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Biodegradation Potential of *Aspergillus niger* on Crude Oil Contaminated Soil

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ABSTRACT

The biodegradation potential of *Aspergillus niger* was investigated on soil experimentally contaminated with crude oil. The fungal load dynamics of *Aspergillus niger* and some chemical parameters of the contaminated soil were determined using standard microbiological and analytical methods. The parameters were determined at weekly intervals and at varying levels of crude oil concentration (10%, 20%, 30% and 40%) for a period of six weeks. There was a progressive increase in the fungal load dynamics ($3.6x10^4$±0.05cfu/g -$1.43x10^5$±0.06cfu/g; $4.2x10^4$±0.02cfu/g - $1.41x10^5$±0.02cfu/g; $2.6x10^4$±0.04cfu/g - $1.32x10^5$±0.05cfu/g and $3.5x10^4$±0.01cfu/g - $1.42x10^5$±0.02cfu/g) from week one to week six at 10%, 20%, 30% and 40% crude oil concentrations respectively. The highest fungal load ($1.43x10^5$±0.06cfu/g) was recorded on 10% crude oil concentration at week six while the lowest fungal load ($2.6x10^4$±0.04cfu/g) was recorded on 30% crude oil concentration at week one. The result of the total petroleum hydrocarbon (TPH) showed a progressive decrease (1642.02±0.047mg/kg - 600.52 ± 0.047 mg/kg; 1286.80 ± 0.047mg/kg-397.98±0.047mg/kg; 1908.3±0.047mg/kg - 759.28±0.047mg/kg and 2228. 7±0.047mg/kg - 778.88±0.047mg/kg) from week one to week six at 10%, 20%, 30% and 40% crude oil concentrations respectively. Other parameters such as pH (5.74±0.124 - 6.50±0.047) and percentage nitrogen (0.103±0.020% - 0.266±0.020%) showed progressive decrease and increase respectively as observed on the crude oil concentrations from week one to week six. All tests were done in triplicates. The result indicates the potential of *Aspergillus niger* in the degradation of crude oil as evidenced in the progressive reduction in the total petroleum hydrocarbon. The use of *Aspergillus niger* in the biodegradation of crude oil polluted soil is therefore recommended.

Keywords: Biodegration, Crude oil, *Aspergillus niger*, TPH, Soil.

I. INTRODUCTION

Oil production has continued to play a dominant role in the Nigerian economy, ranging from generation of foreign exchange to serving as a source of energy to run the nation's economy. Pollution caused by petroleum and its derivatives is the most prevalent problem in oil producing regions of the world (Millioli *et al*., 2009). Incidence of environmental pollution due to high rate of petroleum related activities in the Niger Delta area of Southern Nigeria and other oil producing areas of the world has been associated with frequent oil spills, especially through oil wells blow outs, tanker accidents, bunkering, rupture of pipelines and sabotage (Akpoveta *et al*., 2011). Crude oil is a major contaminant of soil and water in oil producing countries as a result of extraction and processing of the oil. The Niger Delta region of Nigeria supports a lot of petroleum exploration and exploitation activities, which had subsequently led to a wide scale of pollution of its rivers, swamps, farmlands with petroleum hydrocarbon (Okpokwasili and Odokuma, 1994; Okpokwasili, 2006).

Crude oil contamination in the soil has adverse effects on plants, thus soil contamination with hydrocarbons and other organic chemicals pose a variety of ecological

problems. Conventional methods such as physical removal are the first response option. It is worthy to note that they do not achieve complete clean-up of spills. Current mechanical methods typically recover no more than 10-15% of crude after a major spill and almost always leave the receiving body in worse conditions (Abu and Dike, 2008). Natural attenuation is the reliance on natural processes to achieve site-specific remedial objectives (USEPA; 1999). The need to remediate these sites has led to the development of new technology that emphasize on the detoxification and destruction of the contaminants rather than the conventional approach of disposal.

