# Evaluation of bioremediation efficacy of *A.flavus* HQ010119 and *Pencillium sp*. KJ415574.1 strains in used engine oil degradation

**Sriraksha H G, Drishya T D, Durgashree C, Sisira Anil, Manjunatha B K*, Praveen Kumar S V and Valarmathy K**

Department of Biotechnology, The Oxford College of Engineering, Hosur Main Road, Bommanahalli, Bengaluru, 560068, Karnataka, India

**Abstract:** In the present paper, we report the hydrocarbon degrading ability of *A.flavus* HQ010119 and *Pencillium sp*. KJ415574.1 fungal strains in used engine oil. Parameters viz., %Total petroleum hydrocarbon (TPH) analysis, BOD,COD analysis and ability to produce biosurfactant were assessed. Among the strain tested, *A.flavus* was able to degrade 50% of hydrocarbons followed by *Pencillium sp*. 46.42% degradation after 20 days of incubation.

Keywords: Used engine oil, TPH, fungal isolates, biosurfactant

## INTRODUCTION

The increase in the consumption of petroleum fractions has led to the rapid increase in the pollution of soil by used motor oil (UMO). The environment (soil and water) is highly contaminated with hydrocarbons by the disposal of used oils (engine oil, diesel or jet fuels). In today's world, oil spills at auto-mechanic workshops have been left uncared for over the years in many countries, and continuous accumulation of the oil is of high environmental concern as a result of hazard associated with it[Abdulsalam *et al.*,2012]. The attention of researchers have shifted towards the remediation of the environment (soil and water) polluted with hydrocarbons especially the polycyclic aromatic hydrocarbons (PAHs) due to the fact that most of the PAHs causes cancer, gene mutation and are very toxic [Clemente *et al.*,2001].PAHs are toxic, carcinogenic and mutagenic so their presence in environment is of great concern and has deleterious effect on human health. The Release of persistent, bioaccumulative and toxic chemicals (benzene, toluene, ethylbenzene, xylene and polycyclic aromatic hydrocarbon) cause health and environmental hazards. These pollutants find their way into plant tissues, animals and human beings by the movement of hazardous constituents in the environment [Ebenezer, 2013]. Soil polluted with spent and fresh motor oil also create a serious effect on plant tissues, soil components, and its microorganisms, human and other animal health [Stephen, E. *et al.*,2011]. Excess spillage of the oil causes fire hazards which lead to loss of lives and properties

Bioremediation is the naturally occurring process by which microorganisms transform environmental contaminants into harmless endproducts, in order to obtain the sources of carbon and energy. During the process of bioremediation, which involves the activity of microorganisms to remove pollutants, environmental parameters such as temperature, pH, oxygen and moisture content, are optimized to achieve accelerated biodegradation. Basically, there are two different approaches to bioremediation technologies, depending on the pollution situation and type of micro-organisms being used. The first is the one which involves the activation of the indigenous microflora in the polluted area by addition of nutrients and forming the best conditions of other chemical, physical and biological factor, or known as biostimulation. The second (bioaugumentation) is the one which involves the addition of oiloxidizing micro-organisms isolated from other sites, or addition of genetically engineered micro-organisms [Amund O *et al.*, 1987]

Although many species of bacteria and algae have been found to be efficient in degradation of low molecular weight hydrocarbons, for degradation of high molecular weight hydrocarbons, fungal species are preferred (Potin *et al.*, 2004). This is because use of fungi is economical since they grow on inexpensive substrates like forest and agricultural wastes. Also, fungi have the ability to produce many extracellular enzymes that can degrade a range of hydrocarbons (Vanishree *et al.*, 2014) and they can produce sufficiently large quantities of biosurfactant, which help in increasing the rate of biodegradation.

## MATERIALS AND METHODS

### 3.1 Sample collection and Isolation of fungi:

The soil samples were collected from different localities of Western Ghats of Karnataka State, covering the oil spilled areas and the hydrocarbon degrading fungi were isolated using R2A media followed by serial dilution against protocols.

