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ARTICLE

Assessment of diesel degrading potential of fungal and bacterial isolates from Egypt

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Abstract

Two naturally occurring fungal and bacterial species, *Aspergillus flavus* and *Bacillus* sp. H6 strains, were capable of utilizing diesel oil as a sole source of carbon in synthetic microcosoms. The initial diesel oil contamination of 1666 mg kg^{-1} dry soil was reduced to 166.667 mg kg^{-1} after 150 days of incubation in fungal-bacterial consortium microcosm. That is mean 89.9%, of the initial oil concentration was removed. Abiotic process reduced the diesel oil contamination to about 616 mg kg^{-1}dry soil at the end of the experiment. Seven microcosms were set up to fulfill the experiments. The decontamination activity follow this order; *Bacillus* sp. H6 + *A. flavus* consortium > *Bacillus* sp. H6 > natural control > *A. flavus* > cycloheximide treated > benzyl Penicillin-Streptomycin treated > poisoned control. Gas chromatographic analysis data revealed that both *A. flavus* and *Bacillus* sp. H6 treatment led to complete utilization of carbon-17 compounds. Other biodegradation products such as C-15, 16, 20, 21, and 24 appears in the chromatogram after 150 days incubation. Increase of C-20, 21 and C-24 compounds also noticed. The fungal- and bacterium consortium treatment depicted a decrease of all detected n-alkanes. The microbial success in biodegradation was evaluated by determining the number of germinating seeds of *Phaseolus vulgaris*. The highest level of germination (92 %) was detected in consortium microcosms after 150 days incubation. The treatment with *A. flavus* and *Bacillus* sp. H6 separately led to a lower percentage of germination (86%). The other treatments showed variable results except the poisoned control that showed negative germination and minor chemical degradation of diesel oil. Thus, bacterial-fungal consortium treatment is effective in bioremediation of contaminated oils than separate treatment.

Key words – Diesel, biodegradation, *Aspergillus flavus*, *Bacillus* sp. H6.

Introduction

Diesel oil is one of the major contaminants of soil and groundwater. This is almost due to industrial wastes and oil spill accidents. Consequently, littoral marshes ecosystems and other coastal entertainment are known to severely damage from the impacts of large amounts of noxious compounds, especially petroleum oil spills (Mille *et al*. 1998; Teal *et al*. 1992; Duke and Watkinson 2002).

Several methods can be used to remove hydrocarbons from soil and groundwater, including air spraying, soil vapor extraction, and bioremediation. Strategies for inexpensive and clean in situ bioremediation include natural attenuation, biostimulation, bioventing, bioaugmentation, land farming, composting, and phytoremediation. These methods can be reviewed in numerous publications reporting on the bioremediation diesel oil (Richard and

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Vogel 1999; Olson *et al.* 1999; Gallego *et al.* 2001; Bento *et al.* 2004; EL-Morsy 2005; EL-Morsy *et al.* 2006) in soils.

Many isolated bacterial and fungal species have been reported to be capable of effectively in biodegradion of petroleum hydrocarbons and even polynuclear aromatic hydrocarbons (Giraud *et al.* 2001; Márquez-Rocha *et al.* 2005; Xiaojun *et al.* 2008; You-Qing *et al.* 2008). Petroleum products contain a mixture of several hydrocarbons, which are difficult to degrade by any one bacterium. Short-chain alkanes are toxic to many microorganisms and are difficult to degrade. Intermediate chain lengths (C10-C24) are degraded most rapidly. Very long chain alkanes become increasingly resistant to biodegradation. Monoxygenases and dioxygenases are a group of the enzymes involved in the degradation of alkanes. The aromatic hydrocarbons present in petroleum are also difficult to degrade.

Bioremediation is being used or proposed as a treatment option at many hydrocarbon-contaminated sites (Braddock *et al.* 1997). The effectiveness of bioremediation is often a function of the microbial population or consortium and how it can be enriched and maintained in an environment (Márquez-Rocha *et al.* 2001). The ability of fungi to transform a wide variety of hazardous chemicals has aroused interest in using them in bioremediation (Alexander 1994). Biodegradation of oil spills is a major problem because it usually occurs in marine water surface and seeding with bacteria becomes difficult.

It is well known that very few organisms are able to degrade a single compound completely. Degradation of organic compounds in the natural environments is often the result of a community-interacting microbial population, generally termed as a 'consortium'. Fungi and bacteria have been shown to biodegrade organic pollutants via both non-enzymatic and enzymatic processes. Therefore, a synergistic effect is expected in the biodegradation by simultaneous use of bacteria and fungi. Chávez-Gómez *et al.* (2003) stated that several fungal and bacterial co-cultures exhibited synergism for phenanthrene removal, reaching about 70% in 18 days. Likely, You-Qing *et al.* (2008) found a significant synergistic effect between Cladosporium and Mycobacterium resulted in approximate complete degradation (99%) of diesel oil. However, Xiaojun *et al.* (2008) stated that microbial consortia could degrade polycyclic aromatic hydrocarbons (PAHs), but the highest PAH removals were detected by fungi only.

