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RESEARCH ARTICLE

Extraction of Carotenoids from *Phaffia rhodozyma*: A Comparison between Different Techniques of Cell Disruption

Mariano Michelon, Thais de Matos de Borba, Ruan da Silva Rafael, Carlos André Veiga Burkert, and Janaína Fernandes de Medeiros Burkert

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Abstract The yeast *Phaffia rhodozyma* is known for producing carotenogenic pigments, commonly used in aquaculture feed formulation as well as in cosmetic, pharmaceutical, and food industries. Despite the high production of carotenoids from microorganisms by biotechnology, their use has limitation due to the cell wall resistance, which constitutes a barrier to the bioavailability of carotenoids. Therefore, there is a need to improve carotenoids recovering technique from microorganisms for the application of food industries. This study aimed to compare mechanical, chemical, and enzymatic techniques of cell disruption for extracting carotenoids produced by *P. rhodozyma* NRRL Y-17268. Among the techniques studied, the highest specific concentration of carotenoids (190.35 μg/g) resulted from the combined techniques of frozen biomass maceration using diatomaceous earth and enzymatic lysis at pH of the reaction medium of 4.5 at 55ºC, with initial activity of β-1,3 glucanase of 0.6 U/mL for 30 min.

Keywords: β-1,3 glucanase, enzymatic lysis, dimethyl sulfoxide, biomass, diatomaceous earth

Introduction

Carotenoids are natural pigments responsible for yellow, orange, and red in many foods such as fruits, vegetables, egg yolks, and fish like salmon and shellfish. Besides coloring, carotenoids have important biological activity due to their provitamin A activity and properties resulting in potential health benefits such as strengthening the immune system and decreasing the risks of degenerative diseases (1). Furthermore, carotenoids have antioxidant action by neutralizing free radicals as electron donors. Thus, antioxidants prevent the damage caused on living cells by free radicals (2).

Interest in carotenoids has recently increased due to the growing demand for such compounds in pharmaceutical, cosmetic, food, and feed industries (3). The global market demand for carotenoids grows 2.9% per year, with estimated annual sales of about US$ 300 million in synthetic carotenoids (4,1). Commercial production of carotenoids from microorganisms competes mainly with the synthetic production by chemical procedures. Carotenoids used industrially are mainly obtained chemically or by extraction of plants or algae. However, due to the concern about the use of chemical additives in foods, there is increasing interest in carotenoids naturally obtained by biotechnological processes (1). Therefore, the yeast *Phaffia rhodozyma* stands out as a natural source of carotenoids (5-8).

Carotenoids synthesized by *P. rhodozyma* are of intracellular kind, which hampers their use as an additive color on an industrial scale. This fact points out the need of developing and implementing techniques for cell disruption which are adequate to recover the carotenoids produced in order to enable future applications in food products. Since the cell wall composition of the yeast *P. rhodozyma* has β-glucose, α-glucose, and a complex heteropolysaccharide consisting of L-arabinose, D-xylose, D-glucuronic acid, and D-galactose (9), several studies have aimed to elucidate and implement cell disruption techniques for extraction of

Mariano Michelon (✉), Thais de Matos de Borba, Ruan da Silva Rafael, Carlos André Veiga Burkert, Janaína Fernandes de Medeiros Burkert
Bioprocess Engineering Laboratory, School of Chemical and Food, Federal University of Rio Grande, Rua Alfredo Huch 475, 96201-900, Rio Grande, RS, Brazil
Tel: +55-53-32338755; Fax: +55-53-32338720
E-mail: marianomichelon@hotmail.com

microbial carotenoids (4,10-14). Moreover, the use of commercial lytic enzymes obtained from micro-organisms to promote cell disruption and facilitate carotenoid extraction is still little explored.

Therefore, this study aimed to compare the efficacy of different mechanical, chemical, and enzymatic techniques of cell disruption for extraction of carotenoids produced by the yeast *P. rhodozyma.*

Materials and Methods

Microorganism The yeast *Phaffia rhodozyma* NRRL Y-17268 was used for production of carotenoids. It was provided by the Northern Regional Research Laboratory (Peoria, IL, USA) and certified as 'generally recognized as safe' (GRAS). Before the experiments, the yeast was maintained on yeast malt (YM) agar and stored at 4ºC.

Inoculum A microbial culture tube was used to obtain the inoculum previously incubated at 25ºC for 48 h. The culture was scraped with 10 mL of peptone water 0.1%(w/v) and the suspension obtained was transferred to a 500-mL Erlenmeyer flask containing 90 mL YM broth supplemented with 0.2 g/L of KNO_3 (6). The suspension was maintained at 25ºC in rotary incubator at 150 rpm and growth was monitored by counting in a Neubauer chamber (15).

