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Studies on Distribution of Biosurfactant Producing Bacteria in Contaminated and Undisturbed Soils of Kanchipuram

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Abstract: Ever increasing environmental concern about chemical surfactants triggers attention to microbial derived surface-active compounds, essentially due to their low toxicity and biodegradable nature. At present, biosurfactants are predominantly used in remediation of pollutants, in the enhanced transport of metabolites in bacteria, in enhanced oil recovery, as cosmetic additives, in biological control. However, little is known about the distribution and prevalence of biosurfactant-producing bacteria in the environment. The primary objective of this study was to determine how common culturable surfactant producing bacteria are present in contaminated and undisturbed soil samples in and around Kanchipuram (12°50'23"N 79°42'0"E), Tamilnadu, India. A series of each 5 contaminated and undisturbed soils were collected and plated on R2A agar. Totally, 155 morphologically different bacterial isolates were obtained and qualitatively screened for biosurfactant production in mineral salts medium containing 2% glucose. Out of 155 isolates, eight isolates were positive for biosurfactant production, representing most of the soils tested. Quantitative estimation of surface activity identified two potent biosurfactant producing strains *Bacillus sp.*BS3 and *Pseudomonas sp.* Maximum surface activity was observed to be 26.58 x 10^{-3} nm^{-1} and 20.60 x 10^{-3} nm^{-1} respectively for *Bacillus sp.*BS3 and *Pseudomonas sp.* BS5. The present study is a preliminary demonstration that the Indian soils are rich in biosurfactant producing bacteria, which can be exploited for industrial production of biosurfactants.

Key words: Biosurfactants, contaminated soil, undisturbed soil, Kanchipuram, Drop collapse test, *Bacillus sp. Pseudomonas sp.*

INTRODUCTION

Surfactants are amphiphilic compounds containing both hydrophobic (nonpolar) and hydrophilic (polar) moieties that confer ability to accumulate between fluid phases such as oil/water or air/water, reducing the surface and interfacial tensions and forming emulsions (Desai and Banat, 1997). The surface active properties, make surfactants one of the most important and versatile class of chemical products, used on a variety of applications in household, industry and agriculture (Deleu and Paquot, 2004). Microbial surfactants are categorized by their chemical composition and microbial origin. It suggested that biosurfactants can be divided into low molecular mass molecules, which efficiently lower surface and interfacial tension, and high molecular mass polymers, which are more effective as emulsion stabilizing agents (Banat, 1995; Rosenberg and Ron, 1999; Karanth *et al*., 1999; Youssef *et al*., 2005). The major classes of low mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high mass includes polymeric and particulate surfactants. Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acid derivatives whereas the hydrophilic portion can be a carbohydrate, aminoacid, phosphate or cyclic peptide.

The spontaneous release and function of biosurfactants are often related to hydrocarbon uptake; therefore,

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they are predominantly synthesized by hydrocarbon degrading microorganisms. Some biosurfactants, how ever, have been reported to be produced on water soluble compounds, such as glucose, sucrose, glycerol or ethanol (Cooper and Goldenberg, 1987; Guerra *et al.,* 1986; Palejwala and Desai, 1989; Passeri, 1992; Hommel and Huse, 1993). In some instances, these compounds have antibiotic properties which may serve to disrupt membranes of microorganisms competing for food. Examples of these include the lipopeptides of the iturin family produced by *Bacillus subtilis*, which have powerful anti-fungal properties (Sandrin *et al.,* 1990; Thimon *et al.,* 1992), *Candida antarctica*, which have antimicrobial activity (Kitamoto *et al.,* 1993), and *Bacillus licheniformis*, which inhibit bacteria, yeast and filamentous fungi (Fiechter, 1992).

Chemically synthesized surfactants have been used in the oil industry to aid the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to the environment. Biosurfactants, however, have been shown in many cases to have equivalent emulsification properties and are biodegradable. Thus, there is an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil pollution control, cleaning oil sludge from oil storage facilities, soil/sand bioremediation and microbially enhanced oil recovery, which offers advantage over conventional enhanced oil recovery, that lower capital and chemical/energy costs required (Sarker *et al.,* 1998).

