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Influence of some operational parameters on the bioconversion of sitosterol with immobilized whole cells in organic medium

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Abstract

Mycobacterium sp. NRRL B-3805 cells immobilized on Celite were used to selectively cleave β-sitosterol to 4-androstene-3,17-dione (AD), with bis(2-ethylhexyl)phthalate as the bioconversion medium. The amount of water retained in the immobilization matrix proved to be a key parameter for biological activity retention, 0.5 to 0.8 g water/g dry support giving the best results. Mechanically stirred batch reactors were effectively used for sterol biotransformation, no increase in bioconversion rates being obtained for stirring speeds above 100 rpm. Kinetic studies were performed with β-sitosterol concentrations up to 30 mM, with different support particle sizes. Kinetic control of the biotransformation process becomes apparent for support particle sizes below 0.12–0.2 mm.

Keywords: Immobilized cell; Organic solvent; Sterol; Bioconversion; *Mycobacterium* sp.

1. Introduction

The combined use of organic solvents and biocatalyst immobilization provides a proven tool for performing bioconversions when hydrophobic substrates and products are involved, since high substrate concentrations can be used and a protective environment against solvent toxicity is provided [1,2]. Nevertheless, this approach is very often limited by mass transfer resistances [1,2]. Therefore, a careful choice of both agitation conditions and immobilization matrix is necessary in order to minimize this limitation without compromising process productivity. Although much work has been reported on biocatalysis in organic media [2], most of the studies are focused on catalysis with isolated enzymes and single-step whole-cell bioconversions [3–5]; not much data are available for multi-step pathways, such as sterol side chain cleavage [6], carried out using whole cells in so-called monophasic organic solvent systems [7].

In the present work, Celite-adsorbed *Mycobacterium* sp. NRRL B-3805 cells are used to selectively cleave β-sitosterol to 4-androstene-3,17-dione (AD), with bis(2-ethyl-hexyl)phthalate as the bioconversion medium. The aim of the study was to evaluate the overall reaction

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system behaviour in what concerns the balance between kinetic and diffusional, internal or external, control. For this, several operational parameters were investigated, namely: the amount of Celite-adsorbed water, mixing conditions, substrate concentration, particle size diameter and biocatalyst intrinsic activity.

2. Materials and methods

2.1. Cell growth and immobilization conditions

Mycobacterium sp. NRRL B-3805 cells were grown in 1 l erlenmeyer flasks, with 200 rpm orbital shaking at 30°C, containing 250 ml of a medium consisting of fructose (10 g l^{-1}), ammonium chloride (2 g l^{-1}), magnesium sulphate (0.14 g l^{-1}), Tween 20 (0.8 g l^{-1}) and, in some trials, sitosterol (0.1 g l^{-1}) as an activity inducer, in pH 7 potassium phosphate buffer (17.6 mM). When an optical density of 0.4–0.8 was reached, 20 g of celite 545 (particle size 20–45 μm), celite 80–120 mesh (particle size 0.12–0.2 mm) or celite 30–80 mesh (particle size 0.2–0.5 mm) were added to each flask, with fermentation proceeding for a further 20–24 h period. The inoculum was a 8 ml portion of a 24 h culture grown in the described conditions, in a medium composed of yeast extract (10 g l^{-1}) and Tween 20 (0.8 g l^{-1}) in pH 7 phosphate buffer. Immobilized cells were harvested by filtration, thoroughly washed with pH 7 phosphate buffer and stored at -20°C until further use. Some of the immobilized cells were freeze dried in a Micromodulyo 1.5 K Freeze Dryer (Edwards, UK), for removal of Celite-adsorbed water to values under 0.5 g/g support dry weight.