Bioremediation, the use of microorganism or microbial process to detoxify and degrade environmental contaminants is among these new technologies. The goal of bioremediation is to at least, reduce pollutant levels to undetectable, non-toxic or acceptable levels (Pointing, 2001) or ideally completely mineralize organo-pollutants to carbon dioxide. From environmental point of view this total mineralization is desirable as it represents complete detoxification (Gan and Koskinen, 1998).

Regardless of the exact nature of the treatment technology, all bioremediation techniques depend on having the right microbes in the right place with the right environmental conditions for degradation to occur (Atlas and Bartha, 1992). Efforts to achieve biodegradation of oil products have involved bacteria and fungi, since they are the only biological species which have the metabolic capability of utilizing petroleum carbon for cell synthesis (Jobson *et al*,. 1974). There are approximately 70 genera of known oil-degrading microorganisms, including bacteria such as *Achromobacter, Acinetobacter, Actinomyces, Bacillus, Burkholderia, Exiguobacterium, Klebsiella, Microbacterium, Nocardia, Pseudomonas, Spirillum, Streptomyces and Vibrio,* and fungi or yeast such as*Allescheria, Aspergillus, Candida, Debaromyces, Mucor, Penicillium, Saccharomyces* and *Trichoderma.* Under natural conditions, these microorganisms in most areas comprise very few, compared with the total number of identified microorganisms. However, at petroleum hydrocarbon polluted sites, these populations may grow and increase because they use petroleum hydrocarbon as a carbon source (Ahn *et al*., 1999; Aldrett *et al*., 1997; Altas, 1981; Bento *et al*., 2005; Chaerun *et al*., 2004; Das and Mukherjee, 2007; Gallego *et al*., 2001; Hua *et al*., 2003; Mohanty and Mukherji, 2008; Palittapongarnpim *et al*., 1998; Supaphol *et al*., 2006).

Fungi are also capable of influencing metal transformation in several types of media such as industrial wastes, low grade ores and metal bearing minerals (Gadd, 1993). In many instances, these organisms are already present in the soil as indigenous microorganisms. In other circumstances, such as in bioreactors used for treating wastes with high concentration of toxic materials, there may be a need to add exogenous microorganisms to the material. In order for microbes to degrade the contaminants, they must be in close proximity to the contaminants (Baker and Diana, 1994). Once the right microorganisms are present in the right place, the environmental conditions must be controlled or altered to optimize the growth and metabolic activity of the microorganisms. Such environmental factors are temperature, inorganic nutrients (primarily nitrogen and phosphorus), electron acceptors (oxygen, nitrate, sulphate and pH). This research therefore studied the potentials of *Aspergillus niger* in the degradation of a crude oil polluted soil.

II. MATERIALS AND METHODS

Sample collection

Soil samples were collected with a soil auger at surface depth of 0-15cm from the forested area of Imo State Polytechnic, Umuagwo in Ohaji Egbema Local Government Area of Imo State, South Eastern Nigeria, with no pollution history and devoid of crude oil contamination. The soil sample for control analysis was collected using calico soil bag, while the soil samples for microbiological analyses were collected aseptically using 200ml capacity sterile glass sampling container. All samples were labeled with a permanent waterproof marker. Crude oil was obtained from Adax Petroleum Company at Izombe Imo state. All samples were transported to the Laboratory immediately after collection.

Isolation, characterization and identification of fungi.

The method described by Fawole and Oso (1988) was used. One gram of the soil for microbiological analysis was mixed with 9ml sterile distilled water. Ten-fold serial dilution of the mixture was prepared using sterile distilled water up to 10^{-3}. 0.1ml of each diluted sample was placed on Potato Dextrose Agar (PDA) medium by spread plate method and incubated for 2 to 5 days at a temperature of 25^{o}C for the enumeration of fungi. Fungal colonies formed on PDA were sub-cultured on PDA plates, incubated at 25^{o}C and observed daily for growth. On establishment of growth, the cultures were observed for distinct colonies. These distinct colonies were made on fresh sterile PDA plates. Subcultures with uniform growth were considered to be pure. The pure fungal isolates were examined macroscopically and microscopically using the needle mounts technique as described by Fawole and Oso (1988). Their identification was performed according to the scheme of Barnet & Hunter (1987).

