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Bacterial Hemoglobin Gene (*Vgb*) Transformed into *Escherichia coli* Enhances Lead-uptake and Minimizes It's Adsorption

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Abstract: In *Escherichia coli* transformed with bacterial hemoglobin gene (*vgb*) has been shown to increase lead (Pb) uptake, growth yield of the cells grown in LB containing different concentration of Pb. The maximum Pb biosorption of *vgb*-containing and parental cells were determined to be 51 and 30.8 ppm Pb g^{-1} biomass respectively. Using growth curves by liquid and solid media the inhibitory effect of Pb on parental strain was detected at lowest concentration (10 ppm). However, in *vgb*-containing cells, Pb was lethal at 100 ppm. The optimum aeration that required for the cells-containing hemoglobin gene (*vgb*) was lower than that of the same strain without *vgb* on a growth yield, Pb uptake and Pb adsorption basis. The quantity of the Pb uptake inside the cells appears to be independent on the concentration of bacterial cells. On the other hand, in *E. coli* strain VHb the ability of the cells to uptake Pb was primarily a function of the hemoglobin content. The higher the Pb uptake the lower the Pb adsorption. The opposite results was achieved for parental cells in which the higher Pb adsorption the lower Pb uptake.

Key words: *E. coli*, Lead, Pb biosorption, Pb uptake, adsorption and *vgb*

Introduction

Metal ions are important requirements for microbial life. However, some of these needed ones are toxic to microorganisms at high concentration (Gadd and Griffiths, 1977). Lead is one of the most common elements toxic to microorganisms (Gadd and Griffiths, 1977). It show that toxic effects in different means, such as binding to proteins and nucleic acids or altering normal binding sites of the native metals (Dressler *et al*., 1991) and consequently changing its conformation. It might affect oxidative phosphorylation as well. Moreover, the toxicity of leads organoderivatives is much greater than that of simple inorganic derivatives. This occurred by binding of lead to an organo group by a lead-carbon bond. However, there are conflicting studies on which of the organolead compounds are most toxic to microorganisms. Fore example, in *E. coli*, tripropyllead compound is more liposoluble than the triethyl analogue and was more toxic. A conflicting finding is confused by the results observed from culture respiration rates as a measure of growth (Mergeay *et al*., 1985).

Lead as a cation may cause a threat to trophic chain as disturbing plants (Bewley and Campbell 1980; Cappenberg, 1976; Jernelov and Martin, 1975; Pado *et al*., 1994). The most harmful are its salts: Nitrates, Sulfates and leads organoderivatives as mentioned above (Pado *et al*., 1994). When Sulfate reduced by mixed bacteria cultures coexisted with heavy metals (Fe^{+2}, Pb^{+2}, Zn^{+2}, Cu^{+2}), lead was one of the best detoxificated by these bacteria in long term incubation (Cowen and Silver, 1984). Bacteria have often been applied in phase changes, concentration, deposition, redox transformations and in the transfer of heavy metals to higher trophic levels (Cowen and Silver, 1984; Bewley and Campbell 1980; Cappenberg , 1976; Jernelov and Martin, 1975; Pado *et al*., 1994).

Bacterial hemoglobin plays important roles in biotechnology by transferrring oxygen from surroundings to intracellular acceptor molecules (Khosla and Bailey, 1988). Although VHb can reconstitute oxygen-starvation *in vivo*, it is likely that the mechanism of action of VHb is substantially more complex *in vitro*. Many studies have implicated the participation of the VHb in bacterial and fungal metabolic pathways. VHb appears to activate oxygen-starved cells indirectly (De Mondena *et al*., 1993). Modification of cell response by VHb might provide an explanation for the association of VHb with the cell metabolism and its role in cell growth and increasing valuable products. It has been demonstrated by genetic engineering, that intracellular expression of a bacterial hemoglobin from *Vitreoscilla* (VHb) in different hosts elicits *in vivo*, effects of hypooxic conditions for strictly aerobic microorganisms. Also increased formation of usefully products and consequently improved cell growth (De Mondena *et al*., 1993; Khosravi *et al*., 1990; Khosla and Bailey, 1988). The aims of the present study were (1): To determine changes in growth curves of *E. coli* strain αDH5 and transformed *E. coli* strain αDH5 with the *Vitreoscilla* hemoglobin gene (*vgb*) on pb-containing media. As mentioned above, the genetically engineered bacteria have been produced to enhance their ability to grow, survive and thrive under extreme conditions. Therefore they can metabolize target chemicals compounds under these condition (Khosravi *et al*., 1990; Mains and Kappas, 1977; De Mondena *et al*., 1993; Kallio *et al*., 1994; Khosla and Bailey, 1988; Moat and Foster, 1988; Dressler *et al*., 1991). (2): To determine Pb uptake, biosorption and adsorption in both strains. Based on this and other information, a research was carried out in order to determine how far bacterial hemoglobin enhancing dissimulating lead and lower poisonous levels of lead, from industrial water. This will be compared with same bacterial hemoglobin-free cells.

