

Production of glycolipids containing biosurfactant by *Pseudomonas* species

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Microorganisms, that degrade hydrocarbon were isolated and screened for their biosurfactant activity. A total of 68 strains were isolated and tested for their glycolipid activity of which 4 isolates showed good glycolipid activity. Isolate K₁₀ gave the maximum biosurfactant production in medium A (containing kerosene as a sole carbon source) as compared to medium B (containing glucose as a sole carbon source). Characterization of isolate K₁₀ showed that it belongs to *Pseudomonas* species.

Surfactants are hydrophilic hydrophobic molecules that partition preferentially at the interface. These surface active compounds widely possess various functional properties like surface and interface activities, emulsification, wetting, foaming, phase separation and viscosity reduction of heavier crude oils^{1,2}. Biosurfactants are structurally a diverse group of surface active molecules, which are more effective, selective, environmentally friendly and stable than many synthetic surfactants. These are extracellular products secreted by microbial cells during growth and offer some distinct advantages like lower toxicity, higher biodegradability, higher selectivity and specific activity at extreme temperatures, pH and salinity^{3,4}. For production of biosurfactants, substrates like hydrocarbons, carbohydrates or a combination of both are required⁵. Mechanism of action involved in biosurfactant is the surface adsorption where tension between microbial and hydrocarbon phases is inevitably lowered and the phases acquire a greater tendency to intermix with each other. Biosurfactants enhance the biodegradation of hydrophobic compounds within micelle structures, effectively increasing the apparent aqueous solubility of the hydrocarbon phase thus making it available for adsorption. Biosurfactant production was reported from different microorganisms like *Bacillus*⁶, *Pseudomonas*⁷, *Acinetobacter* and *Arthrobacter* species^{8,9}.

Considerable attention has been given in recent years for isolation of biosurfactant producing microorganisms because of their potential commercial utilization. Hence the present work was aimed to isolate hydrocarbon utilizing bacterial strains from oil con-

taminated soil samples and to evaluate their capability to produce biosurfactant from two different media.

Isolation of biosurfactant producing bacteria—For the present study, the soil samples were collected from 4 different places, viz. petrol bunk (P), kerosene depot (K), railway yard (R) and garage (G) respectively in Visakhapatnam, India. These samples were continuously exposed to various oils for longer periods.

Each soil sample (1 g) was added separately to 50ml of sterile water in 250 ml, Erlenmeyer flask (EM flask) and kept on rotary shaker for 30 min. The clear solution obtained after settlement of suspension was used as the inoculum. A 2 ml of inoculum was added to a mixture of 48 ml of kerosene-yeast extract medium (medium A)¹⁰ having the following composition (g/l) : NaNO₃, 2; KH₂ PO₄, 0.1; MgSO₄.7H₂O, 0.2; yeast extract, 0.5 and kerosene, 20ml with pH 7.0. The flasks were incubated at 30°C on a rotary shaker (220 rpm) for 6 days. The samples were then serially diluted with sterile water, plated on nutrient agar plates and incubated at 37°C for 24 hr. Selected colonies of the organisms were picked up from each plate and transferred to nutrient agar slants.

Detection of biosurfactant—Glycolipids are the most commonly isolated and studied biosurfactants. The cell suspension was prepared by scrapping the growth from each slant in 5 ml of sterile water. This was added to 45 ml of nutrient broth in EM flask (250 ml) and incubated at 30°C for 24 hr. A 5 ml of the inoculum was transferred into 45ml of medium A and medium B¹⁰ separately in EM flasks (250 ml) and again incubated at 30°C for 6 days on a rotary shaker (220 rpm). Composition of medium B comprised

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(g/l): KH_2PO_4 , 3; $(\text{NH}_4)_2\text{SO}_4$, 1; K_2HPO_4 , 7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 and glucose, 20 (pH 7.1). The contents of each flask at the end of the incubation period were centrifuged at 5000 rpm for 20 min. The cell-free supernatant was used for the detection of glycolipids.

Detection of glycolipids containing biosurfactant—Two methods used for the detection of glycolipids in the supernatant were thin layer chromatography (TLC) and phenol: H_2SO_4 .

