Reduction of Cr(VI) using indigenous *Aspergillus* spp. isolated from heavy metal contaminated sites

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Abstract

Fifteen (15) fungal morphospecies isolated from heavy metal contaminated sites were assayed for Cr(VI)-tolerance in nutrient scarce PDB medium. Of the 15 isolates, *Aspergillus* sp. MSG7 and *Aspergillus* sp. BP3 tolerated 1440 mg/L Cr(VI) and 1800 mg/L for *Aspergillus* sp. BP2 and *Aspergillus* sp. BP5. Further, *in vitro* Cr(VI) reduction using mycelial balls in reactor tubes with 1/10 strength PDB were assayed at varying Cr(VI) concentrations (90, 180, 360, 720 and 1440 mg/L) at pH 2.0 and at varying pH (1.0 – 4.0) with 360 mg/L Cr(VI). Reactor tubes were incubated at room temperature with shaking at 150 rpm and residual Cr(VI) was quantified using UV-Vis spectrophotometer at OD_{540nm} following 1,5-Diphenylcarbazide (DPCZ) method. Isolates showed Cr(VI) reduction of 30–65% with culture factors as not significant ($p > 0.05$). This signified that isolates were able to tolerate and reduce Cr(VI) within the utilized pH levels and Cr(VI) concentration effectively. The findings indicates the potential biotechnological application of the four fungal isolates in reducing Cr(VI) in broth medium

Key words – *Aspergillus* sp. – Cr (VI) – reduction

Introduction

Heavy metal pollution is one of the most prevalent environmental problems. It is due to rapid industrialization and unsystematic dumping of industrial wastes directly to terrestrial and aquatic systems (Blacksmith institute 2007, Doble & Mishra 2007). Of the common heavy metal contaminants, hexavalent chromium or Cr(VI) is prevalent in various river systems in the Philippines due to effluents coming from leather tanning companies. Cr(VI) predominantly exists in nature as chromates (CrO_4^{2-}) and dichromates ($Cr_2O_7^{2-}$) and are used in industrial processes like metallic alloy manufacturing, leather tanning, chromate preparation, electroplating, metal finishing, and mining (Francisco et al. 2002, Srivastava & Thakur 2006). Since Cr(VI) is easily absorbed, it is approximately 100-fold more toxic amongst known Cr oxidation states, thus, given attention for its mutagenic, carcinogenic, teratogenic and other long-term adverse effects (Darrie 2001, Francisco et al. 2002). Various conventional methods like chemical precipitation, membrane separation, ion exchange and evaporation are employed to remove Cr(VI) in the environment. However, these

technologies are costly and generate secondary pollution (Prigione et al. 2009). Through the emerging technologies of bioremediation, fungal species are effective biomaterials of heavy metal biotransformation and biosorption by converting their toxic state to lesser or non-toxic forms and complete metal absorption (Park et al. 2005, Srivastava & Thakur, 2006). Amongst recent biomaterials, fungi receive increased attention for heavy metal removal and recovery for it produces high biomass yield and are easily manipulated. Further, recent studies showed diverse strains isolated from contaminated sites with excellent ability to remove significant metal quantities both on aqueous solutions and effluents (Bennett et al. 2013, Ezzouhri et al. 2009). Thus, this study isolated, characterized and identified Cr(VI) resistant fungi from different heavy metal contaminated sites and tested their ability for Cr(VI) tolerance and reduction at varying pH and Cr(VI) concentration.

Materials & Methods

Isolation of Cr(VI) resistant fungi
Heavy metal contaminated soil samples were collected at Coto Chromite Deposit in Masinloc, Zambales and Motolite Battery Plant in Novaliches, Quezon City. Samples were placed in sealed bags kept at room temperature and labeled properly. One gram of each soil sample was serially diluted to 10^{-3} and spread plated to ½ strength Potato Dextrose Agar (PDA) amended with 500 mg/L streptomycin sulphate and 1 mg/L Cr(VI). Plates were incubated at room temperature for 3–5 days in triplicates. Fungal colonies on PDA plates were successively purified in PDA slants using spore touch method and incubated at room temperature for 3–5 days. Axenic fungal isolates in Czapex-Dox agar or PDA were characterized microscopically at $400\times - 1000\times$ for identification.

