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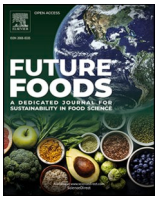

Integrating bead milling and alkaline solubilization for enhanced protein recovery from microalgae: A comprehensive approach

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ABSTRACT

Microalgae, *Chlorella vulgaris* exhibits substantial potential as a sustainable food ingredient, but its robust cell wall and limited protein solubility hinder industrial scale protein recovery. A comprehensive solution was devised, integrating dry bead milling and alkaline solubilization. In this method, cells were initially disrupted with bead milling followed by alkali (NaOH) treatment. Without bead milling, protein extraction yield was very low (5.0 % with water, 16.8 % with 0.1 M NaOH) at a biomass loading of 20 g/L. However, the integrated approach significantly improved these results, achieving a maximum protein extraction yield of about 47.3 % at a biomass loading of 100 g/L. The optimized conditions for both dry bead milling (frequency 26 Hz, duration 1.0 h) and alkaline solubilization (biomass weight to NaOH molar ratio 200–250 (g/M), 37 °C, mixing time 1.0 h) played a pivotal role in realizing these improved results. This integrated approach effectively addresses the challenges and holds industrial relevance, offering a more efficient way to extract microalgal protein.

1. Introduction

Microalgae, particularly *Chlorella vulgaris* emerge as a highly promising and sustainable source of alternative proteins for food and feed due to its impressive protein content ranging from 48 % to 60 % (w/w) complemented by an excellent amino acid profile (Becker, 2007). Despite their protein richness, a significant challenge arises from the fact that proteins within microalgae are typically confined within cells, shielded by robust cell walls. This resilient cell structure poses obstacles to accessing microalgae proteins, necessitating innovative approaches for efficient extraction and utilization.

To address the challenge of protein accessibility, various cell disruption techniques have been explored and can generally be categorized as either mechanical or non-mechanical. Mechanical approaches, including high-pressure homogenization (Jubeau et al., 2013; Spiden et al., 2013), ultrasonication (Gerde et al., 2012), and bead milling (Balasundaram et al., 2012; Doucha and Lívanský, 2008; Postma et al., 2015), employ physical forces to break down cell walls and membranes, facilitating the release of intracellular components. In contrast, non-mechanical methods rely on the use of chemicals or expensive enzymes to hydrolyze cell membranes or enhance their permeability (Günerken et al., 2015; Lee et al., 2017; Roux et al., 2017).

Among these techniques, bead milling has emerged as a promising and effective approach for disrupting the rigid cell walls of microalgae (Postma et al., 2017; Lu et al., 2019; Liu et al., 2021; Safi et al.,2015). Doucha and Lívanský (2008) reported that passing *Chlorella* cells through bead mills can achieve more than 90 % disruption efficiency. While previous studies predominantly focused on the impact of bead milling operating parameters on the efficiency of microalgae cell disruption (Doucha and Lívanský, 2008; Montalescot et al., 2015; Weber et al., 2022), limited attention has been given to protein solubilization or extraction from the milled biomass. For example, Montalescot et al. (2015) explored the impact of various parameters on hydrodynamics within the bead milling grinding chamber and assessed the efficiency of microalgae cell disruption using Residence Time Distribution Modeling. However, the study did not specifically address the influence of cell disruption on protein extraction.

In addition to the challenge of protein accessibility, the limited solubility of microalgal protein in neutral water further impedes the extraction and recovery processes. Traditionally, a chemical approach involving very mild alkaline water (with a pH up to 9.5) is employed to solubilize plant proteins for the industrial-scale production of protein isolates or concentrates, as seen with soy protein. However, our recent findings revealed that employing mild alkaline water treatment (pH 9.0) was ineffective in extracting protein from *C. vulgaris*, yielding only about 6 % of protein in the liquid phase. While Safi et al. (2015) explored

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protein solubilization from *Chlorella* biomass (not treated with bead mill) using alkaline water with an elevated pH of 12, yet the solubilization yield remained low at approximately 26 % of the total protein. These observations prompted our exploration into integrating bead milling with alkaline solubilization as a strategy to enhance protein extraction efficiency. However, there is a lack of comprehensive research investigating the combined application of dry milling and alkaline solubilization for microalgae protein extraction.

