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Microencapsulation by lyophilization of carotenoids produced by *Phaffia rhodozyma* with soy protein as the encapsulating agent

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Abstract

Carotenoids are pigments that can be applied to food but they are unstable towards certain food intrinsic conditions, as well as processing ones. Microencapsulation is an alternative to increasing their stability. This study aimed to produce carotenoids by *Phaffia rhodozyma* crops and promote their microencapsulation by lyophilization with soy protein as the wall material, in different proportions. High process yield of 96% and encapsulation efficiency of around 65% were observed at the ratios under study. Well-defined and separate micrometer-scale particles with different shapes and sizes were formed and the protection of the compounds of interest was confirmed by differential scanning calorimetry which showed that the endothermic event – typical of the free extract after encapsulation – did not occur.

Keywords: pigments; microbial cultures; microcapsules.

Practical Application: Microcapsules protecting microbial carotenoids that allow the application in food.

1 Introduction

Carotenoids are natural pigments with polyene chains which have from 3 to 15 conjugated double bonds. They are considered bioactive compounds due to their antioxidant capacity, since they neutralize free radicals through the reception of electrons from reactive species (Valduga et al., 2009a; Gonnet et al., 2010). Although these pigments are found in plants, animals and microorganisms, most carotenoids destined to the food industry are obtained by chemical processes (Valduga et al., 2009b), a fact that suggests growing interest in studies of carotenoid production by biotechnological processes (Valduga et al., 2009a).

Phaffia rhodozyma is a GRAS (Generally Recognized as Safe) yeast which has the ability to produce carotenoids intracellularly (Frengova & Beshkova, 2009). By comparison with other microorganisms and other natural sources, it has advantages, such as the possibility of using alternative inexpensive media, since it requires simple sources of carbon and nitrogen for its growth and little physical space when it comes from crops on an industrial scale, besides being independent of bioproducts seasonality (Cipolatti et al., 2015; Valduga et al., 2009b).

However, regardless of the production source, carotenoids are unstable compounds at high temperatures in the presence of light and oxygen. Thus, it is difficult to maintain their characteristics when they are subjected to certain intrinsic conditions of some products (Bagetti, 2009). An alternative to increasing stability in storage and processing is the use of microencapsulation methods, which consist in the entrapment of compounds in extremely small capsules obtained by different techniques. As a result, encapsulated material may be protected and released in a controlled way under specific conditions so as to expand its application to food (Favaro-Trindade et al., 2008).

Therefore, this study aims to promote the encapsulation of carotenoids produced by *P. rhodozyma* yeast by the lyophilization method with soy protein as the encapsulating agent.

2 Materials and methods

2.1 Microorganism and inoculum preparation

Phaffia rhodozyma NRRL-Y 17268, a GRAS (Generally Recognized as Safe) yeast from the Northern Regional Research Laboratory (Peoria, USA), was used by this study. The microorganism was kept on sloping agar in malt and yeast (YM) medium with 3 gL^{-1} yeast extract, 3 gL^{-1} malt extract, 5 gL^{-1} peptone and 10 gL^{-1} glucose. Then, 0.2 gL^{-1} KNO_3 was added at 4 °C (Parajó et al., 1998). For reactivation, stock culture tests were performed in test tubes with the same medium and incubated at 25 °C for 48 h. Cell resuspension was performed in 1 mL peptone water (0.1%), added to 9 mL YM medium and incubated under the previously described conditions.

2.2 Production of carotenoids

The inoculum used for yielding pigments was prepared in 500 mL Erlenmeyer flasks with 90 mL YM broth and 10 mL culture from the reactivation. It was incubated at 25 °C, 150 rpm for 48 h or the period of time needed to reach 1×10^8 $cel.mL^{-1}$, counted by the Neubauer chamber (Rios et al., 2015). Bioproduction of carotenoids was carried out in 500 mL Erlenmeyer flasks with 153 mL YM production medium, at initial pH of 6.0, and 10% inoculum (10^8 $cel.mL^{-1}$). The operating conditions of the process were 25 °C, 180 rpm for 168 h (Rios et al., 2015). The resulting biomasses were dried in an oven at 35 °C for 24 h. Then, they

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were macerated with mortar and pestle and particle sizes were standardized by a Tyler 115 sieve, corresponding to > 125 mm (Cipollati, 2012).

2.3 Extraction and recovery of carotenoids

Cell disruption was performed by the ultrasonic wave method with 0.01 g dry biomass (at 35 °C for 48h); 6 mL acetone was added to facilitate carotenoid extraction. Ultrasound (4 cycles of 40 kHz) was applied for 10 min and water from water baths was replaced at every cycle, according to the method adapted from Medeiros et al. (2008). Each sample was centrifuged at 1745xg for 10 min. The solvent was separated and the bursting procedure was repeated until the whole cell bleached. In the solvent phases, obtained by centrifugation, 10 mL 20% NaCl solution (w/v) and 10 mL petroleum ether were added. After shaking and phase separation, excess water was removed with sodium sulfate (Na_2SO_4), yielding carotenogenic extracts (Bonfim, 1999). In order to obtain the total volume of carotenogenic extracts to be encapsulated, 5 extractions were performed in each treatment.