### 3.2 Hydrocarbon utilization studies:

Equal numbers of Erlenmeyer flasks (50/150ml) were taken, to which artificial seawater and Full strength media were added (Austin, 1993). All the flasks were autoclaved and cooled to room temperature. To each of the flasks, 2% test hydrocarbon was added. The test hydrocarbon was used Engine oil. Finally, a loop full of the pure fungal colony was inoculated into the media in aseptic conditions.

**3.2.1 Biomass estimation:**

After the incubation period, the biomass of inoculated strain is expected to increase significantly, which would indicate that the strain has the potential to use the test hydrocarbon as energy source, and thus multiply in number. To determine the increase in biomass, the flask weight was taken before and after incubation period.

**3.2.4 Gravimetric method of % TPH analysis:**

After incubation period, the concentration of residual hydrocarbons in the flasks was evaluated using gravimetric method (Al-Nasrawi, 2012; Ijah *et al.*, 1992; Bartha *et al.*, 1984). Each sample was added to separating funnel along with 10ml of petroleum ether, and shaken thoroughly. It was then allowed to settle and the organic phase containing solvent and residual hydrocarbons was collected into a pre-weighed petriplate. The petriplates were allowed to air dry for 24-48hrs, and the final weight was measured. The difference in final and initial weights was calculated, and this value was considered as weight of test. The same process was repeated for control flask, and the subtracted value was considered as weight of control. Percentage degradation of TPH was calculated as:

$$\%\, degradation = \frac{Weight of control - Weight of test}{Weight of control} \times 100$$

**3.2.2 Biological Oxygen Demand:**

Tests for Biological Oxygen Demand in the incubated flasks were performed according to the method described by IS: 3025 (Part 44). Finally BOD level was calculated as:

$$BOD\left(\frac{mg}{L}\right) = \frac{(\mathrm{Do} - \mathrm{D5} - \mathrm{BC}) Volume of the diluted sample}{Volume of sample taken}$$

Where $D_o$ is the initial dissolved oxygen (DO) for the diluted sample (in mL), $D_5$ is the dissolved oxygen (DO) at the end of 5 days for the diluted sample, BC is Blank Correction ($C_o$-$C_5$ i.e. initial DO of blank – DO of blank after 5 days).

**3.2.3 Chemical Oxygen Demand:**

Tests for Chemical Oxygen Demand in the incubated flasks were done according to the method described by IS: 3025 (Part 58). COD was calculated as:

$$COD\left(\frac{mg}{L}\right) = \frac{(A - B \times N \times 8 \times 1000)}{Volume of sample taken}$$

Where A= Volume of Ferrous Ammonium Sulphate for blank, B= Volume of Ferrous Ammonium Sulphate for sample, N= Normality of Ferrous Ammonium Sulphate

**3.2.5 UV-Visible Spectrophotometer analysis:**

After gravimetric estimation, the residues from the petri plates were re-extracted using petroleum ether (2ml), and added into tubes, and analysed using UV-visible spectrophotometer. The spectra were recorded in (Thermo Evolution 201) UV/VIS spectrophotometer ranging 200-800 nm. Since the wavelength is unknown, the whole range of UV and visible wavelength (200-800nm) was analysed. For blank, the solvent (petroleum ether) was used.

**3.3 Screening for biosurfactant production:**

A loop full of pure culture was inoculated in a series of test tubes containing 10ml of Sabouraud Dextrose Broth (SDB) each. After incubation for 24hrs, the cultures were centrifuged at 10000 rpm for 20 minutes and the supernatant (cell free broth) was retained for further tests.

**3.3.1 Emulsifying index test ($E_{24}$):**

2mL of the used engine oil was mixed with 2mL supernatant in a test tube, and vortexed at high speed for 2 minutes. The test tubes were allowed to stand for 24 hours following which the height of the emulsion formed and the total height of the solution was measured (Suganya, 2013; Sarubbo, 2006). Emulsifying index was calculated as:

$$E_{24} = \frac{Heightofemulsionformed(cm)}{Totalheightofthesolution(cm)} \times 100$$

**3.3.2 Oil dispersion test:**

About 20mL of distilled water was taken in a petri plate. 2mL of the used engine oil was added, followed by the addition of 1mL of supernatant to the center. Formation of clear zones was considered as positive result (Nalini *et al.,* 2013).

