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Asymmetric hydrolysis of a *meso*-diester using pig liver esterase immobilised in hollow fibre ultrafiltration membrane

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Abstract

Pig liver esterase (PLE) was physically immobilised in a polysulphone ultrafiltration hollow fibre membrane reactor and used for the repetitive batch two-phase hydrolysis and separation, on a multigram scale, of the *meso*-diester dimethyl *cis*-cycloxex-4-ene-1,2-dicarboxylate **1** to enantiomerically pure (1*S*,2*R*)-cyclohex-4-ene-1,2-dicarboxylic acid monomethyl ester **2**. After 25 days, the enzyme still retained its initial activity, which corresponds to 62% of its activity in the free form, and the enantiomeric purity of monoester **2** was still higher than 97%. Simple experimental conditions were established for the large laboratory scale preparation of substrate **1** and isolation of product **2** from the aqueous phase.

1. Introduction

Pig liver esterase (PLE) is a versatile biocatalyst for asymmetric transformations.[1–3] This enzyme has been used mainly in aqueous media due to low stability and activity in organic solvents.[3] However, many substrates have low solubility in water and in order to reduce mass transfer limitations a good dispersion of these substrates or the use of immobilised enzyme is necessary.[4] The hydrolysis of the *meso*-diester **1** into the mono ester **2** by PLE with high enantiomeric purity (e.e. 94–97%) is well known (Scheme 1).[4–8] The enantiomerically pure mono ester **2** has also been successfully used as a versatile chiral substrate in asymmetric syntheses.[1,9–15]

Liquid and polymeric (solid) membranes have been used in different applications, including separation,[16] resolution[17–20] and enzyme immobilisation/separation.[21–24] The possibility of associating the advantages of enzyme immobilisation in membranes to the operation of a biphasic reactor seems useful, since the use of a hollow fibre module, a well established technology that provides a compact system with a high surface area/volume ratio (up to 10 000 m^2/m^3), will greatly intensify the mass

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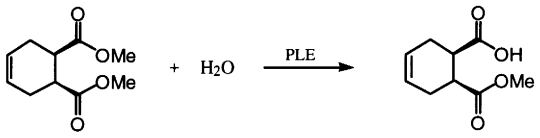


1 2

Scheme 1.

transfer process of the substrate from the organic to the aqueous phase. The enzyme, when physically immobilised in the pores of a non-symmetric membrane, can be easily replaced by fresh enzyme, when deactivated. We performed the physical immobilisation of PLE in polysulphone ultrafiltration hollow fibres, which allows repetitive batch two-phase conversion of **1** into **2** and separation on a multigram scale. Simple experimental conditions were also established for the large laboratory scale preparation of substrate **1** and isolation of product **2** from the aqueous phase.

