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Sustainable one-pot platform for the green recovery of carotenoids from *Phaffia rhodozyma* yeast and their use as natural additives in soap formulation

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ABSTRACT

Microbial-based pigments have attracted the interest of the cleaning and cosmeceutical industries as greener and more effective alternatives to the synthetic antioxidants and antimicrobials used in commercially colored soaps. Astaxanthin and β-carotene are natural carotenoids with strong antioxidant activity produced by yeast *Phaffia rhodozyma* (*P. rhodozyma*), but its use in industrial-scale processes is still very scarce. The optimization of downstream processing operations for the recovery of carotenoids from *P. rhodozyma* using environmentally and energetically favorable processes has not yet been fully achieved. In the present work, we propose a simple and sustainable one-pot process using biocompatible and renewable solvents (ethanolic carboxylic acids mixtures) for the recovery of astaxanthin and β-carotene from *P. rhodozyma* biomass and direct formulation of bioactive soaps, thus minimizing the processing units. For this purpose, carotenoids-enriched ethanolic carboxylic acids solutions were directly ("one-pot") used in the following saponification process for the formulation of bioactive soaps without the need for additional purification units. The recovery aptitude of ethanolic carboxylic acids solutions and the corresponding solvation mechanisms of different solvent mixtures for astaxanthin and β-carotene were screened using the COSMO-SAC model. Operating parameters (*e.g.*, solvent ratio, time extraction, temperature, and re-extraction cycles) were optimized for the solvent mixture (ethanol:lauric acid) that exhibited the highest recovery capacity for both carotenoids. The incorporation of microbial carotenoids in formulated soaps decreases the moisture content (from 10.04 to 6.85%), the pH (from 8.83 to 8.68), and the surface hardness (from 0.68 to 0.60 mm) and increase the foam height (from 5.83 to 7.07 cm). The biological activity of carotenoids-rich soaps was confirmed by antioxidant tests (60.53%). This work demonstrated that mixing natural

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yeast-based carotenoids with biosolvents is a simple, effective and sustainable solution for the formulation of functional and colored soaps.

1. Introduction

Soaps have been widely used in several commercial applications, such as household and personal cleaning products, cosmetic soaps, luxury soaps or deodorant soaps, which have maintained an undisputed place in the market (Lin et al., 2005; Schumann and Siekmann, 2000). These compounds comprise surface-active metallic salts of aliphatic carboxylic acids composed of a hydrophobic hydrocarbon chain and hydrophilic head groups in their structure (Rambabu et al., 2020). Soaps are commonly formulated through a saponification reaction, namely, alkaline hydrolysis of microbial, vegetable, and animal fats and oils (Atolani et al., 2016). However, many soaps prepared from these sources are vulnerable to chemical and physical degradation caused by sunlight, heat, and exposure to microorganisms and chemicals, which can result in color deterioration and/or reduced shelf-life (Atolani et al., 2016; Rambabu et al., 2020). To overcome these drawbacks some approaches have been proposed, for example, the incorporation of antimicrobial and antioxidants additives in soaps (Adigun et al., 2019; Galanakis et al., 2018a; Nadaroglu and Baran, 2020). Most commercial soaps available on the market use synthetic chemicals such as triclosan and parabens to increase antimicrobial activity, while butylated hydroxytoluene is added to enhance antioxidant properties (Galanakis et al., 2018b). However, the industry has recently started to move away from using synthetic chemicals.

In order to avoid the "synthetic nature" of some chemical soap additives (sometimes obtained through "unfriendly chemical" processes) and health concerns related to skin allergies, there is a need for alternative natural antioxidants agents, such as microbial carotenoids (Mussagy et al., 2019a). Carotenoids are pigments naturally biosynthesized by microorganisms and plants, with antibacterial and antioxidant activities, widely used in commercial formulations ranging from cosmetics to medicines (Mussagy et al., 2019a, 2022b). In nature, the most promising sources of astaxanthin are microorganisms, such as *Paracoccus carotinifaciens* (bacteria), *P. rhodozyma* (yeast) and *Haematococcus pluvialis* (microalgae). Among these, the red yeast *P. rhodozyma* appears as one of the most promising biological systems for obtaining natural carotenoids, since it can accumulate higher levels of astaxanthin and β-carotene in the biomass (Mussagy and Dufossé, 2023; Mussagy et al., 2021b; Schmidt et al., 2011). The natural pigments are intracellularly biosynthesized by *P. rhodozyma*, remaining inside yeast cells after the production step, requiring a proper integration with subsequent downstream operation units for their efficient recovery (Monte et al., 2020).

Currently, the most common procedures for the recovery of yeast-based carotenoids (astaxanthin and β-carotene) are solid–liquid extractions (SLE) with volatile organic solvents (VOCs), some of which are considered toxic and harmful to human health and the ecosystem (Yara-Varón et al., 2016). Therefore, the growing concern of consumers with the safety, especially for cleaning products, has opened a window of opportunity for sustainable alternatives for the recovery of these natural carotenoids, for instance, using less toxic and low-energy extraction platforms, as well as "greener" solvents (Khoo et al., 2021, 2019). Recently, our research team demonstrated that carotenoids can be effectively extracted and purified from yeast biomasses applying integrated downstream platforms with biosolvents (Mussagy et al., 2021c, 2020), ionic liquids (Mussagy et al., 2022a, 2019a) and eutectic solvents (Mussagy et al., 2021a). Following the search for eco-friendly alternatives, bio-based carboxylic acids have been suggested as promising unconventional nonpolar solvents for the recovery of astaxanthin and β-carotene (nonpolar compounds), mainly due to their exceptional characteristics such as high ability to solvate non-polar compounds, as well as remarkable thermal and chemical stabilities (Moghadasian and Shahidi, 2017). However, the high viscosity of these compounds is a limitation for their use in different biotechnological/chemical industries, mainly due to mass-transfer issues (Mussagy et al., 2019a). Although viscosity issues can be overcome by increasing temperature, from a bioprocessing point of view, this approach can lead to degradation of biomolecules. Therefore, an alternative to reduce the viscosity of the extractant can be the use of mixtures of polar solvents (such as ethanol) with non-polar bio-based carboxylic acids to facilitate the miscibility with wet biomass and, consequently, favoring the yeast cell wall permeabilization and the solubilization of astaxanthin and β-carotene. The ability of different renewable carboxylic acids to extract carotenoids from several matrices has been reported (Diacon et al., 2021; Mussagy et al., 2022a, 2021a). Mixtures of choline chloride (cation) and carboxylic acids (anions) were used to obtain ionic liquids and eutectic solvents for the recovery of astaxanthin and/or β-carotene (Mussagy et al., 2022a, 2021a), with the observation that increasing the alkyl chain length of the carboxylic acids increases the extraction yields of both carotenoids. Unfortunately, as demonstrated in these previous studies, solvent recycling appears as the main bottleneck in the process due to the low vapor pressure of these solvent mixtures.

