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Research Article

Study on heavy metals toxicity biomarkers in *Aspergillus niger*

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ABSTRACT

Aspergillus niger has shown production of metal chelating compounds mainly oxalic acid and citric acid. Production of oxalic acid by *Aspergillus niger* was found maximum in presence of Pb^{2+}, followed by Cu^{2+}+Pb^{2+} and Cu^{2+}. With increase in concentration of Pb^{2+} to 50, 100 and 250 mg/L, the production of oxalic acid was increased. Whereas increase in concentration of Cu^{2+}+Pb^{2+} and Cu^{2+}, resulted in decreased production of oxalic acid. Except Cu^{2+}+Pb^{2+}, the production of citric acid was increased with increase in Pb^{2+} and Cu^{2+} concentration. Increased synthesis of proline, malondialdehyde and catalase enzymes by metal treated *A. niger* indicates its significance in heavy metals detoxification. Catalase activity increased with increase in metal concentration up to 100 mg/L. Synthesis of malondialdehyde (MDA) was increased up to 100 mg/L metal concentration after 96 hr of growth period. Increased proline content was observed in *A. niger* treated with Pb^{2+} compared to Cu^{2+} and Cu^{2+}+Pb^{2+}.

Key words: *Aspergillus niger*, Heavy metals, Detoxification, Biomarkers.

INTRODUCTION

Metabolites produced by fungi are associated with solubilisation of insoluble compounds containing metal. Fungi immobilize potentially toxic metals through the formation of insoluble metal-oxalate complex. This process is an important detoxification mechanism in fungi [1]. Different organic molecules particularly 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 and over excretion of oxalic acid probably contributed to the metal tolerance exhibited by *Beauveria caledonica* [2]. Wild type *A. niger* exhibit biochemical response against arsenate toxicity [3].

Aspergillus niger is distributed ubiquitously in nature and is an important microorganism in biotechnological applications [4]. *Aspergillus niger* is used to produce organic acids such as gluconic acid, citric acid and extracellular enzymes like glucoamylas, pectinase, acidic lipase, feruloyl esterase, and xylanase. Citric acid and several enzymes produced by *A. niger* are considered as GRAS (Generally regarded as safe) by United States Food and Drug Administration (USDA). *A. niger* is used in biotransformation of toxic compounds like ferrulic acid, progesterone, diperpenoid, terpene, linaloo, gereniol, nirol and citral. In the last two decades, *A. niger* has been developed as an important transformation host to express different useful enzymes. *A. niger is* also ecologically important in biodegradation of toxic chemicals. Waste biomass of *A. niger* from fermentative industry is used to remove hazardous heavy metals such as cadmium, lead, chromium, and copper from aqueous solution. The organic acids production property of *A. niger* can be used to bioleach metals from mining ores and recovery of essential metals [5]. Fungi thrive well in inhospitable habitats with environmental extremes because of their enzyme system. Fungi are involved in the biodegradation of undesirable compounds and convert them into harmless and useful products.

Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites [6]. This study understands the possible cellular strategy such as production of enzymes and acids involved in detoxification of heavy metals by *A. niger*.

MATERIALS AND METHODS

Production of Oxalic acid, Citric acid and Enzymes

The medium used for acid and enzyme production contained composition of, (gL^{-1}): sucrose, 120; NH_4Cl, 4.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; and (mgL^{-1}): Fe (as $FeSO_4 \cdot 7H_2O$), 0.1; Zn (as $ZnSO_4.7H_2O$), 0.1; Mn (as $MnSO_4.2H_2O$), 0.1; and Cu (as $CuSO_4.5H_2O$), 0.05 [7].

Aspergillus niger was grown for 5 days in 250 ml conical flask contained 100 ml of potato dextrose broth prepared by 2% glucose and 20% fresh potato extract in distilled water. A loop full of 5 days old *Aspergillus niger* culture was inoculated in to 100 ml of acid production medium in 250 ml conical flask added with 50mg/L, 100mg/L and 250mg/L concentrations of Cu, Pb and combination of Cu and Pb metals. Control was maintained without containing heavy metals in the medium. All flasks were incubated in rotary shaker for 5 days at 100rpm for 30ºC temperature. After incubation period, cultures were filtered and supernatant was used for the 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), 0.198 ml of sulphuric acid (1 M), 0.176ml of potassium dichromate (100 mM) and 4.8 ml of distilled water was taken in test tube. The reaction mixture was placed in water bath at 60ºC and quenched by adding 0.5ml sodium hydroxide solution (0.75M) after 10min. The absorbance was measured at 600nm using spectrophotometer and medium was used as blank. Concentration of oxalic acid was calculated by comparing the obtained values with standard curve. Results were expressed as µg oxalic acid mg^{-1}dry wt of mycelium [8].

