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SCREENING AND IDENTIFICATION OF POTENTIAL BIOSURFACTANT PRODUCING MICROORGANISMS ISOLATED FROM OIL CONTAMINATED SOILS

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ABSTRACT

Biosurfactant are surface active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. Hundred hydrocarbon saturated soil samples were collected from thirty different areas in Chennai, TamilNadu, India. Seventy two bacterial strains were isolated and cultured by enriching carbon (Glycerol) source. Each culture medium was screening to confirm the ability of biosurfactant production. These were conducted using surface tension and emulsification activity methods. The result reveals that fifty four strains of bacteria showed surface tension in the range between 0.026Nm^{-1} to 0.072Nm$^{-1.}$ The emulsifying capacity evaluated by the E24 emulsification index range from 36-85% EA. Among 54 isolates, 9 isolates displayed highest activity after detection with emulsification index method. They were identified for potent biosurfactant production. These were conducted using series of screening methods.

KEYWORDS: **Biosurfactant, Bioremediation, Surface tension, Emulsification Index.**

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INTRODUCTION

Pollution is the introduction of contaminants into a natural environment that causes instability, disorder, harm or discomfort to the ecosystem. Among the pollutants, oil pollution is a significant one. Accidental and deliberate crude oil spills have been, still continue to be a significant source of environmental pollution and poses a serious environmental problems due to the possibility of soil contamination[1].Oil contamination in soil has been a major threat to the environment because of poor solubility. Soil contamination with petrol, diesel and engine oils are becoming one of the major environmental concern. To remove oils contaminated with soil, Bioremediation provide an effective and efficient strategy to speed up the clean-up processes. One of the approaches to enhance biodegradation of oil is to use Biosurfactants. Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing oil recovery [2].

The biosurfactants have unique properties of structural diversity, possibility of cost effective production and biodegradability make them a promising choice for application in enhancing hydrocarbon bioremediation. The first step in the microbial degradation of oil is to use biosurfactants which could increase solubility of oil in water to enhance the bioavailability of the hydrophobic substrates, leading to higher oil degradation rates. A variety of new biosurfactants respectively producing strains are the key issue in overcoming the economic obstacles of the production of biosurfactants. The principle aim in screening for new biosurfactants is findings new structures with strong interfacial activity, low critical micelle concentration (CMC), high emulsion capacity, good solubility and activity in a broad pH-range[3]. Besides these physicochemical properties, commercial variable biosurfactants have to be economically competitive. Therefore, increased efforts in the discovery of new biosurfactant producing microbes must be made by applying a range of different screening methods.

MATERIALS AND METHODS

(i) Sampling area and Sampling

Composite soil samples saturated with hydrocarbons were collected from various gas stations (Petrol pumps) and automobile stations located in the city of Chennai, TamilNadu, India. Thirty different areas (Fig-1) having contaminated with Petrol, Diesel and Kerosene were selected and One hundred soil samples were collected in sterilized plastic bags.

