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Screening of Biosurfactant/Bioemulsifier Producing Bacteria from Petroleum Contaminated Soil

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ABSTRACT

The release of impurities in the environment, containing petroleum and petroleum cogitated products, is engenders of global being taint. It is also a hazardous for human and animal health, since many of these impurities have evidenced to be toxic and oncogenic. Hydrocarbon particles that are secreted into the environment are hard to get rid of, since they change state to surfaces and are captured by surface tension in a water immiscible stage. Bioremediation has tested to be an alternate to lessen the effects caused to impureness of soil and water, applying the metabolic abilities of microorganisms that can apply hydrocarbons as source of carbon and energy, or that can alter them by co metabolism. The proficiency of removal is directly related to the compound's chemical structure, to its bioavailability (deliberation, harmfulness, flexibility and approach) and to the physicochemical situation present in the atmosphere.

KEYWORDS: *Screening, PetroleumSoil, Biosurfactant, Bioemulsifier, Contaminated, Bacteria*

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INTRODUCTION

Surfactants are surface active agents that reduce the interfacial enmity between two liquids, or liquid and a solid. Surfactants contain both hydrophobic entities (head part) which are water unsoluble i.e. water repellent groups and hydrophilic entities (tail part) which are water soluble i.e. water loving group. Currently, almost all surfactants are being inferred from petroleum sources by the procedures such as ethoxylation, sulfonation, fractional refinement, hydro formulation. Most of the surfactants have side sets that are branched as a consequence, they accumulate in environment and are barely degraded by microorganisms. When concentration of imitative surfactants in soil is high they incline to release toxic pollutants like polychlorinated biphenyls. Some of these impurities have effects like endocrine on marine organisms and these synthetic surfactants are toxic and incline to accrete which are hardly degraded by microorganisms. In recent years due to association of synthetic emulsifiers with environment perils microbial production of surfactants has received ample attention.

Microbial consequent surface active biomolecules called biosurfactants (BS) either cohere to cell surface or are released extracellularly in growth unit. Most commonly assorted biosurfactants are glycolipids and lipopeptides in kind. Glycolipids are lipids in nature with a hydrophilic carbohydrate impounded by a glycosidic bond. When microorganisms are grown on hydrophobic surfaces they have difficulties in using water incongruous substrates like oil as acarbon source because of their low measure. They are either scooped directly by efficient transportation through cell membrane or by releasing certain extracellular factors that turns upinto solubilization or emulsification which leads to hydrocarbon uptake within the cell. One of such extracellular solubilizing intercessor is biosurfactant. Some of the instance of biosurfactants are Rhamnolipids exuded by Pseudomonas Aeruginosa, Saphorolipids produced by Candida spp. as well as Surfactin and Iturin from Eubacteria strains.

Microbial surfactants are anatomically different group of surface-active combinations produced by variety of microorganisms and are getting considerable attention due to their peculiar properties. Biosurfactants has useful properties when compared to their chemically synthesized similitudes such as biodegradability, low toxicityconcerning humans, easy availability of raw materials for production, stability over various range of physical factors (Temperature, pH, ionic intensity), deduction in surface tension and interface activity, biocompatibility and edibility.

Majorly, biosurfactants were considered only as hydrocarbon solvents but now their applications have been greatly extended to replace chemical surfactants such as carboxylates, sulphonates also sulphate acid esters especially in oil, pharmaceutical and food industry. Currently, biosurfactants have great market requirement but these biomolecules do not contend economically with synthetic equivalents because of their higher creation cost. Aim of this study was to classify potent biosurfactant producing bacteria from petroleum contaminated soil samples for the making of biosurfactant at industrial level.

Materials and Techniques

Biosurfactant producing bacteria

Sample collection:

Four different bacterial samples used in this study were obscure from soil samples.

Enrichment and isolation of bacterial colonies:

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

Screening for biosurfactant creation

Isolated clusters were obtained in sample and tested for their biosurfactant production by the following methods:

Qualitative methods

A. Blood hemolysis test

Bacterial cultures were spot enclosed on to superimposed blood agar (SIBA) plates and fostered at 30°C for 48hrs. The plates were visually remarked for hemolysis around the settlement. The diameter of the purestructure was recorded. It is a qualitative signal of biosurfactant production.

