mossé (1990) and Sriperm et al. (2011). It should be mentioned that ammonia was added to compensate the value of some less resistant amino acids that disappeared after the strong acid hydrolysis. In addition, the strong hydrolysis of the peptide bonds generates one molecule of water from each amino acid and therefore during the quantification of total amino acid one molecule of water was subtracted from each amino acid in order to get the total amino acid residue which represents the exact quantity of all amino acids (Lourenço et al. 1998; Fujihara et al. 2001).

Confocal laser scanning microscopy

Cells were observed with an SP2-AOBS confocal laser-scanning microscope (Leica). The fluorochrome calcofluor white that binds to the $\beta{\to}1$–4 linkages in the cell wall polysaccharides was added to the samples. Excited at 488 nm, the cells are identified coloured in light blue.

Statistical analysis

Three experiments were conducted separately on all micro-algae and their protein extract. Statistical analyses were carried out on Microsoft Excel. Measurements of three replicates for each sample were reproducible for ±5 % of the respective mean values.

Results

An amino acid analyser was used to obtain the amino acid profile of the crude microalgae (Table 1) and their protein extract (Table 2). The protein primary composition was reconstituted in order to find the conversion factor (Table 3) that takes into account only the protein nitrogen. The highest conversion factor evaluated for the crude biomass was 6.35 for *C. vulgaris* and *P. cruentum* and the lowest was 6.25 for *H. pluvialis*. If we compare the NTP value of the crude microalgae and the protein extract, we observe that there is no significant difference for *P. cruentum* and *A. platensis*. However, a significant difference was measured for the other three species, which correspond to green microalgae (Table 3).

Table 1 Results of total amino acids of five microalgae expressed in grams per 100 g of algal protein representing three replicates for three experiments±SD (n=3)

Amino acids	Crude microalgae amino acid composition				
	P. cruentum	*A. platensis*	*C. vulgaris*	*N. oculata*	*H. pluvialis*
Aspartic acid	11.21±0.45	11.82±0.11	10.09±0.02	9.14±0.05	8.85±0.10
Threonine	6.25±0.25	6.16±0.10	5.62±0.01	5.91±0.03	5.21±0.06
Serine	8.11±0.29	6.85±0.02	7.17±0.04	6.52±0.01	6.63±0.05
Glutamic acid	8.17±0.29	10.50±0.09	8.37±0.01	10.30±0.02	9.47±0.11
Glycine	6.86±0.28	7.76±0.06	7.93±0.01	9.00±0.01	9.05±0.09
Alanine	6.67±3.67	9.91±0.08	10.05±0.03	10.92±0.01	11.28±0.12
Cysteine	0.33±0.01	0.18±0.02	0.18±0.01	0.19±0.01	0.22±0.01
Valine	2.50±0.10	2.86±0.02	2.85±0.01	3.29±0.02	3.32±0.04
Methionine	2.78±0.11	1.72±0.02	0.60±0.01	1.50±0.01	0.64±0.01
Isoleucine	5.25±0.24	0.12±0.01	0.09±0.01	0.11±0.01	4.53±0.04
Leucine	5.83±0.21	7.02±0.02	6.91±0.02	8.11±0.05	8.09±0.10
Tyrosine	4.43±0.18	4.83±0.05	7.78±0.01	3.40±0.02	2.80±0.04
Phenylalanine	5.00±0.20	4.82±0.04	5.36±0.01	5.05±0.01	4.92±0.07
Histidine	1.11±0.04	0.90±0.01	1.16±0.01	0.94±0.01	0.90±0.01
Lysine	5.50±0.21	5.10±0.62	6.30±0.07	5.70±0.01	5.72±0.08
Arginine	7.78±0.29	7.69±0.07	6.81±0.03	5.93±0.02	6.10±0.08
Tryptophan	1.39±0.05	1.22±0.01	2.04±0.01	1.24±0.01	1.72±0.02
Ornithine	0.27±0.01	0.16±0.09	0.12±0.01	0.16±0.01	0.07±0.01
Proline	2.53±0.17	1.95±0.05	2.74±0.07	4.20±0.07	2.94±1.15
Ammonia	8.02±0.30	8.41±0.09	7.82±0.02	8.38±0.08	7.52±0.08

Table 2 Results of total amino acids of the protein extract extracted at pH 12 and 40 °C of five microalgae expressed in grams per 100 g of algal protein representing three replicates for three experiments±SD ($n=3$)

