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ASSESSMENT OF THE MYCOREMEDIATION POTENTIAL OF *ASPERGILLUS FLAVUS* ON *ZEA MAYS* CULTIVATED SOIL PURPOSELY CONTAMINATED WITH BITUMEN.

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ABSTRACT
This study aimed to evaluate Aspergillus flavus' potential in remediating bitumen-contaminated soil. Aspergillus flavus, previously isolated from a crude oil-contaminated soil sample in Nigeria's Niger Delta region, was sourced from the Old Biology laboratory of Ajayi Crowther University, Oyo state. Bitumen was purchased from Ogunpa market, Ibadan and soil samples from the university's Old Biology Laboratory area. Twenty-four soil samples were collected, with eighteen intentionally contaminated with bitumen and six kept as uncontaminated controls. Aspergillus flavus broth was inoculated into eight contaminated samples. Over three months, microbial and physico-chemical parameters of the soil samples were analyzed using standard methods, including monitoring changes in total petroleum hydrocarbon levels. Microbial counts were enumerated bi-weekly and compared. Field mycoremediation lasted for 12 weeks. In contaminated, sterilized soil treated with Aspergillus flavus, fungal counts initially increased until the 8th week, followed by a decline. Initial contamination yielded a fungal count of 2.5 x 10^6 cfu/g, decreasing to 2.3 x 10^5 cfu/g after 3 months. Total petroleum hydrocarbon in treated soil decreased from 4.60 ppm to 1.06 ppm, indicating a progressive reduction in oil concentration. The Aspergillus flavus isolate exhibited potential for bitumen degradation, suggesting its viability for environmental cleanup of bitumen-contaminated soil.

Keywords: Bitumen; Mycoremediation; Aspergillus flavus; Soil contamination; Environmental cleanup.

1.0 INTRODUCTION

The progress of industrialization, globalization and development has contributed immensely to economic growth, fulfilled basic human needs and made life and work easier, but these activities are not without adverse effects as they continue to generate immense amounts of pollution that contaminate the natural environment around the world. Pollution is a plague that threatens the existence of ecosystems and, if not properly addressed, can gradually lead to the destruction of terrestrial and aquatic habitats. These are due to heavy industrial activities such as the extraction, exploration, processing and transportation of crude oil and petroleum products (Anekwe and Isa, 2023). Bitumen is a general term for a variety of dark, viscous, heavy hydrocarbons found in petroleum, which occur naturally or are formed as a residue during the fractional distillation of petroleum or coal (Akpasi et al.,2022). It is a dense mixture of heterogeneous hydrocarbon compounds produced from the temporal degradation of lighter crude oil (Yang et al., 2022; Stoyanovich et al., 2022). Bitumen was discovered in Nigeria in the 1900's and the first exploration efforts made in 1905 (Orire, 2009).

Key effects on the soil include impaired soil fertility, reduced air and water permeability, disrupted microbial activity, alteration of the physicochemical properties of soil, contamination of ground water and presence of toxic compounds (Zeng *et al*., 2020). Seepages from bitumen deposits in these areas have been observed on land and in water bodies for decades causing impairment to their soil quality (Atojunere, 2021).Therefore, the remediation of PHC-contaminated soils is presently a significant task, and it is vital to develop eco-friendly and effective approaches to uphold soil ecosystems (Cui *et al*., 2020). Various remediation technologies have been proven and deployed to treat petroleum-polluted soils. An actual polluted site may require optimal remediation through a combination of processes for prevalent conditions (Dindar *et al.,* 2013). Recent PHC remediation methods include physical, chemical, and biological approaches (Shi *et al.,* 2022). Physical and chemical pollutant detoxification technologies are often expensive and pose the risk of extra air or water pollution. Biological approaches, owing to the miscellaneous metabolic capabilities of microorganisms and plants, are progressing techniques for the degradation of various environmental pollutants containing petroleum products (Singha and Pandey, 2021).

Bioremediation is an effective, economical, and eco-friendly approach that has gradually become popular for mineralizing organic contaminants in wastewater (Aragaw,2021) and soil

environments (Sayara and Sánchez, 2020). In particular, bioremediation of PHC-contaminated soil has attracted the attention of researchers owing to its eco-friendliness (Roy *et al.*, 2018). Microbes play a crucial role in the biotransformation of intricate pollutant mixtures during the restoration of contaminated soils (Macaulay and Rees, 2014). Evidence in the literature has shown that a large number of microbes (bacteria, fungi, and some algae) and various plant species can mineralize PHCs completely into CO_2, H_2O, inorganic compounds, cell proteins, and simple organic compounds found in soil and water (Das and Chandran, 2011). However, the biological activity of microorganisms is influenced by environmental factors, including energy sources, bioavailability, bioactivity, biochemistry, oxygen and nutrient availability, pH, temperature, and metabolite inhibition (Kebedee *et al*., 2021).

