Selective recovery and purification of carotenoids and fatty acids from *Rhodotorula glutinis* using mixtures of biosolvents

Cassamo U. Mussagy, Daniela Remonatto, Ariela V. Paula, Rondinelli D. Herculano, Valéria C. Santos-Ebinuma, João A.P. Coutinho, Jorge F.B. Pereira

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- Extraction
- Carotenoid
- Fatty acids
- Antioxidant
- Biosolvent

**Abstract**

Selective recovery and purification of carotenoids and lipids from oleaginous red yeast *Rhodotorula glutinis* require a complex integration of different downstream operations. Envisioning a future industrial application in the bioeconomy framework, an experimental planning was used to design ternary mixtures of bio-based solvents (biosolvents) for simultaneous recovery and selective separation of carotenoids (i.e., β-carotene and torularhodin) and fatty acids (i.e., margaric acid, stearic acid and pentadecylic acid) from untreated (fresh wet) and freeze/thaw pre-treated (wet and dry) biomass of *Rhodotorula glutinis* CCT-2186. Two ternary systems of biosolvents, (i) ethanol:ethyl lactate:water and (ii) ethanol:ethyl acetate:water, were investigated in solid–liquid extraction procedures to permeabilize/disintegrate the yeast cells and to enhance the selective recovery of β-carotene, torularhodin and fatty acids from the intracellular environment. Optimization of the ternary mixture composition for recovery of carotenoids and fatty acids from the three types of biomass allowed to obtain yields higher than 75% (w/w) from pre-treated dry biomass using mixture of ethanol:ethyl acetate:water (67:33:00% w/w). The best systems were then integrated with a liquid–liquid extraction for the selective separation of carotenoids and fatty acids and solvent recycling. The mixtures of biosolvents were reused up to three consecutive extraction cycles, maintaining high extraction efficiency yields of both carotenoids (>45% w/w) and lipids (>30% w/w). At the end, the biological activity of the carotenoids extracts was confirmed by determining the antioxidant activity using Electron Paramagnetic Resonance (EPR) and the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The highest antioxidant activity (93%) was obtained with torularhodin (250 μg/mL). This study confirms that extraction platforms using mixed biosolvents are simple, efficient and sustainable solutions for the selective recovery and separation of carotenoids and fatty acids from microbial biomass.

1. Introduction

*Rhodotorula glutinis* is a yeast able to intracellularly accumulate high content of microbial lipids in the form of free fatty acids (e.g., margaric acid, stearic acid and pentadecylic acid) [1,2] and carotenoids (e.g., β-carotene and torularhodin) [3–5]. These bioproducts are highly valuable for commercial purposes due to their natural biological activities for pharmaceutical, cosmetic, chemical and food applications [6,7]. Carotenoids and lipids are intracellularly synthesized by *R. glutinis*, remaining inside the yeast cells [3] to guarantee their structural and functional properties [8]. Unfortunately, from a “bioprocess” point of view, the recovery of these intracellular biomolecules requires the sequential integration of various downstream operations, of which, due to the rigid cell wall, the extraction operation appears as the most costly step [3,8]. Since microbial carotenoids are hydrophobic in nature, e.g., carotenones (β-carotene) and xanthophyll’s (torularhodin), these are usually extracted from dry biomass using volatile organic solvents (VOCs) such as dimethyl sulfoxide, acetone, chloroform, hexane and petroleum ether [9]. Likewise, the most common procedures for the recovery of...
intracellular lipids from yeast are solid–liquid extractions using mixtures of VOCs (namely, hexane, chloroform and methanol) [10–12]. Despite of being very efficient, these VOC-based extraction procedures have been regarded as toxic to human health and harmful to the environment [1], which is prompting a move towards alternative extraction platforms using non-conventional methods such as high pressure or microwave assisted extraction and/or biocompatible solvents [13].

It is today crucial to develop more biocompatible and environmentally friendly extraction procedures, which guarantee not only the sustainability of industrial processes but also the biological activity of the molecules recovered from natural sources [14]. Anyway, from an industrial perspective, as recently shown by our group [1], if obtained (i.e., extracted, synthesized, or derived) from natural and renewable sources and efficiently recycled/reused, the use of VOC-based solid–liquid extraction procedures is probably the most effective and sustainable solution for extracting lipophilic compounds (such as carotenoids and lipids) from complex natural sources (like microbial and plant biomass). Extractive platforms using less toxic and more environmentally friendly solvents (pure or mixed), such as ethanol, ethyl acetate, water, and isopropanol, have been successfully applied for the recovery of carotenoids and lipids from microbial sources [1,15–17]. On a recent work [1], we demonstrated that conventional SLE procedures using a mixture of ethanol, ethyl acetate and water are effective (high recovery efficiencies) and sustainable (low carbon footprint) for the selective recovery of β-carotene, torularhodin, torulene and lipids from R. glutinis wet biomass under mild conditions. The combination of polar and non-polar solvents is crucial to enhance the recovery of intracellular biomolecules, namely: i) the presence of water to facilitate the miscibility with the wet biomass; ii) water and ethanol to help on permeabilization/disintegration of cell wall; iii) the relative hydrophobicity of the ethyl acetate to allow the dissolution of non-polar carotenoids and lipids.

Although the use of solvent mixtures in conventional SLE is a simple and economic solution for the recovery of intracellular compounds from yeast biomass [1,18], for example, as alternative to the non-conventional assisted methods (e.g., ultrasound assisted extraction, microwave assisted, pressurized liquid extraction) [19–21], the choice of the most suitable solvents mixture and composition is still very challenging. In addition, other processual parameters, such as temperature, biomass type and pretreatments that can also strongly influence the SLE performance [22,23], must be considered when carrying out extraction studies. Some researchers have evaluated the potential of biosolvents (pure and mixed) to extract carotenoids and lipids from R. glutinis [3,24,25], mostly focusing on the SLE of these products from wet biomass. The use of dry or pre-treated biomass will certainly affect, not necessarily negatively, the subsequent extraction using biosolvents. On the other hand, the incorporation of physical and chemical pre-treatments (e.g., sonication, enzymatic treatment, freeze thaw) [26,27] to improve the yields of conventional solvent extraction technologies has also been proposed in the extraction of carotenoids and lipids from yeast biomass [1,28–30]. A good example of how adding a simple pre-treatment can contribute positively for the recovery of intracellular biomolecule(s) is freezing-thawing of microbial cells [31], which, can help on the disintegration of the cell wall/membrane through the formation and accretion of ice crystals, dehydration, as well as increasing the solute concentration [32] and subsequent dissolution of target solute (s). Nevertheless, the implementation of additional pre-treatments of the microbial material can both impact the properties of target-biomolecule (s) and the costs of the process [26], making critical a careful balance between these two aspects and the effective increase of recovery yields.

Considering the advantages of using mixed biosolvents, aiming at understanding the impact of the biomass nature (wet vs. dry and treated vs. untreated) and the need of an additional pre-treatment stage, in this work we performed a very comprehensive study for the recovery of β-carotene, torularhodin and fatty acids using two ternary mixtures of biosolvents (i) ethanol:ethyl lactate:water and (ii) ethanol:ethyl acetate: water and three distinct samples of R. glutinis CCT-2186 yeast biomass, namely: a) fresh wet biomass (untreated) used as control; b) wet biomass (pre-treated with one freezing/thawing cycle); and c) dry biomass (pre-treated with one freezing/thawing cycle). Considering that different mixture ratio of solvents led to distinct solvent properties, we focused on the design and formulation of an optimal solvent mixture for both ternary systems and each of the three biomass samples. After defining the most efficient solvent mixture for extraction (both systems) and envisioning the circularity of the entire process, a liquid–liquid extraction using ethanol:ethyl acetate:water (25:25:50% w/w/w) as solvent was integrated with the polishing and recycling operations. At this final stage, the carotenoid and fatty acids extraction performance towards the reuse of solvent mixtures in up to three consecutive extraction stages from wet biomass (pre-treated with one freezing/thawing cycle) was evaluated. Finally, to evaluate if the integrated extractive platform did not negatively affect the biological activities of the carotenoids-rich extracts, the antioxidiant activity of extracted carotenoids was evaluated using free-radicals DPPH, (2,2-diphenyl-1-picrylhydrazyl) by Electron Paramagnetic Resonance (EPR).