Shaken flasks cultivation *P. rhodozyma* NRRL Y-17268 was cultivated in Erlenmeyer flasks of 500-mL containing 153 mL of culture medium proposed by Silva (16), with the following composition: 16.25 g/L malt extract, 8.75 g/L peptone, 15 g/L sucrose, 87.5 g/L rice parboilization waste water, and initial pH of 5.0. Each flask was inoculated with 17 mL of yeast suspension previously prepared, in order to achieve 1×10^7 cells/mL at the beginning of cultivation. The flasks were maintained in rotary incubator at 25ºC and 150 rpm for 168 h. The biomass of *P. rhodozyma* was collected from the fermented medium by centrifugation at 1,745×*g* for 10 min. The sediment was washed twice with distilled water and subjected to drying operation at 35ºC for 48 h (17).

Techniques of cell disruption For the extraction of carotenoids produced by the yeast *P. rhodozyma,* different mechanical, chemical, and enzymatic techniques of cell disruption were used, with dry biomass subjected or not to freezing at −18ºC for 48 h (17).

***Mechanical techniques*:** (1) Ultrasonic waves: This technique was performed by adapting the method proposed by Medeiros *et al.* (18). Tubes containing 0.5 g of biomass and 6 mL of acetone were subjected to 4 ultrasonic cycles of 40 kHz for 10 min. (2) Maceration with diatomaceous earth: This technique was performed by adapting the method proposed by Valduga *et al.* (13). An amount of 0.5 g of biomass and 0.5 g of diatomaceous earth was macerated for 10 min using a pestle in a mortar, to which 6 mL of acetone were added. (3) Abrasion with glass pearls: This technique was performed by adapting the method proposed by Medeiros *et al.* (18). Tubes containing 0.5 g of biomass and 6 mL of acetone were added with a load of 1.1 g/mL glass pearls (0.5-0.59 mm) and agitated vigorously for 10 min in vortex agitator. (4) Immersion in liquid nitrogen: Tubes containing 0.5 g of biomass were added with 6 mL of acetone, followed by immersion in liquid nitrogen and subsequent maceration of the frozen mixture using pestle in a mortar for 1 min. After applying the techniques of cell disruption, the supernatant was separated by centrifugation (1,745×*g* for 10 min) for subsequent extraction of carotenoids.

***Chemical techniques*:** (1) Sodium bicarbonate: Tubes were filled with 0.5 g of biomass and 18 mL of 0.1 M of sodium bicarbonate, and the mixture was subjected to a bath at a temperature of 40-45ºC for 24 h (19). Centrifugation was performed (1,745×*g* for 10 min) and the supernatant was discarded. The precipitate was washed twice using 18 mL of distilled water to eliminate residues of sodium bicarbonate. (2) Dimethyl sulfoxide (DMSO): This technique was performed by modifying the method proposed by Sedmak *et al*. (20). Tubes containing 0.05 g of biomass were added with 2 mL of DMSO at a temperature of 55ºC and agitated vigorously in a tube agitator for 1 min, and then left undisturbed for 1 h, this procedure was repeated until the maximum discoloration of cells. (3-5) Hydrocloridric acid, lactic acid, or acetic acid: Tubes were filled with 0.5 g of biomass and 7.5 mL of 4 M hydrochloric acid, acetic or lactic acid solution and were subjected to an agitated bath for 5 min at 35ºC (12). Centrifugation was performed (1,745×*g* for 10 min) and the supernatant was discarded. The sediment was washed twice using 7.5 mL of distilled water to eliminate acid waste. Subsequent to cell disruption, all chemical techniques were added with 6 mL of acetone and the supernatant containing the carotenoids was separated by centrifugation (1,745×*g* for 10 min).

***Enzymatic techniques*:** The enzymatic lysis of the cell wall of *P. rhodozyma* for carotenoid extraction was performed using the commercial enzyme compound Glucanex® (Novozymes S.A., Bagsvaerd, Denmark) containing the enzyme β-1,3 glucanase obtained from the fungus *Trichoderma harzianum* (21). In order to determine the optimum condition for lysis, 2_{IV}^{4-1} fractional design was carried out. The variables studied included pH of the reaction medium, temperature, initial activity of β-1,3 glucanase enzyme, and reaction time. In addition, relative lytic activity was evaluated. Under the condition of cell lysis, the enzymatic technique of cell disruption was

applied. Tubes containing suspended biomass with absorbance value of 1.68 at 660 nm (22), corresponding value of 0.011 g of dry weight of *P. rhodozyma* subjected or not to freezing, sodium acetate buffer (0.2 M, pH 4.5) and enzyme extract were added so as to match an initial activity of β-1,3 glucanase equal to 0.6 U/mL. The final mixture (4 mL) was incubated at 55ºC in agitated bath for 30 min. Centrifugation was performed (1,745×*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 acetone was added and the supernatant containing the carotenoids was separated by centrifugation (1,745×*g* for 10 min). The same procedure was performed in ultrasonic bath with a frequency of 40 kHz by replacing the agitated bath in order to evaluate the influence of ultrasonic waves on the enzymatic lysis of the cell wall.

