ISOLATION AND CHARACTERIZATION OF POTENTIAL OIL DEGRADING BACTERIA FROM OIL CONTAMINATED SITES BY *PSEUDOMONAS Spp.*

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ABSTRACT: *The discharge of used engine oil from vehicles by motor mechanics is a major source of oil pollution. This study intended to isolate and assess bacteria capable of effectively degrading and cleaning up used engine oil in this locality and also to ascertain the influence of temperature and nutrient concentration on the rates of engine oil degradation by these bacterial isolates. The samples were collected from different mechanical workshops. The microorganisms present in sample were isolated by enrichment technique using Bushnell Haas Broth with used engine oil as sole carbon source. The isolated bacteria were identified by biochemical characterization. The identified bacterium was Pseudomonas species. Oil degradation was studied for various parameters such as different temperature, pH, substrate concentration, salt concentration, Carbone sources, and nitrogen sources. From that highest activity is given by, in temperature at 50 0C and is 79.66%; In pH is 92.25% at pH 4; from substrate concentration, 5% and is 85.63% From salt concentrations, MgCl2 and is 93.34%; From Carbone sources, mannitol and is 91.50%; From nitrogen sources, urea and is 77.75% within 7 days. In vitro study indicates, these isolate V7 is potential for oil degradation. The future prospects are checking for the degradation of other organic compounds like chemical fertilizers with the isolated bacteria and also for the production of bio surfactants using the isolated bacterial strain.*

Key Words: *Oil degradation, Engine oil, Pseudomonas spp.*

I. Introduction

Environmental pollution with petroleum and petroleum products has been recognized as one of the most serious current problems especially when associated with accidental spills on large-scale. The presence of different substrates and metabolites in hydrocarbon contaminated soils has no doubt provided an environment for the development of a quite complicated microbial community (Udeani *et al* 2008; Butler and Mason, 1997). Waste engine oil which is also known as used motor oil is produced when fresh engine oil is subjected to high temperature and high mechanical strain during running of the vehicle for a stipulated time. It is brown-to-black liquid mixture consisting of low to high mol wt (C_{16} to C_{36}) aliphatic and aromatic hydrocarbons, polychlorinated biphenyls, chlorodibenzofurans, lubricative additives, and decomposition products along with heavy metal contaminants, such as, zinc, lead and chromium, coming from engine parts. Thousand million gallons of waste engine oil are generated annually from mechanical workshops, which is not recycled but spilled and dumped by automobile and generator mechanics into runoff, gutters, water drains and open vacant plots and farmlands. Out of this, 1 L is enough to contaminate 1 million gallons of fresh water. The illegal dumping of used engine oil is dangerous to the environment and constitutes a potential threat to human, animals and vegetations (Bhattacharya and Biswas, 2014). Bushnell Haas media was used as enrichment media for the isolation of diesel oil degrading bacteria. Hydrocarbons found in crude oil are metabolized by a large number of bacterial communities into nontoxic biodegradable products. These are the constituents of simple hydrocarbons to polycyclic aromatic hydrocarbons. The release of such petroleum products into the environment shows a great impact on biotic and abiotic communities of that environment if not treated at time (Vignesh *et al*2016). Long term damage to marine ecosystem as well terrestrial: life, human health and natural resources. Contamination of the environment with crude oil results in pollution. Wide scale production, transport use and disposal of petroleum globally have made it a major contamination in both prevalence and quantity in the environment. The oil gets mixed with the river or marine water by many ways as accidental spills or discharge of refineries in river or other water bodies (Singh *et al*2014). Biodegradation is a most effective biological method for treating oil contamination. Bacterial degradation has played a vital role in the management of petroleum contaminants in contrast to conventional approaches that rely upon human labour (Mulani *et al* 2017). Biodegradation is defined as the

