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Phylogenetic relationship of hydrocarbon degrading fungi species in bioremediation

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ABSTRACT: The study focussed on the phylogenetic relationship of hydrocarbon degrading fungi species in bioremediation. Hydrocarbon degrading fungi species were isolated from soil polluted with crude oil and treated with varying concentrations of agro-wastes. DNA sequencing was done on the consortia of fungi isolated. The evolutionary history was inferred by using the Maximum likelihood method based on the Tamura-Nei model. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic relationship between the fungal species revealed that the organisms were grouped into two clusters, cluster 1 consist of *Aspergillus oryzae, Aspergillus flavus, Talaromyces solicola, Penicillium purpurogenum, Penicillium citrium* and *Penicillium daleae* while cluster 2 consist of *Cunninghamella polymorpha* and *Cunninghamella bertholletiae.* The results revealed that *Aspergillus oryzae* differs from *Aspergillus flavus*, while other organisms had high relationship with each other. The *Cunninghamella polymorpha* and *Cunninghamella bertholletiae* were highly related. The sequencing and phylogenetic of the fungi species revealed the specific identity of the organism and showed the similarity between the identified species.

Key words: Bioremediation, fungi, hydrocarbon, phylogenetic, pollution.

INTRODUCTION

The biodegradation of pollutants in the environment is a complex process whose quantitative and qualitative aspects depends on the nature and amount of the pollutant present, the ambient and the seasonal environmental condition, and the constitution of the indigenous microbial community (Leahy and Colwell, 1990). Microbial biodegradation of crude oil in the environment is said to be comparatively slow because it is influenced by a number of factors such as proximity of oil pollutants to microorganisms, population of hydrocarbon biodegraders, temperature and nutrient availability (Atlas and Bartha, 1983). During microbial degradation of petroleum hydrocarbons, the n-alkane chain length is one of the most important factors because shorter-chain petroleum hydrocarbons are generally degraded more rapidly than longer-chain hydrocarbons (Jonge et al., 1997; Seklemova et al., 2001; Mohanty and Mukherji, 2008). In addition to chain length, petroleum hydrocarbon decomposition efficiency is also determined by structures of oil hydrocarbons (for example, polyclic structures) as well as by basic soil system characteristics including water, pH, temperature, mineral nutrients, nitrogen, phosphorus, and organic compounds. Although petroleum is a source of abundant carbon, its lack of nitrogen and phosphate makes it far less biodegradable than other bio-wastes such as food waste, sewage sludge, livestock manure and so on (Atlas, 1981; Michel et al., 1993; Beaudin et al., 1999; Head and Swannell, 1999; Namkoong et al., 2002; Supaphol et al., 2006).

Microbial degradation is a natural mechanism by which

one can clean up the petroleum hydrocarbon pollutants from the environment (Atlas and Bartha 1992; Amund and Nwokoye, 1993). The population of microorganisms found in a polluted environment will degrade crude oil differently and at a different rate than microorganisms in a relatively clean environment (Obire and Okudo, 1997). Crude oil utilizing bacteria can tolerate oil-contaminated environment because they possess the capacity to utilize oil as energy sources (Song et al., 1990). Other species may not and are gradually eliminated. The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones et al. (1983). They studied the extensive biodegradation of alkyl-aromatic hydrocarbons in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and microorganisms such as, *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas* and *Rhodococcus* were found to be involved in alkylaromatic degradation. Microbial degradation also appears to have been a major factor in the disappearance of oil stranded within the littoral zones (Betts, 1998). In soil, the fate of crude oil is a function of both abiotic and biotic processes, which include transport and sorption, chemical catalysis, photooxidation and biodegradation. However, oxidation by microorganisms and photooxidation are probably the major ways hydrocarbons are removed from the environment. Microbial utilization of crude oil in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye et al. (2006). Nine bacterial strains viz. *Pseudomonas fluorescens, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus* sp*., Alcaligenes* sp*., Acinotobacter Iwoffi, Flavobacterium* sp*., Micrococcus roseus and Corynebacterium* sp. were isolated from the polluted stream which could degrade petroleum hydrocarbons. Thus, this study seeks to evaluate the potentials of agricultural wastes in enhancing the proliferation of hydrocarbon degrading fungi and to determine the similarities between the fungi species isolated.

