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Process for selective extraction of pigments and functional proteins from *Chlorella vulgaris*

Sayali Kulkarni, Zivko Nikolov*

Biological and Agricultural Engineering, Texas A&M University, College Station, TX, USA

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ABSTRACT

Extraction of multiple high-value products is recommended for sustainability of the microalgal production platform. This study proposes a process for selective extraction of carotenoids and chlorophylls with ethanol, followed by alkaline pH extraction of proteins from wet, freeze-thawed *Chlorella vulgaris* biomass. A biomass-to-solvent ratio of 1:5 and 3 extraction stages were required to achieve maximum extraction yield of chlorophylls and carotenoids. The main compounds in the ethanol extract were identified as lutein, chlorophyll *a* and chlorophyll *b*. The lutein and total chlorophyll yield in the extracts were 5.4 mg/g and 15.4 mg/g dry biomass respectively. Effective protein release from freeze-thawed biomass was contact-time dependent and > 76% of total protein could be extracted in 15 min via bead-milling, and in 6 min by high-pressure homogenization at 15000 psi. Ethanol extraction of pigments affected protein solubility, and an alkaline pH was required to release the same total protein. Concentration and fractionation of proteins was carried out using a two-stage membrane filtration process and 78–80% of proteins remained in the 300 kDa retentate. Ethanol treatment and higher pH conditions did not negatively impact membrane filtration, nutritive value, or the emulsification properties of protein concentrates.

1. Introduction

Microalgae are photosynthetic microorganisms and are rich sources of lipids, proteins, carbohydrates, and high-value compounds such as pigments, anti-oxidants and vitamins [1]. The various uses of microalgae for food, feed, and energy were identified decades ago [2]. Numerous studies have been conducted focusing on enhancing biomass growth [3], and optimizing processes to obtain a single product from microalgae like lipids or carbohydrates for biofuels production [4,5], proteins for food and feed [6], and pigments for nutraceutical applications [7]. However, life-cycle and techno-economic analyses published in the past ten years indicate that the algal platform would not be sustainable unless multiple high-value products can be generated from algal biomass [8–10]. Therefore, it is important to develop processes aimed at optimizing multiple product extraction which can maximize value of algal feedstock. Based on cell accumulation levels and current market value, proteins, chlorophylls, and carotenoids from microalgae hold promise as potential co-product candidates, especially if they could be selectively and inexpensively extracted.

The microalga *Chlorella vulgaris* is established as a good source of protein and carotenoids [11]. The protein content of *C. vulgaris* can amount to 58% of the cell dry weight (DW) [11], and essential amino acid profile of extracted proteins compares well to the standards recommended by WHO/FAO [12,13]. *C. vulgaris* protein fractions obtained after high-pressure homogenization and membrane filtration displayed emulsification properties comparable to soy protein isolate and sodium caseinate [14]. The lutein (carotenoid) content in *Chlorella* sp. can reach concentrations as high as 7 mg per gram cell dry weight [15]. Dietary intake of lutein helps with preventing early atherosclerosis, decreasing the rate of age-related macular degeneration [16], and ameliorating the onset or progression of cataracts [17]. Chlorophyll, a major pigment in plants and phototrophic algae like *Chlorella* [11], when isolated as a co-product, could also provide a revenue stream. Cited chlorophyll health-benefits include immune system stimulation, blood and liver detoxification, and relief from sinusitis, fluid buildup, and skin rashes [18].

To capture the potential health benefits and value of algal co-products, one has to evaluate the suitability of extraction methods related to their impact on product yield, quality, and overall production cost. Various cell disruption techniques such as sonication, high-pressure homogenization and bead-milling have been tested for protein extraction from *C. vulgaris* biomass [19,20]. High-pressure homogenization [19] and bead-milling [20] performed the best in terms of cell lysis releasing up to 66% and 96% of the total protein respectively.

* Corresponding author.
E-mail address: znikolov@tamu.edu (Z. Nikolov).

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Sonication wasn't as effective, releasing only up to 16% of total protein. Microscopic examination of lysed biomass supported protein release data. Although the initial the condition (dried, frozen, or wet) of tested algal biomass was not always clearly stated, bead-milling and high-pressure homogenization were more effective than sonication in releasing intracellular proteins by disrupting the rigid cell wall [21] of *Chlorella sp*. The state of the harvested biomass (wet or frozen) and the subsequent drying method are also important variables to consider when comparing the effectiveness of cell disruption and protein extraction. First, drying processes caused morphological changes in *Chlorella* [22]. Spray drying of *Chlorella* lead to formation of globular particles. Each particle/granule had a diameter of approximately 60–80 μm, composed of 3000–7000 cells with a void space in the center. Freeze-dried *Chlorella* powders formed sheet-like structures of fused cells. Also, the development of large ice crystals in the intercellular spaces due to freeze-drying lead to the displacement of the constituent parts [22]. Second, the extractability (solubility) of algal proteins could be substantially affected by heat-drying. In the preliminary studies conducted by our group, protein extraction from oven dried biomass was reduced 5–6 fold compared to wet biomass. A direct comparison between protein extraction from heat-dried biomass and wet biomass could not be found in literature. And third, drying of algal biomass is an energy intensive process that would increase processing costs [23–26].

Carotenoids and chlorophylls have previously been extracted from *Chorella* sp. biomass using organic solvents such as ethanol. Most of the reported processes utilized dried algal biomass as a source material [25–27] and, in some cases, high temperatures for pigment extraction [25,26], which are not optimal conditions if recovery of protein co-products are desired. Thus, developing processing conditions that would allow the selective extraction of lutein and chlorophylls before functional and nutritive protein fraction would advance our understanding and assessment of a multiproduct *Chlorella vulgaris* platform.

