# Biosorption of lead (II) and cadmium (II) using Escherichia coli genetically engineered with mice metallothionen I

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# BIOSORPTION OF LEAD (II) AND CADMIUM (II) USING *Escherichia coli* GENETICALLY ENGINEERED WITH THE MICE METALLOTHIONEIN I

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## ABSTRACT
Contamination taken place by heavy metals in waste waters has a high potential of risk because these penetrate with easiness in the trofic chain accumulating as compounds organometallic. In this work, we examined the expression of mice Metallothionein in *E. coli* as a strategy to enhance metal biosorption efficiency of bacterial biosorbents for lead and cadmium. The results showed that MT expression led to significant increase in overall biosorption capacity, especially for biosorption of Pb. Isotherms and kinetic of biosorption were evaluated in this system designed.

**KEY WORDS:** Biosorption, Metallothionein I, *E. coli*

## INTRODUCTION
Global industrialization has increased significantly throughout latter decades of the last century, leaving in its wake a huge conflict of contamination and the need to investigate all areas of research for the recovery of aquatic ecosystems; this mainly because of our growing dependence on many agricultural activities. The contamination with heavy metals is a significant problem. Industrial effluents are considered to be the major sources of the heavy metal contamination. The heavy metals in aquatic ecosystems cannot be degraded and their persistence in the nature is cause of accumulation in the food chain, it is necessary to remove toxic heavy metals from wastewater. The most commonly used procedures for removing metal ions from aquatic systems include chemical precipitation, coagulation, reverse osmosis and ion exchange solvent extraction (Rich & Cherry, 1987). It is searching for new technologies involving the removal of toxic metals from wastewater using environmentally friendly methods, in which the biosorption fits; know as the ability to bind metal ions from different biological materials. The biosorption can be defined as the ability of biological materials has to accumulate heavy metals from wastewater by a metabolic processes or pathways of physicochemical adsorption (Fourest and Roux, 1992). Biological materials such as algae, bacteria, fungi and yeasts have been widely studied for metal removal (Volesky, 1986). The removal of heavy metals from wastewater through the use of biological materials is highly influenced by physicochemical parameters such as pH, concentration of organic and inorganic species that can compete in the biosorption process and the affinity for the metal ion. The use of recombinant bacteria to remove heavy metals has been studied recently in order to increase the sorption capacity of different metals and their removal systems. Such is the case of the present study where we applied the genetically modified *E. coli* to the removal of cadmium and lead. The bacteria has modified with the mice metallothionein I gene in order to increase sorption capacity.
The metallothionein (MT) are intracellular cysteine-rich proteins which are low in molecular weight, ubiquitous and capable of join metals. Recently, substantial research has demonstrated the ability of the mice MT 1 to have the ability to remove heavy metals in solution. (Wei-Chen Kao, et. al. 1998; Cols N, et. al. 2001)The mice MT1 has shown that it is capable to remove metals such as mercury, and yield better zinc (Liu Y, et. al. 2002) cadmium 90% synthetic solutions during the first 4 contact hours (Reyes-Dávila H.P. 2005).

## MATERIALS AND METHODS

*Molecular Cloning of Metallothionein of Mice.*

Genes encoding Mice metallothionein 1 (Mus musculus MT1, genbank accession no.NM_013602), was cloned from adult mice liver by means of reverse transcription-polymerase chain reaction (RT-PCR). The cloning of MT cDNA as well as the construction for expression vector encoding Thio-MT fusion protein was done followed the method described by Wei-Chen Kao et. al. (2006). Briefly, the total RNA was extracted from liver tissues from adult mice, followed by reverse transcription using reverse transcriptase (Promega, WJ) and random hexamers to generate first strand cDNA pools as templates for subsequent PCR reactions. PCR was performed to amplify the DNA fragment spanning the full length open reading frame (ORF) of metallothionein genes using gene-specific primer pairs for mice MT1, sense: **GGGGTACCT**ATGGACCCCAACTGCTCC and anti-sense **GCTCTAGAGG**TCAGGCACAGCACGTGC. The ~204 bp amplified DNA fragment, containing the full-length ORF metallothionein genes flanked by the *Kpn*I and *Xba*I respectively, were cloned into pThioHis A cloning vectors following the manufacturer instructions (Invitrogen™).

*Recombinant Bacterial Biosorbent (pMt-Thio)*

The expression plasmid encoding Thio-MT fusion protein was transformed into *E. coli* Top10 strain according to the $CaCl_2$ mediated heat-shock protocol (Sambrook, 2001). The expression of Thio-MT fusion protein in *E. coli* was under the control of the inducible "*lac*" promoter and was induced to expression by addition of IPTG at a final concentration of 1 mM. Cells samples were collected at various time points after IPTG induction and equal amounts of these samples were subjected to SDS-PAGE to confirm the induction of Thio-MT fusion protein with an approximate molecular weight of 21 kDa.

