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Research Article

BIOSURFACTANT PRODUCTION AND DIESEL DEGRADATION BY BACTERIAL CONSORTIUM ISOLATED FROM CRUDE OIL POLLUTED SOIL

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ABSTRACT
Biosurfactants are surface active compounds produced by microorganisms. The objective of this study is to isolate and identify the biosurfactant producing bacteria from oil contaminated soil. The 5 organisms were selected to determine the ability to produce biosurfactant. The organisms identified were *Bacillus* sp (B1), *Bacillus* sp (B2), *Pseudomonas* sp (B3), *Micrococcus* sp (B4), *Klebsiella* sp (B5).. From the screening test such as hemolytic assay, oil spreading assay and lipase assay, all the 5strains were showed positive results. Diesel degradation studies using organisms has found to be able to degrade the diesel oil of different concentrations.

Keywords: Biosurfactants, diesel degradation, hemolytic assay.

INTRODUCTION

Biosurfactants are surface active compounds produced by microorganisms are broadly use in many environmental applications and are used for the degradation of hydrocarbons due to its high toxicity, and low degradability. In larger ecosystems , the production cost of synthetic surfactants is expensive . Biosurfactants, lead to an increasing interest on these microbial products as alternatives to chemical surfactants [1] and are advantageous characteristics such as structural diversity, low toxicity, higher substrate selectivity, higher biodegradability and better environmental compatibility etc . Numbers of studies reported the synthesis of various types of biosurfactants by microorganisms using water soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates[2] and are directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequent biodegradation of the hydrocarbons by direct cell contact[1, 3,4, 5]. There are different screening methods are used to screen biosurfactant producing microbes such as hemolytic assay [6,7], bacterial adhesion to hydrocarbons (BATH) assay [8,9] drop collapse assay, oil spreading assay[10,11] , emulsification assay [12,13] surface tension measurement, titled glass slide test, blue agar plate and hydrocarbon overlay agar assay[13] .

Hydrocarbon degradation by microorganisms is the conversion of hazardous substances into less or non-toxic and also petroleum and diesel products are removed from the environment inexpensively [4,14,15].One of the most important characteristics of hydrocarbon-degrading bacteria is the ability of emulsifying hydrocarbons in solution by producing surface-active agents such as biosurfactants [2,3,16,17,18].

MATERIALS AND METHODS

Sample Collection

Diesel polluted soil samples were collected from nearby areas of our College. Soil was collected randomly 5-10 cm beneath the surface with sterile spatula and aseptically packed in polybags and transfer to the laboratory . The isolation of organisms was done by using serial dilution method and stock cultures were maintained.

Identification Of Selected Organisms

The selected organisms were identified based on morphological, biochemical and physiological characters according to Bergey's manual of determinative bacteriology.

Screening for Biosurfactant Production

5different Bacterial species were selected and subjected to the following screening tests of biosurfactant production.

Hemolytic assay

The blood agar plate was prepared ,single colonies from each selected cultures were inoculated and incubated for 48-72 hours at 37ºC. The bacterial colonies were then observed for the presence of clear zone around the colonies. This clear zone indicates the presence of biosurfactant production ability of organisms.

Oil spreading assay

Oil spreading assay experiment was performed using the method described [19]. 20 ml of distilled water added to a petridish followed by the addition of 20 µl of crude oil.10 µl of cell free culture broth was then added to the oil surface, the oil will be displaced with an oil free clearing zone when the presence of biosurfactant in the cell free culture broth and diameter of the clear zone indicates the surfactant activity.

Lipase assay

The single colony was inoculated on tributyrin agar plates and were incubated for 24 hr at 37⁰C. The bacterial colonies were observed for the presence of clear zone around the colonies. This clear zone indicates the presence of biosurfactant producing organisms.

EXTRACTION OF BIOSURFACTANT

Biosurfactant extraction was done by preparing nutrient broth which was inoculated with the isolates such as *Bacillus* sp (B1), *Bacillus* sp (B2), *Pseudomonas* sp (B3), *Micrococcus* sp (B4), *Klebsiella* sp (B5) were taken in conical flasks and incubated for 5 days at 30ºC. After the incubation time, the broth was centrifuged at 6000 rpm for 20 minutes at room temperature to separate the bacterial cell and supernatant was taken in a separate conical flask. The pH of the supernatant was adjusted to 2 using HCl. The extraction of the supernatant using 2:1ratio of chloroform and isopropyl alcohol . The upper layer was discarded and bottom layer was kept for evaporation and the volume was measured.

