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# Protic ionic liquids as cell disrupting agents for the recovery of intracellular carotenoids from yeast *Rhodotorula glutinis* CCT-2186

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**ABSTRACT**

*Rhodotorula glutinis* yeasts are natural sources of intracellular carotenoids such as β-carotene, torularhodin and torulene. Since these yeasts are constituted by a rigid cell-wall structure, the use of energy-saving and high-efficiency cell disruption procedures is critical for carotenoids recovery. A new technology using protic ionic liquids (PILs) was here evaluated as alternative platform to permeabilize the *R. glutinis* cells and to improve the extraction of β-carotene, torularhodin and torulene. The cell disruption ability of twelve highly concentrated aqueous solutions of ammonium-based PILs was determined, evaluating the influence of the relative ions hydrophobicity, solid-liquid ratio (SLR), water content, and temperature. Carotenoid extraction yields increased with the hydrophobicity of the PILs (*i.e.* increase of alkyl chain length of anion or cation), temperature (from 25 ºC to 65 ºC) and PIL concentration (from 75% to 90% v/v). Additionally, to demonstrate the potential of PILs in carotenoids recovery, solvent recycling and carotenoids polishing were carried out using a three-phase partitioning (TPP) system. The results demonstrate that the use of PILs as cell disrupting agents can be a simple, efficient, sustainable and feasible method to recover intracellular carotenoids from microbial biomass.

**Keywords:** *Rhodotorula glutinis*; carotenoids; extraction, protic ionic liquids; β-carotene; torularhodin; torulene.

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# INTRODUCTION

Carotenoids are a group of yellow, orange and red polyisoprenoid pigments, that can be naturally synthesized by different microorganisms, including microalgae, bacteria, yeasts and filamentous fungi [1,2]. These biopigments exhibit a plethora of biological attractive properties, such as antioxidant, antiobesity, antidiabetic, anticancer (particularly those that results from cardiovascular diseases and macular degeneration) and antimicrobial activities [3–5]. As a result of their interesting biological properties, natural carotenoids have been attracting great interest from academic and industrial partners for applications in pharmaceuticals, cosmetics and functional foods formulations, as well as the most common uses in food industries [6–8].

*Rhodotorula glutinis* are aerobic yeasts [9] able to synthesize, in an easy and natural way, several industrial high-added value metabolites, *e.g.* lipids, carotenoids, and enzymes. Particularly, these yeasts can produce and accumulate approximately 50% of their dried cellular biomass as fractions of carotenoids and lipids [10,11]. Among the carotenoids, *Rhodotorula glutinis* are able to synthesize β-carotene, torulene, and torularhodin, exhibiting variable production yields according to the specific cultivation conditions [11] and fast grow [12].

However, since the carotenoids from *R. glutinis* yeast are produced intracellularly, appropriate cell-disrupting methodologies are always required for their recovery [13]. A large number of cell disruption techniques, including conventional and non-conventional procedures, have been reported in literature [2,14]. Traditionally, *Rhodotorula glutinis* yeasts are disrupted by using conventional solid-liquid extraction procedures with volatile organic solvents (VOCs), such as petroleum ether, dimethyl sulfoxide (DMSO), acetone, chloroform and hexane [15]. Among the common VOCs, the use of DMSO, in general, lead to the highest release rates of intracellular carotenoids, but their use in industrial processes is limited by health and environmental concerns [16,17].

Therefore, the search for more biocompatible and environmentally friendly solvents, as for example, biosolvents, ionic liquids (ILs) and deep eutectic solvents, is of great importance. Among these, ILs, commonly defined as salts with a melting point lower than 100 °C [18], have been suggested as one of the most interesting

classes of alternative solvents, mainly due to their unique features like high hydrolytic activity, low volatility, high solvation capacity, and excellent thermal and chemical stabilities [19].

Some studies evaluated the capability of different ILs for the extraction of carotenoids [19–21]. Choi and collaborators [19] extracted astaxanthin and lipids from *Haematococcus pluvialis* cyst cells using ten 1-ethyl-3-methylimidazolium ($[Emim]^+$)-based ILs, obtaining complete astaxanthin recoveries (> 99%) and high lipid extraction yields (~82%) at optimum operating conditions, *i.e.* 6.7% (v/v) of IL in water, 30 °C of temperature and 60 min of extraction time. Desai and collaborators [20] recovered more than 70% of intracellular astaxanthin from intact *H. pluvialis* cells using an aqueous solution of 1-ethyl-3-methylimidazolium di-butyl-phosphate ([Emim][DBP]) (40 wt%). Vieira and collaborators [21] recovered fucoxanthin from *Sargassum muticum* cells using aqueous solutions of different surface-active ILs. These examples demonstrate that ILs are effective solvents to extract carotenoids from natural sources; however, most of these works have been performed using "classic" aprotic ILs (AILs). Recent studies have indicated that AILs are not the most adequate for biological-based applications due to their cytotoxicity [22], which led to a growing interest in the third-generation of more benign and biocompatible ILs, *i.e.* protic ionic liquids (PILs) [23].

PILs are a subset of ILs prepared through the stoichiometric neutralization reaction of certain Brønsted acids and Brønsted bases. They have a proton available for hydrogen bonding, and differ from the classic AILs in having both cationic and anionic counterparts formed by low molecular weight organic compounds, usually substituted (or poly-substituted) amines as cations, and organic acids as anions [23].

PILs are more favourable than AILs in terms of toxicity and biodegradability [24]. This fact, coupled with its low production cost, easier synthesis and more benign nature (*i.e.* can be obtained fully or partly from natural raw materials) make them attractive solvents for wider use in industrial bioprocesses [25].

Considering these assumptions, the aim of this study was to evaluate the capability of PILs to disrupt (permeabilize) the cell wall of *Rhodotorula glutinis* yeasts and to increase the recoveries of intracellular carotenoids. Twelve different PILs,

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obtained from the neutralization of three different propylamines and four different carboxylic acids, were used to assess the effect of the relative hydrophobicity of both cation and anion on cell disruption and carotenoids extraction. The carotenoids extraction efficiencies exposed to different aqueous solutions of the twelve PILs (ranging from 75 to 90% (v/v)) at three different temperatures (25 °C, 45 °C and 65 °C) and four different SLR (0.05, 0.1, 0.2 and 0.5 g/mL) were determined. To obtain further information about the mechanisms and the disruption ability of the PILs over the *R. glutinis* cell walls, samples of cells after the different treatments were also analysed by scanning electron microscopy (SEM). The overall sustainability of the proposed technology was assessed in terms of PIL recyclability and carotenoids polishing.

# EXPERIMENTAL SECTION

## Materials

β-carotene and torularhodin standards were acquired from Carbosynth® (San Diego, CA, USA). Dimethyl sulfoxide (DMSO) (P.A), ethyl acetate (P.A) and glutaraldehyde (25 wt% in water) were acquired from Exodo Cientifica (Sumaré, SP, Brazil) and potassium phosphate tribasic ($K_3PO_4$) (P.A) from LS Chemicals (Ribeirao Preto, SP, Brazil). All the amines and carboxylic acids used in the PILs synthesis were purchased from Sigma-Aldrich (St. Louis, MO, USA), namely: Propylamine (PA) (98%), 3-Dimethylamino-1-propylamine (DMAPA) (99%), 3-Diethylamino-propylamine (DEAPA) (99%) as cations; hexanoic acid (Hex) (99%), butyric acid (But) (99%), propanoic acid (Pro) (99.5%), and acetic acid (Ac) (99.8%) as anions. PILs were synthesized *via* neutralization reaction of the base with the appropriate acid, according to the procedure previously reported [26].

All details about the synthesis, as well as the corresponding structures and purities obtained by proton nuclear magnetic resonance ($^1$H NMR) analysis (Bruker Avance III HD 600 (14.1T)) (Massachusetts, USA) are reported in the **PILs synthesis** section from **Electronic Supporting Information (ESI).**

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## Microorganism and cultivation conditions

*Rhodotorula glutinis* CCT-2186 yeasts, isolated from the leaf of kaki fruit (Diospyros), were acquired from the Tropical Culture Collection *André Tosello* (Campinas-SP, Brazil). A stock culture of the microorganism (50% (v/v) glycerol) was maintained at -80 °C. Cells were grown for 48 h at 30 °C and 150 rpm in 100 mL Erlenmeyer® type flasks containing 25 mL of yeast extract–peptone–dextrose (YPD) medium. An aliquot of 0.2 mg/mL of yeast cells was used as inoculum. For production, batch fermentations were carried out in 500 mL Erlenmeyer® type flasks, each one containing 100 mL of modified Czapek Dox medium, using a rotary orbital shaker (Tecnal, model TE- 421 Piracicaba, SP, Brazil) at 30°C/ 150 rpm for 72 h. After 72 h of cultivation (maximum cell biomass concentration of 5.6 g/L), the fermented medium was centrifuged at 2500x*g* for 10 min at 4 °C, using a Hitachi CR-22N centrifuge (Tokyo, Japan). The supernatants of all fermented media were then discarded, and the cellular pellets containing carotenoids were collected and stored for the next carotenoid extraction studies. Further details about carotenoids production can be found in the sections **Inoculum and growing conditions** and **carotenoids production** from the **ESI**.