*Fusion protein optimal expression*

The recombinant strains were grown in Luria Bertani (LB) broth (Difco) added with 100µg/mL ampicillin at 37°C with 250 rpm agitation. When $OD_{600}$ ~ 0.4, 0.25mM of IPTG added into the culture. To analyzed fusion protein localized to the cytosolic fraction or periplasmic space in *E. coli* Top 10 a factorial design $2^3$ was used to obtain the optimal expression of the fusion protein being the variables the temperature, agitation and IPTG concentration in two levels like it is shown in the table 1. Bacterial lysates were collected at 2 h after IPTG induction and equal amounts of total proteins the samples were analyzed by SDS-PAGE.

Table 1. Experimental conditions to optimal expression and localization of fusion protein

| Exp | T (°C) | Shaker (rpm) | [IPTG] (mM) |
|---|---|---|---|
| 1 | 37 | 250 | 0.25 |
| 2 | 37 | 100 | 0.25 |
| 3 | 30 | 250 | 0.25 |
| 4 | 30 | 100 | 0.25 |
| 5 | 37 | 250 | 0.13 |
| 6 | 37 | 100 | 0.13 |
| 7 | 30 | 250 | 0.13 |
| 8 | 30 | 100 | 0.13 |

*Biosorbent production*

In this study we have two biosorbents: *E. coli* Top 10 and *E. coli* genetic modified with induction to production the fusion protein (*pMt-Thio*). The recombinant strains were grown in Luria Bertani (LB) broth (Difco) amended with 100 ug/mL at 37°C and 250 rpm. For fussion protein expression, was necessary to add the IPTG, a final concentration of 0.25 mM and a temperature of 30°C; when $OD_{600} \sim 0.4$ was reached.

*Kinetic study*

In the first series of experiments for the adsorption of Cd(II) the biosorbents were used metabolically active, adding the solution of Cd(II) by separating to the means of cultivation to have 100 $mg \cdot L^{-1}$ as final concentration. It were took samples at different times by 24 hours. The biosorbents prepared were used after 6 hours ($OD_{600}$ ~ 1.2) of to start the growth to Cd(II) sorption. For sorption metal was necessary to add the metal standard (AccuStandard®) to culture media. In the second series of experiments the adsorption of cadmium the biosorbents were used metabolically inactive; 24 hours after IPTG induction, cells were harvested by centrifugation (3500 rpm, 15 min) from the culture media, which had an $OD_{600}$ ~ 3.0. After washing twice with 0.05 M Tris-HCl buffer (pH=5; BioRad®), cells were resuspended in 100 $mg \cdot L^{-1}$ Cd(II) solutions prepared in the same Tris buffer for the biosorption experiments. The same methods were used to study sorption capacity for Pb(II). In both, *E. coli* Top 10 strain was used as blank.

*Adsorption Isotherms*

Four hundred milligrams of wet biosorbent was added to 100 mL of metal solution in Erlenmeyer flasks for the biosorption assay. The flasks were agitated on the shaker at 23±2 °C for 6 hours. The concentrations of metals ions in the biosorption medium varied between 5 and 300 $mg \cdot L^{-1}$. Samples of 5 mL were taken at the beginning of adsorption and at 6 hours. The biosorbent used was *pMt-Thio* inactive. Then the samples were centrifuged at 3,500 rpm for 5 min and the metals ions concentration in supernatants was measured by AAS.

*Analytical Procedure*

All metal analyses were performed by flame atomic absorption spectroscopy (FAAS) using GBC model 932AA with deuterium background subtraction. Analytical wavelengths used for the metals were as follows: lead, 217 nm and cadmium, 229 nm. An impact bead was used to improve the sensitivity and samples were each read three times and the mean value computed. Calibrations were performed within a linear calibration range of each metal and the correlation coefficients for the calibration curves were 0.98 or better. Biomass-free controls of each of the metal solutions were analyzed to detect any possible metal precipitation or contamination. The difference between the initial metal concentration and the remaining concentration in supernatants and effluents was assumed to be taken up by the biomass. The amount of metal ions adsorbed per unit mass of biosorbents (mg metal ions · (g $biosorbent^{-1}$) was obtained using the following expression:

$$q_t = \frac{(C_o - C\ )V}{\mathrm{M}} \qquad (1)$$

where $q_t$ is the amount of metal ions adsorbed onto the unit mass of the adsorbent ($mg \cdot g^{-1}$), $C_o$ and $C$ are the concentrations of the metal ions before and after biosorption ($mg \cdot L^{-1}$), $V$ is the volume of the aqueous phase (L), and M is the amount of the adsorbent (g).

