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Synthesis of eugenyl acetate by enzymatic reactions in supercritical carbon dioxide

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ABSTRACT

Supercritical carbon dioxide (SC-CO_2) as reaction medium has gained attention in the production of terpenic esters catalyzed by lipases. Therefore, this work investigated the production of eugenyl acetate by esterification of eugenol and acetic anhydride in SC-CO_2 using two commercial lipases (Lipozyme 435 and Novozym 435) as catalysts. The influence of enzyme concentration (1–10% weight/weight), substrates' molar ratio (1:1 to 5:1), temperature (40–60 °C) and pressure of SC-CO_2 (10–30 MPa) on the esterification rate (X; %) and specific productivity (SP; kg of product/kg of catalyst x hour) were evaluated. A home-made high-pressure stirred-batch reactor (100 ml) was used in the experiments. The use of Novozym 435 achieved higher conversion and specific productivity of eugenyl acetate than Lipozyme 435. An excess of acetic anhydride (5:1 M/M) and high enzyme concentration (10%) achieved higher esterification rates than the lowest conditions (1% and 1:1 M/M). The optimal temperatures and pressure for the synthesis of eugenyl acetate in SC-CO_2 were 50 and 60 °C at 10 MPa, respectively. The phase behavior of the reaction system and the synthesis in organic medium were also studied. Kinetic experiments performed at 40, 50 and 60 °C indicated that the reaction follows the simple Ping-Pong Bi-Bi mechanism and the affinity of acetic anhydride to enzyme was larger than that of eugenol.

1. Introduction

Low molecular weight esters represent an important class of aroma, consisting of compounds derived from short chain acids such as acetates, propionates and butyrates, which are often responsible for fruity aroma [1–3]. These esters, known for their flavoring properties, are present in essential oils of natural matter, which are technically difficult to extract, isolate and purify. Furthermore, conventional chemical synthesis leads to the formation of undesirable products to the food and pharmaceutical industries. Therefore, biocatalyzed chemical synthesis becomes of great interest due to the high chemo-, regio- and stereo-selectivity of the enzymes, which provide possible in vitro synthesis of naturally existing single enantiomers of specific compounds [2]. Moreover, the esters can be considered as natural [2–4], since the process meets the required conditions by the legislation. For example, the substrates or raw materials used in process are natural and only physical or biotechnological processes must be employed for the isolation and purification of the formed products [5].

Lipases (glycerol ester hydrolases, EC 3.1.1.3) belong to the hydrolase group and are responsible for catalyzing the hydrolysis of glycerol esters and long-chain fatty acids, producing alcohol and acid [6]. In many research works, lipases have been employed as catalysts for the synthesis of esters, such as isoamyl acetate (banana flavor) [3,7], isoamyl butyrate (pear flavor) [8,9] and cinamyl acetate (a compound of cinnamon essential oil) [10]. Besides, a recent research showed that lipases are stable in pressurized fluids, which increased their potential use in esterification reactions [11]. Among the supercritical fluids (SC) used in industrial processes, carbon dioxide (CO_2) is the most common due to its advantages, such as low cost, nontoxicity, non-flammability, inertness, full recovery and moderate critical properties (P_c = 7.38 MPa, T_c = 304.2 K) when compared to other green solvents. Therefore, reactions in supercritical CO_2 can be carried out with low energy cost for pressurization, and at temperatures that do not damage the enzymes [12,13]. Moreover, if SC-CO_2 cannot improve reaction rate, the adjustable solvent power of the fluid allows the design of a production process

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with integrated downstream separation of products and unreacted substrates [11].

Eugenyl acetate is an aroma ester generally found in the essential oil of clove buds (*Syzygium aromaticum*). Besides eugenyl acetate, clove oil is rich in eugenol, beta-carophyllene, alfa-humulene and other minor compounds. The European Food Safety Authority (EFSA) evaluated and considered the application of eugenyl acetate safe as aromatic substance in food products. Actually, eugenyl acetate is listed on the database of the European Union as authorized substance to be used by the food industry [14]. Besides the flavoring property of eugenyl acetate, several works have reported other properties of industrial interest, such as antioxidant capacity [15], antimicrobial [16] and anticancer properties [17].

In this context, the aim of the present work was to investigate the synthesis of eugenyl acetate through enzymatic reactions in SC-CO_2 media. The effects of enzyme and substrates' concentration, temperature, pressure and number of reuse cycles of the enzymes were evaluated for two commercial immobilized lipases. The differences between two commercial immobilized lipases from *C. antartica* were shown. Moreover, experiments were performed to determine the kinetic parameters, and finally the phase behavior of the reaction system and the synthesis in organic medium were studied.

2. Materials and methods

2.1. Materials

Two commercial lipases from *Candida antarctica* (Lipozyme 435 and Novozym 435), both immobilized on a macroporous anionic resin, were kindly supplied by Novozymes Brazil (Araucária-PR/Brazil). The reagents eugenol and eugenyl acetate were obtained from Sigma Aldrich. Acetic anhydride, *n*-hexane and ethyl acetate were supplied by Synth (Diadema-SP/Brazil). All chemicals were analytical grade. Carbon dioxide (99.9%) was purchased from White Martins S.A. (Campinas-SP/Brazil).

2.2. Characterization of the enzymes

The activity of the immobilized enzymes was determined as the initial rate of the esterification reaction of oleic acid (Sigma Aldrich) with propanol (Sigma Aldrich) at a molar ratio of 3:1, with hexane (Synth) as reaction medium and enzyme concentration of 5% (w/w) in the substrates. The mixture was kept at 50 °C in a shaker incubator (TE-421, Tecnal, Piracicaba-SP/Brazil) at 150 orbitals per minute (OPM) for 30 min. Then, the oleic acid content was determined by titration with KOH 0.1 M in ethanol (Synth). A unit of activity (U) was defined as the amount of enzyme needed to consume 1 μmol of oleic acid per minute. All determinations of lipase activity were replicated at least three times. The residual activity (%) of the lipase was defined as the ratio between the activity of the untreated enzyme (U_0) and that of the lipase treated with SC-CO_2 (U), as stated in Eq. (1).

$$\textit{Residual Activity}\,(\%) = \left(\frac{U}{U_0}\right) x100 \quad (1)$$

The protein content of the enzymes was determined by the Lowry method [18], but the original method is not suitable for immobilized lipase forms, so a preliminary desorption step was executed as according to the method proposed by Petry et al. [19]. A known amount of immobilized lipase (0.1–0.2 g) was stirred in 2–4 ml of the extraction buffer/solvent, 10% formic acid in 45% acetonitrile (in water), for 2 h, at room temperature (22–25 °C). The material was washed three times with the same buffer/solvent for 10 min in each step, and finally with distillated water. The supernatant and washings were collected for protein analysis by the Lowry method [18], described as follows.