Extraction and determination of carotenoids The supernatants obtained after applying the techniques of cell disruption were transferred to amber flasks. Next, 10 mL of 20%(w/v) NaCl and 10 mL of petroleum ether were added, and the ether phase was filtered through sodium sulfate. The concentration of carotenoids was determined by spectrophotometric reading of the filtrate at 474 nm and calculated using Eq. 1 (23,24), as follows:

$$\text{Concentration of carotenoids (}\mu\text{g/g dry biomass)} = (10^4 AV)/(2100W) \quad (1)$$

where, A is the absorbance value of diluted extraction at 474 nm, V (mL) is the volume of acetone (filtrate), 2,100 is the molar absorptivity coefficient (25), and W (g) is the weight of dried biomass. The carotenoid extractability was calculated by Eq. 2 (26) modified, as follows:

$$\text{Carotenoids extractability (\%)} = (CA)/(CT)*100 \quad (2)$$

where, CA is the concentration of carotenoids (μg/g) contained in the cell for analysis of cell disruption technique under study and CT is the total carotenoid concentration (μg/g) contained in the cells of *P. rhodozyma*. The CT was obtained using cell disruption with DMSO (26).

Activity of β-1,3 glucanase The reaction mixture (0.5 mL) containing 0.25 mL of enzyme solution and 0.25 mL of laminarin solution 1%(w/v) obtained from the seaweed *Laminaria digitata* (Sigma-Aldrich) in sodium acetate buffer (0.1 M, pH 5.5) was incubated at 55ºC for 30 min. The reaction was stopped by heating at 100ºC for 5 min (27). The reducing sugars were determined by the method of 3,5-dinitrosalicylic acid using glucose as standard sugar (28). For the control the reducing sugars present in the enzyme solution were determined using distilled water instead of laminarin solution. One activity unit of β-1,3 glucanase (U) was defined as the release of 1 μmol of glucose/min/mL of enzyme solution.

Proteolytic activity The proteolytic activity was determined according to the method proposed by Daroit *et al*. (29), using azocasein (Sigma-Aldrich) as substrate. The reaction mixture (0.5 mL) contained 0.1 mL Tris-HCl buffer (100 mM, pH 8.0), 0.3 mL of 1%(w/v) azocasein (in Tris-HCl buffer), and 0.1 mL of conveniently diluted enzyme. After incubation at 40ºC for 30 min the reaction was stopped by adding 0.6 mL of 10%(w/v) trichloroacetic acid. After centrifugation (1,745×*g* for 20 min) of the reaction mixture, 0.8 mL of supernatant was mixed with 0.2 mL of 1.8 M NaOH, and the absorbance at 420 nm was measured. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions.

Relative lytic activity Relative lytic activity was determined using a reaction mixture containing 2 mL of cell suspension of the yeast *P. rhodozyma* with absorbance equal to 1.68 at 660 nm (22) and 2 mL of enzyme solution diluted in appropriate buffer. At the same time, a white tube was prepared as a reference, where instead of enzyme solution only buffer was added. The relative lytic activity was calculated by modifying the method described by Obata *et al.* (30), using Eq. 3, as follows:

$$\text{Relative lytic activity (\%)} = ((AR - AM)/AR_i)100 \quad (3)$$

where, AR is the absorbance of reference at 660 nm, AM is the absorbance of reaction mixture at 660 nm, and AR_i is the initial absorbance of reference at 660 nm.

Protein content Protein content was determined by the Kjeldahl method, using a factor of 6.25 to convert nitrogen into protein content, according to AOAC official method 962.10 (31).

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

Results and Discussion

Mechanical techniques of cell disruption As shown in Table 1, the extraction of carotenoids from biomass not subjected to freezing prior to cell disruption showed no significant differences ($p<0.05$) on the concentration of carotenoids and extractability when used the techniques of

Table 1. Concentration of total carotenoids using different mechanical techniques of cell disruption

Technique	Concentration of carotenoids (μg/g)		Carotenoid extractability (%)	
	Freezing			
	Absence	Presence	Absence	Presence
Maceration with diatomaceous earth	88.80±1.10$^{aB1)}$	93.13±1.96^{aA}	57.03±0.71^{aB}	59.81±1.26^{aA}
Abrasion with glass pearls	70.51±8.10^{bA}	75.42±2.90^{bA}	45.28±5.19^{bA}	48.43±1.86^{bA}
Ultrasonic waves	88.38±2.51^{aA}	79.20±2.66^{bB}	56.75±1.61^{aA}	50.86±1.71^{bB}
Immersion in liquid nitrogen	84.68±2.79^{aA}	82.05±7.21^{bA}	54.38±1.80^{aA}	52.70±5.64^{bA}
Control$^{2)}$	11.58±1.61^{cB}	15.83±0.86^{cA}	7.44±1.04^{cB}	1.17±0.56^{cB}

