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Effect of phase composition on the whole-cell bioconversion of β-sitosterol in biphasic media

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Abstract

The multi-step bioconversion of β-sitosterol by *Mycobacterium* sp. NRRL B-3805 for the formation of androstenedione (AD) as major product, a key intermediate in the production of therapeutic steroids, is a well established industrial application of a biocatalytic process. In the present work, this sterol side-chain cleavage was selected as model system for a study of whole-cell biocatalyst operational stability in prolonged bioconversions in two-phase aqueous-organic media. The aim was to evaluate the effect of phase composition in the process yields by using four high $\log P_{\text{octanol}}$ phthalates as organic phases and phosphate buffer and nutritional media as aqueous phases. The effect of a non-ionic surfactant added to the aqueous phase was also studied. Results show no relation between bioconversion yields and solvent $\log P_{\text{octanol}}$ values, suggesting that, in the used range, solvent molecular structures and the resulting specific interactions with the cell envelope are determining for the biocatalyst behaviour. Different responses were obtained from the use of different aqueous media, the complex nutritional media leading to the highest conversion yields, while the lowest were observed with the mineral nutritional media. The effect of surfactant addition in the bioconversion yields was apparently not significant. In the systems with bis-(2-ethylhexyl) and bis-(3,5,5-trimethylhexyl) phthalates as the organic phases, the fed-batch operational mode allowed the maintenance of stable biocatalytic activity levels up to at least 6 days of operation, with high androstenedione yields.

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

1. Introduction

The selective cleavage of the side-chain of natural phytosterols by *Mycobacterium* sp. mutants to produce androstenedione (AD) and androsta-diene-dione (ADD), which are important intermediates for the pharmaceutical industry, is an industrial biotransformation currently with a world wide market [1].

The steroids involved in this bioconversion are markedly hydrophobic substances with low water solubilities [2], this being the major factor limiting process productivity. One of the technical solutions to this problem can be the use of two liquid-phase reaction media with sterol-solubilising organic solvents [1]. This however requires a careful selection of the solvent, for in whole-cell processes solvent toxicity is often the limiting factor. Biocompatibility indicator parameters like $\log P_{\text{octanol}}$ can be used as a first approach to this issue [3], but do not seem to quantify specific solvent characteristics which could

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be involved in their interaction with microbial cells [4–7].

The cell wall in the *Mycobacterium* genus has a very hydrophobic nature [8], leading to strong cell-solvent affinity and conditioning the bioconversion process through specific interactions with the different lipophilic compounds present in the medium [9]. Therefore, when an aqueous-organic two-phase bioconversion system is considered for this whole-cell biocatalyst, the choice of compositions for both phases is a relevant issue in the process performance.

With the aim of studying phase composition effects, the present work uses as a model bioconversion the β-sitosterol side-chain degradation with *Mycobacterium* sp. NRRL B-3805 cells in 1:1 (v_{org}/v_{aq}) biphasic systems, with varying aqueous and organic phase components. Four highly hydrophobic phthalates were used as organic phases and the aqueous phases were based on phosphate buffer, supplemented with complex and mineral nutrients sources and also with a dispersive non-ionic surfactant.

2. Experimental

2.1. Chemicals

The organic solvents, Tween-20 and salts used were synthesis grade from Merck (Germany). Sterol and steroids were obtained from Sigma (USA). Yeast extract was supplied by Difco (USA). Other chemicals were analytical grade from various suppliers.

2.2. Cell growth

Mycobacterium sp. NRRL B-3805 cells were obtained as described by Cruz et al. [4]. Free cells were harvested by filtration, washed with phosphate buffer and stored at −20 °C as a wet cell paste (approximately 120 mg dry cell weight/g).

2.3. Analytical methods

Protein assays, substrate and product solubility determinations and HPLC analysis (with an associated error <10%) were performed as described by Cruz et al. [4].

2.4. Organic solvents

The selection of di-isoheptyl, bis-(2-ethylhexyl), di-isodecyl and bis-(3,5,5,-trimethylhexyl) phthalates was based on high substrate solubilisation capacity and expected biocompability, as predicted by their respective $\log P_{octanol}$ parameter values, which were calculated as described by Rekker and de Kort [10].