Inoculation and experimentation

A modified method of Adenipekun and Fasidi (2005) was employed. Two hundred grams of sterilized moistened soil was weighed into 350cm^3 sterilized jam bottles. Varying concentrations (10, 20, 30 and 40% w/w) of crude oil was added and mixed thoroughly with the soil. These bottles were autoclaved at 121^{o}C for 15 minutes. After cooling, each bottle was inoculated with the isolated fungi in an inoculating chamber. The bottles were incubated at 25^{o}C for 6 weeks. Samples were collected at weekly intervals to determine the fungal population dynamics and the changes in the chemical parameters of the samples. The control samples were contaminated with the crude oil at the different concentrations but were not inoculated with the fungal isolates. Chemical characteristics such as pH, percentage nitrogen and total petroleum hydrocarbon (TPH) were determined before fungal inoculation and at weekly intervals after inoculation for six weeks. Aseptic sampling techniques were used to avoid contamination. pH values were obtained by direct reading using a pH meter (ATC pH meter HI 8915).

Total Nitrogen Determination

Total Nitrogen was determined by semi-micro Kjeldahl method. 0.1g of the sample was weighed into the digestion flask; 1tablet of Selenium catalyst was added and moistened with a little quantity of distilled water. 5ml of Conc. H_2SO_4 was added and placed on the digestion block. The sample was heated over a fume cupboard until the sample is digested. The digest was made up in a 50ml volumetric flask for semi-micro distillation. The MARKHAM distillation apparatus was switched on and 10ml of the digest was introduced into the distillation chamber. 10ml of 45% NaOH was added gently and the sample allowed distilling into a 10ml of 4% boric acid. About 50ml distillate was collected and titrated with 0.02N H_2SO_4 to get back a pinkish-red end point.

Determination of Total Petroleum Hydrocarbon (TPH)

The total hydrocarbon was determined by Spectrophotometric method. 1.0g of the sample was weighed into a test tube, after addition of 10ml of toluene, the mixture was placed on a water bath in a fume cupboard and digested for about 30minutes. 5ml of the digest was pipette into a 50ml flask and made up to volume. The absorbance was measured in a Spectrophotometer (model APEL PD-303UV) at 570nm wavelength.

Data Analysis: Fungal load dynamics, Total Petroleum Hydrocarbon, pH and percentage Nitrogen were subjected to analysis of variance (ANOVA) and treatment means were compared for significant difference. Variability was considered significant only when the calculated F value was greater than the table F when P is less than or equal to 0.01.

III. RESULTS

After the pure fungal isolates were examined macroscopically and microscopically using the needle mounts technique as described by Fawole and Oso (1988), the isolated strain was identified as *Aspergillus niger* (Table 1). Changes occurred in fungal load of *Aspergillus niger* after the 6weeks incubation and at the different levels of crude oil contamination. This is presented in Table 2 and the result shows that more

Aspergillus niger was found in the soil contaminated with crude oil at the sixth week of crude oil degradation for all the levels of crude oil concentration and was least found in the soil not contaminated. This implies that *Aspergillus niger* increased in growth as the period of crude oil degradation increased. One important parameter to substantiate the efficacy of *Aspergillus niger* in crude oil degradation is the Total Petroleum Hydrocarbon (TPH). At 570nm absorbance substantial reduction occurred in the concentration of TPH from week one to week six and at the different levels of crude oil contamination (Table 3). Significant difference exists between each week and the different oil concentration.

