Application of *Cupriavidus metallidurans* and *Ochrobactrum intermedium* for Copper and Chromium Biosorption

A Thesis

Presented to

the Faculty of the Department of Environmental Engineering

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Environmental Engineering

by

Jingjing Fan

May 2013

Application of *Cupriavidus metallidurans* and *Ochrobactrum intermedium* for Copper and Chromium Biosorption

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Acknowledgements

I wish to express my sincere gratitude to Dr. Debora F. Rodrigues for her kind guidance, supervision, unconditional support and encouragement throughout the entire period of this study. I would also like to thank Dr. Catherine Santos for her helpful suggestions during the course of this project. I am also grateful to the thesis committee for giving valuable suggestions on this research project. Additional thanks go to my lab mates in my research group: Tugba Onal Okyay, Farid Ahmed, Sean Smith, and Nuygen Hang.

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Abstract

Heavy metals at high concentrations are toxic to the environment and living organisms. The present study aims to investigate the best conditions for the removal of $CuSO_4.5H_2O$ and $KCrO_4$ by two bacterial species: *Ochrobactrum intermedium* and *Cupriavidus metallidurans.* The minimum inhibitory concentrations of *C. metallidurans* and *O. Intermedium* were determined to be 750 and 300 ppm for Cu^{2+}, and 100 ppm and 1000 ppm for Cr^{6+}, respectively for each microorganism. Biosorption experiments were also performed with dead and live biomasses of *C. metallidurans* and *O. intermedium*. The results show that dead biomass presented better Cu^{2+} biosorption capacity than live cells. Chromium was removed more efficiently by live cells of *O. intermedium* than dead biomass; while *C. metallidurans* dead biomass biosorbed better than live biomass. The biosorption results fitted well with the Langmuir isotherm model. The main mechanism of live biomass adsorption was determined to be through carboxylic, hydroxyl, and amino functional groups.

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1. Introduction

Rapid industrialization has led to increasing environmental impacts. For instance, effluent discharges from textile, electroplating, and battery industries are commonly contaminated with heavy metals (Dang et al., 2009). Unlike organic pollutants, heavy metals are non-biodegradable, accumulate in living organisms via the food chain, and some of these metals are extremely toxic in relatively low concentrations. Therefore, environmental contaminations with heavy metals are of great concern to human health and diverse ecosystems (Ahmad et al., 2006; Celik & Demirbas, 2005; H. Li et al., 2010). Chromium, which can exist as hexavalent, is commonly associated with cancer and hemorrhaging in the digestive tract (Faisal & Hasnain, 2004). Exposure to high concentrations of Cu^{2+}causes anemia, liver, and kidney damage (Kiran et al., 2005). Therefore, problems arising from heavy metal pollution require urgent removal solutions.

Conventional methods have been used to remove heavy metals from water and wastewater, including chemical reduction and precipitation (Esalah et al., 2000), coagulation, flotation (Zouboulis et al., 1997), activated carbon adsorption (Ravindran et al., 1999), ion exchange, reverse osmosis and electro dialysis (Canet et al., 2002). However, chemical precipitation is only effective when the metal concentration is low. Other methods are, in most cases, expensive and inefficient in terms of energy and chemical products consumption, especially at low metal concentrations between 1~ 100 mg/L (H. Li et al., 2010; J. L. Wang & Chen, 2009). Therefore alternative methods,

which are both economical and efficient, are highly desirable.

Diverse studies have suggested the efficacy of bacterial biomass for use as an inexpensive biosorbent for the removal of metal ions (Ahmad et al., 2006; Gabr et al., 2008; Tangaromsuk et al., 2002). In these investigations, microorganisms were shown to resist, detoxify and adsorb heavy metals (W. M. Chen et al., 2008; Gabr et al., 2008; Huang et al., 2013). The mechanisms of microbial resistance to heavy metals include the formation and sequestration of heavy metals in complexes, reduction of a metal to less toxic species, and direct efflux of metal from the cell (Teitzel & Parsek, 2003). However, these resistance mechanisms were shown to be dependent in the bacterial growth phase. In recent studies, stationary-phase cells presented higher resistance to a variety of antimicrobial agents, including heavy metals, due to their different membrane lipid compositions (Chandran & Love, 2008; Spoering & Lewis, 2001; Yang et al., 2007).

In addition to resistance, biosorption also plays a crucial role in bioremediation of heavy metals. Biosorption can be defined as the capacity of biomass to bind to selected ions or other molecules present in aqueous solutions (Ajjabi & Chouba, 2009; Chang et al., 1997). The biosorption process may involve intricate mechanisms, such as complexation, ion exchange, adsorption, and micro-precipitation. Metal ion biosorption could be due to electrostatic interaction between metal cations and negatively charged sites, such as phosphoryl, phosphate, carboxyl, sulfate, amino, and hydroxyl groups, on the biosorbent surface (Gabr et al., 2008; H. Li et al., 2010). Furthermore, bacteria may

produce macromolecules outside their own cell wall, commonly called extracellular polymeric substances (EPS). Studies have shown that EPS are also responsible for biosorption of heavy metals (Quintelas et al., 2011) .

In the literature, more and more microorganisms have been employed as effective biosorbents in the removal of metal ions (Scott & Palmer, 1990; Tunali, Cabuk, et al., 2006). Other studies have demonstrated that dead biomass can be more effective in removing heavy metals than live biomass (Doshi et al., 2007; Mane et al., 2011; Munoz et al., 2012). However, to the best of our knowledge, detailed comparative mechanistic investigations on heavy metal biosorption by dead and live biomasses have not been fully assessed for their potential use for bioremediation. Furthermore, all investigations so far on microbial biomass biosorption were performed under the microorganims' optimum pH and temperature (Ahmad et al., 2006; Faisal & Hasnain, 2004; Isik, 2008; Lu et al., 2006). In real environmental applications, microorganisms are not usually kept at their optimum conditions for the removal of heavy metals; therefore, it is imperative to investigate their heavy metal removal performance under environmental conditions.

In the present study, two bacterial species isolated from sites contaminated with heavy metals, *Ochrobactrum sp*. and *Cupriavidus metallidurans,* were investigated. *Ochrobactrum sp*., a new isolate from Brazil, was obtained from leachate samples generated by a lysimeter containing organic wastes and heavy metals. *C. metallidurans* was selected as a model organism for comparison with the latter, because it has been

extensively described as one of the best biosorbents (W. M. Chen et al., 2008; Guine et al., 2007; Ledrich et al., 2005; Monsieurs et al., 2011; Rojas et al., 2011). This study aims to investigate their resistance to Cu^{2+} and Cr^{6+}, maximum adsorption capacity, the effect of growth phases, their behavior under different pHs and temperatures, and their potential mechanisms of adsorption to heavy metals. Competitive biosorption assays with both Cu^{2+} and Cr^{6+} were also investigated.

2. Literature Review

2.1 Background

Heavy metals released into the environment from metal plating facilities, mining operations, fertilizer industries, tanneries, batteries, paper industries and pesticide activities pose a significant threat to the environment and public health (Musico et al., 2013; G. Ozdemir et al., 2003). Unlike organic pollutants, heavy metals are non-biodegradable, have the tendency to accumulate in living organisms via the food chain, and present extreme toxicity at relatively low concentrations.

