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Isolation and Characterization of Potent Biosurfactant Producing Bacteria from Petroleum Contaminated Soil and Sea Water

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***Abstract*— Biosurfactant are surface active biomolecules possessing unique and beneficial properties as compared to the synthetic ones. The objective of this study was to isolate and characterized potent biosurfactant producing bacteria from petroleum contaminated soil samples and sea water samples. Sunflower oil was used as a sole source of carbon for the bacterial growth. In all 39 oil degrading bacteria were recovered from six different sites in Mumbai which were subjected to biosurfactant screening tests. After performing various qualitative and quantitative tests, SWSF1 strain, identified as *Pseudomonas aeruginosa,* reduced surface tension of media by 32% and exhibited potent biosurfactant producing ability. Chemically it was found to be rhamnolipid type. As this bacterium gave high yields of biosurfactant it can be possible to use this isolate for industrial production of rhamnolipids.**

Keywords—Biosurfactant;PseudomonasAeruginosa; unflower Oil; Rhamnolipid

I. INTRODUCTION

Surfactants are surface active agents that reduce the interfacial tension between two liquids, or liquid and a solid. Surfactants contain both hydrophobic moieties (head part) which are water insoluble i.e. water repellent groups and hydrophilic moieties (tail part) which are water soluble i.e. water loving group. Currently, almost all surfactants are being derived from petroleum sources by the processes such as ethoxylation, sulfonation, fractional distillation, hydroformylation. Most of the surfactants have side chains that are branched as a result they accumulate in environment and are hardly degraded by microbes [1]. When concentration of synthetic surfactants in soil is high they tend to release toxic pollutants like polychlorinated biphenyls. Some of these pollutants have harmone like effects on marine organisms and these synthetic surfactants are toxic and tend to accumulate which are hardly degraded by microorganisms [2]. In recent years due to association of synthetic surfactants with environment hazards microbial production of surfactants has received considerable attention [3].

Microbial derived surface active biomolecules called biosurfactants (BS) either adhere to cell surface or are secreted extracellularly in growth medium. Most commonly isolated biosurfactants are glycolipids and lipopeptides in nature. Glycolipids are lipids in nature with a hydrophilic carbohydrate attached by a glycosidic bond. When micro-organisms are grown on hydrophobic substrates they have difficulties in using water immiscible substrates like oil as a carbon source because of their low solubility in water. They are either taken up directly by efficient transportation across cell membrane or by secreting certain extracellular factors that result into solubilization or emulsification which results into hydrocarbon uptake inside the cell. One of such extracellular solubilizing mediator is biosurfactant. Some of the examples of biosurfactants are Rhamnolipids released by *Pseudomonas aeruginosa* [4], Saphorolipids produced by *Candida spp* [5] as well as Surfactin and Iturin from *Bacillus subtilis* strains [6].

Microbial surfactants are structurally different group of surface-active compounds produced by variety of microorganisms and are receiving considerable attention due to their unique properties [7]. Biosurfactants has beneficial properties when compared to their chemically synthesized counterparts such as biodegradability, low toxicity towards humans, easy availability of raw materials for their production, stability over various range of physical factors (Temperature, pH, ionic strength), reduction in surface tension and interface activity, biocompatibility and digestibility.

Primarily, biosurfactants were considered only as hydrocarbon dissolving agents but now their applications have been greatly extended to replace chemical surfactants such as carboxylates, sulphonates and sulphate acid esters especially in oil, pharmaceutical and food industry [8]. At the present day, biosurfactants have great market demand but these biomolecules do not compete economically with synthetic counterparts because of their higher production cost. Aim of this study was to isolate potent biosurfactant producing bacteria from petroleum contaminated sea water and soil samples for the production of biosurfactant at industrial level.

II. MATERIALS AND METHODS

1. *Isolation of biosurfactant producing bacteria*

Sample collection:

Bacterial cultures used in this study were isolated from soil samples collected from Mumbai from four different sites; Dombivali petrol pump, Matunga petrol pump, oil refinery, Karjat. Sea water samples were also collected from Gateway of India located in Mumbai and Karanja sea located near Alibaug.

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Enrichment and isolation of bacterial colonies:

1% (w/v) of each soil samples were inoculated in 100ml of Mineral Salt Medium (MSM) [NaNO3 1.5g/L, KH2PO4 1.0 g/L, MgSO4.7H2O 0.5 g/L, MnSO4 1.5g/L, CaCl2 0.02g/L, (NH4)2SO4 1.5g/L and FeSO4 0.01 g/L] with 1% (v/v) sunflower oil. All the flasks were incubated at 30°C for 7 day. Isolation was done on MSM agar plate containing 1% sunflower oil. After 2 days of incubation morphologically distinct colonies were selected. They were gram stained and used for further studies.

