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PETROLEUM CONTAMINATED SOIL AS RICH SOURCE OF BIOEMULSIFIER PRODUCING *ENTEROBACTER CLOACAE*

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ABSTRACT

Bioemulsifier producing bacteria is a promising solution to address petroleum-derived hydrocarbon contamination of soil. Microorganisms can produce bioemulsifier to increase bioavailability, degradation and utilization of contaminating hydrocarbons as sole carbon source. Herein, we report isolation and characterization of bioemulsifier producing bacterial strains isolated from oil contaminated soils like garage and temple. Oil spreading assay was used for screening, and positive strains were grown in minimal medium supplemented with oil and emulsification index and activity was evaluated in cell-free supernatant. A total of 9 bacterial strains were tested, and 5 were positive for the bioemulsifier production. Isolate TS4 showed 27.64 unit/mL, 22.77 unit/mL, 15.23 unit/mL and 8.93 unit/mL emulsification activity against coconut oil, mustard oil, engine oil and groundnut oil, respectively. Isolate TS5 showed 33.87 unit/mL, 35.51 unit/mL, 37.59 unit/mL and 23.48 unit/mL emulsification activities against coconut oil, mustard oil, engine oil and groundnut oil, respectively. Similarly they showed efficient emulsification index against all of the above mentioned oil. Media optimization studies with Plackett Burman design indicated the significant effect of K_2HPO_4, NH_4Cl and oil concentration on bioemulsifier production. The potent bioemulsifying bacteria TS4 and TS5 were identified as *Enterobacter cloacae*. Hence we conclude that the bacterial isolates exhibiting superior emulsification activity can be used for bioremediation in the oil spill affected soil.

KEYWORDS: *Enterobacter cloacae*, Bioemulsifier, Emulsification index, Emulsification activity, Plackett Burman design.

INTRODUCTION

Bacteria produce bioemulsifier to grow on water-insoluble carbon sources by emulsifying the water-insoluble substrate in the culture medium.[1] Such amphipathic molecules from bacteria lower interfacial tension at the oil-water interface forming a microemulsion enhancing the bioavailability of the hydrophobic substrates. Moreover, these bioemulsifiers have got potential applications in food, cosmetic, pharmaceuticals and agriculture. Moreover, they are also used for oil recovery. Low toxicity, high biodegradability, better environmental compatibility, high selectivity and specific activity at extreme temperature, pH and salinity makes them ideal candidates for industrial applications and drug delivery.[2] Similarly, diverse functional properties including wetting, emulsification, foaming, antimicrobial and anti-adhesive activities make them more attractive. These high molecular-weight bioemulsifiers are polymers of proteins, polysaccharides and lipid, such as glycoprotein, lipopolysaccharides and lipoproteins.[3] Bioremediation involving the acceleration of natural biodegradation processes in oil contaminated environments is accomplished strategically mainly by biostimulation and bioaugmentation. Diverse groups of microbes produce bioemulsifiers with very different chemical structures and surface properties. The low molecular weight molecules efficiently lower surface tension and interfacial tension, while the high molecular weight polymers bind tightly to surfaces. Rhamnolipids produced by *Pseudomonas* species, trehalolipids of *Rhodococcus*, *Arthrobacter*, *Mycobacterium* and sophorolipids by *Candida* and *Torulopsis*, all belong to the first group of bioemulsifier. Second group consists of high molecular weight biopolymers with high emulsification activity even at low concentration along with remarkable substrate specificity. Polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixture of these biopolymers belongs to the second group.[4] Herein we report the isolation, characterization, identification of bioemulsifying bacteria from oil contaminated soil. Further we report detail process optimization for maximum bioemulsifier production.

MATERIALS AND METHODS

Isolation, characterization and identification

One gram of oil contaminated soil from garage and temple from Pune, India was suspended in 10 mL of sterile saline separately and incubated at room temperature under shaking

condition for 1 h. 0.1 mL of the above suspensions were inoculated in 10 mL of sterile Luria Bertani broth which were further incubated for 24 h at 30 °C at 120 rpm. The cultures were then serially diluted and 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread on sterile Luria agar and incubated for 24 h at 30 °C. On the basis of distinct colony characteristics isolation was carried out. Bioemulsifier producing isolates were identified using standard Biomérieux VITEK® 2 System.

