ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 523–530

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Conversion of β-sitosterol by *Mycobacterium* sp. NRRL B-3805 cells immobilized on Celite supports

N. Llanes [a,b], P. Fernandes [b], R. Léon [b], J.M.S. Cabral [b], H.M. Pinheiro [b,*]

[a] *Departamento de Procesos Biotecnológicos, Centro Nacional de Investigaciones Científicas, Ave. 25 y 158, 6990 Havana, Cuba*
[b] *Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*

Abstract

Mycobacterium sp. NRRL B-3805 cells immobilized on Celite were effectively used for the selective side-chain cleavage of sitosterol to androstenedione (AD) in organic media (phthalate derivatives). Kinetic studies were performed with sitosterol concentrations up to 24 mM, with different Celite materials and particle sizes. Higher activity levels were observed when a larger pore size Celite was used as immobilization matrix. Substrate inhibition was observed for sitosterol concentrations above 6 mM. Toxicity effects were not apparently correlated with the high log P solvents (> 9) here used as bioconversion media. The use of immobilized cells in repeated batch biotransformations did not prove effective, mainly due to biocatalyst desorption during the periodical, aqueous washing steps used for nutrient delivery to the cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Mycobacterium sp.; Celite; Organic media; Side-chain cleavage; Sitosterol

1. Introduction

The use of water-immiscible organic solvents as media for the biotransformation of hydrophobic substrates has been reported since the late 1970s [1] and the advantages of such biocatalytic systems, as well as their main drawbacks, have been reviewed [2,3]. Their use can lead to highly productive processes due to the increased solubility of the substrates, as compared to aqueous media, although the solvent may exert a toxic effect on the biocatalyst. The latter disadvantage may however be minimised if the biocatalyst is used in immobilized form [4]. On the other hand, this approach can introduce limiting partition and mass transfer resistances [5]. Reduction of the liquid-stagnant layer surrounding the immobilized biocatalyst, through an adequate choice of the liquid phase flow rate [6] or of the stirring speed [7] in bioreactors, decreases external mass transfer resistances. Also, the use of small support particle sizes can be a way of overcoming internal mass transfer limitations [5,8]. Finally, the use of hydrophobic immobilization matrices may allow for more favourable partition and diffusion conditions [8,9]. These operational conditions have to be carefully optimised since high stirring speeds or liquid flow

* Corresponding author. Tel.: +351-1-841-9065; fax: +351-1-841-9062.
E-mail address: qhmrvp@alfa.ist.utl.pt (H.M. Pinheiro).

1381-1177/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S1381-1177(00)00010-2

rates can cause the rupture of the immobilization matrix [5,7] and small particle diameters tend to lead to increased biocatalyst exposure to the organic solvent [10]. Lipophilic solvents have been effectively used as media for the bioconversion of sitosterol to androstenedione (AD) [9,11], bis(2-ethylhexyl)phtalate giving molar conversion yields in excess of 50% for substrate concentrations of 12 mM [11]. Other phtalate derivatives have also been assayed with both free [12] or immobilized *Mycobacterium* sp. cells [11], leading also to good results.

In this work, Celite-adsorbed *Mycobacterium* sp. NRRL B-3805 whole cells were employed to selectively convert β-sitosterol to 3-androstene-4,17-dione (AD) with 1,3-androstadiene-4,17-dione (ADD) as a minor product, using different phthalate derivatives as bioconversion organic media. The main goal of this work was to evaluate the relative contributions of external and/or internal diffusion and intrinsic kinetics to the control of the overall reaction rate. For this, bioconversion runs were performed using different stirring conditions and substrate concentrations, and several particle size diameter ranges from two types of Celite. The operational stability of immobilized cells in organic medium was also evaluated.

2. Materials and methods

2.1. Materials

Mycobacterium sp. NRRL B-3805 cells were maintained on potato dextrose agar slants (42 g l^{-1}), at room temperature. Celite R630 (Fluka, Switzerland) and Celite 560 (Johns-Manville, USA) particles were screened in a series of standard sieves (Retsch, Germany) to particle size diameter batches in the ranges 0.841–2.38, 0.193–0.55, 0.067–0.193, 0.037–0.067 and under 0.037 mm. β-Sitosterol and AD were supplied by Sigma (USA). Tween-20 was obtained from ICI (Spain). Di-*iso*-heptyl-phthalate, bis(3,3,5-trymethylhexyl)phthalate and bis(2-ethylhexyl)phtalate were for synthesis grade (Merck, Germany). Didecyl phtalate was technical grade (BDH, UK). All other reagents were analytical- or HPLC-grade from varied sources.