MATERIALS AND METHODS

The research was carried out in the Environmental Biotechnology unit in the Department of Genetics & Biotechnology, University of Calabar. Six kilograms of dry soil samples each were filled into one hundred and fifty plastic buckets and artificially polluted with 300 ml of Bonny light crude oil. The plastic buckets containing the polluted soils were treated with 0%, 3%, 6% and 10% of groundnut husk powder ($GnH_{14}P$), maize cob powder ($MaC_{14}P$), cassava peels powder ($CasP_{14}P$) and oil palm husks powder ($EFBOP_{14}P$) in single and combined forms and allowed for a duration of 30 days, 60 days and 90 days. Isolation of fungi was carried out in the Department of Microbiology (University of Calabar) and DNA extraction was performed in the Nigerian Institute of Medical Research (Lagos State), while sequencing analysis was done at Inqaba Biotechnology Pty South Africa.

DNA extraction

DNA Extraction was performed at the Anaerobe Laboratory, Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, Yaba Lagos. Methodology was based on PCR and metagenomics analysis. While sequencing analysis was done at a Inqaba Biotechnology Pty South Africa.

DNA extraction was carried out using 24 hours cultured microbial isolates in nutrient broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells were washed three times in 1 ml of Ultra-pure water by centrifuging at 12,000 rpm for 5 minutes. DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps, Model D6005 (Zymo Research, California, USA). 50-100 mg of bacterial cells were re-suspended in 200 µl of sterile water. This was transferred into a ZR Bashing Bead™ Lysis Tube. Exactly 750 µl Lysis Solution was added to the tube. The bead containing the solution was secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR Bashing Bead™ Lysis tube was Centrifuged in a micro centrifuge at 10,000 x g for 1 minute. 400 µl of the supernatant was pipeted into a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1 minute. This was followed by the addition of 1,200 µl of Fungal/Bacterial DNA Binding Buffer into the filtrate in the collection tube. After this 800 µl of the mixture was transferred into a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

The flow through was discarded from the collection tube and the process was repeated to obtain the remaining products. The 200 µl DNA Pre-Wash Buffer was added into the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 x g for 1 minute. This was followed by the addition of 500 µl Fungal/Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred into a clean 1.5 ml micro centrifuge tube and 100 µl of DNA Elution Buffer was then added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in an ice packed container to the laboratory for sequencing.

DNA Sequencing

DNA sequencing was performed by Sanger (dideoxy) Sequencing Technique and was used to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied

Biosystems (Russell, 2002; Metzenberg, 2003). The results were obtained as nucleotides when amplified using sequencing primer -16S: 27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 518R: 5'-ATTACCGCGGCTGCTGG-3' (Weisburg et al., 1991). Sequence data from resultant nucleotides base pairs were downloaded and read using FinchTv software followed by direct nucleotide blasting (http://blast.ncbi.nlm.nih.gov). For every set of isolate, a read was BLASTED and the resultant top hits for every BLAST result showing species name was used to name the specific organism. Corresponding gene back accession number and query length of sequences blasted was also recorded.

Statistical analysis

Data collected were subjected to a three-way analysis of variance using SPSS soft-ware and significant means were separated using Least significant difference test at 5% and 1% probability level.

