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Effects of *Chlamydomonas reinhardtii* (Dangeard) Proteins Extracts on Storage Stability of Freshwater Shrimp *Macrobrachium nipponense* (De Haan)

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Abstract

In this study, the extraction of *Chlamydomonas reinhardtii* Dangeard (*C. reinhardtii*) protein was optimized through response surface methodology. The optimal extractions parameters were 0.091 mol/L sodium chloride concentration, 31.02 mL/g liquid to solid ratio, a homogenization frequency of 10.17 times per second, and a homogenization time of 12.82 minutes. The optimized protein extraction yield was 52.78%. The *C. reinhardtii* protein extract at different concentrations (0.1-0.5%) was used to dip-coat East Asian river prawn (*Macrobrachium nipponense* De Haan). Over a 4-day storage period, the pH value and total viable counts (TVC) of the prawn samples were determined every 24 hours. Results showed that a 0.2% *C. reinhardtii* protein extract effectively delayed increases in pH and TVC of the prawn samples, suggesting that *C. reinhardtii* protein extract could be a promising natural preservative.

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Protein Extraction;
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Total Viable Count.

Introduction

Macrobrachium nipponense (De Haan), commonly known as East Asian river prawn, is a well-known freshwater cultured shrimp. Due to its high fecundity and strong adaptability, its habitat is not limited to freshwater, it can also survive in coastal low-salinity waters. This freshwater prawn has a stable market value; however it is highly susceptible to oxidation and enzymatic reactions, as well as microbial attacks.[1,2] With a high content of free amino acids and water, the pH value of the shrimp increases after death, promoting microbial growht[3] and causing a 25%–30% loss in quality.[4] Moreover, the occurrence of various oxidation reactions significantly affects the quality of fresh shrimp. The primary oxidation product, hydroperoxide, and secondary oxidation

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products, such as aldehydes, ketones, epoxides, oligomers, hydroxyl groups, and other compounds, interact with proteins, amino acids and vitamins, leading to unpleasant odors, discoloration, and loss of protein functionality.[5, 6, 7]

To mitigate the impact of microorganisms, chemicals, and enzymes on the quality of fresh shrimp, the development of efficient fresh-keeping technology and preservatives is critically important. Currently, chemically synthesized preservatives are widely used to extend shelf life of seafood. However, the accumulation of certain chemical compounds in synthetic preservatives not only adversely affects human health[8] but also alters the nutritional composition of the products. Worst still, the long-term use of large amounts of synthetic preservatives can lead to microbial resistance,[9] significantly weakening their preservation efficacy. Consequently, there is a growing demand to replace synthetic preservatives with biological alternatives, which are typically rich in bioactive compounds and have fewer adverse effects on human health. Moreover, natural preservatives often demonstrate comparable, if not superior, antioxidant and antimicrobial properties compared to artificial preservatives.

C. reinhardtii is a single-celled, green freshwater microalga that belongs to the Chlorophyta family. It is commonly used as a model organism in research due to its simple cellular structure and ease of cultivation.[10] *C. reinhardtii* is rich in polysaccharides, proteins, fiber, vitamins and many trace elements, such as potassium, calcium, iron phosphorus, selenium, iodine and cobalt, with protein content up to 30%-50%.[14] Although there is limited research on *C. reinhardtii* protein as a preservative for food products, some studies have shown that its protein exhibit good antibacterial and antioxidative activity, supporting its potential as a food preservation agent. For instance, crude extracts of *C. reinhardtii* obtained using methanol: chloroform: water (5:2:2) blend as a solvent demonstrated strong antibacterial activity against *B. subtilis, P. aeruginosa, K. pneumoniae* and antifungal activity against *C. albicans*.[11] Additionally, other researchers[12, 13] reported that *C. reinhardtii* extracts might prevent the growth of various bacterial species, including *Pseudomonas aeruginosa, E. coli,* and *Staphylococcus aureus*, while also being effective against Candida albicans. β-Carotene and rutin in *C. reinhardtii* have been suggested to retard seafood spoilage.[14]

Moreover, several studies have shown that the use of microalgae extracts in the seafood industry can positively impact product shelf life. The bioactive compounds in these extracts can disrupt microbial cell membrane integrity, increasing membrane permeability and causing the leakage of critical cellular substances (such as potassium and other cytoplasmic contents), ultimately leading to microbial cell death by lysis.[15,16] In addition, polyphenols, algal bile proteins and vitamins are highly effective water-soluble antioxidants in microalgae. For example, a 96% ethanol extract of *Spirulina obtusa* and *Chlorella vulgaris* at a 0.1% concentration significantly inhibited free fatty acid, peroxide value, and thiobarbituric acid in frozen *Oncorhynchus mykiss*.[17] Edible coatings incorporating phycocyanin extracted from *Arthrospira sp* into gelatin exhibited significant DPPH radical scavenging activity and inhibited the growth of gram-positive and gram-negative bacteria.[18] Similarly, bioactive coatings prepared by incorporating spirulina extract into chitosan derived from spider crab (Maja crispata) showed comparable preservative effects.[19]