These factors can be displayed in the diagram below.

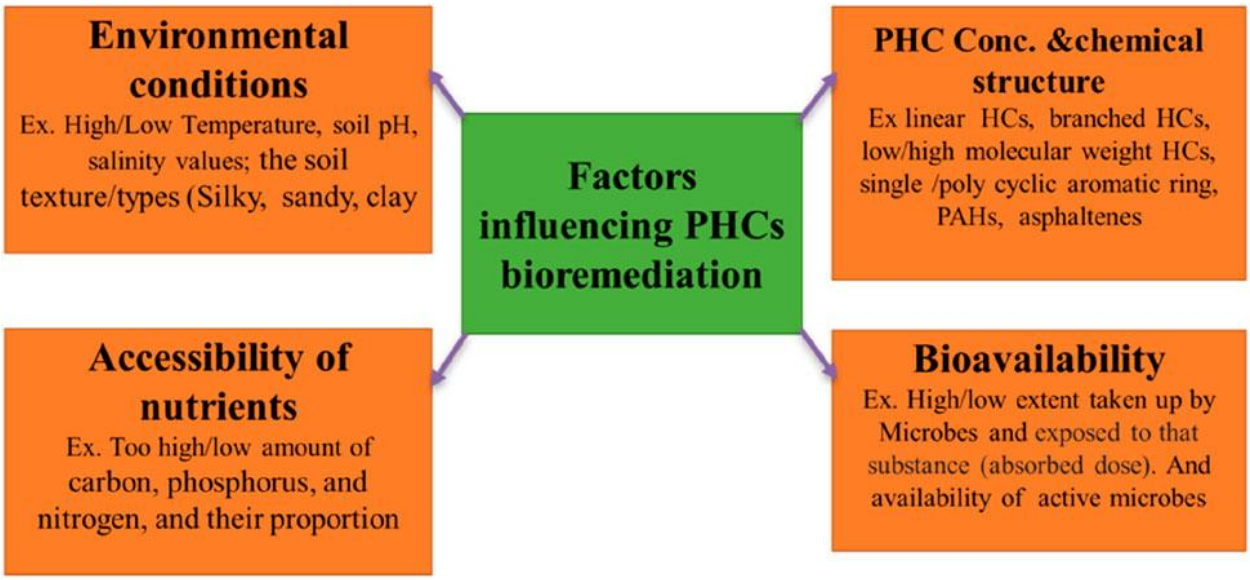

Fig. 1: Major factors influencing the bioremediation of PHC-polluted soil (Kebedee *et al.,* 2021)

And fungi, specifically *Aspergillus flavus* (Al-Dossary et al., 2019) has proven most effective in PHC degradation owing to its ability to produce biosurfactants thereby mycoremediating contaminated soil and water.

Previous research has extensively demonstrated the efficacy of fungi, particularly *Aspergillus flavus*, in the degradation of petroleum hydrocarbons (PHCs) due to their capacity to produce biosurfactants, thereby facilitating mycoremediation in contaminated soil and water. For instance, Ghanem et al. (2015) evaluated the degradation of kerosene using various fungal isolates in liquid medium. The study observed the ability of different local fungal isolates to degrade kerosene in liquid medium. The results showed that the most efficient fungus in the

kerosene degradation was *Aspergillus flavus*). Optimal conditions obtained in this work provided a solid foundation for further use of A. flavus in treatment of kerosene-polluted soil. The optimized conditions were applied to bioremediate 2.5% (v/w) kerosene-polluted soil by A. flavus, and the fungus efficiently degraded kerosene after 35 days of incubation.

The study by Benguenab and Chibani (2021) on the biodegradation of petroleum hydrocarbons by filamentous fungishowed that species belonging to the genus *Aspergillus* are tolerant to hydrocarbon pollutants, *A. flavus* and *A. versicolor* were found resistant to crude oil contamination within the range of 1% to 5% (*v*/*w*) in Minimal Salt Agar and PDA medium. *A. terrus* CCS2B was able to grow and tolerate up to 1000 mg of PAH mixture l−1 in surface culture while *Aspergillus* sp. showed the highest oil removal ability when cultured in PDA media plates containing 2%, 4%, 6% and until 15% (*v*/v) of crude oil.Similarly, Al-Dossaryet al., 2019 investigated the Biodegradation of crude oil using Aspergillus species. Using three fungal species which are *Aspergillus flavus*, Aspergillus fumigatus and Aspergillus versicolar isolated from a soil contaminated with crude oil in the oil fields in Basrah. The results showed that the fungus A. flavus was the best biodegrader, with biodegradation ability reaching 60% in 15 days and 80% in 30 days. This also agreed with results obtained by Keta et al., 2021which indicates that *Aspergillus flavus* possibly possesses an effective enzyme to foster the removal of PAHs.

