Isolation of Crude Oil Degrading Marine Bacteria and Assessment for Biosurfactant Production

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Abstract

Ten bacterial isolates recovered from the crude oil contaminated sea water samples from a ship yard in Alang coast, Gujarat showed optimum growth in presence of crude oil. The crude oil degrading isolates were identified as species of genera Acinetobacter, Marinococcus, Micrococcus, Planococcus, Methylobacterium, Rhodococcus and Noccardia. All the ten isolates degraded 1% crude oil in Bushnell Haas broth. Highest biodegradation of crude oil was found to be 70% by the isolate M2 which belonged to genus Marinococcus. Other isolates showed biodegradation in the range of 55-30%. On addition of 1% Tween 80 biodegradation reduced in majority of cases. 70% of the bacterial isolates scored positive for biosurfactant production and emulsified toluene however emulsion stability varied drastically among the isolates. None of the biosurfactant producing bacterial isolates was hemolytic.Key words: Biodegradation, Biosurfactant, Emulsion, Hemolytic.

INTRODUCTION

In quantitative terms, crude oil is one of the most important organic pollutants in marine environment and it has been estimated that worldwide somewhere between 1.7- 8.8×10^6 tons of petroleum hydrocarbons impact marine waters and estuaries annually (1). Reports have been appearing since last three decades on the biodegradability of crude oil by bacteria which can use hydrocarbons as source of carbon and energy (2). When micro organisms grow in environment rich in hydrocarbon, they undergo many adaptations. One such adaptation is biosurfactant production which is a frequently encountered feature in hydrocarbon degrading bacteria or sometimes even a prerequisite for growth on hydrocarbons (3). Biosurfactant production helps the hydrocarbon degrading bacterium to gain better access to their hydrophobic substrates as it brings about changes like reduction of surface tension of the environment around the bacterium, reduction of interfacial tension between bacterial cell wall and hydrocarbon molecules, membrane modifications like increasing the hydrophobicity of cell wall by reducing the lipopolysaccharide content of cell wall, enhancing the dispersion of hydrocarbon by encapsulation of the hydrocarbon into micelles etc (4,5,6,7). Amongst the above changes reduction in surface and interfacial tension is a universal phenomenon displayed by almost all types of biosurfactant whereas changes like membrane modifications and emulsion formation strictly depends upon type of biosurfactant for e.g. glycolipids, lipopepetides, polymeric or particulate etc. Enhancement in hydrocarbon degradation may occur by cumulative effect of above changes.

Here we present a report on isolation of crude oil degrading marine bacteria from oil contaminated sea water, their screening for crude oil degradation and biosurfactant production.

METHODS

COLLECTION OF SAMPLE

Sea water samples were collected in sterilized glass bottles from a ship yard at Alang coast, Gujarat.

ISOLATION AND SCREENING OF CRUDE OIL DEGRADING BACTERIA

Isolation of crude oil degrading marine bacteria was carried out by spreading 100 µL of serially diluted seawater samples on mineral salt medium (MM2). An ethereal solution of crude oil (10% w/v) was uniformly sprayed over the surface of agar plate. The ether immediately vaporized and thin layer of oil remained on the entire surface. The crude oil was obtained from ONGC plant at Chandkheda near Ahmedabad, Gujarat. The plates were incubated at 25°C for 20 days. The bacterial isolates which appeared on MM2 plate after incubation were screened for crude oil degradation by overlay technique (8).

CRUDE OIL BIODEGRADATION

Crude oil biodegradation experiment was performed by modifying the technique described by Pirnik et al. (9) by adding the inoculating cells of density 10^8 mL^{-1} to BHM with 1% crude oil added as sole carbon source. To study effect of Tween 80 on biodegradation of crude oil a similar set of experiment as described was performed with 1% Tween 80 added to all the flasks (10). The estimation of crude oil degradation was accomplished by Gravimetric analysis. The residual crude oil was extracted in a preweighed flask with hexane in a seperating funnel. Extraction was repeated twice to ensure complete extraction. After extraction hexane was evaporated in a hot air oven at 68-70^{o}C, the beaker cooled down in a desiccator and weighed. The % degradation was calculated as follows (11):

