ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 12 , December 2016

Biosurfactant Production using Diesel Oil Degrading Bacteria

T. SIVAGAMASUNDARI, Dr. N. JEYAKUMAR

Department of Microbiology and Biotechnology, N.M.S.S.Vellaichamy Nadar College, Nagamalai, Madurai, Tamilnadu.
Department of Microbiology, Government Arts College, Surandai, Tamilnadu.

ABSTRACT: Biosurfactant producing micro organisms are naturally present in the oil contaminated soil. Oil contaminant environment contain large amount of hydrocarbons. The present studies was aimed to determine biosurfactant producers from diesel oil contaminated soil collected from automobile workshop in Madurai, Tamilnadu, India. Bacillus sp and Enterobacter hormaechei were isolated, identified and screened for biosurfactant production using diesel oil as sole carbon source. The isolated biosurfactants were detected by CTAB method and oil spreading technique and was analyzed by using TLC method.

KEYWORDS: Biosurfactant, Bacillus sp, Enterobacter hormaechei, oil polluted soil.

I. INTRODUCTION

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of bacteria and filamentous fungi (Chen et al., 2007). The main role of biosurfactant in microbial cells is emulsification of water insoluble substrates such as hydrocarbons and facilitates its transport into the cell to stimulate the growth. A large variety of microorganisms construct effective surface active agents, biosurfactants, which differ in their chemical properties and molecular size. The main applications of biosurfactant is in several areas such as public health, agriculture, health care, waste utilization and environmental pollution control such as in degradation of hydrocarbons in soil. Hydrocarbons are composed of complex chemical structure i.e., aliphatic and aromatic hydrocarbons. Microorganisms show emulsifying activity by generating biosurfactants and use the hydrocarbons as substrate and frequently mineralizing them or translate them into harmless products. The present study focused on the biosurfactant production by Bacillus sp and Enterobacter hormaechei isolated from oil contaminated soil using diesel oil as substrate and biosurfactant production was screened by standard methods viz CTAB method, hemolysis of erythrocytes, oil spreading technique, drop collapsing test and TLC method.

II . MATERIALS AND METHODS

A. Isolation and Identification of Biosurfactant producing Bacteria

Diesel oil contaminated soil sample was collected from the oil spilled area from automobile workshop, Madurai. The soil contaminated with diesel oil was serially diluted using nutrient agar medium and incubated for 48 hrs at 37^{o}C. Then the individual colony was selected and purified using streak plate technique. The isolated bacteria were identified using standard bio chemical tests and Gram's staining.

B. Screening of biosurfactant activity by the isolates

The following screening methods were used to detect biosurfactant production by the isolates

i. Hemolysis of erythrocytes
ii. Formation of dark blue halos in CTAB plates.

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 12 , December 2016

i) **Detection of biosurfactants by the hemolysis of erythrocytes**

Ten micro litre (10µl) of the concentrated culture supernatants were spotted onto agar plates containing 5% blood and incubated at room temperature for two days and the zones of hemolysis was observed (Rashedi et al.,2005).

ii) **Detection of biosurfactans by CTAB method**

Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml) and methylene blue (MB: 0.2 mg/ml) were used for the detection of anionic biosurfactant (Satpute et al., 2008). Thirty microlitre (30µl) of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm in diametre). The plates were then incubated at 37° C for 48-72 hrs. A dark blue halo zone around the well was considered positive for anionic biosurfactant production.

C. **Oil spreading technique**

A loopful of Bacterial isolates was inoculated into 100 ml of nutrient medium. The cultures were incubated on rotary shaker (150 rpm) for 3 days at 37^0 C. The culture suspension was screened for biosurfactant production (Priya and Usharani, 2009; Anandaraj and Thivakaran, 2010). Thirty millilitre of (30ml) of distilled water was taken in the Petriplate. One milliliter (1ml) of diesel oil was added at the centre of the plates containing water. Then twenty micro litre (20µl) of the supernatant of the cultures isolated from the diesel oil spilled soil was added to the centre. The biosurfactant producing organism can displace the oil and spread in the water. The diameter and area of clear halo was visualized under visible light. It was measured and calculated after one minute (Ali et al., 2013)

D. **Production of biosurfactant**

The isolates were inoculated into the production Medium and it was incubated at 37º C for 72 hrs and optical density was measured at 620nm for every twelve hours.

