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Chrysotile as a support for the immobilisation of *Mycobacterium* sp. NRRL B-3805 cells for the bioconversion of β-sitosterol in an organic–aqueous two-liquid phase system

R. Wendhausen[a,b], M. Frigato[b], P. Fernandes[a,c,*], C.C.C.R. Carvalho[a], A. Cruz[a], H.M. Pinheiro[a], J.M.S. Cabral[a]

[a] *Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*
[b] *Fundação Universidade Regional de Blumenau, Rua Antonio da Veiga 140, Blumenau SC, Brazil*
[c] *Universidade Lusófona de Humanidades e Tecnologias, Av. Campo Grande 376, 1749-024 Lisboa, Portugal*

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Abstract

The use of chrysotile, a plentiful and low-cost inorganic material, as an effective immobilisation matrix for mycobacterial cells was evaluated. The side-chain cleavage of β-sitosterol to 4-androstane-3,17-dione (AD), in an organic–aqueous two-liquid phase system, was chosen as model system. Bis(2-ethylhexyl) phthalate (BEHP), a biocompatible organic solvent, was used as organic phase. The biotransformation was carried out with either growing or previously grown cells. Mycobacterial cells were shown to adsorb onto chrysotile in both aqueous buffered system and in organic–aqueous two-liquid phase systems. Cells tend to aggregate among the chrysotile fibres during and after growth. The formed chrysotile/cells complex retained catalytic activity under bioconversion conditions for 1 week, at least. No mass transfer hindrances were observed when the immobilised form of the biocatalyst was used, as compared to the free form. However, among the several approaches to produce the most efficient immobilised biocatalyst, the use of previously grown cells in contact with chrysotile led to the highest product yields as well as highest bioconversion rates. The feasibility of this approach was further evidenced since it allowed the implementation of a continuous mode of operation. A constant product yield of 50% was observed for a 1-week period with this experimental set-up. This work clearly highlights the ability of chrysotile to provide an efficient immobilisation matrix for whole cells to be used as biocatalysts in organic–aqueous two-liquid phase bioconversion systems.

Keywords: Chrysotile; Cell immobilisation; Non-conventional media; Bioreactor

1. Introduction

The bioconversion of hydrophobic substrates in aqueous media is often impaired by low volumetric productivity, due to the poor water solubility of such substrates and related products. The use of an organic–aqueous two-liquid phase system provides an effective and well-established approach to overcome such drawback, since the organic pool allows the solubilisation of high amounts of lipophilic substrates and products [1,2]. Related works have been reported since the early 1980s [3]. Cell membrane integrity may however be tampered with in organic–aqueous two-phase systems. Biocatalyst immobilisation provides a tool to reduce the deleterious effect of the organic phase, by providing cells with a protective environment [4,5]. This approach additionally facilitates biocatalyst separation from the bioconversion media.

This work aims at evaluating the use of chrysotile, a mineral material, as a support for whole cell immobilisation in an organic–aqueous two-liquid phase system. Chrysotile, a very inexpensive and plentiful material, has a high surface charge

* Corresponding author. Tel.: +351 21 8419065; fax: +351 21 8419062.
E-mail address: pedroefe@megamail.pt (P. Fernandes).

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density with a positive zeta potential for pH values between 3 and 12, and a large surface area (14 $m^2\ g^{-1}$). This material has been effectively used as a support for yeast immobilisation [6–8]. The bioconversion of β-sitosterol to androst-4-ene-3,17-dione (AD), two sparingly water soluble chemicals, promoted by *Mycobacterium* sp. NRRL B-3805 cells adsorbed onto chrysotile, was chosen as model system. Sitosterol side-chain cleavage is a key step for the production of therapeutic steroids, with an estimated world market above a thousand tonnes per year, and is currently carried out by a conventional fermentation system [2]. Bis(2-ethylhexyl)phthalate (BEHP) was used as organic phase, since this solvent was previously described as biocompatible for this system, allowing high AD yields [9,10]. Still, only batch mode of operation, using celite-adsorbed mycobacterial cells, has been reported [5,9] and the repeated use of this immobilised biocatalyst in successive batches did not prove effective [11]. This work also evidences that an organic–aqueous two-liquid bioconversion system based in the use of chrysotile immobilised mycobacterial cells can be performed in a continuous mode.