2.5. Aqueous media

Five different media were used. Aqueous medium 1 was phosphate buffer pH 7, aqueous medium 2 was phosphate buffer pH 7 containing yeast extract (10 g/l) (complex medium), aqueous medium 3 was medium 2 supplemented with Tween-20 (0.8 g/l), aqueous medium 4 was phosphate buffer pH 7 containing NH_4Cl (2 g/l), $MgSO_4{\cdot}7H_2O$ (0.14 g/l) and fructose (10 g/l) (mineral medium) and aqueous medium 5 was medium 4 supplemented with Tween-20 (0.8 g/l).

2.6. Bioconversion runs

Two-phase bioconversion systems containing 5 ml of aqueous medium, 5 ml of a β-sitosterol (5 g/l) solution in phthalate and a cell load of 10 mg wet cell paste per millilitre of total system were incubated in orbitally agitated flasks at 200 rpm and 30 °C. Media additions (2 ml) were done every 48 h, maintaining the liquid phase ratio and a substrate supply to the system of around 12 μmol/ml per day. Along the 144 h of process, samples were collected and AD formation monitored. Duplicate runs were carried out.

2.7. Biocatalyst growth tests

In conditions identical to those of the bioconversion trials, parallel assays were performed with bis-(3,5,5-trimethylhexyl) phthalate in which the total flask content was collected and centrifuged at each sampling time. The cell pellet was used for protein content determination, the aqueous phase pH was monitored and the AD content in the organic phase was determined. The tests were carried out for incubation periods up to 72 h, in batch mode (no media additions). Duplicate runs were carried out.

Table 1
Solvent $\log P_{octanol}$ values and substrate and product solubilities in the tested organic solvents

Organic solvent	$\log P_{octanol}$	Solubility (mM)	
		Sitosterol	AD
Di-isoheptyl phthalate	6.9	58 ± 4	112 ± 5
Bis-(2-ethylhexyl) phthalate	9.6	68 ± 4	108 ± 5
Di-isodecyl phthalate	10.4	52 ± 5	65 ± 2
Bis-(3,5,5-trimethylhexyl) phthalate	10.6	78 ± 2	92 ± 3

3. Results and discussion

The high $\log P_{octanol}$ phthalates selected for this work (Table 1) show a high capacity to solubilise both the substrate and the product. The values obtained reveal no apparent correlation with the solvent hydrophobicity parameter, in agreement with previous studies [4], and anticipate a virtually complete substrate and product partition to the organic phase, as the measured β-sitosterol and AD solubilities in the aqueous phases were always below 1.2 mM, even in the presence of surfactant. The reservoir effect of these phthalates can thus allow the prolonged operation of the bioconversion process in fed-batch mode, with the accumulation of solubilised product in the organic phase (Fig. 1). Still, under the same reaction conditions the biocatalyst behaviour is strongly affected by the solvent used as organic phase, as reflected in the different product yields obtained in the assays presented in Fig. 1. However, increasing yields do not consistently result from increasing phthalate $\log P_{octanol}$ values. Di-isohephyl and di-isodecyl phthalates with more linear chain substituents are apparently less biocompatible than the two phthalates with the more branched chain substituents. This struc-

