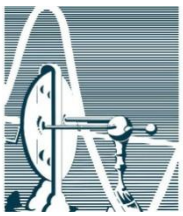

Universidade Federal do Rio de Janeiro
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Dissertação submetida ao Instituto de Biofísica Carlos Chagas Filho da Universidade Federal do Rio de Janeiro para a obtenção do grau de Mestre em Pesquisa Biomédica.

Rio de Janeiro

2016

Universidade Federal do Rio de Janeiro

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Biossorção de cádmio e níquel pela bactéria geneticamente modificada *Cupriavidus metallidurans* MTαβ4.

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Jankovic, Nemanja
J33b Biosorption of cadmium and nickel by genetically modified bacterium Cupriavidus metallidurans MTαβ4 / Nemanja Jankovic. -- Rio de Janeiro, 2016.
49 f.

Orientador: Mauro de Freitas Rebelo.
Dissertação (mestrado) - Universidade Federal do Rio de Janeiro, Instituto de Biofísica, Programa de Pós-Graduação em Formação para a Pesquisa Biomédica, 2016.

1. Biosorption of heavy metals. 2. Synthetic metallothionein . 3. Cupriavidus metallidurans . 4. Cadmium and Nickel. I. de Freitas Rebelo, Mauro , orient. II. Título.

ACKNOWLEDGEMENTS

This project would not have been possible without the support of many people. Many thanks to my adviser, prof. Mauro de Freitas Rebelo, who read my numerous revisions and helped make some sense of the confusion. I am indebted to prof. Francesco Dondero of University of Eastern Piedmont in Italy, for his help with statistical analysis of the experimental data. Many thanks go to prof. Silvana Allodi and the stuff of Laboratory of Comparative Neurobiology and Development for sharing of the equipment and resources. Special thanks go to Tatiana Américo for her help with conducting Western blot experiments. Thanks to CNPq for awarding me a Dissertation Fellowship and providing me with the financial means to complete this project. Many thanks go to my colleagues of Laboratory for Molecular Environmental Biology (BioMA): Juliana Américo, Milica Markovic and Henrique Bomfim for all the help, support and advice throughout these two years. And finally, thanks to my wife, parents, and my family for their patience, trust and love.

To Giovanna and Nikola, my brightest stars in the sky.

CONTENTS

LIST OF ABBREVIATIONS

CrMT - *Crassostrea rhizophorae* metallothionein

CrMTαβ4 – Synthetic metallothionein αβ4

I – Ionic force of solution

ind-MTαβ4- *Cupriavidus metallidurans* ind-MTαβ4 strain

K – stability constant of MT-metal complex

LMG 1195 - *Cupriavidus metallidurans* LMG 1195 strain

mrgA – *Bacillus subtilis* metal-regulated gene A

MT – metallothionein

MTαβ4 - *Cupriavidus metallidurans* MTαβ4 strain

PBS – Phosphate-buffered saline solution

TSA / TSA-CM – Tryptic soy agar / Tryptic soy agar complemented with chloramphenicol

TSB / TSB-CM – Tryptic soy broth / Tryptic soy broth complemented with chloramphenicol

TSM / TSM-CM – Tris salt medium / Tris salt medium complemented with chloramphenicol

ABSTRACT

In order to comply with increasingly strict environmental regulations, effluents containing heavy metals must be treated in order to be safely discarded. Conventional treatment methods, like precipitation, may not be efficient in metal removal and can be expensive. On the other hand, metals can be removed using microbial biomass and, for that, of special biotechnological interest are recombinant microorganisms engineered to express metal-binding proteins, such as metallothioneins, for their improved biosorption capacity. Gram-negative bacterium *Cupriavidus metallidurans* LMG 1195, resistant to at least 20 heavy metallic ions, was engineered in a previous work, to express a synthetic, multi-domain metal binding protein metallothionein from oyster *Crassostrea rhizophorae*. The resulting recombinant strain, named *Cupriavidus metallidurans* MTαβ4, was used in this work to determine its biosorption capacity in 3 independent experiments with sampling times ranging from 3 to 24 h. The expression of the recombinant metallothionein was confirmed by Western blot technique. The biosorption results have demonstrated that Cd^{2+} and Ni^{2+} uptake increased up to 6.5 and 20-fold, respectively, compared to parental strain *C. metallidurans* LMG 1195. Additionally, a possibility of cadmium recovery from the loaded biomass was assessed with three metal-recovery agents: 1 M HCl, 1 mM EDTA and CO_2-saturated water. It was shown that the bound cadmium could be removed from the biomass, with 1 M HCl particularly efficient, removing ~90% of the bound metal resulting in overall efficiency of cadmium recovery of up to circa 70%. These results demonstrated high biotechnological potential of *Cupriavidus metallidurans* MTαβ4 for removal and recovery of metals from industrial effluents.

RESUMO

A fim de cumprir com as regulamentações ambientais cada vez mais rigorosass, os efluentes contendo metais pesados são tratados de modo a serem descartados com segurança. Métodos de tratamento convencionais, como a precipitação, podem não ser eficientes e caros. Por outro lado, metais podem ser removidos a partir de efluentes através do uso de biomassa microbiana. O uso de microrganismos recombinantes desenvolvidos para expressar proteínas de ligação a metais, tais como metalotioneínas, é de especial interesse biotecnológico, a fim de aumentar a biossorção.. A bactéria Gram-negativa *Cupriavidus metallidurans* LMG 1195, resistente a pelo menos 20 íons de metais pesados, foi utilizada em trabalho anterior, para expressar uma proteína sintética de ligação a metais, uma metalotioneína multi-domínio derivada da ostra *Crassostrea rhizophorae*. A cepa recombinante resultante, nomeada *Cupriavidus metallidurans* MTαβ4, foi utilizada neste trabalho a fim de determinar sua capacidade de biossorção, em 3 experimentos independentes, com tempos de amostragem variando de 3 a 24 h. A expressão da metalotioneína recombinante foi confirmada utilizando a técnica de *Western blot*. Os resultados de biosssorção demonstraram que a captação de Cd^{2+} e Ni^{2+} aumentou até 6,5 e 20 vezes, respectivamente, em comparação com a cepa parental *C. metallidurans* LMG 1195. Além disso, a possibilidade de recuperação de cádmio a partir da biomassa carregada foi avaliada usando três agentes de recuperação de metal: 1 M HCl, 1 mM EDTA e água saturada com CO_2. Mostrou-se que o cádmio ligado pode ser removido da biomassa com 1 M HCl sendo particularmente eficiente, removendo ~90% do metal ligado, resultando numa eficiência total de recuperação de cádmio de até ~70%. Estes resultados demonstram o alto potencial biotecnológico de *Cupriavidus metallidurans* MTαβ4 para a remoção e recuperação de metais a partir de efluentes industriais.

1. INTRODUCTION

Metals and minerals represent a major category of nonrenewable resources that are extracted from and returned to the natural ecosystem. As human populations and economies have increased, metals and industrial minerals consumption have increased concurrently. During the 1970 – 2004 period, world population and gross domestic product (GDP), increased by about 72 and 225%, respectively. Simultaneously, the global extraction of major metals grew by over 75% and industrial minerals by 53% (Rogich and Matos, 2008).

In the same period, aluminum consumption increased by more than three-fold, while copper and zinc consumption increased about twofold. Lead consumption increased about one and a half times during the same period. Global consumption of steel increased slowly until the late 1990s, after which it started to increase rapidly to about one billion tons per year, about twice the 1974 level. The rapid increase in consumption that steel experienced was largely due to the increased demand from China. During those 34 years, global chromium and nickel consumption rates increased by factors of nearly 2 and 0.75, respectively. Similarly to steel, Chinese consumption of cadmium was low until 1997, after which it grew more than tenfold by 2004. Other fast developing countries also contributed to high demand for metals. India consumption of basic metals and steel increased in the same period by 6 and 4.5 times, respectively. The extraction of each of these two metal groups in South American countries multiplied 4 times during the studied period (Rogich and Matos, 2008).

Rapid growth in metals and minerals use has serious implications for the future availability of these resources and the health of the environment. The efforts associated with the extraction and initial processing of these commodities can create considerable environmental impacts, particularly where the most advanced technology is not employed. Metal and mineral commodities are nonrenewable resources, and therefore their total availability, while not readily definable, is finite and prone to decline (Rogich and Matos, 2008). For instance, copper content in ores mined in 1900 in the United States was around 4%, but by 1982 it was reduced to around 0.6% (Manahan, 1999). Low grade ores require large equipment able to move and process proportionally bigger quantities of rock and earth with lesser percentage of metal. The result of these activities is higher energy and water consumption and greater waste byproduct generation. Additionally, the disturbed rock material is more prone to erosion, landslides and water pollution with heavy metals (Manahan, 1999).

Metallurgical activities also contribute to contamination by heavy metals (Veglio and Beolchini, 1997). For example, Nieminen *et al.* (2007) reported 75 to 400 times higher concentrations of nickel in ambient air in the vicinity of a Ni-Cu smelter in Finland, compared to non-industrialized areas. They also reported 100 fold increase in nickel concentration in the soil nearby the Ni-Cu smelter, compared to the concentration

in remote areas. According to Moore and Ramamoorthy (1984) nickel levels in unpolluted freshwater are in the range from 1 to 3 $\mu g \cdot L^{-1}$ while mixed industrial sources known to increase these values to 10-50 $\mu g \cdot L^{-1}$. The emissions from nickel smelters resulted in high aerial (200-2000 $\mu g \cdot L^{-1}$) and dissolved concentrations (1 - 183 $\mu g \cdot L^{-1}$) of this metal in the area of The Great Lakes (Moore and Ramamoorthy, 1984). Significant cadmium emissions come from increased use of phosphate fertilizers, in which cadmium is present as an impurity, from waste burning and the improper deposition of Cd-containing batteries (Schulte-Schrepping and Piscator, 2005). Cadmium was found in high concentrations (> 900 $mg \cdot kg^{-1}$) in surface sediments near the discharge site of a Ni-Cd battery factory. While unpolluted marine sediments contain as little as 0.01 mg Cd kg^{-1}, in industrial areas cadmium levels may exceed 50 $mg \cdot kg^{-1}$ (Moore and Ramamoorthy, 1984).

Heavy metals are potential health hazards due to their toxicity. Cadmium, for instance, has no biological role in animals (Maret and Moulis, 2013) and is also a known human carcinogen (Hartwig, 2013). Toxicity of most nickel compounds is regarded as less severe[1] and noncumulative, however, the prolonged exposure to low doses can have detrimental effects on health and lead to cancer development (Kasprzak and Salnikow, 2007). The effluents containing heavy metals must pass a specific treatment in order to be safely discarded because municipal sewage plants are not designed for metal removal from wastes (Volesky, 2001). In Brazil the regulative organization, CONAMA (resolution Nº 430/2011) determines the maximum allowed concentrations of Cd^{2+} and Ni^{2+} in effluents as 0.2 $mg \cdot L^{-1}$ and 2.0 $mg \cdot L^{-1}$, respectively.

There are several conventional methods to mitigate the presence of heavy metals in effluents such as precipitation, adsorption, ion exchange, membrane and electrochemical technologies (Ahluwalia and Goyal, 2007). Precipitation, the most common of them all, is relatively simple but may not be effective due to various drawbacks: efficiency affected by low pH and the presence of other ions, lack of selectivity and generation of large volumes of metal-bearing sludge (Ahluwalia and Goyal, 2007; Volesky, 2001). For example, precipitation of Cu^{2+} and Cd^{2+} from solution as hydroxides generates as much as ten and nine times more solid sludge, respectively (Eccles, 1995). The sludge needs special handling due to its relatively high metal content leading to increased treatment costs and hinders metal re-use (Volesky, 2001). More advanced technologies like reverse osmosis, ion exchange and electrochemical treatment can recover metals and purify effluents to a degree that they can be recycled; however, these methods are inefficient for treating effluents with low metal concentrations (1 – 100 $mg \cdot L^{-1}$). Besides that, they are very expensive, especially when large volumes of effluents need to be treated (Ahluwalia and Goyal, 2007; Volesky, 2001). Therefore, the search for efficient, eco-friendly and cost effective wastewater treatment has been initiated (Vijayaraghavan and Yun, 2008).

[1] Nickel carbonyl is an exception. Its extreme toxicity is comparable to that of hydrogen cyanide (Kasprzak and Salnikow, 2007).

According to Volesky (2001) biosorption can be considered as competitive method for heavy metal removal when compared to conventional methods. It can be defined as a process of capturing heavy metals from effluents by biologically-based materials, via metabolic processes or physicochemical adsorption (Almaguer-Cantú *et al*., 2011). Kratochvil and Volesky (1998) identified main advantages of biosorption over conventional treatment methods to be: minimization of produced chemical sludge, high efficiency of metal removal from diluted solutions and the possibility of metal recovery.

Microorganisms such as bacteria, cyanobacteria, algae, yeasts and fungi have been investigated for metal removal because of their good performance and a possibility to be grown in large quantities (Michalak *et al.,* 2013). The immobilization of metals on cells' surface is mediated by different functional groups such as carboxyl, phosphate, hydroxyl or amino groups (Michalak *et al.,* 2013), and is an important protective mechanism for many microbes (Pazirandeh *et al.*, 1995). Due to their metal binding properties microorganism were used in development of several proprietary biosorption processes that were commercialized in the early 1990s (Volesky, 2001).