2.2. Cell growth and immobilization conditions

Mycobacterium sp. cells were grown in a synthetic medium composed of fructose (20 g l^{-1}), ammonium chloride (4 g l^{-1}), magnesium sulphate (0.07 g l^{-1}), Tween-20 (0.8 g l^{-1}) and β-sitosterol (0.5 g l^{-1}; activity inducer) in pH 7 potassium phosphate buffer (17.6 mM). The autoclaved Celite size batches were added to the fermentation medium at the early exponential growth phase and the immobilized cells were harvested and stored, as described previously [10]. The resulting immobilized cell load was between 15.9 and 24.1 mg cell protein/g dry support, unless referred otherwise. The water content of the immobilized preparation was in the range 0.4–0.9 g/g dry support.

2.3. Solubility determinations

The solubility of β-sitosterol and AD in the phthalate solvents were measured as described by Pinheiro and Cabral [13]. Samples were diluted with a solution of progesterone (0.2 g l^{-1}, internal standard) in *n*-heptane and analysed for sterol/steroid content by HPLC.

2.4. Bioconversion trials

Bioconversion runs were performed in 100 ml screw-capped Erlenmeyer flasks as previously described [10] using 5 ml of organic medium and 250 mg (wet weight) of immobilized biocatalyst, at 30°C with 200 rpm orbital shaking except if referred otherwise. Samples of 70 μl were periodically taken, diluted 10-fold with a solution of progesterone (0.2 g l^{-1}) in *n*-heptane and analysed for steroid content by HPLC. Bioconversion rates were determined from the zero-time slope of the AD concentration vs. time plot. Values are averages from at least two independent runs. In these conditions, the unit of catalytic activity (U) is equivalent to 1 μmol of AD formed per hour. Apparent kinetic parameters were obtained using a software program developed by Cornish-Bowden [14].

2.5. Operational stability tests

A wet weight of 1.2 g of Celite 560 immobilized cells (0.067–0.193 mm size batch) was added to 10 ml of a 2.4 mM solution of sitosterol in bis(2-ethylhexyl)phtalate and incubated at 30°C with 200 rpm orbital shaking in 100 ml screw-capped bottles. Each successive bioconversion was carried during 24 h, after which the immobilized cells were harvested by filtration, washed with 10 ml of a medium composed of yeast extract (10 g l^{-1}) and Tween-20 (0.8 g l^{-1}) in pH 7 phosphate buffer, recovered by filtration and added to fresh bioconversion medium. The amount of water retained on the support was kept in the range 0.45–0.55 g/g dry support.

2.6. Analytical methods

Quantification of the protein content of the cell-loaded support (Lowry method), of the amount of Celite-adsorbed water (Karl–Fischer titration) and of substrate/product concentrations in medium samples (HPLC) were performed as described [10]. Substrate was detected at 215 nm using *n*-heptane containing 1.5% (v/v) ethanol as mobile phase. Products were detected at 254 nm using *n*-heptane containing 4% (v/v) ethanol as mobile phase.

2.7. Scanning electron microscopy

Samples of Celite with immobilized cells were fixed in 4% (v/v) glutaradehyde in pH 7.4 cachodilate buffer (0.1 M) for 3 h at 4°C and dehydrated in diethyl ether (from 30% to 70%) and then in acetone (from 70% to 100%). The samples were dried by the critical point method and covered with a gold film. Sample observations were done on a Philips XL-30 scanning electron microscope.

