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Original Research Article

Biosorption of lead from aqueous solution by a lead tolerant strain *Aspergillus foetidus* MTCC 8876: energy dependence of lead efflux

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Keywords

Aspergillus foetidus, Bioremediation, Lead biosorption, Lead tolerant strain, Bioleaching

A B S T R A C T

A lead-tolerant strain of *Aspergillus foetidus* was isolated from wastewater and toxic effects of lead (II) (Pb) on the same strain were investigated in respect of its growth. Potentialities of the strain for removal of Pb and efflux mechanism of intracellular Pb were studied. Results indicated that the strain could tolerate up to 3700 mg/L and 8500 mg/L Pb in liquid and solid Czapek-Dox (CD) media, respectively. The decrease in pH of the spent media supports the view that the strain can overproduce organic acid under lead stress, which may be act as a first line defense mechanism against lead toxicity. The membrane permeability of the fungal cell was also hampered due to lead toxicity and electrolytic leakage took place. Through this process the strain could also efflux the toxic metal from the cellular environment via chelation. The strain was found to be efficient in removing Pb maximally from liquid growth medium while the strain was growing in the same medium containing 200 mg/L Pb. The strain showed removal of Pb significantly at higher Pb stress (1000 mg/L) condition also. The Pb efflux mechanism of the strain was found to be energy dependent. These data indicated that the strain could show potentiality for acting as a suitable candidate for Pb bioremediation from Pb contaminated aqua-environment.

Introduction

Rapid urbanization and industrialization have led to increased deposition of heavy metals into the environment. Heavy metals that have been identified in the polluted environment include As, Cu, Cd, Pb, Cr, Ni, Hg and Zn (Lone et al., 2008). Among these, Pb is extremely toxic, which makes it a hazardous environmental pollutant (Mouflih et al., 2006). Mining, smelting activities, disposal of municipal sewage and industrial wastes, together with usage of leaded gasoline cause widespread Pb pollution (Peng et al., 2005).

Major adverse impacts of Pb pollution on public health can be as follows - it causes brain and nervous system damage, and particularly impairment of mental development in children; interference of reproductive system including effects such as premature baby and low births; damage of circulatory system such as decrease in O_2 absorption and increase in blood pressure

(Kalra et al., 2000). It can also interfere with the production of oxygen carrying components of blood called haemoglobin, kidneys malfunctioning and it may also lead to respiratory tract, kidney, lung and stomach cancer (Kane and Kumar 1999). The effects of lead on animal are found to be similar to those found in humans, with effects on blood, kidney, nervous, immune and cardiovascular system. Responses of plants to Pb exposure reflected stunted growth (Sengar et al., 2008) reduction in photosynthesis (Xiao et al., 2008), and inhibition of several enzymes (Sharma and Dubey, 2005).

Conventional physico-chemical elimination methods of heavy metal from aquatic environment as well as from soil such as electrochemical treatment, ion exchange, precipitation, osmosis, evaporation and sorption (Gupta and Rastogi, 2008) of heavy metal from waste stream are either much expensive as well as not much efficient (Wang and Chen, 2009; Kadirvelu et al., 2002). Nowadays biosorption is an attractive and economically feasible option for the treatment of effluents contaminated with heavy metals. Biosorption is an innovative technology that employs inactive and dead biomass for the recovery of heavy metals from aqueous solutions (Romera et al., 2007). As an alternative of traditional methods, this technique is found to be very promising showing remarkable as well as attractive results against the bioremediation of heavy metals by biosorbents or by biomass from various microbial sources, moss, aquatic plants (Lodeiro et al., 2005; Sari et al., 2007; Martins and Boaventura, 2011) and it can be considered as more efficient and cost-effective metal-removal biosorbent technique. Some hyper-tolerant strains of *Aspergillus* sp. isolated from Pb-free soil, was found to volatilize Pb from liquid medium up to a significant extent. Several *Aspergillus* species have also been found to be efficient in bioleaching of several compounds of heavy metals (Aung and Ting, 2005; Santhiya and Ting, 2006).

The effects of both biotic and abiotic stress on the antioxidant systems induce oxidative stress that produces ROS in the cell (Shützendübel et al., 2001). Heavy metals like lead toxicity also induce the production and accumulation of toxic oxygen species such as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). These reactive oxy radicals have been found to cause a major oxidative damage to cell permeability by hampering the functional groups of lipids, proteins and nucleic acids. Pb^{2+} ions also were found to be responsible for alterations of membrane integrity in different cellular systems. Lead induced membrane lipid peroxidation causes membrane damage which was followed by electrolyte leakage (Quartacci et al., 2001) which may be another mechanism to efflux the toxic metal outside the cell.

