

An eco-friendly and up-scalable approach to extract canthaxanthin from yeast cells

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Abstract

Canthaxanthin is a naturally occurring ketocarotenoid pigment present in plants, algae, bacteria and some fungi. In addition to its coloring role, canthaxanthin has an excellent antioxidant activity, thus having additional market demands in the feed, food, cosmetic and pharmaceutical industries. Canthaxanthin can be directly isolated from its natural source or produced by chemical synthesis, but these strategies either result in low yields, or use hazardous solvents, respectively. Therefore, the biosynthesis of canthaxanthin using microbial cell factories is becoming an advantageous alternative. Furthermore, microbial synthesis represents an economic and sustainable approach as it enables the use of agriculture and industrial wastes as substrates. In this work, the extraction, recovery and purification of canthaxanthin from modified yeasts using food grade solvents and up-scalable methodologies was studied. The resulting canthaxanthin-enriched extract was characterized (UV-Vis, PXRD and SEM) and quantified (HPLC), resulting in a canthaxanthin purity of 43.7% (w/w).

Keywords: Food pigments; Canthaxanthin; Biotechnological production; Extraction; Scalable methodologies, Structural characterization

1. Introduction

Carotenoids are a group of more than 1100 pigmented terpenoids (Yabuzaki 2017) exhibiting red, yellow, orange and purple colors (Maoka 2020), naturally synthesized by photosynthetic microorganisms, as well as by some non-photosynthetic bacteria and fungi (Britton 1993; Rebelo et al. 2020). As colorants, carotenoids have been widely used by food and cosmetic industries due to the importance of visual appearance in the acceptance and commerciality of a myriad of products. In addition, their economic value is further increased due to health benefits in animals and humans, which obtain carotenoids through their diet, as they cannot *de novo* synthesize them (Rebelo et al. 2020). Amongst a plethora of biological properties, carotenoids display photoprotector, antioxidant, anti-inflammatory and immunomodulatory activities (Maoka 2020), thus having gained a remarkable significance in feed, nutraceutical, and pharmaceutical industries (Rebelo et al. 2020).

Carotenoids can be divided into two main groups: carotenes and xanthophylls. Whilst carotenes, such as β -carotene or lycopene, are composed only of hydrogen and carbon atoms, xanthophylls such as astaxanthin and canthaxanthin also contain oxygen atoms as part of a functional group (*e.g.*, aldehyde, hydroxyl, carbonyl, carboxylic, epoxide), which is believed to be related with the higher bioavailability of the latter carotenoid group (Rebelo et al. 2020). Within xanthophylls, canthaxanthin (β,β -carotene-4,4'-dione) is a ketocarotenoid with two ketone groups (Esatbeyoglu and Rimbach 2017) with a strong red-orange color, widely used as a feed additive for poultry and ornamental fish and birds. Although some concerns about canthaxanthin with some adverse effects on human health, namely canthaxanthin retinopathy (Challiol et al. 2018; Sujak 2009) this ketocarotenoid has been considered safe by the European Food Safety Authority (EFSA) and by the Food and Drug Administration (FDA) as food and feed additive (EFSA 2010; FDA 2011; EFSA 2014), with a concentration of 0.03 mg/kg bw/day being established as an acceptable daily intake (ADI). Canthaxanthin is a strong antioxidant molecule with the capacity to scavenge reactive oxygen species (ROS) and quench singlet oxygen ($^1\text{O}_2$), thus preventing skin ageing, lipid peroxidation, and DNA and protein oxidative damage (Esatbeyoglu and Rimbach 2017). Additionally, canthaxanthin has been reported to enhance the immune response and possess anticancer activity (Bendich and Shapiro 1986; Palozza 1998; Esatbeyoglu and Rimbach 2017). Due to its health benefits and increasing demand in cosmetics and animal feed applications, canthaxanthin market size is rapidly growing and has been valued, in 2023, at USD 135 million. Indeed, a detailed market analysis revealed that canthaxanthin is expected to have a compound annual growth rate (CAGR) of

2.6%, thus resulting in a market size of USD 444 million in 2032 (“Canthaxanthin Market Trends | Growth Outlook 2024-2032” 2024).

Much like other carotenoids, canthaxanthin can be directly isolated from its natural sources or chemically synthesized. However, canthaxanthin is produced in low concentrations by its natural sources, which usually results in an inefficient and time-consuming extraction process. Indeed, this is the underlying reason why the vast majority of commercially available canthaxanthin is chemically synthesized. At least three synthetic routes have been described to produce canthaxanthin, with different levels of complexity and yield, but inevitably resorting to the use of hazardous solvents, such as dimethylformamide, dichloromethane, and chloroform, leading to potentially toxic wastes (Mata-Gómez et al. 2014; Rebelo et al. 2020).

