Isolation and Screening of Biosurfactant Producing Bacterial Species from Petroleum Oil Contaminated Sites

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Abstract: Bacterial isolates from petroleum contaminated soil sample were screened and evaluated for biosurfactant production in this study. Using culture –dependent technique, one hundred and eighty eight (188) bacterial isolates were isolated from 58 different petroleum contaminated sites. The dominant species were mainly found to be Bacillus and Pseudomonas; with an occurrence rate of approximately 25% to 28% each. The isolates were subjected to conventional biosurfactant screening tests: qualitatively (drop collapse, lipase test and hemolysis test) and quantitatively (emulsification activity). In all the isolated bacterial species, Bacillus and Pseudomonas species were found to be showing positive results for all the tests and they had positive clearing zone and an emulsification capacity in range of 85.45% and 87.5% respectively. This confirms their ability to produce biosurfactants that reduce interfacial and surface tension thereby leading to increase in solubility and emulsification of these oils.

Keywords: Biosurfactant, B.subtilis, P.aeruginosa, Emulsification activity, Drop collapse test.

I. INTRODUCTION

Predominantly biosurfactants belongs to a grade of biomolecules procured from microbial pedigree that possess surface active properties parallel to the chemically synthesized surfactants. Biosurfactants being classified as molecule with properties of both a hydrophobic and a hydrophilic group, have an outspread of advantages over their chemical analogues. They are more precise in action along with being environment friendly in nature, and robust under variegated environmental circumstances. They have a considerable range of potential in bioremediation of contaminated areas(1). They are produced and processed in a multifarious type by divergent classes of microbial species. Some of the pre-eminent known examples of biosurfactant producers include *Pseudomonas* sp. (rhamnolipids), *Bacillus sp*. (lipopeptides), *Rhodococcus sp*. (trehalolipids), *Acinetobacter sp* (phopholipids), *Corynebacterium sp*. (sulphonylipids), *Arthrobactersp*. and *Alcanivorax sp*. (glycolipids) and yeasts like *Candida sp*. and *Starmarella* (sophorolipids). [(12)]

Biosurfactants have great potency to reduce the surface tension and emulsify hydrocarbons, thereby, improving the bioavailability quotient of the contaminating hydrocarbons. It becomes rather uncomplicated for the aboriginal bacterial species to assimilate and metabolize the hydrocarbon, for their idiosyncratic nutrition. The whole process is natural and non hazardous, safe by-products are formed during these reactions. A chemical surfactant dramatically differs in this perspective. The related products from the reactions may result in the production of a more harmful product, which might have a higher rate of persistence in the environment. [(13)]

They also have wide range of usage in the food industry as food formulation constituents and antiadhesive substances. [(15).] They find a large application in commercial enterprise such as the medical industry as antibacterial agents, immunomodulatory molecules, adhesive agents and in gene therapy. [(17)] Due to the manifold areas of application, there has been growing concern and research taking place in the direction of the large-scale production of biosurfactants in a cost effective feasible way. Biosurfactants accomplish a notable rate of hydrocarbon degradation because the aboriginal bacterial species have a greater area of contact at the hydrocarbon-water interface. [(12)] Specific contaminated sites have bacterial species well acclimatized to metabolize the hydrocarbons found at that site. The biodegradation pursuit may be due to the outcome of individual bacterial activities. It may be also due to the synergistic activities of the consortium of the various bacterial species residing in that area. Numerous data found until now have proven the applicability of bacterial species for the biodegradation of hydrocarbons. [(2) (3) (8) (10) (11) (20)]

II. MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from Petroleum oil contaminated sites such as garages, automobile repairing shops, petrol pumps, truck parking area, etc. by grab method in sterile air tight polythene zip lock pouches which were labeled appropriately and taken to the laboratory for further study. Bacterial samples were acclimatized in nutrient medium.

