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Comparative *In Vitro* Assessment of Hydrocarbon Degradation Potential of *Pleurotus ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804

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ABSTRACT

Mycoremediation, involving the use of fungus for bioremediation, is one of the promising cost-effective methods for cleaning up the carcinogenic and mutagenic polycyclic aromatic hydrocarbons present in the environment. The present study deals with the isolation of white rot fungus from Chhattisgarh forest, India followed by identification based on ITS sequencing. Identification revealed MP 5 closely related to *Pleurotus ostreatus* isolate 6689 with 99% sequence similarity. Comparative primary screening assay of both the wild isolate and reference strain of *Pleurotus ostreatus* MTCC 1804 was performed by measuring the growth diameter of mycelia on minimal salt media enriched with 2% used engine oil (v/v). The fungal isolate MP 5 showed highest average growth rate. Confirmatory test was conducted via orbital shaking method and spectroscopic study was carried out at 600 nm which displayed degradation within 7 days and percentage of degradation was calculated. Level of degradation was 69.7 ± 0.351% and 64.7 ± 1.153% respectively for *Pleurotus ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804. Degradation potential of both the isolates were evaluated in terms of chemical characterization via Fourier transform infrared spectroscopy (FTIR) which revealed bands formation based on the presence of different functional groups indicating oxidative degradation of hydrocarbons.

INTRODUCTION

Engine oil or motor oil is always on high demand globally and is used all over around the world in vehicles and other power generating engines. Improperly and illegitimately discharging of spent engine oil by automobile industries and other power generating stations into water bodies, farming lands and other arable lands is quite frequent. This disposed and spilled oil left unattended for several years further give rise to pollution of soil, water and air causing lethal environmental hazards. Oil contains more than 30 parent polyaromatic hydrocarbons (PAHs) which again constitutes hundreds of different hydrocarbon compounds such as paraffins, naphthene, aromatics as well as organic sulphur compounds, organic nitrogen compounds and oxygen containing hydrocarbons (Jawhari & Ihsan 2014). Increased use and demand of engines which in turn uses engine oil is the first and foremost reason for soil pollution. Leaving the spent oil unattended for several years reduces the fertility of the soil and converts it into a barren land. Both the used and unused engine oil are similar except that the used one comprises some additional chemicals which may have built up when engine runs by reacting with carcinogenic and mutagenic metals found in used motor oil that comes from engine parts

as they wear down (Adongbede & Sanni 2014). According to various researchers it's a known fact that used motor oil contains metals and heavy polycyclic aromatic hydrocarbons which contribute to chronic hazards including mutagenicity and carcinogenicity (Boonchan et al. 2000, Hagwell et al. 1992, Keith & Telliard 1979). Undesirable presence of petroleum hydrocarbons in the environment is toxic, mutagenic and carcinogenic (Clemente et al. 2001). Therefore, the most ominous problems that the world is grappling today is the environmental pollution by petroleum hydrocarbons and its hazardous effects on ecosystem.

The objective of solving environmental pollution problems with the application of biotechnological processes involving microorganism is rapidly growing in recent decades. Among such application of biotechnological processes, mycoremediation is one which can be defined as the use of fungal systems to catalyse the destruction or transformation of various hazardous chemicals to less harmful forms (Thomas et al. 1998). As it is simple to maintain, applicable over large areas, cost effective and leads to complete destruction of the contaminant, it has been proved out to be one of the fascinating approaches for cleaning up of the petroleum hydrocarbons (Huesemann 1994). The term mycoremediation was coined by Paul Stamets which specifically refers

to the use of fungal mycelia in bioremediation. One of the primary roles of fungi in the ecosystem is decomposition, which is performed by the mycelium. It has been reported by Barr et al. (1994), that fungi secrete nonspecific extracellular enzymes such as laccase, lignin peroxidase and manganese peroxidase, that give these fungi the ability to degrade lignin and also used to degrade a wide range of hydrocarbon pollutants that resemble the lignin structure such as total petroleum hydrocarbons (TPHs), dichlorodiphenyltrichloroethane (DDT), trinitrotoluene (TNT), polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbons (PAHs). Mycelial enzymes can decompose many resistant materials made by humans or nature, because many of the bonds that hold plant material together are like the bonds found in petroleum products including diesel and oil (Stamets 2005).

Application of higher fungi like mushrooms has been known in the remediation of polluted soil for some years now. The application of white-rot fungi in bioremediation is now considered to be economical because the fungi can be cultured on varieties of inexpensive agricultural or forest wastes such as rice straw, corn cobs and sawdust. According to the recent studies, *P. ostreatus* can degrade a variety of polycyclic aromatic hydrocarbons (PAH) (Sack & Gunther 1993). Emuh (2010) proclaimed that the mushroom hypha and mycelia possess the capability to break down and absorb the crude oil and heavy metals present in polluted soil through the secretion of enzymes into environmentally safe levels. Zebulun et al. (2011) reported his work on decontamination of anthracene-polluted soil through a white rot fungus (*P. ostreatus*) which induced biodegradation. Further he divulged that lignolytic enzymes such as lignin peroxidase, laccase and manganese peroxidase by *P. ostreatus* catalysed the degradation of anthracene. Similarly, Okparanma et al. (2011) broadcasted that spent white-rot fungi (*P. ostreatus*) substrate can be utilized to remediate Nigerian oil-based drill cuttings constituting PAHs. The oyster mushroom, *P. ostreatus*, can degrade 80-95% of all PAHs present in soil (Steffen et al. 2007).

The utility of mushroom for biodegradation has beguiling probability because of their potentiality to degrade lignin and other resembling compounds like PAHs. According to the previous explorations involving white rot fungi and their capability to degrade petroleum hydrocarbons, several auspicious results have been reported. Hence the present investigation purposes to isolate white rot fungi and to perform comparative evaluation of hydrocarbon degradation potential of both wild isolate and reference strain of *Pleurotus ostreatus* MTCC 1804 which may prove to be a promising isolate for mycoremediation studies.

MATERIALS AND METHODS

Chemicals and Reagents

All the media used during the experiment were purchased from Himedia Laboratories Pvt. Limited (Mumbai, India). All the fine chemicals used were procured from SRL Chemicals, India with highest purity and analytical grade. Used engine oil of Castrol Company was procured from automobile workshop.

Source of Fungal Cultures

Fruiting bodies of the Basidiomycete samples were collected from Geedam forest spread across Dantewada district, Chhattisgarh, India. The samples were apparently brought to the laboratory in sterilized bags and were refrigerated at 4°C in refrigerator until they were processed. The materials were used for experimental procedures within 24 hours. *Pleurotus ostreatus* MTCC 1804 as reference strain was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungus was propagated on Potato Dextrose Agar (PDA Himedia) at 25ºC for 7 days, maintained in agar slants and sub cultured after every 15 days.

Isolation of White Rot Fungus

The tissues of the wild isolate were inoculated into PDA (Himedia) prepared following the manufacturer's instructions. The pure culture of the test organism was incubated at room temperature for 7 days in biological oxygen demand (BOD) incubator. Culture was maintained in agar slants and subcultured after every 15 days.

Molecular Identification

Molecular characterization of the wild fungal isolate was achieved through ITS sequencing. Identification process was completed by comparing the ITS regions of the fungal isolate with NCBI data available and the closest neighbour with 99% similarity index was assigned. The identification was done by NCMR, Pune, Maharashtra, India.

Comparative Primary Screening

For primary screening, a selective carbon free media known as Mineral Salts Medium (MSM) [v/v] of Mills et al. (1978) as modified by Okpokwasili & Okorie (1988) was prepared which was later supplemented with 50 mg/mL ampicillin and 2% of filtered sterile used engine oil (Vanishree et al. 2014). 1 cm^2 mycelial plug of pure fungal isolate was aseptically inoculated in MSM with the help of cork borer, and incubated at 25°C for 7 days and the growth rates were recorded daily by measuring the diameter of the radial extension of fungal mycelium (Thenmozhi et al. 2013). Pure fungal isolates

were also inoculated on MSM without oil which served as control. All the inoculations were performed in triplicates. The growth rates of both the wild isolate and reference strain were recorded daily by measuring the diameter of the radial extension of the mycelium. Measurements were done by measuring at least two diameters per plate and average of diameters were used as the colony diameter at that time of measurement (cm/day) (dos Santos et al. 2008). According to dos Santos et al. (2008) colony growth rates were further evaluated by regressing the colony diameter against the days after inoculation. Fungal isolates which showed heavy sporulation, more abundant aerial mycelium and greater colony diameter were considered as organisms utilizing hydrocarbons as their carbon source which were later confirmed through confirmatory screening (Thenmozhi et al. 2013).