The biotechnological production of canthaxanthin using genetically modified microbial cell factories (Scaife et al. 2012; Papp et al. 2013; Ravaghi et al. 2017; Gao et al. 2020; Chen et al. 2022; Naz et al. 2021) is a promising alternative to circumvent these limitations, allowing higher yields of the pigment through a more sustainable procedure, due to the ability of microorganisms to use nontoxic and affordable substrates, and even wastes from other industrial processes (Mata-Gómez et al. 2014). Despite its indisputable advantages, large-scale biotechnological production of carotenoids is hindered by the lack of an effective extraction methodology, namely regarding the choice of lysing methodologies and the recovery solvent (Mata-Gómez et al. 2014). Due to their lipophilic nature, carotenoids are commonly recovered with organic solvents such as chloroform, acetone, and methanol, individually or in combination (Machmudah and Goto 2013; Mercadante et al. 2017; Scaife et al. 2012). Despite canthaxanthin being highly soluble in chloroform (Qian et al. 2009), its use in food and pharmaceutical related industries is limited by environmental and health related issues (ATSDR 2002), which demand the use of other solvents. Although canthaxanthin has already been extracted from other microorganisms (Krupa et al. 2010; Scaife, Ma, and Armenta 2012), different structural cell wall compositions imply distinct extraction approaches, and thus published results cannot be directly applied. Thus, there is a need to find the best extraction conditions for yeast, to up-scale the process to greener, sustainable, and economically feasible levels.

In line with this objective, this work reports the optimization of the extraction conditions of canthaxanthin co-produced with β -farnesene, an isoprenoid with biofuel potential, by genetically engineered *Saccharomyces cerevisiae* (Paddon et al. 2018; Benjamin et al. 2016), demonstrating the feasibility of using an environmentally friendly approach using less harmful solvents and scalable methodologies to produce value-added compounds. Since both β -

farnesene and canthaxanthin can be synthesized in *S. cerevisiae* by the mevalonate biosynthetic pathway (Tsegaye et al. 2023), co-production of both molecules could become a good strategy for the valorization of the spent yeast from the β -farnesene production, by further coproducing a high-value carotenoid.

After assessing the efficiency of different feasible extraction solvents and the performance of different hydrolytic enzymes, yeast cell disruption was promoted by combining autolysis and enzymatic hydrolysis procedures, followed by canthaxanthin solvent extraction. Furthermore, a purification methodology was conducted to obtain a final canthaxanthin-enriched extract. To our knowledge, this is the first time that canthaxanthin is not only produced but also extracted from *S. cerevisiae*. Results obtained in this study highlight the potential of producing and extracting canthaxanthin while enabling the valorization of spent *S. cerevisiae*, one of the main by-products obtained in fermentative processes in sectors as the brewing and wine industries.

2. Material and methods

2.1 Biological material and chemicals

Spent *S. cerevisiae* genetically engineered to co-produce canthaxanthin was kindly provided by Amyris, Inc. (Emeryville, California, USA). Alcalase[®] 2.5 L and Promozyme[®] D6 were obtained from Aquitex, Portugal. Viscozyme[®] L, canthaxanthin standard (trans; analytical standard), butylated hydroxytoluene (BHT), ammonium acetate (HPLC grade) and chloroform ($\geq 99.9\%$), were purchased from Sigma-Aldrich, USA. Ethyl acetate ($\geq 99.7\%$) and ethanol ($\geq 99.8\%$) were supplied by Honeywell Riedel-de-Haën, USA. Acetone ($\geq 99.8\%$) was acquired from Fisher Scientific, USA. Isopropanol ($\geq 99.9\%$), *n*-hexane ($\geq 97\%$), methanol ($\geq 99.9\%$) and acetic acid ($\geq 99\%$) were provided by VWR, Portugal.

2.2 Optimization of canthaxanthin extraction

2.2.1 Solvent optimization

Different solvents and mixtures of solvents (**Table 1**; *vide* section 3.1) were assessed for their efficiency to extract canthaxanthin, as compared to that of chloroform. For this evaluation, yeast cell disruption was promoted by the action of glass beads, since this disruption method has been extensively used due to providing the highest carotenoids yields (Schüler et al. 2020; Lopes et al. 2017). Therefore, 500 μ L of yeast cells were transferred to a 15 mL microcentrifuge

tube with approximately 1mL of solid glass beads ($\varnothing = 2$ mm; MP Biomedicals, China), after which 5 mL of the extraction solvent (or mixture of solvents) were added. Thereafter, samples were vortexed 5 times for 1 min, with incubations of 30 s on ice, before being transferred to a multi-rotor (Multi Bio RS-24, BioSan, Latvia) and agitated (100 rpm) for 30 min at room temperature, and then centrifuged for 2 min, at $21500 \times g$ at 4 °C. The organic phases were transferred to clean centrifuge tubes and assessed for canthaxanthin content (section 2.4).