In recent years there has been a growing interest in the isolation and identification of new microbial surfactants that might have application in enhanced oil recovery processes. The possibility of discovering a unique bio-emulsifier like emulsan that possesses novel properties allowing its use as a gelling agent, emulsifier, stabilizer, flocculant, lubricate, or dispersing agent has encouraged this interest. Biosurfactants are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 mN/m to 25 - 30 mN/m. Biosurfactants are of interest because of their broad range of potential industrial applications, including emulsification, phase separation, wetting, foaming, emulsion stabilization, viscosity reduction of heavy crude oils. Potential applications can be envisaged in several industries such as agriculture, food, textiles, cosmetics, petrochemical, petroleum production. The present study aims at assessing the prevalence of biosurfactant producing microorganisms from contaminated as well as undisturbed soil samples in and around Kanchipuram.

MATERIALS AND METHODS

Collection of soil samples:

The soil samples were collected from different places in and around Kanchipuram (12°50'23"N 79°42'0"E) as shown in Table 1. The samples were collected in sterile polythene bags using sterile spatulas. Soils samples were classified as undisturbed or contaminated and the type of contamination was recorded. An arbitrary naming scheme was used to designate soil samples, which uses SC1-SC5 for contaminated soil samples and SU1-SU5 for undisturbed soil samples.

Bacterial Populations:

One gram of soil was diluted in 99 ml of $Na_4P_2O_7$ (1g/l, pH 7.0). Standard serial dilutions followed and 0.1 ml aliquots of dilution were spread on plates. Total culturable aerobic bacteria were enumerated by the spread plate counting method using Nutrient agar (Himedia) medium. The bacterial populations were enumerated as colony-forming units (CFU) from a serial dilution of the soil suspensions. The colonies were counted after incubation for 3 days at 30°C (Huang *et al.,* 2008).

Screening for biosurfactant producing isolates:

Soils were screened for biosurfactants producing isolates by using the following procedure (Bodour *et al.,* 2003). A 5 g sample of each soil was placed into a 250 ml flask containing 50 ml of sterile tap water and incubated at 23°C on a shaker at 200 rpm for 21 days. On days 3, 7, 14, and 21, a sample of each soil slurry was serially diluted, plated on R2A agar (Himedia), and incubated for 1 week. After incubation, plates were enumerated, and morphologically different bacteria were selected for qualitative biosurfactant screening. Isolated colonies were inoculated into 5 ml mineral salts medium (MSM) containing 2% glucose as the sole carbon and energy source. The MSM was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g of $NaNO_3$, 0.4 g of $MgSO_4 . 7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g of $CaCl_2 . 2H_2O$, and 10 ml of concentrated phosphoric acid (85%). This solution was adjusted to pH 7.2 with KOH pellets. Solution B contained (per liter) 0.5 g of $FeSO_4 . 7H_2O$, 1.5 g of $ZnSO_4 . 7H_2O$, 1.5 g of $MnSO_4 . H_2O$, 0.3 g of K_3BO_3,

0.15 g of $CuSO_4 . 5H_2O$, and 0.1 g of $Na_2MoO_4 . 2H_2O$. One milliliter of solution B was added to 1,000 ml of solution A to form the MSM. The broth cultures were incubated with shaking (200 rpm) for 7 to 9 days at 23°C. The cell free supernatants were then tested for the presence of surfactant by using the qualitative drop collapse method.

Qualitative drop collapse tests were performed in the polystyrene lid of a 96 microwell 12.7 by 8.5 cm plate. The lids have 96 circular wells (internal diameter, 8 mm). A thin coat of 20W-40 oil was applied to each well. The coated wells were equilibrated for 24 hours at 23ºC and then 5µl of each supernatant was delivered into the center of each well. If the drop remained beaded after 2 minutes, the result was scored as negative. If the drop spread and collapsed the result was scored as positive for the presence of biosurfactant.

Statistical analysis:

The data from bacterial population studies and qualitative screening were analyzed statistically (Abraham and Ledolter, 1983; Agresti, 1996). Frequency Table was used to determine ratio of biosurfactant producers in general bacterial population of soil samples. T-test was performed to assess the prevalence of biosurfactant producing microbes in undisturbed versus contaminated soils. Analyses were also performed to assess whether there is any spatial or temporal shift in biosurfactant producing population in various locations of sampling or over a period of 21 days in tap water incubation using Two-way ANOVA.