Cr(VI) tolerance assay
A set of 1/10 strength Potato Dextrose Broth (PDB) in triplicates amended with Cr(VI) concentrations from 360 – 1800 mg/L were inoculated with standardized spores ($10^3 - 10^4$ spores/ml) of axenic isolates. Tubes were incubated at room temperature for 3–5 days in rotary shaker at 200 rpm and plated at day 5 in PDA for cell viability test with Cr(VI). Control samples were prepared by inoculating isolates in PDA plates without Cr(VI).

Cr(VI) reduction assay and statistical test
One milliliter of standardized spore suspension (10^3-10^4 spores/ml) was inoculated in 299 ml PDB. Cultures were incubated for 5 days at room temperature with shaking at 150 rpm forming mycelial balls. Mycelia were harvested and placed in 50 ml falcon tubes with sterile distilled water and centrifuged at 10,000 rpm for 5 mins. Supernatant was decanted aseptically and mycelia were re-washed three times with sterile distilled water. A 1 g mycelial ball of each isolate was inoculated in tubes with 1/10 strength PDB amended with 360 mg/L Cr(VI) under varying pH (pH 1.0 – 4.0) incubated at room temperature. In another set up of 1/10 strength PDB, 1 g mycelial ball was added to different tubes with varying Cr(VI) concentrations (180, 360, 720 and 1440 mg/L) at pH 2.0 incubated at room temperature. A 1 ml spent broth medium of both set-up sampled at days 0, 1, 3, and 5 were extracted off and 1000 μl 1,5-Diphenylcarbazide (0.5 g 1,5-DPCZ in 100 ml absolute ethanol and 400 ml 3.6 N H_2SO_4) was added until formation of violet complex analyzed at OD_{540nm} (Lambda 35, perkin elmer UV-Vis spectrophotometer). Percentage Cr(VI) reduction was computed as (Bennett et al. 2013):

$$C_{PR} = (I_{abs} - F_{abs}) / I_{abs} \times 100$$

Where C_{PR} is the percentage Cr(VI) reduction, I_{abs} is the absorbance of control and F_{abs} for the absorbance of experimental samples. All samples were prepared in triplicates. Respective control samples were prepared in all experimental set-ups. Results were analyzed statistically amongst and between mean of data samples with variance analysis at 95% level of confidence.

Results

A total of 15 fungal (Table 1) morphospecies were isolated from two sampling sites (Coto Chromite Deposit in Masinloc, Zambales and Motolite Battery Plant in Novaliches, Quezon City). Of the 15 morphospecies, 2 isolates tolerated 1440 mg/L (*Aspergillus* sp. MSG7 and *Aspergillus* sp. BP3) while another two species (*Aspergillus* sp. BP2 and *Aspergillus* sp. BP5) tolerated 1800 mg/L. Further, cell viability test affirmed isolates viability when cultured in nutrient scarce medium (1/10 strength PDB and PDA) amended with high levels of Cr(VI), thus, suggesting the biotechnological significance of the four isolates.

Table 1 Summary of fungal isolates and Cr(VI) tolerance

Isolate code	Source	Cr(VI) tolerance	Identity
MSG1	Masinloc, Zambales	720	−
MSG2	Masinloc, Zambales	720	−
MSG3	Masinloc, Zambales	720	−
MSG4	Masinloc, Zambales	720	−
MSG5	Masinloc, Zambales	720	−
MSG6	Masinloc, Zambales	720	−
MSG7	Masinloc, Zambales	1440	*Aspergillus* sp.
MSG8	Masinloc, Zambales	720	−
MSG9	Masinloc, Zambales	720	−
BP1	Novaliches, Quezon City	720	−
BP2	Novaliches, Quezon City	1800	*Aspergillus* sp.
BP3	Novaliches, Quezon City	1440	*Aspergillus* sp.
BP4	Novaliches, Quezon City	360	−
BP5	Novaliches, Quezon City	1800	*Aspergillus* sp.
BP6	Novaliches, Quezon City	720	−

