Optimization of Protein Extraction from *Chlorella vulgaris* sp. using Response Surface Methodology

Jose Rafael B. Quidilig[1], Gino Apollo M. Guerrero[1], Denise Ester S. Sanchez[1], Kristel M. Gatdula[1], Ruby Lynn G. Ventura[2], Erwin C. Escobar[3], and Jey-R S. Ventura[3,*]

[1]*Department of Chemical Engineering, College of Engineering and Agro-Industrial Technology, University of the Philippines Los Baños, 4031 Los Baños, Laguna;* [2]*University of the Philippines Rural High School, College of Arts and Science, University of the Philippines Los Baños;* [3]*Department of Engineering Science, College of Engineering and Agro-Industrial Technology, University of the Philippines Los Baños*
* *Corresponding author (jsventura@up.edu.ph)*

Abstract:

Microalgal protein is a potential protein source in food, medicine, cosmetics, and other bio-based products. Hence, the optimization of the protein extraction method from *Chlorella vulgaris* sp. was studied using Response Surface Methodology. The microalgal biomass was subjected to ultrasonication prior to protein extraction by alkali solubilization, and protein recovery by acid precipitation technique. Using a Box-Behnken Design for the alkali solubilization, it was found that the optimum pH, solubilization time, and biomass concentration were pH 11.6, 15.4 minutes, and 1.61% (w/v), respectively. The optimum conditions for acid precipitation using the Face-centered Central Composite Design were pH 3.2 and 39.9 minutes precipitation time. Based on the protein balance, a total of 78.82% (w/w) protein was recovered from the original biomass. Moreover, *C. vulgaris* sp. protein yielded an emulsion stability of 72.71%, which was approximately 16% higher than caseinate and commercial soy peptone. In conclusion, a simple and efficient process to extract the proteins from microalgae like *C. vulgaris* sp. has been established, which could then be used for the development of high-value protein products.

Keywords: microalgae, protein extraction, solubilization, precipitation, response surface methodology

Introduction

The diversity of proteins as a raw material gives it the potential to produce various products. These products include but are not limited to nutraceuticals, bioactive compounds, animal feed, food supplements, or bio-adhesives. One of the main sources of protein could be microalgae. Protein from most microalgal species is found to have around 50% of its body weight (Becker, 2007). Unlike the most popular protein source such as soy or animal protein (Becker, 2007; Elzoghby et al., 2012), the microalgal protein does not compete with food supply. Moreover, microalgae are easier to propagate and require less land compared to terrestrial plant (Gerde et al., 2013; Albarico et al., 2017). Hence, producing protein from this biomass would be more sustainable compared to the other sources.

To extract protein from microalgae, methods such as alkaline and acid precipitation technique, solvent extraction, and the use of enzymes were

proven to be effective (Gerde et al., 2013; Ursu et al., 2014; Parimi et al., 2015). The use of ionic liquid for protein extraction has also been investigated recently (Tang et al., 2012), although its high viscosity limits its use during extraction. The use of supercritical fluids such as CO_2 and pulsed electric field has also been found effective in microalgal protein extraction (Mendiola et al., 2007).

To promote a higher efficiency during extraction, microalgal cell wall disruption is usually employed prior to chemical or enzymatic extraction. The most common technique is the use of ultrasonic wave. Several papers have already discussed the significance of ultrasonication prior to microalgal protein extraction (Safi et al., 2014; Parimi et al., 2015). After ultrasonication, the protein fraction could be then concentrated at its isoelectric point where solubilization at alkaline pH, followed by acid precipitation of the supernatant, were commonly used (Gerde et al., 2013; Parimi et al., 2015).

