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Isolation, screening, and characterization of hydrocarbon-degrading bacterial strains from soil contaminated with engine oil in Kut city

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Abstract

Engine oil contamination is one of the foremost environmental problems caused by diverse human activities. Samples were collected from old garages and auto workshops and placed on a selective medium (Bushnell Haas mineral salt agar), with 1 % engine oil as the growth substrate. Thirty-one isolates from engine oil degraders were identified in eleven samples. Among these isolates, the samples (O9) 68 % and (O10) 64 % showed the highest degradation ability. Based on optical density measurement, biochemical testing, and molecular identification of the local isolate (O9); demonstrated that bacteria isolate (O9) belonging to *Pseudomonas puttida*, showed the highest biodegradation.

Keywords: Bioremediation, Biochemical tests, *Pseudomonas puttida*.

1. Introduction

Soil pollution is chemical, and physical alterations in soil composition. Brought by introducing foreign substances, such as chemical fertilizers and pesticides, acid rain, and spilled petroleum hydrocarbons [1]. Main classes of hydrocarbons are alkanes and aliphatic and aromatic compounds as well as other minor constituents. Hydrocarbons can enter the soil during transportation, storage, and utilization petroleum hydrocarbons compounds, it can accumulate and cause serious issues [2].

Engine oil, also known as lubricating oil, is the most prevalent hydrocarbon pollutant found in soil. Engine oil is a non-volatile liquid that is derived from base oils that are manufactured by distilling petroleum [3]. Engine oil may include heavy metals and other hazardous substances that might have an impact on the

growth of plants [4-5]. The existence of hydrocarbon-using bacteria in the soil, can assist in bioremediation processes to eliminate hydrocarbon pollutants in contaminated environment [6].

Hydrocarbon consuming bacterium can with stand engine oil contaminated environments because they can use hydrocarbon as an energy source while other species may not. Diversity of microorganisms that originate in a contaminated environment will degrade hydrocarbons differently and at a different rate than microorganisms in a moderately clean environment [7].

Numerous studies have demonstrated that *pseudomonas spp.* bacteria can biodegrade hydrocarbon components [8]. The transmission of genetic information between microbial species makes them more resistance, tolerant, and capable to degrade variety chemicals [9].

Hydrocarbon- degrading bacteria generate surface active agents like biosurfactants which may dissolve hydrocarbons into solution. Long chain hydrocarbons are important for microbes because biosurfactants make the molecules more accessible to microbial enzyme systems for utilization.

Additionally, biosurfactants elevate metabolism, and after that biodegradation of hydrocarbons directly by cell [10]. The study objective is to isolate and characterize microorganisms capable of hydrocarbon degradation under laboratory conditions as well as to accelerate isolates rate of biodegradation.

2. Materials and Methods

2.1 Enhancement, and Isolation of Hydrocarbons Degrading Bacteria

Eleven soil samples were collected randomly from old garages and automobiles workshops in different locations from Kut city. Bacteria were extracted from engine oil-contaminated soil samples using a selective enrichment approach, with engine oil used as the only energy and carbon source. Fifty mL of Bushnell- Hass medium broth that consisted of KH_2PO_4 (1 g), K_2HPO_4 (1 g), $MgSO_4$ (0.2 g), $CaCl_2$ (0.02 g), $(NH_4)_2SO$ (1g), and $FeCl_3$ (0.05 g).

These substances were dissolved in one litre of distilled water with a pH of media of 7 was set into 250 mL Erlenmeyer flasks and enhanced with 1 % engine oil. The solution was utilized as a hydrocarbon source and was autoclaved for 15 minutes at 121 ºC. After sterilization (1 g) from the original soil sample was put into the Erlenmeyer flask [11].

The control flask has engine oil and BHM broth, without a soil sample. After 14 days, at 150 revolutions per minute, all

Erlenmeyer flasks were incubated at 30 ˚C in a shaker incubator [11].

