

Tolerance to Heavy Metals by some Fungal Isolates from Petroleum Refinery Effluent in Kaduna, Nigeria.

ABSTRACT

Investigation was carried out to assess the potential of some fungal species to tolerate and grow in biomass invitro in the presence of some heavy metals (Pb, Zn, Cr and Cd) from Kaduna Refinery and Petrochemical Company (KRPC) effluent. This was done by subjecting all the fungal isolates through the tolerant test (biomass production). The isolates were inoculated into replicate 100ml flask containing 50ml of Potato Dextrose Agar (PDA) amended with 5,10 and 15ppm of Pb, Zn Cr, and Cd. Each test isolate was inoculated into replicate flask containing the same medium without the heavy metals to serve as control. All inoculated flasks were incubated aerobically at room temperature on a rotatory shaker for 7days. The mycelial mats were harvested by filtering the cultures through preweighed filter paper (No.1). The filters containing the mycelial mats were dried in an oven at 70°C for 18hours. It was observed that most of the isolates tested tolerated and grew in the medium containing 5 to 15ppm of tested heavy metals. Yield of dry mycelia mats in the heavy metal supplemented medium were also comparable to those grown in heavy metal free PDA medium. Three fungal species- *Aspergillus niger*, *Aspergillus flavus* and *Microsporum nanum* isolated from the refinery were found to be most tolerant to varying concentration of heavy metals (Pb, Zn, Cr and Cd). *A. niger* is most tolerant to 5µg/ml, 10µg/ml, and 15µg/ml concentration of heavy metals followed by *M. nanum*, *A. flavus* and *Penicillium marneffeii*. *Aspergillus fumigatus*, *Chaetomium* sp., *Cunninghamella bertholletiae*, *Trichophyton tonsurans* and *Nigrospora* sp. were less tolerant to the heavy metal

concentrations. **These three most tolerant fungal isolates are important in treating systems contaminated with heavy metals (HMs) because of their bioremediation potential.**

Keywords: Fungal isolates, Heavy metals, Biomass, Tolerance, Petroleum refinery effluent.

Introduction

HMs are present in nature and in industrial wastewater, especially, wastewaters of petroleum, tannery and textile origin.

Environmental pollution by HMs is of global concern". Authors should delete "everywhere to different degrees and specific to certain part of the biogeosphere. At a global scale, approximately 2.4 million tons of liquid effluents containing HMs and other toxic materials are generated per annum (Burgess and Almeida, 2012; Shahramet *et al.*, 2015). **One of the sources by which HMs are introduced into the environment is through anthropogenic activities** (Teitzel and Mathew, 2003).

HMs have been shown to pose significant problems to human health. **They contaminate food sources and environmental systems (soil, water, air).** Also excess loading of hazardous wastes has led to scarcity of potable water and pollution of soil, thus limiting crop production (Karmaludeen *et al.*, 2003). Metals may be accumulated, concentrated and magnified within food chains, causing organisms at higher trophic levels to become contaminated with high concentrations of chemical pollutants and metal contaminants than their prey (Hargrave *et al.*, 2000; Lee *et al.*, 2000). Effluents from **Kaduna Refinery and petrochemical Company (KRPC)**

contain toxic and hazardous materials that settle in rivers as part of the bottom sediment. They pose health hazards to the urban population that depends on the water as source of supply for domestic uses (Rahman *et al.*, 2008). According to Emoyan *et al.*, (2006) and Idiseet *et al.*, (2010), petroleum refining produces large amounts of effluents that are toxic.

Conventional processes for removal of metals from industrial wastewaters include chemical precipitation, oxidation-reduction, filtration, electrochemical techniques and other sophisticated separation procedures using membranes (Wuyepet *et al.*, 2007). These processes are expensive when metals are found in relatively moderate concentrations, such as 1-100 mg/L. Bioremediation through the use of biosorption and bioaccumulation approach is an emerging form of technology in which microbes including fungi are used to clean up contaminated soil and water and to remove or stabilize the contaminants (Strong and Burgess, 2008). Many microbial population including fungi have been identified as superior candidates for metal bioremediation and major advantages of fungi are their significant metal uptake ability at low anticipated price (Bai and Abraham, 2001).

