Algal Research 2 (2013) 145–153

Contents lists available at SciVerse ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Optimizing protein isolation from defatted and non-defatted *Nannochloropsis* microalgae biomass

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ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form 30 January 2013
Accepted 1 February 2013
Available online 27 February 2013

Keywords:
Amino acid content
Amino acid composition
Biofuel co-product
Microalgae
Nannochloropsis spp.
Protein isolation

ABSTRACT

Microalgae are a promising source of lipids for biofuel production. To improve the economic feasibility and sustainability of this biofuel feedstock, one should create value for co-products after lipid extraction. Thus, protein isolation from the defatted biomass presents an opportunity. To extract algae protein, temperature and pH were evaluated to maximize the extraction from *Nannochloropsis* biomass. Maximum quantity of protein was solubilized at 60 °C and pH 11 and recovered at pH 3.2. The isolated protein fractions contained 56.9% and 40.5% protein when using isopropanol (IPA) defatted and non-defatted biomass as the starting materials, with protein yields being 16 and 30%, respectively. The IPA-defatting treatment significantly decreased the protein extraction yield. These values are low compared with soybean protein isolates (>90% protein and ~60% yield). The relatively high protein content (>34%) in the pH 11 insoluble fraction indicates needs for further extraction optimization. The nitrogen and amino acid content of the initial materials and all the fractions were determined and the calculated nitrogen to protein conversion factor was in the range of 4.06–4.70. The possibility of the presence of conjugated protein, i.e., N-containing glycoproteins, is also discussed.

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1. Introduction

Microalgae are a promising source of lipids for the production of biofuels because of their high productivity and efficiency, and their capability to fix CO_2. Lipids can be extracted and converted to fuels [1]. Green algae species (photosynthetic and autotrophic) have been proposed as a means to reduce CO_2 released to the atmosphere by industrial processes [2]. In order to increase the economic feasibility of using microalgae as biofuel feedstock, it is necessary to add value to the co-products derived after lipid extraction. *Nannochloropsis* spp. is a marine green algae belonging to the *Eustigmataceae* family that can yield about 20% lipids by solvent extraction [3]. The type of solvent used for the extraction can have an impact on the quality of the extracted lipids and the defatted biomass. The use of hexanes, typically used for lipid extraction from oilseeds, is impractical for *Nannochloropsis* spp. because of the high amount of polar lipids in the cell [3]. In addition, the presence of water reduces the extraction efficiency; consequently, the use of hexanes requires drying the biomass before the extraction, thus increasing operational costs [4]. The use of chloroform–methanol mixtures can completely extract lipids [5], however because of the toxicity concern they are often only used at a laboratory scale [4]. Lipids can also be extracted using sub- and supercritical fluids [6], however these methods have high energy requirements that impact on the processing cost [4]. The use of isopropanol (IPA) appears to be an effective means to extract lipids from *Nannochloropsis* spp. [4]. The defatted biomass may be used to recover protein and cell wall components for applications as food and feed ingredients [7], cosmetics [8,9], and feedstock for the preparation of antioxidants [10]. Antioxidant peptides have been produced from microalgae protein [10].

Protein isolates from oilseeds, such as soybeans, have been used as functional food ingredients and industrial products. Soy protein isolate is the most common protein isolate used in foods. The production of soy protein isolate is based on protein solubilization in alkaline conditions and isoelectric point precipitation of the proteins from the defatted soybean meal [11]. Unicellular organisms, such as microalgae and yeast, have been proposed as protein sources [7,12] and procedures for protein isolation have been developed for yeast and the protein-producing microalga *Spirulina platensis* [12,13]. In these cases, microorganisms were grown to produce protein as the main product and the protein isolates were recovered by similar procedure as for soy isolate production. In the case of *S. platensis* approximately 80% of the protein nitrogen has been isolated from hexane defatted biomass using water washes [14]. *Nannochloropsis* spp. is an oil producing microalga that was reported to contain a substantial amount of algaenans, which are a recalcitrant insoluble polymer of long chain alkyl units linked by ether bonds and they are resistant to acidic and alkaline hydrolysis [15]. In order to improve the nutritional value and functional properties of defatted *Nannochloropsis* spp. biomass, its protein fraction should be isolated from the rest of the biomass components.

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http://dx.doi.org/10.1016/j.algal.2013.02.001

In order to successfully produce a protein isolate it is necessary to use a reliable protein quantification method. The presence of non-protein nitrogen substances in the microalgae may make the protein quantification erroneous, particularly when the total crude nitrogen is quantified using the Dumas combustion method with the common nitrogen-to-protein conversion factor (same as protein to nitrogen ratio) of 6.25. The non-protein nitrogen and protein contents of microalgae are highly dependent on the species and the physiological stage of the cells [16].

The objectives of the present study were to identify the best parameters for protein extraction from defatted *Nannochloropsis* spp. biomass using the basic pH protein solubilization and isoelectric point (acid) precipitation method; and secondly to characterize the products obtained by the best protein separation conditions. In addition, the contribution of non-protein nitrogen to protein determination was evaluated.

2. Materials and methods

2.1. Microalgae

Nannochloropsis spp. was purchased as a frozen algae paste (12–15% solid content) from Seambiotic Ltd. (Tel Aviv, Israel).

2.2. Solubility curve determination and preliminary protein extraction

Cell mass was defatted by the Folch, Lees and Stanley procedure with chloroform:methanol (2:1) at room temperature (20–25 °C) [5]. The defatted biomass was air-dried at room temperature and suspended in deionized water at 1% solid concentration. To determine the effect of pH on solubility and protein extraction, two biomass dispersions were prepared for pH adjustment. The initial pH was measured and then the pH was raised or reduced with 2 M NaOH or 2 M HCl with 30 min stabilization. Aliquots (1 mL) were sampled and centrifuged at 10,000 *g* for 10 min at 5 °C, and the supernatant was collected and frozen for later protein quantification.

To study protein extraction under very alkaline conditions, 1% biomass suspensions were prepared in water, 0.5 M NaOH, 1 M NaOH, or 1 M NaOH + 0.05% (w/v) β-mercaptoethanol. Protein was extracted at ambient temperature (20–25 °C) under constant shaking (100 rpm) for 1, 5, and 16 h. To study the effect of the temperature, 1% biomass suspensions were prepared and the initial pH was adjusted with 2 M NaOH to 9, 11, and 13. The treatments were incubated for up to 16 h at 30, 45, and 60 °C under constant shaking (100 rpm) for protein extraction with frequent sampling.

Based on the results obtained, another experiment with 50 mL of 1% biomass suspensions was conducted at 60 °C and various pHs using a pH STAT-Titrino 718 (Metrohm AG, Herisau, Switzerland) to maintain the system at the constant pH set. Aliquots (1 mL) were sampled at selected times to evaluate effect of extraction time on protein extraction. In this experiment, extractions from both defatted and non-defatted biomass were evaluated. Sonication was tested as a pretreatment for improving protein extraction from both materials. A laboratory ultrasonicator XL (Misonix, Newtown, CT, USA) equipped with a 1/2 in. tip probe at 100% of amplitude was used for 1-min treatment at 300 W maximum power and 20 kHz frequency for cell disruption. The sonication was done in an ice bath to minimize temperature increase and possible unwanted reactions. Two sonication treatments were applied to the non-defatted material: for one treatment, the entire diluted system was sonicated, and for the other, the algae paste was sonicated before diluting to 1% biomass concentration.

