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Stirred, ultrasound-assisted and microwave-assisted extraction process of β-carotene from *Rhodotorula glutinis* in biorefinery downstream

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ABSTRACT

Due to the general population awareness about the specific health hazards of synthetic chemical dyes, natural colorant materials have been gaining attention in the past several years. Among them, β-carotene is widely used as a soluble orange–red pigment in food industry, while also presenting medical care benefits. Thus, considerable efforts have been dedicated to the development of an efficient serial, cost effective and sustainable extraction process for its recovery. Herein, solid–liquid (SLE), ultrasound-assisted (UAE) and microwave-assisted (MAE) equilibrium separation were carried out using 4 organic solvents for β-carotene isolation of from *Rhodotorula glutinis* yeast. Results suggested that technique-wise, UAE performed similarly to SLE, whereas MAE was not as optimal. According to the solvent applied, the colorant followed the increasing trend: dichloromethane < diethyl ether < dimethyl carbonate < ethyl acetate. The latter resulted in 45 ± 3 μg g^{-1}, 42 ± 2 μg g^{-1} and 41 ± 3 μg g^{-1}, while being extracted with UAE, SLE and MAE, respectively. These operation findings show the positive relationship effect of the greener solvent during the SLE, UAE and MAE activity for the optimisation of high-added value molecular compound, pro-vitamin A, from fungi. Additionally, a diffusion-based model system was implemented for the first time to determine mass transfer phenomena. When correlated with experiments, good measurement accuracy was validated; hence, showing promising results as a predictive model in future research studies, especially mechanistic.

1. Introduction

Synthetic dyes have been used for centuries as food and textile pigments all around the world. However, over the last years, there has been a higher awareness of the population of the health hazards associated with these compounds. Therefore, the demand for natural colorants has been gradually increasing, as proven by its global market value, which is estimated to be *circa* USD 20 billion in 2022. According to the Global Carotenoids Market, carotenoids are expected to grow from USD 1.62 billion in 2021 up to ~ USD 2.37 billion by 2029 [1]. Among these, β-carotene was worth USD 520 million in 2020 and is projected to grow to USD 780 million by 2027 [2].

Carotenoids are well known natural pigments, being responsible for the color of fruits and flowers. In addition to plants, these can also be found in algae, bacteria, fungi and yeast. There are more than 650 carotenoids discovered in nature with some of them presenting very interesting properties, for instance their antioxidant, anticancer, anti-inflammatory, antibacterial and antidiabetic activities. Besides, carotenoids have also demonstrated neuroprotective properties as well as the ability to act as immunomodulators [3–6]. Furthermore, carotenoids can be described as highly conjugated polyprenoid compounds, consisting of two terminal ring systems that are frequently classified based on their structure [4]. β-carotene, for instance, is a tetraterpenoid composed of 8 isoprene units and has 40 carbon atoms in its core. It is generally used in the pharmaceutical, food and cosmetic industries. In addition to the aforementioned properties, β-carotene is mainly used as a colorant and a vitamin A precursor (pro-vitamin A). Due to its properties, it can be used for different shades of red, orange and yellow. Hence, β-carotene can potentially replace the currently used synthetic colorants, namely Tartrazine (E102), Sunset Yellow FCF (E110), Erythrosine (E127) and Allura Red (E129) [7,8]. As such, it could be applied to various products, such as cheese, pastry, ice cream, as well as a colouring agent in pharmaceutic tablets and in cosmetic creams to protect the skin from UV radiation [9]. Likewise, pro-vitamin A activity needs to be emphasized, as it plays an important role in retinol formation [10,11]. Aside from that, it has an effective role in embryonic

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development and normal growth. However, to ensure proper functioning in the human body, vitamin A conversion is obligatory. Only then, it can exert oxidation resistance, anticancer activity and anti-cardiovascular properties [7,11,12].

Hence, it is of great interest to develop more environmentally friendly extraction procedures for β-carotene recovery. This would not only promote more sustainable processes, but would also guarantee an intact biological activity of compounds derived from natural sources [13,14]. Solid-liquid extraction (SLE) procedures represent the most productive and sustainable approach to extract carotenoids from complex natural sources. Application of less toxic and more environmentally friendly solvents, such as ethanol, ethyl acetate and water, has already been successfully applied for the recovery of carotenoids. In particular, Mussagy *et al.* [15] demonstrated the use of greener solvents for the effective and selective recovery of carotenoids, namely β-carotene, torularhodin and torulene from *Rhodotorua glutinis (R. glutinis)* yeast [13,15]. However, the choice of *i)* extraction type, *ii)* process parameters and *iii)* the most suitable solvents remains challenging. Conventional SLE offers simplicity and low cost yet might not always allow the highest yields. On the other hand, ultrasound- (UAE) and microwave-assisted extractions (MAE) can enhance the extraction of intracellular target compounds, resulting in higher yields, minimum thermal effect on targeted compounds, higher extraction of thermo-sensitive bioactive compounds, as well as the need for lower working temperatures [13,16–18]. UAE represents an environmentally friendly method based on acoustic cavitation, which vigorously mixes the solvent and solid particles [17,19]. The latter is attributed to the formation, growth and collapse of bubbles generated by high frequency pressure waves and emerging cavitation forces. Consequently, this disrupts the cell membranes, allowing a greater penetration of the solvent and the release of intracellular compounds [3,19,20]. Recent studies [3,21] have shown the positive effect of ultrasound on the extraction enhancement of carotenoids from various food sources, resulting in higher yield and selectivity, shorter time and reduced amount of chemical hazards. When comparing UAE and MAE, these differ by the energy delivered to the sample. MAE is based on electromagnetic radiation, where cell rupture occurs with the consequent release of bioactive compounds. It represents a fast technique with less time consumption and high extraction efficiency, though with higher costs and a more difficult scale-up process.

