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Isolation, Screening and Identification of Hydrocarbon Degrading Potential of Indigenous Fungus From Oil Contaminated Soil of Modha Para Automobile Shop of Raipur, C.G.

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ABSTRACT

The aim of this present investigation is to isolate, screen and identify hydrocarbon degrading fungi from oil contaminated soil of Modha Para automobiles of Raipur, C.G. During this study, nine pure fungal isolates were obtained through serial dilution of soil in potato dextrose agar. Primary screening tests were done to evaluate the growth diameter of fungal colonies on minimal salt media supplemented with 1% used engine oil (v/v). The fungal isolates MT 19, MT 18, MT 4 showed highest average growth rate. Later, confirmatory test was also performed for detecting the degrading capacity of isolated fungal strains through shake flask method by taking optical density at 600 nm by spectrophotometer. MT 19, MT 18, MT 4 gave higher percentage of oil degradation as compared to other isolates within 7 days. Morphological identification was performed and it was determined that fungal isolate MT 19 Aspergillus flavus gave 0.12 OD, MT 18 Aspergfillus fumigatus gave 0.15 OD and MT 4 Aspergillus nidulans gave 0.18 OD . It was found that MT 19 Aspergillus flavus gave higher % of degradation i.e. 76.9 % as compared to MT 18 Aspergfillus fumigates and MT 4 Aspergillus nidulans which were recorded as 71.1 % and 65.3 %.

Keywords: Aspergillus flavus, biodegradation, fungi, hydrocarbon, oil Aspergfillus fumigates, Aspergillus nidulans .

I. INTRODUCTION

Environmental pollute

on by aromatic hydrocarbons present in oil and petroleum products and its hazardous effects are among the most burning problem that the world is facing today. Crude oil containing poly aromatic hydrocarbons is one of the most significant pollutants in the environment which is continuously causing damages to both the human and the ecosystem including mutagenicity and carcinogenicity [1, 2, 3]. Continuous exposure to high oil concentration containing aromatic hydrocarbon can lead to liver or kidney problems, damages to the eye, bone marrow as well as an increased risk to cancer [4, 5, 6]. As oil and petroleum products contain some gaseous components whose fractions volatize when oil pollution occurs leaving behind the non- volatile components as residues both in and on the soil [7]. Therefore oil spillage alters the physicochemical properties of the soil. Deep impacts of oil pollution on the soil are spoiling the vegetation, soil fertility and soil microbiota. Therefore bioremediation of such oil contaminated soil is necessary in order to keep a balance in the environment and ecosystem.

Biodegradation plays an important ecological role as it contributes to bioremediation. Nowadays the role of fungi has been extensively studied to be most potential degraders of oil and petroleum products than other traditional bioremediation techniques [8]. Filamentous fungi are considered to be better degraders of oil and petroleum than bacteria because they can degrade high molecular weight polycyclic aromatic hydrocarbons whereas bacteria degrade smaller molecules [9]. Another reason which enables them as good potential agents of degraadsation is to produce extracellular enzymes for the digestion of complex carbohydrates which further causes the degradation of hydrocarbon pollutants [9]. Another advantage is that they can easily be grorn in fermenters for large scale productions. Besides all these, the separation of fungal biomass is easy by filtration due to its filamentous structure. They also have capability to under environmentally stress conditions like low pH, poor nutrition, low water activity [9] and are less sensitive towards variations in aeration, temperature.

Raipur, the capital city of C.G state is now considered to be the third worst city in India on the list of top twenty polluted cities in the world when it comes to air pollution. Central pollution control board (CPCB) recently declared it as the country's most polluted city. Several factors are responsible to make it as the most polluted city among which higher concentration of PAHs in the air may be one. The utilization of bioremediation techniques through microorganisms to clean up pollutants is viable and has economic values [10]. Microorganisms possess enzymatic systems for degradation and utilization of oil as a source of carbon and energy [11]. Growth and proliferation of oil utilizing microorganisms in oil contaminated soil is greatly influenced by the presence of nutrients and their hydrocarbonoclastic property [12].Therefore the main objective of the present investigation is to isolate, screen and identify some filamentous fungal flora of oil contaminated soils and to detect their biodegradation potential which may have some future applications in bioremediation techniques.

II. MATERIALS AND METHODS

1.1 Sample collection:

Oil contaminated soil samples were collected in sterile polythene bag after tilling with a sterile scoop from automobile garages located at Modha para , Raipur in April 01/04/2016.

