Research Article

Use of various screening methods for isolation of potential biosurfactant producing microorganism from oil-contaminated soil samples

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ABSTRACT

Introduction: Biosurfactant molecules are low molecular weight surface active compounds produced by microorganisms which reduce the surface and interfacial tension of aqueous solutions and hydrocarbon mixtures. **Aim:** The present study involved different screening tests to select potent bacterial biosurfactant producer from oil-contaminated soil samples. **Materials and Methods:** Number of tests such as hemolytic activity, lipase test, drop collapse test, and oil spreading test was adopted initially for mass screening and also to filter promising isolates. In a second phase, more precise penetration assay, bath test, reduction in surface tension, and emulsification index measurement were used to finalize and score each isolate as per scoring index. In the last step, bacteria were identified as per the 16SrRNA gene sequencing to identify them up to species level. **Result:** Out of 150 isolates collected after enrichment by the 1st phase study, effective 21 isolates were recovered as biosurfactant producer, and by second phase analysis, bacterium Serratia rubidaea strain KAP (Accession number: LC2017792) was recognized as best biosurfactant producer by involving scoring system earn points in every test by the organism. **Conclusion:** Studied methodology put forward the success of combination-based screening tests for selection of potent biosurfactant producers under in vitro conditions.

KEY WORDS: Biosurfactant, Emulsification index, Screening methods, *Serratia* sp., Surface tension measurement

INTRODUCTION

In today's industrial processes, number of surfactants are regularly been implemented and requirement of these is on increase with number of industries growing around. It has been observed that chemical surfactants are now posing severe threat to the environment; making it toxic and pollutant compound whenever it is been used and released into nature.[1] As number of rules are changing with due attention toward eco-friendly approach replacement of synthetic surfactants with eco-friendly biosurfactants are in demand.[2] Biosurfactants are equivalent to chemical surfactant and posses' features such as emulsification, detergency, wetting, foaming, dispersion, and solubilization of number of hydrophobic compounds.[1]

Microbial biosurfactants are in great interest with the host production from bacteria, yeast, and fungi as they results in varied featured biosurfactants.[3]

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These compounds as a biosurfactant successfully had been used to handle environmental bioremediation as a produce by number of microorganisms.[4] Biochemically biosurfactants are low and high molecular weight in nature, among them former is glycolipids and lipopeptides in nature and later one are mostly polymeric biosurfactants.[5,6] Workers advocated its mass use in industrial and other applications as it possess certain biodegradable features along with its low toxicity and prolonged stability in number of environmental conditions such as salinity, extreme pH, and temperature.[4] In the present study, bacterial isolates able to utilize oil as nutrient and capable of producing biosurfactant at its own level under laboratory conditions has been studied when they were sampled from environmental origin to select best-scored biosurfactant producer as per tests result.

MATERIALS AND METHODS

Isolation and Enrichment of Biosurfactant Producing Microorganisms

In a process of sampling, local sites of city of Mumbai such as ration shops, edible oil mills, petrol pumps,

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garage, mangroves, activated sludge, coastal areas, and from areas of petroleum products were selected. During sampling, soil and wash water/effluent from given localities were collected in sterile flasks and brought to laboratory for enrichment and was inoculated in nutrient broth (Himedia) with added 1% coconut oil or machine oil or lubricant as per the sample need as an inducer. Inoculation was set at 1:10 of liquid sample and inoculated in 250 ml of flask which was allowed to incubate at 30°C for 5 days under shaking conditions at 130 rpm.

After enrichment, 50 ml of modified M9 medium in g/L beef extract (0.3); peptone (0.5); KH_2PO_4 (3); Na_2HPO_4 (6); NH_4Cl (1); NaCl (0.5); and pH 7.0 was inoculated with 1 ml enriched sample along with 1% inducer to get incubated at 30°C for 7 days at 132 rpm in shaking incubator. After incubation, obtained growth was diluted up to 10^{-8} in sterile phosphate buffer saline (pH 7.2) and plated on solid medium containing 1% glycerol to obtain colonies which was later on store at 4°C for further study.[7]

Screening of Biosurfactant Producers

Blood agar method

First, potent biosurfactants were screened by inoculating growth on blood agar plates which remained preloaded with fresh human blood (Mackie and Mc Cartney, 14th edition) and let it be incubated at 37°C for 24–48 h. Positive result (Biosurfactant production) recorded on scale as clear zone around the colonies as per scale: ++++ (>3 cm); +++ (>1 and <3 cm); ++ (hemolysis with <1 cm); + (incomplete hemolysis); − (no hemolysis).

