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Concentration and characterization of microalgae proteins from *Chlorella pyrenoidosa*

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Abstract

Background: Many methods are available for the concentration of proteins; however, most are not easily scalable due to costs, the need of specialized instruments and skilled workers or are very time-consuming. Three-phase partitioning (TPP) is a separation technique that has gained a lot of interest due to its rapid, simple and scalable use for concentration, isolation and decontamination of proteins from crude samples with high recovery yields. In the present work, the effect of various parameters of TPP was evaluated to optimize the concentration of proteins from *Chlorella pyrenoidosa* (CP), is green algae that increasingly being used as food supplements because of its positive impacts on human health.

Results: *Chlorella pyrenoidosa* was cultivated in a closed system under controlled conditions. After reaching maximum growth, the microalgae was harvested, dried and powdered. Afterwards, TPP of CP cell lysate was done to concentrate protein content. To maximize protein concentration, various parameters were optimized such as solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), pH (6), incubation time (20 min), slurry to butanol ratio (1:1.5) and enzymatic treatment (combination of Stargen™ and Carezyme™). Also, total starch, cellulose and carbohydrate content before and after the enzymatic treatment were determined to comprehend the impact of enzymatic treatment on protein concentration. Using these optimized parameters, 78.1 % w/w protein concentration was obtained in middle protein concentrate phase. This protein concentrate was characterizedfor proximate composition, colour analysis, water holding capacity, oil-holding capacity, foaming capacity, foam stability, amino acid composition, protein quality and thermal properties.

Conclusion: Various process parameters of TPP influence the protein concentration of middle protein concentrate phase. Enzymatically treated biomass also enhanced protein concentration in middle protein concentrate phase. Characterization of protein concentrate revealed the presence high-quality protein. Therefore, it is possible to implement TPP at an industrial scale for protein concentration.

Keywords: Microalgae, Three-phase partitioning, Protein concentrate, Enzymatic treatment, Protein quality

Background

Microalgae proteins have a great potential to be an alternative protein source since they contain all essential amino acids (Becker 2007; Barbarino and Lourenço 2005; Lourenco et al. 2004; Safi et al. 2014a, b). Microalgae have created immense interest due to their unconventional growth requirements such as being able to grow in salt water in the presence of CO_2 and sunlight on unfertile land. This makes microalgae a potentially sustainable source of feedstock for fuel, food, chemical, textile, polymer and even the pharmaceutical industry (Viegas et al. 2015). Microalgae contain lipids, protein and carbohydrate as their main components and their protein concentrates can be used in the food, feed and bulk chemical industry (Chacon-Lee and Gonzalez-Marino 2010).

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Chlorella pyrenoidosa (CP) is a species of the freshwater green algae that is widely used as food supplements (Garcia et al. 2012; Wang and Zhang 2012). Each year, the United States, Japan, China, Taiwan and Indonesia produce over 2500 tons of dried Chlorella (Bishop and Zubeck 2012) because it is not only considered a good source of nutrients but also a functional food due to its positive health effects (Gao 1998; Richmond 1990). Various clinical and animal studies have shown that CP is a safe source of protein for consumption and dietary supplementation with Chlorella may reduce high blood pressure, lower serum cholesterol and glucose levels, accelerate wound healing, and enhance immune functions (Viegas et al. 2015; Cherng and Shih 2005; Halperin et al. 2003; Lee et al. 1967; Merchant and Andre 2001; Merchant et al. 2002; Mizoguchi et al. 2008; Lisboa et al. 2014). Microalgae proteins also have high techno-functional potential such as being usable as an emulsifier (Ursu et al. 2014). Since proteins from *Chlorella* can act as an animal protein replacement, its use can have a positive impact on the environment (Jones 2016).

Many methods have been used for fractionation/concentration of proteins from microalgae such as ultrafiltration, precipitation, chromatography, dialysis and centrifugation. Even though these methods are effective, they are not easily scalable due to the costs of the process, requirement of a specialized instrument, time-consuming and the need of skilled operators. The drawbacks of ultrafiltration include the susceptibility of rapid membrane clogging, prolonged processing time and the use of expensive membrane filters (Walsh 2007). Precipitation is an industrially suitable method for protein concentration and is performed by salting out, isoelectric focusing and solvent precipitation. However, each of these methods has their limitation such as the low recovery during salting out and irreversible denaturation of proteins after isoelectric focusing and solvent precipitation (Ustunol 2015). Chromatography, dialysis and centrifugation are preferable methods for final-stage purification of proteins but are very much time-consuming (Bio-Sciences 2010). Three-phase partitioning (TPP) is a separation technique useful for initial purification of proteins with high recovery yields. It has gained a lot of interest due to its rapid, simple and scalable use for concentration, isolation and decontamination of proteins from crude samples (Harde and Singhal 2012). TPP can efficiently fractionate non-polar components in solvent phase (upper phase), polar components in aqueous (lower phase) and proteins in the middle phase. This technique uses a combination of ammonium sulphate and *t*-butanol to precipitate proteins from crude extracts. Precipitated protein binds to *t*-butanol, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt phase. Optimum pH, temperature, ammonium sulphate and *t*-butanol concentrations can selectively precipitate proteins at the interface of the organic and aqueous phases. Kosmotropic, salting out, co-solvent precipitation, isoionic precipitation, osmotic electrostatic forces, conformation tightening and protein hydration shifts all contribute to protein precipitation at this interface (Vetal and Rathod 2014). It has been reported that TPP can even enhance enzyme activity compared to conventional methods (Garg and Thorat 2014; Ketnawa et al. 2014; Ozer et al. 2010; Şen et al. 2011). Furthermore, the protein recovery yield is almost perfect reaching values near 100 % of the total protein present (Phongthai and Rawdkuen 2015).