The survival of a fungus in raw untreated effluents from petroleum refining, effluent retention pond and water bodies impacted by the effluent would require that ; (i) it will be able to grow at the expense of hydrocarbons (HCs) as the main source of carbon and energy (Cerniglia *et al.*, 1992; Ulfiget *et al.*, 2003). (ii) It withstands the toxicity of a wide range of polycyclic aromatic hydrocarbons (PAHs) that are components of untreated effluents (Kari *et al.*, 2003, Bakoet *et al.*, 2008). (iii) Will be able to resist and grow in the presence of high levels of toxic heavy metal ions typical of all petroleum refinery effluents (Ezeonuegbu *et al.*, 2014).

This investigation was conducted to assess the potentials of some fungal isolates, isolated from KRPC to remove some heavy metals from the effluents.

Materials and Methods

Collection of Samples

Samples of industrial effluents were collected in twelve (12) sterilized containers; 3 per sampling point from treatment plants at the Kaduna Refinery and Petrochemical Company (KRPC), The effluent samples were collected from four locations; at Point A (Untreated waste water channel), Point B (waste water retention pond), Point C (upstream of River Romi, where the discharge passes through), and Point D (downstream of River Romi). The samples were properly labeled before transporting them in an ice box to the laboratory. Isolation and analysis of the initial heavy metals were carried out immediately to avoid any contamination or deterioration in the samples.

Fungi culture and isolation

The media prepared was used to culture the fungi, the method of Thippeswamy *et al.*, (2012) was modified to isolate and identify the fungi of interest. Exactly 0.1ml of each effluent sample was collected, dropped and spread on Potato Dextrose Agar (PDA) plate containing Chloramphenicol. Inoculated plates were incubated at room temperature - 30°C for five days. After five days, colonies of various kinds were observed with different colours.

Identification of fungi

Isolated fungi were identified by using; macromorphological characteristics such as colour, texture, colour of the reverse side of the colony growing on the medium were observed and

recorded. The micromorphological characteristics such as characteristics of the sexual reproductive structures presence or absence of septation, presence of foot cells and chlamydospores were observed and recorded. Small portion of the growing region was mounted on clean grease free slide with a drop of lacto phenol cotton blue and covered with a cover slip and then examined by microscope using $\times 40$ objective lens. Each fungus was thereafter identified using appropriate taxonomic guide (Klich, 2002; Nagamaniet *al.*, 2006; Murugesan and Mahaswani, 2011; and Thippaswamyet *al.*, 2012). Pure cultures of the isolates were maintained in agar slants and stored in a refrigerator. Sub culturing was carried out as appropriate to maintain its viability. The identification was aided by reference to the description of fungi using identification keys of Barnett and Hunter (1972), Larone (2002), Klich (2002) and Samson *et al.* (2004). Seven (9) fungi species, *Aspergillusniger*, *aspergillusflavus*, *Aspergillusfumigatus*, *Microsporumnanum*, *Penicilliummarneffe*, *Chaetomium* spp, *cunninghamellaberthollliae*, *Trychophytontonsurans* and *Nigrosporaspp* belonging to seven (7) genera were identified.

Preparation of metal solution

Stock solution of 1000mg/l Pb, Zn, Cd and Cr were prepared by dissolving analytical grade salts of $(\text{CH}_3\text{COO})_2\text{Pb} \cdot 3\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$, CdCl_2 and $\text{K}_2\text{Cr}_2\text{O}_7$ Separately in 1 L sterile distilled water. The desired (5, 10 and 15 $\mu\text{g}/\text{ml}$) concentrations of heavy metal solutions were prepared from stock solutions (Thippesswamyet *al.*, 2012).

Screening for the tolerance fungi to heavy metals

The fungal isolates were screened for their capabilities to tolerate and grow in the presence of 5, 10 and 15 $\mu\text{g}/\text{ml}$ of the test heavy metal ions invitro. The yields of biomass in liquid shake

cultures were used as index of tolerance and growth in the presence of the formulated concentrations of the heavy metals. (Bennetet *al.*, 2002).

Each test isolates was inoculated in replicate conical flasks containing 100ml of freshly prepared potato dextrose agar and amended with 5, 10 and 15 µg/ml of Pb, Zn, Cr and Cd. The inoculated flasks were incubated at room temperature (30°C) aerobically on a rotatory shaker for 14 days.

