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Phytase activity of fungi from oil polluted soils and their ability to degrade bonnylight crude oil

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Fungi were isolated from contaminated soil samples taken from three selected automobile workshops, screened for phytase activities and biodegradative abilities. Physicochemical and total petroleum hydrocarbon analyses were carried using standard chemical and gas chromatography procedures, respectively. There was significant increase (at P≤0.05) in the potassium, sodium, calcium, magnesium, pH and organic matter of all contaminated soil samples. The fungi isolated were *Aspergillus niger*, *Aspergillus saprophyticus, Aspergillus fumigatus, Aspergillus flavus, Trichoderma viride, Penicillium italicum, Articulospora inflata* and *Neurospora crassa*. Of all the fungal isolates, *A. flavus* had the maximum phytase activity at the 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation. Phytase activity of *A. flavus* and *A. saprophyticus* were found to be most active at pH 5.0 and 50°C. *A. niger* had the highest degrading ability on crude oil and spent engine oil at all days of incubation while *N. crassa* had the least degrading ability on crude and spent engine oil. The high total petroleum hydrocarbon (TPH) concentration in contaminated soil may be as a result of consistent exposure of the soil to spent engine oil which could make the soil conditions unsatisfactory for microbial growth.

Key words: Fungi, biodegradation, bonny light crude oil, phytase.

INTRODUCTION

Crude oil accounts for approximately 35% of total global energy usage and consists of several hydrocarbons (Metman et al., 2010). Crude oil spills from pipelines and refineries leads to oil pollution which causes damage to the environment (Ogbe et al., 2006). Petroleum hydrocarbon compounds bind to different soil components and these are difficult to remove or degrade (Erdogan and Karaca, 2011). Oil pollution is a major environmental concern in many countries (Nikolopoulou et al., 2013) and this has led to a concerted effort in studying the feasibility of using oil degrading fungi and bacteria for biodegradation (Akoachere et al., 2008). Many microorganisms have the ability to utilize hydrocarbon as the sole source of carbon and energy. It is known that greater degradation of oil pollutants is carried out *in situ* by a consortium of microorganisms and more than 200 species of bacteria, fungi and even algae can biodegrade hydrocarbons (Onifade and Abubakar, 2007). The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (Chaudhry et al., 2012).

Microbes associated with several soil samples are rich sources of new enzymes (Akpan, 2004). Phytic acids (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) are a group of organic phosphorus compounds found

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Abbreviations: MSM, Minimal salts medium; **GC,** gas chromatographic; **TPH,** total petroleum hydrocarbon.

widely in nature (Chang et al., 2004). Phytases are commonly found in nature and can be derived from a number of sources including plants, animals and microorganisms (Krovuo et al., 2002; Singh et al., 2013). There has been a great deal of interest on the study of microbial phytase production and the optimization of media and conditions for maximum production of the enzyme with the aim to increase yields to make it economical as a commercial product. There are many applications of phytic acid, including industrial use as a corrosion inhibitor on metals, a rust remover and an additive to lubricating greases, use as a food additive, and medical applications, including use in the prevention of dental caries, use as an imaging agent for organ scintography and an X-ray enhancement contrasting agent (Chu et al., 2001). It is being used as a hypocholestromic agent, used to reduce gastric secretion for treatment of gastritis, gastroduodenitis, gastric duodenal ulcers and diarrhea, and used as an antidote for toxic metal absorption. It is therapeutically used in the prevention and dilution of calcium deposits associated with various diseases and for reducing calcium concentration in urine (thus checking the formation of renal calculi), It is also used as a preventive agent against severe poisoning with pressurized oxygen and preventing thirst during exercise, as a taste-improving agent in orally administered antibiotics, and in the treatment of multiple sclerosis (Chu et al., 2001). The objectives of this study were therefore, to isolate and identity fungal flora of oil contaminated soils and determine if there would be any correlation between phytase production and degradative abilities of the indigenous fungal isolates.

MATERIALS AND METHODS

Collection of sample

The contaminated and non-contaminated soil samples were collected from three different mechanic workshops at three different spots using a soil auger. Soil samples for physico-chemical analysis were collected in polyethylene bags while those for microbiological analysis were collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. Unused samples were refrigerated at 4°C.

Physicochemical analysis of the soil samples

Physicochemical properties of the soil samples such as pH, total nitrogen, organic carbon, organic matter, calcium, magnesium, potassium, sodium, phosphorus and moisture content were determined according to Ibitoye (2006).

Isolation, enumeration and identification of fungi from uncontaminated and contaminated soil samples

1 g of soil sample was taken from the sterile screw-capped bottles into 10 ml sterilized water in a test tube to form stock solution. This was repeated until the 8th dilution; 1 ml of the diluents from the two to four fold dilutions were pour plated on sterilized potato dextrose agar for fungi, allowed to solidify and incubated at 28±2°C for 72 h. Colonies that developed were counted and recorded as spore forming unit per gram of soil (sfu/g) for fungi. The isolates were subcultured repeatedly to obtain pure isolates. Isolated fungi were characterised by macroscopic (physical appearance on agar plates) and microscopic techniques (under light microscope) including colour of aerial and substrate mycelia comparing them with those of known taxa (Domsch and Gams, 1970).