In conclusion, research on the preservation effects of microalgae has primarily focused on species of *Spirulina* and *Chlorella*, with fewer studies exploring other species. Additionally, most studies have concentrated on fatty acids, polyphenols, pigments or crude extracts, while little attention has been given on algal proteins, despite their protein content being as high as 30-50%. In this study, the preservation effects of *C. reinhardtii* protein extracts as natural preservation agents for freshwater shrimp will be elucidated.

Materials and Methods

Shrimps were purchased from Fanhu Market, Foshan, Guangdong Province. *C. reinhardtii* powder was a commercial product produced by Shanxi Touyun Biotechnology Co., Ltd.. All chemicals used were of analytical grade. Sodium hydrogen phosphate by Tianjin Dengfeng Chemical Reagent Factory, Sodium dihydrogen phosphate by Tianjin Dengfeng Chemical Reagent Factory, ammonium sulfate by Tianjin Juhengda Chemical Co., Ltd., PCA agar by Beijing Aoboxing Co., Ltd., sodium

chloride, by Tianjin Juhengda Chemical Co., Ltd., chitosan, biochemical reagent (BR), from Sinopharm Group Co., Ltd., glacial acetic acid, from Tianjin Damao Chemical Reagent Factory, BCA Protein Concentration Determination Kit from Shanghai Biyuntian Biotechnology Co., Ltd.. were used in this study.

Single-Factor Experiments Design for protein extraction

The concentration of sodium chloride solution was explored first. *C. reinhardtii* powder (2 g) was added to 60 mL of sodium chloride solutions at various concentrations (0.02, 0.04, 0.06, 0.08, and 0.10 mol/L) at 25 °C. Protein extraction was conducted with a sterile homogenizer 12 times per second for 10 min. The best concentration level with highest extraction yield was fixed and proceeded to second factor exploration. In order to evaluate the effects of liquid to solid ratio, different liquid to solid ratio samples were prepared by adding 2 g *C. reinhardtii* powder to different volumes of 0.04 mol/L (prior determined) sodium chloride solutions (40, 50, 60, 70, and 80 mL) at 25°C. Protein extraction was carried out for 10 minutes using a sterile homogenizer at a frequency of 12 times per second for 10 min. Similarly, ideal liquid to solid ratio was determined based on extraction yield. To explore the effects of homogenization frequency, 2 g *C. reinhardtii* powder was added to 60 mL (prior determined) of sodium chloride solution with a concentration of 0.04 mol/L at 25°C, and sterile homogenizer were operated at different frequencies (4, 6, 8, 10, and 12 times per second) for 10 minutes. Once the optimal homogenization frequency was identified, experiment was proceeded to determine the best homogenization time, whereby 2 g *C. reinhardtii* powder were added to 60 mL of 0.04 mol/L sodium chloride solution and homogenized with a sterile homogenizer at a frequency of 12 times per second for various durations (6, 8, 10, 12, and 14 min) at 25 °C.

The prepared *C. reinhardtii* protein extracts under different extraction conditions were centrifuged at 2240 g for 10 min, then the supernatant was taken to determine the protein content and calculated as extraction rate.

Response Surface Methodology (RSM) for Protein Extraction

Following the results from the single-factor experiment, response surface methodology (RSM) was employed to optimize the enzymatic hydrolysis conditions. Model building and data analysis were conducted using Design Expert 11.1.1.0. The independent variables in this study were sodium chloride concentration (A), liquid-to-solid ratio (B), homogenization frequency (C), and homogenization time (D), with protein extraction rate as the response variable. A randomized Box-Behnken design was utilized to optimize these parameters, with each variable coded at three levels (-1, 0, +1) (Table 1). The optimized protein extraction conditions were further validated by verifying the protein extraction rate under the optimal conditions.