Lipase test

Inoculated isolates were streaked on tributyrin agar plates (Himedia) when incubated at 30°C for 24–48 h; a defined zone of clearing around colonies represents biosurfactant production.

The diameter of the clear zones depends on the concentration of the biosurfactant and recorded[8] as "++" complete and large; "+" as incomplete and small clearance, and "-" no clearance around the colony.

Drop collapse method

Initially, 2 µl of mineral oil was loaded in 96 well microtiter plate and allowed to equilibrate for 1 h at 30°C and then 5 µl of the culture was added to the surface of oil.[9] The change in drop of surface oil was checked at 1 min. If biosurfactant is there, a flat drop was recorded with characteristics increase in activity as + to ++++ to record partial-to-complete spreading on oil surface and other as negative.

Oil spreading technique

In a clean Petri plate (25 cm diameter), about 50 ml distilled water was added which was then surface loaded with 20 µl of crude oil followed by 10 µl of culture on oil surface. The positive result was recorded as displacement of oil in diameter of surface.[10,11]

CTAB agar method

This method is used to detect anionic surfactants when isolates were inoculated on modified minimal salt agar medium supplemented with CTAB (0.2 g/L) along with methylene blue dye (0.005 g/L).[7,12] In a positive result after incubation at 37°C for 72 h, appearance of bluish halo around the colonies showcases biosurfactant presence.

Penetration assay

Assay involves 96 well plate filled with 150 µl of hydrophobic paste made of oil and silica gel. The paste was then layered with 20 µl of oil. Later on, 90 µl of culture supernatant supplemented with 10 µl of 1% safranin was prepared of which 20 µl was gently placed on the surface of the preparation and then the microtiter plates were allowed to incubate at 30°C for 15 min. In a result, with the presence of only biosurfactant positive sample been able to pass through hydrophilic liquid to cross the oil layer and that result in color change from red to cloudy white. In a result, response rate was recorded as "++" highly positive, that is, complete color change from red to cloudy white; "+": weakly positive, that is, partial change of color from red to cloudy white and negative with no change in color.[13,14]

Bath test

All isolates were allowed to grow on modified M9 medium and growth was centrifuged at 10,000 g for 15 min to obtain pellet. These pellets were washed with phosphate buffer saline. About 2 ml of cell suspension was then added with 100 µl of n-hexane. The preparation was vortexed thrice at high speed and left for 30°C under static conditions for 60 min. The setup forms two layers, out of which aqueous layer was sampled and results of absorbance recorded at 600 nm under visible spectrometry. The percentage hydrophobicity was then calculated as follows:[15]

$$[1-\{O.D\ (A)\ O.D \div (A_0)\}] \times 100$$

A_0= The O.D of the initial cell suspension

A= The O.D of the cell suspension after incubation

Measurement of surface tension

In a cell-free supernatant of broth, surface tension values in triplicate were recorded as per the Du Nouy ring method by involving tensiometer. In a study,

distilled water was taken as reference control to determine surface tension change.

Measurement of emulsification index

As per Cooper and Goldenberg (1987),[16] 2 ml kerosene was added to the 2 ml of culture broth made cell free and kept in a medium size test tube. Test tube then vortexed for 2 min at high speed and then kept to record emulsion stability at 24 h using the formula:

Emulsification Index = (Height of emulsion layer/ Total height) ×100

Bacterial Identification by 16s rRNA

In a last step, promising biosurfactant producer was identified up to species level by targeting 16s rRNA gene. The service was availed from Sai Biosystems Private Limited, Nagpur, India.