Emulsification Index

The ability of emulsification process was measured using the emulsification index (E24). The emulsification index of culture samples was determined by adding 2 ml of diesel to the same amount of culture media, mixing with a vortex for 2 min and it stand for 24 hr. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm)[2]. Percentage of emulsification index was calculated using the formula:

$$E24 = \frac{\text{Height of emulsion formed} \times 100}{\text{Height of total solution}}$$

Diesel Degradation Studies

5 organisms were inoculated in to each respective tubes containing 3ml of nutrient broth and incubated for 24 hr at 37⁰C which were used as inoculum. 100 ml of nutrient broth was added into twenty, 250 ml conical flasks and sterilized.10 flasks were taken as one set and added with 10 ml of diesel and other one set of 10 flasks were added with 20 ml of diesel. All the flasks were inoculated with 1ml of 24hr culture. 5 flasks each from both concentration of diesel were incubated for 7 days at 37⁰C and another flasks were incubated for 14 days at 37⁰C and the diesel degradation studies were assayed. The rate of bacterial utilization of diesel measured by taking height of diesel layer, appearance of diesel layer, volume of diesel layer, OD at 560 nm and dry weight in 7th and 14th day.

RESULTS AND DISCUSSION

Isolation of Bacteria From Diesel Polluted Soil

5 different bacterial species which were repeated in all dilution plates were selected for the present study and identified as *Bacillus* sp (B1), *Bacillus* sp (B2), *Pseudomonas* sp (B3), *Micrococcus* sp (B4), *Klebsiella* sp (B5).

Screening for Biosurfactant Production

Hemolytic Assay

From 5 bacterial strains tested , all strains were able for hemolysis and B3, B4 showed the maximum hemolytic activity. B1, B2 showed less zone than B5 (Table 2). Hemolytic assay was performed in 5% sheep blood agar plates [20]. Bacterial culture produced clear zone on blood agar plates after incubation at 48 hr at 37°C, the diameter of the clear zone is a qualitative method used as an indicator of biosurfactant production[20,21].

Oil Spreading Assay
In the oil displacement test all the strains were produced a clear zone B3and B5 were produced a clear zone in the maximum level. B1, B2 showed medium spreading of oil and B4 produced a low spreading of oil (Table 3).

Lipase Assay
All organisms were showed lipase activity , comparatively B1 and B2 produced high lipase activity. B3 showed medium zone, B4 and B5 showed lesser zone (Table 4).

Extraction of Biosurfactant
B3 produced higher amount of biosurfactant than other organisms. B1 and B4 produced lesser amount of biosurfactant (Table 5).

Emulsification Index
From the results of emulsification index, B5 showed high emulsification index of about 57.14%. Emulsification index of B3, B1 and B4 is 52.38% and of B2 is 42.85% (Table 6). A study on microorganisms from oil contaminated soil samples with biosurfactant producing ability by hemolytic assay, emulsification index, *etc* and found to be that *Pseudomonas aeruginosa* was a more potent strain [22].

Diesel Degradation Studies
The results of Diesel degradation studies showed that all the organisms used in study were able to degrade the diesel of 10ml and also 20ml. Diesel degradation in 7th and 14th day were measured, the height of diesel layer (Table 7), appearance of diesel layer (Table 8), volume of diesel layer (Table 9), OD (Table 10), dry weight (Table 11) were measured in the 7th and 14th day of incubation. The diesel degradation abilities of *Streptomyces griseus* [23], *Candida rugosa* (DSM 2031) [24] and *Staphylococcus* sp, *Pseudomonas* sp, 2 *Bacillus* sp [25] from oil contaminated soil at various environmental parameters. A diesel degradation experiment using *Bacillus* sp, *Pseudomonas* sp, and *Proteus* sp from cow dung samples and *Pseudomonas* sp was found to give maximum activity[26]. *Bacillus subtilis, Bacillus cereus, Trichoderma harzanium* and *Trichothercium roseum* were hydrocarbon degraders , isolated from diesel oil polluted soil [27] . The growth potential of hydrocarbon utilization of bacteria on petrol and diesel and emulsification activity was studied by using *Micrococcus* sp[28].