Composition	Gram/Litre
Glucose	40
Ammonium dihydrogen phosphate	0.14
Disodium hydrogen phosphate	4.84
Potassium Dihydrogen phosphate	4.08
Ferrous sulphate	0.0152
Manganese sulphate	0.002
Magnesium sulphate	0.196
Calcium chloride	0.001
Distilled Water	1lt.
pH	7.2

E. **Extraction and purification of Lipopeptide from Bacillus** sp **(Isolate -1)**

The surfactant produced by Bacillus sp was extracted by acid precipitation method (Cooper et al., 1981). The production medium was prepared, sterilized and inoculated with the Bacillus Culture. After inoculation it was incubated at 37º C for 72 hours. During incubation the lipopeptide was produced and released into the medium. This was extracted by the acid precipitation method. First the medium was centrifuged at 5000 rpm for 15minues. The cell free broth containing lipopeptide was collected in a separate tube.

The lipopeptide in the broth was precipitated at pH 2 by adding concentrated hydrochloric acid. The broth was again centrifuged at 5,000 rpm for 15 minutes. The lipopeptide was extracted with dichloromethane. Further purification was achieved by recrystallization. The dichloromethane extract was dissolved in distilled water containing

ISSN: 2350-0328
International Journal of Advanced Research in Science, Engineering and Technology
Vol. 3, Issue 12 , December 2016

sufficient Whattman filter paper and it was adjusted to pH 2 with concentrated Hydrochloric acid. The white solid was collected as a pellet after centrifugation.

F. Extraction and purification of Rhamnolipid from Enterobacter hormaechei (Isolate-2)

The surfactant produced by Enterobacter hormaechei was extracted using ammonium sulfate. The production medium was prepared, sterilized and inoculated with the culture. After inoculation it was incubated at 37º C for 72 hrs during incubation the rhamnolipid was produced and released into the medium. This was extracted by using ammonium sulfate. The medium was centrifuged at 8000rpm for 40 minutes the supernatant was collected in the separate tubes. The rhamnolipid in the broth was precipitated by adding 50% sulfate, which was then maintained at 4º C for 24hrs. The broth was again centrifuged at 8000 rpm for 40 minutes then it was suspended in water and dialyzed against distilled water for 2 days (at least five replacements of water). Further purification was achieved by recrystallization.

G. Dry weight quantification of biosurfactants

Sterile Petriplate was taken and the weight of the plate was measured. Then the sediment was poured on the plates. They were placed on the hot air oven for drying at 100º C for 30 minutes. After drying the plates were weighted. The dry weight of the biosurfactants was calculated by the following formula:

Dry weight of biosurfactant = Weight of the plate after drying – Weight of the empty plate

H. ANALYSIS OF BIOSURFACTANT
Thin Layer Chromatography

The preliminary characterization of biosurfactant was done by using TLC method (Anandaraj et al.,2010). A portion of the crude biosurfactant was separated on a silica gel plate using Chloroform: Methanol: water ($CHCl_3$:CH_3OH:H_2O) in the ratio70:10:0.5, v/v/v as an eluent with different color developing reagents. Ninhydrin reagent (0.5 g ninhydrin in 100ml anhydrous acetone) was used as a locating reagent to detect lipopeptide biosurfactant as red spots and anthrone reagent (1g anthrone in 5ml sulfuric acid mixed with 95ml ethanol) to detect rhamnolipid biosurfactant as yellow spots (Yin et al.,2008).

III . RESULT

A. Isolation and Identification of Biosurfactant producing Bacteria

Bacillus sp and Enterobacter hormaechei were isolated from diesel oil contaminated soil. Bacillus sp is a Gram positive, motile, endospore forming bacteria. Enterobacter hormaechei is a Gram negative, Coccibacilli, non motile and non spore forming bacteria.

B. Detection of biosurfactants production

To directly detect surface activity of biosurfactants, different tests were carried out. These include detection by hemolysis of erythrocytes and formation of dark halos of CTAB agar plates around the wells containing biosurfactants.

C. Hemolysis of erythrocytes by biosurfactants

Biosurfactants possess hemolytic properties, biosurfactants producing organism was added on the well of an agar plate containing 5% blood. Figure 1 shows that there is biosurfactant production. The hemolytic zone differs upon isolates.

