Science World Journal Vol. 19(No 4) 2024 https://dx.doi.org/10.4314/swj.v19i4.43
www.scienceworldjournal.org
ISSN: 1597-6343 (Online), ISSN: 2756-391X (Print)
Published by Faculty of Science, Kaduna State University

Full Length Research Article

SCREENING OF MUTANT FUNGI ISOLATED FROM ROMI RIVER FOR POTENTIAL TO DEGRADE REFINERY EFFLUENT

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ABSTRACT
Environmental pollution implies any alterations in the surroundings; but it is restricted in use, especially to mean any deterioration in physical, chemical and biological qualities of the environment. This present study was carried out with the aim to screen and mutate fungi consortia isolated from Romi river for potential to degrade refinery effluent. Refinery effluent samples were collected aseptically into sterile bottles from under the storage tank in Kaduna Refining and Petrochemical Company (KRPC) Kaduna State, Nigeria using aseptic technique into sterile bottles. Physicochemical analysis was carried out using standard methods. Mycological analyses of the samples including the mutation of the fungal isolates were carried out using standard techniques. Identification of the mutated fungi isolated was achieved using both conventional and molecular technique. Results of the physicochemical properties of the samples indicated that most of the parameters were within the acceptable limit set by standard organizations, and parameters supported the survival and proliferation of the fungi. It was observed that *Aspergillus niger* had the highest percentage occurrence across the sampling sites (29.55%), followed by *Aspergillus quadrilineatus* (27.77%), *Aspergillus fumigatus* (22.73%), and *Aspergillus versicolor* (20.45%). In this study, *A. versicolor* and *A. quadrilineatus* were sensitive to UV radiation. There was moderate to maximum growth observed after eight days of screening.

Keywords: Screening, Mutant Fungi, Consortia, Biodegradation, Refinery Effluent.

INTRODUCTION

Environmental pollution implies any alterations in the surroundings; however it is restricted in use especially to mean any deterioration in physical, chemical and biological quality of the environment (Mosley *et al.,* 2014; Ferguson *et al.,* 2020). Large volumes of wastewater are released into the environment. In most developing countries, industries discharge their wastewater without treatment due to high cost of existing treatment. Refinery effluent is highly toxic and poses an incredible threat to the nearby communities due to the presence of petroleum hydrocarbon in it. Therefore, petroleum refinery wastewater has to be sufficiently treated to meet quality standard of established regulations before being discharged into the stream (Musa *et al*., 2015; Santo *et al*., 2015).

Bioremediation (or microbial decomposition) of petroleum and petroleum products, is of considerable economic and environmental importance. Petroleum is a rich source of organic matter and the hydrocarbons that is readily attacked aerobically by a variety of microorganisms (Ataikiru *et al.,* 2017). Filamentous fungi play an important role in degrading diesel and kerosene by producing capable enzymes, because of their aggressive growth, greater biomass production and extensive hyphal growth in soil. Therefore, fungi offer potential for biodegradation technology (Blasi *et al.,* 2016). It has been reported by Ezeonugbu (2015) that single culture of fungi have been found to be better than mixed cultures of fungi and bacteria. Fungi have been found to be better degraders of petroleum than bacteria in traditional bioremediation techniques including bacteria. The persistence of petroleum pollution depends on the quantity and characteristics of hydrocarbon mixture and on the properties of the affected ecosystem. The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are active degraders of that environment (Blasi *et al.,* 2016). Petroleum effluents are of critical concern to environmental health as they are extremely toxic to biological systems, especially the immediate inhabitants of such habitats and therefore requires closed attention through bioremediation. Fungi are notably aerobic and can also grow under environmentally stressed conditions such as low pH and poor nutrient status, where bacterial growth might be limited (Ataikiru *et al.,* 2017). This present study was carried out with the aim to screen and mutate fungi consortia isolated from Romi river for potential to degrade refinery effluent.