biologically catalyzed reduction in complexity of chemical compound. Biodegradation of petroleum hydrocarbons in the environment may be limited by a large number of factors. An important limiting factor in the biodegradation of polluted soils is often the low bioavailability and solubility of the hydrocarbon (Latha and Kalaivani, 2012). Microbial biodegradation of hydrocarbons depends on the following factors such as potential of the indigenous microorganisms, temperature, pH, oxygen and nutrient availability and hydrocarbon concentration (Gopinathan *et al* 2012). Biological degradation method is an advantageous method then other degradation techniques. Biological degradation is considered as safe, effective and an economic alternative method, is a process of decay initiated by biological agents, specifically in this case by microorganisms (Swarnakaran and Panchanathan, 2011). Bioremediation is non invasive and cost effective method. This method showed a number of advantages for remediating oil-polluted and with shorter treatment times, a greater potential efficiency, lower impact on the environment, and easier public acceptance (Doan *et al* 2016).

II. Materials and Methods

Sterilization

Media and glass wares were sterilized in an autoclave at 121 °c with 15 lbs pressure for 20 minutes.

Sample collection

Oil contaminated soil samples were collected from different garages nearby Navsari region, Gujarat. Samples were used for analyze the physico-chemical parameters and to isolate the bacteria. They were then carefully transferred to the laboratory for the analysis and stored under refrigeration condition.

Media

Bushnell Haas broth, Bushnell Haas Agar, Nutrient Broth, Nutrient Agar, Gram's reagent, Tryptone Broth, Glucose phosphate broth, Cimmon citrate slant, Peptone broth, Urea broth, TSI slant, starch agar

Enrichment of microorganisms

10 ml of contaminated soil sample were inoculated into 250 ml of Bushnell Haas broth in 500 ml conical flask and placed it in shaker for 2-3 days for enrichment of microbes.

Isolation and screening of isolates for oil degrading bacteria

Primary screening:-

After enrichment of the sample and take 0.5 ml of enriched culture and perform serial dilution, spread 0.1 ml in each dilution on nutrient agar plate with oil. Incubate it at 37 °c for 24 hours. After incubation period observe the isolated colonies and streak it on nutrient agar plate and put it in incubator at 37 °c for 24 hours.

Secondary screening:-

After primary screening, perform cup borer method for screening of oil degrading bacteria with the use of Bushnell Haas agar plate containing oil as a substrate. After incubation period, clear zone was observed on the plate. These isolates were preserved for future studies.

Identification of the Bacteria:-

The bacterial isolates were identified by morphological and biochemical methods. Various parameters were studied for the degradation.

Effect of pH

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Adjust pH 4, 7, 9 with the help of HCl and NaOH in broth. Incubate the each flask at 37 °c in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

Effect of Substrate concentration

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Adjust substrate concentration 1%, 5%, 10% in broth. Incubate the each flask at 37 °c in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

Effect of Salt

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Take different salts like NaCl, KCl, MgCl2. Incubate the each flask at 37 °c in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

Effect of Temperature

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Take different temperatures 30 °c, 50 °c, 80 °c. Incubate the each flask at respective temperature in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

Effect of Carbon source

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Take different carbon sources like Xylose, Lactose, and Mannitol as a carbon source. Incubate the each flask at 37 °c in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

Effect of Nitrogen source

Take 100 ml of Bushnell Haas broth, autoclave it and inoculate it with 0.5 ml of culture and 20 ml of diesel oil as a substrate. Take different nitrogen sources like Urea, Peptone Beef extract as a nitrogen source. Incubate the each flask at 37 °c in shaker at 150 rpm for 7 days. After incubation, take O.D. of broth at 600 nm.

III. RESULTS AND DISCUSSION

The bacteria were isolated from three different types of samples on nutrient agar medium. Further the samples were screened for the presence of hydrocarbon degrading bacteria on mineral salt medium with 1% of the hydrocarbons as the sole carbon source. Hydrocarbons which are needed as a carbon source can be toxic to microorganisms due to solvent effects of diesel and petrol, which may destroy bacterial cell membrane. Many biodegradation studies were reported on diesel are carried out using lesser diesel concentrations ranging from 0.5-1.5%.