Therefore, the primary objective of this study is to assess the influence of diverse parameters on the integrated process involving dry bead milling and alkaline solubilization. The goal is to establish a scalable methodology for extracting protein from *C. vulgaris* cell biomass. We systematically optimize both dry milling and alkaline solubilization conditions to enhance protein extraction yield. The acid precipitation conditions were optimized to recover the solubilized protein.

2. Materials and methods

2.1. Materials

The dried microalgae *Chlorella vulgaris* biomass was purchased from GO SUPERFOODS LTD (United Kingdom) and used without any treatment. Bradford reagent and bovine serum albumin (BSA) (≥ 98 % purity) were from Sigma-Aldrich. All other chemicals and solvents utilized in the study were of analytical grade.

2.2. Extraction of *C. vulgaris* protein

The extraction process for *C. vulgaris* protein involved a two-step procedure. Firstly, dry milling was performed to disrupt the *C. vulgaris* cell wall, followed by alkaline solubilization using sodium hydroxide (NaOH). Both dry milling and alkaline solubilization processes were optimized as illustrated in Section 2.4 and 2.5. After solubilization, microalgae biomass suspension was centrifuged at 26,000 × *g* using Sorvall Lynx 4000 centrifuge (Thermo Scientific, United States). The solubilized protein in the supernatant was measured using Bradford assay (Bradford, 1976) as illustrate in Section 2.3. The protein extraction yield (%) was calculated using following formula:

$$\text{Protein extraction yield (\%)} = \frac{\text{Solubilized protein in supernatant}}{\text{Total protein in biomass}} \times 100$$

2.3. Bradford assay for measurement of protein in liquid sample

After centrifugation, the solubilized protein in the supernatant was measured by Bradford assay (Bradford, 1976) as used by other researchers for microalgae protein (Chia et al.,2019). In brief, 50 μL of sample was mixed with 1.5 mL of Bradford reagent and incubated for 10 min at room temperature. Subsequently, 250 μL of mixture was added to a 96 micro-well plate and absorbance at 595 nm was measured. Protein was quantified using calibration curve prepared using bovine serum albumin (BSA) as standard.

$$\text{Protein recovery (\%)} = \left(1 - \frac{\text{Protein in supernatant after adjusting pH}}{\text{Protein in supernatant before adjusting pH}}\right) \times 100$$

2.4. Optimization of dry bead milling

2.4.1. Dry milling frequency

Dry milling of *C. vulgaris* biomass was conducted using steel jars and 0.5 mm zirconium beads in Retsch Mixer Mill MM 400 (Retsch, Germany) for 30 min (3 runs of 10 min with 5 min break between the single runs). The different dry milling frequency, namely 0, 8, 12, 16, 20, 24, 26, and 30 Hz were tested. The resulting broken biomass (100 mg) were then mixed with 5 mL of 0.1 M NaOH under a fixed condition (stirring duration 1.0 h, stirring speed 400 rpm and temperature 37 °C) and the protein extracted in aqueous solution was measured by Bradford assay. The most effective milling frequency was selected based on extraction yield (%) measured as illustrated in Section 2.2.

2.4.2. Dry milling duration

The biomass was subjected to dry milling at a best selected frequency of 26 Hz for various duration (10, 20, 30, 60, 90, 120 min), with 5 min intervals applied every 10 min. The resulting broken biomass was subsequently treated with 0.1 M NaOH to extract protein under the same condition mentioned in Section 2.4.1. The best milling duration was selected based on extraction efficiency (%).

2.5. Optimization of alkaline solubilization

2.5.1. Broken biomass loading

Following dry milling, different amount of milled biomass (2–17 % w/v) were mixed with 5 mL of 0.1 M NaOH at stirring speed of 400 rpm and 37 °C for 1.0 h. The mixture was centrifuged, and the protein solubilized in the supernatant was quantified using Bradford assay to calculated extraction yield (%) using the formula stated in Section 2.2.

