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Ionic liquids or eutectic solvents? Identifying the best solvents for the extraction of astaxanthin and β-carotene from *Phaffia rhodozyma* yeast and preparation of biodegradable films

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Experimental Section

Reagents, standards and solvents

β-carotene and astaxanthin standards were obtained commercially by Sigma-Aldrich (St. Louis, MO, USA). Commercial grade granular corn starch (Maizena®) Unilever (Mogi Guaçu, SP, Brazil) was acquired in the local trade. Ultrapure water (purified through a Millipore Milli-Q ion-exchange system) was used for the solvent mixtures. A description of the supplier and purity of the compound's lactic acid, butyric acid, choline bicarbonate, choline chloride and dimethyl sulfoxide is provided in **Table S1**. All other chemicals were of analytical grade and purchased from common sources.

Table S1. Properties and acronyms of the solvents used in the extraction processes.

Compounds	Formula	Acronym.	CAS Number	Purity (%)	Molecular weight (g/mol)	Supplier
Lactic acid	$C_3H_6O_3$	Lac	-	P.A	90.08	Exodo Cientifica (Sumaré, SP, Brazil)
Butyric acid	$C_4H_8O_2$	But	107-92-6	≥99.0%	88.11	Sigma-Aldrich (St. Louis, MO, USA)
Choline bicarbonate	$C_5H_{14}NO \cdot HCO_3$	[Ch]Bic	78-79-9	~80% in H_2O	165.19	Sigma-Aldrich (St. Louis, MO, USA)
Choline chloride	$C_5H_{14}ClNO$	[Ch]Cl	67-48-1	≥99%	139.62	Sigma-Aldrich (St. Louis, MO, USA)
Dimethyl sulfoxide	C_2H_6OS	DMSO	-	P.A	78.13	Exodo Cientifica (Sumaré, SP, Brazil)
Water	H_2O	H_2O	-	Ultrapure	18.01	-

Preparation of ionic liquids and eutectic solvents

The ILs were synthesized *via* neutralization reaction of the base with the appropriate acid, according to the procedure previously reported.[1] Briefly; 1 M of the choline bicarbonate was placed in a 100 mL synthesis-flask, which was cooled to 4 °C, under continuous stirring, using an ice water bath. Then, the corresponding carboxylic acid (1 M) (acetic or butyric acid) was added. Before use, all the ILs were purified and dried for 48 h at constant agitation (≈ 100 rpm) and moderate temperature (≈ 298 K) under vacuum (300 mbar). The choline chloride-based eutectic mixtures were prepared by adding different mole ratios (1:1 and 1:2) of hydrogen bond donors (HBDs) (lactic and butyric acids) to choline chloride (HBA) to form a homogeneous transparent

liquid at 80 °C with constant stirring for 2 h (**Table S2**). The chemical structures of the ILs and eutectic mixtures produced were confirmed by Fourier transform infrared spectroscopy with an attenuated total reflectance (FTIR-ATR) (TENSOR 27, Bruker®, Germany) (**Fig. S1**). The ILs and eutectic mixtures were kept in sealed glass vials in the dark, at ambient temperature until use.

Table S2. List of ionic liquids and eutectic mixtures investigated, acronyms and concentrations used for the initial screening.

Compounds	Acronym.	Mole ratio	Appearance	Concentration (w/w
Choline lactate	[Ch][Lac]	1:1	Transparent liquid	80
Choline butanoate	[Ch][But]	1:1	Transparent liquid	80
Choline chloride + Lactic acid	[Ch]Cl:Lac	1:1	Transparent liquid	80
Choline chloride + Lactic acid	[Ch]Cl:Lac	1:2	Transparent liquid	80
Choline chloride + Butyric acid	[Ch]Cl:But	1:1	Transparent liquid	80
Choline chloride + Butyric acid	[Ch]Cl:But	1:2	Solid	-

Water content and pH determination

The water content of the ILs and eutectic mixtures was measured by volumetric titration at 25 °C using a Karl Fischer Metrohm® 803 TI-Stand titrator (Herisau, Switzerland), Hydranal-Methanol Rapid (reagent for accelerated volumetric one-component KF titration), and Hydranal-Composite 5 (reagent for volumetric one-component Karl Fischer titration; methanol free), both supplied by Sigma-Aldrich (St. Louis, MO, USA) as titrants. The pH (± 0.02) of ILs and eutectic mixtures was also determined using a MS Tecnopon® mPA-210 (Piracicaba, SP, Brazil). The calibration of the pH meter was carried out with two buffers (pH values of 4.00 and 7.00). The corresponding pH values obtained are provided in **Table S3**.

