EXTRACTION OF ASTAXANTHIN FROM THE YEAST *Phaffia rhodozyma* WITH SUPERCRITICAL CARBON DIOXIDE

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Supercritical carbon dioxide extraction of astaxanthin from *Phaffia rodozyma* was carried out, for several experimental conditions, using a semi-continuous apparatus. The yeast was previously freeze-dried and ground with a ball mill. The effects of the pressure (200 and 300 bar), temperature (40, 50 and 60 ºC) and supercritical solvent superficial velocities of 1.2 and 2.4 cm/min, as well as the use of ethanol, as co-solvent (10 %), on the extraction efficiency were assessed. Organic solvent extractions, using acetone, dimethyl sulfoxide (DMSO) and a mixture of methanol and dichloromethane, were also carried out on whole and ground cells. The extraction with acetone of astaxanthin from ground *Phaffia* led to the highest yield. Supercritical extraction was compared with the organic extraction, and the highest recovery (75%) was achieved at the pressure of 300 bar, the temperature of 40 ºC and using ethanol as co-solvent. The lowest recovery of supercritical extraction was obtained at the pressure of 200 bar and the temperature of 50 ºC, without co-solvent. Moreover, the extraction yield increased with the pressure at constant temperature. On the contrary, the increase of temperature at constant pressure led to a decrease of the yield at 200 bar and to a slight decrease at 300 bar. Furthermore, the yield decreased with the flow rate.

INTRODUCTION

Astaxanthin is the carotenoid responsible by the orange-red colour of many living organisms, widely distributed in the animal kingdom, in particular marine seafood, such as salmonid fishes (trouts and salmons) and crustaceans (shrimps and lobsters). The high conjugated carbon-carbon double bonds give to astaxanthin unique properties, such as colourant and antioxidant. Astaxanthin is the highest potent antioxidant (super vitamin E) and the association of carotenoid intake and the reducing risk of certain cancers and cardiovascular diseases [1, 2], as well as astherosclerosis, cataracts, macular degeneration [3] and enhancing of the immune resistances to viral, bacterial, fungal and parasitic infections. In fact, it has been used in the development of new attractive food industry products, with an important impact on new market niches (e.g. beverages, oil-in-water emulsions [4], soybean oil stability [5]), as well other nutraceutical and pharmaceutical applications. Moreover, the main application of astaxanthin is still in marine fish farming.

This high astaxanthin consumption all over the world and the tendency to replace artificial (synthetic) by natural one, has led to explore the capacity of producing astaxanthin in large scale, through microorganisms, namely the microalgæ *Chlorella vulgaris* [6], *Chlorella*

zofingiensis [7], *Haematococcus pluvialis* [8], the yeast *Phaffia rodozyma* [9] and the marine bacteria *Agrobacterium aurantiacum* [10].
For many applications in food and health areas, there is an increase of legal restrictions to the use of toxic organic solvents. So, it is important to obtain the carotenoids, for those purposes, free of such solvents. SFE with carbon dioxide is an appropriate technique for this goal, and there are several works in this field for the separation of carotenoids from plants [11] and microalgae [12, 13, 14, 15], of astaxanthin from crustaceans [16, 17], and from the red yeast *Phaffia rodozyma* [18].
The aim of this work is to study the potential of *Phaffia rhodozyma* as astaxanthin producer, using organic solvents and supercritical CO_2 to evaluate the extraction yields obtained from several experimental conditions.

