Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 21, No. (4) : 2019 : 991-996

ISSN-0972-3005

DEGRADATION OF CRUDE OIL COMPONENTS AND POLYCYCLIC AROMATIC COMPOUNDS IN A BIOAUGMENTATION STUDY USING *SCHIZOPHYLLUM COMMUNE*

KANIKA SHARMA*[1], NIRMAL SUDHIR KUMAR HARSH[1] AND VINAY KUMAR VARSHNEY[2]

[1]Forest Pathology Division, Forest Research Institute, New Forest, Dehradun 248 006, Uttarakand, India
[2]Chemistry Division, Forest Research Institute, New Forest, Dehradun 248 006. Uttarakand, India

(Received 16 January, 2019; accepted 22 March, 2019)

Key words : Mycoremediation, Soil, S. Commune*, GROs, DROs, TPHs, PAHs*

Abstract – A feasibility study was conducted to evaluate the capability of *Schizophyllum commune* a white rot fungus to degrade crude oil components {Gasoline Range Organics (GROs), Diesel Range Organics (DROs), Total Petroleum Hydrocarbons (TPHs) and Polycyclic aromatic hydrocarbons (PAHs)} under *in vitro* conditions and its analysis using Gas Chromatography-Flame Ionization Detector (GC-FID). Samples from oil spill site of Oil and Natural Gas Corporation Limited (O.N.G.C. Ltd.) Ghandhar Asset, *Group Gathering Station*(GGS-II) site, Ankleshwar, Gujarat collected from different distances, i.e. near the well (NW), 20 m, 40 m, 60 m and 80 m distances were treated with *S. commune* in a bioaugmentation experiment. After two weeks of incubation the fungus was found to be effective in degrading GROs (26.4%-71.1%), DROs (20.28%-75.82%), PAHs (15.21%-71.29%) and TPHs (21.82%-74.66%).

INTRODUCTION

Crude oil pollution is a serious concern to protect the biotic as well as the abiotic components of the ecosystem for their harmful effects when spilled accidently. Crude oil is one of the most important resources of energy in the modern industrial world. Oils are used to run many types of engines, lamps, heaters and stoves. The invention of the internal combustion engine and its fast adoption in all transport forms enlarged the employment of this natural resource, thus increasing its demand, production, transport, stockpiling, and distribution, as well as the raw oil and its by-products. All these activities involve pollution risks that can be minimized, but not totally eliminated, causing several problems for the environment (Pala *et al*., 2006).

Oil spills have become global problem particularly in industrialized and developing countries. Contamination of soils and aquifers by oil spills is a persistent and widespread pollution problem ravaging almost all compartments of the environment and imposing serious health implications and ecological disturbances (Bundy *et al*., 2002; Okoh, 2006). The quality of life on earth is linked, inextricably, to the overall quality of the environment. Releases of persistent, bioaccumulative and toxic chemicals have a detrimental impact on human health and the environment. These contaminants find their way into the tissues of plants, animals and human beings by the movement of hazardous constituents in the environment (Vidali, 2001). Petroleum contaminants are typical examples of these hazardous constituents.

There has been increasing interest by researchers in the application of fungi and nutrients to contaminated soils for effective mycoremediation of crude oil components. Although several studies have been conducted on mineralization or degradation of hydrocarbon by microorganisms (Ojumu *et al*., 2005) and exotic mushrooms, very little work has been done on white-rot fungus *S.*

Abbreviations: GROs=Gasoline Range Organics, DROs= Diesel Range Organics, TPHs= Total Petroleum Hydrocarbons, PAHs= Polycyclic aromatic hydrocarbons, GC-FID= Gas Chromatography-Flame Ionization Detector, O.N.G.C. Ltd.= Oil and Natural Gas Corporation Limited, GGS-II=*Group Gathering Station, NW=Near the well.*

commune. In view of this, the present work is aimed at studying the ability of *S. commune* to degrade crude oil components (GROs, DROs, PAHs and TPHs) of the contaminated soil.