2.4 Microencapsulation of carotenoids and characterization of microcapsules

Microencapsulation of carotenoids was performed by the lyophilization method, with soy protein as the coating material, according to Pralhad & Rajendrakumar (2004) and Laine et al. (2008). To prepare the microparticles, the solvent was rotaevaporated from the carotenogenic extracts at 35 °C, followed by dissolution in aqueous solution with the wall material, in the ratios of 1:1 and 1:2 (carotenoids:wall material) in relation to the solid content. The mixture was stirred for 3 h and then subjected to freezing at -80 °C, followed by the lyophilization process.

Microcapsule formation, as well as morphological analysis and particle size, was observed by scanning electron microscopy (SEM), according to Castro (2002). Mean particle size was determined by at least 100 measurements performed by the Sigma Scan Pro 5 software.

Carotenoid encapsulation efficiency (EE) was performed according to the spectrophotometric method described by Sutter et al. (2007), based on the estimation of total carotenoids found inside and outside microparticles. To quantify the carotenoids on the surface of the microparticles, 0.1 g sample and 5 ml hexane were mixed and kept in a vortex shaker for 10 seconds, followed by centrifugation at 3420xg for 10 minutes. The supernatant was collected. To quantify total carotenoids found inside and outside microparticles, they were dispersed in 5 ml hexane, stirred vigorously for the removal of total carotenoids and filtered with cotton into a 10 ml volumetric flask. The residue was washed with hexane. Both collected fractions were evaluated spectrophotometrically at 470 nm to obtain total carotenoid content. Results are expressed as a percentage of encapsulated carotenoids by Equation 1:

$$\%EE = \frac{\text{Total Carotenoids} - \text{Surface Carotenoids}}{\text{Total Carotenoids}} \times 100 \quad (1)$$

Calculation of the yield of the encapsulation process was based on the mass of the initial and final solids followed by conversion to percentage. Confirmation of the encapsulation was performed by differential scanning calorimetry (DSC) analysis according to Rutz (2013) with rate of 10 °C min^{-1} between 25 and 280 °C, with a nitrogen flow of 40 $mL.min^{-1}$.

Experiments were performed in triplicate and results were statistically evaluated by the analysis of variance. When differences were detected at 5% ($p < 0.05$), the T test was applied.

3 Results and discussion

Results of the microencapsulation yield of the lyophilization process with soy protein as the encapsulating agent are shown in Table 1. The highest yields of the microcapsule yields based on the mass of solids in each treatment was 96.40% in the ratio of 1:1 and 97.46% in the ratio of 1:2. It shows that losses of compounds of interest in the process are very small. The same is not observed regarding the efficiency of encapsulation, which considers the percentage of carotenoids protected by the encapsulating agent, since about 65% of total carotenoids was actually protected by the soy protein. Remaining amounts were dispersed among the microcapsules that were formed and, thus, susceptible to alterations caused by the external environment. The ratio of carotenogenic extract:encapsulating agent did not influence the efficiency of encapsulation, since the percentage of entrapped carotenoids did not differ significantly, suggesting that there is no need to add higher proportion of cover material.

Scanning electron micrographs (SEM) (Figure 1) show characteristic microparticle formation, regardless of the ratio under investigation. Granular material with small, well defined and separate particles, but with quite different shapes, was formed. This fact results from the preparation of the sample and the technique which does not enable particle sizes to be standardized.

Microcapsules had an average size of 12.11 ± 4.60 μm for "a", with variation from 3.73 to 24.10 μm and to 11.34 ± 5.16 μm for "b", ranging from 2.69 to 23.21 μm.

According to Rutz (2013), the acquisition of spherical microparticles depends on the process conditions and the coating. Studies conducted by Sousdaleff et al. (2013), Zuanon et al. (2013) and Rutz et al. (2016) applied the lyophilization method to the encapsulation of different compounds and showed the acquisition of non-characteristic particles. They also showed that microparticle spheres were not formed, suggesting that, depending on the wall material, such conditions are not suitable for obtaining microcapsules, although the method has been commonly used.

DSC thermograms (Figure 2) show endothermic and exothermic events by the representation of downward and upward curves, respectively.