2. Experimental

2.1. Material

Ethyl acetate (>99.9%) and ethanol (>99.9%) were acquired from Exodo Científica (Sumaré, SP, Brazil); ethyl lactate (>99.9%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (95%), monoolein (>99.9%), diolein (>99.9%), triolein (>99.9%), oleic acid (>99.9%) and Supelco 37 Component FAME mix standards (analytical standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (purified through a Millipore Milli-Q ion-exchange system) was used for the solvent mixtures. β-carotene and torularhodin standards were acquired from Carbosynth (San Diego, CA, U.S.A).

2.2. Microorganism and cell growing conditions

Rhodotorula glutinis CCT-2186 was acquired from the Tropical Culture Collection André Tosello (Campinas, SP, Brazil), which was isolated from the leaf of a kaki fruit ( Diospyros). The inoculum was prepared by the activation of a stock culture of the microorganism (50% v/v in glycerol aqueous solution maintained at ~80 °C) in Yeast Extract-Peptone-Dextrose (YPD) medium (composed of (g/L in deionized water) peptone bacteriological (20), yeast extract (10), glucose (20)). The inoculum cell culture was prepared in 100 mL Erlenmeyer® type flasks containing 25 mL of the YPD medium. Cells were grown for 48 h at 30° C and 170 rpm in an orbital shaker (Tecnal, model TE- 421 (Piracicaba, SP, Brazil)). To produce the target biomolecule(s) (i.e., β-carotene, torularhodin and lipids), a culture medium composed of (g/L in deionized water) glucose (10), KH₂PO₄ (0.52), MgSO₄ (0.52), NH₄NO₃ (4) and asparagine (10) was used. Batch cultures with 5 L of the culture medium were grown in a 7 L stirred-tank bioreactor (Tecnal®, model Tec-Bio-7.5VI (Piracicaba, SP, Brazil)), equipped with two Rushton turbines radial flow impellers, oxygen and pH electrodes. The initial pH of the medium was adjusted to 5.0 by adding 2 mol/L HCl or NaOH before autoclaving. The production process was then started by transferring 90 mL of the inoculum culture of R. glutinis (at 0.2 mg/mL) to the bioreactor, and the bioprocess conducted at 30 °C, 300 rpm and 1vvm (air volume/medium volume/minutes) over 120 h (antifoam was added as needed). After the cultivation, R. glutinis biomass was separated from the fermented supernatant by centrifugation at 2500 × g for 10 min at 4 °C using a Hitachi CR-22N (Tokio, Japan) centrifuge. The supernatants of all fermented media were then discarded, and the cellular biomass samples containing intracellular carotenoids and lipids were collected and used in the subsequent pre-treatment and/or solid–liquid extraction studies.
2.3. Carotenoids isolation and characterisation

Carotenoids isolation from R. glutinis cells followed the modified conventional method described by Mussagy et al. [3]. In summary, this method starts with a series of successive extractions of carotenoids from wet biomass using a mixture of ethanol:ethyl acetate:water (80:10:10 w/w/w), followed by a separation using column liquid chromatography (with a mobile phase composed of hexane:ethyl ether:acetic acid). The two main fractions of the extracted carotenoids were collected, dry and then identified by RP-HPLC on a chromatographic column Shimadzu® Shim-pack C_{18} (Japan), 4.6 × 250 mm, using as mobile phase methanol: acetonitrile:dichloromethane (60:10:30, v/v/v) eluting isocratically for 15 min. The flow rate was 1 mL/min, and the column temperature was set at 30 °C. The corresponding carotenoids were determined using UV–Vis detector at λ_{max} 450 nm. The identification of β-carotene and torularhodin (yellow and red fraction respectively) was carried out by comparison with previous standards retention time (with high purity level) under the same experimental conditions.

2.4. Lipids isolation and characterization

Lipids were extracted from R. glutinis wet biomass using the two most efficient mixtures of biosolvents, namely, ethanol:ethyl acetate:water (80:10:10% w/w/w) and ethanol:ethyl lactate (33:67% w/w/w). After the extraction, the lipids were separated by thin layer chromatography (TLC) using hexane:ethyl ether:acetic acid as the mobile phase. Lipids were methyl esterified and the fatty acid composition of the structured lipids determined by gas chromatography (GC) using a Shimadzu gas chromatograph mass spectrometer (GC–MS–QP2010) (Japan) equipped with a flame ionization (FID) detector. Supelcowax column (30 m length, 0.32 mm diameter, and 0.5 μm film thickness) was used to separate the fatty acid methyl ester at a flow rate of 0.55 mL/min and the injector temperature was set to 250 °C. The GC oven program was the following: started with 60 °C and the temperature was increased at 20 °C/min to 210 °C for 7 min, then at 20 °C/min to 250 °C for 14 min, with a total run duration of 21 min. The fatty acids were identified as the peak areas relatively to the retention times of Supelco 37 Component FAME Mix standard.

2.5. Preparation of R. glutinis biomass

To evaluate the influence of three distinct types of microbial biomass treatments in the SLE procedure, R. glutinis cellular pellets were subject to different procedures. After cultivation, the cellular pellets containing carotenoids and lipids were washed three-times using 10 mL of phosphate buffer (pH 7) to remove impurities from fermented supernatant. Three distinct biomass samples were then prepared from centrifuged cell pellets, according to the following protocols:

1. Fresh and untreated wet biomass – R. glutinis cell pellet was directly used in the subsequent SLE procedures;
2. Freezing/thawing cycle + wet biomass: R. glutinis cell pellet was suspended in deionized water (25:75 w/w) and subjected to one freezing (at −80 °C) / thawing (at 25 °C) cycle; the wet biomass was separated from the aqueous supernatant by centrifugation at 2500 ×g for 10 min at 4 °C and the wet biomass used in the subsequent SLE procedures;
3. Freeze/thaw cycle + dry biomass: R. glutinis cell pellet was suspended in deionized water (25:75 w/w) and subjected to one freezing (at −80 °C) / thawing (at 25 °C) cycle; the wet biomass was separated from the aqueous supernatant by centrifugation at 2500 ×g for 10 min at 4 °C and dried in an oven (Nova Instrument, NI 1510, Piracicaba, SP, Brazil) at 60 °C for 24 h; the dry and powderd R. glutinis biomass was stored in capped dark glass flasks and used in the subsequent SLE.