This works leverages the beneficial properties of carboxylic acids and carotenoids to develop integrated platforms for the recovery of carotenoids from yeast-based biomass. Since carboxylic acids with longer alkyl chain lengths (from $C_{8:0}$ to $C_{18:1}$) are not efficient for solubilizing wet biomass, the use of ethanolic solutions of carboxylic acids to improve the solvent-biomass miscibility was evaluated. In this phase, additional insights into carotenoids' recovery mechanisms using ethanolic solutions of carboxylic acids were obtained with the COSMO-SAC (COnductor-like Screening MOdel for Segment

Activity Coefficient) computational model. After finding the solvent with the highest recovery yield, other processing parameters (*e.g.*, solvent mixture mass ratio, time, temperature, and re-extraction ability) were then adjusted. In the end, the incorporation of microbial β-carotene and astaxanthin in soap formulations was evaluated to impart color and increase antioxidant activity, proposing an innovative, simple and ecological solution for its formulation. Therefore, after solid–liquid extraction, the colored ethanolic solution of lauric acid (containing carotenoids) was directly used ("one-pot") to obtain soaps with antioxidant activities. Physicochemical parameters such as foam height, moisture content, pH, hardness and antioxidant capacity of bioactive soaps were also measured and analyzed.

2. Experimental section

2.1. Reagents, standards and solvents

The carboxylic acids [Octanoic acid (caprylic acid) - $C_{8:0}$($\geq$99%), decanoic acid (Capric acid) - $C_{10:0}$($\geq$99%), dodecanoic acid (Lauric acid) - $C_{12:0}$($\geq$98%), hexadecanoic acid (Palmitic acid) - $C_{16:0}$($\geq$99%) and cis-9-Octadecenoic acid (Oleic acid) - $C_{18:1}$($\geq$99%)] and 2,2-diphenyl-1-picrylhydrazyl ($\geq$95%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide was obtained from Synth (Diadema, SP, Brazil) and ethanol (>99%) from Exodo Cientifica (Sumaré, SP, Brazil). β-carotene and astaxanthin standards (>99%) were purchased from Carbosynth (San Diego, CA, U.S.A) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2. Cultivation of microorganisms and production of carotenoids

The red yeast *P. rhodozyma* NRRL Y-17268 was preserved in 50% glycerol at −80 °C. The pre-inoculum was cultivated in 100 mL of Yeast Extract-Peptone-Dextrose (YPD) medium containing 10, 20 and 20 g/L of yeast extract, peptone and glucose, respectively, in 500-mL Erlenmeyer glass flasks at 22 °C, 300 rpm for 48 h. The medium used for yeast cultivation was composed (in g/L): glucose (20), malt extract (3), xylose (10), yeast extract (3) and peptone (5). The production of β-carotene and astaxanthin was achieved after 144 h of cultivation *P. rhodozyma* yeast cells using stirred tank bioreactor (model minifors-2 bioreactor, InforsHT, New Jersey, USA) at 22 °C, 300 rpm and 1 vvm (air volume/medium volume/minute). After cultivation, the colored biomass containing β-carotene and astaxanthin were harvested by centrifugation (2500 *xg*, 5 min at 4 °C), washed three times with phosphate buffer (pH = 7.2) and stored at − 80 °C. Wet biomass (80% w/w in water) was then used in the following SLE assays.

2.3. Solid–liquid extraction of carotenoids

To find the best conditions for the extraction of carotenoids from yeast biomass, an initial solid–liquid extraction (SLE) screening was performed using different ethanolic solutions of carboxylic acids (from $C_{8:0}$ to $C_{18:1}$) and pure ethanol (EtOH) as control. Samples (in triplicate) of wet *P. rhodozyma* biomass were homogenized with mixtures of solvents (ethanol + carboxylic acid) in a solid–liquid ratio of 0.2 $g_{wetbiomass}/mL_{solvent}$ for 1 h at 65 °C and 300 rpm (stirring speed) using a stirrer hot plate mixer (model K40-1810H, Kasvi, Sao Jose dos Pinhais, PR, Brazil). The initial conditions were defined according to the previous work of Mussagy et al. (2022a,c).

The ethanolic carboxylic acid solution (*viz.*, EtOH + $C_{12:0}$), for which the highest recovery yields of β-carotene and astaxanthin were obtained, was then selected for a second study focused on finding the best SLE process conditions, *i.e.*, temperature, mass ratio and extraction time. After SLE, the carotenoids-rich colored extracts were centrifuged in a Thermo Fisher Scientific-Fresco 17 centrifuge (Waltham, MA, USA) at 2500 *xg* and 4 °C for 10 min. The colorless pellet was discarded, while the colored supernatant fraction was collected and filtered with a hydrophobic PTFE membrane (0.45 μm pore size).

To achieve a complete recovery of carotenoids from wet cells of *P. rhodozyma*, a set of re-extraction cycles was performed. After an SLE extraction (solid–liquid ratio of 0.2 $g_{wetbiomass}/mL_{solvent}$, at 65 °C, for 1 h, 300 rpm of stirring speed), the biomass was recovered and submitted to the next SLE cycle (under the same conditions of processing) with fresh $C_{12:0}$ ethanolic solution. The re-extraction procedure was performed up to 5 successive cycles. After each re-extraction cycle, the mixture was centrifuged at 2500 *xg*, 4 °C, for 10 min, and the colored fraction was isolated from the colorless pellets, filtered and used to measure concentration of each carotenoid.

To quantify the carotenoids, the absorption spectra of each extract were measured in the range between 350 and 600 nm in a Thermo Scientific - Genesis10S UV–Vis spectrophotometer. The yield of β-carotene and astaxanthin were determined according to the Beer–Lambert law, following the methodology previously reported by Mussagy et al. (2022a). The recovery yield (μg/g) was determined following Eq. (1):

$$Yield\left(\frac{\mu g}{g}\right) = \frac{Conc\left(\frac{\mu g}{mL}\right)}{Biomass\,(g)} \times V(mL) \quad (1)$$

where *Conc* is the concentration of each carotenoid (in μg/mL), *Biomass* is the amount of wet biomass used (g) and *V* is the solvent volume (mL).

2.4. Solubility prediction using COSMO-SAC

COSMO-SAC model, using JCOSMO software, was used to predict the solubility of carboxylic acids and ethanol in the solvation of astaxanthin and β-carotene. The computational modeling was applied following the standard procedure developed by Gerber and Soares (Gerber and Soares, 2010) with the GMHB1808 multi-hydrogen bond parametrization. Sigma-profiles were obtained using the GAMESS Quantum Chemistry package following the procedure described above (Ferrarini et al., 2018; Soares et al., 2020).