2.3. Algae protein isolation procedure

Protein isolation was performed on both defatted and non-defatted material for comparison. Under the first scheme, the cells were sonicated in several 10 mL aliquots using the laboratory ultrasonicator XL as described above, but with a 1/8 in. tip probe at 50% of full amplitude. The total sonication time was 4 min, conducted as 30-s pulses followed by 2 min resting periods in ice bath. The sonicated biomass was immediately mixed with IPA to a final ratio of 70:30 (w/w) IPA: water and 5.7% (w/w) final total solids content to minimize enzyme activity and to extract oil. The mixture was refluxed at 80 °C for 1 h to extract total lipids [4]. While still hot, the mixture was centrifuged at 3000 *g* for 10 min and the pellet was dispersed in IPA:water (88:12, w/w) for a second lipid extraction at 80 °C for 30 min. The resulting pellet after centrifugation was allowed to dry in a fume hood overnight to about 34% solids content. Starting with 20 g of (dry weight) IPA-defatted biomass, a 5% solid suspension was prepared. This concentration is typically used by the industry for soybean protein isolate preparation [11]. Protein was extracted under continuous stirring at 60 °C and pH 11 for 5 h using the pH STAT-Titrino 718 with a thermostat-controlled jacketed vessel according to the procedure illustrated in Fig. 1.

For the second protein isolation scheme for non-defatted biomass, the *Nannochloropsis* spp. paste was blanched at 80 °C for 5 min to minimize possible enzymatic proteolysis before the ultrasonication cell disruption treatment. Sonication was done similarly as the first scheme. The sonicated paste was adjusted to 5% solids and protein was extracted at pH 11 under conditions similar to those of the first scheme but without the solvent-defatting process.

For both extraction schemes, the mixture was centrifuged at 20,000 *g* at 20 °C for 15 min after incubation. The pellet (pH 11 insoluble fraction) was washed with water at 60 °C and pH 11, and re-centrifuged. The supernatants from both centrifugation steps were combined, cooled to 5 °C by immersing the container in chilled water, and the pH was adjusted to 3.2 with 2 M HCl. The material was then centrifuged at 20,000 *g* and 5 °C for 15 min. The supernatant

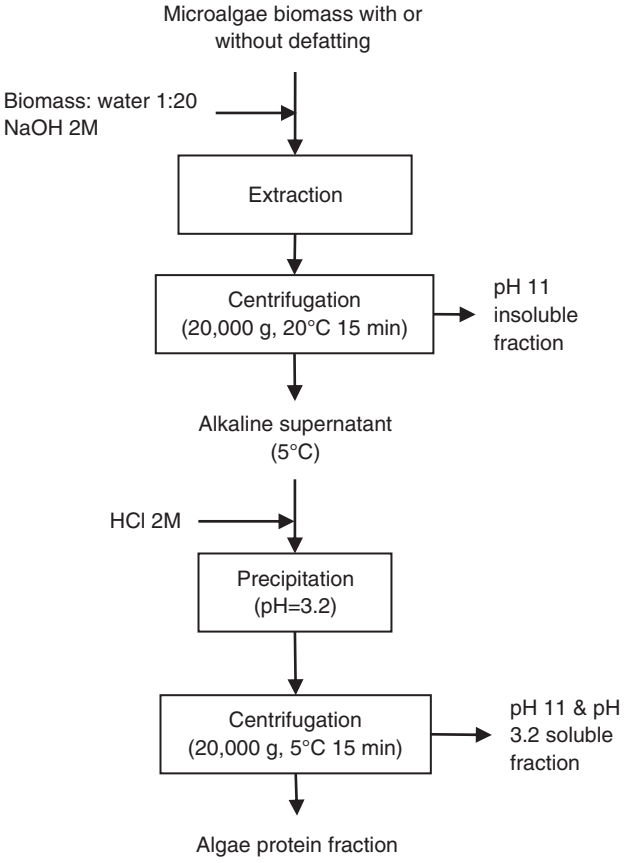

Fig. 1. Flow diagram of the *Nannochloropsis* spp. protein isolation process.

(pH 11 and pH 3.2 soluble fraction) was separated from the pellet (algae protein fraction) and the pH of both fractions was adjusted to 7. All the fractions were freeze-dried and stored at −22 °C until further analysis. Two replicates for each processing scheme were conducted.

2.4. Protein content determination

The protein contents of all fractions from the protein isolation process were determined by using three different methods.

2.4.1. Soluble protein

Soluble protein was determined according to Lowry et al. [17] and measured spectrophotometrically at 750 nm. For this method, sodium deoxycholate and trichloroacetic acid (TCA) were used to precipitate the protein and remove any potential interfering substances in the supernatant after centrifugation at 10,000 *g* for 10 min at 5 °C [18]. The precipitated protein was re-solubilized with 0.1 mL 1 M NaOH. Sodium deoxycholate and TCA precipitated bovine serum albumin (BSA) were used to prepare a standard curve for protein quantification.

2.4.2. Total nitrogen

Total nitrogen content was determined by using the Dumas method [19] using a Rapid N III Nitrogen Analyzer (Elementar Americas, Mt. Laurel, NJ, USA).

2.4.3. Amino acid content and composition

Amino acid content and composition were determined by the Chemical Laboratories of University of Missouri's Agricultural Experiment Station (Columbia, MO) [20]. Based on the amino acid composition, the amino acid residue content for each fraction was calculated. The N-protein conversion factor was then determined as the ratio between the total mass of amino acid residues divided by its nitrogen content as measured by the Dumas combustion method. The total amount of protein nitrogen was also calculated from the amino acid residue composition and the nitrogen contribution of each residue (i.e., true protein nitrogen) and it was compared with the total nitrogen content determined by the Dumas method. In this way, the non-protein nitrogen present in each fraction was determined as the difference between the total nitrogen and protein nitrogen.

2.5. Protein visualization by electrophoresis

The proteins in the different fractions of the protein isolation process were characterized by using SDS-PAGE and a Bio-Rad Miniprotean System™ with Any kD™ gels (Bio-Rad Laboratories, Hercules, CA, USA) according to the procedure of Laemmli [21]. Suspensions containing 0.2–0.5 mg of nitrogen/mL were prepared in sample buffer and after reduction at 95 °C for 10 min, they were spun at 10,000 *g* for 2 min. Each lane of the gel was loaded with 10 μL of the supernatant. Gels were run at constant voltage (100 V) and stained for 2 h in 0.22% Coomassie blue in 4:5:1 water:methanol:acetic acid solution. Overnight de-staining was performed with 15:3:2 water:methanol:acetic acid.

