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CHARACTERIZATION OF BIOSURFACTANT PRODUCING MICROORGANISMS FROM OIL CONTAMINATED SOIL

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ABSTRACT

Biosurfactants are surface active compounds released by various microorganisms. These are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases. Biosurfactants have a wide variety of applications in the petroleum, food and pharmaceutical industries as emulsifiers and wetting agents. They also help in clearing petroleum spills and reducing environmental pollution. *Bacillus* sp. showing efficient degradation of hydrocarbons was isolated and characterized. High emulsification activity was observed on a wide range of hydrocarbons and oils in 24hrs the highest being more than >88% emulsification index(EI_{24}) with Coconut oil, Xylene, Olive oil Engine oil, and Kerosene with BS_2. The activity was associated with the production of high molecular weight bioemulsifier with potential applications.

KEYWORDS : Biosurfactants, *Bacillus sp*, emulsification index, oil degrading.

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INTRODUCTION

Surfactants reduce the surface tension between two liquids and cause emulsification. Surfactants have both water-soluble and water insoluble parts in the same molecule and hence are amphiphathic[1]. These are of both chemical and biological origins and several bacteria, fungi and yeast excrete biosurfactants[2] . Structurally, they are diverse which are grouped based on the chemical nature. The important groups include- lipopeptides, glycolipids, phospholipids, neutral lipids, polymeric surfactants, particulate biosurfactants, fatty acids, and lipopolysaccharides[3,4].Biosurfactants and bioemulsifiers have a wide variety of applications as detergents, emulsifiers, as foaming and wetting agents in cosmetics, petroleum, food and pharmaceutical industries. Natural biosurfactants offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability, bioremediation [5]. They also help in clearing petroleum spills on soil, water and in the cleaning the oil tanks. They help in reducing environmental pollution. However, due to limitations owing to their high production cost of majority biosurfactants, interest is continuing in isolation and identification of novel microorganism producing biosurfactants and optimizing the production of novel compounds. The present study reports the isolation and identification of novel biosurfactant producing organisms.

MATERIALS AND METHODS

(i) Sample Collection

Soil sample were collected from the petroleum contaminated, oil spilled surfaces, petrol bunks and automobile workshops in and around Tirupati and Chittoor. The samples collected in screw cap vials were transported to laboratory and stored in refrigerator at 4°C for further processing. Minimal media and nutrient media with 2% crude oil and blood agar (5% blood was added to nutrient media) were used for screening[6]. To isolate biosurfactant producing bacteria one gram was serially ten- fold diluted and 0.1ml sample of appropriated dilution was plated on media pre coated with oil and plates were incubated at 37°C, for 48 hrs. After incubation colonies which showing clear zones were selected and sub-cultured on nutrient agar and stored for further studies. Biosurfactant producing soil bacteria were analyzed by hemolytic assay, oil displacement test, lipase activity. Biosurfactant production was confirmed with drop collapsing method and emulsification index was calculated. *Bacillus subtilis* MTCC 2423 and *Pseudomonas aeruginosa* MTCC 2297 known biosurfactant producers were used as positive control for comparison in all the tests.

(ii) Haemolytic assay

Haemolytic assay was performed in blood agar plates [7, 8]. 50µl broth culture of each organisms was spot-inoculated on to blood agar plates and incubated 48 hrs at 37°C. The plates were visually observed for the zone of clearance (haemolysis) around the colony and the diameter of the zone of clearance is measured.

(iii) Lipase Activity

The isolates producing lipase were identified on Tributyrin agar plates[9]. A loop of inoculums were streaked on to the agar plates and incubated at 37°C for 48 hr. After incubation, the plates were examined for the formation of clear zone around the colonies.

(iv) Oil displacement test

50 ml of distilled water was added to the large petri-plat followed by 20 µl of Castrol CRB plus engine oil. 10 µl of culture supernatant was then added to the surface of oil [10]. The diameter of the lysis was measured and compared with 1% sodium dodecyl sulfate.

(v) Drop Collapsing Method

Drop collapse test was performed to confirm the production of biosurfactant as well as a semiquantitative method for detection of biosurfactant production [11], 7µl of mineral oil was added to 96-well micro titer plate and 10 µl of the culture was added to the surface of the

oil. After 1 minute, the shape of the drop on the surface of the oil was observed.

(vi) Emulsification Index

The emulsification index was measured using the method of [12]. 4 ml of kerosene and 1 ml of cell free supernatant 6 ml water was taken in a test tube and homogenized in a vortex mixer at high speed for 2 min. After 24 hrs, the emulsification index was calculated. Following formula E I 24 (%) = total height of the emulsified layer / total height of the liquid layer x 100. The results were compared with the positive controls such as SDS and positive cultures.