3. Results and discussion

The overall kinetic behaviour of immobilized biocatalysts can be affected by concentration gradients developing within phase boundary layers around the biocatalyst and/or inside the porous support of an immobilized biocatalyst [15]. External mass tranfer limitations can, however, be prevented if adequate hydrodynamic conditions are chosen [15]. Bioconversion experiments were thus performed at different stirring speeds, using an intermediate size particle range among those available for both Celite types, bis(2-ethylhexyl)phthalate as bioconversion medium and different substrate concentrations (Fig. 1). External mass transfer was apparently not a limiting factor in the employed conditions, since no significant increase of reaction rates was observed for stirring speeds above 200 rpm. A trend to a decrease in catalytic activity was however noted for substrate concentrations above 12 mM, suggesting substrate-inhibited kinetics. Higher activity levels were also consistently observed when Celite 560 was used as immobilization matrix, as compared to Celite R630, although smaller particle sizes were used in the latter case (see legend to Fig. 1), which may indicate larger exposure to the toxic action of the solvent.

Further experiments were performed with 200 rpm stirring speed and using a series of particle diameter size ranges, from both Celite 560 and Celite R630, in order to measure apparent kinetic constants. The substrate inhibition for substrate concentrations in excess of around 7 mM was confirmed, an inhibition model (Eq. 1) providing a better fit to the results

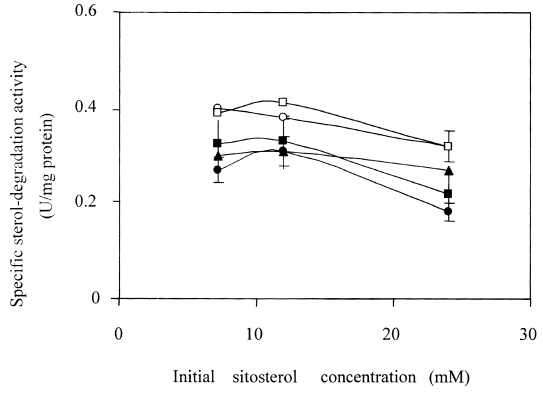

Fig. 1. Effect of the stirring speed on the specific sitosterol degradation activity of *Mycobacterium* sp. cells immobilized on Celite 560 particles in the range 0.067–0.193 mm (○, □) and Celite 630 in the range 0.037–0.067 mm (●, ■, ▲). Stirring speeds of 200 rpm (○, ●), 300 rpm (□, ■) and 370 rpm (▲) were used.

as compared to pure Michaelis–Menten kinetics (Figs. 2 and 3).

$$v = \frac{V_{max} S}{S + K_M + S^2/K_I} \quad (1)$$

Apparent kinetic parameters, V_{max}, K_M and K_I, were determined from Eq. 1, using a software package, as referred in Materials and Methods, while the theoretical substrate concentration resulting in the maximum reaction rate for substrate-inhibited biotransformations ($[S]_{Max.\ theor.}$) was determined by Eq. 2, as suggested by Schuler and Kargi [16] (Table 1).

$$[S]_{Max.\,theor.} = (K_M K_I)^{1/2} \quad (2)$$

The substrate concentration allowing for the maximum reaction rate is roughly seven times lower than the substrate solubility in bis(2-ethylhexyl)phtalate (45 mM, Table 2), thus limiting the process productivity. A trend towards overall kinetic control seems to arise for particle sizes below 0.067–0.193 mm, as taken from the similarity of the apparent K_M values. The reduction in the apparent V_{max} for smaller particle sizes may result from a solvent toxicity effect. For similar particle sizes, Celite 560-adsorbed cells allow for higher V_{max} values as compared to R630-adsorbed cells. Although in both supports, cells tend to be adsorbed on the surface of the immobilization matrix (Fig. 4a and b), the larger pore size of Celite 560 (roughly 1 μm, 2-fold the pore size for Celite R630) may have allowed for part of the cells to grow inside the pores, therefore reducing the contact with the organic solvent. The reduction of K_I with decreasing partice size, for Celite 560, also indicates increased substrate accessibility to the biocatalyst. A heterogeneous cell distribution in the immobilization matrix was also observed, particularly with Celite R630, randomly dispersed biofilm layers being formed at the surface of the support, possibly preventing substrate diffusion to the full extent of the biocatalyst. Presumably, these biofilm layers would be thinner on the smaller particle sizes, due to increased surface area per unit weight of the support,