CONCLUSION
Bacteria are the main agents responsible for the degradation of diesel fuel and also producing biosurfactants. The present project work describes the study on the isolation, identification and biosurfactant production of diesel degrading bacteria from oil polluted soil samples. From the results, clearly understood that all the organisms used this study were showed the diesel degradation ability and also the biosurfactant activity. These organisms may used as a diesel degrading agents in the oil contaminated soil samples to decrease the soil contamination.

Table 1: Hemolytic Assay

Sl no	Test Organism	Results
1	B1	+
2	B2	+
3	B3	+++
4	B4	+++
5	B5	++

\+ small zone,++ medium zone,+++ large zone

Table 2: Oil Spreading Assay

Sl no	Test Organism	Results
1	B1	++
2	B2	++
3	B3	+++
4	B4	+
5	B5	+++

Table 3: Lipase Assay

Sl no	Test Organism	Results
1	B1	+++
2	B2	+++
3	B3	++
4	B4	+
5	B5	+

Table 4: Extraction Of Biosurfactant

Sl no	Test Organism	Quantity of Biosurfactant (ml)
1	B1	50
2	B2	51
3	B3	56
4	B4	50
5	B5	54

Table 5: Emulsification Index

Sl no	Organism	Height of the emulsifying layer (mm)	Height of the liquid column(mm)	Percentage of emulsification
1	B1	110	210	52.38%
2	B2	90	210	42.85%
3	B3	110	210	52.38%
4	B4	110	210	52.38%
5	B5	120	210	57.14%

Table 6: Diesel Degradation Studies (Height Of Diesel Layer)

Sl no	Organism	Concentration of diesel(ml)	Height of the diesel layer (mm)	
			7th day	14th day
1	B1	10	60	60
		20	70	70
2	B2	10	60	50
		20	70	70
3	B3	10	50	40
		20	50	50
4	B4	10	50	20
		20	60	50
5	B5	10	40	40
		20	70	60

Table 7: Diesel Degradation Studies (Appearance of Diesel Layer)

Sl no	Organisms	Concentration of diesel(ml)	Appearance of diesel layer	
			7th day	14th day
1	B1	10	White cloudiness	White cloudiness
		20	White cloudiness	White cloudiness
2	B2	10	Yellow colour with bubble	Yellow colour with bubble
		20	Yellow colour with bubble	Yellow colour with bubble
3	B3	10	Yellow colour with bubble	Yellow colour with bubble
		20	Yellow colour with bubble	Yellow colour with bubble
4	B4	10	Yellow colour with bubble	Yellow colour with bubble
		20	Yellow colour with bubble	Yellow colour with bubble
5	B5	10	Yellow colour with bubble	Yellow colour with bubble
		20	Yellow colour with bubble	Yellow colour with bubble

Table 8: Diesel Degradation Studies (Volume of Diesel)

Sl no	Organisms	Concentration of diesel(ml)	Volume of diesel(ml)	
			7th day	14th day
1	B1	10	8	7
		20	18	17
2	B2	10	6	5
		20	17	15
3	B3	10	9	7.5
		20	16	14
4	B4	10	9	3
		20	15	14
5	B5	10	8	7
		20	19	17

Table 9: Diesel Degradation Studies (Optical Density)

Sl no	Organisms	Concentration of Diesel (ml)	OD at 560 nm	
			7th day	14th day
1	B1	10	0.48	0.67
		20	0.65	1.09
2	B2	10	0.67	0.89
		20	0.76	1.15
3	B3	10	0.72	1.04
		20	1.04	1.17
4	B4	10	0.76	1.0
		20	0.95	1.04
5	B5	10	1.04	1.09
		20	1.04	1.15

Table 10: Diesel Degradation Studies (Dry Weight)

Sl no	Organisms	Concentration of diesel (ml)	Dry weight (gm)	
			7th day	14th day
1	B1	10	0.013	0.018
		20	0.016	0.02
2	B2	10	0.02	0.026
		20	0.016	0.018
3	B3	10	0.002	0.003
		20	0.002	0.004
4	B4	10	0.004	0.02
		20	0.002	0.009
5	B5	10	0.002	0.004
		20	0.012	0.013