## Carotenoids isolation and characterization

Intracellular carotenoids were firstly isolated by using a conventional method described by Park and collaborators [15]. Briefly, *R. glutinis* wet biomass was subjected to chemical treatment with successive solvent extractions using DMSO. After the extraction with DMSO, the samples were centrifuged at 2500 x*g* for 10 min at 4 °C, using a Hitachi CR-22N centrifuge (Tokyo, Japan). The supernatant containing carotenoids were then lyophilized. Lyophilized extracts were solubilized in acetone and separated by liquid chromatography in a column filled with silica gel 60 (Merck®, Pinheiros, SP, Brazil), and fractioning the carotenoids using a mobile phase composed of hexane/ethyl ether/acetic acid (70:29:1 v/v/v). A qualitative analysis of the extracts by thin layer chromatography (TLC), using pre-coated TLC sheets ALUGRAM® (silica gel 60, Macherey-Nagel, Germany), was also carried out (hexane/ethyl ether/acetic acid (70:29:1 v/v/v) as mobile phase).

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The three fractions of the carotenoid lyophilized extracts separated in the column liquid chromatography were collected in different glass test tubes, and then identified by Reverse Phase High Performance Liquid Chromatography, RP-HPLC, (using a column chromatography Shimadzu® Shim-pack $C_{18}$ (Japan), 4.6×250 mm and methanol/acetonitrile/dichloromethane (60:10:30, v/v/v) as mobile phase. The chemical structures and purities of the three purified fractions of carotenoids were also confirmed by $^{1}$H NMR. Further details can be found in the section **conventional methodology for separation and characterization of carotenoids** from the **ESI**.

## Quantification of carotenoids concentration

After the identification and characterization of the chemical structures of carotenoids produced by *R. glutinis* yeasts, the respective visible light absorption spectra were obtained using Thermo Scientific® (Genesis 10S) UV-Vis spectrophotometer (accuracy: ±0.005 A at 1.0 A) (China). The visible light spectra from 380 to 600 nm were obtained and the respective carotenoids calibration curves acquired at 450 nm (β-carotene), 480 nm (torulene) and 500 nm (torularhodin). The carotenoids concentration (µg/mL) were determined according to pre-established calibration curves obtained from the pure β-carotene and torularhodin standards, and pure torulene obtained from the cells of *R. glutinis*. All details of carotenoids quantification are fully described in the section **Carotenoids quantification** from the **ESI**.

## Carotenoids solid-liquid extraction (SLE) using PILs

Aqueous solutions of the twelve ammonium-based-PILs were prepared and used for the disruption of the *R. glutinis* cells, and subsequent carotenoids recovery. As a control, following previous reports that used VOCs as chemical solvents for the extraction of carotenoids from *R. glutinis* cells, [15,27,28] a conventional method using DMSO was also carried out. All assays were performed according to the following procedures: 1) to remove impurities, the *R. glutinis* wet cells were washed 3-times using 3 mL of phosphate buffer (pH 7); 2) after washing, different amounts of wet biomass (0.05, 0.1, 0.2, 0.5 g) were added in 2 mL Eppendorf® type tubes; 3) the tubes were filled with 1 mL of different aqueous solutions of all ammonium-based

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PILs (at 75%, 80%, 85% and 90% (v/v)), or with 1 mL of DMSO for the control assay; 4) samples were homogenized using a rotatory orbital sample shaker (Norte Científica, NH 2200, Araraquara, SP, Brazil) for 1 h at 30 rpm; 4) after homogenization, all the samples were centrifuged at 2500*xg* and 25 ºC for 5 min using an Eppendorf® 5415r centrifuge (Willow Springs, NC, USA); 5) after centrifugation, all cell lysate supernatants were filtered using a Millipore® filter membrane (0.22 μm) and stored for further quantification of carotenoids, while the pellets containing the cellular *debris* were stored for further SEM analysis (further details in **Scanning electron microscopy (SEM) analysis** section from the **ESI**).

**Recycling of the PILs and carotenoids polishing**

The PIL providing the highest purification yield was chosen for the solvent recycling and carotenoids polishing studies. In this stage, samples containing 1 g of wet biomass were prepared in 15 mL conical centrifuge tubes, which were filled with 10 mL of 90% (v/v) of [DEAPA][Hex] in water. The samples were then homogenized in rotatory orbital sample shaker for 1 h, at 30 rpm and 25 ºC. After homogenization, all samples were centrifuged at 2500 *xg* at 25 ºC for 5 min. After centrifugation, cell lysate supernatants were filtered using a Millipore® filter membrane (0.22 μm) and the biomass-containing pellets discarded. Therefore, for the carotenoids polishing and PILs recycling, a three-phase partitioning (TPP) system composed of 60 wt% of [DEAPA][Hex] (with carotenoids), 14 wt% of $K_3PO_4$ and 26 wt% of $H_2O$ was prepared. The three-phase system (liquid-solid-liquid) was then centrifuged at 2500 *xg* at 25 ºC for 10 min. Most of the carotenoids were precipitated as a solid fraction in the interface between both liquid (top and bottom) phases. The solid fraction was carefully removed and both the PIL (top)-rich phase and $K_3PO_4$ (bottom)-rich phase were reused for consecutive carotenoids extraction steps. The carotenoid-rich solid fraction was then subjected to a cold acetone precipitation (5 mL) to remove contaminant proteins. The protein-rich precipitates were removed, and the carotenoids dissolved in the acetone recovered by evaporation under constant vacuum at 300 Mbar, 60 °C for 20 min using a Heidolph (Hei-VAP) rotaevaporator (Schwabach, Germany) coupled to ultrathermostatic bath Solab-SL 152 (Piracicaba, SP, Brazil). The amount and purity of carotenoids were determined according to the

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above-described methods. The recycling and polishing procedure was repeated three times. The complete process for PIL recycling and carotenoids polishing is depicted in **Figure S4** from the **ESI**.

### Statistical analysis

Experiments were performed in triplicate and the results presented as the mean of three independent-assays with the corresponding errors at 95% confidence level. Statistical analyses were performed using the software R Statistic® version 3.5.3 (Vienna, Austria). The results were evaluated by analysis of variance (ANOVA) and Tukey's test in order to verify the existence of significant differences between the groups, with a 95% confidence level.

## RESULTS AND DISCUSSION

### Purification and characterization of the carotenoids

Carotenoids from *R. glutinis* were isolated and characterized qualitatively and quantitatively using different chromatographic and spectroscopic analyses. The three purified pigments (red, light red and yellow) identified as, 1: torularhodin; 2: torulene and 3: β-carotene by NMR spectroscopy, were eluted with hexane/ethyl ether/acetic acid (70:29:1 v/v/v) by column liquid chromatography, and the corresponding peaks determined by RP-HPLC chromatography, as depicted in **Figure 1A**. The TLC separation of the carotenoid extracts using the same hexane/ethyl ether/acetic acid (70:29:1 v/v/v) mobile phase confirmed the presence of the three major carotenoids (**Figure 1B**).

