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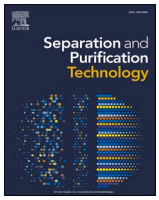

Carotenoids production and extraction from *Yarrowia lipolytica* cells: A biocompatible approach using biosolvents

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ABSTRACT

The use of carotenoids, pigments with significant importance in the food and pharmaceutical industries, has been widely recognized because of their functions as colorants and antioxidants. The production of carotenoids from oleaginous yeasts is an efficient and eco-friendly alternative to that of synthetic carotenoids. *Yarrowia lipolytica*, due to its ability to synthesize high-value-added compounds, including carotenoids and lipids, has emerged as a promising biotechnological platform for this purpose. This study aimed to evaluate the efficiency of biosolvents for carotenoid extraction with the goal of replacing toxic organic solvents that pose risks to both human health and the environment. The Solid-Liquid Extraction (SLE) technique was employed to disrupt the rigid cell wall and extract intracellular pigments. Subsequent extractions were conducted using a 2:3:1 (w/w/w) ratio of ethanol:ethyl acetate:water, resulting in a total β-carotene concentration of approximately 5 mg β-carotene equivalent/g dry biomass. The COSMO-SAC model was utilized to elucidate the solute–solvent affinity, and the results obtained were consistent with the experimental findings. To improve the process, various ratios of biosolvents were investigated, and a subsequent Solid-Liquid-Liquid Extraction (SLLE) was introduced. Process integration and adjustments to the mixed biosolvent composition achieved yields ranging from 50 to 100 % in just four consecutive extractions. The proposed integrative platform not only enhances the efficiency of the extraction process but also enables the fractionation and purification of bioproducts from *Y. lipolytica* cells, thereby maximizing their biotechnological potential.

1. Introduction

Natural or genetically engineered strains of *Yarrowia lipolytica* have proven to be excellent hosts for the biosynthesis of various high-value-added products. *Y. lipolytica* stands out for its ability to grow rapidly, adapt to a wide range of aeration conditions, temperatures and pH values, as well as metabolize diverse substrates while demonstrating tolerance to various inhibitors [1]. Notably, *Y. lipolytica* can accumulate intracellular lipids primarily composed of fatty acid chains similar to those in vegetable oils [2]. This characteristic holds significant industrial potential, particularly for applications like biofuels [3,4]. The present need for biofuels arises from the growing global energy demand and concerns regarding environmental pollution associated with fossil fuel use [5].

In addition to its lipid accumulation capabilities, *Y. lipolytica* can produce carotenoids through heterologous gene expression [6,7]. These carotenoids, including lycopene, β-carotene, and astaxanthin, can be synthesized under conditions similar to those employed for lipid production[8]. Interestingly, the biosynthesis pathways for lipids and carotenoids share acetyl-CoA as a precursor, a compound that *Y. lipolytica* produces in abundance [9]. These advantages have spurred significant interest in genetically modifying this oleaginous yeast to overproduce lipids and carotenoids, despite the existence of other microorganisms capable of producing these pigments naturally. Carotenoids are polyisoprenoid pigments that are of great interest to various industries, such as the pharmaceutical, cosmetic and food sectors, owing to their

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valuable biologically intrinsic properties [10–14]. Some carotenoids also have the potential to serve as nutraceuticals, including precursors of vitamin A, and have been linked to the prevention of degenerative diseases such as cardiovascular diseases, cataracts, and cancer [10].

However, the recovery of these high-value compounds from *Y. lipolytica* presents several challenges. One major obstacle is the need to break down the rigid cell wall of yeast, which can make the extraction process complex and costly [15,16]. Additionally, carotenoids and lipids are difficult to extract because of their varying polarities, complicating their simultaneous recovery using standard Solid-Liquid Extraction (SLE) methods. The relatively hydrophobic nature of carotenoids, along with the use of non-polar solvents for their solubilization, further complicates their extraction from wet biomass and intracellular water-rich environments. Although drying pre-treatments (*e.g.*, oven drying and microwave drying) have been proposed to overcome these issues, the low thermal stability of these compounds remains highly limiting [17]. Commonly used thermal dehydration methods can lead to thermal degradation of carotenoids, making freeze-drying a potential solution, albeit at the cost of time and resources. Furthermore, the sensitivity of these metabolites to harsh conditions, such as high temperature, light, and oxidative environments, adds complexity to the extraction process [17].

To extract these hydrophobic compounds, chemically synthesized volatile organic compounds (VOCs), such as hexane, petroleum ether, chloroform, acetone, and dimethyl sulfoxide are typically used [12,17]. Similarly, non-environment-friendly solvents are often employed for lipid extraction, with *n*-hexane (highly toxic) being a common choice on an industrial scale [15]. These common solvents are not only derived from non-renewable sources but are also highly flammable, volatile, and environmentally hazardous [17], primarily due to their human animal health toxicity [18]. Consequently, there is growing demand for more sustainable extraction methodologies [12].

Recently, Mussagy *et al.* [19] showed an inspiring and straightforward approach to recovering carotenoids and lipids from wet biomass of the yeast *Rhodotorula glutinis* CCT-2186 using a conventional SLE method. Importantly, this approach employs environmentally friendly biosolvents, namely water (H_2O), ethanol (EtOH), and ethyl acetate (EtOAc), offering a more sustainable alternative [12] to the conventional use of toxic fossil-derived organic solvents [9], as well as energy-intensive extraction methods like ultrasound-assisted [20,21], microwave-assisted [20], and pressurized liquid [22] extractions. While this biosolvent-based SLE appears to be a promising solution for carotenoid recovery, its applicability to different microbial biomasses, especially those with distinct cell walls, requires optimization of the operational conditions.

This study presents a comprehensive examination of the utilization of environmentally friendly solvents for carotenoid recovery with the aim of replacing conventional fossil-derived organic solvents. The performance of a biosolvent mixture composed of EtOH, EtOAc, and H_2O was evaluated and compared with that of a control using pure acetone for carotenoid extraction from wet biomass via SLE. This study also explored the adjustment of ternary mixture composition to enhance carotenoid recovery performance, and process intensification was attempted through the development of an SLLE integrative platform. To gain a deeper understanding, we investigated the interaction mechanisms between the solutes of interest and the solvent, shedding light on the role of each solvent in the ternary mixture applied to the oleaginous yeast *Y. lipolytica*, a strain not previously tested for this sustainable extraction process.

2. Material and methods

2.1. Microorganism, growth conditions and inoculum preparation

The *Y. lipolytica* strain employed in this study was genetically engineered, as described by Larroude *et al.* [9], and proven to efficiently produce carotenoids and lipids from different carbon sources [23]. Potato dextrose agar (PDA) plates were prepared to cultivate these yeast cells. *Y. lipolytica* cells were cultivated on these solid PDA plates for 48–72 h at a controlled temperature of 33 °C within a temperature-controlled chamber. Subsequently, yeast colonies from the PDA plates were transferred into 250 mL flasks containing 50 mL liquid potato dextrose broth (PDB) medium. The yeast cells were cultivated in this liquid medium for approximately 24 h under constant temperature conditions at 33 °C, with continuous agitation at 150 rpm in an orbital shaker to obtain the pre-inoculum for subsequent carotenoid production experiments.

