Effect of Biostimulation-Bioaugmentation on saturate and aromatic hydrocarbon degradation

Leyla Gheyrati Arani[1]* and Sonakebriaeezade[2]

[1]Faculty member of Agriculture Department, Payame Noor University, Tehran, Iran
[2]Department of Environmental Management, Science and Research Branch,
Islamic Azad University, Tehran, Iran

*Corresponding author

KEYWORDS	A B S T R A C T
Degradation, *Rhizopus*, Saturate, Aromatic	This work presents a study of the effect of combined biostimulation-bioaugmentation system applied to a silty-loam soil polluted with 60,400mg kg_1 of a complex mixture of total petroleum hydrocarbons (TPH) especially engine oil, which comprises 58% saturate hydrocarbons (sat) and 29% polycyclic aromatic hydrocarbons (PAH) and 13% polar compound. The bioaugmentation was performed with *Rhizopus oryzae*, isolated from aged soils contaminated with 60,400 mg of TPH per kilogram of dried soil. The native fungi were able to grow in a complex solid mixture of hydrocarbons of high molecular weight, after previous acclimatization in liquid culture. The related fungus was able to remove more PAH in comparison with biostimulation alone.

Introduction

In order to fulfill the energy requirement of the world economic, various natural resources have been exploited. However, petroleum continues to serve as the principle source of energy. Wide scale production, transport, various production storage and use of petroleum globally has lead frequent organic pollutants of soil and ground water (Vaajasaari *et al.,* 2002; Booth *et al.,* 2005) and is classified as hazardous waste (Grazyna *et al.,* 2008).

Bioremediation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic and environmentally sound treatment. Bioremediation techniques accelerate the naturally occurring biodegradation by optimizing conditions for biodegradation through aeration, addition of nutrients and control of pH and temperature. About 1% of total mineral oil consumption is used to

formulate lubricants and the total consumption of lubricating mineral oils in 1995 exceeded 36 million tones worldwide and significant amounts of lubricants were lost into environment. Most of these lubricants are used in loss lubrication frictional contacts and in circulation systems, which are disposed improperly. Additionally, lubricants are emitted from leaks and significant amounts remaining in filters and containers have to be taken into account. For these reasons, the distribution, biodegradability and toxicity of lubricants are important factors with respect to environmental management. In spite of the large number of works on petroleum, petroleum derivatives and hydrocarbon biodegradation, little information is available on lubricant oils biodegradation (Nocentini *et al.,* 2000).

Recent studies have reported several bacteria with the capacity to mineralize PAHs (Boonchan *et al.,* 2000). However, most hydrocarbon degradation studies have been carried out using white rot fungi such as *Phanerochaete chrysosporium, Pleurotus ostreatus* and *Trametes versicolor* (Mollea *et al.,* 2005). It would be interesting to develop the bioremediation process further using filamentous fungi, because of their capacity to incorporate rapidly into the soil matrix. Furthermore, they have the ability to grow in environments with low nutrient concentrations, low humidity and acidic pH (Potin *et al.,* 2003).

Fungi are more tolerant to high concentrations of polluting chemicals than bacteria. Their mycelial growth habit allows them to colonize insoluble substrates. Fungi can hydroxylate a wide variety of these compounds because of their degradative enzymes that are frequently nonspecific, also because of their extracellular enzymes that enable them to tolerate high concentrations of toxic chemicals, once those microorganisms ramifies quickly on the substratum, digesting it through the secretion of extracellular enzymes which break down potential food sources, which are then absorbed back into the fungal colony. This capability also allows fungi to oxidize a broad range of toxic organic compounds into nontoxic metabolites and CO_2. These compounds include, among others, various petrochemicals, such as polycarbons and polychlorinated biphenyls (Rama *et al.,* 2001).

Their ecological role is significant considering that the resultant polar intermediates can be mineralized by bacteria or detoxified to innocuous compounds. Besides, the fungi are capable to grow under environmental conditions of stress, for example: environment with low pH values or poor in nutrients and with low water activity.