2. Screening for biosurfactant production

Isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following methods:

2.1 Qualitative methods

a) Blood hemolysis test

Bacterial cultures were spot inoculated on to superimposed blood agar (SIBA) plates and incubated at 30ºC for 48hrs. The plates were visually observed for clear zone (hemolysis) around the colony. The diameter of the clear zone was recorded. It is a qualitative indicator of biosurfactant production [9].

b) Oil displacement assay and Emulsification assay

100 ml of MSM broth containing sunflower oil 1% (v/v) was inoculated with 1% inoculum (0.08 OD at 540nm) of the isolates showing β hemolysis and incubated at 30ºC at 200rpm for 7 days. After incubation broth was centrifuged at 8000rpm at 4ºC for 15 minutes. Supernatant was used for following screening procedures:

Oil displacement assay

20ml of distilled water was taken in petridish and 20 µl of sunflower oil was added on the surface of the water. 10 µl of cell free culture broth was then added to the oil surface. Immediate oil displacement is the indicator of biosurfactant production. Distilled water and Tween-20 were used as negative and positive controls respectively [10].

Emulsification assay

1ml of cell free culture broth was added to 5ml of 50mM-Tris buffer at pH 7. 2ml of sunflower oil was added to this mixture and vortexed for 2 mins and incubated at room temperature for 24 hrs. Emulsification activity was measured as E24 (emulsification after 24hrs) [9].

$$E24 = \frac{\text{Height of emulsion}}{\text{Total height}} \times 100$$

Distilled water and Tween-20 were used as negative and positive controls respectively.

c) CTAB agar plate method

This is semi quantitative assay to detect production of anionic biosurfactant. CTAB agar plate were prepared by adding N,N,N-trimethyl ammonium bromide(0.5mg/ml) and basic dye methylene blue (0.2mg/ml) in MSM with 2% glycerol as a carbon source. Cultures were spot inoculated on CTAB agar plates at 30ºC for 4 to 5 days. If an anionic biosurfactant is secreted by microbes growing on the plate they form ion pair with CTAB and methylene blue giving dark blue halos [11]. Which can also be observed under UV trans illuminator.

d) Foaming activity

Isolates were inoculated in nutrient broth and incubated at 30ºC at 200rpm for 72 hrs. Foam produced in inoculated nutrient broth after 72hrs can be used as an indicator for constitutive production of biosurfactant even in the absence of hydrophobic substrate.

2.2 Quantitative method

Measurement of surface tension

100ml of MSM broth containing sunflower oil 1% (v/v) was inoculated with 1% inoculum (0.08 OD at 540nm) of isolates showing β hemolysis and incubated at 30ºC at 200rpm for 7 days. After incubation, broth was centrifuged at 8000rpm at 4ºC for 15 mins. Surface tension of supernatant was measured by surface tensiometer based on Wilhelmy principle and compared with uninoculated broth. The results were expressed in millinewton per meter (mN/m). The surface activity of the biosurfactant was expressed in terms of percentage reduction in surface tension which was calculated by the following formula [12],

$$\text{Percentage of the reduction in Surface tension (\%)} = \frac{(\gamma m - \gamma c)}{\gamma m} \times 100$$

ɣm = surface tension of uninoculated control medium and

ɣc = surface tension of test supernatant.

3. IDENTIFICATION OF POTENT BIOSURFACTANT PRODUCER

Isolate showing highest activity was identified based on 16srRNA analysis. Gene sequencing was performed at Metropolis, India to identify the isolate. Genomic DNA was extracted from isolated bacterial colony by using commercial kit (GenElute bacterial genomic DNA kit, sigma, USA). PCR was carried out using the universal primers such as XB4 (10 pm/ µl) and PSL (10 pm/ µl). 2X KAPA Mix contained KAPA Taq DNA polymerase (1U/50 µl), KAPA Taq buffer, dNTPs (0.2 mM of each dNTP at 1X), MgCl2 (1.5 mM at 1X) and Stabilizer 3 µl of extracted DNA and HPLC grade water. ProFlex™ PCR system was used for PCR with the following cycle program, 95ºC for 5 minutes, 30 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds, 72 ºC for 45 seconds and final extension was carried out at 72 ºC for 7 minutes and sample was held at 4ºC. PCR product was then subjected to sequencing PCR by ProFlex™ PCR system (Applied Biosystem). Total volume of reaction mixture contained HPLC grade water, 5X Sequencing buffer 1.75 µl, Big Dye™ Terminator v3.1(Sequencing RR mix) 0.5 µl, primer XB4 (3.33 pm/ µl), PCR product 0.3 µl (3-5ng).Total reaction mixture volume was subjected to sequencing PCR, which consisted of stage 1, 96ºC for 3 minutes, 25 cycles of stage 2 : 96ºC for 10 seconds, 50 ºC for 5 seconds, 60 ºC for 1 minute and finally held at 4 ºC.

