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Continuous kinetic resolution of aliphatic and aromatic secondary alcohols by sol-gel entrapped lipases in packed bed bioreactors

Anamaria Todea, Paula Borza, Adinela Cimporescu, Cristina Paul, Francisc Peter*

University Politehnica Timişoara, Faculty of Industrial Chemistry and Environmental Engineering, Carol Telbisz 6, 300001 Timisoara, Romania

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ABSTRACT

The efficiency of tailored sol-gel entrapment using a ternary mixture of silane precursors was demonstrated for the continuous-flow kinetic resolution of aliphatic and aromatic secondary alcohols catalyzed by lipases. The main factors (temperature, substrate concentration, flow rate) influencing the enantioselective acylation of two model compounds, *rac*-2-octanol (*rac*-**1**) and *rac*-1-phenylethanol (*rac*-**2**), catalyzed by *Candida antarctica* B lipase in a packed-bed reactor were optimized by experimental design. The optimal values resulted for the *rac*-**1**, 0.5 M substrate concentration, 50 °C reaction temperature and 0.8 ml/min flow rate, were validated by long-term processing (144 h) in the continuous mode, resulting a productivity of about 145 μmol min^{-1} g^{-1} and enantiomeric ratio E higher than 280. For *rac*-**2**, the optimal parameter values were calculated as 40 °C reaction temperature, 0.45 ml/min flow rate and 0.2 M substrate concentration, yielding a productivity of about 40 μmole min^{-1} g^{-1} and enantiomeric ratio above 200. For both substrates, the productivity was considerably higher compared to data reported by other authors, demonstrating the robustness, efficiency, and stability of the selected biocatalyst. The kinetic resolution of the third substrate, *rac*-2-chloromandelic acid (*rac*-**3**) was first studied in batch mode, selecting the best lipase (*Candida antarctica* A), immobilization method (sol-gel entrapment with a ternary silane mixture combined with adsorption on Celite 545), temperature (40 °C), and reaction medium (diisopropylether). These optimal parameters were successfully upgraded to the continuous-flow process, resulting a productivity of 1.7 μmol min^{-1} g^{-1}.

1. Introduction

Despite the impressive new progress in asymmetric synthesis, the dominant production method to obtain a single enantiomer in large-scale synthesis consists of kinetic resolution of racemates [1]. Lipases are the most intensively utilized enzymes, since they can perform kinetic resolution and synthetic reactions in aqueous medium or in organic solvents in the absence of any cofactors [2]. As a result, lipases remain the choice of enzymes for the resolution of chiral drugs, preparation of various substituents of cocoa butter, production of biofuels, modification of fats, synthesis of cosmetics, as flavor enhancers and to speed-up waste degradation [3].

Immobilization of enzymes and whole cells is a key strategy for developing efficient and economically viable biocatalytic processes. In case of lipases, immobilization frequently promotes an increase in catalytic activity in organic solvents as an extra advantage [4] and may modify the enantioselectivity [5].

The increasing demand for rational design of novel, more efficient and tailor-made biocatalysts favored the development of sol-gel entrapment of enzymes, mainly lipases, as a possible generic immobilization method. This technique allows the fine-tune of the physical properties of the matrix, which can be correlated with the structure of the substrate, as well as the activity, selectivity and stability of the enzyme [6,7]. Sol-gel encapsulated lipases are currently used for the industrial production of various specialty esters, aroma compounds, and cosmetic agents [8].

In addition to immobilization, reactor technology may offer further means of upgrading the efficacy of biotransformations [9], reducing the impact of catalyst costs on the product [10]. Continuous-flow reaction systems gained increasing importance in biocatalytic processes performed with immobilized enzymes and they are strongly influenced by the characteristics of the selected carrier. Enzymes are already used in the synthesis of about 2/3 of chiral products produced on large industrial scale [8]. Most of the continuous-mode biocatalytic syntheses of optically active chiral intermediates at relatively large scale are performed using immobilized lipases in packed-bed reactors [11].

* Corresponding author.
E-mail addresses: francisc.peter@upt.ro, Francisc52@gmail.com (F. Peter).

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In this research, tailor-made immobilized lipases from *Candida antarctica* A and *Candida antarctica* B (*Cal*A and *Cal*B) have been used for the continuous kinetic resolution of racemic substrates of practical importance. Among readily available lipases, *Cal*A and *Cal*B have become the most widely used biocatalysts in both industrial applications and scientific research [9,12]. The selected aliphatic (*rac*-2-octanol, *rac*-**1**) and aromatic (*rac*-1-phenylethanol, *rac*-**2**) model substrates were already subjects of intensive investigations concerning their kinetic resolution in lipase-catalyzed reactions, performed by our group [13] and elsewhere [11], but continuous-flow processes using sol-gel entrapped lipases were not yet reported for these substrates. Chiral 1-phenylethanol derivatives are important building blocks for synthetic intermediates in fine-chemical and pharmaceutical industries, agrochemicals and natural products [14,15]. For the first time, the optimal immobilization and reaction conditions for the kinetic resolution of *rac*-2-chloromandelic acid (*rac*-**3**) were determined at batch level and applied for the continuous process. The relevance of pure (*R*)-2-chloromandelic acid is due to its importance in the industrial synthesis of the anti-thrombotic agent (*S*)-clopidogrel [16]. Uhm et al. performed the transesterification of methyl 2-chloromandelate using vinyl acetate and Chirazyme L-5 in a batch system in toluene, obtaining a conversion of 44.8%, enantiomeric excess of 65.7% and E value of 18.5 [17], but the continuous enzymatic kinetic resolution of 2-chloromandelic acid by enantioselective transesterification was not yet reported.

