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Heavy Metals Resistance Potential of Some *Aspergillus spp.* Isolated from Tannery Wastewater

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ABSTRACT
Wastewater from three functional tanneries within sokoto metropolis, Nigeria, was investigated to determine the physicochemical properties as well as the distribution of fungal species in the untreated wastewater samples. Samples collected were designated (a-c) representing three sampling points. The isolated fungal species were; *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus tamarii* and *Aspergillus oryzae*. Of the fungal species, *Aspergillus flavus* was the most prevalent (17%) of the total count on the isolation plate, followed by *Aspergillus niger* (12%) and the least was *Aspergillus oryzae.* High levels of pH (9.6±1.7-9.9±1.2); electrical conductivity (4656 µs/cm ±886-5933µs/cm± 228); total dissolved solids (2024 mg/l±514-2934 mg/l±113) and nitrate (88.7 mg/l±2.2-94.7 mg/l±4.5) was observed in all the sampling points. The fungal species were screened for their ability to resist and grow in the presence of different concentrations of Pb (lead), Cr (chromium) and Cd (cadmium) in the laboratory. The result revealed that the majority of the isolates were resistant to Pb and Cr, whereas to Cd, only a few were able to resist and grow. *A. niger*, *A. flavus* and *A. terreus* had the highest level of resistance and tolerance to all the heavy metals, with a strong growth often exceeding the control (PDB without test heavy metals). Therefore, it was concluded that these species of *Aspergillus* could be performing an essential role in the mycoremediation of these metals present in the tannery wastewater during their period of acclimatization through bioaccumulation.
Keywords: *Aspergillus sp.*, Bioaccumulation, Mycoremediation, NESREA.

INTRODUCTION
The tanning industry is known to be source of pollutions especially through the discharge of effluents that possess organic and inorganic dissolve solids accompanied by propensities for high oxygen demand containing potentially toxic metal residues. A significant part of the chemical used in processing the leather is not actually absorbed in the process but it is discharged into the environment (Durai and Rajasimman, 2011). The quantity of heavy metals released into the environment is on the increase as a result of industrialization and technological advancement (Amini *et al*., 2008). This is of great concern to environmentalist as most of the metals are toxic, accumulate in food chains and more importantly non-degradable and persistent in nature and exerts a selection pressure on soil micro-biota (Emmanuel *et al*., 2015).

Lead, chromium and cadmium are included as one of the most detrimental heavy metals because of their high toxicity (Graz *et al*., 2011; Salinas *et al*., 2000).With the sporadic rise in industrial development, diseases and several struggles arising from heavy metal pollution requires safe and effective remediation approach as most if not all of the physiochemical methods of waste treatment are affected with many problems (Ahluwalia and Goyal, 2007). According to Rao *et al*. (1982) effluents from tanneries are known to have higher concentration of heavy metals. Rabah and Ibrahim (2010) reported high chromium content in soil polluted with tannery wastes in Sokoto.

Fungi are a ubiquitous group of microorganisms found in sub-aerial and subsoil of environments and often become a dominant group of metal-rich or metal polluted environments (Gadd, 1990). Today studies have shown that the strains isolated from contaminated area have the potential to tolerate such toxic conditions. Microbes have been shown to have the capacity to survive by adapting at high concentrations of heavy metals (Anahid et al., 2011; Yuan et al., 2007). Therefore, there is the need to explore newcomer resistant isolates as eco-friendly alternative means of heavy metal removal from tannery wastewater and other industrial effluents.

This research was carried out to isolate fungi from untreated tannery wastewater in Sokoto metropolis and to assess their resistance level towards cadmium, lead and chromium.

MATERIALS AND METHOD

Sample collection

Wastewater samples were collected from three functional tanneries: A (Unguwar Rogo I), B (Unguwar Rogo II) and C (Tudun Wada). In all, three samples i.e. one sample from each sampling point was collected in clean plastic containers according to standard procedures (Reza and Singh, 2010).The samples were collected in duplicate by lowering the plastic containers 30 cm deep into the mixed section of sampling point. All sample containers containing 500 ml of the sample were properly labelled to indicate the sample code, collection point, date and sampling time. After collection, the bodies of the plastic containers were rinsed thoroughly with sterile distilled water before transporting them in ice box to the laboratory for microbiological and the following physicochemical analysis: pH, nitrate, sulphate, phosphate, biological oxygen demand, and oil and grease contents.

