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Isolation, Identification and Evaluation of Hydrocarbon Degradation Capabilities of Diesel Degrading Bacteria Isolated from Mechanic Workshop in Anambra State.

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Abstract- ***This work was aimed at isolating bacteria species capable of degrading diesel from hydrocarbon polluted soil. Mineral salt agar was used for the isolation by vapour phase transfer of diesel as the only source of carbon. Two morphologically different colonies that gave the best growth were identified using 16S rRNA sequence as Pseudomonas aeruginosa strain SI64S and Stenotrophomonas maltophilia strain H258. Shake flask degradation of 1% diesel showed that the two organisms can utilize diesel as the only source of hydrocarbon as indicated by their growth measured by optical density (OD) 600nm; 2.025 and 1.821 for Pseudomonas aeruginosa and Stenotrophomonas maltophilia respectively. On the effect of incubation time, the optimum growth for both Strains was observed at the 10th day of incubation with P. aeruginosa strain showing higher density of growth than Stenotrophomonas maltophilia strain. The optimization of growth conditions showed that pH 7 was the best for the growth of Pseudomonas aeruginosa strain S164S while Stenotrophomonas maltophilia strain H258 grew best at pH 8. The two isolates showed remarkable growth in all the nitrogen sources tested; with Ammonium nitrate giving the optimal growth for both strains. The ability to grow in higher concentrations of diesel up to 10% was also tested. The result showed that Pseudomonas aeruginosa can degrade up to 10% diesel as shown by its growth (OD 600nm, 0.452), while Stenotrophomonas maltophilia grew in up to 6% diesel with the optical density 0.316. The isolates were also tested for their ability to grow in different fractions of crude oil at 1% concentration. The result showed that Pseudomonas aeruginosa can utilize kerosene, crude oil, engine oil and condemn oil as the only source of carbon; while Stenotrophomonas maltophilia strain was able to utilize crude oil and kerosene but not engine oil and condemn oil. The result of this research shows that these two organisms isolated from our environment can conveniently be applied for bioremediation of hydrocarbon contaminated soil.***

Key words -Bacteria, Degradation, Diesel, Isolation, Mechanic-Workshop.

I. INTRODUCTION

Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce. Apparently inevitable spillage which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field (Okoh, 2003). Oil spill have become a global problem in industrialized and developing countries. Attention has been focused on the land and marine environment, because of the largest and most dramatic spills. Petroleum products such as diesel, fuel, Engine oil and Kerosene are widely used in various forms in mechanic workshops. These product tend to harden and change the colour of the soil, which have untold health hazard on technicians and artisans (Udeani et al., 2009). Used motor oil contains more metals and heavy polycyclic aromatic compound (PAH_s) that would contribute to chronic hazard (Broogmans et al, 2009). Prolonged exposure and high oil concentrations may cause the development of liver and Kidney diseases, possible damage to bone marrow.

Several bacteria have exhibited great abilities in utilizing the hydrocarbon substrate (Bogan et al., 2003; Chikere et al., 2009). These bacteria species are wide spread in pristine and oil polluted environment (Hamanura et al., 2006; Cappello et al., 2007; Van Beilen and Funhoff, 2007). Some organisms that are capable of degrading the diesel oil include Bacillus Subtilus, Bacillus spp., Alcaligenes spp., Flavobacterium spp., Micrococcus roseus,

Corynebacterium spp., Citrobacter freundii, klebsiella, Pseudomonas, Arthrobacter, Acromobacter, Alcaligenes, Nocardia and Actinomycetes (Amund, 2000, Wackett and Hershberger 2001; Parales et al 2002).

The problems of hydrocarbon contamination are known to be widespread in oil producing areas, and are of public health concern. Attempt to remediate hydrocarbon-polluted sites using physiochemical methods had been in existence since (Okoh, 2003), but, due to other implication, their application were discouraged. Bioremediation using microorganisms capable of degrading hydrocarbon is now believed to be more proving, effective, and environmental friendly. Search for specific microorganisms with such ability have been undertaken and their distribution and diversity are thus widespread (Onifade and Abubakar, 2007; Nkwelang et al., 2008).

Biodegradation has become one of the most popular and promising technologies with growing demand for the remediation of petroleum – contaminated soil because pollutants can be completely removed at low cost. This study will be significant as it will add more to the available literature on biodegradation potentials of isolates. It will also lay a good foundation for further research into the subject of study. The scope of the present work is to assess the degree at which soil isolated bacteria can degrade high concentration of diesel and other petroleum products. The main objectives of the study are to isolate hydrocarbon degrading bacteria from hydrocarbon contaminated soil, characterize and identify the microbial isolates, determine the time Course for diesel degradation, the effect of pH on the growth of the isolates in diesel, the effect of Nitrogen Source on the growth in diesel and to screen the microbial isolates for ability to degrade different concentrations of diesel in shake flask.