The unique cellular structure and inimitable antioxidant mechanism of fungi makes it a prime tool for bioremediation. Generally low molecular weight organic acids released by different plants, bacteria and fungi were found to be enhanced under stress conditions. Among them oxalate, ascorbate, citrate and malate are the strongest metal chelators (Jones, 1998). Gadd (1993) reported organic acids released extracellularly from fungi, was associated with their metal tolerance capacity by extracellular chelation of metal. Ascorbic acid, present in cell wall was reported to be the most ubiquitous soluble antioxidant in plant as well as in fungi, and is vital for reducing substrate for H_2O_2 detoxification (Singh and Sinha, 2005). There were several reports that ascorbic acid can serve as a free radical scavenger against O_3 and ROS (El-Khatib, 2003) generated by metal stress that cause pervasive damage to membranes and associated molecules. So ascorbate performs a

first-line defense system against ROS and super oxides.

Not only ascorbic acid, oxalic acid was also found to play a vital role in metal detoxification by precipitation of metal ions outside of the fungal cell, or by formation of metal–organic acid complex in a form of metal oxalate or crystal oxalate or by solubilizing the metal (Clausen and Green, 2003). In case of copper tolerant wood rot fungi it was reported (Sutter and Jones, 1985) to produce insoluble form copper oxalate in inert form to detoxify copper based wood preservatives.

In the present study, a lead tolerant strain of fungus, *Aspergillus foetidus (A. foetidus* MTCC 8876) has been used to evaluate its potentiality for bioremediation of Pb from Pb-contaminated liquid media. The effects of metabolic inhibitors on Pb uptake were conducted to explicate the energy dependence of Pb uptake by the Pb resistant strain. It is well known (Zhan, 2012) that metabolic inhibitors like Vanadate and 2,4-dinitrophenol suppress the electrical response for metal uptake, and it is assumed that plasma membrane H^+–ATPase is involved in the establishment of an electrochemical proton gradient acting as a driving force for active uptake. But this type of metabolic inhibitors inhibits the activity of plasma membrane H^+–ATPase. For that reason metal uptake by the strain could be altered. Therefore effects of metabolic inhibitors on Pb uptake or efflux by the fungal cells were also studied in order to find out the scientific reason of Pb uptake or efflux by the test strain.

Materials and Methods

Samples

Sewage sediment sample was collected from the flowing sewage water of treatment center, Kalyani, West Bengal, India, using a sediment grab sampler. The sample was preserved at 0°C until use. The sample was diluted serially with a sterile 145 mM NaCl solution and thoroughly shaken. To prepare pure culture the conidia of the preserved strain was taken in sterile water containing one/two drops of Tween 80 and shaken vigorously. The properly diluted sample was used to spread onto solid Czapek Dox (CD) plates containing 1000mg/Lt $Pb(NO_3)_2$. The fungus *Aspergillus sp.* isolated from wastewater treatment center, Kalyani was identified as a lead (Pb^{+2}) tolerant strain by supplementation experiments with high concentrations of Pb and finally it was identified as *Aspergillus foetidus* MTCC8876 by The Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Isolation of microorganisms

The isolation and enumeration of microorganisms were carried out in solid CD medium as described by Raper and Thom (1949), that contained (per liter): KH_2PO_4 (1g), $NaNO_3$ (2g), $MgSO_4$ (0.5g), KCl (0.5g), $FeSO_4$ (0.01g), $ZnSO_4$ (0.01g), and glucose (40gm). Here glucose-1- phosphate was used instead of KH_2PO_4 as phosphate source because lead phosphate precipitation was formed in presence of inorganic phosphate in the medium. The pH of the medium was adjusted to 8 before autoclaving. The medium was solidified with 2% agar as solid CD medium (CDA). Streptomycin was added to the medium for arresting the bacterial growth. All of the plates were allowed to incubate at 30°C in an incubator for 72 h for the fungal growth. The best grown fungal with black conidia was primarily identified as high Pb(II) ion tolerant strain, *Aspergillus foetidus* MTCC8876 by The Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH),

Chandigarh, India and the same was preserved in CDA slant or CD containing 1000 mg/Lt $Pb(NO_3)_2$ for further purification. Slant cultures were routinely sub-cultured every 1 month prior to experimental use on the same medium; 8 day old spore suspension was used as inoculums.

Preparation of fungal biomass

The *Aspergillus* strain was grown aerobically in the shake flask method. Liquid CD broth was used for the study of growth of fungus. 100 ml of CD medium was transferred into a series of 500 ml conical flasks; the flasks were cotton plugged and autoclaved. After cooling down the CD medium to room temperature, equal amounts of streptomycin were added to the experimental and control flasks. Five different concentrations of Pb (II), 200, 400, 600, 800 and 1000 mg/L were added separately to growth media. The conical flasks were then inoculated with Spore suspension of the test strain *A. foetidus* spores (10^{10} conidia/L) and shaken at 175 rpm at 32ºC in an orbital shaker. Biomass was harvested after 96 h growth period, filtered and washed with de-ionized water. Biomass was kept at -20ºC until used.

