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Short Communication

Sitosterol bioconversion with resting cells in liquid polymer based systems

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ABSTRACT

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. The growing environmental and public health awareness is nevertheless leading to restrictions in the use of organic solvents in industrial processes, in order to render these more environmentally friendly. Different approaches are hence being assessed for the design of alternative bioconversion media, involving the use of supercritical fluids, ionic liquids and natural oils and liquid polymers, among others.

In this work, the use of liquid polymers as key components in the bioconversion media for a multi-step microbial bioconversion was assessed. The model system used was the selective cleavage of the side-chain of β-sitosterol by free resting cells of *Mycobacterium* sp. NRRL B-3805, a well established industrial multi-enzymatic process involving the use of nine catabolic enzymes in a fourteen-step metabolic pathway. High product yields were obtained when silicone B oil was used as substrate carrier/product pool, both in single oil and in oil:buffer two liquid phase system.

1. Introduction

The use of organic solvents as substrate and/or product reservoir, in order to improve the efficiency of bioconversion systems, is possibly the most widely chosen approach to overcome the toxicity and/or low solubility of useful compounds (Heipieper et al., 2007; Kim et al., 2007). Most of the organic solvents commonly used as carriers present nevertheless some drawbacks, namely the damaging effects on microbial cells, along with their explosive and environmentally hazardous nature (Schmid et al., 1998; Déziel et al., 1999; Kim et al., 2007). Alternative approaches that retain the advantages of organic solvents, namely solubilization of hydrophobic compounds to high concentration, while avoiding the drawbacks have thus been sought after and presented (Kim et al., 2007; Wang, 2007). One of these is based in the use of liquid polymers as components of the bioconversion media, either in polymer:polymer systems or in polymer:salt solution systems (Zijlstra et al., 1998). This strategy, which provides a mild environment to the biocatalysts and is also environmentally friendly (Zijlstra et al., 1998; Raghavarao et al., 2003), has been scarcely applied to sterol/steroid bioconversions, namely to Δ^1-dehydrogenation of cortisol (Kaul and Mattiasson, 1986; Santos et al., 1991) and side-chain cleavage of cholesterol (Flygare and Larsson, 1989) and of phytosterols (Kutney et al., 2000; Stefanov et al., 2006). Flygare and Larsson (1989) performed cholesterol bioconversions using immobilized resting cells in a polymer:polymer:detergent media and an initial substrate concentration of only 1.0 g l^{-1}, whereas Kutney et al. (2000) and Stefanov et al. (2006) performed phytosterol bioconversions using growing cells in a polymer:aqueous media system. In the present work the feasibility of using free resting *Mycobacterium* sp. cells for sitosterol side-chain cleavage to 4-androstene-3,17-dione (AD), in a polymer based medium is addressed. The phytosterol used was recovered from tall-oil (Dias et al., 2002) and cells were occasionally grown using waste glycerol recycled from a lab-scale biodiesel production systems as part of the carbon source. When a given bioconversion can be performed by either growing or resting cells, and provided cell viability is not compromised, in case this feature affects catalytic activity, the use of resting cells can be advantageous over growing cells. The former approach allows for a more focused evaluation of the bioconversion itself, given that the complex metabolic pathways involved in microbial cell growth are not present and thus do not have to be taken into consideration, and there is no need for sterile environment, the whole leading to a simpler system (Mutafov et al., 1997; Wang et al., 2005; Cánovas et al., 2007). The use of a single liquid phase allows on the other hand higher volumetric productivities, and in the overall simplifies the design of reaction media, selection of operational conditions and downstream processing. High product yields were observed for initial sitosterol concentrations of 5.0 g l^{-1} (12 mM) both in silicone oil media and in silicone oil:buffer two-phase systems. The poly(methylphenylsiloxane) oil, henceforth termed silicone B oil,

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proved thus to provide a suitable media for sitosterol side-chain cleavage with microbial cells.

2. Methods

2.1. Reagents

Yeast extract and potato dextrose agar were obtained from Difco (Detroit, MI, USA). Ammonium chloride and dioctyl phthalate (DOP) was supplied by Merck-Schuchardt (Hohenbrunn, Germany), glycerol was from Riedel-de-Häen (Seelze, Germany). Sitosterol (for preparation of analytical standards) was from Acros (Geel, Belgium), otherwise it was obtained from tall-oil as described elsewhere (Dias et al., 2002). Tween 20, 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione (ADD) and progesterone were obtained from Sigma (St Louis, MO, USA). Silicone oils (high temperatures), namely poly(dimethylsiloxane), henceforth termed silicone A, and silicone B, were obtained from Acros Organic (New Jersey, USA). Polypropylene glycol (PPG) 425 and 2000 were also obtained from Acros Organic (New Jersey, USA). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade and purchased from various suppliers.

