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Degradation Of Non-petroleum Based Natural And Synthetic Oil By Lipase Producing Fluorescent *Pseudomonas Spp*. Isolated From Petroleum Based Hydrocarbon Saturated Soils Of Shillong, Meghalaya, India

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Abstract

The capital city of India's eastern state of Meghalaya, i.e. Shillong often referred to as the "Scotland of East " due to its striking resemblance with the rolling hills of the Scottish countryside is now facing extensive rise in population. This rise in population and the related anthropogenic activities has resulted in a serious problem of sewage and effluent disposal in this hilly city. The study therefore aimed at isolating potential soil bacteria that might serve as possible tools for bioaugmentation of the biodegradation processes of sewage and effluents rich in fats, oils and greases that are of primarily non-petroleum origin. Deviating from the normal approach, soils saturated with petroleum based hydrocarbons were taken as the source material to isolate bacterial species showing effective biodegradation potential for non-petroleum based oils. This was done in order to establish a possible link between the biodegradation of petroleum based hydrocarbons to that of the non-petroleum based ones. Our study reveals that soil bacteria that were able to tolerate significant amounts of petroleum based hydrocarbons in the environment, are also capable of effectively degrading non-petroleum based hydrocarbons of natural and synthetic origin through the production of lipases. Three isolates of fluorescent *Pseudomonas spp*. namely P7, D15 and M23 were able to degrade Mustard oil, Olive oil and Tween 80 in minimal media. All the three isolates also showed extensive extra-cellular lipase production in two variants of the plate based lipase assay. Isolates D15 and M23 were shown to harbour a single plasmid, whereas isolate P7 showing the highest utilization ability for the supplemented hydrocarbons interestingly, showed the absence of plasmids. It is therefore, likely that the phenomenon of horizontal gene transfer through extra-chromosomal DNA exchange among natural populations of soil bacteria, may not be always responsible for the bio-degradative ability they exhibit against hydrocarbons of both petroleum and non-petroleum origins. Moreover, these soil bacteria may serve as potential candidates for bioaugmentation of sewage and effluent treatments in the near future.

INTRODUCTION

Oil spills and effluents of petroleum industries and gas stations create major problem of soil and water contamination. Pristine ecosystems and the few remaining world's biodiversity hotspots are now at a threat due to anthropogenic activities and vehicle pollution. The capital city of the state of Meghalaya (India) i.e. Shillong often referred to as the "Scotland of East " due to its striking resemblance with the rolling hills of the Scottish countryside and its cool climate, is now facing serious environmental pressures due to an ever increasing population and pollution. Being a city located among the hills, the problem of a rapid surge in human population, mechanization and automobile activities over the last few decades, has also given rise to effluent and sewage disposal related problems in the once famed hill station of the eastern Himalayan region of India. The small rivers of Umkhrah, Umshyrpi and Umjasai in Shillong are now extensively polluted by domestic sewage (**Acharya**, 2008).

A wide variety of industries produce effluents rich in fats, oils and greases (FOGs). Effluents p**roduced by the restaurant trade (Stoll and Gupta, 1997; Wakelin and Forster,** 1997), the dairy industry (**Vidal *et al.,*** 2000) and food processing (**Cammarota *et al.,*** 2001) are a small sample of those that present potential problems in terms of wastewater management due to their high FOG content. Fats often solidify causing pipes and sewer lines to become blocked (Baig & Grenning, 1976).Grease traps may also fail to retain dissolved and emulsified fats efficiently allowing them to enter the water treatment system. These lipids may then interfere with aerobic biological wastewater treatment processes by reducing oxygen transfer rates (**Chao** & Yang 1981) and reduce the efficacy of anaerobic treatment processes by reducing the transport of soluble substrates to the bacterial biomass (Rinzema ***et al.*** 1994). FOGs not properly treated by sewage works may enter rivers and oceans with potentially detrimental environmental impacts (**Stams** & **Oude** 1997). Reduction in the levels of FOGs in effluents is thus highly desirable for proper waste management. Numerous microorganisms capable of degrading FOGs have been identified, which are potential candidates for bioaugmentation products. **Mudge** and **Pereira** 1999, demonstrated that biodiesel derived from vegetable oils as a potential candidate for removing crude oil from contaminated beaches.