Considering the aforementioned, the aim of this work was to compare the use of SLE, UAE and MAE techniques for the extraction of β-carotene from *R. glutinis* via bioremediation of olive mill wastewater, while using different organic solvents. Even though many studies have compared the extraction performance of these three techniques for several bioactive compounds [22,23], including β-carotene, there is still little information concerning this colorant extraction from the oleaginous red yeast [13,15,24–28]. A few studies reported the influence of different types of solvent (volatile organic solvents, ionic liquids and biosolvents) upon β-carotene extraction from *R. glutinis* [13,15,27,28] yet, these were performed using conventional SLE. Thus, there is still a lacuna regarding the influence of different techniques. Furthermore, herein the effect of a pretreatment was also studied, as well as the influence of various process parameters. Alongside the experimental analysis, a diffusion-based mathematical model was implemented and solved numerically for the first time. The implemented model represents a useful tool in terms of predicting and understanding experimental results; additionally, it can significantly decrease the experimental expenses, due to optimization of experimental parameters and process design on both industrial and laboratory scales. In this case, modelling separation/isolation of β-carotene offers investigation of mass transfer phenomena dictating rate and efficiency of the separation procedure. Additionally, it can be used for a deeper understanding of the process, its limitations and potential.

2. Materials and methods

2.1. Experimental section

2.1.1. Chemicals

β-carotene (≥97 %), purchased from Sigma Aldrich (USA), was used as a standard for HPLC analysis. The organic solvents used during the extractions were purchased from different suppliers: ethyl acetate (≥99.9 %), methanol (≥99.8 %) and acetonitrile (≥99.9 %) from Honeywell (USA), dichloromethane (≥99.5 %) and diethyl ether (≥99.5 %) from Merck (USA) and dimethyl carbonate (≥99 %) from Sigma Aldrich (USA).

2.1.2. Rhodotorula glutinis yeast fermentation

Rhodotorula glutinis DSM 70398 was purchased from Deutsche Sammlung von Mikrooganismen and Zellkulturen GmbH (DSM, Braunschweig, Germany). The yeast was firstly cultivated on agar slants with olive mill wastewater (OMW) from Olive Oil Company, Laleli, Burhaniye, Turkey, as it was used for the bioremediation of this waste. OMW was previously sterilized to ensure that biodegradation was performed only by *R. glutinis*. To this end, the pre-grown cultures on agar-OMW slant were inoculated into liquid-OMW medium, while maintaining ca. 0.03 g L^{-1} biomass. The batch experiments were carried out in 500 mL flasks filled with 200 mL culture medium at 15 °C, 150 rpm and 10 L min^{-1} airflow in orbital shaker (Shel Lab S16R-2, USA). More specific details can be found in [22]. Once the fermentation was over, the cells were subjected to centrifugation and separation by Alfa Lava Cross Flow separator at 5000 rpm. Afterwards, these were washed using deionized water, centrifuged and separated once more. Lastly to facilitate the biomass storage until usage, it was frozen with nitrogen and subjected to lyophilisation in an miVac SpeedTrap Lyo, Genevac, United Kingdom lyophilisation system under complete vacuum.".

2.1.3. Pretreatment

Pretreatment was carried out by grinding the lyophilized *R. glutinis* for 2 min using pestle and mortar, until a powder was formed.

2.1.4. Scanning electron microscopy (SEM) analysis

R. glutinis yeast was structurally characterized using scanning electron microscopy, FE-SEM SUPRA 35 VP, Carl Zeiss, Jena, Germany. SEM analysis was carried out to obtain information about the size and/or morphology of the cell structure before and after pretreatment and for the mathematical modelling (the estimated size of yeast particles represents a relevant model input).

2.1.5. Solid-liquid extraction

SLE of β-carotene from *R. glutinis* yeast was carried out in 100 mL reactors on a Carousel 6 Plus Reaction Station, Radleys, United Kingdom, at 30 °C and under constant stirring rate (200 rpm). Here, 1 g of lyophilized *R. glutinis* yeast was mixed with 20 mL of organic solvent (ethyl acetate, diethyl ether, dichloromethane or dimethyl carbonate) and subjected to the chosen conditions. Solvent selection followed a previous study [28], where 10 organic solvents were screened, with the most promising ones being evaluated here . Samples of 1 mL were collected at different times, depending on the parameter evaluated, filtered through 0.2 μm pore size cellulose filter and analysed through HPLC, as described in section 2.1.10. When the extraction time was studied, samples were taken at 1, 3, 5, 10, 20, 30 and 60 min at 30 °C, using ethyl acetate as an extracting solvent; technique-wise, the samples were taken at 5, 10 and 20 min at 30 °C for all 4 organic solvents (ethyl acetate, dichloromethane, diethyl ether, dimethyl carbonate). Lastly, when the extraction temperature was evaluated, SLE was carried out at 30 and 50 °C, with the samples being taken at 10, 20, 30 and 60 min, using ethyl acetate as a solvent.

2.1.6. Ultrasound-assisted extraction

UAE was carried out in an open rectangular ultrasonic bath (Cole Parmer SS, 24.1 × 14 × 15.2 cm internal dimensions), that allows a stable ultrasonic field. The maximum working power was 220 W at a frequency of 40 KHz and the temperature set to 30 °C. The process remained the same, weighing 1 g of lyophilized *R. glutinis* yeast into a 250 mL reactor and adding 20 mL of each organic. The samples were collected at different times and temperatures, depending on the parameter evaluated, filtered through 0.2 μm cellulose pore size filter and quantified using HPLC, as described in section 2.1.10. Similar to SLE, for optimal time selection, samples were taken at 1, 3, 5, 10, 20, 30 and 60 min at 30 °C, using ethyl acetate. When comparing extraction techniques, samples were taken at 5, 10 and 20 min at 30 °C, comparing all 4 organic solvents (ethyl acetate, dichloromethane, diethyl ether, dimethyl carbonate). Lastly, for temperature determination, UAE was carried out at 30 and 50 °C and the samples were taken at 10, 20, 30 and 60 min, using ethyl acetate. Considering the overall working time range from 1 to 60 min, the energy consumption using this ultrasound device varied from 0.66 Gj m^{-3} to 39.6 Gj m^{-3}. This was determined according to Nogueira and co-workers [29].