MATERIALS AND METHODS

Sample collection

Crude oil contaminated soil samples were collected from oil spill site of O.N.G.C. Ltd.Gandhar Asset. GGS II site, Ankleshwar, Gujarat. As the spill occurred from the oil producing well and covered an area of 40 x 90 m (3,600 sq m) therefore, samples were collected at different distances from the well i.e. near the well, at 20 m distance, at 40 m distance, at 60 m distance and at 80 m distance from the well. Distances were measured by using measuring tapes. Samples were collected in wide mouth glass jar with Teflon lined cap, brought to the laboratory and stored in deep refrigerator at -20ºC as recommended by Weisman (1998).

Fungus

Schizophyllum commune Fr. was obtained from the National Type Culture Collection (NTCC), Forest Pathology Division, Forest Research Institute, Dehradun for this study. Fungal culture was maintained on potato dextrose agar (PDA) slants and plates.

Amendments/ supplements

Molasses was obtained from Doon Valley Distilleries, Kuanwala, Dehradun. Yeast extract (RM 027) of Himedia Laboratories (India) make was used as a nitrogen source in the present study. These nutrient supplements were used in the bioaugmentation study. Molasses (1% w v^{-1}) and yeast extract (1% w v^{-1}) were prepared in distilled water and autoclaved at 121ºC, 15 psi for 20 min which were than cooled down to 45-50ºC.

Preparation of standard solutions of GROs, DROs and PAHs for gas chromatographic analysis

Gasoline range organics, diesel range organics and polycyclic aromatic hydrocarbons standard solutions were prepared at a concentration of 10 mg ml^{-1} of dichloromethane(DCM) by following the procedure given by Wisconsin (1995). Gasoline rangestandards were: Methyter butyl ether, benzene, toluene, ethyl benzene, m- xylene , p-xylene, o- xylene, 2,2,4- Trimethylpentane. Diesel range standards were: Decane (≥ 99%), dodecane (≥ 99%), tetradecane (Olefine free, ≥ 99%), hexadecane (≥ 99%), octadecane (99%), eicosane (99%), docosane (99%), tetracosane (99%), hexacosane (99%), octacosane (99%). Polycyclic aromatic standards were: Napthalene (≥ 99%), acenapthene (99%), fluorene (98%), phenanthrene (Sublimed, >99.5%), anthracene (99%), pyrene (98%). Individual GRO standard solutions (10 mg mL^{-1}) were divided in 10 mL of DCM in a volumetric flask. One mL of each of the GROstandard solution was then mixed in a 10 ml volumetric flask and used for GC-FID analysis to detect its retention time, peak area, peak height, etc.Similarly, diesel range organics (DRO) standard solutions and polycyclic aromatic hydrocarbons (PAH) standard solutions were prepared.

GC-FID Analysis

The GC analysis of the extracted crude oil was performed on a gas chromatograph Trace GC 600 Series (Thermo Fisher Scientific), equipped with FID, split/ splitless injector (1:20 split ratio), a BP5 (5% phenyl and 95% dimethyl polysiloxane) capillary column (30 m x 0.25 mm; film thickness 0.25 μm). Temperature programming was from 40ºC (1 min) @ 5ºC min^{-1} to 100ºC 8ºC to 240ºC (90 min). The injector and detector temperatures were 220°C and 260°C. Nitrogen (99.99%) was used as a carrier gas at a constant flow of 1.5 mL min^{-1}. Total peak area was determined directly from the chromatogram after adjusting the areas of the solvent and internal standard peak. The identity of the petroleum hydrocarbons and polycyclic aromatic compounds in the crude oil contaminated soil sample was established by comparison of retention time of the peak with that of the reference standard.

Bioaugmentation experiment

In this experiment, sterilized (autoclaved) crude oil contaminated soil from near well, 20 m, 40 m, 60 m and 80 m distances were used to study the effect of addition of nutrients and test fungus in degradation of GROs, DROs, TPHs and PAHs as per modified protocol of Adenipekun and Fasidi (2005). Crude oil contaminated soil (25 g) was collected in 100 mL volumetric flask and autoclaved at 121ºC, 15 psi for 20 min to remove any indigenous microorganism if present in the soil sample. To this soil, 3 mL yeast extract (1%, w v^{-1}) and 2 mL molasses (1%, v v^{-1}) was added. The soil samples were then inoculated with 3 discs of 1 cm^2 culture of *S. commune* grown on PDA. Soil samples without test fungus was used as

control.

