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Full Length Research Paper

Effect of biosurfactant from two strains of *Pseudomonas* on germinating seedlings of *Cicer arietinum* L and *Phaseolus mungo* Roxb.

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Two *Pseudomonas* strains isolated from oil-contaminated soil which produce biosurfactant were studied. The biosurfactant containing broth formed stable emulsions with liquid light paraffin, cooking medium vegetable oil and toluene. The strains under study produce extra cellular biosurfactant in the culture media. Biosurfactant was extracted from the culture broth by acetone-HCl precipitation. Critical micelle concentration (CMC) of biosurfactant from *Pseudomonas* sp I and *Pseudomonas* sp II were found to be 120 and 140 mg/l, respectively. The biosurfactants from the two species were found to have no effects on germinating seedlings of *Phaseolus mungo* Roxb and *Cicer arietinum* L when treated at concentration 1, 2, 5 and 10%, suggesting a potential use in surfactant aided bioremediation and for even distribution of fertilizers and pesticides in agricultural sector. This is the first report on effect of biosurfactant on total length proliferation of germinating seedlings.

Key words: Biosurfactant, *Pseudomonas*, emulsification, *Cicer arietinum* L, *Phaseolus mungo* Roxb., bioremediation.

INTRODUCTION

The term biosurfactant has been widely referred to any compound obtained from microorganisms that exert some influence on interfaces in both aqueous solutions and hydrocarbon mixtures. These properties cause micro-emulsions in which micelle formation occurs where hydrocarbons can solubilize in water, or water in hydrocarbon (Banat, 1995). The structures of various biosurfactants are elaborately reviewed (Cooper and Zajie, 1980). Generally biosurfactants are classified into five major groups; glycolipids, phospholipids and fatty acid, lipopeptides (lipoprotein) and polymeric biosurfactant and particulate biosurfactant. *Pseudomonas* sp. produces rhamnolipid, a glycolipid type of biosurfactant (Hiratsuka et al., 1971; Lang and Wagner, 1987; Cooper and Zajic, 1980). Recently biosurfactant has been found to get a place in health science research. An antitumor lipopeptide biosurfactant purified from *Bacillus natto* TK-1 was able to inhibit the proliferation of MCF-7 human breast-cancer cells in a dose- and time-dependent manner (Cao et al, 2009). Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans* was recently studied by Das et al. (2008).

Biosurfactant production is generally associated with utilization of hydrocarbon by microbial community. Various taxonomic groups of microorganisms capable of utilizing hydrocarbon was reviewed (Atlas, 1981). There is large number of reports of biosurfactant production from aliphatic hydrocarbon. Recently, Deziel et al. (1996) reported the production of biosurfactant by a *Pseudomonas* strain when grown on aromatic hydrocarbon-naphthalene. However, there are also reports on biosurfactant production by microbes when grown on water soluble substrates like glucose, sucrose, starch, glycerol and ethanol. These reports contradicted the concept that biosurfactant are produced by the microbes to facilitate the uptake of water insoluble substrates.

Biosurfactant is extensively used worldwide as it is preferred to chemical surfactants for it being non toxic and easily biodegradable (Desai and Banat, 1997). The cost of biosurfactant is much cheaper as compared to

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chemical surfactants. Biosurfactants are found to have lower CMC value as compared to chemical surfactant. In recent years, interest on biosurfactant has been generated due to their possible application in environment protection, crude oil drilling, in pharmaceutical and food processing industries (Kosaric et al., 1987; Mulligan and Gibbs, 1993).

One of the major biosurfactant applications in environmental protection is bioremediation. The most cost effective methods include *in-situ* bioremediation. *Pseudomonas aeruginosa* SB30 biosurfactants were tested for the ability to remove oil from Exxon Valdez Alaskan contaminated gravel in laboratory. Another major use of biosurfactant is in agricultural sector. Surface active agents are needed for the hydrophilization of heavy soil for obtaining good wettability and also to achieve equal distribution of fertilizers and pesticides in the soils (Banat et al., 2000). Most of the pesticides are water soluble and are marketed in powder or in liquid concentrate form. Before spraying in field, these are diluted with water and mixed with surface active compound for spontaneous distribution of the water insoluble pesticides in the aqueous phase as well as an equal distribution on wetting of the treated areas.

