

## BIOACCUMULATION POTENTIALS AND FT-IR ANALYSIS OF HEAVY METALS RESISTANT RHIZOMUCOR PUSILLUS, RHIZOPUS ORYZAE, AND TRICHOPHYTON EQUINUM IN THE REMOVAL OF SOME HEAVY METALS FROM TANNERY EFFLUENT

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### ABSTRACT

Heavy metals resistant *Rhizomucor pusillus*, *Rhizopus oryzae* and *Trichophyton equinum* were isolated from tannery effluent and screened for their ability to tolerate and bioaccumulate chromium, cadmium, copper, manganese and lead present in the tannery effluent analyzed in this study. *Rhizomucor pusillus* recorded the highest mean uptake of chromium (0.153 mg/g) per unit biomass while *Trichophyton equinum* recorded the least mean uptake of lead (0.007 mg/g) per unit biomass. Also, out of the three heavy metals fungi species isolated and used in this study, *Rhizomucor oryzae* had the highest percentage removal efficiencies of 73% of chromium while *Trichophyton equinum* had the least percentage removal efficiency of 21% of cadmium. Fourier Transform -Infra red (FT-IR) spectroscopy analysis was also conducted on the test isolates to determine the likely functional groups responsible for the uptake of the heavy metals during the bioaccumulation experiment. It was revealed that amines, hydroxyl, amides and carboxyl groups were the main functional groups present on the surface of the fungi cell wall that were responsible for the uptake of these heavy metals present in the tannery effluent during the bioaccumulation process. Finally, the result from this study further proves the potentials of these heavy metals resistant fungi species isolated from sites contaminated with heavy metals for the treatment of heavy metals in industrial effluent.

### INTRODUCTION

Pollution of the environment with toxic heavy metals is spreading throughout the world along with increased industrialization and human activities which had been reported to have negative impact on the environment (Aurelio et al., 2012). The problem of water pollution due to tanneries is also a serious environmental threat especially in developing countries (Smiley and Piyush, 2013). Leather processing in a tannery generally comprises three categories: beam house operations (pretreatment of skin/hide), tanning operation (chrome or vegetable tanning of skin/hide) and finishing operations (Stoop, 2003; Thanikaivelan et al., 2004). Suthanthararajan et al. (2004) reported that an approximate of 30m<sup>3</sup> of wastewater is generated during processing of one tone of raw

skin/hide. These wastewater usually contains high level of heavy metals and other pollutants such as large quantities of biological oxygen demand (BOD), low dissolved oxygen (DO), color, sodium sulphates, nitrate, phosphates, chromium, total suspended solids (TSS) and total dissolved solids (TDS) (Szyrkowicz et al., 2001).

The continuing introduction and accumulation of these heavy metals into the aquatic systems had often resulted into the deterioration of the ecosystem (Bueno et al., 2008). Also, the toxicity produced by the presence of these heavy metals ions in industrial wastewaters is a subject of public concerns, since these heavy metal ions can reach food chain and persist in nature (Basurco et al., 2009).

Previous researchers had also reported that the discharge of heavy metals in the aquatic system has become a global problem due to their carcinogenic and mutagenic nature (Mahiva *et al.*, 2008). According to the water standards used in most countries, levels of heavy metals ions in waste waters must be controlled and reduced to permissible limits (Dursun *et al.*, 2003). Beyond certain limits, heavy metals are toxic to living organisms and may cause serious hazard to public health. For these reasons the removal of excess heavy metals from wastewaters has generated a considerable interest in recent past (Dursun *et al.*, 2003).

Most heavy metal salts are soluble in water and form aqueous solutions and consequently cannot be separated by ordinary physical means of separation (Say *et al.*, 2003). Several methods usually employed for the removal of these heavy metals include chemical precipitation, membrane filtration and ion exchange (Bueno *et al.*, 2008). However, these conventional treatment techniques are either inefficient or expensive and may also be associated with the generation of secondary environmental pollutants in the process of removing these heavy metals from the industrial effluent (Kiran *et al.*, 2005). Therefore, there is need for an efficient, economical and environmentally friendly technology for the removal of heavy metals present in both industrial effluent and polluted environment (AL-Garni, 2005; Svecova *et al.*, 2006).

The ability of microorganisms to accumulate heavy metal ions from aqueous solutions has been widely reported (Tangaromsuk *et al.*, 2002; EL-Sherif *et al.*, 2008; Zamil *et al.*, 2009). The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in the

microbial communities (Vadkertiova and Slavikova, 2006), hence exerting a selective pressure on the microbiota (Verma *et al.*, 2001). Strains isolated from contaminated sites have an excellent ability of removing significant quantities of heavy metals from industrial effluents (Malik, 2004). Fungi are recognized for their superior ability to produce a large variety of extra cellular proteins, organic acids, enzymes for the bioremediation of heavy metals from contaminated sites (Dwivedi *et al.*, 2012).