B. Oil displacement assay and Emulsification assay

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

Oil displacement assay

20ml of water was kept inPetri dish and 20µl of sunflower oil was supplemented the surface of the water. 10µl of cell free cultivation victuals was then added to the oil surface. Contiguous oil displacement is the indicator of biosurfactant creation. Distilled water and Tween-20 were used as negative and positive controlsseverally.

Emulsification assay

1ml of cell free culture victuals was added to 5ml of 50mM-Tris buffer at pH 7. 2ml of sunflower oil was added to this mixture and stream for 2 mins and nurtured at room temperature for 24 hrs. Emulsification was evaluated as E24 (emulsificationafter 24hrs).

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

Distilled water and Tween-20 were used as negative and positive ensures severally.

C. CTAB agar plate method

This is semi valued assay to detect creation of anionic biosurfactant. CTAB agar plate were inclined by appending N, N, N-trimethyl ammonium bromide(0.5mg/ml) and plain dye methylene blue (0.2mg/ml) in MSM with 2% glycerol as a carbon foundation. Cultures were pointenclosed on CTAB agar plates at 30°C for 4-5 days. If an anionic biosurfactant isreleased by microbes producing on the plate they form ion match with CTAB and methylene blue yielding dark blue halos. Which can be detected under UV trans illuminator.

D. Foaming activity

Assorts were enclosed in nutrient stock and incubated at 30°C at 200rpm for 72 hrs. Foam produced in enclosed nutrient stock after 72hrs can be used as an indicator for constituent production of biosurfactant even in the deficiency of hydrophobic substrate.

Quantitative method

Measurement of surface tension

100ml of MSM stockincluding sunflower oil 1% (v/v) was enclosed with 1% substance (0.08 OD at 540nm)

of insulates showing β hemolysis and altered at 30oC at 200rpm for 7 days. After incubation, stock was centrifuged at 8000rpm at 4°C for 15 mins. Surface tension of supernatant was evaluated by surface tensiometer based on Wilhemy principle and matched with uninoculatedstock. The results were conveyed in mill newton per meter (mN/m). The surface activity of the biosurfactant was denoted in terms of percentage fall in surface tension which was calculated by the subsequent formula,

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

ɣm = surface tension of uninoculated control component and

ɣc = surface tension of measurement supernatant

Identification of Potent Biosurfactant Producer

Discrimination showing highest action was identified settled on 16srRNA analysis. Gene sequencing was executed at Metropolis, India to recognize the isolate. Genomic DNA was educed from isolated bacterial settlement by using commercial kit. PCR was carried out utilizing the universal primers such as XB4 (10 pm/ µl) and PSL (10 pm/ µl). 2X KAPA Mix included KAPA Taq DNA polymerase (1U/50 µl), KAPA Taq cache, dNTPs (0.2 mm of each dNTP at 1X), MgCl2 (1.5 mm at 1X) and Preservative 3 µl of extracted DNA and HPLC rank water. ProFlex PCR arrangement was used for PCR with the following cycle sequence, 30 cycles of 95 ºC for 30 seconds,95ºC for 5 minutes, 60 ºC for 30 seconds, 72 ºC for 45 seconds and final elongation was carried out at 72 ºC for 7 minutes and sample was detained at 4ºC. Total volume of reaction substance contained5X Sequencing buffer 1.75 µl, HPLC mark water, primer XB4 (3.33 pm/ µl),Big Dye Terminator v3.1(Sequencing RR mix) 0.5 µl, PCR product 0.3 µl (3-5ng).Total substance volume was impacted to sequencing PCR, which comprised of stage 1, 96ºC for 3 minutes, 25 cycles of stage 2 - 96ºC for 10 seconds, 50º C for 5 seconds, 60ºC for 1 minute and finally held at 4ºC.

Finally the product was laden on sequence-3500DX Genetic unit for Sanger sequencing and so DNA sequence was linked with NCBI GenBank entries by applying BLAST algorithm.

