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Short communication

Application of a *Burkholderia cepacia* lipase-immobilized silica monolith micro-bioreactor to continuous-flow kinetic resolution for transesterification of (*R*, *S*)-1-phenylethanol

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ABSTRACT

Burkholderia cepacia lipase was immobilized in silicates forming from *n*-butyl-substituted precursors within a silica monolith from methyl-substituted precursors. The resultant preparation gave about 12 times higher rates of transesterification of (*R*, *S*)-1-phenylethanol with vinyl acetate and an approximately two-fold increase in the enantioselectivity toward (*R*)-1-phenylethanol, as compared to a non-immobilized counterpart. The highest enzymatic activity and enantioselectivity (reaching 250) were found at a low water activity of 0.11. The continuous-flow kinetic resolution of (*R*, *S*)-1-phenylethanol was successfully conducted using lipase-immobilized silica monolith micro-bioreactors with various inside diameters ranging from 0.25 to 1.6 mm. The reactor performance during continuous operation was consistent with the prediction from the batch reactor. A steady state conversion of 40% and enantiomeric excess more than 98% were maintained over a time period of 15 days.

1. Introduction

The use of enzymatic micro-reactors is expected to be a powerful approach that will lead to intensified process design for the production of both fine and commodity chemicals. Reduced channel sizes offer increased reaction efficiency due to their higher surface area to volume ratios and therefore shorter diffusion path lengths. In addition, flow-through micro-bioreactors enable the consumption of minute amounts of reagents, thus are environmentally friendly processes, and they also allow high throughput continuous processing [1–5]. Capillary micro-bioreactors are probably the easiest and most facile to manufacture and operate because they do not require specialized micro-fabrication techniques such as photolithography. A silica-based monolithic column, which was initially introduced as an efficient stationary phase for liquid chromatography, is an attractive support for enzyme immobilization. The use of enzyme-immobilized silica monolith micro-bioreactors offers several benefits, including very low back-pressure, high contacting efficiency (rapid mass transfer due to convective flow through micro-scaled interstices within the monolith), and mechanical durability, as compared with conventional packed-bed bioreactors [6–11].

Lipase is a versatile enzyme that catalyzes a variety of bioconversions, including hydrolysis, acidolysis, alcoholysis, esterification, and transesterification. This enzyme has recently been immobilized in capillary tube micro-bioreactors, where *Pseudomonas cepacia* lipase has been immobilized by adsorption in mesoporous silica thin films on the inner walls of a borosilicate tube (0.2 mm i.d.) [12,13]. A silica microstructured optical fiber has been used to covalently immobilize *Candida antarctica* lipase on the inner surfaces of 4–5 μm holes [14].

A silica-monolith can be prepared from a 4:1 mixture of two precursors, methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS), using a sol–gel method. This consists of an aggregate of spherical particles of several micrometers in diameter due to phase separation during the sol–gel transition, resulting in a macroporous structured support, without shrinkage. Interparticle gaps formed within the monolith serve as flow paths for the substrate solution flowing through the column. A silica monolith micro-reactor, in which *C. antarctica* lipase A was adsorbed, was used to perform an aqueous–organic biphasic reaction, the hydrolysis of 4-nitrophenyl butyrate [15].

In previous studies, we prepared a silica monolith micro-bioreactor from methyl-substituted precursors containing protease or lipase by using a one step sol–gel method and used it for

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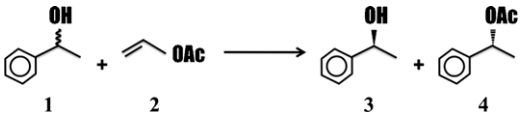

Scheme 1. *Burkholderia cepacia* lipase-catalyzed enantioselective transesterification of (*R*, *S*)-1-phenylethanol (1) with vinyl acetate (2) to form (*S*)-1-phenylethanol (3) and (*R*)-1-phenylethyl acetate (4).