Preparation of inoculating medium for the growth of culture

Cornstarch medium consisted: Cornstarch (Hubinger), 80 g; glucose, 30 g; $MgSO_4$. $7H_20$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; K_2HPO_4, 0.2 g; pH 5.0. The solution was mixed to effect complete dissolution following the addition of sodium phytase into each flask. The conical flask containing each medium was sterilized by autoclaving at 121°C for 15 min. Inoculum for the moulds was prepared by transferring 2 x 10^7 spores per ml from stock slants to 50 ml of cornstarch medium in 250-ml Erlenmeyer flasks which were incubated for 3 days at 28°C on an orbital shaker (270 rev/min).

Preparation of crude extract

A set of twelve (12) 100 ml conical flasks was labeled A to L to cover the 72 h incubation period. Each flask contained 30 ml optimized basal medium. All the flasks with their contents were sterilized in an autoclave at 121°C for 15 min, the solution was allowed to cool and inoculum for the moulds was prepared by transferring 2 x 10^7 spores per ml from stock slants was introduced, respectively, into each of the incubating flasks and then incubated at 37°C in shaking water bath for the period of 72 h. Five mililitre (5 ml) of the enzyme solution was withdrawn from each of the cultured medium after 72 h of the incubation period. The enzyme solution obtained daily was centrifuged at 6,000 rpm to get clear supernatant which will be used for phytase assay. The supernatant obtained was used for the analysis of phytase activity and the physicochemical properties of phytase.

Determination of phytase production through assay of phytase activity

Phytase activity was determined by measuring the initial rate of phosphorous, as indicated by an increase in absorbance at 415 nm. The sample test tube contained 1 ml of the enzyme solution, 2 ml of substrate solution incubated at 37°C for 65 min using a regulated Gallenhamp water bath and the reaction was stopped with 2 ml of colour stop mix to precipitate the enzymes. Blank sample contained only 1 ml of sodium acetate buffer, 2 ml of substrate and 2 ml of colour stop mix. Colour stop mix consist mixture of ammonium molybdate stock solution, ammonium vanadate stock solution, nitric acid ($HN0_3$) and distilled H_20. One enzyme unit (IU) was defined as 1 µmol phosphate liberated per minute.

Effect of pH on the activity of phytase

Two fungi, *Aspergillus saprophyticus* and *Aspergillus flavus* were selected for this assay. The optimal pH was determined by measuring the activity pH range 4.0-8.0, using 0.1 M acetate (pH 4.0-5.0), 0.1 M phosphate (pH 6.0-7.0) and 0.1 M Tris (pH8.0) buffers. Maximum activity was taken as the optimum pH for phytase for activity expressed in comparison with maximum activity (Gulati et al., 2007).

Effect of temperature on activity of phytase

The assay mixture was incubated at different temperature from 30-60°C to determine the effect of temperature on enzyme activity. At

65 min incubation period, 2 ml colour stop mix was added and the enzyme activity was measured according to the standard assay method at 5°C internal for each of the different temperature.

Determination of rates of utilization of crude oil and used engine oil by fungal isolates

Minimal salts medium (MSM) of Zajic and Supplison (1972) containing; 1% refined petroleum product (crude oil and used engine oil) as the only source of carbon, 0.27 g K_2HPO_4, 0.6 g NH_4Cl, 0.03 g $MgSO_4.7H_2O$, 0.015 g NaCl, 0.0015 g $NaSO_4.7H_2O$ was used. Crude oil and used engine oil were tested directly for the ability of fungal and bacterial isolates to degrade them using the method earlier described by Okpokwasili and Okorie (1988), as their sole sources of carbon and energy by the determination of growth turbidity. This was carried out by dispensing 100 ml of MSM into conical flask (Zajic and Supplison, 1972). Following sterilization by autoclaving and cooling, 0.1 ml of the isolates from 10^{-4} to 10^{-6} dilutions were seeded in 1,000 ml of minimal salt medium, pH 7.4, medium, followed by 0.1 ml filter-sterilized (0.45 µm pore size filter, Millipore) crude oil and used engine oil. The cultures were then incubated at room temperature for seven days. For each isolate, a control was set up in which no organism was seeded. At the end of the incubation, the optical density (OD) of each culture was measured at 650 nm (Eja et al., 2003) using spectronic 20 Genseys spectrophotometer. In this case, the OD was an index of growth reflecting the potential for the biodegradation of the petroleum products by the respective fungal species.