This paper thus seeks to investigate the biodegradative ability of *Aspergillus flavus* on bitumen contaminated soil.

2.0 MATERIALS AND METHODS

This study aimed at investigating the biodegradative ability of *Aspergillus flavus* in bitumen contaminated soil. The experiment was carried out at the nursery section located behind the Old Biology Laboratory, Department of Microbiology at Ajayi Crowther University Oyo state, Nigeria within latitudes 7° 50′ 57″N and longitudes 3° 56′ 51″E.

Samples were taken using a sterile hand trowel at various locations on site at a depth of about 10cm to ensure a broad spectrum of naturally occurring microorganisms. Bulk soil from the site was collected in a sterile basin and taken to the laboratory for weighing. One gramme of the sample was removed and taken to Laboratory for pre-physiochemical analysis. Eight kilogram each, of the soil samples were weighed and transferred into 24 pots in total. The soil samples in 12 out of the 24 pots were sterilized in the autoclave at 121^{o}C for 30 minutes, the other 12 were

left unsterilized. 16 of the 24 bags were purposely contaminated with 300mls of bitumen and the remaining 8 served as the control pots, which is the uncontaminated reference pot. The bitumen was thoroughly mixed with the soil samples and the samples were labeled appropriately.

The experiment was implemented using a 2 by 3 randomized complete block design in duplicates, as shown in Table 1 below. The potted experiment was carried out for a period of 12 weeks from December to February under natural environmental conditions. The bulk soil from the site was measured out in 8kg each into polypropylene sandbags. The pots were then divided into two. The first set of 12 represented sterile soil while the other twelve were unsterilized. They were further divided into 8 sets (in duplicates), the first 8 represented sterile soil purposely contaminated with 300 ml each of bitumen. The second set of 8 pots represented its non- sterile counterpart, while the third set of 3 pots (in duplicates) represented sterile soil also purposely contaminated with 300ml each of bitumen and inoculated with 300 ml of *Aspergillus flavus* broth and the fourth set of the other 3 pots represented its non- sterile counterpart. The final set of 4 pots (in duplicates) represented sterile normal soil and the replicate 4 represented non-sterile normal soil, which served as control. Five seeds were sown in each bag on 15th November, 2023. A week after planting, the seedlings were thinned to four plants per bag.

Twelve of the soil samples were sterilized at 121°c for 15 minutes for three consecutive days in order to exclude all visible microorganisms present. This was done to ensure that only the inoculated fungal isolate will degrade the bitumen present in the soil in order to determine the mycoremediative ability. The physicochemical properties of the soil samples collected were determined using the procedure in the method of soil analysis (Wagh *et al.,* 2013). Isolate was identified Morphologically and Microscopically.

Then the isolate was introduced into PDA broth and left for 24 hours, thereafter the number of cells was counted with a hemocytometer and standardized to obtain an inoculum size of about 5.0×10^5 per ml. 300 ml of the *A. flavus* broth was inoculated in eight of the soil bags which had already been purposely contaminated with bitumen. The inoculated soil were then thoroughly mixed to ensure uniform distribution of the added fungi cells.

Soil samples were taken bi – weekly for 12 weeks from each pot of each treatment option by scooping the soil at the top, middle and bottom with a sterile spoon so that the sample taken was a true representation of the treated soil sample. The biodegradation process was determined

by the following methods:

The total viable microbial count of soil sample was determined for the period of 12 weeks in order to relate the microbial load to the changes in the total petroleum hydrocarbon content. The pour plate technique was used, incubation at room temperature for 24 – 48 hours and 72 hours for Nutrient Agar and potato dextrose agar respectively. Colonies that developed were counted and recorded for 2^{nd}– 12^{th}weeks.

About 2g of the sample was taken into a 50ml conical flask, 25ml of n-Hexane was added and the sample was vortex 2-Genie. The conical flask was later transferred onto an ultra-sonic bath and sonicated for about 30 minutes. The clear organic layer was decanted and centrifuged at 1000RPM for 5 minutes. The clear supernatant is taken into a pre-weighed beaker and evaporated to dryness inside a fume cupboard. The weight difference is calculated and expressed in mg/Kg.

