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Biomass Production and Petroleum Hydrocarbon Degradation by *Aspergilus niger* Tiegh Isolated from the Root Zone of *Helianthus annuus* L.

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Abstract Soil and water contamination with crude oil is a major global problem that can be addressed by using phytoremediation. The technology makes use of the synergy between the plant and its microbial population to achieve complete mineralization of contaminants. This research focuses on the role the fungal rhizosphere community can play in this relatively new technology. The aim of this study is to investigate the possibility of *Aspergillus niger* isolated from the rhizosphere of *Helianthus annuus* a known phytoremediant to convert petroleum hydrocarbons to its own mycelia biomass. *A. niger* isolated from the rhizosphere of *H. annuus* was cultured in crude oil contaminated minimal salt broth and control broth without crude oil and biomass production monitored for a period of 30 days. Two methods were used for biomass estimation; the dry weight method and also the spectrophotometric method at 540nm. The concentration of residual petroleum hydrocarbons in the treated and control broths were got using GC-FID. The growth indices got indicate that the fungus *A. niger* used crude oil as its sole carbon source converting it to its own biomass. There was gradual increase in biomass production until the 30th day, where senescence set in. There was a concomitant reduction in the concentration of total petroleum hydrocarbons from 3867mg/l in the control to 504.99mg/l in the *A. niger* treated medium. There was no significant difference between the dry weight and spectrophotometric methods. The spectrophotometric method is preferred to the dry weight as it is faster and easier to complete. *Aspergillus niger* in the root zone of *Helianthus annuus* has the potential to degrade up to 70% of petroleum hydrocarbons and can therefore be used for bio-augmentation during phytoremediation of crude oil polluted sites with the plant *Helianthus annuus.*

Keywords: *phytoremediation, rhizosphere, crude oil, minimal salt broth, biomass*

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1. Introduction

The use of crude oil and its products worldwide is widespread and comes with a lot of environmental problems. Exploration for Crude oil and its refining process comes with a lot of risks and dire consequences. There could be accidental spills of crude oil and its products into the environment. Petroleum hydrocarbons are mostly harmful to both man and the environment. The wastes generated from these processes have to be disposed properly as improper handling of oil sludge can lead to environmental pollution and introduction of these harmful chemicals to the environment. High toxicity profiles have generally been shown for petroleum hydrocarbons to microorganisms, plants, animals and humans. It is established that these petroleum hydrocarbons can be carcinogenic and mutagenic [1,2] (Anderson et al., 2014; Cohen et al., 2014). There are now quite a number of methods used for the treatment of pollution from the oil industry. One of such is bioremediation, a process that makes use of biological systems for the clean-up or decontamination of environmental pollutants. The biological systems could be bacteria, fungi, plants, algae or combination of two or three of these organisms. There are highly specialized methods that involve a synergy of soil inhabitants. One of the most proficient and widely accepted methods of remediation is phytoremediation. Phytoremediation is a cost effective and environmentally acceptable method that can be used for the clean-up of crude oil contaminated sites [3] (Kickpatrick et al., 2008). The biotechnology makes use of plants that are able to withstand and degrade contaminants in a complex process called rhizodegradation. It is clearly evident that growing plants in crude oil contaminated sites has the potential to decontaminate such sites and some of such plants used are *Cyperus brevifolius* and *Helianthus annuus* [4,5] (Basumatary et al., 2012; Chirakkara and Reddy, 2015). The plants are able to secrete exudates (with oxidative enzymes) from their roots, that can break down these contaminants into non-toxic compounds [6,7] (Siciliano and Germida, 1998; Macek et al., 2000). Plants however have a limitation when it comes to degrading polycyclic