2.2. Microorganisms, growth conditions and bioconversion

Mycobacterium sp. NRRL B-3805 cells were maintained in potato dextrose agar slants (42 g l^{-1}) and grown in 1 l Erlenmeyer flasks containing 200 ml of a defined medium composed of (%, w/v) glycerol (2), NH_4Cl (0.4), Tween® 20 (0.08), $MgSO_4 \cdot 7H_2O$ (0.02), β-sitosterol (0.1), prepared in pH 7 di-sodium/potassium phosphate buffer (100 mM). Incubation was performed for 48 h at 30 °C and 200 rpm in orbital shakers Aralab Agitorb 2001C (Portugal) with 25 mm shaking diameter. Active microbial cells were harvested by filtration in qualitative filter paper, thoroughly washed with pH 7 di-sodium/potassium phosphate buffer (100 mM), and stored at −20 °C as a wet cell paste (roughly 200 mg dry cell weight per gram wet paste) until use (Staebler et al., 2004).

Bioconversion runs were performed at 35 °C in 15 ml magnetically stirred (800 rpm) reactors, closed with sealing tape, containing 1 ml of bioconversion media, to which 25 mg of wet cell paste were added. The bioconversion media was composed either by 1 ml of a 12 mM solution of sitosterol in a given liquid polymer or in DOP, or by a two-phase system composed of similar volumes of the sterol rich solution, in either polymer or in DOP, and a Tris-HCl, pH 7.0, 100 mM, buffered phase. A sacrificial vessel approach, based on the methodology described by Ferreira-Torres et al. (2005), was used. Periodically, the whole volume of two replicate reactors was collected and centrifuged (14.000 rpm × 5 min). A given volume of the DOP or polymer phase was collected and diluted in a solution of progesterone (0.2 g l^{-1}, internal standard) in *n*-heptane. For the two-phase systems, a given volume of the aqueous phase was extracted twice with a three-fold volume of the internal standard solution. Triplicate samples were prepared for each reactor. Steroid quantification was performed by HPLC.

2.3. Partition and solubility data

In order to assess the ability of the selected polymers to solubilize the substrate, given amounts (100 mg) of sitosterol were added to 5 ml of each liquid polymer until saturation occurred, as checked visually by formation of insoluble phase. The process was performed in magnetically stirred vessels, under controlled temperatures of either 35 °C or 100 °C. For each polymer, eight replicate runs were made. The supernatant was collected and diluted in a solution containing the internal standard for assessment of sitosterol concentration. Sterol solubility was determined based on an average value of the eight runs for each polymer. Occasionally, saturated solutions incubated at 100 °C were allowed to cool to 35 °C, to check for substrate crystallization or similar phenomena.

Partition of substrate and product between oil and aqueous phases were assessed as follows: a volume of 5 ml of polymer containing 8 mM of AD and/or sitosterol was added to 5 ml of aqueous media. After overnight magnetic stirring (at 30 °C), phase separation was achieved by centrifugation (4500 rpm, 30 min, 30 °C). The aqueous phase was collected and the amount steroid/sterol was determined by HPLC analysis. Triplicate runs were carried out.

2.4. Sterol/steroid analysis

HPLC analysis (Lichrospher Si-60 column, 5 m particle size, Merck, Germany) with 1 ml min^{-1} isocratic elution was performed to determine products concentration, with UV detection at 215 nm (sitosterol) and 254 nm (AD and ADD). The mobile phase was composed of *n*-heptane and ethanol (90:10, v/v). Samples were extracted with *n*-heptane containing *n*-heptane, containing 0.2 g l^{-1} progesterone as internal standard, prior to injection into the HPLC column.

3. Results

Given the promising results obtained with silicone oil and PPG as substrate carriers in AD production from phytosterols with growing *Mycobacterium* sp. cells (Kutney et al., 2000; Stefanov et al., 2006), the feasibility of using specific types of such liquid polymers as substrate carriers was tentatively addressed. Polyethers displayed a far higher sterol solubilization capability, particularly at higher temperatures, although this feature is not particularly noticeable at 35 °C (Table 1).

Free resting *Mycobacterium* sp. cells were introduced into the different liquid polymers tested as reaction media (Fig. 1). Since the threshold for sitosterol solubilization at 35 °C was about 10–12 mM (Table 1), 12 mM sitosterol solutions used in bioconversion trials were prepared by incubating at higher temperatures, transferred to the individual reactors and allowed to cool to 35 °C prior to addition of the biocatalyst. Crystallization was not observed in the process. In the bioconversion runs performed, Silicone B clearly emerged as the most suitable substrate carrier among the liquid polymers tested. Given the promising results, silicone B was also tested in a two-liquid phase system and compared to the performance of the well established DOP-buffer bioconversion system (Staebler et al., 2004) (Fig. 2). Both substrate and AD were shown in preliminary trials to partition preferably to the silicone phase, since partition coefficient between oil and aqueous phase of 44 (±6) and 1.2×10^2 ($\pm 2 \times 10^1$) were observed for sitosterol and AD, respectively. Similar partition coefficients were tentatively

Table 1
Solubility of sitosterol in the liquid polymers tested.

Polymer	Sitosterol solubility (mM)	Temperature (°C)	Comments
PPG 425	12(±2.5)	35	
	84(±5.9)	100	Crystallization of sitosterol after addition to the aqueous phase (2)
PPG 2000	12(±1.8)	35	(1)
	72(±2.9)	100	(2)
Silicone A	10(±1.0)	35	(1)
	20(±2.5)	100	(2)
Silicone B	10(±1.7)	35	(1)
	44(±3.5)	100	(2)