Different cell disruption methods for obtaining carotenoids by *Sporidiobolus pararoseus* and *Rhodotorula mucilaginosa*

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Abstract Since carotenoids are synthesized inside the cell, it is desirable to find an efficient method to extract carotegenic pigments. This study aimed at comparing the effectiveness of different chemical and mechanical techniques to disrupt the cell wall of *Sporidiobolus pararoseus* and *Rhodotorula mucilaginosa* yeasts isolated from environmental samples. Among the techniques under study, the ultrasonic bath and the abrasion with glass beads yielded the most promising results for *S. pararoseus* (84.8 ± 2.3 and 76.9 ± 2.1 µg/g, respectively). The ultrasonic bath yielded the highest specific concentration of carotenoids for *R. mucilaginosa* (193.5 ± 25.8 µg/g), while the biomass freezing process improved neither the extractability nor the specific concentration of carotenoids. Lyophilization increased the specific concentrations of carotenoids from *S. pararoseus* and *R. mucilaginosa* by 20 and 13.7%, respectively, while the freezing process did not significantly affect ($p > 0.05$) the recovery of carotenoids from both yeasts; thus, it may be eliminated from the process.

Keywords Yeast · Carotenoids · Ultrasonic bath · Biomass · Freezing

Introduction

Carotenoids have been considered one of the most important classes of pigments [1], since they account for colors ranging from yellow to red [2]. Besides acting as colorants,

they have biological activities with beneficial effects on health, such as pro-vitamin A activity [3]. Interest in carotenoids has increased in recent years due to growing demands for the application of these compounds to food, pharmaceutical, cosmetics and animal feed industries [4].

Consumers' concern for health has become an incentive in the search for functional foods and has triggered increase in the demand for carotenoids in the food industry. However, microbial-derived pigments have higher commercial value than those derived from synthetic sources. β-carotene produced by a bacterial source costs twice as much (US\$ 1000/kg) as the synthetic one (US\$ 500/kg). Even though most commercially available carotenoids derive from a chemical synthesis and do not satisfy consumers' desire to use natural pigments, such as microbial carotenoids, they compete in the market because they are natural sources, despite being more expensive [5, 6].

Microbial carotenoids have advantages, such as independence of seasonality and availability of raw material, over the ones extracted from plants [7]. The high cost of production can be minimized by the optimization of the process and efficient use of industrial by-products as alternative nutrient sources [8]. Yeasts *R. mucilaginosa* and *S. pararoseus* are capable of growing in alternative medium with agro industrial products, such as parboiled rice water, corn steep liquor, raw glycerol and sugar cane molasses [9–11], and have promising antioxidant activity due to the presence of β-carotene. On the other hand, carotenoids are produced by these yeasts intracellularly and the rigidity of the cell wall limits their extraction. Thus, mechanical, physical, chemical and/or enzymatic techniques are needed for cellular rupture so as to achieve the best recovery of the biocompounds [12].

Cellular rupture can be performed by different techniques, but only a few are applicable on a large scale.

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Mechanical methods have been the most widespread in the industry, although enzymatic and chemical ones are of great interest [13]. Cell rupture by abrasion with the use of glass beads and ultrasonic waves, in addition to sodium carbonate and dimethyl sulfoxide in *Phaffia rhodozyma* [14] and the use of supercritical CO₂ combined with the use of dimethyl sulfoxide in *Sporodiobolus salmonicolor* [15], was evaluated. However, the application of these techniques to *S. pararoseus* and *R. mucilaginosa* has been poorly studied. Therefore, this study aimed to compare the efficiency of different chemical and mechanical techniques in cell rupture for the recovery of carotenoids produced by *S. pararoseus* and *R. mucilaginosa*.

Materials and methods

Microorganisms

S. pararoseus CCT 7689 and *R. mucilaginosa* CCT 7688 were previously isolated [9] from environmental samples obtained in the Escudo Sul-Rio Grandense region (Rio Grande do Sul, Brazil). They were identified and deposited in the André Toselo Tropical Culture Collection (CCT).