Many factors are identified in the solubilization and precipitation of proteins. Studies suggested that pH, biomass concentration, temperature, and mixing time are the parameters that have significant roles in protein extraction (Abas Wani et al., 2006; Gerde et al., 2013; Parimi et al., 2015; Awaludin et al., 2016). pH is known to affect the solubility of microalgal protein by affecting the net charges of amino acids (Parimi et al., 2015). At isoelectric point, the protein is said to have no net charge where its solubility is at the lowest. Therefore, beyond this point, the protein will be soluble. An increasing temperature, on the other hand, could weaken the hydrogen bond of the protein molecule which in turn decrease its polarity (Awaludin et al., 2016). Hence, the decrease in polarity will allow non-polar compounds to dissolved in water. Biomass concentration and mixing time can contribute also to the overall efficiency of the protein extraction process. Depending on the biomass size, shape, and molecular composition; the concentration of biomass may impede or enhance the protein extraction capacity of the process. Variations in conditions could therefore be observed in different species of microalgae. Meanwhile, mixing time will allow utmost interaction of the extractant to the available protein in the microalgal biomass.

Response surface methodology (RSM) is a very popular statistical tool for optimizing various processes in biotechnology (Parimi et al., 2015; Albarico et al., 2017). This approach reduces the number of experiments hence requiring less time for the analysis while maintaining high statistical interpretation capability. Several investigations have already implemented the use of RSM for the optimization of microalgal protein extraction methods on different microalgae species (Gerde et al., 2013; Parimi et al., 2015; Yucetepe et al., 2018). In the study of Parimi et al. (2015) using *Spirulina platensis*, it was found that the optimum solubilization is at pH 11.38 and 35 minutes using a 3.60% (w/w) biomass concentration. Likewise, during acid precipitation, pH of 4.01 and precipitation time of 60 minutes could provide the optimum condition. Meanwhile, using the same microalgal species, Yucetepe et al. (2018) reported the optimum protein solubilization at pH 7.46, mixing time of 120 minutes, and temperature of 45°C. For the *Nannochloropsis microalgae*, the maximum protein recovery was found at solubilization temperature of 60°C and pH 11.0, and precipitation at pH 3.20 (Gerde et al., 2013).

In this study, protein extraction from *C. vulgaris* sp. was optimized. Specifically, the effects of pH, time, and biomass concentration on protein solubilization and the effects of pH and time on protein precipitation were evaluated and optimized using the Box-Behnken and Face-centered Central Composite Designs. In addition, since higher protein stability means a longer shelf-life and better usability of the protein-based product (Lam and Nickerson, 2013; Ursu et al., 2014), the emulsion stability of the extracted protein was also determined and compared to commercially available protein.

Materials and Methods

Materials

A commercial food grade *C. vulgaris* sp. biomass was obtained from SuperFoodGrocers, Inc. (Manila, Quezon City, Philippines). Based on the product label, this microalgal biomass contain approximately 65% (w/w) of protein. Analytical grade NaOH (Macron Fine Chemicals, USA) and

HCl (RCI Labscan Limited, Thailand) were used in protein extraction. Alkali CuSO$_4$ solution and Folin-Ciocalteau analytical reagents and bovine serum albumin (BSA) standard were purchased from Sigma-Aldrich (Sigma-Aldrich, USA).

pH-recovery curve and protein extraction optimization

To determine the desired pH range for the optimization of protein extraction from *C. vulgaris* sp. biomass, a pH-recovery (PR) curve was made. The PR curve was constructed to determine the optimum ranges in the solubilization and precipitation of proteins according to the method of Parimi et al. (2015). Specifically, the cell was pretreated first via ultrasonication (Qsonica Q55, USA) at 20% amplitude for 40 minutes in an ice bath. The cell-disrupted biomass slurry (6% w/v) was then separated into different fractions. These fractions were dissolved using either 1 M NaOH or 1 M HCl for 30 minutes at a pH range of 2-13 in one-unit interval. The mixture was centrifuged (RevSpin 200T, USA) at 8000 g for 15 minutes to separate the protein-rich alkali supernatant from the residue. Finally, the protein-rich supernatant of all the samples was analyzed for its protein content. As a response parameter, the protein recovery was calculated in terms of protein recovered from the alkali supernatant after centrifugation, over the indicated actual product amount of protein in the biomass sample.

