

*Full Length Research Paper*

# Degradation of diesel oil in a polluted soil using *Bacillus subtilis*

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Diesel oil, left standing in a laboratory for six months, was used as source for the isolation of *Bacillus subtilis*, *Bacillus cereus*, *Trichoderma harzanium* and *Trichothercium roseum*. These organisms were found to be hydrocarbon degraders. On further testing, it was found that *B. subtilis* had higher potential to utilize diesel oil as carbon source. Soil samples were polluted with diesel oil at a loading rate of 5% (v/w) (oil/soil). These soil samples, together with the unpolluted control samples, were seeded with the *B. subtilis* isolate. The degradation of the diesel oil was monitored over a twenty-seven -day period, using gravimetric method. The rates of degradation of diesel oil by the isolate at the end of day one, day twelve and day twenty-seven were  $5.8 \times 10^{-4}$ ,  $1.83 \times 10^{-3}$  and  $1.05 \times 10^{-3}$  g/h, respectively.

**Key words:**Degradation, diesel oil, *Bacillus subtilis*, *Bacillus cereus*, *Trichoderma harzanium*, *Trichothercium roseum*.

## INTRODUCTION

Nigeria is a major producer of crude oil in the world and pollution of the environment due to oil spillage has steadily increased. In the Niger Delta Area alone, there have been over 550 reported cases of crude oil spillage since 1976, releasing about 2.8 million barrels of crude oil into the environment (Korie-Siakpere, 1998; Odieta, 1999). Crude oil originating from different parts of the world will differ considerably in their physical and chemical properties. These differences become important in relation to the behaviour of spilled oil in hot environment and subsequent clean up techniques (Awabajo, 1981).

Diesel oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel oil by some vehicles and generators, greater quantities are being transported over long distances. Therefore diesel oil can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanics (Hill and Moxey, 1960). Diesel oil spills on agricultural land generally reduce plant growth. Sugges-

ted reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants (Baker, 1982) and reduced germination (Udo and Fayemi, 1975) to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel oil.

Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods (Ijah and Okang, 1993). This is possible because microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy (Ijah and Antai, 1988; Ezeji et al., 2005; Antai and Mgbomo, 1993). This study was designed to isolate, characterize and identify diesel oil-degrading microorganisms. Diesel oil degradation by the isolates was further monitored to determine their potentials as bioremediation agents.

## MATERIALS AND METHODS

### Isolation of microorganisms

Microorganisms capable of degrading diesel oil were isolated from diesel oil left standing for six months in the Microbiology Laboratory,

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Federal University of Technology, Owerri. One ml of this contaminated diesel oil was serially diluted up to  $10^{-8}$  dilution. An aliquot (0.1 ml) of the  $10^{-8}$  dilution of the contaminated diesel oil was plated in duplicates onto modified diesel medium using the pour plate method. The modified diesel medium comprised of 1.4 gm  $K_2HPO_4$ , 0.2 gm  $(NH_4)_2SO_4$ , 0.6 gm  $KH_2PO_4$ , 0.6 gm  $MgSO_4 \cdot 7H_2O$ , 4 gm agar – agar and 2 ml distilled water. The mineral components of the medium were dissolved in 200 ml of distilled water and mixed with 4 gm agar – agar and 2 ml diesel oil. The medium was autoclaved at  $121^\circ C$  for 15 min according to Okpokwasili and Ananchukwu (1988). The plates were incubated at  $37^\circ C$  for 48 h. After incubation, the plates that were between 30 to 200 colonies were used. Each bacterial colony type was subcultured repeatedly into nutrient agar plate to obtain a pure culture. The isolates were characterized based on cultural characteristics, cell morphology and biochemical characteristics. The organisms were further identified using the methods of Gerharelt et al. (1981) and screened for their utilization of diesel oil using the methods of Abu and Ogiji (1996).

#### Hydrocarbon utilization test

The isolates which include 2 bacteria and 2 fungi were each tested for their ability to utilize diesel oil as sole source of carbon and energy for growth. Each isolate was steak-inoculated unto modified mineral salt agar medium which contain a filter paper soaked with diesel oil. The plates were incubated for 7 days at room temperature using the methods of Abu and Ogiji (1996).

#### Determination of microbial colony numbers for degradation studies

Using a sterile pipette, 5 ml of nutrient broth were transferred into a bÿou bottle and aseptically inoculated with a loopful of pure stock culture of *Bacillus subtilis* isolate and incubated at  $37^\circ C$ . Five percent (v/w) of the inoculum was transferred into another bÿou bottle and incubated at  $37^\circ C$  from where samples were taken in a cuvette at 6 h intervals beginning from zero and their corresponding absorbances measured at 540 nm using Comspec visible spectrophotometer. This procedure was continued till a 36-h inoculum was prepared and inoculated, whose absorbance remained consistent for triplicate measurements. Microbial inoculum (0.1 ml) was used to inoculate the polluted and control soil samples for degradation studies. The population of *B. subtilis* used for seeding purposes was calculated using the relationship: Colony forming unit (cfu) = (number of colonies x dilution factor) / volume of inoculum used.

