

Nannochloropsis Protein as Potential Fish Feed

Saif Al Ghais¹, Vibha Bhardwaj²

Environment Protection & Development Authority (EPDA), Government of Ras Al Khaimah, UAE

Abstract: Recently, micro-algal biomass has attracted much attention due to wide diversity of compounds synthesized from different metabolic pathways. A rapid, convenient small scale method for micro-algal protein extraction in an optimized sequential extraction in trichloroacetic acid (TCA) and NaOH to achieve chemical lysis. Incubation of lyophilized material in 20% (w/v) TCA at 65°C followed by hot alkaline treatment. Extraction process can be complete in 3h and protein concentration was determined by Lowry method, requiring further 30min of incubation. The results showed that the protein concentration of the crude extract was 28.8%. The highest concentration of protein fraction was indicated by the fraction 0-80%. These results demonstrate that inexpensive *Nannochloropsis* sp. could be a new alternative to produce protein.

Keywords: Nannochloropsis, protein, extraction, micro-algal, Lowry method

1. Introduction

The global population is expected to increase by over a third (2.3 billion people) by 2050, requiring an estimated 70% increase in food production (Godfray et al 2010). A combination of improved agricultural food production methods and an increase of average per capita income have led to a decrease in global hunger over the last half-century, despite a doubling of the world's population (Godfray et al 2010). However, worldwide food production is now facing a greater challenge than ever before. Previously utilized methods of intensifying agriculture will soon no longer be an option due to the high impact trade-offs they have on the environment, including fragmenting natural habitats and threatening biodiversity, production of greenhouse gases from land clearing, fertilisers and animal livestock production, and nutrient run-off from fertilizer damaging marine, freshwater and terrestrial ecosystems (Tilman D et al 2011). In particular, protein is one of the main nutrients that will be in short supply in the future. Alternative protein sources and production methods are required to fulfil the demand of consumers and to meet predicted global protein requirements. Algae are a diverse group of species which can be broadly described as oxygen-producing, photosynthetic, unicellular or multicellular organisms excluding embryophyte terrestrial plants and lichens (Cavalier-Smith, T., 2007). Seaweed and microalgae are considered a viable source of protein. Some species of seaweed and microalgae are known to contain protein levels similar to those of traditional protein sources, such as meat, egg, soybean, and milk (Gouveia, L et al., 2008). Algae use for protein production has several benefits over traditional high-protein crop use in terms of productivity and nutritional value. Seaweed and microalgae have higher protein yield per unit area (2.5–7.5 tons/Ha/year and 4–15 tons/Ha/year, respectively) compared to terrestrial crops, such as soybean, pulse legumes, and Foods **2017**, 6, 33; doi:10.3390/foods6050033
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wheat (0.6–1.2 tons/Ha/year, 1–2 tons/Ha/year, and 1.1 tons/Ha/year, respectively) (Van Krimpen, M et al., 2013). Terrestrial agriculture already requires approximately 75% of the total global freshwater with animal protein in particular requiring 100 times more water than if the equivalent amount of protein was produced from plant

sources (Wallace, J. et al., 2000). However, widespread use of seaweed and microalgae is limited by a number of factors including; harvesting access and rights, seasonality and geographical location of algae, as well as the availability of scalable production methods for protein isolation from algae. Current processes of algal protein isolation are time-consuming and economically unviable (Wijffels, R.H. et al., 2010).

Micro-algal protein in particular, has potential for animal feed or human consumption, recombinant protein technology and as a valuable by-product of biofuel production (Becker, 2007; Potvin and Zhang, 2010; Williams and Laurens, 2010). Marine strains could avoid conflicts with agriculture for freshwater supplies, e.g. *Nannochloropsis*, used both in fish-farm aqua-feed and large scale biofuel production (Day et al., 2012; Radakovits et al., 2012; Rodolfi et al., 2003). In order to evaluate protein levels in novel strains and micro-algal collections, a rapid but generally applicable extraction procedure was needed. Lyophilized biomass was preferred as the starting material to avoid inaccuracies associated with measuring FW (fresh weight) in micro-algae due to liquid carry-over. Dye-based procedures such as Bradford and Lowry largely overcome these problems and are more appropriate when applied to a wide variety of strains such as high-throughput screening procedures (Bradford, 1976; Lowry et al., 1951). Extraction procedures that incorporate TCA precipitation of protein can also remove TCA-soluble factors that may interfere with estimation (Clayton et al., 1988). Nevertheless, micro-algal extraction procedures for dye-based protein assays generally require prior extraction of protein by homogenization, which can add to processing time (Bergeset et al., 1993; Clayton et al., 1988; Conover, 1975; Rausch, 1981). Since lyophilized material is generally harder to extract protein from, most methods tend to start with fresh material (Walker, 2002). Recent procedures have been described for lyophilized micro-algae but also require labor intensive disruption steps either