2. Material and methods

2.1. Material

Mycobacterium sp. NRRL B-3805 cells were maintained on potato dextrose agar slants (42 $g\,l^{-1}$) at room temperature. Natural chrysotile 5R type was obtained from SAMA Ltd. (Brazil) and used after being washed with tap water and activated by sonication (25 Hz, 30 min) in phosphate buffer pH 7. BEHP was for synthesis grade from Merck-Germany. β-Sitosterol and AD were supplied by Sigma (USA), Tween 20 was obtained from ICI (Spain).

2.2. Cell growth and immobilisation

Mycobacterium sp. NRRL B-3805 cells were obtained as described by Cruz et al. [12]. The cells were grown either in the presence or absence of chrysotile by adding an innoculum to a growth medium composed of the following: glycerol (10 $g\,l^{-1}$), yeast extract (10 $g\,l^{-1}$), Tween 20 (0.8 $g\,l^{-1}$) and sitosterol (0.12 mM), in pH 7 phosphate buffer (17.6 mM). Cells were immobilised by adsorption onto activated chrysotile fibres, added to the fermentation medium in a weight to volume ratio of roughly 1–6%. After growth, the cells, either free or adsorbed onto chrysotile fibres, were harvested by filtration, washed with phosphate buffer pH 7 and either used immediately for bioconversion or stored at −20 °C until use. Cells that were to be used in bioconversions in continuous mode were grown in the presence of chrysotile inside the same reactor to be used for the biotransformation step, a 80 ml volume jacketed glass vessel. Growth was performed with forced aeration (3 vvm) and magnetic stirring (250 rpm) and the medium was continuously fed with a 0.03 ml min^{-1} flow rate.

2.3. Bioconversion

Bioconversion trials were carried out either with growing or previously grown cells, in simple aqueous medium or in an organic–aqueous biphasic system, with or without chrysotile. The aqueous biotransformation medium consisted of the following: glycerol (10 $g\,l^{-1}$), yeast extract (10 $g\,l^{-1}$), Tween 20 (0.8 $g\,l^{-1}$) and β-sitosterol (2.6 mM), in pH 7 phosphate buffer (17.6 mM). The biphasic system used in consisted of a 1:1 or a 1:4 organic to aqueous volumetric phase ratio, with 30 ml total volume (shake flask runs) or 70 ml (magnetically stirred tank runs). Either a 2.6 or 5.0 mM solution of β-sitosterol in BEHP was used as organic phase. The aqueous phase composition was as described above, except no β-sitosterol was present. Bioconversion runs were performed in 100 ml orbitally agitated flasks at 200 rpm, or in a 80 ml volume jacketed glass vessel, both at 30 °C. This latter set-up was operated with 250 rpm magnetic stirring and forced aeration (roughly 3 vvm). The organic:aqueous dispersion was fed to the reactor in an up-flow mode using a peristaltic P-1 pump from Pharmacia (Sweden). Flow rates of 0.03 and 0.05 ml min^{-1}, corresponding to 39 and 23 h of residence time, respectively, were assayed. The immobilisation matrix was kept inside the reactor with the aid of a metallic grid (250 Tyler mesh), placed on top of the reactor. A second peristaltic P-1 pump collected the excess liquid in the top of the reactor. Samples were taken periodically for evaluation of product formation.

2.4. Analytical methods

AD formation was monitored by HPLC analysis as described by Staebler et al. [13].

2.5. Microscopy analysis

Cell aggregation and adsorption onto chrysotile were observed by microscopy under visible and fluorescent light. In the latter case, LIVE/DEAD® Backlight™ viability kit from Molecular Probes (USA) was used. The microscope was an Olympus CX40 (Japan), with an Olympus U-RFL-T burner and U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515).

2.6. Adsorption onto chrysotile

The maximum amount of cells retained by gram of support was evaluated gravimetrically during the time course of the fermentation. Samples were filtered off, the immobilisation support was thoroughly washed with water, dried at 80 °C until constant weight and matched to the previously weighed cell free support, the difference corresponding to the amount of immobilised biocatalyst. Cell attachment during growth was monitored by microscopy analysis. For the biphasic system the chrysotile fibres were first filtered from the reaction medium, dried and extracted with diethyl ether to fully elim-

