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Whole-cell bioconversion of β-sitosterol in aqueous–organic two-phase systems

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Abstract

The selective cleavage of the β-sitosterol side-chain by free *Mycobacterium* sp. NRRL B-3805 cells was used as a model system for the study of solvent effects in a whole-cell bioconversion in two phase aqueous–organic media. This multi-step degradation pathway leads to the production of 4-androstene-4,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) as a minor product. In an attempt to correlate the substrate and cell partition effects and solvent hydrophobicity (log P) with biocatalytic activity, 15 carboxylic acid esters with log P values between 3 and 10 were screened. The results indicated that the toxicity of the tested solvents in this system could not be correlated to their log P, but seemed to depend on their ability to accumulate in the cells, as these showed a strong affinity towards the organic phase. Different solvent/aqueous ratios and hydrodynamic conditions were further tested in the solvent systems (phthalates) showing significant biodegradation activity. The bioconversion rate was generally not much affected by the stirring speed in the employed range (150–300 rpm) but was strongly influenced by the aqueous/organic phase ratio. Results suggest that the bioconversion takes place at the interphase, its rate being possibly limited by mass transport inside the organic phase.

Keywords: Two-phase systems; Organic solvents; *Mycobacterium* sp.; β-Sitosterol; Whole-cells

1. Introduction

Solvent toxicity effects are a major drawback in the application of non-conventional media in whole-cell biotransformations of hydrophobic substrates. Still, these processes are gaining importance as they present all the potential advantages of biocatalysis in organic media [1], along with those offered by the use of whole-cells, namely in reactions involving cofactor regeneration, and in those requiring multi-enzymatic metabolic pathways. The selective cleavage of the side-chain of β-sitosterol by *Mycobacterium*sp. cells is an example of the latter situation. Performing this bioconversion in a two-liquid phase system reduces potential substrate and product inhibitory effects, and enables the in-situ removal of the product [2], considering the organic phase as a reservoir. In whole-cell systems, the solvent might be incorporated within membrane lipids causing disruption of membrane functions, inactivation or denaturation of membrane bound enzymes, collapse of transport mechanisms, and even cell lysis [3]. Therefore, in order to achieve all the advantages previously described, biocompatibility becomes the solvent most important requirement. The logarithm of the partition coefficient of the solvent in the oc-

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tanol–water two-phase system (log P; Hansch parameter) is a commonly used parameter for establishing a correlation between the biocompatibility and a solvent physicochemical property, in this particular case, hydrophobicity [1]. This relationship is not completely understood and does not hold true for all the organic solvents. Vermüe et al. [4] concluded that the toxicity of organic solvents is also related to their molecular structure, and Bruce and Daugulis [5] pointed out the importance of the characteristics of the cell membrane.

The main goal of the present work was to evaluate the effect of carboxylic acid esters with different log P values on the sitosterol biodegradation activity of *Mycobacterium* sp. NRRL B-3805. For this purpose, several determinations were carried out, namely the solubilities of the substrate and the main product (4-androstene-4,17-dione, AD), cell and substrate partition coefficients in solvent–aqueous systems, solvent solubility in the aqueous phase, and the amount of solvent accumulated by the cells in the presence of a solvent-saturated aqueous phase. In biphasic systems, the influence of stirring speed and phase ratio on the bioconversion rate was also examined.

2. Experimental

2.1. Chemicals

All the organic solvents are listed in Table 1 (except biodiesel, a mixture of sunflower oil methylic esters, kindly supplied by INETI, Portugal), Tween 20 and salts were of synthesis grade from Merck (Germany). Sitosterol, AD and 1,4-androstadiene-3,17-dione (ADD) were supplied by Sigma (USA) and yeast extract was obtained from Difco (USA). Ethanol and *n*-heptane were of analytical grade from different suppliers.