Maintenance and reactivation of microorganisms

Microorganisms were maintained in inclined test tubes with GYMP agar (2.0 g/L glucose, 1.0 g/L malt extract, 0.5 g/L yeast extract, 0.2 g/L NaH₂PO₄ and 1.8 g/L agar) with mineral oil at 4 °C [14] for 3 months. To carry out reactivation, samples were transferred from stock cultures to fresh test tubes with the same medium and incubated at 25 °C for 48 h. Cell resuspension (pre-inoculum) was performed in 1.0 mL peptone water (0.1%). Nine milliliters of modified YM medium was added, in agreement with Parajó et al. [16] (3.0 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose and 0.2 g/L KNO₃). The resulting mixture was incubated under the previously described conditions.

Inoculum preparation

For each microorganism used in this study, 1 mL cell suspension was prepared in sterile peptone water (0.1%) from the tubes with the microorganism in inclined YM agar; 9 mL of modified YM broth was added to it. The resulting mixture was incubated at 25 °C for 48 h. The inoculum was grown in 500 mL Erlenmeyer flasks with 90 mL YM medium, previously sterilized in an autoclave (Fabbe model 103, São Paulo, Brazil) at 121 °C for 15 min. The cell suspension was added to it and incubated at 25 °C with orbital shaking (Tecnal model TE 425,

Piracicaba, Brazil) at 150 rpm for either 48 h or the length of time needed to achieve density of 1×10^8 cells/mL, concentration counted by a Neubauer chamber (Optik Labor, Lancing, UK) [12, 14].

Incubation of cultures

Cultures for the bioproduction of carotenoids were prepared in 500 mL Erlenmeyer flasks with 225 mL YM culture medium at initial pH 6.0. Then, 10% of the inoculum was added (cultivation started at 1×10^7 cells/mL). They were incubated at 25 °C for 168 h at 180 rpm, without light [12].

Recovery of biomass

Biomasses of yeasts were collected from the fermented medium by centrifugation (1745×g for 10 min). The sediment was washed twice with distilled water and then dried (at 35 °C for 48 h) (Eletrolab, São Paulo, Brazil), as reported by Fonseca et al. [14]. The resulting biomass was macerated in a mortar and sieved (115 mesh), in agreement with Cipolatti et al. [17]. Samples selected for freezing were frozen at −18 °C for 48 h [18] (Consul, Joinville, Brazil).

Cell disruption techniques

In the extraction of carotenoids produced by *S. pararoseus* and *R. mucilaginosa* yeasts, different mechanical and chemical techniques were used for disrupting cells. Dried biomass was selected for freezing at −18 °C for 48 h [18].

Chemical techniques

The technique with dimethyl sulfoxide (DMSO) (Synth, Diadema, Brazil) was performed by modifying the method proposed by Sedmak et al. [19]. Two milliliters DMSO was added to tubes with 0.05 g biomass at 55 °C (Fanem model 102, São Paulo, Brazil). The tubes were agitated vigorously by a tube agitator (1 min) (Biomixer model QL-901, Ningbo, China) and then left undisturbed for 15 min. This procedure was repeated either for 1 h or until maximum cell discoloration was reached [14].

Techniques with hydrochloric acid (Synth, Diadema, Brazil), lactic acid (Synth, Diadema, Brazil) and acetic acid (Synth, Diadema, Brazil) were performed as follows: 0.5 g biomass and 7.5 mL 4 mol/L hydrochloric, acetic or lactic acid solution were mixed. Resulting solutions were subjected to a 35 °C bath for 5 min [20]. Centrifugation (Cientec CT 5000 R, Belo Horizonte, Brazil) was performed (1745×g for 10 min) and the supernatant was discarded. The sediment was washed twice with 7.5 mL

distilled water to eliminate acid waste. Subsequent to cell disruption, 6 mL acetone (Synth, Diadema, Brazil) was added to all samples subjected to chemical disruption. The supernatant containing the carotenoids was separated by centrifugation ($1745\times g$ for 10 min).

Mechanical techniques

The ultrasonic wave technique was adapted from the method proposed by Medeiros et al. [21]. Tubes with 0.5 g biomass and 6 mL acetone were subjected to 4 ultrasonic cycles of 40 kHz (Quimis, Diadema, Brazil) for 10 min.

The sonic ruptor technique used the method proposed by Lemes et al. [22]. Tubes with 0.5 g biomass and 6 mL acetone were subjected to ultrasonic homogenization (Omni International Sonic Ruptor 250, Kenesaw, USA) for 20 min under constant cooling at 20 kHz frequency and maximum power of 150 W, using a micro titanium ferrule with 40% power.

