International Journal of Research in BioSciences
Vol. 2 Issue 4, pp. (10-16), Oct 2013
Available online at http://www.ijrbs.in
ISSN 2319-2844

Research Paper

Isolation and screening of hydrocarbon degrading bacteria from soil near Kadi (Gujarat) region

Patel Nilesh K.* **and Pethapara Hardik**
Dept. of Biotechnology, Kadi sarva vishwavidhyalaya, Gandhinagar, Gujarat, INDIA

(Received July 29, 2013, Accepted September 30, 2013)

Abstract

Ten bacterial isolates were recovered from the Hydrocarbons contaminated soil samples from an ONGC well and petrol pump near Kadi region, Gujarat. These organisms showed optimum growth in presence of hydrocarbons like crude oil, Diesel, Kerosene and 2T oil. The Hydrocarbons degrading isolates were identified as species of genera *Acinetobacter, Micrococcus, Methylobacterium, Pseudomonas sp, Rhodococcus and Noccardia.* All the ten isolates degraded 1% Hydrocarbons (Petrol, Diesel, Kerosene and 2T oil) in Bushnell Haas broth. Highest biodegradation of Hydrocarbon was found to be 70% by the isolate KD1 which belonged to genus *Pseudomonas sp.* Other isolates showed biodegradation in the range of 30%-55%. On addition of 1% Tween 80 biodegradation reduced in majority of cases.

Keywords: Hydrocarbons, Biodegradation, Crude oil, Tween 80, Bushnell Haas broth.

Introduction

In quantitative terms, hydrocarbons (Crude oil, Diesel, Kerosene, and 2T oil) are one of the most important organic pollutants in land and it has been estimated that worldwide somewhere tons of petroleum hydrocarbons impact land annually [5]. Reports have been appearing since last three decades on the biodegradability of hydrocarbons (crude oil, Diesel, Kerosene, and 2T oil) by bacteria which can use hydrocarbons as source of carbon and energy [7]. 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 [13]. 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 [1,2,4,15].

The objective of this study were focused on isolation and screening of hydrocarbons degrading bacteria from oil contaminated soil and their characterization in terms of hydrocarbon degrading ability in presence and absence of detergent tween 80.

Materials and Methods

Collection of sample

Soil samples were collected from three different oil contaminated sites: (A) ONGC well, near kadi (B) Rajshri Garage, kadi (C) Keshav petrol Pump, kadi thol road. Soil was collected randomly 5-10 cm beneath the surface using spatula and were packed in sterile polybags and transferred to the laboratories. Used Engine Oil: Collected from Rajshri Garage, kadi.

Isolation and screening of crude oil degrading bacteria

Isolation of hydrocarbon degrading bacteria was carried out by spreading 100 µL of serially diluted soil samples in sterile water on six different plates containing tri butyrins agar (TBA). 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 KADI village near Maheshana, Gujarat. The plates were incubated at 25^{o}C for 20 days. The bacterial isolates which appeared on TBA plate after incubation were screened for crude oil degradation by overlay technique [3].

Hydrocarbon biodegradation

Hydrocarbon biodegradation experiment was performed by modifying the technique described by Pirnik *et al*. [11] by adding the inoculating cells of density 10^{8} mL^{-1} to BH (Bushnell Haas) with 1% hydrocarbons like crude oil, Diesel, Kerosene, 2T oil added as sole carbon source. To study effect of Tween 80 on biodegradation of crude oil or hydrocarbons a similar set of experiment as described was performed with 1% Tween 80 added to all the flasks [10]. The estimation of hydrocarbon degradation was accomplished spectrophotometer method. The flacks media were centrifuged and supernatant was measured at 410 nm and growth of bacteria was found that showing degradation of hydrocarbons.

Biochemical test for identification of bacteria:

Citrate Utilization Test

Citrate utilization test was carried out to detect the ability of bacteria to use citrate as the sole source of carbon and energy. For this test Simmon's Citrate agar slants were inoculated with bacterial culture and incubated at 18 to 24 hours. Positive results gave growth was visible on the slant and the medium turns in Prussian blue. Negative results gave no growth was visible and No color change in Simmon's Citrate agar mediam[14].