RESULTS AND DISCUSSION

Crude-oil contamination reduces the population of non-hydrocarbon utilizing fungal species in soil and survival of the hydrocarbon utilizing fungal is reduced. The degradation process would take a longer time to complete, as a mean of accelerating the rapid growth of hydrocarbon-utilizing fungi left in soil, agro-wastes at varying levels were applied in the soils. The total fungi (TF) in soil treated with 6% and 10% $EFBOP_{14}P$, 10% $CasP_{14}P$ + $EFBOP_{14}P$ and soil treated with 6% and 10% $GnH_{14}P$ + $CasP_{14}P$ had more counts than the pristine soil (PS +ve), crude oil polluted soil (COPS -ve) and other treatment groups. These were followed by soils treated with 6%, 10% $GnH_{14}P$, 3%, 6%, 10% $MaC_{14}P$, $GnH_{14}P$ + $MaC_{14}P$, $CasP_{14}P$ + $MaC_{14}P$, $MaC_{14}P$ + $EFBOP_{14}P$, 3% and 6% $GnH_{14}P$ + $EFBOP_{14}P$ and 3% $GnH_{14}P$ + $CasP_{14}P$ with no variation in the mean TF counts of the soils. These counts were also more than the counts obtained in the PS and COPS soil. The results implied that the treatment of the polluted soil with agro-wastes increase the TF counts of the soils than the counts obtained in the controls. The PS and COPS had no significant variation in the mean TF counts of the soil. Figure 1 showed the results of the TF counts of the soil. Due to duration of the study, it was observed that the average TF counts of the soil treated with $CasP_{14}P$ + $MaC_{14}P$ at 60 days were more than other amended groups and durations. These were also followed by soil ameliorated with $CasP_{14}P$ + $MaC_{14}P$ at 30 days of soil treatment. The soil treated with $CasP_{14}P$ + $EFBOP_{14}P$ was next, higher ($P<0.05$) than the counts obtained from soil enhanced with $EFBOP_{14}P$ at 60 days of soil treatment. These were followed by other treated soils that showed no variation in the mean TF counts of the soils. The increased in fungal counts were observed to be significantly higher at 60 days of the study than other study period. These results could imply that the complete acclimatization of the fungal population exposed to the agro-wastes was achieved at 60 days of soil study. The fungal population in soil treated with $CasP_{14}P$ + $MaC_{14}P$ were higher than other treatment groups, while the combined form of the different agro-wastes resulted in a high fungal counts than the single treatments. However, the soils treated with 10% $CasP_{14}P$ + $MaC_{14}P$ had increased crude oil-utilizing fungi (CUF) counts, these were followed by the counts obtained in soils treated with 6% $CasP_{14}P$ + $MaC_{14}P$, further followed by soils treated with 10% $GnH_{14}P$ + $MaC_{14}P$ and 10% $MaC_{14}P$ + $EFBOP_{14}P$ with insignificant difference ($P>0.05$) in the mean values obtained. These were more than the count obtained in soils treated with 3% $CasP_{14}P$ + $MaC_{14}P$ and 6% $GnH_{14}P$ + $MaC_{14}P$ which had insignificant differences ($P>0.05$) in the average CUF counts obtained in the soils. The soils treated with 3%, 6% $GnH_{14}P$, 3%, 6% $MaC_{14}P$, 3% $GnH_{14}P$ + $MaC_{14}P$, 10% $EFBOP_{14}P$, 6%, 10% $CasP_{14}P$ + $EFBOP_{14}P$, 6% and 10% $GnH_{14}P$ + $EFBOP_{14}P$, and 3%, 6% $MaC_{14}P$ + $EFBOP_{14}P$ had insignificant differences ($P>0.05$) in the average fungal counts obtained in soils but significantly higher than the CUF counts obtained in soils treated with 3%, 6%, 10% $CasP_{14}P$, and 3%, 6%, 10% $EFBOP_{14}P$ and the pristine and crude oil polluted soils (Table 1). The results as presented on Table 1 showed that no variation existed between the CUF counts obtained in the pristine and the crude oil polluted soils. The results obtained for the crude oil-utilizing fungi at different duration of study indicate that there were differences in the mean values obtained. The soils treated with $CasP_{14}P$ + $MaC_{14}P$ at 60 days of study showed significantly higher ($P<0.05$) mean fungal counts. These were followed by the fungal counts obtained in soil treated with $GnH_{14}P$ + $MaC_{14}P$ at 60 days, $CasP_{14}P$ + $MaC_{14}P$ at 30 days and 90 days and $MaC_{14}P$ + $EFBOP_{14}P$ at 60 days with no variation in the mean CUF counts obtained in the soil but more than the mean fungal counts obtained in soils treated with $GnH_{14}P$ + $MaC_{14}P$ at 30 days, $MaC_{14}P$ + $EFBOP_{14}P$ and $GnH_{14}P$ + $CasP_{14}P$ at 30 days of soils treatment. The results as presented in Figure 2 indicate that the crude oil utilizing fungal population obtained from soils treated with the single amendment was reduced as compared to some of the amendments in combined form. Figure 2 also showed that the CUF counts obtained at 60 days were more than the counts obtained at 30 days and 90 days.

