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'Gelozymes' in organic synthesis: synthesis of enantiomerically pure (*S*)-2-hydroxy-(3-phenoxy)phenylacetonitrile with lipase immobilised in a gelatin matrix†

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Abstract

Lipase from *Pseudomonas cepacia* (Amano PS and PS Lipase, Fluka) immobilised in microemulsion-based organogels formed by gelatin solubilisation and crosslinking with glutaraldehyde ('Gelozyme') has been used for the alcoholysis of the butanoate ester of racemic 2-hydroxy-(3-phenoxy)phenylacetonitrile with 1-butanol in hexane to obtain (*S*)-2-hydroxy-(3-phenoxy)phenylacetonitrile. The immobilised enzyme can be used over 25 days (25 cycles) without significant loss of enzyme activity (<10%).

1. Introduction

Optically active cyanohydrins are important starting materials for the synthesis of a number of chiral pharmaceuticals and agrochemicals because they can quite easily be transformed into multifunctional chiral synthons such as β-hydroxy amines, α-hydroxy carboxylic acids and α-hydroxy ketones.[1–8] One of the most important enantiomerically pure cyanohydrins of commercial interest is the cyanohydrin (*S*)-2-hydroxy-(3-phenoxy)phenylacetonitrile **2**, since many of the presently commercially important pyrethrin analogues such as Deltamethrin (Hoechst) and Esfenvalerate (Sumimoto Chemicals) are esters of **2**. The annual demand for these synthetic pesticides is as high as 7000 TPA. Several enzymatic approaches have been developed for the synthesis of enantiomerically pure (*S*)-cyanohydrins such as the enantioselective addition of HCN to 3-phenoxybenzaldehyde catalysed by the enzyme oxynitrilase from

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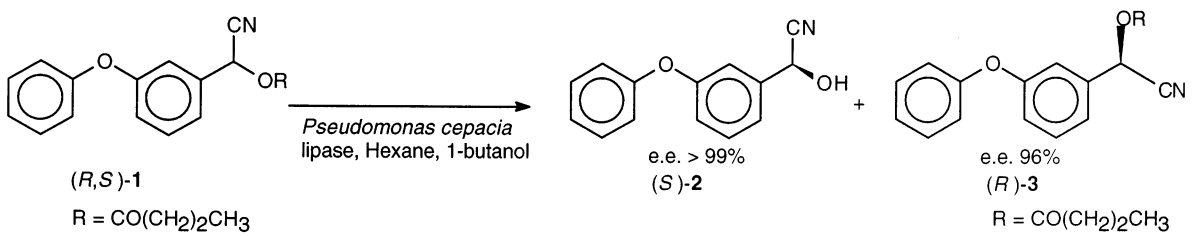
Sorghum bicolor or *Haevea brasiliens*[2,3] or the lipase-catalysed kinetic resolution coupled with in situ formation and racemization of the cyanohydrin in an organic solvent[4] and the lipase-catalysed enantioselective hydrolysis or transesterification of a racemic cyanohydrin ester using an (*S*)-specific lipase to obtain a mixture of (*R*)-ester and (*S*)-cyanohydrin.[5–9] For recycling purposes the (*S*)-specific enzyme, lipase from *Pseudomonas* sp., has been immobilised on Celite[6] and DEAE-Sephadex[9], but the enzyme activity was found to deplete over long periods.

Recently, we have reported a novel technique of enzyme immobilisation in a gelatin matrix using a bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT)–isooctane–water microemulsion system[10] to obtain immobilised enzyme preparations, 'Gelozymes'. Here we report an important application of such a 'gelozyme' in the preparation of (*S*)-2-hydroxy-(3-phenoxy)phenylacetonitrile **2**. The enzyme lipase from *Pseudomonas cepacia* (Amano PS) is immobilised in a glutaraldehyde-crosslinked gelatin matrix with a loading of 20% (w/w) and is used for the alcoholysis of (*RS*)-cyano(3-phenoxyphenyl)methyl butyrate **1** ($R = CO(CH_2)_2CH_3$) with 1-butanol in hexane (Scheme 1). On a small scale the immobilised enzyme has been used for 25 recycles over a period of 25 days without serious loss of enzyme activity (<10%) at room temperature. In comparison, the native enzyme powder used as a suspension lost 50% of its activity after 10 recycles. The product (*S*)-**2** (e.e.>99%) and unreacted (*R*)-**3** (e.e. 96%) were isolated in 85 and 95% theoretical yield, respectively, after column chromatography in the cold.[11] Large scale reaction (30 g reaction scale, reaction volume 550 ml) was carried out in a stirred tank reactor with reproducible results. Although the commercial enzyme is sold as an enzyme preparation already supported on diatomaceous earth (30 units/mg), it was far more useful after immobilization in the gelatin gel. To compare the results with another enzyme source, the experiments were repeated with lipase obtained from Fluka (PS lipase, 50 units/mg). The results obtained were similar in both cases after giving due consideration for differences in enzyme activities.