1.2 Isolation of fungi:

Collected soil samples were mixed homogenously and sieved to remove stones, soil debris. Sieved soil samples were serially diluted upto $\mathbf{10^{-5}}$ dilution and approx. Potato Dextrose Agar media was prepared and autoclaved at 121°C for 15 min which was later supplemented with 50 mg/ml ampicillin and 10% of filtered sterile used engine oil [13]. 1 ml of dilution from $\mathbf{10^{-3}}$, $\mathbf{10^{-4}}$ and $\mathbf{10^{-5}}$ were poured on potrato dextrose agar plates (PDA) by pour plate method and left for incubation at 27°C for a period of three days. After three daysof incubation, each pure fungal isolate were then subcultured on a fresh PDA medium both in plates and slants for further analysis

1.3 Primary Screening:

For primary screening of oil degrading fungi, a selective media i.e. oil agar medium also known as Mineral Salts Medium (MSM) [v/v] of Mills *et al* [14] as modified by Okpokwasili and Okorie [15] was prepared. MSM was later supplemented with 50 mg/ml ampicillin and 1% of filtered sterile used engine oil [13]. 1cm^2 mycelial plug of pure fungal isolates were inoculated in oil agar medium, and incubated at 27°C for 7 days and the growth rates were recorded daily by measuring the diameter of the radial extension of fungal mycelium [16]. Pure fungal isolates were also inoculated on MSM which served as control. All the inoculations were carried out in duplicates. Average of the diameter of fungal growth at that particular time of measurement (cm/day) was calculated by the regression of the colony diameter against the day after inoculation [17]. Fungal isolates which gave heavy sporulation, more abundant aerial mycelium and greater colony diameter were considred as organiosms utilizinhydrocarbons which were later confirmed thrpugh confirmatory screening [16].

1.4 Confirmatory screening *(shaking flask method)*:

For confirmatory screening of the biodegradability of the selected fungal strain a modified technique based on the redox indicator 2, 6- dichlorophenol indophenol (DCPIP) was performed [18]. For this method Bacto Bushnell- Hass broth medium was prepared [18]. A control flask without organism was also prepared. Two agar plugs from 7 days old culture of pure fungal isolates were picked from the surface of the petridish and inoculated carefully into 50 ml of sterilized BH broth medium using 150 ml Erlynmeyer flask. Later, 0.1 % (v/v) Tween 80, 1% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol indophenols) were incorporated into the BH medium. All the flasks were incubated in shaking incubator at temperature 28°C with constant shaking at 180 rpm for 7 days. The aliquots inside the flask were monitored daily for colour change from deep blue to colourless. After 7 days of incubation, broth inside the flasks were filtered with the help of filter paper for the separation of fungal biomass, followed by centrifugation at 8000 rpm for 15 minutes. Supernatant obtained after centrifugation was analyzed spectrophotometrically at 600 nm and the percentage of biodegradation was calculated by the following equation [18].

% of Degradtion = [1- Absorbance of treated sample/ Absorbance of control] x 100

1.5 Biodegradation assay of hydrocarbon by isolated fungal cultures:

Three agar discs of fresh pure cultures of selected fungal isolates of MT 4, MT 18, MT 19 were transferred into 50 ml of BH broth in Erlynmeyer flask of 150 ml containing 0.1 % (v/v) Tween 80 and 1% (v/v) crude oil. Simultaneously control flask was also prepared accordingly with no fungal culture. All the flasks were incubated in shaking incubator at temperature 28°C with constant shaking at 180 rpm for 7 days. After 7 days of incubation broth inside the flasks 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 [18].

1.6. Morphological Identification:

Morphological identification was performed for the selected fungal strains. Both microscopic (lactophenol cotton blue staining) and macroscopic (cultural characteristics) examinations were performed and confirmed by comparing their morphology and cultural characteristics with descriptions given by Barnett, 2003 [19].

II. RESULT

2.1.Isolation of fungi:

In this present investigation 9 pure fungal isolates were obtained from mother cultures of $\mathbf{10^{-3}}$, $\mathbf{10^{-4}}$ and $\mathbf{10^{-5}}$ dilutions during isolation of funfi through serial dilution of oil contaminated soil with 10% used engine oil.