2.6. Carbohydrate determination

All fractions were hydrolyzed under acidic conditions according to the NREL Laboratory Analytical Procedure NREL/TP-510-42618 [22]. The total amount of simple sugars in the acid hydrolysates was determined by the phenol–sulfuric acid method [23].

2.7. Acyl lipid quantification

Acyl lipid distribution in the different fractions from the non-defatted biomass separation scheme was determined by direct esterification and transesterification of acyl lipids into methyl esters by reacting 0.1 g of material and 5 mg heptadecanoic acid (17:0, internal standard) with 6 mL 6% H_2SO_4 in methanol. The reaction was allowed for 20 h in an oven at 60 °C in sealed vials. The resulting material was extracted with 20 mL of hexanes and washed with deionized water. The hexane extracts were analyzed by GC using a Hewlett Packard Model 5890 Series II (Agilent, Santa Clara CA, USA) GC with a flame ionization detector and a Supelco SP-2340 (Bellefonte, PA, USA) capillary column of 60 m (length) × 0.25 mm (I.D.) × 0.2 μm (film thickness). The injector and detector were maintained at 250 °C and the oven temperature program was: 100 °C initial temperature for 1 min, 4 °C/min heating rate to 230 °C final temperature, and 12 min-hold at 230 °C. The carrier gas (helium) flow rate was 2.9 mL/min and the split ratio was 50:1. The total FAME content in each fraction was calculated by using the total FAME area relative to the area of the internal standard.

2.8. Ash content

Ash contents of all fractions were determined using a modification of the AOCS Bc 5-49 standard method [24] by incinerating overnight in a muffle furnace at 550 °C.

2.9. Mass balance determination

All fractions were quantified and mass balances were calculated on a moisture-free basis in order to estimate the recovery of each individual biomass component at each step.

2.10. Statistical analysis

All treatments and analyses were run in duplicate, the results were analyzed using one-way ANOVA, and means were compared with Proc GLM by using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The significance level was established at $P = 0.05$ unless otherwise noted.

3. Results and discussion

3.1. Protein solubility curve

The amount of protein dissolved in water at ambient temperature from a 1% suspension of defatted biomass at selected pH ranging between 1.9 and 13 is illustrated in Fig. 2a. The unadjusted pH of the suspension was 7.2. An increase in protein solubility was observed with increasing pH. Minimum solubility was observed at pH 3.2, and this was used as the isoelectric point for this algae protein. After this solubility result, basic pH extraction conditions were further explored since only 2.5% of the biomass was solubilized as protein at the most alkaline condition, which was much lower than expected.

Alkaline conditions were explored to enhance protein extraction. Very alkaline (0.5 and 1 M NaOH) and reducing (0.05% β-mercaptoethanol) conditions were used to increase protein extraction compared with extraction with deionized water (Fig. 2b) at ambient temperature. There were no differences among the alkaline treatments and with the reducing agent. However, as expected, the protein extractions with all alkaline conditions were much greater than that with water. Extraction time was shown to be a very important factor, with greatly increased extraction after 5 h. For all treatments, maximum extraction was approximately 12% of the biomass, however, these pHs are too high to be applicable because of potential amino acid degradation [25].

3.2. Effects of pH and temperature on protein extraction

For all extraction temperatures (30, 45, and 60 °C), pH 13 resulted in the greatest extraction (Fig. 3). At 45 °C and 60 °C, extraction plateaued at approximately 12% of the biomass. However, at 60 °C

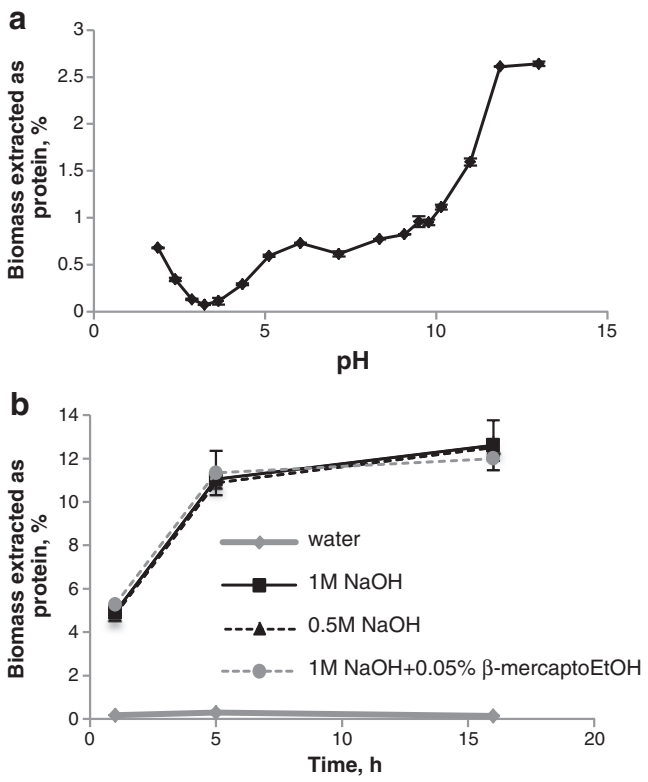

Fig. 2. (a) Protein solubility curve of a 1% defatted *Nannochloropsis* spp. biomass at ambient temperature; (b) protein extraction from 1% dispersions of defatted *Nannochloropsis* spp. biomass at selected NaOH and reducing conditions, and extraction time at ambient temperature. Error bars represent standard deviation of the mean.

the plateau occurred after 2–5 h and at 45 °C after 8–16 h, indicating a much faster extraction at higher temperature. The pH at the end of the extraction decreased from the initial 11 and 9 to the final of 6.8 to 6.2 for all three temperature treatments. For the treatment at pH 13, the final pH did not change significantly. These changes in pH were probably the result of protein dissolution and buffering. At pH 13, the amount of protein dissolved was probably not sufficient to greatly reduce the concentration of hydroxide ions.

3.3. Effect of biomass pretreatment on protein extraction

Because pH had a significant effect on protein extraction, more extractions were performed using an automatic titrator, i.e., pH Stat, to maintain a constant pH. All extractions were done at 60 °C because at this temperature the extraction process was faster than at 45 °C and more effective than at 30 °C. When the extraction pH was held constant, the extraction at pH 11 was much greater than that at pH 9 (Fig. 4) and also greater than when the pH was only initially adjusted to 11 (not maintained by automatic titrator, Fig. 3). The effect of sonication on the extraction was investigated at pH 11. With 1 min sonication of the defatted biomass mixture, there was no difference in protein extraction after 5 h. However, when non-defatted cells were used with either sonicating the concentrated cell paste or sonicating the dispersed 1% solid suspension, the extraction was much greater than when using defatted material. One reason may be that the presence of high concentration of polar lipids (glycolipids and phospholipids) [3] may improve protein extraction because of their surfactant activity [26]. A second possible reason for greater extraction is the presence of more soluble proteins in the non-defatted biomass. It is well known that during IPA or chloroform:methanol lipid extraction, some soluble proteins can be co-extracted with lipids [4,27,28]. So the defatted materials may have contained less soluble proteins. A third possible reason is that there may be some degree of protein denaturation by organic solvents during

