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Full Length Research Paper

Phaffia rhodozyma disruption methods for astaxanthin recovery in different cells

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Astaxanthin (3,3'-dihydroxy-b,b'-carotene-4,4'-dione) is carotenoid of high market value whose demand has increased in such fields as aquaculture, pharmaceutical supplements and natural coloring. Cell disruption is the first step for isolating intracellular materials and it depends on the cell wall permeability. In order to maximize the recovery of astaxanthin from *Phaffia rhodozyma* NRRL-Y17268, drying and freeze pretreatments were tested by different cell disruption methods: abrasion with celite, glass pearls in vortex agitator, ultrasonic waves, sodium carbonate (Na_2CO_3) and dimethyl sulfoxide (DMSO). The method with Na_2CO_3 was not effective; meanwhile, the agitator with glass pearls, the abrasion with celite and the ultrasonic waves were found as promising for future studies. As a result, the DMSO in freeze-dried biomass with 4 process cycles and biomass/DMSO relation of 0.025 g/ml was found to be the most efficient for analytical determination, increasing about up to 25 times the astaxanthin recovery.

Key words: Carotenoids, yeast, chemical disruption, dimethyl sulfoxide.

INTRODUCTION

Astaxanthin (3,3'-dihydroxy-b,b'-carotene-4,4'-dione) is an oxygenated carotenoid that has a high market value and an increasing demand. Interest in such a pigment is found in various fields as aquaculture, pharmaceutical supplements and natural coloring (Liu and Wu, 2006; Moriel et al., 2005; Dutta et al., 2005).

According to Liu and Wu (2006) and (Moriel et al. (2005)), the pink color of the salmon and of the peels of cooked crustaceans is due to the astaxanthin presence, which also contributes to the aroma of these meats after cooking. In aquaculture, rearing salmonids and crustaceans in pen culture systems requires a diet containing astaxanthin to obtain the appropriate coloration (Nakano and Takeuchi, 1995), as they cannot synthesize carotenoids (Liu and Wu, 2006; Moriel et al., 2005). Besides pigmenting the fish muscle, astaxanthin improved the health of fishes, increasing the performance of liver and tissues, possibly inhibiting the generation of reactive oxygen and preventing lipoproteins from oxidizing. Astaxanthin has various fundamental biological functions for these fishes, such as: protection against oxidation of polyunsaturated fatty acids; protection from the effects of UV rays; immune answer; reproductive behavior and improving reproduction (Guerin et al., 2003). In domestic bird rearing, astaxanthin is used for increasing the coloration of egg yolk, resulting in a better quality and acceptance in the consumer market (Liu and Wu, 2006; Moriel et al., 2005).

Carotenoids have considerable nutritional benefits due to its antioxidant effect and the potential to impede or delay degenerative diseases such as arteriosclerosis, cancer and aging; as well as it increases the immune answers in animals and humans. The antioxidant properties of astaxanthin play an important role in protection against photo-oxidation from light UV, inflammations, cancer, infectious ulcers from *Helicobacter pylorii*, aging and diseases related to age or in health promotion,

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Abbreviation: DMSO, Dimethyl sulfoxide.

Table 1. Pretreatments of *P.rhodozyma* biomass to evaluate different methods of disruption.

Assay	Pretreatment 1 (drying*)	Pretreatment 2 (freeze**)
1	Absence	Absence
2	Absence	Presence
3	Presence	Absence
4	Presence	Presence

* Period, 48 h; Temperature, 35ºC.
** Period, 48 h; Temperature, -18ºC.

the cells were washed with distilled water and centrifuged again in the same conditions, being discarded the supernatant. The precipitate biomass was submitted for pretreatments described in Table 1, for subsequent cellular disruption evaluating the physical methods, mechanics and chemical.

Assays of cell disruption

Assays of cell disruption were carried out in triplicate at least, after the biomass pretreatments resulting in the astaxanthin specific concentration. Next was disruption cell in the mechanical methods, the biomass was then centrifuged at 1745 x g for 10 min (Persike et al., 2002) and the astaxanthin specific concentration was determined in the supernatant.

Mechanical methods

Abrasion with celite

In mortar and pistil, 0.5 g of cells was triturated with 0.5 g of celite, to which 6 ml of acetone were added in order to extract the carotenoid (Valduga et al., 2007).

Abrasion with glass pearls in vortex agitator

Flasks containing 0.5 g of biomass were put in vortex agitator with 0.2 g of glass pearls of 0.25 mm diameter and 6 ml of acetone and agitated for 10 min.