2.2.Primary Screening:

All the 9 pure fungal isolates were screened to test their ability to degrade the used engine oil based on their average growth rate by calculation of the diameter of radial extension of the fungal mycelium on minimal MSM media supplemented with 1% (v/v) used engine oil as tabulated in Table no. 1. Among 9 fungal isolates the highest average growth rate were observed for MT 19, MT 18 and MT 4 which were recorded as 3.85, 3.6, 3.2 cm/day, respectively. Remaining fungal isolates MT 17, MT 11, MT 8, MT 5, MT 1, MT 7 grew poorly on MSM which were recorded as 2.2, 1.05, 0.2, 0.1, 0.03, 0.03 cm/day.

2.3. Confirmatory screening (shaking flask method):

For confirming the degradation potential of fungal strains shaking flask method using a redox indicator dye 2, 6 Dichlorophenol indophenols (2, 6 DCPIP) used to detect the potential of most efficient isolates to degrade oil was performed. BH broth inoculated with different fungal isolates changed colour from blue to colourless meant that the isolate is potential hydrocarbon oxidizer. Out of nine fingal isolates, only three fungal isolates MT 19, MT 18 and MT 4 showed change in colour from blue to colorless as tabulated in Table no. 2.

Table no. 3 shows that these total colour change can be meadsured as an absorbance value using spectrophotometer which correlates to the ability of the isolate to utilize hydrocarbon.

Percentage of degradation of oil of all the nine fungal isolates were calculated on the basis of the absorbance values of filtrates obtained spectrophotometrically after centrifugation. Higher percentage of degradation of oil was shown by the fungal isolate MT 19 which was recorded as 76.9 %. Percent of degradation of MT 18 and MT 4 were recorded as 71.1 % and 65.3 % which was higher as compared to other isolates as tabulated in Table no.4

2.4. Biodegradation assay of hydrocarbon by isolated fungal cultures:

On the basis of biodegradation assay in terms of fungal biomass, it was resulted out that at concentration of 1% (v/v) oil in BH broth, MT 19 gave good results in terms of biomass, which was estimated out by their bulk mass of 0.460 gm followed by MT 18 and MT 4 with a biomass of 0.390 gm and 0.381 gm, tabulated at Table no. 5.

2.5.Morphological Identification:

On the basis of microscopic (lactophenol cotton blue staining) and macroscopic (culutural characteristics) examinations, three selected fungal strains MT 19, MT 18 and MT 4, fungal isolates were identified and tabulated in Table no.6. On the basis of cultural characteristics tabulated in Table no. 6, fungal isolates identified were MT 19= *Aspergillus flavus* , MT 18 = *Aspergfillus fumigates,* MT 4 = *Aspergillus nidulans* as shown in fig no. 1, 1.1, 2, 2.1, 3, 3.1.

III. DISCUSSION

For the isolation, screening and identification of oil degrading fungus, primary screening method was performed. According to R. Thenmozhi *et al.* [9] result of primary screening based on colony growth rate on selective meadia (MSM) amended with 1% used engine oil made it clear that the fungal isolates which yielded bigger colony diameter, heavy sporulation, and abundant aerial mycelium were considered as hydrocarbon degraders on their capability to utilize used engine oil for their growth [9]. Among 9 fungal isolates the highest average growth rate were observed for MT 19= *Aspergillus flavus* , MT 18 = *Aspergfillus fumigates,* MT 4 =

Aspergillus nidulans which were recorded as 3.85, 3.6, 3.2 cm/day, respectively. Mycelium of all these fungal isolates proliferated rapidly on MSM supplemented with 1% used engine oil forming heavy sporulation and dense hyphae. In the present study *Aspergillus flavus*, *Aspergfillus fumigates*, *Aspergillus nidulans* were better adapted to the culture condition in comparison to MT 17, MT 11, MT 8, MT 5, MT 1, MT 7 also plotted graphically in graph no. 1 and . Similarly R. Thenmozhi *et al.*, 2013 [9] reported JJF3 *Aspergillus niger* and JJF9 *Aspergillus luchiencis* for producing greater average colony diameter as 2.7 and 3.72 cm/day as compared to other fungal isolates.Similarly Sakineh *et al*., 2012 reported *Aspergillus niger* to show highest growth diameter of fungal mycelium in PDA media supplemented with 20% kerosene.