For the optimization of the protein extraction procedure, the alkali solubilization and acid precipitation technique was applied. The pretreated biomass slurry was made to the specified concentration (1.0 % w/v, 3.0 % w/v, 5.0 % w/v) using distilled water. One molar NaOH was used to adjust the pH (pH 10, 11, and 12) and solubilize the mixture during the specified duration (15, 30, and 45 minutes) at 240 rpm. The solid and liquid components were then separated by centrifugation at 8000 *g* for 15 minutes. The protein-rich supernatant was subjected to acid precipitation. One molar HCl was used for precipitation of the proteins at the desired pH values (pH 2, 3, and 4). The precipitating mixture was mixed at 240 rpm until the specified precipitation time (15, 30, and 45 minutes) was

reached. The precipitate was then separated by centrifugation at 8000 *g* for 15 minutes.

Design of experiment

The design of the experiment aims to optimize conditions present in protein extraction. Biomass concentration, pH, and time (solubilization and precipitation) were the optimized parameters of the experiment. Temperature was maintained at room temperature to reduce denaturation. Optimization was done in two segments, alkali solubilization and acid precipitation, using Design Expert® v11.0 (Stat-Ease Inc., USA). The experimental design for RSM was made based on the Box-Behnken design for alkali solubilization and Face-centered Central Composite design for acid precipitation from which equations containing quadratic, linear and intercept terms were fitted. For alkali solubilization, pH, biomass concentration, and solubilization time were optimized. A pH range indicating maximum solubility was determined from the PR curve. Solids concentrations ranging from 1.0-5.0% (w/v) were used since it is within the typical range for harvesting microalgal biomass (Gerde et al., 2013; Parimi et al., 2015). A solubilization time of 15-45 minutes was used for solubilization since near 60 minutes will produce no significant effect on the solubilized proteins (Parimi et al., 2015).

After optimization of the alkali solubilization step, acid precipitation was done. Unlike the alkali solubilization step, the acid precipitation step used pH and precipitation time as parameters for optimization. The pH ranges from the PR curve indicating minimum solubilization was used in optimization. Similar to protein solubilization, the precipitation time adopted 15-45 minutes as its range of values. Protein recovery was measured in terms of the amount of protein recovered in both the solubilization and precipitation steps.

Analytical methods

A modified Lowry method using BSA as standard was used for the analysis of all hydro-soluble proteins (Lowry et al., 1951). Alkali CuSO$_4$ was added to the protein samples at a 1:10 ratio.

The samples were then mixed thoroughly using a vortex mixer and incubated for 10 minutes at 25°C. After incubation, diluted Folin-Ciocalteau reagent was added to the samples and incubated for 30 minutes at 25°C. The samples were then read for their absorbance values using UV-Vis Spectrophotometer (Shimadzu UV-1800, Japan) at 660 nm.

For the emulsion stability determination, an emulsion of 2% (w/v) proteins treated in alkaline conditions mixed with an equal volume of palm oil was prepared by vigorous mixing at 10,000 rpm for 1 minute (Ursu et al., 2014). The emulsion was heated to 80°C for 30 minutes, cooled with tap water to room temperature and left to stand for 24 hours. Using a graduated cylinder, the emulsion stability was measured as the ratio of the emulsion height to the total height of the mixture after the 24-hour decantation. Protein from soy peptone (BD Biosciences, USA) was used as reference during the test.