There are several heavy metals like Cu^{2+}, Ni^{2+}, Pb^{2+}, and Cr^{6+} that can cause serious impact (Ahmad et al., 2006; Celik & Demirbas, 2005; H. Li et al., 2010). For example, chromium, specifically the hexavalent species, is commonly associated with cancer and hemorrhage in the digestive tract (Faisal & Hasnain, 2004). Exposure of excessive concentrations of Cu^{2+} will cause anemia, liver, and kidney damage (Kiran et al., 2005). Lead damages many body organs, especially the central and peripheral nervous systems and the kidneys. Lead can also cause stillbirth or otherwise affect the fetus (L. Deng et al., 2007). Nickel has an appreciable phytotoxicity, which can cause damage in plant and fish species. Table 1 shows other heavy metals of concern and their major effects. The fact that heavy metals can cause serious health and environmental issues led to various regulatory agencies to set maximum limits for their discharge in the environment. The permissible limits of heavy metals by international regulations are

also given in Table 1. Even though there are strict regulations for heavy metal disposal and release into the environment, especially in U.S., there are several activities like industrial and agricultural activities or wastewater irrigations that frequently lead to release of heavy metals at levels that are hazardous to health and the ecosystem.

Table 1: Permissible limits and health effects of various toxic heavy metals (Sud et al., 2008).

Metal contaminant	Permissible limits by international bodies (µg/L)		Health hazards
	WHO	USEPA	
Arsenic	10	50	Carcinogenic, produce liver tumor, skin and gastrointestinal effects
Mercury	01	02	Corrosive to skin, eyes and muscle membrane, cause dermatitis, anorexia, kidney damage and severe muscle pain
Cadmium	03	05	Carcinogenic, cause lung fibrosis, dyspnea and weight loss
Lead	10	05	Suspected carcinogen, loss of appetite, anemia muscle and joint pains, diminish IQ, cause sterility, kidney problems and high blood pressure
Chromium	50	100	Suspected human carcinogen, produce lung tumor and allergic dermatitis
Nickel	-	-	Cause chronic bronchitis, reduce lung function, cause lung cancer and sinus problems
Zinc	-	-	Cause short-term illness called " metal fume fever" and restlessness
Copper	-	1300	Long term exposure cause irritation of the mouth and eyes, headache, stomachache, dizziness, diarrhea

Heavy metal pollution urgently requires clean-up strategies to maintain environmental safety. Conventional methods have been used to remove heavy metals, including reduction and precipitation (Esalah et al., 2000), coagulation, flotation (Zouboulis et al., 1997), adsorption on activated carbon (Ravindran et al., 1999), ion exchange and reverse osmosis (Canet et al., 2002). However, chemical precipitation is only partially effective when the metal concentration is low. Other methods are, in most cases, expensive in terms of energy and chemical product consumption, and ineffective, especially at low metal concentrations ranging from 1-100 mg/L, which is still generally much higher than the levels recognized by the WHO, etc (H. Li et al., 2010; J. L. Wang & Chen, 2009). Another limitation includes the generation of toxic sludge waste products that require careful disposal. These drawbacks of traditional metal removal techniques have led researchers to investigate alternative methods, which are both economical and efficient. The comparison among conventional metal removal technologies is presented in Table 2.

2.2 Definition of biosorption

Biosorption is defined as a passive metal uptake by biomass, which may be non-living (Holan & Volesky, 1994). In recent years, investigations on the mechanisms of biosorption have intensified since biomass can be employed to sequester heavy metals from industrial effluents or recover precious metals from processing solutions (Davis et al., 2003). Metal sequestration by different parts of the cell can occur via complexation,

coordination, and chelation of metals, ion exchange, adsorption and inorganic microprecipitation. The comparison of the conventional technologies and the biosorption technology is presented in Table 3. The major advantages of biosorption over conventional treatment methods include: low cost, high efficiency, minimization of chemical or biological sludge, no additional nutrient requirement, and regeneration of biosorbents and possibility of metal recovery (Sud et al., 2008). This cost advantage of the biosorption technology can facilitate the wide implementation and acceptance by heavy metal producing and polluting industries (Demirbas, 2008).

Table 2: Conventional metal removal technologies from wastewater treatment (O'Connell et al., 2008).

Method	Disadvantage	Advantage
Chemical precipitation	(a) Difficult separation (b) Disposal of resulting toxic sludge (c) Not very effective	(a) Easy to operate (b) Relatively cheap
Electrochemical treatment	(a) Applicable for high metal concentrations (b) Sensitive to specific conditions	(a) Metal recovery
Reverse osmosis	(a) Application of high pressures (b) Membrane scaling/fouling (c) Expensive	(a) Pure effluent/permeate
Ion exchange	(a) Sensitive to the presence of particles (b) Expensive resins	(a) Effective (b) Possible metal recovery
Adsorption	(a) Not very effective for certain metals	(a) Conventional sorbents

2.3 Biosorbents

2.3.1 Waste materials of food and agricultural by-products as biosorbents

Agricultural by-products have been widely studied for metal removal from water. Peat, wood, pine bark, and cotton hull have been demonstrated to remove heavy metals from wastewater (Aman et al., 2008; Fu & Wang, 2011; Kaczala et al., 2009; H. J. Park et al., 2007). These agricultural waste biosorbents are inexhaustible, cheap non-hazardous, selective for different heavy metals, and can be easily disposed by incineration (Das et al., 2008). Other forms of inexpensive, non-living plant material widely investigated as potential biosorbents for heavy metal removal (Fu & Wang, 2011) are potato peels (Aman et al., 2008), sawdust (Kaczala et al., 2009), eggshell (H. J. Park et al., 2007), and citrus peels (Schiewer & Patil, 2008).

Table 3: Comparison of various metal-removal technologies (Kapoor & Viraraghavan, 1995).

Technology	Properties of each technology					
	Concentration dependence	pH	Suspended solids	Effluent concentration (mg/L)	Regeneration	Sludge generation
Biosorption	Yes	Yes	Yes	<1	Yes	No
Hydroxide ppt	No	No	Yes	2-5	No	Yes
Sulfide ppt	No	No	Yes	<1	No	Yes
Ion exchange	Yes	Some	No	<1	No	Yes
Reverse osmosis	No	Some	No	1-5	No	No
Activated carbon	-	-	-	-	No	No
Adsorption	Yes	Some	Yes	1-5	Yes	No
Evaporation	Yes	Yes	Yes	1-5	Yes	No

2.3.2 Eukaryotes as biosorbents

Algae are eukaryotic organisms that contain chlorophyll and carry out oxygenic photosynthesis. These organisms are considered to be renewable natural biomasses that proliferate ubiquitously and abundantly. These characteristics have attracted the attention of many investigators to test and use these organisms as new adsorbents of metal ions (Fu & Wang, 2011). Advantages in applying algae as biosorbents include the wide availability, low cost, high metal sorption capacity and reasonably regular quality. In the study of Romera and collaborators (Romera et al., 2007), six species of algae, namely, *Codium vermilara, Spirogyra insignis, Asparagopsis armata, Chondrus crispus, Fucus spiralis* and *Ascophyllum nodosum* were tested as biosorbents to remove five different heavy metals: cadmium, nickel, zinc, copper and lead. They concluded that the six species achieved effective removal of the five metals. Three brown algae: *Macrocystis pyrifera, Kjellmaniella crassiforia,* and *Undaria pinnatifida* were shown to effectively biosorb Cd^{2+} (Seki & Suzuki, 1998). The biosorption of Cu^{2+} and Zn^{2+} was also accomplished by dried marine green macroalga (*C. linum*) at pH 5 (Ajjabi & Chouba, 2009).

Fungi and yeast are also eukaryotic organisms that are easy to grow, produce high yield of biomass and at the same time can be manipulated genetically and morphologically (Kapoor & Viraraghavan, 1995). The fungi are also widely used in a variety of large-scale industrial fermentation processes. Fungi biosorbents include

Penicillium spinulosum, which has high removal capacity for Cu^{2+} and Zn^{2+} (Townsley et al., 1986), *Rhizopus arrhizus*, which is capable to uptake Cr^{6+} and Cu^{2+} (Tobin et al., 1984), *Aspergillus terrus* and *Mucor ramanniunus,* which also exhibit high sorption capacities for Ca^{2+} than activated carbon and ion-exchange resins (Azab & Peterson, 1989). Furthermore, there are a number of reports describing the removal of copper ions using NaOH treated *Rhizopus oryzae* biomass in batch reactors (Bhainsa & D'Souza, 2008). The maximum copper adsorption capacities of viable or pretreated biomasses were 19.4 and 43.7 mg/g, respectively (Fu & Wang, 2011).