Microscopic studies of the fungus

Lactophenol–cotton blue staining technique was followed to stain the freshly grown mycelia and the morphology of the fungal strain was examined under phase contrast microscope.

Changes in pH of spent media

Before autoclaving the pH of the medium was adjusted to 8 but after autoclaving the pH of the CD broth was found to be maintained at 5 which were followed by the addition of lead for different treatment group. The media were inoculated with fungal spore suspension of the test strain *A. foetidus* spores (10^{10} conidia/L) and biomass was harvested after 96 h growth period. The ph of the spent media was measured for each treatment group.

Determination of Oxalic acid (OA) content

Oxalic acid (OA) was determined by the following method of Xu and Zhang (2000) with some modifications. 0.2 g of fresh fungal mycelia (grown at different metal concentrations) for each sample was taken and ground in a mortar and pestle with 0.6 g of alumina and extracted with 4 ml of 50 mM ice-cold phosphate buffer (pH 7.0) at room temperature. The suspension was centrifuged at 3,000g for 20 min at room temperature and the supernatant was collected. The reaction mixture consists of 50 μL sample (or standard OA solution), 27.5 μL (1mM) bromophenol blue (BPB), 49.5 μL (1M) sulfuric acid (H_2SO_4), 44 μL (100mM) potassium dichromate ($K_2Cr_2O_7$), and 1.2 mL MilliQ water. The mixture was vortexed and incubated in a water bath at 60°C for 10 min, the reaction was quenched with 110 μL 2 M sodium hydroxide (NaOH).

The absorbance was read at 600 nm against a blank (MilliQ water) in a spectrophotometer. OA concentration was expressed in mg/100 g FW by comparison with an OA standard curve (0.1–10 μg/mL) and absorbance of the reaction mixture was measured at 600 nm by which the concentration of oxalic acid was estimated.

Determination of total ascorbic acid content (AA) content

Total ascorbate was analyzed following the method of Hodges and Lester (2006) using L-ascorbic acid as a standard. 0.2 g of fresh fungal mycelia (grown at different metal concentrations) for each sample was taken and ground in a mortar and pestle with 0.6 g of alumina and extracted with minimum required volume of ice-cold, freshly prepared

5% (w/v) *m*-phosphoric acid. The homogenate was then centrifuged at 10,000 g for 15 min at 4°C. Total ascorbate was determined by initially incubating 100 µL supernatant, 500 µL (150mM) KH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA, and 100 µL (10mM) dithiothreitol (DTT) at room temperature for 50 min. Then 100 µL of 0.5% (w/v) N-ethylmaleimide (NEM) was added to remove excess DTT. In order to develop color in reaction mixtures, reagent solutions were added in the following order 400 µL 10% (w/v) trichloroacetic acid (TCA), 400 µL 44% *o*-phosphoric acid, 400 µL 4% (w/v) α-α1-dipyridyl and 200 µL 30 g/L ferric chloride ($FeCl_3$). The reaction mixtures were incubated at 40°C for 60 min in a shaking water bath and absorbance was read at 525 nm. The results were expressed as µmol/g FW.

Electrolytic leakage

Electrolytic leakage of the media was measured according to the method described in IPPAS (1999). 0.1 g of fresh fungal mycelia (grown at different metal concentrations) for each sample was taken in a conical flask with 20 ml distilled water. The flask was shaken for 24 hours. Now the conductance of the sample was measure. Now the same flakes were autoclaved and after cooling the conductance were measured again. Relative conductivity (RC) was calculated according to the formula % EL = Ci/Cf x100, where Ci is the conductivity of the vacuum-treated samples and Cf is the conductivity of the heat-killed samples.

Effects of metabolic inhibitors

In order to study the effects of metabolic inhibitors, e.g., ouabain, vanadate $(VO_4)^{3-}$, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP), the test strain was grown in liquid CD medium containing 200 mM Pb. After 96 h growth, mycelia was harvested and washed repeatedly with de-ionized sterile distilled water in order to ensure that least or no Pb is attached to the mycelial surface. Equal amount of mycelia was then incubated in de-ionized distilled water along with sub-lethal concentrations **o**f the metabolic inhibitors. After 72 hrs incubation, spent media were taken and used for AAS studies to measure the Pb efflux in order to assess the role of these metabolic inhibitors on Pb efflux from the cells of the test strain.

Statistical analysis

All determinations were carried out in three replicates in each case. Statistical analysis was done by one-way ANOVA followed by post-hoc multiple comparisons by Duncan's method. The difference was considered as significant when $P<0.05$.

