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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

cell disruption with dimethyl sulfoxide (standard method of disruption) and extraction with dichloromethane (Xiao et al., 2009).

The same procedure described above was performed using an ultrasonic bath with a frequency of 40 kHz instead of the agitated bath to evaluate the influence of ultrasonic waves on the enzymatic lysis of the cell wall.

Runs were performed in triplicate, and data were analyzed with Statistica 5.0 software (StatSoft Inc., United States). The results were evaluated by analysis of variance (ANOVA) and Tukey's test to verify the existence of significant differences among the techniques studied at the 95% confidence level.

2.6. Procedure and experimental apparatus for precipitation

The experimental apparatus used in this work for the precipitation of astaxanthin from *H. pluvialis* with PHBV was the same employed in previous works by this group. A more detailed description of the apparatus and the experimental procedure can be found elsewhere (Franceschi et al., 2008, 2009).

For the precipitation of compounds present in the extracts, some parameters were followed based on previous work (Franceschi et al., 2008, 2009): a PHBV concentration of 20 mg mL^{-1} in an organic solution, a temperature of 35 °C, a solution flow rate of 1 mL min^{-1} and an anti-solvent flow rate of 20 mL min^{-1}. The precipitation pressures were 80 and 100 bar. The extracts were obtained from *H. pluvialis* biomass using the technique established in the previous step. The biomass to dichloromethane ratios (B/DCM) in the extraction step were 5, 8 and 10 mg mL^{-1}.

2.7. Percentage of encapsulation and encapsulation efficiency

The two parameters were determined as described by Franceschi et al. (2008). A sample of astaxanthin in PHBV was weighed (between 94 and 138 mg) using an analytical balance with a precision of 0.0001 g (Shimadzu, Model AY220, Japan) and added to 20 mL of ethanol to remove non-encapsulated (free) astaxanthin. Ethanol was used as a washing solvent because astaxanthin has relatively low solubility in this solvent, making it possible to remove the non-encapsulated material without damaging the polymer wall. The precipitates in ethanol solutions were manually agitated for approximately 20 s at room temperature (~25 °C), and then, all samples were filtered using a membrane filter with a pore diameter of 0.22 μm (Millipore, Model FGLP, United States) in a vacuum pump (Prismatec, Model 131B, Brazil).

After filtration, the retained material was dried (De Leo, Model B5CBE, Brazil) at 35 °C for 24 h. After this, the dried powder was dissolved in 10 mL of dichloromethane, and the solution was analyzed in a spectrophotometer (Femto, Model 800XI, Brazil) with an absorbance at 455 nm for astaxanthin, which was determined by a standard curve for the solution. Comparing the results with a standard curve of absorbance vs. concentration of astaxanthin in the solvent, the percentage of encapsulation (PE) and encapsulation efficiency (EE) of astaxanthin in each run were evaluated by Eqs. (4) and (5) and (Priamo et al., 2010):

$$\mathrm{PE}(\%) = \frac{\text{mass of astaxanthin encapsulated}}{(\text{mass of astaxanthin} + \text{mass of PHBV})\text{ after filtration}} \times 100 \quad (4)$$

$$\mathrm{EE}(\%) = \frac{\text{mass of astaxanthin encapsulated}}{\text{theoretical loading percentage of astaxanthin encapsulated}} \times 100 \quad (5)$$

where the theoretical loading percentage of astaxanthin encapsulated is the ratio between the mass of astaxanthin and the total mass of astaxanthin and PHBV used in the precipitation experiments.

2.8. Particle characterization

The precipitated particles were analyzed by a scanning electron microscope (JEOL JSM-6390LV, Tokyo, Japan) to determine the shape and particle morphology. The particle size was determined using Meter Size software (version 1.1), using at least 600 particles for each experiment.

