

## Fungal Biodegradation of Diesel by *Ulocladium Atrum*

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### Abstract

Interest in the cleanup of oil contamination in the use of microorganisms has existed for many years, early studies showed that hydrocarbons oxidizing bacteria were widespread in all sites polluted by crude oil. Biodegradation means to use a biological remedy to abate or cleanup site contamination, microorganisms are often used to remedy environmental problems found in the contaminated site. For the process of biodegradation, it is necessary that the microorganisms should be active, so that they can perform their duty efficiently. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, Bioaugmentation, biostimulation and biotransformation. On the basis of removal and transportation of oil for treatment, basically there are two methods, in situ and exsitu, and there are potential advantages and disadvantages for both methods. The microorganisms act well only when the hydrocarbons materials help them to generate energy and nutrients to build up more cells. A diesel degrading fungus was isolated from a diesel contaminated site in Riyadh city, Saudi Arabia, and tentatively identified as *Ulocladium atrum*. Optimization studies using different concentrations of diesel in mineral liquid medium showed that the fungus can grow in all diesel concentrations. Diesel biodegradation and the effect of application frequency were investigated in soils artificially contaminated by diesel amended with mineral liquid medium. The contaminated soil was inoculated by the tested fungus and incubated at 37°C for seven weeks. Supplementation of diesel degradation with mineral liquid medium resulted in a significant increase in diesel biodegradation. The characteristics of this fungus suggest that it is suitable for bioremediation of diesel contamination. The significance of this study will give more characters of the microbial degradation of diesel in Saudi Arabia and this will help in the study of bioengineering of these microorganisms and also gives more information about microbial biodegradation of diesel. The potential impact of conducting this research will appear clear in the cleaning-up of petroleum contamination in Saudi Arabia, but the limitation of the study is that we need more extensive studies in the Arabia Gulf Region and especially in Saudi Arabia.

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**Keywords:** bioremediation, biotechnology, contamination, hydrocarbons, soil, treatment

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### INTRODUCTION

Hydrocarbons industrialization has produced large quantity of waste to which current physical and chemical treatment of the wastes are generally expensive and are not able to treat all wastes; so, bioremediation is a cheaper alternative and could remove most of the pollutant. To date, numerous bioremediation of hydrocarbons waste have been reported successful (Shukor *et. al.*, 2009; Padayachee and Lin, 2011; Martinho *et. al.*, 2012).

Bioremediation, biostimulation and bioaugmentation of petroleum contaminated soil is a treatment technology that utilizes naturally occurring microorganisms to degrade hydrocarbons to carbon dioxide, water and humus (Binsadiq and Alharbi, 2000; Binsadiq, 2004A; Binsadiq, 2007).

Most of the Eastern region of Saudi Arabia including coastal area, became polluted with crude oil during the recent Gulf war. Microorganisms including bacteria and fungi could degrade petroleum as a carbon and energy source and could be converted into

useful products by biodegradation activity of these microorganism (Molnaa and Grubbs, 1990).

There have been many reports on the isolation of diesel degrading bacteria in different places of the world, but little attention have been paid to the fungal degrading diesel contamination in the Gulf Region and especially in Saudi Arabia.

The application of highly sensitive analytical techniques to environmental analysis has provided the society with disturbing information. The air we breathe, the water we drink and bath in, the soil in which our crops are grown, and the environments in which the populace, animals and plants grow, are contaminated with a variety of different chemicals and petroleum products such as diesel.

The major agents causing the biological transformation in soil, sediment, wastewater, surface and groundwater, and many other sites are the microorganisms with different techniques such as biodegradation, bioremediation, biostimulation,

bioaugmentation, bioavailability, biocenteration and biotreatment (Jose *et al.*, 2001; Shukor *et al.*, 2009).

Several conditions must be satisfied for biodegradation to take place in an environment; these include the following:

- a – microorganisms must exist which have the necessary enzymes to bring about biodegradation.
- b – microorganisms must be present in an environment containing the pollutant.
- c – the pollutant must be accessible to microorganisms having the requisite enzymes.
- d – conditions in the environment must be conducive to allow the proliferation of the potentially active microorganisms (Binsadiq, 2004C).

The most important role of microorganisms in the transformation of pollutants such as diesel is their ability to bring out detoxification, which refers to the change in a molecule that renders it less harmful to one or more susceptible species. Detoxification results in inactivation, with the toxicologically active substance being converted to an inactive product (Alexander, 1994).

The ability of microorganisms to degrade petroleum and its products such as diesel is well known (Udeme and Antai, 1988; Baker and Herson, 1999). The phenomenon of petroleum products degradation by microorganisms in different places of the world have also been reported (Kilbane *et al.*, 2000; Ruberto *et al.*, 2003).

Diesel fuel is a hydrocarbon product that boils approximately between 150 and 400°C, with carbon chain length of C15 to C22; various classes are available and classification differs from country to country. A variety of additives may be used to improve the stability of fuel; these include compounds such as aliphatic amines, chelating agents, detergents, and corrosion inhibitors, which can act as a nutrient source for microorganisms (Gaylarde *et al.*, 1999). Diesel, on the other hand, is a good and suitable media for microbial growth and acts as a source of carbon and energy (Mohanani *et al.*, 2005; Nwaogu *et al.*, 2008).