Results and Discussion

Construction of pH-recovery curve

The pH-recovery curve in Figure 1 showed that protein recovery is high at alkaline conditions while the acidic conditions drastically decrease protein solubility. The smallest value of protein recovery (% w/w) was observed at pH 4.0 yielding 22.19% while the largest value was observed at pH 11.0 (87.04% protein) followed by 75.26% at pH 10.0. A decrease in protein recovery was observed after pH 11.0 as evident by a drop from 87.04% to 42.61% at pH 12.0.
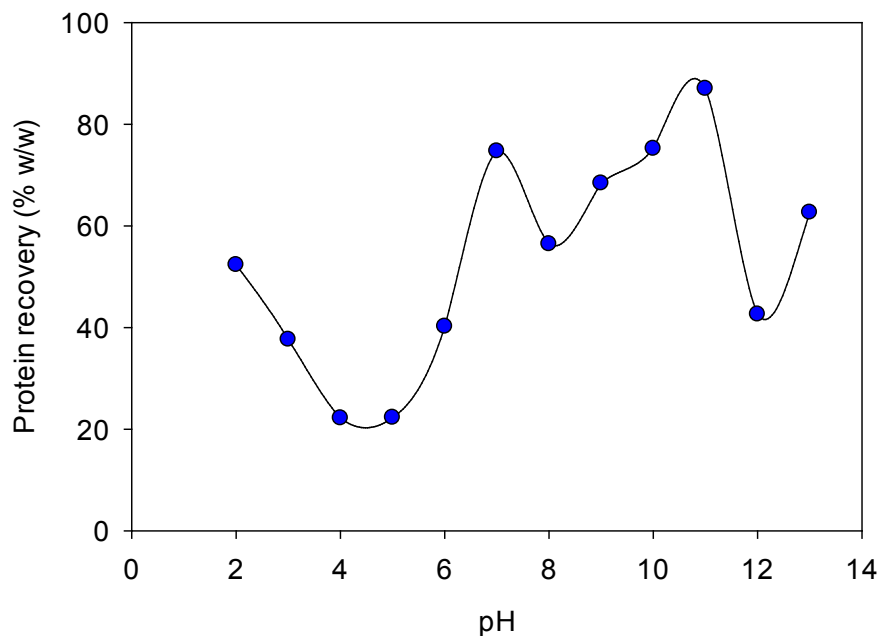

Figure 1. Recovery of *C. vulgaris* sp. proteins at different pH values.

The lower recovery at these values could be explained by the denaturation and clustering of proteins at extremely alkaline conditions (Gerde et al., 2013; Ursu et al., 2014; Parimi et al., 2015). The fluctuations in protein recovery could be due to the samples passing through the different isoelectric points of the microalgal proteins. Proteins at their isoelectric points are said to be least soluble (Ursu et al., 2014), which would explain the fluctuating recovery values observed. Hence, the pH ranges used for the optimization of protein solubilization and precipitation steps were pH 10-12 and pH 2-4, respectively.

Our study showed similar pH ranges for protein recovery of other green microalgae as reported in literatures (Gerde et al., 2013; Ursu et al., 2014; Parimi et al., 2015). As reported, *S. platensis* was observed to have a peak protein recovery at pH 11.0 (Parimi et al., 2015). A similar study on *C. vulgaris* explains that proteins are more likely to solubilize at alkali conditions (pH 12.0) than in neutral conditions (pH 7.0) (Ursu et al., 2014). For the *Nannochloropsis* sp., recovery was said to increase until pH 13.0 (Gerde et al., 2013).

Optimization of protein solubilization using Box-Behnken RSM

The optimization of the protein solubilization process using a Box-Behnken model yielded fifteen runs with two center points. Table 1 shows the results of each run at different values of pH (pH 10-12), solubilization time (15-45 minutes), and biomass concentration (1-5% w/v) with protein recovery as the response variable of the experiment. Optimum concentration of 95.62% protein extract was observed at pH 12.0, 30 minutes solubilization, and 1.0% (w/v) biomass concentration (Table 1, Run 2). In contrast, the lowest protein concentration of 64.26% was observed at pH 12.0, 30 minutes solubilization, and 5.0% (w/v) biomass concentration (Table 1, Run 4).

Table 1. Optimization data for the solubilization of proteins from *C. vulgaris* sp.