MATERIALS AND METHODS

Study Area

The Kaduna Refining and Petrochemical Company (KRPC) is located to the west, off Kachia Road in Kaduna (10.4896º N, 7.4188ºE). The Refinery occupies a land area of 2.89 square kilometers. It is approximately 15km Southeast of Kaduna city. The area has an approximate elevation of 615m above mean sea level. Kaduna Refinery was constructed by the Chiyoda Chemical Engineering and Construction Company (now Chiyoda Corporation) and was commissioned in 1980 with an initial capacity of 100,000 barrels per stream day (BPSD) as the third Refinery in Nigeria in order to meet the tremendous and growing demand for petroleum products (Ezeonuegbu, 2015).

Collection of Samples

Water samples from Romi River were collected under aseptic condition. The samples were placed into polyethylene bags using sterile scooper kept in sterile containers and transported in ice chests to the Postgraduate Laboratory at Department of Microbiology, Kaduna State University (KASU) in ice chests. The water samples were collected from different points of the river (discharge, upstream and downstream) into 4L containers placed in ice block cooler and transferred to the laboratory immediately for analysis.

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Physicochemical Analysis of the Refinery Effluent and Romi River Water Samples

The physicochemical analysis was carried out as outlined in the methods of Thipeswamy *et al.* (2021). This was done to assess the natural conditions of the habitats, extent of pollution, physical and chemical conditions under which the autochthonous potential-biodegrading microorganisms exist. The physicochemical parameters analyzed were pH, temperature, electrical conductivity, dissolved oxygen (DO), biological oxygen demand (BOD), total dissolved solids, NO_3, SO_4, PO_4 and chemical oxygen demand (COD) (Thipeswamy *et al.,* 2021).

Isolation of Fungi from Water Samples of Romi River
Enumeration of fungal isolates

Samples were mycologically analyzed on the same day of collection in order to eliminate any possible form of microbial spoilage of the samples. Samples were collected and allowed to stand at ambient temperature (28±2°C) on a sterile workbench. Nine (9) mL of each sample in duplicates were aseptically dispensed in sterile centrifuge tubes at a speed of 250 rpm for 10 minutes order to obtain concentrates of the samples. After decanting the supernatant, 0.1 mL of the residue of each sample was spread-plated on Potato Dextrose Agar (PDA) in duplicate. With the aid of bent glass rod, 50µg/L of chloramphenicol was spread onto the surface of the medium (Ezeonuegbu, 2015). The inoculated plates were incubated at an ambient temperature for 3-5 days in a dust-free cabinet.

Isolation and Identification of Fungi from Refinery Effluents

Macromorphological characteristics of the fungal isolates, such as colour, texture, colour of the reverse side of the fungal isolates were observed and recorded. For the micromorphological characteristics, small tincture of the fungal growth was mounted on clean grease-free slide with a drop of lacto phenol cotton blue, covered with a cover slip and examined using 40x objective lens of the compound light microscope. Characteristics of the sexual reproductive structures, presence or absence of septate, aerial and substrate mycelia, presence of foot cells and chlamydospores were observed and recorded. Taxonomic guide as described by (Cheesbrough, 2012; Thipeswamy *et al.,* 2021) were used to identify each of the fungal isolate. The Isolated pure cultures were maintained on agar slants and stored in a refrigerator.

Mutation of Fungal 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 (Ghimire and Wang, 2018).

Mutant Fungal Bioremediation of Refinery Effluent

The fungal isolates were screened for their potential to utilize refinery effluent following the method described by Ghimire and Wang (2018). Each fungus isolate were inoculated into sterile Potato Dextrose Broth (PDB) and incubated at room temperature (25±2°C) for 48 hours. Mineral Salt Medium (MSM) used consisted 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 Ghimire and Wang (2018) was prepared containing 0.1% of refinery effluents and was sterilized by autoclaving at 121°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°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.

