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# MICROALGAE AS POTENTIAL PROTEIN SOURCES: EVIDENCE FROM PROTEIN EXTRACTION AND AMINO ACID PROFILING OF *Chlorella vulgaris* AND *Scenedesmus* SP.

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The high protein contents in green microalgae can be used for the generation of bioactive peptides for feed, food, and health. Many methods are available for the extraction of microalgal protein but they are not easily scalable due to the requirement of special techniques and instruments. In the present work, the nitrogenous component of green microalgae *Chlorella vulgaris* and native species *Scenedesmus* sp. were quantified by the Lowry method and their amino acid composition was analyzed and compared. Proteins extraction was done by milling algal biomass, solubilization at various concentrations of NaOH, and precipitation at acidic pH. In results, both *Scenedesmus* sp. and *C. vulgaris* showed the maximum specific growth rate i.e. 0.653 and 0.589 g $L^{-1}$ $d^{-1}$, respectively in Bold's Basal Medium. Grinding biomass and then alkaline (1N) treatment at 100 °C for 10 min resulted in the maximum protein extraction from both species. Under optimum conditions, an increase of 19 and 18% in protein contents of *Chlorella vulgaris* and *Scenedesmus* sp., respectively compared to control treatment was noted. Regarding amino acid profiling, glutamic and aspartic acids were found in the highest concentrations in *Scenedesmus* sp. (2.9 and 2.5%) and *C. vulgaris* (3.2 and 2.8%), respectively. Six amino acids (alanine, aspartic acid, glutamic acid, leucine, lysine, and glycine) were responsible for 50% or more of the total detected amino acid contents in both microalgal species. In conclusion, the consolidating activity of freeze-drying and milling the dry biomass along with alkaline extraction significantly boosted the protein yield.

**Keywords:** Microalgae; Protein extraction; Amino acids; *Scenedesmus* sp.; *Chlorella vulgaris.*

## INTRODUCTION

Algae are autotrophic plants with vast diversity, divided into two forms, unicellular and multicellular (Bibi *et al.,* 2017). Besides this, microalgae biomass contains fundamental supplements such as proteins, sugars, lipids, and polysaccharides, which are otherwise regularly disregarded during pigment recovery (Becker, 2007; Pignolet *et al.,* 2013; Katiyar and Arora, 2020; Khemiri *et al.,* 2020). Tokusoglu and Unal (2003) have demonstrated that edible microalgae after being cultured have the potential for food supplement and food additives for animals. From their nutritional compositions, exceptionally high protein contents (normal 63%) were found in *Spirulina platensis* (Tokusoglu and Unal, 2003; Soni *et al.,* 2021). The commercial benefits of microalgae are their quick growth e.g. microalgae can produce 50 times more biomass compared to the higher plants (Li *et al.,* 2008). The maximal conversion efficiencies of solar radiation into biomass are 4.6 % for $C_3$ plants and 6.0 % for $C_4$ plants at 30 °C, which drops to 2.9 % and 4.2 % respectively when measured under field conditions (Zhu *et al.,* 2010). For example, the sugarcane field has an average energy conversion efficiency of 10 ton $ha^{-1}$ $year^{-1}$ while microalgae translate into expected maximal productivity of 280 ton of algal biomass $ha^{-1}$ $year^{-1}$ (Chisti, 2007).

For optimum growth of microalgae, light, carbon source, water, and nutrients (phosphorous and nitrogen as major nutrients) are required. Light provides energy for photosynthesis. Carbon dioxide is a carbon source of microalgae cell development. There is great flexibility and adaptability of microalgae to grow on non-arable land and therefore competition with agriculture for food production can be avoided (Khemiri *et al.,* 2020). This makes microalgae a potentially sustainable source of feedstock for next-generation biofuel, chemical, textile, polymer, and even the pharmaceutical industries (Ursu *et al.,* 2014; Ghosh *et al.,* 2016).

Cultivation of microalgae on an industrial scale is increasing worldwide for its enhanced nutritional aspects (Spolaore *et*

*al.,* 2006). However, the production of microalgal biomass on large scale involves high costs due to the requirement of a high volume of seed culture (Das *et al.,* 2015; Sayedin *et al.,* 2020). However, the sustainable and economical production of algae biomass on large scale is in progress (Di Lena *et al.,* 2020). A huge diversity of species (50,000) exists with a set number of around 30,000 adopted and examined (Richmond, 2004). Among these, microalgae with significant commercial value include green algae (Chloreophycea) *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina*, *Secenedesmus obliqus*, and (Cyanobacteria) *Spirulina maxima* (Ursu *et al.,* 2014).