aromatic hydrocarbons. This is because they are generally incapable of assimilating highly adsorbent compounds [8,9] (Anderson and Coats, 1994; Pichtel and Liskanen, 2001). A school of thought is however of the opinion that the rhizoremediation, (another term for phytoremediation) process is a synergistic one between the microorganisms in the root zone and the plants [10,11] (Banks et al., 2003; Chang et al., 2014). In fact, the microbial population particularly fungi and bacteria play a major role in breaking down these contaminants to acceptable and available forms for the plant roots to pick up. Records show that the population of PAH-degrading microorganisms in rhizosphere soil were three to four high than those in non-rhizosphere soil [3] (Kickpatrick et al., 2008). This indicates that the activities of plant roots in the soil encourage the growth of PAH-degrading organisms, as well as other microorganisms that can degrade other contaminants. Therefore, one of the known ways of improving remediation of crude oil contaminated sites is the growth of plants which have the potential to increase petroleum degrading microorganisms and as such enhance the the rate and extent of remediation at such sites [12] (Afzal et al., 2013). Fatima et al., (2015) [13], showed that rhizospheric bacteria in grasses and trees were able to degrade petroleum hydrocarbons by 78%. Bioremediation for a long time was attributed to bacteria but in recent years (last twenty), attention has shifted to fungi. Fungal bioremediation or mycoremediation became very attractive alternative for most researchers involved in bioremediation. This is because of the chemical resemblance between lignin and PAHs and some other environmental contaminants. Lignolytic fungi have been regarded as one of the most promising candidates for degradation of PAHs. Fungi secrete extracellular enzymes and are thus able to degrade a lot of compounds within their environment indiscriminately. They are abundant in the root zone of most plants forming sometimes beneficial associations called mycorrhizae. These associations help improve yield in plants and also ensure that plants survive adverse environmental conditions like petroleum hydrocarbon contamination [14] (Xun et al., 2015). Some fungi are host specific with their associations and as such aid only in certain functions that the plants are involved in. The plant that is involved in degradation of PAH or uptake of certain metals will have fungi that are able to mineralize such compounds and metals and get them into available forms for the plants to pick up through bio-absorption. They are therefore involved in sequestration of heavy metals and also petroleum hydrocarbons from the environment. Plants that have been implicated in phytoremediation have both bacteria and fungi within their root zones. These plants rely on the microbial population to degrade harmful compounds in their root zone and make them less toxic and also available for plant uptake. The degradation of compounds is a natural process by which fungi achieve growth. They are able to break down complex hydrogen-carbon bonds and use these for metabolic activities like growth. Evidence of growth is seen by biomass production or accumulation. This peculiar mode of living is harnessed in mycoremediation, particularly when using white rot fungi.

It is established already that these fungi can make heavy metals in soil bioavailable and cleanup of heavy metal contaminated sites using fungi has been achieved [15] (Joshi et al., 2011). *Helianthus annnuus* commonly called sunflower is a hyper-accumulator of heavy metals and the plant has been implicated in the degradation of petroleum hydrocarbons [5] (Chirakkara and Reddy, 2015). This, the plant is able to do with aid of the microbial population, as these can break down complex molecules and into mineral elements and harmless products of petroleum hydrocarbons that are bio-absorbed by the plant.

The hypothesis in this study is that the fungi within the root zone of this plant play a major or significant role in the degradation of petroleum hydrocarbons and sequestration of heavy metals. One of the most abundant fungus found within the root zone of this plant is *Aspergillus niger* a filamentous fungus ubiquitous in soil and commonly called black mould. It grows fast and has been isolated from crude oil polluted sites indicating that it can withstand crude oil pollution. *Aspergillus* sp. and *Penicillium* sp. strains have been implicated in lipase production in a series of fermentation studies and also the ability to convert oils into its own biomass [16] (Ohnishi et al., 1994). It therefore has a lot of promise in the degradation of petroleum hydrocarbons. The fungus here is isolated and grown in a medium that is deplete of all nutrients but crude oil. It is important to know the microorganisms around the root zone of plants that show phytoremediation potential and also be able to determine the extent to which these organisms can degrade particular contaminants. This will help in determining exactly what organisms should be used for bio-augmentation of the plants to enhance optimal removal of contaminants. The process also makes nutrients available to the plant by making sure that all bound biologically unavailable minerals are made available. This way the organism has taken care of both the plant and the soil and also its own growth requirements.

The study aims to monitor biomass formation under the stress condition of crude pollution. The main aim is to find out if the fungus isolated from the root zone of a plant known to bio-remediate is able to use crude oil as its sole carbon source converting it to its own biomass. The ultimate goal of the investigation is to find out if the fungus can be used to improve the plant's remediation potential and also explain its presence in the rhizosphere of the plant.

2. Materials and Methods

2.1. Test Soil

The plant *Helianthus annuus* was harvested from a fallow plot at the University of Ibadan, Oyo State Nigeria. The soil around the roots of the plant was shaken into polyethylene bags and stored in the refrigerator at 4°C until needed. The test soil was loamy soil - 17% Clay, 42% loam and 39% sand, 5.6% organic matter, 1.9g/l^{-1} -N, 14mg/l -P, 105mg/l -K with a pH of 8.5.