2.6. Solid-liquid extraction (SLE) of carotenoids and lipids using mixtures of biosolvents

The selection of ethanol (EtOH), ethyl acetate (EtOAc) and ethyl lactate (EtOLuc) as biosolvents for SLE experiments was carried out based on: i) the Pfizer Solvent Selection Guide [33] and GSK Sustainable Guide [34] (in which these solvents are classified as biocompatible and eco-friendly alternatives); ii) the possibility to obtain them from bio-based renewable sources; iii) their ability to solubilize the different types of R. glutinis biomass. The solid–liquid extraction of lipids and carotenoids experiments were carried out in 20 mL capped glass test tubes. 1 g of each type of R. glutinis biomass (route (1), (2) or (3)) and 5 mL of each of the two biosolvents’ systems (pure or mixtures of water (H_{2}O), EtOH, and EtOAc / EtOLuc - ratios and acronyms of the different systems are detailed in Table S1 in the Supplementary Material and in Figs. 2 and 3 in the Results and Discussion section). The tubes were then homogenized using a magnetic stirrer hot plate mixer (Norte Científica, NH 2200, Araraquara, SP, Brazil) for 1 h at 65 °C and 300 rpm (operating conditions based on Mussagy et al. [3]). After homogenization, the tubes were cooled to 25 °C, all samples were collected and centrifuged (at 2500 ×g and 25 °C) for 5 min. After centrifugation, cell lysate supernatants were taken, passed through a 0.22 μm Millipore® PTFE filter membrane and the remaining solvents evaporated at 60 °C and 100 mbar using a Heidolph (Hei-YAP) rotavaporator (Schwabach, Germany). The samples were carefully stored (without light exposure) and used in the following carotenoid and lipid content analysis.

Carotenoids were re-dissolved in acetone and these extracts filtered using PTFE membrane (0.22 μm pore size) and quantified according to the methodology described below. The recovery yields (%) of each carotenoid (β-carotene and torularhodin) were calculated as the ratio between the amount (in mass) of carotenoid extracted with each solvent system relatively to the initial amount of carotenoid present in fresh wet biomass (untreated), pre-treated wet biomass (treated with freeze/thaw cycle) and pre-treated dry biomass (treated with freeze/thaw cycle), according to Eq. (1). On the other hand, the lipids were determined gravimetrically (i.e., lipids in each extract were dried and weighed) following the standard Bligh and Dyer protocol. Similarly, the recovery yields (%) of lipids were defined as the ratio between the amount (in mass) of lipids extracted after each SLE and the initial amount of lipids accumulated in the three distinct R. glutinis biomass samples, as described by Eq. (2).

\[
\text{Carotenoids extracted} = \left( \frac{\text{Carotenoids} \times 100}{\text{Total carotenoids in R. glutinis biomass}} \right)
\]

\[
\text{Lipids extracted} = \left( \frac{\text{Lipids} \times 100}{\text{Total lipids in R. glutinis biomass}} \right)
\]

2.7. Quantification of total carotenoid content

The β-carotene and torularhodin content were determined following the conventional extraction method described by Mussagy et al. [3]. Briefly, 1 g of each R. glutinis biomass was mixed with 5 mL of DMSO and disrupted by maceration (5 min ON/15 min OFF for a total 1 h). After the procedure, the supernatant was recovered, and the procedure repeated until the cells become fully bleached. The supernatants of the cell lysates were mixed with 10 mL of a sodium chloride aqueous solution (at 20% (w/v)) and 10 mL of petroleum ether. After the formation of a biphasic system, the non-polar phase was collected, and the excess of water removed with sodium sulfate aqueous solutions. The carotenoids-rich extracts were redissolved in acetone and filtered with a PTFE membrane (0.22 μm pore size). The quantification of the three carotenoids was obtained from the visible-light absorption spectra using a Microplate reader® UV–Vis spectrophotometer (model Biotek, Synergy...
HT, Germany). The visible-light spectra from 380 to 600 nm were acquired, and the respective carotenoids calibration curves at 450 nm (β-carotene) and 490 nm (torularhodin) determined. The carotenoid concentrations (μg/g) were quantified according to the β-carotene and torularhodin standard calibration curves.

### 2.8. Quantification of total lipids content

To determine the total lipid content accumulated in the *R. glutinis* cells, intracellular lipids were extracted from the (1) fresh untreated wet biomass, (2) pre-treated (with one freeze/thaw cycle) wet biomass, and (3) pre-treated (with one freeze/thaw cycle) dry biomass, further dried and weighed by using a modified Bligh and Dyer procedure [10]. Briefly, the cells were harvested by centrifugation (2500 × g, 10 min), washed and dried (50 °C, 24 h) to achieve constant weight. Dry yeast cells were mixed with chloroform, MeOH and H2O to reach 1:2:1 ratio (v/v/v) and homogenized for 1 h at 300 rpm and 25 °C. Afterwards, chloroform and H2O were added to reach a final volume ratio of 2:2:2 (chloroform/MeOH /H2O). The ternary mixture was homogenized for 10 min, centrifuged at 2500 × g and 25 °C for 5 min. After the phase separation, coexisting aqueous and organic phases were carefully separated, and filtered to remove residual cellular debris.

After the separation, the bottom phase containing chloroform and lipids was transferred to rotary evaporator flasks (previously weighed, W1), which were dried under vacuum (at 300 mbar for 30 min) until the organic phase was fully evaporated, and the final weight of the flask measured (W2). Lipid content, expressed as mg/g dry cell weight per initial weight of the pellet (W) was determined according to Eq. (3):

\[
\text{Total lipids content (mg/g) } = \frac{W_2 - W_1}{W}
\]

### 2.9. Recycling of the biosolvents and polishing of carotenoids and lipids

To demonstrate the circularity of the two best biosolvent systems (i.e., with highest yields) for recovery of carotenoid and lipids from pre-treated (with one freeze/thaw cycle) wet biomass were chosen for the solvent recycling and polishing studies. Samples (50 mL) containing 20% (w/w) of wet biomass and 80% (w/w) of the mixtures EtOH:EtOAc:H2O (67:33:00% in w/w/w) or EtOH:EtOAc:H2O (80:10:10% in w/w/w) were added in a 100 mL glass test tubes. The samples were homogenized in a magnetic stirrer hot plate mixer for 1 h, 65 °C at 300 rpm, and subsequently centrifuged at 2500 × g at 25 °C for 5 min. After centrifugation, biomass solid pellets were discarded, and cell lysate supernatants were passed through a 0.22 μm Millipore® PTFE filter membrane. To recycle the solvents of both mixtures an evaporation operation (at 65 °C and 80 mbar) was carried out, and the biosolvents mixtures reused in following SLE procedures. To separate the proteins from carotenoids and lipids, cold acetone (1:1 w/w) was added to each colored solid fraction (remaining after the solvent evaporation). The addition of cold acetone induced the precipitation of proteins, while carotenoids and lipids remained in the acetone supernatant. Acetone was then evaporated (at 65 °C and 80 mbar) and reused in subsequent precipitation’s steps. Afterwards, the solid colored fraction (with carotenoids and lipids) was used in a liquid–liquid extraction to separate carotenoids (β-carotene and torularhodin) from lipids (fatty acids), in which 20 mL of EtOH:EtOAc:H2O mixture (at a percentage weight ratio of 25:25:50) was added to the solid colored fraction forming a biphasic system. The biphasic system was homogenized for 10 min and further centrifuged at 2500 × g at 25 °C for 10 min (to guarantee the full phases splitting). The carotenoids were preferentially partitioned in the EtOAc-rich phase, while the fatty acids were separated in the EtOH/H2O–bottom rich phase. Note that the solvent mixtures of this LLE stage (i.e., top and bottom phases) were also recycled, namely, the EtOAc-rich phase were evaporated and reused in a consecutive LLE and the carotenoids recovered as a solid-colored fraction; EtOH/H2O-rich phase was...
evaporated and reused as mixed solvent into a subsequent LLE and the fatty acids recovered as a solid fraction. To demonstrate the reproducibility and reusability of the solvents, extraction, recycling and polishing studies were repeated for three consecutive times, in which the evaporated solvents from the SLE were mixed with *R. glutinis* pre-treated (with one freeze/thaw cycle) wet biomass for a next SLE, while the top and bottom phases of LLE were reused in a following LLE cycle. The recovery yields (%) of β-carotene, torularhodin and fatty acids in each step were quantified according to the above-mentioned protocols.

