Biosurfactant Production by *Pseudomonas aeruginosa* BN10 Cells Entrapped in Cryogels

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Production of a rhamnolipid biosurfactant by cells of *Pseudomonas aeruginosa* strain BN10 immobilized into poly(ethylene oxide) (PEO) and polyacrylamide (PAAm) cryogels was investigated under semicontinuous shake flask conditions and compared to biosurfactant secretion by free cells. The biosurfactant synthesis was followed over 9 cycles of operation of the immobilized system, each cycle comprising 7 days at ambient temperature and neutral pH. Type and quantity of the carrier were optimized for the rhamnolipid production. The highest rhamnolipid yield of 4.6 g l^{-1} was obtained in the 6th cycle for the immobilized system with 3 g PEO compared to 4.2 g l^{-1} obtained for the free cells, thus immobilization provided physiological stability of the cells. Scanning electron microscopy revealed preservation of the cell shape and regular distribution of the cells under the matrix surface. The polymer matrices possessed chemical and biological stability and very good physico-mechanical characteristics which are a prerequisite for a high life span of these materials for the production of rhamnolipids.

Key words: Pseudomonas aeruginosa, Biosurfactant Production, Cell Immobilization, Cryogel

Introduction

Naturally occurring surface-active compounds (biosurfactants) are produced by a wide variety of bacteria and fungi (Desai and Banat, 1997). They are amphipathic molecules with a hydrophilic and a hydrophobic domain that can accumulate at interfaces, form micelles, lower the surface tension, and thereby enhance the solubility of poorly soluble compounds. A wide spectrum of microbial compounds, including glycolipids, lipopeptides, and fatty acids, have been found to possess surface activity (Morikawa *et al*., 2000). Compared to synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, and better environmental compatibility (Karanth *et al*., 1999).

Glycolipids are the most common low-molecular weight biosurfactants, and among them rhamnolipids are one of the best-studied structural subclasses. Rhamnolipids are produced predominantly by various *Pseudomonas* species as mixtures of homologous species containing L-rhamnose and 3-hydroxy fatty acids (Lang and Wullbrandt, 1999). They are produced in high yields as compared to other biosurfactants and cause a remarkable reduction of the surface tension of water from 72 to 30–32 mN m^{-1} (Mata-Sandoval *et al*., 1999). Rhamnolipids also display high emulsifying activity with a variety of hydrocarbons and vegetable oils (Abalos *et al*., 2001). These properties make rhamnolipid surfactants of potential use in several industrial applications such as petrochemical, food, and pharmaceutical industries, as well as in the bioremediation of pollutants (Lang and Wullbrandt, 1999; Maier and Soberon-Chavez, 2000; Mulligan, 2005).

At present, biosurfactants are unable to compete with synthetic surfactants due to their high production costs, and their use is much restricted. There are several approaches to reach a low-cost product that is easy to obtain and apply: (1) use of cheap and waste substrates, (2) development of overproducing strains, and (3) development of efficient fermentative bioprocesses.

An effective immobilization procedure could help to achieve this goal. In the past decade, considerable interest has focused on the immobilization of various cells and biomolecules into supermacroporous polymer cryogels. These materials

are attractive for many applications, especially in biomedicine and biotechnology (Lozinsky, 2002; Lozinsky *et al.*, 2003). In particular, the synthesis of cryogels via UV irradiation appears to be a very fast and efficient approach which can be set up at low cost (Doycheva *et al.*, 2004; Petrov *et al.* 2006, 2007). This method allows preparation of various biocompatible, biodegradable, and/or thermosensitive supermacroporous cryogels by irradiation of moderately frozen aqueous systems with UV light (irradiation dose rate of 950 J m^{-2} s^{-1}) for 2–5 minutes in the presence of a photoinitiator. Such materials have been exploited for immobilization of yeast cells (Velickova *et al.*, 2010), bacteria capable to degrade xenobiotics (Satchanska *et al.*, 2009; Topalova *et al.*, 2011), fibroblast cells (Petrov *et al.*, 2011a), enzymes (Petrov *et al.*, 2011b, 2012), and drugs (Petrov *et al.*, 2010; Kostova *et al.*, 2011).