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 12 , December 2016

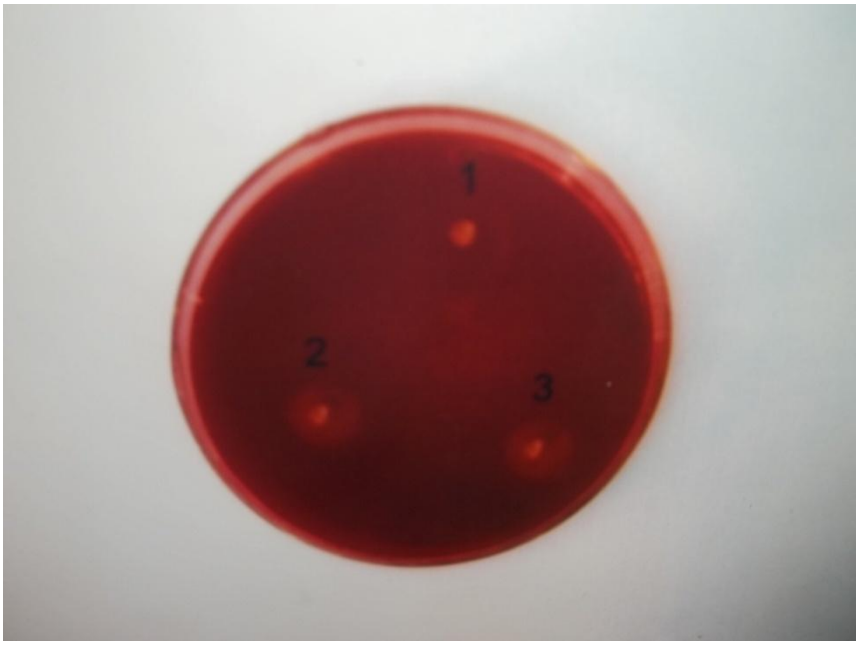

Figure 1: Hemolytic activity of biosurfactant from the isolates

1. Control
2. Biosurfactant form Bacillus sp
3. Biosurfactant from Enterobacter hormaechei

D. Formation of dark blue halos on CTAB plates

The blue agar plates with cetyl trimethy1 ammonium bromide and methylene blue were used to detect the biosurfactant production and the formation of dark blue halos around the well containing biosurfactant was observed in Figure 2.

ISSN: 2350-0328
International Journal of Advanced Research in Science, Engineering and Technology
Vol. 3, Issue 12 , December 2016

