IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 11, Issue 8 Ver. II (August. 2017), PP 01-09
www.iosrjournals.org

Assessment of bioaugmentation efficiency of *Penicillium chrysogenum* and *Aspergillus nudilans* in bioremediation of crude oil spill soil

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Abstract: *Assessment of bioaugmentation efficiency of two fungal species Penicillium chrysogenum and Aspergillus nudilans species on crude oil spill site (surface and underground soil) in Qio Tai, Ogoni land was evaluated. Penicillium chrysogenum and Aspergillus nudilans were used to augment the indigenous microorganisms present in the soil to speed up the degradation rate for a period of 28days at weekly interval (1, 7, 14, 21, and 28 days). The indigenous fungi isolates from the soil were identified to be of the following genera, Penicillium sp., Aspergillus sp., Histoplasma sp., Cladosporium sp., Mucor sp. and Alternaria sp. while the hydrocarbon utilizing bacteria were also identify to be Alcaligenes, Pseudomonas and Klebsiella. During the bioremediation process, it was observed that the augmenting organisms used were able to degrade the petroleum hydrocarbon in the soil. The initial Total Hydrocarbon Content (THC) of the un-augmented crude oil spill soil samples used as control (day 1) were: polluted surface soil (142422.14mg/kg), polluted underground soil (74779.29mg/kg); while on the day 28, the residual value were as follows: polluted soil with Penicillium chrysogenum (79279.28mg/kg) < polluted surface soil with Aspergillus nudilans (79422.14mg/kg) < surface soil control (92279.28mg/kg), polluted underground soil were as follows: polluted surface soil with Aspergillus nudilans (44636.43mg/kg) < polluted soil with Penicillium chrysogenum (47636.42mg/kg) < underground soil control (53993.59mg/kg). The percentage bioremediation rates of the fungal species for surface soil were as follows: polluted surface soil with Penicillium chrysogenum (36%) > polluted surface soil with Aspergillus nudilans (35%) > and control polluted surface soil (29%); while underground soil: polluted underground soil with Aspergillus nudilans (38%) > polluted underground soil with Penicillium chrysogenum (35%) > control polluted underground soil (27%). Comparatively, Penicillium chrysogenum (36%) express higher bioremediation potential than Aspergillus nudilans (35%) in the crude oil polluted surface soil while in the underground soil; Aspergillus nidulans (38%) had higher bioremediation potential than Penicillium chrysogenum (35%). This type of bioremediation shows that biologically cultured organisms aid the degradation of soil polluted with hydrocarbon and this method could be adopted for the remediation of a crude oil spill site. Summarily, bioremediation with the use of fungal isolate can effectively remove the petroleum hydrocarbons and shorten the remediation period, with the use of Penicillium chrysogenum for surface soil crude oil spill clean-up and Aspergillus nidulans for crude oil polluted underground soil bioremediation process.*

***Keywords**: Bioaugmentation, Bioremediation, Total Hydrocarbon Content, Penicillium chrysogenum and Aspergillus nudilans*

Date of Submission: 14-08-2017 Date of acceptance: 28-08-2017

I. Introduction

Most organic chemicals and many inorganic ones are subject to enzymatic attack through the activities of living organisms. Most of modern society's environmental pollutants are included among these chemicals, and the actions of enzymes on them are usually lumped under the term "biodegradation". The productive use of this biodegradative processes to remove, remediate or detoxify pollutants that have found their way into the environment and thereby threatening public health, usually as contaminations of soil, water, or sediments is Bioremediation. The term Bioremediation is the use of microorganism and their products to remove contaminant from the environment (USEPA 2000, 2012). It uses living system such as microorganisms mainly bacteria and fungi to restore polluted environment.

Soil contaminated with petroleum has a serious hazard to human health, causes organic pollution of ground water which limits its use, causes economic loss, environmental problems and decreases the Agricultural productivity of the soil (Wang, *et al.,* 2008). A great part of the oil pollution problem results from the fact that the major oil producing countries are not the major oil consumers. It follows that massive movement of petroleum have to be made from areas of high production to those of high consumption. Some types of

microorganisms are able to degrade petroleum hydrocarbons and use them as source of carbon and energy. The specificity of the degradation process is related to the genetic potential of the particular microorganisms to introduce molecular oxygen into hydrocarbon and to generate the intermediate and subsequently enter the general energy yielding metabolic pathway of the cell (Millioli, *et. al.,* 2009). Fungal potential in bioremediation processes is eminent as they act as primary decomposers. They are usually there to do the work when anything such as plants, animals or even non-living objects is ready to be broken down again into its molecular constituents. Fungi are found in soil, in fresh and sea water, inside the body of plants and animals and travelling through the air as spores. While they often are found functioning together with bacteria and are array of microorganisms, it is fungi that can handle or breakdown some of the largest molecules that are present in nature (Fermandez-Luqueno *et. al*., 2010). Potential of *Penicillium* species in the bioremediation field; fungi have been harnessed by humans for several applications for thousands of years. They are well known to degrade, or cause deterioration, respectively (Sing *et. al.,* 2006). *Penicillium* belongs to the phylum *Ascomycota* (Grimm *et. al.,* 2005). On the other hand, fungi are more versatile and more suitable in what concerned to the breadth of substrates they can use. Unlike fungi, bacteria are unable to degrade efficiently polycyclic aromatic hydrocarbons (PAH's) with more than four aromatic rings. The morphology and growth characteristics of fungi are responsible for the rapid colonization of substrates (Cox *et. al.,*1998).

