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Studies on Mycoremediation of used engine oil contaminated soil samples

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ABSTRACT

The used engine oil contaminated soil samples were collected from various automobile workshops in Pudukkottai, Tamilnadu, South India. From the collected samples indigenous fungi were isolated using Malt extract medium. Totally sixteen isolates were isolated among which Aspergillus and Rhizopus sp were found to be predominant. The morphological and microscopical studies were done. Morphological studies of isolated fungal isolates showed different growth pattern on different media. Preliminary hydrocarbon degradation analyses were done to evaluate the growth diameter of fungal colonies on Minimal media supplement with used engine oil. Turbidity assay using Minimal medium was done to confirm the hydrocarbon degradation. The fungal isolates JJF3 and JF9 showed better results; hence they were selected for further analysis. The gravimetric analysis revealed that the both isolates degraded used engine oil 40.5% and 51.6% respectively at 30 days. The FT-IR spectrum of control and treated samples with fungal isolates revealed differences in band formation indicating microbial oxidation of hydrocarbons. When conditions such as pH requirement and nutrient availability are taken into consideration during a bioremediation project, the rate of microbial degradation could be conveniently achieved within a much shorter time than even what was obtained in this study. The rigours of bioaugumenting contaminated soils for the purpose of bioremediation can be avoided and effective remediation still achieved if suitable conditions that will enhance indigenous microbial activities for optimum degradation are satisfied.

Key words: Fungal isolates, Used engine oil, Bioremediation

INTRODUCTION

Petroleum like all fossil fuels primarily consists of a complex mixture of molecules called hydrocarbons. In large concentrations, the hydrocarbon molecules that make up crude oil and petroleum products are highly toxic to many organisms, including humans [2]. Used motor oil contains metals and heavy polycyclic aromatic hydrocarbons that could contribute to chronic hazards including mutagenicity and carcinogenicity [9, 12, 18]. Prolonged exposure to high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow, and an increased risk of cancer [20, 22, 27]. The most rational way of decontamination of the environment loaded with petroleum derivatives is an application of methods based mainly on metabolic activity of micro organisms [19]. However, single cultures of fungi have been found to be better than mixed cultures [26] and more recently, fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria [7]. The filamentous fungi possess some attributes that enable them as good potential agents of degradation, once those microorganisms ramifies quickly on the substratum, digesting it through the secretion of extracellular enzymes. Besides, the fungi are capable to grow under environmental conditions of stress, for example: environment with low pH values or poor in nutrients and with low water activity. In accordance with several scientific publications, can be pointed out that, amongst the filamentous fungi *Trichoderma* and *Mortierella spp* are the most

common ones isolated from the soil. *Aspergillus* and *Penicillium spp* have frequently been isolated from marine and terrestrial environments. The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (such as incineration, thermal desorption, extraction) [6]. More so, their utilization is a gentle non-aggressive approach. The application of bioremediation capabilities of indigenous organisms to clean up pollutants is viable and has economic values [8]. The objectives of this study were therefore, to isolate and identity some of the indigenous fungal flora of oil contaminated soils and evaluate the biodegradation efficiencies of the potent isolates.

MATERIALS AND METHODS

Microbial isolation and growth condition

The fungal strains were isolated from used motor oil contaminated soil samples using standard pour plate method. one hundred micro liters of used motor oil , and 0.1 g of the top five centimeters of motor oil contaminated soil were poured in to a separate empty Petri dishes, respectively .The autoclaved Malt extract agar was then cooled down to 50 °C, and poured on to the samples in Petri dishes. The Petri dishes were immediately swirled to ensure adequate mixing. Five replicates were prepared for each sample and incubated at 27 °C for a period of three days after which fungal growth were observed. Each pure fungal isolate was then subcultured on to a fresh Sabouraud agar medium containing 50 mg/ml Ampicillin in order to prevent bacterial contamination.