## Results and Discussion

*Recombinant Bacterial Biosorbent (pMt-Thio)*

The expression plasmid encoding Thio-MT fusion protein was transformed into *E. coli* Top10 strain according to figure 1 where show that the fusion protein is localized in the modified cells. The level of expression of Thio-MT fusion protein (~21 kDa) in *E. coli* increases as it increases the time of growth showing a level of more expression (figure 1).

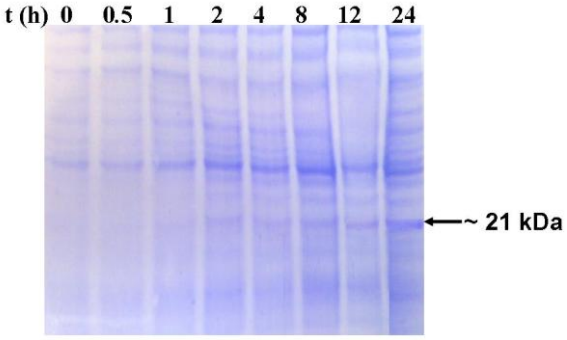


**Figure 1**. Kinetics of expression of the fusion protein during 24 hours of growth after the induction with IPTG. Proteins were detected in Coomasie Blue-stained SDS-PAGE gels

*Fusion protein optimal expression (Mt-Thio)*

The fusion protein was located in the cells with more expression level to 100 rpm and 30 °C, that indicates us that the protein is not secreted to the culture medium it remains expressed in the cell.

The fusion protein only was localized in periplasm (Figure 2a) in all experimental conditions, shows apparently different expression levels with an expected molecular weight of 21 kDa. In according to results obtained the conditions of optimal expression of fusion protein were at 100 rpm, 30 °C and 0.25mM [IPTG]. The MT´s mice fused to the MBP was reported by Wei-Chen Kao (2006) encoded by the pMAL- p2X vector will be targeted to the periplasmic space and this system was applied to remove different heavy metals reported high levels of efficiency with respect to the MT´s mice fused and expressed in the cytoplasm encoded pMBP-TEV vector.

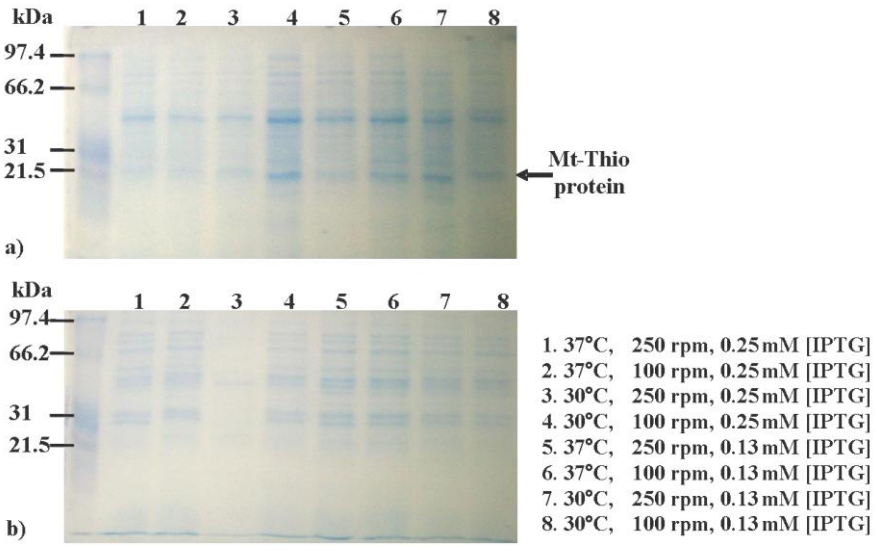


**Figure 2.** Localization of Mt-Thio fusion protein a) periplasmic space b) cytoplasmic fraction. Bacterial lysates were collected at 2 h and equal amounts of these lysates were subjected to 12 % SDS-PAGE. Arrowhead indicates the position of the induced ~ 21 kDa periplasmic Mt-Thio fusion proteins.

*Kinetic study*

The effect of active and inactive biosorbent with respect to time on adsorption was studied and the results are shown in figure 3. With active biosorbent, the removal percentage of Cd(II) and Pb(II) was small during 24 hours. The removal percentage increased rapidly with using inactive biosorbent, and reached a plateau around 39 % to Cd(II) and 99% to Pb(II). The data obtained shown that the use on inactive biosorbents have better sorption capacity inclusive to control (*E.coli* Top 10) and that *pMt-Thio* become the maximum removal around 6 hours (Figure 3).