2.2.2 Yeast cell wall disruption – enzymatic hydrolysis optimization

Although widely used, scalability of cell lysis mechanism using glass beads is often a complex endeavour, especially due to its high energy consumption to avoid thermal degradation of the bioactive compounds (Liu et al. 2016; Saini and Keum 2018). Within other extraction procedures available, autolysis and enzymatic hydrolysis are advantageous methodologies, due to their high recovery yields and cost-effectiveness (Zeghloul et al. 2021; Saini and Keum 2018). Accordingly, thermally induced autolysis was performed at 50 °C in a water bath (SW22, Julabo GmbH, Germany), under agitation at 100 rpm for 6 h, and with the addition of ethyl acetate (0.5%, v/v) and BHT (0.1%, w/v). At the end of each incubation period, pH was adjusted to 5.5, and the enzymatic hydrolysis process was carried out with the addition of commercial enzymes. Three different combinations of enzymes were assessed: Alcalase[®] (A), Alcalase[®] + Viscozyme[®] (AV) and Alcalase[®] + Promozyme[®] (AP). Alcalase[®] and Promozyme[®] enzymes were added to a final concentration of 3% (v/v), while Viscozyme[®] was added to a final concentration of 2% (v/v), and enzymatic hydrolysis occurred at 55 °C for 4 h under agitation (100 rpm). Samples were diluted in twice the volume of organic solvent. After vigorous shaking, samples were centrifuged, and canthaxanthin was quantified from the organic phase (as described in 2.4).

2.2.3 Optimal conditions assortment

Thermally induced autolysis and enzymatic hydrolysis using the most promising combination of enzymes, Alcalase[®] + Viscozyme[®] (AV), were carried out as described in section 2.2.2. The resulting samples were diluted in twice the volume of organic solvent – chloroform and ethyl acetate. After vigorous shaking, samples were centrifuged, and canthaxanthin was quantified from the organic phase (as described in 2.4). Additionally, the organic phase was dried under N₂ atmosphere in a pre-weighed recipient in order to measure the extract weight.

2.3 Enrichment of canthaxanthin extract

To enhance the canthaxanthin content of the final extract, a purification methodology was conducted as described herein. Canthaxanthin-producing *S. cerevisiae* cells were lysed as previously described in section 2.2.2, using 3% (v/v) Alcalase[®] and 2% (v/v) Viscozyme[®] in the enzymatic hydrolysis step. Cell lysate was resuspended in two equivalent volumes of ethyl acetate containing 0.1% BHT and agitated for 4 h. The organic and aqueous phases were allowed to separate overnight, at room temperature. After this period, the organic phase was collected and centrifuged at $3500 \times g$ for 20 min at room temperature. The organic phase was then evaporated in a rotary evaporator at 26 °C and 120 rpm, under vacuum (100 mbar). Following solvent evaporation, the round bottom flask containing the canthaxanthin extract was left at 4 °C, protected from light for 48 h. This procedure resulted in the appearance of a dark precipitate, which was rinsed from the flask using a solution of ethanol:acetone (5:2), and the resulting suspension was filtered. The precipitate was transferred to a glass test tube and lastly dried under N₂ atmosphere. Canthaxanthin content was determined by HPLC (*vide* section 2.4) after solubilization in chloroform.

2.4 Canthaxanthin quantification

Canthaxanthin content was analyzed by reverse-phase high performance liquid chromatography (HPLC) in an Agilent 1260 Infinity II system (Agilent Technologies, California, USA) equipped with a diode array detector (DAD), using a Zorbax Eclipse Plus C8 column (2.1 x 100 mm, 3.5µm; Agilent Technologies, California, USA). Mobile phase A (A) composed of methanol:0.1% acetic acid (1:1, v/v) in 5 mM ammonium acetate, and mobile phase B (B) composed of methanol:isopropanol:0.1% acetic acid (1:8:1, v/v/v) in 5 mM ammonium acetate, were eluted using the following gradient: 98% A in the beginning and maintaining for 0.5 min, decreased to 65% A at 1.0 min, 50% A at 1.50 min, 40% A at 2.0 min and maintained until 7.0 min, further decreased to 10% A at 7.5 min and maintained until 13.50 min. Further decreased to 5% A at 14.0 min and finally increased to 98% A at 14.50 min and maintained until 15.50 min. All solvents and samples were filtered through 0.45 µm polyester filters and mobile phases were degassed before use. Standard canthaxanthin solutions were prepared under anoxic conditions to generate a calibration curve in the range 1-25 µg/mL. Samples and standard solutions were injected at a 2.0 µL volume, and chromatograms were collected at 250 nm, 454 nm, and 471 nm. The retention time of canthaxanthin is 6.0 min.

