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A porous vessel bioreactor for gel entrapped biocatalysts: Kinetic resolution of *trans*-methyl (4-methoxyphenyl)glycidate by Lecitase® Ultra in gelatin organogel (Gelozyme)

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ABSTRACT

Preparation of *trans*-(2*R*,3*S*) methyl (4-methoxyphenyl)glycidate with enantiomeric excess (e.e.) of >99% has been carried out by enantioselective hydrolysis of the racemic glycidate ester by Lecitase® Ultra immobilized in macroporous gelatin organo-gel (gelozyme) in 47% yield. Effects of water content and particle size of the gel, different solvents, and flow rate of reaction medium on observed reaction velocity lead to optimum conditions. The reaction performed in a porous vessel bioreactor using toluene as preferred reaction medium, and gelatin-immobilized enzyme with average particle size of 1–1.2 mm, water content of 16.5% (w/w), and flow rate of 20 mL min^{-1}, followed a typical Michaelis–Menten kinetics with apparent $V_{max,app}$ of 38 mM min^{-1} g^{-1} and apparent K_m of 0.53 M. Theoretical considerations suggest that the bioreactor functions as a plug-flow reactor with complete recycle without serious pore diffusion and external mass transfer diffusion limitations. The immobilized enzyme can be used continuously for several recycles without significant loss of activity for at least two weeks.

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1. Introduction

Enzymes are interesting biocatalysts with increasing applications in the fine chemicals industry such as pharmaceuticals, agrochemicals, and health care products because of the regio- and stereo-control that can be achieved under mild and environmentally benign conditions. These biotransformations are preferably performed in reactors in which the biocatalyst is immobilized [1]. Apart from providing a simple technique to separate and recycle the biocatalyst, immobilization is also known to provide additional stability to the biocatalyst [2]. Consequently, several types of bioreactors have been developed to suit the needs of a particular process. Most popular equipment for using immobilized biocatalysts are, continuous stirred tank reactors (CSTR), packed bed reactors, fluidized bed, and membrane reactors.

In the stirred tank reactors, attrition of immobilized enzyme due to mechanical friction with stirrer blades is a major concern and it is necessary that immobilization matrix is of high mechanical strength. In case of packed bed reactors the degree of reaction is proportional to the length of reactor column, thus a turbulent flow of reaction mixture through the column is preferred as it improves mixing. However, colloids or precipitates formed during the reaction may clog up packed bed reactors. Due to compact packing, excessive pressure drops are encountered which form the major bottleneck for the packed bed reactors. Channelling is also encountered which leads to improper contact between the biocatalyst and the reactants [3,4] The fluidized bed bioreactors need very high flow rates causing attrition of the biocatalyst and loss of the enzyme activity. The technique of enzyme immobilization in ultrafiltration membrane [5] is useful only in cases of enzymes that have long term stability in solutions and are relatively inexpensive and hence expendable.

We have developed a novel porous vessel bioreactor for reactions with immobilized enzymes and microbial cells which overcome most of the disadvantages encountered with conventional reactors [6]. The apparatus broadly consists of a catalytic zone, which is inside the porous vessel submerged in a tank containing the circulating liquid (Fig. 1). Herein, we report the performance of a biocatalytic reaction in such a bioreactor. As a model case, we have carried out preparation of enantiomerically pure methyl *trans*-(2*R*,3*S*)-3-(4-methoxyphenyl) glycidate 1a (Scheme 1), an important chiral intermediate in the production of (+)-*cis*-(2*S*,3*S*)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one (*cis*-lactam) 6, which in turn is used in manufacture of Diltiazem, a drug used to treat hypertension (Scheme 2).

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Nomenclature

A	area of column (cm^2)
D	diffusivity ($cm^2\,h^{-1}$)
E	Enzyme
k	first order rate constant (h^{-1})
K_m	Michaelis constant (M)
L	length of the reactor (cm)
n	exponent in Eq. (4)
P	product
Q	flow rate ($mL\,min^{-1}$)
r_A	rate of reaction ($mol\,min^{-1}\,g^{-1}$)
S_0	initial substrate concentration (M)
t	time (h)
u	velocity of the recycled stream ($cm\,h^{-1}$)
V_{obsd}	observed initial rate ($M\,min^{-1}\,g^{-1}$)
V_{max}	maximum reaction rate ($M\,min^{-1}\,g^{-1}$)
W	weight of the enzyme (g)
Z	height of packing (cm)
δ	conversion
ε	porosity
φ	Thiele modulus

