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Optimization of androstenedione production in an organic–aqueous two-liquid phase system

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Abstract

A systematic evaluation of the effect of key operational parameters on the selective cleavage of sitosterol to 4-androstene-3,17-dione (AD) with *Mycobacterium* sp. NRRL B-3805 in a dioctyl phthalate: aqueous buffer two-liquid phase system was performed. Of the parameters assessed, buffer composition, biomedium pH, temperature, and biomass and substrate concentration were those that mostly affected overall bioconversion rate. The optimum pH was 7.5 with Tris buffer. The highest bioconversion rate was observed at 35 °C, although at 40 °C bioconversion activity was virtually lost. Michaelis–Menten type kinetics adequately described the bioconversion system. Increasing biomass concentration from 10 to 70 $g_{wet\ cell\ weight}\ l^{-1}$ favored AD final yield, although the specific AD yield slightly decreased.

Keywords: Androstenedione; Biotransformation; Sitosterol side-chain cleavage; *Mycobacterium* sp. NRRL B-3805; Organic–aqueous phase system

1. Introduction

The use of a biocompatible water-immiscible organic phase as a substrate and product pool has been acknowledged as an effective tool to overcome the low volumetric productivity of aqueous bioconversion systems involving hydrophobic compounds [1–3].

This approach has been successfully used for the specific side-chain cleavage of sitosterol to 4-androstene-3,17-dione (AD), with 1,4-androstadiene-3,17-dione (ADD) as side product, using *Mycobacterium* sp. NRRL B-3805 cells [4]. Extensive work has been performed regarding the selection of a biocompatible organic solvent, the evaluation of the mechanisms of solvent interaction with microbial cells and the organic–aqueous volumetric phase ratio [4–6]. However, little information is available on the influence of other system parameters on the reaction rate of this bioconversion system in an organic–aqueous two-phase system [4]. As for AD production from sitosterol by conventional fermentation methods, using *Mycobacterium vaccae* (or mutants of this strain, such as the one used in this work [7]), some data on the influence of temperature and pH on bioconversion yield is available. Thus, this bioconversion is usually performed in media with an initial pH of 7 [7,8–13], although some works also report a pH medium of 6.45 [14] or 6.0–6.2 [15]. However, no thorough assessment of the effect of the medium pH on bioconversion activity has been reported. Srivastava and Patil reported a peak of activity at pH 6, but for cholesterol side-chain cleavage, with *Mycobacterium fortuitum* [16]. The fermentation process is currently carried out at 30 °C [11,13], 31 °C [9], 32 °C [7,15] or 35 °C [12,14]. Although it has been stressed that maintaining the temperature in the range of 30–35 °C during the fermentation process is a key factor for a successful bioconversion [14], no comprehensive evaluation on the effect of the temperature on bioconversion activity has been reported.

The present work aims to contribute to fill in some of these blanks. Thus, a systematic evaluation of the effects of system parameters on the overall rate of AD formation from sitosterol, using *Mycobacterium* sp. NRRL B-3805 cells in

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dioctyl phthalate:aqueous buffer two-phase system, was performed. Namely buffer composition, pH and concentration, stirring speed, substrate and biomass concentration were assayed. Such data provide the basis for the setup of a highly effective system for AD production from sitosterol, exploiting the advantages of a two-phase system. The optimized bioconversion system can be used as a model system for the future development of a strategy for AD recovery from the organic phase, possibly based on chromatographic methods. The use of resins (e.g. Amberlite XAD-7) has been effectively used for AD(D) recovery from the fermentation broth, followed by chromatography [17], although liquid extraction of the fermentation broth, followed by-product isolation by crystallization [12,18], by chromatography [11] or by the two combined methods [19] are more commonly used.

2. Materials and methods

2.1. Materials

Yeast extract and potato dextrose agar were obtained from Difco (USA). Synthesis grade dioctyl phthalate was supplied by Merck (Germany), glycerol p.a. grade was from Riedel-de Haën (Germany), Tween 20, β-sitosterol, AD, ADD and progesterone were obtained from Sigma (USA). All other chemicals were analytical- or HPLC-grade from various suppliers.

2.2. Cell growth

Mycobacterium sp. NRRL B-3805 cells were maintained in potato dextrose agar slants (42 g l^{-1}). *Mycobacterium* sp. NRRL B-3805 cells were grown in a complex medium composed of yeast extract (10 g l^{-1}), glycerol (10 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.14 g l^{-1}), sitosterol (0.5 g l^{-1}), Tween 20 (0.8 g l^{-1}) in pH 7 potassium phosphate buffer (20 mM) at 30 °C. Cells were harvested by centrifugation (5 min, 4 °C, 5000 rpm) in the late exponential growth phase, thoroughly washed with pH 7 potassium phosphate buffer. The wet cell paste (roughly 120 $mg_{dry\ cell\ weight}$ g^{-1}) was either immediately used in bioconversion trials or stored at −20 °C until use.