In Egypt, attention has been devoted to the application of crude oil degrading microorganisms in the bioremediation of crude oil spills left at the end of 1967 war in Suez Gulf area (Ammar *et al.* 1997; Ammar and Shady 2001). The aim of this work is to bioremediate the diesel contaminated soil by using *Aspergillus flavus* and *Bacillus* sp. H6.

Materials and Methods

2.1. Soil microcosm's processes

2.1.1. Selected species

Aspergillus flavus and *Bacillus* sp. H6 were selected for bioremediation study based up on their abundance and lipolytic activities (EL-Morsy *et al.* 2006).

2.1.2. Microcosms preparation

Soils from the coastal beaches of New Damietta were collected. The soils are sandy and alkaline (pH 8.8), with organic matter content of 5.9%, and total nitrogen of 0.2 1% and an available phosphate content of 24.8 %. The soil (0–10 cm depth) was collected cleaned from wood drafts soil crustaceous skeleton, and sieved at 0.02 mm sieve. The soil for laboratory experiments was dried at 80 ^{o}C overnight (this may serve to eliminate indigenous bacteria from the soil). Once dried, About 10 kg of soil was selected for use, sprinkled with 0.166 g diesel oil/g dry soil until form a very thin layer of oil over the soil and left for 7 days. After adsorption of diesel, the humidity was approximately 43 %. The pH was adjusted to 7.5-7.8 and to avoid any acidic shift in pH the soil was limed with 2 g $CaCO_3$ / kg dry soil. The nitrogen level was also adjusted to 150 ug /g soil. Ratio of oil: N: P was 100: 10: 1. After thorough mixing, about 2 kg of

soil was placed into each of seven microcosms. Each microcosm consisted of plastic box (length, 25 cm; width, 15; depth, 15). The water content was adjusted regularly to 60 % water retention capacity. The seven microcosms were set up as follow. The microcosms M1, M2 and M3 were amended with *Bacillus* sp. H6 (6.1 x 10 5 / g dry soil), *Aspergillus flavus* (6.1 x 10 5 / g dry soil), and a consortium of *Bacillus* sp. H6 plus *A. flavus* (6.1 x 10 5 / g dry soil) respectively. The fourth (M4) was a natural control microcosm without any treatment. The fifth microcosm (M5) was sterilized by addition of fungicide (cycloheximide 75 mg / kg) antibiotic. The third (M6) microcosm was weekly treated with bactericide (benzyl Penicillin G, 37.5 mg / kg; Streptomycin, 37.5 mg / kg). A poison control (M7) made by monthly addition of $AgNO_3$ (0.3 % W/W). The experiment has been done in triplicate. Sampling was done immediately after the microcosms were set up and at regular intervals of 15 d and through out a 5 month at 28 ±2ºC. The soil in the microcosms was thoroughly mixed, and approximately 5-g samples, equally distributed, were taken from each box. Samples were analyzed in triplicate.

2.2. Hydrocarbon biodegradation

Total petroleum hydrocarbons (TPH) were measured in ten grams of soil. The soil dehydrated with Na2SO4 and mixed for 30 min with 10 ml of 1, 1, 2-trichlorotrifluoroethane; the TPH content of the filtrate was quantified as described in (American Public Health Association 1985).

2.3. Gas chromatography

5 g of soil were extracted twice with 50 ml of dichloromethane. The organic phase was passed through $NaSO_4$ and concentrated to 0.2 ml and then analyzed by gas chromatography (GC 17-A, Shimadza – Jaban model) equipped with the fame ionization detector and capillary column. The column oven found at 60 ^oC, which rise to 270 ^oC by 7 ^oC at 10 minutes. The injections temperature 280 ^oC, the detector (FID) temperature 300 ^oC and the injections volume is 1 micro millimeters. Peaks from 5 to 35 minutes were used for total diesel determination in all experiments.

2.4. Germination of plants

Known weight of bioremediation soil (10 g) was mixed with fifteen seeds of *Phaseolus vulgaris* in pots. The plants were irrigated with water and the germination of the seeds was examined daily for a week. The results were taken in duplicates.

