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Effect of lead on growth, protein and biosorption capacity of *Bacillus cereus* isolated from industrial effluent

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Abstract

A bacterial strain (*Bacillus cereus*) with the ability to grow under conditions of high concentrations of lead was isolated from the industrial effluent collected from Peenya Industrial Area, Bangalore. The effect of lead on growth, protein content and lead biosorption capacity of *Bacillus cereus* was investigated. The results revealed that with increase in lead concentration (100, 200, 300, 400 and 500 mg l^{-1}) there was a decrease in growth, protein content (10.6, 8.2, 6.7, 3.8 and 1.9 mg g^{-1} d. wt.) and lead biosorption (90.3, 57.8, 48.94, 31.3 and 22.24 %) *Bacillus cereus,* signifying toxic effect of lead on the bacterial strain. Plasmid DNA was isolated from *Bacillus cereus* to study its resistance mechanism. The size of the plasmid was approximately 33kb. Transformation results suggest that lead resistance gene may be present on the chromosomal DNA rather than the plasmid DNA as the transformants did not show lead resistance.

Key words

Bacillus cereus, Industrial effluent, Lead biosorption, Plasmid DNA

Introduction

Heavy metals are generally not abundant in biosphere. However, due to industrial activities and deliberate, as well as accidental discharge, these heavy metals are causing environmental pollution problem (Soltan *et al*., 2008). The concentration of lead in the atmosphere is of serious concern. The high input of lead is especially through automobile exhausts, mining and smelting (Jung *et al*., 2004; Poikolainen *et al*., 2004). Lead mobilized and released in the environment by human activities tends to persist indefinitely, circulating and eventually accumulating into the food chain, thus resulting in serious ecological and health problems (Malik, 2004; Coral *et al*., 2005). The heavy metals exert toxicity through five general mechanisms: displacing the essential metal ions from their native binding sites; blocking the essential functional groups of biomolecules such as proteins and enzymes; changing the conformation of biological molecules (i.e. proteins and nucleic acids); decomposing essential metabolites and changing the osmotic balance around the cells (Rajendran and Gunasekaran, 2007).

Pollution by heavy metals like lead affects every aspect of microbial metabolism and activity, including respiration, membrane transport and the synthesis and activity of ribosomes, in some cases resulting in death of the cells (Rathnayake *et al*., 2009). Selective pressures from metal containing environments have led to the development of resistance systems in microorganisms to virtually all toxic metals. Cervantes *et al*. (2001) reviewed the interactions of bacteria, algae, fungi and plants with chromium and stated that the presence of chromium in the environment has influenced certain selected microbial variants to tolerate high levels of chromium compound. According to Jjemba (2004), tolerance and resistance are distinct, the former referring to the ability of the organism in question to cope with the toxicity based on its intrinsic properties while the latter refers to the ability of the organism to survive metal stress by using some detoxification mechanism induced in direct response to the metal. They accumulate metals through various mechanisms including complexation, adsorbtion, precipitation and active transport into the cells. Because of a large surface to volume ratio, microorganisms provide a large contact area which can interact

with metals in the surrounding environment (Ledin, 2000). Alluri *et al.* (2007) carried out an extensive review on the biosorption of heavy metals, resulting in identification of some biomass types that show very promising uptake of metallic ions. While isolating six copper resistant bacterial strains from industrial effluents, Shakoori and Muneer (2002) found these bacterial strains were capable of adsorbing (50-80%) and accumulating (30-45%) Cu^{2+} inside their cells. A heavy metal resistant bacteria *Bacillus circulans* strain EB1 isolated from heavy metal contaminated soil was capable of biosorption of Mn, Zn, Cu, Ni and Co (Yilmaz, 2003). Congeevaram *et al.* (2007) studied bioaccumulation of Cr and Ni by heavy metal resistant fungi and bacteria isolated from soil samples of electroplating industry and evaluated their applicability for heavy metal removal.