*Chlorella vulgaris* and *Scenedesmus* sp. ($UKM_9$) are amongst the most commercially important model of microalgae. *C. vulgaris* is a photosynthetic microorganism with an enhanced growth rate (Phukan *et al.,* 2011) and composed of lipids in addition to high protein contents suitable for biofuel production and human dietary requirement (Becker, 2007; Morris *et al.,* 2009; Seyfabadi *et al.,* 2011). *Chlorella* sp. is surrounded by a rigid cell wall, which results in lower permeability of intracellular components, especially the extraction of proteins (Callejo-Lópeza *et al.,* 2020). Therefore, cell lysis methods are accomplished before protein extraction. Various methods such are mechanical activity (high-pressure homogenizers, bead mills), ultrasounds, enzymatic or compound medicines, thermal or osmotic stuns (continued freezing/defrosting) have been used for the extraction and transforming extracted proteins from microalgae into concentrated form (Doucha and Lívanský, 2008; Sari *et al.,* 2013; Schulze *et al.,* 2019; Callejo-Lópeza *et al.,* 2020). Even though these methods are effective, however, these are not easily practicable due to the high costs involved in the form of specialized instruments, time consumption, and the skills required. Alkaline media has been found the most suitable for protein extraction in microalgae (Horax *et al.,* 2011; Callejo-Lópeza *et al.,* 2020). During alkali treatments, consideration must be paid to keep away from the saponification of intracellular lipids.

Besides this, algal biomass contains high protein contents (46.8% approximate) with a good amino acid profile and low metal, which make them a promising feedstock for marine fish (Safari *et al.,* 2008). The utilization of microalgae in the commercial industry can expand if an improved cultivation system with optimum production of algal biomass rich in active substances (proteins and amino acids) is adopted. Moreover, an efficient protein extraction system would enhance the recovery of proteins and amino acids. Based on this hypothesis, the present study was conducted to evaluate different alkaline treatments for the recovery of protein fraction and amino acid compositions from *Chlorella vulgaris* and *Scenedesmus* sp., which can be used in food, feed, health, and bulk chemical market. Up to our knowledge, no study has elucidated the comparative effectiveness of different alkaline pretreatments for protein extraction from microalgae.

## MATERIALS AND METHODS

Green microalgal species i.e., *Chlorella vulgaris* (CHV) and *Scenedesmus* sp. ($UKM_9$) were collected from the Algal culture Laboratory of Universiti Kebangsaan Malaysia-Yayasan Sime Darby (YSD). Among them, *Scenedesmus* sp. ($UKM_9$) was a native species, isolated from palm oil mill effluent (POME) sources collected from Carrey Island, while *C. vulgaris* was commercial species. Both algal species were grown individually in 2 L Erlenmeyer flasks using two different fresh algal mediums i.e. Bold's Basal Medium (BBM) (Edris *et al.,* 2014) and Blue-green medium (BG11) (Patterson *et al.,* 1991) to observe the maximum biomass productivity. The recipe of BBM was as $NaNO_3$ (240 mg $L^{-1}$), $K_2HPO_4$ (76 mg $L^{-1}$), $KH_2PO_4$ (176 mg $L^{-1}$), NaCl (26 mg $L^{-1}$), $MgSO_4.7H_2O$ (76 mg $L^{-1}$), $CaCl_2.2H_2O$ (27 mg $L^{-1}$), $FeSO_4.7H_2O$ (4.99 mg $L^{-1}$), $H_3BO_3$ (11.32 mg $L^{-1}$), EDTA (51 mg $L^{-1}$), KOH (33 mg $L^{-1}$), $ZnSO_4.7H_2O$ (8.72 mg $L^{-1}$), $MnCl_2.4H_2O$ (1.34 mg $L^{-1}$), $MoO_3$ (0.73 mg $L^{-1}$), $CuSO_4.5H_2O$ (1.67 mg $L^{-1}$) and $Co(NO_3)_2.6H_2O$ (0.5 mg $L^{-1}$). Similarly, Blue Green medium (BG11) contained $NaNO_3$ (1.6 g $L^{-1}$), $K_2HPO_4$ (0.05 g $L^{-1}$), $MgSO_4.7H_2O$ (0.075 g $L^{-1}$), $CaCl_2.2H_2O$ (0.036 g $L^{-1}$), citric acid (0.007 g $L^{-1}$), ammonium ferric citrate green (0.007 g $L^{-1}$), EDTA $Na_2$ (0.002 g $L^{-1}$), $Na_2CO_3$ (0.03 g $L^{-1}$), $H_3BO_3$ (2.76 mg $L^{-1}$), $MnCl_2.4H_2O$ (1.28 mg $L^{-1}$), $ZnSO_4.7H_2O$ (0.12 mg $L^{-1}$), $Na_2MoO_4.2H_2O$ (0.29 mg $L^{-1}$), $CuSO_4.5H_2O$ (0.09 mg $L^{-1}$) and $Co(NO_3)_2.6H_2O$ (0.06 mg $L^{-1}$). The pH of both media was adjusted to 7.0 and autoclaved for sterilization at 121 °C for 20 min.

An air pump (Aqua King AK-548, Big Bay B-10200) was used for aeration, and flasks were illuminated with cool white fluorescent light using tungsten lamps (330 W) placed 45 to 60 cm above the culture. The culture was illuminated by 12:12 (L:D) continuous light. For all experimental runs, the temperature was maintained at 23 °C, pH 6.5-7.0, and the cultivation period was 20 days.

