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Production and characterization of biosurfactant from bacterial species isolated from oil contaminated soil

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Abstract

Biosurfactants are surface active substance synthesized by living cells. Micro-organisms that degrade hydrocarbon were isolated and screened for their activity. A total of 20 samples of oil contaminated soil were collected and three organisms were isolated and tested with tests like detection of glycolipid, surface tension measurement, emulsification index, heavy metal, antagonistic test, hemolysis, drop collapsing test, and thin layer chromatography for the degradation activity. The three organism's *viz. Bacillus subtilis, Pseudomonas aeruginosa and Pseudomonas fluorescens* degraded the oil in soil.

Keywords: Biosurfactant, Bioremediation, surface active substance, hydrocarbon.

1 Introduction

Bioremediation is a process that uses microorganisms and their enzymes to return the environment altered by contaminants to its original condition [1-6]. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. The term *surfactant* is a blend of *surface active agent*. Surfactants are compounds that lower the surface tension of a liquid, the interfacial tension between liquids or between liquid and solid [1]. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants [1].

Biosurfactant are surface-active substances synthesized by living cells. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable [7]. Several biosurfactants like Glycolipids, Rhamno-lipids, Sophorolipids, Terhalolipids, Lipo protein and Lipopeptides, Fatty acids, Phospholipids, Polymeric biosurfactant exist in the environment, each produced by specific micro organisms [8]. Biosurfactant enhances the emulsification of hydrocarbons, has the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation[9]. The use of chemicals for the treatment of a hydrocarbon polluted site may contaminate the environment with their by-products, whereas biological treatment may efficiently destroy pollutants, while being biodegradable themselves [9]. Thus, biosurfactant producing microorganisms play an important role in bioremediation. The compounds enhance oil recovery and may be considered for other potential applications in environmental protection [9]. Other applications include herbicides and pesticides formulations, detergents, health care and cosmetics, pulp and paper, coal, textiles, ceramic processing and food industries, uranium ore-processing and mechanical de-watering of peat [10]. Quite a few microorganisms synthesize surface-active agents most of them are bacteria and yeasts [11]. Biosurfactant production was reported from different microorganisms like *Bacillus* and *Pseudomonas* sp. [12].

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2 Materials and methods

Isolation of organisms – For the study, the soil samples were collected from four different localities *viz*. workshop, petrol bunk, mechanic shed and ration shop in and around Vadapalani, Chennai which were continuously exposed to oils like Petrol, The soil was transferred into a sterile polythyne bags and stored at 4 °C until using. The soil samples (10 g) were suspended in 100 ml of sterilized Saline/ double distilled water in 250 ml conical flask, the samples were then kept in shaker at 200 rpm for 48 h to ensure complete mixing of soil and subsequently 1 ml of this suspension was added to 9 ml saline to obtain desired dilutions up to 10. 0.1 ml of 10^{-5} dilution of each samples were spread on nutrient agar media. The inoculated plates were incubated at 37 °C for 24 h and observed for pigmented colonies [13]. Isolated colonies of *Bacillus* sp. were named as SCB1 to SCB5, and *Pseudomonas* sp. as SCPA1 to SCPA5, were streaked on selective medium and incubate at 37 °C for 24 h. From the sub cultured samples Gram's staining and biochemical tests were carried out to identify the organisms.

Screening of Biosurfactant - The organisms were inoculated in the minimal salt medium broth and it was incubated at 37 ºC for 24 h and the inoculums were precipitated to collect biosurfactant solution. The screening of biosurfactants was carried out for the isolates using following methods:

Detection of Glycolipid - Blue agar plates were prepared using minimal salt medium with the addition of CTAB and methylene blue. After solidification of agar, well was made in the middle of the plate and the biosurfactant solution was loaded on it, and the plates were incubated for 24 h at 37 ºC [14].

Surface tension measurement - Surface tension was determined by the ring method. Biosurfactant solution was loaded in tensiometer and allowed to drop uniformly. Numbers of drops were counted and calculated using the formula, *Mass of one drops the medium, m = W2-W1 / total droplet* (W2: weight of the sample with beaker, W1: weight of the empty beaker) [15].

Emulsification index - The emulsification index of culture samples was determined by adding 3 ml of oil and 3 ml of the cell-free broth in test tube, vortex at high speed for 2 min and allowed to stand for 24 h. One ml of fermentation medium was added to each tube and a control (Tween 20) was also maintained. The emulsification index was measured by tabulating the amount of degradation of oil. *Emulsification index = (height of the emulsified layer/ total height of the hydrocarbon) x 100* [15].