The mycelia mats produced were harvested by filtering the cultures through preweighed Whatman filter paper (No 1). The filter paper containing the mycelial mats were dried in an oven at 70°C for 48 hours and reweighed. The yield of dry mycelia biomass was obtained by subtracting the weight of the filter paper alone from the weight of the filter paper and the mycelia biomass (Bennetet *al.*, 2002).

Results

The mean biomass yield of fungal isolates, tolerant to 5µg/ml, 10µg/ml and 15µg/ml of heavy metals are presented in Figures 1, 2 and 3 respectively. The highest mean biomass production was observed in *C. bertholletiae* at 5µg/ml of Zn with 81.33mg but it could not withstand 5µg/ml of Cr. *T. tonsurans* was also found not to grow at this concentration of Zn (Figure 1). The lowest biomass production was observed in *Chaetomium* sp. with 21.33mg/g of Cr.

The highest mean biomass production was observed by *A. niger* at 10µg/ml of Zn with 64.64mg/g. It was observed that *Chaetomium* sp. and *C. bertholletiae* could not grow at 10µg/ml of Cr, the lowest biomass (24.00mg/g) of Zn was recorded for *Chaetomium* sp. (Figure 2).

The highest mean biomass production (50.55mg) was observed with *C. bertholletiae* at 15µg/ml of Zn. whereas, the lowest biomass production was observed with *Nigrospora* sp. (Figure 3). No

growth was observed in many species (*Chaetomium* sp., *C. bertholletiae* and *T. tonsurans*) at this concentration of heavy metals, Zn, Cd and Cr (Figure 3).

Table 1 shows the overall tolerance of the various fungal isolates to different concentrations of the heavy metals. The most tolerant fungi were *M. nanum*, *A. niger*, *P. marneffei* and *A. flavus* with total mean biomass production of 48.03mg/g, 46.71mg/g, 44.14mg/g and 38.15mg/g respectively. Whereas, the least resistant fungi are; *Chaetomium* sp., *Nigrospora* sp., *T. tonsurans*, *C. bertholletiae* and *A. fumigatus* with biomass production of 18.00mg/g, 23.93mg/g, 27.92mg/g, 35.14mg/g and 36.19mg/g respectively. There was no significant ($P > 0.05$) difference between the biomass production of *M. nanum* and *A. niger* as well as *P. marneffei* and *A. flavus* during the tolerance test. This forms the basis for selecting the three most tolerant fungi used in this study.

Discussion

Fungi have been identified as superior candidates for metal bioremediation (Bai and Abraham, 2001). Heavy metals and their bioavailability exert main selective pressure on fungi to tolerate the toxic effects of high heavy metal concentration and to accumulate the heavy metals (Jaekel et al., 2005). The metal tolerant via biomass production from the fungi isolated from heavy metal contaminated effluent of the refinery have shown good growth in the presence of Pb, Zn, Cd and Cr (Figure 1, 2 and 3).

Our result from the tolerance test showed that increasing amount of heavy metal had different influence on the fungal biomass yield and heavy metal accumulation. All the fungi showed tolerance to Pb, Zn, Cd and Cr at high concentration except few that could not tolerate or accumulate the heavy metals at higher concentrations. Zetic et al., (2001) reported that the effect

of heavy metals on fungal growth was variable and dependent on the type of metal and its concentration in the medium. *A. niger*, *A. flavus*, *P. marneffeii*, *M. nanum* and *A. fumigatus* tolerated heavy metals at all level of concentrations tested. Though, the biomass reduced as the concentration increased (Zeticet *al.*, 2001). However, *Chaetonium* sp., *C. bertholletiae*, *T. tonsurans* and *Nigrosporasp.* could not survive some heavy metals at some concentrations. The observed toxicity of some heavy metals like Cr and Cd on fungi was due to their strong affinity complex with cell membrane constituents causing loss of cell integrity and impairment of cell functions (Chen and Wang, 2007). In all, the increased biomass was observed at low concentration and little or decreased biomass was observed at high concentration of heavy metals. This agrees with the work of Thippeswamy *et al.*, (2012), where they reported that the biomass of *A. niger* and *A. flavus* decreased with increasing metal concentration. The fungal biomass growths observed with some species of fungi after increasing the concentration of these metals indicated the importance of these fungi to grow in the presence of these heavy metals and hence suggest their capabilities in bioremediation of heavy metal contaminated effluents. The fungi capabilities of surviving in high concentration of heavy metals may be due to formation of oxalate precipitation (Sintuprapa *et al.*, 2000).