Considering the importance of the removal and elimination of heavy metals from tannery effluent and other industrial effluent in order to preserve the ecosystem, this study was designed to elucidate the capability of indigenous filamentous fungi isolated from sites contaminated with tannery effluent from Challawa industrial estate of Kano state, Nigeria, to explore their potentials in the bioaccumulation of heavy metals and also to obtain information on the functional groups present on the surface of the fungi cell wall and possibly responsible for the bioaccumulation of these heavy metals present in the tannery effluent.

## **MATERIALS AND METHODS**

### **2.1 Sample Area**

The sample area used for this study was a tannery industry located at Challawa industrial estate of Kano state. Challawa ( Lat 11°52m 41sN, long 08°28m 09sE) is 515m above sea level originate from the Challawa gorge dam in Challawa village and stretches down to River Kano where it empties into lake Chad. The tannery industry discharges its effluents into canals, which converge at a point and flow into river Challawa as shown in the map below.

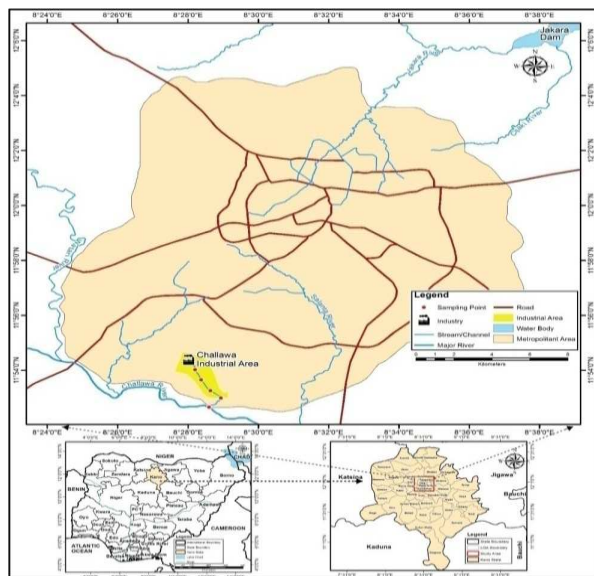


Figure 2.1: Satellite Image of Challawa Industrial Estate of Kano State Showing Challawa River Source: Google Image 2015



Figure 2.2: Map of Kano Showing Tannery Industry and Sampling Points in Challawa Industrial Estates of Kano, Nigeria.

Source: Adapted and modified from the

## 2.2 Sample Collection

Tannery effluent samples were collected at five sampling points comprising of, the upstream of Challawa river (A), tannery effluent from discharge point 1 of the tannery industry (B), tannery effluent from discharge point 2 of the tannery industry (C), entry point of the tannery effluent into Challawa river (D) and downstream (Challawa river) (E). An approximate interval of at least 50 meters apart was maintained from one sampling point to the other as shown in the map above. A total of 50 tannery effluent samples were collected for this study according to the method described by Ezike *et al.* (2012).

## 2.3 Assessment of Heavy Metals and Physicochemical Parameters of the Tannery Effluent

Assessment of heavy metal contents of the tannery effluent sample was carried out using standard methods described by APHA, (1992), Ademoroti, (1996); Abida *et al.* (2009); Thippeswany *et al.* (2012).

## 2.4 Isolation and Preservation of Fungal Isolates from Tannery Effluent

Isolation of fungi isolates was carried out according to the method described by Ijah (1998) using potato dextrose agar (PDA) supplemented with 100mg/100ml of chloramphenicol to suppress bacteria growth

and also to suppress each of the five heavy metals used in this study. The experiment was carried out in duplicates. While identification and characterization of the fungi isolate was carried out by carefully comparing macromorphological and micromorphological characteristics of the fungi isolates with appropriate taxonomic guide as described by Barnett and Hunter, (1999); Larone, (2002); Ellis *et al.* (2007); John and Roland, (2007); (Thippaswamy *et al.*, 2012). The pure identified and characterized fungi isolates were subcultured and preserved on PDA slant for further analysis (Liu *et al.*, 1997).

## 2.5 Bioaccumulation Experiment

The most heavy metal resistant fungi isolates were used to assess the ability of the fungi isolates to bioaccumulate chromium, copper, cadmium, manganese and lead present in the tannery effluent sample and Potato dextrose broth was also used to serve as nutrient (Dwivedi *et al.*, 2012). The Potato dextrose broth and the tannery effluent were mixed in the ratio of 4:1 (120ml of the Potato dextrose broth and 30ml of the sterilized tannery effluent sample) in 250ml Erlenmeyer flask and there after sterilized using an autoclave at 121°C under 15lb/sq for 15minutes (Joshi, 2011; Dwivedi *et al.*, 2012).