RESULTS AND DISCUSSION

In the present study, number of sampling points in Mumbai city were selected (wash water/soil/effluent samples), and those were rich in hydrocarbon content and also promising for hydrocarbon degrading bacteria producing biosurfactant. Worker Jennings *et al*.[17] also put forward the success of biosurfactant producing organisms when they were sampled from hydrocarbon-rich soil and wash water or effluent.

Hydrocarbon degrading rich flora when serially diluted and plated on the nutrient agar when supplemented coconut oil, diesel, machine oil, and lubricant, about 150 isolates appeared with their typical colonies. In a similar approach, biosurfactant producing microorganisms' were successfully recovered when they were supplemented with inducers such as ethanol-blended gasoline, diesel, coconut oil, sunflower oil, waste frying oil, and others.[18,19]

As per early four tests, when screening was done with 150 isolates, marker 21 isolates which had shown positive tests for hemolytic activity, lipase activity, oil drop collapse test, and oil displacement test were selected and considered further. In a similar approach, worker Satpute *et a*l. (2010)[20] adopted the tests such as tilted glass slide test, drop collapse test, oil spread method, hydrocarbon overlay agar plate, blue agar/CTAB agar plate, emulsification index, and emulsification assay when investigated on marine bacteria. Another worker Elazzazy *et al.*[21] implemented drop collapse test, oil displacement test, blood hemolysis test, blue agar test, and others to successfully study biosurfactant producer.

Further, in the present study, testing of these 21 isolates reported positive hemolysis activity with different capabilities [Table 1]. It is referred that careful screening by hemolysis test certainly increases the chances for better selection of biosurfactant producer as this test is recognized as the gold standard in preliminary screening process.[1,22] Carrillo *et al*. demonstrated the association of hemolytic activity and surfactant production. Screening through positive hemolytic activity isolates for the potent biosurfactant production has commonly been recommended by workers; Carrillo *et al*.[23,24] In response similar to our study, Nalini and Parthasarathi, Satpute *et al*., and Elemba *et al*.,[12,25,26] has isolated number of hemolytic positive isolates as *Serratia marcescens*, *Bacillus* sp., *and Pseudomonas* sp. with biosurfactant capabilities.

In the present study, selection of 21 isolates for biosurfactant capabilities was achieved by recording the combining reports of more than one test. This approach of using more than one screening test to finalize the biosurfactant producer has also been advocated by other workers like Satpute *et al* (2008) and Saravanan and Vijaykumar (2012)[12,27], as one test base (e.g., hemolysis) may lead to false-positive test for detection of biosurfactant producer.[28] Hence, it has been decided to consider aforementioned collective tests results to select best performing biosurfactant and worked effectively.

Further, for lipase activity, all isolates did not turned positive which was earlier recorded positive in hemolysis as in Table 1. Hence, those giving positive results in tributyrin agar and hemolysis were further checked for biosurfactant production. In one of the report, Deepa *et al.* (2015)[29] suggested to consider lipase assay result as potent biosurfactant producer,

Table 1: Screening results for hemolytic activity, tributyrin agar hydrolysis, and oil drop collapse test of the 21 isolates

Bacterial isolate	Hemolytic activity	Tributyrin agar hydrolysis	Oil drop collapse test
W-13	++	+	++
W-15	+	+	+
W-16	+++	++	+++
W-19	++	+	+
W-20	++	+	+
W-28	++++	++	+++
W-31	++	+	++
W-37	++	+	++
W-44	+	+	+
W-46	++	+	++
W-49	++	+	++
W-51	+	+	+
W-85	+	-	+
W-86	+++	-	++
W-87	+++	-	+++
W-88	+++	+	++
W-89	+++	+	++
ISL-01	+++	++	+++
ISL-02	+++	++	++
IE-02	+++	++	+++
SS-04	++	+	++

especially for *Streptomyces* sp. It is well studied that ability of lipase expression in biosurfactant-producing microorganisms related with the production of many compounds such as fatty acids, lipopeptides, glycolipids, phospholipids, neutral lipids, and lipopolysaccharides.[3] It is supportive to know that lipase and biosurfactant can be produced concurrently by involving solid state bioprocess and submerge culture system.[30] Here, it is mentioned that lipases keeps industrial importance in number of processes, especially to resolve racemic mixtures and for the treatment of residues containing oils and fats.[31] The present study demonstrated to produce lipase by many bacterial species using tributyrin agar [Table 1].

