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RESEARCH ARTICLE **AJHSE040109**

REMOVAL OF Cr (VI) from NORKRAN'S LIQUID MEDIUM USING *Aspergillus fumigatus* and *Rhizopus* sp.

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ABSTRACT

Soil and water pollution caused by heavy metals can result in health challenges such as cancer. Therefore, this study was aimed at testing the ability of *Aspergillus fumigatus* and *Rhizopus* sp. isolated from cassava grinding mill environment to reduce, bio-accumulate and tolerate Cr (VI) in Norkran's medium. Fungal isolates were obtained from soil samples using 2 % malt extract agar. Cr (VI) reduction potential of the fungal cells was done using shake flask method. The tolerance of fungal isolates to the different concentrations of Cr (VI) used for the bio-sorption experiment was determined by collecting 1 ml of Norkran's sample from each experimental flask on days 0, 5, 10 and 20 followed by inoculation in 2 % malt extract agar plates. The ability of fungal isolates to adapt to increasing Cr (VI) concentrations was ascertained by inoculating mycelial ball on 2 % malt extract agar amended with 16.1 mg/L of Cr (VI). There were reductions of Cr (VI) for all the treatments amended with *A. fumigatus* and *Rhizopus* sp. at 16.1, 8.1, 4.0 and 2.0 mg/L concentrations of Cr (VI) while 50 % decrease in Cr (VI) concentrations was observed for treatments inoculated with *Rhizopus* sp. on days 20, 10 and 5 at concentrations of 8.1, 4.0 and 2.0 mg/L respectively. The amounts of Cr (VI) observed at a concentration of 16.1 mg/L on day 20 for treatments inoculated with *Rhizopus* sp. were observed to be significantly less than ($P<0.05$) those of *A. fumigatus*. The fungal tolerance assay demonstrated increase in fungal abundance throughout the experimental period for all the treatments indicating that the different concentrations of Cr (VI) used were not toxic to the fungal species. Induce-tolerance assay suggested that Cr (VI) was able to induce metal tolerance potential in these fungal species. Findings from this investigation revealed that these fungi can be further explored biotechnologically to clean-up chromium contaminated environment.

Keywords: *Bioaccumulation, Biotechnology, Chromium (VI), Contaminants, Fungi, Tolerance assay*

INTRODUCTION

Heavy metals such as chromium cause environmental pollution that can negatively impact humans by causing diseases like cancer (Simonescu and Ferdes, 2012; Fashola *et al*., 2015; Fashola *et al*., 2016; Ayangbenro and Babalola, 2017; Ndeddy-Aka and Babalola, 2017; Vendruscolo *et al*., 2017). The ejection of these heavy metals into the environment can also affect the structural and functional diversity of microorganisms in of arable land (Igbinosa and Igiehon, 2015; Igiehon and Babalola, 2017), thereby reducing food production. Several researches have resorted to exploring biological systems as an eco-friendly alternative to remove heavy metals from the environment. Fungi are examples of microorganisms that are effective in removing metals from the ecosystem because of their high surface area and solid-liquid demarcation (Mishra and Malik, 2013). Fungi use different mechanisms to degrade different contaminants, and as such, they can render metals less toxic by various mechanisms including valence conversion, cellular precipitation, bio-absorption, developing impermeable cell wall, ejection and sequestration (Muñoz *et al*., 2012). Researchers are trying to develop better bioremediation strategies by exploring the ability of environmental microbiomes to breakdown, bio-absorb or remove pollutants from the environment and species recovered from different sites have been reported to possess this ability (Iram *et al*., 2012). To put this in context, the absorption of cadmium and chromium by filamentous fungi such as Aspergillus and Rhizopus species isolated from agricultural soil has been reported (Iram *et al*., 2012; Sivakumar, 2016).

In a polluted environment, microbial response to heavy metal is influenced by different factors such as the concentration and accessibility of metal, the type of metal and the nature of the environment. Some species of fungi cum yeast have been reported not to be susceptible to heavy metal and strive under different extreme environments of temperature, heavy metal concentrations, pH and nutrient availability. Metal tolerance refers to the capability of a microbial species to resist or survive metal toxicity by developing mechanism in response to the metal of concern (Chen *et al*., 2017). These metal species are toxic in the soil where they negatively affect microbial diversity and functions (Xie *et al*., 2016). Heavy metals can destroy microbial species by different means, depending on the type of metal and prevailing environmental factors. For instance, heavy metals can affect soil fungal species by transforming their morphological structure, physiology, growth pattern and reproductive process.