The fungi isolates were inoculated into the Erlenmeyer flasks and later placed in a shaker for seven days at room temperature (Iram *et al.*, 2012<sup>a</sup>). A similar experiment was set up without inoculating fungi isolates to serve as control. The experiment was carried out in duplicate. After the incubation period of one week, the content of each Erlenmeyer flask was aseptically filtered through a pre-weighed sterile Whatman's No. 1 filter paper to separate the mycelia from the culture filtrate. The concentration of chromium, copper, cadmium, manganese and lead left in the filtrate after the bioaccumulation experiment were analyzed using standard methods as described by APHA, (1992), Ademoroti, (1996); Abida *et al.* (2009); Thippeswany *et al.* (2012).

### **2.6 Estimation of Heavy Metals Uptake by Fungi per Unit Mycelium Biomass**

The heavy metal uptake of each of the heavy metals by the fungi isolates per unit mycelium biomass used in this study were calculated using the formula given below, in accordance with the methods described by Viraghavan *et al.* (1999) and Joshi *et al.* (2011).

$$Q = \frac{[V \times C_i - C_f]}{W} \times 1000$$

Where;

Q = Concentration of heavy metal uptake bioaccumulated per unit mycelium biomass by the fungal isolates (mg/ g).

V = Volume of heavy metal solution (tannery effluent) (ml)

C<sub>i</sub> = Initial concentration of heavy metal in the effluent before bioaccumulation (ppm)

C<sub>f</sub> = Final concentration of heavy metal in the effluent after bioaccumulation (ppm)

W = Dry weight of the fungal mycelial biomass (g).

### **2.7 Estimation of Percentage Removal Efficiency of Heavy Metals by the Selected Fungal Biomass**

The percentage removal efficiency of each of the fungi isolates mycelia used in this study in the removal of the heavy metals present in the tannery effluent were calculated using the formula given below, in accordance with the method of Kumar *et al.* (2011).

%removal efficiency

$$= \frac{\text{Con initial} - \text{Con final}}{\text{Con initial}} \times 100$$

Where;

Con<sub>initial</sub> = Initial concentration of heavy metals in the effluent before bioaccumulation (ppm)

Con<sub>final</sub> = Final concentration of heavy metals in the effluent after bioaccumulation (ppm)

### **2.8 Fourier Transform Infra Red Spectroscopy (FT-IR) of Fungal Mycelial Biomass**

FT-IR analysis on the heavy metal resistant fungi isolates were carried out as a tool to describe the phenomenon of fungi biomass in the bioaccumulation of heavy metals (Kumar *et al.*, 2009; Sahar and Hala, 2012). This is to obtain information on the possible functional groups (nature of the possible cell-metal interaction) responsible for the bioaccumulation of heavy metals in the tannery effluent (Rezael *et al.*, 2011). The analysis was conducted at the National Research Institute for Chemical Technology, Basawa- Zaria Kaduna state, Nigeria. The powdered fungi mycelia biomass before and after the bioaccumulation of the heavy metal experiment was air dried at 60°C using an oven for 18 hours in order to remove the moisture in the fungal biomass. Pellets method was used by mixing 0.1g of the fungal biomass with potassium bromide (KBr) and analyzed within the range of 3400  $\text{cm}^{-1}$  to 4500  $\text{cm}^{-1}$  (Schimadzu model 8400). The change in the absorbance peaks of the functional groups on the surface of the fungal cell wall as a result of exposure to the presence of heavy metals during the bioaccumulation experiment were observed and recorded. (Lata and Suman, 2012).

### **2.9 Statistical Analysis**

Data generated in this study were subjected to statistical analysis using statistical package for the social sciences (SPSS) version 20.0 one-way analysis of variance (ANOVA  $\leq 0.05$ ) to establish significant differences at 95% confidence limit of various parameters determined in the study.

## **RESULTS**

### **3.1 Characterization, Identification and Percentage Occurrence of Fungi Isolated from Study Sites**

A total of twenty five fungi species were isolated in this study comprising of three genera; *Rhizomucor pusillus*, *Trichophyton equinum* and *Rhizopus oryzae*

These fungi isolates were characterized and identified based on macromorphological and micromorphological features of the fungal isolates that were observed, recorded and later carefully compared with fungal atlas for proper identification of the fungal species in the tannery effluent samples as described by Barnett and Hunter, (1999), Larone (2002), and Ellis et al. (2007) and John and Roland, (2007).

