Screening, Production, Optimization and Characterization of Biosurfactant Producing Bacteria from Petroleum Contaminated Soil

Hina A. Rathod[1], Hetal K. Panchal[2]

[1,2]Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad, 396001, India

Abstract

Biosurfactant are amphiphilic compounds produced by various bacteria and fungi which reduce surface tension and interfacial tension. In this investigation Petroleum contaminated soil samples were collected from 5 different garages of Valsad. Mineral salt medium supplemented with 1% crude oil as the hydrocarbon source for isolation. 14 different isolate were obtained based on their colony characteristics and gram reaction. 10 isolates were selected for their ability to produce biosurfactant based on screening assay, including hemolytic activity, drop collapse test, oil displacement test, emulsification activity and cell surface hydrophobicity studied. In which 6 isolates selected for biosurrfactant production. From 6 isolates B5 isolate were produced in highest amount of biosurfactant (60g/100ml). By using B5 isolate optimization study was carried out. Various parameters such as pH, temperature, carbon sources, nitrogen sources, hydrocarbon sources, and trace elements were studied and optimized. The extraction of biosurfactant and emulsification activity was studied. The mannitol was best carbon source and ammonium chloride best nitrogen source. The environmental factor such as pH 7 and temperature 37°C were found to be optimum for the production.

Among the hydrocarbon sources motor engine oil were good source. The biosurfactant yield was higher in without using $MnSO_4$ as trace elements. Characterization of biosurfactant done by FTIR spectra and it is indicated the presence of functional group and all 6 biosurfactants are lipopeptide in nature.

Key words: Biosurfactant, crude oil, emulsification activity, extraction, optimization, characterization.

INTRODUCTION

One of the important properties of hydrocarbon degrading bacteria is the production of surface active agents (biosurfactant) can be applied to soils contaminated with petroleum hydrocarbons to enhance bioremediation [1]. Bioremediation is a process used for the degradation of hydrophobic organic compounds (HOCs) in oil-contaminated water and soil due to its environmental and economic advantages over other physicochemical remediation method [2]. So far, the main approach for enhancing the bioavailability of HOCs is the use of synthetic or natural surfactants [3], which promote the solubility of HOCs and improve the biodegradation process [4]. During the process of crude petroleum biodegradation, petroleum-degrading strains utilize petroleum hydrocarbons as sole source of carbon and energy [5, 6]. Biosurfactant are classified based on their chemical configuration as glycolipids, Lipopolysaccharides, lipopeptides, and are produced by diverse bacterial genera [7].

Biosurfactant are amphiphilic molecules mainly produced by microorganisms as a secondary metabolite, they posses both hydrophilic and hydrophobic moieties and are able to display a variety surface activities and help to solubilize hydrophobic substrates [8]. Biosurfactant producing microorganisms were naturally present in the oil contaminated soil. Oil contaminated environment contain large amount of hydrocarbons. Hydrocarbon are composed of complex chemical structure i.e., aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activities by producing biosurfactant and utilize the hydrocarbon as substrate. Biosurfactant have been widely used to facilitate biodegradation of petroleum pollutant [9]. Biosurfactant have advantages over their chemical counterparts in biodegradability, low toxicity, better environmental compatability, high selectivity at extreme temperature, pH, salinity and the ability to be synthesized from renewable feedstock [10]. Because of this properties, biosurfactant have a variety of potential application, including in pharmaceutical, cosmetics, detergent, and food industry. Rhamnolipid (class of glycolipids) is the most frequently used in bioremediation and oil recovery, but the major problem for commercial application of the biosurfactant has its low yield and high production cost. Thus there is a need to develop a new efficient biosurfactant producers species/or strain that can produce large amount, and effective biosurfactant are desired to address the challenges [11].

In recent decades, several novel biosurfactant producers have been discovered and was used as no-cost complete nutrient medium viz. distillery wastewater, curd whey waste, fruit processing waste for biosurfactant production [12]. Some microorganisms, mainly from genera *pseudomonas* and *mycobacterium*, were capable of oxidizing petroleum products and trace of organic acids [13].

The main natural hydrocarbon removal mechanisms are photo-oxidation, evaporation and microbial degradation, which may take years to stabilize contaminated location. It is therefore imperative that low cost, high efficiency remediation processes be developed [14].

MATERIALS AND METHODS

A. Materials

Mineral salt broth medium supplemented with 1% crude oil, mineral salt agar plates, blood agar plates, 0.002 M sodium phosphate buffer, 4M ammonium sulphate, potassium phosphate buffer were purchased from Finar Limited (Mumbai), 96-well Microtiter plate were purchased from Himedia (Mumbai), methanol and chloroform were purchased from Thermo fisher scientific India. Pvt. Ltd.

B. Sample collection

5 different soil samples were collected from depth of 6 inches from different garages of valsad. Soil sample were then packed in plastic bags until further processing.