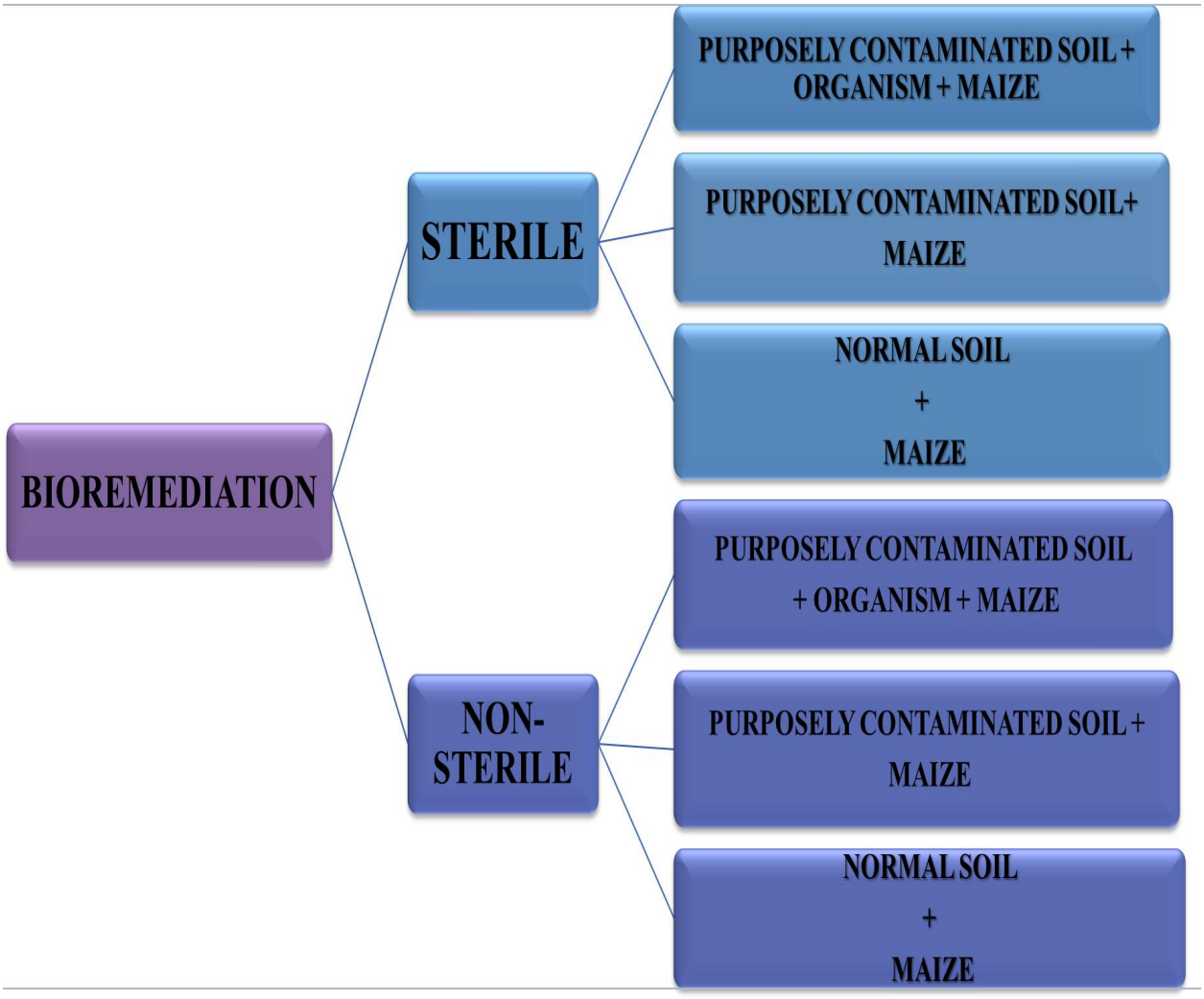

Fig. 2: Typical Biotreatment Design Set-up

3.0 RESULTS AND DISCUSSION

3.1 Physiochemical Properties of the soil

The study recorded physical and chemical parameters of the soil sample, including pH, organic carbon, total nitrogen, exchangeable bases, and micronutrients, for pre-physiochemical and post-physiochemical analysis, with results presented in Table 3.1.