Analysis in terms of percentage occurrence of the fungi species isolated from the study sites shows that, out of the twenty five of the fungi species isolated, *Rhizomucor pusillus* and *Rhizopus oryzae* had the highest percentage occurrence of 16%(4) while *Rhizopus oryzae* and *Trichophyton equinum* recorded the least percentage occurrences of 4%(1) as presented in figure 3.1 and 3.2.

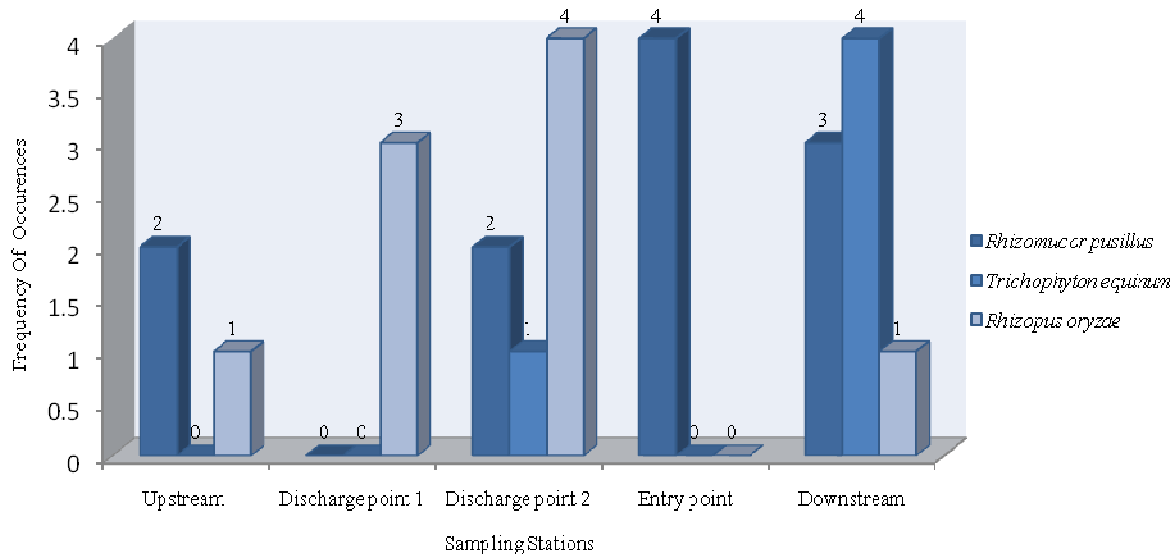


Figure 3.1 Frequency Occurrences of Fungi Species Isolated Across Sampling Points

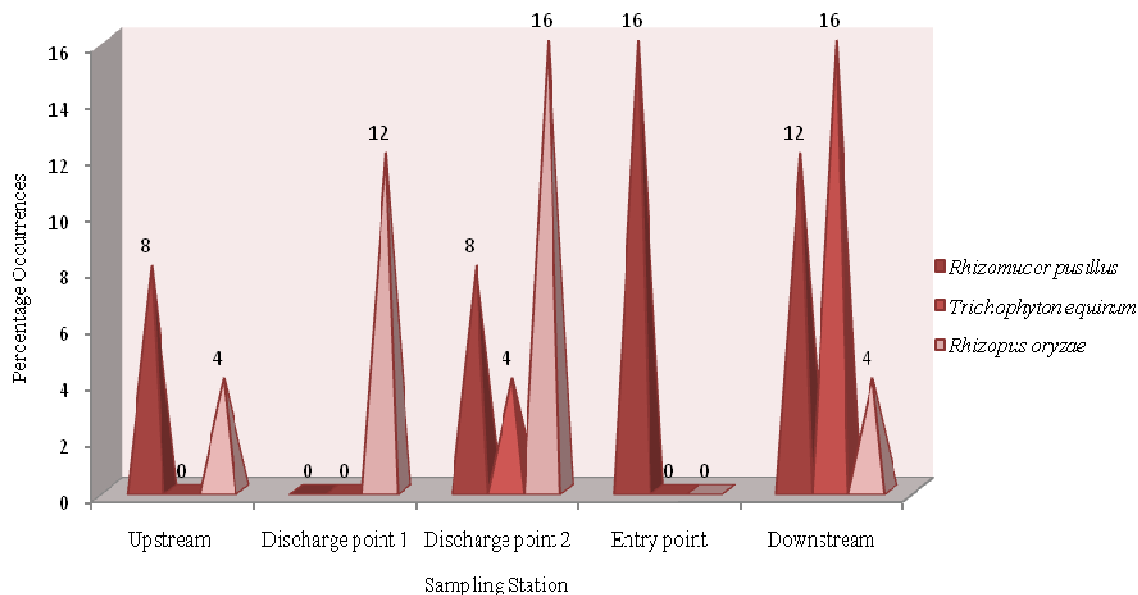


Figure 3.2 Percentage Occurrences of Fungi Species Isolated Across Sampling Points