Confirmatory Screening *(Orbital Shaking Method)*

To confirm the hydrocarbon degrading capability of the both the wild isolate and reference strain a modified technique based on the redox indicator 2, 6- dichlorophenol indophenol (DCPIP) was performed and purposely Bacto Bushnell- Hass (BH) broth medium was prepared (Hanson et al. 1993). A control flask without inoculum was also prepared. Three agar plugs from 7 days old culture of pure fungal isolates were picked from the surface of the Petri dish with the help of cork borer and aseptically inoculated into 50 mL of sterilized BH broth medium using 150 mL Erlenmeyer flask and later, also incorporated with 0.2 % (v/v) Tween 80, 2% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol indophenols). All the flasks were incubated in orbital shaking incubator at 25°C with constant shaking at 180 rpm for 7 days. The aliquots inside the flask were supervised daily for colour change from deep blue to colourless. After 7 days of incubation period, filtration of the broth of all the flasks was accomplished with the help of filter paper for the separation of fungal biomass, proximately followed by centrifugation at 8000 rpm for 15 minutes. Supernatants obtained after centrifugation were assayed spectrophotometrically at 600 nm with the help of UV-VIS spectrophotometer (Systronics Double Beam Spectrophotometer 2203) and the percentage of biodegradation was evaluated via the following equation (Hanson et al. 1993, Tiwari et al. 2017)

Percentage (%) of Degradation = [1- Absorbance of treated sample/ Absorbance of control] x 100

Biodegradation Assay of Hydrocarbon by Fungal Cultures

Three agar discs of fresh pure cultures of duo fungal isolates were inoculated into 50 ml of BH broth in Erlenmeyer flask (150 mL) comprising 0.2 % (v/v) Tween 80 and 2%

(v/v) crude oil. Meanwhile control flask was also prepared accordingly but with no inoculum. Later all the flasks were incubated in orbital shaking incubator at temperature 25°C with constant shaking at 180 rpm for 7 days. Again after 7 days of incubation, broths were filtered with the help of filter paper to separate the biomass which was weighed for the dry weight of fungal biomass by constant weighing (Hanson et al. 1993).

FTIR Analysis

Two-three mycelial plugs of the fungal isolates were inoculated in Czapeck dox broth with 2 % v/v used motor oil in a 250 mL Erlenmeyer flask. Control experiment containing same medium contents but without inoculum was performed. The flasks were agitated at 200 rpm at 25°C for a period of 30 days and the content of each test including the control were harvested and subjected to FT-IR analysis (Thenmozhi et al. 2013). After harvesting all the samples including the control, were dried at 70°C overnight before analysis to convert them into powdered form. KBr pellet was used as a background reference (Velioğlu et al. 2015). Approximately 1 mg of each of the samples were mixed with approximately 100 mg of dried KBr separately and then pressed to form a pellet for measurement. The FTIR spectra were broadcasted on the Perkin Elmer Inc. – Spectrum BX FTIR spectrometer, in the 4000-400 cm^{-1} spectral region.

Statistical Analysis

An analysis of variance (ANOVA) was performed to establish if there were any significant differences between the growth rates of the strains and their control plates at 95% confidence level (P £ 0.05). T test was also performed to statistically compare the means of colony diameter (cm/day) of both the isolates, percentage of degradation (%) and biodegradation assay of hydrocarbon in terms of fungal biomass (g/50 mL) at 95% confidence level (P ≤ 0.05).

RESULTS

Isolation of White Rot Fungus and Cultivation of *Ostreatus* MTCC 1804

Tissue culture of wild isolate MP 5 on PDA followed by 7 days of incubation gave pure culture of cottony white coloured mycelium with glossy surface (Fig. 1). Likewise procured culture, *Pleurotus ostreatus* MTCC 1804 also gave the mycelium with same appearance (Fig. 2).

Molecular Identification

The isolated white rot fungus was identified based on ITS sequencing and sequence of the identified isolate was

generated using NCBI data base, and the confidence in identification was limited by both the availability and the extent of homology shown by the ~550 bp sequence of the sample with its closest neighbour in the database. Molecular characterization of the wild isolate which exhibited high sequence similarity (~99%) to that of *Pleurotus ostreatus* isolate 6689 (accession No. AY450345.1). Fig. 3 shows the nucleotide sequence in FASTA format which was resulted out based on ITS sequencing. After identification process, MP 5 isolate was also got deposited in NCMR Pune, in general deposition repository under accession No. MCC 1815.

Colony Growth Evaluation

Colony growth rate evaluation was done to investigate the primary screening of hydrocarbon degradation potential of both the isolates based on colony diameter. As the colony growth rates (cm/day) were calculated by regressing the colony diameter against the days after inoculation it was resulted out that the growth curves of the isolates, based on colony diameters as a function of time, were typical of fungal growth. The correlation coefficients (r^2) were greater than 0.95 (r^2 for MP 5 = 0.99, r^2 for MP 5 control = 1 and r^2

for *Pleurotus ostreatus* MTCC 1804 = 0.99, r^2 for *Pleurotus ostreatus* MTCC 1804 = 0.97), which indicated that the linear regression satisfactorily explained the variation of the colony diameter as a function of time.

The mean values and standard deviations for the growth rates of MP 5 and *Pleurotus ostreatus* MTCC 1804 strain including control are represented in Table 1. According to Table 1, it was resulted out that both the strains showed highest average colony growth each day (cm/day) in terms of diameter (mean ± sd) on MSM plates supplemented with 2 % oil as compared to their control MSM plates which was not supplemented with oil also explained graphically in Fig. 4 (for MP 5) and Fig. 5 (for *P.ostreatus* MTCC 1804). All these differences between the strains and their control plates was found to be significant at $p \leq 0.05$ at 95% confidence level as calculated by ANOVA.

On comparison it was also evaluated out that the wild strain MP 5 showed highest average colony growth rate (cm/day) with mean ± sd 3.2 ± 0.057 more than *Pleurotus ostreatus* MTCC 1804 which exhibited mean ± sd 1.2 ± 0.115 as depicted graphically in Fig. 6. This comparison was also significant at $p \leq 0.05$ at %95 confidence level as calculated by t-test.

Fig. 1: *P. ostreatus* MP 5.

Fig. 2: *P. ostreatus* MTCC 1804.

GGAAGtTTGTTGCTGGCCTCTAGGGGCATGTGCACGCTTCACTAGTCTTTCAACCACCTGTGAACTTT
TGATAGATCTGTGAAGTCGTCTCTCAAGTCGTCAGACTTGGTTGCTGGGATTTAAACGTCTCGGTGTGACTAC
GCAGTCTATTTACTTACACACCCCAAATGTATGTCTACGAATGTCATTTAATGGGCCTTGTGCCTTTAAACCATAA
TACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCCTTGGTATTCCGAGGGGCATGCC
TGTTTGAGTGTCATTAAATTCTCAAACTCACTTTGGTTTCTTTCCAATTGTGATGTTTGGATTGTTGGGGGCTGC
TGGCCTTcGACAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTGCCTCTGCGCATGATGTGATAA
TTATCACTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAATCGTCCGCAAGGACAATTTGACAATTTGACC
TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAGAT

Fig. 3: The~550 bp nucleotide sequence of fungal isolate MP 5.

Table 1: Average colony growth rates± standard deviations (cm/day) (n = 3) and statistical analysis of data of MP 5 and *Pleurotus ostreatus* MTCC 1804 on MSM + 2% oil and their control plates after 7 days:

No. of days	*Pleurotus ostreatus* MP 5		*Pleurotus ostreatus* MTCC 1804	
	MSM + oil plate Mean ± SD	Control plate Mean ± SD	MSM + oil plate Mean ± SD	Control plate Mean ± SD
Day 1	0.2 ± 0.057	0 ± 0	0.1 ± 0.057	0 ± 0
Day 2	0.8 ± 0.1	0.2 ± 0.057	0.3 ± 0.1	0 ± 0
Day 3	1.4 ± 0.057	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.057
Day 4	1.8±0	0.6 ± 0.057	0.7 ± 0.057	0.2 ± 0.1
Day 5	2.2 ± 0.152	0.8 ± 0.152	0.9 ± 0.057	0.3 ± 0.1
Day 6	2.8 ± 0.208	1.0 ± 0.1	1.0 ± 0.057	0.4 ± 0.152
Day 7	3.2 ± 0.057	1.2 ± 0.057	1.2 ± 0.115	0.5 ± 0.152