Keeping these factors in mind, the overall goal of this study was to develop optimal processing methods and conditions to permit selective co-extraction of pigments (lutein and chlorophylls) and quality protein from wet, freeze-thawed *C. vulgaris* biomass. The ancillary objectives of the experiential work were to determine: (i) the efficiency of solvent extraction to obtain a high-value pigment fraction from *C. vulgaris*, (ii) the best cell lysis method(s) for complete disruption and protein release from wet, freeze-thawed *C. vulgaris* biomass, and (iii) the impact of pigment removal on extractability of protein as well as nutritive value (determined by amino acid analysis) and emulsification properties of protein fractions.

2. Methodology

2.1. Ethanol extraction of carotenoids and chlorophylls

Frozen *C. vulgaris* biomass with 24% (w/w) solids content was supplied by Global Algae Innovations (HI, USA). Biomass was stored at −80 °C and thawed at room temperature for use. Biomass was mixed with 95% ethanol at a wet, freeze-thawed biomass-to-solvent (w/v) ratio of 1:3, 1:5 and 1:10, for 30 min at room temperature (22–25 °C). Ethanol was recovered by centrifugation at 7500 ×*g* at 4 °C for 10 min and supernatant absorbance at 470 nm, 649 nm, and 664 nm were recorded. Ethanol extraction of the same biomass sample was repeated two more times - a total of 3 extraction stages. Chlorophyll and carotenoid concentrations in the supernatants were estimated using the following equations [28]:

$$\text{Chlorophyll a } (\mu g/mL) = (13.36 \times A_{664}) - (5.19 \times A_{649}) \quad (1)$$

$$\text{Chlorophyll b } (\mu g/mL): (27.43 \times A_{649}) - (8.12 \times A_{664}) \quad (2)$$

$$\text{Total carotenoids } (\mu g/mL): (1000 \times A_{470} - 2.13 \times \text{Chlorophyll a} - 97.64 \times \text{Chlorophyll b})/209 \quad (3)$$

A total of 3 replicates were carried out for each experiment.

2.2. RP-HPLC analysis of ethanol extracts

Lutein, chlorophyll *a* and chlorophyll *b* standards were purchased from Sigma Aldrich. A Dionex HPLC (Thermo Fisher) system equipped with an ASI-100 automated sample injector, PPA-100 photodiode array detector, and P680 HPLC pump was used for analysis of ethanol extracts. 20 μL aliquots were injected onto a reverse phase (RP) C-18 column (Thermo Scientific Acclaim™ 4.6 × 250 mm, 5 μm). A gradient elution of 0–40% dichloromethane in methanol was started immediately after injection and was run for a total of 30 min. Absorbance was measured at 450 nm and 652 nm.

2.3. Cell disruption methods for protein extraction

Frozen *C. vulgaris* biomass with 24% (w/w) solids content was stored at −80 °C and thawed at room temperature for use. Three cell lysis methods were investigated and compared: high pressure homogenization (Emulsiflex C3, Avestin), ultra-sonication (Q55, Qsonica Sonicator with 1/8″ diameter probe, 20 kHz), and bead milling (GenoGrinder 2000, SpexSamplePrep). High-pressure homogenization was performed at 15000 psi for a total of 5 passes, bead milling was done using 0.5 mm glass beads at 1500 strokes/min, and sonication was performed using 30 s on/off intervals at 50% amplitude. Biomass was dispersed in RO water (reverse osmosis purified) at pH 7 with a wet, freeze-thawed biomass-to-water (w/v) ratio of 1:10. The concentration of the starting biomass suspension was 2×10^9 cells/mL. The varying volumetric capacity of cell lysis equipment determined the amount of processed cell suspension; 10 mL of suspension was used for ultra-sonication, 1 mL for bead beating and 100 mL for high-pressure homogenization. For cell lysis experiments at pH 12, cell suspensions were adjusted to pH 12 with 2 M NaOH. Initial temperature of cell suspension was 10 °C and the final temperature of cell lysate was maintained below 25 °C. Cell lysates were clarified by centrifugation for 10 min at 15000 ×*g*, and supernatants analyzed for total soluble protein. After high-pressure homogenization at pH 7 and 12, the remaining intact cells were counted using a hemocytometer (Bright Line, Hausser Scientific). A total of 3 replicates were carried out for each experiment.

2.4. Concentration of protein extracts by tangential flow ultrafiltration

After ethanol treatment, cells were lysed using high-pressure homogenization at pH 7 and pH 12. The cell lysates were clarified by centrifugation at 15000 × *g*, 4 °C for 15 min. Clarified algal extracts were fractionated and concentrated by two-stage tangential flow ultrafiltration (Fig. 1), using the Spectrum KMPi Tangential Flow Filtration system. A 300 kDa molecular weight cut off (MWCO) hollow fiber module (Spectrum Laboratories) was used in the first stage. The 300 kDa permeate was concentrated by a 3 kDa MWCO hollow fiber module in the second stage. Both modules were made of hydrophilic modified polyethersulfone (mPES) membrane that provides higher flux rates and low protein binding for better product yields. Protein retentates (300 kDa and 3 kDa) were first concentrated 4-fold and then diafiltered with three volumes of RO water adjusted to either pH 7 or 12. Membrane filtration was conducted at a constant transmembrane pressure (TMP) of 7 psi. The permeate flux (measured as $L/m^2/h$ or LMH), normalized average flux (permeate flux/TMP) and protein concentrations in both membrane retentates and permeates were recorded for all samples. Membrane filtration was performed at the recommended Spectrum Labs shear rate of 2000 s^{-1}. Membranes were regenerated by washing with 1% Tergazyme (Alconox Inc.) and 0.1 M NaOH. Control protein concentrate samples (from algal biomass not