Phaseolus mango Roxb and *Cicer arietinum* L. belong to the family leguminosae. These are grown mainly around the Mediterranean, in Ethiopia and in India, the latter producing 80% of world production. The seeds not only have high levels of protein, but also are rich in carbohydrates and fats, making them a highly nutritious food. More recently the beans have become a popular item on salad bars everywhere.

In this work we report the method of extraction (standardized in our laboratory) and the effect of biosurfactant from two *Pseudomonas* isolates on germinating seedlings of *P. mungo* Roxb and *C. arietinum* L.

MATERIALS AND METHODS

All the chemicals were of analytical grade and were obtained from Himedia, India. Acetone was obtained from Merck, India. Liquid light paraffin was obtained from Loba Chemie, India. The seeds of *P. mungo* Roxb and *C. arietinum* L. were obtained from a local nursery.

Enrichment culture and isolation of microbes

Four oil contaminated soil samples were collected from Gauhati Refinery, Assam, India, each having a varied level of oil contamination. One gram each of the soil samples was added to 100 ml of defined medium with 2% (v/v) liquid light paraffin and incubated at 37°C for 7 days at 200 rpm. The composition of the defined medium was (per liter) $NaNO_3$ - 3 g; KH_2PO_4 - 1.5 g; Na_2PO_4 - 1.5 g; $MgSO_4.7H_2O$ -2 g; $CaCl_2.2H_2O$ - 0.005 g; $FeSO_4$ - 0.001 g; $ZnSO_47H_2O$ - 70 µg; $CuSO_4.5H_2O$ – 50 µg; H_3BO_3 - 10 µg; MoO_3 - 10 µg.; pH 7 ± 0.2. After 7 days 1 ml inoculum was transferred to fresh medium with hydrocarbon and incubated for another 7 days. The process was repeated for three times and 1 ml inoculum from the third enrichment culture was appropriately serially diluted and 100 µl aliquots from the last two dilutions were streaked on nutrient agar plates. Isolated pure colonies were picked up and maintained on nutrient agar slants containing hydrocarbon.

Screening for biosurfactant production

The isolated pure cultures were transferred separately to big culture tubes containing 30 ml defined medium with 2% (v/v) liquid light paraffin. One tube was kept as control with no inoculum. The tubes were then incubated for 168 h at 37°C and 200 rpm. After the incubation period the tubes were vortexed for 2 min and foaming, turbidity and reduction of surface tension of the growth medium were recorded. Two best isolates were selected for further studies.

Identification of the selected culture

The two isolates in the study were identified according to the standard microscopical, cultural and biochemical tests as described in Microbiology: A laboratory Manual by Cappuccino and Sherman (1999) and comparing the results in Bergey' Manual of Systematic Bacteriology (1984).

Surface tension and critical micelle concentration (CMC)

The surface tension of biosurfactant was measured by the Ring method using a DuNouy Tensiometer (S.C.Dey Co., Kolkata, India) at room temperature. The concentration at which micelle formation occurs is represented as the CMC; CMC was determined by the sudden break in the curve obtained by the plot of surface tension versus the plot of log of concentration of biosurfactant.

Emulsification assay

Emulsification assay was done as described by Cooper and Goldengerg (1987). Briefly, emulsification activity was measured by adding 6 ml of kerosene to 4 ml of aqueous sample and vortexing the mixture at high speed in SPINIX vortex for 2 min. The emulsification index (E_{24}) is the measurement of the height of the emulsion layer after 24 h, divided by the total height multiplied by 100. Here in this study we have replaced kerosene by liquid light paraffin, cooking medium vegetable oil and toluene.

Extraction of biosurfactant

Method of extraction of biosurfactant has been optimized in our laboratory. Extraction was done by using acetone and then precipitation by HCl, overnight at 4°C. The product recovered was dried under vacuum and preserved.