2.2 Microbiological Analysis

After the incubation period optical density with a spectrophotometer (600 nm) was measured for each flask and a loop full from each flask (sample and control) was cultured on plates of MSM agar composed from. These components were dissolved in (1 L) distilled water and the pH was changed to 7. Then, 2 % (v/v) agar-agar was added, by streaking method, three replicates of plates were made for each flask, and all plates were incubated at 30 ˚C for 48 hours After incubation, plates were observed for bacterial growth [11].

2.3 Primary Screening for Engine Oil Degradation

Pure bacterial isolates were reactivated on nutrient agar medium and incubated at 30 ˚C for 24 hours. Then bacterial inoculum of each isolate was prepared using L broth and incubated at (30˚C for 18 hours). From 250 mL Erlenmeyer flasks filled with (50 mL) of liquid BHM with the pH set at 7.0.

Flasks were sterilized, after sterilization 1 % engine oil was added as a substrate. Bacterial inoculum (5 mL) from each reactivated bacterial isolate was used to inoculate the Erlenmeyer flasks and the flasks incubated at 30 ˚C for seven days in a shaker incubator. After the end of the incubation period, optical density, biomass, and the percentage of hydrocarbon decomposition were measured to determine the most efficient bacterial isolates in decomposition [12].

2.4 Secondary Screening

2.4.1 Formation of Clear Zone

A technique was used to validate the findings from the previous experiment. First, the engine oil was diluted with ether, and the ether solution as evenly poured over the surface of the solid MSM plates. The ether quickly evaporated, leaving behind a thin layer of oil on the agar surface. In the centre of solid MSM plates, a loop full of pure bacterial isolates was cultivated using a spreading method over a 1 cm area.

Plates were then incubated at 30 ˚C from 24 to 144 hours. For individual bacterial strain, all experiments were run in triplicate, and the diameter of the clear zone surrounding colonies was measured [13].

2.4.2 Growth on Mineral Salt Medium Agar Plates

Pure isolates were grown on mineral salt medium agar supplemented with 1 % v/v engine oil at pH 7 ± 0.2. Following sterilization, the medium was injected with three replicates of each bacterial isolate in

the centre of the agar plate. Isolates were in the centre of the agar plate and incubated for seven days at 30 degrees Celsius. Isolates can grow on this medium depends on the degree of microbial growth strong, weak, medium, or no growth. This method used to determine which isolates can grow in this medium [14].

2.5 Characterization and Identifications of Isolates

Bioremediation-capable bacterial isolates were selected based on their phenotypic characters are based on the colony's overall cultural features (colour, shape, texture, and size) and the Gram stain response [15]. Biochemical tests were performed in accordance with the standard procedures outlined in Bergey's manual of systematic bacteriology.

Testes including methyl red test, Voges Proskauer test, indole test, starch hydrolysis, catalase, oxidase, urease test, gelatine utilization test, motility test, and Simon citrate test. Isolates identified genotypically using the PCR technique to determine *P. putida* using specified primers forward and reverse of the 16S rRNA gene.

In PCR method made use of a specific primer for each gene (alkB, alkA, PAH) genes with a total volume (of 25µL) containing a master volume (of 25 µL) containing a master mix of 12.5 µL, DNA (5 µL), forward (1 µL), reverse (1 µL), and distilled water (5.5 µL). The specific primer for each gene was used in the PCR technique. The control tube consists of all PCR materials except target DNA. Positive results could be distinguished when DNA bands were the same size as the target product and the electrophoresis findings were detected using a UV-Transilluminator system.

3. Results and Discussion

3.1 Isolation and Identification Bacteria.

The study involved thirty-one isolates of hydrocarbon-degrading bacteria from engine oil-contaminated soil. These isolates included different species of bacteria that characterized bacterial isolates through morphological and biochemical techniques. Diversity of hydrocarbon compounds that contaminated soil samples in the study area, as well as environmental factors like temperature, pH, biomarkers, maturity level, and the presence or availability of other organic materials. All contribute to the variation in the amount of spilled petroleum compounds and diversity of isolated bacteria [16].