Morphological identification

All the pure fungal isolates were grown on different media (Potato Dextrose Agar, Czapek Dox Agar ,Sabouraud Agar) plates for a period of 7 days at 27 °C .The isolated fungal strains were identified by visual observation and micro-morphological techniques [25]. According to the general principles of fungal classification based on the nature of mycelium and growth patterns. In addition, the fruit bodies of these isolated fungi that contained the sporangium and spores were harvested and stained using acid Fuschin followed by morphological examination under light microscope.

Preliminary screening of hydrocarbon degrading fungal isolates

All pure fungal isolates were screened for their ability to utilize used motor oil by visual assessment of the fungal growth on selective media. The fungal isolates were grown according to Santos *et al*. [29] in minimal salt medium containing g/l : KH_2PO4 0.05, $(NH4)_2SO4$ 0.05 , MgSO4.2 H2O 0.05 and agar 15, adding 1% v/v of filtered sterile used motor oil .The oil agar plates were then inoculated with $1cm^2$ mycelial plug from each of the pure culture followed by incubation at 27 ºC for a period of 7 days. The fungal isolates were also grown on minimal media that served as control. All the experiments were carried out in triplicates. The growth rates of the fungal isolates were recorded daily by measuring the diameter of the radial extension of the mycelium .The average growth of the diameter of growing colony was determined by measuring atleast two diameter per plate .The average growth diameter at that particular time of measurement (cm/day) was calculated by the regression of the colony diameter against the day after inoculation [29]. The fungal isolates that yielded heavy sporulation, greater colony diameter or more abundant aerial mycelium on plates containing used motor oil were selected for further examination.

Confirmatory Test for Hydrocarbon Utilization Potentials of the Isolated Fungi

The enrichment procedure as described by Nwachukwu [14] was used in the estimation of hydrocarbon utilizers. A minimal salt broth (MSB) containing 2.0g of NA2HPO4, 0.17g of K2SO4, 4.0g of NH4NO3, 0.53g of KH2PO4, 0.10g of MgSO4, 7H2O and 5.0g of agar – agar dissolved in 1000ml of distilled water was prepared. The solution was sterilized by autoclaving. Twenty-eight test tubes were sterilized and placed in test tube racks, where there were four test tube racks containing seven test tubes each. 10ml of the minimal salt broth was measured into each of the test tube. 2ml of either crude-oil or diesel or engine oil or spent engine oil or the seed oil extract was measured and added to 10ml of minimal salt broth in the first 6 test – tubes in each rack respectively with the exception of the last test – tubes, that is, the seventh test tube in each rack, making 12ml in twenty – four test tubes and 10ml of only minimal salt broth in four test tubes which served as controls. The isolated fungal strains from soil were added to the test tubes in three racks with the test tubes in the last rack serving as control without fungi. Each of the test tubes was plugged with sterile cotton wool wrapped with aluminium foil so as to ensure maximum aeration and prevent cross – contamination. All the test tubes were then incubated at room temperature (28 °C - 31 °C) for 40 days. The test tubes were shaken constantly throughout the duration of the experiment to facilitate oil-cell phase contract. The ability to degrade the petroleum products (based on the growth rate of the organisms in the MSB medium) was measured every 5days using the visual method which is based on the turbidity of the MSB using the photoelectric colorimeter. The experiment was carried out twice.

Degradation studies and Hydrocarbon analysis

The potential organisms were subjected to gravimetric assay. The fungal isolates (JJF3,JJF9) cultivated in a SDA for 7 days, then 1×1cm mycelial plugs was inoculated in Czapeck dox broth with 1 % v/v used motor oil in a 250 ml Erlenmeyer flask . Control experiments containing same medium without addition of 1% (v/v) used motor oil .The flasks were agitated at 200 rpm at room temperature the used motor oil experiment was carried for a period of 30 days and the content of the each test including the control were harvested and subjected to hydrocarbon analysis by FT-IR analysis.