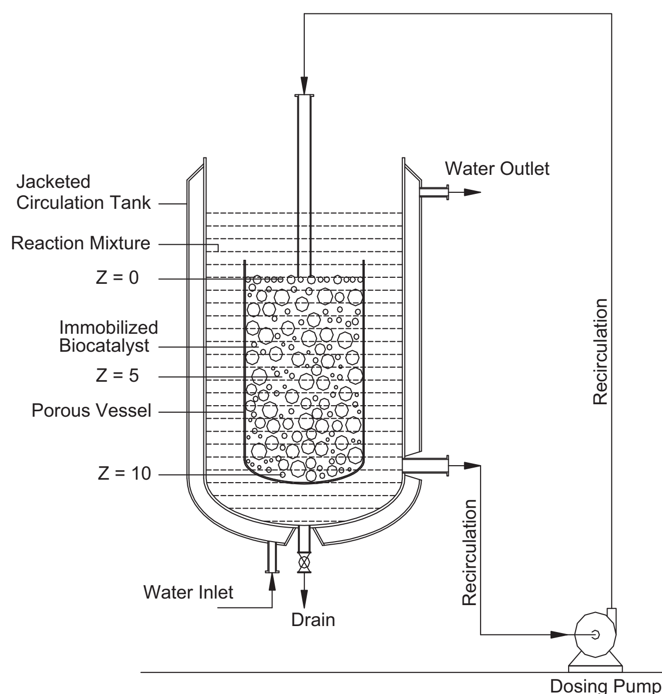
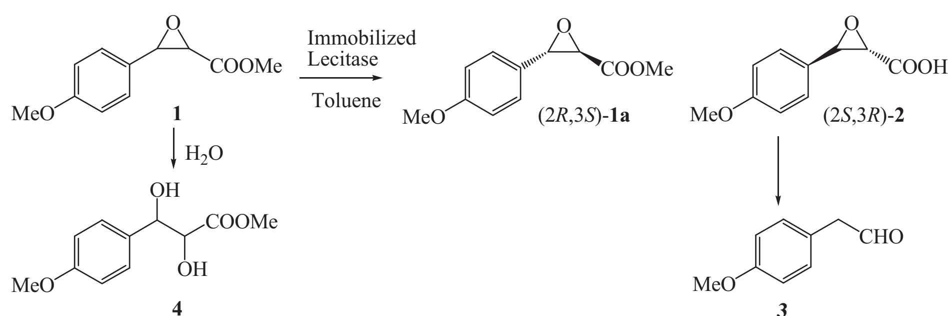
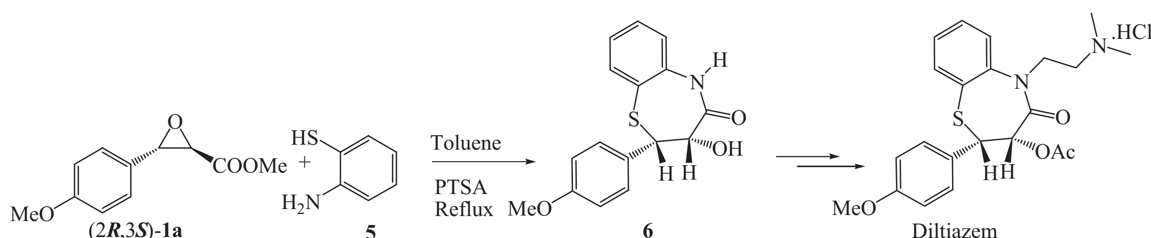


Fig. 1. A schematic representation of porous vessel bioreactor.

Enzymatic resolution of racemic glycidate esters and alcohols into enantiomerically pure forms is an important field of research due to the use of their enantiomerically pure forms in production of pharmaceuticals. For example, both (*R*)-and (*S*)-glycidyl butyrates are versatile chiral synthons [7] and are employed for synthesis of several drugs [8]. Various methodologies have been explored to achieve the resolution. Cao and co-workers have described an elegant two-step enzymatic resolution process for production of (*R*)- and (*S*)-glycidyl butyrates employing two enzymes with opposing enantioselectivity, porcine pancreatic lipase (*S*-selectivity) and Novozym 435 (*R*-selectivity) to obtain products with e.e. 98% in 36 and 42% yield, respectively [9]. A hydrolytic route to resolution of glycidyl butyrate using a lipase-like enzyme from porcine pancreas immobilized on DEAE–Sepharose has been described by the group of Guisan and Fernaĭndez-Lafuente [10]. Recently, resolution via enantioselective transesterification with vinyl butyrate has been described using a recombinant lipase BSL2 (*Bacillus subtilis* lipase 2) with excellent results [11]. Similarly, synthesis of the enantiomer **1a** via resolution of racemic glycidate esters by an esterase/lipase catalyzed enantioselective hydrolysis is also well known [12]. However, the glycidic acid 2, which forms as the hydrolysis product is unstable and quickly decomposes to aldehyde **3** (Scheme 1). This aldehyde acts as an inhibitor of the enzyme and it is necessary to design the bioreactor that allows a continuous removal of the aldehyde as a bisulfite adduct [13]. Recently, we have discovered that resolution of racemic glycidate ester **1** can be performed by employing Lecitase® Ultra immobilized in gelatin organogel (gelozyme)



Scheme 1. Enzymatic resolution of glycidate ester **1**.



Scheme 2. Synthesis of *cis*-lactam **5**.

wherein the aldehyde formed during the reaction did not inhibit the enzyme, the side reaction of epoxide ring opening and formation of diol **4** (Scheme 1) was minimum and it was possible to obtain the product **1a** with e.e. > 99% and excellent yield in a single batch operation [14] (Scheme 2). The reaction was however carried out on a very small scale (200 mg) in a magnetically stirred round bottom flask with purified enzyme. We would like to report here that the reaction can be carried out with equal efficiency with commercial enzyme after simple dialysis to remove salts. Considering the commercial importance of the product, we have carried out the reaction on a larger scale (7.2 g batch) in the porous vessel bioreactor and several parameters such as solvent effect, catalyst particle size, flow rate, water content and enzyme recycle have been studied in detail.

