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Crude Oil Bioremediation Efficiency of Indigenous Soil Fungal Community Spiked with Cassava Peels in Niger Delta Region, Nigeria

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Abstract:
The bio-stimulation potential of cassava peels (CP) on crude oil degradation by indigenous fungal flora was investigated in soil microcosm. Air dried, sieved (2.00 mm) composite top soil (0-20 cm depth) samples were measured into four plastic buckets (2 kg each) and artificially contaminated by applying 200 ml of crude oil and left for one week. Each contaminated soil sample was then mixed thoroughly with pulverised pre-sieved (2.00 mm) cassava peels (CP) at various concentrations (100 - 400 g) and incubated at room temperature with periodic addition of moisture and thorough mixing for 42 days. Soil sampling was done at 14 days' interval post one-week soil acclimatization period for hydrocarbon degradation, total heterotrophic fungi counts (THFC) and utilization (HUFC), and pH using standard techniques. Results revealed that the percentage of crude oil hydrocarbon degradation was higher in CP amended crude oil treated soils. The highest hydrocarbon degradation (78.67 %) recorded was observed in the 400 g CP amendment as against 45.21 % biodegradation in un-amended control. The THFC and HUFC were significantly ($P < 0.05$) higher in CP spiked soils than in the un-amended controls. A wider pH variation was observed in the un-amended soil (6.88 – 7.92) than the CP amended soils (6.76 – 6.98) after 42 days. The indigenous hydrocarbon degrading fungal genera isolated were Rhizopus sp., *Fusarium* sp., *Apergillus* sp, *Mucor* sp., *Geotrichum* sp., *Curvularia* sp., *Penicillium* sp., *Aternaria* sp., *Cladosporium*sp., *Trichoderma* sp., and *Botrytis*sp.*The results of this study suggest that cassava peels, like some other agricultural waste, could be considered as a potential bio-stimulating agent for bioremediation of crude oil polluted soils in Niger Delta where these wastes are massively generated and dumped indiscriminately.*

Keywords: *Myco-remediation, Bio-stimulation, Crude oil, Cassava Peels*

1. Introduction

The resultant contamination of soils, ground water, surface water, sediments, swamps, vegetation and air with hydrocarbon compounds contained in spilled crude products is a major environmental challenge in oil producing communities, especially the Niger Delta region of Nigeria (UNEP, 2011; Adekunle et al*.,* 2012). The impact of crude oil pollution is further aggravated by sabotage and deliberate vandalization of pipelines in this region. The consequences of these have been enormous financial loss, extensive environmental degradation, and poverty leading to the continuous crises in the Niger Delta Area, that have culminated into kidnapping of oil workers, and even children (Imoobe and Iroro, 2009). The devastating effects of oil spill on the environment and ecobiota calls for measures to remediate affected environments (Imoobe and Iroro, 2009).

Generally, remediation of polluted systems could be achieved by physical, chemical or biological methods. However, the attendant negative consequences of the physicochemical methods make the biological alternative or bioremediation more attractive (Okoh, 2013). Bioremediation, which is the application of biological agents (microbes, plants, animals or their products/enzymes, is a green technology with great potential if the techniques are well understood and applied. One of the techniques currently explored by environmentalist is bio-stimulation based on the fact that crude oil polluted environments is usually nutrient limiting, among other factors. Lack of essential nutrients such as nitrogen and phosphorus is one of the major factors affecting biodegradation of hydrocarbon by microorganisms in soil and water environment (Okoh, 2006; Abioye et al., 2012). Therefore, the addition of inorganic or organic nitrogen-rich nutrients (biostimulation) is an effective means to enhance the bioremediation process(Omoni et al., 2015). Previous researchers had reported that some of our agricultural wastes or left over could be potential sources of bio-stimulating agents in bioremediation of crude oil in the trophics (Agarrya et al., 2010; Adams et al., 2014; Omoni et al., 2015, Romanus et al., 2015a, c))

Cassava (*Manihot esculenta crantz*) also known as tapioca, is a native of tropical South America and is a major root crop grown for its starchy roots in the humid tropics(Henery and Hershey, 2002).Nigeria stands as one of the world's foremost cassava producer with about twenty-six million tonnes annual prodcution (Aderemi and Nworgu, 2007). In Nigerian communities, the waste usually generated during cassava processing includes majorly the peels which are often disposed indiscriminately on open dump sites near

local processing factories usually located close to residential areas (Ayansina et al., 2014). The peels pile up at those dump sites thereby constituting significant environmental pollution and aesthetic nuisance (Ubalua, 2007).
Considering the relatively highly nutrient content of cassava peels and the fact that they are generated abundantly during processing as 'waste' in Nigeria, we decided to study its potential as nutrient supplier and bulking agent for the fungal biodegradation of hydrocarbons in soil samples artificially polluted with crude oil. This will go a long way to presenting an array of possible alternative bio-enhancing agents needed for crude oil bioremediation

