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ISOLATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIA AND THEIR APPLICATION AS AN ANTIBACTERIAL AGENT

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ABSTRACT

Biosurfactants are the compound which helps in reducing surface tension. These compounds help in stabilizing emulsions, promoting, foaming and reducing surface tension etc. Eight bacterial isolates were isolated from Auranga River, District of Valsad, Gujarat. The isolates were screened for biosurfactant production using different screening methods as oil spreading assay, blood agar hemolysis, emulsification assay, emulsification activity and confirmation by phenol sulfuric method. The biosurfactant was investigated for potential antimicrobial activity by using the cup borer method against *Bacillus subtilis, Escherichia coli and Staphylococcus aureus.* Antimicrobial studies of the surface active molecules indicated the presence of biocontrolling potential against human bacterial pathogen.

KEYWORDS: *Auranga River, Biosurfactant, Antimicrobial activity, Screening.*

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INTRODUCTION

Biosurfactants are surface active compounds produced by microorganisms. They reduce surface and interfacial tension by accumulating at the interface of immiscible fluids and thus increase the solubility, bioavailability and subsequent biodegradation of the hydrophobic or insoluble organic compounds.[1] They consist of heterogeneous group of organic compounds including glycololipids, lipopolysaccharides, oligosaccharides and lipopeptides.[2] Biosurfactants vary in chemical properties and molecular size. They contain hydrophobic and hydrophilic moieties that give them the ability to interact between fluid phases. Thus minimizing surface and interfacial tension at the surface and interface respectively.[3, 4] Microorganisms make use of a wide range of organic compounds as a source of carbon and energy for their growth: when the carbon source is in an insoluble form like a hydrocarbon, microorganisms make possible their diffusion into the cell by producing a variety of biosurfactants. Some of the bacteria and yeasts excrete ionic surfactants which emulsify the hydrocarbon substance in the growth medium. A few examples of this group of biosurfactant are rhamnolipids.[5] or sophorolipids.[6] Microorganisms producing biosurfactants help to amplify the bioavailability of hydrocarbons by enhancing the contact between pollutants and the microorganisms in the presence of the biosurfactant which help in the accelerated bioremediation of hydrocarbon contaminated sites.[7] The properties of biosurfactants include excellent detergency, emulsification, foaming, wetting, cleansing, phase separation, surface activity and reduction in viscosity of crude oil, makes it feasible to utilize them for many application purposes.[8]

MATERIAL AND METHODS

Sample collection

The study was carried out using Auranga River water resources, District, Valsad. The water samples from Auranga River were collected and placed an ice thermo insulated container (temperature inside was not higher than 7^0 C), and brought to laboratory where they were immediately analysed.

Isolation

The collected water sample was inoculated on to sterile mineral salt medium (MSM) containing 2% of substrate. The substrate selected for the purpose was mustard oil, sunflower oil, kerosene, petrol, diesel. These plates were incubated at 37^0c for 48 hour. The plates were examined for varying types of colonies. All the different types of colonies were isolated based on their colony characteristics. Pure isolates were obtained by repeating sub culturing on fresh mineral salt medium. The pure isolates were maintained on mineral salt agar slant in a refrigerator (4°C). The isolates were identified by biochemical characterization.

Biosurfactant production

Isolates were grown in 500 ml Erlenmeyer flasks containing 100 ml mineral salt medium adjusted to pH 7.0 and the flasks were incubated at 37° C on shaker for 7 days and then screening was performed with following method.