Results and Discussions

39 different oil degrading bacterial assorts were isolated from soil tainted with hydrocarbon. All the classified strains were then examined for their ability to make biosurfactant. Biosurfactant evolving strain of Pseudomonas aeruginosa MTCC 7925 was utilized as a positive mechanism. More than one examining procedures were applied for studying the manufacture of biosurfactant by assorts.

Out of 39 isolated values 14 were detected gram positive and 25 were detected gram negative. As shown in in table 1, 12 strains examined positive for β hemolysis. In this study hemolytic attribute was used as a primary measure for biosurfactant production because biosurfactant being surfactant in nature induces form change in RBC's resulting in lysis which can be found as a zone of clearance over the colony. The chemical action of biosurfactants was firstset off by Avigad and Bernheimer who detectedh emolysis of RBC's by biosurfactant made by B. subtilis. Carrillo et al invented blood agar lysis as a primary technique to examine of biosurfactant production. None of the studies in the profession reported non hemolytic attribute of biosurfactant. However, hemolysis does not perpetually mean biosurfactant production chemicals other than biosurfactants may induce hemolysis.

Hence in the present study in accumulation to hemolysis test, oil transposition assay, emulsification assay, foaming action and surface tension capacities were included to confirm biosurfactant creation.

To confirm the biosurfactant creation by hemolytic strains oil transposition assay was applied. Morikawa covered that the area of oil transposition directly correlates to the sum of the biosurfactant in the solvent. However, in this study oil transposition assay was applied as qualitative measure to check occurrence of biosurfactant.

Out of 12 hemolytic strains, 8 strains verified positive for oil transposition assay. Principle of this method is based on the capacity of biosurfactant to modify the contact angle at water oil surface.

Biosurfactant creation by assorts was further corroborated by emulsification assay. All the strains showing oil transposition were found to give emulsification thru sunflower oil in emulsification assay once 24hrs.

Table 1: Hemolysis results

Sr. No.	Isolate name	Type of hemolysis	Zone of clearance in mm	Sr. No.	Isolate name	Type of hemolysis	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.aerugin osa MTCC 7925	β	28

Results shown in table 2. Extremum emulsification was detected in case of SWSF1 strain

To find out if biosurfactant made by isolates is anionic in type CTAB agar plate technique was used. Dark blue halos was detected around only colonies of 3 assorts namely SWSF1, SWSF2, SWSF3 out of 8 assorts which indicates that SWSF1, SWSF2, SWSF3 formed anionic biosurfactant such as rhamnolipid. For the simpler detection of halos, plates were detected under UV trans illuminator. Rhamnolipid presence an anionic biosurfactant can form a complex with the cationic compounds (CTAB) that were recognized by the development of dark blue areas across colonies on the agar plates as presented in figure 1 and 2.

Essential manufacture of biosurfactant was determined by observing foam in enclosed nutrient broth after 72hrs of activity. After phase the enclosed nutrient stock with 8 positive values flasks were hand stirred and foam was detected as shown in figure 3 and 4. All the cultures were found to yield foam after 72 hrsexcluding LP820BX and LP85A. Foam stability was also tested for and detected that foam formed by three SWSF strains was steady for more than 3days as shown in figure 5 and via two AN strains was steady upto 2 days as shown in figure 6.

Table 2: Emulsification activity of the assorts

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 action is tested to find the value'scapacity to produce biosurfactant even in the lack of hydrophobic substrates i.e. constitutively. Pseudomonas aeruginosa MTCC 7925 was also verified as a positive mechanism.

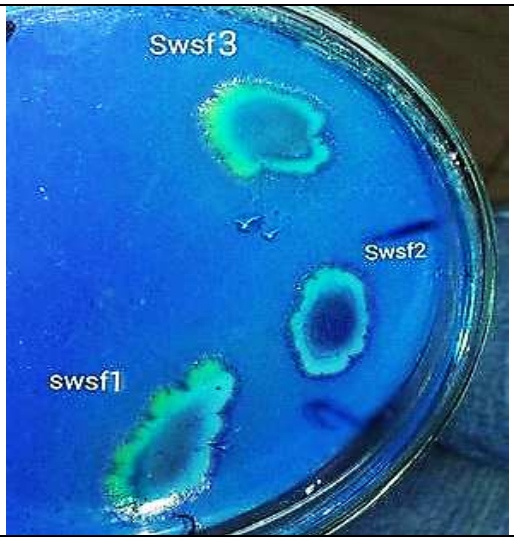

Figure 1. Colonies growing on CTAB agar plate presenting dark blue precipitation outline.