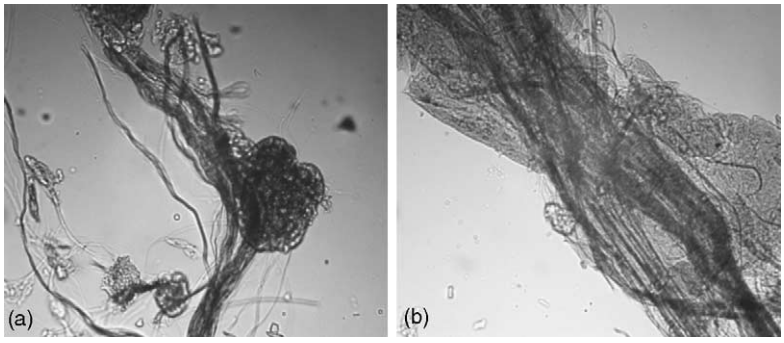

Fig. 1. Microscopy images of *Mycobacterium* sp. NRRL B-3805 cells attached to chrysotile fibbers under brightfield transmitted light. Samples collected after 48 h of biotransformation in a single aqueous biotransformation system (a), and in a biphasic system (b). Lens: ocular-15×; objective-10× (a) and 20× (b).

inate phthalate and only then submitted to the gravimetric determination procedure described above.

3. Results and discussion

Microscopy images of the immobilised biocatalyst show that mycobacterial cells remain attached onto chrysotile fibres under bioconversion conditions, either an aqueous or a two-phase system is evaluated (Fig. 1). In a free suspension these cells tended to form aggregates, due to the hydrophobic nature of the cell wall [14]. In the presence of chrysotile fibres, the trend for mycobacterial cells to form aggregates remained. These aggregates apparently tended to strongly attach to the chrysotile surface (Fig. 1a). In the two-phase systems, cells were more evenly distributed on the chrysotile fibres, probably due to the small solvent droplets retained within the fibres (Fig. 1b). An interfacial interaction between mycobacterial cells and the surface of phthalate droplets was previously reported [12].

The time course of cell loading onto chrysotile fibres during mycobacterial cell growth is shown in Fig. 2. The total amount of immobilised cells was gravimetrically quantified and a maximum of 1:3 chrysotile to cell mass ratio was observed, corresponding to the saturation of the support. This adsorption yield shows a considerable increase as compared to a yield of 1:1, previously reported in the literature, although for chrysotile adsorbed yeast cells [6].

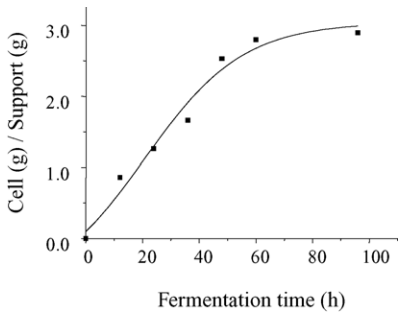

Fig. 2. Progression of mycobacterial cell load onto chrysotile fibbers during a typical fermentation run for immobilised biocatalyst production.

The performance of free and immobilised grown cells in the bioconversion system is shown in Fig. 3. No significant differences were observed in the trend of the time course of product formation, that ultimately led to 1.5 mM AD, which corresponds to a final product yield of 60%, irrespectively of the cell preparation used (Fig. 3). This behaviour suggests that cell adsorption onto chrysotile fibres during growth does not affect significantly the metabolic pathway for β-sitosterol side-chain cleavage.

The performance of immobilised and free cells after thawing in a biphasic system was matched by assessing the formation of AD during the time course of the bioconversion (Fig. 4). An increase of 11% in the initial reaction rate reac-

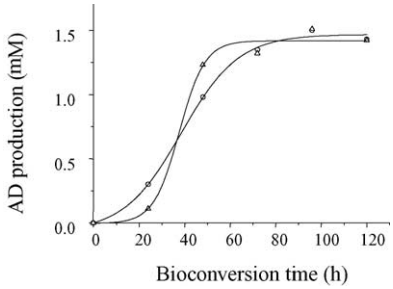

Fig. 3. Time course of AD formation with free (◯) or chrysotile adsorbed (△) *Mycobacterium* sp. NRRL B-3805 growing cells, in an organic–aqueous two-liquid phase system. The initial concentration of β-sitosterol was 2.4 mM.

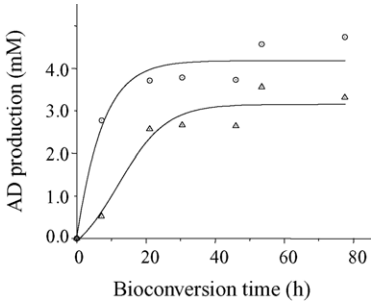

Fig. 4. Time course of AD production during bioconversion runs in biphasic systems, with immobilised (◯) or free (△) *Mycobacterium* sp. NRRL B-3805 cells previously grown. The initial concentration of β-sitosterol was 5 mM.