Weight of residual crude oil = Weight of beaker containing extracted crude oil – Weight of empty beaker

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

% degradation = Amount of crude oil degraded/ amount of crude oil added in the media × 100

BIOSURFACTANT PRODUCTION

All the isolates obtained through overlay technique were screened for biosurfactant production in mineral medium added with 2% glucose as carbon source and incubating for 7 days in shaking condition at room temperature. After incubation media was centrifuged at 15,000 rpm for 10 min to obtain a cell free supernatant. The culture supernatant was tested for the presence of biosurfactant by the Drop collapsing assay as described by Bodour and Miller (12). Mineral oil was used in place of Pennzoil® as described in the report. The isolates which scored positive in the drop collapsing assay were checked for emulsification activity (13). Emulsification activity (%) was calculated as follows:

Emulsification activity = Height of the emulsion layer/ Total height of mixture × 100

Oil displacement assay was performed in which 15 µL of crude oil was placed on the top of 40 mL of distilled water in a 150 mm diameter petri plate. Then 10 µL of culture supernatant was gently added to the centre of the oil film. The diameter of the halo formed in the middle was measured after 30 sec (14).The bacterial isolates were also checked for haemolytic activity of biosurfactants on blood agar prepared by adding 5% v/v human blood to the blood agar base. Stab inoculation was done at the centre of the agar to check the haemolysis activity (15).

STATISTICAL ANALYSIS

The statistical analysis was performed using MS office Excel 2003 for calculating mean, standard deviation and standard error.

RESULTS AND OBSERVATION

ISOLATION AND SCREENING OF CRUDE OIL DEGRADING BACTERIA

Both fungal and bacterial colonies were observed on MM2 agar plate after incubation of 20 days at 25C on which contaminated sea water sample was spread and sprayed with ethereal solution of crude oil, only bacterial colonies were chosen for the study. A total of eighteen bacterial isolates could be distinguished on the basis of colony morphology and colour. Ten out of eighteen isolates showed profuse growth on the overlay plates and were considered crude oil degraders.

ESTIMATION OF CRUDE OIL BIODEGRADATION

The results from control flasks indicated 15% abiotic loss of crude oil from the medium. Highest crude oil biodegradation was observed with isolate M2 (70%) followed by M1 and M8, both degrading 55% of the added 1% crude oil in the medium. A comparatively lower biodegradation of 40-50% was

found in isolates M3, M4, M5, M6 and M7. Least biodegradation of 30% was recorded with M9 and M10.

The effect of 1% Tween 80 on biodegradation of crude oil (1%) by the bacterial isolates varied drastically. On addition of 1% Tween 80 highest increase in biodegradation was shown by M9 which was otherwise the least efficient degrader. Biodegradation among other isolates in presence of Tween 80 ranged from 25-55%. Enhancement in crude oil biodegradation was observed only with two other isolates namely, M3 and M10. In these isolates the increase in biodegradation was 37 and 50% respectively. However, most of the isolates showed decrease in biodegradation in the range of 10 to 44% after addition of Tween 80. Isolate M8 was indifferent to presence of Tween 80 in this regard (Table 1).

Figure 1

Table 1: Crude oil degradation by bacterial isolates

Bacterial isolate	Crude oil degradation (%)	
	With Tween 80	Without Tween 80
M1	50*	55
M2	45	70
M3	55	40
M4	40	50
M5	35	40
M6	45	50
M7	25	45
M8	55	55
M9	65	30
M10	45	30

* Values represent mean of triplicates

BIOSURFACTANT PRODUCTION

Seven out of ten isolates scored positive for biosurfactant production in drop collapsing assay. On addition of the culture supernatant of isolate M2, M3 and M8 on mineral oil, beaded drops were observed even after 24 hrs indicating lack of biosurfactant production. In all other isolates addition of culture supernatant on the mineral oil lead to formation of flat drop after 60 sec and/or 24 hrs varying according to the isolate and therefore they were considered biosurfactant producers (Table 2).