Results and Discussion

Effect of Pb on growth of the strain

The toxic effect of Pb (II) on the test strain was detected by analyzing the dry weight of mycelia grown in liquid CD medium and it was found that the growth of the strain was slightly stimulated in liquid CD medium at 200 mg/L Pb treatment compared with the growth of the strain in control CD medium only and then the growth of the strain was found to be decreased gradually with further increase in Pb concentration. Growth was severely stunted at 1000 mg/L Pb treatment, resulting in 75% growth reduction in comparison to the control. In solid CDA plates, visible growth was found up to 8500 mg/L Pb treatment. However, colony diameter gradually decreased with the increase in Pb treatment (Figure 1 and 2).

Microscopic studies revealed that sporulation capability of the strain gradually decreased with the increase in Pb treatment as reflected in Figure 3. However, significant sporulation was noted up to 600 mg/L Pb treatment.

Changes in pH of spent media

The Initial pH of the CD broth before inoculation was maintained at 5.For the control group the pH of the spent media was found to be changed from 5 to 2.99 after 96 hour growth (Figure 4). So it could be assumed that the strain was probably capable of producing organic acids which accumulated in the growth media and the pH of the media declined. With the increase of Pb treatment the pH of the spent media were found to be decreased. For the 200 mg/L treatment group pH of the spent media was decreased by more than 1 unit (3.99 to 2.60) from that of the control group. For 1000mg/L treatment group the pH of the spent media decreased by more than 2 units from that of control group (3.99 to 1.14)

Changes in Oxalic acid (OA) content

The Oxalic acid content Of the test strain was found to be gradually increased with increase in Pb(II) content. It was observed that OA content was maximum at 1000 mgL^{-1} that is almost about 4.45 fold greater than that of control (Figure 5.).

Changes in ascorbic acid content

The total ascorbate content was measured in test strain. It was noticed that there was a linear increase in ascorbic acid concentration .At 1000 mgL^{-1},(here highest Pb treatment group) AO concentration was found to be increased almost 87.25% higher with respect to control (Figure 6).

Changes in electrolytic leakage

The toxic effect of Pb^{2+} treatment on test strain resulted in significant changes in electrolytic leakage as metal concentration increased. After 24 h of exposure the electrolytic leakage for the 200,400, 600 and 800 mg L^{-1} Pb treatment groups was found to be increased by 1.38, 1.81, 2.16, 2.50 fold respectively in comparison to control (Figure 7). Maximum extent of electrolytic leakage was found at 1000 mg/L^{-1} Pb treatment with increase by almost 2.77 fold in comparison to control.

Effects of metabolic inhibitors on Pb uptake / efflux

The effects of metabolic inhibitors like ouabain, orthovanadate ($VO4^{3-}$), CCCP and 2,4-DNP were studied to elucidate the energy dependence of Pb efflux by the cells of the test strain.

In case of Vanadate the IC_{50} (half maximal inhibitory concentration) was found to be 5 mM for the test strain. Efflux of Pb decreased significantly in presence of vanadate in efflux experiments (Table 1). After 24 h, efflux in presence of vanadate was found to be decreased by almost 67% with respect to control (no metabolic inhibitor).

The IC_{50} of 2,4-DNP was found to be 13 μM for the test strain. Efflux of Pb decreased in presence of 2,4-DNP. The efflux values decreased by almost 75%, 69% and 66% after 24, 48 and 72 h, respectively with respect to control (Table 1). From these data it may be concluded that Pb efflux mechanism of the test strain is an energy requiring process and is dependent on the activity of plasma membrane H^+ ATPases. The IC_{50} of CCCP was found to be 7 μM for the test strain. Pb efflux decreased significantly in presence of CCCP (Table 1).

The IC_{50} of ouabain was found to be 13 μM for the test strain. In the present study, the introduction of ouabain caused a marked decrease in Pb efflux. The efflux values were found to decrease by almost 85%, 83% and 82% after 24, 48 and 72 h, respectively with respect to control (Table 1).

On the basis of results obtained in the present work, it was found that the fungal strain can withstand such a high concentration of lead and the growth of the biomass was slightly stimulated at 200mg/L Pb concentration as found in growth stimulation in *Aspergillus nidulans* (Cooley et al., 1986; Guelfi et al., 2003) and *Aspergillus niger* (Mukherjee et al., 2010) in presence of toxic substances like cadmium. There is a possibility of growth stimulation at low concentration of lead as it may form complexes with constituents of the culture medium which could allow essential trace elements to become available to the fungus. Alternatively, Pb may provoke an Arndt–Schultz effect on the test fungal strain. Results in the accumulation of toxins in non-lethal concentrations at the cell surface is to cause alteration in the cellular permeability and this in turn leads to a free flow of nutrients within the cells and thus metabolic activity of the strain increases (Babich and Stotzky, 1980; Ahonen-Jonnarth et al., 2004).

