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Studies on Heavy Metals Detoxification Biomarkers in Fungal Consortia

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Abstract

Fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. have shown the production of metal chelating compounds mainly oxalic acid and citric acid. Fungi show evoked responses to counter metal toxicity by suitable alterations of certain enzymes and several biomolecules. Increased synthesis of proline, malondialdehyde and catalase enzymes by metal treated fungi indicates its significance in heavy metals detoxification. The concentration of these enzymes increased with increase in metal concentration. But at very high metal concentration, the synthesis of these enzymes gets reduced.

Production of oxalic acid by fungal consortia was more in presence of $\text{Cu}^{2+}+\text{Pb}^{2+}$ followed by Pb^{2+} and Cu^{2+} . Fungal consortia showed decreased citric acid production with increasing in concentration of Pb^{2+} , Cu^{2+} and $\text{Cu}^{2+}+\text{Pb}^{2+}$. Except Pb^{2+} , the production of proline was high in Cu^{2+} and $\text{Cu}^{2+}+\text{Pb}^{2+}$ treated fungal consortia up to 250 mg/L metal concentration. The increased synthesis of malondialdehyde (MDA) was observed after 96 hr growth of fungal consortia in heavy metals containing medium. MDA content was increased up to 100 mg/L metal concentration and above this concentration production of MDA remains constant. Increased catalase activity was observed at 50 mg/L metal concentration and very low catalase activity was found at 100 mg/L and above concentration.

Introduction

Fungi have capable to produce metal chelating compounds such as siderophores and organic acids. Organic acids, mainly oxalic acid and citric acid are common metabolites produced by several fungi and their production is associated with the solubilisation of insoluble compounds containing metal. Organic acids immobilize potentially toxic metals through formation of insoluble compounds such as metal-oxalate complexes. This process is an important heavy metals detoxification mechanism involved in fungi¹. Different organic molecules, in particular di and tricarboxylic acids that do not belong to the matrix of the cell wall are excreted by fungal cells to chelate metal ions. The induction of oxalic acid efflux correlated closely with Cu tolerance in brown rot fungi. Over excretion of oxalic acid probably contributed to the metal tolerance exhibited by *Beauveria caledonica*².

A. niger wild-type strain shows evoked responses to counter arsenate toxicity by suitable alterations of certain enzymes and several biomolecules. *A. niger* cause possible cellular strategy towards tolerance of arsenate-induced toxicity and thereby to evaluate its application for bioremediation of arsenic from arsenic-contaminated aqua-environment³. This study was made to detect the cellular mechanisms involved in *A. niger* to detoxify heavy metals toxicity. This study understand the possible cellular strategy towards tolerance of arsenate-induced toxicity and thereby to evaluate the possibility of application of the test strain for bioremediation of arsenic from arsenic-contaminated aqua-environment.

MATERIALS AND METHODS

Isolation of *Aspergillus niger*

A. niger, *Penicillium* sp. and *Rhizopus* sp. was isolated by standard plate count method from paper effluent by serial dilution method on Potato dextrose agar (PDA) medium.

Production of Oxalic acid, Citric acid and Enzymes

The medium used for acid and enzyme production contained the composition of, (g^l⁻¹) Sucrose, 120; NH₄Cl, 4.0; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.25; and (mg⁻¹): Fe (as FeSO₄·7H₂O), 0.1; Zn (as ZnSO₄·7H₂O), 0.1; Mn (as MnSO₄·2H₂O), 0.1; and Cu (as CuSO₄·5H₂O), 0.05⁴.

A. niger, *Penicillium* sp. and *Rhizopus* sp. culture was grown for 5 days in 250 ml conical flask contained 100 ml potato dextrose broth prepared by 2% glucose and 20% fresh potato extract in distilled water. A loop full of 5 days old each culture was inoculated in to series of 100 ml acid production medium each added with 50mg/L, 100mg/L and 250mg/L concentrations of Cu, Pb and combination of Cu and Pb metals taken in 250 ml conical flask. A loop full of 5 days old each culture was inoculated in to medium without containing heavy metals and it was maintained as control. All flasks were incubated in rotary shaker for 5 days at 100rpm in 30°C temperature. After incubation period, cultures were filtered and the supernatant was used for estimation of citric acid and oxalic acid. The protein and enzyme concentration of mycelia fractions were determined.

