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Organic-solvent-free extraction of carotenoids from yeast *Rhodotorula glutinis* by application of ultrasound under pressure

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ABSTRACT

The extraction of *Rhodotorula glutinis* carotenoids by ultrasound under pressure (manosonication) in an aqueous medium has been demonstrated. The influence of treatment time, pressure, and ultrasound amplitude on *R. glutinis* inactivation and on the extraction of carotenoids was evaluated, and the obtained data were described mathematically. The extraction yields were lineal functions of those three parameters, whereas inactivation responded to a more complex equation. Under optimum treatment conditions, 82% of carotenoid content was recovered. Extraction of carotenoids in an aqueous medium was attributed to the capacity of ultrasound for cell disruption and emulsification. Cavitation caused the rupture of cell envelopes and the subsequent formation of small droplets of carotenoids surrounded by the phospholipids of the cytoplasmic membrane that would stabilize the emulsion. Analysis of the dispersed particle size of the extracts demonstrated that a fine, homogeneous emulsion was formed after treatment (average size: 230 nm; polydispersity < 0.22). This research describes an innovative green process for extracting carotenoids from fresh biomass of *R. glutinis* in which only two unit operations are required: ultrasonic treatment, followed by a centrifugation step to discard cell debris. The extract obtained thanks to this procedure is rich in carotenoids (25 mg/L) and could be directly incorporated as a pigment in foods, beverages, and diet supplements; it can also be utilized as an ingredient in drugs or cosmetics.

1. Introduction

Carotenoids are liposoluble pigments naturally synthesized by plants and microorganisms, and they have industrial applications in food, cosmetic, and pharmaceutical product formulations [1,2]. In addition to their coloring properties, carotenoids have been shown to prevent cancer, macular degeneration, and cataracts when they are ingested in human diet [3]. Moreover, carotenoids can act as an antioxidant agent, and protect cells against oxidative damage [4].

Many carotenoids exploited in the industry are currently obtained through chemical synthesis [1,5]. However, unfavorable reports published by regulatory agencies (FDA, EFSA), combined with growing consumer concerns regarding artificial additives, are leading to an increased search for natural colorants, which may be healthier than synthetic colorants [6,4,7].

Compared with plants as a carotenoid source, microbial carotenoid production only requires a small production area, and is independent of changes in climate, seasonality, and soil composition [8]. Apart from microbial sources of carotenoids such as algae including the *Dunaliella* or *Haematococcus* species, yeast such as *Phafia rhodozyma* and *Rhodotorula glutinis* are likewise of commercial interest [9–12]. These yeasts have been considered as potential sources of natural carotenoids because they can produce high yields while growing in low-cost substrates such as agro-industrial waste [13,14].

The carotenoids produced by *R. glutinis* are synthesized intracellularly and remain inside the cell, where they have structural and functional properties [10,15]. Therefore, the recovery of carotenoids from yeast requires a series of downstream operation units in which extraction is critical [4].

Owing to the hydrophobic nature of carotenoids, they are traditionally extracted after dehydration of yeast biomass by applying a mixture of organic solvents [16]. Moreover, to achieve effectiveness, treatments designed to disrupt cell walls and other physical barriers are required before or during the extraction process [17–20]. However, drying out produces thermal degradation, while conventional solvent extraction requires the utilization of a large amount of harmful solvents in multiple extraction steps [16]. After extraction, the toxic solvents (benzene, ether, hexane, etc.) are evaporated and the carotenoids are re-suspended in food-grade solvents, thereby generating a considerable amount of pollutants [21]. Many of these solvents have been shown to be highly toxic and detrimental to the environment; the European Union is therefore implementing stricter rules for their use, resulting in

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increased costs for storage and disposal, even downright prohibition [22,23].

In view of these environmental regulations and health concerns, the search for more ecological extraction methodologies has become imperative to ensure the sustainable development of industrial processes designed to exploit yeast as a promising source for pigments.

Ultrasound is a non-thermal technology that has been shown to be very effective in improving the extraction of heat-labile compounds owing to the phenomenon of acoustic cavitation [24,25]. Cavitation consists in the formation, growth, and collapse of microbubbles inside a liquid submitted to high-frequency sound waves (> 20 kHz) [26]. As a consequence, molecules violently collide with one another, giving rise to shock waves and creating spots of very high temperature (5500 °C) and pressure (up to 50 MPa) for short periods of time (10^{-9} s) [27]. Ultrasound-assisted extraction does not act through one mechanism alone, but by different independent or combined mechanisms stemming from cavitation such as fragmentation, erosion, capillarity, detexturation, and sonoporation [25]. These mechanical effects may enhance the release of intracellular compounds by disrupting the cell, and by facilitating the penetration of the solvent. The effect of ultrasound on the extraction of lipids [28–30], carotenoids [31,32], and other high-value components [33] from different microalgae has been investigated. Likewise, the ultrasound-assisted extraction of lipids [34], carotenoids [35], and polysaccharides [36] from yeast has been reported. Moreover, the extraction of lipophilic compounds in a hydrophilic media thanks to ultrasound was described by Adam et al. (2012) [28]. Ultrasound can be applied to the fresh biomass avoiding drying step and thus reducing the energy consumption of the extraction method. Furthermore, as this technology speeds up mass transfer, it could impact in the reduction of the use of organic solvents leading to the reduction of wastes and pollution [37].

In the present investigation, ultrasound treatment was applied under pressure (manosonication). This combination has been shown to increase the effect derived from cavitation [38]. Manosonication drastically increases the inactivation effect of ultrasound on microorganisms [39,24,40], and it has also proven to be effective in the extraction of carotenoids from tomato pomace [41]. However, the effect of combining ultrasound and pressure on the extraction of biocompounds from yeast has not yet been investigated.