The phylogenetic relationship between the fungal species revealed that the organisms as presented on Table 2 were grouped into two clusters, cluster 1 consist of *Aspergillus oryzae, Aspergillus flavus, Talaromyces solicola, Penicillium purpurogenum, Penicillium citrium* and *Penicillium daleae* while cluster 2 consists of *Cunninghamella polymorpha* and *Cunninghamella bertholletiae.* The results revealed that *Aspergillus oryzae* differs slightly from *Aspergillus flavus*, while other

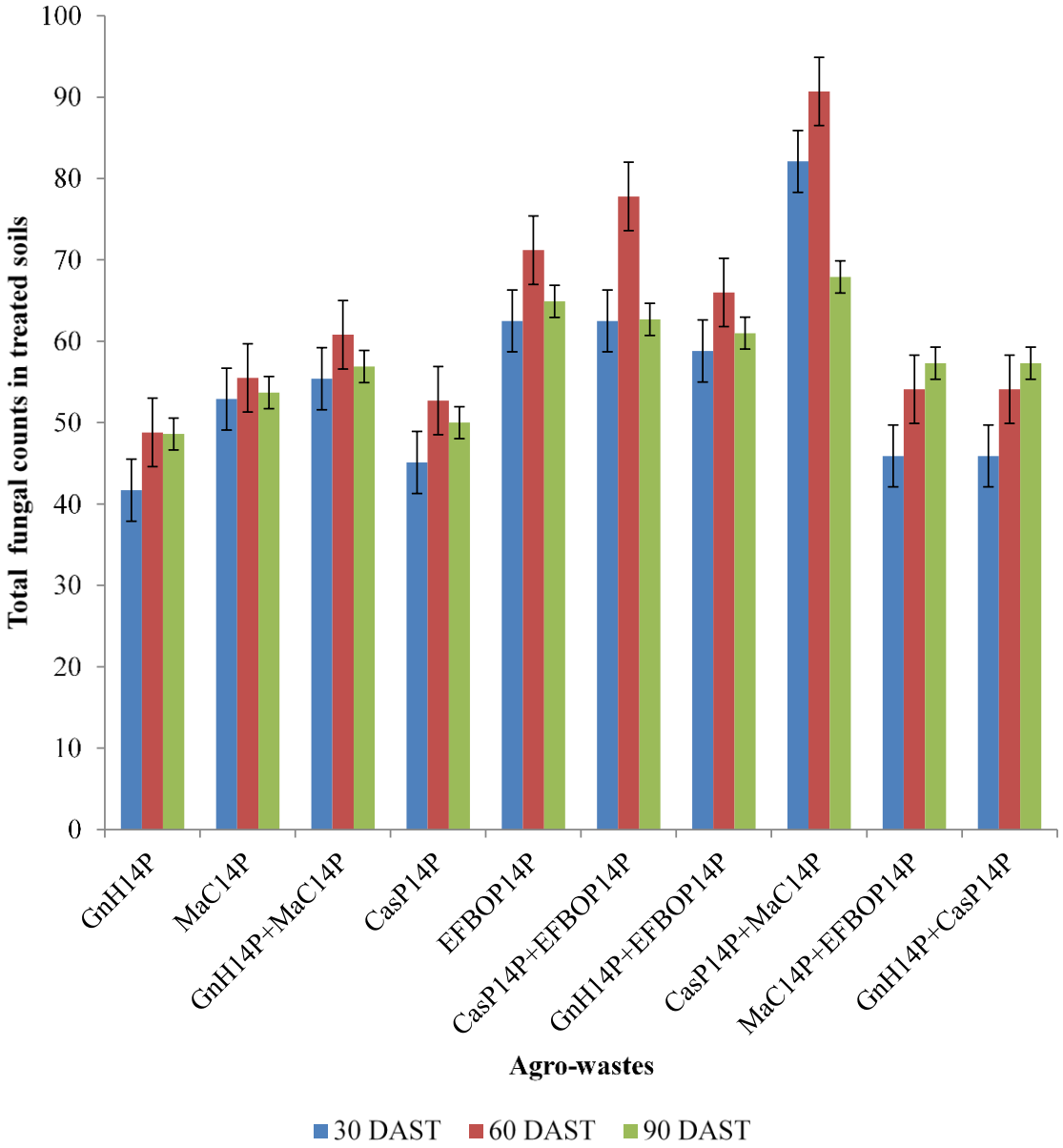

Figure 1. Total fungal counts in polluted soils treated with agro-wastes. $MaC_{14}P$, Maize cob 2014 powder; $EFBOP_{14}P$, Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$, Cassava peels 2014 powder; DAST, Days after soil treatment.

