Biochemical Engineering Journal 87 (2014) 1–7

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular Article

An immersed hollow fiber membrane bioreactor for enhanced biotransformation of indene to *cis*-indandiol using *Pseudomonas putida*

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ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 6 March 2014
Accepted 22 March 2014
Available online 30 March 2014

Keywords:
Pharmaceuticals biotransformation
Substrate inhibition
Immersed hollow fiber membrane bioreactor
Pseudomonas putida

ABSTRACT

An immersed hollow fiber membrane bioreactor (IHFMB) was fabricated for production of pharmaceutical intermediates *cis*-indandiol from indene by *Pseudomonas putida* ATCC 55687. At 2–6 g/L indene, significant increase of 160–400%, corresponding to 240–400 mg/L of *cis*-indandiol was achieved as compared to that obtained from suspension culture bioreactors (SCB). The IHFMB volumetric productivity of 10.1–12.9 mg/(L h) and molar yield of 0.050–0.093 mol/mol indene was also higher than the 4.3–8.3 mg/(L h) and 0.01–0.030 mol/mol indene, respectively, in the SCB. The hollow fiber membranes in the IHFMB facilitated a more effective biotransformation through reactant sequestration and cell immobilization, with equal contribution at low indene concentrations, albeit a lower contribution by the latter at 6 g/L indene. The performance of the IHFMB was further improved by process optimization and the highest product titer of 487 mg/L, overall volumetric productivity of 24.6 mg/(L h) and molar yield of 0.126 mol/mol indene were achieved with a module comprising 28 membranes (168 cm^2/150 mL working volume) at a medium circulation rate of 5 mL/min and aeration rate of 0.5 vvm. Reusability of the membranes has also been successfully demonstrated through 5 successive operations of batch biotransformation.

1. Introduction

Green chemistry, through bio-mediated reactions, is the future of sustainable pharmaceutical manufacturing. Given that regio- and stereo-selective hydroxylation and oxygenation of alicyclic compounds yield many important and interesting pharmaceutical intermediates and compounds, research in these biotransformation reactions using microorganisms (so-called whole cell-based biotransformation) has become very exciting in recent years [1–5].

The bio-oxidation of indene to *cis*-indandiol, an important intermediate in the synthesis of CRIXIVAN (indinavir sulfate, a potential HIV-1 protease inhibitor), by *Pseudomonas putida* 421-5 (ATCC 55687) is one example [6]. Indene is asymmetrically converted, in the absence of toluene, to *cis*-indandiol with greater stereospecificity than is possible through traditional chemical synthesis. Biohydroxylation of pyrrolidine using *Sphingomonas* sp. HXN 200 to various pharmaceutical intermediates is another example [7,8]. While it is been found that such biotransformation reactions are propitious with regards to efficiency and yields, their large-scale applications are limited by substrate and product inhibitions experienced by the microorganisms. Low *cis*-indandiol titer and yield have been reported due to substrate and product inhibitions, as well as significant accumulation of inhibitory by-products such as 1-indenol and 1-indanone [9]. For the case of biohydroxylation, Li et al. [8] reported that the yield (close to 80%) and activity (in some cases up to 24 U/g dcw) of the hydrolyzed products was found to be high only at low substrate concentrations of up to 3 mM. At higher substrate concentrations, severe substrate and product inhibitions set in. As a result, bioactivity is compromised with a concomitant decrease in reaction rates and overall yields. It is noteworthy that substrate and product inhibitions were also experienced in many other processes such as microbial fermentation and degradation of toxic compounds by microorganisms [10–13].

Liquid–liquid two-phase partitioning bioreactor (TPPB) using silicone oil as a secondary phase was a promising avenue to control sparingly water soluble toxic reactants such as indene [6]. At the same time, inhibitory products could also be removed to some extent by the secondary phase and it was therefore considered an

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http://dx.doi.org/10.1016/j.bej.2014.03.011
1369-703X/

in situ product removal (ISPR) process. However, the use of silicone oil or soybean oil resulted in emulsification problems and complicated downstream processing; cell migration into the oil phase also resulted in decreased nutrient availability and lower productivity [14]. Recent studies have shown that enhanced biosynthesis of *cis*-indandiol could be accomplished using a solid–liquid TPPB that permitted simultaneous in situ substrate addition (ISSA) and ISPR. The emulsion problem was avoided by using adsorbent polymer beads to replace the immiscible secondary phase as the sequestering phase [15]. The use of a large number of solid polymer beads in the TPPB, however, may interfere with the biotransformation process in addition to increasing the operating cost and energy consumption associated with agitating the more complex solid–liquid–gas multiphase system.

Another technique that has been shown to overcome substrate/product inhibitions was cell immobilization. Here, diffusion limitations minimized direct exposure of the immobilized cells to high, inhibitory substrate/product concentrations. Operation of immobilized-cell hollow fiber membrane bioreactors, in which cells were entrapped in the porous regions, has been successfully demonstrated for phenol wastewater treatment [16–18].