2.5. Formulation of bioactive soaps

Ethanolic-$C_{12:0}$ solutions containing carotenoids obtained after the SLE were directly used for the formulation of bioactive soaps. For this purpose and to improve the carboxylic acid content of the soaps, 60 mL of the carotenoids-rich extracts (*i.e.*, colored ethanolic-$C_{12:0}$ solutions) were mixed with 50 g of pure $C_{12:0}$ in glass tubes and stirred for 10 min at 300 rpm and 30 °C (Solution 1). After enrichment in carboxylic acids, 50 mL of each Solution 1 was mixed with 10 mL of NaOH (1M in water) and stirred continuously to ensure efficient formulation. The mixture was then transferred into molds to solidify. After 30 min, the solid soaps were removed from the molds and kept in a dark place (at 25 °C) for a curing period of 15 days. To obtain soaps for comparative purposes, a soap without the addition of carotenoids-rich extract was prepared and used as control.

The pH values of formulated soaps were determined by adding soap samples (0.5 g) in distilled water (10 mL) and measured using a MS Tecnopon® pH meter (model mPA-210, Piracicaba, SP, Brazil). The same solution was vigorous stirred for 10 min, and left to stand for 10 min to estimate the height of the foam.

Soap hardness was calculated following the procedure described by Rambabu et al. (2020). Briefly, the needle (4.0 cm *x* 0.99 mm) was loaded with 350 g magnetic bar on the upper surface and softly penetrated the soap for approximately 25 s. Soap hardness was then estimated in terms of needle penetration depth.

Soap moisture content (MC) in % by weight (W) was measured by drying 5 g of soap samples for 24 h at 60 °C and calculated as detailed in Eq. (2):

$$MC\ (\%) = \frac{W_1 - W_2}{W_1} \times 100 \tag{2}$$

where W_1 and W_2 are the initial and final weights of the soap samples (g).

2.6. Antioxidant activity of soaps

The bioactive character of carotenoid-rich soaps was evaluated in terms of their antioxidant capacities. The antioxidant activity was determined using the modified DPPH$^{\cdot}$ free-radical-scavenging activity assay (40 μg/mL in methanol). Briefly, 2 g of each carotenoid-rich soap sample (*i.e.*, containing 21 and 66 $\mu g/g_{soap}$ of astaxanthin and β-carotene, respectively) was mixed with 2 mL of DPPH$^{\cdot}$ solution. The same conditions were used for a control sample using methanol. After 15 min, the absorbance was recorded at 518 nm (UA) in a UV–Vis spectrophotometer (Thermo Scientific®, Genesis 10S). Antioxidant activity (%) was determined according to the percentage reduction of the DPPH$^{\cdot}$ absorbance following the Eq. (3):

$$\text{Antioxidant activity}\ (\%) = \frac{A_{DPPH} - A_{Sample}}{A_{DPPH}} \times 100 \tag{3}$$

where A_{DPPH} is the absorbance (UA) at 518 nm of the initial DPPH$^{\cdot}$ and A_{Sample} corresponds to the absorbance after the addition of soaps (carotenoid-rich soaps and control soap).

2.7. Statistics

All experiments were performed in triplicate. Results were compared using one-way ANOVA, and for multiple comparisons, Tukey's test was applied to determine significantly different treatments effects. Statistical approaches were evaluated using the Origin Lab software 9.1 version (Origin Lab Corp., Northampton, MA) for data analysis.

3. Results and discussion

This section will be dedicated to *(i)* understand the carotenoid-solvent molecular affinity, evaluation of carboxylic acid:ethanol mixtures to find the best solvent for the extraction of astaxanthin and β-carotene from yeast biomass, as well as understanding the intramolecular interactions responsible for the selective recovery of carotenoids; *(ii)* optimization of extraction procedures and understand the influence of mixture composition (by mass), temperature, extraction time and re-extraction cycles on the recovery of astaxanthin and β-carotene by the solvent mixture with higher recovery yields; and *(iii)* development of simple, integrated and eco-friendly process for the extraction of carotenoids from yeast biomass and preparation of bioactive and colored soaps without further purification.

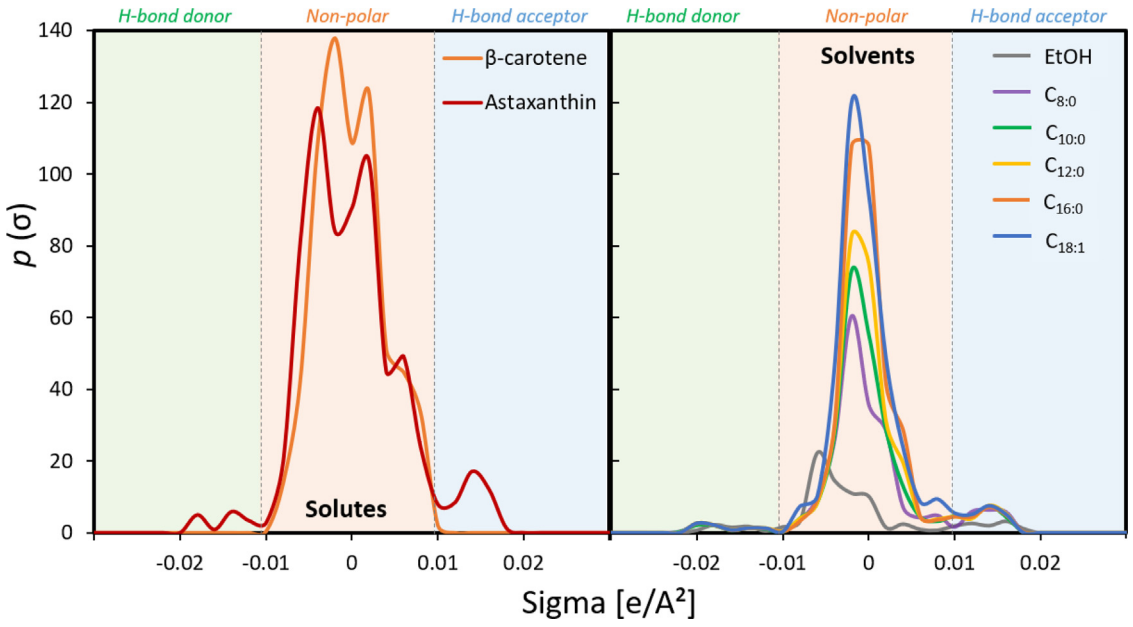

Fig. 1. The σ-profiles obtained from COSMO-SAC of solutes (astaxanthin and β-carotene) and each solvent (carboxylic acids and EtOH).