2.2. Isolation of Fungi from Rhizosphere Soil

Soil got from the root zone of the plant *H. annuus* was filtered with a 4mm sieve to remove debris and rocky particles. Ten grams of soil sample was poured into an Erlenmeyer flask to which 100ml of sterilized water had

been added and shaken at intervals for 30minutes. Four-fold serial dilution were prepared and plated in malt extract agar medium. Six replicates were made and incubated at room temperature. The total fungal colony got was recorded. The isolated fungi were identified by examining colony morphology and hyphal arrangement and phialiade formation under the microscope. The fungus that was most abundant was *Aspergillus niger*. This was selected from the mixed culture and pure cultures of this was got by plating separately in malt extract agar.

Crude oil used was Escravos crude oil collected from Chevron Nigeria Limited, Lekki, Lagos, Nigeria.

2.3. Effect of CRUDE OIl on Biomass Production

Growth of *Aspergillus niger* in crude oil contaminated medium was estimated by monitoring biomass formation over a period of 30days. The entire growth process was tracked using dry weight and optical densities simultaneously [17] (Banerjee et al., 1993).

The fungus *Aspergillus niger* isolated from the root zone of the test plant was maintained on PDA slants at 4°C before they were used. Test cultures were prepared in 250ml Erlenmeyer flasks containing 50ml medium of the following composition 1.25g of $NaHPO_4$, 0.29g of KCl, 10.0g of NaCl, 0.42g of $NaNO_3$, 0.83g of KH_2PO_4, 0.42g of $MgSO_4.7H_2O$, 5.0g of Agar dissolved in 1000ml of distilled water and 0.5g of yeast extract. The pH of the medium was adjusted to 5.5 with 1M HCL prior to sterilization at 121°C for 30minutes.

A set of 87 shake flasks (250ml) containing 50ml of the earlier specified sterilized medium were inoculated with (10% v/v homogenized biomass of *Aspergillus niger*) and held on a rotary shaker (room temperature 25-30°C, 200rpm) until desired. Each of the flasks were plugged with sterile nonabsorbent cotton, which was wrapped with aluminum foil so as to prevent cross contamination. All the flasks were incubated at room temperature for 30days and constant shaking of the flasks was ensured.

Of these a set of 42 flasks made up the positive control with 50ml medium and these had no crude oil but *A. niger* only. Another set of 3 flask made up the negative control and had crude oil but no fungus in the medium.

The second set of 42 flasks was contaminated with crude oil had 2.94ml of Escravos crude oil added to 50ml of medium to make a 5% level of crude oil contamination and 10% v/v homogenized biomass of *A. niger*. There were seven sampling periods (1, 5, 10, 15, 20, 25 and 30days) and this was done by harvesting the mycelial. Flasks were removed from the shaker and stored at 4°C if necessary until convenient to take readings.

At sampling times six flasks from both control and the treatment was sampled making a total of twelve. Three of these were homogenized and optical densities at 450nm were read. Homogenization of biomass was done aseptically with a blender (Waring Commercial Blender 7011, Dynamics Corporation America, New Hartford, CT) at low setting for 2minutes. Two blanks were used during spectrophometry; one was the crude oil contaminated treatment without the fungus and the second was the medium without either fungus or crude oil.

The other set of sampled three had the broth filtered out under suction through a 25µm pore" Nitex" nylon cloth and the mycelia washed with several sample volumes of deionized water after rinsing with n-hexane and dried overnight at 95°C. The dry weights of the mycelia were recorded.