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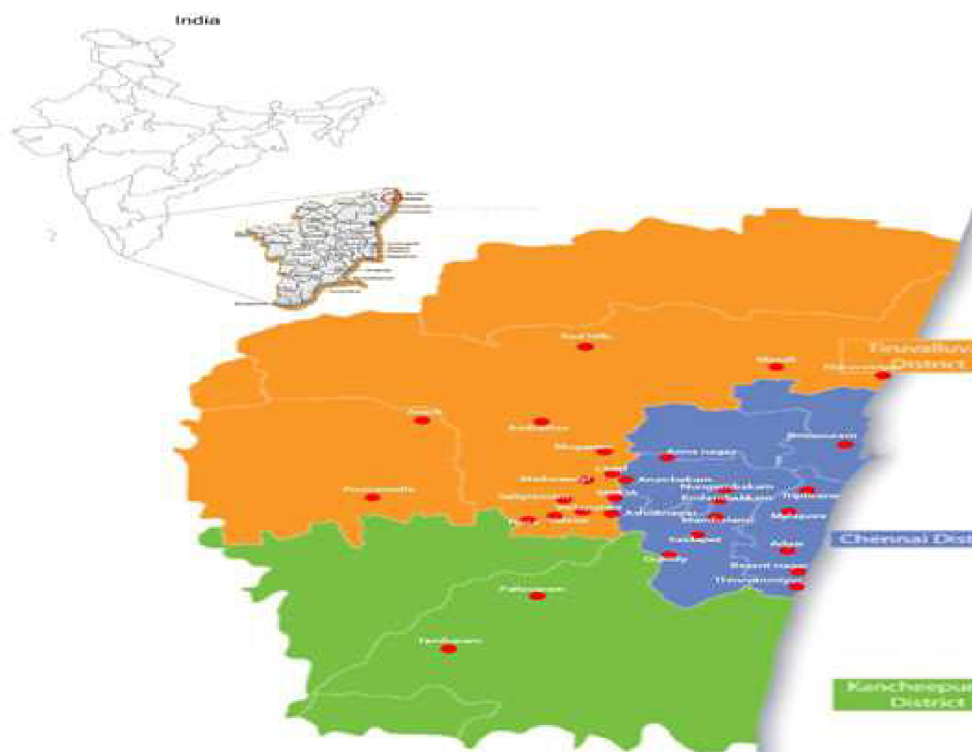

Figure 1
The areas choosen for sampling:

(ii) Isolation and enumeration of bacteria from samples

1 gram of soil sample was mixed with 99 ml of sterile distilled water. The samples were kept in shaker at 200 rpm for 24-48 hrs. After incubation, samples were serially diluted from 10^{-2} to 10^{-6} in sterile distilled water. From the dilutions 0.1ml was spreaded over that 20ml of sterile Nutrient agar, Cetrimide agar, Trypticase soy agar[4] (37^0C for 24 hrs) and Starch casein agar[5] (30^0C for 7-10 days).The number of total aerobic bacteria were recorded and colonies showing promising growth on selective media were then subsequently isolated as pure culture and maintained in the same media as slants. The bacterial isolates were then characterized by standard procedures.

(iii) Extraction of biosurfactants

The growth and biosurfactants production were studied in the screened carbon sources mineral salt media. Erlenmeyer flasks of 1000ml capacity containing 250 ml of the mineral salt medium with glycerol as the carbon source were individually inoculated with 5ml of particular inoculum[6]. The flasks were incubated in a rotary shaker incubator at 30^0C for 24-48 hrs. The culture obtained was used for the extraction of biosurfactants. The culture medium was centrifuged at 350g for 20 min and then the supernatant was adjusted to pH of 2.0 by adding 5mol/H_2SO_4 for the biosurfactant precipitation. The precipitates were extracted with two volumes of diethely ether/methanol (1:1,v/v) mixture. Evaporation of the solvent yielded crude biosurfactants[7].

(iv) Screening Methods

Biosurfactants are structurally a very diverse group of biomolecules, e.g., glycolipids, lipopeptides, lipoproteins, lipopolysaccharides or phospholipids. Therefore, most methods for a general screening of biosurfactant producing strains are based on the physical effects of surfactants. Alternatively, the ability of strains to interfere with hydrophobic interfaces can be explored. The screening methods can give qualitative and quantitative results.

1. Surface Tension test:[8]

The majority of screening methods for biosurfactant producing microbes were based on the interfacial or surface activity. The

surface tension can be determined on the basis of the number of drops which fall per volume and the density of the sample. Surface tension for all the samples were calculated. Those samples, which showed lowering of the surface tension, were further processed
.

Surface tension (T) = mg/3.8rNm^{-1}

Where, m: Mass of one drop of the liquid,

g: acceleration due to gravity,

r: radius of the capillary tube

To determine the surface tension, mass of the medium has to be calculated and simply weighing the drop of the medium.

Mass of one drop of the medium, m= W2-W1/total droplet

Where, W2- Weight of the sample with beaker,

W1- Weight of the empty beaker.