Results

3.1. Bioremediation

Figure 1 demonstrates the effect of different treatments on hydrocarbon removal for a period of 150 days and at 28 ± 2 ^oC. The initial activity was variable and the decontamination activities are low after 15 and 30 days in all microcosms. The initial oil concentration 1666 mg / kg dry soil was reduced to 633.33, 800, 166.667, 633.33, 1100, 830 and 850 mg / kg dry soil, in *Bacillus* sp. H6, *A. flavus*, *Bacillus* sp. H6 + *A. flavus*, natural control, cycloheximide-treated, streptomycin-benzyl penicillin treated and poisoned control microcosms after 150 days, respectively. More than 1000 mg hydrocarbons were eliminated after 105 days in the microcosm that contains *Bacillus* sp. H6 plus *A. flavus*. Moreover, biodegradation by *Bacillus* sp. H6 plus *A. flavus* was reached to 166.7 mg / kg dry soil of the added oil after 150 days incubation. About 50 % of the oil added was removed in the bacterial less microcosms.

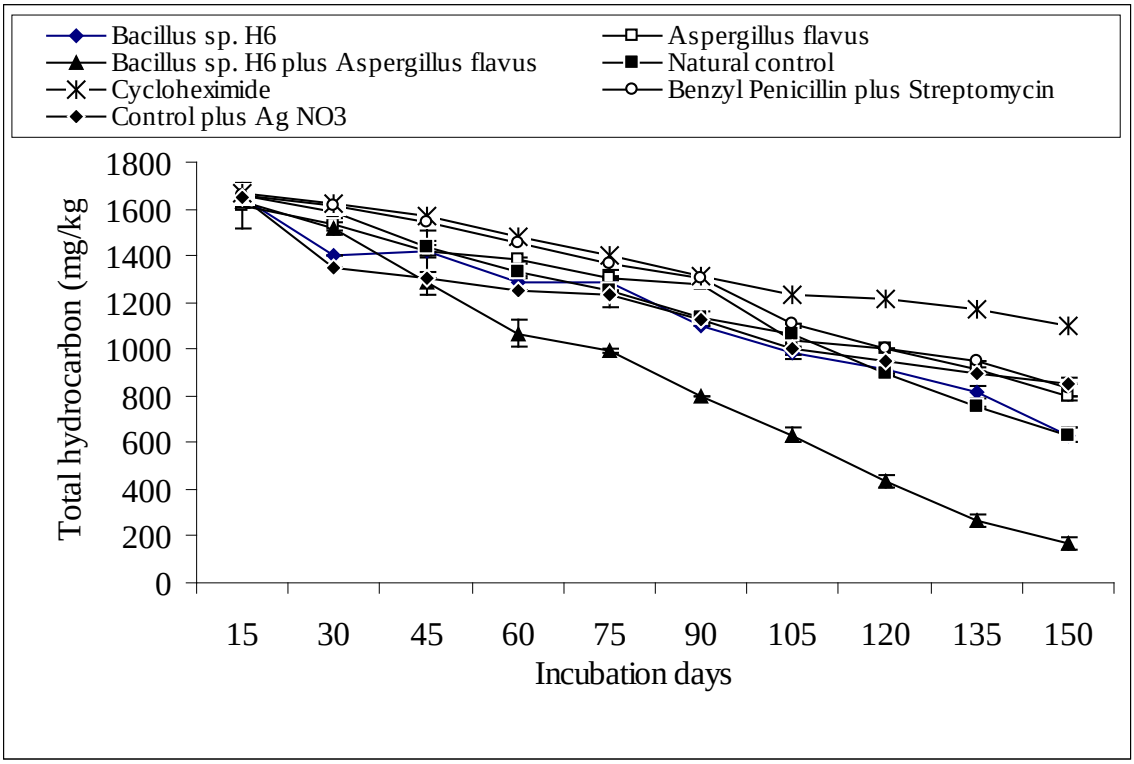

Fig. 1- Biodegradation of diesel oil incubated in soil microcosms at 28 ± 2 ^{o}C.

