

## Biosurfactant Production by a New *Pseudomonas putida* Strain

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Observation of both tensio-active and emulsifying activities indicated that biosurfactants were produced by the newly isolated and promising strain *Pseudomonas putida* 21BN. The biosurfactants were identified as rhamnolipids, the amphiphilic surface-active glycolipids usually secreted by *Pseudomonas* spp. Their production was observed when the strain was grown on soluble substrates, such as glucose or on poorly soluble substrates, such as hexadecane, reaching values of  $1.2 \text{ g l}^{-1}$ . When grown on hexadecane as the sole carbon source the biosurfactant lowered the surface tension of the medium to  $29 \text{ mN m}^{-1}$  and formed stable and compact emulsions with emulsifying activity of 69%.

### Introduction

Biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous-hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms (Gerson, 1993). Among the best studied biosurfactants are rhamnolipids that belong to the glycolipid class. Rhamnolipids have been identified predominantly from *Pseudomonas aeruginosa* (Burger *et al.*, 1963; Zhang and Miller, 1995; Beal and Betts, 2000).

We show here that the newly isolated strain *Pseudomonas putida* 21BN produces a surfactant which substantially changes the surface tension of the culture medium when grown on hexadecane.

### Materials and Methods

#### Microorganisms

Hydrocarbon-utilizing microorganisms were isolated at this laboratory from industrial waste water samples. Isolates were plated on mineral salt agar containing 2% hexadecane as the sole carbon source. To confirm their ability to grow on hydrocarbons, single colonies obtained were transferred into 100 ml Erlenmeyer flasks containing 10 ml liquid minimal salt medium supplemented with 2% hexadecane or n-paraffins and kerosene, respectively, and cultivated at 28 °C and

130 rpm. The isolates were maintained on Nutrient Broth (NB) agar (Merck) slants at 4 °C and subcultures were made every 2 weeks. Inocula were prepared by growing cells at 28 °C for 18 h in NB in an orbital incubator at 130 rpm.

#### Morphological and physiological characterization of isolates

Isolates were examined at different time for Gram reaction and cell morphology. The Vitek system (bioMerieux, Montalieu-Vercieu, France) was used for isolate characterization depending on the results of morphological identification. Other biochemical tests were performed following directions of the latest edition of Bergeys Manual (Holt *et al.*, 1994).

#### Growth conditions

The composition of the basal mineral salt medium (MS) used in this study was the following ( $\text{g l}^{-1}$ ):  $\text{K}_2\text{HPO}_4$ , 3  $\text{H}_2\text{O}$ , 4.8;  $\text{KH}_2\text{PO}_4$ , 1.5;  $(\text{NH}_4)_2\text{SO}_4$ , 1.0;  $\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7)$ , 2  $\text{H}_2\text{O}$ , 0.5;  $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2; yeast extract, 0.1. For biosurfactant production the medium (BMS) was supplemented with trace elements solutions with the following composition ( $\text{mg l}^{-1}$ ):  $\text{CaCl}_2$ , 2  $\text{H}_2\text{O}$ , 2.0;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.4;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.4;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.2;  $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$ , 0.2; and 2% hexadecane or 2% glucose as sole carbon

source, pH 7.2. Hexadecane was sterilized through 0.2  $\mu\text{m}$  membrane filters (Milipore Corp., Bedford, Mass.). Growth was monitored by measuring the  $A_{600}$ .

#### *Detection for biosurfactant activity*

Samples of the culture media of each selected strain were centrifuged at  $8\,000\times g$  for 20 min. Surface tension (ST) of the supernatant fluid of the culture was measured by the Wilhelmy method with filter paper as a sensing element on a surface tensiometer model 5000 (Advanced Technologies Ltd., Sofia, Bulgaria). Prior to the measurements calibration was done against clean water. 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 a stable emulsion. Emulsification power was measured by vortexing equal volumes of the centrifuged culture with kerosene for 1 min and determining the percentage of volume occupied by the emulsion. The mixture was allowed to settle for 24 h and the height of the emulsion was measured.

Blue agar plates containing cetyltrimethylammonium bromide (CTAB) ( $0.2\text{ mg ml}^{-1}$ ; Sigma Chemical Co., Poole, UK) and methylene blue ( $5\text{ mg ml}^{-1}$ ) were used to detect extracellular glycolipid production (Siegmund and Wagner, 1991). Biosurfactants were observed by the formation of dark blue halos around the colonies.