Indol utilization test

Bacteria that produce the enzyme tryptophanase can convert the amino acid tryptophan to by-products that include indole. This test is among a suite of tests (Indole, Methyl-Red, Vogues-Proskauer, and Citrate) that are used to differentiate among the Gram-Negative bacilli in the family Enterobacteriaceae. When indole is combined with Kovac's Reagent (which contains hydrochloric acid and dimethylaminobenzaldehyde in amyl alcohol) the solution turns from yellow to cherry red. Because amyl alcohol is not water soluble, the red coloration will form in an oily layer at the top of the broth [14].

Vogues-Proskauer test (VP Test)

If glucose is fermented by the butylenes glycol pathway, the main product is acetylmethyl-carbinol with small amounts of mixed acids. The resulting pH of the medium is not as acidic as in the mixed acid pathway, and the MR test is negative. The principle of the VP test is to detect the presence of

acetylmethylcarbinol. After adding alpha-naphthol and 40% KOH, the carbinol is converted to diacetyl, which is a red complex [14].

Methyl-Red test (MR Test)

If glucose is fermented via the mixed acid pathway, strong acids are produced and a low pH of 4.0 is maintained for at least 48 hours. This low pH is detected by adding MR, which turns yellow to red at pH 4.4 [14].

Triple Sugar Iron Test

Enzymatic Digest of Casein, Enzymatic Digest of Animal Tissue, and Yeast Enriched Peptone provide the nitrogen, carbon, and vitamins required for organism growth. Triple Sugar Iron Agar contains three carbohydrates, Dextrose, Lactose and Sucrose. When the carbohydrates are fermented, acid production is detected by the Phenol Red pH indicator. Sodium Thiosulfate is reduced to hydrogen sulfide, and hydrogen sulfide reacts with an iron salt yielding the typical black iron sulfide. Ferric Ammonium Citrate is the hydrogen sulfide (H_2S) indicator. Sodium Chloride maintains the osmotic balance of the medium. Agar is the solidifying agent [14].

Starch agar test

Beef extract provides the nitrogen, vitamins, carbon and amino acids in Starch Agar. Starch reacts with Gram Iodine to give a blue color. Organisms hydrolyzing starch through amylase production will produce a clearing around the isolate while the remaining medium is blue. Agar is the solidifying agent [14].

Catalase Test

Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Hydrogen peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell, because of this, any cell that uses O_2 or can live in the presence of O_2 must have a way to get rid of the peroxide. One of those ways is to make catalase [14].

Casein Hydrolysis

The enzyme caseinase is secreted out of the cells (an exoenzyme) into the surrounding media, catalyzing the breakdown of milk protein, called casein, into small peptides and individual amino acids which are then taken up by the organism for energy use or as building material. The hydrolysis reaction causes the milk agar, normally the opacity of real milk, to clear around the growth area as the casein protein is converted into soluble and transparent end products—small chains of amino acids, dipeptides, and polypeptides [14].

Tributyrin agar test (TBA)

Tributyrin agar is a differential medium that test the ability of an organism to producer an exoenzyme called lipase that hydrolyzes tributyrin oil. Lipases break down lipids. Tributyrin oil is a type of lipid called a triglyceride. Other lipase tests use different fat sources such as corn oil, olive oil, peanut oil, egg yolk, and soybean oil. Tributyrin oil forms an opaque suspension in the agar. When an organism produces lipase and breaks down the Tributyrin, a clear halo surrounds the areas where the lipase-producing organism has grown [14].

Results and Discussion

Isolation and screening of hydrocarbon degrading bacteria

Bacterial colonies were observed on TBA agar plate after incubation of 3 days at 37^0C on which contaminated soil sample was spread and sprayed with ethereal solution of crude oil, only bacterial colonies were chosen for the study. A total of twenty bacterial isolates could be distinguished on the basis of colony morphology and colour. Ten out of twenty isolates showed profuse growth on the overlay plates and were considered crude oil or hydrocarbons degraders.

Estimation of Hydrocarbon biodegradation

The results from control flasks indicated 15% abiotic loss of Hydrocarbons (crude oil, Diesel, Kerosene and 2T oil) from the medium. Highest Hydrocarbon biodegradation was observed with isolates KD1 (75%) followed by KD4 and KD7, both degrading 50% of the added 1% hydrocarbons (crude oil, Diesel, Kerosene and 2T oil) in the medium. A comparatively lower biodegradation of 40-50% was found in isolates KD2, KD9, KD5, KD6 and KD10. Least biodegradation of 30% was recorded with KD3and KD8.