GC analysis of the extracted crude oil for the estimation of total TPHs, GROs, DROs and PAHs compounds

Teng of soil sample from near the well, 20 m, 40 m, 60 m and 80 m distanceswas dissolved in 40 ml of DCM containing heptane (1 g l^{-1}) as an internal standard and kept on shaker for 24 h. The supernatant was taken and centrifuged at 4,000 rpm for 10 min to remove any soil pellet, if present. The supernatant was transferred to 10 mL volumetric flask and stored at –20°C until analyzed by GC-FID (Adebusoye *et al*., 2007; Adebusoye *et al*., 2010).

Concentration of the analyte was calculated using the following formula:

$$\text{Conc. of analyte (mg/g)} = \frac{\text{Area ratio x Extraction volume}}{\text{Weight of sample}}$$

Area ratio = Peak area/ Area of internal standard

Using this formula, concentrations of crude oil components (GROs, DROs and PAHs) in original soil, sterilized soil and treated soil (with *S. commune*) were calculated for bioaugmentation treatment. In the present study, the following formula was used to calculate the percentage degradation of individual components of crude oil (GROs, DROs and PAHs) in bioaugmentation experiment:

$$\text{Percentage degradation (\%)} = \frac{X_1 - [(X_1 - X_2) + X_3] \times 100}{X_1}$$

Where, X_1= Original soil concentration, X_2= Sterilize soil concentration, X_3= Treated soil concentration.

Statistical analysis of data

Experiments were conducted using three independent replicates. Data were subjected to analysis of variance (ANOVA) and the averages were compared by Tukey's test at $p < 0.01$.

RESULTS

The results of bioaugmentation, expressed as percentage degradation of the compounds constituting in GROs, DROs and PAHs are given in Table 1. These compounds were selected on the basis of prior analysis of crude oil contaminated soil for detection of individual compounds present in the sample. Crude oil contaminated soil samples collected from different distances were treated with *S. commune*.

In the present study, fungal spore count at day zero was 1.498 x 10^8 spores ml^{-1}. Colony forming units of test fungus after 14 days of incubation was found to be: 0.8 x 10^5, 1.2 x 10^5, 2.0 x 10^5, 2.2 x 10^5, 2.8 x 10^5, respectively at near well, 20 m, 40 m, 60 m and 80 m distances.

Percentage degradation of individual components of GROs, DROs and PAHs increased as the distance increased from well (Table 1) i.e. maximum degradation was achieved at 80 m distance followed by 60 m, 40 m, 20 m and near well.

At near well, significant maximum percentage degradation of dodecane (34.77%) and minimum percentage degradation of hexadecane (11.65%) was observed with *S. commune*. At 20 m distance, significant maximum percentage degradation of dodecane (54.12%) and significant minimum percentage degradation of tetradecane (17.76%) was observed. At 40 m distance, maximum value of percentage degradation was observed with tetracosane (67.07%) and minimum value was observed with 1, 2, 4 Trimethylbenzene (TMB) (42.38%). At 60 m distance, maximum percentage degradation was observed with benzene (78.19%) which was not significantly different from the percentage degradation value of octacosane (77.52%). Significant minimum percentage degradation of 1, 2, 4 TMB (49.02%) was observed at this distance. At 80 m distance, percentage degradation of benzene was 79.67% which was not significantly different from the percentage degradation values of naphthalene (80.01%), tetradecane (81.43%), hexadecane (79.74%), fluorene (81.08%), tetracosane (77.23%) and octacosane (82.31%). Significant minimum percentage degradation was observed with 1, 2, 4 TMB (53.62%) at this distance.