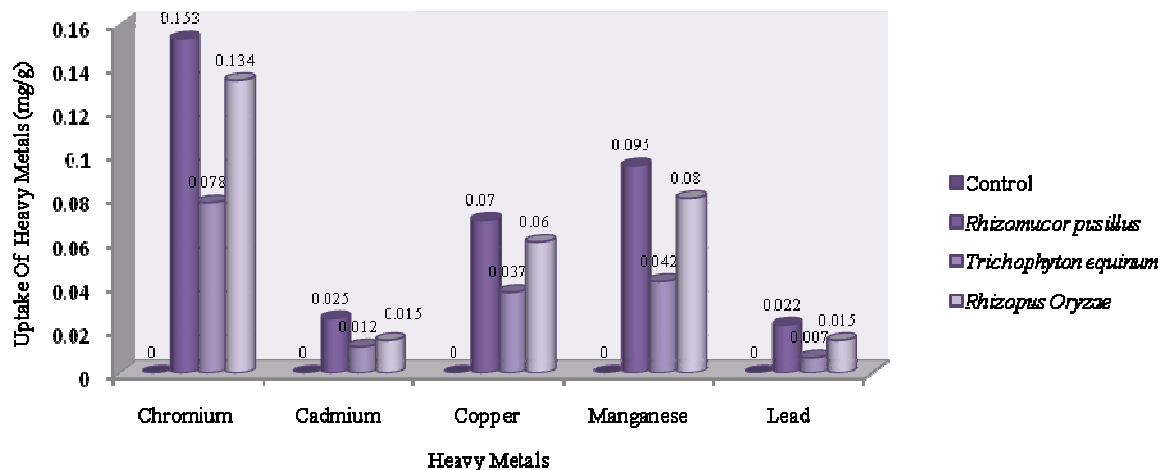
**3.2 Uptake of Heavy Metals by the Heavy Metal Resistant Fungi Isolates**

Figure 3.3 present result of the mean uptake of the five heavy meals used in this study. The highest mean uptake per unit biomass of 0.153

mg/g (of chromium) was recorded by *Rhizomucor pusillus* isolated at the discharge point 1 of the tannery industry while the least mean uptake per unit biomass of 0.007 mg/g (of lead) was recorded by *Trichophyton equinum*

isolated at the downstream of the Challawa river. Using analysis of variance (ANOVA  $\leq 0.05$ ), there were significant differences among the tested fungi isolates used in this study (p

value=0.01) in terms of the quantity of heavy metals that were bioaccumulated per unit biomass of the tested fungi isolates.



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Figure 3.3: Uptake of Heavy Metals by the Heavy Metal Resistant Fungi Isolates

### 3.3 Percentage Removal Efficiency of Heavy Metals in Tannery Effluent by Heavy Metal Resistant Fungi Isolates

Mean percentage removal of the five heavy metals by the three fungi isolates used in this study ranges from 21% to 73% of cadmium and chromium by *Rhizomucor pusillus* and *Trichophyton equirum* isolated at the downstream of Challawa river and discharge

point 2 of the tannery industry respectively as presented in figure 3.4. Using analysis of variance (ANOVA  $\leq 0.05$ ), there were significant differences among the tested fungi isolates used in this study (p value=0.00) in terms of the percentage quantity of heavy metals that were removed from the tannery effluent during the bioremediation process.