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1, 2, 5 and 10% concentration of biosurfactant extracted from the culture broth of two *Pseudomonas* strains under study were made with glass double distilled water, making the final volume to 10 ml. Healthy seeds of the above species were presoaked in the respective concentration for 24 h. A control was taken with no surfactant. The seeds were spread in filter paper (soaked in respective concentration of biosurfactant) on petridishes. The total length (cm) proliferation of the germinating seedlings was recorded after 4 days.

Statistical analysis

Experiments were setup in a Randomized Block Design (RDB) and each experiments had 5 replicates. Observations were recorded on total length proliferation after 4 days of treatments. The analysis of variance (ANOVA) was carried out to detect the significance of differences between the treatment means at 5% probability level.

RESULTS AND DISCUSSION

Isolation of biosurfactant producing microbes

The production of surfactants by microbial cells often results in foaming, a decrease in surface tension and emulsification of hydrophobic substrates (Banat et al. 2000). These properties were used in screening for surfactant producing strains. By enrichment culture, altogether 17 isolates were isolated in pure form from four oil contaminated soil samples. These 17 isolates were capable of growing in mineral medium containing 2% (v/v) liquid light paraffin as the sole carbon source. The media was a slight modification of Baruah and Deka (1995). On the basis of the growth, foaming and reduction of surface tension of the growth medium, the two best isolates were considered for further investigation. These two isolates could reduce the surface tension of culture medium from 69 mN/m to around 32 mN/m. The enrichment culture particularly makes the selection of hydrocarbon (liquid light paraffin) degrading bacteria which was focused here for the production of biosurfactant.

Characterization and identification of the selected cultures

Strain *Pseudomonas* sp. I and *Pseudomonas* sp. II proved to be gram negative non-sporing rods with a width of 0.9 to 1.2 μm and 0.7 to 1 μm and a length of 3.2 to 4.5 μm and 3.0 to 4.0 μm, respectively. Both were oxidase negative. They showed negative for tests of indole production and denitrification. They were also negative for MR-VP and ammonium production. *Pseudomonas* sp. I could hydrolyze gelatin, while *Pseudomonus* sp. II could not. Both could hydrolyze casein and starch. Both were positive for citrate reduction and proved to be motile and were negative for tests for acid production in milk and gas and alcohol production from sugars like glucose, sucrose, mannitol, glycerol and lactose. No growth was observed at 45 and 4°C. Optimum growth was found at 37°C. Both the *Pseudomonas* strains produced fluorescent green pigment in the mineral salt culture media.

Extraction of biosurfactant

The biosurfactant was extracted from the 168 h old culture broth by hexane acetone method followed by precipitation in 6 N HCl. It consists of just one solvent namely acetone added to the broth in excess ratio as shown in the flow chart (Figure 1). The treatment with hexane at a ratio of 1:3 to the culture broth removed the residual hydrocarbon, which would otherwise have been difficult to remove by centrifugation. The yield of biosurfactants of *Pseudomonus* I and *Pseudomonas* II were 4.5 and 4.9 g/l, respectively. A maximum yield of around 5.5 g/l was reported by Pruthi and Cameotra (1995) by three *Pseudomonas* species grown on n-dodecane.

Dry weight determination

Biomass was determined by centrifuging at 10,000 rmp for 10 min, washing the cells three times at PBS (phosphate buffer saline); the biomass was dried overnight at oven in 100^{o}C and weighed. The biomass of *Pseudomonas* sp. I has a higher biomass compared to that of *Pseudomonas* sp II, the weight being 1.36 and 1.01 g/l, respectively.

Emulsification index (E_{24})

The cell free culture broth of *Pseudomonas* sp. I had an emulsification activity of 80% with cooking medium vegetable oil, 75% with liquid light paraffin. The emulsification activity of cell free culture broth of *Pseudomonas* sp. II was 78% with cooking medium vegetable oil, 72% with liquid light paraffin. Both the *Pseudomonas* isolates showed equal emulsification activity of 50% in case of toluene.