2.1.7. Microwave-assisted extraction

A domestic microwave oven (Candy CMXW 20 DW) was used for MAE, capable of operating at a maximum power of 800 W at a frequency of 2450 MHz. The microwave was modified, removing the turn table and selecting the program of desired power (following the manual's instructions). The latter was set to 10 % of maximum power (80 W) in order to maintain a stable temperature of 30 °C. A 100 mL Radleys Carrousel reactor containing lyophilized *R. glutinis yeast* (1 g) and organic solvent (20 mL) was placed in the microwave oven cavity. As for all previous experiments, the samples were collected at different times, depending on the parameter evaluated, filtered and analyzed on HPLC (described in section 2.1.10). For time duration evaluation, samples were taken at 1, 3, 5, 10, 20, 30 and 60 min at 30 °C, using ethyl acetate; whereas technique-wise, samples were taken at 5, 10 and 20 min at 30 °C, using all 4 organic solvents (ethyl acetate, dichloromethane, diethyl ether, dimethyl carbonate). MAE was not considered to study the extraction temperature as it was not possible to maintain a stable temperature above 30 °C. Considering the overall working time range from 1 to 60 min, the energy consumption using this microwave device and at the specified programme varied from 0.24 Gj m^{-3} to 14.4 Gj m^{-3}. This was determined according to Nogueira and co-workers [29].

2.1.8. Optimization of the experimental conditions

The experimental conditions, namely time and temperature, were further optimized using ethyl acetate. For the temperature optimization, SLE and UAE were carried out at 30 and 50 °C and samples were taken at 10, 20, 30 and 60 min. Regarding time optimization, SLE, UAE and MAE were carried out and the extraction was prolonged to 60 min. Here, samples were taken at 1, 3, 5, 10, 20, 30 and 60 min, being then filtered and analysed on HPLC (described in section 2.1.10).

2.1.9. HPLC analysis

Carotenoid concentrations were determined using an ultra-high performance liquid chromatography, UHPLC Fisher Thermo Scientific Ultimate 3000 and a Kinetex C18 column (5 μm, 100 mm × 4.6 mm). Mobile phase was formed by acetone (A) and water (B) in a gradient method [30]: 0 – 5 min 80 % A, 5 – 10 min 80 % A, 10 – 15 min 86.7 % A, 15 – 20 min 93.3 % A, 20–35 min 100 % A, 35 – 40 min 80 % A, 40 – 45 min, 80 % A. Column oven temperature was 30 °C with a flow rate 1 mL min^{-1} and the injection volume was 15 μL. The detection was performed at a wavelength of 450 nm. It is important to note that all experiments were performed in triplicate, hence all the results presented correspond to their average. Standard deviation was also determined and presented.

2.2. Modelling section

The developed modelling framework for isolation of β-carotene involves two essential steps. The first step is to model the diffusion-dictated mass transfer within the yeast particle, which is characterized by the diffusivity coefficient (denoted as *D)*. The second considered step in the model is the convective mass transfer of β-carotene molecules from the yeast particles to the solvent. Due to the differences in the characteristic times of the two individual steps – diffusion being the limiting process – the model can be simplified in terms of the convective mass transfer at the surface of the yeast particles.

Considering the first part of the designed model – diffusion step, a known second order partial differential equation (PDE) describing diffusion of a component in a solid particle can be derived (Eq. (1)). For this, application an ideal spherical shape of the yeast particles with radius R was assumed, which was in turn supported by photos taken with a scanning electron microscope (SEM). Hence, the governing model equation (*i.e.*, the diffusion equation) shown in Eq. (1) is set in the spherical coordinate system [31].

$$\frac{dc(r,t)}{dt} = D\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial c(r,t)}{\partial r}\right) \tag{1}$$

The spherical yeast particle is assumed to be effectively non-porous, which is a simplified view of the otherwise porous yeast structure. In order to solve Eq. (1), the corresponding initial (Eq. (2)) and two boundary conditions (Eqs. (3) and (4)) for this diffusion problem are specified, which altogether render the problem as well-posed and thus solvable.

$$c(t=0,r) = c_o \tag{2}$$

$$\frac{\partial c(t,r=0)}{\partial r} = 0 \tag{3}$$

$$c(t,r=R) = 0 \tag{4}$$

In Eqs. (1)–(4), variable c denotes the concentration of β-carotene in the yeast, which is dependent on time, t, and radial position, r. The latter can take values from 0 to the total radius R. D is the diffusion coefficient, which among other quantities, is assumed to be time and position independent and thus considered as constant during the extraction process. Furthermore, additional simplifications were introduced in the model, namely, the concentration of β-carotene in the solvent is assumed to be much smaller than the concentration in the starting yeast material, since the volume of the yeast is much smaller than the volume of the solvent used in the extraction – thus the concentration of β-carotene is set to be zero at the surface of the spherical yeast particle at $r = R$, as suggested in Eq. (4). Additional considerations and derivation regarding this simplification can be found in the SI (*cf.* Fig. S1-S4).