Confirmatory screening also known as shaking flask method in the present study depends on the principle of change in the colour of BH broth from blue to colourless of BH broth treated with fungal isolates, 0.1 % (v/v) Tween 80, 1% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol indophenols) [20]. This screening method is also known as redox indicator technique [20] which is userd to detect the ability of fungal isolates to degrade crude oil in the prence of redox indicator 2, 6- DCPIP. The main mechanism behind this degradation using redox reagent is that when we incorporate an electron acceptor such as DCPIP to the broth inoculated with fungi and supplemented with oil and tween 80 , it becomes possible to assess the ability of fungi to utilize the substrate by seeing the the colour change of DCPIP from blue (oxidized) to colurless (reduced) [21]. Tween 80 is an emulsifier which facilitates active contact between hydrocarbon and the isolate [20].Three indication which confirms the ability of fungi in biodegradation process is first, the change in colour of broth from dark blue to colourless, second is the the diasappearence of crude oil from rhe broth and third is developing a mass of fungal growth in the bottom of the broth [20].

Colour changing from blue to colourless during biodegradation process supports the fact that isolates are potential hydrocarbon oxidizers which can be measured as an absorbance value using spectrophotometer [22].The present investigation confirms the ability of MT 19*Aspergillus flavus*, MT 18 *Aspergfillus fumigates* and MT 4 *Aspergillus nidulans* to degrade crude oil by causing colour change from blue to colourless as shown in fig no.4, 4.1, 4.2, 4.3, 5, 5.1, 5.2, 5.3, 6, 6.1, 6.2, 6.3 after seven days of incubation in shaking incyubator.

Absorbance at a wavelength of 600 nm was recorded for fungal isolates because a peak in absorbance was observed at 600 nm as reported by Yoshida *et al*., 2001 [23]. According to Undugoda *et al*., 2016 [24] the higher fungal hydrocarbon degraders had low absorbance values after the incubation period as compared to the control whereas high absorbance values were shown by the low hydrocarbon degrading fungi compared to the control. In the present work, the lowest absorbance value was observed for *Aspergillus flavus* gave 0.12 OD, *Aspergfillus fumigates* gave 0.15 OD and *Aspergillus nidulans* gave 0.18 OD. Absorbnce values of all the fungal isolates are also plotted in graph no. 2. Saroj *et al*., 2013 [25] worked according to the same method applied for taking absorbance values of potential hydrocarbon degraders and reported that the bacterial isolate PS06 *Pseuodomonas sp*. gave 0.730 OD at 600 nm which was considered as the potential hydrocarbon degraders.

On the basis of absorbance value obtained percentage of degradation of each fungal isolate was calculated after applying the formula, according to which *Aspergillus flavus* gave higher percebtage of degradation which was calculated as 76.9 % which is also plotted graphically in graph no. 3.Saroj *et al*., 2013 [25] also calculated percentage of degradation of oil and reported *Fusarium sp.* which gave 42 % of naphthalene degradation.

Result of the present study in which *Aspergillus flavus* resulted out to be the most potent oil degrader agrees with the result of Shreyasri et al. [26] who reported *Aspergillus flavus* among those fungal species capable of degrading oil. Shamiyan *et al.*, 2015 also reported some fungal species which possessed higher degradation capacity among which *Aspergillus sp*. was also one which caused biodegradation of petroleum products [27]. According to Kapoor *et al.,* 1999 [28] large number of studies have been reprted from Nigeria on the biodegradation of hydrocarbons using strains of *Aspergillus*.

R. Thenmozhi *et al.*, 2013 [9] reported *Aspergillus niger* and *Aspergillus luchiencis* which showed promising result for hydrocarbon degradation.

According to Hussein *et al*., 2012 [21] developing a mass of fungal growth in the bottom of culture medium supplemented with oil which is one of the indication of those fungi which are capable of degrading fungi. Therefore the biodegradation assay of the selected fungal strains in terms of fungal biomass was also performed according to which it was found out that at concentration of 1% oil, *Aspergillus flavus* gave good results in terms of biomass, which was estimated by their bulk mass of 0.460 gm followed by *Aspergfillus fumigates* and *Aspergillus nidulans* with a biomass of 0.390 gm and 0.381 gm also plotted graphically in graph no…. Saroj *et al*., 2013 [25] reported *Rhizopus sp.* which gave good result in terms of bulk mass of 0.467 gm followed with *Fusarium sp.* with a biomass of 0.385 gm which proved to be potential degraders of hydrocarbon.