Growth rates between the fungal isolates MP 5 and *Pleurotus ostreatus* MTCC 1804 and their respective control plates are significantly different from each other at the 95% confidence level and significant at $p \leq 0.05$, p value was calculated by one-way ANOVA. Comparison of the average colony growth rates (cm/day) between MP 5 and *Pleurotus ostreatus* MTCC 1804 in terms of diameter is also significant at $p \leq 0.05$ level which was calculated by t-test.
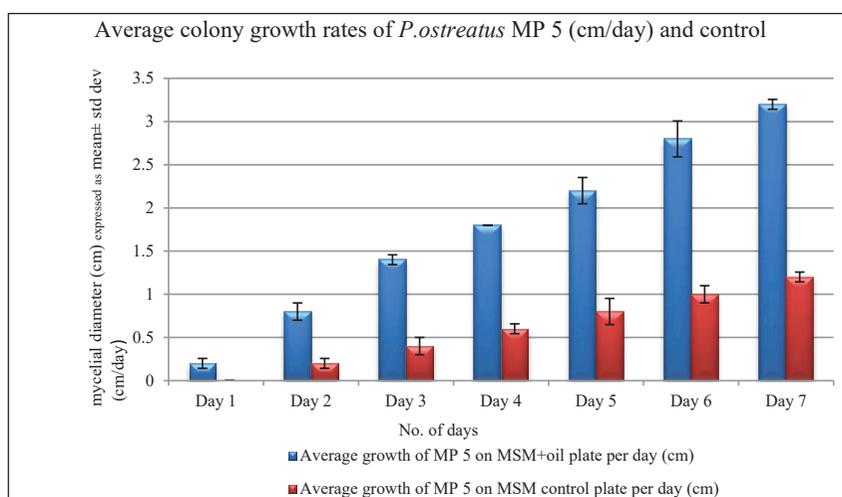

Fig. 4: Average colony growth rates (cm/day) of *Pleurotus ostreatus* MP 5 and control plates. Each bar represents Mean ± SD of two separate observations (n = 2). The results were statistically significant at $p \leq 0.05$.

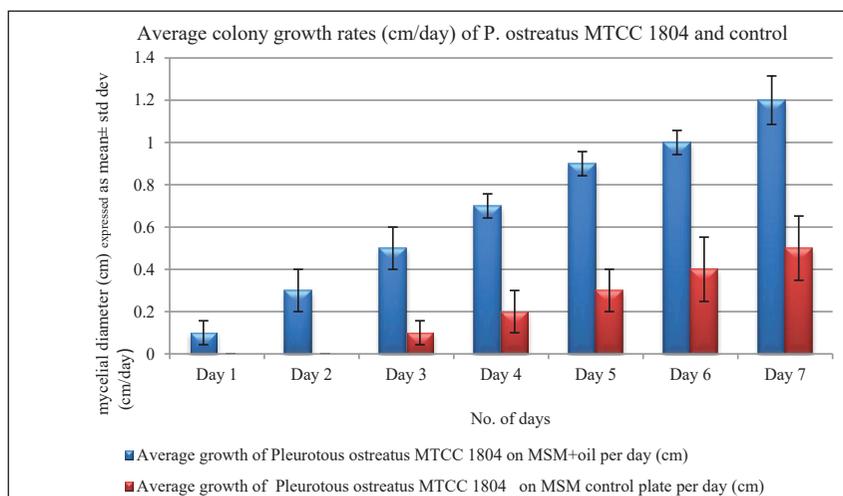Fig. 5: Average colony growth rates (cm/day) of *Pleurotus ostreatus* MTCC 1804 and control plates. Each bar represents Mean ± SD of two separate observations (n = 2). The results were statistically significant at $p \leq 0.05$.

Confirmatory Screening (Orbital Shaking Method)

2, 6-dichlorophenol indophenols (2, 6-DCPIP) test or redox indicator test was performed to detect the potential of most efficient strain to degrade oil (hydrocarbon) revealed that BH broth inoculated with each fungal isolates changed colour from blue to colourless and gave 100 % response as tabulated in Table 2 which can be visualized in Figs. 7(a) and (b). Table 3 describes the measurement of total colour change as an absorbance value in terms of mean ± SD using spectrophotometer recorded as 0.459 ± 0.005 and 0.536 ± 0.017 for *Pleurotu sostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804 found to be significant at p ≤ 0.05 calculated by T test which correlates to the potentiality of the isolate to utilize hydrocarbon.

Based on the absorbance values of filtrates obtained spectrophotometrically after centrifugation, percentage or level of hydrocarbon degradation of the duo fungal isolates were calculated which are expressed in Table 4. Higher percentage of degradation of oil was shown by the fungal isolate MP 5 which was recorded as 69.7±0.351 (mean±SD). Percent of degradation recorded for *Pleurotus ostreatus* MTCC 1804 was 64.7 ± 1.153 (mean ± SD) which was less as compared to MP 5, also elaborated graphically in Fig. 8. Differences in percentage of degradation of both the isolates was found to be significant at p ≤ 0.05 calculated by t-test.

Biodegradation Assay of Hydrocarbon by Fungal Cultures
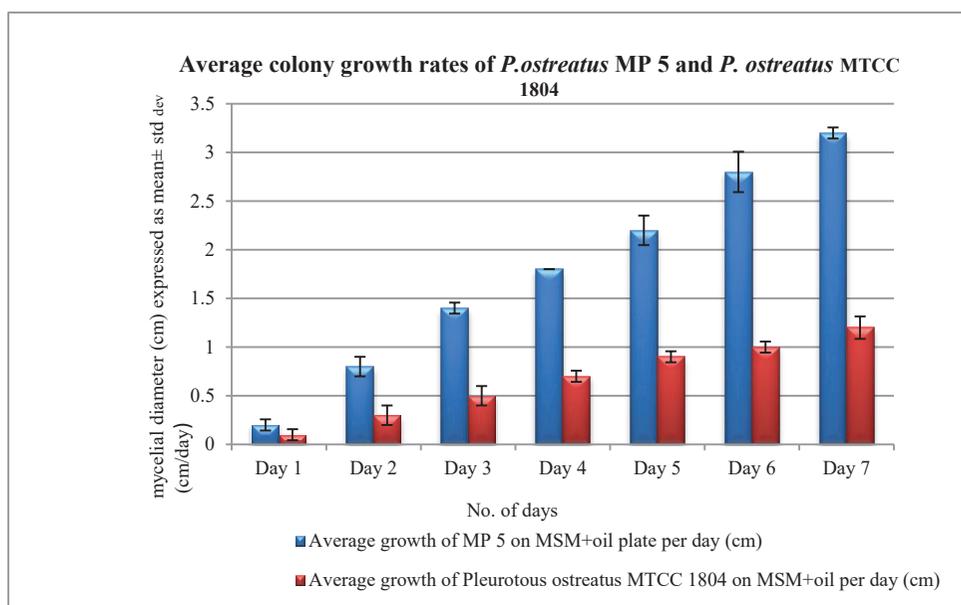
Biodegradation assay in terms of fungal biomass revealed

Fig. 6: Average colony growth rates (cm/day) of *Pleurotus ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804. Each bar represents Mean ± SD of

Table 2: DCPIP test of fungal isolates for colour change with 2% DCPIP (BH broth + 0.2 % tween 80 +2 % crude oil + 2 % DCPIP).

S.No.	Fungal isolates	Colour change (blue to colourless)			Percentage of response (%)
		flask I	flask II	flask III	
1.	Control	-	-	-	0
2.	*Pleurotus ostreatus* MP 5	+	+	+	100
3.	*Pleurotus ostreatus* MTCC 1804	+	+	+	100

Table 3: Absorbance values of fungal isolates with 2 % DCPIP (50 ml BH broth + 0.2 % tween 80 + 2% crude oil + 2 % DCPIP) in terms of Mean±SD (n=3).

| S.No. | Fungal isolates | Optical density (OD) (at 600 nm) |
		Mean ± SD
1.	Negative Control	1.523 ± 0.001
2.	MP 5	0.459 ± 0.005
3.	*Pleurotus ostreatus* MTCC 1804	0.536 ± 0.017

Comparison of the absorbance (od) between MP 5 and *Pleurotus ostreatus* MTCC 1804 at 600 nm is also significant at p ≤ 0.05 level which was calculated by t-test.

Table 4: Percentage of oil degradation of fungal isolates represented as Mean±SD (n = 3) of fungal isolates with 2% DCPIP (50 mL BH broth + 0.2% tween 80 + 2% crude oil + 2% DCPIP).

