Enhanced biosurfactant synthesis by cryogel entrapped bacteria

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INTRODUCTION AND OBJECTIVES

A wide spectrum of microbial substances, including glycolipids, lipopeptides and fatty acids possess surface activity (Morikawa 2000). Surface-active compounds 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. Biosurfactants have several advantages over synthetic surfactants for potential industrial applications. The most important advantage is their ecological acceptance, owing to their low toxicity and biodegradable nature (Prieto 2008). Some of these biosurfactants have been investigated for their ability to act as biologically active compounds for pharmaceuticals (Yilmaz 2005). Rhamnolipids also display high emulsifying activity with a variety of hydrocarbons and vegetable oils. Microorganisms used for their synthesis and used in industrial processes are selected to provide the best possible combination of characteristics and conditions for best performance of the specific process. An effective immobilization technology involving the right microorganism with appropriate carriers could lead to enhanced production of the desired product. Immobilization of various cells and biomolecules into super macroporous polymer cryogels has attracted much attention in the recent years because they can be used in many areas, especially in biomedicine and biotechnology (Lozinsky 2003).

MATERIALS AND METHODS

The bacterial cells for immobilization were harvested by centrifugation at 8000 x *g* and re-suspended in phosphate buffer (0.06 M, pH 7.0 at 20 °C) to obtain a cell density of 65 · 10^9 g $^{-1}$. Then 0.12 g of poly(ethylene oxide) (PEO-Union Carbide Corporation) of $1 \cdot 10^6$ g mol^{-1} 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 cross-linking agent (*N,N'*-methylene bisacrylamide) and 0.006 g photoinitiator [(4-benzoylbenzyl) trimethylammonium chloride-Sigma-Aldrich], dissolved in 1 mL water, were introduced and the obtained mixture was poured into Teflon dishes of 1 mL, 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) for 5 min at a dose of 2.85 .10^5 J m^{-2} and an input power of 930 W m^{-2}.

Pseudomonas aeruginosa strain BN10 was maintained on nutrient agar slants (Difco) at 4 °C . Cells from the storage culture were put into 250-mL flasks containing 50 mL of nutrient broth and incubated at 30 °C and 150 rpm on a rotary shaker. For biosurfactant synthesis, 1 mL of a mid-exponential phase culture was inoculated into a 1-L flask containing 200 mL of mineral salt medium with 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 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. For the 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). (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. For direct assessment of the amount of glycolipids in the sample, the orcinol assay (Chandrasekaran 1980) was used. Rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE) in mg mL^{-1}.

RESULTS AND DISCUSSION

The production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* free and entrapped in Poly(ethyleneoxide) cryogel cells was followed. The immobilized system showed better performance in the rhamnolipid production. The highest biosynthetic activity and biosurfactant yield was reached by the cryogel entrapped bacteria - 4.6g L^{-1}, while the free cells synthesized 4.0g L^{-1} of rhamnolipid.

The kinetics of biosurfactant production by free and immobilized cells was 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. 1a). The rapid drop of the ST 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 significantly reaching 4.0 g L^{-1} at 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 cryogel matrix, the ST of the medium declined after about 36h of cultivation, and its lowest value was at the 48h (Fig. 1b). Reduction of the ST and formation of stable emulsions indicated biosurfactant production. Although the maximum amount of biosurfactant was achieved after 96h, it exceeded that obtained with the 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 a greater physiological stability of the cells as reported by other authors (Chukwudi 2013).

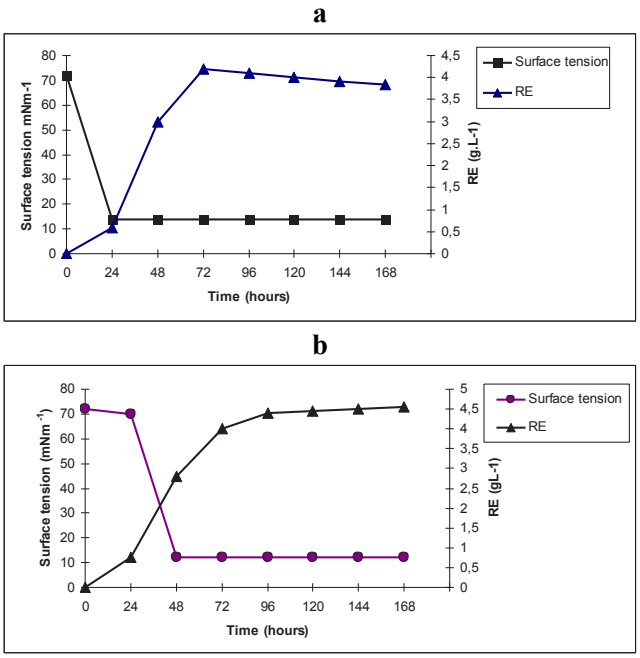

Fig. 1 Kinetics of biosurfactant production by free (a) and entrapped (b) *Pseudomonas aeruginosa*

During the experiment the bacterial cells were mixed with the reagents in aqueous media and then the polymer network was formed by UV-induced crosslinking in the frozen state. 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. 2a). 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. The scanning Electron Microscopy observations provided the proof that immobilization lead to physiological stability of the cells, which shape was well preserved and they were regularly distributed under the matrix surface (Fig. 2 b). Estimating their biosynthetic capability, we prove that their viability is kept for a long period.

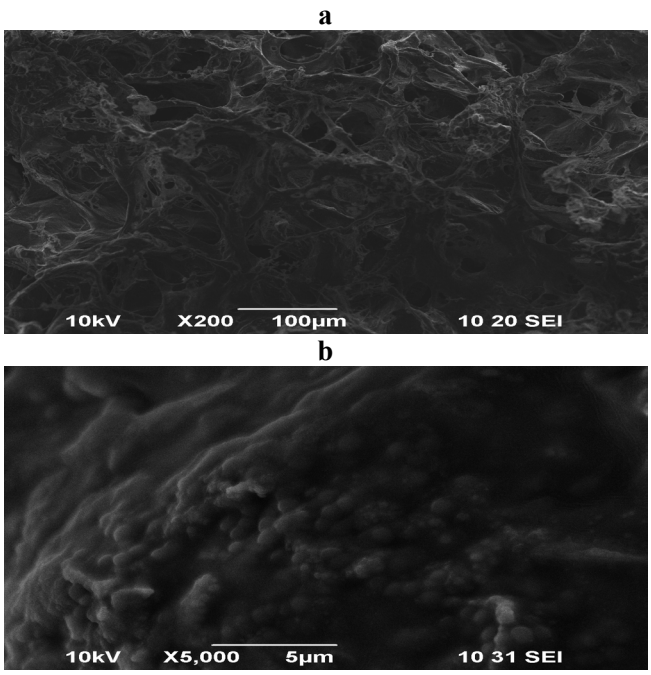

Fig. 2 SEM observations: (a) PEO matrix; (b) cells entrapped in the PEO matrix.

The immobilized system was tested for its operational stability for a period of 84 days (12 cycles), revealing its long-term stability. Performing the fermentation with free cells, a significant loss of activity was registered after the 9th cycle of operation (data not shown).

CONCLUSIONS

Entrapment in a nontoxic and inexpensive matrix and performing a rapid and versatile procedure for rhamnolipid yield enhancement was successfully accomplished. The yield of rhamnolipids obtained by the immobilized system exceeded that of the free bacterial cells, distinguishing an effective bioprocess.

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