The total canthaxanthin content present in the yeast was determined by first bead beating the yeast to completely disrupt the cell wall, followed by the extraction of canthaxanthin using

15 equivalent volumes of chloroform. This is considered the value of total content of canthaxanthin in the yeast and was used for the determination of canthaxanthin recovery yield.

2.5 Structural Characterization

2.5.1 Ultraviolet-Visible spectroscopy – UV-Vis

Sample and standard solutions were prepared by dissolution in chloroform and dilution in the same solvent until an appropriate absorption range was attained. The solutions were scanned from 250 to 650 nm on a Shimadzu UV-1900 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan).

2.5.2 Powder X-Ray Diffraction (PXRD) Analysis

Powder X-ray Diffraction Analysis (PXRD) was performed on Rigaku MiniFlex 600 diffractometer with Cu α radiation, with a voltage of 40 kV and a current of 15 mA ($3^\circ \leq 2\theta \leq 60^\circ$; a step of 0.01 and speed rate of 2.0°/min). The evaluation of the crystallinity index (%CI) was performed using the program PDXL2 from Rigaku® (version 2.8.1.1) by the adjustment of the baseline and the profile of the diffractograms. The CI value is then obtained by considering the ratio of the total areas of crystalline peaks to the total area of all peaks. The measurements were performed in duplicate.

2.5.3 Scanning Electron Microscopy – SEM

The morphology of the canthaxanthin enriched powder extract was evaluated by Scanning Electron Microscopy on a Thermo Scientific™ Pro Scanning Electron Microscope. Prior to the analysis, an aliquot of the sample was placed into observation stubs covered with double-sided adhesive carbon tape (NEM tape, Nisshin, Japan), and coated with Au/Pd (target SC510-314B from ANAME, S.L., Madrid) using a Sputter Coater (Polaron, Bad Schwalbach, Germany). Observations were performed in high vacuum mode with an acceleration voltage of 5 kV. The specimen was observed using 1000x and 4000x magnification. The images were found to be representative of the morphology of the extract.

2.6 Statistical Analysis

The T-student test were carried out in association with Welch's correction and one-way ANOVA for multiple comparisons in association with Tukey's post test were performed in

GraphPad Prism 8.0.2 software. Results are represented as mean values $\pm$ SD (standard deviation), and $p < 0.05$ was considering statistically significant.

3. Results and Discussion

3.1 Extraction solvent optimization

Due to their hydrophobic nature, most carotenoids are insoluble in water, and thus, their extraction (recovery) is generally performed using organic solvents. The solubility of carotenoids in these organic solvents depends on a combination of factors such as their polarity, the presence of different functional groups and chain length, among others. Although canthaxanthin is particularly soluble in chloroform, the high toxicity of the solvent led the Food and Drug Administration (FDA) federal agency to ban its use in drugs, cosmetics and food packaging (ATSDR 2002). Thus, since the use of chloroform as an extraction solvent for canthaxanthin can be controversial and generate safety concerns, alternative solvents (and a mixture of solvents) were assessed for their capacity to efficiently extract canthaxanthin from the yeast extract, as compared with chloroform (**Table 1**). The solvent selection was performed in accordance with their polarity index (Sadek 2002), capacity to solubilize canthaxanthin (evaluated according to Hansen Solubility Parameters, Hansen, 2007), and safety: substances generally recognized as safe (GRAS) in food (ethyl acetate), substances affirmed as GRAS in food (ethanol), or secondary direct food additives permitted in food for human consumption (*n*-hexane and acetone), according to FDA (FDA 2011). From the results obtained (**Table 1**), it can be observed that those solvents (or mixtures) with a polarity index closer to that of chloroform were those with a higher efficiency, as expected. Thus, ethyl acetate was selected as an alternative to extract canthaxanthin from the yeast extract, as it allows a yield of extraction only 10% lower than the one obtained with chloroform.

Table 1. Solvents and mixtures of solvents evaluated for their efficacy to extract canthaxanthin. Results are expressed as efficiency percentage (%) calculated by comparison with chloroform (100% efficiency), n= 2.

Solvents	Polarity Index	Ratio (v/v)	Efficiency (%)
Chloroform	4.1	n.a.	100
Ethyl Acetate	4.4	n.a.	90.1±3.0
Ethyl Acetate:Ethanol	4.8	1:1	89.9±2.5
Ethyl Acetate:Acetone:Ethanol	4.8	2:1:1	87.7±1.1
Ethyl Acetate:Acetone	4.7	1:1	66.9±0.1
<i>n</i> -Hexane:Ethanol	2.6	1:1	58.2±3.3
<i>n</i> -Hexane	0.0	n.a.	50.9±0.7
<i>n</i> -Hexane:Acetone:Ethanol	2.6	2:1:1	45.7±1.2
<i>n</i> -Hexane:Ethanol:Ethyl Acetate	2.4	2:1:1	43.2±0.5
<i>n</i> -Hexane:Acetone	2.6	1:1	37.8±0.1

n.a.: not applicable. Polarity index (PI) for solvent mixtures was calculated as a balanced value between the PI values of the individual solvents used and their proportions in the mixture.