One of them is AlgaSORB™, a biosorbent based on the freshwater alga *Chlorella vulgaris*, immobilized in a silica gel matrix. This biosorbent was employed in removing uranium and mercury from ground waters with reported efficiency of about 95% for both metals. Another example is AMT-BIOCLAIM™, a biosorbent based on *Bacillus subtilis* treated with caustic solution. This biosorbent, able to immobilize Au^{+}, Cd^{2+} and Zn^{2+}, is considered suitable for secondary treatment processes (polishing technique) due to high operating costs of treating effluents with metal concentrations above 250 $mg \bullet L^{-1}$. Bio-Fix™ is an example of mixed-biomass biosorbent whose composition includes bacteria, fungi, peat moss and algae, immobilized onto polysulfone as beads. Bio-Fix™ was used for treatment of acid mine drainage liquor. This biosorbent was found to be selective for heavy metals (Cd^{2+}, Zn^{2+} and Mn^{2+}) in the presence of high concentrations of alkaline earth metals (Vijayaraghavan and Yun, 2008; Eccles, 1995). However, neither of these commercial biosorbents managed to attract widespread adoption. This was primarily due to a lack of understanding of the underlying mechanism of metal sorption, proper selection of industrial effluents for pilot testing and scaling up of the process (Ahluwalia and Goyal, 2007).

Biological sludge obtained from sewage treatment plants is an example of biosorbent that is easily available in adequate amounts and at very low cost. However, the metal-sorbing capacities of sludges, based on very mixed and heterogeneous microbial populations, are usually low and pH dependent. It is reportedly possible to improve their metal sorbing capacity but the heterogeneity of the biomass makes the process difficult and doubtful to validate (Hammaini *et al*., 2007; Volesky, 2001).

A promising strategy for development of biosorbents with enhanced metal sorption capacity is, therefore, the overexpression of metal-binding peptides, such as metallothioneins in bacteria (Bae *et al*., 2000).

1.1 Metallothioneins – efficient metal binding proteins

Metallothioneins (MTs) belong to low-molecular weight (~6-7 kDa) family of metalloproteins that reportedly take part in the homeostasis of essential metals (zinc and copper), protection against oxidative stress and detoxification of heavy metals in eukaryotes (Sutherland and Stillman, 2014; Romero-Isart and Vasak, 2002). The first biochemical and biophysical characterizations of MTs were published by Kagi and Vallee who coined the term "metallothionein", referring to high metal and sulfur content in the purified protein (Kagi & Vallee, 1960; Kagi & Vallee, 1961). Typically, MTs primary structure is composed of repeating Cys-X-Cys and Cys-X-X-Cys motifs, where Cys is cysteine and X any other amino acid. The primary MT structure comprises about 30% of cysteine residues with the notable absence of aromatic amino acids. The metals are shared between two metal–thiolate clusters: α- and β-domain, giving MT a dumbbell shape. The two are concatenated by a flexible linker made of highly conserved Lys-Val-Lys sequence. Metals are bound *via* covalent bonds for bridging and terminal thiolates of cysteine residues. Bridging cysteins coordinate two, while terminal cysteines bind one metal ion, respectively. The mammalian α-domain comprises eleven cysteine residues capable of binding four divalent metals; β-domain comprises nine cysteine residues and is capable of binding three divalent metals (Sutherland and Stillman, 2014).

MTs are "promiscuous" *i.e.* they can bind different metals at the same time (Palacios *et al*., 2011). Up to 12, 7 and 7 of mono-, di- and trivalent ions, respectively, can be held by a single MT molecule (Sutherland and Stillman, 2014; Ngu *et al.,* 2010; Li and Otvos, 1996). Difference in the binding affinity of MTs for heavy metals generally follows the order: $Hg^{2+} > Bi^{3+} > Cu^{+} > Ag^{+} > Cd^{2+} > Pb^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+}$ (Nielson *et al.,* 1985). However, quantitative information on the metal ion affinity of MTs is limited and binding affinities were reported only for Cd^{2+} and Zn^{2+}, the two most studied metals: log K_{Cd} ~17 and log K_{Zn} ~14 (Blindauer, 2014). Differences in metal binding affinities are reflected in the measured pH of half-displacement[2]: pH = 4.5 for Zn^{2+} and pH = 3.0 for Cd^{2+} (Blindauer, 2014).

Binding properties of adsorbents like granulated activated carbon, suitable for treatment of low metal concentration (<10 $mg \cdot L^{-1}$) effluents (Eccles, 1995), can be compared to binding of MTs to metals. The removal of metals by physical adsorption, as with activated carbon, is based on van der Waals forces that are effective over long range but that are weaker compared to covalent bonds. The enthalpy of physical adsorption is, therefore, relatively small, typically around 20 $kJ \cdot mol^{-1}$ (Atkins and de Paula, 2006). Comparatively, Chang and co-workers (1998) estimated the contributions of individual cysteines in metal binding affinity and calculated the total binding energy[3] between MTs and metal ions *viz*. Zn^{2+} and Cd^{2+}. Depending on the distribution of metals in metal-protein complex, the calculated binding energy was in

[2] Titration of metal-protein complex with acid until 50% of metal ions are displaced from the complex.

[3] The total binding energy was estimated as the sum of contributions of all cysteines involved in metallic complexes. Calculations were based on measuring heat of formation in a molecular teasing process.

the range of ~1900 to ~2700 $kJ \cdot mol^{-1}$ (Chang *et al*., 1998) meaning that binding of metals by MTs is >100 times stronger than by physical adsorption.

1.2 Genetic modifications to enhance microbial biosorption

High binding energies of MTs have been exploited to develop recombinant microorganisms as more efficient biosorbents. In one of the earliest studies where MTs were heterologously expressed in bacteria by Romeyer *et al*. (1988), who cloned a synthetic, human hepatic MT fraction, expressed as an inducible fusion protein in *E*. *coli*. Metal bioaccumulation was measured in cells which were grown in the presence of 20 μM Cd^{2+}. After 4 h, the metal uptake by the recombinant strain was 20% of the total bioavailable metal, while the wild type strain removed only some 3%. The same group of authors (Jacobs *et al*., 1989) reported the expression of the recombinant MT on the cell membrane afterwards. The bioaccumulation of Cd^{2+} by the recombinant strain improved by 66-fold compared to MT-nonproducing cells. However, the maximum uptake was still only 20% of total Cd^{2+} content in the culture, and, despite the demonstrated cellular accumulation of this metal, the genetically-modified strain did not improve its Cd^{2+} tolerance compared to the parental strain (Jacobs *et al*., 1989).

Following this work, Pazirandeh and co-workers (1995) cloned the gene coding for the *Neurospora crassa* metallothionein in *E. coli*. They studied Cd^{2+} uptake by the recombinant strain in the presence of different concentrations of Ca^{2+} or Mg^{2+} and compared it to that of AlgaSORB™, which is considered highly selective among available biosorbents[4]. The Cd^{2+} uptake by the metallothionin-expressing strain was rapid (75% of the final uptake of Cd^{2+} after 20 min) and highly selective: 10^5-fold excess of alkaline-earth metals did not affect Cd^{2+} uptake. Comparatively, it was reported for AlgaSORB™ that the presence of alkaline-earth metals under concentrations 250 times higher than that of the heavy metal reduced the uptake of the heavier elements by around 30% (Pazirandeh *et al.,* 1995). In another study (Brower *et al.,* 1997), metal binding properties of the same recombinant strain were compared with another commercial biosorbent, Bio-Fix™. The recombinant strain was able to remove 87% of the initial (0.1 μM) Cd^{2+} concentrations while Bio-Fix™ removed around 33%. The metal uptake by the cyanide process from wastewater showed that the metallothionein-expressing strain sequestered 3- and 5 times more Ni^{2+} and Cd^{2+} compared to Bio-Fix™, respectively (Brower *et al.,* 1997). The results demonstrated superior selectivity of metallothioneins for heavy metals over commercially available biosorbents (Pazirandeh *et al.,* 1995), and indicated that MT-based biosorbents can be efficient polishing treatment for electroplating wastewaters (Brower *et al.,* 1997).

More recently, Kao *et al*. (2006) constructed *E. coli* strains that expressed mouse, human or fish metallothionein isoforms. After 24 h incubation with 0.9 mM Cd^{2+} the best results were obtained with

[4] Alkaline and alkaline-earth metals are often present in wastewaters in high concentrations; though non-toxic, they can compete for the available binding sites and reduce the efficiency of the process (Pazirandeh *et al.,* 1995).

strains expressing fish MT in periplasmic space. The enhancement in metal uptake was 213% compared to the parent strain.

Results from different studies (Pazirandeh and Mauro, 2001; Bae *et al*., 2000; Pazirendah *et al*., 1995) suggest that in order to maximize bioaccumulation, metal-binding proteins should be expressed on or near the cell surface. Another strategy to create better bacterial biosorbents is the expression of multiple copies of metal-binding domains such as engineered metallothioneins. Compared to cells expressing a single copy peptide, there are several advantages of this strategy including increased binding capacity, improved stabilization of the recombinant protein and a possibility to incorporate binding domains for different metals in a single protein (Mauro and Pazirandeh, 2000). In their work, Mauro and Pazirandeh (2000) expressed multiple copies (up to 12) *in tandem* of *N. crassa* MT subunits in *E. coli*. The cells that expressed a single copy of metal-binding domain showed tenfold increase in Cd^{2+} binding compared to the non-modified strain. On the other hand, cells expressing nine copies of MT had their metal-binding capacity enhanced about 65 times relative to the parental strain cells.

Other metal-binding peptides, like phytochelatins (Bae *et al*., 2000) or poly-hystidine peptides (Sousa *et al.*, 1996) have also shown to enhance metal accumulation when expressed in multiple copies compared to cells that expressed single copies of these polypeptides. Bae *et al*. (2000) constructed *E. coli* strains displaying 8, 11 and 20 copies of synthetic phytochelatin on their outer membranes. The respective increase in metal uptake, compared to their parental strain, was 4.5, 7.5 and 15-fold. At the same time, *E. coli* strains expressing one or two copies of hexa-His peptides on their cell surfaces, accumulated 5 and 11-times more Cd^{2+} than their wild type counterparts (Sousa *et al*., 1996).

From the previous sections it can be depicted that *E. coli* is an attractive host for heterologous expression of different metal-binding proteins, including metallothioneins. However, by itself, this bacterium is not an ideal microorganism for bioremediation purposes due to its relatively low metal resistance. More realistic approaches to the designing of detoxifying agents have to count on more robust microorganisms (Valls *et al.,* 2000). One of them is soil-borne, β-proteobacterium *Cupriavidus metallidurans* LMG 1195, which harbors particular functions to survive tough conditions imposed by high (milli-molar) levels of heavy metals (Janssen *et al.,* 2010).

1.3 *Cupriavidus metallidurans* as host for expression of metal-binding peptides

Gram-negative, *Cupriavidus metallidurans* LMG 1195 is a very good candidate for biotechnological applications due to a variety of favorable traits (Diels *et al.*, 2009). Contrary to the majority of soil-borne bacteria, *C. metallidurans* LMG 1195 is easily cultivated in laboratory and rapidly forms small colonies that can be easily examined for interesting phenotypes (Craig *et al.,* 2009). Secondly, this species can survive in heavy metal contaminated habitats (Text Box 1). One adaptation to life in these environments

derives from its ability to produce siderophores, compounds that can solubilize and sequester iron and other metals from the extracellular environment. This property has been exploited by Diels *et al.* (2009) to treat sandy soils contaminated with heavy metals. Concentration of metals in the tested soil *viz.* Zn^{2+}, Pb^{2+} and Cu^{2+} decreased 8-, 8- and 16-fold, respectively, demonstrating the potential of this bacterium for bioremediation purposes (Diels *et al*., 2009). Finally, *C. metallidurans* LMG 1195 cells are easily transformable (Craig *et al*., 2009). This ability, to accept and express foreign genes, is said to be the adaptation to harsh environments where genetic flexibility is necessary to survive (Diels *et al.*, 2009). Despite its biotechnological potential, genetic manipulations (e.g. expression of metal-binding proteins) of *C. metallidurans* have been explored in a limited number of studies.

Text Box 1. Basic physiology and morphology of *Cupriavidus metallidurans* LMG 1195.

C. metallidurans LMG 1195 is a facultative chemolithotroph that can grow autotrophically in a mineral medium under a gas mixture of H_2, O_2 and CO_2 at various ratios, or heterotrophically assimilating acidic sugar-derivatives like D-gluconate, pyruvate or lactate (Janssen *et al.*, 2010), but it cannot use D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl D-glucosamine or maltose as carbon sources because it does not possess a functional 6-phosphofructokinase (Goris *et al.*, 2001). *C. metallidurans* LMG 1195 can oxidize inorganic sulfur compounds like thiosulfates (HS^-) and sulfites (HSO_3^-) and organosulfates such as: aromatic sulfonates, alkanesulfonates, and sulfate esters to generate energy (Schmalenberger and Kertesz, 2007).

The cells are short, motile and rod-shaped (0.8 x 1.2 – 2.2 μm) that grow as single cells, in pairs or in short chains. The colonies are round, smooth, convex and transparent. The size of the colonies, after 24 h of incubation on Tryptic Soy Agar at 30°C, is about 0.5 mm in diameter. The cells can grow at 20 and 37°C but not at 4 or 41°C (Goris *et al.*, 2001). This temperature range sets *C. metallidurans* LMG 1195 in a group of mesophilic microorganisms. However, owing to its innate ability to survive in heavy metal contaminated environments it can be considered as extremophile (Nies, 2000) or even metallophile (Diels *et al.,* 2009).

