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Monoculture Degradation of *Aspergillus Flavus* and *Penicillium Chrysogenum* of Varying Concentration of Spent Engine Oil (Spent Motor Oil) in Oba, Anambra State-Nigeria

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ABSTRACT

This study investigates the degradative potential of *Aspergillus flavus* and *Penicillium chrysogenum* monocultures on varying concentrations of spent engine oil. In order to do this, two species of fungi were cultivated in BHA medium supplemented with spent engine oil at concentrations of 1%. The ability of each monoculture to degrade the spent engine oil was then assessed by measuring its concentration in the solution over a period of 28 days.The results revealed that both *A. flavus* and *P. chrysogenum* demonstrate significant potential for degradation of spent engine oil, with the greatest degradative capacity observed at 1% and 3% concentrations. In addition, *P. chrysogenum* demonstrated a higher overall biomass than *A. flavus*, suggesting that it is more suitable for biodiesel production. Furthermore, the results show that both *A. flavus* and *P. chrysogenum* are able to significantly reduce the concentration of spent engine oil in the presence of sufficient carbon sources. Overall, this study provides a better understanding of the potential of *A. flavus* and *P. chrysogenum* monocultures for degradation of spent engine oil and highlights their potential for biodiesel production. Moreover, it also indicates that both species are able to degrade spent engine oil even in the presence of varying concentrations.

Key words: Spent Engine oil, *Aspergillus flavus* and *Penicillium chrysogenum*

Introduction

Crude oil is currently Nigeria's and of course, the world's main energy source (Agu *et al.,* 2015; Agu and Odibo, 2021; Agu *et al.*, 2022; Orji *et al.,* 2022). Engine oil which is a component of crude oil is a complex mixture of hydrocarbons that are used to lubricate parts of an automobile engine to avoid excessive wearing out (Mbachu *et al.,* 2014; Andem *et al.,* 2019). Motor engine oil contains metals and Polycyclic Aromatic Hydrocarbons (PAHs) and these could contribute to chronic hazards including mutagenicity and carcinogenicity- This is because it contains a mixture of different chemicals including low to high molecular weight (C_{15}-C_{21}) compounds, lubricants, additives and decomposition products and heavy metals which have been found to be harmful to the soil and human health. Engine oil picks up a variety of extra compounds from engine wear when it is utilized in automobiles. Iron, steel, copper, zinc, lead, barium, cadmium, sulfur, filth, and ash are among them. Used motor oil disposal can be more harmful to the environment than crude oil pollution due to additives and pollutants (Abioye *et al*., 2012). If these chemicals and toxins are permitted to reach the ecosystem through waterways or soil, they can have both short and long-term effects (Anoliefo and Vwioko, 2001).

Increasing industrialization and technological expansion, rapid urbanization, increased energy utilization and waste generation from domestic and industrial sources have ravaged the environment by discharge of industrial wastes into the environment (Ojiagu et al.,2018). A wide range of hydrocarbon utilizers (HCUs) is found to be useful in the soil and it includes the following species such as; *Pseudomonas spp, Rhodococcus spp., Mycobacterium spp., Bacillus spp., Acinetobacter spp., Providecin spp., Flavobacter spp., Carynebacterium spp., Streptococcus spp.* (Bhatace *et al.,* 2002). Other organisms like fungi are also capable of degrading the hydrocarbon in engine oil to a certain extent, but they take longer period of time to grow when compared to their bacterial counterparts (Prenafeta *et al.,* 2001).

Bioremediation is an environmental clean-up technique involving the use of naturally occurring microorganisms in the decontamination process (Ifediegwu *et al.,* 2015;). Petroleum based products are the major source of energy for industries and daily living. It is at present Nigerians and indeed, the world's most important derived energy source (Agu *et al*., 2015).

The utilization of the bioremediation technique that involves employing the use of microorganisms to detoxify or degrade pollutants depending to their varied metabolic capabilities is an evolving technique for the elimination and degradation of many environmental pollutants including the products of petroleum industry (Stephen *et al*., 2020). Some microorganisms capable of degrading hydrocarbon substrates excrete biosurfactants which aids the microorganism by increasing the surface area and bioavailability of the water-insoluble substrates (Anaukwu et al., 2016). Again, it is imperative to

reduce greenhouse gas emissions since the amount, since the amount of carbon released into the atmosphere is growing as the world's population grows (Oriyomi *et al.*, 2022)

This research paper is aimed at examining the monoculture degradative potential of *Aspergillus flavus* and *Penicillium chrysogenum o*n varying concentration of spent engine oil.