2.4. Percentage Degradation of Peroleum Hydrocarbon by Fungi

The residual petroleum hydrocarbons were recovered using chloroform at a ratio of 1:1 (MSM medium: Chloroform) [18] (Chaillan et al., 2004). After 30days, the extent of Petroleum hydrocarbon degradation by *Aspergillus niger* was determined using GC-FID, Gas Chromatography with–Flame Ionization Detector. The gas chromatography equipment used was Hewlett Packard HP. The analytical conditions were as follow: carrier gas, helium, makeup nitrogen gas (flow rate -22ml/min), fuel-air flow rate 45ml/min, fuel-H_2 flow rate 45ml/min, injector temperature at 220°C, initial and final oven temperature70-200°C. The detector type was flame ionized with temperature at 250°C. The amount of PAH before and after were seen at the peak height of the chromatographic run. The concentration of the standard and peak area of the standard was then used to quantify the concentration of the test sample with respect to the peak area. The percentage degradation of samples was calculated as follows;

$$\text{Sample peak area} = \text{Total peak area} - \text{Solvent peak area}$$

$$\text{Concentration of sample} = \frac{\left(\begin{array}{l}\text{Sum total}\left(\text{Peak area of sample}\right)\\ \times\text{Conc. of the standard}\end{array}\right)}{\text{um total}\left(\text{Peak area of standard}\right)}.$$

NOTE: The result divided by 10 (Dilution Factor)

2.5. Statistical Analysis

The data obtained were statistically analyzed using SPSS statistical package version 16.0. the mean and standard deviation of the mans were got and significant difference between means was got using Duncan's multiple range test, $P<0.05$.

3. Results

There was significant increase in the biomass of *Aspergillus niger* in crude oil contaminated medium (Figure 1). The contamination had no negative effect on the growth of the fungus. The fungus used up the crude oil as its sole carbon source and this was evident as there was growth in the contaminated medium and also minimal growth in the control medium. The optical densities for each sampling period showed a progressive increase in the biomass content or concentration. The biomass in the crude oil contaminated medium was significantly higher than those in the control that had no crude oil but the fungus only. This confirms the fact that crude oil was used up for growth –biomass generation or build up.

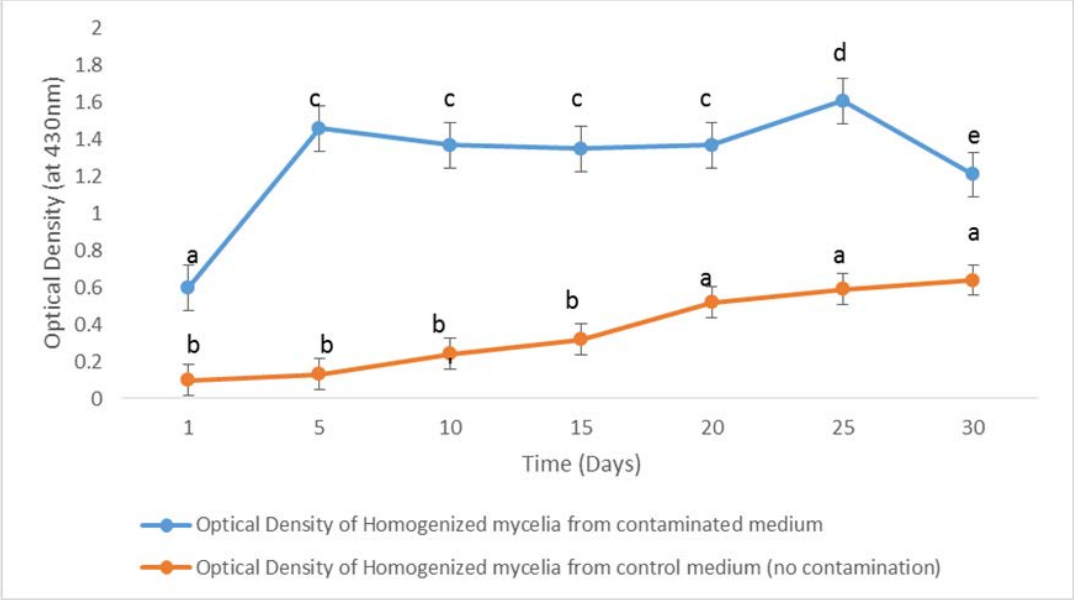

Figure 1. Optical densities of homogenized mycelia of *Aspergillus niger* grown in crude oil contaminated medium (Means ±SD Different letters above bars indicate significant differences based on Duncan's multiple range test)

The dry weight of the fungus also showed similar trend as seen in the optical density readings showing a progressive increase in the biomass of the fungus with growth indicated by biomass build up been significantly higher in the contaminated medium (Figure 2).