C. Isolation of biosurfactant producing bacteria

One gram of soil sample was mixed to 9 ml of sterile distilled water. After that 1 ml mixture was transferred to sterile mineral salt broth supplemented with 1% crude oil for enrichment and incubated at room temperature for 24-48 hours. After enrichment serial dilution was done up to 10^{-6} and spread plated into mineral salt agar plate containing NH_4Cl 2.0, KH_2PO_4 5.0, Na_2HPO_4 4.0, $MnSO_4$ 0.2, $MgSO_4$ 0.2, $FeCl_3$ 0.05, $CaCl_2$ 0.001, yeast extract 0.01 with pH 7.2. The plates were incubated at 37°C for 24-48 hours. The isolated colonies were further purified by sub culturing. The purified colonies were studied for gram reaction.

D. Screening for biosurfactant production

I. Hemolytic Activity

Hemolytic activity was used to determine screening of isolated biosurfactant-producing strain. 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 heamolysis to confirm production of biosurfactant [16].

II. Drop Collapse test

All bacterial strain was cultured in mineral salt medium with 1% crude oil incubated for 5 days. Screening of biosurfactant production was performed using the qualitative drop collapse test described by Bodour and Miller-Maier (1998). 2µl of oil was applied to the well regions deliminated on the covers of 96- well microtiter plate and these were left to equilibrate for 24h. 5µl of the 48 h old culture supernatant was transferred to the oil-coated well regions. The results were monitored visually after 1 h. Deionized water used as negative control [17].

III. Oil displacement test

10 µl of crude oil is 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 are gently placed on the oil layer. Oil is displaced and a clearing zone is formed [18].

IV. Emulsification test

Cell free culture broth was used as the biosurfactant source to check the emulsification of crude oil. Culture broth was centrifuged at 10,000 rpm for 15 minutes. 3 ml of supernatant were mixed with 3 ml kerosene and vortexed at high speed for 2 minutes. This was left undisturbed for 1 h to separate the aqueous and hydrocarbon phases [19]. The emulsification index was calculated by the following equation:

$$E24 = \frac{\text{Height of emulsion layer} \times 100}{\text{Total height}}$$

V. BATH assay

Bacterial cells were washed twice with the equal volume of buffer salt solution (K_2HPO_4, KH_2PO_4) and then were resuspend in the same buffer salt solution and OD taken at 620nm. Crude oil (100 µl) was added and vortex it for 3 minutes in test tubes. After vortex-shaking the crude oil and aqueous phase were allowed to separate for 2 hours. OD of aqueous was then again measured at 620nm in spectrophotometer [20]. Percentage of cell adherence calculated by as following equation:

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

VI. SAT

Sodium phosphate (0.002M pH 6.8) was used to dilute a solution of 4 M $(NH_4)_2SO_4$ in 0.002 M sodium phosphate pH 6.8. Serial dilutions were made giving $(NH_4)_2SO_4$ concentration ranging from 4.0 to 0.2 M differing by 0.2 M per dilution. A bacterial suspension of 25µl in 0.002 M sodium phosphate buffer pH

6.8 was mixed with an equal volume of salt solution into 24-well tissue culture tray. The bacterial/salt mixture was gently rocked for 2 min at 25 ℃, and visual reading was performed against a black background. The results were expressed as the lowest molarity of ammonium sulphate causing bacteria. [20]

E. Production and extraction of Biosurfactant:

Production of biosurfactant was carried out using Erlenmeyer flask of 250ml in which 100 ml of Mineral salt medium with 1% crude oil as a substrate was prepared (pH 7.2) and the isolate capable of producing biosurfactant were inoculated in the MSM broth and kept on rotatory shaker for 7 days at 37°C. the production medium was centrifuged at 7000 rpm for 10 minutes and the supernatant was collected in the sterile flasks. 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 [21].

F. Optimization of biosurfactant production [22]

VII. Effect of pH on Biosurfactant production

100ml of mineral salt medium was prepared of varying pH 5.0, 6.0, 7.0, 8.0. It was added with 1% crude oil. MSM broth inoculated with bacterial culture and incubated for 5 days at 37°C. After incubation period extraction and emulsification assay was done.

VIII. Effect of Temperature on Biosurfactant production

100ml of mineral salt medium was prepared with 1% crude oil inoculated with bacterial culture. The flask was incubated at two different temperatures such as 37°C and 25°C for 5 days. After incubation extraction was done and emulsification assay was performed.

IX. Effect of Carbon sources on Biosurfactant production

100ml of mineral salt medium was prepared with 3 different carbon sources like glucose, mannitol, and starch. It was added with 1% crude oil and inoculated with bacterial culture. Incubate flask at 37°C for 5 days. After incubation extraction was done and emulsification assay was performed.