In the present work, the effect of various parameters of the TPP method was evaluated to optimize the concentration of proteins from CP. The effect of an enzymatic treatment on protein concentration efficiency was evaluated by quantifying initial and final starch, cellulose and total carbohydrate concentrations. Also, the protein concentrate obtained using optimized conditions was characterized to understand the influence of the TPP method.

Methods

All chemicals, salts, solvents and reagents used in this study were of analytical grade and were purchased from SD Fine, Mumbai, India. Stargen™ 002 was gifted from Genencor International, Danisco US and Carezyme™ was procured from Sigma-Aldrich, USA. Corn oil was gifted by Kamani oils, Mumbai, India.

Culture growth conditions and harvesting

The species *Chlorella pyrenoidosa* strain NCIM 2738 is a freshwater green alga from the genus Chlorella. This strain was obtained from National Centre for Industrial Microorganisms (NCIM), Pune, India. Batch size of 40 L (20 flasks) was cultivated in 3-L glass conical flasks containing 2 L modified Chlorella medium in each flask and sterilized in an autoclave at 120 °C, 15 bar pressure for 20 min. Modified chlorella medium, adjusted to pH 7.5, consisted of following components (g/l): KNO_3 (4), $NaNO_3$ (0.1), $MgSO_4{\cdot}7H_20$ (0.1), $CaCl_2{\cdot}H_2O$ (0.05), H_3BO_3 (0.00286), $MnCl_2{\cdot}4H_2O$ (0.00181), $ZnSO_4{\cdot}7H_2O$ (0.00022), $NaMoO_4{\cdot}2H_2O$ (0.0004), $CuSO_4{\cdot}5H_2O$ (0.00008), $FeSO_4{\cdot}7H_2O$ (0.00557), and Na_2EDTA (0.00745). The flasks were maintained in a plant growth chamber where the illumination (800–1000 lux) was provided by cool white fluorescent lamps (Philips) and light intensity was measured with an LUX meter. The temperature was maintained at 25 ± 2 °C and flasks were agitated twice daily for 2 min at 60 revolutions per min. Ten-day-old cultures were inoculated in the media-containing flasks and incubated during 45 days. The biomass

was harvested by sedimentation and further centrifuged (Backman J2-MC, USA) at 5000×*g* for 10 min to collect the thick algal slurry. The algae slurry was washed with distilled water to remove salts. This slurry was dried in an oven at 50 °C for 24 h. Dried biomass was ground and passed through 36-mesh-size sieve to obtain uniform particle size then stored at −20 °C in an air-tight seal container until used. Afterwards, cell disruption of dried biomass was performed using ultrasonication (Branson Ultrasonifier 450, USA) and disruption was confirmed by microscopic (Motic BA310, with Moticam 480, China) observation at 40×.

Three-phase partitioning

TPP of cell lysed CP biomass was carried out to concentrate its proteins. In 100 mL glass beakers, 20 mL cell lysed biomass was mixed with 8 g of ammonium sulphate followed by addition of 20 mL *t*-butanol. This mixture was stirred for 20 min at 28 ± 2 °C. Immediately, three distinct phases were separated carefully using Pasteur pipette. Initially, the upper organic phase was pipetted then lower aqueous phase was pipetted by piercing middle phase. The middle phase, containing the concentrated proteins (see "Background" section), was used for further study. Salt was removed from protein concentrate using Hi-media dialysis membrane-50 for 12 h in 1 L distilled water (fresh distilled water was replaced after 6 h). Dialysed protein concentrate was precipitated by adjusting the pH to 5.0 followed by centrifugation at 8000×*g* for 10 min to remove water. This protein concentrate was dried at 50 °C for 12 h and used for analysis. Effect of various process parameters was evaluated such as solvent, ammonium sulphate, solid load, pH, incubation time, slurry to butanol ratio and enzymatic treatment. The protein concentration of middle phase proteins was represented as a concentrate in % w/w.

Protein quantification

Protein concentration in middle phase was determined by the modified Kjeldahl method (Persson et al. 2008). This method mainly involved digestion, distillation and titration (Kel-Plus, Elite Ex 8L, Pelican equipments, India). For microalgae and cyanobacteria undergoing rapid growth, the recommended Kjeldahl nitrogen-to-protein conversion factor used was 5.95 instead of 6.25 (Lopez et al. 2010). In the case of enzymatically treated biomass, the amount of enzyme used was subtracted from total protein concentration found, to eliminate the contribution of enzymes in protein quantification. In every run, two digestion tubes were kept as blanks. All samples were analysed in triplicate and mean with standard deviation was reported.