3. Results and discussion

3.1. Lytic activity

Table 1 shows the matrix of the 2_{IV}^{4-1} fractional factorial design for the relative lytic activity of the three commercial enzyme preparations tested, totaling eight runs plus three replications at the central point for each enzyme preparation. Relative lytic activity ranged from 2.83% in Run 4 (pH 8.5, 55 °C, 0.2 U mL^{-1} of β-1,3-glucanase and 30 min) to 17.73% in Run 7 (pH 4.5, 55 °C, 0.6 U mL^{-1} of β-1,3-glucanase and 30 min) when the commercial enzyme preparation Glucanex® was tested. Relative lytic activity ranged from 3.90% in Run 4 (pH 8.5, 55 °C, 0.2 U mL^{-1} of β-1,3-glucanase and 30 min) to 9.94% in Run 7 (pH 4.5, 55 °C, 0.6 U mL^{-1} of β-1,3-glucanase and 30 min) when the commercial enzyme preparation Lyticase® was tested. Relative lytic activity ranged from 2.16% in Run 1 (pH 4.5, 35 °C, 0.2 U mL^{-1} of β-1,3-glucanase and 30 min) to 7.98% in Run 5 (pH 4.5, 35 °C, 0.6 U mL^{-1} of β-1,3-glucanase and 90 min) when the commercial enzyme preparation Driselase® was tested.

The use of experimental design enables the study on influence of the levels of one variable on the response variable. Thus, the main effects of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the variable and the average of measurements at the low level (−1) (Rodrigues and Iemma, 2014).

According to Table 2, similar behavior was observed for all enzyme preparations. Only the initial activity of β-1,3-glucanase had a significant effect ($p < 0.05$) on the relative lytic activity. The change from level −1 (0.2 U mL^{-1}) to +1 (0.6 U mL^{-1}) of this variable caused an increase in relative lytic activity of 6.24%, 3.56% and 3.21% for Glucanex®, Lyticase® and Driselase®, respectively. Temperature, pH and reaction time had no significant effects ($p > 0.05$) on the relative lytic activity.

Therefore, by analyzing the effects, it was possible to confirm that the best conditions for cell wall lysis were a temperature of 55 °C, a pH of 4.5, a reaction time of 30 min and an initial β-1,3-glucanase activity of 0.6 U mL^{-1} using Glucanex® (Run 7, Table 1), resulting in a relative lytic activity of 17.73%, which was approximately twice the maximum values obtained with the other enzyme preparations. Similar results were observed by Michelon et al. (2012), reaching a relative lytic activity of 22% with the same enzyme acting on *Phaffia rhodozyma* cells. Fleuri and Sato (2010) used a β-glucanase of *Cellulosimicrobium cellulans* 191 in the cell wall lysis of different microorganisms. They observed a wide range of relative lytic activity (from 8.43% for *Candida glabrata* to 100% for *Pachysolen tannophillus*), related to the different composition of the cell wall and its organization.

According to Fleuri and Sato (2005), β-1,3-glucanase from *T. harzianum* has an optimum temperature of 55 °C and an optimum pH of 4.5, whereas the protease produced by this microorganism has an optimum temperature of 40 °C and an optimum pH of 8.0 (Marco and Felix, 2002). Thus, the selected conditions of pH and temperature for the lysis of *H. pluvialis* cells in this study corresponded to the optimum pH and temperature of the β-1,3-glucanase enzyme.

Table 1
Matrix of the 2_{IV}^{4-1} fractional factorial design with actual levels (coded levels) and responses for relative lytic activity.

Run	X1	X2	X3	X4	Y1	Y2	Y3
1	4.5 (−1)	35 (−1)	0.2 (−1)	30 (−1)	3.21	4.12	2.16
2	8.5 (1)	35 (−1)	0.2 (−1)	90 (1)	4.27	4.91	3.76
3	4.5 (−1)	55 (1)	0.2 (−1)	90 (1)	4.61	4.70	3.29
4	8.5 (1)	55 (1)	0.2 (−1)	30 (−1)	2.83	3.90	3.97
5	4.5 (−1)	35 (−1)	0.6 (1)	90 (1)	4.75	5.13	7.98
6	8.5 (1)	35 (−1)	0.6 (1)	30 (−1)	7.29	6.81	6.74
7	4.5 (−1)	55 (1)	0.6 (1)	30 (−1)	17.73	9.94	7.16
8	8.5 (1)	55 (1)	0.6 (1)	90 (1)	10.13	9.81	4.16
9	6.5 (0)	45 (0)	0.4 (0)	60 (0)	6.48	7.96	6.12
10	6.5 (0)	45 (0)	0.4 (0)	60 (0)	6.09	7.17	6.08
11	6.5 (0)	45 (0)	0.4 (0)	60 (0)	5.78	7.36	6.10

X1, initial pH of the reaction medium; X2, temperature (°C); X3, initial activity of β-1,3-glucanase (U mL^{-1}); X4, reaction time (min); Y1, relative lytic activity (%) for Glucanex®; Y2, relative lytic activity (%) for Lyticase®; Y3, relative lytic activity (%) for Driselase®.