Abrasion with glass pearls was adapted from the method proposed by Medeiros et al. [21]. Tubes with 0.5 g biomass and 6 mL acetone were loaded with 1.1 g/mL glass pearls (0.5–0.59 mm) and agitated vigorously for 10 min by a vortex agitator.

Maceration with diatomaceous earth was adapted from the method proposed by Valduga et al. [23]. A mixture of 0.5 g biomass and 0.5 g diatomaceous earth was macerated for 10 min with a mortar and pestle. Then, 6 mL acetone was added to it.

Six milliliters acetone was added to tubes with 0.5 g biomass. It was followed by immersion of the tubes into liquid nitrogen and subsequent maceration of the frozen mixture with a mortar and pestle for 1 min. After cell disruption, the supernatant was separated by centrifugation ($1745\times g$ for 10 min) for subsequent carotenoid extraction [24].

Lyophilization

The biomass was partitioned in two; a partition was subjected to freeze-drying in an equipment which holds 1.5 L (Edwards). Samples were kept at $-70\text{ }^{\circ}\text{C}$ (Liotop UFR30, São Carlos, Brazil) for 15 h before being lyophilized (Liotop L101, São Carlos, Brazil) for 24 h [25]. The other partition was subjected to conventional drying by a forced air circulation oven ($35\text{ }^{\circ}\text{C}/48\text{ h}$).

Microscopy

Visualization of cell disruption of *S. pararoseus* and *R. mucilaginosa* was carried out by light microscopy (Nikon ECLIPSE E200, PR China) with dye (crystal violet/fuchsin combination) and magnified 1000 times.

Determination of total carotenoids

After each cell disruption method, samples with acetone were centrifuged ($1745\times g$ for 10 min) and the supernatant was used for extracting carotenoids.

Ten milliliters NaCl (Synth, Diadema, Brazil) 20% (p/v) and 10 mL petroleum ether were added to the solvent phases that resulted from centrifugation. After the formation of both phases, the polar phase was collected and excess water was removed with sodium sulfate (Na_2SO_4) (Synth, Diadema, Brazil), thus, forming carotenogenic extracts [12].

Carotenoids were quantified by spectrophotometry (BIOSPECTRO SP-220, Zhejiang, PR China) using their tabulated absorption coefficients. Both the wavelength maximum and specific absorptivity were significantly affected by the solvent [26]. Concentrations of total carotenoids in the extracts were determined by a spectrophotometer (Biospectro SP-220, PR China) with average maximum absorbance of 448 nm, expressed as its major carotenoid product (β -carotene in petroleum ether with specific absorptivity = 2592), using Eq. 1 [27]:

$$\text{TC} = \frac{A \times V \times 10^6}{A_{1\text{cm}}^{1\%} \times 100 \times m_{\text{sample}}} \quad (1)$$

where TC is the total concentration of carotenoids ($\mu\text{g/g}$), A is the absorbance, V is the volume (mL), m_{sample} is the dry cell mass (g) and $A_{1\text{cm}}^{1\%}$ is the specific absorptivity.

Carotenoid extractability (EC) was calculated by Eq. 2 [28], as follows:

$$\text{EC}(\%) = \frac{\text{CR}}{\text{CT}} \times 100 \quad (2)$$

where CR is the concentration of carotenoids ($\mu\text{g/g}$), obtained by the cell disruption technique under study and CT is the total carotenoid concentration ($\mu\text{g/g}$) found in the cells of each yeast. CT was obtained by cell disruption with DMSO.

Statistical analysis

All assays were performed in triplicate. Results were evaluated by the analysis of variance (ANOVA) and the Tukey's test to verify the existence of significant differences among the techniques at 95% confidence level.

Results and discussion

Chemical techniques of cell disruption

DMSO used for obtaining carotenoids showed the highest values among the chemical techniques. Specific concentrations

(Table 1) of *S. pararoseus* in the presence and absence of freezing were 86.9 ± 5.3 $\mu\text{g/g}$ and 87.3 ± 4.4 $\mu\text{g/g}$, respectively. Regarding *R. mucilaginosa*, its behavior in the presence and absence of freezing was similar and reached 297.6 ± 27.7 $\mu\text{g/g}$ and 234.1 ± 4.0 $\mu\text{g/g}$, respectively, thus, achieving 100% extractability for both microorganisms.