3.2. Gas chromatography analysis

The gas chromatography analyses of biodegraded diesel oil were illustrated in chromatograms of figs. 2 to 8. The chromatographic data produced in this analysis is separated in several peaks. Fig. 2b to 8b, representing residual diesel after 150 days (end of the biodegradation study) depict changes. Fig. 2a to 8a representing diesel at the start point. Figure 2 revealed that before inoculation with *A. flavus* and after 150 days incubation, meian chain C-17 compound was completely eliminated and other biodegradable compounds (C-20, C-21 and C-24) would increase. Alike, Fig. 3 demonstrated the appearance of C-15 and C-16 biodegradation product, before inoculation with *Bacillus* sp. H6 and after 150 days of incubation. It also confirmed the elimination of C-17 and increase of C-20, C-21 and C-24 biodegradation carbon product. Moreover, Fig. 4 illustrated the decrease of all detected n-alkanes that detected before inoculation with *Bacillus* sp. H6 and *Aspergillus flavus*. Likely, Fig. 5 demonstrated that soil with indigenous microfungi showed a low consuming rate of C-17, 18, 20 and C-24 compounds. Whereas Fig. 6 showed a prominent elimination of C-15, C-16, and C-17, besides the release of more C-20, C-21 and C-24 biodegradation product, in soil with indigenous bacteria. Furthermore, GC analyses in Fig. 7 demonstrated also the induction of more C-17, C-18, C-19, C-20, C-21 and C-24 biodegradation products, in natural soil microcosm, before and after 150 days of incubation. Conversely, in poisoned microcosm the oil was degraded chemically as appear in Fig. 8.

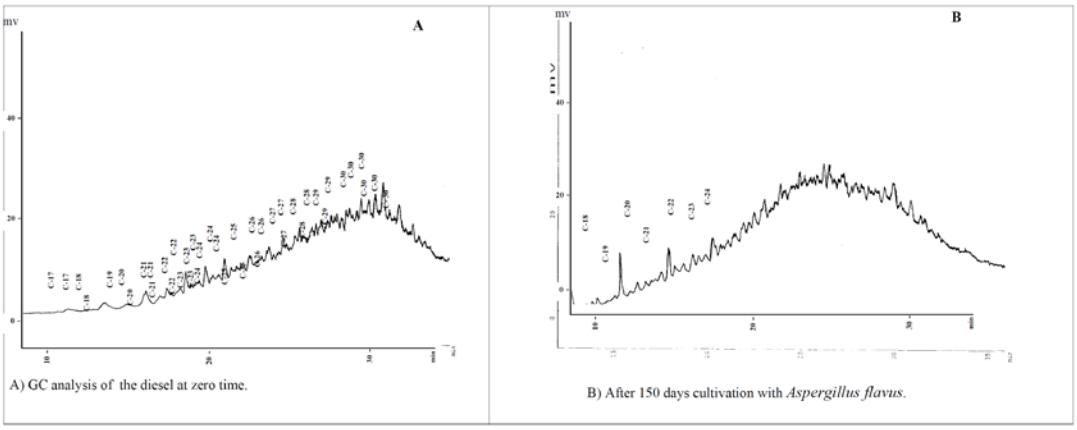

Fig. 2- Gas chromatography (GC) analyses of the remainder diesel when *Aspergillus flavus* was grown in the microcosm.

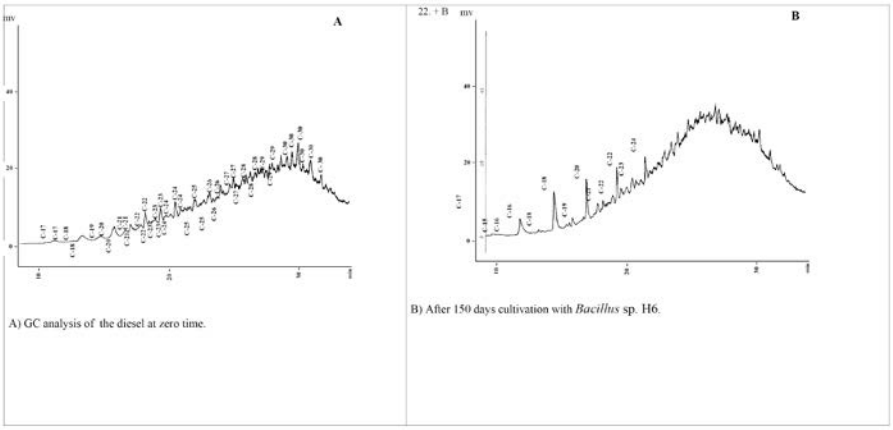

Fig. 3- Gas chromatography (GC) analyses of the remainder diesel when *Bacillus* sp. H6 was grown in the microcosm.