RESULTS AND DISCUSSION

In this study, sixteen pure fungal isolates with different morphological characteristics has been successfully isolated from the five different types of soil samples contaminated with used engine oil. Table.1. listed all the fungal isolates that were successfully grown, identified and characterized morphologically. The fungal isolates showed difference in morphological appearance, pigmentation and sporulation in different media. Based on the macroscopic and microscopic morphological characteristics, the sixteen fungal isolates were belonging to the genera *Rhizopus sp* and *Aspergillus sp* respectively. All of the pure fungal isolates were sequentially subcultures on to SDA for an incubation period of 7 days, when the mycelia started to grow and formed on the surface of SDA. Among all the fungal isolates obtained *Aspergillus* species were the most common fungi that were successfully isolated in all the samples. The filamentous fungi can grow on hydrocarbons with *Aspergillus sp* were being the most frequently reported [5,10,11].

Colony growth rate evaluation a selective media have been extensively used to investigate the growth of filamentous fungi in most research studies on screening and isolating the hydrocarbon and their derivatives degrading fungi [4,21,28,29,30]. Also, Okerentugba and Ezeronye [26] demonstrated that *Penicillium spp., Aspergillus spp.* and *Rhizopus spp*. were capable of degrading hydrocarbons especially when single cultures were used. Batelle [7] showed that fungi were better degraders than traditional bioremediation techniques including bacteria. Also, the ability of the white-rot fungus – *Pleurotus tuberregium* to ameliorate crude oil polluted soil has been reported by Isikhuemhen *et al.* [15].

All the fungal isolates were screened for their ability to degrade the used motor oil based on their average growth rate by calculating the diameter of radial extension of the fungal mycelium, on minimal media amended with 1% (v/v) used motor oil (Table. 2). The highest average growth rates were observed for JJF3 and JJF9 recorded as 2.7, 3.72 cm/day, respectively. In addition, the mycelium of these isolates grew rapidly on the minimal media forming dense hyphal matrix and became fimbriate. Black exudates were also observed on the fungal mycelium. Meanwhile, another two fungal isolates JJF8 and JJF6 also demonstrated high average growth rate (cm/day) among all the fungal isolates. The average growth rate of and are recorded as 0.90 and 1.0 cm/day, respectively. The mycelium of both spread rapidly on the minimal media amended with 1% (v/v) used motor oil. The remainder fungal isolates demonstrated poor average growth of between 0.14 to 0.79 cm/day. The results observed by Husaini *et al.* [13] coincides the present observation. The fungal isolates that yielded heavy sporulation, greater colony diameter, and more abundant aerial mycelium formation were selected for further analysis on their capability to utilized used motor oil. In this study JJF3 and JJF9 appeared to be adapted better to the culture condition compared to the other fungal isolates were selected for further analysis.

The growth pattern of fungi in minimal salt solution is represented on Table.3. It shows that the growth rate of each fungus had different maximum growth peaks. There was fluctuation in the growth of each fungus. JJF3 and JJF9 had the highest growth rates on the 30th days with optical densities 1.03, 0.91 respectively. There was a marked decrease in the growth rates of JJF3 and JJF9 on the every 10 days. Remaining fungal isolates showed least optical densities. This was probably due to the difference in growth rates of each fungus with each fungi attaining a maximum growth peak and declining after some days, probably as a result of exhaustion of nutrients and release of toxic materials into the medium. Similar studies were done by Adekunle and Adebambo [1]. In addition, the black color of the crude oil and spent engine oil samples resulted in the maximum optical density of 1.0 possessed by both samples at the beginning of the experiment. Therefore, the measure of degradation was indicated by a lightness in colour (as the hydrocarbon molecules were broken down) which was equivalent to a reduction in optical density. Therefore, the organism that gave the lowest optical density was taken as the best degrader of both crude oil and spent engine oil in minimal salt broth respectively.