The aim of this study was to evaluate the potential of ultrasound under pressure for extracting carotenoids from *R. glutinis* in an aqueous medium. Response surface methodology was used to evaluate the potential of manosonication for the optimization of the carotenoid-extraction yield. The ultimate objective was to design an ecofriendly and sustainable process for obtaining carotenoids from fresh yeast cells while avoiding the use of organic solvents.

2. Material and methods

2.1. Strain, medium, and culture conditions

A commercial strain of *Rhodotorula glutinis var. glutinis* (ATCC 2527), provided by *Colección Española de Cultivos Tipo* (CECT), was used. The yeast cultures were grown at 25 °C in 500 mL glass flasks containing 250 mL of Potato-Dextrose broth (PDB, Oxoid, Basingstoke, UK) under orbital shaking at 185 rpm (Heidolph, Schwabach, Germany). Yeast culture growth was monitored by measuring absorbance at 600 nm (correlated with cellular density) and the number of cells, using a Thoma counting chamber and the plate-counting method in Potato-Dextrose-Agar (PDA, Oxoid, Basingstoke, UK). Dry weight (d.w.) of yeast was determined by vacuum drying (GeneVac, Ltd, UK) at 60 °C until constant weight.

2.2. Ultrasound under pressure treatment (Manosonication)

Manosonication (MS) treatments were carried out in a specially designed resistometer similar to one previously described in the literature [39]. However, in our case, a 100 mL treatment chamber pressurized with nitrogen was used for the extraction experiments. A manometer connected to the pressure circuit ensured the pressure in the treatment chamber. The equipment allowed us to monitor the effect along time of ultrasound treatments on carotenoid extraction at different pressures and amplitudes. The tip of a sonication horn (13 mm diameter) connected to a 2000 W Digital Sonifier® ultrasonic generator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) with a constant frequency of 20 kHz was used. The wave amplitude values of this equipment range from 34 to 145 μm. Once treatment amplitude has been selected, the equipment supplies the required power: therefore, the greater the applied pressure, the higher the power supplied by the ultrasonic generator to maintain the amplitude of the selected vibration. A cooling coil placed in the treatment chamber was used to dissipate the heat generated by ultrasound and to maintain the temperature below 30 °C by circulating a cooled water-ethylene glycol mixture.

Extraction experiments were performed on cells after 72 h of culture, which corresponded with the highest carotenoid content. Prior to treatment, fresh biomass of *R. glutinis* was centrifuged at 3000 × g for 5 min (MiniSpin Plus, Eppendorf Ibérica, Madrid, Spain) at room temperature and re-suspended in a citrate-phosphate pH 7.0 McIlvaine buffer to a final concentration of approximately 10^8 cells/mL (10 $g_{d.w.}$/L). The medium used to propagate ultrasound waves was the own buffer. The chamber was filled with the suspension through the valve arranged for this purpose. Manosonication treatments were performed at three different amplitudes of 70, 90, and 120 μm at an atmospheric pressure, 100 or 200 kPa. Samples of 3 mL were collected each 30 s along 180 s. These treatments correspond to energies between 36.2 and 376.56 kJ/kg based on calorimetric measurements of power output [42].

2.3. Evaluation of yeast inactivation after treatment

After ultrasound treatments under pressure, serial decimal dilutions in peptone water (Oxoid, Basingstoke, UK) of the suspensions were pour-plated in PDA. The number of viable cells, expressed in colony forming units (CFU), corresponded to the number of colonies counted after 72 h of incubation at 25 °C. Longer incubation times did not affect the number of survivors (data not shown).

2.4. Evaluation of carotenoid extraction

2.4.1. Total content of carotenoids in suspensions

Throughout all the procedures, samples were protected from light as much as possible. An aliquot of 5 mL of suspension was centrifuged, and the pellet was re-suspended in 5 mL dimethyl sulfoxide (DMSO), vortexed for 1 min, and incubated in a shaking incubator (Unimax 1010; Heidolph, Schwabach, Germany) at a velocity of 200 rpm at room temperature for one hour. After that, 5 mL of hexane and 1 mL of diethylether were added to the tubes, vortexed, and incubated in shaking for 30 min. Then, 1 mL of NaCl saturated solution was added to the mixture, and tubes were vortexed for 1 min. Finally, tubes were centrifuged (4000 × *g*, 10 min) and the colored upper phase was collected. This procedure was repeated until the collected hexane phase became transparent. Pooled together, the collected extracts were evaporated under a continuous nitrogen flux and dissolved with a known volume of hexane for spectrophotometric quantification of carotenoids at 474 nm as described in Martínez et al. (2018) [43].

2.4.2. Quantification of extracted global carotenoids

Carotenoid extraction was carried out in the treatment medium itself (McIlvaine buffer) in the course of manosonication treatment. For subsequent quantification, 1 mL of the untreated or US-treated suspension of *R. glutinis* was centrifuged at 10.000 × *g* for 2 min at room temperature in order to separate the pellet-containing cells and the

supernatant. Carotenoid extraction was calculated by the difference between the total carotenoid content of the suspension and the carotenoids remaining in the pellet.

2.4.3. Specific analysis of carotenoids extracted

Specific analysis of carotenoids in the extracts obtained after method described in Section 2.4.1 and after US-treatments were performed. HPLC analysis was performed using a Varian ProStar high performance liquid chromatograph (Varian Inc., Walnut Creek, CA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler and a ProStar 335 Photodiode Array Detector. The system was controlled with a Star chromatography workstation v.6.41 (Varian). Separation was achieved on a reverse-phase column (LC Luna® 100 Å C18 250 × 4.6 mm; 5 µm particle size, Phenomenex) with a precolumn (LC Luna 50 × 4.6 mm; 5 µm particle size, Phenomenex) of the same material.