Another study microorganisms were isolated from the study area, which was polluted with crude oil spilled, were capable of surviving and living in

hydrocarbon-contaminated soil [17]. These bacterial isolates were subjected to a primary and secondary examination to determine the ability of bacteria for bioremediation of contaminated soil with used engine oil, enumeration of microbes in oil-contaminated soil, as shown in (table 1).

Table 1: Enumeration of microbes in oil-contaminated soil.

Isolates (n)	Used engine oil samples
2	O1
1	O2
4	O3
3	O4
2	O5
2	A6
2	O7
3	O8
6	O9
4	O10
2	O11
31	Total number isolates

3.2 Screening of Bacterial Isolates That Degraded Compounds

3.2.1 Primary Screening for Engine Oil Degradation

Bacteria were isolated from soil samples contaminated with engine oil were cultured in liquid BHM containing 1 % engine oil as the only carbon source. Samples then incubated for seven days at 150 rpm at 30 ºC in a shaker incubator. Samples (O1) and (O10) were more active and, isolate O9 was quite successful at engine oil degrading. Isolated bacterial O9 optical density, biomass, and hydrocarbon degradation % were 0.978 nm, 0.57 g/L, and 68 % respectively as demonstrated in (table 2).

Hydrocarbons in soil can be broken down more efficiently by native species that have been isolated from areas polluted by motor oil. Bacteria that develop on polluted soil have been shown to have a better capability to break down oil than microorganisms found in uncontaminated oil sites because they can adapt and thrive on hydrocarbon contamination sites [17].

According to Naeem [18], removal efficiencies of soil hydrocarbons C12-C40 achieved by bioremediation methods were 70 %, 55 %, and 45 %. Gathered nineteen strains of diesel-degrading bacteria from various sample locations, including soil garage sites [19]. Fourteen bacterial isolates were selected for further screening tests utilizing a clear zone and the capacity to grow on mineral salt agar combined with motor oil (1%).

Identification of bacteria that grew on the used engine oil at the most rapid rate. Results of the screening test were *pseudomonas sp.* and *stenotrophomonas maltophilia*. *Bacillus cereus* is more dominant, with the highest resistance to hydrocarbons and highest growth rate. *Bacillus cereus* may be more able than the others to use hydrocarbons as a carbon and

energy source, while some microorganisms can mineralize hydrocarbons [19-20].

Table 2: Variations between bacterial isolates in primary screening.

Isolates	Optical Density 600 nm	Biomass g/L	Hydrocarbon Degradation %
O1	0.247	0.08	10 %
O2	0.13	0.25	56 %
O3	0.513	0.34	58 %
O4	0.088	0.092	8 %
O5	0.168	0.08	28 %
O6	0.447	0.44	58 %
O7	0.997	0.45	50 %
O8	0.325	0.212	42 %
O9	0.978	0.57	68 %
O10	0.563	0.55	64 %
O11	0.17	0.16	52 %
O12	0.246	0.67	29 %
O13	0.218	0.44	28 %
O14	0.19	0.217	26 %

3.2.2 Result of Clear Zones

Experiments were done on isolates (O1-O11) that showed the highest degrading capability in the initial screening to create clear zones. The clear zone formed on solid MSM plates was used to quantify the amount of engine oil deterioration by fourteen different bacterial strains, as shown in (table 3).

Table 3: Clear zones.

Bacterial isolate	Clear Zones (mm) on BHM plates
O1	17
O2	42
O3	44
O4	51
O5	53
O6	43
O7	45
O8	41
O9	60
O10	55
O11	36
O12	39
O13	27
O14	22

3.3 Molecular Identification

3.3.1 Biochemical Characterization of Bacteria

Morphological and biochemical characterization results *s* is the most active bacterial isolates. Morphological and biochemical properties of isolate O9, gram reaction, gram negative, colony shape circular colony, colour (Creamy Mucous Texture), surface smooth, colony elevation convex, colony edge zigzag edges, methyl red (-), Voges Proskauer test (-), indole test (-), Gelatin test (-), starch hydrolysis (-), catalase test (+), oxidase test (+), urease test (-), motility test (+), Simon citrate test [22].