Screening for bioemulsifier production

Screening for bioemulsifier production was performed by oil spreading assay as per method described by Morikawa et al.[5] In brief overnight grown cultures were inoculated in minimal medium containing 1% of different oils like engine oil, coconut oil, groundnut oil and mustard oil and incubated at 30°C for 48 h under shaking condition. Thereafter culture broths were centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to oil spreading assay. 20 mL of distilled water was added to a Petri dish followed by addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free supernatant of each culture was then added to the oil surface. The time taken for displacement of oil with oil free clearing zone was noted which indicates the presence of bioemulsifier in the cell free broth. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and sodium dodecyl sulphate (SDS) was used as the positive control.

Emulsification index

The cultures positive for oil spreading assay were induced for bioemulsifier production by supplementing 2% engine oil as sole carbon source in the growth medium. After 4 days of incubation under the above mentioned culture condition the culture supernatant was recovered and emulsification index was evaluated against four different oils. The emulsification capacity was determined by adding 2 mL of engine oil, coconut oil, groundnut oil and mustard oil separately to the same amount of cell free culture broth, mixed for 2 minutes on vortex mixer which was then allowed to stand for 24 hour. E24 index is defined as the percentage of height of emulsified layer divided by the total height of liquid column.[6]

Emulsification activity

The isolates positive for oil spreading assay were further checked for emulsification activity. Supernatant of the cultures induced as earlier for the bioemulsifier production were subjected to emulsification activity.[1] In short, 3mL of cell free supernatant was vortexed vigorously

with 0.5 mL of engine oil, coconut oil, groundnut oil and mustard oil, separately for 2 minutes and incubated at 30°C for one hour for phase separation. After removal of aqueous phase carefully, its absorbance was recorded at 400 nm. Appropriate blank was prepared similarly by replacing the cell free supernatant by sterile medium. An absorbance of 0.010 units at 400 nm multiplied by dilution factor, was considered as one unit of emulsification activity per ml (EU/mL).

Media optimization for bioemulsifier production

In order to assess the role of various parameters on the bioemulsifier production the optimization was carried out using minimal media 1 (3 g/L NH_4Cl, 3 g/L NaCl, 0.4 g/L K_2HPO_4) and minimal media 2 (3 g/L K_2HPO_4, 2 g/L KH_2PO_4, 3 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$) with 1% of engine oil. The effect of most critical parameters on bioemulsifier production was studied using the Plackett–Burman design.

RESULTS

Characterization and identification

Nine bacteria were isolated and sub cultured altogether from both garage soil and temple soil based on their colony characteristics (Table 1). Seven bacteria were found to be Gram negative while two were Gram positive. Except GS1 all other bacteria were found to be motile. GS1, GS2, GS3 and TS2 were short rods. TS4 and TS5 were Gram negative motile rods with raised cream colour colony on Luria Bertani Agar medium. Among nine cultures two cultures showing most superior bioemulsifier production, were identified using standard Biomérieux VITEK® 2 System. According to the biochemical test data (Table 2) the cultures were identified as *Enterobacter cloacae* with 99% similarity which were assigned the name *E. cloacae* TS4 and *E. cloacae* TS5 and represented likewise throughout the manuscript.

Table 1: Colony characteristics of the isolated bacteria.

Characters	GS1	GS2	GS3	GS4	TS1	TS2	TS3	TS4	TS5
Size	1mm	1mm	2mm	1mm	1mm	1mm	1mm	1mm	1mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Colour	Cream	Cream	Cream	Cream	Faint yellow	Faint yellow	Milky white	Cream	Cream
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Opacity	Opaque	Translucent	Translucent	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Elevation	Flat	Convex	Convex	Convex	Convex	Convex	Convex	Raised	Raised
Gram Character	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative rods	Gram positive cocci	Gram negative short rods	Gram positive cocci	Gram negative rods	Gram negative rods
Motility	Non motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile

Table 2: Identification using the Biomérieux VITEK® 2 System.