by pestle and mortar with inert ceramic particles (López et al., 2010) or use of the Potter's homogenizer (Barbarino and Lourenço, 2005). Furthermore, some commonly studied strains such as *Chlorella* or members of *Eustigmatophyceae* (e.g. *Nannochloropsis*) can present general extraction

difficulties possibly due to small cell size or resilient cell walls (Chiu et al., 2009; Doucha and Lívansky, 2008). These difficulties could be compounded when extracting dried material from these strains and this requires investigation. In some reported procedures, it was evident that extraction at high temperature in alkaline solution (2 N NaOH at 95 °C; Pruvost et al., 2011) or hot-TCA (Price, 1965) could achieve chemical lysis without the need for homogenization but this was only shown for fresh material and with a limited number of strains. The method of Price (1965) employed very brief incubation in hot-TCA (6%, w/v) followed by extraction of the TCA pellet in 0.1 N NaOH (55 °C). This relatively mild treatment was sufficient for *Euglena gracilis* which is fragile and easy to extract protein from (Price, 1965) but not for other micro-algae (Rausch, 1981). Both steps were prolonged by Conover (1975) to extract from the diatom *Thalassiosira fluviatilis* but a homogenization step was also included. Conditions for extraction solely with alkaline solution were investigated by Rausch (1981), where short incubations at 80–100 °C, with concentrations up to 0.5 N NaOH were found to be optimal and could avoid losses by hydrolysis. It was concluded that this method gave higher yields than the TCA-based methods. Nevertheless, it was apparent from this earlier literature that the sequential hot-TCA and alkaline solution extraction steps described in the original Price procedure could be developed into a widely applicable procedure for (Price, 1965; Rausch, 1981). Therefore the aim of this study was to develop a rapid, small scale and relatively simple protein extraction method suitable for micro-algal screening and to produce algal proteins as a source of human nutrition, functional foods and animal feed, as well as to describe a novel processing technology that is used to make algae a viable source of protein ingredients.

2. Methods

Micro-algal strains and culture

The micro-algal *Nannochloropsis* sp. used was obtained from the Arabian Gulf Culture Collection of Algae of EPDA. Starter cultures of 100 mL were incubated under a 12 h/12 h L/D (Light/Dark) regime at 50–80 IE/m²/sec at 20 °C for 7–10 d, without shaking (Westpoint Int. WPX-287.TG, China). Once the culture reached stationary phase, they were harvested by centrifugation at 4000g for 15 min (Sigma 3-30KS centrifuge, Germany). The harvested cells were then dried for 3 days in hot air oven. The dried algae biomass was then transferred to individual glass vials, with a Teflon-lined stopper, and stored in the dark.

Protein extraction

The small-scale method developed for protein extraction of micro-algal dry-weight (DW) was based on that used by Price (1965) with extensive modifications. 5 mg ($\pm 10\%$) of freeze-dried micro-algae material was weighed out. Three separate extractions were carried out for each experimental condition to determine variation in yield. Samples were resuspended by vortexing in either 250 μ L 10% (w/v) TCA or 200 μ L 20% (w/v) TCA. Homogenates were incubated in a water bath at either 65 °C or 95 °C, for 15 min, in screw-capped micro-centrifuge tubes and allowed to cool to RT. The samples containing 20% (w/v) TCA were diluted to 10% (w/v) with ultrapure water. The homogenates were

centrifuged at 15,000g for 20 min at 4 °C (Centrifuge, 3-30KS, Germany) and their supernatants discarded. The pellets were resuspended in 0.5 mL Lowry Reagent C (see in protein quantification section) by repeated pipetting or vortexing and incubated over a series of time-points (10 min to 22 h) at 55 °C. Samples were then cooled to RT, spun at 15,000g for 20 min RT and the supernatant retained; samples could be frozen at -20 °C for further analysis.