C. metallidurans LMG 1195 is efficiently adapted to living in environments with a high metal content (Nies, 2016). The list of metal ions to which the bacterium is resistant includes, but is not limited to: Ag^+, Au^+, Au^{3+}, AsO_2^-, Bi^{3+}, Cd^{2+}, Co^{2+}, Cs^+, Cu^+, Cu^{2+}, CrO_4^{2-}, $HAsO_4^{2-}$, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sr^{2+}, SeO_3^{2-}, SeO_4^{2-}, Tl^+ and Zn^{2+} (Janssen *et al.*, 2010). At least 30 different metal resistance mechanisms, like efflux ATPase pumps, protect *C. metallidurans* LMG 1195 against these metals (Janssen *et al.,* 2010; Mergeay *et al.*, 2003). Due to its resistance to concentrations of heavy metals as high as milli-molar, *C. metallidurans* LMG 1195 is considered a model system for research on heavy metal homeostasis in bacteria (von Rozycki and Nies, 2009; von Rozycki

Valls *et al.* (2000) expressed mouse MT as a hybrid protein on the surface of *C. metallidurans* LMG 1195. When grown in the presence 300 μM Cd^{2+}, recombinant cells accumulated 3 times more Cd^{2+} compared to wild type cells. It is noteworthy that the growth rate of the recombinant bacteria was not affected neither by the production of the hybrid MT nor by the increased biosorption of Cd^{2+}. The tests with *Nicotiana bentamiana* growing on cadmium-containing soil have shown that inoculation of the soil with the MT-expressing recombinant resulted in immobilization of ~70% of bioavailable metal, which was reflected by a positive effect on a plant growth (Valls *et al.*, 2000).

Rojas *et al*. (2011) engineered recombinant *C. metallidurans* with improved resistance to organic and inorganic mercury. The growth of recombinant strain was not affected by addition of 0.04 mM Hg^{2+} (which prevented parental *C. metallidurans* to multiplate). The transformant cells exhibited 2.4 and >16-fold increased tolerance to Hg^{2+} and CH_3Hg^+, respectively, and were able to completely remove Hg^{2+} from 0.10 and 0.15 mM synthetic solutions.

Lastly, Biondo *et al*. (2012) expressed synthetic, multidomain phytochelatin in *C. metallidurans* LMG 1195. The same gene, coding for 20 *in tandem* copies, was previously expressed in *E. coli* (Bae *et al* 2000). The resulting strain was especially efficient in removing Pb^{2+} and Zn^{2+} from their respective solutions (>3-fold higher than by wild strain cells for both metals). The growth kinetics of modified *C. metallidurans* cells showed no significant difference with that of control cells, indicating that the genetic modification did not affect normal cell functioning.

It is possible to envisage that the expression of protein that binds metals with higher specificity and affinity in *C. metallidurans* LMG 1195 would generate an even more efficient biosorbent.

1.4 Metal recovery from the loaded biomass

A desirable requirement for wastewater treatment by a biosorption process is the regeneration of the sorbent which can be of crucial importance for keeping the process costs low as well as for reclaiming the metals originally removed from the liquid. Desorption should yield metal solutions in a concentrated form so that they can be extracted, usually via electrochemical methods such as electrowinning (Volesky, 2001). It is worth recollecting that these methods, despite their shortcomings, can be used to recover metals but, in order to operate efficiently, high metal concentrations (> 100 $mg \cdot L^{-1}$) are needed[5]. It is also desirable that the as minimum as possible damage occurs to the biomass so as to allow its re-use in subsequent adsorption-desorption cycles (Veglio and Beolchini, 1997). Alternatively, if biomass recycling is not considered worthwhile, its incineration yields ash with a high concentration of the desired metal (Vijayaraghavan and Yun, 2008) that can be extracted by conventional techniques like filtration and acid leaching (Bunge, 2015).

Ethylenediaminetetraacetic acid (EDTA) is one of the most common metal-chelating agents studied in the literature (Peters, 1999). Its propensity to form stable complexes with multivalent metal ions has been explored for treatment of contaminated soils and metal recovery from immobilized biosorbents and activated sludges (Nunez-Lopez *et al.,* 2008; Vijayaraghavan and Yun, 2008; Hammaini *et al*., 2007; Peters, 1999). The stability constants of EDTA complexes with different transition metals are given in Table 1 (Housecroft and Sharpe, 2005; Auld, 1995).

[5] Please, refer to the discussion on p. 4.

Table 1. Stability constants of some EDTA-metal complexes, given as log *K* (Housecroft and Sharpe, 2005; Auld, 1995)

Metal	Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}
log *K*	13.8	14.3	16.3	18.6	18.7	16.1	16.4

In many cases, treatment with EDTA allowed reusing of the biosorbent over several cycles (Vijayaraghavan and Yun, 2008; Hammaini *et al*., 2007). However, some environmental concerns exist regarding its use because of its low biodegradability and toxicity to plants and microorganisms (Nunez-Lopez *et al.,* 2008; Peters, 1999).

Auld (1995) studied the withdrawal of metal ions from different metallopeptidases with chelating agents, including EDTA. He concluded that because of the high stability of complexes with bound metals, 1mM EDTA concentration should be sufficient to remove the metal from most of the enzymes (Auld, 1995). Metallothioneins stability in presence of metal-chelators was also investigated *in vitro* with the results showing that stoichiometric quantities of EDTA are sufficient to remove all metals bound to MTs (Blindauer, 2014).

Another option for the recovery of biosorbed metals is washing with strong, inorganic acids (Vijayaraghavan and Yun, 2008). Aqueous solutions of hydrochloric acid (HCl) have been efficiently employed on a number of occasions (Hammaini *et al.,* 2007; Chen *et al.*, 2005; Costley and Wallis, 2001; Chang *et al.,* 1997). The dissociation of HCl in water increases the concentration of H^+ ions. At sufficiently low pH levels, H^+ ions can dislodge the metals bound to the biomass in an ion-exchange process (Okafor *et al.,* 2012; Hammaini *et al.,* 2007; Kratochvil and Volesky, 1998; Fourest and Roux, 1992; Matheickal *et al.,* 1991).

Stability of MT-metal complexes depends on pH and the ionic strength of the medium (Blindauer, 2014). As mentioned in Section 1.1, high thermodynamic stability of MT-metal complexes requires low pH if all metal ions are to be dissociated from the protein. In these demetallation reactions, H^+ acts as an electrophile attacking thiolate sulfur. Though metal-bound thiolates are much less reactive than the metal-free ones, strong electrophiles can react with them, ultimately leading to the ejection of the metal ion. The usual result of the electrophilic attack is oxidation of adjacent thiol groups and formation of disulfide bridges. Stability of MT complex is expected to be reduced with an increase in ionic strength, and this has been demonstrated for bacterial single domain MT (*Synechococcus elongates*) and for plant MT (wheat). When ionic strength of the medium, *I*, increased from *I* = 4 mM to *I* = 10 mM, the stability constants of bacterial MT, changed from log K_{Zn} ~13 to log K_{Zn} ~10, and wheat MT from log K_{Zn} = 10.6 to log K_{Zn} = 8.6, respectively (Blindauer, 2014).

The pH of the solution can also be lowered by dissolving gasses that produce acid in contact with water. Carbon dioxide, when dissolved in water, produces carbonic acid according to the reaction (Earnshaw and Greenwood, 1997):

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$
$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Carbonic acid is weakly dissociated (K_1 = 4.5 x 10^{-7} M) and at standard conditions of pressure and temperature carbon dioxide is only partially dissolved in water. However, if introduced under pressure the solubility of CO_2 will increase in compliance with Henry's law:

$$p\mathrm{CO}_2 = x_{\mathrm{CO}_2} K_{\mathrm{CO}_2}$$

where: *p*, *x* and *K* refer to partial pressure, mole fraction and Henry's constant of CO_2, respectively (Atkins and de Paula, 2006). Under pressures found in a can of soda, for example, $p\mathrm{CO}_2$ = 2.5 bar, and at 25°C the dissociation of carbonic acid yields pH ~3.7 (Brooker, 1994).

Carbon dioxide has already been used in ion-exchange processes. Silva and Brunner (2006) used commercial ion-exchange columns to capture heavy metals (Cd^{2+}, Cu^{2+} and Pb^{2+}) from their single-species solutions. For regeneration of the columns after adsorption sub-critical solution of carbon dioxide and water (CO_2/H_2O) was used. It was concluded that the CO_2/H_2O system was suitable for removal of Cu^{2+} and Cd^{2+} from the tested commercial columns.

2. OBJECTIVES

2.1 General objective

The objective of the present work was to establish the growth conditions for bacterium, *Cupriavidus metallidurans* MTαβ4, genetically modified to express synthetic metallothioneins in laboratory scale and its suitability as Cd^{2+} and Ni^{2+} biosorbent.

2.2 Specific objectives

1. Select the optimal medium and parameters for cultivation of *C. metallidurans* MTαβ4;
2. Determine biosorption fractions of Cd^{2+} and Ni^{2+} from synthetic, single-species solutions;
3. Compare metal uptake of *C. metallidurans* MTαβ4 to that of parental strain *C. metallidurans* LMG 1195;
4. Determine the effects of exposure time on Cd^{2+} and Ni^{2+} biosorption; and
5. Determine and compare the recovery efficiency of Cd^{2+} from metal-loaded biomass using HCl, EDTA and CO_2/H_2O as meal-recovery agents.

3. MATERIAL AND METHODOLOGY

3.1 Bacterial strains

Cupriavidus metallidurans LMG 1195, obtained from BCCM/LMG bacteria collection of University of Gent, Belgium, and *Cupriavidus metallidurans* MTαβ4, resulted from the genetic manipulations of *C. metallidurans* LMG 1195, were used in the present work. Genetic engineering of *C. metallidurans* MTαβ4 is described in Text Box 2.

Text Box 2. Engineering of *Cupriavidus metallidurans* MTαβ4.

The gene that codes for *Crassostrea rhizophorae* (mangrove oyster) metallothionein, CrMT, was sequenced and characterized (Americo, 2014). It was discovered that its canonical form comprises two metal-binding domains, α and β that, similarly to those of mammalian species, can bind up to 7 divalent metals: 4 *via* its α-domain and 3 *via* β-domain. The two domains are connected by a short linker composed of three amino acid residues, Lys-Val-Lys (Americo, 2014).

The CrMT sequence served as the basis for construction of a synthetic gene, CrMTαβ4, which was heterologously expressed in *C. metallidurans* LMG 1195. The construction CrMTαβ4 comprises 4 copies *in tandem* of the original CrMT gene. In the CrMTαβ4 sequence, the short linker (Lys-Val-Lys) concatenates all four copies in one single protein (Americo, 2014).

In order to effectively express CrMTαβ4 gene in *C. metallidurans* LMG 1195, the bacterium was transformed with plasmid denominated pBBR1-SD-MTαβ4. The construction of this plasmid was based on pBBR1MCS plasmid, which is widely used for gene cloning in Gram-negative bacteria (Kovach *et al.*, 1994). A gene cassette was constructed *in silico* and synthesized *de novo*. The cassette was composed of a metal-regulated gene A (*mrg*A) promoter from *Bacillus subtilis* and an open reading frame (ORF) containing the following sequences: signal peptide *pel*B, synthetic gene CrMTαβ4, E-tag and β-domain of IgA protease. This cassette was inserted in plasmid pBBR1MCS thus generating recombinant plasmid named pBBR1-SD-MTαβ4 that was used to transform the *C. metallidurans* LMG 1195 strain. The recombinant strain was named *Cupriavidus metallidurans* MTαβ4. Chloramphenicol-resistance gene for selection of transformants was part of the unmodified pBBR1MCS plasmid.

The *mrg*A promoter, expressed constitutively, is inducible when heavy metals such as Cd^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} are added in the culture (Ribeiro-dos-Santos *et al.*, 2010). The signal peptide sequence of *pel*B of *Erwinia carotovora* directs newly synthesized protein to periplasmic space of the cell. The E-tag is an epitope used to further confirm the expression of recombinant protein and is recognized by commercial Anti-E-tag antibody. The β-domain of IgA protease from *Neisseria gonorrhoeae* is an autotransporter domain with functions to transport and anchor fusion protein on the outer surface of the cell membrane. The properties of wild type and recombinant gene products are summarized in Table 2.

More detailed information on the engineering of *C. metallidurans* MTαβ4 can be found in Americo (2014).