(vii) Production of biosurfactants in the culture media

The culture of the four positive cultures were inoculated in 50 ml of mineral broth with 2% Castrol CRB plus. The culture was incubated at 37°C for 48hrs on shaking incubator at 180rpm. After incubation the bacteria cells were pelleted by centrifugation at 8000rpm, 4°C for 15 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 6 N HCl. Equal volume of chloroform, methanol (2:1) was added. This mixture was shaken well and left overnight at 4°C. The crude biosurfactant in the form of white colored material was recovered and washed sterile water and the material was weighed[6,11].

(viii) Characterization of biosurfactant producing organism

The biosurfactant producing organism was then characterized by using morphological and different biochemical tests like Gram staining, Spore Staining, Motility Test, IMViC test , Starch hydrolysis, Catalase test., Oxidase test Casein hydrolysis, Gelatin hydrolysis using standard protocols [13] . 16srRNA typing was carried using universal primers (Forward primer 5' AGAGTTTGATCCTGGCTCAG 3'and Reverse primer 5' ACGGCTACCTTGTTACGACTT 3'). The purified PCR products were sequenced and phylogenetic tree was constructed by using the BLAST and isolates were characterized[16].

RESULTS AND DISCUSSION

Screening of the isolates for biosurfactant activity

10 soil samples collected from various locations were screened for the production of biosurfactants and about 20 organisms found positive showing zone of clearance on oil coated plates were isolated in pure culture and retested for production of biosurfactant. Of these 12 best isolates were tested for oil displacement activity, drop collapse test, hemolytic activity, lipid hydrolysis and emulsification activity. The results are tabulated as shown in Table: 1. All the isolates exhibited oil displacement activity except BS_8 and BS_{11}. Maximum oil displacement was exhibited by BS_1 and BS_4. Drop collapse test was positive for all the twelve isolates with BS_1, BS_2 BS_4 BS_9 showing maximum activity. The isolates BS_2, BS_4 and BS_9 showed complete and clear hemolysis whereas remaining isolates exhibited partial hemolysis. BS_{10} and BS_{11} was negative for lipid hydrolysis the others showed complete lipid hydrolysis. Maximum lipid hydrolysis was observed with BS_1 , BS_2 and BS_9. All the isolates were positive for emulsification test with BS2 and BS_4 showing maximum activity. Based on these results BS1, BS_2, BS_4 and BS_9 showing good activity in more than three tests were selected for further investigation of production of biosurfactants. The emulsification index of the four BS isolates was tested against nine oils and 1% SDS was taken as control. BS_2 showed the maximum emulsification of 88-95% for five of the nine substrates tested. For the other substrates the emulsification index is between 30-62 (Table-2). This was followed by BS_1, BS_9 and BS_4. 1% SDS taken as positive control showed as emulsification index of 99.5%. The emulsification index of the isolates tested was significantly higher than the *Bacillus subtillus* MTCC 2423 culture used as positive controls suggesting that the organisms have good potential of biosurfactant production. The production of crude biosurfactant was carried out in mineral broth with 2% Castrol CRB plus. The culture was incubated at 37°C for 48hrs on shaking incubator at 180rpm. The supernatants were taken for extraction of crude biosurfactant in the form of dry white powder by chloroform, methanol (2:1) extraction.

Table1
Screening for biosurfactant producing bacteria

Isolates	Oil displacement activity	Drop collapse test	Hemolytic test	Lipid hydrolysis	Emulsifiction activity
BS1	++++	++	+	++++	+++
BS2	+++	++	++++	++++	++++
BS3	+	+	+	+	+
BS4	++++	+	++++	++	++++
BS5	+	+	++	+	++
BS6	+	+	++	+	+
BS7	++	+	_	+	+
BS8	-	+	+	+	+
BS9	++++	++	++++	+++	++
BS10	+	+	+	-	+
BS11	-	+	+	-	+
BS12	+	+	+	+	+
B. subtilis MTCC 2423	++	+	+	+	+
P. aeruginosa MTCC 2297	+++	++	+	++	++

Efficient degradation was obtained by BS_2 as seen in Figure-1. The yield of BS_2 with 6.3 g/l was highest compared with other three isolates which were 5.6g/l, for BS_4 and BS_9 and 4.6 g/l for BS_1 as shown in Table-3. The control *Bacillus* strain tested also produced a lower yield of 3.5 g/l. The yield was higher than several of the biosurfactant *Bacillus sp.* reported earlier[10-16].