Effect of enzymatic treatment on protein concentration

Enzymatic treatment

Effect of enzymatic treatment on protein concentration was studied using Stargen™ 002 (570 GAU/g from Genencor International) and Carezyme™ (1000 U/g from Sigma-Aldrich). Cell disrupted microalgae slurry was treated separately with each individual enzyme and also, both enzymes were used together to evaluate the combined effect. Simultaneously, non-enzymatic treated cell lysates were used as controls. The condition during Stargen™ 002 treatment was pH (4.5), temperature (50 °C) and enzyme loading (10 µg for 0.75 g of cell lysate in 20 mL distilled water) for 1 h with gentle stirring (300 rpm) using a magnetic stirrer. For, Carezyme™ treatment conditions were pH (5), temperature (37 °C) and enzyme loading (10 µg for 0.75 g of cell lysate in 20 mL distilled water) for 1 h with gentle stirring (300 rpm) using a magnetic stirrer. The combined effect of Stargen™ 002 and Carezyme™ was studied by first treating the cell lysate with Stargen™ 002 followed by Carezyme™ treatment both in the conditions described above for each individual enzyme. Effect of enzymatic treatment was assessed by determining protein concentration, starch, cellulose and total carbohydrate of TPP middle phase protein concentrate powder as described below.

Total starch quantification

Starch content was determined using a modified method of Takeshita et al. (2014). Briefly, 0.5 g of each sample was homogenized in 80 % ethanol at 50 °C to remove sugars. The residue was separated by centrifugation at 5000×*g* for 10 min then washed repeatedly with 80 % ethanol at 50 °C until the washings did not give a colour with anthrone reagent and removed all pigments. This residue was completely dried over a water bath then 2.5 mL of water and 2.5 mL of 60 % perchloric acid were added. These tubes were kept at 0 °C for 20 min for starch extraction. The supernatant was separated by centrifugation at 5000×*g* for 10 min and stored. The extraction was repeated using fresh 60 % perchloric acid and the supernatant was separated by centrifugation at 5000×*g* for 10 min. From the supernatant, 1 mL of extract was pipetted and 4 mL of anthrone reagent was added to each tube. Afterwards, the test tubes were heated in a boiling water bath for 10 min then cooled to room temperature, and the absorbance at 630 nm was measured using a spectrophotometer. Glucose was used as a standard for calibration graph to determine glucose concentration in samples. Starch content was calculated by multiplying the value by a factor 0.9.

Cellulose quantification

Cellulose was quantified as described by Thayumanavan and Sadasivam (1984). Acetic/nitric reagent (3 mL of a solution consisting of 10 volumes of 80 % acetic acid and 1 volume of concentrated nitric acid) was added to 0.5 g of the sample in a test tube and mixed using a vortex mixer. The tubes were then placed in a water bath at 100 °C for 30 min, cooled and centrifuged at 5000×*g* for 15 min. The supernatant was discarded and cellulose-containing residue was washed with distilled water. To this residue, 10 mL of 67 % sulphuric acid was added and allowed to stand for 1 h. Further, 1 in 100 dilution was made and 1 mL of this diluted solution combined with 10 mL of anthrone reagent (200 mg anthrone in 100 mL chilled concentrated sulphuric acid) was added and mixed well. The tubes were heated in a boiling water bath for 10 min. Further, tubes were cooled and the absorbance at 630 nm was measured. Blank was prepared with anthrone reagent and distilled water and the calibration curve was prepared using cellulose in the range of 40–200 µg.

Total carbohydrate quantification

A sample (100 mg) was taken in a test tube and 5 mL of 2.5 N HCl was added and kept in boiling water bath for 3 h for hydrolysis. This solution was allowed to cool and was neutralized using sodium carbonate until effervescence ceases. Volume was adjusted to 100 mL with distilled water and centrifuged at 8000×*g* for 20 min. The supernatant was collected and 1 mL aliquot was taken for analysis to which 4 mL of anthrone reagent was added followed by heating for 10 min in boiling water bath. After cooling to room temperature, absorbance was measured at 630 nm. Glucose was used as a standard for the preparation of calibration graph (Thayumanavan and Sadasivam 1984) and distilled water was used as blank in place of sample.

Characterization of protein concentrate

Proximate composition of dried biomass and protein concentrate

Samples were analysed for moisture and total ash contents following standard AOAC (2001) method. Total protein content was determined using the micro-Kjeldahl procedure with a nitrogen-to-protein conversion factor of 5.95 (Lopez et al. 2010). Fat content was determined by the Soxhlet method (using Instant Soxhlet apparatus-Socs Plus, Pelican Equipments, Chennai, India) using petroleum ether (B. P. 60–80 °C) as the solvent. Carbohydrate was estimated by difference [Percentage carbohydrate content = 100 − (% moisture + % ash + protein + % fat)] and also using the method with anthrone as described above.

Color analysis

Color of the dried biomass and protein concentrate were measured using Hunter Lab colorimeter model DP-9000 D25A (Hunter Associates Laboratory, Reston, VA, USA) in terms of Hunter L (lightness, ranging from 0 to 100 indicating black to white), a (+a, redness and –a, greenness) and b (+b, yellowness and –b, blueness). The determination of color was done on six different samples. Standardisation of the instrument was done using a black and white standard plates.

