

Improved Production of Rhamnolipids by a *Pseudomonas aeruginosa* Mutant

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ABSTRACT

A *Pseudomonas aeruginosa* mutant derived by random mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, producing high levels of the rhamnolipid biosurfactants was selected on Siegmund-Wagner (SW) plates. The mutant designated *P. aeruginosa* PTCC1637 produces rhamnolipids at concentrations 10 time more than parent strain. NMR analysis and surface tension measurement showed that the biosurfactants produced by the mutant were identical to those produced by the wildtype strain. The biosurfactants exhibited a low surface tension of 28.0 mN m⁻¹ and a low critical micelle concentration of 9 mg l⁻¹. Similar to the wildtype strain, the mutant produced biosurfactants at the stationary phase. *Iran. Biomed. J.* 8 (1): 25-31, 2004

Keywords: Biosurfactant, Rhamnolipid, *Pseudomonas aeruginosa*, Mutagenesis

INTRODUCTION

Biosurfactants are produced on microbial cell surfaces or excreted extracellularly and contain both hydrophilic and hydrophobic moieties. Like chemical surfactants, these compounds can be used in many processes involving emulsification, foaming, detergency, wetting, and dispersing or solubilizing [1].

Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability [2], better environmental compatibility [3], higher foaming [4], high selectivity and specific activity at extreme temperatures, pH, and salinity [5], and the ability to be synthesized from renewable feedstock [1].

Several biosurfactants have high surface activities and low critical micelle concentrations (CMC) and are, therefore, promising substitutes for synthetic surfactants [3]. Biosurfactants are ideal surfactants for environmental applications due to their biodegradability and low CMC [6]. Compared to synthetic compounds, bio-surfactants also offer the advantages of little or no environmental impact and the possibility of *in situ* production. Recent studies have demonstrated the successful use of biosurfactants for facilitating the degradation of

organic pollutants in soil and for the dispersion of oil from oil spills [7].

Although biosurfactants have many interesting properties, their industrial importance is dependent upon ease of production [8]. Low yields of biosurfactant are a major factor jeopardizing its popularity. Recently, efforts have been made to increase yields by focussing on nutritional and environmental factors [9, 10].

Pseudomonas species is well known for its ability to produce rhamnolipid biosurfactants with potential surface active properties when grown on different carbon substrates [11, 12] and therefore is a promising candidate for large scale production of biosurfactants. In 1949, Jarvis and Johnson [13] described this compound. Then, in 1963, a biosynthetic pathway for synthesis of rhamnolipids by sequential glycosyl transfer, has been proposed [14]. In 1994, Ochsner *et al.* [15] isolated and analysed a gene involved in regulation of rhamnolipids biosynthesis.

Four different rhamnolipid homologues, produced by *Pseudomonas aeruginosa*, have been identified and characterized [16, 17] (Fig. 1). The rhamnolipids consist of one or two L-rhamnose units and one or two units of β -hydroxydecanoic acid. RL1 and RL3 are the principal rhamnolipids

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produced. RL2 and RL4 are biosynthesized under certain cultivation conditions only.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) is an effective mutagenic compound that induces mutation by an error-prone DNA repair pathway [18] and is more effective in inducing mutation than UV light. In this study, we described the effects of mutagenesis by NTG on the production of rhamnolipid biosurfactants by *P. aeruginosa* that can be used for enhancing production of biosurfactants.