S. No.	Fungal isolates	Percentage of degradation (Mean±SD)
1.	*Pleurotus ostreatus* MP 5	69.7 ± 0.351
2.	*Pleurotus ostreatus* MTCC 1804	64.7 ± 1.153

Comparison of percentage of degradation of both the isolates is found to be statistically significant at p ≤ 0.05 level as calculated by t-test.
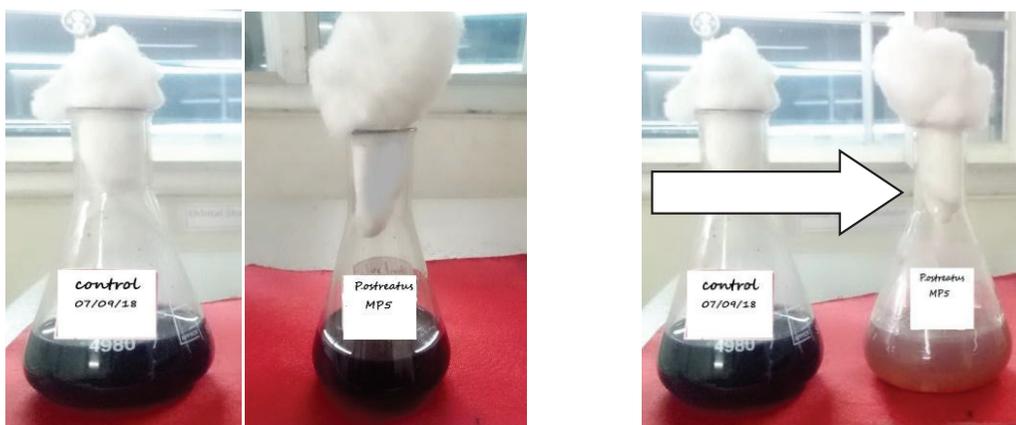

Fig. 7 (a): depicting DCPIP test, visualizing colour change from blue to colourless after biodegradation.

Fig. 7 (b): depicting DCPIP test, visualizing colour change from blue to colourless before after biodegradation.
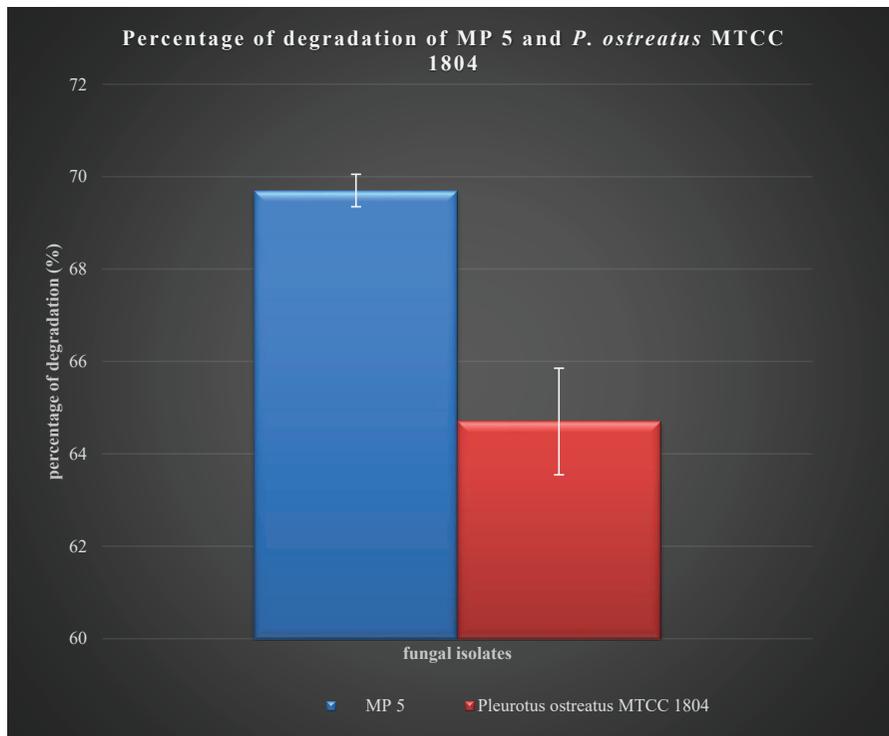
Fig. 8: Percentage degradation of MP 5 and *P. ostreatus* MTCC 1804. Each bar represents Mean ± SD of two separate observations (n = 2). The results were statistically significant at p ≤ 0.05.

that at concentration of 2% (v/v) oil in BH broth, MP 5 gave better result than the reference strain on the basis of its bulk mass which was estimated out as 0.334 ± 0.009 g/50 mL as tabulated in Table 5 and graphically depicted in Fig. 9. Fungal biomass estimated out for reference strain was 0.308 ± 0.007 g/50 mL. This differences between dry weight of fungal biomass was found to be significant at p ≤ 0.05 as calculated by T test.

FTIR Analysis

FTIR analysis of both the isolates including control described hydrocarbon degradation potential of MP 5 and *Pleurotus ostreatus* MTCC 1804 via the formation of various peaks indicating the presence of different functional groups formed during degradation (Figs. 10, 11 and 12).

DISCUSSION

The main aim of this investigation was to perform comparative study between the hydrocarbon degradation potential of two white rot fungal isolates for which primary screening, confirmatory screening, biodegradation assay, molecular identification and FTIR analysis were performed. Morphological and biochemical uniqueness of fungi are globally used for their identification, but divergence of close homologous cultures requires extensive molecular techniques (Shahriarinour et al. 2011). Promising techniques to identify a diversified range of fungi to the species level involves PCR amplification with universal primers targeted to conserved regions within the rRNA complex and subsequent DNA sequencing of the internal transcribed spacer (ITS) regions (Chen et al. 1999). According to White et al. (2001) PCR primer sets are routinely used for amplification of ITS regions. Therefore, the identification of the potential wild isolate was performed by NCMR, Pune, Maharashtra, India based on molecular technique involving ITS sequencing. To study the molecular identification of the selected wild isolate able to degrade benzo[a]pyrene based on ITS sequencing. Sourav et al. (2011) reported *Pleurotus ostreatus,* which is somewhat like the finding of the present investigation of molecular identification based on ITS sequencing.

dos Santos et al. (2008) confirmed the use of colony growth rate evaluation to investigate the growth rate of filamentous fungi in different hydrocarbons and oil derivatives and suggest this methodology as a tool to demonstrate the biodegradation potential of fungal strains. Present analysis demonstrated that the correlation coefficients (r^2) were greater than 0.95 which indicated that the linear regression

Table 5: Dry weight of fungal isolates represented in terms of Mean±SD (n = 3) (50 mL BH broth + 0.2 % tween 80 + 2% crude oil).

S. No.	Fungal isolates	Dry weight of fungal biomass (mean±sd) (g/50mL)
1.	*Pleurotus ostreatus* MP 5	0.334 ± 0.009
2.	*Pleurotus ostreatus* MTCC 1804	0.308 ± 0.007

Dry weight of both the fungal isolates shows statistically significant differences at P ≤ 0.05 level as calculated by t-test.
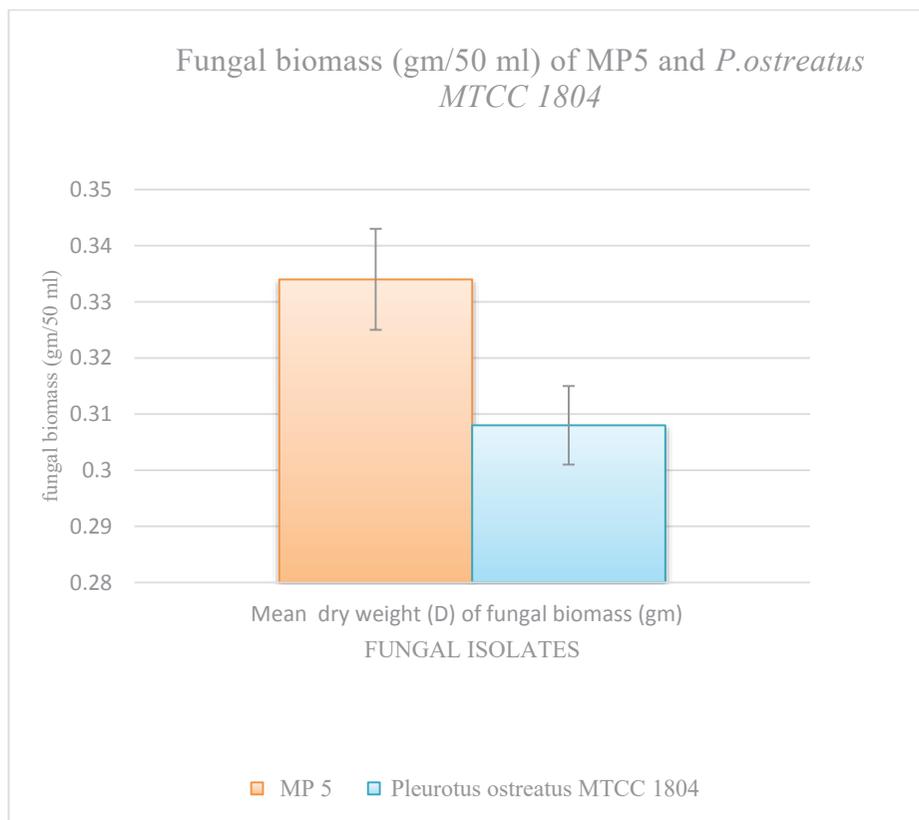

Fig. 9: Fungal biomass of MP 5 and *P. ostreatus* MTCC 1804. Each bar represents Mean±SD of two separate observations (n = 2). The results were statistically significant at p ≤ 0.05.

satisfactorily explained the variation of the colony diameter as a function of time. dos Santos et al. (2008) supports the present analysis by reporting that during his experiments of colony growth evaluation of four fungal isolates on plates containing a specific hydrocarbon or petroleum derivative as the only carbon source, correlation coefficients (r^2) were greater than 0.95 (data not shown), indicating that the linear regression adequately explained the variation of the colony diameter as a function of time.