2. Materials and Methods

2.1. Source of Samples and Processing

The Escravos light crude oil (ELCO) (dark brown in colour) used in this study was obtained from NPDC, NNPC subsidiary, Warri, Delta State, Nigeria**.** The mineral salts medium components were purchased from Chris Colon Scientific Company and Equator Laboratory, Port-Harcourt, Rivers State, Nigeria. The Soil samples used were pooled from farm land around Novena University, Amai campus using hand trowel after clearing debris from the soil surface. Samples for physico-chemical analyses were collected in polyethylene bags, while those for microbiological analysis were collected in clean black polythene bags. The soil sample were spread on the laboratory work bench and allowed to air dry for some days. It was later filtered using a 2.00 mm sieve mesh to obtain the working stock. The cassava peels were randomly collected from major refuse dumps within Amai town in Ukwuani Local Government Area of Delta State. The peels were air dried on the laboratory workbench, pulverized using Electric Blender, sieved (2.00 mm) and stored for further use.

2.2. Physico-Chemical Analysis of Soil and Cassava Peels

The moisture content of each sample was determined by gravimetric method as described by Black (1965). pH was determined using the pH electrode meter (Jenway 3015 model) as described earlier (Mclean,1982).The soil textural class was determined by hydrometer methodas described by Sheldrick and Wang (1993). The total organic carbon content was done according to the procedure method of Nelson and Sommers (1982). Total nitrogen was determined by the microkjeldahl digestion method (Bremner and Mulvancy, 1982). Available phosphorus was measured using alkaline oxidation method as described by Dick and Tabatabai (1977).

2.3. Myco-Degradation Experimental Design and Study

The study was carried out during the rainy season between themonths of April and August 2015. The air dried, sieved (2.00 mm) composite top soil (0-20 cm depth) samples were measured into four plastic buckets (4 kg each) and artificially contaminated by applying 200 ml of crude oil and left for one week. Each contaminated soil sample was then mixed thoroughly pulverised cassava peels (CP) at various concentrations (100, 200 and 400g) and control all labelled $T_0 - T_3$as shown in Table 1. The various treatment buckets and controls were then incubated at room temperature (30 ± 2^0C) in the laboratory for 42 days. The water content of the soil in each bucket was adjusted with sterile distilled water to a field moisture holding capacity of 50%. In order to avoid anaerobic condition, the contents in each bucket were thoroughly mixed every 3 days. Soil sampling was done on day 14th, 28th and 42nd post one-week acclimatization period for analyses of residual total petroleum hydrocarbon, total heterotrophic fungi count and hydrocarbon utilizing fungi andpH using standard procedures

Soil Microcosm	**Treatment**
T_0	Un-amended crude oil polluted soil [2 kg soil + 200 ml CrO + 0 g CP]
T_1	Amended crude oil polluted soil [2 kg soil + 200 ml CrO + 100 g CP]
T_2	Amended crude oil polluted soil [2 kg soil + 200 ml CrO + 200 g CP]
T_3	Amended crude oil polluted soil [2 kg soil + 200 ml CrO + 400 g CP]

Table 1: Experimental design and treatment
CP = Cassava peel, CrO = Crude oil

2.4. Determination of Residual Total Petroleum Hydrocarbon (TPH)

The amount of residual total petroleum hydrocarbon in the amended and un-amended treated soil samples was estimated using the gravimetric method as described earlier[7] by Omoni et al. (2015). 10 g of soil samples (triplicates) was taken from each microcosm and transferred into a 50-ml flask and the hydrocarbon content in oil polluted soil was extracted using 20 ml of n-hexane. The mixture was shaken vigorously for 30 min and allowed to stand for 10 min until the hexane extract completely separate the oil from the soil sample. The solution was then filtered using a Whatman filter paper and the liquid phase extract (filtrate) diluted by taking 1 ml of the extract into 50 ml of hexane. The absorbance of this solution was measured spectrophotometrically at a wavelength of 420 nm spectrophotometer using n-hexane as blank. The total hydrocarbon in soil sample was estimated with reference to a standard curve derived from fresh crude oil of different concentration diluted with n-hexane. Percentage crude oil biodegradation was calculated using formula:

$$\% \text{ Crude oil degradation} = [(A-B) \times (B) \times 100] / (A) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(i)}$$

Where A and B are the initial and residual total hydrocarbon contents respectively.

2.5. Enumeration of Total Heterotrophic and Total Hydrocarbon Utilizing fungi

The total heterotrophic fungal counts of the samples were determined by making ten-fold serial dilution of the samples on normal saline (0.85 % w/v) sterile NaCl. 0.1 ml aliquot portion was then spread plated (using sterile glass spreader) on pre-sterilized gelled Potato Dextrose Agar (Hi-media, India) previously augmented with 30 mg/l streptomycin and incubated at ambient temperature (30 ± 2 ^{0}C) for 24-72 hrs (Pelczaret al., 2005). Thereafter emerging colonies were counted.