3.1. Solvent screening for solid–liquid extraction of carotenoids

An initial solvent screening was performed to find out which is the best solvent for SLE of carotenoids (*i.e.*, astaxanthin and β-carotene) from wet biomass of *P. rhodozyma*. Considering that the focus is on finding the best carboxylic acid:ethanol mixture that maximizes SLE performance, we initially investigated which carboxylic acids have high affinity for the two target carotenoids (astaxanthin and β-carotene). The carboxylic acids studied were: octanoic acid (caprylic acid) - $C_{8:0}$; decanoic acid (capric acid) - $C_{10:0}$; dodecanoic acid (lauric acid) - $C_{12:0}$; hexadecanoic acid (palmitic acid) - $C_{16:0}$; *cis*-9-octadecenoic acid (oleic acid) - $C_{18:1}$. It is important to note that for the SLE each carboxylic acid was mixed with EtOH to improve miscibility of the wet biomass in the respective solvent mixture. These solvents were selected due to their biocompatibility and greater affinity with astaxanthin and β-carotene molecules. Thus, to determine the carotenoid-solvent affinity, the σ-profile (sigma profile) of astaxanthin/β-carotene and pure carboxylic acids were obtained with the COSMO-SAC computational model, as shown in Fig. 1.

As depicted in Fig. 1, the σ-profile allows the determination of the charge distribution and its correlation with the polarity of each molecule. Through this analysis, the polarity is evaluated in three main regions, namely: *(i)* regions below -0.01 e/A^2 - polar region associated with the hydrogen bond donor (H-bond donor); *(ii)* regions between -0.01 and $+0.01$ e/A^2 - corresponding to the nonpolar region; and *(iii)* region above $+0.01$ e/A^2 - polar region associated with the hydrogen bond acceptor interactions (H-bond acceptor, HBA). The two solutes are different in terms of molecular structure, *i.e.,* β-carotene does not contain oxygen while astaxanthin does. Therefore, as shown in the σ-profile, a large peak in the nonpolar region is common for both carotenoids, but a remarkable peak in the polar region is only observed in the astaxanthin molecule.

On the other hand, the solvents, carboxylic acids and EtOH, exhibit high affinity for nonpolar regions and HBA groups. According to the height of the peaks in the nonpolar region, the following solvent nonpolarity scale can be found: $C_{18:1}$> $C_{16:0}$> $C_{12:0}$> $C_{10:0}$> $C_{8:0}$> EtOH. The recovery/solubilization process of various biomolecules is generally associated with the "like-dissolve-like" concept (Mussagy et al., 2020). Therefore, from the analysis of the σ-profile and nature of both carotenoids, it is expected that higher recovery yields will be obtained using more hydrophobic solvents, mainly because of favorable nonpolar interactions for β-carotene and astaxanthin, although some HBA interactions may also be beneficial for the recovery of astaxanthin.

However, to establish a correlation between the theoretical predictions of COSMO-SAC and the experimental results of SLE, some aspects must be considered, *cf.*, the existence of synergistic effects generated by proteins, lipids and carbohydrates present in *P. rhodozyma* cells, and the impact of mass transfer and solvent viscosity on the complexity of SLE procedures, which are not predicted in the theoretical model.

Following the theoretical insights and to obtain further evidence about their relation to the performance of each SLE procedure, an experimental screening was carried out. This included SLE trials were performed using different mixtures composed of EtOH and each carboxylic acid (in a 2:1 mass ratio) and under the following conditions: 0.2

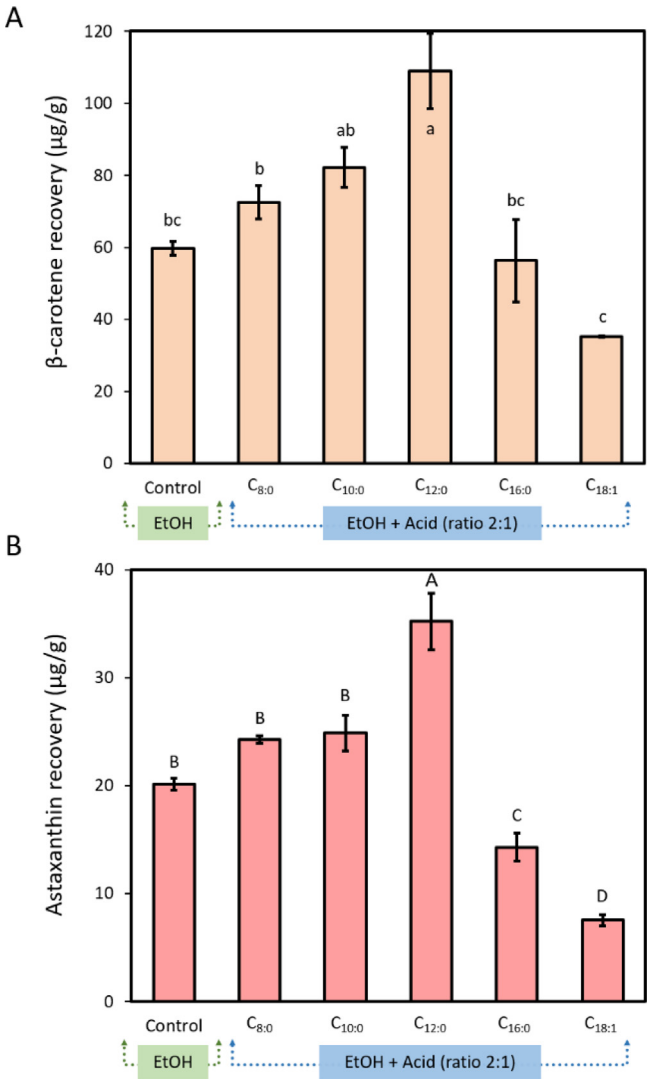

Fig. 2. A- β-carotene and **B-** astaxanthin recovery using ethanolic carboxylic acids solution (2:1 mass ratio) and pure EtOH (as control) at 0.2 $g_{wetbiomass}/mL_{solvent}$, at 65 °C, 60 min, 300 rpm. The error bars correspond the mean of three independent assays, where the same letters represent the group that do not differ significantly (95% confidence interval).

$g_{wetbiomass}/mL_{solvent}$, at 65 °C, 1 h, 300 rpm. The ability of each ethanolic carboxylic acid solution to recover β-carotene and astaxanthin (μg/g) was determined and the corresponding results shown in Fig. 2.

As depicted in Fig. 2, and according to the theoretical insights of COSMO-SAC, all ethanolic carboxylic acid mixtures were able to recover both pigments, achieving recovery values of 7.53 to 35.19 μg/g for astaxanthin and 35.15 to 108.9 μg/g for β-carotene. It should be noted that the lower extraction values for astaxanthin are related to the lower biosynthesis of this pigment by *P. rhodozyma* under the cultivation conditions established for this study. The recovery performance trend of each solvent mixture was similar for both carotenoids, namely: EtOH + $C_{12:0}$> EtOH + $C_{10:0}\cong$ EtOH + $C_{8:0}$> EtOH > EtOH + $C_{16:0}$> EtOH + $C_{18:1}$. Interestingly, of the five ethanolic carboxylic acid mixtures, the highest recovery yield was obtained with EtOH + $C_{12:0}$, which allowed to obtain about 2 times more astaxanthin (35.19 μg/g) and β-carotene (108.9 μg/g) than the control (*i.e.*, 20.12 and 59.79 μg/g of astaxanthin and β-carotene were extracted using pure ethanol, respectively).