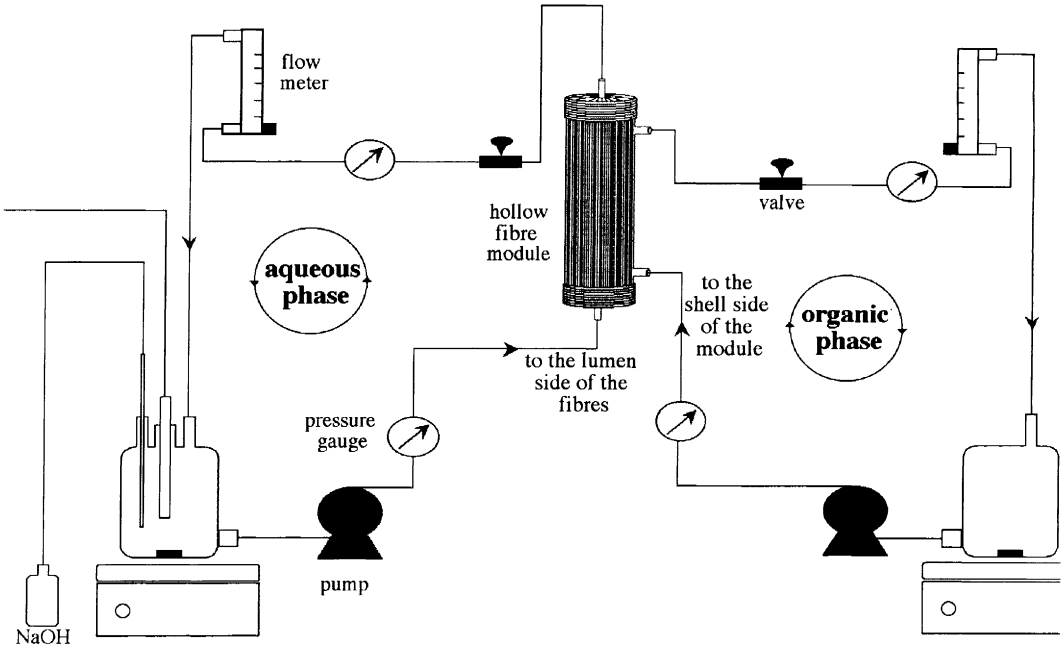
2. Results and discussion

The physical immobilisation was tested using polysulphone ultrafiltration hollow fibres (30 kD). Due to the porous structure of the membrane (asymmetric, with an external diameter of the pore larger than its internal diameter), the enzymatic immobilisation was accomplished by the circulation of an enzyme solution from the outside to the inside of the hollow fibres, which forced the enzyme to be retained in the interior of the membrane porous structure. The enzyme molecule is too large (~150 kD), in relation to the pore dimension of the membrane, and therefore it cannot diffuse through the membrane to the lumen side of the fibres, while the low enzymatic solubility in hexane prevents its back-diffusion to the shell side of the module. The substrate **1** has a low solubility in 0.1 M aqueous phosphate buffer, pH 7.0 (42 mM), while the salt of **2** is almost insoluble in hexane (<0.1 mM). These solubility properties allow simultaneous reaction and separation processes to take place in the hollow fibre module, by the hydrolysis of the hydrophobic substrate **1** into the polar product **2** in the interface, which is then transferred to the aqueous phase.

The process was performed by circulating the hexane phase containing the substrate **1** and the aqueous receiving phase of the product **2** as presented in Scheme 2.

The conversion of **1** into **2** was complete after 24 h with a high enantiomeric excess (see Fig. 1). After each cycle, both phases were replaced by fresh solutions and the process was repeated again. The evolution of the reaction rate with time, as presented in Fig. 2, gives information on the evolution of enzyme activity. The initial activity observed for the immobilised enzyme corresponds to 62% of its activity in the free form. It was observed that there is an activity increase during the first hours of operation, which is probably due to the fact that the enzyme is acquiring a more favourable position when contacting with both aqueous and organic solutions. A similar observation was reported using lipases in inverted micelles.[25,26] After the increase of initial enzymatic activity, it stabilises for a period of about 200 h, after which it starts decreasing. After 25 days[1] of repeated operation the enzyme still presents an activity similar to its initial immobilisation value, and the enantiomeric purity of **2** was kept higher than 97%. Starting from a combined mixture of collected aqueous phases (one-litter scale), the product **2** was isolated with an enantiomeric excess of 98.9% by simple evaporation of aqueous phase, small

[1] Each hollow fibre module immobilised with PLE was tested under continuously operating conditions during the maximum time of 25 days.



Scheme 2. Schematic representation of the hollow fibre ultrafiltration biphasic membrane system

volume extraction and further crystallisation. The enzyme was replaced in the hollow fibre module by fresh enzyme. This process was repeated three times without any observation of decreasing performance of the module.