Water-holding capacity (WHC) and oil-holding capacity (OHC)

To determine WHC, 1 g protein concentrate was dispersed in 10 mL of distilled water and placed in centrifuge tubes. The dispersions were stirred for 5 min, held for 30 min, followed by centrifugation at 3000×*g* for 25 min. The supernatant was eliminated, the excess of water was removed by draining for 25 min at 50 °C and the sample was reweighed. For OHC, 0.5 g of sample was mixed with 6 mL of corn oil and allowed to soak for 30 min. Then, tubes were centrifuged at 3000×*g* for 25 min. The separated oil was then removed with a pipette and the tubes were inverted for 25 min to drain the oil prior to reweighing. The WHC and OHC were expressed as grams of water or oil bound per 100 g of the sample on dry weight basis (Ghribi et al. 2015).

Foaming capacity (FC) and foaming stability (FS)

Samples were dispersed in 50 mL distilled water at a concentration of 3 % (w/v) and homogenized at 8000×*g* for 3 min. The blend was immediately transferred into a graduated cylinder. The volume was recorded before and after whipping. FC was expressed as the volume (%) increase due to whipping.

$$\text{Foaming capacity (\%)} = \frac{(\text{Volume after} - \text{Volume prior})}{\text{Volume prior}} \times 100.$$

For the determination of FS, foam volume changes in the graduated cylinder were recorded after 180 min of storage and expressed in percentage (Cano-Medina et al. 2011).

Amino acid composition and protein quality

Amino acid composition was determined using high-pressure liquid chromatography (HPLC). The sample of protein concentrate was dissolved in 6 mL of 6 N HCl and was subjected to hydrolysis in boiling water bath for a period of 24 h. The tubes were vortex mixed after every 1 h for proper hydrolysis. After 24 h of hydrolysis, the tubes were centrifuged at 3500×*g* for

15 min. The supernatant was filtered and was neutralized with 1 N NaOH. Then, the filtered solution was diluted to 1:100 with HPLC grade water and was used for estimation of protein amino acids in HPLC (Huesgen 1999; Sonawane et al. 2015). Protein quality was evaluated by the essential amino acid index (EAAI), which is based on the content of all essential amino acids compared to a reference protein and human requirements as mentioned in FAO/WHO/UNU standard (2007). The EAAI is a rapid method to evaluate and optimize the amino acid content of food formulations (Smith and Nielsen 2010). CP protein concentrate by TPP was comparatively evaluated with literature data on best protein sources such as soy protein and casein. However, EAAI does not include any estimate of protein digestibility, which could be affected by processing method.

Essential Amino Acid Index (EAAI) was calculated using the following equation:

$$= \sqrt[9]{\left(\frac{\text{mg of lysine in 1g of the test protein}}{\text{mg of lysine in 1g reference protein}}\right) \times (\text{etc. For other 8essential AA}).}$$

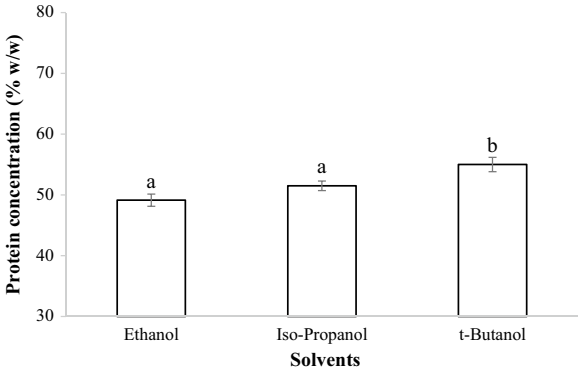

Fig. 1 Effect of various solvents on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

Thermal properties

To study thermal properties, differential scanning calorimetry (DSC) analysis was done using DSC-60 (Shimadzu Scientific Instruments, Kyoto, Japan) instrument fitted with TA 60 WS detector and computer-aided data analysis. The empty sample and reference pans were taken of equal mass for analysis. 5.00 ± 0.25 mg of sample was weighed in the DSC pan, sealed hermetically and placed on the sample side. A hermetically sealed identical empty pan was placed on the reference side. Scans were performed between 30 and 200 °C at a heating rate of 5 °C/min with 70 mL/min nitrogen gas purging.

Statistical analysis

The significant difference between mean values was assessed by one-way analysis of variance (ANOVA). Tukey test was carried out using SPSS 16.0 software to determine whether there was any significant difference at the level of $p < 0.05$.

Results and discussion

TPP of *Chlorella pyrenoidosa* cell lysate for protein concentration

Effect of solvent on protein concentration

As shown in Fig. 1, the effect of ethanol, isopropanol and *t*-butanol on protein concentration were studied by keeping other parameters constant such as ammonium sulphate (20 % w/v), solid load (1 g/20 mL), pH (7), incubation time (10 min) and slurry to solvent ratio (1:1). It was found that *t*-butanol was the best solvent for TPP of cell lysate to obtain the maximum protein concentration. In the presence of *t*-butanol, 55 % w/w of protein concentration was obtained in the middle phase. Solvents act in TPP by increasing the buoyancy of the precipitated protein by binding to it, resulting in a middle phase that locates itself above the denser aqueous salt phase. It was previously shown that *t*-butanol enhanced the buoyancy of precipitated *Aloe vera* L. protein more efficiently than other alcohols studied (Tana et al. 2015).