Considering the aforementioned strategy of metal detoxification and removal, it was envisaged that screening for environmental fungal species may provide species with bio-absorption ability as only limited reports of fungal remediation potentials are available in literatures. Thus, this study the highlights isolation and phenotypic characterization of environmental fungi and their bio-absorption potentials. A lot of studies have been done on the isolation of microbial species from heavy metal contaminated sites with the hope that such species have evolved mechanisms to tolerate and bio-absorb metal. Therefore, this study was aimed at testing the ability of *Aspergillus fumigatus* and *Rhizopus* sp. isolated from cassava grinding mill environment to reduce, bio-accumulate and tolerate Cr (VI) in Norkrans medium.

MATERIALS AND METHODS

Sampling:

Using a soil auger, 5 soil samples (20 g each) were obtained (5 cm from the surface soil) from cassava grinding mill environment and placed in labeled clean plastic bags. The samples were collected at Eyean in Benin City, Edo State, Nigeria and immediately stored in the refrigerator. Soil samples were later compounded before isolation.

Preparation of malt extract agar:

Two % (2%) malt extract agar was prepared as described by Igiehon and Babalola (2019)

Isolation of fungi from cassava grinding mill environment:

Fungi were isolated using the method employed by El Hameed *et al*. (2015). Ten g (10 g) of soil samples was mixed with 90 ml of distilled water and shaken thoroughly. This was serially diluted and used for the inoculation of the 2 % malt extract medium. Culture plates were incubated in static incubator at room temperature (28±2 ºC). Pure cultures of fungal biomass was obtained by sub-culturing on a freshly prepared medium, microscopic identification using lactophenol cotton blue (to detect fungal hyphae) was done (Barnett and Hunter, 1972) and the purified isolates were preserved for bio-sorption assay.

Preparation of bio-sorption medium:

The medium used for bio-sorption assay was Norkran's liquid medium. Norkran's liquid medium was prepared according manufacturer's instructions. Fifty ml (50 ml) of the medium was dispensed in flasks and autoclaved and these flasks were used for the Cr (VI) bio-sorption assay.

Cr (VI) reduction potential of fungal mycelia:

It was done according to the method employed by Chen *et al*. (2017) with little modifications. Fungal mycelia were multiplied by sub-culturing in 2 % malt extract agar with pure fungal isolates. Five Norkrans flasks (each set representing a treatment) were amended with 16.1, 8.1, 4.0 and 2.0 mg/l of Cr (VI). Two mycelial mass (0.5 cm in diameter) from the plates were used to inoculate each of the flasks of the Norkrans liquid medium and the pH was adjusted using a pH to 5± 2 by adding HCl and subjected to incubation in a shaker incubator at 150 rpm (Babalola, 2011) under room temperature (28 ± 2 ºC). Control experiments containing Norkrans liquid medium and Cr (VI) only, Norkrans liquid medium and fungal biomass only and Norkrans liquid medium only were similarly set up. Flask samples were collected on days 0, 5, 10 and 20, centrifuged at 3700 rpm for 8 min and the concentrations of Cr (VI) in the fungal biomass-free supernatants were quantified using atomic absorption spectroscopy (AAS) (Merck, South Africa).

Cr (VI) bioaccumulation assay:

This was done following the methods of Chen *et al*. (2017) and Bennett, Cordero *et al*. (2013). On the 20th day, the remnant fungal biomass was dried in the oven at 70 ºC for 2 h and the dry weights were taken. The bio-sorption potential of Cr (VI) by the fungal cells was deduced using the formula below (Simonescu and Ferdes, 2012).

$$Q = (Ko - Kf)\ V \div W \quad (1)$$

Where Ko: initial Cr (VI) concentration, Kf: final Cr (VI) concentration, V: volume of flask content, W: dry weight of fungal cells

Fungal tolerance assay to different concentrations of Cr (VI):

The tolerance of fungal isolates to the different concentrations of Cr (VI) used for the bio-sorption experiment was determined by collecting 1 mL of Norkrans sample from each experimental flask on days 0, 5, 10 and 20 followed by

inoculation in 2 % malt extract agar (pH 4.7 ± 0.2) plates. Plates were incubated at room temperature (28±2 ºC) for 5 days followed by colonial counts.