Growth of these species was monitored every 24 h by measuring the optical density of the cultures at 689 and 650 nm for *Scenedesmus* sp. and *C. vulgaris*, respectively using a UV-visible spectrophotometer (Jenway 6850 Stone, Staffordshire, UK). Algal sample (5 mL) was collected daily and microscopic observations were performed by using Core Parmer Microscope (Vernon Hills, Illinois 60061) to monitor the growth of algal species as shown in Fig. 1. For this purpose, a drop of oil was placed on a glass slide containing an algal sample, shielded the slide with a coverslip, and viewed under a microscope. The slide was observed in the range of 100X magnification.

The concentration of algal biomass was measured by calculating the dry weight. After 24 hours, 10 mL of each

sample was taken for screening. Whatman glass microfibers (Grade GF/C1.2 µm) were used for filtering under vacuum and washed two times with purified water. The collected microalgal samples were dried in an oven at 105 °C for 24 h and dry weight was measured. The microalgal biomass was determined by the combined weight of filter paper and microalgal dry biomass deducted with the weight of filter paper (Rice *et al*., 2012). The trial setup was repeated three times to avoid handling errors. Then the developmental profile was evaluated.

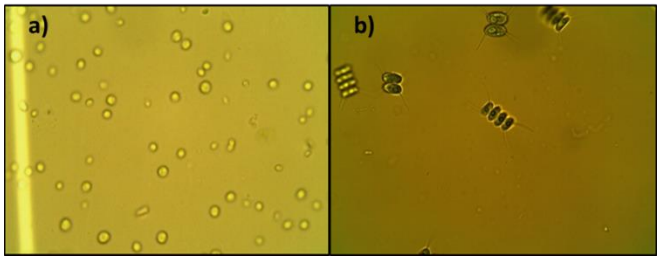


**Figure 1: Microscopic observation of microalgae strains a) *Chlorella vulgaris* and b) *Scenedesmus sp.* (UKM9)**

***Specific Growth Rate*:** The equation used for the calculation of specific growth rate per day was applied according to Mo *et al*. (2015).

$$\text{Specific growth rate (g day}^{-1}) = \frac{\ln(X_2/X_1)}{T_2 - T_1}$$

Where $X_2$ denotes algal biomass (g) after a particular time, $X_1$ represents algal biomass at the initial time, and $T_2 - T_1$ is a change in time in days.

***Pretreatment of algal species*:** About 30 g of the frozen paste of crude microalgae was directly introduced into a freeze dryer. The temperature was reduced to -80 °C while pressure was reduced to 0.01 bar. This practice of freeze-drying was conceded under vacuum for a total of 72h. Dry biomass was stored at -20 °C for further analysis. For control, an aggregate of 25 mg of each algal strain was disintegrated in distilled water (20 mL) for 1h. The slurry was separated by utilizing a centrifuge machine at 10,000 rpm for 10 min at room temperature. Pellet was disposed of and the supernatant was recovered for protein investigation. The treatment was reflected as a control.

***Grinding and milling with ceramics*:** To measure the effect of pretreatment methods for protein quantification, dry biomass (25 mg) of each strain i.e., *C. vulgaris* and *Scenedesmus* sp. was physically milled by using pestle and mortar for 5 min. Then the powdered biomass was transferred into glass tubes (15 mL) and hydrolyzed with three different concentrations of NaOH (0.5, 1.0, and 2.0 N). After that, dry algal biomass (25 mg) was ground with ceramics ($Al_2O_3$) and placed into another set of glass tubes (15mL). Then the samples were again hydrolyzed with three different concentrations of NaOH. (0.5, 1.0 and 2.0 N). For this purpose, 3 mL of each concentration of NaOH was added to the respective test tube (15 mL). At this point, the samples were heated at 100 °C for ten minutes in a preheated oven, cooled down, and centrifuged at 4000 rpm for eight minutes to get extracts for protein examination (Lowry *et al.*, 1951).

***Measurement of proteins by the Lowry method*:** Total intracellular protein contents were determined by Lowery *et al*. (1951) utilizing bovine serum albumin (BSA) as standard. To estimate the protein contents, 0.2 mL of every standard was pulled back, and afterward, Lowry reagent (2 mL) was introduced. Each sample was then vortexed and hatched at room temperature for 10 min in dark. After hatching, 0.2 mL of 1N Folin-Ciocalteu reagent were added, vortexed and incubated for 30 min in dark. The absorbance was measured at 750 nm using a UV-visible spectrophotometer (Jenway 6850 Stone, Staffordshire, UK). Follin reagent is very sensitive to light, so to avoid the possible degradation, the test was conducted in dark. Spectroscopic absorbance was transformed into protein concentration using a calibration curve established with BSA (1 mg $mL^{-1}$). The protein substance of biomass was determined by the following equation:

Y = ax-b

Where 'a' denotes the slope, 'b' represents the y-intercept while x and y are holding the place of coordinates (x, y) of any point that lies on the line. The protein concentration of *C. vulgaris* and *Scenedesmus* sp. ($UKM_9$) was measured using the equation produced from the standard curve i.e., y = 0.43x-0.005.