2.3.3 Prokaryotes as biosorbents

Microbial removal of diverse heavy metal ions from wastewater has been indicated as a highly effective method (Veglio & Beolchini, 1997). Bacteria show a panoply of responses to metal ions; furthermore diverse bacterial groups, such as anaerobic, aerobic and thermophilic, have developed abilities to cope with these toxic elements in a variety of environments. With respect to pollution control, these microbes show great promise for: (1) hastening the mobilization of metals (with possible application in biomining) (2) designing metal-tolerant strains that are better adapted to performing biodegradation of organic pollutants, and (3) metal bioremediation or mitigation through the breeding of natural or engineered strains (Valls & de Lorenzo, 2002). Bacteria including *Bacillus cereus* (Pan et al., 2007), *Escherichia coli* (Munoz et al., 2012; Tuomanen et al., 1986) and *Pseudomonas aeruginosa* (Aksu & Donmez, 2006; Gabr et al., 2008) were widely

reported to be good biosorbents.

Table 4: Biosorption of Cu^{2+} using different adsorbents.

Adsorbents	pH	Q_{max} (mg/g)	Reference
Algae			
Spirogyra sp.	5.0	151.575	(Gupta & Rastogi, 2008)
Cladophora fascicularis	5.0	99.36	(L. P. Deng et al., 2006)
Sargassum fluitans	5.0	108	(Leusch et al., 1995)
Ascophyllum nodosum	5.0	77	(Leusch et al., 1995)
Fucus spiralis	6.0	70.9	(Romera et al., 2007)
Fungi			
P. chrysosporium	6.0	20.23	(Say et al., 2001)
Aspergillus niger	6.0	4.46	(Kapoor et al., 1999)
Trametes versicolor	6.0	44.16	(Bayramoglu et al., 2003)
R. arrhizus	4.0	53.4	(Sag & Kutsal, 1996)
Bacteria			
Myriophyllum spicatum	5.5	4.56	(G. X. Li et al., 2010)
Enterobacter sp. J1	2.0	32.5	(Lu et al., 2006)
Thuja orientalis	7.7	19.23	(Nuhoglu & Oguz, 2003)
Pantoea sp.	5.0	31.3	(G. Ozdemir et al., 2004)
Geobacillus toebii	4.0	41.5	(G. Ozdemir et al., 2004)
Other			
Carrot residue	4.5	32.74	(Nasernejad et al., 2005)
Rice bran	6.0	33.58	(X. S. Wang & Qin, 2005)
Chestnut shell	5.0	12.56	(Yao et al., 2010)
Mimosa tannin gel	5.0	24.39	(Sengil & Ozacar, 2008)
Wheat shell	6.0	10.84	(Basci et al., 2004)

Table 4 summarizes the different types of sorbents used for Cu^{2+} biosorption. As seen in table 4, algae exhibit a Cu^{2+} removal capacity significantly larger than other biosorbents. Bacteria and fungi had similar removal capacities.

2.4 Biosorption mechanisms

Many microbial species are known to be capable of concentrating metal species from diluted aqueous solutions, accumulating them inside or on the surface of their cell structures. The complexity of the microorganism's cell structure implies that there are many ways for the metal to be captured by the cell. Therefore, metal ion uptake can result from several mechanisms, such as physical adsorption, ion exchange and coordination binding to functional groups on the surface of living or non-living cells. These mechanisms may be classified by two criteria. The first criterion is according to the cell metabolism, which can be divided into (1) metabolism dependent; and (2) metabolism independent (Veglio & Beolchini, 1997).

The second criterion classifies the biosorption mechanisms by the location in the cell where the metal is accumulated after adsorption, which can be (1) extracellular, (2) on the cell surface, or (3) intracellular (Veglio & Beolchini, 1997). Figure 1 summarizes the relationships among all the different biosorption mechanisms.

2.4.1 Transport across the cell membrane

The transport of heavy metals across the cell membrane is the least understood mechanism. Heavy metal transport across microbial cell membranes is conjectured to be

similar to mechanisms used to convey metabolically essential ions like potassium, magnesium and sodium (Veglio & Beolchini, 1997). Many studies have reported that biosorption by living microorganisms are comprised by two basic steps. Firstly, the metal binds to high affinity binding proteins in the cell walls or cell membranes and then is actively transported across the cell membrane into the cell (Joner et al., 2000; H. Li et al., 2010; Veglio & Beolchini, 1997; Vijayaraghavan et al., 2006).

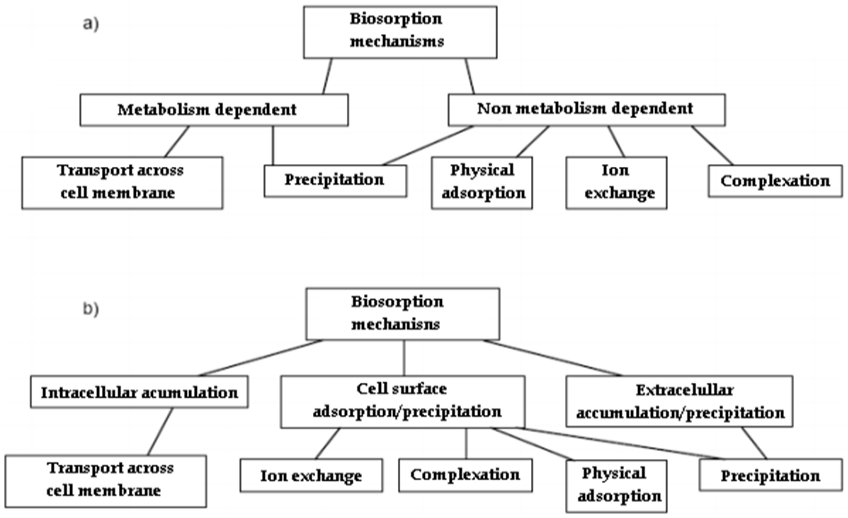

Figure 1: Biosorption mechanisms (a) Classified according to cell dependence on metabolism; (b) Classified according to the metal location on the biosorbent.

Figure 2: Hypothesis of mechanisms of heavy metal transport across the cell membrane.

2.4.2 Physical adsorption

This phenomenon is associated with the presence of van der Waals' forces. Volesky et al. pointed out that radionuclides present in aquatic environments are accumulated by marine microorganisms through direct adsorption from water (Tsezos et al, 1981). In the comparison with chemisorption, in which the electronic structure of bonding atoms or molecules form covalent or ionic bonds, physiosorption, generally speaking, can only be observed in the environment at low temperatures and in the absence of any relatively strong chemisorbent (Blackwell et al., 1995). As a result, compared with other mechanisms, physical adsorption does not significantly influence biosorption.

The hypothesis that uranium, cadmium, zinc and copper biosorption by dead biomass of fungi, algae, bacteria and yeast happens through electrostatic interactions between ions in solution and cells walls has been introduced (Kuyucak et al, 1988). Tsezos et al. also confirmed that thorium and uranium biosorption by *Rhizopus arrhizus* is based on the physical adsorption in the cell-wall structure (Tsezos et al, 1997). Electrostatic interactions have been demonstrated to be responsible for copper biosorption by *Neurospora crassa* (Kiran et al., 2005). Similarly for lead and cadmium biosorption by the green algae, *Ulva lactuca,* has also been proven to be due to electrostatic interactions (Sari & Tuzen, 2008). Physical adsorption has also been described for biosorption of copper, nickel, zinc, cadmium and lead by *Rhizopus arrhizus*

(Bhainsa & D'Souza, 2008; Tobin et al., 1984; Zafar et al., 2007).