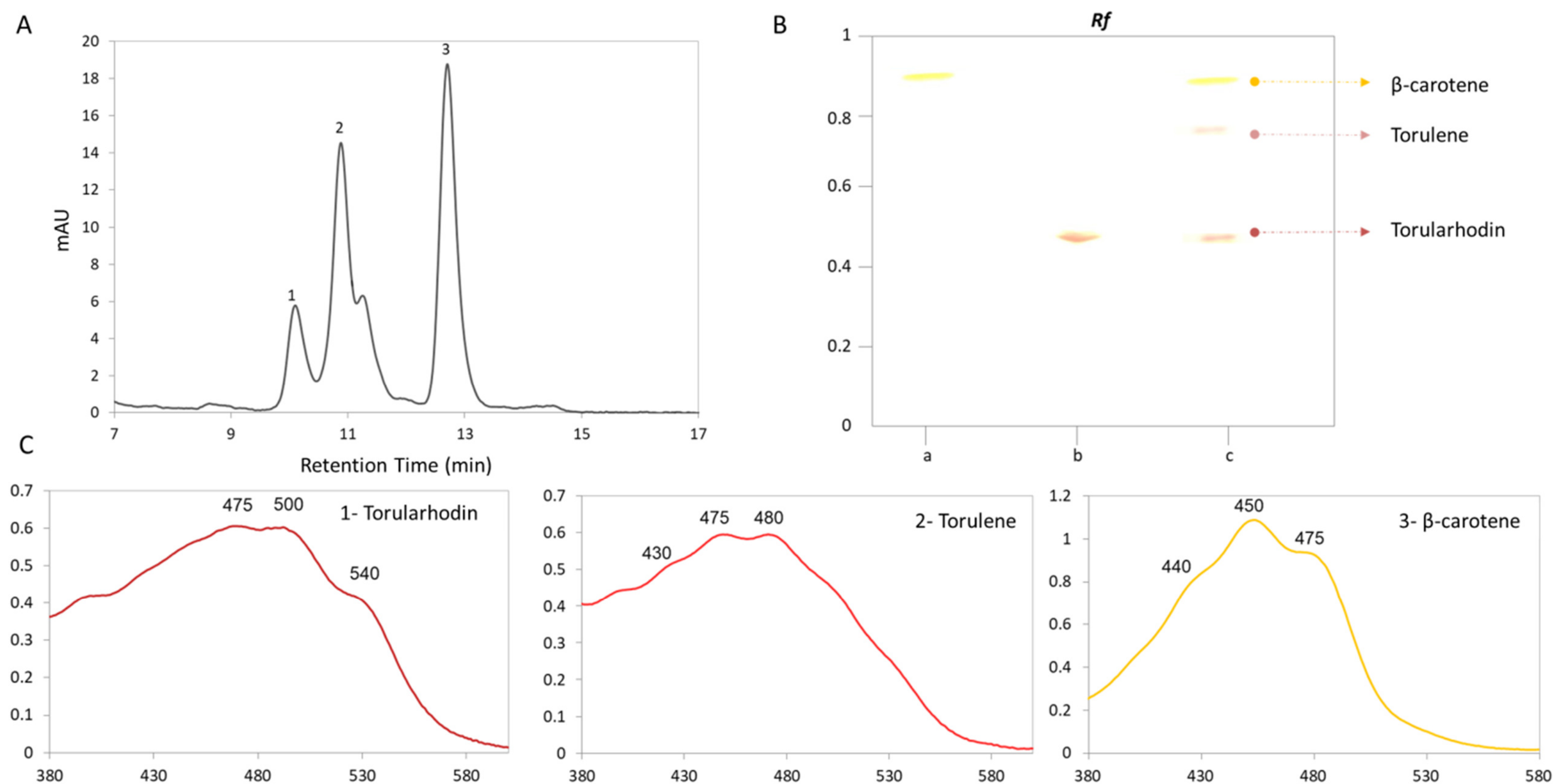


**Figure 1. A-** HPLC analysis of the three major carotenoids of *R. glutinis* CCT-2186 (1: torularhodin; 2: torulene; 3: β-carotene). **B-** Analytical TLC of *R. glutinis* cells' extracts: a- β-carotene standard, b-Torularhodin standard, c- Sample (cells' extracts). **C-** UV absorption spectra of the three major pigments of *R. glutinis*.

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As shown in **Figure 1B**, β-carotene has the highest mobility ($R_f$ = 0.97), followed by torulene ($R_f$ = 0.80), and lastly torularhodin with half of mobility ($R_f$ = 0.40). The respective $R_f$ values are in accordance with RP-HPLC chromatogram of the carotenoids (depicted in **Figure 1A**), which eluted the carotenoid fractions in an isocratic reverse phase mode, with retention times following the trend of decreasing polarity of each carotenoid fraction. Carotenoids were also identified according to their visible light spectra characteristics (**Figure 1C**). Both β-carotene and torulene carotenoids (more apolar) spectra showed a maximum absorbance at wavelengths of 450 nm ($\lambda_{max=450nm}$) and 480 nm ($\lambda_{max=480nm}$), respectively. On the other hand, torularhodin, the more polar carotenoid, exhibited a maximum absorbance at 500 nm ($\lambda_{max=500nm}$). The spectroscopic and chromatographic characteristics of the carotenoids isolated in this work are in agreement with those previously obtained by Jeevaratnam and Latha [29] and Park et al. [15].

### Effect of the PILs chemical structure on the extraction of carotenoids

After identifying the major carotenoids produced by *R. glutinis*, these were extracted using different PILs aqueous solutions. In the first set of experiments, the influence of the PIL ion nature on the carotenoid extraction was evaluated, *i.e.* the effect of increasing the anion and cation alkyl chain length. This initial experimental set was carried out at constant concentration of PILs (90% v/v), wet cell concentration of 0.2 g/mL, 1 h of stirring at 30 rpm and 25 °C. The respective values are shown in the **Figure 2** and detailed in the **Table S4** from **ESI**.

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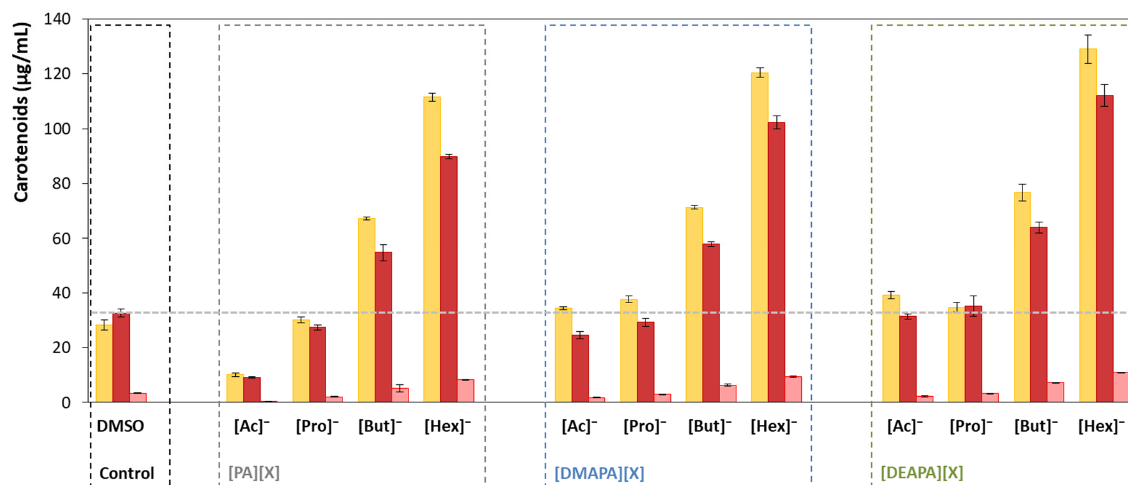


**Figure 2.** Recovery of β-carotene (■), torularhodin (■) and torulene (■) using DMSO and aqueous solutions of PILs (90% v/v) at a concentration of 0.2 g/mL of wet cells after 1 h stirring (30 rpm) at 25 °C. The error bars represent 95% confidence levels for the mean of three independent assays.

The results depicted in **Figure 2** demonstrate that the PILs exhibit high capacity to extract β-carotene (yellow bars) followed by torularhodin (dark red bars), with the lowest recoveries for torulene (pink bars). The lower recovery of torulene is in accordance with the lower abundance of this pigment in the wet biomass. Contrarily to the DMSO control assay that presents a similar aptitude to extract both β-carotene and torularhodin pigments, PILs are more selective towards β-carotene. The selectivity of PILs can be attributed to the highest hydrophobicity of this pigment that, despite of being largely produced by *R. glutinis* cells, was not effectively recovered with DMSO. Interestingly, excep [PA][Ac], all the other PILs were able to extract more carotenoids than DMSO (in the control assay (28.97 ± 1.86 µg/mL of β-carotene, 32.50 ± 1.48 µg/mL of torularhodin and 3.34 ± 0.15 µg/mL of torulene were recovered). The highest extraction rates were achieved by using [Hex]$^{-}$-based PILs, *i.e.* 6-fold more carotenoids than the control.

Analysing the effect of PIL anion nature, independently of the target carotenoid, the three families of PILs, *i.e.* [PA][X], [DMAPA][X] and [DEAPA][X], exhibit a similar pattern, where increasing the anion alkyl chain length promote higher carotenoid recovery yields.. As shown in **Table S4**, in general the extraction aptitude was significantly ($p \leq 0.05$) affected by the anion nature, according to the

