Bioremediation of Hexavalent Chromium in Potassium Dichromate Solution by *Botrytis aclada fres* and *Chrysonilia sitophila*

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Abstract: Bioremediation of hexavalent chromium, Cr(VI) by *Botrytis aclada fres* and *Chrysonilia sitophila* was studied. The organisms were isolated from decaying onion bulb and apple fruit respectively, purified in Potato Dextrose Agar, and grown for 144 hours in solutions of potassium dichromate of concentrations ranging from 5-20 mg/l at 40^0C. Effective reduction of Cr(VI) was observed at 5-20 mg/l compared to 25 mg/l dichromate treatments in both organisms. The results showed significant decrease ($P < 0.05$) in biomass concentration in the two fungi used with increasing concentration of the dichromate treatment (5-25 mg/l). Significant increase ($P < 0.05$) in residual glucose concentration was also observed in the culture media with increase in concentration of the dichromate treatment. However, at 20 mg/l dichromate treatment, Cr(IV) concentration, 1.36 ± 0.02 and 1.71 ± 0.03 ($P < 0.05$) were revealed in the culture media of *B. aclada fres* and *C. sitophila* respectively. Also, 0.71 ± 0.03 and 0.94 ± 0.03 Cr(IV) concentration ($P < 0.05$) were observed in the fungal mycelia at 20 mg/l dichromate treatment in *B. aclada fres* and *C. sitophila* respectively. After 144 hours of growth, Cr(VI) reduction of 89.65% in *B. aclada fres* and 86.75% in *C. sitophila* at 20 mg/l dichromate treatment were revealed. This investigation suggests that the two fungi adopted a process of reduction to tolerate the toxicity of hexavalent chromium. The results indicate the potentials of the fungi in bioremediation particularly in the treatment of waste water containing hexavalent chromium.

Keywords: Chromium contamination, Cr(VI) reduction, detoxification, waste water treatment, *Botrytis aclada fres, Chrysonilia sitophila.*

INTRODUCTION

Chromium is a naturally occurring heavy metal widely distributed in the earth's crust. Chromium is considered as an essential nutrient and as a health hazard. It occurs mainly in two valence states. Hexavalent chromium [Cr(VI)] is harmful even in small dose, while Cr(III) is essential for good health in moderate intake [1, 2]. Chromium and its salts are used in the leather tanning industry, the manufacture of catalysts, pigments and paints, fungicides, the ceramic and glass industry, and in photography, and for chrome alloy and chromium metal production, chrome plating, and corrosion control [3]. Improper disposal of industrial wastes has lead to the contamination of the environment with serious health consequences [4]. Potential health effects of chromium exposure include respiratory tract problems, ulcer, anemia, sperm damage, and cancer [5].

Various physical, chemical and biological methods are used for remediation of polluted environments. Physical and chemical process have disadvantages like high operational cost, produces secondary contamination, high energy consumption which needs a cost effective approach for remediating such polluted environments [6]. Bacteria, fungi and plants have been used for the bioremediation of chromium. Balaji *et al.* 2015 [7] reported the phycoremediation of industrial effluents contaminated with Cr(VI) using the cyanobacterium, Arthrospira platensis. The aquatic carnivorous plant, *Utricularia gibba L*. has been applied in the removal of chromate when it grows in water that has been polluted by the metal [8]. Similarly, *Pseudomonas aeruginosa* and *Serratia marcescens* have been used in the bioremediation of chromium contaminated river sediments [9].

The transformation of Cr(VI) to Cr(III) by sulphate-reducing bacteria has been reported [10,11]. The uptake and metabolic transformation of hexavalent chromium by cultures of *Aspergillus niger* and *Aspergillus parasiticus* was also reported [12]. Various cellular responses such as oxidation-reduction reactions, biosorption by cell biomass, bioaccumulation, binding by cytosolic molecules and protein-DNA adduct formation have been characterized to fungi, bacteria and other microbes in the tolerance of stress generating environment caused by heavy metals [13,14]. The present work reports the detoxification of simulated industrial waste effluents containing hexavalent chromium by *Botrytis aclada fres* and *Chrysonilia sitophila*.