Table 2. Comparison between wild type (CrMT) and recombinant (CrMTαβ4) metallothioneins (Americo, 2014)

Gene	Number of amino acid residues in protein	Organization of domains	Number of cysteine residues	Number of divalent metal ligands (theoretical)	Molecular mass (kDa)
CrMT	75	αβ	21	7	7.40
CrMTαβ4	309	(αβ)4	84	28	30.61

3.2 Cultivation media

The following media were used for cultivation of *C. metallidurans* LMG1195 and *C. metallidurans* MTαβ4: Tris-salt mineral medium (TSM), Tryptic Soy Broth (TSB, from BD) and Tryptic Soy Broth with addition of 15 $g \cdot L^{-1}$ of Agar (TSA). TSM contained 20 $g \cdot L^{-1}$ of sodium gluconate, 6.06 $g \cdot L^{-1}$ of tris(hydroxymethyl)aminomethane, 8 $g \cdot L^{-1}$ of NaCl, 1.49 $g \cdot L^{-1}$ of KCl, 1.07 $g \cdot L^{-1}$ of NH_4Cl, 0.43 $g \cdot L^{-1}$ of Na_2SO_4, 0.2 $g \cdot L^{-1}$ of $MgCl_2 * 6H_2O$, 0.03 $g \cdot L^{-1}$ of $CaCl_2 * 2 H_2O$, 0.23 $g \cdot L^{-1}$ of $Na_2HPO_4 * 12H_2O$, 0.005 $g \cdot L^{-1}$ of Fe(III)(NH_4) citrate (Meregeay *et al*., 1985) complemented with 0.1% (v/v) of the trace element solution

SL 7 (Biebl & Pfennig, 1981). Additionally, several custom formulated media, labeled medium I, II and III were also prepared (Table 3). In all instances the media used for cultivation of *C. metallidurans* MTαβ4 were amended with 250 $\mu g \cdot mL^{-1}$ of chloramphenicol (Sigma-Aldrich).

Table 3. Composition of the custom formulated media and the concentrations of the compounds ($g \cdot L^{-1}$): Corn steep liquor (21.40); Tryptone (5.45); Casein (5.45); NaCl (5.00); Na_2HPO_4 (2.03); Na-gluconate (20.0). The *x* labels compounds used in each of the formulations.

Compound	Medium		
	I	II	III
Corn steep liquor	x	x	
Tryptone	x		x
Casein	x		
NaCl	x	x	x
Na_2HPO_4	x	x	x
Na-gluconate	x	x	x

3.3 Growth conditions

3.3.1 *C. metallidurans* LMG 1195. Petri dishes with TSA were streaked with a loopful of thawed glycerol stock of *C. metallidurans* LMG 1195 at 30°C for 48 h in order to obtain single colonies. One single colony was then transferred to 50 mL conical tube with 10 mL of TSM and the cultures were grown at 30°C and 180 rpm for 48 h. This volume was then added to 190 mL of TSM in 1 L Erlenmeyer flask. The cells were incubated at 30°C and 180 rpm for 48 h and the cell viability was checked according to Miles and Misra serial dilution method (1938) on TSA. The plates were incubated at 30°C for 72 h and the colonies were counted. The average cell viability was scored from colony counts from duplicate plates with the final dilution factor of up to $1*10^{12}$.

3.3.2 *C. metallidurans* MTαβ4. Plates with solid chloramphenicol-containing TSA (TSA-CM) were streaked with a loopful of thawed glycerol stock of *C. metallidurans* MTαβ4. After incubation at 30°C for 48 h a single colony was then transferred to 50 mL conical tube with 10 mL of liquid chloramphenicol-containing TSM (TSM-CM). The bacteria were grown at 30°C and 180 rpm for 48 h and then transferred to 190 mL of TSM-CM in 1 L Erlenmeyer flask. The cells were incubated at 30°C and 180 rpm for 52 h and the cell viability was checked by plating on TSA-CM as described by Miles and Misra (1938). The colonies were counted after 72 h incubation at 30°C. The average cell viability was scored from colony counts from duplicate plates.

3.3.3 Expression induction of synthetic metallothionein. Based on the previous work (Biondo *et al*., 2012). *C. metallidurans* MTαβ4 cells were grown in presence of 0.3 mM $CdCl_2$ and are referred in this text as *Cupriavidus metallidurans* ind-MTαβ4. Analytical-grade $CdCl_2$ (Sigma-Aldrich) was used to prepare 480 mM stock metal solution which was filter-sterilized. Appropriate amounts of this solution were added to 200 mL of TSM-CM reaching a final concentration of 0.3 mM $CdCl_2$ in medium. At the same time, the cultures were cultivated in the presence of increasingly concentrations of $CdCl_2$ namely: 0.003, 0.03, 3 and 30 mM. This was done so as to compare the effect of different inducer concentrations on the survival of the strain during the growth phase. All cultures were incubated at 30°C and 180 rpm for 24 h. The viability was checked by counting the number of colony-forming units after 72 h incubation on TSA-CM at 30°C. The average cell viability was scored from colony counts from duplicate plates.

3.4 Growth curve determination

Bacterial growth in liquid cultures was monitored as a function of number of viable cells with time. First, *C. metallidurans* MTαβ4 cells were incubated on TSA-CM plates at 30°C for 48 h in order to obtain spread colonies. A single colony was then transferred to 10 mL of TSM-CM in 50 mL conical tubes and the culture was incubated at 30°C and 180 rpm for 48 h. The volume of 5 mL of this culture was used to inoculate 20 mL of TSM-CM in 125 mL Erlenmeyer flask and the cells were incubated for additional 48 h. Finally, 20 mL of this culture was added to 380 mL of TSM-CM in a 2 L Erlenmeyer flask and the liquid culture was incubated for six days. The samples were taken in regular intervals of 4 h in order to determine the viability of the bacterial cells in the culture. In case of *C. metallidurans* ind-MTαβ4 this final culture volume (400 mL) was supplemented with 0.3 mM Cd^{2+}, as aforementioned. Cell viability on TSA-CM was checked as described earlier using duplicate samples. The plates with the final dilution factor of up to $1*10^{12}$ were incubated at 30°C for 72 h, and colonies were counted.

3.5 Recombinant protein expression analysis

Western blot assay was performed in order to confirm the expression of CrMTαβ4 gene in transformed *C. metallidurans* cells. Cells of *C. metallidurans* LMG 1195 and *C. metallidurans* MTαβ4 strains were grown in 10 mL of TSB and TSB-CM, respectively, at 30°C for 24 h. The cells were then pelleted by centrifugation at 10000•g for 1 minute. Supernatants were discarded and the pellets were resuspended in 1 ml of phosphate-buffered saline solution (PBS) and centrifuged again at 10000•g for 1 minute. Supernatants were discarded again and pellets resuspended in 50 µL of cracking buffer 2X (100 mM Tris-Cl pH 6.8, 4 % SDS, 0.02% bromophenol blue, 20% glycerol) (Barnard *et al.,* 2004). Samples were immediately boiled at 100°C for 10 minutes. A total of 15 µL of each sample was resolved in a 10% SDS-PAGE gel along the ladder Spectra Multicolor Broad Range (Thermo Scientific) in Tris/glycine/SDS running buffer as described by Sambrook and Russell, 2001.

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by wet transfer method using transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol), at 200 mA for 2 h. Membranes were blocked in skimmed milk (5%, diluted in Tris-buffered saline with Tween 20 (TBS-T), pH 7.6, TBS 50 mM, 150 mM NaCl, 0.05% Tween 20) for 2.5 h with agitation. Membranes were incubated with the primary antibody (Anti E tag, Abnova PAB12765, diluted 1:1000 in 5% skimmed milk in TBS-T) overnight with agitation, at 4°C. Membranes were washed three times with TBS-T for 5 min with agitation and then incubated with the secondary antibody (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling, #7074, diluted 1:2000 in 5% skimmed milk in TBS-T) for 1 h with agitation at room temperature. Membranes were washed three times with TBS-T for 5 minutes with agitation. Finally, the blot was developed using the reagent Luminata Forte Western HRP substrate (Millipore) and the imaging system ChemiDoc MP (BIO-RAD).

3.6 Biosorption of cadmium and nickel

Three independent biosorption experiments were conducted and each determination was carried out in triplicates. The cells were cultivated as described earlier and harvested by centrifugation at 4000•g, 4°C, for 10 min. The biomass was washed with sterile PBS, and re-centrifuged for additional 10 min (4000•g, 4°C). Supernatant was discarded and the resulting biomass was re-suspended in sterile, deionized water to produce a concentrated biomass suspension.

Analytical-grade $CdCl_2$ and $NiCl_2$ salts (Sigma-Aldrich) were used to prepare single metal solutions of 1 mM nominal concentration. The salts were dissolved in deionized water and then heat-sterilized in as described by Benson (2001). Conical tubes (50 mL) were then filled with 10 mL of single metal solution.

Equal volumes (1 mL) of the concentrated cell suspension were used for inoculation of those single metal solutions at 30°C and shaking at 180 rpm. Sampling intervals varied between experiments. In the first experiment, all strains were incubated with metals for 12 and 24 h. In the second experiment, samples of *C. metallidurans* ind-MTαβ4 were taken after 12 and 24 h, while the samples for *C. metallidurans* LMG 1195 and *C. metallidurans* MTαβ4 were taken after 24 h of incubation. In the third experiment, all strains were incubated for 3, 6, 9 and 12 h. At every sampling time the cell viability was checked as previously described on TSA-CM (*C. metallidurans* MTαβ4 and *C. metallidurans* ind-MTαβ4) and TSA (*C. metallidurans* LMG 1195). The plates were incubated at 30°C for 72 h, when the colonies were counted.

The biosorption samples were centrifuged at 4000•g, 4°C, for 10 min. Supernatants were separated from the biomass (pellet) by decantation and both phases were preserved by freezing and stored at -20°C until the time of analysis. The metal content was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) in accordance with Standard Methods for Examination of Water and Wastewater

(SMEWW) procedure 3120 (Greenberg *et al.*, 1992). Metal analyses of samples were completed in Laboratório de Análises Químicas e Ambientai Ltda – Epp (Rio de Janeiro, RJ).

3.7 Cadmium recovery from the loaded biomass

Concentrated *C. metallidurans* ind-MTαβ4 biomass suspension and Cd^{2+} solutions were prepared as previously described. Equal volumes of the concentrated biomass (1 mL) were transferred to 50 mL conical tubes containing 10 mL of 1 mM Cd^{2+} solution and incubated at 30°C and 180 rpm for 3 h. The samples were centrifuged at 4000•g, 4°C, for 10 min. Supernatants were separated from the biomass (pellet) by decantation and discarded. Obtained metal-loaded biomasses were treated with three metal-recovery agents: 1 M hydrochloric acid (HCl) solution, 1 mM ethylenediaminetetraacetic acid (EDTA) solution and CO_2-pressurized deionized water. The metal recovery, expressed as relative percentage, was calculated using the following formula:

$$(\%)\ \text{Recovery} = \frac{m_{ads} - m_{rec}}{m_{ads}} * 100$$

where: m_{ads} and m_{rec} (in mmol Cd^{2+} kg^{-1} biomass) stand for cadmium content in biomass before and after the metal recovery cycle, respectively. This allows for the efficiencies of different metal-recovery treatments to be compared. Another parameter that was monitored in all experiments was the cell viability. Viability was determined at different stages of the metal-recovery cycle: before loading the cells with cadmium, before the biomass exposure to metal-recovery agents and upon the completion of the recovery treatment. The control cells were incubated with the metal solution but were not exposed to the metal-recovery agents. Three separate experiments were performed in triplicates for each metal-recovery procedure. All samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) as per Standard Methods for Examination of Water and Wastewater (SMEWW) procedure 3120 (Greenberg *et al.*, 1992). Determination of metal contents in samples was performed in Laboratório de Análises Químicas e Ambientais Ltda – Epp (Rio de Janeiro, RJ).

3.7.1 HCl and EDTA. Hydrochloric acid (PA, Merck) and analytical grade EDTA (PA, Sigma-Aldrich) were diluted in deionized water to the concentrations of 1 M and 1 mM, respectively and sterilized in autoclave (Benson, 2001). One milliliter of each reagent was added to metal-containing biomass samples (pellets) and incubated for 15 min. The samples were centrifuged at 4000•g, 4°C, for 10 min; the pellets were frozen and kept at -20°C until being analyzed. Metal analysis was conducted by ICP-AES as already described.

3.7.2 CO_2/H_2O. The metal-recovery properties of CO_2/H_2O solution were investigated in Drechsel's flask (gas-scrubber bottle). Metal-loaded biomasses were first suspended in 50 mL of deionized water and then quantitatively transferred to 250 mL Drechsel's flasks. This type of vessel was chosen because of its

design that features a porous end of the gas inlet tube that causes bubbling of the injected gas which enhances the contact between gaseous and liquid phases. The volume of bacterial suspension in the flask was sufficient to keep the porous end of the gas-inlet tube submerged all the time during the CO_2 injection. Carbon dioxide (White Martins) was injected at the gauge pressure of 0.01 bar for the period of 15 min. The treated bacteria suspension was centrifuged at 4000•g, 4°C, for 10 min. The pellets were preserved by freezing at -20°C before being analyzed by ICP-AES, as aforementioned.

3.8 Statistical analyses

In order to determine whether the observed differences in metals concentrations in biomass and in supernatant were statistically significant, a nonparametric Kruskal-Wallis One-way Analysis of Variance was applied, followed by a pair-wise comparison using Dwass-Steel-Chritchlow-Fligner test. Time dependent relationships were tested by linear regression coefficients. All results were considered significant when $p < 0.05$. The statistical analysis was performed using Statistica v.8 software pack.

4. RESULTS AND DISCUSSION

Here we present a metal-sorption analysis of the genetically modified bacterium, *C. metalidurans* MTαβ4, engineered to express four copies *in tandem* of *Crassostrea rhizophorae* metallothionein (Text Box 2). This multidomain MT harbors high binding capacity of divalent metals ions (up to 28 per MT molecule). Because of the increased number of metal binding sites, it is expected that the resulting biosorbent outperforms not just *C. metallidurans* LMG 1195 as a parent strain but also *C. metallidurans* engineered strains previously reported in the literature (Valls *et al*., 2000; Biondo *et al*., 2012). To this end, *C. metalidurans* MTαβ4 biosorption properties were investigated in this work.