REFERENCES

1. Banat IM, Makkar RS and Cameotra SS Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol. 2000;53:495-508.
2. Desai JD and Banat IM. Microbal production of surfactants and their commercial potential. Microbiol Mol Biol Rev. 61: 47-64.
3. Hommel RK. Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation. 1990;1:107-119.
4. Leahy JG and Colwell RJL. Microbial degradation of hydrocarbons in the environment. Microbiol Rev. 1990;54: 305-315.
5. Deleu M, Paquot M, Jacques P, Thonart P, Adriaensen Y and Dufrene YF. Nanometre scale organization of mixed surfactin/phosphatidyl- choline monolayers. Biophys J. 1999;77: 2304-2310.
6. Banat IM. The isolation of a thermophilic biosurfactant producing Bacillus sp. Biotechnol Lett . 1993;15: 591-594.
7. Yonebayashi H, Yoshida S, Ono K and Enomoto H. Screening of microorganisms for microbial enhanced oil recovery process. Sekiyu Gakkaishi. 2000;43:59-69.
8. Volchenko NN, Karasev SG, Nimchenko DV and Karaseva EV. Cell hydrophobicity as a criterion of selection of bacterial producers of biosurfactants. Microbiology. 2007;76:112-114.
9. Tuleva BK, Ivanov RG and Christova NE. Biosurfactant production by an new Pseudomonas putida strain. Z Naturforsch. 2002;57: 356-360.
10. Bodour AA and Miller-Maier R. Application of a modified dropcollapse technique for surfactant quantification and screening of biosurfactant-producing microorganisms. J Microbiol Methods. 1998;32:273-280.
11. Youssef NH, Duncan KE, Nagle DP, Savage KN and Knapp RM. Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Methods. 2004;56:339-347.
12. Afshar S, Lotfabad TB, Roostaazad R, Najafabadi AR and Noghabi AK. Comparative approach for detection of biosurfactant-producing bacteria isolated from Ahvaz petroleum excavation areas in south of Iran. Ann Microbiol. 2008;58:555-560.
13. Satpute SK, Bhawsar BD, Dhakephalkar PK and Chopade BA. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J Mar Sci. 2008;37:243-250.
14. Atlas RM. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 1981;45:180-209.
15. Lidderdale T. Demand, supply, and price outlook for low-sulfur diesel fuel. In Energy Information Adminismtion. Short term energy outlook annual supplement DOE-E&0202. 1993;93.
16. Neu TR. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol Rev. 1996;60:151-166.
17. Bredholt H, Josefsen K, Vatland A, Bruheim P and Eimhjellen K. Emulsification of crude oil by an alkane-oxidizing Rhodococcus species isolated from sea water. Can J Microbiol. 1998;44:330-340.
18. Das M, Das SK and Mukherjee RK. Surface active properties of the culture filtrates of a Micrococcus species

grown on n-alkanes and sugars. Bioresour Technol. 1998;63:231-235.

19. Morikawa M, Hirata Y and Imanaka TA. A study on the structure-function relationship of lipopeptide biosurfactants. Biochim Biophys Acta. 2000;1488:211- 218.
20. Mulligan CN, Cooper DG and Neufeld RJ. selection of microbes producing biosurfactants in media without hydrocarbons. J Ferment Technol. 1984;62:311-314.
21. Rodrigues LR, Teixeira JA, Van Der Mei HC and Oliveira R. Isolation and partial characterization of a biosurfactant produced by Streptococcus thermophilus A. Colloids Surf B Biointerfaces. 2006;53:105-112.
22. Saravanan V and Vijayakumar S. Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil . J Acad Indus Res. 2012;1(5).
23. Vishnupriya B, Sundaramoorthi C, Kalaivani M and Selvam K. Production of lipase from Streptomyces griseus and evaluation of Bioparameters. International journal of chemtech research. 2010;1380-1383:2.
24. Lakshmi B. S, Kangueane P, Abraham B and Pennathur G. Effect of vegetable oils in the secretion of lipase from Candida rugosa (DSM 2031) , Letters in Applied Microbiology. 1999;29(1):66–70.
25. Nisha. P, Nayana. M and Viji Varghese. Degradation Studies on Diesel Oil Using Bacterial Consortium Isolated from Oil Polluted Soil. Advanced Biotech. 2013;13:02 .
26. Joshi PA and Pandey GB. Screening of petroleum degrading bacteria from cow dung. Research journal of agricultural sciences. 2011;2(1):69-71.
27. Waogu LA, Onyeze GOC and Nwabueze RN. Degradation of diesel oil in a polluted soil in using Bacillus subtilis African journal of Biotechnology. 2008;7(12):1939-1943.
28. Santhini K, Myla J, Sajani S and Usharani G. Screening of Micrococcus sp from oil contaminated soil with reference to Bioremediation Botany research international. 2009;2(4):248-252.