A standard curve was prepared with bovine serum albumin (BSA) powder (Sigma Aldrich). Samples, supernatant and washings were diluted in order to fit within the BSA standard curve range (0.02–0.6 mg/ml). 50 μl of sample and 450 μl of distilled water were placed in each tube. Next, 5 ml of biuret reagent was added to each tube and mixed thoroughly with a vortex. The biuret reagent was prepared by mixing three solutions: solution A: cupric sulfate at 1%; solution B: sodium potassium tartrate at 1%; solution C: 2% sodium carbonate in 0.1 M of NaOH with ratio of 1:1:50 (A:B:C). The mixture was then let incubating for 10 min prior to the addition of 500 μl per tube of 1.0 N Folin & Ciocalteu's reagent (Dinâmica, Diadema/SP), and the samples were mixed immediately. Color was developed for 30 min in the dark at room temperature and the absorbance was measured at 650 nm. All absorbance determinations were made using a UV–vis spectrophotometer (Hach, DR/4000U, Colorado, USA). All experiments were replicated at least three times.

The water content of immobilized lipases was determined by Karl Fischer titration using a model 701 Metrohm apparatus (Herisau, Switzerland) equipped with a 5 ml burette and an extractor, which was operated at 120 °C and a nitrogen (White Martins S.A., Campinas-SP/Brazil) flow rate of 50×10^{-9} ml/min. The Karl Fischer reagent used in the titration was from Merck (Darmstadt, Germany).

The mean particle size distributions of Lipozyme 435 and Novozym 435 were determined based on the static light scattering method using a Multi-Angle Static Light-Scattering Mastersizer (Malvern Instruments, Worcestershire, UK). The real densities of immobilized enzymes were measured by helium pycnometry, whereas bulk density was measured by weighing a known volume of solid material. Finally, the ratio between real and bulk density determined the porosity of the packed enzyme bed.

2.3. Synthesis of eugenyl acetate in SC-CO_2

The experimental homemade apparatus used in all reaction experiments consists in a CO_2 booster (Maximator M-111, Zorge/Germany), a solvent reservoir, a cooling (Solab SL152/18, Êxodo Científica, Hortolândia/SP, Brazil) and a heating thermostatic (Marconi S.A., Campinas-SP/Brazil) baths. Manometers (Zurich, São Paulo-SP/Brazil), a magnetic stirrer (IKA, RCT Basic, Staufen/Germany), thermocouples, control valves (Autoclave Engineers), a micrometric valve (Autoclave Engineers, Erie/PA, USA) and a stainless steel vessel of 100 ml. Fig. 1 shows the schematic flow diagram of the high-pressure stirred-bath reactor unit.

First, an amount of immobilized lipase was placed inside the high-pressure stirred-bath reactor. After 30 min of thermal stabilization and to remove the residual superficial water in the catalyst and the wall reactor, the reaction mixture formed by eugenol and acetic anhydride was introduced in the stirred-batch reactor. Next, the reactor was pressurized with CO_2 at a rate of 10 MPa min^{-1}. After the end of the established reaction time (1 h), the system was depressurized at 1 MPa min^{-1}. In all experiments the stirring rate was fixed at 600 rpm. The evaluated process variables were pressure (10–30 MPa), temperature (40–60 °C), enzyme concentration (1–10%), concentration of substrates (molar ratio from 1:1 to 5:1 of acetic anhydride: eugenol) and the reuse of the enzyme (1, 2 and 3 times). In the kinetic experiments each point of the curve at the same process condition represents the mean of the performed experiments at the established reaction time. All experiments were replicated at least three times.

From the amount of eugenol and eugenyl acetate after the reaction, the mass balance and the reaction stoichiometry, it was possible to determine the esterification rate or conversion (X, %),

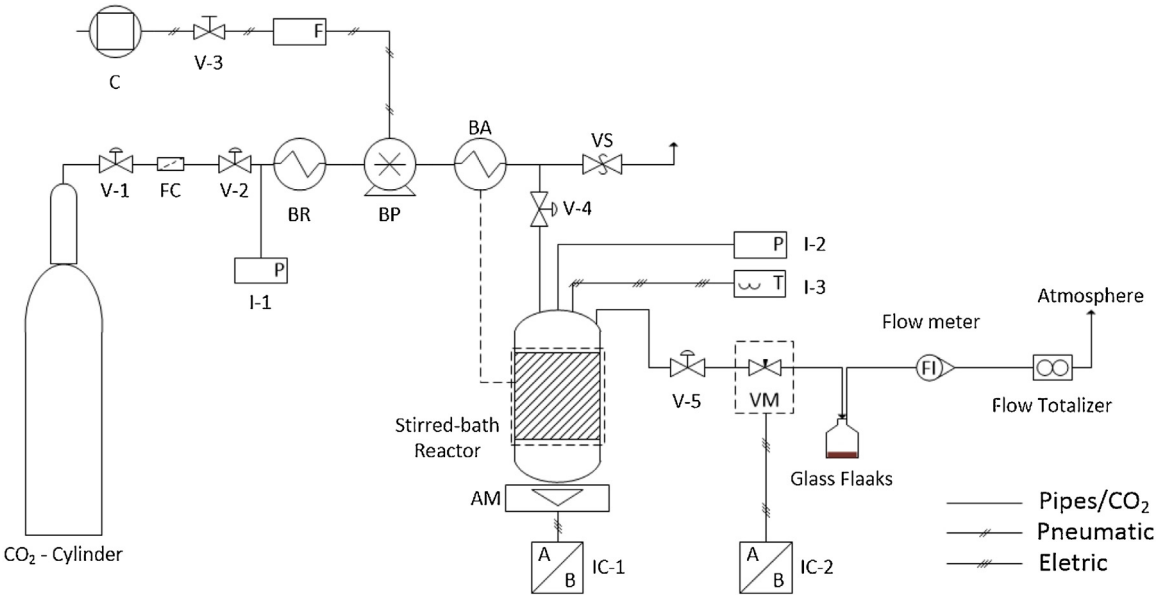

Fig. 1. Diagram of the homemade unit for high-pressure stirred-batch reactor containing: V-1, V-2, V-3, V-4 and V-5– Control valves; VM – Micrometer valves; VS – Safety valve (Pmax = 30 MPa); C- Compressor; F-Compressed air filter; FC – CO_2 Filter; BR – Cooling bath; BP- Pump (Booster); BA – Heating bath; I-1 and I-2– Pressure indicators; I-3 – Temperature indicators; IC-1–Indicators and controllers of magnetic stirrer; IC-2–Indicators and controllers of temperature of micrometer valve; AM – Magnetic stirrer.