Determination of Oxalic acid

For determination of oxalic acid in the culture medium, reaction mixture contained 0.2 ml of sample (or standard oxalic acid solution), 0.11ml of bromophenol blue (BPB, 1 mM), 0.198ml of sulphuric acid (1 M), 0.176ml of potassium dichromate (100mM) and 4.8 ml of distilled water was taken in test tube. The reaction mixture was placed in a water bath at 60°C and quenched by adding 0.5ml sodium hydroxide solution (0.75M) after 10min. The absorbance was measured at 600nm by means of spectrophotometer and medium was used as blank. Concentration of oxalic acid was calculated by using standard curve and results were expressed as µg oxalic acid produced by mg⁻¹ dry wt of mycelium⁵.

Determination of Citric acid

Citric acid was determined by colorimetric method. 1.0 ml. of test sample, citric acid standard contained 25 to 200µg of citric acid, and water as the reagent blank was taken in clean test tube. To this solution 1.30ml of pyridine was added and the tube was swirled briskly. All the tubes were added with 5.70 ml of acetic anhydride and the tubes were swilled well for proper mixing. Immediately all the tubes were placed in constant temperature water bath of 32°C. Color developed after 30 minutes of incubation and remained stable in another 30 minutes at room temperature. The intensity of color was read at 420nm with the blank set at 100% transmission. Citric acid content in the samples was estimated with reference to the standards value⁶.

Screening for Acid Production

5 days old culture of *A. niger*, *Penicillium* sp. and *Rhizopus* sp. was inoculated into series of Petriplates filled with potato dextrose agar contained Bromophenol blue (50mg ml⁻¹) and 100mg/L concentration of Cu, Pb, and mixture of Cu and Pb. Plates were incubated for 5 days at 25°C for media colour change from blue to yellow⁵.

Assay of Catalase Activity

0.5g of fungal mycelia was freeze dried with liquid nitrogen and ground using mortar and pestle by suspending in 8mL of 50mM potassium phosphate buffer with pH 7.5. The homogenate was centrifuged at 15,000g for 20min at 4°C. The supernatant was used for assay of catalase activity and lipid peroxidation.

The catalase activity was measured by taken reaction solution (3mL) contained 50mM phosphate buffer (pH 7.0), 15mM H₂O₂, and 0.1mL of sample. Catalase activity was determined using spectrophotometer to measure the decrease in absorbance at 240nm during decomposition of H₂O₂ by the enzyme catalase³.

Protein Estimation

Protein concentration was determined by Bradford method. The amount of protein in the sample was calculated using a standard graph drawn with Bovine serum albumin (10 mg/100 mL)⁷.

Assay of Lipid Peroxidation

The content of malondialdehyde (MDA), a final product of lipid peroxidation was determined by taken 0.5 mL aliquot of sample to 1mL of 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The mixture was heated in water bath at 95°C for 30 min. The content was cooled to room temperature and centrifuged at 10,000g for 10 min. The supernatant was read for absorbance at 532nm and 600nm. The absorbance for non specific absorption at 600nm was subtracted from the value at 532nm to obtain the amount of MDA present in sample^{8,9}.

Assay of Proline

0.2 g fresh mycelium was ground in mortar and pestle and extracted with 4mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 2000g for 20 min. 2mL of the supernatant was pipetted into 10mL test tube and 2 mL each of glacial acetic acid and acid ninhydrin reagent were added to the supernatant. The content was heated to 100°C for 45min. Subsequently tubes were placed in an ice bath to cool at room temperature and 4mL of toluene was added to each tube. The tubes were shaken vigorously for 2 min and allowed to stay for 15 min, for complete phase separation. Upper toluene layer was separated and kept in room temperature for 10 min and the red color intensity was read at 520 nm against toluene blank. Concentration of proline was determined from standard curve³.