METHODOLOGY

Description of Study Area

The study will be carried-out in Oba, Idemili South Local Government, Anambra State which is geographically located at latitude 6° 4'0'' N and longitude 6° 50' 0'' E. The study will be carried-out in five selected filling station (Alignment workshops) in the Oba Idemili South Local Government. The filling station are located at three different sides; (1) ones along Onitsha Owerri road, Oba, (2) along Oba - Nnewi old road and (3) along Oba - Nnewi new road Oba.

SAMPLE COLLECTION

Contaminated soil samples will be collected using a Dutch soil auger in a randomized method from five (5) different locations within the study area, these sample points will be constantly receiving spent engine oil and will be labeled samples SA, SB, SC, SD, SE. At each of these sampling locations, auger-boring instrument will be used to bore holes of depths 0 – 5cm depth (top soil) and subsoil 5 – 15cm. Two (2) samples will be collected 2km away from the study area as control and will be labelled CF and CG; they will not receive SEO or any other type of contaminant (undisturbed forest). The samples will be homogenized in a clean plastic bucket and a composite sample will be drawn from each. This process will be repeated for all the experimental units. All the composite samples will be air dried and allowed to pass through a 2 mm sieve, which will then be poured into polythene bags, label adequately and transport to the laboratory immediately for analyses.

Preliminary Screening of Spent Engine Oil Degraders:

One gram (1g) of the soil samples will be weighed out aseptically and introduced into 100ml of sterile Bushnell Haas Broth medium containing 2ml/L of spent engine oil for the screening of spent engine oil degrading bacteria and placed on a rotary shaker at a revolution speed of 90rpm for 7days.

Bushnell Haas Medium Broth composition (gL^{-1})

Magnesium sulphate 0.200g, Calcium chloride anhydrous 0.020g, Potassium dihydrogen phosphate 1.000g, Dipotassium hydrogen phosphate 1.000g, Ammonium nitrate 1.000g, Ferric chloride 0.050g, Final pH (at 25°C) 7.0±0.2.

Bushnell Haas Medium Agar composition (gL^{-1})

Magnesium sulphate 0.200g, Calcium chloride anhydrous 0.020g, Potassium dihydrogen phosphate 1.000g, Dipotassium hydrogen phosphate 1.000g, Ammonium nitrate 1.000g, Ferric chloride 0.050g, Agar 20.000g Final pH (at 25°C) 7.0±0.2.

Degradation by Monocultures

2 fungal plugs of the isolates will be inoculated into a separate 50ml of BHA broth containing varying concentration (1%, 3% and 5%) of spent engine oil in 100ml flask and incubated at 150rpm in a rotary shaker for 35days at 30ºC. The level of degradation by spectrophotometric analysis at 680nm, pH and temperature will be considered every 7days interval period.

Degradation of Spent Engine Oil by the Isolates

The extent of degradation of spent engine oil by the isolates will be studied by using mass spectrophotometery (MS) analysis of the oil residue from the degradation at every 7days interval at a wavelength of 680nm.

Total Oil Degrading Fungi Count (TODFC)

A 10-fold serial dilution of each of the sample in the rotary shaker will be carried out using peptone water as the diluents. 0.1ml of appropriate dilutions (10^{-2}) of the samples were pour plated in sterile plates of Sabouraud Dextrose Agar (SDA) plates for the isolation of spent crude oil degrading fungi. The culture plates will be incubated at 25°C aerobically for 72hours in duplicates. Developing colonies on SDA agar were counted to obtain TODF count. Discrete colonies of the bacteria will be obtained by sub culturing into SDA plates and will be subsequently identified using biochemical and molecular identification methods.

Isolation and Characterization of the Fungi

This will be done based on the description of the gross morphological appearance of fungal colonies on the SDA culture medium and the modified slide culture technique using lactophenol cotton blue stain for the microscopic evaluation under X10 and X40 magnification of the microscope (Agu and Chidozie, 2021); with reference to the Manual of Fungal Atlases (Barnett and Hunter, 2000; Watanabe, 2002; Ellis *et al.*, 2007).

PHYSICOCHEMICAL ANALYSIS OF SOIL SAMPLE

Determination of pH (AOAC, 1984)

10g of air dried and sieved soil samples will be weighed into a beaker, then 100ml of water solution will be added and thoroughly mixed. The mixture will be left to stand for 1 hour before being read using a pH meter.