which was obtained by the molar ratio between the products (n_p) and substrates (n_s), using Eq. (2):

$$X\,(\%) = \left(\frac{n_p}{n_s}\right) x100 \tag{2}$$

The specific productivity of eugenyl acetate (*SP*) was expressed as the mass ratio between product (m_p) and catalyst (m_c) per unit time (t), as shown in Eq. (3):

$$SP\left(kg/kg \times h\right) = \frac{m_p}{m_c \times t} \tag{3}$$

The esterification of eugenol was also performed in *n*-hexane for comparison with SC-CO_2. The reaction was carried out at atmospheric pressure and temperature, substrate molar ratio (5:1 acetic anhydride/eugenol) and concentration of enzyme (1%) equal to those used in the reactions in SC-CO_2 at the best condition of conversion and specific productivity. The reaction volume was completed to 100 ml of hexane and the mixture was incubated in a shaker incubator (TE-421, Tecnal, Piracicaba-SP/Brazil) under agitation of 150 orbitals per minute (OPM). After one hour an aliquot of 1.5 ml was filtered (Chormafil Xtra PA-20/25, Macherey-Nagel, Düren/Germany) and analyzed by gas chromatography (see Section 2.5.) to determine the concentrations of eugenol and eugenyl acetate. The experiments were replicated three times.

2.4. Phase behavior experiments

The solubility of substrates in SC-CO_2 depends on CO_2 density, which can be tuned by changes in temperature and/or pressure, and an increase in solubility of substrates in SC-CO_2 can lead to an increase in the reaction rate. On the other side, a decrease on reaction rate can occur due to formation of two phases (liquid and gas) [20]. Therefore, phase behavior experiments were performed through a static method without sampling in a high-pressure variable-volume view cell. Basically, the apparatus consists of a view cell with two sapphire windows for visual observations, a magnetic stirrer to promote the agitation in the mixture, an absolute pressure transducer (Novus, TP-Model 520, Brazil), a "PT 100" type thermocouple inside the cell, a CO_2 pump (Thermo Separation Products, Model Constametric 3200 P/F, Waltham/USA) that was coupled to a cooling ultra-thermostatic bath (Marconi S.A., Model MA-184, Campinas-SP/Brazil) and a metallic jacket on the cell coupled with a heating thermostatic bath (Polyscience, Illinois/USA). The equilibrium cell includes a movable piston, which allows pressure control inside the cell.

Phase behavior was visually observed through the pressure manipulation using the pump with CO_2 as pneumatic fluid. First, the amount of substrates and enzyme were introduced inside the view cell. The cell was closed and loaded with a determined amount of CO_2. After the desired temperature was reached, the pressure was increased until the observation of a single phase. From this point, the pressure was slowly decreased until the apparition of a new phase. Three temperatures were evaluated (40, 50 and 60 °C) with enzyme concentration and substrates' molar ratio of 1% and 5:1 (acetic anhydride/eugenol), respectively, which are the same used in the reaction experiments.

2.5. Analytical methods

After the reaction the reactant (eugenol) and product (eugenyl acetate) contents were determined using a gas chromatograph with a flame ionization detector (GC-FID). The method was based according to Zabot et al. [21]. A GC–FID chromatograph (Shimadzu, CG17A, Kyoto/Japan) equipped with a capillary column of fused silica DB-5 (J&W Scientific, 30 m × 0.25 mm × 0.25 μm, Folsom,/USA) was used. Each sample was filtered (Chormafil Xtra PA-20/25, Macherey-Nagel, Düren/Germany) and diluted to 5 mg/ml in ethyl acetate, and 1 μl was injected into the chromatograph. The sample split ratio was 1:20. The carrier gas (Helium, 99.9% purity, White Martins, Campinas,/Brazil) flowing at 1.1 ml/min. The injector and the detector temperatures were 220 and 240 °C, respectively. The column was heated from 60 °C to 246 °C at 3 °C/min. At these conditions the retention times of eugenol and eugenyl acetate peaks were 24 and 32 min, respectively. Quantification was performed using external standard calibration curves (R^2 = 0.999 to eugenol and R^2 = 0.996 to eugenyl acetate).

2.6. Kinetic model

The experimental data of conversion versus time were adjusted with a second-order model, as shown in Eq. (4). The quality of this adjustment was evaluated from the determination coefficient (R^2) using the MATLAB software (2014Ra, MathWorks, Natick, MA, USA)

$$X(\%) = at^2 + bt + c \quad (4)$$

where $X$is the conversion (%), t is time (h) and a,b and c are adjustable parameters.

Through the analytical derivative of the adjusted model for eugenol conversion, the eugenol conversion rate at each time t was calculated, as shown in Eq. (5).

$$v = C_{e,0}\left(\frac{dX}{dt}\right) \quad (5)$$

where v is the reaction rate at the time t (mol/l s), $C_{e,0}$ is the initial ($t = 0$) concentration of eugenol (mol/l) and dX/dt is the analytical derivative of the conversion calculated with Eq. (4).

Next, the reaction rate was adjusted with the Ping-Pong Bi-Bi model without inhibition, as described by Eq. (6).

$$v = \frac{V_{\max}[A][B]}{K_m^B[A] + K_m^A[B] + [A][B]} \quad (6)$$

where $V_{\max}$ is the maximum reaction rate (mol/l g s), $[A]$ and $[B]$ are the acetic anhydride and eugenol concentrations (mol/l), respectively, and K_m^A and K_m^B are the Michaelis-Mentem constants for acetic anhydride and eugenol (mol/l).

