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Extraction of intracellular protein from *Chlorella pyrenoidosa* using a combination of ethanol soaking, enzyme digest, ultrasonication and homogenization techniques

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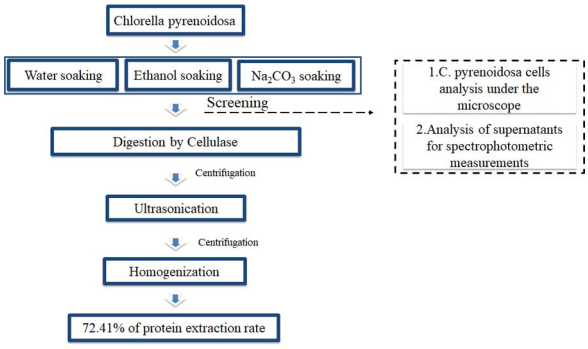
GRAPHICAL ABSTRACT



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ABSTRACT

Due to the rigid cell wall of Chlorella species, it is still challenging to effectively extract significant amounts of protein. Mass methods were used for the extraction of intracellular protein from microalgae with biological, mechanical and chemical approaches. In this study, based on comparison of different extraction methods, a new protocol was established to maximize extract amounts of protein, which was involved in ethanol soaking, enzyme digest, ultrasonication and homogenization techniques. Under the optimized conditions, 72.4% of protein was extracted from the microalgae *Chlorella pyrenoidosa*, which should contribute to the research and development of Chlorella protein in functional food and medicine.

1. Introduction

Microalgae is recognized as novel green sources of multiple components like proteins, carbohydrates, lipids and pigments for food, pharmaceutical, cosmetic and energy products (Becker, 2007). However, it is still challenging to effectively release all intracellular components from the microalgae due to the rigid cell wall of Chlorella species (Morris et al., 2008). To resolve this drawback, the first step is to break the cell wall and cell membranes and to facilitate the release of high value added components, particularly, extraction of proteins. Ultrasonication is usually suggested as an approach to breaking algal cells. High frequency sonic waves generate intense local shock waves equivalent to thousands of atmosphere pressure to bring about implosive collapse of the gas-filled cavitation bubbles and ultimately cause cell wall disruption (Zheng et al., 2011). In terms of energy and bioactivity, enzymatic hydrolysis is an advantageous approach, because

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it exhibits high selectivity with fewer side products and operates at lower temperatures (Fu et al., 2010; Gerken et al., 2013). In addition, many other methods have been developed for cell disintegration, such as bead milling, acid or alkali treatment, microwaves, pulsed electric field and high-pressure homogenization (Garcia, 2014; Grimi et al., 2014; Safi et al., 2014).

Notably, many of these cell disintegration techniques are involved in harsh conditions like high pressures, high shear levels or high temperatures, which negatively affect the quality and purity of the extracts. To maintain the functionality of proteins, cell disintegration should be done under mild conditions. However, mild methods frequently result in low extraction yields of the target components (Grimi et al., 2014). On the other hand, some techniques could be very energy intensive, for example, bead milling is known to be a high-energy consumption technique (Postma et al., 2015). These unresolved questions inspired many inventions for extraction of intracellular proteins from microalgae, and different cell disruption approaches have been already investigated on microalgae (Safi et al., 2017; Yang et al., 2017). However, most of these methods were related to high pressures, high shear levels, high temperatures or high energy consumption.

Therefore, there is a growing interesting in finding mild cell disintegration methods without use of high pressures, high shear levels, high temperatures or high energy consumption, but effective enough release of the valuable intracellular proteins. The aim of this work is to compare the extraction efficiency of proteins from *C. pyrenoidosa* using different techniques, then, a new protocol was designed and the process is optimized based on protein extraction yield.

2. Methods

2.1. Materials and chemicals

C. pyrenoidosa powder (62.4% ± 1% of total protein contents) were presented by Professor Zhang Daojing, East China University of Technology, Shanghai, China. BCA Protein Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute. Other reagents were analytical grade and commercially available.

2.2. Protein extraction methods

2.2.1. Chemical techniques of cell disruption (DWSE, ESE, SCSE)

The *C. pyrenoidosa* powder was soaked in different solvents for protein extraction, including distilled water-soaking extraction (DWSE), 60% ethanol-soaking extraction (ESE), 5% sodium carbonate (Na_2CO_3)-soaking extraction (SCSE), with liquid to solid ratio (10:1) and extraction time 24 h for all extraction.

2.2.2. Physical techniques of cell disruption (FTE, UE, HE)

Freeze-thawing extraction (FTE) was performed as below: one gram of *C. pyrenoidosa* powder was dissolved in 10 mL of distilled water, frozen at −20 °C for 30–100 min and thawed, 3–10 freeze-thaw cycles in total.

