China Petroleum Processing and Petrochemical Technology

Catalyst Research Volume 23, Issue 2, November 2023 Pp. 3253-3261

ISOLATION AND IDENTIFICATION OF BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED SOIL

Kalyani Pullapukuri [1, a], G. Sravan Kumar[2, b]

Author Affiliations

[1]Dept. of Microbiology, Bio Enviro Chemical Solutions, Visakhapatnam, Andhra Pradesh, India

[2]G. Sravan Kumar, Assistant Professor, Dept. of Biotechnology, Gayatri Vidya Parishad College for Degree and PG Courses, Visakhapatnam, Andhra Pradesh-530017.

Author Emails

[a] Corresponding author: pkalyani156@gmail.com

[b] sravang2010@gmail.com

Abstract

Biosurfactant is a structurally diverse group of surface-active molecules, synthesized by microorganisms. They are capable of reducing surface and interfacial tension with low toxicity, high specificity and biodegradability. The samples were enriched in nutrient agar containing diesel oil, serially diluted and poured on nutrient agar plate. Biosurfactant producing organisms were screened by using oil spreading method, Blood hemolysis test, Emulsification index (E_{24}) and Drop collapse method. Out of 50 isolates, ten strains showed positive biosurfactant production. Another interesting finding in this study was the use of crude oil as hydrophobic substrate for the isolation of biosurfactant producing bacterial strains; this approach may be useful during the initial isolation of biosurfactant producing bacteria to reduce the number of strains to be screened. These strains were characterized by using different biochemical tests like Gram's staining, IMVIC etc.

Keywords: Biosurfactant, Biodegradability, Hydrocarbons

Introduction

Oil pollution and remediation technology has become a global phenomenon of increasing importance. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms have an important role in combating environmental pollution. Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size which are surface active compounds which increases the surface tension of the hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation. Almost all surfactants currently produced are chemically derived from petroleum. These synthetic surfactants are usually toxic themselves and are hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in
recent years, drawn much attention to the microbial production of surfactants or biosurfactants (Urum and Pekdemir, 2004).

Microorganisms have adopted different strategies to enhance the bioavailability and gain access to hydrophobic compounds, such as hydrocarbons, including biosurfactant mediated solubilization, direct access of oil drops and biofilm-mediated access (Hommel, 1990). The production of biosurfactants and bioemulsifiers is generally involved, although to different

degrees, in all the above strategies. Biosurfactant structural uniqueness resides in the coexistence of a hydrophilic (a sugar or peptide) and a hydrophobic (fatty acid chain) domain in the same molecule, which allows them to occupy the interface of mixed phase systems (e.g., oil/water, air/water, oil/solid/water) and consequently to alter the forces governing the equilibrium conditions. This is the prerequisite for a broad range of surface activities to take place including emulsification, dispersion, dissolution, solubilization, wetting and foaming (Banat et al., 2000; Desai and Banat, (1997).

Biosurfactants are used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others. They can be used as emulsifiers as well as demulsifiers, wetting agents, foaming agents, spreading agents, functional food ingredients and detergents. The interfacial surface tension reducing ability of biosurfactants made them to play important role in oil recovery and bioremediation of heavy crude oil (Volkering et al., 1998). Screening and isolation of most potent biosurfactant producing organism was studied from oil contaminated soil.

Materials and Methods

Reagents and Chemicals

All the medias and chemicals are used in this study are purchased from High Media laboratories, Sigma and Merk

Collection of soil sample

Soil samples were collected from various oil contaminated soil places like garage, petrol bunks, diesel sheds in Visakhapatnam, Andhra Pradesh, India. The collected soil samples are placed in sterilized glass bottles and properly sealed with foil to avoid light reaction and transferred laboratory carefully. The collected soil samples were stored at room temperature for further use.

Isolation of biosurfactant producing bacteria

The isolation of biosurfactant producing bacteria from oil contaminated soil by Enrichment culture method. Soil sample 1gm was serially diluted in sterile saline up to 10^{-5} and1ml of the suspension was transferred to 100ml of BH broth and incubated at 37ºC for one week. After incubation the culture suspension was speeded on sterile nutrient agar plate and incubated at 37 ºC for 48hrs, after incubation the colonies were observed. Each colony again straked on individual nutrient agar plate and then incubated 37ºC for 48hr. The isolated colonies were subjected for further identification.

Screening of isolates for biosurfactant production

Primary screening of isolates for the production of biosurfactants, the selected isolates were introduced in to nutrient agar medium and then incubated at 37ºC for 48hr. After incubation, the culture was transferred for centrifugation at 3000rpm for 30min. After centrifugation, the supernatant was collected and used for various screening tests.