Environmental assessment of ogoni land by UNEP reveals that Ogoni land has a tragic history of pollution from oil spills and oil wells fibres, although no systematic scientific information has been available about the ensuing contamination. The environmental assessment of Ogoni land covers contaminated soil, groundwater, surface water, sediment, vegetation, air pollution, public health, industry practices and institutional issues.

This study reports the bioaugumention effiency of two fungal species *Penicillium chrysogenum* and *Aspergillus nudilans* species on crude oil spill site (surface and underground soil) in Qio Tai, Ogoni land

II. Materials and Methods

Source of sample/collection

The soil samples collected for this study were taken from crude oil spill site in Gio Tai in Tai Local Government Area of Rivers State, Nigeria. The soil sample was taken from the surface and from the underground, with the use of shovel into two sterile black polythene bags containing the surface and the underground soil. The mouth of the polythene bag was kept loosely open so as to allow air penetration for the survival of the naturally occurring organisms present in the soil. After the collection of the soil sample, it was immediately transported to the laboratory for immediate analyses.

Sources of organism

The organisms used were fungi specifically *Penicillium chrysogenum* and *Aspergillus nudilans.* These organisms were isolated from the soil sample using a selective media for fungi (sabouroud dextrose agar). After which pure cultures obtained were inoculated into Modified Sabouraud dextrose broth in 500ml Erlenmeyer flask loosely plugged with sterile cotton wool for the growth of the augmenting test organisms. Broth cultures with optical density of 0.2 were used for augmentation.

Sample (soil) preparation and application of organisms

Soil (crude oil spill polluted soil from site) were collected in two locations, one batch is surface soil while the other is sub-surface soil tagged underground soil about 3 feet deep. The soil samples were collected in two batches, from the surface and from underground soil. Two thousand five hundred gram (2500g) each of the surface and underground soil were weighed into six test troughs (plastic rubber); three set for each soil. For a proper monitoring and interpretation; from each batch, a test trough is used as control in which organism was added while others both surface and underground were augmented with organisms (*Penicillium chrysogenum* and *Aspergillus nudilans*). 100ml of the broth cultured organisms were added to each of the setup sample and it was properly stirred with a sterile spatula for the organism to thrive successfully and have more oxygen. 50ml of sterile distil water were added to each experimental setup once in two days and stirred to enhance soil moisture content and organism activity. NOTE: The soil from the crude oil spill site were already heavily polluted with crude oil, hence no additional crude oil was used to contaminate the soil.

Batch A: *Crude Oil Polluted Surface Soil*		*Setup label*
(i)	2500g Control surface soil without added organism	CTRL[ss].
(ii)	2500g Polluted surface soil + 100ml *Aspergillus nidulans* broth culture	PS+AN[ss]
(iii)	2500g Polluted surface soil + 100ml *Penicillium chyrosogenum* broth culture	PS+PC[ss]
Batch B: *Crude Oil Polluted underground Soil*		
(i)	2500g Control underground soil without added organism	CTRL[us].

(ii) 2500g Polluted underground soil + 100ml *Aspergillus nidulans* broth culture PS+AN[us]
(iii) 2500g Polluted underground soil + 100ml *Penicillium chyrosogenum* broth culture PS+PC[us]

Total Hydrocarbon Content (THC) analysis

Total Hydrocarbon Content (THC) analyses were carried out on all the six setups using spectrophotometric method for Day 1, 7, 14, 21 and 28.

Media Used

Sabouroud Dextrose Agar: It is use for the isolation of fungi isolates. The preparation of this media is 65g into 1000ml of distill water, so you used the manufacturer specification to calculate depending on the number of plate to be prepared. After the preparation it is then autoclave at 121°C for 15 minutes and then aseptically poured the media into the plates for inoculation.

Nutrient Agar: It is a general purpose medium supporting the growth of a wide range of non fastidious organisms. Nutrient agar was used for the isolation of total heterotrophic bacteria with a normal calibration of 28 grams into 1000ml of distilled water.