To prepare the pre-inoculum for further experiments, the cells were centrifuged using a Frontier™ series multi pro (FC5816 230 V) at 3500 rpm for 10 min. After centrifugation, the supernatant was discarded and the cellular pellet was washed twice with 0.9 % (g/100 mL) NaCl saline solution. To estimate the quantity of biomass for inoculation, a few milliliters of distilled water were added to the pellets, and the optical density (OD) was measured at 600 nm. To prevent contamination, the culture medium and all the materials were autoclaved at 121 °C for 20 min before use.

2.2. Yeast growth conditions for carotenoids production

The culture medium used for carotenoid production contained the following compounds *per* liter of distilled water: 5 g of KH_2PO_4; 2.5 g of Na_2HPO_4; 1.5 g of $MgSO_4$, 1 g of $(NH_4)_2SO_4$, and 0.1 g of $CaCl_2$. Additionally, approximately 40 g/L of glucose was added as the carbon source, and the initial pH of the medium was adjusted to 6, using HCl 2 M and NaOH 2 M aqueous solutions. Flasks (500 mL) containing 200 mL of the culture medium were sterilized in an autoclave at 121 °C for 20 min. The flasks were then inoculated with pre-inoculum to achieve an initial OD_{600nm} of 0.8. The flasks were placed in a thermostatic room at 33 °C and continuously agitated at 150 rpm for 168 h. During the culturing process, we regularly monitored the biomass growth and glucose consumption, as described in Section 2.5.

Following cultivation, the contents of the 15 flasks were combined and the entire volume was centrifuged at 4000 rpm for 10 min at 15 °C using a Hitachi CR22N centrifuge. The supernatant was discarded, and the cellular biomass was washed twice with phosphate-buffered saline (PBS) at pH 7.1. The samples were then stored at −20 °C in glass tubes until subsequent extraction and analysis experiments were conducted.

2.3. Solid-Liquid extraction of carotenoids

SLE of carotenoids was carried out using acetone or a mixture of biosolvents (specific compositions in Fig. 1A). The SLE experimental approach was adapted from methods previously described by Mussagy *et al.* [12]. In brief, these extractions were performed in 10 mL capped glass tubes. A mixture of ethanol (EtOH), ethyl acetate (EtOAc), and water (H_2O) (which varies according to the ratio presented in Fig. 1 A), totaling 1.125 mL, was added to a specific quantity of wet biomass, with pure acetone used as a control. The tubes were then homogenized using a magnetic stirrer hot-plate mixer (IKA C-MAG HS7) for 1 h at 65 °C with agitation. The samples were then centrifuged at 3800 rpm for 10 min, and the resulting cell lysate supernatants were retrieved, stored, and used for carotenoid content analysis. SLEs were performed at various points with different EtOH, EtOAc, and/or H_2O mixtures (see points A to E in Fig. 1) located in the monophasic region.

To determine the SLE profiles, successive extractions were conducted until no carotenoids were found, and the total amount of β-carotene equivalent in the supernatants was properly quantified.

2.4. Solid-Liquid extraction (SLE) followed by Solid-Liquid-Liquid extraction (SLLE) for phase separation

The same SLE steps were repeated as in the previous section;

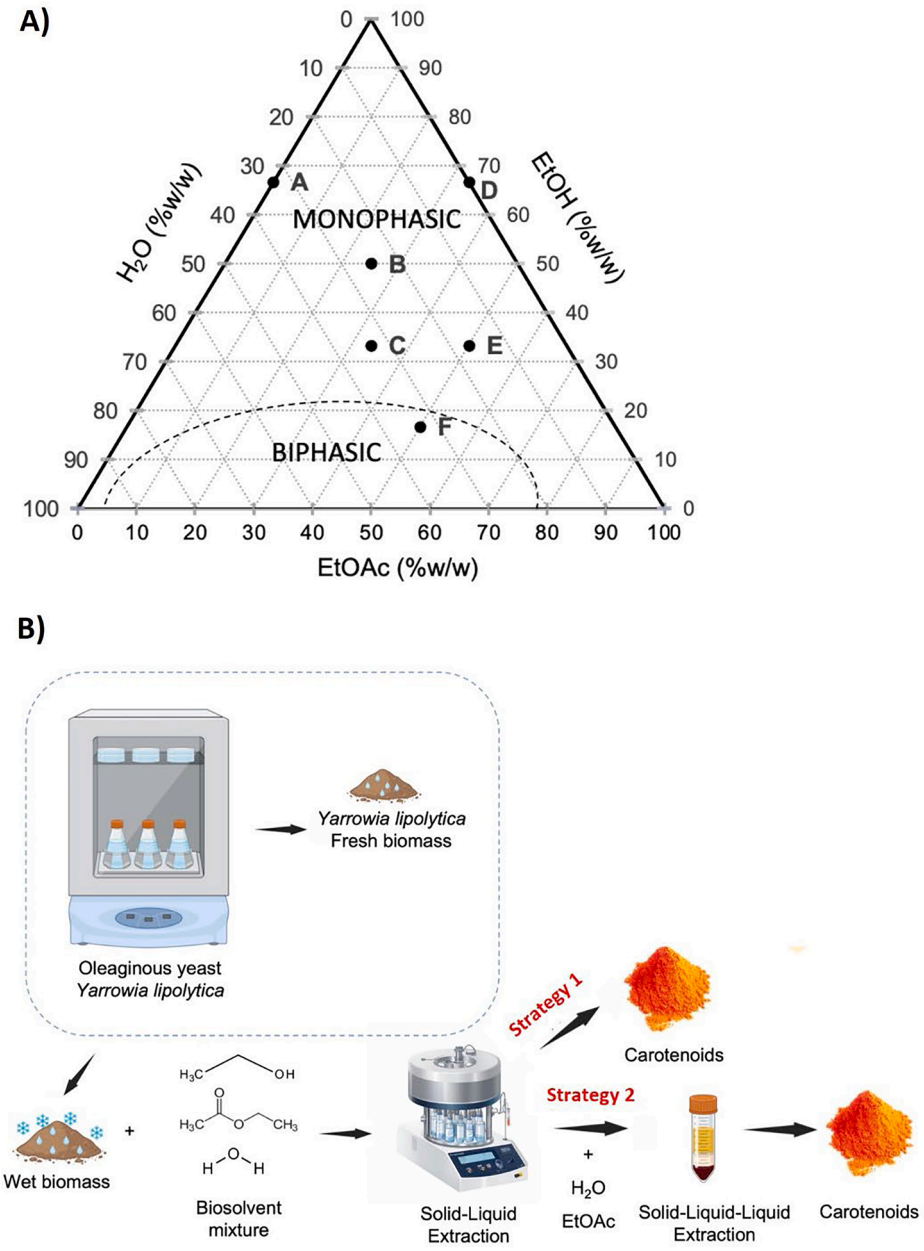

Fig. 1. A) Ternary phase diagram of EtOH/EtOAc/H_2O and solvent mixture composition (% w/w) used to perform different SLEs (points A–E) and solvent mixture composition used for the subsequent SLLE (point F). The hypothetical binodal solubility curve, distinguishing the monophasic and biphasic regions, was represented by the internode curve and was constructed based on previous studies by Resa et al. [24] and Trofimova et al. [25]. B) Schematic representation of experimental protocol including both SLE (Strategy 1) and SLLE (Strategy 2) approaches.

however, after heating for 1 h at 65 °C and stirring, the samples underwent a second stage, SLLE. This involved the addition of more H_2O and EtOAc to the tubes to transition from the monophasic to a biphasic region (*i.e.*, point F in Fig. 1). The samples were then vortexed for 5 min and centrifuged at 3800 rpm for 10 min. The coexisting phases were meticulously separated, and the carotenoids in the upper phase were quantified (the bottom phase was colorless; therefore, the carotenoid concentration was not determined).

