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Full Length Research Article

SCREENING OF FUNGI ISOLATED FROM KADUNA REFINERY AREA FOR PETROLEUM HYDROCARBON BIOREMEDIATION POTENTIALS

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ABSTRACT
Bioremediation relies upon microbial enzymatic activities to degrade the offending contaminants. The aim of this study was to isolate and screen fungi from a Petroleum refinery environment for oxidase enzyme. Soil, effluent, surface water, sediment and ground water samples were analysed for hydrocarbon utilizing fungi (HUF). The HUF were isolated by the standard plate count method and the hydrocarbon vapour phase transfer technique. Oxidase enzyme activity was determined spectrophotometrically with acetaldehyde as substrate. Sixty fungi were isolated. Out of the 60 fungal isolates 52 (87%) were positive for oxidase enzyme. The colonial and microscopic characteristics indicated that the probable fungi from the sites were *Penicillium*, *Fusarium*, *Aspergillus flavus Monilia*, *Cephalosporium*, *Verticillium, Phytophora,* and *yeast*. *Penicillium* and *Fusarium* were the dominant fungi. The results of the oxidase enzyme activity indicated that majority of fungi in the study site possessed the ability to transform or degrade, contaminants.

Keywords: Bioremediation, Fungi, Enzyme, Oxidase, Isolates, Kaduna Refinery.

INTRODUCTION

Bioremediation is a new technology for the mitigation of contaminated sites. It relies upon microbial enzymatic activities to transform or degrade the offending contaminants. Bioremediation is cost effective. Other methods of disposal have drawbacks, incineration or burial in landfills are very expensive, if the amount of contaminants are large. Chemical and mechanical means used to remove hydrocarbons from contaminated sites have limited effectiveness and can be costly (Philp *et al.,* 2005).

Many microorganisms had been investigated for bioremediation processes. Notable among them are bacteria and fungi. However, fungi have been rated as better pollutant degraders (Obire *et al.,* 2008; Hadibarata et al., 2009; Qianwei *et al.,* 2020). This is because of their aggressive growth, greater biomass production, ability to grow under environmentally stressed conditions and extensive hyphal penetration in the environment (Obukohwo *et al.,* 2020).

Fungi are capable of degrading petroleum hydrocarbons by secreting enzymes (such as laccases, tyrosinases, manganese peroxidases, cytochrome P450 monoxygenases, and reductive dehalogenases) and they affect metal speciation by excretion of many other metabolites such as organic acids, amino acids, siderophores, extracellular proteins (Qianwei *et al.,* 2020).

Fungi utilize petroleum hydrocarbons as a carbon and energy source and assimilate them into fungal biomass. Some species can oxidize pollutants extracellularly by the production of laccases manganese peroxidasese, or lignin peroxidases (Grossart and Rojas-Jimenez, 2016; Falade *et al.,* 2017; Qianwei *et al*., 2020).

It has been reported that fungal cell membranes are permeable to many organic pollutants and these can be degraded by intracellular enzymes e.g. cytochrome P450, reductive dehalogenases and nitroreductases to simpler organic compounds, followed by further metabolism and entry into the tricarboxylic acid (TCA) cycle. (Xu and Zhou, 2016; Varjani, 2017; Tripathi *et al.,* 2017; Qianwei *et al*., 2020*).*

Fungi also possess many mechanisms or properties that influence metal toxicity and mobility, such as the production of metal-binding proteins, organic and inorganic precipitation, active transport and intracellular compartmentalization, while cell walls and associated pigments and polysacchandies have significant metal-binding abilities (Gadd, 1993; Gadd, 2007, Qianwei *et al*., 2020*).*

In spite of these capabilities of fungi, not much is known about the potential of fungi in sites that are contaminated with petroleum pollutants, while a lot of research has been performed on bacterial bioremediation of environments contaminated with petroleum hydrocarbons (Qianwei *et al.,* 2020). This work was undertaken to screen fungi from the study area for oxidase enzyme as part of site characterization. Oxidases catalyse, the introduction of oxygen and are often the key enzyme involved in the initial attack on hydrocarbon. Hence this study will provide information on capability of fungi in the site to degrade petroleum pollutants.

MATERIALS AND METHODS

Study sites

The site for the study was the Kaduna Refinery and Petrochemical Company (KRPC), in Kaduna, Nigeria. It lies between Latitudes 10^0 24' 05" N and 10^0 25' 20" N and Longitude 7^0 28' 49" E and 7^0 30' 01" E. The Company occupies an area of 2.89 square kilometres and is located on an undulating land about 700m above sea level (Buggu *et al*., 2020).

