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CELLULAR DISTRIBUTION OF BIOACCUMULATED TOXIC HEAVY METALS IN ASPERGILLUS NIGER AND RHIZOPUS ARRHIZUS

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ABSTRACT

A cadmium tolerant strain of *Aspergillus niger* and a lead tolerant strain of *Rhizopus arrhizus* were studied for distribution of accumulated toxic heavy metals in the mycelia in comparison to wild type strains. Accumulated cadmium in the cell wall fractions of cadmium tolerant *Aspergillus niger* was more than the wild type strain. Cytosolic fraction contained the next highest load of metal. Similar was the observation with lead tolerant *Rhizopus arrhizus* during bioaccumulation of lead. The results indicated contribution of the surface property of the fungus in metal bioaccumulation. Involvement of the cellular metabolism during metal bioaccumulation and distribution in the sub-cellular compartments was substantiated by the use of some metabolic inhibitors during growth in presence of metals. It was also observed that about 65% of the cell wall bound cadmium in *Aspergillus niger* and about 79% of the cell wall bound lead in *Rhizopus arrhizus* were recovered by Ethylene diamine tetra acetic acid (EDTA) treatment, which indicated that the accumulated metals generally reside in the outer surface of the fungi, which could be recovered easily for possible commercial applications.

KEYWORDS

Bioaccumulation, lead, cadmium, *Aspergillus niger*, *Rhizopus arrhizus*

INTRODUCTION

Microorganisms like bacteria, fungi, algae and yeasts concentrate heavy metals at different capacities in inactivated or alive conditions, which are utilized for removal of heavy metals from various types of water bodies[1,2]. It has been established previously from our laboratory that, metabolically active, preferably metal-resistant fungus like *Aspergillus niger* and *Rhizopus arrhizus* ensure better accumulation of toxic metals like cadmium and lead[3,4]. These are all energy-dependent cellular activities, which need metabolic energy that could be replenished by the use of appropriate macro- or micro-nutrients[5,6].

It is generally assumed that microorganisms concentrate accumulated metals in the cell surface. Such phenomenon results from complexation and/or ion-exchange reactions between metal ions and the charged chemical constituents of cell-walls[7]. This was thoroughly studied for inactivated *Rhizopus arrhizus* during uranium biosorption[8].

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However, in case of metabolically active fungus, the situation is not completely understood till date. In the present study, that aspect has been addressed comprehensively by fractionating mycelia of two common fungus, viz. *Aspergillus niger* and *Rhizopus arrhizus* after accumulation of cadmium and lead, respectively from aqueous culture medium, and determining metal contents in different fractions.

One another aspect of metal uptake studies with microbes is the recovery of the accumulated metal by easy leaching processes. In the present study, a simple leaching method was employed for extraction of the accumulated metal in the cell surface, which could be utilized commercially for recovery of precious heavy metals accumulated by common fungus like *Aspergillus niger* and *Rhizopus arrhizus*.

MATERIALS AND METHODS

***Chemicals* – All the chemicals used in the present study were purchased from Merck, India or BDH, India (AR grade). All the culture media preparations were done using double-distilled water.**

Microorganisms and culture conditions – High concentration of cadmium tolerant strain of *Aspergillus niger* (viz. *Aspergillus niger* AB_{10}) and lead tolerant strain of *Rhizopus arrhizus* (viz. *Rhizopus arrhizus* M1) was developed and maintained as described previously[9]. Composition of the synthetic culture media for metal uptake experiments as well as other culture conditions was also described earlier[5,6]. Briefly, Experimental flasks containing synthetic media composed of (in g/L of medium) – Sucrose 50, NH_4NO_3 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.5 and $MnSO_4.7H_2O$ 0.1, (initial pH 4.5) were inoculated with spore suspension having a concentration of $4.5x10^7$ spores/ml for *Rhizopus arrhizus*. Lead was added to the fermentation medium at a concentration of 1 µg/ml in the form of $Pb(NO_3)_2$ and incubated for 7 days at $28^o±2^oC$. For experiments with *Aspergillus niger*, the composition of the media was (in g/L of medium) – Sucrose 60, NH_4NO_3 2, KH_2PO_4 1, KCl 0.2, $MgSO_4.7H_2O$ 0.01, $FeSO_4.7H_2O$ 0.05 and $MnSO_4.7H_2O$ 0.1; initial pH 4.5 and the spore concentration was $2x10^5$ spores/ml. Cadmium was added at a concentration of 4 µg/ml in the form of $Cd(NO_3)_2$ and incubated for 8 days at $28^o±2^oC$. Control cultures were devoid of the metals.