It was found that the pH of the spent media was gradually decreased turning the growth media more acidic, at highest concentration Pb, the pH was found to be decreased more than 2 unit in comparison to control. This result suggested that the increase in Pb content in the medium induced the fungi to over produce organic acids extracellularly which may chelate the metal into its insoluble form to convert the toxic metal into its rather nontoxic form.

In this study it was found that the pH of the spent medium after growth was declined with increase in Pb treatment which may be due to the overproduction of organic acids, which in turn act as good chelating agents for heavy metals like Pb. *Aspergillus* spp has been known to produce organic acid commercially such as ciric acid, oxalic acid, gluconic acid, ascorbic acid (Angumeenal et al., 2002; Baby et al., 1996).

In this present study the oxalic acid content was found to be gradually increased with increase in Pb treatment. From our previous work (Chakraborty et al., 2012) SEM and energy dispersive X-ray spectroscopy of lead induced fungal mycelia confirmed the presence of crystals of Pb along with carbon (C) and oxygen (O). Determination of molecular formula has been revealed that those crystals may be lead oxalate crystals. From these findings, it is evident that the strain was capable of converting Pb (II) ions into insoluble lead oxalate crystals and to retain those crystals on its mycelial surface. Same results were depicted by Clausen and Green (2003) and Jarosz- wilkolazka et al. (2006) where under Cu and Cd toxicity over production of oxalate was found in brown rot and white rot fungi. However in this study decrease in pH of the spent medium supports this finding. The production of water insoluble lead oxalate crystals by this strain may be definitely an efficient way to remove the toxic metal from the cellular environment.

Ascorbic acid is a well known small secondary antioxidant molecule which directly works upon heavy metal induced superoxide radicals (O_2^-), singlet oxygen (O^-) or hydroxyl radical (OH^-) (Singh and Sinha 2005) to neutralize them. It also can reduce transition metals converting them to a pro-oxidant character (Poljšak et al., 2005). Ascorbic acid performs a fist line defense system to organism intoxicated by heavy metals. In this study it was observed that the total ascorbic acid content was gradually increased with increase in Pb content in growth media. Hence from this study it was evident that the over expression of ascorbic acid may be induced by Pb toxicity.

In this study the electrolytic leakage was found to be increased with increase in Pb treatment. Heavy metal generated ROS directly damage the membrane permeability

of intoxicated organism causing a distortion of the lipid bilayer (Bačkor et al., 2007) of the membrane proteins and disruption of the membrane by the Fenton reaction, which in turn leads to electrolytic leakage. Heavy metals like lead induce peroxidation to poly-unsaturated fatty acids present in cell membrane as well as in the cytosolic environment and produce different types of cytotoxins like malondialdehyde (MDA)—a secondary lipid peroxidation product (Biswas et al., 2008). Increased lipid peroxidation was also reported in this strain in presence of different Lead treatment (Chakraborty et al., 2012).

As reported in the earlier paper (Chakraborty et al., 2012) the Pb removal efficacy of the strain, *Aspergillus foetidus* was found to be maximum at 200 mg/L treatment (94.57 %) where as at the 400 & 600 mg/L lead treatment groups, the strain could remove almost 93.45% & 87.79% P,b respectively and at 1000 mg / L treatment group, although little growth of the strain was found about 28.67% of Pb was removed by the same fungal biomass.

In this present study the effects of the following metabolic inhibitors have been elucidated to prove that the Pb accumulation by the strain could be biphasic. Though the surface accumulation of the metal by the test strain is an passive process there is no need of energy but the transport of metal is strictly an energy dependent mechanism. So in presence of metabolic inhibitors the metal binding capacity of the strain was found to be not affected but the influx or efflux mechanism was found to be seriously altered.

In presence of Vanadate the efflux of the metal was found to be decreased. This may be due to the fact that the plasma membrane H^+ ATPases of the test strain are responsible for removing Pb from the fungal cells as a part of cellular detoxification mechanisms. Inhibition of the plasma membrane H^+ ATPases causes Pb to remain inside the fungal cells and less efflux values are obtained. Hence efflux of Pb from the fungal cells may depend on the activity of plasma membrane H^+ ATPases. Vanadate acts as an inhibitor of the plasma membrane H^+ ATPase in plant and animal cells. Inhibition by vanadate has become an important criterion for identifying ion-translocating ATPases associated with plant, fungal, and animal cell membranes and thus has become an important tool for understanding the biochemistry of membrane (Bennett et al. 1983; Gallagher and Leonard (1982) .