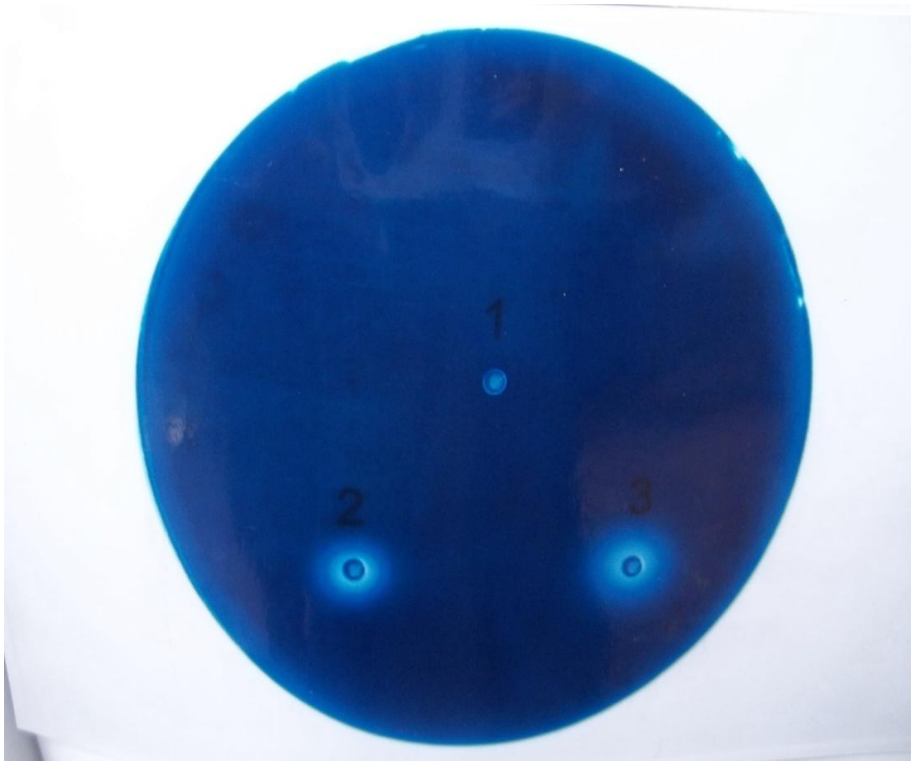

Figure 2: Biosurfactant activity of the isolates on CTAB agar plate

1. Control
2. Biosurfactant from Bacillus sp
3. Biosurfactant from Enterobacter hormaechei

E. Oil spreading technique

The colonies were centrifuged and added to the oil containing plates. The culture showed a zone of displacement in the oil (Figure 3). The biosurfactant producing organism can only able to displace the oil. The zone of displacement was measured and tabulated (Table-1). In this Enterobacter hormaechei shows larger zone of displacement than Bacillus sp.

Table: 1 Oil displacement method

Isolates	Zone formation (mm)
Bacillus sp	8
Enterobacter hormaechei	12

ISSN: 2350-0328
International Journal of Advanced Research in Science, Engineering and Technology
Vol. 3, Issue 12 , December 2016

Figure 3: Zone formation by biosurfactants producing organisms using oil spreading technique

F. Dry weight of biosurfactants

The dry weights of the biosurfactants were measured and estimated (Table 2).

Table 2: Dry weight of Biosurfactants

Isolates	Plate weight (g)	After drying of biosurfactants in the plate (g)	Dry weight of biosurfactant (g)
Bacillus sp	47.121	47.264	0.143
Enterobacter hormaechei	47.121	47.286	0.165

G. Analysis of biosurfactants

Biosurfactants isolated by acid precipitation at pH 2 was analysed by TLC method. lipopeptide from Bacillus sp was identified by red color spot (Figure 4) where as rhamnolipid from Enterobacter hormaechei was identified by yellow color spot (Figure 5). These results conclude both the organisms uses diesel oil a source for biosurfactant production.

ISSN: 2350-0328
International Journal of Advanced Research in Science, Engineering and Technology
Vol. 3, Issue 12 , December 2016

IV.DISCUSSION

Figure 4: Analysis of lipopeptide from Bacillus sp

Figure 5: Analysis of rhamnolipid from Enterobacter hormaechei

The biosurfactant producing organisms were isolated from oil spilled soils of workshop (Anandharaj et al., 2010 and Thavasi et al 2010). The isolated bacterial cultures from oil polluted soil were identified as Bacillus sp and Enterobacter hormaechei. Bacillus sp is a Gram positive rod shaped endospore forming bacteria. Enterobacter hormaechei is a Gram negative rod shaped bacteria. These oil degrading bacteria were used to produce biosurfactants. Huszcza et al., (2006) had used Bacillus sp for their studies on biosurfactant production. Various biosurfactant producing microbes were detected from oil contaminated soil sample (Batista et al., 2006). Schulz et al., (1991) used mineral media with C14 and C15 n-alkanes for isolation of biosurfactant producing strains.

The cultures Bacillus sp and Enterobacter hormaechei showed β- haemolytic activity on blood agar plate. It correlates with the studies of Krepsky et al., (2007), Rashedi et al., (2005), who screened biosurfactant producing organisms by blood hemolysis test. In CTAB method, the biosurfactant producing organisms showed dark blue halos around the blue agar plate. The micro organism isolated from oil contaminated soil sample showed biosurfactant activity. It is similar with the studies of Satpute et al., (2008). In oil spreading technique the zone of displacement was measured. Both the organisms produce the zone of displacement in diesel oil. This oil spreading method was also studied by Priya et al., (2009), Anandaraj et al., (2010) and Jaysree et al., (2011). The dry weight of biosurfactant also measured and tabulated. Among the isolates Enterobacter hormaechei yield higher amount of biosurfactant that was 0.165 g/ml and Bacillus sp produces 0.143g/ml by measuring its dry weight. It is also followed by B.Anandaraj and P.Thivakaran, 2010. The extracted biosurfactants were analyzed by using TLC method. The lipopeptide produced by Bacillus sp was detected by using ninhydrin as red spot and rhamnolipid biosurfactant produced by Enterobacter hormaechei as yellow spots was observed by using anthrone. This method was also used by Yin et al., (2008) for the analysis of lipopeptide and glycolipid. Ali et al., (2013) extracted the biosurfactant by conventional way and characterized by thin layer chromatography (TLC) and reported the presence of glycolipid and lipopeptide in nature.