Carotenogenic extracts exhibit an endothermic event at 59 °C. Wall materials have different behavior, since soy protein has two endothermic events at 171 °C and 226 °C (a and b). When all encapsulated carotenogenic extracts, regardless of the concentration, are observed, there are no endothermic

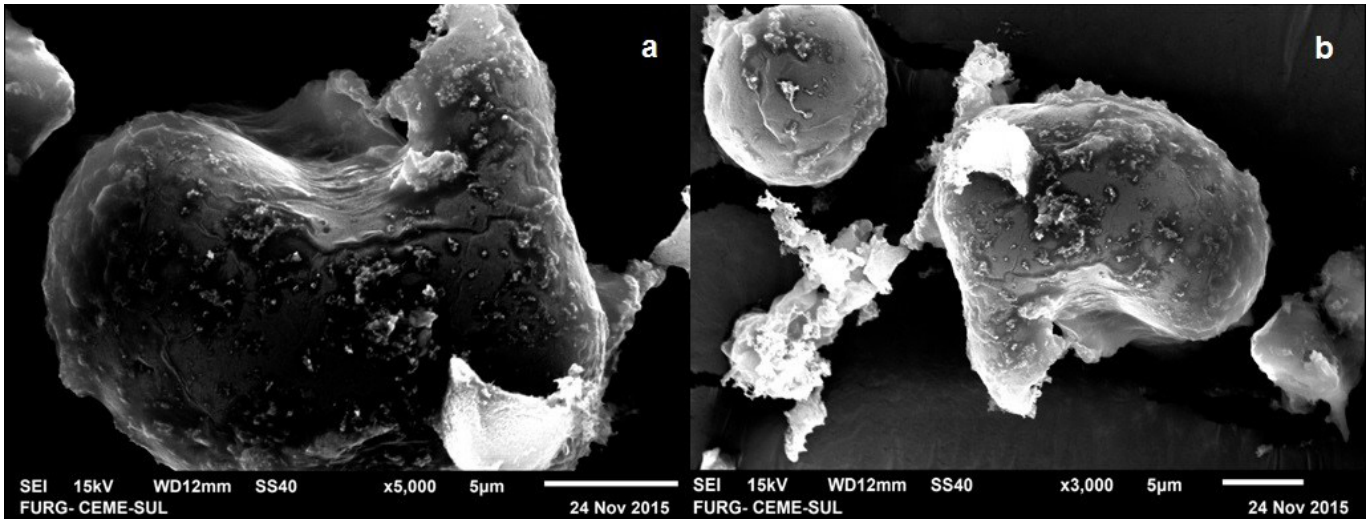

Figure 1. SEM micrographs of carotenoid microcapsules using soy protein, in different proportions, as encapsulating agent: (a) (x 5000) - soy protein 1:1; (b) (x 3000) – soy protein.

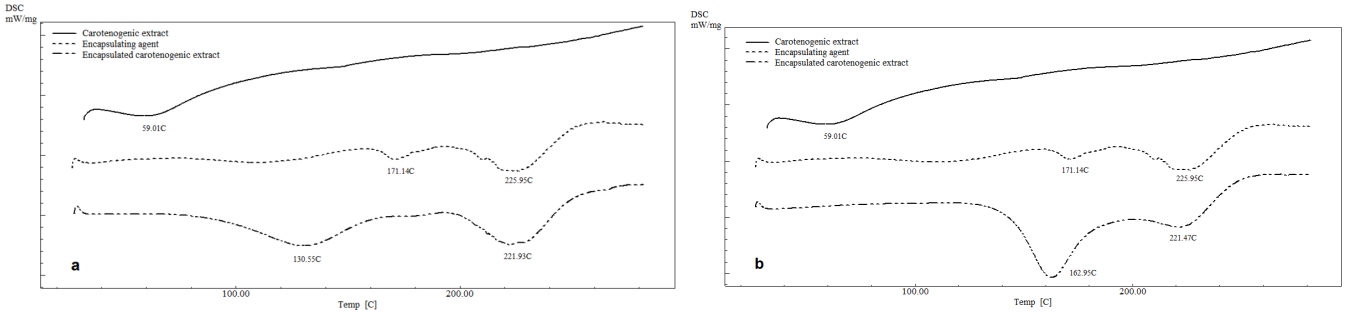

Figure 2. DSC thermograms of the carotenoid microcapsules using soy protein as a coating material in different proportions: (a) soy protein 1:1; (b) soy protein 1:2.

Table 1. Encapsulation Efficiency of the lyophilization method.

Encapsulating agent	Yield of microcapsules (%)	Encapsulation Efficiency (%)
Soy protein (1:1)	96.40	65.38 ± 1.36[a]
Soy protein (1:2)	97.46	65.22 ± 1.85[a]

Different lowercase letters in the column indicate significant difference by the T Test ($p < 0.05$).

events at temperatures around 59 °C, which is typical of the free carotenogenic extract. It shows a behavior that resembles the wall materials, suggesting the protection of the compounds of interest and consequent encapsulation.

4 Conclusion

Microencapsulation of carotenoids produced by *P. rhodozyma* yeast with soybean protein as the encapsulating agent by the lyophilization technique was carried out. High process yield and encapsulation efficiency around 65% were reached, resulting in the formation of well-defined and separate microparticles. Protection of the compounds of interest was successful, indicating the possibility of their application to food.

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