II. MATERIALS AND METHODS

Collection of Sample

Soil was collected from Trucks mechanic workshop that has been polluted for over 15 years due to vehicle emissions and hydrocarbon polluted soil in Nanka, Orumba North LGA of Anambra State, Nigeria. The contaminated soil was collected in clean polythene bags from two different parts. The soil sample showed dark colour with fine texture.

Media Used

Mineral salt medium (MSM) containing $NaNO_3$ (7g/L), K_2HPO_4 (1g/L), KH_2PO_4 (0.5g/L), KCl (0.5g/L), $MgSO_4.7H_2O$ (0.5g/L), 18g agar, 1% diesel were used for the isolation.

Isolation of Diesel Degrading Bacteria

10g of the soil sample was suspended in 100ml of sterile water in 250ml Erlenmeyer flask and shaken properly. O.1ml of 10^{-3} dilution of the soil suspension was inoculated into culture plates of sterile mineral salt agar using spread plate method. Sterile filter papers saturated with sterile diesel was placed on the cover of the plates containing the medium. The diesel served as the sole carbon and energy source for the growth of the microorganisms by vapour phase transfer. The plates were incubated at 37^o for 7 days. Colonies with different morphological appearance were selected carefully and sub-cultured two times in nutrient agar plates for purification. The inoculum was then transferred into nutrient agar slants supplemented with 1% diesel and stored for further experimental studies.

Shake Flask Degradation of Diesel

The ability of the isolates to grow in mineral salt medium (MSM) supplemented with 1% diesel was determined in a shake flask. The isolates were inoculated in 100ml Erlenmeyer flask containing 30ml mineral salt medium of pH 7.0, and incubated along with a blank flask containing mineral salt medium and 1% diesel without organism in a rotary shaker at 140 rotation per minute (rpm) at 35^OC for 7 days. The growth of the isolates was determined by optical density (OD) measured at 600nm in a Spectrophotometer. The optical density of the blank was subtracted from OD of the isolates in order to obtain the actual growth of the isolates.

Identification of the Isolates

Two of the isolates that gave the highest O.D. result were molecularly characterized and Identified using 16S rRNA sequences.

The primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTTGTT ACG ACT T) 3' were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30$\mu\ell$ reaction mixture by using a EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95 °C for 2minutes, 35 cycles of 95 °C for 1minutes,
55°C, and 72 °C for 1min each were performed, finishing with a 10-minute step at 72 °C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

Determination of Time Course for Diesel Degradation

The optimum time for diesel oil degradation was determined using 100ml of mineral salt medium inoculated with the isolates in a 250ml flasks, 5ml was taken after 2days interval for 15days and the O.D at 600nm in UV- spectrophotometer was determined.

Effect of pH on Diesel Degradation by the isolates.

In order to determine the optimum pH required for the microbial growth on diesel oil, mineral salt medium containing 1% diesel was prepared and sterilized. The pH was adjusted to different level (4, 5, 6, 7, 8, 9 and 10) using NaOH and HCl. Filter sterilized 1% diesel oil was added aseptically to the medium. The flasks were inoculated with the isolated bacterial cultures as usual, and incubated at 35^0C. After incubation, the growth for O.D at 600nm in UV Spectrophotometer was determined.

Effect of Nitrogen Sources on Diesel degradation

In order to determine various nitrogen sources required for microbial growth on diesel oil, mineral salt medium was prepared using different nitrogen sources (soy bean meal, Urea, Ammonium nitrate, peptone, Ammonium chlorine, yeast extract) in place of $NaNO_3$ and sterilized. The pH was adjusted to optimum. After sterilization 1% filter sterilized diesel oil was added aseptically to the medium. The isolated bacterial cultures were incubated for 7days at 35^0C in a rotary shaker at 140rpm. After incubation, the growth O.D. at 600nm in UV Spectrophotometer was measured.

