J. Exp. Zool. India Vol. 18, Supplement 1, pp. 13-21, 2015 *ISSN 0972-0030*

ROLE OF FUNGI IN THE BIODEGRADATION OF CRUDE OIL

Babita Kumari*, S. N. Singh and D. P. Singh[1]

Plant Ecology and Environmental Science Division, CSIR-National Botanical Research Institute, Lucknow - 226 001, India.
[1]School of Environmental Science, Babasahab Bhimrao Ambedkar University, Lucknow - 226 025, India.
*e-mail: babita.bit86@gmail.com

ABSTRACT : Role of bacteria has been extensively exploited in the area of degradation of petroleum hydrocarbon or petroleum crude oil. However, only a few fungal species were reported for the degradation of petroleum hydrocarbon. Lignolytic enzymes induced by fungi i.e. lignin peroxidase, manganese peroxidase and laccase play an important role in the mineralization of petroleum hydrocarbons. Laccases enzymes are capable of catalysing the oxidation of phenols, polyphenols, and anilines, coupled to the 4-electron reduction of molecular oxygen to water. In present study, seven fungal strains were isolated from crude oil contaminated soil, collected from Barauni Oil Refinery, Begusari, Bihar. Among the isolated fungal strains, *Aspergillus* sp. PS9 degraded maximum (31%) of total petroleum hydrocarbon in MSM supplemented with 2% of crude oil after 10 days of incubation period. During the degradation of crude oil, laccase activity was found significantly highest in PS9 (1025 ± 21.5 çmol mg^{-1}) as compare to other fungal strains. When this strain was applied with different bacterial strains in soil contaminated with crude oil, highest degradation of crude oil was recorded by this strain with *Pseudomonas* sp. BP10. This study recommends the use of fungal strains for the decontamination of petroleum hydrocarbons from our environment as bacteria and fungi are ubiquitous and co-exist in soil.

Key words : Fungi, laccase, bacteria, crude oil.

INTRODUCTION

Crude oil is a major source of energy and various petroleum products. Besides, it is also a source of petroleum hydrocarbons contamination of soil or water. Crude oil spillage is very common incident and frequently occurs worldwide during its exploration, production, transportation, storage and refining processes. Impact of oil spill can be understood easily by the fact that one barrel of crude oil can make one million barrels of water undrinkable (Petroleum Review, 1998; Ercoli *et al*, 2001). Oil spills involve the release of dangerous hydrocarbons, such as benzene and poly nuclear aromatic hydrocarbons into the soil and water sources. These spillages affect vast stretches of land and waterways, thus polluting not only crops, but also marine life and the sources of water for domestic uses.

Recovery of spilled crude oil by physical and chemical methods over the marine water is possible only to 10-15% of oil. As compared to physical and chemical methods, such as booms, skimmers, adsorbents, chemical surfactants, oxidants etc., bioremediation is thought to be a self-driven, economical and eco-friendly method.

However, bacteria, fungi (Dawoodi *et al*, 2015; Kota *et al*, 2014) or algae (Aditi *et al*, 2015) were already reported as petroleum hydrocarbon degrader, fungi was considered as better than bacteria because of fungal hyphae and potential hydrolytic enzyme that can penetrate deeply in soil resulted in the degradation of petroleum hydrocarbons (Messias *et al*, 2009; Venkatesagowda *et al*, 2012).

Several fungi species which have been recorded as biodegraders belong to the genera: *Alternaria, Amorphoteca, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Graphium, Mucor, Paecilomyces, Penicillium, Rhizopus, Rhodotorula, Sphaeropsidales, Talaromyces* and *Trichoderma* (Elshafie *et al*, 2007; Al-Nasrawi, 2012; Reyes-César *et al*, 2014). Crude oil is a complex mixture of many petroleum hydrocarbons, like alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen.

The purpose of this investigation is to compare the ability of the native fungal strains to degrade different hydrocarbon fractions of crude oil. Simultaneously, an induction of catabolic enzymes i.e. laccase and metabolite formation in terms of resorcinol formation were also investigated for their involvement in crude oil degradation. Environmental parameters like Temperature, pH, agitation (aeration) and concentration of contamination in the form of different concentration of crude oil was also optimize to get better biomass development. Selected fungal strains

Proceeding of the *National Seminar on Current Trends in Biological Sciences; Advances and Challenges* organised by Dept. of Zoology, Janta College, Bakewar, Etawah; 13-14 December 2014

were incubated with different hydrocarbon degrader bacterial strain (tested earlier, Kumari *et al*, 2012) in soil to find a combination of bacterial and fungal strains as bacteria and fungi are ubiquitous and co-exist in soil.

MATERIALS AND METHODS

Materials

Crude oil was procured from Barauni Oil Refinery, Barauni, India.

Isolation of fungal strains

2 g of crude oil was added to 100 ml sterilized minimal salt medium (MSM) (composition: 7 g di potassium phosphate, 2 g mono potassium phosphate, 0.5 g sodium citrate, 1 g ammonium sulfate, 0.1 g magnesium sulfate in 1 L medium pH= 7.0 ± 0.2) and then incubated at 37°C and 150 rpm in an orbital shaker for the enrichment of petroleum hydrocarbon degrading bacterial strains. After 1 week of incubation, 1 ml of active inoculum was transferred to the flasks having fresh sterilized MSM supplemented with oil (2% w/v). After 3 successive transfers in MSM (100ml) with 2% (w/v) of crude oil, the active inoculum was used for the isolation of TPH degrading fungi by serial dilution method and spreading over the potato dextrose agar (PDA, composition: 200 g potato infusion, 20 g dextrose and 15 g agar in 1.0 L medium with pH 5.6 ± 0.2). Separate colonies were picked up for the isolation of fungi on MSM agar plates supplemented with 1% (w/v) crude oil.