Experiments performed by Thenmozhi et al. (2013) revealed that that the fungal isolates JJF3 *Aspergillus niger* and JJF9 *Aspergillus luchiencis* with average colony diameter as 2.7 and 3.72 cm/day as compared to other fungal isolates exhibited bigger colony diameter, heavy sporulation, and abundant aerial mycelium on selective media (MSM) amended with 1% used engine oil. These evaluations foresighted those as hydrocarbon degraders which further elaborated their capability to utilize used engine oil as a carbon source for their growth. Likewise, in the present study, the highest average colony growth rate in terms of colony diameter (cm/day) observed for *Pleurotus ostreatus* MP 5 on MSM amended with 2 % oil was recorded as 3.2 ± 0.057 cm (mean±day) respectively which interpreted that mycelium of this fungal isolate proliferated rapidly forming heavy sporulation and dense hyphae than *Pleurotus ostreatus* MTCC 1804 (0.308 ± 0.007). Similarly, Popa et al. (2013) evaluated the ability of two isolates of *P. ostreatus* (P50, P421) and a sample of *P. ostreatus* collected from Chitila woods to use crude-oil
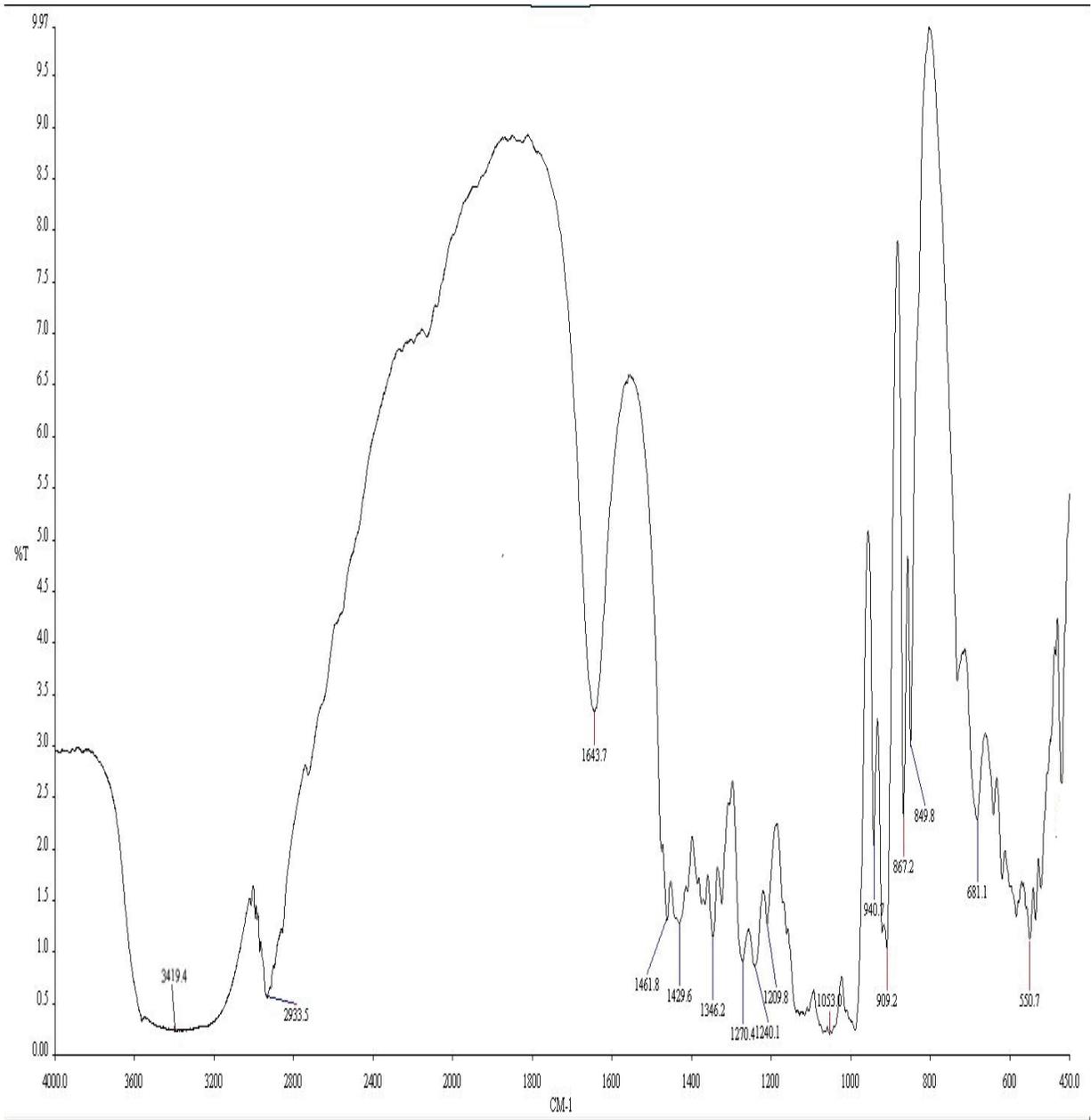
Fig. 10: FTIR spectrum of control sample.

as a carbon source for which he inoculated mycelia inoculum on MSM plates with crude oil (1 mL) spread on the entire carbon free mineral salt medium (MSM) surface and in plates with pieces of filter paper soaked in crude oil and placed around the inoculum. Evaluation done every day for 14 days, by measuring the radial growth of mycelia (in cm) and extrapolated as growing percentage to culture plate diameter (9 cm) revealed that both isolates of *P. ostreatus*

as well as *P. ostreatus* originated from Chitila forest were able to utilize the crude oil as a source of carbon and energy in their metabolism.

Confirmatory screening also known as orbital shaking method also known as redox indicator test and DCPIP test in the present study is based on the principle of change in colour of BH broth from blue to colourless incorporated with fungal isolates, 0.2 % (v/v) Tween 80, 2% (v/v) crude oil and
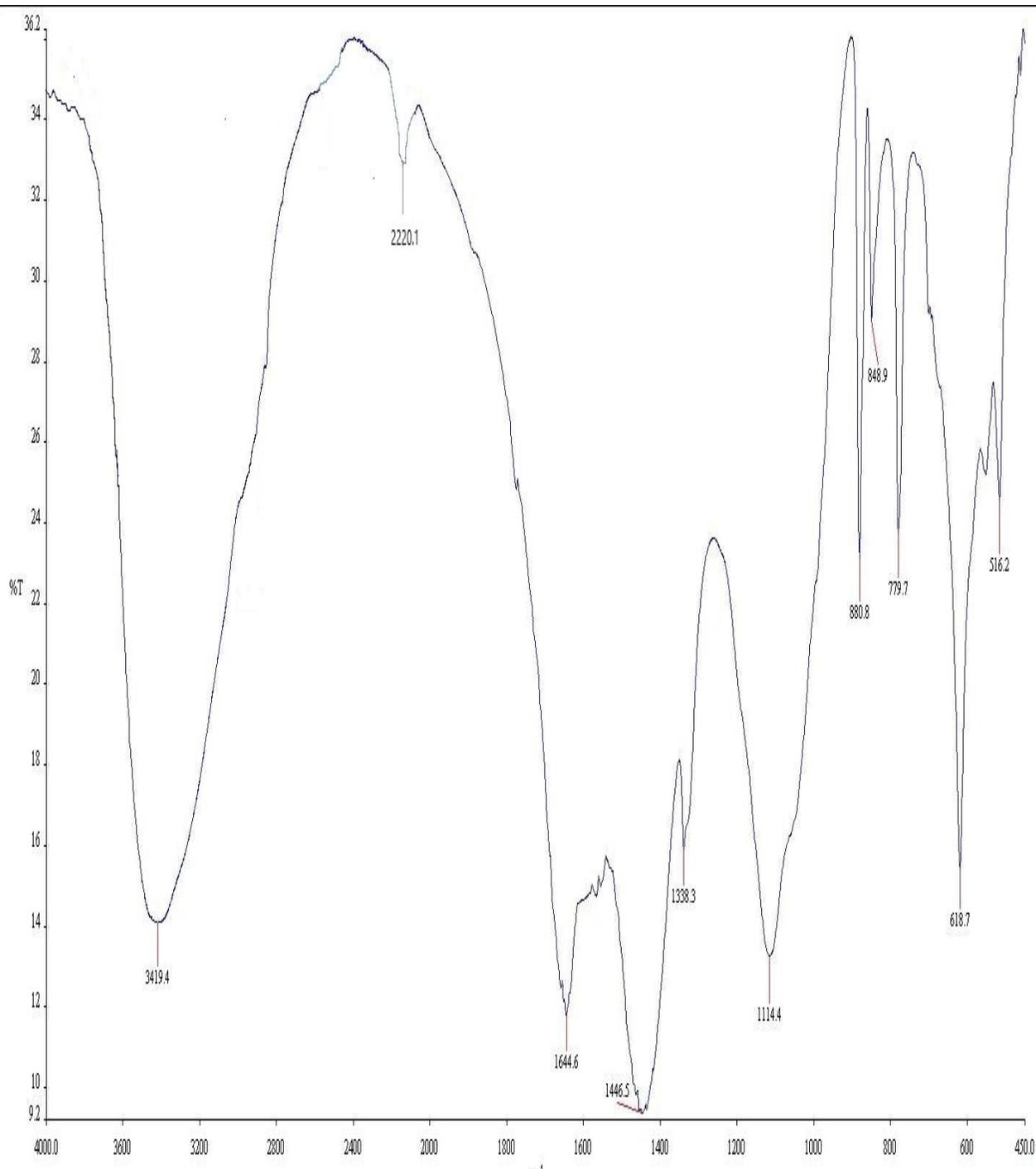
Fig. 11: FTIR spectrum of *P. ostreatus* MP 5.