Biodegradation represents an important route by which these oils can be removed from the environment. The first step in the microbial degradation of oil is its hydrolysis to release the component fatty acyl groups. Triacylglycerols and their partially hydrolysed products, di- and mono-acylglycerols, are not assimilated as such (**Ratledge**, 1994).The present study therefore aimed at isolating lipase producing bacteria from petroleum based hydrocarbon saturated soils i.e. the soils in the vicinity of petroleum and lubricant distribution and automobile units in the city of Shillong, Meghalaya, India and to study their biodegradation potential on non-petroleum based vegetable oils, to sustain their metabolic carbon demand and growth.

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MATERIALS AND MEDHODS

Collection of soil samples

Composite soil samples saturated with hydrocarbons were collected from various gas stations (petrol pumps) and automobile stations located in the city of Shillong, Meghalaya, India. Three different contaminated sites, having contamination with different types of petroleum fractions i.e. petrol, diesel and lubricating oil (Mobil) were selected for the soil sampling purpose. Soil samples were then collected in sterilized sample bottles and plastic bags with spatula as per the procedure described by **Singh *et al.,*** 2003.

Serial dilution of soil sample

One gram of soil sample of each of the three composite samples was then diluted with 10 ml of autoclaved distilled water. From that 1 ml was taken and inoculated into 9 ml autoclaved distilled water to get subsequent dilutions of 10^{-1} to 10^{-8}. From each of the diluted tubes 50 µl was inoculated in minimal agar media plates supplemented with the respective petroleum fraction.

Isolation of soil bacteria showing degradation potential against hydrocarbons

The number of total aerobic bacteria was then recorded and colonies showing promising growth in the minimal agar plates (Peptone: 10.0 g/L, Sodium Chloride: 5.0 g/L, Calcium Chloride Hydrated: 0.1g/L, Agar, 15.0g/L) supplemented with 100-1000 µl of the respective petroleum based hydrocarbon fractions (i.e. Petrol, Diesel and Mobil), were then subsequently pure cultured and maintained in the same minimal agar media as slants. Three isolates namely P7, D15 and M23 (one from each of the soil samples), showing vigorous growth in minimal media were then selected for further analysis to ascertain their vegetable oil degrading capacity and lipase production.

Morphological, cultural and biochemical tests:

The bacterial isolates were then characterized morphologically and biochemically as per the standard protocols described by Cappucino & **Sherman,** 1996. These isolates were also checked for their fluorescence under UV light.

Lipase Assay

Lipase production is an important phenomenon of microbes in the degradation of hydrocarbon or fatty acid substances. Two assays for the estimation of the lipase producing activity were performed. In the first experiment; we dissolved 0.15 g of tetrazolium red powder in 3 ml of DMSO. Tween agar media (containing 10% Tween) was added with 5% tetrazolium solution. Bacterial cultures were then inoculated over the plates and incubated at 37 ^{0} C for 48 hours. Lipase activity can be visualized as a mild yellow/pink coloured halo around the isolate. In the second experiment, minimal media (without adding peptone) was used .The minimal media therefore contained only salt and the respective oil (10%).50µl of the liquid culture broth of the bacterial isolates were then inoculated into the wells punctured at the centre of the minimal agar plates. Production of a ring like precipitate around the well is indicative of lipase production.

Culture of the bacterial isolates in Minimal Media supplemented with non petroleum based hydrocarbons.

Minimal media comprised of peptone as protein and amino acid, sodium chloride and calcium chloride as salt composition. Tween 80, Mustard oil and Olive oil were supplemented as carbon source in three different sets. The bacterial cultures were then streaked over the minimal agar plates and kept for incubation at 37°C for 24 hours.