Materials and Methods

The strains used were *Escherichia coli* αDH5 as parental cell and the same strain transformed with *Vitreoscilla* hemoglobin gene (*vgb*) (denoted strain VHb). Plasmid used was pUC8:16 contains *Vitreoscilla* fragment of 1.4 kb which has been cloned into pUC8. Resulting recombinant strain was denoted as *E. coli* strain VHb.

Growth curve: Medium for growth experiments was LB medium supplemented with 10, 25, 50, 75, 100, 125 and

Table 1: Shows the biosorption, adsorption and uptake of Pb by *E. coli* á DH5 (Parental strain). Data were obtained by incubation of different Pb concentration (ppm in 12 ml solution) with bacterial cells (50 mg each) for 2 h as mentioned in Material and Methods. Data were averages of three independent trials

Pb Concentration (ppm)	Pb biosorption ppm Pb g^{-1}	Pb uptake ppm Pb g^{-1}	Pb adsorption ppm Pb g^{-1}
0	0.001	0.0	0.0
25	6.0	5.1	0.9
50	20.4	13.8	0.6
100	30.8	24.2	5.8
125	32.0	24.9	7.1
150	31.5	24.8	6.7

Table 2: Shows the biosorption, adsorption and uptake of Pb by *E. coli* αDH5 strain VHb (transformed). Data were obtained by incubation of different Pb concentration (ppm in 12 ml solution) with bacterial cells (50 mg each) for 2 h as mentioned in Material and Methods. Data were averages of three independent trials

Pb Concentration (ppm)	Pb biosorption ppm Pb g^{-1}	Pb uptake ppm Pb g^{-1}	Pb adsorption ppm Pb g^{-1}
0	0.01	0.0	0.0
25	9.00	7.1	1.9
50	30.00	26.9	3.1
100	51.00	48.0	3.0
125	60.00	54.0	6.0
150	56.00	51.1	4.9

Table 3: Effect of aeration on yield, Pb biosorption and its uptake by *E. coli* α DH5 strain VHb (Transformed) grown in LB-Ap. Pb concentration used was 75 ppm. Data were averages of four independent trials

RPM	Yield (g wet wt l^{-1})	Pb biosorption ppm Pb g^{-1}	Pb adsorption ppm Pb g^{-1}	Pb uptake ppm Pb g^{-1}
0	1.9	6.0	3.0	3.0
50	2.9	18.0	3.8	14.8
100	6.2	42.5	5.5	37.0
150	6.0	51.8	11.7	40.1
200	5.9	49.0	12.8	36.2
250	5.4	48.2	13.0	35.2
300	5.2	40.2	10.5	29.7

Table 4: Effect of aeration on yield, Pb biosorption and its uptake by untransformed *E. coli* αDH5 (parental cells). Pb concentration used was 75 ppm. Data were averages of four independent trials

RPM	Yield (g wet wt l^{-1})	Pb biosorption ppm Pb g^{-1}	Pb adsorption ppm Pb g^{-1}	Pb uptake ppm Pb g^{-1}
0	1.4	6.0	3.9	2.1
50	2.7	16.0	11.0	5.0
100	3.0	24.0	19.0	5.0
150	5.2	32.0	26.8	5.2
200	5.1	29.1	24.2	4.9
250	5.7	28.0	24.0	4.0
300	4.1	23.4	20.2	3.2

150 ppm lead nitrate. Medium for the transformed strain also contained ampicillin at 100 µg ml^{-1}. Experiments were performed in two ways. In the first set of experiments overnight cultures grown in a GFL Model 3032 shaker at 37°C, 150 rpm, in LB of the untransformed and transformed strains. Samples were taken at intervals and assayed for viable counts on LB plates and optical density at 600 nm. In the second set of experiments the effect of aeration on the yield, efficiency of lead uptake, biosorption and adsorption by both, transformed and parental strains was investigated. Lead-free media were used as control growth medium for both strains.