2.3. Bioconversion trials in two-liquid phase systems

Bioconversion trials were performed either in 25 ml screw-capped vessels with 7 ml reaction volume or in 250 ml reactors with 50 ml reaction volume. All experiments were performed using a 1:1 organic–aqueous volumetric phase ratio [6]. A given amount of cells was added to a given volume of 100 mM buffer solution, corresponding to 10 $g_{wet\ cell\ paste}$ l^{-1}, unless stated otherwise. One volume of a 12 mM solution of β-sitosterol in dioctyl phthalate was then added and the whole incubated at 30 °C with 200 rpm orbital shaking or 300 rpm mechanical stirring, unless stated otherwise. Orbital shaking experiments were carried out using 7 ml of emulsion in 25 ml screw-capped flasks. Stirred tank experiments were performed using 50 ml of emulsion in 300 ml vessels. Mechanical stirring was promoted with a 45° pitched four-bladed turbine. Samples of 300 μl were collected periodically, centrifuged and 80 μl of the organic phase diluted with 720 μl of a solution of progesterone (0.2 g l^{-1}, internal standard) in *n*-heptane, whereas the aqueous phase was extracted with the same solution. The samples were then analyzed for sterol/steroid content by HPLC. Bioconversion rates were determined from the zero-time slope of the AD concentrations versus time plot. Values are averages of at least two independent runs. Apparent kinetic parameters were estimated using a software program developed by Cornish-Bowden [20].

2.4. Bioconversion trials in aqueous phase systems

All experiments were performed in 25 ml screw-capped bottles, with 100 mg of a wet cell paste, 10 ml of buffer solution and 200 μl of a 10 g l^{-1} solution of sitosterol in ethanol. The flasks were incubated at 30 °C in a rotary shaker (200 rpm). Cells were also incubated in the absence of sitosterol, in order to evaluate pH profile along time. Samples were collected periodically and extracted as described above for steroid quantification.

2.5. Analytical methods

HPLC analysis (Lichrospher Si-60 column, 10 μm particle size, Merck, Germany) with 1 ml min^{-1} isocratic elution was performed to determine substrate and products concentration, with UV detection at 215 and 254 nm. The mobile phase was composed either of *n*-heptane and ethanol (98:2, v/v), for detection at 215 nm, or of *n*-heptane and ethanol (95:5, v/v), for detection at 254 nm. With the more hydrophobic mobile phase was used, the following retention times (min) were obtained: β-sitosterol (10.5), progesterone (23.9), AD (41.8) and ADD (60.8). When the less hydrophobic mobile phase was used, the following retention times (min) were obtained: progesterone (9.3), AD (14.4), ADD (25.4).

3. Results and discussion

AD production after 24 h bioconversion trials in either aqueous or two-phase systems was not affected by phosphate buffer concentration up to 100 mM. An increase to 200 mM, corresponding to 0.38 M ionic strength, proved deleterious for biocatalytic activity (data not shown), probably due to excessive osmotic pressure. Incubation of resting mycobacterial cells with activity for sitosterol degradation in aqueous media was performed in carbonate, Tris–HCl and phosphate buffers along pH values ranging from 6 to 11 (Table 1). Such preliminary trials clearly show that AD

Table 1
Effect of buffer composition in AD production in aqueous media, using resting *Mycobacterium* sp. NRRL B-3805 cells, after 24 h bioconversion trials at 30 °C

Buffer	Bioconversion			No bioconversion	
	Initial pH	AD (mM)	Final pH	Initial pH	Final pH
Phosphate	6.0	0.059	6.0	6.0	6.2
	6.5	0.045	6.3	6.5	6.7
	7.0	0.046	6.9	7.0	7.1
	8.0	0.046	7.4	8.0	8.1
	8.5	0.030	7.4	8.5	8.5
Tris–HCl	7.0	0.246	6.5	7.0	7.0
	7.5	0.221	7.2	7.5	7.6
	8.0	ND	ND	8.0	8.1
	8.5	0.206	8.3	8.5	8.5
	9.0	0.211	8.3	9.0	8.9
Carbonate	9.0	0.043	8.6	ND	ND
	10.0	0.004	10.0	ND	ND
	11.0	0	10.9	ND	ND

Buffer solutions with 20 mM concentration were used. The influence of AD production in pH shift during the incubation of mycobacterial cell in biconversion medium is also assessed, by comparing media with and without sitosterol.