Determination of Citric acid

Citric acid was determined by colorimetric method. 1.0 ml of test sample, citric acid standard containing 25µg to 200µg of citric acid, and water as the reagent blank was taken separately in clean test tube. To this solution, 1.30ml of pyridine was added and the tube was swirlled briskly. All the tubes were added with 5.70 ml of acetic anhydride. The tubes were again swrilled and immediately placed in constant temperature water bath of 32°C. Colour developed after 30 minutes of incubation and remained stable in another 30 minutes at room temperature. The intensity of colour was read at 420nm with the blank set at 100% transmission. Citric acid content in the samples was estimated with reference to the standards value [9].

Screening for Acid Production

5 days old culture of *Aspergillus niger* was inoculated on Petri plate filled with potato dextrose agar containing Bromophenol blue (50 mg mL^{-1}) and 100mg/L concentration of Cu, Pb, and mixture of Cu and Pb. Plates were incubated for 5 days at 25ºC for colour change of media from blue to yellow [8].

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 4ºC. The supernatant was used for assay of catalase activity and lipid peroxidation.

The catalase activity was measured by taking reaction solution (3 mL) containing 50 mM phosphate buffer (pH 7.0), 15mM H_2O_2, and 0.1mL of sample. Catalase activity was determined by using spectrophotometer to measure the decrease in absorbance at 240nm during decomposition of H_2O_2 by the enzyme [3].

Protein Estimation

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

Assay of Lipid Peroxidation

The content of malondialdehyde (MDA), a final product of lipid peroxidation was determined by taking 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 on water bath at 95ºC for 30 min. The content was cooled to room temperature and centrifuged at 10,000g for 10 minutes. The supernatant obtained after centrifugation was read for absorbance at 532 nm and 600 nm. The absorbance for non specific absorption at 600 nm was subtracted from the value at 532 nm.

The amount of MDA present in red pigment form was calculated [11, 12]

Assay of Proline

0.2 g fresh mycelium was ground in mortar and pestle. Fine ground material was extracted with 4mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 2000g for 20 min. 2mL of the supernatant was pipette into a 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 100ºC for 45min. Subsequently tubes were placed in an ice bath to cool at room temperature and 4mL 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 intensity of red colour was read at 520 nm against toluene as blank. Concentration of proline was determined from standard curve [3].

Statistical Analysis of Results

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

RESULTS

Acid production

Presence of yellow halo around 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 fungi for detoxification of heavy metals. Production of oxalic acid by *Aspergillus niger* in presence of heavy metals was observed (Table 1). Production of oxalic acid by *Aspergillus niger* was maximum in presence of Pb^{2+}, followed by Cu^{2+}+Pb^{2+} and Cu^{2+}. With increase in Pb^{2+} concentration to 50, 100 and 250 mg/L, the production of oxalic acid was increased. Whereas increase in concentration of Cu^{2+}+Pb^{2+} and Cu^{2+}, the production of oxalic acid was decreased. In *Aspergillus niger* treated metals except Cu^{2+}+Pb^{2+}, the production of citric acid was increased with increase in the concentration of Pb^{2+} and Cu^{2+} (Table 2).

Production of oxalic acid and citric acid in metal treated *Aspergillus niger* when compared to control showing the absence of acid production indicate the significance of acid in detoxification of heavy metals. Concentration of enzymes involved in detoxification of heavy metals was increased with increase in metal concentration. But at very high metal concentration, the synthesis of these enzymes gets reduced. The production of these enzymes in metal untreated fungi was found less compared to metal treated fungi.

Proline Assay

Synthesis of proline by *Aspergillus niger* treated with heavy metals was observed (Fig 1). Proline accumulated during growth of *Aspergillus niger* at different concentrations of Cu^{2+}, Pb^{2+} and Cu^{2+}+Pb^{2+} up to 100mg/L concentration after 96 hr of growth period. Increased proline synthesis was observed in *Aspergillus niger* treated with Pb^{2+} compared to Cu^{2+} and Cu^{2+}+Pb^{2+}.

