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BIOREMEDIATION OF PETROLEUM REFINERY EFFLUENTS USING MUTATED FUNGAL CONSORTIA: A CASE STUDY FROM KADUNA, NIGERIA

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ABSTRACT

Bioremediation is a process of using naturally occurring species to break down hazardous substances into less harmful or non-toxic substances. Effluents from petroleum refinery pose a serious health hazard on the people who depend on the water as source of supply for domestic uses. To avoid health hazard, it is imperative for these toxic compounds to be removed from waste water before its disposal. The primary goal of this study was to carry out bioremediation of petroleum refinery effluents using mutant strains of fungal consortia. Refinery effluents were collected under the storage tank in Kaduna Refining and Petrochemical Company, Kaduna, Nigeria. Effluent samples were analyzed for total petroleum hydrocarbons using Gas Chromatography and Mass Spectroscopy (GC-MS). Standardization of the fungal consortium inoculum was prepared following standard method. The culture medium of each isolate of the mutated fungi *Aspergillus niger*, *A. fumigatus*, *A. versicolor* and *A. quadrilineatus* was carried out following standard procedure. The result of the GC-MS analysis revealed that cis-13-Octadecenoic acid, methyl ester; 7-Hexadecenoic acid, methyl ester; 10-Octadecenoic acid, and Methyl stearate; showed the highest percentage quality of (99%) respectively. Bioremediation of Kaduna refinery effluents in this study indicated that, there was decline in the bioremediation parameters in the second week up to the fourth week whereby the COD, BOD all showed significant decrease. Based on the Gas Chromatographic (GC) analysis of the fungi consortium, the degradative ability of the mutants of *Aspergillus versicolor and Aspergillus quadrilineatus* were observed to be prominent.

Keywords: Bioremediation, Refinery effluent, Mutation, Fungi consortia, Total Petroleum Hydrocarbon

INTRODUCTION

Bioremediation is a process of using naturally occurring species to break down hazardous substances into less harmful or non-toxic substances (Ławniczak *et al.,* 2020). All substances in nature eventually break down, decay, or transform. In order to obtain energy for their growth, micro-organisms break down many organic compounds in the environment. In another definition by Magdalene *et al.* (2020), bioremediation is said to be defined as a process by which microorganisms are stimulated to rapidly degrade hazardous organic pollutants to environmentally safe levels in ground water. Microorganisms find food in the oil or water where they live. However if a contaminant is present it can become an additional food source for the microorganisms. The contaminants serve two useful purpose for the microbes. First the contaminants provide a source of carbon needed for growth. Second, the microbes obtain energy by breaking chemical bonds and transferring electrons away from the contaminants. This is known as an oxidation reduction reaction. The contaminant that loses electron is oxidized while that which accepts is reduced (Ezekoye *et al.,* 2017). The petroleum hydrocarbons oil spill disaster has an impact on the marine environment and ecosystem (Al-Hawash *et al.,* 2018). As TPH is discharged directly into water bodies by oil spills, petroleum hydrocarbons float on the surface of water and establish thin oily layer. Seabirds are particularly vulnerable because oil contact inhibits the ability to fly. The resulting intake of infected food, inhalation, and repeated encounters with the interface of the oil water result in severe personal poisoning with high mortality rates (Al-Hawash *et al.,* 2018). Ingested or dissolved oil in the body via membranes, e.g., gill surfaces cause direct lethal toxicity, sub-lethal effects and marine organisms' reproductive failure (Fingerman, 2016).

Petroleum hydrocarbon contamination in Kaduna is released to the environment through oil leakages during transportation of petroleum products by tankers, industrial releases of effluents by recent activities of the Kaduna Refinery and Petrochemical Plant or by-products of private or commercial uses (Al-Hawash *et al.,* 2018). Microbial decomposition of petroleum and petroleum products is of considerable economic and environmental importance. Because petroleum is a rich source of organic matter and the hydrocarbons within it are readily attacked aerobically by a variety of microorganisms, bioremediation employs microorganisms capable of degrading toxic contaminants (Margesin *et al.,* 2017). Effluents from petroleum refinery and petrochemical companies are characterized by the presence of large quantities of crude oil products, aromatic hydrocarbons, phenols, metal derivatives, sulphides, heavy metals and other chemicals, that contains toxic and hazardous materials, which when discharged settled in receiving rivers as part of the bottom sediment. They pose a serious health hazard on the people who depend on the water as source of supply for domestic uses, but most importantly, affect the general composition and behavior of microorganisms inhabiting such water body. Most hydrocarbon substances bind with soil particles and remain in the soil for a long time, while microbes that are present in the soil break down some hydrocarbons. Secondly, contact may occur via dermal constant contact, inhalation, and ingestion, depending on the properties of the chemical or media (i.e., air, water, soil, food) in which the chemical affects human activity in and around that material (Speight, 2018).