Screening on fungal strains

Isolated 7 fungal strains were incubated on MSM agar supplemented with crude oil as a sole source of carbon. Their utilization of crude oil as carbon source was observed on the base of their radial growth. Three fastest strains were screened out for further study on the in situ degradation of crude oil in MSM broth.

Experimental set up for the degradation of crude oil by fungal strains in MSM broth

Three best crude oil utilizing fungal strains were finally screened for the degradation of crude oil (2% w/v). Inoculum of PS9 was prepared by suspending the biomass of freshly grown PS9 in sterilized water so that inoculum density became approx. 106 spores ml^{-1}. One bit (0.8 cm) of each strain from PDA plate was added to 50 ml of sterilized MSM broth with 1 g of crude oil. Uninoculated medium was treated as control. Inoculated medium was kept for 15 days incubation period at 30°C at 150 rpm in an orbit shaker. Samples were harvested at every 5 days intervals for the control and inoculated flasks during the incubation period of 15 days.

Fractionation of TPH

Extracted TPH was dissolved in n-pentane. The undissolved fraction of TPH was separated by syringe filter which represents asphaltenes. Dissolved fraction was loaded in silica gel column and then its different fractions, such as alkane, aromatic, and NSO were eluted by using 100 ml of organic solvents; hexane, benzene and methanol+chloroform, respectively.

Analysis of Alkane and aromatic compounds in TPH

Alkane fraction of TPH was analyzed by GC (Agilent 7890A) with flame ionization detector using capillary BP-5 column (5% phenyl-95% methyl polysiloxane, 30 m × 0.32 mm × 0.25 µm) to quantify the different components of alkanes against the standard mixture of C12-C28. Both injection and detector temperatures were maintained at 280°C. The initial oven temperature was kept 80°C for 2 min and increased to 300°C with 10°C increase per min. The injection volume of sample was 5µl for GC analysis. On the other hand, aromatic fraction was analyzed by HPLC using UV detector (Dionex Ultimate 3000).

Aromatic

Fractions, collected in benzene containing aromatic compounds of crude oil, were concentrated to 2 ml and analyzed with GC-FID. The injection volume was 1 µl. The injection temperature and detector temperature were maintained at 280°C. Oven temperature was initiated with 60°C for 3 min and increased up to 280°C with the rate of 10°C min^{-1}. After holding for 1 min at this temperature, oven temp was again increased with the same rate to 300°C. Oven temperature was put on 5 min at 300°C.

Fungal biomass

Fungal growth was assessed in the form of biomass (µg ml^{-1}). Fungal biomass was seperated through filtering and analysed gravimetrically.

Extraction and estimation of extracellular enzyme

Fungal biomass was separated from 10 ml of harvested sample of MSM by centrifuging at 5,000g at 4°C for 15 min. Cell mass was washed and resuspended in the phosphate buffer (0.1 M, pH=7.0) and subjected to ultrasonic disruption at 003 RMS (at 0-4°C ten times, each time for 30 seconds followed by 2 minutes of incubation in ice (Okungbowa *et al*, 2007) Then sonicated sample was centrifuged at 10000 g for 15 min at 4^0C to separate out the cell debris. Supernatant was used for protein estimation and enzyme assays.

Protein was estimated by following the method of Lowry *et al* (1951). Standard Curve was prepared on

the basis of OD_{660} of different concentration of BSA (10-750 µg ml^{-1}).

Laccase activity

The specific activity of laccase was assayed spectrophotometrically (Perkin-Elmer Lambda 35 UV-VIS spectrophotometer) by monitoring an increase in absorbance at 530 nm ($\varepsilon = 65\ mM^{-1}\ cm^{-1}$) due to oxidation of syringaldazine. 3 ml of assay mixture contained 500 µl enzyme extract, 2.2 ml phosphate buffer (pH 6.5) and 300 µl of 0.216 mM syringaldazine in absolute methanol.

Resorcinol equivalent

The formation of hydroxylated aromatic compounds as indicator of PAH degradation intermediates were evaluated as resorcinol equivalent by using folin-ciocalteau reagent (Silva *et al*, 2009). 10 ml of medium was sonicated for 5 min in an ice bath with an addition of 1.5 ml of 200 g l^{-1} Na_2CO_3. After the addition of 0.5 ml of folin–ciocalteau reagent, the tubes were incubated for 30 min at 20°C. OD of the reaction mixture was measured at 750 nm by UV –Vis spectrophotometer (Perkin Elmer Lamda 35). Different concentrations of resorcinol prepared (from a 100 µg ml^{-1} stock solution) were used to prepare a standard curve for the quantification of resorcinol equivalent (RE) values.

Growth optimization for PS9

Growth of PS9 was monitored in the form of biomass (µg ml^{-1}) against the variable environmental factors like pH, temperature, dissolved oxygen (agitation) in MSM with 2% of crude oil. Autoclaved MSM (20 ml) supplemented 2% (w/v) of crude oil as carbon source in 100 ml conical flask was inoculated with PS9 and incubated in an orbital shaker for 6 days. At a time, one parameter was kept variable where as others were kept constant as described in table 1.

Testing of degradation capacity of PS9 in presence of bacteria

PS9 were tested for the degradation of crude oil in soil either in isolation and in combination of two other crude oil degrading bacteria i. e. BP10 and NJ2 (Kumari *et al*, 2012). 10 g sieved (2mm) soil was taken on the glass petri dishes spiked with 2% (w/w) crude oil. Three replicates of each spiked soil were inoculated with PS9, BP10+PS9 and NJ2+ PS9, seperatly. Soils were incubated at room temperature (25±2°C) and moisture level was maintained at 60-70% by using sterilized double distilled water. Soil was mixed with the help of sterilized sticks on alternate days to maintain aerobic condition.