IV. FIGURES, GRAPHS AND TABLES

4.1.Figures:

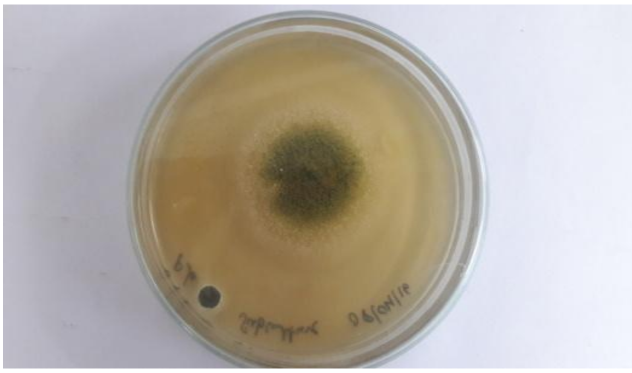

Fig No.1 MT 4. Aspergillus nidulans

Fig No. 1.1 MT 4. Aspergillus nidulans

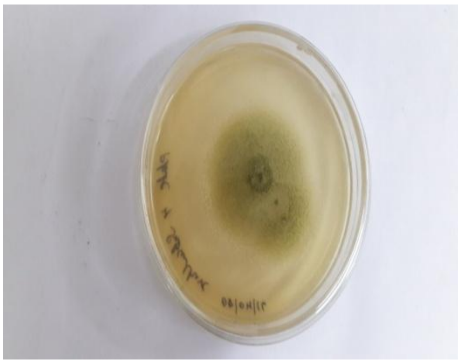

Fig No.2 MT 18 *Aspergillus fumigates*

Fig No. 2.1 MT 18 *Aspergillus fumigates*

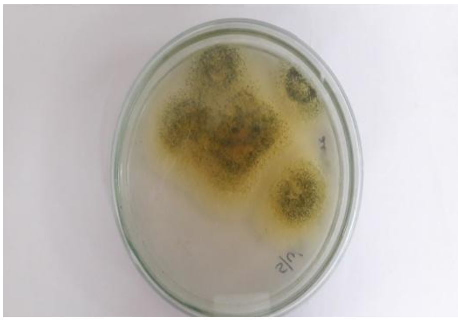

Fig No.3 MT 19 *Aspergillus flavus*

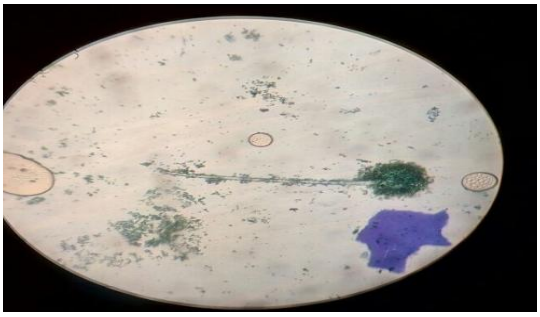

Fig No. 3.1 MT 19 *Aspergillus flavus*

Fig 4 showing left flask as control without fungus and right flask containing BH broth, fungal isolates, 0.1 % (v/v) Tween 80, 1% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol indophenols)

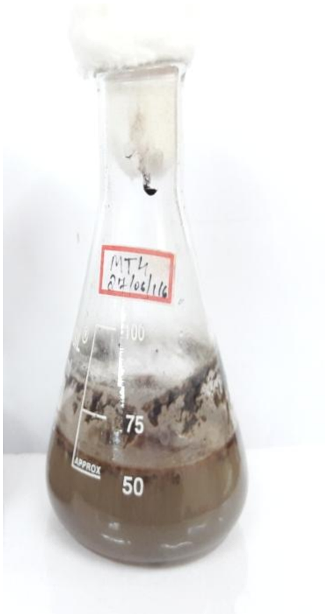

Fig 4.1 showing change in colour of BH broth MT 4 from deep blue to colourless after biodegradation.