Protein extract amino acid composition					
Amino acids	*P. cruentum*	*A. platensis*	*C. vulgaris*	*N. oculata*	*H. pluvialis*
Aspartic acid	10.71±0.02	9.70±0.02	6.81±0.28	4.47±0.05	6.54±0.07
Threonine	4.45±0.01	5.54±0.04	4.16±0.19	3.18±0.04	3.70±0.02
Serine	7.49±0.02	7.25±0.03	5.73±0.27	3.16±0.06	5.66±0.01
Glutamic acid	9.05±0.01	11.65±0.02	11.63±0.51	22.60±0.19	13.55±0.01
Glycine	7.68±0.01	8.42±0.02	9.75±0.42	8.79±0.10	11.00±0.01
Alanine	10.46±0.02	10.94±0.02	16.82±0.75	14.02±0.13	19.12±0.03
Cysteine	0.27±0.01	0.20±0.01	0.24±0.01	0.38±0.01	0.40±0.01
Valine	3.15±0.01	3.31±0.01	3.66±0.15	2.36±0.03	2.80±0.01
Methionine	2.37±0.01	1.71±0.01	1.28±0.04	1.23±0.03	1.04±0.01
Isoleucine	5.34±0.02	0.12±0.01	2.32±1.87	1.08±1.44	3.06±0.01
Leucine	7.30±0.01	8.02±0.02	7.15±0.33	4.11±0.04	4.99±0.03
Tyrosine	3.69±0.01	4.33±0.01	2.56±0.10	2.15±0.07	1.91±0.02
Phenylalanine	4.12±0.01	4.26±0.01	2.74±0.12	1.63±0.02	2.63±0.01
Histidine	0.79±0.03	0.73±0.01	0.53±0.03	0.28±0.01	0.43±0.01
Lysine	5.60±0.01	5.39±0.01	6.16±0.27	2.35±0.01	3.48±0.01
Arginine	6.63±0.02	6.84±0.01	5.85±2.71	1.96±0.09	6.88±0.01
Tryptophan	0.72±0.02	1.07±0.01	0.49±0.02	0.18±0.01	0.31±0.01
Ornithine	0.22±0.01	0.23±0.01	0.50±0.02	0.55±0.03	0.43±0.01
Proline	1.87±0.02	1.86±0.08	2.11±0.13	14.41±0.33	1.61±0.04
Ammonia	8.05±0.04	8.39±0.01	9.47±0.44	11.09±0.31	10.37±0.06

The total nitrogen content of crude microalgae was determined by elemental analysis. The hydro-soluble proteins were quantified by the Lowry method. By using the NTP values from Table 3, the total proteins and the corresponding fraction of hydro-soluble proteins were accurately determined starting from the results of elemental analysis (Table 4).

A small difference was observed between the essential and non-essential amino acids total percentage for *P. cruentum*, *A. platensis* and *C. vulgaris* and their protein extract, and an important difference was observed for *N. oculata* and *H. pluvialis* with a noticeable increase in non-essential amino acids percentage in the protein extract and a decrease in essential amino acids percentage (Table 5).

Finally, the laser scanning confocal microscopic images presented in Fig. 1 showed that in the case of *P. cruentum* and *A. platensis* a total disruption of the cell wall occurred after the alkaline treatment, whereas *C. vulgaris*, *N. oculata* and *H. pluvialis* maintained their globular form indicating that at least a part of their cell wall was intact.

Discussion

The present study contributes original individual NTP conversion factors for five current species of microalgae. Indeed, direct comparison of our results with literature values can only be done for *N. oculata* (Lourenço et al. 1998); the report from Gonzalez Lopez et al. (2010) combines all the cyanobacteria and *P. cruentum* in a global result. To our knowledge, NTP data for *C. vulgaris* and *H. pluvialis* have never been reported. Moreover, according to Mossé (1990), there are three kinds of NTP conversion factors called k, k_A and k_P (Yeoh and Truong 1996; Sriperm et al. 2011). In this study, k_A was calculated for all species, and this kind of conversion factor is larger than k_P and k. For this reason, there is a significant difference between the conversion factors calculated for *N. oculata* in this study (6.28) and the one calculated for the same microalga (4.87) in the Lourenço et al. (1998) study.

Table 3 Nitrogen-to-protein conversion factors for the crude microalgae and their protein isolate based on three replicates for three experiments±SD ($n=3$)

NTP			
Microalgae	Crude microalgae	Protein extract	% Relative difference
P. cruentum	6.35±0.03	6.34±0.04	0.16
A. platensis	6.27±0.02	6.21±0.07	0.96
C. vulgaris	6.35±0.07	5.96±0.23	6.14
N. oculata	6.28±0.06	5.86±0.32	6.69
H. pluvialis	6.25±0.07	5.63±0.18	9.92