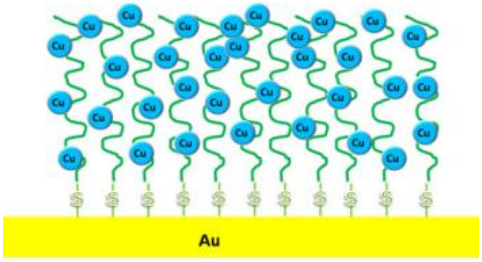

Figure 3: Cu^{2+} ions adsorbed on the Au surface by physical adsorption.

2.4.3 Complexation

Metal complex, consist of an atom or ion, and a surrounding array of bound molecules or anions, that are in turn know as ligands or complexing agents (Canet et al., 2002; Das et al., 2007). Complex formation of metal ions with organic molecules involves ligand centers in the organism. Figure 4 shows the formation of complexation. The metal removal from a solution may take place through complex formation on the cell surface after interaction between the metal and active functional groups (Das et al., 2008). At least one study has reported that the heavy metal biosorption by *Rhizopus arrhizus* is not only based on physical adsorption, but also on complex formation (Tsezos et al., 1981). Other studies have also hypothesized that biosorption of Cu^{2+} by *Chlorella vulgaris* happened through the adsorption and coordination of bonds between metals and amino and carboxyl groups of cell wall polysaccharides (Aksu & Donmez, 2006). The uptake mechanism of calcium, magnesium, chromium, zinc and copper accumulated by *Penicillium sp.* (Ahmad et al., 2006) were also determined to be due to complexation.

Chelation, one type of complexation, refers to more than one atom binding to a central metal ion to from a ring structure. In general, since a chelating agent may bind to a metal ion in more than one place simultaneously, chelation is more stable than other types of complexes.

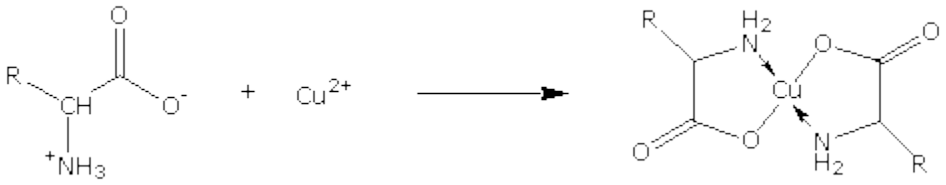

Figure 4: Copper chelation complex formation.

2.4.4 Ion exchange

Ion exchange is one of the main mechanisms related to biosorption. Ion exchange happens when heavy metal ions in the solution are exchanged with other protons or cations present in the binding sites of sorbents. These protons or ions can be displaced by heavier ions such as Cu^{2+}, Cd^{2+} and Zn^{2+}, resulting in the biosorptive uptake of metals (Das et al., 2008). This mechanism has been indicated as playing an important role in metal sorption by algal and fungal biomasses (Ajjabi & Chouba, 2009; Akar & Tunali, 2005; Mane et al., 2011). Cell walls of microorganisms containing polysaccharides as basic building blocks present excellent ion exchange properties (Tsezos et al., 1981; Das et al., 2008). Ion exchange was found to be responsible for copper and chromium biosorption by *Aspergillus niger* (Ahmad et al., 2006) and *Neurospora crassa* (Kiran et al., 2005). As a result, ion exchange has a significant influence on biosorption.

It should be pointed out that the term ion-exchange does not explicitly identify the binding mechanism, it rather describes experimental observations. The precise binding mechanisms may range from physical to chemical binding. In the case of heavy metal by brown algal biomass, the mechanisms can be viewed, in principle, as being extracellular, or occurring discretely at the cell wall. Intracellular sorption would normally imply bioaccumulation by a viable organism (Davis et al., 2003).

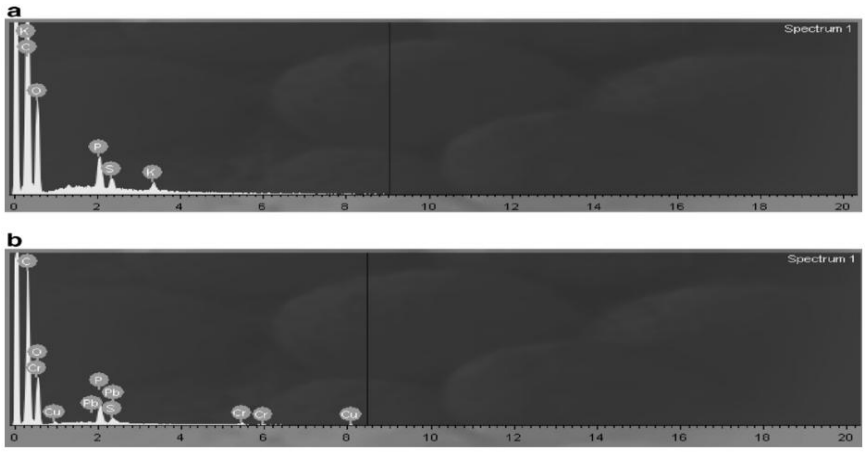

Figure 5: EDS analysis suggests that metallic species replaced K^+ on the bacterial cell wall.

2.4.5 Precipitation

Precipitation may be either dependent on the cellular metabolism or independent. Scott and Palmer found that *Arthrobacter* and *Pseudomonas* species could remove cadmium from solutions by a detoxification system capable of inducing precipitation of cadmium on the cell surface (Scott & Palmer, 1990). It has also been reported that precipitation plays an important role during biosorption of lead and uranium by a brewery yeast (Aksu & Donmez, 2006; Blackwell et al., 1995).

Although there are several biosorption mechanisms described in the literature, these mechanisms are still far from being fully understood. It is evident from the literature that biosorption mechanisms are not only various, but they can also take place simultaneously (Das et al., 2008).

2.5 Factors affecting biosorption

The uptake of heavy metals in biosorption can be influenced by a number of parameters. The investigation of environmental factors affecting biosorption is essential for a better understanding of the biosorption mechanisms, the optimum conditions for biosorption, and potential applications. There are several environmental factors that can affect biosorption, the most important ones are pH, temperature, biomass concentration, and cell age.

2.5.1 Effects of pH

One of the most important environmental parameters for heavy metal removal is pH, which controls the surface properties of sorbents, functional groups and ionic state of metal species (Kiran et al., 2006; H. Li et al., 2010). Many researchers reported that the metal binding ability of biosorbents increase gradually with pH increase. As reported in one study, at low pH (pH<4), H_3O^+ ion competes for the Cu^{2+} binding sites in the biomass preventing the copper ions from adsorbing (Karthikeyan et al., 2007). In addition, some researchers also reported that more negatively charged functional groups become exposed as the pH increases (Kiran et al., 2005). This could explain the increasing

removal capacity of heavy metals from pH 4 to 6. At high pH (pH>6), the binding sites are not active and precipitation is usually the main mechanism for the removal of metal ions. Researchers also suggested that at low pH the driving force for the metal binding is not the main cause for displacement of protons form all binding sites, but a combination of this driving force and the solution chemistry of metals, the activity of the functional groups in the biomass and the competition between ions and metal ions in the solution.

2.5.2 Effects of temperature

Significant effects of temperature on biosorption have been reported by some studies (Congeevaram et al., 2007). Temperature plays a major role in the adsorption of heavy metals because the intracellular uptake of some heavy metals by microorganisms can involve kinetic energy dependent mechanisms. Furthermore, temperature has also been described to influence other factors related to biosorption, which includes: (a) the stability of metal ion species initially placed in solution; (b) the stability of microorganism-metal complexes depending on the biosorption sites; (c) the microorganism cell wall configuration; (d) the ionization of chemical moieties on the cell wall (Sag & Kutsal, 2000). These factors may jointly or individually affect the binding sites for heavy metal removal. On the other hand, some researchers have obseved that temperature had little or no effect on the biosorption of certain heavy metals. For instance, Antunes and collaborators demonstrated that temperature did not affect biosorption of copper in a temperature range between 25^0C and 55^0C (Antunes et al., 2003; Dang et al.,

2009). These findings suggested that effects of temperature on biosorption can be metal dependent for different biosorbents.