TLC—It is a method for qualitative analysis of glycolipids described by Helmut¹¹. The technique employed was two dimensional chromatography. The samples were spotted on TLC plates and developed with a mixture of solvents (CHCl_3 , 65%: CH_3OH , 25%: H_2O , 5%) by placing them in a chromatographic tank previously saturated with the same solvent. Then the solvent front (CHCl_3 , 65%: CH_3OH , 25% : H_2O , 5%) was allowed to run up to three-fourth of the length of the plates. The plates were removed, air-dried and sprayed with anisaldehyde reagent (1 ml of concentrated sulfuric acid is added to a solution of 0.5ml of anisaldehyde in 50 ml acetic acid). Finally the plates were placed in oven at 100-105°C for 15 min until the spots attained maximum color intensity (bluish-green color spots) which indicates the presence of glycolipids.

Phenol: H_2SO_4 method¹²—A 1 ml of 5% phenol was added to the supernatant. To this mixture, 5 ml of concentrated H_2SO_4 was added drop by drop. The development of yellow to orange colour indicated the presence of glycolipids.

Estimation of biosurfactant activity—Medium A in EM flasks was inoculated with isolated strains, incubated at 30°C on rotary shaker for 6 days and tested for the presence of biosurfactants. Extent of emulsification and lowering of surface tension were taken as the criteria for estimation of biosurfactant producers. All the experiments were carried out in triplicate.

Fermentation broth was used to measure the surface tension quantitatively at air/water interfaces by using a Troube Stalagmometer¹³. Stalagmometer was filled with sample and the liquid was allowed to flow from it at a rate of 15 drops per min. Rate of flow was adjusted by attaching a piece of rubber tubing with a screw clip at the tip of stalagmometer.

Number of drops formed while the level of culture sample falls from upper to lower mark on stalagmometer was counted. This determination was repeated thrice until reproducible results were obtained.

Emulsifying ability of isolates were measured by vortexing 1 ml of broth, 4 ml of water and 6 ml of kerosene until maximum emulsification was achieved. This was allowed to settle for 48 hr. Percentage of emulsification after 48 hr was calculated by measuring the height of the foam formed. Maximum biosurfactant activity can be achieved by optimizing various environmental conditions, viz. inoculum level, initial pH and incubation temperature. All these parameters are evaluated in terms of surface tension. Various inoculum levels (2.5, 5, 10 and 15%), initial pH (6, 6.5, 7, 7.5 and 8) and incubation temperature (21°, 32°, 37° and 45°C) were studied.

Identification of isolate—Isolate that gave the highest biosurfactant activity was subjected to further characterization studies. Morphological, cultural and biochemical tests were studied as per Bergey's Manual of Determinative Bacteriology¹⁴ and Medical Microbiology¹⁵.

Screening of biosurfactant producers—Screening programme resulted in isolation of 18, 21, 13 and 16 isolates from P, K, R and G soils samples respectively having the ability to use hydrocarbon as a sole carbon source.

Detection of glycolipids containing biosurfactants—Out of 68 bacterial isolates, only four isolates showed good biosurfactant activity, 19 isolates showed mild activity while the rest showed no activity. Four isolates, which showed good biosurfactant activity, were designated as P_{18} , K_3 , K_{10} and R_{11} and they were further subjected for estimation of biosurfactant activity.

Estimation of biosurfactant activity—Isolate K_{10} showed maximum biosurfactant activity with surface tension (52.29 dynes/cm) and emulsifying ability (61%) followed by isolates R_{11} (63.20 dynes/cm, 29%), K_3 (55.17 dynes/cm, 38%) and P_{18} (60.55 dynes/cm, 31%) respectively, in medium A (Table 1). However, this isolate showed maximum biosurfactant activity with surface tension (51.21 dynes/cm) and emulsifying ability (58%) when compared to the other three isolates which cause negligible reduction in surface tension and low emulsifying ability in medium B (Table 1). Inoculum level of 10% with initial pH 7 and incubation temperature 37°C were found to be optimum for maximum production of biosurfactant (Fig. 1). Since the isolate K_{10} showed maximum biosurfactant activity in both media, it was subjected for further characterization studies.

Identification of isolate K_{10} —An attempt was made to characterize the above selected isolate K_{10} .

