Astaxanthin Extraction from *Paracoccus carotinifaciens* Employing Fatty Acid based Natural Deep Eutectic Solvents

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Astaxanthin is a red-orange pigment widely used as a natural dye, additive in animal feed (salmonids) and for human consumption as dietary supplements. Recently, the consumers' demand for natural-based products has encouraged to exploit the high added value bioactive compounds obtained by biomass, for applications in food industry, was explored. Researchers made an effort to develop sustainable extraction processes to guarantee the biocompatibility and safety of the products.

In the present work, the possibility of employing fatty acids based NaDES (Natural Deep Eutectic Solvents) as a non-toxic alternative to traditional hydroalcoholic solvents for the extraction of astaxanthin from *Paracoccus Carotinifaciens* was evaluated. Furthermore, the extraction kinetic was studied by using mass transfer and reaction kinetic models. In addition, the antioxidant activity of the extracts was evaluated at each extraction time by ABTS assay. Experimental results show that the extraction yield was up to 60% and the reaction kinetic model provided a slightly better fitting of the data, when compared to the mass transfer model.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red-orange ketocarotenoid pigment. Due to the presence of oxygen atoms in its chemical structure, it belongs to the Xanthophylls' family. The potential benefits of astaxanthin on human health include antioxidant, anticancer, antilipid peroxidant, anti-inflammatory, antidiabetic and skin protection properties, among many others (Brotosudarmo et al., 2020). Therefore, astaxanthin is widely used in pharmaceutical, cosmetic and functional foods additives. Due to its particular pigmentation action, it is widely used as an additive in fish and crustacean feed, especially for salmonids. In fact, animals are unable to synthesize carotenoids so that the accumulation of these substances relies exclusively on the animal diet. An astaxanthin-rich diet has also been shown to improve several vital functions of animals, especially those associated with their reproductive system (Putman et al. 1991). Astaxanthin can be produced both by chemical synthesis and by extraction from natural matrices, which is considered as safer for human consumption as dietary supplements, as reported on EFSA (2020). Crustacean- processing by-products are an important source of natural astaxanthin, but its content depends on species, feed and different growth conditions (Özogul et al., 2018) and extracting astaxanthin from them requires processing large amounts of wastes. Various alternative sources, like microalgae, yeast, and bacteria have been proposed to simplify the process of extracting astaxanthin from natural sources. Microalgae, like *Haematococcus pluvialis sp.*, produce this pigment during their growth under specific stress conditions (e.g high irradiance, limiting nutrients and extreme temperatures) (Mularczyk et al., 2020). Astaxanthin is also produced through controlled fermentation of yeasts, like *Phaffia rhodozyma*, and bacteria, like *Paracoccus carotinifaciens*. (Du et al., 2020; Hayashi et al., 2020). Soxhlet extraction is the conventional extraction method that allows the most efficient recovery of astaxanthin. However, it is a time-consuming methodology that requires the use of high amounts of volatile organic solvents at temperatures that are often not suitable for the recovery of thermosensitive molecules. Furthermore, they are generally toxic and products may require high purification costs.

In recent years, researchers focused their attention on unconventional methods such as ultrasound- or microwave- assisted, supercritical fluid or pressurized liquid, pulse electric field, and enzyme-assisted extraction (Rodrigues et al., 2020). Despite the high extraction yields obtained, these methods generally require high energy expenditures. For this reason, researchers attempted at developing an extraction method with the aim to reduce energy consumption. Over the last few years, Natural Deep Eutectic Solvents (NaDES) have emerged as a good alternative for conventional solvents. NADES are mixtures of natural compounds such as organic acids and bases, amino acids, sugars, sugar alcohols, and polyalcohols that interact through hydrogen bonding and liquefy if combined in specific molar ratios. They represent a novel and environmentally friendly class of ionic solvents, as they have a non-volatile nature, are of simple formulation and require mild operating conditions (Pavia et al., 2014). For these reasons, the interest in NaDES has rapidly expanded particularly into the study of their application in the extraction of (targeted) bioactive compounds from natural sources (Dai et al., 2018).

Furthermore, according to NaDES formulation, extracts obtained by employing this solvent can be potentially applied to several fields such as cosmetic, nutraceutical and phytosanitary.In the light of these observations, the aim of the present contribution was to carry out a preliminary study of the astaxanthin extraction kinetics from *Paracoccus carotinifaciens* employing a fatty acid based NaDES formulated by Florindo et al. (2018) and tested on microalgal biomass by Sed et al. (2018). In addition, the antioxidant activity of the extracts was evaluated at each extraction time.