Collection of samples

Samples were collected aseptically using sterile containers from soil, surface water, sediment and effluent in the refinery environment. The sampling sites were:

Wastewater treatment facility and effluent discharge channel. The stations were designated RPE (Retention pond effluent), ORE (Outlet of retention pond effluent), IMFO (100 meters from outlet of retention pond), and 5MFO (500 meters from outlet of retention pond).

Wastewater receiving water body (Romi River). The surface water sampling stations were coded RU (100m upstream of effluent

discharge point on Romi River), and RD (100m downstream of effluent discharge point on Romi River). The corresponding sediment sampling sites were coded RUSD and RDSD respectively.
Sludge dumpsite (Area W) within the KRPC premises. The station for soil sample in this area was coded SDSL.
Farmland by Romi River upstream, Romi River downstream and Chidunu village respectively. The stations were coded RUSL, RDSL and CVSL respectively for soil samples.

Isolation of fungi
Serial ten-fold dilutions of the samples were carried out using quarter strength Ringers solution. Alquot of 0.1ml of the appropriate dilution was plated in two (2) replicates in minimal salt medium containing (g/l: NaCl 10.00, $MgSO_4$ $7H_20$, 0.42, KCl 0.29 kH_2PO_4 0.83, $Na_2HPO_4H_2O$ 1.25 $NaNO_3$ 0.42, Agar 15.00, distilled and deionized water to 1000ml mark (pH 7.2). Crude petroleum sterilized by filtration (millipore size 0.45µm) jointly served as the carbon and energy source and was made available to the culture in vapour phase as described below. Sterile filter paper (Whatman No. 1) saturated with sterile crude oil was placed on the inside cover of each petri dish kept in an inverted position. These filter papers supplied the hydrocarbon by vapour - phase transfer to the inocula (Odokuma and Okpokwasili, 1993; Nwachukwu, 2000). The two replicates were incubated at room temperature (25^{Oc}) for 4-8 days. At the end of incubation the isolates were streaked for purity on Potato Dextrose Agar. They were then transferred onto Potato Dextrose Agar slants in Bijou bottles and stored for characterization.

Characterization and identification of mould isolates
Stored mould isolates were recultured on acidified Sabouraud Dextrose Agar plates and incubated at room temperature (25^oC) for 4-8 days. The appearance and pigmentation of the colonies on each plate were observed. Rapidity and luxuriance of growth, texture of growth and gross topographic characteristics were equally noted. The cultural morphology of the distinct colonies were examined following the methods of Barnet and Hunter, (1972). A small piece of mycelium was removed with a mounted needle and teased out on a slide with a drop of alcohol. When the alcohol had almost evaporated, a drop of lactophenol blue was added, and a cover slip was applied. The slide was left for 30 minutes before examination. Identification of the moulds was on the basis of their colonial characteristics and morphological appearance when viewed under the microscope. Structures of fungi for instance phialides, sporangia, collumellae, sporangiospores and conidiospores were observed to further characterize the moulds. Each fungal species identified was confirmed using cultures already identified by the International Mycological Institute (I.M.I) Egham, Surrey at United Kingdom (U.K.). The cultures were examined at the Departmental Repository in the Crop Protection Department, Institute of Agriculture Research (IAR), Ahmadu Bello University (A.B.U.) Zaria.

Characterization and identification of yeast isolates
Characterization and identification of the yeast isolates were based on the methods described by Barnet *et al.* (1983), Almedida and Pais, (1996).

Screening of isolates for oxidase enzyme activity
Each of the sixty fungal isolates was grown in 5 mL of Sabouraud Dextrose broth and incubated aerobically at room temperature for 3 days. At the end of incubation period, each culture was centrifuged at 5000 revolutions per minute (rpm) for five (5) minutes. The supernatants were transferred into sterile bottle, while the cells were transferred into 2 mL Eppendorf safe-lock tubes and stored in the refrigerator. Test tubes containing 100µl phosphate buffer, 20 µL methylene blue, 100 µL of supernatant and substrate (acetaldehyde), 0.1 M, 0.3 M, 0.5 M and 0.01 M) were shaken and covered with paraffin oil to avoid oxidation of methylene blue and incubated at $40^{0\ C}$ for five minutes. Control was also set up without the supernatant. Absorbance was read using spectrophotometer at 450 nm.