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 KD1 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, KD2 and KD 7. 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).

Table 1: Hydrocarbon degradation by bacterial isolates

Bacterial Isolates	Hydrocarbons degradation (%)	
	Presence of Tween 80	Absence of Tween 80
KD1	70	80
KD2	40	70
KD3	50	35
KD4	30	35
KD5	20	29
KD6	35	55
KD7	30	50
KD8	45	45
KD9	70	60
KD10	45	35

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 2 & 3).All isolates have different colony morphology and pigmentation .KD1,KD2,KD3,KD4 and KD5 were shown a different colour pigmentation likewise Green, Yellow, Orange, Pink and Light Pink. KD 6, KD7, KD8, KD9 and KD10 do not shown any Pigmentation. Most of the isolates were Gram positive. Only four isolates namely KD1, KD6, KD7 and KD10 were gram negative.

The appearance of colonies on the tri butyrins agar (TBA) plate sprayed with solution of crude oil or hydrocarbon contaminated soil has a hydrocarbon degrading bacteria. The bacterial isolates were designated as hydrocarbon utilizes [8].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 area near ONGC well, Petrol Pump, Auto service center at the population of hydrocarbon degrading degraders was dominating [17]. Ten out of twenty two isolates showed profuse growth on screening through overlay technique.

Table 2: Characterization of the bacterial isolates

Isolates	Size	Shape	Margin	Elevation	Surface	Consistency	Opacity	Pigment	Gram reaction
KD1	Midium	Irregular	Erose	Flate	Smooth	Viscous	Opaque	Green	Gram Negative
KD2	Pinpoint	Round	Entire	Rasied	Smooth	Moist	Opaque	Yellow	Gram Positive
KD3	Small	Round	Entire	Rasied	Smooth	Viscous	Opaque	Orange	Gram Positive
KD4	Pinpoint	Irregular	Un-entire	Convex	Smooth	Moist	Opaque	Pink	Gram Positive
KD5	Big	Irregular	Entire	Convex	Smooth	Viscous	Opaque	Light orange	Gram Positive
KD6	Small	Regular	Entire	Convex	Smooth	Moist	Opaque	-	Gram Negative
KD7	Pinpoint	Regular	Entire	Convex	Smooth	Moist	Opaque	-	Gram Negative
KD8	Midium	Regular	Entire	Rasied	Smooth	Moist	Opaque	-	Gram Positive
KD9	Midium	Round	Un-entire	Convex	Smooth	Moist	Opaque	-	Gram Positive
KD10	Midium	Irregular	Un-entire	Convex	Smooth	Dry	Opaque	-	Gram Negative

Table 3: Biochemical characteristics of the bacterial isolates

Isolates	Citrate utilization test	Indol utilization test	TSI	VP test	Methyl Red test	Starch agar	Catalase	Casein Agar	TBA
KD1	+VE	-VE	-VE	-VE	+VE	-VE	+VE	+VE	+VE
KD2	-VE	-VE	+VE	-VE	-VE	+VE	+VE	+VE	+VE
KD3	-VE	-VE	-VE	+VE	+VE	+VE	+VE	+VE	+VE
KD4	-VE	-VE	-VE	-VE	-VE	+VE	-VE	+VE	+VE
KD5	-VE	-VE	+VE	+VE	+VE	+VE	+VE	-VE	+VE
KD6	-VE	-VE	-VE	+VE	-VE	+VE	+VE	+VE	+VE
KD7	+VE	-VE	-VE	+VE	-VE	+VE	+VE	+VE	+VE
KD8	+VE	-VE	+VE	-VE	+VE	+VE	+VE	+VE	+VE
KD9	-VE	-VE	+VE	-VE	+VE	+VE	+VE	+VE	+VE
KD10	-VE	-VE	-VE	-VE	+VE	+VE	+VE	+VE	+VE

Colonies of most of the isolates were mucoid or moister 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 hydrocarbons [17]. The biodegradation of hydrocarbons by bacterial isolates was on a very wide scale. Where on one hand 70% of hydrocarbons was degraded by isolate KD1, the isolates KD4 and KD7 degraded only 50% of the added hydrocarbond. This might account for the varying ability of the isolates to survive in a single concentration of crude oil [17]. The 1% hydrocarbons added to the medium might be higher than the tolerance limit of KD2, KD9, KD5, KD6 and KD10 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% .

