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Isolation of Engine Oil Degrading Microorganisms

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Microbial remediation of oil contaminated site is accomplished by diverse group of microorganisms known as petrophiles, particularly indigenous bacteria present in soil. Hydrocarbons have widespread occurrence in various ecosystems and the illegal dumping of used engine oil is an environmental hazard with global ramification. Thus it must be reduced, and for this, there is a need for isolation and screening of engine oil degrading microbes from soil. Inoculation of the serially diluted sample soil taken from Khushboo Auto traders boring road, Patna were done on Nutrient agar and Potato dextrose agar plates, for bacteria and fungi respectively. Then the screening of isolated bacteria and fungi on Bushnell-Haas broth and Czapek-Dox agar media containing 0.1%engine oil as a sole source of carbon was done, no fungus could be screened. Of the eleven isolates on NA five showed turbidity in the BH broth and screened as engine oil degrading bacteria, and identified as Flavobacterium sp, Moraxella sp, Pseudomonas sp and Streptococcus sp(moderate degraders) Bacillus sp(slow degrader) by Gram staining and several biochemical tests. Optimal oil concentration was found to be 1.0 %in Bushnell-Haas agar media. The isolated bacterial species can be used for in situ bioremediation of polluted sites as indigenous bacteria; also they can help environmental protection agencies for proper application of such agents.

Key words: *Engine-oil, Bioremediation, Bushnell-Haas media, Flavobacterium sp, Bacillus sp, Moraxella sp, Pseudomonas sp, Streptococcus sp.*

Introduction : Engine oil is a complex mixture of oil degrading hydrocarbon and some organometallics (Butler & mason, 1997). During fractional distillation, petroleum is separated into different fraction having different boiling ranges, this process is also known as "refining"(Chang, 1998). Products are gaseous hydrocarbons, petrol, kerosene oil, gas oil engine oil, wax and Bitumen. Boiling point of engine oil ranges between 544-673^{0}C, Carbon number varies fromC_{15}-C_{18}. The most important characteristic of lubricating oil is its viscosity and, hence, it is used to lubricate pats an automobile engine (Hagwell et al, 1992). Petrophiles are oil-consuming unique organisms that can naturally degrade and utilize them as a source of Carbon and energy (Harder, 2004). They degrade oil using enzyme in their metabolism and can be useful in cleaning up oil contaminated sites (Alexender, 1999). This process, known as bioremediation, has been shown to be effective method that stimulate the biodegradation of contaminated soil (McLaughlin, 2000; Harder2004). It has been estimated that bioremediation accounts for 5-10% of all pollution treatment and has been used for successfully cleaning up dumping of used Mobil. Microbial remediation of a Hydrocarbon contaminated site is accomplished with the help of a diverse group of microorganisms (Petropiles), particularly indigenous bacteria present in soil. Bioremediation, which is accomplished by adding exogenous microbial population or adding indigenous ones, attempts to raise rates of degradation found naturally to significant higher rates. Multiple regressions showed that the effectiveness of bioremediation depend on the Nitrogen delivered, the concentration of oil, and time. These microbes can degrade a wide range of target constituents present in oily sludge (Barathi and Vasudevan, 2001; Mishra et al, 2001. A large number of *Pseudomonas* strains capable of degrading bacteria that had been isolated from soil (Johnson et al 1996). *Polaromonas* strain such as (*P.vacuolata, P.hydroenivorans*) can biodegrade important groundwater contaminants including petroleum hydrocarbons. Other petroleum hydrocarbon degrading bacteria that had been isolated are *Bacillus* spp, *Corynebacterium* spp, *Providencia* spp,

Stenotrophomonas spp, *Streptococcus* spp, *Spingobacterium* spp, *Yokenella* spp, *Alcaligenas* spp, *Moraxella* spp, *Rosemonas* spp (Bhattacharya et al 2002). Fungi are also capable of degrading hydrocarbon in engine oil to certain extent. However, they take longer period to grow (Prenafeta Bolder et al, 2001). Marine bacteria capable of biodegrading hydrocarbon have been isolated from various marine areas. The study focused on isolation of engine oil degrading bacteria and fungi using enrichment method by using engine oil as a sole source of carbon and energy in culture medium and further characterization of these isolates in order to get knowledge of using indigenous microflora for in situ bioremediation of contaminated soil.

Materials and methods :

Sampling

Four top layer (0-10 cm) soil samples were collected aseptically from oil polluted site-Khushboo auto traders, Boring road, Patna. Serially diluted soil sample was plated on Nutrient agar media (10^{-7}) and Potato Dextrose agar media (10^{-5}) for primary isolation of Bacteria and fungi respectively.