Optimization of the biosurfactant synthesis using cells of *Pseudomonas aeruginosa* strain BN10 immobilized into poly(ethylene oxide) (PEO) and polyacrylamide (PAAm) cryogels was the goal of this study. The polymer matrices were proven to possess chemical and biological stability and very good physico-mechanical characteristics which are a prerequisite for a high life span of these materials for the production of rhamnolipids.

Material and Methods

Microorganism and cultivation

Pseudomonas aeruginosa strain BN10 (Christova *et al.*, 2011) was maintained on nutrient agar slants (Difco Laboratories, Detroit, MI, USA) at 4 °C. Starting cultures were prepared by transferring bacterial cells from the storage culture to 250-ml flasks containing 50 ml of nutrient broth and incubation at 30 °C and 150 rpm on a rotary shaker. For biosurfactant synthesis, 1 ml of a mid-exponential phase culture was inoculated into an 1-l flask containing 200 ml of mineral salt medium with the following composition (g l^{-1}): $K_2HPO_4 \cdot 3H_2O$ (7.0); KH_2PO_4 (3.0); $(NH_4)_2SO_4$ (1.0); $MgSO_4 \cdot 7H_2O$ (0.2). The pH value of the medium was adjusted to 7.0. Cultures were incubated with shaking at 150 rpm at 30 °C for 7 d. The carbon source was glycerol (2% v/v). Growth was monitored by measuring the optical density at 610 nm (OD_{610}).

Detection of biosurfactant production

Two simple preliminary methods were used for detection of biosurfactant production: (1) The surface tension (*ST*) of the supernatant fluid was measured, after centrifugation at 8000 x *g* for 20 min, by the du Noüy ring method using a tensiometer (Krüss, Hamburg, Germany). Before each measurement, the instrument was calibrated against triple distilled water. (2) The emulsifying activity of the culture supernatant was estimated by adding 0.5 ml of sample fluid and 0.5 ml of kerosene to 4.0 ml of distilled water. The tube was vortexed for 10 s, held stationary for 1 min, and then visually examined for turbidity of the stable emulsion.

The orcinol assay (Chandrasekaran and Bemiller, 1980) was used for direct assessment of the amount of glycolipids in the sample. Rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (*RE*) in mg ml^{-1}.

In situ immobilization into polymer cryogels

Cells were harvested by centrifugation at 8000 x *g* and resuspended in phosphate buffer (0.06 M, pH 7.0 at 20 °C) to obtain a cell density of $65 \cdot 10^9$ g^{-1} carrier material. Then 0.12 g of 10^6 g mol^{-1} poly(ethylene oxide) (PEO; Union Carbide Corporation, Danbury, CT, USA) was added to 5 ml of the cell suspension under stirring at 20 °C and kept overnight to ensure complete dissolution of the polymer. Then, 0.006 g crosslinking agent (*N,N'*-methylene bisacrylamide; Merck, Darmstadt, Germany) and 0.006 g photoinitiator [(4-benzoylbenzyl) trimethylammonium chloride; Sigma-Aldrich, Schnelldorf, Germany], dissolved in 1 ml water, were added, and the obtained mixture was poured into Teflon dishes (portions of 1 ml in 5 dishes with a diameter of 20 mm) forming a 4 mm thick layer. The samples were frozen at –20 °C for 2 h and irradiated with the full spectrum of UV-Vis light from a 400-W metal halide flood lamp (Dymax 5000-EC; Dymax Corporation, Torrington, CT, USA) for 5 min at a dose of $2.85 \cdot 10^5$ J m^{-2} and an input power of 930 W m^{-2}.

The cells were immobilized in polyacrylamide (PAAm) cryogels following the same procedure, except that 0.3 g of acrylamide (Merck) and 0.03 g of *N,N'*-methylene bisacrylamide were dissolved in 1 ml water and mixed with 5 ml cell suspen-

sion. Finally, 0.075 ml initiator (30 vol.-% H_2O_2; Merck) were added prior to freezing.

The diameter of each matrix disc was 1.6–1.7 cm and its weight was 1.0–1.1 g for the PAAm and PEO carriers, respectively.