2. Materials and methods

2.1. General

Lecitase® Ultra was a gift from Novozymes, Denmark. All other reagents were purchased from M/s Loba Chemie, India. IR spectra were recorded on a Perkin–Elmer RX-1 FT-IR system. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on Bruker Avance-300 MHz spectrometer. Optical rotations were measured with Horiba-SEPA-300 digital polarimeter. HPLC analyses were carried out on Hewlett Packard HP1090 unit with diode array detector and HP Chem Station software. Chiral HPLC columns were obtained from Daicel, Japan. Racemic glycidate ester **1** was synthesized by standard technique. Mathematical fittings were performed with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA and MATLAB, The MathWorks, Inc. USA.

2.2. Immobilization of Lecitase® Ultra in gelatin

Commercial Lecitase solution (50 mL) was first dialyzed against distilled water solution for 24 h before use to remove salts (presence of inorganic salts in enzyme solution or gelatin prevents gel formation). Activity of dialyzed Lecitase was assayed using tributyrin [14] which showed activity of 1725 units/mL. Determination of protein content by Lowry's method (54 mg mL^{-1}) reflected enzyme activity of 32 units/mg.

Gelatin (50 g) was heated with distilled water (85 mL) at 60 °C for 15 min to complete gelation and cooled to 30 °C. Cetyl trimethylammonium bromide (CTAB) solution (350 mL, 0.3 M in 1:1 chloroform–isooctane) was added to gelatin solution with vigorous stirring. The viscous and turbid gel so obtained was cooled in ice with shaking for 10 min to get a transparent free flowing liquid. The enzyme solution (50 mL) was slowly added to the cold solution with vigorous stirring to achieve a uniform distribution of the enzyme. The cooling bath was removed and glutaraldehyde (10 mL, 25% solution) was added. The contents were stirred at room temperature with glass rod till the contents start to become viscous, poured into open Petri dishes and left at room temperature overnight under a fan. The dry gel was cut into small pieces, washed several times with chloroform and air dried (58 g). Assay of enzyme activity by tributyrin showed lipase activity corresponding to 1034 units/g, indicating a loading of 32 mg active protein/g (60% of original enzyme activity). Karl-Fisher titration of the gel showed that water content of the gel was 16.5% (w/w). The gel was then frozen in liquid nitrogen, powdered with pestle and mortar and sieved with sieves of varying mesh size to obtain immobilized enzyme granules of average particle sizes of 2.36, 1.70, 1.40, 1.18, 1.00, and 0.85 mm which were stored in refrigerator.

2.3. Preparation of gels with varying water contents

To prepare gels of different water contents, the air dried gel was freeze dried and powdered. Several tubes containing enzyme powder (100 mg) were prepared, buffer (1 mL, 0.05 M containing 15 mM $CaCl_2$, pH 8.5) was added to each tube, the gel was allowed to swell for 1 h in cold, then frozen in liquid nitrogen and placed for freeze drying. Sample tubes were removed at intervals of 30 min from the freeze drier. In this manner, sample tubes containing equal quantity of immobilized enzyme (100 mg, dry wt.) but varying amounts of water uniformly distributed throughout the gel matrix were obtained. Water content of the samples was determined by Karl-Fisher titration and the gel samples with required water content (10–50% w/w) were selected for hydrolytic reaction which was performed by adding substrate solution in toluene (0.29 M, 1 mL) and shaking the reactants on an orbital shaker at 200 rpm for 6 h. The reaction mixture was then analyzed by chiral HPLC.

2.4. Porous vessel bioreactor

The porous vessel bioreactor designed in our laboratory consists of a circulating cylindrical tank made of glass of 39 mm diameter and 190 mm length provided with a drain valve and an outlet as illustrated in Fig. 1. The porous vessel is made of sintered glass (15–40 μm, Type G3) with length of 120 mm, diameter of 30 mm and thickness of 2 mm. The vessel is submerged in a double walled glass tank attached to a circulating water bath with temperature control. The inlet to the porous vessel is placed on top as shown in Fig. 1. The immobilized biocatalyst is placed inside the porous vessel as granules and the substrate was added as a solution in toluene. The volume of toluene phase was adjusted in such a way that the liquid level is above the level of the catalyst bed. The outlet from the glass tank is connected to inlet of a dosing pump of capacity ranging from 5 mL min^{-1} to 80 mL min^{-1}. The outlet from the pump is connected to the inlet of the porous vessel bioreactor. In most experiments, the catalyst bed was made of gel particles of average diameter of 1–1.2 mm (17 g). The overall catalyst bed height was 5 cm with the catalyst occupying a volume of 35 mL. The total volume of recirculating reactant solution was 120 mL.

2.5. Hydrolytic reaction

Hydrolytic reaction was carried out in toluene saturated with aqueous buffer. Thus toluene (1 L) was shaken vigorously with Tris–HCl buffer (0.05 M containing 15 mM $CaCl_2$, pH 8.5) for 15 min and the layers were then separated in a separating funnel. The buffer saturated toluene (water content 0.03% v/v) was used to prepare the substrate solution of required concentration. The porous vessel of the bioreactor maintained at 30 °C was loaded with immobilized enzyme (17 g) and the substrate solution was added (120 mL). Reaction was started by pumping the substrate solution with dosing pump at rate of 20 mL min^{-1}. The reaction was monitored by HPLC by taking aliquots at regular intervals.