Effect of ammonium sulphate on protein concentration

Various types of salts can be used for TPP but generally, ammonium sulphate is preferred due to its salting out ability. Ammonium sulphate ions are high in the Hofmeister series and easily soluble in water (Ozer et al. 2010; Şen et al. 2011; Dhananjay and Mulimani 2009; Huddleston et al. 1991; Rawdkuen et al. 2012; Roe 2000; Vinoth Kumar et al. 2011). Hence, ammonium sulphate concentration should play a major role in TPP efficiency. At higher salt concentrations, water molecules are attracted by salt ions resulting in stronger protein–protein interactions and the protein molecules coagulate through hydrophobic interactions (Narayan et al. 2008). In the presence of high concentrations of ammonium sulphate, the effective dielectric constant of water greatly increases making *t*-butanol act in a more lipophilic manner increasing hydrophobicity and exclusion from the water. Different concentrations (ranging from 20 to 50 % w/v) of ammonium sulphate were studied for optimization of protein concentration in the middle phase (see

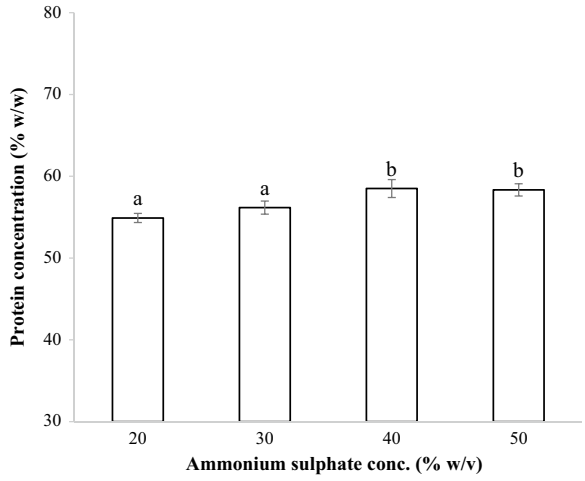

Fig. 2 Effect of ammonium sulphate concentration on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

Fig. 2). The optimum protein concentration of 58.5 % w/v was obtained in middle phase using 40 % w/v ammonium sulphate and keeping the other parameters constant such as solvent (*t*-butanol), solid load (1 g/20 mL), pH (7), incubation time (10 min) and slurry to butanol ratio (1:1).

Effect of solid load on protein concentration

The solid load is an important parameter for any kind of extraction process as it can affect the process cost that is dependent on time and solvent requirements (Eskilsson et al. 1999). Optimum protein concentration (61.8 % w/w) was obtained at maximum solid load of 0.75 g/20 mL (see Fig. 3) where solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), pH (7), incubation time (10 min) and slurry to butanol ratio (1:1) were maintained constant.

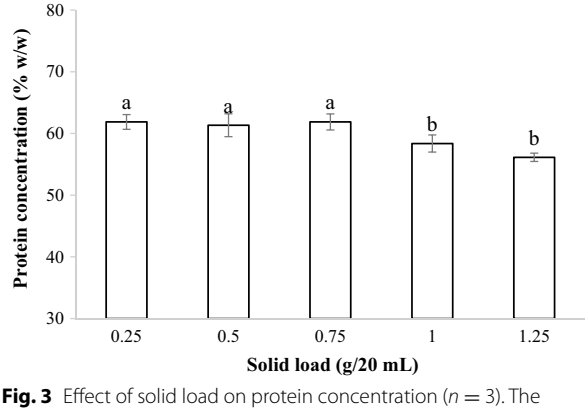

Fig. 3 Effect of solid load on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

Effect of pH on protein concentration

pH significantly influences TPP of protein since it affects the state of ionization of acidic or basic amino acids. Change in pH facilitates changes in the net charge of protein and also affects the partitioning behaviour of the protein. In the present study, different pH values (from 5 to 8) were used to study their effect on protein concentration in protein concentrate phase. As shown in Fig. 4, at pH 6 optimum protein concentration of 64.5 % w/v was obtained where other experimental parameters such as solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), incubation time (10 min) and slurry to butanol ratio (1:1) remained constant with all pH values evaluated. From these results, it can be concluded that in slightly acidic pH conditions, most of the protein concentrated in middle protein concentrate phase. As pH increases to slightly alkaline values, protein concentration was decreased, probably due to slightly increased solubility of protein in aqueous phase.

Effect of incubation time on protein concentration

Preferably minimum time for any process is a prerequisite for any industrial process as a cost-saving measure. Incubation time was varied from 5 to 25 min. Optimum protein concentration (68.0 % w/w) was found to be after 20 min of TPP (Fig. 5). During optimization of incubation time, other parameters were kept constant such as solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), pH (6) and slurry to butanol ratio (1:1).