X. Effect of Nitrogen sources on Biosurfactant production

100ml of mineral salt medium was prepared with 3 different nitrogen sources like peptone, ammonium chloride, and yeast extract. It was added with 1% crude oil and inoculated with bacterial culture. Incubate flask at 37°C for 5 days. After incubation extraction was done and emulsification assay was performed.

XI Effect of Hydrocarbons on the Biosurfactant production

100ml of mineral salt medium was prepared with 1ml different heavy hydrocarbon like xylene, kerosene, motor engine oil. Inoculate it with bacterial culture. Incubate flask at 37°C for 5 days. After incubation extraction was done and emulsification assay was performed.

XII. Effect of trace elements on the Biosurfactant production

100 ml of mineral salt medium was prepared as follows: A) Without $MgSO_4$, B) Without $MnSO_4$, C) Without $FeSO_4$. 1% crude oil was added and bacterial culture was inoculated. Incubate flasks at 37°C for 5 days. After incubation extraction and emulsification assay was done.

G. Structural characterization [23]

To understand the overall chemical nature of the extracted biosurfactants, Fourier Transform Infrared Spectroscopy (FTIR) was used. It was done at Center of Excellence, Vapi. One milligram of dried, partially purified biosurfactant was ground with 100mg of KBr to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Niocolate, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. KBr pellet was used as background reference.

RESULTS AND DISCUSSION

A. Results of isolation

In this research petroleum contaminated soil samples was collected from 5 different garages for isolation and identification of biosurfactant producing bacteria. A total 14 isolates were isolated and were identify by colony characteristics and Gram's reaction. The isolated colonies were further purified by sub culturing. In which 8 were Gram negative and 6 were Gram positive. These isolates were further screened for the biosurfactant production and were used for the following study.

B. Results of screening for biosurfactant producing bacteria

Table 1: Screening test for biosurfactant producing bacteria

Bacterial isolates	Hemolytic Activity (I)	Drop collapse test (II)	Oil displacement test (III)
B1	Hemolytic	+	+
B2	Hemolytic	-	-
B3	Hemolytic	+	+
B4	Hemolytic	+	+
B5	Hemolytic	+	+
B6	Hemolytic	-	-
B7	Hemolytic	+	+
B8	Non-hemolytic	+	+
B9	Non-hemolytic	-	-
B10	Hemolytic	+	+
B11	Non-hemolytic	+	+
B12	Non-hemolytic	-	-
B13	Non-hemolytic	+	+
B14	Hemolytic	+	+

As shown in Table 1 screening test for biosurfactant producing bacteria. In which 9 isolates gave hemolytic activity positive and, 10 isolates gave drop collapse and oil displacement test positive. Therefore from this screening test 10 isolates was selected for further study.

IV. Emulsification activity

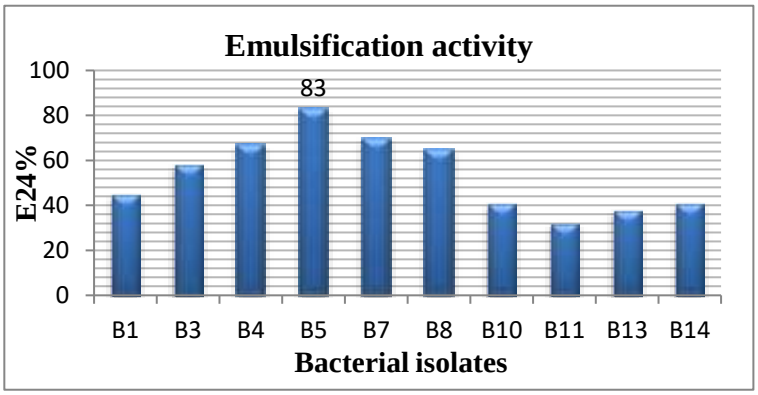


Figure 1: Results of emulsification activity given by different bacterial isolates

Emulsification test used to check emulsifying property of bacterial isolates. Figure 1 showed emulsification index. Stability of emulsion formed with kerosene and cell-free culture medium, in which Stability of emulsion layer was observed after 1 h. Highest stability observed with isolate B5 (E24%=83%).

V. BATH assay (bacterial adherence to hydrocarbon)

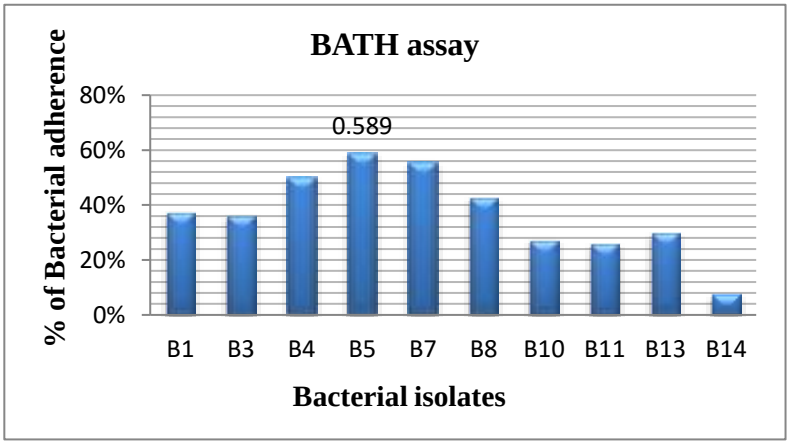


Figure 2: Results of BATH assay

As shown in the above graph (Figure 2) the B5 isolate gave highest % of bacterial adherence to hydrocarbon that is 58.9%. It has been reported that isolate B5 had high affinity towards hydrophobic substrate.