2.10. Determination of carotenoid antioxidant activity

The carotenoid fractions obtained after recycling and polishing studies were used for determination of carotenoid antioxidant activity, namely, by performing a DPPH⁺ free-radical-scavenging activity test using Electron Paramagnetic Resonance (EPR) spectroscopy (also known as Electron Spin Resonance). The experimental protocol for antioxidant activity was carried out according a method adapted from De la Rosa et al., [35]. Briefly, samples were prepared by mixing 1.8 mL of standard DPPH⁺ (40 μg/mL in methanol) with 200 μL of β-carotene (from 100 to 500 μg/mL) and torularhodin (from 125 to 250 μg/mL) methanolic extracts. A control assay pure methanol was used. To evaluate the relative content of the radicals, after 15 min of reaction, all solutions were transferred to a capillary glass tubes, which were sealed and placed on standard EPR quartz tubes (inner diameter 3.00 mm). EPR spectra were recorded on a Bruker E-scan (Cambridge, UK) using a central magnetic field of 3480 G, a scanning field of 3495–3595 G, a scan time 50 s, a modulation amplitude of 3.29 G, a modulation frequency of 86.0 kHz and a microwave power of 10.93 mW. The area of peak-to-peak intensity spectrum was referred to as EPR signal. The reduction of DPPH⁺ EPR signal was calculated according the Eq. (4):

Fig. 2. Contour plots of EtOH:EtOLac:H₂O, solvent mixture composition (% w/w) and the respective recovery yields (% w/w) of [a), d), g)] β-carotene, [b), e), h)] torularhodin and [c), f), i)] lipids at *R. glutinis* biomass [fresh untreated wet, pre-treated (freeze/thaw) wet, and pre-treated (freeze/thaw) dry, respectively] concentration of 0.2 g/mL after 1 h stirring (300 rpm) at 65 °C. The results are presented as the average value of three independent assays with a 95% confidence level.
ESR (%) = 100 × \( \frac{S_{\text{DPPH}} - S_{\text{Sample}}}{S_{\text{DPPH}}} \)  

where ESR correspond to EPR- Signal Reduction, and \( S_{\text{DPPH}} \) and \( S_{\text{Sample}} \) correspond, respectively, to the EPR signals for solution containing DPPH• in the absence (control) and presence of the methanolic extracts (sample) [36].

3. Results and discussion

3.1. Isolation and characterisation of carotenoids and lipids

Carotenoids extracted from \( R. \) glutinis CCT-2186 biomass were isolated and characterized using conventional chromatographic and spectroscopic analytical protocols. The carotenoid-rich fraction was eluted with hexane:ethyl ether:acetic acid (at a volume ratio (%) of 70:29:1) by liquid chromatography. The respective peaks for the two main carotenoids were identified and quantified by RP-HPLC chromatography, as shown in Fig. 1a. The torularhodin and \( \beta \)-carotene peaks correspond to dark red and yellow pigments (Fig. 1d), respectively, which are characteristically identified by two isolated spots in the thin layer.
The results in Fig. 1b demonstrate that β-carotene has a higher mobility ratio (Rf = 0.96) than torularhodin (Rf = 0.42), Rf values, which are in accordance with the HPLC chromatogram of the carotenoid extract (Fig. 1a). Since the carotenoid fractions were eluted in an isocratic reverse phase mode, it should be noted that the retention time follows the order of decreasing polarity of each carotenoid.

Regarding to visible spectrum characteristics of both carotenoids, as shown in Fig. 1C, the more apolar β-carotene exhibit a maximum absorbance at 450 nm (λmax=450nm), while the more polar torularhodin has a maximum absorbance at higher wavelength, namely, 490 nm (λmax=490nm). The spectroscopic and chromatographic characteristics of the two carotenoids are in agreement with previous reports [24,37] as well as our previous work [3]. However, a careful comparison with our previous results [3], where β-carotene, torulene and torularhodin were isolated and identified as main carotenoids from R. glutinis, it is now noted the absence of the peak corresponding to torulene. In fact, the absence of torulene is a direct consequence of increasing the R. glutinis cultivation time in bioreactor from 72 to 120 h, which allow the full conversion of torulene to torularhodin by the microorganism [5]. From an industrial perspective, the existence of only two main carotenoids will facilitate the separation steps.

Similarly, the profile of lipids accumulated by R. glutinis were qualitatively analysed by thin layer chromatography (TLC). As shown in Figure S1a from Supplementary material, the lipidic fraction is mainly composed of free fatty acids (FFA) with a minor trace of triglycerides (TAG) and diglycerides (DAG). The abundance of microbial FFA synthetized by R. glutinis was confirmed by GC, being identified a preferential accumulation of long-chain fatty acids, namely, 75% of margaric acid, 10% of stearic acid, and 8% of pentadecylic acid (as depicted in the FAME profile from Figure S1b-c in the Supplementary material). Despite the increase in the R. glutinis cultivation time, the profile of lipids produced by the yeast remained similar to the previously obtained by us [1].

3.2. Solid-liquid extraction of carotenoids and lipids using mixtures of biosolvents

During the cultivation of R. glutinis, carotenoids and lipids are accumulated intracellularly in the yeast cells, with a distinct profile and composition of commercially valuable biomolecules. From the initial characterization study, β-carotene and torularhodin were identified as the main pigments present, while FFA (mainly, margaric acid) were accumulated as lipidic fraction. According to their intrinsic chemical structure, these carotenoids and FFA are generally regarded as very hydrophobic compounds [3,38]. However, more than the chemical nature of the biomolecule(s), the SLE process is highly dependent on the type and nature of solid biomass containing these target solutes, and how these are accessible to the solvent. Therefore, in this work, the SLE performance of two ternary solvents were firstly screened, (a) EtOH:EtOLac:H2O, (b) EtOH:EtOAc:H2O, and (c) EtOH:EtOAc:EtOLac, with the aim to find the best mixture ratio for the simultaneous recovery of β-carotene, torularhodin and FFA from R. glutinis. Together with the evaluation of the solvent influence, this work aimed to understand how the distinct nature of cellular biomass impact the extractive capacity of each mixed biosolvent, namely, (1) fresh and untreated wet biomass, (2) pre-treated (one freeze/thaw cycle) wet biomass, and (3) pre-treated (one freeze/thaw cycle) dry biomass. The SLE performances of the mixed biosolvents are analysed separately in the next two Sections (3.2.1 and 3.2.2) and compared for better understanding of the SLE mechanisms in the Section 3.2.3.

3.2.1. Ternary mixture of ethanol, ethyl lactate and water

In the first set of experiments, the influence of EtOH:EtOAc:H2O mixture ratio on the recovery of carotenoids and lipids (fatty acids) was evaluated for the three distinct R. glutinis biomass. EtOH and H2O are two environmentally friendly solvents widely used industrially [39]. In addition, due to its “greenness”, EtOLac has also been considered as one of the most promising industrial chemical commodities [40].

Considering the environmentally friendly characteristics of these biosolvents, an initial experimental set was performed using 0.4 mL of each biomass, 1 h of stirring at 300 rpm, and 65 °C, for fourteen monophasic systems selected from the EtOH:EtOAc:H2O ternary phase diagram. The mixture points are identified as A to N in Fig. 2 and detailed in Table S1 in the Supplementary material, while the respective recovery yields (% w/w) of β-carotene, torularhodin and lipids are presented in Table S1 in the Supplementary Material, and depicted in Fig. 2 as the contour plots of the EtOH:EtOAc:H2O systems for the three R. glutinis biomass samples. To facilitate the analysis and discussion of the results, they were combined according to the influence of solvent (pure or mixture) ratio on the recovery of β-carotene, torularhodin and lipids (Fig. 2 from left to right) and the impact of biomass nature, that is, untreated, pre-treated and dry (Fig. 2 from top to bottom).

