JTE
HCMUTE
ISSN: 2615-9740

JOURNAL OF TECHNOLOGY EDUCATION SCIENCE
Ho Chi Minh City University of Technology and Education
Website: https://jte.hcmute.edu.vn/
Email: jte@hcmute.edu.vn

Protein Extraction from Spirulina Platensis with The Cellulase Enzyme Assistance

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ARTICLE INFO

Received: 17/5/2022
Revised: 24/5/2022
Accepted: 15/6/2022
Published: 28/6/2022

KEYWORDS

Spirulina platensis;
Protein;
Enzyme;
Cellulase;
Extraction.

ABSTRACT

Microalgae is a relatively new, sustainable source of protein supplements. Among various microalgae strains, *Spirulina platensis* has shown an effective protein potential source compared with others. A high-performance protein recovery technology is required for this prospect. When the cellulase enzyme supported the recovery process, the condition to get the highest protein recovery efficiency (40.13±2.87%) were 50 UI/g dry algae for enzyme activity, 1:20 for the ratio of dry algae: solvent, 7.0 for pH value with a process temperature of 50^{o}C for 90 minutes. This study succeeded in using cellulase enzymes to support protein extraction with high recovery efficiency from Spirulina platensis grown in Vietnam, opening up an additional source of protein in the trend of plant-based meat production.

Doi: https://doi.org/10.54644/jte.70B.2022.1213

1. Introduction

Animal-derived proteins are crucial in preventing malnutrition and muscle aging in humans, but they're also harmful to the environment since they are typically produced inefficiently [1] – [3]. For manufacturing and delivery, animal-derived proteins occupy a lot of lands, water, fertilizers, and energy, resulting in a lot of greenhouse gas emissions. As a consequence, current research has prioritized the search for alternate protein sources and the transition to a more sustainable diet [1]. Alternative sources of protein in meals of plant origin, insects, single-cell proteins (mycoproteins or microalgae), and artificial meat have all been researched as part of this approach [4].

Microalgae is a relatively new, sustainable source of protein supplements. These microalgae may create a wide range of biologically important chemicals, including proteins, long-chain polyunsaturated fatty acids, carotenoids, vitamins, minerals, and phenolic compounds [5] – [7]. Microalgae do not compete for arable land like other food sources and can be cultivated with non-potable water, sewage, or even saltwater, depending on their purpose. Their high biomass yield per unit area is another advantage [7] – [8]. Microalgae, eukaryotes, and photosynthetic bacteria, commonly known as prokaryotic cyanobacteria, are all microalgae [9]. Because of their physiological similarities and ability to undertake photosynthesis, cyanobacteria, or blue-green algae, are included in this group. Arthrospira sp. is one of the most important plants of green algae, with *Spirulina platensis* and *Spirulina maxima* being the most important species [10] – [11]. *Spirulina platensis*, also known as Spirulina, is a filamentous cyanobacterium found in warm, alkaline lakes with high salt concentrations and pH values [10] – [12]. The helical shape of Spirulina's multicellular cylindrical filaments in an open helix with a length of 0.3 to 1.0 mm is a morphological characteristic [13].

These microalgae species are widely cultivated because of their applications in food, feed, pharmaceuticals, pharmaceuticals, and cosmetics [14]. It is used in human nutrition not only because of its high protein content (up to 70% of the dry matter) but also because of its essential amino acid (AA) composition and good digestibility, making it a potential alternative protein source [15] – [17]. In addition, microalgae proteins also have promising technological properties, which can be used as a foaming, gelling and emulsifying agents [15] – [16]. Several studies have shown that microalgae proteins can compete with some commercial proteins used as emulsifiers such as sodium caseinate, whey protein, and soy protein [15] – [18].

To recover proteins from microalgae, many different extraction methods can be applied, but the ultimate goal is still to break the cell membrane of microalgae to release intracellular content; thereby, the protein would be recovered from the microalgae. Two groups of the assisted methods are applied, which can be listed: mechanical methods (crushing, homogenizing,...); non-mechanical methods. Non-mechanical methods use chemical agents, enzymes, or physicochemical methods to release compounds from biomass [19] – [21]. In general, non-mechanical assisted extraction is less effective than mechanical extraction techniques, so they are used to extract biological compounds that can be degraded in the harsh conditions of mechanical assisted extraction techniques [22]. However, some of the used chemical agents can contaminate the obtained solution, such as the use of surfactants and EDTA that affect the yield. lipid extraction from microalgae [23].