***Elemental Analysis*:** Elemental composition such as carbon, nitrogen, hydrogen, oxygen, and sulfur contents of harvested algae was conceded out by CHNS Elemental Analyzer (Thermo FlashEA 1112, USA).

***Amino Acid Analysis*:** Amino acid composition of the freeze-dried biomass was accomplished according to the 6N hydrochloric acid (HCl) hydrolyzate method (Hirs *et al.*, 1954). A total of 0.2 g of each sample was placed into a hydrolyzate test tube. Then 5 mL (6N) HCl was added. The hydrolyzate test tube was sealed tightly with a stopper, placed into a container, and closed tightly. Then the samples were heated in an oven at 110 °C for 24 h. When heating was over, algal biomass was cooled, and then transferred into a volumetric flask (100 mL) using a filter funnel. A total of 400 µL alpha aminobutyric acid (50 µM) was added into the volumetric flask as an internal standard, made the volume up to 100 mL with deionized water, and filtered an aliquot through syringe filter into screw neck vial. 10 µL sample was pipetted into a centrifuge tube and injected into the derivatization sample. High-performance liquid chromatography (HPLC) was used for amino acid profiling of both microalgal species. The chromatographic conditions for this method were column: AccQ Tag Column., 3.9 × 150

mm., Mobile phase; AccQ Tag Eluent A, concentre, AccQ Tag Eluent B or 60% Acetonitrile, flow rate 1mL $min^{-1}$, column temp was 36 °C, fluorescent detector (Ex λ = 250 nm, Em λ = 395 nm, filter = 1.5 s).

***Statistical analysis*:** The statistical analysis and data processing were performed using Origin Pro 8.5, Graph pad Prism 8.0 software, and Microsoft Excel 2016. All the experiments were conducted in triplicates. Results are represented as mean ± standard deviation. Percentages are expressed as weight/weight on dry biomass basis. One-way analysis of variance (ANOVA) and Tukey's HSD analysis was done to compare the differences between algal species regarding biomass yield.

## RESULTS AND DISCUSSION

***Biomass yield and productivity*:** Optical density and dry weight have been appropriate means for the estimation of biomass. Microalgal species were grown in Bold Basal medium (BBM) and BG11, to find out the medium based on the production of the maximum biomass yield and productivity (Fig. 2a). Both *Scenedesmus* sp. ($UKM_9$) and *C. vulgaris* demonstrated the maximum specific growth rate (0.653 and 0.589 g $L^{-1}$ $d^{-1}$) in BBM which shows that BBM could be a reasonable medium for the optimum growth of both species compared to BG11. Generally, microalgae grow in six different stages (stationary phase, lag phase, linear phase, exponential phase, death phase, and declining phase) during batch culture, which is similar to the other microorganisms. As clear from Figure 2a, the lag phase was observed from day 1 to 4, which might be due to the physiological adjustments to start utilizing nutrients. With time from day 4 to day 11-12, boosted the growth of both algal species, i.e. exponential growth stage was observed. On the fifteenth day, the dry weight of *Scenedesmus* sp. ($UKM_9$) grown in the BBM reached the maximum (0.737 ± 0.058 g $L^{-1}$) compared to that observed in the case of BG11 medium (0.623 ± 0.006 g $L^{-1}$). The dry weight values on the fifteenth day for *Scenedesmus* sp. ($UKM_9$) represented a statistically significant change between the media. Tukey's HSD test demonstrated a significant difference between BBM and BG11 ($p < 0.05$). Likewise, on day 15, the dry weight of *C. vulgaris* developed in BBM was 0.677 ± 0.001 g $L^{-1}$ compared to that observed in the BG11 medium (0.530 ± 0.006 g $L^{-1}$) on day 13. The dry weight on day 15 for *C. vulgaris* represented a statistically significant ($p = 0.05$) difference between the media tested.

The development of algal growth was also monitored based on the change in optical density (OD) of the growth medium (Figure 2b). A sharp rise in the optical density was observed in case of *Scenedesmus* sp. ($UKM_9$) cultured in BBM compared to that observed under the BG11 medium. A similar trend in optical density was observed in the case of *C. vulgaris* cultured in BBM. On day 15, BBM indicated the maximum optical density, which showed the maximum growth and development of both microalgal species. Earlier, Ilavarasi *et al*. (2011) and Wong *et al*. (2017) also recorded similar results as observed in the present study.

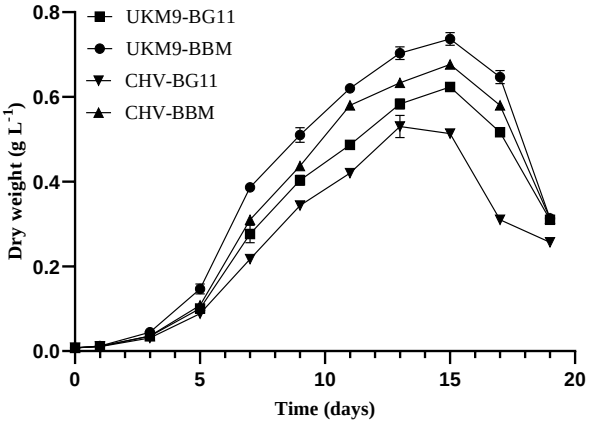


**Figure 2a. Dry weight of *Chlorella vulgaris* (CHV) and *Scenedesmus* sp. (UKM-9) in BBM and BG11 media. Note: The error bars represent the standard error of mean where n = 3**

