COMPARATIVE ANALYSIS OF CELL DISRUPTION METHODS FOR *Spirulina* sp. TO OBTAIN HIGH CONCENTRATIONS OF PHYCOCYANIN

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ABSTRACT

The use of products derived from microalgae as additives in the food, pharmaceutical, and cosmetics industries has intensified due to their high nutritional value and bioactivity. Phycocyanin, a protein pigment extracted from cyanobacteria, is increasingly used as a food coloring and holds high added value due to its nutritional composition. The process of obtaining this pigment involves disrupting the cyanobacterium cell and extracting the pigment using a suitable buffer. Several disruption methods are described in papers and in industry, but there is limited information on the standardization and efficiency of these methods. The aim of this study was to evaluate two cell disruption methods for obtaining phycocyanin, using fresh and dried *Spirulina* sp. biomass. One method was physical, the classical freezing and thawing, while the other was enzymatic, utilizing hen lysozyme. In both cases, a 50 mM sodium phosphate buffer was utilized as the solvent. Following the cell disruption procedure, the extracted pigment was quantified, resulting in a maximum concentration of 16.5 mg mL^{-1} and yield of 65.6 mg of phycocyanin per gram of dry biomass, obtained using the successive enzymatic method extraction.

Keywords: Phycocyanin. Cyanobacteria. Extraction. Pigment. Cell disruption.

1 INTRODUCTION

Cyanobacteria are a group of microorganisms characterized by the production of phycobiliproteins, protein pigments that are part of their light absorption system for photosynthesis. *Spirulina* (*Arthrospira*) sp. is a cyanobacterium genus that usually forms helical and multicellular filaments, which can be around 50 to 300µm long and 10µm in diameter, on average (1,2). It is used as a food and nutraceutical supplement in human diets and has also been explored for its potential to produce phycocyanin, a blue-colored phycobiliprotein used commercially as a natural color additive in food, cosmetics, and pharmaceuticals (3,4). Since it is an intracellular product, a cell disruption process is necessary to extract the pigment. Cell disruption techniques are categorized as mechanical/physical (e.g., ball mills, ultrasound) or non-mechanical (e.g., chemical, enzymatic methods). Generally, non-mechanical and less invasive methods, such as enzymatic treatment, are recommended for recovering more sensitive products with a high degree of purity, especially for proteins (5–8).

The most commonly used cell disruption methods to obtain phycocyanin include ultrasonication, the use of enzymes, and freezing and thawing. In all cases, specific buffers like sodium or potassium phosphate with a pH between 6 and 7 are recommended to maintain protein stability after extraction, as the leaked cellular content may alter the medium pH. The ultrasonication and enzymatic methods promote cell disruption and progressive dissolution of the cell wall, respectively. Freezing and thawing, on the other hand, are based on freezing the intracellular liquid, creating ice crystals, which can perforate the cell and leak the intracellular content upon thawing. The cycles are repeated, increasing the likelihood of cell breakage due to the crystal's action. Some authors suggest a higher pigment yield after 3 to 4 cycles of freezing and thawing, considering increased leakage of proteins and other contaminating molecules after more than 5 cycles (5,8,9). The aim of this study was to compare two methods of cell disruption, enzymatic and freezing and thawing, using both fresh and dried *Spirulina* sp. biomass, and to evaluate a method of successive enzymatic extraction in dry biomass. The objective was to evaluate and determine which method yields the highest extraction of pigment.

2 MATERIAL & METHODS

The cultures of Spirulina sp. LEB 52 were maintained in Zarrouk medium (1966), under standardized conditions, illuminated between 44 ±3 µmol photons m^{-2} s^{-1} by 10W tubular LED lamps, with a 12-hour photoperiod. The temperature was maintained at 30 ± 3°C, along with constant aeration using air pumps at an approximate flow rate of 0.9 vvm (volume of air per volume of medium per minute) in each culture. After 15 days of cultivation, the biomass was separated by vacuum filtration, using a glass fiber filter membrane with an average pore size of 0.7 µm. The retained biomass was then collected by scraping, and a portion of it was dried in an oven at 40°C for 24 hours.