Viscosity determination

The viscosity of ILs and eutectic mixtures were determined in a variable at 65 °C (optimal extractions temperature) under atmospheric pressure using Anton Paar® SVM 3000 viscometer-densimeter (Graz, Austria). The viscosimeter was previously calibrated using standard solutions.

Phaffia rhodozyma growing conditions

Phaffia rhodozyma NRRL Y-17268 (*P. rhodozyma*) yeast was acquired from the Northern Regional Research Laboratory (also known as NRRL) (Peoria, USA). The pre-inoculum was prepared by the activation of a stock culture of the yeast (50% v/v in glycerol aqueous solution maintained at −80 °C) in YPD (Yeast Extract-Peptone-Dextrose) medium [composed of (g/L in deionized water): peptone bacteriological (20), yeast extract (10), glucose (20)]. The inoculum cell culture was prepared in 100 mL Erlenmeyer® type flasks containing 25 mL of the YPD medium. Cells were grown for 48 h at 22 °C and 300 rpm in an orbital shaker New Brunswick Innova 40R (Eppendorf, Inc., USA).

For astaxanthin and β-carotene production, a culture medium composed of (g/L in deionized water): xylose (12.5), yeast extract (3), malt extract (3) and peptone (5) was used. Batch cultures with 4 L of the culture medium were grown in a 5 L stirred-tank bioreactor (Minifors II (INFORS, New Jersey/USA), equipped with two Rushton turbines radial flow impellers, oxygen and pH electrodes. The initial pH of the medium was adjusted to 5.0 by adding 2 mol/L HCl or NaOH before autoclaving. The production process was then started by transferring 10% (v/v) of the inoculum culture of *P. rhodozyma* (at 0.2 mg/mL) to the bioreactor, and the bioprocess conducted at 22 ºC, 300 rpm and 1 vvm (air volume/medium volume/minutes) over 120 h (antifoam was added as needed). After the cultivation, *P. rhodozyma* cell biomass was separated from the fermented supernatant by centrifugation at 2500 xg for 10 min at 4º C using a Hitachi CR-22N (Tokio, Japan) centrifuge. The supernatants of all fermented media were then discarded, and the cellular biomass samples containing astaxanthin and β-carotene were collected and used in the following solid-liquid extraction procedures.

Solid-liquid extraction of carotenoids using ILs and ESs

ILs and eutectic mixtures aqueous solutions were prepared and used for the recovery of astaxanthin and β-carotene from *P. rhodozyma* biomass. As control, DMSO was used as extractant agent following previous reports regarding the extraction of carotenoids from *P. rhodozyma* cells.[1] The solid-liquid extraction of astaxanthin and β-carotene experiments were

carried out according to the following procedures: *i)* to remove impurities generated in the upstream stage, the *P. rhodozyma* wet cells were washed twice using 5 mL of phosphate buffer with pH 7; *ii)* after removing impurities, 0.05, 0.1, 0.2, 0.5, 1 g and 0.2 g of wet biomass were added in 4 mL capped glass test tubes for solid-liquid ratio and initial screening evaluation respectively; *iii*) the tubes were filled with 1 mL of different aqueous solutions of ILs and eutectic mixtures (with 10%, 20%, 30%, 40% and 50% (v/v) of water) to evaluate the effect of water in extractions and 20% (v/v) of water for the initial screening, or with 1 mL of DMSO for the control assay; *iv*) samples were then homogenized using a magnetic stirrer hot plate mixer (Norte Científica, NH 2200, Araraquara, SP, Brazil) for 1 h at 65 °C and 300 rpm. After homogenization, the tubes were cooled to 25 °C, all samples were collected and centrifuged (at 2500 *xg* and 25 ºC) for 5 min. *v)* after centrifugation, all cell lysate supernatants were filtered using a PTFE Millipore® membrane (0.22 µm pore size) and stored (without light exposure) for further quantification of astaxanthin and β-carotene according to the methodology described below. The recovery yields (%) of astaxanthin and β-carotene were calculated as the ratio between the amount (in w/w) of carotenoid extracted with each solvent system (IL or eutectic mixture) relatively to the initial amount of carotenoid present in wet biomass, according to **Eq. 1** and **Eq. 2**.