Effect of slurry to butanol ratio on protein concentration

Slurry to *t*-butanol ratio was varied to 1:0.5, 1:1, 1:1.5 and 1:2 (see Fig. 6). Optimum protein concentration was found to be 71.3 % w/w when slurry to *t*-butanol ratio was set to 1:1.5, where all other parameters were kept

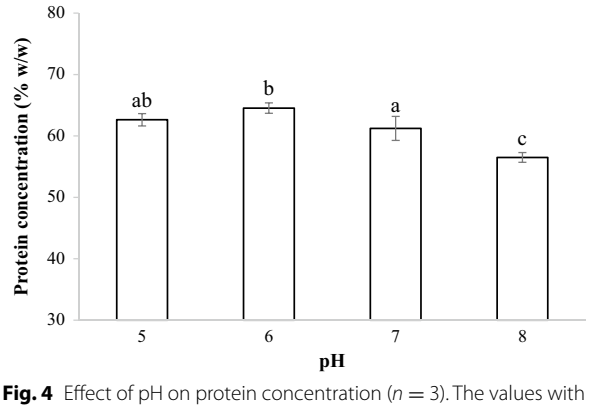

Fig. 4 Effect of pH on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

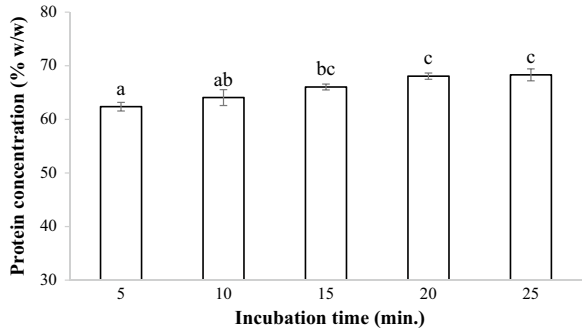

Fig. 5 Effect of incubation time on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

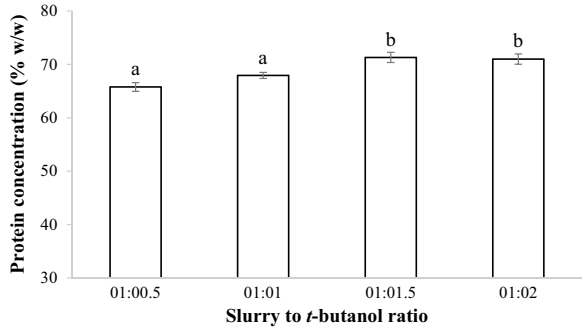

Fig. 6 Effect of slurry to butanol ratio on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

constant such as solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), pH (6) and incubation time (20 min). As *t*-butanol concentration increases more amount of water fascinates from aqueous phase resultant in better precipitation of protein at the interface. A similar trend was observed previously by Ozer et al. (2010).

Effect of enzymatic treatment on protein concentration

Previous reports indicate that TPP can be significantly affected by enzymatic treatment (Harde and Singhal 2012). In this study two enzymes were used: (1) Stargen™ 002 is enzyme mixture that contains *Aspergillus kawachi* alpha-amylase expressed in *Trichoderma reesei* and a glucoamylase from *Trichoderma reesei* that work synergistically to hydrolyse starch to glucose at optimum conditions (pH 4.5, temperature 50 °C) and (2) Carezyme™ which is cellulase from *Aspergillus* sp. that hydrolyse cellulose to glucose at optimum conditions (pH 5, temperature 37 °C). Enzymatic treatments were carried out at optimum conditions for the enzymes and processed for TPP. The enzymatic treatment increased protein concentration of middle phase (Fig. 7). This might be due to the fact that even though the cells had been lysed by a physical method of cell disruption, most of the carbohydrates remain bonded with protein matrix that precipitates in the middle phase. This had been proven by quantification of starch, cellulose and total carbohydrate of TPP protein concentrate powder.

Since the enzymatic treatment with Stargen™ was given before TPP, this decreased the starch content of protein concentrate (Table 1). A similar trend was observed with Carezyme™ on cellulose content in protein concentrate. It is also possible to observe that a higher content of carbohydrate is found in the control compared to the enzymatically treated samples. This might be due to hydrolysed carbohydrate partitioned into the aqueous phase (polar phase) as explained by Harde and Singhal (2012). Most importantly, it was observed that protein concentration in the concentrate increased after enzymatic treatment. Optimum protein concentration in middle protein concentrate phase of 78.1 % w/w was obtained with the combination of both commercial enzyme preparations (Stargen™ and Carezyme™) as observed when all of the parameters of TPP were maintained constant such as solvent (*t*-butanol), ammonium sulphate concentration (40 % w/v), solid load (0.75 g/20 mL), pH (6), incubation time (20 min) and slurry to butanol ratio (1:1.5).

Characterization of protein concentrate

Proximate composition of dried biomass and protein concentrate

Proximate composition of dried biomass and protein concentrate partitioned by TPP is given in Table 2. From proximate composition, it can be seen that the obtained protein content was 78.3 % w/w. Since the resulting protein mixture contained less than 80 % protein, it conforms to the term "protein concentrate" (Ghribi et al. 2015). Fat and carbohydrate content of dried biomass was reduced after TPP whereas proteins were concentrated.