Table 1: Factors and levels designed in response surface experiment

Independent variables	Coded symbols	Coded level		
		-1	0	+1
concentration of sodium chloride solution (mol/L)	A	0.06	0.08	0.10
liquid to solid ratio (mL/g)	B	25	30	35
homogenization frequency (times per second)	C	8	10	12
homogenization time (min)	D	10	12	14

Determination of Protein Content

The protein content was determined using a Bicinchoninic Acid Kit (BCA1-1KT, Sigma-Aldrich). The centrifuged protein supernatant from *C. reinhardtii* was diluted in a 100 mL volumetric flask, and a sufficient volume was taken to prepare a 10-fold diluted extract sample. A 20 µL aliquot of this diluted sample was combined with 200 µL of BCA reagent in a well of a 96-well plate, thoroughly mixed, and incubated at 37°C for 30 minutes. Absorbance was measured at 640 nm using a microplate reader The extraction rate of *C. reinhardtii* protein was estimated using the formulae as follows:

$$Y = \frac{nx \times V}{1000M} \times 100\%$$

where Y is the *C. reinhardtii* protein yield (%); n is the dilution factor, x is the *C. reinhardtii* protein concentration in the extract(mg/mL); V is the extract volume (100 mL); M is the weight of *C. reinhardtii* powder (g).

$$T = \frac{Y}{H} \times 100\%$$

where T is the protein extraction rate of *C. reinhardtii* (%); H is the protein content of *C. reinhardtii* (%) (H is provided by Shanxi Touyun Biotechnology Co., Ltd., which was 40.6%).

Determination of Cell Breaking Rate

An aliquot of 1 mL of *C. reinhardtii* suspension before and after treatment with the combination of optimal conditions were taken and diluted at a ratio of 1:100(v/v). High-definition electron microscope (FA2104, Shanghai Anting, China) was used to observe the changes taken place and cell breaking rate was calculated according to the formula as follows:

$$R = \frac{c - c'}{c} \times 100\%$$

where R is the cell breaking rate of *C. reinhardtii* (%); c is the number of intact cells before treatment; c' is the number of intact cells after treatment.

Preparation of *C. Reinhardtii* Protein Samples

Chlamydomonas reinhardtti powder suspension was prepared based on the optimized extraction conditions pre-determined by the RSM experiments. After soaking at 25 oC for 2 hours, the suspension was stored in a freezer at -80 °C for 5 hours and thawed at 37 oC in a water bath. The freezing and thawing process was repeated 3 times to destroy the cell wall and dissolve the *C. reinhardtii* protein. After the last thawing, samples were homogenized at the optimized frequency and time pre-determined by RSM. After that, suspension was centrifuged at 8950 g for 10 min at 4 °C, ammonium sulfate was added to the supernatant until saturated, then the mixture was put in a refrigerator (4°C) overnight. To collect the protein precipitate, supernatant was centrifuged at 8950×g for 15 min at 4 oC. Chlamydomonas reinhardtti protein pellet collected was subjected to freeze drying for 72 hours.

Preparation of Shrimp Samples

After washing with double distilled water and draining, shrimp heads and shells were removed, and the tail muscles were collected as shrimp samples. A part of the shrimp samples was taken to determine the initial pH value and total number of colonies. The rest of the fresh shrimp samples was refrigerated at 4 °C for later use. The shrimp samples were divided into test groups and control group, from which several shrimps were taken to measure the relevant fresh-keeping indicators.

***reinhardtii* Protein Treatment on Shrimps**

Approximately 25 g of drained shrimps were soaked in 100 mL of 0.10%, 0.20%, 0.30%, 0.40%, and 0.50% *C. reinhardtii* protein extracts solution for 30 min. Double distilled water was used as control. All treated shrimps were put into storage bag and stored. Changes in pH value and total viable counts were determined at every interval of 24 hours up to 4 days of storage.

Determination of pH Value

Approximately 25 g of shrimp samples were crushed and mixed well with freshly boiled and cooled water (50 mL), soaked for 30 min at 25°C, and centrifuged at 1430 g for 10 min. Supernatant was collected for pH determination using a pH meter. Measurements were repeated three times.

Determination of Total Viable Count (TVC)

A 5 g portion of shrimp sample was crushed and placed in a sterile bag with 45 mL of sterile normal saline, then homogenized for 2 minutes to obtain a 1:10 dilution. This initial dilution was further diluted to 1:100 and 1:1000 ratios. From each dilution, 1 mL of shrimp suspension was transferred into sterile plates, followed by the addition of 20 mL of agar medium cooled to 40 °C. Each dilution was plated in triplicate. Sterile normal saline served as the control. Once the agar solidified, the plates were inverted and incubated at 30 °C for 72 hours. Plates with colony counts between 30 and 300 CFU, without overlapping colonies, were selected for enumeration.