In this study, the best result was expected with the use of dimethyl sulfoxide since this is one of the most common techniques to estimate total carotenoids on a laboratory scale [12]. Similar results to the ones found by this study of cell disruption with the application of DMSO were achieved for *P. rhodozyma* [12], with high carotenoid recovery (153.91 ± 2.57 and 155.72 ± 2.34 $\mu\text{g/g}$, in the absence and presence of freezing). Among different solvents, DMSO, petroleum ether and acetone were used together to disrupt the cell wall of *R. glutinis* [29] and recover about 258.5 $\mu\text{g/g}$. These results were significantly higher than the others under investigation. However, the presence of toxic compounds in these extracts makes it impossible to apply them on an industrial scale [13].

Concerning cell disruption of *S. pararoseus*, the highest pigment recoveries were found with hydrochloric acid, followed by acetic and lactic ones (Table 1). Although they were lower than the ones found with dimethylsulfoxide, they were advantageous due to their low toxicity. Because of the absence of chemical additive residues, they can be used in industries and be applied to food [8, 12]. According to Ni et al. [20], this phenomenon may be linked to the values of acid strength (pKa). The stronger the acid, the greater the disruption efficiency in the cell and the extractability of the carotenoid. However, results differ for *R. mucilaginosa*, i.e., hydrochloric acid, whose pKa is -7 , was less efficient at cell disruption. The hydrochloric acid action in the cell wall could have been so intense that, in addition to breaking it, it may have caused the degradation of the carotenoids that were released, harming the recovery

of carotenogenic pigments. Hydrochloric acid, in relation to acetic and lactic ones, had better results for carotenoid recovery: for *S. salmonicolor*, it was 94.30 ± 6.1 $\mu\text{g/L}$ [15] whereas for *P. rhodozyma*, it was 61.22 ± 1.27 $\mu\text{g/g}$ and 65.37 ± 1.99 $\mu\text{g/g}$, in the absence and presence of freezing, respectively [12].

The freezing process did not increase the recovery of carotenoids when chemical disruption methods were applied to both yeasts, except for the extractability of lactic acid in *R. mucilaginosa* (Table 1), in which case it decreased. Therefore, the freezing step may be eliminated from the process when these chemical methods of cell disruption are used.

Mechanical techniques of cell disruption

Regarding *S. pararoseus*, mechanical techniques of cell disruption, such as ultrasonic bath and abrasion with glass beads (Table 2) were better than the others and did not show significant differences ($p > 0.05$) among themselves in the absence of freezing. However, extractability of carotenoids decreased significantly ($p < 0.05$) when freezing was combined with these mechanical methods (Table 2). The formation of ice crystals during the freezing process causes damage to the cell wall, resulting in loss of cytoplasmic fluid and reduction in particle size [12]. This phenomenon may have occurred because the cells had lower volume than eddies formed by the ultrasonic waves and the glass beads. Thus, biomass freezing can also be eliminated from the carotenogenic recovery process by ultrasonic bath and abrasion with glass beads. As a result, energy is saved and processing time is decreased.

Other techniques, such as the use of an ultrasonic ruptor and diatomaceous earth, which were evaluated for *S. pararoseus*, showed similar values in the recovery and

Table 1 Concentrations of total carotenoids using different chemical techniques of cell disruption on *S. pararoseus* and *R. mucilaginosa*