The Ping-Pong Bi-Bi model was fitted to the calculated reaction rate (v) using a minimum of constrained nonlinear multivariable function algorithm from the MATLAB software. The objective function was defined as sum of the squares, as shown in Eq. (7).

$$f_{obj} = \sum_{i=1}^{n}\left[v_{exp} - v_{sim}\right]^2 \quad (7)$$

where v_{exp} is the experimental reaction rate (mol/l s), v_{sim} is the simulated reaction rate (mol/l s) and n is the total of experimental points.

The activation energies were obtained from the slope of the straight line of $ln\left(\frac{k_B T}{hK_m}\right)$ *vs* reciprocal of absolute temperature (1/T), as described by the Arrhenius Eq. (8) and the Eyring absolute rate equation [22,23].

$$\ln\left(\frac{k_B T}{hK_m}\right) = -\frac{E_a}{RT} + \ln(A) \quad (8)$$

where K_m is the Michaelis-Menten constant,h is the Planck constant (6.6256×10^{-34} J s), k_B is Boltzman constant (1.3805×10^{-24} J/K), A is the frequency factor, E_a is the activation energy and R is the molar gas constant (8.314 J/K mol).

2.7. Statistical analysis

The results of conversion and specific productivity of eugenyl acetate were statistically evaluated by ANOVA, using the software Statistica for Windows 6.0 (Statsoft Inc., USA) in order to detect significant differences. The significant differences at level of 5% ($p < 0.05$) were analyzed by the Tukey test.

3. Results and discussion

3.1. Effect of the enzyme concentration

In industrial applications the process variables should be controlled to obtain the maximum specific productivity with minimum cost, specifically in biocatalyzed processes where high esterification and low enzyme concentrations should be achieved. Therefore, the effects of enzyme concentration and substrates' molar ratio on the conversion and specific productivity of eugenyl acetate using Lipozyme 435 and Novozym 435 were evaluated. In these experiments the temperature and pressure were fixed at 40 °C and 10 MPa, respectively. In these conditions the immobilized lipases have shown their highest residual activities [24–26]. Moreover, the stirring rate was fixed in a high value to ensure that the reaction would be kinetically controlled and the inter-particle diffusion and external mass transfer effects were negligible [27,28]. The results are shown in Table 1.

The ANOVA results show that the effect of enzyme concentration on the conversion was statistically significant ($p < 0.05$ and $F_{cal} = 37.97$) using immobilized lipase Lipozyme 435, and not significant for Novozym 435 ($p = 0.362$ and $F_{cal} = 1.14$). It can be observed that, for Lipozyme 435, the highest conversion of eugenyl acetate was obtained with high concentration of the enzyme (10%) at the three evaluated substrate molar ratios, but for Novozym 435 this behavior was not observed in any condition. Moreover, at the highest substrate molar ratio 5:1 (acetic anhydride/eugenol) for Lipozyme 435, the concentration of lipase did not affect the conversion. The same behavior was found to the specific productivity of eugenyl acetate, but the magnitude of the specific productivity decreases with the amount of enzyme increase, even with an increase of reaction rate.

The increase of enzyme loading results in higher reaction rate, as exposed on Table 1. Although the reaction time was kept constant at one hour, the highest rate and specific productivity for both commercial lipases were obtained with 1% enzyme and 5:1 molar ratio. Eventually, with longer reaction time some experimental runs would overlap for larger enzyme content. However, in the industrial point of view it is interesting to use lower amount of catalyst (enzyme), which results in reduced cost. Other works using SC-CO_2 as reaction medium obtained similar results [29,30]. Chiaradia et al. [16], studying the eugenyl acetate esterification in a solvent-free system found a significant effect of the enzyme (Novozym 435) concentration on the conversion, and concluded that lower concentrations can be used without affecting the reaction conversion. It must be emphasized that the conversion increased with the substrates' concentration, but at the same molar ratio, 5:1, the increase of enzyme concentration from 5.5 to 10% did not affect the reaction rate (conversion). This probably happened because in these conditions the amount of substrates was the controlling factor of the reaction rates instead of the enzyme concentration. Furthermore, the reaction rates achieved in these experiments were not large enough to compensate the amount of used immobilized lipase, thus the lowest specific productivities were obtained.

3.2. Effect of acetic anhydride and eugenol molar ratio

Acetic acid has been traditionally used as acyl donor to flavor ester reactions (direct esterification) [31]. Moreover, several woks have reported an inhibition of enzyme by high concentration of acid [3], probably due to the decrease of pH in the reaction system. In addition, acetic acid can be a lipase inhibitor, reacting with serine in the active site of the enzyme [32]. Alternatively, the synthesis of esters can be performed by transesterification with acetates and by acylation with acetic anhydride [7]. Table 1 shows the conversions and specific productivities obtained with three acetic anhydride concentrations and three different enzyme loadings.

A positive effect of molar ratio on the conversion and specific productivity of eugenyl acetate was observed, and the ANOVA results showed that the acetic anhydride/eugenol molar ratio was statistically significant in the conversion using both immobilized lipases, Lipozyme ($p < 0.05$ and $F_{cal} = 104.20$) and Novozym 435

Table 1
Effect of enzyme concentration and substrates' molar ratio on the conversion and specific productivity of eugenyl acetate using two different lipases in SC-CO_2.

Enzyme (%)[1]	Molar Ratio[2]	Conversion (%)		Specific productivity (kg/kg h)	
		Lipozyme 435	Novozym 435	Lipozyme 435	Novozym 435
1	1:1	4.42 ± 0.18^{eA}	1.63 ± 0.02^{eB}	3.29 ± 0.02^{abA}	1.21 ± 0.04^{cdB}
1	3:1	6.50 ± 0.25^{deB}	15.60 ± 0.69^{cdA}	2.59 ± 0.10^{bcB}	6.73 ± 0.45^{bA}
1	5:1	19.34 ± 1.34^{aB}	33.23 ± 0.26^{aA}	6.22 ± 0.41^{aB}	10.09 ± 0.07^{aA}
5	1:1	12.27 ± 2.53^{bcA}	5.11 ± 0.54^{eB}	1.89 ± 0.39^{cdA}	0.78 ± 0.08^{dB}
5	3:1	15.70 ± 0.27^{abB}	19.61 ± 0.85^{cA}	1.37 ± 0.02^{deB}	1.71 ± 0.07^{cA}
5	5:1	18.50 ± 1.20^{aB}	26.58 ± 0.36^{bA}	1.13 ± 0.12^{deB}	1.62 ± 0.02^{cA}
10	1:1	10.34 ± 0.95^{cdB}	14.45 ± 2.92^{dA}	0.80 ± 0.07^{eA}	1.11 ± 0.21^{cdA}
10	3:1	16.30 ± 0.01^{abB}	19.37 ± 0.99^{cA}	0.72 ± 0.01^{eB}	0.84 ± 0.04^{dA}
10	5:1	20.27 ± 1.00^{aA}	19.51 ± 0.49^{cA}	0.64 ± 0.03^{eA}	0.60 ± 0.01^{dA}