Determination of Total Organic Carbon (AOAC, 1984)

1. The moisture content of the air – dry soil will be determined which will be grinded to pass through a 0.42 sieve. Accurately amount of sample will be weighed to contain between 10g and 20mg of carbon into a dry tarred 20ml conical flask (between 0.5g and 1g for 1g for top soil and 2g and 4g for subsoil).
2. 10ml of 0.1N $K_2Cr_20_7$ will be added and swirl the content in the flask gently to disperse the soil in the solution. 20ml concentration of H_2SO_4 will be added, directing the stream into the suspension. Immediately the flask will be swirled until the soil and the reagent are mixed. A 200^0C thermometer will be inserted and mixture will be heated while swirling the flask and the content on a hot plate or over a gas burner and gauze until the temperature reaches 139^0C.
3. The set– up will be set aside to cool slowly on an asbestos sheet in a fume cupboard. Two blanks (without soil) will be run in the same way to standardized $FeSO_4$ solution.
4. When the set– up cools to (20 – 30mins), the mixture will be diluted to 200ml with deionised water, and the mixture will be titrated with the $FeSO_4$ using either the ferroinN indicator or potentiometrically with an expending scale pH/MV meter or auto titrator. **Ferroin Titration**
5. 3 or 4 drops of ferroin indicator will be added and titrate with 0.4N $FeSO_4$. As the end points is being approached, the solution will take on a greenish colour and then changes to a dark green. At this point, the $FeSO_4$ will be added drop- by- drop until the colour changes from blue – green to reddish – grey. If the end point is overshot, 0.5 or 1.0ml of 1N $K_2Cr_20_7$ will be added and to re-approach the end point.

Total Nitrogen Determination (AOAC, 1984)

Principle: the method is the digestion of sample with hot concentrated sulphuric acid in the presence of a metallic catalyst. Organic nitrogen in the sample is reduced to ammonia. This is retained in the solution as ammonium sulphate. The solution is made alkaline, and then distilled to release the ammonia. The ammonia is trapped in dilute acid and then titrated.

Procedures

Exactly 1g of sample will be weighed into a 30ml Kjedahl flask (gently to prevent the sample from touching the walls of the side of each and then the flasks were stoppered and shaken. Then 1g of the Kjedahl catalyst mixture will be added. The mixture will be heated cautiously in a digestion rack under fire until a clear solution appeared.

The clear solution will then be allowed to stand for 30 minutes and allowed to cool. After cooling about 100ml of distilled water will be added to avoid caking and then transferred to the Kjedahl digestion apparatus.

A 500ml receiver flask containing 5ml of boric acid indicator will be placed under a condenser of the distillation apparatus so that the tap will be about 20cm inside the solution. 10ml of 40% sodium hydroxide will be added to the digested sample in the apparatus and `distillation commenced immediately until distillation reaches the 35ml mark of the receiver flask, after which it will be titrated to pink colour using 0.01N hydrochloric acid.

Calculations

% Nitrogen = Titre value x 0.01 x atomic mass of nitrogen x 4

Where 0.01 = normality of the acid.

Loss of organic matter Determination (AOAC, 1984)

5g of soil and soil sample will be placed into a tarred 20-ml beaker, the set up will be allowed to dry for 2 hours or longer at 105^0C weight of sample will be recorded to ± 0.001g**.** The temperature of the oven will be brought to 360^0C samples must remain at 360^0C for 2 hours.

The set – up will be cool to < 150^0C and weighed to ± 0.001g, in a draft – free environment

Calculation

$$\text{Loss of organic matter} = \frac{(\text{wt. at } 105^0C) - (\text{wt. at } 360^0C) \times 100}{\text{wt. at } 105^0C}$$

Where,

wt: weight

Heavy Metal Analysis of Soil Samples

Heavy metal analysis will be conducted using Varian AA240 Atomic Absorption Spectrophometer according to the method of APHA 1995 (American Public Health Association)

Working principle: Atomic absorption spectrometer's working principle is based on the sample being aspirated into the flame and atomized when the AAS's light beam is directed through the flame into the monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wavelength, a source lamp composed of that element will be used, making the method relatively free from spectral or radiational interferences. The amount of energy of the characteristic wavelength absorbed in the flame will be proportional to the concentration of the element in the sample.

Dry Digestion

Digestion

2g of the sample will be weighed into a crucible and put into a muffle furnace for ashing at a temperature of 550^0c for 3hours. The sample will be removed from the furnace and allowed to cool. The dry ash will be emptied into a 250ml beaker 20ml of 20% H_2SO_4 will be added , heated in a water bath for 20mins, filtered and made up to 50ml with distilled water and stored in a sample bottle for AAS macro and micro nutrient analysis.