2. Experimental

2.1. Materials

Lipases *Candida antarctica* B (*Cal*B lipase) and *Candida antarctica* A (*Cal*A) were products of C-Lecta (Leipzig, Germany) and ChiralVision (Leiden, The Netherlands), respectively. Lipases from *Pseudomonas fluorescens*, *Burkholderia cepacia*, hog pancreas, *rac*-2-chloromandelic acid, vinyl acetate, decane (>99%), were purchased from Sigma – Aldrich. The silane precursors vinyltrimethoxysilane (VTMOS) and phenyltrimethoxysilane (PhTMOS), as well as tris-(hydroxymethyl)-aminoethane, 2-propanol, 1-octyl-3-methyl-immidazolium tetrafluoroborate ([Omim]BF_4), Celite 545, *rac*-2-octanol, vinyl acetate, *n*-hexane, were purchased from Merck. Methylene chloride was purchased from Roth. Sodium fluoride, *rac*-1-phenylethanol, *n*-hexane, *n*-heptane, methyl-tertbuthylether, diisopropylether, *tert*-butanol, acetonitrile, acetone, diethylether, toluene (all HPLC grade), cyclohexane, tetrahydrofuran, tetramethoxysilane (TMOS), were products of Fluka (Buchs, Switzerland).

2.2. General procedure for immobilization by sol-gel entrapment

2.2.1. Method A (with NaF as catalyst)

*Cal*A and *Cal*B were immobilized by sol-gel entrapment, as previously described [13]. The initial lipase suspension in TRIS/HCl buffer 0.1 M, pH 8.0 contained 120 mg mL^{-1}, while binary or ternary mixtures of silane precursors and 1-octyl-3-methylimidazolium tetrafluoroborate as additive were employed to build up the immobilization matrix.

2.2.2. Method B (using a prepolymer sol obtained with HCl)

*Cal*A lipase suspension (120 mg/mL) in TRIS/HCl buffer 0.1 M, pH 8.0 was stirred at room temperature for 30 min, centrifuged, and the supernatant was utilized for immobilization. 1 ml of this solution was mixed with 200 μl [Omim]BF_4 and 200 μl isopropyl alcohol in a 4 ml glass vial and magnetically stirred for 30 min. In a second 4 ml glass vial, the silane precursors (binary or tertiary mixture, total 6 mmol), 0.4 ml water and 60 μl 0.04 M HCl were stirred for 30 min at room temperature. Subsequently, the two solutions were mixed, 0.2 μl ammonia (25% solution) has been added, and the whole mixture was stirred magnetically until the gelation occurred. The resulting gel was kept at room temperature for 24 h to complete polymerization. The bulk gel was processed using the same procedure as in *Method A*.

2.2.3. Combined sol-gel entrapment and adsorption

Until the start of gelation, the method was identical to the normal sol-gel entrapment (*Method A* or *Method B*). Then, 0.5 g Celite 545 were blended with the gelling mixture. Subsequently, the obtained solid preparates were processed as described above.

2.3. Kinetic resolution of 2-chloromandelic acid in the batch process

An Eppendorf Thermomixer Comfort (Eppendorf, Germany) heating shaker was used for all batch experiments. 2-Chloromandelic acid (10 mM), was acetylated with vinyl acetate (30 mM), in the appropriate reaction medium. The reaction was initiated by the addition of immobilized *Candida antarctica* A lipase (40 mg) to 1 ml reaction mixture and was carried out under continuous shaking at 800 rpm and 40° C. At definite times, the reactions were stopped by centrifugation of the enzyme and the samples were analyzed by chiral HPLC-UV. All experiments were made in duplicate and the HPLC analyses in duplicate, as well. The reproducibility was excellent, as the relative standard deviation from the mean values (given in Table 6) was less than 5% for all immobilized preparates.

2.4. General procedure for continuous acylation of aliphatic and aromatic alcohols in the packed-bed bioreactor

Sol-gel immobilized *Cal*B lipase (1063 mg for *rac*-**1** and 1278 mg when *rac*-**2** was used as substrate) was packed into a stainless steel column (150 × 4.6 mm). Before packing, the column was washed with water, ethanol, *n*-hexane and acetone.

The acylation reaction of *rac*-**1** with vinyl acetate was performed using a solution of *rac*-**1** (different substrate concentrations, in the range of 0.05–1.25 M) and vinyl acetate (at 1:1, 1:3 or 1:5 molar ratio), in *n*-hexane as organic medium. The appropriate solution was pumped through the lipase-filled column set at different temperatures (40, 50 and 60 °C), at a flow rate set between 0.1–1.25 ml/min.

The acylation of *rac*-**2** with vinyl acetate was performed basically by the same experimental methodology. The solution of *rac*-**2** (0.05–1.25 M) and vinyl acetate (1:3 molar ratio) in *n*-hexane was pumped through the lipase-filled column, set to different temperatures (40, 50 and 60 °C), at a flow rate in the range of 0.3–1.5 ml/min.

In both cases, samples were collected at specific time intervals and submitted to chiral GC analysis.

For the continuous kinetic resolution of *rac*-**3**, 844 mg immobilized *CalA* lipase (sol-gel entrapment combined with adsorption on Celite 545, silane precursors PhTMOS: VTMOS:TMOS, at 1.6:0.4:1 molar ratio) were packed into a stainless steel column (95 × 3 mm). During the continuous experiments, the column temperature was maintained at 40 °C, the flow rate was set to 0.3 ml/min or 0.5 ml/min, and different substrate concentrations were tested. Samples were collected at specific intervals and were analyzed by chiral HPLC.

2.5. Monitoring the experiments by chiral chromatographic analysis

Transesterifications of *rac*-**1**, as well as of *rac*-**2**, were monitored by chiral gas-chromatography, on a Varian 450 instrument

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