The results plotted in Fig. 2a to c), namely, for the recovery from fresh untreated wet biomass, demonstrated that mixtures with equilibrated composition of all the solvents (i.e., concentration (w/w) of EtOH from 33 to 66%, EtOAc from 17 to 33% and H2O from 17 to 33%) are more favourable for the recovery of β-carotene and torularhodin, while low concentrations of EtOH (from 17 to 33%) are preferable to extract the FFA accumulated in R. glutinis biomass. Among the fourteen systems, the highest carotenoid recovery yields (of circa 57.11 ± 0.42 w/w of β-carotene and 55.46 ± 2.45 w/w of torularhodin) were achieved in point K, containing (in mass) 66.7% of EtOH, 16.7% of EtOAc and 16.7% of H2O, while the highest concentration of lipids (64.10 ± 0.10 w/w) was obtained with the mixture point L (composed of 16.7%, 66.7% and 16.7% (w/w) of EtOH, EtOAc and H2O). These results clearly demonstrate that, independently of the target solutes, the use of ternary mixtures of solvents is more favourable for the recovery of these solutes from untreated wet R. glutinis biomass. In fact, an increased polarity of the mixed solvent is crucial for increasing the carotenoid and lipids extraction yields from wet biomass, firstly to improve the miscibility and biomass permeabilization, and second to facilitate the access of the solvent to the intracellular yeast environment for subsequent solubilization of the hydrophobic carotenoids and fatty acids [1]. Anyways, a relationship between the characteristics of solute and solvent (i.e., “like-dissolve-like” principle) [41] also has an impact on the SLE process, particularly, as demonstrated with the high concentrations of EtOH or EtOAc (the solvents with non-polar nature) that are necessary for the recovery of carotenoids and lipids, respectively.

Fig. 2d to f show the results relatively to the SLE using pre-treated (freeze/thaw) wet biomass. Through a general analysis of their contour plot diagrams, after one pre-treatment cycle of the R. glutinis biomass, systems with low amount, or absence, of H2O (i.e., binary mixture of EtOH and EtOAc) are more favourable for the extraction of β-carotene and torularhodin. The EtOH:EtOAc binary mixtures (2:1 or 1:2) maximized the recovery of the two carotenoids, in which, approximately 60.06 ± 0.35%, 53.38 ± 0.08% (w/w) of β-carotene and torularhodin were extracted, respectively. Although the binary biosolvent mixture also enhanced the extraction of FFA, with recovery yields higher than pure or ternary mixture systems, it should be noticed that the best systems are EtOH:EtOAc and EtOAc:H2O in the ratio 2:1 in weight. This second set of results shows that when the yeast cells were subjected to an initial freezing/thawing pre-treatment, the disintegration of the yeast cells occurs due to the formation and detachment of ice crystals on the cell wall, and further dehydration, facilitating the subsequent access to the solutes by the solvents as well as their subsequent solubilization [32]. However, even considering the total disintegration of the cell structure after pre-treatment, the hydrophilic environment around the target biomolecules still makes a certain polarity degree essential in the mixed biosolvent mixture, which can be obtained from the remarkable hydrogen bonding abilities of EtOH or H2O in the binary mixtures.

The third set of experiments evaluated the performance of EtOH:
EtoLac:H$_2$O in the recovery of carotenoids and lipids but in this case from a pre-treated (freeze/thaw) dry biomass. Firstly, as detailed in Table S1 in the Supplementary Material, no significant increase in recovery yields were observed, compared to the two other wet biomass samples. In the case of carotenoids, the optimal condition (point K - system composed of 66.7% (w/w) of EtOH, and 16.7% (w/w) of both EtoLac and H$_2$O) allowed recoveries of β-carotene and torularhodin to be around 64.05 ± 1.34% and 62.00 ± 0.74% respectively, whilst 59.00 ± 1.06% (w/w) of the lipids were extracted at the point N mixture (EtOH:EtoLac:H$_2$O at 80:10:10 in weight %). Despite of similar recovery yields, an overall analysis of the counter plots depicted in Fig. 2 g) to i) demonstrates a change on the mixture ratio profile comparatively to the pre-treated wet biomass. In fact, due to the use of dry biomass, systems with high EtoLac content were most efficient for the recovery of both non-polar carotenoids. Interestingly, for lipids, intermediate ternary mixtures still presented the highest recovery yields. Although the biomass is dry and the most hydrophobic solvent should extract the highest amount of lipids, an increased polarity to extract lipids is needed, obtained from the mixing of polar solvents (EtOH and H$_2$O) with EtoLac, first, to facilitate the release lipids from protein-lipid complexes [42], and second, to enhance the hydrogen bond and electrostatic misfit interactions with the carboxylic groups of the fatty acids [1].

Together the counter plots depicted in Fig. 2 reveal clear differences in the SLE performance using EtOH:EtoLac:H$_2$O mixtures for the three types of biomass. Despite the interactions and extraction mechanisms for the recovery of carotenoids and FFA being different for each type of biomass (further discussion about these mechanisms is presented below in Section 3.2.3), recovery yields higher than 50% (w/w) were always guaranteed.

### 3.2.2. Ternary mixture of ethanol, ethyl acetate and water

In the previous section, biosolvent mixtures containing EtoLac were successfully used for the recovery of intracellular carotenoids and fatty acids for all studied samples of R. glutinis biomass (i.e., treated, untreated, wet or dry). Regardless of their “greenness”, these EtoLac-based mixtures present a critical disadvantage in their further implementation, which is the non-formation of two-phase systems when mixed with H$_2$O and EtOH. From a processual perspective the formation of a biphasic regime will facilitate the selective separation and purification of carotenoids and lipids by means of subsequent liquid-liquid extraction (LLE). Therefore, to find a system that in addition to the recovery also allows the selective separation of carotenoids and fatty acids, ternary mixtures of EtOH:EtoLac:H$_2$O were evaluated, exploring EtoAc as potential alternative to EtoLac. This choice is based on the idea that, if obtained from the conversion of “bio-ethanol” [43], EtoAc can also be considered as a biosolvent. Moreover it has the ability to create a liquid-liquid equilibria when mixed with EtOH and H$_2$O above certain concentrations [44], allowing not only the SLE of carotenoids and lipids in the monophasic region, but also their separation and purification in LLE using mixtures at the biphasic region.

The second set of extractions was carried out using EtOH:EtoAc:H$_2$O under the same processual conditions as before (i.e., biomass subject to three types of treatments at a concentration of 0.2 g/mL, 1 h of stirring at 300 rpm and 65 °C). For comparison purposes the SLE was only carried out using mixtures at the monophasic region (mixtures points from A to P and H, K, N identified in Fig. 3). All solvents compositions together with the obtained recovery yields and the results from SLE performance are detailed in Table S1 in the Supplementary Material. The SLE performance is depicted in Fig. 3, as the contour plots of the EtOH: EtoAc:H$_2$O of the recovery yields (% w/w) of β-carotene, torularhodin and lipids (Fig. 3 from left to right) respectively for the fresh untreated, pre-treated (freeze/thaw) wet and pre-treated (freeze/thaw) dry biomasses (Fig. 3 from top to bottom).