Haemolysis test

Catalyst Research Volume 23, Issue 2, November 2023 Pp. 3253-3261

Screening of biosurfactant bacteria by using Haemolysis test. The selected pure culture of bacteria was streaked on blood agar plates and incubated at 48-74hr. After incubation the clear zone was observed.

Drop Collapsing test

Two ml of oil was applied to the wells of 96-well microplate and the plate left to 24hr for equilibration. 5ml of 24hr bacterial culture after centrifugation was transferred on oil coated 96-well microplate and left for one min. After that the drop size was observed by using magnifying glass. The drop was flat, the bacteria considered as positive for the production of biosurfactant

Oil spread method

Twenty millilitre of distilled water was added on the petriplate by the addition of 20 millilitre of crude oil on the surface of the water and then 10 millilitre of cell free culture broth was added on the surface of the oil. If the biosurfactant was present in the culture broth, the clear zone was observed. The diameter of the zone of inhibition indicates the surface activity.

Emulsification Test

For measuring the emulsion index, 2ml of culture was placed in test tube by the addition of 2ml of diesel. The mixture was vortexed at high speed for 2 min and placed a side for 24hr. After 2hr, height of the stable emulsion layer was measured. The emulsion index E was calculated as the ratio of the height of the emulsion layer and the total height of liquid

$$E_{24} = \frac{\text{Height of emulsion}}{\text{Total height}} \times 100$$

Biochemical characterization of selected isolates

The selected bacterial isolates were subjected to cultural and morphological characteristics. The tests include Gram's staining, IMVIC tests, catalase test, oxidase test, starch hydrolysis test, urease test, Hydrogen sulphide test, urease test, gelatin hydrolysis test, motility test, fermentative test and utilization of carbon sources.

Production of biosurfactant

Luria Bertani (LB) broth was used to make the inoculum, which was then incubated at 37°C for the duration of the night with 100 rpm of agitation. To produce biosurfactant, a fresh overnight culture was employed as an inoculum.1% inoculum was added aseptically to 100 ml of production medium containing 2% diesel oil, and it was then incubated at 370C for 48 hours at 120 rpm in a shaking incubator.

Catalyst Research Volume 23, Issue 2, November 2023 Pp. 3253-3261

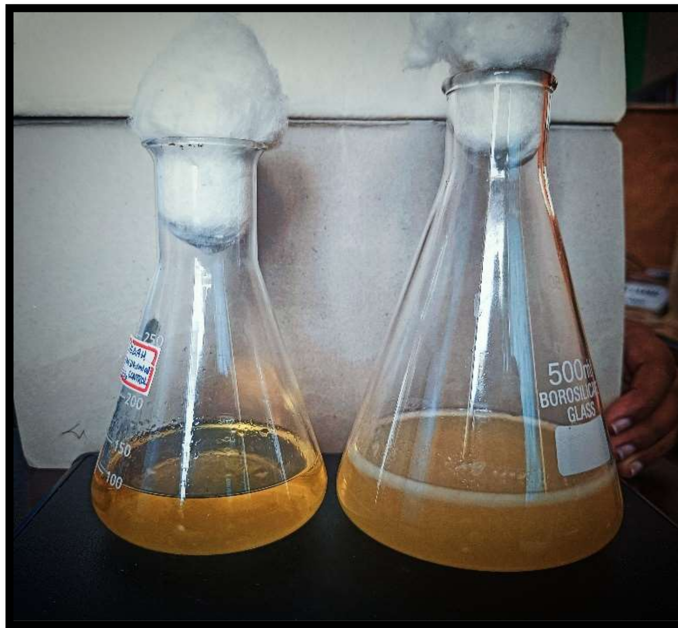


Fig. 2 Production of biosurfactant

Extraction of biosurfactant

Centrifugation at 10,000 rpm for 15 minutes yielded the culture supernatant of the bacterial isolate, which was subsequently filtered via a Millipore membrane system. By using cold acetone precipitation, which involves adding three volumes of chilled acetone and letting it sit for 10 hours at 4°C, the biosurfactant was extracted from the cell-free culture supernatant. Centrifugation was used to separate the precipitate, which was then evaporated to eliminate any remaining acetone before being redissolved in sterile water. For further investigation, the end result was regarded as the crude biosurfactant.