Subcellular fractionation of the fungi – A previously established method for fractionation of the fungal mycelia was employed[10]. Briefly, separated and washed mycelia (before and after metal accumulation) were suspended in Tris-HCl buffer (20 mM, pH 7.5, containing 5 mM 2-mercaptoethanol) preheated to 75^oC and incubated for 20 minutes. Mycelia were then lysed and the homogenates were centrifuged at 600g for 10 minutes to separate cell wall components. The supernatant was then further centrifuged at 15,000g for 5 minutes to separate mitochondrial fraction from cytosol.

Extraction of cell surface bound metal – This was done according to a published method[11]. Briefly, metal exposed mycelia of *Aspergillus niger* AB_{10} and *Rhizopus arrhizus* M1 were harvested by centrifugation and washed several times with double distilled water to remove loosely bound metals. The mycelia were then suspended in a solution of 10 mM EDTA in ammonia buffer for 15 mins with occasional stirring. Mycelia were then separated by centrifugation and the metal contents in the EDTA extractives were determined by atomic absorption spectrophotometer.

Effect of metabolic inhibitors on bioaccumulation – The metabolic inhibitors tested were sodium azide, 2,4-dinitophenyl hydrazine (DNPH), sodium fluoride and sodium arsenite. They were added in the growth medium at concentrations of 10^{-3} M on the 0th day, 4th day and 6th day of incubation in case of cadmium accumulation by *Aspergillus niger* and

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on the 0th day, 2nd day and 4th day of incubation in case of lead accumulation by *Rhizopus arrhizus*.

Analytical determination of lead and cadmium – Whole mycelia, fractionated components from mycelia and the EDTA extractives were digested with 6(N) HNO_3 and the metal contents were analyzed by Atomic Absorption Spectrophotometer (Varian SP-20 BQ) according to a previously described method[9].

Statistical analyses – All the data, in the present study, were either analyzed by one-way ANOVA followed by Dunett's post-hoc test for multiple comparisons of the treatment means, or analyzed by Student's paired '*t*'-test. A *p*-value of 0.01 or less was taken as criteria for a statistically significant difference. All statistical calculations were done with the software 'SPSS', version 7.5.

RESULTS

In the present study, it was observed that maximum amount of accumulated cadmium by a cadmium tolerant strain of *Aspergillus niger* (*Aspergillus niger* AB_{10}) and maximum amount of accumulated lead by a lead tolerant strain of *Rhizopus arrhizus* (*Rhizopus arrhizus* M1) were concentrated on the cell wall of the fungus (Table 1). Concentration of accumulated cadmium in the cell wall of *Aspergillus niger* AB_{10} was 65.82±4.28 % after optimum incubation period of 8 days in a synthetic medium containing cadmium at a concentration of 4 µg/ml. The value was significantly higher ($p<0.01$) than the cell wall content of cadmium (56.17±2.55 %) of wild type strain of *Aspergillus niger* after bioaccumulation of the metal (Table 1). The next highest concentration of the metal was found in the cytosolic fraction (28.87±3.26 %). The rest of the metal was distributed in the mitochondrial and nuclear fractions with greater amount present in the mitochondrial fraction (Table 1).

Table 1.

Distribution of cadmium in Aspergillus niger and lead in Rhizopus arrhizus in different sub-cellular fractions after bioaccumulation from aqueous culture medium. Incubation period was 8 days for Aspergillus niger and 5 days for Rhizopus arrhizus. Results are expressed as mean (n=4) ± SD.