2.5.3 Biomass concentration effect

The concentration of the biomass can influence the biosorption of heavy metals. As concentrations of biomass increases, heavy metal removal also increases (Lu et al., 2006). However, excess biomass can lead to interference between binding sites (Gadd et al., 1988). Additionally, other studies reported that chromium and lead uptake kinetics were not significantly improved using biomass concentration higher than 4.5 g/L (Teitzel & Parsek, 2003). This phenomenon was found to be related to the increase in electrostatic interaction that could inhibit metals biosorption. These properties must be taken into account in any application of biomass as an adsorbent.

2.5.4 Cell age effect

The growth phase of the biomass can affect the metal uptake quantitatively. In several studies, researchers have described that the removal capacity of bacterial cells in log phase was higher than in stationary phase. Daughney and co-authors explained that microorganisms change their structure and cell wall composition depending on their growth phase, and therefore, affected the proton adsorption (Daughney et al., 2001). Several studies have shown that in the exponential phase, the cell wall presented higher densities of favorable binding sites and better metal uptake capacity (Daughney et al., 2001; Kiran et al., 2005; Macaskie & Dean, 1984). The change in metal sorption

properties with different growth phases was also attributed to the change in ultrastructure and excretion of exoplysaccharides (Daughney et al., 2001). However, Simmons and Singleton indicated that the intracellular components play a more important role than cell wall in the binding capacity of heavy metals (Simmons et al., 1996). Hence, the microorganism growth phase plays an essential role in the biosorption capacity of heavy metals.

2.5.5 The effects of cell pretreatment

Pretreatment techniques have been shown to be effective in increasing the biosorption capacity. Many investigators have successfully pretreated microbial cells with HCl, $HClO_4$, H_2SO_4, HNO_3 (Akar & Tunali, 2005; Aravindhan et al., 2004; Mane et al., 2011; Tunali & Akar, 2006), NaOH (H. J. Park et al., 2007) and thermal treatment (Paul et al., 2012) to improve the surface properties for metal sorption. Other inorganic chemicals, such as $CaCl_2$ (Fourest et al., 1994) and also organics like ethanol, methanol, acetone and chloroform (Kuyucak et al., 1989; Kuyucak et al., 1988) were also utilized. These pretreatments aim to improve the binding surface characteristics of the microorganisms, by increasing the negative charges on the cell surface and as a consequence enhance the ion exchange capacity. These pretreatments of the biomass not only increased the biosorption ability, but also improved the chemical and mechanical stability of the biosorbent. Therefore, pretreatment of cells is commonly used in recent studies to improve the biosorption capacity for biosorbents.

2.5.6 Other factors

Other factors, such as solution mixing and presence of other cations, can substantially affect the adsorption ability of the biosorbent. For instance, the speed of the mixing of the solution containing the heavy metal and the adsorbent have been reported to affect the biosorption kinetics significantly (Nuhoglu & Oguz, 2003). In addition, since heavy metals are cations, any type of cations could potentially compete for the metal binding sites on the microbial surface. Therefore, the increase in concentration of competing cations could decrease the heavy metal removal (Aksu & Donmez, 2006). For instance, researchers described that biosorption of Zn^{2+} and Cu^{2+} by *Rhizopus arrhizus* is greatly affected by sodium salt of EDTA, sulphate and chloride ions (Zhou et al., 1999; Zhou et al., 1991).

2.6 Kinetics of biosorption

Biosorption kinetics have been divided into two steps: a very rapid initial adsorption step followed by a slow adsorption step (S. Ozdemir et al., 2009). The kinetics of biosorption is essential to determine the efficiency of the sorbent. Kinetic models have been applied in experimental data to predict the biosorption kinetics. These methods include the pseudo-first-order and the pseudo-second-order (Tunali, Akar, et al., 2006).

The pseudo-first –order rate equation can be described as follows,

$$\frac{1}{q_t} = \frac{1}{q_1} + \frac{k_1}{q_1 t}, \quad (1)$$

Where q_1 and q_t are the amount of heavy metal biosorbed at equilibrium (mg/g); K_1 is the pseudo first-order-rate constant (min^{-1})

The pseudo-second-order kinetic model is expressed as follows,

$$\frac{t}{q_t} = \frac{1}{k_2 {q_2}^2} + \frac{1}{q_2}\, t, \qquad (2)$$

where q_2 is the maximum biosorption capacity (mg/g); q_t is the amount of heavy metal biosorption at a certain equilibrium (mg/g); k_2 is the equilibrium rate constant (g/mg min)

The pseudo-second-order rate expression was used to describe chemisorption involving valence forces through the sharing or exchange of electrons between the adsorbent and adsorbate through covalent forces and ion exchange. In recent years, the pseudo-second-order rate expression has been widely applied in the adsorption of pollutants from aqueous solutions. The advantage of using this model is that there is no need to know the equilibrium capacity from the experiments, as it can be calculated from the model.

In the report of Tunali, it was found that the correlation coefficient for the pseudo-first-order model was lower than that of the pseudo-second-order model, meaning that under the experimental conditions of their study, which were 20^0C and pH 5, the adsorption of lead by *Cephalosporium aphidicola* did not follow the pseudo-first-order kinetics (Tunali, Akar, et al., 2006). However, according to Kiran, who investigated the biosorption properties of AR57 dye by *Cephalosporium aphidicola,* the results indicated that the biosorption system followed the pseudo-second-order kinetic model (Kiran et al.,

2006). Hence, the accuracy of the kinetic model largely depends on the biosorption system and conditions tested.

2.7 Equilibrium models of biosorption

For single metal biosorption systems, experimental equilibrium data are usually described by biosorption isotherms, which is a plot of the sorption uptake (Q_{max}, mg metal/g dry cell) against the final equilibrium concentration of the metal ion in solution (C_e, mg metal/L). Langmuir and Freundlich adsorption isotherm models are widely used to describe the relations between Q_{max} and C_e in biosorption systems. Other models like Brunauer-Emmett-Teller (BET) isotherm model, Redlich-Peterson model are applied to predict biosorption performace (Basha & Murthy, 2007).

2.7.1 Langmuir isotherm model

The Langmuir model considers that the sorption has a monolayer mechanism and assumes that all the active sites on the sorbent surface have the same affinity for the sorbate. Furthermore, the Langmuir isotherm model assumes that the sorbent surface has uniform energies of adsorption (Colak et al., 2009; H. Li et al., 2010). The Langmuir adsorption isotherm has traditionally been used to quantify and contrast the performance of different biosorbents. The Langmuir equation is:

$$Q_e = \frac{q_{max} b C_e}{1 + bC_e}, \quad (3)$$

where Q_e represents the maximum monolayer biosorption capacity of the biosorbents

(mg/g); b (L/mg) is the Langmuir adsorption constant related to the affinity of the binding sites; C_e is the adsorbed quantities of the metal ion per gram of dried biomass at equilibrium (mg/g); and q_{max} is the Langmuir adsorption constant representing the maximum amount bound at C_e (mg/g)

The parameter b is equal to $1/K_d$ where K_d is the dissociation constant. Therefore, b measures the stability of the complexes formed between metal ions and biomass cells wall under specific experimental conditions. The larger the b value, the stronger the binding of metal ions to the biomass.