3.2 Yeast cell disruption exploitation

Different extraction approaches have been used to disrupt yeast cell walls and membranes aiming to recover intracellular carotenoids (Mussagy et al. 2019; Šovljanski et al. 2022; Saini and Keum 2018; Takaloo et al. 2020), namely, mechanical, physical, chemical, and enzymatic procedures. Although low time-consuming, physical disruption methodologies such as ultrasonic frequencies, bead mills, or high-pressure homogenization present several limitations, as they require expensive technology, consume high amounts of energy, and often generate undesirable heat that can cause thermal degradation of carotenoids (Saini and Keum 2018; Takaloo et al. 2020). Differently, and despite being biased and seen respectively as time-consuming and expensive approaches, cell disruption by endogenous (autolysis) or exogenous (enzymatic hydrolysis) enzymes are eco-friendly processes that occur under mild reaction conditions and with minimal usage of solvents (Zeghlouli et al. 2021; Saini and Keum 2018). Moreover, the highly specific nature of enzymes usually leads to higher recovery yields of the target molecules (Zeghlouli et al. 2021), thus enabling a more cost-effective strategy.

Following the above considerations, a two-step methodology to recover the intracellularly produced canthaxanthin was selected, comprising a thermally induced autolysis step followed by an enzymatic hydrolysis process.

Autolysis optimum conditions are difficult to establish, as these are influenced by the combination of a significant number of variables, such as the addition of promoters, temperature, pH, and incubation periods. Since long autolysis periods strongly enhance the

promotion of unwanted bacterial contamination (Alves, Souza, and Oliva Neto 2021), a reduction of this extent was decided. Additionally, autolysis was carried out with the addition of 0.5% (v/v) ethyl acetate, as this simple ester has been reported to act as an autolysis promoter by increasing cell membrane permeability (Takaloo et al. 2020). Autolysis was carried out at 50 °C for an incubation period of 6 h. BHT was also added to avoid canthaxanthin oxidation (Paradiso et al. 2020).

After the autolysis step, an enzymatic hydrolysis was performed using three commercial enzymes: Alcalase[®] (a serine endopeptidase that consists primarily of subtilisin A), Viscozyme[®] (an enzymatic cocktail containing several carbohydrases such as arabanase, cellulase, β -glucanase, hemicellulose and xylanase) and Promozyme[®] (a pullulanase that catalyses the debranching of cell wall oligosaccharides). Aiming to obtain the highest amount of canthaxanthin in the hydrolysis process, three combinations of enzymes were assessed: Alcalase[®] (A), Alcalase[®] + Viscozyme[®] (AV) and Alcalase[®] + Promozyme[®] (AP). While Alcalase[®] and Viscozyme[®] have been frequently used in the enzymatic hydrolysis of yeast (Marson et al. 2020), Promozyme[®] (pullulanase) could potentially be an interesting choice since this enzyme specifically hydrolyses the α -1,6-glycosidic bonds present in the cell wall of *S. cerevisiae* (Orlean 2012). Hydrolysis was performed at 55 °C since this temperature is within the temperature range at which the activity of the three enzymes is optimal, and the potential of the above-mentioned combination of enzymes was assessed in the hydrolysis step over an incubation period of 4 h. After solvent extraction, the concentration of canthaxanthin in the organic phase was determined by HPLC, as detailed in previous sections. The canthaxanthin recovered after this procedure was normalized to the control (0 h) and is depicted in **Fig. 1** as fold change. Canthaxanthin content in the control was determined in a yeast sample in which glass beads were used for cell wall disruption and chloroform was used as the extraction solvent. The results clearly show that the hydrolysis using a combination of 3% (v/v) Alcalase[®] and 2% (v/v) Viscozyme[®] at 55 °C (**AV**) for 4 h, preceded by autolysis at 50 °C for 6 h, provides the highest canthaxanthin extraction yields. While A and AP presented similar fold increases (1.2 ± 0.1 and 1.1 ± 0.2 , respectively) AV increased the canthaxanthin concentration by 2.0 ± 0.2 , representing a recovery yield of 39.5 ± 2.7 %.

