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Standardization of used engine oil concentration for analysis of biodegradation potential of *Pleurotus* sp. MP 5 MCC 1815 - A Scanning Electron Microscope based analysis

Madhavi Tiwari[1], Ashish Saraf[2] and Akhilesh Kumar Pandey[3]

[1]School of Sciences, MATS University, Raipur 494 001, C.G., India
[2] Faculty of MATS School of Sciences, MATS University, Raipur 494 001, C.G., India
[3] Vice Chancellor, Vikram University, Ujjain 456 001, M.P., India

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ABSTRACT

To broaden the understanding of mycoremediation mechanisms of white rot fungus *Pleurotus* Sp. MP 5 MCC 1815, an attempt was made in the present work for standardization of used engine oil (UEO) for analysis of its biodegradation potential. Standardization of UEO concentration proceeded via radial plate assay method which revealed that MP 5 displayed highest average colony growth rate (cm/day) than *Pleurotus ostreatus* MTCC 1804 (Positive test control) recorded as 5.9±0.1 cm/day ($P \leq 0.05$ under t-test) at 2 % (v/v) UEO on 7th day) as compared to that of 0.5, 1, 1.5 and 2.5% UEO ($P \leq 0.05$ under one-way anova), therefore 2% UEO was standardized for further studies. SEM based analysis was later performed in order to study the surface characterization of the degraded oil sample which indicated the surface changes occurred during degradation in fungal treated oil sample and were compared to that of control.

Key words : *Mycoremediation, Used Engine Oil (UEO), Pleurotus ostreatus, Biodegradation, Radial Plate Assay, Scanning Electron Microscope (SEM)*

Introduction

Engine oil is always on high demand globally and is used all over the world in vehicles and other power generating engines. improper and illegitimate discharge 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 serious environmental hazards like reduction in soil fertility, changes in normal biotas of environment, disturbances of geochemical cycle etc. 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; Keith and Telliard, 1979).

In line with the previous explorations involving white rot fungi and their potentiality to degrade petroleum hydrocarbons, several auspicious results have been reported. Therefore, our previous investigation purposed to isolate white rot fungi and to per-form comparative evaluation of hydrocarbon degradation potential of both wild isolate and refer-

ence strain of *Pleurotus ostreatus* MTCC 1804 based on FTIR analysis in order to provide a promising isolate for mycoremediation studies (Tiwari *et al*., 2020).

As many researchers claimed the fact that there occurs arrest in biodegradation of crude oil above a certain concentration may be due to some unfavourable environmental conditions exerted by volatile hydrocarbons, therefore the main aim of the present investigation is to standardize the concentration of used engine oil (UEO) required for analysis of biodegradation potential of *Pleurotus* Sp. MP 5 MCC 1815 via Scanning Electron Microscopy (SEM) based analysis.

Materials and Methods

Chemicals and Reagents

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

Source of Fungal Cultures

As published in our previous investigation isolated white rot fungus via tissue culture method was sent to Microbial Identification Report, National Centre for Microbial Resource (NCMR)-National Centre of Cell Science (NCCS), Pune, India for ITS sequencing as per their requirement. 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. After identification was completed, cultures were deposited at NCMR-NCCS, Pune, India under general deposition repository following various guidelines. During deposition process the strain was assigned with accession No. No. MCC 1815 under general deposition repositories

Procurement of Fungal Cultures

Pleurotus ostreatus MTCC 1804 as reference strain or positive test strain was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungus was propagated on Potato Dextrose Agar (PDA Himedia) at 25 ºC for 7 days, maintained in PDA slants and sub cultured after every 15 days.

Standardization of UEO Concentration

Radial plate assay method

Mineral Salts Medium (MSM) was prepared and later supplemented with 50 µg/ml ampicillin and for standardization purpose (Bhattacharya *et al*., 2012). MSM was further supplemented with 0.5 % (v/v). 1.0 % (v/v), 1.5 % (v/v), 2.0 % (v/v) and 2.5 % (v/v) of filtered sterile used engine oil (Vanishree *et al*., 2014). Meanwhile, another MSM was also prepared without supplementation of oil which served as control. 5 mm mycelial plug of wild fungal isolate designated with sample id MP 5 as well as the mycelial plug of reference strain *Pleurotus ostreatus* MTCC 1804 (Positive test control) were aseptically inoculated in oil agar plates of varying concentration with the help of sterilized 5 mm 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 (in cm) (Popa *et al*., 2013). Both the fungal isolates were also inoculated on control MSM plates without oil. All the inoculations were carried out in triplicates. 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).