Run	pH	Time (minutes)	Biomass concentration (% w/v)	Protein recovery (% w/w)
1	11.0	15.00	1.0	94.80
2	12.0	30.00	1.0	95.62
3	12.0	45.00	3.0	80.19
4	12.0	30.00	5.0	64.26
5	10.0	30.00	1.0	87.04
6	11.0	30.00	3.0	88.97
7	10.0	15.00	3.0	82.61
8	11.0	45.00	5.0	77.29
9	10.0	30.00	5.0	73.09
10	12.0	15.00	3.0	88.59
11	11.0	30.00	3.0	92.50
12	11.0	45.00	1.0	93.71
13	11.0	30.00	3.0	91.66
14	10.0	45.00	3.0	89.54
15	11.0	15.00	5.0	69.68

The following equation (Equation 1) was created to predict the protein recovery (W) of the solubilization process in terms of pH (X), solubilization time (Y), and biomass concentration (Z). Statistically, the model equation is significant and sufficient for the optimization of the process.

$$W = -641.7545 + 120.1730X + 2.8928Y + 25.6316Z - 0.2554XY - 2.1759XZ + 0.0725YZ - 4.8379X^2 - 0.0043Y^2 - 1.5502Z^2$$

(Equation 1)

The analysis of variance (ANOVA) results of the regression model indicates that the model is statistically significant (p-value = 0.0004) with biomass concentration (Z) being the most significant parameters to the model (p-value < 0.0001). The interaction effects (XY, XZ, and YZ) were all significant, which means that the effectiveness of protein solubilization depends on the combination of these parameters. Likewise, the quadratic terms X^2 and Y^2 were found to be significant. The R^2 value for the model is 0.9866 adjusted to 0.9626. The predicted R^2 value is at 0.8566 which is in reasonable agreement with the adjusted R^2, as the difference was less than 0.2.
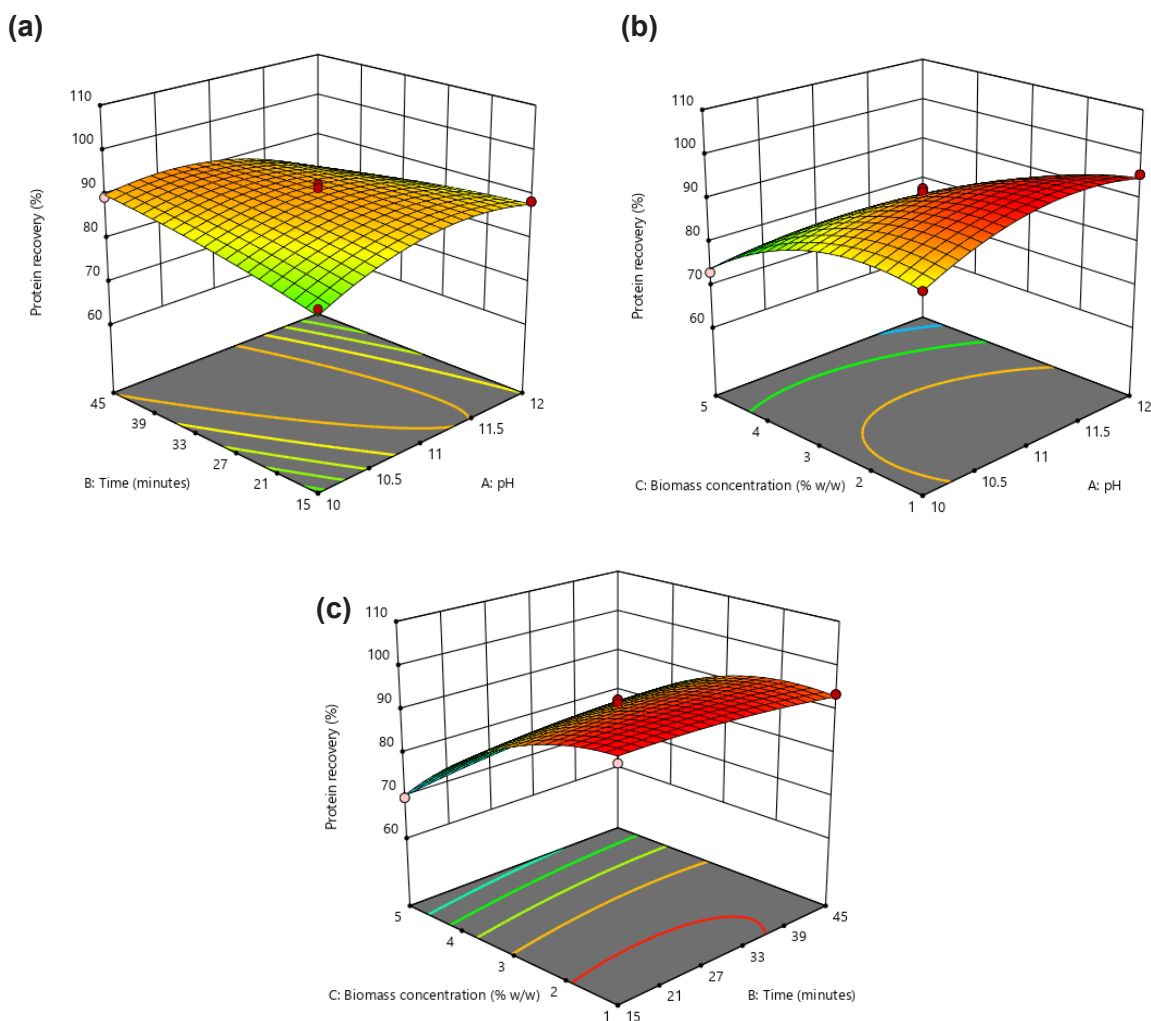