After 30 days of incubation period, TPH was extracted from soil samples using soxhlet extraction method and the extracts were analyzed by the gravimetric method. 5g of soil sample was taken in a porous cellulose thimble (25 × 70 mm) and placed in a soxhlet extractor. The extractor was consecutively loaded with equal volumes of hexane, dichloromethane, methanol and chloroform (100 ml each). Extracted solutions were pooled in pre-weighted beakers and evaporated for quantification of different hydrocarbon fractions.

Statistical analysis

All the data were collected in triplicates and represented with the mean value in graph and their standard deviation (as error bar in figure). One way analysis of variance (ANOVA) and least significant difference (LSD) at $p \leq 0.05$ were calculated, specially, to determine the significance differences in the degradation of total petroleum hydrocarbon (TPH) due to augmentation of different microbial strains either in isolation or combination and treatment of different biostimulants.

RESULTS

Screening of fungal strains

Seven fungal strains were isolated from the crude oil contaminated soil through the enrichment method and these strains were coded as PS1, PS2, PS3, PS5, PS6, PS8 and PS9. When they were grown on MSM agar supplemented with crude oil (1%w/v) for 7 days at 28°C, maximum radial growth was observed in PS9 (3.1 cm), followed by PS8 (2.3 cm) and PS6 (2.1 cm) and least growth was recorded for PS1 (0.7 cm) as shown in Fig 1. Hence, these three strains were selected for *in situ* degradation study of crude oil in MSM broth.

Degradation of TPH

When three potent strains i.e. PS9, PS8 and PS6 were incubated individually in MSM broth supplemented with 2% (w/v) crude oil for 10 days, PS9 degraded TPH of crude oil with faster rate as shown in Fig 2. After reduction of abiotic degradtion of TPH i.e. 4% as reflected by control, PS9, PS8 and PS6 contributed 27, 16 and 12%, respectively.

Among the different fractions of TPH, alkane was degraded maximumally in all treatment ation follwed by aromatic and NSO-compound while least degradation was recorded in asphaltene (Fig. 2). Similar to the TPH degradation, alkane (Fig. 3) and aromatic (Fig. 4) fraction were degradaded higest in PS9. After 10 days of incubation, PS9 could degrade 38.4% alkane, 31.2% aromatic, 25.7% NSO and 12.9% of asphaltene.

Fungal biomass

Among these three strains, maximum enhancement

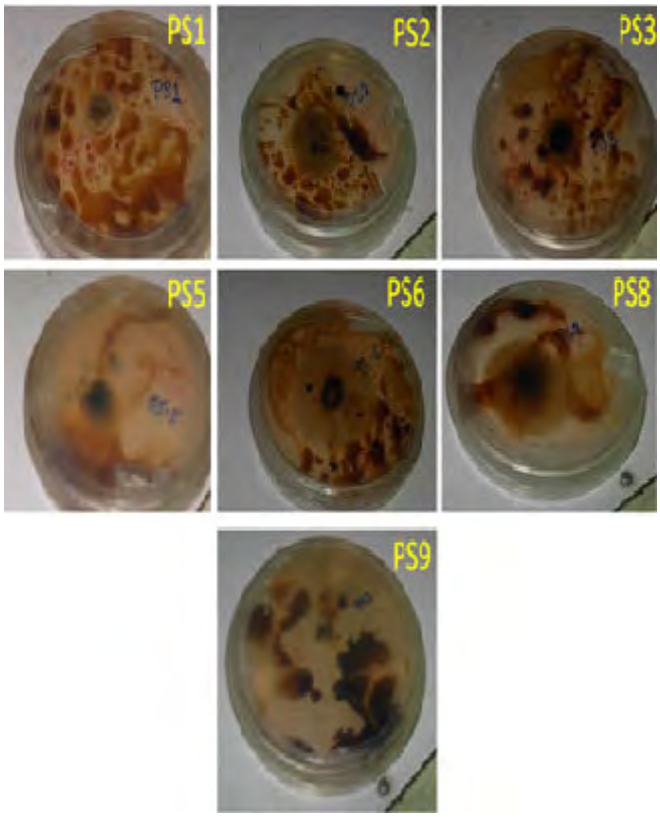

Fig. 1 : Radial growth of isolated fungal strains on MSM agar with crude oil.

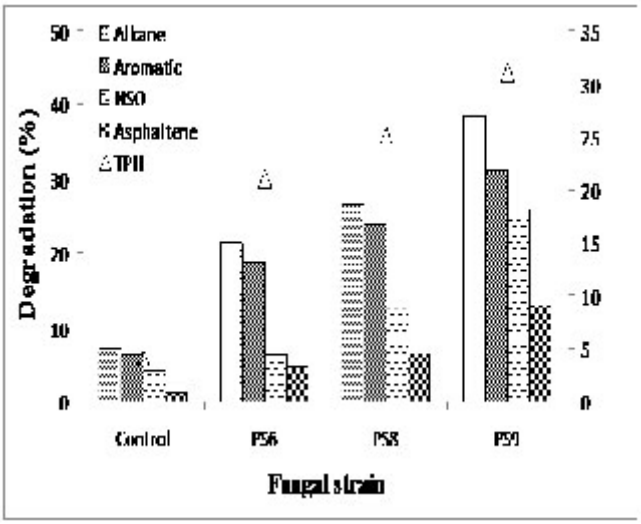

Fig. 2 : Degradation of TPH and its fraction by different fungal strains.

in fungal biomass was observed in PS9 (35.3 µg ml^{-1}) followed by PS6 (27.4 µg ml^{-1}) and least was observed in PS8 (18.7 µg ml^{-1}) after 10 days of incubation in MSM media supplemented with 2% crude oil as shown in fig 5. Fungal biomass of all fungal strains increases with increasing the incubation period. Hence, peak of fungal count to all strain was on 10^{th} day of incubation.