Microorganisms respond to microbial growth by the addition of soil petroleum products and causes the added substrate to disappear from the community (Aitken *et al.*, 2004; Miranda, 2007; Santos *et al.*, 2008). The present investigation aims to study the fungal that is potentially useful in biodegradation of diesel in liquid culture and transfer the results obtained to the applied field laboratory in the site contaminated with diesel.

In Saudi Arabia a huge amounts of waste hydrocarbons been added to soil and sea water which causing contaminated environments .

There is far more involved with biodegradation projects in Saudi Arabia than simply adding microorganisms, various factors need to be considered to insure the success of these programs, the proper engineering to facilitate biological growth is crucial step in the process of biodegradation a site , in the Saudi Arabia little attention have been paid to the role of microbial biodegradation of diesel contamination , this study part of extensive research for microbial decomposition of petroleum and its products and also study microbial criteria of diesel biodegradation , so the present out line may give a data base for more and future studies in Saudi Arabia and in the Gulf Region .

## MATERIALS AND METHODS

Soil samples each measuring approximately 10 gm contaminated by diesel were randomly collected from Riyadh Saudi Arabia, each to a depth of 10 cm from the topsoil and stored in sterile screw-capped tubes ( 5 samples). Soil samples were resuspended in 10 ml of sterile distilled water and vigorously shaken for 5 min. Mineral liquid medium was used as a basal medium ( $\text{g L}^{-1}$ ) ( $\text{K}_2\text{HPO}_4$  1.71;  $\text{KH}_2\text{PO}_4$  1.32;  $\text{NH}_4\text{Cl}$  1.26;  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$  0.011;  $\text{CaCl}_2$  0.02 and 1 ml of trace mineral solution) to which spread plate technique was used for culture isolation and enumeration. The cultures were then incubated at 37°C; isolates exhibiting distinct colonial morphologies were isolated by repeated subculturing into the medium until purified fungus was obtained .

*Ulocladium atrum* isolated from diesel contaminated soil was identified (Figure 1) (Moubasher, 1993).

The tested fungus was grown on PDA plates, and discs of mycelium were cut from the margin of actively growing colonies using 4 mm cork borer, and transferred to 250 ml conical flask (1 disc/flask) containing 50 ml of the liquid mineral medium to which diesel was added to the flask at 0, 3, 6, 8 and 10 ml; the medium was adjusted to pH 6 before being sterilized (vacuum filtration membranes 0.22  $\mu\text{m}$ ) while flasks were incubated at 37°C and harvest were taken at 7, 14 and 21 days. At harvest, mycelium was transferred to preweighed filter paper, thoroughly washed with hexane, oven dried at 80°C for 24 h and reweighed. The pH of the residual media was also measured.

The soil for laboratory experiments was dried at 80°C overnight to eliminate microbial growth. Once dried, 0.2 g diesel (dry soil)<sup>-1</sup> was uniformly placed on the soil and it was allowed to be adsorbed for 30 min. After adsorption of diesel, the humidity was adjusted

to approximately 45%; the soil had an organic matter content of 5.8%, nitrogen content of 0.23% and an available phosphate content of 25%, control soil samples and sterilized and non-sterilized soil samples were included.

Seven grams of soil with adsorbed diesel were placed in a flask, and inoculated with *U. atrum* and incubated at 37°C. The control was carried out without addition of the tested fungus (n=3) and the sampling was programmed once a week for seven weeks.

## RESULTS AND DISCUSSION

In Saudi Arabia, petroleum products are responsible for the generation of large amounts of organic residues, as well as for the pollution of air, soil and water. So, one of the best approaches to restore contaminated environments is to make use of the physiological potential of microorganisms to degrade the pollutants through biotreatment process. An attractive approach has been used in different studies to clean up hydrocarbons pollutants because it is simple to maintain, applicable over large areas, cost effective and leads to the complete destruction of the contamination (Bento *et. al.*, 2005).

Mycelial growth rate of the tested fungus at different concentrations of diesel after 7, 14 and 21 days are given in Table 1. At all diesel concentrations, the tested fungus was able to grow with different rates. It is clear that the fungus was capable of growing and utilizing the diesel and this is in agreement with the results of different investigations (Bento *et. al.*, 2005; Mohanan, 2005; Nwaogu *et. al.*, 2008).

Mycelial growth rate of the tested fungus at different concentrations of diesel after 21 days of growth are given in Figure 2. From the present results, it could be observed that the biodegradation potential of the tested fungus varied within the different concentrations as mentioned (Santos, 2007; Binsadiq, 2012).

It is also observed from Figure 3 that *U. atrum* shifts pH toward acidity and this is in agreement with different investigations (Binsadiq and Al-Obaid, 1996; Binsadiq, 2012; Miranda *et. al.*, 2007).