It is important to notice that the bacteria used in this study: *C. metallidurans* LMG 1195, *C. metallidurans* MTαβ4 and *C. metallidurans* ind-MTαβ4 are in the further text referred to as: LMG 1195, MTαβ4 and ind-MTαβ4, respectively.

4.1 Growth of MTαβ4 in different media

First, we set to demonstrate the possibility to cultivate MTαβ4 in laboratory conditions. The successful growth was observed both in chemically defined and in custom prepared formulations based on inexpensive simple reagents (Figure 1). For all tested media the number of viable cells after 24 h cultivation was around 1 x 10^9 CFU•mL^{-1} except in medium II. In that case the cell viability was approximately 10 times lower. MTαβ4 does not use dextrose as a C-source (Text Box 1) which may explain better growth in dextrose-free TSB compared to TSB. The highest viability was observed for cells grown in medium I (1.7 x 10^9 CFU•mL^{-1}) followed by medium III and TSM (1.3 x 10^9 CFU•mL^{-1}). In another study, sodium gluconate as a component of medium I, II and III was substituted with sodium lactate of the same concentration as the sole C-source, while the rest of the composition was left unchanged. The growth in lactate-containing media was very poor; the number of counted colonies was 5 to 6 orders of magnitude lower than in gluconate-containing counterparts (data not shown).

Despite the better results from cultivation in medium I and III, TSM [amended with chloramphenicol (250 µg•mL^{-1}) when required] was used in the further experiments (*i.e.*, biosorption and metal recovery). The decision was based on the following criteria:

- TSM has well defined composition that permits uniformity of the experimental conditions;
- TSM is a mineral medium with sodium gluconate as the only C-source, thus the possibility for interactions between metal ions and organic matter is minimized; and
- The phosphate content in TSM is sufficient to allow unrestricted cell growth but is low enough to prevent precipitation of heavy metals added in millimolar concentrations (Nies, 2016). The

precipitation would reduce the concentration of bioavailable metal and could interfere with the metal effect on bacterial cells.

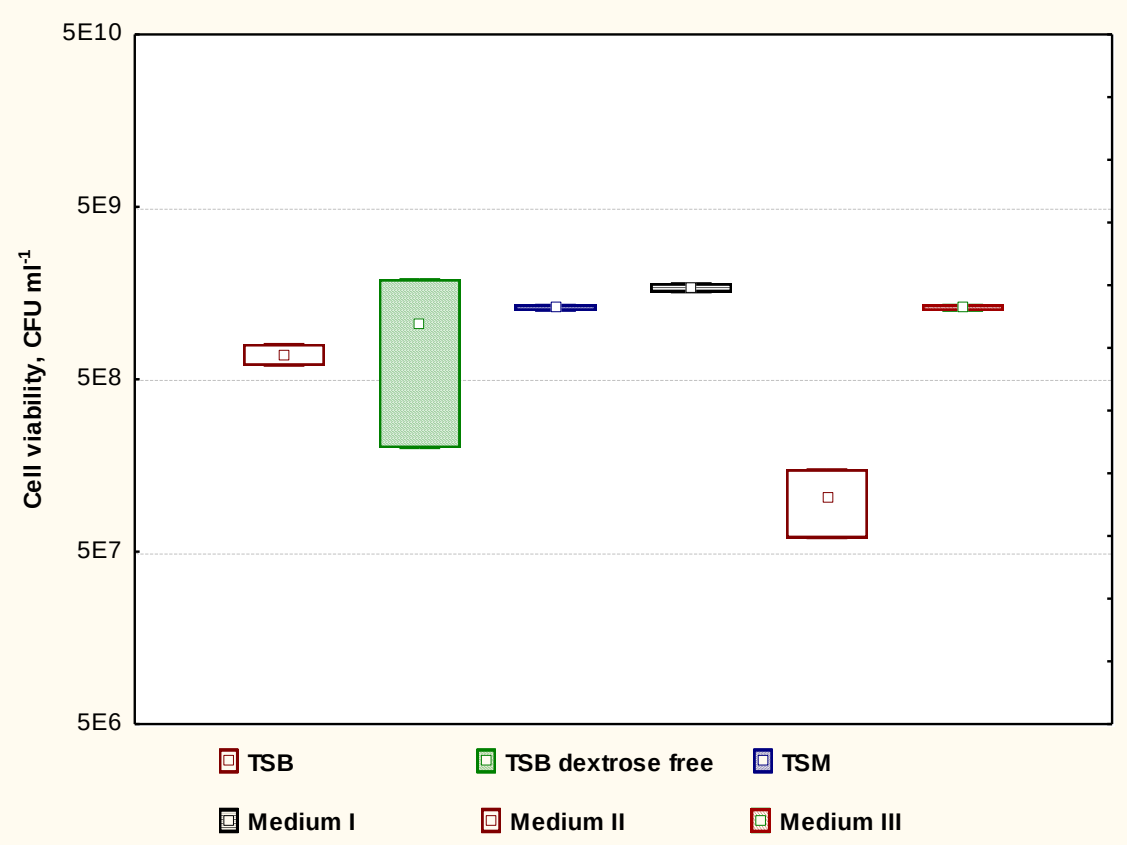

Figure 1. Viability of *C. metallidurans* MTαβ4 cells in different media determined after 24 h cultivation. The box plots indicate median values of two replicates with 25th and 75th percentiles. The compositions of media I, II and III are given in Table 1.

Medium I and III are composed primarily of readily obtainable simple reagents, such as corn steep liquor or casein (Table 3). Nevertheless, the growth of MTαβ4 cells was supported comparably well with other tested media (Figure 1). Therefore, these media look promising for future scaled-up cultivation of MTαβ4 biomass.

4.2 MTαβ4 viability in the presence of Cd^{2+} as inducer

The viability of LMG 1195 and MTαβ4 cells in the presence of different Cd^{2+} concentrations is shown in Figure 2. Both strains proliferated less in the presence of higher Cd^{2+} concentrations in the cultivation medium. LMG 1195 was the more resilient strain in this aspect: the cell growth was observed even at Cd^{2+} concentrations as high as 30 mM. In comparison, the highest tested metal concentration at which

the MTαβ4 cells were viable was 3 mM of Cd^{2+}. The number of viable cells of both strains in the presence of 0.003 or 0.03 mM Cd^{2+} was approximately the same, around 10^9 CFU•mL^{-1}.

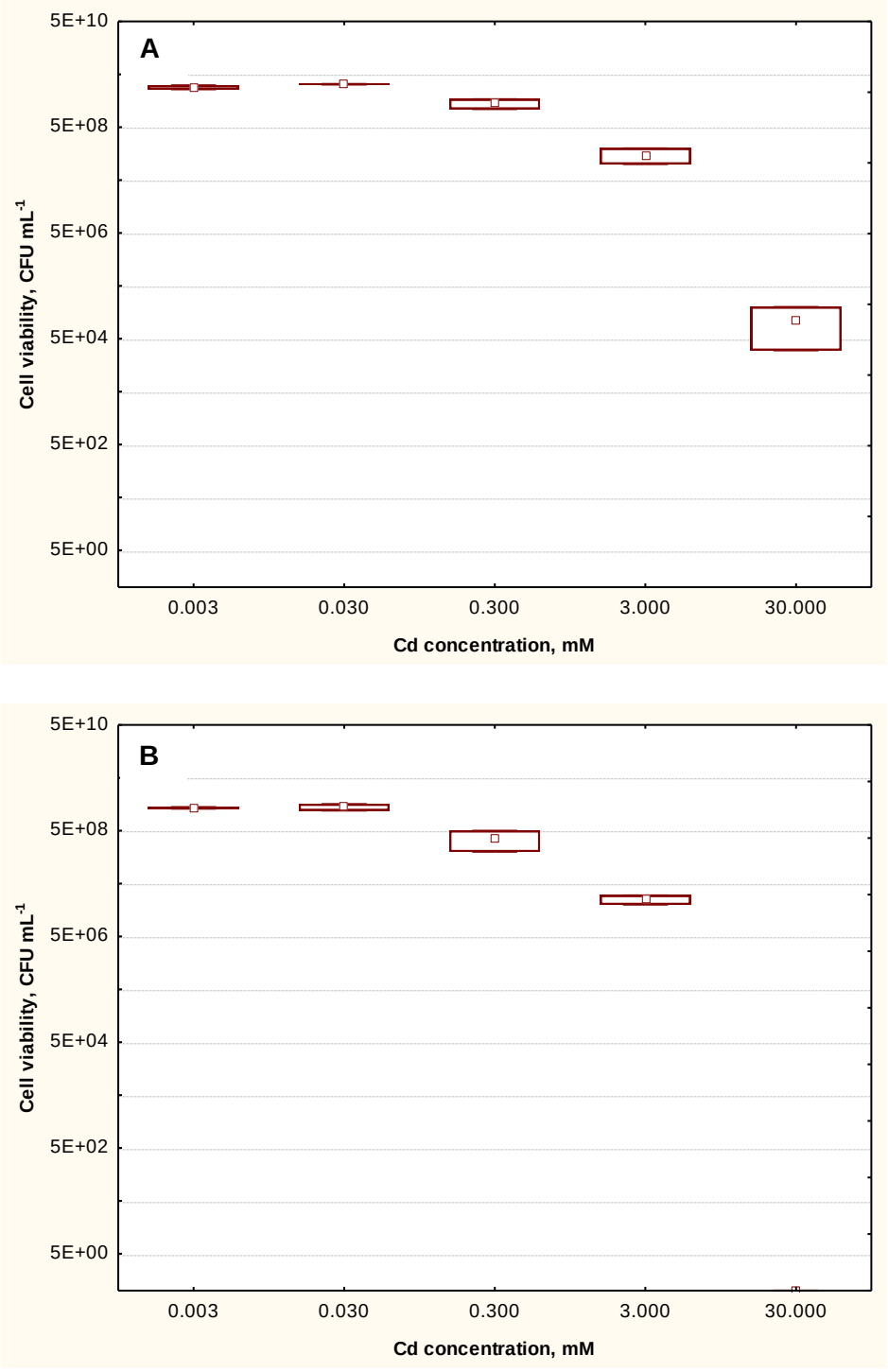

Figure 2. Cell viability of *C. metallidurans* cultivated for 48 h in presence of different Cd^{2+} concentrations: (A) LMG 1195; (B) MTαβ4. The box plots indicate median values of two replicates with 25th and 75th percentiles.

4.3 Growth curves of MTαβ4 and ind-MTαβ4

In order to better understand the growth profile of ind-MTαβ4 and MTαβ4 cells, determination of their growth curves were conducted and are showed in Figure 3. After monitoring bacterial growth for six days, it was shown that the growth dynamics of ind-MTαβ4 and MTαβ4 cells had similar profiles. Peak growth in 400 mL of medium (TSM) was reached at approximately 52 h (>10^9 CFU•mL-1). Therefore, these strains were cultivated for this period of time when biomass was prepared for biosorption and metal recovery experiments.

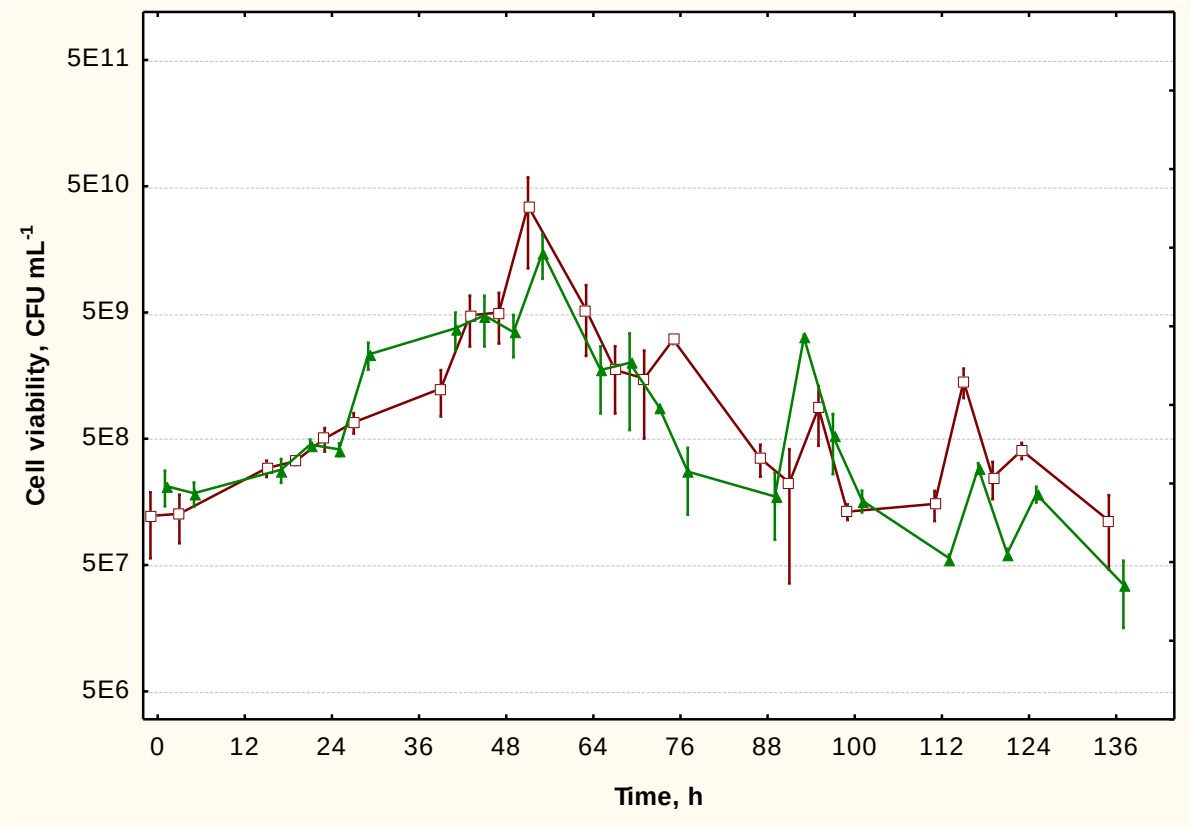

Figure 3. Growth curves of *C. metallidurans* ind-MTαβ4 (red) and *C. metallidurans* MTαβ4 (green) during 136 h. The plots represent the mean of duplicate samples ± standard deviation.