#### *Detection and quantification of rhamnolipids*

Three independent tests were used for the detection of rhamnolipids. They included detection of rhamnolipids by thin-layer chromatography (Koch *et al.*, 1988), the hemolysis of erythrocytes by rhamnolipids (Johnson and Boese-Marazzo, 1980) and the growth inhibition of *Bacillus subtilis* by rhamnolipids (Itoch *et al.*, 1971). 10 ml of the culture supernatants (pH 6.5) were concentrated by the addition of  $\text{ZnCl}_2$  to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml sodium phosphate buffer (pH 6.5) and extracted twice with equal volumes of diethyl ether. The pooled organic phases were evaporated to dryness and the pellets dissolved in 100  $\mu\text{ml}$  of methanol. 10  $\mu\text{l}$  of the concentrated culture super-

natants were spotted on paper filter discs (6.0 mm, Whatman AA) and then put onto agar plates containing 5% sheep blood or onto plates with freshly grown on NB agar *B. subtilis* cells ( $10^9\text{ ml}^{-1}$ ). The blood agar plates were incubated at room temperature for 2 days and *B. subtilis* plates were put at  $37\text{ }^\circ\text{C}$  for 1 night and then the zones of hemolysis and growth inhibition were measured.

The orcinol assay (Chandrasekaran and Bemiller, 1980) was used for direct assess of the amount of glycolipids in the sample. Extracellular glycolipids concentration was evaluated in triplicate by measuring the concentration of rhamnose: 333  $\mu\text{ml}$  of the culture supernatant was extracted twice with 1 ml diethyl ether. The ether fractions were evaporated to dryness and 0.5 ml of  $\text{H}_2\text{O}$  was added. To 100  $\mu\text{ml}$  of each sample 900  $\mu\text{ml}$  of a solution containing 0.19% orcinol (in 53%  $\text{H}_2\text{SO}_4$ ) was added. After heating for 30 min at  $80\text{ }^\circ\text{C}$  the samples were cooled at room temperature and the  $\text{OD}_{421}$  was measured. The rhamnolipid concentrations were calculated from a standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (RE)( $\text{mg ml}^{-1}$ ).

#### *Infrared spectra (IR)*

The biosurfactant was extracted from the supernatant fluid (2 ml) with chloroform (2 ml), dried with  $\text{Na}_2\text{SO}_4$  and evaporated on a rotary evaporator. In order to avoid band saturation spectra were obtained with the ATR technique. The IR spectra were recorded on the Bruker IFS113vFTIR-spectrometer, in the  $4\,000\text{--}400\text{ cm}^{-1}$  spectral region at a resolution  $2\text{ cm}^{-1}$ . 100 scans for each spectrum, using a 0.23 mm KBr liquid cell.

#### *Cell surface hydrophobicity test*

The bacterial adhesion to hydrocarbons (BATH) assay was used to determine changes in cell surface hydrophobicity during growth on minimal salt medium with glucose or hexadecane. (Rosenberg *et al.*, 1985).

Bacteria were harvested from growth cultures by centrifugation at  $8000\times g$  for 10 min at  $4\text{ }^\circ\text{C}$ , washed twice, and suspended in PUM buffer (22.2 g  $\text{K}_2\text{HPO}_4\cdot 4\text{H}_2\text{O}$ ; 7.26 g  $\text{KH}_2\text{PO}_4$ ; 1.8 g urea and 0.2 g  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  in 1 l distilled water, pH 7.2) to an initial absorbance at 400 nm to 1.0. Hexadecane (0.5 ml) and cell suspensions (2.0 ml)

were vortexed in a test tube for 2 min and equilibrated for 15 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and the  $A_{400}$  was measured. The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension prior mixing.

## Results and Discussion

### Screening for glycolipid biosurfactant producers

From 14 isolates, 5 bacterial strains were able to grow with hexadecane as the sole carbon source. Two of them decreased the culture medium surface tension below  $35 \text{ mN m}^{-1}$  and formed kerosene-water emulsions. They formed halos on blue agar plates, which detect the production of extracellular glycolipids by *Pseudomonas* spp. (Siegmond and Wagner, 1991). When cultured in liquid BMS medium supplemented with 2% hexadecane, indications of biosurfactant production were seen within 3 to 5 days of incubation, depending on the strain.

The strain which displayed the highest biosurfactant production was selected for a more detailed analyses. It was identified as *Pseudomonas putida* 21BN.

### Detection and quantification of the surface active glycolipids

To directly detect and quantify the surface active glycolipids three independent tests that have been previously used were carried out. These included detection by thin-layer chromatography (Koch *et al.*, 1988), hemolysis of erythrocytes by rhamnolipids (Johnson and Boese-Marazzo, 1980) and growth inhibition of *B. subtilis* by rhamnolipids (Itoch *et al.*, 1971).

In the thin layer analyses the concentrated culture supernatant was applied to a silica gel thin-layer plate and three typical glycolipid spots were revealed after the orcinol-sulfuric staining at  $R_f$  0.78, 0.60 and 0.38. Further identification of the sugar moiety after acidic hydrolysis confirmed it as rhamnose. This result suggests that *Pseudomonas putida* 21BN produces a mixture of rhamnolipids, the amphiphilic surface-active glycolipids usually secreted by *Pseudomonas* spp.