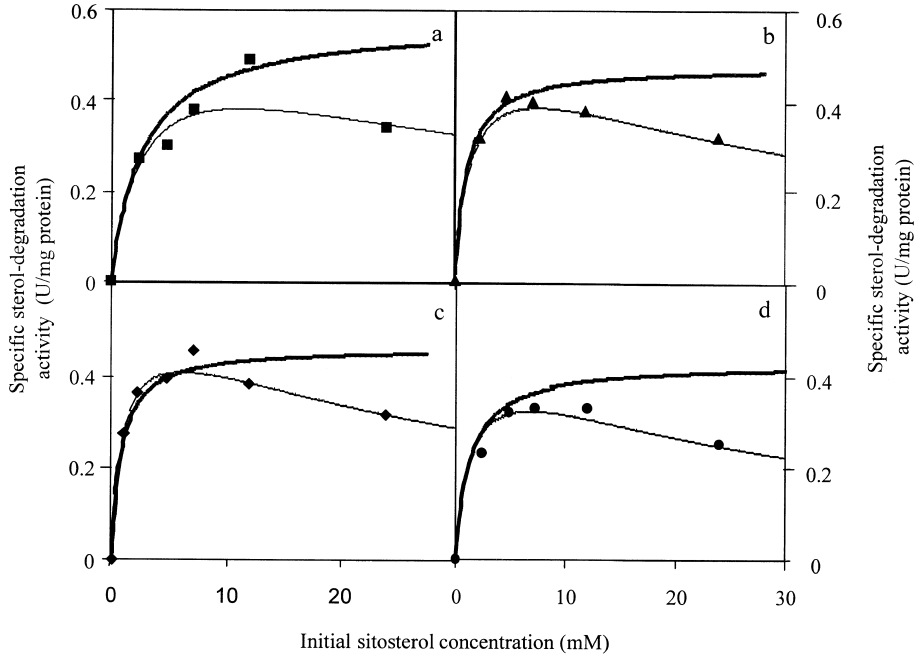

Fig. 2. Experimental (●, ■, ▲, ◆) and predicted effect of substrate concentration on the specific degradation activity of sitosterol to AD, using Celite 560-adsorbed *Mycobacterium* sp. cells and bis(2-ethylhexyl)phtalate as bioconversion medium. Particle sizes in the range 0.193–0.55 mm (a), 0.067–0.193 mm (b), 0.037–0.067 mm (c) and lower than 0.037 mm (d) were used as immobilization supports. Michaelis–Menten (——) and substrate inhibition (——) kinetic models were used.

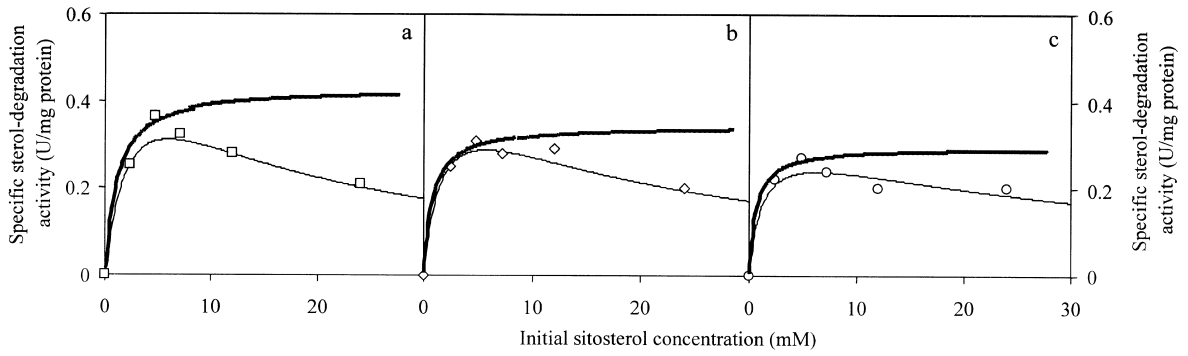

Fig. 3. Experimental (□, ◇, ○) and predicted effect of substrate concentration on the specific degradation activity of sitosterol to AD, using Celite R630-adsorbed *Mycobacterium* sp. cells and bis(2-ethylhexyl)phtalate as bioconversion medium. Particle sizes in the range 0.841–2.38 mm (a), 0.037–0.067 mm (b) and lower than 0.037 mm (c) were used as immobilization supports. Michaelis–Menten (——) and substrate inhibition (—✠ kinetic models were used.