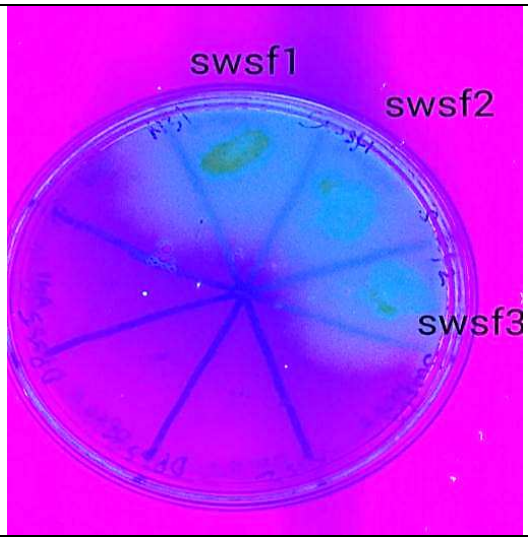

Figure 2. Fluorescence across colonies on CTAB agar plate below UV trans illuminator.

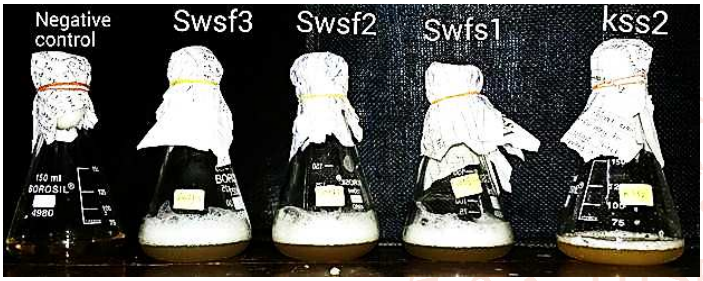

Figure 3. Foam produced in enclosed nutrient broth after 3 days of incubation.

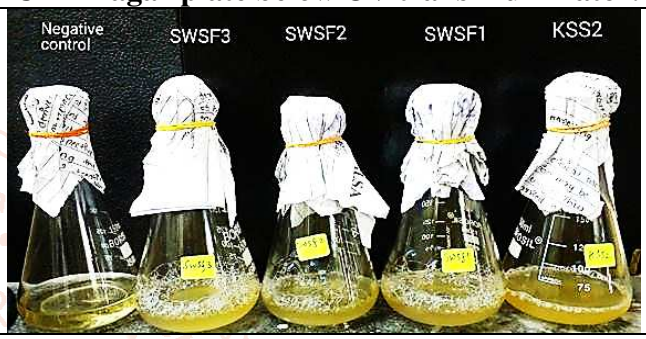

Figure 4. Foam stability of three SWSF and one KSS2 values after 3 days.

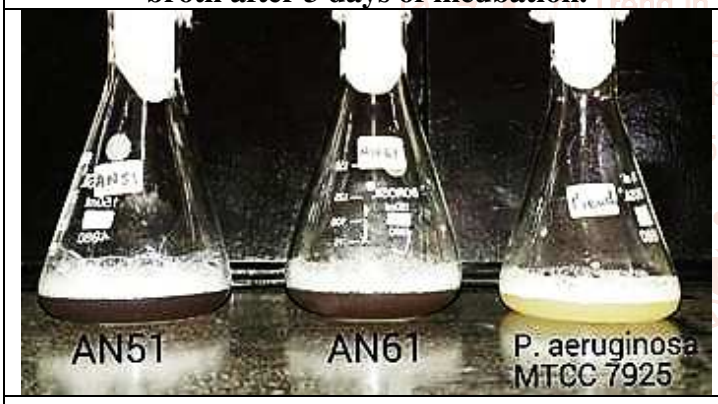

Figure 5. Foam created in enclosed nutrient broth after 3 days of incubation.