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6 trend: $[Hex]^-$ > $[But]^-$ > $[Pro]^-$ > $[Ac]^-$. The results from **Figure 2** seem to indicate that
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8 the hydrophobic character of the PIL anion is a key factor for the extraction of the
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10 intracellular carotenoids.
11 Afterwards, to infer if the anion relative hydrophobicity is governing the
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13 carotenoids extraction, the concentration of carotenoids recovered with [PA][X],
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15 [DMAPA][X] and [DEAPA][X] were correlated with the logarithmic function of the
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17 octanol/water partition coefficient values, log $K_{ow}$, of the corresponding acids used
18 in the PILs synthesis (Figure 3), namely: Hex (log $K_{ow}$ = 1.84) > But (log $K_{ow}$ = 0.78)
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20 > Pro (log $K_{ow}$ = 0.25) > [Ac] (log $K_{ow}$ = -0.28). The log $K_{ow}$ values (obtained from
21 ChemSpider[45]) corresponds to a relative measurement of the hydrophobicity of the
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23 molecules, with higher values indicating higher hydrophobicity of the compounds.
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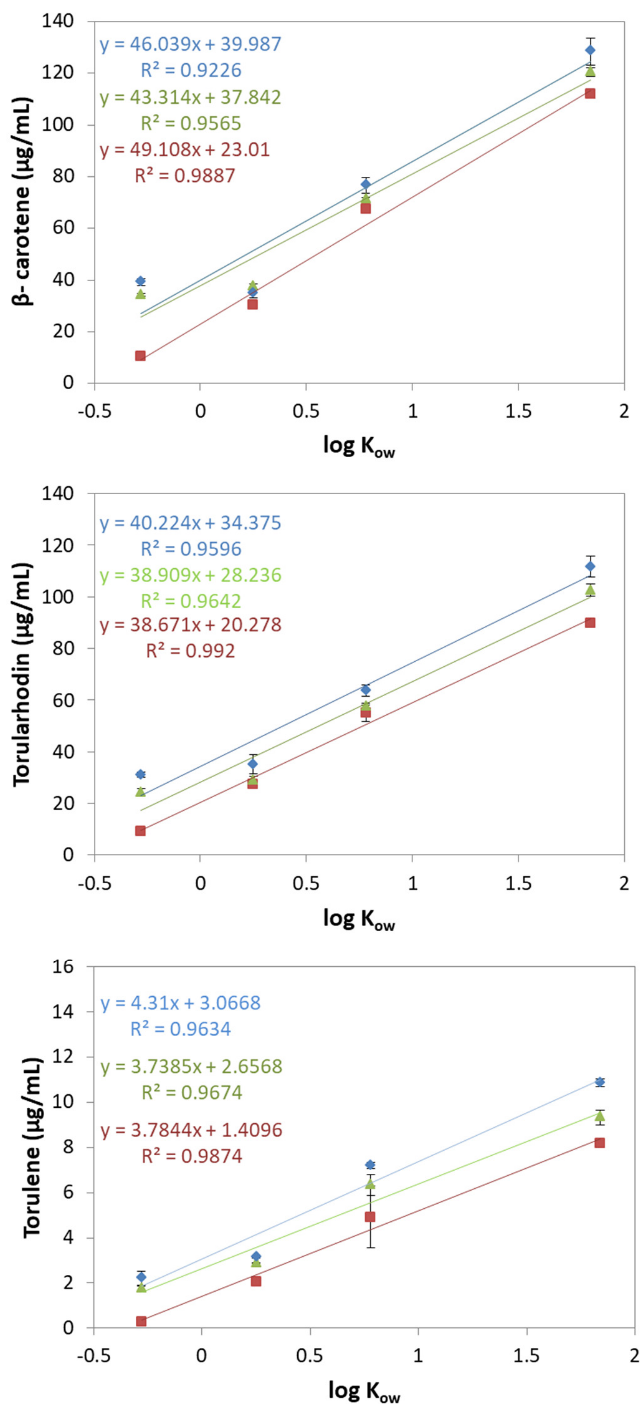


**Figure 3.** Linear relationship between log $K_{ow}$ of the anions versus A- β-carotene; B- torularhodin and C- torulene recovery at a concentration of 0.2 g/mL of wet cells after 1 h of stirring (30 rpm) at 25 °C for different PILs-based cations families: [PA][X] (■), [DMAPA][X] (▲) and [DEAPA][X] (♦).The error bars represent 95% confidence levels for the mean of three independent assays.

As can be seen in **Figure 3**, regardless of the cation used, a linear dependency between the relative hydrophobicity of the PIL anion and the carotenoids recovery yields was observed. PILs constituted by $[Ac]^-$ or $[Pro]^-$ anions, the most hydrophilic ones, showed the lowest extraction aptitude (almost equal). The similar positive slopes obtained for the three types of carotenoids confirm the increase of the recovery yields with the hydrophobicity of the PIL anion, as demonstrated by the good correlation of the linear least-squares regression obtained for all the PILs ($R^2 > 0.92$). Although it is not the focus of the work, the slight negative deviation in the linearity observed with $[Pro]^-$-based PILs is probably a result of an odd-even effect previously observed by some authors[32,48], where the length of the alkyl chain spacer of the anion caused a reduction of the carotenoids recovery. **Figure 3** also depicts the following cation trend for the recovery of the three carotenoids: [DEAPA][X] > [DMAPA][X] > [PA][X]. The influence of the cation in the recovery of carotenoids will be extensively analysed in the following sections for the $[Hex]^-$-based PILs at different operating conditions.

The results of this section demonstrated that a strong hydrophobic character of the anion favours the carotenoids extraction, which is in accordance with previous studies [30-31] that defined the hydrophobicity as a good indicator for the choice of the most adequate carotenoids extractants. Mojaat et al.[30] evaluated the extraction of β-carotene from the microalgae *Dunaliella salina* using organic solvents, observing that only the solvents with the highest log $K_{ow}$ values (log $K_{ow} > 5$) (more hydrophobic) are efficient in the recovery of β-carotene. Absalan et al.[31] studied the aptitude of two hydrophobic ILs (1-butyl-3-methylimidazolium hexafluorophosphate $[BMIM][PF_6]$ and 1-butyl-3-methylimidazolium tetrafluoroborate $[BMIM][BF_4]$) to recover 3-indole butyric acid (IBA) from pea plants, demonstrating a higher affinity of IBA to the hydrophobic ILs. Interestingly, the authors have also shown a predominant effect of the anionic part of the IL on the extraction of IBA, in comparison with the cationic counter-part.

**Effect of temperature on the viscosity of PILs**

The previous section supports thatthe more hydrophobic (more viscous) $[Hex]^-$-based PILs present the highest extraction efficiencies to recover intracellular

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carotenoids. However, the high viscosity of the PILs is a limitation for their use at industrial scale, hindering the carotenoids mass transfer process from the intracellular to the extracellular environment, as well as increasing the energy processing requirements in terms of mixing and pumping costs[33]. From a thermodynamic point of view, viscosity issues can be simply overcome by increasing the temperature of the process. Therefore, considering that the carotenoids are fairly thermostable[34], the second series of experiments evaluated the association between increasing temperature and the solid-liquid extraction using the PILs solutions.

The viscosity dependence with temperature of the $[Hex]^-$-based PILs aqueous solutions (90%, 85%, 80% and 75% (v/v)) from 25 to 70 °C (defined as maximum temperature to avoid the thermal degradation of the PILs) were assessed. As shown in **Figure S3** from **ESI**, both the increase of the temperature and water concentration in $[Hex]^-$-based PILs (90% v/v) significantly decreased the viscosity, namely from 6.19 to 1.89 mPa.s for [PA][Hex]; from 10.08 to 2.64 mPa.s for [DMAPA][Hex]; and from 9.79 to 2.51 mPa.s for [DEAPA][Hex]. As previously reported[35–37], the viscosity of the PILs decreases exponentially with the increase of temperature [36]. The viscosities of the PILs are more comparable to those of oils than to organic solvents, because of the hydrogen bonding and van der Waals interactions[33]. Therefore, by increasing the temperature, the intermolecular distances are decreased, the attraction forces between the molecules are reduced, and consequently their viscosities are also reduced[33,35].

Regarding the water influence, the viscosity decreased gradually with the increase of the water content for the three PILs, *i.e.* with the highest viscosities at 90% (v/v) of PILs and with the lowest values at 75% (v/v). As expected, the presence of the water molecules reduced the electrostatic attraction between the ions, and thus, decreased the cohesive energy of the system and its viscosity [38].

Moreover, it was observed that the viscosity is strongly influenced by the chemical structure of the PILs cations, with higher viscosity index for $[DEAPA]^+$ and $[DMAPA]^+$ cations, the bulkier ions. On the other hand, the [PA][Hex] aqueous solutions exhibited the lowest viscosities. Our results are in agreement with those by

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Zhao et al.[39], which demonstrated a close relation between the diethanolamine-based PILs viscosity and their relative mobility.

**Effect of PIL concentration and temperature on the extraction of carotenoids**

The previous section illustrates that the temperature increase, high water content and the use of shorter PILs cations reduced the viscosity index of PILs. However, as shown above, both the water content and PILs cations appeared as key parameters to control the carotenoid extraction yields. Thus, to assess the relative influence of each operating parameter, the extraction of the three carotenoids using [PA][Hex], [DMAPA][Hex] and [DEAPA][Hex] as a function of concentration (v/v) (90%, 85%, 80% and 75%) and temperature (25, 45 and 65 ºC) was performed. The results are depicted in **Figure 4** and detailed in the **Table S5** from **ESI**.