Sr.no.	Biochemical	TS4	TS5	Sr.no.	Biochemical	TS4	TS5
1	Ala-Phe-Pro-ARYLAMIDASE	(-)	(-)	25	SACCHAROSE/SUCROSE	(+)	(+)
2	ADONITOL	(-)	(-)	26	D- TAGATOSE	(-)	(-)
3	L-Pyrrolydonyl-ARYLAMIDASE	(-)	(-)	27	D-TREHALOSE	(+)	(+)
4	L-ARABITOL	(-)	(-)	28	CITRATE(SODIUM)	(+)	(+)
5	D-CELLOBIOSE	(+)	(+)	29	MALONATE	(+)	(+)
6	BETA-GALACTOSIDASE	(+)	(+)	30	5-KETO-D-GLUCONATE	(-)	(-)
7	H2S production	(-)	(-)	31	L-LACTATE alkalinization	(+)	(+)
8	BETA-N-ACETYL-GLUCOSAMINIDASE	(+)	(+)	32	ALPHA-GLUCOSIDASE	(-)	(-)
9	Glutamyl Arylamidase Pna	(-)	(-)	33	SUCCINATE alkalinization	(+)	(+)
10	D-GLUCOSE	(+)	(+)	34	Beta-N-ACETYL-GALACTOSAMINIDASE	(+)	(+)
11	GAMMA-GLUTAMYL-TRANSFERASE	(+)	(+)	35	ALPHA-GALACTOSIDASE	(+)	(+)
12	FERMENTATION/GLUCOSE	(+)	(+)	36	PHOSPHATASE	(+)	(+)
13	BETA-GLUCOSE	(+)	(+)	37	Glycine ARYLAMIDASE	(+)	(+)
14	D-MALTOSE	(+)	(+)	38	ORNITHINE DECARBOXYLASE	(+)	(+)
15	D-MANITOL	(+)	(+)	39	LYSINE DECARBOXYLASE	(-)	(-)
16	D-MANNOSE	(+)	(+)	40	L-HISTIDINE assimilation	(-)	(-)
17	BETA-XYLOSIDASE	(+)	(+)	41	COURMARATE	(-)	(-)
18	BETA-Alanine arylamidase Pna	(-)	(-)	42	BETA-GLUCURONIDASE	(-)	(-)
19	L-Proline ARYLAMIDASE	(-)	(+)	43	O/129 RESISTANCE (comp.vibrio.)	(+)	(+)
20	LIPASE	(-)	(-)	44	Glu-Gly-Arg-ARYLAMIDASE	(-)	(-)
21	PALATINOSE	(+)	(+)	45	L-MALATE assimalation	(-)	(-)
22	Tyrosine ARYLAMIDASE	(+)	(+)	46	ELLMAN	(-)	(-)
23	UREASE	(+)	(+)	47	L-LACTATE assimalation	(-)	(-)
24	D- SORBITOL	(+)	(+)				

Screening for bioemulsifier production

In order to assess the most suitable hydrocarbon for induction of bioemulsifier production all nine cultures were incubated separately with four different oils as sole carbon source (Table 3). It was observed that all cultures except GS1 and TS1 showed excellent result with engine oil. GS4, TS4 and TS5 took least time for oil spreading indicating the production of maximum bioemulsifier. However, GS1 and TS1 showed positive result with groundnut oil and mustard oil. Coconut oil could induce bioemulsifier production in five cultures while

groundnut oil and mustard oil induced seven and six cultures, respectively for bioemulsifier production as evident from the oil spreading assay. Further, the positive cultures with high bioemulsifier producing potential were checked for the emulsification index and emulsification activity.

Table 3: Screening for bioemulsifier production by oil spreading assay.