2.5. Analytical methods

2.5.1. Biomass determination

Yeast growth was determined spectrophotometrically using a Hitachi Model U-200 (Pacisa & Giralt, Madrid, Spain) during cultivation, as previously described. The OD_{600nm} of the 1 mL sample was measured, allowing for the construction of growth curves based on a prior calibration line of OD_{600nm} *vs.* dry cell weight (mg/mL).

2.5.2. Determination of glucose consumption

To measure glucose consumption over time during yeast cultivation, high-performance liquid chromatography (HPLC) was used for sugar quantification. It was equipped with two detectors: a diode array detector and a refractive index detector, with a detection wavelength of 210 nm. Samples (1 mL) were taken from the flasks and centrifuged for 5 min at 7000 rpm in an Eppendorf tube, and the aqueous supernatant was filtered through a 0.22 μm polytetrafluoroethylene (PTFE) filter before injection into the HPLC. Sulfuric acid (0.005 M) at a flow rate of 0.80 mL/min was used as the mobile phase. The sample volume injected into the Agilent Hi-Plex H 300 x 7.7 mm column was 20 μL at 30 °C.

2.5.3. Determination of the lipid content

The lipid content was determined according to the method described by Larroude *et al.* [9]. First, the samples were centrifuged for 10 min at 4000 rpm and the supernatant was discarded. Distilled water (1 mL) was added and homogenized by vortexing, and the samples were freeze-dried for 24–48 h. Then, 10–30 mg of dry biomass was subjected to transesterification using a solution of methanol and sulfuric acid (40:1, v/v). Quantification was performed using gas chromatography (GC) (Thermo Fisher Scientific).

The GC was equipped with a flame ionization detector (FID) and Agilent vf-23 ms column (60 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas and the initial oven temperature was set to 120 °C. This temperature was held constant for 1 min, then increased to 200 °C at 25 °C/min, and again increased by 4 °C/min to 230 °C, which was maintained for 1 min. Fatty acids were identified by comparison with standard solutions of fatty acid methyl esters (FAMEs). Lipid quantification was performed using the internal standard method by adding 25 mg of commercial C12:0 to FAME.

2.5.4. Determination of the carotenoids content

Spectrophotometry was used to determine the equivalent concentration of β-carotene. Calibration curves were established at 455 nm and carotenoid concentrations were determined using these curves. After extraction, the supernatants were collected and analyzed for carotenoid content using a spectrophotometer (V-550/560/570). The visible light spectrum was measured from 380 to 600 nm, and a β-carotene calibration curve was established at 455 nm. Carotenoid concentration was calculated in terms of the β-carotene equivalent concentration (mg/g) based on the standard β-carotene calibration curve.

2.6. COSMO-SAC approach

The COSMO-SAC model was applied using JCOSMO software developed by Gerber and Soares [26] with GMHB1808 multi-hydrogen bond parameterization, available for free at (https://doi.org/10.5281/zenodo.3613786) [27]. The sigma profiles were obtained using the GAMESS Quantum Chemistry package [28] following the procedure described by Ferrarini *et al.* [29]. The prediction of the activity coefficient in infinite dilution of β-carotene in the solvent mixtures in the monophasic region at 65 °C was also evaluated.

3. Results and discussion

The primary goal of this study was to assess the efficacy of various biosolvents in extracting carotenoids and other potential bioproducts from *Y. lipolytica*. This approach aims to replace the traditional, less sustainable organic solvents known for their detrimental environmental and health impacts. Hence, yeast cell growth and carotenoid production ability were evaluated. Additionally, this study sought to quantify the lipid content accumulated by yeast to provide a comprehensive overview of its production capabilities.

3.1. Production of lipids and carotenoids: Growth and glucose consumption by Yarrowia lipolytica

To achieve these objectives, we conducted fermentation using glucose as the substrate. Glucose is a widely used carbon source for lipid [30] and carotenoid [31] production. The initial fermentation conditions involved a total glucose concentration of 36.6 g/L and a carbon-to-nitrogen (C/N) ratio of 69, which aligns with recommended values for most oleaginous microorganisms [32]. As shown in Fig. 2, we monitored the growth of *Y. lipolytica*, depicted as dry cell weight (DCW) values, along with glucose consumption during cultivation and the concentration of total lipids and carotenoids obtained at the end of fermentation.

Fig. 2 shows that after seven days of cultivation, the yeast consumed more than 90 % of the initially available glucose, prompting us to conclude the experiment. *Y. lipolytica* exhibited robust growth in this medium, with minimal observed lag phase (lower than 6 h). The biomass concentration peaked 143 h post-inoculation, with a maximum dry cell weight of 8.93 g/L, corresponding to a glucose consumption of 29.6 g/L at that time. The glucose consumption rate was 0.2208 g/L.h. The substrate uptake rate were in the same range as those reported by Pereira *et al.* [33], who achieved glucose uptake rates of 0.32 g/L.h and 0.19 g/L.h in batch cultures of *Y. lipolytica* NCYC 2904 when using glucose as the sole carbon source or volatile fatty acids (VFAs) supplemented with glucose, respectively.

When practically all the glucose was consumed (33 g/L), after 168 h, fermentation was stopped and the lipid content accumulated in yeast cells was determined, as outlined in section 2.3.3. The results depicted in Table 1 reveal a lipid content of 20.2 % *per* gram DCW, with a

Table 1
Lipid content, lipid concentration and lipid profile obtained with *Yarrowia lipolytica* with 33 g/L glucose consumed, after 168 h of cultivation at 33 °C and 150 rpm.

Lipid content % (g_{lipids}/g_{DCW})	Lipid concentration (g/L)	Relative amount of total fatty acids (%, g/g)				
		C16:0	C18:0	C18:1	C18:2	Other
20.2	1.39	18.3	4.79	49.3	14.7	12.9

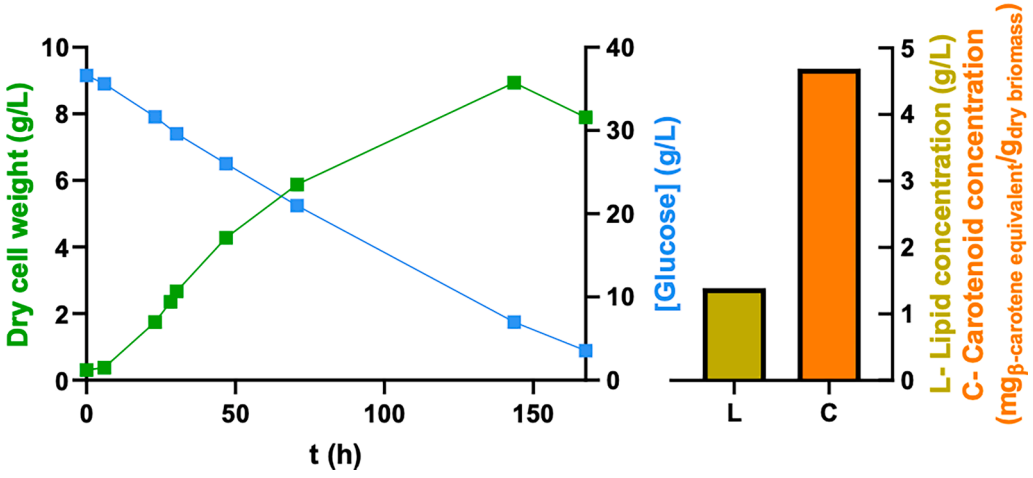

Fig. 2. Growth of *Y. lipolytica* (■, DCW (g/L)) and glucose consumption (■, g/L) during cultivation, and lipid (■, g/L) and carotenoid (■, mg β-carotene equivalent/g dry biomass) concentrations were obtained at the end of fermentation.