Isolation of bacterial species from soil samples

Isolation was carried out by pour plate technique. The 5 gm of each soil sample inoculated in 100 ml of Mineral Salt Medium (MSM) for 72 hours at 37˚C. After incubation the bacterial sample were serially diluted in normal saline solution. The pour plate technique was performed to isolate the bacterial species. [9]

Mineral Salt Medium (MSM) NaNO3 0.5**.,** K2HPO4 0.5., KH2PO4 0.5, MgSO4.7H2O 0.5, KCL 0.1., FeSO4 0.01, (NH4) MO7O24.4H2O 0.01, CaCl2.2H2O 0.03, Citric acid 0.40, Yeast extract 0.01. Trace element 2 (mL), 1ml engine oil added to the flasks, 1% Glucose was added to the flasks as a sole source of carbon. Final pH (at 25°C) 7.4±0.2

Nutrient Broth : Ingredients in Gms / Litre: Peptic digest of animal tissue 5, Sodium chloride 5, Beef extract 1, Yeast extract 1.5, Final pH (at 25°C) 7.4±0.2

Nutrient Agar : Ingredients in Gms / Litre

Peptic digest of animal tissue5, Sodium chloride 5, Beef extract 1.5, Yeast extract 1.5, Agar 15, Final pH (at 25°C) 7.4±0.2

Identification Disc for bacterial identification using HiDetectTM Rapid Universal identification disc:

Newly emerged diagnostic HiDetectTM Rapid Identification Disc was used for the direct identification of isolated bacterial species from soil samples. This HiDetectTM Rapid Identification Disc eliminated the use of Selective Medias for confirmation of bacterial isolates. This is a reliable and simple technique for detection of bacteria.

The method for detection of the bacterial isolates is target bacteria comprise of following steps:

Step I: Isolation, Inoculation of the bacterial sample from the culture tubes on surface plating medium and incubation of bacterial species at 35˚C-37˚C for 18-24 hours was carried out. Plates were observed for any bacterial growth after incubation period.

Step II: Replication and Identification based on staining property.

HiDetectTM Rapid Identification Disc was placed on the surface of the agar plates for maximum time span of 30 seconds. Corresponding orientation was marked on the HiDetectTM Rapid Identification Disc paper for convenience. This was called as replication technique. The replicated identification discs were incubated on the empty sterile petri plates at 35˚C-37˚C for 1-4 hours. After incubation the discs were observed for the development of color and the results were interpretated. (9) Identified morphologically by special staining methods and then plated for further studies.

Isolation and Screening of biosurfactant producing bacterial species:

Various different methods of screening were employed to evaluate the efficiency of production of biosurfactant by the 188 isolates. The isolated bacterial species were further screened for the ability of biosurfactant production. The bacterial isolates were named IHD. To confirm the production of biosurfactant by the bacterial isolates (IHD), different screening tests were carried out.

The several screening tests were assessed to investigate for the potential for biosurfactant production by the isolated bacterial species. The various screening tests included Emulsification test (emulsification index E24), Drop Collapse test, Lipase test, Blood Agar Haemolysis test.

Emulsification test:

Emulsification test was carried out using engine oil. Centrifugation of the bacterial cell culture was performed at 12000 rpm for 15 minutes. 2 ml of engine oil was taken in a sterile tube to which 1 ml of cell free supernatant obtained after the centrifugation of the bacterial cell culture was added to it and it was vortexed for about 2 minutes for the proper mixing of both the liquids. (OU, 2003) The emulsification activity was observed after 24 hours. The emulsification index (Emulsification index 24) E24 was calculated by using following formula:

Total height of the emulsified layer / Height of the aqueous layer x 100

The calculations were done for each of the bacterial samples separately and their emulsification indexes were compared with each other.

Drop Collapse Test:

The isolated bacterial species were inoculated in mineral salt medium with 0.1% engine oil and incubated at 37 ˚C for 48 hours. Drop collapse test was performed to screen for the ability of biosurfactant production. 2 µl of engine oil was applied to the glass slide and these slides left to equilibrate for 24 hours.

The 48 hours culture was centrifuged at 12000 rpm for 15 minutes at 24 hours at 25˚C. The centrifugation was done to remove the cells. 5µl of the supernatant was transferred to the engine oil coated glass slide. The size of the drop was observed after 1 minute with the help of magnifying glass.

The results were considered to be positive when the diameter of the drop was increased by 1 mm from which were produced by distilled water which were taken as the negative control for the drop collapse assay.

Lipase activity:

Ingredients in Gms / Litre

Peptic digest of animal tissue 5, Yeast extract 3, Agar 15, Final pH (at 25°C) 7.5±0.2 [6][7]

The bacterial isolates that are known to produce lipase enzyme which degrades the lipid molecules in fatty acid molecules shows lipolytic activity. The isolated bacterial species were screened for their lipolytic activity using the lipase test. The medium used for screening of lipolytic activity was Tributyrin Agar medium. A loop full of the isolated bacterial species was streaked onto the Tributyrin Agar Media Plates. The Tributyrin Agar plates were then incubated at 37˚C for 24 hours. Peptic digest of animal tissue and yeast extract in the medium provide nutrients to the organisms. The lipase activity was examined by the ability of bacterial species to form zone of clearance around the bacterial colonies.