The solvents were HPLC grade methanol (VWR, Paris, France) and methyl-tert-butylether (MTBE; Fisher Scientific, Pittsburgh, PA, USA). A gradient system was used involving two separately mixed mobile phases. Mobile phase A was methanol/MTBE/water (81:15:4) and mobile phase B was methanol/MTBE (9:91). The initial values were 100% of A and 0% of B, to 50% A and 50% B in 45 min, followed by 100% B within 25 min. The flow rate was 1.0 mL/min throughout the entire run. Before the HPLC analyses, samples were prepared by a saponification protocol in order to break ester bonds. All samples were injected via a 20 µL loop using a 100-µL syringe. On the basis of the absorbance maxima for the carotenoids of *R. glutinis*, detection was done at 450 and 485 nm by the Photodiode Array Detector. The elution profile of ß-carotene and torularhodin standards with the C18 column was obtained and standard curves were constructed by plotting HPLC peak absorbance area versus concentration of the carotenoids in the injected sample.

2.5. Microscopic observation after treatment

Untreated cells and cells subjected to a manosonication treatment were observed under optical microscopy (Nikon Eclipse 6400, Nikon, Tokyo, Japan) in order to monitor morphological changes and effective cell disruption.

2.6. Experimental design and statistical analysis

Response surface methodology (RSM) was used to evaluate the effect of MS parameters: amplitude (70–120 µm), time (30–180 s) and pressure (0–200 kPa) on the inactivation of *R. glutinis* and on the associated carotenoid extraction yield.

The data obtained after treating the cells were fitted to the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \quad (1)$$

in which Y is the response variable to be modeled, Xi and Xj are independent factors, β_0 is the intercept, βi is the linear coefficient, βii is the quadratic coefficient, βij is the cross-product coefficient, and k is the total number of independent factors. A backward regression procedure was applied to determine the models' parameters. It systematically removed the effects that were not significantly associated ($p > 0.05$) with the response until a model with a significant effect was obtained.

Experiments were performed in triplicate, and the presented results are means ± standard deviation. One-way analysis of variance (ANOVA) using Tukey's test was performed to evaluate the significance of differences among the mean values. Differences were considered significant at $p < 0.05$. Multiple regression analysis was conducted to fit Eq. (1) to the experimental data, and significant terms of the model were determined by ANOVA. Root-mean-square error (RMSE) was used to measure differences between values predicted by the model and the values observed. The RMSD represents the square root of the second sample moment of the differences between predicted values and observed values, or the quadratic mean of those differences.

Central composite design and the corresponding data analysis were carried out with the software package Design-Expert 10 (Stat-Ease Inc., Minneapolis, MN, USA).

2.7. Analysis of emulsions

The supernatants were analyzed using the dynamic light scattering (DLS) technique with Zetasizer Nanoseries equipment (Malvern, Worcestershire, UK). The size of the particles dispersed in the liquid was analyzed, and results were expressed in Z-Average Size and polydispersity (Pd). The Z-Average Size term is defined as the harmonic intensity averaged particle diameter. Pd is a parameter calculated from an analysis of the DLS-measured autocorrelation function. In that analysis, a single particle size mode is assumed, and a single exponential fit is applied to the autocorrelation function. Pd describes the width of the assumed Gaussian distribution, and a Pd lower than 0.25 indicates that the sample is monodisperse. Three biological replicates of each treatment condition were performed, and each sample was measured twice. Results represent the mean ± standard deviation.

3. Results and discussion

3.1. Carotenoid extraction from R. glutinis by application of ultrasound under pressure

Fig. 1 illustrates the effect of pressure along time on the extraction of carotenoids from *R. glutinis* assisted by ultrasound. Independently of the external pressure applied, an exponential kinetic of extraction was observed, and the ultrasound treatment was more efficient when applied at 200 kPa rather than at atmospheric pressure. For example, after 120 s of treatment, extraction efficiency increased by 65%. At 200 kPa, 231 µg of carotenoids/$g_{d.w.}$ were extracted in comparison with 149 µg of carotenoids/$g_{d.w.}$ extracted at atmospheric pressure.

The application of ultrasound with the purpose of improving the extraction of compounds from microbial cells has been widely investigated [44–47]. The positive effect of ultrasound on extraction yields is attributed to the mechanical breakage of cells, and to heightened mass transfer produced by cavitation, causing high shear stresses, microstreaming, and turbulence [28,48]. In this investigation,

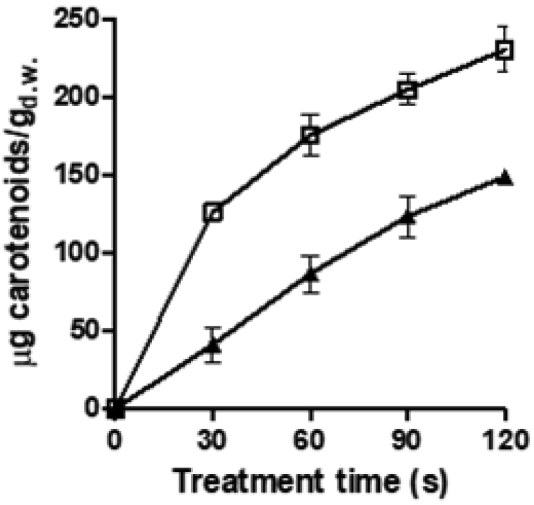

Fig. 1. Carotenoid extraction curve from *Rhodotorula glutinis* cells treated by ultrasound (96 µm amplitude) under different pressures: 0 kPa (▲) and 200 kPa (□).