In this study the fungi isolate had increased growth rates in the media containing petroleum and petroleum products compared to only when minimal salt broth was used. This might be due to the fact that the fungi isolated were able to use the hydrocarbons as substrates for growth by probably releasing extra cellular enzymes and acids which are capable of breaking down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and

carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth and nutrition of the fungi. After being completely broken down, the reaction releases Carbon (IV) oxide, water and energy used to create cellular biomass [17]. However, it must be noted that there were also nutrients present in the minimal salt broth though more of it could have been present in the oil which stimulated the growth of each fungus. In view of this then, the additional nutrients present in the minimal salt broth helped in overcoming nutrient limitation to microbial growth to a certain extent and also helped in creating a favourable environment for the rapid development of the fungi especially at the times when the fungi had not started breaking down the hydrocarbons into simpler molecules.

Since the fungal isolates JJF3 and JJF9 showed promising result in preliminary and confirmatory test for hydrocarbon degradation, they were selected for gravimetric and FT-IR analysis. The gravimetric analysis of JJF3 and JJF9 indicated that they have degraded the used engine oil 40.5% and 51.6% respectively, after 30 days incubation. The ability of selected fungal isolated in degrading used motor oil also revealed by swollen hyphae. In previous studies similar results were shown by Husaini *et al*. [13] coincides the present work.

Table.1. Sampling locations and the isolated Fungal isolates

Fungal Isolates	OD at 570-700 nm					
	10th day		20th day		30th day	
	M+F	O+F	M+F	O+F	M+F	O+F
JJ F3	**0.19**	**1.24**	**0.26**	**1.09**	**0.09**	**1**
JJ F5	0.6	0.57	0.2	0.37	0.26	0.56
JJ F6	0.36	1.02	0.17	0.81	0.14	1.25
JJF8	1	0.66	1.03	0.49	1	0.68
JJF9	**0.19**	**1.19**	**0.26**	**1**	**0.09**	**0.91**
JJF10	0.41	0.53	0.52	0.65	0.71	1.32
JJ F11	1	1.41	0.54	0.71	0.8	0.79
JJ F13	0.02	0.29	0.4	0.54	1.34	0.82
JJF14	0.15	0.2	0.24	0.48	0.3	0.69
JJ F16	0.36	1.02	0.17	0.81	0.14	1.25

Table.2. Average growth rate (cm/day) of fungal isolates on Minimal media after 7 days

Sl. No.	Soil samples	Fungal isolates	Identified species
1	Sampling site 1	JJ F1	*Aspergillus candidus*
		JJ F2	*Aspergillus terrus*
		JJF 3	*Aspergillus niger*
2	Sampling site 2	JJ F4	Sterile mycelium
		JJ F5	*Rhizopus nigaricans*
		JJ F6	*Aspergillus terrus*
		JJ F7	*Rizopus sp*
		JJ F8	*Aspergillus niger*
3	Sampling site 3	JJ F9	*Aspergillus luchiencis*
		JJF 10	*Aspergillus niger*
		JJF11	*Aspergillus sp*
4	Sampling site 4	JJ F12	*Aspergillus fumigates*
		JJF13	*Aspergillus ochraceous*
		JJ F14	*Aspergillus niger*
5	Sampling site 5	J JF 15	*Aspergillus niger*
		J JF16	*Aspergillus sp*

The FT-IR analysis of untreated used engine oil revealed bands 3902 cm^{-1}, 3787.97 cm^{-1} and 3432.72 cm^{-1} indicating the presence of hydroxyl stretching, 2932.57 cm^{-1} signifying to -C-H stretching ,2349.16 cm^{-1} corresponding to C-O stretching; 1617.45 cm^{-1} indicates carbohydrates and at 729.62 cm^{-1} showed presence of alkyl halides (Fig.1). The FT-IR spectrum of treated sample with JJF3 after 30 days of microbial incubation indicated that formation of new bands at 1288 cm^{-1} corresponding C=O in carboxyl esters and at 667.0 cm^{-1} (Fig.2). However, the FT-IR microbial oxidation of used engine oil by JJF9 spectrum indicated the new band formation at 674.6cm^{-1} (Fig.3). After 30 days the high degradation of hydrocarbon compounds by the microbial incubation, maximum bands observed after treatment with the fungal strains. Similar studies by Mohammad Yunus *et al*. [24] strongly supports the present work.Moreover, FTIR spectroscopy can 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[23]. Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil. Although some studies have reported their removal at high rates under optimal conditions, high molecular weight aromatics, resins and asphaltenes are generally considered to be recalcitrant or exhibit only low