Table 2
Emulsification index of the isolates against various substrates

Different oil	Emulsification Index (EI_{24}) %					
	BS_1	BS_2	BS_4	BS_9	*B.subtilis* (Positive control)	Culture free broth
Engine oil (Castrol)	64	97.5	40	66	60	0
Olive oil	40	95	60	16.6	16	0
Soya oil	42	62	43	33.3	20	0
Kerosene	10	88	15	12	15	0
Petrol	15	61	10	15	18	0
Diesel	13	30	16	17	13	0
Coconut oil	88	90	70	90	0	0
Xylene	22	90	00	15	0	0
Palm oil	55	60	33	33.3	40	0

Table3
Yield of crude biosurfactant

Isolates	Quantity of product in g/l
BS_1	4.6
BS_2	6.3
BS_4	5.6
BS_9	5.6
B.subtilis	3.5

Figure 1
Degradation of Castor oil CBR by BS_2

A) Before degradation B) After degradation by BS_2

Identification of the isolates

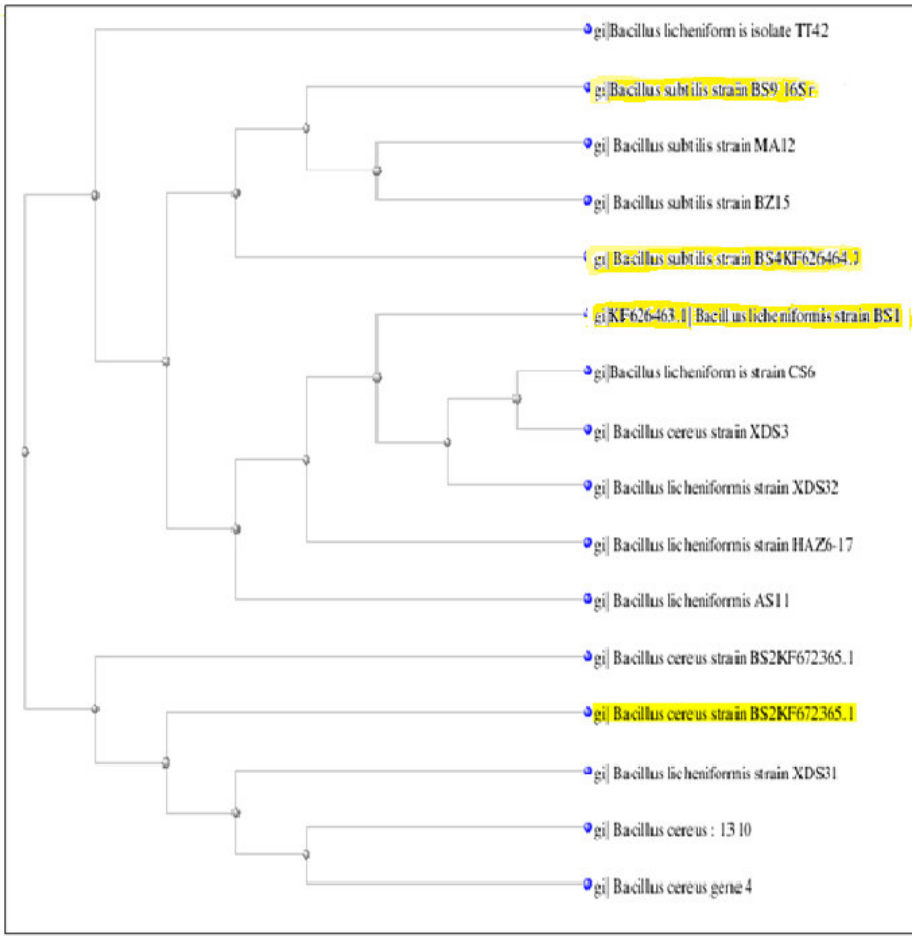
Biochemical characterization was performed for all the four isolates BS_1 BS_2 BS_4 and BS_9. The results are tabulated as given in the Table-4. All four isolates utilized galatose and xylose sugar. The four isolates did not utilize dextrose, lactose, and sorbitol. BS_1, BS_2 and BS_9 utilized trihalose and sucrose whereas BS4 did not utilize. BS_1 and BS_9 did not utilize maltose and arabinose while BS_2 and BS_4 utilized them. BS_2 failed to utilize mannitol which was utilized by BS_1, BS_4 and BS_9. All the isolates were gram positive aerobic rods that were indole negative, VP positive. Three were MR negative and one is positive, and all the four isolates were citrate positive as well as oxidase and catalase positive. Considering all the other characters studied the isolates were grouped under genus *Bacillus*. Further identification of the four isolates was carried with 16srRNA sequencing and sequences are deposited with accession no KF626463.1to KF626465.1 and KF672365.1. The results (Figure-2) revealed that BS_1 was *Bacillus licheniformis* showing 99% similarities with *Bacillus licheniformis* strain CS6, XDS32, BS_2 was *Bacillus cereus*, showing 99% similarities with *Bacillus cereus* strain 1310, BS_4 and BS_9 were *Bacillus subtilis* and grouped with *Bacillus subtilis* strain MA12, BZ15.