**3.3.3 Drop collapse test:**

Single drops of used engine oil, when added to the wells of a microtitre plate develop into a dome shaped droplets. To each of the wells, 10µL of the supernatant was added on top of the drop of hydrocarbon. If the shape of the drop of hydrocarbon flattens, it was considered positive result (Chandran *et al.,* 2010).

**3.4 Detection of Enzyme activity:**

Microorganisms produce certain enzymes that catalyse the degradation process, thereby reducing the time. The activity of such enzymes is to be identified. One of such enzymes of importance for degradation studies is dehydrogenase enzyme. For the detection of dehydrogenase activity, 5g of soil sample was mixed with 5mL of 2, 3, 5-triphenyl tetrazolium chloride (TTC) and incubated for 24hrs at 37$^{o}$ C. TTC solution was prepared by mixing 5 g/L of TTC with 0.2 M Tris–HCl buffer, pH 7.4. After incubation, two or three drops of concentrated $H_2SO_4$ were added in order to stop the reaction. It was then mixed with 5mL of hydrocarbon, mixed thoroughly, and allowed to stand for 30mins at room temperature. Later, the contents were centrifuged at 1000 RPM for 20mins. Absorbance of the extract was measured at 492 nm. Finally, the presence of dehydrogenase is said to be confirmed if the OD value is above 0.5 (Soleimani *et al.,* 2010; Cheema *et al.,* 2009).

**RESULTS AND DISCUSSION**

**1. Gravimetric method of % TPH analysis:**

Bioremediation study of used engine oil using*A.flavus*HQ010119 and *Pencillium sp*. KJ415574.1 with incubation of 20 daysis presented here.Gravimetric method of % TPH analysis study revealed that**,***A.flavus*(BNG-05) showed 50% degradation and *Pencillium sp*(T-2) showed 46.42% degradation(table 2) ,(fig 2)

**2. BOD:**

Measurement of consumed oxygen by aquatic microorganisms to decompose or to oxidize organic matter is analyzed. High BOD has high pollution potential if discharged untreated into a water course because it can result in severe depletion of oxygen content of the water and thus kill aquatic animals. The analysis revealed that the BOD level decreased after every five days of incubation (fig 3).

**3. COD:**

The requirement of dissolved oxygen for the oxidation of organic and inorganic constituents is measured . Usually cod results are typically higher than BOD values and it decreased after the bioremediation process. The chemical oxygen demand (COD) for fungal isolates inoculated with used engine oil, after every 5 days of incubation, was assessed by APHA method 5210 B.(fig 4).

**4. UV analysis:**

After gravimetric analysis of the residual hydrocarbons, the residues are re-extracted and the absorbance is measured using a UV-visible spectrophotometer. The maximum wavelength ($\lambda_{max}$) is given in the bracket for each sample. The change in $\lambda_{max}$ value indicates the loss of conjugation and breakdown of molecular structure of oil thereby proving the degradation of used engine oil by the isolates

**5. Biosurfactant analysis:**

Biosurfactant can increase the surface area of hydrophobic materials, such as pesticides and other hydrocarbons in soil and water environment, thereby increasing their water solubility. Hence, the presence of surfactant may increase microbial degradation of pollutants. In emulsification test ($E_{24}$) it is observed that *A.flavus* and *Pencillium sp*. showed emulisification index nearby to 50% . **Drop collapse test**, when an oil drop was added to the wells of a microtitre plate, it formed a dome shaped convex droplet. The ability of the biosurfactant to disturb this structure and cause it to collapse was considered as positive result. The collapsed structure appeared flat in shape, compared to the non-collapsed structure after the addition of biosurfactant produced *A.flavus* and *Pencillium sp*.(table 3) .Oil dispersion test depicts the capacity of biosurfactant produced by the potent organism to disturb the surface of oil, by altering the surface tension. This is important because it can help in dispersing large droplets of oil, making them smaller and more available to the microorganisms for degradation. The ability of the biosurfactant to disturb the oil surface

is considered positive result. (Nalini *et al.*, 2013), (table 4).The results for oil dispersion test reveals the biosurfactant produced by *A.flavus* and *Pencillium sp*. which were capable of dispersing oil surface.