transesterifications in organic media [16,17]. The activities of some types of lipases, however, are more significantly enhanced when immobilized in silicates forming from precursors with longer chain alkyl-groups than by methyl-groups, such as propyl- or butyl-groups, probably due to the stronger hydrophobic interaction leading to more opened lid structure [17–20]. We thus used a two-step sol–gel method to immobilize *Rhizopus oryzae* lipase. The silica monolith was prepared from a mixture of MTMS and TMOS in the absence of the enzyme and then the inner surface of the resultant monolith was coated with a sol mixture composed of *n*-butyltrimethoxysilane (BTMS) and TMOS containing lipase [17,18]. The lipase immobilized in the layer of butyl-substituted silicates exhibited approximately ten times higher activity than lipase immobilized in a methyl-substituted silica monolith. In the present study, we prepared a *Burkholderia cepacia* lipase-immobilized silica monolithic bioreactor using a similar methodology to that described above, and applied it to the continuous-flow kinetic resolution of racemic compounds. The test reaction used was the transesterfication of (*R*, *S*)-1-phenylethanol (1) with vinyl acetate (2) to produce (*S*)-1-phenylethanol (3) and (*R*)-1-phenylethyl acetate (4) in isooctane (Scheme 1). The kinetic resolution of (*R*, *S*)-1-phenylethanol has been used in many studies to evaluate the performance of *B. cepacia* or *Candida rugosa* lipases immobilized in sol–gels [20–22], adsorbed on hydrophobic materials [23–25], coated with ionic liquid [26], and variously modified and suspended in ionic liquids [27].

2. Materials and methods

2.1. Materials

Lipase PS-SD originating from *B. cepacia* was purchased from Amano Enzyme Inc. (Nagoya, Japan) and used without further purification. The protein content of this sample was determined to be 0.69% (w/w) by Bradford assay using a commercial Bio-Rad dye and bovine serum albumin as the protein standard. (*R*)-, (*S*)-, and (*R*, *S*)-1-phenylethanol were purchased from Sigma–Aldrich Japan Co. (Tokyo, Japan). (*R*, *S*)-1-phenylethyl acetate was obtained from Merck Chemicals Japan Co. (Tokyo, Japan). Vinyl acetate, tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS) and other chemicals were of reagent grade and were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). *n*-Butyltrimethoxysilane (BTMS) was kindly supplied by Dow Corning Toray Co., Ltd. (Ichihara, Japan). PEEK (poly(ether ether ketone)) tubes with four different inside diameters of 0.25, 0.5, 1.0 and 1.6 mm were purchased from GL Sciences Inc. (Tokyo, Japan).

2.2. Preparation of a silica monolith derived from a mixture of MTMS and TMOS

A silica monolith support was first prepared by the sol–gel method reported previously [17,18]. At room temperature, a mixture of 950 μL of MTMS and 247 μL of TMOS (molar ratio of MTMS to TMOS, 4:1), 290 μL of distilled water and 12.5 μL of 40 mM HCl were mixed in a test tube to form a homogeneous sol. The mixture was cooled to 4 °C and then 2.7 mL of 100 mM phosphate buffer (pH 7.5) was added. Under this condition, the molar ratio of silanes to water was 5:100. Gelation was allowed to proceed at room temperature for one day and the hydrogel formed was lyophilized for one day. This provided a non-shrinkable silica monolith in the test tube that was then used as a support for immobilization of lipase.

2.3. Preparation of a lipase-immobilized silica monolith coated with silicates derived from a mixture of BTMS and TMOS

A mixture of 91.0 μL of BTMS and 17.7 μL of TMOS (molar ratio of BTMS to TMOS, 4:1), 41.0 μL of distilled water, and 5.5 μL of 40 mM HCl were mixed to form a homogeneous solution. The mixture was cooled to 4 °C and then 3.17 mL of enzyme solution (100 mM phosphate buffer, pH 7.5) containing 400 mg lipase was mixed with the silane solution. Under this condition, the molar ratio of silanes to water was 0.33:100. The resultant mixture (3.32 mL) was poured slowly into the MTMS-based silica monolith formed in the test tube. The liquid–solid mixture was degassed under reduced pressure, before gelation took place, so that the sol solution could be infiltrated into the interstices of the monolith. Thereafter, gelation was allowed to proceed at room temperature for one day and the lipase-immobilizate coated with BTMS-based silicate was dried *in vacuo* for one day. The lipase-immobilized silica monolith was retrieved and crushed in a mortar. The prescribed amount of the crushed gels was equilibrated for one day in a desiccator containing various saturated salt aqueous solutions at 30 °C to adjust the water activity of the preparations. The salts used were LiCl (water activity, $a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), K_2CO_3 ($a_w = 0.43$), $CuCl_2$ ($a_w = 0.71$), and KCl ($a_w = 0.84$). The samples thus hydrated were used for batch reaction experiments.