Extracellular protein and laccase activity

During the degradation of TPH in liquid media, induction of fungal protein showed an increasing trend till 10 days of incubation period (Fig. 6). Highest induction of protein was observed in PS9 (0.558 mg ml^{-1}) followed by PS8 (0.378 mg ml^{-1}) and PS6 (0.398 mg ml^{-1}).

Laccase activity was found significantly higher in PS9 (1025 ± 21.5 çmol mg^{-1}) than other two strains i.e. PS8 (803 ηmol mg^{-1}) and PS6 (705 ηmol mg^{-1}) during the degradation of crude oil in MSM broth**.** At 10^{th} days of incubation, PS9 showed 1.45 and 1.27 fold higher induction than PS6 and PS8, respectively (Fig. 7).

Resorcinol formation

Formation of PAHs intermediate in the form of resorcinol was also found in increasing trends with incubation period (Fig. 8). Its trends is very similar to degradation rate and recorded highest i.e. 82.1 µg ml^{-1} for PS9 followed by 62 µg ml^{-1} for PS8 and 58.5 µg ml^{-1} for PS6 after 15 days of incubation period where as it was only 14.7 µg ml^{-1}.

Growth optimization for PS9

Optimum conditions for the growth of PS9 were recorded as pH 5.5, temperature 30°C, 150 rpm agitation and 2% of crude oil concentration (Fig. 9).

Degradation capacity of PS9 in presence of bacteria

When PS9 was incubated in soil spiked with 2% crude oil, degradration rate of TPH was boosted in presence of bacteria especially with BP10. After reduction of natural degradation due to abiotic and biotic factor, PS9 in combination with BP10 and NJ2 enhanced the degradation of TPH by 22.4 and 17.3%, respectively, while in isolation enhancement was only 15.1%.

DISCUSSION

Sustainable development becomes need for economical growth of any country that allows the use of natural resources with minimum damage of our environment. The same was applied for the use of crude oil. Demand of crude oil can't be denied as it is a major source of energy (production of electricity, cooking gas and facilitate transportation) and raw materials for various petroleum products like solvents, fertilizers, plastics, paints, pesticides etc.

Petroleum hydrocarbon degrading fungi are found natuarlly in environment (Dawoodi *et al*, 2015) and their biomass increases by addition of petroleum hydrocarbon like through oil spilling (Garapati and Mishra, 2012). However, use of autochthonus microbes are always preffered for bioremediation as they are expected to be

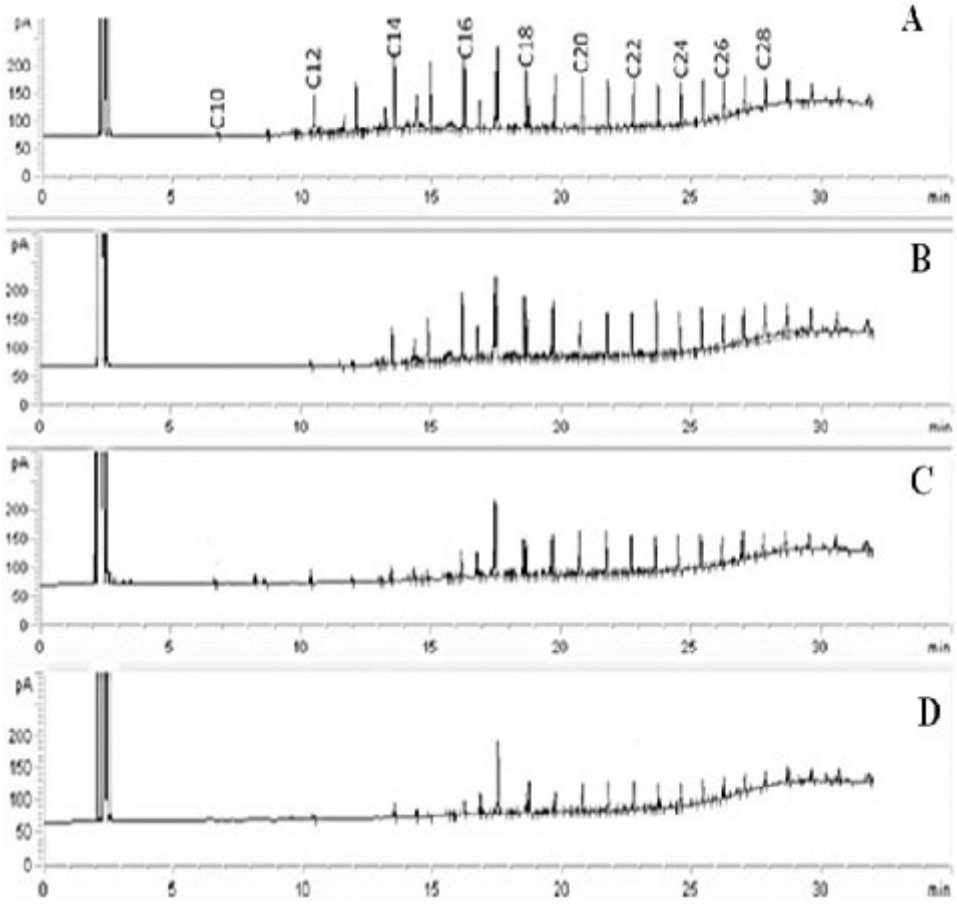

Fig. 3 : GC chromatogram of residual alkane fraction in control (A), PS6 (B), PS8 (C) and PS9 (D).