Isolation of fungi

This was done according to method of Ezeonuegbu *et al*. (2014). Nine (9) millilitres of each sample was transferred into duplicate sterile centrifuge tubes and spun at 250 rpm for 10 min to concentrate the fungal propagules present in the samples. An equivalent amount of 0.1 ml aliquot of each sample suspensions was spread inoculated on triplicate plates of different freshly prepared solidified media (Potato dextrose agar (PDA) and Potato carrot agar) containing 50 ug/L of chloramphenicol using a sterile bent glass rod. All plates were incubated aerobically at 28 ^{0}C in disinfected dark cupboard for 7 days and monitoring daily for evidence of fungal colonies.

The fungal colonies observed were distinguished based on their cultural characteristics and recorded as (%) frequency of occurrence. The distinct colonies were isolated into slants of potato dextrose agar to obtain pure isolates and for further laboratory analysis.

Identification of fungal isolates

All the fungal isolates obtained from each of the samples were identified based on macro-morphological characteristics such as surface colour, texture, elevation, colour of the reverse side, margins and growth was recorded. For the micro-morphological characteristics, a small portion of the growing region was mounted on clean grease free slide with a drop of Lactophenol cotton blue, covered with a cover slip and examined by microscopy using x40 objective lens. The presence of reproductive structures, septation, conidia and conidiophores were observed and recorded (Samson *et al*., 1981).

Preparation of stock solution of heavy metals

Stock solutions of lead nitrate, potassium chromate and cadmium sulphate were prepared by dissolving 0.1g of each analytical grade salt in separate conical flask containing 500 ml of distilled water. The flasks were warmed on a hot plate simultaneously with gentle shaking to obtain a clear solution of 200 ug/ml concentrations and sterilized at 121 ^{0}C for 15 min. The solutions were stored in a refrigerator at 4 ^{0}C until needed.

Screening of fungal isolates for heavy metals resistance

The fungal isolates were screened for their ability to resist and grow in the presence of 5, 10 and 15 ug/ml of the test heavy metals in vitro. The yield of biomass in liquid static cultures was used as index of resistance and growth in the presence of test concentration of the heavy metals (Bennet, *et al*., 2002; Ezeonuegbu, *et al*., 2014). Each test isolate was inoculated in duplicate conical flasks containing 50 ml of freshly prepared potato dextrose broth supplemented with different concentrations of the test heavy metals. Also, medium inoculated with test isolates without heavy metals was used as controls and for comparative evaluation. The inoculated flasks were incubated at ambient temperature (28 ^{0}C) aerobically for 7 days. The mycelia mats produced were harvested by filtration through pre-weighed Whatman filter paper (No. 1) and aluminium foil. The filter paper bearing the mycelia mats was dried in an oven at 70 ^{0}C for 48 hours and re-weighed. 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 (Bennet, *et al*., 2002; Ezeonuegbu, *et al*., 2014).

Data analysis

Descriptive statistics was used to analyse physicochemical data using Prism software (Graphpad version 16.0).

RESULTS AND DISCUSSION

As presented in Table 1, the values of temperature in the study sites varies from 20.0 to 23.9 which were within the permissible limit (<40) of NESREA, Nigeria. The lower temperature in this study could be attributed to sampling time and weather condition as at the time of sampling. The Electrical conductivity recorded for the sampling sites range from 4656-5933 µs/cm and was above the recommended limit (200 µs/cm) of NESREA (2007). The variations observed could be attributed to method of analysis adopted by the researchers and possible difference of the dissolved salts used in the tannery industry. The higher conductivity could pose a threat when discharged into water bodies by altering the chelating properties of the water body and create an imbalance of free metal availability for both flora and fauna (Akan *et al*., 2008).