Numerous analytical and numerical approaches for solving PDE-s of similar types are developed in literature; in this work the finite difference method approach was used as a numerical tool – further details about discretized model equations are shown in SI [31,32]. In addition to the numerical solution, a solution of Eq. (1) in the form of the Fourier series is presented in Eq. (5). The exact solution was also implemented to verify the results of the numerical simulation; however, the derivation of the analytical solution is explained elsewhere [31].

$$c(t,r) = c_0 + (c_0 - c(t,R))\left(1 + \frac{2R}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\sin\left(\frac{n\pi r}{R}\right)\exp\left(-\frac{Dn^2\pi^2 t}{R^2}\right)\right) \tag{5}$$

In any case, regardless of the solution approach – numerical or exact, the instantaneous concentration profile inside a single yeast particle is to be translated to the concentration of β-carotene in the solvent, which is again described in detail in the Supporting Information (SI). Besides, a thorough explanation of numerical and analytical solutions of the model equations can also be found in SI, together with the effects of individual process parameters on the evolution of concentration.

3. Results and discussion

3.1. Pretreatment effect

Pretreatment of raw material plays an important role for extraction improvements [33]. Grinding enhances the disruption of the cell walls and membranes, facilitating the release of intracellular compounds [34]. Herein, the target intracellular compound is β-carotene. SEM analysis of *R. glutinis* yeast has been performed before and after grinding to show the chemical disruption of cells, as presented in Fig. 1. The comparison of cells in Fig. 1.A) and 1.B) clearly shows the change of shape, from perfect round cells to suppressed, grinding cells, where disruption of the cell wall occurred.

In order to evaluate the influence of the pretreatment on the extraction of β-carotene, SLE was chosen due to its simplicity. Fig. 2 presents the obtained results while using ethyl acetate, dimethyl carbonate, diethyl ether and dichloromethane as the extraction solvents. These results show a different behaviour according to the solvent used, being the main difference observed at the beginning of the extraction, namely around 5 min. Here, grinding significantly increases the β-carotene concentration in dimethyl carbonate from 18 ± 1 μg g^{-1} yeast to 26 ± 2 μg g^{-1} yeast and in diethyl ether from 16.0 ± 0.4 μg g^{-1} yeast to 20.0 ± 0.2 μg g^{-1} yeast. Considering 20 min of extraction, that is when the extraction seems to have reached a plateau, the concentration before and after grinding in each solvent varies less: in ethyl acetate from 38 ± 2 μg g^{-1} yeast to 42 ± 2 μg g^{-1} yeast, in dimethyl carbonate from 29 ± 4 μg g^{-1} yeast to 35 ± 3 μg g^{-1} yeast, in diethyl ether from 19 ± 1 μg g^{-1} yeast to 21 ± 1 μg g^{-1} yeast and in dichloromethane from 11.9 ± 0.3 μg g^{-1} yeast to 13 ± 2 μg g^{-1} yeast. Due to the obvious decrease in cell size and morphology (as shown by SEM), simplicity of grinding and the ~ 10 % difference in concentration for all solvents, a pretreatment step was applied in the next experiments.

Similarly, the implemented diffusion model is capable of accounting for the effect of pretreatment on the extraction profile based on the yeast particle size despite the fact that the perfect spherical shape of the particles is a simplification, which is not observed in reality (see Fig. 1). In this sense, the pretreatment of the material affects the distribution size of the yeast particles resulting in a larger specific surface area, since the average particle size is decreased. In the model, a monodisperse distribution of yeast particles is assumed with a particle size R – at the same yeast mass. When applying the pretreatment, a smaller R results in a higher number of particles and thus increased surface available for solvent to wet the surface of the yeast particles. Since the model is simplified to not account for the porosity of the particles, but only models the mass transfer at the surface of the spherical particles, the effective surface area is responsible for the faster extraction in the case of the grinded material (Fig. 2B) as opposed to the ungrinded material (Fig. 2A).

Furthermore, both figures show 4 sets of lines corresponding to the model and 4 sets of experimental points, where color coding is used to denote different types of solvent used for the extraction. Results show that the net amount of β-carotene, which can be extracted using a particular solvent, depends on the type of solvent. Extraction capabilities of different solvents are related to their physical and chemical properties, such as the solubility of β-carotene, density, viscosity, wetting characteristics and the ability to penetrate the pores of the yeast particles. However, in the model, a simplified approach is used to model all these contributions by estimating an effective solvent-dependent initial concentration, c_0, which can represent the maximum possible concentration of the extracted β-carotene upon completion of extraction. This value only depends on the type of solvent used, but is independent of the pretreatment, temperature, extraction technique and other process parameters.

Additionally, due to a lack of literature information, diffusivity coefficient, D, is another important unknown process variable in the model, which generally depends on temperature. Furthermore, in this simplified approach it was also assumed that the diffusivity coefficient depends on the type of extraction, since using an assisted extraction procedure (*i.e.,* MAE and UAE) affects the mobility of the species and thus its diffusivity coefficient. Table 1 presents the estimated parameters of the model, namely the effective initial concentration and the effective diffusivity coefficient for the plots shown in Fig. 2. Sufficiently good agreement between the model and the experimental measurements is again observed, confirming relevant functional dependencies of the unknown process parameters on the operating conditions.