Figure 2. 3D-surface plot of the interaction of **a)** pH and solubilization time, **b)** pH and biomass concentration, and **c)** solubilization time and biomass concentration with respect to protein recovery.

Figure 2 shows the three-dimensional (3D) surface plots of each interaction namely, pH versus solubilization time, pH versus biomass concentration, and solubilization time versus biomass concentration with respect to protein recovery. As compared to the other variables, biomass concentration exhibited the greatest effect on the solubilization of proteins in *C. vulgaris* sp. (Figures 2b & 2c). This is expected, as the formation of biomass aggregates in the slurry could hinder its exposure to the extraction conditions at higher biomass concentrations (Ursu et al., 2014). It should however be noted that an extremely low concentration would yield low recoveries due to low biomass loading (Figure 2b).

Optimization of the protein solubilization step revealed 113 iterated solutions. Thus, the optimum values were selected based on the lowest solubilization time and highest attainable microalgal biomass concentration. These chosen optimal values were at pH of 11.6, solubilization time of 15.44 minutes, and a biomass concentration of 1.61%, which gave a protein recovery of 97.54%. It should be noted, however, that the other optimized solutions would yield similar results at pH 10.9-11.9, 15.00-34.23 minutes solubilization time, and 1.00 to 1.91% (w/v) biomass concentration, for a protein yield between 95.63% to 97.97%.

Verification of optimal conditions indicate a 92.79% protein recovery for the selected optimal conditions which corresponded to a -4.87% error from the 97.54% predicted recovery. Hence, the result of the experimental verification is within the allowable 10% margin of error, verifying the selected values as the optimum conditions.

Optimization of protein precipitation using Face-centered Central Composite Design RSM

Optimization of the protein precipitation step using a Face-centered Central Composite Design required 10 runs with two center points. Table 2 provides a summary on the results of the experiment for optimization. The highest recovery value during precipitation was observed to be 81.09% at pH 3.0 and 45 minutes precipitation (Table 2, Run 5). Conversely, the lowest value, 73.48% was observed at pH 2.0 and 45 minutes precipitation (Table 2, Run 10).

The following equation (Equation 2) was created to predict the protein recovery (*T*) of the precipitation process in terms of pH (*U*) and precipitation time (*V*).

$$T = 29.7787U + 28.8099V + 0.02899UV - 4.6748U^2 - 0.0043V^2$$

(Equation 2)

Table 2. Optimization data for the precipitation of proteins from *C. vulgaris* sp.