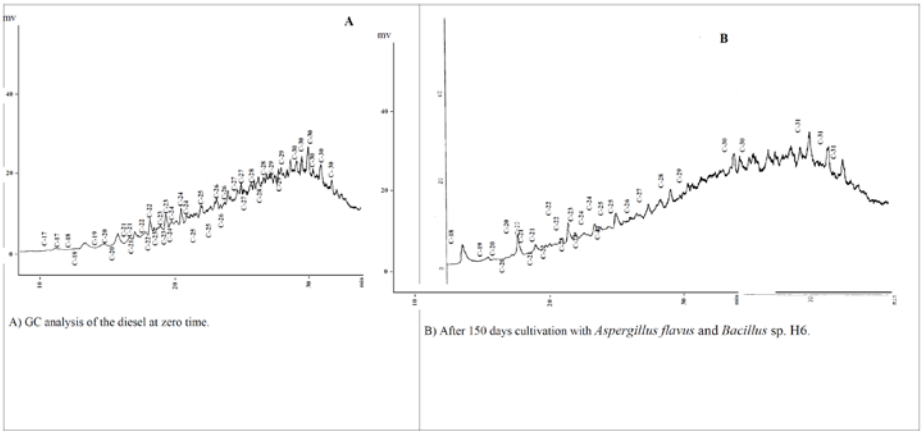

Fig. 4- Gas chromatography (GC) analyses of the remainder diesel when *Aspergillus flavus* and *Bacillus* sp. H6 were grown in the microcosm.

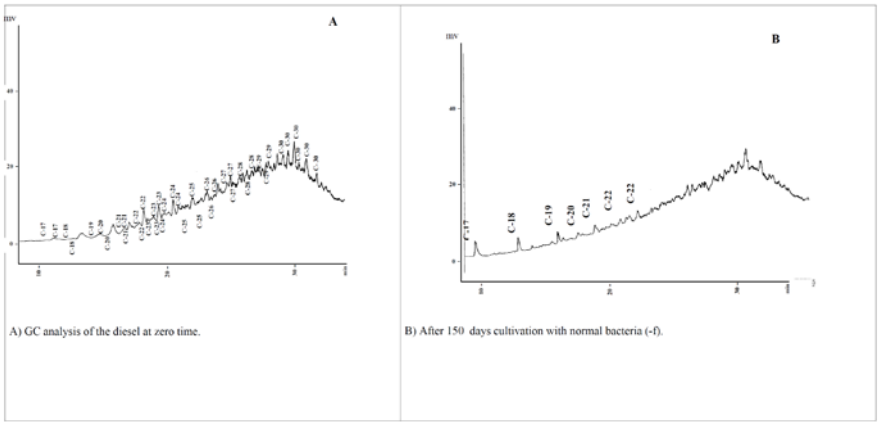

Fig. 5- GC analyses of the remainder diesel when normal bacteria were growing in the microcosm.

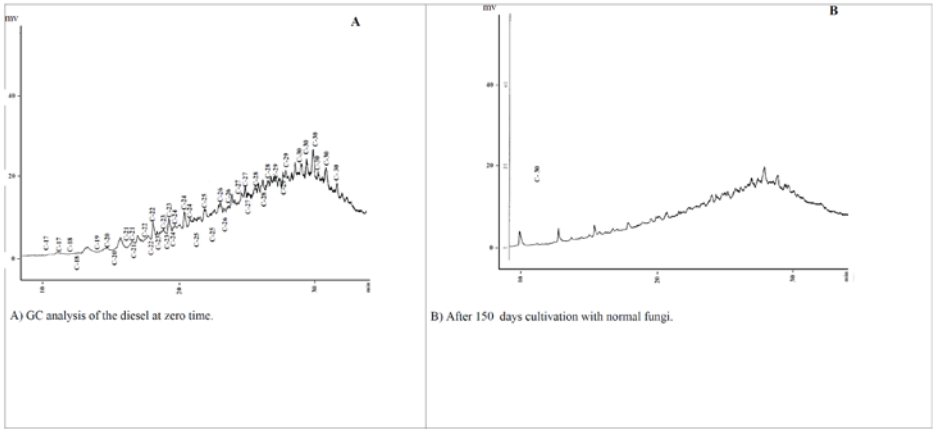

Fig. 6- GC analyses of the remainder diesel when normal fungi were growing in the microcosm.

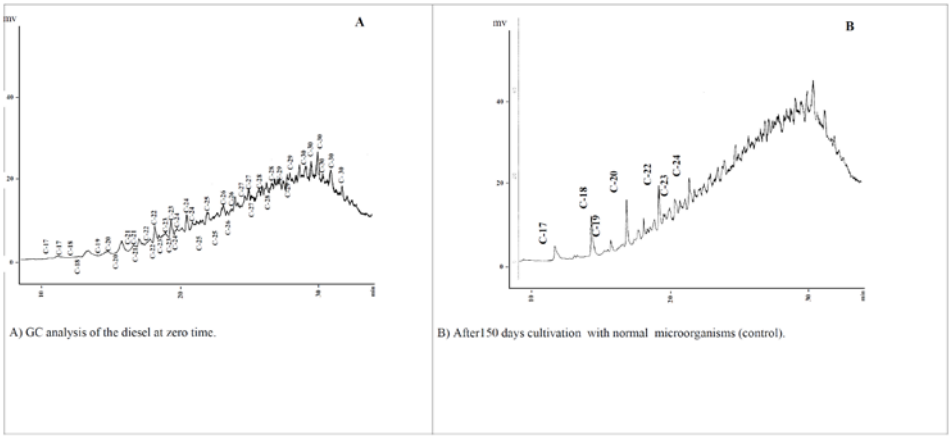

Fig. 7- GC analyses of the remainder diesel when normal microorganisms (control) were grown in the microcosm.