thus reducing mass transfer hindrances. However, this would also expose the cells more to the solvent, which could account for the reduction in V_{max}. It should also be noted that, with Celite R630, apparent K_I values increase with decreasing support particle size (Table 1). If the cells' exposure to the solvent is increased, one or more of the sitosterol degradation pathway enzymes, particularly membrane-associated enzymes, could have suffered conformational shifts, possibly favouring substrate binding to the active site, in relation to non-catalytic, inhibitory binding.

Kinetic control of the bioconversion can thus be achieved at low particle size, although the system is overall not very sensitive to internal mass transfer limitations.

In order to possibly minimize the toxicity effect of the organic solvent on the biocatalyst, further bioconversion trials were carried out using other phtalate derivatives with different log *P* values, Celite 560-adsorbed cells (0.037–0.067 mm particle size) and a substrate concentration of 6 mM, so as to allow for overall kinetic control of the catalytic system (Table 2). Since the solubility of both substrate and products in the solvents used was roughly 5- to 20-fold above the concentration used in these experiments, the possibility of precipitate formation was discarded. Among the phtalate derivatives tested for use as bioconversion medium with Celite-adsorbed *Mycobacterium* sp. cells, bis(2-ethylhexyl)-phtalate gave the higher molar conversion yields

Table 1
Apparent kinetic parameters obtained from non-linear regression analysis using software package *Leonora* [14]. Bioconversion runs were performed using Celite 560 and Celite 630-adsorbed *Mycobacterium* sp. NRRL B-3805 cells and bis(2-ethylhexyl)phtalate as bioconversion medium. Bioconversions were performed in the 0–24 mM substrate concentration range

Immobilization support	Particle size range (mm)	K_M (mm)	V_{max} ($U/mg_{protein}$)	K_I (mM)	$[S]_{Max.\ theor.}$ (mM)
Celite 560	0.193–0.550	2.8 ± 0.5	0.57 ± 0.05	46 ± 9	11 ± 3
	0.067–0.193	1.8 ± 0.3	0.57 ± 0.02	32 ± 2	8 ± 1
	0.037–0.067	1.2 ± 0.2	0.56 ± 0.03	32 ± 1	6 ± 1
	< 0.037	1.8 ± 0.5	0.45 ± 0.03	26 ± 7	6 ± 1
Celite R630	0.841–2.38	2.8 ± 0.6	0.60 ± 0.05	13 ± 2	6 ± 1
	0.037–0.067	1.8 ± 0.4	0.47 ± 0.04	18 ± 3	6 ± 1
	< 0.037	1.2 ± 0.2	0.33 ± 0.03	33 ± 3	6 ± 1

Table 2
Sitosterol side-chain activity of Celite-adsorbed *Mycobacterium* sp. NRRL B-3805 using different phtalate derivatives as bioconversion media. Celite 560 particles in the range 0.037–0.067 mm were used for cell immobilization. A sitosterol initial concentration of 6 mM was used in all trials

Organic solvent	Solubility (mM)		log *P*	24 h AD molar conversion yield (%)
	Sitosterol	AD		
Di-*iso*-heptyl-phthalate	58[a]	112[a]	6.9	11.6 ± 0.9
Bis(2-ethylhexyl)phthalate	45	78	9.6	45.8 ± 0.6
Bis(3,5,5-trimethylhexyl)phthalate	78[a]	92[a]	10.6	35.6 ± 0.2
Didecyl phthalate	55	66	11.7	20.0 ± 1.0

[a] Data obtained by Cruz et al. [12].

(Table 1). Although more lipophilic solvents have been generally associated to higher cell viability and/or catalytic activity retention [17,18], this trend does not fully apply in the present study, when solvents with log *P* values above 9 are considered. This points to the need for searching for more specific effects, such as substrate partitioning and solvent–cell interactions in order to find the most suitable solvent.