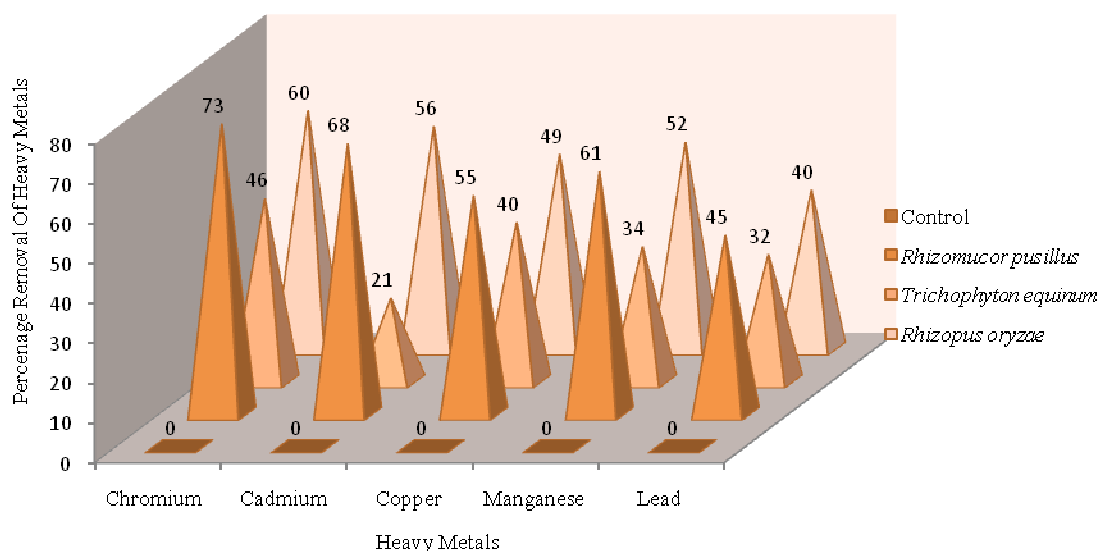


Figure 3.4: Percentage Removal Efficiency of Heavy Metals in Tannery Effluent by Heavy Metal Resistant Fungi Isolates

### 3.4 Fourier Transform Infra Red Spectroscopy (FT-IR) Analysis of Heavy Metal Fungal Biomass

Results of the FT-IR analysis of the three screened heavy metal resistant fungi isolates used during the bioremediation experiment is presents in table 3.1. This is aimed at determining the major functional groups responsible for the bioaccumulation of the heavy metals during the bioremediation process. The FT-IR analysis revealed that four major functional groups which include amines

(N-H), hydroxyl (O-H), alcohols (C-O), amides (C=O) played major roles in the bioaccumulation of the heavy metals during the bioremediation experiment except in the case of *Rhizopus oryzae* where the absorption bands (peak) for alcohols (C-O) was not detected. These functional groups were characterized by comparing the peaks obtained during the FT-IR analysis using the mycelium fungal biomass isolates with the standard FT-IR interpretation chart.

**Table 3.1: Fourier Transform Infra red Spectroscopy (FT-IR) Analysis of Heavy Metal Fungal Biomass**

Fungal Mycelium Biomass	Functional Groups Peaks (1/cm)							
	N-H (before)	N-H (after)	O-H (before)	O-H (after)	C-O (before)	C-O (after)	C=O (before)	C=O (after)
<i>Trichophyton equinum</i>	3437.26	3417.98	2938.65	2929.97	1092.71	1083.07	1646.30	1646.30
<i>Rhizopus oryzae</i>	1529.60	1514.17	2838.35	2885.60	-	-	1686.81	1687.77
<i>Rhizomucor pusillus</i>	3418.94	3419.90	2943.47	2942.51	1060.88	1068.60	1641.48	1642.44

Key: N-H; Amines, O-H; hydroxyl, C-O; Alcohols, C=O; Amides.

## DISCUSSION

### 4.1 Identification, Characterization and Percentage Occurrence of Fungi Isolated from Study Sites

Isolation of *Rhizomucor pusillus*, *Rhizopus oryzae* and *Trichophyton equinum* from the different stations in this study could be attributed to the ability of these isolates to survive in the tannery effluent due to the possible transfer of heavy metal resistant genes from one isolates to the other during storage of the tannery effluent from one settling reservoirs tank to the other during the production of leather. Malik and Ahmad, (2004) reported that, tanning process which include various activities such as soaking, liming, tanning of the hides and skin of animal produce high quantity of sludge and nitrogenous compound containing heavy metals. Also, in a similar study by Ahmad *et al.* (2005) reported that the occurrences of the fungi species in tannery effluent could be due to the ability of these organisms to tolerate and resist the high concentration of heavy metals usually present in tannery effluent.

### 4.2 Bioaccumulation of Heavy Metals in Tannery Effluent using Heavy Metal Resistant Fungal Isolates

Heavy metal tolerance microorganisms have been studied in different areas not only for their ability to remove heavy metals from polluted sites, but also to act as tools for bioremediation of polluted wastewater (Parameswari *et al.*, 2010). In the present study, it was observed that the three fungi species isolated from the tannery effluent were able to bioaccumulate the five heavy metals (chromium, copper, cadmium, manganese and lead) at different capacity. The difference in terms of their bioaccumulation potentials may be ascribed to the differences in the chemical compositions of each of the fungi cell wall of the isolated fungi species isolated in this study and also the various types of interaction between the heavy metals and the fungi cell wall. According to Kumar *et al.* (2011) reported that, the ability of fungi to resist and bioaccumulate heavy metals results from various mechanisms, such as, active transport of heavy metal ions into the cell, masking metals by chelating and enzymatic transformation of metal ions.