Effect of *S. commune* in degradation of total GROs, total DROs, total PAHs and total petroleum hydrocarbons (TPHs) were studied and presented in Table-2 and Fig.1(a-d). Percentage degradation of these compounds when treated with *S. commune* increased as the distance increased from the well. Minimum percentage GROs degradation (26.41%) was observed at near well, while maximum degradation of GROs was observed at 80 m distance (71.10%). Similarly, with DROs minimum percentage degradation (20.28%) was observed at near well distance while maximum percentage

*Table 1. Percentage degradation of crude oil components when treated with *S. commune* [Values are means ± SE of 3 replicates]

S. No	Compounds	Percentage Degradation (%) at Near well	Percentage Degradation (%) At 20 m distance	Percentage Degradation (%) at 40 m distance	Percentage Degradation (%) At 60 m distance	Percentage Degradation (%) at 80 m distance
Gasoline Range Organics						
1	Benzene	26.92±0.464^{f}	33.64±0.015^{f}	46.84±0.377^{ab}	78.19±0.765^{i}	79.67±0.887^{fg}
2	Toluene	0	0	0	0	0
3	Ethylbenzene	0	0	0	0	0
4	o-xylene	0	0	0	0	0
5	1,2,4 TMB	29.03±0.550^{g}	32.83±0.771^{def}	42.38±0.198^{a}	49.02±0.277^{a}	53.62±0.968^{a}
6	Napthalene	23.28±0.708^{e}	32.03±0.218^{def}	62.24±0.561^{efg}	74.57±0.449^{gh}	80.01±0.039^{fg}
Diesel Range Organics						
7	Dodecene	34.77±0.587^{h}	54.12±0.688^{h}	54.84±3.002^{c}	70.39±0.513^{ef}	74.87±1.766^{de}
8	Tetradecane	12.56±0.219^{a}	17.76±0.458^{a}	57.40±0.346^{cd}	62.91±1.628^{c}	81.43±0.102^{fg}
9	Hexadecane	11.65±0.038^{a}	33.00±0.108^{ef}	62.17±0.699^{efg}	63.55±0.427^{c}	79.74±0.585^{fg}
10	Octadecane	26.03±0.031^{f}	31.48±0.156^{de}	63.72±0.228^{fgh}	65.67±0.203^{cd}	69.35±0.139^{c}
11	Eicosane	23.74±0.076^{e}	30.91±0.220^{d}	65.04±0.324^{gh}	67.55±0.768^{de}	72.09±0.307^{cd}
12	Docosane	19.17±0.018^{cd}	26.27±0.093^{bc}	65.69±0.543^{gh}	66.10±0.370^{cd}	72.49±0.073^{cd}
13	Tetracosane	20.97±0.002^{d}	32.35±0.455^{def}	67.07±0.219^{h}	71.26±0.511^{fg}	77.23±0.573^{ef}
14	Hexacosane	15.20±0.422^{b}	24.80±0.152^{b}	59.95±0.439^{def}	70.06±0.092^{ef}	72.88±0.154^{cde}
15	Octacosane	18.45±0.015^{c}	28.17±0.458^{c}	58.89±0.435^{cde}	77.52±0.226^{hi}	82.31±0.273^{g}
Poly cyclic Aromatic Compounds						
16	Acenapthene	11.96±0.605^{a}	31.02±0.422^{d}	49.03±0.269^{b}	58.80±1.292^{b}	59.28±1.158^{b}
17	Anthracene	20.56±0.118^{d}	33.84±0.172^{f}	62.04±0.583^{defg}	72.03±0.258^{fg}	73.51±0.997^{cde}
18	Fluorene	13.11±0.628^{a}	37.70±0.118^{g}	58.14±0.916^{cde}	74.45±0.345^{gh}	81.08±1.874^{fg}

*Values in each column are means along with standard errors. Superscripts have been used in each column based on grouping of means (Tukey's test $p \leq 0.01$). Means with same superscripts are not significantly different from each other. Values of standard errors have been rounded off.