$^{1)}$Mean±SD (n=3); Different letters (a-c) and (A-B) represent significant differences in the column and row, respectively ($p<0.05$).
$^{2)}$Carotenoid extractions performed in the absence of cell disruption techniques

ultrasonic waves (88.38 μg/g and 56.75%), immersion in liquid nitrogen (84.68 μg/g and 54.38%) and maceration with diatomaceous earth (88.80 μg/g and 54.03%). The extraction of carotenoids from biomass subjected to freezing prior to cell disruption, using the technique of maceration with diatomaceous earth, resulted in the highest concentration of carotenoids and extractability (93.13 μg/g and 59.81%), differing statistically from the other techniques. Cell freezing prior to disruption caused a significant increase ($p<0.05$) when the concentration of carotenoids used the technique of maceration with diatomaceous earth. This was not observed when the techniques of immersion in liquid nitrogen and abrasion with glass pearls were used, since the freezing cell had no influence on the disruption.

The use of ultrasonic waves in biomass subjected to freezing prior to cell disruption decreased significantly ($p<0.05$) the extraction of carotenoids. This fact can be related with cavitation phenomena, i.e., the shear stress developed by viscous dissipative eddies arising from shock waves produced by imploding cavitation bubbles. Thus, larger cells experience more disruptive eddies than smaller cells (10). The formation of ice crystals during freezing possibly caused lesions in the cell wall, resulting in the loss of cytoplasmic fluids and therefore a reduction in size. So, the cells showed a smaller size than eddies formed by ultrasonic waves.

The control experiments, where the biomass was subjected to extraction without the step of cell disruption, showed carotenoid concentration of 11.58 and 15.83 μg/g in the absence and presence of previous biomass freezing, respectively. This occurred probably because the carotenoids produced by the yeast *P. rhodozyma* have a strong link to the cell, or they suggest the need for an efficient cell disruption due to the high rigidity of such yeast wall.

Chemical techniques of cell disruption With respect to chemical techniques for cell disruption in the presence or absence of biomass prior freezing, the Table 2 shows that the highest concentrations of carotenoids and extractabilities were found for cell disruption with DMSO. This result was expected because such technique has been used in various studies as an analytical technique for quantification of microbial carotenoids (20). However, the presence of toxic compounds, possibly derived from the DMSO, in the carotenogenic extract and the compliance with the existing laws for food are factors that hinder the application of this technique for industrial production of microbial carotenoids.

Table 2 shows that the biomass prior freezing caused a significant increase ($p<0.05$) in the concentration of carotenoids when using techniques with sodium bicarbonate and hydrochloric acid of approximately 56 and 6% respectively. The opposite effect was observed when using

Table 2. Concentration of total carotenoids using different chemical techniques of cell disruption

Technique	Concentration of carotenoids (μg/g)		Carotenoid extractability (%)	
	Freezing			
	Absence	Presence	Absence	Presence
Sodium bicarbonate	16.43±2.71$^{eB1)}$	36.96±1.57^{cA}	10.55±1.74^{eB}	23.74±1.01^{cA}
Latic acid	47.72±3.65^{cA}	20.20±2.72^{deB}	30.65±2.34^{cA}	12.97±1.75^{deB}
Acetic acid	30.55±1.13^{dA}	24.63±2.58^{dB}	19.62±0,72^{dA}	15.82±1.66^{dB}
Hydrocloridric acid	61.22±1.27^{bB}	65.37±1.99^{bA}	39.32±0,81^{bB}	41.98±1.27^{bA}
Dimethyl sulfoxide	153.91±2.57^{aA}	155.72±2.34^{aA}	ND	ND
Control$^{2)}$	15.12±0.55^{eB}	17.30±1.06^{eA}	9.71±0.36^{eB}	11.11±0.68^{eA}

$^{1)}$Mean±SD (n=3); Different letters (a-e) and (A-B) represent significant differences in the column and row, respectively ($p<0.05$); ND, not detected
$^{2)}$Carotenoid extractions performed in the absence of cell disruption techniques

techniques with lactic acid and acetic acid, where freezing caused a significant reduction ($p<0.05$) at concentrations of approximately 55 and 20% respectively.

Regarding acids for cell disruption, when hydrochloric acid was used there was larger concentration of carotenoids than with the use of lactic and acetic acids. This phenomenon can be explained by the values of constant acidity (pKa). The stronger the acid, the greater the efficiency of the yeast cell disruption showed (12). Hydrochloric acid is a strong acid (pKa= −7), whereas lactic acid (pKa=3.83) and acetic acid (pKa=4.74) are weak acids. The acidity constant of this study was proportional to the increase in the extraction of carotenoids.

The technique with sodium bicarbonate is classic for cell disruption of *Saccharomyces cerevisiae* for the extraction of proteins related to some cell wall carbohydrates. However, the disruption caused by this technique was not effective to break the cell wall of *P. rhodozyma*, probably because its structure is mainly composed of insoluble glucan, which is responsible for the hardness against mechanical shock and osmotic imbalance (32), thus differing from the *S. cerevisiae* yeast cell wall.