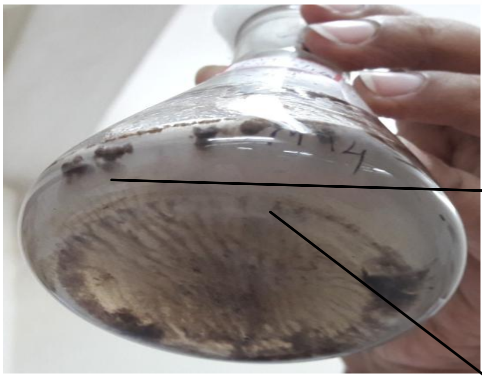

Fig 4.2 oil biodegradation by *Aspergillus Nidulans.*

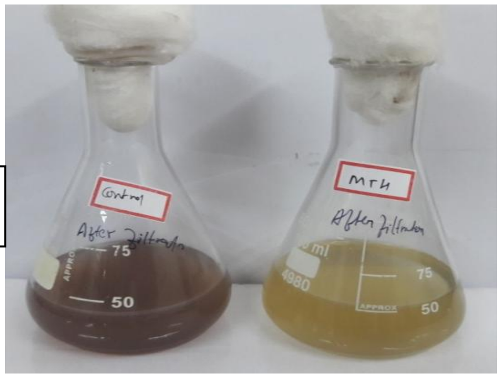

Fig 4.3 biodegraded MT 4 after filteration.

Fig 5 showing left flask as control without fungus and right flask MT 18 containing BH broth, fungal isolates, 0.1 % (v/v) Tween 80, 1% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol

.

Fig 5.1 showing change in colour of MT 18 from dark blue to colourless after biodegradation

Fig 5.2 biodegradation of oil

by *Aspergillus fumigates.*

Fig 5.3 biodegraded MT 4 after filteration.

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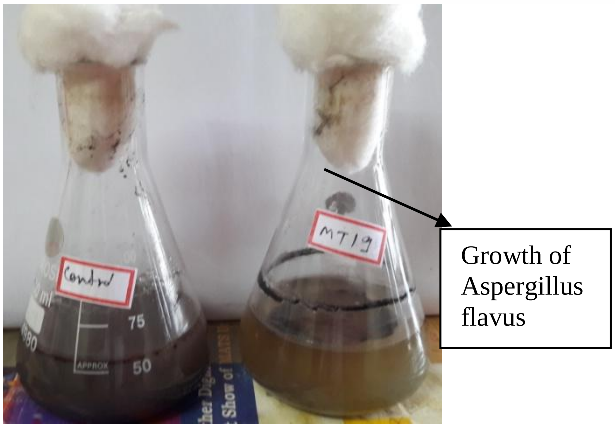

Fig 6 showing left flask as control without fungus and right flask MT 19 containing BH broth, fungal isolates, 0.1 % (v/v) Tween 80, 1% (v/v) crude oil and redox reagent (2% 2, 6- dichlorophenol indophenols) before biodegradation

Fig 6.1 showing change in colour of MT 19 from dark blue to colourless after biodegradation.

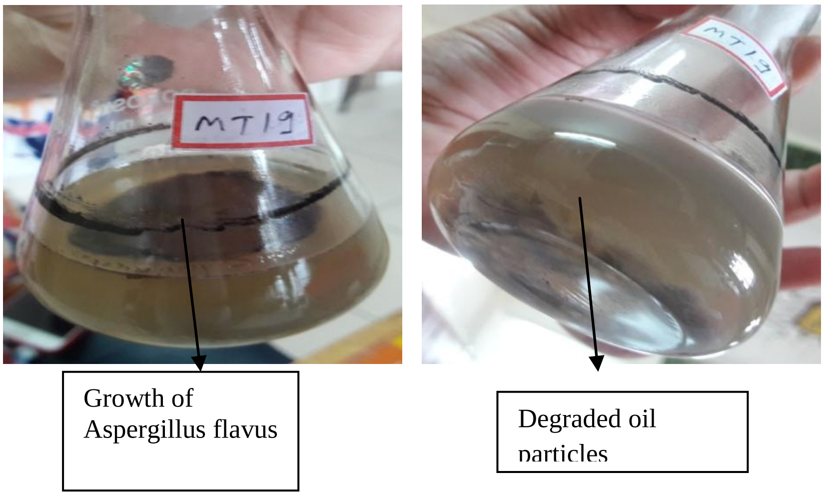

Fig 6.2 biodegradation of oil by Aspergillus fumigates.