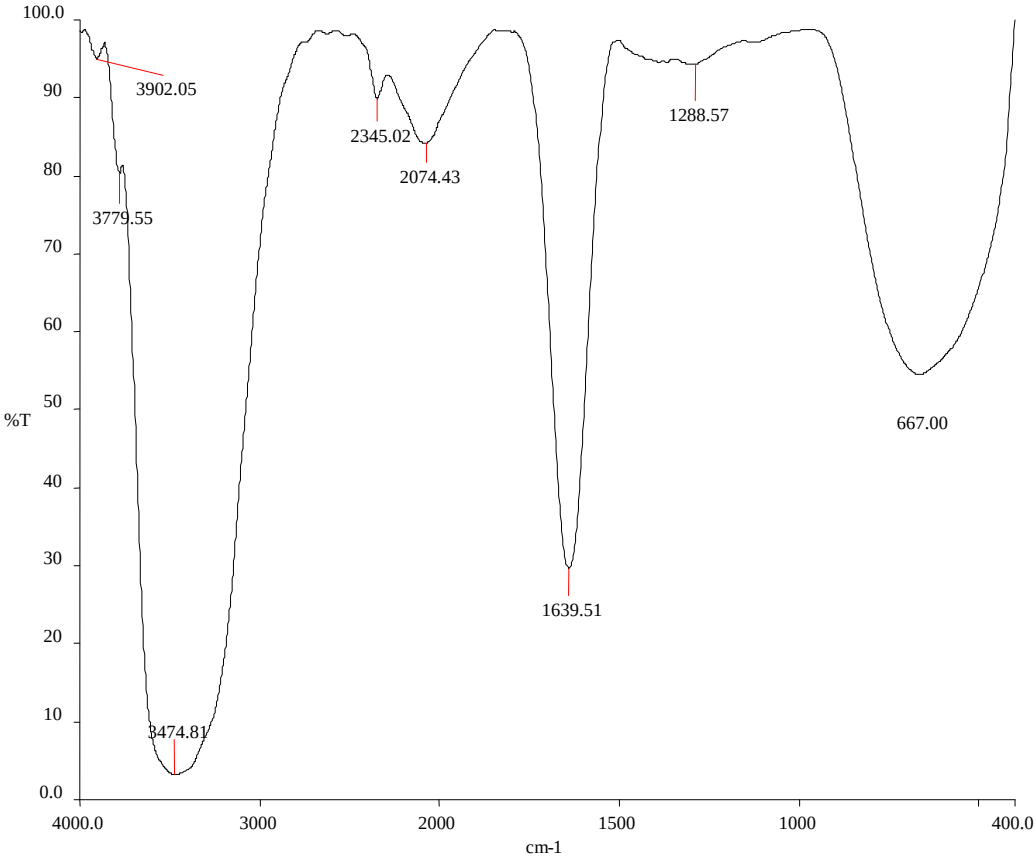
rates of biodegradation. Amund and Akangbou [3] showed that crude oil fractions with lower amount of saturated hydrocarbons were more resistant to microbial degradation than the fraction(s) containing higher amount(s) of saturated hydrocarbons.