Oil Agar: Oil agar medium was also prepared for the isolation of hydrocarbon utilizing bacteria. The medium was prepared with a composition of k_2HPO_4 (0.5g), $MgSO_4.7H_2O$ (0.3g), $NaCl_2$ (0.3g), $MnSO_4.H_2O$ (0.2g), $FeSO_4.6H_2O$ (0.02g), $NaNO_3$ (0.03g), $ZnCl_2$ (0.3g) and agar (15g) into 1litre of distilled water. 1% of pure Bonny light crude oil was added to the mixture and then autoclave at 121°C for 15 minutes.

Isolation and enumeration of total heterotrophic bacteria

Total heterotrophic bacteria for each bioremediation set up were enumerated by spread plate method. 0.1ml aliquot of the 10^{-6} was transferred unto well-dried nutrient agar plates and incubated at 37^{0}C for 24 to 48 h after incubation; the bacterial colonies that grew on the plates were counted and sub-cultured unto fresh nutrient agar plates using the streak plate technique. Discrete colonies on the plates were aseptically transferred into agar slants, properly labeled and stored as stock cultures for preservation and identification (Nrior and Odokuma, 2015).

Isolation and enumeration of total fungal count

The total fungi population in the bioremediation set up were enumerated and isolated by inoculating 0.1ml aliquot of the mixture unto well-dried Sabouraud dextrose agar containing antibiotics (Tetracycline, Penicillin and Ampicillin) to inhibit bacterial growth. Pure cultures of the fungi isolates were enumerated and transferred unto Sabouraud dextrose agar slants as stock cultures for preservation and identification (Odokuma and Okpokwasili, 1992).

Isolation and enumeration of Petroleum utilizing bacteria

Enumeration of Petroleum Utilizing Bacteria was performed by inoculating 0.1ml aliquot of the dilutions unto mineral salt agar plates. (Odokuma and Okpokwasili, 1992, Nrior and Odokuma, 2015). Colonies were counted after 48 to 72 h incubation at ambient temperature. The bacterial colonies on the plates after incubation were counted and sub-cultured onto fresh mineral salt agar plate.

Identification of Bacterial and Fungal isolates

The cultural, morphological and biochemical characteristics of the discrete bacterial isolates were compared with the recommendation in Bergey's manual of determinative bacteriology (1994). The morphological and biochemical test include; gram staining, motility, catalase, oxidase, citrate utilization, hydrogen sulphide production, indole production, methyl red and voges proskauer tests. The presence or absence of septa in the mycelium, type of spore, presence of primary or secondary sterigmata, and other microscopic characteristics as well as cultural characteristics were used in the identification of the fungal isolates of the biodegradation flask set up (Cheesbrough, 2006).

Preparation of stock culture:

Ten percent glycerol solution was prepared and dispensed in a McCartney bottles and autoclave for 15 minutes and then inoculate the pure cultures into the bottles to prevent contamination and preserved for a long time in a refrigerator.

Percentage (%) Bioremediation analysis

The percentage (%) bioremediation rate is calculated from the la used by Nrior and Echezolom (2016) as follows; ***Step 1***: Amount of pollutant remediated equals to Initial concentration of pollutant (Day 0 or 1) minus Final concentration of pollutant at end of experiment (Last day). ***Step2***: Percentage (%) Bioremediation

equals to Amount of pollutant remediated divided by Initial concentration of pollutant (Day 0 or 1) multiplied by 100.

$$B_C = I_C - F_C$$

Where;
B_C = Amount of pollutant remediated
I_C = Initial concentration of pollutant (Day 0 or 1)
F_C = Final concentration of pollutant at end of experiment (Last day)

$$\% \text{ Bioremediation} = \frac{B_C \times 100}{I_C}$$

III. Results and Discussion

Total heterotrophic bacteria were highly populated in the soil with the count of 10^8cfu/g throughout (Fig. 1). The hydrocarbon utilizing bacteria remain relatively low with the count of 10^3. These hydrocarbon utilizing bacteria are generally few in the soil and not as numerous as hydrocarbon degrading fungi in that same soil (Fig.2-3). The hydrocarbon utilizing bacteria count decreased steadily maybe due to the competition bacteria population of the soil. Also, the reduction in the hydrocarbon utilizing bacteria count could be due to petroleum hydrocarbon concentration degradation hence reduction in nutrients source for the hydrocarbon utilizing bacteria resulting to a decline count of 10^5 to 10^3. The characterization of fungi isolate was carried out base on their morphology and microscopic appearances were also identified to be of the following genera; *Penicillium, Aspergillus, Mucor, Histoplasma, Cladosporium* and *Alternaira* species.

Nrior and Echezolom, 2016 made an observation that petroleum based contamination increase due to anthropogenic activities and not only affect the environment but also the mycobiota associated with soil and water. Hence, fungi isolated from oil spill environment can reduce oil pollution. Nevertheless, interest in fungi has received a considerable attention for their bioremediation potential of soil contamination.