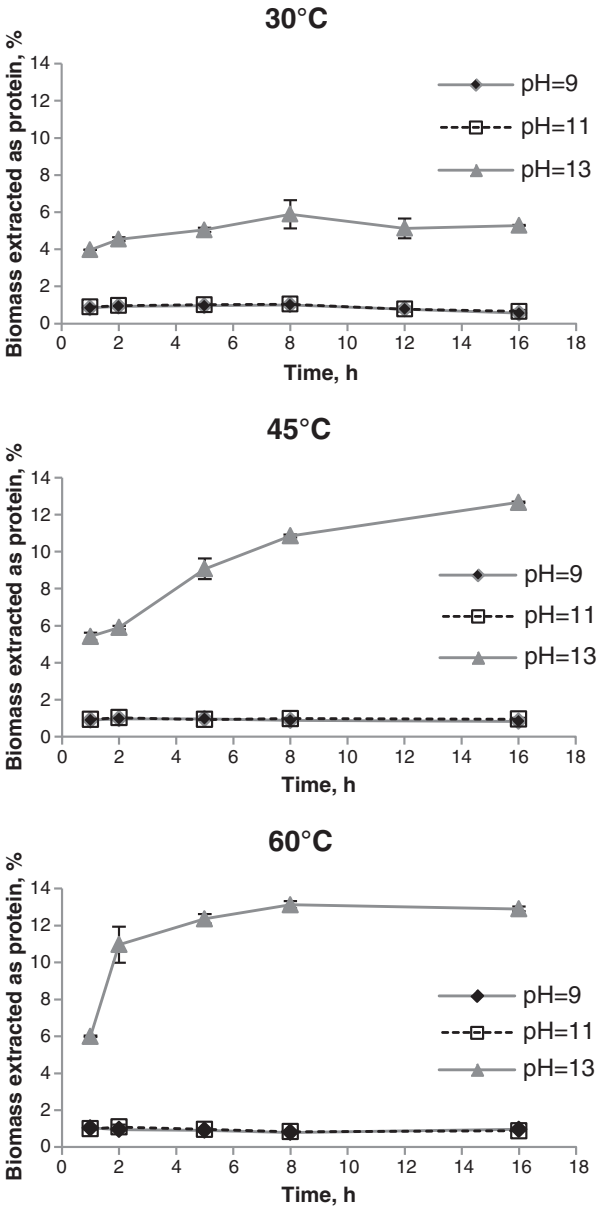

Fig. 3. Protein extraction from 1% dispersion of defatted *Nannochloropsis* spp. biomass at selected pH, temperature, and time. Error bars represent standard deviation of the mean.

lipid extraction, and such denatured proteins could not be easily solubilized even at high pH [17]. Probably, all three reasons are contributing factors to the greater protein yield from the non-defatted biomass.

Sonication of the concentrated algae paste seemed to be more effective in increasing protein extraction than sonication of the final extraction system (same amount of cells, greater volume). More optimization in cell breakage is needed to maximize protein extraction.

3.4. Mass balance of algae components during protein extraction

3.4.1. Total solid mass balance

The distribution of all solid fractions from protein extraction process is shown in Fig. S1. For IPA-defatted material, most of the solids (54%) remained in the pH 11 insoluble fraction, while from the non-defatted material the pH 11 and pH 3.2 soluble fractions accounted for a majority (52%) of the biomass. This was probably in part the result of protein denaturation during IPA lipid extraction at elevated temperature and/or protein dissolved in IPA that was lost during the defatting process (Fig. S1a).

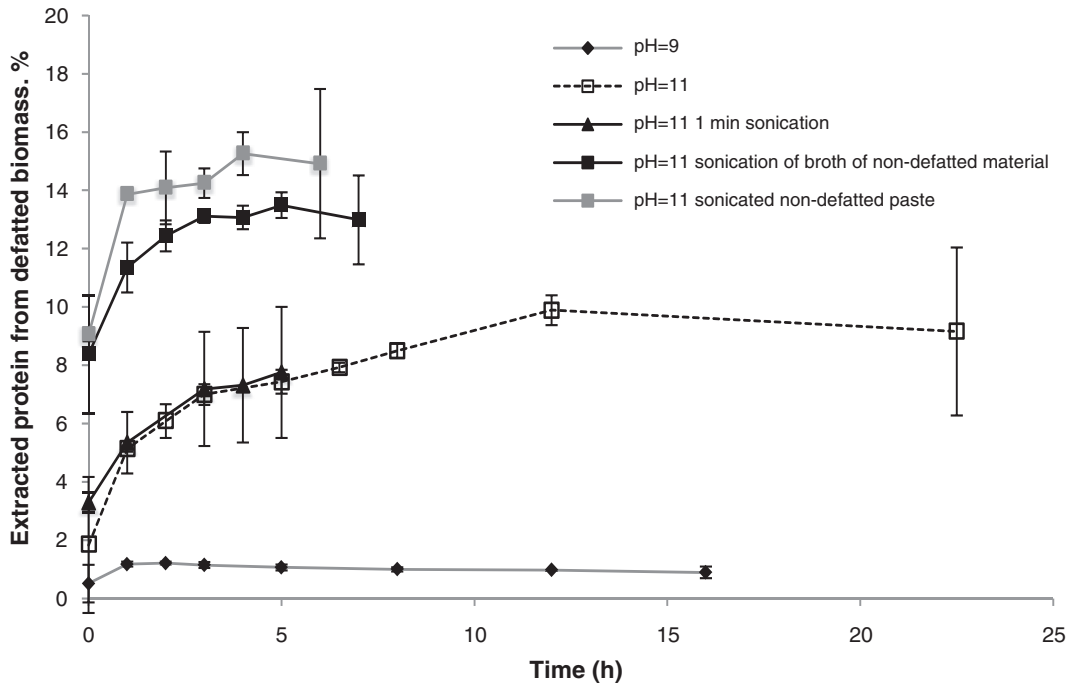

Fig. 4. Protein extraction from 1% dispersion *Nannochloropsis* spp. biomass at selected and constant pH at 60 °C. Error bars represent standard deviation of the mean.

3.4.2. Nitrogen mass balance

For nitrogen recovery, there was no significant difference between the two treatment schemes (Fig. 5a). However, the actual content of nitrogen in the protein fraction from the IPA-defatted biomass (14.0%) was greater than that from the non-defatted biomass ($P<0.05$) (Table 1). The lower nitrogen content in the protein fraction from the non-defatted material (8.94%) was in part the result of the presence of lipids. Total acyl lipids were distributed equally between the insoluble fraction and the protein fraction from the non-defatted treatment (Fig. S1b), and they contributed to the decrease of the nitrogen concentration in the protein fraction.

The total protein recovery determined by the Lowry method was greater than 100% and the variability was substantially higher than that with other methods, especially in the protein fractions (Fig. 5b). This is because to determine the protein content by Lowry method, all the fractions had to be treated in 0.1 M NaOH for protein solubilization. For the initial material, not all the protein present in the biomass was solubilized, leading to over estimating the protein recovery in the subsequent fractions because all the percentages were relative to the value for the initial material. On the other hand, the pH 11 insoluble fraction was mostly exhausted of proteins that could be further solubilized in alkaline conditions during the Lowry extraction, leading to underestimating the total protein present in this fraction. Therefore, Lowry protein quantification in such algae extraction applications is not a suitable method.