Surface tension and CMC

Surface tension reduction and CMC are effective and efficient criteria of a surfactant. The effectiveness is the measurement of minimum value to which the surface tension of the growth medium could be reduced. Effectiveness is the measurement of the surfactant concentration required to produce a significant reduction in the surface tension of water. The CMC can be obtained from a semi logarithmic plot of the surface tension of a solution against the surfactant concentration. The surface tension at CMC is designated as Y^{CMC}.

The biosurfactant extracted from two *Pseudomonas* species was dissolved in distilled water and the surface tension was measured at various concentrations. As shown in Figures 2 and 3, the CMC of biosrfactant extracted from *Pseudomonas* sp. I and *Pseudomonas* sp. II were 120 and 140 mg/1 and Y^{CMC} value were 33.3 and 33.0 dynes/cm, respectively. The result indicates that the biosurfactant from *Pseudomonas* sp. I is comparatively more effective and efficient though the yield is a little low

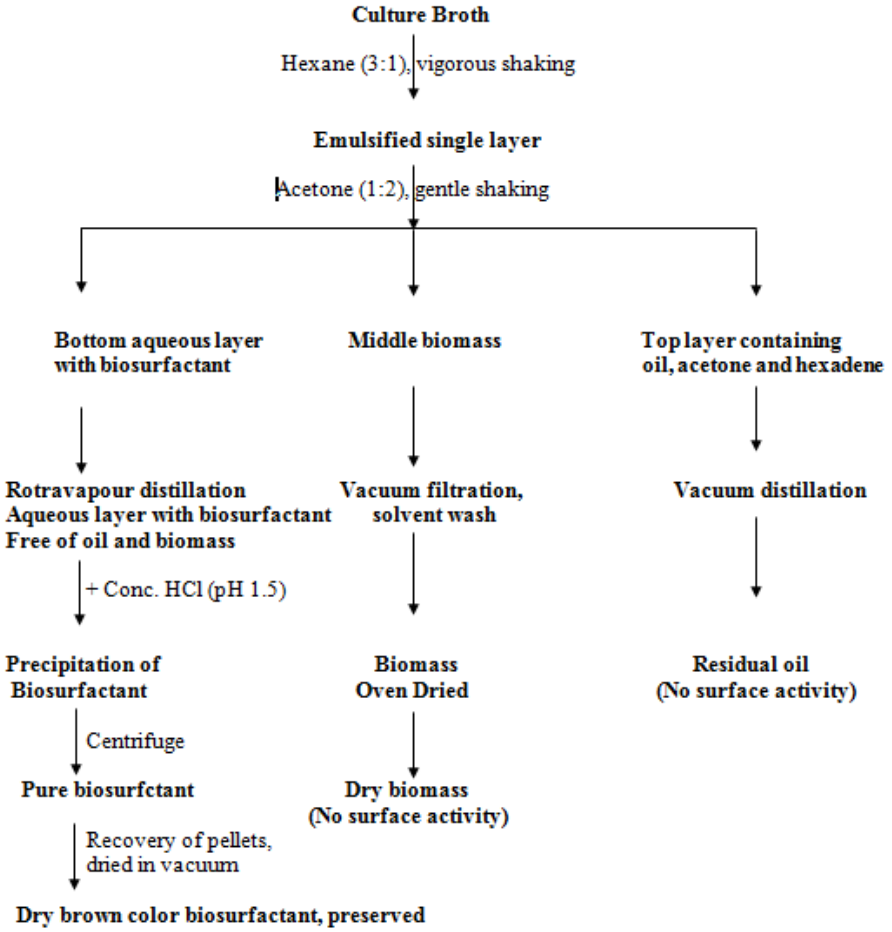

Figure 1. Scheme for extraction of biosurfactant from culture broth

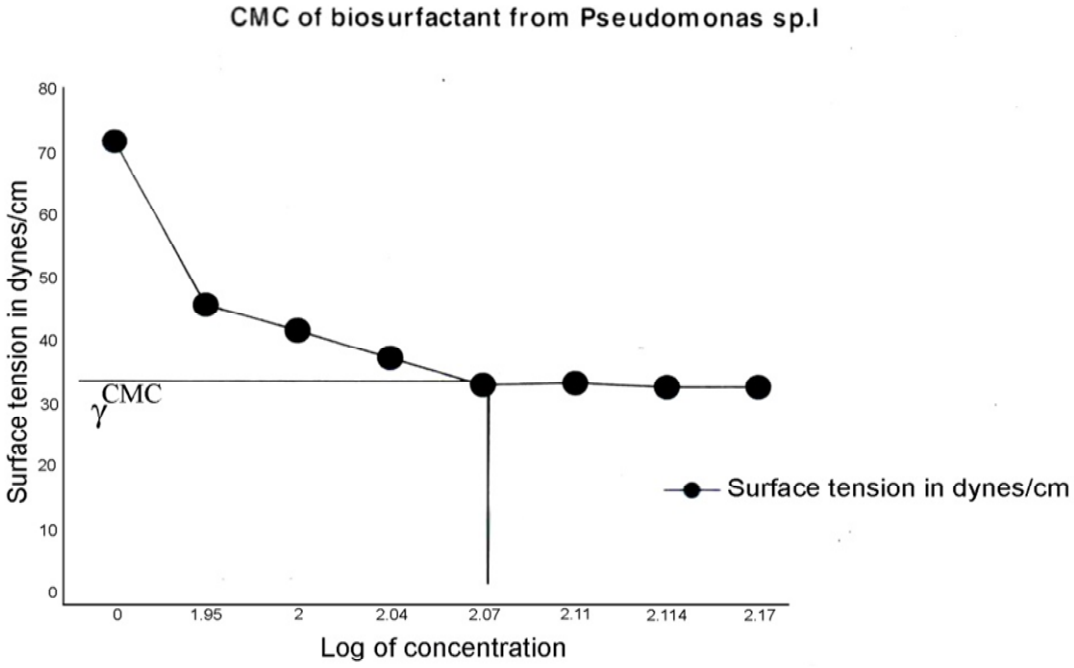

Figure 2. CMC of biosurfactant from *Pseudomonas* sp. I.