VI. SAT (salt aggregation test)

Table 2: Results of SAT (salt aggregation test)

Bacterial Isolates	Ammonium sulfate concentration in M
B1	1.0
B3	1.3
B4	2.0
B5	1.0
B7	1.2
B8	2.0
B10	0.25
B11	1.0
B13	2.0
B14	0.5

Salt aggregation test was performed to check that, the lowest molarity of ammonium sulphate which caused bacterial aggregation. Table 2 showed molarity of ammonium sulphate that caused bacterial cell aggregation.

From the emulsification index and BATH assay it was observed that 6 bacterial isolate were gave highest activity for biosurfactant production. Therefore 6 bacterial isolate was selected for biosurfactant production.

C. Results of extracted biosurfactant :

Table 3: Results of dry extracted crude biosurfactant

Bacterial Isolates	Characteristics of isolates	Extracted dry biosurfactant (g/100ml)
B1	Hemolytic	0.47g
B3	Hemolytic	0.51g
B4	Hemolytic	0.40g
B5	**Hemolytic**	**0.60g**
B7	Hemolytic	0.53g
B8	Non-hemolytic	0.56g

Figure 3: Dry extracted biosurfactant

Table 3 shows the dry weight of biosurfactant. Highest biosurfactant was produced by B5 isolate (0.60g/100ml). Out of 6 isolates, B5 isolate were selected for further optimization process based on production results.

D. Results of optimization for biosurfactant production of B5 isolate

I) Effect of pH on biosurfactant production

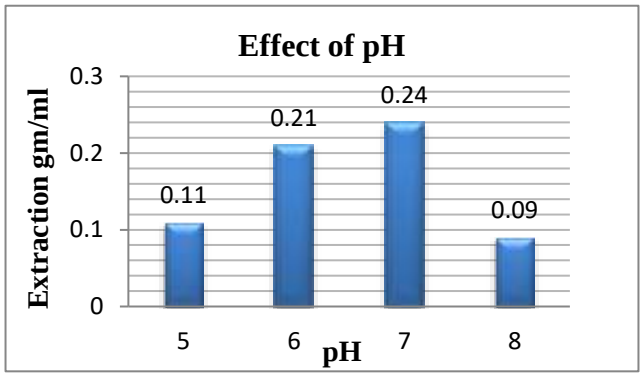


Figure 4: Effect of pH on biosurfactant production

Table 4: Results of emulsification assay

Isolate	pH range	Emulsification index (E24%)
B5	5	–
	6	51%
	7	54%
	8	38%

II) Effect of temperature on biosurfactant production

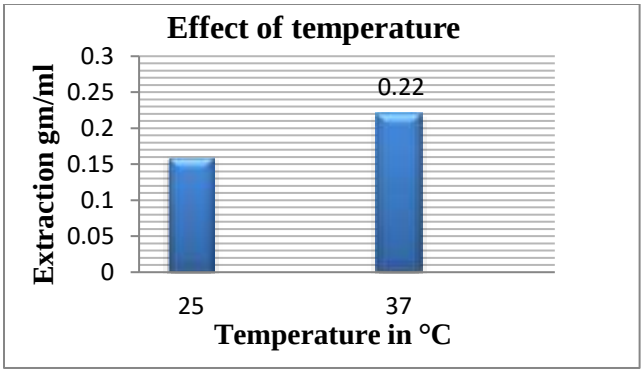


Figure 5: Effect of temperature on biosurfactant production

Table 5: Results emulsification assay

Isolate	Temperature	Emulsification Index (E24%)
B5	37°C	41%
	25°C	38%

Optimization of pH and temperature: The pH ranges from 5 to 7 and temperature 37°C & 25°C for the optimization. The maximum biosurfactant production obtained at pH range 7, and temperature range 37°C.