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Finally the product was loaded on sequence-3500DX Genetic analyzer for Sanger sequencing and then DNA sequence was compared with NCBI GenBank entries by using BLAST algorithm.

III. RESULTS AND DISCUSSIONS

39 different oil degrading bacterial isolates were isolated from soil contaminated with hydrocarbon and from sea water. All the isolated strains were then screened for their ability to produce biosurfactant. Biosurfactant producing strain of *Pseudomonas aeruginosa MTCC 7925* was used as a positive control. More than one screening procedures were employed for studying the production of biosurfactant by the isolates [13].

Out of 39 isolated cultures 14 were found to be gram positive and 25 were found to be gram negative. As shown in in table 1, 12 strains tested positive for β hemolysis. In this study hemolytic property was used as a primary criterion for biosurfactant production because biosurfactant being surfactant in nature causes conformation change in RBC's resulting in lysis which can be observed as a zone of clearance around the colony [14]. The hemolytic activity of biosurfactants was first discovered by Bernheimer and Avigad who reported lysis of RBC's by biosurfactant produced by *B. subtilis*. Carrillo et al devised blood agar lysis as a primary method to screen for biosurfactant production. None of the studies in the literature reported non hemolytic property of biosurfactant. However, hemolysis does not always mean biosurfactant production compounds other than biosurfactants may cause hemolysis.

Hence in the present study in addition to hemolysis test, oil displacement assay, emulsification assay, foaming activity and surface tension measurements were included to confirm biosurfactant production.

To confirm the biosurfactant production by hemolytic strains oil displacement assay was used. Morikawa et al reported that the area of oil displacement directly correlates to the amount of the biosurfactant in the solution. However, in this study oil displacement assay was used as qualitative measure to check presence of biosurfactant.

Out of 12 hemolytic strains, 8 strains tested positive for oil displacement assay. Principle of this technique is based on the ability of biosurfactant to alter the contact angle at water oil interface [15].

Biosurfactant production by isolates was further confirmed by emulsification assay. All the strains showing oil displacement were found to give emulsification with sunflower oil in emulsification assay after 24hrs.

Table 1: Hemolysis results

Sr. No.	Isolate name	Type of hemoly-sis	Zone of clearance in mm	Sr. No.	Isolate name	Type of hemoly-sis	Zone of clearance in mm
1.	DPSS1	ɣ	-	21.	AN41	ɣ	-
2.	DPSS2	β	25	22.	AN51	β	-
3.	DPSS3	ɣ	-	23.	AN61	β	22
4.	DPSS4	ɣ	-	24.	AN62	ɣ	13
5.	DPSS5	β	30	25.	MSSD1	ɣ	-
6.	SWSF1	β	35	26.	NS1	β	18
7.	SWSF2	β	35	27.	LP1	β	13
8.	SWSF3	β	35	28.	LP2	ɣ	-
9.	MPSS1	ɣ	-	29.	LP3	ɣ	-
10.	MPSS2	ɣ	-	30.	LP4	ɣ	-
11.	MPSS3	ɣ	-	31.	LP5	ɣ	-
12.	MPSS4	ɣ	-	32.	LP6	ɣ	-
13.	KSS1	ɣ	-	33.	LP7	ɣ	-
14.	KSS2	β	30	34.	LP85A	β	20
15.	KSS3	ɣ	-	35.	LP820bX	β	21
16.	KSS4	ɣ	-	36.	LP9	β	22
17.	AN31	ɣ	-	37.	LP10	ɣ	-
18.	AN32	ɣ	-	38.	LP11	ɣ	-
19.	AN41	ɣ	-	39.	LP12	ɣ	-
20.	ANB	ɣ	-	40.	*P.aeruginosa MTCC 7925*	β	28

Results are shown in table 2. Maximum emulsification was observed in case of SWSF1 strain

To determine if biosurfactant produced by isolates is anionic in nature CTAB agar plate method was used. Dark blue halos was observed around only colonies of 3 isolates namely SWSF1, SWSF2, SWSF3 out of 8 isolates which indicates that SWSF1, SWSF2, SWSF3 produced anionic biosurfactant such as rhamnolipid. For the easier detection of halos, plates were observed under UV trans illuminator. Rhamnolipid being an anionic biosurfactant can form a complex with the cationic compounds (CTAB) that were identified by the formation of dark blue areas around colonies on the agar plates as shown in figure 1 and 2.