− not determined

Cr(VI) reduction assay

Statistically, there were no significant difference ($p > 0.05$) for Cr(VI) reduction when mycelial balls were subjected to varying pH levels (pH 1.0 – 5.0) at 360 mg/L Cr(VI) (Figure 1) and at varying Cr(VI) concentrations, pH 2.0 (Figure 2) both incubated at room temperature.

In Figure 1, acidic pH was inferred as important factor for fungal Cr(VI) reduction due to conversion of anionic Cr(VI) complex at pH ≤ 5.0 (Bennett et al. 2013). Based from our results, fungal isolates showed no significant difference ($p > 0.05$) regardless of pH, as grouping factor, in media amended with 360 mg/L Cr(VI). However, based on rank, pH 1.0 showed highest reduction followed by pH 2.0. Further, amongst four fungal species as statistical grouping factor, it showed no significant difference ($p > 0.05$) indicating similar Cr(VI) reduction abilities. This indicates that regardless of species, reduction was shown efficient [30 – 68% Cr(VI) reduction] and that binding properties of fungal cell wall played an important factor in Cr(VI) reduction.

Based on Figure 2 with Cr(VI) concentration as grouping factor, Cr(VI) reduction showed no significant difference ($p > 0.05$). This indicates that Cr(VI) did not affect growth and viability of fungal species within the utilized Cr(VI) concentration levels (180 – 1440 mg/L). Further, amongst four species, Cr(VI) was found non-significant ($p > 0.05$). This indicates that cell wall properties of fungal isolates play an important factor in Cr(VI) reduction process. Thus, Cr(VI) reduction [35 – 65% Cr(VI) reduction] was efficient regardless of isolates and Cr(VI) concentration.
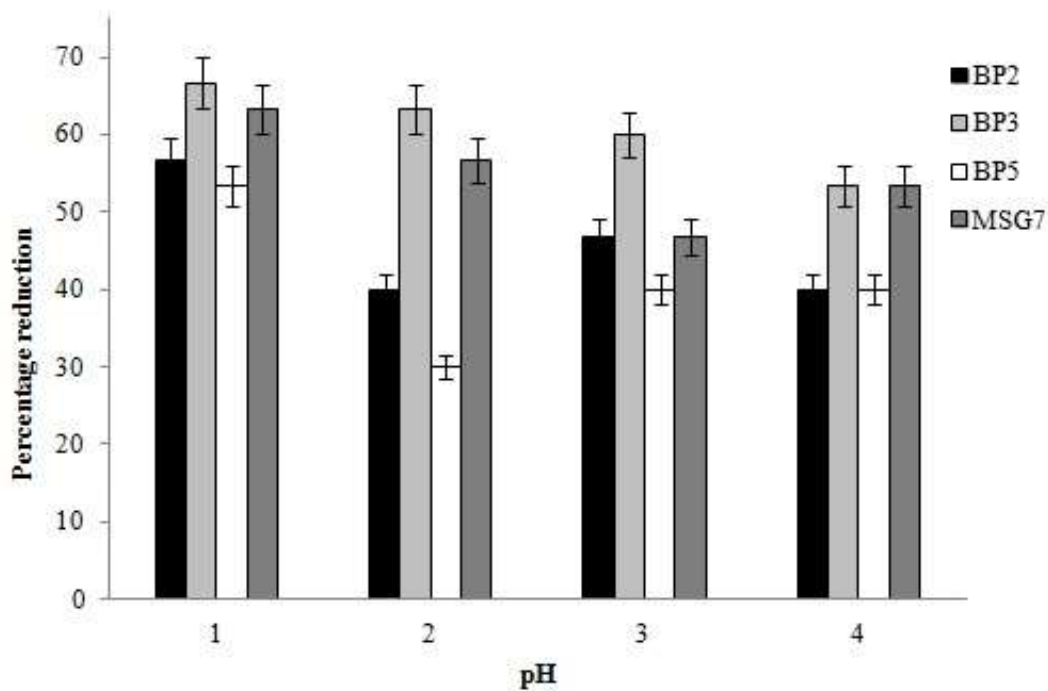
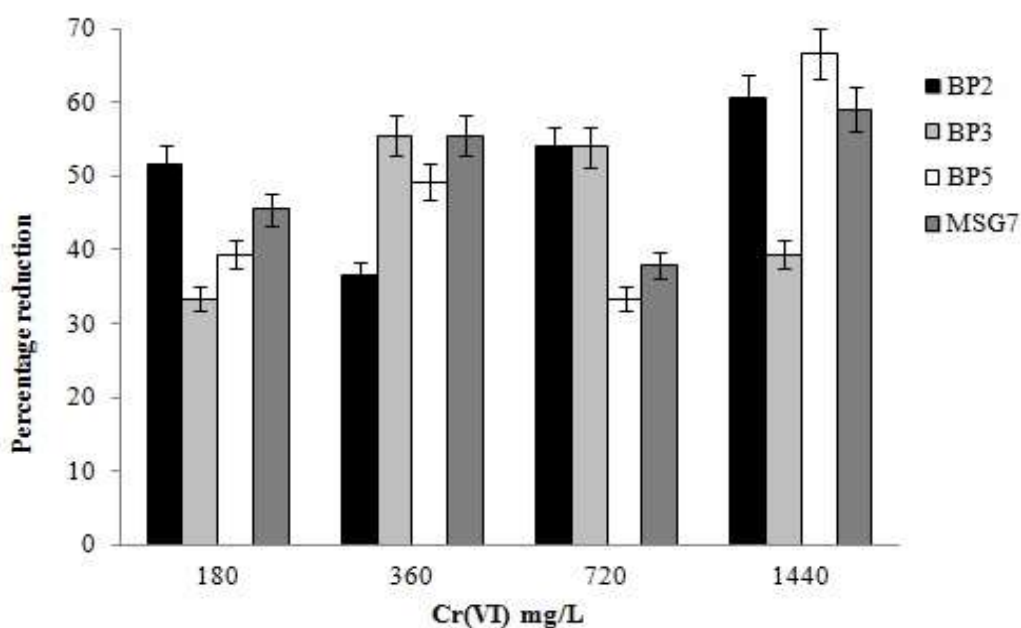**Fig. 1** − Percentage reduction of 360 mg/L Cr(VI) at varying pH levels