**6. Enzyme screening**

The activity of dehydrogenase enzyme was assessed by measuring the reduction of 2, 3, 5 - triphenyl tetrazolium chloride (TTC) to 1, 3, 5 - triphenyl formazan (TPF). The $OD_{492}$ values for the fungal strains were used to determine the presence or absence of the enzyme. OD values above 0.5 were considered to be positive result.Both *A.flavus* and *Pencillium sp* showed positive for the test.

**Table 1: List of potent isolates used for degradation studies along their percentage of biodegradation.**

| Fungal strain | 5th day | 10th day | 15th day | 20th day |
|---|---|---|---|---|
| BNG-05 | 18.51 | 27.7 | 41.66 | 50 |
| T-2 | 13.70 | 18.94 | 33.33 | 46.42 |

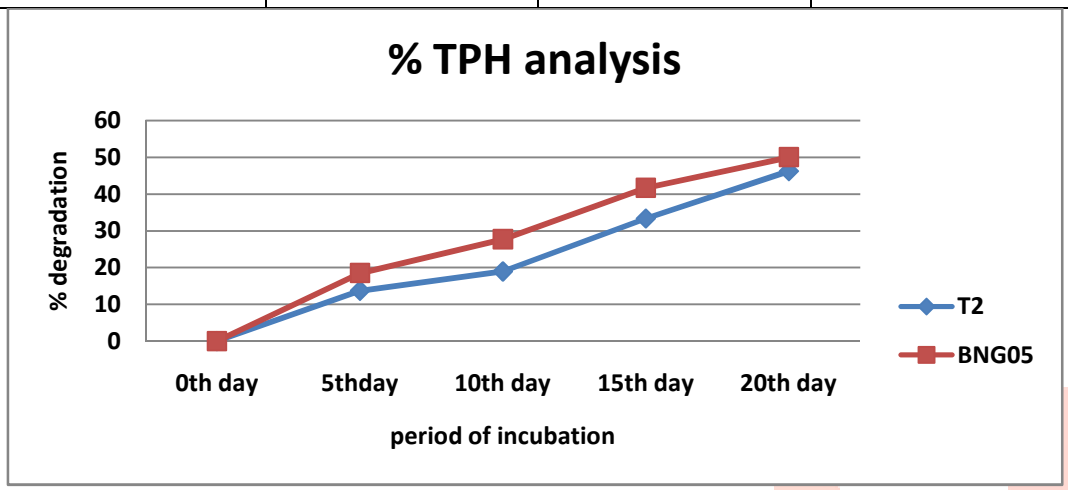



**Fig 1: Day wise degradation of used engine oil by potent fungi, where graph show %TPH of all the isolates up to 20 days of incubation**