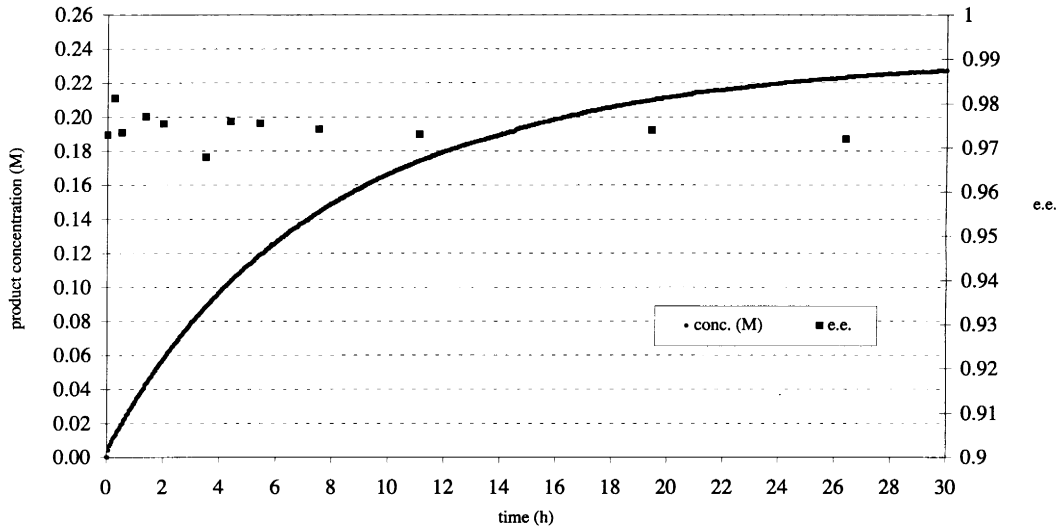


Fig. 1. Typical cycle evolution of the conversion of substrate **1** into the product **2** and of the enantiomeric excess of **2**. General conditions: fresh aqueous phase (0.1 M phosphate buffer at pH 7.0), organic phase containing **1** (0.23 M in hexane) and PLE immobilised in a hollow fibre ultrafiltration biphasic membrane module with an external effective area of 0.49 m^2

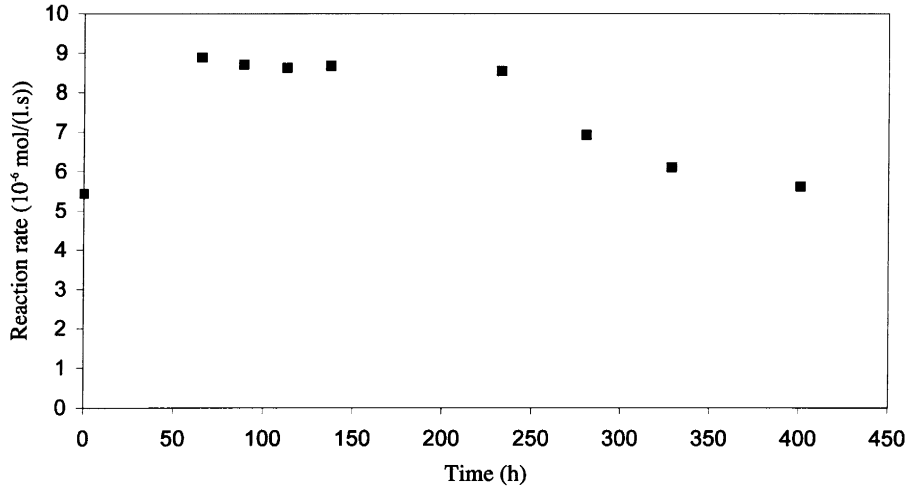


Fig. 2. Observed evolution of the reaction rate over time for PLE immobilised in a hollow fibre ultrafiltration biphasic membrane module with an external effective area of 0.49 m^2

3. Conclusion

The optimised conditions presented here allow the use of PLE physically immobilised in polysulphone ultrafiltration hollow fibre membrane for the efficient hydrolysis of *meso*-diester **1** into the versatile chiral substrate **2** in repetitive batch enzymatic two-phase transformation and separation for a long operating period with high enantiomeric excess. The method used for physical immobilisation also allows replacing the less active enzyme by fresh enzyme. We believe that this process should also be potentially useful for other comparable biocatalyst transformations of substrates into products with opposite solubility properties.

4. Experimental

Reagent quality solvents were distilled prior to use. PLE (EC 3.1.1.1) was supplied from Sigma (ref. E3019). Analytical thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (aluminium-backed silica gel Merck 60 F_{254}). Melting points (uncorrected) were determined on an Electrothermal Mod. IA 6304 capillary melting point apparatus. Infrared spectra (IR) were recorded on a Buck Scientific Mod. 500 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker ARX 400 spectrometer. Observed rotations at the Na-D line were measured at 25°C using an Optical Activity polarimeter Mod. AA-1000. The concentration of **1** and the e.e. of **2** in aqueous solution were determined by HPLC using the chiral column Chiralcel OJ-R; eluent: 0.5 M perchlorate buffer at pH 2 and acetonitrile (22:78); 0.5 ml/min; detection wavelength λ=225 nm; **2** t_R (1*S*,2*R*)=10.2 min; t_R (1*R*,2*S*)=12.0 min. Automatic titrator (Radiometer) and control system (data acquisition LabPc+ and Labview 4.0 software) were used to continuously keep the pH constant during reaction. Membrane module: polysulphone ultrafiltration hollow fibre module (ref. SPS 6005-4), with a 30 kD cut-off, was supplied by Fresenius: internal diameter 500 μm; effective length 22.5 cm; number of fibres 1200. These modules have an external effective area of 0.49 m^2.

