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PRODUCTION, EXTRACTION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY MARINE MICROBIAL ISOLATES

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Abstract: Biosurfactants are extracellular surface active compounds produced by bacteria, fungi and yeast. Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharides protein complexes, fatty acids and phospholipids. The main criteria used in this study were isolation of biosurfactants producer were hemolytic assay, bacterial adherence to hydrocarbons (BATH), visualization of bacteria in oil droplets, drop collapse test and emulsification assay. Isolation and screening of biosurfactant producing bacteria were performed from marine water and sand samples collected from Tithal beach of Valsad district and Dumas beach of Surat District, Gujarat, India and isolation was done on nutrient agar plates and Sabouraud's agar plates, from which 9 bacterial isolates and 4 fungal isolates were obtained. Isolates were characterized and analyzed, based on different screening methods like growth on mineral salt agar medium, hemolytic assay, BATH assay, visualization of bacteria in oil droplets, drop collapse test and emulsification assay. 7 isolates were able to grow on MSM. 8β (hemolytic) and 5ɣ (non- hemolytic) isolates were obtained on blood agar plates. In BATH assay from 9 isolates, 2 isolates shown high affinity with engine oil. 6 isolates had shown the red dot of TTC absorption in visualization of bacteria in oil droplates. In drop collapse test only 1 isolate using supernatant had shown great activity. In emulsification assay only 1 isolate was found promising biosurfactant producer and is selected based on its highest emulsification activity and is used for production and extraction process. Production of biosurfactant was carried out by single isolate which one is showing high zone as well as passing all the screening test. Finally production of biosurfactants was carried out by only 1 isolate. The biosurfactant was extracted from the cell- free supernatant by centrifugation. Antibacterial activity of biosurfactant was done against the test organisms and zone of inhibition was observed. Also the qualitative analysis of the extracted biosurfactant was done by TLC and FTIR to check presence of possible functional group.

***Keywords*- Biosurfactant producing microbes, Haemolytic activity, Triphenyl tetrazolium chloride, Bacterial Adhesion to Hydrocarbon (BATH) assay, organic solvent (chloroform- methanol), Antibacterial activity**

1. INTRODUCTION

Biosurfactant are amphiphilic compounds produced by bacteria, fungi and yeast. They belong to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides. Accumulating at the interface, these chemicals can decrease the surface tension (ST) and the interfacial tension between aqueous and other immiscible solutions. Most biosurfactants are either anionic or neutral and the hydrophilic moiety can be a carbohydrate, an amino acid, a phosphate group, or some other compounds. The hydrophobic moiety is mostly a long carbon chain fatty acid. Surfactants are synthesized chemically from petrochemical and oleochemical resources. Nowadays, due to global environmental awareness, the utilization of biological surface-active agents produced from living organisms has attracted scholarly attention (Ghasemi *et al., 2019*). Rosenberg et al. (1999) suggested that biosurfactants can be divided into low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high molecular-mass polymers, which are more effective as emulsion stabilizing agents.

Biosurfactants enhance the emulsification of hydrocarbons, have the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation. 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. Biosurfactant production should involve using cheap substrates in order to decrease the overall costs of fermentative processes. In other words, an effective BS-productive process develops an economic system that makes use of low-cost materials while it provides a high-product yield.

In oil and gas industries, biosurfactants are used as agents in the recovery of oil trapped inside reservoir rocks. In addition, they are eco-friendly. EOR (Enhanced oil recovery) is expected to be one of the major technologies in the future to improve oil recovery. The interactions between the surface active molecules and oil-water interface and reservoir rock surface determine the amount of oil which could be produced as a result of an EOR process.

Biosurfactants are widely used in food industries, cosmetic industries and medical industries. Eshrat-Gharaei-Fathabad in 2011 elucidated on the wide range of applications of biosurfactants in medicine which include; antimicrobial activity, immunological adjuvants, gene delivery. There are many advantages of using biosurfactants which are biodegradability, low toxicity, physical factors and surface and interface activity (Fakruddi, 2012).

2. MATERIALS AND METHODS

2.1 Sample collection and Isolation of bacteria

Biosurfactant producing bacteria were isolated from 2 marine water and 6 marine sand samples collected from the Tithal beach situated in Valsad district and Dumas beach situated in Surat. 3 different types of sand sample were collected from the both of the beach. Samples were collected in clean containers. Sand samples were collected from the depth of around 5cm and were utilized after collection.

2.2 Screening of isolates for biosurfactant production

Screening was performed in order to get biosurfactant producing microorganisms.

2.2.1 Primary screening of isolates from obtained samples

For isolation, 1:10 dilution was prepared. The samples were streaked on sterile Nutrient agar plates and sterile sabouraud's agar plates. Plates were incubated at 37°C for 24hours and at room temperature for 2-3 days respectively. The plates were examined for various types of colonies and colony characteristics were noted. For bacteria, gram's stain was performed and for fungi, mounting was performed with lactophenol picric acid. Then, bacteria and fungi were subsequently streaked on sterile nutrient agar and sterile sabouraud's agar plates respectively, until the pure isolates were obtained. Then, purified isolates were maintained on sterile nutrient agar slants and sterile sabouraud's agar slants at 4°C.

2.2.2 Secondary screening of isolates obtained from samples

In secondary screening all the isolates were subjected to check their growth on sterile MSM agar and check their hemolytic activity, BATH assay, visualization of bacterial in oil droplets, drop collapse test and emulsification assay.

2.2.2.1 Cultivation of isolates on MSM agar

All the isolates were streaked on sterile MSM agar plates and were incubated for 24 to 48 hours at 37°C.