Microorganism	Acid	Concentration of carotenoids ($\mu\text{g/g}$)		Carotenoid extractability (%)	
		Freezing			
		Presence	Absence	Presence	Absence
<i>S. pararoseus</i>	Acetic	$13.7 \pm 1.7^{\text{bA}}$	$17.0 \pm 4.2^{\text{aA}}$	$15.8 \pm 1.7^{\text{bA}}$	$19.6 \pm 5.5^{\text{aA}}$
	Hydrochloric	$20.2 \pm 1.5^{\text{aA}}$	$18.8 \pm 3.3^{\text{aA}}$	$23.2 \pm 0.4^{\text{aA}}$	$21.6 \pm 4.8^{\text{aA}}$
	Lactic	$9.5 \pm 0.4^{\text{cA}}$	$11.0 \pm 1.4^{\text{aA}}$	$11.0 \pm 0.9^{\text{cA}}$	$12.7 \pm 2.2^{\text{aA}}$
<i>R. mucilaginosa</i>	Acetic	$93.2 \pm 8.9^{\text{aA}}$	$89.2 \pm 5.8^{\text{aA}}$	$31.6 \pm 5.4^{\text{aA}}$	$38.1 \pm 2.1^{\text{aA}}$
	Hydrochloric	$78.4 \pm 7.3^{\text{aA}}$	$63.8 \pm 8.5^{\text{bA}}$	$26.5 \pm 3.4^{\text{aA}}$	$27.3 \pm 3.9^{\text{bA}}$
	Lactic	$76.9 \pm 8.5^{\text{aA}}$	$81.7 \pm 7.3^{\text{aA}}$	$25.7 \pm 2.3^{\text{aB}}$	$34.9 \pm 2.6^{\text{aA}}$

Mean $\pm$ SD ($n = 3$); Different letters (a–c and A–B) represent significant differences among the columns and rows, respectively ($p < 0.05$); DMSO_{*S. pararoseus*}: 86.9 ± 5.3 $\mu\text{g/g}$ in the presence and 87.3 ± 4.4 $\mu\text{g/g}$ in the absence of freezing; DMSO_{*R. mucilaginosa*}: 297.6 ± 27.7 $\mu\text{g/g}$ in the presence and 234.1 ± 4.0 $\mu\text{g/g}$ in the absence of freezing; EC = 100% in the presence and absence of freezing (DMSO_{*S. pararoseus*} and DMSO_{*R. mucilaginosa*})

Table 2 Concentrations of total carotenoids using different mechanical techniques of cell disruption on *S. pararoseus*

Technique	Concentration of carotenoids (µg/g)		Carotenoid extractability (%)	
	Freezing			
	Presence	Absence	Presence	Absence
*Glass beads	75.1 ± 1.8 ^{bA}	76.9 ± 2.1 ^{aA}	66.2 ± 3.6 ^{bB}	88.3 ± 6.7 ^{aA}
*Ultrasonic bath	88.0 ± 3.3 ^{aA}	84.8 ± 2.3 ^{aA}	77.5 ± 3.6 ^{aB}	97.3 ± 6.2 ^{aA}
*Ultrasonic ruptor	51.9 ± 2.6 ^{cA}	39.0 ± 5.5 ^{bB}	45.8 ± 4.1 ^{cA}	44.9 ± 7.6 ^{bA}
**Diatomaceous earth	45.8 ± 2.7 ^{c,dA}	33.1 ± 8.5 ^{b,cA}	55.0 ± 3.3 ^{cA}	39.7 ± 10.2 ^{bA}
**Liquid nitrogen	43.0 ± 2.3 ^{dA}	24.0 ± 2.4 ^{cB}	55.1 ± 3.7 ^{cA}	33.7 ± 8.1 ^{bB}

Mean ± SD (n = 3); Different letters (a–d and A–B) represent significant differences among the columns and rows, respectively ($p < 0.05$)

* DMSO: 113.6 ± 4.6 and 87.3 ± 4.4 µg/g

** DMSO: 78.2 ± 3.3 and 72.7 ± 9.7 µg/g in the presence and absence of freezing, respectively

extractability of pigments, but lower than those of the sonic bath and glass beads. Freezing with liquid nitrogen resulted in low performance for all treatments. Higher results than the ones found by this study were obtained in different conditions of the production process for the recovery of carotenoids by *S. salmonicolor* [23]. They reached 913 µg/L with the use of liquid N₂ and DMSO in cell rupture. The recovery of astaxanthin from *Phaffia rhodozyma* [14] with ultrasonic bath application and abrasion with glass beads reached 2198.4 µg/g and 305.3 ± 27.7 µg/g, respectively. Other biocompounds, such as β-galactosidase enzyme, were also recovered by ultrasonic bath and abrasion with glass beads [21, 22].