2.6. Chiral HPLC analysis

Chiral HPLC analysis was carried out using Chiralcel AD-H column (250 mm × 5 mm), Daicel Corporation, Japan. The mobile phase consisted of 15% 2-propanol in hexane at a flow rate of 0.7 mL min^{-1}. The products were detected at 230 nm. Retention times (2*S*, 3*R*) 9.8 min, (2*R*, 3*S*) 11.0 min. The racemic diol **4** gave 4 peaks at 20.2, 20.9, 22.5 and 24.6 min (configurations not assigned since product was racemic).

Table 1
Enantioselective hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** by immobilized Lecitase Ultra in various water-miscible solvents.[a]

Solvent	C_{50}, M (%, v/v)	Log $P_{octanol}$	[Diol] **4** (%)	e.e. of **1a** (%)
THF	2.96 (24)	0.46	72	8.5
DMSO	7.6 (54)	−1.35	94	77
Ethanol	6.85 (40)	−0.32	79	30
Acetonitrile	5.74 (30)	−0.34	76	67
1,4-Dioxane	4.22 (36)	−0.27	57	59
DMF	5.55 (43)	−1.01	77	3

[a] Reaction conditions: [substrate] = 0.048 M. Immobilized Lecitase 1 g containing varying quantities of water. Reaction volume 10 mL. Temperature 30 °C.

2.7. Product isolation

The reaction mixture after enzymatic conversion containing enantiomerically pure **1a** along with the aldehyde 3 was converted to *cis*-lactam 5 (47 g, 90%) as described earlier [9]. The precipitated *cis*-lactam 5 was filtered and the filtrate containing aldehyde was vigorously stirred with 30% sodium bisulfite solution (50 mL). The bisulfite adduct of 2-(4-methoxyphenyl)acetaldehyde separated as a white solid which was filtered, washed with methanol and dried under vacuum (36 g, 90%).

3. Results and discussion

3.1. Effect of solvent on reaction rate

Hydrolytic resolution of the glycidate ester **1** cannot be carried out in aqueous buffer due to low solubility of the substrate and rapid hydrolysis of the epoxide ring to diol **4** (Scheme 1), hence it is necessary to perform the reaction in an organic solvent. However, solvents cause differences in reaction rates due to several factors such as differences in polarity, interactions with the enzyme and the immobilization matrix, partitioning of the substrate between bulk solvent phase and the immobilization matrix. To select the most appropriate solvent for performing the kinetic resolution, we have studied the stability and reactivity of Lecitase in binary mixtures of various solvents in aqueous buffer.

3.1.1. Effect of water-miscible co-solvents

Typically, the immobilized enzyme (1 g) was incubated for 6 h in co-solvent mixtures of varying composition (10 mL), and the residual activity was determined. In general, the enzyme was found to be stable up to a solvent concentration of 25% (v/v) in most cases and its stability decreased at higher solvent concentrations. As observed in case of α-chymotrypsin and other enzymes [15], the plots of enzyme activity against co-solvent concentration were typically sigmoidal and the enzyme activity was lost in a narrow range of solvent concentration. The values of solvent composition at which the enzyme activity decreases to 50% (C_{50}) are given in Table 1.

The C_{50} values for Lecitase dissolved in various water miscible organic co-solvents follow their log $P_{octanol}$ values and decrease with increasing hydrophobicity of the co-solvent (Fig. 2). Such dependence of enzyme activity on solvent hydrophobicity is a general phenomenon and has been well reviewed [16]. Taking into account possible denaturation of the enzyme at high cosolvent concentrations, reactions with substrate **1** were carried out for 6 h in solvent mixtures containing 20% (v/v) solvent, a solvent composition at which the enzyme activity is stable for at least 6 h in all cases. The enantiomeric purity of the residual ester (ee_s) was determined from the peak areas of individual enantiomers. Excepting dimethyl formamide and tetrahydrofuran, hydrolysis of the ester did take place enantioselectively in all co-solvent mixtures, but the enzymatic reaction was accompanied by spontaneous hydrolysis of epoxide due to large excess of water present in the reaction mixture.

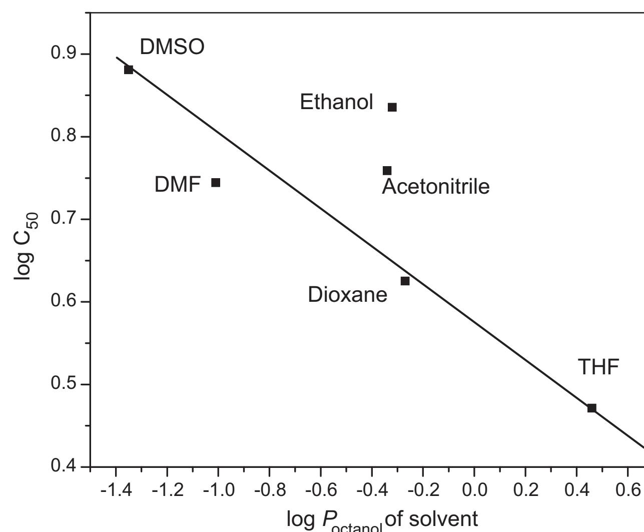


Fig. 2. Plot of log $P_{octanol}$ for various water miscible cosolvents *vs* log C_{50} for activity of immobilized Lecitase in binary aqueous mixtures at 30 °C.