The statistical analyses revealed that there was no significant difference ($P > 0.05$) between *A. niger* and *M. nanum* in biomass production. **This may probably be due to the fact that** both organisms produced the same metal complex with thiol species and in turn stored in the vacuoles (Chen and wang, 2007; Abhishek *et al.*, 2010). It was observed that fungi of the same genus were significantly different in biomass production as Baldian and Gabriel (2002) also reported that various genera and also isolates of the same genus did not necessarily have the same heavy metal tolerance.

Most fungi belonging to the genera, *Aspergillus*, *Penicillium* and *Microsporum* resident in the refinery effluent impacted sites had high level of resistance and tolerated Pb, Zn, Cd and Cr. To survive and grow at the tested concentration of the metals used, the test isolates may have developed mechanisms to circumvent the toxicity of these metals. These mechanisms include exclusion by permeable barriers (Nies and Silver, 1995), extracellular sequestration (Nilenjana *et al.*, 2008) and enzymatic modification of the ions to less toxic form (Keong, 2003).

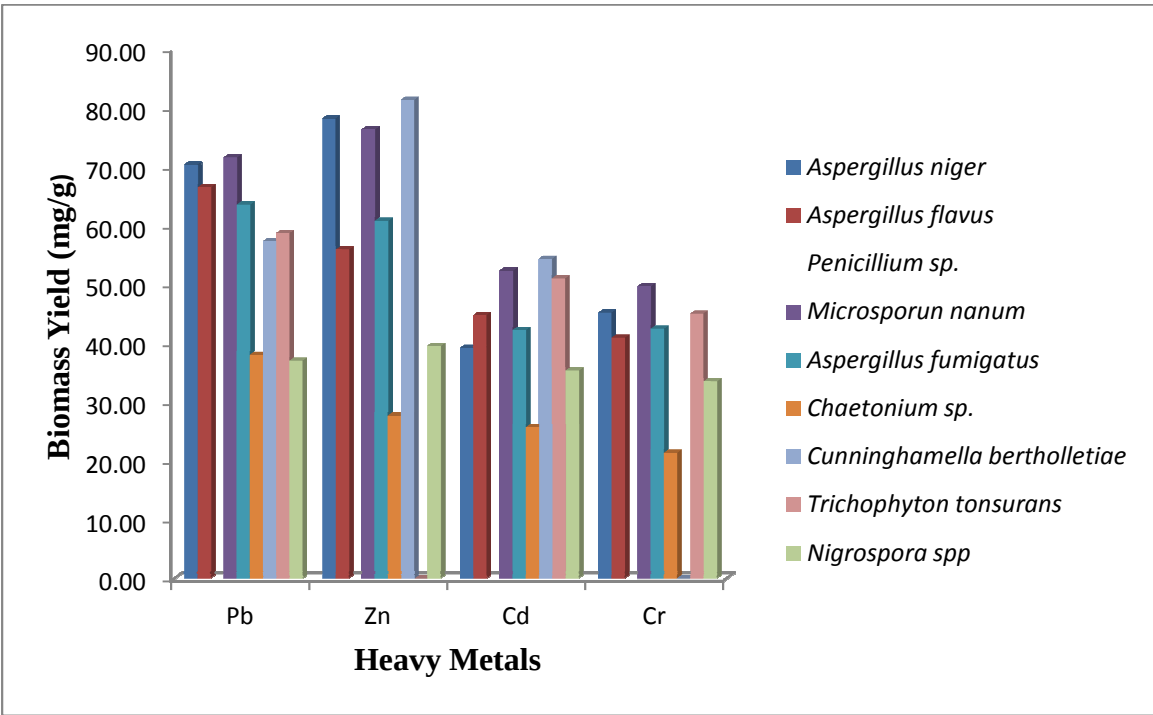


Figure.1: Mean biomass yield of fungal isolates tolerant to 5 µg/ml of heavy metals