2.5.2. NaOH concentration

NaOH concentration (0–0.5 M) was varied for solubilizing the protein from milled biomass of 2, 5 and 10 % w/v under the condition mentioned in Section 2.4.1. The NaOH volume was 5 mL. The best NaOH concentration was selected based on extraction yield (%) measured as described in Section 2.2.

2.5.3. Mixing (protein solubilization) time

Both broken (milled at 26 Hz for 1.0 h) and unbroken (control) biomass (5 % w/v) were mixed with 5 mL of 0.1 M NaOH for varying time lengths of 10, 20, 30, 40, 50, 60, and 90 min. The mixing temperature and speed were set at 37 °C and 400 rpm, respectively. The solubilized protein content was then measured using Bradford assay and the optimal solubilization time was selected based of extraction efficiency (%).

2.6. The recovery of protein from alkaline solution by pH induced precipitation

The protein solubilized in alkaline (NaOH) solution was recovered by pH induced precipitation. For this, microalgae biomass slurry in NaOH solution was centrifuged and the pH of the supernatant was subsequently adjusted to different pH (up to 1.0) by lowering pH using hydrochloric acid (HCl). To determine the amount of protein recovered or precipitated, the protein in the supernatant before and after adjusting pH was measured by Bradford assay. Protein recovery (precipitate) was estimated using following formula:

2.7. Determination of protein content in dry solid sample (protein concentrate)

Freeze drying was performed at −56 °C and 0.021 mbar using a freeze dryer (Labconco, United States) after neutralizing the

precipitated protein. The total protein content of the freeze-dried solid sample (protein concentrate) was measured using Dumas combustion method in DUMATHERM instrument (Gerhardt Analytical Systems, Germany). A nitrogen-to-protein conversion factor of 6.25 was used (Chen et al., 2019).

2.8. Statistics

Each experiment was conducted in triplicate under identical conditions. All the data were reported as mean ± standard deviation. Error bars on charts represent standard deviation of mean value of the data. The data were analyzed by analysis of variance (ANOVA) and Turkey's multiple comparisons using SPSS software (IBM, USA) to identify significant difference between two mean values at 95 % confidence level (p-value < 0.05).

3. Results and discussion

3.1. Optimization of bead milling conditions for breaking C. vulgaris cells

3.1.1. Effect of dry milling frequency

Bead milling is a mechanical cell disruption technique that involves agitating cells with beads in a chamber to break them open. One critical aspect influencing the efficiency of bead milling is the frequency at which the milling process is conducted. Hence, we examined the impact of dry bead milling frequency on breaking *C. vulgaris* cells by assessing the release of cellular proteins into a subsequent extraction media of 0.1 M NaOH and water. The results depicted in Fig. 1 illustrate that protein extraction yield increased with higher frequencies, reaching 43.4 % at 26 Hz, beyond which it plateaued. In the absence of bead milling, the protein extraction yield with 0.1 M NaOH alone was only 16.8 %. These findings suggest that higher frequencies of dry milling led to more effective disruption of *C. vulgaris* cells, resulting in increased protein release into the 0.1 M NaOH solution. However, dry milling alone may not be sufficient to completely disrupt the rigid cell wall of *C. vulgaris*. Thus, the integration of bead milling with alkaline treatment is crucial for enhancing protein extraction efficiency. The increased milling frequency enhances collisions between beads and cells, thereby generating greater shear force and impact stress on the cells. This intensified mechanical stress facilitates more efficient cell disruption, leading to a higher release of proteins into the subsequent extraction medium of 0.1 M NaOH, thereby improving the protein extraction yield. Excessively high milling frequencies may have detrimental effects on the quality of the extracted proteins. Hence, a dry milling frequency of 26 Hz was chosen for further optimization of *C. vulgaris* protein extraction.