Table 1
Log P values, substrate and product solubilities and bioconversion screening for different organic solvents (esters)
The log P values were calculated from hydrophobic fragmental constants as determined by Rekker and de Kort [8]. Specific product yields are given per unit weight of cell protein (prot)

Solvent	Log P_{oct}	24-h Bioconversion product yield			Solubility (mM)	
		Organic media (μM AD/mg prot)	Biphasic media (μM AD/mg prot)	Solvent-saturated aqueous media (%)[a]	Sitosterol	AD
Biodiesel		≅ 0	≅ 0	43.0 ± 9.4	128.6 ± 2.5	43.9 ± 0.9
Esters of dicarboxylic acids						
Dibutyl succinate	3.1	≅ 0	≅ 0	3.4	54.8 ± 0.7	116 ± 8.0
Diallyl phthalate	3.6	0.06 ± 0.001	≅ 0	27.9 ± 0.1	39.8 ± 2.5	123 ± 1.4
Benzylbutyl phthalate	5.5	≅ 0	≅ 0	112 ± 2.7	47.4 ± 6.1	215 ± 4.8
Diisoheptyl phthalate	6.9	0.25 ± 0.05	1.0 ± 1.1	96.3 ± 0.1	58.3 ± 4.6	112 ± 4.5
Bis (2-ethylhexyl) adipate	8.2	≅ 0	≅ 0	29.9	59.9 ± 6.6	49.2 ± 2.7
Bis (2-ethylhexyl) phthalate	9.6	2.21 ± 0.08	14.1 ± 0.6	242 ± 19.0	67.9 ± 3.9	108 ± 5.4
Bis (3,5,5-trimethylhexyl) phthalate	10.6	0.89 ± 0.15	3.3 ± 1.1	135 ± 11.6	78.1 ± 2.4	91.9 ± 2.9
Esters of monocarboxylic acids						
Methyl nonanoate	3.8	0.01	≅ 0	3.9 ± 0.1	79.8 ± 8.5	78.8 ± 0.6
Ethyl nonanoate	4.3	≅ 0	≅ 0	4.6 ± 0.8	132 ± 11.0	151 ± 1.2
Ethyl decanoate	4.8	≅ 0	≅ 0	5.0 ± 0.6	136 ± 3.5	133 ± 3.4
Ethyl undecanoate	5.3	≅ 0	≅ 0	3.3 ± 1.1	73.6 ± 2.3	46.0 ± 0.3
Ethyl laurate	5.9	≅ 0	≅ 0	4.3 ± 0.2	103 ± 13.2	108 ± 3.9
Ethyl myristate	6.4	≅ 0	≅ 0	3.6 ± 0.4	125 ± 1.6	90.4 ± 4.5
Isopropyl myristate	7.4	0.05 ± 0.01	≅ 0	10.7 ± 4.3	67.4 ± 5.5	27.0 ± 0.1

[a] Percentage relative to a blank assay in pH 7 phosphate buffer, where the specific, 24-h product yield was 0.61 ± 0.04 μM AD/mg prot.

2.2. Cell growth

The *Mycobacterium* sp. NRRL B-3805 active cells were obtained as described by Pinheiro et al. [6], grown in a medium of pH 7 phosphate buffer containing NH_4Cl (4 g/l), $MgSO_4 \cdot 7H_2O$ (0.14 g/l), fructose (20 g/l), β-sitosterol (0.5 g/l) and Tween 20 (0.8 g/l). The free cells were harvested by filtration, washed with phosphate buffer and kept at −20°C until use.

2.3. Analytical methods

The assays for protein content in aqueous cell suspensions were performed according to Pinheiro et al. [6].

HPLC analysis (Lichrosorb Si-60 column) with 1 ml/min isocratic elution was performed to determine substrate, products and solvent contents, with UV detection at 215 and 254 nm. The mobile phase was an *n*-heptane/ethanol mixture (volumetric ratio 96:4 for products and solvents, 98.5:1.5 for substrate). Organic or aqueous samples were diluted or extracted, respectively, with *n*-heptane prior to injection into the HPLC column.

2.4. Substrate and products solubilities in organic solvents

Amounts above the solubility limit of sitosterol, AD and ADD were added to vials with 1 ml of each organic solvent. The vials were shaken at 200 rpm and 30°C for 1 week, followed by centrifugation and sterol and steroids HPLC analysis. Previous solvent saturation with buffer was obtained by 1-min vigorous agitation of equal parts of phosphate buffer and pure solvent, and centrifugal phase separation (4500 rpm, 10 min).