Screening for Biosurfactant Production

Oil displacement method

It is a method used to determine the diameter of the clear zone, which occurs after adding surfactant containing supernatant collected by spinning the 24 hour culture at 1000 rpm for 10 min on an oil water interphase. The diameter evaluation allows the surface tension reduction efficiency of a given biosurfactant. In this test, 15 ml distilled water was added to a petridish of 90 mm, 100 micro liter of test oil was added to the water surface followed by the addition of 20micro liter of cell culture supernatant on the oil surface. The diameter and the clear halo visualized under visible light was measured after 30 seconds.[9]

Blood hemolysis method

Blood agar hemolysis method is used to screen biosurfactant producing bacteria. This method is based on the fact that biosurfactants are able to hemolyse the red blood cell present in the blood. Culture of selected isolated were spot inoculated on blood agar plates. These plates were incubated for 24-48 hours at 37°C. After incubation, zones of hemolysis were measured. Alpha hemolysis showed partial hemolysis, beta hemolysis showed complete hemolysis and gamma hemolysis showed no hemolysis.[10]

Emulsification assay

The Emulsifying capacity was evaluated by an emulsification index (E_{24}). The E_{24} of culture samples was determined by adding 2 ml of the oil (Engine oil, crude oil, kerosene, olive oil) to 2 ml of the culture supernatant in test tube, vortexed at high speed for 2 min and allowed to stand for 24 hour. The E_{24} index is given as percentage of the height of the emulsified layer (cm). The percentage of the emulsification index was calculated by using the equation. The results were compared with tween 80 as positive control.[11]

$$E_{24} = \text{Height of emulsion formed/ total height of solution} \times 100$$

Emulsification activity

5ml of supernatant was taken in a 30 ml screw capped test tube. Emulsification activity was tested against crude oil. To the above solution 5 ml of hydrocarbon was added and shaken well for 20 min in a shaker at 150 rpm and the mixture was allowed to stand for 20 min. The optical density of the mixture was measured at 610 nm and the results were expressed as D_{610}.[12]

Confirmatory method

Phenol sulfuric acid method

Biosurfactant producing bacteria selected from above screening methods were inoculated in mineral salt

medium broth and incubated at 37° C on rotatory shaker for 4-5 days. After incubation, broth was centrifuged at 10,000 rpm for 15 min and supernatant was collected while pellet was discarded. 1 ml collected supernatant was mixed with 1 ml of 5% phenol then 5ml of conc. H_2SO_4 was added in drop wise manner. Presence of biosurfactant in supernatant produces yellow to orange colour.[13]

Extraction of biosurfactant

The whole culture broth was centrifuged at 400 rpm for 1 minute. The supernatant was dispensed into sterile test tubes using a sterile pipette, and then 0.5 ml of hydrochloric acid (HCL) was added. 1 ml of the organic solvent (chloroform-methanol) in the ratio of 2:1 (v/v) was also dispensed into the test tube and allowed to stay for 30 minutes and then centrifuge at 400 rpm for 1 minute. Then the supernatant was collected using a sterile pipette and dispensed into a sterile test tubes and then place in oven at 40°C to obtain the dried crude biosurfactant.[14]

Characterization of biosurfactant using thin layer chromatography

The components in the extracted biosurfactant were separated on aluminium silica gel 60F254 Plates. using a solvent system chloroform-to-methanol-to-water in the ratio 65:25:4 . The separated components were detected by iodine vapour and under UV light. Ninhydrine reagent was sprayed to detect lipopeptide biosurfactant as red spots. Throne reagent was sprayed to detect glycolipid biosurfactant as yellow spots. [15]

Determination of antimicrobial activity of biosurfactant

The crude biosurfactant was dissolved in methanol and kept in a sterile glass tube. Antimicrobial activity of the biosurfactant was performed by agar well diffusion method against three test organisms: *Escherichia coli, Staphylococcus aureus*, *Bacillus Subtilis*. Fresh bacterial culture of 0.1 ml was spread on nutrient agar plate and allowed to dry for few minute. Methonol is used as a control in one well. Wells of 6 mm in diameter were punched off into medium with sterile cork borer and filled with 50 µl of biosurfactant suspension prepared in methanol is used as a control in one well (1 mg/ml). All the plates were kept in refrigerator to allow prediffusion of extract for 30 min and further incubated at 37°C for 24 hour the antimicrobial activities were evaluated by measuring the zone of inhibition.

RESULTS

8 isolates with different colony morphology were observed on mineral salt medium plate to obtain pure culture and they were used for further study.