The hydrocarbon utilizing bacteria identified were of the following genera; *Klebsiella, Pseudomonas* and *Alcaligenes.* This was confirmed by Gogoi *et. al.* (2003), that these genera mentioned above were majorly users of diesel oil, a part of refined crude oil.

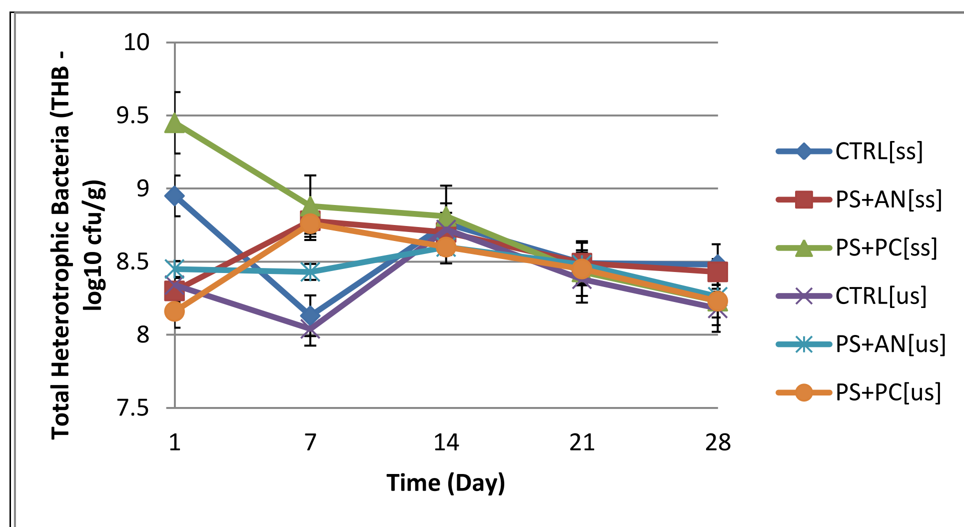

Figure 1: Total Heterotrophic Bacteria (log10 cfu/g) count during bioremediation of polluted soil samples

Key: Control surface soil without added organism CTRL[ss], Polluted surface soil + *Aspergillus nidulans* PS+AN[ss], Polluted surface soil + *Penicillium chyrosogenum* PS+PC[ss], Control underground soil without added organism CTRL[us], Polluted underground soil + *Aspergillus nidulans* PS+AN[us], Polluted underground soil + *Penicillium chyrosogenum* PS+PC[us]

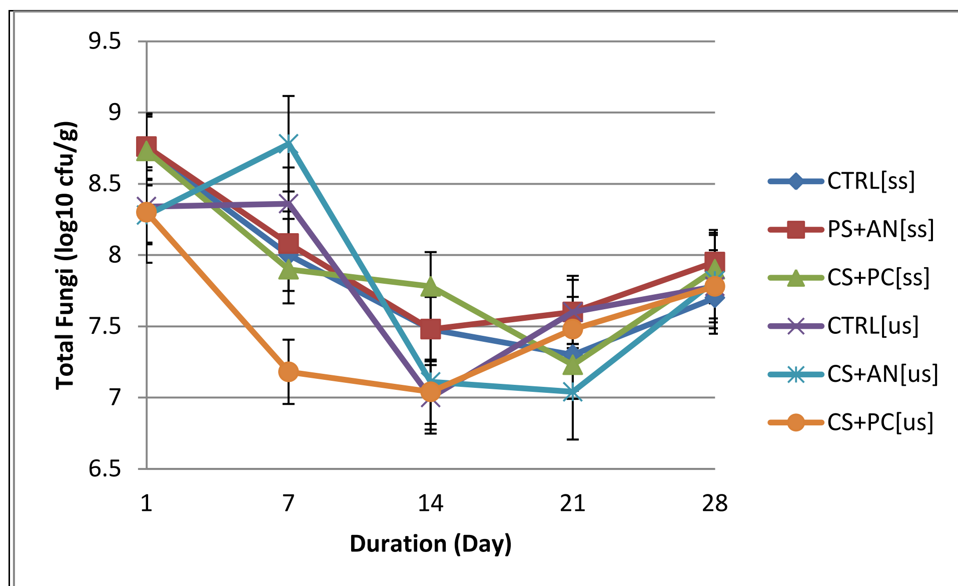

Figure 2: Total Fungi (log10 cfu/g) count during bioremediation of polluted soil samples

Key: Control surface soil without added organism CTRL[ss], Polluted surface soil + *Aspergillus nidulans* PS+AN[ss], Polluted surface soil + *Penicillium chyrosogenum* PS+PC[ss], Control underground soil without added organism CTRL[us], Polluted underground soil + *Aspergillus nidulans* PS+AN[us], Polluted underground soil + *Penicillium chyrosogenum* PS+PC[us]