#### Sample collection and preparation

Top soil sample was collected from the premises of the Federal University of Technology, Owerri, in sterilized plastic containers and taken to the laboratory. Soil sample meant for degradation studies was sterilized using autoclave at  $121^\circ C$  for 15 min, after which it was allowed to cool to room temperature for further treatments.

#### Description and treatment of samples

The soil samples in each group were treated as follows:

**Group A:** 33 samples of 20 g sterilized soil mixed with 1 ml (0.85 g) diesel oil plus 0.1 ml ( $4.2 \times 10^6$  cells/ml) *B. subtilis*.

**Group B:** 6 samples of 20 g sterilized soil mixed with 1 ml (0.85 g) diesel oil plus 0.1 ml distilled water.

**Group C:** 6 samples of 20 g sterilized soil mixed with 1 ml distilled water plus 0.1 ml ( $4.2 \times 10^6$  cells/ml) *B. subtilis*. Groups B and C

served as controls.

#### Diesel oil degradation studies

The ability of *B. subtilis* to degrade diesel oil was demonstrated in terms of reduction in the quantity of diesel oil introduced to pollute the soil samples. The rate of utilization was monitored on the first day (day zero) of the study and subsequently at 3-day interval for 27 days. Carbon tetrachloride was employed as the extractant. On each day, three samples per single treatment were analyzed for the quantity of residual diesel oil using the methods of Udeme and Antai (1988).

Each of the 20 g soil treatment samples was mixed with 40 ml of carbon tetrachloride, placed in a separating flask, shaken vigorously for 3 min and allowed to settle for 5 min. The liquid phase was separated by allowing the mousse (diesel oil – carbon tetrachloride) to pass gradually through a funnel fitted with filter paper (Whatman No 1). Anhydrous sodium sulphate spread on the filter paper was employed to remove any moisture in the mixture. The liquid phase was collected in a 50-ml pre-weighed pyrex beaker. The beaker containing the extract was placed in an oven and the extractant, allowed to evaporate at  $50^\circ C$ . The beaker with the residual diesel oil was allowed to cool to room temperature and weighed to determine the quantity of residual diesel oil by difference, according to Udema and Antai (1988).

The percentage of diesel oil degraded at three days interval was determined from the equation: % diesel oil degraded = (weight of diesel oil degraded / original weight of diesel oil introduced)  $\times$  100. Where the weight of diesel oil degraded was determined as original weight of diesel oil minus weight of residual diesel oil obtained after evaporating the extractant. Rate of degradation = weight of diesel oil degraded (g) / time taken (h).

## RESULTS AND DISCUSSION

Tables 1 and 2 show that, using cultural characteristics, cell morphology and biochemical characteristics, two *Bacillus* species; *B. subtilis* and *Bacillus cereus* and two fungal species; *Trichoderma harzanium* and *Trichothercium roseum*, were identified. Total viable counts of bacteria and fungi isolates in diesel oil yielded the results presented in Table 3. From this table, it is seen that the number of viable bacteria especially *B. subtilis* is greater than the other isolates (*B. cereus*, *T. harzanium* and *T. roseum*). Figure 1 shows the pattern of growth of *B. subtilis*. The pattern of microbial growth differs from organism to organism. The factors that determine this pattern of growth include the incubation period, the nature and composition of the nutrient in which the organism is growing. The ability of *B. subtilis* to utilize and degrade diesel oil within the 27 days is shown in Figure 2 while the quantity of diesel oil extracted at 3-day interval is shown in Table 4.

In the present study, *B. subtilis* proved to be a better hydrocarbon degrader than the other isolates. This observation agrees with that of Atlas (1984) and Bartha and Atlas (1977) who reported that refined petroleum product supply only carbon and energy to resident microorganisms while crude oil supplies, in addition to carbon and energy, mineral nutrients such as nitrogen, sulphur and heavy metals. *B. cereus*, *T. harzanium* and

**Table 1.** Morphology and biochemical characteristics of bacterial isolates.

Character	Isolates	
	B1	B2
Colony morphology on diesel oil and nutrient agar	Cream, big, spreading, finely wrinkled and slimy	Cream, big, flat Irregular white colonies
Gram stain	Rod	Rod
Spore	Central spores	Central spores
Motility	+	+
Catalase	+	+
Oxidase	-	-
Citrate	+	+
Indole	-	-
Gelatin	+	+
Glucose	Acid production	Acid production
Lactose	Gas production	Acid production
Sucrose	Acid production	Gas production
Mannitol	-	+
Organism	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>