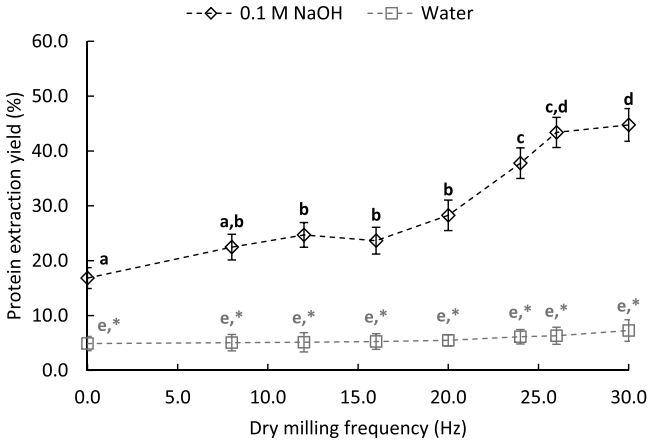

Fig. 1. Effect of dry milling frequency on *C. vulgaris* protein extraction yield under a fixed milling time of 0.5 h. The same superscript letters indicate values with no statistically significant difference (p-value < 0.05). Asterisks mark (*) indicates a significant difference between the solvents (0.1 M NaOH and water) used.

It is noteworthy that, unlike in 0.1 M NaOH, the protein extraction yield in water without bead milling is notably low at 5.0 % (at a milling frequency of 0.0 Hz), and it only marginally increases to 7.3 % with bead milling, even at a very high frequency of 30 Hz. The minimal protein extraction yield (7.3 %) in water suggests that *Chlorella* protein solubility in water is inherently low. An alkaline solution promotes cell disruption and breaks down hydrogen bonds (Cui et al., 2017), thereby resulting in a significant increase (about 7-fold higher) in the extraction yield observed in 0.1 M NaOH at 26 Hz compared to that in water (6.3 %). The integration of bead milling and alkaline solubilization thus emerges as an effective technique for microalgal protein extraction.

3.1.2. Effect of dry milling time

Fig. 2 presents the protein extraction efficiency for different milling times of at a constant milling frequency of 26 Hz. It can be observed that the protein extraction efficiency increased with the increase in dry milling time and reached 46.0 % at 60 min (1.0 h), after which the extraction efficiency plateaued, remaining almost the same. The increase in milling time could result in longer contact between beads and microalgae cells, thereby breaking more cells and releasing more protein during alkaline solubilization. The lack of considerable change in the extraction efficiency with prolonged milling time of more than 1.0 h suggests that the microalgal cell was either completely broken or reached the maximum level under the bead milling condition of frequency 26 Hz and milling time 1.0 h. Shehadul Islam et al. (2017) reported that longer milling time may lead to protein degradation and aggregation due to prolonged exposure under strong shearing force. Hence, the milling time of 1.0 h was chosen for subsequent studies.

3.2. Optimization of alkaline protein solubilization

3.2.1. Effect of mixing (protein solubilization) time

Alkaline solubilization typically use NaOH, to break down cell walls and release proteins from biological matrices. This method is favoured due to its simplicity, cost-effectiveness, and ability to extract a wide range of proteins. One critical parameter that significantly influences the protein extraction efficiency is mixing (protein solubilization) time. For evaluating the influence of mixing time on protein extraction yield, both bead-milled (broken) and unmilled (unbroken) microalgae biomasses (2 % w/v) were mixed with 0.1 M NaOH solution and protein extraction yield at different mixing time was shown in Fig. 3. It can be seen that the extraction yield increased with increasing mixing time and reached maximum of 46.0 %, and 16.8 % for broken and unbroken biomass respectively after one hour of mixing, after which the yield remained the same. This observed trend aligns with findings from previous studies by Lu et al. (2019) and Sun et al. (2022), in which the protein yield of both

Fig. 2. Effect of dry milling time on *C. vulgaris* protein extraction yield. The same superscript letters indicate values with no statistically significant difference (p-value < 0.05).