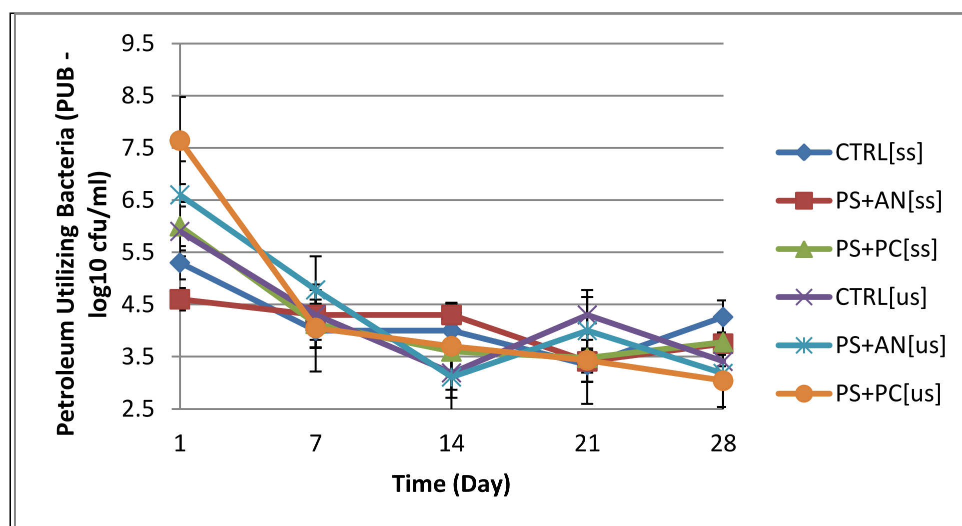

Figure 3: Petroleum Utilizing Bacteria (log10 cfu/g) count during bioremediation of polluted soil samples

Key: Control surface soil without added organism CTRL[ss], Polluted surface soil + *Aspergillus nidulans* PS+AN[ss], Polluted surface soil + *Penicillium chyrosogenum* PS+PC[ss], Control underground soil without added organism CTRL[us], Polluted underground soil + *Aspergillus nidulans* PS+AN[us], Polluted underground soil + *Penicillium chyrosogenum* PS+PC[us]

Gardner (1965) observed that when pure cultures of fungi were inoculated, the inoculums have to compete with the indigenous micro flora. The completion between the two decides the fate of treatment process. Similar results were also observed in the experiments conducted by Kaviyarasan *et. al.,* (2003) and Kamra (1998). Based on the analysis carried out on the polluted soil, it was found out that the concentration of the pollution decreases with the time interval.

The remediation processes were carried out with the use of fungi isolates (*Aspergillus nudilans* and *Penicillium chrysogenum*) to augment the indigenous microorganism in the polluted soil. The augmentation with these organisms helped to speed up the remediation rate since hydrocarbon content in the soil decreases with time. The analysis were done on a weekly interval, day1, 7, 14, 21 and 28 (Fig. 4-5) to observe the potential of organisms for the remediation of petroleum hydrocarbon pollution on soil and the result was achieved as the organisms were able to degrade the soil pollution. The Total Hydrocarbon Content (THC)

remediation determined showed decrease in hydrocarbon concentration from 142422.14mg/kg in the day 1 control surface soil to 92279.28mg/kg in day 28 (Fig. 4-5), as the day 1 was generally higher than the day 7 down to day 28 respectively. At day1, hydrocarbon concentration remains the same in all the treatment, this could be that the augmenting microbes were acclimatizing to the system, significant remediation process was noticed from the day 7 in setups augmented with organisms.