2.7.2 Freundlich isotherm model

The Freundlich isotherm suggests that as the concentration of the heavy metal increases, the metal biosorption will also increase. This model is not only based on a heterogeneous energetic distribution of active sites, but also in the interaction between sorbed metals and the sorbent, as there may be multilayer reversible binding sites. Biosorption can be explained by the following empirical equation,

$$Q_e = kC_e^{1/n}, \quad (4)$$

where k is the Freundlich adsorption constant related to the adsorption capacity; n is the Freundlich adsorption constant related to the adsorption intensity; C_e is the residual concentration of the metal ion at equilibrium (mg/L). The Freundlich model is an empirical method used for data description, and was developed for heterogeneous

surfaces.

2.7.3 Brunauer-Emmett-Teller (BET) isotherm model

The BET isotherm represents a multi-layer adsorption model that assumes the Langmuir isotherm applies to each layer. A further assumption is that a given layer may not need to be completely formed before the next layer forms. The BET equation is represented as follows,

$$Q_{eq} = \frac{BQ^0 C_{eq}}{(C_s - C_{eq})[1 + (B-1)(C_{eq}/C_s)]}, \quad (5)$$

where C_s is the saturation concentration of the adsorbed component; B is a constant indicating the energy of interaction between the solute and the adsorbent surface; Q^0 is a constant indicating the amount of solute adsorbed forming a complete monolayer.

2.7.4 Dubinin-Radushkevitch (D-R) isotherm model

The Dubinin-Radushkevich (D-R) isotherm is a more general model than the Langmuir isotherm model since it does not assume a homogeneous surface or constant biosorption potential. It is commonly applied to distinguish between the physical and chemical biosorption mechanisms (Benhammou et al., 2005),

$$q_e = q_m e^{-\beta\varepsilon^2}, \quad (6)$$

where β is a constant related to the mean free energy of biosorption per mole of the biosorbate (mol ^{2} J $^{-2}$); q_m is the theoretical saturation capacity; ε is the Polanyi potential,

which is equal to RT (1+ $1/C_e$), where R (J $mol^{-1}K^{-1}$) is the gas constant and T(K) is the absolute temperature.

In the literature there are several examples of biosorbents' best fit being obtained by each of these different models. For instance, in the study of Schmuhl et al., they demonstrated that the Cu^{2+} removal, over the concentration range of 10 to 1000 ppm, was fit best by the Freundlich isotherm (Schmuhl et al., 2001). Aksu indicated that the Fe^{3+}-Cr^{6+} binary system could be described by both Langmuir and Freundlich adsorption models (Aksu et al., 2002). Similar results were obtained for the biosorption of Cr^{6+}-Ni^{2+} binary mixture on dried *Chlorella vulgaris* (Aksu & Donmez, 2006). On the other hand, the Dubinin-Radushkevich was used to describe the adsorption of Cu^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+} in another report (Tunali, Akar, et al., 2006). The BET isotherm model was applied for biosorption of phenol on *A. niger* biomass pretreated with sulfuric acid (Rao & Viraraghavan, 2002). In the latter study, the authors compared the Langmuir and Freundlich isotherm model with the BET isotherm model and concluded the BET model presented a higher correlation coefficient (Rao & Viraraghavan, 2002).

Many other isotherm models have also been widely used to present the equilibrium of biosorption, their equations and theories are shown in Table 7. Additionally, Table 5 and Table 6 show the summary of the Cr^{6+} and Cu^{2+} biosorption by different bacteria. As can be seen in Table 5 and Table 6, different microorganisms have different optimum condition for Cu^{2+} and Cr^{6+} biosorption.

2.8 Binding sites of biosorption

The microbial cell structure complexity makes it difficult to identify the binding sites for heavy metals. In general, to simplify, the binding sites for biosorption are generically classified as extracellular and intracellular. In the case of intracellular biding sites for fungal biomass, it has been demonstrated that the uptake of chromium by live cell involves intracellular accumulation. Transmission electron microscopy and energy dispersive X-ray analysis (TEM-EDX) confirmed that chromium can be found on the cell surface (Das & Guha, 2009). Other studies, also using TEM-EDX with three bacterial species provided information that palladium, silver, yttrium, and nickel, were adsorbed by different parts of the cells. These results indicated that heavy metal was retained inside the microbial cell (Tsezos et al., 1997).

The first part of the cell that interacts with the metal solution is the cell surface. Hence, the functional groups present on the cell surface play an important role in the biosorption of heavy metals. The microbial cell wall is mainly composed of polysaccharides, proteins and lipids, offering abundant metal binding functional groups, such as carboxylic, hydroxyl, sulphate, phosphate and amino groups (Akar & Tunali, 2005; Tunali & Akar, 2006; Tunali, Akar, et al., 2006). These finds were further confirmed by the decrease in biosorption capacity after esterification of the carboxylic groups and methylation of the amino groups on the cell wall (Mashitah et al., 1999). Other studies also showed that the carboxylic groups were the major functional groups

for biosorption of metal ions after chemical modifications of carboxyl and amino groups on the algal cell surface (Ajjabi & Chouba, 2009; Sari & Tuzen, 2008; Selatnia et al., 2004).

Table 5: Summary of the work done by various researchers using bacteria as biosorbents for the removal of Cr^{6+}.

Bacteria strain	Metal ion	pH	Temperature	Langmuir model	Freundlich model	Maximum uptake (mg/g)	reference
Ochrobactrum anthropi	Cr^{6+}	2.0	27^0C	0.93	0.98	86.2	(G. Ozdemir et al., 2003)
Staphylococcus xylosus	Cr^{6+}	1.0	Room temperature	0.96	0.97	143	(Ziagova et al., 2007)
Pseudomonas sp.	Cr^{6+}	4.0	Room temperature	0.94	0.99	95	(Ziagova et al., 2007)
Mucor hiemalis	Cr^{6+}	2.0	50^0C	0.96	0.83	53.3	(Tewari et al., 2005)
Pseudomonas aeruginosa	Cr^{6+}	7.0	37^0C			38.6	(Kang et al., 2007)
Pantoea sp.	Cr^{6+}	3.0	27^0C	0.96	0.99	204.1	(G. Ozdemir et al., 2004)
Saccharomyces cerevisiae	Cr^{6+}	1.0	35^0C	0.99		26.7	(Ozer & Ozer, 2003)
Bacillus coagulans	Cr^{6+}	5.5	37^0C	0.97	0.97	19.45	(Quintelas et al., 2006)

Table 6: Summary of work done by various researchers using bacteria as biosorbents for the removal of Cu^{2+}.

Bacteria strain	Metal ion	pH	Temperature	Langmuir model	Freundlich model	Maximum uptake (mg/g)	reference
Geobacillus toebii	Cu^{2+}	4.0	60^0C	0.99	0.87	41.5	(S. Ozdemir et al., 2009)
Geobacillus Sp.	Cu^{2+}	4.0	60^0C	0.89	0.98	48.5	(S. Ozdemir et al., 2009)
Pseudomonas sp.	Cu^{2+}	6.0	35^0C			24.79	(Andreazza et al., 2010)
Myriophyllum sp.	Cu^{2+}	5.5	25^0C	0.96	0.89	0.19	(G. X. Li et al., 2010)
Enterobacter sp. J1	Cu^{2+}	2	37^0C	0.99	0.99	32.5	(Lu et al., 2006)
Thuja orientalis	Cu^{2+}	7.7	37^0C	0.99	0.93	19.23	(Nuhoglu & Oguz, 2003)
Pantoea sp.	Cu^{2+}	5.0	27^0C	0.99	0.82	31.3	(G. Ozdemir et al., 2004)
Pseudomonas sp.	Cu^{2+}	5.0	27^0C	0.98	0.8	23.0	(Chang et al., 1997)
Sphaerotilus natans	Cu^{2+}	5	30^0C	0.99			(Pagnanelli et al., 2003)

Table 7: Isotherm models used to represent the equilibrium of biosorption.