Results and Discussion

The present study was designed to isolate the biosurfactant producing bacteria from oil contaminated soil. The soil sample was collected from different places like petrole bunks, Disele sheds, railway stations and carefully transferred the samples to microbiology laboratory for further processing. Standard morphological and biochemicals methods were used to isolate the biosurfactant producing bacteria from oil contaminated soil. From the soil samples 20 isolates were observed on nutrient agar plates. On characterization, 10 isolates were selected for the different type of screening tests for the production of biosurfactant. The selected isolates were tested for the different biochemical characterization and the results were presented in Table-1. Out of 10 isolates, seven isolates are gram positive and the three isolates were gram negative. All the isolates showed catalase positive (Fig. 1).

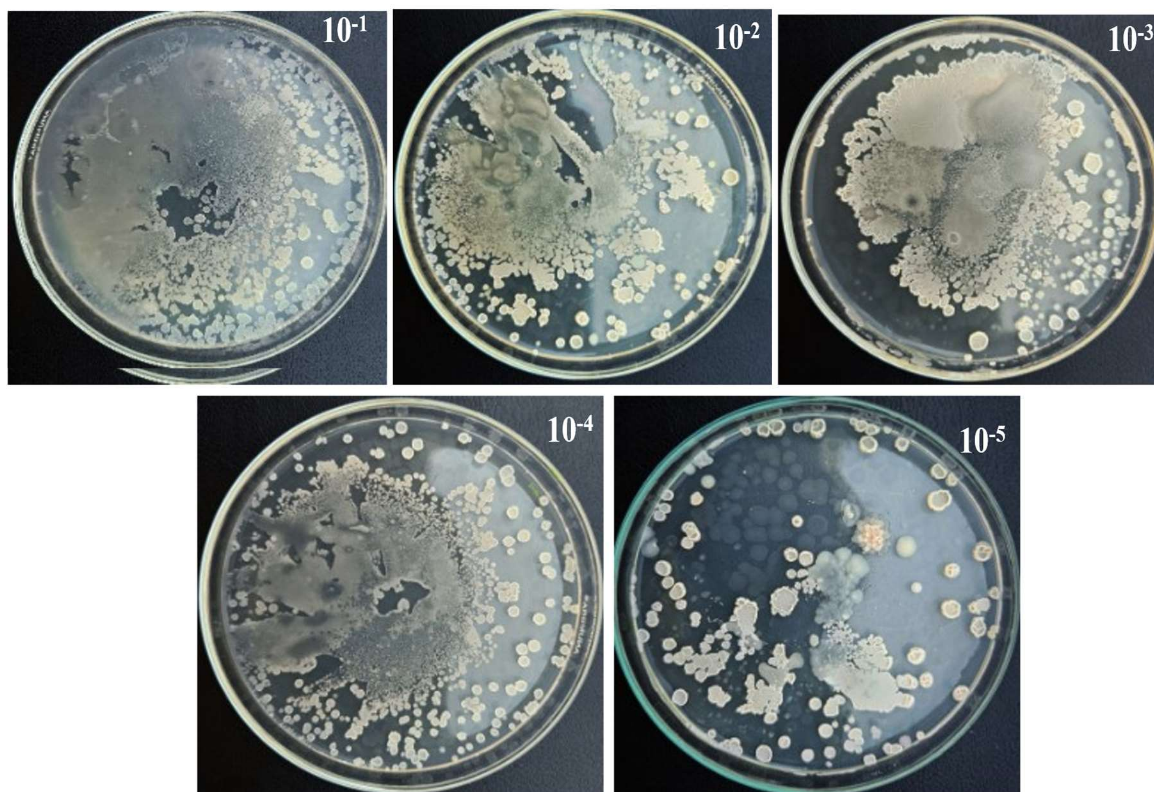


Fig. 1 colonies appear on Nutrient agar plate along with oil

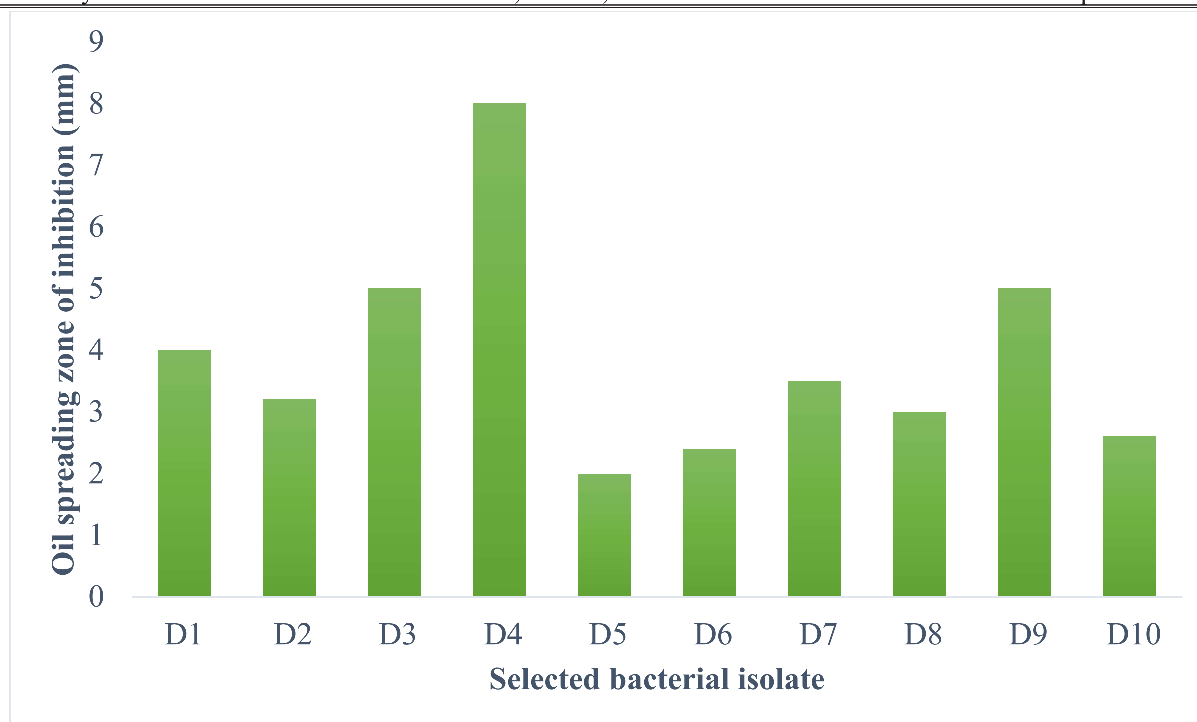
Table-1 Biochemical characterization of selected isolates

S. No	Selected isolate	Gram's staining	Indole test	MR test	VP test	Simmon citrate agar test	Catalase test	Oxidase test	Urease test
1	D_1	+	-	+	+	_	+	+	+
2	D_2	+	-	+	+	+	+	+	+
3	D_3	+	-	+	+	+	+	+	-
4	D_4	-	-	-	+	-	+	+	-
5	D_5	+	-	+	+	+	+	-	+
6	D_6	-	-	-	-	-	+	-	+
7	D_7	+	-	+	-	+	+	+	+
8	D_8	+	-	+	-	+	+	+	-
9	D_9	+	-	+	+	-	+	-	+
10	D_{10}	-	-	-	+	-	+	+	+

Catalyst Research Volume 23, Issue 2, November 2023 Pp. 3253-3261

Table-2 Screening of selected bacterial isolates

S. No	Blood hemolytic test	Oil spreading method (mm)	Drop collapse method	Emulsification Test
D_1	α-hemolysis	4	+	38
D_2	β-hemolysis	3.2	+	34
D_3	β-hemolysis	5	+	39
D_4	α-hemolysis	8	+	48
D_5	β-hemolysis	2	+	33
D_6	β-hemolysis	2.4	+	32
D_7	γ-hemolysis	3.5	+	40
D_8	γ-hemolysis	3	+	36
D_9	β-hemolysis	5	+	41
D_{10}	β-hemolysis	2.6	+	29



Graph. 1 Oil spreading method of selected bacterial isolates