On addition of 1% Tween 80 the bacterial isolates KD2, KD6 and KD7 increase their activity against hydrocarbons and it's degrades hydrocarbons faster than their original activity. Then the other isolates decrease their activity due to some toxic effect on the bacterial cell. So probably the concentration of Tween 80 was added (1%) in the media which affect on the growth of hydrocarbon bacteria and the rate of degradation of hydrocarbons.

On the basis of colony characters, morphology, staining and biochemical characteristics KD1 were a member of genus *Psudomonas.* Similarly KD3 and KD4 belonged to *Micrococcus* genera. KD2,KD5, KD7 were belonged *Acinetobacter* genera. They were different at spices level due to some different in biochemical properties. KD6 and KD8 belonged to *Noccardia* genera. KD9 and KD10 were belonged followed by *Rhodococcus* and *Methylobacterium* genera*.*

References

1. Al-Tahhan RA, Sandrin TR, Bodour AA, Maier RM, Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: Effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiology, 66 (8):3262-3268 **(2000).**

2. Barkay T, Navon–Venezia S, Ron EZ, Rosenberg E. Enhancement of solubilisation and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiology, 65 (6):2697-2702 **(1999).**

3. Bogardt AH, Hemmingsen BB. Enumeration of phenanthrene degrading bacteria by an overlayer technique and its use in evaluation of petroleum contaminated sites. Appl Environ Microbiology, 58 (8):2579-2582 **(1992).**

4. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev, 61(1):47-64 **(1997).**

5. Head IM, Swanell RPJ. Bioremediation of petroleum hydrocarbon contaminants in marine habitats. Curr Opin Biotechnol, 10(3): 234-239 **(1999).**

6. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinative bacteriology. 9th edition. Baltimore: Williams and Wilkins **(1994).**

7. Horowitz A, Gutnick D, Rosenberg E. Sequential growth of bacteria on crude oil. Appl Microbiology, 30(1):10-19 **(1975).**

8. Iwabuchi N, Sunairi M, Anzai H, Nakajima M, Harayama S. Relationships between colony morphotypes and oil tolerance in Rhodococcus rhodochrous. Appl Environ Microbiology, 66 (11):5073-5077 **(2000).**

9. Kiyohara H, Nagao K, Yana K. Rapid screening for bacteria degrading water insoluble solid hydrocarbon on agar plates. Appl Environ Microbiology, 43(2):454-457 **(1982).**

10. Krieg NR, Holt JG. Bergey's manual of systemic bacteriology. Vol. 1. Baltimore: Williams and Wilkins **(1984).**

11. Leahy JG, Colwell RR. Microbial Degradation of hydrocarbons in the environment. Microbiology Rev, 54(3):305-315 **(1990).**

12. Phillips GJM, Stewart JE. Effect of four dispersants on biodegradation and growth of bacteria on crude oil. Appl Microbiology, 284 (40):547-552 **(1974).**

13. Pirnik MP, Atlas RM, Bartha R. Hydrocarbon metabolism by Brevibacterium erythrogenes: Normal and Branched alkanes. J Bacteriol, 119(3):868-878 **(1974).**

14. Rakesh Patel., experimental microbiology, vol 1, india, **(2000)**

15. Sneath PA, Mau NS, Sharpe ME, Holt JG. Bergey's manual of systemic bacteriology. Vol II. Baltimore: Williams and Wilkins **(1986).**

16. Whyte LG, Slagman SJ, Pietratonio F, Bourbonniere L, Koval SF, Lawrence JR et al. Physiological adaptations involved in alkane assimilation at a low temperature by Rhodococcus sp. Strain Q15. Appl Environ Microbiology, 65(7):2961-2968 **(1999).**

17. Zhang Y, Miller RM. Enhanced octadecane dispersion and biodegradation by a Pseudomonas rhamnolipid surfactant (biosurfactant). Appl Environ Microbiology, 58(10): 3276-3282 **(1992).**