Characterization of crude oil and used engine oil

The crude oil sample (Bonny light), used engine, contaminated and non-contaminated soil sample were subjected to total hydrocarbon analysis using gas chromatographic (GC) method to determine total petroleum hydrocarbon (TPH) content of the crude oil and engine oil sample according to the method of Adesodun and Mbagwu (2008).

Statistical analysis

Data obtained were subjected to a single factor analysis of variance (ANOVA) while the significant means were separated with the Duncan's multiple range test (DMRT) at 5% confidence level (P = 0.05) using SPSS (16).

RESULT

Physicochemical properties of soil samples

The physicochemical properties of contaminated and uncontaminated soil sample immediately after collection are as shown in Table 1. The values of pH in contaminated soils ranged from 6.61 to 7.58 while pH of uncontaminated soils ranged from 6.40 to 6.64. The organic carbon content and organic matter ranged from 0.04 to 3.89% in contaminated soils and 0.03 to 0.20% in uncontaminated soils. The calcium concentration in contaminated soil ranged from 0.92 to 4.13 mg/kg while those in uncontaminated soils ranged from 1.70 to 2.11 mg/kg. Magnesium ion concentrations were higher in contaminated soils than in uncontaminated soils. It ranged from 0.70 to 3.22 mg/kg while those in uncontaminated soils ranged from 0.91 to 1.82 mg/kg. Sodium ion concentration in contaminated soils ranged from 0.05 to 0.42 mg/kg and it ranged from 0.10 to 0.15 mg/kg in uncontaminated soils. Potassium ion concentration in contaminated soils ranged from 0.09 to 0.41 mg/kg while those in uncontaminated soils ranged from 0.09 to 0.11 mg/100g. The phosphorus content in contaminated and uncontaminated soils ranged from 6.18 to 33.33 mg/kg and 1.99 to 3.38 mg/kg, respectively. Nitrogen level in contaminated soils ranged from 0.05 to 0.38% while in uncontaminated soils it ranged from 0.05 to 0.09%.

The results obtained from physicochemical analysis revealed that the contaminated soil contained high appreciable essential nutrients phosphorus in all the contaminated soils compared to non-contaminated soil. There was significant difference in the potassium, nitrogen; sodium, calcium, magnesium, pH, organic carbon and matter of all contaminated soil samples while there were no significant difference in that of all the non-contaminated soil samples.

Total plate count of fungi in uncontaminated and contaminated soils

The total plate count of fungi in contaminated soils ranged from 1.0×10^5 to 2.0×10^5 sfu/g, while in uncontaminated soils, it ranged from 2.0×10^5 to 3.0×10^5 sfu/g. Eight fungal isolates; *Aspergillus niger, A. saprophyticus, A. flavus, Aspergillus fumigatus*, *Trichoderma viride, Penicillium italicum, Articulospora inflata* and *Neurospora crassa* were obtained from both contaminated and non-contaminated soil samples.

Growth profile of isolated fungi

In Figure 1, there was an exponential phase at 38 to 45 h of incubation, *A. fumigatus* and *A. saprophyticus* had 18 to 45 h of incubation, *A. niger* had 40 to 60 h for incubition and *T. viride* had 22 to 50 h of incubation.

Production of phytase

Result of phytase activity from isolated fungi is presented in Figure 2. From this study, it was observed that of all the fungal isolates, *A. fumigatus* had the maximum phytase activity at 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation. Phytase activity of *A. flavus* and *A. saprophyticus* were found to be most active at pH 5.0 (Figure 3). Also, at 50°C, phytase activity of *A. flavus* and *A. saprophyticus* were found to be optimum (Figure 4).

Utilization of bonny light crude oil and used engine oil by fungal and bacteria isolates

The degradative abilities of fungal isolates on crude and used engine oil are as shown in Figures 5, 6, 7 and 8.

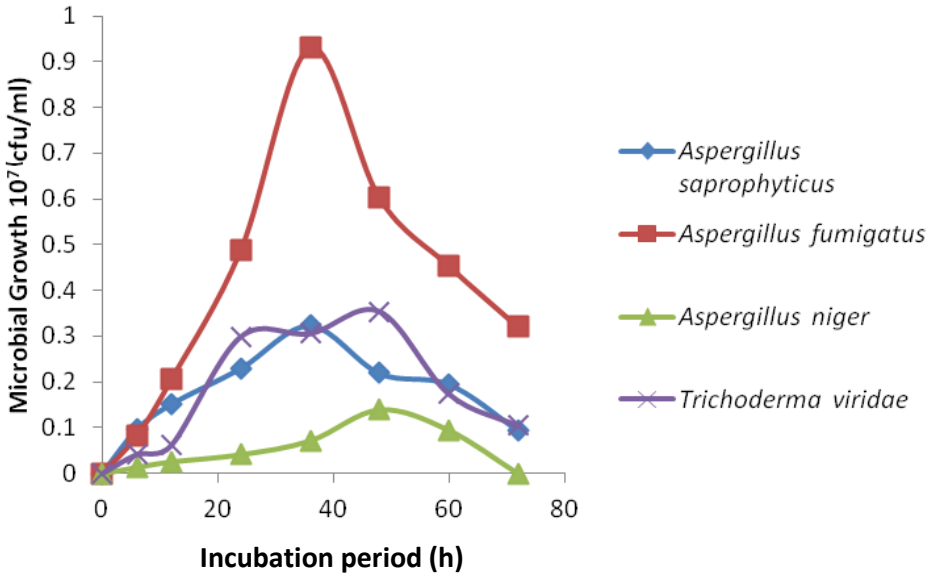

Figure 1. Growth profile of selected fungal isolates in optimized nutrient medium.