From the above study it was observed that Bacillus sp and Enterobacter hormaechei isolated from oil contaminated soil sample showed biosurfactant activity. It concluded that both the organisms are capable of producing biosurfactants that would help in degrading diesel oil in the environment.

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 12 , December 2016

V.CONCLUSION

Biosurfactant are produced by many different bacteria genera. The chemical structure of biosurfactant varies widely but all biosurfactant described thus anionic or non-anionic. Biosurfactant such as lipopeptide and rhamnolipid are effectively used in waste water treatment, anti dandruff shampoos, hair gels, deodorant sticks, after shave lotion, nuclear fuel processing plants, paints, crop protection formulation, corrosion inhibition, textile detergents and cleaning agents. Biosurfactant are amphiphilic compound of microbial origin with considerable potential in commercial applications with various industries.

REFERENCES

[1] Ali, S.R., Chowdhury, B.R., Mondal, P. and Rajak, S. (2013): Screening and characterization of biosurfactants producing microorganism from natural environment (Whey spilled soil). J.Nat.Sci.Res., 3(13): 34 – 64.

[2] Anandaraj, B. and Thivakaran, P. (2010). Isolation and production of biosurfactant producing organism from oil spilled soil. J.Biosci.Tech., 1(3): 120 – 126.

[3] Batista, S., Mounteer, A., Amorim F., et al. (2006). Isolation and characteraization of biosurfactant / bioemulsifier – producing bacteria from petroleum contaminated sites. Bioresour. Technol., 97 (6): 868 – 875.

[4] Chen, S.Y., Wei, Y.H. and Chang J.S. 2007. Repeted pH - stat fed-batch fermentation for rhamnolopid production with indigenous Psedomonas aeroginosa S2. Applied microbial Biotechnol., 76 (1): 67 – 74.

[5] Cooper D.G., MacDonald C.R., Duff S. J.B., and Kosaric N. (1981), Enhanced production of surfactant from Bacillus subtilis by continuous product removal and metal cation additions. Appl. Environ, Microbio, 42, 408 – 412.

[6] Huszcza E. and Burczyk B. (2006), Sufactin isoforms from Bacillus coagulans. Z.Naturforsch. 61c, 727 – 733.

[7] Jaysree,R.C., Basu,S., Singh, P.P., Ghosal, T., para, A.P., Keerthi,Y., and Rajendran (2011). Isolation of biosurfactant producing bacteria from environmental samples, Pharmacologyonline, 3, 1427 – 1433.

[8] Krepsky, N., et al. (2007), Alternative methodology for isolation of biosurfactant producing bacteria, Brazilian Journal of Biology, Vol. 67 No: 1, February 2007.

[9] Plaza, G., Zjawiony, I. and Banat, I. (2006), use of different methods for detection of thermophilic biosurfactant producing bacterial from hydrocarbon contaminated bioremediatied soils. J.Petro.Sci.Eng., 50(1): 71 – 77.

[10] Priya, T. and Usharani, G. (2009). Comparative study for biosurfactant production by using Bacilus subtilis and Pseudomonas aeruginosa. Bot. Res. Int., 2(4): 284 – 287.

[11] Rashedi. H., Jamshidi, Mazaheri Assadi M., and Bonakdarpour.B., 2005. Isolation and production of biosurfactant from Psedomonas aeroginosa isolated from Iranian southers wells oils. Int. Environ. Sci. Tech. 2(2); 121 – 127.

[12] Satpute, S.K., Bhawsar, B.D., Dhakephalkar, P.K. and Chopade, B.A. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J.marine Sci., 37: 243 – 250.

[13] Schulz, D., passeri, A. and Schmidit, M., et al., (1991). Marine biosurfactas screening for biosurfactans among crude oil degrading marine microorganisms from the North Sea. Z.Naturforsch, 9C., 46 (3 – 4): 197 – 203.

[14] Thavasi, R., Subramanyam Nambaru, V.R.M., Jayalakshmi, S., Balasubramanian, T., Ibrahim M.2008. Biosurfactant production by Azotobacter chroococoum Isolated from the marine Environment. Marine Bitechnol.

[15] Yin H., J.Qiang, Y.Jia, J.Ye, H.Peng, H. Qin, N.Zhang and B, 2008. Characteristics of biosurfactant produced by Pseudomonas aeruginosa S6 isolated from oil containing waste water. Process Biochemistry. 44: 302 – 308