MATERIALS AND METHODS

The yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) ATCC 24202 used in this study was cultivated and offered by the Laboratory of Enzimology and Technology of Fermentation, Paraná Federal University, Brasil.
The freeze dried cells of the yeast *Phaffia rhodozyma* were extracted by organic solvents: acetone, a mixture of dichloromethane and methanol (50:50 v/v) (DiCl:MeOH) and dimethyl sulfoxide (DMSO), on whole and ground cells (obtained with a ball mill), and by SFE with CO_2 at different experimental conditions.
Approximately 100 mg of biomass were used for carotenoids extraction tested by four different methods:
1) Acetone method: 10 ml acetone was added to the dry biomass, vigorously homogenized (vortex for 1 min, twice). The mixture was centrifuged and the pellet was re-extracted with further 10 ml portions of acetone until complete extraction, which was evaluated by the absence of colour in the solvent [6].
2) DiCl:MeOH: 10 ml dichlorometane:methanol 50:50 (v/v) were added to the dry yeast using the same method as mentioned above.
3) DMSO: after the addition of 2 ml DMSO solvent, dry yeast samples were stored for 30 min. Then, 6ml of acetone were added, homogenized and centrifuged (3.500 rpm, 5 min). The pellet was re-extracted until complete pigments extraction. 10ml NaCl solution (20%) and 10ml petroleum ether (40-60^o) were added to the collected solvent extractions, filtered under anhydrous Na_2SO_4, and make up to mark 25 ml with petroleum ether (40-60^o), as described by Moriel et al [19].
4) Acetone plus glass balls: dry biomass was submitted to extraction with small portions of acetone (6 ml), with 425-600 μm glass balls (2ml) alternately in an ice bath and in a vortex agitation (1 min), until no colour was obtained.
All extractions with the yeast were done in triplicate.
The SFE was carried out in a semi-continuous apparatus already previously described [13].
The liquid CO_2 (99.998 % purity) from a cylinder was compressed to the working pressure using a metering pump. The pressure was controlled by a back-pressure regulator and the fluid, before reaching the extractor (5 ml), passed through a coil immersed in the water bath at a temperature above the critical one. After flowing through the yeast bed, contained in the extractor, the supercritical fluid was expanded to atmospheric pressure through a three-way valve and the solutes were collected, in cooled glass U-tubes filled with glass wool. Gas flow rate was monitored with a rotameter and the total volume of gas was measured with a wet test meter.

At the end of each run, the extracted carotenoids were collected washing the glass wool, the inside of the three-way valve and the expansion tubing with the acetone.
Fractions of 5 to 20 L of expanded gas were collected along time.
The supercritical CO_2 extractions were done at different experimental conditions, on two grams of ground freeze-dried *P. rodozyma*, in order to study the effect of pressure (200 and 300 bar), temperature (40º, 50º and 60ºC), CO_2 superficial velocity (1.2 and 2.4 cm/min), as well as the effect of co-solvent (10 mol% of eyhanol) on the extraction efficiency.
Reversed-phase analysis of extracts was performed on a HPLC (Perkin Elmer) with a Vydac colunn (201TP54, 250mm/4,6mm) and a detector UV/VIS Waters 481 (λ=477 nm), with acetronitrile:methanol (10:90v/v), as eluent. The pigments were eluted over 20 min with a flow rate of 1ml/min.
In order to determine the amount of astaxanthin a calibration curve was obtained using an astaxanthin standard (Sigma, 98% purity).
Total carotenoids concentration was determined, in astaxanthin equivalents, by comparing total and astaxanthin areas.

RESULTS AND DISCUSSION

Supercritical Fluid Extraction

Using CO_2 as supercritical solvent, the extraction yield increased with pressure, at constant temperature, and decreased with temperature, at constant pressure, as can be seen in Figure 1. The solubility of the solutes is influenced by two factors: the density of the solvent, which increases with the pressure at constant temperature and the vapour pressure of the solutes, which increases with the temperature, at constant pressure. The solubility will change according to the predominant factor. At 300 bar, for this system, the initial yields are about the same (the curves almost overlap), for the three studied temperatures.

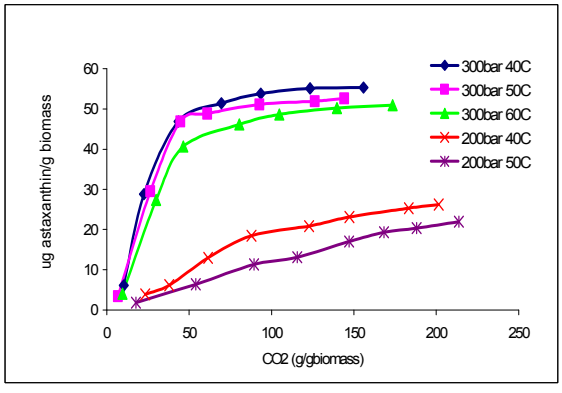

Fig. 1 Effect of pressure and temperature on Supercritical CO_2 Extraction of astaxanthin from the yeast *Phaffia rodozyma*