Also, sterilized prepared plates of mineral salt agar medium (per litre of 1.5g K_2HPO_4, 1.0g NH_4NO_3, 0.5g $MgSO_4$, $7H_2O$, 0.2g $CaCl_2$, 30g NaCl, 0.3g KCl and 0.02g $FeCl_3$) was prepared (Odjadjare et al., 2008) and used for the enumeration of hydrocarbon utilizing fungi. Sterile 9 cm Whatman no 1 filter paper soaked in the crude oil and aseptically placed in the cultured plates served as the sole carbon source supplied to the inoculums by vapour-phase transfer. The plates were incubated for 3-7 days at room temperature and the expressed colonies enumerated accordingly (Romanus et al., 2015). The expressed colonies were further sub-cultured and characterized based on their macroscopic and microscopic characteristics as described by (Fawole and Oso, 1988). The fungal isolates were also identified by matching their macroscopic and microscopic features, and biochemical properties with those of known species described in standard mycological texts (Onions *et al*., 1981; Ellis, et al., 2007).

2.6.Determination of pH

The method described by Osuji and Nwoye (2007) was followed. Five grams (5.0 g) of each amended and unamended treatment sample added 50 ml of distilled water. The lumps of the soil were then stirred to form homogenous slurry, then pH meter (Jenway 3015 model probe was immersed into the sample and allowed to stabilize at 25^{o}C and pH of the sample then recorded.

2.7. Determination of Crude OilUtilizing Fungi

The ability of the isolates to utilize crude oil was confirmed by inoculating each isolate into separate cotton plugged 250 ml Erlenmeyer flasks containing sterile Mineral Salts Medium (MSM). The MSM broth and crude oil were autoclaved separately at 121 °C for 15 min. Sterile crude oil which served as source of carbon and energy was added at 1 % (v/v) to make up a final volume of 100 ml sterile liquid MSM. Each isolate was subsequently inoculated into separate flask of the medium. Control flask containing the MSM and 1 % (w/v) of crude oil but without organism was also prepared. The flasks were monitored and agitated daily for a period of 21 days. Utilization of crude oil was assessed by monitoring the cell density at 600 nm wavelength with spectrophotometer after 21 days' period (Khan and Shukla, 2011).

2.8. Statistical Analysis

Calculation of means and standard deviations, and regression analysis were performed using Microsoft Excel office 2007 version. Correlations and test of significance were performed using SPSS 16.0 version for Windows program (SPSS, Inc.)

3. Results and Discussion

Bioremediation is currently an attractive technology that researchers have been studying to understand the process and mechanisms with a view to developing an optimized technique for the management of toxic pollutants in the environment. In this study, the crude oil bioremediation efficiency of indigenous soil fungal community spike with cassava peels at various concentrations was study. Preliminary physicochemical analyses of the soil sample and cassava peels utilized in the study revealed are shown in Table 1. It was observed that the soil texture was sandy loam and with pH that was slightly acidic in nature (6.71). Soil pH is important because most microbial species can survive only within a certain pH range. Furthermore, soil pH can affect the availability of nutrients in the soil. Interestingly, the soil contained appreciable percentage of moisture content (8.45) and phosphorus (8.98 mg/kg), but with relatively low amount of Nitrogen (0.17%), a reflection of the source of the soil sample. Nitrogen and phosphorus are the two vital limiting factors in biodegradation potentials of most microorganisms, hence the need for supplements. The cassava peel was slightly acidic (pH 6.56) but contain relatively high content of Phosphorus (22.08 mg/kg) and Nitrogen (2.45%) content, hence the rational for the use of the bio-waste in in bio-stimulation of indigenous soil fungi community.

Parameter	Soil	Cassava Peel
pH	6.71	6.56
Moisture (%)	7.45	2.23
Organic carbon (%)	2.92	36.91
Nitrogen (%)	0.17	2.45
Phosphorus (mg/kg)	10.98	22.08
C: N ratio	11:01	15:01
Soil Textural class	Sandy Loam	--

Table 1: Physicochemical Properties of soil and cassava peels used for myco-remediation

The biodegradation study of crude oil in soil throughout the 42 days' period are detailed in Figure 1. The results revealed a high percentage of biodegradation of the crude oil by the native fungi in soil supplemented with cassava peels (CP) when compared to the control soil treatment. The CP had a significant influence on the biodegradation process, though in a concentration dependent pattern, with the highest CP (400 g) soil treatment yielding the best biodegradation of 78.67 % (Fig 1). The other CPs (100 g and 200 g) amended crude oil polluted soils yielded 52.02 and 71.45 % biodegradation respectively after 42 days. While the crude oil

biodegradation observed in the un-amended crude soil polluted control was 45.21 % after 42 days. These observations suggest that CP must have stimulated the biodegradation capacity of the indigenous fungi in the amended soil. The CP could have acted as nutrient (N and P) supplier, soil bulking agent and or emulsifying agent, which had been reported to partly encourage microbial growth, cellular uptake and break down of crude oil components. Previous researchers had reported that most agro-waste could be potential nutrient suppliers and soil bulking agents for the bioremediation of hazardous chemicals in agricultural soils (Agarrya et al., 2010; Omoni et al., 2015; Romanus 2015a, b). These interesting findings on the biostimulation potentials of CP further confirmed the report of Romanus et al. (2015b), who recently submitted that cassava peels possessing ability to enhance crude oil biodegradation by bacterial population in soil. Its thus indicates that CP can stimulate autochthonous bacterial and fundal communities for crude oil bioremediation.