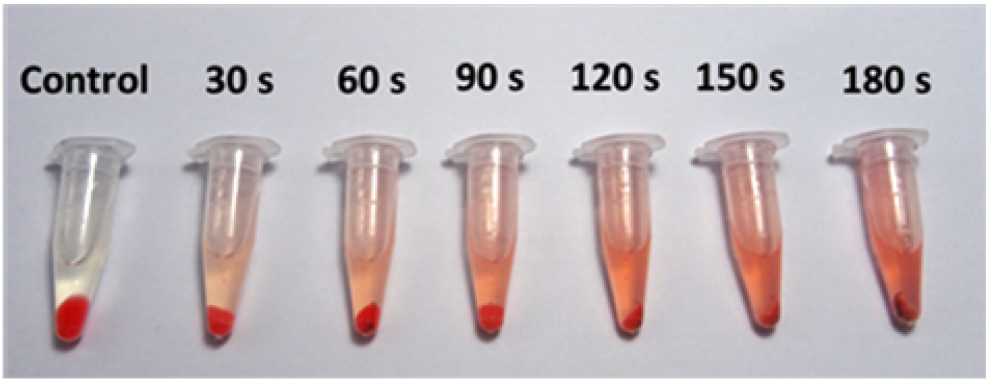

Fig. 2. Supernatant observation along treatment time (96 μm amplitude, 200 kPa) of *R. glutinis* suspension after manosonication.

Table 1
Inactivation of *R. glutinis* and carotenoid extraction yield after ultrasound treatments with different hydrostatic pressures, amplitudes and treatment times. Mean ± standard deviation.

Pressure (KPa)	Amplitude (μm)	Time (s)	Log_{10} survival fraction		Extraction (%)	
			Mean	SD	Mean	SD
0	70	30	−0.15	0.11	23.21	6.00
0	70	180	−0.83	0.04	49.12	4.51
0	96	105	−0.75	0.26	43.42	2.20
0	120	30	−0.26	0.13	24.71	6.42
0	120	180	−1.17	0.09	58.54	7.76
100	70	105	−0.67	0.20	40.85	4.55
100	96	30	−0.14	0.09	15.94	13.41
100	96	105	−0.70	0.09	43.96	7.11
100	96	180	−1.31	0.06	62.19	1.67
100	120	105	−1.14	0.21	63.52	14.32
200	70	30	−0.67	0.46	26.61	4.23
200	70	180	−1.94	0.41	65.35	2.05
200	96	105	−1.44	0.09	59.36	2.89
200	120	30	−0.54	0.06	36.58	0.25
200	120	180	−2.41	0.21	81.80	2.82

ultrasound was applied under moderate pressure (manosonication), because it is well known that if ultrasound is applied at sufficiently high intensity, external pressure increases the effects of cavitation [38,49]. Although the improvement of extraction thanks to the application of ultrasound under pressure has been previously demonstrated in substrates such as dried tomato pomace [41], the positive effect of this strategy on the extraction of carotenoids from yeast is demonstrated here for the first time.

It is worth noting that in this investigation, as previously reported by Adam et al. (2012) [28], ultrasound enables the extraction in aqueous medium of lipophilic compounds such as carotenoids (Fig. 2). Therefore, in addition to facilitating extraction by breaking up the cell envelopes of *R. glutinis* cells observed microscopically (data not shown), cavitation likewise permits the formation of a stable mixture of immiscible compounds such as carotenoids and water. It is well known that the main carotenoids produced by *R. glutinis* (torularhodin, torulene and β-carotene) are highly soluble in organic solvents, but do not dissolve in water [12]. A stable mixture of immiscible compounds requires the formation of an emulsion. Emulsification involves the formation of small droplets of the dispersed phase in the continuous phase, and the subsequent stabilization of the droplets by applying surface-active substances (emulsifiers). The formation of small droplets of carotenoids requires a certain amount of mechanical energy that could be supplied by the cavitation brought about by ultrasound: the emulsifying capacity of ultrasound technique has been widely described [50–52]. Generally, however, when ultrasound is used to form an emulsion, emulsifiers are added to stabilize the system. For example, Amiri-Rigi and Abbasi (2016) [53] extracted lycopene from tomato pomace treated enzymatically in an aqueous medium by applying ultrasound, and they used saponin as an emulsifier. In our investigation, a stable mixture was obtained without having to add an external emulsifier, which indicates that some of the yeast's own compounds could exert that function. It is well known that phospholipids, which are the main components of cytoplasmic membranes, are good emulsifiers due to their amphiphilic structure [54,55]. Therefore, in a first step, cavitation would cause the breakage of the cell and the release of carotenoids, and, in a second step, it would lead to the formation of small droplets of carotenoids, which would be stabilized by the phospholipids of the cytoplasmic membrane acting as emulsifiers.

3.2. The influence of pressure, amplitude, and treatment time of manosonication on the extraction of carotenoids from R. glutinis

After having demonstrated that manosonication treatment allowed carotenoid extraction in aqueous medium from *R. glutinis*, we evaluated the influence of sonication hydrostatic pressure (0–200 kPa), amplitude (70–120 μm), and treatment time (0–180 s) on cell inactivation and on the extraction of carotenoids from fresh biomass of *R. glutinis*.