PCR Detection of 18S rDNA of Mutant Fungal Isolates

The fungal isolates were grown at 20±2°C in a Sabouraud Dextrose Broth in an Erlenmeyer flask for seven days and shaken at 200rpm using a centrifuge (Hesham *et al*., 2012). To collect the mycelium, the centrifuged broth was filtered with a nytal mesh (42 µL pore size), then washed with distilled water, blotted with paper towels (Hesham *et al*., 2012). The collected mycelium was frozen with liquid nitrogen (4°C) and ground into a fine powder with a mortar and pestles (Hesham *et al*., 2012). Two (2) mL of extraction buffer 80.2 m (Tris. HCl, pH 8.0, 0.25 m NaCl, 25 mm EDTA, 0.5% SDS) were added to the powder and incubated at 65°C for an hour (Hesham *et al*., 2012). The genomic DNA was extracted with a DNA extraction kit following the manufacturer's instructions (Hesham *et al*., 2012). The rDNA Internal Transcribed Region (ITS) containing the ITS 1 and 2 and also the intervening 5.8S rRNA gene was amplified, using a Perkin Elmer 2400 thermal cycler. The primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used. DNA amplification was begun with initialization step that is, holding the target DNA at 94-96°C for 5min. Denaturation of the double strands took 30 seconds and 30 cycles at 95°C. Annealing of products was at 50°C for a minute. Next is the elongation step at 72°C for a minute, then the final holding time at 72°C for 7 minutes to complete the final extension (Hesham *et al*., 2012). The final products of the PCR were resolved by electrophoresis in agarose MP gel. Then it was cleansed following the GENECLEAN II protocol (BIO101). Molecular weights of the amplified DNA were estimated by comparing them with 100bp DNA ladder (Gibco.BRL) (Hesham *et al*., 2012).

Dynamic ET Terminator Cycle Sequencing Kitprotocol was used for sequencing. Each sequencing reaction was assembled according to the manufacturer's instruction with a calculated volume of template DNA (0.1–0.2 pmol), primer (5 pmol), water, sequencing reagent premix in microliter using a formula provided by the manufacturer (Hesham *et al*., 2012).

RESULTS

The physicochemical parameters of Romi river are shown Table 1. The pH of river water was 6.70, total dissolved solid (128.00 Mg/L), electrical conductivity (0.20 ds/m) total nitrate (0.84 Mg/L), phosphate (4.61 Mg/L), sulphate (8.44 Mg/L), turbidity (0.30 NTU), and temperature (25.80°C) respectively. The BOD and COD of the water analysis had values of (115 Mg/L) and (120 Mg/L) respectively (Table 1).

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Table 1: Physicochemical Properties of Romi River Water Sample

Parameters	Value	WHO Standard
pH	6.70	6.5-8.5
Total Dissolved Solids (Mg/L)	128.00	50-150
Total Nitrate (Mg/L)	0.84	≤10
Available Phosphate (Mg/L)	4.61	≤10
Available Sulphate (Mg/L)	8.44	0-630
Electrical Conductivity (ds/m)	0.20	50-1500
Turbidity (NTU)	0.30	0.5-1.0
Temperature (°C)	25.80	-
Biological Oxygen Demand (Mg/L)	115	≤80
Chemical Oxygen Demand (Mg/L)	120	200

Table 2 shows the cultural and microscopic characteristics of the fungal isolates. Growth features were observed morphologically, culturally, and microscopically with Greenish coloration observed to be common amongst the isolates. The presence of septate and conidiosphores of the fungal isolates were as well viewed under 40x objective lenses of a compound light microscope.