4.4 Analysis of recombinant protein expression

In order to confirm the heterologous expression of the recombinant CrMTαβ4, Western blotting experiments were conducted with the LMG 1195 and MTαβ4 strains. The band of protein with the expected size (~79 kDa) appeared in the MTαβ4 extract but not in the LMG 1195 one (Figure 4) demonstrating that successful expression of the CrMTαβ4 gene occured in *C. metallidurans*. Therefore,

the enhancement of biosorption capacity in modified strain should be ascribed to chelation properties of synthetic metallothioneins and their interaction with tested metals.

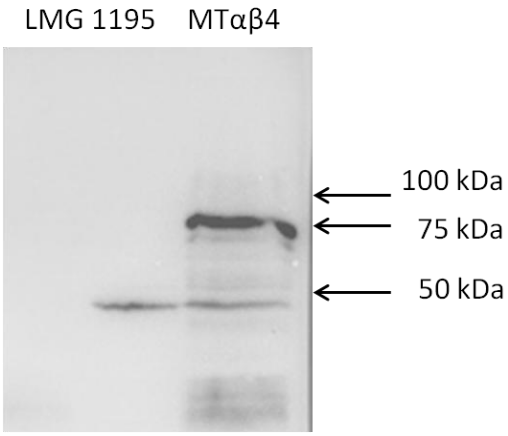

Figure 4. Western blot of the recombinant CrMTαβ4 protein (~79 kDa) expressed in *C. metallidurans*: LMG 1195 (left) and MTαβ4 (right). Cells were grown in TSB and TSB-CM, respectively, at 30°C for 24 h.

4.5 Biosorption of cadmium and nickel

Resistance of MTαβ4 strain to milli-molar Cd^{2+} levels that may be inhibitory to other bacteria such as *E. coli* has been discussed in section 4.2. However, the ability to survive in the presence of, and biosorption of heavy metals are two independent properties. Therefore, an increase in Cd^{2+} tolerance of the cells does not necessarily lead to increased biosorption of this metal (Sousa *et al.*, 1996). To determine whether the expression of recombinant protein increase the biosorption capacity of the engineered strain, MTαβ4 bacteria were exposed to Cd^{2+} and Ni^{2+} solutions. The concentrations of the metals in solution and in biomass were monitored along different time intervals in three independent experiments.

In the first, preliminary biosorption experiment, metal uptake by MTαβ4 and by ind-MTαβ4 bacteria was compared to that of LMG 1195 during a period of 24 allowing us to compare our results of biosorption with similar studies (e.g. Biondo *et al*., 2012). Figure 5 shows the change in metal concentration (Cd^{2+} or Ni^{2+}) in solution and in biomass for LMG 1195 (Fig. 5A, 5B), MTαβ4 (Fig. 5C, 5D) and ind-MTαβ4 (Fig. 5E, 5F). The data were fitted with linear regression method. The regression coefficients (*r*) were determined for the p-value of $p < 0.05$, and are presented on the biosorption plots.

From Figures 5A and 5B, it can be seen that biosorption of both metals by LMG 1195 increased with time. The final uptakes of Cd^{2+} and Ni^{2+} after 24 h were 5 and 3 $mmol \cdot kg^{-1}$ biomass, respectively. There was no clear evidence on how much was the removal of the two metals from solution by LMG 1195. Since Ni^{2+} was not detected in LMG 1195 biomass after 12 h it was not possible to calculate the relative increase in metal capture by MT-expressing cells for this condition.

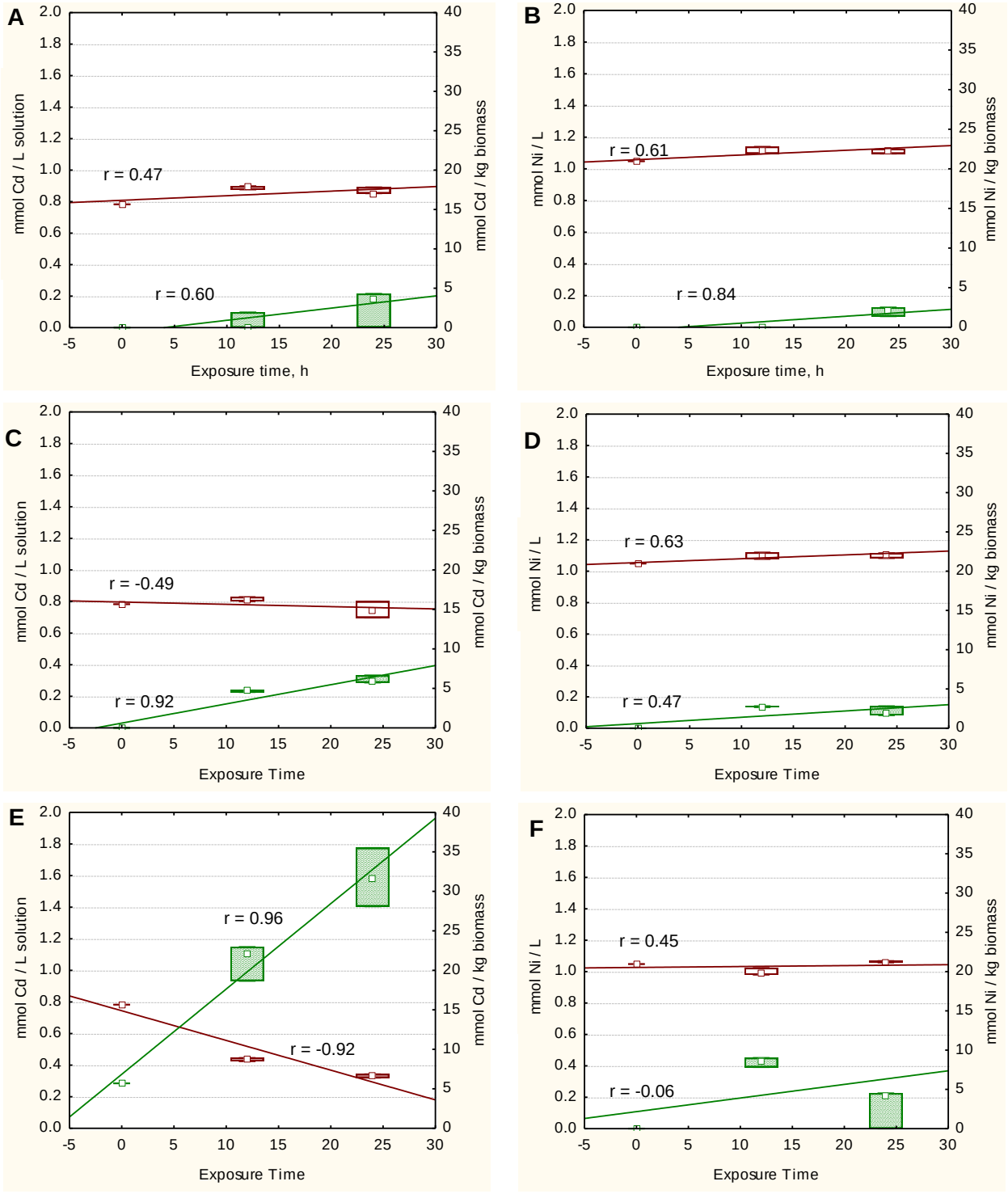

Figure 5. Metal concentration in solution (red) and in biomass (green) during the first biosorption test. The cells were incubated for up to 24 h in respective 1 mM metal solutions: LMG 1195 (A = Cd^{2+}; B = Ni^{2+}); MTαβ4 (C = Cd^{2+}; D = Ni^{2+}); ind-MTαβ4 (E = Cd^{2+}; F = Ni^{2+}). The box plots show median values of three replicates with 25^{th} and 75^{th} percentiles.

After 24 h, around 2 times more Ni^{2+} was biosorbed by ind-MTαβ4 than by LMG 1195. The amounts of Cd^{2+} found in MTαβ4 cells, following 12 and 24 h incubation, were 150 and 50% higher compared to LMG 1195, respectively. For the same conditions, ind-MTαβ4 biosorption was 6.5- and 6-fold higher. The shape of the adsorption curves (Fig. 5E) and linear regression coefficients (r = 0.96, Cd^{2+} in biomass; r = -0.92, Cd^{2+} in solution) suggest that longer exposure favors removal of Cd^{2+} by the ind-MTαβ4 strain. The absence of an adsorption curve plateau, even after 24 h, suggests that saturation of biosorbent did not occur. Removal of Cd^{2+} from solution was 44 and 57%, after 12 and 24 h, respectively.

Linear correlation was also established for capture of Cd^{2+} by engineered MTαβ4 (r = 0.92). The absolute amount of Cd^{2+} sequestered by the ind-MTαβ4 strain was higher relatively to the MTαβ4 one. Correspondingly, the removal of Cd^{2+} from solution was better by ind-MTαβ4 than by MTαβ4 (Fig. 5C, 5E). Regarding Ni^{2+} biosorption by MTαβ4 and by ind-MTαβ4 strains, r values were 0.47 and -0.06, respectively ($p < 0.05$). In both cases the concentration of this metal in biomass was higher after 12 h than after 24 h (Fig. 5D, 5F). Difference in binding affinity of metallothioneins is seemingly reflected in the results of biosorption, as out of the two tested metals, Cd^{2+} was adsorbed more efficiently than Ni^{2+} by both ind-MTαβ4 and MTαβ4 cells.

In the second biosorption experiment (Figure 6), the sampling times were repeated for the “best-performer” (ind-MTαβ4 strain), whereas LMG 1195 and MTαβ4 strains were analyzed after 24 h exposure only. It is worth noting that the metal concentrations in solution (data for LMG 1195, and MTαβ4 strains, to an extent) apparently increased with time. This is an impossible scenario since there was no additional input of these ions. The likely reason for this result is the lack of replicates at zero hour that led us to present a single value that did not incorporate the technical variability of the biosorption experiment samples. Nevertheless, the removal of Cd^{2+} and Ni^{2+} from solution by MTαβ4 and LMG 1195 after 24 h could not be determined accurately.

Linear correlation for biosorption by the LMG 1195 strain was established with good fit (Figure 6A, 6B), partially at least, because of the small number of sampling points. The enhancement in Ni^{2+} capture by metallothionein-expressing cells, compared to LMG 1195, was determined as 9-fold for ind-MTαβ4 and 2.5-fold for MTαβ4 strains. It was observed that the biosorption of Cd^{2+} by ind-MTαβ4 was higher after 12 h than after 24 h (16 and 10 mmol•kg^{-1} biomass, respectively). Therefore, the enhancement of biosorption properties after 24 h was less pronounced but still significant: induced cells adsorbed about two- and three times more Cd^{2+} than MTαβ4 and LMG 1195 strains, respectively, for the same incubation interval.