The total dissolved solids of the samples analyzed range from 2024 to 2934 mg/L as presented in Table 1. These values in all sampling sites were higher than the permissible limit (900 mg/L) of NESREA (2007). The high values recorded in this study might be attributed to high salt content which further renders the tannery wastewater unsuitable for irrigation, suggesting the need for treatment (Goel, 1997; Awofolu *et al*., 2005). Earlier analysis by Adamu *et al*., (2015) substantiated this study by reporting values for conductivity (13586-15500 µs/cm) and TDS (170-943.5 mg/l) that were higher than permissible limit of NESREA (2007) in Sokoto.

The results of this study shows that the levels of pH range from 9.6 to 9.9. Of the sampling sites, (A) shows a higher pH value than (B) and (C) which was basic and above the standard value set by NESREA (2007) (6-9). Akan *et al*. (2008) in Kano and Wosnie and Wondie (2014) from Ethiopia reported values that fell within the permissible limit. The pH outside permissible limit adversely affects the availability of heavy metal concentration and microorganisms (Akan *et al*., 2008). The high values in this study might be as a result of lime, soda ash and sodium sulphide used in the hides and skin processing and also due to the presence of carbonates and bicarbonates. The values in this study were similar to the findings of Amanial (2016). Discharge of untreated wastewater with such a high pH into rivers or on lands for any purpose could be detrimental to soil fauna and aquatic biota (Sugasini and Rajagopal, 2015).

The average dissolved oxygen along the sampling sites range from 5.9-6.6 mg/L and were within the standard permissible limit (>2.0) of NESREA (2007). DO is a measure of the degree of pollution by organic matter. The concentration of DO in water and wastewater is affected by physical, chemical and biological factors. Decomposition of large quantities of organic matter can severely depletes the water of oxygen. The standard for sustaining aquatic life is stipulated at 5mg/L a concentration below this value adversely affects aquatic biological life, while concentration below 2mg/L may lead to death for most aquatic life forms (Rossi *et al*., 2015).

The biological oxygen demand (6.0-8.1 mg/L) and chemical oxygen demand (7.8-9.0 mg/L) analyzed in this study were within the permissible limits of NESREA indicating a low microbial load. These low values might be attributed to the low biodegradable organic and inorganic matter present in the wastewater, which coincides with the fact that the lesser the BOD the higher the dissolved oxygen in any water or wastewater sample. The result in this study shows that the wastewater samples from the study site do not constitute a serious pollution problem with regards to the organic and inorganic matter content and therefore the discharge of wastewater with such a low BOD and COD poses no threat to the survival of aquatic life.

Nitrate concentrations in the sampling sites range from 84.7 to 94.7 mg/L respectively and were above the permissible limit (20 mg/L) of NESREA (2007). Nitrate is highly leaching, therefore, excessive rainfall or over-irrigation of soil with untreated wastewater with such a high nitrate content could lead to leaching of nitrate below the plant's root zone and may eventually reach groundwater. In addition, disposal of such wastewater with excessive concentration of nitrates into lakes and streams can cause excessive growth of algae and other plants, leading to accelerated eutrophication of the water bodies and occasional loss of dissolved oxygen (Knepp and Arkin, 2006).

The wastewater samples contained (4.3-5.0 mg/L) oil and grease which was above the permissible limit (0.1 mg/L) of NESREA respectively. These high values might be attributed to the fact that during leather manufacturing, natural oils and grease is released from within the skin structure. If surface waters are contaminated with grease or thin layers of oil, oxygen transfer from the atmosphere is reduced. If these fatty substances emulgate, they create a very high oxygen demand on account of their biodegradability (Bosnic *et al*., 1996).