Response surface methodology (RSM), a widely accepted statistical tool for the optimization of extraction processes [56], was used to study the influence exerted by those factors. Experimental conditions corresponding to a central composition design, as well as results obtained from the inactivation of *R. glutinis* and the extraction of carotenoids, are shown in Table 1. Inactivation response is expressed as Log_{10} cycles of survival fraction, and extraction response is listed as extraction percentage of total carotenoids. Depending on the intensity of the applied treatment, the percentage of extracted carotenoids ranged from ca. 16% when a treatment of 30 s, ultrasonic amplitude of 96 μm, and hydrostatic pressure of 100 kPa was applied, to ca. 82% when treatment time was increased to 180 s, and amplitude and pressure were increased to 120 μm and 200 kPa, respectively. As shown in Table 1, the conditions that produced the highest and lowest carotenoid extraction also led to the highest and lowest inactivation of *R. glutinis* respectively. The relation between the percentage of extracted carotenoids and the percentage of dead cells is shown in Fig. 3. The locations of the dots below the equivalence line show that extraction did not match with inactivation. This observation seems to indicate that the extraction of carotenoids not only depends on the destruction of *R. glutinis* cells by ultrasound, but also on other effects generated by ultrasound, such as the disassembling of carotenoids from the yeast structure, as well as emulsification. For that reason, carotenoid extraction continued to increase, even when manosonication treatments that destroyed more than 99% of the population had been applied. For example, a 65% rate of extraction was achieved with a treatment that inactivated around 99% of the population (70 μm, 200 kPa, 180 s), while with a treatment that inactivated 99,9% of the population (96 μm, 200 kPa, 180 s) extraction

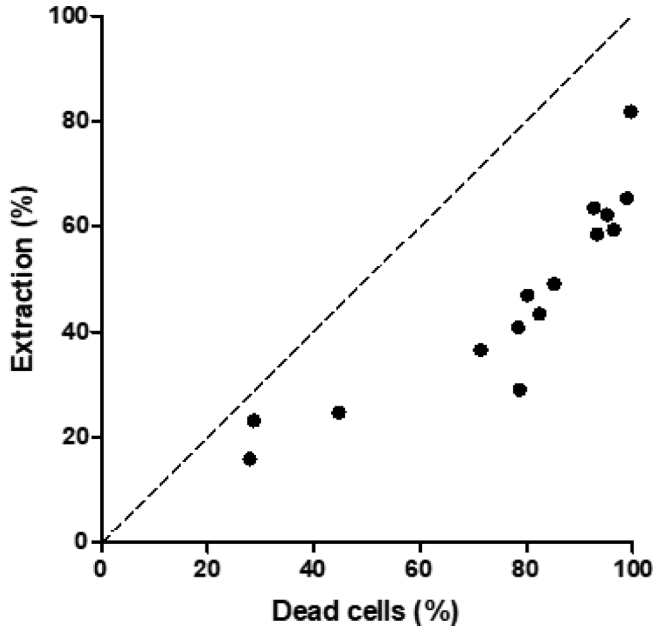

Fig. 3. Relationship between the percentages of extraction of carotenoids from *R. glutinis* cells against the percentages of dead cells. To show the degree to which each treatment causes carotenoid extraction, a theoretical straight line with slope = 1 and intercept = 0, is included. Data shown as mean, n = 3.

increased to 82%. Therefore, in order to maximize carotenoid extraction, it is necessary to extend the duration or increase the intensity of the manosonication treatment to bring about the release of carotenoids attached to yeast structures and/or emulsify the extracted carotenoids.

In order to quantify the effect of hydrostatic pressure, amplitude, and treatment time on the inactivation and extraction of carotenoids from *R. glutinis,* data presented in Table 1 were fitted to a quadratic mathematical equation using multiple regression analysis. After removing non-significant terms ($p > 0.05$), the relation between independent variables (hydrostatic pressure, amplitude, and time) and dependent variables (Log_{10} cycles of survival fraction and carotenoid extraction yield) are shown in Eqs. (2) and (3), respectively.

$$I = -0.1472 + 3.3422 \times 10^{-3} \times P + 7.61 \times 10^{-4} \times A - 3.8667 \times 10^{-5} \times t - 2.2490 \times 10^{-5} \times (P)^2 - 2.565 \times 10^{-5} \times P \times t - 5.54 \times 10^{-5} \times A \times t \quad (2)$$

$$CEY = -8.7443 + 0.0731 \times P + 0.2295 \times A + 0.2501 \times t \quad (3)$$

in which I is the inactivation expressed in Log_{10} cycles of survival fraction; CEY is the carotenoid extraction yield; P corresponds to the hydrostatic pressure (kPa); A to the amplitude (μm); and t to the treatment time (seconds). Stepwise regression with backward elimination removed the squared terms of amplitude and time, as well as the interaction between pressure and amplitude, from the quadratic equation of inactivation (Eq. (2)). Squared terms and interactions were removed from the extraction equation (Eq. (3)). In order to show the two equations' goodness of fit, Table 2 shows the results of the analysis of variance (ANOVA) for the significant terms of the two models obtained, along with the statistics used to test their adequacy. In both cases, the obtained F-values of the equations indicate that the equations were significant ($p < 0.0001$); therefore, the terms in the equations have a significant effect on the response. The determination coefficient (R^2) of the inactivation equation (Eq. (2)) was 0.98, thereby indicating that < 2% of the total response variation cannot be explained by the model. In the case of the extraction equation (Eq. (3)), the determination coefficient (R^2) was 0.90, indicating that the percentage of total variation observed in dependent variable parameters not explained by the equation is around 10%. On the other hand, the adjusted R^2 values

Table 2
F-values and p-values of the ANOVA analysis for the mathematical equations developed to describe the influence of hydrostatic pressure, amplitude and treatment time on the inactivation and extraction of carotenoids from *R. glutinis*. R^2: determination coefficient; RMSE: root mean square error.