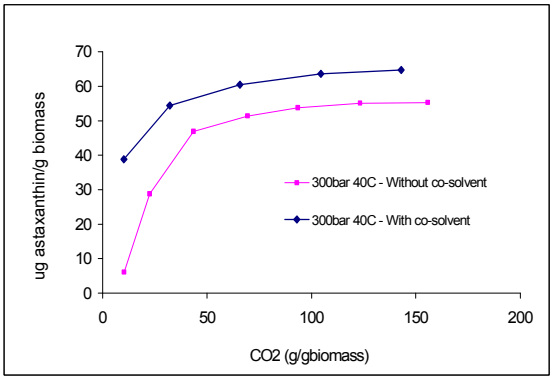

Fig. 2 Effect of the co-solvent ethanol (10%) on Supercritical CO_2 Extraction of astaxanthin from the yeast *Phaffia rodozyma*

Figure 2 shows the effect of the presence of ethanol, as co-solvent, and it shows that it increases the SFE yield. The improvement of the yield (about 25%) by the ethanol can be due to several effects: the increase of astaxanthin solubility in supercritical CO_2 plus co-solvent, due to the polar character of the carotenoid, which eases the formation of hydrogen bonds with CO_2, the swelling of the biomass pores, easing the release of astaxanthin, and the disruption of matrix structures [21].

The effect of the solvent flow rate is shown in Figure 3 and it can be verified that the yield decreases with the superficial velocity. Initially, the curves almost overlap, meaning that the extraction of astaxanthin more accessible is carried out at almost equilibrium conditions. However, after this initial period, it seems that resistance to mass transfer inside yeast particles is predominant.

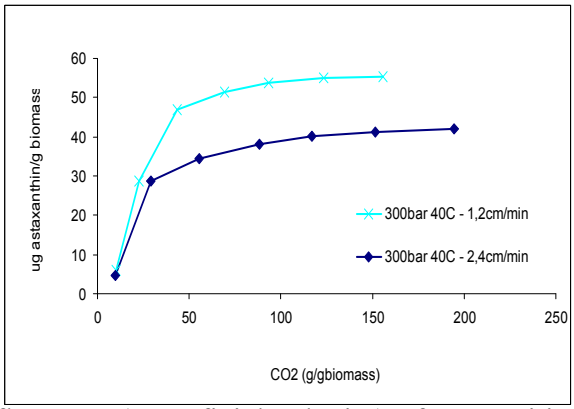

Fig. 3 Effect of the flow rate (superficial velocity) of supercritical CO_2 in SFE of astaxanthin from the yeast *Phaffia rodozyma*

Comparison between organic solvent and supercritical fluid extraction

Figure 4 shows the total carotenoids extractions by the organic solvents used and the highest yield (µg astaxanthin/g dry biomass) was obtained with acetone+glass balls, for both whole and ground cells, followed by (DiCl:MeOH). Furthermore, the acetone was efficient only for ground cells, while the yield for DMSO was similar for both whole and ground cells and lower than that obtained with acetone, since DMSO and methanol are able to break the cellular wall of the yeast.

The highest yield obtained with acetone+glass balls is probably due to the increase of disrupted cells as a consequence of the physical effect of the balls (breaking the cell wall and the carotene-proteins bonds) [20].

With supercritical extraction using pure CO_2 at 300 bar and 40 ºC, it is possible obtaining 85 % of carotenoids (recoveries compared with the yield before mentioned obtained with acetone plus glass balls), while with CO_2 mixed with the co-solvent, at the same conditions of pressure and temperature, the recovery is about 100 % (Figure 5).

Figure 6 shows that the best yield of astaxanthin by organic solvents was obtained using acetone+glass balls, followed respectively by (DiCl:MeOH), acetone and DMSO. The reason for this behaviour is probably the same as above. On the other hand, the lowest value of acetone+glass balls for the whole cells is possibly due to less free astaxanthin available, when the cells are not broken.

With supercritical extraction (Figure 7), the highest recoveries of astaxanthin, 63 and 72 %, were obtained at the pressure of 300 bar and temperature of 40 ºC, without and with the co-solvent, respectively. These values are lower than those obtained for the total carotenoids; so, some degradation of astaxanthin must have occurred. Lim et al. [18] obtained recoveries of carotenoids and astaxanthin, at 40 ºC and 500 bar, of 84 and 90 %, respectively. However, these authors don't report the absolute yields (wt/wt), either of the organic solvent extraction or the supercritical one. On the other hand, the extraction with organic solvent was carried out only using acetone on the ground yeast.