In the third test (oil drop collapse test) isolates W-16, W-28, W-87, ISL-01, and IE-02 recorded to give positive results [Table 1]. In a similar result, worker Hanen *et al*.[32] suggested that positive cultures for collapse of the oil drop resulted in better biosurfactant producer and certainly been involved in lowering the surface and interfacial tension between oil and water.[33] We observed that given test is easy to perform with its high sensitivity and quantitative capabilities as referred by the Bodourand and Miller-Maier[8] also. However, low biosurfactant production reduces its sensitivity and gives most of them negative test.[28]

In the present study, by involving oil displacement test which is based on the biosurfactants altering the angle of contact at the oil and water, which resulted in surface pressure exerted by the biosurfactant and displacement of the oil drop[34] and results put forward that all the isolates remain positive for oil displacement test. Thus, oil displacement test has been used by past researcher to screen and identify the primary potential of various biosurfactant producing isolates. Among them, isolates W-16, W-28, W-86, W-87, ISL-01, and IL-02 were the key performers, but isolate ISL-01 recorded maximum displacement as given in [Figure 1. Similar to isolate ISL-01, worker Nalini and Parthasarathi[25] reported positive displacement test for the bacterium, *Serratia rubidaea* SNAU 02 and also *Staphylococcus hominis* found to be positive for oil displacement.[35]

In the next test, Siegmund and Wagner[36] reported a biosurfactant detection with blue agar or CTAB agar plate, especially to detect glycolipids and rhamnolipids production. In the present study, isolates W-16, W-28, W-87, and ISL-01 found to be positive for the said test [Table 2]. As per results, certainly, these isolates contain anionic biosurfactant since they can form insoluble ion pair with the cationic CTAB and methylene blue and formed blue halo around growth.[12] Similar to our isolates, *Pseudomonas* sp. also possesses this feature as reported by Nisanthi *et al*. and Sumathi and Yogananth.[37,38]

Maczek *et al*.[13] developed an assay suitable for high throughput screening for biosurfactant production called the penetration assay. This assay relies on the fact that if biosurfactant is present, the hydrophilic liquid will cross the oil layer and result in change in color from red to cloudy white. Out of the 21 isolates selected for the screening studies, only 16 bacterial isolates gave a positive result for penetration assay. The best result was obtained for the isolates W-16, W-28, ISL-01, ISL-02, and IE-02 [Table 2]. Vandana and Peter,[39] screened *Pseudomonas aeruginosa* for biosurfactant production using penetration assay. Similar results were obtained by Nisanthi *et al*. and Sumathi and Yogananth.[37,38]

In a bath test, isolates W-13, W-28, W-31, W-46, W-87, ISL-01, and ISL-02 resulting as best scorer [Table 2]. In one of the report published by Thavasi *et al*.,[40] as many as 91 bacterial species out of 105 marine isolates found to be positive for bath test and linked then with affinity toward hydrophobic substrates. Bath test was developed by Rosenberg *et al*.[15] There is a direct correlation between cell surface hydrophobicity and biosurfactant production. Cell bound biosurfactant production is associated with hydrocarbon uptake. Depending on the hydrocarbon uptake behavior, microorganisms may have high- or low-surface hydrophobicity. Those microbes which can take hydrocarbon by direct uptake mode do show high surface hydrophobicity.[41] In a study carried out by Sumathi and Yogananthan,[38] they found that biosurfactant produced by *Pseudomonas aeruginosa* presented 50% hydrophobicity. Thavasi *et al*.[40] screened 105 marine isolates using BATH assay. They found that 91 bacterial strains were positive for the BATH assay, which indicated the affinity of the bacterial cells toward hydrophobic substrate. Maximum cell attachment was found with *Pseudomonas aeruginosa* followed by *Lactobacillus delbrueckii*. Bacterial strains with high cell hydrophobicity are reported as potential biosurfactant producers.[42,43]