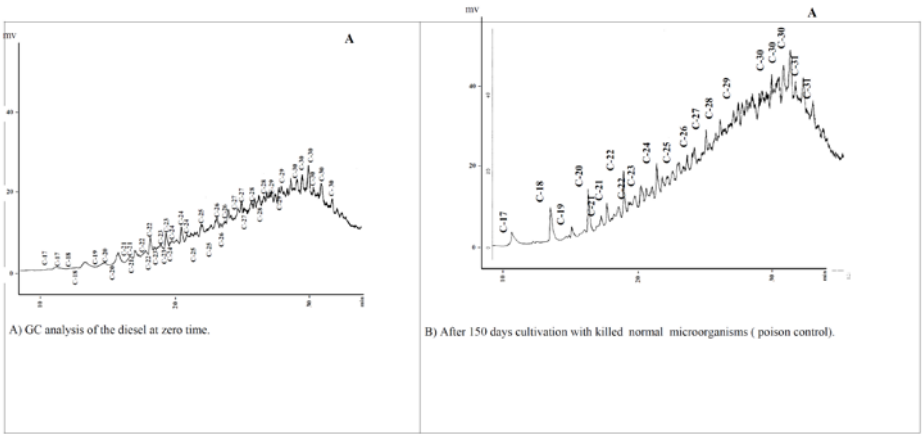

Fig. 8- GC analyses of the remainder diesel when all normal microorganisms were killed (poison control) in the microcosm.

3.3. Germination test

The results in figure 9 illustrated that the percentage of germinating seeds increases with incubation time in soil collected from all microcosms except poisoned one. At the beginning of the experiment the percentage of the germination is very low. The highest level of germination was detected after 150 days incubation (92 %) consorium microcosms (M3). whereas, the treatment with *A. flavus* and *Bacillus* sp. H6 separately led to a lower percentage of germination (86%) at the end of the experiment. Analogously antibacterial (M6), antifungal (M5) treated and natural (M4) microcosms have good percentage of seed germination of 73 %, 66 % and 60 % respectively, after 150 days incubation. Conversely, in the poisoned treatment, seed germination was completely absent.

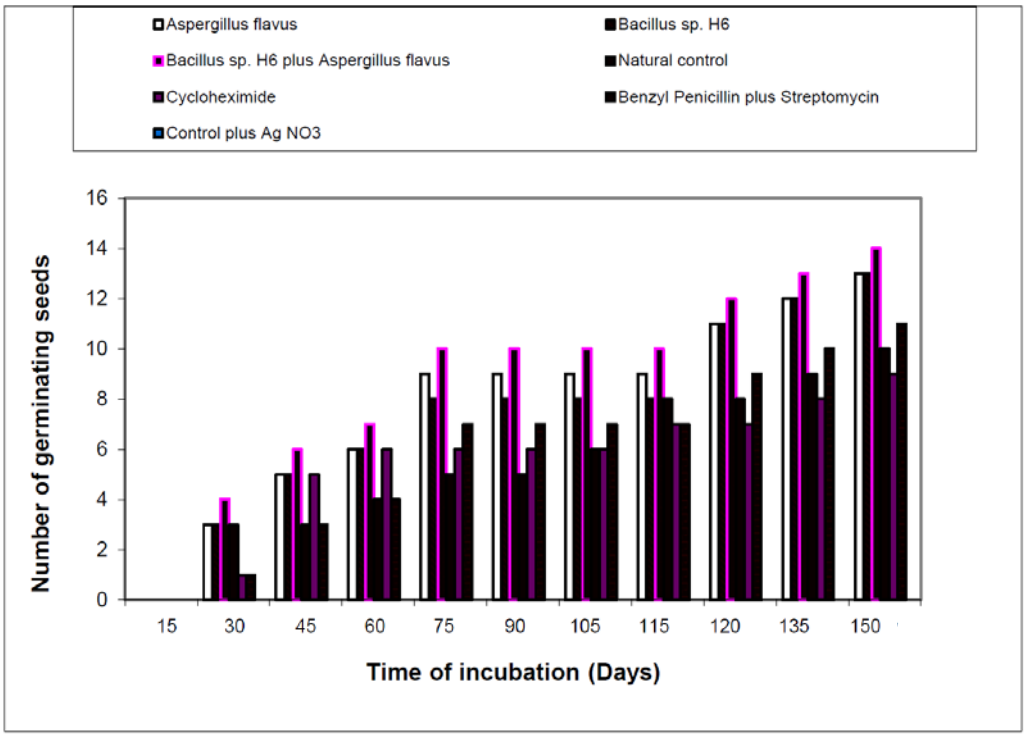

Fig. 9- Effect of different treated soils on the germination of the *Phaseolus vulgaris* seeds through out the whole experiments.