Statistical Analysis

One-way analysis of variance (ANOVA) was conducted for statistical analysis of the experimental data using Origin 8.1 software. Design Expert11.1.1.0 was used for RSM model building. Significantly different means ($p < 0.05$) between treatments were determined by SPSS.

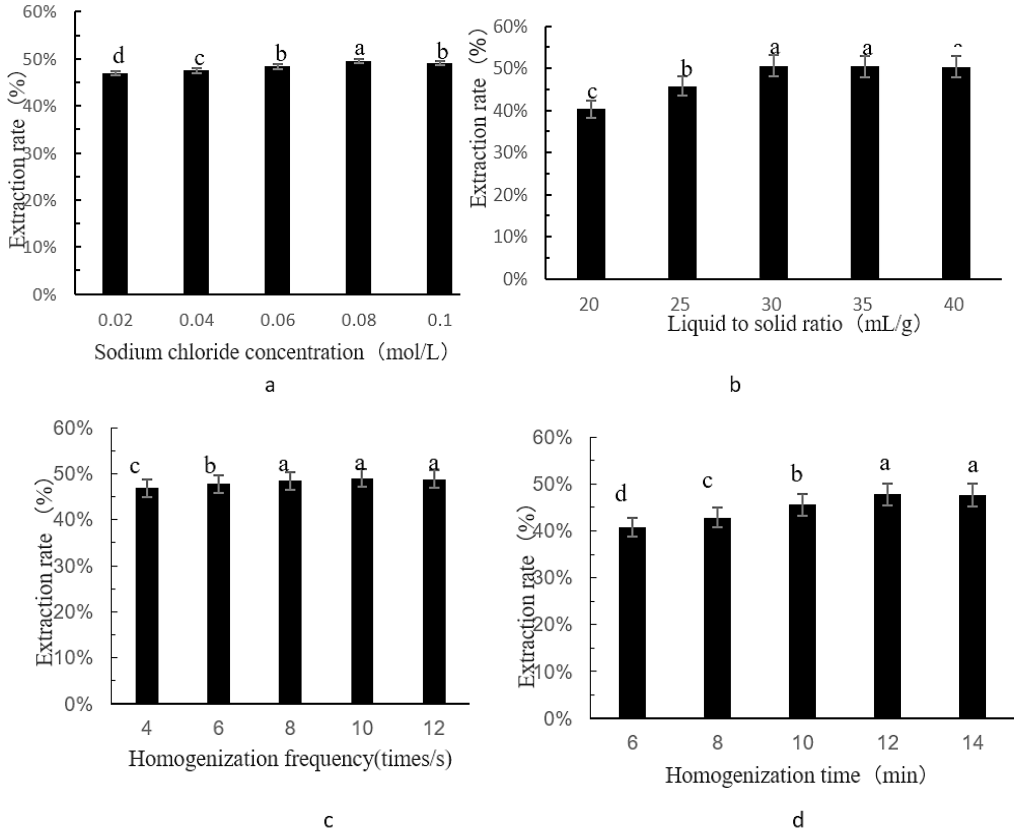

Fig. 1 Effect of different extraction conditions on protein extraction rate. Values are expressed as mean ± standard deviation. Lower case letters (a–d) mean different groups are significantly different

Results

Single Factor Experiments

Figure 1 shows the effects of different conditions on the extraction rate of *C. reinhardtii* protein. It can be seen from Figure 1a that different concentrations of sodium chloride solutions have little effect on the extraction rate of *C. reinhardtii* protein, for the extraction rate ranged from 46.99% to 49.52% among which the highest extraction rate appear at the concentration of 0.08 mol/L. As shown in Figure 1b, varying the liquid-to-solid ratio affects the protein extraction rate of *C. reinhardtii*. When the liquid to solid ratio was 30, 35 and 40 mL/g, there was no significant difference in protein extraction rate, and the highest extraction rate was 50.60% at the ratio of 30 mL/g. The protein extraction rate of *C. reinhardtii*

was increased from 40.27% to 45.71% when the liquid to solid ratio was lower than 30 mL/g. The impact of beating frequency on protein extraction rate of *C. reinhardtii* is depicted in Figure 1c. *C. reinhardtii* had the greatest protein extraction rate, which was 49.05%, at a beating frequency of 10 times per second. The figure illustrates that when the beating frequency is less than 10 times per second, the protein extraction rate increased with beating frequency, while the extraction rate declined when the beating frequency is greater than 10 times per second. The impact of beating time on protein extraction rate of *C. reinhardtii* is shown in Figure 1d. Protein extraction rate of *C. reinhardtii* peaked at 47.78% when the beating period was 12 min. The figure shows that when the beating duration is shorter than 12 min, the longer the beating time, the higher the *C. reinhardtii* protein extraction rate.