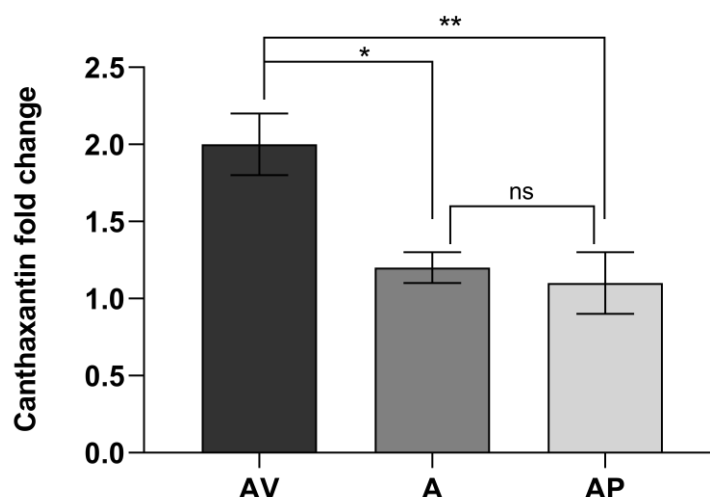


Fig. 1. Canthaxanthin content is depicted as the fold change in relation to the initial canthaxanthin obtained by simple solvent extraction (time-point 0, pre-autolysis). Autolysis was performed at 50 °C for 6 h for all samples followed by enzymatic hydrolysis using one of three combination of enzymes – Alcalase® + Viscozyme® (AV), Alcalase® (A), and Alcalase® + Promozyme® (AP) – at 55 °C for 4 h. Bars indicate the mean of the replicates $\pm$ standard deviation (SD, $n = 2$). The statistical analysis revealed significant differences between the enzymatic hydrolysis expressed by the symbol ** ($p < 0.01$), * ($p < 0.05$) and “ns” indicates non-significant statistical differences.

3.3 Final optimal conditions evaluation

The most favorable conditions of autolysis (50 °C) and enzymatic hydrolysis (AV; 55 °C) were used to investigate canthaxanthin recoveries and contents with two solvents – the standard extraction solvent, chloroform, and ethyl acetate, the most promising GRAS solvent. The procedure followed was described in section 2.2.3. The resulting canthaxanthin concentration (% w/w) and yields (%) are depicted in **Fig. 2**, where it is clearly shown that, even though the resulting extracts present similar canthaxanthin concentrations (0.544 ± 0.002 % and 0.489 ± 0.016 % (w/w)) for chloroform and ethyl acetate, respectively, the yields are significantly different. The extraction using chloroform resulted in a yield of 56.5 ± 0.2 % whereas ethyl acetate only yielded 22.8 ± 0.7 % of canthaxanthin. Despite the fact that ethyl acetate only reduced the extraction efficiency by ten percent in the conditions of the solvent optimization study (section 3.1), the current extraction conditions led to a more pronounced reduction. Whereas the solvent optimization study was conducted employing a mechanical approach to the cell disruption, the current study employed softer disruption methodologies – autolysis and enzymatic hydrolysis. This discrepancy in the disruption mechanism may result in canthaxanthin being entrapped in the cell and shifting the extraction to a process increasingly

dependent on cell permeabilization by the solvent. Furthermore, the solvent-to-yeast ratio was 10 and 2 in the solvent optimization and current study, respectively, which also explains the differences attained.

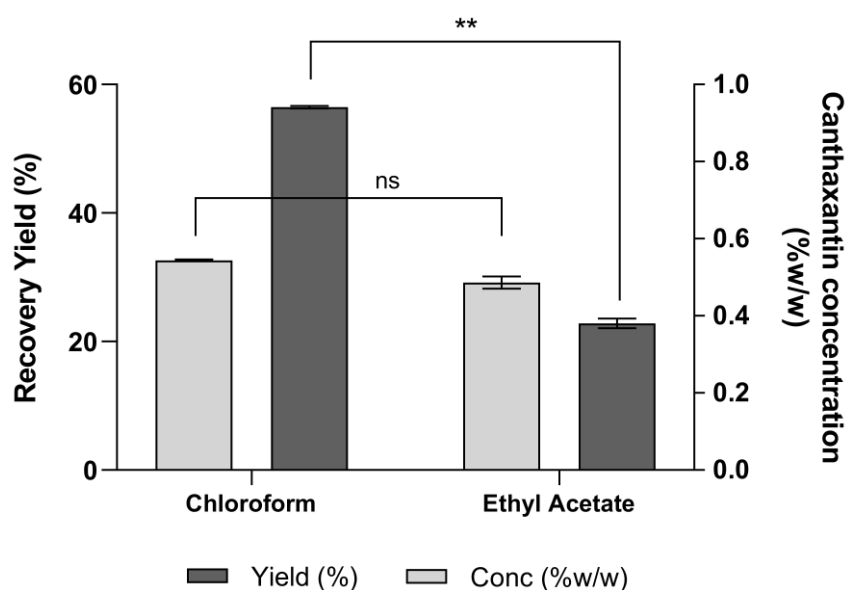


Fig. 2. Canthaxanthin concentration expressed as % w/w and recovery yield expressed as %, $n = 2$. The statistical analysis revealed significant differences between the extraction solvents expressed by the symbol ** ($p < 0.01$) in terms of yield. The symbol “ns” indicates non-significant statistical differences.