These findings were further analysed using the fact that rhamnolipids possess hemolytic properties. For this purpose  $10 \mu\text{l}$  of 100-fold concentrated culture supernatant with initial concentration of  $1 \text{ mg ml}^{-1}$  glycolipids were spotted on filter paper discs on top of an agar plate containing 5% sheep blood. Fig. 1A shows that the culture supernatant contained abundant amounts of hemolysin as the diameter of the hemolytic zone was 11 mm. The same overall pattern was seen in the *B. subtilis* inhibition test shown in Fig. 1B. A clear growth inhibition zone with a diameter of 36 mm was quantified when the concentrated culture supernatant of *Ps. putida* 21BN was spotted on filter discs on top of an agar plate with freshly grown *B. subtilis* cells.

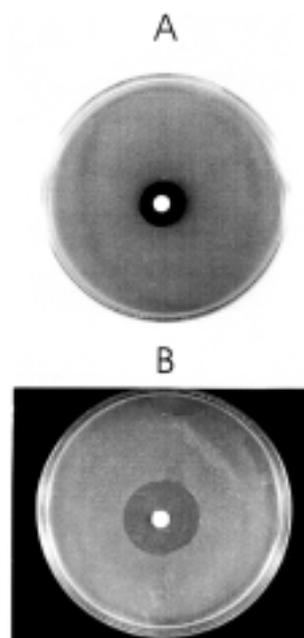


Fig. 1. Quantification of rhamnolipids produced by *Pseudomonas putida* 21BN. (A) Hemolytic activity of a 100-fold concentrated culture fluid. (B) Inhibition of *Bacillus subtilis* by a 100-fold concentrated culture supernatant.

### Infrared spectra analyses

Findings listed above were confirmed also by infrared spectra analyses of the extracts from noninoculated control media and from media inoculated with *Ps. putida* 21BN on the 5 day of cultivation (data not shown). New characteristic bands were found in the IR spectrum of the inoculated culture

fluid. In the region  $3000\text{--}2700\text{ cm}^{-1}$  were observed several C-H stretching bands of  $\text{CH}_2$  and  $\text{CH}_3$  groups. The deformation vibrations at  $1467$  and  $1379\text{ cm}^{-1}$  also confirm the presence of alkyl groups. Carbonyl stretching band was found at  $1745\text{ cm}^{-1}$  which is characteristic for ester compounds. The ester carbonyl group was also proved from the band at  $1250\text{ cm}^{-1}$  which corresponds to C-O deformation vibrations. Lack of characteristic bands for organic acids that usually appear at

$3500\text{--}2700\text{ cm}^{-1}$  and  $1720\text{--}1680\text{ cm}^{-1}$  and  $950\text{--}900\text{ cm}^{-1}$  indicates the presence of an ester compound.

#### Biosurfactant production

Biosurfactant production was observed when the strain was grown on soluble substrates like glucose and glycerol or on poorly soluble substrates as hexadecane. Fig. 2 shows the profile of biosurfactant production obtained when the strain was cultivated in BMS with 2% glucose. Since biosurfactants are secondary metabolites maximal glycolipid production (expressed as rhamnose equivalents) of  $1.2\text{ mg ml}^{-1}$  was reached in the stationary growth phase.

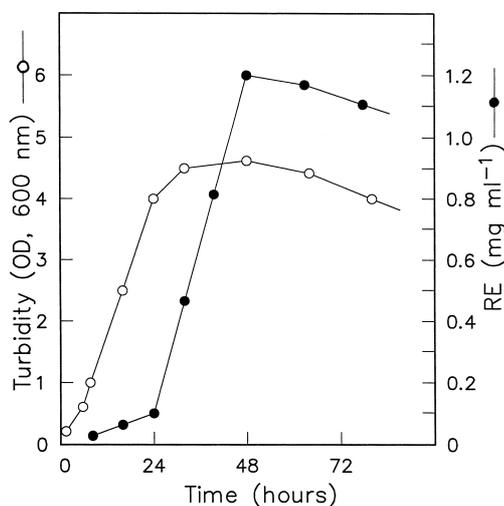


Fig. 2. Production of biosurfactants (glycolipids) by *Ps. putida* 21BN in BMS medium with 2% glucose. Incubation was done at  $28\text{ }^{\circ}\text{C}$  with shaking at 130 rpm. OD, optical density. Biosurfactant levels are expressed as rhamnose equivalents (RE). Values are averages from three cultures.