RSM Analysis

According to the response surface optimization, the protein extraction rates ranged from 43.81% to 51.02%. The optimal extraction conditions for *C. reinhardtii* protein were liquid to solid ratio of 31.02 mL/g, homogenization frequency of 10.17 times per second, NaCl concentration of 0.091 mol/L, and homogenization time of 12.82 min. Verification experiment was performed under the optimal conditions with approximation (liquid to solid ratio 31 mL/g, homogenization frequency of 10 times per second, NaCl concentration of 0.09 mol/L, and homogenization time of 13 min.), and the protein extraction rate of 53.23% was obtained while the predicted protein extraction rate from the model was 52.78%.

The quadratic regression models in correlation of protein extraction rates with four variables are shown by the following second-order polynomial equation: $Y=29.22+1.28A+0.8925B+0.1917C+0.8000D+0.0800AB+0.0775AC-0.0350AD-0.0575BC+0.0050BD-0.1150CD-3.17A^2-1.11B^2-0.6958C^2-0.6883D^2$ ($R^2=0.8114$)

where Y were protein extraction rate, and A, B, C and D were the coded values for sodium chloride solution, liquid to solid ratio, homogenization frequency, and homogenization time, respectively.

Table 2. ANOVA analysis of the response surface quadratic model for optimizing protein extraction rate

Source	Sum of squares	df	Mean square	F value	p value
Model	104.13	14	7.44	9.60	< 0.0001**
A	7.68	1	7.68	9.92	0.0071**
B	19.53	1	19.53	25.22	0.0002**
C	0.4408	1	0.4408	0.5692	0.4631
D	9.56	1	9.56	12.34	0.0034**
AB	0.0049	1	0.0049	0.0063	0.9377
AC	0.0529	1	0.0529	0.0683	0.7976
AD	0.0001	1	0.0001	0.0001	0.9911
BC	0.0240	1	0.0240	0.0310	0.8627
BD	0.0256	1	0.0256	0.0331	0.8583
CD	0.0132	1	0.0132	0.0171	0.8979
A^2	3.07	1	3.07	3.97	0.0662
B^2	65.06	1	65.06	84.01	< 0.0001**
C^2	3.14	1	3.14	4.06	0.0637
D^2	7.95	1	7.95	10.27	0.0064*
Residual	10.84	14	0.7745		
Lack of fit	10.62	10	1.06	19.07	0.8960
Pure error	0.2228	4	0.0557		
Cor total	114.98	28			

**:p<0.001; *:p<0.05

Table 2 displays the analysis of variance for the fitted quadratic polynomial model for optimization of protein extraction parameters. The model's low level of significance (p 0.0001) suggested that the anticipated value and experimental value agreed well.[20] The results were not significantly affected by unknown factors because the Lack of Fit item's significance threshold was p = 0.8960. The quadratic regression model's coefficient of multiple determinations (R^2) was 0.8114, which indicates that the model could account for 81.14% of the variability. Further evidence that this model may be utilized to examine and forecast changes in protein extraction rate under various extraction settings came from the fact that adjusted determination coefficients and R^2 values were both near to 1 (R^2 adj = 0.8114 and R^2adj = 0.9065, respectively).[21]

Ph Value of Fresh Shrimp in Storage

Under optimal extraction conditions, varying concentrations of *C. reinhardtii* protein extracts (0.1%-0.5%) were applied for fresh shrimp preservation. The pH changes of the shrimp during cold storage for 4 days are illustrated in Figure 2. A positive correlation was confirmed between pH and quality acceptability of seafoods, and pH<7.70 was attributed to good quality.[22] The initial pH value of the shrimp samples determined was 6.89, this value is in good agreement with other researchers [23] (pH 6.87) , and it was obviously lower than the value reported earlier, such as pH 7.04,[24] pH 7.61[25] and pH 7.30.[26] It was noted that pH values changed slightly on the 1st day and significantly decreased on the 2nd day of all samples. Differences appeared on the 3rd day, except for the control group and the group treated with 0.3% protein extracts, the pH value of other groups was found to decrease progressively over storage from day 0 to day 3. On the 4th day, the pH values of all groups were found to increase, indicating that the shrimps began to enter the spoilage stage, but the 0.2% protein extracts group still showed a good fresh-keeping effect, whose pH value (6.98) was significantly lower than that of the control group (7.06). In addition, the pH value of 0.1% protein extracts group showed no significant difference from that of the control group, while the pH value of 0.3%-0.5% protein extract groups was significantly higher.