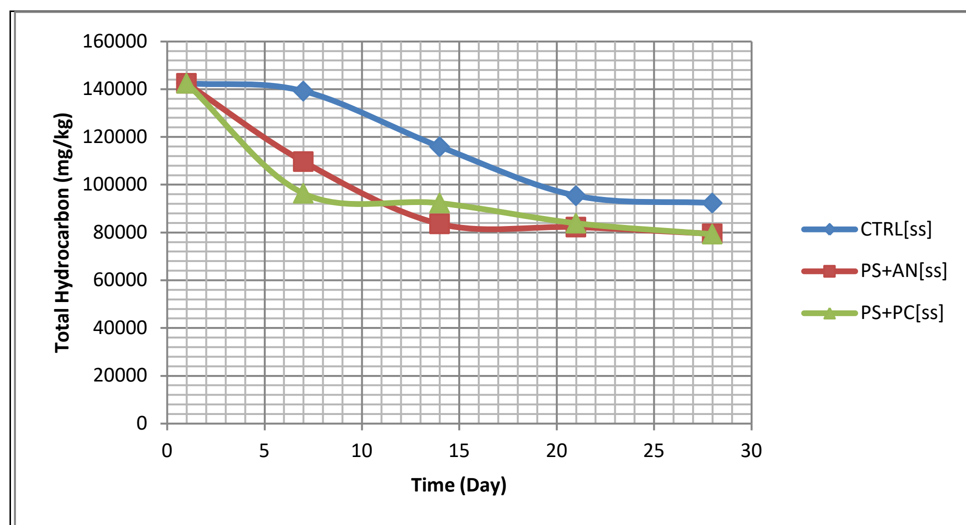

Fig. 4: Total Hydrocarbon Content (THC – mg/kg) of bioremediated oil polluted surface soil using *Aspergillus nudilans* and *Penicillium chrysogenum* (**Key**: CTRL[ss] = Control surface soil without added organism, PS+AN[ss] = Polluted surface soil + *Aspergillus nidulans*, PS+PC[ss]= Polluted surface soil + *Penicillium chyrosogenum*)

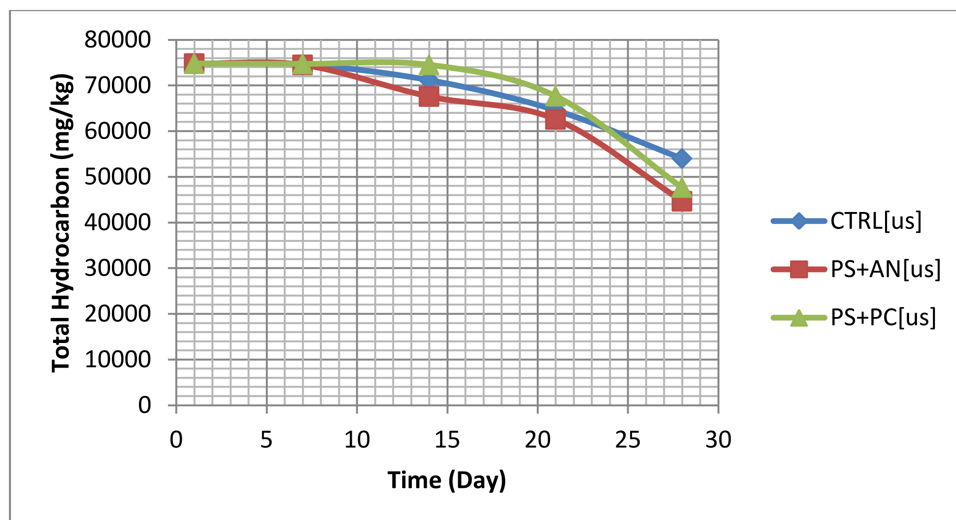

Fig.5: Total Hydrocarbon Content (THC – mg/kg) of bioremediated oil polluted underground soil using *Aspergillus nudilans* and *Penicillium chrysogenum* (**Key**: CTRL[us] = Control underground soil without added organism, PS+AN[us] = Polluted underground soil + *Aspergillus nidulans*, PS+PC[us]= Polluted underground soil + *Penicillium chyrosogenum*)

The Total Hydrocarbon Content (THC) as at the first day was relatively constant. The surface soil was heavily polluted than the underground soil (Fig. 8-9). The polluted surface soil plus *Penicillium chrysogenum* surface soil demonstrated much rapid rate of degradation (79279.28mg/kg) than polluted surface soil plus *Aspergillus* (79422.14mg/kg) on the last day for the surface soil while in the underground, polluted soil plus *Aspergillus nudilans* degraded faster than *Penicillium chrysogenum* (44636.43mg/kg) on the last day. This indicates that *Aspergillus nudilans* and *Penicillium chrysogenum* are potential organisms for bioremediation of hydrocarbon polluted soil according to the reading recorded in Fig. 6-7. On the day 28, the residual value were as follows: polluted surface soil with *Penicillium chrysogenum* (79279.28mg/kg) < polluted surface soil with *Aspergillus nudilans* (79422.14mg/kg) < surface soil control (92279.28mg/kg), underground soil were as

follows: polluted soil with *Aspergillus nudilans* (44636.43mg/kg) < polluted soil with *Penicillium chrysogenum* (47636.42mg/kg) < underground soil control (53993.59mg/kg).