Induced–tolerance adaptation:

The ability of fungal isolates to adapt to increasing Cr (VI) concentrations was ascertained by inoculating mycelial mass (0.5 cm in diameter) on 2 % malt extract agar amended with 16.1 mg/l of Cr (VI). This concentration was the maximum Cr (VI) concentration used for the tolerance assay. Control 2 % malt extract plates were also prepared and all inoculated plates were incubated at ambient temperature (28 ± 2 ◦C) for a week. Isolates from the growing fungal culture plates were later sub-cultured to fresh 2 % malt extract agar containing higher concentration of Cr (VI) (18 mg/L, 20 mg/L and 25 mg/L) to "induce tolerance adaptation." Tolerance index was calculated at the end of the experiment. Tolerance index is the total number of fungal biomass in the malt extract medium containing increasing concentrations of Cr (VI) divided by the total number of fungal biomass in the malt extract medium without Cr (VI) (Control).

Statistical analysis:

Each treatment had five replicates. Using Microsoft excel, normality test was done for data before they were imported to Statistical Package for the Social Sciences (SPSS) where they were subjected to analysis of variance (ANOVA). Means and standard errors were extracted from the ANOVA output. Correlation analysis was also done. $P<0.05$ was considered significant (Babalola, 2007; Babalola, 2010; Dytham, 2011; Osamwonyi *et al*., 2013; Igiehon, 2015).

RESULTS

Aspergillus fumigatus and *Rhizopus* sp. were isolated from soil collected from cassava grinding mill environment. These fungal species were further used for the bio-reduction, bioaccumulation and fungal tolerance assay.

On day 0, the concentrations of Cr (VI) for the control experiments (with Norkran's liquid medium and Cr (VI) only) and treatments amended with Cr (VI), *A. fumigatus* and *Rhizopus* sp. were the same at concentrations of 16.1, 8.1, 4.0 and 2.0 mg/L (Fig. 1a, b, c and d). This supports the idea that the Cr (VI) concentrations read by the AAS were not influenced by the fungal biomass, metabolites and Norkrans medium. There were steady reductions of Cr (VI) in all the treatments amended by *A. fumigatus* and *Rhizopus* sp. at the different concentrations of Cr (VI) (Fig. 1a, b, c and d). Fifty percent (50 %) decrease in Cr (VI) concentrations was observed for treatments inoculated with *Rhizopus* sp. on days 20, 10 and 5 at concentrations of 8.1, 4.0 and 2.0 mg/L respectively. Reduction of Cr (VI) was highly significant ($P<0.01$) for treatments incorporated with 2.0 mg/l of Cr (VI), however *A. fumigatus* was more effective in reducing chromium at that concentration as depicted on days 10 and 20 (Fig. 1d). Also at a concentration of 4.0 mg/L of Cr (VI), no significant difference ($P>0.05$) was observed (day 5) between the reduction rate of Cr (VI) inoculated with *A. fumigatus* and *Rhizopus* sp.; although there was a significant difference ($P<0.05$) between these fungal treatments and control treatment on this day.