3.2. Extraction duration

To facilitate the optimization of the extraction duration, ethyl acetate was used as it is the most performant solvent, being the results displayed in Fig. 3. SLE, UAE and MAE were analysed during 60 min, with samples being taken at 1, 3, 5, 10, 20, 30 and 60 min. These results show two different trends, *i.e.* up to 20 min of extraction, there is an exponential increase in the β-carotene concentration, while after this, a plateau is reached. In more detail, the techniques follow the increasing trend in the first 20 min of extraction: MAE (41 ± 3 μg g^{-1} yeast) < SLE (42 ± 2 μg g^{-1} yeast) < UAE (45 ± 3 μg g^{-1} yeast). After SLE at 20 min, the concentration of β-carotene resulted in 42 ± 2 μg g^{-1} yeast, whereas at 60 min it resulted in 42 ± 2 μg g^{-1} yeast. The same negligible difference was observed for UAE, where the concentration resulted in 45 ± 3 μg g^{-1} yeast to 45 ± 1 μg g^{-1} yeast. MAE showed a slightly higher deviation in β-carotene concentration between 20 and 60 min, namely 41 ± 3 μg g^{-1} yeast and 45 ± 2 μg g^{-1} yeast, though still not significant. Overall, the difference between 20 and 60 min is minor for all techniques, proving that 20 min of extraction are enough to reach a plateau. Goula *et al.* [3] performed a similar study using UAE and reported that the extraction yield increased rapidly from 10 to 30 min, whereas until 60 min, it slowed down. The steady concentration was due to the extraction being presented in two stages: at first there is a rapid stage, in which the solvent penetrates the cell walls and membranes, allowing the

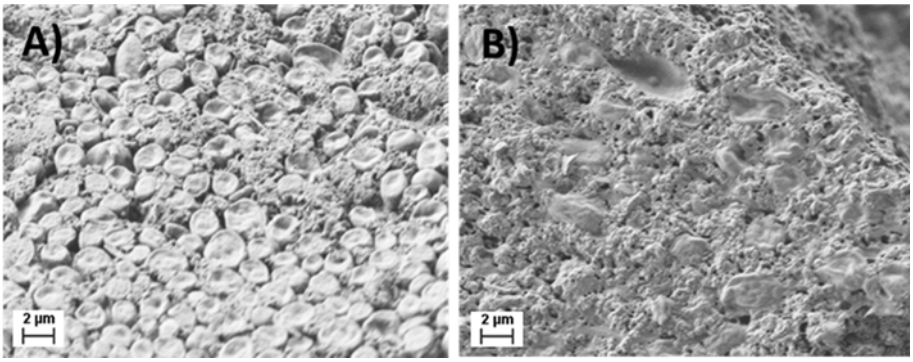

Fig. 1. SEM images of *R. glutinis*: **A)** before grinding; **B)** after grinding.

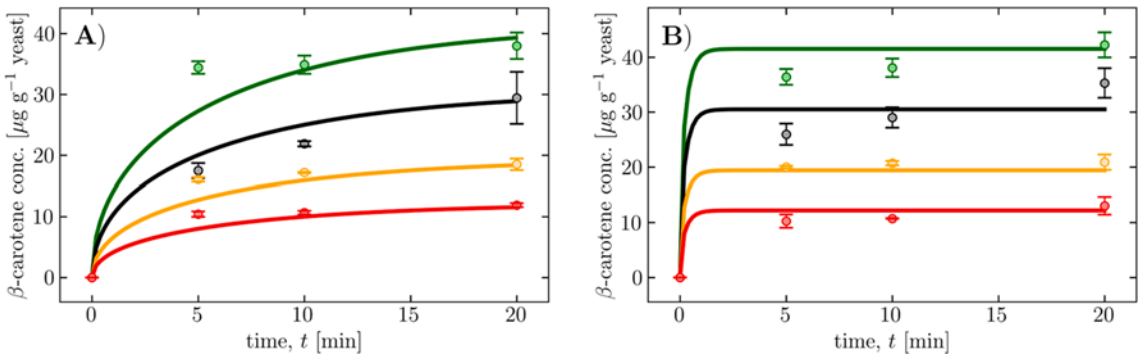

Fig. 2. Influence of a pretreatment step during the β-carotene extraction from *R. glutinis* using SLE and different solvents: ethyl acetate (green), dimethyl carbonate (black), diethyl ether (orange) and dichloromethane (red). **A)** using ungrinded *R. glutinis*. **B)** using grinded *R. glutinis*. The points represent the experimental extractions whereas the lines correspond to the modelling developed here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Reported values of the estimated parameters effective initial concentrations for different types of solvent, the estimated diffusivity coefficient and the model performances for SLE when assessing the effect of pretreatment.

Pretreatment	Yeast particle size (radius), R [μm]	Estimated effective diffusivity coefficient, D [$\mu m^2/s$]	Type of solvent	Estimated effective initial concentration, c_0 [μg/g]	Coefficient of determination, R^2 [-]
Ungrinded	2.5	1.29×10^{-3}	Ethyl acetate	41.5	0.944
			Dimethyl carbonate	30.5	0.965
			Diethyl ether	19.4	0.945
			Dichloromethane	12.2	0.931
Grinded	1		Ethyl acetate	41.5	0.967
			Dimethyl carbonate	30.5	0.937
			Diethyl ether	19.4	0.987
			Dichloromethane	12.2	0.934

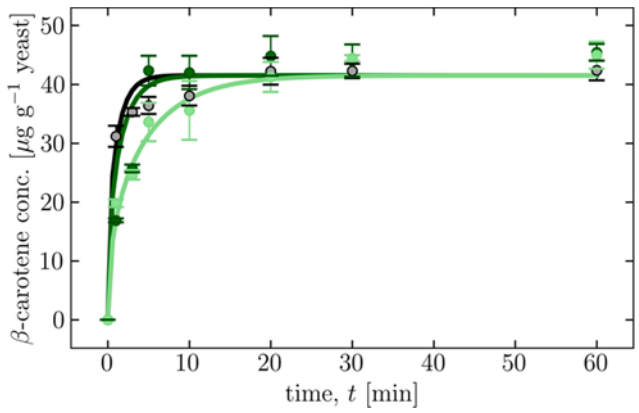

Fig. 3. Influence of the extraction time while using SLE (black), UAE (dark green) and MAE (light green) with ethyl acetate at 30 °C for 60 min. Analysis was carried out for 5, 10, 20, 30 and 60 min samples. The points represent the experimental extractions whereas the lines correspond to the modelling developed here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