Other mechanisms that also aid the bioaccumulation potentials of fungi include creating of vacuoles in which the metal ions are gathered and immobilized in the form of polyphosphates, increased in the production of melanin and other pigments and also production of specific metal binding compounds inside the cell (Kumar *et al.*, 2011).

#### **4.3 Fourier Transform Infra Red Spectroscopy (FT-IR) Of Fungal Biomass Used for the Bioremediation Of Heavy Metals In Tannery Effluent**

The result of the FT-IR analysis conducted on the heavy metal resistant fungi mycelium biomass in this study revealed that four dominant functional groups were detected on the surface of the fungi cell wall; these functional groups include the amines, hydroxyl, alcohols and amides groups. Also the slight shifts or changes in the dominant peaks of the functional groups observed in this study may be attributed to the involvement and interaction of these functional groups (amines, hydroxyl, alcohols and amides) on the surface of the fungal cell wall with heavy metals during the bioremediation process. According to Rao *et al.* (2005); Akthar *et al.* (1996) all reported that the changes in the position and intensity of the peaks noticed in the FT-IR analysis of the fungal mycelium biomass in this study (before and after the bioaccumulation experiment) can be attributed to complexation of the functional groups on the surface of the fungi mycelium biomass which resulted from the hydrolysis of some polysaccharides on the fungal cell wall to shorter saccharides such as oligosaccharides, dioses and manoses as a result of the interaction of these functional groups with the heavy metals during the bioremediation process. In a similar finding, Razael *et al.* (2011) reported that the presence of hydroxyl group along with carbonyl group confirmed the presence of carboxylic acid groups in the cell wall of the fungal mycelial biomass. Also the presence of amines group and hydroxyl group along with carbonyl group might be attributed to the presence of amino acid groups in the cell wall of the fungal mycelium biomass. The result of this study is also in agreement with similar findings by Niu *et al.* (1993); Yan and Viraraghavan, (2003) and Ozsoy *et al.* (2008),

they all reported that bioaccumulation of heavy metals process occurred at amino, hydroxyl, carboxyl and amide groups by creating a negatively charged surface on the cellulose and chitin of fungal cell wall as a result of the ionization of the functional groups thereby enhancing the binding of the heavy metal ions. Also, the FT-IR analysis further revealed that the presence of functional groups such as amines, amides, carboxyl and hydroxyl groups were the main functional groups responsible for chemical reactions such as hydrogen bonding, electrostatic interaction and complexation of metal ions during the bioaccumulation experiment as previously being reported by Kumar *et al.* (2009); Lata and Suman, (2012). Lodeiro *et al.* (2006) also reported that functional groups such as carboxyl, hydroxyl, amides, amines have been identified as potential adsorption sites to be responsible for binding of metallic ions to fungal cell wall and that their potential for metal uptake depends on factors such as abundance of sites accessibility, chemical states and affinity between adsorption site and the heavy metals. Finally, according to Ahluwalia and Goyal, (2007), they reported that fungi are specific in heavy metal bioaccumulation which is attributed to the genera, species variability, functional groups, surface area and cell division. This phenomenon was also evident from the result of the bioaccumulation experiment recorded in this study which revealed that the heavy metal percentage removal efficiency by the three fungi mycelium biomass isolates were not the same across the three fungi isolates used for the bioremediation experiment. According to Das *et al.* (2008), they reported that bioaccumulation process by living cells is a two-step process; in the first step, metal ions are adsorbed to the surface of cells by interactions between metals and functional groups displayed on the surface of cells, while the second step involved the uptake or transportation of these metals into the cell membrane and cell cytoplasm.

#### **CONCLUSION**

Total of 25 fungi species were isolated from tannery effluent comprising of three fungi genera (*Rhizopus oryzae*, *Rhizomucor pusillus* and *Trichophyton equinum*).



Out of the three fungi species isolated in this study, *Rhizomucor pusillus* had the highest potential to bioaccumulate and removed chromium (73%) present in tannery effluent while *Trichophyton equinum* had the least capacity to bioaccumulate lead from the tannery effluent (21%)

FT-IR spectroscopy results further revealed the possible physico-chemical interactions between heavy metal ions and functional groups present on the surface of the fungal cell wall during the bioaccumulation of the heavy metals present in the tannery effluent. It was discovered that amines, hydroxyl, amides and carboxyl groups were the main functional groups present on the surface of the fungi cell wall and possibly responsible for the uptake and bioaccumulation

of the heavy metals present in the tannery effluent during the bioremediation process.

**RECOMMENDATIONS**

1. In order to further understand the phenomenon behind heavy metals interaction with fungal cell wall biomass, Scanning Electron Microscope (SEM) and Energy Dispersive X-ray (EDX) analysis should be conducted on the fungal isolates in future research as this will further revealed how these heavy metals interact with the fungi cell wall during the bioaccumulation process.
2. Advanced molecular techniques should be employed in other to find out the possible genes responsible for the heavy metals resistance by the fungi isolates.