Gram positive bacteria are known to possess high metal sorption capacities than Gram-negative cells. Carboxyl groups are the main agents in the uptake of heavy metals. The sources of these carboxyl groups are teichoic acids, associated to peptidoglycan layers of the cell wall as observed by Issazadeh *et al*. (2011). While working on a lead resistant *Bacillus* strain isolated from a slag disposal site, Pandey *et al*. (2011) found them to be capable of efficiently accumulating lead. Similar studies carried out by Singh *et al*. (2010) on protease producing halotolerant *Bacillus cereus* SIU1 strain isolated from a non-saline environment was also found to be resistant to heavy metals (As, Pb and Cs). Costa *et al*. (2001) studied the bioaccumulation of Cu, Zn, Cd and Pb by *Bacillus* sp., *Bacillus cereus, Bacillus sphaericus* and *Bacillus subtilis* and concluded that the best results were obtained by *Bacillus subtilis* and *Bacillus cereus*. Murthy *et al*. (2012) reported the biosorption capacity of *Bacillus cereus* through scanning electron microscopy and energy dispersive X-ray spectroscopy studies where in it was found that lead was adsorbed to the cell surface of the bacteria.

Resistance to metals in prokaryotes is mostly mediated by plasmids and is highly specific for a particular metal. Metal resistance systems have been found on plasmids of every bacterial group tested (Silver and Phung, 1996, 2005). However, some resistance, particularly for essential metals, is chromosome mediated. Plasmid mediated resistance to metals can be moved rapidly between cells within a population. In this manner microorganisms reduce the burden of carrying the genes for resistance to non-essential metals since they are only needed on certain occasions (Bruins *et al*., 2000).

The present study was undertaken to study the effect of lead on growth, protein content and lead biosorption capacity of *Bacillus cereus.* Besides, the study also aimed to investigate the resistance mechanism in *Bacillus cereus* by isolating Plasmid DNA.

Materials and Methods

Bacterial strain and growth curve : The bacterial isolate employed in the present study was isolated from the Vrishabhavathi River, which flows through Peenya Industrial area, Bangalore, carrying industrial effluent and urban sewage. Initially 60 lead resistant strains were isolated from the water samples out of which one isolate could tolerate very high concentrations of lead (500 mgl^{-1}). The isolate was identified and confirmed as *Bacillus cereus* (GenBank Accession Number: EF488087) at Bangalore Genei, Bangalore based on 16S rDNA data. The isolate was maintained by weekly subculturing on tryptone glucose extract agar and stored at 4°C.

Growth curve experiment was carried out as per the procedure described by Aneja (2001). 1 ml aliquot of *Bacillus cereus* suspension (24 hr old) was inoculated in 250 ml Erlenmeyer flask with 50 ml of lead free medium and incubated on a shaking incubator at optimum temperature (30°C) of the isolate at 120 rpm. Optical density was measured at 600nm after every 2 hr for 30 hrs. Then a graph was plotted between O.D. and time in hours for determining phases of growth of the isolate on lead free medium.

Analysis of growth and protein content : 1 ml aliquot of *Bacillus cereus* suspension (24 hr old) was inoculated in 250 ml Erlenmeyer flask with 50 ml of lead supplemented (100, 200, 300, 400, 500 mgl^{-1} $Pb(NO_3)_2$) medium and incubated on a shaking incubator at optimum temperature with 120 rpm shaking. Optical density was measured at 600nm after every 2 hr for 30 hr (Soltan *et al*., 2008).

In order to quantify the protein concentration of *Bacillus cereus*, the isolate was grown in the medium amended with lead (100,200,300,400 and 500 mgl^{-1}) along with a control. The protein content of *Bacillus cereus* was isolated and detected following the method of Lowery *et al*. (1951).

Study on biosorption capacity of *Bacillus cereus* : A batch equilibrium method was used to determine the biosorption capacity of lead by *Bacillus cereus* (Al-Garni, 2005). One ml aliquots of *Bacillus cereus* suspension (24 hr old) was inoculated in 100ml tryptone glucose extract broth medium containing different concentrations of lead (100, 200, 300, 400 and 500 mgl^{-1}). The Erlenmeyer flasks were incubated at 30°C in an orbital shaker at a speed of 120 rpm for 48 hrs. The flasks were taken out after 48 hr of incubation and were centrifuged at 10,000 rpm for 10 mins at 4°C. After centrifugation both supernatant and pellet (three times washed with NaOH 1N) were digested with HNO_3 67% and H_2O_2 (30% v/v), and metal concentration was determined by atomic absorption spectrometry. All experiments were conducted in triplicate.