The recovery of the amino acids in the different fractions (Fig. 5c) shows that in the protein fraction and pH 11 and pH 3.2 soluble fractions from the non-defatted material, there were more amino acids partitioned than in those fractions from the IPA-defatted material. Also, the amino acids in the pH 11 insoluble fraction of the IPA-defatted treatment was greater than that of the non-defatted material ($P<0.05$). This may be because of the denaturing effects of IPA and heat on the proteins, thus affecting protein solubility and extraction. IPA-defatted biomass was exposed to the alcohol and heat (80 °C) for 1 h during oil extraction, while the non-defatted cells were exposed to heat (80 °C) in aqueous system for only 5 min. Another possibility is that the presence of polar lipids (with surfactant activity) could have helped solubilize and extract the protein in the non-defatted material [26].

The algae protein isolation method used in this study was a modification of the method used for the preparation of soy protein isolates. Soy protein isolates are produced from hexane-defatted soy flakes (white flakes). Soy protein isolate accounts for approximately 60% of the total protein present in the defatted white flakes and their protein content is generally >90% [11]. Protein isolate produced from *Tetraselmis* sp. had a protein content of 64% with a protein yield of only 7% [29]; however the methodology to produce it included not only pH treatment but also ion exchange chromatography for purification. Yeast protein isolates have also been produced using the same technique as for soy protein, achieving 65% protein recovery in the alkaline extract and protein contents of >83% in the isolate [12]. Recoveries of protein from *Nannochloropsis* spp. biomass in this study were 16 and 30% for the IPA-defatted and non-defatted treatments. The lower protein yields compared with those of soybean or yeast protein isolates were probably the result of the type of proteins present and their actual physiological function in the cell. Storage proteins that are present in protein bodies, such as those in soybeans, are usually easily extractable, while proteins that are associated with cell walls may be very difficult to extract. Protein isolate of *S. platensis* produced by alkaline solubilization and isoelectric precipitation contained about 68% protein, however, the starting non-defatted material contained about 63% protein, so the concentration process increased the protein content by only 5% [13]. For the *Nannochloropsis* spp. protein extraction, the protein content of the isolated protein (40.5%) from the non-defatted material was lower than that from the IPA-defatted material (56.9%) because of the presence of approximately 20% lipids (quantified as fatty acid methyl esters) (Table 2). The increase in protein content in the isolated protein compared with the starting material was about 7% for the IPA-defatted material, and about 8% for the non-defatted material.

3.4.3. Algae N-protein conversion factor

It is generally assumed that N-protein conversion factor for plant material is 6.25 (6.25 g of protein per g of total nitrogen)[30]. This factor is typically used in estimating the protein content of soybean-based materials, although the actual N-protein factor for soybeans is 5.71, and also for most food products [11]. For microalgae, there was evidence

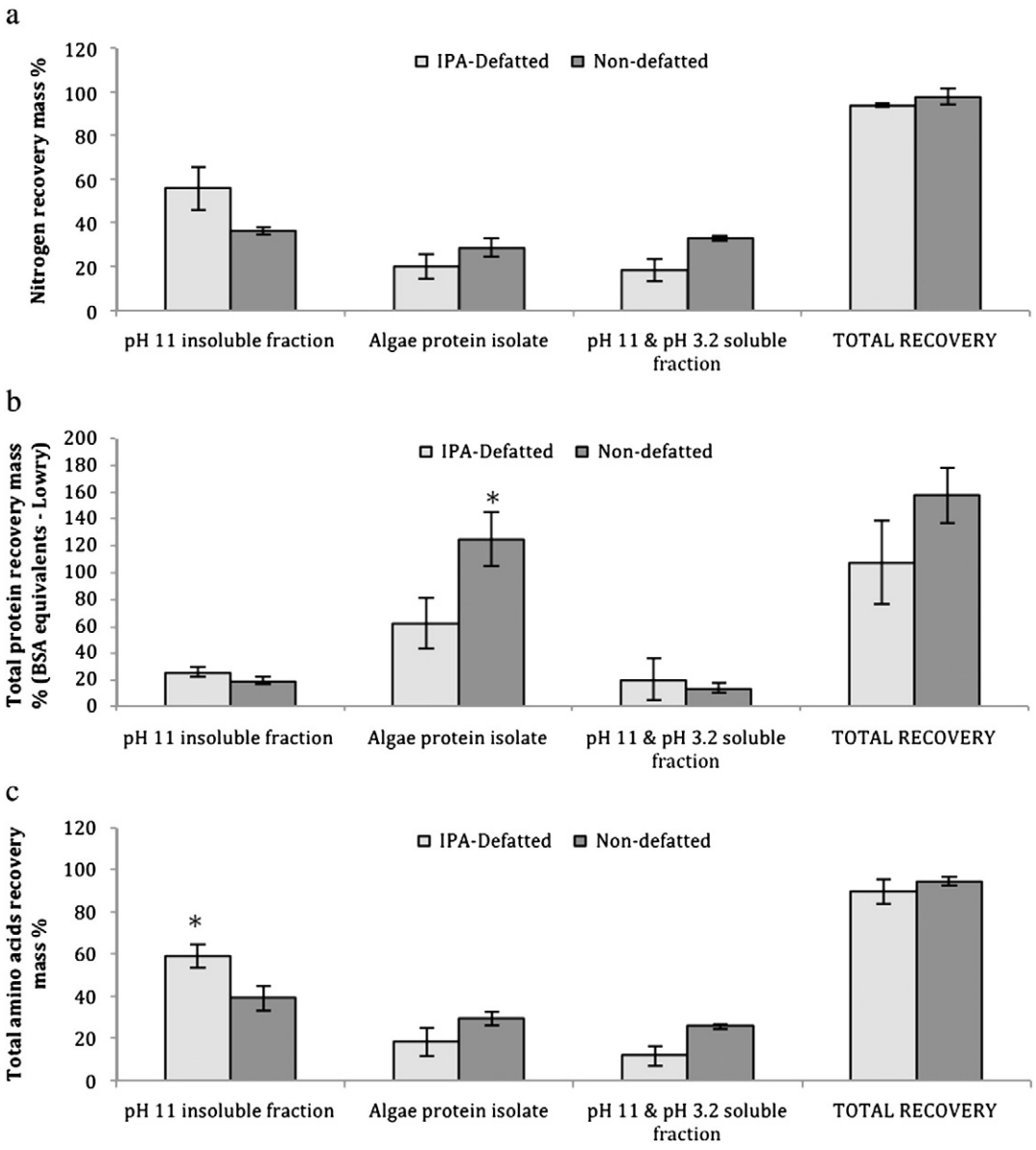

Fig. 5. Mass balance and total recovery of total crude nitrogen (a), Lowry protein (b), and total amino acids (c) of IPA-defatted and non-defatted *Nannochloropsis* spp. biomass from the algae protein isolation procedure. *Indicates significant differences between treatments within a fraction with $P<0.05$. Error bars represent standard deviation of the mean.