2.2. Bioconversion trials

A given amount (around 50 mg dry weight per milliliter) of immobilized cells was added to a 8 mM solution (except if otherwise stated) of β-sitosterol in bis(2-ethylhexyl)phthalate. Bioconversions were carried out at 30°C with 200 rpm orbital shaking, in screw-capped 100 or 500 ml flasks containing 10 or 80 ml, respectively, of bioconversion medium, or in a 6 cm diameter 270 ml vessel stirred with a 45° pitched four-bladed turbine, containing 80 ml of bioconversion medium and thermostated at 30°C. Duplicate runs (at least) were performed for each trial. Samples of 0.1 ml were taken periodically, diluted with a solution of progesterone (0.2 g l^{-1}) in *n*-heptane and analyzed for steroid content by HPLC.

2.3. Analytical methods

Samples of the cell-loaded support were dried and assayed for protein content according to the method of Lowry et al. [8], following cell hydrolysis by heating at 100°C for 20 min in 1 M NaOH [9]. The amount of water retained in the support and in the bioconversion medium was determined using a Karl–Fischer titrator (Mettler DL18, Switzerland). Steroid analysis was performed by HPLC using a Lichrosorb Si-60 column (250 mm $\times$ 4 mm; 10 mm particle diameter), with *n*-heptane containing 6% (v/v) ethanol as the mobile phase at a flow rate of 1.0 ml min^{-1}. The products were detected at 254 nm and matched to pure AD and ADD. The unit of catalytic activity (U) is equivalent to 1 μmol of AD formed per hour.

2.4. Chemicals

Sitosterol, AD and ADD, were supplied by Sigma (USA). Tween 20 and bis(2-ethylhexyl)phthalate were of synthesis-grade from Merck (Germany). Yeast extract was from Difco (USA). All other reagents were HPLC- or analytical-grade from varied sources.

3. Results and discussion

The low solubility of sitosterol in water (below 1 μM [10]), suggests that sitosterol diffu-

sion in the immobilization matrix could be enhanced if the water retained in the support was reduced to a minimum. Indeed, an effective increase in the AD formation rate was obtained when the amount of Celite-adsorbed water was decreased from 2 to around 0.6 g/g dry support weight (Fig. 1). For values below 0.4 g water/g dry support weight, a steep decrease in catalytic activity is observed, which could be attributed to excessive cell dehydration or to the loss of the water barrier between the biocatalyst and the solvent.

Preliminary bioconversion trials performed in a mechanically stirred reactor in which the water level in the bioconversion medium was not monitored, led to low conversion yields, as compared to trials with orbital shaking. In order to ascertain whether this was due to water loss or to some other effect (cell desorption and/or shear stress), some runs were carried out in larger, closed or open vessels (Fig. 2). AD formation shows a similar trend, irrespective of the mixing conditions, and the measured level of cell desorption was minimal in both cases (results not shown). Water level determinations (Fig. 2) showed that the loss of catalytic activity was clearly related to the rapid decrease in the water amount retained in the bioconversion medium.

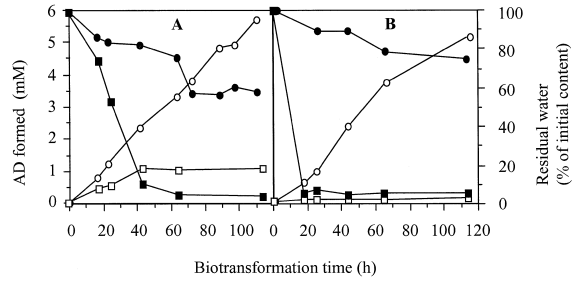

Fig. 2. Effect of water loss on β-sitosterol degradation with *Mycobacterium sp*. NRRL B-3805 cells immobilized on Celite 545. Bioconversions were carried out in closed (○, ●) or open (■, □) reactors at 30°C. Mechanical stirring of 100 rpm (A) or 200 rpm orbital shaking (B) were used. AD formation (○, □) and residual water in the system (●, ■) are shown.

Experiments carried out in the turbine-stirred reactor at different stirring speeds showed that external mass transfer was apparently not the limiting factor at stirring speeds of 100 rpm (corresponding to an impeller Reynolds number of 70) or above (Fig. 3).