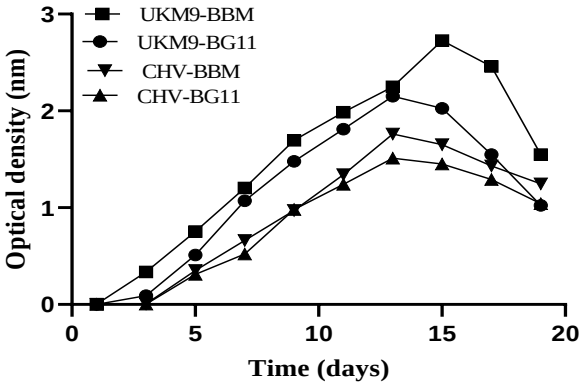


**Figure 2b. Optical density Chlorella vulgaris (CHV) and Scenedesmus sp. (UKM-9) in BBM and BG11. Note: The error bars represent the standard error of mean where n = 3**

***Quantification of proteins*:** Proteins are large polypeptides formed by linking many α-amino acids via amide bonds. The presence of many functional groups like $–NH_2$ groups, -COOH groups, -OH groups, and (–S-S-) disulfide groups makes protein a promising material for the production of bio-based polymeric materials (Schulz and Schirmer, 1979). The rise in the global population has led to the exploitation of alternative sources of energy and food. Feedstock that contains high protein contents is also abundant in nature, including microalgae, soybean protein products, soy flour, defatted soybean meal, soy protein concentrates, and soy protein isolates (Kumar *et al.,* 2002; Callejo-Lópeza *et al.,* 2020; Khemiri *et al.,* 2020). Because corn and soybean are

staple food crops for humans, their common use as the main source of dietary energy and proteins in animal feed does not look reasonable. Alternately, de-fatted microalgal biomass may be a viable replacement for corn and soybean meal due to their high levels of proteins, well-balanced amino acid profile, and rich contents of minerals and vitamins.

Traditionally, algal proteins were extracted by water, acidic, or alkaline methods with the process of centrifugation and saving techniques including ultrafiltration, precipitation, or chromatography (Hildebrand *et al.,* 2020). Synthetic way of extraction technique includes two-stage acid and salt treatments, found proficient for separation of protein from *A. nodosum* and *Ulva* sp. The differentiation of protein concentration in a sample was observed in the constructed standard curve (Kadam *et al.,* 2017). Bovine serum albumin (BSA) is considered a dominantly used standard as it is highly pure and less expensive. In earlier studies, it is added as a referenced protein for the formation of a linear graph (Indriani *et al.,* 2018). The blank sample was used as control and the spectrophotometer was zeroed for a blank. The presence of interference enhanced the absorbance of a blank reagent. These interferences are due to nucleic acids, drugs, amino acid derivatives, sugars, lipids, salts, and certain buffers. The calibration curve was observed straight with a high correlation coefficient ($R^2$ = 0.997) within the absorbance scope of 0.2 to 1 mg $mL^{-1}$ by utilizing BSA.

Three different concentrations of alkali (NaOH) were used to hydrolyze the protein from algal species i.e., 0.5, 1, and 2N NaOH (Fig. 3). Comparing the three treatments, 1N NaOH for 10 min at 100 °C was found the best treatment for protein extraction in the case of both microalgal species i.e., *C. vulgaris* (0.877 ± 0.006 g $L^{-1}$) and *Scenedesmus* sp. ($UKM_9$) (0.717 ± 0.035 g $L^{-1}$). However, when extreme conditions were applied i.e., 2N NaOH, 10 min, 100 °C, the protein yield was decreased; indicating a possible degradation of extracts.

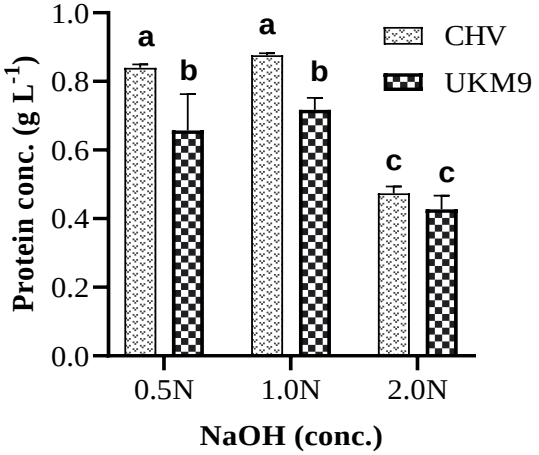


**Figure 3. Extraction of protein from *C. vulgaris* and *Scenedesmus sp.* (UKM-9) by using different concentrations of alkali (NaOH). Means sharing the same letter above columns do not differ significantly ($p < 0.05$). Note: The error bars represent the standard error of mean where n = 3**

Similar results were observed by Gerde *et al*. (2013) during the optimization of protein extraction from microalgae. For accurate estimation of microbial proteins, it is a prerequisite for the cells to be pretreated for a thorough extraction of intracellular proteins by physical or synthetic methods (Barbarino and Lourenço, 2005). In our study, pestle and mortar were used for initial processing. Moreover, processing with aluminum oxide for 5 min brought absolute algal cell disturbance and in limited time, the grouping of protein in cell homogenates was observed. Similar observations were claimed by Ceron *et al*. (2008). Soluble protein segment released in NaOH after disruption of each microalgal strain is demonstrated in Fig. 4a, b.