Isotherm model	Equation	Theory	Reference
Langmuir	$Q_e=(Q_{max})bC_e/(1+bC_e)$	Established relationship between the amount of gas sorbed on a surface and the pressure of the gas. Assumes monolayer coverage of the adsorbate over a homogenous adsorbent surface.	(Ozer & Ozer, 2003)
Freundlich	$Q_e=K_fC_e^{1/n}$	This exponential equation assumes that as the adsorbate concentration increases so does the concentration of adsorbate on the adsorbent surface. Can be applied to non-ideal sorption surfaces with heterogeneous or multilayer surfaces.	(Antunes et al., 2003)
BET	$Q_e = \frac{BQ^0C_{eq}}{(C_s - C_{eq})[1 + (B-1)(C_{eq}/C_s)]}$	This isotherm represents the multi-layer adsorption model. It assumes that a Langmuir isotherm applies to each layer. A further assumption is that a given layer may not need to be completely formed before the next layer forms.	(Quintelas, Fernandes, et al., 2008)
Dubinin-Radushkevich	$Q_e = q_m\, e^{-\beta\varepsilon^2}$	The characteristic sorption curve is related to the porous structure of the sorbent.	(Kiran et al., 2006)
Redlich-Peterson	$Q_e=(K_rC_e)/(1+a_sC_e^{1/bs})$	This isotherm model incorporates features of both the Langmuir and Freundlich isotherms and may be used to represent adsorption equilibrium over a wide concentration range.	(Quintelas, Fernandes, et al., 2008)
Sips	$Q_e= (K_SC_e^{1/bs})/(1 +a_SC_e^{1/bs})$	Is also called Langmuir–Freundlich isotherm, and the name derives from the limiting behavior of the equation. At low sorbate concentrations it effectively reduces to a Freundlich isotherm and thus does not obey Henry's law. At high sorbate concentrations, it predicts a monolayer sorption capacity characteristic of the Langmuir isotherm.	(Quintelas, Femandes, et al., 2008)
Toth	$Q_e= (K_tC_e)/[(at+C_e)^{1/t}]$	Derived from potential theory, it is used in heterogeneous systems. It assumes a quasi-Gaussian energy distribution, i.e. most sites have adsorption energy lower than the peak of the maximum adsorption energy.	(Roane et al., 1996)

3. Material and Methods

3.1 Microorganisms and culture conditions for DNA extraction

Two Gram-negative strains, *C. metallidurans* and *O. intermedium,* were investigated in the present study. *C. metallidurans* CH34 was isolated from sludge of a zinc decantation tank (Monsieurs et al., 2011). An isolate resistant to several heavy metals was obtained from leachate samples generated by a lysimeter containing organic wastes and heavy metals in Brazil. The new isolate was initially grown in Tryptic Soy Agar (TSA) at 27^0C (Difco Laboratories, Detroit, MI) to obtain single colonies. Then, a single colony from the plate was transferred to 5 mL of Tryptic Soy Broth (TSB, Oxoid Ltd., Basingstone, Hampshire, England) and grown at 27^0C and 80 rpm for 24 h to obtain enough biomass for DNA extraction.

3.2 DNA extraction and polymerase chain reaction (PCR) amplification of DNA

Total DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) as described by the manufacturer. The final extracted DNA was quantified using a spectrophotometer (EL800 universal microplate reader; Bio-Tek Instruments, Inc., Winooske, VT) at 260 nm absorbance. The DNA was stored at -20^0C prior to PCR amplification. Primers for 16S rRNA gene were used for the initial identification of the new isolate as *Ochrobactrum* sp. The amplification of the DNA was performed with universal 16S rRNA primers, namely, 27F (5′-AGATTTGATCMTGGCTCAG-3′) and

1401R (5′-CGGTGTGTACAAGACCC-3′). PCR amplification was performed in a total volume of 50 µL containing 25 µL of 2X AmpliTaq Gold PCR master mix (Promega, Madison, WI), 50 ng/mL of genomic DNA, 0.2 µM of each primers, and 23 µL of nuclease-free water. The amplification was performed according to the following protocol: initial denaturation at 95^0C for 3 min, followed by 30 cycles of denaturation at 94^0C for 1 min; annealing at 55^0C for 30 s; extension at 72^0C for 1 min and a single final extension at 72^0C for 3 min (Rodrigues et al., 2009). The negative control contained all the reaction components of the reaction mixture with the exception of the template DNA.

In order to identify the strain of *Ochrobactrum sp.*, PCR amplifications using specific pairs of primers for *O. anthropi* or *O. intermedium* were used (Scholz et al., 2008). The primers for *O. anthropi* were Anth-f (5'-GCAAGCTGGGTGTCGATCTGG-3') and Anth-r (5'-TTCTCGACGACACCGGCCTTTA-3'), and for *O. intermedium*, the primers were Inter-f (5'-CGGCGTTGGTGGCTTGCCTAA-3') and Inter-r (5'-GGAACGAGAGATAGACGCGGTA-3').

The PCR mixtures (25 µL) for each pair of primers contained 0.2 µM of each primer, 50 ng/mL of template DNA, 10.5 µL nuclease-free water and 12.5 µL 2X of PCR Master Mix (applied biosystems, California). Amplification was carried out with a 9600 thermal cycler (Perkin-Elmer Gene Amp PCR system; Perkin-Elmer, Nowark, CT, USA) as follows: 3 min of initial denaturation at 95^0C, followed by 30 cycles of DNA

denaturation at 94 ℃ for 30 s, primer annealing at 62 ℃ for 1 min, and an elongation step at 72 ℃ for 1 min. A final step included a final extension at 72 ℃ for 4 min (Scholz et al., 2008).

To determine the successful amplification of the new isolate with one of these two primers, 10 µL of the PCR product was run in 1% agarose (OmniPur, EMD) gel with 1 x Tris-acetate-EDTA (TAE) buffer (Fermentas Life Sciences) at 100 V for 1.5 h, then stained with 5 µL of Sybr Safe DNA gel dye (Invitrogen). The bands were visualized with Compact Digimage System with 10.0 megapixel digital camera and built-in 254 nm UV Transilluminator (Major Science, USA) instrument.

3.3 Heavy metal growth culture conditions

Prior to the experiments, *C. metallidurans* and *Ochrobactrum sp.* were grown in TSA at 27^0C (Difco Laborarotires, Detroit, MI) to obtain single colonies and check for purity. All plates were kept at 4^0C for no longer than one month, after which they were transferred to fresh plates. A single colony from each plate was transferred to Tris medium (TSM) for subsequent experiments. The TSM medium contained 6.06 g/L base Tris, 4.68 g/L NaCl, 1.49 g/L KCl, 1.07 g/L NH_4Cl, 0.43 g/L Na_2SO_4, 0.2 g/L $MgCl_2.6H_20$, 0.03 g/L $CaCl_2.2H_20$, 0.23 g/L $Na_2HP0_4.12H_20$, 0.005 g/L of Fe(III)(NH_4) citrate (Mergeay et al., 1985). The pH of the medium was adjusted to 7.0 ± 0.5 with 1M HCl and 1M NaCl prepared with deionized water. The medium was autoclaved for 15 min. The sterilized medium was allowed to cool at room temperature and 1 mL of a filter

sterilized trace element solution SL7 of Biebl and Pfennig (Lakshmi et al., 2009) was added to the sterile TSM medium. The SL7 trace element solution was prepared by adding 2 g/L $MnSO_4.H_2O$, 0.8 g/L $CoCl_2.6H_2O$, 0.4 g/L $ZnSO_4.7H_2O$, 0.4g/L $Na_2MoO_4.H_2O$, 0.2 g/L $CuCl_2.2H_2O$, 0.1g/LH_3BO_3, and 10 mL of concentrated HCl (37%), and then sterilized with a 0.2 μm filter (PTFE Milipore filter, KTGR04FH3).