The results presented in Fig. 3 a) to c), using fresh untreated wet biomass, show that EtOH concentrations of 67 to 80% (w/w), EtoAc of 10 to 33% (w/w) and concentrations of H$_2$O up to 10% (w/w) maximized the recovery of β-carotene and torularhodin, while a decrease in the concentration of EtoAc (from 0 to 17% w/w) is necessary to improve the performance of SLE for lipids. For carotenoids, the highest recovery yields were obtained at Point N composed of, by weight, 80% of EtOH, 10% of EtoAc and 10% of H$_2$O, which allowed the recovery of approximately 56.53 ± 0.40% and 56.95 ± 1.48% (w/w) of both β-carotene and torularhodin. Concerning the lipids, 65.27 ± 3.31% (w/w) of the lipids accumulated in R. glutinis fresh biomass were recovered in the 2:1 EtOH:H$_2$O binary mixture (i.e., point E). These results are in agreement with our previous work [1], where, under similar conditions, carotenoid and lipids recovery yields up to 60% (w/w) were also achieved. Similar to the EtoLac-based mixtures (in the previous section), an increased polarity of the mixed solvent (mainly due to the high concentrations of EtOH) is necessary for the recovery of the three main solutes present in R. glutinis cells. It favours the miscibility with wet biomass and contributes positively to the permeabilization/disintegration of the cell wall and the penetration of the solvent through the hydrophilic environment surrounding the target biomolecule [46].

Interestingly, as depicted in the contour plots of Fig. 3 d) to f), a very similar SLE profile of the mixed EtOH:EtoAc:H$_2$O was obtained for the recovery of carotenoids and lipids from pre-treated (freeze/thaw) wet biomass. In fact, the only difference relatively to the untreated biomass is a slight intensification on the extractive performance of the mixed biosolvent, namely: from ≈56.53 ± 0.40% to ≈61.60 ± 0.50% for β-carotene (at point N); from ≈56.06 ± 0.51% to ≈58.29 ± 0.12% for torularhodin (at point D); and from ≈65.27 ± 3.31% to ≈49.53 ± 3.31% (at point E) for lipids. As expected, the addition of an initial freezing/thawing pre-treatment intensified the yeast cell disruption, facilitating the SLE, as clearly noted with the increase of recovery yields. Regardless the existence of an initial pre-treatment, the extraction ability of a solvent is again observed to be enhanced for mixed biosolvents with balanced polar/non-polar characteristics.

The last mixture design was carried out for the recovery of carotenoids and lipids from pre-treated (freeze/thaw) dry biomass. As confirmed by the results detailed in Table S1 from Supplementary Material and represented by the intensification of the contour plots in the Fig. 3 g) to j), the drying of the R. glutinis biomass increased the SLE performance of most of EtOH:EtoAc:H$_2$O mixtures, in comparison with the other biomass samples. The recovery yields of the β-carotene and torularhodin increased up to 75.80 ± 0.66% (w/w), which were achieved in the binary mixture (point F) composed of 33.3% (w/w) of EtOH and 66.7% (w/w) of EtoAc, while a maximum recovery yield of 75.04 ± 0.33% (w/w) for lipids was obtained in the binary mixture composed of 66.7% (w/w) EtOH and 33.3% (w/w) H$_2$O (i.e., point E). These results demonstrated that the drying of biomass allowed to intensify the extraction of the carotenoids (of extremely non-polar nature) by solvents with a more hydrophilic nature (i.e., EtoAc) [1] and, therefore systems with high EtoAc content are more effective for the recovery of these biomolecules. However, as above-mentioned, for FFA, due to the presence of carboxylic acids and the existence of protein-lipid complexes, systems with low quantity, or even without EtoAc, are preferable.

### 3.2.3. Comparison and understanding of the SLE mechanisms: Mixed biosolvents properties versus biomass nature

In the two previous sections, it was demonstrated that mixed biosolvents (binary or ternary mixtures) are effective solutions for the recovery of intracellular carotenoids, torularhodin, β-carotene, torularhodin and fatty acids accumulated in the R. glutinis biomass. It was evident that a mixture design is important to adjust the solvation mechanisms for the target solute(s) as well as to intensify the cell disruption/permeabilization and biomass dissolution abilities of the solvent. However, in our opinion, what must be emphasized is that, regardless of the nature of the biomass and the target solute, mixtures of biosolvents (binary or ternary) allow recovery rates higher than those of pure solvents. The differences in the recovery yields of β-carotene, torularhodin and lipids using pure
biosolvents and the best mixtures can be clearly seen in Fig. 4. As shown in Fig. 4 all pure biosolvents are still able to extract some β-carotene, torularhodin and lipids from the three *R. glutinis* biomass samples, with the carotenoids/lipids recovery yields increasing as follows: EtOLac > EtOAc ≃ EtOH > H2O. Due to the amphiphilic nature of EtOLac (with both polar and non-polar domains), this is the biosolvent with highest extraction aptitude to release the intracellular compounds from the yeast cells. The more hydrophobic is the nature of the solvent (log Kow values > 0), as it is the case of EtOAc, the lower are the recovery yields, being the hydrophobicity of the solvent a diffusion barrier, i.e., making difficult the solvent diffusion through the cell wall in wet cells and in the penetration in the hydrophilic environment surrounding the intracellular solutes. Similarly, the lack of non-polar character in the solvent also negatively impacts the recovery of these biomolecules (particularly carotenoids). As demonstrated with the very low recoveries obtained using H2O, due to the poor solubility of the carotenoids (practically insoluble in water) [1].

Therefore, as confirmed with higher recovery yields with pure EtOLac (in comparison with the other pure solvents), solvents with both polar and non-polar moieties are more effective in the SLE of carotenoids and lipids from the three distinct samples of yeast biomass. However, as much as the solvent has both polar and non-polar characteristics, it will be limited by its intrinsic chemical structure. Alternatives approaches using ionic liquids [3,47] and deep eutectic solvents [48,49] have been successfully applied for the recovery of hydrophobic molecules from biomass. Of course, as confirmed with the results compiled in Fig. 4, a much simpler and cheaper approach is to mix polar and non-polar solvents, widening the hydrophobic-hydrophilic window of the solvent as well as to increase the recovery yields of biomolecules with extremely well as to increase the recovery yields of biomolecules with extremely non-polar (like carotenoids), slight non-polar (like FFA) and polar (like proteins) characteristics. After careful design, the mixed biosolvents can allow carotenoids/lipids recovery yields of two or three-fold higher than the corresponding pure solvents. These results clearly demonstrate the advantage of using mixed biosolvents for recovery of biomolecules, since the interactions that maximize the SLE performance can be finely adjusted as a function of the type of biomass or the treatment it has suffered.

The results reported in Fig. 5 allow an evaluation of the biomass treatment on the SLE performance. Regardless of the solvent used (pure or mixture), the pre-treatment of the *R. glutinis* biomass with one freeze/thaw cycle improved the recovery yields, confirming the positive effect of pre-treatment in the extraction. In that case, prior to the implementation of these SLE processes at a large scale, what is important to assess if the increase in recovery yields is enough to offset the energy costs from the pre-treatment stage. Reports about carotenoids and lipids extraction from *R. glutinis* are inexistent in literature. Imatoukene et al., [23] evaluated the extraction of lipids using a high-pressure homogenizer combined with isooamyl acetate, butyl acetate, and ethyl acetate for cell disruption of *Yarrowia lipolytica* (wet and dry), obtaining high recoveries of lipids for the extraction from dry *Y. lipolytica* biomass. Despite of the differences in the extractive performances, the authors stated that each biomass has its own advantages and disadvantages, namely: high extraction yields of non-polar lipids achieved with dry biomass but the drying process contributed most for the total process energy; the extraction from a wet biomass reduced the processing cost but also provided lower extraction yields due to the water content [23].