Table 1: Pre physicochemical and post physicochemical analysis of the sterilized and unsterilized soil samples

SOIL PROPERTIES	Normal soil (Pre-physicochemical analysis) (PRE-NSM)	Purposely contaminated +No organism (Pre-physico chemical analysis) (PRE-PCM)	Purposely contaminated+ No organism (Post-physicochemical analysis) (POST-PCM)	Purposely contaminated + Organism (Post-physicochemical analysis) (POST-PCAM)	Sterilized + Purposely contaminated Organism (Post-physicochemical analysis) (POST-PCAMS)
Organic Carbon (g/kg)	11.20	16.85	17.7	19.00	20.00
Total Nitrogen	1.000	1.020	1.090	2.000	2.000
Available Phosphorus (mg/kg)	15.00	9.500	13.00	23.50	35.00
Exchangeable Bases (cmol/kg)					
Calcium	4.000	3.990	3.960	4.700	5.330
Magnesium	0.790	12.40	0.590	0.710	1.000
Potassium	0.400	0.200	0.170	0.200	0.220

Sodium	0.110	0.260	0.290	0.090	0.120
Exchangeable acidity	0	1.120	0.000	0.000	0.000
ECEC	5.300	4.310	4.810	5.920	6.650
Base saturation (g/kg)	1000	981.0	1000	1000	1000
Micronutrients (mg/kg)					
Manganese	65.00	68.00	107.0	114.0	198.0
Iron	46.00	60.00	60.00	77.00	82.00
Zinc	6.290	8.090	8.590	8.630	13.04
Copper	0.550	0.520	0.040	0.050	0.110
Particle Size Distribution (g/kg)					
Sand	902.0	902.9	958.0	958.0	938.0
Silt	80.00	80.00	41.00	41.00	41.00
Clay	18.00	18.00	21.00	21.00	21.00

From the results,

3.1.1 Characterization

The fungal isolate's identity was confirmed through colony, conidia, and conidiogenous cell morphology, as well as microscopic characteristics, as shown in Table 3.1.

Microscopic: Under a basic biological light microscope, biseriate with philiades, globose conidia, roughened conidiophore, septate and hyaline hyphae, and long, smooth conidiophore were observed.

Macroscopic: The mycelium spread across the agar plate, forming greenish colonies encircled by a white border.

Table 2: Macroscopic and Microscopic Characteristics of Fungal Isolates

Probable organism	*Aspergillus flavus*
Vesicle Shape	Subglobose
Conidial head	Radiate

Special reproductive	Phialides borne
Structure	Directly on the vesicle, Sclerotia
Asexual spore	Globose conidia
Special vegetative structure	Footcell
Nature of hyphae	Septate
Type of Soma	Filamentous
Colony colour	Dense felt yellow – green colony

3.2 Screening For Biosurfactant Production

The study prepared Aspergillus broth and Fusarium for control, and used cell free culture broth for drop collapse, oil spreading, emulsification, surface tension measurement, and qualitative assay. All screening experiments were performed in triplicates, with results recorded.

3.3 Microbial Count

The microbial count of soil samples was conducted, with Nutrient Agar plates having a higher microbial load than Potato Dextrose Agar plates, and fungal load increasing until declining in week 8.

Table 3.3: Total bacterial count of soil samples during the period of biotreatment (Log10CFU/g)

Soil samples	Total bacteria count (cfu/g)						Standard Deviation
	WK 2	WK 4	WK 6	WK 8	WK 10	WK 12	
PCAMS	36.00±1.00	38.00±1.00	40.00±3.00	46.00±9.00	48.00±11.00	14.00±23.00	11.12
PCAMS	32.00±1.00	34.00±1.00	42.00±3.00	44.00±9.00	46.00±11.00	17.00±23.00	11.12
P2CAMS	30.00±4.50	26.00±8.50	30.00±4.50	36.00±1.50	38.00±3.50	47.00±12.50	6.87
PCAMS	28.00±4.00	24.00±8.00	32.00±0.00	36.00±4.00	36.00±4.00	36.00±4.00	4.62
PCAM	40.00±1	46.00±6.	48.00±4.	50.00±2.	52.00±0.6	80.00±27.	12.78