Table 2 presents the mycoflora isolated from the study sites which had a predominance of six (6) fungal species belonging to the genus Aspergillus. This observation strongly indicates that members of this fungal genus have the capacity to survive, adapt and colonize environments polluted with tannery effluent. The difference between the sampled sites regarding their richness in microbial isolates appears to be closely linked to the high level of pH, TDS, and EC. The percentage and frequency of occurrence of fungal isolates (Table 3) was higher in (B) than in (C) and (A) i.e. B>C>A respectively. The source of pollutants as well as long periods of exposure could be among factors regulating fungal stress and adaptation (Zafar *et al*., 2007). Several reports have shown that organisms in tannery effluent utilize phenols and other hydrocarbons as source of energy (Mythili and Karthikeyan, 2011). The ability of fungi to secret extracellular enzymes in enormous could probably influence their ability to grow on a different hydrocarbon source (Steffen *et al*., 2003) as well as resist high levels of heavy metals due to their ability to bioconvert (Lubertozzi and Keasling, 2009), bioadsorb (Gorgeevaram *et al*., 2007; Nilanjana *et al*., 2008) or bioaccumulate (Lubertozzi and Keasling, 2009) the metal ions.

The results obtained from this study revealed that resistance to the toxicity of Pb, Cr and Cd was common among species of the mycoflora of tannery wastewater under study. At 5 µg/ml, *A. fumigatus, A. flavus*, *A. niger* and *A. terreus* were the most resistant to Pb (Fig. 1). *A. oryzae, A. tamarii* and *A. niger* were observed to be the most resistant to Cr at the same concentration. However, 5 µg/ml concentration of Cd was found to suppress the growth of all the isolates except for *A. niger* and *A. tamarii*. Increasing the concentration of the test metals to 10 µg/ml yielded a different resistant pattern among the isolates to Pb, Cr and Cd (Fig. 2). *A. niger* was observed to be the most resistant to Pb as well as Cr at the same concentration. It was also noted that *A. flavus and A. niger* were the most resistant to Cd. However, the mean yield of dry biomass was generally lower at 10 µg/ml than at 5 µg/ml. Increasing the levels of the test metals to 15 µg/ml resulted in a significant decrease in the biomass yield (Fig. 3) and Pb was better resisted at this concentration compared to Cr and Cd.

Table 1: Physicochemical Properties of the Wastewater from the three tanneries in Sokoto Metropolis

Physicochemical Parameters	Sampling Sites (n=3)			
	A	B	C	Permissible Limits NESREA
pH	9.9±1.2	9.7±1.5	9.6±1.7	6.0-9.0
Temperature	20.1±0.9	22.5±0.7	23.9±1.3	<40
Electrical Conductivity(µs/cm)	5845±34.0	5933±228	4656±886	200
Total Dissolved Solids (mg/L)	2893±16.5	2934±113	2024±514	900
Dissolved Oxygen (mg/L)	6.6±1.8	6.0±1.2	5.9±1.1	>2.0
Biological Oxygen Demand (mg/L)	8.1±0.6	6.0±0.3	7.0±0.4	30
Chemical Oxygen Demand (mg/L)	9.0±0.7	7.8±1.1	8.5±0.9	80
Phosphate (mg/L)	0.4±0.0	0.5±0.0	0.4±0.0	
Nitrate (mg/L)	84.7±2.2	94.7±4.5	88.5±8.0	20
Sulphate (mg/L)	2.9±0.4	2.5±0.4	3.5±0.5	500
Oil and grease (mg/L)	5.0±0.4	4.3±0.4	4.7±0.8	0.1

A=Unguwar Rogo I; B=Unguwar Rogo II and C=Tudun Wada

The ability for the fungi species to survive and grow in the presence of different concentrations of the test heavy metals suggest that they must have developed mechanisms to resist the toxicity of the metals. Several of such mechanisms have been reported to be employed by fungi growing in environments with high concentrations of heavy metals. These include; metal sequestration or accumulation (Teitzel and Mattew, 2003; Nilanjana, *et al*., 2008) and enzymatic modification of the metal ions to less toxic form (Ramarao *et al*., 1997). In addition, it has been reported that fungi possess specific genes for resistance to heavy metal ions. Mostly, these genes are chromosomal but others are plasmid borne (Nies, 1999). The plasmid encoded genes are mostly induced by the presence of specific metal ions (Rosen, 2002). These form the basis of bioaccumulation as a mechanism of resistance to heavy metal ions among fungi (Ezeonuegbu, 2014).