MATERIALS AND METHODS

Materials

Atomic Absorption Spectrophotometer (200A Analyst) and Heating mantle (EMA 250) were products

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of Thermo Fisher Scientific, Waltham, Massachusetts, United States of America. Oven (50/DIG) and Incubator (G200) were products of Gen Lab, Wildness, Cheshire, United Kingdom. UV/Visible Spectrophotometer (CE7000) was a product of X–rite, Michigan, United States of America. Centrifuge (E.5427-R) was a product of Eppendorf, Hamburg, Germany. Analytical Digital Balance (Rs7000/piece) was a product of Texcare Instrument, Uttam Nagar, New Delhi, India. Potassium-dichromate ($K_2Cr_2O_7$), Nicotinamide adenine dinucleotide, (NADH), Diphenylcarbazide and Glucose oxidase reagents were products of Pacific Trading Co. Ltd, Changzhou, China. Glucose and Ethanol were products of BDH Chemical Ltd, Poole, England. All other chemicals and reagents used were of analytical grade.

Isolation of Organisms

The fungi, *Botrytis aclada fres* and *Chrysonilia sitophila* were isolated from decaying onion bulb and apple fruit respectively obtained from a local market in Maiduguri, Nigeria. The samples were processed and sub-cultured on Potato Dextrose Agar, PDA at 40^0C for 72 hours. The fungi were identified by their macroscopic characteristics which include color of colony and pattern of growth of colony. The microscopic structures used for identification as observed under the electron microscope include, patterns of arrangement of stolen on rhizoid as well as patterns of growth of rhizoid. Other microscopic characteristics include, shape of conidia, shape of spores, shape of conidiophores and patterns of arrangement of spores on conidia.

Preparation of Growth Medium and Culture Conditions

Various concentrations of Cr(VI), 5-25 mg/l were prepared by dissolving corresponding mass of potassium dichromate, $K_2Cr_2O_7$ in 1000 cm^3 of sterile modified Vogel's mineral salts medium [15] fed with 10 g of glucose. The pH of the medium was adjusted to 5 using concentrated HCl. The solution was then divided into portions of 100 cm^3 each in Erlenmeyer flask, and inoculated with 50 mg/l inoculum of pure culture of individual fungus used. The culture media were incubated at 40^0C for 144 hours. Culture medium without Cr(VI) treatment was used as control.

Measurement of Biomass Concentration

The fungal biomass concentration was determined after 144 hours of growth by separating the cells from the media using Whatman No. 1 filter paper of a known mass. The cells were dried in an oven for 24 hours at 70 °C [16]. The mass of the cells were read on analytical digital balance, and evaluated as:

$$\text{Biomass concentration (mg / l)} = \frac{Y - X}{V} \quad \text{(i)}$$

Where,

X = mass of the filter paper (mg)

Y = mass of the filter paper + dry cells (mg)

V = volume of culture media (l)

Measurement of Residual Glucose Concentration in Culture Media

The residual glucose concentration in the culture media was determined by preparing a blank of 3 ml of glucose oxidase reagents mixed with 1.5 µl of distilled water to zero the absorbance reading of the uv/visible spectrophotometer used. A standard containing 100 mg/l glucose mixed with 3ml glucose oxidase reagents was prepared. Also, 1.5 µl of the unknown sample was reacted with 3ml of the reagents. The two reaction mixtures were separately incubated at 30 °C for 10 minutes according to standard method, the residual glucose concentration was calculated as follows:

$$\text{Glucose concentration (mg / dl)} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of calibrator}} \times \text{Calibrator value (mg / dl)} \quad \text{(ii)}$$

Measurement of Total Chromium Concentration in Fungal Mycelia

Dry cells of known mass were used after being separated by filtration and dried in an oven at 70 °C for 24 hours. The dry cells were digested in a known volume of concentrated nitric acid, HNO_3 for 20 minutes at 25 °C in line with Munoz *et al*. [17], heated gradually on a heating mantle to a temperature of 60 °C for 15 minutes and then allowed to cool. Hydrogen peroxide, H_2O_2 of about half the volume of the acid used was added and heated again to 60 °C for 10 minutes. The supernatant was obtained through centrifugal at 3000 rpm. The absorbance of the sample was taken at 358 nm using Atomic Absorption Spectrophotometer, AAS. The concentration of the sample was extrapolated from the absorbance/ concentration curve of the standard.