Ultrasonic waves

Amber flasks containing 0.5 g of biomass and 6 ml of acetone were taken in the sonicator bath (power of 40 W RMS and frequency of 20 kHz) with cold water level at half of the recommended limit and 4 cycles of 10 min each were performed (Medeiros et al., 2008).

Chemical methods

Sodium bicarbonate

In Erlenmeyer flasks of 500 ml, 18 ml of Na_2CO_3 0.1 M was added with 6 g of biomass until a homogeneous solution. The container was closed with a cotton wad and bathed at 40 to 45°C for 24 h. Next, centrifugation was performed at 290 x g for 15 min for the cell debris to silt up and the astaxanthin specific concentration was determined in the supernatant. The supernatant volume was measured and frozen (Sabaj, 1979) for determining the astaxanthin specific concentration.

Dimethyl sulfoxide (DMSO)

Centrifuge tubes were filled with 2 ml of DMSO, pre-warmed at 55°C for 30 min in 0.5 g of biomass and strong shaking was carried out in vortex agitator for 1 min and then, left for 30 min at rest. Next, it was added 6 ml of acetone and the solution was homogenized and centrifuged at 1745 x g for 10 min and the supernatant was collected (Persike et al., 2002). The same procedure was repeated for the precipitate. Supernatants were gathered for determining the astaxanthin specific concentration (Sedmak et al., 1990).

Extraction and determination of astaxanthin

In the supernatant obtained were added 10 ml of NaCl 200 g/l and 10 ml of petroleum ether. After the agitation, the solvent phase was separated. To ensure extraction, 5 ml of petroleum ether were added. The solvent phase was filtrated with Na_2SO_4 to eliminate the moisture. Absorbance was measured at 474 nm and the astaxanthin specific concentration was calculated by Equation 1 (Moriel et al., 2004; Moriel et al., 2005).

$$C = (A * V * 10^6)/(A^{1\%}_{1\,cm} * 100 * m) \quad (1)$$

Statistical analysis

All the assays of cell disruption were carried out in triplicate and treated by analysis of variance (ANOVA), followed by Tukey's test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). The results (averages ± standard deviations) followed by equal letters indicate that, no significant difference was found (Tukey's test). All analyses were performed considering a confidence level of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Cell disruption by chemical method with Na_2CO_3 was not effective in any of the pretreatments under study, presumably due to the cell wall structure and composition of the yeast *P. rhodozyma*, which is mainly composed of glucan and responsible for the cell rigidity before mechanical shocks and osmotic disequilibria (Fleuri and Sato, 2005). Although, Na_2CO_3 is a classic method for the cell