The concentrations of diol formed in various binary mixtures and e.e. values of the unhydrolyzed ester are given in Table 1. It appears that in most cases the *E* values are >200 except in cases of THF and DMF. Unfortunately, it was not possible to determine the enantioselectivity *E* of the reactions since the epoxide hydrolysis created uncertainty in calculating the enzymatic conversion ‘*c*’.

3.1.2. Effect of water-immiscible solvents

To overcome the problems with water-miscible cosolvents, stability of the immobilized enzyme was studied in three different water-immiscible solvents namely, diisopropyl ether, toluene and dimethyl carbonate (DMC). Air dried enzyme gel containing 16.5% (w/w) water was suspended in the solvent and stirred at 100 rpm in a round bottom flask on a magnetic stirrer at room temperature. It was observed that the loss of enzyme activity was less than 5% even after incubation with the solvents for 72 h in all the three cases, and it was possible to perform reactions with immobilized enzyme suspended in the solvent containing the substrate. To select the most appropriate solvent, reactions were performed in the three solvents under identical reaction conditions and the enantiomeric purity of the unhydrolyzed ester was determined every 6 h till it reached 99% (Table 2). It was observed that the reaction was faster (∼54 h) in diisopropyl ether in comparison to that in toluene and dimethyl carbonate. From the peak heights in HPLC analysis, it was also apparent that the enantioselectivity of the enzyme in all the three solvents was comparable ($E > 200$). However, evaporation losses were high due to low boiling points of diisopropyl ether and dimethyl carbonate, hence toluene was chosen as a solvent for further studies.

Table 2
Enantioselective hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** by immobilized Lecitase Ultra in water-immiscible solvents.[a]

Solvent	Maximum solubility of substrate (% w/v)	Boiling point of solvent (°C)	Reaction period (h) to reach e.e. > 99%
Diisopropyl ether	5	68	54
Toluene	11	110	210
Dimethylcarbonate	12	90	270

[a] Reaction conditions: [substrate] = 0.048 M. Immobilized lecitase 1 g containing 16.5% water (w/w). Reaction volume 10 mL. Temperature 30 °C.

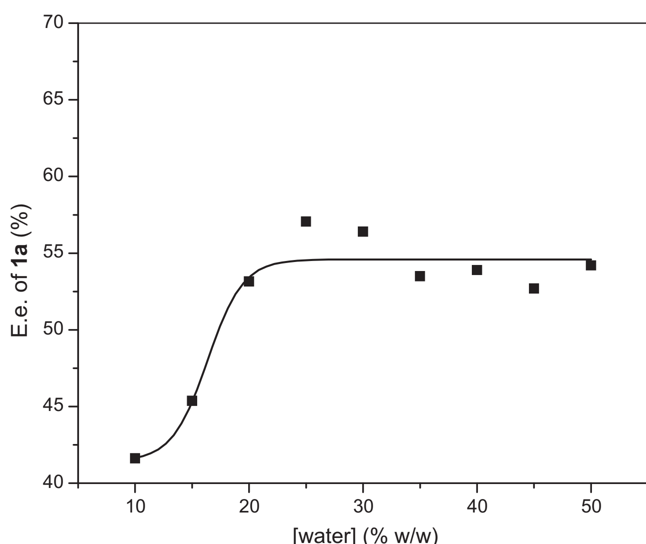


Fig. 3. Effect of water content in Lecitase organo-gel on rate of enantioselective hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** in toluene. Wt. of gel 100 mg, reaction volume 1 mL. [**1**] = 0.29 M, reaction volume 1 mL, reaction period 6 h.

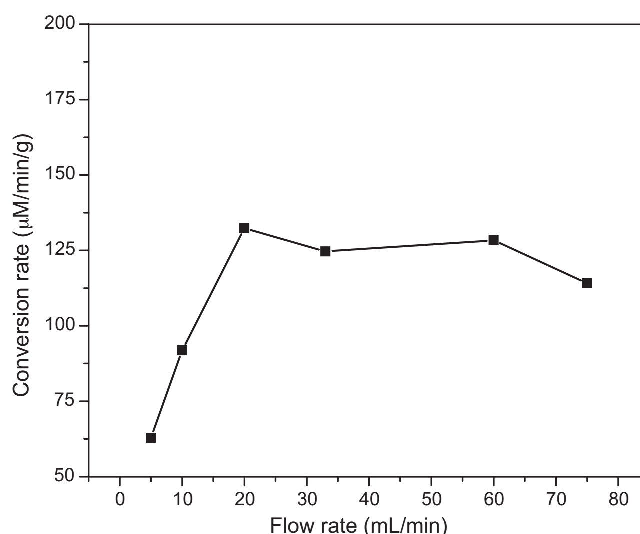


Fig. 4. Effect of flow rate on hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** by immobilized Lecitase Ultra in porous vessel bioreactor at 30 °C. Reaction conditions: [substrate] = 0.29 M in toluene. Reaction volume 120 mL. Immobilized Lecitase 17 g. Reaction period 6 h.