Isolation of Plasmid DNA : Two flasks with 50ml Luria Bertani broth with one of them containing 500 mgl^{-1} of $Pb(NO_3)_2$ and the other control were inoculated aseptically with *Bacillus cereus* and incubated at 35°C for 24 hrs. Plasmid was isolated from both the flasks by Enzene Plasmid Mini – Prep Kit (Cat. No. EZRK18). The procedure was followed as described by manufacturer for

isolation of plasmid DNA.

DNA concentration was determined as follows : Plasmid DNA was diluted in phosphate-buffered saline (PBS) (1:100), and the concentration and purity of the extracted plasmids were determined spectrophotometrically. The optical density (OD) of the DNA was measured at 260 and 280 nm. The OD 260 allowed calculation of the DNA concentration in the sample, where an OD 260 of 1 corresponds to approximately 50 $\mu g ml^{-1}$ of double stranded DNA. The ratio of the OD 260 nm OD 280 provides an estimate for the purity of the DNA.

Plasmid DNA was separated by electrophoresis on a 0.7% agarose gel (w/v) at 50 volts overnight. 33500/24500 to 500 bp molecular marker was used in the gel as marker. The gel was stained with ethidium-bromide, visualized under UV trans-illumination and photographed.

An aliquot of plasmid DNA to be transformed (1µl) was transferred into a cold sterile 1.5 ml microcentrifuge tube and was placed on ice. Then 100 µl of competent *E.coli* XLI Blue cells were transferred into the microcentrifuge tube with the plasmid DNA, mixed carefully and kept on ice for 20 mins. After 20 mins, the tube was transferred to 42°C water bath for 90 sec and immediately placed on ice (Sambrook and Russell, 2006). The cells were spread plate on LB agar plate containing lead and tetracycline and incubated at 37°C overnight until colonies develop.

Results and Discussion

To study the effect of lead on growth of *Bacillus cereus*, the isolate was grown in medium with and without lead. Fig. 1 shows that the growth of *Bacillus cereus* was affected by the presence of lead in the culture media. The growth curves with and without lead treatment were determined for the time of incubation and optical density. Fig. 1 clearly shows the characteristic phases during the growth of culture. The control growth curve showed lag phase of 3- 5 hr and log phase of 12-22 hrs. After 24 hr, the O.D of the isolate remained constant, this was the stationary phase. Whereas, *Bacillus cereus* under metal stress of 100 mgl^{-1} of lead showed 3-10 hr of lag phase and 14-20 hr of log phase and after 24 hr it attained a stationary phase. In case of 200 mg l^{-1} lead concentration *Bacillus cereus* showed lag phase of 3-5 hr, and log phase of 9-18 hr and attained a stationary phase after 22 hr. A metal stress of 300 mg l^{-1} lead on *Bacillus cereus* showed a lag phase of 2-4 hr, 5-16 hr of log phase and after 22 hr it attained a stationary phase. A lead concentration of 400 mgl^{-1} showed a lag phase of 4-9 hr, 12-20 hr of log phase and after 24 hr it attained stationary phase. In case of 500 mg l^{-1} lead concentration *Bacillus cereus* showed a very slow growth rate. Hence, the results have amply proved that with increase in concentration of lead there is decrease in growth rate. The result is in accordance with the earlier studies carried out by Maldonado *et al*. (2010); Castillo-Zacarías *et al*. (2011) and Roane, (1998). The drastic decrease in growth rate might have occurred due to consumption of metabolic energy to resist metal, which are permeable to cell membrane and may enter into cell where its reduction occurs (Salnikow *et al*., 1992; Kawanishi and Hiraku, 1995; Sudgen and Wetterhahn, 1996). On treatment of *Bacillus cereus* with lead, cells need longer time to prepare themselves to grow in the stress condition and divide at very slow rate.