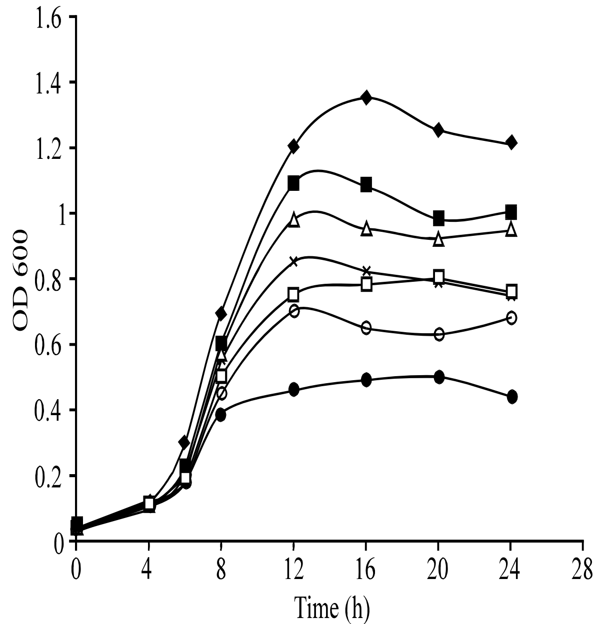

Fig. 1: Growth of *E. coli* (untransformed) on LB medium containing different concentration of Pb (ppm) as indicated above. The control for this strain had no added Pb. Points plotted are means of triplicate cultures: error bars indicate standard deviation

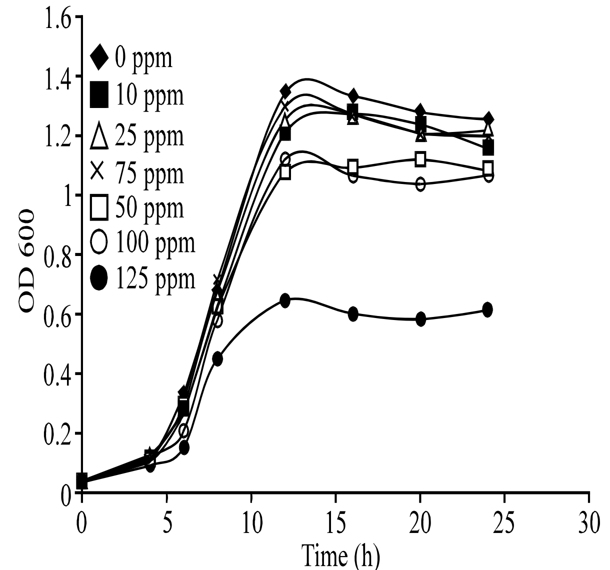

Fig. 2: Growth of *E. coli* transformed with bacterial hemoglobin gene (*vgb*) in LB medium containing different concentrations of Pb (ppm) as indicated above. The control for this strain had no added Pb. Points plotted are means of triplicate cultures. Error bars indicate standard deviations; where not visible they are smaller than the diameters of the points

Total Pb biosorption, adsorption and uptake: Biosorption analyses were made according to the procedure of Scott

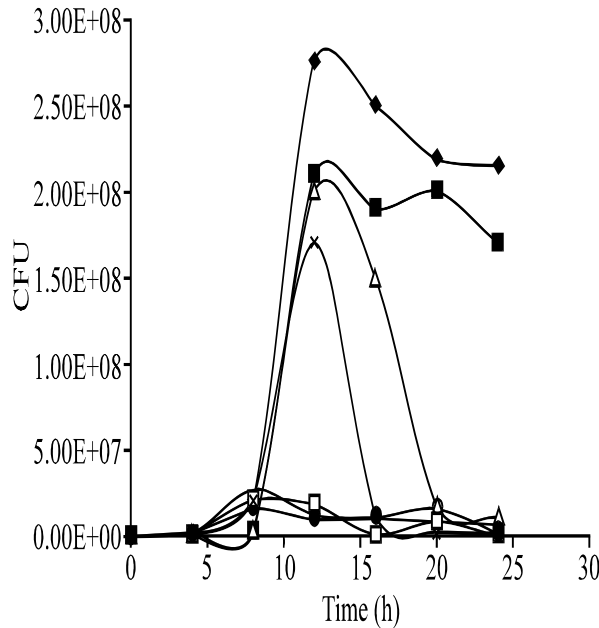

Fig. 3: Growth (viable cells) of *E. coli* (parental cells) in LB containing different concentration of Pb as indicated above. The control for this strain had no added Pb..Values are the averages of three independent trials; error bars indicate standard deviation. CFU; colony forming unit