	Eq. (1) (Inactivation)		Eq. (2) (Extraction)	
	F value	p value	F value	p value
Equations	69.10	< 0.0001	34.17	< 0.0001
Pressure	108.26	< 0.0001	12.50	0.0047
Amplitude	11.68	0.0091	7.70	0.0181
Time	254.38	< 0.0001	82.31	< 0.0001
Pressure $_x$ Time	21.64	0.0016		
Amplitude $_x$ Time	6.31	0.0364		
$(Pressure)^2$	12.32	0.0080		
R^2	0.9811		0.9031	
Adjusted R^2	0.9669		0.8767	
RMSE	0.0857		5.6723	

that correct the R^2 according to the number of responses and terms in the equations were close to R^2 values, thereby indicating that there was good agreement in both equations between experimental and predicted values.

Yeast inactivation was described by a more complex equation that included not only the linear relationships between inactivation and the evaluated factors, but also some of their interactions. Thus, amplitude and time, pressure and time, and the square of pressure were significant factors. However, in the case of extraction, only the linear effects of the factors were significant. Based on the linear effect of hydrostatic pressure, ultrasound amplitude, and treatment time on the extraction of carotenoids from *R. glutinis* in aqueous medium, it could be considered that ultrasound, within the investigated range of conditions, would not cause an observable carotenoid degradation, because increased intensity of treatment led to greater extraction yield.

Evaluating in greater detail the F-values of the equation parameters displayed in Table 2, the significance of the variables effects can be reported. Thus, according to those F-values, in the case of inactivation, the manosonication time linear term (F = 254.38) and the hydrostatic pressure linear term (F = 108.26) were the two most significant variables, thereby indicating that changes in those factors exerted the greatest influence on inactivation (Log_{10} cycle of survival fraction). The fact that the squared hydrostatic pressure term (F = 12.32) was also a significant term indicated that, beyond a certain pressure value, inactivation significantly increased within the studied range. Although this increment occurs between certain pressure values, the pressure increment would probably hinder cavitation beyond a critical value, and therefore the effect of ultrasound would remain constant or might even decrease thereafter. The fact that further increments in pressure do not increase the percentage of inactivation has been observed by other authors [57,58]. Finally, the influence of interaction terms (P × t; A × t) was also significant, but had lower F-values. The presence of those interaction terms implied that the effect of pressure and amplitude on inactivation depended on treatment time. With respect to F-values of the terms in the extraction equation (Eq. (3)), treatment time (F = 82.31) was the most significant parameter, followed by hydrostatic pressure (F = 12.50) and amplitude (F = 7.70).

Fig. 4 shows the response surface plots illustrating the influence of the most significant parameters (treatment time and hydrostatic pressure) on the inactivation (4A) and extraction of carotenoids (4B). In both cases, it is represented the influence of those two factors when sonication amplitude corresponds to the intermediate value of the assayed experimental range (96 μm). As indicated, these figures illustrate the conclusions derived from the analysis of the parameters of multiple regression Eqs. (2) and (3). Fig. 4A shows that when time increases independently of pressure, *R. glutinis* inactivation increases linearly, whereas when pressure increases within any time value, the increment

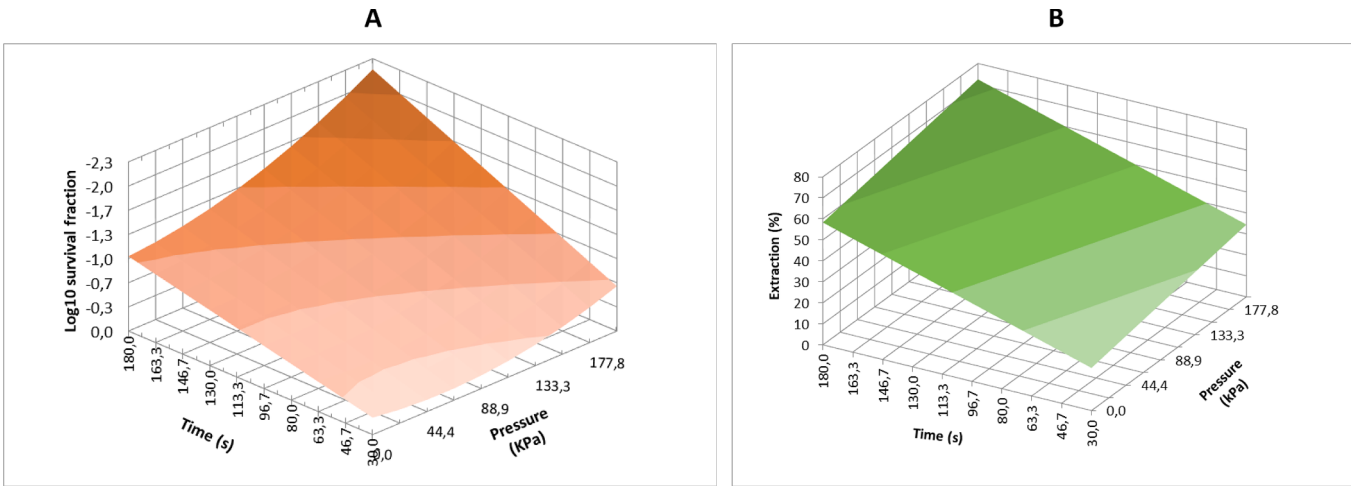

Fig. 4. Three-dimensional response surface plots of the influence of treatment time and pressure of manosonication treatments at 96 μm amplitude on inactivation (A) and carotenoid extraction (B) from *R. glutinis*.

in response is not linear. It is thus necessary to apply hydrostatic pressures above 100 kPa in order to be able to observe that this parameter meaningfully increases the inactivation of *R. glutinis*. On the other hand, Fig. 4B shows that time and hydrostatic pressure linearly increase carotenoid extraction. Thus in the assayed range, the greater the pressure and the more extended the time, the more efficient is the release of these compounds to the aqueous medium.