Table 2. Cultural and Microscopic Characteristics of the Fungi Isolates

Isolates Codes	Features	A.species
A_1	Smoky green and velvely-powdery growth on PDA medium. Septate hyphae, flask-shaped vesicles- at 40X magnification.	*Aspergillus fumigatus*
A_2	Initial yellow growth which quickly turned bright to dark- Yellow-green, lime and olive green. Septate hyphae with long- conidiosphores, numerous conidia at 40X magnification.	*A. versicolor*
A_3	Growth of cinnamon brown on PDA after 3-5 days of incubation. Septate hyphae, short conidiosphores, round and smoothconidia at 40X magnification.	*A. quadrilineatus*
A_4	Surface granular in texture with whitish coloration that rapidly turned- black at the centre. Three (3) long conidiosphores arising from septate- hyphae. The central vesicles are completely covered with conidia 40X magnification.	*A. niger*

The frequency distribution of the fungal isolates is presented in Table 3 below. A total of forty-four fungal isolates were isolated in this study. It was observed that *Aspergillus niger* had the highest percentage occurrence across the sampling sites (29.55%), followed by *Aspergillus quadrilineatus* (27.77%), *Aspergillus fumigatus* (22.73%), and *Aspergillus versicolor* (20.45%).

Table 3: Percentage Distribution Occurrence of Fungal Isolates across the Sampling Sites (N= 44)

Organisms	Sampling Sites			
	A	B	C	Total (%)
Aspergillus niger	5	2	6	13 (29.55)
Aspergillus versicolor	3	3	3	9 (20.45)
Aspergillus quadrilineatus	2	6	4	12 (27.27)
Aspergillus fumigatus	3	4	3	10 (22.73)

Key: A: Discharge point; B: Upstream part of the river; C: Downstream part of the river.

The growth characteristics for mutant *Aspergillus niger* in duplicates isolated for growth in refinery effluent taken for one minute, five and ten minutes of screening respectively was observed. The fungus showed the highest turbidity growth after 8 days at (60 and 623 NTU) respectively. The highest pH value of the growth medium after eight days was 8.11 and the lowest was 6.99. It was equally observed that the organism recorded a maximum growth at 1 and 10 minutes of the screening respectively (Table 4).

Table 4: Growth Characteristics of Mutated *Aspergillus niger* from Refinery Effluent

Coded Isolate	Turbidity growth medium after 8 days (NTU)	pH growth	Dry weight (g)
F0	0	-	0
A_1	58	8.01	0.0168
A_2	56	7.97	0.0164
B_1	49	8.11	0.0163
B_2	52	8.05	0.0176
C_1	62	7.01	0.0233
C_2	60	6.99	0.0235

A_1-A_2= *Aspergillus niger* (1 minute of screening), B_1-B_2 =A. *niger* (5 minutes of screening), C_1-C_2= *A. niger* (10 minutes of screening), +++ Maximum growth, ++ Moderate growth, + Minimum growth, - No growth, NTU= Nephelometric Turbidity Unit.

The screening test for *Aspergillus versicolor* in duplicates isolated for growth in refinery effluent taken for one minute, five and ten minutes of screening respectively. Generally, a minimum growth was observed visually after eight days with the highest turbidity value recorded at (21 NTU) while the lowest was (12 NTU) respectively. Similarly, the pH values of the growth medium were observed, and the highest value was 6.81 which is slightly neutral, and the lowest pH value was 5.88 a weak acidity respectively (Table 5) below.

Table 5: Growth Characteristics of *Aspergillus versicolor* Isolated from Refinery Effluent

Coded Isolate	Turbidity after 8 days (NTU)	pH growth	Dry weight (g)
f0	-	0	0
F_1	17.5	6.81	0.0161
F_2	13.0	6.70	0.0140
G1	20.2	6.49	0.0153
G_2	21.0	6.41	0.0175
H_1	12	6.09	0.0134
H_2	16.5	5.88	0.0162

f0= Control; F_1-F_2= *Aspergillus flavus* (1 minute of screening), G_1-G_2 =*A. versicolor* (5 minutes of screening), H_1-H_2= *A. versicolor* (10 minutes of screening), +++ Maximum growth, ++ Moderate growth, + Minimum growth, - No growth, NTU= Nephelometric Turbidity Unit.