*Table 2. Effect of *Schizophyllum* commune in degradation (%) of GROs, DROs, PAHs and TPHs [Values are means ± SE of 3 replicates]

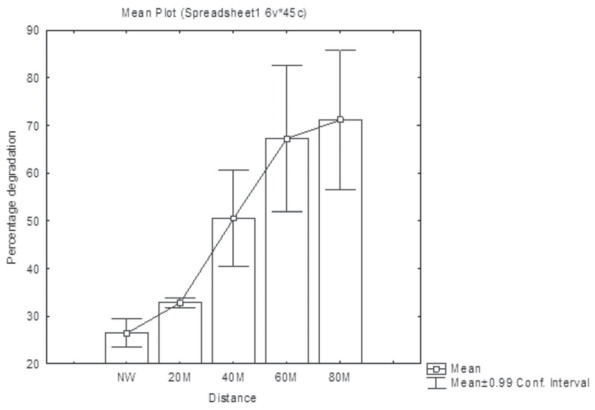
Distance (m)	*Schizophyllum commune*			
	Percentege degradation (%) of GROs	Percentege degradation (%) of DROs	Percentege degradation (%) of PAHs	Percentege degradation (%) of TPHs
Near well	26.41±0.889^{a}	20.28±1.34^{a}	15.21±1.37^{b}	21.82±1.117^{bc}
20m	32.83±0.328^{a}	30.98±1.83^{b}	34.18±0.97^{c}	31.45±1.378^{de}
40m	50.49±3.01^{c}	61.64±0.82^{c}	56.40±1.95^{d}	58.86±1.249^{hi}
60m	67.26±4.59^{b}	68.34±0.86^{d}	68.43±2.46^{a}	68.07±1.276^{jk}
80m	71.10±4.38^{b}	75.82±0.86^{e}	71.29±3.27^{a}	74.66±1.279^{kl}

*Values in each column are means along with standard errors. Superscripts have been used in each column based on grouping of means (Tukey's test $p \leq 0.01$). Means with same superscripts are not significantly different from each other. Values of standard errors have been rounded off.

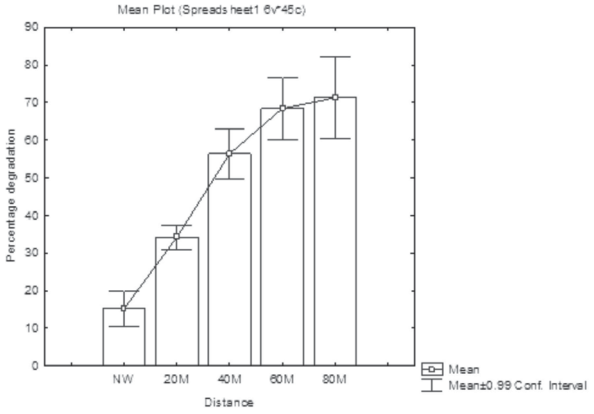
degradation (75.82%) was observed at 80 m distance. With PAHs, minimum percentage degradation (15.21%) was observed at near well distance while maximum percentage degradation (71.29%) was observed at 80 m distance. With TPHs, maximum percentage degradation (74.66%) was observed at 80 m distance which was not significantly different from the percentage degradation (68.07%) observed at 60 m distance. Minimum value of TPHs (21.82%) was observed at near well distance with *S. commune*.

DISCUSSION

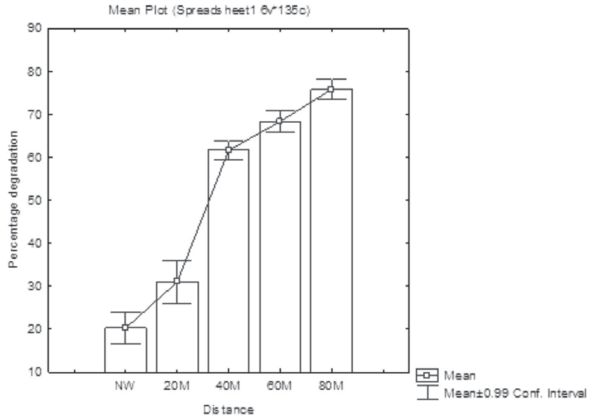
Mycoremediation potential of *S. commune* in PAHs