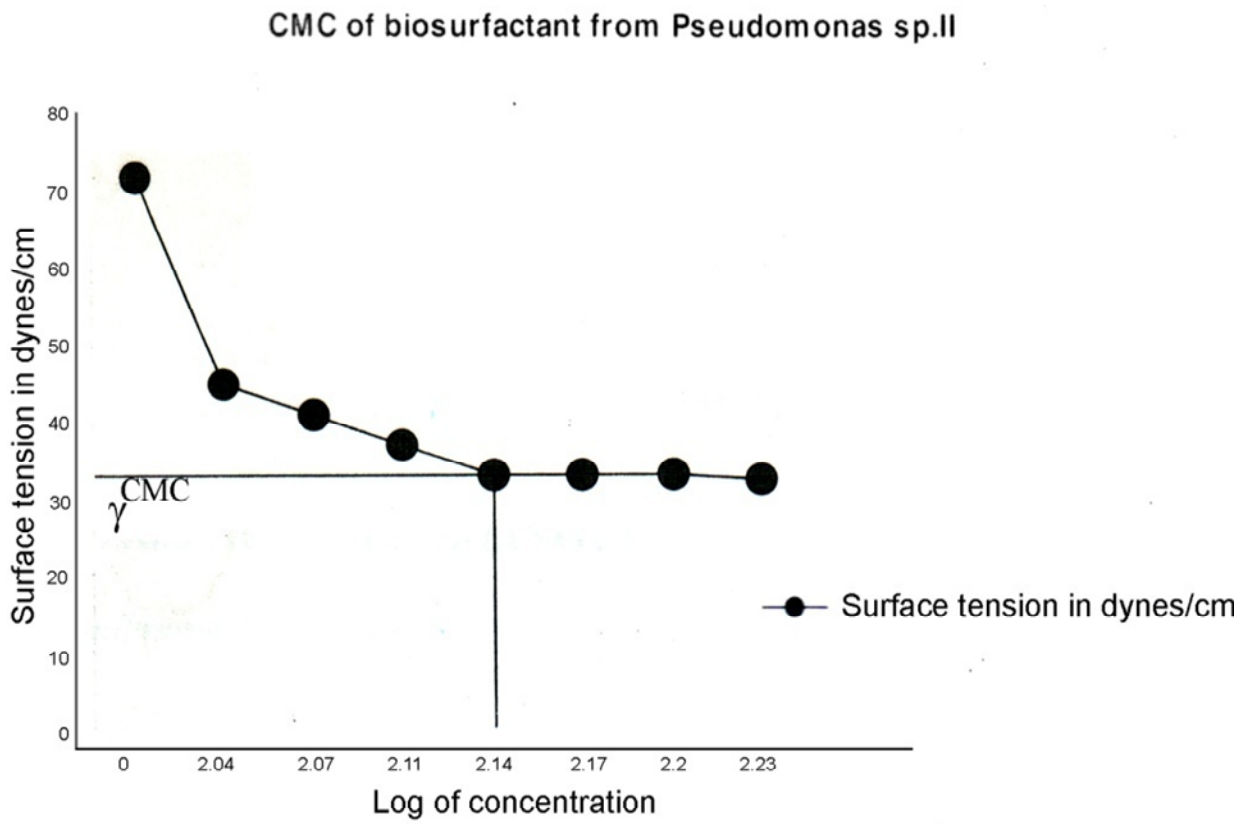

Figure 3. CMC of biosurfactant from *Pseudomonas* sp. II.

Table 1. Analysis of variance (ANOVA) on the effect of biosurfactant produced by *Pseudomonas* sp. I and *Pseudomonas* sp II on total length proliferation on the germinating seedling of *C. arietinum* L.

Parameter	Treatment				
	Control (0%)	1%	2%	5%	10%
Biosurfactant of *Pseudomonas* sp. I	6.6*	4.16	8	6.16	5
Biosurfactant of *Pseudomonas* sp. II	5.3	7.6	8.6	7.3	6.6
Source of Variance	Df	SS	MSS=SS/Df	F = MSS/EMSS	E = Tabulated at 5% level
Varieties	1	3	3	2.04	7.708
Treatment	4	8.92	2.23	1.51	6.3884
Error	4 x 1 = 4	5.88	1.47		
Total	9				

*Shoot length. Each value represents the mean of five (5) replicates in cm.

as compared to Pseudomonas sp. II.

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The total proliferation seedling length was measured after 4th day of treatment. The calculated value for varieties and treatment (Tables 1 and 2) were found to be much smaller than the tabulated value (7.708 and 6.3884) at 5% probability level, hence the test is insignificant. So it can be concluded that the biosurfactant from the two strains of *Pseudomonas* have no effect on the total length proliferation on the germination seedlings of *C. arietinum* L and *P. mungo* Roxb. The concentration of biosurfactant used in this study ranged from 1 - 10% and 10% is the maximum concentration which is suggestible for practical application of biosurfactants in surfactant aided bioremediation of cultivated land. Also, the biosurfactant could possibly be used in hydrophilization of heavy soil and even distribution of pesticides and fertilizers in agriculture.

Table 2. Analysis of variance (ANOVA) on the effect of biosurfactant produced by *Pseudomonas* sp. I and *Pseudomonas* sp II on total length proliferation on the germinating seedling of *P. mungo* Roxb.

Parameter	Treatment				
	Control (0%)	1%	2%	5%	10%
Biosurfactant of *Pseudomonas* sp. I	6.6*	4.16	8	6.16	5
Biosurfactant of *Pseudomonas* sp. II	5.3	7.6	8.6	7.3	6.6
Source of Variance	Df	SS	MSS =SS/Df	F =MSS/EMSS	E = Tabulated at 5% level
Varieties	1	0.007	0.007	0.0379	7.7086
Treatment	4	3.58	0.895	0.485	6.3884
Error	4 x 1 = 4	7.3	1.845		
Total	9				

*Shoot length. Each value represents the mean of five (5) replicates in cm.

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