Table 4
Biochemical tests characterization of biosurfactant producing bacteria

Test	BS_1	BS_2	BS_4	BS_9
Indole test	-	-	-	-
Methyl red test	-	+	-	-
Vogus -Proskauer test	+	+	+	+
Citrate test	+	-	+	+
Oxidase test	+	+	+	+
Catalase test	+	+	+	+
Urease test	+	+	-	-
Triple sugar test	+	+	+	+
Starch hydrolysis	+	+	-	+
Casein Hydrolysis	-	+	+	-
Gelatin hydrolysis	+	+	-	+
Sugar Fermentation Test				
Dextrose	-	-	-	-
Sucrose	+	+	-	+
Arabinose	-	+	+	-
Sorbitol	-	-	-	-
Lactose	-	-	-	-
Maltose	-	+	+	-
Galatose	+	+	+	+
Xylose	+	+	G	+
Fructose	-	+	-	+
Mannitol	+	-	+	+
Trihalose	+	+	-	+
Nitrate reduction	+	-	+	+

G= Gas production was observed in sugar utilization test. + = Positive - = Negative

Figure 2
Phylogentic analysis of the biosurfactant producing Bacillus sp



REFERENCES

1. Cooper DG, and Golden berg BG, surface-active agents from two *Bacillus species*, Applied and Environmental Microbiology, 53: 224 - 229, (1986).
2. Kumar M, Leon V, Materano ADS, Biosurfactant production and hydrocarbon degradation by halotolerant and thermotolerant Pseudomonas sp, World J Microbiol Biotechnol, (7): 1047 - 1057, (2008).
3. Pornsunthorntawee N, Arttaweeporn N,.Paisanjit S, SomboonthanateAbe P, M.R. Rujiravanit MR, and Chavadej S, Isolation and comparision of biosurfactants produced by Bacillus subtilis Pt2 *and Pseudomonas aeruginosa* SP4 for Microbial surfactant-enhanced oil recovery, Biochemical Engineering Journal. 42: 172 - 179, (2008).
4. Thavasi R, Jayalakshmi S, Balasubramanian T, and Ibrahim MB, Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* uing economically cheaper sources, World. J. Microbiol. Biotechnol, 24: 917 - 925, (2008).
5. Patel RM and Desai AJ, Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses, Lett. Appl. Microbiol, 25: 91 - 94, (1997).
6. Abalos A Vinas M Sabate J, Manresa MA and Solanas AM Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Psedudomonas aeruginosa AT10*. Biodegradation, 15: 249 - 260, (2004).
7. Mulligan CN, Cooper DG and RJ, Selection of microbes producing biosurfactants in media without hydrocarbons. Journal of Fermentation Technology. 62: 311 - 314, (1984).
8. Rodrigues L Banat IM Teixeria J and Oliveira R Biosurfactants: Potential applications in medicine, Journal of Antimicrobial Chemotherapy,57: 609 - 618, (2006).
9. Kuiper I, Lagendijk EL, Pickford R, Derrick JP, Lamers.GEM., Thomas-Oates JE, Lugtenberg BJJ, and. Bloemberg GV, Characterization of two Pseudomonas putida lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms, Molecuralr Microbiology, 52: 97 - 113, (2004.)
10. Jain D, Collins-Thompson D and Lee H, A drop-collapsing test for screening surfactant-producing micro-organisms,J Microbiol Methods, 13 (4): 271 - 279, (1999).
11. Cooper DG and BG Goldenberg, Surface-active agents from two Bacillus species, Applied and Environmental Microbiology, 53: 224 - 229, (1987).
12. Vater J, Kablitz.Wilde B,C, Franke P, Mehta N and Cameotra S, Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactant in whole cells and culture filtrates of B. subtilis C-1 isolated from petroleum sludge, Appl Environ Microbiol, 68: 6210 - 6219, (2002).
13. Mackie and McCartney Practical Medical Microbiology. (Collee, G.,Duguid, JP, Fraser AG and Marmion B.P.Eds.) 13th Edition. Churchill Livingstone, London. 2: 43 - 160, (1989)
14. Chavez G and Martinez A Characterization of the lip A gene encoding the major lipase from strain IGB83, Appl. Microbiol. Bitoechnol. 56: 731 - 735, (2001).
15. Marsudi S, unoo H, Hori K Palm oil utilization for the simultaneous production of polyhydroxyalksnoates and rhamnolipids, Appl. Microbiol. Biotechnolgy, 78: 955-961, (2008).
16. K.Danashekar D.Natarajan and N.Mathiyazhagan. Amplification of biosurfactant producing gene (rhlb) from pseudomonas aeruginosa isolated from oil contaminated soil International Journal of Pharma and Bio Sciences 2:498-504 (2011).