organisms had very high cordial relationship with each other. The *Cunninghamella polymorpha* and *Cunninghamella bertholletiae* were strongly related (Figure 3). Mycoremediation is an important aspect of bioremediation, it is strongly reflected in the ability of specific fungi species to show degradation potentials in reducing petroleum hydrocarbons in soil environment. Molecular methods have been widely applied in the identification of a large number of *Aspergillus* spp. DNA amplification followed by DNA sequence analysis is a powerful tool in taxonomy studies. *Aspergillus* are among the best studied fungi genetically. The complete genome of *A. flavus* is now completely sequenced and has been released to the NCBI in July 2005. However, *A. flavus* and *A. oryzae* isolated from the bioremediated soils are genetically similar and difficult to differentiate. They possess high degrees of DNA relatedness and similar genome size (Kurtzman et al., 1987). The phylogram obtained in the study showed that *A. flavus* and *A. oryzae* are genetically similar with no much divergence. Gersinde et al. (2008) and El-shafie et al. (2007) reported that *Aspergillus sp*, *Penicillium sp* and *Rhizopus* sp isolated from soils environment possessed degrading properties for petroleum hydrocarbons. The degradation of hydrocarbon by fungi has also been

Table 1. Fungal population in crude oil amended soils.

Parameters	Treatment levels	TFC (CFU/g)	CUFC (CFU/g)
	PC	$3.92^{d}\pm1.49 \times 10^{6}$	$4.21^{f}\pm0.99 \times 10^{6}$
	COC	$3.43^{d}\pm1.23 \times 10^{6}$	$3.43^{f}\pm1.07 \times 10^{6}$
$GnH_{14}P$	3%	$4.48^{d}\pm1.49 \times 10^{6}$	$5.16^{f}\pm3.91 \times 10^{6}$
	6%	$5.57^{c}\pm1.88 \times 10^{6}$	$6.06^{e}\pm3.39 \times 10^{6}$
	10%	$5.79^{c}\pm1.83 \times 10^{6}$	$6.81^{e}\pm3.36 \times 10^{6}$
$MaC_{14}P$	3%	$5.17^{c}\pm1.24 \times 10^{6}$	$4.80^{f}\pm1.61 \times 10^{6}$
	6%	$6.78^{c}\pm2.33 \times 10^{6}$	$6.69^{e}\pm1.58 \times 10^{6}$
	10%	$7.72^{c}\pm1.40 \times 10^{6}$	$7.37^{e}\pm2.17 \times 10^{6}$
$GnH_{14}P+MaC_{14}P$	3%	$6.29^{c}\pm2.23 \times 10^{6}$	$7.10^{e}\pm4.40 \times 10^{6}$
	6%	$7.03^{c}\pm1.26 \times 10^{6}$	$8.26^{d}\pm5.37 \times 10^{6}$
	10%	$8.18^{c}\pm2.33 \times 10^{6}$	$9.71^{c}\pm4.9 \times 10^{6}$
$CasP_{14}P$	3%	$5.01^{c}\pm3.01 \times 10^{6}$	$4.41^{f}\pm1.92 \times 10^{6}$