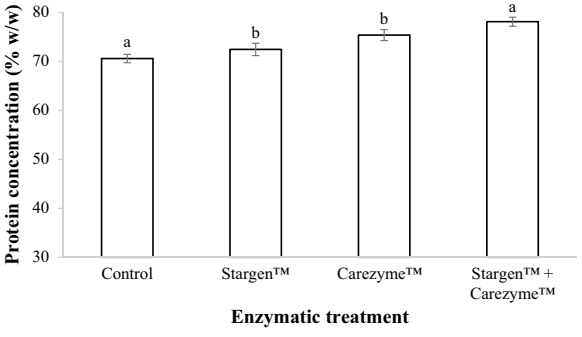

Fig. 7 Effect of enzymatic treatment on protein concentration ($n = 3$). The values with different superscripts on *bar graph* differ significantly ($p < 0.05$)

Table 1 Effect of enzymatic treatment on starch, cellulose and total carbohydrate content in protein concentrate ($n = 3$)

Enzymatic treatment	Starch (% w/w)	Cellulose (% w/w)	Total carbohydrate (% w/w)
Control (untreated)	5.30 ± 0.52	10.16 ± 1.11	22.43 ± 0.94
Stargen™	2.10 ± 0.25	10.13 ± 1.00	19.91 ± 0.80
Carezyme™	5.03 ± 0.45	1.40 ± 0.41	16.06 ± 1.30
Stargen™ + carezyme™	2.20 ± 0.20	1.23 ± 0.40	12.80 ± 0.83

All the data are expressed as mean ± SD of three determinations

Table 2 Physiochemical characterization of dried biomass and protein concentrate ($n = 3$)

Parameters	Dried biomass (% w/w)	Protein concentrate (% w/w)
Moisture	6.42 ± 0.34	4.43 ± 0.02
Ash	4.56 ± 0.26	4.10 ± 0.13
Protein	45.02 ± 2.73	78.30 ± 0.93
Fat	22.89 ± 1.78	4.12 ± 0.12
Carbohydrate (by difference)	21.11	9.05
Carbohydrate (by anthrone method)	23.32 ± 1.05	12.80 ± 0.83
Water-holding capacity (WHC)	ND	3.09 ± 0.01
Oil-holding capacity (OHC)	ND	2.02 ± 0.04
Foaming capacity (FC)	ND	95.00 ± 1.14
Foaming stability (FS)	ND	97.45 ± 0.46
Thermal characteristic		
T_0 (°C)	ND	43.29 ± 1.91
T_d (°C)	ND	60.10 ± 2.43

All the data are expressed as mean ± SD of three determinations

ND not determined

Colour analysis

Colour analysis of dried biomass and protein concentrate was comparatively studied since it is an important parameter for improving overall appearance. From Table 3, it was found that L^* value changed from 28.3 to 34.8 indicating improved lightness of protein concentrate over dried biomass. Decreased value of a^* indicates decreased intensity of green colour in protein concentrate. This might be due to the extraction of green colour pigment in upper *t*-butanol phase. b^* values increased from 1.1 to 4.7 showing a decrease in blue colour in protein concentrate.

Table 3 Colour analysis of dried biomass and protein concentrate ($n = 6$)

Parameter	Dried biomass	Protein concentrate
L^*	28.26 ± 0.05	34.78 ± 0.02
a^*	−0.48 ± 0.38	0.07 ± 0.01
b^*	1.07 ± 0.03	4.72 ± 0.03

All the data are expressed as mean ± SD of six determinations

Water-holding capacity and oil-holding capacity

Water-holding capacity of protein concentrate can be explained by the presence of polar amino acid and lower amounts of water-soluble protein. WHC of the optimized protein concentrate was 3.1 g/mL. The presence of non-proteinaceous material in protein concentrate also contributes WHC (Kaur and Singh 2007). The OHC is of great importance from an industrial viewpoint since it reflects the emulsifying capacity, a highly desirable characteristic in products such as mayonnaise. The presence of several non-polar side chains may bind the hydrocarbon chains of fats, thereby resulting in higher absorption of oil (Kaur and Singh 2007). OHC of protein concentrate was 2.1 g/mL which is within the range previously published for various protein concentrate of 1.3–4.1 g/mL. High OHC is desirable for food formulation (Kaur and Singh 2007).

Foaming capacity (FC) and foaming stability (FS)

Foamability is linked with flexible protein molecules that reduce the surface tension which keeps air bubbles in suspension and slows down the rate of coalescence. Globular proteins are relatively stable to surface denaturation which gives low foam. Previously, foaming capacity of winged bean protein concentrate (36 %), soya isolates (235 %) and mucuna bean protein concentrate (58 %) were reported. In this study, the microalgae protein concentrate had an FC of 95 %. FS is important since the usefulness of whipping agents depends on their ability to maintain the whip as long as possible. In the case of microalgae protein concentrate, foaming stability was 97 % after 180 min of storage. This excellent FS suggests that the native proteins that were soluble in the continuous phase (water) were very surface-active in microalgae proteins (Kaur and Singh 2007).

Amino acid composition and protein quality

The amino acid composition of CP protein concentrate partition by TPP was characterized (Table 4). From its amino acid profile, all essential amino acids such as lysine, methionine, threonine, tryptophan, histidine, leucine, isoleucine, valine and phenylalanine were present in the protein concentrate at substantial concentration (Smith and Nielsen 2010). Furthermore, amino acids composition of CP protein concentrate was comparatively studied and evaluated for the essential amino acid index (EAAI). Higher EAAI indicates the presence of high concentration of essential amino acid. As shown in Table 5, higher EAAI of CP protein concentrate was obtained compared to soy protein whereas casein EAAI was higher. EAAI indicates that CP protein concentrate have higher nutritional values compared to soy protein (Bertol et al. 2001; Han et al. 2015; Yi et al. 2013).