2.5. Biocatalytic activity screening

The bioconversions were performed in an orbital shaker (200 rpm, 30°C), in duplicate runs. In organic media, 200 mg of wet cell paste (18.4 mg cell dry weight, 2.7 mg protein) were added with 200 μl of phosphate buffer to 5 ml of a β-sitosterol (5 g/l, 12 mM) solution in each of the organic solvents in 100 ml screw-capped flasks. For the tests in 1:1 (V_{org}/V_{aq}) two-phase systems, the cell load was 200 mg wet cell mass in a 10-ml total reaction volume and the aqueous phase was pH 7 phosphate buffer. In aqueous solvent-saturated media, 4-ml vials were used with 30 mg of wet cell paste, 20 μl of a 10 g/l sitosterol solution in ethanol and 1 ml of solvent-saturated phosphate buffer. The latter was obtained as described above with collection of the aqueous phase.

2.6. Substrate partition ratios

Substrate partition ratios, were determined by HPLC analysis of the aqueous and organic phases of 1:5 (V_{org}/V_{aq}) two-phase systems, which were submitted to 1 min vortex agitation followed by overnight incubation in a rotary shaker (200 rpm, 30°C) and centrifugal phase separation (4500 rpm, 10 min). The aqueous phase was phosphate buffer pH 7 and the organic phase was a 5 g/l sitosterol solution in organic solvent. In the runs with biomass, cells were previously inactivated by heat (20 min, 100°C).

2.7. Cell partition ratios

The cell partition coefficients between the aqueous phase and the organic phase (the latter including the interphase) were estimated by 30-min shaking (200 rpm, 30°C) of 10 ml of phosphate buffer with 1 ml of pure organic solvent and 100 mg of wet cell paste, followed by phase separation and protein quantification in the aqueous phase. Biomass in the organic phase plus interphase was estimated through a mass balance.

2.8. Solvent distribution in aqueous cell suspensions

Solvent solubility in the aqueous phase (cell-free phosphate buffer) was determined by HPLC analysis of the aqueous phases of 1:5 (V_{org}/V_{aq}) two-phase systems, after vigorous vortex agitation, centrifugal phase separation (4500 rpm, 15 min) and exhaustive extraction into *n*-heptane. In the presence of cells, successive additions of small amounts of organic solvent (under the solubility limit) were made to a

Table 2
Cell partitioning in the organic–aqueous two-phase systems. This estimation was based on protein determination in the aqueous phase and overall mass balance to the biomass

Solvent	P (org + int/aq)[a]
Biodiesel	260
Dibutyl succinate	330
Diallyl phthalate	190
Benzylbutyl phthalate	220
Diisoheptyl phthalate	230
Bis (2-ethylhexyl) adipate	180
Bis (2-ethylhexyl) phthalate	180
Bis (3,5,5-trimethylhexyl) phthalate	320
Methyl nonanoate	210
Ethyl nonanoate	150
Ethyl decanoate	180
Ethyl undecanoate	170
Ethyl laurate	220
Ethyl myristate	260
Isopropyl myristate	200

[a](Organic phase + interphase)/aqueous phase.

cell suspension (8 mg wet cell paste/ml) in phosphate buffer until saturation of the aqueous phase. The amount of solvent retained by the cells was estimated by subtracting the amount of solvent measured in the buffer after cell removal (centrifugation) from the amount of solvent added.

2.9. Bioconversions in two-phase systems

Trials of 24-h duration were performed in a 250-ml turbine stirred vessel at 30°C, with a total biotransformation medium volume of 50 ml and a cell load of 10 mg wet cell paste per ml total medium. For the study under different hydrodynamic conditions, two-phase systems with 25% and 75% (V_{org}/V_{total}) organic phase were used at 150, 200, 250 and 300 rpm. Five different solvent/aqueous ratios (10–90% organic phase) were tested at 200 rpm.