Table 2
Main effects of variables for relative lytic activities of Glucanex®, Liticase® and Driselase® for the factorial fractional design 2_{IV}^{4-1}.

Variable	Effect	Standard error	$t(6)$	p-value
Glucanex®				
X1	−1.44	2.16	−0.67	0.53
X2	3.94	2.16	1.82	0.12
X3	6.24*	2.16*	2.88*	0.03*
X4	−1.82	2.16	−0.84	0.43
Lyticase®				
X1	0.43	1.10	0.39	0.71
X2	1.89	1.10	1.71	0.14
X3	3.56*	1.10*	3.22*	0.02*
X4	−0.01	1.10	−0.01	0.99
Driselase®				
X1	−0.49	1.00	−0.49	0.64
X2	−0.51	1.00	−0.51	0.63
X3	3.21*	1.00*	3.19*	0.02*
X4	−0.21	1.00	−0.21	0.84

X1, initial pH of the reaction medium; X2, temperature (°C); X3, initial activity of β-1,3-glucanase (U mL^{-1}); X4, reaction time (min).
* $p < 0.05$.

3.2. Enzymatic lysis of the cell wall with Glucanex®

Table 3 shows that the best values for total carotenoids and extractability were found using enzymatic lysis with Glucanex® assisted by ultrasound, with no significant differences ($p > 0.05$) between the use of freezing (Run 4) and the absence of freezing (Run 3).

Similar behavior was observed for enzymatic lysis with Glucanex® without using ultrasound. The previous freezing (Run 2) did not affect the extractability compared with the run without freezing (Run 1). However, a significant increase (∼2%) in total carotenoids was observed compared to Run 1. The use of enzymatic lysis with Glucanex® assisted by ultrasound using biomass without freezing (Run 3) caused an increase of 5% in total carotenoids compared with enzymatic lysis in the absence of ultrasound (Run 1). The same behavior was observed with biomass submitted to freezing, comparing Run 4 and Run 2. Therefore, it can be stated that the freezing of the biomass can be neglected, reducing energy costs.

Similar results were observed by Michelon et al. (2012) for *P. rhodozyma*, who noted an increase in extractability and total carotenoids approximately 12% using enzymatic lysis assisted by ultrasound.

The control experiments, in which the biomass was subjected to extraction without the step of cell disruption, showed total carotenoids of 503.63 and 532.59 μg g^{-1} and carotenoid extractability of 34.20 and 36.16% in the absence and presence of previous biomass freezing, respectively. This behavior occurred because the carotenoids produced by the microalgae *H. pluvialis* are enveloped by a tough cell wall, resulting in the need for an efficient cell wall disruption method.

Thus, enzymatic lysis assisted by ultrasound without freezing using Glucanex® was chosen as the most adequate technique to obtain a carotenoid extract from *H. pluvialis* biomass. With this cell disruption technique, it was possible to obtain total carotenoids of 1235.89 μg g^{-1} and an extractability of 83.90%.

The enzymatic lysis of microalgae has gained interest across many fields in the recovery of high-value intracellular compounds. Different cell disruption methods, such as enzymatic lysis by snailase, enzymatic lysis by lysozyme and enzymatic lysis by cellulase, have been applied in the recovery of lipids produced by *Chlorella vulgaris* (Zheng et al., 2011). Other intracellular high-value products from a variety of microalgae require cell disruption techniques that may be recovered (Mendes-Pinto et al., 2001; Grima et al., 2003; Cerón et al., 2008; Moraes et al., 2010). With the ability of the commercial enzyme preparation Glucanex® to act on the lysis of the cell wall of *H. pluvialis* demonstrated in this study, an expansion in the application fields of enzymatic lysis of microalgal biomass for the recovery of carotenoids and other bioproducts of commercial interest is expected.