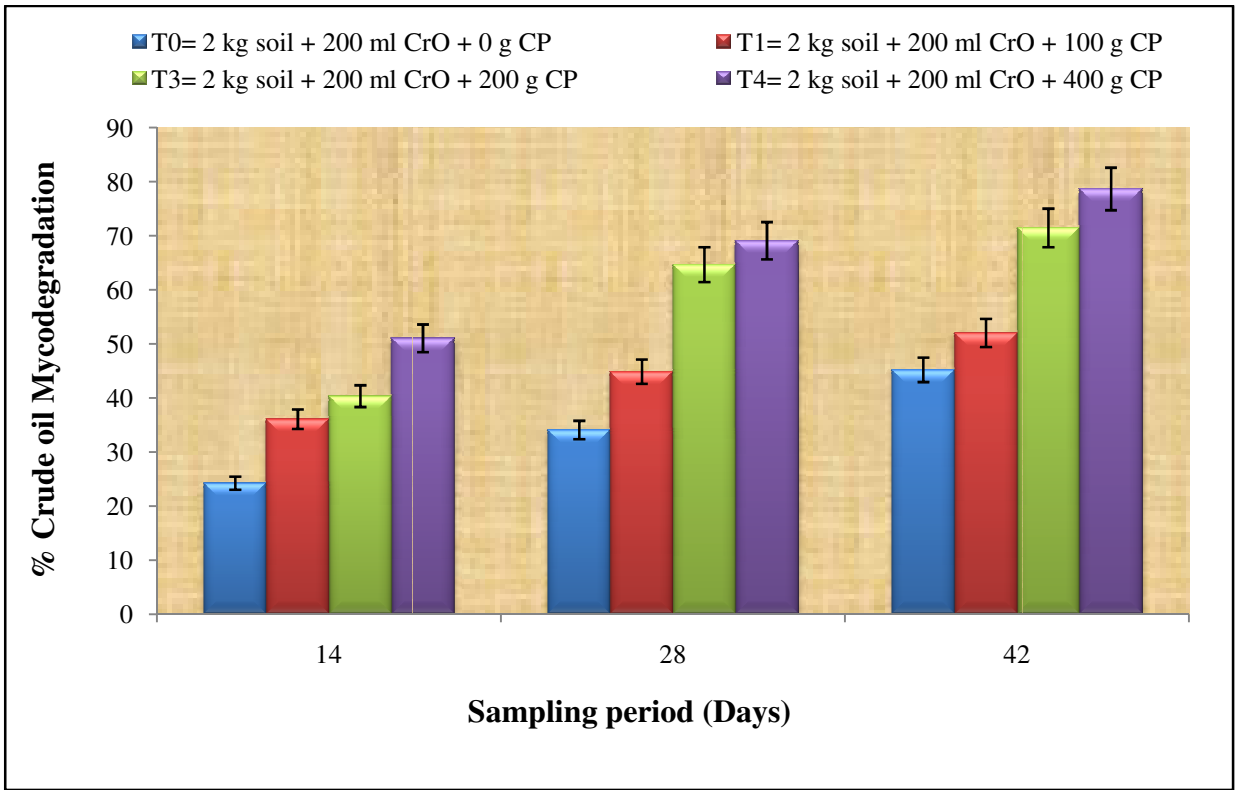

Figure 1: Myco-degradation of the cassava peel amended crude oil polluted soil microcosm

The results of total heterotrophic fungal counts (THFC) and hydrocarbon utilizing fungal counts (HUFC) are depicted in Figs 2 and 3. The THFC in soil contaminated with crude oil and amended with CPs ranged between 36.45 x 10^5 cfu/g and 219.08 x 10^5 cfu/g.The increase in the fungal count was observed to significantly ($P<0.05$) increase with increase in concentration of the amendment used. Of course this was expected considering the fact that the added CP boosted the limiting nutrient content which are essential for indigenous aerobic fungal multiplication. Nutrient availability has been reported as one of the major factors that stimulates the growth and maintenance of microbial community in crude oil contaminated ecosystems (Okoh, 2006). The un-amended control soil had THFC ranging between 20.45 x 10^5 cfu/g and 57.89 x 10^5 cfu/g. The effect of the increased fungal growth reflected in the increased rate of hydrocarbon biodegradation in the treated soils (Fig 1). This is an indication that the indigenous fungal population could grow in the presence of crude oil hydrocarbon.