Blood Hemolysis Test:

Blood was drawn from sheep which had been fasted for 18 to 24 h. The neck area was shaved and swabbed with tincture of iodine. The blood was collected in sample bottle containing sodium citrate for each 100 ml of blood to be drawn. The bottle was inverted occasionally to facilitate proper mixing. The blood was stored in the laboratory at 4˚ C prior to use. In blood agar base 5% of sheep blood was aseptically added and the blood agar was the cooled agar to give a final volume of blood agar was dispensed into sterile petri dishes and the plates were allowed to solidify. All prepared plates were stored at 4^0C. Single fresh colony from the isolated bacterial culture was taken and it was streaked onto the Blood Agar Plates. These plates were then incubated for 48 hours to 72 hours at 37˚C. After the incubation period the blood agar plates were observed for the presence of clear zone of hemolysis or partial zone of hemolysis around the bacterial colonies. (5)

III. RESULTS AND DISCUSSION

Microbial enrichment of soil sample:

One hundred and eighty-eight bacterial isolates were isolated after enrichment of the soil samples collected from petroleum contaminated soil. These specimens were selected after a series of serial dilution steps and plating procedures. These bacterial strains were further subjected to a series of screening tests to select the efficient biosurfactant producers.

Emulsification index (EI_{24}%):

TABLE 1 : EMULSIFICATION INDEX (EI_{24}%)

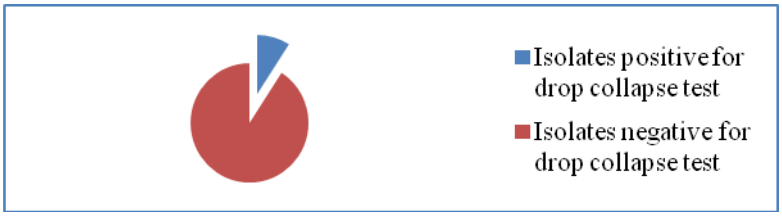
Bacterial isolates (IHD)	(EI_{24}%)	Bacterial isolates (IHD)	(EI_{24}%)	Bacterial isolates (IHD)	(EI_{24}%)	Bacterial isolates (IHD)	(EI_{24}%)
IHD1	37.5	IHD51	22.5	IHD101	57.5	IHD151	50
IHD2	25	IHD52	5	IHD102	12.5	IHD152	80
IHD3	50	IHD53	15	IHD103	32.5	IHD153	42.5
IHD4	30	IHD54	12.5	IHD104	45	IHD154	52.5
IHD5	40	IHD55	27.5	IHD105	17.5	IHD155	45
IHD6	42.5	IHD56	20	IHD106	32.5	IHD156	40
IHD7	22.5	IHD57	27.5	IHD107	32.5	IHD157	68
IHD8	7.5	IHD58	47.2	IHD108	15	IHD158	45
IHD9	5	IHD59	7.5	IHD109	25	IHD159	37.5
IHD10	37.5	IHD60	20	IHD110	27.5	IHD160	15
IHD11	22.5	IHD61	30	IHD111	17.5	IHD161	17.5
IHD12	30	IHD62	32.5	IHD112	58	IHD162	15
IHD13	85.45	IHD63	17.5	IHD113	37.5	IHD163	27.5
IHD14	20	IHD64	12.5	IHD114	32.5	IHD164	15
IHD15	20	IHD65	25	IHD115	17.5	IHD165	22.5
IHD16	35	IHD66	20	IHD116	7.5	IHD166	52.5
IHD17	30.5	IHD67	20	IHD117	20	IHD167	47.5
IHD18	42.5	IHD68	42.5	IHD118	15	IHD168	40
IHD19	87.5	IHD69	7.5	IHD119	40	IHD169	37.5
IHD20	17.5	IHD70	20	IHD120	12.5	IHD170	30
IHD21	30	IHD71	10	IHD121	20	IHD171	20
IHD22	30	IHD72	7.5	IHD122	25	IHD172	17.5
IHD23	27	IHD73	12.5	IHD123	25	IHD173	20