3.4 Canthaxanthin-enriched extract

Considering the low canthaxanthin concentration in the organic phase obtained from its extraction with ethyl acetate ($0.489 \pm 0.016\%$ (w/w)), the need for an additional purification step became obvious. The purification procedure previously described in section 2.3 allowed an increase in the canthaxanthin content to $43.7 \pm 7.7\%$ (w/w), around 90-times higher than the content of the extract recovered from *S. cerevisiae* cells after the autolysis and hydrolysis procedure. Chromatograms of both canthaxanthin standard and canthaxanthin-enriched extract solution are depicted in **Fig. 3**.

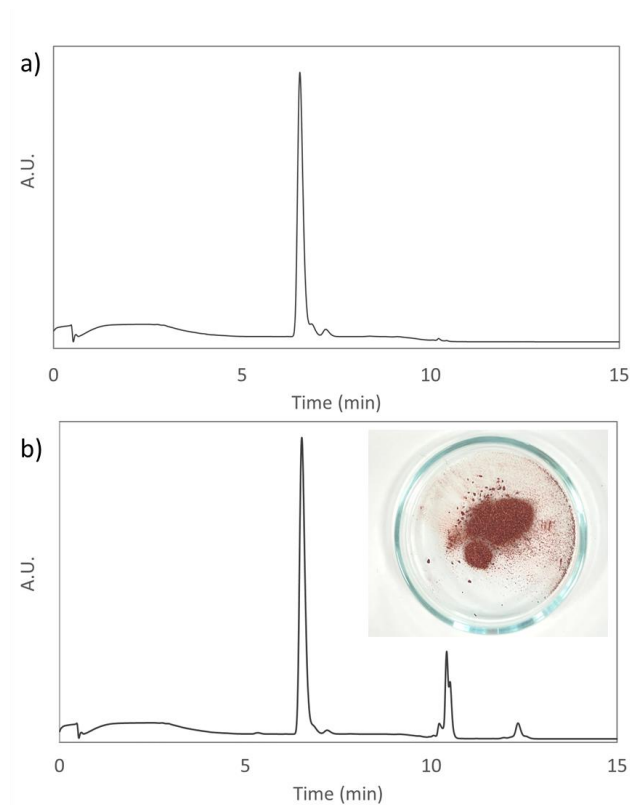


Fig. 3. Chromatograms collected at 471 nm obtained in the analysis of a) canthaxanthin analytical standard (Sigma-Aldrich 32993) and b) canthaxanthin-enriched powder extract with a photo of the powder on the right top corner.

It is clear from the chromatogram of the canthaxanthin-enriched extract in **Fig. 3b** that the most significant peak corresponds to canthaxanthin, but additional peaks can be seen eluting at 10.4 min and 12.3 min. Since the HPLC method employed encompasses a reverse-phase column, the molecules are eluting in order of decreasing polarity, and therefore, the additional peaks correspond to less polar molecules, such as, potentially, carotenes.

An overall canthaxanthin recovery of $13.7 \pm 3.7\%$ was obtained, when dividing the canthaxanthin in the enriched extract by the canthaxanthin content in the initial yeast (section 2.4).

3.5 Structural Characterization

The structural analysis of the canthaxanthin-enriched powder extract involved the following techniques – UV-Vis spectroscopy, Powder X-ray diffraction (PXRD), and Scanning Electron Microscopy (SEM), whose results are depicted in **Fig. 4**