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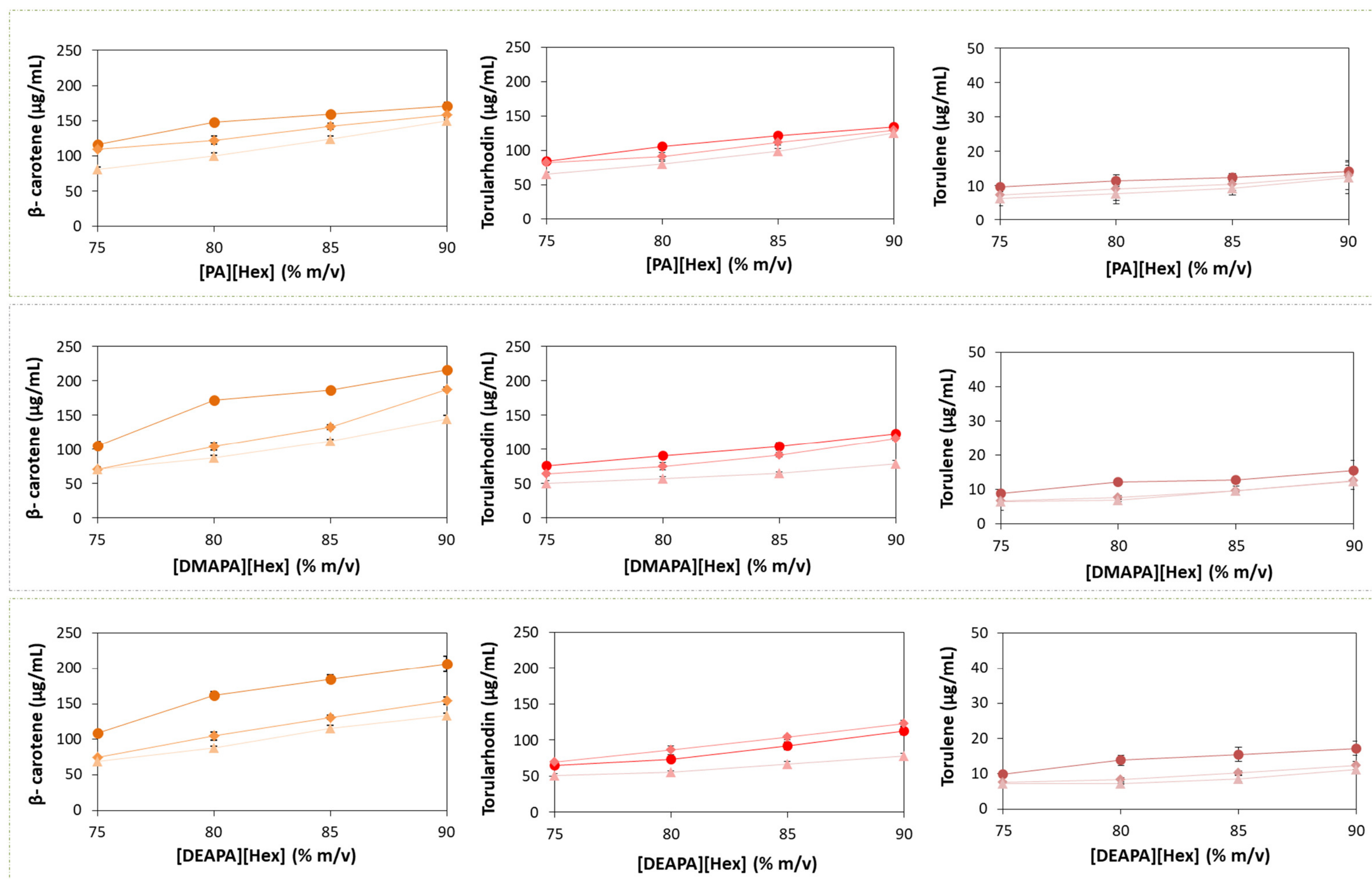


**Figure 4.** Effect of temperature [(-▲-) 25 °C; (-♦-) 45 °C; (-●-) 65 °C] as a function of PIL concentration [75%, 80%, 85% and 90% (v/v)] on the release of β-carotene, torularhodin and torulene from *R. glutinis* wet cells (0.2 g/mL), after 1 h of stirring (30 rpm).The error bars represent 95% confidence levels for the mean of three independent assays, but in some cases are smaller than the markers.

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As shown in **Figure 4** the rates of carotenoids recoveries follow the trends [PA][Hex] < [DMAPA][Hex] < [DEAPA][Hex] (from top to bottom) and β-carotene > torularhodin > torulene (from left to right). The increase of temperature and PILs concentration favoured significantly ($p \leq 0.05$) the release of β-carotene and torularhodin, but it was not significant for the torulene ($p \geq 0.05$) (**Table S5**). For all the assays, an increase of cell disruption processing temperature favoured the carotenoids release, *i.e.* 65°C > 45°C > 25°C. For example, the extraction at 25 ºC using 90% (v/v) of [DEAPA][Hex] recovered 133.06 ± 9.74 μg/mL, 78.15 ± 3.83 μg/mL and 11.25 ± 1.47 μg/mL; meanwhile, at 65 ºC recovered 206.65 ± 10.75 μg/mL, 112.82 ± 6.09 μg/mL, 17.21 ± 1.99 μg/mL of β-carotene, torularhodin and torulene, respectively, which corresponds to an increase of approximately 35.62%, 30.74% and 34.64% of the respective carotenoids concentration.

The increase of temperature reduced the viscosity of the solvents, favouring the diffusion of the solvent into the biomass [14], as well as increasing the carotenoids solubility. The solubility of the material extracted and its diffusivity increased with temperature, and consequently, higher extraction yields were achieved. Furthermore, the temperature can also favour the "destruction" of cellular structure [40], allowing a greater release of the intracellular carotenoid content from the *R. glutinis* yeasts.

Similarly, the increase of PIL concentration from 75 to 90% (v/v), at optimum conditions (*i.e.* [DEAPA][Hex] at 65 ºC), increased the carotenoids concentration from 108.60 ± 5.48 to 206.65 ± 10.75 μg/mL (β-carotene), from 64.83 ± 2.96 μg/mL to 112.82 ± 6.09 μg/mL (torularhodin), and from 9.84 ± 0.81 μg/mL to 17.21 ± 1.99 μg/mL (torulene), which correspond to increases of 47.45%, 42.54%, and 42.83% in the extraction yields, respectively. Note that the increase of carotenoids recoveries are linearly dependent on the increase of PILs concentration. The treatment with concentrated PILs confers a more hydrophobic character to the solvent enhancing the carotenoids solubilization, as well as weakening the cell wall structure and promoting the release of intracellular carotenoids to the extracellular environment. These results are in agreement with the studies of astaxanthin recovery from intact *H. pluvialis* cells using 1-ethyl-3-methylimidazolium di-butylphosphate

([EMIM][DBP]), carried out by Desai et al.[20], where it was demonstrated that the extraction yield of astaxanthin increases from ~2% to 18% when IL concentration increases from 20% to 80%.

The results from this section showed that the decrease of the viscosity, by increasing the temperature, is beneficial for the recovery of the intracellular carotenoids. However, although more diluted solutions are less viscous, the increase of the PILs concentration is also critical for the cell disruption and the increase of the relative hydrophobicity of the solvent. Therefore, it seems that the balance between both diffusion and solubility parameters is the key for the success on carotenoids recoveries.

**Effect of SLR and temperature on the extraction of carotenoids**

In the previous section, it was demonstrated that the increase of the extraction of intracellular carotenoids can be enhanced by increasing both the temperature and concentration of the PIL. However, one of the most important processing parameters in the design of cell disrupting/biomass solid-liquid extraction industrial procedures is the solid/liquid ratio (SLR). Therefore, the next set of assays was conducted to find the best SLR (*i.e.,* wet *R. glutinis* cell mass/PIL solution volume) for the release of the intracellular carotenoids. $[Hex]^-$-based PILs solutions at 90% (v/v) with different SLR (0.05, 0.1, 0.2 and 0.5 g/mL of wet cells) were prepared and used to determine the concentration of the carotenoids released at 25, 45 and 65°C, as shown in **Figure 5** (detailed values listed in **Table S6** from **ESI**).

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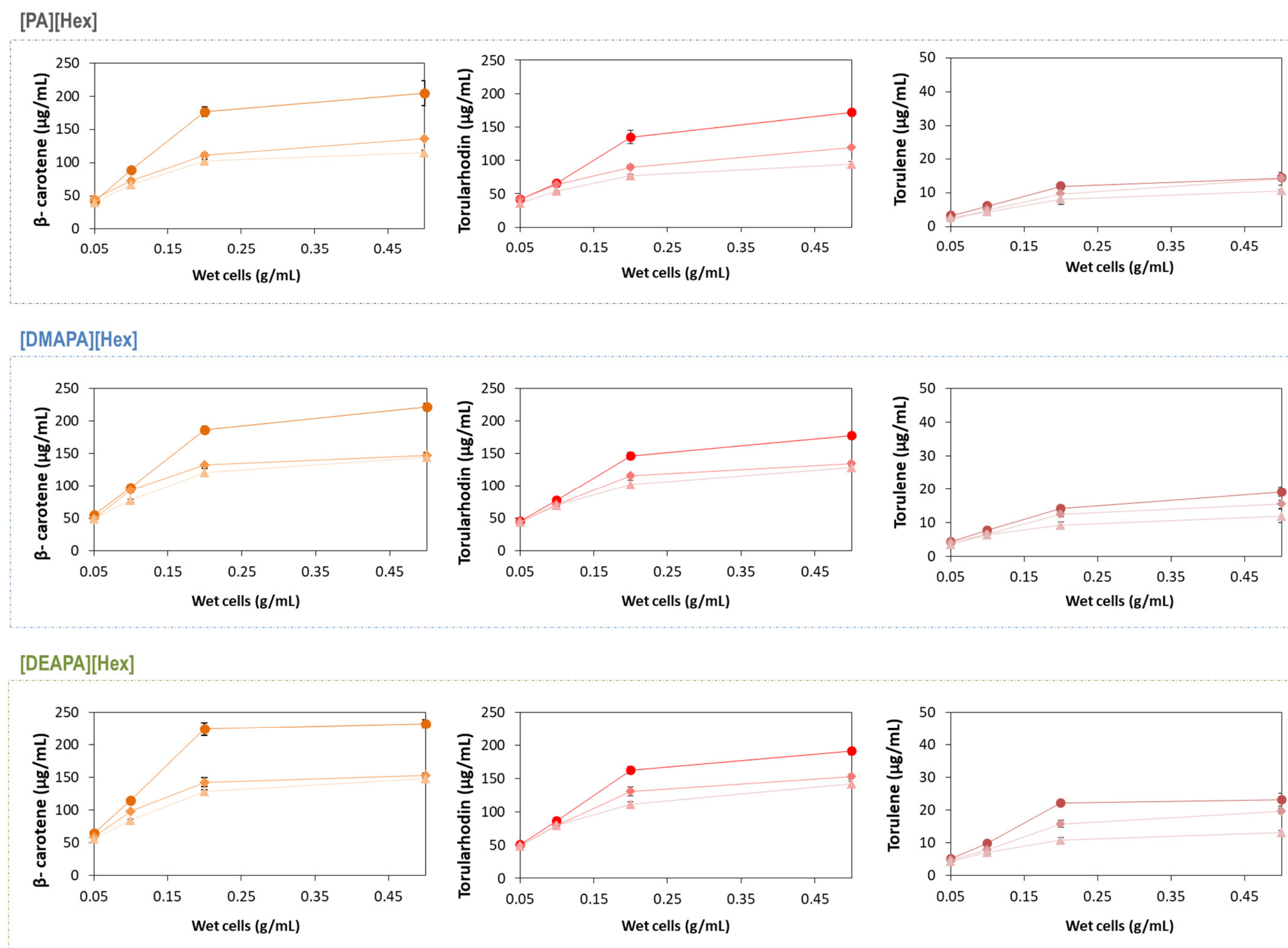