Scheme 1.

2. Results and discussion

2.1. Enzyme loading

In our previous communication we used clear solutions of native enzyme powders dissolved in aqueous buffer for immobilisation with low enzyme loading in the gelatin matrix.[10] We have now found that it is possible to immobilise as much as 20% (w/w) of the lipase available commercially as an enzyme powder bound to diatomaceous earth (30–50 units/mg) in the gelatin matrix. A slurry of the native enzyme can be mixed with gelatin solution in isooctane–water

microemulsion and crosslinked with glutaraldehyde without any significant loss of activity compared with native enzyme powder (<10%). This type of double immobilisation provides extra stability to the enzyme without serious diffusional problems with the added advantage that the particles are now larger and heavier for easier separation from the reaction mixture.

2.2. Choice of substrate

In the earlier reports, the cyanohydrin acetate **1** ($R=COCH_3$) is used as the substrate for lipase-catalysed resolution. However, it is somewhat tedious to follow the reaction by GC since the cyanohydrin has to be derivatised due to its instability.[6,9] We have observed that both the (*R*)- and (*S*)-enantiomers of the butanoate ester, the (*R*)- and (*S*)-enantiomers of cyanohydrin, and 3-phenoxybenzaldehyde are well separated on a Chiralcel OJ column in the same run and it is very convenient to follow the course of reaction by chiral HPLC.[11] Besides, we have also observed that the immobilised enzyme shows excellent enantioselectivity ($E>200$) and the reaction practically stops at 49% conversion giving the (*S*)-cyanohydrin with e.e.>99%. In comparison, hydrolysis of the corresponding acetate in aqueous buffer proceeds with lower enantioselectivity ($E=88$).[8] Hence, in all the experiments we have chosen the butanoate ester **1** ($R=CO(CH_2)_2CH_3$) as the substrate.

2.3. Effect of alcohol concentration on reaction rate

Earlier studies[6,9] have shown that 1-butanol is an excellent reagent for alcoholysis, while hexane and diisopropyl ether are the preferred solvents. Since the immobilisation matrix used by us is different from that reported earlier, we have re-investigated the effect of alcohol concentration on reaction rate in hexane for the enzyme immobilised in gelatin matrix. The results are given in Table 1. Earlier, Effenberger and co-workers[6] observed that in the case of the enzyme immobilised on Celite the molar ratio of alcohol to substrate played an important role. We have observed that the reaction was very fast when the molar ratio of alcohol to substrate was 0.5. The rate of reaction became gradually slower in proportion to the increase of the molar equivalents of butanol, probably due to the denaturation of the enzyme and/or destruction of the structure of the gel matrix. The use of excess butanol was, however, compensated for by another beneficial effect. Indeed, the increased amount of butanol (for example, 3 equivalents) increased the solubility of the cyanohydrin, the product, and helped the progress of the

Table 1
Amano PS catalysed alcoholysis of (*RS*)-**1** (0.17 M) with 1-butanol in hexane at 30°C

[1-Butanol]/[(*RS*)-**1**]	Reaction rate[a] (mM/h/g)
0.5	62.6
1	47.2
2	43.3
3	45.9
4	39.6

[a] The reactions were carried out with 60 ml reaction mixture containing the ester **1** and 1-butanol in hexane, and 1 g of lipase immobilised in 5 g gelatin. Product concentration is based on chiral HPLC analysis. Rates are given per gram of native enzyme used before immobilisation.

transesterification. When the reaction was carried out with low alcohol equivalence, the cyanohydrin separated out of the reaction mixture and deposited itself on the surface of enzyme preparation, which caused a serious retardation of the reaction, especially in the large scale preparations.

2.4. Effect of substrate concentration on reaction rate

Typically, the alcoholysis of (*RS*)-**1** proceeded with a pseudo zero-order rate up to at least 70% conversion (correlation coeff. 0.98–0.99). From the Lineweaver–Burke plot (Fig. 1) the apparent Michaelis constant $K_{m,app} = 0.14$ M and the apparent maximum velocity $V_{max,app.} = 91.4$ mM/h/g enzyme were calculated.