3. Results and discussion

Previous works [6,7] had established bis(2-ethylhexyl)phthalate as a suitable organic solvent for the

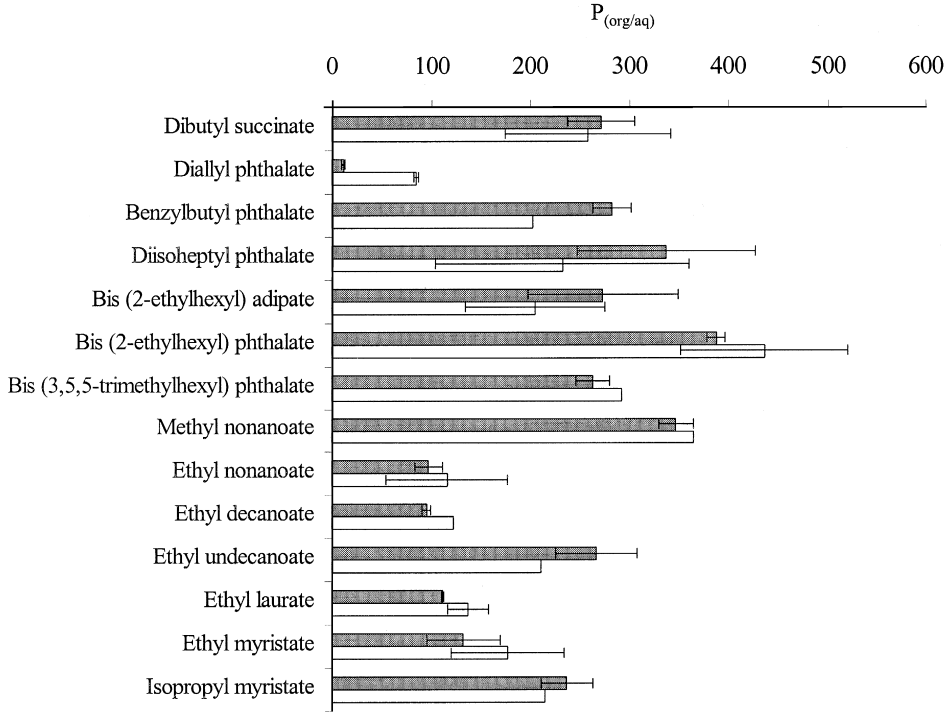

Fig. 1. Sitosterol partition coefficients ($P_{org/aq}$) in organic–aqueous two-phase systems in the presence (□) and in the absence (■) of cells.

sitosterol side-chain cleavage in both free and immobilized *Mycobacterium* cells. This result led to the selection of a group of mono- and di-carboxylic acid esters as potential organic phases of the biphasic systems for the same biotransformation. Apart from these, a biodiesel was also tested, as this commercial mixture of sunflower oil methylic esters could be available in large amounts at low prices. The esters listed on Table 1 have log P values between 3 and 10 (calculated from hydrophobic fragmental constants determined by Rekker and de Kort [8]), an interval which, according to Laane et al. [1], would include possibly toxic ($\log P = 2$–4) and non toxic ($\log P > 4$) solvents. However, an observation of the log P values and the sitosterol bioconversion yields after 24 h in organic, biphasic and aqueous saturated media presented in Table 1, does not confirm this prediction. This result is in agreement with that obtained by Vermüe et al. [4] who only found a general solvent tolerance at log P values above 5. In the present case, only diisoheptyl phthalate, bis(3,5,5-trimethylhexyl) phthalate and bis (2-ethylhexyl) phthalate allow for significant sitosterol degradation activity. Substrate or product precipitation could be excluded as the solubility values determined for all the solvents (Table 1) are far above the 12 mM sitosterol concentration used in the organic reaction phases. Cell partition results in Table 2 point out the strong cell affinity for the hydrophobic phases tested, in accordance with the generally reported high cell surface hydrophobicity of *Mycobacteria*[7]. This would presumably facilitate substrate transport to the cells as the sitosterol partitions almost completely to the organic phase (Fig. 1), the presence of biomass not having a significant effect on this partitioning. However, even though some cells could be visually detected in the organic phase, biomass mostly accumulated at the interphase, after phase separation by centrifugation.