Biostimulation consists of the activation of native soil microorganisms through the addition of nutrients, is the most often bioremediation strategies used(Lee *et al.,* 2006). It is possible that even if the native microorganism population is large enough, it does not have the ability to degrade components of high molecular weight or to emulsify insoluble compounds. Bioaugmentation could be used for this case. This technique is defined as the addition of pre-grown microbial cultures to perform a specific remediation task in a given environment. The microbial cultures must have the ability to withstand different soil environmental conditions and to survive in the presence of other microorganisms. Most bioaugmentation studies have been carried out using filamentous fungi inoculated into model soil systems and using contaminants of low molecular weight PAHs with up to four rings (D'Annibale *et al.,* 2006). For the

breakdown of complex aromatic structures, fungi bacteria consortia are preferred due to the successful results reported. For example, the consortium comprising *S. maltophilia–P. janthinellum* degraded 44–80% of a chrysene, benzo[a]anthracene, benz[a]-pyrene and dibenz[a,h]anthracene mixture, in 100 days (Boonchan *et al.,* 2000). This success has rekindled an interest in treating solid wastes generated by the petroleum industry.

The objective of this study was to determine the effect of a combined biostimulation–bioaugmentation treatment of a soil contaminated with a complex mixture of weathered engine oil hydrocarbons. The bioaugmentation was performed with *Rhizopus* oryzae isolated from areal soil polluted with weathered hydrocarbons.

Material and Methods

Contaminated soil

The material selected for this study was from the site used as a repairing yard for vehicles during 10 years. During this time, hydrocarbon-containing materials were used extensively including diesel, lubricant oils, and other hydrocarbon products.

Soil was grounded and sieved to 2mm and it was carefully mixed and homogenized with a spatula. The total nitrogen (N), available phosphorus (P), total organic carbon (C), water retention capacity (WRC), pH, microbial count and total petroleum hydrocarbon (TPH) concentration were measured and recorded before the samples were stored (Table 3).

Hydrocarbon Analyzes

The total extractable material (TEM) and concentration of each fraction were determined by column chromatography (Ryuji *et al.,* 2003). The lubricant was extracted by soxhlet using chloroform as a solvent. The extract that contained total oil compounds was estimated by weighing the dry residue after evaporation of the solvent. The dry extracts were suspended in 60 ml of hexane. The hexane-insoluble fraction containing polar compound were determined after filtration of the hexane solution on the solvent-washed pre-weighed Whatman GF/A glass—microfiber filters. Filters retaining the polars were dried and weighed. The hexane soluble fraction was separated by solid–liquid chromatography on a 15 cm length×1 cm diameter activated (overnight, at 120°C) silica-gel (100–200 mesh) column. Successive elution was performed with 60 ml of hexane, 60 ml of a 60:40 (v/v) mixture of hexane–dichloromethane. The fractions eluted with these solvents are named saturates, aromatics, respectively. After evaporation of the solvents, the residual hydrocarbon was determined by gravimetrically.

Isolation of fungal strain

Fungal strain was isolated by serial dilution (from 1×10^{-1} to 1×10^{-7}) of 1 g hydrocarbon-polluted soil previously dried and grounded. A sample of 200 µL of each dilution was distributed in Petri dishes previously prepared with solid medium potato dextrose agar. Fungal colony was selected and plated on the same medium until pure colonies were obtained. The Petri dishes were incubated at 28°C for 5 days. The criteria for selecting the fungi was the capacity of this strain to grow in a liquid mineral medium augmented with a complex mixture of lubricant oil as carbon source (Chavez-Go´mez *et al.,* 2003). Fungal strain was identified by micro culture.

Inoculums Preparation

A selected fungus was cultivated in Petri dishes previously prepared with solid medium of nutrient potato dextrose agar.

The Petri dishes were incubated at 28°C until total sporulation of the culture was observed. Spores were suspended in 10mL of sterile distilled water.

The suspension was then washed and filtered with sterile glass wool and stored in 20mL amber vials at 6°C (Verdin *et al.,* 2004). The spore stock was carried out in triplicate in sterile conditions. The spore concentration in each vial was calculated by direct count in a Neubauer counting chambers.