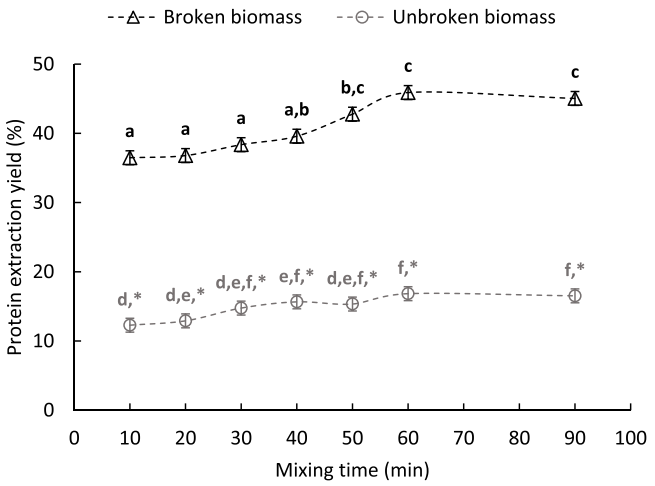

Fig. 3. Effect of mixing time on *C. vulgaris* protein extraction yield under a fixed biomass loading of 2 % (w/v). The same superscript letters indicate values with no statistically significant difference (p-value < 0.05). Asterisks mark (*) indicates a significant difference between the biomass types (broken and unbroken) used.

C. pyrenoidosa and *S. dimorphus* exhibited an increasing trend up to a specific extraction time length but decreased when mixed for a prolong period. Mixing time facilitates the diffusion of alkaline reagents into the cellular matrix, promoting the disruption of cell walls and enhancing the release of proteins into the extraction medium. Insufficient mixing time results in incomplete disruption of cell structures, leading to low protein yields. Gerde et al. (2013) reported that some proteins, particularly cell-wall associated proteins, are difficult to extract, which can take longer times to release into the extraction medium. Excessive mixing time can cause protein denaturation or degradation, reducing overall yield and compromising protein quality. Hence, mixing time of 1.0 h was selected for subsequent protein optimization.

3.2.2. *Effect of microalgae biomass loading*

Optimizing biomass loading in alkaline protein extraction processes is crucial for industrial applications. However, previous studies have not thoroughly examined the relationship between biomass loading and protein extraction yield. Therefore, our aim is to fill this gap by investigating the influence of biomass loading on alkaline protein extraction from *C. vulgaris* cell biomass. Fig. 4 depicts a decreasing trend in protein extraction efficiency across the biomass loading range of 2 % to 17 %

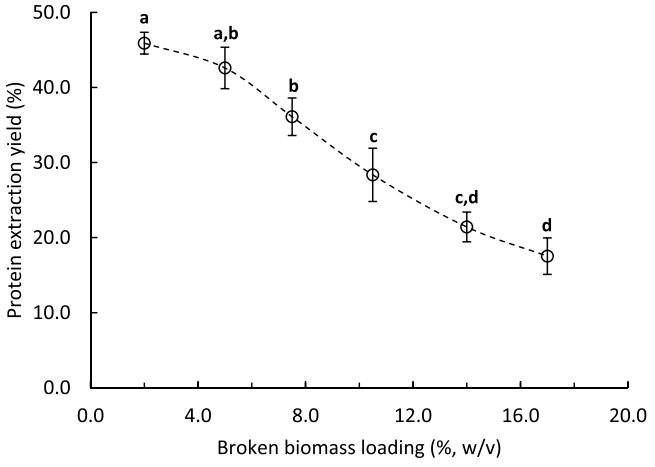

Fig. 4. Effect of broken biomass loading on *C. vulgaris* protein extraction yield. The same superscript letters indicate values with no statistically significant difference (p-value < 0.05).

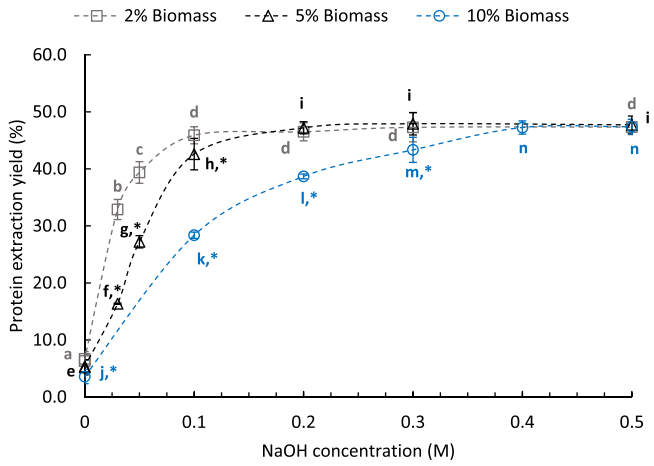

Fig. 5. Effect of NaOH concentration on *C. vulgaris* protein extraction yield for 2, 5 and 10 % biomass loading. The same superscript letters indicate values with no statistically significant difference (p-value < 0.05). Asterisks mark (*) indicates a significant difference between the biomass loadings employed.

