Antimicrobial Resistance of Fungi Isolated from Oil-Producing Vicinity

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***Abstract*: - Antimicrobial resistance is a complex problem that affects all society. Some species of fungi are naturally resistant to treatment with certain types of antifungal agents. This study therefore investigated the resistant of fungi to antimicrobial agents in oil producing industry. Fungi were isolated from oilfield wastewater enriched soils obtained from the vicinity of an onshore oil producing platform using standard methods. The total fungi counts ranged between 1.15 x $10^4$to 4.09 x 10^4 CFU/g, while the hydrocarbon utilizing fungi ranged between 1.7 x 10^4 to 5.4 x 10^4 CFU/g. The fungal isolates were identified as *Aspergillusniger, Penicillium* sp, *Saccharomyces* sp, *Fusariumlichenicola* and *Aspergillussydowii*. Antifungal sensitivity testing showed that *Aspergillusniger, Aspergillussydowii* and *Fusariumlichenicola* were all resistant to Nystatin, Clotrimazole, Fluconazole and Ketoconazole. On the other hand, Penicillium was sensitive to Clostrinazole, Ketoconazole and Fluconazole but intermediate to Nystatrin while Saccharomyces was resistant to all the antifungal agents except Fluconazole with which it recorded an intermediate reaction. The results obtained revealed the occurrence of antifungal resistant in the oil producing vicinity. In order to control the spread of antifungal resistance, it is important to have an understanding of the environments in which such spread is likely as to help mitigate its occurrence to protect public health.**

Keywords: **Oil producing industry, antifungal Resistance, Clotrimazole, Ketoconazole**

I. INTRODUCTION

Fungal infections are caused by eukaryotic organisms, and it is therefore more difficult to ascertain their presence and apply the appropriate therapeutic treatment compared to bacterial infections. The cell wall of fungi maybe considered as the prime target for selectively toxic antifungal agents because of its chitin structure which is absent in human cells. Chemical treatments are largely effective but resistant species can be developed (Filomena *et al*., 2017). Antifungal resistant organisms pose a great burden on human population and are becoming a significant concern to clinicians who are taking care of patients. Incidence of fungal infections has drastically increased over the past three decades and was simultaneously accompanied by increased acquired and innate resistance to antifungal drugs (Patrick *et al*., 2012). Science daily (2018) reports that many drugs used to treat fungal infections in plants and animals are in danger of becoming ineffective and fear the same could happen to those treatments used for human infections. According to the authors, overuse of existing antifungal chemicals is helping resistance to spread and is increasingly rendering treatment ineffective,which could lead to a global increase in human fungal diseases as well as increased loss of food crops and livestock to fungal pathogens. The threat of antimicrobial resistance is well established in bacteria compared to fungi. Therefore the study is aimed at investigating the antimicrobial resistance in oil producing vicinity.

II. MATERIALS AND METHODS

Collection of Oilfield wastewater and soil samples

Oilfield wastewater was collected from Ogbogu Flow Station; an onshore oil production platform located in Ogba Egbema Ndoni local government Area (ONELGA) of Rivers State, Nigeria. The Oilfield wastewater samples were collected using 4 Litre capacity plastic bottles and stored in an ice packed cooler.

The soil samples were collected 80 meters away from the discharge pond at a depth of 0-15cm with a sterile spatula into sterile polythene bags and stored in an ice packed cooler. The collected and appropriately labeled oilfield wastewater and soil samples were immediately transported to the laboratory for analysis within 24 hours for processing and analyses.

Soil enrichment with Oilfield Wastewater

Soil enrichment with oilfield wastewater was carried out by inoculating various concentrations (10%, 25%, 50% and 75%) of oilfield wastewater into separate soil samples and incubated in a rotary shaker. Samples were withdrawn at different time intervals or incubation periods and analyzed for fungal count and hydrocarbon-utilizing fungi as described below.

Determination of total fungi count of enriched soils

The total count of fungi in the samples was determined by the spread plate technique. An aliquot (0.1ml) of serial dilution (10^{-2}) of each of the various samples was plated onto separate Potato dextrose agar plates to which 0.1 ml of streptomycin solution was incorporated to suppress bacterial growth. The plates were incubated at 28°C for 5-7 days and the discrete colonies that developed were enumerated as the viable counts (CFU) of fungi in the oilfield wastewater and soil samples (Obire and Wemedo, 1996).

Hydrocarbon utilizing fungal count (HUF) of samples

Total hydrocarbon utilizing fungal count of the enriched soil samples was determined by inoculating 0.1ml of the serially diluted samples -1 on mineral salt agar. The mineral salt medium will be supplemented with streptomycin (0.1ml) to

suppress bacterial growth (Obire and Wemedo, 1996). The Vapour Phase Transfer method was adopted by the use of sterile filter paper discs that were soaked in filter sterilized crude oil which served as the only carbon source in the mineral salt agar. The sterile crude oil-soaked filter papers were aseptically transferred to the inside cover of the inoculated Petri dishes and incubated for 5 days at room temperature. Colonies that develop were counted, average of duplicate colonies calculated colony forming units pergram soil calculated.