RESULTS
Table 1 shows the result of oxidase enzyme activity (OEA) of fungal isolates from effluent samples. All the fungal isolates (23) expressed oxidase enzyme. The OEA ranged between $6.00x10^{-5}$mM and $1.48\ x10^{-2}$mM. The highest ($6.00\ x\ 10^{-5}$mM) was from 1MFO (100 m from outlet of retention pond site).
The range of OEA from surface water samples is depicted in Table 2. The OEA varied between $2.94\ x\ 10^{-3}$ mM and $6.06\ x\ 10^{-3}$mM. Surface water from RU (100m upstream of effluent discharge point on Romi River) had the highest OEA. Two fungal isolates were negative for OEA. Table 3 shows the OEA of fungal isolates from soil. Out of fifteen (15) fungal isolates, ten (10) expressed OEA which ranged between $2.40\ x\ 10^{-4}$mM and $2.00\ x\ 10^{-2}$mM. Fungi isolate from RDSL (farmland soil by Romi River downstream) had the highest ($2.00\ x\ 10^{-2}$mM). The OEA of fungal isolates from sediment samples is shown in Table 4. Sixteen (16) out of the seventeen (17) isolates expressed oxidase enzyme which varied between $3.00\ x\ 10^{-4}$mM and $7.59\ x\ 10^{-3}$mM. The highest was OEA from Romi River Upstream sediment (RUSD).
In summary, 52 (87%) out of 60 fungal isolates were positive for oxidase enzyme activity. The overall highest OEA ($2.00\ x\ 10^{-2}$mM) was by fungal isolate from farmland soil at Romi River downstream.

Table 1: Oxidase enzyme activity of fungi isolated from effluent at Kaduna Refinery and Petrochemical Company

S/No	Probable organism	Site reference	Sample description	Absorbance	Enzyme activity (mM)
1.	NI	IMFO	Effluent	0.493	$1.48x10^2$
2.	NI	IMFO	Effluent	0.459	$1.38x10^2$
3.	NI	RPE	Effluent	0.418	$1.25x10^2$
4.	*Fusarium*	IMFO	Effluent	0.237	$7.11x10^3$
5.	Yeast	RPE	Effluent	0.233	$6.99x10^3$
6.	NI	IMFO	Effluent	0.194	$5.82x10^3$
7.	NI	RPE	Effluent	0.179	$5.37x10^3$
8.	*Monilia sitophila*	RPE	Effluent	0.167	$5.01x10^3$
9.	NI	RPE	Effluent	0.138	$4.14x10^3$
10.	NI	RPE	Effluent	0.131	$3.93x10^3$
11.	*Penicillium*	RPE	Effluent	0.12	$3.60x10^3$
12.	*Cephalosporium*	IMFO	Effluent	0.113	$3.39x10^3$
13.	NI	IMFO	Effluent	0.112	$3.36x10^3$
14.	*Monilia sitophila*	RPE	Effluent	0.109	$3.27x10^3$
15.	*Penicillium*	RPE	Effluent	0.104	$3.12x10^3$
16.	Yeast	5MFO	Effluent	0.096	$2.88x10^3$
17.	Trichoderma	RPE	Effluent	0.092	$2.76x10^3$
18.	*Fusarium*	RPE	Effluent	0.081	$2.43x10^3$
19.	*Penicillium*	IMFO	Effluent	0.045	$1.35x10^3$
20.	NI	PRE	Effluent	0.024	$7.20x10^4$
21.	Yeast	RPE	Effluent	0.013	$3.90x10^4$
22.	NI	RPE	Effluent	0.007	$2.10x10^4$
23.	NI	5MFO	Effluent	0.002	$6.00x10^5$

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Key:
RPE – Retention pond effluent
1MFO – 100M from outlet of retention pond
5MFO – 500m from outlet of retention pond
NI – Not identified.

Table 2: Oxidase enzyme activity of fungi isolated from water at Kaduna Refinery and Petrochemical Company

S/No	Probable organism	Site reference	Sample description	Absorbance	Enzyme activity (mM)
1.	*Fusarium*	RU	Surface water	0.202	$6.06x10^3$
2.	*Verticillium*	RD	Surface water	0.177	$5.31x10^3$
3.	*NI*	RD	Surface water	0.098	$2.94x10^3$
4.	*Monilia sitophila*	RD	Surface water	NIL	
5.	Yeast	RD	Surface water	NIL	

Key:
RU – 100m upstream of effluent, discharge point on Romi River
RD – 100m downstream of effluent, discharge point on Romi River
NI – Not identified.