corresponding lipid concentration of 1.39 g/L. *Y. lipolytica* is considered an oleaginous species capable of accumulating lipids at levels of at least 20 % of DCW, and the accumulation obtained in our study is in line with other studies. Gao *et al.* [34] achieved a lipid percentage of 37.3 % and a lipid concentration of 0.88 g/L working with *Y. lipolytica* CICC 31596 and glucose as the substrate. They primarily focused on the use of more economical carbon sources, with the highest lipid content of 31.6 % when acetic acid was the sole carbon source. Pereira *et al.* [35] conducted batch experiments with VFAs co-utilizing glucose to obtain lipids. They obtained a lipid content of 12.4 % when butyric acid and glucose were used with *Y. lipolytica* W29 strain. However, it is noteworthy that they managed to enhance the lipid content up to 25.4 % in two-stage batch cultures, where the yeast was first grown on glucose (20 g/L) and then a mixture of VFAs (18 g/L) was added sequentially. Considering these results, it can be affirmed that the modification of the strain used in the present study to produce carotenoids was successful, as it also produces lipids efficiently.

The lipid composition obtained in our study, as presented in Table 1, included oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), and stearic acid (C18:0). These four acids account for 87 % of the total acids obtained, with oleic acid representing 49 %, making it an attractive choice for biodiesel production.

At the same time that lipids accumulate, carotenoids are also synthesized. Since carotenoid production takes place within cells, the "productivity" is significantly influenced by the amount of carotenoids quantified after cell disruption and extraction, as well as the efficiency of the solvents used as extractants. The specific methods for extracting carotenoids are discussed in subsequent sections, but it can be anticipated that the maximum amount of total carotenoids accumulated was approximately 5 mg $_{\beta\text{-carotene equivalent}}/g_{\text{dry biomass}}$ under the optimized extraction conditions.

3.2. Extraction of carotenoids using mixed biosolvents

3.2.1. Solid-Liquid extraction of carotenoids

To conduct the SLE tests, we used a ternary mixture consisting of EtOH, EtOAc and H_2O. Our choice was inspired by previous work with *R. glutinis* CCT-2186 conducted by Mussagy *et al.* [12]. In their study, they sought to substitute ethyl lactate (EtOLac), which is also considered an environmentally friendly solvent, with EtOAc. The latter method has the advantage of forming a biphasic regime when combined with specific quantities of H_2O and EtOH. The possibility of forming a system with two immiscible liquid phases in equilibrium greatly facilitates the separation and purification of the target metabolites [12]. Furthermore, while H_2O is conventionally regarded as a biological solvent for convenience [36], both EtOH and EtOAc are included in the category of green solvents as *per* the solvent selection guides [36,37], and EtOAc is listed as a biosolvent if derived from bioethanol conversion [38].

The efficiency of the mixed biosolvents (EtOH, EtOAc, and H_2O) in carotenoid extraction was evaluated by considering the influence of biomass type and nature as well as how the solvent could access these compounds [12]. Different ternary mixtures were tested using various proportions of biosolvents and different amounts of initial wet biomass, considering their dry weights. The aim of this study was to determine optimal extraction conditions for carotenoids. An extraction solvent volume of 1.125 mL was established, and the experiments commenced with 0.225 g of dry weight biomass, equivalent to a concentration of a 0.2 g dry biomass *per* mL solvent. However, at this concentration, the biosolvents become saturated, hindering the efficient recovery of carotenoids. After multiple trials, we found that reducing the concentration to 0.05 g dry biomass *per* mL of solvent or 0.025 g dry biomass *per* mL of solvent yielded consistent results; the same amount of carotenoids was consistently extracted without saturation. Subsequently, the total carotenoid content of the wet samples was determined. For this purpose, we conducted successive SLEs using a 2:3:1 mixing ratio of EtOH:EtOAc:H_2O, until the extracts became colorless. As a control, the same procedure was performed using acetone. The corresponding results are presented in Fig. 3 as the concentration of total carotenoids, quantified as β-carotene equivalent (mg) *per* gram of dry biomass.

As shown in Fig. 3, the total quantities of carotenoids recovered with acetone were 3.14 $mg_{\beta\text{-carotene equivalent}}/g_{\text{dry biomass}}$ for 0.056 g of dry biomass, decreasing to 2.80 $mg_{\beta\text{-carotene equivalent}}/g_{\text{dry biomass}}$ for 0.028 g of dry biomass. In contrast, when employing the biosolvent mixture, recoveries of 4.78 $mg_{\beta\text{-carotene equivalent}}/g_{\text{dry biomass}}$ and 4.69 $mg_{\beta\text{-carotene equivalent}}/g_{\text{dry biomass}}$ were achieved for 0.056 g and 0.028 g of dry biomass, respectively. The disparities in the efficiency of carotenoid extraction between acetone and mixed biosolvents can be attributed not only to the capacity of the solvent to disrupt cells and dissolve intracellular carotenoids but also to the intrinsic stability of carotenoids in each solvent. The latter effect can be more pronounced when attempting to deplete biomass by conducting successive ($n > 10$) extractions with fresh solvents, which results in extended contact times between carotenoids and solvents, ultimately leading to the degradation of these highly unstable compounds.

Microbial carotenoids are inherently hydrophobic and are typically extracted from dry biomass using organic solvents such as acetone, DMSO, among others [17]. In this study, it was feasible to use wet biomass rather than dry biomass. This approach is very interesting, as it could significantly reduce costs by reducing the need to dry the cells prior to analysis, by reducing one unit of operation. Working with wet biomass made acetone less effective than the biosolvent mixture. Interestingly, these results clearly revealed that using mixed biosolvents, carotenoid recovery can be significantly enhanced, with an increase of almost 2-fold in carotenoid extraction in comparison with pure acetone. In fact, the use of more polar and water-miscible biosolvents, such as EtOH, enhances cell-solvent miscibility and subsequent cell wall disruption [39,40]. It has been previously demonstrated that the extraction of carotenoids from wet samples is accomplished using a mixture of polar and non-polar solvents. Hydrophilic solvents assist in removing water, facilitating the extraction of pigments in the nonpolar solvent [41], in this case, EtOAc. Specifically, H_2O solubilizes wet yeast biomass, EtOH permeabilizes the membrane, and EtOAc dissolves carotenoids. This mixture was essential because H_2O and EtOAc exhibit limited miscibility. However, when combined with EtOH in certain