Measurement of Cr(VI) and Cr(III) Concentration in Fungal Mycelia

A known mass of dried mycelia was used for each sample. The supernatant was obtained after acid digestion, centrifugation and filtration as previously described. The supernatant was reacted with 0.2% w/v 1,5-diphlcarbazide reagent and 0.1mM NADH at room temperature according to Acevedo-Aguilar *et al*. [18]. The absorbance reading was taken at 540 nm using UV/Visible spectrophotometer. The concentration was extrapolated from the calibration curve. Trivalent chromium concentration in fungal mycelia was evaluated by deducting Cr(VI) concentration from total chromium uptake by the fungi.

Measurement of Cr(VI) and Cr(III) Concentration in Culture Media

The concentration of Cr (VI) in the culture media was determined as described by Filipovic-Kovacevic, and Aksu *et al*. [19,20]. One milliliter of the sample was reacted with 0.2% w/v 1,5-diphenylcarbazide reagent. The intensity of the purple colour of the reaction mixture was read at 540 nm spectrophotometrically. The concentration of Cr(VI) in the unknown sample was obtained from the absorbance/concentration of the calibrator.

The concentration of Cr(III) in the culture media was evaluated as:

$$Y = A – (B + C) \quad \text{(iii)}$$

Where,

Y = Cr(III) concentration (mg/l) in culture media

A = Cr(VI) treatment (mg/l)

B = Total chromium, C_T (mg/l) in fungal mycelia

C = Cr(VI) concentration (mg/l) in culture media

Statistical Analyses

One-way analysis of variance (ANOVA) and the student t-test were used to analyze the data obtained using GraphPad InStat Statistics software. Differences between means were considered significant at value of $P < 0.05$.

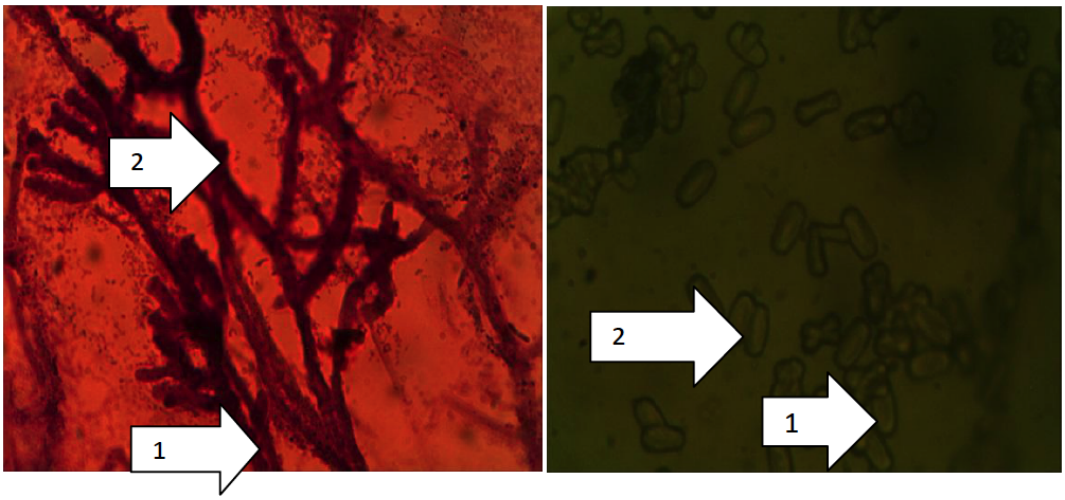
RESULTS AND DISCUSSION

Botrytis aclada fres is one of the primary causal agents of neck rot disease of onion. Eventhough infection occurs in the field, the symptoms of the disease typically develop after bulbs have been harvested and placed in storage [21]. *Chrysonilia sitophila* has a wide distribution. It has been isolated from soils of roots of mangrove forest plants; and was found to be useful in degrading petroleum hydrocarbon compounds [22]. Figure **1** shows the isolates.

Table **1** shows the effects of Cr(VI) concentration on the growth of *Botrytis aclada fres* and *Chrysonilia sitophila*, revealing significant ($p < 0.05$) decrease in biomass concentration with increase in the concentration of Cr(VI) treatment. At 20 mg/l Cr(VI) treatment, reduction in biomass concentration of about 35.0% and 48.9% compared to the control were observed in *B. aclada fres* and *C. sitophila* respectively. Also, reduced biomass concentration of about 61.8% and70.7% were revealed in *B. aclada fres* and *C. sitophila* respectively at 25mg/l Cr(VI) treatment. Inhibition of growth by Cr(VI) toxicity and the ability of the ion to cross the cell membrane with the aid of non-specific anion carriers have been reported previously [23,24]. It may be suggested that the toxicity of Cr(VI) has led to decreased synthesis of biochemical substances such as enzymes and nucleic acids necessary for growth and metabolism. The reduction in biomass yield may also be due to reactions of Cr(VI) with some reductants within the cell, thereby generating reactive oxygen species, ROS according to Stearns and Wetterhahn [25] leading to inhibition of cell division and multiplication.