Shake Flask Experiment with Fungal Inoculum

Shake flask experiment with preparation of Bacto Bushnell- Hass (BH) broth medium was carried out. As in our previous experiment *Pleurotus* sp. MP 5 MCC 1815 was proved out to be more hydrocarbon degrading potential isolate, therefore it was selected out for performing shake flask experiment and SEM analysis. Two-three mycelial plugs (5mm) of potential white rot fungal isolate were inoc-ulated in 50 ml BH broth with optimized percentage of used engine oil in a 250 mL Erlenmeyer flask. Control experiment containing same medium contents but without inoculum was also performed simultaneously. All the flasks were agitated at 200 rpm at 27ºC for a period of 28-30 days and the content of each test including the control were harvested (Thenmozhi *et al*., 2013). Before harvesting visual observation of fungal treated UEO samples were compared with control to visualize oil over the surface of medium contents and fungal growth (Ishfaq *et al.,* 2015). During harvesting, contents inside the flasks were filtered with the help of Whatman No.1

filter paper and the filtrates obtained were centrifuged at 10000 rpm for 30 minutes (Soma Prabha and Jayachitra, 2018). After centrifugation all the supernatants (liquid containing biodegraded oil) including the control were collected in 100 ml beaker and were dried in hot air oven at 70 ºC overnight to convert them into powdered form. Surface analysis of this dried form of the supernatant (liquid containing biodegraded oil), converted into powdered form was analysed by SEM analysis.

Analysis by scanning electron microscopy (SEM)

The surface morphology of the degraded UEO by both the white rot fungal isolates obtained in the dried powdered form was analysed through scanning electron microscopy after shake flask experiment. Samples were coated with thin layer of gold to make them conductive via the use of sputter coater. The micrographs were obtained on Quanta FEG 450, TECNAI G2 Spirit (FEI, Netherland) Scanning Electron Microscope equipped with Gatan digital camera operated at an accelerating voltage at 80 Kv. The images of control and test samples were observed and recorded.

Statistical Analysis

An analysis of variance (ANOVA) was performed at 95% confidence level ($P \leq 0.05$) to establish if there were any significant differences while performing the comparison of the average colony growth rate (cm/day) of potential white rot fungal isolate MP 5 and *Pleurotus ostreatus* MTCC 1804 (positive control) on MSM +0.5 %, 1.0 %, 1.5 %, 2.0 % and 2.5% UEO for 7 days.

Results and Discussion

Standardization of UEO Concentration

Radial Plate Assay Method

Pleurotus sp. MP 5 MCC 1815 and reference strain *Pleurotus ostreatus* MTCC 1804 (Positive test control) were standardized to test their ability to degrade the used engine oil based on their average colony growth rate (cm/day) by calculation of the diameter of radial extension of the fungal mycelium for 7 days on MSM supplemented with 0.5%, 1%, 1.5 %, 2 and 2.5% (v/v) used engine oil. According to the results obtained MP 5 and *Pleurotus ostreatus* MTCC 1804 (Positive test control) displayed highest average colony growth rate (cm/day) recorded as 5.9±0.1 cm/day and 3.20±0.1 cm/day at 2 % (v/v) UEO on 7th day as compared to that of 0.5, 1, 1.5 and 2.5% UEO ($P \leq 0.05$ under one-way anova) (Table 1 and Fig. 1), therefore 2% UEO was standardized for further studies.

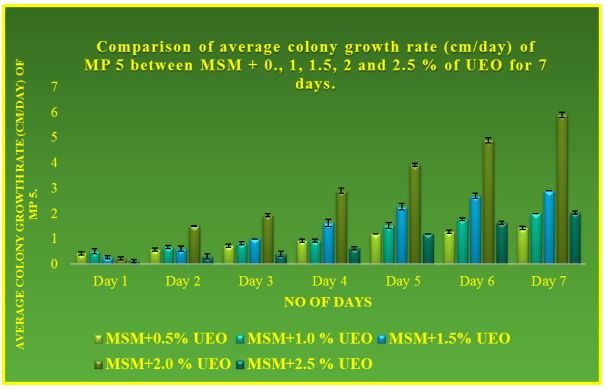

Fig. 1. Comparison of the average colony growth rates (cm/day) in terms of mycelial diameter (cm/day) of MP 5 between MSM with different concentration of UEO i.e., MSM + 0.5, 1.0, 1.5, 2.0 and 2.5 % UEO for 7 days. Each bar represents Mean ± SD of three separate observations (n=3). The results were statistically significant at $p \leq 0.05$.