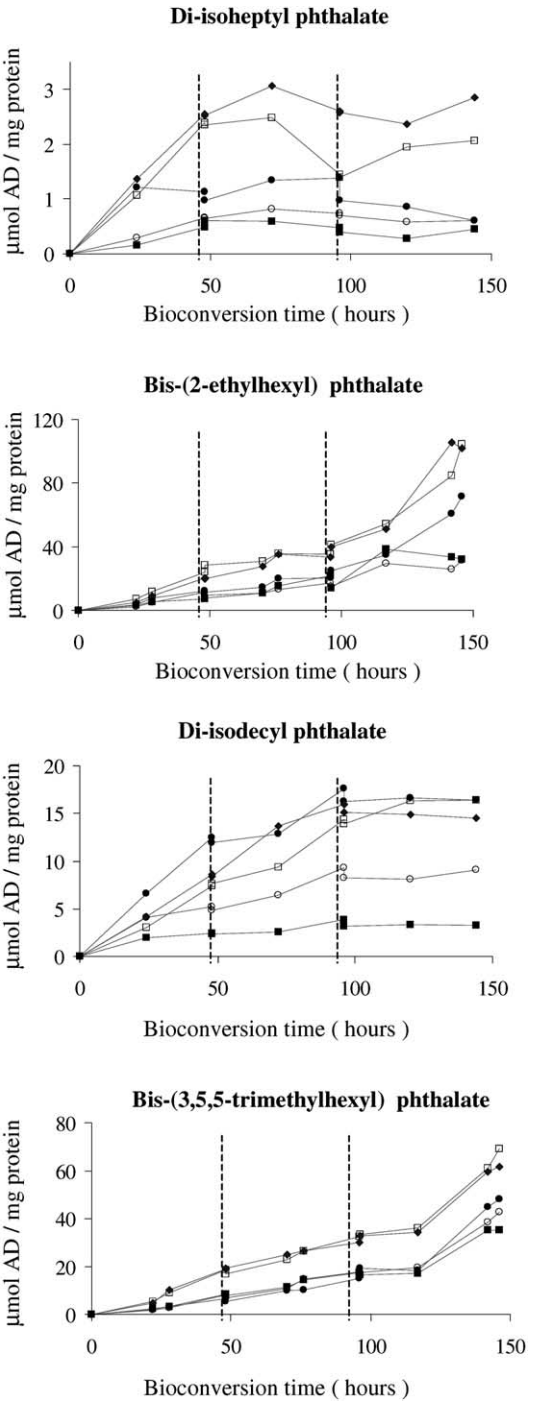

Fig. 1. Effect of phase composition on the fed-batch bioconversion of β-sitosterol by *Mycobacterium* sp. in 1:1 (v_{org}/v_{aq}) two-phase aqueous-phthalate systems. The organic phases were di-isoheptyl, bis-(2-ethylhexyl), di-isodecyl and bis-(3,5,5,-trimethylhexyl) phthalates. The aqueous phases were composed of phosphate buffer pH 7 (●), complex medium (□), complex medium with Tween-20 (◆), mineral medium (■) and mineral medium with Tween-20 (○). Trials were performed in duplicate runs in an orbital shaker (200 rpm, 30 °C). Dashed lines indicate additions of fresh phases (with substrate in the organic phase), maintaining the phase ratio.

tural difference may affect their specific interactions with the cell envelope, thus determining biocatalyst behaviour.

The AD formation profiles throughout the fed-batch bioconversions are similar, showing a progressive product accumulation. However, an almost parabolic profile is observed in the more linear substituted phthalates, with a lower bioconversion rate towards the end of the runs, whereas the other two solvents show an apparent increase in biocatalytic activity in that same period.

The effect of nutrient sources in the aqueous media is more marked with the solvents showing higher conversion rates, although it can be observed in all four cases. In fact, when the aqueous phase is composed of mineral buffered media the AD yields are lower than those obtained with complex nutrient media aqueous-phases, but have identical evolutions in time.

The aqueous-phase components were chosen as they constitute the nutritional media used for biocatalyst inoculum maintenance and cell growth [4], and so they are equally able to provide a source of nutrients to the biocatalyst in the long term bioconversion. However, the mineral media led to lower AD yields than phosphate buffer, whereas the complex aqueous media gave higher or comparable yield values. These results show that the nature of the nutrient source strongly affects biocatalyst behaviour, possibly through its ability to maintain cell energy levels and ease co-factor regeneration. Other authors refer the enhancement of sterol side-chain degradation in the presence of glycine [11] and lecithin [12] in the bioconversion medium, however relating it to induced changes in the *Mycobacterium* cell envelopes.