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The screening test for *Aspergillus fumigatus* in duplicates isolated for growth in refinery effluent taken for one minute, five and ten minutes of screening respectively. Moderate growth of the fungus was observed with much of the growth recorded at the first minute of the screening. However, unlike the fungus *Aspergillus versicolor*, the turbidity growth medium of *A. fumigatus* recorded after eight days was observed to have high turbidity values with the highest value at (37.5 NTU) while the lowest value being (12.0 NTU). Similarly, the pH values recorded were slightly acidic, neutral and weak base (6.50, 7.02, and 8.2) respectively (Table 6)

Table 6: Growth Characteristics of *Aspergillus fumigatus* Isolated from Refinery Effluent

Coded Isolate	Turbidity after 8 days (NTU)	pH growth	Dry weight (g)
L0	0	-	0
I_1	37.5	8.2	0.0172
I_2	32.5	7.99	0.0168
J_1	27.02	7.35	0.0181
J_2	27.58	7.02	0.0186
K_1	12.0	6.79	0.0132
K_2	16.03	6.50	0.0159

L0= Control; I_1-I_2= *Aspergillus fumigatus* (1 minute of screening), J_1-J_2 =*A. fumigatus* (5 minutes of screening), K_1-K_2= *A. fumigatus* (10 minutes of screening), +++ Maximum growth, ++ Moderate growth, + Minimum growth, - No growth, NTU= Nephelometric Turbidity Unit.

Table 7 shows the screening test for *Aspergillus quadrilineatus* in duplicates isolated for growth in refinery effluent at one, five and ten minutes of analysis respectively. There was moderate-maximum growth observed after eight days of screening with maximum growth taken at the first minute. High values were equally recorded for the turbidity growth medium of the isolates (54, 61, and 63 NTU) respectively. A pH range of weak acidity-neutral and basic values were as well recorded (6.82, 7.90, and 9.0).

Table 7: Growth Characteristics of *Aspergillus quadrilineatus* Isolated from Refinery Effluent

Coded Isolate	Turbidity after 8 days (NTU)	pH growth	Dry weight (g)
L0	0	-	0
L_1	50	7.92	0.0158
L_2	54	7.90	0.0161
M_1	31.0	8.85	0.0157
M_2	27.0	9.0	0.0142
N_1	63	6.82	0.0179
N_2	61	6.50	0.0174

L0= Control;L_1-L_2= *Aspergillus quadrilineatus* (1 minute of screening), M_1-M_2 =*A. quadrilineatus* (5 minutes of screening), N_1-N_2= *A. quadrilineatus* (10 minutes of screening), +++ Maximum growth, ++ Moderate growth, + Minimum growth, - No growth, NTU= Nephelometric Turbidity Unit.

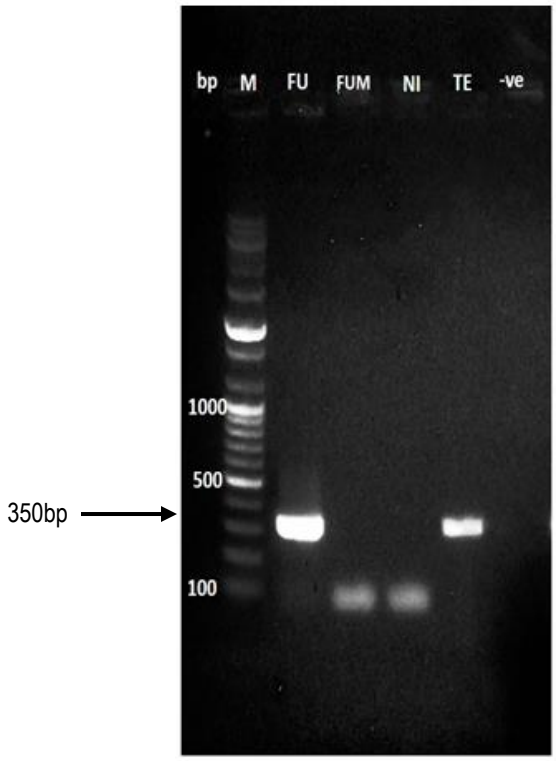

Plate 1: Gel electrophoretogram of mutant strains of *A. versicolor* and *A. quadrilineatus*