The application of 2,4-DNP, a common metabolic inhibitor believed to inhibit the formation of ATP by uncoupling oxidative phosphorylation. In presence of 2,4-DNP the efflux values were found to be decreased by almost 75%, 69% and 66% after 24, 48 and 72 h, respectively with respect to the control. Hence it could be concluded that like vanadate, 2,4-DNP also acts as an inhibitor of the plasma membrane H^+ ATPase (Tripathi and Srivastava, 2007).

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an H^+ ionophore which dissipates the H^+ gradient and thus uncouples electron transport from ATP synthesis. It can transport protons into the cell without the participation of ATP synthase (Schlegel, 1986). In presence of CCCP, Pb efflux has been found to be decreased. Similar result has been observed in case of potassium efflux from maize roots (Baker, 1973). Hence it is clear that efflux of Pb from the cells of the test strain is an energy requiring process.

Ouabain is considered to be a potent Na^+/K^+-ATPase inhibitor (Farber et al., 1989; Morant-Avice et al., 1997). The large decrease in Pb efflux due to the blockage of Na^+/K^+-ATPase

pump may reflect its significant role in Pb efflux by the test strain. From these data, it may be concluded that Pb efflux from the cells of the test strain is mainly mediated by plasma membrane Na^+/K^+-ATPase and H^+ ATPases pumps and that the efflux process is energy dependent as inhibition of ATP synthesis leads to marked decrease in Pb efflux.

It has been reported that *A.foetidus* is an suitable strain for bioremediation of heavy metals like nickel (Le et al., 2006; Valix et al., 2000) and chromium (Prasenjit and Sumathi, 2005). However reports are scanty for removal of Pb using *A. foetidus*. But in our earlier work (Chakraborty et al., 2012) it has already been confirmed the presence of tightly bound Pb as insoluble crystals onto fungal mycelia from the analysis of the images obtained in Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopic (EDS) studies. In most of the reports, dead or immobilized biomass of microorganisms has been used to remove the metals from heavy metal media contaminated liquid media. However a microbial strain growing in highly heavy metal toxic situation, it bioaccumulate as well as biosorb heavy metals from the heavy metal contaminated liquid media which definitely indicated a leading role as a bioremediation tool.

Table.1 Pb efflux by *A. foetidus* mycelia (mg/gFW) in Czapek-Dox broth containing 200 mg/L Pb and metabolic inhibitors in sub-lethal concentrations

Time (hr)	Control	2.0 mM vanadate	5.0 µm 2,4 DNP	4.0 µm CCCP	9.0 µm Ouabain
24	**269.73 ± 21.9**	**89.88 ± 8.61**	**66.206 ± 5.45**	**73.837 ± 9.11**	**40.311 ± 3.15**
48	**381.197 ± 38.22**	**157.912 ± 17.57**	**119.713 ± 9.13**	**133.491 ± 16.77**	**62.231 ± 9.86**
72	**474.457 ± 45.32**	**215.02 ± 14.52**	**161.553 ± 23.97**	**174.375 ± 20.98**	**82.581 ± 9.05**

Efflux was measured from the Pb contents of the incubation media for efflux studies. Data were found to be significant at $P<0.05$

Figure.1 Colony diameter of *A. foetidus* strain grown in solid Czapek Dox media with different Pb(II) concentrations; □ control; ▲ 200 mg/L; ■ 400 mg/L; × 600 mg/L; + 800 mg/L; Δ 1000 mg/L. Data were found to be significant at $P < 0.05$

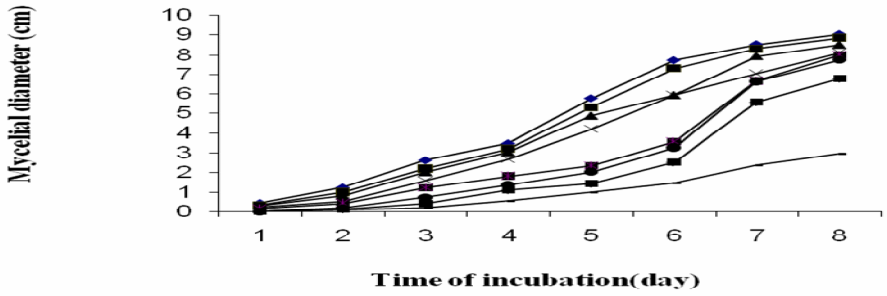


Figure.2 Fungal mycelia grown in CDA medium with different Pb concentrations; (a) control, (b) 200 mg/L Pb, (c)400mg/L Pb, (d) 600mg/L Pb, (e) 800mg/L Pb, (f) 1000mg/L Pb