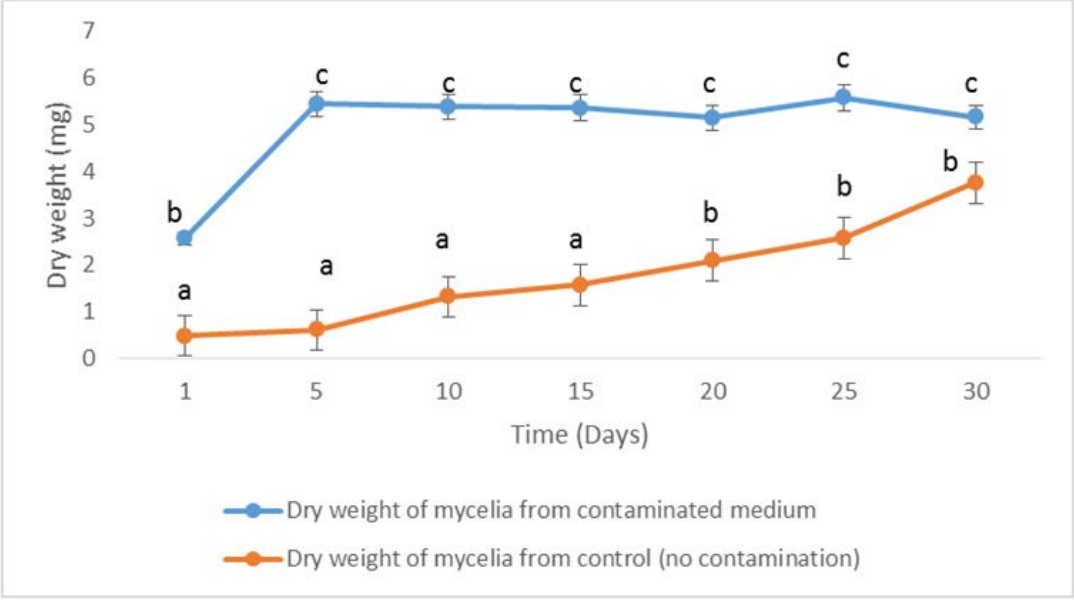

Figure 2. Dry weight of mycelia of *Aspergillus niger* grown in crude oil contaminated medium (Means ±SD Different letters above bars indicate significant differences based on Duncan's multiple range test)

There was no significant difference between the two methods used for estimating the biomass content of the treated and untreated medium. The trend line shows similar pattern of growth with both the dry weight method and the spectrophotometric methods (Figure 3). There was however slight variation in the trend in the control media as the trend lines were slightly apart for both the dry weight and the optical density indicating that the fungus mycelia showed more variation in the absence of nutrient (Figure 4).

Figure 5 and Figure 6 shows the chromatographic profile of crude oil in both control media that had crude oil but no fungal treatment and the crude oil contaminated medium treated with *A. niger*. The concentration of crude oil or petroleum hydrocarbons in control was 3867mg/l and there was the presence of more petroleum hydrocarbons not degraded when compared to the profile of the treated medium (Figure 5 & Figure 6). The control had petroleum hydrocarbons from ranging from C_2 to C_{28} (Figure 5).

There was noticeable reduction in the peak areas of the chromatogram in the treated medium (Figure 6). The concentration of petroleum hydrocarbons in the treated medium was 504.99mg/l. The treated crude oil showed reduction in the compounds present and complete mineralization of C_6, C_8, C_{22}, C_{23}, C_{24}, C_{25}, C_{26}, C_{27} and C_{28} compounds. There was therefore degradation of up to 78% in the *Aspergillus niger* treated crude oil contaminated medium.