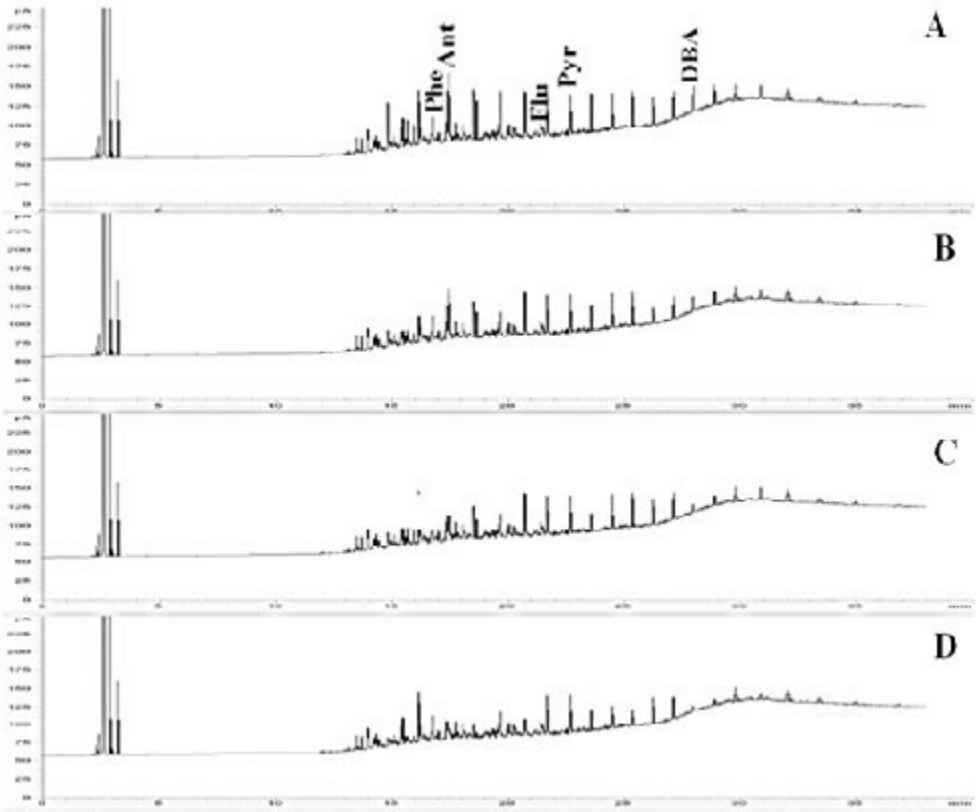

Fig. 4 : GC chromatogram of residual aromatic fraction in control (A), PS6 (B), PS8 (C) and PS9 (D).

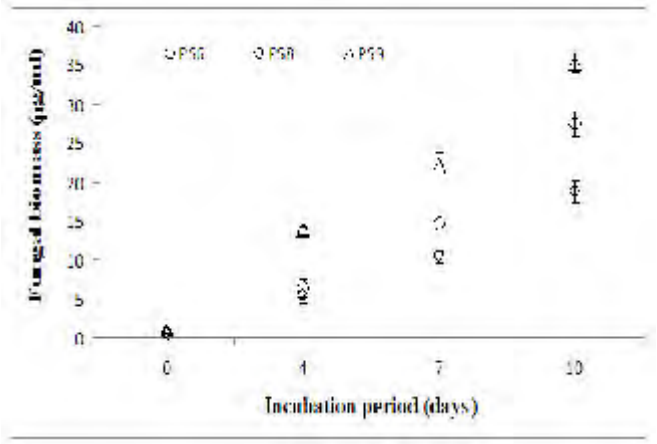

Fig. 5 : Fungal biomass (µg ml^{-1}) by different fungal strains during degradation of crude oil.

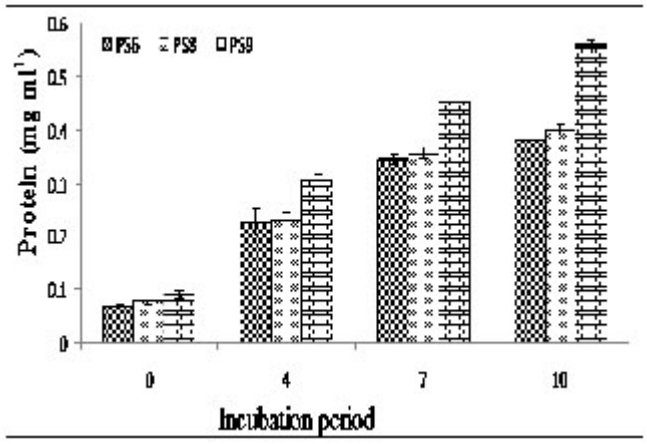

Fig. 6 : Induction of protein (mg ml^{-1}) by different fungal strains during degradation of crude oil.

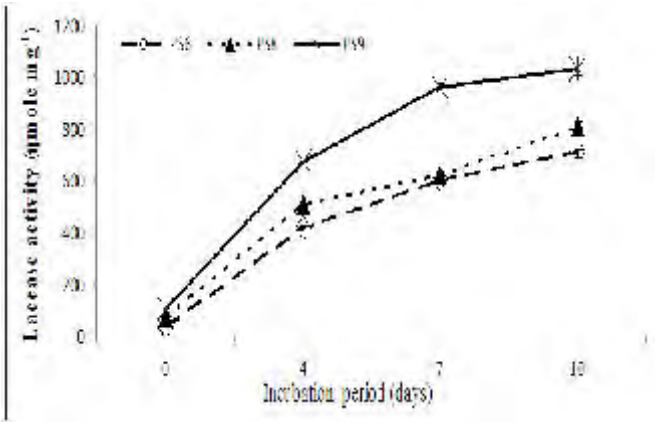

Fig. 7 : Specific activity of laccase by different fungal strains during degradation of crude oil.