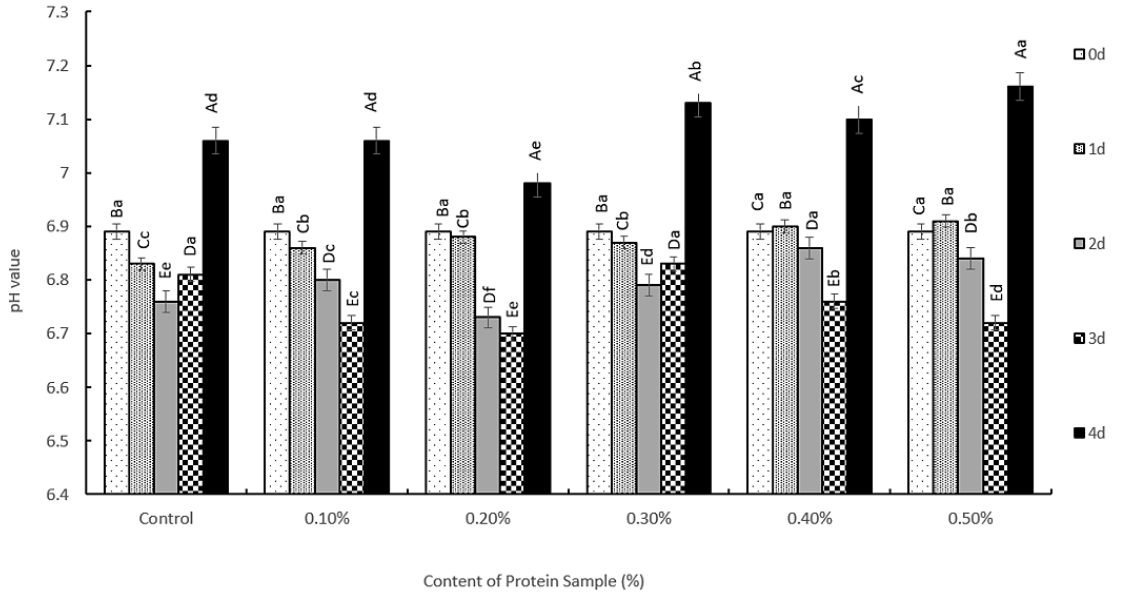

Fig. 2 Changes in pH value of shrimp treated by *C. reinhardtii* protein extract at different concentration. Lower case letters (a–f) mean in the same time, different groups are significantly different, and superscript capital letters (A–E) mean in the same group, different days are significantly different (p< 0.05).

The total Viable Count (TVC) of Fresh Shrimp in Storage

Figure 3 shows the changes in TVC of shrimp treated with different concentrations of protein extracts. The initial TVC of 0.8 log CFU/g in each group was determined. Compared with TVC of shrimp reported in previous researches, as 2.42±0.13 log CFU/g,[27] 3.32 log CFU/g,[28] and 3.79 log CFU/g,[29] the value in the present study was considered lower, and the freshness of shrimp used in this study was estimated as good. Generally, the total number of colonies in all groups increased progressively with storage time; however, the increase in TVC of the shrimp in the control group and the protein extracts treated groups showed different trends. The 0.1% and 0.2% protein extract solutions performed better in the first 3 days of storage. In day 4, the TVC significantly increased in all the groups, but it is important to note that the TVC in the groups treated with various protein extract solutions (<4.6 log CFU/g) was significantly lower than that in the control group (5.1 log CFU/g), and 0.2% protein extracts treated group showed the lowest TVC (3.2 log CFU/g). This may suggest that the protein extracts solution's antibacterial ability took a longer time to manifest, and if the measurement period was extended, the protein extracts was more likely to display a better impact. Moreover, the least TVC on day 4 displayed in the 0.20% protein extracts group, indicating the lowest degree of spoilage, was substantiated by the pH value results presented above.

