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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, %),