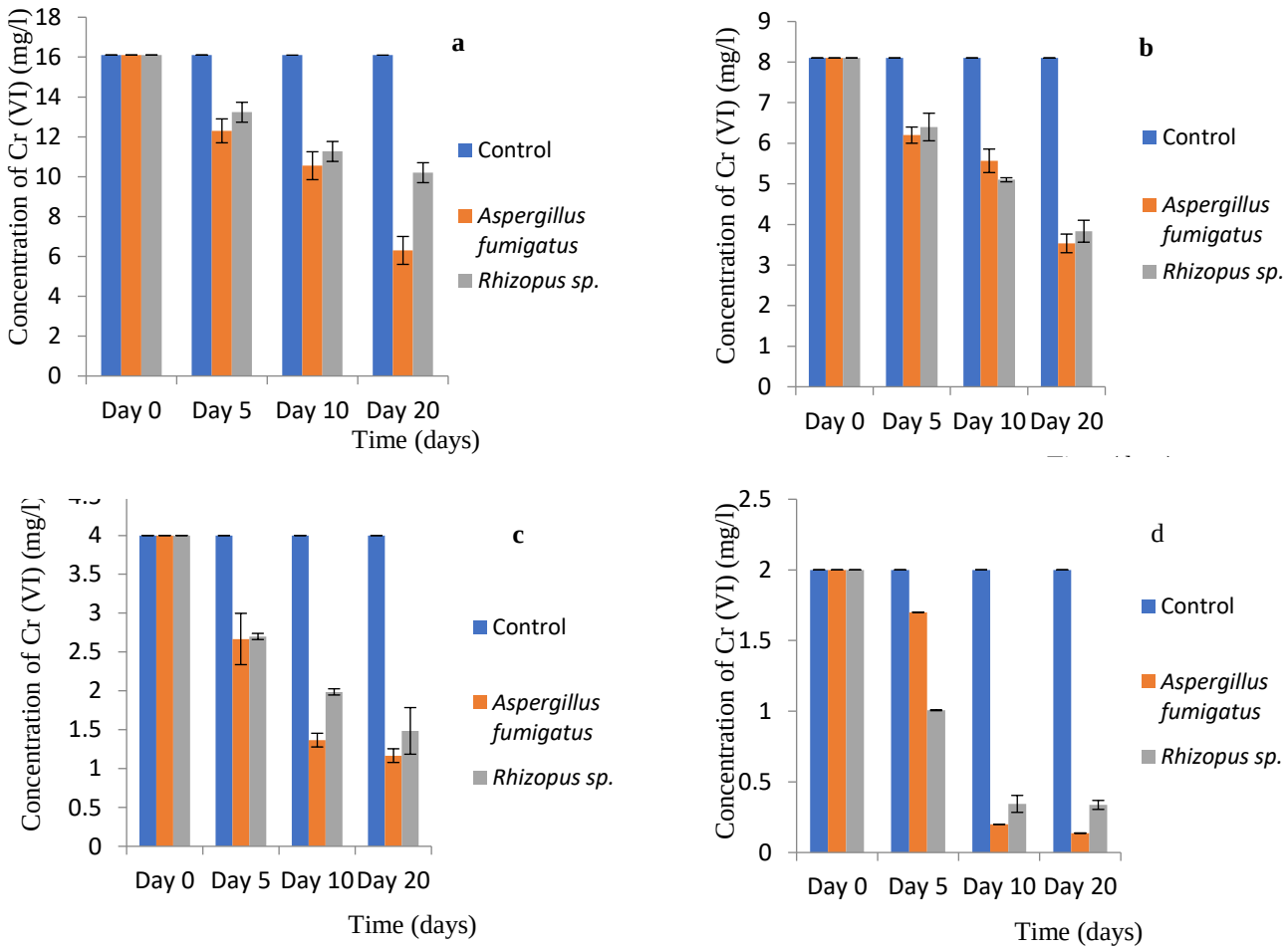

Figure 1. Chromium reduction profile in Norkrans flask experiments amended with fungal biomass at Cr (VI) concentrations of (a) 16.1 mg/L (b) 8.1 mg/L (c) 4.0 mg/L (d) 2.0 mg/L

The fungal species, *A. fumigatus* and *Rhizopus* sp. demonstrated promising bio-sorption and or bioaccumulation abilities as depicted in Table 1. *A. fumigatus* had higher amounts of bio-absorbed Cr (VI) in all the treatments compared to *Rhizopus* sp. The highest amount (6.0 mg/L) of bio-absorbed Cr (VI) by *A. fumigatus* was observed in treatments amended with 16.1 mg/L of Cr (VI) while in the same treatment, 4 mg/L was the maximum amount of Cr (VI) bio-absorbed by *Rhizopus* sp.