Sterilized and non-sterilized soil preparation

The soil was divided into two batches and one batch was autoclaved at 121∘C for 30 min to remove the indigenous microbial population. The other batch was left in natural condition. No crude oil was further added into the soil. As controls, one set of sterile soil and another of the non-sterile soil were left un-inoculated.

Treatments for each soil batches

For Each soil batches, three different treatments were considered.

A. Soil without any stimulation but humidified to 70% of its WHC.
B. Soil with nutrient amendment in order to providing C: N ratio of 25: 1.
C. Soil stimulated with nutrient and bulking agent (wheat bran).

In order to easy the interpretation of the result, according to different bioremediation strategy, controls in treatment A, B and C has been named as natural attenuation, nutrient stimulation, nutrient plus bulking agent stimulation respectively (Table 1).

For treatment A (fungal augmentation without stimulation), the humidified soil (50g) was added to a 500 ml sterilized vial and it was inoculated with 10^7 of the corresponding inoculums propagated in liquid culture; total water added to the solid mixture was 70% of its WRC.

For treatment B (fungal augmentation plus nutrient stimulation), in order to have a C: N: P ratio of 250:10:1, soil was humidified (70%, w/w) with a source of nitrogen $((NH_4)_2SO_4$, and phosphorous (K_2HPO_4) solution and then inoculation with different fungi was conducted.

For treatment C (fungal augmentation plus nutrient and bulking agent stimulation): about 20 g of barley grain, soaked in water for 48 hours and drained, were placed in each bottle. The bottles were plugged before being sterilized at 121°C for 15 min (15 Ib psi) in an autoclave. Each bottle was aseptically inoculated with selected fungal isolate by slicing the PDA colonized by the organisms into small 5mm pieces. The bottles were incubated in the dark for three weeks at 25°C to allow complete colonization of the grains. The colonized grains were then inoculated into the contaminated soil at the rate of 5 g of barley to 50 g of soil (fresh weight).

Bioaugmentation treatment

Bioaugmentation treatment was done with 100 g polluted soil and moistened (70% of the WRC) with nutrient solution as described above. Each bottle was inoculated with 10^7 of the inoculums propagated in liquid culture. The vial content was mixed under sterile conditions to obtain a homogeneous solid mixture.

Jars of all above treatment were covered and incubated at 30°C for 50days and passively

aerated for 15 min, under sterile conditions, every 2 days. Residual TPH as well as saturate, aromatic and polar fraction were determined as described in determination of hydrocarbon. A biotic hydrocarbon removal was performed with a sterilized soil without inoculums.

Bioremediation with pure fungi

In the present work, bioaugmentation with pure fungi is of interest because of their ability to synthesize unspecific enzymes that can degrade aromatic structures of high molecular weight. The same ''bioremediation by Bioaugmentation Treatment'' methodology was applied, except that the polluted soil added was previously sterilized. The sterile polluted solid matrix was tested by serial dilution for culture using solid medium of PDA.

Result and Discussion

Soil characteristic

The soil contaminated with oil was analyzed for various physico-chemical parameters to know the status of properties. Table 2 shows characteristic of composite soil sample which used for bioremediation experiments. Sterilization of the soil was done to know the soil characteristic. Soil showed 0.08 % of nitrogen and 0.02 % of phosphorous, having water holding capacity (WHC) 50 to 53%, while total petroleum hydrocarbon (TPH) was 4.58–4.83 % (There was a loss of TPH in sterilized soil i.e., 0.25 % may be due to heat).

The contents of sand, silt, and clay were 63% sand, 25% silt and 12% clay respectively. Soil texture analysis indicated that this soil has moderate air permeability and water holding capacity.

TPH Removal by bioaugmentation

Gradual and homogeneous mycelia colonization of the selected fungal strain was observed on the soil polluted with 60,400 mg TPH kg^{-}1. Such colonization indicates the capacity of fungal re adaptation to a highly polluted soil (Canet *et al.,* 2001).

