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Biocatalytic epoxidation of α-pinene to oxy-derivatives over cross-linked lipase aggregates

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ABSTRACT

Lipase-based cross-linked aggregates were investigated for a non-specific reaction, *i.e.* the epoxidation of α-pinene to its oxygenated derivatives. The activity of the biocatalysts has been evaluated in a green context, *i.e.* ethyl acetate as both acetate-supplier and organic solvent with H_2O_2/UHP/TBHP as oxidant. Screening of the lipase sources indicated *Aspergillus niger* lipase as the most efficient biocatalyst for this reaction. Different immobilization protocols ((i) cross-linked enzyme aggregates (CLEA), (ii) cross-linked enzyme aggregates onto magnetic particles (CLEMPA) and (iii) covalent immobilized enzyme (CIE) onto magnetic particles (MP)) were evaluated considering the activity as main parameter. Thus, CLEA and CLEMPA afforded better epoxidation yields of α-pinene towards CIE. The investigated biocatalytic systems allowed to transform α-pinene into oxigenated derivatives with industrial and commercial applications (*e.g.* α-pinene oxide, camphene, pinanediol and camphonelic aldehyde). FTIR investigations on the biocatalysts revealed the effects of the immobilization protocol on the enzyme secondary-structure. Additionally, textural characterizations were performed by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) analysis.

1. Introduction

α-Pinene is the main component of the monoterpene fraction in some essential oils (*e.g.* mastic oil) and turpentine, which is a paper and pulp industry residue available in bulk quantities at a low price. Approximately 350,000 tons of turpentine are produced annually worldwide [1]. Commonly, its applicability is limited to fuels for the recovery boilers, although it could be effectively utilized in other processes on site. On the other side, α-pinene is considered as a renewable raw resource with a great potential for the production of pharmaceuticals, agrochemicals and other fine chemicals [2].

Epoxidation may provide an efficient route to achieve the sustainability in the valorization of α-pinene and its derivatives (*e.g.* α-pinene oxide, verbenone, verbenol, campholenal, pinanediol, camphene, etc). It affords the introduction of an oxygen atom into the olefinic terpene skeletons [3] leading to oxidation products that are usually found in the plant kingdom [4,5], but at low concentration. Molecules incorporating these structures exhibit important flavor/fragrance (sandalore (Givaudan) and polysantol (Firmenich)) and anti-cancinogenic behavior [6]. Also, verbenol is a well-known aggregation pheromone of the bark beetle that is utilized in the forestall pest control. However, to achieve them in an efficient way new challenging synthetic routes are necessary [7].

Biocatalytic methods offer a fair balance between efficiency, environmental friendly and cost aspects [8]. α-Pinene treated with an oxidation agent (*e.g.* hydrogen peroxide – H_2O_2 or ureea-hydrogen peroxide – UHP) and carboxylic acids in an organic medium using lipases (*e.g. Candida antarctica B*), chloroperoxidases (*e.g. Caldariomyces fumago*) or laccases (*e.g. Trametes versicolor, Trametes hirsuta* or *Botrytis cinerea*) as biocatalysts led to the desired epo-oxidation products [9–12]. Epoxidation is achieved under mild conditions by employing peroxy-carboxylic acids produced continuously *in situ* by lipase-catalyzed perhydrolysis of the corresponding carboxylic acid [10]. Additionally, derivatives of the carboxylic acids, such as ethyl acetate, phenyl acetate and dimethyl carbonate were also used as a perhydrolysis substrate and solvent [13–15]. Noteworthy, for lipase, the biocatalyst performances have even been improved by the immobilization of the enzyme either in organic-modified clay or in smectite nanoclay [16,17]. Thus, the immobilization of the lipase onto the hydrophobic surfaces resulted as a promising strategy for the preparation of an efficient biocatalyst for the epoxidation of α-pinene.

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Literature reported several alternatives for immobilization of enzymes [18]. They include both physical adsorption and covalent interaction. However, the covalent approach (covalent immobilized enzyme – CIE, cross-linked enzyme aggregates – CLEA, and cross-linked enzyme aggregates onto magnetic particles – CLEMPA) shows more advantages that entails its larger applicability. CLEA and CLEMPA are related [18–20], both involving the precipitation of an enzyme from aqueous solutions. However, for CLEA the enzyme aggregates are linked themselves, while for CLEMPA they are connected together with magnetic particles (*e.g.* MP-NH_2). Glutaraldehyde is the cross-linker reagent in both cases. Previous studies demonstrated that the behavior of the cross-linked biocatalysts is influenced by the size of the network. The diameter of the biocatalyst is the result of the interaction between the enzyme aggregates and magnetic particles [21]. The efficiency of such lipase-based CIE, CLEA and CLEMPA biocatalysts has already been demonstrated in several reactions of interest, including the glycerol conversion to glycerol carbonate [22].

This study reports new abilities of the lipase cross-linked enzyme aggregates (CLEA/CLEMPA) as "heterogeneous" recoverable biocatalysts for the epoxidation of α-pinene to oxygenated derivatives. The epoxidation process has been developed using H_2O_2/UHP/*t*-butyl hydroperoxide (TBHP) as the oxidative agent and ethyl acetate with double role, i.e. acetate-supplier and organic solvent. With this scope, all the three (i) CIE; (ii) CLEA and (iii) CLEMPA protocols have been compared. The biocatalytic systems were evaluated in terms of activity (measured as TON), conversion of α-pinene and selectivity to α-pinene epoxide. The biocatalyst behavior was also correlated with its textural characteristics and the enzyme-secondary structure which is a quite novel aspect for the research on CLEA/CLEMPA biocatalysts.