2.2.2.2 Hemolytic activity

Isolates were spot inoculated on blood agar plates. These plates were incubated for 24 to 48 hours at 37°C. After incubation plates were observed for zone of hemolysis to confirm production of biosurfactant.

2.2.2.3 BATH assay

Bacterial cells were suspended in distilled water to give an optical density at 630nm of ~ 0.5. Then the bacterial cells were washed twice and suspended in a buffer salt solution (g/l 16.9 K_2HPO_4, 7.3 KH_2PO_4). 100µl engine oil was added into 2ml of cell suspension and was vortex-shaken for 3 minutes in the test tube. After shaking, engine oil and aqueous phase were allowed to separate for 1 hour. Optical density of the aqueous phase was then measured at 630nm in a colorimeter. Hydrophobicity is expressed as the percentage of cell adherence to engine oil calculated as follows: 100x (1- OD of the aqueous phase/OD of the initial cell suspension) (Rengathavasi Thavasi et al. (2011).

2.2.2.4 Visualization of bacteria in oil droplets

1% of Triphenyl tetrazolium chloride (TTC) solution was added to the BATH assay culture broth and observed under the microscope. The TTC turned red if it was reduced inside the cells, which indicates the viability and adherence of cells with engine oil droplets.

2.2.2.5 Drop-collapse test

All the isolates were cultured in mineral salt medium with 0.1% engine oil for 48 hours. 2 ml of engine oil was added in the small petri plates. All the plates were equilibrated for 1 h at 37°C and 5µl of 48 hour old bacterial culture supernatant (after centrifugation at 10000 rpm for 10 minutes to remove cells) was added to the surface of the oil and the shape of the drop was observed after 1 minute. Deionized water was used as negative control.

2.2.2.6 Emulsification assay

Cell free culture broth was used as the biosurfactant source to check the emulsification of engine oil. Culture broth was centrifuged at 10000rpm for 15 minutes. 3ml of supernatant was mixed with 0.5ml of hydrocarbon and vortexed vigorously for 2 minutes. This was left undisturbed for 1 hour to separate the aqueous and hydrocarbon phases. Uninoculated broth was used as blank. The optical density of aqueous phase was measured at 400nm.

2.3 Biosurfactant production and extraction

Isolate was grown in 500 ml Erlenmeyer flask containing 100 ml mineral salt medium adjusted to pH 7.0 and the flask was incubated at 37˚C on shaker for 7 days and then extraction was performed. The whole culture broth was centrifuged at 10000 rpm for 10 minute. The supernatant was dispensed into sterile test tubes using a sterile pipette, and then 0.5 ml of hydrochloric acid 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 centrifuged at 10000 rpm for 10 minute. Then the supernatant was collected using a sterile pipette and dispensed into a sterile petri plates and then placed in oven at 40˚C to obtain the dried crude biosurfactant.

2.4 Characterization of biosurfactant

2.4.1 Thin layer chromatography (TLC)

The components in the extracted biosurfactant were separated on silica gel using a solvent system having 2:1 ethyl acetate to n-hexane ratios. To observe the resultant spots on the TLC plates, the plate was dried at 100˚C in a hot air oven; Ninhydrin (0.02 w/v in acetone) was sprayed on to the plates which were further dried for 3 minutes at 100˚C and spots were observed. The separated components were detected by U.V light (260 nm).

The spots were marked and reference value (Rf) of the spots were calculated using the following formula:

Rf value= distance travelled by solute from origin/ distance travelled by solvent from origin (in cm)

TLC sheet used for the purpose were readymade TLC silica gel sheets manufactured by Merck Pvt. Ltd.

2.4.2 Fourier transform infrared spectroscopy

To understand the overall chemical nature of the extracted biosurfactants, Fourier Transform Infrared Spectroscopy (FTIR) was used. It was done at Centre of Excellence, Vapi. 2 to 3 milligram of dried, partially purified biosurfactant was grind with 100mg of KBr. The mixture was taken in the accessory and pressed in hydraulic pellet and with 15 psi pressure. Then the pellet was transferred to sample compartment with the help of sample holder and the sample was scanned.

2.5 Antibacterial activity assessment of biosurfactants

1% and 10% of the extracted active compound was utilized for the purpose. The activity was performed against test organisms *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi.* 0.l ml of fresh bacterial culture was spreaded on nutrient agar plates and allowed to dry for few minute. Then paper disc was dipped into the biosurfactant suspension prepared in the phosphate buffer and put on the petri plate, respectively. Phosphate buffer (pH-7.4) was used as control in all plates. All the plates were incubated at

37˚C for 24 hours. The antibacterial activities were evaluated by measuring the zone of inhibition with the help of zone reader in millimeter (mm).

3. RESULTS AND DISCUSSION

3.1 Sample collection for biosurfactant production

Total 8 samples were collected and the samples were tested for its physiological properties.

Table: 3.1 The physiochemical properties of obtained samples

Physiological properties	**Water sample**		**Sand sample**					
Place of sample collected	Tithal beach, Valsad	Dumas beach, Surat	Tithal beach, Valsad			Dumas beach, Surat		
No. of sample	T1	D1	T2	T3	T4	D1	D2	D3
Volume	10 ml	10 ml	1g	1g	1g	1g	1g	1g
Ph	8.0	8.3	8.2	8.1	8.26	8.4	8.2	8.24
Color	Brown	Muddy black	Black	Black	Black	Black	Black	Black
Consistency	-	-	Tightly bound	Semi-tightly bound	Loose	Tightly bound	Semi-tightly bound	Loose

The pH of the beaches samples varies from 8.0 to 8.4.

The color of water sample of Tithal beach was brown whereas the color of water sample of Dumas beach was muddy black. The color of sand samples were black of both the beaches.