2.4. Preparation of a lipase-immobilized silica monolith micro-bioreactor

In order to obtain capillary columns loaded with the MTMS-based silica monolith, twelve PEEK tubes with length 5 cm and four different inside diameters (three tubes for each diameter) were placed into a test tube and filled with the sol mixture containing MTMS and TMOS as described above. This resulted in the formation of an MTMS-based silica monolith both in the test tube and in the PEEK tubes. In the second sol–gel coating step, 3.7 mL of a sol mixture containing 110 μL BTMS, 21 μLTMOS and 1 g of lipase was slowly poured into the MTMS-based silica monolith formed in the test tube, in which the silica monolith-containing PEEK tubes were still embedded. The mixture was then degassed under reduced pressure to permeate the sol into the interstices of the monolith. After gelation and vacuum drying as described in the previous section, the PEEK tubes were retrieved and the masses of gels inside and outside the PEEK tubes were measured. The mass of lipase immobilized within each PEEK tube was calculated by assuming a uniform distribution throughout the whole gel. The mass of the silica monolith including lipase and the enzyme content was 1.4 mg and 62% (w/w), respectively, for the 0.25 mm i.d. × 5 cm length PEEK tube. The silica monoliths were hydrated at a water activity of 0.11 according to the procedure described above. The silica monolith PEEK tubes thus prepared were used for flow-through reaction experiments.

2.5. Batch reaction experiments

The reaction mixture contained 20 mM (*R*, *S*)-1-phenylethanol and 0.4 M vinyl acetate in 10 mL isooctane as the organic solvent (Scheme 1). Batch reactions were initiated by the addition of 25 mg lipase powder or 70 mg particles of crushed silica monolith containing 25 mg crude, immobilized lipase, and were allowed to proceed at a temperature of 35 °C in 20 mL screw-capped vials on a shaking incubator maintained at 1500 min^{-1}. Reactions were carried out in duplicate or triplicate, and the results were represented as mean ± standard deviation.

2.6. Continuous-flow kinetic resolution in a silica monolith micro-bioreactor

The lipase-immobilized silica monolith micro-bioreactor (15 cm in total length) consisted of the three PEEK tubes (5 cm each) connected in series. It was provided with end fittings, attached to an HPLC pump (GL-7410, GL Sciences Inc., Tokyo, Japan), and placed in a constant temperature bath maintained at 35 °C. The substrate solution contained 20 mM (*R*, *S*)-1-phenylethanol and 0.4 M vinyl acetate in isooctane equilibrated at a water activity of 0.11 and this was fed at flow rates of 1.0–15.7, 3.2–47.4, 11.5–173, and 28.4–427 mL h^{-1} to the 0.25, 0.5, 1.0 and 1.6 mm i.d. bioreactors, respectively, by the HPLC pump. The steady state at each flow rate was confirmed when the exit concentration of product became independent of the process time.

The silica monolith bioreactor with 0.25 mm i.d. and 15 cm length was continuously operated for 15 days. The substrate solution was fed to the inlet of the micro-bioreactor at a constant volumetric flow rate of 0.72 mL h^{-1}.

2.7. Sample analysis

Organic samples were analyzed using an HPLC (GL-7400, GL Sciences Inc., Tokyo, Japan) equipped with a Chiracel OD-H column (Daicel Chemical Industries, Ltd., CPI Co., Tokyo, Japan). The eluent consisted of 97% (v/v) isooctane and 3% (v/v) 2-propanol at a flow rate of 1 mL min^{-1} and detection was carried out at 250 nm with a UV detector. The concentrations of (*R*)- and (*S*)-1-phenylethanol and (*R*)- and (*S*)-1-phenylethyl acetate were determined from calibration curves, which were prepared using the corresponding reference compounds and benzyl alcohol as the internal standard. The conversion of (*R*, *S*)-1-phenylethanol and enantiomeric excess of (*R*)-1-phenylethyl acetate and corresponding enantioselectivity were calculated from the concentrations of (*R*)- and (*S*)-1-phenylethyl acetate.