The average colony growth rate (cm/day) of highest potential test organism and reference strains were increasing from 0.5 % to 2% UEO concentration but a decrease in mycelial diameter (cm/day) was observed at 2.5 % UEO which may be due to some toxic effects exerted by hydrocarbons at certain level of concentration and insufficient oxygen. Hence, this observation was in total accordance with the results obtained by Mahalingam and Nithya (2014) who confirmed that diesel oil is needed as a carbon source but with certain level of contamination it can be toxic to microorganisms due to the solvent effect which destroy fungal and bacterial cell wall. According to them many biodegradation studies on diesel were carried out using lesser diesel contamination ranging from 0.5 to 1.5 % whereas in our study we carried out biodegradation studies ranging from 0.5 to 2.5 % UEO contamination. The findings of present study were in partial agreements with those obtained by Mahalingam and Nithya (2014) which revealed that 1% diesel oil supports excellent growth for all three bacterial isolates with significant difference ($p<0.005$) whereas in our study we reported that at 2 % UEO the potential *Pleurotus* Sp. MP 5 MCC 1815 showed excellent growth statistically significant at $p<0.005$. Moreover, Rahman *et*

Table 1. Comparison of the average colony growth rate (cm/day) in terms mycelial diameter (cm/day) of potential white rot fungal isolate MP 5 and *P. ostreatus* MTCC 1804 (positive control) between MSM +0.5 %, 1.0 %, 1.5 %, 2.0 % and 2.5% UEO for 7 days

S. No.	MSM+% of oil concentration	Average colony growth rate (cm/day) at Day 1		Average colony growth rate (cm/day) at Day 2		Average colony growth rate (cm/day) at Day 3		Average colony growth rate (cm/day) at Day 4	
		Fungal isolates		Fungal isolates		Fungal isolates		Fungal isolates	
		MP 5	*P.ostreatus* MTCC 1804	MP 5	*P.ostreatus* MTCC 1804	MP 5	*P.ostreatus* MTCC 1804	MP 5	*P.ostreatus* MTCC 1804
1.	MSM+0.5% UEO	0.43±0.06	0.00±0	0.57±0.06	0.17±0.06	0.73±0.06	0.30±0	0.93±0.06	0.47±0.06
2.	MSM+1.0 % UEO	0.50±0.1	0.03±0.06	0.67±0.06	0.23±0.06	0.83±0.06	0.37±0.06	0.93±0.06	0.50±0.1
3.	MSM+1.5% UEO	0.27±0.06	0.07±0.06	0.6±0.1	0.27±0.06	1.0±0	0.43±0.06	1.63±0.15	0.57±0.06
4.	MSM+2.0 % UEO	**0.23±0.06**	**0.07±0.06**	**1.50±0.01**	**0.3±0.1**	**1.93±0.06**	**0.5±0.1**	**2.90±0.1**	**1.17±0.06**
5.	MSM+2.5 % UEO	0.13±0.06	0±0	0.30±0.1	0.07±0.06	0.40±0.1	0.30±0.1	0.63±0.06	0.50±0.1

S. No.	MSM+% of oil concentration	Average colony growth rate (cm/day) at Day 5		Average colony growth rate (cm/day) at Day 6		Average colony growth rate (cm/day) at Day 7	
		Fungal isolates		Fungal isolates		Fungal isolates	
		MP 5	*P.ostreatus* MTCC 1804	MP 5	*P.ostreatus* MTCC 1804	MP 5	*P.ostreatus* MTCC 1804
1.	MSM+0.5% UEO	1.20±0	0.63±0.06	1.27±0.06	0.83±0.06	1.43±0.06	0.93±0.06
2.	MSM+1.0 % UEO	1.53±0.12	0.73±0.06	1.77±0.06	0.87±0.06	2.00±0	1.0±0
3.	MSM+1.5% UEO	2.27±0.12	0.77±0.06	2.7±0.1	0.93±0.06	2.90±0	1.07±0.06
4.	MSM+2.0 % UEO	**3.93±0.06**	**2.1±0.1**	**4.9±0.1**	**2.73±0.06**	**5.9±0.1**	**3.20±0.1**
5.	MSM+2.5 % UEO	1.20±0	0.73±0.06	1.63±0.06	0.9±0	2.03±0.06	1.0±0