2. Emulsification Index (E_{24})[9]

Biosurfactants has the ability to emulsify various hydrocarbons. The emulsifying property of the crude biosurfactant was carried out with petrol, diesel and kerosene. The emulsification index on hydrocarbons was calculated by standard method .

E24= (Height of the emulsified layer/Total height of the hydrocarbon) x 100.

3. Drop collapse assay: [10]

In drop collapse method, 2µl of mineral oil was added to 96 well micro titer plate and 5 µl of the crude biosurfactant was added to the surface of the oil. After 1 minute, the shape of the drop on the surface of the oil was observed..

4. Microplate assay: [11]

The surface activity of individual strains can be determined qualitatively with the microplate assay. A 100 µl sample of the crude biosurfactant of each strain was taken and put into a 96-microwell plate. The plate was viewed using a backing sheet of paper with a grid. If biosurfactant was present, the concave surface distorts the image of the grid.

5. Penetration assay: [12]

The wells of a 96 well microplate were filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste is covered with 10µl of oil. Then, 90µl of crude biosurfactant was colored with adding 10µl of a red staining solution. The colored supernatant is placed on the surface of the paste. If biosurfactant present, the hydrophilic liquid was break through the oil film barrier into the paste. The Silica was penetrated the hydrophilic phase and the upper phase were changed clear red to cloudy white within 15 min.

6. Oil spreading assay: [13]

10 µl of crude oil was added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer. Then, 10 µl of crude biosurfactant was gently placed on the centre of the oil layer. The diameter of the clearing zone on the oil surface was measured.

7. Bacterial adhesion to hydrocarbons assay (BATH):[14]

The method was based on the degree of adherence of cells to various liquid hydrocarbons. A turbid, aqueous suspension of washed microbial cells was mixed with a distinct volume of a hydrocarbon After mixing for 2 minutes, the two phases was allowed to separate. The hydrocarbon layer was removed and the turbidity of the aqueous phase was measured and calculated by H= [1- A/A_0] x 100. Whereas A_0 was the mixing without absorbance of the bacterial suspension without hydrophobic phase added and A the

absorbance after mixing with hydrophobic phase.

8. Salt aggregation assay: [15]

The salt aggregation test provides a simple means for identifying bacteria associated with the production of biosurfactants. The bacterial suspension was mixed with an equal volume of salt solution on glass depression slides. The suspension was mixed for 2 minutes at 20^0C, then visual reading against black background was carried out. A positive reaction shows a clear solution and white aggregates with a diameter of app.0.1mm. A bacterial suspension mixed with 0.002 M sodium phosphate without addition of salt is used as negative control.

9. CTAB agar plate assay: [16]

The isolated strains were inoculated on a light blue mineral salt agar plate containing the cationic surfactant Cetyltrimethylammonium bromide (CTAB) and the basic dye methylene blue. If anionic surfactants were secreted by the strains, they form a dark blue, insoluble ion pair. The productive colonies were surrounded by dark blue halos.

10.Haemolysis: [17]

The isolated strains were streaked into blood agar plate and incubated. The plates were visually observed for the zone of clearness around the colony. The concentration of biosurfactant was depends on the diameter of the clear zone.

11. Lipase assay: [18]

The isolates that produce lipase were screened using tributrin agar plates. A loopful of inoculums was streaked on the tributrin agar and incubated .After incubation, the plates were examined for the formation of clear zone around the colonies.

12. Heavy metal assay: [19]

Heavy metal salt solutions were used for evaluating the heavy metal resistance of the isolates. The isolates were swabbed on selective medium .Using a sterile cork borer (7 mm width), wells were made on the surface of the medium. To each well 500 µl of the heavy metal salt solution were added and incubated and the inhibition area (mm) was measured.

13. Antagonistic assay: [20]

20 µl of biosurfactants were inoculated to the well made in the freshly grown bacterial cultures on Muller hinton agar plates. The plates were incubated and observed for zone of inhibition.