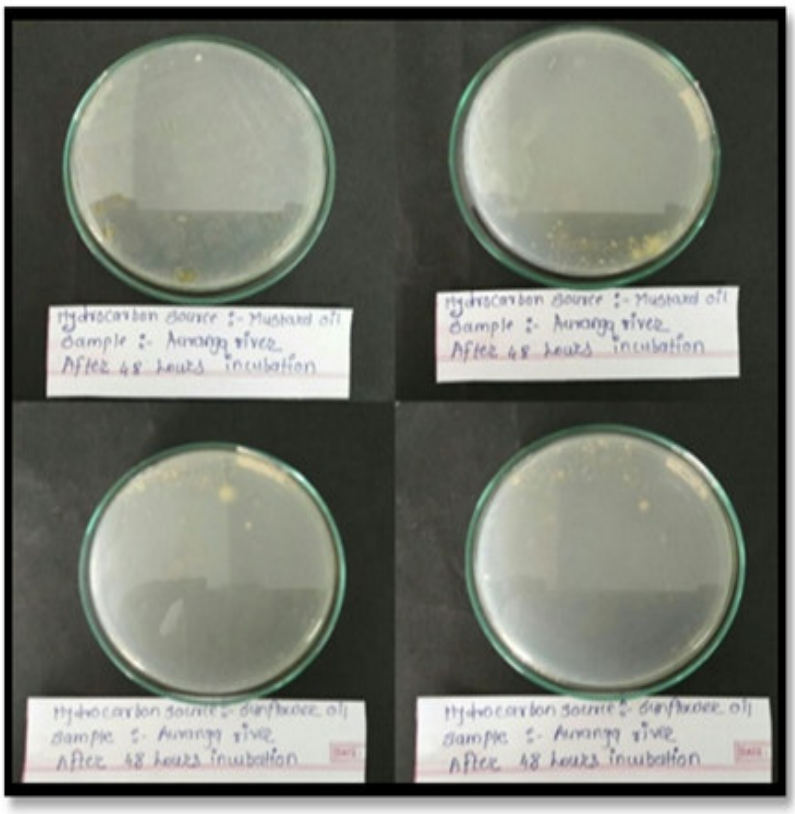


Figure 1
Isolated bacterial colony grown on mineral salt medium plate.

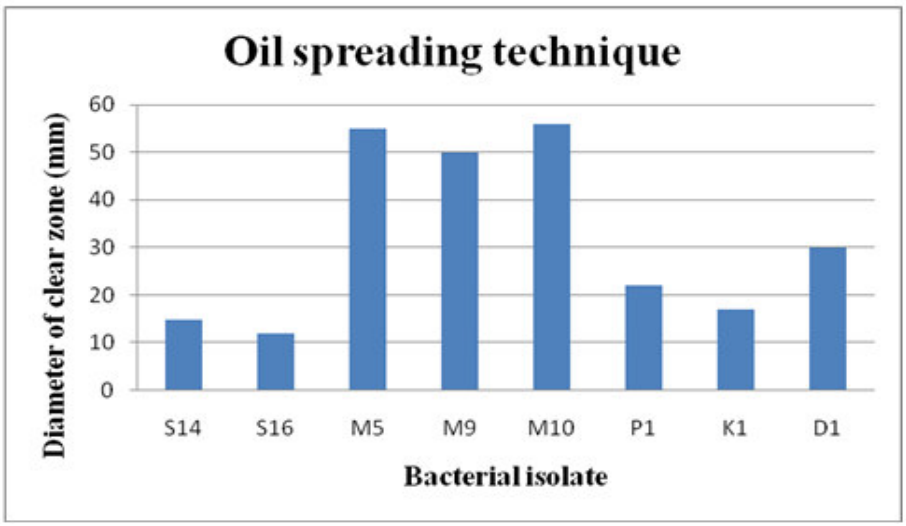
Table 1
Morphological and biochemical characteristics of bacteria isolated from Auranga River.