III) Effect of carbon source on biosurfactant production

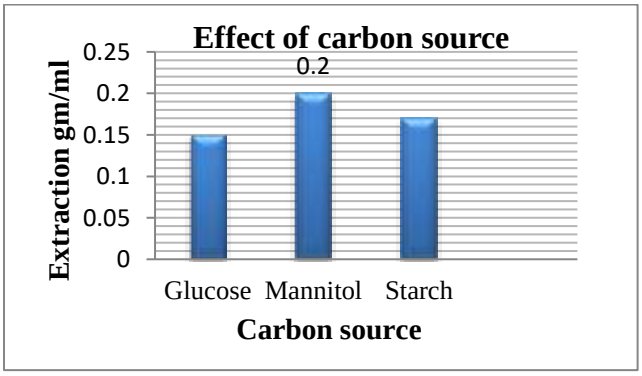


Figure 6: Effect of carbon source on biosurfactant production

Table 6: Results of emulsification assay

Isolate	Carbon source	Emulsification Index (E24%)
B5	Glucose	32%
	Mannitol	51%
	Starch	41%

IV) Effect of nitrogen source on biosurfactant production

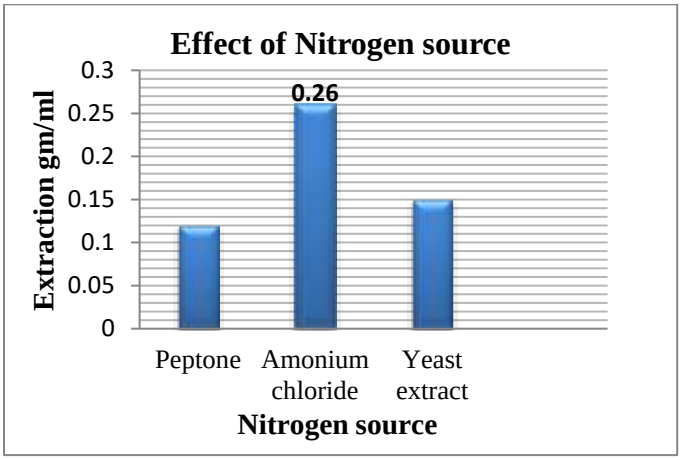


Figure 7: Effect of nitrogen source on biosurfactant production

Table 7: Results of emulsification assay

Isolates	Nitrogen source	Emulsification index (E24%)
B5	Yeast extract	_
	Peptone	_
	Ammonium chloride	35%

Optimization of carbon source and nitrogen sources: The carbon sources like glucose, mannitol, starch and nitrogen sources such as peptone, ammonium chloride, and yeast extract. It is observed that mannitol is optimum carbon source and, ammonium chloride is optimum nitrogen source for biosurfactant production.

V) Effect of hydrocarbon on biosurfactant production

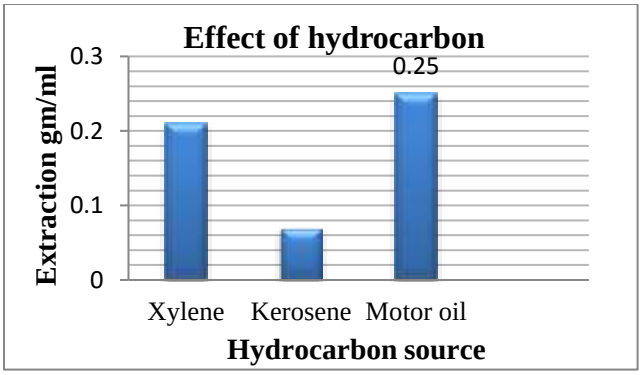


Figure 8: Effect of hydrocarbon on biosurfactant production

Table 8: Results of emulsification assay

Isolates	Hydrocarbon sources	Emulsification index (E24%)
B5	Kerosene	43%
	Xylene	45%
	Motor oil	48%

VI) Effect of trace elements on biosurfactant production

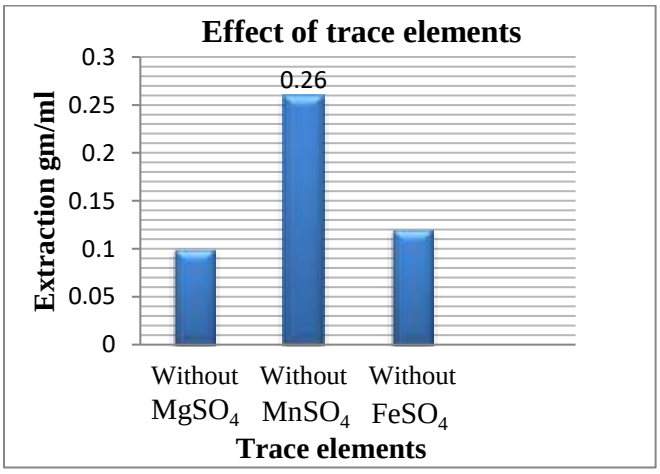


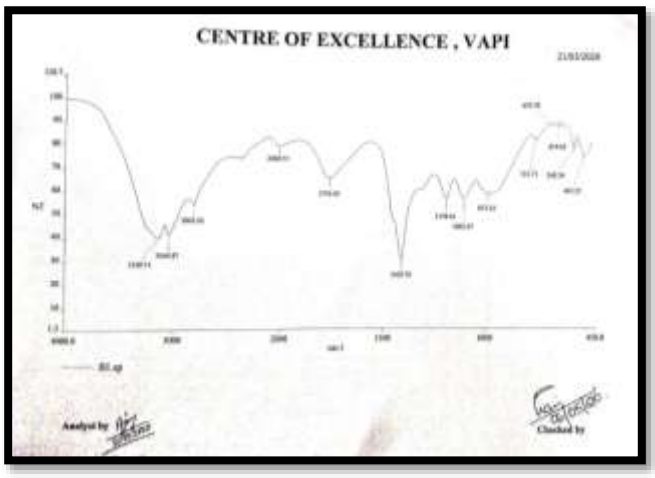
Figure 9: Results of effect of trace elements on biosurfactant production