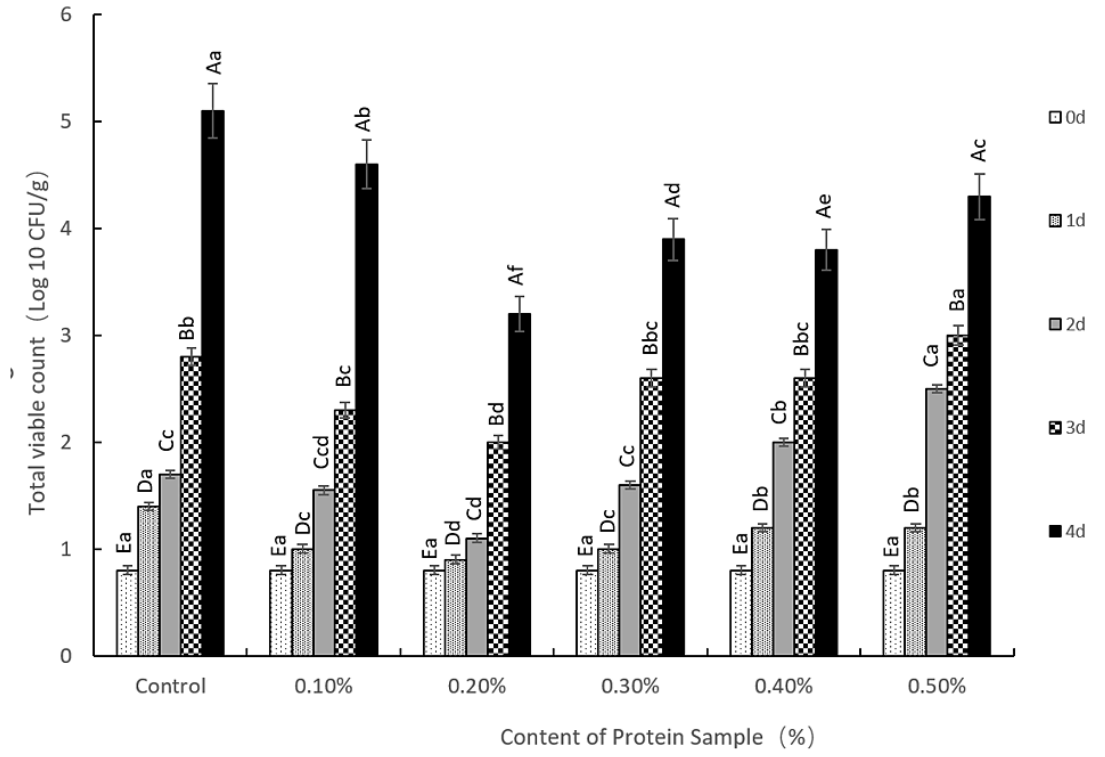

Fig. 3 Changes in total viable count of shrimp treated by protein extract at different concentration. Lower case letters (a–f) mean in the same time, different groups are significantly different, and superscript capital letters (A–E) mean in the same group, different days are significantly different ($p < 0.05$).

Discussion

Research30 stated that the addition of salt solution can enhance the permeability of cells and promote the dissolution of proteins from cells. It was also stated in a study of fish protein extraction that 0.08 mol/L sodium chloride was the best solvent concentration.[31] A study showed that when the concentration of sodium chloride increased from

0 to 1 mol/L, the solubility of papaya seed protein increased.[32] The reason may be attributed to an appropriate increase in the concentration of chloride ions promoting the combination between chloride ions and protein groups with positive charge, resulting in the electrostatic repulsion between proteins enhanced and the solubility increased. The liquid-to-solid ratio is a crucial factor in extraction efficiency. A low ratio can hinder full contact between the algal powder and the solvent, negatively affecting the dispersion of the powder and dissolution of algal protein. A similar trend was reported by other researchs,[33] where it was found that the yield of crude polysaccharides from Rubus chingii increased as the liquid-to-solid ratio rose from 10 to 30 mL/g, with no significant yield increase at ratios above 35 mL/g. This suggests that increasing the solvent volume beyond an optimal ratio does not necessarily enhance the extraction process. Conversely, an excessively high ratio increases the total extraction volume, leading to higher energy consumption and lower extraction efficiency. As for the beating frequency and beating time, they positively correlated with the protein extraction rate within a certain range. However, the aseptic homogenizer produced a considerable amount of shear force throughout the homogenization procedure. Rapid and long-term beating can result in the instrument heating up during extended shearing, which can cause protein foaming and denaturation and lower the rate of *C. reinhardtii* protein extraction.[34]

Solvent extraction is an efficient and environmentally friendly method for obtaining bioactive ingredients from plant materials. As shown in Table 4, A, B, D, B2, and D2 all significantly affected the rate of protein extraction ($p<0.001$). Variable B, “Liquid to solid ratio”, was commonly studied in numerous research. Some studies believed that it has significant impact on the extraction rate, while others reported that the liquid to solid ratio mattered only under certain conditions. For example, it was reported[35] in research that increased liquid to solid ratio in the range of 10-30 mL/g contributed to enhancing polysaccharide yield in brown algae *Ascophyllum nodosum*. Moreover, a rise was observed in bioactivity extracts in sage as liquid to solid ratio was increased from 6:1 to 18:1.[36] However, several researchers agreed that liquid to solid ratio only affected the extraction rate of bioactive compounds significantly in a certain range of temperature. For example, in high temperatures between 89 and 93°C, 35ml/g was found to be the optimal ratio for the yield of polysaccharides extraction of mulberry leave;[37] and the highest bioactive compounds yield of pomegranate peel was achieved with a liquid to solid ratio of 23 mL/g in 60°C) in an ultrasound-assisted extraction.38