Comparison of the average colony growth rates (cm/day) in terms of mycelial diameter (cm/day) of the potential white rot fungal isolate MP 5 and *Pleurotus ostreatus* MTCC 1804 (positive test control) between MSM with different concentration of UEO i.e., MSM + 0.5, 1.0, 1.5, 2.0 and 2.5 % UEO for 7 days are significantly different from each other at 95% confidence level, hence statistically significant at p £ 0.05, p value was calculated by one-way anova.

al. (2002) agreed with the fact that arrest in biodegradation of crude oil above a certain concentration might be caused by unfavourable changes in environmental conditions as nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons hence supports the present investigation. In our investigation during primary screening lower average colony colony growth rate for both micro and macro fungal isolate was observed when the initial concentration of oil was lower i.e., at 0.5, 1.0, 1.5 % of oil and higher when the concentration increased up to 2 % which was satisfied with the condition reported by Vyas and Dave (2007) who revealed that degree of biodegradation was lower when the initial concentration of oil was lower i.e., at 2g/l and become higher when the concentration increases at 5 g/l.

Shake Flask Experiment with Fungal Inoculum

Shake flask experiment was performed for visual comparison of fungal treated UEO samples with standardized concentration of UEO i.e., 2 % to that of control which indicated that oil colour was faded and completely disappeared from the surface of the BH broth medium and fungal growth was also observed above the surface (Fig. 2 a and b).

Hanson *et al*. (1993) ruled out three indications which confirm the ability of fungi in biodegradation process i.e., first, the change in colour of broth from dark to colorless, second is the disappearance of crude oil from the broth and third is developing a mass of fungal growth in the bottom of the broth. Since the present investigation satisfies all the above-mentioned indications for the potential wild isolate during the tenure of work, hence confirms the degradation property of *Pleurotus* Sp. MP 5 MCC 1815 with different percentage of UEO degradation.

Analysis by scanning electron microscopy (SEM)

Surface morphology of the powdered form of the of the supernatant i.e., the liquid containing biodegraded oil collected from the BH broth + UEO treated with *Pleurotus* sp. MP 5 MCC 1815 during shake flask experiment was analysed through SEM. Analysis indicated the surface changes which occurred during degradation in fungal treated oil samples incubated for 30 days and compared with that of control where no surface changes were analyzed. SEM analysis of oil samples (powdered form) treated with wild fungal isolate indicating surface changes like cracks, pits and holes could be visualized clearly in Fig. 3 (b). SEM analysis of control indicating no surface changes could be visualized in Fig. 3 (a)

The SEM photographs visualized that surface roughness, wrinkled texture increased in fungal treated sample due to degradation as compared to control. Even some holes were observed in biodegraded samples of oil after 30 days. A similar pattern of biodeterioration of paint was observed earlier by Breitbach *et al*. (2011) and Ishfaq *et al*. (2015) while studying the surface changes of fungal treated