Growth Characteristics of bacterial isolates and oil degradation pattern

Growth characteristics of the bacterial isolates were then studied spectrophotometrically at 540 nm in liquid minimal media supplemented with Tween 80, Mustard oil and Olive oil in three different sets. The hydrocarbon utilization pattern i.e. utilization of Tween 80, Mustard oil and Olive oil in our particular case, was then indirectly found out by the estimation of the release of free fatty acids into the liquid growth medium in relation to time. This was achieved by addition of a 1 ml of base (1M NaOH) to the growth medium. Upon addition of the base, saponification reaction occurred leading to formation of soap molecules, characterised by a muddy white colour of the media. To correlate bacterial growth with the release of free fatty acids into the media, the wavelength for taking the readings was kept the same i.e. 540 nm. Records for growth as well as the hydrocarbon utilization pattern were taken at the same time intervals in a CECIL UV visible spectrophotometer.

Isolation of Plasmid DNA from the bacterial isolates

Isolation of plasmid DNA was carried out as per the protocol described by Kado & Liu, 1981. Electrophoresis was performed on 1.5% of agarose gel at 80 Volts for 1 hour. The electrophoretic profile of the isolated plasmid DNA was observed under UV gel documentation system GEL Logic 100 (Kodak).

Results and Discussion

The total bacterial population in petrol contaminated soil was found to be 5.97 x $10^{2.}$ The populations in diesel and mobil (lubricant) contaminated soils were found to be 6.45x 10^{4} and 6.45x 10^{4} respectively (Table 1).

Morphological tests showed that the isolates were all gram negative short rods. All the three isolates also showed fluorescence upon exposure to UV light. Further, biochemical tests were performed on the fluorescent isolates P7, D15 and M23 isolated from petrol, diesel and Mobil (lubricant) contaminated soils respectively. Upon comparison of the biochemical characters with standard bacteriological manuals like the Bergey's Manual of Determinative Bacteriology, the bacterial isolates were identified to be species of fluorescent *Pseudomonas (*Table 2).

Growth of the isolates were observed in minimal agar plates supplemented with Tween-80, mustard and olive oil .Growth pattern was monitored after every one hour for isolates P7, M23 and D15 simultaneously with the release of free fatty acids into the culture medium. It was observed that the amount of free fatty acids released into the media increased along with the increase of the growth of the bacterial cells. (Figure: 1-3).

The total bacterial population was seen to be the least in petrol contaminated soils as compared to the diesel and Mobil (lubricant) contaminated sites. Mobil contaminated soil was seen to support the highest bacterial population among the contaminated soils under study. The best growth in the liquid minimal medium was shown by isolate M23, while the most promising ability to utilize the hydrocarbons as source of carbon was shown by isolate P7. (Figure: 1-3). This result can be correlated with the total bacterial counts in the three contaminated soils. The Mobil (lubricant) contaminated soil was shown to support a large population of bacteria whereas the least bacterial count was recorded from petrol contaminated soil (Table 1). This effect may be due to the fact that the stress conditions exerted by a particular fraction of petroleum based hydrocarbon such as petrol in the soil, leads to the selective amplification of particular biodegrading bacterial species.

Furthermore, all the three bacterial isolates were also shown to possess considerable lipase producing ability, as proved by the two variants of the plate based lipase assay (Fig*ure* 4-7). In the first lipase assay, tetrazolium red reacted with the lipase enzyme produced by the bacterial species and a yellow coloured circular zone was seen around the inoculum due to the formation of formazon. (Figure , 4,5).In the second assay a prominent halo of precipitation was observed around the bacterial colony (Figure 6, 7).The two minimal agar plate based assays to determine the lipase producing ability of the isolates P7, D15 and M23 thereby showed that each of the isolates were able to produce lipid degrading enzymes i.e. lipases. **Haba *et al.,*** 2000, also isolated lipase-secreting bacteria by deploying used frying oil as selective substrate. From the 47 strains of bacteria and yeast they had screened, the genera *Pseudomonas*, *Bacillus*, *Candida*, *Rhodococcus*, and *Staphylococcus* grew on the waste olive and sunflower oils and produced lipolytic activity. Amongst the studied strains they found that the highest lipase producers were *Pseudomonas sp* 3AT (2748 U/L) and *Pseudomonas aeruginosa* ATCC 111 (1703.8 U/L).Plasmids have been reported by various workers to possess genes responsible for the production of extra-cellular lipases (**Paparaskevas *et al.,***1992). Single plasmids were isolated from two of the isolates namely D15 and M23. Isolate P7 showing the highest utilization ability for the supplemented hydrocarbons interestingly did not posses even a single plasmid . It is therefore, likely that that the gene responsible for the lipase production may therefore be present in the chromosomal DNA in case of isolate P7. (Figure 8.)

