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C-PHYCOCYANIN EXTRACTION FROM *Spirulina platensis* WET BIOMASS

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Abstract - C-Phycocyanin is a natural blue dye used in food and pharmaceutical industry. In the present study, a simple and efficient method to extract C-phycocyanin from *Spirulina platensis* wet biomass is reported. The extractions were carried out using six different methods, including chemical (organic and inorganic acid treatment), physical (freezing and thawing, sonication, homogenization) and enzymatic (lysozyme treatment) methods. The extraction using ultrasonic bath in the presence of glass pearls in the biomass proved to be the most efficient method, 56% higher than using freezing and thawing (the method most frequently used), and presented a extraction yield of 43.75 $mg.g^{-1}$ and a C-phycocyanin concentration of 0.21 $mg.mL^{-1}$.
Keywords: *Spirulina*; Wet biomass; Extraction; C-Phycocyanin.

INTRODUCTION

Phycobiliproteins are accessory photosynthetic pigments that participate in an extremely efficient energy transfer chain in photosynthesis (Róman et al., 2002), responsible for about 50% of light capitation from cyanobacteria and red algae (Williams et al., 1980). These proteins are assembled into complex structures called phycobilisomes that are attached to the outer surface of the thylakoid membranes (Yu and Glazer, 1982), because chlorophyll absorbs light energy only in a region of the solar spectrum. The excitation energy is posteriorly transferred to the reaction centers placed in the photosynthetic membranes, causing the photosynthetic process. The three main groups of phycobiliprotein are phycocyanins, allophycocyanins and phycoerythrins (Bennett and Bogorad, 1973).

C-phycocyanin (C-PC) could be extracted from cyanobacteria such as *Spirulina platensis,* which has been widely used in commercial applications in the food and cosmetic industry as a natural blue dye. Recent studies have demonstrated the hepatoprotective (Romay et al., 2003), anti-inflammatory (Romay et al., 2003; Reddy et al., 2003; Bhat and Madyastha, 2001) and antioxidant (Estrada et al., 2001; Bhat and Madyastha, 2000) properties of C-PC.

Each microorganism has particular characteristics refering to the location of intracellularly produced proteins, meaning that the molecule of interest might be located in the cytoplasm, periplasm or even be stored in some cellular organelle, such as in the mitochondria. Hence, the extraction protocol could vary according to the desired protein.

In general, the extraction method is the key for maximum recovery of phycobiliproteins in the natural state from algae (Niu et al., 2006). The extraction of phycobiliproteins involves cell rupture and release of these proteins from within the cell. The cell walls of cryptophytes are easily disrupted, but those of cyanobacteria are extremely resistant (Siegelman and Kycia, 1978). Thus, the use of variations in the osmotic pressure, abrasive conditions, chemical treatment, freezing-thawing and

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sonication, amongst other disruption methods, are necessary. Mechanical cell disintegration methods are currently preferred for large-scale operations (Gacesa and Hubble, 1990; Kula and Schütte, 1987) since a complete disintegration of the biomass is desired, with high product and activity yields.

Some papers report C-PC extraction from cyanobacterium. Moraes et al. (2010) and Silveira et al. (2007) studied the optimization of extraction from dried biomass. The reported methods to extract C-PC from wet biomass include freezing and thawing (Sony et al., 2008; Sarada et al., 1999; Abalde et al., 1998; Bermejo et al., 2006), sonication (Bermejo et al., 2006; Abalde et al., 1998), homogenization (Sarada et al., 1999), lysozyme treatment (Bermejo et al., 2006; Stewart and Farmer, 1984) and acid treatment (Sarada et al., 1999; Bermejo et al., 2006).

Considering the uses of C-phycocyanin, the aim of this study was to evaluate some methods previously reported to extract C-phycocyanin and other bioproducts, in order to find the best procedure to extract C-phycocyanin from wet biomass of *Spirulina platensis* considering the extraction yield.