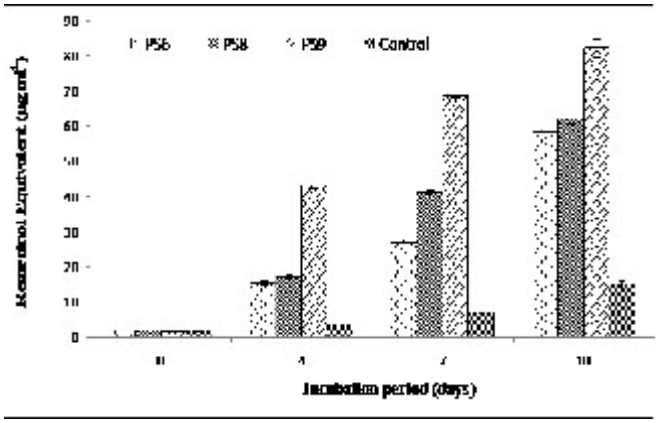

Fig. 8 : Resorcinol formation by different fungal strains during degradation of crude oil.

Table 1 : Parmaters optimized for the growth of PS9.

S. No.	Constant parameters				Parameter tested for optimization
	Agitation(rpm)	Temp.(°C)	pH	Conc. of crude oil(% w/v)	
1.	150	30	-	2	pH 5.0, 5.5, 6.0, 6.5 and 7.0
2.	150	-	5.5	2	Temp. 20°C, 25°C, 30°C, 35°C and 40°C
3.	-	35	5.5	2	Agitation 100, 150, 200 and 250
4.	150	35	5.5	-	Conc. of crude oil 1, 2, 5 and 10%

more adapted to particular soil environment than exotic species (Silva *et al*, 2009). Ekundayo *et al* (2012) isolated different fungal strains like *Aspergillus niger*, *Mycotpha microspora*, *Penicillium italicum*, *Botryris cinerea*, *Gliocladium deliquescence* and *Verticillium albo-atnum* from petroleum contaminated soil, collected from two automobile workshops in Akure, Nigeria. Among them, *Aspergillus* was reported as best degrader of crude oil. Microbes induced extracellular and intracellular enzyme but extracellular enzymes play a major role in the degradation of petroleum hydrocarbons (Barnabas *et al*, 2013). Extracellular enzyme break down oil into simpler compounds and easily taken/utilized by microbes.

Trichoderma strains when exposed to oil, the maximum fungal growth was achieved at 96 h (Argumedo-Delira *et al*, 2012). Hamzah *et al* (2012a) found 40% degradation of TPH, 100% of pristine and 74% of phytane compounds by *Trichoderma viren* UKMP-12 after 9 days of incubation under optimal physical and nutrient parameters. In present study, *Aspergillus* PS9 degraded about 1.68 and 2.25 fold higher TPH than PS8 and PS6, respectively. Similarly, Al-Jawhari (2014) isolated different species of genera *Aspergillus, Alternaria, Fusarium, Penicillum* and *Rhizopus*, but found that *A. flavus* and *P. notatum* were capable of to utilize crude oil more than other tested fungi.