Petroleum effluents are of critical concern to environmental health due to their high occurrences' in soluble form, with extremely toxic to biological system, especially the immediate inhabitants of such habitats. To avoid health hazard, it is imperative for these toxic compounds to be

removed from waste water before its disposal. Fungi mycelia are known with the ability to penetrate oil and increase the surface area available for degradation by other microbes. Bioremediation has an edge over other treatment methods because it can efficiently degrade oil and phenol from the waste water and doesn't allow the contaminant to accumulate. Mutant strains of fungi are known for maximum performance on environmental pollutants whereby their genes are able to withstand harsh conditions in the degradation of these pollutants (Magdalene *et al.,* 2020). This present study therefore, focused on using mutant strains of fungal consortia for the bioremediation of petroleum refinery effluents in Kaduna, Nigeria.

MATERIALS AND METHODS

Sample Collection

Refinery effluents were collected under the storage tank in Kaduna Refining and Petrochemical Company (KRPC) Kaduna State (10.4896^{o} N, 7.4188^{o}E), Nigeria using sterile trowel into polyethylene bags, kept in sterile containers. The wastewater samples from different concrete wastewater reservoirs in Kaduna Refinery and Petrochemical Company were collected into four liters plastic bottles and stored in an ice block cooler and transferred to the laboratory immediately for analysis and transported to the Department of Microbiology Postgraduate Laboratory, Kaduna State University in ice chests. Analysis commenced immediately upon arrival.

Determination of Total Petroleum Hydrocarbons (TPHs)

Effluent samples were analyzed for total petroleum hydrocarbons using Gas Chromatography-Mass Spectrometry (GC-MS). Gas chromatography was performed with a Hewlett Packard (HP, Palo Alto USA) HP5890-II. MS-DOS compactable workstation was used for the system control as well as data acquisition. For the extraction, approximately 5.0mL of the effluent sample was used and thoroughly mixed with 150 mL of extraction solvent and extracted over 4h. The extraction was performed in 1,1,2-trichloro-1,2,2- trifluoroethane for IR and pentane for GC-FID analyses, respectively. After the extraction, 0.3g of silica gel was added to adsorb the polar material, such as vegetable oils and animal fats. The USEPA method 8440 (USEPA, 1996c) regards all "oil and grease" materials that are not eliminated by silica gel adsorption as "petroleum hydrocarbons". The extracts were filtered through Whatman GF/C filters (UK) using a DINKO D-95 vacuum pump (Barcelona, Spain). Sodium sulphate was added to the samples during the extraction procedure and in the filtration process to eliminate residual water. The extracts thus obtained were analyzed by GC-MS (Wei *et al.,* 2019).

Mutagenesis of Fungi Isolates using UV Light

A prepared spore suspension of the isolated fungi was discharged into sterile plates that were located under UV exposure (254 nm) for a defined time of exposure (1, 5 and 10 min) respectively the diameter of the plates was measured (Nahide *et al.,* 2017). Fungi isolates were screened for their potential to utilize refinery effluent following the method described by Ezekoye *et al.* (2017). Each fungi isolate were inoculated into sterile potato dextrose broth (PDB) and incubated at room temperature (25±2^{o}C) for 48 hours. Mineral Salt Medium (MSM) used consist of Na_2HPO_4 (0.2g), K_2SO_4 (0.017g), NH_4NO_3 (0.4g), KH_2PO_4 (0.003g), $MgSO_4.7H_2O$ (0.05g) as described by Ezekoye *et al.* (2017) was prepared containing 0.1% of refinery effluents and was sterilized by autoclaving at 121^{o}C for 15minutes. Ten millilitres of MSM were transferred into fifteen test tubes and 0.2ml of PDB grown isolates was transferred into fourteen tubes of the sterile MSM, mixed properly and incubated at room temperature (25±2^{o}C) for 8 days. Test tube of MSM with 0.1% refinery effluent (without inoculum) served as control. The tubes were monitored for growth indicated by the level of turbidity, dry weight and change in pH of the medium.