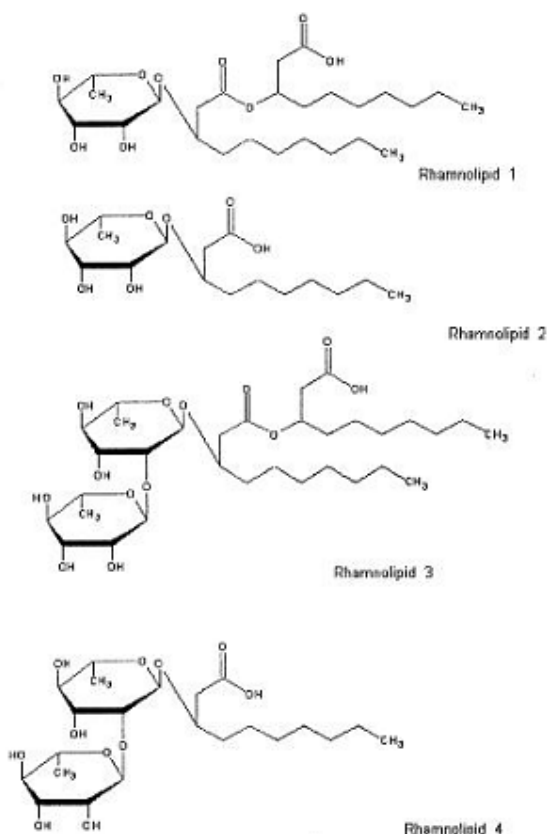


Fig. 1. Structure of Rhamnolipids

MATERIALS AND METHODS

Bacteria and culture conditions. *P. aeruginosa* that used as parent strain was obtained from Persian Type Culture Collection (PTCC). The strain was confirmed by PTCC Identification report No. 1011 as *P. aeruginosa*, therefore is designated as *P. aeruginosa* MM1011.

The parent and mutant strains were maintained on nutrient agar slants at 4°C and subcultured every

two weeks. Every 3 months a new frozen culture used for preparation of slant cultures.

These frozen stocks were prepared by transferring a loopful bacteria from slant to a 250-ml Erlenmeyer flask containing 50 ml of mineral salts medium and 2% (v/v) corn oil as carbon source. After growing on a shaker incubator (200 rpm and 30°C) for 3 days, 30 ml sterile glycerol was added and mixed thoroughly. Then, 2 ml aliquots was dispensed into sterile vials and stored at -70°C. Frozen cultures were recovered by transferring whole vials of thawed culture to 25 ml of sterile mineral salts medium containing 25% (v/v) corn oil as carbon source and incubated on a shaker incubator at 30°C for 3 days.

Random mutagenesis. *P. aeruginosa* MM1011 was grown in 500 ml Erlenmeyer flasks containing 100 ml sterile mineral salts medium and a 6% glucose as carbon source at 200 rpm at 30°C for 12 h. The mineral salts medium used throughout this study was made according to Lindhardt *et al.* [19], contained (g L⁻¹): NaNO₃, 15; KCl, 1.1; NaCl, 1.1; FeSO₄.7H₂O, 0.00028; KH₂PO₄, 3.4; K₂HPO₄, 4.4; MgSO₄.7H₂O, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g L⁻¹): ZnSO₄.7H₂O, 0.29; CaCl₂.4H₂O, 0.24; CuSO₄.5H₂O, 0.25; MnSO₄.H₂O, 0.17.

The trace element solution was filter-sterilized through a 0.22-μm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

The mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (Fluka) was added into 2 ml of the *P. aeruginosa* MM1011 culture in a 15-ml centrifuge tube to a final concentration of 0.1 g L⁻¹ and the culture suspension was further incubated in a water bath at 37°C for 10 minutes. The final concentration of treated cells was about 5 × 10⁸ cfu ml⁻¹. The treatment was subsequently terminated by diluting the culture 20 times with pre-chilled, fresh mineral salts medium.

Screening method. We used Siegmund-Wagner (SW) medium [20], a semi-quantitative agar plate medium, previously developed for the detection of anionic extracellular rhamnolipids produced by *Pseudomonas aeruginosa* for the screening of the *P. aeruginosa* MM 1011 hyperproducer mutants. The culture obtained from random mutagenesis was diluted with fresh medium and replica plated to cetyltrimethyl-ammonium bromide (CTAB)-methylene blue agar plates prepared by adding 0.2 g CTAB (Merck), 0.005 g methylene blue, and 15 g

agar in 1L of mineral salts medium.

Rhamnolipid-producing colonies on SW agar plates were identified following the formation of dark blue halos around the colonies on a light blue-plate background [20].