14. Stability characterisation: [21]

To determine the thermal stability of the biosurfactant, cell free broth was also maintained at a constant temperature range of $20\text{-}100^0$C for 15 minutes, and cooled at room temperature. To determine the effect of pH on activity, the pH of the biosurfactant was adjusted (4.2 -9.2) prior to filter sterilization. The effect of addition of different concentration of NaCl on the activity of the biosurfactant was investigated. The biosurfactant was re-dissolved after purification with distilled water containing the specific concentration of NaCl (5-15%,w/v). The surface tension values of each treatment were performed as described above.

RESULTS

A total of 72 isolates of *Pseudomonas spp* and *Actinomycetes spp* were isolated from 100 soil samples. The density of *Pseudomonas spp* and *Actinomycetes spp* were in the range of 5.2×10^4 to 4.2×10^6. The bacterial isolates from soils were identified as follows; 32 isolates (44.4%) were *Pseudomonas aeruginosa*, 25 isolates (34.7%) were *Pseudomonas fluorescence,* 7 isolates (9.7%) were *Pseudomonas putida* and 8 isolates (11.1%) were *Actinomycetes spp*.(Table- 1). Area wise incidence indicated that 90% (27/30) of sampling area were positive.(Table -2) Extracted biosurfactants were found to be turbid and yellowish brown in colour. The surface tension of the medium were reduced from $0.026Nm^{-1}$ to $0.072Nm^{-1}$. Among 72 isolates, 54 isolates have reduced the surface tension effectively (Table- 3 a & b). Emulsification index were calculated and tabulated (Table-4). They were also effectively emulsifying the Petrol than Diesel and Kerosene. Among 54 isolates, nine isolates displayed highest activity after detection with emulsification index method.(Table 5).The selected nine were tested against the series of

screening methods. The results were observed and tabulated (Graph 1 and 2, Table 6)

DISCUSSION

To isolate beneficial bacteria to biotechnology and bioremediation, the samples were collected from hydrocarbon contaminated soils located in the city of Chennai, TamilNadu, India. A high prevalence of *Pseudomonas species* were found, with a high average when compared with other genera of bacteria isolated from the same samples. This may be due to moisture and warmness of the soil characteristics. Some species of *Pseudomonas* has been use as bioremediation tool which is able to clear the environmental pollution and improve the hygienic measures by partially or completely degrade Pollutants[22]. The fermentation of *Pseudomonas aeruginosa* was first investigated by using a couple of carbon sources. Rhamnolipid production by glycerol is much higher than that of other substrates including glucose, vegetable oil and liquid paraffin. So, glycerol is the most effective substrate for the production of biosurfactants than other substrates. The fermentation of biosurfactant was carried out using 30g/L glycerol as the sole carbon source [23]. Biodegradation of crude oil by microbial appears to be the natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds or transformed into the other organic compounds such as biosurfactants [24].

Table 1
The percentage of different bacterial isolates from hydrocarbon contaminated soils

Bacterial isolates	No. of isolates out of 72	(%)
Pseudomonas aeruginosa	32	44.4
Pseudomonas fluorescence	25	34.7
Pseudomonas putida	7	9.7
Actinomycetes spp	8	11.1

Table 2
Area wise incidence of bacterial isolates from hydrocarbon contaminated soils.

Bacterial isolates	No. of isolates out of 30 areas	(%)
Pseudomonas aeruginosa	23	76.6
Pseudomonas fluorescence	21	70.0
Pseudomonas putida	7	23.3
Actinomycetes spp	8	26.6

Table 3 a
The reduction of surface tension values of the samples

Name of the samples	Surface tension Nm^{-1}
Control	0.072
Pseudomonas aeruginosa-2642	0.026
Pseudomonas fluorescence-1749	0.028
Pseudomonas putida-7525	0.030
Rhodococcus rhodochorous-3552 .	0.028
Pseudomonas aeruginosa Samples (25)	0.026 to 0.049
Pseudomonas fluorescence Samples (19)	0.028 to 0.049
Pseudomonas putida Samples (5)	0.030 to 0.044
Actinomycetes spp. Samples (5)	0.028 to 0.048

Table 3 b
Non reduced surface tensions values of the samples

Name of the samples	Surface tension Nm^{-1}
Control	0.072
Pseudomonas aeruginosa Samples (7)	0.071 to 0.072
Pseudomonas fluorescence Samples (6)	0.071
Pseudomonas putida Samples (2)	0.071 to 0.072
Actinomycetes spp. Samples (3)	0.071

Table 4
Emulsification index (E_{24}) of the samples.