Bioremediation Studies of Refinery Effluents

Fungi which showed maximum growth during the screening test was molecularly characterized and selected for bioremediation study. Standardization of the fungal consortium inoculum was prepared following the method described by Ijah (2017). The culture medium of each isolate of the mutated fungi *Aspergillus niger*, *A. fumigatus*, *A. versicolor* and *A. quadrilineatus* was mixed with 500 mL of distilled water in a blender. The spores were counted in Neubauer's chamber and five suspensions of fungal isolates were pooled, resulting in a final volume of 2.5 L. The control was without fungi inoculum. Bioremediation study was carried out in accordance with the methods of Ijah (2017). by monitoring the following parameters: fungi count, pH, Temperature, COD, BOD of the effluent. A total of 16 conical flasks (250 mL) were used in duplicate, 4 mL of each consortium was inoculated into 200 mL of previously sterilized refinery effluent supplemented with mineral salt media. The samples were incubated at room temperature at 150 rpm for 28 days and were analyzed at an interval of 7 days for a period of four weeks. Fungi count was carried out using Neubauer chamber (hemocytometer) where 0.2 mL of the remediating medium was taken on a weekly basis and observed using 40X objectives of the light microscope. The pH of the medium was also determined on a weekly basis using a pH meter. The control did not include fungal inoculum. Instrument calibration and reproducibility testing were carried by following standard method.

RESULTS AND DISCUSSION

The GC-MS analysis of the refinery waste water discharge in order of retention time (seconds), area Pct, compound identified and percentage quality of the compounds. It was revealed that cis-13-Octadecenoic acid, methyl ester; 7-Hexadecenoic acid, methyl ester; 10-Octadecenoic acid, methyl ester; 11-Octadecenoic acid, methyl ester and Methyl stearate; showed the highest percentage quality of (99%) respectively. Other compounds identified include; 7-Hexadecenoic acid, methyl ester, (Z) (98%), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (98%), 11-Octadecenoic acid, methyl ester (98%) and Z,Z-10,12-Hexadecadien-1-ol acetate (90%) respectively (Table 1) below.

Table 1: Chemical Composition of the untreated Refinery Effluent determined using GC-MS

Retention time	Area Pct	Compound	% Quality
18.2038	0.2736	A	97
22.469	0.259	B	92
23.8445	0.62	C	96
25.3909	0.4398	D	97
26.122	0.1672	E	97

24.604	1.0128	F	98
26.2256	1.0555	G	97
26.6581	9.2763	I	98
27.5561	0.2479	J	99
27.7964	0.1241	K	99
28.2301	0.2673	L	91
28.5423	0.1034	M	95
29.2638	8.1056	N	99
29.3786	1.1953	O	99
29.4357	1.067	P	99
29.6896	4.7258	Q	98
35.0421	9.7015	R	90

Keys:
B= Diethyl Phthalate; C= Methyl tetradecanoate; D= Tetradecanoic acid, 12-methyl-, methyl ester, (S)- E= 13-Hexyloxacyclotridec-10-en-2-one; F= 7-Hexadecenoic acid, methyl ester, (Z)-; G= 9-Hexadecenoic acid, methyl ester, (Z)-; H= Pentadecanoic acid, 14-methyl-, methyl ester; I= 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; J= cis-13-Octadecenoic acid, methyl ester; K= 7-Hexadecenoic acid, methyl ester, (Z)-; L= Hexadecanoic acid, 14-methyl-, methyl ester; M= 9,12-Octadecadienoic acid, methyl ester; N= 10-Octadecenoic acid, methyl ester; O= 11-Octadecenoic acid, methyl ester; P= Methyl stearate; Q= 11-Octadecenoic acid, methyl ester; R= Z,Z-10,12-Hexadecadien-1-ol acetate

Table 2 shows the bioremediation study of selected mutated fungi consortium for the period of 28days. Parameters such as fungal counts, pH, temperature, COD and BOD were determined at weekly basis throughout the study time.

Table 2: Bioremediation Potential of the Selected Mutated Fungi Consortia

Isolate code	**Parameters**				
	Fungal count	**pH**	**Temperature**	**COD (Mg/L)**	**BOD (Mg/L)**
Week 1					
Control	0	6.72	24	116	112
A_1+B_2	$0.48X10^{-4}$	6.68	22.70	109	110
C_1+D_2	$1.16X10^{-4}$	6.63	22.72	106	107
$A_1+B_2+C_1+D_2$	$1.27X10^{-4}$	6.53	22.81	102	105
Week 2					
Control	0	6.72	24	116	112
A_1+B_2	$1.14X10^{-4}$	6.61	22.63	104	106
C_1+D_2	$1.28X10^{-4}$	6.57	22.57	102	105
$A_1+B_2+C_1+D_2$	$1.43X10^{-4}$	6.53	22.52	98	102
Week 3					
Control	0	6.72	24	116	112
A_1+B_2	$1.18X10^{-4}$	6.70	21.96	105	111
C1+D2	$1.36X10^{-4}$	6.67	22.48	103	108
$A_1+B_2+C_1+D_2$	$1.41X10^{-4}$	6.65	22.50	101	106
Week 4					
Control	0	6.72	24 116	112	
A_1+B_2	$0.92X10^{-4}$	6.69	21.61	114	109
C_1+D_2	$1.15X10^{-4}$	6.68	22.54	108	107
$A_1+B_2+C_1+D_2$	$1.29X10^{-4}$	6.70	22.70	102	110