Assay of Lipid Peroxidation

Increased synthesis of malondialdehyde (MDA) was observed after 96 hr growth of *Aspergillus niger* in medium contained heavy metals. The content of malondialdehyde was found high in metal treated *Aspergillus niger* culture compared to control without treated with metals (Fig 2). MDA content was increased up to 100 mg/L metal concentration and then remained constant at 250 mg/L concentration of heavy metals.

Assay of Catalase Activity (CAT)

The catalase activity in *Aspegillus niger* increased with increase in metal concentration up to 100 mg/L (Fig 3). The increased metal concentration above 100 mg/L resulted in decreased catalase activity.

DISCUSSION

A. niger has shown evoked responses to counter metal toxicity by suitable alterations of certain enzymes and several biomolecules. 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 vacuole [13].

High synthesis of proline in presence of Pb^{2+} indicate that Pb^{2+} acts as chief inducer for proline synthesis compared to other metals. Hence *Aspergillus niger* has increased resistance to Pb^{2+}. Increase in proline

content indicates its protective mechanism of *Aspergillus niger* to detoxify heavy metals by acting as a scavenger of hydroxyl radicals formed due to metal toxicity inside the cell. However, decrease in proline synthesis at 250 mg/L metal concentration indicates the disturbance in cellular heavy metals detoxification mechanism. Because high heavy metals concentration is very drastic for *Aspergillus niger* to cope up with metal toxicity.

The gradual increase in MDA content with increase in metal concentration to 100mg/L indicate the increased reactive oxygen species (ROS) generation by increased doses of heavy metals and hence causes membrane damage by peroxidation of membrane lipids. Increased catalase activity up to 100mg/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 suggests that reactive oxygen species generated in presence of heavy metals doses provoked the inhibition of catalase activity. This study understands the possible cellular strategy involved in tolerance of metal toxicity and thereby to evaluate the possibility of *Aspergillus niger* in bioremediation of heavy metals. 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 signalling [14].

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 biosorbtion ultimately rendering the effluents more eco-friendly [6]. 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 concentrations [15].

The release of phytochelatins and non-protein thiol content as biomarkers against copper and cadmium heavy metals toxicity is observed in maize plant [16]. This study is helpful to know the mechanisms involved in detoxification of toxic heavy metals by *Aspergillus niger* and can be made applicability of these mechanisms for large scale applications.

CONCLUSION

Aspergillus niger is appeared to be most appropriate in the treatment of metallic effluents by producing various extracellular proteins, organic acids and other metabolites. Fungi are ubiquitous in nature and their enzymes producing activity makes them effective to use fungi for removal toxic metals by biosorbtion and bioaccumulation process. This made ultimately rendering the effluents more ecofriendly. In feature research work, optimization of parameters and use of gene manipulation technology in fungi made increased detoxification of heavy metals by fungi.

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

Fungi	Oxalic acid (µg/mg dry wt. mycelium)								
	Control	Pb^{2+} (mg/L)			Cu^{2+} (mg/L)			$Pb^{2+}+Cu^{6+}$ (mg/L)		
		50	100	250	50	100	250	50	100	250
A.niger	80±2.7	-	-	-	-	-	-	-	-	-
	-	110±3.3	127±3.5	147±3.7	95±2.9	117±3.1	125±3.7	119±3.7	136±2.9	123±2.5

Note: Results are expressed in Mean, ± Standard Error (n=3).

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

Fungi	Citric acid (µg/mg dry wt. mycelium)									
	Control	Pb^{2+} (mg/L)			Cu^{2+} (mg/L)			$Ni^{2+}+Cr^{6+}+Pb^{2+}$ (mg/L)		
		50	100	250	50	100	250	50	100	250
A.niger	89 ± 2.4	-	-	-	-	-	-	-	-	-
	-	121 ±3.2	132±3.5	147±4.1	117±2.9	129±2.3	136±2.9	141± 3.3	136 ± 3.1	119 ± 2.1

Note: Results are expressed in Mean, ± Standard Error (n=3).