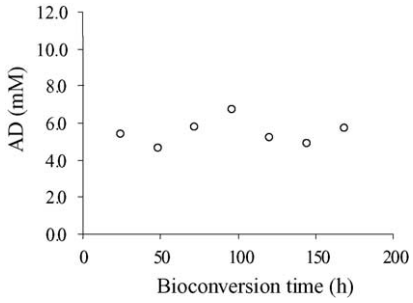

Fig. 5. Time course of AD production using mycobacterial cells immobilised onto chrysotile, in a continuous stirred tank reactor, with a flow rate of 0.031 ml min^{-1}.

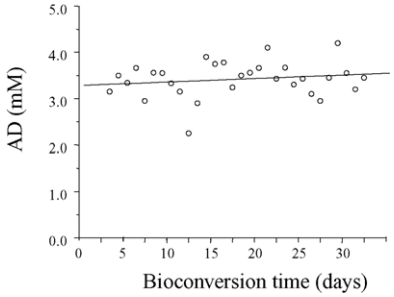

Fig. 6. Time course of AD production using mycobacterial cells immobilised onto chrysotile, in a continuous stirred tank reactor, with a flow rate of 0.050 ml min^{-1}.

tion rate (from 0.24 to 0.35 mM AD h^{-1}) and final product yield (from roughly 60% to 90%) was observed when the immobilised system was compared with free cells. This may suggest that the freezing/thawing process may be relatively harmful for the cells, although immobilisation may provide a relatively protective environment.

Microscopy images of the immobilised system taken after biotransformation showed adequate retention of the cell integrity. Images obtained by fluorescence microscopy suggested that more than 80% of the cells were still viable (data not shown), which is in accordance with the results of de Carvalho et al. [15], while assessing the effect of dioctyl phthalate in *Mycobacterium* sp. B-3805 cells.

Given the promising results obtained in shake flask experiments, further trials were performed in a magnetically stirred tank reactor, an experimental set-up more amenable for scale-up. This experimental approach may also be used in a continuous operation mode. The ability to maintain a stable dispersion in the three-phase system in the presence of different amounts of chrysotile loaded with mycobacteria (from 0.7 to 4 g) was evaluated. Within the range evaluated, only the use of 1.5 g of support enabled a stable dispersion for more than a week. The other amounts of support tested led to inadequate mixing. Aeration of the bioconversion medium enhanced mixing besides providing the oxygen required for the biotransformation to proceed [16]. The need for aeration in this experimental set-up was suggested by the work of Angelova and coworkers [17] and confirmed in preliminary experiments.

A molar product yield ranging from 40% to 56% was observed throughout the time course of the bioconversion trial for a flow rate of 0.031 ml min^{-1} (Fig. 5). Variations in yield may be associated to observed fluctuation in the feed flow rate. Increasing the flow rate to 0.05 ml min^{-1} led to a concomitant decrease in the residence time to 22 h and in AD yield to roughly 20% (Fig. 6).

Nevertheless, this system displayed a significant operational stability, since catalytic activity remained apparently unchanged for a 30-day period. The stability is related not only to product yield, but to mechanical integrity of the support as well.

4. Conclusions

Mycobacterium sp. NRRL B-3805 cells were effectively adsorbed onto chrysotile fibres either during or after growth to provide an effective immobilised biocatalyst for the side-chain cleavage of β-sitosterol to AD. A maximum cell load to chrysotile fibres mass ratio of 3:1 (g/g) was obtained. The formed chrysotile/cells complex is stable under typical bioconversion conditions, e.g. incubation at 30 °C and 200 rpm orbital shaking in an organic–aqueous two-liquid phase systems.

In the biotransformation systems where growing cells were used, no differences in either yield or bioconversion rate were observed, when free and immobilised cells were used. However, when previously grown cell were used, an increase in both product yield and bioconversion rate was observed when the performance of the immobilised cell was assessed, as compared to the free cell system. No decrease in bioconversion rate was observed when the immobilised cell system was used, as compared to free cell, which may suggest that the former may not be significantly hampered by diffusion limitations. An effective batch bioconversion system for the production of AD from β-sitosterol was thus developed, based on the use of chrysotile fibres as an immobilisation matrix for *Mycobacterium* sp. NRRL B-3805. The immobilised biocatalyst was also used in continuous mode of operation for time periods ranging from 1 week to 1 month, with a roughly constant product yield, which although relatively low, suggest this is a valid approach and further work could be developed aiming at its optimisation.

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