Experiments were carried out at fixed conditions of time (1 h), stirring (600 rpm), pressure (10 MPa) and temperature (40 °C). Different indexes (a, b, c, d) in the same column (lowercase) or in the same line (uppercase) indicate that the means differ significantly by Tukey's test ($p \leq 0.05$). For example, lowercases compares the means at different experimental conditions with the same enzyme and uppercases compares the means of different enzymes at the same experimental condition.

[1] Enzyme concentration expressed by weight of substrates, w/w (%).

[2] Acetic anhydride/eugenol molar ratio (M/M).

Table 2
Characteristics of two different immobilized lipases from *Candida antarctica*.

Characteristic	Novozym 435	Lipozyme 435
Enzymatic Activity (U/g)	167.1 ± 0.39	173.9 ± 0.36
Protein Content (mg/g of particle)	85.4 ± 5.41	108.2 ± 6.38
Specific Enzymatic Activity (U/mg)	1.95 ± 0.07	1.60 ± 0.01
Water Content (g/g, %)	1.62 ± 0.04	2.71 ± 0.06
Bulk Density (g/cm^3)	0.35 ± 0.01	0.35 ± 0.01
Real Density (g/cm^3)	0.96 ± 0.01	1.21 ± 0.06
Porosity (ad.)	0.64 ± 0.01	0.71 ± 0.01
Mean Diameter (μm)	350.3 ± 2.5	452.4 ± 12.6

($p < 0.05$ and $F_{cal} = 447.40$). In fact, the major effect on the conversion was that of acetic anhydride/eugenol molar ratio. The same behavior was observed by Chiaradia et al. [16], who concluded that the molar ratio of acetic anhydride/eugenol presented a positive effect, followed by the effect of temperature on eugenyl acetate production in a solvent-free system using Novozym 435. It is also observed on Table 1 that the conversion and specific productivity of eugenyl acetate using Novozym 435 was higher than that of Lipozyme 435 at all experimental conditions, except under the lowest molar ratio with 1 and 5% of enzyme, which is probably due to experimental errors. In order to explain the reasons why Novozym 435 outperformed Lipozyme 435 in the eugenol conversion, the characterization of both lipases was performed and the results are exposed on Table 2.

According to the enzyme supplier, Lipozyme 435 is food grade while Novozym 435 is analytic or technical grade, information also reported by Melgosa et al. [24]. This can be confirmed by the specific enzymatic activity (SA), which represents the ratio between the enzymatic activity and protein content. The SA of Lipozyme 435 was 1.6 U/mg while Novozym 435 achieved SA about 18% higher, 1.9 U/mg. The water content of Novozym 435 was similar to that found by Habulin et al. [33] (1.44%) and lower than the obtained by Yadav and Devi [34] (3%), but the titrable water content for Lipozyme 435 was about four times higher than that reported by Melgosa et al. [24] ($0.7 \pm 0.2\%$).

Particle size, porosity and density are important characteristics of catalysts, which can influence mass transfer and intraparticle diffusion of substrates and products, and consequently the reaction rate and specific productivity. The measured bulk density for both immobilized lipases was the same as reported by the enzyme supplier, and closer to that reported by Yadav and Devi [34] for Novozym 435 (0.45 g/cm^3). However, the real density of Lipozyme was higher than that of Novozym 435. In fact, the immobilization material is the same for both lipases (macroporous resin of poly-methyl methacrylate (Lewatit VP OC 1600)) [24,35], but since Lipozyme 435 contains more protein with lower SA, these proteins may also have been immobilized during the immobilization process, increasing the real density of the particles. Moreover, the mean particle diameter of Lipozyme is higher than that of Novozym 435. According to Yadav and Devi [34] the bead size range of Novozym 435 is 0.3–0.9 mm, and the same particle distribution was observed for both immobilized lipases. Despite the higher specific enzymatic activity of Novozym 435, the study of the effects of SC-CO_2 pressure and temperature on the conversion and specific productivity of eugenyl acetate was performed with Lipozyme 435 because few papers dealing with enzymatic reactions using a food grade immobilized lipase (Lipozyme 435) are found in literature.

3.3. Effect of pressure and temperature of SC-CO_2

Based on the results shown on Table 1, the effects of temperature and pressure were evaluated at the conditions that achieved high conversion and specific productivity. Thus, the enzyme concentration and acetic anhydride/eugenol molar ratio were kept constant at 1% and 5:1, respectively. According to Oliveira et al. [25] for Novozym 435 and Melgosa et al. [24] for Lipozyme 435 and Lipozyme RM IM, the increase of temperature in a supercritical reaction process causes a decrease of the residual activity of enzymes. The same behavior was reported to pressure by several works [24,25,36]. Indeed, according to Knez, Habulin and Primožič [37] the pressure-induced deactivation of enzymes occurs mostly above 150 MPa. Based on the literature survey, the experiments were performed at temperatures from 40 to 60 °C and pressures from 10 to 30 MPa. Table 3 shows the effects of temperature and pressure on the conversion and specific productivity of eugenyl acetate using the Lipozyme 435 as catalyst.

The ANOVA analyses showed that the effects of pressure and temperature were statistically significant ($p < 0.05$ and $F_{cal} > F_{tab}$) on the conversion and specific productivity of eugenyl acetate. A positive effect of pressure at 40 °C and a negative effect at 50 and 60 °C can be observed on Table 3. The positive effect can be related to the increase of CO_2 density with pressure, which improves its solvation power in the reaction medium [20]. In turn, the negative effect on the conversion can be connected with the dilution of the substrates caused by the greater amount of CO_2 pumped into the reactor, causing an increase in the molar ratio between SC-CO_2 and substrates [20,38]. Knez et al. [38] studied the effect of pressure on the conversion of free fatty acid into fatty acid ester using lipase from *Rhizomucor miehei* (Lipozyme RM IM) in dense CO_2. According to the authors, at pressures above 10 MPa the conversion decreased due to the dilution effect caused by larger CO_2 amount, which reduced the substrates' molar fraction in the reaction bulk, slowing down the esterification.