redox reagent (2% 2, 6- dichlorophenol indophenols). This screening method is also known as redox indicator technique which is applied to assess the ability of fungal isolates to degrade crude oil in the presence of redox indicator 2, 6-DCPIP (Hanson et al. 1993). The main principle behind this

degradation using redox reagent is that after incorporation of an electron acceptor such as DCPIP to the broth inoculated with fungi supplemented with oil and tween 80, it becomes possible to assess the ability of fungi to utilize the substrate by visualizing the colour change of DCPIP from blue (ox-
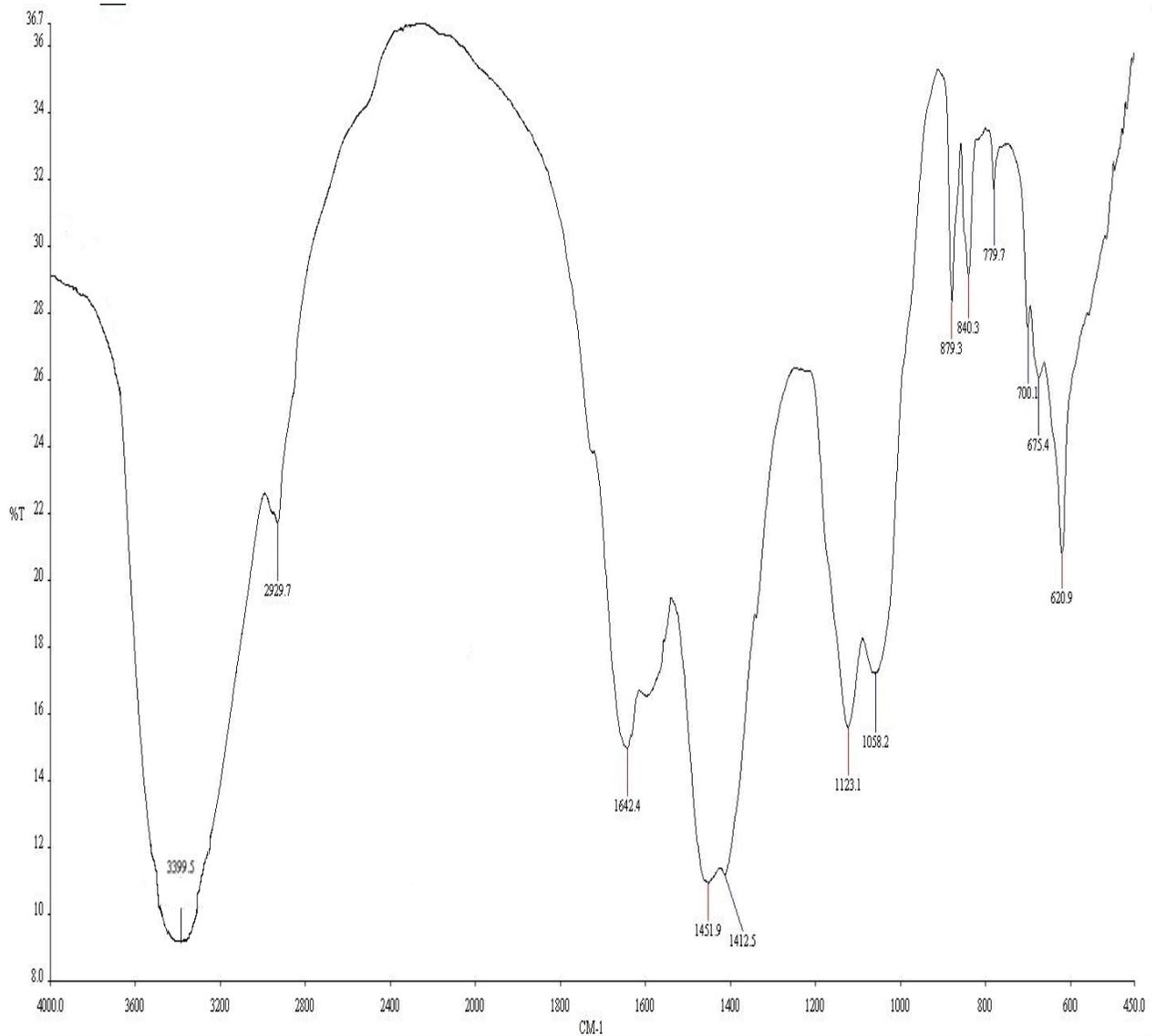
Fig. 12: FTIR spectrum of *P. ostreatus* MTCC 1804.

idized) to colourless (reduced) (Al-Nasrawi 2012). Tween 80 acts as an emulsifier which facilitates active contact between hydrocarbon and the isolate (Hanson et al. 1993). Three indication which verifies the ability of fungi involved in biodegradation process is firstly the change in colour of broth from dark blue to colourless, secondly the disappearance of crude oil from the broth and thirdly developing a mass of fungal biomass growth in the bottom of the broth (Hanson et al. 1993).

Colour transformation from blue to colourless during biodegradation process involving the specific isolates ramparts the fact that isolates are potential hydrocarbon

oxidizers and the colour transformation can be measured as an absorbance value using spectrophotometer (George et al. 2009). The present investigation confirms the ability of *Pleurotus ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804 to degrade used engine oil by displaying colour change from blue to colourless after seven days of incubation in shaking incubator.

As Yoshida et al. (2001) observed a peak in absorbance at 600 nm therefore; absorbance at a wavelength of 600 nm was recorded for fungal isolates. According to Undugoda et al. (2016) the higher hydrocarbon degrading fungi have low absorbance values after the incubation period as compared

to the control whereas lower hydrocarbon degrading fungi possess high absorbance values. The present comparative assessment reported that wild isolate *Pleurotus ostreatus* MP 5 (OD = 0.459 ± 0.005) has proved itself as a more potent hydrocarbon degrader than *Pleurotus ostreatus* MTCC 1804 (OD = 0.536 ± 0.017) by displaying the fastest onset colour disappearance (decrease in absorbance of broth medium) and hence, highest capability of biodegradation. Thus the present investigation is supported by the reports of Khan et al. (2015) who reported that Aspergillus sp., Penicillium sp. and Rhizopus sp. displayed the colour transformation from blue to colourless in the BH broth incorporated with the fungal isolates comprised with 0.1% (v/v) Tween 80 and 1% (v/v) petrol, kerosene and diesel and 0.008 mg/50 mL of redox indicator and hence showed the highest capability of biodegradation. The high rate of petroleum products (hydrocarbon) degradation by the wild isolate may be due to their enzyme production responses during their growth phases which could be supported by the reports and findings of Bogan & Lamar (1996) according to which during the growth phases of white rot fungi, extracellular ligninolytic enzymes are produced in response to their growth phases.