For all evaluated methodologies, the recovered biomass was resuspended in 0.5 M sodium phosphate buffer at pH 6.8, at a ratio of 1:4 (m/v) (5). The first assay involved the action of lysozyme at a concentration of 0.4 mg mL^{-1}. The samples were then incubated for 24 hours in an oven at 35 ± 2°C as this is the optimum temperature for lysozyme activity. The second disruption method assessed was freezing and thawing, carried out through 4 cycles for each step, with freezing at -18 ± 2°C and thawing in an oven at 35 ± 2°C. In both cases, samples were then centrifuged at 2260 xg for 15 minutes. Phycocyanin quantification has been made

by spectrophotometer reading at wavelengths 620 and 652, and calculated using the method described by Bennett and Bogorad (1971) (10,11).

A test utilizing enzymatic cell disruption methodology was conducted on dry biomass, employing successive extractions. Following the initial extraction, the extract was retrieved and set aside. Subsequently, Sodium phosphate buffer solution was added to the residual biomass in the same volume as the extract recovered in the first extraction, and then homogenized. Another centrifugation at 2260 xg for 15 minutes was performed, and the new supernatant was recovered and combined with the supernatant from the initial extraction. This process was repeated twice more, in a total of 3 successive extractions. The graphs were plotted using unweighted means, sample standard deviation, and 95% confidence interval by OriginPro 8.5® software.

3 RESULTS & DISCUSSION

Following the disruption processes, the samples were examined under the microscope to visualize the disruption cells. Figures 1 and 2 illustrate the contrast between the filaments broken by freezing and thawing, from dry and fresh biomass (Figures 1a and 1b, respectively), and by enzymatic action on dry and fresh biomass (Figures 2a and 2b, respectively). The images demonstrate a noticeable difference in the cell fragments resulting from enzymatic disruption in dry and fresh biomass. The cell disruption in the dry biomass produced smaller cell fragments when subjected to enzymatic treatment, whereas with the fresh biomass, the trichomes remained relatively intact. Similarly, in both the dry and fresh biomass subjected to freezing and thawing, the trichomes remained practically intact in both cases.

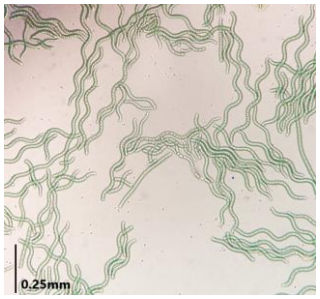

Figure 1a Cell disruption by freezing/thawing of fresh biomass.

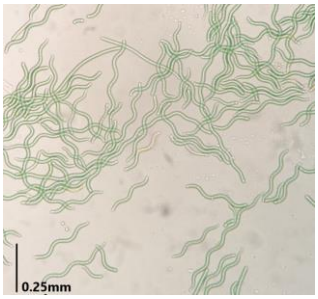

Figure 1b Cell disruption by freezing/thawing of dry biomass.

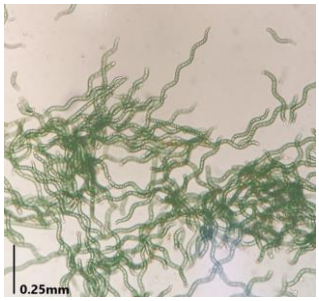

Figure 2a Enzymatic cell disruption of fresh biomass.

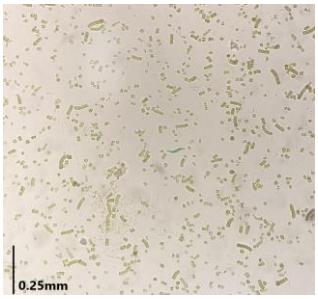

Figure 2b Enzymatic cell disruption of dry biomass.

The results of the readings to determine the phycocyanin concentration were calculated using the method described by Bennett and Bogorad (1971), with PC (phycocyanin) in mg mL^{-1}:

$$PC = \frac{(OD_{620} - 0{,}474.(OD_{652}))}{5{,}34}$$

In terms of the phycocyanin yield obtained in each procedure, figure 3 depicts the concentrations in mg of phycocyanin per g of biomass, showing that enzymatic disruption in dry biomass produced the highest amount of pigment. The tests were carried out using 90 mg of biomass. In the freezing and thawing method, both with fresh and dry biomass, yielded the lowest amounts, approximately 10.03 ± 1.04 mg g^{-1} (of fresh biomass) and 10.20 ± 0.69 mg g^{-1} (of dry biomass). From enzymatic disruption a yield of 29.17 ± 1.40 mg g^{-1} of phycocyanin was obtained with fresh biomass. A high concentration of phycocyanin was achieved through enzymatic disruption in dry biomass, with a yield of 41.89 ± 5.49 mg g^{-1}.