Hemolysis - Hemolytic activity was tested using blood agar plate. Blood agar was prepared with sheep blood (5%) and blood agar base. The blood agar base was sterilized by autoclaving at 121 ºC at 15 lbs pressure for 15 min. Prior to pouring blood was added and allowed to solidify. The isolates were streaked on the blood agar and the plates were incubated at 28 ºC for 24 h following [16].

Antagonistic test - The cell free supernatant was separated from the mixture and inoculated as 20 µl to the well made in the freshly grown cultures of *Escherichia coli, Vibrio cholerae, Staphylococcus aureus, Salmonella typhi, Klebsiella pneumonia* on Muller Hinton agar plate. Then the plated were incubated at 37 ºC for overnight and observed for zone of inhibition[13].

Heavy metal test - Screening for heavy metal resistance was carried out using heavy metal salt solutions, zinc sulphate, potassium dichromate. The concentration of the standard heavy metal solutions was 1000 ppm. The concentration of heavy metal salt solutions was ranged from 10, 50, 100, 500, 1000 mM. The salt solutions were sterilized separately for 15 min at 110 °C [16]. Pour the prepared salt solution about 500 µl into the 5 ml of MSM broth containing biosurfactant solution and incubate it 37 ºC for 24 h**.**

Drop collapsing method - Oil (2 µl) was added to 96-well micro titer plate and equilibrated for 1 h at 37 ºC. The culture supernatant (5 µl) was added to the surface of the oil in the well. The shape of drop on the oil surface was noted after 1 min. The culture supernatant that collapsed the oil drop was indicated as positive and the other which failed to collapse the oil drop as negative. Distilled water act as negative control [16].

Thin Layer Chromatography - A thin uniform layer of stationary phase was made on a glass plate. The plate was air dried for 15 min and then over dried for 15 min then over dried for 10-15 minutes and 100 ºC. Twenty ml of each ample and standard sugars were spotted on line drawn 1.5-2.0 cm from the bottom. The TLC plate was gently in a mobile phase contained in a chromatographic tank and allowed for solvent development. As the solvent front reached about 1-2 cm from the top of the plate, the plate was removed and air dried. The plates were sprayed with the spraying reagent and treated at 100 ºC for 10 min. Stationary phase: Silica gel, Mobile phase: Solvent = $CHCl_3$:CH3OH: H2O (70: 10: 0.5 V/V/V), Spraying agent: Ninhydrin reagent was used to detect lipopeptide biosurfactant as red spots. Anthrone reagent to detect glycolipid biosurfactant as yellow spots [9].

3 Results and discussion

All the ten isolates produced Glycolipid on the plate and drop collapsing test confirmed the presence of biosurfactant. Extraction of biosurfactant and their production was controlled by Thin Layer Chromatography. *Bacillus* sp. displayed red color spots with ninhydrin as a sprayer reagent. The *Pseudomonas* sp. displayed yellow color spots with anthrone as a sprayer reagent. Isolates reduced the surface tension, and emulsified the oils. And the *Pseudomonas* isolate produced β-hemolysis and *Bacillus* isolates produced α- hemolysis in the heamolysis test. In antagonistic test, *Bacillus* sp. inhibited the growth of *Klebsille pneumonia* and *Pseudomonas* sp. inhibited the growth of *E. coli* and both the species inhibited other species at a moderate level. Heavy metal test makes clear that all the isolates were resistant to heavy metal salts.

Table 1: Antagonistic Test

Name of the Isolates	*(a)* (mm)	*(b)* (mm)	*(c)* (mm)	*(d)* (mm)	*(e)* (mm)
SCB1	1	1	1	1	1.4
SCB2	0.9	0.8	1.1	0.8	1.1
SCB3	0.9	0.7	0.6	0.7	0.7
SCB4	-	1	0.6	0.8	0.8
SCB5	1.1	1	0.9	0.9	1.1
SCPA1	0.8	0.8	0.9	1	1
SCPA2	0.7	0.9	0.8	0.9	0.7
SCPA3	0.8	0.9	0.9	1	1
SCPF4	0.6	0.8	0.9	0.8	0.6
SCPF5	1.1	0.9	1	1	0.8

(a) Escherichia coli
(b) Vibrio cholerea
(c) Staphylococcus aureus
(d) Salmonella typhi
(e) Klebsille pneumonia

In Blue agar method, *Bacillus* sp. and *Pseudomonas spp.* produce high amount of glycolipid (Table 2, Fig.2). Surface tension test: For detection of surface of medium the value were determined and compared. The surface of medium was found in *Bacillus* sp. to reduce a range of 0.062 to 0.074. The surface of medium was found in *Pseudomonas* sp. 0.0362 to 0.062 which indicates the surface active agent in the medium was high in *Pseudomonas* sp. than *Bacillus* sp. (Table 2, Fig.2).