The results of the activity tests performed in aqueous, solvent-saturated media (Table 1) indicate also that molecular toxicity played an important role in the majority of the tested systems. According to Bar [9], molecular solvent toxicity is evidenced by a retardation of the biphasic bioconversion process with respect to the same process carried out in the absence of organic solvent, due to solvent molecules dissolved in the aqueous phase. Under this definition, only the higher log P phthalates seem not to exhibit molecular toxicity. In fact, the bioconversion yield is higher in media pre-saturated with this solvents than in solvent-free aqueous medium, suggesting an incorporation of the organic solvent in the cells, altering substrate transport rates, and/or enzyme activities. Table 3 presents the solvent solubilities at 30°C in the aqueous phase and also a mass balance estimation, for some of the tested esters, of the amount of solvent stored in the cells, in equilibrium with the saturating aqueous phase concentrations. The phthalates allowing higher sitosterol degradation activities are those with lowest saturation concentrations both in aqueous medium and in the cells. This is in agreement with the Osborne et al. [10] critical membrane concentration concept, suggesting that there is a critical solvent concentration in the cell membrane, independent of the solvent type, at which complete loss of biocatalytic activity occurs. Due to the extremely low water solubility of the phthalates of the present study, they would never reach this critical concentration in the cells, while for the other tested esters the amount of solvent present in the pre-saturated aqueous media (Table 3) was

Table 3
Solvent quantification in the organic–aqueous two-phase systems

Solvent	Solubility in the aqueous phase(mM)	Solvent in the cells (mmol solvent/ mg dry cells)
Dibutyl succinate	1.57 ± 0.34	n.d.
Diallyl phthalate	0.82 ± 0.02	n.d.
Benzylbutyl phthalate	0.34 ± 0.01	n.d.
Diisoheptyl phthalate	0.25 ± 0.04	0.19
Bis (2-ethylhexyl) adipate	0.94 ± 0.05	1.36
Bis (2-ethylhexyl) phthalate	0.03 ± 0.01	0.02
Bis(3,5,5-trimethylhexyl) phthalate	0.06 ± 0.01	0.06
Methyl nonanoate	1.84 ± 0.24	n.d.
Ethyl nonanoate	1.71 ± 0.10	n.d.
Ethyl decanoate	2.56 ± 0.15	n.d.
Ethyl undecanoate	4.01 ± 0.21	5.47
Ethyl laurate	2.89 ± 0.31	n.d.
Ethyl myristate	2.02 ± 0.39	n.d.
Isopropyl myristate	1.54 ± 0.43	2.04

The approximate solvent amount retained in the cells was obtained considering the solvent added until saturation of the aqueous phase (see Section 2). The solvent solubilities were measured in cell-free phosphate buffer.
n.d. — Not determined.

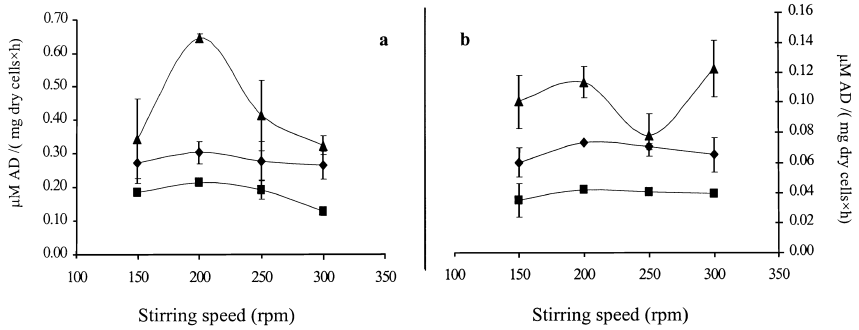

Fig. 2. Effect of the stirring rate (turbine mixing) on the specific sitosterol degradation activity of free *Mycobacterium* sp. cells in three aqueous–phthalate systems with 25% (a) and 75% (b) organic phase content (V_{org}/V_{total}). (▲) Bis(2-ethylhexyl)phthalate; (♦) bis(3,5,5-trimethylhexyl)phthalate; (■) diisohepthyl phthalate.

apparently enough to approach or surpass the critical membrane concentration, thus resulting in the loss of activity reported on Table 1. On the other hand, this implies that, even in two-phase systems and with the cells accumulating at the interphase, a water film is still retained around the cells to serve as a barrier against direct solvent-cell contact.