(w/v) for a fixed NaOH concentration of 0.1 M. The highest protein extraction yield of 46.0 % was observed at 2 % (w/v) of biomass loading, while the lowest protein extraction efficiency of 17.5 % was noted at 17 % (w/v) biomass loading. Lu et al. (2019) investigated the influence of *Chlorella pyrenoidosa* biomass loading within a low biomass loading range of 0.18–1.8 % and observed a similar trend. The observed negative effect of biomass loading on protein extraction yield could potentially be attributed to the decrease in the biomass to NaOH weight ratio as biomass loading increased.

To better understand this relationship, we have explored the effect of increasing NaOH concentration for biomass loadings of 2 %, 5 % and 10 %. Fig. 5 demonstrates that the protein extraction yield increased with rising NaOH concentration and reached a plateau for all biomass loadings. The maximum protein extraction yield was found to be 46.0, 47.2 and 47.3 % with the biomass loadings of 2 %, 5 % and 10 %, respectively. The optimal NaOH concentration for 2 %, 5 % and 10 % biomass loadings were 0.1 M, 0.2 M and 0.4 M, respectively, corresponding biomass weight to NaOH molar ratio (g/M) of 200, 250 and 250, respectively. These findings suggest that the biomass weight to NaOH molar ratio remained almost the same regardless of biomass loading. Hence, the NaOH concentration alone may not be the main critical factor, but rather the biomass weight to NaOH molar ratio for achieving maximum protein extraction yield.

Table 1 illustrated the comparison of our findings with previous results reported by other research groups for microalgae protein extraction using different techniques. Our study achieved a notable extraction yield of 47.3 % through integration of bead milling and alkaline solubilization, while other methods efficacy varied from 4 % to 42 %. Our results highlight the potential of this integrated approach for enhancing protein extraction from microalgae, offering a more efficient and effective solution for microalgae cell breaking and protein recovery.

3.3. *Effect of precipitation pH on protein recovery from alkaline solution*

Protein recovery from alkaline solutions is a vital process in various industries, including food, pharmaceuticals, and biotechnology. One key aspect that significantly affects this recovery process is the precipitation pH. Fig. 6 demonstrates that 100 % of the protein remained in the alkaline solution at pH 13.2. As the solution pH was gradually reduced by the addition of HCl, the solubilized protein began to precipitate slowly. It is evident from Fig. 6 that approximately 98.0 % of the protein precipitated (recovered), leaving only about 2.0 % of the protein in the alkaline solution at a pH of 4. The protein recovery remained consistent with further reduction of the pH. This result strongly suggests that the

Table 1
Comparison of other studies using different cell disruption and protein extraction methods for different microalgae species with our study.

Study	Microalgae	Extraction technique	Protein extraction yield
This study	*Chlorella vulgaris*	Dry bead milling followed by alkaline extraction	47.3 %
Postma et al. (2016)	*Chlorella vulgaris*	Pulse electric field in water	4.4 %
Sun et al. (2022)	*Scenedesmus dimorphus*	Ultrasonication in alkaline solution	40.13 %
Anjos et al. (2022)	*Tetraselmis chuii*	Wet bead milling in buffer	27 %
Schwenzfeier et al. (2011)	*Tetraselmis* sp.	Wet bead milling in buffer	21 %
Gerde et al. (2013)	*Nannochloropsis spp.*	Ultrasonication in alkaline solution	16 % in defatted biomass 30 % in non-defatted biomass
da Silva et al. (2024)	*Tetradesmus obliquus*	Dry ball milling	14.11 %
		Ultrasonication in water	19.95 %
		High pressure homogenization in water	15.68 %
Postma et al. (2015)	*Chlorella vulgaris*	Wet bead milling in buffer	42.0 %
Postma et al. (2017)	*Chlorella vulgaris*	Wet bead milling in buffer	36.3 %
	Neochloris oleoabundans		~32.0 %
	Tetraselmis suecica		~23.0 %
Günerken et al. (2016)	*Neochloris oleoabundans*	Wet bead milling in buffer	34.0 %
Alavijeh et al. (2020)	*Chlorella vulgaris*	Wet bead milling in water	39.0 %