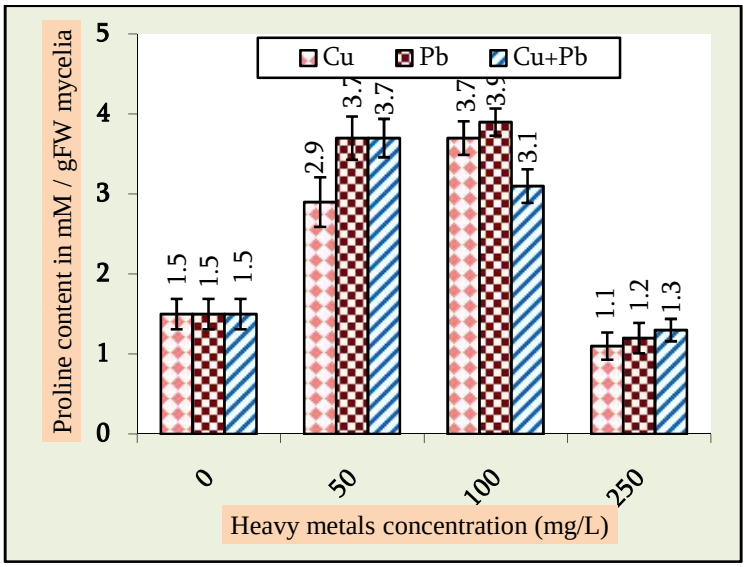

Figure 1: Assay of proline in metal untreated and metal treated (50, 100, and 250 mg/L) *Aspergillus niger*.

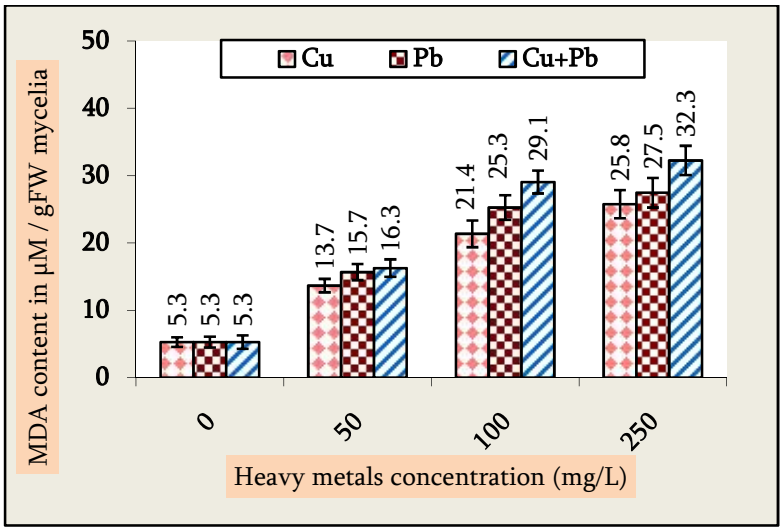

Figure 2. Assay of malondialdehyde in metal untreated and metal treated (50, 100, and 250 mg/L) *Aspergillus niger*.

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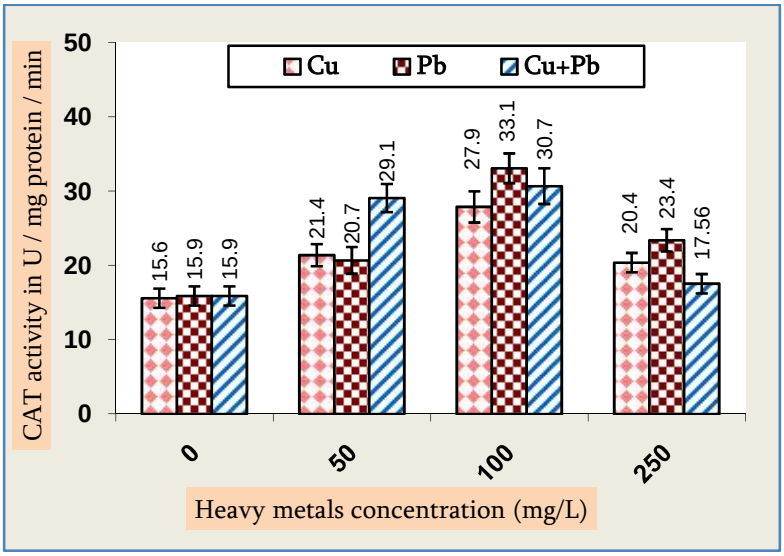

Figure 3. Assay of catalase activity in metal untreated and metal treated (50, 100, 250 mg/L) *Aspergillus niger*.

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