Fig 6.3 biodegraded MT 19 after filteration.5.2 Graphs:

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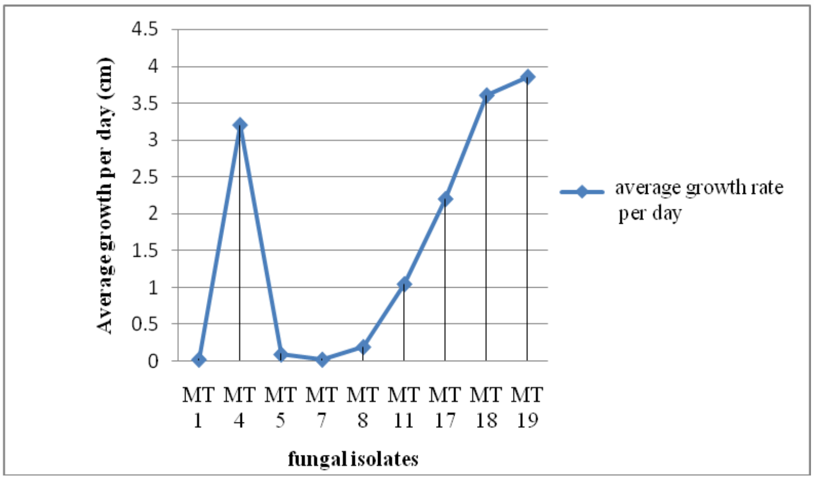
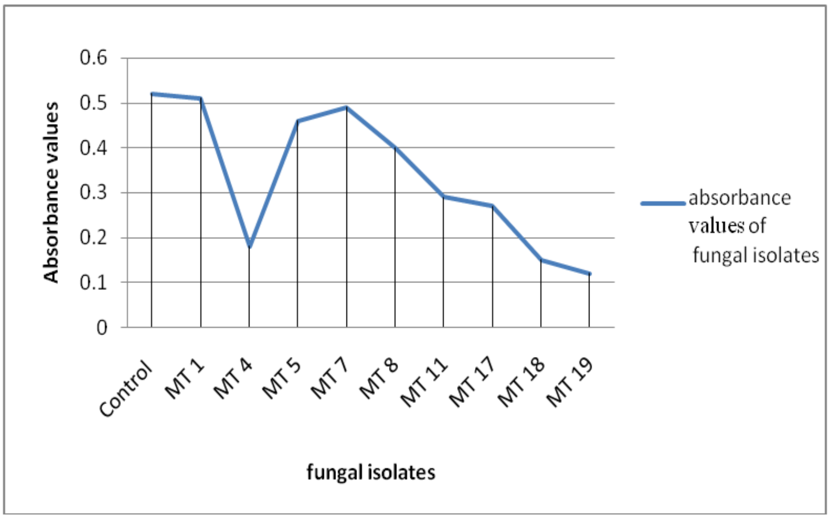
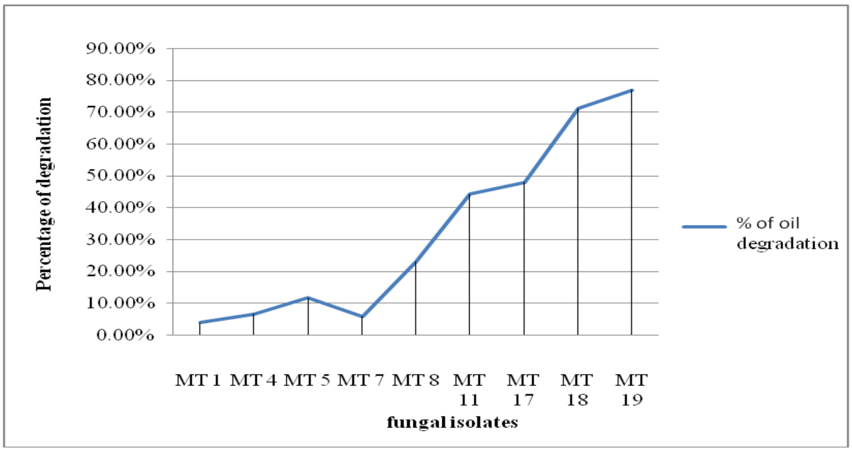


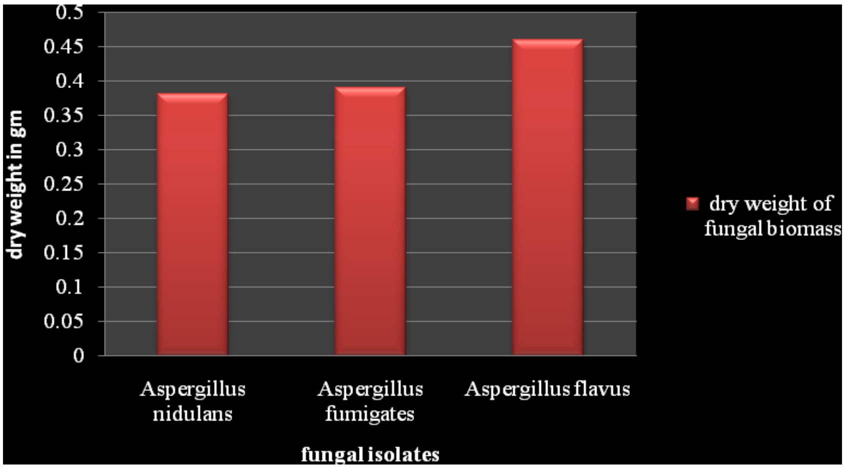
Graph no. 1 showing average growth rate of mycelium per day in MSM media