Percentage nitrogen increased progressively both in weeks and in oil concentration as the degradation lasted (Table 4). The percentage nitrogen content of the soil inoculated with *Aspergillus niger* was highest at 40% level of concentration at week six and least at week two at 30% level of oil concentration. Graphic diagrams of Table 2, 3 and 4 as represented in Figure 1, 2 and 3 respectively puts these results in a better perspective. Changes in pH were recorded after week 1, week 2, week 3, week 4, week 5 and week 6 days of treatment with *Aspergillus niger* at various concentrations of crude oil. Figure (4) depicts the variations in the pH of the medium during the treatment period and was found to be decreasing gradually especially as concentration increased from 30% to 40% resulting in an acidic environment indicating the degradation of the crude oil. The minor increment witnessed from week 4 to week 6 could be attributed to basic intermediates produced during the course of the biodegradation. All the tested parameters were statistically significant at P less than or equal to 0.01($P \leq 0.01$)

Table 1: Characterization of the Fungus Isolate

Cultural Characteristics	**Microscopic characteristics**	**Inferences**
Black colony with granular surface and black reverse	Septate hyphae. Dark brown large globose conidial heads. Hyaline smooth-walled conidiophores which turn dark toward the vesicle. Conidial heads are biseriate.	*Aspergillus niger*

Table 2: Fungal load dynamics of crude oil polluted soil after treatment at weekly intervals

% of Crude oil	C Control cfu/g	Week 1cfu/g	Week 2cfu/g	Week 3cfu/g	Week 4cfu/g	Week 5cfu/g	Week 6cfu/g	ANOVA F-statistics
10	1.9x104 ±0.03	3.6x104 ±0.05	5.1x104± 0.08	7.2x104± 0.03	1.18x105 ±0.05	1.36x105± 0.03	1.43x105±0.06	53.3508
20	1.9x104 ±0.03	4.2x104 ±0.02	5.7x104± 0.03	7.3x104± 0.05	1.16x105 ±0.03	1.30x105± 0.08	1.41x105±0.02	8.17
30	1.9x104 ±0.03	2.6x104 ±0.04	4.1x104± 0.08	6.4x104± 0.03	1.02x105 ±0.02	1.22x105± 0.01	1.32x105±0.05	12.898
40	1.9x104 ±0.03	3.5x104 ±0.01	5.0x104± 0.03	7.2x104± 0.02	1.12x105 ±0.21	1.32x105± 0.04	1.42x105±0.02	19.351

Table 3: Values of TPH after treatment with *Aspergillus niger*

Percentage of crude oil	Control mg/kg	Week 1 mg/kg	Week 2 mg/kg	Week 3 mg/kg	Week 4 mg/kg	Week 5 mg/kg	Week 6 mg/kg
10	386.2±0.039	1642.02±0.047	1158.6±0.047	989.50±0.047	979.60±0.047	870.32±0.047	600.52±0.047
20	374.5±0.038	1286.80±0.047	1200.3±0.047	900.7±0.047	882.69±0.047	603.0±0.047	397.98±0.047
30	416.6±0.042	1908.3±0.047	1664.4±0.047	1248.0±0.047	836.16±0.047	973.44±0.047	759.28±0.047
40	196.5±0.020	2228.7±0.047	1998.2±0.047	1518.5±0.047	1047.4±0.047	1038.50±0.047	778.88±0.047
ANOVA F-statistic	9.085	352.189	13.173	7.397	123.054	328.908	355.821

Table 4 : Values of % Nitrogen after treatment with *Aspergillus niger*.

Percentage of crude oil	Control %	Week 1%	Week 2 %	Week 3 %	Week 4 %	Week 5 %	Week 6 %
10	0.140±0.021	0.103±0.020	0.126±0.020	0.116±0.020	0.348±0.020	0.294±0.020	0.266±0.020
20	0.28±0.023	0.084±0.020	0.132±0.020	0.098±0.020	0.294±0.020	0.300±0.020	0.308±0.020
30	0.126±0.021	0.103±0.020	0.064±0.020	0.064±0.020	0.112±0.020	0.280±0.020	0.350±0.020
40	0.28±0.023	0.182±0.020	0.070±0.020	0.168±0.020	0.114±0.020	0.210±0.020	0.364±0.020
ANOVA F-statistic.	8.258	4.284	5. 094	8.865	9.835	9.127	7.341