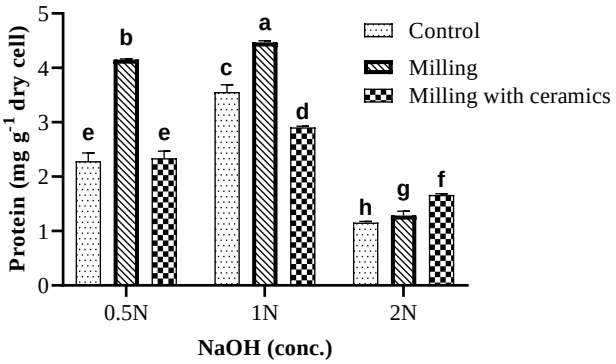


**Figure 4a. Influence of pretreatment on protein contents (mg $g^{-1}$ dry cell) of Scenedesmus sp. (UKM-9) under different concentrations of NaOH. Means sharing the same letter above columns do not differ significantly ($p < 0.05$). Note: The error bars represent the standard error of mean where n = 3**

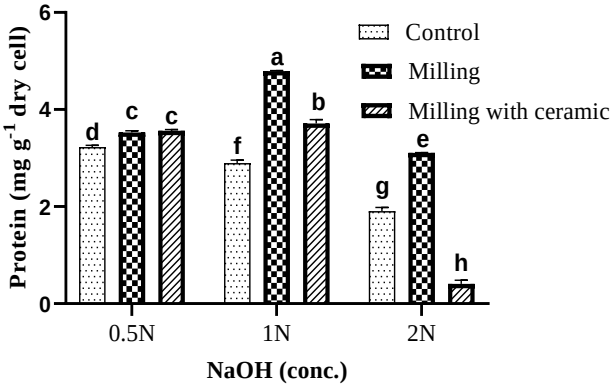


**Figure 4b. Effect of pretreatment on protein contents (mg $g^{-1}$ dry cell) of *Chlorella vulgaris* (CHV) under different concentrations of NaOH.**

**Means sharing the same letter above columns do not differ significantly ($p < 0.05$). Note: The error bars represent the standard error of mean where n = 3**

The 25 mg of freeze-dried microalgal species were ground for 5 min. by using pestle and mortar. Two types of pretreatments were followed i.e., milling and milling with ceramics ($Al_2O_3$). Then the ground species were treated with three different concentrations of NaOH (0.5N, 1N, 2N) to solubilize microalgal protein. Fig. 4a indicates that both milling and milling with ceramic raised greater protein yield. In the case of *Scenedesmus* sp. ($UKM_9$), the maximum protein recovery (18%) was obtained by milling and then extraction with 0.5N NaOH, which showed a 7% increase over control. Fig. 4a and b indicated significant values ($p = 0.05$) for 0.5N and 1N solution of NaOH while the least protein (non-significant) recovery resulted with milling with ceramic and 2N NaOH, which indicates that harsh chemical condition had denatured amount of protein (Fig. 4a, b). A minor increase in protein concentration was noticed for *C. vulgaris* in ceramic grinding with the 2N NaOH method, which might be due to the rigid cell wall of *C. vulgaris*. For *C. vulgaris*, 19% more protein yield compared to the control was obtained with physical milling and extraction with 1N NaOH. Milling with ceramic also showed maximum yield i.e. 9% more than control, which indicates that the cellulosic structure of *C. vulgaris* had been disrupted by ceramics. In experiments, *Chlorella* sp. showed better productivity of protein compared to the *Scenedesmus* sp. ($UKM_9$).

Fig. 5a and 5b are showing the linear relation between dry weight and protein contents with increasing time in both microalgal species. The dry weight and protein contents were measured regularly after two days of time intervals for 12 days to find out either protein liberation show an increasing or decreasing trend. An increasing trend was noted in protein contents of *C. vulgaris* with increasing dry weight up to the exponential stage as shown in Fig. 5a. Both species have different cell wall compositions. Therefore, changed protein contents were observed between 0.2 to 1.4 g $L^{-1}$ of dry mass. *C. vulgaris* exhibited higher extraction efficiency over the range of 0.02 to 0.16 g $L^{-1}$ with an almost linear yield of protein contents. In contrast, *Scenedesmus* sp. showed an optimal protein yield between 0.02 to 0.13 g $L^{-1}$ (Fig. 5b). A correlation of relative protein yields (values communicated in % of most extreme yield at 100 °C) uncovers lower relative yields for *Scenedesmus* sp. i.e., 7.8-10.0% while relatively higher yields from *C. vulgaris* i.e., 11-12% protein contents were liberated. Overall, the treatments at 100 °C, 10 min. and 1N NaOH results in significant protein liberation ($p = 0.05$) compared to 0.5 N and 2 N NaOH, possibly due to incomplete protein liberation below 100 °C and protein denaturation at harsh conditions.