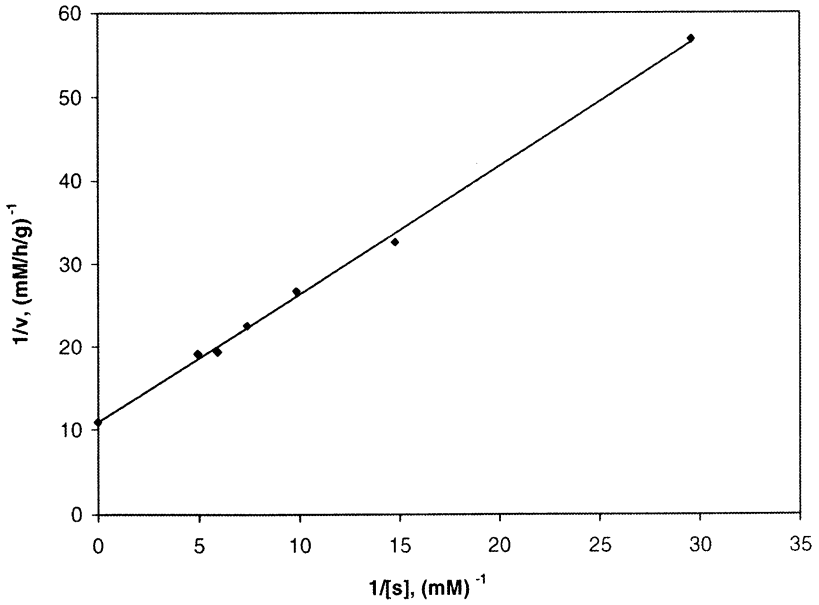


Figure 1. Lineweaver–Burke plot for enantioselective alcoholysis of (*RS*)-cyano(3-phenoxyphenyl)methyl butyrate **1** with 1-butanol in hexane catalysed by immobilised *Pseudomonas cepacia* lipase (1 g of lipase immobilised in 5 g gelatin) at 30°C. [**1**] = 0.034–0.2 M, [1-butanol]/[**1**] = 3. Reaction volume 60 ml

During the course of the reaction, formation of 3-phenoxybenzaldehyde due to decomposition of the cyanohydrin was also observed. The rate of decomposition of the cyanohydrin was found to be approximately 10 mM/h/g of the enzyme. This was significant ($\sim$10%) compared with the rate of cyanohydrin formation. Addition of a small amount of glacial acetic acid (0.1%) effectively suppressed the rate of cyanohydrin decomposition to an acceptable value of 0.86 mM/h/g without significant change in the alcoholysis rate.

2.5. Enzyme recycle

Enzyme recycle studies were carried out with a substrate concentration of 0.17 M (5%), 3 molar equivalents of 1-butanol in hexane and 1 g of immobilised enzyme in 60 ml reaction volume (substrate to enzyme ratio 1:3, w/w). The enzyme was recycled 25 times with less than 10% loss of activity after 25 runs. The overall activity of the enzyme powder before and after

immobilisation was comparable for the first two runs (Fig. 2), but the activity of the native enzyme powder decreased rapidly and after the 6th cycle 50% of the activity was lost.