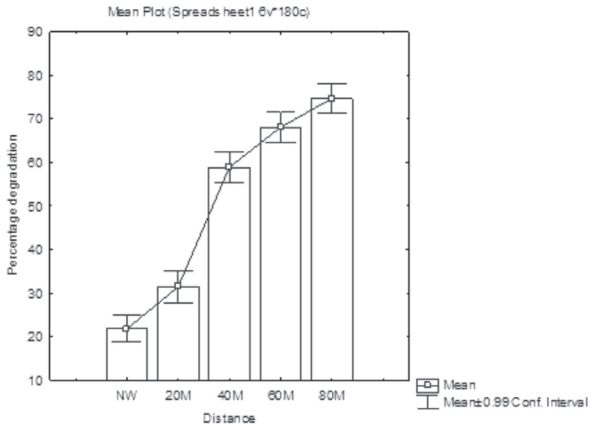
(a) Mean error effect plot of percentage degradation of GROs at different distances



(c) Mean error effect plot of percentage degradation of PAHs at different distances



(b) Mean error effect plot of percentage degradation of DROs at different distances



(d) Mean error effect plot of percentage degradation of TPHs at different distances

Fig. 1(a-d). Percentage degradation patterns of GROs, DROs, PAHs and TPHs at different distances of contaminated soil

degradation was reported by Matsubara *et al*. (2006) and Kim *et al*. (2010). However, role of *S. commune* in degradation of crude oil components (GROs, DROs and TPHs) is being reported for the first time in this study.Percentage degradation of gasoline range organics (toluene, ethylbenzene and o-xylene) could not be determined in sterilized soil and treated soil because these compounds are highly volatile in nature which might have caused them to escape into the atmosphere during the sterilization procedures and hence could not be detected in gas chromatogram. It has already been reported that TPHs in soils can be lost due to abiotic processes, such as volatilization, as well as due to the biotic activity of microorganisms as reported by Margesin *et al*. (2007). In another study, abiotic loss of TPHs has been shown to be less than 10% of total TPHs at 25°C within the first 30 days (Margesin and Schinner, 1997).

Increase in the population of petroleum degrading fungusafter 14 days of incubation in this study is an indication of the adaptation of the fungus to petroleum contamination which is in accordance with the findings of Song and Bartha (1990); Delille *et al*. (1997) and Amadi and Braide (2003).

In the present study, efficient degradation of GROs, DROs, TPHs and PAHs was observed in sterilized contaminated soil which was inoculated with *S. commune* and supplemented with molasses and yeast extract. Addition of molasses is applicable and effective biostimulation method to enhance the soil bioremediation rate. Al- Hadhrami*et al*. (1996) showed that addition of an alternative carbon and nutrient source such as molasses increased respiration and n-alkane degradation in synthetic sea water. Furthermore, addition of cane molasses can also improve the efficiency of TPHs

biodegradation. Cosgrove *et al*. (2010) reported that biostimulation with yeast extract increased degradation of aromatic compounds by 45% as compared to control soil. Yeast extract allowed a larger degrading population to exist on the surface, leading to enhanced degradation by inducing enzymes responsible for degradation.

CONCLUSION

S. commune was found to be effective for the *in-vitro* degradation of GROs, DROs, TPHs and PAHs components of crude oil when supplemented with nutrients in a bioaugmentation study. The degradation capacity of the fungus increased as the distance increased from the well, when the soil was treated for 14 days.

ACKNOWLEDGEMENT

Authors are thankful to O.N.G.C,Ankleshwar, Ghandhar Asset. for providing contaminated soil sample for carrying out crude oil analysis. The study was supported by Forest Research Institute, Dehradun and funded by Department of Science and Technology-Innovation in Science Pursuit for Inspired Research (DST-INSPIRE), Government of India under the research fellowship program.

REFERENCES

Adebusoye, S.A., Ilori, M.O., Obayori, O.S., Oyetibo, G.O., Akindele, K.A. and Amund, O.O. 2010. Efficiency of cassava steep liquor for bioremediation of diesel oil-contaminated tropical agricultural soil. *Environmentalist.* 30 : 24-34.