Approximately four screening methods were used and results were given in Table-2. Most researchers have used maximum 2 to 3 screening methods before selecting biosurfactant producers. It is suggested that a single method is not suitable to identify all types of biosurfactants (Yousef et al., 2004). Therefore, combination of various methods is required for effective screening. Occurrence of biosurfactant producing bacteria in hydrocarbon polluted environments was reported by many researchers (Yateem et al.,2002., Bodour et al., 2003., das & Mukherjee., 2005). Single screening method is unsuitable for the isolation of biosurfactant producing microorganisms and more than two screening methods are must be included for the primary screening of biosurfactant producing microorganisms reported by Kiran et al., (2010).

Blood haemolysis is most commonly used primary screening method for the isolation of biosurfactant producing microorganisms. Mulligan et al., (1984) reported that the biosurfactant can cause the lysis of erythrocytes. From the present study six isolates showed complete lysis β-hemolysis, whereas two isolates showed α- hemolysis and remaining two isolates showed γ-hemolysis. . Mulligan et al., (1984) reported that the blood agar method as a preliminary screening method which should be supported by other techniques based on the surface activity measurement. All isolates gave positive results of drop collapse and tilted glass slide. Biosurfactants are suitable alternatives to chemical surfactants due to their properties like less toxicity, eco-friendly, high specificity, biodegradability and synthesis from cheaper renewable substrates (Mohan et al., 2006). Therefore, search of biosurfactant producing microorganisms is an important area of research. According to Bodour and Miller, (2000); two or three screening methods were used for selection of biosurfactant producer. Single screening method is not suitable to identify all types of

Catalyst Research Volume 23, Issue 2, November 2023 Pp. 3253-3261

biosurfactants. Using more screening methods most efficient organisms can be selected for biosurfactant production.