Production, isolation and purification of biosurfactants. Mutants and the parent strain of *P. aeruginosa* MM1011 were grown aerobically in 200 ml mineral salts medium (described above) in 1L Erlenmeyer flasks at 200 rpm at 37°C. Culture samples (3 ml) were withdrawn periodically for biomass and biosurfactant quantification in aseptic conditions. Total viable counts were determined by plate count of suitable dilutions of culture medium.

For isolation of rhamnolipids, the pH of the culture (3 ml) supernatant fluid, obtained after removal of cells by centrifugation (10,000 ×g, 10 min), was adjusted to 2.0 and allowed to stand overnight at 4°C. This was followed by extraction with a mixture of CHCl₃ and CH₃OH (2:1 v/v). The solvent was evaporated and the residue was dissolved in 0.1 mol L⁻¹ NaHCO₃ (3 ml).

For analytical purposes, rhamnolipid samples were solubilized in chloroform (0.3 g in 1 L) and approximately 100 µl applied to 20 × 20 cm glass plates. The plates were developed in a chloroform:methanol: acetic acid solvent system (65:15:2 v/v/v) and a small portion of plate, 3 cm from the edge, was sprayed with α-naphtol solution in acidified ethanol. The portions of the plates not sprayed were scraped, corresponding to the bands visualized in the sprayed area. The silica gel scrapings of the bands were collected and the rhamnolipids extracted twice with 8 ml of chloroform:methanol (1:2 v/v).

For separation of more rhamnolipids and better purification of them from the possible contaminating molecules we used column chromatography. A column 30 × 4 cm (diameter) was prepared with 60 g of activated silica gel (230-400 mesh) chloroform slurry. A 1-g sample of crude rhamnolipid extract was prepared in 20 ml of chloroform and loaded with a Pasteur pipette. The column was washed with chloroform until neutral lipids were completely eluted. Chloroform: methanol mobile phases were then applied in sequence: 50:3 v/v (1400 ml); 50:5v/v (250 ml) and 50:50 v/v (150 ml) at a flow rate of 1 ml min⁻¹ and 15 ml fractions were collected.

Analytical methods. Rhamnolipids were quantified in triplicate by weight and by the colorimetric determination of sugars with orcinol [21].

A modified orcinol method was used to assess the amount of glycolipids in the sample: 333 µL of the culture supernatant was extracted twice with 1 ml of diethyl ether. The ether fractions were pooled and evaporated to dryness, and 0.5 ml of H₂O was added. To 100 µL of each sample, 900 µL of a solution containing 0.19% orcinol (in 53% H₂SO₄) was added. After heating at 80°C for 30 min, the samples were cooled at room temperature for 15 min and the A₄₂₁ was measured by UV spectrophotometer (NOVASPEC II, Pharmacia Biotech).

The concentration of rhamnolipids was calculated by comparing the data with those of rhamnose standards between 0 and 50 µg/ml [21]. Standards, blanks and unknowns were analyzed in triplicate and the linear correlation was demonstrated between the quantity of rhamnolipid and optical density.

Surfactant properties of the samples were determined by the capillary rise method [22]. A 0.1-ml pipette having a 0.001-ml incremental scale was modified such that the tip was polished and the mouth connected to a syringe so that liquid could be released in a controlled manner. The critical micelle concentration and the ability of the rhamnolipids to reduce the surface tension of water were calculated according to Martin *et al.* [22].

The separated rhamnolipids were identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS). Analytical equipment used in the identification were as follows: Varian 400-MHz unity plus (Varian, Sugarland, TX, USA) for ¹H NMR and ¹³C NMR; Finnigan TSQ-70 (Finnigan, Austin, TX, USA) for MS.

RESULTS AND DISCUSSION

Selection of a mutant. Treatment of the wildtype *P. aeruginosa* MM1011 with NTG at a concentration of 0.1 mg ml⁻¹ for 10 min terminated by dilution led to a survival rate of 10%. It is believed an intermediate dose allows survival of 10% of the treated cell population generates more mutants with improved titer [23].