Revealed that the isolate exhibiting the highest capacity for oil breakdown was a catalase positive, Gram-negative *Pseudomonas sp*. and *Escherichia coli* strain. *Pseudomonas putida* because of its

remarkable resistance to a variety of xenobiotics, including hydrocarbons, and its superior internal metabolism, *Pseudomonas putida* seems to be particularly well-suited to create natural products [17]. Show that two isolates of *Pseudomonas spp*. and one isolate of *Rhodococcus erythropolis* from soil polluted were able to degrade 90 % of hydrocarbons in six weeks [22].

3.3.2 Polymerase Chain Reaction-Based Molecular Identification of Local Isolate (O9).

Presto Mini gDNA Bacteria kit was used to extract the genome of the local bacterial isolate O9. Confirming the existence and identifying catalytic genes. Three catabolic genes (alkA, alkB, PAH) that encode enzymes involved in the breakdown processes of alkanes and polycyclic aromatic hydrocarbons were identified in the purified DNA, and the 16S rRNA.

Throughout the genomic DNA was identified by a genotyping test employing universal primers. Moreover, (table 4), represent catabolic genes in isolates and reports a variety of alkane dioxygenases. that actively take part in alkane decomposition [22].

Table 4: Catabolic genes in isolates.

Isolates	Dioxygenase Enzyme Genes		
	AlkA	AlkB	PAH
O1	-	+	-
O2	+	-	-
O3	+	+	-
O4	+	+	+
O5	+	+	+
O6	-	+	+
O7	+	+	-
O8	+	+	-
O9	+	+	+
O10	+	+	-
O11	+	-	+
O12	-	+	-
O13	-	+	-
O14	-	+	-

Dioxygenase genes responsible for PAH-degrading [23].

The study observed the presence of three catalytic genes in the isolate O9, Isolate O4 and O5 with different levels as shown in (figure 1). Genes (alkA, alkB, PAH), were found in the DNA sequence of *Pseudomonas putida*. that certainly adapted to hydrocarbon-containing environments and able to degrade engine oil.

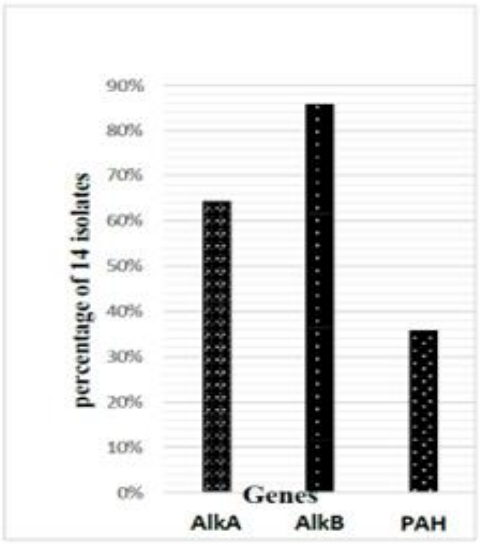

Figure 1: Percentage of catabolic gens (alkA, alkB, PAH) in isolates.

These bacterial isolates were identified pseudomonas putida by molecular identifications based on DNA extraction, amplification, and sequence analysis of 16S rDNA gene segments. The PCR reaction for the local bacterial isolate appears in (figure 4), that represents results of electrophoresis for the extracted PCR result. This data agrees with identification results determined by the biochemical tests. The genotype test uses universal primers to detect the 16S rRNA in the whole genomic DNA of the local bacterial isolate O9. The important step of the molecular analysis is to detect the 16SrRNA and alkB gene in the local isolate O9.

Results in (figure 2) showed that Gel electrophoresis of alkB gene PCR product, 1.6 % agarose gel, 100 v for 50 min. Lane M molecular marker, lanes 1-7 and 6 shows a positive test and a single band of about 641 bp. sequence to verify that the local bacterial isolate was *P. putida*. The presence of catabolic genes in the local isolate of *Pseudomonas putida* was discovered and validated by using the primer pair specific to the alkane hydroxylase (alkB) gene fragment for *Pseudomonas putida* [17].