Despite resulting in extensive system emulsification and preventing cell aggregation, as also described by Angelova and Schmauder [9], the surfactant had no significant effect in the bioconversion yields (Fig. 1).

The higher product accumulation yields in the presence of a nutritional aqueous-phase could be caused simply by cell growth. Considering this hypothesis, parallel runs were carried out in similar experimental conditions for the determination of cellular protein (harvested by centrifugation) total content along the process. Fig. 2 presents the results obtained in the assays carried out in aqueous-bis-(3,5,5,-trimethylhexyl) phthalate systems, together with the AD formation profile during 72 h of incubation. It can be observed that even in the presence of nutritional media no net cell growth occurs. In batch fermentations for the production of the biocatalyst, cell growth is always accompanied by a pH shift to more acidic values [13]. In the aqueous phase of the two-phase bioconversion assays, no pH variation was observed along the process, supporting the idea that no cell growth occurred. The effect of nutrients seems, therefore, related to cell maintenance and to providing substrates for co-factor regeneration in the redox steps involved in sitosterol side-chain cleavage [14].

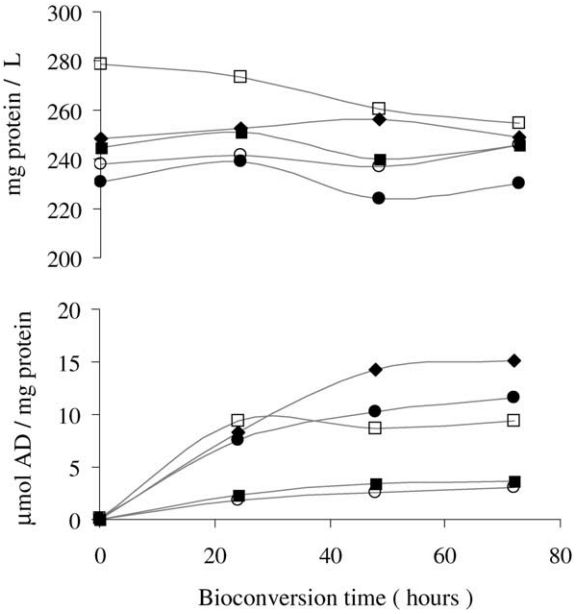

Fig. 2. Evolution of cell protein concentration (associated standard error <10%) and AD accumulation in batch bioconversions carried out in 1:1 (v_{org}/v_{aq}) aqueous-bis-(3,5,5-trimethylhexyl) phthalate systems for 72 h in multiple flasks (200 rpm, 30 °C). The aqueous phases were composed of phosphate buffer pH 7 (●), complex medium (□), complex medium with Tween-20 (◆), mineral medium (■) and mineral medium with Tween-20 (○).

The fed-batch operational mode allowed the maintenance of high biocatalyst activity levels up to at least 6 days of operation with the two more branched phthalates as organic phases. An activity increase even

seems to occur with these two solvents towards the end of the experimental period (Fig. 1), which could be an indication of cell adaptation. Non-growing cell adaptation mechanisms to organic solvents have been previously described by Isken and de Bont [15].

4. Conclusions

The selective side-chain cleavage of β-sitosterol with *Mycobacterium* sp. whole-cells was achieved in high yield, with aqueous-organic systems using hydrophobic phthalates as organic phases. The composition of the aqueous phase has a strong effect in the bioconversion outcome, particularly in what concerns the type of nutrients made available. The use of complex nutritional aqueous media results in an enhanced conversion yield, with no net cell growth being apparently required. The presence of a surfactant had no significant effect, suggesting that interphase mass transfer of substrate/products or eventual toxicity from enhanced cell-solvent contact are not determining factors in the bioconversion yields. The $\log P_{\text{octanol}}$ parameter was not a sensitive indicator for the organic solvent relative biocompatibility in this system, for the higher range of values considered. With bis-(2-ethylhexyl) and bis-(3,5,5-trimethylhexyl) phthalates as organic phases the bioconversion yields improved after 96 h of reaction, especially in the presence of a complex nutritional aqueous medium. This could indicate a biocatalyst adaptation effect.

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