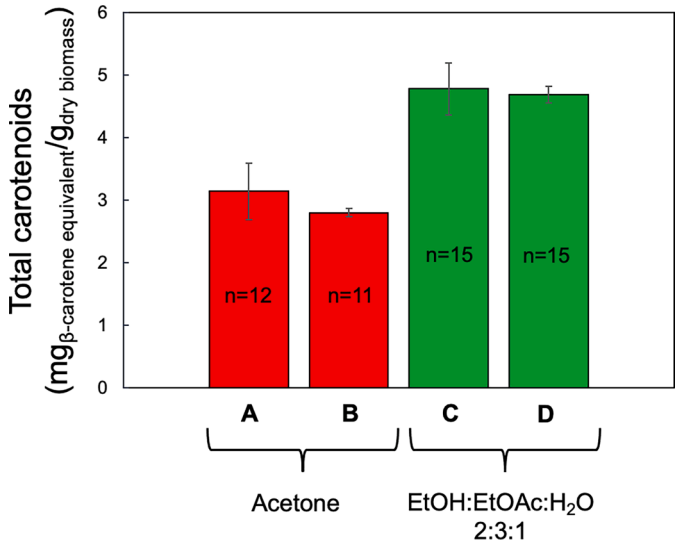

Fig. 3. Total amount of carotenoids extracted with acetone and the ternary mixture of biosolvents (EtOH:EtOAc:H_2O, 2:3:1). Results A and C were obtained using a 0.05 g dry biomass/mL solvent, while 0.025 g dry biomass/mL solvent was used for B and D. "n" represents the number of SLEs carried out until the complete extraction of carotenoids was achieved (*i.e.*, supernatant becoming colorless). The plotted data are based on tests performed in triplicate, and the error bars represent the mean ± standard deviation of triplicate experiments.

proportions, a miscible system was formed.

To investigate the extraction process further, we constructed an extraction profile involving up to eight successive SLEs, as shown in Fig. 4. For both acetone and biosolvents, approximately 90 % and 80 % of the total carotenoids were extracted with eight successive SLEs, respectively. The remaining SLEs did not significantly contribute to the extraction of the minimal residual amounts of these metabolites. Beyond 5–6 cycles, no further extractions were deemed necessary as the extraction yields decreased considerably.

The profiles obtained using acetone and the biosolvent mixture, although following similar patterns from the first to the second extractions, exhibited some differences for the following extractions, which should be properly discussed. In the initial SLE, both solvents faced challenges in penetrating the wet cells and extracting carotenoids. Interestingly, for acetone, independent of the dry biomass concentration, maximum extraction efficiency was achieved during the second SLE. This could be attributed to its role as a dehydrating agent in the first extraction, which removed water from the wet sample. In contrast, for mixed biosolvents, experiments with 0.056 g dry biomass required three SLEs to attain maximum extraction efficiency, because the higher biomass amount poses challenges for biosolvents to penetrate the cell wall effectively. The maximum recovery rates were reached later than in the experiments with 0.028 g dry biomass. It is worth noting that more extraction cycles were required for biosolvent extraction compared to acetone, as in each cycle, a smaller amount of the hydrophobic solvent (EtOAc) was used with the same biomass amount, unlike in the acetone experiments. In the acetone trials, 1.125 mL of pure acetone was employed in each cycle, whereas in the ternary mixture extractions, EtOAc accounted for 50 % (w/w). As the solid–liquid ratio (SLR) increased, the same amount of solvent was distributed among a larger number of cells, leading to delayed maximum recovery. Considering the limitations of increasing the SLR for carotenoid recovery, subsequent experiments were conducted using 0.025 g of dry biomass *per* mL of solvent.

In the case of acetone, only four consecutive extractions were required to extract 75 % of carotenoids. In the biosolvent experiments, which significantly outperformed acetone in terms of β-carotene equivalent recovery, approximately 65 % of the carotenoids were extracted after four consecutive SLEs, regardless of the initial biomass amounts.

While previous similar assays with *Y. lipolytica* are lacking, these trials align with previous studies using other yeast cells. For instance, Mussagy *et al.* [19], working with *R. glutinis* CCT-2186, demonstrated the feasibility of recovering various carotenoids (β-carotene, torularhodin, and torulene) and lipids from the wet biomass of this strain using EtOH, EtOAc and H_2O. Moreover, recent studies have delved into the use of biocompatible and renewable solvents with the related yeast *Phaffia rhodozyma* [42,43], in which biosolvents, including EtOH and EtOAc, exhibit better performance for carotenoid recovery, suggesting the possibility of replacing conventional organic solvents such as acetone. Furthermore, Mussagy *et al.* [44] proposed a sustainable process using mixtures of ethanolic carboxylic acids as biocompatible and renewable solvents for carotene extraction, with the ethanol and lauric acid mixture showing the highest capacity for recovering astaxanthin and β-carotene.

To the best of our knowledge, no studies have been conducted on the recovery of carotenoids from *Y. lipolytica* using green solvents. However, for comparison, we compared the results of the present investigation with those obtained by Larroude *et al.* [9], who achieved one of the highest β-carotene contents reported in the literature using the same strain of *Y. lipolytica* and glucose as the carbon source. In their study, they used fed-batch fermentation to achieve a maximum β-carotene content of 90 mg/g after 122 h of fermentation, initiated at a rate of 6 g/L glucose after 6 h. It is worth noting that the higher yield obtained by Larroude *et al.* may be attributed to the higher amount of substrate consumed by *Y. lipolytica* and the cultivation fed-batch mode employed, which involves feeding small amounts of substrate at regular intervals, allowing for better adaptation of the yeast to the culture medium and

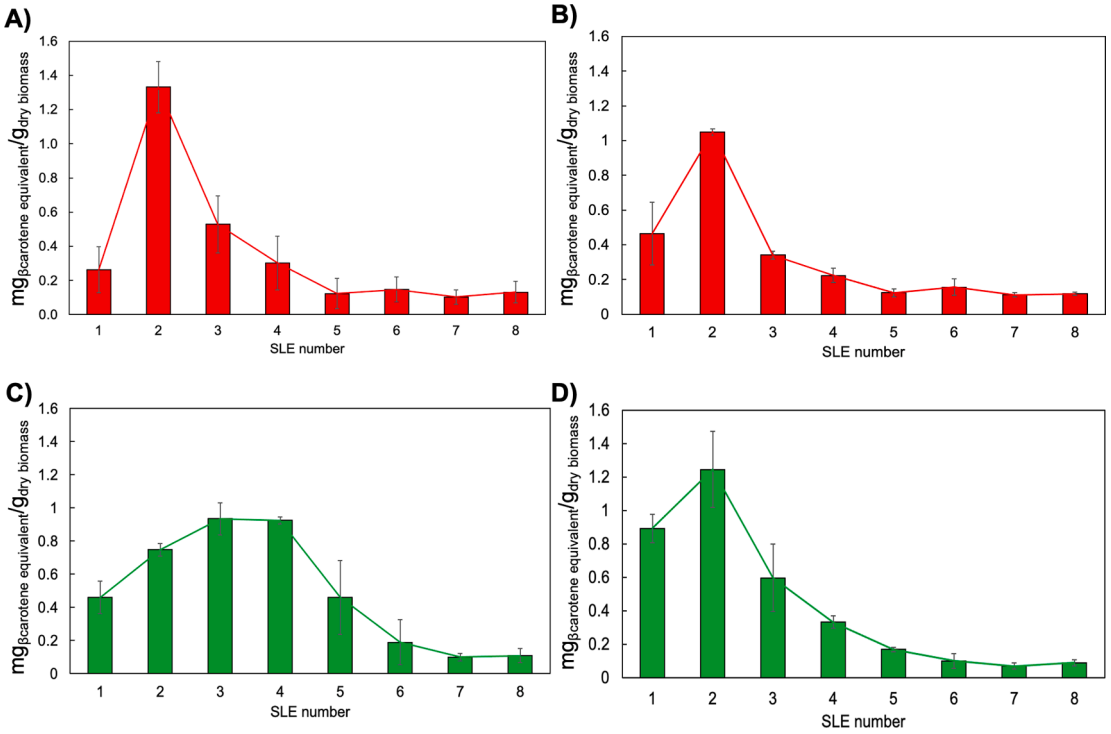

Fig. 4. Extraction profiles were obtained with acetone and with a ternary mixture of biosolvents (EtOH: EtOAc:H_2O, 2:3:1) up to eight SLE. The results for A and C obtained using a 0.05 g dry biomass/mL solvent, while for B and D 0.025 g dry biomass/ mL solvent was used. ■ Red squares represent the experiments with acetone; ■ green squares represent the experiments with mixed biosolvents. The results were obtained from tests performed in triplicate, and the error bars represent the mean ± standard deviation.