Percentage of degradation of each fungal isolate was calculated on the basis of absorbance value obtained after applying the formula, according to which *Pleurotus ostreatus* MP 5 gave higher percentage of degradation as compared to that of *Pleurotus ostreatus* MTCC 1804. Percentage of hydrocarbon degradation calculated for *Pleurotus ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804 was 69.7 ± 0.351% and 64.7 ± 1.153%. Similarly, Ekundayo (2014) worked on comparative Studies on biodegradative abilities of *Pleurotus ostreatus* and *P. pulmonarius* in soils contaminated with crude and used engine Oils and investigated that *P. ostreatus* reduced the initial total hydrocarbon content (THC) to 8% and 9% in soils polluted with 20% of crude and used engine oils, respectively, which was lower than that of *P.pulmonarius*. Mineralization of various PAHs by white rot fungus *Pleurotus ostreatus* was reported by Bezalel et al. (1995) according to whom, *Pleurotus ostreatus* was able to mineralize to 7.0% of [14C]catechol, 3.0% of[14C] phenanthrene, 0.4% of [14C]pyrene, and 0.19% of [14C] benzo[*a*]pyrene followed by incubation of 11 days. Besides, the implication of the wild strain MP 5 as a more potent hydrocarbon degrader than that of the reference strain *Pleurotus ostreatus* MTCC 1804 from our result is similar to the findings of Sourav et al. (2013) while testing the potentiality of the isolate *P. ostreatus* PO-3 and *P.ostreatus* MTCC 142 to degrade 1 µg/mL of Benzo[*a*]pyrene BaP, a high molecular weight polycyclic aromatic hydrocarbon (HMW PAH) under liquid condition, reported that isolate *P. ostreatus* PO-3 has

better degradation potential than *P. ostreatus* MTCC 142 and concluded that the level of degradation were 32% and 29% respectively for *Pleurotus ostreatus* isolate PO-3 and *Pleurotus ostreatus* MTCC 142.

Proliferation of a mass of fungal growth in the bottom of culture medium supplemented with oil is one of the indications of those fungi which are capable of degrading hydrocarbon (Al-Nasrawi 2012). Therefore, biodegradation assay of both the fungal strains in terms of fungal biomass was performed according to which it was found out that at concentration of 2% oil, *P. ostreatus* MP 5 gave better performance in terms of biomass as compared to that of the reference strain *Pleurotus ostreatus* MTCC 1804. The bulk mass weighed out for *P. ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804 was estimated as 0.334±0.009 g/50 mL and 0.308±0.007 g/50 mL respectively. Experiments performed by Sourav et al. (2013) documented that in the proximity of PAH, the biomass proliferation by the PO-3 isolate was higher than that of *P. ostreatus* MTCC 142 which was calculated as about 51 and 43 mg/50 mL of media which reinforces our present findings in terms of biodegradation essay.

The capability of the fungus to degrade PAH is related to its ability to produce lac case and similar enzymes (Sourav et al. 2013). Therefore, it could be concluded that *P. ostreatus* MP 5 isolate could degrade PAH better than *P. ostreatus* MTCC 142 possibly due to its ability to synthesize higher titers of laccase and manganese peroxidase. Likewise, a native isolate of *P. ostreatus* HP-1 (Genbank Accession No. EU420068) was discovered to comprise an excellent laccase producing ability (Rubailo et al. 2008).

Since the trends of hydrocarbon degradation were assessed overtime and FTIR was utilized and applied to elucidate the degradation of oil spilled from vehicles in the contaminated soil therefore, FTIR spectroscopy has been prove to be a very useful tool in performing preliminary tests in order to predict remediation performance so as to select an appropriate approach for clean- up technologies (Bhat et al. 2011). The present study on comparison of the hydrocarbon degradation potential of the two fungal isolates was finally confirmed by spectral analysis using FTIR to assess biodegradation experiments. Identification and interpretation of peaks indicating various functional groups were done with the reference of available literatures (Bhat et al. 2011, Sohal et al. 2019, Srivastava & Saraf 2019).

The FTIR spectrum monitored for untreated sample, i.e. control as depicted in Fig. 10 indicated a broad peak at 3419.4 cm^{-1} corresponding to O-H stretching vibrations of alcohols. Absorption band around 2933.5 cm^{-1} is assigned to C-H stretching in alkanes (aliphatic compounds), at

1643.7 cm^{-1} indicating C=C stretching of alkenes (aliphatic compounds). Another prominent spectrum which appeared between 1346.2 cm^{-1} to 1461.8 cm^{-1} can be ascribed to the bending vibration of C-H in alkenes (aliphatic compounds). The presence of carboxylic acid functional group in the molecule was confirmed by medium intensity band in the region of 1270.4 cm^{-1} and 1240.1 cm^{-1} for C-O stretching of carboxylic acids. Another bands revealed at 1209.8 and 1053.0 cm^{-1} indicating C-O stretching of alcohols; at 940.7 cm^{-1} indicates S-O stretching in sulphonates; at 909.2 cm^{-1} indicating C-H bends in alkenes; at 867.2 cm^{-1} indicating S-O stretching in sulphonates; at 849.8 cm^{-1} corresponding to C-H bends in aromatics; at lower wave length of 681.1 cm^{-1} and 550.7 cm^{-1} indicating C-Cl stretching signifying the presence of alkyl halides.

The FT-IR spectrum of treated sample with *Pleurotus ostreatus* MTCC 1804 (Fig. 12) after 30 days of microbial incubation indicated the formation of new bands at 3399.5 cm^{-1} indicating O-H stretching vibrations of alcohols; at 2929.7 cm^{-1} corresponding to C-H stretching of alkanes; at 1642.4 cm^{-1} signifying N-H bend in amides. C=C stretching in aromatic compound ring was indicated at 1451.9 cm^{-1}. Spectrum at 1412.5 cm^{-1} can be ascribed to the bending vibration of O-H in carboxylic acids (-COOH). Absorption peaks at 1123.1 and 1058.2 cm^{-1} signified C-O stretching of alcohols (OH). Modification of functional groups at these absorption spectrum indicated the conversion of C-O stretching of carboxylic acids at 1270.4 cm^{-1} and 1240.1 cm^{-1} as depicted in control into C-O stretching of alcohols (OH) at 1123.1 cm^{-1} and 1058.2 may be due to the microbial oxidation of used oil which is supported with the findings of Bhat et al. (2011) who reported the same at 2363.2 cm^{-1}. Formation of new bands at 879.3 cm^{-1}, 840.3 cm^{-1} and 779.7 cm^{-1} indicated C-H bending in aromatics. The appearance of another prominent peak at 700.1 cm^{-1} confirmed C-H bending in alkenes. The absorption peak around 675.4 and 620.9 cm^{-1} was reported as C-Cl stretching in alkyl halides. Besides, it was observed that there is significant change in transmittance which increased in the sample treated with *P. ostreatus* MTCC 1804 as compared to that of control representing degradation of complex mixtures.

The FTIR spectrum obtained to assess the biodegradation capability of P. *ostreatus* MP 5 (Fig. 11) also showed the formation of new bands with different intensity of wavelength which at first indicated a broad spectrum at 3419.4 cm^{-1} corresponding to O-H stretching vibrations of alcohols. Another prominent spectrum which appeared at 2220.1 cm^{-1} can be ascribed to C-N stretching in nitriles. Here the conversion of -C-H stretching in aliphatic compounds as depicted in control or the untreated sample into

nitrile groups may be due to the hydrocarbon degradation by wild isolate MP 5 which was in total accordance with the result of FTIR analysis performed by Bhat et al. (2011) for the mycoremediation of hydrocarbon contaminated soil who also concluded the conversion of aliphatic compounds into nitrile groups during hydrocarbon degradation. Another characteristic absorption band at 1644.6 cm^{-1} showed C=C stretching in alkenes. Absorption band around 1446.5 cm^{-1} is assigned to the bending vibration of asymmetric C=C stretching in aromatic. Another prominent absorption peak around 1338.3 cm^{-1} reported as C-H bending in alkenes. The presence of another absorption band at wavelength 1114.4 cm^{-1} indicated C-O stretching of alcohols may be due to the microbial oxidation of used oil. Another new band revealed at 880.8 cm^{-1} and 848.9 cm^{-1} indicated the presence of C-H bending (meta) and C-H bending (para) in aromatics which further indicated the conversion of alkenes as reported in control at 909.2 cm^{-1} into aromatic compounds. The absorption peak around 779.7 cm^{-1} was reported as C-Cl stretching in alkyl halides. At lower wave length of 618.7 and 516.2 cm^{-1} occurrence of C-Br stretching of alkyl halides was revealed out. Apart from all these modifications of functional groups which occurred during degradation process, the percent transmittance increased as compared to that of control and *P. ostreatus* MTCC 1804 which finally confirmed the higher degradation potential of wild isolate MP 5.