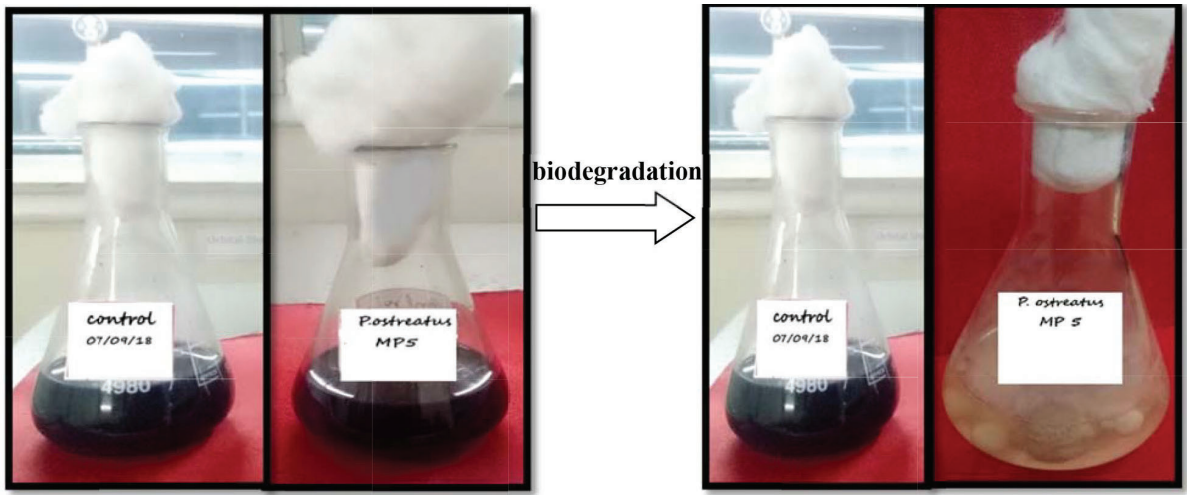

Fig. 2(a). Showing left flask as negative control without fungus and right flask MP 5 containing BH broth, fungal isolate and 2% (v/v) used engine oil before biodegradation.

Fig. 2(b). Showing left flask as negative control without fungus and right flask MP 5 containing BH broth, fungal isolate and 2% (v/v) used engine oil after biodegradation.

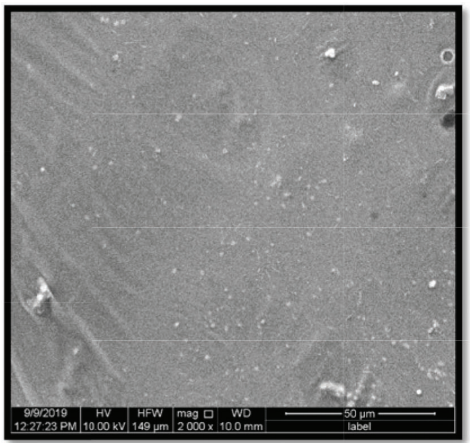

Fig. 3(a). SEM photograph of untreated oil sample (control)

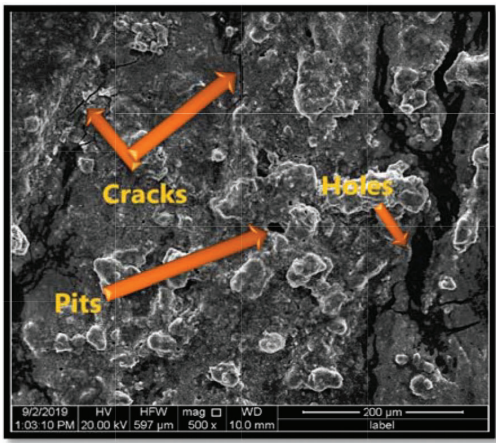

Fig. 3(b). SEM photograph of oil sample treated with *Pleurotus ostreatus* MP 5 arrows indicating the cracking, holes and pits.

paint samples during degradation by fungal species which supported the conclusion of our investigation Surface roughness and distortion was found to be more prominent in sample of oil treated with *Pleurotus* sp. MP 5 MCC 1815. SEM analysis evidenced hyphal penetration and oil disruption and confirmed that interactions between fungal hyphae and oil might have taken place which initiated the breakdown of oil film which was in total accordance with findings of English *et al*. (2003) who performed SEM analysis which showed interactions between the fungal hyphal of *A. pullans* and the paint flakes and concluded the same.

Conclusion

Present work made an attempt to standardize the UEO concentration required by white rot fungus *Pleurotus* sp. MP 5 MCC 1815 to analyze its biodegradation potential based on SEM analysis for bioremediation purpose as the utility of fungi for biodegradation has beguiling probability because of their potentiality to degrade polycyclic aromatic hydrocarbon (PAH) present in the oil and other petroleum products. Present manuscript demonstrated the fact that degree of biodegradation was lower when the initial concentration of oil was lower and become higher when the concentration increases but agreed with the fact that arrest in biodegradation of crude oil above a certain concentration might be caused by unfavourable changes in environmental conditions as nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. Further, biodegradation process was supported by studying the surface characterization of biodegraded oil via SEM analysis.

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Conflict of Interest

No conflict of interest.

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