Ni *et al*. (12) studied chemical methods for cell disruption de *P. rhodozyma* Past -1 strain using lactic acid, acetic acid, and hidrochloric acid and verified carotenoids extractability of 84.1, 77.3, and 78.9%, respectively. Xião *et al*. (26) using different techniques for *P. rhodozyma* 7B12 cell disruption, like spray drying, autolysis, and other treatment ways: acid, basic, autoclaving, and acid/autoclaving. The best results were the acid/autoclaving treatment (HCl 0.5 M and autoclaved 5 min, 121ºC), which promoted 85% of carotenoids extractability. Gu *et al*. (4) found high concentrations of carotenoids of *Rhodobacter sphaeroides* using hydrochloric acid (3 M) at 28ºC and an acid/biomass contact time of 30 min.

Enzymatic techniques of cell disruption Glucanex® showed specific enzymatic activity of 0.49 U/mg protein for β-1,3 glucanase and of 2.52×10^5 U/mg protein for protease. This confirmed the active presence of both enzymes needed for cell wall lysis of the yeast *P. rhodozyma*.

***Evaluation of cell wall enzymatic lysis of P. rhodozyma by fractional design*:** Table 3 shows a matrix of a 2_{IV}^{4-1} fractional design with 3 replications at the central point, where relative lytic activity ranged from 0.16% in Assay 4 (pH 8.5, 55ºC, 0.2 U/mL of β-1,3 glucanase and 30 min) to 22.44% in Assay 7 (pH 4.5, 55°C, 0.6 U/mL of β-1,3 glucanase and 30 min).

An estimate of a main effect is obtained by evaluating the difference in process performance caused by a change from low (−1) to high (+1) levels of the corresponding variable (33). In Table 4, all variables showed significant effect ($p<0.05$) on the relative lytic activity, where the initial activity of the enzyme β-1,3 glucanase showed the greatest influence on enzymatic lysis followed by temperature, pH of the reaction medium and reaction time. The transition from level 1 (4.5) to +1 (8.5) in the pH of the reaction medium and from level −1 (30 min) to +1 (90 min) in the enzymatic reaction time caused a decrease in relative lytic activity of 2.87 and 2.15% respectively. The increase of 20ºC in temperature and 0.4 U/mL in the initial activity of β-1,3 glucanase caused the increase in relative lytic activity of 4.09% and 9.31 respectively.

Therefore, the effects analysis allowed to obtain the best conditions for cell lysis of 55ºC, pH 4.5, after 30 min of reaction, with initial activity of β-1,3 glucanase of 0.6 U/mL, resulting in a relative lytic activity of 22.44%, according to Assay 7 in Table 3. The enzyme β-1,3 glucanase from *Trichoderma harzianum* has optimum temperature of 55ºC and pH 4.5 (32), but the protease produced by the such microorganism has optimum temperature of 40ºC and pH 8.0 (34). Although the enzymatic lysis occurs by synergism of the enzymes protease and β-1,3 glucanase (32), the optimum condition of pH and temperature for lysis of *P. rhodozyma* in this study corresponds to the optimum pH and temperature of

Table 3. Matrix of 2_{IV}^{4-1} fractional design in actual levels (coded)[1)]

Assay	X1	X2	X3	X4	X5
1	4.5 (-1)	35 (-1)	0.2 (-1)	30 (-1)	4.67
2	8.5 (+1)	35 (-1)	0.2 (-1)	90 (+1)	4.00
3	4.5 (-1)	55 (+1)	0.2 (-1)	90 (+1)	7.60
4	8.5 (+1)	55 (+1)	0.2 (-1)	30 (-1)	0.16
5	4.5 (-1)	35 (-1)	0.6 (+1)	90 (+1)	6.10
6	8.5 (+1)	35 (-1)	0.6 (+1)	30 (-1)	12.09
7	4.5 (-1)	55 (+1)	0.6 (+1)	30 (-1)	22.44
8	8.5 (+1)	55 (+1)	0.6 (+1)	90 (+1)	13.05
9	6.5 (0)	45 (0)	0.4 (0)	60 (0)	10.15
10	6.5 (0)	45 (0)	0.4 (0)	60 (0)	9.04
11	6.5 (0)	45 (0)	0.4 (0)	60 (0)	9.55

[1)]X1, pH of the reaction medium; X2, temperature (ºC); X3, initial activity of β-1,3-glucanase (U/mL); X4, reaction time (min); X5, relative lytic activity (%)

Table 4. Effect estimates for relative lytic activity

Factor[1)]	Effect (%)	Standard error	*t* (2)	*p*-value
Media	8.98	0.16	53.64	<0.01
X1	-2.87	0.39	-7.32	0.01
X2	4.09	0.39	10.42	<0.01
X3	9.31	0.39	23.70	<0.01
X4	-2.15	0.39	-5.47	0.03