2. Materials and methods

2.1 Chemical and biological materials

Octanoic Acid (C8), Dodecanoic Acid (C12), ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), ethanol (purity ≥ 96%) and potassium persulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). Astaxanthin was purchased from Merck (Darmstadt, Germany). Demineralized water was used for the experiments. All reagents were used without further purification. The biomass used in this work was obtained as a courtesy of DPhar S.p.A (Anagni, FR, Italia). It is obtained from the fermentation of *Paracoccus carotinifaciens* NITE SD 00017, a carotenoids-rich bacterium. The bacterium is grown in aerobic bioreactors and fed with glucose as a substrate. The carotenoids content is monitored until the process specification is reached. The suspension is sterilized, concentrated, and dried. The final product, a very fine deep red powder, is sold under the commercial name of Panaferd®-AX. In a work by Boris et al. (2007), the composition of *Paracoccus carotinifaciens* was studied and the results showed that this product is made up of 4 % carotenoids, which consist of 50 % of astaxanthin.

2.2 NaDES preparation and characterization

The solvent was prepared by heating method. Lauric acid (C_{12}) and caprylic acid (C_8) were mixed together with a molar ratio equal to 0.33 in a capped beaker. The mixture was heated at 50°C for 20 minutes until a homogeneous and colourless liquid was formed. This NaDES is highly hydrophobic and exhibits a solidification temperature of 9°C, compared to 16°C and 43.8°C of the individual fatty acids (Sed et al., 2018). The reology of NaDES C_{12}: C_8 was investigated with a rotoviscosimeter (Haake Rotovisco RV12) and the dependence of the viscosity upon temperature was characterised and the results were correlated by using Arrhenius equation:

$$\eta = \eta_0 \times e^{E_a/_{RT}} \ (mPa * s)$$

(1)

Where R is equal to 8.314 J mol^{-1} K^{-1}.

2.3 Design of extraction experiments

Astaxanthin extraction from Panaferd®-AX with NaDES C12:C8 was carried out using a solid to liquid ratio equal to 0.03 g/mL in 250 mL Erlenmeyer flasks kept under stirring conditions in a thermostated orbital shaker (B. Braun CERTOMAT H.). The temperature was kept constant to 25 °C during all the extraction experiments and the extraction stirring rate was 250 rpm. The kinetic factors were investigated by carrying out the extraction at different contact times from 5 to 360 minutes. After the extraction step, the solid-liquid separation was carried out by centrifugation at 1165 g for 10 minutes. The extracts obtained were stored at 4°C for further analyses. The antioxidant activity was evaluated by the ABTS assay (Handayani et al., 2008) and the results were expressed as trolox equivalents antioxidant capacity (TEAC) on dry basis, using a calibration curve obtained with standard solutions of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

2.4 Astaxanthin quantification

After the extraction, the astaxanthin in the supernatant obtained from the centrifugation was quantified by UV-VIS spectrophotometry (Shanghai Mapada Spectrophotometer UV- 1800 PC) by reading the absorbance at 489 nm and using a calibration curve obtained with standard solutions of astaxanthin in NaDES C_{12}:C_8.

Nine astaxanthin solutions in NaDES (1.4–14.4 mg/L) were prepared and analysed spectrophotometrically. The intensity peak for each solution was recorded at a wavelength of 489 nm. The extraction yield was obtained by dividing the astaxanthin content by the carotenoid content (31 g/kg) in Panaferd®-AX according to the biomass datasheet. In the results section, the extraction yield (Y%) is expressed as weight of astaxanthin equivalents on a dry basis (DB). The results were compared to those obtained by employing organic solvents, namely, acetone and ethanol, in the same experimental conditions.

2.5 Extraction kinetics

Experimental data were described by using the kinetic model and the reaction kinetic model. Given the lack of information regarding the content of esterified and free astaxanthin in *Paracoccus carotinifaciens*, for preliminary investigations, the astaxanthin composition of this bacteria, was assumed to be that of *Haematococcus pluvialis* sp. In particular, in *Haematococcus pluvialis sp*, astaxanthin is mainly found in the esterified form with fatty acid (monoester, 70 %, and diester, 25 %) and just 5 % free (Miao et al., 2006). Mass transfer kinetic model was proposed to describe the extraction data of esterified astaxanthin, ready to dissolve in a fatty acid based solvent and the mass transfer from solid to bulk liquid is considered the controlling mechanism. In term of concentration, the rate of mass transfer of astaxanthin from the solid phase to liquid bulk can be written as:

$$\frac{VdC_A}{dt} = K_L \times a[C_{Ae} - C_A]$$

(2)

Where: $K_L a$ is the volumetric mass transfer coefficient and C_{Ae} is the astaxanthin concentration in bulk liquid at equilibrium.