In the last paragraphs we provided a discussion regarding the different roles of the biosolvents and pure solvents for the recovery of carotenoids and fatty acids from the three biomass samples. Since the main objective of this work was not only to evaluate and understand the extractive performance of two mixtures of biosolvents, i.e., EtOH:EtOLac:H2O and EtOH:EtOAc:H2O, but also to find the best mixture ratios for the recovery of the target solutes and how these are influenced by the three types of biomass pre-treatment. In addition to optimize the SLE of the biomolecules, it is thus essential to elucidate the influence of each biomass pretreatment and the respective solvent mixture ratio. Considering the number of experimental parameters analyzed in the previous two sections and to obtain a more clear picture of the main mechanisms behind the recovery of carotenoids and lipids, in Fig. 5 we compared the ratios of the EtOH:EtOLac:H2O and EtOH:EtOAc:H2O systems, in which the highest carotenoids / FFA recovery yields were achieved, as a function of each type of *R. glutinis* biomass, i.e.: FRESH untreated wet; pre-treated WET; pre-treated DRY biomasses. Considering that the β-carotene and torularhodin recovery yields were obtained in the same mixture point and to facilitate the understanding of mechanisms, they are depicted and discussed together as carotenoids.

Fig. 5 clearly demonstrates that the best composition ratio for the recovery of intracellular carotenoids and lipids is not the same for the mixtures containing EtOLac or EtOAc. It is particularly relevant that for EtOAc-based mixtures, regardless of the *R. glutinis* biomass nature, the highest recovery yields of all solutes are always obtained using systems rich in EtOH, namely, composed of weight concentrations higher than 66.7%, followed by lower amounts of EtOAc (for carotenoids) or H2O (for FFA). It should be noted that for the recovery of carotenoids the absence of H2O is recommended (specifically for dry biomass), while for lipids the extractions are maximized in systems without the EtOAc (that has the highest non-polar nature). These results are in agreement with our previous observations obtained using COSMO-RS modelling [1], in which it was demonstrated that carotenoids due to non-polar characteristics are preferentially dissolved in solvents with non-polar nature (solvents with low water content), while the extraction of FFA is mainly governed by hydrogen bond and electrostatic misfit interactions between the solute and the solvent (with low amount of EtOAc). It should be stated that the lipids accumulated in *R. glutinis* CCT-2186 biomass are preferentially FFA [margaric acid (75%), stearic acid (10%) and pentadecylic acid (8%)] (Figure S1 from Supplementary Material), thus prone to form hydrogen bonds with solvents like EtOH and H2O. However, it is important to remember that these FFA have long non-polar moieties which also promote dispersive interactions with the hydrophobic portions of the solvent [1].

On the other hand, a general analysis of the best EtOH:EtOLac:H2O mixtures for the three biomass samples clearly reveals that systems with low water content (<10% w/w) are the most efficient in the recovery of carotenoids and FFA. Two distinct trends are observed, high amount of EtOLac to maximize the extraction of carotenoids (mainly in the SLE from the two pre-treated biomass samples) and high amount of EtOH for lipids recovery. These two trends are in line with solute–solvent mechanisms described for the EtOAc-based mixtures.

In summary, for the EtOLac and EtOAc mixtures, the results seem to indicate that using mixtures containing a more non-polar character, that is EtOAc (log Kow = 0.73 [50]), the solutes recoveries yields are enhanced by using EtOH:H2O binary systems, or with ternary mixtures with low EtOAc content. On the other hand, for mixtures containing EtOLac, which is a solvent with slightly non-polar nature (as confirmed by the log Kow = 0.2) [50], EtOH:EtOLac binary mixtures or ternary mixtures with low amount of H2O are the most efficient in the recovery of both biomolecules. Therefore, it seems that a subtle balance on the relative hydrophobicity (which can be obtained by adding a minor amount of EtOAc or higher amount of EtOAc and/or EtOH) is the key to increase the release of intracellular compounds from *R. glutinis* biomass. Of course, having always in mind that the mixed biosolvent should be designed to guarantee at same time high diffusion rates and solubility capabilities.
Fig. 4. Recovery yields (% w/w) of \( \beta \)-carotene (a), torularhodin (b) and lipids (c) at \( R. \ glutinis \) biomass [fresh untreated wet, pre-treated (freeze/thaw) wet, and pre-treated (freeze/thaw) dry, respectively] concentration of 0.2 g/mL after 1 h stirring (300 rpm) at 65 °C. The results are presented as the average value of three independent assays with a 95% confidence level.
In summary, the presence of a minimal H$_2$O content in the mixture is important for the recovery of biomolecules from the fresh untreated and pre-treated wet biomasses. Particularly, it contributes positively to the miscibility of the solvent with wet cells and the permeabilization of the cell wall (i.e., helping to solubilize the main constituents of the cell wall (proteins and lipids) [1,51]). However, the H$_2$O concentration should be low, in order to prevent a decrease of the recovery yields as a result of the limited solubility of bioactive compounds with extremely hydrophobic nature (specially, carotenoids) [52]. However, since the main objective of the SLE is the recovery of all intracellular compounds from R. glutinis cells, the mixture design should consider a balance between the miscibility of biomass (different when the biomass is wet or dry), the diffusion of the solvent through the cell wall for fatty acids solubilization, and the solubilization of intracellular highly hydrophobic carotenoids and less hydrophobic fatty acids in the intracellular hydrophilic environment of the cells (even after drying a minor water content will still surround the intracellular solutes).

### 3.3. Recycling of biosolvents and polishing of carotenoids and lipids

In this work, two different ternary systems composed of EtOH:EtOLac:H$_2$O (System a) and EtOH:EtOAc:H$_2$O (System b) were optimized through an experimental planning for the extraction of β-carotene, torularrhodin and fatty acids from three different R. glutinis biomass samples. Having in mind the future implementation of this technology at industrial level, it is crucial to evaluate the recyclability and reuse of the biosolvents as well as the subsequent integration with polishing stages. From a sustainable perspective, if the isolation/purification of biomolecules and the efficient reuse of solvents is guaranteed, the technology will be closer to the market.

Therefore, the processual integration of the proposed technology for the recovery of carotenoids and lipids from yeast biomass was carried out, by evaluating the use of subsequent operations for recycling the biosolvents and separate carotenoids from fatty acids. Despite the high recovery yields obtained with the pre-treated dry biomass, as reported in literature [23], the incorporation of an additional drying unit in the process will be responsible for most of the energy consumption on the process. Considering that wet biomass also allowed to obtain good recovery yields of carotenoids and lipids (recovery yields > 60% w/w) and to decrease the implementation costs of this technology, all the recycling studies were then performed using pre-treated wet biomass. The extracts obtained using the mixture point F (w/w) composed of 67% of EtOLac and 33% of EtOH and the mixture point N (w/w) composed of 80% of EtOH, 10% of EtOAc and 10% H$_2$O were recovered and subjected to following recycling and polishing studies. After the SLE from each
After the precipitation, a LLE using a system composed of EtOH:EtOAc:H₂O was carried out for the selective separation of carotenoids and fatty acids (with a reuse of the mixed solvent in the next LLE). Note that the initial evaporation of the mixed biosolvents used in the SLE (before the LLE) was included to allow a comparison between the recycling procedures for the EtOAc and EtOAc mixtures. However, if the mixture EtOH:EtOAc:H₂O is selected for the SLE, the following LLE can be easily achieved by adding further amounts of the biosolvents to reach the biphasic regime, avoiding the incorporation of an extra evaporation unit (this integrative approach was recently demonstrated by us [1]). A schematic diagram of the proposed process is depicted in Fig. 6, which includes two main stages: (i) Solid-liquid extraction procedures, were the β-carotene, torularhodin and fatty acids are recovered from the pretreated wet R. glutinis biomass by applying two different systems composed of biosolvent mixtures (EtOH:EtOLac and EtOH:EtOAc:H₂O). (ii) Solvent recycling, selective separation and polishing of β-carotene, torularhodin and fatty acids were performed using LLE.

