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Technological process for cell disruption, extraction and encapsulation of astaxanthin from *Haematococcus pluvialis*

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ABSTRACT

In this work, the effectiveness of different enzymatic techniques for cell wall disruption of *Haematococcus pluvialis* for the extraction of carotenoids and subsequent encapsulation of extracts in the co-polymer poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) using the Solution Enhanced Dispersion by Supercritical fluids (SEDS) technique was investigated. Glucanex® performed best compared with Lyticase® and Driselase®. The conditions for enzymatic lysis using this enzyme preparation were established as a pH of 4.5, a temperature of 55 °C, an initial activity of β-1,3-glucanase of 0.6 U mL^{-1} and a reaction time of 30 min. Enzymatic lysis assisted by ultrasound without biomass freezing was shown to be a promising and simple one-step technique for cell wall disruption, reaching 83.90% extractability. In the co-precipitation experiments, the highest encapsulation efficiency (51.21%) was obtained when using a higher biomass to dichloromethane ratio (10 mg mL^{-1}) at the carotenoid extraction step and a lower pressure of precipitation (80 bar). In these conditions, spherical particles in the micrometer range (0.228 μm) were obtained.

1. Introduction

Astaxanthin is an orange pigment used in aviculture and aquaculture, especially in the production of feed for the captive breeding of fish and crustaceans, which mainly uses synthetic astaxanthin (Rodríguez-Sáiz et al., 2010). However, due to the concern about the use of chemical additives in foods, there is increasing interest in astaxanthin obtained from natural sources such as yeasts (Silva et al., 2012) and microalgae (Domínguez-Bocanegra et al., 2004; Rodríguez-Sáiz et al., 2010). In addition, this carotenoid exhibits strong antioxidant activity (Cipolatti et al., 2015).

Haematococcus pluvialis is a microalgae that forms non-motile resting cells, called aplanospores, in response to stress conditions. The thick cell wall of aplanospores is characterized by an exceptional resistance to mechanical and chemical agents, as well as a very low permeability. These are factors to be considered because they contribute to a reduction in the bioavailability of carotenoids when the cell is used intact. Thus, considering the presence of mannans in the cell wall of *H. pluvialis,* the use of lytic enzymes is a promising alternative for cell wall rupture and recovery of carotenoids produced by this microalgae (Hagen et al., 2002).

Several studies have explored different cell disruption methods to recover carotenoids from yeast biomass (Fonseca et al., 2011; Michelon et al., 2012; Monks et al., 2013). However, studies to elucidate and implement cell disruption techniques for extracting carotenoids from green alga *H. pluvialis* are scarce.

In contrast, when handling substances with high instability, such as astaxanthin, an encapsulation process is generally performed by forming a polymeric matrix or coating layer around the substances to protect their biological activity from environmental factors (Higuera-Ciapara et al., 2004). It has been reported that the thermal stability of astaxanthin was greatly improved upon encapsulation (Tachaprutinun et al., 2009).

Several researchers have demonstrated that supercritical fluids are useful as solvents or anti-solvents for particle precipitation, especially supercritical carbon dioxide, to modify material properties such as particle size, size distribution and morphology (Reverchon et al., 2003). The application of supercritical fluids as an alternative to the conventional process of encapsulation, such as spray-drying, coacervation, freeze-drying and

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interfacial polymerization, can overcome the drawbacks of these traditional techniques, such as the poor control of particle size and morphology, degradation of thermo-sensitive compounds, low encapsulation efficiency, and/or low yield (Franceschi et al., 2008). Another advantage of this technique to other industrial techniques is the efficient separation of the solvent and anti-solvent of the particles after precipitation, preventing organic solvent residues in the final product and permitting reutilization of solvent and anti-solvent (Rantakylä et al., 2002). Although there are some industrial-scale plants using this technology, already in operation, several academic investigations still are needed in order to improve the process parameters (Priamo et al., 2013). In recent years, we have reported the precipitation of bioactive compounds using the Solution Enhanced Dispersion by Supercritical fluids (SEDS) technique (Franceschi et al., 2008; Franceschi et al., 2009; Priamo et al., 2010; Boschetto et al., 2013).

In this context, the main goal of this work was to evaluate the enzymatic lysis for cell wall disruption to obtain an extract from *H. pluvialis* biomass to be used in the co-precipitation of astaxanthin in poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) by the SEDS technique, with supercritical carbon dioxide as the anti-solvent and dichloromethane as the organic solvent.

2. Experimental

2.1. Materials

The enzyme preparations tested were Driselase® (Novozymes S.A., Tokyo, Japan), which contained β-1,3-glucanase and xylanase obtained from the fungus *Basidiomycetes* sp., Glucanex® (Novozymes S.A., Bagsvaerd, Denmark), which contained β-1,3-glucanase and protease obtained from the fungus *Trichoderma harzianum*, and Lyticase® (Novozymes S.A., Franklinton, United States), which contained β-1,3-glucanase and protease from the bacteria *Arthrobacter luteus*.