Ultrasonication extraction (UE) was performed in the following: one gram of *C. pyrenoidosa* powder was dissolved in 10 mL of distilled water, ultrasonicated by Biosafer 1200DT Ultrasonic cell crusher with power 600 W for 6 s (9 s interval) for 30 min.

Homogenization extraction (HE) was performed as below: one gram of *C. pyrenoidosa* powder was dissolved in 10 mL of distilled water, the solution was homogenized by shanghai ANgni instruments AD200S-H homogenizer with 8000 rpm for 10 min (2 min interval).

2.2.3. Biological techniques of cell disruption (EDE)

Enzyme digestion extraction (EDE): one gram of *C. pyrenoidosa* powder was dissolved in 10 mL of distilled water. The sample was adjusted to pH 5.0 with buffer NaH_2PO_3 and incubated at 50 °C with 1% (w/w, protein basis) cellulase (Sigma, St. Louis, MO, USA), digested for 3.0 h.

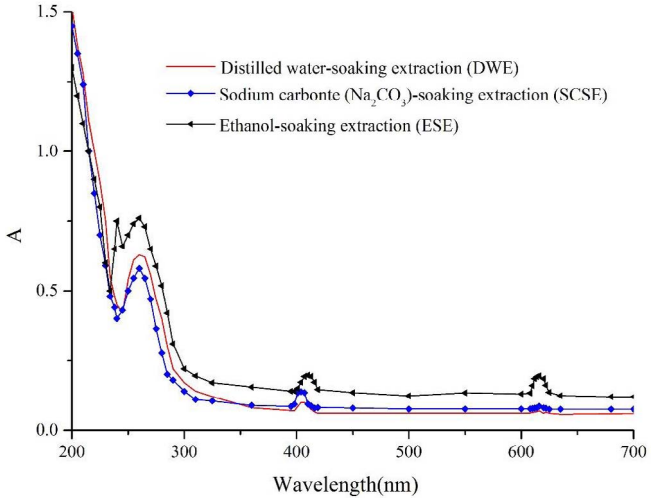

Fig. 1. Full-wavelength scan (200–800 nm) of *C. pyrenoidosa* supernatant soaked in (a) distilled water, (b) ethanol, (c) Na_2CO_3.

2.2.4. Combination of different methods (ESE, EDE, UE and HE) and optimization

The experimental operations used for optimal extraction of *C. pyrenoidosa* proteins are presented in Fig. 1. Specifically, ethanol-soaking extraction (ESE) was firstly used, a factorial design based on Box-Behnken method was carried out in 17 runs (12 factorial point and 5 replicates of central point). Based on initial experiments, three factors were chosen for independent variables: A, ethanol content (0%, 20%, 40%); B, soaking time (60, 66, 72 h); C, Liquid to solid ratio (25, 27.5, 30 g/g); and protein extraction rate was used as response variable (Table 1). After ethanol soaking, the solution was centrifuged at 5000 rpm and 4 °C for 10 min for protein analysis. Secondly, EDE was applied, i.e. cellulase was introduced for 3.0 h at pH 5.0. Thirdly, ultrasonication extraction (UE) was used. Three factors controllable were selected for single factor experiment: power (W), cycle time and liquid to solid ratio. Similarly, a factorial design based on Box-Behnken method was carried out in 11 runs (8 factorial point and 3 replicates of central point). Three factors were selected for independent variables: A, Power (800, 900, 1000 W); B, time (20, 30, 40 min, 6 s each time, 9 s interval); C, Liquid to solid ratio (30, 35, 40 g/g); and protein extraction rate was used as response variable (Table 2). Then, homogenization extraction (HE) was used for all samples, 8000 rpm for 2 min (2 min interval). Finally, each of them was centrifuged by Allegra™ 25R Centrifuge at 5000 rpm (8694*g*) and 4 °C for 10 min.

2.3. Analysis

The total protein content of the extraction supernatant before and after each treatment was determined by BCA Protein Assay Kit. For analysis of the protein extraction rate, the supernatant was collected by centrifugation and washed 3–5 times by distilled water.

$$Y = \frac{c * V}{1000 * T} \quad (1)$$

where Y is the protein extraction rate (%), c is the protein content of supernatant (mg/mL), V is the volume of supernatant (mL) and T is the total protein (g).

For spectrophotometric measurements, the absorption spectra of supernatant were recorded with a full wavelength (200–800 nm) Shimadzu UV-2550 spectrophotometer at room temperature. For *C. pyrenoidosa* cells analysis, some sediment was collected by centrifugation and observed under the microscope (Nikon ECLIPSE E100).