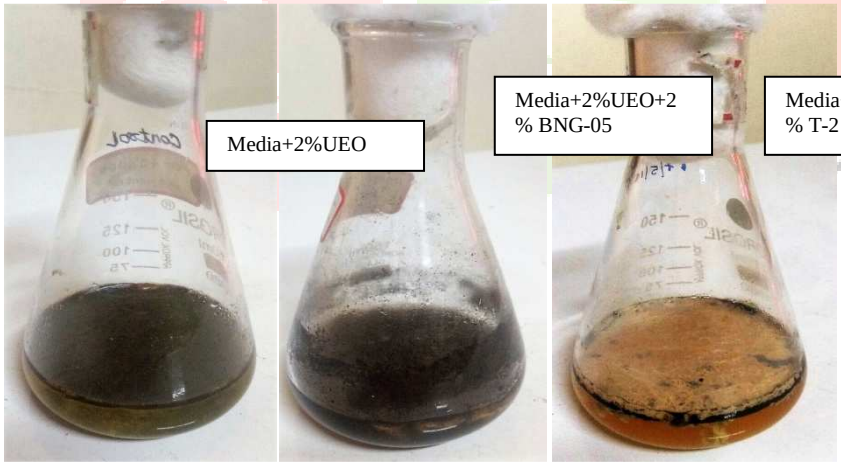



**Fig 2: Degradation of used engine oil by fungal isolates BNG O5 andT-2 after 20 days of incubation**

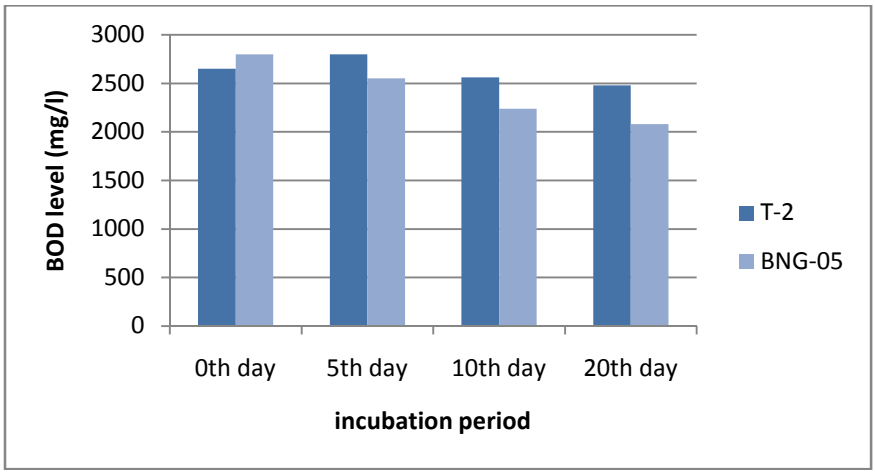



**Fig 3: BOD level of fungal isolates inoculated with used engine oil after 20days of incubation**

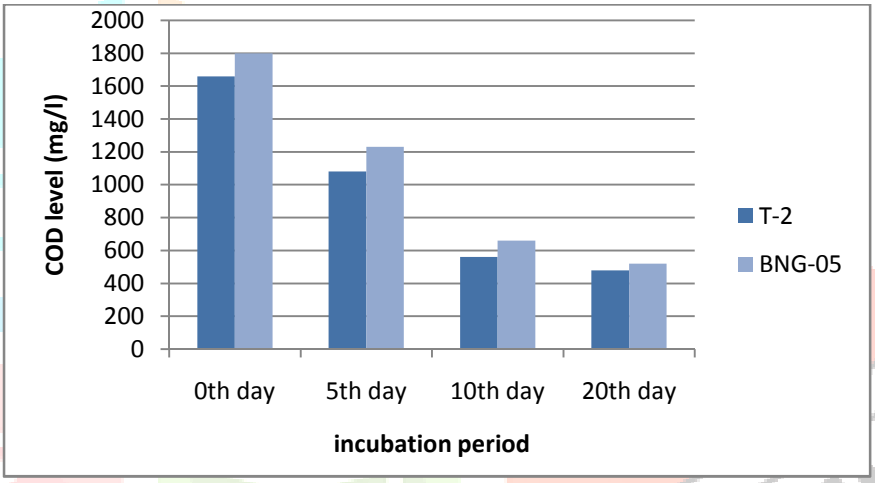
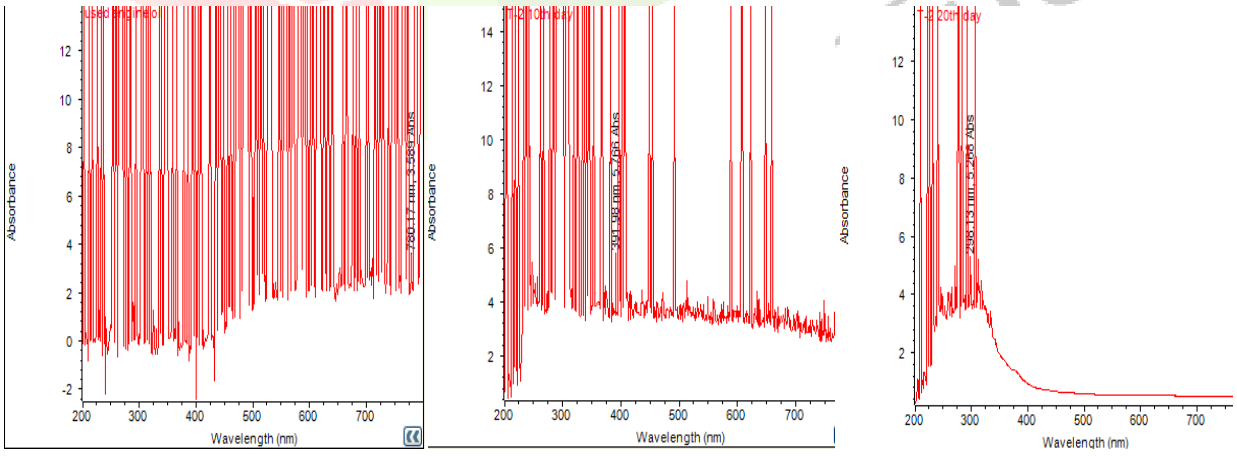
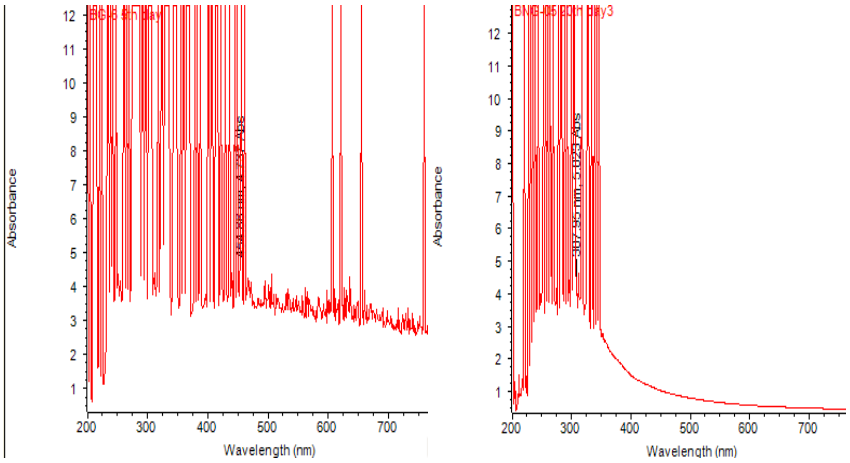



**Fig 4: COD level of fungal isolates inoculated with used engine oil after 20days of incubation**




**(a)** **(b)** **(c)**




**(d)** **(e)**

**Fig 5 :Uv analysis for (a) used engine oil$\lambda_{max=780nm}$, test incubated withA.flavus (b) 5$^{th}$ day$\lambda_{max=391.98nm}$,(c) 20$^{th}$ day$\lambda_{max=298.13nm}$,test incubated withPencillium sp(d) 5$^{th}$ day$\lambda_{max=454.88nm}$ ,(e) 20$^{th}$ day$\lambda_{max=307.95nm}$**

**Table 2:Emulsification test**

| Sl. No. | Sample name | Emulsification layer length (cm) | Height of the solution (cm) | Emulsification index ($E_{24}$ in %) |
|---|---|---|---|---|
| 1 | T2 | 1.1 | 2.6 | 42.31 |
| 2 | BNG05 | 1.3 | 2.8 | 46.43 |

**Table 3: Results for drop collapse test**

| Sl. No | Sample name | Shape of drop for Engine oil |
|---|---|---|
| 1 | T2 | Flat |
| 2 | BNG05 | Flat |

## CONCLUSION

The present study reveals the potency of *A.flavus*HQ010119 and *Pencillium sp*. KJ415574.1 isolated from different localities of West Coast of Karnataka, to degrade used engine oil components and convert into less toxic components. Further optimization of process parameters and immobilization studies need to be undertaken to enhance the degradation in both ex-situ and in-situ environment.