One of the advantages of using immobilized biocatalysts is the possibility of their reuse in repeated biotransformations, which requires however a high operational stability [19] or the reactivation of the biocatalyst between batches [19,20]. In order to evaluate the possibility of reactivation of the sterol-degradation capacity of the biocatalyst, Celite 560-adsorbed cells (0.037–0.067 mm particle size) were used in repeated, batch biotransformations of sitosterol (2.4 mM) in bis(2-ethylhexyl)phtalate. A complex nutrient solution was used as washing medium between batches since it had been shown before to allow for a partial reactivation of the sterol-degradation capacity in free cells [21]. A decrease in AD yield was nevertheless observed upon repeated use of the biocatalyst (Fig. 5), limiting its effectiveness. Although a loss of catalytic activity of free *Mycobacterium* sp. cells between repeated biotransformations has been observed both in aqueous and organic media [21], in the present work this effect is essentially due to cell desorption observed when immobilized cells were incubated in the washing medium. This desorption, not occurring with freshly grown cells, indicates a change in cell surface adhesion properties resulting from physiological alterations, possibly including contact with the solvent. Delivering nutrients to the immobilized biocatalyst

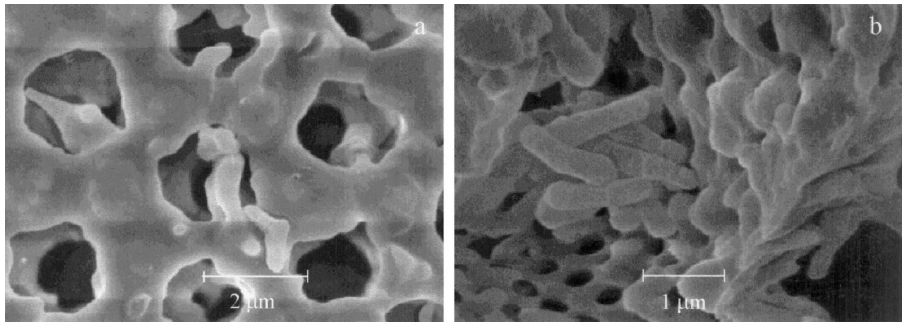

Fig. 4. Scanning electron micrographs of Celite-immobilized *Mycobacterium* sp. NRRL B-3805 cells. Surface attached cells to Celite 560 (a) and Celite R630 (b) are shown.

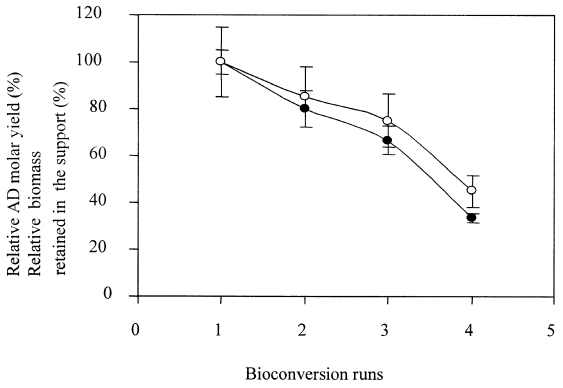

Fig. 5. Repeated batch biotransformations of sitosterol to AD in bis(2-ethylhexyl)phtalate with Celite 560 (0.067–0.193 mm)-adsorbed *Mycobacterium* sp. NRRL B-3805 cells. Immobilized cells were washed with a yeast extract solution between biotransformations. Each biotransformation lasted for 24 h. An initial cell load of 1.3 mg cell protein/g dry support was used. AD molar yield (●) and cell protein retained in the support after the aqueous washing steps (○) are presented, as a percentage of their values on the first run.

without periodic transfer to an aqueous medium, possibly using lipophilic nutrients, could be a way of prolonging catalytic activities in the present system.