Microorganism	Strain	Per cent (w/w) metal in cell-wall fraction	Per cent (w/w) metal in cytosolic fraction	Per cent (w/w) metal in mitochondrial fraction	Per cent (w/w) metal in nuclear fraction
Aspergillus niger	AB_{10}	65.88±4.28*	31.87±3.26*	1.52±0.18*	0.35±0.06
	Wild type	56.16±2.55	24.35±2.17	1.05±0.12	0.44±0.08
Rhizopus arrhizus	M1	61.41±4.07*	27.33±3.61*	0.88±0.06	0.26±0.04
	Wild type	52.40±3.85	21.13±2.66	0.74±0.08	0.33±0.03

*$p<0.01$, compared to wild type strains (One-way ANOVA followed by Dunett's post-hoc test)

In case of *Rhizopus arrhizus* M1, concentration of accumulated lead in the cell wall was 75.51±4.07 % after optimum incubation period of 5 days in a synthetic medium containing 1 µg/ml of lead. The value was significantly higher ($p<0.001$) than the cell wall content of lead (59.44±3.85 %) of wild type strain of *Rhizopus arrhizus* after bioaccumulation of the metal (Table 1). The next highest concentration of the metal was found in the cytosolic fraction (19.33±5.61 %). The rest of the metal was distributed in the

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mitochondrial and nuclear fractions with greater amount present in the mitochondrial fraction (Table 1). Metal bioaccumulation characteristics in the two fungi thus follow similar pattern.

To prove that metal bioaccumulation was energy dependent, metabolic inhibitors was added to the synthetic culture medium at specific interval of time during growth. It was observed that metabolic inhibitors inhibited metal bioaccumulation, which was reflected in their metal bioaccumulation capacities (Figs 1 and 2). Bioaccumulation ceased as and when the inhibitors were added to the culture medium, indicating involvement of cellular energy during metal bioaccumulation. The pattern was identical for both the fungi.

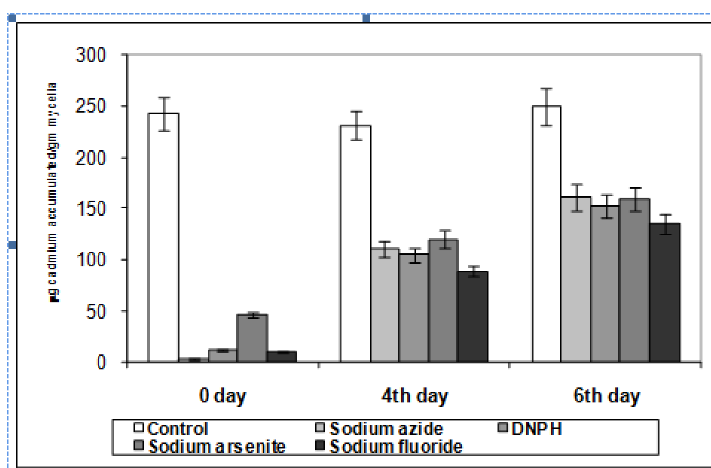

Fig. 1 ***Effect of metabolic inhibitors (added at a concentration of 10^{-3} M on different days of incubation) on bioaccumulation of cadmium by Aspergillus niger AB_{10}. Results are expressed as mean (n=4) ± SD.***

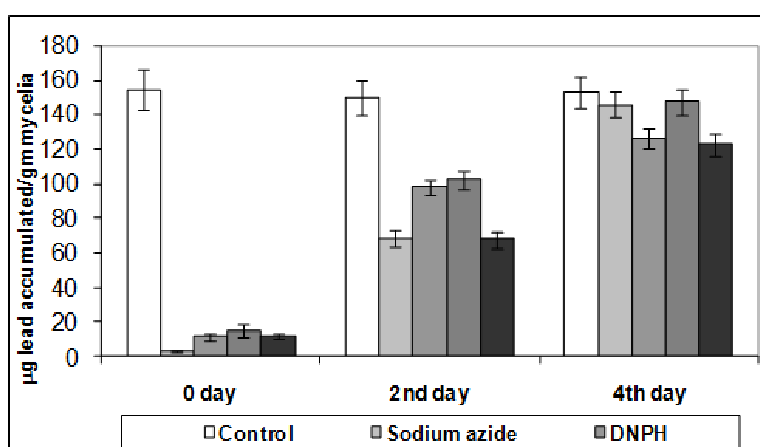

Fig. 2 ***Effect of metabolic inhibitors (added at a concentration of 10^{-3} M on different days of incubation) on bioaccumulation of lead by Rhizopus arrhizus M1. Results are expressed as mean (n=4) ± SD.***