Figure 2

Table 2: Biosurfactant studies on bacterial isolates

Bacterial isolate	Biosurfactant production	%Emulsification		Area of the halo (cm^2)
		10 min	24 hrs	
M1	+	56.75±0.40*	10.65±0.30	1.275±0.002*
M2	–	NA	NA	NA
M3	–	NA	NA	NA
M4	+	68.23±0.40	66.75±0.18	0.26±0.003
M5	+	47.75±0.46	6.5±0.17	0.14±0.003
M6	+	68.7±0.35	68.46±0.17	0.34±0.001
M7	+	63.85±0.30	65.65±0.35	0.275±0.004
M8	–	NA	NA	NA
M9	+	49.35±0.17	Emulsion disappears	No oil displacement observed
M10	+	62.44±0.27	14.9±0.25	0.37±0.002

* Mean values indicated with standard error

NA: Not Applicable

Each of the seven isolates which scored positive for biosurfactant production also emulsified toluene but to a varying extent. Not all the emulsions formed by these isolates were stable after 24 hrs. Highest emulsifying index of 68% was observed with M6 after 10 min which remained stable after 24 hours. Similarly isolates M4 and M7 registered stable emulsification activity of 68.23% and 63.85% respectively. Emulsification activity of isolates M1, M5 and M10 was 56.75%, 47.75% and 62.44% respectively after 10 min which drastically declined after 24 hrs. M9 showed emulsification activity of 49.35% only after 10 minutes while M2 did not show any such activity.

The seven biosurfactant producers tested for oil displacement activity did not yield appreciable activity. No displacement activity was observed in M9, despite scoring

positive for production of biosurfactant and emulsification of toluene (Table 2). Biosurfactant produced by none of the isolates had hemolytic properties. This was evident as no zone of clearance was observed in the blood agar plates inoculated with the isolates

CHARACTERIZATION OF THE BACTERIAL ISOLATES

The ten isolates recovered from oil contaminated sea water varied in their characteristics. There were similarities observed in colony morphology and pigmentation of few isolates but they differed in biochemical properties (Table 3). Isolates like M3, M5 and M6 were found to have similar colony morphology and pigmentation. Likewise, M7 and M10 were also similar in appearance, shape and color. Cocci and coccobacilli were the dominant cell morphology and most of the isolates were Gram positive. Only two isolates namely M1 and M8 were gram negative. Pigmentation of colonies varied from yellow, orange, and pink to beige.

Figure 3

Table 3: Biochemical characteristics of the bacterial isolates

Isolate Biochemical characteristics	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
Gram reaction	–	+	+	+	+	+	+	–	+	+
Dextrose fermentation	+	+	–	–	–	–	–	–	+	+
Sucrose fermentation	+	–	–	–	–	+	–	–	+	–
Lactose fermentation	–	–	–	–	–	+	+	–	–	–
Motility	–	+	+	–	+	+	–	–	–	–
TSI	+	–	–	–	–	+	–	–	+	+
Starch hydrolysis	+	+	–	+	+	+	–	–	+	–
Gelatin hydrolysis	+	+	–	–	+	–	–	+	+	+
Oxidase	–	–	+	–	+	–	–	–	–	–
Lysine decarboxylation	–	+	+	+	+	+	+	+	+	+
Phenylalanine deamination	–	–	–	–	–	–	–	–	–	–
Citrate utilization	–	+	–	+	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–	–	+
Methyl red test	–	+	–	–	–	+	–	–	–	+
Voges prausker tst	+	+	+	+	+	+	+	+	+	+

DISCUSSION

The appearance of colonies on the MM2 agar plate sprayed with ethereal solution of crude oil showed that contaminated sea water at ship yard in Alang coast harboured crude oil degrading bacteria. The bacterial isolates were designated as crude oil utilizers (16).

The overlay technique also confirmed that hydrocarbon degrading bacteria were ubiquitously present; their population size might be small in non-polluted area but in the hydrocarbon polluted area like the ship yards where crude oil pollution is common the population of crude oil degraders was dominating (17). Ten out of eighteen isolates showed profuse growth on screening through overlay technique.