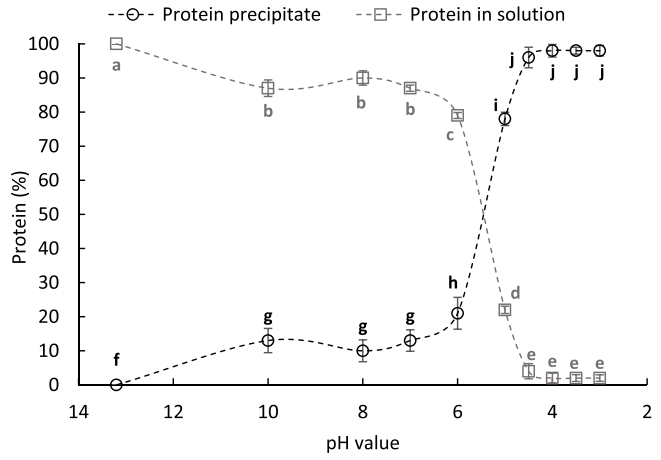

Fig. 6. Effect of pH on *C. vulgaris* protein precipitation. The same superscript letters indicate values with no statistically significant difference (p-value < 0.05).

isoelectric point of the protein in *C. vulgaris* is around 4.0. Protein precipitation is governed by various mechanisms, including changes in protein charge, solubility, and conformation. At the pI of a protein, where the net charge is zero, protein solubility is typically low, resulting in precipitation. Adjusting the pH above or below the pI can modulate protein charge, leading to electrostatic repulsion or attraction between protein molecules and influencing their aggregation behavior. Further research into the mechanisms underlying *C. vulgaris* protein precipitation will continue to advance protein recovery technologies and their applications in various industries.

The protein precipitate was separated via centrifugation and then neutralized using a NaOH solution. Subsequently, the neutralized protein precipitate underwent freeze-drying. Analysis via the Dumas method revealed that the protein content in the resulting freeze-dried sample was 69.1 % (w/w). This notably high protein content suggests its suitability for use as a protein concentrate.

4. Conclusion

An integrated process involving dry bead milling followed by mixing the milled biomass with alkali (NaOH) solution was optimized for efficient protein extraction from microalgae *C. vulgaris*. The significance of bead milling lies in its ability to disrupt the strong and rigid cell wall of microalgae, thereby improving protein release into the extraction medium. Alkaline treatment further breaks down the cells and promotes protein solubilization. The integrated process has proven to be effective in maximizing the extraction of protein from *C. vulgaris* cell biomass. Additionally, we uncovered a phenomenon: the optimal *C. vulgaris* biomass weight to NaOH molar ratio of 200–250 (g/M) remained consistent regardless of biomass concentration. Acid precipitation resulted in a protein recovery rate of 98.0 % from the liquid phase. The protein content in the recovered freeze-dried protein concentrate was 69.1 % (w/w).

Future research should focus on addressing the challenges associated with separating non-proteinaceous components, particularly insoluble polysaccharides, from the protein concentrate to further enhance protein purity.

Ethical statement – studies in humans and animals

I testify on behalf of all co-authors that our manuscript submitted to the Special Issue "The potential of algal protein as a sustainable functional food ingredient" of Future Foods

CRediT authorship contribution statement

Jun Wei Ng: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Tong Mei Teh:** Methodology, Investigation. **Weingarten Melanie:** Writing – review & editing. **Md. Mahabubur Rahman Talukder:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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