Constitutive production of biosurfactant was determined by observing foam in inoculated nutrient broth after 72hrs of incubation. After incubation the inoculated nutrient broth with 8 positive cultures flasks were hand shaken and foam was observed as shown in figure 3 and 4. All the cultures were found to produce foam after 72 hrs except LP820BX and LP85A. Foam stability was also checked for and found that foam produced by three SWSF strains was stable for more than 3days as shown in figure 5 and by two AN strains was stable upto 2 days as shown in figure 6.

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Table 2: Emulsification activity of the isolates

Sr. no.	Test cultures	E24 %
1.	AN51	29.89
2.	AN61	28.78
3.	SWSF1	37.77
4.	SWSF2	28.88
5.	SWSF3	33.33
6.	KSS2	32.50
7.	LP820BX	27.88
8.	LP85A	28.09
9.	*P. aeruginosa MTCC 7925*	33.00
10.	POSITIVE CONTROL (Tween20)	65
11.	NEGATIVE CONTROL (distilled water)	-

Foaming activity is tested to find the culture ability to produce biosurfactant even in the absence of hydrophobic substrates i.e. constitutively. *Pseudomonas aeruginosa MTCC 7925* was also tested as a positive control.

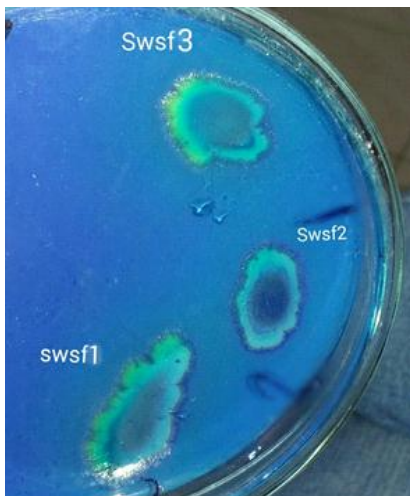

Figure 1. Colonies growing on CTAB agar plate showing dark blue precipitation outline.

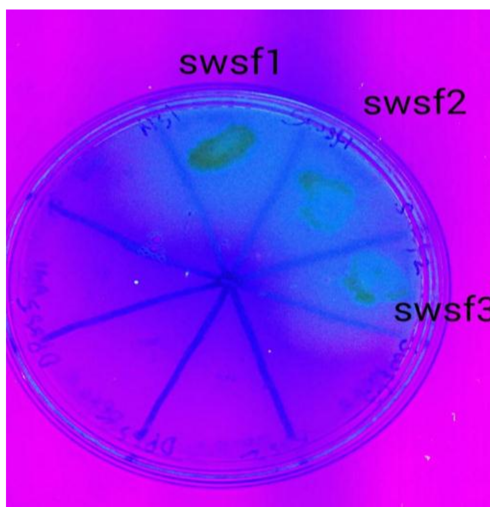

Figure 2. Fluorescence around colonies on CTAB agar plate under UV trans illuminator.

Figure 3. Foam produced in inoculated nutrient broth after 3 days of incubation.

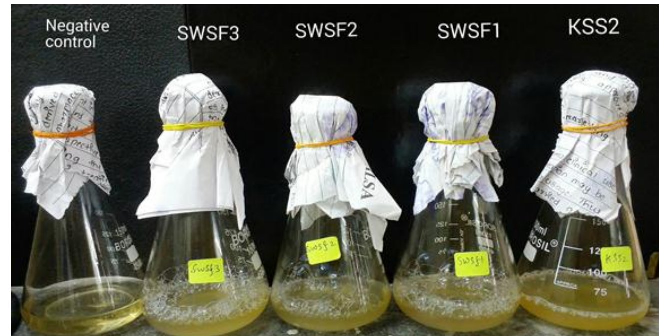

Figure 4. Foam stability of three SWSF and one KSS2 cultures after 3 days.