3.3. Co-precipitation of astaxanthin in PHBV

Table 4 presents the results for the mean particle size (X), minimum particle size (X_{min}), maximum particle size (X_{max}), standard deviation (σ) and variation coefficient (VC) for the particle size, percentage of encapsulation and encapsulation efficiency obtained by the experimental precipitation runs of astaxanthin from *H. pluvialis* in PHBV. With regard to X, X_{min} and X_{max}, results are presented only for the three experimental conditions investigated in which the spherical form of the particles were found, as seen in Fig. 1 and revealed by SEM micrographs.

Comparing Run 2 and 3 (Table 4), it was possible to verify that the increase of the B/DCM ratio from 8 to 10 mg mL^{-1}, with an increase in the solute concentration, at constant pressure and temperature, led to a reduction in the mean particle size from 0.396 to 0.228 μm. This behavior was also observed by Priamo et al. (2010), who studied β-carotene precipitation by the SEDS technique. In contrast, when applying the Supercritical Anti-Solvent (SAS) process with fixed conditions of pressure, temperature and CO_2 molar fraction, an increase in solute concentration produces, in general, an increase in spherical microparticle diameter (Reverchon et al., 2008). In addition, the reduction in particle size was accompanied by a narrowing of the particle size distribution (Table 4).

Unfortunately, it was not possible to visualize clearly the effect of pressure on the particle size in this work. In relation to some other works (Franceschi et al., 2009; Priamo et al., 2010; Boschetto et al., 2013), in most cases, a pressure decrease causes an increase in particle size and an enlargement of the particle size distribution.

Table 3
Total carotenoids and extractability using different enzymatic cell disruption techniques with Glucanex®.

Run	Technique	Freezing	CE (%)[2]	TC ($\mu g\,g^{-1}$)[2]
1	Enzymatic lysis	Absence	79.34 ± 2.71 [b]	1,173.54 ± 6.25 [c]
2	Enzymatic lysis	Presence	81.34 ± 1.85 [a,b]	1,198.74 ± 3.13 [b]
3	Enzymatic lysis assisted by ultrasound	Absence	83.90 ± 1.84 [a,b]	1,235.89 ± 5.41 [a]
4	Enzymatic lysis assisted by Ultrasound	Presence	85.06 ± 2.05 [a]	1,253.26 ± 2.52 [a]
5	Control[1]	Absence	34.20 ± 1.65 [c]	503.63 ± 16.97 [e]
6	Control[1]	Presence	36.16 ± 1.24 [c]	532.59 ± 7.35 [d]

CE, carotenoid extractability; TC, total carotenoids.
[1] Control, carotenoid extractions performed in the absence of a cell disruption technique.
[2] Mean ± SD ($n = 3$); Different superscripts represent significant differences in the column ($p < 0.05$).

Table 4
Results of particle size, percentage of encapsulation and encapsulation efficiency of astaxanthin extract in PHBV by the SEDS technique.

Run[a]	P (bar)	B/DCM ($mg\,mL^{-1}$)	X (μm)	X_{min} (μm)	X_{max} (μm)	s (μm)	VC (%)	PE (%)	EE (%)
1	80	5	–	–	–	–	–	6.25	23.78
2	80	8	0.396	0.150	0.800	0.012	3.03	7.28	29.97
3	80	10	0.228	0.157	0.406	0.063	27.63	17.06	51.21
4	100	5	0.224	0.106	0.364	0.050	22.32	5.29	26.43
5	100	8	–	–	–	–	–	7.57	26.47
6	100	10	–	–	–	–	–	11.05	33.14

P, pressure; B/DCM, biomass to dichloromethane ratio; X, mean particle size; X_{min}, minimum particle size; X_{max}, maximum particle size; s, standard deviation; VC, variation coefficient; PE, percentage of encapsulation; and EE, encapsulation efficiency.
Experimental conditions: temperature at 35 °C; solution flow rate at 1 $mL\,min^{-1}$; anti-solvent flow rate at 40 $mL\,min^{-1}$; PHBV concentration into organic solution at 20 $mg\,mL^{-1}$.
[a] Experiments 1, 5 and 6 also formed particles, but the particles formed did not have a spherical shape, which made determination of particle diameter impossible.