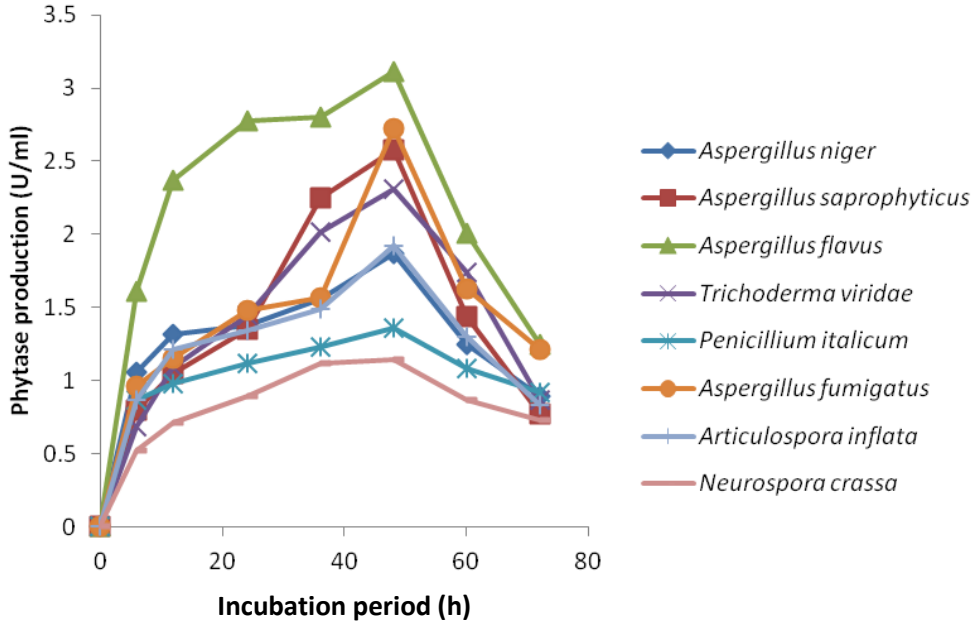

Figure 2. Phytase activity of fungal isolates.

Degradative abilities of crude oil were observed to be in the following order: *A. niger* > *A. fumigatus* > *T., viride* > *A. flavus* > *A. saprophyticus* > *A. inflata* > *P. italicum* > *N. crassa.* On the other hand, the abilities of fungi to degrade used engine oil were observed from the study to be in the following order: *A. niger* > *A. fumigatus* > *A. saprophyticus* > *T. viride* > *A. flavus* > *P. italicum* > *A. inflata* > *N. crassa. A. niger* was observed to be the best fungus for rapid degradation of crude oil and used engine oil while *N. crassa* was the least degrader of crude oil and used engine oil at all time of incubation.

Total petroleum hydrocarbon content of the crude oil and soil sample

The total petroleum hydrocarbon content showed that soil sample A had the highest with a total of 856,328 Mg/kg,

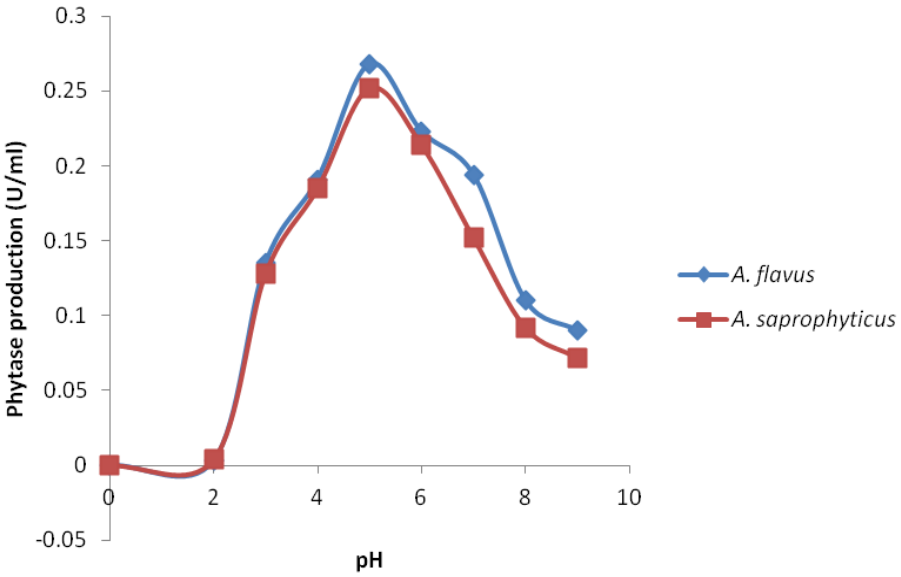

Figure 3. Effect of pH on the activity of phytase from *A. flavus* and *A. saprophyticus*.