Results

Influence of carrier type and quantity on biosurfactant production

We reported previously that free cells of *Pseudomonas aeruginosa* strain BN10, when grown in a mineral salt medium with different carbon sources, accumulate surface-active rhamnolipids (Christova *et al*., 2011). Cells of this strain were immobilized *in situ* into the biocompatible PEO and PAAm cryogels. First, the cells were mixed with the reagents in aqueous media and then the polymer network was formed by UV light-induced crosslinking in the frozen state. According to the accepted mechanism (Lozinsky, 2002), during freezing the major portion of water forms large polycrystals, and cells, polymer (monomer), photoinitiator, crosslinking agent, and bound water (non-freezable solvent) accumulate in a non-frozen liquid microphase where the formation of network takes place. After thawing, the cryogels consisted of smooth polymer walls, resulting from the microphase, which were surrounded by interconnected pores, filled mainly with free water from the melted ice crystals (Fig. 1a). It has been established (Velickova *et al*., 2010) that the immobilization procedure employed in the present study preserves the cells' viability and biosynthetic capability. Considering both the thickness of cryogel walls (2–3 μm) and the size of the cells (0.5–0.7 μm), cells can be considered partly embedded in the polymer matrix (Fig. 1b). Moreover, scanning electron microscopy studies confirmed that the cells preserved their shape and their regular distribution beneath the matrix surface after many cycles of use.

The matrix type and its quantity influenced the obtained rhamnolipid concentration in the case of both matrices (Fig. 2). The use of three discs (3 g) of the matrix with immobilized bacterial cells resulted in the highest biosurfactant yield (4.6 g l^{-1} in the 6th cycle of operation). With two discs, 4.3 g l^{-1} were obtained, and with a single disc only a considerably reduced concentration of 2.6 g l^{-1} was achieved. The PEO matrix appeared to be more suitable. The observed increase in the rhamnolipid production in the medium with a larger quantity of the carrier may have resulted in an increase in the diffusion surface and consequently in a better oxygen and nutrient supply. The immobilized preparations had a high operational stability, and production of the biosurfactant was maintained for 9 cycles of operation, each comprising 7 days. When repeated batch experiments with free cells were performed, a rapid decrease in the biosurfactant production was observed in the 3rd cycle (data not shown).

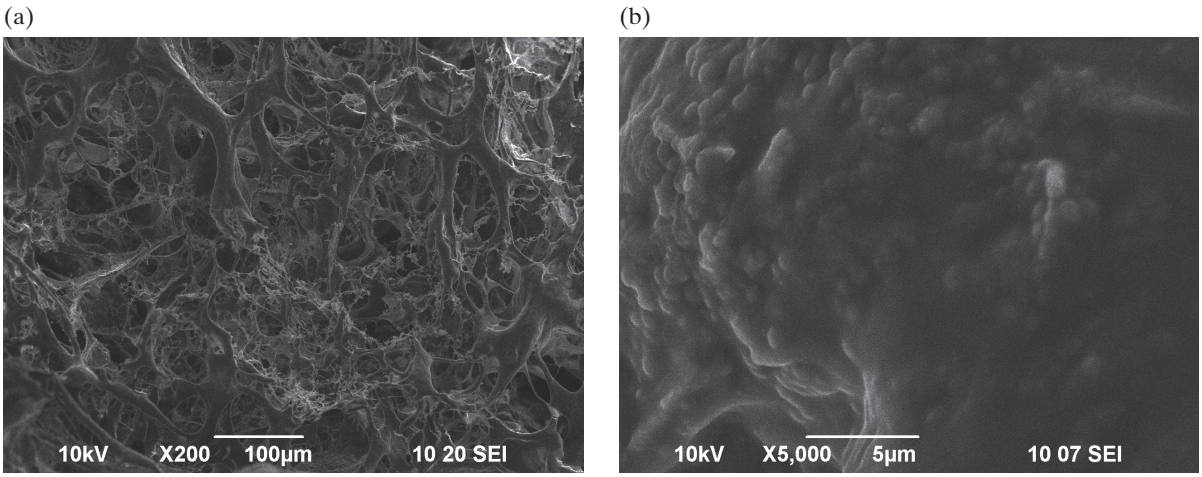

Fig. 1. Scanning electron micrographs: (a) matrix surface; (b) cells entrapped in the PEO matrix.