For emulsification activity petrol, diesel, and kerosene were tested. The study indicates that both the organisms have the emulsifying capacity, but third isolate of *Pseudomonas* sp. alone produced highest emulsifying activity in diesel. Thus, *Pseudomonas* sp. has higher emulsifying capacity than *Bacillus* sp. (Table 2, Fig.2).

All the *Pseudomonas* sp. produce ß heamolysis surrounding the bacterial colonies indicates the ability of biosurfactant to lysis the erythrocytes and *Bacillus* sp.

produce α heamolysis, which produce partial lysis of erythrocyte. Thus the present study indicates that both the organisms has its ability to heamolysis the blood agar, and *Pseudomonas* sp. has higher ability than *Bacillus* sp..

Antagonistic test revealed that *Bacillus* sp. inhibits the growth of tested organisms except one isolate of *Bacillus* sp. that did not inhibited the growth of *Escherichia coli*. *Pseudomonas* sp. inhibits the growth of organism tested (Table 1, Fig.1).

Table 2: Detection of Glycolipid, Surface Tension Test, Emulsification Index, TLC

S. No	Name of the Isolates	Zone of measurement	OD value (Nm^{-1})	Kerosene EI	Petrol EI	Diesel EI	TLC-RF value
1	SCB1	4.2	0.062	81	70	45	0. 61
2	SCB2	3.3	0.074	72	80	54	0.65
3	SCB3	3.5	0.063	72	80	54	0.76
4	SCB4	3.2	0.062	63	70	54	0.68
5	SCB5	3.8	0.072	81	70	45	0.72
6	SCPA1	4.1	0.053	81	80	54	72
7	SCPA2	3	0.062	81	70	54	0.8
8	SCPA3	3.2	0.051	72	70	27	0.66
9	SCPF4	3.3	0.045	63	70	45	0.57
10	SCPF5	3.6	0.036	72	90	54	0.55
11	Control	Nil	0.082	81	70	27	Nil

Table 3: Heavy metal test

Name of the Isolates	10mM		50 mM		100 mM		500mM		1000mM	
	$ZnSO^4$	$K^2Cr^2O^7$	$ZnSO^4$	$K^2Cr^2O^7$	$ZnSO^4$	$K^2Cr^2O^7$	$ZnSO^4$	$K^2Cr^2O^7$	$ZnSO^4$	$K^2Cr^2O^7$
SCB1	0.27	0.06	0.24	0.05	0.13	0.04	0.11	0.03	0.09	0.03
SCB2	0.26	0.05	0.23	0.05	0.14	0.04	0.12	0.04	0.07	0.03
SCB3	0.22	0.06	0.2	0.05	0.15	0.05	0.1	0.05	0.06	0.04
SCB4	0.24	0.06	0.21	0.06	0.18	0.05	0.15	0.04	0.1	0.04
SCB5	0.25	0.05	0.22	0.05	0.19	0.04	0.14	0.04	0.11	0.03
SCPA1	0.35	0.06	0.29	0.06	0.14	0.05	0.11	0.04	0.07	0.03
SCPA2	0.32	0.05	0.26	0.05	0.21	0.04	0.15	0.04	0.11	0.03
SCPA3	0.3	0.06	0.23	0.05	0.2	0.04	0.16	0.03	0.1	0.03
SCPF4	0.28	0.06	0.26	0.05	0.2	0.05	0.18	0.04	0.14	0.04
SCPF5	0.31	0.06	0.27	0.06	0.22	0.05	0.19	0.04	0.15	0.03

*ZnSO4 – Zinc Sulphate, K2Cr2O7 – Potassium Dichromate

The heavy metal tolerance test indicates that when the concentration of heavy metal salts increases the tolerance of *Bacillus* sp. decreases. The tolerance of *Pseudomonas* sp. moderately decreases (Table 3, Fig.3).

