MECHANICAL CELL DISRUPTION TO RECOVERY OF PHYCOCYANIN FROM *Phormidium autumnale*

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ABSTRACT

The cultures for microalgal biomass have a wide range of applications. Pigments extracted from these microorganisms can be used in various industries, including food, pharmaceutical and cosmetics. Phycocyanin, found only in cyanobacteria, is a protein of approximately 20 kDa and consists of two subunits α and β. In solution, it is found in a complex mixture of monomers, trimers, hexameters, and other oligomers. *Phormidium autumnale* is the cyanobacterium that has been gaining notoriety in research into wastewater treatments via heterotrophic metabolism and obtaining bioproducts, mainly because it is recognized as a robust species and tolerant to high concentrations of nutrients and temperature extremes. In this sense, the research aims to evaluate the cell disruption conditions for obtaining phycocyanin from *P. autumnale* biomass. The results indicate that the highest phycocyanin content (around 0.16 mg mL^{-1}) was obtained after 15 minutes of cell disruption at 55ºC and mixing at 1600 rpm, with higher productivity in terms of pigment around 8 µg mL^{-1} min^{-1}. The reduction of the filaments facilitated the extraction of the pigment and the mechanical disruption method presents potential for a cultivation system with this cyanobacterium due to its efficiency and ease of scaling-up.

Keywords: Cell disruption. Phycocyanin. *Phormidium*. Downstream processes. Cyanobacteria.

1 INTRODUCTION

Microalgae are considered as a sustainable alternative feedstock for food, feed, fuels, pigments and bioactive molecules. Both microalgae growth conditions and downstream steps must be developed to value the different biomass fractions for high yield and robustness of large-scale production[1,2]. Downstream unit operations are harvesting, cell disruption, phase separation, extraction and purification. As there are no general and standardized protocols for downstream steps, it is always necessary to evaluate separation methods depending on the type of cell and biomolecule. The costs of these steps using currently available technologies can represent more than 50% of global exploration costs. Mechanical cell disruption, in particular bead milling, is a promising technique for industrial application due to its efficiency of disruption and the commercially available at different scales [1].

Phormidium autumnale is a filamentous cyanobacterium that has been recognized in several studies. This microalga has filaments unbranched, and is capable of surviving for long periods in desert environments and freezing, becoming metabolically active after rehydration [3]. Furthermore, due to filamentous morphological, *P. autumnale* tends to facilitate the separation of biomass from the cultivation media. This characteristic is fundamental considering that biomass recovery is a main technological bottleneck in large-scale microalgae production. Studies have demonstrated the potential of this cyanobacteria by heterotrophic growth, in bioremediation processes from effluents with high organic and nutritional loads, in addition to generating biomass with commercial value [4,5,6]. However, there are no reports of research in the literature that explore the potential for obtaining phycocyanin by this species of cyanobacteria, nor is it associated with the use of byproducts in systems called microalgal “biorefineries” [6].

There is a growing demand for naturally produced pigments due to food safety concerns due to the harmful elements present in synthetic sources. Among the various sources, microalgae are great producers of natural pigments due to their ability to synthesize them in high concentrations, and can be cultivated in brackish waters or wastewater from the consumption of nutrients, reducing dependence on chemical products [7]. The classes of pigments found in microalgae are called chlorophylls, carotenoids and phycobiliproteins. The most commercially valuable are carotenoids and phycobiliproteins, which are widely used in cosmetics, food additives, biocompounds, pharmaceutical industries and food colorings [8].

Cyanobacteria have phycobiliproteins, which make up granular structures called phycobilisomes and are found in the outer membranes of the thylakoids. There are four phycobiliproteins in cyanobacteria: phycocyanin (blue color), phycoerythrin (red color), allophycocyanin (blue color) and phycoerythrocyanin (red color). These pigments are generally produced in autotrophic cultures; however, some species also perform in heterotrophic growth [9]. Phycobilisomes of cyanobacteria are located in photosynthesis reaction centers and perform the function of transferring energy to photosystems. From there, phycobiliproteins increase the spectrum of light capture by photosynthesis or act as a nitrogen reserve. If a situation arises where cyanobacteria suffer from a lack of nitrogen, the proteins that make up phycobiliproteins are degraded and phycobilisomes are eliminated, resulting in the release of nitrogen for the most essential metabolic processes. And if nitrogen is introduced into the medium, phycobilisomes can be reconstituted. The concentration of phycobiliproteins in cyanobacteria can reach up to 40% of the total soluble protein content. It is worth mentioning that the presence of a carbon source in the medium in photoautotrophic cultivation can lead to the inhibition of other proteins, favoring the synthesis of phycobiliproteins. As the carbon/nitrogen ratio of the culture