3.2 Screening of isolates for biosurfactant production

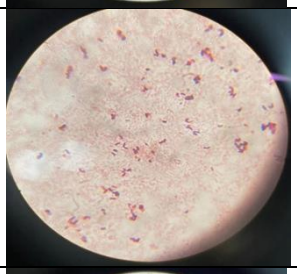
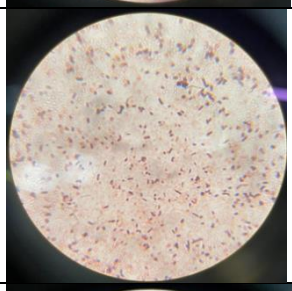
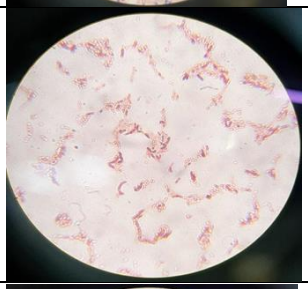
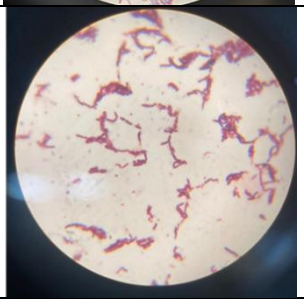
3.2.1 Primary screening of isolates from obtained samples

From 8 samples (Tithal beach and dumas beach) 9 bacterial isolates were obtained on sterile nutrient agar plates. From which 5 were isolated from Tithal beach, Valsad and 4 were isolated from Dumas beach, Surat. 4 fungal isolates were obtained on sterile sabouraud's agar plates from Tithal beach, Valsad.

On performing morphological and colony characteristics (table 3.2), it was found that 6 isolates were Gram positive- rods and 3 isolates were Gram negative-short rods.

Organisms were purified on nutrient agar plates, all the isolated colonies were obtained and maintained on nutrient agar slants at 4°C till its use.

Table 3.2: Results of colony characteristics and gram reaction of obtained isolates on nutrient agar medium

Isolates	**Colony characteristics on nutrient agar**	**Gram reaction & morphological characteristics**	**Figure**
B1	Round, Entire, Flat, Smooth, Moist, Translucent, Non pigmented colonies	**Gram positive, short rods occurring singly and in chain**	
B2	Pinpoint, Entire, Flat, Smooth, Moist, Translucent, Non pigmented colonies	**Gram positive, short rods occurring singly and in chain**	
B3	Round, Entire, Flat, Smooth, Moist, Sabaceous, Non pigmented colonies	**Gram positive, coccobacillus occurring singly and in pair**	
B4	Round, Irregular, Flat, Smooth, Moist, Opaque, Non pigmented colonies	**Gram positive, thick rods occurring singly and in chain**	
B6	Round, Entire, Raised, Smooth, Viscous, Translucent, Non pigmented colonies	**Gram positive, long rods, occurring singly and in chain**	
B7	Round, Repand, Flat, Smooth, Moist, Opaque, Non pigmented colonies	**Gram positive, short rods, occurring in chain and singly**	

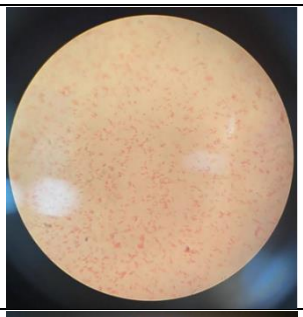
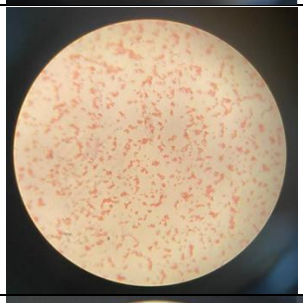
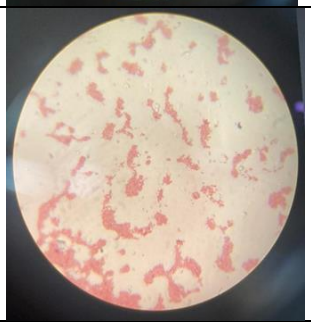
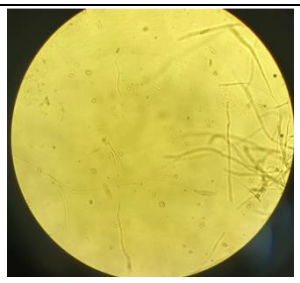
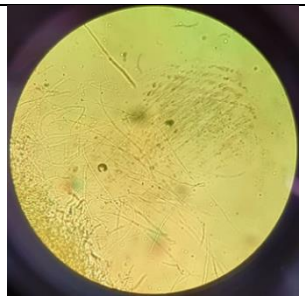
B8	Round, Entire, Flat, Smooth, Moist, Sebaceous, Non pigmented colonies	**Gram negative, short rods, occurring singly**	
B9	Round, Entire, Flat, Smooth, Moist, Sebaceous, Non pigmented colonies	**Gram negative, Coccobacilli, occurring singly**	
B10	Pinpoint, Entire, Flat, Smooth, Moist, Transparent, Non pigmented colonies	**Gram negative, short rods occurring singly and in pair**	

Table 3.3: Results of mounting and colonial characteristics of obtained isolates on sabouraud's agar plates

Isolates	**Observation**	**Figure**
F1	Greenish white filamentous structure	
F3	White filamentous structure	

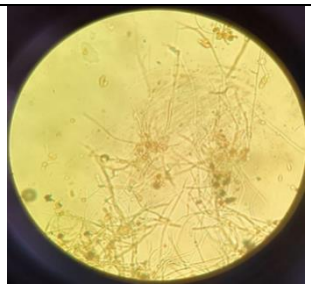
F4	Green colored filamentous structure	
F5	Brownish white, filamentous structure	

Table 3.3 shows the result of 4 fungal isolates which were isolated on sabouraud's agar medium. This fungal isolates were subcultured until purified and maintained on sabouraud's agar slants at 4°C until further studies.