Adenipekun, C.O. and Fasidi, I.O., 2005. Bioremediation of oil-polluted soil by *Lentinussubnudus*, a Nigerian white-rot fungus. *African Journal of Biotechnology*. 4 (8) : 796-798.

Al-Hadhrami, M.N., Lappin, S.H.M. and Fisher, P.J., 1996. Effects of addition of organic carbon source on bacterial respiration and *n*-alkane biodegradation of Omami crude oil. *Marine Pollution Bulletin.* 32 : 351-357.

Bundy, J.G., Paton, G.I. and Campbell, C.D. 2002. Microbial communities in different soil types do not converge after diesel contamination. *Journal of Applied Microbiology.* 92 : 276-288.

Cosgrove, L., McGeechan, P.L., Handley, P.S. and Robson, G.D., 2010. Effect of biostimulation and bioaugmentation on degradation of polyurethane buried in Soil. *Applied and Environmental Microbiology*. 76 (3) : 810-819.

Delille, D., Basseres, A. and Dessomme, A. 1997. Seasonal variation of bacteria in sea ice contaminated by diesel fuel and dispersed crude oil. *Microbial Ecology.* 33 : 97-105.

Kim, M.-J., Lee, H., Choi, Y.-S., Kim, G.-H., Huh, N.-Y., Lee, S., Lim, Y.W., Lee, S.-S. and Kim J.-J., 2010. Diversity of fungi in creosote-treated crosstie wastes and their resistance to polycyclic aromatic hydrocarbons. *Antonie van Leeuwenhoek*. 97 : 377–387.

Kulshreshtha, S., Mathur, N. and Bhatnagar, P. 2014. *AMB Expr* 2014. 4 : 29. https://doi.org/10.1186/s13568-014-0029-8

Margesin, R. and F. Schinner., 1997. Effect of temperature on oil degradation by a psychrotrophic yeast in liquid culture and in soil. *FEMS Microbiology Ecology*. 24 : 243-249.

Margesin, R., Hämmerle, M. and Tscherko, D. 2007. Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: Effects of hydrocarbon contamination, fertilizers, and incubation time. *Microbial Ecol*ogy. 53 : 259-269.

Matsubara, M., Lynch, J.M. and De Leij, F.A.A.M. 2006. A simple screening procedure for selecting fungi with potential for use in the bioremediation of contaminated land. *Enzyme and Microbial Technology.* 39 : 1365-1372.

Ojumu, T.V., Bello, O.O., Sonibare, J.A., Solomon, B.O., 2005. Evaluation of microbial systems for bioremediation of petroleum refinery effluents in Nigeria. *African Journal of Biotechnol*ogy. 4 (1): 31-35.

Okoh, A.I. 2006. Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. *Biotechnology and Molecular Biology Reveiws.* 1: 38-50.

Pala, M., Carvalho, D.D., Pinto, J.C. and Sant Anna Jr, G.L. 2006. A suitable model to describe bioremediation of a petroleum-contaminated soil. *International Biodeterioration & Biodegradation.* 58 (6): 254-260.

Reddy, C.A. 1995. The potential for white-rot fungi in the treatment of pollutants. *Current Opinion in Biotechnology*. 6 : 320-328.

Song, H.G. and Bartha, R. 1990. Effects of Jet Fuel Spills on the Microbial Community of Soil. *Applied and Environmental Microbiology*. 56 (3) : 646-651.

Vidali, M. 2001. Bioremediation. An overview. *Pure Appl. Chem*. 73 : 1163-1172.

Weisman, W. 1998. Analysis of Petroleum Hydrocarbons in Environmental Media. Total Petroleum Hydrocarbon Criteria Working Group Series Volume 1. Amherst Scientific Publishers. pp. 97.

Wisconsin, 1995. DNR Modified DRO method for determining Diesel Range Organics. PUBL-SW-141 September 1995. http://dnr.wi.gov/regulations/labcert/documents/methods/DROSep95.pdf