PCMS	30.00±0.67	28.00±2.67	30.00±0.67	32.00±1.33	32.00±1.33	32.00±1.33	1.49
P2CMS	27.00±5.83	30.00±2.83	32.00±0.83	36.00±3.17	36.00±3.17	36.00±3.17	3.48
P2CMS	20.00±6.00	24.00±2.00	26.00±0.00	28.00±2.00	28.00±2.00	30.00±4.00	3.27
PCM	30.00±6.67	32.00±4.67	36.00±0.67	38.00±1.33	40.00±1.33	44.00±7.33	4.71
PCM	32.00±6.67	36.00±4.67	38.00±0.67	40.00±1.33	44.00±1.33	48.00±7.33	4.79
P2CM	32.00±2.50	34.00±0.50	36.00±1.50	38.00±3.50	40.00±5.50	27.00±7.50	4.23
P2CM	28.00±8.00	30.00±6.00	34.00±2.00	38.00±2.00	42.00±6.00	44.00±8.00	5.89
NSMS	10.00±14.00	18.00±6.00	22.00±2.00	26.00±2.00	30.00±6.00	36.00±12.00	8.37
NSMS	8.00±15.33	20.00±3.33	24.00±0.67	28.00±4.67	28.00±4.67	32.00±8.67	7.80
N2SMS	12.00±14.00	22.00±4.00	26.00±0.00	30.00±4.00	32.00±6.00	34.00±8.00	7.39
N2SMS	16.00±13.33	24.00±5.33	28.00±1.33	34.00±4.67	36.00±6.67	38.00±8.67	7.63
NSM	58.00±5.67	60.00±2.67	62.00±1.67	64.00±0.67	68.00±4.67	70.00±6.33	4.23
PCAM	42.00±8.00	46.00±4.00	48.00±2.00	50.00±0.00	54.00±4.00	60.00±10.00	5.77
P2CAM	40.00±9.00	42.00±7.00	46.00±3.00	52.00±3.00	56.00±7.00	58.00±9.00	6.81
P2CAM	38.00±8.33	44.00±2.33	46.00±0.33	46.00±0.33	50.00±3.67	54.00±7.67	4.96
PCMS	26.00±5.33	26.00±5.33	30.00±1.33	32.00±0.67	34.00±2.67	40.00±8.67	4.85

	7	±3.67	67	33	3	3	
NSM	54.00±11.00	58.00 ±7.00	60.00±5.00	66.00±1.00	74.00±9.00	78.00±13.00	8.62
N2SM	62.00±5.50	64.00 ±3.50	66.00±1.50	68.00±0.50	72.00±4.50	73.00±5.50	3.99
N2SM	60.00±6.00	61.00 ±5.00	65.00±1.00	68.00±2.00	70.00±4.00	72.00±6.00	4.43

Note: Mean±standard error of mean (SEM) and a probability value less than 0.05 was considered to be statistically significant

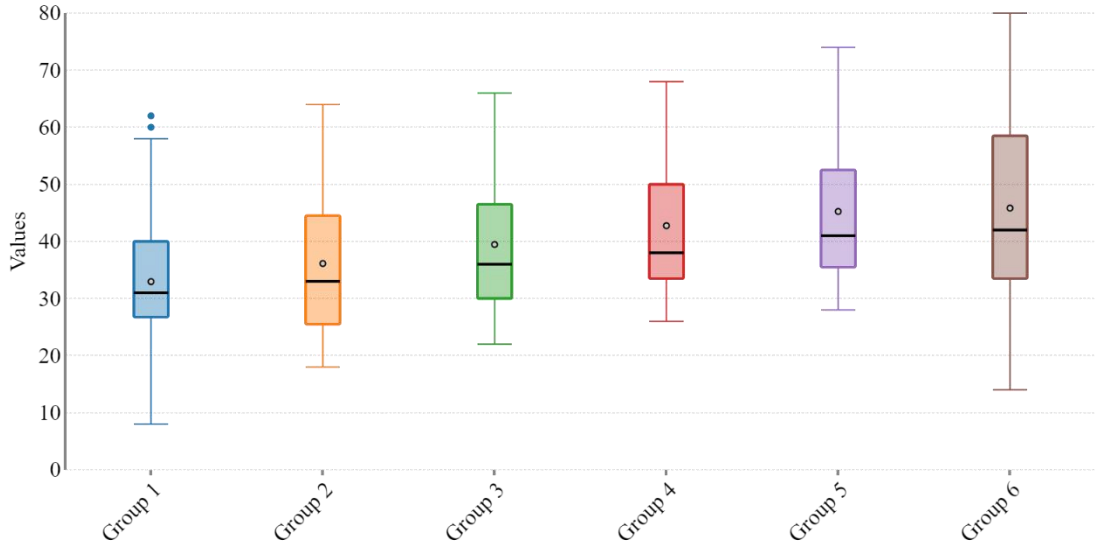

Fig. 3: Total heterotrophic bacteria count in the soil samples for the period of biotreatment