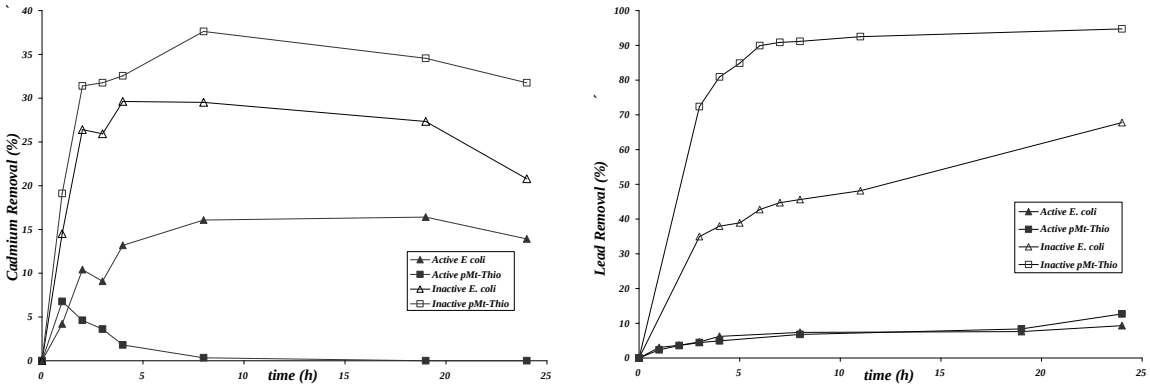


**Figure 3.** Removal efficiencies of Cd(II) and Pb(II) by *E. coli* (control) and *pMt-Thio* at different times.

Figure 4 shows the changes in the amount of Cd(II) and Pb(II) ions biosorbed with time, which were calculated by using Equation 1. The biosorption conditions are given in the figure legend. The initial slope of the curve reflects the biosorption rate. It should be noted that there was no precipitation in these groups of experiments. As seen here, high biosorption rates are observed at the beginning, and then plateau values (i.e. adsorption equilibrium) are gradually reached within 3 minutes.

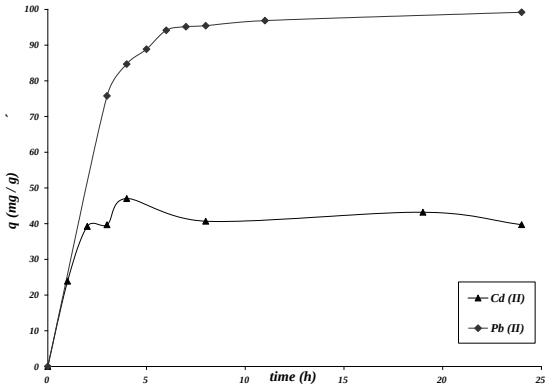


**Figure 4.** Adsorption rates of a) Cd(II) ions and b) Pb(II) ions by *pMt-Thio*. Adsorption conditions: volume of the biosorption medium: 50 mL; initial concentration of metals ions 100 $mg \cdot L^{-1}$; pH 5.5; temperature: 23°C.

Data on the adsorption kinetics of heavy metal ions by various sorbents have shown a wide range of adsorption rates. For example Sobhan and Sternberg (1999) observed high degrees of cadmium biosorption during the first 48 h by green microalgae. The biosorption of cadmium onto pretreated biomass of *Durvillaea potatorum* has been studied and the biosorption process was very fast, with 90% up take taking place within 30 min. Note that there are several parameters which affect the biosorption rate; such as structural properties of the biosorbent, the properties of the ion under study (i.e. ionic radius), the initial concentrations of ionic species and more. Therefore, it is too difficult to compare the data reported. However some researchers have using models that have been developed to describe the kinetics of heavy metal biosorption (Yang and Volesky, 1999; Hashim and Chu, 2004; Gupta et al., 2006). The

pseudo-second order kinetic model based on the sorption capacity of solid phase can be used in this case assuming that measured concentrations are equal to cell surface concentrations. The linearized form of the pseudo-second order model was proposed by Ho and McKay (1998, 1999, 2000) and has been widely applied to the sorption of metal ions, dyes, herbicides, oil, pesticides, and organic substances from aqueous solutions (Ho, 2005). The pseudo-second order kinetic rate equation is expressed as:

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \qquad (2)$$

Integrating for the boundary conditions $qt = 0$ at $t = 0$ and $qt$ at time $t$, the linearized form of pseudo-second order model is obtained:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} \qquad (3)$$

where $k_2$ is the second order biosorption rate constant (g/mg/min); $q_e$ and $q_t$ are the amounts of adsorbed metal ions on the biosorbent at the equilibrium and at any time $t$, respectively. The removal rate of $Cd^{2+}$ and $Pb^{2+}$ by *pMt-Thio* was rapid in the first 5 hours, and leveled off after 6 h (figure 4). The kinetic data were analyzed in term of the pseudo-second order. Figure 5 showed the plots of $t/q$ vs. $t$ with different conditions of biosorbent. The values of $k_2$ and $q_e$ were presented in Table 2. The adsorption of $Cd^{2+}$ and $Pb^{2+}$ onto *pMt-Thio* followed the second order model very well ($R^2$ = 0.9959 and 0.9989 respectively), and based on the assumption that the rate limiting step may be chemisorption involving valence forces through sharing or exchange of electrons between sorbent and sorbate.

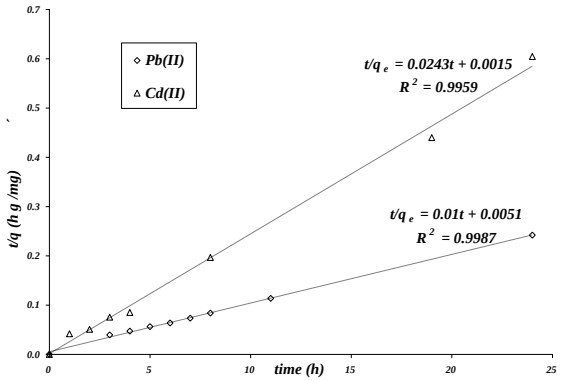


**Figure 5.** Linearized pseudo-second order kinetics Cd(II) and Pb(II) biosorption by *pMt-Thio*.

**Table 2.** The pseudo-second order adsorption constants

| Metal | $q_e$ (mg·L$^{-1}$) | $k_2$ (g·h·mg$^{-1}$) | $R^2$ |
|---|---|---|---|
| Cd(II) | 41.15 | 0.394 | 0.9959 |
| Pb(II) | 100.00 | 0.020 | 0.9987 |

*Adsorption Isotherms*

Two adsorption models, the Langmuir model and Freundlich equation, were applied to the experimental data. The Langmuir model, which was initially developed to study physical adsorption, is a useful tool for the interpretation of biosorption profiles. The Langmuir model can be explained as follows:

$$q = \frac{q_{\max} b C_e}{1 + b C_e} \quad (4)$$

where $q_{max}$ (mg·g$^{-1}$) is the maximum sorbate ($Cd^{2+}$) uptake under the given conditions and $b$ (L·mg$^{-1}$) is the Langmuir constant related to the affinity between the sorbent and sorbate. For the fitting of experimental data, the model was linearized as follows:

$$\frac{1}{q} = \frac{1}{q_{\max} b} \cdot \frac{1}{C_e} + \frac{1}{q_{\max}} \quad (5)$$

The Freundlich equation deals with heterogeneous surface adsorption and can be explained as follows:

$$q = k \cdot C_e^{1/n} \quad (6)$$

where $k$ and n are Freundlich constants. This equation is easily linearized by plotting it in a log-log format.

$$\log q = \log k + \frac{1}{n} \cdot \log C_e \quad (7)$$

The correlation coefficients obtained from the Langmuir model and Freundlich equation were 0.9528 and 0.9244 respectively by Cadmium sorption and 0.9735 and 0.9354 respectively by Lead sorption. Langmuir model and Freundlich equation parameters found from the fitting of experimental points from Figure 6 are shown in the Table 3.

**Table 3.** Isotherm model constants and correlation coefficients for biosorption of Cd(II) and Pb(II) ions from aqueous solution.

| Metal | Langmuir | | | Freundlich | | |
|---|---|---|---|---|---|---|
| | $q_{max}$ (mg·g$^{-1}$) | $b$ (L·mg$^{-1}$) | $R^2$ | $k$ (L·g$^{-1}$) | $n$ | $R^2$ |
| Cd(II) | 24.2718 | 0.0194 | 0.9693 | 0.3119 | 1.1400 | 0.9673 |
| Pb(II) | 28.1409 | 0.1125 | 0.9735 | 5.0909 | 2.9087 | 0.9354 |

The Langmiur model makes several assumptions, such as monolayer adsorption and constant adsorption energy, while the Freundlich equation deals with heterogeneous surface adsorption (Zhou, J.L. and Kiff, R.J., 1991). The agreement of the experimental data with the Langmiur model implied that the monolayer adsorption existed for the experimental conditions used.