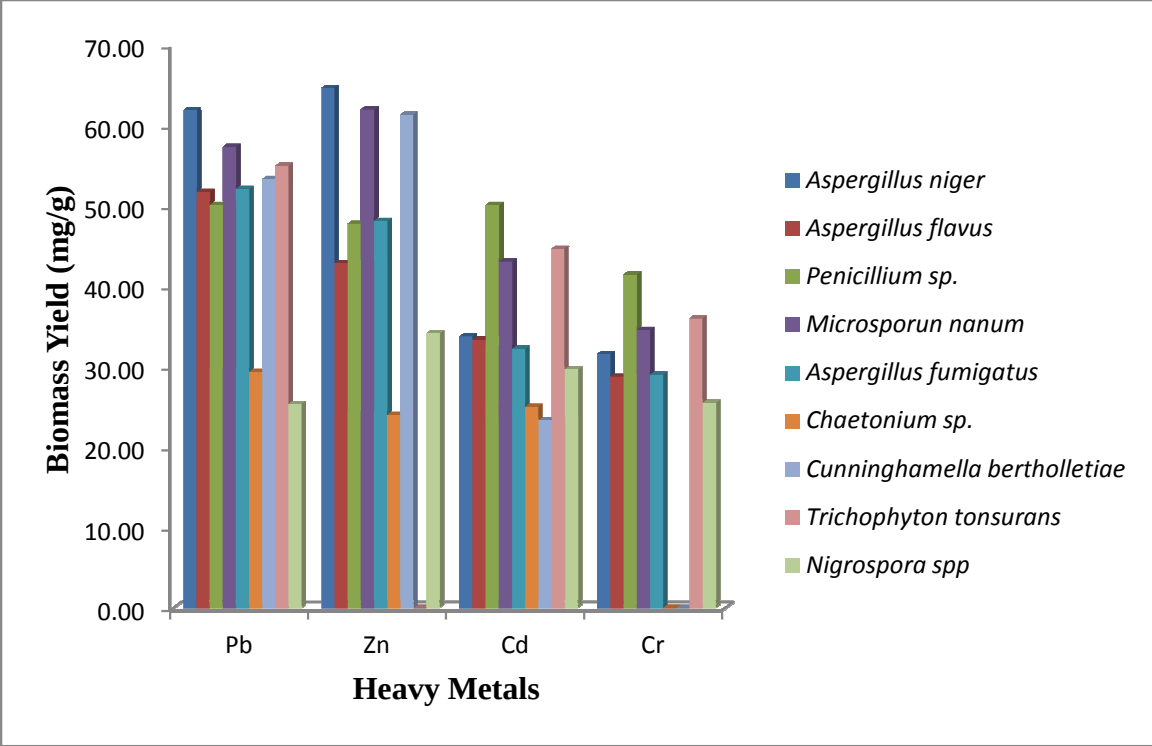


Figure 2: Mean biomass yield of fungal isolates tolerant to 10µg/ml of heavy metals