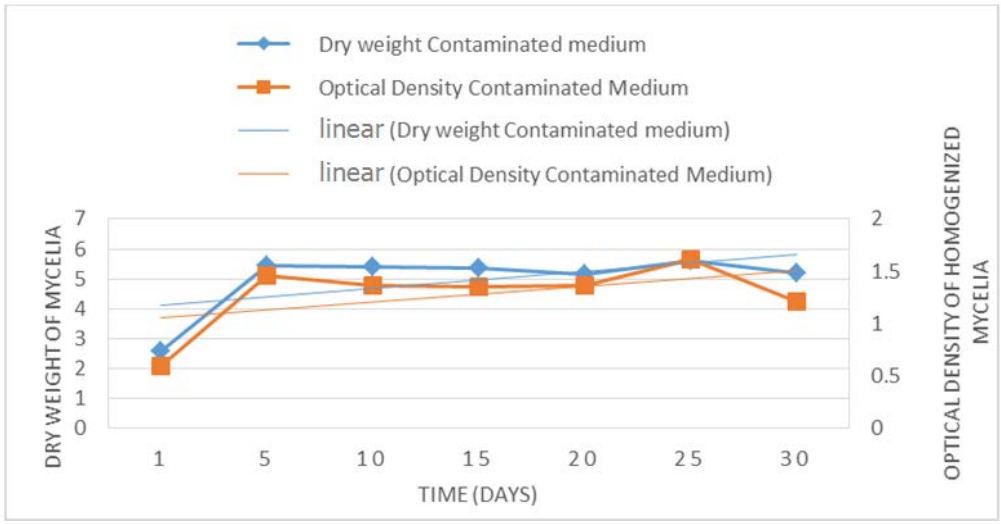

Figure 3. Comparison of *Aspergillus niger* growth profiles determined by dry weight and optical density in crude oil contaminated medium

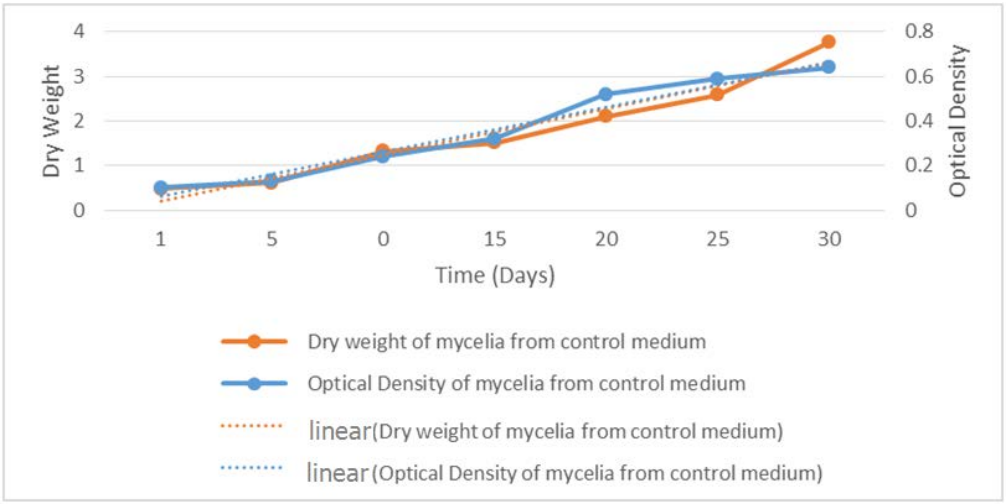

Figure 4. Comparison of *Aspergillus niger* growth profiles determined by dry weight and optical density in control medium (no crude oil)