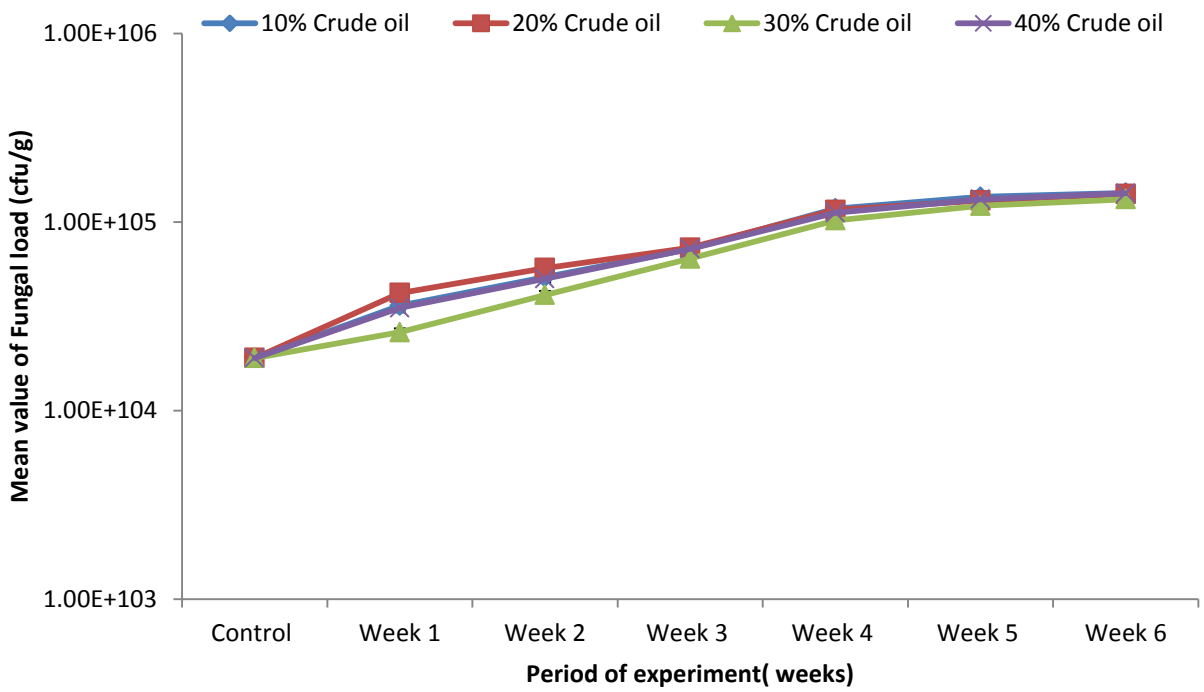

Figure 1 : Fungal load dynamics of crude oil polluted soil after treatment at weekly intervals