Microalgae disruption using enzymes require low energy and gentle operating conditions, but the high costs of enzymes may limit the adoption of this technology in some commercial segments of microalgae products since cocktails of enzymes could be required for efficient hydrolysis [19] – [24]. On the other hand, the employment of enzymes may make downstream procedures such as protein isolation easier. Enzymes have high specificity for biological components, resulting in selective cell breakdown and the release of unwanted bio compounds [22] – [25]. When opposed to mechanical approaches, enzymes require a longer treatment time and well-controlled settings to achieve high conversion rates. Therefore, this study was conducted to determine the protein extracted conditions using cellulase enzyme from *Spirulina platensis* grown in Vietnam. In which factors such as enzyme activity (UI/g dry algae), raw dry algae: solvent ratio, pH of solvent, temperature (^oC), and extraction time (minutes) were investigated to determine the highest protein recovery.

2. Materials and Methods

2.1. Materials

Spirulina platensis was provided by Vinh Hao Spirulina Algae Corporation (Binh Thuan, Vietnam). Algae biomass was dried and freeze-dried to a moisture content of 5% and stored in the refrigerator with zip bags for later processing. The cellulase enzyme was obtained from *Trichoderma* sp. (activity 10,000 UI/g), supplied by Leaf Cleantech (India) manufacturer. The chemicals used to prepare buffer solutions and in the analysis of protein content have purity > 98%, meeting analytical standards.

2.2. Enzyme-assisted extraction of algae

Water-soluble protein was extracted from raw dry algae (1 g) with cellulase enzyme in potassium phosphate buffer. The factors of the experiment were investigated such as the enzyme activity (20 - 80 UI/g dry algae), the ratio of dry algae: solvent (1:10 - 1:30), pH value (5.5 - 7.5), temperature (30 - 70^oC) and extraction time (30 - 150 minute). After the end of the extraction process, the obtained solution was centrifuged at 4,000 rcf for 10 minutes and collected the supernatant to determine the soluble protein content.

2.3. Analysis methods

The moisture content of the raw materials was determined by drying to constant mass. The contents of ash, fat, and carbohydrate were analyzed following the AOAC procedures [26]. Protein content was determined by the Kjeldahl method on UDK 139 (Velp, Italy), approximately 1 g of samle was hydrolyzed mixing 15 mL of H_2SO_4 and copper tablest (as catalyzer) at 420^oC for 2 h; then, the samles

were neutralized and titrated for the protein content using a 6.25 value as nitrogen conversion factor. The protein recovery (%) was calculated by the extracted protein amount over the protein content in the raw dry algae.

2.4. Statistical analysis

All the analyzed were done in triplicate (n = 3) and the results obtained were expressed as mean with standard deviation (SD). To deterimine statistical differences, the results were analyzed with one-way ANOVA and Tukey's multiple comparisons test ($p < 0.05$) using Minitab 18.

3. Results and Discussion

3.1. Composition of Spirulina platensis

The chemical composition of the freeze-dried spirulina biomass is presented in Table 1. The main ingredient found in the raw algae is a protein (76.45%) followed by fat content (9.83%). This result is similar to the obtained studies with Spirulina cultivated in many parts of the world [27]. This high protein content in algae biomass shows the potential to become a great source of protein in the future development of the food industry. Low-fat content has also been reported with different references. However, when growing conditions are different (media composition, culture technique), the fat content can be increased up to 25% [28]. The ash content of algae cultured in Vinh Hao has lower values (7.68%) than those recorded in other research (8-18%).

Table 1. *Composition of Spirulina platensis (based on dry biomass)*

Composition	Amount (%)
Moisture	4.16 ± 0.12
Ash	7.68 ± 0.07
Protein	76.45 ± 0.15
Fat	9.83 ± 0.02
Carbohydrate	1.72 ± 0.36

3.2. Effect of enzyme activity

The effect of enzyme activity on protein recovery from dried Spirulina was investigated. The experiment was carried out with fixed factors: pH of the solvent for 7; the ratio of material: solvent for 1:20; temperature for 50°C; extracted time for 60 minutes. While enzyme activity was changed from 20 UI/g dry algae to 80 UI/g dry algae. The obtained results are shown in Figure 1.