A similar trend was recorded among the HUFC, though their population were lower than the THFC in the soil sample (Figure 3). From the analysed results, the HUFC ranged from 45.89 x 10^4 cfu/g in crude oil contaminated soil amended with 100 g CP through 55.68 x 10^4 cfu/g in the 200 g CP treatment to 60.23 x 10^4 cfu/g in 400 g amendment after 14 days' incubation period. After the 42 days of biodegradation, the counts were significantly higher; 72.9 x110^4 cfu/g, 89.34 10^4 cfu/g and 92.89 x 10^4 cfu/g respectively. The HUFC for un-amended crude oil soil treatment rose from 31.00 x 10^4 cfu/g and peaked at 49.30 x 10^4 cfu/g after 28 days and later decreased to 45.89 x 10^4 cfu/g.

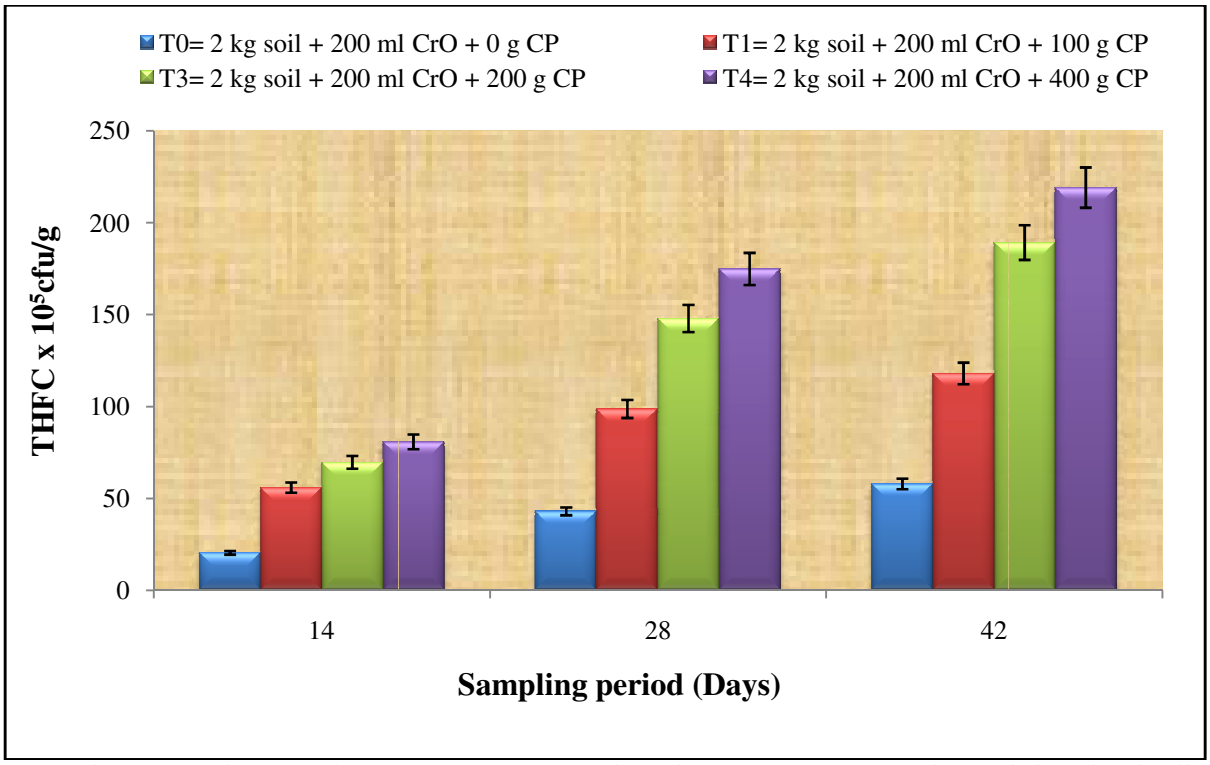

Figure 2: Total Heterotrophic Fungal Count (THFC) of Myco-degradation of cassava peel amended crude oil polluted soil