Isolates	Engine oil		Coconut oil		Groundnut oil		Mustard oil	
	(+/-)	Time(s)	(+/-)	Time(s)	(+/-)	Time(s)	(+/-)	Time(s)
GS1	(-)	18	(-)	(-)	(+)	10	(+)	1
GS2	(+)	10	(+)	43	(+)	82	(+)	24
GS3	(+)	9	(+)	120	(+)	28	(-)	(-)
GS4	(+)	4	(-)	(-)	(+)	23	(+)	30
TS1	(-)	(-)	(-)	(-)	(+)	25	(+)	121
TS2	(+)	128	(+)	95	(-)	(-)	(-)	(-)
TS3	(+)	82	(+)	111	(-)	(-)	(-)	(-)
TS4	(+)	8	(+)	29	(+)	12	(+)	11
TS5	(+)	8	(-)	(-)	(+)	12	(+)	37

Emulsification index

Among the five isolates, TS4 and TS5 showed excellent bioemulsifier production as reflected by the emulsification index (Figure 1). TS4 showed emulsification index up to 83.3 % with engine oil followed by 66.7 % with coconut oil. TS4 showed 56 % emulsification activity with both groundnut and mustard oil. Similarly TS5 also showed high emulsification activity up to 88.9 %, 74.1 %, 60% and 55.6% with mustard oil, coconut oil, engine oil and groundnut oil, respectively. Both GS2 and GS3 showed 70% emulsification activity with engine oil. GS3 exhibited higher activity with coconut and engine oil compared to groundnut and mustard oil.

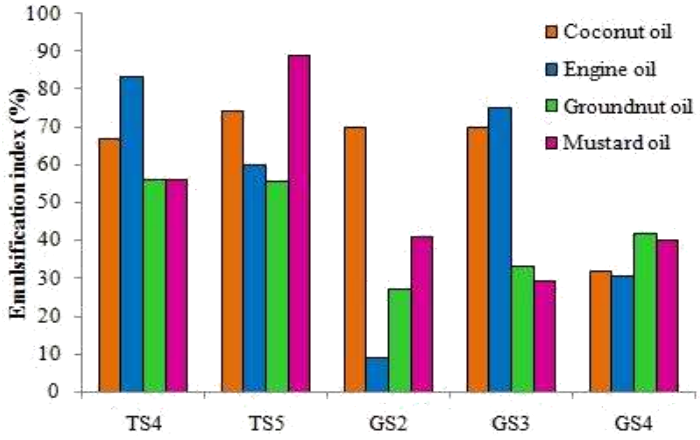

Figure 1: Emulsification index of superior bioemulsifier producing cultures.

Emulsification activity

TS5 showed the highest emulsification activity against all 4 oils. It showed 37.59 EU/mL, 35.71 EU/mL, 33.87 EU/mL and 23.48 EU/mL with engine oil, mustard oil, coconut oil and groundnut oil, respectively. Similarly, TS4 showed 27.64 EU/mL, 22.77 EU/mL, 15.23 EU/mL and 8.93 EU/mL with coconut oil, mustard oil, engine oil and groundnut oil, respectively. GS4 showed higher activity with mustard oil (24.22 EU/mL) and coconut oil (22.53 EU/mL) while GS3 exhibited almost identical activity with mustard oil (19.65 EU/mL) and coconut oil (18.58 EU/mL). GS2 showed comparable activity with all three edible oils, namely coconut, groundnut and mustard oil.

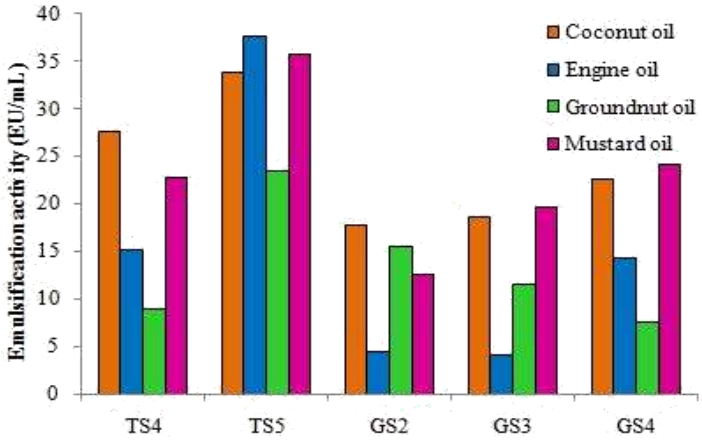

Figure 2: Emulsification activity of superior bioemulsifier producing cultures.