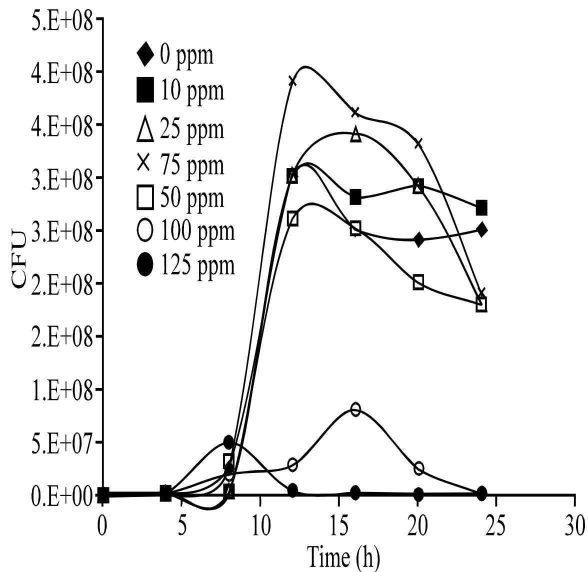

Fig. 4: Growth (viable cells) of *E. coli* strain VHb in LB-Ap containing different concentration of Pb as indicated above. The control for this strain had no added Pb. Values are the averages of three independent trials; error bars indicate standard deviations. CFU; means colony forming unit

(1990) with slight modifications. Cultures were incubated in LB medium in a rotary shaker at 37°C and 150 rpm. Cells were harvested by centrifugation at 8000 rpm for 20 min in a Sorval GSA Rotor. Cells were washed twice with 0.85% saline. Harvested cells (biomass) 50 mg, was placed in 12 ml solutions of 0, 10, 25, 50, 75, 100 and 125 ppm Pb for 3 h. The above solutions were filtered by Millipore filter (0.45 µM in pore size), to separate the biomass from the filtrate. The resulted biomass was washed with either distilled water or 0.01 N (N-CH_3-$COONH_4$) solutions, pH 4.0 according to the method described by Kanazawa and Mori (1996). After washing, the biomass reweighed in the dry state and decomposed by 1% nitric acid for 24 h. The Pb content quantified by use of a flame atomic absorption spectrophotometer using spect AA 800 (Varian) with a deuterium lamp background correction (Scott, 1990). The biomass washed with (N-CH_3COONH_4) was used for measuring the uptake and biomass washed with distilled water for measuring the biosorption. Pb adsorption was determined by taking off the amount of Pb uptake from the amount of Pb biosorption. All 3 measures were made by use of a flame atomic absorption spectrophotometer as described above (Scott, 1990; Scott and Palmer, 1988).

Results and Discussion

Growth curves: Cells grown at temperature 37°C in LB-containing different Pb concentrations at 150 rpm showed significance difference in log phase, stationary phase and counted viable cells. A liquid medium (Fig. 1 and 2) allowed more precise study of a bacterial growth. The inhibitory effect of Pb on parental strain was detected at lowest concentration (10 ppm). Whereas in vgb-containing cells, pb was lethal at 100 ppm. However, at 10, 25, 50 until 75 ppm lead was shown to enhance growth of *E. coli* strain VHb in LB-Ap when compared with controls (VHb strain had no Pb in LB-Ap) (Fig. 1) a result was difficult to explain. In contrast, in parental cells, 10 ppm lead was shown to inhibit the growth when compared with control (parental cells with no Pb in LB (Fig. 2). In viable account (Fig. 3) less than 10% of the parental strain where able to grow on agar medium supplemented with 75 ppm Pb when compared with the same parental cells without Pb. However, the transformed cells (*vgb*-containing cells) were able to grow to more than 50% when grown at the same conditions. In solid media, Pb bioavailability or its chemical form were probably varied from those in liquid media (Moat and Foster, 1988; Babich and Stotzky, 1986). However, the existence of bacterial hemoglobin gene (*vgb*) was dramatically affected the growth rate and the viability of cells in both types of media when comparing with parental cells (untransformed) (Fig. 1, 2, 3 and 4). The changes in growth patterns and lead utilization by transformed cells could be due to direct effects of bacterial hemoglobin (VHb) or more subtlety and indirect effects of (VHb) on cell metabolism (De Mondena *et al*., 1993; Kallio *et al*., 1994; Khosla and Bailey, 1988; Khosravi *et al*., 1990).