Table 9: Results of emulsification assay

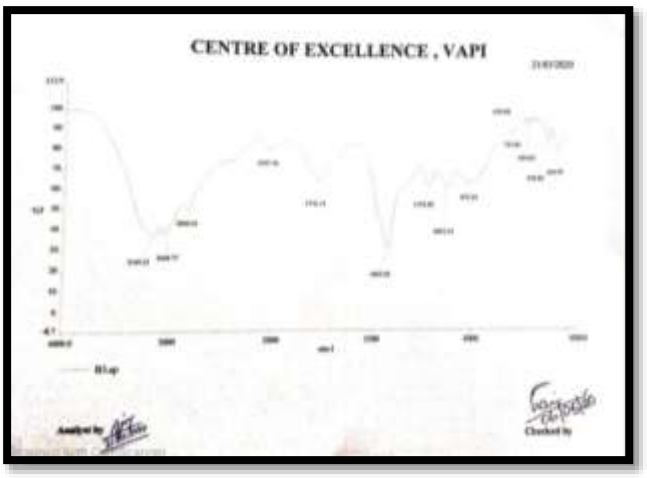
Isolate	Trace elements	Emulsification index (E24%)
B5	Without $FeSO_4$	32%
	Without $MnSO_4$	48%
	Without $MgSO_4$	38%

Optimization of hydrocarbon sources and trace elements: The hydrocarbon sources like xylene, kerosene, motor oil and, trace elements such as without $FeSO_4$, without $MnSO_4$, without $MgSO_4$. It is said that motor oil is best hydrocarbon source and without $MnSO_4$ biosurfactant produced in good amount.

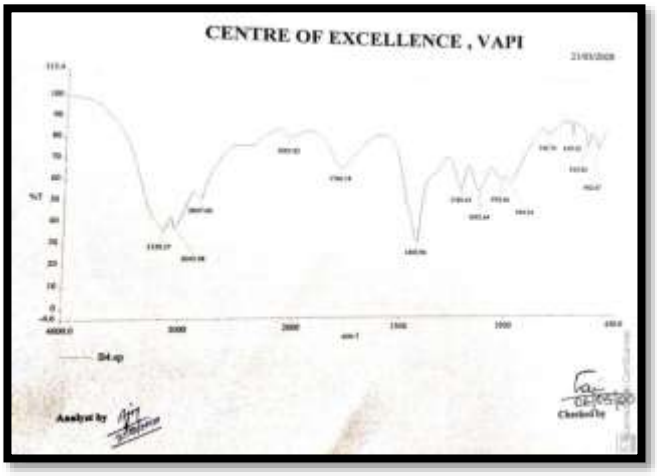
E. Fourier transform infrared spectroscopy: 6 bacterial isolates Biosurfactants were characterized by FTIR. A common stretching and bending was observed in all 6 biosurfactant. As a result C-H stretching vibration and N-H stretching vibration was observed, with wave number ranging from 3600 cm^{-1} to 3100 cm^{-1}(Figure 10). This is typical of carbon-containing compound with amino groups. Sharp absorbance peaks are observed at 2854.74 cm^{-1} and 1405.45 cm^{-1} indicative of aliphatic chains (-CH_3 and –CH_2-). These peaks reflect the presence of alkyl chains in the compound. A strong band was also observed at1754 cm^{-1} to 1764.03 cm^{-1}. This is due to a carbonyl group. The presence of C=O stretching causing C=O stretching vibration. Aliphatic fluro compound observed at 1100.18 cm^{-1} to 1094.36 it indicated the C-F stretch. Aliphatic iodo compound observed at639.76 cm^{-1} to 497.65 cm^{-1}. The FTIR spectrum implied the production of a lipopeptide biosurfactant.