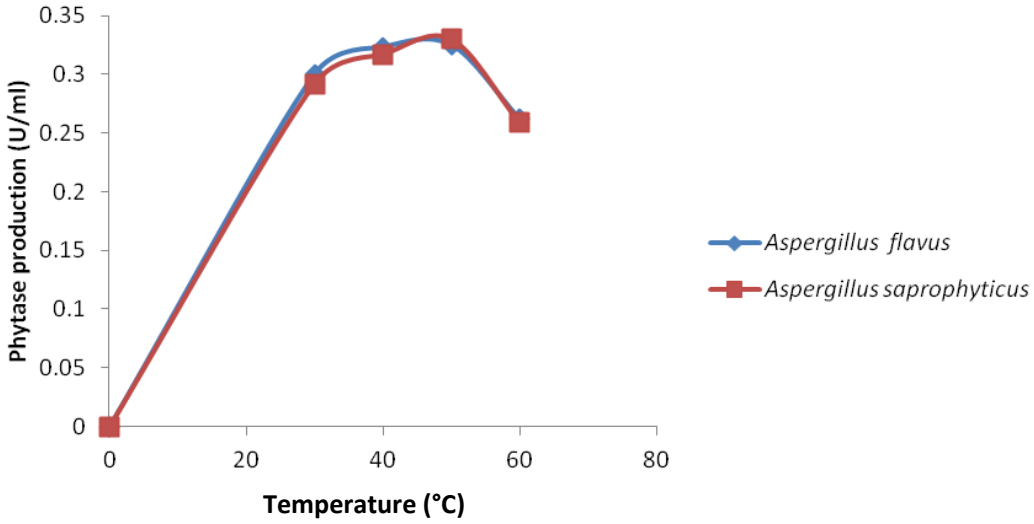

Figure 4. Effect of temperature on the activity of phytase from *A. flavus* and *A. saprophyticus.*

sample B had 642,302 Mg/kg sample C had the least with 545, 168 Mg/kg, that of the uncontaminated sample was 4.90 Mg/kg, while that of the bonny light crude oil and used engine oil was 98,346.102 Mg/l and 35,726.80 Mg/l.

DISCUSSION

The relative high pH in contaminated soil as compared to the non-contaminated soil could be as a result of the production of some organic acids in the course of the biodegradation process (Dennis, 2009). The slow biodegradation of organic compounds is often associated with low concentration of one or more inorganic nutrients needed for microbial growth in natural environments (Lewis et al., 1986; Swindoll et al., 1988; Coveney and Wetzel, 1992). The addition of nitrogen and phosphorus may increase the biodegradation of a compound (Pritchard and Costa, 1991). The results obtained revealed that the contaminated soil contained appreciable essential nutrients like nitrogen, phosphorus, potassium, magnesium, calcium and sodium. The calcium and magnesium ion concentrations in contaminated soils were higher than those in uncontaminated soils. This is believed to be the action of phytase on the phosphorus complexes, known as phytate.