that the N-protein factor was not only dependent on the type of algae, but also on cell growing stage [16]. The N-protein factor values calculated for the initial biomass, the pH 11 insoluble fraction, and the isolated algae protein obtained from both extraction schemes (Table 1) are within the range of those previously reported [16]. However, the N-protein factor values obtained for the pH 11 and pH 3.2 soluble

Table 1
Nitrogen composition in different fractions from the protein isolation process.[a]

	N (%)		Lowry protein (BSA%)		AA (%)		AA residues (%)		Nitrogen recovered from AA residues (%)		N (%)		N-protein factor	
											Protein	Non protein		
IPA-defatted														
Initial material	11.4	(0.12)	13.9	(0.52)	57.9	(1.30)	49.6	(1.12)	8.04	(0.18)	70.5	29.5	4.35	(0.05)
pH 11 insoluble fraction	10.0	(0.58)	5.59	(0.24)	54.5	(0.88)	46.7	(0.73)	7.51	(0.08)	74.9	25.1	4.66	(0.34)
Algae protein fraction	14.0	(0.08)	53.5	(1.03)	66.3	(2.97)	56.9	(2.55)	8.95	(0.35)	63.8	36.2	4.06	(0.21)
pH 11 & pH 3.2 soluble fraction	5.32	(0.21)	6.40	(3.27)	17.2	(1.03)	14.7	(0.90)	2.37	(0.11)	44.5	55.5	2.76	(0.28)
Non-defatted														
Initial material	7.46	(0.17)	7.82	(0.19)	38.0	(0.84)	32.5	(0.72)	5.26	(0.12)	70.6	29.4	4.36	(0.19)
pH 11 insoluble fraction	7.24	(0.20)	3.96	(0.15)	39.7	(1.19)	33.0	(1.02)	5.40	(0.16)	74.6	25.4	4.70	(0.27)
Algae protein fraction	8.94	(0.33)	41.0	(3.84)	47.3	(1.53)	40.5	(1.31)	6.36	(0.19)	71.2	28.8	4.53	(0.02)
pH 11 & pH 3.2 soluble fraction	4.00	(0.23)	1.73	(0.35)	15.9	(0.08)	13.5	(0.06)	2.12	(0.02)	53.0	47.0	3.39	(0.18)

[a] Values in parentheses are standard deviations of the means.

fractions of both schemes were much lower. This is the result of the presence of N-containing substances that were non-protein. These substances may be inorganic nitrogen-containing salts (nitrates and nitrites), nucleic acids, free ammonium ion, and/or amino sugar derivatives [16,31]. Most of these substances are very soluble, so they are expected to concentrate in the pH 11 and pH 3.2 soluble fractions, thus decreasing the proportion of protein nitrogen and consequently leading to the low N-protein factor.

In the isolated algae protein, the non-protein nitrogen accounted for 28.8 and 36.2% of the total nitrogen from the non-defatted and defatted materials. In these presumed pure protein fractions, the non-protein nitrogen probably originated from post-translational modification of the proteins with N-containing compounds such as N-acetyl glycosamine. Structural proteins are known to be glycosylated in order to provide cellular structure stability and assist membrane anchoring. In legumes and oilseeds, proteins are stored in protein bodies to be mobilized during seed germination, and these proteins are not extensively glycosylated. However, in oil-accumulating microalgae, the extracted proteins are most likely the functional and structural proteins. The additional sugar functional groups they carry may lend these proteins great functional properties that are to be further explored.

The similarity in the molecular weights of the proteins or peptides (Fig. S2), as well as the similarity in amino acid composition as discussed later (Section 3.5) support the theory that the proteins extracted in the isolated protein fraction and the proteins remaining in the pH 11 insoluble fraction, i.e., the presumed cell wall fraction, may be from the same cellular structure. In addition, the similarity of the proportions of protein and non-protein nitrogen in the starting materials, the pH 11 insoluble fraction and the isolated algae protein from both extraction schemes (Table 1) is an evidence of the non-protein nitrogen being closely related to the protein nitrogen, thus further suggesting the non-protein nitrogen is part of the protein structure, and possibly in the amino sugar forms.

3.4.4. Ash mass balance

The recovery of ash in the isolated protein was similar for both treatment schemes (Fig. S1c). The pH 11 insoluble fraction from the defatted biomass had greater ash content than the non-defatted material. On the other hand, the ash partitioning into the pH 11 and pH 3.2 soluble fraction was greater for the non-defatted treatment. The starting IPA-defatted material had about 5% lower concentration of ash than the non-defatted material. It is possible that many soluble minerals may have been removed during the aqueous-IPA lipid extraction, leaving biomass with not only a lower ash content, but also enriched with minerals, such as calcium and magnesium, with low solubility under alkaline conditions. This explains the greater ash retention in the pH 11 insoluble fraction of the defatted treatment.

3.4.5. Carbohydrate mass balance

The distribution of carbohydrates as determined by using the phenol–sulfuric acid method is shown in Fig. S1d. The glucans partitioned relatively equally between the pH 11 and pH 3.2 soluble fractions and the pH 11 insoluble fraction. Although they were not fully characterized, the carbohydrates in the pH 11 and pH 3.2 soluble fractions were possibly soluble sugars and small oligosaccharides. The carbohydrates remaining in the pH 11 insoluble fraction were probably insoluble polysaccharides with structural functions in the cell wall. It is important to note that the calibration curve used for the carbohydrate quantification was prepared with glucose and that different carbohydrates may have different responses to the spectrophotometric method [23]. Therefore, this result may not accurately represent the total content of carbohydrates in each fraction if the hydrolysates contained a complex mixture of simple sugars. In a preliminary HPLC analysis of the hydrolyzed carbohydrates, no other simple sugar than glucose was observed. However, other acidic and basic sugars might not have been detected with this quantification method. In future studies, a HPLC quantification method for derived sugars, particularly for the nitrogen-containing sugars, should be established.

3.5. Molecular weight distribution and amino acid composition of isolated algae protein

For both treatments, presence of a high molecular weight protein of approximately 250 kDa size was evident. The intensity of this band was much greater in the pH 11 insoluble and protein isolate fractions, when compared with the initial material (Fig. S2). This high molecular weight band may have been produced during the protein extraction process since alkaline conditions may induce protein cross-linking through the formation of lanthinonine, lysinoalanine, or dehydroalanine [32,33]. In the pH 11 and pH 3.2 soluble fractions, there was no clear band, however, there was greater intensity of the dye in the low molecular weight zone, indicating the presence of small peptides that were not precipitated during the acidification of the process.

The similarity of the SDS-PAGE band profiles between the isolated protein and pH 11 insoluble fraction also suggests that the protein extraction may not be complete under the conditions used in this study. Further investigation using modified extraction conditions is needed to ensure full protein isolation from microalgae biomass.