Table 1: The concentration of Cr (VI) bio-accumulated by fungal species

Isolate	Mean intracellular Cr (VI) (mg/L)			
	Treatments	16.1	8.1	4.0	2.0
A. fumigatus		6±0.001	3.0±0.001	1.5±0.000	0.5±0.000
Rhizopus sp.		4±0.000	2.8±0.001	1.0±0.00	0.4±0.000

Legend: Values are mean ± standard error

The fungal tolerance assay results showed an increase in fungal abundance in all the experimental set-up (Fig. 2a, b, c and d). A. fumigatus was more abundant on the 20th day with a count of 2.2 x 103 Cfu/ml while Rhizopus sp. was more abundant on that same day for treatments amended with 8.1, 4.0 and 2.0 mg/L of Cr (VI) with biomass counts of 3.25 x 104, 3 x 104 and 4.4 x 103 Cfu/ml respectively. A significant negative correlation ($P<0.05$) was observed between fungal abundance and Cr (VI) reduction rate. Fungal growth was not observed for the control experimental set-up that contained only Norkrans liquid medium and Cr (VI) only and thus data are not reflected in the graphs.

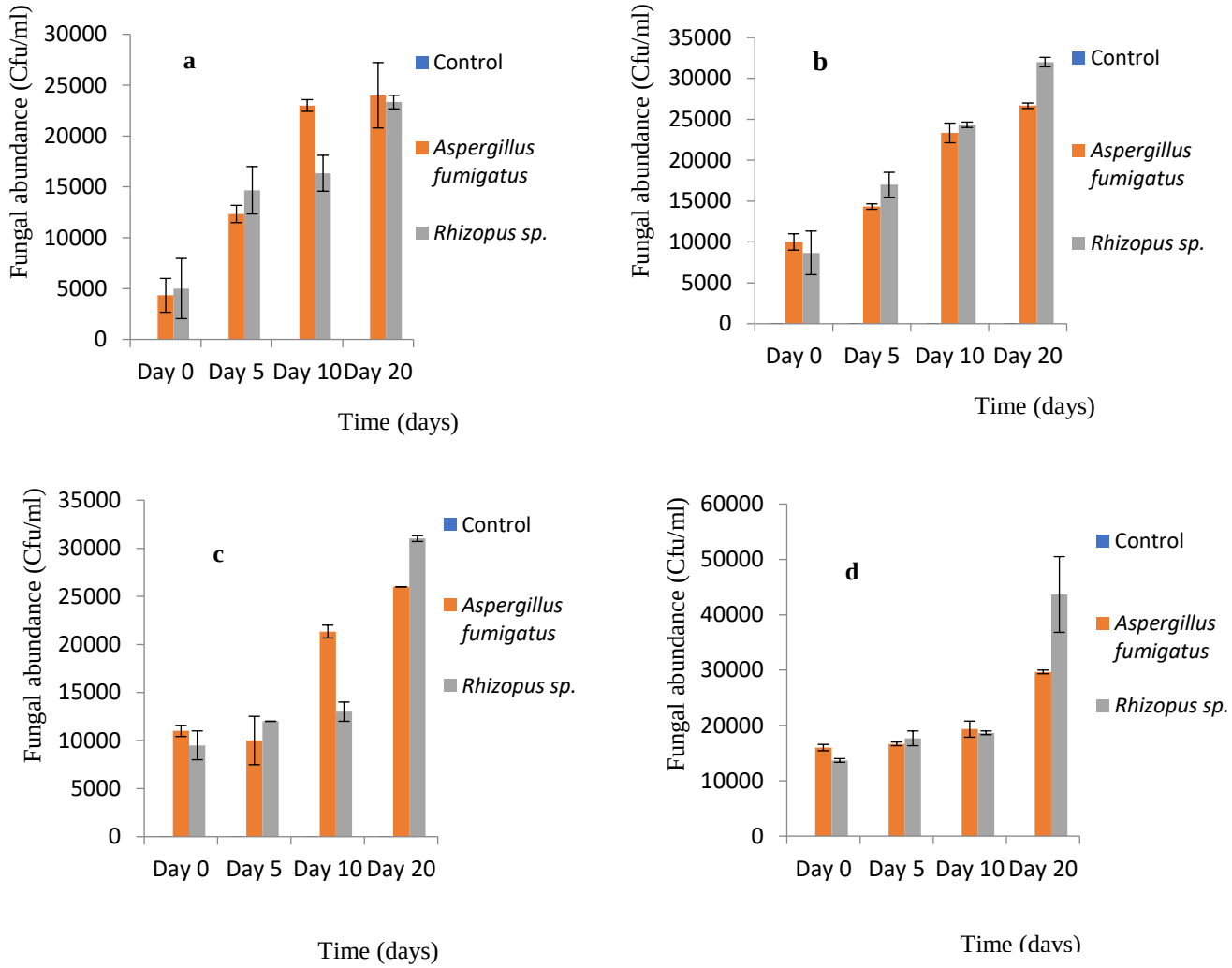

Figure 2. Fungal abundance of Cr (VI) Norkrans flask experiments at concentrations of (a) 16.1 mg/L (b) 8.1 mg/L(c) 4.0 mg/L (d) 2.0 mg/L