**Figure 5.** Effect of the temperature [(-▲-) 25 °C; (-♦-) 45 °C; (-●-) 65 °C] as a function of solid liquid ratio, SLR (0.05, 0.1, 0.2 and 0.5 g/mL of wet cells), on the release of β-carotene, torularhodin and torulene using different solutions of PILs at 90% (v/v) after 1 hour of stirring (30 rpm). The error bars represent 95% confidence levels for the mean of three independent assays, but in some cases are smaller than the markers.

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**Figure 5** shows a similar pattern between carotenoids concentration and SLR for the three $[Hex]^-$-based PILs (top to bottom) and the three carotenoids (left to right) studied. Namely, the concentration of each carotenoid increased linearly with the SLR up to a concentration of 0.2 g/mL of *R. glutinis* wet cells. Above that SLR, the carotenoids concentration values remained constant. These results demonstrate that the carotenoid release capacity increases with the increase of the SLR up to a concentration where the solvent is fully saturated with carotenoids. According to Tan et al[41], higher SLR favour the concentration gradient by increasing the diffusion rate and the biomolecules contact with the solvent, allowing higher solids extraction yields. This is in full agreement with the mass transfer principle that defines the concentration gradient between solid and solvent as the driving force of solid/liquid extraction[42]. These previous studies[41] also show that the increase of the SLR only benefited the extraction yields up to the equilibrium concentration, after which the concentration of carotenoids remained constant.

Moreover, from **Table S6,** it is possible to infer the significant effect of temperature as a function of the SLR ($p \leq 0.05$) for both the β-carotene, torularhodin carotenoids. Although the extraction trends are maintained, higher temperatures favoured the increase of carotenoid release. The increase of the temperature reduced the viscosity of the PILs solutions, enhancing the permeability of the cell wall for the PILs, and consequently, increasing the solubility of the carotenoids in the PILs solutions[37], supporting the results of the previous section.

## Comparison and understanding of the PILs effects on the extraction of carotenoids

The main aim of this work was to evaluate and understand the capacity of different PILs for the intracellular recovery of carotenoids (β-carotene, torularhodin and torulene) from the yeast *R. glutinis*. The experimental assays evaluated several process variables, namely temperature, concentration and type of PIL and SLR. Considering the number of parameters and for a better understanding of the PIL characteristics and their effect on the carotenoids release, **Figure 6** shows the absolute results obtained for β-carotene, torularhodin and torulene extractions at 25, 45 and 65 ºC at 0.2 g/mL of wet cells using aqueous solutions 90% (v/v) of different

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6 [Hex]$^-$-based PILs (those with the highest extraction capacity) (detailed values are
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8 listed in **Table S5** from **SI**). As a control, the results were compared with the
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10 extraction values obtained with DMSO at similar operating conditions.
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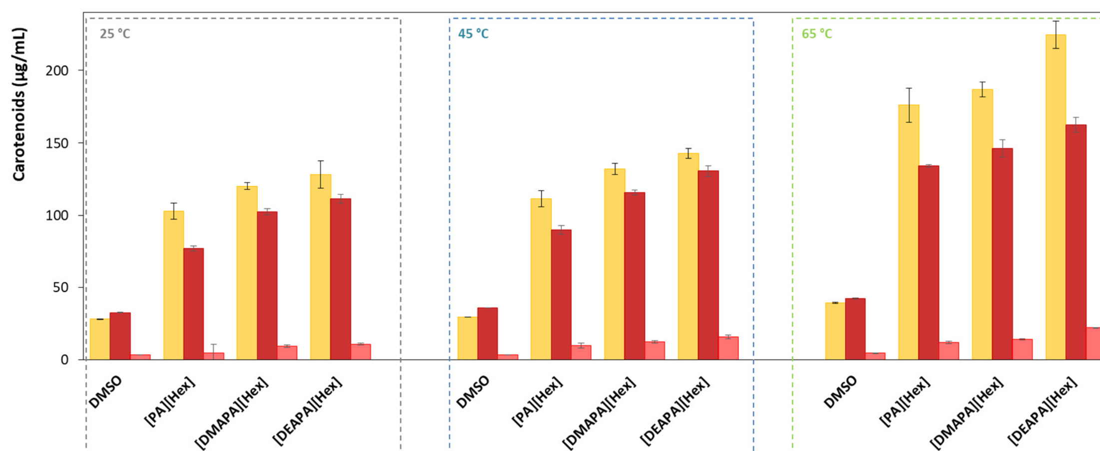


28 **Figure 6.** Recovery of β-carotene (■), torularhodin (■) and torulene (■) using DMSO (control) and
29 aqueous solution of [Hex]$^-$-based PILs (90% v/v) at a concentration of 0.2 g/mL of wet cells after 1 h
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31 of stirring (30 rpm) at 25, 45 and 65 °C. The error bars represent 95% confidence levels for the mean
32 of three independent assays.
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34 As shown in **Figure 6,** the increase in temperature favoured the extraction,
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36 with the highest yields achieved at 65 °C, exhibiting an increase according to the
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38 following trend: [DEAPA][Hex] > [DMAPA][Hex] > [PA][Hex]. Oppositely to the PILs
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40 aqueous solution, the increase of temperature did not significantly ($p \geq 0.05$) favour
41 the extraction of intracellular carotenoids using DMSO (similar carotenoids
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43 concentrations for the three temperatures). All [Hex]$^-$-based ILs were able to recover
44 up to 4-fold (25 ºC) or 6-fold (at 65 ºC) more carotenoids than the DMSO. The highest
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46 carotenoids recoveries were obtained at 65 °C using [DEAPA][Hex], where 206.65
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48 ± 10.75 µg/mL of β-carotene, 112.82 ± 6.09 µg/mL of torularhodin and 17.21 ± 1.99
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50 µg/mL of torulene were extracted.
51 On the other hand, from Figure 6 it was possible to confirm that all PILs are
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53 more selective for the recovery of β-carotene and torularhodin, with a lower capacity
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56 torularhodin than β-carotene. Both PILs and DMSO exhibited a low capability to
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recover torulene, probably because of the low production of *R. glutinis* for this type of carotenoids.

To obtain further information about the mechanisms behind the carotenoids extraction, as well as PILs effects on the cell structure, some samples of cellular debris undergoing different treatments were analysed using scanning electron microscopy (SEM) (9000x resolution) and compared with whole yeast *R. glutinis* cells (without any treatment). The morphology of the cells without any treatment and cell debris after exposure to DMSO (control), [PA][Hex], [DMAPA][Hex] and [DEAPA][Hex] concentrated (90% v/v) solutions at 25, 45 and 65 °C are compared in **Figure 7**.