Table 4 Different protein contents in crude microalgae

	Nea[a] (%)	Total protein=Nea×NTP[b] (%)	Hydro-soluble protein=P_{Lowry}[c] (%)	Proportion of hydro-soluble protein in total protein=$\frac{P_{Lowry}}{Nea \times NTP} \times 100$(%)
P. cruentum	9.04±0.69	57.33±3.84	46.06±0.97	80.34±1.69
A. platensis	8.53±0.20	53.51±1.10	37.19±2.67	69.50±5.00
C. vulgaris	7.81±0.18	49.59±1.04	21.50±0.34	43.35±0.62
N. oculata	7.41±0.39	46.55±2.14	15.52±0.42	33.34±0.90
H. pluvialis	8.27±0.07	51.73±0.43	14.22±0.69	27.48±1.34

[a] Nea, total nitrogen % (dw) obtained by elemental analysis

[b] NTP, nitrogen-to-protein conversion factor from Table 3

[c] P_{Lowry}, hydro-soluble protein % (dw) at pH 12 and 40 °C calculated by Lowry method

The NTP conversion factor of the crude microalgae and their protein extract depended on the type of microalgae. If we calculate the relative difference between these two values for a single species, we observe that there is almost no difference in the case of red algae and cyanobacteria, whereas it reached almost 10 % for green microalgae (Table 3). These differences can be correlated to the rigidity of the cell wall. Indeed, it is generally accepted that green microalgae possess a more rigid cell wall than red algae or cyanobacteria. More in detail, *P. cruentum* does not have a true cell wall but instead encapsulated by a layer of sulfurized polysaccharides (Arad et al. 1985, 1988; Adda et al. 1986; Geresh and Arad 1991; Geresh et al. 2002; Sobczuk et al. 2006). *A. platensis* has a relatively fragile cell wall mainly composed of murein without cellulose (Lee 2008; Lu et al. 2006). As far as the green microalgae, the chlorophycean *C. vulgaris* and the eustigmatophycean *N. oculata*, both have a cell wall mainly composed of cellulose and hemicelluloses (Payne and Rippingale 2000). The highest difference perceived was for *H. pluvialis*, which has a thick trilaminar cell wall composed of cellulose and sporopollenin in the aplanospore stage (Mendes-Pinto et al. 2001; Montsant et al. 2001; Aflalo et al. 2007). The composition of its cell wall makes this microalga less permeable and extremely mechanically resistant (Hagen et al. 2002).

Taking into account the standard deviation of the three samples considered for each microalga, we could affirm that, at 95 % of confidence level, all the values of total protein content shown in Table 4 are statistically equivalent. This fact is noteworthy, because when we consider the extracted hydro-soluble proteins (Table 4), we observe that their amounts decrease following the same correlation than the differences in NTP value: high extraction for the microalgae with no real cell wall and very low extraction for the most rigid of all. Therefore, the hydro-soluble protein capable to be extracted under alkaline conditions from inside the cell is correlated to the cell wall characteristics and to the freeze-drying process that conserves well the samples but it makes the protein extraction more difficult for some algal species. In addition, the high values of hydro-soluble proteins extracted especially for *P. cruentum* and *A. platensis* could be explained by the possible presence of peptides and free amino acids because no precipitation was carried out (Nguyen and Harvey 1994; Barbarino and Lourenço 2005).

Further evidence was found in the variation in composition relative to essential and non-essential amino acids (Table 5) in the protein extract of the green microalgae. There was a significant drop in the percentage of essential amino acids ranging from 13.9 % for *C. vulgaris* to 49.1 %

Table 5 Percentage (dw) of essential and non-essential amino acids for each microalga and its protein extract