3.2. Effect of water content on hydrolytic reaction

In a two-phase system involving enzyme in water-immiscible organic solvents, it is necessary to maintain a critical amount of water in the reaction system to achieve proper ionization states of various amino acid residues at the active site of the enzyme [17]. In present case, water acts also as a reactant in the hydrolysis of the glycidate ester. However, excess water causes unwanted ring opening of the epoxide to give corresponding diol. Thus to optimize the water content, reactions were carried out at different water contents of the gel (determined by Karl-Fisher titration) under identical reaction conditions. Analysis of the reaction mixture by chiral HPLC showed that the rate of conversion (expressed in terms of % e.e. of the product) increases with increasing water content of the gel up to 20% (w/w) and then remains constant even up to 50% (w/w) (Fig. 3). However, addition of too much water (>30%) caused swelling of the gel and its slow disintegration. In addition, formation of diol as a side reaction was observed when the water content of the gel exceeded 30% (w/w). After several experiments, we established that it was best to use air dried gelatin matrix holding 16.5% (w/w) water in toluene saturated with Tris–HCl buffer (0.05 M containing 15 mM $CaCl_2$, pH 8.5). Under these conditions, small changes in water content due to partitioning/transfer of water between bulk organic phase and gel phase did not cause any appreciable changes in observed reaction rates, the gel appeared to be mechanically sturdy and diol formation was not observed.

3.3. Reactions in porous vessel bioreactor

Although the enzymes immobilized in gelatin matrix (gelozymes) display excellent stability in organic solvents and can be recycled several times [18], the soft gel matrix suffered severe attrition due to stirring in a stirred tank reactor. Similarly, in a packed bed reactor the gel particles agglomerated and caused high pressure drop. The porous vessel bioreactor was been found to be most suitable for such a gel-type matrix. Since the flow of the liquid from the bioreactor occurs both radially and axially, fouling or blocking of the pores and the pressure drop across the column was avoided. In addition, the reactor worked as a loop reactor operated under a complete recirculation of the liquid stream (Fig. 1).

3.3.1. Effect of particle size of the biocatalyst on reaction rate

Substrate diffusion in the pores can play an important role in determining the reaction velocity in case of solid catalysts and the resistance to pore diffusion can be eliminated by selection of appropriate size of catalyst [19]. To study the importance of pore diffusion resistance in the present case, reactions were carried out under identical conditions with equal catalyst weight (17 g), substrate concentration of 0.29 M in buffer saturated toluene, at a flow rate of 20 mL min^{-1} for 10 h with catalyst particles of sizes 2.36, 1.70, 1.40, 1.18, 1.00, and 0.85 mm. The conversion was followed by HPLC analysis. Very little variation (<5%) in the typical conversion rate of 132 μM min^{-1} g^{-1} was found in all the cases indicating that in the particle size range of 0.85–2.36 mm, pore diffusion resistance is negligible. This is not surprising since scanning electron microscopic studies of the gelozymes prepared in our laboratory show an ordered macroporous structure with channels of 30–40 μM diameter [18] which are sufficiently large to allow a free passage of reactants through them.

3.3.2. Effect of flow rate on rate of reaction

To study the external mass transfer limitation which can be minimized by operating a reactor at high velocities, the hydrolytic reaction was studied at a fixed substrate concentration of (0.29 M) and fixed enzyme loading (17 g, particle size 1–1.2 mm) in the porous vessel bioreactor at 30 °C at different flow rates. It was observed that the rate of conversion increased with the flow rate up to a flow rate of 20 mL min^{-1} and remained unchanged (within experimental error of ±5%) at least up to a flow rate of 75 mL min^{-1} (Fig. 4). The experimental data was analyzed in terms of Thiele modulus (φ), which quantifies the ratio of the reaction rate to the diffusion rate of the immobilized enzyme. The Thiele modulus is generalized as in Eq. (1),

$$\varphi = \frac{(-r_A)L}{\left\{2D\int_{S_0}^{S}(-r_A)\mathrm{d}[S]\right\}^{1/2}} \tag{1}$$

For first-order reaction, the Thiele modulus is given by Eq. (2),

$$\varphi^2 = \frac{k[S]^2L^2}{D\{[S]^2-[S_0]^2\}} \tag{2}$$

Table 3
Thiele modulus values for different flow rates.[a]

Flow rate ($mL\,min^{-1}$)	Thiele modulus (φ)
5	0.089602636
10	0.098956457
20	0.114855886
33	0.146191927
60	0.202941661
75	0.244382816

[a] [Substrate] = 0.29 M in toluene, immobilized Lecitase 17 g, reaction volume 120 mL.

where, r_A is the rate of reaction, k is the first order rate constant, L = radius/3 (assuming spherical shape of catalyst particle), D is the diffusivity calculated by method of Wilke and Chang [20], and $[S_0]$ and $[S]$ are the initial and final concentrations of the substrate. It was observed that the values of Thiele modulus increase from 0.09 at flow rate of 5 $mL\,min^{-1}$ to 0.24 at 75 $mL\,min^{-1}$ (Table 3). These values of φ are fairly low, suggesting that substrate molecules are able to diffuse far into the pore structure before they get converted into product. Thus, the concentration of substrate in this case is nearly uniform throughout the catalyst and the entire accessible surface area participates in the reaction. In this regime it is not seriously affected by mass-transfer limitations.