In a surface tension estimation, isolates W-16, W-28, W-86, W-87, ISL-01, and IE-02 over scored with maximum value as 30 mN/m recorded with isolate ISL-01 [Figure 2] which resulted in reduction of surface tension up to 52.38%. In a similar finding, *S. rubidaea* SNAU 02 observed with reduction value of 34.4 mN/m very close to our ISL-01 isolate.[25] *Isolate S. marcescens* UCP 1549 reported with surface tension as 33 mN/m[44] and in other study *Bacillus sp*. strain MTCC 5877 surface tension was recorded as 72 to 30.06 mN/m [45] and both the data remained comparable with the present study values. Ibrahim [45] isolated two biosurfactant producers from engine

Table 2: Results of CTAB agar test, penetration assay, and bath test of different isolates

Bacterial isolate	CTAB agar test (mm)	Penetration assay	Bath test (% hydrophobicity)
W-13	19	+	49
W-15	14	+	25
W-16	28	++	30
W-19	20	+	31
W-20	22	+	26
W-28	30	++	58
W-31	23	+	49
W-37	20	+	24
W-44	12	-	11
W-46	22	+	49
W-49	23	+	27
W-51	13	–	13
W-85	0	–	10
W-86	25	+	22
W-87	26	+	45
W-88	26	-	11
W-89	14	-	14
ISL-01	28	++	58
ISL-02	22	++	48
IE-02	26	++	38
SS-04	25	+	37