Discussion

The cultures, *A. flavus* and *Bacillus* sp. H6, in separate or in consortium demonstrated very good n-alkane degradation characteristics and could simultaneously degrade n-alkanes with a wide range of carbon numbers. As observed in this study, good degradation of the n-alkane fraction in various types of oil is often reported in the literature. Olson *et al.* (1999) reported 75% loss for the n-alkane fraction of total extractable petroleum hydrocarbons in diesel oil after 35 days in batch flask experiments.

Diesel oil consists mostly of linear and branched alkanes with different chain lengths and contains a variety of aromatic compounds. Many of these compounds, especially linear alkanes, are known to be easily biodegradable. However, diesel oil has low water solubility and so their biodegradation is limited by slow rate of dissolution, desorption, or transport and also transport processes to the cell (Márquez-Rocha *et al.* 2001; Sticher *et al.* 1997). Moreover, both biotic and abiotic processes are dependent on the chemical structure of the substrate to be degraded. In our experiment, diesel biodegradation does not appear to be limited by metabolic capabilities of the used bacterium, since that long chain alkanes (>C12) were biodegraded after bacterial growth. It seems to be limited due to mass transfer in bacteria, whereas fungi are better adapted because they grew well through soil particles.

Fungi are seemed to play a major role in biodegradation of aromatic compounds in soil. In this respect, Xiaojun *et al.* (2008) used three microbial consortia (bacteria, fungi and bacteria–fungi complex) and stated that they could degrade polycyclic aromatic hydrocarbons (PAHs), and the highest PAH removals were found in soil and slurry inoculated with fungi (50.1% and 55.4%, respectively. Moreover, *Cladosporium* was found to be strongly biodegraded diesel oil with a real degradation ratio of up to 34% after 5 days treatment (You-Qing *et al.* 2008). When the culture of *Cladosporium* was used with *Mycobacterium hyalinum* this led to a significant synergistic effect between them resulted in almost complete degradation of diesel oil, achieving a total diesel removal of 99% over 5 days of treatment. Comparable, it is reported that after 13 and 50 days of treatment with bacterium, all of the n-alkanes were degraded in the culture inoculated with the consortium of *Pseudomonas* and the major identified residues consisted of branched chain alkanes like pristane and phytane. It is also reported that no aromatic compounds were detected at the end of the experiment (Richard and Vogel 1999; Márquez-Rosha *et al.* 2001).

Generally, different microorganisms exhibit different abilities to degrade hydrocarbons. Some microorganisms can effectively degrade aliphatic alkanes, some have strong ability to degrade aromatic hydrocarbons, and some can degrade both aliphatic alkanes and aromatic hydrocarbons (Atlas 1995). The maximum decontamination activity occurred in the treatment of *Bacillus* sp. H6 and *A. flavus* is corresponding to 89.9 %. Alike, *Bacillus* sp. H6 has a decontamination rate corresponding to 61.9 %. In the same order, *A. flavus* has a decontamination activities corresponding to 51.9 %. *A. flavus* was found to be the most active fungus in degradation of oil in soil microcosms (EL-Morsy 2005).

The rate and extent of biodegradation was interpreted based on the GC chromatograms for residual diesel. The resolved n-alkane peaks in the diesel chromatograms were found to be in the carbon number range C15–C31. The fungus and bacterium either separately or in synergistic pattern act are consuming the carbon 17 compound in their metabolism and their biodegradation activity led to release of other short carbon compound such as C-15, C-16, C-20, C-21 and C-24. Ward *et al.* (2003) reported maximum degradation rate (mg/h) inversely related to chain length for C8–C11, in crude oil for mixed and pure cultures. Consequently, *A. flavus* alone can degrade both aliphatic hydrocarbons and aromatic hydrocarbons in diesel, though the degradation ability is not so strong. When the *Bacillus* sp. is mixed with *A. flavus*, the latter degrades aromatic hydrocarbons in diesel oil, permitting better growth of the former. Correspondingly, You-Qing *et al.* (2008) stated that the diesel biodegradation is significantly promoted by the mixed strains,

resulting in the observed synergistic effect. He deduced that, degradation of aromatic hydrocarbons by *Cladosporium* favors the growth of *Mycobacterium*, enhancing the degradation of saturated hydrocarbons in diesel that promotes in turn the degradation of aromatic hydrocarbons by *Cladosporium*, increasing greatly the bioavailability of diesel oil. Alike, Sharma and Rehman (2009) stated bacterial consortium degraded long chain hydrocarbon into short chain one and so it is the better option for biodegradation of diesel oil in soil.