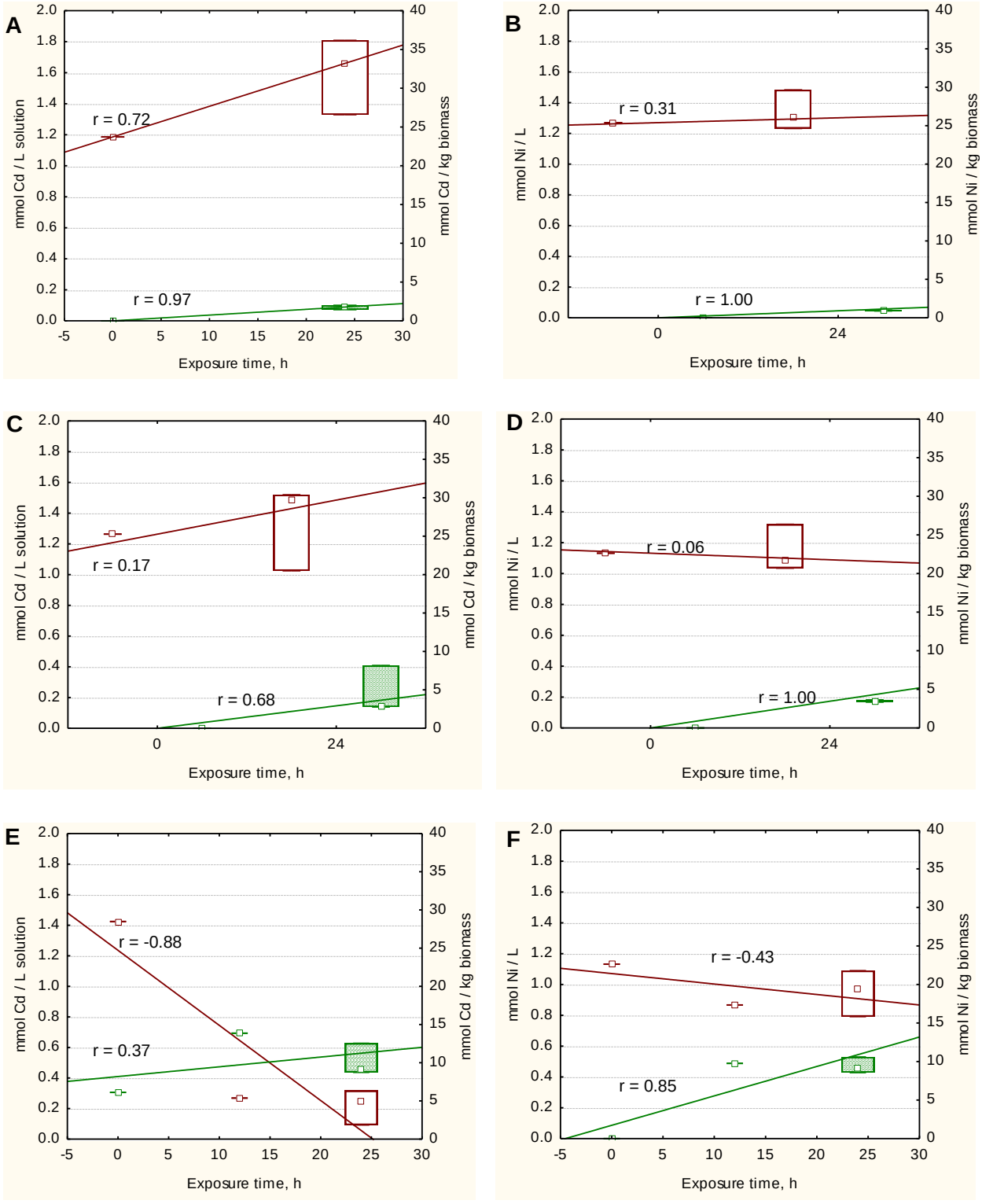

Figure 6. Metal concentration in solution (red) and in biomass (green) during the second biosorption test. Cells were incubated for up to 24 h in respective 1 mM metal solutions: LMG 1195 (A = Cd^{2+}; B = Ni^{2+}); MTαβ4 (C = Cd^{2+}; D = Ni^{2+}); ind-MTαβ4 (E = Cd^{2+}; F = Ni^{2+}). The box plots represent median values of three replicates with 25th and 75th percentiles.

It was noted earlier (in the first biosorption assessment) that genetically modified *C. metallidurans* cells showed greater affinity for binding Cd^{2+} over Ni^{2+}. This trend was observed again for the ind-MTαβ4 strain. After 12 and 24 h, respectively, residual concentrations in solution were reduced to around 0.27 and 0.22 mmol Cdf^{2+} L^{-1}, which corresponded to removal of 81 and 89%. Meanwhile, for the same respective intervals, the concentration of Ni^{2+} was reduced by ~30 and ~25%. The concentration curve of Cd^{2+} in solution showed good linearity (r = -0.88, Figure 6E), which was in agreement with previous experiment findings that longer exposure favors the sorption of this metal.

Finally, after the two "long exposure" experiments, we set to determine if shorter contact time between metal and bacteria could be beneficial for metal uptake. For that reason, in this part of the research, the biosorption time was limited to 12 h. Figure 7 shows the results of the third biosorption assay.

The results for the removal of Cd^{2+} by the ind-MTαβ4 strain agree with both previous tests which indicated that biosorption was favored by prolonged contact time between the metal and bacteria. The solution concentration was continuously reduced along the time reaching its lowest point at 12 h (r = -0.90, Fig. 7E). By this point, ~31% of Cd^{2+} was removed from the solution. In comparison, the maximal Cd^{2+} removal by the LMG 1195 strain was only around 5%. The reduction of metal content in solution was accompanied by its increase in biomass. After three hours, biosorption of Cd^{2+} by ind-MTαβ4 and MTαβ4 strains was 6 and 5-fold higher compared to LMG 1195, respectively. After 12 h the average amount of Cd^{2+} found in ind-MTαβ4 biomass was 5 times greater than that in LMG 1195 (5.3 and 1.1 mmol kg^{-1} biomass, respectively).

The shorter exposure to Ni^{2+} seemed to have benefitted the biosorption of this metal by MTαβ4 and ind-MTαβ4 strains with the maximal Ni^{2+} capture at 3 h, in both cases. For this sampling time the Ni^{2+} biosorption by MTαβ4 and ind-MTαβ4 strains were 15 and 20 times greater than that of LMG 1195, respectively. Further exposure times to metal ions resulted in desorption of Ni^{2+} in samples containing MTαβ4 and ind-MTαβ4 strains. After 12 h, the uptake by these strains was 2-fold higher, comparing to LMG 1195.

Despite the observed variability in results for both ind-MTαβ4 and MTαβ4 strains, it can be said, as a general conclusion, that the accumulation of metals by ind-MTαβ4 and MTαβ4 was greater for Cd^{2+} than for Ni^{2+}. The absolute amount of cadmium (given as mmol Cd^{2+} kg^{-1} biomass) varied from 15 to 30, for ind-MTαβ4 cells, and from 5 to 7, for MTαβ4 cells. At the same time the absolute amount of nickel (given as mmol Ni^{2+} kg^{-1} biomass) varied from 5 to 10, for ind-MTαβ4 cells, and from 3 to 7, for MTαβ4 cells. The absolute amounts of Cd^{2+} and Ni^{2+} accumulated by LMG 1195 (mmol metal kg^{-1} biomass) varied from 0.6 to 4, and from 0.4 to 2, respectively.

The differences in metal uptake also reflected in the biosorption relative enhancement. Considering the three experiments, cadmium biosorption by MTαβ4 cells increased 0.5- to 6-fold; cadmium biosorption by ind-MTαβ4 increased 1.5- to 6.5-fold, relatively to LMG 1195.

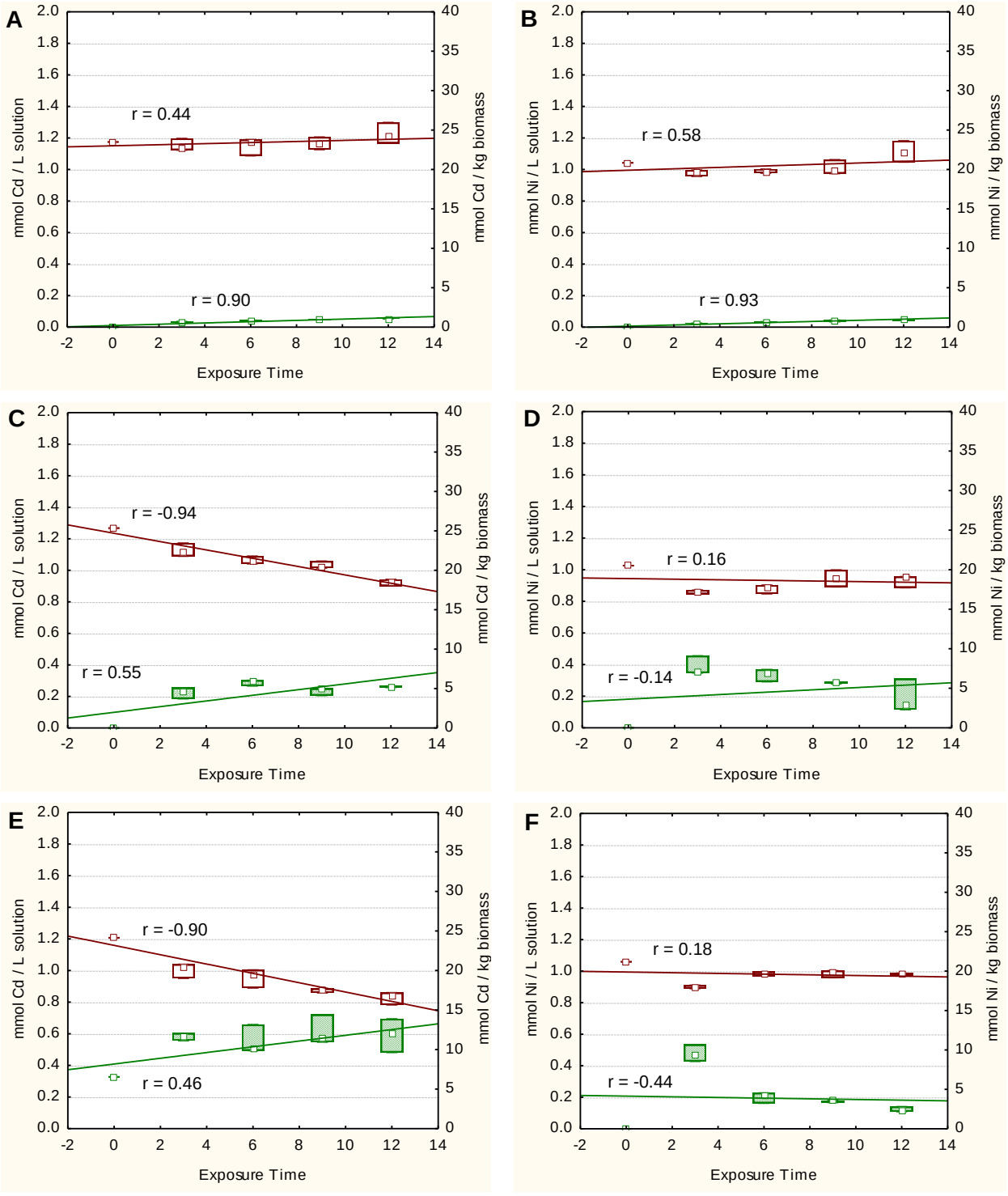

Figure 7. Metal concentration in solution (red) and in biomass (green) during the third biosorption test. The cells were incubated for up to 24 h in respective 1 mM metal solutions: LMG 1195 (A = Cd^{2+}; B = Ni^{2+}); MTαβ4 (C = Cd^{2+}; D = Ni^{2+}); ind-MTαβ4 (E = Cd^{2+}; F = Ni^{2+}). The box plots represent median values of three replicates with 25th and 75th percentiles.

Relative enhancements in Ni^{2+} biosorption by MTαβ4 and ind-MTαβ4 strains increased up to 15 times and up to 20 times, respectively. These values were reached after 3 h of metal contact with bacteria, indicating that, following this interval, a fraction of the bound Ni^{2+} was released from the cells' surface back to the solution. No difference in viability between the LMG 1195 and MTαβ4/ind-MTαβ4 strains for the tested time intervals was observed (data not shown). These results suggest that the different biosorption dynamics of Ni^{2+} and Cd^{2+} are not the result of increased toxicity of metals but more likely reflect the difference in binding affinity of MTs with the two metals. Therefore, even though the absolute capture of Cd^{2+} was bigger, the relative enhancement of Ni^{2+} biosorption was better.

Overall, the highest uptake of either metal was obtained with ind-MTαβ4 rather than by MTαβ4. This is a meaningful result for biotechnological purposes because the presence of the metallic species themselves can further enhance metal binding by bacteria. At the same time, it eliminates the need for addition of external inducers which can limit scalability of the process.

The use of recombinant bacteria for metal removal is not a new concept. Several groups of authors reported attempts to explore metal binding properties of metallothionein-like proteins and some have already been discussed earlier in the text. For example, Sousa *et al*. (1996) engineered *E. coli* in which poly-His peptides were transformed to express them on the cell surface, resulting in 11-fold increase in cadmium accumulation compared to the wild type strain. The same group of co-workers expressed human MT on the surface of *E. coli* (Sousa *et al.,* 1998) which further increased the bioaccumulation relative to the wild type *E. coli* parent strain (up to 20-fold).

However, Valls *et al.* (2000) were among the first ones to realize that, due to the low metal tolerance, *E. coli* is not the satisfactory choice for bioremediation applications, and that some other bacterial host should be explored for expression of metal-binding peptides. Therefore, they expressed mouse-MT in both *E. coli* and *C. metallidurans* LMG 1195 in order to compare the sorption capacities of the two strains producing the same poly-His peptide. The biosorption assays with *E. coli* were performed with 30 μM Cd^{2+}. More robust *C. metallidurans* recombinants were exposed to 30 and 300 μM Cd^{2+}. The uptake of cadmium by MT-expressing *E. coli* increased tenfold comparing its wild type strain. MT-expressing *C. metallidurans* had its binding capacity increased 3-fold for both 30 μM and 300 μM Cd^{2+} concentrations, though the total amount of bound metal varied proportionally to the initial metal content. It is important to observe that despite the difference in relative increases between the two MT-recombinants, the final amount of metal accumulated in both strains was approximately equal (14 mmol Cd^{2+} kg^{-1} dry biommas). That is because of higher metal-binding capacity of wild-type *C. metallidurans* strain comparing to wild-type *E. coli* strain. For example, metal uptake by the respective wild-type strain cells when grown with 30 μM Cd^{2+} was 1.5 and 5 mmol Cd^{2+} kg^{-1} dry biomass.