The induced-tolerance assay result indicated that *A. fumigatus* and *Rhizopus* sp. expressed tolerance towards increasing concentrations of Cr (VI) (Table 2). For both fungal species, the tolerance index values for trained fungal isolates were higher than those of fungal isolates without training suggesting that Cr (VI) concentrations at 18, 20 and 20 mg/L were able to induce tolerance on fungal species.

Table 2: Tolerance index of *A. fumigatus* and *Rhizopus* sp. with and without "induced tolerance adaptation" at different concentrations of Cr (VI)

Isolate	Cr (VI) concentration (mg/L)	Tolerance index (TI)	
		With adaptation	Without adaptation (16.1 mg/L)
A. fumigatus	18	0.660± 0.001	0.5600 ± 0.011
Rhizopus sp.	18	0.670±0.011	0.5000± 0.000
A. fumigatus	20	0.710±0.001	0.7000± 0.000
Rhizopus sp.	20	0.770±0.000	0.6100± 0.001
A. fumigatus	25	0.777±0.000	0.7440± 0.002
Rhizopus sp.	25	0.854±0.001	0.5300± 0.000

Legend: Values are mean ± standard error

DISCUSSION

The fungi isolated from the cassava grinding mill environment in this study were *Aspergillus fumigatus* and *Rhizopus* sp. In a related study, Igbinosa and Igiehon (2015) reported the presence of fungi (*Aspergillus, Penicillium* and *Rhizopus* species) and bacteria (*Bacillus subtilis, Bacillus macerans, Pseudomonas aeruginosa, Klebsiella aoxytoca* and *Escherichia coli*) in cassava effluent impacted milieu. The isolated fungi in this study were further tested for their ability to remove and or bio-accumulate chromium from the environment by conducting in vitro laboratory experiments using Norkrans liquid medium. *A. fumigatus* was observed to reduce Cr (VI) more than *Rhizopus* sp. (Fig. 1a, b, c and d) almost through-out the experimental period. This observation corresponds to the amount of Cr (VI) bio-absorbed by *A. fumigatus* (Table 1) and there was also significant negative correlation ($P<0.05$) between the amount of Cr (VI) bio-accumulated and the concentrations of Cr (VI) on the 20th day. There was no significant difference ($P>0.05$) in Cr (VI) concentrations between *A. fumigatus* and *Rhizopus* sp. inoculated treatments on days 5 and 10 (Fig. 1a), but a difference was observed significantly ($P<0.05$) between the above-mentioned treatments on the 20th day (Fig. 1a). This significant difference may be attributed to the extended sampling time between day 10 and 20. The amount of Cr (VI) bio-absorbed by both fungi was directly proportional to the concentration of chromium incorporated into the flasks (Table 1) since the bio-absorbed Cr (VI) increased from treatments amended with 2.0 mg/L of Cr (VI) all through to those incorporated with 16.1 mg/L of Cr (VI). According to Sivakumar (2016), *Aspergillus niger, A. nidulans, A. foetidus, A. viridinutans, A. fumigatus* and *A. heteromorphus* were able to absorb 18.1 mg/L of Cr (VI) from industrial effluent. Similarly, Singh *et al*. (2016) revealed 16.1 mg/L as the maximum concentration of Cr (VI) that was absorbed by *A. fumigatus* and suggested that this fungus can be utilized in model plants for the management of chromium-contaminated effluents.