The present investigation establishes a relatively cheap and simple protocol to study the biodegradation of non-petroleum based hydrocarbons both of natural and synthetic origin, with the key concept of the basic saponification reaction at its core and the sole requirement of a basic laboratory spectrophotometer for colorimetric data acquisition. Our results show that the fluorescent *Pseudomonas* spp. isolated from petroleum based hydrocarbon saturated soils were able to degrade non-petroleum based hydrocarbons i.e. vegetable oils (mustard and olive) and Tween 80.These *Pseudomonas spp.* were also found to produce extra-cellular lipid degrading enzymes (lipases) for causing the hydrolytic breakdown of the lipid molecules. These bacteria may become potential candidates in the development of microbial bioaugmentation products for the treatment of vegetable oil rich sewage/effluents, particularly adapted to the hilly region of Meghalaya, India. However, molecular studies are still needed to find out the genes responsible for the phenomenon, leading to a better understanding of the complex biochemical pathways/ lipase based enzyme systems employed by these bacteria for exhibiting their biodegradative ability.

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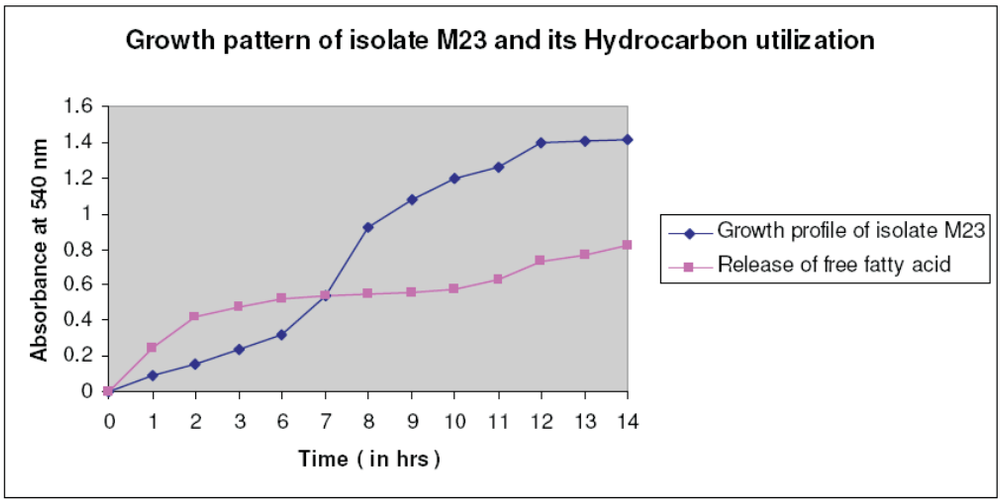

Figure1: Growth characteristics of the bacterial isolate M23 and its subsequent utilization of hydrocarbon (Mustard oil).