Features	S14	S16	M5	M9	M10	K1	P1	D1
Gram stain	+	-	+	-	-	-	-	-
Motility	Motile	Non- motile	Non-motile	Non-motile	Non- motile	Non- motile	Non-motile	Non-motile
Iodole test	-	-	-	-	-	-	+	+
MR test	+	-	-	-	-	-	+	+
VP test	+	-	+	-	-	+	-	-
Lead acetate test	-	-	-	-	-	-	-	+
Urea test	-	-	-	-	-	-	-	-
Citrate test	-	-	-	+	+	+	+	+
Gelatine test	-	-	+	+	+	+	-	-
TSI test	-	-	-	+	+	+	+	+
PN water test	+	+	+	+	+	+	+	+
Oxidase test	+	+	-	+	+	+	+	+
Catalase test	+	+	-	+	+	+	+	+
Sugar utilization test Xylose	+	-	+	-	-	+	+	+
Sugar utilization test Maltose	+	-	+	-	-	+	+	+
Sugar utilization test Mannitol	+	+	+	-	+	+	+	+

Screening of isolated bacteria
Isolates were screened for the confirmation of biosurfactant and these were used for the further study.

Oil spreading technique
Supernatant of isolates were added to the plate containing mineral oil. It was added to the centre of oil layer. All isolates displaced the oil and showing a clear zone. The biosurfactant producing organism can only able to displace the oil. Hence the results were noted down.

Graph 1
Results of oil spreading technique



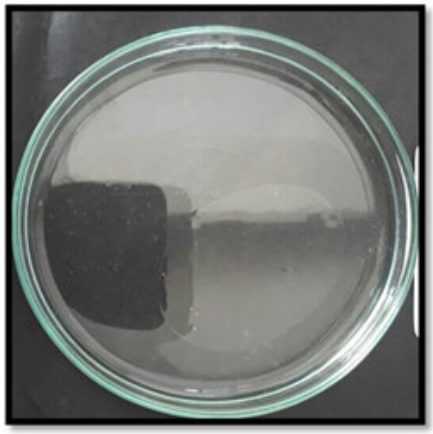


Figure 2
Oil spreading technique.

Blood hemolysis test

The isolated were spot inoculated on blood agar plate. In which the isolates S14, S16, M9, M10 showed β hemolysis, whereas M5, P1, D1 showed γ hemolysis and K1 showed αhemolysis.

Table 2

Results of blood hemolysis test

Bacterial isolate	Diameter of clear zone	Interpretation of blood hemolysis test
S14	35mm	βhemolysis
S16	36mm	β hemolysis
M5	-	γhemolysis
M9	39mm	β hemolysis
M10	30mm	β hemolysis
K1	11mm	α hemolysis
P1	-	γhemolysis
D1	-	γ hemolysis