***Elemental analysis and amino acid profiling:*** The results of elemental analysis are presented in Table 1. The higher percentage of oxygen in CHV (33.77%) and UKM9 (40.08 %) indicated that products produced from this biomass would be highly oxygenated compounds and it results in low quality of biofuels produced from such type of biomass. Microalgae are deliberated as an alternative source of amino acids, which combine to form thousands of different proteins.

**Table 1. Elemental analysis of microalgal species used in the study**

| Elements | *Chlorella vulgaris* | *Scenedesmus* sp. |
|---|---|---|
| Carbon (%) | 49.44±0.13* | 45.86±0.13 |
| Hydrogen (%) | 7.00±0.04 | 6.63±0.03 |
| Nitrogen (%) | 8.68±0.01 | 6.20±0.01 |
| Sulphur (%) | 1.45±0.05 | 1.44±0.03 |
| Oxygen (%) | 33.43±0.11 | 39.87±0.11 |

*The values following mean values represent the standard error of the mean (SEM) where n = 3

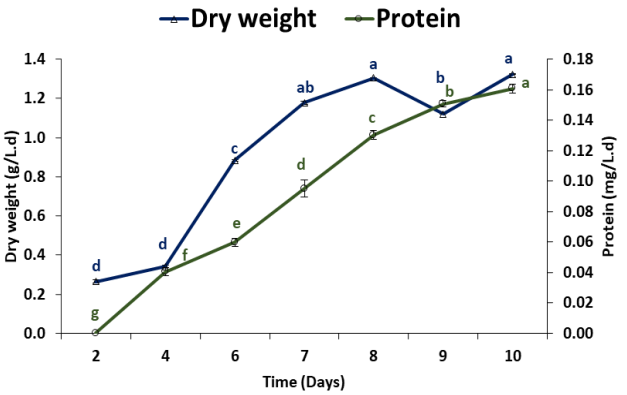


**Figure 5a: Dependence of protein yield (mg $L^{-1}$) on extraction time (2-12 days) with dry weight (0.2-1.4 g $L^{-1}$) for *Chlorella vulgaris* cells. Means sharing the same letter above columns do not differ significantly ($p < 0.05$). Note: The error bars represent the standard error of mean where n = 3**