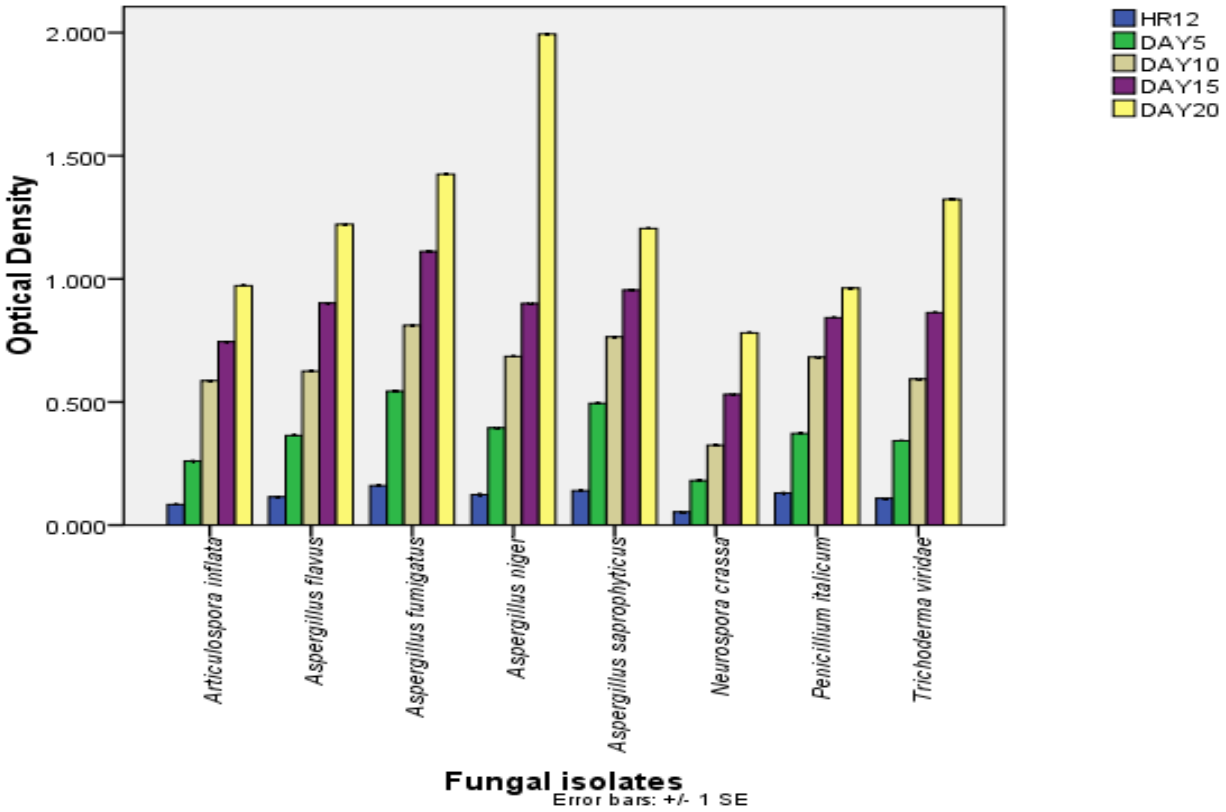

Figure 5. Optical density of fungal isolates from contaminated soil at different hours of biodegradation of bonny light crude oil.

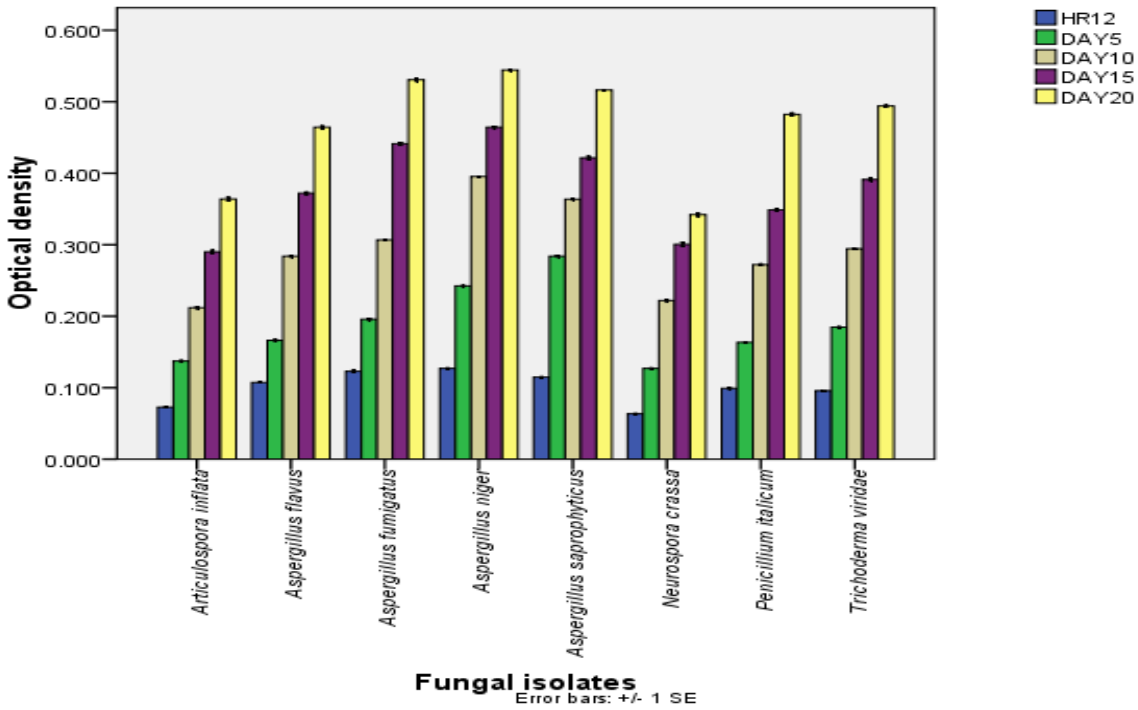

Figure 6. Optical density of fungal isolates from contaminated soil at different hours of biodegradation of used engine oil.