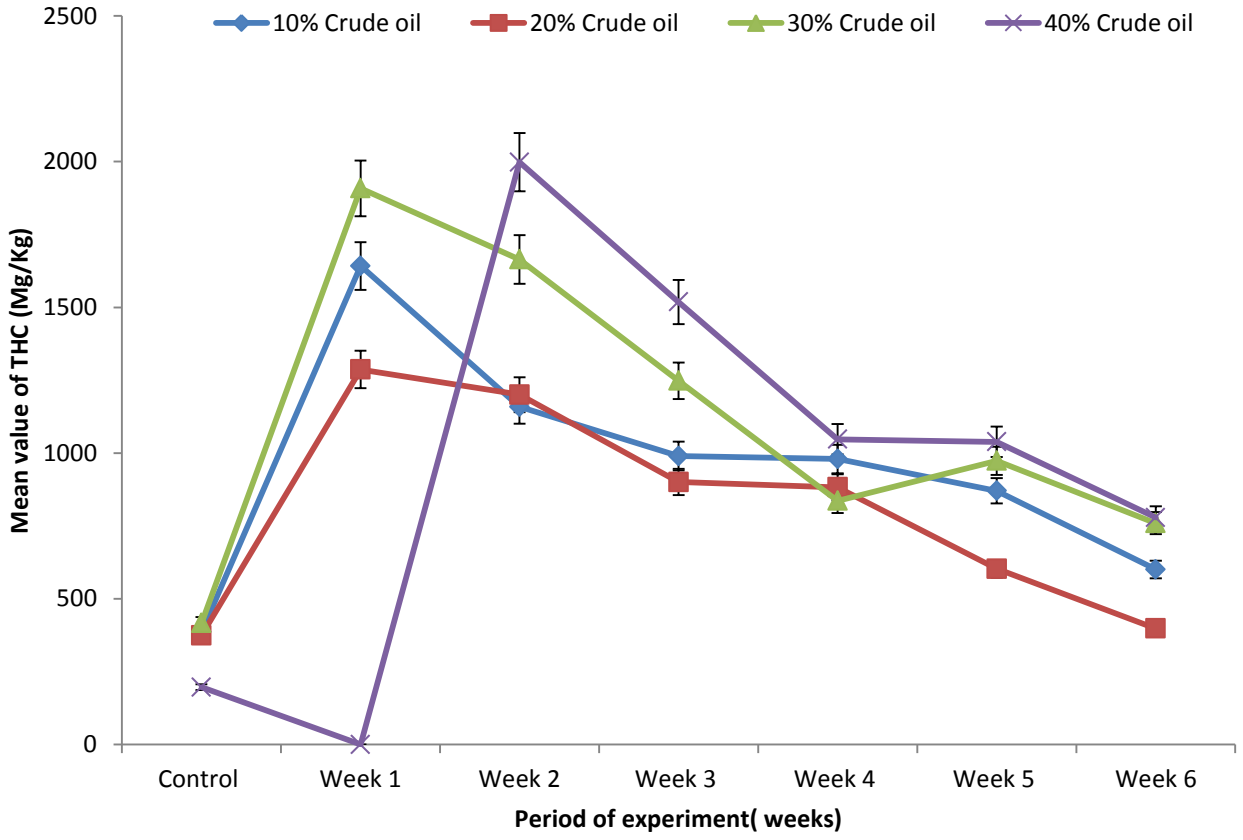

Figure 2: TPH against period of experiment.