At extreme acidity, biodegradation tends to be fastest; the effect of pH on biodegradation of diesel contamination has received scant attention. Biodegradation is the cleaning up of contaminated sites by exploiting diverse metabolic abilities for microorganism such as fungi to convert diesel contamination to less harmless products by mineralization and generation of carbon oxide and water or by conversion into microbial biomass.

The results obtained for the tested fungus can be used to draw a biodegradation protocol involving the optimization of parameters, such as biostimulation and bioaugmentation for the recuperation of areas contaminated by diesel as well as by other petroleum products.

Manipulating the treatment site to favor microbiological growth promotes the degradation and hence alleviates the actual or potential pollution (Binsadiq and Al-Obaid, 1996; White *et. al.*, 2006). The results of laboratory experiments indicated that the enhancement of the microbial activity in hydrocarbon contaminated soil can be achieved with the combination of stepwise soil inoculation and nutrient additions, and it is also clear that *U. atrum* have been reported to utilize diesel (Rocha *et. al.*, 2001).

*Ucladium* species have also been frequently reported as a degrader of various petroleum products as well as diesel (Leahy and Colwell, 1990). In contrast, the tested fungus was able to destroy the diesel and grow very well in the soil contaminated with diesel, to which the soil became nearly free of the diesel contamination after seven weeks, while there was no change in the control.

The application of microorganisms activities dominate present day biotechnology, there different environmental and economically important uses of microorganisms, such as biotreatment of petroleum contamination (Binsadiq, 2004B).

This study might establish a simple scale up procedure with good predictability by using fungal isolation in liquid, and then inoculation of the contaminated soil in Saudi Arabia. This procedure might also enhance the capabilities of a fungal species to degrade and mineralize oil products such as diesel.

In conclusion in Saudi Arabia, petroleum and its products are responsible of soil, water and air contamination, so one of the best approaches to restore contaminated environments is to make use of the physiological potential microorganisms to degrade the pollutants such as diesel. The result of this research indicates that the isolated fungi from diesel contaminated soil can grow and transfer the diesel to their cells as a source of carbon and energy and this may help the contamination removal in Saudi Arabia to get out of the problems of hydrocarbons where the Gulf Region and Saudi Arabia are considered one of the main crude oil export in the world.

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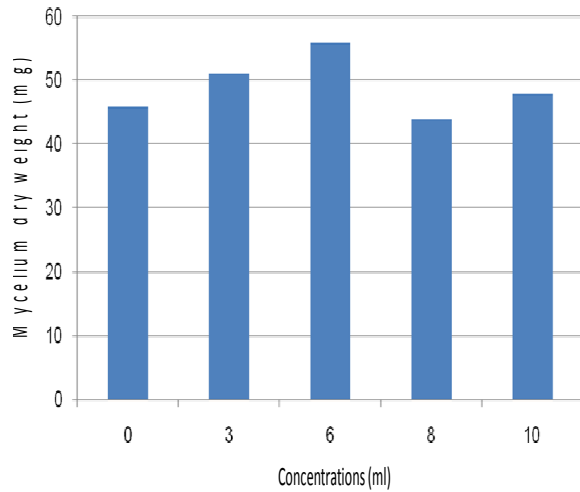
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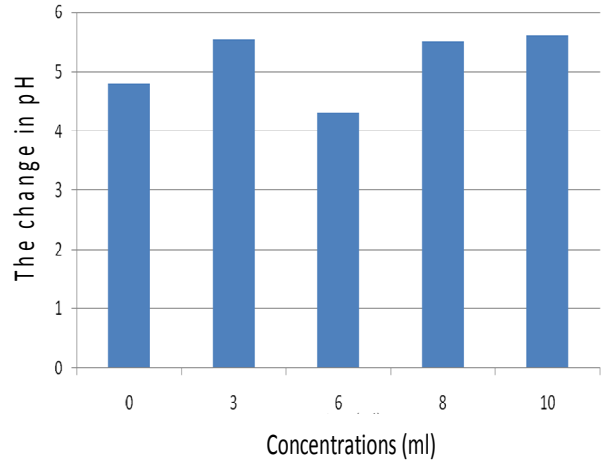
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**Figure 1.** *Ulocladium atrum*, conidiophores and conidia.



**Figure 2.** Mycelial growth rate of the tested fungus at different concentrations of diesel after 21 days of growth



**Figure 3.** The changes in pH of the filtered medium after 21 days of *U. atrum* growth in different concentrations of diesel (ml).

**Table 1.** Mycelial growth rate of the tested fungus at different concentrations of diesel (ml) n=3, ±= standard deviation, W= dry weight (mg), start pH=6.

Harvest days	Diesel concentrations (ml)									
	0		3		6		8		10	
	W	pH	W	pH	W	pH	W	pH	W	pH
7	30±0.3	4.8	33±0.15	5.11	48±0.3	5.6	53±0.6	5.1	50±0.11	5.11
14	35±0.11	4.5	48±0.3	5.8	51±0.6	5.1	42±0.11	5.8	53±0.03	4.8
21	46±0.6	4.8	51±0.5	5.53	56±0.3	4.3	44±0.9	5.5	48±0.5	5.6

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