medium is fundamental in heterotrophic growth, it appears to play an important role in the concentration of these proteins. Specifically, about phycocyanin, this pigment has its main applications as a natural dye, although it is also used in the pharmaceutical sector as an antioxidant, anti-inflammatory and neuroprotective agent. Phycocyanin has been used as a food pigment in gums, candies, soft drinks, and dairy products. Due to its spectral properties and high fluorescence yield, it is also widely used as a fluorescent agent. Phycocyanin is stable at pH 5.5 - 6 and at temperatures below 47°C, with degradation of the pigment due to denaturation in conditions outside these ranges [10]. In this context, it is essential to evaluate the conditions of the recovery and purification process of phycocyanin extracted from cyanobacteria, with cell disruption being one of the most critical steps.

2 MATERIAL & METHODS

Cyanobacterium *Phormidium autumnale* isolated from the Cuatro Cienegas Desert, Mexico, was kindly provided by the Federal University of Santa Maria (UFSM), maintained and propagated in standard medium [11] at 25ºC and 12-hour light-dark photoperiod.

For the recovery of phycocyanin, the identification of the best conditions for cell disruption of this cyanobacteria in disruptor bead mill TE-099 and set up experiments for establish the experimental conditions of mixing, temperature and disruption time with glass spheres, as bead mills. 5 mL of a concentrated suspension (around 18 mg mL^{-1}) containing the *Phormidium antumnale* inoculum in BG11 medium was evaluated under various cell disruption conditions in bead mills. After the tests, the rupture efficiency was evaluated based on the phycocyanin content extracted with phosphate buffer 0,2 M and measured by spectrophotometry [12]. The total protein content was estimated after cell disruption by absorbances at 260 and 280 nm [13]. Furthermore, the cell microalgal disruption was analyzed using microscopic images using the Leica LAS EZ Software to measure and compare the filaments.

3 RESULTS & DISCUSSION

Cell disruption of the *Phormidium antumnale* inoculum suspension was assessed by the amount of phycocyanin released into the medium. Therefore, Figure 1a presents a phycocyanin profile measured in the liquid fraction after cell disruption at 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70ºC. Despite what is reported in the literature for *Spirulina´*s phycocyanin [10], that is, the use of temperatures below 47ºC to avoid denaturation, in the case of our results, the temperature that benefited the release of the pigment was 55ºC (0.16 mg mL^{-1}). These pigment contents are similar to those reported in the literature [14]. The thermal effect intensifies at higher temperatures, as the results indicate for 60, 65 and 70ºC. These results may indicate a slightly superior thermal stability for phycocyanin from *P. antumnale*. This highest phycocyanin value was around 0.98% (mass) of the total amount of biomass and, as a comparison, this sample presented 48.4% of total cellular proteins. Furthermore, Figure 1b indicates at 55ºC, mixing at 1600 rpm for 15 min the greater release than for 25 min (phycocyanin productivity around 8 µg mL^{-1} min^{-1}). This suggests that thermal stability can be maintained at this temperature as long as the downstream process occurs in a shorter time interval.