avoiding the potential inhibitory effects of high substrate concentrations. Additionally, Larroude *et al.* used a non-environmentally friendly extraction mixture (50:50 v/v; hexane–ethyl acetate; 0.01 % butyl hydroxyl toluene) for carotenoid recovery. Matthäus *et al.* [45] used the latter mixture to extract lycopene from lipid bodies produced using genetic modification techniques in *Y. lipolytica*, reaching a yield of 16 mg/g.

Most studies conducted on the accumulation of lipids and carotenoids in this particular yeast have primarily focused on increasing production yields. However, little research has been conducted on the potential to improve efficiency from an environment-friendly perspective. This study provides valuable data and a comprehensive analysis of the feasibility of using green solvents for the extraction of products synthesized by *Y. lipolytica*.

3.2.2. SLE extraction integrated with a subsequent SLLE

In the previous section, we demonstrated a significant enhancement in carotenoid extraction from *Y. lipolytica* using the SLE technique with a mixture of EtOH, EtOAc and H_2O. In this section, we evaluate the enhancement of the extraction performance. An essential consideration is the effect of the composition of the biosolvent mixture on the extraction of the target compounds [19]. It should also be noted that the formation of a biphasic regime using Liquid-Liquid Extraction (LLE) post-SLE substantially aids in the separation and purification of carotenoids and lipids [12].

Hence, we conducted different SLEs by randomly varying the initial proportions of biosolvents and integrating this stage with an SLLE within the biphasic region (as shown in Fig. 1). In this instance, we opted for SLLE instead of LLE, that is, the cells were not removed, saving time and process costs as it eliminates the need for prior centrifugation and subsequent recovery of the liquid supernatant. The extraction was carried out using a *Y. lipolytica* biomass concentration of 0.025 $g_{dry\ biomass}/mL_{biosolvent}$, mixed for 1 h with stirring at 65 °C, and the biomass was mixed with different mixed biosolvents (five mixture points, A to E in Fig. 1, in the monophasic region of the EtOH/EtOAc/H_2O phase diagram). Regardless of the initial proportion of mixed biosolvents, the same biphasic mixture (point F in Fig. 1), namely EtOH:EtOAc:H_2O (in a 1:3:2 ratio), was obtained by adding more H_2O and EtOAc to the systems.

The concentration of total carotenoids extracted ($mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) and the corresponding carotenoid Recovery Yields (%) for all biosolvent compositions employed in the SLE were determined and are presented in Table 2. To calculate the recovery yields, we considered the total carotenoid concentration in the samples to be 4.69 $mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$. This value corresponds to the mean of the concentrations obtained following successive SLEs with 0.025 $g_{dry\ biomass}/mL_{solvent}$.

The results presented in Table 2 reveal that the optimal conditions for the SLE of total carotenoids (in $mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) from *Y. lipolytica* cells were achieved with high EtOH concentrations (ranging from 33 % to 67 % w/w), intermediate concentrations of EtOAc (ranging from 33 % to 50 % w/w), and low (17 % w/w) or even zero H_2O concentrations. These conditions yielded carotenoid recovery rates of up to 100 % after four consecutive extractions.

Table 2
Concentration of carotenoids extracted ($mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) and the corresponding Recovery Yields (%) of β-carotene equivalent for *Y. lipolytica* wet cell concentration of 0.025 $g_{dry\ biomass}/mL_{solvent}$. Extraction was performed after 1 h of stirring at 65 °C (points A to E from Fig. 1), with subsequent phase separation achieved at point F (see Fig. 1). The concentrations and recoveries were determined after four consecutive SLEs, each integrated with a subsequent SLLE. The results were based on tests conducted in triplicates.

	$mg\beta_{carotene\ equivalent}/g_{dry\ biomass}$	Recovery Yields (%) of β-carotene equivalent
A→F	2.31 ± 0.87	49.30 ± 18.66
B→F	2.88 ± 0.43	61.40 ± 9.25
C→F	2.74 ± 0.42	58.43 ± 8.94
D→F	4.70 ± 0.05	100.15 ± 1.12
E→F	4.73 ± 0.30	100.89 ± 6.43

Interestingly, as presented in Fig. 5, integrating an SLLE after SLE allows for the recovery of 49–100 % of the carotenoids present in the samples in four stages. This is a highly promising outcome compared to the 15 SLEs required for carotenoids recovery (as discussed in Section 3.3.1). For more detailed information, please refer to Fig. 5, which illustrates the extraction profiles and concentration of total carotenoids obtained after four double cycles ($mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$).

Fig. 5 shows how inclusion of a second SLLE after each SLE significantly altered the extraction profile trends. In contrast to SLE, in which at least two consecutive extractions are required to peak carotenoid recovery (Fig. 4C and 4D), for all tested mixed biosolvent ratios, the highest recovery of carotenoids was achieved in the first extraction cycle. This outcome offers notable advantages in terms of efficiency, simplicity, cost-effectiveness, and reduced extraction times. As previously mentioned, this increase can be attributed to the higher concentration of EtOAc in the biphasic region, which enhances carotenoid recovery [12]. For the two most effective SLE+SLLE systems, D and E, the initial biosolvent composition played a pivotal role, recovering 84 % and 85 % of the total carotenoids, respectively, after the first two integrated extractions. Even with just a single integrated step, systems D and E managed to recover 61 % (2.86 $mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) and 50 % (2.35 $mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) of the total carotenoids, showing a highly promising result.

Mussagy *et al.* [42] also found that these high carotenoid recovery yields are attributed to the excellent compatibility of EtOH and EtOAc with both H_2O and nonpolar compounds, respectively. This compatibility enhances the ability of the solvents to interact with the cell wall components of this type of wet cell, facilitating solvent penetration inside the cell and subsequent solubilization of carotenoids. By integrating the first SLE with a second SLLE by simply increasing the concentration of the solvents in the mixture, we maximized the contact of the nonpolar solvent (EtOAc) with the hydrophobic carotenoids, enhancing their solubilization. Fig. 5 clearly indicates that the recovery extraction yields were higher when the amount of H_2O used in the SLE was lower. Systems A, B, and C, which contain higher H_2O content, yield lower carotenoid recovery rates than systems D and E, in which the presence of H_2O is more limited.