Quantitative measurement of surface activity:

All the 8 isolates that tested positive in the drop collapse test were then tested quantitatively for biosurfactant production with the drop weight method described by Sabesan *et al*., in 2002 (Sabedan *et al.,* 2002). The isolates were grown in 5ml of mineral salt medium amended with 2% glucose. Cell suspensions were centrifuged at 5000 rpm for 15 minutes and the cell free supernatant was poured in to a burette. The bottom of the burette consists of a rubber tube attached with glass tube of 3 mm diameter. An empty pre-weighed beaker was placed under the burette and the supernatant was released slowly drop by drop. 50 drops were poured in to the beaker and it was weighed to determine the weight of 50 drops.

The mass of one drop was calculated by using the formula

$$\text{Mass of one drop (M)} = \frac{\text{Beaker + Sample weight} - \text{Beaker Weight}}{\text{Number of drops}}$$

Then the surface tension of the supernatant was calculated by using the formula

$$\text{Surface tension (T)} = \frac{Mg}{\pi r} \times 10^{-3} \text{ x nm}^{-1}$$

Where
M = Mass of one drop
g = Gravity
r = Radius of glass tube

Surface activity of each isolate was calculated by the following formula:

Surface activity = Surface tension of uninoculated medium – surface tension of supernatant.

Identification of Potent Isolated Strains:

Standard microbial identification procedure (Cappuccino and Sherman, 1999) were used to characterise the isolated bacterial strains and compared with Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994).

RESULTS AND DISCUSSIONS

The ten soil samples screened for biosurfactant producers were collected primarily from different places in and around Kanchipuram (Table 1). Five sample were collected from metal and petro-products contaminated sites and five sample from undisturbed area. Each soil sample was enumerated for bacterial population, the

total number of culturable, aerobic, bacteria per gram of soil sample was measured and their counts are given in Table 2.

Soils were screened for biosurfactant producing isolates by drop collapse test (Bodour *et al.,* 2003). The initial screening on R2A agar yielded a total of 155 isolates which were grown in MSM glucose broth for a week and then tested qualitatively for biosurfactant production with the drop collapse test employing 96 well microplate lid and 8 positive strains were identified. The surface active isolates obtained in this study were designated as BS1 to BS8. Of the 10 soils tested, 5 contaminated soils contained 8 biosurfactant producers and interestingly none of the undisturbed soils contained biosurfactant producers (Table 2). Bodour *et al*., (2003), obtained 45 biosurfactant producing isolates out of 1305 isolates screened from southwestern Arizona soils. Most of the biosurfactant producing colonies were obtained from undisturbed soils. Only two of the six hydrocarbon contaminated soils yielded biosurfactant producers. It is interesting to note that in the present study, all 8 biosurfactant producers were obtained from hydrocarbon and metal contaminated soil samples contrary to the previous findings. These differences may be attributed to variations in soil type and different pattern of microbial evolution. Similarly, Tabatabaee *et al*., (2005) have reported isolation of 45 biosurfactant producing bacteria from petroleum hydrocarbon contaminated site in west Iran.

Table 1: Collection of soil samples in Kanchipuram District

Soil type/Location	Sample No	Contamination	Geographical coordinates	Soil Texture
Contaminated				
Fuel bunk	SC1	Petrol and diesel	$12^0$50'39.10" N $79^0$41'58.38" E	Fine
Welding workshop	SC2	Metal and waste oil	$12^0$50'06.58" N $79^0$42'28.17" E	Fine
Motor mechanic shed	SC3	Motor oil	$12^0$50'55.89" N $79^0$42'22.06" E	Fine
Ground nut oil factory	SC4	Waste oil	$12^0$50'08.41" N $79^0$42'05.38" E	Fine
Kerosene oil shop	SC5	Kerosene	$12^0$50'22.58" N $79^0$41'51.72" E	Fine
Undisturbed				
Palar river basin I	SU1	None	$12^0$47'46.04" N $79^0$42'12.55" E	Sand
Palar river basin II	SU2	None	$12^0$46'46.25" N $79^0$45'11.99" E	Sand
Forest area	SU3	None	$12^0$36'08.07" N $80^0$11'24.40" E	Sand
Mountain I	SU4	None	$12^0$44'41.51" N $79^0$39'54.38" E	Fine
Mountain II	SU5	None	$12^0$44'22.25" N $79^0$39'56.68" E	Fine

Table 2: Enumeration of soil microbial counts and qualitative screening for biosurfactants