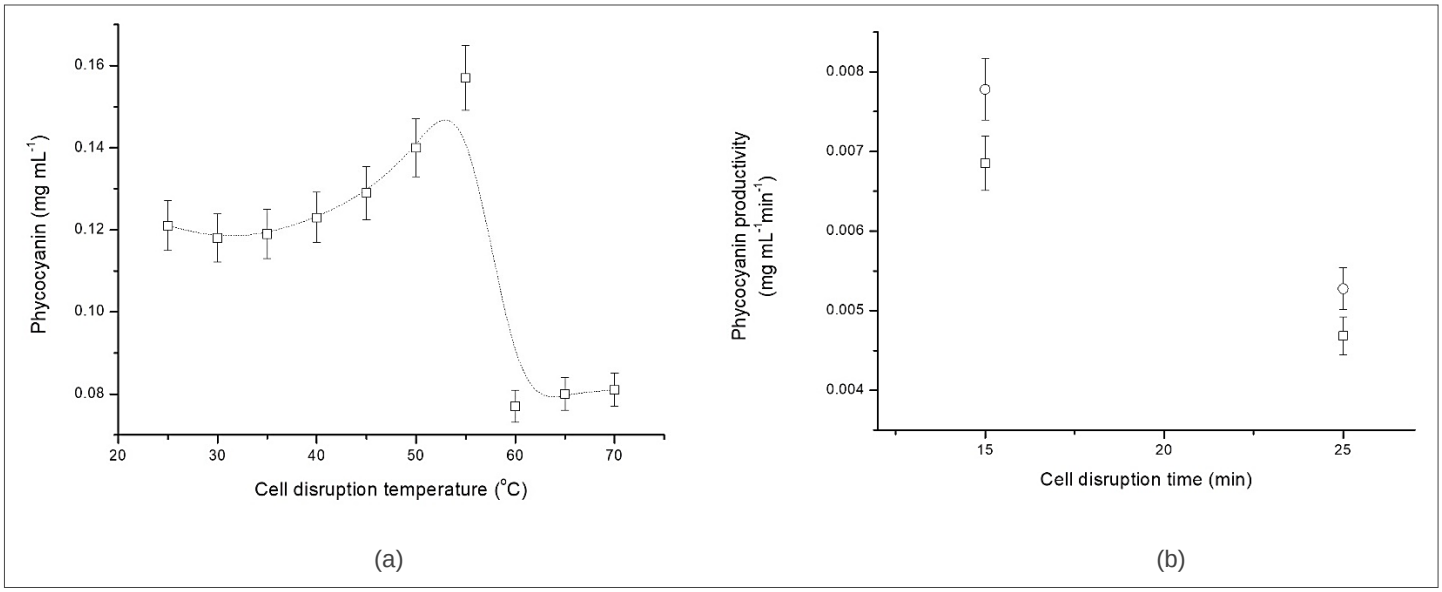
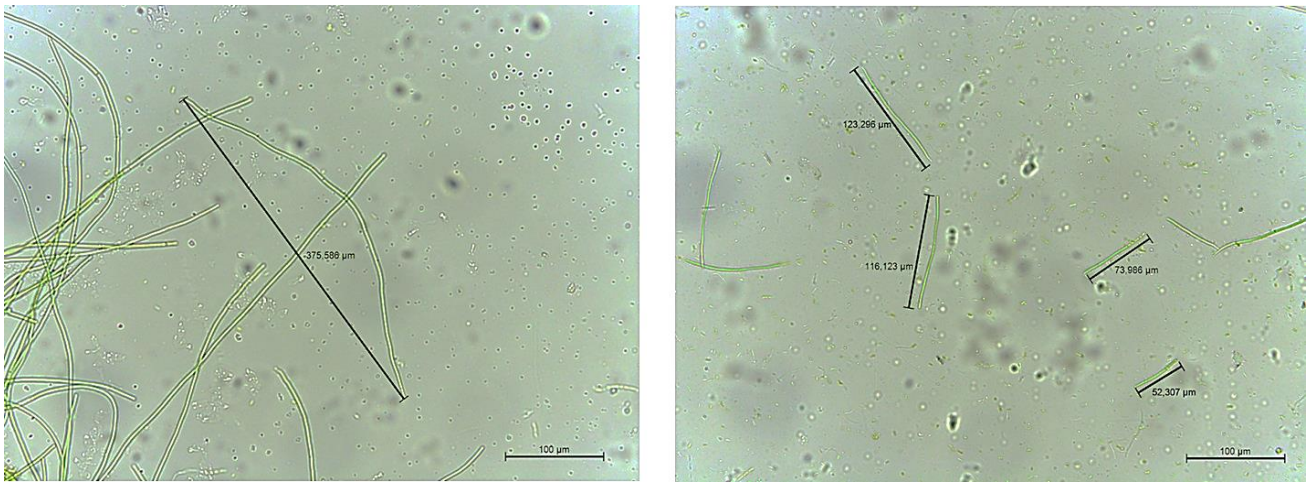


Figure 1 Phycocyanin content of *Phormidium autumnale* extracted at different temperatures (a) and phycocyanin productivity at 55ºC for 1200 (□) and 1600 rpm (○)

Comparison of the rupture behavior of different microalgae species reveals that rupture kinetics in ball milling are influenced by cell size, composition and stiffness of the cell membrane and cell wall [1]. Microalgae cells are normally distributed in different size classes corresponding to different growth stages. Cells in the growth phase tend to accumulate the first pigments and proteins and then energy storage metabolites such as lipids and starch until the division step. In fact, the resilient structure of the cell wall of some species of microalgae acts as a barrier to industrial exploitation of microalgae. The composition of the cell wall is dependent on the species and can be used as an identification marker for its taxonomy [2].