The result suggests that lead inhibits protein synthesis of *Bacillus cereus*. The protein inhibition percentage caused by lead was 26.7, 43.3, 53.7, 73.7 and 86.9% at 100, 200, 300, 400 and 500 mg l^{-1} lead respectively (Fig.2). Khan *et al*. (2007, 2010) reported a negative correlation between the amount of lead and the enzyme activities in soil bacterial community. Early reports suggest that some species of *Pseudomonas, Rhodobacter and Rhizobium* showed a decrease in total protein content when treated with different concentrations of Cd. The decrease in protein content could be due to the toxic effect of lead on the cellular metabolism (Soltan *et al*., 2008).

The *Bacillus cereus* could efficiently process lead from the medium. The concentration of lead remaining in the medium

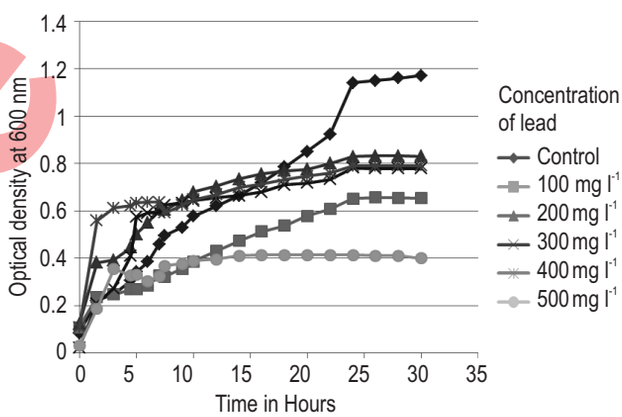

Fig. 1 : Effect of different concentration of lead on the growth of *Bacillus cereus*

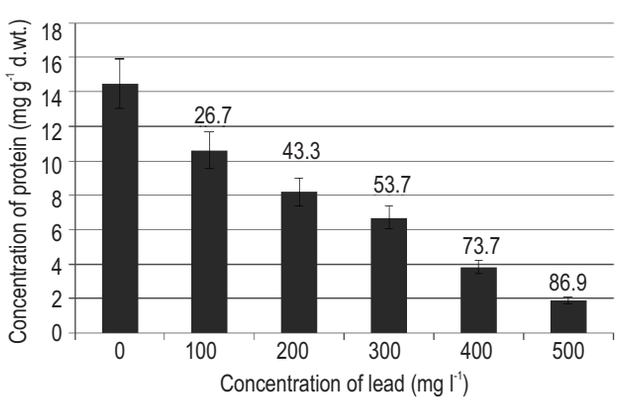

Fig. 2 : Effect of different concentrations of lead on the total protein content of *Bacillus cereus*. Values shown on bar is percent inhibition

was 9.7 mg l^{-1}, 84.4 mg l^{-1}, 153.2 mg l^{-1}, 274.8 mg l^{-1} and 388.8 mg l^{-1} for 100 mg l^{-1}, 200 mg l^{-1}, 300 mg l^{-1}, 400 mg l^{-1} and 500 mg l^{-1} respectively, thus indicating that *Bacillus cereus* reduces 90.3% of 100 mgl^{-1} lead, 57.8% of 200 mgl^{-1}lead, 48.94% of 300 mgl^{-1}lead, 31.3% of 400 mgl^{-1}lead and 22.24% of 500 mgl^{-1}lead from the medium after 48 hr (Fig.3).The result clearly illustrated that the percentage of lead uptake by *Bacillus cereus* decreased with increasing concentrations of lead. The reduction in metal sorption could be due to increase in electrostatic interactions involving sites of progressively lower affinity for metal ions (Al-Asheh and Duvnjak, 1995; Puranik and Pakniker, 1999). The data indicated that lead uptake by *Bacillus cereus* was chemically equilibrated and saturation was attained at a lead concentration of 400 mgl^{-1}. Thus, there was no increase in metal uptake as long as the binding sites were saturated by the metal ions.