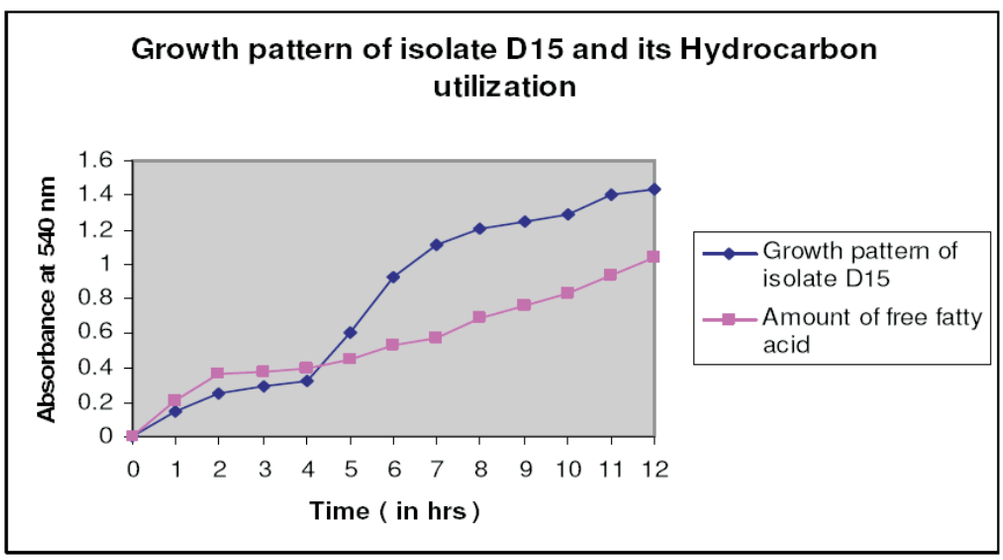

Figure2: Growth characteristics of the bacterial isolate D15 and its subsequent utilization of hydrocarbon (Mustard oil).

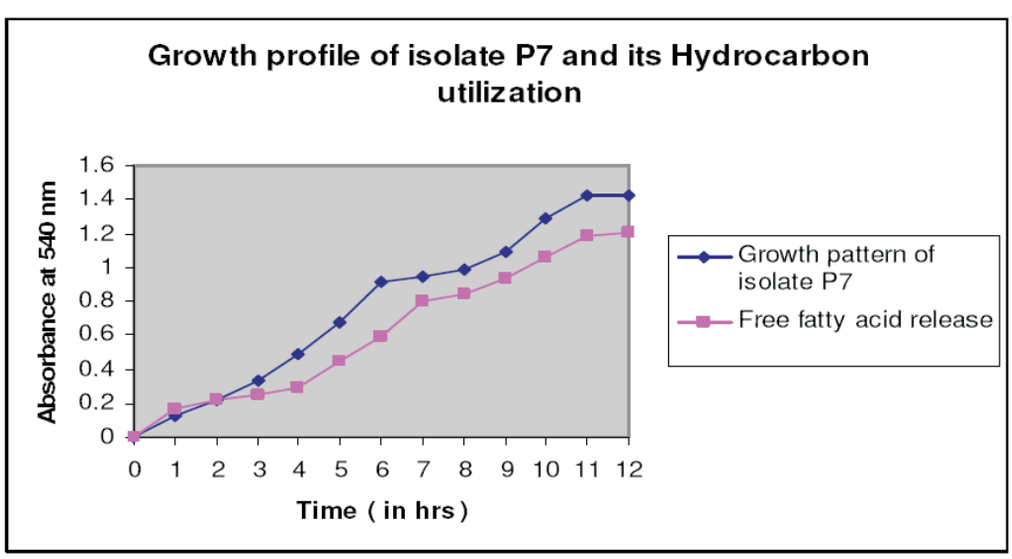

Figure3: Growth characteristics of the bacterial isolate P7 and its subsequent utzation of hydrocarbon (Mustard oil).

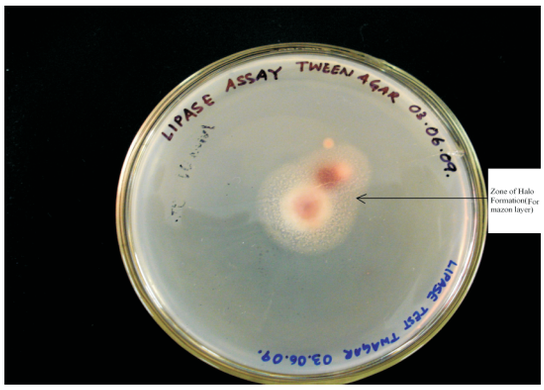

Figure4: Lipase Assay (I) tetrazolium based lipase assay showing zone of formazon formation after 24 hours of incubation of strain P7 on Tween 80 supplemented minimal agar media