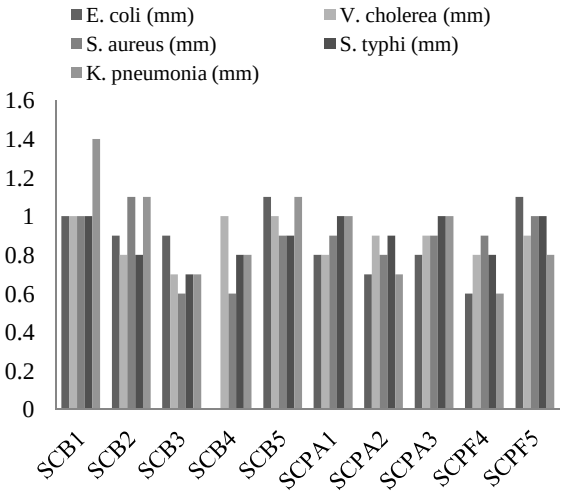

Figure 1: Antagonistic Test

Biosurfactant isolates by acid precipitation method at pH 2 was identified by TLC method. Surfactin from *Bacillus subtilis* was identified in red color spot where rhamnolipid from *Pseudomonas spp.* was identified by yellow color spot. The RF value of *Bacillus* sp. surfactin ranges from 0.61 to 0.72. The RF value of *Pseudomonas* sp. rhamnolipid ranges from 0.72 to 0.55.

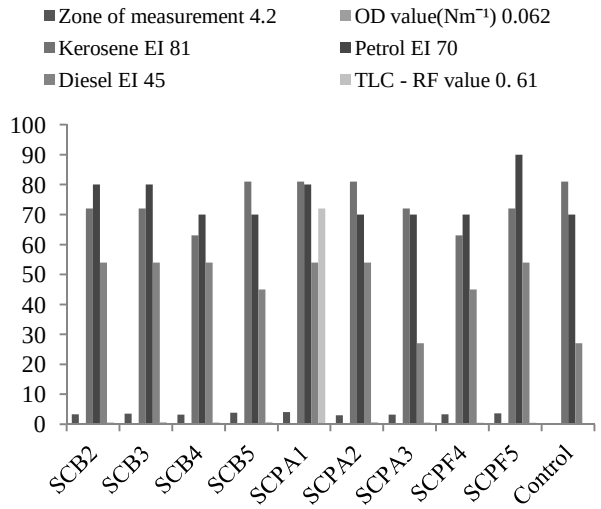

Figure 2: Detection of Glycolipid, Surface Tension Test, Emulsification Index, TLC

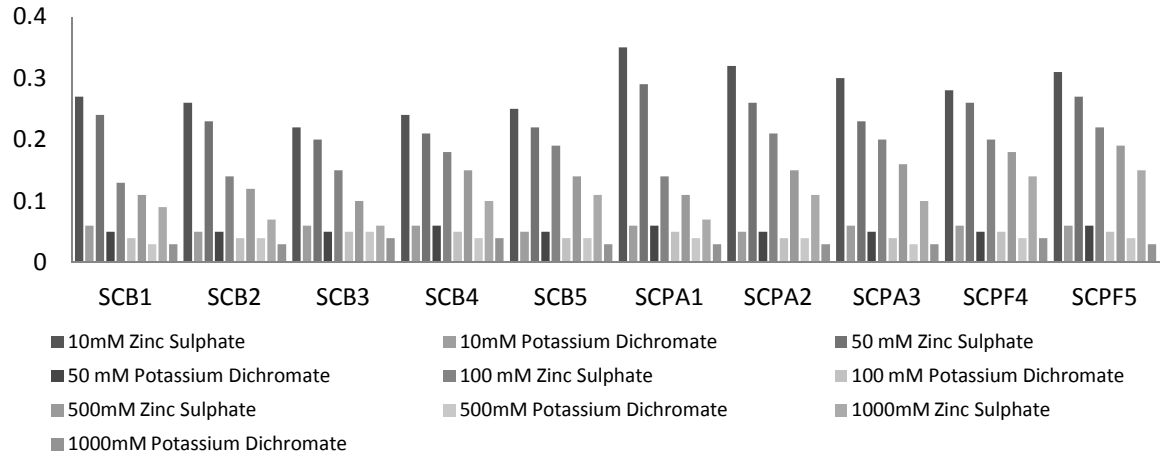

Figure 3: Heavy metal test

4 Conclusions

Finally this study concluded that both organisms produce biosurfactant. Comparatively *Pseudomonas* sp. produces higher biosurfactant than *Bacillus* sp. (Table 2, Fig.2).

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