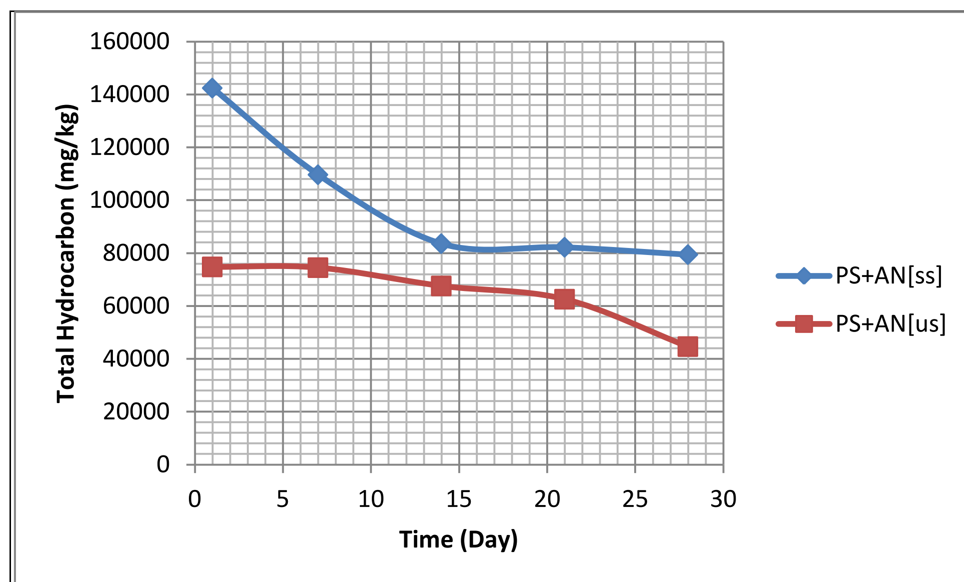

Fig. 6: Comparative Total Hydrocarbon Content (THC – mg/kg) of bioremediation potential of *Aspergillus nudilans* on surface and underground soil (Key: PS+AN[ss] = Polluted surface soil + *Aspergillus nidulans*, PS+AN[us]= Polluted underground soil + *Aspergillus nidulans*)

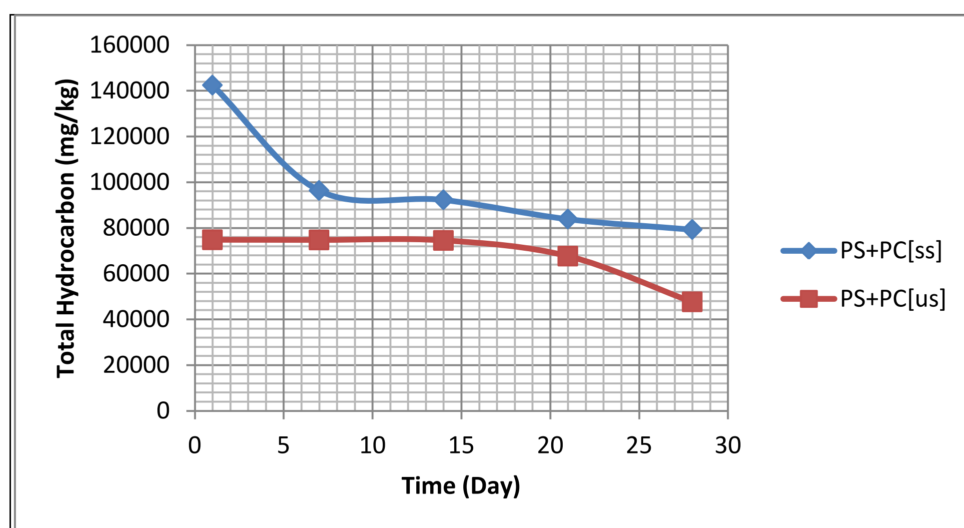

Fig. 7: Comparative Total Hydrocarbon Content (THC – mg/kg) of bioremediation potential of *Penicillium chrysogenum* on surface and underground soil (Key: PS+PN[ss] = Pollutedsurface soil + *Penicillium chrysogenum*, PS+PC[us]= Polluted underground soil + *Penicillium chyrosogenum*)

The results gotten from the day 1 down to the 28th day showed increase in remediation as the hydrocarbon concentration of the experimental polluted soils decreased over the 28 day period. The degradation rates in percentage for the surface polluted soil were as follows: control (29%), polluted soil plus *Aspergillus nudilans* (35%), polluted soil plus *Penicillium chrysogenum* (36%) and the polluted underground soil were as follows: control (27%), polluted soil plus *Aspergillus nudilans* (38%) and polluted soil plus *Penicillium chrysogenum* (359%) (Fig. 8-9).