The recycling/polishing procedures were repeated three times, and the respective recovery yields (% w/w) of β-carotene, torularhodin and FFA determined. All results are listed in Table 1, including a comparison between using “fresh” (non-reused) solvent mixture (control) and the reused mixtures.

As detailed in Table 1, both mixtures can be recycled up to three times, guaranteeing high extraction efficiencies, as demonstrated by the losses lower than 10% for carotenoids and 2% for lipids in comparison with the control (fresh non-reused mixed solvent). Namely, after three recycling cycles, the recovery yields (% w/w) were higher than 48.92 ± 0.67, 43.28 ± 0.57, 29.15 ± 0.83 (System a - SLE using EtOH:EtOLac) and 49.18 ± 0.34, 47.14 ± 0.65, 52.33 ± 1.04 (System b – SLE using EtOH:EtOAc:H₂O) for β-carotene, torularhodin and fatty acids, respectively.

The biosolvents-based extraction technology proposed in this work is simple and effective for the recovery and selective separation of β-carotene, torularhodin and fatty acids from R. glutinis pre-treated wet biomass. However, considering that the recycling and polishing step were performed without treatment, it is advised for further scale-up studies, to evaluate the make-up of the solvent (by adding fresh solvent solution), which will not only restore solvent losses during the evaporation but also to improve the solvent extractive performance of this integrated platform.

### Table 1

<table>
<thead>
<tr>
<th>Recovery yields ± σ (% w/w)</th>
<th>Mixed Solvent</th>
<th>First reuse</th>
<th>Second reuse</th>
<th>Third reuse</th>
</tr>
</thead>
<tbody>
<tr>
<td>System a (EtOH:EtOLac)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td>56.84 ± 3.62</td>
<td>50.87 ± 1.10</td>
<td>49.53 ± 0.16</td>
<td>48.92 ± 0.67</td>
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<tr>
<td>Torularhodin</td>
<td>52.75 ± 1.62</td>
<td>48.58 ± 1.61</td>
<td>46.40 ± 1.64</td>
<td>43.28 ± 0.67</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>31.88 ± 0.04</td>
<td>29.84 ± 0.41</td>
<td>29.09 ± 0.39</td>
<td>29.15 ± 0.57</td>
</tr>
<tr>
<td>System b (EtOH:EtOAc:H₂O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-carotene</td>
<td>60.17 ± 5.67</td>
<td>53.47 ± 0.94</td>
<td>51.07 ± 0.94</td>
<td>49.18 ± 0.34</td>
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<tr>
<td>Torularhodin</td>
<td>51.81 ± 0.78</td>
<td>49.83 ± 0.77</td>
<td>46.96 ± 0.31</td>
<td>47.14 ± 0.65</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>53.36 ± 0.03</td>
<td>53.54 ± 0.77</td>
<td>53.14 ± 0.31</td>
<td>52.33 ± 0.65</td>
</tr>
</tbody>
</table>

* The results represent 95% confidence levels for the mean of three independent assays.

### 3.4. Antioxidant activity of extracted carotenoids

In the previous section we demonstrated that the mixtures of biosolvents are efficient for the recovery of intracellular carotenoids and fatty acids from yeast cells as well as for their selective separation. Despite the high recovery yields of both biomolecules, it is important to guarantee that at the end of the downstream processing these compounds still exhibit their biological activities [53,54]. Thus, in the final stage of this work, the antioxidant activity of the extracted carotenoids was evaluated, evaluating if the proposed integrated process is carotenoid-friendly, i.e., did not affect their biological activity. For that purpose, the DPPH* radical scavenging method by UV spectrophotometry is one of the most frequently used to assess antioxidant activity due to its low-cost and simplicity [55,56]. However, one of the main limitations of the spectrophotometric DPPH* assay is the overlapping spectra of carotenoids, which absorb in the same wavelength range as the DPPH* (450–550 nm), causing interferences with the results [57]. To overcome this issue, the antioxidant activity of extracted carotenoids was measured using Electron Paramagnetic Resonance (EPR) spectroscopy, which directly measures the concentration of DPPH* radical at a submicromolecular level [58]. The antioxidant activities of carotene (β-carotene) and xanthophyll (torularhodin) evaluated in this study are...
presented in Table S2 in the Supplementary Material and Fig. 7 a) and b) as the concentration-dependent evolution of the EPR spectrum of the methanolic DPPH⁺ solution using *R. glutinis* extracts of β-carotene (concentration from 100 to 500 μg/mL) and torularhodin (125 to 250 μg/mL), respectively.

The DPPH solution showed a reduction in the intensity of EPR signal after addition of different concentrations of both carotenoids, indicating that the carotenoids preserve the antioxidant properties (Fig. 7), and the use of the integrative platform using a ternary mixture of EtOH:EtOAc: H₂O is a milder and efficient alternative for recovering this class of bioactive compounds from *R. glutinis* cells. Carotenoids extracts showed positive results in DPPH⁺ radical scavenging assay, with a percentage inhibition increased with the concentration (Fig. 7 a and b). Relatively to the antioxidant capacities of carotenes and xanthophylls, it is clearly demonstrated that torularhodin extract at 250 μg/mL allowed the highest antioxidant activity (93%), approximately 2-fold higher than the β-carotene, at same concentration. This difference is a result of the structural features of each carotenoid, particularly, the length of conjugated double bonds of carotenes and xanthophylls, which regulate the scavenging ability [53]. The β-carotene have 11 conjugated double bonds while torularhodin have 13 [6]. Therefore, the decrease of β-carotene scavenging ability is a result of the two-double bonds in cyclohexene rings that are not planar with the rest of the molecular backbone [53,56]. This result confirms that *R. glutinis* yeast are a good source of powerful antioxidants (carotenoids), which can be further used in formulation of functional foods, as well as pharmaceutical, nutraceutical and cosmeceutical preparations.

4. Conclusions

In this work, an integrated process for the recovery and polishing of torularhodin, β-carotene and fatty acids from three samples of *R. glutinis* CCT-2186 biomass subjected to different pre-treatments and using two systems of bio-based solvent mixtures were studied. The results show that independently of the biomass pre-treatment, a subtle balance on the relative hydrophobicity of the solvent mixture is the key to increase the release of intracellular compounds from *R. glutinis* biomass. The pre-treatment stage with one freeze/thaw cycle was found to improve the recovery yields, especially for dry biomass, confirming not only the positive effect of the pre-treatment in the recovery but also the positive effect of drying. However, the incorporation of an additional drying unit can increase the costs of the process, requiring a careful assessment of the technology cost before implementation. The integrative process of the cell disruption using SLE and separation/polishing with LLE allows the efficient and selective separation of carotenoids from fatty acids, and the biosolvents reuse, as demonstrated up to three cycles, without affecting the recovery yields. The antioxidant potential of torularhodin and β-carotene extracted from *R. glutinis* CCT-2186 biomass was determined, confirming that the integrative extraction platform using these mixed biosolvents is carotenoid-friendly, without negatively affecting their biological activity. These findings confirmed the potential of using bio-based solvent mixtures in the extraction of biomolecules with non-polar character at mild conditions as well as their use in SLE-LLE integrative platforms as environmentally friendly and biocompatible alternatives for cost-effective and efficient recovery of high-added value compounds from biomass sources.

CRediT authorship contribution statement

Cassamo U. Mussagy: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. Daniela Remonatto: Investigation. Ariela V. Paula: Methodology. Rondinelli D. Herculano: Methodology. Valéria C. Santos-Ebinuma: Conceptualization, Visualization. João A.P. Coutinho: Conceptualization, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. Jorge F.B. Pereira: Conceptualization, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Fig. 7. Concentration-dependent evolution of EPR spectrum of the methanolic DPPH⁺ solution of β-carotene (a) and torularhodin (b) extracted from *R. glutinis*.

Conceptualization, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References