4.1. Preparation of dimethyl cis-cycloxex-4-ene-1,2-dicarboxylate **1**

Methanol (3 000 ml) was added to a round-bottomed flask (5 000 ml) equipped with mechanical stirrer, condenser and drying tube ($CaCl_2$). Anhydrous hydrogen chloride (prepared from concentrated sulphuric acid (50 ml) and 37% hydrochloric acid (33 ml)) was bubbled and then *cis*-1,2,3,6-tetrahydrophthalic anhydride (Aldrich, 1 500 g, 9.859 mol) was added in small portions. The mixture was heated slowly until complete dissolution and then refluxed for 24 h. The mixture was allowed to reach room temperature, sodium hydroxide (pellets) was added in small portions until the pH was 6.5 and then sodium bicarbonate, in excess, was added. The mixture was filtered, the solvent was evaporated under vacuum and distilled under reduced pressure (column: diameter 3.5 cm, length 35 cm, 110–140°C/0.2–0.4 mmHg) to give **1** (1 774 g, 91%) as a colourless oil, pure by HPLC and spectral data identical to those reported.[5,27]

4.2. Physical immobilisation

An enzyme solution (840 ml) of 8.82 units/ml in 0.1 M phosphate buffer, pH 7, after a prior filtration through a 0.2 μm membrane pore to remove impurities, at room temperature, was recirculated through the ultrafiltration polysulphone membrane module. This recirculation was performed from the outside to the inside of the fibres using the two inlet shell sides and two outlet lumen sides of the membrane, using a pressure of 1.2 bar and for a period of 1 h. The enzyme immobilised per membrane unit area was 1.28 units/cm^2 which was determined from activity measurements of the enzyme solution before and after its recirculation through the module using ethyl butyrate as substrate.

4.3. Continuous hydrolysis of diester **1** to (1S,2R)-cyclohex-4-ene-1,2-dicarboxylic acid monomethyl ester **2** (Fig. 1)

One cycle (module of 0.49 m^2 external effective area): a solution of **1** (220 ml, 0.23 M) in hexane were recirculated (270 ml/min) through the shell side of the module containing immobilised PLE (1.28 units/cm^2), and a solution of 0.1 M phosphate buffer at pH 7.0 (220 ml) was recirculated (102 ml/min) through the lumen side of the module at 25°C. The two phases were equilibrated exerting a minimum pressure difference of 0.3 bar between the organic and aqueous phases. After a conversion higher than 98% (>24 h), the aqueous and the organic phases were replaced by fresh solutions for the next cycle which was performed as described before. The automatic titration of the product in the aqueous phase with aqueous solution of sodium hydroxide was used to measure the immobilised enzyme activity and keep the pH at 7.0.

4.4. Reuse of the module

A solution of aqueous NaOH (500 ml, 0.5 M), after prior filtration through a 0.2 μm membrane pore, at room temperature, was circulated through the ultrafiltration polysulphone membrane module in the opposite direction used for physical immobilisation until the outlet solution became discoloured. Distilled water was circulated and then the module was emptied by gravity.

4.5. Isolation of (1S,2R)-cyclohex-4-ene-1,2-dicarboxylic acid monomethyl ester **2** from aqueous phase

Aqueous phosphate buffer solution of **2** (1 220 ml, pH 7–6; 25.9 mg of **2**/ml; e.e. 97%) was extracted with hexane (500 ml). The aqueous phase was evaporated under vacuum until a total volume of approx.

225 ml. The mixture was acidified with 37% hydrochloric acid (pH 1), extracted with diethyl ether (3×200 ml), the combined organic layers were dried ($MgSO_4$) and evaporated to dryness to give a liquid residue (23.35 g). Distilled water (30 ml) was added and left in the fridge (+6°C) to give **2** (18.45 g, e.e. 98.9%) as a white crystalline solid; mp 62–63.5°C; mp of commercial sample (Aldrich) 63.5–64.5°C, lit.[27] 65.5–66.0°C (Et_2O–hexane); $[\alpha]_D^{27}$=+16.6 (c 1.0, EtOH); lit.[27] $[\alpha]_D^{20}$=+17.7 (c 1.0, EtOH); spectral data identical to those reported.[5,27] The water fraction was evaporated again to dryness and the residue (3.01 g) was crystallised as before to give **2** (2.05 g, e.e. 82.5%). The monoester **2** (e.e. 34.8%) was still present in the residual water fraction.