Using 16s rDNA bacterial universal primers, bacterial isolates demonstrated a positive polymerase chain reaction (PCR) amplification product size of 1500 bp. AlkB gene expression was enhanced during incubation in every simulation. *Pseudomonas sp* alkane hydroxylase's alkB genes particularly control the breakdown of alkanes [24]. Eighteen bacterial isolates tested positive for the *Pseudomonas* species- specific PCR amplification product [25].

The PAH gene was also detected by the genotyping test, PCR product, 1.6 % agarose gel, 100 volts for 50 minutes. lane M molecular marker, lanes 1, 6, 7, 8, and 9 exhibit a single band of about 803 bp and a positive test result. Tests in lanes 2–5 is negative. As shown in (figure 3). PAH genes encode enzymes responsible for the breakdown of polycyclic aromatic hydrocarbons contaminants, can promote the formation of certain PHC degraders in the soil, which increases the amount of PHC breakdown genes as PHC [17].

PCR amplification using 16s rDNA primers yielded positive product sizes of around 1283 bp, with Lane M showing positive results.

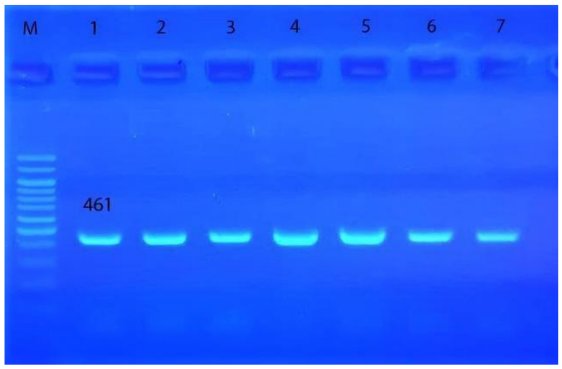

Figure 2: Gel electrophoresis of alkB gene.

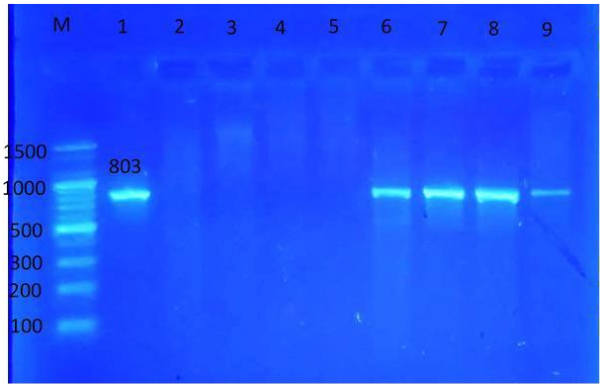

Figure 3: Gel electrophoresis of alk PAH PCR product.

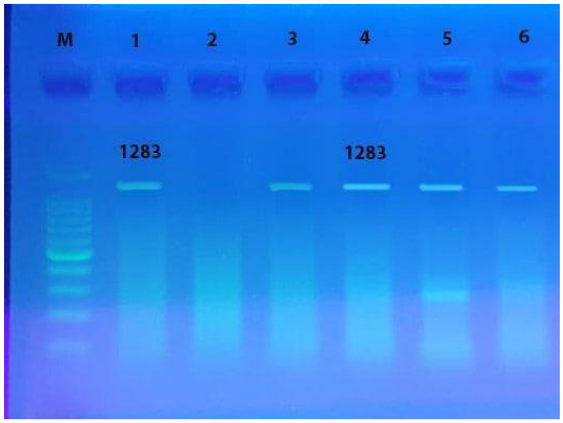

Figure 4: Gel electrophoresis of 16SrRNA gene PCR product.

4. Conclusion

Obtained results from the research showed that *Pseudomonas putida* isolated from the contaminated soil with engine oil can be used for the biodegradation process. *Pseudomonas putida* can degrade engine oil at 64 % and 68 %. The highest percentage compared to other isolates and would be more efficient in the decomposition of different components of hydrocarbons. *Pseudomonas putida* strains may be engineered to produce genetically modified species very effective in the bioremediation of soil contaminated with used engine oil.

5. References

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