Identification of fungal isolates from samples

The fungi were identified molecularly using internal transcribed spacer (ITS)

Internal Transcribed Spacer (ITS) Amplification of Fungi

The ITS region of the rRNA genes of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of10ul, the components included 0.25 ulBigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Antifugal Susceptibility Testing

Disk diffusion assay was used. The disks contained nystatin (100 units), clotrimazole (10µg/ml), fluconazole (25µg/ml) and ketoconazole (10µg/ml). Inoculums were prepared by picking five distinct colonies of approximately 1 mm from each 24 hours old culture grew on potato dextrose agar and incubated at 35^0C. Colonies were suspended in 5ml of sterile 0.85% normal saline. The suspension is vortex and turbidity adjusted to yield 1 x 10^6 – 5 x 10^6cells/ml (0.5 McFarland standards). A sterile cotton swab moistened with the inoculums suspension was used to apply to a plate containing Muller Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue. The plates were allowed to dry for 5-15 minutes before disks were placed in the center of the agar. The plates were incubated for 18-24 hours at 37^0C and slowly growing isolates were again read after 48 hours incubation. Zone sizes were measured in millimeters. The result interpretation was done using National Committee for Clinical Laboratory Standards (NCCLS, 2000) zone interpretative criteria for susceptible (diameter, ≥ 19 mm), resistant (diameter, ≤ 14 mm) or dose dependant (diameter between 15 and 18mm) for fluconazole (CLSI, 2012).

III. RESULTS

The result of the total fungal count (CFU/g soil) in soils treated with various concentrations of oilfield wastewater is as shown in Table 1 below. The counts of total fungi of various treatments ranged from 1.15×10^4 to 4.09×10^4

Table 1: Total Fungi Count from various Concentration of the Oilfield Wastewater on Soil

Sample	24hrs	Day 7	Day 14	Day 21	Day 28
Soil + 10ml of Oil Field Wastewater	1.65×10^4	1.45×10^4	1.77×10^4	1.51×10^4	1.60×10^4
Soil + 25 ml of Oil Field Wastewater	1.53×10^4	1.71×10^4	2.3×10^4	2.5×10^4	3.2×10^4
Soil + 50 ml of Oil Field Wastewater	1.39×10^4	1.56×10^4	2.8×10^4	3.2×10^4	4.01×10^4
Soil + 75 ml of Oil Field Wastewater	1.15×10^4	1.29×10^4	3.7×10^4	4.02×10^4	4.09×10^4

The result of the total hydrocarbon utilizing fungal count (CFU/g) in soils treated with various concentrations of oilfield wastewater is as shown in Table 2 below. The counts of total hydrocarbon utilizing fungal ranged from 1.7×10^4 to 5.4×10^4

Table 2: Hydrocarbon Utilizing Fungi from various Concentration of the Oilfield Wastewater on Soil

Sample	24hrs	Day 7	Day 14	Day 21	Day 28
Soil + 10ml of Oil Field Wastewater	3.8×10^4	3.1×10^4	4.7×10^4	3.6×10^4	3.4×10^4
Soil + 25 ml of Oil Field Wastewater	2.87×10^4	3.8×10^4	4.2×10^4	3.2×10^4	3.0×10^4
Soil + 50 ml of Oil Field Wastewater	2.2×10^4	3.2×10^4	3.6×10^4	3.0×10^4	2.8×10^4
Soil + 75 ml of Oil Field Wastewater	1.7×10^4	2.53×10^4	3.0×10^4	4.0×10^4	5.4×10^4

The antifungi sensitivity profiles of isolates from various concentration of oilfield wastewater are shown in Table 3. The most resistant fungi were *Aspergillusniger*, *Fusariumlichenicola* and *Aspergillussydowii*.

Table 3: Anti-Fungi Sensitivity Profile of Fungi Isolated from Soil treated with various Concentration of Oilfield Wastewater

Fungi	**NY**	**C**	**K**	**F**
*Penicillium*sp	I	S	S	S
Fusariumlichenicola	R	R	R	R
Saccharomycescerevisiae	R	R	R	I
Aspergilliussydowi	R	R	R	R
Aspergilliusniger	R	R	R	R

KEY: R- Resistance, S- Sensitivity, I-Intermediate, NY-Nystatrin, C-Clostrinazole, K-Ketoconazole, F-Fluconazole

IV. DISCUSSION

The present study had revealed the fungi population and resistance sensitivity profile in soils treated with various concentrations of oilfield wastewater. The soil treatment with 75ml concentration had the least fungi count on 24hours. The fungi count in all the treatment options increased progressively during the incubation period apart from 10ml treatment option which had fluctuation in counts during the incubation period. The hydrocarbon utilizing fungi were higher on day 28 compare to the total fungi. 75ml treatment option recorded progressive increase throughout the period of experiment. The prevalence of hydrocarbon utilizing fungi indicated that indigenous populations of soil heterotrophic microbes with particular reference to fungi have been displaced by active indigenous hydrocarbon utilizers. This is attributed to the proliferation of hydrocarbon utilizers as a result of the enrichment of their growth in the presence of the oilfield wastewater. All the fungal isolates showed resistance to Nystatrin, Clostrinazole, Ketoconazole and Fluconazole except *Penicillium* sp which was intermediate to Nystatrin, Fluconazole and susceptible to Clostrinazole and Ketoconazole, while *Saccharomycescerevisiae* showed intermediate to Fluconazole. The highest level of fungal resistance pattern to all tested antifungal was observed in *Fusariumlichenicola*, *Aspergillusniger* and *Aspergillussydowii.* The high level of resistance of fungi in this study in oil producing vicinity is of major concern. The resistance shown in this study could be attributed to the protective mechanisms developed by the fungi that are used to withstand toxic substance produced by hydrocarbon pollution. It has also been noted that susceptibility to antimicrobial agents is not static and could be altered by environmental impact and human activities (Abu and Egenonu, 2008).

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