Preparation of Reference Solution:

A series of standard metal solutions in the optimum concentration range will be prepared, the reference solutions will be prepared daily by diluting the single stock element solutions with water containing 1.5ml concentrated nitric acid/litre. A caliberation blank will be prepared using all the reagents except for the metal stock solutions.

Caliberation curve for each metal will be gotten by plotting the absorbance of standards versus their concentrations.

Table 1: table showing the various colonial morphology and microscopy of the isolates

Isolates	Colony morphology	Microscopy	Identity
F1	Colonies appear on SDA a powdery mass of yellow-green spores on the surface and light yellow in the reverse side. Incubated 30ºC for 5 days	Conidial heads are large (up to 3 mm by 15 to 20 µm in diameter), globose, dark brown, becoming radiate and tending to split into several loose columns with age. Hyphal growth are thread-like Branching and producing Mycelium (3-6 µm in diameter) Conidiophore stipes are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae.	*Aspergillus flavus*
F2	Cultures on SDA are fluffy, bright yellowish green with bluish green tint, funiculose with bundles of hyphae, reverse yellowish pink with reddish purple tint. Rather good in growth	Conidiophores hyaline, erect, developed from aerial hyphae, branched penicillately at the apexes with primary and secondary metulae, verticillate phialides and catenulate conidia in each phialide, forming rather open-spaced yellowish green conidial heads: phialides lanceolate or abruptly sharpened.	*Penicillium chrysogenum*

Table 2: Table showing physic-chemical parameters of the various soil samples

Parameters	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Lead ppm	0.081	0.028	0.037	0.037	0.042
Cadmium ppm	0.001	0.002	0.00	00.001	0.001
Manganese ppm	0.025	0.007	0.018	0.011	0.008
Cobalt ppm	0.010	0.006	0.015	0.012	0.011
pH	7.20	6.50	5.60	5.90	5.50
Organic matter %	58.815 – 58.746 x 100 2.089 = 3.303%	60.378 – 60.280 x 100 2.086 = 4.698	59.014 – 58.96 x 100 2.098 = 2.574	59.915-59.417 x 100 2.078 = 23.965	59.470-59.362 x 100 2.088 = 5.172
Nitrogen %	Titre = 5.4 % N = 5.4 X 0.01 X 14 X 4 = 3.024	Titre =5.9 % N = 5.9*0.01*14*4 = 3.304	Titre = 5.7 % N = 5.7 *0.01*14*4 = 3.192	Titre = 6.6 % N = 6.6 *0.01*14* 4 = 3.696	Titre = 5.9 % N = 5.9 *0.01*14*4 = 3.304
TOC %	0.058	0.364	0.127	0.711	0.783
Nickel ppm	0.012	0.020	0.022	0.017	0.015

Table 3: Table showing pH **Reading of Degradation at Concentration 1%**

	0	7	14	21	28
F1	7.0	6.71	6.32	5.67	
F2	7.0	6.12	5.98	5.49	
Control	7.0	7.0	7.0	7.0	7.0

Table 4: **Table showing** pH **Reading of Degradation at Concentration 3%**

	0	7	14	21	28
F1	7.0	6.82	6.64	6.27	
F2	7.0	7.24	6.91	7.88	
Control	7.0	7.0	7.0	7.0	7.0

Table 5: Table showing pH **Reading of Degradation at Concentration 5%**

	0	7	14	21	28
F1	7.0	7.13	7.15	7.20	
F2	7.0	7.44	7.31	7.26	
Control	7.0	7.0	7.0	7.0	7.0

Table 6: Table showing Temperature Reading of Degradation at Concentration 1%

	0	7	14	21	28
F1	30.42	31.74	31.96	28.91	
F2	30.41	31.89	31.97	28.90	
Control	30.41	30.86	30.95	28.46	31.11

Table 7: **Table showing Temperature Reading of Degradation at Concentration 3%**

	0	7	14	21	28
F1	30.42	31.09	31.44	29.20	
F2	30.41	31.43	31.80	29.30	
Control	30.41	30.86	30.95	28.46	31.11

Table 8: Table showing Temperature Reading of Degradation at Concentration 5%

	0	7	14	21	28
F1	30.42	31.00	31.02	28.80	
F2	30.41	31.15	31.29	28.85	
Control	30.41	30.86	30.95	28.46	31.11

Table 9: **Table showing Spectrophotometry Reading of Degradation at Concentration 1%**

	0	7	14	21	28
F1	1.135	1.115	1.007	0.931	
F2	1.135	0.988	0.734	0.457	
Control	1.135	1.132	1.131	1.125	1.120