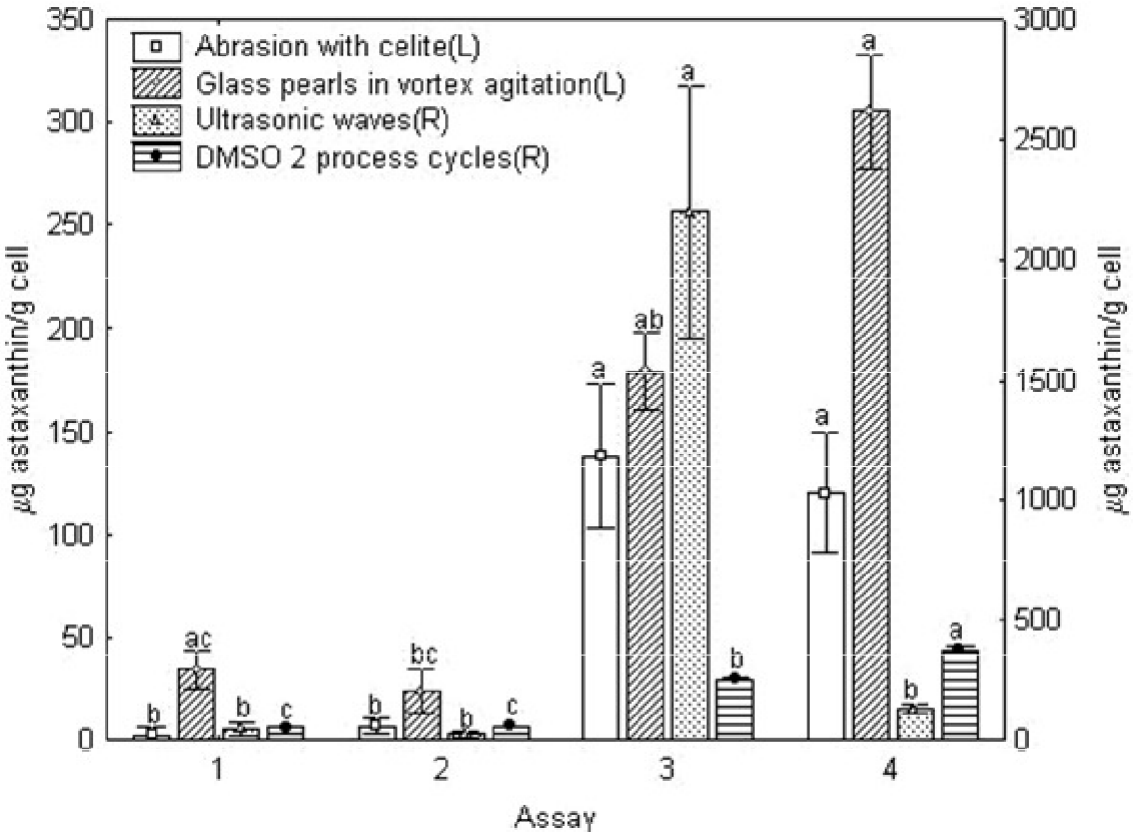

Figure 1. Astaxanthin specific concentration ± standard deviation for disruption method by abrasion with celite, glass pearls in vortex agitator, ultrasonic waves and DMSO in 2 process cycles. Assay 1, (fresh biomass); assay 2, (frozen biomass); assay 3, (dry biomass); assay 4 (dry and frozen biomass). L, left-axes; R, right-axes. The coincidence of one or more letters in the same method of cell disruption indicates that there is no difference at the significance level of 5% (Tukey test).

wall disruption of the yeast *Saccharomyces cerevisae* at concentration of at least 30 mM, it is obtained by extraction of protein covalently linked to some carbohydrates of the cell wall (Fleuri and Sato, 2005), which has glucan in much lesser amount than in *P. rhodozyma*.

Results for the astaxanthin specific concentration from cell disruption by abrasion with celite are shown in Figure 1. Tests of differences among the averages was able to verify that the cell disruption was most effective on the drying pretreatment (assay 3), as evidenced by the significant increase in the astaxanthin specific concentration (138.1 ± 35.0 µg/g). Recovery of such carotenoid through cell disruption by abrasion was not influenced by the freeze pretreatment, since the astaxanthin concentration with fresh (assay 1) and frozen (assay 2) biomass were statistically the same, with a similar occurrence effect when compared with assays 3 and 4. However, when comparing the assays 1 and 3 and 2 and 4, between them is the drying pretreatment found as effective for the cell wall disruption.

The astaxanthin specific concentration from cell disruption by vortex agitator with glass pearls is shown in Figure 1. Results were found similar to the ones of disruption by celite. It may be explained because both methods have the disruption principle based on the physical abrasion of cells with their respective agents. The maximum astaxanthin specific concentration obtained for the disruption using the drying and freeze pretreatments was 305.3 ± 27.7 µg/g. Such behavior may have occurred as the drying pretreatment decreased the water outside the cell, thus, increasing the surface contact with the abrasive and favoring the disruption. Also, since carotenoids are liposolubles, water absence facilitates its extraction.

As shown in Figure 1, the use of ultrasonic waves for cell disruption was efficient for the drying pretreatment only (assay 3), which reached an astaxanthin specific concentration of 2198.4 ± 523.7 µg/g. However, Tukey's test was expected not to show statistical difference between the assays 3 and 4, where the biomass was submitted to drying and freeze pretreatments, resulting in 128.5 ± 19.2 µg/g of astaxanthin. The mechanism of cell disruption is associated with the cavitation phenomena, a shear stress developed by viscous dissipative eddies

tical determination.

The best astaxanthin results were found from the DMSO used as disruption agent in freeze-dried biomass with 4 process cycles and biomass/DMSO relation of 0.025 g/ml, with an optimization of about up to 25 times in astaxanthin recovery, being more efficient for analytical determination than the other methods under study.

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Symbols: C, Astaxanthin specific concentration (µg/g); rpm, rotations per minute; a, absorbance; v, volume (ml); m, cell mass (g); $A^{1\%}_{1cm}$, specific absorption coefficient of 1 g/100 ml, being used the specific absorptivity for xanthophyll, which is 1600 (Davies, 1976).

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