

NOTE

***Pseudomonas* Strains as Source of Microbial Surface-Active Molecules**

S. BHATTACHARYYA, M. GHOSH and D.K. BHATTACHARYYA*

*Department of Chemical Technology, (Oil Technology Division) Calcutta University
(92, A.P.C. Road, Kolkata-700 009, INDIA)*

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Abstract: Isolation of two new strains of *pseudomonas* with characteristic surface-active properties were isolated from soil contaminated with natural surfactant. Observation of surface tension properties indicated that biosurfactants were produced by these newly isolated and promising strains of *Pseudomonas sp.* (SB 3 and SB 5). The isolation of the microbes, their harvesting in the nutrient media, extraction of the bacterial lipid and composition of those lipids extracted, are discussed in the present study.

Key words: *Pseudomonas sp.*, phospholipid, biosurfactant

1 Introduction

Microbial surface-active molecules, also known as biosurfactants, are unique in their character for reducing interfacial surface tension and also for stabilizing emulsions. These surface-active molecules include a wide range of natural surfactants and emulsifiers, such as glycerophospholipids, glycolipids, glycosphingolipids, bile acids, saponins, proteins, polysaccharides etc.(1) and have remarkable applications as food additives, in petroleum recovery and in pharmaceutical and cosmetic applications.

A number of economically interesting biosurfactants are, in fact obtained from microorganisms, such as *Nocardia corynebacterides* SMI (2). *Rhodococcus erythropolis* DSM 43215 (3). *Pseudomonas rubescans*, *Gluconobactor cerinus*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*, *Candida petrophilum*(3), *Serratia marcescens* (4), *Rhodococcus sp.* (5). *Pseudomonas aeruginosa* (6), *Rhodococcus erythropolis* (7). A species of *Arthrobacter* (8) isolated from soil and designated as H13 A produced a variety

of glycolipid surfactants when grown on a hydrocarbon substrate and *Cornebacterium lepus* (8) produced phospholipid surfactants. Bandyopadhyay *et al.* also isolated *mortierella* strains which could produce biosurfactants in the form of phospholipid and glycolipid (9).

Pseudomonas strains are already very well known for their capability of biosurfactant production. A lot of work already have been done on various strains of *Pseudomonas* to isolate and identify the biosurfactants produced by them. The strains *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas rubescans* are very well studied. This was not unexpected, because these are common soil organisms, and all species identified have literature support for their ability to produce a biosurfactant. Results from the FAME analysis were similar to the results of the Biolog analysis. Future work in the area of biosurfactants can include obtaining additional evidence of biosurfactant producers indigenous to various soils. Additionally, more research is needed in the area of gram positive biosurfactant producers.

This study does bring to the forefront the isolation of

*Correspondence to: D.K. BHATTACHARYYA, Department of Chemical Technology, (Oil Technology Division) Calcutta University, 92, A.P.C. Road, Kolkata-700 009, INDIA
E-mail: dkb@cucc.ernet.in

newer strains of *Pseudomonas* isolated from natural surfactant induced media which can produce bio-surfactant. We show here that these two newly isolated strains *Pseudomonas putida* SB3 and SB5 produces a surfactant which substantially changed the surface tension in non-aqueous medium.

2 Materials and Methods

2.1 Materials

Variety of bacterial strains were collected from soil. Soyalecithin was collected from M/s S.M. Dyechem (Indore, India). The solvents and chemicals used were of A.R. grade supplied by S.D. Fine Chemicals, Mumbai, India.

2.2 Isolation and Purification of Bacterial Strain

Soil samples were collected from crop fields and gardens. There were altogether 15 samples of soil out of which 8 were selected for the experiment and microbes were collected from it by *enrichment test* with soyaphospholipid. Different bacterial strains were inspected under microscope and two *Pseudomonas* strains were purified after examining their ability in producing biomass containing biosurfactant.

2.3 Morphological and Physiological Characterization of Isolates

Isolates were examined at different time for Gram reaction and cell morphology (identification was done at National Institute of Cholera and Enteric Diseases, Kolkata). Other biochemical tests were performed following directions of the latest edition of Bergeys manual (10).

2.4 Incubation Condition and Media

The purified cultures were incubated in the sterilized nutrient medium (60 ml medium in 250 ml conical flask) for 3-15 days at 37 °C (consisting of 1g beef extract, 2 g yeast extract, 5 g peptone, and 0.5 g sodium chloride in 1000 ml of distilled water) with continuous shaking (120 stokes/min). The pH of the medium was kept nearly at 7.0. Samples from the cultures as well as from sterile controls were collected and analysed.

2.5 Separation of Bacteria from Media

The culture medium was centrifuged at 10,000 r.p.m.

for 30 mins. The cells were precipitated out from the media. The supernatant were decanted off. The whole cells were washed with buffer of pH 7.0 and again centrifuged to free from medium completely.

2.6 Extraction of Bacterial Lipid

Cells were taken in a homogenizer and cellular lipid were extracted with the mixture of chloroform : methanol : water (2 : 1 : 0.8) in a standard procedure (11). After extraction, the solvent was removed under a stream of nitrogen, and finally the extract was dried under vacuum. Then the percent total lipid of bacterial cell is estimated gravimetrically and phospholipid is estimated spectrophotometrically according to the standard method (12).

2.7 Thin Layer Chromatography

The different fractions of the bacterial lipid were separated by column chromatography and measured gravimetrically. The bacterial lipid was tested for the presence of glycolipid and phospholipid by *Molisch's test* (13), and phosphate stain test (11), respectively. The fatty acid composition of the total lipid and phospholipid fraction was determined with a gas-liquid chromatography (GLC) method after conversion into methyl esters (14).