Table.1 Different treatments prepared for bioremediation

Sterile soil					
augmentation	Stimulation + augmentation		control	stimulation	
augmentation	augmentation + nutrient stimulation	augmentation + nutrient as well as bulking agent stimulation	Control (natural Attenuation)	nutrient stimulation	nutrient and bulking agent stimulation

Table.2 Physical and chemical properties of soil composite sample (All values is in percentage)

Soil Condition	Total Nitrogen	Total Phosphorous	TEM	pH	WHC	TPH	Organic Carbon	C:N ratio
NON-ST	0.08	0.02	6.04	5.8	53	6	12.2	610 :4 :1
ST	0.08	0.02	5.8	6	50	5.8	11.8	590 :4 :1

NON-ST: Non-sterilized   ST: Sterilized

The contents of sand, silt, and clay were 63% sand, 25% silt, 12% clay, respectively. Soil texture analysis indicated that this soil has moderate air permeability and water holding capacity.

Table.3 Initial composition of hydrocarbon in contaminated soil (g/kg)

Soil condition	Saturate H.	Aromatic H.	Polar fraction	TPH(g/kg)
NON-ST	35.032	17.516	7.852	60.4
ST	34.944	17.472	5.824	58.2

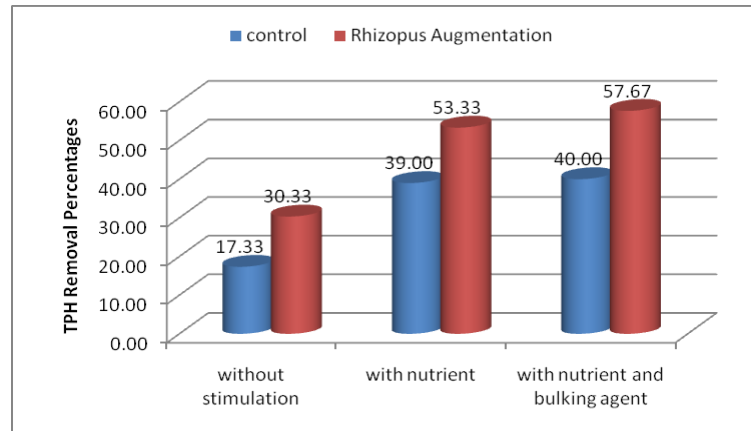**Figure.1** Percentage of TPH removal in different treatments for *Rhizopus* augmentation

Figure 2.Percentage of hydrocarbon fraction removal in different treatments for non- sterilized soil
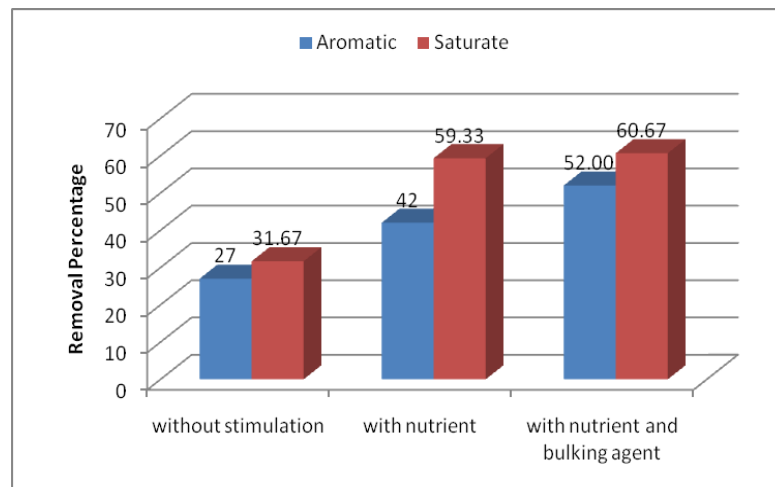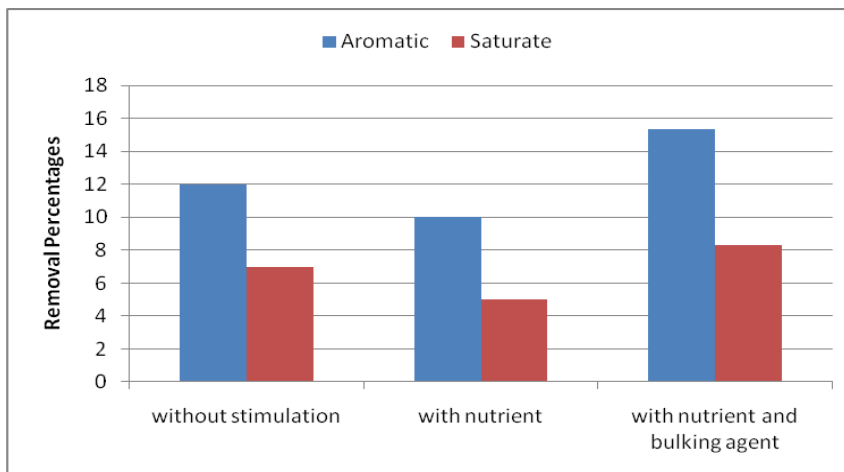
Figure.3 Percentage of hydrocarbon fraction removal in different treatments for sterilized soil