In the natural control, the decontamination rate is similar to the treatment of *Bacillus* sp. H6. Other treatments, however, had low decontamination activities. In cycloheximide and the streptomycin-benzyl penicillin treated microcosms the decontamination activities were corresponding to 33.9 % and 49.8, respectively and this can be attributed to that the fungal activities of removal of the pollutant is higher than that of the bacterial activities. Conversely, poison control showed a decontamination activity. This abiotic loss of hydrocarbons in poisoned microcosm could be evaluated as a result of physical processes, as adsorption on soil particles and to evaporation of the volatile diesel oil fractions (Atlas and Bartha 1992; Bragg *et al.* 1994; Margesin and Schinner 1997). Additionally, it is observed that the hydrocarbon content cannot be reduced to zero and always reduction obtained was between 10 to 30 % (Chaineau *et al.* 1995; Groβ *et al.* 1995; Hollerbach *et al.* 1992; Margesin and Schinner 1997). Even when the incubation was prolonged; the reduction was slightly increased (Chaineau *et al.* 1995; Groβ *et al.* 1995). This could be due to low bioavailability of the contaminant, the accumulation of recalcitrant components and inhibiting metabolites, and to the lack of microbial growth factors. Conversely, You-Qing *et al.* (2008) deduced that it is quite possible to achieve a complete degradation of diesel oil by appropriately increasing the degradation time.

The measured gas chromatograms of the diesel oil samples biodegraded was illustrated in figures from 2 to 8. From the figures it is obvious that, the degradation treatment caused a uniform decrease of the GC peaks, indicating that all of the components in diesel oil, including both saturated hydrocarbons and aromatic hydrocarbons, were significantly degraded at almost the same degradation rate. This strongly supports the above explanation. Moreover, Fig. 4B shows low level of residual diesel oil after a 150-day treatment.

The effects of the above treatments on the availability of oil-contaminated soil for agriculture were obvious in *Phaseolus vulgaris* seed germination test. In facts, all treatments enhanced the rate of seed germination except the poisoned treated one. The rate increased from 0-germination before the treatment in all microcosms to 92 %, 86 %, 86 %, 73 %, 66 % and 60 % germination in soil collected from *A. flavus*-*Bacillus* sp. H6, *A. flavus*, *Bacillus* sp. H6, streptomycin benzyl-Penicillin, cycloheximide and natural treated microcosms respectively, at the end of the experiments. Silva *et al.* (2015) reported that diesel oil toxicity was found to be reduced after seven days in bioreactor trials where lettuce seeds (Lactuca sativa) and beans (*Phaseolus vulgaris* L.) showed germination rates of 41- 54 %, respectively. Also, Roy *et al.* (2014) observed that under microcosm, soil quality was improved significantly in the treatments of crude oil by bacterial consortium as confirmed by maximum increase in seedling dry weight for both rice (*Oryza sativa*) (54 %) and mung (*Vigna radiata*) (63 %). Likely, it was reported that oil pollution is a serious obstacle to photosynthesis where albinos seedlings of *Avicennia marina* have been observed with increasing oil concentration in sediments, some plants die, but others survive and have possible sub-lethal responses (Duke *et al.* 1997; Duke and Watkinson 2002). It is obvious that the concentration of oil used was toxic to the seeds and raising their germination rate progressively with increasing incubation period may be attributed to the microbial biodegradation of diesel oil to non toxic intermediates and other changes in soil physico-chemical properties (Atlas 1981; Leahy and Colwell 1990; Banks *et al.* 2000).

Conclusion

Based on the present work, the following conclusions could be drawn: the rate and extent of diesel biodegradation was significantly increased by using fungal and nacterial consortium. Diesel pollutants in soil microcosms could be almost completely removed (89%) by the synergistic effect of *Bacillus* sp. H6 and *A. flavus*. The chemical tolerance of the microorganisms is very essential for achieving highly efficient degradation of organic pollutants when the mixed microorganism consortium is used. Thus, these microorganisms can be used effectively in the treatment of oil polluted areas.

Conflict of Interest

The authors do not have any conflicts of interest.

Acknowledgements

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