[1)]X1, pH of the reaction medium; X2, temperature (ºC); X3, initial activity of β-1,3-glucanase (U/mL); X4, reaction time (min)

Table 5. Concentration of total carotenoids using different enzymatic techniques of cell disruption

Assay	Technique[1)]	Freezing	Concentration of carotenoids (μg/g)	Carotenoid extractability (%)
1	Enzymatic lysis	Absence	143.90±3.65[b2)]	92.42±2.34[b]
2	Enzymatic lysis	Presence	156.96±4.55[a]	101.81±2.92[a]
3	Enzymatic lysis + Ultrasonic waves	Absence	163.12±5.54[a]	104.76±3.56[a]
4	Enzymatic lysis + Ultrasonic waves	Presence	114.86±1.68[c]	73.76±1.08[c]
5	Control	Absence	17.34±1.57[d]	11.11±1.01[d]
6	Control	Presence	24.12±1.07[d]	15.49±0.68[d]

[1)]Control, carotenoid extractions performed in the absence of cell disruption techniques
[2)]Mean±SD (n=3); Different lowercase letters represent significant differences in the column ($p<0.05$).

the enzyme β-1,3 glucanase. Depending on the molecular weight and 3-dimensional conformation of the enzyme, glucanases may lyse yeast cells in the absence of protease and other products that carry the role of hydrolysis of the external layer of mannan-protein (35).

Values lower than those reported in this study were described by Fleuri and Sato (36), who used the purified enzyme β-1,3 glucanase from the bacteria *Cellulosimicrobium cellulans* 191 in the enzymatic lysis of various yeasts at pH 6.5 and 30ºC for 60 min of enzymatic reaction, and found relative lytic activity of 15.12% for *S. cerevisiae* KL 88, 8.43% for *Candida glabrata* NCYC 388, 10.87% for *Saccharomyces capensis*, 20.64% for *Kluyveromyces drosophilarum,* and 21.93% for *Pichia membranaefaciens*.

The enzymatic lysis of yeast cells has a wide field of application in the production of recombinant proteins, enzymes, hormones, functional cell wall polymers, degradation of cell wall for using extract in food and animal feed as well as post-treatment for mechanical disruption (37). The susceptibility of the yeast *P. rhodozyma* to enzymatic lysis by Glucanex® as verified in this study enables a widening of the fields of application of this yeast lytic enzyme to obtain carotenogenic extracts through biotechnology.

***Cell disruption using enzimatic lysis and ultrasonic waves*:** With respect to the techniques of cell disruption of the yeast *P. rhodozyma* using enzymatic lysis with Glucanex®, Table 5 shows that the highest concentration of carotenoids and extractability (163.12 μg/g and 104.76%) was found using the enzymatic lysis in the presence of ultrasonic waves without biomass prior freezing (Assay 3). This did not differ significantly from Assay 2, where enzymatic lysis was applied in the absence of ultrasound and the step of biomass prior freezing was included (156.96 μg/g and 101.86%).

The step of biomass prior freezing before enzymatic lysis with ultrasound (Assay 4) caused a reduction in the concentration of carotenoids of about 30% compared with the same technique without freezing (Assay 3). Similar pattern was already found in experiments of mechanical cell disruption using ultrasonic waves, according Table 1. The opposite effect was observed in enzymatic lysis without using ultrasound, where freezing (Assay 2) promoted an increase of approximately 8% in the concentration of carotenoids when compared to Assay 1.

The use of ultrasound on enzymatic lysis of the biomass not frozen (Assay 3) caused an increase of 11% in the concentration of carotenoids compared with the technique of lysis enzyme in biomass not subjected to freezing (Assay 1). The same pattern was not observed in the biomass subjected to freezing, where ultrasound (Assay 4) caused a decrease in the concentration of carotenoids of about 27% compared with the enzymatic lysis assay (Assay 2).

Combined techniques In order to increase the extraction of carotenoids, disruption of the cell wall of *P. rhodozyma* was performed using the most efficient mechanical technique followed by enzymatic lysis. These experiments were accomplished only on biomass subjected to the thermal process of freezing, since for both techniques this step had a positive influence on the extraction of carotenoids. The concentration of carotenoids obtained from each technique separately and their combination is shown in Table 6.

The combined technique of cell disruption resulted in the highest concentration of carotenoids and extractability (190.35 μg/g and 122.25%). The combination of techniques showed an increase of approximately 65 and 18% in the concentration of carotenoids for maceration with diatomaceous

Table 6. Concentration of total carotenoids using the techniques of maceration with diatomaceous earth, enzymatic lysis, and the combination of both in the biomass subjected to freezing prior to cell disruption

Technique	Concentration of carotenoids (μg/g)	Carotenoid extractability (%)
Maceration with diatomaceous earth	93.13±1.96[c1)]	59.81±1.26[c]
Enzymatic lysis	156.96±4.55[b]	101.81±2.92[b]
Maceration with diatomaceous earth + Enzymatic lysis	190.35±4.39[a]	122.25±2.82[a]

[1)]Mean±SD (n=3); Different lowercase letters represent significant differences in the column ($p<0.05$).

earth and enzymatic lysis, respectively. When comparing the combination of techniques for cell disruption with the chemical technique using DMSO, there was an increase in the concentration of carotenoids of about 18%.