The residual glucose concentration in the culture media of *B. aclada fres* and *C. sitophilia* is presented in Table **2**. Residual glucose concentration is significantly ($p < 0.05$) higher in all Cr(VI) treated cultures compared to the control. Also, glucose concentration increased between the various Cr(VI) treated media ($P < 0.01$) with increase in concentration of Cr(VI) treatment. Glucose is required for growth [26], impaired glucose uptake by the fungi may be due to the toxic effect of Cr(VI) in relation to the concentration, thereby affecting general metabolic processes of the organisms.

Table **3**, shows the total chromium concentration in the mycelia of *B. aclada fres* and *C. sitophilia.* At 25 mg/l Cr(VI) treatment, about 11.8% and 14.5% total chromium were revealed in *B. aclada fres* and *C. sitophilia* respectively. Total chromium in the mycelia of both organisms increased significantly ($P < 0.05$) as the concentration of Cr (VI) treatment increased. According to reports, [27-29], all Cr(VI) anions will be absorbed by the cell from their surroundings. On the



a **b**

Figure 1: a. *Botrytis aclada fres* (Mag. x 1000) 1=Rhizoid, 2=Stolen. **b**. *Chrysonilia sitophyla* (Mag. *x* 1000) 1=Conidium, 2=Hyaline strand.

Table 1: Biomass Concentration of *B. aclada fres* and *C. sitophila* in Hexavalent Chromium Treated Cultures after 144 Hours of Growth

	Botrytis aclada fres	***Chrysonilia sitophila***
Cr(VI) treatment (mg/l)	**Biomass Concentration (mg/l)**	**Biomass Concentration (mg/l)**
0	481.73 ± 1.12^{a}	492.07 ± 0.94^{ab}
5	471.90 ± 0.88^{b}	434.20 ± 1.10^{bc}
10	453.90 ± 1.94^{c}	382.83 ± 1.13^{cd}
15	404.60 ± 1.97^{d}	355.70 ± 0.48^{de}
20	313.13 ± 0.78^{e}	251.20 ± 1.02^{ef}
25	184.17 ± 1.69^{f}	144.37 ± 1.13^{fg}

Values are mean ± S.D. of three determinations.
Means with different subscripts down a column are significantly different ($p < 0.05$).
Means with different subscript along a row are significantly different ($p < 0.05$).

Table 2: Glucose Concentration in Hexavalent Chromium Treated *B. aclada fres* and *C. sitophila* Cultures after 144 hours of Growth

	Botrytis aclada fres	***Chrysonilia sitophila***
Cr(VI) treatment (mg/l)	**Glucose Concentration (mg/l)**	**Glucose Concentration (mg/l)**
0	123.58 ± 1.00^{a}	96.48 ± 1.79^{ab}
5	294.33 ± 2.69^{b}	263.22 ± 0.93^{bc}
10	500.68 ± 1.81^{c}	484.07 ± 0.72^{cd}
15	613.96 ± 1.80^{d}	608.43 ± 1.83^{de}
20	664.95 ± 1.42^{e}	665.81 ± 1.76^{e}
25	712.13 ± 2.13^{f}	718.33 ± 1.80^{f}

Values are mean ± S.D. of three determinations.
Means with different subscripts down a column are significantly different ($P < 0.05$).
Means with different subscript along a row are significantly different ($P < 0.05$).

other hand, Cr(III) is taken up by the cell as a complex with biologically relevant ligand molecules [30].

Table **4** shows the concentration of hexavalent chromium and trivalent chromium of *B. aclada fres* and *C. sitophila* in culture media after 144 hours of growth.