$$Recovery\ of\ astaxanthin\ (\%\ w/w) = \frac{Astaxanthin\ extracted}{Total\ Astaxantin\ in\ P.\ rhodozyma\ \ biomass} \times 100 \quad \textbf{(Eq. 1)}$$

$$Recovery\ of\ \beta - carotene\ \%\ (w/w) = \frac{\beta - carotene\ extracted}{Total\ \beta - carotene\ in\ P.\ rhodozyma\ \ biomass} \times 100 \quad \textbf{(Eq. 2)}$$

Quantification of total carotenoid content

Astaxanthin and β-carotene total content were determined following the modified conventional extraction method described by Mussagy et al.[1] Briefly, 0.2 g of each *P. Rhodozyma* biomass was mixed with 1 mL of Acetone and disrupted by maceration (5 cycles of 10 min ON/10 min OFF). After the extraction, the supernatant was recovered, and the procedure repeated until the cells become fully bleached. The acetone-rich supernatants were recovered and evaporated. After the solvent evaporation, the colored extracts containing astaxanthin and β-carotene were redissolved in 1 mL of acetone and filtered with a PTFE membrane (0.22 µm pore size). The

quantification of astaxanthin and β-carotene was obtained from the visible-light absorption spectra using a Thermo Scientific® (Genesis 10S) UV-vis spectrophotometer). The visible-light spectra from 380 to 600 nm were acquired, and the respective carotenoids calibration curves at 455 nm (β-carotene) and 480 nm (astaxanthin) determined. The carotenoid concentrations (μg/mL) were quantified according to the astaxanthin and β-carotene standard calibration curves.

Preparation of biofilms

The biofilms were prepared using the eutectic mixtures [Ch]Cl:But (1:5) containing carotenoids-rich extracts ([Ch]Cl:But-carotenoids) at two-different concentrations [astaxanthin (0.8 and 4 μg/mL) and β-carotene (3 and 15 μg/mL)] mixed with corn starch (CS). Briefly, 1.8 g of CS were mixed with 60 mL of water and 5 mL of [Ch]Cl:But-carotenoids in a beaker at 80 °C and continuously stirred at 300 rpm for 1 h. The obtained liquid solution was dried at 45 °C for 48 h in a silicone plate produced in our lab (6 g biofilm-forming solution in 6 cm diameter silicone plate). A control biofilm was prepared using pure [Ch]Cl:But with the same molar ratio (1:5) instead of the [Ch]Cl:But-carotenoids. The [Ch]Cl:But acted as a green plasticizer and provided the acidic medium fundamental for dissolving the CS and form the colored biofilms.

FTIR and tensile behaviour of biofilms

Fourier transform infrared spectroscopy (FTIR) spectra of the dried biofilms samples were analyzed using a Total Attenuated Reflectance (ATR) method on the Fourier Transform Infrared (FTIR) spectrometer (TENSOR 27, Bruker®, Germany) in the region between 4000-400 cm^{-1}, with a HeNe laser source, DLaTGS detector, 4 cm^{-1} resolution and 32 scans. Tensile tests on the specimens (25 mm length, 19 mm width and 0.12 mm thickness) were carried out on a DL-2000 (EMIC®, Brazil) testing machine, with a 50 kgf load cell, initial grip separation of 10 mm and crosshead speed of 0.83 mm/s, at 25°C. The tensile strength and elongation at break values were collected from the stress-strain curves and the Young's modulus was calculated from the slope of the initial (linear elastic deformation) part of the stress-strain curve (0-5% elongation).