release of the compounds. Afterwards, in a second stage, there is the external diffusion of soluble compounds through the pores. Identical results were reported by Purohit *et al.* [35], that presented a 50 % and a 10 % extraction yield increased from 10 to 30 min and from 30 to 50 min, respectively. In the last 10 min of extraction, the β-carotene extraction yield decreased and was attributed to the longer periods of ultrasonic treatment, possibly leading to the colorant degradation. Hiranvarachat *et al.* [36] also noticed the decrease in β-carotene and total carotenoids content when prolonging time during MAE. Though this is not exactly observed in our case, but a steady state. This might be due to two reasons: *i)* the extraction is complete or *ii)* the solvent is saturated. Either way, extending the extraction over 20 min is not useful so this time was chosen owing to economic and ecological reasons.

When the experimental data is compared with the modelling results, both seem to be in accordance, as shown in Fig. 3. However, at a closer look at the results in Table 2, it can be observed that the SLE diffusivity coefficient is the largest, even though this technique represents the benchmark compared to other two techniques, which facilitate ultrawaves or micro-waves to enhance the extraction process. The plausible explanation relies in the fact that SLE was carried out in a magnetic stirred vessel, while for UAE and MAE this was not the case. Thus, it led to more turbulent mixing conditions than MAE and UAE, where no mixing was employed. Consequently, the SLE effective D is higher than in the remaining techniques.

3.3. Comparison of different techniques for β-carotene extraction

SLE, UAE and MAE were carried out with pure solvents in order to evaluate their performance for β-carotene extraction from *R. glutinis* yeast. These results are displayed in Table 3. Depending on the different extraction techniques, β-carotene concentration increased in the following trend: MAE < SLE ≈ UAE, for most solvents. The main difference is observed after the first 5 min in ethyl acetate, where the concentration of β-carotene after UAE resulted in 42 ± 3 μg g^{-1} yeast in comparison to 36 ± 1 μg g^{-1} yeast of SLE and 34 ± 7 μg g^{-1} yeast of MAE. At 20 min, the concentration after UAE, SLE and MAE increased to 45 ± 3 μg g^{-} yeast, 42 ± 2 μg g^{-1} yeast and 41 ± 3 μg g^{-1} yeast, respectively. These results show the highest recovery of β-carotene with UAE, though with a negligible difference in comparison with SLE. In dimethyl carbonate, UAE enhanced the β-carotene concentration after 5 min, resulting in 28 ± 2 μg g^{-1} yeast in comparison to SLE (26 ± 2 μg

Table 2
Reported values of the estimated parameters, namely the effective initial concentrations for different types of solvent, the estimated diffusivity coefficient and the corresponding model performances.

Extraction technique	Yeast particle size (radius), R [μm]	Estimated effective diffusivity coefficient, D [$\mu m^2/s$]	Type of solvent	Estimated effective initial concentration, c_0 [μg/g]	Coefficient of determination, R^2 [-]
SLE	1	1.29×10^{-3}	Ethyl acetate	41.5	0.964
UAE		8.95×10^{-4}			0.837
MAE		3.12×10^{-4}			0.910

Table 3
Influence of different solvents: ethyl acetate, dimethyl carbonate, diethyl ether, dichloromethane, and techniques: SLE, UAE, MAE upon β-carotene extraction from *R. glutinis*. All extraction techniques were carried out at the same extraction conditions, except SLE, which was subjected to stirring (200 rpm).

Technique	Solvent	Concentration (μg g^{-1} yeast)		
		Time (min)		
		5	10	20
SLE	Ethyl acetate	36.4 ± 1.45	38.1 ± 1.68	42.2 ± 2.29
	Dimethyl carbonate	26.0 ± 1.94	29.0 ± 1.85	35.3 ± 2.70
	Diethyl ether	20.0 ± 0.23	20.7 ± 0.39	20.9 ± 1.39
	Dichloromethane	10.3 ± 1.20	10.7 ± 0.06	13.0 ± 1.60
UAE	Ethyl acetate	42.3 ± 2.54	42.0 ± 2.83	44.8 ± 3.38
	Dimethyl carbonate	28.4 ± 2.39	31.7 ± 1.95	35.2 ± 2.08
	Diethyl ether	19.0 ± 0.08	20.7 ± 0.29	21.8 ± 0.66
	Dichloromethane	11.5 ± 1.95	13.3 ± 0.10	14.4 ± 0.54
MAE	Ethyl acetate	33.6 ± 7.16	35.6 ± 5.01	41.3 ± 2.55
	Dimethyl carbonate	12.6 ± 0.35	18.6 ± 1.92	25.3 ± 1.91
	Diethyl ether	20.2 ± 2.10	22.0 ± 1.88	23.8 ± 2.07
	Dichloromethane	12.6 ± 3.40	13.8 ± 0.41	13.9 ± 1.24

g^{-1} yeast) and MAE (12.6 ± 0.4 μg g^{-1} yeast), whereas at 20 min the concentration was almost equal for UAE and SLE, namely 35 ± 2 μg g^{-1} yeast and 35 ± 3 μg g^{-1} yeast, whereas for MAE (25 ± 2 μg g^{-1} yeast) it was considerably lower. In diethyl ether, UAE did not have an enhanced effect on β-carotene extraction, as it resulted in ~ 20 μg g^{-1} yeast at 5 and 20 min, being identical to SLE. In contrast, the concentration range in dichloromethane varied from 10 ± 1 μg g^{-1} yeast after 5 min of SLE to the highest observed after 20 min of UAE, 14 ± 1 μg g^{-1} yeast. Interestingly, dichloromethane was the only solvent where microwave radiation enhanced the release of β-carotene after 5 min when compared to the remaining techniques.