3.2.2 Secondary screening of isolates from obtained samples

Various tests and assays were carried out for screening biosurfactant producing isolates from obtained isolates.

3.2.2.1 Cultivation of isolates on mineral salt agar

On MSM agar plates 7 (B1, B2, B3, B4, B7, B8, B9) isolates were able to grow (figure 3.1) out of 9 isolates. From which 6 (B1, B2, B3, B4, B6, B7) were gram positive rods and 2 (B8, B9) were gram negative coccobacilli and rods.

Fungal isolates were not able to cultivate on MSM medium.

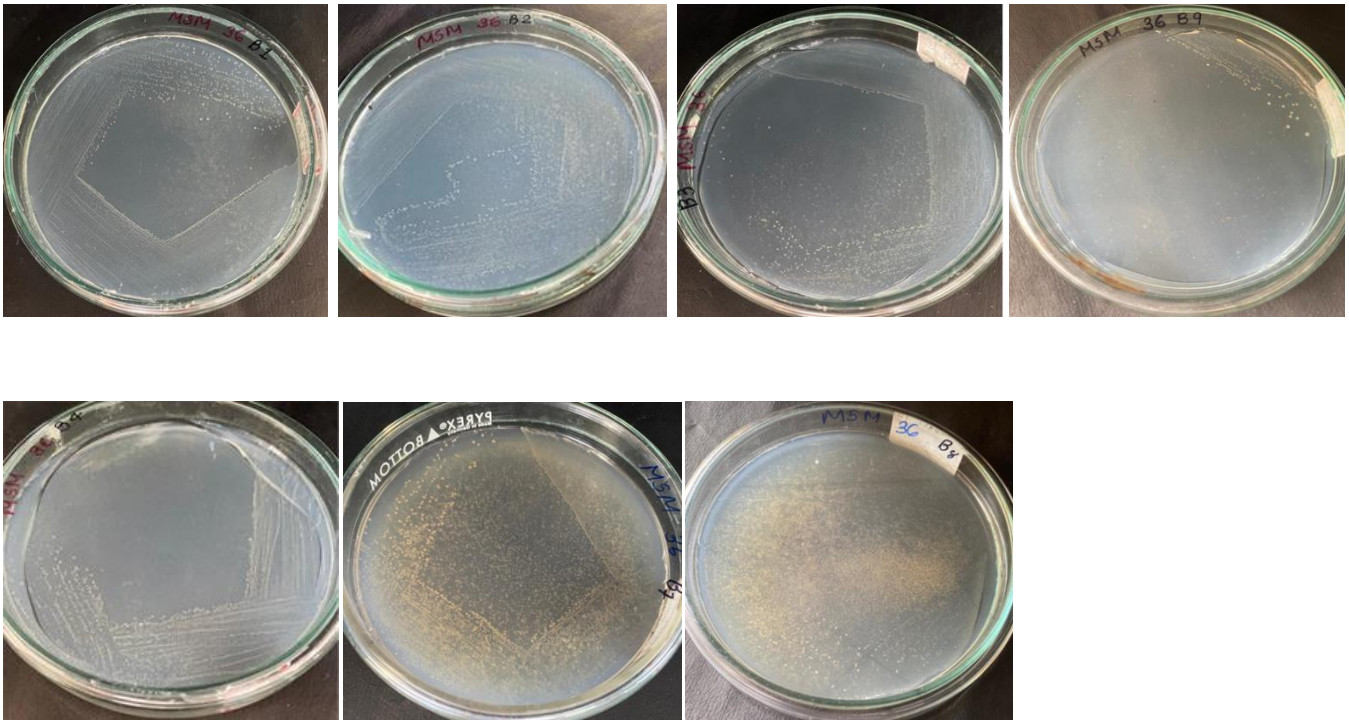


Figure: 3.1 Growth of isolates on MSM agar plate

3.2.2.2 Hemolytic activity

Isolates were spot inoculated on blood agar plates in which, 8 β (hemolytic) and 5 ɣ (non- hemolytic) isolates were obtained on hemolytic assay (figure 5.2). Hemolytic assay was used in this study as a criterion for biosurfactant production because it is a widely used method to screen biosurfactant production and in some reports it is the sole method used to screen biosurfactant production.

Similar work done by Aziz and his co-worker in 2014 and they observed hemolysis on blood agar plate. The hemolytic activity of biosurfactant was first discovered when Bernheimer and Avigad in 1970 reported that the biosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells.