Name of the samples	Emulsification Index(%)		
	Petrol	Diesel	Kerosene
Control –Tween -80	85	82	83
Pseudomonas aeruginosa-2642	82	80	78
Pseudomonas fluorescence-1749	80	79	75
Pseudomonas putida-7525	80	82	81
Rhodococcus rhodochorous-3552 .	79	82	81
Pseudomonas aeruginosa Samples (25)	36-82	42-79	38-75
Pseudomonas fluorescence Samples (19)	37-79	44-73	43-74
Pseudomonas putida Samples (5)	49-70	51-69	49-71
Actinomycetes spp. Samples (5)	42-73	49-71	52-65

Table 5
Potent biosurfactant producers based on Surface Tension and Emulsification Index.

Name of the Samples	Surface Tension mN-m1	Emulsification Index		
		Petrol	Diesel	Kerosene
PSPA3	**26**	**82**	**78**	**75**
PSPA15	**26**	**83**	**79**	**77**
PSPA16	**26**	**84**	**75**	**73**
PSPA22	**26**	**80**	**74**	**70**
PSPF2	**28**	**79**	**72**	**74**
PSPF16	**28**	**78**	**73**	**71**
PSPF17	**28**	**78**	**73**	**72**
PSPP6	**30**	**71**	**69**	**70**
PSAS6	**28**	**73**	**71**	**65**

Graph 1
Antagonistic properties of potent biosurfactant producers against selected bacteria

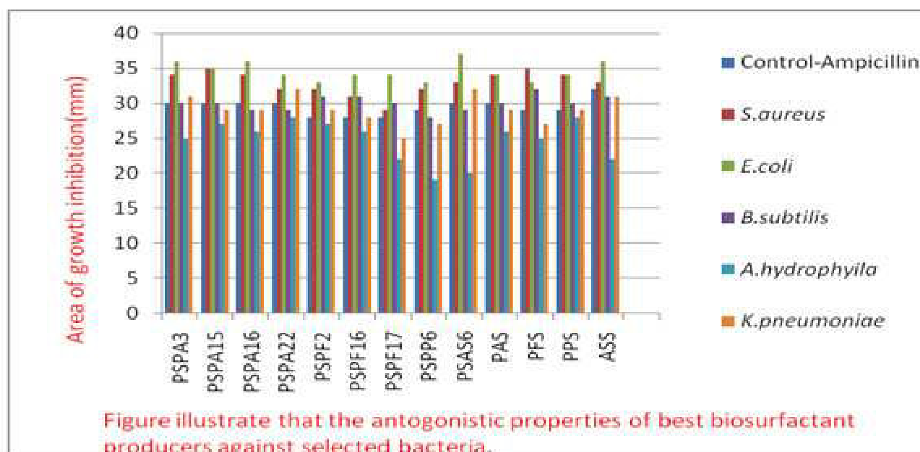

Figure illustrate that the antogonistic properties of best biosurfactant producers against selected bacteria.

Graph 2

Heavy metal resistance exhibited by potent biosurfactant producers against metal salt solutions.