Since objective of the present work was to evaluate the degradation rate of hydrocarbon fractions as well as total petroleum hydrocarbon in different bioremediation treatments, also to evaluate degradation rate through indigenous fungal culture in two soil batches (sterilized and non-sterilized soil). The result were converted the residual hydrocarbon concentration to percentage of reduction through following expression.

% of degradation = [(initial TPH – TPH concentration after treatment)
/ Initial TPH] $\times$ 100.

Pure fungal culture was of interest because of its ability to synthesize unspecific enzymes that can degrade aromatic structures of high molecular weight (Colombo *et al.* 1996.). Non-sterilized soil augmentation also was of interest in order to evaluate the syntrophic metabolism activity between fungi and bacteria.

Figure 1 shows rate of TPH removal in nutrient stimulation and nutrient, bulking agent stimulation in compare to natural attenuation (without stimulation). It shows that nutrient as well as nutrient, bulking agent stimulated the hydrocarbon removal in

control treatments as well as *Rhizopus* augmented culture.

Nitrogen and phosphorus are the usual nutrients, which have been used in bio-stimulation process in order to support microbial growth (Liebeg and Cutright, 1999). Percentage of TPH removal in *Rhizopus* augmentation plus stimulation increased 2 times rather than *Rhizopus* augmentation alone.

Figure 2 shows the percentage of saturate and aromatic hydrocarbon removal in *Rhizopus* augmentation treatment in compare to control. It shows that nutrient stimulation is more effective for saturate degradation than aromatic whereas nutrient plus bulking agent stimulation degrade saturate and aromatic both in same rate.

Figure 3 shows the percentage of saturate and aromatic hydrocarbon removal in *Rhizopus* augmentation treatment in compare to control for sterilized soil. It shows that nutrient plus bulking agent stimulation is more effective in aromatic degradation than saturate. Nutrient stimulation showed inhibitory effect on saturate as well as aromatic degradation when *Rhizopus* augmented in sterilized soil.

In the present work aromatic removal with pure fungi augmentation was more than 9%. This was higher than result which Mancera-Lopez *et al.* (2007) obtained in filamentous fungal augmentation of soil polluted with hydrocarbon (9%).

Fungal augmentation in sterilized soil resulted in more aromatic hydrocarbon degradation than saturate fraction whereas in non-sterilized soil, saturate degradation was more than aromatic (Figure 2 and 3).

Conclusion

Nutrient stimulation is more effective in saturate degradation than aromatic whereas nutrient, bulking agent stimulation degrade saturate and aromatic both in same rate. Also co- metabolism between fungi and bacteria were higher in presence of nutrient and bulking agent than their absence.

References

Boonchan, S., Britz, M.L., Stanley, G.A., 2000. Degradation and mineralization of high molecular-weight polycyclic aromatic hydrocarbons by defined fungal–bacterial cocultures. *Appl. Environ. Microbiol.,* 66: 1007–1019.