IHD24	15	IHD74	7.5	IHD124	32.5	IHD174	30
IHD25	32.5	IHD75	22.5	IHD125	7.5	IHD176	72.5
IHD26	25	IHD76	17.5	IHD126	17.5	IHD177	40
IHD27	10	IHD77	30	IHD127	20	IHD178	47.5
IHD28	15	IHD78	22.5	IHD128	12.5	IHD179	40
IHD29	25	IHD79	20	IHD129	15	IHD180	45
IHD30	10	IHD80		IHD130	27.5	IHD181	17.5
IHD31	20	IHD81	15	IHD131	37.5	IHD182	17.5
IHD32	32.5	IHD82	12.5	IHD132	30	IHD183	20
IHD33	12.5	IHD83	20	IHD133	20	IHD184	5
IHD34	27.5	IHD84	12.5	IHD134	15	IHD185	15
IHD35	12.5	IHD85	10	IHD135	25	IHD186	32.5
IHD36	77.5	IHD86	17.5	IHD136	7.5	IHD187	5
IHD37	20	IHD87	12.5	IHD137	22.5	IHD188	82.5
IHD38	20	IHD88	22.5	IHD138	27.5		
IHD39	15	IHD89	68	IHD139	27.5		
IHD40	27.5	IHD90	25	IHD140	30		
IHD41	25	IHD91	15	IHD141	20		
IHD42	12.5	IHD92	30	IHD142	47.5		
IHD43	27.5	IHD93	20	IHD143	27.5		
IHD44	74	IHD94	32.5	IHD144	37.5		
IHD45	30	IHD95	42.5	IHD145	12.5		
IHD46	25	IHD96	68	IHD146	15		
IHD47	7.5	IHD97	50	IHD147	32.5		
IHD48	7.5	IHD98	45	IHD148	64		
IHD49	20	IHD99	15	IHD149	7.5		
IHD50	32.5	IHD100	47.5	IHD150	20		

Drop Collapse Test:

Out of that 188 bacterial isolates, 17 bacterial isolates showed positive results. IHD3, IHD13, IHD19, IHD21, IHD36, IHD44, IHD58, IHD80, IHD89, IHD96, IHD112, IHD148, IHD152, IHD157, IHD176, IHD178, IHD188 gave positive results for Drop Collapse test after 48 hours that means they were producing bio-surfactant. The Drop Collapse test has shown that the area of displacement by a surfactant containing solution signifies the production of biosurfactant in the solution. The results were considered to be positive when the diameter of the drop was increased by 1 mm from which were produced by distilled water which were taken as the negative control for the drop collapse assay. The drop collapse test suggests the surface activities of crude biosurfactant. Drop collapse test were highly positive for crude biosurfactant produced by isolated bacterial species than commercial surfactants, Tween 80 and SDS, which indicated their high surface activities.

FIGURE 1: DROP COLLAPSE TEST OF BACTERIAL ISOLATES

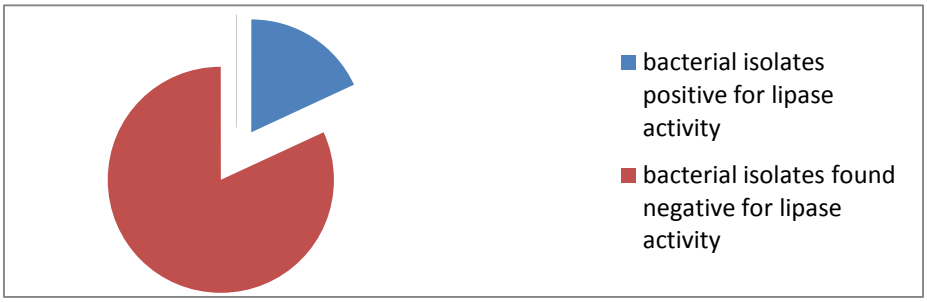


Lipase activity:

The lipase activity was considered to be positive by the ability of bacterial species to form zone of clearance around the bacterial colonies. Out of total 188 isolates, 34 bacterial isolates were found to show lipolytic activity. Isolates IHD3, IHD13, IHD 19, IHD28, IHD36, IHD44, IHD48, IHD56, IHD58, IHD61, IHD64, IHD73, IHD79, IHD80, IHD89, IHD96,

IHD107, IHD112, IHD124, IHD125, IHD133, IHD140, IHD148, IHD152, IHD156, IHD157, IHD158, IHD159, IHD161, IHD165, IHD167, IHD171, IHD176, IHD18-0, IHD184, IHD188 displayed lipase activity on Tributyrin agar plates.