Run	pH	Time (minutes)	Protein recovery (%)
1	3	30	80.66
2	4	45	78.05
3	3	30	79.35
4	2	15	71.96
5	3	45	81.09
6	4	30	77.40
7	3	15	78.70
8	2	30	75.01
9	4	15	74.79
10	2	45	73.48

The ANOVA results (not shown) indicates that the model is statistically significant (*p*-value = 0.0043) with quadratic term of pH (U^2) being the most significant to the model (*p*-value < 0.0010). The R^2 value for the model is 0.9683 adjusted to 0.9286. The predicted R^2 value is at 0.8009, which agrees statistically with the adjusted R^2.

Figure 3 shows the 3-D surface plot of the pH and precipitation time with respect to protein recovery. Both pH and precipitation time exhibited prominence in the acid precipitation step. From the figure, it is shown that as pH approaches certain pH values, more proteins precipitate from the system and vice-versa. This is due to the precipitation of microalgal proteins at the isoelectric points of the amino acids in the proteins (Parimi et al., 2015). Furthermore, as precipitation time increases, the protein recovery increases. This is in contrast with the report of Ursu et al. (2014) where precipitation time did not change much the protein recovered after reaching a certain optimum time.
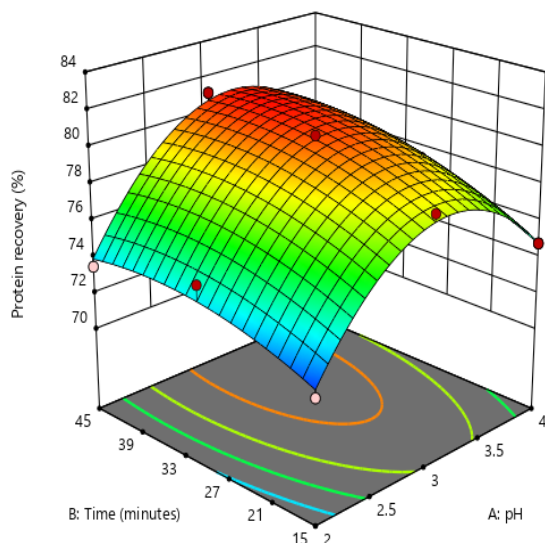
Figure 3. 3-D surface plot of pH and precipitation time with respect to protein recovery.

Optimal conditions were calculated and observed to be at pH 3.2, 39.86 minutes precipitation yielding a recovery of 81.0%. Verification resulted in a yield of 84.84% with a difference of 4.74%, which is within the allowable 5.0% error. The optimum conditions for the same process done by Parimi et al. (2015) to *S. platensis* sp. were found to be at pH 4.01 and with a precipitation time of 60.00 minutes. The lower optimum precipitation pH of *C. vulgaris* sp. could be attributed to the difference in amino acid composition. *C. vulgaris* sp. contains more aspartate and glutamate (Ursu et al., 2014) than *S. platensis* (Alvarenga et al., 2011), hence, a lower isoelectric point and consequently a lower precipitation time.

C. vulgaris sp. proteins emulsion stability

An important parameter in protein emulsions is the stability of protein in maintaining its form as dictated by the interaction of polar and non-polar functional groups. The stability of *C. vulgaris* sp. protein emulsions was compared with emulsions made from a commercial soy peptone. From the comparison, it was observed that *C. vulgaris* sp. proteins maintained their emulsions over a 24-hour period, yielding a stability of 72.71%. In comparison, soy peptone proteins were found to be stable at the same period with a value of 60.95% while commercial emulsifying proteins like sodium caseinate exhibited a stability of 62.00% (Ursu et al., 2014). This contributes to a difference of 15.91% (sodium caseinate) and 17.60% (soy peptone) in favor of the microalgal proteins.

Microalgal proteins are said to contain high molecular weight proteins or protein aggregates, which contribute to the stearic interactions as compared to surfactants (Ursu et al., 2014). This explains why *C. vulgaris* sp. protein emulsions have high values of emulsion stability. Moreover, proteins treated in alkaline conditions could induce denaturation thereby decreasing the emulsion stability. This scenario does not hold true for microalgal proteins since high molecular weight proteins tend to form aggregates in alkaline conditions. Microalgal protein emulsions are therefore expected to be more stable than emulsion made from soy proteins.