Figure 2 presents the micrographs of *P. autumnale* suspensions before and after cell disruption under optimized conditions (55ºC, 1600 rpm for 15 min. The illustrations indicate that cell rupture *sensu stricto* does not appear to occur, but the reduction of the

filaments by approximately three to six times the original value, allowing technologically viable phycocyanin levels and productivity as shown in Figure 1. The images in Figure 2 indicate that the reduction of *P. autumnale* filaments appears to have facilitated the extraction of phycocyanin and the mechanical rupture method presents potential for a cultivation system with this cyanobacterium due to its efficiency of bead mills and ease of scaling-up.



(a) (b)

Figure 2 Micrographs of *Phormidium autumnale* filaments before (a) and after (b) the cell disruption process (55ºC, 1600 rpm, 15 min)

4 CONCLUSION

The mechanical cell disruption presents a potential for filament reduction and phyconicanin release from *Phormidium autumnale* suspensions with selected optimal conditions of 55ºC, 1600 rpm for 15 minutes. The results indicate a preliminary potential use of a cyanobacteria little reported in the literature in obtaining biopigments, and other conditions and analyzes will indicate the viability of the proposed mechanical method of cell disruption

REFERENCES

[1] ZINKONÉ, T.R., GIFUNI, I., LAVENANT, L., PRUVOST, J., MARCHAL, L. 2018. Bead milling disruption kinetics of microalgae: Process modeling, optimization and application to biomolecules recovery from *Chlorella sorokiniana*. Bioresource Technology. v 267. 458-465.

[2] ALHATTAB, M., KERMANSHAHI-POUR, A., BROOKS, M.SL. 2019. Microalgae disruption techniques for product recovery: influence of cell wall composition. Journal of Applied Phycology. v. 31. 61–88.

[3] STRUNECKÝ, Otakar, ELSTER, Josef, KOMÁREK, Jiří. 2012. Molecular clock evidence for survival of Antarctic cyanobacteria (*Oscillatoriales, Phormidium autumnale*) from Paleozoic times. FEMS Microbiology Ecology. v. 82 (2). 482–490.

[4] FRANCISCO, E.C., FRANCO, T.T., ZEPKA, L.Q., JACOB-LOPES, E. 2015. From waste-to-energy: The process integration and intensification for bulk oil and biodiesel production by microalgae. Journal of Environmental Chemical Engineering. v. 3 (1). 482–487.

[5] FRANCISCO, E. C., JACOB-LOPES, E., VIEIRA, K.R. 2019. Nitrogen Starvation of Assessment in the Production of Single Cell Oils and Biodiesel Quality in Heterotrophic Cultures of Cyanobacteria *Phormidium autumnale*. Journal of Advanced Chemical Engineering. v. 9 (192). 1-7.

[6] VISENTIN, T.G., GUIMARÃES, B.M., BASTOS, R.G. 2024. Effects of temperature, pH, and C/N ratio of sugarcane wastewater processing (vinasse) on *Phormidium autumnale* heterotrophic cultivation. Algal Research-Biomass Biofuels and Bioproducts. 77. 103349.

[7] KOYANDE, A. K., CHEW, K.W., RAMBABU, K. 2019. Microalgae: A potential alternative to health supplementation for humans. Food Science and Human Wellness. v. 8 (1). 16–24, 2019.

[8] MORANÇAIS, M., MOUGET, J., DUMAY, J. 2018. Proteins and Pigments. In: Microalgae in Health and Disease Prevention. Elsevier Inc. 145–175.

[9] MORAIS, D.V.; BASTOS, R.G. 2019. Phycocyanin Production by *Aphanothece microscopica* Nägeli in Synthetic Medium Supplemented with Sugarcane Vinasse. Applied Biochemistry and Biotechnology. 187. 129-139.

[10] CHAIKLAHAN, R., CHIRASUWAN, N., BUNNAG, B. 2012. Stability of phycocyanin extracted from *Spirulina sp:* influence of temperature, pH and preservatives. Process Biochemistry. v.47.659–664.

[11] RIPPKA, R., DERUELLES, J., WATERBURY, J.B., HERDMAN, M., STANIER, R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology. 111. 1-61.

[12] BENNETT, A., BOGOBAD, L. 1973. Complementary chromatic adaptation in a filamentous blue-green alga. Journal of Cell Biology, v. 58 (2). 419–435.

[13] WARBURG, O., CHRISTIAN, W. 1942. Isolation and Crystallization of Enolase. Biochemische Zeitschrift. 310. 384-421.

[14] NAIR, M.S., RAJARATHINAM, R., VELMURUGAN, S., SAM, K.J., ARUMUGAM, L. 2023. Chemical Enginering Technoogy. 46 (2). 279–289.

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