It was found that, despite the high concentrations of phycocyanin obtained through the enzymatic rupture of the cells in the dried biomass, part of the pigment was still retained in the rehydrated residual biomass. Therefore, a successive extraction method was implemented to achieve greater recovery of the residual phycocyanin, aiming to obtain a concentrated extract with high recovery and purity. The first extract recovered had an average concentration of 26.8 ± 5.8 mg mL^{-1} and yield of 29.9 ± 5.57 mg g^{-1} of phycocyanin per dry biomass, a recovery content of 2.97% (w/w), and purity of around 0.28. After 3 successive extractions (subsequently cell rupture), a phycocyanin concentration of 16.5 ± 3.07 mg mL^{-1} and yield of 65.6 ± 5.67 mg g^{-1} of dry biomass was obtained, with an average purity of 0.46 and a recovery rate of 6.57% (w/w). Therefore, to obtain a crude phycocyanin extract with high concentration, yield, and purity, it is possible to carry out successive extractions with the addition of sodium phosphate buffer at 50 mM and pH 7, in the same volume as the extract recovered after cell rupture.

The total reported concentration of phycocyanin present in *Spirulina* sp. biomass was around 17.5% (w/w) (12). Although the recovery of the phycocyanin was not complete, the addition of solvent in stages can facilitate the gradual release of the pigment from the cells, allowing for a concentrated extract to be obtained with greater utilization of the extracted pigment. This method also opens up the possibility of obtained biomass with considerable residual protein content, especially as a supplement for animal feed. In other studies, a maximum yield of 36.54 mg g^{-1} of *Spirulina maxima* dry biomass and purity of 1.4 were achieved using 0.1 M phosphate buffer in a 1:4 ratio (m/w), lysozyme at 0.4 mg mL^{-1}, and an 8-hour incubation period at 35 ± 2°C (5). A yield of 73.73 mg g^{-1} of phycocyanin, with a purity of 0.66, was achieved from the dried biomass of *Spirulina platensis* using a solvent ratio of 1:6 (m/w), 0.1 M phosphate buffer at pH 6.8, and 4 freezing and thawing cycles (-40 ± 2 °C / 27 ± 2 °C) (13). Although the

concentration of phycocyanin obtained was higher with 4 freezing and thawing cycles, the purity of the extract was lower compared to the purity achieved by enzymatic disruption.

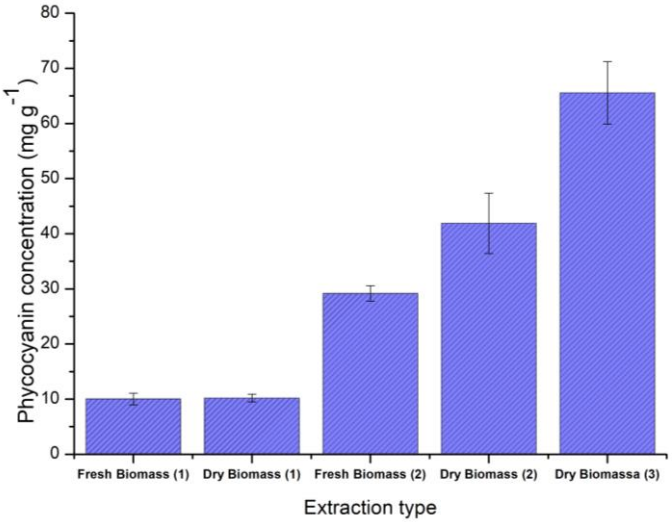

Figure 3 Graph relating the type of cell disruption and the concentration of phycocyanin, on different methods: (1) Freezing and thawing, (2) Enzymatic (3) Enzymatic (successive extraction)

These results indicate that enzymatic cell rupture was more efficient than freezing and thawing. There is considerable uncertainty regarding whether drying biomass promotes cell rupture. Some authors suggest that the drying process induces resistance to rupture (5,8). However, in the case of enzymatic rupture in dry biomass, it resulted in a higher yield of phycocyanin compared to wet biomass. One drawback of the drying process is the potential for protein denaturation, but under controlled conditions, up to 40°C, no adverse effects on pigment quality have been observed.

4 CONCLUSION

The enzymatic disruption of cells proved to be more efficient, particularly in dry biomass, when employing the successive extraction method. The advantage of utilizing dried biomass is that it is better preserved and stored compared to fresh biomass. Additionally, residual biomass can be used as fertilizer or animal feed, supporting the circular economy. Further experiments on the total pigment content of Spirulina biomass are needed.

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