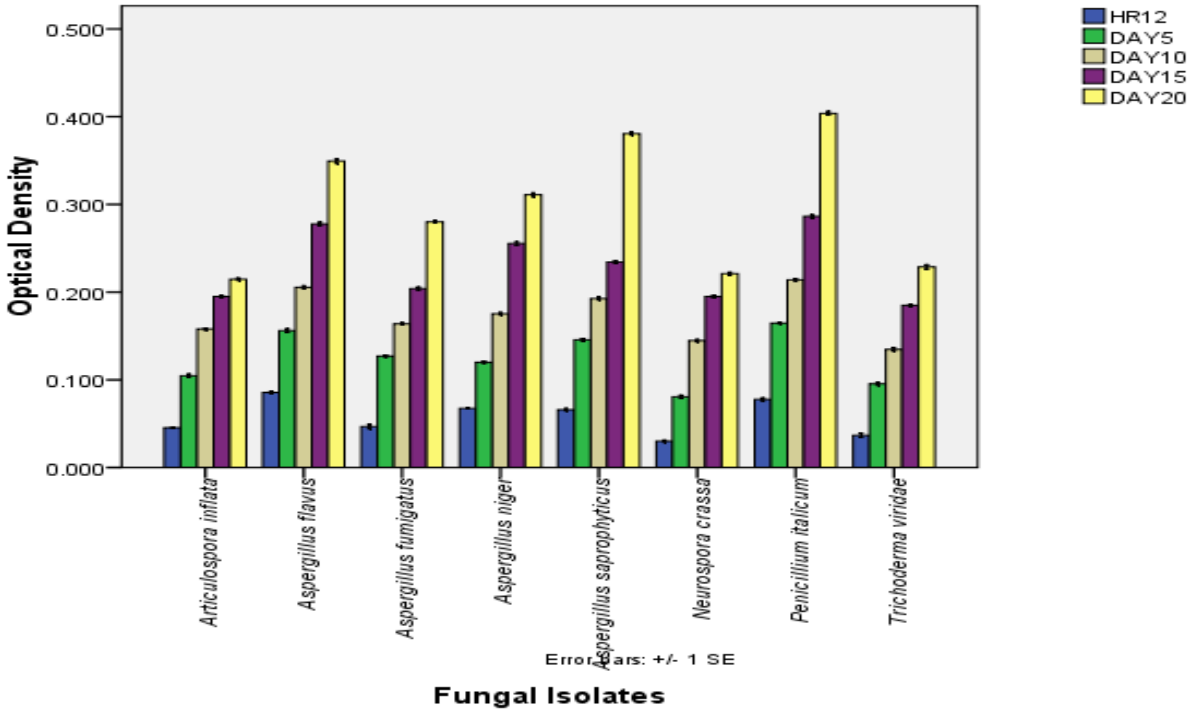

Figure 7. Optical density of fungal isolates from non-contaminated (control) soil at different hours of biodegradation of bonny light crude oil.