Therefore, it is important to understand the metabolism of *Y. lipolytica*. This oleaginous yeast is a natural lipid producer, making it suitable for β-carotene production, as this hydrophobic and lipophilic compound accumulates in lipid droplets produced by these microorganisms. Similarly, *R. glutinis* forms intracellular lipid droplets rich in carotenoids [46]. *P. rhodozyma* also accumulates these compounds in lipid droplets [47]. The presence of H_2O, although needed to solubilize the wet biomass, hinders the miscibility and solubilization of these biomolecules with organic solvents while also reducing cell wall permeabilization. To overcome this limitation, a strategy can be to increase the miscibility of H_2O and EtOAc by adding EtOH or using only EtOH to provide the polarity required for wet biomass solubilization. These findings align with the study by Mussagy *et al.* [42], which extracted β-carotene and astaxanthin from the wet biomass of *P. rhodozyma* using a binary mixture of EtOH and EtOAc. In their one-step extraction, they achieved a total amount of 42.81 μg/g DCW and 63.11 μg/g DCW, respectively, despite the hydrophobic nature of these biomolecules. Furthermore, another study of Mussagy *et al.* [12] examined the recovery of carotenoids from *R. glutinis* biomass using biosolvent mixtures with the SLE procedure. The most favorable results were obtained from pretreated dry biomass using a mixture of EtOH, EtOAC, and H_2O (67:33:00, w/w/w), with yields exceeding 75 % (w/w).

In contrast, while the use of LLE simply favors the selective separation and purification of metabolites [12,19,43], our study shows that SLLE, where the biomass remains in constant contact with biosolvents,

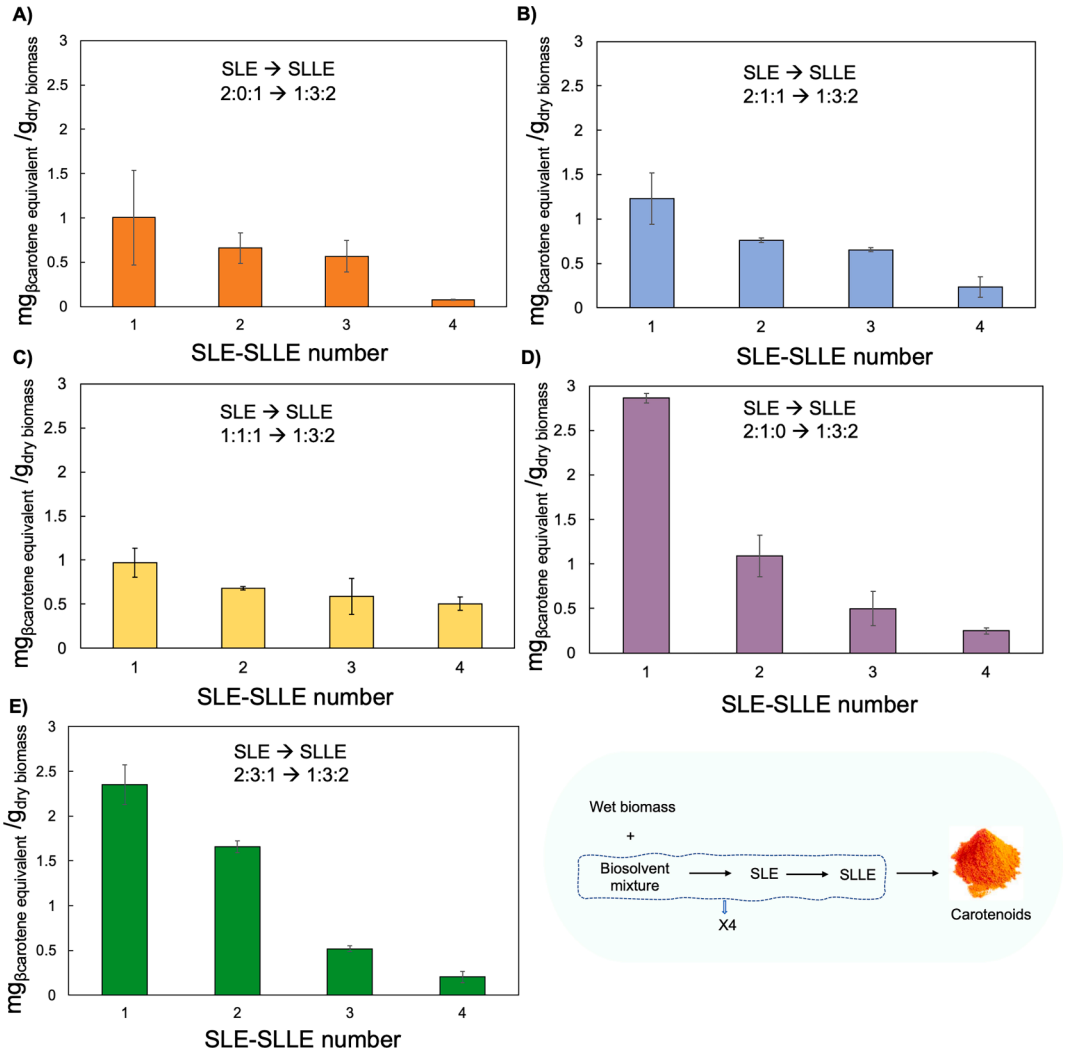

Fig. 5. Extraction performance of sequential SLE + SLLE for various biosolvents mixtures, denoted as initial SLE in the mixtures A to E (*i.e.*, EtOH:EtOAc:H_2O, 2:0:1; 2:1:1; 1:1:1; 2:1:0 and 2:3:1, respectively) followed by SLLE at the biphasic region (point F, *i.e.* EtOH:EtOAc:H_2O, 1:3:2). All the results correspond to the extraction obtained after two consecutive cycles. The experiments were conducted using 0.025 $g_{dry\ biomass}/mL_{solvent}$ after 1 h of stirring at 65 °C. The reported results were derived from triplicate experiments, and the error bars represent the mean ± standard deviation of triplicate measurements.

not only allows for separation and purification but also significantly enhances the extraction efficiency of the preceding SLE operation. The innovative extraction technology proposed in this study is characterized by its simplicity and efficiency in the selective recovery and separation of carotenoids from wet *Y. lipolytica* biomass. Additionally, this study stands out, as there have been no previous investigations evaluating the efficacy of eco-friendly solvents for the extraction of carotenoids produced by this yeast. Most studies have used conventional solvents or combinations of toxic solvents. For instance, Bruder *et al.* [6] used a solvent mixture of *n*-hexane:EtOH:acetone (50:25:25); Ma *et al.* [48] used DMSO, and Larroude *et al.* [9], as discussed above, a mixture of hexane:EtOAc (50:50) with 0.01 % butyl hydroxyltoluene. Given that *Y. lipolytica* can also produce other valuable metabolites, future studies employing greener solvents to assess the simultaneous recovery of carotenoids, lipids, proteins, or other potential products are warranted to fully exploit this integrated platform.

3.3. Insights on mechanisms behind the carotenoids' recovery using COSMO-SAC

The experimental findings indicate that the relative non-polarity of solvents plays a pivotal role in the effective recovery of carotenoids. To gain a deeper insight into the influence of the relative solubility of β-carotene in solvent mixtures, we employed computational modeling using COSMO-SAC and compared it with the experimental results. Thus, COSMO-SAC was utilized to generate σ-profiles of the compounds, providing insights into the degree of compatibility of β-carotene and EtOH:EtOAc:H_2O mixtures. The σ-profile facilitated the inference of the affinity between carotenoids and solvents, as molecules with similar charge distributions and polarities would exhibit affinity for each other (enhancing solubility), whereas differences would lead to a lack of affinity (reducing solubility) (Fig. 6A). The σ-profile can be categorized into polar (values lower than −0.01 and higher than +0.01 e/A^2), associated with the hydrogen-bond acceptor (HBA) and hydrogen-bond donor (HBD) regions, respectively, and the nonpolar region (ranging between −0.01 and +0.01 e/A^2). Notably, the peaks of β-carotene are distinctly prominent solely in the nonpolar region of the σ-profile, underscoring the nonpolar nature of this carotenoid and its pronounced affinity towards nonpolar solvents such as EtOAc. Conversely, EtOAc and EtOH exhibited some attraction to nonpolar regions, whereas H_2O demonstrated a marked affinity towards the HBA and HBD groups (Fig. 6A).