Protein quantification

Protein quantification followed the method of Lowry et al. (1951) as modified by Price (1965). A stock of Lowry Reagent C was made up daily in a 48:1:1 ratio of Lowry Reagents A (2% (w/v) Na₂CO₃ (anhydrous) in 0.1 N NaOH); B (1% (w/v) NaK Tartrate tetra hydrate and 0.5% (w/v) CuSO₄.5H₂O in H₂O), respectively. Reagents A, B, and C can be stored at RT. The Lowry assay also employs Folin-Ciocalteu phenol reagent (Sigma). A stock of a 1:1 ratio of 2 N Folin-Ciocalteu phenol reagent: ultra-pure water was made daily. An appropriate volume (up to 50 μ L) of the protein extract was added to individual 1.5 mL microfuge tubes in triplicate, and make up volume up to 0.5 mL by Lowry Reagent A, followed by immediate mixing. Then add 2.5 mL of Lowry reagent C. Shaked well by vortexing. Samples were then incubated for 10 min at RT. Next, 0.25 mL of the diluted Folin-Ciocalteu phenol reagent was added to each tube and vortexed immediately. After 30 min at RT, the absorbance of each sample was read at 600 nm (Spectrophotometer-Jenway 7315, Bibby scientific Ltd, UK). Calibration curves were prepared for each assay with a bovine serum albumin (BSA) stock solution (200 mg/mL; Sigma P5369) and using a polynomial line of best fit generated in Microsoft Excel 2010.

Fractionation

The crude extract was fractionated by using ammonium sulphate at saturation level of 0-20%, 20-40%, 40-60%, and 0-80%. Protein concentration of each fraction was determined by Lowry method using bovine serum albumin (BSA) solution as a standard.

Statistical analysis

Experimental error was determined for triplicate assays and expressed as standard deviation (SD). The significance of differences in the protein yield for micro-algal species subjected to varying TCA treatments and alkaline solution incubation.

3. Result and Discussion

In order to develop a rapid small-scale protein quantification method for lyophilized micro-algal material, a method based on hot-TCA extraction was chosen as a starting point (Price, 1965). The original method harvested fresh algal material by filtration and extracted without homogenization in 6% (w/v) hot-TCA (temperature unspecified) for 1 min. This was followed by centrifugation and incubation of the precipitated material with Lowry Reagent C (which contains 0.1 N NaOH, Section 2.3) at 55 °C for 3 min. Extracted protein could then be conveniently measured with a modified-Lowry assay, as described (Price, 1965). For the method developed here, harvesting of cells by centrifugation of cells was carried out in place of filtration to avoid potential losses

due to adsorption, highlighted by Rausch (1981). This also avoided the possible need to homogenize the filter and reduced handling time. Both the hot-TCA incubation and alkaline solution pellet-resuspension steps were considered to be important in determining yields in this procedure. It was also anticipated that extraction efficiency would vary according to species-specific factors.

Optimizing hot-TCA extraction

To define optimal conditions for extraction of proteins from lyophilized micro-algal material in hot-TCA, incubation temperature and TCA concentration were investigated in a matrix of four different conditions (Fig.). Hot-TCA extraction was tested at the original TCA concentration of Price (1965) at 10% (w/v) and twofold higher at 20% (w/v). In the latter case, the TCA was diluted back to 10% (w/v) prior to centrifugation. It was found that lyophilized material

could be readily resuspended in TCA by vortexing prior to the incubation steps. Incubation in TCA was tested at two different temperatures, 65 and 95 °C, for 15 min. In Fig.1, the effect of these treatments is shown in terms of protein yield improvement. Considerable increases in protein yield were observed by raising TCA concentration to 20%(w/v) TCA at incubation temperature to 65 °C (Fig.1). Therefore increasing TCA concentration and incubation temperature enhanced yields to differing degrees depending on the micro-algal species. Taken together, the most stringent hot-TCA extraction condition(20% (w/v) TCA at 65 °C) produced substantial improvements in yield compared with milder treatments. This suggested that potentially, this treatment could be widely applicable across micro-algal taxa.