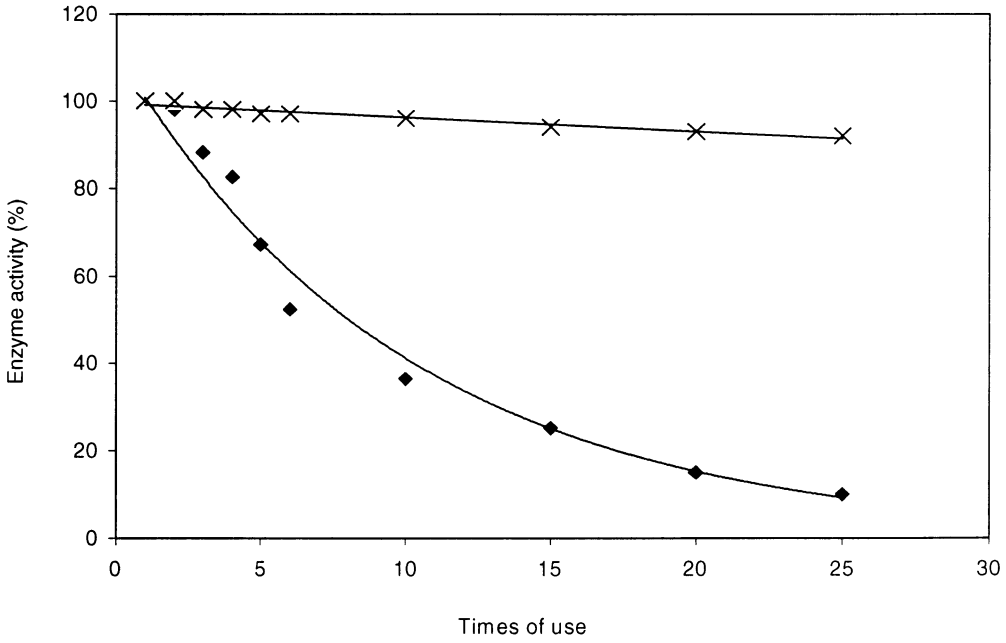


Figure 2. Recycling of *Pseudomonas cepacia* lipase for enantioselective alcoholysis of (*RS*)-cyano(3-phenoxyphenyl)methyl butanoate **1** with 1-butanol in hexane at 30°C. ◆ native enzyme powder; × immobilised enzyme. [**1**] = 0.17 M, [1-butanol] = 0.51 M, 1 g immobilised lipase in 60 ml reaction volume

2.6. Enzyme inhibition studies

Fishman and Zviely[9] have shown that the enzyme immobilised on DEAE-Sephadex is inhibited by the product cyanohydrin up to 34% when 0.5 equivalents of (*S*)-cyanohydrin are added to the reaction mixture and by about 20% in the presence of 1 equivalent of 3-phenoxybenzaldehyde. We have observed that the enzyme immobilised in gelatin matrix is inhibited by only 10% by one equivalent of 3-phenoxybenzaldehyde. Also, the (*R*)-enantiomer practically does not act as a competitive inhibitor for the alcoholysis of the (*S*)-enantiomer. This was evident from the observation that the rate of reaction for the enantiomerically pure (*S*)-ester[12] was similar to the rate for the racemic ester within an experimental error of 5%.

3. Conclusion

The present study provides an excellent methodology to produce (*S*)-2-hydroxy-(3-phenoxy)phenylacetonitrile on a large scale using the lipase from *Pseudomonas cepacia* immobilised in gelatin matrix. Compared to the techniques of adsorption on Celite or Sephadex, the methodology of entrapment and covalent binding of the enzyme to gelatin matrix provides excellent protection to the enzyme active site, which allows repeated use of the enzyme without serious loss or inhibition of activity. In addition, the use of butanoate ester simplifies the

analytical procedure for following the progress of the reaction by chiral HPLC, and this methodology can be applied for industrial scale preparation. The separation of the unreacted (*R*)-ester and the (*S*)-cyanohydrin by solvent extraction technique[9] is presently being studied. The data presented here are not only potentially useful for the industrial production of the title compound, but also open up several possibilities of using such gelozymes in large scale enzymatic reactions, especially in organic solvents.

4. Experimental

Lipase PS was resourced from Amano Pharmaceutical Co. Ltd., Japan and lipase from *Pseudomonas cepacia* PS lipase (50 units/mg) was purchased from Fluka, Switzerland. Dioctylsulfosuccinate sodium salt (AOT) was obtained from Sigma, USA; and gelatin from Hi-Media, India. All other reagents were A.R. grade obtained from Qualigens, India. NMR spectra were recorded on a Varian FT-200 MHz (Gemini) using tetramethylsilane (TMS) as the internal standard. Elemental analyses were carried out on a Vario EL, Elementar, Germany. Infrared spectra were scanned on a Perkin–Elmer 1310 spectrophotometer with sodium chloride optics. HPLC analyses were carried out on a Hewlett-Packard HP 1090 unit with diode array detector and HP Chem Station. Optical rotations were measured on JASCO DIP-370 digital polarimeter.