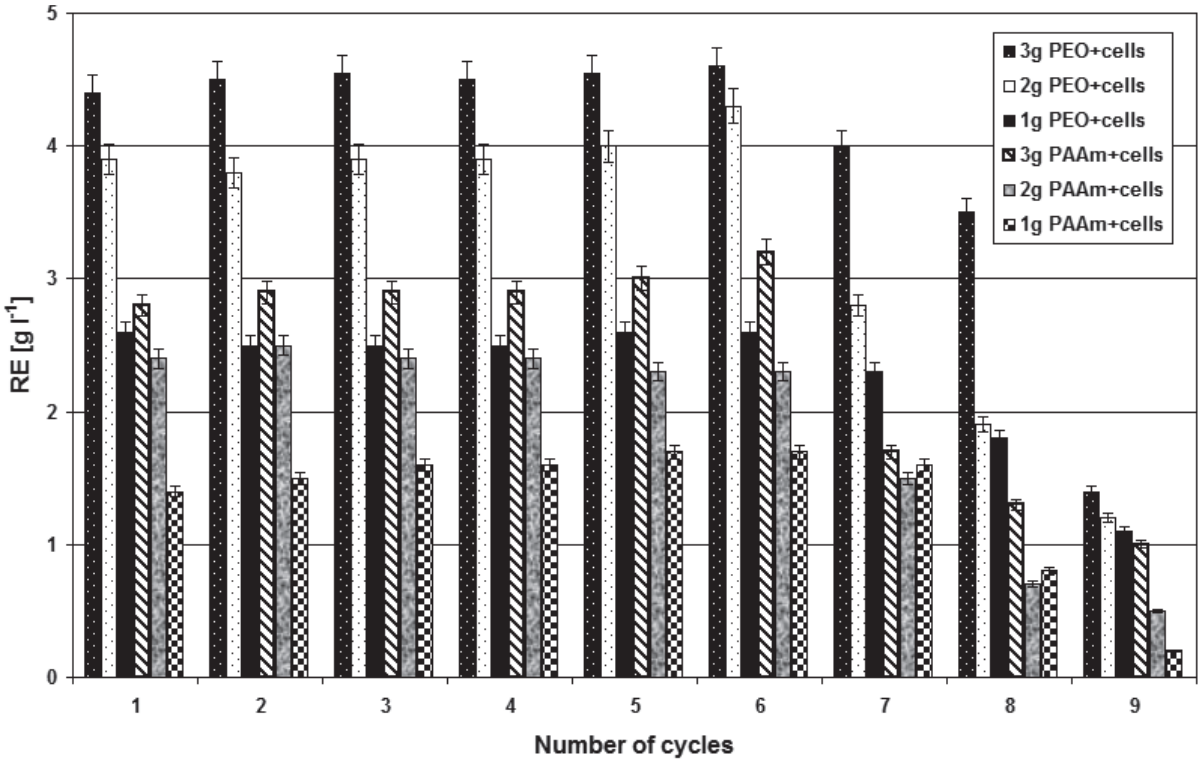

Fig. 2. Repeated batch cycles of rhamnolipid production by cells immobilized in PEO and PAAm matrices.

Kinetics of biosurfactant production

The kinetics of the biosurfactant production by free and immobilized cells were followed in 7-day batch cultures under optimal conditions. During growth of the bacterium in the presence of 2% glycerol, the surface tension of the medium decreased from 70 mN m^{-1} to 27.5 mN m^{-1} in the middle of the exponential growth phase, *i.e.* after 24 h of cultivation (Fig. 3). The rapid drop of the surface tension was accompanied by the formation of stable emulsions of the cell-free culture broth with kerosene, both parameters indicating biosurfactant production. The rhamnolipid production was low during the initial 24 h, after which it increased drastically reaching 4.2 g l^{-1} after 72 h. High levels of rhamnolipids were observed in the late stationary phase suggesting that the biosurfactant was produced as a secondary metabolite.

In the case of cells immobilized in the PEO matrix, the surface tension of the medium declined after about 36 h of cultivation, and its lowest value was 29.0 mN m^{-1} (Fig. 4). Reduction of the surface tension and formation of stable emulsions indicated biosurfactant production. Although the maximum amount of biosurfactant was achieved after only 96 h of culture, it exceeded that obtained with free cells. The observed delay in biosurfactant production could be due to some mass transfer limitations. On the other hand, the optimal production of rhamnolipids (4.6 g l^{-1}) clearly showed that the immobilization procedure led to greater physiological stability of the cells as reported by other authors (Abouseoud *et al*., 2008).