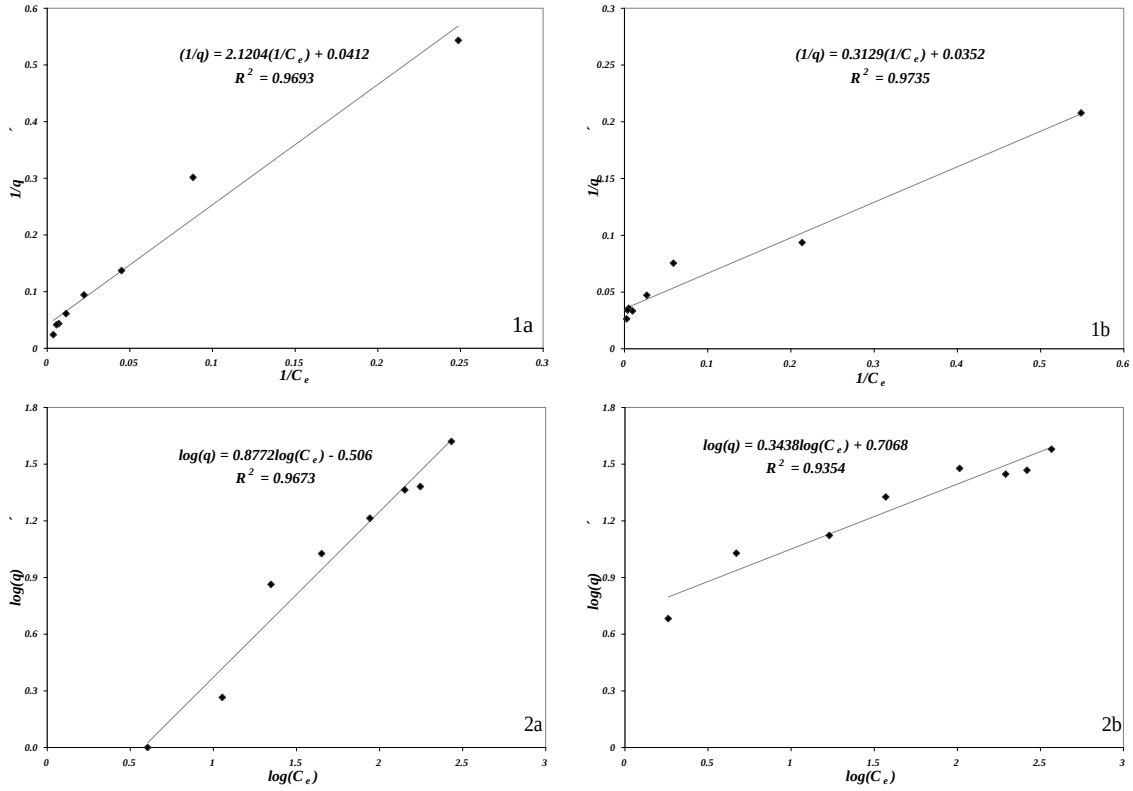


**Figure 6.** Application of 1) Langmuir model and 2)Freundlich equation to experimental data for biosorption of a) Cd(II) and b) Pb(II) ions from aqueous solution.

## Conclusions

In conclusion, it was obtained a genetically modified strain of *Escherichia coli* with the recombinant Methalothionein I of mice that was able to remove heavy metals showing a higher efficiency when it was applied to lead than to cadmium.

The kinetic results using active biosorbent at 100 $mg \cdot L^{-1}$ solution of Cd(II) and Pb(II) showed that the residual concentration of metal is lower in Wild Type *E. coli* than in recombinant strain, however the inactive biosorbent showed higher efficiency in biosorption process for both cases. Data obtained were adjusted to model of pseudo-second order and affinity of the biosorbent to each metal was calculated.

Isotherms of adsorption showed that the biosorption adjuste in a better way to the model of Langmuir that which suggests a superficial adsorption.