FIGURE 2: LIPASE ACTIVITY OF BACTERIAL ISOLATES



Blood Hemolysis Test :

On Blood Agar plate out of 188 bacterial isolates 40 bacterial isolates were found to be positive for α-hemolysis activity. The results were considered to be positive if the bacterial species showed the clear zone of hemolysis or the partial zone of hemolysis around the colonies. 19 bacterial isolates showed positive results by showing β- hemolysis activity and the remaining 129 bacterial isolates showed gamma hemolysis. After screening test culture slants of bacterial isolates that were found to be positive for biosurfactant production were maintained in pure culture form for further study.

FIGURE 3: BLOOD HEMOLYTIC ACTIVITY OF BACTERIAL ISOLATES

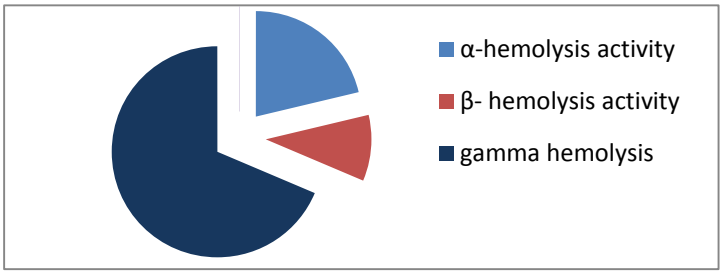


FIGURE 4: IDENTIFICATION OF BACTERIAL ISOLATES ON HIDETECT™ RAPID IDENTIFICATION DISC

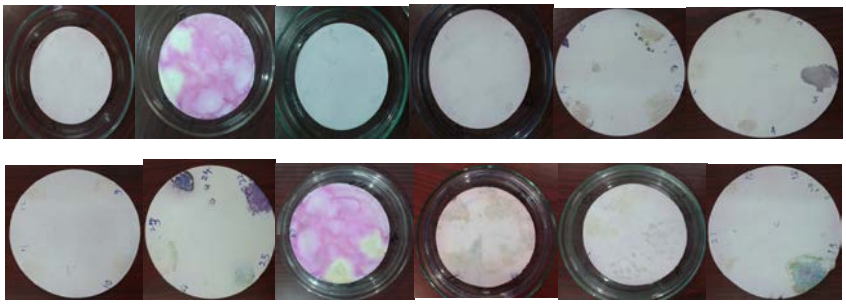


FIGURE 5: EMULSIFICATION TEST

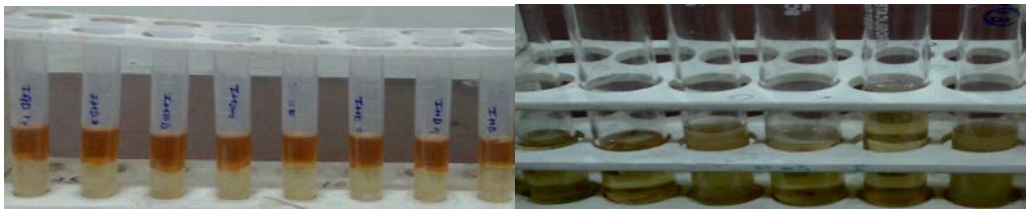


FIGURE 6: DROP COLLAPSE TEST

FIGURE 7: LIPASE ACTIVITY

FIGURE 8: HEMOLYTIC ACTIVITY

The screenings of biosurfactant production by various bacterial species were investigated by hemolytic assay, drop collapse test, emulsification index, lipase test. The present results were found to be similar to the studies reported by Saravanan.V, 2012. [18]

IV. CONCLUSION

The current study comprised the enrichment of bacterial species from a petroleum-contaminated site and isolation of a bacterial isolate, showing potential biosurfactant-producing ability. Soil contaminated with petroleum oil contains large amount of hydrocarbons i.e., aliphatic and aromatic hydrocarbons. This study has revealed that bacterial species exhibit a high emulsifying activity for production of biosurfactants by utilizing hydrocarbons as substrate often mineralizing them or converting them into harmless products.

These bacterial species with the potential for biosurfactant production have wide range of applications in petrochemical and oil industries, pharmacy, medical, cosmetics, food and pharmaceutical.

V. FUTURE SCOPES

To study and examine the robustness of the biosurfactant produced from bacterial species found in petroleum contaminated site under extreme physical conditions and the feasibility of using biosurfactants for enhanced oil recovery operations.

VI. REFERENCES

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