Table 3
Effect of temperature and pressure on the conversion and specific productivity of eugenyl acetate using Lipozyme 435.

Temperature (°C)[1]	Pressure (MPa)[2]	Conversion (%)	Specific productivity (kg/kg h)
40	10	19.34 ± 1.34^{c}	6.22 ± 0.41^{b}
40	20	20.76 ± 0.25^{c}	6.38 ± 0.08^{b}
40	30	22.80 ± 0.08^{c}	7.01 ± 0.02^{b}
50	10	32.26 ± 1.25^{a}	9.77 ± 0.36^{a}
50	20	21.17 ± 0.85^{c}	6.23 ± 0.26^{b}
50	30	24.77 ± 3.80^{bc}	7.53 ± 1.15^{b}
60	10	30.75 ± 0.74^{ab}	9.47 ± 0.23^{a}
60	20	20.17 ± 1.85^{c}	6.05 ± 0.35^{b}
60	30	24.70 ± 0.26^{bc}	7.51 ± 0.19^{b}

Experiments were carried out at fixed conditions of time (1 h), agitation (600 rpm), enzyme concentration (1%) and acetic anhydride/eugenol molar ratio (5:1). Different indexes (a, b, c, d) in the same column (lowercase) indicate that the means differ significantly by Tukey's test ($p < 0.05$).
[1] Temperature.
[2] Pressure of SC-CO_2.

The effect of temperature from 40 to 50 °C was positive for the conversion, from 19 to 32% at 10 MPa, from 20 to 21% at 20 MPa and from 22 to 24% at 30 MPa. Besides, the increase of temperature from 50 to 60 °C caused a decrease on the conversion, which can be related to the changes in the physical properties of the solvent, such as limitations in mass transfer, viscosity, surface tension and due to the decrease of the solvation power of the substrates at lower solvent density. The esterification of eugenol was also carried out in *n*-hexane for comparison with the results obtained at supercritical conditions. The conversion in *n*-hexane was $15.3 \pm 1.8\%$, whereas in SC-CO_2 (10 MPa and 40 °C) it was near 19%. According to Laudani et al. [39], the higher diffusivity and lower viscosity and surface tension of SC-CO_2 are responsible for the reduction of interphase transport limitations, thus increasing the reaction rate and esterification of eugenol.

Researches dealing with esterification reactions in supercritical media have shown that the conversion can be influenced by the solubility of substrates in SC-CO_2, which depends on the system's temperature and pressure. The solubilities of eugenyl acetate and eugenol in SC-CO_2 were reported by Cheng et al. [40], Souza et al. [41] and Guan et al. [42], who showed that the solubility of these compounds increases with CO_2 density. However, few publications dealing with phase equilibrium of the whole enzymatic esterification system have been found in the literature. Phase equilibrium experiments of the system eugenol/acetic anhydride/Lipozyme 435/SC-CO_2 were performed to observe the behavior at each experimental condition exposed on Table 3, and the results are shown in Appendix A (Supplementary data, Fig. S1).

At 10 MPa and 60 °C the reaction mixture contained solid (enzyme), liquid and gas phases, and at the other conditions the equilibrium between solid and supercritical phase was observed. The transition from three-phase (solid-liquid-gas) to two-phase (solid-supercritical) system at 40, 50 and 60 °C was observed at pressures around 8.0–8.2, 9.1–9.3 and 11–13 MPa, respectively. At the end of all phase equilibrium experiments a white and opaque solid material was observed on the view cell. This material can be observed in Fig. S1 (Appendix A: Supplementary data) at conditions of 50–60 °C and 20–28 MPa, where the reaction bulk became more opaque. This solid material may be a small particle displaced from the support of the immobilized lipase. Moreover, this can help elucidating the decrease of conversion at high temperature and pressure observed in the reactions using Lipozyme 435.

3.4. Reuse of Lipozyme 435

The main advantage of immobilized lipases over the free form is that the enzyme can be reused many times in the process, thus reducing the cost of catalyst. Several works have shown that the enzyme activity decreases continuously with the increasing number of cycles, so the reusability of Lipozyme 435 was analyzed at 10 MPa and 40 °C, as shown on Table 4. The substrates and products were extracted from the high-pressure vessel at 2 h with a CO_2 flow rate of 2.84×10^{-4} kg/s, which was enough to remove all the compounds from the reactor, as established by previous experiments. The data on Table 4 show that the conversion and specific productivity of eugenyl acetate decreased continuously with the increasing number of cycles. The catalytic ability of the lipase CALB (*Candida antarctica* lipase B) in an immobilized form decreases with the increase of the utilization cycles [20] or depressurization/pressurization cycles [24,25]. The reduction of enzyme activity after SC-CO_2 exposure has been related to several reasons. One of them is the water content needed to preserve the conformation of enzyme, and thus its optimal activity [24,43]. Therefore, the effect of the number of depressurization/pressurization cycles on the residual activity (%) and water content (%) of Lipozyme 435 treated with SC-CO_2 was evaluated. Each cycle was carried out at 40 °C, 10 MPa, 1 h of exposure time, and the depressurization rate was fixed at 1 MPa min^{-1}. The results are presented in Fig. 2.

Table 4
Influence of reuse cycles of Lipozyme 435 on the conversion and specific productivity of eugenyl acetate in SC-CO_2 at 10 MPa and 40 °C.

Number of Cycles	Conversion (%)	Specific productivity (kg/kg h)
1	19.34 ± 1.34	6.22 ± 0.41
2	17.60 ± 0.29	5.45 ± 0.60
3	13.10 ± 0.06	4.43 ± 0.02

Experiments were carried out at fixed conditions of time (1 h), agitation (600 rpm), enzyme concentration (1%) and acetic anhydride/eugenol molar ratio (5:1).