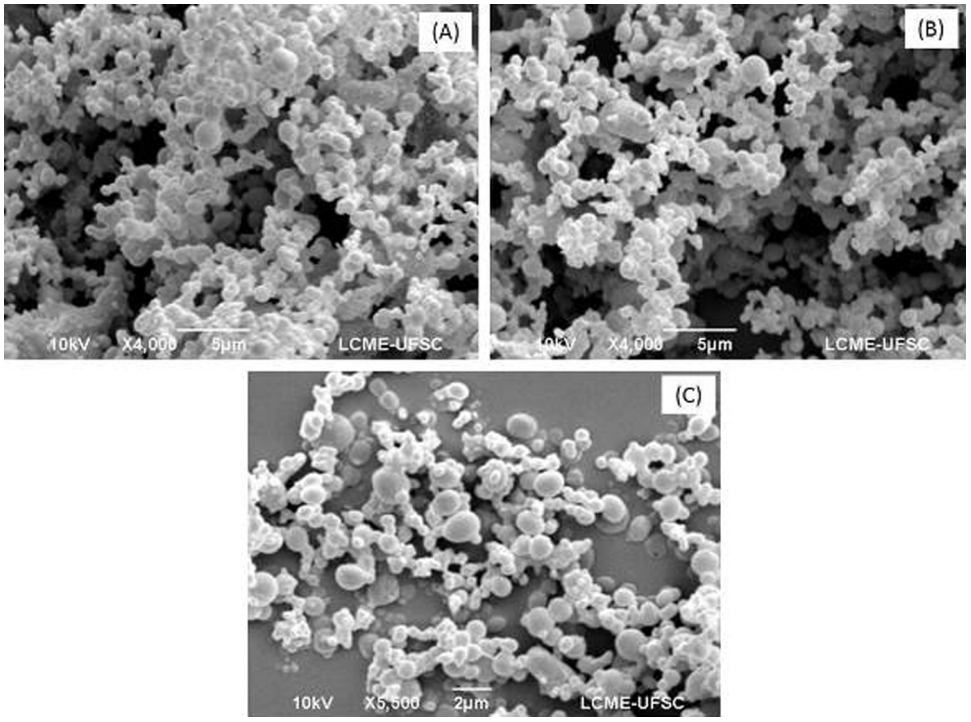

Fig. 1. SEM micrographs of co-precipitation of astaxanthin from *H. pluvialis* in PHBV. (A) 80 bar and 8 $mg\,mL^{-1}$, with 10,000 X magnification, (B) 80 bar and 10 $mg\,mL^{-1}$, with 4,000 X magnification, (C) 100 bar and 5 $mg\,mL^{-1}$, with 10,000 X magnification.

However, in some other cases, the influence of pressure produced the opposite effect.

Bustos-Garza et al. (2013), studying the thermal and pH stability of spray-dried encapsulated astaxanthin, found microcapsules with rounded outer surfaces with particle sizes from 1 to 10 μm. Higüera-Ciapara et al. (2004), studying the microencapsulation of astaxanthin in a chitosan matrix, found microcapsules with a non-homogeneous size and a diameter of 5–50 μm. Kittikaiwan et al. (2007), studying the encapsulation of cells from *H. pluvialis* in chitosan to protect the astaxanthin and its biological activity against oxidative environmental conditions, found capsules with a mean diameter of 0.43 cm and a total film thickness of approximately 100 μm. Hong et al. (2009), studying the precipitation of astaxanthin from *H. pluvialis* in supercritical fluid, found values of the particle size between 0.5 and 3 μm at experimental conditions of 200 bar and 35 °C. Tachaprutinun et al. (2009) reported similar results, a particle size of 0.312 μm, when studying the prevention of thermal degradation of com-