Sample No	Total aerobic bacterial count X10^5 CFU/g	CFU after Days of incubation in water (X10^3 CFU/g)				Number of isolates screened	Biosurfactants producing isolates
		3 days	7 days	14 days	21 days		
Contaminated							
SC1	33.3 ± 1.5	154.0 ± 2.0	22.6 ± 2.0	21.3 ± 1.1	8.0 ± 1.0	35	3
SC2	46.0 ± 1.0	93.6 ± 1.5	17.0 ± 1.0	14.3 ± 1.1	12.0 ± 1.0	18	1
SC3	44.0 ± 1.0	113.6 ± 1.5	12.0 ± 1.0	8.0 ± 1.0	13.0 ± 1.0	22	2
SC4	63.3 ± 1.5	67.0 ± 2.0	11.3 ± 0.5	22.3 ± 0.5	11.3 ± 1.1	12	1
SC5	53.0 ± 2.6	31.6 ± 0.5	7.0 ± 1.0	16.0 ± 1.0	8.0 ± 1.0	8	1
Undisturbed							
SU1	46.0 ± 0.5	34.0 ± 2.6	25.0 ± 1.0	18.0 ± 1.0	11.6 ± 0.5	10	0
SU2	27.0 ± 2.0	37.6 ± 2.3	30.6 ± 1.1	26.3 ± 0.5	15.0 ± 1.0	11	0
SU3	26.0 ± 1.0	43.6 ± 1.5	30.3 ± 1.5	23.3 ± 1.5	19.0 ± 1.0	14	0
SU4	46.0 ± 1.0	46.3 ± 1.5	25.3 ± 1.5	28.6 ± 1.5	21.0 ± 1.0	13	0
SU5	63.0 ± 2.0	36.6 ± 1.5	27.6 ± 1.5	25.0 ± 1.0	18.3 ± 1.5	12	0

Based on the statistical analysis, the average bacterial population of soil sample was found to be 44.76X10^5 CFU/g of soil, in which ~ 0.0000018 % is biosurfactant producing isolates, while the computed value for contaminated soil alone is ~ 0.0000033 %. The probability of isolating a biosurfactant producer from contaminated soil is approximately two fold greater when compared to undisturbed soil. A two-way ANOVA was performed using location of soil sample as one variable and duration of incubation in water as another variable to asses whether there is any spatial or temporal shift in biosurfactant producing population. The calculated F-Ratio for location of the sample was 1.44 while that of duration of incubation was 1.2 and both these values were found to be less than table values. Based on ANOVA, the present study clearly demonstrates that there is no change in number of biosurfactant producing microbes over a space or period, even though, the raw data showed 6 out of 8 isolates were recovered only after 14 days of incubation. The results of T-test demonstrated that the biosurfactant producing microbes are abundant in contaminated soils and extremely meager in undisturbed soils. It was also previously reported that the biosurfactant producing isolates were obtained generally after 14-21 days of incubation in tap water (Bodour *et al.,* 2003).

All the 8 isolates that tested positive in the drop collapse test were then tested quantitatively for biosurfactant production with the drop weight method (Sabesan *et al.,* 2002). The results are shown in Table 3. Maximum surface activity was observed in BS3 (26.58 x 10^{-3} nm^{-1}) followed by BS5 (20.60 x 10^{-3} nm^{-1}).

These two potential bacterial isolates BS3 and BS5 were characterized morphologically and biochemically and identified as strains of *Bacillus sp.* (BS3) and *Pseudomonas sp.* (BS5) as described in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994).

Table 3: Quantitative measurement of surface activity of biosurfactant producing isolates using drop weight method

S.No	Inoculated Isolate	Surface tension of MSM x 10^{-3} nm^{-1}	Surface activity x 10^{-3} nm^{-1}
1	Uninoculated control	68	-
2	BS1	48.92	19.08
3	BS2	50.80	17.20
4	BS3	47.40	**20.60**
5	BS4	52.65	15.35
6	BS5	41.42	**26.58**
7	BS6	49.80	18.20
8	BS7	47.42	20.58
9	BS8	54.34	13.66

Conclusions:

The present study is the first preliminary assessment on the distribution of biosurfactant producing bacterial population in Kanchipuram soils. The results showed that biosurfactants producing bacteria are widely distributed in contaminated soils. In addition, two potent biosurfactant-producing bacterial isolates *Bacillus sp.* BS3 and *Pseudomonas sp.* BS5 were chosen as research targets for biosurfactant production, to be described in a further work.

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