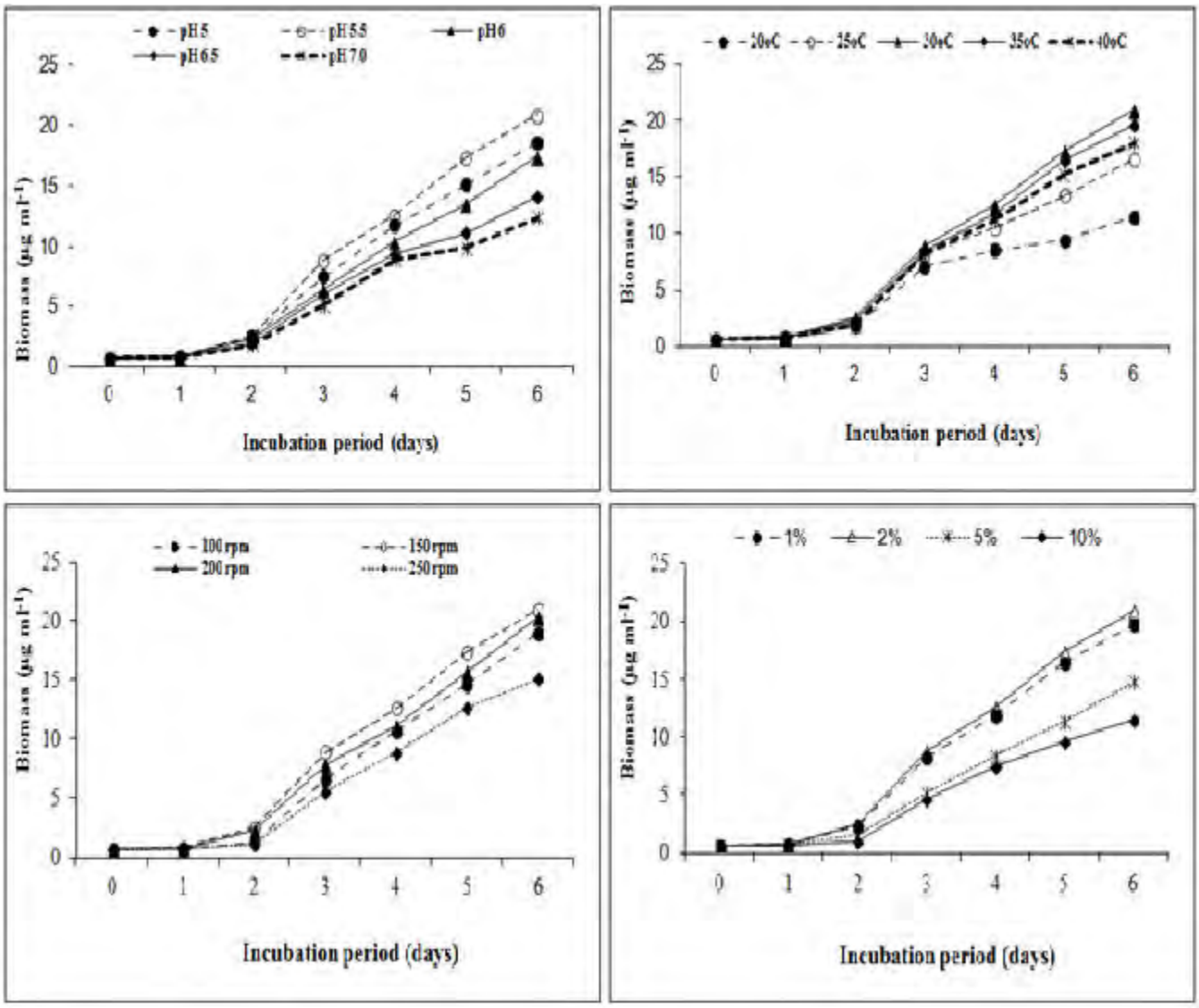

Fig. 9 : Fungal Biomass of PS9 on different pH (A), temperature (B), agitation (C) and concentration of crude oil (D).

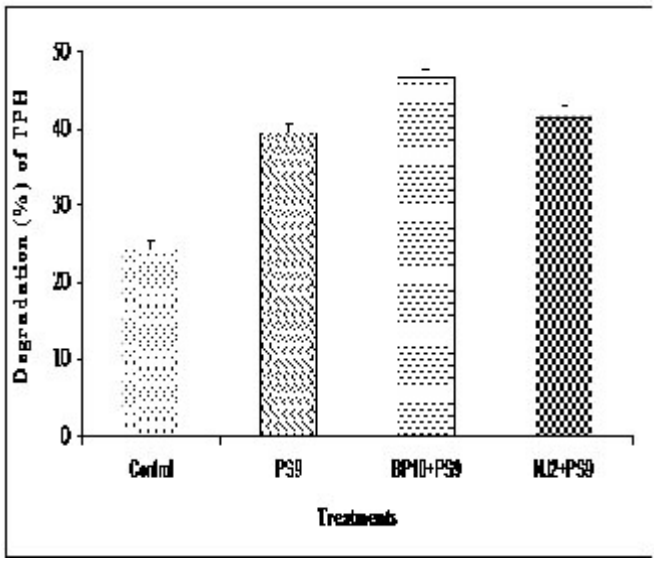

Fig. 10 : Degradation of TPH of crude oil in soil by PS9 individually and in combination of other bacterial strains.

Depending on the susceptibility to microbial degradation, different components of crude oil may be arranged as follow: linear alkanes > aromatics > asphaltenes (Kumari *et al*, 2012). More hydrophobic compounds are generally recalcitrant and not easily degraded by microbes.

Laccase (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) require molecular oxygen for catalysis resulting in the transformation or immobilization of a wide variety of phenolic compounds including lignin and humic substances (Baldrian, 2006). Ascomycetes were frequently reported for laccase production (Scherer and Fischer, 1998). High biomass did not ascertain the high yield of laccase (Xavier *et al*, 2001)like in present study fungal biomass of PS8 was less than PS6, but induction was higher in PS8 that may directly correlate with the degradation of petroleum hydrocarbon. Balaji *et al* (2014)

reported highest laccase enzyme production 79 and 73 U ml^{-1} by *P. chrysogenum* and *Aspergillus fumigates*, respectively.

Biodegradation is a natural process that can be harnessed or optimized by providing an optimal living environment for the microbes. Microbial growth and biodegradation of petroleum hydrocarbon depends on various environmental factors. Ferreira *et al* (2012) found a positive correlation of temperature, agitation and initial cell concentration with degradation rate where as initial petroleum concentration may be negatively correlated.

This study showed that bioaugmentation of the potent fungal strains with potent bacterial strain boosted the degradation processs. Study of Chen *et al* (2009) also supports our observation. The remediation *via* inoculating the fungal-bacterial consortium removed 92.6% of TPH in 60 days while the control experiment with the indigenous microorganisms removed 21.9% (Chen *et al*, 2009).

CONCLUSION

Using of microbial agents for the decontamination of petroleum hydrocarbons contaminated sites is ecofreindly and economic technique that completely detoxify these xenobiotic compounds in CO_2 and H_2O. Native fungal strains that are present in the contaminated site are accmetised to the environment and potent for the degradation of petroleum hydrocarbons. Laccase enzyme induced by fungi play an important role in degradation of crude oil. As bacteria and fungi are ubiquitous and co-exist in environment, combination of these two may be better option to boost the bioremediation process.

ACKNOWLEDGEMENT

Authors are thankful to the Director, National Botanical Research Institute, Lucknow and Head, Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow for their active support. We are also grateful to CSIR for financial support in the form of research project NWP019. Help rendered by Dr. Pankaj Srivastava, Scientist, CSIR-NBRI and Mrs. Aradhana are also duly acknowledged.