**Table 2.** Cultural characteristics of fungal isolates.

Isolate number	Colonial morphology	Microscopy	Organism
F1	Whitish green colony in tufted collodial areas with reverse side being colourless	Well branched conidiophore with short branches at the apex	<i>Trichoderma harzanium</i>
F2	Light, brown relvely margin and a central tuft with fungilose all over the surface, with the reverse side being light brown in colour	Conidiophore unbranched which bears basipetal in zig-zag-chain like fashion. The candia had obliquely base scares.	<i>Trichothercium roseum</i>

*T. roseum* were not further used because their degradation potentials were low compared to that of *B. subtilis* (Table 3). This study was therefore limited to degradation of diesel oil in polluted soil using *B. subtilis*.

Microorganisms (bacteria and fungi) have different rates at which they utilize and degrade hydrocarbons in the soil or water. This rate is reflected in the multiplication and colony forming units (cfu) for the isolated organisms. The use of microorganisms to degrade petroleum hydrocarbon resulting from oil spillage has been a subject of extensive research since the first publication of bacterial growth on petroleum hydrocarbons (Atlas, 1981; Gerson, 1985). Several petroleum hydrocarbon degrading microorganisms have been isolated from both soil and marine sources, which are the two major environments affected by petroleum hydrocarbon pollution (Bossert and Bartha, 1984; Antai and Mgborno, 1989).

However, Stanbury and Whitaker (1989) highlighted that different organisms have different incubation periods, which range from minutes to several hours. It was observed from this study that the organisms isolated increased in the number of their colonies with time

**Table 3.** Microbial isolate loads on diesel oil.

Isolate	Colony forming units (cfu)
<i>Bacillus subtilis</i>	$5.1 \times 10^5$
<i>Bacillus cereus</i>	$2.8 \times 10^3$
<i>Trichoderma harzanium</i>	$1.2 \times 10^2$
<i>Trichothercium roseum</i>	$1.1 \times 10^2$

(Figure 1). This was apparent from the measurement of the absorbance at 540 nm for *B. subtilis*.

Atlas and Bartha (1972) reported that the application of crude oil to Arctic tundra soil caused overall increase in microbial numbers compared to un-oiled reference (control) soil. Atlas and Bartha (1972) also reported obtaining  $7.5 \times 10^5$  colony forming unit from un-oiled reference control soils while  $41 \times 10^7$  colony forming unit was obtained from a 5 L crude oil polluted soil after 14 months.

In this study, polluted soil challenged with *B. subtilis* showed a reduction in the quantity of diesel oil with time.

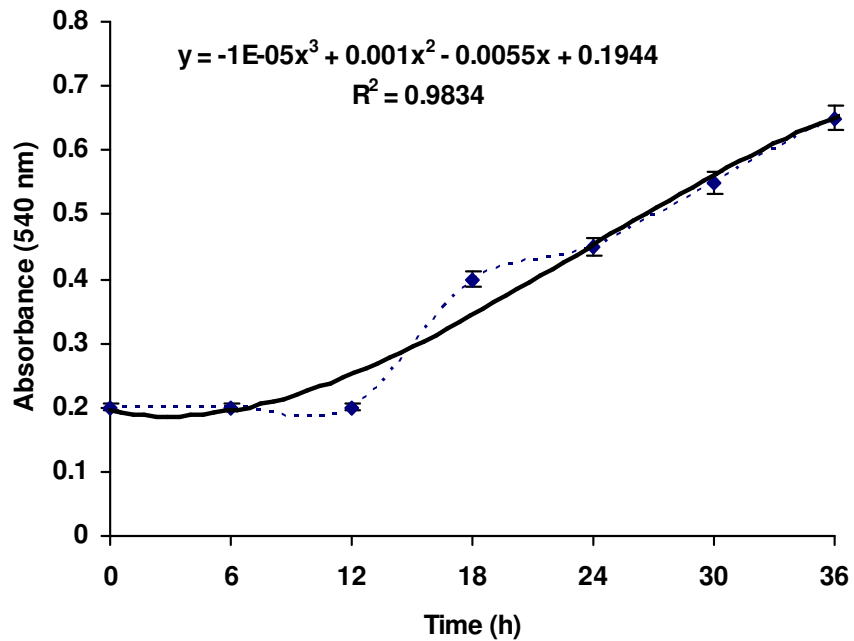


Figure 1. The pattern of growth of *Bacillus subtilis* in a given nutrient medium.