Ultrasound-assisted cell disruption also achieved the best performance in the recovery of carotenoids produced by *R. mucilaginosa* (Table 3). It resulted in recovery of 202.4 µg/g with 72.9% extractability from frozen biomass; values did not differ statistically ($p > 0.05$) from the ones found with unfrozen biomass (193.5 µg/g and 77.4% extractability), showing that the freezing process did not influence the recovery of carotenoids.

The application of diatomaceous earth to the cell disruption process did not differ from ultrasonic bath except when freezing was used. *Rhodotorula* strain used by this study achieved higher results than *R. mucilaginosa* AY-01 [30], which obtained 98.4 ± 0.8 µg/g of total carotenoids with the same mechanical techniques of ultrasonic waves. Michelon et al. [12] evaluated techniques of cell disruption for the extraction of carotenoids produced by *P. rhodozyma* and found the best results with combined techniques which reached 163.12 µg/g (maceration of unfrozen biomass with ultrasonic waves and enzymatic lysis) and 190.35 µg/g (maceration of frozen biomass with diatomaceous earth and enzymatic lysis).

Visualization of *S. pararoseus* [Fig. 1(B)] and *R. mucilaginosa* [Fig. 1(D)] ruptured cells by light microscopy, by comparison with intact yeast cells [Fig. 1(A, C)], showed the success of the ultrasonic bath-assisted cell disruption. This method had the best results of carotenoid recovery in this study: in the absence of biomass freezing, values of 97.3% for *S. pararoseus* and 77.4% for *R. mucilaginosa* were found. They were close to those

Table 3 Concentrations of total carotenoids using different mechanical techniques of cell disruption on *R. mucilaginosa*

Technique	Concentration of carotenoids (µg/g)		Carotenoid extractability (%)	
	Freezing			
	Presence	Absence	Presence	Absence
*Glass beads	144.2 ± 2.4 ^{bA}	118.9 ± 4.5 ^{cB}	51.8 ± 4.2 ^{bA}	47.2 ± 1.9 ^{bA}
*Ultrasonic bath	202.4 ± 21.6 ^{aA}	193.5 ± 25.8 ^{aA}	72.9 ± 11.2 ^{aA}	77.4 ± 16.1 ^{aA}
*Ultrasonic ruptor	152.4 ± 11.7 ^{bA}	127.9 ± 8.9 ^{b,cB}	54.5 ± 0.7 ^{bA}	50.9 ± 6.9 ^{bA}
**Diatomaceous earth	124.1 ± 13.9 ^{bB}	159.3 ± 12.3 ^{a,bA}	57.1 ± 4.9 ^{a,bA}	70.3 ± 9.1 ^{a,bA}
***Liquid nitrogen	139.0 ± 11.9 ^{bA}	142.7 ± 6.6 ^{b,cA}	49.2 ± 4.4 ^{bB}	60.9 ± 1.7 ^{a,bA}

Mean ± SD (n = 3); different letters (a–c and A–B) represent significant differences among the columns and rows, respectively ($p < 0.05$)

* DMSO: 279.4 ± 18.6 and 252.5 ± 17.8 µg/g

** DMSO: 216.9 ± 10.1 and 228.7 ± 27.1 µg/g

*** DMSO: 282.6 ± 1.8 and 234.4 ± 4.4 µg/g in the presence and absence of freezing, respectively

Fig. 1 Yeast cells *S. pararoseus* (A) and *R. mucilaginosa* (C) without disruption; cells of *S. pararoseus* (B) and *R. mucilaginosa* (D) after disruption via ultrasonic bath

obtained with DMSO (100% extractability). Ultrasonic baths are advantageous since they are simple techniques which do not need constant manual work, produce toxic-free residue extract—enabling them to be used in industries [13]—and do not require expensive equipment [22].

According to Gerde et al. [31], when ultrasound is applied to a liquid state, it produces a cavitation phenomenon in the structure of cells, thus, contributing to cell disruption. In addition, sonication can increase the permeability of the cell, thereby increasing extraction of components. The treatment with ultrasonic bath (exposure time of 40 min and frequency of 40 kHz) showed higher results than the ultrasonic ruptor (exposure time of 20 min and frequency of 20 kHz) when applied to the cell wall of both yeasts (Tables 2, 3), because both exposure time and frequency used by this method were higher.