Graph no. 2 showing absorbance values of fungal isolates at 600 nm.



Graph no. 3 showing percentage of oil degradation of fungal isolates.



Graph no. 4 showing fungal biomass of *Aspergillus nidulans*, *Aspergillus fumigates*, *Aspergillus flavus* 5.3

Tables:

Table no. 1: Average growth of fungal isolates per day

S.No.	Fungal isolates	Control (cm)	Plate I (cm)	Plate II (cm)	Average growth per day
1.	MT 1	4	0.3	0.2	0.03
2.	MT 4	2	3	3.4	3.2
3.	MT 5	1.5	0.1	0.1	0.1
4.	MT 7	4	0.2	0.3	0.03
5.	MT 8	0.5	0.1	0.3	0.2
6.	MT 11	0.5	1.2	0.9	1.05
7.	MT 17	1.2	1.9	2.5	2.2
8.	MT 18	1	4	3.2	3.6
9.	MT 19	0.5	4.1	3.6	3.85

Table no. 2 DCPIP test of fungal isolates for colour change

S.No.	Fungal isolates	Colour change (blue to colourless)
1.	Control	-
1.	MT 1	-
2.	MT 4	+
3.	MT 5	-
4.	MT 7	-
5.	MT 8	-
6.	MT 11	-
7.	MT 17	-
8.	MT 18	+
9.	MT 19	+

Table no. 3 absorbance values of fungal isolates

S.No.	Fungal isolates	Optical density (at 600 nm)
1.	Control	0.52
2.	MT 1	0.50
3.	MT 4	0.18
4.	MT 5	0.46
5.	MT 7	0.49
6.	MT 8	0.40
7.	MT 11	0.29
8.	MT 17	0.27
9.	MT 18	0.15
10.	MT 19	0.12

Table no. 4: percentage of oil degradation of fungal isolates:

S.No.	Fungal isolates	Percentage of degradation
1.	MT 1	3.84%
2.	MT 4	6.53 %
3.	MT 5	11.5%
4.	MT 7	5.76 %
5.	MT 8	23%
6.	MT 11	44.2%
7.	MT 17	48%
8.	MT 18	71.1%
9.	MT 19	76.9%

Table no. 5: dry weight of fungal isolates

S. No.	Fungal isolates	Macroscopic and microscopic characteristics	Fungal species
1.	MT 4	Green colony, conidial heads are short, columnar. Conidiohores stripes are short, brownish and smooth walled. Conidia are globose and rough walled.	*Aspergillus nidulans*
2.	MT 18	Bluish green colony, conidial heads are typically columnar. Conidiophore stipes are short, smooth walled . conidia globose to subglobose, green and finely roughned.	*Aspergillus fumigatus*
3.	MT 19	Green colony, conidial heads were typically radiate, conidia were globose to subglobose, pale green.	*Aspergillus flavus*

Table no. 6: macroscopic and microscopic characyeristics:

S.No.	Fungal isolates	Dry weight of fungal Biomass (gm)
1.	*Aspergillus nidulans*	0.381
2.	*Aspergillus fumigates*	0.390
3.	*Aspergillus flavus*	0.460

VI. CONCLUSION

This study has revealed that the higher biodegradation efficiency was shown by *Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus nidulans* isolated from the oil contaminated soil of Modha para automobiles workshop.

Under this investigation among nine pure fungal isolates isolated from soil, *Aspergillus flavus* gave higher percentage of degradation of oil which was recorded as 76.9 %, *Aspergfillus fumigates* and *Aspergillus nidulans* gave 71.1 % and 65.3 %. Thus in future they can be effectively utilized for the degradation of oil and petroleum products as well as for biodegradation of soil already polluted or contaminated with oil especially those located nearby the oil refinery plant, petroleum processing and disposing sites.

VII. ACKNOWLEDGEMENTS

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