Keys: Group 1: Week 2; Group 2: Week 4; Group 3: Week 6; Group 4: Week 8; Group 5: Week 10; Group 6: Week 12; Values: Total Bacteria count (Log 10 CFU/g)

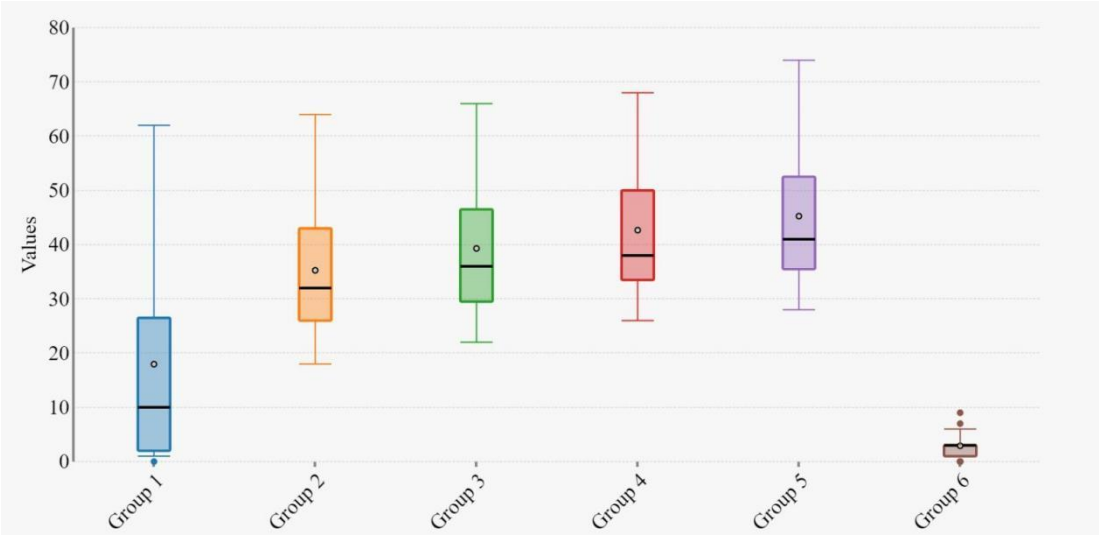

Fig 4: Total fungal count in the soil samples for the period of biotreatment (cfu/g)

Keys: Group 1: Week 2; Group 2: Week 4; Group 3: Week 6; Group 4: Week 8; Group 5: Week 10; Group 6: Week 12; Values: Total Fungal Count (Log 10 CFU/g)

3.4 Total petroleum hydrocarbon count

The study assessed the effectiveness of *Aspergillus flavus* in polluted soil by determining the total petroleum hydrocarbon count of the soil sample over 12 weeks, as depicted in Figure 5.