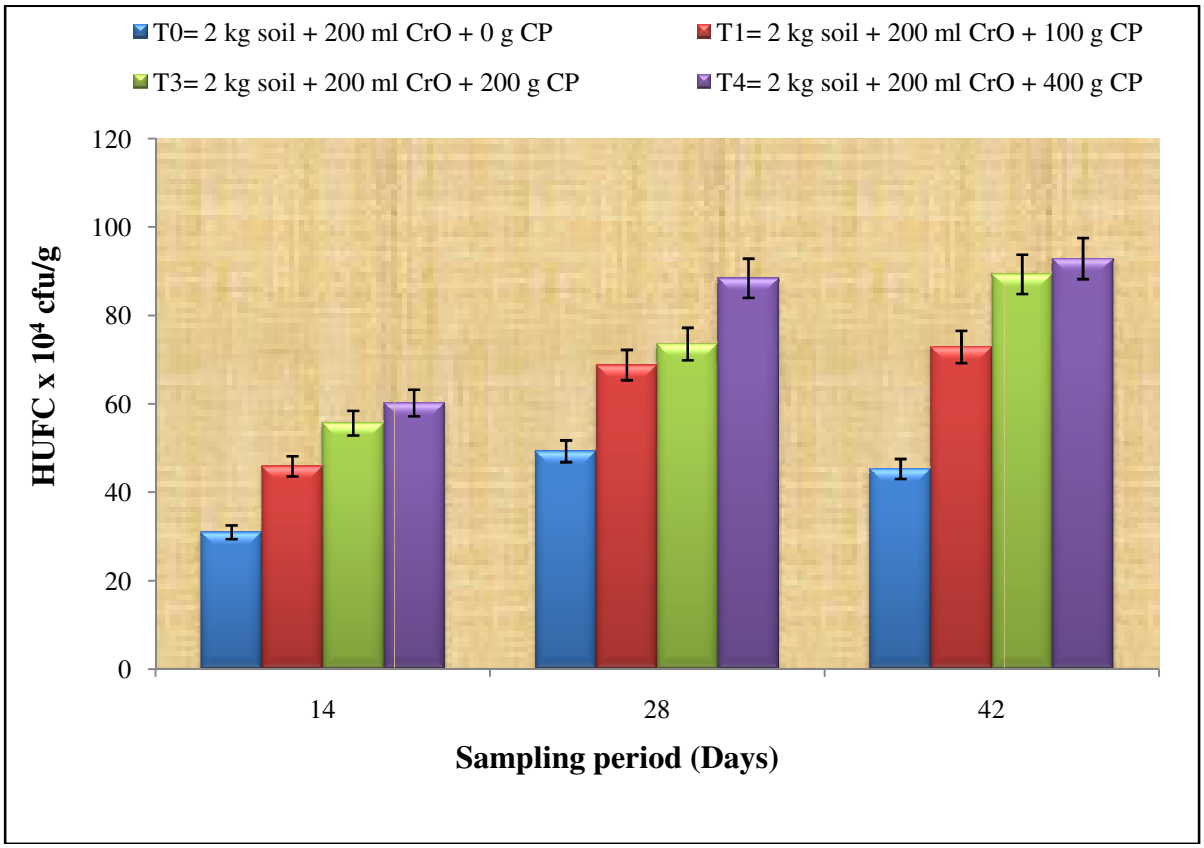

Figure 3: Hydrocarbon Utilizing Fungal Count (HUFC) of Myco-degradation of cassava peel amended crude oil polluted soil

A comparison of these HUFC with the observed rate of biodegradations (Figure 1) showed that the fungal growth increased with increased in the rate of hydrocarbon utilization for all the CP amendments. For instance, an increase in HUFC from 45.89 x 10^4 cfu/g (100g CP amendment) after 14 days to 92.89 x 10^4 cfu/g (400g CP amendment) resulted in increase an in crude oil biodegradation from 36.09 % to 78.67 % after 42 days respectively. This was not the case with the un-amended control soil, where despite the

decrease in the HUFC after 28 days, there was still an increase in their percentages crude oil biodegradation. A possible reason for this growth pattern is marked competition, antagonism and or predation by the individual crude oil degradation fungal community. Mixture of different crude oil utilizing fungal species could result in competition with others for the available resources (nutrients and prevailing environmental conditions) leading to loss of part or all of their populations within the new microbial community. The observation is in agreement with the report of Atlas and Bartha (1998) who stated that the populations might achieve lower maximal due competition. Another possible suggestion is that added CPs might have neutralized the toxic effect of the crude oil on the fungal population through rapid improvement of the soil physicochemical properties, such as buffering the effect of extreme pH change. A similar submission was reported by previous studies where guinea corn shaft, banana, plantain, and melon peels were utilized as bio-stimulant for the bioremediation of hydrocarbon contaminated soil (Abioye, et al., 2009; Agarrya et al., 2010; Omoni et al., 2015; Romanus 2015a, b).

The results of pH analysis during the bioremediation study showed that the amended crude oil contaminated soil had pH of (6.76 – 6.98), while the unamended recorded pH of 6.88 – 7.92 after 42 days (Figure 3). This further reaffirms the capacity of CP to buffer the effect of extreme pH changes. Extreme pH change is one of the key physicochemical properties that affect the bioremediation of petroleum hydrocarbon in the environment (Atlas, 1991; Okoh, 2006). Hence, CP application did not significantly ($P < 0.05$) affect the pH of the bioremediation study, unlike the unamended control after 42 days. Moreover, the pH variations observed were within the favourable range for activities of soil fungi. Previous researchers had reported similar pH trends hence, this finding is in line with their reports (Abioye et al 2009; Agarry and Jimoda, 2013; Romanus et al., 2015b).