From the fungal tolerance assay, it was observed that there was an increase in fungal abundance throughout the experimental period for all the treatments indicating that the different concentrations of Cr (VI) used were not toxic to *A. fumigatus* and *Rhizopus* sp. Tolerance is the capacity to resist toxicity and is ascribed to the inherent features (such as cell surface functional groups) of the cell wall of fungi as well as their capability to release protein molecules that have affinity for metals. Such functional groups like amino and hydroxyl groups are largely present on cell wall

proteins, glucan and lipids (Das *et al*., 2008). According to Chen *et al*. (2017), metal tolerance of fungi is ostensibly through sequestration into cell compartments and these authors similarly reported bio-sorption potential for Cr (III) as well as Pb (III) by Trichoderma asperellum and Simplicillium chinense in liquid matrix. Rhizopus sp. was virtually more abundant throughout the sampling points for treatments containing 8.1 and 2.0 mg/L of Cr (VI) even though the rate of Cr (VI) reduction and bio-sorption at these concentrations was relatively less than that of the Aspergillus species counterpart. This goes to show that the increase in biomass of these fungi may not be dependent on the Cr (VI) absorbed from the environment but rather on the Norkrans medium recipe. Fungal species tolerant to heavy metals such as chromium, lead and nickel have previously been isolated from heavy metal-contaminated environmental samples (Dwivedi 2012). Experimental outputs have also shown fungal candidates that exhibited better growth at minimal heavy metal concentrations but declined in growth when the concentrations of the metal were increased (Iram *et al*., 2012).

Tolerance of Aspergillus niger, Penicillium sp. and Saccharomyces sp. at 16.1, 8.1, 4.0 and 2.0 mg/L Cr (VI) concentrations has been observed previously (Igiehon and Babalola, 2019); but these species were isolated from different environmental sources. They were also observed to reduce and bio-absorb Cr (VI) and could therefore serve as biological systems for the removal of contaminants from soil and liquid matrices if fully explored. The quest for substitutes to the well-known conventional technologies for the elimination of Cr (VI) paves the way for the utilization of fungi, bacteria and yeast as bio-sorption and bioremediation agents. Comparatively, this biological system is cheaper, more effective and more ecofriendly than the conventional clean-up techniques (Carol *et al*., 2012; Igiehon, 2015). These biological agents can be used to clean-up sites contaminated with industrial effluents, hydrocarbon polluted sites and even agricultural land deteriorated by heavy metals since according to Ndeddy Aka and Babalola (2017) metal such as chromium, cadmium and nickel are the main pollutants that compromise soil quality, plant production as well as human health globally.

Induced-tolerance adaptation is said to improve fungal species tolerance towards metal if tolerance index of the trained isolates (inoculated in a medium with increasing metal concentration, 18, 20 and 25 mg/L) is higher than those of the isolates without training (inoculated in a medium with 16.1 mg/L). "With induced tolerance adaptation", enhanced tolerance at a relatively higher Cr (VI) concentration was observed (Table 2). This was noticeable with the increase in tolerance index for both fungal species (*A. fumigatus* and *Rhizopus* sp.) at 18 to 25 mg/L Cr (VI) concentrations. For *A. fumigatus* enhancement to Cr (VI) tolerance was observed with a tolerance index ranging from 0.660 to 0.854 corresponding to 18 to 25 mg/L Cr (VI) concentrations respectively. Similarly, for *Rhizopus* sp. improvement to Cr (VI) tolerance was also detected at 18 to 25 mg/L with corresponding tolerance index of 0.670 to 0.854 respectively (Table 2). However, the tolerance index observed for *Rhizopus* sp. with induced tolerance adaptation" were higher than those observed for those without training (Table 2). "These observations supported the fact that induced tolerance training encouraged build-up of fungal tolerance towards increasing metal concentrations, allowing it to adapt in an otherwise toxic metal concentration (adaptive tolerance behavior)" (Valix and Loon, 2003; Chen *et al*., 2017). According to Chen *et al*. (2017) elongated "induced-tolerance adaptation" could result to species acclimatization to high a concentration of metal caused by selective pressure when exposed to metal for a long period. This might similarly trigger ADP/ATP translocase, cysteine synthase, glucoamylase and enolase expression which function as oxidative stress amelioration (Sugimoto *et al*., 2004).

CONCLUSION

This present study has shown that *A. fumigatus* was more proficient in reducing Cr (VI), however both fungi were able to bio-accumulate and tolerate Cr (VI) at different concentrations of 16.1, 8.1, 4.0 and 2.0 mg/L. Findings from this investigation revealed that *A. fumigatus* and *Rhizopus* sp. can be explored biotechnologically to clean-up chromium contaminated soil and water.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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