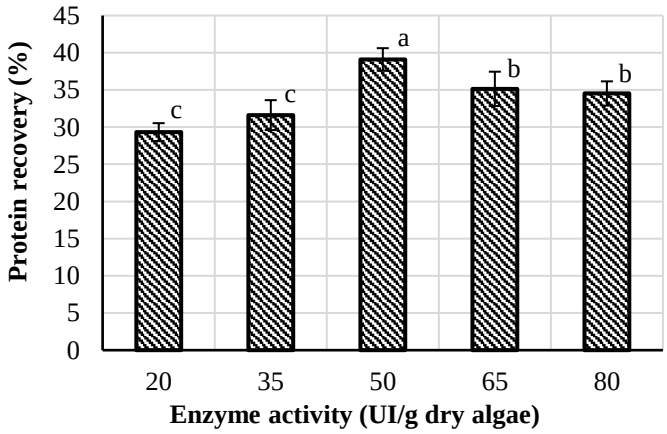

Figure 1. *Effect of enzyme activity on protein recovery*
([a,b,c]The different letters correspond to the significant data)

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When cellulase enzyme activity increased from 20 UI/g dry algae to 50 UI/g dry algae, the protein recovery increased and reached the maximum value of 39.09±1.52%. However, this value decreased with further increase of cellulase enzyme concentration up to 80 UI/g dry algae, therefore, cellulase enzyme concentration 50 UI/g dry algae will be applied for the further experiments. The amount of protein obtained depends on the rate of diffusion of intracellular fluid through the cell wall into the extraction solvent and on the stability of the substances after being extracted. The cell wall of *Spirulina platensis* is mainly composed of cellulose [8], so under the action of the cellulase enzyme, cellulose will be hydrolyzed into short-chain cellulose promoting the release of intracellular biomolecules and the increasing amount of protein in the extract. In general, increased enzyme content increases the rate and efficiency of protein extraction. However, when the amount of enzyme was saturated, the decreased yield of useful substances from the intracellular content was observed due to the reverse inhibition of hydrolysis due to the high enzyme concentration to saturation [29].

3.3. The ratio of dry algae: solvent effect

With fixed factors such as cellulase enzyme activity for 50 UI/g dry algae, the pH solvent for 7.0, temperature for 50ºC, the extraction time for 60 minutes, the effect of dry algae: solvent ratio was evaluated for protein recovery when varying from 1:10 to 1:30. The obtained results are shown in Figure 2.

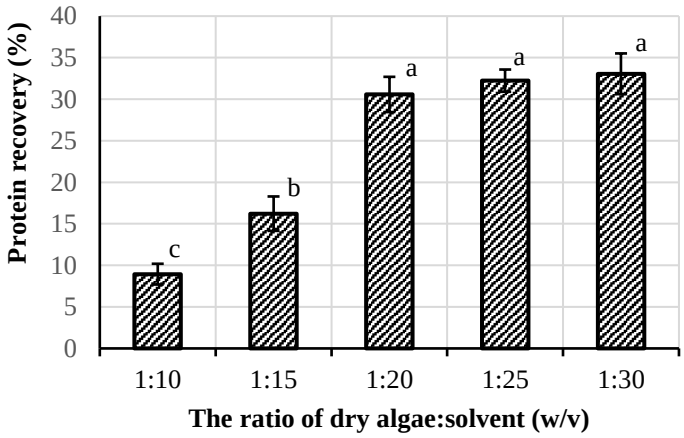

Figure 2. *The ratio of dry algae: solvent (w/v) on protein recovery*
([a,b,c]The different letters correspond to the significant data)

With the ratio of dry algae: solvent from 1:10 to 1:20, the protein recovery increased from 8.94±1.25% to 30.56±2.72%. When this ratio was further increased to 1:30, the protein recovery efficiency was almost unchanged. Based on this result, the 1:20 ratio will be the appropriate value to be used for further experiments of protein extraction. During the extraction process, the buffer solution not only acts as the extraction solvent, penetrating the cell to help dissolve the intracellular substances but also increases the mobility of enzymes, supporting the hydrolysis reaction, increasing the diffusion of proteins into the extract. However, when the dry algae: solvent ratio is high, the enzyme activity decreases, the contact ability between the enzyme and the raw dry algae decreases; on the other hand, the high water content increases the protein's ability to be hydrolyzed and oxidized, reducing the protein content in the extracted solution.

