ISOLATION, SCREENING AND PRODUCTION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED SOIL, MANGROOVE SEDIMENT AND MARINE WATER

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***Abstract*:** Pollution is one of the major serious problem that is faced globally. Hydrocarbon contaminated sites possess a major threat to the environment. Release of hydrocarbon into the environment whether accidentally or due to human activities is the main cause of water and soil pollution. Biosurfactant are used in bioremediation of hydrocarbon contaminated sites and this could be a promising approach. With this view, isolation, screening and production of biosurfactant producing bacteria was investigated. In the present study primary screening was carried out using Mineral Salt agar plate containing 2% used engine oil as a substrate and a total of 12 morphologically distinct bacterial strains were isolated from different samples: Oil contaminated soil, Mangrove sediment and Marine water. Out of these 3 were Gram positive and 9 were Gram negative. S1, S2, S3, S4, S5, S6 isolates were obtained from oil contaminated soil, M1, M2 isolates from mangrove sediment and W1, W2, W3, W4 isolates were obtained from marine water. All of these isolates were then subjected for secondary screening: BATH assay, Drop collapse test, Oil displacement assay, Emulsification index and Phenol-sulfuric acid method. On the basis of all these test results best isolate from each type of sample was selected for the biosurfactant production. Then biosurfactant was extracted using acid-precipitation method and dry extracted biosurfactant was obtained.

***Index Terms*-** Biosurfactants, BATH assay, Oil displacement, Phenol sulfuric acid, Extraction.

I. INTRODUCTION

Pollution is one of the major serious problem that is being faced globally. Oil contaminated sites possess a major threat to the environment. Soil contaminated with oil has a serious hazard to human health and causes serious environmental problems as well. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution. Soil contamination with hydrocarbons causes extensive damage of local system since accumulation of pollutants in animals and plant tissue may cause death or mutations. When oil spreads in an environment, low-molecular-weight hydrocarbons are volatilized while polar components are dissolved in water. However, most of the oil hydrocarbons remain on the water surface or adhere to soil particles due to their low solubility. Evaporation and photo-oxidation play an important role in oil detoxification, with ultimate and complete degradation being accomplished mainly by microbial activity.

Surfactants increase the surface area of hydrophobic contaminants in soil or water and thus increase their aqueous solubility and consequently their microbial degradation. So luckily Surfactants especially biosurfactants play a major role in this whole scenario. Surfactants are an important category of industrial chemicals and are widely used in almost every modern industrial process.

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals. Due to toxic and non-biodegradable nature of chemical surfactants there is a great emphasis on biosurfactants.

Biosurfactants are the surfactants obtained through microbial source. Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi from various substances including sugars, oils and wastes. Biosurfactant-producing microorganisms universally inhabit fresh water, soil, sediment, and sludge.

Biosurfactants are classified according to their chemical structure and microbial origin. Low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants. Biosurfactants are widely used in various fields including cosmetics, food processing, pharmaceuticals, bioremediation, agriculture, enhanced oil recovery.

II. MEDIA

1. Mineral Salt Medium (MSM)
2. Reagents for Phenol sulfuric acid method: -5% phenol
   - concentrated H_2SO_4
3. Reagents for Extraction: -6N HCL
   -Organic solvents (Chloroform: Methanol-2:1v/v)

III.RESEARCH METHODOLOGY

3.1 Sample collection

Three Oil contaminated soil samples were collected from the depth of 6 inches from different garages of Valsad. One Mangrove sediment sample was collected from Udwada, Valsad and Two Marine water samples were collected from Tithal Beach and Nargol Beach, Valsad. Soil samples were collected in clean plastic bags and plastic stopper bottles were for the collection of marine water samples**.**

3.2 Enrichment

Initially, the bacteria consortium was enriched by adding small quantity of samples into 30ml of Mineral Salt Medium (MSM) containing 2% used engine oil and pH 7.4. This mixture was shaken (150rpm) at 37 °C for 5 days. After incubation 1ml aliquot of the culture broth was then transferred to 30ml of fresh Mineral Salt Medium and was incubated under same conditions.

3.3 Isolation and Screening

3.3.1 Primary Screening

All the samples after enrichment were streaked onto Mineral Salt Agar (MSA) plate containing 2% used engine oil as a substrate. Plates were incubated at 37°C for 24-48hours. Morphological distinct colonies were selected and purified. The isolates obtained were then studied for Gram reaction and Motility test.