Isolation of biosurfactant overproducer mutants with blood agar plate test has been previously reported by Mulligan *et al.* [24, 25]. However, the extent of hemolytic zone formation on blood agar plates is not solely dictated by the concentration of rhamnolipid and may be affected by divalent ions and other hemolysins produced by the microbe under investigation. An alternative approach

previously developed for the detection of extracellular rhamnolipids and other anionic glycolipids [20] were employed in this study for the screening of rhamnolipid biosurfactants production by *P. aeruginosa* MM1011 and mutants. The assay was developed based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substances. The formation of insoluble ion pair precipitate in the agar plate containing methylene blue exhibited dark blue color against the light blue background. The diameter of the dark blue region previously has been shown to be semiquantitatively proportional to the concentration of the rhamnolipid biosurfactants [20]. The cationic chemical selected in the assay was CTAB. A colony containing the largest dark blue halo further cultivated as *P. aeruginosa* PTCC 1637 and was selected for further investigation.

Structural analysis and properties of rhamnolipids. To recover the biosurfactants from fermentation broth and determine the identities of glycolipidic biosurfactants, a systematic study combining TLC, NMR and surface tension measurement was conducted.

The surface tensions of the culture supernatants of *P. aeruginosa* MM1011 and *P. aeruginosa* PTCC1637 were measured to estimate the extent of biosurfactants production. The surface tension of *P. aeruginosa* MM1011 and mutant of PTCC1637 were in the range of 28 mN m⁻¹ indicating the production of effective biosurfactants (Table 1).

Rhamnolipid biosurfactants precipitate at pH below 2 and can be extracted by organic solvents efficiently. In this study, we repeat the extraction 5 times to insure complete product recovery. The surface tension of the extraction retentates was 67.9. It showed that all biosurfactants were retained by extraction.

Like most secondary metabolites, the biosurfactants produced by *P. aeruginosa* consist of a family of glycolipids with similar structures.

Table 1. The surface properties of culture supernatants of the *P. aeruginosa* MM1011 and mutant *P. aeruginosa* PTCC1637 measured at 25°C.

	Surface tension mN m ⁻¹	CMC dilution	Rhamnolipid g L ⁻¹
Fresh medium	72.0	-	0.0
<i>P. aeruginosa</i> MM1011	28.2	50-100	1.2
<i>P. aeruginosa</i> PTCC1637	27.9	500-1000	12.5

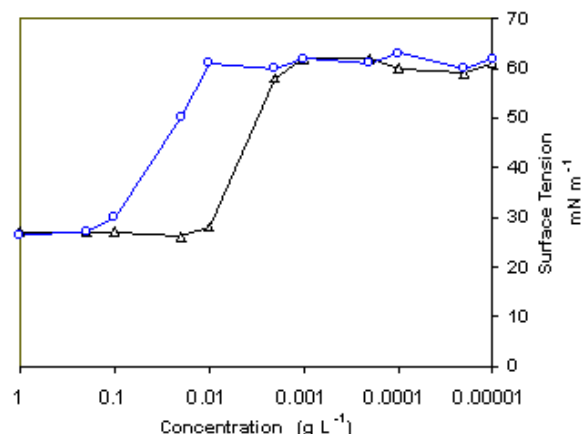


Fig. 2. The surface tensions of the serially diluted solutions of two main biosurfactants produced by *P. aeruginosa* MM1011. Δ , First fraction isolated by column chromatography (RL1); O, Second fraction isolated by column chromatography (RL3).

These compounds composed of rhamnose and β -hydroxycarboxylic acid subunits and are excreted into the culture medium [16, 26]. For separation of different rhamnolipids and their purification in sufficient quantity, we used column chromatography as described above. By this method, two main fractions were isolated and purified.

Surface tension measurement was conducted to demonstrate that the two main fractions were indeed biosurfactants. The two column chromatography fractions were individually dissolved in water for surface tension measurement. The relationship between biosurfactant concentration and surface tension were shown in Figure 2.

Solutions of biosurfactant fractions 1 and 2 had a low surface tension of 26.5 mN m⁻¹. Critical micelle concentration of fraction 1 was around 30 ppm, while fraction 2 showed critical micelle concentration 10 times more than fractional (around 250 ppm). These values agree with those previously reported for rhamnolipids by Parra *et al.* [17].