Statistical Analysis of Results

The data were statistically analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) software. Results were expressed as the mean ± standard error (SE). The means obtained from each set were compared using the Duncan's multiple range tests at 0.05 confidence level.

Results

Acid production

Presence of yellow halo around the fungal colony was observed in the medium supplemented with metals and bromophenol blue as pH-indicator. This result is considered as an evidence for acid released by the fungi for heavy metals detoxification.

Production of oxalic acid was observed in fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. The concentration of oxalic acid production was decreased with increasing in metal concentrations. Production of oxalic acid by fungal consortia was more in presence of Cu²⁺+Pb²⁺ followed by Pb²⁺ and Cu²⁺ at 50 mg/L metal concentration. With increase in Pb²⁺, Cu²⁺ and Cu²⁺+Pb²⁺ to 100 and 250 mg/L concentration, the oxalic acid production was decreased (Table 1). The fungal consortia has showed decreased citric acid production with increase in Pb²⁺, Cu²⁺ and Cu²⁺+Pb²⁺ metal concentrations to 50mg/L, 100mg/L and 250mg/L (Table 2).

Increased oxalic acid and citric acid production in metal treated fungal consortia compared to control fungal culture without treated with metals indicate the significance of acid production in detoxification of heavy metals.

Proline Assay

The synthesis of proline in fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. treated with heavy metals was observed (Fig 1). Proline synthesis was maximum up to 100mg/L concentration of Cu²⁺, Pb²⁺ and Cu²⁺+Pb²⁺ metals after 96 hr of growth period. Except Pb²⁺, the production of proline was high in Cu²⁺ and Cu²⁺+Pb²⁺ treated fungal consortia at 100mg/L metal concentration. Decreased proline synthesis was observed at 250mg/L metal concentration.

Assay of Lipid Peroxidation

Increased synthesis of malondialdehyde (MDA) was observed after 96 hr growth of fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. in heavy metals contained medium (Fig 2). MDA content was increased up to 100 mg/L and then remained constant at 250 mg/L concentration of heavy metals.

Assay of Catalase Activity (CAT):

Catalase activity was observed in fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. treated with different concentration of Cu^{2+} , Pb^{2+} and $\text{Cu}^{2+}+\text{Pb}^{2+}$ (Fig 3). The catalase in fungal consortia was high at 50 mg/L concentration of metal. Increased metal concentration above 50 mg/L resulted in decreased catalase activity. Decreased catalase activity was noticed with further increase in heavy metals concentration to 50 mg/L and above concentration. Very low catalase activity was found at 100 mg/L and above concentration.

Discussion

Increased production of oxalic acid by fungal consortia at low metal concentration of 50 mg/L $\text{Cu}^{2+}+\text{Pb}^{2+}$ suggested that all the test fungi in consortia have increased resistance to this metal system. Increase proline synthesis in presence of Pb^{2+} indicates that Pb^{2+} acts as chief inducer for proline synthesis compared to other metals. Increase in proline synthesis indicates its protective mechanism in fungi to detoxify heavy metals by acting as scavenger of hydroxyl radicals formed due to metal toxicity inside the cell. However, decrease in proline synthesis at 250 mg/L metal concentration indicates disturbance in cellular heavy metals detoxification mechanism because this concentration of heavy metals is very drastic for the fungi to cope up.

Gradual increase in MDA content with increase in metal concentration to 100mg/L indicates the increased reactive oxygen species (ROS) generation and this cause the membrane damage by peroxidation of membrane lipids. This suggests that heavy metals made induced anti-oxidative response in the fungal strains to detoxify heavy metals toxicity. Increased catalase activity up to 100 mg/L metal concentration suggests that heavy metals treatment induced anti-oxidative response in the fungal strains to detoxify heavy metals. Very low catalase activity observed at 100 mg/L and above concentration suggested that reactive oxygen species generated in presence of heavy metals doses provoked the inhibition of catalase activity.

Amino acids and amino acid derived molecules have high significance in plant to adapt in heavy metal stress conditions. N-containing metabolites majorly proline is frequently synthesized under heavy metal stress such as Cd, Cu, Ni, and Zn. Proline has three major functions in metal detoxification namely metal binding, antioxidant defence, and signalling¹⁰.