When MT-expressing *C. metallidurans* cells were cultivated in presence of higher Cd^{2+} concentration (300 µM), the absolute metal accumulation was 42 mmol Cd^{2+} kg^{-1} dry biomass. Even though the metal uptake was higher in comparison to 30 µM Cd^{2+}, the result corresponded to metal removal of only ~5%.

Almaguer-Cantú *et al.,* (2011) investigated Cd^{2+} biosorption properties of *E. coli* engineered to express mouse metalothionein in periplasimc space. The metal concentration (0.9 mM Cd^{2+}), the contact time (up to 24 h) together with the fact that the metal capture was performed from synthetic metal solutions instead of being added to growing bacteria, made this metal-binding assays comparable with the conditions used in our study. Maximal reported removal by MT-expressing *E. coli* strain was 39%. This result was observed after 6 h when an uptake plateau was reached indicating that saturation of biosorbent had occurred. However, the statistical analysis showed no significant difference between the recombinant and wild-type strain, indicating that the removal of Cd^{2+} might have happened because of unspecific binding rather than because of MT expression in periplasmic space.

Biondo *et al.,* (2012) introduced synthetic phytochelatins in *C. metallidurans* that were expressed on the outer surface of the cells. The cells were incubated in pure metal solutions (Cd^{2+} and Ni^{2+}) of the same nominal concentration as used in this work (1mM), in order to assess the enhancement of bioaccumulation in transformants. The obtained results are important because several studied parameters were similar to our work. This permitted comparison of biosorption efficiencies of the two recombinant biosorbents. For example, Biondo *et al.* (2012) used the same metal-inducible promoter and autotransporter for expression of phytochelatins explored here for expression of CrMTαβ4. Another similarity with our work is that the biosorption tests were carried out in synthetic aqueous solutions and not in culturing media amended with the target heavy metal. As noticed by Biondo *et al*. (2012) there is conflicting information in the literature regarding wild-type strain *C. metallidurans* metal-binding capacity. Therefore, better indication on biosorption enhancement would probably depend on the comparison of the results against the control strain of the same study and then to compare this relative increase with the ones from the previous studies. In their study, Biondo *et al*. (2012) reported enhancement in bioaccumulation of Cd^{2+} and Ni^{2+} after 24 h contact with respective metal solutions to be 59 and 45%, respectively.

Comparatively, we report here a novel biosorbent, *C. metallidurans* ind-MTαβ4 strain, for which the ability to immobilize Ni^{2+} and Cd^{2+} improved up to 20 and up to 6.5 times, respectively, relatively to the parental strain, *C. metallidurans* LMG 1195. This enhancement is much higher than what has been previously reported for *C. metallidurans* transformed to express metal-binding proteins.

4.6 Metal recovery from the biomass

The biosorption results have demonstrated that the ind-MTαβ4 strain was able to bind both tested metals, with cadmium seemingly bound more tightly. This led to a new set of experiments where recovery of this metal from the ind-MTαβ4 strain biomass was conducted. The two parameters that were monitored in this study were the percentage of the recovered metal from loaded biomass (Figure 8) and the cell viability after the treatment with metal-recovery agents (Figure 9). Monitoring of cell viability was carried out in order to see how the metal-recovery treatment affected the survival of the bacteria and therefore, their possible re-use. Importance of this step has been discussed earlier in Section 1.5. The number of viable cells before and after metal loading (steps which preceded the metal-recovery) basically remained unchanged in all experiments, which was ascribed to the high tolerance of the bacterium to Cd^{2+} (data not shown).

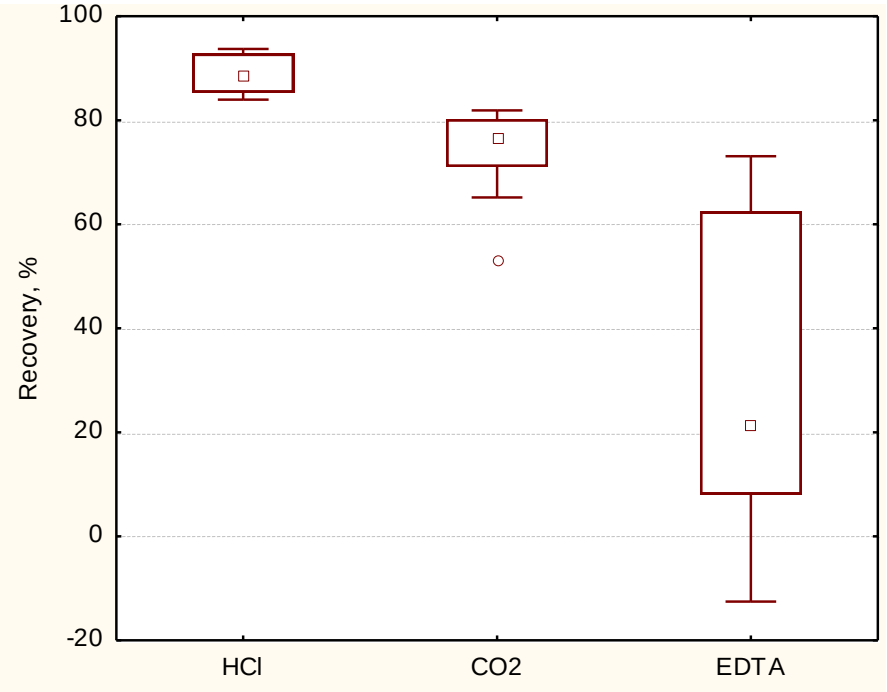

Figure 8. Cadmium recovery from metal-loaded *C. metallidurans* ind-MTαβ4 biomass. The cells were incubated in 1mM metal solution for 3 h, followed by 15 min treatment with the following metal-recovery agents: CO_2-saturated water, 1 M HCl, 1 mM EDTA. The results correspond to the median of the three experiments with interquartile ranges.

EDTA solutions have been frequently reported as efficient metal-recovery agents (Nunez-Lopez *et al.,* 2008; Vijayaraghavan and Yun, 2008). However, over three experiments the least reliable results were obtained with this compound (Figure 8). Variability between the results meant that the amount of recoverable cadmium could not be accurately determined (possibly between ~20 to ~60%). EDTA is a chelating agent with strong metal binding properties that competes with metallothionein molecules for Cd^{2+} ions. It also binds strongly other divalent metals like Ca^{2+} and Mg^{2+} and can cause the

destabilization of the outer membrane in Gram-negative bacteria (Vaara, 1992). However, as observed from Figure 9, there was no significant difference in the number of viable colonies between cells treated with EDTA and non-treated ones, which suggests that cells were unaffected by the treatment. In a similar study, Sousa *et al*. (1998) engineered MT-expressing *E. coli* strain. They preloaded recombinant cells with Cd^{2+} and subsequently exposed them to 0.5 mM EDTA for 15 min. Viability of recombinant cells was not affected either, and metal desorption was around 40%.

The efficiency of the acid wash in stripping metals off the biomass is well documented (e.g. Hammaini *et al.,* 2007; Costley and Wallis, 2001). Across the three recovery experiments of Cd^{2+} with HCl there was small variability in results with the average metal recovery of approximately 90% (Figure 8). Exposure to 1 M HCl however, led to a complete disintegration of the biomass after 15 min incubation. Cellular disruption, in addition to the ion-exchange process, was the probable reason for the highest observed efficiency (~90%) among the tested metal-recovery methods. This finding is in agreement with a previous report where HCl was used as an efficient eluent of metals from the activated sludge (Hammaini *et al.,* 2007). Metal recovery in the mentioned study was >90%; however, the aggressive effect of HCl on biological structures limited its efficient use as an eluent to only one sorption–desorption cycle (Hammaini *et al.,* 2007).

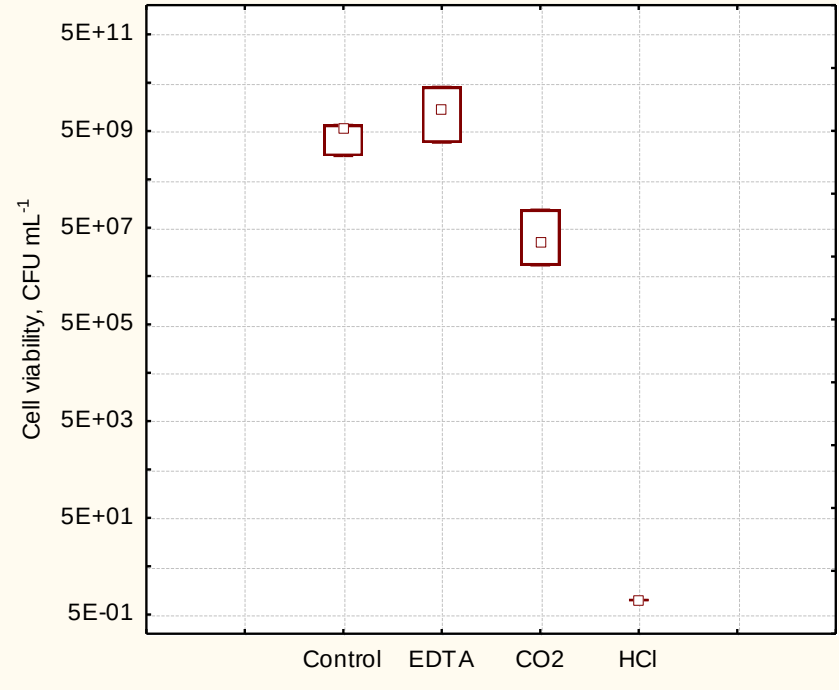

Figure 9. Cell viability after 15 min treatment with metal-recovery agents: non treated cells (inverted triangles); 1 mM EDTA solution (squares); CO_2 (circles); 1 M HCl solution (triangles). The results correspond to the median of the three experiments with interquartile ranges.

Recovery of Cd^{2+} with CO_2/H_2O solution also showed small variation between samples. Carbonic acid, produced when CO_2 dissolves in water, is a weaker acid than hydrochloric acid, hence less aggressive. Consequently, greater number of cells managed to survive the metal recovery process: the number of viable colonies was reduced by about 100-fold which is much less than the 10^9 reduction seen in the case of HCl (Figure 9). With the average amount of metal extracted from the biomass of approximately 70%, the CO_2/H_2O-based recovery represents a compromise between the efficiency of HCl and the cell survival of EDTA treatment. The lack of the data in literature did not allow us to make a comparison on CO_2-based desorption efficiency with other studies.

Therefore, out of the three metal-recovery agents used in this work the most efficient one was 1 M HCl wash with average metal recovery of approximately 90% but was also the most cellular degrading. The average metal recovery of the CO_2-based method was approximately 70% and represents a compromise between the efficiency of HCl and the cell survival of EDTA treatment. Finally, the lack of consistency makes EDTA the least reliable of the tested metal-recovery agents in this study. It should be noted that the 'less efficient' one-step CO_2/H_2O-mediated recovery process, compared to HCl-wash recovery, allows the reutilization of the ind-MTαβ4 strain; this, in turn, may make the overall CO_2/H_2O-based process to be more efficient than the HCl-wash recovery one, because of the added advantage that is the possible reuse of the engineered strain.

As a concluding remark of this study, it has been shown that heavy metals can be removed from solution with an efficiency of over 80%, whereas the captured metals can be recovered in the metal-biosorption process with an efficiency of up to about 90%. Therefore, for one complete biosorption cycle, where it is desired to remove metals from solution and recover metals back for reuse, the combined efficiency of adsorption-desorption process can be in excess of 70%. This means that more than two thirds of dissolved metal, which may have been previously considered as waste and/or pollutant, could be recovered with *Cupriavidus metallidurans* MTαβ4 reinforcing this strain's biotechnological potential for the cleanup of metal-bearing wastewaters and effluents.

5. CONCLUSIONS

1. *C. metallidurans* MTαβ4 engineered strain was successfully cultivated in laboratory in volumes up to 400 mL. Induction with 0.3 mM Cd^{2+} concentration was chosen as the best to express MTαβ4, and optimal time for cell growing after 52 h.
2. *C. metallidurans* MTαβ4 strain can biosorb both Cd^{2+} and Ni^{2+} from the single-species solutions.
3. *C. metallidurans* MTαβ4 strain has enhanced biosorption properties compared to parental *C. metallidurans* LMG 1195. Up to 6.5 times more Cd^{2+} and up to 20 times more Ni^{2+} were captured by MTαβ4 strain compared to LMG 1195 strain.
4. The uptake of Cd^{2+} by *C. metallidurans* MTαβ4 strain was favored by longer exposures to metal; shorter intervals suited better for Ni^{2+} uptake.
5. The most efficient method for recovery of biosorbed Cd^{2+} from loaded *C. metallidurans* MTαβ4 biomass was HCl-wash, while the biggest potential for the re-use of the biomass was the CO_2/H_2O treatment.

6. PERSPECTIVES

Even though it has been demonstrated that *C. metallidurans* MTαβ4 can capture cadmium and nickel ions form their synthetic solutions, variability in results of the independent experiments imply that more biosorption test should be carried out in order to demonstrate the repeatability of the biosorption results. For these, the adequate biosoption isotherms will be established. Additionally, the capture of other industrially interesting metals, such as lead, cobalt and zinc from their respective pure solutions will be tried and tested. As a mid-term plan, the scalability of the bacteria cultivation will be conducted in bioreactors (working volume of up to 10 L) so as to demonstrate that the cultivation of bigger amounts of biomass is feasible. The ultimate aim is to test the biosorption capacity of *C. metallidurans* MTαβ4 when exposed to real metal-bearing solutions such as effluents from smelting factories.

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