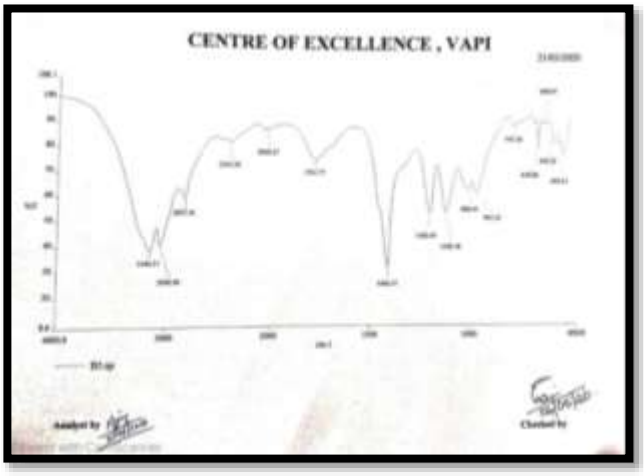
Isolate B1



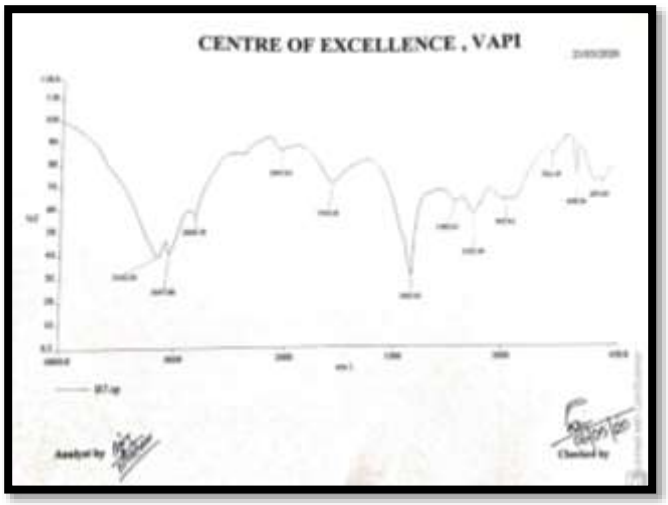
Isolate B3



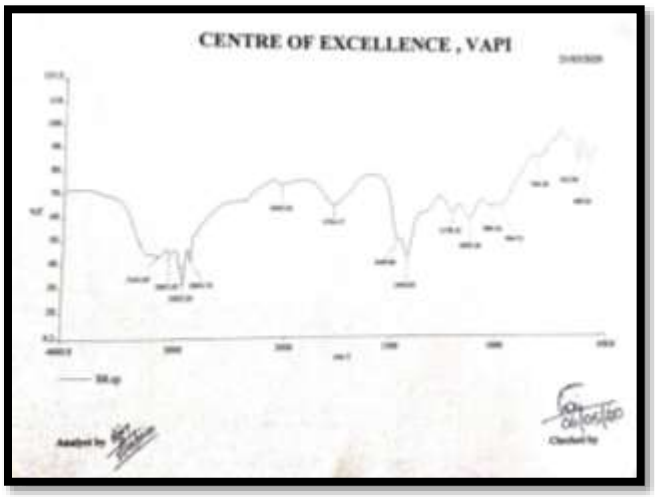
Isolate B4



Isolate B5



IsolateB7



Isolate B8

Figure1.11: FTIR results of the biosurfactant produced by 6 isolates (B1, B3, B4, B5, B7, B8,)

CONCLUSION

In the present investigation, the study represented surfactant activity of the bacterial strain isolated from petroleum contaminated soils from different garages. The majority of bacterial isolates were Gram-negative. However out of 14 isolates 10 isolates were identified as capable to produced biosurfactant, from the various screening test. The screening of biosurfactant producing bacterial isolates was investigated by hemolytic activity, drop collapse test, oil displacement test, emulsification activity, BATH assay, and salt aggregation test. On the basis of screening test results, 6 isolates was gave highest activity and it is selected for biosurfactant production. From 6 isolates B5 isolate produced highest amount of biosurfactant (60g/100ml) and it is selected for optimization process. In optimization process Temperature 37°C and pH 7 was optimum for biosurfactant production. Mannitol was better carbon source than glucose and starch. Ammonium chloride was good nitrogen

source than yeast extract and peptone. In case of hydrocarbon source, motor engine oil was best hydrocarbon source for production of biosurfactant than xylene and kerosene. Optimization of trace elements without $MnSO_4$ biosurfactant produced in good amount than the $MgSO_4$ and $FeSO_4$. The extracted crude biosurfactant was characterized by FTIR spectroscopy to confirm its chemical nature, and it concluded that all 6 biosurfactants are lipopeptide in nature. From the above observation, it was concluded that bacterial isolates have the ability to secrete surface active agents it is gain more important in future for industrial and environmental applications.