Table 3: Oxidase enzyme activity of fungi isolated from soil at Kaduna Refinery and Petrochemical Company

S/No	Probable Organism	Site reference	Sample description	Absorbance	Enzyme activity (mM)
1.	NI	RDSL	Soil	0.665	$2.00x10^2$
2.	*Penicillium*	RDSL	Soil	0.221	$6.63x10^3$
3.	NI	RDSL	Soil	0.195	$5.85x10^3$
4.	*Cephalosporium*	CVSL	Soil	0.163	$4.89x10^3$
5.	NI	RDSL	Soil	0.082	$2.46x10^3$
6.	*Aspergillus flavus*	SDSL	Soil	0.07	$2.10x10^3$
7.	*Fusarium*	SDSL	Soil	0.04	$1.20x10^3$
8.	*Aspergillus flavus*	SDSL	Soil	0.03	$9.00x10^4$
9.	*Penicillium*	RDSL	Soil	0.02	$6.00x10^4$
10.	*Cephalosporium*	RUSL	Soil	0.01	$2.40x10^4$
11.	NI	RUSL	Soil	NIL	
12.	*Penicillium*	RDSL	Soil	NIL	
13.	NI	RDSL	Soil	NIL	
14.	NI	RDSL	Soil	NIL	
15.	NI	CVSL	Soil	NIL	

Key:
RUSL – Farmland soil by Romi River upstream
RDSL – Farmland soil by Romi River downstream
CVSL – Chidunu village soil
SDSL – Sludge dump site soil
NI – Not identified

Table 4: Oxidase enzyme activity of fungi isolated from sediment at Kaduna Refinery and Petrochemical Company

S/No	Probable Organism	Site reference	Sample description	Absorbance	Enzyme activity (mM)
1.	*Penicillium*	RUSD	Sediment	0.253	$7.59x10^3$
2.	NI	RUSD	Sediment	0.23	$6.90x10^3$
3.	NI	RUSD	Sediment	0.175	$5.25x10^3$
4.	*Cephalosporium*	RDSD	Sediment	0.164	$4.92x10^3$
5.	*Penicillium*	RUSD	Sediment	0.145	$4.35x10^3$
6.	*Penicillium*	RUSD	Sediment	0.133	$3.99x10^3$
7.	NI	RUSD	Sediment	0.121	$3.63x10^3$
8.	*Phytophora*	RDSD	Sediment	0.108	$3.24x10^3$
9.	*Penicillium*	RUSD	Sediment	0.096	$2.88x10^3$
10	NI	RUSD	Sediment	0.096	$2.88x10^3$
11	*Fusarium*	RDSD	Sediment	0.071	$2.13x10^3$
12.	*Yeast*	RDSD	Sediment	0.063	$1.89x10^3$
13.	*Penicillium*	RUSD	Sediment	0.056	$1.68x10^3$
14.	*Fusarium*	RUSD	Sediment	0.046	$1.38x10^3$
15.	NI	RUSD	Sediment	0.032	$9.60x10^4$
16.	NI	RDSD	Sediment	0.008	$3.00x10^4$
17.	*Penicillium*	RUPS	Sediment	NIL	

Key:
RUSD – Romi River upstream sediment
RDSD – Romi River downstream sediment
NI – Not identified

DISCUSSION

The major degradative pathways for both saturated and aromatic hydrocarbons involve oxidases (Philp *et al.,* 2005; Adnan *et al*., 2018). Oxidases activate hydrocarbon by inserting one or two oxygen atoms. Such activations which are unique to hydrocarbon allow the hydrocarbon to enter the standard cellular pathways for metabolism. Dioxygenases catalyse the addition of both atoms of oxygen from molecular oxygen into hydrocarbon substrate. Monoxygenases incorporate only one atom of molecular oxygen into the hydrocarbon substrate; the other atom of oxygen is reduced to water.it has been reported that the overall ability to degrade oil largely depends upon the enzymes produced by the hydrocarbon degrading species. Since 87% of fungal isolates in the study site possessed the oxidase enzyme, by implication their ability to transform or degrade contaminants is important. Indigenous microbes are ideal candidates for use in the bioremediation of hydrocarbon pollutants. Some researchers are of the opinion that addition of microbes (bioaugmentation) for remediation purposes can only be effective in the laboratory and not in the field. The reasons are numerous. Foreign strains of microorganisms may not be able to compete with indigenous population in contaminated sites. Secondly, the concentration of contaminants may not be sufficient to support their growth. Furthermore, the environment may contain substances that inhibit growth, predation by protozoa, also the fact that the introduced microbe may not be able to penetrate the soil to reach the contaminant. It had been reported that in recent times bioaugmentation had more success using activated soil rather than pure culture (Neethu *et al*., 2019). The activated soils are those soils containing indigenous microbial populations recently exposed to the contaminants.