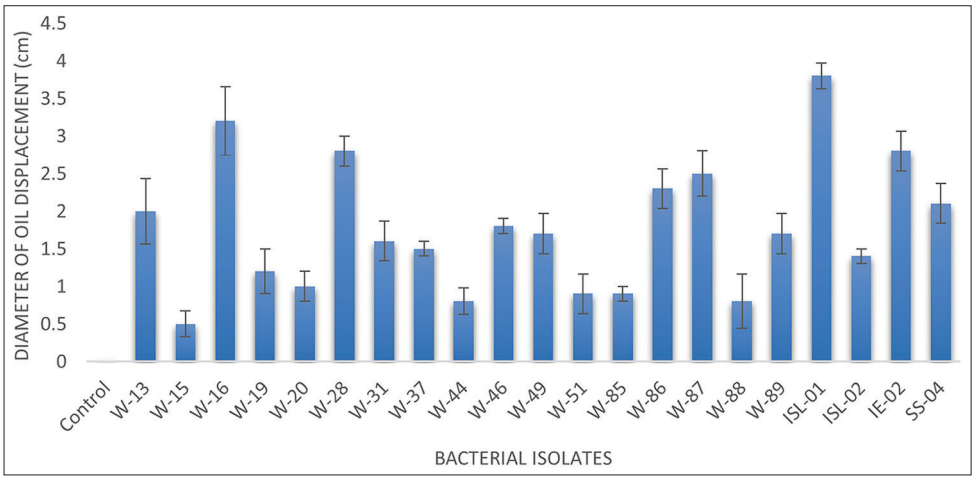

Figure 1: Result of oil displacement test of different bacterial isolates

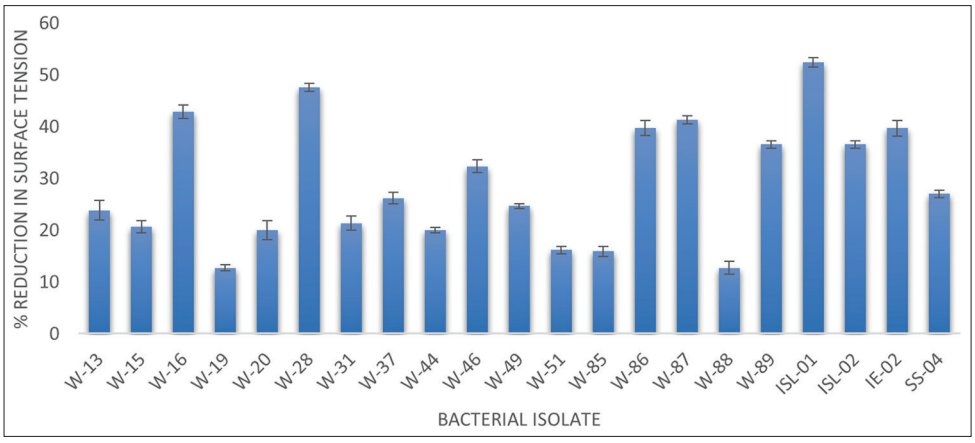

Figure 2: Result of surface tension reduction by different isolates

oil-contaminated soil *Ochrobactrum anthropi* HM-1 which gave a surface tension reduction (30.8 ± 0.6 mN/m) and *Citrobacter freundii* HM-2 (32.5 ± 1.3 mN/m), while the control culture broth medium and distilled water gave a surface tension value of 70 ± 0.9 and 72 ± 0.7 mN/m.

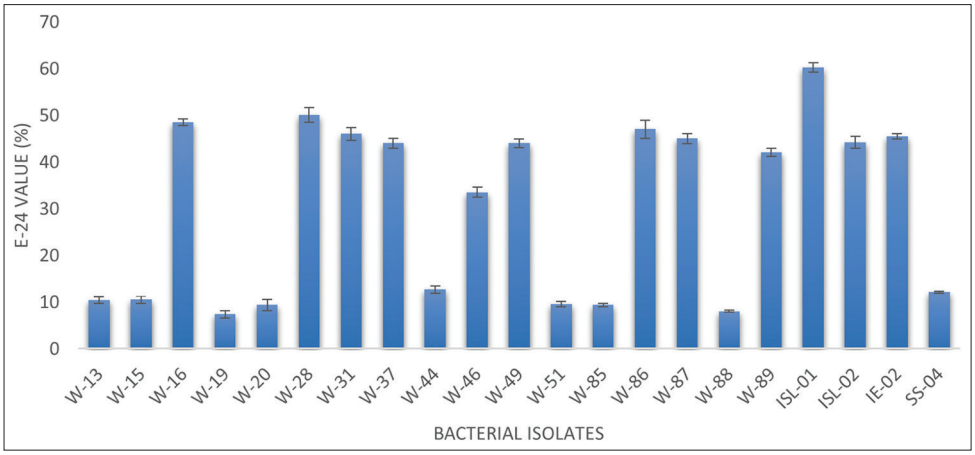

Figure 3: Emulsification index of different isolates

As per E-24 value or emulsification index, E-24 above 30% considered as base value for efficient biosurfactant producer[12] and in results, isolates W-16, W-28,W-31, W-37, W-46, W-86, W-87, ISL-01, ISL-02, and IE-02 found to be efficient in activity more than 30% [Figure 3]. In a comparison, isolate ISL-01 once again recorded higher with E-24 value as 60.25% which was even higher than *S. marcescens* (58%) as reported by Gumma *et al*.[46] In few studies, some higher value of E-24 recorded with species, *S. marcescens* UE015 (78.90%) and 79.92% for strain UCP1549.[47]

Overall, tests analysis indicated the positive response from isolate ISL-01 as it acted as best performer in most of the tests which was further identified by 16s rRNA gene homology as *S. rubidaea* KAP (Accession number: LC201792). Here, it is important to note that *Serratia* sp. already been linked with biosurfactant production capabilities,[25] and especially *S. rubidaea* SNAU02 isolated from oil-contaminated soil resembles in its features.

CONCLUSION

In the present study, carefully selected number of soil and water samples were found to harbor potent biosurfactant producers and those have been easily detected by applying screening tests such as oil drop collapse, oil displacement, CTAB agar, penetration assay, bath test, and measurement of surface tension along with emulsification index. Among the isolates, ISL-01 identified as *S. rubidaea* strain KAP (NCBI Accession no. LC 201792), which is found to be the best performer as biosurfactant producer and hence adopted methodology puts forward the success of isolation, selection, and screening protocols for biosurfactant producer.

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