Table.3. Growth pattern of fungal isolates in Minimal salt broth

Sl. No.	Fungal isolates	Control cm	Plate 1 cm	Plate 2 cm	Average growth per day
1	JJ F3	2	3.5	4	**3.72**
2	JJ F5	3	0.9	0.9	0.9
3	JJ F6	1	2	2	2
4	JJF8	0.6	0.8	0.6	0.7
5	JJF9	1	3.2	2.2	**2.7**
6	JJF10	1.6	0.8	0.8	0.8
7	JJ F11	1.5	1.5	1.2	1.3
8	JJ F13	0.5	0.4	0.3	0.3
9	JJF14	0	1	0.8	0.9
10	JJ F16	0.5	0.6	0.7	0.6

M-Media, F-Fungal isolate, O- used engine Oil

FIGURE .2. FTIR SPECTRUM OF TREATED SAMPLE BY *Aspergillus niger*

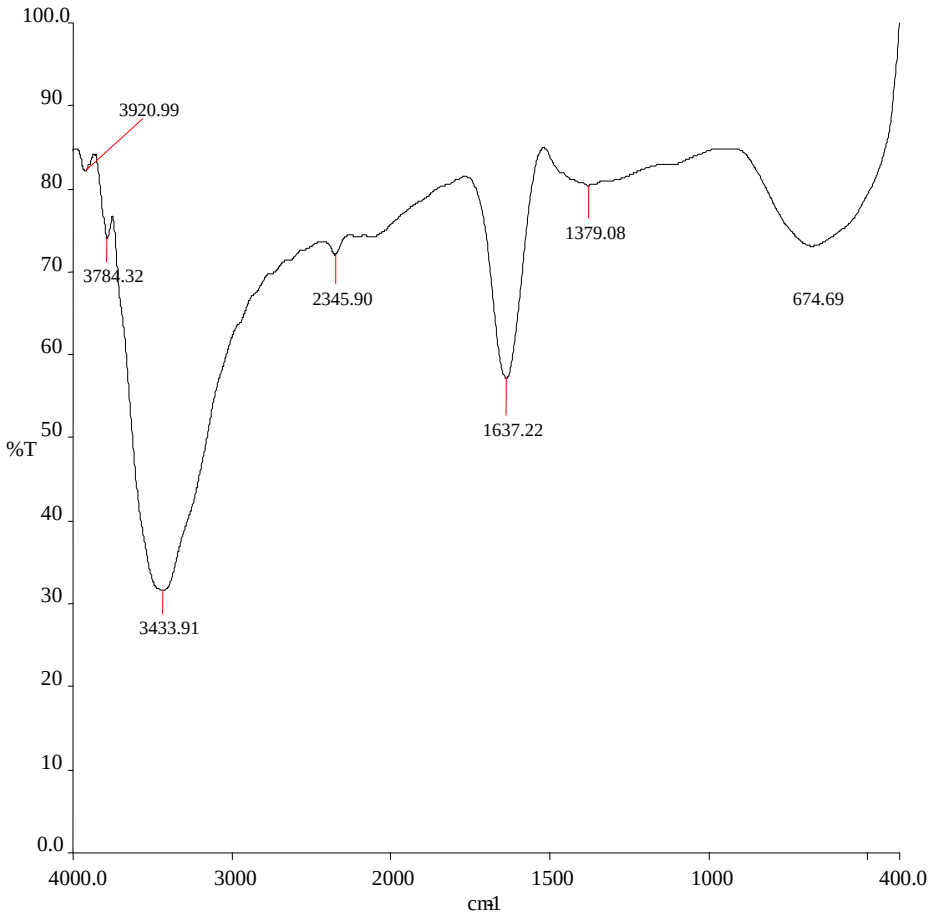


L-Sample-A.pk

L-SAMP~3.SP 1801 4000.00 400.00 3.17 100 4.00 %T 15 1.00

REF 4000 98.49 2000 87.06 600
3902.05 94.99 3779.55 80.27 3474.81 3.17 2345.02 89.92 2074.43 84.21
1639.51 29.70 1288.57 94.23 667.00 54.55