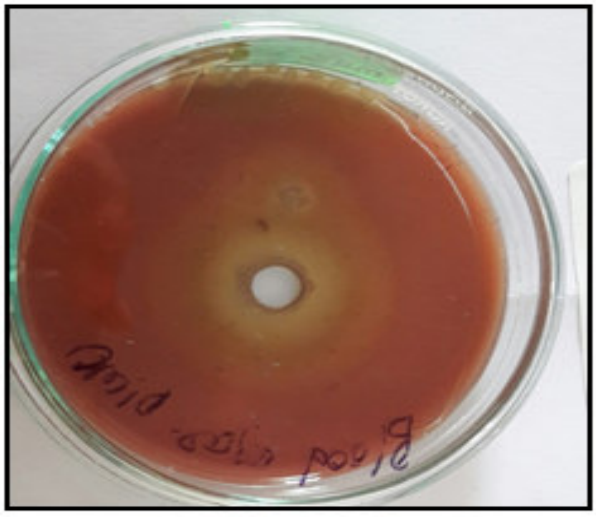


Figure 3

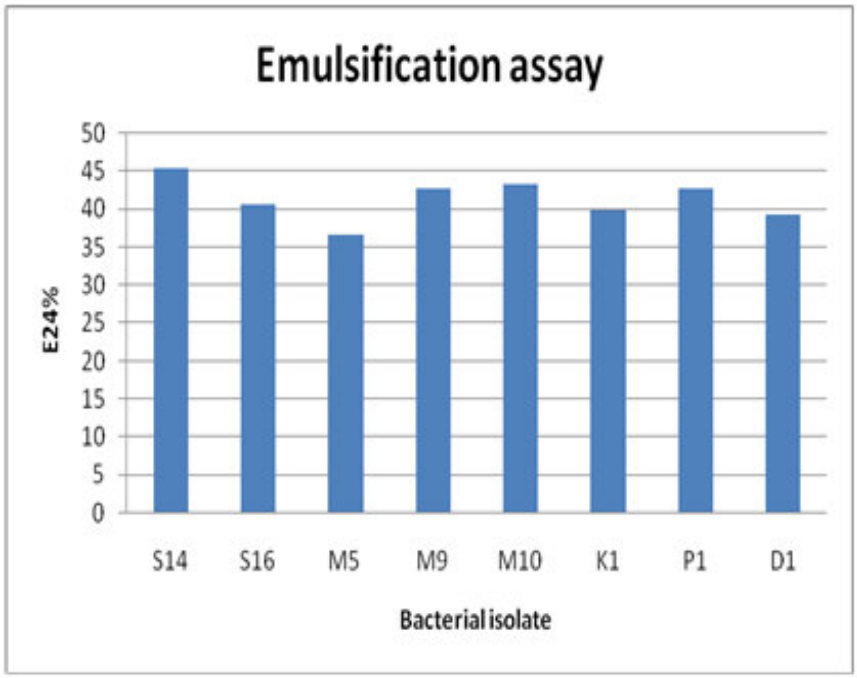
Results of blood hemolysis test isolate no S14.

Emulsification assay

The isolated showing positive results were tested for their abilities to emulsify crude oil and in this study, kerosene was used for the emulsification assay. Test was done by adding 2 ml of supernatant and kept overnight. After then results were recorded.

Graph 2

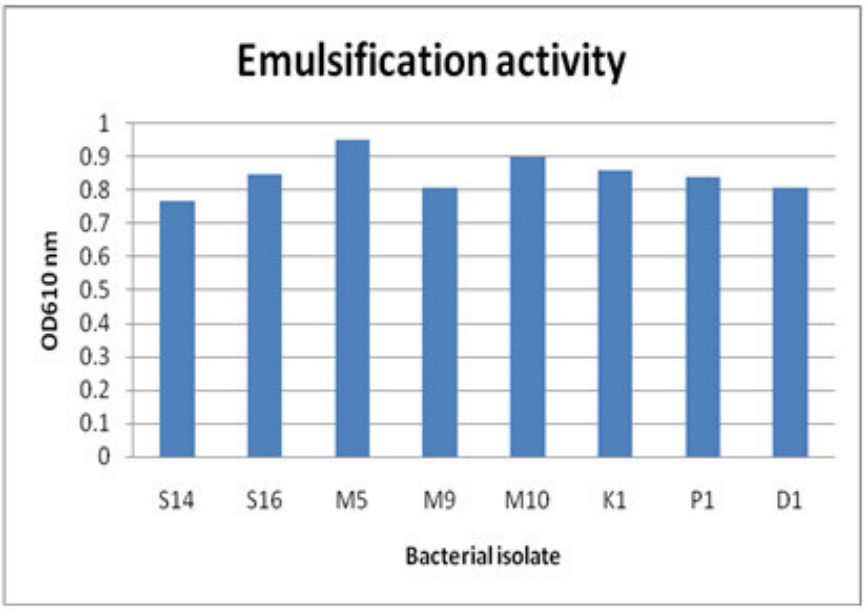
E_{24} assay of bacterial isolate



Emulsification index

Emulsification index is an indirect method used to screen biosurfactant production. It was assumed that if the cell free culture broth used in this assay contains biosurfactant, then it will emulsify the hydrocarbons present in the test solution. In this study mineral oil was used as the hydrophobic substrate. Results observed in this study, showed that M10 and M5 has the highest emulsification activity of D_{610}.