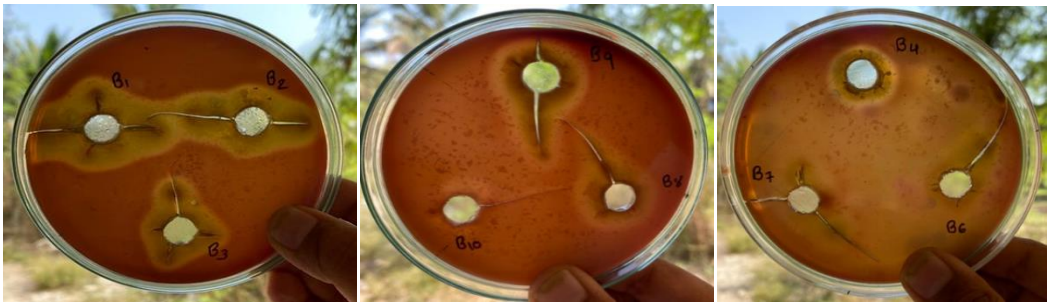


Figure: 3.2 Results of hemolytic activity of obtained isolates

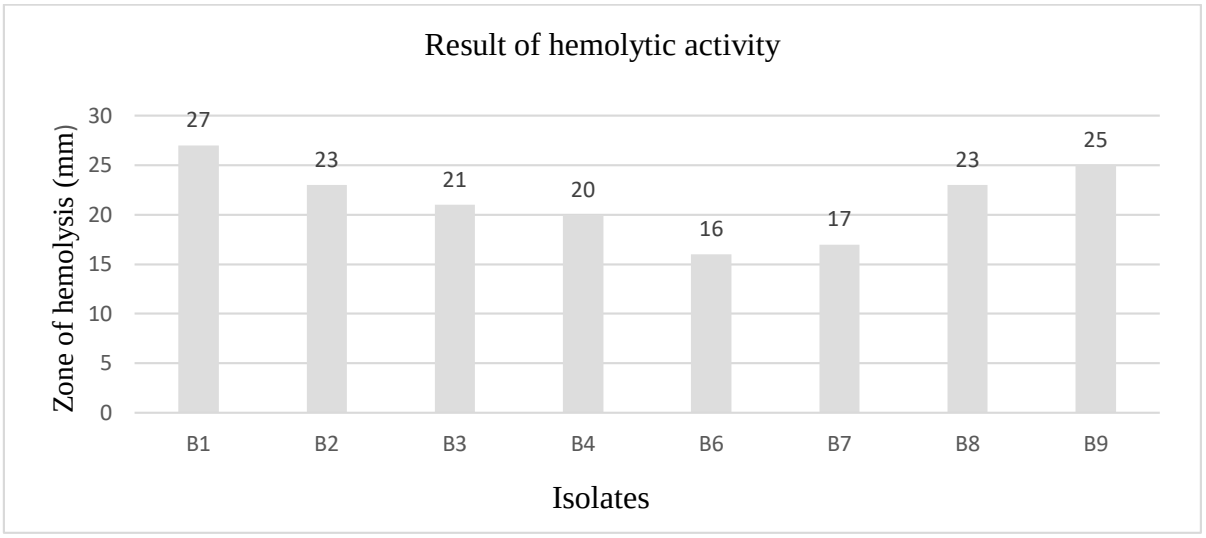


Figure: 3.3 Graphical representation of hemolytic activity of obtained isolates

From the figure 3.3 it can be stated that B1 gave highest zone of hemolysis (27 mm) followed by B9 (25 mm) and lowest zone was found of B6 (16 mm).

3.2.2.3 Bacterial adhesion to hydrocarbons (BATH assay)

Cell hydrophobicity was measured by BATH assay according to a method similar to that described by Rosenberg and his colleagues in 2006. Cell adherence with hydrophobic compounds like engine oil is considered as an indirect method to screen bacteria for biosurfactant production, because cells attach themselves with oil droplets by producing surface active compounds called biosurfactants. BATH assay conducted in this study with all 9 isolates revealed that isolates B10 and B9 gave high affinity (86% and 74%) respectively with engine oil and B4 gave lowest affinity (8.3%) with engine oil, which indicated the affinity of the bacterial cells towards hydrophobic substrate. Similar work done by Youssef and his co-workers in 2004 revealed that *P.aeruginosa* showed high affinity (95.3%) with crude oil followed by *Corynebacterium kutscheri* (49.7%).