Oil spreading is easy to carry out and it is an indirect method. This oil spreading method required small amount of sample. If the supernatant contains surfactant, it clears the oil and forms the clear zone (Rodrigues et al., 2006). Oil spreading method is most effective method to identify the biosurfactant producing microorganisms by According to Plaza et al., (2006). Whereas drop collapse technique to be considered as chief screening method for biosurfactant production. If the liquid contains surfactants, the drop collapses because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. Bodour et al., (2003) reported drop collapse is efficient technique for detection of biosurfactant producing microorganisms for various samples.

Conclusion

In this study four different screening methods were used to screen bacterial isolates for the production of biosurfactant (Graph-1). Drop collapse and Oil spreading methods are most suitable methods for the production of biosurfactant because these methods require small amount of sample. Biosurfactant producing microbes discussed either as a single method or combination of two or three methods like haemolytic assay, drop collapse method, oil spreading assay, tilted glass slide and emulsification index measurement. For large scale production of biosurfactant different industrial agro wastes were used as substrates. Based on the results the selected bacterial isolates have the ability to produce biosurfactant from oil contaminated soil microorganisms.

References

1. Banat IM, Makkar SR, Cameotra SS (2000) Potential commercial application of microbial surfactants. Appl Microbiol Biotechnol, 53: 495–508.
2. Desai, J. D., and Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol., 61: 47–64.
3. Volkering, F., A.M. Breure and W.H. Rulkens, (1998). Microbiological aspects of surfactant use for biological soil remediation. Biodegradation, 8: 401-417.
4. Mohan, P.K.; Nakhla, G. and Yanful, E.K. (2006): Biokinetics of biodegradability of surfactants under aerobic, anoxic and anaerobic conditions. Water Research, 40: 533–540.
5. Bodour AA, Drees KP, Rain M, Maier RM. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl Environ Microbiol. 2003; 69(6): 3280-3287.
6. Rodrigues LR, Teixeira JA, Van der Mei HC, Oliveira R (2006) Physiochemical and functional characterization of a biosurfactant produced by Lactococcus lactis 53. Colloids Surf B, 49: 79–86.
7. N.K. Bordoloi & B.K. Konwar, Microbial surfactant-enhanced mineral oil recovery under laboratory conditions, Colloids and Surfaces B: Biointerfaces, 63(2008)73–82I.

8. O. Pornsunthorntawee, N. Arttaweeporn, S. Paisanjit, P. Somboonthanate, M. Abe, R. Rujiravanit, S. Chavadej, Isolation and comparison of biosurfactants produced by Bacillus subtilis PT2 and Pseudomonas aeruginosa SP4 for microbial surfactant-enhanced oil recovery, Biochemical Engineering Journal 42 (2008) 172–179.
9. E.J. Gudina, J.F.B. Pereira, L.R. Rodrigues, J.A.P. Coutinho, J.A. Teixeira, Isolation and study of microorganisms from oil samples for application in Microbial Enhanced Oil Recovery, International Biodeterioration & Biodegradation 68 (2012) 56-64.
10. P. Sivasankar, G. Suresh Kumar, Influence of pH on dynamics of microbial enhanced oil recovery processes using biosurfactant producing Pseudomonas putida: Mathematical modelling and numerical simulation, Bioresource Technology, 224(2017)498–508.
11. V.M. Alvarez, D. Jurelevicius, J.M. Marques, P. Macedo de Souzaa, L. Vieira de Araujo, T. G. Barros, R.O.M. Alves de Souza, D.M.G. Freire, L. Seldin, Bacillus amyloliquefaciens TSBSO 3.8, a biosurfactant-producing strainwith biotechnological potential for microbial enhanced oil recovery, Colloids and Surfaces B: Biointerfaces,136 (2015) 14–21.
12. S.K. Nandwani, N.I. Malek, V.N. Lad, M. Chakraborty, S. Gupta, Study on interfacial properties of Imidazolium ionic liquids as surfactant and their application in enhanced oil recovery, Colloids and Surfaces A: Physicochemical and Engineering Aspects, Colloids and Surfaces A: Physicochem. Eng. Aspects, 516 (2017) 383–393.