Overall, these results show that UAE and SLE performed almost identically and that MAE is the least promising technique for β-carotene extraction at the chosen conditions yet, this is probably due to the use of domestic equipment. It is also known that MAE's main obstacle is the rapid increase in temperature, which can cause the termination of extraction too fast due to the boiling of solvent. This leads to not sufficiently diffused compounds and thus lower extraction yields [36–38]. Herein, preliminary studies were carried out to select the right potency to keep the temperature constant, though some oscillations might occur. Chutia *et al.* [17] compared UAE, MAE and the conventional SLE method and showed that the recovery of carotenoids was significantly higher after UAE than MAE. Authors emphasized the effect of energy consumption in an extraction process and explained how it affects the power dissipated to the sample medium and further to the system type, input power, viscosity of solvent and temperature. Similar studies comparing the different techniques have also been carried out by Chuyen *et al.* [38,39]. Once again, MAE executed at 120 W resulted in lower carotenoid yield than conventional SLE. On the other hand, UAE at 200 W performed the best, which may be because of greater cell wall disruption of initial material. When increasing ultrasound power, degradation can happen and it can result in lower extraction yields. The most important advantage of UAE and MAE is the time reduction, as the yield does not differ that significantly in comparison to conventional SLE. These extraction techniques have also been compared for the isolation of antioxidants, dyes and pigments [40]. Sivakumar *et al.* [41] extracted the natural beetroot dye, using UAE. They compared it with a conventional magnetic stirring process and showed a significant improvement in the extraction efficiency while using UAE. Shirsath *et al.* [40] reviewed the extraction of natural products, such as essential oils, aromatic compounds, sugars, proteins, etc. Authors emphasized that ultrasound can effectively be used to enhance the isolation of aforementioned natural products as it helps with the cell wall disruption due to cavitation, enabling the release of compounds from the cells [3,14].

Considering the performance of the solvent, it was verified that the solvent's efficiency is independent of technique and follows the tendency: dichloromethane < diethyl ether < dimethyl carbonate < ethyl acetate. Even though the trend was the same for all techniques, the maximum yield achieved varied. The highest concentration obtained was achieved with ethyl acetate, which evidently facilitates access to the intracellular yeast cell environment, as already reported by us [28] and other authors [15,42]. Therefore, it was chosen for further optimization of extraction conditions. Modelling was also carried out to predict the experimental results (Table S1 and Fig. S5) and shows a good accordance between the experimental and theoretical data.

3.4. Effect of extraction temperature

As aforementioned, ethyl acetate was the most performant solvent, thus being used for further studies. In order to evaluate the temperature influence over the β-carotene extraction, 30 and 50 °C were selected. However, it should be highlighted that this was only performed for SLE and UAE since it was not possible to maintain a stable temperature using microwave at 50 °C. These results are presented in Fig. 4, in which Fig. 4. A) and B) represent SLE and UAE, respectively, at both temperatures, while C) and D) directly compare the influence of the technique at a fixed temperature, *i.e.* 30 and 50 °C, respectively. It was evident that, independently of the method used, the temperature rise led to some improvements in β-carotene extraction. When considering SLE, β-carotene concentration increased from 42 ± 2 μg g^{-1} yeast to 50 ± 2 μg g^{-} yeast with temperature, which represents a 15 % increase. A similar behaviour was observed for UAE, with a concentration increase from 45 ± 1 μg g^{-1} yeast to 51 ± 2 μg g^{-1} yeast, accounting for a 10 % increase. Purohit *et al.* [35] came to a similar conclusion, when authors increased the temperature of β-carotene extraction from 20 to 50 °C in an ultrasonic bath, the extraction yield increased only ~ 20 %. Moreover, Umair *et al.* [43] performed a response surface methodology with central-composite design study to improve the extraction of various carotenoids (β-carotene, lutein and lycopene) from carrot pomace, and concluded that the optimal temperature for β-carotene extraction was 29 °C. Under all the optimal conditions, it was possible to extract 14.9 ± 0.4 μg g^{-1} pomace.

The effect of temperature on the extraction was expected to primarily influence the dynamics of extraction, since the diffusivity coefficient is temperature dependent, as evident in Table 4. Additionally, temperature also affects the viscosity of the solvent, which can have an additional effect on the diffusivity coefficient, as expected when using the Einstein-Stokes relation (further considerations regarding the effect of temperature can be found in the SI, *cf.* Fig. S6). Experimental results correspond to this conclusion, since in the initial phases of the extraction, a higher