Discussion

An effective immobilization technology with appropriate carriers could lead to enhanced production of the desired product. Immobilization of living cells releasing biosurfactants into the supermacroporous supports offers enormous advantages for their continuous production. Several attempts have been made with this purpose in mind. Thus, Wilson and Bradley (1996) used a suspension of free cells and immobilized cells of *Pseudomonas fluorescence* on a commercial bio-

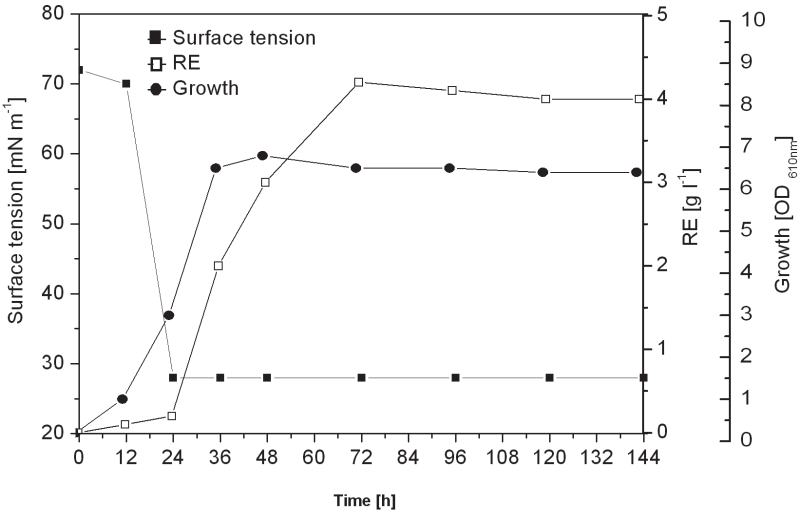

Fig. 3. Kinetics of biosurfactant production by free *Pseudomonas aeruginosa* BN10 cells.

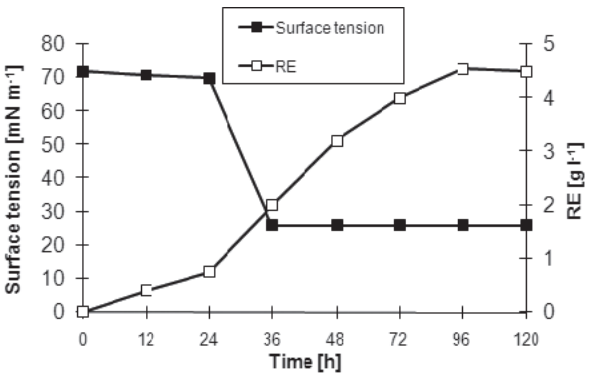

Fig. 4. Kinetics of biosurfactant production by cells immobilized in a PEO matrix.

support as bioremediation agents in an aqueous system with petrol as the carbon source. Data from this study showed that immobilization resulted in enhanced rhamnolipid production and increased the contact between the cells and hydrocarbon droplets. It was also demonstrated that the entrapment of the marine bacterium *Pseudomonas aeruginosa* BYK-2 in 10% poly(vinyl alcohol) beads yielded 0.1 g rhamnolipid per hour in an airlift bioreactor (Jeong *et al.*, 2004). Furthermore, the capacity for rhamnolipid production by both free and alginate-entrapped cells of *Pseudomonas fluorescence* was investigated in batch cultures, and it was confirmed that immobilization increased the biosurfactant recovery (Abouseoud *et al.*, 2008). Heydl *et al.* (2011) reported a new integrated process for continuous rhamnolipid production by *Pseudomonas aeruginosa* DSM 2874 entrapped in magnetic alginate beads, reaching a final amount of 70 g rhamnolipid after four production cycles in a 10-l bioreactor.

To the best of our knowledge, the entrapment of *Pseudomonas aeruginosa* BN10 in cryogels has been carried out for the first time in this study and proven to be a non-toxic, inexpensive, rapid, and versatile tool for rhamnolipid yield enhancement, with easy manipulation of the immobilized system. Thus, environmental and economic issues have motivated the implementation of this task. The yield of rhamnolipids in the immobilized system exceeded that of the free bacterial cells, distinguishing an effective bioprocess.

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