	6%	$5.89^{c}\pm2.89 \times 10^{6}$	$5.04^{f}\pm2.30 \times 10^{6}$
	10%	$6.39^{c}\pm1.94 \times 10^{6}$	$5.93^{e}\pm1.64 \times 10^{6}$
$EFBOP_{14}P$	3%	$6.0^{c}\pm1.29 \times 10^{6}$	$5.12^{f}\pm2.28 \times 10^{6}$
	6%	$9.38^{a}\pm2.41 \times 10^{6}$	$5.84^{e}\pm2.8 \times 10^{6}$
	10%	$1.04^{a}\pm3.37 \times 10^{7}$	$6.30^{e}\pm3.25 \times 10^{6}$
$CasP_{14}P+ EFBOP_{14}P$	3%	$7.14^{c}\pm2.24 \times 10^{6}$	$4.84^{f}\pm1.56 \times 10^{6}$
	6%	$8.43^{c}\pm6.05 \times 10^{6}$	$5.86^{e}\pm2.01 \times 10^{6}$
	10%	$1.09^{a}\pm5.69 \times 10^{7}$	$6.31^{e}\pm1.89 \times 10^{6}$
$GnH_{14}P+ EFBOP_{14}P$	3%	$5.79^{c}\pm1.72 \times 10^{6}$	$8.38^{d}\pm2.87 \times 10^{6}$
	6%	$8.08^{c}\pm2.37 \times 10^{6}$	$1.08^{b}\pm4.6 \times 10^{7}$ $1.32^{a}\pm6.69 \times 10^{6}$
	10%	$9.78^{a}\pm2.44 \times 10^{6}$	$1.29^{d}\pm7.16 \times 10^{7}$
$CasP_{14}P+MaC_{14}P$	3%	$5.01^{c}\pm3.01 \times 10^{6}$	$1.32^{d}\pm9.22 \times 10^{7}$
	6%	$5.89^{c}\pm2.37 \times 10^{6}$	$1.52^{c}\pm10.95 \times 10^{7}$
	10%	$6.39^{c}\pm1.94 \times 10^{6}$	$1.74^{a}\pm14.18 \times 10^{7}$
$MaC_{14}P+ EFBOP_{14}P$	3%	$5.2^{c}\pm2.92 \times 10^{6}$	$6.40^{e}\pm2.59 \times 10^{6}$
	6%	$6.53^{c}\pm2.85 \times 10^{6}$	$7.90^{e}\pm3.76 \times 10^{6}$
	10%	$7.12^{c}\pm2.89 \times 10^{6}$	$1.01^{c}\pm6.19 \times 10^{6}$
$GnH_{14P}+CasP_{14}P$	3%	$7.97^{c}\pm3.04 \times 10^{6}$	$3.97^{f}\pm1.30 \times 10^{6}$
	6%	$9.02^{a}\pm5.50 \times 10^{6}$	$4.69^{f}\pm1.72 \times 10^{6}$
	10%	$1.04^{a}\pm4.61 \times 10^{7}$	$5.08^{f}\pm1.60 \times 10^{6}$

$MaC_{14}P$, Maize cob 2014 powder; $EFBOP_{14}P$, Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$, Cassava peels 2014 powder; DAST, Days after soil treatment.

Table 2. Sequence identification of fungi species detected in agro-wastes amended soil samples.

Sample No.	Query No.	Gene bank accession no.	Identity of isolate obtained
1.	891	KJ767060.1	*Penicillium daleae*
2.	558	HQ596918.1	*Penicillium citrinum*
3.	490	KF619561.1	*Aspergillus oryzae*
4.	951	KM067097.1	*Aspergillus flavus*
5.	757	JN585934.1	*Penicillium sp. Cs/2/5*
6	979	KF983475.1	*Cunninghamella polymorpha*
7.	993	DQ681328.1	*Talaromyces purpureogenus* *Or Penicillium purpurogenus*
8	833	FJ654434.1	*Aspergillus flavus*
9	974	AF252930.1	*Cunninghamella bertholletiae*
10.	572	DQ155288.1	*Cunninghamella bertholletiae*