Eq(2) was integrated with the following boundary conditions

1) at t=0, C_A=0;

2) at any time the astaxanthin content in bulk liquid is C_A=C_{Ae}.

$$C_A = C_{Ae}[1 - e^{-k_L at}]$$

(3)

Re-writing Eq(2) in terms of extraction yield:

$$Y = Y_e[1 - e^{-k_L at}]$$

(4)

The free form of astaxanthin is characterized by the hydroxyl groups without esterification. During the extraction step, esterification between hydroxyl groups in astaxanthin and fatty acids in NaDES Y may occur. Lagergren pseudo-second-order model was adopted to describe experimental data (Ho and Ofomaja, 2006).

$$\frac{dC_A}{dt} = k_A[C_{Ae} - C_A]^2$$

(5)

where k_A is the reaction constant. Eq(5) was integrated with the boundary conditions:

1) at t=0, C_A=0;

2) at any time the astaxanthin content in bulk liquid is C_A=C_{Ae}.

$$c_A = \frac{C_{Ae}^2 \times k_A \times t}{(1 + C_{Ae} \times k_A \times t)}$$

(6)

Re-writing Eq(6) in term of extraction yield Y (%):

$$Y = \frac{Y_e^2 \times k_A \times t}{(1 + Y_e \times k_A \times t)}$$ (7)

Where the extraction yield was expressed as:

$$Y = \frac{C_{At}}{C_{A0}} \times 100$$ (8)

For the first model, Ye, $k_L a$ and k_A were estimated by nonlinear least squares fit of Eq(4) and Eq(7) to experimental data.

3. Results and discussion

NaDES C_{12}: C_8 was never found to be a function of shear rate, thereby showing that this NaDES is a Newtonian fluid. The viscosity reduction is 56%, from 8.33 mPa*s to 12.76 mPa*s, by increasing the temperature from 20°C at 60°C and at the extraction temperature (25°C) it was equal to 7.78 mPa*s. This result is similar to that obtained by Florindo et al. (2017).
The calibration curve obtained with standard solutions of astaxanthin (2-15 mg/L) in NaDES. The mass attenuation coefficient (ε) was 0.1185 L mg^{-1} cm^{-1}.
The extraction yield obtained after 360 minutes of orbital shaking was 60%. This result is 50% higher than those obtained with acetone and ethanol (30% and 20%, respectively). These results could be explained by the significant hydrophobic nature of NaDES. In particular, due to the presence of hydroxyl groups of astaxanthin, this biomass is probably easily released in the fatty acid based NaDES. The experimental kinetic data of astaxanthin extraction obtained with NaDES and the predicted data of mass transfer kinetic model is displayed in Figure 1. This model provides a good correlation of the experimental data. However, for extraction times higher than 240 minutes a deviation of the model from the experimental data is observed. This behaviour could be attributed to the forms that astaxanthin (free and esterified) assumes in the biomass. In particular, as the extraction time increases, the esterification of hydroxylic groups in free astaxanthin and fatty acids in NaDES may occur. This involves an increase of astaxanthin amount in the liquid bulk and the model gives lower extraction yield values than those obtained experimentally.
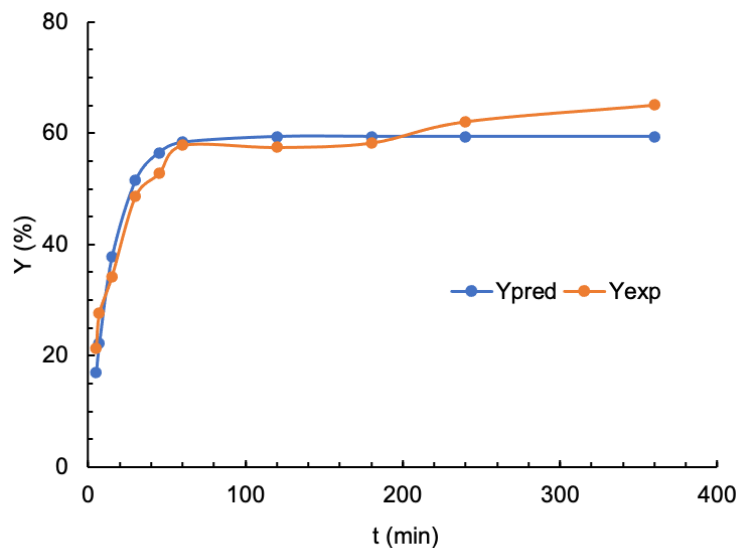