3.3.2 Secondary Screening

All the isolates were then inoculated in Mineral Salt Medium containing 2% used engine oil and this mixture was incubated at 37°C on rotatory shaker for 4-5days. After incubation, broth was centrifuged at 10,000 rpm for 15 mins and the supernatant was collected which was used for secondary screening. Secondary screening was performed using various test.

3.3.2.1 BATH (Bacterial Adherence To Hydrocarbon) assay

Bacterial cells were washed twice with the equal volume of buffer salt solution (K_2HPO_4, KH_2PO_4) and then were resuspended in the same buffer salt solution and Optical density was taken at 620nm. 100 µl of crude oil was added and vortex it for 3mins in test tubes. After vortex-shaking the crude oil and aqueous phase were allowed to separate for 2 hours. OD of aqueous phase was measured at 620nm in spectrophotometer. (Sarwar *et al.*,2018). Percentage of cell adherence to crude oil was calculated as follows:

$$\{1 - (\text{OD of aqueous phase} \div \text{OD of initial cell suspension})\} \times 100$$

3.3.2.2 Drop-Collapse test

Screening was performed using drop collapse test described by Bodour and Miller-Maier (1998).2µl of oil was applied to the well regions delaminated on the covers of 96 well microplate and these were left for equilibrated for 24 hours. 5µl of the culture supernatant was transferred to the oil coated well regions. The results were monitored visually after 1 hour. Deionised water was used as a negative control.

3.3.2.3 Oil Displacement assay

10µl 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 culture supernatant was gently placed at the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed (Morikawa *et al.*,1993)**.**

3.3.2.4 Emulsification index (EI)

This assay was developed by Cooper and Golderberg, 1987.Cell free culture broth was used to check the emulsification of crude oil. 3ml of the supernatant was mixed with 3ml of kerosene and the mixture was vortex at high speed for 2 minutes. This was left undisturbed for 24 hours to separate the aqueous and hydrocarbon phases. And the emulsification index was calculated by the following formula.

$$E_{24}(\%) = (\text{Height of emulsion} \div \text{Total height}) \times 100$$

3.3.2.5 Phenol sulfuric acid method

1ml of the supernatant was mixed with 1ml of 5% phenol and 5ml of concentrated H_2SO_4 was added drop by drop. Presence of biosurfactant in supernatant was confirmed by change in yellow to orange colour which indicates the presence of glycolipid type of biosurfactant (Kalyani *et al.*, 2014).

3.4 Production of Biosurfactant

Production of biosurfactant was carried out using Erlenmeyer flask of 250ml which contain 100ml of Mineral Salt Medium (MSM) supplemented with 2% used engine oil as a substrate and the isolates capable of producing biosurfactant were inoculated into MSM Broth. The flasks were then incubated for 7 days at 37°C on rotatory shaker.

3.5 Extraction of Biosurfactant

The production medium was centrifuged at 10,000rpm for 15 minutes and the supernatant was collected in the sterile test tubes. 0.5ml of 6N HCL and organic solvent chloroform and methanol (2:1v/v) was added to the supernatant and kept at room temperature for 30 minutes. Again, after centrifugation the supernatant was collected in the sterile flask and placed on the evaporator to obtain the dried crude biosurfactant. (Arora S. K. *et al.*, 2015).

IV. RESULT AND DISCUSSION

4.1 Physiochemical characteristics of collected samples

Table 4.1: Result of analysis of samples

Sample No.	Type of sample	Place of sample collection	Physiochemical characteristics			
			Colour	Texture	Temp	pH
1	Oil contaminated soil sample-1	Near Halar talav, Valsad	Dark brown	Sandy loam	30°C	6
2	Oil contaminated soil sample-2	Besides mango market, Valsad	Blackish brown	Sandy	29°C	7
3	Oil contaminated soil sample-3	Opp RPF ground, Valsad	Brown	Clay-sandy	31°C	6
4	Mangrove sediment-soil sample	Udwada beach	Brown	Clayey	28°C	8
5	Marine water sample-1	Tithal beach, Valsad	-	-	28°C	7
6	Marine water sample-2	Nargol beach, Valsad	-	-	29°C	8

4.2 Enrichment of samples

All samples collected were immediately inoculated into sterile Mineral Salt Medium (MSM) containing 2% used engine oil and pH 7.4 was incubated for 5days at 37°C for the purpose of enrichment so as to obtain sufficient growth of bacterial consortium.

4.3 Isolation of biosurfactant producers from obtained samples

The enriched culture broth was streaked onto Mineral Salt Agar (MSA) plates containing 2% used engine oil as a substrate for the purpose of primary screening.