Throughout storage, the shrimp's overall pH initially decreased and then rose, a pattern consistent with previous studies.[22, 23, 24] Early in storage, glycogen and ATP degradation in shrimp muscle generated acidic compounds, including lactic, phosphoric, and succinic acids,[39] leading to a pH decrease. During extended cold storage, microbial activity and enzymatic processes broke down nitrogenous substances like proteins, forming alkaline by-products such as ammonia and histamine, which leads to an increase in the pH value.[40] The initial pH values may vary depending on the species, diet, season, and level of stress experienced during harvesting. A study about shrimp treated by green tea leaves extracts41 showed decreasing pH values from 6.9 to 6.6 within the first 2 days and then increased progressively in subsequent storage. To some extent, the result in the present study indicates that the freshness of shrimp treated with protein extracts could be enhanced during storage. A study stated that 0.1% microalgae extracts displayed better fresh-keeping potential than other concentration of microalgae extracts, for it significantly controlled the increase of lipid peroxidation.[17] Similar result was reported wherein pH values of pacific white shrimp samples were found to increase when treated with 0.2% green tea extracts, as compared to those treated with 0.5% and 1.0% group during a 4-day storage.[41] This indicated that a higher concentration of bioactive extracts does not always guarantee better preservation effect, in fact it may accelerate the spoilage stage in contrary.

TVC is a main basis for judging spoilage of aquatic products. Microbial metabolites generated from protein breakdown, such as peptides or amino acids, contribute significantly to unfavorable sensory alterations in marine products unwanted odor, texture, and appearance signs of food deterioration.[42, 43] In a relative study,[41] 1.0% green tea extracts and 1% fenugreek seeds extracts were proved to exhibit better preservation effect on pacific white shrimp than lower concentration fractions;

whereby shrimp samples showed 5.1 log CFU/g and 6.23 log CFU/g under respective treatment. Results of other research revealed that shrimp treated with 0.5% cinnamaldehyde possessed the lowest aerobic plate count compared with counterparts of lower concentration.[24] These results underlined the fact that antimicrobial activity of plant extracts depended largely on the concentration of the solution prepared.

Conclusion

In this study, *C. reinhardtii* protein was extracted using a simple, time-efficient, and environmentally friendly method involving an aseptic homogenizer. The fresh-keeping effect of *C. reinhardtii* protein extracts on freshwater shrimp was also investigated. Under optimal extractions conditions - 0.091 mol/L sodium chloride concentration, a 31.02 mL/g liquid to solid ratio, a homogenization frequency of 10.17 times per second and a homogenization time of 12.82 minutes - the protein extraction yield reached 52.78%.Additionally, it was observed that a 0.2% *C. reinhardtii* protein solution could extend the shelf life of shrimp by delaying the increase in pH and total viable counts (TVC) during refrigerated storage. This finding highlights the potential of *C. reinhardtii* protein as a natural preservative, addressing issues associated with synthetic antibacterial agents, such as risks to human health, impacts on food nutrition, and microbial resistance.

This study contributes to the growing field of natural food preservation by presenting an innovative application of *C. reinhardtii* protein extracts. It not only provides a sustainable alternative to synthetic preservatives but also paves the way for further exploration of algal proteins in enhancing the quality and safety of perishable food products. However, research on *C. reinhardtii* remains relatively limited, and the chemical indicators assessed in this study are not yet comprehensive. Further studies could incorporate additional indicators, such as total volatile basic nitrogen and polyphenol oxidase, and extended the storage period to 2-3 weeks to further explore the food preservation potential of *C. reinhardtii* protein extracts.

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Conflict of Interest

All authors declare no conflict of interest regarding this subject matter.

Data Availability Statement

The data will be available from the corresponding author upon reasonable request.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

- **Ke Ying Su:** Methodology, Data Curation and Formal Analysis, Writing – Original Draft Preparation.
- **Qian Li:** Data Curation and Formal Analysis, Investigation.
- **Yi Yang Zhong:** Investigation.
- **Xue Wu Zhang:** Funding acquisition, Supervision.
- **Lai Hoong Cheng:** Supervision, Conceptualization, Writing – Review and Editing

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