Shake Flask Degradation of Different Concentrations of Diesel

Mineral salt medium (MSM) supplemented with different concentrations of diesel was used in the experiment. About 200ml mineral salt medium was prepared and distributed 30ml each into 100ml conical flasks labeled with different concentrations (2%, 4%, 6%, 8% and 10%) of Diesel sterilized in an autoclave and allowed to cool. Each of the distribution was inoculated aseptically, followed by the addition of diesel according to their percentages. The inoculated flasks were shaken well and covered with cotton wool. The flasks were incubated in a rotary shaker at 140 rotations per minute (rpm) at 35°C for 7 days. Blank flasks that contained mineral salt medium and 1% diesel were used as control. The growth of the isolates was determined by optical density (OD) at 600nm in a Spectrophotometer as before.

Degradation of Different Fractions of Crude Oil

Mineral salt medium (MSM) supplemented with 1% of five (5) different fractions of crude oil namely: kerosene, petrol (pms), diesel oil, condemn oil (used engine oil) and engine oil (unused) was used for the experiment.

About 200ml mineral salt medium was prepared and distributed 30ml each into 100ml conical flasks labeled with the different fractions and sterilized in an autoclave. The flasks were allowed to cool after sterilization. Each of the flasks was inoculated aseptically with 0.5ml of culture of the isolates in a nutrient broth. This was followed by the addition of 1% of the different fractions to their respective flasks.

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The flasks were gently shaken to allow for proper mixture and then covered with cotton wool and fool. The flasks were incubated in a rotary shaker at 140 rotations per minute (rpm) at 35°C for 7 days. Blank flasks that contained only mineral salt medium (MSM) and 1% of the different fractions served as control. The growth of the isolates was determined by optical density (OD) at 600nm in a Spectrophotometer.

III. RESULTS

The eight (8) isolates purified showed good growth in 1% diesel. The result of shake flask degradation of diesel by the isolates is shown in Table 1. Two of the isolates (N1and P1) that gave the highest optical density values of 1.821 and 2.025 were identified by genetic method to be Stenotrophomonas maltophilia strain H2S8 and Pseudomonas aerugirosa strain S164S respectively. It was observed that the highest growth of both isolates occurred between 8th and 10th day of incubation as shown by optical density in Figure 1. The optimum pH for the degradation was observed to be between 7 and 8 for both isolates as shown in Figure 2. Different Nitrogen sources tested were all utilized by the two isolates as shown in Figure 3. The two bacterial isolates grew well in different concentrations of diesel, but growth decreases as concentration increases (Figure 4). Pseudomonas aeruginosa grew well in all the fractions of hydrocarbon tested while Stenotrophomonas maltophilia grew well in only two of the fractions as shown in Figure 5.

Table 1: Growth of the isolates in 1% diesel.

Isolates	Optical Density (O.D.) at 600nm
B1	0.765
B2	0.687
E1	0.985
E2	0.750
N1	1.821
N2	1.672
P1	2.025
P2	1.098