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References

1. Ohno, M.; Otsuka, M. *Organic Reactions* **1989**, *37*, 1–55.
2. Zhu, L.-M.; Tedford, M. C. *Tetrahedron* **1990**, *46*, 6587–6611.
3. Ruppert, S.; Gais, H.-J. *Tetrahedron: Asymmetry* **1997**, *8*, 3657–3664 and references cited therein.
4. Heiss, L.; Gais, H.-J. *Tetrahedron Lett.* **1995**, *36*, 3833–3836 and references cited therein.
5. Mohr, P.; Waespe-Sarcevic, N.; Tamm, C.; Gawronska, K.; Gawonski, J. *Helv. Chim. Acta* **1983**, *66*, 2501–2511.
6. Gais, H.-J.; Lukas, K. L.; Ball, W. A.; Braun, S.; Lindner, H. J. *Liebigs Ann. Chem.* **1986**, 687–716.
7. Hamilton, G. H.; Huang, Z.; Yang, X.-J.; Patch, R. J.; Narayanan, B. A.; Ferkany, J. W. *J. Org. Chem.* **1993**, *58*, 7263–7270.
8. Ito, Y. N.; Ariza, X.; Beck, A. K.; Bohác, A.; Ganter, C.; Gawley, R. E.; Kühnle, F. N. M.; Tuleja, J.; Wang, Y. M.; Seebach, D. *Helv. Chim. Acta* **1994**, *77*, 2071–2110.
9. Boland, W.; Nidermeyer, U.; Jaenicke, L.; Görisch, H. *Helv. Chim. Acta* **1985**, *68*, 2062–2073.
10. Houpis, I. N.; Molina, A.; Reamer, R. A. *Tetrahedron Lett.* **1993**, *34*, 2593–2596.
11. Kobayashi, S.; Shibata, J.; Shimada, M.; Ohno, M. *Tetrahedron Lett.* **1990**, *31*, 1577–1580.
12. Hamilton, G. H.; Huang, Z.; Yang, X.-J.; Patch, R. J.; Narayanan, B. A.; Ferkany, J. W. *J. Org. Chem.* **1993**, *58*, 7263–7270.
13. Baldwin, J. E.; Adlington, R. M.; Mitchell, M. B. *J. Chem. Soc., Chem. Commun*. **1993**, 1332–1335.
14. Borzilleri, R. M.; Weinreb, S. M. *J. Am. Chem. Soc.* **1994**, *116*, 9789–9790.
15. Kocienski, P.; Stocks, M.; Donald, D.; Perry, M. *Synlett* **1990**, 38–39.
16. *Membrane Separations Technology. Principles and Applications*; Noble, R. D.; Stern, S. A., Eds.; Dordrecht: Kluwer Academic Publishers, 1995.
17. Pirkle, W. H.; Bowen, W. E. *Tetrahedron: Asymmetry* **1994**, *5*, 773–776.
18. Yoshikawa, M.; Fujisawa, T.; Izumi, J.-I.; Kitao, T.; Sakamoto, S. *Anal. Chim. Acta* **1998**, *365*, 59–67.
19. Shinbo, T.; Yamaguchi, T.; Yanagishita, H.; Sakaki, K.; Kitamoto, D.; Sugiura, M. *J. Membr. Sci.* **1993**, *84*, 241–248.
20. Bryjak, M.; Kozolowski, J.; Wieczorek, P.; Kafarski, P. *J. Membr. Sci.* **1993**, *85*, 221–228.
21. Risson, S.; Beliczey, J.; Giffels, G.; Kragl, U.; Wandrey, C. *Tetrahedron: Asymmetry* **1999**, *10*, 923–928.
22. Cauwenberg, V.; Vergossen, P.; Stankiewicz, A.; Kierkels, H. *Chem. Eng. Sci.* **1999**, *54*, 1473–1477.
23. Lopez, J. L.; Matson, S. L. *J. Membr. Sci.* **1997**, *125*, 189–211.
24. Meindersan, G. W.; Augeraud, J.; Vergossen, F. H. P. *J. Membr. Sci.* **1997**, *125*, 333–349.
25. Crools, G. E.; Rees. G. D.; Hobinson, B. H.; Svensson, M.; Stephenson, G. R. *Biotechnol. Bioeng.* **1995**, *48*, 190–196.
26. Fletcher, P. D. I.; Freedman, R. B.; Oldfield, C.; Robinson, B. H. *J. Chem. Soc., Faraday Trans. 1* **1985**, *81*, 2667–2679.
27. Kobayashi, S.; Kamiyama, K.; Ohno, M. *Chem. Pharm. Bull.* **1990**, *38*, 350–354.