Analyzing in detail the performance of each carboxylic acid, independently of the target solute, an increase in the relative hydrophobicity of the ethanolic carboxylic acid solution (by increasing the fatty acid chain length) only promotes an increase in the recovery yields from $C_{8:0}$ to $C_{12:0}$, while longer carboxylic acids led to a subsequent decrease in recovery yields. The observed increase from $C_{8:0}$ to $C_{12:0}$ corroborates with the activity coefficient at infinity dilution ($\ln \Upsilon \infty$) estimated by COSMO-SAC for an ethanol:carboxylic acid mixture (at 2:1), namely: the smaller the $\ln \Upsilon \infty$ values, the greater the attractive forces between the molecules. Thus, the increase in recovery is a result of similarities in the polarity

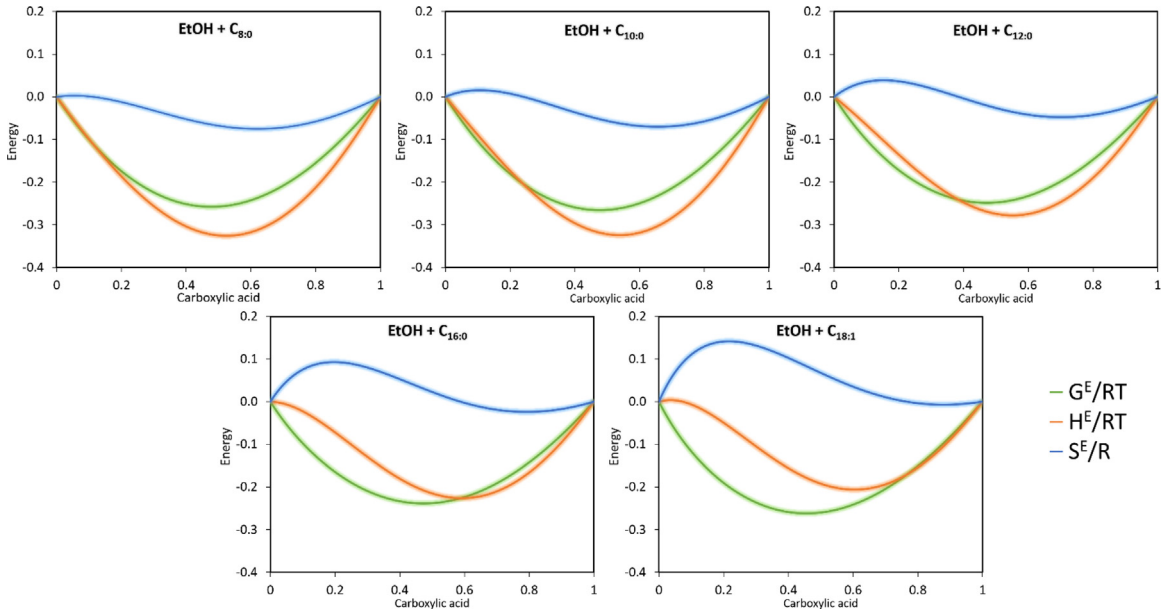

Fig. 3. Comparison of Excess Enthalpy (H^E), Gibbs Free Energy (G^E) and Entropy (S^E) of EtOH + carboxylic acids mixtures (mole fractions) predicted by COSMO-SAC.

of solutes and solvents, *i.e.*, the ln $\Upsilon \infty$ values (for a mixture EtOH:carboxylic acid at 2:1) obtained for β-carotene follow the tendency: EtOH + $C_{12:0}$(ln $\Upsilon \infty = 1.85$) < EtOH + $C_{10:0}$(ln $\Upsilon \infty = 1.97$) < EtOH + $C_{8:0}$(ln $\Upsilon \infty = 2.02$). The ln $\Upsilon \infty$ values showed a lower affinity of both carotenoids with mixtures containing higher carboxylic acids ($C_{16:0}$ and $C_{18:1}$).

For further clarification on the influence of ethanolic carboxylic acid solutions on carotenoids recovery, particularly, to reveal the mechanisms behind the decreases observed in the mixtures using carboxylic acids > $C_{12:0}$, the behavior of solvent mixtures was also examined. Thus, to reveal the primary molecular interaction energies that occur between EtOH and carboxylic acids, estimation of the excess properties of Excess Enthalpy (H^E), Gibbs Free Energy (G^E), and Entropy (S^E) of each mixture was performed using COSMO-SAC, as shown in Fig. 3.

Molecular interaction energies of EtOH and carboxylic acid molecules play a significant role in the solubility performance. The standard thermodynamic properties for the mixtures under study are summarized in Fig. 3. Note that the entropy change of the liquid mixture (S^E) is calculated by COSMO-SAC using the difference between G^E and H^E. In the systems composed of EtOH and $C_{8:0}$, $C_{10:0}$ or $C_{12:0}$, an exothermic enthalpy contribution results from the strong interaction between ethanol and the respective carboxylic acid. The values of $S^E \cong 0$, revealed the equilibrium of the mixture, which agree with the good recovery yields of carotenoids using these ethanolic carboxylic acid solutions. The enthalpic contribution decreased with increasing length of the carboxylic acid alkyl chain. On the other hand, for mixtures containing $C_{16:0}$ and $C_{18:1}$, positive values of S^E(> 0) indicate endothermic and entropy-driven processes, *i.e.*, the solvation of the carboxylic acid solvation by the alcohol is not favorable, affecting the extraction performance of the solvent mixture (Fig. 3). These unfavorable entropy changes could explain why mixtures containing $C_{16:0}$ and $C_{18:1}$ showed the worst performance for the recovery of carotenoids compared to mixtures with $C_{8:0}$, $C_{10:0}$ or $C_{12:0}$. These results allow us to state that the weak interaction in the mixture, will primarily affect the solvent recovery performance.

These observations allow us to infer that the improvement of carotenoids recoveries strongly depends on the nature and relative affinity of solvent mixtures for solutes. However, rather than assessing the change of nonpolar character of the solvent mixture, the intermolecular interactions between the solvent constituents must always be considered together with the extraction process conditions, *i.e.*, temperature, and type of biomass (wet or dry) among other processing parameters.