The amino acid compositions of the initial material, the isolated algae protein, and the pH 11 insoluble fraction of both treatment schemes were similar for most amino acids (Table 3). Changes in amino acid composition were most evident in the pH 11 and pH 3.2 soluble fractions. The proportion of proline in both treatments increased in the pH 11 and pH 3.2 soluble fractions, especially in the non-defatted treatment. The proportion of hydroxy-lysine also increased in the pH 11 and pH 3.2 soluble fractions. As the result of the dramatic increase of proline, the content of the other amino acids in the pH 11 and pH 3.2 soluble fractions decreased. Lanthionine, an amino acid typically formed during the degradation of cystine in alkaline conditions [32] was found only in the isolated fractions. Its concentrations were the greatest in the fractions with greatest cysteine concentration in both treatments: the pH 11 and pH 3.2 soluble fractions (Table 3). Arginine can degrade into ornithine in alkaline conditions [34]. The concentration of ornithine increased while the concentration of arginine decreased in all isolated fractions compared with the initial material in both treatments. The formation of ornithine and lanthionine during the protein isolation process shows protein degradation. This is an

Table 2
Ash, carbohydrate and lipid composition of the different fractions from the algae protein isolation process.[a]

	IPA-defatted				Non-defatted					
	Ash (%)		Glucose equiv. (%) (acid hydr. + pH H_2SO_4)		Ash (%)		Glucose equiv. (%) (acid hydr. + pH H_2SO_4)		FAME (%)	
Initial material	12.4	(–)	15.3	(0.63)	17.8	(–)	12.6	(0.33)	10.5	(0.64)
pH 11 insoluble fraction	16.5	(2.79)	10.7	(1.17)	14.0	(0.42)	8.77	(0.41)	15.2	(0.67)
Algae protein fraction	8.23	(1.30)	6.68	(2.34)	7.37	(1.04)	5.59	(0.52)	20.5	(0.43)
pH 11 & pH 3.2 soluble fraction	51.5	(3.55)	15.3	(4.32)	52.2	(1.91)	9.15	(1.14)	0.00	(0.00)

[a] Values in parentheses are standard deviations of the means.

Table 3
Amino acid compositions (%) of the different fractions from the protein isolation process.[a]

Amino acid	IPA-defatted								Non-defatted							
	Initial material		Isolated algae protein		pH 11insoluble fraction		pH 11 & pH 3.2 soluble fraction		Initial material		Isolated algae protein		pH 11 insoluble fraction		pH 11 & pH 3.2 soluble fraction	
Taurine	0.01	(0.00)	0.03	(0.02)	0.01	(0.01)	0.11	(0.02)	0.04	(0.02)	0.04	(0.00)	0.03	(0.00)	0.18	(0.05)
Hydroxyproline	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.06	(0.09)
Aspartic acid	9.49	(0.18)	10.4	(0.24)	9.45	(0.08)	9.84	(1.10)	8.25	(0.23)	10.0	(0.26)	9.73	(0.29)	5.54	(0.17)
Threonine	4.74	(0.10)	4.84	(0.06)	4.95	(0.08)	4.50	(0.66)	4.32	(0.09)	4.97	(0.09)	5.10	(0.15)	3.06	(0.18)
Serine	3.74	(0.14)	3.57	(0.04)	3.92	(0.20)	3.91	(0.49)	3.33	(0.02)	3.83	(0.00)	3.89	(0.12)	2.49	(0.19)
Glutamic acid	12.1	(0.32)	13.1	(0.21)	11.7	(0.12)	13.9	(0.65)	10.1	(0.17)	12.1	(0.25)	11.4	(0.39)	7.70	(0.20)
Proline	5.94	(0.06)	4.69	(0.23)	4.82	(0.14)	14.6	(2.48)	14.8	(0.22)	5.60	(0.16)	5.41	(0.32)	38.4	(1.32)
Lanthionine	0.00	(0.00)	0.18	(0.01)	0.14	(0.03)	0.37	(0.00)	0.00	(0.00)	0.12	(0.08)	0.11	(0.12)	0.47	(0.06)
Glycine	5.45	(0.13)	5.57	(0.12)	5.37	(0.14)	6.90	(0.01)	5.15	(0.13)	5.48	(0.08)	5.32	(0.07)	4.50	(0.24)
Alanine	6.72	(0.11)	6.79	(0.38)	7.00	(0.18)	6.59	(0.72)	6.42	(0.16)	6.94	(0.25)	7.00	(0.16)	5.97	(0.02)
Cysteine	0.99	(0.01)	0.53	(0.03)	0.52	(0.04)	1.68	(0.41)	0.88	(0.02)	0.44	(0.06)	0.51	(0.09)	0.97	(0.04)
Valine	6.39	(0.17)	6.64	(0.55)	6.77	(0.37)	4.46	(1.12)	5.63	(0.15)	6.84	(0.35)	6.79	(0.21)	3.47	(0.15)
Methionine	1.98	(0.03)	2.02	(0.03)	2.33	(0.04)	1.11	(0.30)	2.04	(0.06)	2.52	(0.12)	2.67	(0.07)	0.78	(0.00)
Isoleucine	4.73	(0.13)	4.91	(0.48)	4.98	(0.33)	2.96	(0.79)	4.20	(0.11)	5.05	(0.27)	5.14	(0.11)	2.23	(0.07)
Leucine	9.50	(0.23)	9.91	(0.90)	10.5	(0.59)	5.45	(1.66)	8.39	(0.23)	10.3	(0.50)	10.4	(0.27)	4.17	(0.21)
Tyrosine	3.98	(0.13)	4.28	(0.32)	3.90	(0.05)	2.63	(0.57)	3.29	(0.10)	4.04	(0.14)	4.07	(0.22)	1.97	(0.14)
Phenylalanine	5.23	(0.13)	5.53	(0.53)	5.80	(0.27)	2.99	(0.73)	4.70	(0.14)	5.60	(0.23)	5.80	(0.11)	2.21	(0.09)
Hydroxylysine	0.52	(0.02)	0.05	(0.02)	0.05	(0.01)	3.11	(1.12)	2.30	(0.00)	0.15	(0.09)	0.16	(0.02)	6.63	(0.69)
Ornithine	0.27	(0.02)	0.97	(0.30)	0.81	(0.27)	1.69	(0.10)	0.39	(0.02)	0.93	(0.11)	0.87	(0.01)	1.32	(0.01)
Lysine	7.18	(0.17)	6.55	(0.15)	6.27	(0.13)	7.69	(0.52)	6.84	(0.17)	6.67	(0.15)	6.34	(0.35)	5.88	(0.26)
Histidine	1.98	(0.02)	1.94	(0.16)	2.16	(0.05)	1.39	(0.35)	1.77	(0.04)	2.14	(0.09)	2.24	(0.10)	0.76	(0.04)
Arginine	6.65	(0.18)	4.96	(0.08)	6.34	(0.30)	3.57	(0.32)	5.85	(0.16)	4.56	(0.03)	5.42	(0.12)	0.85	(0.10)
Tryptophan	2.45	(0.01)	2.54	(0.08)	2.24	(0.26)	0.50	(0.70)	1.29	(0.08)	1.59	(0.14)	1.54	(0.02)	0.34	(0.48)
Total	100	(2.29)	100	(4.96)	100	(3.70)	100	(14.8)	100	(2.30)	100	(3.46)	100	(3.30)	100	(4.79)

[a] Values in parentheses are standard deviations of the means.

important consideration if the protein isolate is to be used in food or feed formulations.