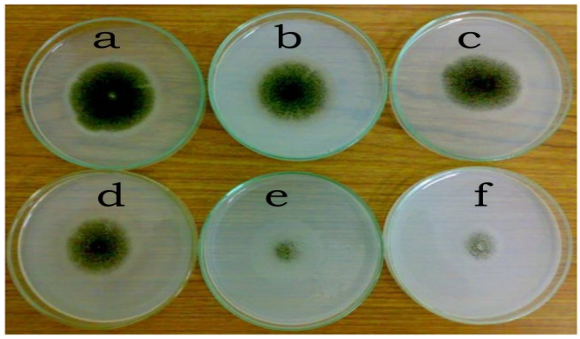


Figure.3 Microscopic studies of sporangiophore *of A. foetidus* grown in CDA medium with different Pb concentrations; (a) control, (b) 200 mg/L Pb, (c) 1000 mg/L Pb

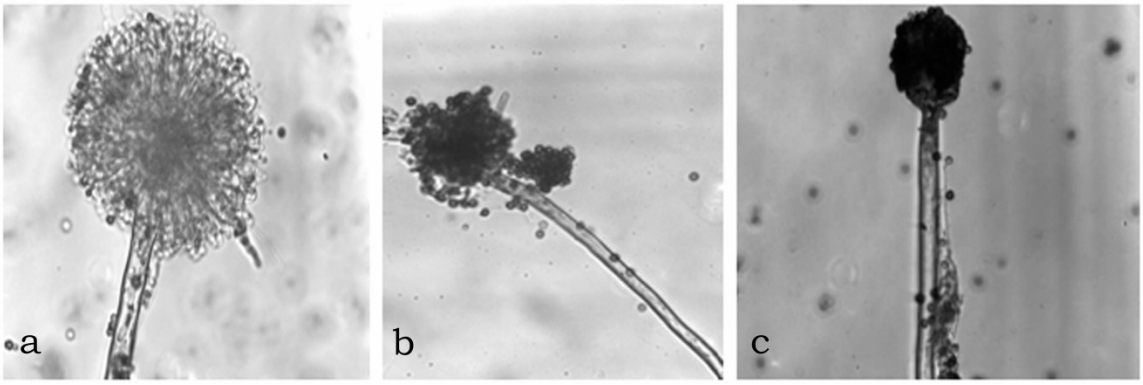


Figure.4 Changes in pH of the spent medium after 96 h growth in liquid CD broth with different Pb (II) concentrations. Data were found to be significant at P<0.05

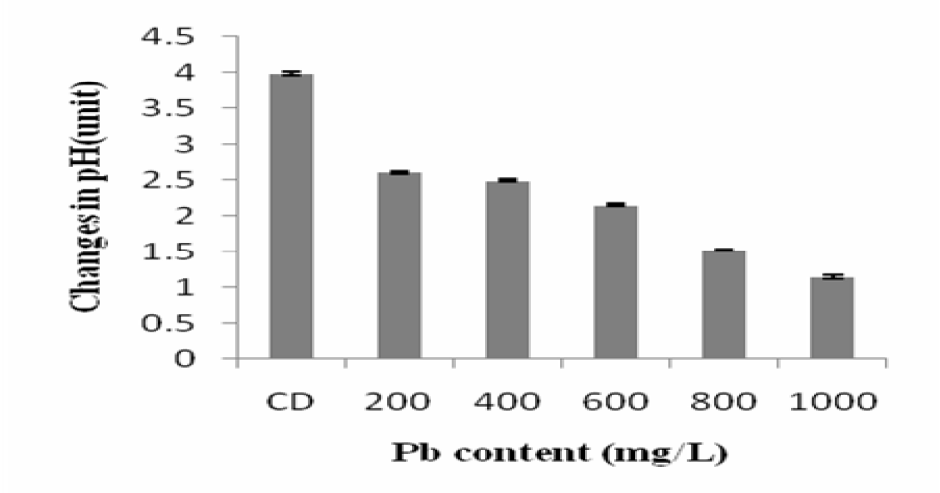


Figure.5 Changes in oxalic acid content of the strain in response to Pb (II) treatment. Data were found to be significant at $P < 0.05$