FIGURE .3. FTIR SPECTRUM OF TREATED SAMPLE BY *Aspergillus Sp.*

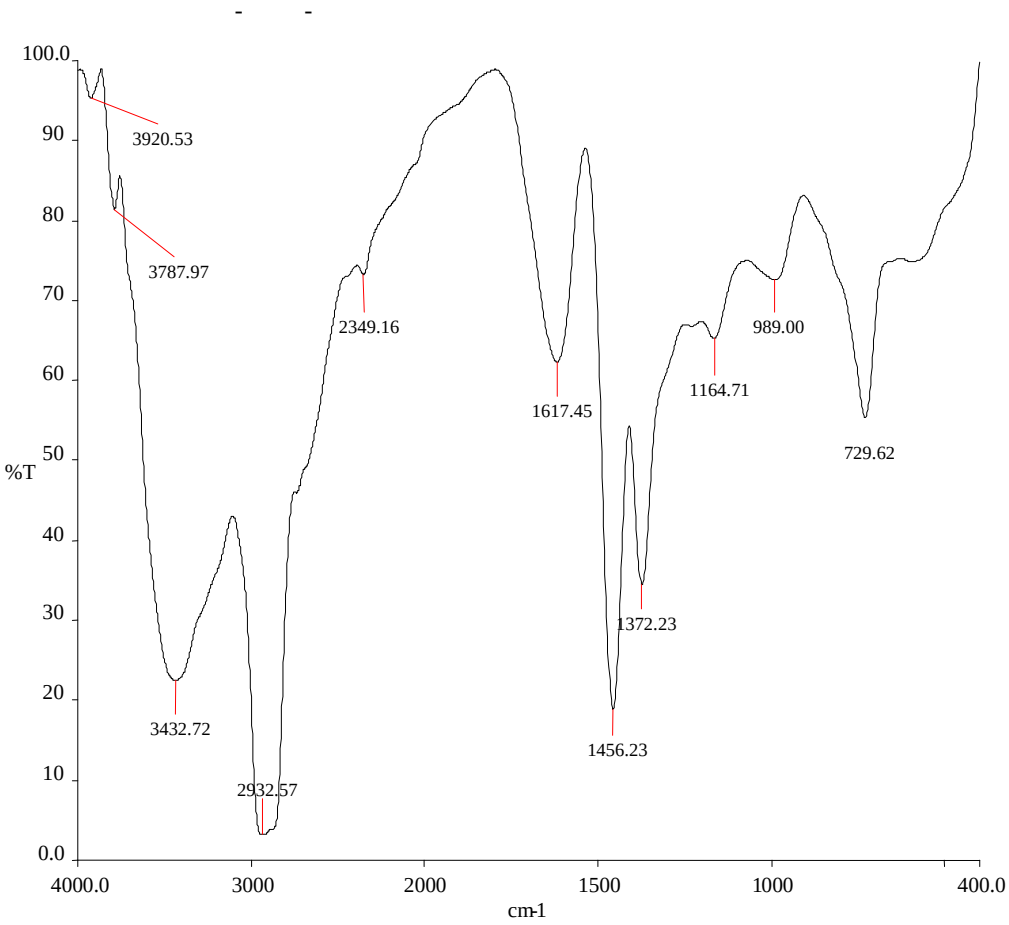


L-Sampl€.pk

L-9B61~1.SP 801 4000.00 400.00 31.62 100.00 4.00 %T 15 1.00

REF 4000 84.67 2000 75.73 600
3920.99 82.06 3784.32 74.06 3433.91 31.62 2345.90 72.07 1637.22 57.17
1379.08 80.39 674.69 73.09

FIGURE .1. FTIR SPECTRUM OF UNTREATED SAMPLE



L-SampleF.pk

L-AB6D~1.SP 1801 4000.00 400.00 3.17 99.88 %T 1.00 1.00

REF 4000 98.72 2000 90.67 600
3920.53 95.25 3787.97 81.42 3432.72 22.48 2932.57 3.17 2349.16 73.19
1617.45 62.30 1456.23 18.86 1372.23 34.46 1164.71 65.22 989.00 72.54
729.62 55.33

CONCLUSION

In conclusion, the result here shows that fungi isolated from the used engine oil contaminated soil samples can be exploited in the biodegradation of crude petroleum oil spill and bioremediation of the environment.

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