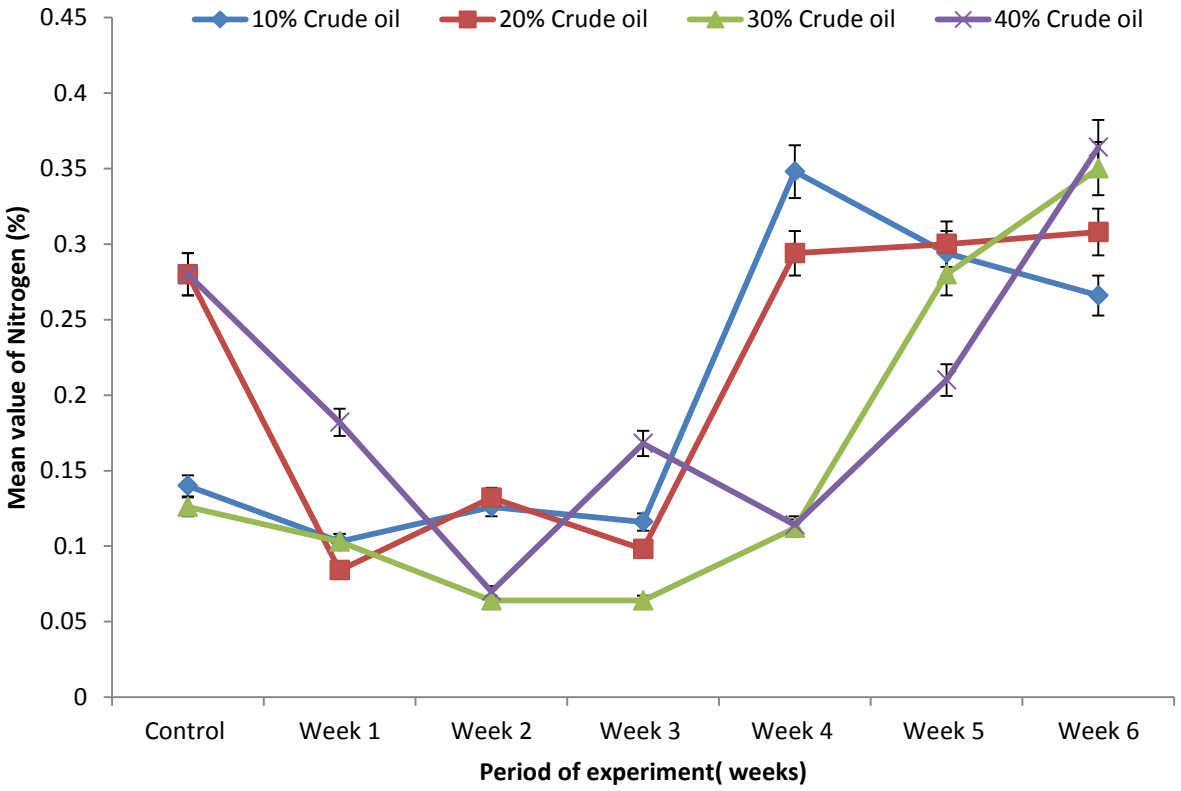

Figure 3 : % Nitrogen property after treatment with *Aspergillus niger*.

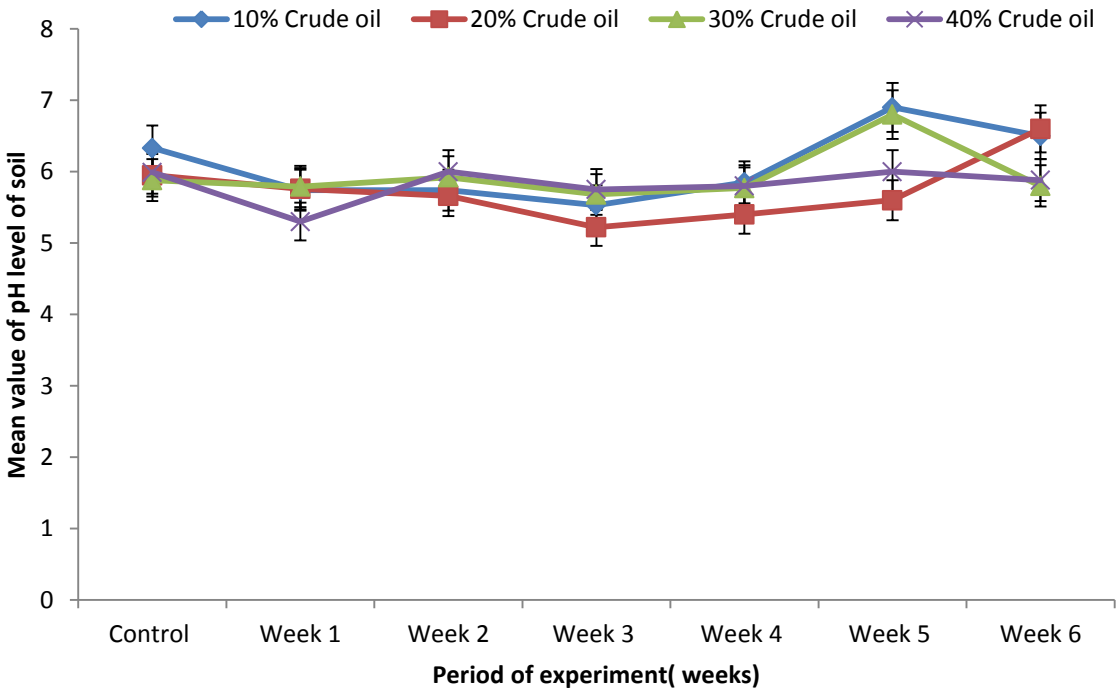

Figure 4: pH level of soils contaminated with crude oil after treatment with *Aspergillus niger*.