3.2. Effect of processing parameters on solid–liquid extraction of carotenoids

The highest recovery yields for both carotenoids from the wet biomass of *P. rhodozyma* were obtained with the EtOH + $C_{12:0}$ mixture. This ethanolic carboxylic acid solution was then used in a set of studies to evaluate the influence of mixture composition (in mass ratio) on the recovery of astaxanthin and β-carotene. Different mixtures of EtOH:$C_{12:0}$ (mass ratios of 3:1, 2:1, 1:1, 1:2, and 1:3) were prepared and used for the recovery of both carotenoids from *P. rhodozyma* under the same experimental conditions as the initial screening (*i.e.*, 0.2 $g_{wetbiomass}/mL_{solvent}$, at 65 °C, 60 min, and 300 rpm). Fig. 4A shows the results concerning the aptitude of each EtOH:$C_{12:0}$ mixture for the recovery (μg/g) of β-carotene

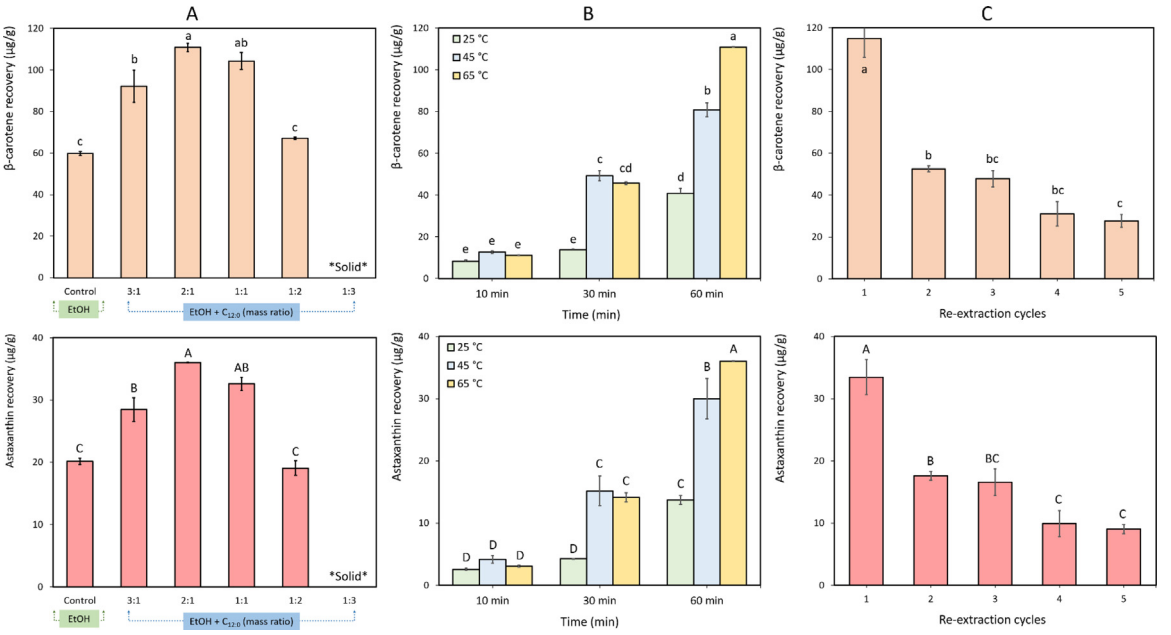

Fig. 4. β-carotene and astaxanthin recovery yields using ethanolic $C_{12:0}$ solutions as a function of **A-** mass ratio; **B-** temperature (25, 45, and 65 °C) and time extraction (10, 30 and 60 min) and **C-** recovery yields through different re-extraction cycles using ethanolic $C_{12:0}$ solution (mass ratio 2:1) at 0.2 $g_{wetbiomass}/mL_{solvent}$, at 65 °C, 60 min, 300 rpm. The error bars correspond the mean of three independent assays, where the same letters represent the group that do not differ significantly (95% confidence interval).

and astaxanthin and how the EtOH:$C_{12:0}$ mass ratio influences the recovery of both carotenoids from *P. rhodozyma* wet biomass.

Interestingly, the extraction performance trend as a function of the mixture composition is similar for both carotenoids, with the highest recovery yields achieved in the mixture with 2:1 EtOH:$C_{12:0}$ mass ratio, (*i.e.*, 36.06 and 110.91 μg/g of astaxanthin and β-carotene, respectively), while an increase or decrease of the mass ratio reduced the extraction performance. These results confirm that the use of polar and water-miscible solvents (such as EtOH) mixed with the nonpolar carboxylic acid $C_{12:0}$ is crucial to enhance the recovery of nonpolar intracellular biomolecules (such as astaxanthin and β-carotene) from wet microbial biomass. The use of EtOH facilitates the miscibility of the wet biomass with the nonpolar carboxylic acid, facilitating the diffusion of the nonpolar solvent in the intracellular environment and the subsequent solubilization of the target biomolecules. However, it is important to keep in mind that a certain hydrophobicity degree must be ensured, otherwise the extraction performance will be diminished. This evidence becomes clear with the increase in the polarity of the solvent mixture, namely, using the mixture of EtOH:$C_{12:0}$ with mass ratio of 3:1 (Fig. 4A), which did not favor the recovery of both carotenoids. On the contrary, it caused a decrease in extraction efficiencies compared to EtOH:$C_{12:0}$ at 2:1 and 1:1 mixtures.

On the other hand, an increase in the carboxylic acid content will lead to an increase in the viscosity of the solvent mixture and, consequently, will decrease the recovery of carotenoids from the intracellular environment. The limiting effect of mass-transfer caused by the use of highly viscous solutions is well reported (as previously observed for other eutectic mixtures), mainly associated with the decrease in the effect of solvents on membrane permeation. A further increase of EtOH:$C_{12:0}$ to a mass ratio of 1:3 led to the formation of a solid mixture, precluding its use for biomass solubilization and SLE of carotenoids.

Considering that astaxanthin and β-carotene are heat-sensitive molecules (Kaur et al., 2021; Mussagy et al., 2022a), the next set of experiments evaluated the influence of temperature (from 25 to 65 °C) and extraction time (from 10 to 60 min) in the recovery of both carotenoids using the EtOH:$C_{12:0}$ solution (at 2:1 mass ratio) (Fig. 4B). As exposure to higher temperatures for longer periods can degrade both carotenoids, 60 min and 65 °C were established as the maximum parameters for SLE assays. As depicted in Fig. 4B, the simultaneous increase of extraction time and temperature significantly ($p < 0.05$) favored the recovery of both pigments from the wet biomass. In fact, the combination of these two process parameters seems to be crucial to enhance carotenoids extraction, since after 60 min, there is a clear correlation between the increase in carotenoids concentration and temperature, following the trend: 25 < 45 < 65 °C. In this condition (*i.e.*, 65 °C at 60 min), the concentration of β-carotene and astaxanthin recovered were 100.91 and 36.01 μg/g respectively, corresponding to a 60% increase in relation to the extraction of β-carotene and astaxanthin (40.82 and 13.7 μg/g, respectively) at 25 °C in the same extraction time (60 min).

The increase in carotenoids recovery yields with increasing temperature is expected, as it allows for a decrease in solvent viscosity and, thus, favors mass transfer and solubilization of pigments (Ghatee et al., 2010; Marsh et al., 2004).