production is favored by the use of either Tris–HCl or phosphate buffer solutions. The more extreme pH environment, related to the use of carbonate buffer, is likely to be inhibitory for the enzymes of the side-chain cleavage pathway. Along the time course of the biotransformation, the bioconversion medium became slightly more acidic. This effect became increasingly noticeable when a more basic media was used. Such pH shift, which was not observed when cells were incubated in the absence of sitosterol, is most likely caused by acidic metabolites released during sitosterol side-chain cleavage [8]. The effect of the pH medium in the bioconversion rate became much more evident when an organic–aqueous two-phase system was used for sitosterol bioconversion (Fig. 1). The pH optimum shifted from 7.5 to 8.0 when Tris–HCl buffer was replaced with phosphate buffer. The former buffer also favored biocatalytic activity. Ionic strength is lower in Tris buffered media, roughly half of buffer concentration, whereas ionic strength in phosphate buffered media is about twice the concentration, due to the higher charge numbers of the ionic species in phosphate buffer [21].

The bioconversion system shows an optimum at 35 °C, irrespectively of the buffer used as aqueous media (Fig. 2). A further increase of the temperature to 40 °C led to a steep activity decrease. Total loss of the sterol degradation activity of Celite-adsorbed *Mycobacterium* sp. NRRL B-3805 cells in dioctyl phthalate media at 40 °C was reported previously [5]. Although a broad shaped curve could be expected, since the bioconversion involves several enzymes [22], a sharp peaked activity curve was obtained. This may suggest a relevant influence of one of the enzymes of the pathway on the bioconversion activity. The catalytic activity increase observed between 20 and 35 °C could also be partially related to the concomitant decrease in phthalate viscosity, roughly 40% [5,23], which could have enhanced mass transfer and hence the overall bioconversion rate.

External mass transfer resistances are overcome for stirring speeds above 300 rpm. Increasing stirring speed up to

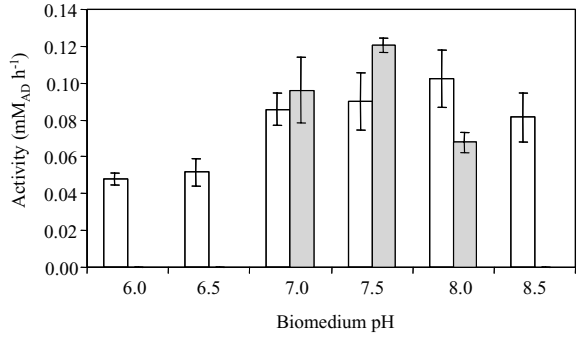

Fig. 1. Effect of the pH of the bioconversion medium in the side-chain cleavage activity of *Mycobacterium* sp. NRRL B-3805 cells in a two-phase system. Aqueous phase was composed of 100 mM phosphate (□) or Tris–HCl (■) buffers. Trials were performed at 25 °C, in 300 ml vessels under 300 rpm mechanical stirring.

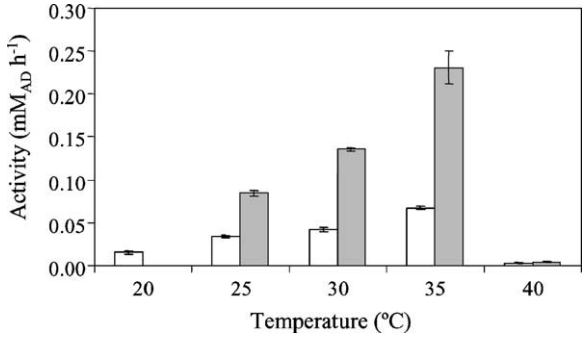

Fig. 2. Influence of the temperature in the side-chain cleavage activity of *Mycobacterium* sp. NRRL B-3805 cells in a two-phase system. Aqueous phase was composed of 100 mM pH 8 phosphate (□) or pH 7.5 Tris–HCl (■) buffers. Trials were performed in 300 ml vessels under 300 rpm mechanical stirring.

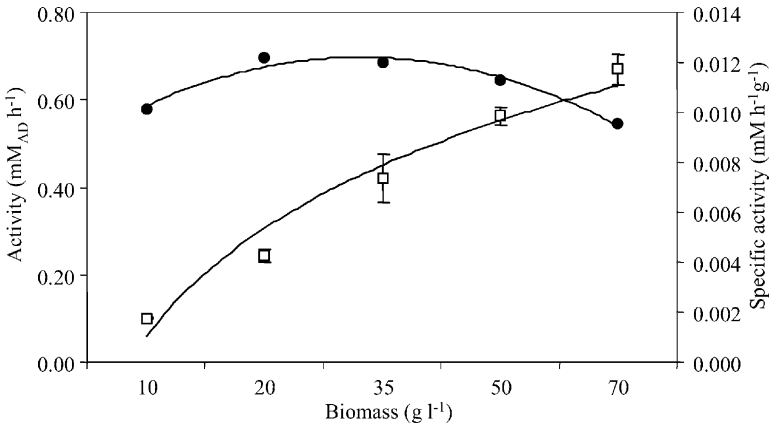

Fig. 3. Influence of biocatalyst concentration in the side-chain cleavage activity (□) and specific activity (●) of *Mycobacterium* sp. NRRL B-3805 cells in a two-phase system. Aqueous phase was composed of 100 mM pH 7.5 Tris–HCl buffer. Trials were performed at 35 °C in 300 ml vessels under 300 rpm stirring speed.