Although previous articles claimed that ultrasound increases degradation of natural products [24,25,37], results of specific carotenoids analysis obtained after DMSO multi-step method and after US under pressure treatment reveal that degradation is not produced under the range of conditions assayed. In order to detect if ultrasound treatment caused any change in the carotenoids from *R. glutinis,* the extracts obtained by DMSO multi-step method (reference of total extraction) and after the most effective US under pressure treatment (200 kPa, 100 μm, 180 s) were analysed by reverse-phase HPLC. Similar chromatogram profiles were obtained for the extracts obtained (data not shown). On the other hand Table 3 shows that statistically significant differences were not observed between the proportions of the two carotenoids detected after the US treatment in comparison to DMSO multi-step method ($(p < 0.05)$. Proportion of the two main carotenoids in the extracts obtained with both procedures contained around 65% of Torularhodin and 35% of β-carotene. Therefore these results seems to indicate that the ultrasound treatment did not affected the extraction of a selected carotenoid and no evidence of carotenoids degradation was observed.

Fig. 5 shows combinations of time and pressure of ultrasound treatments to obtain different Log_{10} cycles of inactivation (A) and carotenoid extraction yields (B) according to Eqs. (2) and (3). An increase in treatment pressure allowed for a significant reduction of the treatment time required for *R. glutinis* inactivation and for carotenoid extraction, but in different ways. In the case of inactivation, pressure increments hardly reduced processing time when they were lower than 100 kPa during the first moments of the treatment; however, with longer treatment times, pressure increments reduced processing time almost linearly. For example, the increment of pressure from 0 to 200 kPa reduced the time required to inactivate 1 Log_{10} cycle *R. glutinis* population by 60% (from 170 to 70 s). Regarding carotenoid extraction yields, any increment in pressure linearly reduced processing time. For example, the application of sonication treatments under 200 kPa, instead of at atmospheric conditions, allowed a reduction of treatment time from 145 to 87 s (40% reduction) for the extraction of 50% of carotenoid content.

Similarly to our results, Adam et al. (2012) [28] observed a linear influence of ultrasound treatment time on the extraction of lipids from fresh microalgae cells in aqueous medium. Time was identified as the second most significant term, only preceded by the biomass/solvent ratio, which inversely correlated with the yields. However, little was known until now regarding the effect of hydrostatic pressure in ultrasound treatments on the extraction of compounds of interest from microorganisms. In our results, pressure was identified as the second most influential parameter on inactivation of *R. glutinis* and on carotenoid extraction. Luengo et al. (2014) [41] similarly observed the improvement of carotenoid yields from tomato waste by increasing pressure from 0 to 100 kPa when applying ultrasound. The effect of vibration amplitude was the less significant term affecting *R. glutinis* inactivation and carotenoid extraction. However, amplitude did exert a certain amount of influence, which can be explained by the circumstance that, at higher vibration amplitudes, the effective size of the zone of the liquid undergoing cavitation and the range of bubble size undergoing cavitation also increase [27].

3.3. Analysis of dispersed particle size of the carotenoid extracts

In order to characterize the emulsion obtained after manosonication treatments, the particle size of droplets dispersed in aqueous medium was evaluated. Table 4 shows the Z-Average size and polydispersity of the droplets in the aqueous supernatants of *R. glutinis* suspensions

Table 3
Proportion of the two main carotenoids detected in *R. glutinis* extracts obtained by the total extraction method using DMSO multi-step procedure or after US treatment (200 kPa, 100 μm, 180 s). Results represent mean ± SD. Symbols in brackets represent significant differences ($p < 0.05$).

Peak n°	Retention time (min)	Absorbance maxima (nm)	Pigment	Extraction method	
				DMSO % of total	US % of total
1	13.5	495	Torularhodin	65.52 ± 5.23(a)	63.72 ± 5.93(a)
2	30.5	485	β-carotene	34.48 ± 3.54(b)	36.38 ± 4.94(b)