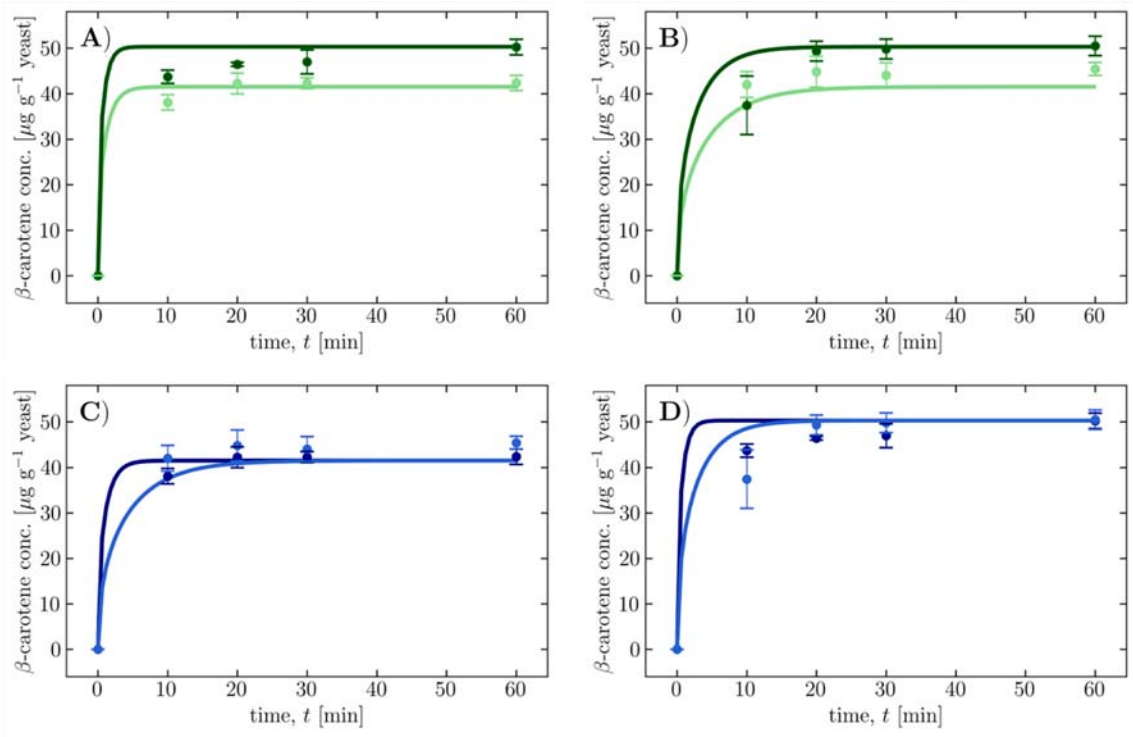

Fig. 4. Optimization of temperature conditions for SLE and UAE in ethyl acetate at 30 and 50 °C: **A)** SLE at 30 (light green) and 50 °C (dark green), **B)** UAE at 30 (light green) and 50 °C (dark green), **C)** SLE (dark blue) and UAE (light blue) at 30 °C, **D)** SLE (dark blue) and UAE (light blue) at 50 °C. The points represent the experimental extractions whereas the lines correspond to the modelling developed here. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Reported values of the estimated parameters effective initial concentrations for different types of solvent, estimated diffusivity coefficient and model performances when assessing the effect of temperature.

Extraction technique	Yeast particle size (radius), R [μm]	Temperature, T [°C]	Effective diffusivity coefficient, D [$\mu m^2/s$]	Estimated effective initial concentration, c_0 [μg/g]	Coefficient of determination, R^2 [-]
SLE	1	30	1.29×10^{-3}	41.5	0.990
		50	2.00×10^{-3}	50.3	0.961
UAE		30	1.06×10^{-3}	41.5	0.963
		50	4.84×10^{-3}	50.3	0.933

operational temperature leads to a higher net extracted mass of β-carotene (Fig. 4A and 4B). However, experimental data also suggests that, at a higher temperature not only the rate of extraction, but also the total amount of extracted β-carotene is affected. Therefore, the effective initial concentration was again estimated using experimental data for the experiments performed at a higher temperature. It should be here stressed that the diffusivity coefficient and its temperature-dependency was not re-estimated, but was calculated directly from the Stokes-Einstein relation (*cf.* SI for further insight).

Furthermore, and in order to facilitate the analysis of different techniques, Fig. 4.C) and D) compare SLE and UAE at a fixed temperature. Here, it is clearly seen that technique does not play such an important role.

Overall, a proof of concept study comparing the efficiency of SLE, UAE and MAE upon β-carotene extraction from *R. glutinis* was here reported. Among these techniques, SLE and UAE have displayed similar results, yet, due to its simplicity, SLE represents the easiest technique for scale – up implementation. Moreover, when solvents are compared, ethyl acetate was the most efficient solvent. This is of particular interest in terms of environmental awareness, as it is considered a greener solvent. Hence, showing a promising feasibility for process scale-up.

A microbial biorefinery using *R. glutinis* has not been implemented yet. Nonetheless, it has immense potential as *R. glutinis* can be used for the bioremediation of several agricultural and food wastes (some of which present a considerable toxicity like OMW), while also producing several high-added value compounds (carotenoids, lipids, enzymes) [44]. Mussagy *et al.* [26,45] reported the efficient production of different carotenoids by *R. glutinis* at lab scale and in a 5 L stirred – tank bioreactor, showing the high feasibility in scaling-up the upstream process of this microbial biorefinery. On the other hand, the downstream process of oleaginous yeasts is more challenging, owing to the high costs and unfeasibility of some tasks [24], thus requiring more efforts. Nevertheless, the industrialization of this microbial biorefinery could become a reality in the near future when key factors are considered and balanced, namely productivity, efficiency, recyclability, cost-efficiency and sustainability [44].

4. Conclusion

In this work, a comparison of different extraction techniques for the recovery of intracellular β-carotene from *R. glutinis* was established, using various solvents. It was proven that ethyl acetate was the best solvent considering its higher aptitude to interact with the yeast cell walls and membranes, and release and solubilize β-carotene. Technique-wise, SLE and UAE performed similarly yet, due to its simplicity and cost SLE seems the most promising technique for β-carotene isolation. To

gain additional insight into the investigated extraction process, a diffusion-based mathematical model was derived and solved both numerically and analytically, for the first time. The model can, despite the many simplifications introduced in the model equations, mechanistically describe the effects of various process parameters, such as the pretreatment, the temperature and the extraction technique on the progress and performance of the extraction.

CRediT authorship contribution statement

Lucija Hladnik: Data curation, Formal analysis, Writing – original draft. **Filipa A. Vicente:** Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Andraž Košir:** Data curation, Formal analysis, Software, Writing – original draft. **Miha Grilc:** Validation, Formal analysis, Supervision, Writing – review & editing. **Blaž Likozar:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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

All data has been presented in the manuscript and supporting information.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2023.123293.

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