Table 1—Surface tension and emulsification measurement of selected strains grown in medium A and B

Isolate used	Density (g/ml)	Average number of drops	Surface tension (dynes/cm)	Emulsion after 48 hr. (%)
Medium A				
P ₁₈	0.9783	18.23	60.55	31
K ₃	0.9740	19.93	55.17	38
K ₁₀	0.9732	21.00	52.29	61
R ₁₁	0.9766	17.43	63.20	29
Distilled water	0.9590	15.03	72.00	—
*Control	0.9606	15.00	72.76	0
Medium B				
P ₁₈	0.9776	16.00	69.29	4
K ₃	0.9698	15.00	71.42	8
K ₁₀	0.9754	21.60	51.21	58
R ₁₁	0.9744	15.40	71.76	12
Distilled water	0.9586	15.10	72.00	—
*Control	0.9622	15.10	72.06	0

*Control—Un-inoculated medium

- Average number of drops per min. The flow of liquid was adjusted at a rate not greater than 15 drops per min. with water.

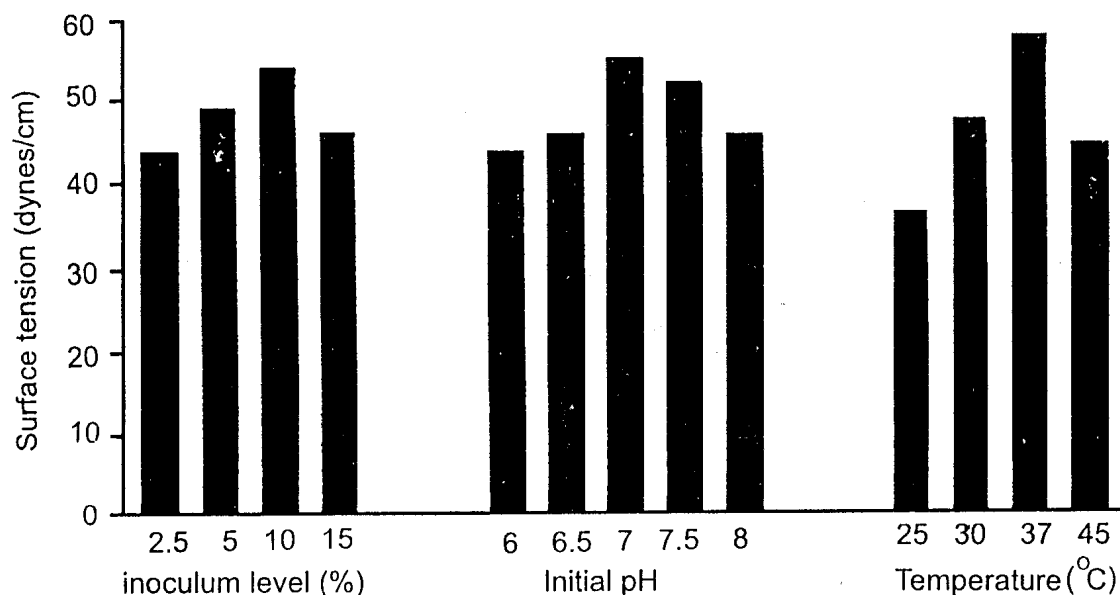


Fig. 1—Effect of inoculum level, pH and temperature on biosurfactant production

Morphological, cultural and biochemical tests were studied. Characteristics of the isolate K₁₀ showed that the cells were straight rod shape with occasional coccoids, Gm-ve and motile. Other biochemical tests indicated that it belonged to the genus, *Pseudomonas*, under the family *Psuedomonodaceae*.

Comparing the biosurfactant activities obtained in the medium A and medium B, it can be concluded that hydrocarbons were better carbon substrate than carbohydrates for the isolate K₁₀.

Baruah *et al.*⁶ have reported a good growth and maximum production of biosurfactant by *Bacillus laterosporous* (11g/l, 27 dynes/cm) and *Pseudomonas aeruginosa* (7 g/l, 30 dynes/cm) in a medium containing n-paraffins with inorganic salts. Singh and Thomas⁵ have reported that biosurfactants are produced in media containing hydrocarbons or a mixed feed containing carbohydrates and hydrocarbons/fatty oils.

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