KEYS: A_1 (*Aspergillus niger*), B_2 (*A. fumigatus*). C_1 (*A. versicolor*), and D_2 (*A. quadrilineatus*)

Table 3: Chemical Composition of Treated Effluent with Consortium of A+B+C+D Determined using GC-MS

Retention time	**Area Pct**	**Compound**	**% Quality**
15.4899	0.2618	A	95
17.8281	0.5705	B	87
18.9541	1.299	C	91
22.4512	0.2813	D	96
23.8243	0.1672	E	97
24.604	0.2422	F	96
26.1237	17.9251	G	98
26.6889	5.5101	I	98
28.2284	1.6324	J	91
28.5376	0.5178	K	97
28.7442	0.4686	L	97

Keys: A_1 (*Aspergillus niger*), B_2 (*A. fumigatus*). C_1 (*A. versicolor*), and D_2 (*A. quadrilineatus*)

A= 1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene- B= Undecanoic acid, 10-methyl-, methyl ester; C= Ledol; D= Methyl tetradecanoate; E= Methyl 13-methyltetradecanoate; F= Pentadecanoic acid, methyl ester; G= 13-Docosenoic acid, methyl ester; H= 7-Hexadecenoic acid, methyl ester, (Z)-; I= Pentadecanoic acid, 14-methyl-, methyl ester; J= Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester; K= Hexadecanoic acid, 15-methyl-, methyl ester; L= 13-Hexyloxacyclotridec-10-en-2-one;

Table 4: Chemical Composition of Treated Effluent with Consortium of A+B Determined using GC-MS

Retention Time	Area Pct	Compound	(%) Quality
10.9306	0.8882	A	64
30.231	1.0342	B	72
30.3155	3.0334	C	94
30.6224	34.1586	D	98
31.3023	3.3067	E	96
32.1363	8.6049	F	97
35.618	6.3508	G	96
36.592	6.7039	H	97

KEY:
A = Octane, 2-methyl; B= 1-Decanol, 2-hexyl; C= Nonadecane; D= Hexadecanoic acid, methyl ester; E= Eicosane; F= 11-Octadecenoic acid, methyl ester; G= Hexacosane; H= Heptacosane

Table 5: Chemical Composition of Treated Effluent with Consortium of C+D Determined using GC-MS

Retention Time	Area Pct	Compound	(%) Quality
13.370	0.3383	A	66
22.539	1.0141	B	84
22.4516	2.0276	C	92
22.2781	28.1261	D	91
23.2065	1.1063	E	97
24.1806	3.6202	F	94
26.341	5.1036	G	97
29.421	5.3518	H	98

KEY:
A = Eiocasane, 2-methyl; B= 11-Docosenoic acid; C= Hexadecanoic acid; D= Hexadecanoi acid, 11-methyl-, methyl ester; E= Methyl, 1,2 tetradecanoate; F= Methyl, 11-methyltetradecanoate; G= Hexadecanoic acid, 13-methyl ester; H= Nonadecanoic acid.

Discussion

The total petroleum hydrocarbons (TPHs) assessed in this study indicated that the percentage quality of most compounds were extremely high as well as their occurrences. This could be attributed to capacity of the fungi breaking down the large/heavy complex of the hydrocarbons into smaller/simpler ones, which are released into the environment. There present in water bodies pose a great danger to the aesthetic nature of the river, creating unpleasant odor, causing harm to both the micro and macro fauna of such water body. These petroleum products gets themselves attached to growing plants along the bank of the river thereby transferring its effects on humans as they consumed these irrigated farm produce, as well as the sea foods in general (Zhang *et al.,* 2019).