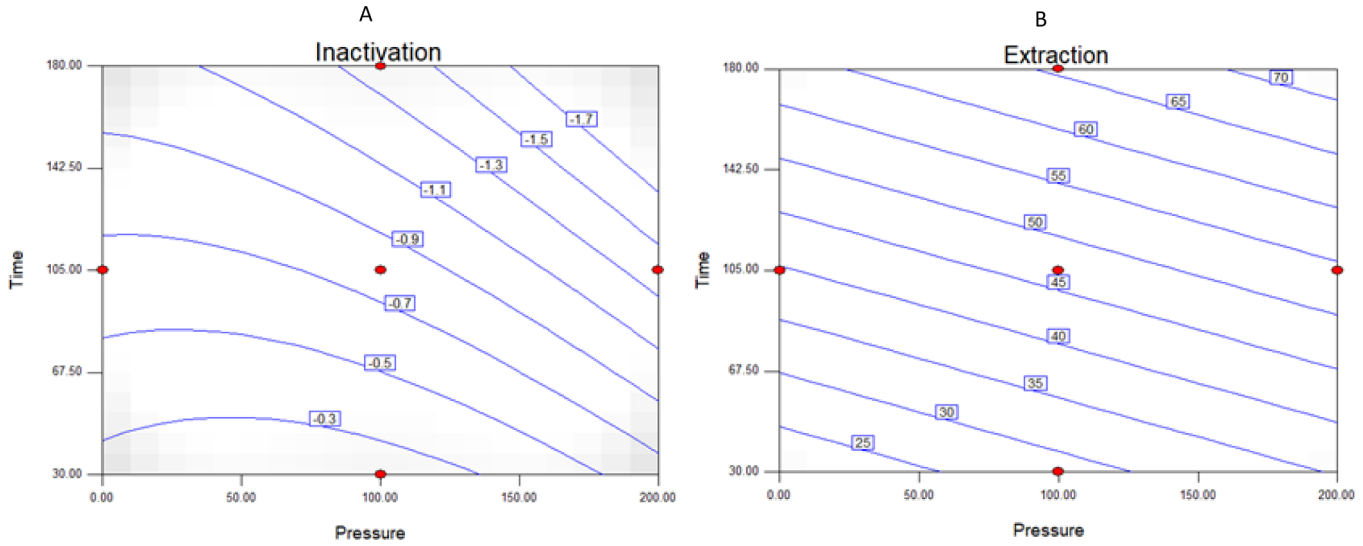

Fig. 5. Fitted iso-$\log_{10}$ cycles of cell inactivation (A) and carotenoid extraction percentage (B) contour plots of *R. glutinis* after manosonication treatments with 96 μm amplitude at different pressures and times.

Table 4
Z-Average Size and polydispersity (Pd) values of the supernatants of *Rhodotorula glutinis* suspensions treated by ultrasound under different conditions.

Pressure (KPa)	Amplitude (μm)	Time (s)	Z-Average Size (nm)		Pd	
			Mean	SD	Mean	SD
0	96	105	238.2	3.31	0.221	0.01
100	96	105	228.0	11.53	0.191	0.02
200	96	30	220.4	3.54	0.213	0.01
200	96	105	223.8	5.36	0.201	0.02
200	96	180	235.1	3.61	0.214	0.01

treated by ultrasound under pressure at different conditions. The histograms representing size mainly exhibited a single peak. For all treatment conditions, the deviations in Z-Average size were very low, indicating that particle size was quite homogeneous. Likewise, the Pd values of approximately 0.20 indicate the existence of a single particle size mode with Gaussian distribution of narrow width (monodisperse) in the extracts. Droplet size for all conditions assayed was around 230 nm, and no statistically significant differences were found among sizes after the application of treatments of different intensities. The formation of carotenoid emulsion in aqueous medium after ultrasound treatments had been previously reported by several authors [50,52,53,59]. The small size of the droplets formed in the *R. glutinis* extracts (around 230 nm) would explain the emulsion's notable stability along time. Kanafusa et al. (2007) [60] reported an oil-in-water emulsion containing β-carotene particles of similar size (93–310 nm) after microfluidization under pressure, and de Paz et al. (2013) [61] described a micellar particle size of less than 200 nm obtained by ultrasound emulsification of β-carotene.

Although no significant differences were observed in Z-Average size when amplitude, pressure, or time were varied, parallel tendencies among processing parameters and Z-Average size could be observed. Thus, when increasing pressure, size decreased from 238 to 223 nm, and Z-Average size increased with processing time from 220 to 235 within the range of the conditions investigated. These tendencies could be connected with the mechanisms of action of ultrasound and their interaction with processing parameters, as discussed above. An increment in pressure would limit cavitation; however, when it occurred, a great amount of energy would be released, thereby reducing droplet size. On the other hand, longer processing times would result in increased liberation of carotenoids and phospholipids, thereby incrementing the possibilities of coalescence and enlarging the size of the droplets.

Despite the different carotenoid concentrations of the *R. glutinis* extracts in the present research, droplet size was similar after different manosonication treatments. Therefore, it seems that the higher effectivity of more intense treatments (higher pressure, time, and amplitude) in terms of extraction yields could be due to the greater number of droplets formed, and not to the increment in droplet size. Furthermore, it is important to point out that a large amount of carotenoid emulsification was achieved in a very short time, especially when ultrasound was combined with the application of pressure. This is of great interest, since carotenoids are easily degraded in the presence of light, heat, and oxygen. Emulsions would protect the active compound and overcome its low bioavailability due to its low solubility in aqueous media. Furthermore, the use of carotenoids as colorants in beverages requires an appropriate formulation in order to stabilize the carotenoid particles in water suspensions and to provide the desired color. The addition of the extracts containing the carotenoid emulsions obtained after manosonication treatment would solve this issue.

4. Conclusion

Conventionally, carotenoid production from yeast involves cultivation, harvesting, extraction, and purification. In addition to the environmental, health, and safety hazards associated with the solvents usually applied, the cost of several required unit operations including drying, solid-liquid extraction, filtration, and solvent evaporation hamper the upscaling and the economic viability of *R. glutinis* as a source of carotenoids.

This research describes an innovative green-solvent extraction process assisted by ultrasound under pressure in which only two unit operations are required: the treatment of the yeast solution, followed by a centrifugation step to discard cell debris. This treatment enables the extraction of carotenoids from *R. glutinis* yeast in aqueous medium while avoiding the drying of biomass and the use of organic solvents. However, ultrasound units able to apply treatments under pressure should be developed in order to apply the treatment conditions identified in this research at industrial scale. Cavitation leads to the formation of small droplets of carotenoids surrounded by the phospholipids of the cytoplasmic membrane. An analysis of dispersed particle size of the extracts supports the assumption that a considerably fine, homogeneous, and stable emulsion is formed after treatment.

The extract obtained by this procedure could be directly incorporated as a pigment in foods, beverages, and diet supplements, and can be used as an ingredient in drugs or cosmetics. The emulsion would protect the active compound against degradation, and increase its bioavailability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2019.104833.

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