4. Conclusions

Celite-adsorbed *Mycobacterium* sp. NRRL B-3805 were effectively used for the selective side-chain cleavage of β-sitosterol with bis(2-ethylhexyl)phtalate as conversion medium. External mass transfer limitations could easily be overcome by adjustment of stirring conditions. Kinetic control of the bioconversion was apparently achieved for particle size ranges below 0.067–0.193 mm for both types of Celite tested. Substrate inhibition was however observed in both bioconversion systems, the substrate concentration allowing for maximum reaction rates being around 6 mM, roughly seven times lower than the substrate solubility in this organic solvent, thus limiting the system productivity. A solvent toxicity effect seemed to affect apparent V_{max} values particularly since cells are preferentially adsorbed on the surface of the support. The use of larger pore-sized Celite apparently made it possible for cells to grow inside the pores, thus providing some protective effect. Catalytic activity of Celite 560-immobilized cells in different phthalate derivatives with high log P values (above 9), did not show a clear relation to the latter values, calling for further investigation into cell–solvent interaction mechanisms. The repeated use of the immobilized biocatalyst for batch biotransformations of sitosterol in phtalate did not prove to be effective due to cell desorption during the aqueous washing steps for delivering nutrients to the cells.

Acknowledgements

N. Llanes acknowledges a grant from UNESCO-MCBN (Global Network for Molecular and Cell Biology). The authors wish to acknowledge Prof. Francisco Galvan and the Microscopy Service from the Universidad de Sevilla for the scanning electron microscopy analysis.

References

[1] M.D. Lilly, J. Chem. Technol. Biotechnol. 32 (1982) 162.
[2] P. Nikolova, O.P. Ward, J. Ind. Microbiol. 12 (1993) 76.
[3] R. Léon, P. Fernandes, H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 23 (1998) 483.
[4] M.D. Hocknull, M.D. Lilly, Appl. Microbiol. Biotechnol. 33 (1990) 148.
[5] B. Katzbauer, M. Narodoslawski, A. Moser, Bioprocess Eng. 12 (1995) 173.
[6] M. Zaiat, A.K.A. Cabral, E. Foresti, Water Res. 30 (1996) 2435.
[7] N. Chitra, A. Baradarajan, Bioprocess Eng. 7 (1992) 265.
[8] T. Yamané, H. Nakatani, E. Sada, T. Omata, A. Tanaka, S. Fukui, Biotechnol. Bioeng. (1979) 2133.
[9] H.-J. Steinert, K.D. Vorlop, J. Klein, in: C. Laane, J. Tramper, M.D. Lilly (Eds.), Biocatalysis in Organic Media, Elsevier, Amsterdam, 1987, p. 51.
[10] P. Fernandes, J.M.S. Cabral, H.M. Pinheiro, J. Mol. Catal. B: Enzym. 5 (1998) 307.
[11] A.C.P. Dias, H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 16 (1994) 708.
[12] A. Cruz, P. Fernandes, J.M.S. Cabral, H.M. Pinheiro, J. Mol. Catal. B: Enzym., submitted for publication.
[13] H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 37 (1991) 97.
[14] A. Cornish-Bowden, Analysis of Enzyme Kinetic Data, Oxford Science Publications, Oxford, 1995.
[15] A. Ballesteros, L. Boross, K. Bucholz, J.M.S. Cabral, V.

Kasche, in: J.M.S. Cabral, D. Best, L. Boross, J. Tramper (Eds.), Applied Biocatalysis, Harwood Academic Publishers, Reading, 1993, p. 237.
[16] M.L. Schuler, F. Kargi, Bioprocess Engineering, Prentice-Hall, New Jersey, 1992, p. 72.
[17] S.J. Osborne, J. Leaver, M.K. Turner, P. Dunnill, Enzyme Microb. Technol. 12 (1990) 281.
[18] M. Vermuë, J. Sikkema, A. Verheul, R. Bakker, J. Tramper, Biotechnol. Bioeng. 42 (1993) 747.
[19] K.A. Koshcheenko, G.V. Sukholdskaya, V.S. Tyurin, G.K. Skryabin, Eur. J. Appl. Microbiol. Biotechnol. 12 (1981) 161.
[20] S. Jovetic, J. Tramper, F. Marinelli, Enzyme Microb. Technol. 22 (1998) 117.
[21] P. Fernandes, J.M.S. Cabral, H.M. Pinheiro, in: A. Ballesteros, F.J. Plou, J.L. Iborra, P.J. Halling (Eds.), Stability and Stabilization of Biocatalysts, Elsevier, Amsterdam, 1998, p. 625.