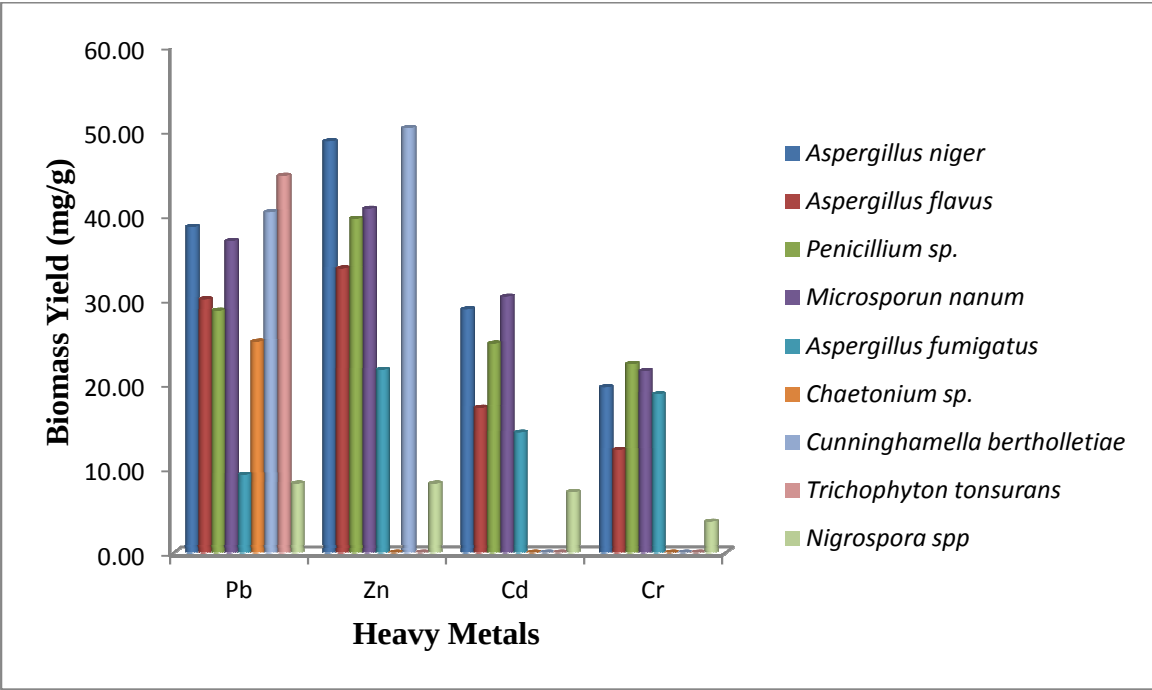


Figure 3: Mean biomass yield of fungal isolates tolerant to 15µg/ml of heavy metals

221 **Table 1: Overall effect of the heavy metal concentration on biomass production of the**
222 **different fungal isolates from KRPC refinery effluent**

Fungal Isolate	Biomass Yield (mg/g)				Average
	Zinc	Cadmium	Chromium	Lead	
<i>Aspergillusniger</i>	63.86±5.05 ^a	33.92±5.14 ^{ab}	32.11±4.81 ^a	56.94±5.59 ^a	46.71±2.80 ^a
<i>Aspergillusflavus</i>	44.14±5.27 ^{bc}	31.75±4.92 ^{ab}	27.28±4.40 ^a	49.42±3.38 ^{ab}	38.15±2.37 ^{ab}
<i>Penicilliummarneffeii</i>	47.93±3.71 ^{ab}	44.11±4.30 ^a	38.67±3.28 ^a	45.89±4.31 ^{ab}	44.14±1.96 ^{ab}
<i>Microsporunnum</i>	59.69±4.93 ^{ab}	41.92±5.24 ^a	35.25±4.91 ^a	55.28±5.06 ^a	48.03±2.62 ^a
<i>Aspergillusfumigatus</i>	43.52±4.08 ^{bc}	29.56±5.45 ^{ab}	30.07±5.17 ^a	41.63±5.35 ^{ab}	36.19±2.56 ^{bc}
<i>Chaetoniump.</i>	17.22±4.39 ^{de}	16.89±4.23 ^{bc}	7.11±3.56 ^{bc}	30.78±1.99 ^{bc}	18.00±2.23 ^e
<i>Cunninghamella berth</i>	64.33±4.73 ^a	25.89±7.89 ^{ab}	0.00±0.00 ^c	50.33±2.82 ^a	35.14±4.74 ^{bc}
<i>Trichophytonsurans</i>	0.00±0.00 ^a	31.89±8.09 ^{ab}	27.00±6.91 ^a	52.78±2.23 ^a	27.92±4.11 ^{cd}
<i>Nigrospora</i>	27.28±3.90 ^{cd}	24.06±4.47 ^{ab}	20.89±4.22 ^{ab}	23.50±3.21 ^c	23.93±1.97 ^{de}
P value	0.000**	0.081ns	0.001**	0.000**	0.000**

223 **Means across the same column with different superscripts are significantly different at**
224 **p<0.05.**
225 ****= Highly significant ($\alpha = 1\%$), ns = not significant**

226
227 **Conclusions**
228 All the fungal isolates produced substantial amount of biomass and tolerated heavy metal
229 concentrations; *A. niger*, *M. nanum* and *A. flavus* were the most tolerant to heavy metals. Those
230 isolated fungi from the refinery could be good candidates for bioremediation of polluted water
231 for heavy metals removal.

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233 **References**

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