4.3.1 Results of Primary Screening

Organism capable of utilizing oil as a substrate were grown on Mineral Salt Agar plate containing 2% used engine oil. 12 isolates were grown on Mineral Salt Agar (MSA) plates. These 12 isolates were further purified by re-streaking onto Mineral Salt Agar (MSA) plates. The purified isolates were then studied for gram reaction and motility test from which 9 isolates were Gram negative, 3 isolates were Gram positive and 8 isolates were motile and 4 isolates were non-motile.

Table 4.2: Isolates obtained on Mineral Salt Agar (MSA)

Serial Number	Sample	No. of Isolates obtained	Isolates obtained
1	Oil contaminated soil-1	2	S1, S2
2	Oil contaminated soil-2	3	S3, S4, S5
3	Oil contaminated soil-3	1	S6
4	Mangrove sediment	2	M1, M2
5	Marine water-1	2	W1, W2
6	Marine water-2	2	W3, W4

4.3.2 Results of Secondary screening

All of the 12 isolates were then subjected to secondary screening where the isolates were screened using various tests.

4.3.2.1 BATH Assay- Bacterial adherence to hydrocarbon

It is a simple photometrical assay for measuring the hydrophobicity of bacteria. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cells. Uninoculated medium was used as control and the results of test are shown. The results of Bacterial Adherence to Hydrocarbon isolates as shown in Below graph (fig 4.2), are as follows: S1-8%, S2-52%, S3-6.6%, S4-11.1%, S5-25%, S6-5.7%, M1-12.5%, M2-30%, W1-3.6%, W2-23%, W3-32.7%, W4-7.7%.

Table 4.3: Optical density of BATH Assay

Serial Number	Bacterial isolates	Initial OD at 620 nm	OD of aqueous phase at 620 nm
1	S1	0.5	0.46
2	S2	0.5	0.24
3	S3	0.30	0.28
4	S4	0.45	0.40
5	S5	0.48	0.36
6	S6	0.52	0.49
7	M1	0.08	0.09
8	M2	0.10	0.07
9	W1	0.55	0.53
10	W2	0.52	0.40
11	W3	0.52	0.35
12	W4	0.39	0.36

Control Test

Figure 4.1: BATH assay

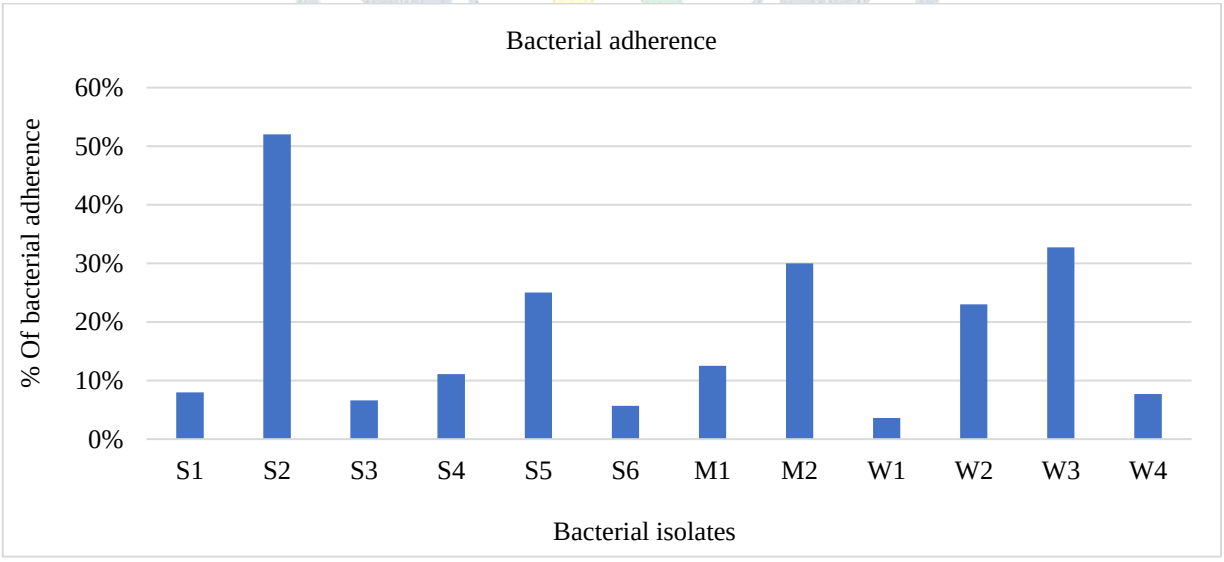

Figure 4.2: Graph of BATH assay

4.3.2.2 Drop collapse test

96-well micro-titre plate was used for drop collapse test. This test relies on the destabilization of oil droplet by surfactant. If culture supernatant contains surfactant, the drop spread or collapse because the force between the supernatant and the hydrophobic surface is reduced. As a control deionised water was used. As shown in table4.4: 8 isolates gave Drop Collapse test positive.