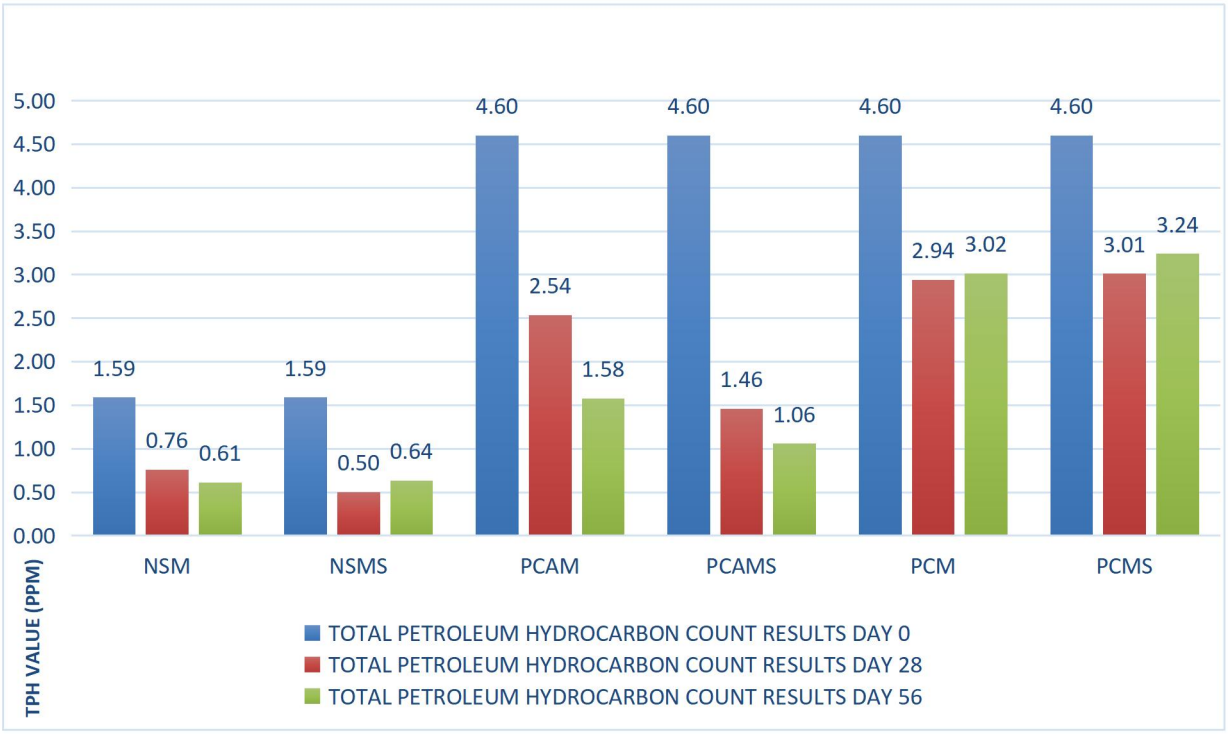

Fig. 5: Total Petroleum Hydrocarbon values in Bitumen contaminated soil samples over 12 weeks (ppm)

Keys: Blue- Total petroleum hydrocarbon count day 0; Orange- Total petroleum hydrocarbon count day Day 28; Grey- Total petroleum hydrocarbon count day Day 56.

Figure 5 depicts the reduction in total petroleum hydrocarbon (TPH) values in bitumen-contaminated soil samples inoculated with *Aspergillus flavus* after 12 weeks from 4.60ppm to 1.06ppm suggests that *Aspergillus flavus* is capable of degrading bitumen. This supports the development of fungal bioremediation methods as a promising approach for soils heavily contaminated with persistent organic compounds like polyaromatic hydrocarbons.

The bioremediation analysis provides valuable insights into the dynamics of microbial communities during the remediation process. The steady increase in bacterial counts aligns with previous studies, while the fungal counts peaked at week 8 and then declined due to nutrient

limitations.

Aspergillus flavus has the potential to act as a biosurfactant producer, enhancing the solubilization and desorption of soil petroleum hydrocarbons. This mechanism is thought to be responsible for the observed reduction in turbidity and residual oil by week 12.

4.0 CONCLUSION AND RECOMMENDATIONS

From this study, it has been investigated that *Aspergillus flavus* can degrade bitumen in bitumen-contaminated soil with maize, reducing soil TPH values over the course of the 12 weeks from 4.60ppm to 1.06ppm. The observed increase in fungal counts correlated with a significant reduction in total petroleum hydrocarbon levels, indicating their role in bitumen degradation.

While the fungal counts showed a peak and subsequent decline around week 8, possibly due to nutrient limitations, the continuous reduction in TPH throughout the 12-week period suggests sustained degradative activity, potentially with other microbial communities also contributing, or the initial significant degradation by *Aspergillus flavus* being sufficient.

The increasing bacterial counts observed, also suggest a role for the broader soil microbial community in the overall bioremediation process, working in concert with the inoculated *Aspergillus flavus*. The high initial fungal count of 2.5 x 10^6 cfu/g and its subsequent activity contributed to the rapid initial decline in TPH.

This study conclusively demonstrates that *Aspergillus flavus* effectively degrades bitumen in contaminated soil, evidenced by the significant reduction in soil TPH values which coincided with the proliferation and activity of the fungal population, likely facilitated by its biosurfactant production.

While this study conclusively demonstrates the significant potential of *Aspergillus flavus* in degrading bitumen in contaminated soil, certain limitations should be acknowledged for future research considerations. The experiment was conducted under natural environmental conditions over a 12-week period, which introduces variability that might not be present in a strictly controlled laboratory setting. Although soil samples were taken bi-weekly, continuous real-time monitoring of all physicochemical parameters and microbial activity was not feasible, potentially missing some short-term fluctuations. The study focused on a single fungal isolate, *Aspergillus flavus*, and a specific concentration of bitumen contamination (300ml). Therefore,

the findings may not be directly generalizable to other fungal species, different types of petroleum hydrocarbons, or varying levels of contamination without further investigation.

Further research efforts could be carried out by varying the concentration of the pollutant and varying the measure of the inoculum to assess for optimum bioremediation. Further research could be directed towards understanding and harnessing the mycoremediative potential of this fungi and its potential use as a bio-inoculant in agriculture.

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