Table 3: Total Chromium Concentration in Fungal Mycelia after 144 hours of Growth

	Botrytis aclada fres	*Chrysonilia sitophila*
Cr(VI) treatment (mg/l)	**Concentration (mg/l)**	**concentration (mg/l)**
5	0.04±0.01^{a}	0.12±0.02^{ab}
10	0.41±0.04^{b}	0.34±0.05^{bc}
15	0.64±0.02^{c}	1.16±0.03^{cd}
20	1.34±0.02^{d}	1.92±0.06^{de}
25	2.94±0.05^{e}	3.62±0.08^{ef}

Values are mean ± S.D. of three determinations.
Means with different subscripts down a column are significantly different (P < 0.05).
Means with different subscript along a row are significantly different (P < 0.05).

Cr(III) concentration increased (P < 0.05) as the concentration of Cr (VI) treatment increased in both organisms. Although, low concentration of Cr(VI) was revealed in all the treatment suggesting the possibility of Cr(III) oxidation back to Cr(VI), meanwhile the reduction potential of *B aclada fres* is higher in all the Cr(VI) treated cultures which may be due to different adaptation techniques and responses by various organisms to heavy metals [31,32]. It has been reported previously [33] that Cr(VI) reduction occurs intracellularly and may also proceed outside the cell. However, rapid decrease in Cr(VI) tolerance at 25mg/l as compared to 20mg/l in both organisms may be due to alteration in the genes responsible for Cr(VI) uptake or glutathione metabolism [34,35,36] leading to overbalancing of the oxidation-reduction system of the cells.

Table 4: Concentrations of Hexavalent Chromium and Trivalent Chromium in Culture Media after 144 Hours of Growth.

Cr(VI) treatment (mg/l)	*Botrytis aclada fres*		*Chrysonilia sitophila*	
	Concentration (mg/l)		**Concentration (mg/l)**	
	Cr (VI)	**Cr(III)**	**Cr (VI)**	**Cr(III)**
5	0.16±0.01^{a}	4.80±0.41^{a}	0.03±0.02^{ab}	4.85±0.62^{a}
10	0.08±0.05^{b}	9.51±0.76^{b}	0.09±0.03^{b}	9.57±0.42^{b}
15	0.67±0.03^{c}	13.69±0.31^{c}	0.84±0.10^{cd}	13.00±0.48^{cd}
20	1.36±0.02^{d}	17.30±0.28^{d}	1.71±0.08^{de}	16.31±0.65^{de}
25	4.85±0.13^{e}	17.21±1.04^{e}	6.17±0.09^{ef}	15.21±1.01^{ef}

Values are mean ± S.D. of three determinations.
Means with different subscripts down a column are significantly different (P < 0.05).
Means with different subscript *along* a row are significantly different (P < 0.05).

Table 5: Concentrations of Cr (VI) and Cr(III) in Fungal Mycelia after 144 Hours of Growth.

Cr(VI) treatment (mg/l)	*Botrytis aclada fres*		*Chrysonilia sitophila*	
	Concentration (mg/l)		**Concentration (mg/l)**	
	Cr(VI)	**Cr(III**	**Cr(VI)**	**Cr(III)**
5	0.03±0.01^{a}	0.09±0.01^{a}	0.04±0.01^{a}	0.11±0.01^{a}
10	0.07±0.03^{b}	0.32±0.02^{b}	0.09±0.01^{b}	0.25±0.03^{b}
15	0.31±0.02^{c}	0.33±0.06^{c}	0.49±0.04^{cd}	0.67±0.02^{cd}
20	0.71±0.03^{d}	0.63±0.04^{d}	0.94±0.03^{de}	1.04±0.01^{de}
25	1.47±0.07^{e}	1.50±0.07^{e}	2.14±0.05^{ef}	1.48±0.02^{e}

Values are mean ± S.D. of three determinations.
Means with different subscripts down a column are significantly different (P < 0.05).
Means with different subscript along a row are significantly different (P < 0.05).

Concentration of Cr(VI) and Cr(III) in fungal mycelia after 144 hours of growth is presented in Table **5**. The ability of chromium uptake by both organisms significantly ($p < 0.05$) decreased with increase in Cr(VI) concentration. The organisms exhibited a low accumulation potential of the hexavalent chromium similar to the reports of [37,38] on Cr(VI) –resistant mutants of *S. cerevisiae*.

These results revealed the potentials of the fungi in the bioremediation of hexavalent chromium by oxidation-reduction process. Further research needs to be carried out with the objective of applying the organisms in association with other agents of bioremediation for the treatment of chromium contaminated waste water.

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