Bacillus cereus showed (Fig.4) a well-defined plasmid above 33kb. Zolgharnein *et al*. (2007) reported that the frequency of occurrence of plasmids in heavy metal resistant bacteria was more than that in the common bacteria. The study also demonstrated that about 66% of heavy metal (Cd, Cu, Zn and Pb) resistant bacteria carried large (38-62kb) and/or small sized (4->2 kb) plasmids and the highest plasmid incidence (84.6%) was detected from industrial wastewater. There are reports illustrating plasmid encoded lead resistance (Taghavi *et al*., 2009) however in the present study when *E.coli* XLI Blue was transformed with the isolated plasmid transformant did not show lead resistance. This suggests that lead resistance gene may be present on the chromosomal DNA rather than plasmid DNA, which is in agreement with earlier reports of Taghavi *et al*. (2009); Raja and Selvam (2009); Gummersheimer and Giblin (2003) respectively.

Our studies revealed that with increase in lead concentration, the growth, protein content and lead biosorption capacity of *Bacillus cereus* decreased indicating the toxic effect of lead. Owing to lead resistance and biosorption capacity, *Bacillus cereus* could be considered as a promising candidate for the removal of lead from industrial effluents. Further studies on

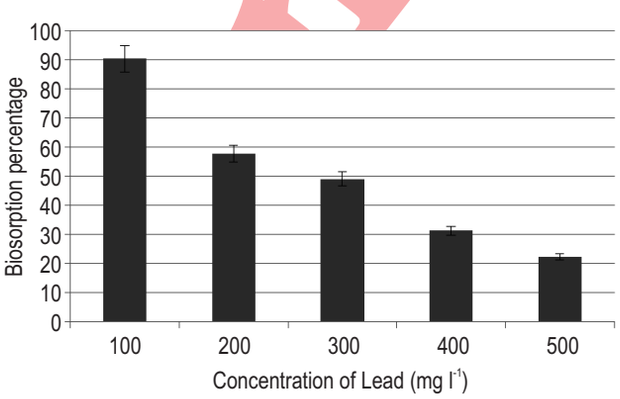

Fig. 3 : Effect of different concentrations of lead on the biosorption capacity of *Bacillus cereus*

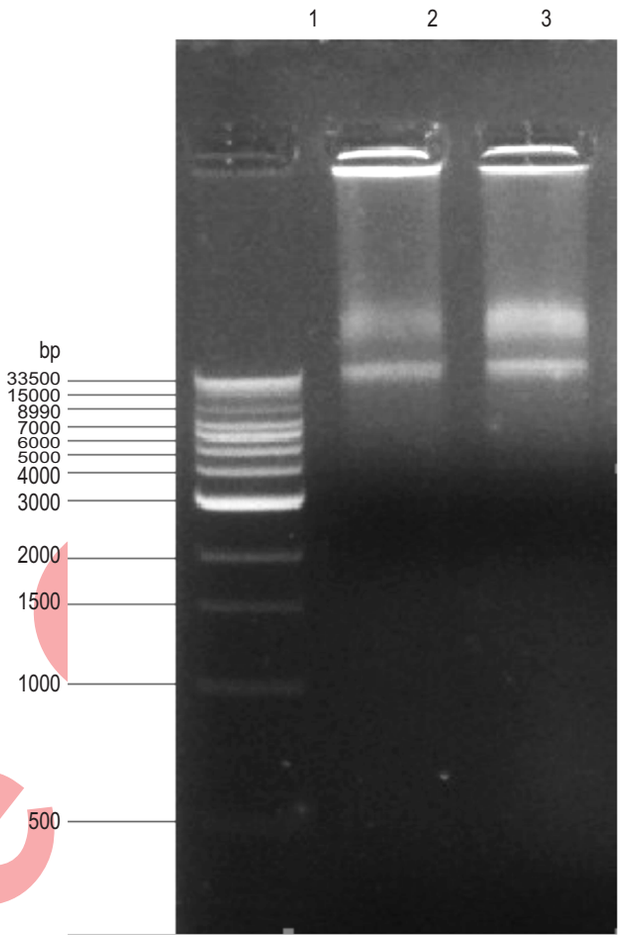

Fig. 4 : Gel photograph showing Plasmid DNA of *Bacillus cereus*: Lane 1-Supermix DNA, Lane 2 and 3- Plasmid DNA of *Bacillus cereus*.

mechanism of lead resistance and location of gene responsible for survival in high lead concentration are under investigation.

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