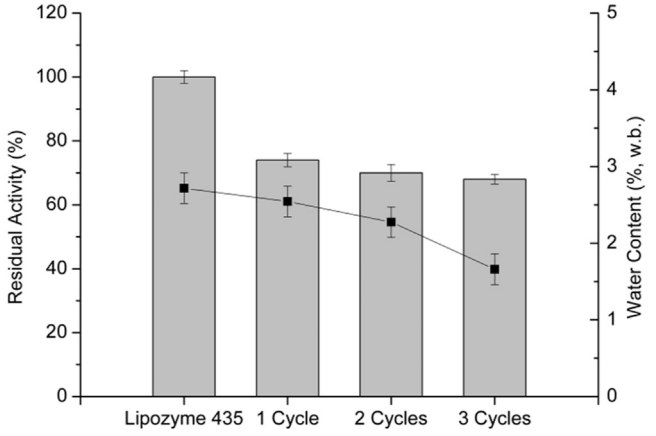

Fig. 2. Effect of the number of depressurization/pressurization cycles on the residual activity (bar chart, %) and water content (■, %) of Lipozyme 435 treated with SC-CO_2 at 10 MPa and 40 °C.

It can be noted that the residual enzyme activity and the water content decreased with the increase of the number of depres-

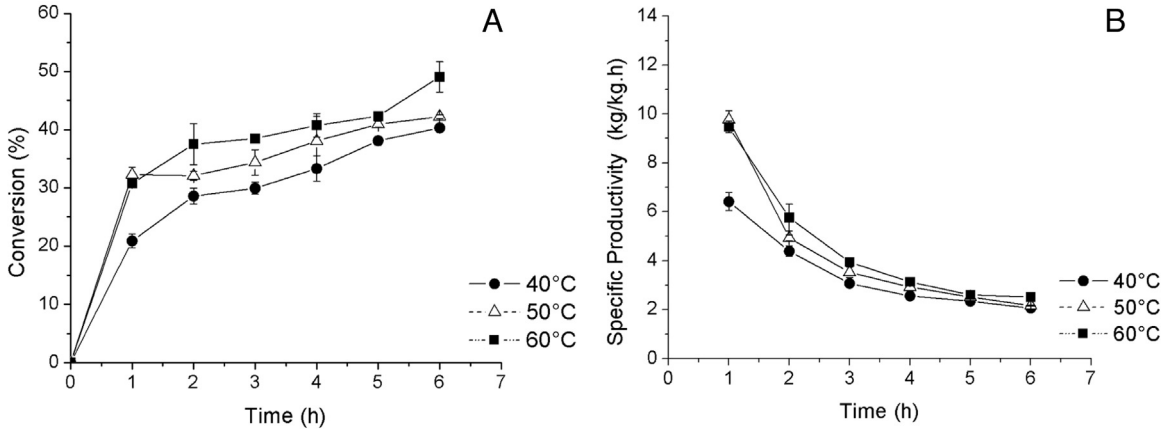

Fig. 3. Kinetics of eugenyl acetate conversion (A) and specific productivity (B) in SC-CO_2 at 40, 50 and 60 °C. Experiments were carried out at fixed conditions of pressure (10 MPa), agitation (600 rpm), enzyme concentration (1%) and acetic anhydride/eugenol molar ratio (5:1).

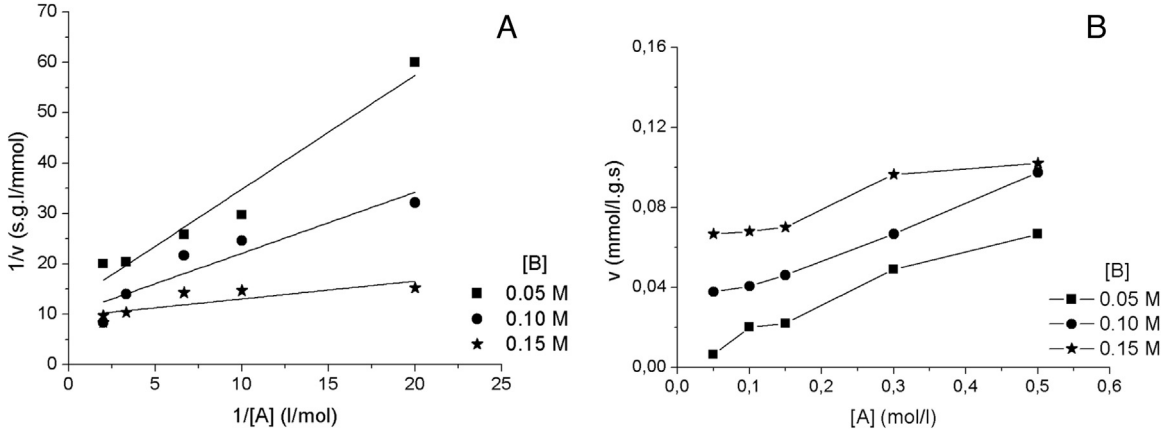

Fig. 4. (A) Lineweaver-Burk plot of reciprocal of the reaction rates versus reciprocal acetic anhydride [A] concentration at different eugenol [B] concentrations. (B) Effect of acetic anhydride [A] concentration on the reaction rate at different eugenol [B] concentrations. Experiments were carried out at fixed conditions of agitation (600 rpm), enzyme concentration (1%), time (1 h), temperature (40 °C) and pressure (10 MPa).

surization/pressurization cycles. The initial titrable water content of Lipozyme 435 was 2.71 ± 0.06%, and after exposure to SC-CO_2 it decreased to 2.54 ± 0.20, 2.27 ± 0.22 and 1.66 ± 0.19%, for 1, 2 and 3 cycles respectively. These results of residual activity are in accordance with those reported by Melgosa et al. [24] for Lipozyme RM IM and Lipozyme 435, in which several depressurization/pressurization steps led to activity loss of both immobilized lipases. Moreover, the authors did not report the water content of the Lipozyme 435 after treatment with SC-CO_2, because of the low values found. Habulin et al. [33] related the decrease of residual activity to the extraction of essential water from the immobilized lipase Novozym 435, in which the water content fell from 1.44% for the untreated enzyme to 0.88% for lipase treated with SC-CO_2.