The dichloromethane utilized (DCM, 99.5%) was purchased from Merck (Darmstadt, Germany). The carbon dioxide (99.9% in liquid phase) was supplied by White Martins S.A. (Florianópolis, Brazil). PHBV, with a molar mass of 196,000 and a polydispersity index of 1.85 (specified and measured for the supplier by GPC using a calibration curve obtained from polystyrene standards), was supplied by PHB Industrial S.A. (Passo Fundo, Brazil). All materials were used as received.

2.2. Microorganism

The microorganism used in this work was the microalgae *H. pluvialis*, provided by the Elisabeth Adair Microalgae Collection (Federal Fluminense University-CMEA/UFF, Rio de Janeiro, Brazil). The inoculum was cultivated in Bold Basal Medium (BBM) (Domínguez-Bocanegra et al., 2004) in 1 L photobioreactors containing 700 mL of medium and kept under a constant illuminance of 1.5 klx and a temperature of 25 °C (Domínguez-Bocanegra et al., 2004).

2.3. Cultivation

The cultivation of microalgae was performed using BBM and sodium acetate medium (Tripathi et al., 1999) with 0.30 L min^{-1} aeration at a temperature of 25 °C with an illuminance of 6 klx over 15 days. Initially, the pH was adjusted to 7.0, and the media were inoculated with 10% of the volume of the pre-culture. The biomass of *H. pluvialis* was collected from the medium by centrifugation at 1745 × *g* for 10 min. The sediment was washed twice with distilled water and dried (De Leo, Model B5CBE, Brazil) at 35 °C for 48 h (Moraes et al., 2010).

2.4. Lytic activity

To establish the experimental conditions for lysis, a 2_{IV}^{4-1} fractional factorial design was carried out for each enzyme preparation. The variables studied included the pH of the reaction medium (4.5, 6.5 and 8.5), temperature (35, 45 and 55 °C), initial activity of β-1,3-glucanase (0.2, 0.4 and 0.6 U mL^{-1}), and reaction time (30, 60 and 90 min). The relative lytic activity was evaluated as the response.

Relative lytic activity was determined using a reaction mixture containing 2 mL of cell suspension of the microalgae *H. pluvialis* (0.041 g of dried algae) with absorbance equal to 1.68 at 660 nm (Michelon et al., 2012) and 2 mL of enzyme solution diluted in the appropriate buffer. At the same time, a blank tube was prepared as a reference, in which only buffer was added. The relative lytic activity was calculated by modifying the method described by Obata et al. (1977) using Eq. (1):

$$\text{Relative lytic activity}\,(\%) = \left(\frac{\text{AR} - \text{AM}}{\text{AR}_\text{i}}\right) \times 100 \quad (1)$$

where AR is the absorbance of the reference (660 nm), AM is the absorbance of the reaction mixture at 660 nm, and AR_i is the initial absorbance of the reference at 660 nm.

2.5. Enzymatic lysis of the cell wall for carotenoid recovery

Cell disruption techniques were applied under the best conditions of lytic activity. In tubes containing suspended biomass with an absorbance value of 1.68 at 660 nm (Michelon et al., 2012), which corresponds to a value of 0.041 g of *H. pluvialis* (dry weight), buffer and enzyme extract were added to coincide with the initial activity of β-1,3-glucanase established in the experimental design. Both unfrozen and frozen (−18 °C for 48 h) (Moraes et al., 2010) biomasses were tested. The final mixture (4 mL) was incubated in an agitated bath at the temperature and time established by the experimental design. Centrifugation was performed at 1745 × *g* for 10 min, and the supernatant was discarded. The precipitate was washed twice using 4 mL of distilled water to eliminate buffer and enzyme waste. A volume of 6 mL of dichloromethane was added, and the supernatant containing the carotenoids was immediately subjected to centrifugation at 1745 × *g* for 10 min at room temperature.

The supernatants obtained after applying cell disruption techniques were transferred to amber flasks. Next, to assist in the formation of two phases, 10 mL of NaCl 20% (w/v) and 10 mL of dichloromethane were added, and the solvent phase was filtered through sodium sulfate. The concentration of total carotenoids (TC) was determined by absorbance reading of the filtrate at 474 nm and calculated using Eq. (2) (Chumpolkulwong et al., 1997):

$$\text{TC}(\mu\text{g g}^{-1}) = \frac{A \times V \times 10^4}{2{,}100 \times W} \quad (2)$$

where A is the absorbance value of the diluted extract at 474 nm, V (mL) is the volume of dichloromethane (filtrate), 2100 is the molar absorptivity coefficient for dichloromethane (Mendes-Pinto et al., 2004) and W (g) is the weight of the dried biomass.

The carotenoid extractability (CE) was calculated by Eq. (3) (Xiao et al., 2009):

$$\text{CE}(\%) = \frac{C}{C_\text{DMSO}} \times 100 \quad (3)$$

where C is the total carotenoids (μg g^{-1}) recovered from the cells using the disruption technique under study and C_{DMSO} is the total carotenoids (μg g^{-1}) recovered from the cells of *H. pluvialis* using