Colonies of most of the isolates were mucoid and fused together in dense growth areas. This might be because of the exopolysaccharide production which leads to mucoid colony morphology. It has been reported that there is a close relationship between mucoid colony morphology and the ability to grow on crude oil. (18). The biodegradation of crude oil by bacterial isolates was on a very wide scale. Where on one hand 70% of crude oil was degraded by isolate M2, the isolates M9 and M10 degraded only 30% of the added crude oil. This might account for the varying ability of the isolates to survive in a single concentration of crude oil (17). The 1% crude oil added to the medium might be higher then the tolerance limit of M9, M10, M3 and M5 thus slowing down their growth and hence biodegradation, whereas the same concentration might not be high enough to affect the growth of the other isolates negatively and hence they could degrade it efficiently in the range of 50-70% (Table 1).

On addition of 1% Tween 80 the results obtained in case of bacterial isolates namely M10, M3 and M9 were in agreement with the published reports as degradation of crude oil was enhanced in the media of these isolates (19). The enhancement could be owed to the reduction of surface tension of the media and interfacial tension between hydrocarbon and cell surface by Tween 80 being a chemical surfactant (20). But there was decrease as well in degradation of crude oil on addition of 1% Tween 80 in most of the bacterial isolates namely, M2, M5, M4, M7, M1 and M6 which may be due to the toxic effects of Tween 80 on bacterial cells. Tween 80 beyond a certain concentration is poisnous to bacteria (21). So probably concentration of Tween 80 added in media i.e. 1% was inhibitory for these isolates retarding their growth and hence the degradation of crude oil.

70% of the bacterial isolates showed biosurfactant producing ability through drop collapsing assay, emulsification of

toluene and oil displacement assay (Table 2). This accounts for the natural adaptation of biosurfactant production in many hydrocarbon degrading bacteria for better bioavailability of their substrates as hydrocarbons are not easily soluble being hydrophobic (20).

The supernatant obtained on centrifugation of the media contained biosurfactant and supernatant of few biosurfactant producing isolates formed good emulsion with toluene. In almost all isolates the emulsification activity was greater then 50-55% which accounts for good emulsification. However most of the emulsions with toluene were not stable as emulsification activity reduced in most of the isolates in 24 hrs. Differences in emulsification indices reflected different interactions among biosurfactant and hydrocarbon which explained why emulsions of some isolates with toluene were more stable than others. This observation emphasizes upon selection of specific biosurfactant for particular hydrocarbon pollution (22). The results of oil displacement assay indicated a very feeble activity from most of the isolates (Table 2). This could be attributed to the very low concentration of biosurfactant in the supernatant, as this assay is sensitive to as low as 10 nmol of biosurfactant concentration (14).

In our study the results of biosurfactant production could not be correlated to crude oil degradation in all the isolates as the observations made were contradictory. The highest crude oil degrader, M2 did not produce detectable levels of biosurfactant. This result is not in agreement with the published reports which say that biosurfactant producing bacteria are efficient crude oil degraders as well (23). The mode of hydrocarbon uptake is different for different bacteria and biosurfactants mostly enhance the attachment of the hydrocarbon to the substrate .Thus if biosurfactant production does not augment the mode of hydrocarbon uptake by the cell it may not always ensure enhanced biodegradation. Another reason might be that biosurfactants have been mostly reported to desorb the hydrocarbons from soil in mesocosm studies making them more and more bioavailable to the microorganisms in the soil but in experimental liquid media where hydrocarbons are easily available to the bacteria biosurfactants may not have any significant role to play (24).

In the hemolysis assay, none of the isolate gave clear zone on the blood agar. This may be because the biosurfactants falling under the category of lipopeptides and lipoproteins mainly show the property of hemolysis of mammalian blood so none of the bacterial isolates might be producing biosurfactant belonging to this category (6).

On the basis of colony morphology, staining and biochemical characteristics M1 was a member of genus Acinetobacter. Similarly M2 and M6 belonged to genera Marinococcus but they differed at species level due to differences in biochemical properties and pigmentation. Isolate M5 belonged to Micrococcus genera, while M3 was identified as Planococcus. Both M4 and M9 belonged to genus Rhodococcus. M8 was Methylobacterium, M7 and M10 due to their distinct colony morphology and biochemical features were similar with members of Noccardia genus (25, 26 and 27).

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