Booth, L., Heppelthwaite, V., O'Halloran, K. 2005. Effects-based assays in the earthworm *Aporrectodea caliginosa*: Their utilisation for evaluation of contaminated sites before and after remediation. *J. Soils Sediments,* 5(2): 87–94.

Canet, R., Birnstingl, J.G., Malcom, D.G., Lo´ pez-Real, J.M., Beck, A.J. 2001. Biodegradation of polycyclic hydrocarbons (PAHs) by native microflora and combinations of white rot fungi in a coal-tar contaminated soil. *Bioresource Technol.,* 76: 113–117.

Chavez-Go´ mez, B., Quintero, R., Esparza-Garcı´a, F., Mesta-Howard, A.M., Zavala Diaz de la Serna, F.J., Herna´ndez-Rodrı´guez, C.H., Guillen, T., PoggiVaraldo, H.M., Barrera-Corte´, S.J., Rodrı´guez- Va´ zquez, R. 2003. Removal of phenanthrene from soil by co-cultures of bacteria and fungi pregrown on sugarcane bagasse pith. *Bioresource Technol.,* 89: 177–183.

D'Annibale, A., Rosetto, F., Leonardi, Federici, V.F., Petruccioli, M. 2006. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. *Appl. Environ. Microbiol.,* 72: 28–36.

Grazyna A. Płaza, KamleshJangid, KrystynaŁukasik. 2008. Reduction of petroleum hydrocarbons and toxicity in refinery wastewater by bioremediation. *Bull. Environ. Contam. Toxicol.,* 81(4): 329–33.

Lee Sang-Hwan, Seokho Lee, Dae-Yeon Kim, Jeong-gyu Kim. 2006. Degradation characteristics of waste lubricants under different nutrient conditions. *J. Hazard. Mater.,* 143: 65–72.

Liebeg, E.W., Cutright, T.J. 1999. The investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil. *Int. Biodeterioration Biodegrad.,* 44: 55–64.

Mancera-López, M.E., Rodríguez-Casasola, M.T., Ríos-Leal, E., Esparza García, F., Chávez-Gómez, Rodríguez-Vázquez, R., Barrera-Cortésa, J. 2007. Fungi and bacteria isolated from two highly polluted soils for hydrocarbon

degradation. *Acta. Chim. Slov.,* 54: 201–209.

Mollea, C., Bosco, F., Ruggeri, B. 2005. Fungal biodegradation of naphthalene: microcosms studies. *Chemosphere,* 60: 636–643.

Nocentini, M., Pinelli, D., Fava, F. 2000. Bioremediation of a soil contaminated by hydrocarbon mixtures: the residual concentration problem. *Chemosphere,* 41: 1115–1123.

Potin, Catherine Rafin, Etienne Veignie. 2003. Bioremediation of an aged Polycyclic aromatic hydrocarbon (PAH –contaminated soil by filamentous fungi isolated from the soil. *Int. Biodeterioration Biodegrad.,* 54: 45–52.

Rama, R., Sigoillot, J.C., Chaplain, V., Asther, M., Jolivaltand, C., Mougin, C. 2001. Inoculation of filamentous fungi in manufactured gas plant site soils and PAH transformation. *Polycycl. Aromat. Compd.,* 18: 397–414.

Ryuji, S., Yasuhiro, K., Megumi, I., Masayoshi, K., Tomomasa, T., Tetsuyoshi, I., Susumu, K., Kazuhiro, F. 2003. Induction of L-formlike cell shape change of Bacillus subtilis under microculture conditions. *Microbiology,* 149: 2501–2511.

Vaajasaari, K., Joutti, A., Schultz, E., Selonen, S., Westerholm, H. 2002. Comparisons of terrestrial and aquatic bioassays for oil-contaminated soil toxicity. *J. Soils Sediments,* 2(4): 194–202.

Verdin, A., Loune`s-HadjSahraoui, A., Durand, R. 2004. Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *Int. Biodeterioration Biodegrad.,* 53: 65–70.