3.3.3. Enzyme kinetics

The hydrolytic reaction of **1** was carried out in the bioreactor by varying the concentration of substrate from 0.048 M to 0.384 M in toluene at a fixed flow rate of 20 $mL\,min^{-1}$ at 30 °C with biocatalyst particle size of 1–1.2 mm. Samples were collected from the recycle stream at regular intervals during first 5 h and analyzed by HPLC. The results of observed initial reaction velocity, V_{obsd}, as a function of substrate concentration followed a simple Michaelis–Menten kinetics (correlation coefficient r = 0.986, Fig. 5). The values of apparent K_m, $K_{m,app}$; and apparent V_{max}, $V_{max,app}$ calculated by non-linear analysis (GraphPad Prism 5) were 0.53 M and 0.38 $mM\,min^{-1}\,g^{-1}$, respectively. Since the substrate concentration (0.29 M) at which most reactions were carried out is lower than K_m, the reaction follows a typical first-order exponential path with k = 0.088 h^{-1} (correlation coeff. r = 0.99, Fig. 6) at least up to 10% conversion.

For comparison with free enzyme, reactions were carried out in a magnetically stirred round bottom flask using enzyme solution in buffer (1 mL, 1725 units) in contact with substrate solution in toluene (10 mL). The contents were magnetically stirred at 100 rpm and samples were analyzed every

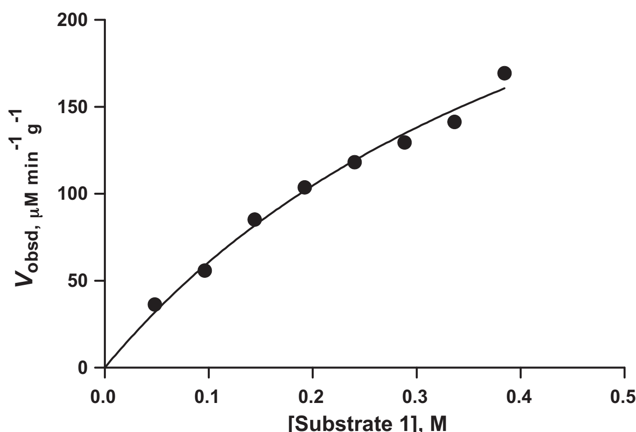


Fig. 5. Plot of V_{obsd} against [substrate] for hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** by immobilized Lecitase Ultra in porous vessel bioreactor at 30 °C. Reaction conditions: [substrate] = 0.048–0.385 M in toluene, immobilized Lecitase 17 g, reaction volume 120 mL, flow rate 20 $mL\,min^{-1}$.

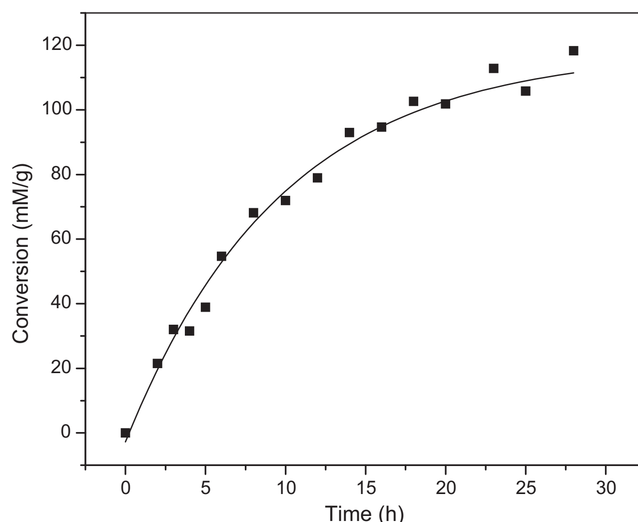


Fig. 6. A typical plot of progress of reaction during hydrolysis of (±)-*trans*-methyl (4-methoxyphenyl) glycidate **1** by immobilized Lecitase Ultra in porous vessel bioreactor at 30 °C. Reaction conditions: $[S]_0$ = 0.29 M in toluene, immobilized Lecitase 17 g, reaction volume 120 mL, flow rate 20 $mL\,min^{-1}$.

30 min for 6 h. The values of $K_{m,app}$ and $V_{max,app}$ were found to be 0.25 M and 0.32 $mM\,min^{-1}\,mL^{-1}$, respectively. The enzyme efficiency calculated as the ratio of $V_{max,app}/K_{m,app}$ for free ($1.28 \times 10^{-3}\,min^{-1}\,mL^{-1}$) and immobilized enzyme ($7.2 \times 10^{-4}\,min^{-1}\,g^{-1}$) are in excellent agreement with enzyme assay with tributyrin which showed that 60% of the enzyme was active after immobilization. As expected for immobilized enzyme, the $K_{m,app}$ is higher than that for free enzyme. Considering that 60% of the immobilized enzyme is actually active, the $V_{max,app}$ value for immobilized enzyme appears to be higher than that for free enzyme. Although such 'activating' effects of immobilization matrix have been reported [2], the substrate concentration that can be reached in the solvent (0.6 M) is much lower than required for saturation kinetics (at least 2 M) in the present case. Thus it is not possible to confirm whether the immobilized enzyme really exhibits a higher activity than free enzyme.