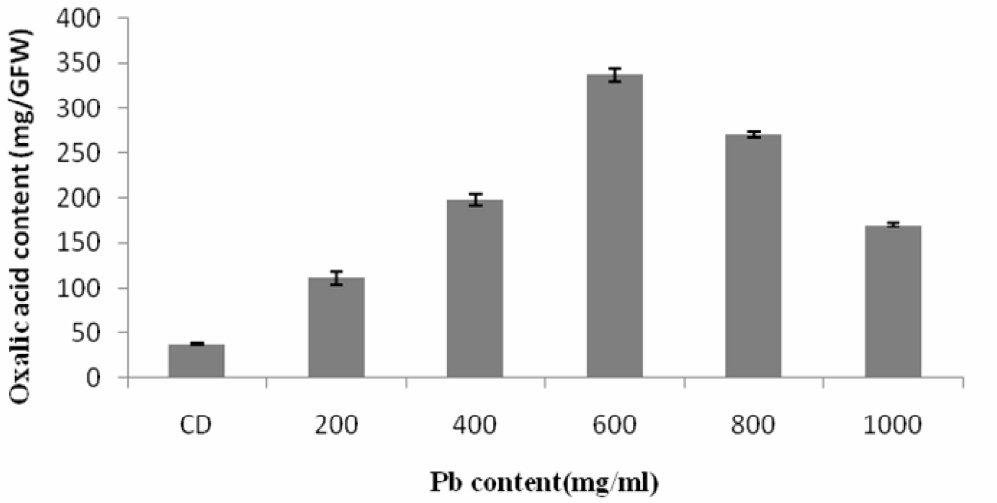


Figure.6 changes in Total ascorbic acid content of the strain in response to Pb (II) treatment. Data were found to be significant at $P < 0.05$

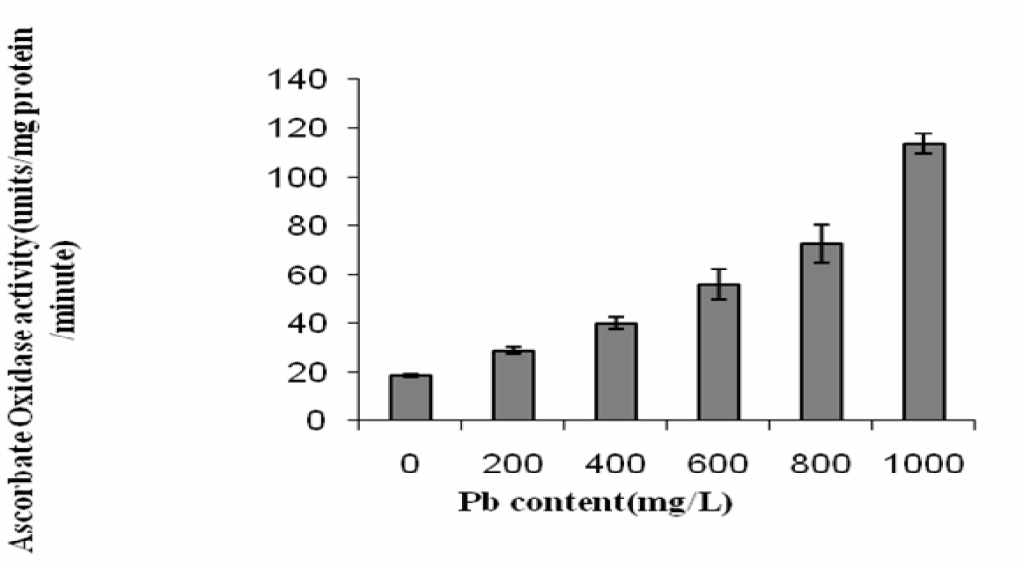
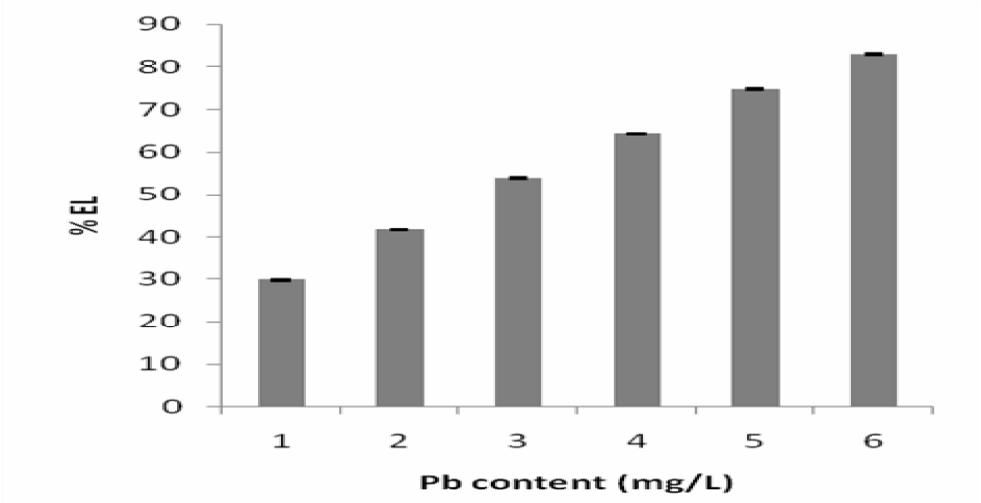


Figure.7 Extent of electrolytic leakage in spent medium in response to Pb (II) treatment. Data were found to be significant at $P < 0.05$



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