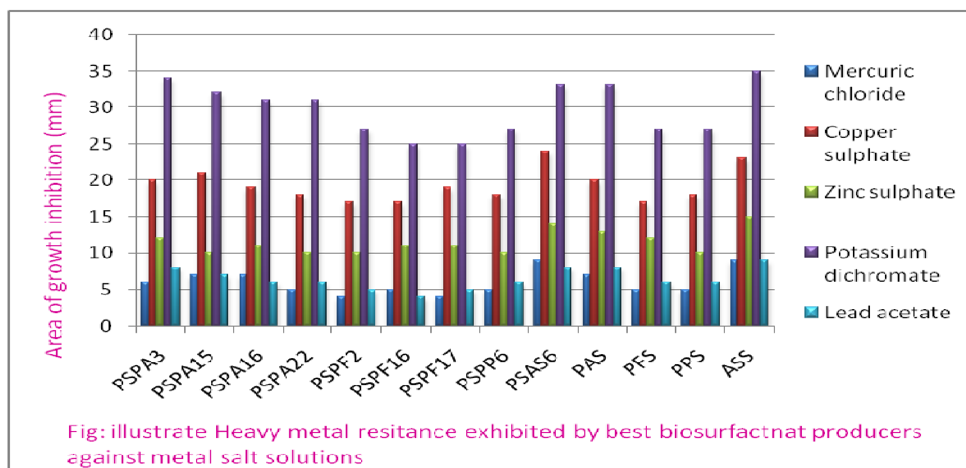

Fig: illustrate Heavy metal resitance exhibited by best biosurfactnat producers against metal salt solutions

Table 6

Different screening methods for the detection of potent biosurfactant producers isolated from hydrocarbon contaminated soils.

Name of the isolates	Drop collapse Assay	Microplate Assay	Penetration Assay	Oil spreading assay	BATH(%)	Salt Aggregation Assay	CTAB	Haemolysis	Lipase activity
PSPA3	***	W/C	CW	***	62.5	+	DBH	++++	+
PSPA15	***	W/C	CW	***	59.4	+	DBH	+++	+
PSPA16	***	W/C	CW	***	59.4	+	DBH	+++	+
PSPA22	***	W/C	CW	***	56.4	+	DBH	++	+
PSPF2	***	W/C	CW	***	47.1	+	DBH	++	+
PSPF16	***	W/C	CW	***	55.9	+	DBH	+++	+
PSPF17	***	W/C	CW	***	55.9	+	DBH	+++	+
PSPP6	***	W/C	CW	***	42.5	+	DBH	++	+
PSAS6	***	W/C	CW	***	52.8	+	DBH	+++	+
PAS	***	W/C	CW	***	54.3	+	DBH	+++	+
PFS	***	W/C	CW	***	51.5	+	DBH	+++	+
PPS	***	W/C	CW	***	47.1	+	DBH	+++	+
ASS	***	W/C	CW	***	46	+	DBH	+++	+

+ - Positive, ++ - Complete haemolysis with a diameter of lysis < 1cm,
+++ - Complete haemolysis with a diameter of lysis > 1cm to < 3 cm ,
++++ - Complete haemolysis with a diameter of lysis > 3cm,
**** - Complete spreading on the oil surface,*
W/C- Wetting edge with Concave surface, CW- Cloudy White, DBH- Dark Blue Halo

Biosurfactants production is associated with uptake of water soluble carbon substrates like glycerol by bacteria. The choosen bacteria does not require any hydrophobic substrates, however, they need soluble carbohydrates in the form of glycerol for their growth. The biosurfactant producing ability of the strain *Streptomyces spp.VITDDK3* was tested by different screening methods. Both Gram(+) and Gram(-)bacteria capable of producing surface active agents and they are amphipathic extracellular lipopeptides[25].Production of lipopeptides biosurfactant producing bacterium *Rhodococcus sp.TW53* was reported from pacific ocean deep sea sediments[26]. Biosurfactant activity of free fatty acids and glycolipids extracted from *Rhodococcus erythropolis (3C-9*-Strain) isolated from sea side soil. Sampling and isolation of bacteria are the basis for screening of biosurfactant producing microbes. In subsequent steps the isolates have to be characterized and identified the strains which were interesting for further investigations. Several techniques have been developed for identifying biosurfactant producing strains. Most of them were directly based on the surface or interfacial activity of the culture supernatant. Apart from that, some screening methods explore the hydrohobicity of the cell surface. This trait also gives identification on biosurfactant production. The development of rapid and reliable methods for

screening potentially active organisms and the subsequent evaluation holds the key to the discovery of new biosurfactants or production strains.