4.1. Immobilisation of the lipase

Gelatin (5 g) was dissolved in distilled water (8.5 ml) by warming to 60°C. A solution of bis(2-ethylhexyl)sulfosuccinate sodium salt, AOT (0.3 M in isooctane, 35 ml) was added; the mixture was stirred for 5 min at 50°C and cooled in ice to obtain a free flowing gelatin solution. Lipase from *Pseudomonas cepacia* (Amano PS or PS Lipase from Fluka 1.25 g) was suspended in 3 ml water and mixed with gelatin solution in cold. After the enzyme was well dispersed, glutaraldehyde (25%, 1 ml) was added with stirring and the solution was then poured onto a petriplate and allowed to dry overnight at room temperature. The thin film of the immobilised enzyme so obtained was frozen in liquid nitrogen, powdered using a mortar and pestle and sieved to obtain immobilised enzyme granules of particle size 1–2 mm. The particles were washed with hexane to remove AOT and dried under vacuum.

4.2. Preparation of (RS)-cyano(3-phenoxyphenyl)methyl butyrate **1**

Preparation of **1** was carried out essentially by the method of Fishman[9] using butyryl chloride instead of acetic anhydride. The residual dark yellow oil of the crude product was extracted with boiling hexane. The hexane extract on removal of solvent gave a pale yellow viscous oil (42 g, 95%). IR (neat) 3064, 2256, 1904, 1760, 1696, 1584, 1488, 1456, 1392, 1248, 1208, 1152, 1072, 688 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.96 (t, 3H), 1.60–1.75 (m, 2H), 2.37–2.47 (m, 2H), 6.40 (s, 1H), 7.02–7.45 (m, 9H). ^{13}C NMR ($CDCl_3$, 200 MHz): δ 13.48, 18.21, 35.55, 62.31, 115.99, 117.57, 119.41, 120.07, 122.00, 124.10, 130.00, 130.64, 133.74, 156.39, 158.35, 171.48.[13] Anal. calcd for $C_{18}H_{17}NO_3$: C, 73.20; H, 5.80; N, 4.74; found C, 72.86; H, 5.72; N, 4.66.

4.3. Chiral HPLC analysis

HPLC analysis was done on Chiralcel OJ (5×250 mm), Daicel Chemical Industries, Japan. Mobile phase 18% isopropanol in hexane; flow rate 0.7 ml/min. and detection wavelength 240 nm. Retention times: 3-phenoxybenzaldehyde 9.2; (*R*)-butyrate 12.7; (*S*)-butyrate 14.9; (*R*)-cyanohydrin 18.2; (*S*)-cyanohydrin 22.7 min.

4.4. Alcoholysis of (RS)-cyano(3-phenoxyphenyl)methyl butyrate *1* with Pseudomonas cepacia lipase immobilised in gelatin gel

In a typical experiment, (*RS*)-**1** (2.95 g, 10 mmol) in hexane (60 ml) and 1-butanol (2.22 g, 30 mmol) were stirred with immobilised enzyme (5 g powder, 1 g enzyme) in a stirred tank reactor with an overhead mechanical stirrer at 80–100 rpm. The progress of the reaction was monitored by chiral HPLC. The reaction was continued until almost all of the (*S*)-ester had reacted (8 h, 49% conversion) and the reaction came to a standstill. The reaction mixture was then decanted from the enzyme and the enzyme was washed with hexane. After removal of hexane under vacuum (without warming) the products were separated by column chromatography at 10°C (solvent 5% ethyl acetate in hexane, R_f values: aldehyde 0.60, ester 0.45, cyanohydrin 0.35) to obtain (*S*)-cyanohydrin **2** (0.96 g, 85%), (*R*)-ester **3** (1.4 g, 95%). The products were characterised by ^{1}H NMR. The absolute configuration of the product cyanohydrin and unreacted butanoate ester was assigned on the basis of known stereochemical preference of the enzyme and comparison of the optical rotation value with literature. (*R*)-Cyano(3-phenoxyphenyl)methyl butanoate **3** $[\alpha]_D^{25}$ −4.9 (*c* 1, chloroform) e.e. 96%; lit.[7] value for corresponding (*R*)-acetate −7.02 (*c* 1, chloroform). (*S*)-2-Hydroxy-(3-phenoxy)phenylacetonitrile **2** $[\alpha]_D^{25}$ −24.3 (*c* 1, chloroform), e.e.>99%; lit.[7] $[\alpha]_D^{25}$ −23.4 (*c* 1, chloroform), e.e. 96%.

Acknowledgements

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13. In the phenoxy ring, the '*ortho*' carbon atoms C-2.2 and C-6.6, and '*meta*' carbon atoms C-3.3 and C-5.5 are equivalent and resonate at δ 119.41 and 130.00 ppm, respectively, in the ^{13}C NMR spectrum giving 16 signals for a molecule with 18 carbon atoms.