4.3.2.3 Oil displacement assay

As seen in fig 4.3 in test oil was displaced and a clear zone was formed, which indicates the presence of Biosurfactant and distilled water was used control. 8 isolates gave Oil Displacement test positive as shown in table 4.4.

Control Test

Figure 4.3: Oil displacement assay

Table 4.4: Results of Drop collapse test and Oil displacement assay

Isolates	Interpretation
S1	+
S2	+
S3	-
S4	+
S5	+
S6	-
M1	+
M2	+
W1	-
W2	+
W3	+
W4	-

4.3.2.4 Emulsification index

Emulsification index (E24) checks the emulsifying capacity of the biosurfactant produced by isolates. As surfactants are able to lower the surface tension of the medium enough to create emulsifications of two phases and thus enhance the solubility of the hydrophobic compound. Buffer was used as control and 8 isolates gave emulsification test positive namely: S1-22%, S2-70%, S4-10%, S5-40%, M1=16%, M2-60%, W2-32%, W3-50% (as sown in fig 4.4).

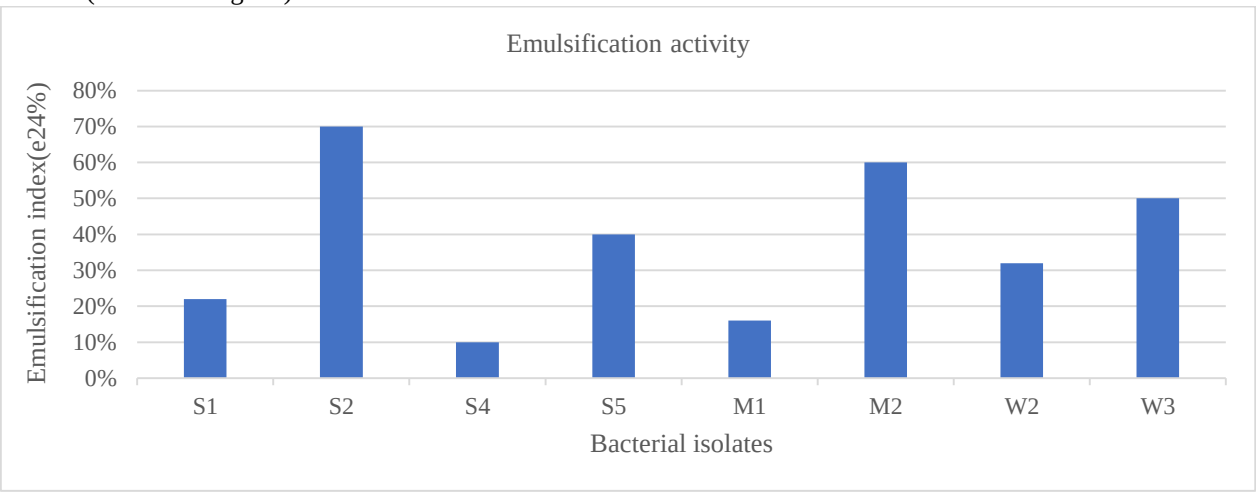

Figure 4.4: Graph of Emulsification index

4.3.2.5 Phenol-sulfuric acid method

Yellow to orange colour was produced in the test which indicates the presence of glycolipid type biosurfactant in the supernatant. The phenol test showed positive results for all 8 isolates namely: S1, S2, S4, S5, M1, M2, W2, W3(as shown in table 4.5).