The variation in resistance to Pb, Cr and Cd observed among the fungal genera isolated from the studied sites suggest the isolated fungal species differ in their sensivity to different metals (Shazia *et al*., 2012; Nies and Silver, 1995; Ezeonuegbu, 2014). The observations made in this study strongly suggest most of the isolates tested probably have the potential as candidates in mycoremediation of tannery effluent (Nies and Silver, 1995; Shazia *et al*., 2012; Thipeswamy *et al*., 2012) thus, may be selected for use in the treatment of heavy metal contaminated environment.

Table 2: Percentage and Frequency of Occurrence of Fungal genera Isolated from the study sites.

Fungal Isolates	Sampling Sites (%)			
	A (%)	B (%)	C (%)	Total (%)
Aspergillus niger	4 (28.6)	3 (16.7)	5 (33.3)	12 (25.5)
Aspergillus flavus	5 (35.7)	5 (27.8)	7 (46.7)	17 (36.17)
Aspergillus fumigatus	0 (0)	1 (5.6)	0 (0)	1 (2.13)
Aspergillus terreus	3 (21.4)	7 (38.9)	0 (0)	10 (21.28)
Aspergillus tamarii	1 (7.1)	2 (11.1)	3 (20)	6 (20.9)
Aspergillus oryzae	1 (7.1)	0 (0)	0 (0)	1 (2.13)
Total	14 (28)	18 (38.3)	15 (31.9)	47 (100)

A= Unguwar Rogo I, B= Unguwar Rogo II and C= Tudun Wada
Values in parenthesis are percentage frequency of occurrence of the fungal isolates.

Table 3: Occurrence of Fungi Isolates in Wastewater Samples from the Study Sites

Sampling Points	Isolates (per 0.5 ml of Sample)					
	A. niger	*A. flavus*	*A. fumigatus*	*A. tamarii*	*A. terreus*	*A. oryzae*
A	(++)	(+++)	-	(+)	(++)	(+)
B	(+++)	(+++)	(+)	(++)	(+++)	-
C	(+++)	(+++)	-	(++)	-	-

(+++)=High (++)= Moderate (+)=Low (-)=No growth

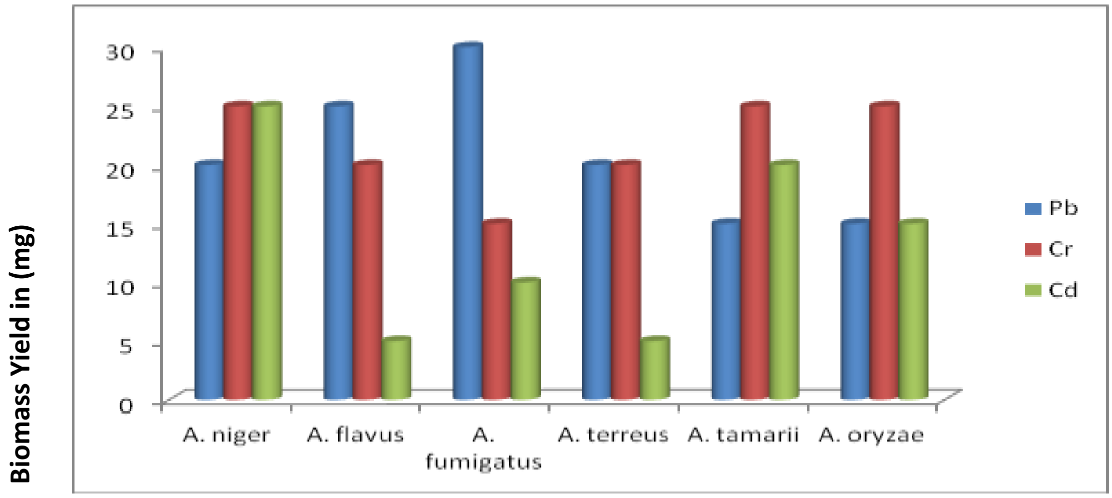

Figure 1: Mean Biomass Yield of the Test Fungal Isolates to 5 µg/ml of Heavy Metals