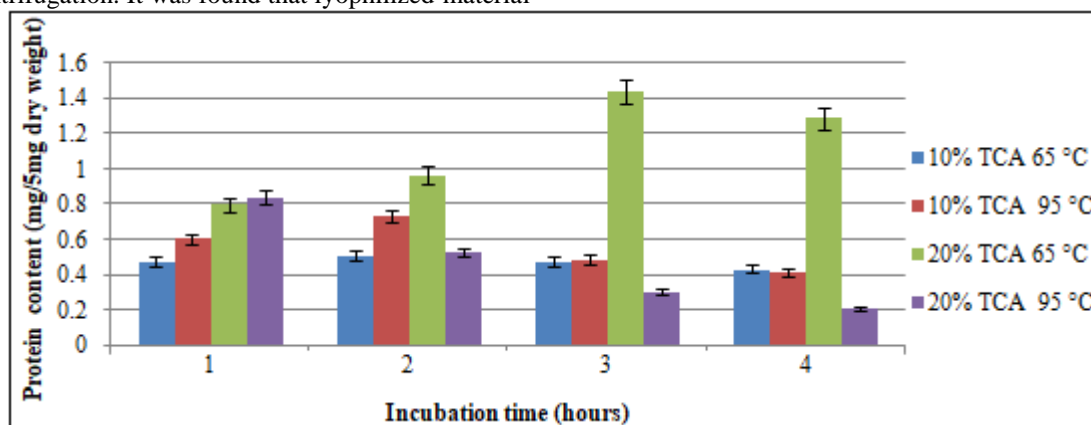


Figure 1: The effect of temperature and TCA concentration on the efficiency of hot-TCA extraction in terms of protein yield. Micro-algal species was subjected to four different combinations of hot-TCA conditions: 10% (w/v) TCA at 65 °C; 6% (w/v) 95°C; 20% (w/v) 65°C and 20% (w/v) 95°C. Incubation was for 15 min followed by a 2 h solubilization of precipitated protein in Lowry Reagent C at 55 °C. Data are mean values of replicate assays, error bars indicate SD

Evaluation of alkaline treatment and protein quantification

The next step in the Price (1965) procedure after hot-TCA extraction was recovery of precipitated protein and other insoluble material by centrifugation. In the original procedure, this was followed by re-solubilization of the centrifugal pellet in the alkaline Lowry Reagent C (0.1 N NaOH), for 3 min at 55°C. It was noted that alkaline solutions are often used as protein extraction reagents in their own right. Therefore in some circumstances, in complete extraction with hot-TCA might be mitigated during the pellet-incubation period leading to yield maximization. Prolonged incubation times could reduce yield through degradation by hydrolysis, however (Rausch, 1981). In the above analysis of the hot-TCA conditions, subsequent incubation of the TCA pellet in Lowry Reagent C was carried out at 55 °C for 2 h. To resolve these issues and optimize the procedure further, the incubation time period in Lowry Reagent C was investigated further. It was previously established that the most stringent hot-TCA extraction condition (20% (w/v) TCA at 65 °C) provided significant improvements (Fig.1). Therefore, this treatment was followed by a range of incubation periods from 10 min to overnight(1320 min) in Lowry Reagent C. These data show that for *Nannochloropsis* sp., on increasing incubation time in Lowry Reagent C from 10 min to 3 h, resulted in

progressive yield increases, but only where the mildest hot-TCA treatment had been applied (Fig 1.).