To investigate the relative importance of kinetic and internal mass transfer rates, further tests were carried out, with orbital shaking at 200 rpm, using cells immobilized on Celite of different particle sizes. The Michaelis–Menten model provided a good description of the system, the measured apparent kinetic parameters being given in Table 1. The similarity of apparent K_M values, estimated for particle diameters

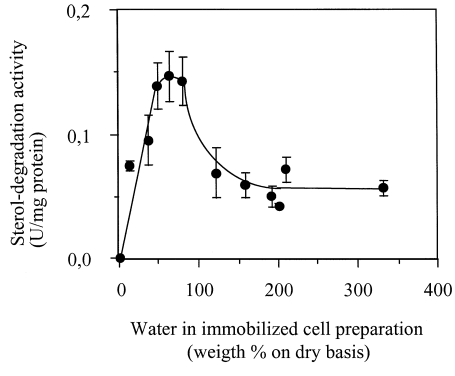

Fig. 1. Effect of water retained in the support on the specific β-sitosterol degradation activity of *Mycobacterium* sp. NRRL B-3805 cells immobilized on Celite 545. Bioconversions were performed in 100 ml erlenmeyer flasks, at 30°C, with 200 rpm orbital shaking.

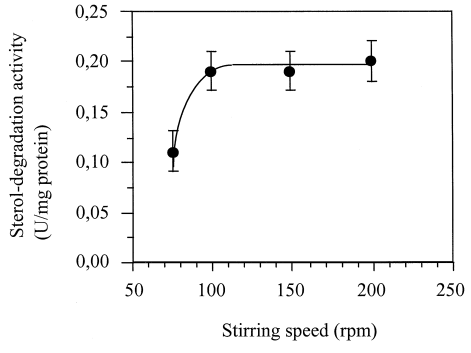

Fig. 3. Effect of stirring speed on the specific β-sitosterol degradation activity of *Mycobacterium* sp. cells immobilized on Celite 545. Tests were performed in a 6 cm diameter, 270 ml vessel stirred with a 4.5 cm diameter, 45° pitched four-bladed turbine.

Table 1
Apparent kinetic parameters, determined as an average from individual values obtained from Lineweaver–Burke and Eadie–Hofstee plots, for the side-chain cleavage of β-sitosterol to AD, using Celite-adsorbed *Mycobacterium* sp. cells and bis(2-ethylhexyl)phthalate as the bioconversion medium. Bioconversion trials were performed in the 0–30 mM substrate concentration range

Particle size range (mm)	Activity induction during cell growth	V_{max} (U/mg protein)	K_M (mM)
0.2–0.5	No	0.12	4.5
0.12–0.2	No	0.13	2.5
0.020–0.045	No	0.09	2.4
0.020–0.045	Yes	0.24	1.9

below 0.12–0.2 mm, show a trend to kinetic control. The slight decrease in the apparent V_{max} value observed for the smallest particle diameter could indicate a solvent toxicity effect. The use of cells grown in the presence of an inducer, with catalytic activity levels (measured in standard aqueous media) roughly twice of those of non-induced cells, led to a definite increase in the V_{max} value, with no significant change in apparent K_M. This result confirms that the system is kinetically controlled in the lowest particle size range.

4. Conclusions

Selective side-chain cleavage of β-sitosterol to AD, using *Mycobacterium* sp. cells immobilized on Celite, with bis(2-ethylhexyl)phthalate as the conversion medium, is strongly influenced by the amount of water adsorbed to the immobilization matrix, the control of water levels during the bioconversion being necessary. Mechanically stirred reactors can be effectively used to carry out this bioconversion, external diffusion limitations being readily overcome. Kinetic control of the process can be achieved for support particle sizes below the 0.12–0.2 mm range, improved productivities resulting from cells with higher intrinsic activity.

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