The production of various metabolites like citric acid, homogeneous proteins, heterogeneous proteins, peroxidases by fungi made them effective for detoxification of heavym metals from industrial effluents. White rot fungi are ubiquitous in nature and their enzymes producing activity makes them effective decolourizers and remove toxic metals by biosorbition ultimately rendering the effluents more eco-friendly¹¹. Exposure to elevated heavy metals concentration in mycorrhizal species of *Pinus sylvestris* made it to produce organic acids. Among different acid production, the level of oxalic acid is significantly high compared to other acids like malonic acid, citric acid, shikimic acid, lactic acid, acetic acid, propionic acid, fumaric acid, formic acid, iso-butyric acid and butyric acid are found in variable concentrations¹².

The release of phytochelatins and non-protein thiol content as biomarkers against copper and cadmium heavy metals toxicity is observed in maize plant¹³. When metal ions are taken up into the cell, properties like high reactivity or limited solubility require their chelation. The metal ions are bound by chelators and chaperons. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations, while, chaperons specifically delivers metal ions to organelles and metal requiring proteins. Metal chelators include phytochelatins, metallothioneins, organic acids and amino acids. The main storage compartment of toxic compound in fungi is the vacuole. In fungi vacuole transporters help to remove potentially toxic cations from the cytosol. Metal/ H^+ antiporters are involved in metal sequestration into the vacuole¹⁴.

Conclusion

This study is helpful to know the cellular mechanisms involved in detoxification of toxic heavy metals by fungal consortia contained *Aspergillus niger*, *Penicillium* sp. and *Rhizopus* sp. The obtained results can be made applicable for large scale application for bioremediation of heavy metals.

Table 1: Oxalic acid concentration in PDB medium containing heavy metals.

Fungi	Oxalic acid ($\mu\text{g}/\text{mg}$ dry wt. mycelium)									
	Control	Pb^{2+} (mg/L)			Cu^{2+} (mg/L)			$\text{Cu}^{2+}+\text{Pb}^{2+}$ (mg/L)		
		50	100	250	50	100	250	50	100	250
<i>A.niger</i> + <i>Penicillium</i> sp.+ <i>Rhizopus</i> sp.	75 \pm 2.9	-	-	-	-	-	-	-	-	-
<i>A.niger</i> + <i>Penicillium</i> sp.+ <i>Rhizopus</i> sp.	-	113 \pm 2.3	119 \pm 2.7	121 \pm 2.5	113 \pm 2.6	109 \pm 1.9	110 \pm 2.4	131 \pm 3.7	129 \pm 3.3	111 \pm 3.0

^aNote: Results are expressed in Mean, \pm Standard Error (n=3).

Table 2: Citric acid concentration in PDB medium containing heavy metals.

Fungi	Citric acid ($\mu\text{g}/\text{mg}$ dry wt. mycelium)									
	Control	Pb^{2+} (mg/L)			Cu^{2+} (mg/L)			$\text{Cu}^{2+}+\text{Pb}^{2+}$ (mg/L)		
		50	100	250	50	100	250	50	100	250
<i>A.niger</i> + <i>Penicillium</i> sp.+ <i>Rhizopus</i> sp.	95 \pm 2.7	-	-	-	-	-	-	-	-	-
<i>A.niger</i> + <i>Penicillium</i> sp.+ <i>Rhizopus</i> sp.	-	127 \pm 1.9	129 \pm 2.5	117 \pm 2.7	125 \pm 3.6	121 \pm 3.1	117 \pm 2.3	132 \pm 2.9	119 \pm 3.4	98 \pm 2.9

^aNote: Results are expressed in Mean, \pm Standard Error (n=3).