4. Concluding remarks

In this study, a protein-rich fraction was isolated from IPA-defatted and non-defatted *Nannochloropsis* spp. algae biomass under high pH and elevated temperature conditions. There was a substantial amount of non-protein nitrogen present in the biomass that partitioned into the various fractions. The isolation process produced a protein-rich product that may have unique functional properties due to the seemingly high degree of glycosylation. However, the overall recovery of the protein is relatively low, especially when compared with sources such as soybean and yeast, and should be further improved.

Acknowledgments

The authors express their sincere gratitude to Phillips 66 for providing financial support and to Charlwit Kulchaiyawat, PhD graduate student, Jun Yi Lio, MS graduate student, and Devin Maurer, Technician, of the Department of Food Science and Human Nutrition at Iowa State University for their assistance with protein extraction, ash determination, and carbohydrate preliminary HPLC analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2013.02.001.

References

[1] E. Suali, R. Sarbatly, Conversion of microalgae to biofuel, Renewable and Sustainable Energy Reviews 16 (2012) 4316–4342.
[2] Y. Chisti, Biodiesel from microalgae, Biotechnology Advances 25 (2007) 294–306.
[3] G. Wang, T. Wang, Characterization of lipid components in two microalgae for biofuel application, Journal of the American Oil Chemists' Society 89 (2012) 135–143.
[4] L. Yao, J.A. Gerde, T. Wang, Oil extraction from microalga Nannochloropsis sp. with isopropyl alcohol, Journal of the American Oil Chemists' Society 89 (2012) 2279–2287.
[5] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipides from animal tissues, Journal of Biological Chemistry 226 (1957) 497–509.
[6] M. Herrero, A. Cifuentes, E. Ibanez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae. A review, Food Chemistry 98 (2006) 136–148.
[7] E.W. Becker, Micro-algae as a source of protein, Biotechnology Advances 25 (2007) 207–210.
[8] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, Journal of Bioscience and Bioengineering 101 (2006) 87–96.
[9] P. Stolz, B. Obermayer, Manufacturing microalgae for skin care, Cosmetics & Toiletries 120 (2005) 99–106.
[10] I.C. Sheih, T.K. Wu, T.J. Fang, Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems, Bioresource Technology 100 (2009) 3419–3425.
[11] N.A. Deak, L.A. Johnson, E.W. Lusas, K.C. Rhee, Soy protein products, processing, and utilization, in: L.A. Johnson, P.J. White, R. Galloway (Eds.), Soybeans: Chemistry, Production, Processing, and Utilization, AOCS Press, Urbana, IL, 2008, pp. 661–724.
[12] P. Vananuvat, J.E. Kinsella, Extraction of protein, low in nucleic acid, from *Saccharomyces fragilis* grown continuously on crude lactose, Journal of Agricultural and Food Chemistry 23 (1975) 216–221.
[13] I.S. Chronakis, A.N. Galatanu, T. Nylander, B. Lindman, The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface, Colloids and Surfaces A: Physicochemical and Engineering Aspects 173 (2000) 181–192.
[14] M. Anusuya Devi, G. Subbulakshmi, K. Madhavi Devi, L.V. Venkataraman, Studies on the proteins of mass-cultivated, blue-green alga (*Spirulina platensis*), Journal of Agricultural and Food Chemistry 29 (1981) 522–525.
[15] F. Gelin, J.K. Volkman, C. Largeau, S. Derenne, J.S. Sinninghe Damsté, J.W. De Leeuw, Distribution of aliphatic, nonhydrolyzable biopolymers in marine microalgae, Organic Geochemistry 30 (1999) 147–159.
[16] S.O. Lourenço, E. Barbarino, P.L. Lavín, U. Lanfer Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors, European Journal of Phycology 39 (2004) 17–32.
[17] O.H. Lowry, N.J. Rosebrough, L.A. Farr, R.J. Randall, Protein Measurement with the Folin Phenol Reagent, Department of Pharmacology, Washington University School of Medicine, 1951, pp. 265–275.
[18] A. Bensadoun, D. Weinstein, Assay of proteins in the presence of interfering materials, Analytical Biochemistry 70 (1976) 241–250.
[19] AOAC, Official Methods of Analysis of the Association of Official Analytical Chemists, 16th ed., 1995. , (Arlington, VA. Method 990.03).
[20] AOAC, Official Methods of Analysis of AOAC International, 18th ed., 2006. (Arlington, VA. Method 982.30 E(a, b, c) chp. 45.3.05).
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, Nature 227 (1970) 680–685.

[22] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of structural carbohydrates and lignin in biomass, in: NREL (Ed.), 2008 (NREL/TP-510-42618, http://www.nrel.gov/biomass/pdfs/42618.pdf).
[23] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Analytical Chemistry 28 (1956) 350–356.
[24] AOCS, Official Methods and Recommended Practices of the American Oil Chemists' Society, 4th ed., 1993, pp. 5–49, (Champaign, Il. Method Bc).
[25] M. Friedman, C.E. Levin, A.T. Noma, Factors governing lysinoalanine formation in soy protein, Journal of Food Science 49 (1984) 1282–1288.
[26] M.A. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Analytical Biochemistry 87 (1978) 206–210.
[27] J. Folch, I. Ascoli, M. Lees, J.A. Meath, F.N. Lebaron, Preparation of lipide extracts from brain tissue, Journal of Biological Chemistry 191 (1951) 833–841.
[28] F. Henriques, R.B. Park, Compositional characteristics of a chloroform/methanol soluble protein fraction from spinach chloroplast membranes, Biochimica et Biophysica Acta 430 (1976) 312–320.
[29] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. Bioresource Technology 102 (2011) 9121–9127.
[30] F. Mariotti, D. Tomé, P.P. Mirand, Converting nitrogen into protein — beyond 6.25 and Jones' factors, Critical Reviews in Food Science and Nutrition 48 (2008) 177–184.
[31] J. Burczyk, B. Smietana, K. Terminska-Pabis, M. Zych, P. Kowalowsky, Comparison of nitrogen content amino acid composition and glucosamine content of cell walls of various chlorococcacean algae, Phytochemistry 51 (1999) 491–497.
[32] M. Friedman, Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins, Journal of Agricultural and Food Chemistry 47 (1999) 1295–1314.
[33] J.A. Gerrard, Protein–protein crosslinking in food: methods, consequences, applications, Trends in Food Science & Technology 13 (2002) 391–399.
[34] K. Murray, P. Stroier Rasmussen, J. Neustaedter, J. Murray Luck, The hydrolysis of arginine, The Journal of Biological Chemistry 240 (1965) 705–709.