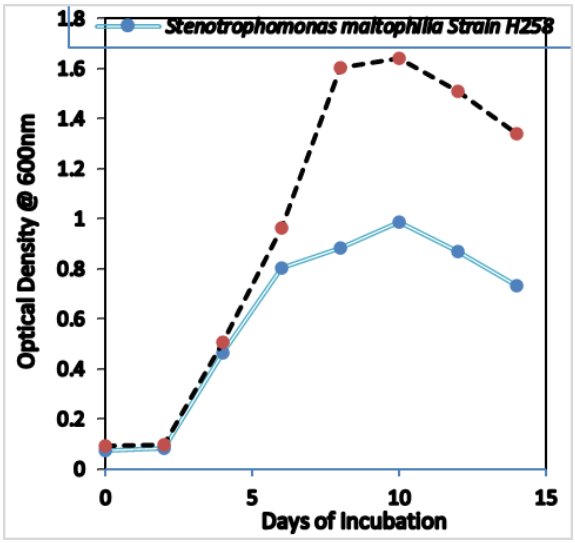

Fig. 1: Time Course for Diesel Degradation by the Isolates

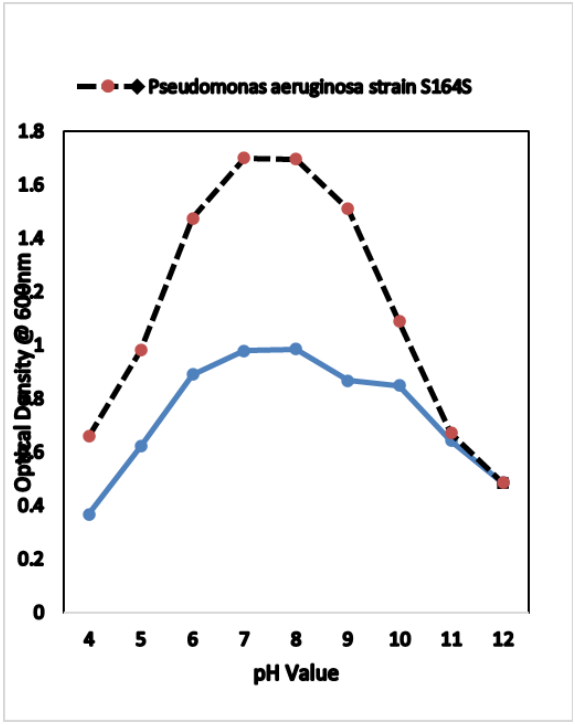

Figure 2: Influence of pH on diesel degradation by the isolates.

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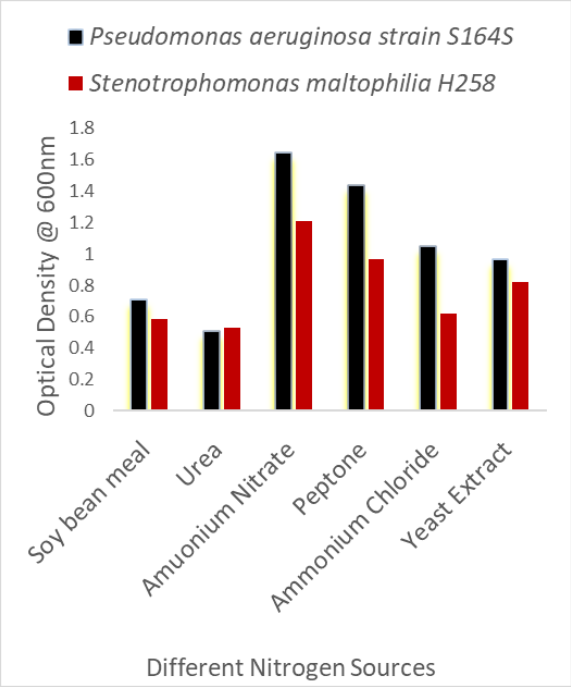

Fig. 3: Effect of Nitrogen Sources on Diesel degradation

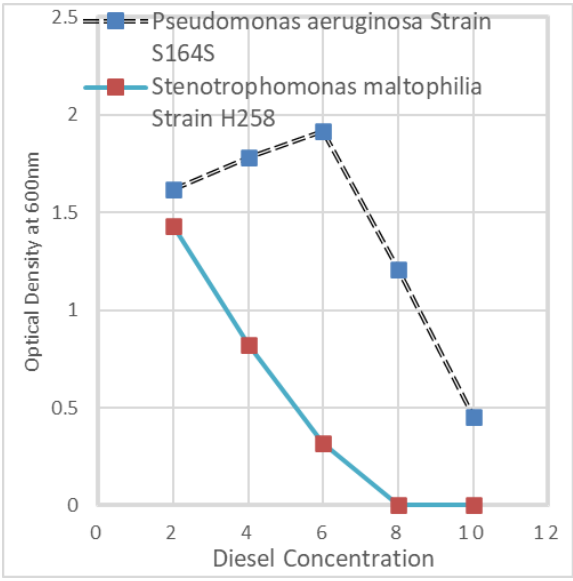

Fig. 4: Growth of the isolates in different concentrations of Diesel

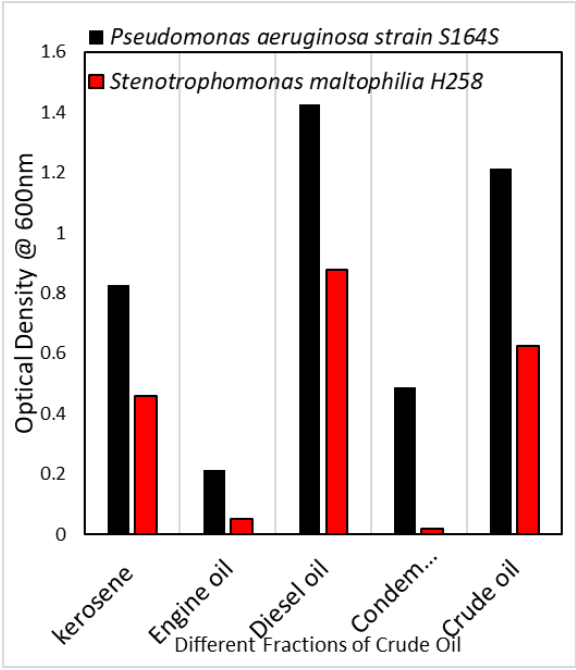

Fig. 5: Growth of the isolates in different fractions of Crude oil (1%)