Higher expression of OEA relatively observed by fungal isolates from effluent (1.48×10^{-2}mM) is probably due to the acidic nature

of the effluent, which is more favourable for fungi growth (Chikere and Okpokwasili, 2004). This could also be part of the reason why fungal isolate from soil (pH 5.1) expressed the highest OEA (2.00 $X10^{-2}$ mM). Relatively higher OEA expressed by fungal isolates in this study over bacteria (unpublished) suggests that fungi are potentially more active hydrocarbon degraders.

The higher number of fungal isolates from effluent that expressed OEA shows also that probably effluent contains substance(s) that favour fungi growth. It had been reported that fungi have a broad substrate range and are active against a wide range of compounds including the chlorinated aliphatics (Kari *et al*., 2003). Fungi also have capacity to grow in the presence of high concentration of toxic heavy metals (Bako *et al.*, 2008; Machido *et al.,* 2014). Furthermore, Ojumu *et al.,* (2005) reported that phenols and its derivate are prominent among pollutants in petroleum refineries and petrochemicals. Kaduna Refinery effluent contains phenolic compounds (NNPC, 1987). Also, Obukohwo *et al.* (2020) observed decline in phenol, lead, cadmium and nickel in the entire bioremediation medium in a study on fungal isolates from KRPC.

It is not surprising that lower number of fungal isolates from surface water expressed OEA. Gruttener and Jensen (1983) reported minor degradation role of fungi in aquatic environment. Bacteria and yeast had been reported to be the prevalent degraders in aquatic ecosystems (Atlas and Bartha 1992). It is a known fact that bacteria thrives better in environment with higher water activity while the converse is true for fungi. This is probably the reason why the highest OEA expressed by fungi in this study was by fungi isolate from soil (2.00 x 10^{-2} mM) and also sixteen (16) out of seventeen (17) fungal isolates from sediments were positive for Oxidase enzyme activity.

The finding of hydrocarbon utilizing filamentous fungi *Aspergillus* and *Penicillium* in the effluent, surface water, soil and sediment in the study area is significant. According to literature filamentous fungi show some advantages in the transport or translocation of essential substances including nutrients and water and pollutants over significant distance (Boswell *et al*., 2003; Qianwei *et al*., 2020). Colonization of soil by the fungal mycelium lead too enmeshment and aggregation of soil particles and improvement of soil structure, which is critical for bioremediation. *Aspergillus* and *Penicillium* spp had been investigated for the degradation of aliphatic hydrocarbons, chlorophenols and polycyclic aromatic hydrocarbons, with the organic pollutants serving as carbon and energy sources (Harms *et al*., 2011). The finding of hydrocarbon utilizing *Fusarium* in the samples is also interesting. *Fusarium* isolated from petrol station soil were investigated for the degradation of pyrene and tolerance to copper and zinc. The organism degraded more than 60% of the supplied pyrene and accumulated copper (cu) and Zinc (Zn) (Hong *et al*., 2010). However, Nilanjana and Chandran (2011) reported that fungal genera and Yeast genera namely *Candida* isolated from petroleum contaminated soil proved to be potential organisms for hydrocarbon degradation. They reported that *Aspergillus*, *Cephalosporium* and *Penicillium* were found to be potential degrade of crude oil hydrocarbons.

Interestingly too, the genus *Verticillium* which had been used successfully for bioremediation in contaminated soil (Hua *et al*., 2008) was also isolated in this study. The presence of hydrocarbon utilizing *Trichoderma* is equally interesting. The genius *Trichoderma* is genetically very diverse with a number of capabilities (Pratibha *et al*., 2013). *Trichoderma* is tolerant to a range of recalcitrant pollutants including heavy metals, pesticides, and polyaromatic hydrocarbons (Pratibha *et al.*, 2013). According to Leonce *et al.* (2020) several works indicated that *Trichoderma*, *Penicillium* and *Aspergillus Spp*_had higher Cu and Cobalt biosorption capacity compared to other fungi spp such as *Geotrichum Monilia* and *Fusarium.* Kumar *et al*., (2014) observed that *Aspergillus* sp and *Chrysosporium* sp could reduce the concentration of heavy metals from effluent. Further Pilot Scale bioremediation studies in the environment is recommended.

Conclusion

Sixty fungi were isolated from the refinery effluent, surface water and sediment. Out of the 60 isolates 52(87%) were positive for oxidase enzyme. The colonial and microscopic characteristics indicated that the probable fungi from the sites were *Penicillium, Fusarium, Aspergillus flavus, Monilia, Cephalosporium, Verticillium, Phytophora* and *yeasts*. *Penicillium* and *Fusarium* were the dominant fungi. The results of the oxidase enzyme activity indicated good potentials for the use of fungi to detoxify and degrade petroleum hydrocarbon in the study area. Further work should be done on bioremediation of the contaminated environments.

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