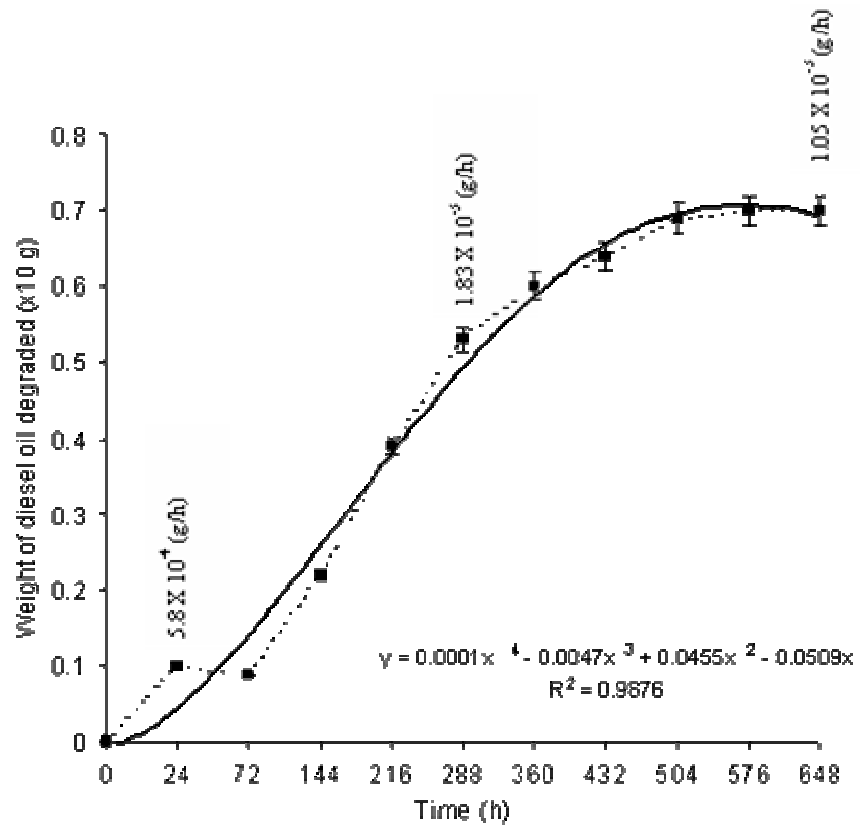


Figure 2. Diesel oil degraded with time (h) using *Bacillus subtilis*.

Udeme and Antai (1988) reported employing fungi singly for the degradation of crude oil in water. Isinguzo and

Odu (1987) on the other hand, reported employing a mutant strain of *Bacillus pumilus* to degrade crude oil in

**Table 4.** Weight of diesel oil extracted (on various days) from 20 g soil samples polluted with 1 ml (0.85 g) of diesel oil.

Day	Sample description	Weight of diesel oil extracted (g)*
0	Sterilized soil mixed with diesel oil plus <i>Bacillus subtilis</i>	0.85 ± 0.01
1	Sterilized soil with diesel oil plus <i>Bacillus subtilis</i>	0.83 ± 0.03
	Sterilized soil plus <i>Bacillus subtilis</i>	0.00 ± 0.00
	Sterilized soil mixed with diesel oil	0.85 ± 0.02
3	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.76 ± 0.01
6	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.63 ± 0.01
9	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.45 ± 0.02
12	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.32 ± 0.01
15	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.24 ± 0.02
18	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.21 ± 0.01
21	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.18 ± 0.01
24	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.16 ± 0.01
27	Sterilized soil plus <i>B. subtilis</i> mixed with diesel oil	0.14 ± 0.02
	Sterilized soil plus <i>B. subtilis</i>	0.00 ± 0.00
	Sterilized soil mixed with diesel oil	0.80 ± 0.03

\* Values are means of triplicate determinations.

soil. The soil they used was mixed with paraffin-supported-nitrogen fertilizer (PSF) as nitrogen source. Rosenberg (1972) in his reports highlighted using mixed cultures of bacteria to degrade crude oil in soil. The soil used was thoroughly mixed with inorganic fertilizer, which served as nitrogen and phosphorus sources.

In this study, the *B. subtilis* was used singly and there was no addition of fertilizer as a source of nitrogen or phosphorus. The results obtained from this study indicate that (i) *B. subtilis* has a high potential to degrade diesel oil at  $5.8 \times 10^{-4}$ ,  $1.83 \times 10^{-3}$  and  $1.05 \times 10^{-3}$  g per hour for day 1, 12 and 27, respectively (Figure 2). and (ii) microbial degradation of petroleum hydrocarbon is not restricted to crude oil, it can occur in other petroleum hydrocarbon cuts such as diesel oil.

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