The objective of this research was to develop an immersed hollow fiber membrane bioreactor (IHFMB) to effect high efficiency whole cell-based biotransformation reactions for pharmaceuticals production. This study endeavors to test the concept of an IHFMB, in which the membranes served not only as a matrix for cell immobilization but also as a secondary phase for sequestering the toxic substrate. This was demonstrated using the model system of biosynthesis of *cis*-indandiol from indene using *P. putida* ATCC 55687. To examine the mechanism in action, the IHFMB operation was studied by employing 2 different types of hollow fiber membranes: cellulose acetate (CA) and polyvinylidene fluoride (PVDF). Optimization of the IHFMB operation was then investigated by varying the number of membranes in the module, medium circulation and aeration rates. Finally, the repeated use of the developed IHFMB was demonstrated through successive operations of biotransformation using the same membrane module.

2. Experimental

2.1. Microorganisms, media and membrane

P. putida 421-5 (ATCC 55687) was obtained from ATCC and maintained in Tryptic Soy Agar (TSA) slants. Cell inoculum was raised in 500 mL conical flasks at a working volume of 150 mL Tryptic Soy Broth (TSB) medium, which had been autoclaved at 121 °C for 20 min. After inoculating with one loop of *P. putida* ATCC 55687 cultures from TSA agar slants, the cells were incubated in an orbital shaking water bath at 200 rpm and 30 °C. Exponentially growing cultures were then used as inoculum for the biotransformation experiments except for those in the IHFMB.

Unless otherwise specified, all chemicals were purchased from Sigma–Aldrich or Merck. Indene used for analytical determination was 98% pure, while that used for bioconversions was >90% pure. *Cis*-indandiol was purchased from Wako Chemicals, USA and used as analytical standards. TSB and LB medium were purchased from BD Biosciences, USA. N-methyl-2-pyrrolidinone (NMP), used for hollow fiber membranes spinning, was purchased from Merck. Cellulose acetate (CA-389-30) was purchased from Eastman Chemical Company, USA.

Polyvinylidene fluoride (PVDF) hollow fiber membrane was purchased from Xiamen Kymem Technology Co., Ltd, People's Republic of China. The PVDF membrane used has an outer diameter of 2.2 mm and has a polyester inner support layer.

Table 1
Spinning conditions for cellulose acetate (CA) hollow fiber membranes.

	Spin conditions
Spinning solution	CA/NMP
Polymer concentration (wt%)	13
Dope solution flow rate (mL/min)	6
Bore fluid flow rate (mL/min)	1.5
Length of air gap (cm)	0
External coagulant	Water
Internal coagulant	90% NMP
Speed of take up roller (rpm)	4.6
Spinneret ID/OD (mm)	0.8/1.2

2.2. Membrane spinning and module fabrication

Cellulose acetate (CA) hollow fiber membranes were prepared based on the spinning process described by Chung et al. [16]. Table 1 shows the wet spinning conditions employed. All polymers were first dried in a vacuum oven at 120 °C overnight to remove moisture, and then dissolved in NMP. The solutions were stirred over 12 h and degassed for 2 days before spinning. Water and 90% NMP served as external and internal coagulants, respectively. After extruding from the spinneret, the nascent fibers directly entered the coagulation bath filled with tap water, and finally wound on a take-up roller. All the spinning experiments were finished at room temperature (25 °C). After formation of the hollow fibers, they were kept in a water bath for at least 2 days and then freeze dried for at least 24 h. The resulting hollow fiber membranes had inner diameter of 0.8 mm and outer diameter of 1.2 mm.

The hollow fiber membrane modules used for biotransformation experiments were fabricated by bunching a fixed number of the hollow fibers (each 16 cm in length) arranged into a cluster. The ends of the membrane module were fixed into short, glass or polypropylene tubing with quick-drying Araldite epoxy adhesive resin. The modules were then placed in an oven at 60 °C for several hours to harden the epoxy, after which the modules were ready for use.

2.3. Indene biotransformation

2.3.1. Suspension cultures

In order to establish baseline studies, *P. putida* ATCC 55687 was grown in suspension cultures in 500 mL conical flasks at a working volume of 150 mL at 30 °C. Fig. 1A is a schematic of the suspension culture bioreactor (SCB) setup. Purified air was filtered through an autoclaved Millex vent filter (Millipore Corp., USA) and then passed into a sealed water bottle. The saturated air was then passed into the inoculated cell culture (inoculum at 5.0 A.U. inoculated at 2.5%) at a flow rate of 0.5 vvm (volume of air per volume of liquid per minute). The cells were grown in MMG medium [6] prepared as follows (per L): 20.9 g MOPS buffer, 1.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 0.4 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeSO_4{\cdot}7H_2O$, and 2.5 mL trace elements solution. Twenty gram of glucose was provided as carbon source, and the final pH was adjusted to 7.2 with 2 M NaOH. The trace elements solution consisted of (per L): 300 mg H_3BO_3, 50 mg $ZnCl_2$, 30 mg $MnCl_2{\cdot}4H_2O$, 200 mg $CoCl_2$, 10 mg $CuCl_2{\cdot}2H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$, and 30 mg $Na_2MoO_4{\cdot}2H_2O$. For the biosynthesis of *cis*-indandiol, a predetermined amount of indene (ranging from 2 g/L to 6 g/L) was added to the culture flask as reactant. Samples (1 mL) were collected at predetermined time points for analysis of cell density and product concentration. Unless otherwise indicated, all experiments were performed in triplicates to ascertain reproducibility.

2.3.2. IHFMB biotransformation

Fig. 1B shows a schematic of the IHFMB setup. The experimental setup was similar to the SCB except for the immersion of a hollow