Temperature is also responsible for weakening the molecular interactions of the *P. rhodozyma* yeast cell wall, resulting in an effective release of intracellular carotenoids (Mussagy et al., 2022a). However, as shown in Fig. 4B, the real impact of temperature will only be fully realized if proper timing and mixing is ensured for the SLE, particularly when using complex biomasses such as yeast cells. For instance, if the extraction time is only 30 min, the maximum recovery yields of both carotenoids will be achieved at 45 °C and 65 °C (recovery yields approximately the same), mainly as a direct result of the decrease in viscosity (*i.e.*, the decrease in solvent mixture viscosity is higher from 25 °C to 45 °C than that observed from 25 °C to 45 °C – *data not shown*). Therefore, the positive influence of temperature on cell permeabilization is only observed after 60 min of extraction, as shown in Fig. 4B, with maximum recovery yields for β-carotene and astaxanthin using EtOH:$C_{12:0}$ solution (mass ratio 2:1).

Furthermore, selection of the best extraction time is also an important parameter in the design of DSP operations as it is highly responsible for increase/decrease of energy consumption and cost of the overall process (Alara et al., 2018). Therefore, it is crucial to determine the minimum time required for maximum recovery of target solutes. In the case of intracellular pigments using EtOH:$C_{12:0}$ solutions, the influence of extraction time (from 10 to 60 min) on the recovery of both carotenoids for the three temperatures was also evaluated. As shown in Fig. 4B, regardless of temperature, carotenoid recovery yields increased with increasing extraction time, *i.e.*, 10 < 30 < 60 min. Maximum releases were reached after 60 min, with the highest values for β-carotene (100.91 μg/g) and astaxanthin (36.01 μg/g) obtained at 65 °C, an increase of about 90% compared to that obtained after 10 min of extraction at the same temperature. An equivalent increase with extraction time was observed for tests performed at 15 and 45 °C. It is generally believed that extraction time enhances the mass transfer process (Wen et al., 2018), leading to effective permeabilization of the cell wall by the solvent and solubilization of intracellular carotenoids. These findings are in line with the results obtained in our previous work (Mussagy et al., 2019a), where maximum carotenoid recovery yields from the yeast *Rhodotorula glutinis* were achieved using an extraction time of 60 min and a temperature of 65 °C.

Once the best extraction parameters (*i.e.*, 60 min and 65 °C) were defined, a next study evaluated the use of following re-extraction cycles for complete recovery of intracellular carotenoids. As depicted in Fig. 4C, complete recovery of intracellular pigments from *P. rhodozyma* is achieved after five extraction cycles. A first cycle using a fresh EtOH:$C_{12:0}$ solution yielded 33.44 μg/g and 114.84 μg/g of astaxanthin and β-carotene, respectively. At this stage, as the intracellular pigments content decreases, the recovery yields of both pigments in the following stages are reduced. Therefore, the proper balance between recovery yields and overall processing costs is crucial from an industrial perspective. In this case, adding more than 2 cycles for recovery of carotenoids will increase processing costs, not only requiring a more significant amount of fresh EtOH:$C_{12:0}$ solution, but also leading to an increase in processing time and in energy consumption. To overcome these concerns, the use of biomass pre-treatment unit (*cf.*, mechanical, physical, and/or chemical) before the SLE procedure can be a solution to overcome some of the above-mentioned drawbacks. The implementation of technologies linked to the circular and sustainable approach led to a sudden development of several bio-based industrial processes.

3.3. Formulation of carotenoids-rich soaps

The recycling of solvents with a high boiling point, such as $C_{12:0}$ (boiling point of 298.9 °C), emerges as the most problematic issue for the implementation of this technology on an industrial scale, as it makes the polishing of carotenoids and recycling of solvents much more complex and less cost-effective. In the literature, some preliminary studies focused on the purification of carotenoids and recycling of low vapor pressure solvents have been reported (Mussagy et al., 2022a; Wang et al., 2021). As an innovative approach, in this work we suggest combining: *(i)* the possibility of using medium-chain carboxylic acid (*i.e.*, lauric acid – $C_{12:0}$) for the formulation of bio-based soaps through a saponification process and; *(ii)* the incorporation of attractive biological properties in soaps using carotenoids-rich extracts as a biocompatible alternative to obtain directly ("one-pot") new commercial carotenoids-based soaps with powerfully antioxidant capacity. At this point, the integration of the extraction process for the recovery of pigments from *P. rhodozyma* using an EtOH:$C_{12:0}$ solution with a subsequent formulation of carotenoids-based soaps was evaluated. For this, carotenoids-rich extracts concentrated in the EtOH:$C_{12:0}$ solution (mass ratio 2:1) were enriched with $C_{12:0}$. The influence of the carboxylic acid, astaxanthin and β-carotene on soap formulation was evaluated by preparing a soap composed of $C_{12:0}$ mixed with EtOH but without carotenoids (used as control) and compared with the soap obtained after the SLE using the EtOH:$C_{12:0}$ solution. The visual appearance, physicochemical parameters and antioxidant activity of the resultant carotenoids-rich soaps are presented in Fig. 5.

The visual observation of both soaps confirms the successful formulation using an ethanolic $C_{12:0}$ solution (EtOH:C12:0, mass ratio 2:1) rich in carotenoids. As depicted in Fig. 5A (table inset on the right), the pH values of formulated soaps were slightly alkaline in nature, mainly due to the short curing period (15 days). These pH values are still considered to be within an acceptable range for human application. Carotenoids-rich soaps containing 21 $\mu g/g_{soap}$ and 66 $\mu g/g_{soap}$ of astaxanthin and β-carotene, respectively, revealed a slight decrease in the pH values (8.68) compared to the control (8.83). The decrease in the pH values for the carotenoids-rich soaps was due to the slight acidic character of the carotenoids, *viz.*, astaxanthin esters (Schmidt et al., 2011). The pH values obtained in this work are in line with those reported by Atolani et al. (2016) and Rambabu et al. (2020) whose soaps formulated with natural extracts exhibited pH values in the range of 8 to 11. The moisture content (MC) in soaps significantly impacts the texture, appearance, shape, and weight, and is commonly used to assess the shelf life of products (Rambabu et al., 2020). Low MC makes soaps more resistant,