2.8 Investigation of Surface Active Property of Bacterial Lipids

Surface active property of cellular lipids of the bacterial strains was determined at different concentrations with du-NOUY tensiometer (made by Jenson & Co., Kolkata, India) graduated to 0.1 mN/m (15).

2.9 Analysis of Polar Lipids

The presence of phospholipids in the lipid isolated was confirmed by phosphate reagent test. The amount of phospholipid in the lipid extract was determined spectrophotometrically.

Column chromatography separation of the lipids showed that neutral lipids formed 70-75%, phospholipids 10-13% and other polar lipids 17-20% of the total lipid.

The lipid responded positively to the *Molisch's test* and therefore indicated the presence of glycolipid. But the amphiphilic surface-active glycolipid i.e. rhamnolipid are usually secreted by *pseudomonas sp.*, and their production was observed when the strain was grown on

soluble substrates (16). Detection of rhamnolipid was done by thin-layer chromatography (17).

3 Result and Discussion

From 8 bacterial strains, 2 varieties of bacterial strains of *Pseudomonas* sp. SB 3 and SB 5 were studied to investigate biosurfactant production. They were identified as *Pseudomonas putida* SB 3 and SB 5 (vide **Table 1**).

The fatty acid compositions of bacterial lipid after harvesting the cells in nutrient media for 15 days at 37 °C are shown in **Table 2**. The table shows that the bacterial lipids are rich in long chain polyunsaturated fatty acids (LC-PUFA). The strain of SB3 synthesizes

high amount of C_{20:5} fatty acid whereas the SB 5 strain synthesises high amount of C_{20:5} fatty acid along with considerable amount of C_{22:6} fatty acids.

The fatty acid profiles of phospholipid of two strains are almost of similar nature. Like the total lipid fraction, the phospholipid fraction is also quite rich in C_{20:5} fatty acid. Measurement of surface tension (**Table 3**) showed that there was an appreciable reduction in surface tension values of water upon the addition of bacterial lipid, and the surface tension was reduced with an increase in the concentration of the lipids from 0.5 to 1%.

Table 1 Identification of Bacterial Strains.

| Characteristics | Strain SB 3 | Strain SB 5 |
|--------------------------------------|-------------|-------------|
| Gram | Positive | Positive |
| Shape | Rod | Rod |
| Number of flagella | >1 | >1 |
| Fluorescent, diffusible pigments | + | + |
| Non-diffusible nonfluoresce pigments | — | — |
| Poly-β-hydroxybutyrate accumulation | — | — |
| Growth at 41 °C | — | — |
| Growth at 4 °C | d | + |
| Oxidase reaction | + | + |
| Denitrification | — | — |
| Gelatin hydrolysis | — | — |
| Starch hydrolysis | — | — |
| Utilisation of: | | |
| Glucose | + | + |
| Trehalose | — | — |
| 2-Ketogluconate | + | + |
| meso-inositol | — | — |
| Geraniol | — | — |
| 1-Valine | + | + |
| β-Alanine | + | + |
| 1-Arginine | + | + |
| Nitrate used as a nitrogen source | + | + |

Table 2 Fatty Acid Composition of Cellular Lipid (Total) and Phospholipid Fractions of Bacteria Grown in Nutrient Media for 15 Days of Incubation.

| Bacterial Strains | Total lipid/ Fractions | Major Fatty Acids, (% w/w) | | | | | | | |
|-------------------|------------------------|----------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------|
| | | C _{16:0} | C _{18:0} | C _{18:1} | C _{18:2} | C _{20:4} | C _{20:5} | C _{22:6} | others |
| SB3 | Total | 18.1 | 2.5 | 13.3 | 23.9 | 0.1 | 37.2 | — | 2.3 |
| | PL | 26.1 | 3.2 | 25.0 | 1.1 | 5.2 | 37.0 | — | 0.6 |
| SB5 | Total | 14.1 | 1.0 | 12.0 | 20.9 | 0.2 | 32.2 | 7.3 | 7.5 |
| | PL | 24.3 | 4.6 | 28.7 | 1.1 | 2.6 | 26.6 | 6.6 | 5.5 |

PL : Phospholipid Fraction

Table 3 Surface Tension of Microbial Lipid Extracted from Bacteria Grown in Nutrient Media for Different Incubation Period.

| Bacterial Strain no. | Time of Incubation in days | Dilution of Solution % | Surface Tension | |
|----------------------|----------------------------|------------------------|-----------------|------------|
| | | | mN/m | Temp at °C |
| SB3 | 10 | 1.0 | 32.7 | 30.0 |
| | | 0.5 | 33.0 | |
| | | 0.1 | 38.7 | |
| SB3 | 15 | 0.1 | 37.3 | 26.0 |
| | | 0.05 | 37.4 | |
| | | 0.025 | 40.8 | |
| SB5 | 10 | 1.0 | 32.0 | 30.0 |
| | | 0.5 | 33.0 | |
| | | 0.1 | 37.0 | |

4 Conclusion

The biosurfactants from the newer strains of *Pseudomonas* which are rich in LC-PUFA and exhibit quite good surface-active properties can be used in food and pharmaceutical applications to a considerable extent. This study does bring to the forefront the additional evidence of biosurfactant producers indigenous to various soils and thus the role of biosurfactant producers in contaminated soil is extremely important because this may be an untapped source of potentially useful microorganisms.

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