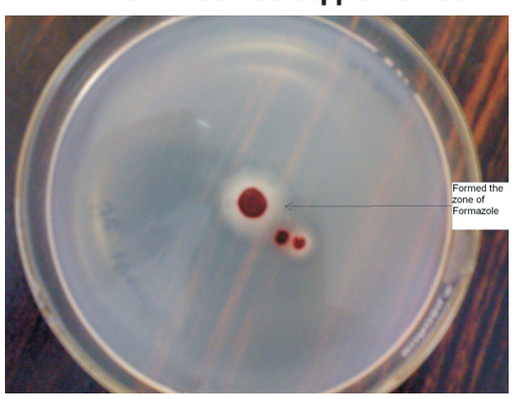

Figure 5: Lipase Assay (I) tetrazolium based lipase assay showing zone of formazon formation after 24 hours of incubation of strain P7 on Olive oil supplemented minimal agar media

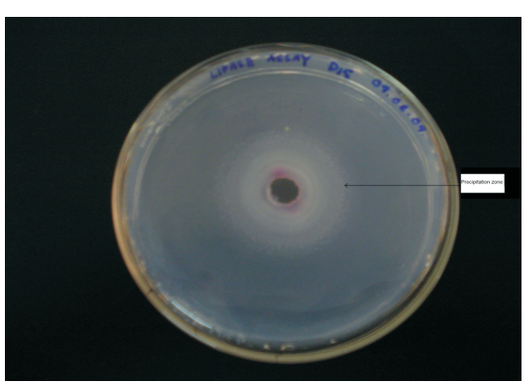

Figure6 : Lipase assay (II) showing halo of precipitation after 24 hours of incubation of strain D15 on Tween 80 supplemented minimal agar media.

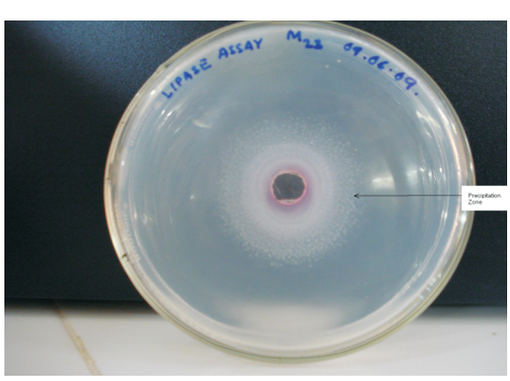

Figure 7: Lipase assay (II) showing halo of precipitation after 24 hours of incubation of strain M23 on Olive oil supplemented minimal agar media.

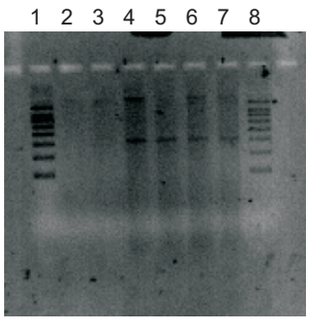

Figure8: Agarose gel electrophoresis of plasmid DNA isolated from the bacterial isolates. Lane 1 and 8: 1 kb DNA ladder, lane 2-3: strain P7, lane 4-5: strain D15, lane 6-7: strain M23.

SUPPLEMENTARY MATERIAL

TABLE: 1. CFU count of the bacterial isolates.

Soil Type	CFU/g of dry soil
Petrol Contaminated	5.97×10^2
Diesel Contaminated	6.45×10^4
Mobil Contaminated	6.45×10^4

*All values are mean of three replicates

TABLE: 2. Morphological & Biochemical analysis

Morphological & Biochemical parameters	Isolate P7	Isolate D15	Isolate M23
Gram Stain	-	-	-
Shape	Short rods	Short rods	Short rods
Pigment Production	-	+ (Red)	+(Orange)
Indole	+	-	+
H_2S Production	+	+	+
Lactose	+	+	+
Glucose	+	+	+
Sucrose	+	+	+
Citrate	-	+	+
Catalase	+	+	+
Oxidase	+	+	+
Urease	+	+	+
Methyl Red	+	+	+
Voges Proskauer	-	+	+