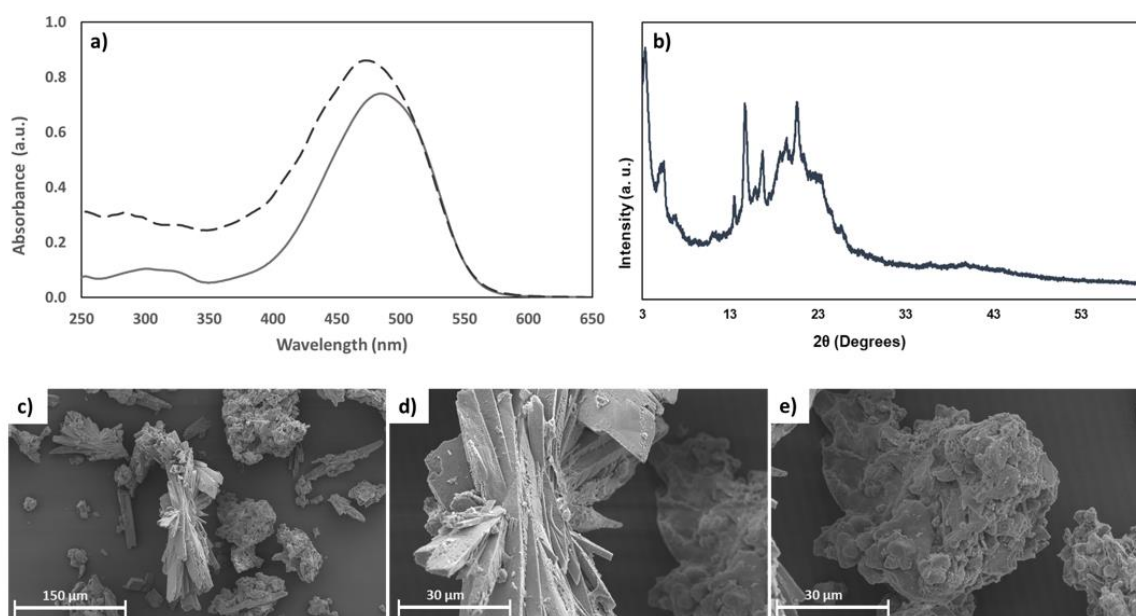


Fig. 4. Canthaxanthin-enriched powder was structurally characterized using different techniques: a) UV-Vis spectroscopy spectra of the extract (dashed line) and standard canthaxanthin (solid line); b) Powder X-Ray diffraction pattern; c), d) and e) Scanning electron microscopy micrographs with c) x1000 and d), e) x4000 magnifications of the canthaxanthin-enriched powder. Higher magnification micrographs reveal the presence of d) crystalline- and e) amorphous-like structures.

The UV-Vis spectra of the extract and the standard canthaxanthin (both prepared in chloroform), depicted in **Fig. 4a** respectively as a dashed line and a solid line, are overlaid, and maxima absorption were found to be 474 nm and 486 nm for the extract and the standard, respectively. The canthaxanthin-enriched extract presents a maximum absorption towards a shorter wavelength, which can be a result of its heterogeneous composition. The PXRD pattern of the canthaxanthin-enriched product is highlighted in **Fig. 4b**. The crystallinity index (CI in %) was also determined, being obtained a CI of $45.2 \pm 0.2\%$, which is in a close agreement with canthaxanthin content as determined by HPLC, $43.7 \pm 7.7\%$ (w/w), suggesting a potential correlation between the crystal fraction of the extract and the canthaxanthin content. The morphology of this sample was also investigated, as portrayed in **Fig. 4c, d** and **e**, being possible to observe the presence of two distinct structures: an amorphous-like one, without

particular distinctive morphology (**Fig. 4e**) and a different one, characteristic of a crystalline structure formed with plaques clustered around a center in a star-like shape (**Fig. 4d**). From the analysis of the three SEM images, we can conclude that the sample can be classified as heterogeneous, as it reveals a well-defined and organized structure, of apparently smooth grouped plates (**Fig. 4d**), united by a single center. The other structures presented appear to be heterogeneous as well, as they exhibit both more rounded and more elongated structures. These structures appear to have a high density and abundance in the sample. (**Fig. 4c** and **e**). This observations further support the results obtained by PXRD and suggest that the crystal structures correspond to canthaxanthin.

4. Conclusions

Spent yeast is one of the main by-products of several industries in need of more conscious waste management. By-products can be valorized and minimized by different approaches, such as the genetic modification of *S. cerevisiae* cells aiming for the co-production of high valued and health beneficial products such as canthaxanthin. In the present work, autolysis and hydrolysis of spent yeast *S. cerevisiae* cells genetically engineered to co-produce canthaxanthin were tentatively optimized. Different extraction solvents were assessed for their capacity to efficiently extract the pigment, aiming for a sustainable and eco-friendly process. Canthaxanthin was successfully recovered through a yeast cell lysis process composed of an autolysis step, a subsequent enzymatic hydrolysis and the use of ethyl acetate as the extraction solvent. Further purification of the canthaxanthin containing extract was achieved by precipitation of canthaxanthin from a saturated solution via temperature reduction. Indeed, when correlating HPLC, PXRD and SEM results, it is possible to assume a cantaxantine content of nearly 50%. This methodology provides a straightforward, up-scalable and eco-friendly process for the recovery of canthaxanthin from modified *S. cerevisiae*. Additional and thorough optimization of the extraction process (cell disruption methods, types of enzymes, incubation time and temperature) and precipitation parameters may further improve the yield and purity of canthaxanthin recovered from yeast cells, while further minimizing the produced wastes, thus increasing sustainability metrics.

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