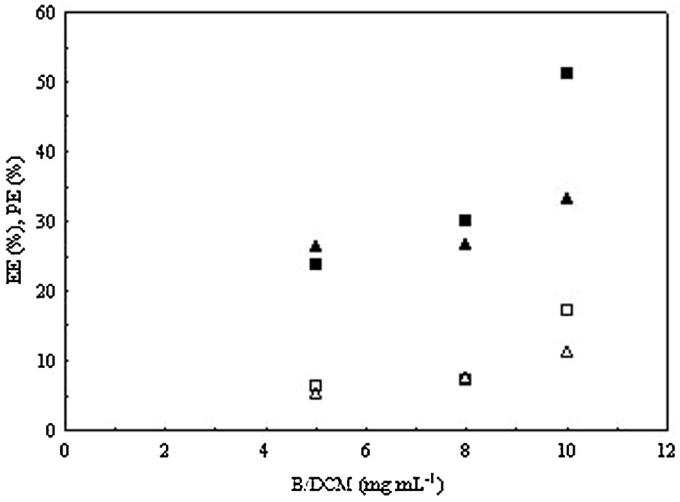

Fig. 2. Influence of the B/DCM ratio on the encapsulation efficiency (EE, closed symbols) and the percentage of encapsulation (PE, open symbol). ■,□ (80 bar); ▲,Δ (100 bar). Other experimental conditions are shown in Table 4.

mercial astaxanthin through encapsulation in the polymers poly(ethylene oxide)-4-methoxycinnamoylphthaloylchitosan, poly(vinyl-alcohol-co-vinyl-4-methoxycinnamate) and ethylcellulose.

With respect to the values of the variation coefficient, they were similar to the ones obtained by Priamo et al. (2010) (25–59%) when they studied the precipitation and encapsulation of β-carotene in PHBV using the same technique.

Concerning the results of the percentage of encapsulation and encapsulation efficiency for the conditions investigated in this work, it was observed that an increase in the B/DCM ratio in the extraction step, with a corresponding increase in astaxanthin concentration in the organic solution, resulted in enhancement of the percentage of encapsulation and encapsulation efficiency values for both pressures tested (80 and 100 bar) (Fig. 2). Similar behaviors were observed by Priamo et al. (2010) for β-carotene and Boschetto et al. (2013) for grape seed extract. The highest values were observed in Run 3 (80 bar pressure, 10 mg mL^{-1} B/DCM ratio), 17.06% (percentage of encapsulation) and 51.21% (encapsulation efficiency).

In the same way, in general, an increase in pressure led to a decrease in the encapsulation efficiency, noted by comparing Run 2 with Run 5 and Run 3 with Run 6 (Fig. 2). This is in agreement with studies of encapsulation of lavandin essential oil with biopolymers using the Particles from Gas-Saturated Solution (PGSS) technique (Varona et al., 2010). These authors argued that this can be explained by considering that the solubility of the extract in CO_2 increases when pressure is increased, becoming completely miscible with CO_2 at pressures above the mixture critical point.

The encapsulation efficiency values obtained in this work appear to be consistent with current scientific literature. Mezzomo et al. (2012) studied the encapsulation of astaxanthin extracted from pink shrimp residue and mentioned an encapsulation efficiency of 42 ± 2% with the use of the SAS technique under experimental conditions of 120 bar and 35 °C. Park et al. (2014), studying the effects of particle size on the physicochemical properties of optimized astaxanthin-rich *Xanthophyllomyces dendrorhous* using an air atomizing system, found microparticles ranged from 10 to 800 μm with a mean particle size of 210.26 μm and an entrapment efficiency with a variance between 68 and 79%. Santos et al. (2012) reported encapsulation of carotene and lycopene suspended in aqueous media that were produced by extraction of the organic solvent from the droplets of an oil-in-water emulsion with supercritical CO_2, with a final particle size of 0.344–0.366 μm and an encapsulation efficiency with a variance between 34 and 89%.

4. Conclusion

In this work, an efficient cell disruption technique for recovery of carotenoids from *H. pluvialis* was established. Enzymatic lysis assisted by ultrasound, a one-step procedure for carotenoid recovery, with no need for biomass freezing, produced an important increase in total carotenoid and extractability compared with the extraction without cell disruption. This biotechnological technique constitutes an alternative for commonly used techniques such as autoclaving, extraction by acids and bases, and others. In addition, the mild process conditions are most appropriate to recover carotenoids because the solvent used in the extraction is removed during the encapsulation process. In the co-precipitation experiments, the results showed that the highest encapsulation efficiency (51.21%) was obtained when using a higher B/DCM 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. Thus, the data obtained in this work support the importance of research and the use of enzymatic lysis and the SEDS technique for the development of new bioproducts from microalgae, with potential applications in the food and pharmaceutical industries.

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