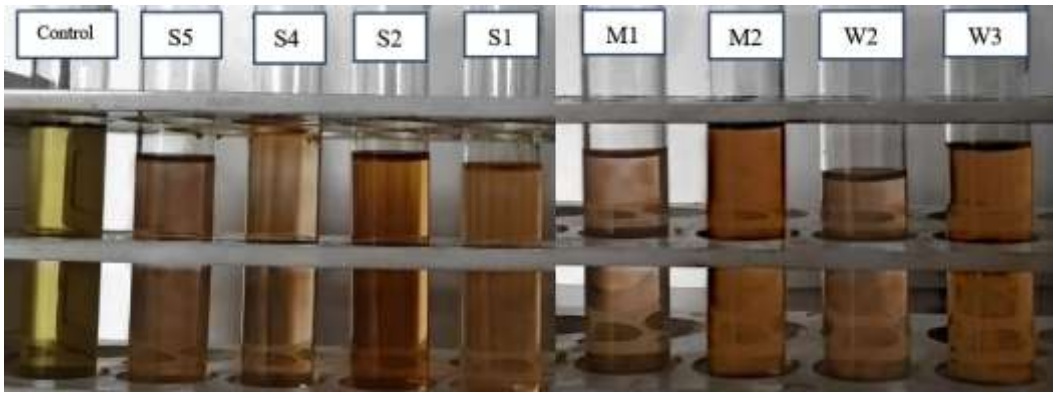

Figure 4.5: Phenol sulfuric acid method

Table 4.5: Results of Phenol sulfuric acid method

Bacterial isolates	Interpretation
S1	+
S2	+ + +
S4	+
S5	+
M1	+
M2	+ +
W2	+
W3	+ +

4.4 Production of Biosurfactant

On the basis of results of BATH assay, Drop Collapse test, Oil Displacement assay, Emulsification index and Phenol Sulfuric acid method results best isolate from each type of sample was selected for the production of Biosurfactant. These were S2, M2 and W3.

4.5 Extraction of Biosurfactant

Table 4.6 indicates that maximum biosurfactant was produced and extracted from S2 isolate which was obtained from oil contaminated soil :0.55g/100ml- light brown colour. Respectively 0.50g/100ml - black colour and 0.47g/100ml- grey colour of biosurfactant was produced and extracted from M2 isolate which was obtained from mangrove sediment and W3 isolate from marine water.

Table 4.6: Colour and Weight of dry extracted biosurfactant

Bacterial isolates	Colour of dry biosurfactant	Extracted dry biosurfactant(g/100ml)
S2	Light brown	0.55g
M2	Black	0.50g
W3	Grey	0.47g

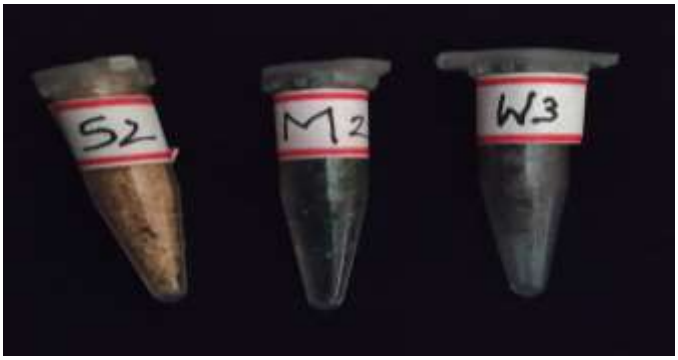

Figure 4.6: Dry Extracted Biosurfactant

V. CONCLUSION

In this study total 12 isolates of bacteria were obtained on Mineral Salt Agar plate which was used for primary screening from the 3 different types of samples: Oil contaminated soil, Mangrove sediment and Marine water. From 12 isolates, 9 isolates of bacteria were Gram negative and 3 isolates were Gram positive and motility test revealed 8 isolates to be motile while 4 isolates to be non-motile. Obtained isolates were inoculated in Mineral Salt Medium incubated for 4-5days on rotatory shaker at 37°C. After incubation broth was centrifuged and bacterial cell culture and cell free supernatant were collected and used for secondary screening which consist of various tests. Firstly, BATH assay was performed using bacterial cells and percentage of cell adherence to crude oil was calculated. Secondly, Drop collapse test and Oil displacement test was performed in which from 12 isolates obtained in primary screening 8 isolates showed positive results: S1, S2, S4, S5, M1, M2, W2 and W3. Further, Emulsification index (E_{24}) was performed in which 8 isolates gave this test positive: S1, S2, S4, S5, M1, M2, W2 and W3. Lastly, Phenol sulfuric acid was performed method which indicates the presence of glycolipid type of biosurfactant in the supernatant. On the basis of secondary screening test results best isolate from each type of sample was selected for the production process. S2 isolate which was obtained from oil contaminated soil, M2 isolate obtained from mangrove sediment and W3 isolate obtained from marine water sample were selected for the production. After production, extraction was carried out using 6N HCL and organic solvents [chloroform: methanol-2:1]. Light brown, black and grey colour biosurfactant was extracted which was produced by S2, M2 and W3 isolates respectively. Further characterization of the extracted biosurfactant can be carried out by: - TLC: Thin Layer Chromatography and FTIPR: Fourier Transform Infrared Spectroscopy.

VI. AKNOWLEDGMENT

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