600 rpm did not damage cell integrity, but mass transfer was not improved (data not shown). Furthermore, for time-extended experimental runs (more than 24 h) considerable water transfer into the vapor phase was observed, thus altering the volumetric phase ratio (data not shown). For stirring speeds below 200 rpm incomplete mixing was observed, therefore preventing effective two-phase systems. Increasing biomass concentration up to 50 g l^{-1} led to a linear increase in sitosterol degradation activity (Fig. 3). A further increase of the biomass concentration to 70 g l^{-1} led to a shift from linearity, possibly due to mass transfer limitations, as suggested by Doig et al. [22]. These authors observed a similar behavior while evaluating the reduction of geraniol to citronellol induced by baker's yeast. This led to a decrease in specific activity for the highest cell load (Fig. 3). On the other hand, the use of 70 g l^{-1} of biomass allowed full bioconversion of the substrate in 24 h, while comparatively reducing by-product (ADD) formation (Fig. 4). A similar product yield, for the same initial sitosterol concentration, is only obtained in roughly 3–7 days when a fermentative bioconversion process is used [10,12,24].

Overall bioconversion rate exhibited a Michaelis–Menten type kinetics (Fig. 5). The apparent K_M, 0.15 mM, is quite

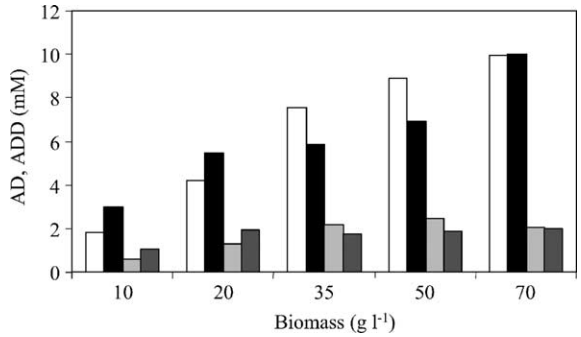

Fig. 4. Influence of biocatalyst concentration on AD yield (□, ■) and ADD yield (▒, ▓) after 24 h (□, ▒) and 48 h (■, ▓) of bioconversion in a two-phase system. Experimental conditions as in Fig. 5.

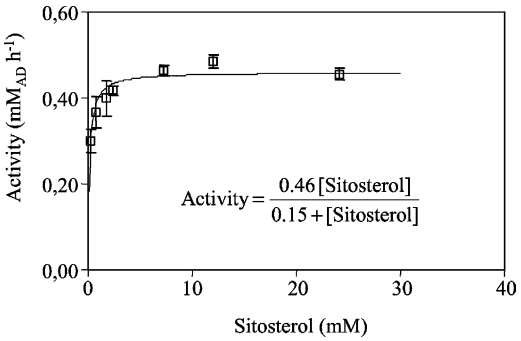

Fig. 5. Experimental (□) and predicted (—) effect of substrate concentration in the side-chain cleavage activity of *Mycobacterium* sp. NRRL B-3805 cells in a two-phase system. Aqueous phase was composed of 100 mM pH 7.5 Tris–HCl buffer. Trials were performed at 35 °C in 300 ml vessels under 300 rpm stirring speed. In the inset data are presented corresponding to the Michaelis–Menten model used.

lower than the apparent values estimated for Celite immobilized cell systems, which was never below 1.2 mM, for the smallest particle diameter evaluated (roughly 40 μm) [25,26]. This could suggest that internal mass transfer resistances may be the bottleneck for the overall reaction rate when immobilized cells are used. A free cell system would thus be more suitable for this bioconversion system.

4. Conclusions

Specific sitosterol side-chain cleavage by *Mycobacterium* sp. NRRL B-3805 in an organic–aqueous two-liquid phase system is influenced by pH and buffer composition and concentration, temperature, hydrodynamic conditions, biomass and substrate concentration. The highest catalytic activity was observed at 35 °C, with an aqueous phase composed of pH 7.5 Tris–HCl buffer (100 mM). Mass transfer limitations can be readily overcome by the use of mechanically stirred tank reactors. The overall reaction rate exhibited

a Michaelis–Menten type kinetics in the substrate range evaluated. Decreasing the incubation period, by increasing biocatalyst concentration favors AD yield, since ADD production is minimized. Full substrate conversion of 12 mM substrate could thus be achieved in a 24 h bioconversion run.

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