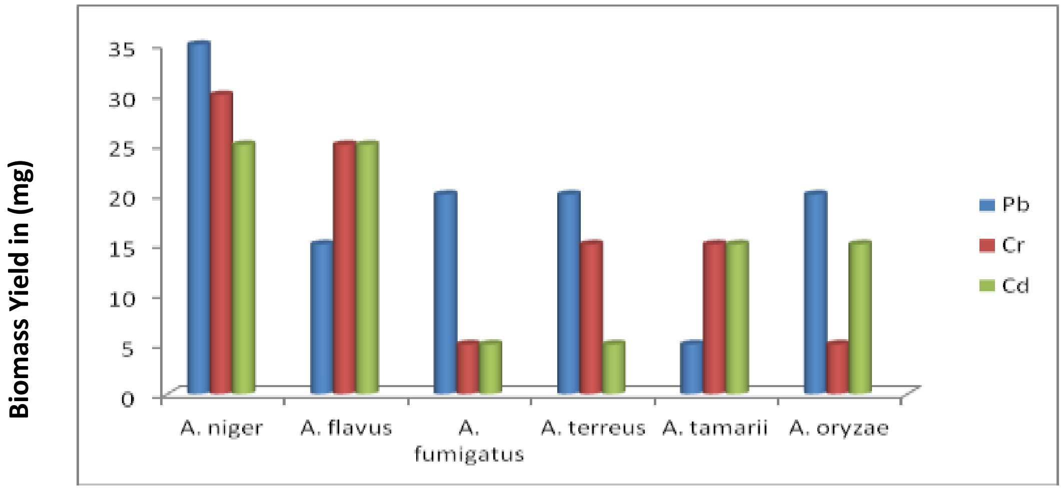

Figure 2: Mean Biomass Yield of the Test Fungal isolates to 10 µg/ml of Heavy Metals

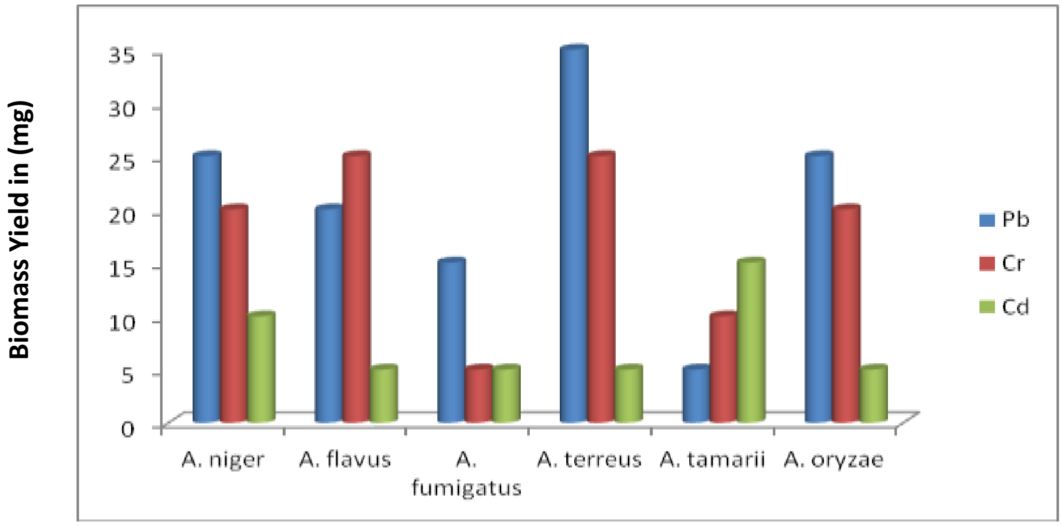

Figure 3: Mean Biomass Yield of the Test Fungal isolates to 15 µg/ml of Heavy Metals

CONCLUSION

The physicochemical quality of the wastewater and other pollutants causes variation in fungal population in the different samples analysed. *A. niger*, *A. terreus* and *A. flavus* were the most resistant to Pb and Cr while Cd was the least resisted by all the fungal species at 15 µg/ml concentration. The fungal species identified could possibly be useful in bioremediation of toxic pollutants as well as biosorption and bioaccumulation of toxic heavy metals.

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