3.4. Modeling of a porous vessel bioreactor as a plug flow reactor (PFR)

As in the plug flow reactor the concentration of the fluid varies from point to point along the flow path, under steady state conditions, the porous vessel bioreactor is assumed to behave as a plug flow reactor. For a continuous plug flow reactor under steady-state condition and negligible mass transfer limitations, the design equation becomes:

$$\left\{\frac{1-\varepsilon}{\varepsilon}\right\}\frac{LV_{max}}{u} = (S_0\delta) - K_m \quad \ln(1-\delta) \qquad (3)$$

where ε is the porosity of the gelozyme, L is the height of catalyst bed, u is the velocity of the recycled stream, V_{max} is the maximum reaction rate of enzyme catalyzed reaction, S_0 is the initial substrate concentration, δ is substrate conversion ($(S_0 - S)/S_0$), and K_m is Michaelis–Menten constant [21]. A plot of $\delta \times S_0$ versus $\ln(1-\delta)$ should give a straight line with slope equal to K_m [22]. Fig. 7 shows a plot of experimental values of δS_0 *vs* $\ln(1-\delta)$ for hydrolysis of **1** (data from Fig. 5) which shows a good linearity (correlation coefficient r = 0.987) supporting the above model. Using the values of L (5 cm), ε (0.45) calculated from relation ε = [1 − (bulk density/particle density)], and u (25.5 $cm\,h^{-1}$), the value of K_m obtained from the slope is 0.46 M which is reasonably close to the value of 0.53 M obtained from non-linear analysis.

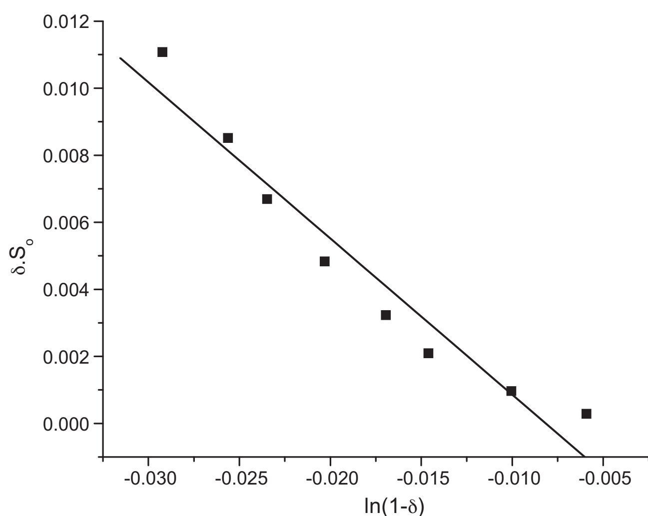


Fig. 7. A plot of (δS_0) *vs* $\ln(1-\delta)$ showing the performance of the porous vessel bioreactor as a plug-flow reactor. Reaction conditions: $[S]_0$ = 0.04–0.36 M in toluene at fixed flow rate of 20 mL min^{-1}. Reaction period 10 h, [enzyme] = 17 g.

3.5. Modeling of a porous vessel bioreactor as a recycling reactor

A material balance for the recycling porous bioreactor gives

$$V\varepsilon^n \times \left\{\frac{\mathrm{d}[S]}{\mathrm{d}t}\right\} = -MV_{\text{obsd, max}} \tag{4}$$

where V is volume of reactor, ε is the porosity of the gelatin matrix, n is an exponent, S is the substrate concentration, t is reaction time, M is the mass of immobilized enzyme and $V_{\text{obsd,max}}$ is the observed maximum reaction rate per unit mass of immobilized enzyme at a given substrate concentration.

Integrating Eq. (4) between the following limits:At $t=0$, $S=S_0$ and at $t=t$, $S=S$

$$V_{\text{obsd}} = \frac{(S_0 - S)V\varepsilon^n}{Mt} \tag{5}$$

From the data of $(S_0 - S)$ as a function of 't' $V_{\text{obsd,max}}$ was calculated using the values of V, ε and M. The calculated values of $V_{\text{obsd,max}}$ were close to experimental V_{obsd} (data in Fig. 5) when $n=2$ in the Eq. (5). A more detailed modeling is being worked out.

3.6. Enzyme recycle

In the recycle studies, the reactants were drained after 24 h reaction, the gel was washed with toluene saturated with buffer (2 × 100 mL) and left in toluene at room temperature for 24 h more. A fresh batch of the substrate was then introduced and the reaction was continued for 24 h. In this way, 15 recycles of the enzyme were conducted over a period of 30 days. It was observed that the immobilized enzyme was quite stable up to 15 days and slowly lost its activity. However, the activity was still about 50% at the end of 30 days.

4. Conclusion

In conclusion, we observe that the porous vessel bioreactor is an excellent solution to the problem of using biocatalyst immobilized in a fragile gel-type matrix. It behaves essentially like a plug flow reactor with recycle and the enzymatic reaction follows a normal Michaelis–Menten kinetics. The enzyme can be recycled several times or used in a continuous mode over a period of at least two weeks without appreciable loss of activity.

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