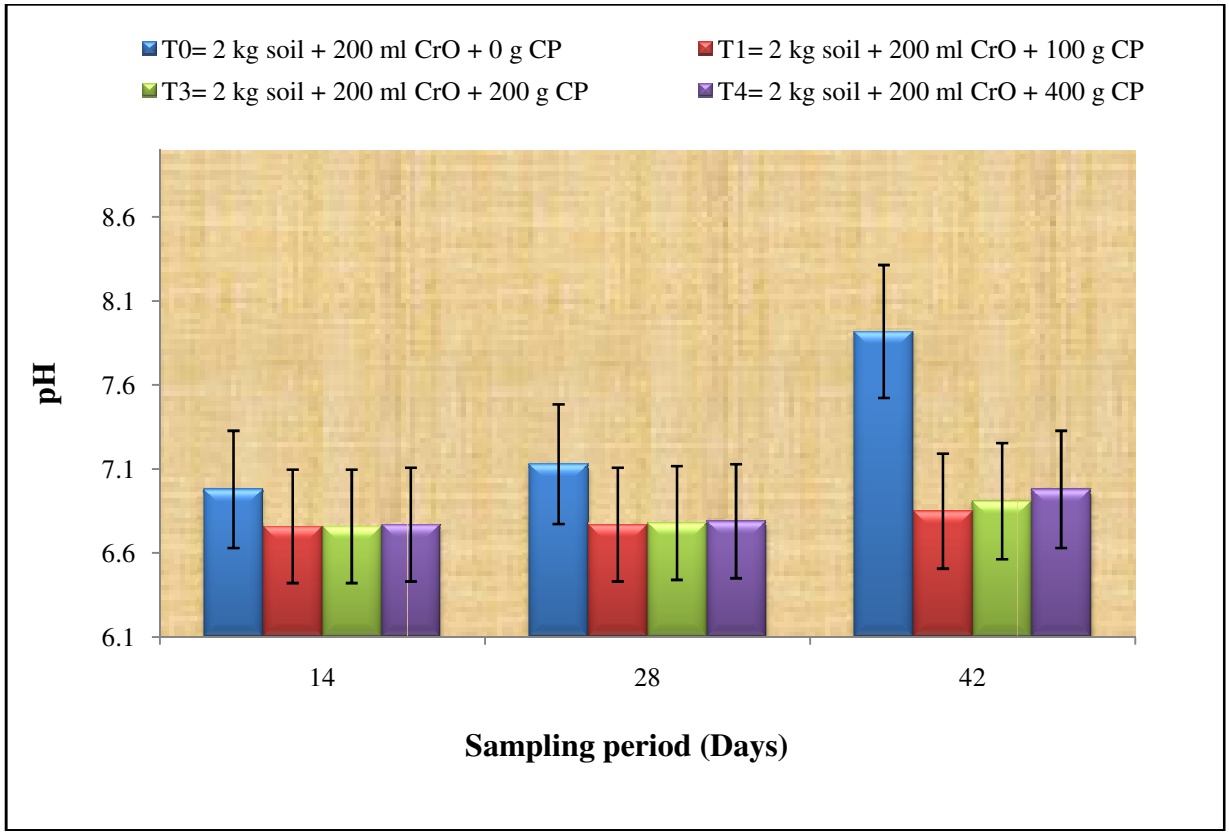

Figure 3: pH of Myco-degradation of cassava peel amended crude oil polluted soil

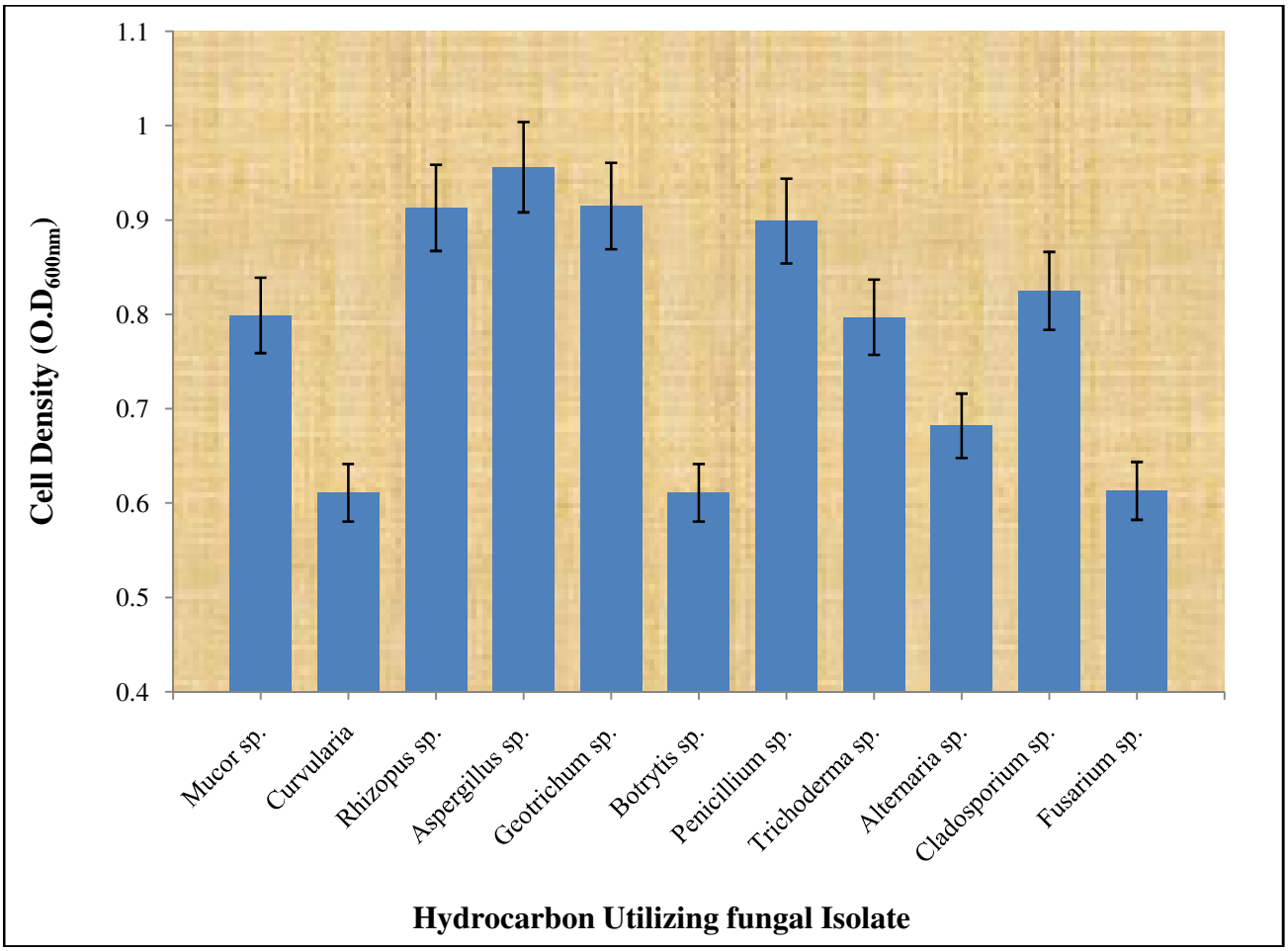

Figure 4: Cell density of the fungal isolates

A total of 11 hydrocarbon utilizing fungi was isolated, namely: *Rhizopus* sp*, Fusarium* sp*, Apergillus* sp*, Mucor* sp*, Geotrichum* sp*, Curvularia* sp*, Penicillium* sp*, Aternaria* sp*, Cladosporium* sp, *Trichoderma* sp, and*Botrytis*sp. The seven most significant (P<0.05) crude oil utilizing fungi based on the increase in their cell biomass were *Apergillus sp*. *Geotrichum* sp, *Rhizopus* sp, *Penicillium* sp, *Trichoderma* sp., *Cladosporium* sp. and *Mucor* sp. The least crude oil utilizers were *Curvularia, Alternaria, Botrytis and Fusarium.* (Figure 4). Some of these hydrocarbon utilizing indigenous fungi had been isolated from crude oil contaminated soils by earlier researchers in the Niger Delta region. In their study, Chikere and Azubuike (2014) reported the isolation of *Aspergillus, Candida, Penicillium, Rhizopus, Saccharomyces, Fusarium, Mucor Cladosporium, Fusarium, and Mucor* from hydrocarbon polluted sediments and water in Portharcourt. In the same vain, *Fusarium* sp., *Zygorrhynchus* sp., *Geotrichum* sp., *Cladosporium* sp., *Aspergillus japonicas, Geotrichum* sp., *Yarrowia lipolytica, Zygorrhynchus* sp., and Yarrowia *lipolytica*were isolated from crude oil contaminated soils in Sakpenwa community, Niger Delta (Iheanacho et al., 2014). Thus, the indigenous fungi isolated in this study are members of groups that have been well reported in the literature as petroleum hydrocarbon degraders. Therefore, these fungal can be used singly or in appropriate combination to clean up petroleum contamination in the environment. Moreover, the significantly high crude oil biodegradation demonstrated by the indigenous fungal community in CP amended crude oil contaminated soil further gave credence to the positive contribution of organic wastes in biorediation of crude oil hydrocarbons.

3.1. Conclusion

It is evident from this study that the myco-remediation potentials of indigenous fungal flora of crude oil contaminated soils could be stimulated for greater efficiency by addition of calculated amount of cassava peels waste. Thus, CP could therefore be considered as an appropriate enhancing bio-agent in the clean-up petroleum contamination soils, especially in many crude oil contaminated sites in Niger Delta, where cassava waste is generated massively and dumped indiscriminately in the environment.

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