It was also observed that leaching of the mycelia of the fungus with EDTA after bioaccumulation of metals could be successfully used for recovery of the accumulated metals. Treatment of the mycelia of *Aspergillus niger*

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AB_{10} with EDTA released 65.30% of the bound metal whereas 65.50% of the bound metal from the wild type strain was released (Table 2). In case of *Rhizopus arrhizus*, lead released by EDTA treatment was 81.00%, which was similar to the amount of metal released from the wild type strain (Table 2). Since, metal adapted strains accumulated greater amounts of metals, the amount of recovered metal after EDTA leaching would also be greater than the wild-type strains. It could be easily assumed from such observations that fungus adapted to higher concentrations of metals could be effectively used for metal bioaccumulation followed by recovery from solutions containing economically important metals.

Table. 2

Recovery of accumulated cadmium from cell wall fraction of Aspergillus niger and lead from cell wall fraction of Rhizopus arrhizus by treatment with EDTA. Incubation period was 8 days for Aspergillus niger and 5 days for Rhizopus arrhizus. Results are expressed as mean (n=4) ± SD.

Microorganism	Strain	Amount of accumulated metal in whole mycelia (µg/g)	Amount of metal in cell-wall fraction (µg/g)	Amount of metal recovered by EDTA treatment (µg/g)	Per cent of metal recovered
Aspergillus niger	AB_{10}	243.20±18.17	160.27±11.33	104.13±8.68	64.97
	Wild type	186.72±10.52	104.94±6.55	68.21±3.26	65.00
Rhizopus arrhizus	M1	154.41±11.64	94.82±8.39	74.68±5.15	78.76
	Wild type	112.76±8.37	59.09±4.45	45.50±3.36	77.00

DISCUSSION

It has been observed, in the present study, that maximum amount of accumulated cadmium by wild type as well as a cadmium tolerant strain of *Aspergillus niger* (*Aspergillus niger* AB_{10}) and maximum amount of accumulated lead by wild type as well as a lead tolerant strain of *Rhizopus arrhizus* (*Rhizopus arrhizus* M1) were concentrated on the cell wall of the fungus. The results indicated that the cell surface functional groups of the fungus might act as ligands for metal sequestration resulting in removal of the metals from the aqueous culture media. Such activities in case of fungus have been reported in the literature during metal bioremoval[12]. However, due to active metabolism of the fungus during growth in presence of the metals, some amount of metal has been internalized and resided in the cytosol. It was interesting to note that the accumulated metal in the cytosol of the metal-adapted strains of the two fungus were significantly higher than the metal contents in the cytosol of the respective wild type strains. This indicated possible involvement of the metabolically controlled processes for metal bioaccumulation. Rest of the metal was distributed in the mitochondrial and nuclear fractions.

To understand whether fungal metabolic processes are involved in metal bioaccumulation and subsequent internalization of the metal, effect of a few metabolic inhibitors on bioaccumulation was also studied. Sodium azide was used as inhibitor of mitochondrial electron transport chain, sodium fluoride was used as inhibitor of glycolysis, sodium arsenite was used as inhibitor of oxidative phosphorylation and DNPH was used as dissipater of mitochondrial membrane potential[13]. Addition of the metabolic inhibitors at different days of incubation also revealed interesting facts. Impairment in metal accumulation was observed as soon as the inhibitors were added in the incubation media, even after a few days of incubation. This clearly indicated a definite link between metal

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accumulation and cellular metabolism of the fungus.

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