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**Figure**

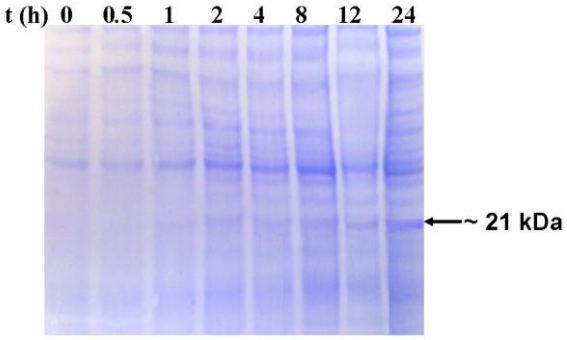
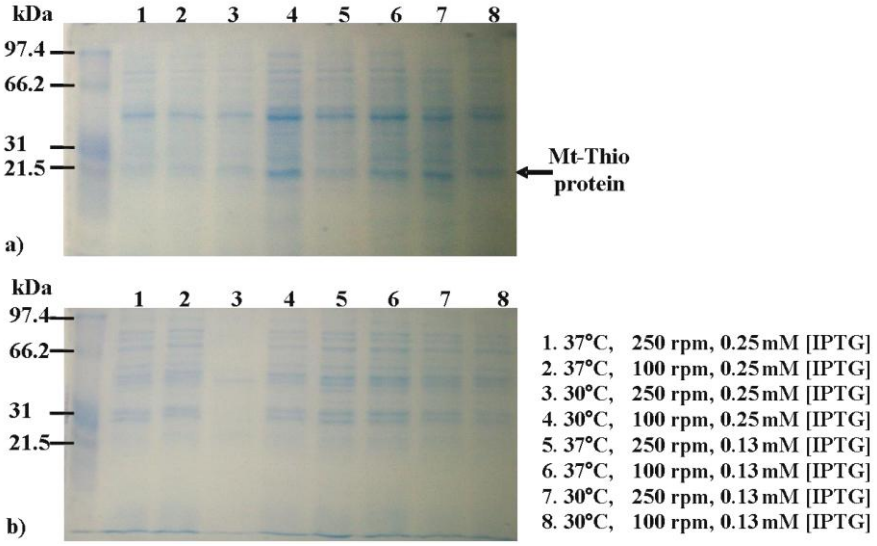



**Figure 1**. Kinetics of expression of the fusion protein during 24 hours of growth after the induction with IPTG. Proteins were detected in Coomasie Blue-stained SDS-PAGE gels

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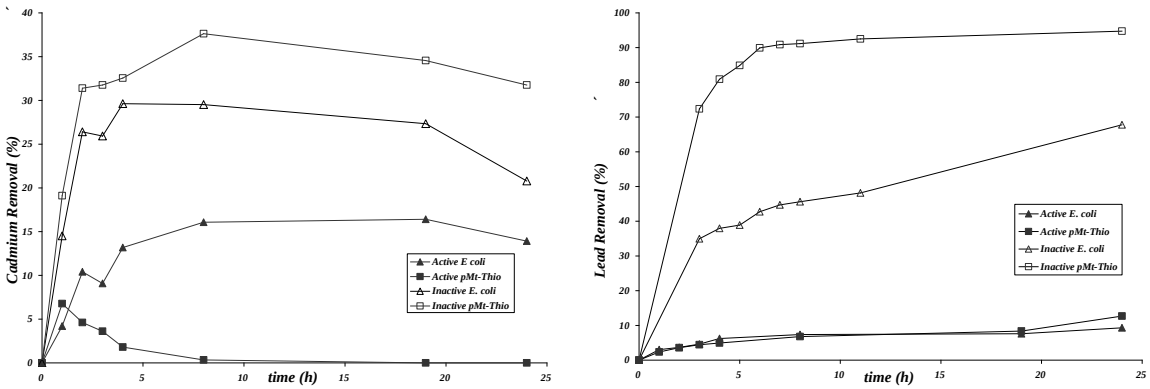
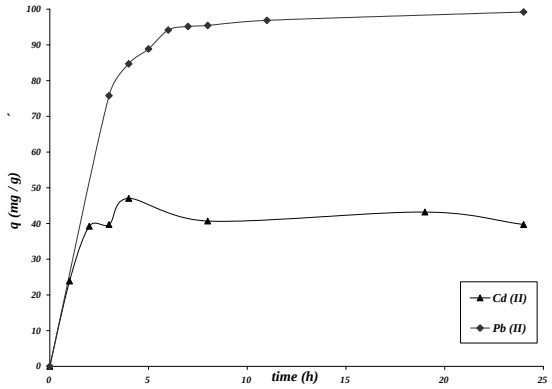



**Figure 3.** Removal efficiencies of Cd(II) and Pb(II) by *E. coli* (control) and *pMt-Thio* at different times.

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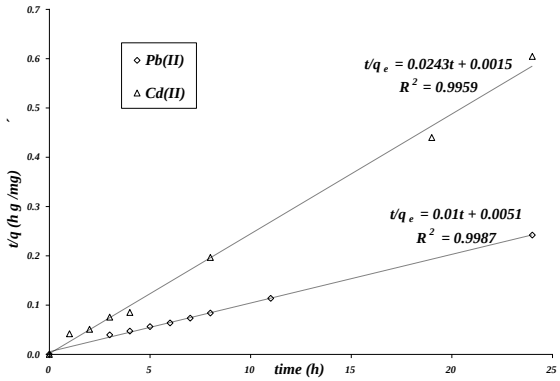