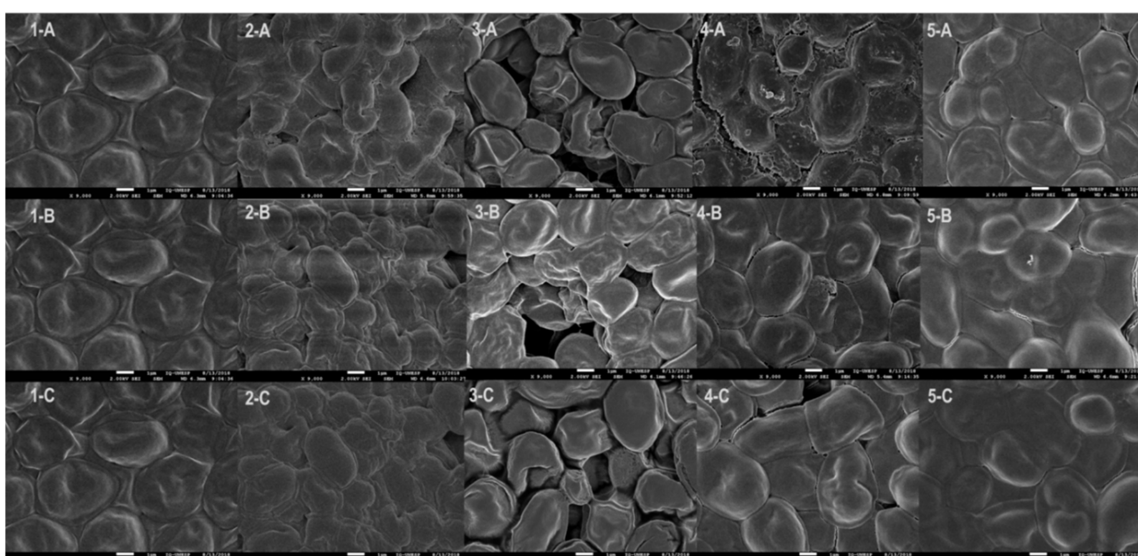


**Figure 7.** Scanning Electron Microscopy (SEM) images of *R. glutinis* CCT-2186 cells (or cell debris) (x9000) at different conditions: A- 25 °C; B- 45 °C; C- 65 °C. 1- without treatment; after treatment with DMSO (2), [PA][Hex] (3), [DMAPA][Hex] (4) and [DEAPA][Hex] (5) at 90% (v/v) and 0.2 g/mL of wet cells.

As expected, the SEM images of the cells without treatment (**Figure 7**: **1-A**, **1-B** and **1-C**) show the characteristic spherical and oval shape of *R. glutinis* yeasts cells [46]. After the treatment with DMSO (**Figure 7**: **2-A**, **2-B** and **2-C**) no significant cell-wall change was observed, but the cells appear dehydrated. For the cells exposed to the PILs aqueous solutions (**Figure 7**: **3, 4** and **5**), it is difficult to distinguish significant changes between the treatments, since some cells remained intact and other exhibit a thin coat around the cells which may be due to some

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"destruction" (permeabilization) of the cell membrane or due to the incomplete removal of the PILs after repeated washing with water. Particularly, the cells exposed to both [DMAPA][Hex] and [DEAPA][Hex] treatments, at 65 ºC, seemed to lose the characteristic shape of the cell wall, inducing the permeabilization of the wall, as well as a more elongated shape of the cell debris. These results corroborate those described by Desai and collaborators[20] where the use of IL permeabilized and/or weakened the cell wall of the microalgae *H. pluvialis*, allowing the recovery of the carotenoid astaxanthin.

From the literature, the carotenoids extraction mechanisms using ILs are still not fully understood[19,20]. Considering the changes of the cell wall structure and the high extraction yields using $[Hex]^-$-based PILs, it is evident that these solvents exhibit a different cell disrupting mechanism than the common organic solvents (such as DMSO). Looking for the yeast cell wall composition, these are constituted of 15-30 % proteins, 5-20 % lipids, 30-60 % polysaccharides, glucans, galactomannan, with a minor content of chitin[39]. Some of the yeast cell components are easily extractable under alkaline conditions, as for example (1,3) β-D-glucan and (1,3) α-D-glucan. Therefore, considering that the pH values of the ammonium-based PILs solutions varied from 7 to 11, it is highly plausible that the alkaline extraction significantly impaired both glucans, leading to a partial exposure of the cell membrane to PILs solution, and consequent permeation of the PILs into the cells. Another hypothesis for the high carotenoid recovery yields can be a mechanism caused by the IL absorption and bilayer disruption by the addition of relative hydrophobic PILs ions. As previously reported in the simulation studies of Benedetto and Ballone[43] using few different phosphatidylcholine lipids in water solutions of imidazolium-based ILs, the cations of the imidazolium-based ILs tend to enter the bilayer as a result of preferential attraction with the negative oxygens in the carbonyl group at the matching point of the hydrocarbon tails, or by binding to the non-ester oxygens of the phosphonium group. Both authors have clearly shown that the cation absorption is apparently favoured by substantial dispersion forces between the IL and phospholipid tails, as well as by the hydrophobic character of the hydrocarbon tail of the IL. The simulation results confirmed an increase of both the IL absorption and

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bilayer disruption with the increase of the alkyl chain length of cation tail. Other computational studies focused on more hydrophobic anions ($I^-$ > $Br^-$ > $Cl^-$ > $F^-$ ions) also confirmed the tendency to segregate at the lipid/water interface, in agreement with the experimental evidences for traditional salts (aqueous solutions of alkali halide salts).[44,47] Further modelling studies that simulate the interactions/solubility of PILs with the *R. glutinis* cell membrane components will allow to better understand the main interactions behind the intracellular carotenoids extraction.

**Recycling of the PILs and carotenoids polishing**

Due to the current market size and the relatively high cost of ILs, the industrial production of ILs is mainly for lab and pilot-scale applications[49]; although a couple of IL-technologies have been successfully commercialized[50]. Regarding the current and future ILs markets, recently, Thomas Schubert[50] stated that "*if the technical performance of an IL is fully demonstrated, it is trivial that at a certain point also the price determines the final commercial success of a product*", but "*to lower costs should never be the only argument that determines the success of technologies*". Thus, from an academic point of view, it is important to demonstrate that the IL-based technology is possible, even without considering economics. Anyway, to make these technologies economically viable in comparison to existing industrial ones, ILs' regeneration, recycling and low purity requirements should be always considered, in order to provide technical solutions or answers to reduce the ILs costs in the process.

Therefore, even no industrial technology is yet available for ILs recycling and reusability, most of the academic publications on ILs have been proposing adequate methodologies for the effective reuse and regeneration of the IL solutions[51]. Many researchers agree that we are only at the very beginning of understanding the ILs recyclability, and the understanding of ILs volatility, purity, stability, biodegradability, and toxicity is still necessary for their recovery, since these determine whether an IL can be sustainably applied for a specific application [49-52].

Therefore, to address the feasibility of the implementation of the proposed technology for a future industrial recovery of carotenoids from yeasts cells, a method for recycling the PILs solutions and to obtain the recovered carotenoids was

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attempted. The development of adequate technologies that allow the reuse of solvents is extremely important for increasing the environmental and economic sustainability of the downstream processes. Thus, an integrative approach for the polishing of carotenoids and recycling of the PILs using a three-phase partitioning (TPP) platform is here proposed, as schematized in **Figure 8**.

The recycling studies were carried out for the [DEAPA][Hex] aqueous solution (90% v/v), which exhibited the highest recovery yields of carotenoids from *R. glutinis* wet cells. After the extraction, the carotenoid-rich [DEAPA][Hex] solution was separated from the residual *R. glutinis* biomass by centrifugation and subjected to a subsequent TPP extraction by adding $K_3PO_4$ concentrated aqueous solution at 25 ºC. After the addition of the $K_3PO_4$ aqueous solution a TPP (liquid-solid-liquid) system was formed, with most of the carotenoids being precipitated as a solid fraction in the interface between both liquid (top and bottom) phases. The TPP was filtered and the carotenoid-rich (solid) fraction recovered as the concentrate, as well both the PIL (top)-rich phase and $K_3PO_4$ (bottom)-rich liquid phases (permeate). PIL-rich phase was recycled for a consecutive recovery of intracellular carotenoids from fresh *R. glutinis* cells and the $K_3PO_4$-rich phase recycled in a subsequent TPP procedure. The recycling procedure was repeated three-times and the respective amounts of carotenoids extracted using the reused PIL solution compared with the fresh PIL (control) (detailed values are listed in **Table 1**).