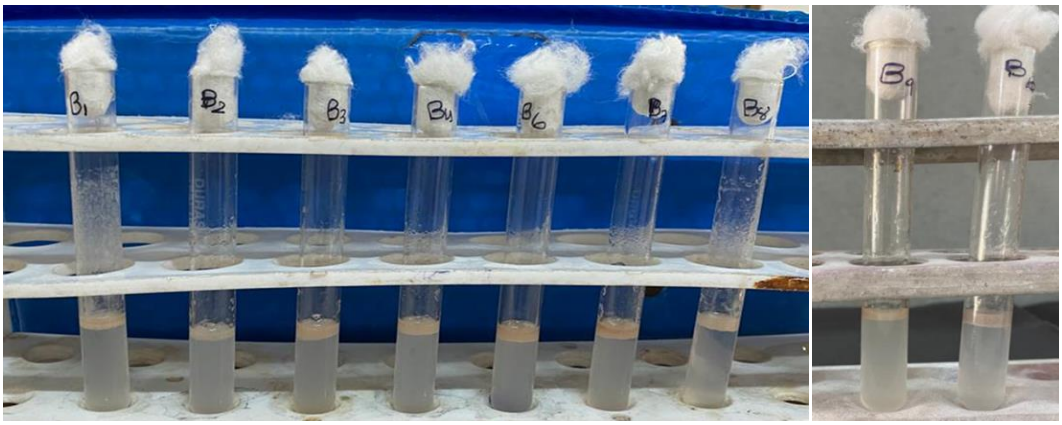


Figure: 3.4 Results of BATH assay of obtained isolates

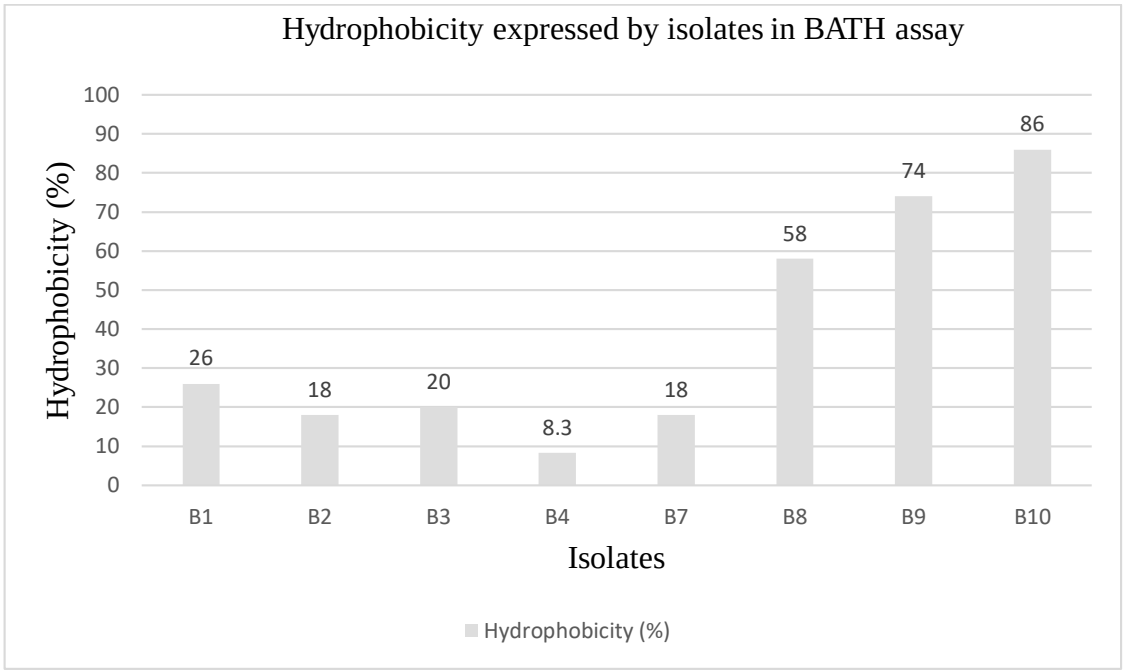


Figure: 3.5 Results of hydrophobicity expressed by isolates in BATH assay

3.2.2.4 Visualization of bacteria in oil droplets

Visualization of bacterial cells adhered with engine oil using TTC (Triphenyl tetrazolium chloride) staining also confirmed the BATH results. In this test 6 isolates (B3, B6, B7, B8, B9, B10) had shown red dot inside the cells, indicating viability and adherence of cells with engine oil droplets. Similar work done by Betts and his colleague in 1989 by using INT stain and also confirmed the BATH assay results, *P.aeruginosa* isolate gave positive result.

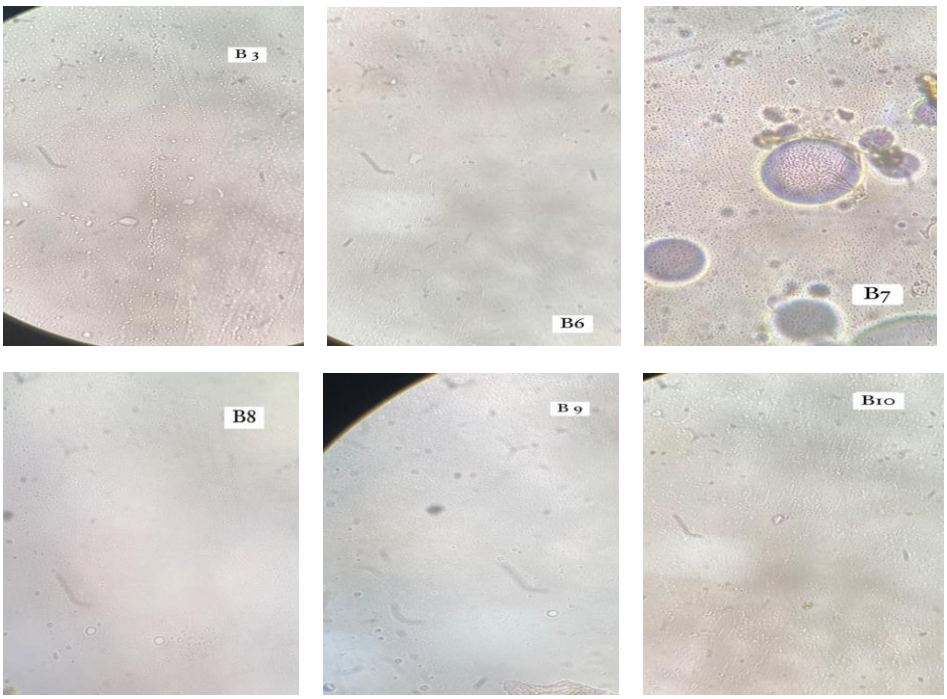


Figure: 3.6 Results of visualization of bacteria in oil droplets

3.2.2.5 Drop-collapse test

Drop collapse test was performed and described by Jain and his colleagues in 1991 and modified by Bodour and Miller-Maier in 1998. Drop collapse method is a sensitive and easy to perform method which requires small volume of culture broth or biosurfactant solution to test the surfactant property. From all the 9 isolates only B9 had shown the test positive by collapsing the drop, which indicates higher biosurfactant production.

B9- Positive test Control Negative test

Figure: 3.7 Results of drop-collapse test of obtained isolates

3.2.6 Emulsification assay

B9 was the promising biosurfactant producer and it was gram negative bacteria. Out of 9 isolates this 1 isolate was selected for biosurfactant production based on results. Similar work done by Patil and Chopade (2001) and in their study all the strains showed positive emulsification activity.