Bioremediation has often been viewed as a potent tool in the cleanup of oil polluted environment, and it is economical, environmentally friendly and minimally disrupts site (Roy *et al.,* 2018). According to Ataikiru *et al.* (2017), microorganisms are the main degraders of petroleum hydrocarbons in contaminated ecosystems. Bioremediation of Kaduna refinery effluents in this study indicated that, there was decline in the bioremediation parameters in the second week up to the fourth week whereby the COD, BOD all showed significant decrease. This implies that the UV mutagenesis of the fungal strains in this study had positive effect on the remediation of the effluents samples. This finding is similar to previous studies which have shown that UV mutagenesis has a positive effect on increasing the biodegradation ability of strains (Al-Khalid and El-Naas, 2018). However, it has also been shown that UV mutagenesis can have no influence on degradation efficiency (Shahi *et al*., 2016). It has been reported that the mutant strain of *Pseudochrobactrum* species XF1 has more tolerant over a wide range of pH values than the wild strain (El-Mahdi, 2017), who observed that UV irradiation and (EtBr) were applied in different doses for inducing mutations in the *P. roquefortii*. The hydrocarbon remediation results showed that mutants strains of *Aspergillus versicolor* had more degradative potential as compared to *A. quadrilineatus* which was confirmed by its biodegradation parameters (pH, temperature, COD, and BOD) respectively. These parameters have also been shown by Zahed *et al.* (2020) to induce or provide a suitable condition for other species of *Aspergillus* where *A. oryzae* is reported to have degradative potential resulting from the hydrocarbon degradation by its higher enzyme production. It has been reported that fungi are the most important eukaryotic microorganisms that are distinguished by their ability to utilize and use wide range natural and industrial media for growth and reproduction due to a multi enzyme systems that support the fungi to use the more complex materials such as hydrocarbons as a source of carbon needed for fungal metabolism to produce energy as ATP (Al-Khalid and El-Naas, 2018). The findings of this study confirmed that fungal tolerance to complex hydrocarbons is a function of their degradation ability accounted by the enzyme secreted as secondary metabolites by fungi which enhanced hydrocarbon degradation faster (Perdigão *et al.,* 2020). It has been reported by Ataikiru *et al.* (2018) that some yeast have great potentials in the field of

bioremediation. Once these organisms are exposed to these hydrocarbons, a series of biochemical and morphological modifications are triggered within the yeast cell especially when alkanes are the sole carbon source. There are modifications on the cell surface due to hydrocarbon transport in cell; induction of cytochrome P450 active in alkane and NADPH-cytochrome (P450) reductase hydroxylation; induction of enzymes involved in oxidation of fatty alcohols and their aldehydes; peroxisomes proliferation, induction of the characteristic beta-oxidative pathway and of the enzymes involved in glyoxylic acid and gluconeogenesis (Ataikiru *et al.*, 2018). More so, other compounds such as phenols, polychlorinated biphenyls (PCBs) and polyurethane can be degraded by fungi (Stella *et al.*, 2017).

Based on the Gas Chromatographic (GC) analysis of the fungi consortium, the degradative ability of the mutants of *Aspergillus versicolor and Aspergillus quadrilineatus* were observed to be prominent. An increase in crude oil degradation corresponded to an increase in cell number during the degradation process demonstrating the isolates' ability of utilizing crude oil. The highest degradability action by the above stated consortium could be attributed to the synergistic catabolic effect of both isolates. This implies that mutated fungi strains of *Aspergillus*, are good hydrocarbon degraders in nature. Previous study has shown that there were decreases in total petroleum hydrocarbons and higher decreases when a consortium was used in bioremediation (Ezekoye *et al.*, 2017). Wei *et al.* (2019), in their bio-augmentation studies used a yeast mixture of five different strains and recorded 80.7% – 98.5% loss of high molecular weight polycyclic aromatic hydrocarbons (PAHs). Ferguson *et al.* (2020) in their studies used *Candida tropicalis* in their bioaugmentation studies and reported 96% (saturated) and 42% (aromatic) hydrocarbon losses at optimum pH. Nrior and Onwuka (2017) investigated the biodegradation of crude oil impacted marshland soil by bioaugmentation using *Candida* and *Penicillium* species singly and as a consortium. They reported that the consortium containing *Candida* and *Penicillium* species exhibited higher bioremediation potentials than the individual species. The reduction of peaks of the GC chromatograph showed a qualitative score of the progress of hydrocarbon degradation (Ezekoye *et al.*, 2017).

CONCLUSION

The reductions in TPH, COD, and BOD levels observed in this study indicates the effectiveness of the mutant strains of fungal consortium used in this study, hence they are good degraders of petroleum products and as such very useful in the bioremediation technique to clean up/break down heavy pollutants in the environment. Findings of this study recommend further research on similar study should be carried out particularly on the enzymatic mechanisms of the organisms to degrade pollutants and the whole genome sequence to know the point of mutation.

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