The results of NMR analysis, infrared spectroscopy analysis (Fig. 3), surface tension measurement, and CMC analysis indicated that the glycolipids produced by the *P. aeruginosa* PTCC 1637 are identical to those by wildtype strain.

Microbial growth and biosurfactants production. The microbial growth and the biosurfactants production profiles of *P. aeruginosa* MM1011 and PTCC1637 were shown in Figures 4A and B. As it shown, *P. aeruginosa* MM1011 started producing biosurfactants at the stationary

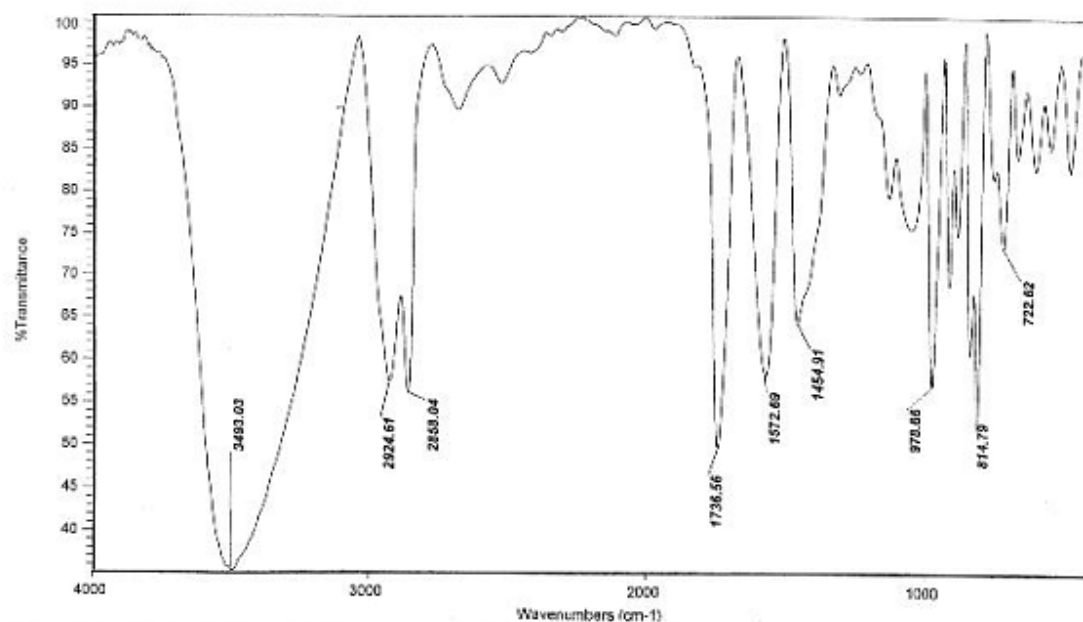


Fig. 3. The infrared spectrum of the glycolipid biosurfactants produced by *P. aeruginosa* PTCC1637 with FTIR Nicolet-550 spectrophotometer (Nicolet, USA)

phase and the concentration of biosurfactants reached its maximum 1.2 g L^{-1} , at the 7th day of the incubation (Fig. 4A) while the mutant PTCC1637 reached to a maximum of almost 10 fold of wild strain, 12.5 g L^{-1} at 5th day of incubation (Fig. 4B).

The results of quantitative analysis of biosurfactants by orcinol method were consistent with surface tension measurements. The surface tensions of the serially diluted *P. aeruginosa* MM1011 and mutant *P. aeruginosa* PTCC1637 supernatants are shown in Figure 5. It can be

estimated from the graph that the concentration of biosurfactants of the original culture broth obtained from *P. aeruginosa* MM1011 at 10 days after inoculation is approximately $100 \times \text{CMC}$. The CMC of wildtype and the mutant *P. aeruginosa* biosurfactants were estimated as 10 mg L^{-1} [17]; therefore the concentration of biosurfactants in the culture supernatant was approximately 1 g L^{-1} by surface tension analysis. This was close to the value (1.2 g L^{-1}) determined by orcinol method.