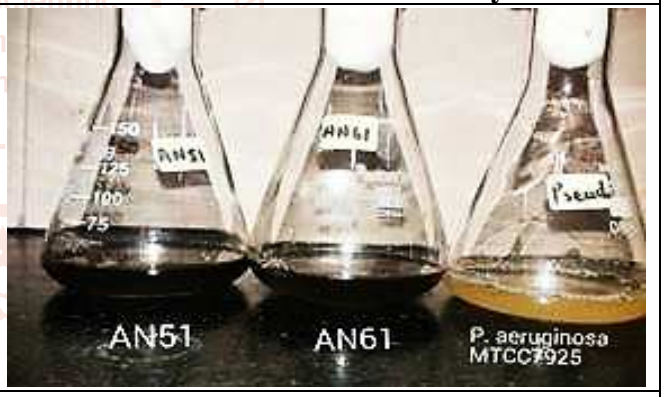

Figure 6. Foam constancy of the two AN culture and P. aeruginosa MTCC 7925 after 2 days.

After all the qualitative experiments, the quantitative test centered on surface tension measurement was conceded by dynamic surface tensiometer supported by Wilhemy principle. To test and select the concentrated biosurfactant manufacturer among all the positive values surface tension of spent stock was measured. Pseudomonas aeruginosa MTCC 7925 was also tested as a positive mechanism.

SWSF1 was detected at lower the surface tension of MSM media by 31.14% which was maximum among the proposed biosurfactant producing assorts.

The results of testing frequently proved the biosurfactant manufacturing property of the isolate. The potent biosurfactant constructing classify belonged to the genus Pseudomonas. The 16S rRNA evaluation alignment shows that the distort SWSF1 was closely interrelated to Pseudomonas aeruginosa.

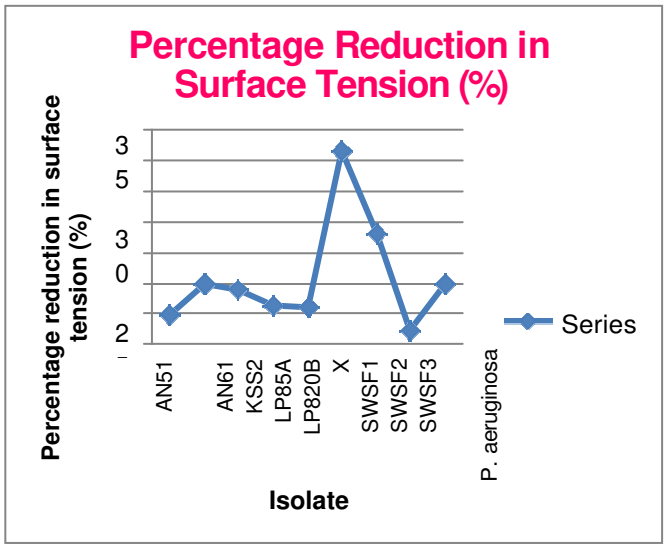

Figure 7 Percentage reductions in surface tension of media by the isolates.

Conclusions

In this study bacteria capacity of developing biosurfactant for the solubilization of hydrocarbon were classified. Bacteria were examined qualitatively and quantitatively for their ability to develop the biosurfactant. Among 39 isolates examined, SWSF1 gave the maximum decrease in the surface tension of media by 32%. In similitude to all the isolates SWSF1 generated more biosurfactant. Isolated variant of Pseudomonas was found to develop more biosurfactant as associated to the standard biosurfactant producing strain of Pseudomonas aeruginosa MTCC 7925. CTAB assay was utilized to detect the existence of anionic biosurfactant as rhamnolipid. SWSF1 pointedchemical reaction over the colony which indicate the capability of the bacterium to develop rhamnolipid. As this bacteria gave richproductions, it can be possible to use this isolate for the industrial production of rhamnolipid. It can be a generator of rhamnose which is valuable sugar. Foaming action also uncovered the potential of the bacterium to yield the biosurfactant even in the lack of hydrophobic substrates which has an imparted advantage in case of industrial manufacture of rhamnolipid.

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