## ACKNOWLEDGEMENT

We would like to thank, Head of the Department. Department of Biotechnology, The Oxford College of Engineering for the infrastructure and encouragement.

## REFERENCES

[1] Abdulsalam, S. Adefia, S. S. Bugaje, I. M. and Ibrahim, S, "Bioremediation of Soil Contaminated With Used Motor Oil in a Closed System," J. Bioremediation Biodegrad., vol. 3, no. 12, pp. 3–9, 2012.

[2]Al-Nasrawi, Hussain. 2013. "Biodegradation of Crude oil by fungi isolated from Gulf of Mexico". J. Bioremed. Biodegrad. 3(4):1-6.

[3]Amund OO, Adebowale AA, Ugoji EO (1987) Occurrence and characteristics of hydrocarbon utilizing bacteria in nigerian soils contaminated with spent motor oil. Indian J Microbiol 27: 63-67.

[4]Bartha, Richard., Lanzilotta, R. P., and Pramer, David. 1966. "Stability and Effects of Some Pesticides in Soil". Applied Microbio. 15(1):67-75.

[5]Chandran, P., Das, N. 2011. "Characterization of sophorolipid biosurfactant produced by Yeast species grown on diesel oil". Int. J. of Science and Nature. 2:63-71.
[6]Clemente, A. R. Anazawa, T. A. and Durrant, L. R, "Biodegradation of Polycyclic Aromatic Hydrcarbons by Soil Fungi," Brazilian J. Microbiol., vol. 32, pp. 255–261, 2001.
[7]Ebenezer, L. A, "Bioremediation of Hydrocarbon Contaminated Soil Using Compost, NPK Fertilizer and Cattle Bile as Amendment Materials," Kwame Nkrumah University of Science and Technology, 2013.
[8]. Ijah,U.J.J.: The potential use of chickendrop microorganisms for oil spillremediation. *The Environmentalist*.,**23**, 8995 (2003).
[9]Nalini, S., Parthasarathi, R., Thandapani, C.M. 2013. "Isolation, Screening and characterization of biosurfactant produced by *Bacillus sp*. from automobile oil contaminated soil". Int. J. of Pharmaceutical & Biological Archives 4(1):130-135.
[10]Potin, Olivier., Catherine Rafin, Etienne Veignie. 2004. "Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil". Int. Biodeterioration & Biodegrad. 54:45 – 52.
[11]Soleimani, Mohsen., Majid Afyuni, Mohammad Hajabbasi, A., Farshid Nourbakhsh, Mohammad Sabzalian, R., Jan Christensen, H. 2010. "Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses". Chemosphere. 81:1084–1090.
[12]Stephen, E. and Ijah, U. J. J, "Comparison OfComparison Of Glycine Max and Sida Acuta in the Phytoremediation Of Waste Lubricating Oil Polluted Soil," Nat. Sci., vol. 9, no. 8, pp. 190–193, 2011
[13]Suganya, R.S. 2013. "Screening Optimization and Production of Biosurfactants from *Bacillus* and *Pseudomonas* Species". Int. J. Curr Pharm Res. 5(1):19-23
[14].Uzoamaka, G.O., T. Floretta and M.O. Florence: Hydrocarbon degradation potentials of indigenous fungal isolates from petroleum contaminated soils. *J. Phy. Nat. Sci.*, 3, 1-6 (2009).
[15]Vanishree, M., Thatheyus, A.J., Ramya, D. 2014. "Biodegradation of petrol using fungus *Penicillium sp.*" Science Int. 2(1): 26-31.