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ORIGINAL ARTICLE

APLICATION OF BACILLUS SUBTILIS IN DEGRADATION OF DIESEL OIL AT POLLUTED SOIL IN GILAN

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Abstract: 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. 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 6.8 x 10-4, 1.73 x 10 -3 and 1.04 x 10 -3g/h,respectively. The results indicate that native strains have great potential for in situ remediation of diesel-contaminated soils in oil refinery sites.

Keywords: Diesel oil, Soil, Trichoderma harzanium, Bacillus

INTRODUCTION

Iran is the first country in the oil-rich Middle East region to start oil operations with current production capacities of over 4 million barrels of crude oil and 80,000 m^3/day of diesel fuel. There are up to 1,500,000 cubic meters of soil contaminated with crude oil around the Tehran refinery, Iran. Soil and ground water are often contaminated with gasoline or diesel fuel from leaking underground storage tanks and also due to accidental spills and leakage from pipelines. Due to their mobility, these compounds may cause considerable damage not only in soils, but also in water intakes or ground water reservoirs [20].

The carbon number of diesel oil hydrocarbons is between 11 and 25 (2000 to 4000 hydrocarbons) and the distillation range is between 180 to 380 °C [11]. Diesel oil is a complex mixture of normal, branched and cyclic alkanes and aromatic compounds with the properties of low water solubility, high adsorption coefficient and high stability of the aromatic ring [10; 23; 21; 27].

Oil spillage and oil pollution in water environment have been a major threat to the ecosystem and human being through the transfer of toxic organic materials including polycyclic aromatic hydrocarbons (PAHs) into the food chain [1].Oil-degrading microorganisms are ubiquitous. They can be isolated from contaminated soils where the oil seepage occurs. In this study, five different bacterial species were isolated from soil contaminated with used engine oil and their ability to degrade used engine oil in vitro was studied.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 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 [16]. Diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants [7]and reduced germination [26] and to unsatisfactory soil condition due to insufficient aeration of the soil because of the displ acementof air from the space between the soil particles by diesel oil. One of the best approaches to restoring contaminated soil is

to make use of bioremediation which is an attractive approach of cleaning up petroleum hydrocarbons because it is simple to maintain, in This paper was designed to isolate, characterize and identify diesel oil-degrading microorganisms.

MATERIALS AND METHODS

Collection of soil samples

Based on the previous studies reported on the presence of oil degrading microbes in oil contaminated soil, soil sample was collected from a mechanic workshop situated in Gomtinagar Soil was collected randomly 5-10 cm beneath the surface using a sterile spatula and were packed in sterile polybags and transferred to the laboratory for further studies**.**

Isolation of microorganisms

Microorganisms capable of degrading diesel oil were isolated from diesel oil left standing for six months in the Microbiology Laboratory, 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 unto modified diesel medium using the pour plate method. The modified diesel medium comprised of 1.4 gm K 0.2 gm (NH 4)2SO4, 0.6 gm KH2PO4, 0.6 gm MgSO4 7HO, 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 o C for 15 min. The plates were incubated at 37 oC for 48 h. After incubation, the plates thatwere 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 biochemicalcharacteristics[2;3]. The organisms were further identified using the methods of [14] and screened for their utilization of diesel oil using the methods of Abu and Ogiji [6;13].

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 modifiedmineral salt agar medium which contain a filter paper soaked with diesel oil. The plates were incubated for 7 days at room temperature [5] Determination of microbial colony numbers for degradationstudies Using a sterile pipette, 5 ml of nutrient broth were transferred into abÿou bottle applicable over large areas, cost-effective an leads to the complete destruction of the contaminant [2].

and asceptically inoculated with a loopful of pure stockculture of ***Bacillus subtilis*** isolate and incubated at 37C. Fivepercent (v/w of the inoculum was transferred into another bÿoubottle and incubated at 37oC from where samples were taken in acuvette 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 wasprepared and inoculated, whose absorbance remained consistentfor triplicate measurements. Microbial inoculum (0.1 ml) was usedto inoculate the polluted and control soil samples for degradationstudies. The population of ***B. subtilis*** used for seeding purposeswas calculated using the relationship: Colony forming unit (cfu) =(number of colonies x dilution factor) / volume of inoculum used.