2. Experimental

2.1. Chemicals and solutions

The screening of enzymes for the investigated epoxidation process was performed with lipases extracted from various sources, *e.g. Aspergillus niger* (0.2 U/mg), *Candida antarctica* (1 U/mg), *Rhizopus arrhizus* (10 U/mg), *Candida cylindracea* (5.3 U/mg), *Pseudomonas cepacia* (46.2 U/mg), *Porcina pancreas* (22.7 U/mg) and *Rhizopus niveus* (36 U/mg). These enzymes were purchased from Sigma-Aldrich company (USA). Magnetic particles with different size and structure were bought from Chemicell company (Rostock, Germany), *e.g.* (i) SiMAG-Amine (MP-NH_2) with a maghemite core, a shell of silica (average hydrodynamic diameter of 500 nm) and external layer of aminosilane polymer, and (ii) fluidMAG-PEA (MP-PEA), with a magnetite core covered by poly-(dimethylamin-co-epichlorhydrin-co-ethylendiamin) and an average hydrodynamic radius of 100 nm. Both types of particles had $-NH_2$ as a terminal active group that has been used for the enzyme immobilization. The immobilization reagent was glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). A solution of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES), pH 7.4 was used as a coupling buffer for the enzyme attachment. Phosphate buffer saline (PBS) of 10 mM concentration (8 g NaCl, 0.2 g KCl, 1.43 g $Na_2HPO_4 \times 2H_2O$ and 0.34 g KH_2PO_4 in 0.1 L distilled water) at pH 7.4 containing 0.1% BSA and 0.05% sodium azide was used for the blockage of the active sites on the solid surface after the immobilization of the enzyme. The other synthesis reagents (α-pinene, hydrogen peroxide (H_2O_2), *t*-butyl hydroperoxide (TBHP), urea hydroperoxide (UHP), glutaraldehyde, ethyl acetate, typical organic solvents) were of the analytic purity and purchased from Sigma-Aldrich.

2.2. Biocatalyst preparation

The enzyme aggregates were prepared using lipase from *Aspergillus niger* source. The CLEMPA immobilization approach was performed according to a previous report [22]. Lipase was precipitated from an enzyme aqueous solution (50 mg/mL lipase in 0.1 M MES) using dimethyl carbonate as precipitation reagent. The resulted enzyme aggregates and magnetic particles (MP-NH_2) were linked together using GA (25% w/v in water) following a gentle agitation of the mixture for 2 h at room temperature. The CLEMPA biocatalyst was easily separated and washed several times with distilled water alternating the dispersion and separation of the enzyme-bead clusters. The fresh biocatalyst was characterized and tested in the catalytic reaction of α-pinene epoxidation. The preparation approach of the CLEA biocatalyst was similar with CLEMPA, except the use of the magnetic particles [21,22].

Covalent biocatalyst (CIE) was prepared following the protocols described in previous papers [23–25]. Lipase enzyme was anchored on MP-NH_2 using the GA approach, while MP-PEA particles were used as support for the EDC/NHS approach. For the MP-NH_2 support a larger cross-linker (GA polymer) was necessary to avoid the steric inhibition of the enzyme after the immobilization. However, this was not necessary for the MP-PEA support where the external layer of the support is constituted from polyethylene amine.

Tests of the stability and recyclability were performed reusing the biocatalysts in successive reaction cycles. The biocatalyst was easily removed from the reaction mixture at the end of the reaction by centrifugation (*e.g.* CLEA) or using a permanent magnet placed close to the reaction vial (*e.g.* CLEMPA and CIE). Then, the biocatalyst was recycled after washing several times with distilled water and at the end with MES (0.1 M).

2.3. Biocatalyst characterization

IR spectra were recorded for all of the biocatalyst models using a FTIR instrument (Thermo Electron Scientific Corporation, USA). Ten scans were collected with a resolution of 4 cm^{-1} in the range 400–4000 cm^{-1}. Transmission Electron Microscopy (TEM) analysis provided information of the particles size and was carried out on a Hitachi H-8100 microscope, operating at 10–15 kV. The morphological characterization of the biocatalysts was performed by SEM analysis in a Hitachi S-2400 equipment, placing a droplet of the MP/biocatalyst suspensions (200 μL) on a conductive aluminum foil support, and let it dry in air. Atomic force microscopy (AFM) was also performed utilizing a Nanoscope IIIa multimode microscope (Digital Instruments, Bruker). The images were obtained in air, in tapping mode, using etched silicon tips of ca. 300 kHz resonance frequency (Bruker) and with a scan rate of 1.0–1.5 Hz. AFM samples were prepared by placing a drop of MP/biocatalyst suspensions on freshly cleaved mica substrate and drying with pure N_2 before analysis. A rinsing step with water was required to remove the salt from the surface.

2.4. Biocatalytic oxidation of α-pinene

Biocatalyst (1 mg lipase free or corresponding immobilized enzyme) was mixed with the substrate (1 mmol α-pinene) and oxidation agent (1 mmol H_2O_2/TBHP/UHP) in 1 mL ethyl acetate solvent. Reaction mixture was incubated for 24 h at room temperature under gentle mixing conditions. Products of the reaction were monitored using a GC–MS system (Thermo Electron Scientific Corporation, USA) incorporating a TR-WAX capillary column and GC-FID system (Schimadzu GC-2014, Schimadzu, USA) equipped with a TG-5SilMS capillary column. Hydrogen was the carrier gas (1 mL min^{-1}). The temperature of the injection was set up at 200 °C, while the column temperature was varied with 5 °C min^{-1} (from 50