Thermal properties

DSC was used for study the thermal denaturation of protein concentrate (see Fig. 8). The thermal stability of the proteins functionally indicates their resistance to aggregation in response to heating Thermal denaturation of protein is affected by various such as extraction method, pre-treatments, heating and solvents. Literature reports illustrated thermal denaturation temperatures in the range of 75–95 °C for various protein concentrates (Ghribi et al. 2015; Ibanoglu 2005). In the present study, we found thermal denaturation temperature (T_d) of 60.10 °C which is significantly lower compared to another protein concentrate. This lower denaturation temperature could be due to various processes involved such as initial drying of biomass, alcohol denaturation during TPP and drying after the partitioning of protein concentrate.

Conclusions

Microalgae have attracted the attention of the food industry mainly because of the need for valuable, sustainable, unconventional, non-climate-dependent protein source for food and feed. Chlorella species have a great potential to be used as an alternative valuable protein source for humans, especially CP. TPP is an interesting method because of its ability to separate proteins from lipids simultaneously and because it is scalable and an economical process to obtain high protein yields. In this study, it was shown that process parameters such as solvent, ammonium sulphate, solid load, pH, incubation time and slurry to butanol ratio were important in TPP for protein concentration. Enzymatically treated cell lysate of CP increased protein concentration of protein concentrate. Using optimized TPP parameters and enzymatic treatment, the protein concentration was 78.1 % w/w. Characterization of this protein concentrate revealed that it was a high-quality protein for human consumption because of its high essential amino acid content. TPP concentrated protein was less intense in colour and also foaming stability was excellent. Thus, TPP concentrated proteins could be easily used in techno-functional applications in pharmaceutical, food and chemical industries.

Table 4 Composition of amino acids in protein concentrate

Amino acids	Protein concentrate (g/100 g of protein)
Aspartic acid	8.12 ± 0.16
Glutamic acid	7.87 ± 0.23
Serine	2.79 ± 0.03
Histidine	1.64 ± 0.01
Glycine	9.73 ± 0.42
Threonine	3.45 ± 0.04
Arginine	5.91 ± 0.07
Alanine	5.08 ± 0.19
Tyrosine	1.22 ± 0.01
Phenylalanine	3.83 ± 0.11
Valine	5.17 ± 0.05
Methionine	3.30 ± 0.02
Cysteine	2.82 ± 0.06
Isoleucine	6.20 ± 0.14
Leucine	3.44 ± 0.06
Lysine	8.14 ± 0.37

All the data are expressed as mean ± SD of two determinations

Table 5 Comparative study of amino acid pattern in CP protein concentrate, soya protein and Casein protein with FAO/WHO/UNU standard and calculation of EAAI

Essential amino acids (EAA)	CP protein concentrate (mg/g protein)	Soya protein (mg/g protein) (Bertol et al. 2001)	Casein (mg/g protein) (Han et al. 2015)	FAO/WHO/UNU standard (2007) (mg/g protein)
Histidine	16	12	30	15
Isoleucine	62	21	49	30
Leucine	34	35	97	59
Lysine	81	34	82	45
Methionine/cysteine	61	7	29	22
Phenylalanine/tyrosine	50	24	107	38
Threonine	35	19	43	23
Valine	52	24	59	39
EAAI	1.35	0.657	1.66	

EAAI essential amino acid index

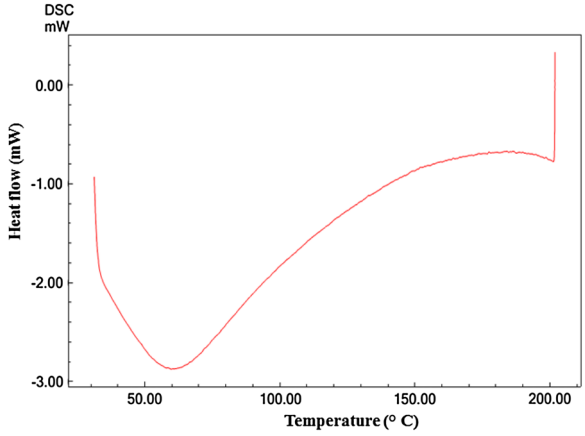

Fig. 8 DSC heating curve of *Chlorella pyrenoidosa* protein concentrate by TPP

Animal trials and other in vivo studies of the protein concentrate of CP obtained by TPP concentration must be performed to ensure safety and digestibility before proposing its introduction in the food chain of humans and other animals.

Abbreviations

CP: *Chlorella pyrenoidosa*; TPP: three-phase partitioning; WHC: water-holding capacity; OHC: oil-holding capacity; FC: foaming capacity; FS: foaming stability; HPLC: high-pressure liquid chromatography; EAAI: essential amino acid index; DSC: differential scanning calorimetry.

Authors' contributions

AGW have conducted an experiment, analysed results and prepared the manuscript. MKS have analysed samples for protein quantification by Kjeldahl method. JGL have provided technical support and assisted in manuscript drafting. SSA have supervised and guided in experimental work as well as in writing manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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