**Figure 5.** Linearized pseudo-second order kinetics Cd(II) and Pb(II) biosorption by *pMt-Thio*.

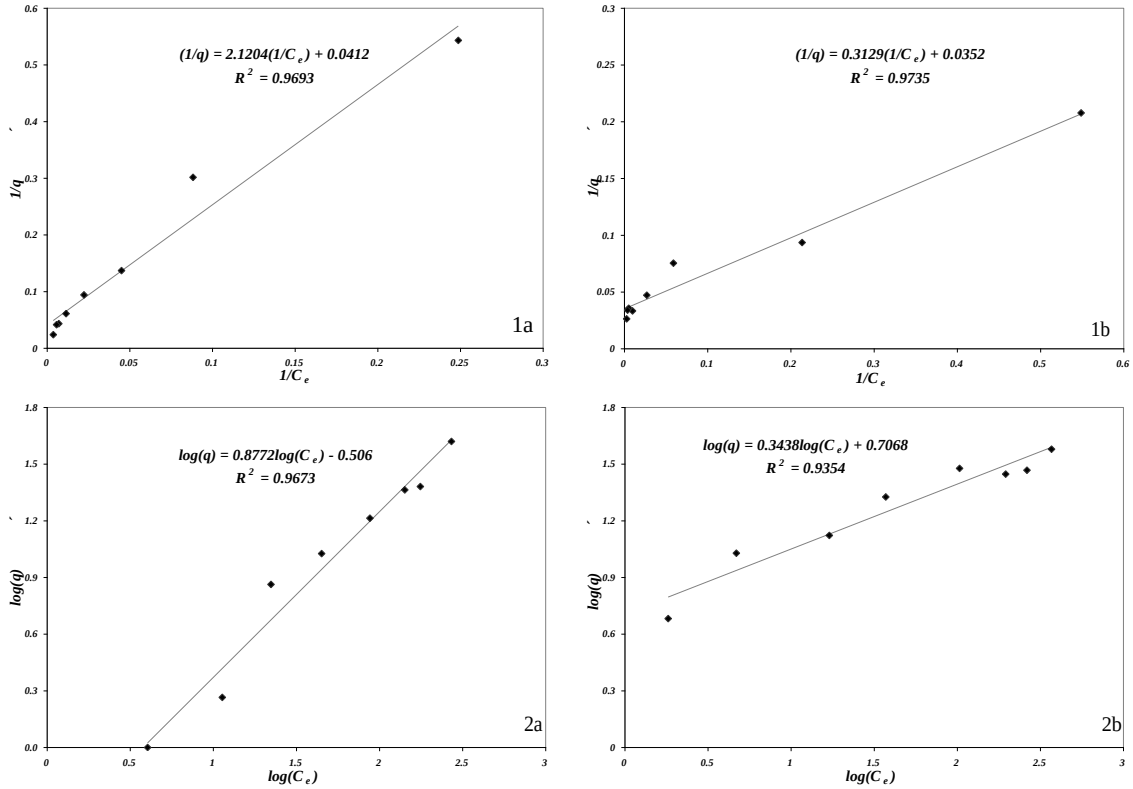


**Figure 6.** Application of 1) Langmuir model and 2)Freundlich equation to experimental data for biosorption of a) Cd(II) and b) Pb(II) ions from aqueous solution.

**Table 1**. Experimental conditions to optimal expression and localization of fusion protein

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| 3 | 30 | 250 | 0.25 |
| 4 | 30 | 100 | 0.25 |
| 5 | 37 | 250 | 0.13 |
| 6 | 37 | 100 | 0.13 |
| 7 | 30 | 250 | 0.13 |
| 8 | 30 | 100 | 0.13 |

**Table 2.** The pseudo-second order adsorption constants

| Metal | $q_e$ (mg·L$^{-1}$) | $k_2$ (g·h·mg$^{-1}$) | $R^2$ |
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| Metal | Langmuir | | | Freundlich | | |
|---|---|---|---|---|---|---|
| | $q_{max}$ (mg·g$^{-1}$) | $b$ (L·mg$^{-1}$) | $R^2$ | $k$ (L·g$^{-1}$) | $n$ | $R^2$ |
| Cd(II) | 24.2718 | 0.0194 | 0.9693 | 0.3119 | 1.1400 | 0.9673 |
| Pb(II) | 28.1409 | 0.1125 | 0.9735 | 5.0909 | 2.9087 | 0.9354 |