Screening for Oil Degrading Bacteria

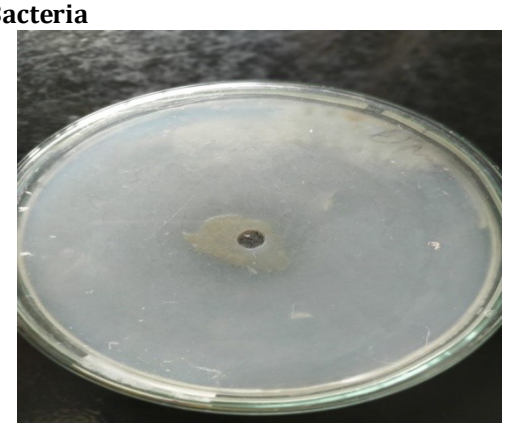

Figure-1 Zone of inhibition of V7 isolate

On the basis of zone of inhibition, various microorganisms were selected.(Fig 1).

Identification of Bacteria

The isolated bacteria was identified by Gram staining, and some biochemical tests. The results of biochemical test were compared to the Bergeys manual. (Sneath *et al* 1996).(Table: 1)

Biochemical test	Media	Indicator	Result
Indole test	1% tryptone	Kovac's reagent	Positive
Methyl Red test	Glucose phosphate broth	Methyl red	Positive
Vogeus-Proskaur test	Glucose phosphate broth	0.6 ml α-napthol + 0.2 ml KOH	Negative
Citrate utilization test	Simmon citrate slant	-	Positive
H2S test	-	-	Negative
Urea utilization test	Urea broth	-	Positive
Triple sugar iron test	-	-	
Carbohydrate utilization test Glucose Lactose Maltose Sucrose Xylose	-	Andrade's indicator	Positive A/G in all the sugar.
Starch hydrolysis test	Starch agar plate	Lugol's iodine	Positive

Table-1 Biochemical characterization of V7 isolate

Characterization of various parameters using V7 isolate

Effect of pH on oil degradation:

In the previous study, some species achieved maximum oil degradation at pH 7.5, but some species shown negative effect at pH 6-9(Chen *et al*2017).Based on literature review *Pseudomonas spp. w*as found to give oil degradation at pH 7(Vignesh *et al* 2016). Optimum oil degradation was found at pH between 6 and 9 (Das *et al* 2011).Figure-2 shows that the optimum pH for oil degrading bacteria was found to be pH 7, which shows 92.25% oil degradation within 7 days of the study.

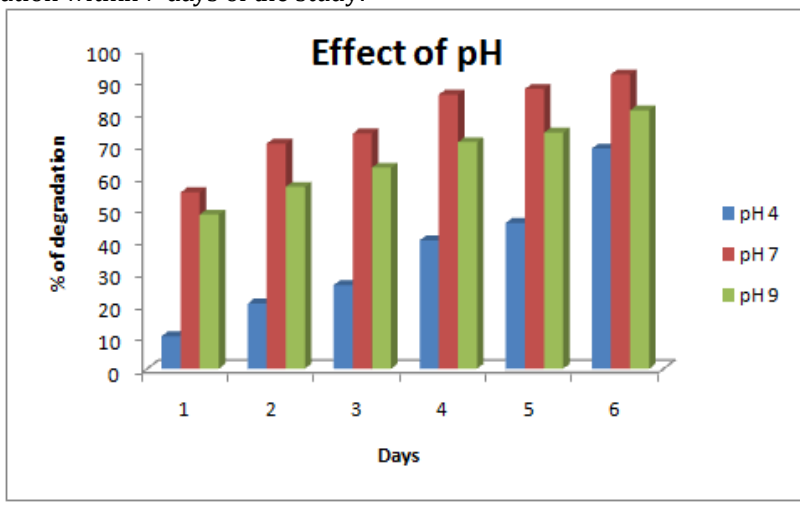

Figure-2 Effect of pH on oil degradation

Effect of Substrate concentration on oil degradation

In the previous study, some species achieved maximum oil degradation at 1% substrate concentration (Chen *et al*2017).Figure-3 shows that the optimum substrate concentration for oil degrading bacteria was found to be 1%, which shows 85.63% oil degradation within 7 days of the study.Many biodegradation studies were reported that oil degradation found in concentration ranging from o.5 to 1.5 %, but Kaplanet *et al*2004 reported that oil degradation at 3.5% and 6% substrate concentration (Diptendu *et al* 2016).