Determination of biofilms antioxidant activity using EPR

Two-biofilms samples (0.5 g), containing extracted astaxanthin and β-carotene were used for the determination of biofilms antioxidant activity, performing a DPPH• free-radical-scavenging activity test using Electron Paramagnetic Resonance (EPR) spectroscopy (also known as Electron Spin Resonance). The experimental protocol for antioxidant activity was carried out according to our previous work[2] with slight modifications. Briefly, biofilms samples were prepared by mixing 1 mL of standard DPPH• (40 μg/mL in methanol) with 0.5 g of biofilm with astaxanthin (0.8 and 4 μg/mL) and β-carotene (3 and 15 μg/mL) methanolic extracts. For the control assay, pure methanol was used. In order to evaluate the relative content of the radicals, after 15 min of reaction, all solutions were transferred to a capillary glass tube, sealed and placed on standard EPR quartz tubes (inner diameter 3.00 mm). The EPR spectra were recorded on a Bruker E-scan (Cambridge, UK) using a central magnetic field of 3480 G, a scanning field of 3495–3595 G, a scan time 50 s, a modulation amplitude of 3.29 G, a modulation frequency of 86.0 kHz and a microwave power of 10.93 mW. The area of peak-to-peak intensity spectrum was referred to as EPR signal. The reduction of DPPH• EPR signal was calculated according the **Eq. 3:**

$$ESR\ (\%) = 100 \times \frac{S_{DPPH} - S_{Sample}}{S_{DPPH}} \quad \textbf{(Eq. 3)}$$

where *ESR* correspond to EPR- Signal Reduction, and S_{DPPH} and S_{sample} correspond, respectively, to the EPR signals for solution containing DPPH• in the absence (control) and presence of the methanolic extracts (sample).

Statistical analysis

All experiments were performed in triplicate, and the results are expressed as the average of three independent assays with the corresponding errors at a 95% confidence level for each dependent variable (astaxanthin and β-carotene). Statistical analyses were performed using the R-Studio Software version 3.5.3 (Vienna, Austria). Values of $p \leq 0.05$ were considered statistically.

Table S3. Recovery of astaxanthin and β-carotene using DMSO, ILs and ES (80% v/v) at a concentration of 0.2 g mL^{-1} of *P. rhodozyma* wet cells after 1 h of stirring (300 rpm) at 65 °C.

Solvent	Recovery yields (% w/w)		pH	Viscosity (mPa.s)
	Astaxanthin	β-carotene		
Control (DMSO)	38.6 ± 0.4	43.1 ± 0.6	10.08	1.16
[Ch][Lac]	12.8 ± 0.1	17.0 ± 0.3	11.32	8.41
[Ch][But]	14.7 ± 1.8	21.2 ± 0.1	12.83	17.62
[Ch]Cl:Lac 1:1	4.0 ± 0.1	5.5 ± 0.1	0.76	15.93
[Ch]Cl:Lac 1:2	8.2 ± 1.2	10.1 ± 1.4	1.46	19.50
[Ch]Cl:But 1:1	-	-	-	-
[Ch]Cl:But 1:2	47.9 ± 0.9	46.1 ± 1.0	1.68	9.99

Table S4. Effect of HBA:HBD molar ratio on cell permeability at a concentration of 0.2 g mL^{-1} of *P. rhodozyma* wet cells after 1 h of stirring (300 rpm) in the recovery of astaxanthin and β-carotene at 65 °C using concentrated solutions of [Ch]Cl:But.

HBA:HBD molar ratio	Recovery yields (% w/w)	
	Astaxanthin	β-carotene
[Ch]Cl:But 1:2	58.7 ± 0.8	60.3 ± 1.9
[Ch]Cl:But 1:3	70.1 ± 0.4	70.7 ± 0.4
[Ch]Cl:But 1:4	65.7 ± 3.2	66.5 ± 3.0
[Ch]Cl:But 1:5	81.8 ± 0.3	82.3 ± 0.4
[Ch]Cl:But 1:6	77.6 ± 0.0	77.4 ± 1.3
[Ch]Cl:But 1:7	70.1 ± 0.8	74.5 ± 2.6
[Ch]Cl:But 1:8	75.0 ± 1.0	78.6 ± 0.1

Table S5. Effect of water content in solvent concentration on cell permeability at a concentration of 0.2 g mL^{-1} of *P. rhodozyma* wet cells after 1 h of stirring (300 rpm) in the recovery of astaxanthin and β-carotene at 65 °C.

Water content (% w/w)	Recovery yields (% w/w)	
	Astaxanthin	β-carotene
0%	81.8 ± 0.3	82.3 ± 0.4
10%	71.4 ± 3.9	78.1 ± 2.2
20%	68.9 ± 2.1	68.0 ± 1.8
30%	54.4 ± 4.7	51.1 ± 3.3
40%	41.1 ± 3.1	42.9 ± 3.1
50%	29.1 ± 0.5	35.7 ± 4.2

Table S6. Effect of SLR on cell permeability of *P. rhodozyma* wet cells after 1 h of stirring (300 rpm) in the recovery of astaxanthin and β-carotene at 65 °C using concentrated solutions of [Ch]Cl:But (1:5).