**FTIR Spectra Of The Three Heavy Metal Resistant Fungi Isolates**

Note:

The black peaks represent FT-IR result of the fungal mycelium biomass before bioremediation

The red peaks represent FT-IR result of the fungal mycelium biomass after bioremediation

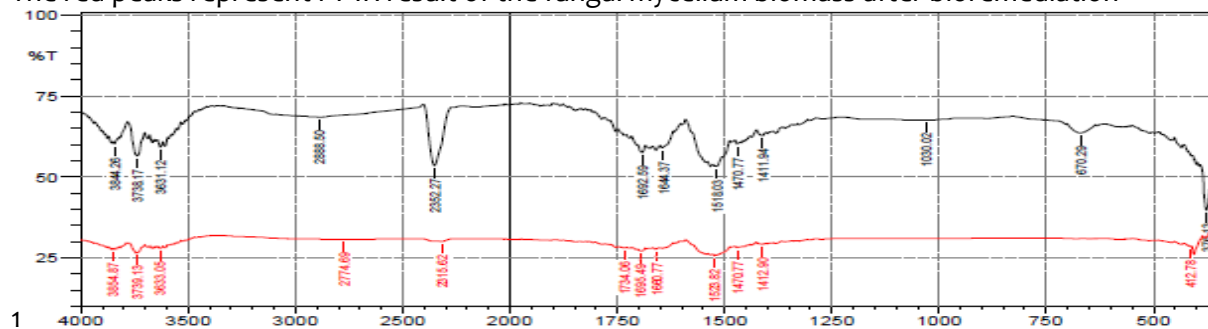


Figure 1: FT-IR Spectra of *Rhizomucor pusillus* fungal biomass

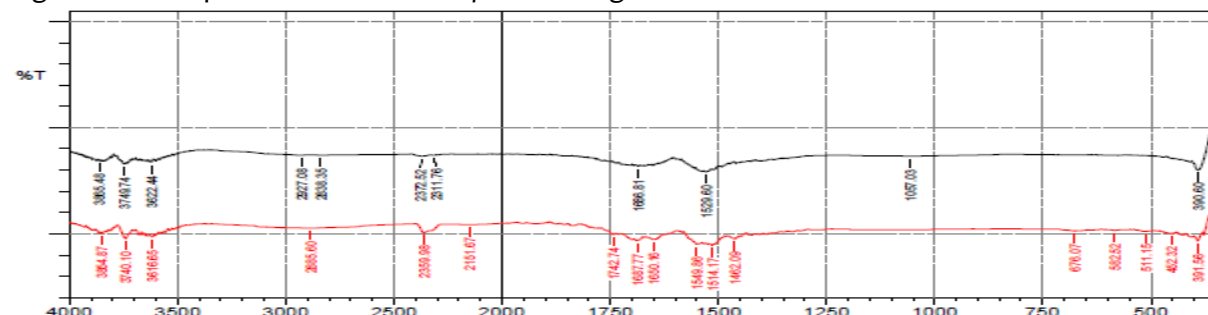


Figure 2: FT-IR Spectra of *Rhizopus oryzae* fungal biomass

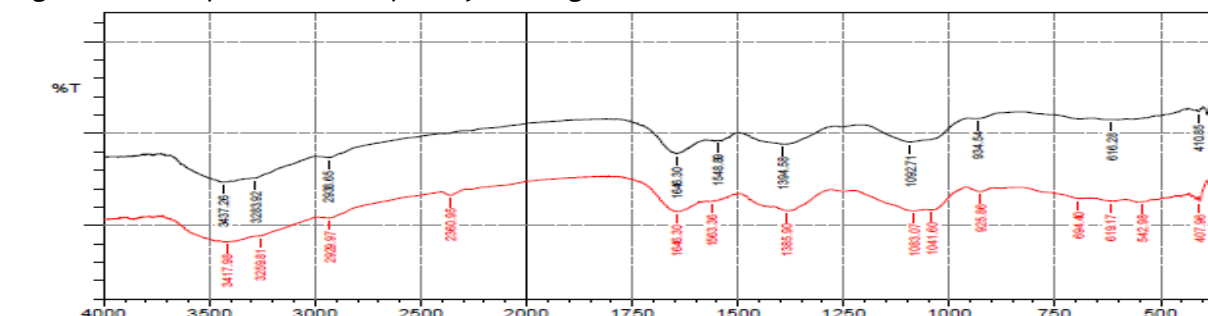


Figure 3: FT-IR Spectra of *Trichophyton equinum* fungal biomass

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