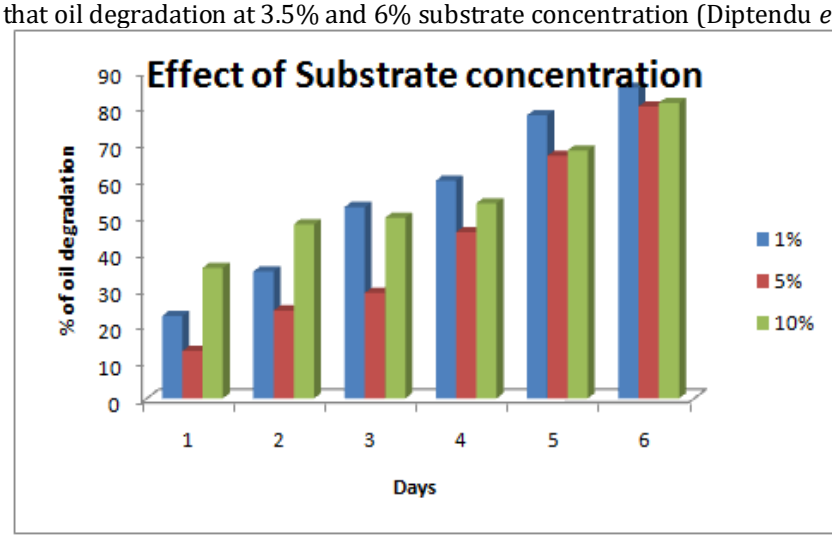

Figure-3 Effect of Substrate concentration on oil degradation

Effect of Salt on oil degradation

In the previous study, some species achieved maximum oil degradation in $MgCl_2$ (Chen *et al*2017).Figure-4 shows that the optimum salt for oil degrading bacteria was found to be $MgCl_2$, which shows 93.34% oil degradation within 7 days of the study.

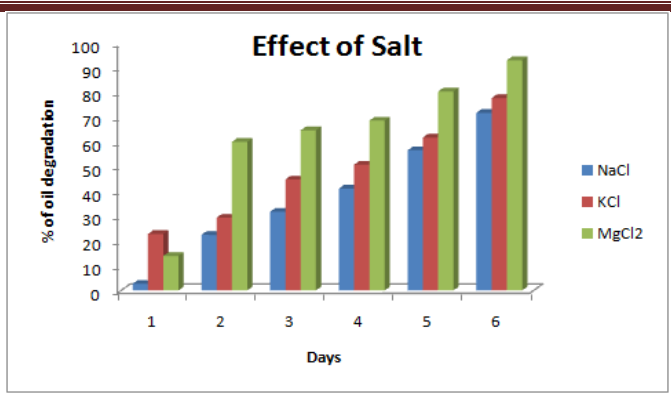

Figure-4 Effect of Salt on oil degradation

Effect of Temperature on oil degradation

In the previous study, some species achieved maximum oil degradation at 45 ˚C(Swarnakaran *et al*2011), but some species achieved maximum oil degradation at 30 ˚Cand at lower temperature shows negative effect (Chen *et al*2017).The degrading potentials of the oil degrading bacteria was observed to be optimum at temperatures of 28 ˚C and 32 ˚C (Umanu *et al* 2013).Figure-5 shows that the optimum temperature for oil degrading bacteria was found to be 30 ˚C, which shows 79.96% oil degradation within 7 days of the study.