ACKNOWLEDGEMNT

The authors are very grateful to Dolat-Usha Institute of Applied sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad, India for allowing us to use all facilities for our work, and their encouragement and support

REFERENCES

1. Banat, I.M., (1995). Biosurfactant production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. *Journal of Bioresource Biotechnology.* 51: 403-410.
2. Silva, E.J., Silva, N.M.P.R., Rufino, R.D., Luna, J.M., Silva, R.O., Sarubbo, L.A., (2014). Characterization of a biosurfactant produced by *Psedumonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Colloidal Surface B.* 117: 36-41.
3. Walter, G.W., Aitken, M.D., (2001). Surfactant-enhanced solubilization and anaerobic biodegradation of 1, 1, 1-trichloro-2, 2-bis (p-chlorophynyl)-ethane (DDT) in contaminated soil. *Journal of Water Environment Resources.*73: 15-23.
4. Zheng, C., Wang, M., Wang Y., Huang Z., (2012). Optimization of biosurfactant-mediated oil extraction from oil sludge. *Journal of Bioresource Technology*. 110: 338-342.
5. Banat, I.M., Samrah, M., Murad, R., Horn, and Benrjee, S., (1991). Biosurfactant production and use in oil tank clean-up. *Journal of Microbiology Biotechnology*. 7: 80-84.
6. Bodour, A.A., Dress, K.P., and Maier, R.M., (2003). Distribution of biosurfactant-producing microorganisms in undistributed and contaminated arid southwestern soils. *Journal of Applied Environmental Microbiology*. 69: 3280-3287.
7. Frenzetti, A., Gandolfi, I., Bestetti, G., Symth T.J., Banat, I.M., (2010). Production and application of trehalose lipid biosurfactants. *European Journal of Lipid Science Technology* 112, 617-627.
8. Manif, I., Elleuch, M., Chaabouni, S.E., and Ghribi, D., (2013). Bacillus subtilis SPB1 biosurfactant: production optimization and insecticidal activity against the carob moth *Ectomyelois ceratoniae*. *Journal of crop protection* 50: 66-72.
9. Maria, P.N., Silva, R., Rufino, R.D., Luna, J.M., Santos, V.A., and Sarubbo, L.A., (2014). Screening of Pseudomonas species for biosurfactant production using low-cost substrates. *Journal of Biocatalyst and Agriculture Biotechnology* 3: 132-139.
10. Thanomsub, B., Puneechockchai, W., Limitrakul, A., Arunrattiyakorn, P., Petchieelaha, W., and Nitoda, T., (2006). Chemical structure and biological activities of rhamnolipid produced by *Pseudomonas aeruginosa* B189 isolated from milk factory waste. *Bioresour. Technol*. 98: 1148-1153.
11. Mukhrjee, S., Das, P., Sen, R., (2006). Towards commercial production of microbial surfactants. *Trends Biotechnol* 24: 509-515.
12. Juwakar, A.A., Dubey, K.V., Nair, A., Singh S.K., (2008). Bioremediation of multi-metal contaminated soil using biosurfactant – a novel approach. *Indian Journal of Microbiology*. 48: 142-146.

13. Zobell, C.E., (1946). Action of microorganisms on hydrocarbon. *Bacterial Rev*. 10 (1):
14. Bongolo, G., (1998). Biosurfactant as emulsifying agents for hydrocarbon. *Physicochemical Engineer Aspect.* 152: 41-52.
15. Tehzibi, A., Kamal, f., and Assadi, M.M., (2004). Improved production of rhamnolipids by a *Pseudomonas aeruginosa* Mutant. *Iran. Biomed. J.* 8(1): 25-31.

16. Carrilo, P.G., Mardaraz C., Pitta-Alvarez S.I., Giulietti, A.M., (1996). Isolation and selection of biosurfactant-producing bacteria. *World Journal of Microbiology Biotechnology.* 12: 82-82.

17. Bodour, A.A., Miler-Maier, R.M., (1998). Application of modified drop collapse technique for surfactant quantification and screening of biosurfactant-producing microorganisms. *Journal of Microbial Methods*. 32: 273-280.

18. Morikawa, M., Hirata, Y., Imanaka, T., (2000). A study on the structure function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta*. 1488: 211-218.
19. Cameron, D.R., Cooper, D.G., Neufeld, R.J., (1998). The mannoprotien of *Saccharomyces cerevisiae* is an effective bioemulsifier. *Applied Environmental Microbiology.* 54: 1420-1425.
20. Pruthi, V., Cameotra, S.S., (1995). Rapid method for monitoring maximum biosurfactant production obtained by acetone precipitation. *Journal of Biotechnology*. 9: 271-276.
21. Desai, J.D., Banat, I.M., (1997). Microbial production of surfactants and their commercial potential. *Journal of Microbial Molecular Biology*. 61: 47-64.
22. Joice, P. A., and Parthasarathi, R., (2014). Optimization of biosurfactant production from *Pseudomonas aeruginosa* PBSC1. *International Journal of Current Microbiology and Applied Sciences. 3*(9): 140-151.

23. Rahaman, K.S.M., Banat, I.M., Thahira, J., Thayumanavan, T., Lakshmanaperumalsamy, P., (2002). Rhamnolipid biosurfactant production by strain of *Pseudomonas aeruginosa* using low-cost raw materials. *Biotechnol Prog*. 18: 1277-1281.