Studies with a focus on maximizing the cell disruption of *P. rhodozyma* using enzymatic lysis combined with other techniques are not found in the literature until this moment. Bjerkeng *et al*. (21) promoted the cell rupture of *P. rhodozyma* with an enzyme preparation produced *Trichoderma harzanium* (Glucanex®), the same used in the development of this work to evaluate the digestibility and muscle retention astaxanthin in Atlantic salmon (*Salmo salar*). The biological assays were performed with 2 formulations of diets containing astaxanthin synthetic and from yeast treated with Glucanex®. The use of microbial carotenoid obtained by enzymatic disruption showed higher muscle retention efficiency (2.56 mg/kg) compared to the synthetic (1.96 mg/kg).

Furthermore, the combination of techniques of biomass maceration subjected to freezing with diatomaceous earth and enzymatic lysis resulted in the highest concentration of carotenoids, surpassing the chemical technique of cellular disruption with DMSO. Obtaining a carotenogenic extract by means of biotechnological techniques using efficient cell disruption represents important scientific advances and allows possible extensions from laboratory to industrial scale.

The studies undertaken allowed to establish that the combined techniques of frozen biomass maceration with diatomaceous earth and enzymatic lysis at the pH of the reaction medium of 4.5 at 55ºC, with initial activity of β-1,3 glucanase of 0.6 U/mL for 30 min, resulted in the highest concentration of carotenoids and extractability (190.35 μg/g and 122.25%), compared to other techniques studied. Therefore, the use of enzymatic lysis for cell disruption is promising for the recovery of intracellular carotenoids produced by the yeast *P. rhodozyma*.

References

1. Valduga E, Tatsch PO, Tiggemann L, Treichel H, Toniazzo G, Zeni J, Luccio MD. Carotenoids production: Microorganisms as source of natural dyes. Quim. Nova 32: 2429-2436 (2009)
2. Campocosío AT. Pigments from yeast. pp. 181-193. In: Yeasts and Their Products as Ingredients in the Food Industry. Wagner JR, Rambala MAO, Legarreta I (eds.). Universidad Nacional de Quilmes Editorial, Bernal, Argentina (2008)
3. Valduga E, Valério A, Tatsch PO, Treichel H, Furigo Jr A, Luccio MD. Optimization of the production of total carotenoids by *Sporidiobolus salmonicolor* (CBS 2636) using response surface technique. Food Bioprocess Tech. 2: 415-421 (2009)
4. Gu Z, Chen D, Han Y, Chen Z, Gu F. Optimization of carotenoids extraction from *Rhodotobacter sphaeroides*. LWT -Food Sci. Technol. 41: 1082-1088 (2008)
5. Fontana JD, Czeczuga B, Bonfim TMB, Chociai MA, Oliveira BH, Guimarães MF, Baron M. Bioproduction of carotenoids: The comparative use of raw sugarcane juice and depolymerized bagasse by *Phaffia rhodozyma*. Bioresource Technol. 58: 121-125 (1996)
6. Parajó JC, Santos V, Vázquez M. Optimization of carotenoid production by *Phaffia rhodozyma* cells grown on xylose. Process Biochem. 33: 181-187 (1998)
7. Liu YS, Wu JY, Ho KP. Characterization of oxygen transfer conditions and their effects on *Phaffia rhodozyma* growth and carotenoid production in shake-flask cultures. Biochem. Eng. J. 27: 331-335 (2006)
8. Frengova GI, Beshkova DM. Carotenoids from *Rhodotorula* and *Phaffia*: Yeasts of biotechnological importance. J. Ind. Microbiol. Biot. 36: 163-180 (2009)
9. Calo P, Gonzáles T. The yeast *Phaffia rhodozyma* as an industrial source of astaxanthin. Microbiologia Sem. 11: 386-388 (1995)
10. Geciova J, Bury D, Jelen P. Methods for disruption of microbial cells for potential use in the dairy industry - A review. Int. Dairy J. 12: 541-553 (2002)
11. Park PK, Kim EY, Chu KH. Chemical disruption of yeast cells for the isolation of carotenoids pigments. Sep. Purif. Technol. 53: 148-152 (2007)
12. Ni H, Chen Q, He G, Wu G, Yang Y. Optimization of acidic extraction of astaxanthin from *Phaffia rhodozyma*. J. Zhejiang Univ. -Sc. B 9: 51-59 (2008)
13. Valduga E, Valério A, Tatsch PO, Treichel H, Furigo Jr A, Luccio MD. Assessment of cell disruption and carotenoids extraction from *Sporidiobolus salmonicolor* (CBS 2636). Food Bioprocess Tech. 2: 234-238 (2009)
14. Fonseca RAS, Rafael RS, Burkert CAV, Kalil SJ, Burkert JFM. Different cell disruption methods for astaxanthin recovery by *Phaffia rhodozyma*. Afr. J. Biotechnol. 10: 1165-1171 (2011)
15. Zhang Y, Rittmann BE, Wang J, Sheng Y, Yu J, Shi H, Qian Y. High-carbohydrate wastewater treatment by IAL-CHS with immobilizied *Candida tropicalis*. Process Biochem. 40: 857-863 (2005)
16. Silva DA. Maximization the production of astaxanthin by *Phaffia rhodozyma* (*Xanthophyllomyces dendrohous*) using rice parboilization water. PhD thesis, Federal University of Rio Grande, Rio Grande, RS, Brazil (2009)
17. Moraes CC, Burkert JFM, Kalil SJ. C-Phycocyanin extraction process for large-scale use. J. Food Biochem. 34: 133-148 (2010)
18. Medeiros F, Alves FG, Lisboa CR, Martins D, Burkert CAV, Kalil SJ. Ultrasonic waves and glass pearls: A new method of extraction of β-galactosidade for use in laboratory. Quim. Nova 31: 336-339 (2008)
19. Sabaj JAL. Caderno de Práticas de Bioquímica Fundamental (Notebook Practices of Fundamental Biochemistry.) Edgrafurg, Rio Grande, Brazil. pp. 45-45 (1979)
20. Sedmak JJ, Weerasinghe DKE, Jolly SO. Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. Biotechnol. Tech. 4: 107-112 (1990)
21. Bjerkeng B, Peisker M, Schwartzenberg KV, Ytrestoyl T, Asgard T. Digestibility and muscle retention of astaxanthin in Atlantic salmon, *Salmo salar*, fed diets with the red yeast *Phaffia rhodozyma* in comparsion with synthetic formulated astaxanthin. Aquaculture 269: 476-489 (2007)
22. Ventom AM, Asenjo JA. Characterization of yeast lytic enzymes from *Oerskovia xanthineolytica* LL-G109. Enzyme Microb. Tech. 13: 71-75 (1991)
23. Chumpolkulwong N, Kakizono T, Nagai S, Nishio N. Increased astaxanthin production by *Phaffia rhodozyma* mutants isolated as resistant to diphenylamine. J. Ferment. Bioeng. 83: 429-434 (1997)
24. Domínguez-Bocanegra AR, Torres-Muñoz JA. Astaxanthin hyperproduction by *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) with raw coconut milk as sole source of energy. Appl. Microbiol. Biot. 66: 249-252 (2004)
25. An GH, Schuman DB, Johnson EA. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl. Environ. Microb. 55: 116-124 (1989)
26. Xião A, Ni H, Cai H, Li L, Su W, Yang Q. An improved process for