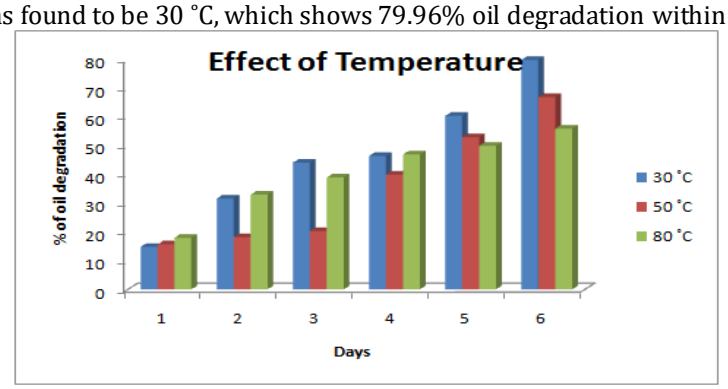

Figure-5 Effect of Temperature on oil degradation

Effect of Carbon sources on oil degradation

Figure-6 shows that the optimum carbon source for oil degrading bacteria was found to be Mannitol, which shows 91.50% oil degradation within 7 days of the study.So from the above result it can be said that the carbon sources can be varied according the various spp. Our result varied and the bacteria Pseudomonas spp. Showed maximum degradation using mannitol as a carbon source and xylose and lactose showed less degradation.

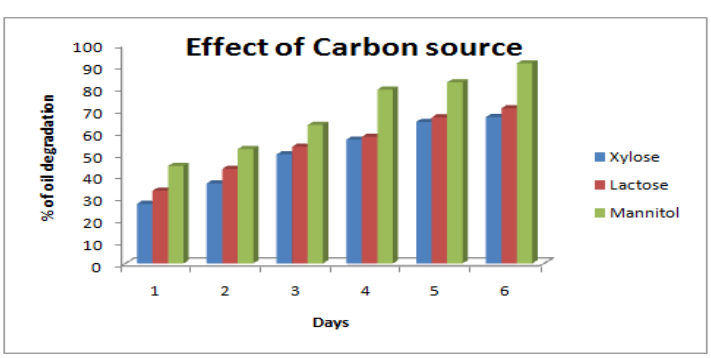

Figure-6 Effect of Carbon source on oil degradation

Effect of Nitrogen sources on oil degradation

The degrading potentials of the oil degrading bacteria were observed to be optimum in yeast extract (Bhattacharya *et al*2014).Figure-7 shows that the optimum nitrogen source for oil degrading bacteria was found to be urea, which shows 92.25% oil degradation within 7 days using the isolate.

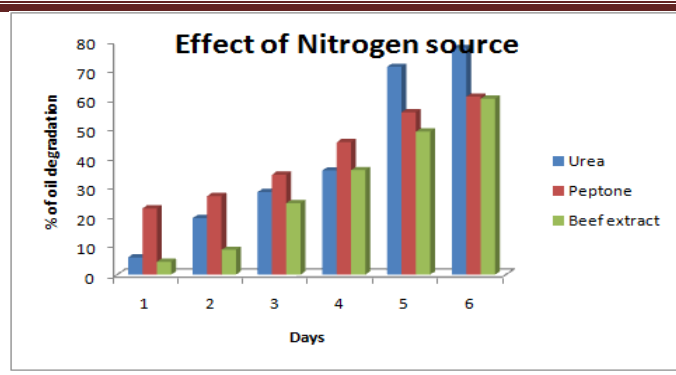

Figure-7 Effect of Nitrogen source on oil degradation

IV. Conclusion

From this study it was concluded that oil contaminated soil is the best source for the isolation of oil degrading bacteria and may be used for the isolation of other microorganisms. The isolated bacteria can be used for the degradation of different hydrocarbon containing oils and it can also be used for the remediation of contaminated sites. *Pseudomonas spp*. is the potential bacteria for oil degradation.

V. Future aspects

The future prospects are checking for the degradation of other organic compounds like chemical fertilizers with the isolated bacteria and also for the production of biosurfactants using the isolated bacterial strain.

VI. Acknowledgment

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