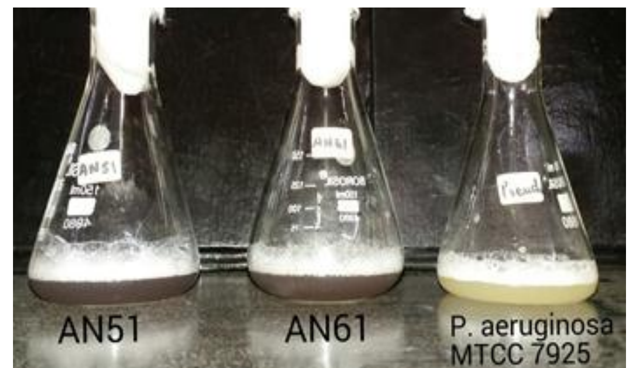

Figure 5. Foam produced in inoculated nutrient broth after 3 days of incubation.

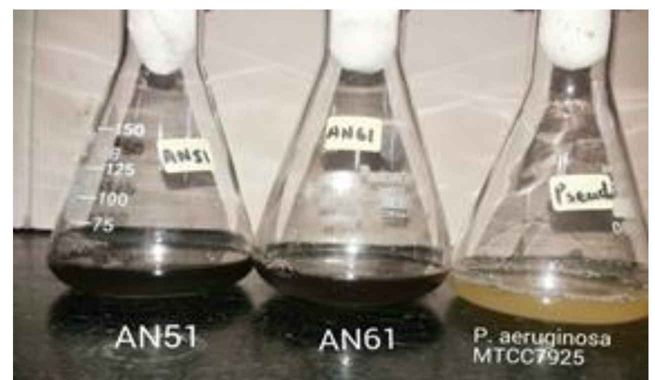

Figure 6. Foam stability of the two AN culture and *P. aeruginosa MTCC 7925* after 2 days.

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After all the qualitative tests, the quantitative test based on surface tension measurement was carried out by dynamic surface tensiometer based on Wilhemy principle. To screen and select the maximum biosurfactant producer among all the positive cultures surface tension of spent broth was measured. *Pseudomonas aeruginosa MTCC 7925* was also tested as a positive control.

SWSF1 was found to lower the surface tension of MSM media by 31.14% which was highest among the selected biosurfactant producing isolates.

The results of screening tests repeatedly proved the biosurfactant producing property of the isolate. The potent biosurfactant producing isolate belonged to the genus *Pseudomonas*. The 16S rRNA sequence alignment shows that the strain SWSF1 was closely related to *Pseudomonas aeruginosa*.

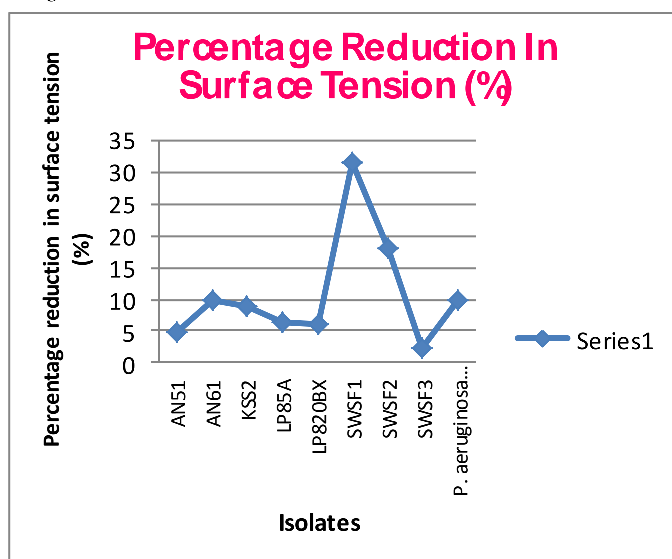

Figure 7. Percentage reduction in surface tension of media by the isolates.

IV. CONCLUSIONS

In present study bacteria capable of producing biosurfactant for the solubilization of hydrocarbon were isolated. Bacteria were tested qualitatively and quantitatively for their ability to produce the biosurfactant. Among 39 isolates screened, SWSF1 gave the maximum reduction in the surface tension of media by 32%. In camparison with all the isolates SWSF1 yielded more biosurfactant. Isolated strain of *Pseudomonas* was found to produce more biosurfactant as compared to the standard biosurfactant producing strain of *Pseudomonas aeruginosa MTCC 7925*. CTAB assay was used to detect the presence of anionic biosurfactant like rhamnolipid. SWSF1 showed precipitation around the colony which indicate the capacity of the bacterium to produce rhamnolipid. As this bacteria gave high yields, it can be possible to use this isolate for the industrial production of rhamnolipid. It can be a source of rhamnose which is an expensive sugar. Foaming activity also revealed the potential of the bacterium to produce the biosurfactant even in the absence of hydrophobic substrates which has an added advantage in case of industrial production of rhamnolipid.

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