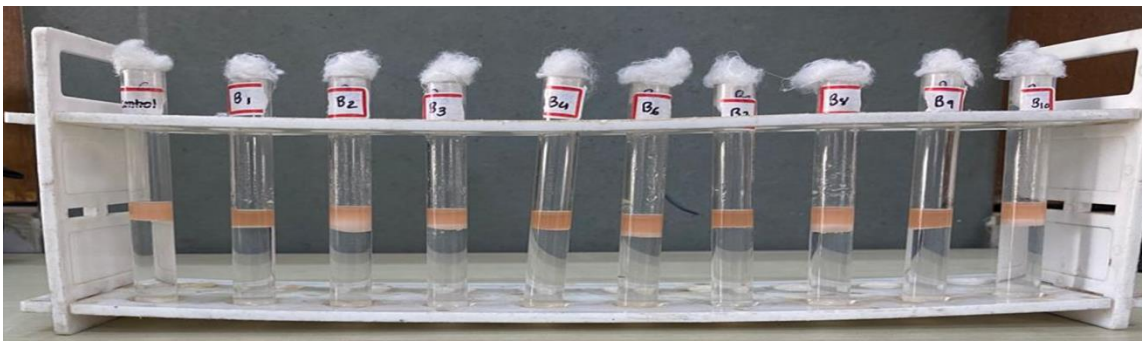


Figure: 3.8 Results of emulsification assay of obtained isolates

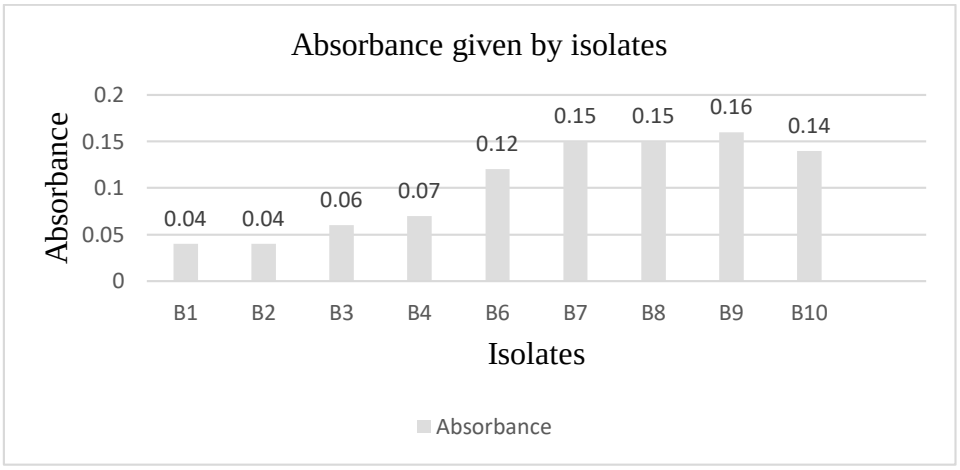


Figure: 3.9 Graphical representation of absorbance given by isolates in emulsification assay

B9 isolate had highest absorbance (0.16) followed by B7 and B8 (0.15 and 0.15) respectively and B1 and B2 gave lowest absorbance (0.04 and 0.04) respectively.

Similar work done by Patil and Chopade in 2001 and in their study all the strains showed positive emulsification activity.

From all tests result, out of all 9 isolates, only B9 isolate was selected for biosufactant production.

3.3 Production and extraction of biosurfactant

Production of biosurfactant was carried out by B9 isolate using MSM medium. The biosurfactant was extracted from the whole cell-free culture by using organic solvent (chloroform-methanol) by centrifugation. 4.38 gram biosurfactant was extracted by performing repetitive cycle of production.

(a) After the production of biosurfactant

(b) After adding organic solvent into cell free culture

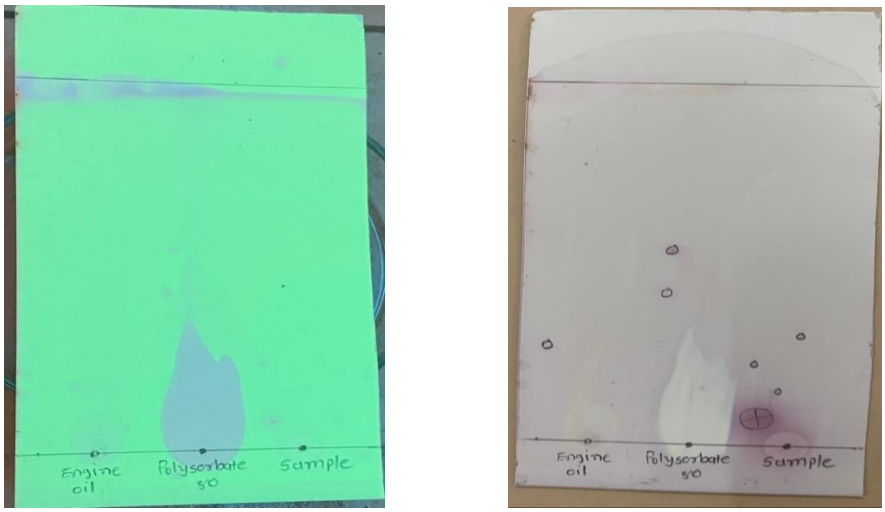
Figure: 3.10 Production of biosurfactant from B9 isolate

Figure: 3.11 Extracted biosurfactant from B9 isolate

3.4 Characterization of biosurfactant produced from B9 isolate

3.4.1 Thin layer chromatography :

The biosurfactant was partially purified from crude extract with ethyl acetate:n-hexane ratio 2:8 by thin layer chromatography for development of spots on TLC plate. The TLC plate can be viewed by both ways, presence of UV rays and with spraying of ninhydrin reagent. First the TLC plate was placed in UV lamp to view the chromatogram, but results were not promising. After that ninhydrin regent was sprayed and allowed to dry. The results obtained, showed various spots.