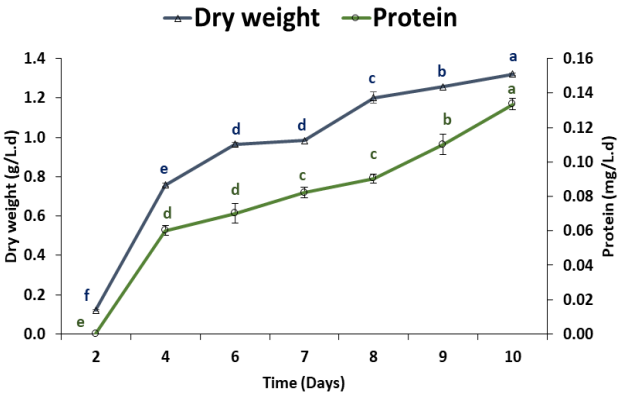

**Table 2. Combined amino acid profile of *Chlorella vulgaris* and *Scenedesmus* sp.**

| Name | Symbol | M.W | Molecular Formula | Retention Time (min) | | Height (%) | | Amount (% DW) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | UKM-9 | CHV | UKM-9 | CHV | UKM-9 | CHV |
| Alanine | Ala | 89.10 | $C_3H_7NO_2$ | 20.580 | 20.349 | 4.71 | 4.97 | 1.955 | 2.525 |
| Arginine | Arg | 174.20 | $C_6H_{14}N_4O_2$ | 18.499 | 18.113 | 1.77 | 1.78 | 1.302 | 1.428 |
| Aspartic acid | Asp | 133.11 | $C_4H_7NO_4$ | 12.172 | 11.961 | 2.94 | 4.52 | 2.503 | 2.817 |
| Glutamic acid | Glu | 147.13 | $C_5H_9NO_4$ | 13.877 | 13.184 | 3.23 | 3.40 | 2.857 | 3.205 |
| Glycine | Gly | 75.07 | $C_2H_5NO_2$ | 14.915 | 14.371 | 3.68 | 3.27 | 1.664 | 1.657 |
| Histidine | His | 155.16 | $C_6H_9N_3O_2$ | 15.585 | 14.701 | 0.89 | 0.85 | 0.524 | 0.561 |
| Hydroxyproline | Hyp | 131.13 | $C_5H_9NO_3$ | 10.680 | 10.652 | - | - | - | – |
| Isoleucine | Ile | 131.18 | $C_6H_{13}NO_2$ | 32.408 | 32.401 | 5.81 | 5.42 | 1.04 | 1.011 |
| Leucine | Leu | 131.18 | $C_6H_{13}NO_2$ | 32.912 | 32.905 | 11.56 | 12.05 | 2.124 | 2.29 |
| Lysine | Lys | 146.19 | $C_6H_{14}N_2O_2$ | 31.493 | 31.488 | 3.22 | 3.13 | 1.717 | 1.698 |
| Methionine | Met | 149.21 | $C_5H_{11}NO_2$ | 29.222 | 29.208 | 1.74 | 1.76 | 0.519 | 0.549 |
| Phenylalanine | Phe | 165.19 | $C_9H_{11}NO_2$ | 33.861 | 33.856 | 8.25 | 8.28 | 1.403 | 1.45 |
| Proline | Pro | 115.13 | $C_5H_9NO_2$ | 23.424 | 23.322 | 1.70 | 1.46 | 1.422 | 1.276 |
| Serine | Ser | 105.09 | $C_3H_7NO_3$ | 13.314 | 12.737 | 2.04 | 2.36 | 1.107 | 1.185 |
| Threonine | Thr | 119.12 | $C_4H_9NO_3$ | 19.232 | 18.916 | 2.27 | 2.13 | 1.208 | 1.268 |
| Tyrosine | Tyr | 181.19 | $C_9H_{11}NO_3$ | 27.625 | 27.603 | 1.89 | 2.00 | 1.219 | 1.35 |
| Valine | Val | 117.15 | $C_5H_11NO_2$ | 28.712 | 28.692 | 7.25 | 7.26 | 1.499 | 1.571 |

**Figure 5b. Dependence of protein yield (mg $L^{-1}$) on extraction time (2-12 days) with dry weight (0.2-1.4 g $L^{-1}$) for *Scenedesmus* sp. cells. Means sharing the same letter above columns do not differ significantly ($p < 0.05$). Note: The error bars represent the standard error of mean where n = 3**

In addition to health benefits, many different industries ranging from pharmaceutical to the food industry rely on amino acids (Seyfabadi *et al.,* 2011; Hildebrand *et al.,* 2020; Soni *et al.,* 2021). For example, threonine can be used to produce herbicide aztreonam and glycine as glyphosate. Cultivation method and growth conditions (nature and composition of culture medium, light quality, salinity, the temperature of algal cells, pH, and turbulence) are two important factors, which play a key role in the amino acid contents of algal biomass. The hydrolysis of protein to amino acids by acid hydrolysis is an eminent process (Tibbetts *et al.,* 2020). Generally, hydrolysis proceeds smoothly if a sufficient amount of water is present. The hydrolysis rate depends upon the temperature, pH, size of anion or cation, and the concentration of additives that are being used. Standard hydrolysis procedures involve 24 h of acid hydrolysis using 6M HCl at 110 °C.

The amino acid profiling of the hydrolysates from the two microalgal species is shown quantitatively in Table 2. Seventeen proteidogenous amino acids were detected from *C. vulgaris* and *Scenedesmus* sp. ($UKM_9$) grown on BBM and these were about 25.841 and 24.063 % of algal biomass used for amino acid profiling. The estimated protein concentration in *C. vulgaris* and *Scenedesmus* sp. ($UKM_9$) calculated from Kjeldahl nitrogen were 53.52 and 45.93%. The remaining portion might include other amino acids. Moreover, the values of estimated protein concentration in both species might be high due to the multiplication of nitrogen contents with a 6.25 factor. This premise is supported by the results obtained from the amino acid profiling of seventeen species of algae that the multiplication factor i.e. 6.25 for protein estimation was found greater compared to the actual measurement (Biancarosa *et al.,* 2017). Only minor differences were found in the levels of specific amino acids in the two species. Glutamic acid (2.9-3.2%) and aspartic acids (2.5-2.8%) were found in the highest concentrations in *Scenedesmus* sp. and *C. vulgaris*, respectively. The contents of sulfur-containing amino acids i.e. histidine and methionine of both microalgal species were unusually low. The results showed that six amino acids (alanine, aspartic acid, glutamic acid, leucine, lycine, and glycine) were responsible for 50% or more of the total detected amino acid contents in both microalgal species. Tryptophan and cysteine were not detected in both clusters, as these two amino acids are especially labile during acid hydrolysis. Apart from these two, the other amino acids constituted about 25.063% in *Scenedesmus* sp. and 25.841% in *C. vulgaris*.

***Conclusions*:** Two different cell disruption techniques were compared along with control with three different concentrations of alkaline solubility (0.5N, 1N, 2N). The recovery yield in *Chlorella vulgaris* and *Scenedesmus* sp. was 19 and 18%, respectively. Among tested techniques, milling with pestle and mortar with 1N NaOH treatment was found to be the best technique for protein extraction from both species. A minor increase in protein concentration was noticed for *C. vulgaris* in ceramic grinding with the 2N NaOH method. Therefore, it is concluded that combining the

action of freeze-drying, pretreatment, and alkaline extraction significantly boosts the protein extraction yield from *Chlorella vulgaris* and *Scenedesmus* sp.

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