3.4. pH solvent effect

With the two fixed factors (the cellulase enzyme activity for 50 UI/g dry algae, the dry algae: solvent ratio for 1:20), the influence of the pH value of solvent during the extraction process is investigated. In addition, the following factors are also kept constant such as the process temperature for 50ºC and the extraction time for 60 minutes. The pH value of the solvent would be changed from 5.5 to 7.5. The obtained survey results are shown in Figure 3.

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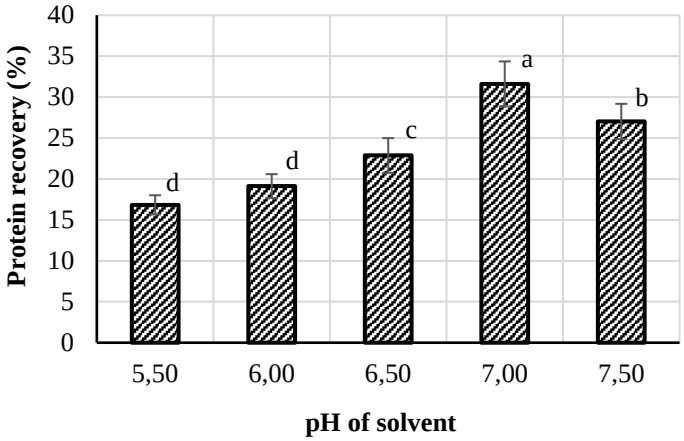

Figure 3. *Effect of pH value on protein recovery*
(^{a,b,c,d}The different letters correspond to the significant data)

When the pH increased from 5.5 to 7.0, the protein recovery increased and reached the maximum value of 31.61±2.75% (at pH 7.0). However, when the pH continued to increase, the recovered protein content decreased. This can be explained by the role of pH in the extraction medium, which greatly affects the degree of substrate ionization through the change in H^+ concentration, thereby affecting the enzyme's impact on the substrate. Any change in pH value also affects the efficiency of extraction and protein recovery. In addition, pH affects the surface charge of the protein molecule and can affect the solubility of the protein. The results of this survey show that a pH of 7.0 will be the best condition when using cellulase enzyme. From this result, it is shown that the pH value of 7.0 will be used in the further experiments of protein extraction.

3.5. The effect of temperature

The investigated results on the influence of temperature on the protein recovery are presented in Figure 4. This experiment was conducted under fixed conditions such as enzyme activity for 50 UI/g dry algae, the dry algae:solvent for 1:20, the pH of solvent for 7.0 and the extraction time for 60 min. The investigated temperature varied from 30ºC (room temperature) to 70ºC. This result shows that when the temperature is increased from 30ºC to 50ºC, the protein recovery will increase clearly, from 29.11±1.17% to 39.51±2.12% and a decrease in protein recovery was observed when the temperature increase was greater than 50ºC. This result is almost equivalent to other studies when using cellulase enzyme in supporting the extraction process.

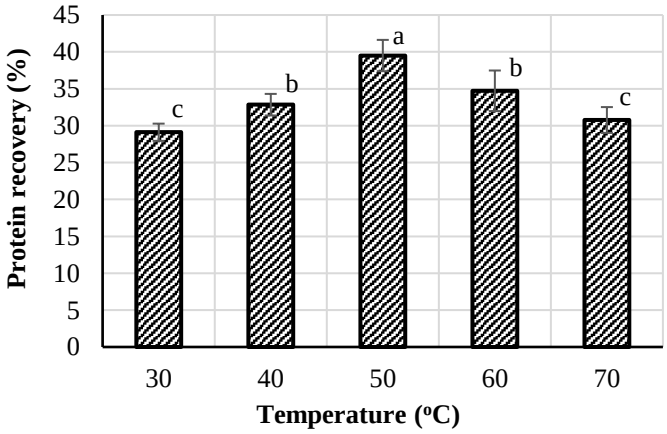

Figure 4. *Effect of temperature on protein recovery*
(^{a,b,c}The different letters correspond to the significant data)