Effect of bacterial hemoglobin (VHb) on bp biosorption: Effect of (VHb) on bp biosorption was conducting using *E. coli* αDH5 as mentioned in Materials and Methods. The results are shown in Table 3 and 4. The maximum Pb biosorption of vgb-containing and parental cells were determined to be 51 and 30.8 ppm bp/g biomass respectively. This optimum biosorption were at 100 ppm Pb-containing LB media. Therefore, the 100 ppm Pb was taken to be the maximum growth. These results shown in growth curves (Fig. 1 and 2).

Pb uptake: Pb uptake by both strains (transformed and

parental cells) is shown in Table 3 and 4. The maximum Pb uptake results were paralleled to results shown in Pb biosorption. The optimum Pb concentration in the media was 100 ppm for both transformed and untransformed cells (Table 3 and 4). In vgb-containing *E. coli*, a surprising effect of VHb on Pb uptake was observed. The Pb uptake was two times more than that of parental cells, whereas the Pb adsorption less than that of parental cells (Table 1 and 2). The increases of Pb uptake results in these cells confirm that the bacterial hemoglobin has a positive role in the bioleaching of heavy metals probably through the activation of bacterial stress enzymes and consequently an advantageous mean in biotechnology.

Effect of aeration on yeild, biosorption and uptake in parental and transformed cells: Surprisingly, the amount of aeration also affected the pb uptake of cells grown in LB (Table 3). In *vgb*-containing cells the optimum yeild , was at a shaking rate of 100 rpm. However, the optimum Pb biosorption and its uptake were at a shaking rate of 150 rpm (Table 3). In parental cells, the optimum yield, Pb biosorption and its uptake were at a shaking rate of 150 rpm (Table 4). Presumably Pb is metabolized by *E. coli* strain VHb to intermediates which are not toxic, while the Pb concentration 100 ppm or above there appeared to be growth disadvantage. Moreover, Pb at that concentration might affect oxidative phosphorylation or electron transport chain. These results also reinforce the theory states that the bacterial hemoglobin enhances bacterial metabolism directly or indirectly (Kallio *et al*., 1994; Khosla and Bailey, 1988; Khosravi *et al*., 1990). In *E. coli* strain VHb grown in LB-Ap, the bacterial hemoglobin did confer a significant growth as measured by yield and Pb uptake. Additionally, this was also determined throughout the course of experiments, in which dry biomass for both strains grown on different concentration of Pb where significantly different. In *E. coli* and other Gram-negative bacteria, cell wall has a complex three-layer structure that binds and immobilizes pb^{+2} and Hg^{+}. Although they are the same bacteria, transformed *E. coli* strain VHb exhibit greater Pb tolerance than Gram-positive bacteria. This was also confirmed by its higher Pb uptake as compared with parental cells, which showed higher adsorption (Scott, 1990; Scott and Palmer, 1988). Respiration activity more sensitive *In situ* of heavy metals toxicity on a sediment microbial community (Babich and Stotzky, 1986; Montuelle *et al*., 1994; Aiking *et al*., 1985 and Balakina *et al*., 1996). Additionally, studies of the heavy metal toxicity versus bacterial population according to environmental parameters including oxygen level causes sometimes problem because of variation in bacterial activity (Babich and Stotzky, 1986; Montuelle *et al*., 1994).

The experiments detailed here, described the effects of *vgb*/VHb on the Pb uptake, biosorption and yield. The results indicate that the effects of *vgb*/VHb on the heavy metal bioleaching can be used in another more effective organisms on a case by case basis. These effects may also reflected in the response of physiological behavioral alterations that enabled transformed cells uptake Pb in unknown mechanism. The results may assure that Pb affect oxidative phosphorylation by which VHb gene (*vgb*) has higher respiration rate. As VHb traps oxygen intracellularely and the extra might help ATP production through oxidative phosphorylation resulting in stimulation of the oxygen requiring step(s) in the Pb detoxification.

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