Media optimization of bioemulsifier production

Optimization studies revealed that among various parameters like media components, carbon source (oil), temperature, pH, aeration and volume, K_2HPO_4 and hydrocarbon (engine oil) concentration played a major role in bioemulsifier production by TS4 in minimal medium 1. On the other hand all other variables showed negligible effects towards bioemulsifier production by TS4 in minimal medium 2. However, K_2HPO_4, $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ exhibited very significant role in bioemulsifier production by TS5 in minimal medium 1. A similar trend comparable to TS4 was observed in case of bioemulsifier production by TS5 in minimal medium 2.

Table 4: Media optimization by Plackett–Burman design.

Trials	A	B	C	D	E	F	G	TS4 (EU/mL)		TS5 (EU/mL)	
								MM1	MM2	MM1	MM2
1	H	H	H	L	H	L	L	8.4	9.6	11.2	22.3
2	L	H	H	H	L	H	L	1.34	10.63	6.14	22.41
3	L	L	H	H	H	L	H	2.62	11.01	7.51	28.33
4	H	L	L	H	H	H	L	4.75	10.51	10.08	27.08
5	L	H	L	L	H	H	H	5.11	10.1	10.7	27.23
6	H	L	H	L	L	H	H	3.18	14.64	11.92	29.24
7	H	H	L	H	L	L	H	3.36	13.45	12.5	28.84
8	L	L	L	L	L	L	L	4.95	12.57	12.92	29.24

Note: MM1=Minimal medium 1; MM2=Minimal medium 2

DISCUSSION

Microbial synthesis of bioemulsifier is advantageous and environmentally friendly approach. Among the nine cultures isolated from the oil contaminated soil two were found to be most potent producers of bioemulsifier. TS4 and TS5 identified as *E. cloacae* might be producing bioemulsifier to disperse the growth substrate into oil in water emulsion in order to increase the interfacial area. This in turn enhances the availability of carbon source as reported for *Pseudomonas nautica.*[7] Adherence and emulsification are considered as two principle modes allowing the transfer of hydrocarbon to the cell surface. Such molecules from bacteria are reported to possess antiviral, antitumor, hemolytic, blood anticoagulant, and fibrinolytic activities apart from other numerous environmental and biotechnological applications. They are also reported to play significant role in oil recovery, remediation of soil contaminated by heavy metals, and biocontrol against phytopathogens and insects.[8] Solid waste oil samples are previously reported as rich source of diverse bacteria like *Bacillus pumilus, Bacillus subtilis, Micrococcus luteus, Alcaligenes faecalis* and *Enterobacter* sp, some of which could emulsify octane, xylene, toluene, mineral oil and crude oil indicating their ability to remove hydrocarbons which has a promising application in bioremediation technologies.[9] Both the bacteria in our study showed efficient bioemulsifier production when induced by engine oil.

Although induction was best by engine oil, the synthesized bioemulsifier could efficiently emulsify edible oils like coconut, groundnut and mustard oil, probably due to ease of internalization, biodegradation and intracellular metabolism. Our results are in close agreement with the earlier reports where *E. cloacae* derived bioemulsifier effectively emulsified corn oil.[10] Similarly, salt tolerant *E. cloacae* mutant was reported for bioaugmentation of petroleum and salt contaminated soil which will ultimately enhance the

bioavailability of oil contaminates and thus enhance the bioremediation process.[11] *E. cloacae* strain TU is also known to secrete an exopolysaccharide bioemulsifier for degradation of hexadecane.[12] Further, genetically engineered *E. cloacae* strain produces exopolysaccharide for microbial enhanced oil recovery.[13] Such bioemulsifiers are of utmost importance as till date, more than 225 patents are available describing these microbial amphiphilic agents, e.g., Emulsan which are extensively used for commercial applications, particularly, in the bioremediation sector.[14] Hence, our study will enable to treat and address soil and water contamination due to accidental spillage of large quantities of crude oil, hydrocarbons, petroleum oil products and halogenated compounds, which are otherwise detrimental to the environment.[15]

CONCLUSIONS

Bioemulsifier producing bacteria were isolated and characterized from garage and temple soils which were identified as *E. cloacae*. The cultures showed superior bioemulsification against coconut oil, engine oil, groundnut oil and mustard oil in terms of emulsification index and emulsification activity. Further optimization studies using Plackett Burman design indicated the role of salts and oil in the bioemulsifier production.

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