REFERENCES

Aditi, Praveen Kumar G and Suneetha V (2015) The role of algae in degradation of petroleum wastes-A study. *J. Chem. Pharm. Res.* **7(1)**, 499-503.

Agrumedo-Delira R, Alaron A, Ferrera-Cerrato R, Almaroz J J and Pena-Cabriales J J (2012) Tolerance and growth of 11 Trichoderma strains to crude oil, naphthalene, phenanthrene and benzo (a) pyrene. *J Env. Mgt*. **95**, S291-S299.

Al-Jawhari I F H (2014) Ability of some soil fungi in biodegradation of petroleum hydrocarbon. *J. Appl. Env. Microbiol*. **2.2**, 46-52.

Al-Nasrawi HA (2012) Biodegradation of crude oil by fungi isolated from Gulf of Mexico. *J Bioremed. Biodegrad.* **3**, 147-152.

Balaji V, Arulazhagan P and Ebenezer P (2013) Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. *J. Env. Biol*. **35**.

Baldrian P (2006) Fungal laccases-occurrence and properties. *FEMS Microbiol. Rev.* **30(2)**, 215–242.

Barnabas J, Saha S, Singh V and Das S (2013) Effect of enzyme extracts on bacterial degradation of garage petroleum oils. *J. Env. Sci. Comp. Sci. Eng. Tech*. **2(2)**, 206-211.

Chen L, Chen Y, Chen L and Chen W (2009) Study on fungi-bacteria augmented remediation of petroleum contaminated soil from Northwest of China. *Food Agr. Environ*. **7(3&4)**, 750-753.

Dawoodi V, Madani M, Tahmourespour A and Golshani Z (2015) The Study of Heterotrophic and Crude Oil-utilizing Soil Fungi in Crude Oil Contaminated Regions. *J. Bioremed. Biodeg.* **6**: 270. doi:10.4172/2155-6199.1000270.

Ekundayo F O, Olukunle O F and Edundayo E A (2012) Biodegradation of Bonnylight crude oil by locally isolated fungi from oil contaminated soils in Akure, Ondo satate, Malaysian. *J Microbio*. **8(1)**, 42-46.

Elshafie A, AlKindi AY, Al-Busaidi S, Bakheit C and Albahry SN (2007) Biodegradation of crude oil and n-alkanes by fungi isolated from Oman. *Mar. Pollut. Bull.* **54**, 1692-1696.

Ercoli E, Galvez J, Calleja C, Calvo V, Cantero J, Videla S, Medaura MC and Dipaola M (2001) Extensive evaluation of aerated accumulation technique for soil treatments. SPE Paper 69445, presented at the SPE Latin American and Caribbean Petroleum Engineering Conf., Buenos Aires, Argentina, 25-28 March.

Ferreira T F, Coelho M A Z and deRochoLeao M H M (2012) Factors influencing crude oil biodegradation by Y. lipolytica. *Braz. Arch. Bio. Tech*. **55(5)**, 785-791.

Garapati V K and Mishra S (2012) Hydrocarbon degradation using fungal isolates: nutrient optimized by combined grey relation analysis. *Int. J. Eng. Res. Appl*. **2**, 390-399.

Hamzah A, Abu Zarin M, Hamid A A, Omar O and Senafi S (2012a) Optimal Physical and Nutrient Parameters for Growth of *Trichoderma virens* UKMP-1M for Heavy Crude Oil Degradation. *Sains Malaysian* **41(1)**, 71-79.

Kota M F, Hussaini A A S A, Zulkharnain A and Roslan H A (2014) Bioremediation of Crude Oil by Different Fungal Genera. *Asian J. Plant Biol*. **2(1)**, 11-18.

Kumari B, Singh S N and Singh D P (2012) Characterization of two biosurfactant producing strains in crude oil degradation. *Process Biochem.* **47(12)**, 2463-2471.

Lowry O H, Roserbrough N T, Farr A L and Randall R J (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265–75.

Messias J M, da Costa B Z, de Lima V M G, Dekker R F H, Rezende M I, Krieger N and Barbosa A M (2009) Screening Botryosphaeria species for lipases: Production of lipase by *Botryosphaeria ribis* EC-01 grown on soybean oil and other carbon sources. *Enz. Microb. Technol.* **45**, 426-431.

Okungbowa F I, Ghosh A K, Chowdhury R, Chaudhuri P, Basu, A and Pal K (2007) Mechanical Lysis of Candida Cells for Crude Protein and Enzymatic Activity Estimation: Comparison of Three Methods. *World J. Med. Sci.* **2(2)**, 101-104.

Reyes-César A, Absalón ÁE, Fernández FJ, González JM and Cortés-Espinosa DV (2014) Biodegradation of a mixture of PAHs by non-ligninolytic fungal strains isolated from crude oil-contaminated soil. *World J. Microbiol. Biotechnol.* **30**, 999-1009.

Scherer M and Fischer R (1998) Purification and characterization of laccase II of *Aspergillus nidulans*. *Arch. Microbiol.* **170(2)**, 78–84.

Silva I S, dos Santo E da C, de Menezes C R, de Faria A F, Franciscon E, Grossman M and Durrant LR (2009) Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia. *Bioresour. Technol*. **100**, 4669-4675.

Venkatesagowda B, Ponugupaty E, Barbosa A M and Dekker R F H (2012) Diversity of plant oil seed-associated fungi isolated from seven oil bearing seeds and their potential for the production of lipolytic enzymes. *World. J. Microbiol. Biotechnol.* **28**, 71-80.

Xavier A M R B, Evtuguin, D V, Ferreira R M P and Amado F L (2001) *Laccase production for lignin oxidative activity*. In: Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry 4–8, Helsinki, Finland.