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At the temperature for 50ºC, the protein recovery was the highest 39.51±2.12%. With this result, it is possible to choose the extraction temperature at 50ºC for the protein extraction from Spirulina algae. Each enzyme will work best at the optimal temperature range; according to the manufacturer, the cellulase enzyme preparation of this research has an operating temperature range of 30-70ºC, so the results obtained have been conceded. In addition, the diffusion rate of the solvent into the cell, of the intracellular substances out of the solvent, and the equilibrium concentration of the extraction process are also affected by temperature. Theoretically, as the temperature increases, the viscosity decreases, making it easier for enzymes and solvents to come into contact with the active ingredients, then increasing hydrolysis. However, continuing to increase the high temperature can cause denaturation of enzymes and substances in the intracellular fluid, causing a decrease in protein concentration in the extract.

3.6. The effect of extraction time

The influence of extraction time on protein recovery is shown in Figure 5. To carry out this investigation, factors were kept constant such as cellulase enzyme activity for 50 UI/g dry algae, the ratio of material: solvent for 1:20, pH of the solvent for 7.0, and the process temperature for 50ºC; with a change in the extraction time factor from 30 minutes to 150 minutes. The obtained results show that the extraction time is an important factor affecting the enzyme activity as well as the protein recovery results.

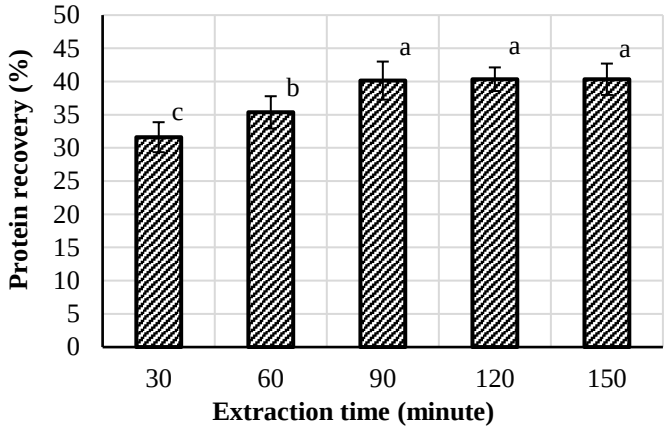

Figure 5. *Effect of extraction time on protein recovery*
([a,b,c]The different letters correspond to the significant data)

According to Figure 5, the protein recovery reached a high value of 40.13±2.87% at 90 minutes for extraction time. Increasing the extraction time will help the enzyme to hydrolyze the cell wall, facilitate the release of protein from the raw dry algae into the solvent, that to enhance the protein recovery. However, when the extraction time further increased, the protein recovery was not significantly increased and there may be the hydrolysis of the obtained protein. Then the time to perform the enzyme-assisted extraction process can be stopped at 90 minutes to benefit the time cost when carrying out protein recovery from *Spirulina platensis*.

4. Conclusions

With the great potential of *Spirulina platensis* grown in Binh Thuan (Vietnam), it is necessary to find a suitable solution to extract and recover valuable components from the intracellular content of algae, including proteins. When the extraction process was supported by cellulase enzyme, the condition to get the highest protein recovery efficiency is 50 UI/g dry algae for enzyme activity, 1:20 for the ratio of dry algae: solvent, 7.0 for pH value with a process temperature of 50ºC for 90 minutes. Under these conditions, the water-soluble protein recovery efficiency was 40.13±2.87%. The obtained protein solution can be further processed to add to food products that need to be supplemented with protein

sources or plant-based meat products. In addition, the results obtained from this study can be used as a basis for further studies needed to improve the protein recovery efficiency from *Spirulina platensis*.

Acknowledgments

This research was supported of Vinh Hao Spirulina Algae Corporation (Binh Thuan, Vietnam).

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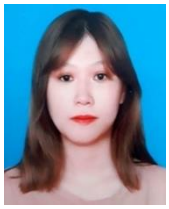

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