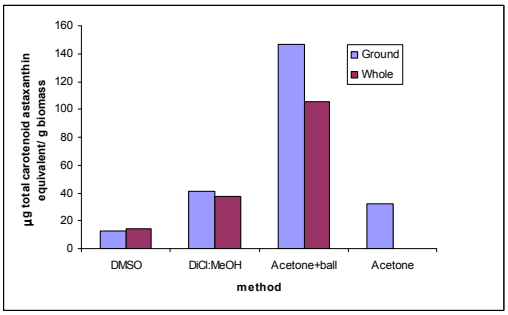

Fig. 4 Total carotenoids yield from the yeast *Phaffia rodozyma* by different solvent extractions methods.

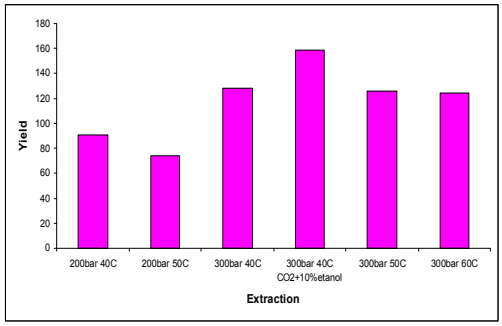

Fig. 5 Total carotenoids yield (µg/g) from the yeast *Phaffia rodozyma* by Supercritical CO_2 Extraction

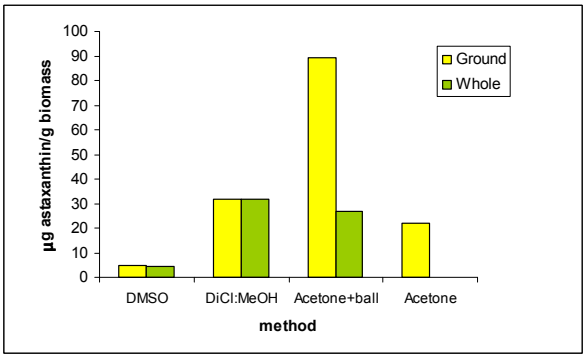

Fig. 6 Astaxanthin yield from the yeast *Phaffia rodozyma* by different solvent extractions methods.

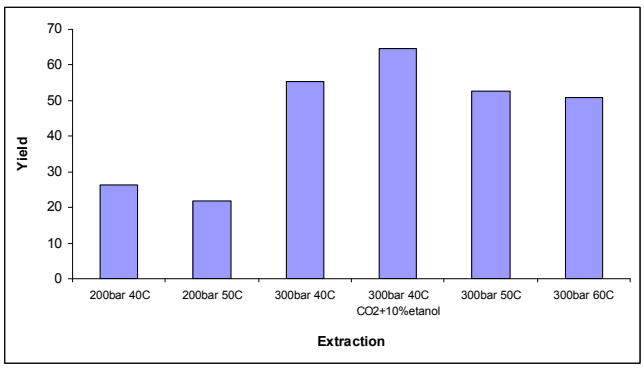

Fig. 7 Astaxanthin yield (µg/g) from the yeast *Phaffia rodozyma* by Supercritical CO_2 Extraction

4. CONCLUSIONS

Phaffia rhodozyma is a producer of astaxanthin (90µg/g dry matter), which can be used in feed, food and/or health applications after the carotenoid extraction. Several organic solvents, safe and unsafe, were tested to obtain the carotenoids from this yeast. The best extraction yield was obtained using acetone plus glass balls using well ground *Phaffia*. The highest recovery of carotenoids (about 100%) by supercritical fluid extraction was achieved at the pressure of 300 bar and the temperature of 40 ºC, with ethanol as co-solvent. The values obtained for astaxanthin were lower (about 75 %). Moreover, the extraction yield increased with the pressure at constant temperature and the increase of temperature, at constant pressure, led to a decrease of the yield at 200 bar and to a slight decrease at 300 bar. Extraction yields decreased also with the flow rate.

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