CONCLUSION

The present study reveals that the molecular identification technique like ITS sequencing was carried out for the potent isolate which revealed that the isolate MP 5 shares a 99% sequence similarity to that of *Pleurotus ostreatus* isolate 6689 (accession No. AY450345.1). Primary screening assay confirmed that the wild isolate *P. ostreatus* MP 5 showed highest average growth rate on MSM supplemented with 2% oil. Confirmatory test conducted for both the isolates through orbital shaking method for detection of biodegradation by spectrophotometric method at 600 nm displayed degradation within 7 days and level of hydrocarbon degradation figured out for *P. ostreatus* MP 5 and *Pleurotus ostreatus* MTCC 1804 was 69.7 ± 0.351% and 64.7 ± 1.153%. The FTIR spectrum of control and treated samples with fungal isolates showed different peaks based on the presence of different functional groups indicating oxidative degradation of hydrocarbons by microbes. Thus, it can be concluded from the present investigation that the wild isolate *P. ostreatus* MP 5 had better potential for degrading hydrocarbon in comparison to *P. ostreatus* MTCC 1804 which can be served as a better potential hydrocarbon degrader for mycoremediation studies.

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REFERENCES

Adongbede, E.M. and Sanni, R.O. 2014. Biodegradation of engine oil by *Agaricus campestris* (a white rot fungus). J. Bioremed. Biodeg, 5(262): 2.

Al-Nasrawi, H. 2012. Biodegradation of crude oil by fungi isolated from Gulf of Mexico. Journal of Bioremediation and Biodegradation, 3(04): 1-6.

Boonchan, S., Britz, M.L. and Stanley, G.A. 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol., 66(3): 1007-1019.

Barr, D.P. and Aust, S.D. 1994. Mechanisms white rot fungi use to degrade pollutants. Environmental Science & Technology, 28(2): 78A-87A.

Bogan, B.W. and Lamar, R.T. 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol., 62(5): 1597-1603.

Bezalel, L. Hadar, Y. and Cerniglia, C.E. 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol., 62(1): 292-295.

Bhat, M.M. Kumar, D. Narain, K. Shukla, R.N. and Yunus, M. 2011. Mycoremediation of hydrocarbon contaminated soil-FTIR based analysis. World Appl. Sci. J., 15: 1547-1552.

Clemente, A. Anazawa, T.A. and Durrant, L.R. 2001. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Brazilian Journal of Microbiology, 32(4): 255-261.

Chen, D.H. and Ronald, P.C.1999. A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Molecular Biology Reporter, 17(1): 53-57.

dos Santos, E.O. da Rosa, C.C. dos Passos, C.T. Sanzo, A.V.L. Burkert, J.F.D.M. Kalil, S.J. and Burkert, C.A.V. 2008. Pre-screening of filamentous fungi isolated from a contaminated site in Southern Brazil for bioaugmentation purposes. African Journal of Biotechnology, 7(9): 1314-1317.

Emuh, F.N. 2010. Mushroom as a purifier of crude oil polluted soil. Inter. J. Sci. Nat.,1(2): 127-132.

Ekundayo, F.O. 2014. Comparative studies on biodegradative abilities of *Pleurotus ostreatus* and *P. Pulmonarius* in soils contaminated with crude and used engine oils. Advances in Microbiology, 4(12): 849.

George-Okafor, U. Tasie, F. and Muotoe-Okafor, F. 2009. Hydrocarbon degradation potentials of indigenous fungal isolates from petroleum contaminated soils. Journal of Physical and Natural Sciences, 3(1): 1-6.

Hagwell, I.S. Delfino, L.M. and Rao, J.J. 1992. Partitioning of polycyclic aromatic hydrocarbons in soil by microbial consortium. International Biodeterioration Biodegradation, 54: 61-67.

Huesemann, M.H. 1994. Guidelines for land-treating petroleum hydrocarbon-contaminated soils. Soil and Sediment Contamination, 3(3): 299-318.

Hanson, K.G., Desai, J.D. and Desai, A.J. 1993. A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnology Techniques, 7(10): 745-748.

Jawhari, A.L. and Ihsan, F.H. 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. Journal of Applied & Environmental Microbiology, 2(2): 46-52.

Keith, L. and Telliard, W. 1979. ES &T special report: Priority pollutants: A perspective view. Environmental Science & Technology, 13(4): 416-423.

Khan, S.R., Ji, N.K., Kumar, R.N. and Patel, J.G. 2015. In vitro study on assessment of petrol, kerosene and diesel degrading potential of indigenous fungal isolates from different petroleum product effected soils. International Journal of Recent Research and Review, 8(1): 8-15.

Mills. A.L. Breuil, C. and Colwell, R.R. 1978. Enumeration of petroleum-degrading marine and estuarine microorganisms by the most-probable number. Canadian Journal Microbiology, 24: 552-557.

Okparanma, R.N., Ayotamuno, J.M., Davis, D.D. and Allagoa, M. 2011. Mycoremediation of polycyclic aromatic hydrocarbons (PAH)-contaminated oil-based drill-cuttings. African Journal of Biotechnology, 10(26): 5149-5156.

Okpokwasili, G.C. and Okorie, B.B. 1988. Biodeterioration potentials of microorganisms isolated from car engine lubricating oil. Tribology International, 21(4): 215-220.

Popa, G. Nicolcioiu, M.B. and Cornea, C.P. 2013. *In vitro* evaluation of crude oil degradation potential of some *Pleurotus ostreatus* isolates. Scientific Bulletin. Series F. Biotechnologies, 17: 143-146.

Rubailo, A.I. and Oberenko, A. V. 2008. Polycyclic aromatic hydrocarbons as priority pollutants. Journal of Siberian Federal University, 4(1): 344-354

Stamets, P. 2005. Mycelium Running: How Mushrooms Can Help Save The World. Random House Digital, Inc.

Sack, U. and Günther, T. 1993. Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. Journal of Basic microbiology, 33(4): 269-277.

Steffen, K.T. Schubert, S. Tuomela, M. Hatakka, A. and Hofrichter, M. 2007. Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi. Biodegradation, 18(3): 359-369.

Shahriarinour, M. Wahab, M.N.A. Ariff, A. and Mohamad, R. 2011. Screening, isolation and selection of cellulolytic fungi from oil palm empty fruit bunch fibre. Biotechnology, 10(1): 108-113.

Sourav, B. Arijit, D. Palaniswamy, M. and Angayarkanni, J. 2013. Molecular characterization of Pleurotus ostreatus PO-3 involved in mycoremediation of benzo[a]pyrene. International Research Journal of Biological Sciences, 2(8): 48-53.

Sohal, J.K. Saraf, A. Shukla, K. and Shrivastava, M. 2019. Determination of antioxidant potential of biochemically synthesized silver nanoparticles using aloe vera gel extract. Plant Science Today, 6(2): 208-217.

Srivastava, M. and Saraf, A. 2019. Antimicrobial activity of bioactive molecules isolated from filamentous fungi. Int. Res. J. Biological Sci., 8(3): 13-18.

Tiwari, M. and Saraf, A. 2017. Isolation, screening and identification of hydrocarbon degrading potential of indigenous fungus from oil contaminated soil of Modha Para automobile shop of Raipur, CG. International Journal of Advance Research in Science and Engineering, 6(10): 782-795.

Thomas, S. Becker, P. Pinza, M.R. and Word, J.Q. 1998. Mycoremediation of aged petroleum hydrocarbon contaminants in soil. Washington State Department of Transport. Publication No. WA-RD 464.1.

Thenmozhi, R. Arumugam, K. Nagasathya, A. Thajuddin, N and Paneerselvam, A. 2013. Studies on mycoremediation of used engine oil contaminated soil samples. Advances in Applied Science Research, 4(2): 1.

Undugoda, L.J.S. Kannangara, S. and Sirisena, D.M. 2016. Aromatic hydrocarbon degrading fungi inhabiting the phyllosphere of ornamental plants on roadsides of urban areas in Sri Lanka. J. Bioremed. Biodeg., 7(328): 2.

Vanishree, M. Thatheyus, A.J. and Ramya, D. 2014. Biodegradation of

petrol using *Aspergillus* sp. Annual Research & Review in Biology, 4(6): 914-923.

Velioğlu, Z. and Ürek, R.Ö. 2015. Biosurfactant production by Pleurotus ostreatus in submerged and solid-state fermentation systems. Turkish Journal of Biology, 39(1): 160-166.

White, N.A. Dehal, P.K. Duncan, J.M. Williams, N.A. Gartland, J.S. Palfreyman, J.W. and Cooke DEL. 2001. Molecular analysis of intraspecific variation between building and "wild" isolates of *Serpula lacrymans* and their relatedness to *S. himantioides*. Mycol. Res, 105(4): 447-452.

Yoshida, N. Hoashi, J. Morita, T. McNiven, S.J. Nakamura, H. and Karube, I. 2001. Improvement of a mediator-type biochemical oxygen demand sensor for on-site measurement. Journal of Biotechnology, 88(3): 269-275.

Zebulun, H.O. Isikhuemhen, O.S. and Inyang, H. 2011. Decontamination of anthracene-polluted soil through white rot fungus-induced biodegradation. The Environmentalist, 31(1): 11-19.