The cell and sitosterol partition coefficients and substrate and product solubilities indicate that the bioconversion probably occurs in the interphase of the biphasic systems used. Further sitosterol partition tests were carried out (data not shown) at the different phase ratios subsequently used for biotransformations, no significant change being noted in the partition coefficients. In these circumstances, an increase of the stirring speed in the biphasic bioconversions (Fig. 2) would presumably result in a consequent increase of the interfacial area, and higher bioconversion activities [11]. This was, however, not observed for the three phthalates tested, with the exception of

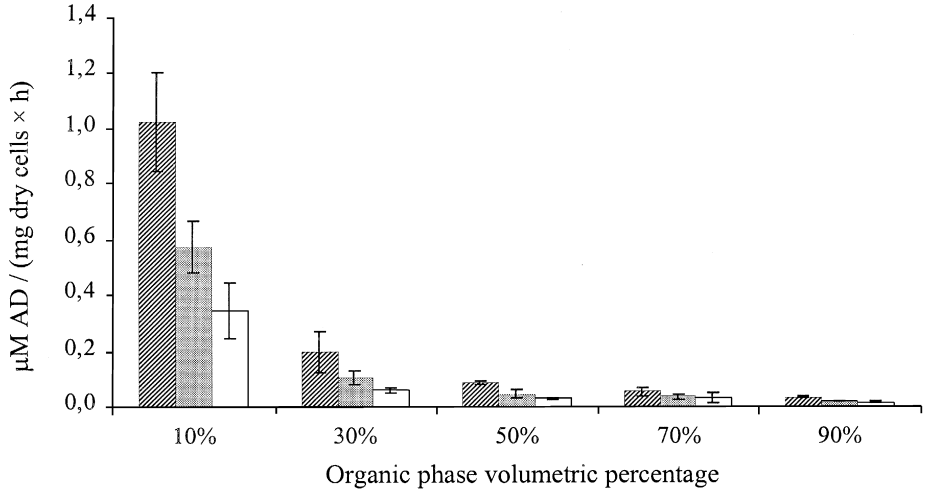

Fig. 3. Effect of solvent content (% by volume) on the degradation activity of sitosterol by *Mycobacterium* sp. in three phthalate–aqueous systems [(box with a bold diagonal line) — Bis(2-ethylhexyl)phthalate; (■) — bis(3,5,5-trimethylhexyl)phthalate; (□) — diisohepthyl phthalate). Stirring speed was always 200 rpm.

bis(2-ethylhexyl)phthalate at the lower range of stirring speeds. It was also observed that stable emulsions were readily formed at the whole range of stirring speeds for the two phase ratios tested, possibly aided by the presence of the cells at the interphase. However, the phase ratio had a marked effect on catalytic activity (Fig. 3). Since the total cell load and organic phase substrate concentration were kept constant, an increase in the volumetric proportion of organic phase brings about higher substrate/biocatalyst weight ratios. The observed decrease in specific activity (Fig. 3) thus indicates that the substrate could be less available to the cells, through reduction of the interfacial area per unit volume of organic phase and possibly mass transfer resistances inside the latter.

4. Conclusions

The selective cleavage of the β-sitosterol side-chain using free *Mycobacterium* sp. NRRL B-3805 cells was successfully achieved in the presence of hydrophobic solvents as substrate and product reservoirs. The suitable solvents, among the esters tested, could not be selected on the sole basis of the hydrophobicity parameter, log *P*. The distribution of the cells, substrates/products and solvents in the organic/aqueous two-phase systems used suggested that the bioconversion takes place either in the organic phase or at the interphase and that low equilibrium solvent concentration in the cells is related to higher catalytic activity. Stirring speed and phase ratio effects on two-phase system performance seem to indicate that the bioconversion takes place at the interphase, with apparent bioconversion rates limited by mass transfer inside the organic phase.

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