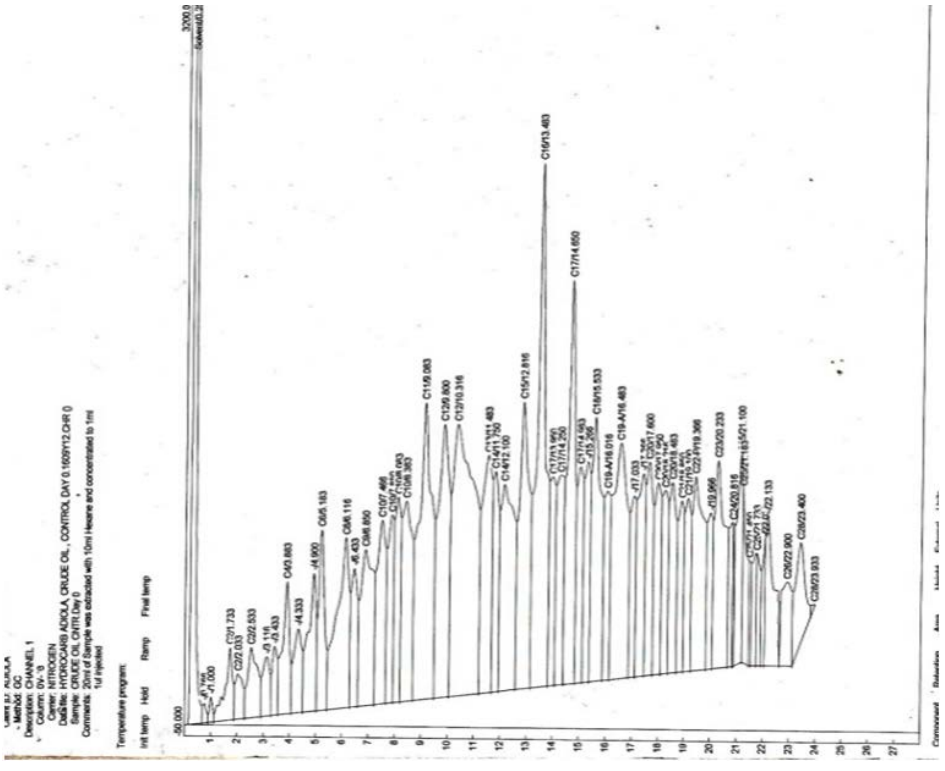

Figure 5. Chromatographic profile of crude oil not treated with Aspergillus niger (Control medium)

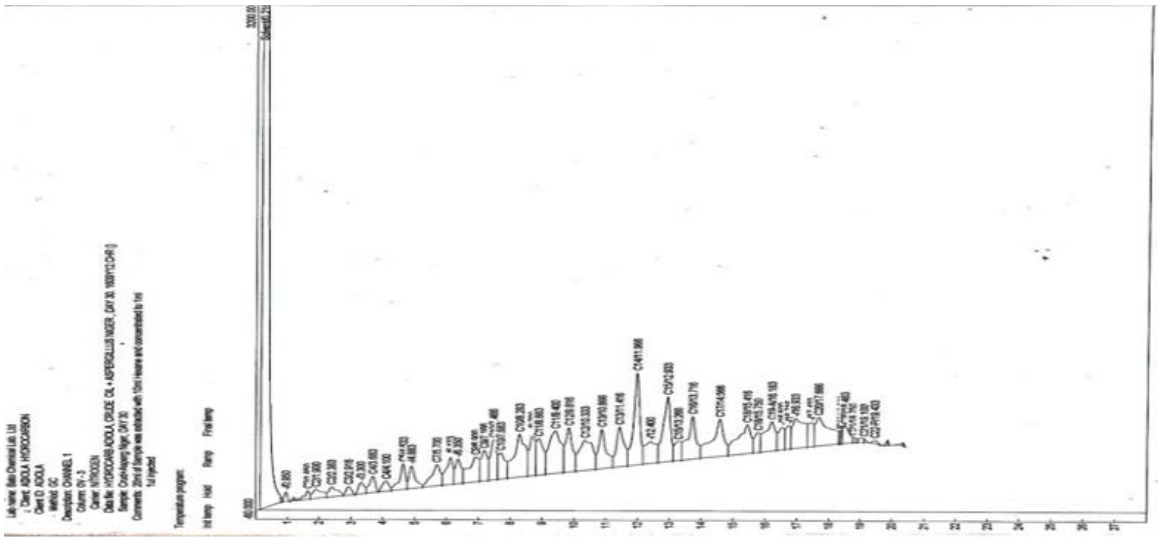

Figure 6. Chromatographic profile of Residual Petroleum Hydrocarbons at the end of 30-day treatment with *Aspergillus niger* mycelia

4. Discussion

In the present study crude oil degradation was achieved by the application of *Aspergillus niger* isolated from the root zone of the plant *Helianthus annuus*. The fungus grew successfully in nutrient deprived medium and was able to use the petroleum hydrocarbons present as its sole carbon source converting it to its own biomass. This was evident from the increase in biomass noticed by the mycelia harvested from the contaminated medium (Figure 1). This was significantly higher than the mycelia biomass harvested from the control media that had no crude oil. The two methods used for estimating biomass production gave similar pattern or trend of growth. The dry weight of mycelia harvested from the contaminated media were significantly higher than that harvested from the control treatments without crude oil or petroleum hydrocarbons (Figure 2). There was also linear correlation between the two methods used and the trend followed the same pattern of growth for the fungus (Figure 3 and Figure 4). This indicates that both methods were effective for the estimation of biomass production. The spectrophotometric method is however faster and more convenient to carry out and is therefore recommended for large scale analysis. This affirms the fact that filamentous fungal growth can be measured using turbidity measurements (Banerjee et al., 1993). The hyphae just have to be homogenized which is quite easy to achieve these days with modern homogenizers now available in the market.