IV. DISCUSSION

In the present study, two diesel-degrading bacteria species were isolated and identified to be Pseudomonas aeruginosa strain S164S and Stenotrophomonas maltophilia strain H2S8 using the 16S rRNA sequence. The result of this study agrees with the report of several researchers who reported that these organisms can degrade hydrocarbon at different rates. Okoh (2006) reported isolation of Pseudomonas aeruginosa in the clean-up of Petroleum hydrocarbon pollutants. Afuwale and Modi (2012) mentioned Stenotrophomonas sp. in the study of bacterial diversity of crude oil degrading bacteria isolated from crude oil contaminated sites. Wook et al (2006) reported Pseudomonas aeruginosa in biodegradation of crude oil. The degrading efficiency of isolated organisms was studied using Mineral salt medium (MSM) supplemented with 1% diesel. Among the two isolated species, Pseudomonas aeruginosa demonstrated a higher biodegradation rate than Stenotrophomonas maltophilia, as indicated by greater optical density measured using a Spectrophotometer at 600nm (Table 1). Increase in Optical density is regarded as utilization of the hydrocarbon as the sole carbon and energy source by

the bacteria species, hence cell multiplication occurred which resulted to increase in turbidity.

On the effect of incubation time, the optimum growth for both Strains was observed at the 10th day of incubation with S. aeruginosa strain showing higher density of growth (Figure 1).

This finding agree with the work of Gessesse, et al (2022), who in their research reported that strains of Pseudomonas aeruginosa and Stenotrophomonas maltophilia showed maximum growth in diesel at the 10th day of incubation. The optimization of growth conditions showed that pH 7 was the best for the growth of Pseudomonas aeruginosa strain S164S while Stenotrophomonas maltophilia strain H258 grew best at pH 8 (Figure 2). The isolates showed an optimum growth requirement indicating that pH control is very important to get optimum results during bioremediation process. Therefore, the optimization of environmental conditions is important for the enhancement of bacterial growth and for designing effective bioremediation strategy. The requirement of neutral or near neutrality for optimal growth of bacteria on diesel is also exhibited by many other diesel-degrading bacteria (Marquez-Rocha et al., 2005; and Rajasekar et al., 2007). The two isolates showed remarkable growth in all the nitrogen sources tested. However, Ammonium nitrate gave the optimal growth for the two strains (Figure 3). This is an indication that the source of nitrogen is an important factor to be considered in biodegradation of hydrocarbons (Kwapisz,et al, 2008). Different organisms may also behave differently with respect to nitrogen sources.

The same organisms were tested for the ability to degrade different percentages (2%, 4%, 6%, 8% and 10%) of diesel under the same conditions. Pseudomonas aeruginosa showed an increase in cellular density with respect to growth tolerance occurring at maximum 6 % (v/v) of diesel concentration with OD of 1.915. The result also showed that Pseudomonas aeruginosa can grow very well at 10% concentration of diesel with OD 0.452, while Stenotrophomonas maltophilia showed highest cell density of OD of 1.432 at 2% Diesel concentration and can only grow up to 6% diesel with OD 0.316 (Figure 4). The selected bacterial strain Pseudomonas aeruginosa Strain S164S was able to tolerate high diesel concentration; this suggests that the strain is a better applicant for diesel biodegradation. Degradation at a much higher concentration (7.5% v/v diesel) has been reported (Arabo et al 2022).

Furthermore, the efficacy of these organisms was tested on its ability to degrade different fractions of petroleum products. The result showed that Pseudomonas aeruginosa strain was able to degrade all the fractions which includes; kerosene, engine oil, crude oil, and condemn oil (Figure 5). This agrees with the work of Hong et al. (2005) who degraded diesel and other fractions using Pseudomonas aeruginosa. The result also showed that Stenotrophononas maltophilia strain was able to degrade Kerosene and Engine oil but showed insignificant growth in crude oil and condemn oil (Figure 5). This is in agreement with the work of Ebrahimi et al. (2012) who reported Stenotrophomonas sp. as a strain with low degradation efficiency of certain fractions of petroleum hydrocarbon. In their study they reported that Stenotrophomonas sp. degraded crude oil and kerosene but not engine oil and condemn oil.

This study therefore proves that microorganisms found in oil – polluted environments degrade hydrocarbons at different rates. It also shows that these two organisms isolated from our environment can conveniently be applied for bioremediation of hydrocarbon contaminated soil.

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