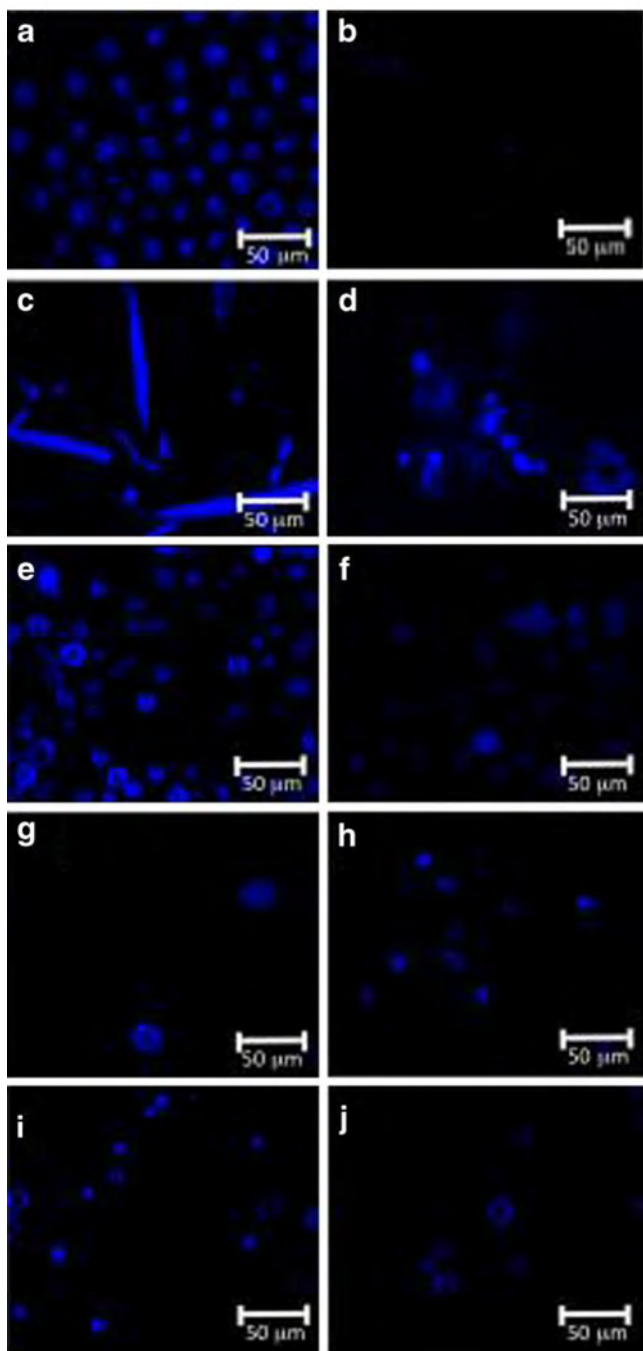|	Crude microalgae			Protein extract		
	Essential	Non-essential	Not identified	Essential	Non-essential	Not identified
P. cruentum	46.81	44.89	8.29	44.58	47.15	8.27
A. platensis	41.76	49.67	8.57	39.87	51.50	8.62
C. vulgaris	41.02	51.03	7.94	35.32	54.70	9.97
N. oculata	41.00	50.46	8.53	20.88	67.47	11.64
H. pluvialis	43.91	48.50	7.58	29.01	60.17	10.81

Fig. 1 Laser scanning confocal microscopic observation before (*left*) and after (*right*) alkaline treatment for each microalga. **a** and **b** *P. cruentum*, **c** and **d** *A. platensis*, **e** and **f** *C. vulgaris*, **g** and **h** *N. oculata*, **i** and **j** *H. pluvialis*

for *N. oculata* and an increase in non-essential amino acids from 6.7 % for the former to 25.2 % for the latter. On the contrary, only small changes in the composition of the proteins were detected for fragile cell wall microalgae, with a percentage difference ranging from 4.5 % for *A. platensis* to 4.8 % for the essential amino acids of *P. cruentum*, whereas non-essential amino

acids difference ranged from 3.5 % for the former to 4.8 % for the latter.

These correlations with the relative hardness of the cell wall can integrate a chemical dimension as well. Indeed, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents (Hagen et al. 2002). But in the case of cellulose-rich cell walls, such as *C. vulgaris* and *N. oculata*, the sodium hydroxide is able to penetrate the microcrystalline structure of cellulose to form alcoholates in a process similar to mercerisation. Sodium hydroxide can also dissolve the hemicelluloses attached to cellulose. The partial permeation of this kind of cell wall can therefore occur by alkaline action. Finally, *A. platensis* has a cell wall rich in amino sugars cross-linked with oligopeptide chains. The former are labile in alkaline conditions by deamidation of the *N*-acetylglucosamine and the latter are soluble in alkaline conditions. The cell wall becomes therefore very permeable allowing the alkaline extraction of proteins. In summary, the chemical action acts in synergy with the mechanical characteristics of the cell wall.

Extraction of proteins together with the evaluation of the conversion factor brought additional evidence that the cell wall of any microalga plays an important role in protein quantification. This means that not taking it into consideration may lead to wrong quantification of the protein content. In addition, for microalgae, there is not a universal conversion factor that can be recommended for all species as demonstrated by comparison of our study with many studies which have been carried out on dozens of different microalgae. Therefore, every time accurate quantification of protein is needed, it will be required to evaluate the conversion factor. In addition, this study showed a correlation between the cell wall rigidity and/or the chemical structure and the differences in NTP conversion value. Microalgae with fragile cell wall did not show significant differences with their protein extract, which was the complete opposite for the microalgae having a rigid cell wall that showed noticeable difference on evaluating the conversion factor of their protein extract, and therefore, breaking or permeabilize the rigid cell wall of *C. vulgaris*, *N. oculata* and *H. pluvialis* is strongly required to prevent underestimation of the protein content after extraction. Logically, there is no need for cell wall breaking for *P. cruentum* since it does not have a cell wall and concerning *A. platensis* soft cell wall disruption technique is needed to make sure that its fragile cell wall will not hinder the extraction of any intracellular components.

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