Sample collection and preparation

Soil sample meant for degradation studies was sterilized using autoclave at 121o 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 X 10 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 x 1062o cells/ml) B. subtilis. Groups B and C

Diesel oil degradation studies

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 o C. The beaker with the residual diesel oil was allowed to cool to room temperature and weighed to deter-mine the quantity of residual diesel oil by difference, according to [26].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) × 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

In the present study, B. subtilis proved to be a better hydrocarbon degrader than the other isolates. This observation agrees [4;8] 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 T. roseum were not further used because their degradation potentials were low compared to that of B. subtilis .

Tables 1 and 2 show that, using cultural characteristics, cell morphology and biochemical , two Bacillus species; B. subtilis and Bacillus cereus and two fungal species; Trichoderma harzanuum and Trichotherciuroseum, were identified. 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 harzanuum and Trichothercium roseum, were identified. Total viable counts of bacteria and fungi isolates in diesel oil yielded the results presented in Table 3.

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.

Table 1. Morphology and biochemical characteristics of bacterial isolates

Caracter	Isolates	
Colony morphology on diesel oil	B1	B2
Gram stain	Rod	Rod
Spore	Central spores	Central spores
Motility	+	+
Catalase	+	+
Oxidase	–	–
Citrate	+	+
Gelatin	+	+
Mannitol	–	+
Glucose Acid	production Acid	production Acid
Lactose Gas	production Acid	production Acid
Sucrose Acid	production Gas	production Gas
Organism	Bacillus subtilis	Bacillus cereus

Table 2. Cultural characteristics of fungal isolates.

Isolate number	Colonial morphology	Microscopy	Organism
F1	Whitish green colony in tufted collidial areas with reverse side being colourless	Well branched conidiophore with short branchesat the apex	Trichoderma harzanium

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.	Trichothercium roseum

Table 3. Microbial isolate loads on diesel oil.

Isolate	Colony forming units (cfu)
Bacillus cereus	2.8×10^3
Bacillus subtilis	5.1×10^5
Trichothercium roseum	1.1×10^2
Trichoderma harzanium	1.2×10^2

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

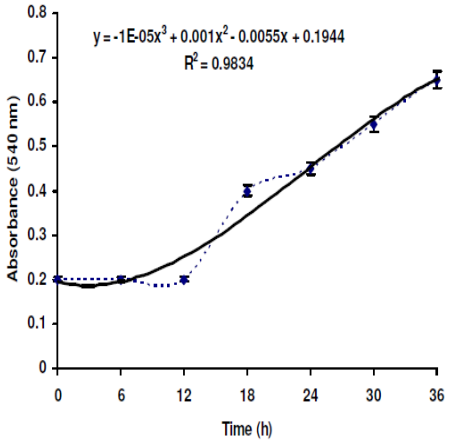

Figure 1. The pattern of growth of *Bacillus subtilis* in a given nutrient medium.

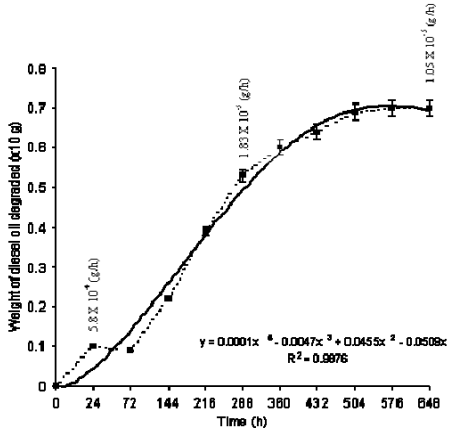

Figure 2. Diesel oil degraded with time (h) using Bacillus subtilis.

The soil they used was mixed with paraffinsupported-nitroge fertilizer (PSF) as nitrogen source. 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 x 10 -4 , 1.83 x 10-3 and 1.05 x 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.

CONCLUSION

Degradation of hydrocarbons by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutant. It has also been shown that bacteria are the most predominant microorganism among other microorganisms in either in situ or ex situ bioremediation processes, indicating that bacteria are the main agents responsible for the degradation of diesel fuel. This paper describes the first study on the isolation and characterization of commercial diesel-degrading bacterium from Iranian soils.

Based on the present study it can be concluded that *Bacillus* species inhabiting the oil contaminated sites can be a good source for the degradation of oil. As oil pollution caused due to any mode is very much harmful for both plant and animal life, bioremediation which is a better mode than the traditional methods discussed earlier can be a very good mode for the cleaning up of environment contaminated with oil. Finally, the selected bacterial isolates could be effective in clearing oil spills and the mixed bacterial culture can efficiently degrade the crude oil components.

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