Conclusion

This study successfully optimized the protein extraction of *C. vulgaris* sp. using the alkaline solubilization and acid precipitation technique. At optimized conditions, a total of 78.82% (w/w) protein was recovered from the microalgal biomass. Overall, the study developed a simple and efficient protein extraction process in *C. vulgaris* sp. with comparable emulsion stability close to commercially-available proteins. Hence, the extracted protein from this biomass could be further explored for the production of bio-based protein products such as in food, medicine, and cosmetic industry.

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References

Abas Wani, A., Sogi, D., Grover, L., & Saxena, D. (2006). Effect of temperature, alkali concentration, mixing time and meal/solvent ratio on the extraction of watermelon Seed proteins - a response surface approach. *Biosystems Engineering*, *94*(1): 67-73.

Albarico, J.S., Detras, M.C.M., Sanchez, P.R.P, Alfafara, C.G., Borines, M.G., Nayve, F.R.P, Dorado, A.A., Escobar, E.C., & Ventura, J.S. (2017). Yield Optimization of Reducing Sugars from Acid Hydrolysis of Waste Microalgae Biomass. *Philippine e-Journal for Applied Research and Development*, *7*: 21-33.

Alvarenga, R.R., Rodrigues, P.B., Cantarelli, V.D., Zangeronimo, M.G., da Silva Júnior, J.W., da Silva, L.R., dos Santos, M., & Pereira, L.J. (2011). Energy values and chemical composition of spirulina (*Spirulina platensis*) evaluated with broilers. *Revista Brasileira de Zootecnia*, *40*(5): 992-996.

Awaluddin, S.A., Thiruvenkadam, S., Izhar S., Hiroyuki, Y., Danquah, M.K., & Harun, R. (2016). Subcritical water technology for enhanced extraction of biochemical compounds from *Chlorella vulgaris*. *BioMed Research International*, 5816974: 1-10.

Becker, E.W. (2007). Micro-algae as a cource of protein. *Biotechnology Advances, 25*(2): 207-210.

Elzoghby, A.O., Samy, W.M., & Elgindy, N.A. (2012). Protein-based nano-carriers as promising drug and gene delivery systems. *Journal of Controlled Release*, *161*(1): 38-49.

Gerde, J. A., Wang, T., Yao, L., Jung, S., Johnson, L. A., & Lamsal, B. (2013). Optimizing protein isolation from defatted and non-defatted *Nannochloropsis* microalgae biomass. *Algal Research*, *2*(2): 145-153.

Lam, R.S. & Nickerson, M.T. (2013). Food proteins: A review on their emulsifying properties using a structure-function approach. *Food Chemistry*, *141*(2): 975-984.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall R.J. (1951). Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry*, *193*: 265-275.

Mendiola, J.A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibañez, E., Señoráns (2007). Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chemistry, 102*:1357–1367.

Parimi, N.S., Singh, M., Kastner, J.R., Das, K.C., Forsberg, L. S., & Azadi, P. (2015). Optimization of protein extraction from *Spirulina platensis* to generate a potential co-product and a biofuel feedstock with reduced nitrogen content. *Frontiers in Energy Research*, *3*: Article 30. doi:10.3389/ fenrg.2015.00030

Safi, C., Ursu, A.V., Laroche, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. *Algal Research*, *3*: 61-65.

Tang, B., Bi, W., Tian, M., & Row, K.H. (2012). Application of ionic liquid for extraction and separation of bioactive compounds from plants. *Journal of Chromatography B*, *904*:1–21.

Ursu, A., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation, and functional properties of protein from the microalgae, *Chlorella vulgaris*. *Bioresource Technology*, *157*: 134-139.

Yucetepe, A., Saroglu, O., Dakskaya-Dikmen, C., Bildik, F., & Ozcelik, B. (2018). Optimisation of ultrasound-assisted extraction of protein from *Spirulina platensis* using RSM. *Czech Journal of Animal Science, 36*(1): 98–108.