Graph 3
Emulsification index of bacterial isolate



Graph 3
Emulsification index of bacterial isolate

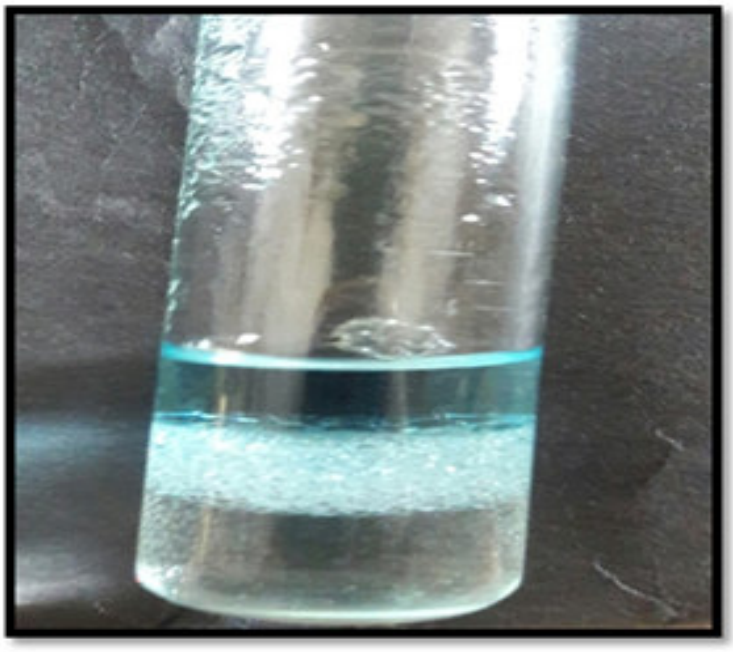


Figure 4
Emulsification activity.

Phenol-sulfuric acid method

The phenol test showed positive result for the 8 isolates, including the positive control by a change in colour from yellow to deep orange.

Table 3
Results of phenol sulfuric acid method.

Bacterial isolate	Interpretation of phenol sulfuric acid method
S14	++
S16	+++
M5	+
M9	++
M10	++
K1	+
P1	+
D1	+

Figure 5
Phenol-sulfuric acid method

Biosurfactant production and extraction
Production of biosurfactant was carried out by 8 isolates. The biosurfactant was extracted from the whole cell-free culture by centrifugation and concentrated by adjusting with sulphuric acid.

Purification of compound by thin layer chromatographic technique
The biosurfactant was partially purified from crude extract with chloroform: methanol: water by thin layer chromatography. 6 spots were observed, as given below.

Table 4
Results of Thin layer chromatographic technique

Sr. No.	Rf value (in mm)
Spot 1	0.71
Spot 2	0.78
Spot 3	0.44
Spot 4	0.42
Spot 5	0.75
Spot 6	0.64

Figure 6
Results of TLC using extracted biosurfactant for lipid content

Antimicrobial activity

Antimicrobial activity of purified biosurfactant showed broad spectrum of activity against the test organisms. The result showed that the highest activity against *Staphylococcus aureus* (40mm) followed by *Escherichia coli.* The least activity observed against *Bacillus Subtilis* (14mm).

Table 5
Antimicrobial activity by well diffusion technique.