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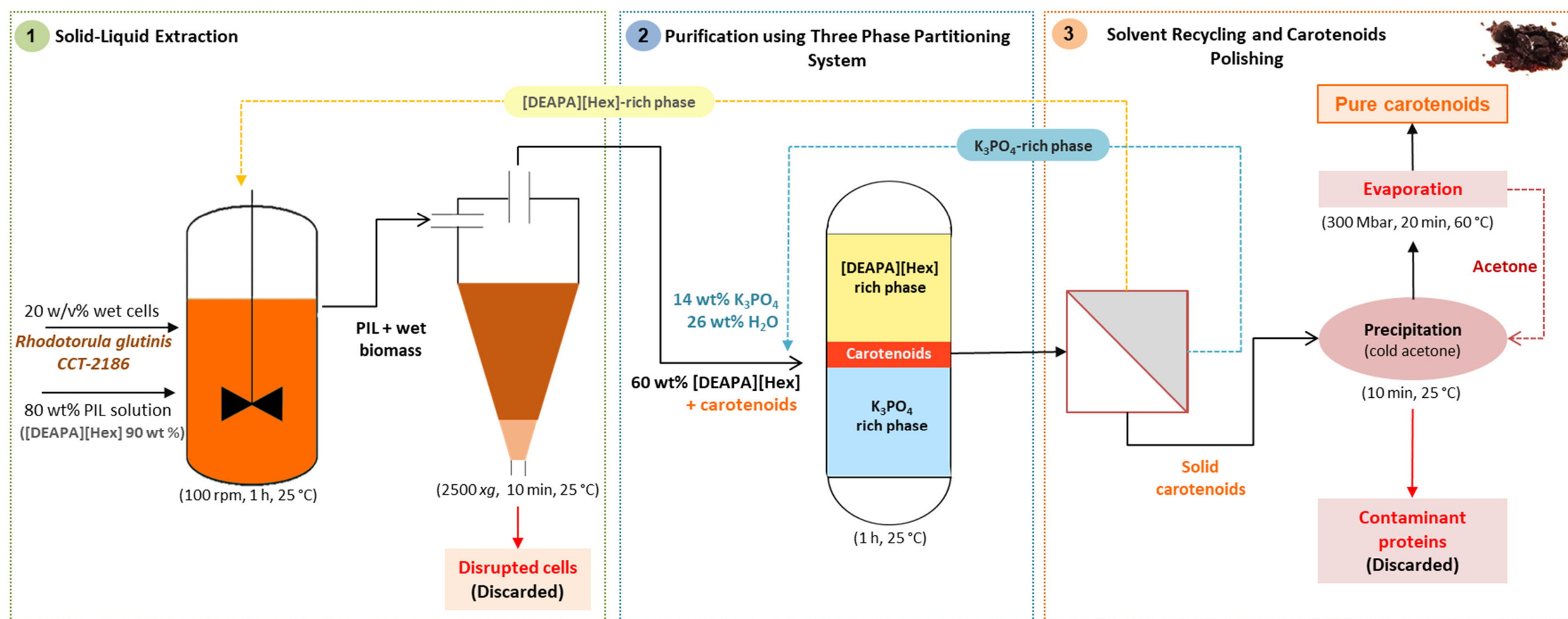


**Figure 8.** Diagram of the integrative process for the extraction of intracellular carotenoids using [DEAPA][Hex] (90 wt% in water), the recycling of the PIL using a three-phase partitioning system (TPP) by adding $K_3PO_4$ aqueous solution and the polishing of the carotenoids.

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**Table 1.** Recovery of β-carotene, torularhodin and torulene using fresh and reused [DEAPA][Hex] solution (90% v/v) at a concentration of 0.2 g/mL of wet cells after 1 h of stirring (30 rpm) at 25 °C (stage 1) and residual carotenoids remaining in the IL solution after the recycling procedure (stage 3). The results represent 95% confidence levels for the mean of three independent assays.

| | Carotenoids Recovered (μg/mL) / (%) | Residual Carotenoids in IL solution after Recycling (μg/mL) / (%) |
|---|---|---|
| **β-carotene** | | |
| **Fresh PIL** | 187.8 ± 0.5 / 100.0 ± 0.0 | 18.4 ± 1.4 / 9.8 ± 0.8 |
| **1st reuse** | 206.3 ± 6.5 / 109.7 ± 4.2 | 37.9 ± 0.1 / 20.2 ± 0.1 |
| **2nd reuse** | 168.8 ± 1.7 / 89.7 ± 0.5 | 60.6 ± 3.6 / 32.3 ± 1.9 |
| **3rd reuse** | 150.7 ± 3.3 / 80.2 ± 1.9 | - / - |
| **Torularhodin** | | |
| **Fresh PIL** | 122.6 ± 4.1 / 100.0 ± 0.0 | 7.5 ± 0.7 / 6.1 ± 0.4 |
| **1st reuse** | 127.7 ± 5.9 / 104.8 ± 1.4 | 12.3 ± 0.4 / 10.0 ± 0.0 |
| **2nd reuse** | 106.3 ± 4.1 / 86.7 ± 6.2 | 23.3 ± 0.7 / 19. 0 ± 1.2 |
| **3rd reuse** | 91.2 ± 3.9 / 74.4 ± 5.7 | - / - |
| **Torulene** | | |
| **Fresh PIL** | 22.9 ± 2.6 / 100.0 ± 0.0 | 9.1 ± 0.8 / 39.7 ± 7.9 |
| **1st reuse** | 27.7 ± 2.8 / 120.8 ± 6.5 | 17.3 ± 0.5 / 75.7 ± 6.6 |
| **2nd reuse** | 15.5 ± 2.4 / 67.5 ± 8.6 | 18.3 ± 0.9 / 79.9 ± 13.2 |
| **3rd reuse** | 13.4 ± 1.2 / 58.8 ± 1.6 | - / - |

As can be seen in **Table 1** the PILs can be recycled up to three times without any treatment, guarantying the maintenance of good extraction efficiencies (> 58.8%). Particularly, after the third reuse of the PIL solution, the recovery efficiencies of 80.2%, 74.4% and 58.8% were achieved for β-carotene, torularhodin and torulene, respectively.The extraction rates decreased after the 2nd reuse as a result of the solvent saturation, *i.e.* the increase of the residual carotenoids concentration (the fraction that did not precipitate with the TPP procedures) in PIL solution with the increase of number of recycling stages (column 2 of **Table 1**). However, it is important to note that in the 1st reuse the PIL solution was not saturated, and the residual content of carotenoids caused an increase of the carotenoid recoveries to values higher than 100%.

Anyway, it was demonstrated the reusability of the PIL by a simple integration of the initial solid-liquid extraction unit with a subsequent TPP process. However, considering that the recycling and polishing steps were carried out without any

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treatment, it should be highlighted that the make-up of the solvent (by adding fresh PIL solution) will improve the solvent recycling capacity of this platform.

Simultaneously with the recyclability of the PIL, the carotenoids polishing was performed. Then, the carotenoids-rich solid fraction, obtained after the TPP, was dissolved in acetone and subjected to a next precipitation step. The precipitation using cold acetone allowed the removal of the intracellular proteins also extracted with [DEAPA][Hex] concentrated solution. After the precipitation, the acetone was removed by evaporation, and the purity of the carotenoids obtained in each recycling step ($1^{st}$, $2^{nd}$ and $3^{rd}$ PIL reuse) assed by FTIR-ATR, as shown in **Figure S3** from **ESI**. The FTIR-ATR spectra of the solid carotenoids fractions after the evaporation have a similar profile than the original carotenoids extracted with DMSO, confirming the non-degradation and purity of the carotenoids, as well as indicating that they can be either used for the formulation of some bioproduct of interest (food or feed purposes) or undergo further biomaterials manufacturing processes.

Summing up, the results of this work indicate that the integrative approach using PILs is an interesting platform to extract “natural” and valuable carotenoids from *R. glutinis* cells. Furthermore, the recovery of carotenoids and recycling of the PILs are also much easier to accomplish, supporting its use as more cost-effective purification strategy. Although for the implementation at industrial scale further scaling-up studies are still required, these results indicate that this platform is promising for the recovery of other microbiologically-produced molecules.

## CONCLUSIONS

In this work, an integrative and effective process for the recovery of intracellular carotenoids from *Rhodotorula glutinis* CCT-2186 cells was established. All PILs allowed the recovery of the three major carotenoids, *i.e.* β-carotene, torularhodin and torulene. The increase of the temperature, as well as the hydrophobicity (either by increasing the anionic or cationic alkyl chain length) and concentration of the PILs favoured the carotenoids extraction. $[Hex]^-$-based PILs were the most efficient in the recovery of intracellular carotenoids with extractions rates 6-fold higher than the

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common volatile organic solvent (DMSO). Additionally, the economic and environmental sustainability of the process was demonstrated, by integrating the cell-disruption stage with a subsequent three-phase partitioning unit, where the carotenoid extraction yields were maintained after reusing up to three-times the [DEAPA][Hex] concentrated solution. This study shows the potential of the use of PILs in the extraction of biologically active molecules (*i.e.,* carotenoids) at mild and accessible conditions, as alternative to environmentally non-favourable volatile organic solvents.

## SUPPORTING INFORMATION

Detailed description of the standard procedure for: PILs synthesis; water content and pH measurements of PILs; viscosity determination; inoculum and growing conditions; carotenoids production; scanning electron microscopy (SEM) analysis; conventional methodology for separation and characterization of carotenoids; carotenoids quantification. Chemical structure, nuclear magnetic resonance ($^{1}$H NMR), pH and purity of the studied PILs. $^{1}$H NMR and UV-Vis spectra of three carotenoids from *R. glutinis* CCT-2186. Detailed experimental data of: all recovery values for β-carotene, torularhodin and torulene recovery; viscosity values of the different aqueous solutions of PILs as a function of temperature. Fourier transform infrared spectroscopy of carotenoids before and after the recycling procedure.

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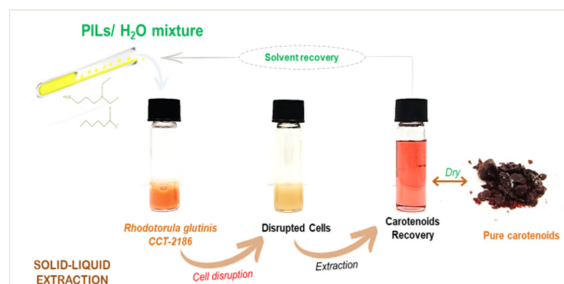


**Sentence:** An alternative and efficient process to isolate intracellular carotenoids from *Rhodotorula glutinis* yeasts.