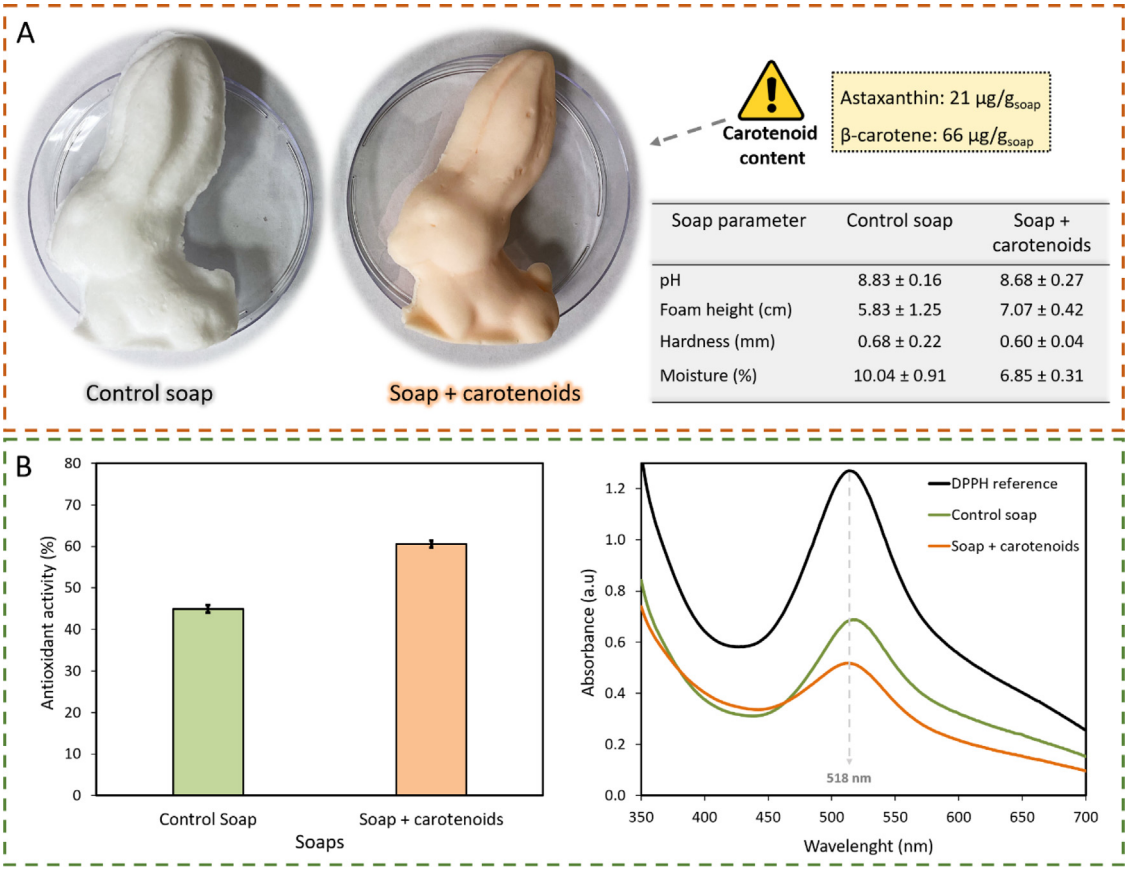

Fig. 5. A- Soaps produced (rabbit shape) using lauric acid + EtOH (control soap), carotenoids-rich soaps (resultant from the SLE procedure) and physicochemical parameters evaluated. **B-** antioxidant activity of soaps.

while high MC would lead to contamination during storage (W. Shreve et al., 2002). Interestingly, carotenoids-rich soaps had the lowest MC values (6.85%) (similar to commercial soaps), while the control had the highest MC (10.04%) (Fig. 5A). Astaxanthin and β-carotene are very hydrophobic molecules (Mussagy et al., 2019b; Novoveská et al., 2019; Saini and Keum, 2018) therefore, due to their low affinity for water, a significant reduction in MC was obtained in carotenoids-rich soaps. In addition, strength stability (surface hardness) tests were carried out for the formulated soaps (carotenoids-rich soaps and control). This parameter is critical, as it directly depends on the MC and fatty acids composition. In this case, the low penetration of the needle reveals a stronger surface hardness of the soap, while high penetration values indicate a weak surface. As depicted in Fig. 5A, the carotenoids-rich soaps have lower penetration values (0.60 mm) compared to the control (0.68 mm), which is associated to the low MC.

The antioxidant activity of soaps prepared without (control) and with astaxanthin/β-carotene pigments was also evaluated using DPPH$^{\cdot}$ free-radical-scavenging activity assay. As shown in Fig. 5B, the DPPH$^{\cdot}$ solution exhibited a reduction in the initial absorbance spectrum after the addition of carotenoids-rich and control soaps. Carotenoids-rich soaps (containing 21 and 66 $\mu g/g_{soap}$ of astaxanthin and β-carotene) showed higher antioxidant activity (60.53%) compared to the control (44.96%). The capacity of the astaxanthin and β-carotene to act as free radical scavengers confirmed the intrinsic antioxidant nature of both carotenoids (Ao and Kim, 2019; Sztretye et al., 2019). In addition to the "extra" antioxidant characteristics, the use of carotenoids also provided a very attractive color for the soaps (orange), without the need of coloring additives, as astaxanthin and β-carotene are natural pigments. Despite the excellent results using carotenoids-rich extracts in the formulation of soaps, attention should also be given to soaps without carotenoids incorporation (*viz.*, lauric acid) due to the human health-related benefits of this fatty acid. Therefore, the use of this carboxylic acid is interesting not only as a solvent/additive for the recovery carotenoids and soap production, but also as a natural antioxidant compound for cosmeceutical purposes (Lappano et al., 2017; Sheela et al., 2019).

In summary, we demonstrate that the carotenoid-rich $C_{12:0}$ solution can be directly used for the formulation of new bioactive soaps, emerging as a sustainable, biocompatible and cost-effective alternative to conventional soap formulations, but some additional studies to evaluate the safety of the proposed technology, as well as techno-economic analysis and the life-cycle assessment are still necessary for later application of bioactive soaps in cosmeceutical industries.

4. Conclusions

In the present work, the use of a integrated and "greener" platform for the recovery of natural carotenoids from *P. rhodozyma* biomass using ethanolic carboxylic acid solutions was evaluated and applied to soap formulation with enhanced antioxidant activity. Increasing the fatty acid alkyl chain length favors the recovery of astaxanthin and β-carotene, but above a certain molecular weight (> $C_{16:0}$) the interaction of carotenoids with the solvents is negatively affected. Furthermore, this work reported for the first time the use of ethanolic $C_{12:0}$ solutions rich in carotenoids directly as a sustainable additive to produce soaps with high antioxidant activities (60.53%). Ethanolic carboxylic acid solutions containing bioactive molecules (such as carotenoids) can be directly used for the formulation of new soaps, emerging as an ecological and simple alternative to conventional soap formulation protocols, some of which still use synthetic and harmful chemicals. Additional studies are still needed to evaluate the safety of these bioactive soaps, as well as techno-economic analysis and life-cycle assessment to confirm the environmental and economic sustainability of the proposed integrative technology. A new functionality of natural yeast-based carotenoids (astaxanthin and β-carotene) has been revealed, demonstrating that these microbial pigments can be converted into a profitable commodity, *i.e.,* for the industrial manufacturing of bioactive soaps.

CRediT authorship contribution statement

Cassamo U. Mussagy: Conceptualization, Investigation, Formal analysis, Data curation, Visualization, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing. **Fabiane O. Farias:** Investigation, Writing – review & editing. **Valeria C. Santos-Ebinuma:** Supervision, Validation. **Jorge F.B. Pereira:** Formal analysis, Writing – review & editing, Visualization. **Adalberto Pessoa Jr:** Writing – review & editing, Supervision.

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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