Table 1: Effect of Incubation time in amount of protein extraction by TCA

S. No	Protein Extraction by TCA	Protein extracted in mg/5mg dried sample				
		Incubation Time in hours	1 hrs.	2 hrs.	3 hrs.	4 hrs.
1	10% TCA 65°C		0.480	0.512	0.480	0.436
2	10% TCA 95°C		0.608	0.736	0.490	0.416
3	20% TCA 65°C		0.800	0.970	1.440	1.290
4	20% TCA 95°C		0.840	0.530	0.306	0.213

This suggested that TCA protein extraction had been incomplete in the mild conditions, but could be completed with extended incubation in Lowry Reagent C. Overall, yield improvements were obtained by extending Lowry Reagent C incubation time but this was found to be species-dependent as noted for the hot-TCA conditions. Incubation of TCA-pellet in Lowry Reagent C beyond 3 h appeared to reduce yields. To summarize, substantial improvements to protein yield could be obtained by altering: (a) hot-TCA concentration and temperature and (b) incubation period in Lowry Reagent C. Use of more stringent hot-TCA extractions (20% (w/v) TCA at 65 °C for 15 min) than those described in Price (1965), substantially increased yields. Provided the most stringent hot-TCA extraction was used,

extended TCA-pellet incubation in Lowry Reagent C incubation beyond 4 h showed indications of yield reduction, possibly due to hydrolysis occurring in the absence of further protein extraction. Therefore, a compromise 3 h period was chosen for incubation in Lowry Reagent C.

Table 2: Effect of Incubation time in % of protein extraction by TCA

SNO	Protein Extraction by TCA	Protein extracted (%)			
	Incubation Time in hours	1 hrs.	2 hrs.	3 hrs.	4 hrs.
1	10% TCA 65°C	9.6	10.24	9.6	8.72
2	10% TCA 95°C	12.16	14.72	9.8	8.32
3	20% TCA 65°C	16	19.4	28.8	25.8
4	20% TCA 95°C	16.8	10.6	6.12	4.26

Protein purification

The purification of proteins was done by using ammonium sulphate fractionation. The fractionation aimed to separate proteins based on their solubility differences in water. The protein fractionation process was carried out by the addition of ammonium sulphate at saturation levels of 0-20%, 20-40%, 40-60%, and 0-80%. The protein precipitation used the principle of salting out, where water binds with ammonium sulphate.

The addition of ammonium sulphate salt from low to high concentration led to different types of protein buildup. The solubility of proteins in water was different and the addition of salt to a certain concentration led to precipitation of particular proteins. The fractionation using ammonium sulphate produced proteins with high salt content, therefore salts remaining in the precipitation process.

Determination of Protein Concentration

The determination of protein concentration was done by using the Lowry method, which is based on the reaction of proteins with phosphotungstate-phosphomolybdate acid at the alkaline atmosphere and would give a blue color where the intensity depended on the concentration of the protein. Furthermore, the absorbance measurements were performed using Spectrophotometry. Based on the measurements that have been done, the concentration and the total of proteins in micro-algae *Nannochloropsis sp.* crude extract and protein fractions at different levels of saturation of ammonium sulphate fractionation are given in Table 3.

Table 3: Protein fractions at different levels of saturation of ammonium sulphate fractionation

S No	Protein Fraction	Volume of Fraction (mL)	Protein Concentration (mg/mL)	Total Protein (mg)
1	Crude Extract	100	1.440	144
2	0-20%	10	0.750	7.5
3	20-40%	10	0.812	8.12
4	40-60%	10	0.851	8.51
5	0-80%	10	2.015	20.15

As seen in Table 3, protein concentration of the crude extract was 1.440 mg/mL and the total amount of protein was 144 mg in 100 mL crude extract. The highest protein concentration was found in protein fraction of 0-80% at 2.015 mg/mL, and the lowest concentration of the protein in fraction 0-20% at 0.750 mg/mL. The different concentration

of each fraction showed that different types of protein were built up. Some proteins had different solubility in water. The higher the solubility, the lesser protein was built up.

Overall, the modified-Price method data correlated with measurements and was similar to other published data using the Lowry method. This was supportive of complete protein extraction and measurement using the hot-TCA protocol developed here.

4. Conclusion

Measuring protein content in micro-algal material can be hampered by extraction difficulties, especially with lyophilized material. Often this is overcome by introducing labor-intensive homogenization steps. The aim was to develop a method with sequential hot-TCA and alkaline solution extractions that avoided this and was simpler to carry out. An optimized procedure was arrived at in terms of reagent concentration, temperature and incubation period.

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