The growth of the fungus in the presence of crude oil and biomass production in the absence of other carbon source indicates that the petroleum hydrocarbons were removed or mineralized. This was confirmed by the GC-FID results as which showed complete mineralization of some compounds.

Phytoremediation is now one of the most accepted means of environmental decontamination as it is seen as been environment friendly. It is also thought to be a very slow process that can be sped up by encouraging microbial degradation around the root zones of plants that can be used for phytoremediation. The process is also termed rhizoremediation, and comprises the incorporation of the microbial component of soil into the remediation process of the plant. The effectiveness of phytoremediation as a bioremediation technique depends on the appropriate selection of both the plant and the fungal partners for process to be effective [19] (Turnau et al.,2006). Fungi and bacterial are fast growing organisms and hence degradation or breakdown of compounds by them is faster. Fungi are peculiar because they secrete extracellular enzymes and breakdown compounds externally and most often indiscriminately with recorded success in mineralizing some of the largest molecules on earth [20] (Fernández-Luqueño et al., 2010). It is therefore imperative that the organisms around the root zone that have degradative potentials be investigated and such organisms' activity encouraged and their number increased to ensure complete mineralization of contaminants. The fungus *Aspergillus niger* isolated from the root zone of the plant *Helianthus annuus* has shown premise in previous experiments as good bio-remediant of crude oil and its product [21] (George-Okafor et al., (2009). In this experiment, same result is recorded and the percentage degradation recorded is also comparable and sometimes even higher. [22] Stephen et al., (2013) also recorded that *Aspergillus niger* degraded alkanes present in lubricating oil successfully as recorded here in this study too. The catch here is that the fungus was isolated from the root zone of a plant that has been implicated in remediation activities, and not from a polluted site. Fungi isolated from soil have shown promise in degradation studies but emphasis has not previously been placed on the rhizosphere fungi particularly from around the root zone of phytoremediants like *Helianthus annuus*. The fungus is able to use up crude oil as its sole carbon source converting it to significant biomass for itself. The ability of this fungus to do this is not surprising as record also show that it can produce bioemulsifiers and therefore able to break down hydrophobic bonds easily [23] (Collin et al., 2010). The plant is therefore recommended for further studies for use in rhizosphere technology studies incorporating *Aspergillus niger* into the technology for total clean-up of crude oil contaminated soils.

The ability of *Aspergillus niger* to convert oil to its own biomass had been recorded by previous researchers like [24] Papanikolaou et al., (2010). *Aspergillus* species have also been successfully used to treat waste waters that from

olive mills and winery [25] (Salgado et al., 2006). They demonstrated that strains of *Aspergillus* and *Penicillium* have the potential to convert cooking olive oil to its biomass thereby removing the waste cooking oil from the environment as seen here in this study. The fungus *Aspergillus niger* has also been shown in previous experiments to be able to utilize petroleum hydrocarbons as its sole carbon source converting it into mycelia biomass [26] (Elshafie et al., 2007).

The success of the mold in crude contaminated medium indicates that it has the necessary enzymes needed for degradation of the compounds present in crude oil utilizing them as source of nutrition and eventually growth. The efficacy of this fungus in degrading petroleum hydrocarbons is clearly demonstrated.

The results shown in this study implies that the fungus has potentials for bioremediation and this has not been fully harnessed. The presence of the fungus in the root zone of the plant indicates that it plays a major role the degradation activity of the plant. It can only improve the degradation potential of the plant. In applying the phytoremediation technology, it is important that this fungus be incorporated into the scheme to achieve maximum degradation of petroleum hydrocarbons. This can be achieved by bio-augmenting with the fungus and also ensuring that the plant stays healthy. Bioaugmentation with A. *niger* has been applied in the treatments of soils contaminated endosulfan with success but not with crude oil contaminated soils [27] (Bhalerao, 2012). There are other parameters that has to be considered like the role that mycorrhizal fungi play and also the role of growth stimulating bacteria within the roots of this plant plays in its remediation activity. It is therefore important to note that the plants used for phytoremediation have to be carefully studied and specific microorganisms that can enhance the process also studied to ensure complete and faster decontamination of polluted sites.

5. Conclusion

This study has clearly shows that *Aspergillus niger* is one fungus that can enhance petroleum hydrocarbon degradation in conjunction with the plant *Helianthus annuus*. Further work still has to be done and is ongoing to get the total microbiome within the root zone of this plant.

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