Bacterial isolate/test organism	*Bacillus Subtilis* (zone size in mm)	*Escherichia coli* (zone size in mm)	*Staphylococcus aureus* (zone size in mm)
S14	23	28	30
S16	32	30	36
M5	24	31	36
M9	29	38	36
M10	14	21	13
K1	-	-	36
P1	23	17	29
D1	30	27	40

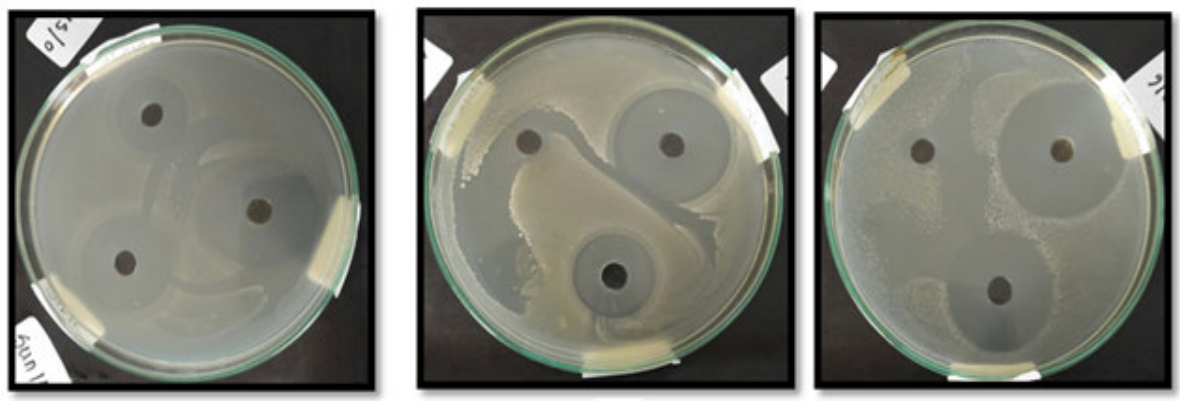


Figure 7
Results of antimicrobial activity of biosurfactant isolate no S14,S16,M5.: (a) against Escherichia coli; (b) against Bacillus subtilis; (c) against Staphylococcus aureus

DISCUSSION

The biosurfactant producing bacteria were isolated from the Auranga River. In this study, 8 isolates were obtained from Auranga River. Detection of biosurfactant producing bacteria was tested by three methods such as hemolysis, emulsification index, oil displacement method. 8 isolates were found to produce biosurfactant.

The supernatant of the isolate, added to the mineral oil containing plate's showed clear zone by displaying their ability to displace the oil. Thus, indicating biosurfactant production. All the 8 isolates showed clear zone by displaying their ability to displace the oil thus indicating biosurfactant production. Table 2 showed the screening methods for biosurfactant producing from isolated bacteria. The results of isolated bacteria on blood agar media. The emulsification activity showed that the stability of the emulsion directly proportional to the quantity of the biosurfactant present in the medium. The phenol sulfuric acid test showed positive result for the 8 isolates, including the positive control by a change in colour from yellow to deep orange that indicates the confirmation of biosurfactant producing bacteria and among these S16 showed highest result. The biosurfactant extracted was characterized by using TLC. The components were obtained as rhamnolipid that is a glycolipid while sprayed at ninhydrine reagent on the TLC sheet. Table 4 results of thin layer chromatographic technique showed that the highest lipid content in S14 in TLC sheet. River water contains not only beneficial microbes, but also various pathogens affecting different hosts. Biosurfactants can benefit the aquatic environment by dual process such as bioremediation and bio-control. Antimicrobial role of biosurfactant produced by 8 isolated bacteria was recorded against *Escherichia coli, Staphylococcus aureus, Bacillus Subtilis.* Antimicrobial activity against these organisms indicated the presence of inhibitory activity against the pathogens.

CONCLUSION

The study represented surfactant activity of the bacterial isolated from Auranga River. This confirms that environment has an influence on the metabolism of the tested microbes. Application of biosurfactant and biosurfactant producing bacteria in environmental cleaning (bioremediation) is a potential area of more research as revealed from the present study. Both organic and inorganic contaminates can be removed through different processes (physico-chemical and biological) in which biosurfactants are involved. Due to their, biodegrading potential and low hazard to the environment and human health. These microbes are very promising focus in environmental biotechnologies. Further study on the utilization of agro industrial wastes as substrates for the large-scale production of biosurfactants is recommended.

CONFLICT OF INTEREST

Conflict of interest declared none.

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