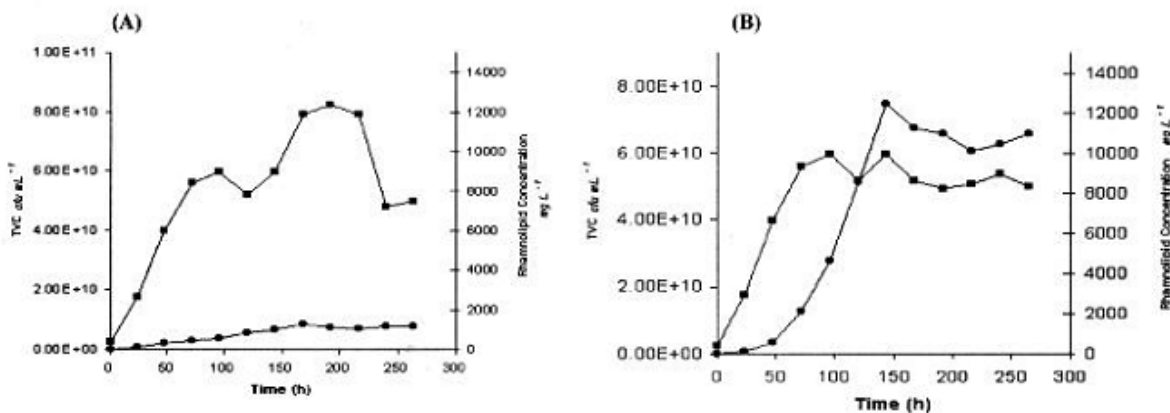


Fig. 4. (A), the typical growth and biosurfactant production profiles of the parent strain *P. aeruginosa* MM1011 [●– Total Rhamnolipid Concentration (mg L^{-1}), ■– Total Viable Count of *P. aeruginosa* MM1011 (cfu mL^{-1})]; (B), the mutant *P. aeruginosa* PTCC1637 [●– Total Rhamnolipid Concentration (mg L^{-1}), ■– Total Viable Count of *P. aeruginosa* PTCC1637 (cfu mL^{-1})].

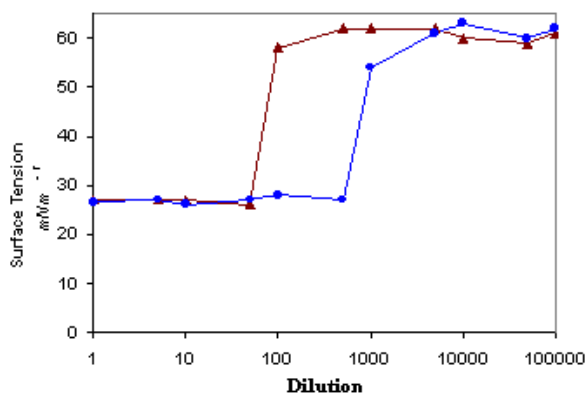


Fig. 5. The surface tensions of the serially diluted culture supernatant of the *P. aeruginosa* MM1011 (▲) and mutant *P. aeruginosa* PTCC1637 (●) after 10 days incubation.

The results of quantitative analysis of biosurfactants by orcinol method were consistent with surface tension measurements. The surface tensions of the serially diluted *P. aeruginosa* MM1011 and mutant *P. aeruginosa* PTCC1637 supernatants are shown in Figure 5. It can be estimated from the graph that the concentration of biosurfactants of the original culture broth obtained from *P. aeruginosa* MM1011 at 10 days after inoculation is approximately 100 CMC. The CMC of wildtype and the mutant *P. aeruginosa* biosurfactants were estimated as 10 mg L⁻¹ [17]; therefore the concentration of biosurfactants in the culture supernatant was approximately 1 g L⁻¹ by surface tension analysis. This was close to the value (1.2 g L⁻¹) determined by orcinol method.

These results indicate that the mutant not only possesses significantly higher biosurfactants productivity but also different metabolic activities at the stationary phase.

P. aeruginosa PTCC1637 exhibits favorable properties including enhanced biosurfactants productivity and reduced biosurfactants degradation over the wild strain. These properties alleviate the difficulty encountered in the production of biosurfactants with *P. aeruginosa* and hence eliminate the need of continuous fermentation for the large-scale production of the glycolipid biosurfactants

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