When grown on hexadecane as the sole carbon source *Ps. putida* 21BN showed similar growth kinetics (Fig. 3A). In the case when the culture was inoculated with an overnight inoculum (on BMS medium with 2% glucose) an adaptation was needed before reaching the stage of maximal surfactant production. This delay in the lag phase was expected since a number of different biochemical

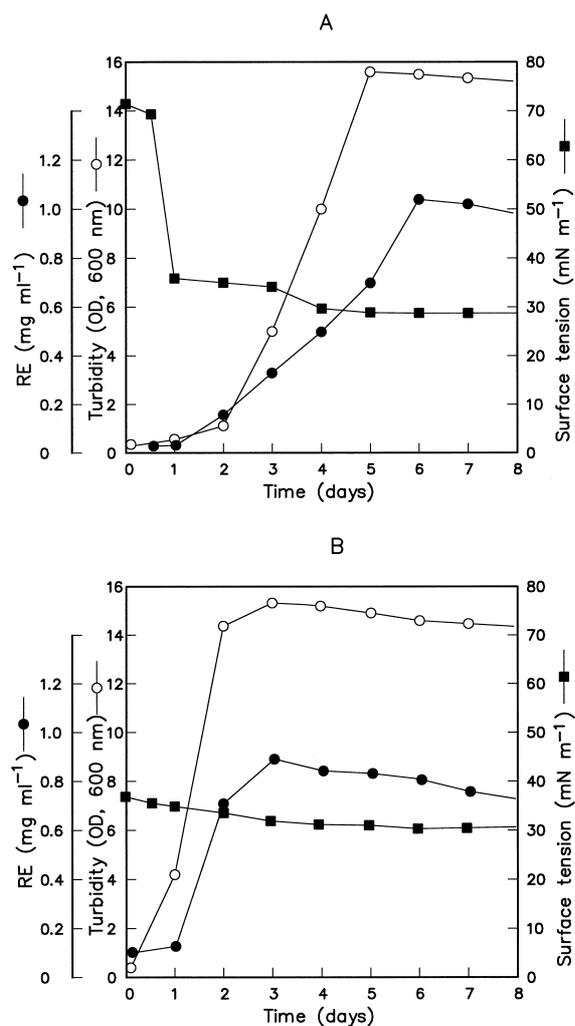


Fig. 3. Production of biosurfactants by *Ps. putida* 21BN grown on BMS medium with 2% hexadecane as substrate. (A) Inoculation with 2 ml from an overnight culture on BMS with 2% glucose. (B) Inoculation with 2 ml from a seven-day-old culture on BMS with 2% hexadecane. Incubation was done at  $28\text{ }^{\circ}\text{C}$  with shaking at 130 rpm. Biosurfactant levels are expressed as rhamnose equivalents (RE). ST, surface tension. Values are averages from three cultures.

reactions are involved in alkane utilization including their terminal hydroxylation and the  $\beta$ -oxydation (Witholt *et al.*, 1990). However, enough rhamnolipids were secreted to cause a drop in the surface tension from 71 to 37 mN m<sup>-1</sup> even after 24 h of incubation. The ST reached a minimum of 29 mN m<sup>-1</sup> in the stationary growth phase and did not decline further on. Stable and compact emulsions of kerosene with the supernatant fluid of the culture were observed after 24 h of cultivation reaching maximal value of 69% at 120h of incubation. Biosurfactant production increased progressively and maximal values of 1.0 mg ml<sup>-1</sup> were reached in the stationary phase again.

Delayed lag phase was avoided when a 7-day-old culture (on BMS medium with 2% hexadecane) was used as inoculum (Fig. 3B). Rhamnolipid production started more rapidly and the ST of the medium started to decrease at 4 h of incubation. The ST decreased further on to 30.6 mN m<sup>-1</sup>, coincidentally with the transition to the stationary growth phase. The shorter lag phase and the very rapid drop of ST may be partially explained by the fact that hexadecane availability for the cells was

enhanced by the concomitant addition of biosurfactants with the inoculum. Moreover, the inoculum culture fluid may have contained diffusible autoinductors which regulate rhamnolipids synthesis in *Ps. aeruginosa* (Ochsner and Reiser, 1995)

Hydrophobicity of the cell surface was tested using the BATH assay. At the beginning of stationary phase hydrophobicity of *Ps. putida* 21BN grown on n-hexadecane was slightly higher (72±2.3%) than when grown on glucose as the carbon source (60±3.2%) and there was not any important change in its values during growth. This suggests that biosurfactant production does not contribute for decreasing or increasing cell surface hydrophobicity.

The exact reason why some microorganisms produce surfactants is unclear. However, biosurfactant-producing bacteria are found in higher concentrations in hydrocarbon contaminated areas (Margesin and Schinner, 2001). These strains represent a valuable source of new compounds with surface-active properties, and potential application for bioremediation.

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