Solid Liquid ratio (g mL^{-1})	Recovery yields (% w/w)	
	Astaxanthin	β-carotene
0.05	52.6 ± 1.6	55.9 ± 1.0
0.10	63.0 ± 1.5	67.5 ± 1.8
0.20	82.3 ± 0.4	81.8 ± 0.3
0.50	44.7 ± 0.9	43.1 ± 1.0
1.0	16.4 ± 0.2	16.3 ± 0.0

Table S7. Effect of solvent nature on cell permeability of *P. rhodozyma* wet cells after 1 h of stirring (300 rpm) in the recovery of astaxanthin and β-carotene at 65 °C using precursors, ILs and ES aqueous solutions.

Solvent (50% w/w)	Recovery yields (% w/w)		pH	Viscosity (mPa.s)
	Astaxanthin	β-carotene		
[Ch]Cl	5.0 ± 0.3	6.5 ± 0.5	4.06	0.84
But	13.8 ± 1.6	15.5 ± 1.6	2.88	1.07
[Ch][But]	13.0 ± 0.3	14.7 ± 0.6	10.68	3.54
[Ch]Cl:But (1:5)	29.1 ± 0.5	35.7 ± 2.2	1.61	1.30

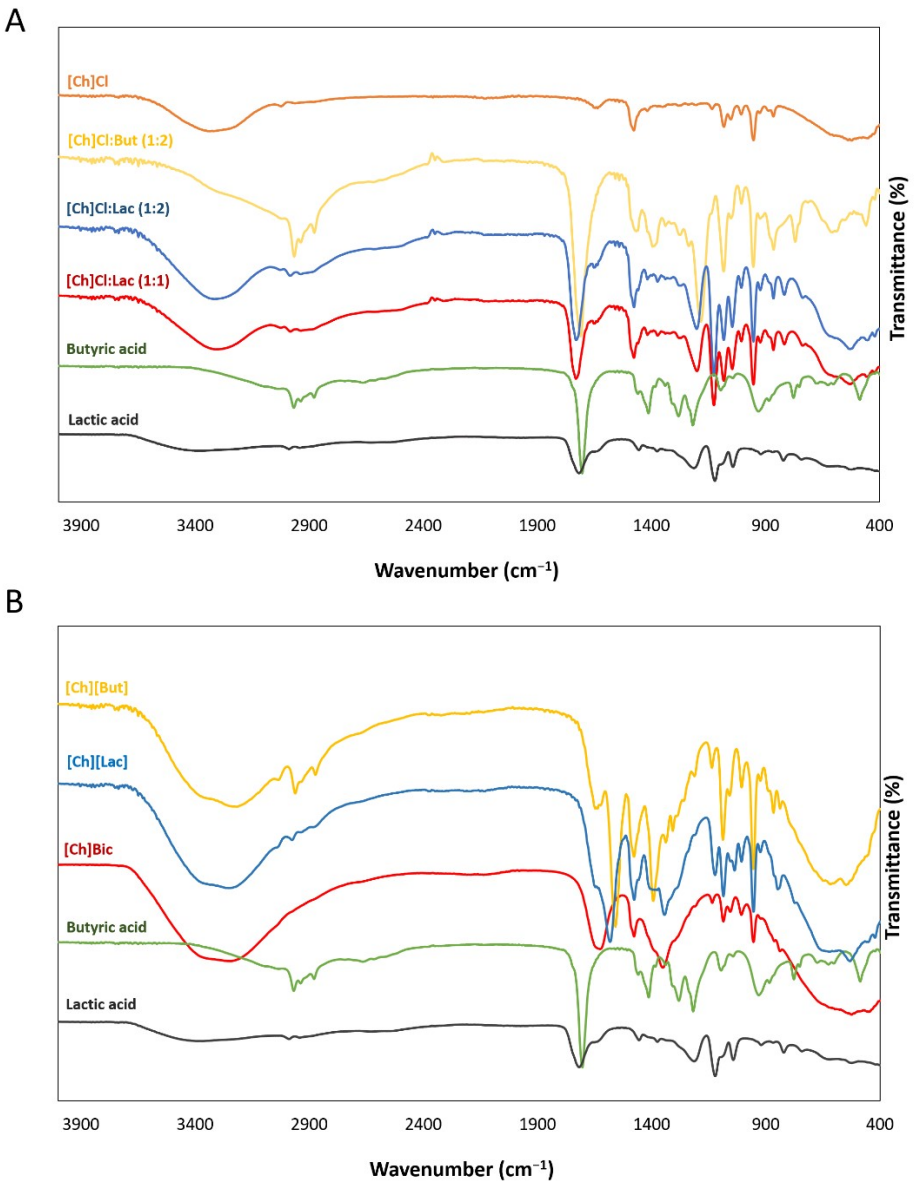

Fig. S1. FTIR spectra of precursors (Lac, But, [Ch]Bic and [Ch]Cl) and synthesized eutectic solvents ([Ch]Cl:Lac and [Ch]Cl:But) and ionic liquids ([Ch][Lac] and [Ch][But]).

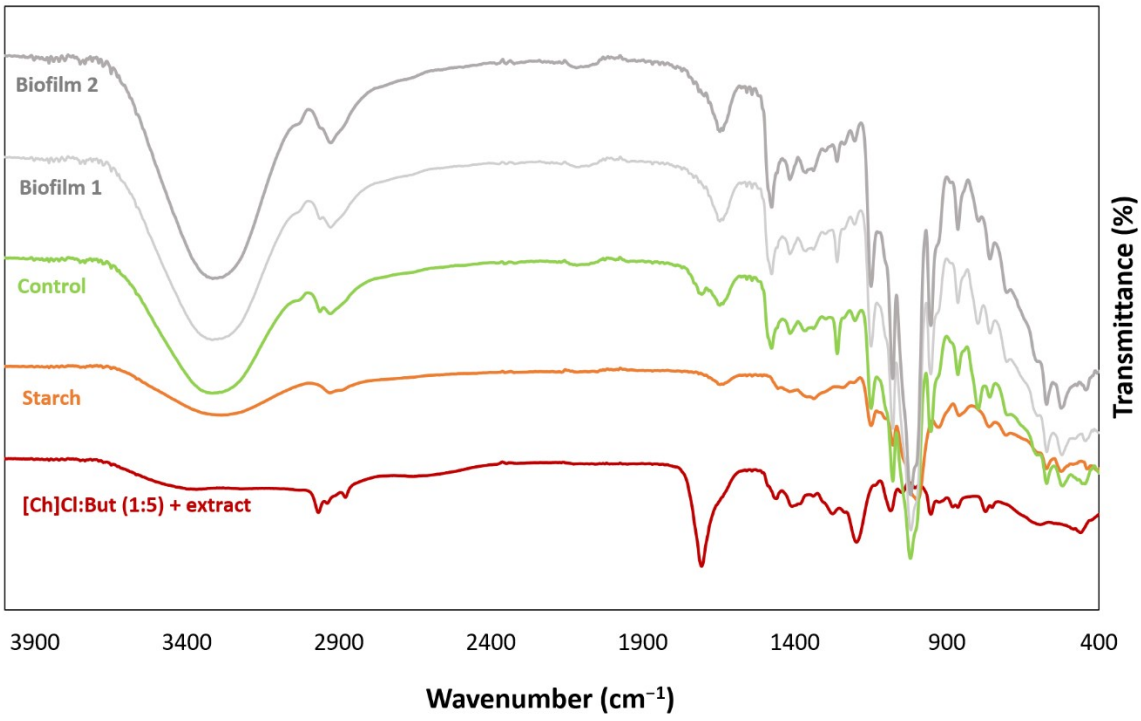

Fig. S2. FTIR spectra of the precursors and starch based-biofilms

References

1. C. U. Mussagy, V. C. Santos-Ebinuma, M. Gonzalez-Miquel, J. A. P. Coutinho and J. F. B. Pereira, *ACS Sustain. Chem. Eng.*, 2019, **7**, 16765–16776.

2. C. Mussagy, D. Remonatto, A. V. Paula, R. D. Herculano, V. C. Santos-Ebinuma, J. A. P. Coutinho and J. F. B. Pereira, *Sep. Purif. Technol.*, 2021, **266**, 118548.