Fig. 2 − Percentage reduction of varying Cr(VI) concentrations at pH 2.0

Discussion

Studies on industrial application of fungal species are of recent relevance due to their ubiquitous and metabolic nature. Fungi can adapt and grow at extreme conditions of pH, temperature, scarce nutrient and high metal concentrations. They have excellent cell wall metal-binding receptors for various heavy metals. Generally, fungal species developed remarkable tolerance to heavy metals when exposed to high metal concentrations leading to mutation and adaptation (Ezzouhri et al. 2009, Valix et al. 2001). This explains why our local isolates tolerated high Cr(VI) levels (1440 and 1800 mg/L) under nutrient-scarce medium, since, our sampling sites

are contaminated with both chromium and lead. It was further supported by Muñoz et al. (2012) that collection sites with extensive metal contaminants contribute to resistance of microorganisms to heavy metals. In the study of Kumar et al. (2012), *Aspergillus niger, A. sydoni, Penicillium janthinellum* and *Trichoderma viridae* were able to tolerate high levels of Ni(II), Zn(II) and Cr(VI). Further, *Penicillum funiculosum, P. simplicissimum* and *Aspergillus foetidus* gained resistance when continuously subcultred in agarised media supplemented with heavy metals (Valix et al. 2001). Fungi can develop high resistance to heavy metals through adsorption to cell surfaces, complexation by exopolysaccharides, intracellular accumulation and precipitation (Massaccesi et al. 2002, Saxena et al. 2006). The ability of fungi as biosorbents has been extensively evaluated and showed excellent metal sequestering abilities for heavy metals such as cadmium, chromium, copper, zinc, lead, iron, nickel, silver and uranium from aqueous solution (Ahluwalia & Goyal 2007, Bennett et al. 2013, Kapoor & Viraraghavan 1995, Mungasavalli et al. 2007). Both living and dead fungi are effective in removing metals, but dead cells are preferable for wastewater treatment since they are not affected by toxic wastes and chemicals (Prigione et al. 2009). Thus, heavy metals passively transports into fungal biomasses. In this study, mycelial balls were utilized as Cr(VI) reduction and sorption agents to test whether a Cr(VI)-metabolic independent interaction transpired (Ahalya et al. 2003). The most important parameter influencing fungal sorption was pH. It changes cell wall charges in presence of excess H^+ or OH^- ions. Some advantages of acidic medium in Cr(VI) reduction are; first, fungal cell wall becomes highly protonated due to excess H^+ ions that binds to functional groups (–OH, $-NH_2$, and –C=O with –NH) (Bennett et al. 2013); and second, Cr(VI) forms its anionic species to $HCrO^{4-}$, CrO_4^{2-} and $Cr_2O_7^{2-}$ (Bennett et al. 2013, Sanghi et al. 2009). In the process of Cr(VI) reduction under acidic condition, key steps were elucidated similar to previous reports; first, formation of anionic species ($HCrO^{4-}$, CrO_4^{2-} and $Cr_2O_7^{2-}$) of Cr(VI); second, binding of negatively charged anionic Cr(VI) species to highly protonated fungal cell wall; third, conversion of Cr(VI) to Cr(III); and lastly, repulsion of Cr(III) back into broth medium due to similarities in charges (Bennett et al. 2013, Sanghi et al. 2009), since Cr(III) has no ability to form its anionic equivalents. In our set-up, it was theorized that Cr(III) transpired in broth medium following the principle of Cr(VI)-Cr(III) biotransformation mechanism as mediated by electron donors present in fungal cell wall. This explain why pH showed no significant effect (*p > 0.05*) when used as grouping factor since, pH 1.0 – 4.0 statistically resulted to similar Cr(VI) coupled reduction and biotransformation mechanism. Our results showed similar data from findings of Bennett et al. (2013) in which *Aspergillus* sp. and *Aspergillus niger* efficiently reduced Cr(VI) within pH 2.0 – 7.0, and pH 2.0 and 5.5 for *Paecilomyces lilacinus* (Kumar et al. 2012, Sharma & Adholya 2011). The study further affirmed and established the Cr(VI)-Cr(III)-fungal cell wall chemistry occurring amongst *Aspergillus* spp. as previously described by several publications. Cr(VI) concentration showed no significant effect (*p > 0.05*) on Cr(VI) reduction in broth medium. Since, fungal isolates functioned as Cr(VI)-sorbing/reducing agent and not as developing mycelia. This infers the metabolic independent type of interaction to Cr(VI) (Ahalya et al. 2003). The functional groups of fungal cell wall and Cr(VI) underwent a physico-chemical interaction as mediated by the conversion of Cr(VI) to Cr(III) via fungal-cell wall sequestration and biotransformation. In this case, our local isolates converted the toxic Cr(VI) to its non- to lesser toxic Cr(III) form. Further, increased surface mycelial biomass efficiently reduce and transform Cr(VI) in broth medium as attributed to large surface area of binding sites of fungal cell wall (Sahin & Ozturk 2004).

Conclusion

Based on the construed data, it showed the ability of *Aspergillus* spp. to tolerate high Cr(VI) concentrations and efficiently reduce Cr(VI) under nutrient scarce condition. Further, pH showed to be an important factor in Cr(VI) reduction and absorption based on statistical data on percentage reduction. With the aforementioned data, it affirms the potential of the isolates in absorbing and reducing Cr(VI) in natural systems.

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