Screening for engine oil degradation capabilities

The isolated bacteria and fungi were initially screened by using enrichment media containing 0.1% engine oil as a sole source of carbon and energy. For enumeration of oil utilizing fungus following modified Czapek Dox agar medium was employed: Sodium nitrate, 2.0g; Dipotassium Hydrogen phosphate, 1.0g; Magnesium sulphate,0.5g; Potassium chloride,0.5g; Ferrous sulphate,0.01g; Engine oil,1 ml; agar,15.0g; distilled water,1000ml.pH was adjusted to 7.3; incubation was carried out at 26^{O}C for 3-4 weeks.

For screening of bacteria, Bushnell-Haas (BH) broth media ($MgSO_4.7H_2O$, 0.2g; K_2HPO_4, 1.0g; KH_2PO_4, 1.0g; $FeCl_3$, 0.05g; NH_4NO_3, 1.0g; $CaCl_2$, 0.02g; Engine oil,0.1ml; Distilled water,1000.0ml; pH 7.2) Bushnell L.D., was & HF Haas (1941) employed. Erlenmeyer flasks were incubated at room temperature (27$\pm$2^{O}C) for 7- 14 days in a shaker-incubator to observe turbidity.

Biodegrading capabilities of screened strain tested with different oil concentration

One loop full of aliquot of each of turbid culture were streaked on BH agar media containing various oil concentration; 0.1%, 0.2%, 0.5%, 0.7%, 1.0%, 2.0%, 5.0%, 10.0%; to observe best growth. Rate of growth of colonies around oil droplet and clearing of oil droplet as well was fastest in 1.0% engine oil containing BH media.

Basic test for identification

Several basic morphological, micro morphological (Gram reaction) and biochemical tests were performed in or investigation including amylase production test, gelatinase test, fermentation of Carbohydrate, litmus milk test IMViC test, catalase test.

Result and Discussions :

Direct plating from soil diluted sample on potato dextrose agar and Nutrient agar resulted two fungal and eleven bacterial isolated respectively. No growth of fungus on enrichment media may be due to their negligible presence in soil or lack of proper growth condition.

Out of eleven, five bacterial isolates were screened on BH broth media as they showed turbidity. Subsequently, streaking these five isolates on BH agar plates, containing different oil concentration, showed best growth in 1.0% engine oil enrichment media. The result of colony morphology of five isolates on BH agar plate is summarized in table 1.

Table 1: Colony characteristics of 5 oil degrading bacteria isolated on oil containing Bushnell-Haas media:

Isolates	RN 1	RN 2	RN 3	RN 4	RN 5
Colour	Yellow	White	Yellow	Yellow	Golde
Growth	Moderate	Slight	Moderate	Moderate	Moderate
Form	Circular	Irregular	Circular	Irregular	Irregular
Margin	Entire	Lobate	Entire	Lobate	Lobate
Elevation	Raised	Convex	Convex	Flat	Raised
Density	Opaque	Opaque	Opaque	Opaque	Opaque

Result of tests for utilization of oil by direct observation for growth of isolates is summarized as four strains (RN1, RN3, RN4, and RN5) showed moderate capacity of oil degradation while one strain (RN2) showed slow capacity towards degradation.

The micromorphological and biochemical results are tabulated in table 2 and table 3 respectively.

Table 2: Gram staining

Isolates	Gram reaction	Shape
RN 1	G –ve	Rod
RN 2	G +ve	Rod
RN 3	G –ve	Cocci
RN 4	G-ve	Coccobacilli
RN 5	G+ve	Cocci

Table3: Biochemical test result

Isolates	RN1	RN2	RN3	RN4	RN5
Glucose fermentation	A+G-	A+G-	A-G-	A-G-	A+G-
Sucrose fermentation	A+G-	A-G-	A+G-	A+G+	A-G-
Lactose fermentation	A+G-	A+G-	A+G+	A+G-	A-G-
Gelatin liquefaction	+	+	+	-	+
Starch hydrolysis	–	+	+	-	-
Litmus milk reaction	cd	Ac/cd	cd	Ac/pep	Ac/cd/rdn
Indole production	_	+	+	_	+
H2S production	+	+	_	_	+
Methyl red test	_	+	_	+	_
Voges-Proskauer test	+	_	+	_	+
Citrare utilization	+	_	_	+	_
Catalase test	+	+	+	+	+

cd- curd formation ; Ac-acid ; Pep – Peptonization ; rdn - reduction

Comparing our result with literature data, a high similarity can be established according to biodegrading capacity and growth characteristics of the similar strains. The genera could possibly belong to *Flavobacterium* sp (RN1), *Bacillus* sp (RN 2), *Moraxella* sp (RN 3), *Pseudomonas* sp (RN 4), and *Streptococcus* sp (RN 5). The identification is based on morphological and biochemical characteristics and not on confirmatory genetic characteristics (16s rRNA homology).

Conclusion :

In this study, different culture media were used. Our results were compiled with the findings of engine oil derading bacteria, their rate of engine oil degradation, morphological, biochemical characteristics. Degradation is reconfirmed by dual inoculation on BH media and optimal oil concentration for growth was found to be 1.0%.

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