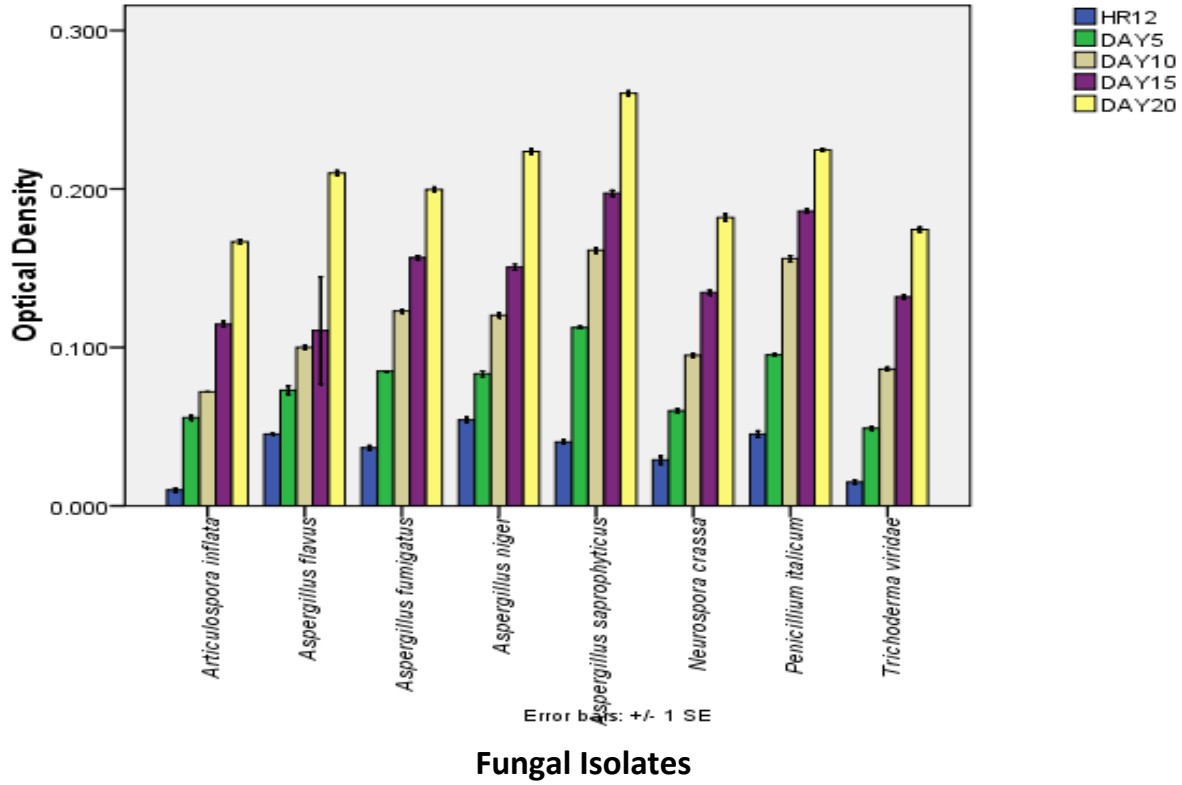

Figure 8. Optical density of fungal isolates from non-contaminated (control) soil at different hours of biodegradation of used engine oil.

This might occur as a result of phytase hydrolysing the phytate that has being chelated to release phosphate as a form of nutrient that will enhance biodegradation process. The significant differences between the concentration of potassium ion in contaminated and uncontaminated soils, indicate that the potassium ion in the contaminated soil is as a result of phytase released by the organism causing the phytate to be hydrolyzed. It was observed from this study that phosphorus concentrations in contaminated soils were higher than those in uncontaminated soils and this might occur as a result of phytase released by the organism causing the phytase to be hydrolyzed.

The total plate counts of fungi were higher in uncontaminated soils than contaminated soils. This is likely to be due to the environmental stress and toxicity caused by the hydrocarbons to the fungi. This finding agreed with the report of Atlas and Bartha (1992) that crude oil products contain hydrocarbon that are toxic to microorganisms. The result shows an obvious influence of waste engine oil and crude oil discharge on the microbiological and physiochemical properties of soil. The significant difference in the total plate count of fungi in contaminated and uncontaminated soil samples may be due to the fact that the fungi thriving in contaminated soils were able to synthesize enzymes capable of digesting the hydrocarbons in the crude oil and used engine oil (Ijah and Abioye, 2003).

The growth profile of the isolated microorganisms showed the growth curve exhibiting the lag, exponential, stationary and decline phase which agrees with the report by Prosser (1983) that the morphology and growth characteristic of the isolated microorganism vary continuously throughout colony development. The curve showed an exponential phase from 12 to 48 h. All the fungal isolated from this study were able to produce phytase in varying degrees. Kumat and Bhat (2011) found out that out of 161 fungal isolated forty soil samples, only 33 were phytase producers. Jared et al. (2010) also noted that *A. fumigatus* was among the fungal isolates that produced phytase. The maximum phytase activity was more prominent at the 48 h of incubation. It was observed that of all the fungal isolates, *A. flavus* had the maximum phytase activity at the 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation.

The pH versus phytase activity profiles of the selected fungi displayed substantial production of phytase at two distinct pH optimum; the highest activity was recorded at pH 6.0 and a second activity peak occur at pH 3.0 which is in accordance with the work of Gibson (1984). Maximum activity for phytase production from the selected fungi was attained at 50°C. According to Mullaney and Ullah (2003), the phytase molecule has a limited thermal stability and studies have demonstrated that losses in activity begin to occur at around 60°C. *A. flavus, A. fumigatus, A. saprophyticus, A. niger, T. viride, N. crassa, A. inflata* and *P. italicum* isolated in this study showed evidence of high ability to degrade crude oil as compared to used engine oil and this can be attributed to the presence of saturated alkane with intermediate chain (C10 – C24) length (Atlas and Bartha, 1996). However, *N. crassa, A. inflata* and *P. italicum* showed the lowest degrading ability on crude oil and used engine oil. Fungi show tremendous diversity and adaptability in utilization of different organic molecules as a carbon source, however, their abilities to degrade a specific hydrocarbon as a source of energy and/or biomass may differ (Hadiba and Tachinaba, 2009). The ability shown by the isolates in contaminated soil is believed to have been enhanced by the sufficient availability of phosphorus which is made available by the hydrolyzing effect of phytase on the complex salts of phosphorus that are in insoluble in the soil, and phosphorus is known as one of the limiting nutrient in biodegradation process.

The high TPH concentration in contaminated soil is as a result of consistent exposure of the soil to used engine oil, this high concentrations of the TPH make soil conditions unsatisfactory for microbial growth (Dejong, 1980). The TPH level in the uncontaminated soil (control) was as low as 4.9 mg/kg indicating that hydrocarbons could be present in uncontaminated soils and sediments, as earlier reported by Geiger and Blumer (1974). This research provides information that would lead to selection of fungal species and physicochemical condition that could be employed for bioremediation of soils polluted with crude and spent engine oil. It is therefore concluded that oil-degrading fungi are abundant in soils collected from mechanic workshop in Akure.

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