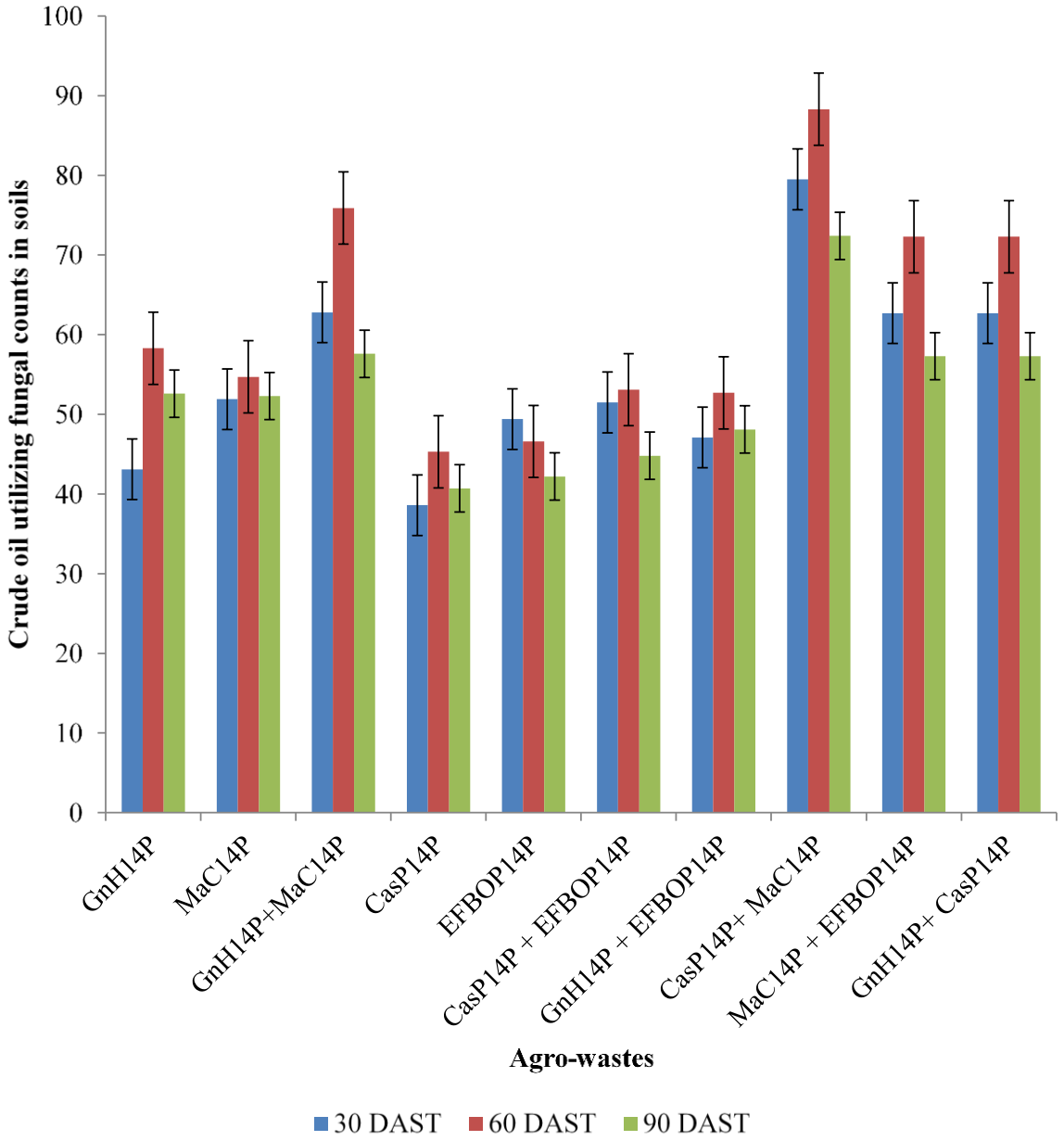

Figure 2. Crude oil utilizing fungal counts in polluted soils amended with agro-wastes. $MaC_{14}P$, Maize cob 2014 powder; $EFBOP_{14}P$, Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$, Cassava peels 2014 powder; DAST, Days after soil treatment.

reported by Adekunle and Oluyode (2002). The degradation ability of *A. flavus* in crude oil polluted environment have been widely reported by different researchers (Adekunle and Oluyode, 2002; Kurtzman et al., 1987). *Penicillium daleae* has been reported to be a strong degrader of monomeric rhamnogalacturonam-11 (mRG-11), a complex pectin polysaccharide ubiquitous in the primary plant cell wall. Polysaccharide as to hydrocarbons contain carbon and hydrogen molecules, the enzyme found in the *Penicillium daleae* that causes it to degrade polysaccharide, suggest that they could also be strong hydrocarbon degraders that aids in the breakdown of the petroleum hydrocarbon in the polluted soil amended with the agro-wastes. Favela-Torres et al. (2006) reported that *Penicillium* and *Aspergillus* species are able to produce pectinase (Pectinase is an enzyme that breaks down pectin, a polysaccharide found in plant cell walls). *Cunninghamella bertholletiae* and *Cunninghamella polymorpha* are species of *Zygomycetous* fungi in the order mucorales. The traditional methods identified this two fungi species as *Rhizopus* and *Mucor* species. The morphological attributes of *C. bertholletiae*