Figure 1: Astaxanthin extraction mass transfer kinetic model.

This discrepancy was less appreciable by using the reaction kinetic model. The results show that this model gives better correlation than mass transfer kinetic model, as displayed in Figure 2, for each extraction time.
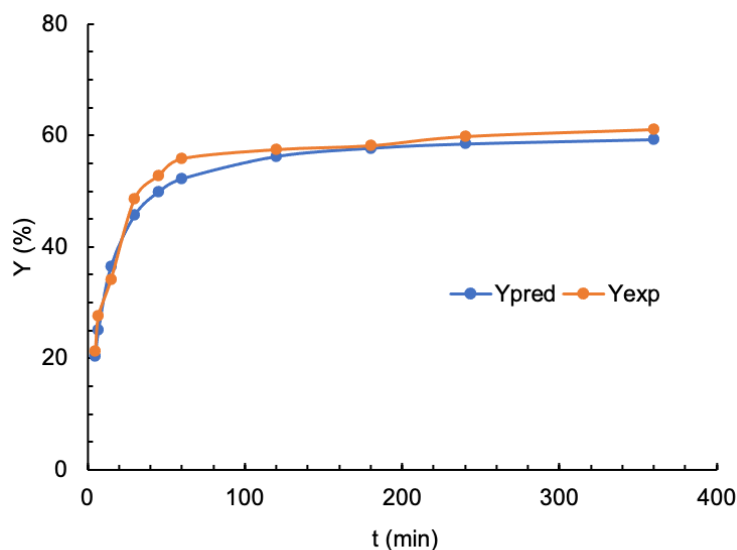

Figure 2: Astaxanthin extraction reaction kinetic model.

The optimal parameters for the fitting of mass transfer kinetic model and reaction kinetic model are summarized in Table 1. According to the values of the coefficients of determination R^2 for kinetic and mass transfer kinetic models, equal to, respectively 0.951 and 0.987, the latter provides a slightly more accurate correlation of data. Similar results were reported by Handayani et al. (2008) for astaxanthin extraction from crustacean by-products.

Table 1: kinetic parameters calculated using mass transfer kinetic model and reaction kinetic model

Kinetic Model	$Y_{e, fitted}$ (%)	$Y_{e,experimental}$ (%)	$k_L a$ (1/min)	k_A (1/min)	R^2
Mass transfer	0.594	0.575	0.067	-	0.951
Reaction	0.601	0.575	-	0.164	0.987

Antioxidant activity, expressed as trolox equivalents, varied linearly with the astaxanthin content of the extracts and was comprised between 4 and 12 mgTE/ g DB.

4. Conclusions

In the present contribution, the extraction of astaxanthin from *Paracoccus carotinifaciens* employing a fatty acid based NaDES was carried out. The extraction yield reached after 360 minutes of room temperature extraction in orbital shaker was 60% and 50% higher than those obtained with organic solvents (acetone and ethanol). In order to carry out a preliminary study of the extraction mechanisms, the experimental results were correlated by using mass transfer kinetic and reaction kinetic models. Both models seem to provide a good correlation of the experimental data, however, according to a slightly higher value of R^2, the latter seems more suitable for the process. According to these preliminary results, the extraction process is likely to be controlled by the reaction occurring between free astaxanthin in *Paracoccus carotinifaciens* and fatty acids used for the solvent formulation. The antioxidant activity measured at each extraction time was up to 12 mgTE/ g DB and it results linearly dependent with astaxanthin content in the extracts. The results encourage further studies on this topic and, in order to exploit the full potential of this application, an optimization of the extraction operating conditions could be carried out to maximize the extraction yield. Furthermore, as reported in previous studies

employing other biomass, it could be interesting evaluating the extraction yield of other bioactive compounds, such as proteins and other metabolites (Cicci et al., 2017).

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