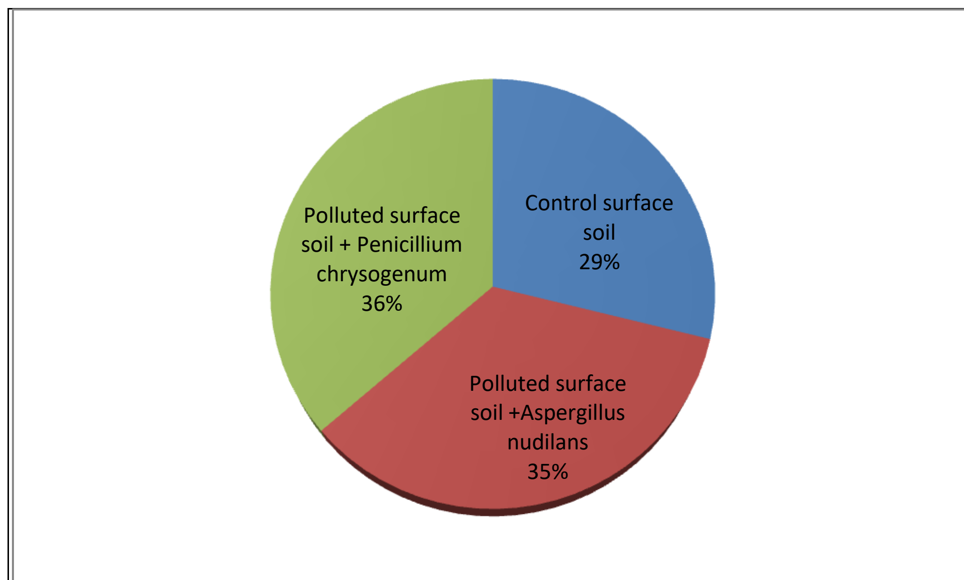

Fig. 8: Bioremediation rate of augmenting organisms in surface soil

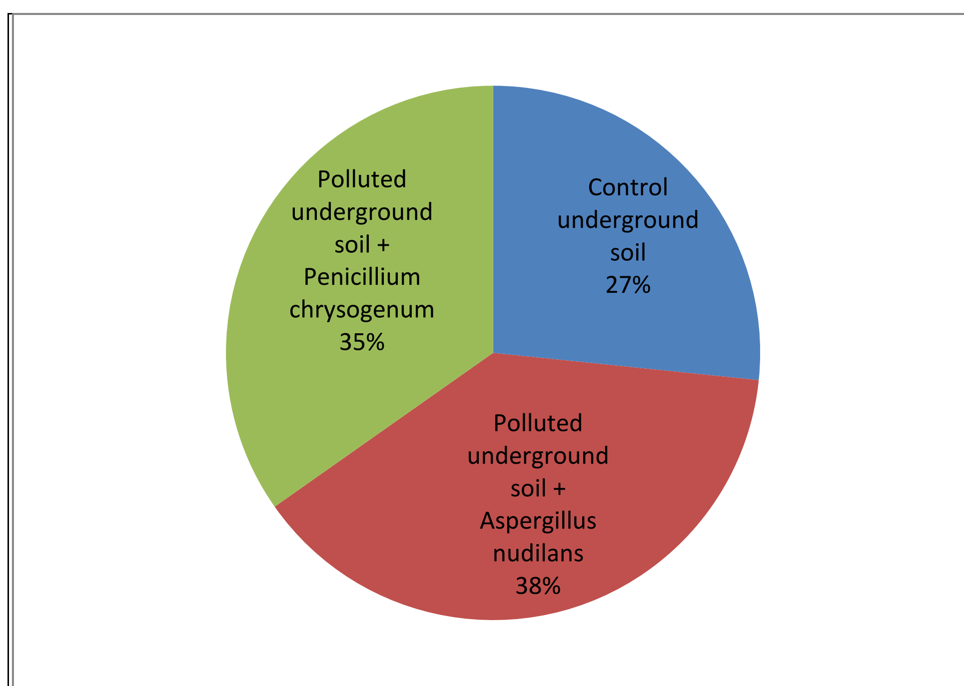

Fig. 9: Bioremediation rate of augmenting organisms in underground soil

The percentage bioremediation rates for surface soil were as follows: polluted soil with *Penicillium chrysogenum* (36%) > polluted soil with *Aspergillus nudilans* (35%) > and control polluted soil (29%); while underground soil: polluted soil with *Aspergillus nudilans* (38%) > polluted soil with *Penicillium chrysogenum* (35%) > control polluted soil (27%) Fig. 8-9. All these observations revealed that indigenous soil fungus *Aspergillus* species was a potential hydrocarbon degraders (Klien, 2000) Comparatively, *Penicillium chrysogenum* (36%) express higher bioremediation potential than *Aspergillus nudilans* (35%) in the crude oil polluted surface soil while in the underground soil; *Aspergillus nidulans* (38%) had higher bioremediation potential than *Penicillium chrysogenum* (35%). Base on all these studies carried out, Bioremediation with the use of fungal isolate can effectively remove the petroleum hydrocarbons and shorten the remediation period, with the use of *Penicillium chrysogenum* for surface soil crude oil spill clean-up and *Aspergillus nidulans* for crude oil polluted underground soil bioremediation process.

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Renner Renner Nrior. "Assessment of bioaugmentation efficiency of Penicillium chrysogenum and Aspergillus nudilans in bioremediation of crude oil spill soil." IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT) , vol. 11, no. 8, 2017, pp. 01–09.