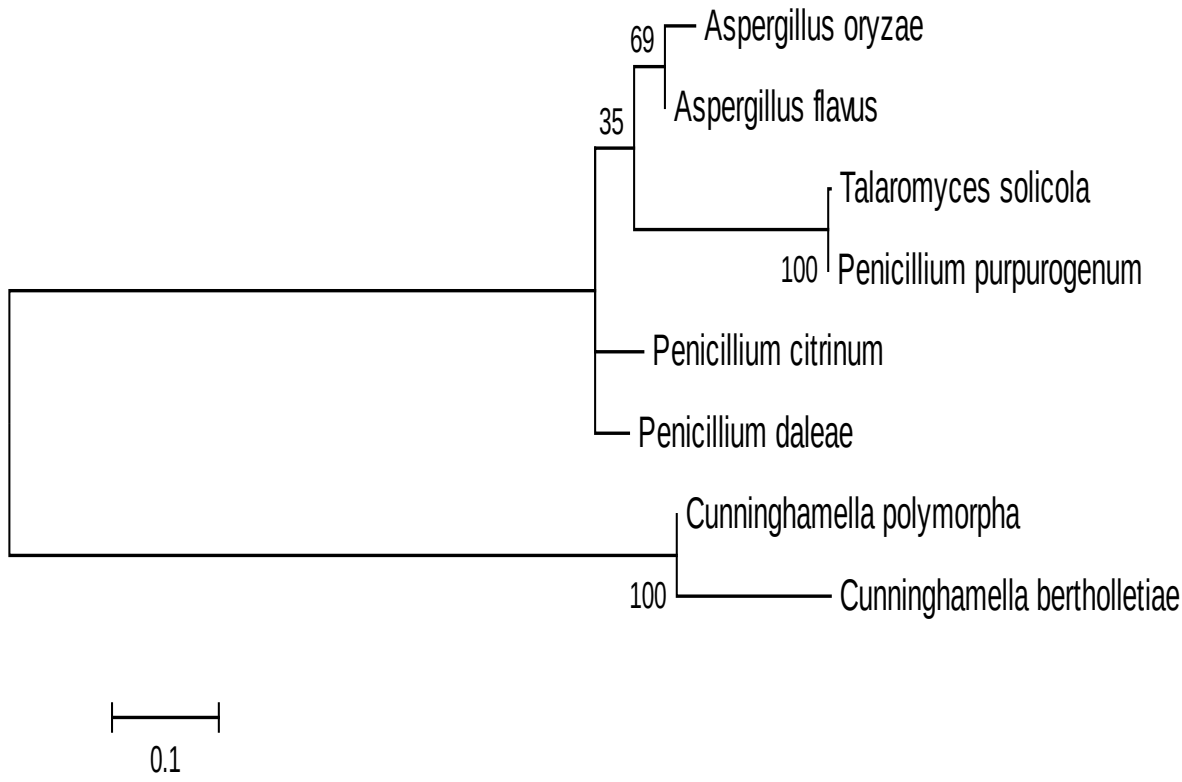

Figure 3. Phylogenetic relationship of hydrocarbon degrading fungal species.

and *C. polymorpha* identified in the crude oil amended soils had 100% similarity in their genetic composition. However, this fungi species grows as mold in soil environment, they grow rapidly on Sabourald agar with white appearance but become grey and powdery when they sporulate.

Conclusion

The results from the findings revealed that the amenability of the polluted soil with the agricultural wastes enhances the growth of hydrocarbon degrading fungi species in the soil. The phylogenetic diagram shows the genetic similarities of the fungi species. It is therefore, concluded that these fungi species should be use in enhancing the degradation process of hydrocarbon polluted soils.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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