The surface tension values of the culture supernatants ranged from 0.026Nm-1 to 0.072Nm-1. The decrease in surface tension indicated the production of extracellular surface active compounds. The investigation showed that among 72 isolates, 54 isolates were effectively reduced the surface tension. They were also emulsifying the Petrol than Diesel and Kerosene. Among 54 isolates, nine isolates displayed highest activity after detection with emulsification index method. In drop collapse assay, all the nine samples collapsed the oil drops, they contain biosurfactant which spread or collapse the oil because of force or interfacial tensions between drops and hydrophobic force. In Microplate assay, all the nine samples caused wetting at the edge of the well, surface became concave and takes the shape of diverging lens. In Penetration assay, all the nine samples were break through the oil film barrier into the paste; silica was entered into hydrophilic phase and changed from clear red to cloudy white. Silica was entering into hydrophobic phase to hydrophilic phase more quickly if biosurfactant present. Biosurfactant free culture turn into cloudy red. In Oil spreading assay, all the nine samples were displaced oil and clear zone were formed. BATH was a simple but indirect method. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cells. The percentage of cell bound to the hydrophobic phase(H) was calculated , all the nine samples were showed 42.5 to 62.5% ability to adhere hydrocarbons. The Salt aggregation test provides a simple means for identifying bacteria associated with the production of biosurfactant. All the nine samples were produced positive aggregation reaction of a clear solution and white aggregates with a diameter of 0.1 mm.

The CTAB agar plate method was a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. If anionic surfactants were secreted by all the nine samples, they form a dark blue halos, insoluble ion pair with cetyltrimethylammoniumbromide and methylene blue. The CTAB agar assay was a comfortable screening method but it was specific for anionic biosurfactants.

The blood agar as a preliminary screening method which should be supported by other techniques based on surface activity measurements. The tested nine samples were caused lysis of blood cells and exhibited a colorless, transparent ring around the colonies. On Tributrin agar plates, all the nine samples were produced a clear zone around the colony indicating lipase production. All the nine samples were tested for their ability to remove heavy metals. Based on agar well diffusion method, the samples produced an inhibition zone of 4-9 mm for Lead acetate and Mercuric chloride. But the samples produced an inhibition zone of 25-35mm for Potassium dichromate, 17-24 mm for Copper sulphate and 10-15 mm for Zinc sulphate. From the results clear that the nine samples were resistant to Lead acetate and Mercuric chloride salt solutions. All the nine samples exhibited marked antagonistic activity against all the bacterial pathogens. They showed significant antimicrobial activity when compared with the standard Ampicillin. This result was correlated with *Pseudomonas aeruginosa* isolated from oil contaminated soil [27] and was tested against the selected microorganisms. The stability of biosurfactant was tested over a wide range of temperature. The nine samples were found to be thermo stable. Heating of the supernatant to 100^0C caused no significant effect on the biosurfactant performance. The surface tension reduction were quite stable at the temperature used (ST=28).The stability of biosurfactant was tested over a wide range of pH. These results indicated that pH increase or decrease were alter the stability but the pH range was 6.2 to 7.2 showed positive stability effect on surface tension(ST=30-26).Effect of sodium chloride indicate that, the stability was maintained at a concentration of 7, little changes were observed with addition of up to 15%w/v Sodium chloride.

CONCLUSION

The results obtained from the study revealed that, nine organisms displayed highest activity against surface tension and emulsification index. Interest in biosurfactants has led to the

development of a multitude of methods for the screening of biosurfactant producing strains. They were screened and identified as potential biosurfactant producers. If new production strains become available, the economic obstacle of biosurfactants may eventually be eased. Further the potent strains will be tested against their efficacy on oil remediation and suitable for use in oil fields such as Soil washing, Microbial Enhanced oil Recovery (MEOR) and removal of heavy metal pollution.

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