Blank 0.173

Wavelength 680nm

Table 10: **Table showing Spectrophotometry Reading of Degradation at Concentration 3%**

	0	7	14	21	28
F1	1.319	1.217	1.140	0.945	
F2	1.319	1.080	0.682	0.451	
Control	1.319	1.310	1.298	1.285	1.274

Blank 0.173

Wavelength 680nm

Table 11: **Table showing Spectrophotometry Reading of Degradation at Concentration 5%**

	0	7	14	21	28
F1	1.441	1.385	1.364	1.287	
F2	1.441	1.401	1.140	0.821	
Control	1.441	1.437	1.418	1.405	1.381

Blank 0.173

Wavelength 680nm

Table 12: Table showing Total Oil Degrading Fungi Count in cfu/ml and isolates distribution on Sabouraud Dextrose Agar (SDA) after 72hrs

X	X(CFU/ml)	Y (CFU/ml)
Sample 1	NG	NG
Sample 2	3.1x 10^4 (*Aspergillus flavus*)	3.0 x 10^4 (*Aspergillus flavus*)
Sample 3	4.1 x 10^4 (*Aspergillus flavus* and *Penicillium chrysogenum*)	4.5 x 10^4 (*Aspergillus flavus* and *Penicillium chrysogenum*)
Sample 4	3.2 x 10^4 (*Penicillium chrysogenum*)	3.0 x 10^4 (*Penicillium chrysogenum*)
Sample 5	NG	NG

Table 13: Table showing Degradation Fungi Based on Qualitative Analysis on SDA Plate After 48/72hrs

x	BEFORE 1%	AFTER 1%	BEFORE 3%	AFTER 3%	BEFORE 5%	AFTER 5%
F1	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus flavus*	NG
F2	*Penicillium chrysogenum*	*Penicillium chrysogenum*	*Penicillium chrysogenum*	*Penicillium chrysogenum*	*Penicillium chrysogenum*	*Penicillium chrysogenum*

NB Fungi inoculation was done with two (2) 8mm fungi plugs of the isolates

Where,

NG No Growth

DISCUSSION

Mycoremediation has played and continue to play a key role in the restoration of many environmentally polluted sites emananting from man's arthropogenic activities mediated by human development and civilization of major concerns are activities of mining of crude oil and spillage of crude oil resources, plastic waste degradation and man industrial wastes such as tie and dyes.

The fungal isolates used in this study is *Aspergillus flavus* and *Penicillium chyrsogenum* which shares similar genus nomenclature with the isolates used by Tudararao-Aherobo,and Mesogboriwon (2020) which shows that there was an increase in the fungi counts from Day 0 to Day 28 in both spent engine oil concentrations (5% and 10%) for the three fungi isolates used, in relation to the counts in the control. On day 0, for the 5% SEO test microcosms, counts ranged from 5.80 x 10^5CFU/g (*Aspergillus glaucus*) to 8.0 x 10^8 CFU/g (*Talaromyces flavus*), while on day 28, fungi counts ranged from 5.0x107CFU/g *Trichoderma polysporum*) to 5.80 x 10^9 CFU/g (*Talaromyces flavus*) and agree with our findings that after 21 days there was a complete utilization by both fungal (*Aspergillus flavus* and *Penicillium chrysogenum*) below the control value at 680nm of the varying concentration of the spent engine oil from table (9-11).

The results of the *in vivo and in vitro* bioremediation assay carried out by Umana *et al.* (2016) using *Penicillium sp.* (has the ability to degrade monocyclic aromatic hydro carbons such as benzene, toluene, ethyl benzene and xylene; BTEX), phenol compounds and heavy metals like lead, nickel and iron using mono-oxygenases, forming a trans-diol) mycelium treated spent engine oil contaminated soil at different concentrations/treatment levels of 20/180, 40/360, 60/540, 80/720 and 100ml/900mm shows that *in vitro* bioremediation assay showed a significant increase in the mycelia growth of *Penicillium sp*. relative to the control which conforms with the findings from this study which shows that penicillium chrysogenum has the highest degradative potential on varying concentration of spent engine oil.

CONCLUSION

The science of bioremediation using fungi (mycoremediation) to treat environmental polluted sites which pose threats to both aquatic and terrestrial organisms inhabiting a particular ecological niche has begun and it will continue for the next several decades as a viable biological approach that leaves zero after-threats to the choice habitat. *Penicillium chrysogenum* and *Aspergillus flavus* from this study will be a handful biotechnological tool to help curb the menace in form of spent motor oil from various automechanic workshops that finds itself through run-off into water bodies and also affects plant growth and development.

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