MATERIAL AND METHODS

Culture Conditions

Spirulina platensis LEB-52 strain was obtained from the Oceanographic Institute, University of Sao Paulo and kept in the Laboratory of Biochemical Engineering of the Federal University of the Rio Grande as LEB-52. The cyanobacterium was grown and maintained in an open outdoor photo-bioreactor, under uncontrolled conditions, in the south of Brazil. During these cultivations, the water was supplemented with 20% Zarrouk synthetic medium (Costa et al., 2000). This medium was also used to prepare the biomass for the initial inoculation of each batch. All the reagents used were of analytical grade, obtained from Merck (Darmstadt, Germany) and Synth (São Paulo, Brazil).

Extraction Procedures

Phycocyanin was extracted from the wet biomass of *Spirulina* by using the following methods, totalizing 6 methods and 11 assays.

1. Homogenization of cells in a mortar and pestle: Frozen biomass was homogenized in a mortar and pestle in the presence of diatomaceous earth, in the proportion of 5:1 (g biomass: g diatomaceous earth).

2. Freezing and thawing: Biomass was subjected to freezing and thawing for 24 or 48 hours. In the second case (48 hrs), the freezing and thawing procedure was repeated twice, with 24 hrs intervals.

3. Inorganic acid extraction: The wet biomass was treated with different concentrations of hydrochloric acid (2, 4, 6, 8 and 12 M) in the proportion 5:1 (g biomass: mL inorganic acid) and then left for 24 hours at room temperature.

4. Organic acid extraction: The procedure was carried out in the same way used in inorganic acid extraction; in this case the wet biomass was treated with 1 M of acetic acid at room temperature.

5. Lysozyme treatment: Lysozyme was added to the biomass in 0.1 mM sodium phosphate buffer (pH 7.0) containing 100 mM sodium EDTA solution, to give a final concentration of 100 $\mu g.mL^{-1}$. The biomass was then incubated for 24 hours at room temperature, according to Boussiba and Richmond (1980).

6. Ultrasonic treatment: Biomass was added to an ultrasonic bath (50 kHz), with glass pearls in the proportion of 1:1.1 (g biomass: g glass pearls) during 40 minutes. (Medeiros et al., 2008).

After extraction, the samples were centrifuged and the supernatant used to verify the extraction yield.

Analytical Procedures

C-phycocyanin concentration: The C-phycocyanin concentration (CPC) in $mg.mL^{-1}$ was calculated from the optical densities at 652 and 620 nm, using Equation 1 (Bennett and Bogorad, 1973):

$$CPC = \frac{(OD_{620} - 0.474OD_{652})}{5.34} \quad (1)$$

Extraction yield: the extraction yield was calculated using Equation 2 (Silveira et al., 2007).

$$Yield = \frac{(CPC)V}{DB} \quad (2)$$

where Yield is the extraction yield of phycocyanin in mg of C-phycocyanin/dry biomass (g), *V* is the solvent volume (mL) and *DB* is the dry biomass (g).

Statistical Analysis

To validate the results reproducibility, each essay was done in triplicate. The results were treated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using *Statistica 6.0* (Statsoft Inc., 1998). All analyses were performed considering a level of 95% of confidence ($p<0.05$).

RESULTS AND DISCUSSION

The different methods used to extract C-phycocyanin from wet biomass showed the presence of this molecule in the supernatant. Comparison was done among different procedures for C-phycocyanin extraction with freshly harvested biomass and the data are presented in terms of mg phycocyanin per g dry weight of *Spirulina platensis,* named the extraction yield.

One of the most important requirements to obtain phycobiliproteins from cyanobacterium is optimizing the extraction and purification steps. Techniques used to extract and purify proteins are also applied to the phycobiliproteins, but a purification procedure that suits a phycobiliprotein from one organism may not be the best method of choice for a corresponding phycobiliprotein from another organism. The release of C-phycocyanin is directly related to cell rupture, but small algae such as *Spirulina* have resistant multilayered cell walls, making the extraction procedure difficult (Stewart and Farmer, 1984).