(a) Observation of TLC plate under UV rays

(b) Observation of TLC plates after spraying ninhydrin reagent

Figure: 3.12 Results of TLC using extracted biosurfactant

Table: 3.4 Results of chromatogram (R_f value) of biosurfactant by thin layer chromatography technique

Sr no.	R_f value
Spot 1 (engine oil)	0.23
Spot 2 (engine oil)	0.15
Spot 3 (polysorbate 80)	0.36
Spot 4 (polysorbate 80)	0.47
Spot 5 (polysorbate 80)	0.14
Spot 6 (sample)	0.20
Spot 7 (sample)	0.07
Spot 8 (sample)	0.14
Spot 9 (sample)	0.27

R_f value of all the spots were studied which is stated in the table 5.4. Sot 6 (sample) matched with spot 1 (engine oil) and spot 8 (sample) matched with spot 2 (engine oil) and spot 5 (polysorbate 80). From the results of R_f value, we can state that the extracted crude may be boisufactant.

3.4.2 Fourier transform infrared spectroscopy (FTIR)

Figure: 3.13 Result of FTIR of boisurfactant produced by isolate B9

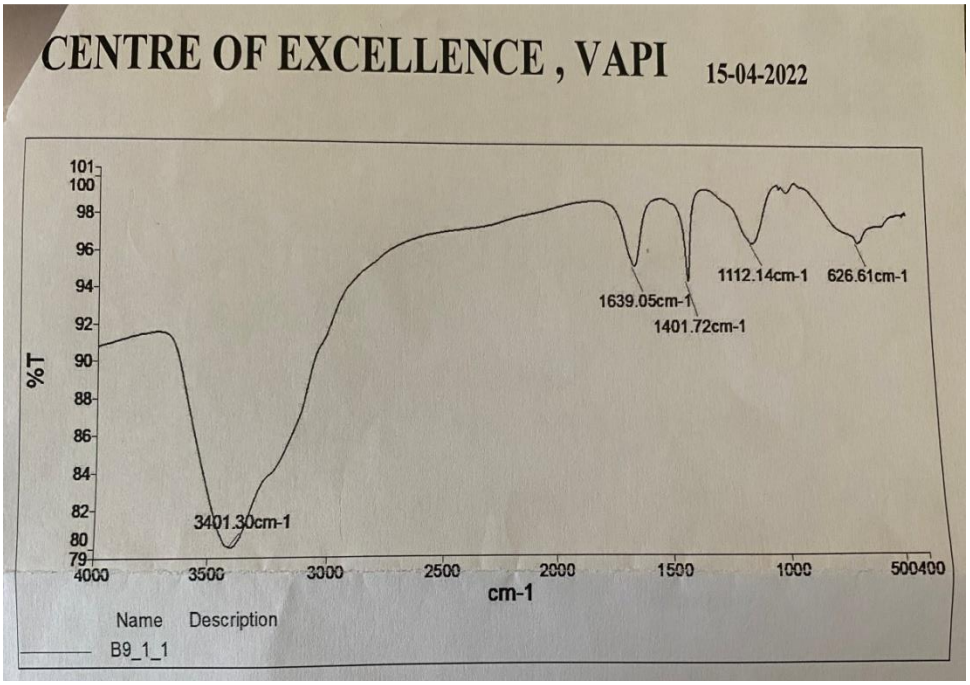


Table: 3.5 Respective stretch and bends given by surfactants in FTIR spectra

Peak No.	IR frequency (cm^{-1})	Bond	Functional group
1	3401.30	N-H	Primary or secondary amine
2	1639.05	C=O	Primary or secondary amide
3	1401.72	O-H or C-H	Alcohol/carboxylic acid/phenol or alkane
4	1112.14	C-O	Vinyl ether/primary, secondary or tertiary alcohol
5	626.61	C-H	Disubstituted or trisubstituted

FTIR spectroscopy is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of unknown mixture. It is rapid and intensive method. FTIR is a physiochemical method based on measuring the vibrations of a molecule excited by IR radiation at specific wavelength range. Figure 5.13 shows the FTIR analysis of biosurfactant isolated from B9 which giving the confirmation of presence of biosurfactant.

3.5 Antibacterial activity of biosurfactant against microorganism

Biosurfactant (1% and 10% concentration) was tested against test organisms for studying antibacterial activity. The antibacterial activity was performed against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*. The highest zone of both the concentration (1% and 10%) of biosurfactant showed 10 mm and 13 mm respectively against *Staphylococcus aureus* and 7 mm and

9 mm respectively against *Eschrichia coli.* Contradictory result was found for *Salmonella typhi* which gave no zone of inhibition in both different concentration of biosurfactant.

Staphylococcus aureus *Escherichia coli* *Salmonella typhi*

Figure: 3.14 Results of antibacterial activity of biosurfactant against microorganisams

Table: 3.6 Result of antibacterial activity

Test organism	Zone of inhibition	
	1% concentrated biosurfactant	10% concentrated biosurfactant
Staphylococcus aureus	10 mm	13 mm
Escherichia coli	7 mm	9 mm
Salmonella typhi	-	-

4. CONCLUSION

From all the 8 different marine samples (water and sand), test performed in secondary screening, B9 isolate was able to grow on MSM agar medium, gave zone of hemolysis, shows 74% affinity in BATH assay, had shown positive result in visualization of bacteria in oil droplets, had shown positive result in drop collapse test and gave highest absorbance in emulsification assay. From all tests result, out of all 9 isolates, only B9 isolate was selected for biosufactant production.

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