As illustrated in Fig. 6B, we employed the same *in silico* method to estimate the solute–solvent affinity by calculating the predicted activity

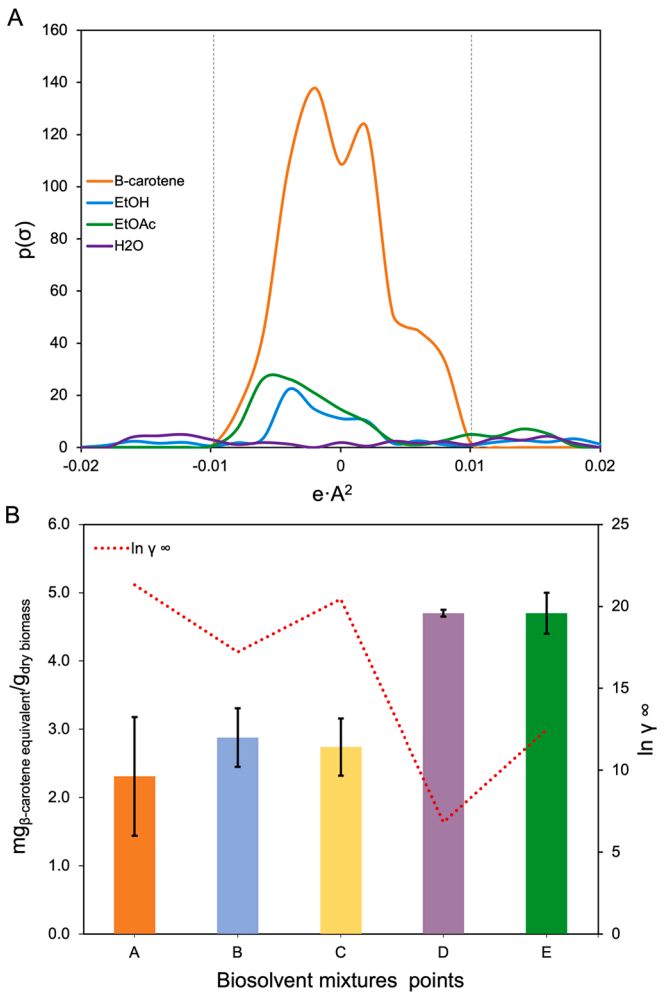

Fig. 6. A- σ-profiles of β-carotene and pure solvents used for the mixtures used in this work generated by COSMO-SAC. B- Concentration of carotenoids extracted ($mg_{\beta\text{-carotene equivalent}}/g_{dry\ biomass}$) for *Y. lipolytica* wet cell concentration of 0.025 $g_{dry\ biomass}/mL_{solvent}$ and the corresponding ln γ^{∞} of β-carotene equivalent and solvent mixtures at different monophasic ternary points (from A to E). Extraction was performed after of 1 h stirring at 65 °C (points A to E in Fig. 1) and ln γ^{∞} values were generated by COSMO-SAC.

coefficients in infinite dilution for β-carotene in bio-based solvent mixtures (monophasic ternary points from A to E). The quantification of solute–solvent affinity was achieved through the determination of ln Υ^{∞} values, wherein a more negative value corresponds to a stronger β-carotene-solvent affinity to the solvent mixture. Based on the ln Υ^{∞} values, the established order for the studied bio-based solvent mixture points was A ≈ B ≈ C > E > D. These computational predictions align with the experimentally obtained results, where D ≈ E > C ≈ B > A, underscoring the efficacy of these tools in predicting carotenoid-solvent interactions.

It is worth noting that the data presented in Table 2 and Fig. 6B were obtained by combining each SLE with subsequent SLLE in the biphasic region (denoted as point F in Fig. 1). At this point, there was a low EtOH concentration (17 % w/w), high EtOAc concentration (50 % w/w), and an intermediate H_2O concentration (33 % w/w). Although the COSMO-SAC was based on monophasic ternary points, it clearly demonstrated that there is a relationship between the initial monophasic system composition and each extraction performance obtained in the biphasic system in which the SLLE occurred.

Upon closer analysis of all experimental and computational results, it became evident that the presence of EtOAc was more critical in the SLLE stage, as it efficiently extracted and recovered carotenoids after breaking down the cell wall in the preceding SLE stage [12]. In contrast, the presence of polar solvents (H_2O and EtOH) played a more important role in the SLE stage, where the combination of both facilitated the solubilization of wet yeast cells and subsequent cell permeabilization. Although carotenoids are hydrophobic, short alcohols and water have been found to aid in dissolving the cell wall membrane, thus facilitating the release of carotenoids from wet oleaginous yeast biomass [19,49]. The addition of polar solvents to nonpolar solvents significantly contributes to the extraction of carotenoids and lipids by promoting cell disruption and permeabilization [19].

4. Conclusions

The use of green solvents for efficient recovery of carotenoids from the wet biomass of *Y. lipolytica* was successfully demonstrated. In addition, the extraction was greatly optimized by performing SLE followed by another SLLE, which allowed a significant increase in the recovery yields of carotenoids and, at the same time, the combination of these techniques represents a truly process integration methodology. In this study, it was shown that the presence of EtOAc is more relevant in the SLLE stage because it is the solvent that recovers the pigments after breaking the cell wall in the previous SLE and that the extraction of pigments is more efficient by increasing the amount of EtOAc and decreasing the amount of H_2O in the ternary mixture used for the recovery of carotenoids.

In conclusion, the results demonstrate high recovery yields of carotenoids using SLE and SLLE with a ternary mixture of EtOAc, EtOH, and H_2O, without the need for techniques or chemical compounds with unfavorable environmental impacts. These findings underscore the increasing demand for technologies prioritizing human health and environmental sustainability, while facilitating the recovery of valuable compounds such as carotenoids. They also highlight the significant potential of yeasts for carotenoid production. Considering the potential of *Y. lipolytica* for both pigment and lipid synthesis, the results of this study offer promising prospects for carotenoid extraction without reliance on fossil-derived organic solvents or energy-intensive mechanical methods. Despite the promising outcomes, further pilot and industrial studies, as well as techno-economic analysis and life-cycle assessment, are necessary to fully demonstrate the competitiveness and viability of the proposed technology. These studies could explore solvent recycling/reuse strategies and optimize purification processes for the obtained biomolecules, aligning with a commitment to sustainability and environmental stewardship.

5. Declaration of generative AI AND AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT-3.5 and Paperpal Copilot in order to improve readability and language. After using these technologies, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Cecilia Naveira-Pazos: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **María C. Veiga:** Validation, Writing – review & editing. **Cassamo U. Mussagy:** Writing – review & editing, Methodology, Investigation. **Fabiane O. Farias:** Writing – review & editing, Software, Methodology, Investigation. **Christian Kennes:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Jorge F.B. Pereira:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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