Key:
bp= base pairs; M= molecular ladder; FU= *A. versicolor;* FUM= *A. fumigatus;* NI= A. *niger*; TE= *A. quadrilineatus;* -ve= negative control

DISCUSSION

In this study, it was revealed that the pH of the effluent ranges 6.53-6.68 and a temperature of 25°C. This by implication suggests that the values of these parameters is supportive to the survival and proliferation fungi since most fungi grows best (optimally) at acidic pH range and temperature values that are within ambient (25-28°C) (Ahmed, 2019). It has been reported in a similar study that temperature affects the distribution and diversity of filamentous fungi, and that there is greater diversity in tropical areas than in temperate waters. In addition, marine fungi require temperatures between 25°C and 30°C to reproduce (Bay *et al.,* 2013), in a research Musa *et al.* (2015) of a river water in Phnom and its sub-urban regions reveals that the average temperature of the Bassac River (29.35°C) is slightly higher than other three rivers followed by the Upper Mekong River (29.10°C), the Tonle Sap (28.62°C) and the Lower Mekong River (28.45°C). The low pH in the range of 3.5–4.5 can affect the aquatic life (Varjani, 2017). The present study showed that pH of Romi river water sample is slightly neutral. However, the dissolved oxygen reveals changes that occur in the biological parameters due to aerobic or anaerobic phenomenon, and this suggest the condition of the river water to be suitable for several of most for aquatic organisms. Physical and chemical factors of any environment is one of the major determinants of the survival and proliferation of microorganisms in such an environment, particularly in environments that are harsh or

depleted of necessary nutrients/ carbon source for the metabolic activities of the organisms (Ghimire and Wang, 2018).

Four species of the fungal genera *Aspergillus* (*niger, versicolor, fumigatus* and *quadrilineatus)* were isolated from this study and their mutated consortium were used for the remediation study. The presence of *Aspergillus* species from the petroleum refinery effluent discharge could be attributed to the ability of *Aspergillus* species to withstand harsh environmental condition due to the production of spores which the organism utilizes as protective mechanism for survival in an unfavorable environment (Bay *et al.,* 2013). Ahmed *et al.* (2019) in a similar study reported that members of the genus *Aspergillus* are cosmopolitan and prevalent components of different ecosystems in a wide range of environmental and climatic zones due to the fact that they can colonize a wide variety of substrates. Fungi genera have an effective ability to break down hydrocarbon compounds into small parts by their extracellular enzymatic systems that secrete special enzymes to break down hydrocarbon compounds (Hesham *et al.*, 2012; Otunkunefor and Obiukwu, 2015). In this study, *A. versicolor* and *A. quadrilineatus* were sensitive to UV radiation. Consequently, lethality increased with radiation time and reached 95% at 5 minutes, after which it become constant. Thus, within 5 to 10 minutes it can be suggested to be an appropriate radiation time for inducing mutagenesis for hydrocarbon degradation. Gogoi *et al.* (2013) reported *P. griseofulvum* and *A. terreus* were sensitive to UV radiation and lethality also increased within the radiation time of 15 minutes at 95%. Therefore, it can be suggested that within 5 to 15 minutes time provided an appropriate radiation doses required to induce mutagenesis for efficient biodegradation of hydrocarbon. In this study, it was found that the fungi consortium tolerated varying concentrations of the refinery effluent samples and utilizes the hydrocarbons present in it as sole carbon source.

Conclusion

The physicochemical properties of the refinery effluent discharge were supportive to the growth and proliferation of the isolated organisms in this study. The isolated fungi from this study were of primarily *Asgergillus* genera (*niger, fumigatus, versicolor* and *quadrilineatus*). Mutations of these organisms were achieved using UV light. The study therefore, recommends that mutant strains of the isolated fungal species should be employed in the bioremediation of petroleum hydrocarbons in areas that are polluted with it.

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