cell disruption and astaxanthin extraction from *Phaffia rhodozyma*. World J. Microb. Biot. 25: 2029-2034 (2009)
27. Saeki K, Iwatw J, Yamazaki S, Watanabe Y, Tamai Y. Purification of a yeast lytic β-1,3-glucanase from *Oerskovia xanthineolytica* TK-1. J. Ferment. Bioeng. 78: 407-412 (1994)
28. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428 (1959)
29. Daroit DJ, Corrêa AF, Brandelli A. Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus*. Int. Biodeter. Biodegr. 63: 358-362 (2009)
30. Obata T, Iwata H, Namba Y. Proteolytic enzyme from *Oerskovia* sp. CK lysing viable yeast cell. Agr. Biol. Chem. Tokyo 41: 2387-2394 (1977)
31. AOAC. Official Method of Analysis of AOAC Intl. 17th ed. Method 962.10. Association of Official Analytical Communities, Arlington, VA, USA (2000)
32. Fleuri LF, Sato HH. Production, purification, cloning, and application of lytic enzymes. Quim. Nova 28: 871-879 (2005)
33. Burkert JFM, Kalil SJ, Maugeri Filho F, Rodrigues MI. Parameters optimization for enzymatic assays using experimental design. Braz. J. Chem. Eng. 23: 163-170 (2006)
34. Marco JL, Felix CR. Characterization of a protease produced by a *Trichoderma harzianum* isolated which controls cocoa plant witche's broom disease. BMC Biochem. 3: 1-7 (2002)
35. Guilloux-Benatier M, Pageault O, Man A, Feuillat M. Lysis of yeast cells by *Oenococcus oeni* enzymes. J. Ind. Microbiol. Biot. 25: 193-197 (2000)
36. Fleuri LF, Sato HH. Protoplasts production and yeast cell wall lysis using β-1,3 glucanase. Cienc. Tecnol. Alimet. 30: 471-476 (2010)
37. Liu LC, Prokopakis GJ, Asenjo JA. Optimization of enzymatic lysis of yeasts. Biotechnol. Bioeng. 32: 1113-1127 (1998)