Biomass freezing caused significant increase ($p < 0.05$) in the recovery of carotenoids when the ultrasonic ruptor and liquid nitrogen were used for *S. pararoseus* (Table 2) and when the ultrasonic ruptor and abrasion with glass beads were used for *R. mucilaginosa* (Table 3). According to Voda et al. [32], rapid freezing leads to growth of small ice crystals within the cell, causing less damage to the cell

wall, while slow freezing allows ice crystals to grow outside the cell, causing damage to the cell structure and promoting disruption. Thus, the size of ice crystals affects the degree of damage caused to the cell membrane.

The use of liquid nitrogen for rapid freezing caused the formation of cracks in the cell walls, thereby increasing damage in the structure and consequently increasing specific concentration and extractability of carotenoids (Table 2). This behavior was not observed by the method that applied liquid nitrogen to the cell wall of *R. mucilaginosa* (Table 3), where freezing did not favor specific concentration and extractability of carotenoids. It may be explained by the fact that ice crystals formed during freezing were not sufficient to increase the disruption by this method for this yeast. Therefore, the yield with respect to freezing seems to depend not only upon the type of yeast used, but also on the type of mechanical disruption technique applied.

In general, among mechanical disruption techniques, the most promising results were obtained by the ultrasonic bath and the freezing process did not affect the recovery of carotenoids for both yeasts. Thus, this step can be discarded and time and energy can be saved.

Several studies have reported the efficient use of ultrasound to obtain different biocompounds. Li et al. [33] used sunflower oil as an organic solvent for carotenoid extraction from carrots and found high recovery of β -carotene (334.75 mg/L after 20 min of ultrasound, intensity 22.5 W/cm² at 40 °C). Gerde et al. [31] studied the extraction of chlorophyll and carotenoids from autotrophic and heterotrophic microalgae. Both species needed approximately 800 J/10 mL of energy to maximize cell disruption, regardless of the cell concentrations under study, reaching 1.0 μ g chlorophyll/cell mg and 0.3 μ g carotenoid/mg.

Biomass drying by lyophilization

Specific concentrations of carotenoids obtained from *S. pararoseus*, with and without the freeze-drying process, were 98.6 ± 2.9 and 93.9 ± 2.4 μ g/g, respectively, showing no significant difference ($p > 0.05$). In conventional drying, concentrations of specific carotenoids were 78.2 ± 3.3 μ g/g (frozen biomass) and 72.7 ± 9.8 μ g/g (unfrozen biomass) (Fig. 2). There was no significant difference ($p > 0.05$), either. The lyophilization process resulted in an increase of approximately 20% in the carotenoid specific concentration from *S. pararoseus*, by comparison with that obtained by the conventional drying process. The increase in carotenoid recovery by lyophilization may be explained by the direct transition from solid to gas, causing change in the physical state by

sublimation and making the product more stable due to decreased water activity [34].

In Fig. 2, the method of drying by lyophilization with frozen and unfrozen biomass showed 296.5 ± 12.3 and 292.8 ± 8.9 μ g/g, respectively, for *R. mucilaginosa*. For conventional drying, values were 279.4 ± 18.6 μ g/g (frozen biomass) and 252.5 ± 17.8 μ g/g (unfrozen biomass). The use of lyophilization, regardless of the fact that the biomass of *R. mucilaginosa* is frozen or not, led to a significant increase ($p > 0.05$) in the recovery of carotenoids, by comparison with the conventional drying, which was about 13.7% with the frozen biomass.

Chemical and mechanical techniques can be used for the disruption of cell walls of *S. pararoseus* and *R. mucilaginosa* yeasts for the recovery of carotenoids. Cell disruption assisted by ultrasonic bath reached the best results with unfrozen biomass and showed 84.79 ± 2.34 and 193.5 ± 25.8 μ g/g for *S. pararoseus* and *R. mucilaginosa*, respectively. Lyophilization promoted gains of 20% and 13.7% in the recovery of carotenoids of *S. pararoseus* and *R. mucilaginosa*, respectively, by comparison with the conventional drying procedure.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Fig. 2 Concentrations of total carotenoids (μ g/g) in treatment 1: Lyophilization (frozen biomass); treatment 2: Lyophilization (not frozen biomass); treatment 3: conventional drying (frozen biomass); treatment 4: Conventional drying (not frozen biomass). Different letters represent significant differences among the columns for the same microorganism ($p < 0.05$)

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