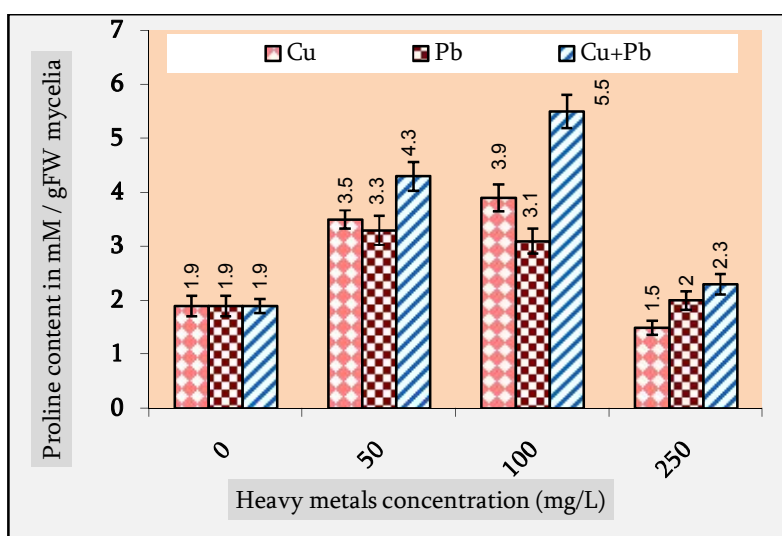


Figure 1: Assay of proline in metal untreated and metal treated (50, 100, and 250 mg/L) fungal consortia.

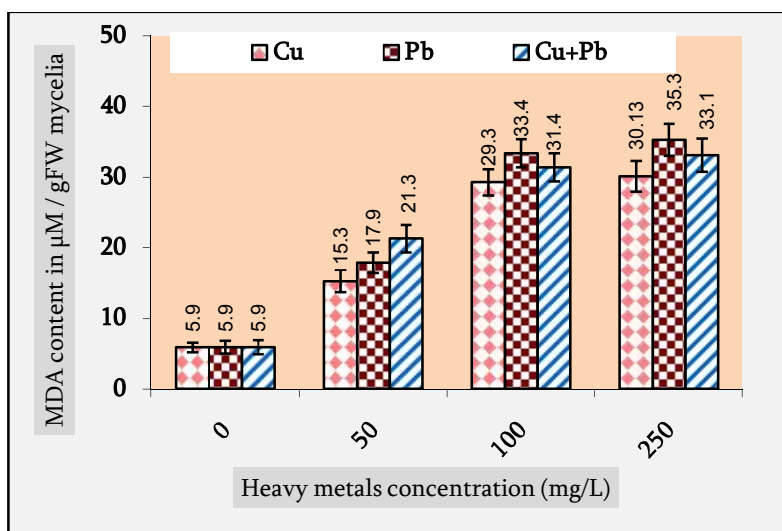


Figure 2: Assay of malondialdehyde in metal untreated and metal treated (50, 100, and 250 mg/L) fungal consortia.

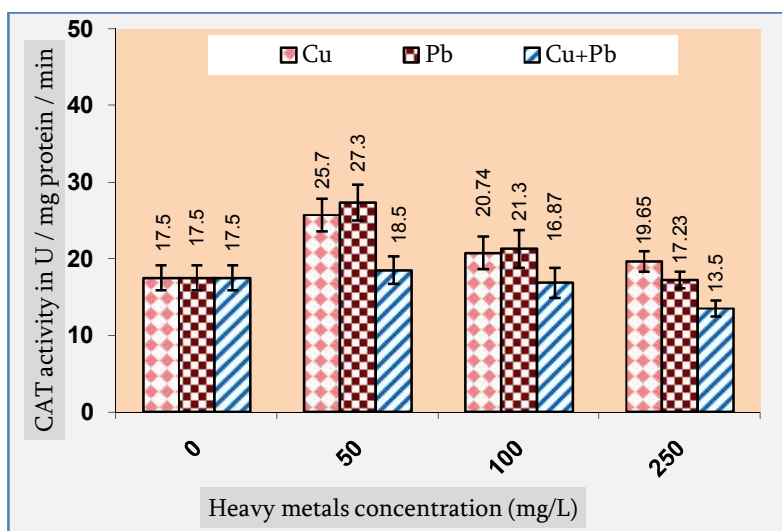


Figure 3: Assay of catalase activity in metal untreated and metal treated (50, 100, 250 mg/L) fungal consortia.

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