3.5. Kinetic experiments

The kinetics of the production of eugenyl acetate was investigated at 10 MPa, with 1% (w/w) of enzyme, 5:1 molar ratio and stirring at 600 rpm. The results are shown in Fig. 3(A) for conversion (%) and (B) for specific productivity (kg/kg h). It can be observed that, at longer times, the conversion increased for the reaction at the three tested temperatures, 40, 50 and 60 °C. However, the specific productivity of eugenyl acetate decreased with time for all temperatures. This behavior can be explained observing Eq. (3), which suggests that the increase of reaction time decreases its specific productivity. To keep the specific productivity at similar values for all reaction times the conversion should be twice the obtained at the previous point. Based on the kinetic experiments and the phase behavior, the optimal conditions for the synthesis of eugenyl acetate were 50 and 60 °C at 10 MPa. Although a two-phase region (liquid and gas) appears at 60 °C and 10 MPa, the reaction rate was almost equal to those of the experiments at 50 °C and 10 MPa.

The kinetics of many reactions have been successfully described by the Ping-Pong Bi-Bi model, such as hydrolysis, esterification and transesterification, using organic solvents [22,44] or supercritical fluids [29,30] as reaction media. In the kinetic study it is first necessary to determine possible inhibitions caused by any substance involved in the reaction [44]. In this work, the substrates are eugenol and acetic anhydride, and the products are eugenyl acetate and acetic acid. A second route of eugenol esterification can occur between acetic acid and eugenol, resulting in eugenyl acetate and water. This second route and the effect of acetic acid on the kinetics were not considered. The reaction routes are shown in Appendix A (Supplementary data, Fig. S2). However, acetic acid is an important inactivating compound, causing an indirect inhibition by the decrease of pH in the reaction medium, changing the isoelectric point of proteins and thus inactivating the enzyme. Moreover, acetic acid can also act as a direct inhibitor of the enzyme, forming a dead-end complex. According to Romero et al. [44], this effect has little importance when the initial concentration of substrates is low. Fig. 4 shows the Lineweaver-Burk plot of reciprocal of the reaction rates versus reciprocal concentrations of acetic anhydride [A] and

Table 5
Kinetic parameters for the production of eugenyl acetate in SC-CO_2 at 40, 50 and 60 °C using Lipozyme 435.

Parameter	40 °C	50 °C	60 °C
V_{max}(μmol/g s)	3.6	4.2	4.5
K_m^a (μM)	17.0	17.5	14.0
K_m^b (μM)	260.2	190.0	147.5
R^2 (%)[a]	97.1	97.5	94.9
f_{obj}[b]	8.0×10^{-14}	0.7×10^{-14}	3.0×10^{-14}

[a] Lack of fit to Eq. (4).
[b] Values for adjustment of Ping-Pong Bi-Bi model (Eq. (6)).

eugenol [B] at 40 °C and 10 MPa (A) and the effect of substrates on the reaction rate (B).

The variation of the concentrations of the substrates acetic anhydride [A] and eugenol [B] caused an increase on the reaction rate, as shown in Fig. 4(B). Moreover, in the plot of Fig. 4(A) the esterification of eugenol followed the classical Michaelis-Menten kinetics. However, the three fitting straight lines in Fig. 4(A) were not parallel to each other, suggesting that the enzymatic reaction did not follow a simple Ping-Pong Bi-Bi mechanism within the range of tested concentrations [22,45]. The kinetic parameters were obtained by fitting the model to the experimental data using a subroutine (*fminsearch*) of the software MATLAB [29]. The values calculated by the Lineweaver-Burk plot of reciprocal of the reaction rates versus reciprocal concentrations were used as initial guess. Table 5 shows the kinetic parameters adjusted with the model for the production of eugenyl acetate in SC-CO_2.

The affinity of the enzyme to the substrates can be determined by the value of $1/K_m$, which indicates that the affinity of the enzyme to acetic anhydride is larger than to eugenol. This suggests that acetic anhydride is the acyl donor that binds to the active sites of lipase. Similar behavior also was observed by Romero et al. [41], investigating the esterification of isoamyl alcohol and acetic anhydride using immobilized lipase in *n*-hexane. According to the authors, the Ping-Pong Bi-Bi mechanism can be divided in two steps: a) First, the acyl donor binds the enzyme, forming an enzyme-anhydride complex (EA). Through a unimolecular isomerization reaction, the EA complex is transformed to an enzyme-acyl complex (EC) and the first product (acetic acid) is released; b) The second substrate (eugenol) binds to the enzyme-acyl complex (EC) forming a ternary complex, enzyme-acyl-eugenol. Again, through a unimolecular isomerization reaction the ternary complex is transformed to an enzyme-ester complex, finally releasing the second product (eugenyl acetate) and the enzyme in its initial conformation.

It can be noted on Table 5 that the affinity of both substrates (K_m^A and K_m^B) to the lipase and the maximum reaction rate increased from 40 to 60 °C, suggesting a positive effect of temperature on the esterification rate. Furthermore, the activation energy for both steps could be determined by the Arrhenius equation (Eq. (8)) and the absolute values of the reaction rate. The activation energy for the first step was 5.8 kJ/mol. However, the activation energy for the second step was about four times higher, 21.4 kJ/mol. This confirms that the second step of the reaction has required more energy than the first step, so it was the rate-limiting reaction.

4. Conclusions

The synthesis of eugenyl acetate in SC-CO_2 using the immobilized lipases Novozym 435 and Lipozyme 435 were investigated at several conditions. Emphasis was given to the differences between the enzymes, the number of the utilization cycles and the effect of temperature and pressure, and the esterification kinetics was also studied. The specific enzymatic activity (SA) obtained for Novozym 435 was about 18% higher than that achieved by Lipozyme 435. Differences were also observed in the particle size distribution and water content of the immobilized lipases. Novozym 435 achieved higher conversion and specific productivity of eugenyl acetate than Lipozyme 435.

The influence of temperature and pressure was verified in reactions conducted with 1% of enzyme and substrate molar ratio of 5:1, and the optimal condition for the synthesis of eugenyl acetate in SC- CO_2 was determined at 50 and 60 °C at 10 MPa, in which the study of phase behavior identified that the reaction mixture contained solid (enzyme), liquid and gas phases. The conversion and specific productivity of eugenyl acetate decreased with the increasing number of cycles, and the residual enzyme activity and the water content decreased with the increase of the number of depressurization/pressurization cycles. The results suggested that the reaction mechanism could be described to follow the simple Ping-Pong Bi-Bi mechanism within the range of studied concentrations. The affinity of acetic anhydride to the enzyme was larger than that of eugenol, and the affinity of both substrates to the lipase and the maximum reaction rate increased with temperature. The second step of the reaction kinetics required more energy that the first step, so it was identified as the rate-limiting reaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.06.018.

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