IV. DISCUSSION

Fungi play a central role in the biodegradation or decomposition of organic compounds and are producers of an array of extracellular enzymes. In particular, filamentous fungi have been implicated in the biodegradation of a wide range of aromatic hydrocarbons and thus they could contribute significantly to bioremediation efforts (Hughes *et al* 2007, Hughes and Bridge 2009). Petroleum contamination is a global problem and in Polar regions these spills result in extensive damage to ecosystems as cold region ecosystem recovery is a much slower process than that of warmer regions (Aislable *et al* 2001).

Okerentugba and Ezeronye (2003) demonstrated that *Penicillium spp*., *Aspergillus spp*. and
Rhizopus spp. were capable of degrading hydrocarbons especially when single cultures
were used. Oboh *et al*., (2006) have reported the abilities of bacterial species such as

Pseudomonas, Bacillus, Alcaligenes, Citrobacter and fungi such as *Aspergillus, Penicillium, Rhizopus* and *Rhodotorula* which can grow on crude petroleum as the sole carbon and energy source when screened for hydrocarbon utilization. In the present study, the fungal isolate *Aspergillus niger*, showed efficiency to reduce the Total Petroleum Hydrocarbon in the crude oil contaminated soil. Uzoamaka *et al*., (2009) has reported that some eight isolates of fungi showing potentials for hydrocarbon biodegradation includes *Aspergillus versicolor, A. niger, A.flavus, Syncephalastrum spp*., *Trichoderma spp., Neurospora sitophila, Rhizopus arrhizus* and *Mucor spp*. In a taxonomic study of fungi, hydrocarbon assimilation is most common in the orders *Mucorales* and *Monilales*, as well as in the genera *Aspergillus* and *Penicillium* which come under the Order Eurotiales (Enabulele and Obayagbona, 2013).

During the degradation of the crude oil, pH was found to decrease gradually from the first week to week four of incubation. The optimum pH for biodegradation of hydrocarbons is around 6-8 (Mentzer and Ebere 1996). Biodegradation of crude petroleum in an acid soil (pH 4.5) could be doubled by limiting to pH 7.4 (Vanishree, *et al* 2014). In the present work, the decrease in pH may be due to the release of organic acids in the medium. Petroleum contaminated soil contains various hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons; they are potentially toxic, mutagenic, and carcinogenic (Jelena *et al.,* 2008). Microorganisms promoting fouling of oil can live in a wide range of pH from 4 up to 9, however, they tend to prefer a neutral pH (Boszczyk-Maleszak *et al* 2006). Ekpenyong *et al*. (2007) reported that in studies involving mixed microbial consortium, the pH depression was not as much as was observed in the yeast or mould consortia studies, but decreased from 7 to 6 gradually suggesting possible neutralizing effect by basic intermediate products mostly from organisms that utilize oxidative biodegradation pathways.

During the treatment period the fungal load indicated the utilization of crude oil as a source of carbon. Growth of the fungus was observed to increase, which indicated the degradation of crude oil increasing with the incubation period. *Aspergillus niger* was found to be efficient crude oil degrader. In pure cultures, specific aromatic hydrocarbons and PAH fractions have been removed by up to 90% and 75%, respectively (Atlas 1991). It is also known that these hydrocarbon removal percentages can diminish or increase depending on the fermentation type (solid, liquid or slurry and microorganisms or micro flora involved (pure cultures or co-cultures; bacteria-bacteria, fungi-fungi or bacteria-fungi), as well as on the characteristics and concentration of the pollutant involved. Eja *et al.,* (2005) investigated the biodegradative potentials of the isolated fungal species from the soil polluted by petroleum products by measuring the optical densities (OD) of the fungal cultures spectrophotometrically and reported for the fungal isolates of the genera, *Saccharomyces, Aspergillus, Cladosporium Rhizopus, Mucor, Penicillium* and *Cladosporium*

V. CONCLUSION

Aspergillus niger tested in the present study can be used in oil bioremediation programmes as it has the ability to grow in even 10% crude oil concentration as evidenced by the increased Fungal growth, decline in Total Petroleum Hydrocarbon (TPH), reduced pH and
an increase in percentage nitrogen.

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