In this study, the yields vary between 0.57 $mg.g^{-1}$ (sonication) and 43.75 $mg.g^{-1}$ (sonication with glass pearls). The biomass treated with sonication (without glass pearls), lysozyme, 2 M HCl, 4 M HCl and 1 M acetic acid showed low extraction yield, under 2 $mg.g^{-1}$ and the supernatants were colorless due the low PC content. For this reason, this data were not considered and the following data evaluation considers only the homogenization in mortar and pestle (1), freezing and thawing for 24 h (2), freezing and thawing for 48 h (3), sonication in the presence of glass pearls (4), 6 M HCl (5), 8 M HCl (6) and 12 M HCl (7). The mean of these results, standard deviation and significant differences among the assays are presented in Figure 1, where the standard deviation is represented by the error bars and equal letters mean results do not differ statistically at a significance level of 5% (Tukey's Test).

The treatment using sonication with glass pearls and 12 M HCl were the ones that reached the highest values for yield, and are statistically equal. Sarada et al. (1999) report the microscopic disintegration of *S. platensis* cell when treated with HCl concentrations higher than 8 M, as we observed in this study. The disintegration promotes the release of C-phycocyanin from inside the cell into the solution. However, because of the low pH, C-phycocyanin precipitated as soon as extracted from the cells. This was not observed when the cells were treated by sonication with glass pearls.

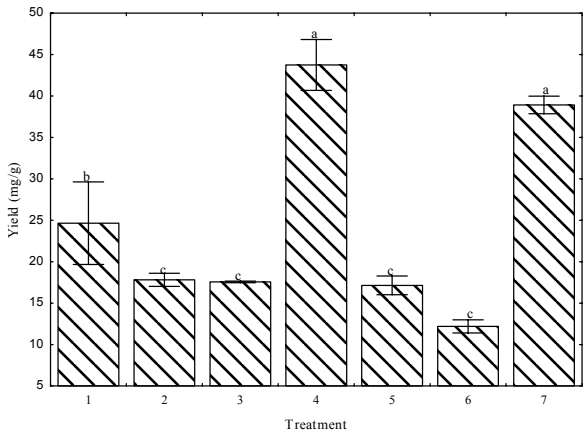

Figure 1: Extraction yield of C-phycocyanin from *S. platensis* cells using different treatments. 1- homogenization in mortar and pestle; 2- freezing and thawing for 24 h; 3- freezing and thawing for 48 h ; 4- sonication in the presence of glass pearls; 5- 6 M HCl; 6- 8 M HCl; 7- 12 M HCl. The standard deviation is represented by the error bars and equal letters means results do not differ statistically at the significance level of 5% (Tukey's Test).

In recent years, some papers reporting the C-phycocyanin extraction from wet biomass were published (Soni et al., 2008; Bermejo et al., 2006; Sarada et al., 1999; Abalde et al., 1998). In all of them, freezing and thawing was considered to be the best, since it showed higher C-phycocyanin content than the others methods studied by the authors. This method presents some advantages such as being simple, quick (10-12 h), reproducible, robust – since is independent of biomass quantity, free of corrosible material and without presenting significant losses of the biological capacity of the protein. Acker and McGann (2003) suggest that, when the cell is frozen, there is an inevitable intracellular ice formation, resulting in damage to the cell, promoting a better extraction of intracellular substances.

In this paper, we used a method that results in an extraction efficiency 57% higher than freezing and thawing, presenting only one inconvenience, the difficulty in scale up. Medeiros et al. (2008) suggest that the cavitation phenomenon associated with the abrasion effect generated by the glass pearls during the sonication favored the intracellular protein extraction.

A comprehensive technique must include quick and efficient disruption for a quantitative extraction and recovery of the released pigment. In the present study, a simple and efficient method to extract C-phycocyanin from wet biomass was obtained, and

presented an extraction yield of 43.75 $mg.g^{-1}$ and a C-phycocyanin concentration of 0.21 $mg.mL^{-1}$.

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NOMENCLATURE

CPC	C-phycocyanin concentration	$mg.mL^{-1}$
DB	dry biomass	g
OD_{620}	optical densities at 620 nm	
OD_{652}	optical densities at 652 nm	
V	solvent volume	mL
Yield	extraction yield of phycocyanin in mg of C-phycocyanin/dry biomass	g

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