For all batch experiments in the present study, unless indicated otherwise, the microorganisms were prepared as follow. The microorganisms were grown in 25 mL of TSM broth in Corning@ 50 mL PP centrifuge tubes (Sigma-Aldrich) and incubated at 27 ^{0}C by shaking at 80 rpm for 36 and 48 h to obtain cells in logarithmic and stationary phases, respectively (Guine et al., 2007). Dead cells were prepared by autoclaving cells grown for 48 h at 121 ^{0}C for 20 min (Kefala et al., 1999). Cells were harvested by centrifugation for 8 min at 8000 rpm and washed once with fresh TSM medium. Finally, the cells were ressuspended in Phosphate Buffer Solution (0.01M PBS, pH = 7.4 at 25 ^{0}C, 0.0027 KCl, 0.137 NaCl, Fisher Scientific, USA), to an optical density of 0.3 at the wavelength of 600 nm (OD_{600}). All the experiments were performed at this O.D. unless indicated otherwise.

3.4 Minimum inhibitory concentration (MIC) determination

Stock solutions (2000 ppm) of $CuSO_4.5H_2O$ and $K_2Cr_2O_7$ (Fisher Scientific) were prepared in TSM medium at pH 7.0 ± 0.5 and sterilized by using 0.2 μm filter (PTFE Milipore filter, KTGR04FH3). Prior to the experiments, the concentrated heavy metal

stock solutions were diluted with TSM without heavy metals to obtain the right concentrations for the experiments. Single colonies grown in TSM broth without heavy metals at 27^0C and 80 rpm for 48 h were used for the MIC experiments. A volume of 20 µL of each cell suspension in PBS at OD_{600} = 0.3 was inoculated into a 96-well microtiter plate (Costar 3370, Corning, NY) containing 200 µL of TSM with triplicate concentrations of Cu^{2+} and Cr^{6+} ranging from 5 ppm to 1000 ppm and 10 ppm to 1000 ppm, respectively. Negative controls for each microorganism and heavy metal concentrations were performed in TSM without inoculation of microorganisms. Positive controls were conducted in metal-free TSM broth inoculated with *C. metallidurans* or *Ochrobactrum sp*. The MIC was determined by measuring the cell growth at 27^0C and 80 rpm with a plate reader spectrophotometer (EL800 universal microplate reader; Bio-Tek Instruments, Inc., Winooske, VT) at 600 nm every hour. Each experiment was performed in triplicate (Teitzel & Parsek, 2003).

3.5 Assessing the relative susceptibilities of logarithmic-and stationary-phases to high heavy metal concentrations

This method was optimized by Teizel (Teitzel & Parsek, 2003). The growth rates (µ) were calculated (h^{-1}) based on the following equation,

$$OD=OD_0e^{\mu t} \quad . \qquad (7)$$

In this equation, OD is the culture optical density at time *t* at the end of the exp

onential phase; and OD_0 is the optical density at time zero (t_0) of the exponential phase of the growth curve.

Cells in logarithmic- and stationary-phases were exposed to 100 ppm filter-sterilized solutions of Cu^{2+} and Cr^{6+} at 27°C for 3 h in TSM media (Teitzel & Parsek, 2003). The control samples were performed in TSM without exposing the cells to heavy metal. After 3 h of exposure to the heavy metal solutions, the cell survival was estimated by the plate counting method in TSA after 24 h incubation at 27^0C (Balestra & Misaghi, 1997).

3.6 Bacterial dry mass determination

Cells in stationary-phase were resuspended in PBS and the OD_{600} was adjusted to obtain the following optical densities: 0.1, 0.3, 0.5, 0,7, and 1. A volume of 2 mL from each suspension was filtered through 0.2 μm pore-size sterile filters (PTFE Milipore filter, KTGR04FH3). After filtration, the filter membranes containing the bacteria were dried at 70°C overnight and kept in the desiccator until measured. The dried biomass was determined by calculating the difference before and after filtering the cells in the filter membranes. Experiments for each concentration were conducted in triplicate and the average biomass of dried cells per filter was compared with the control filter membranes after filtration of the PBS without bacteria, to determine any effect of the buffer constituents retained in the filter membranes.

3.7 Time-course biosorption of live and dead biomass under environmental conditions

Logarithmic- and stationary-phases and dead biomass of *C. metallidurans* and *Ochrobactrum sp.* were washed and then suspended to a cell concentration of 0.3 mg/mL in a 30 mL TSM broth with 100 ppm of Cu^{2+} or Cr^{6+} in a 150 mL flask (Lu et al., 2006). Cell-metal suspensions were incubated at 27^0C, 80 rpm, and at pH 7.0 ± 0.5, which corresponds to the original environmental condition for these microbes (Mitchell & Hamilton, 1949). Heavy metal solutions without microbial biomass were used as controls to take into consideration any heavy metal precipitation. During this procedure, 3 mL samples were taken every 10 min for the first hour, after which samples were taken every 30 min for 400 min. Each sample was filtered through 0.2 μm pore size membrane. The filtrate was analyzed by AAnalyst 200 Atomic Absorption Spectrometer (Perkin Elmer AANALYST200). Standard curves were performed with six standard solutions diluted in deionized water ranging from 5 to 100 ppm prior to each sample measurement (Lu et al., 2006). The capacity of biosorption (q; mg metal/g dry cell) was calculated by

$$q = (C_0 - C_e)/X, \quad (8)$$

where C_0 and C_e is the initial and residual metal concentration (mg/L) and X is the biomass concentration (g/L) (W. M. Chen et al., 2008).

3.8 Optimization of pH and temperature for biosorption

Batch tests were conducted in metal solutions at pH varying from 3.0 to 8.0 (±0.1). The cell concentrations were kept at 0.3 mg/mL. The experiments were all incubated at 27 ^{0}C and 80 rpm for 400 min unless indicated otherwise. The effects of different temperatures on the heavy metal sorption by the biomass were determined at pH 7 in the following temperatures: 22 ^{0}C, 27 ^{0}C, 32 ^{0}C, and 37 ^{0}C. Aliquots of 3 mL were taken from the solution and filtered through a 0.2 μm pore size membrane for all the batch tests. The metal concentrations in the filtrates were measured by Atomic Absorption Spectrometer. Biomass-free heavy metal solutions were also measured as controls.

3.9 Determination of adsorption isotherms

Samples of *C. metallidurans* and *Ochrobactrum sp.* in logarithmic and stationary phases, as well as samples of dead biomasses were suspended in 30 mL TSM media containing Cu^{2+} or Cr^{6+} concentrations ranging from 10 to 100 ppm with 10 ppm intervals. Control experiments were prepared for each metal concentration without biomass. The adsorption experiments to obtain the isotherms were done for a period of 400 min and performed under environmental and optimum conditions. The first condition consisted of a temperature of 27^{0}C and a pH of 7 for both *C. metallidurans* and *Ochrobactrum sp*. The optimum conditions for heavy metal adsorption by each microorganism were determined in the section of "Optimum temperature and optimum pH". After 400 min incubation, 3 mL samples were taken from the solution and filtered

through 0.2 μm membranes. The residual metal concentration was measured with Atomic Absorption Spectrometer. Experimental data were modeled using the Langmuir isotherm to determine the characteristic parameters (Q_{max} and K_d) of biosorption. The Langmuir isotherm equation used was:

$$q=Q_{max}C_e/(K_d+C_e), \quad (9)$$

where q_{max} represents maximum adsorption capacity (mg/g dry cell), and K_d represents the Langmuir constant (mg/L). (H. Li et al., 2010)

3.10 Competitive biosorption assays

Solutions of 30 mL containing both metal ions at 100 ppm with different ratios (4:1, 2:2, and 1:4) were investigated to determine heavy metal biosorption preference by the microorganisms. The cell concentrations in the solution were kept at 0.3 mg/mL and the experimental conditions (temperature, pH and agitation speed) were identical to the experiments performed in the section "adsorption isotherms" under both environmental and optimum conditions. Inoculant-free controls were conducted under the same conditions. After 400 min of incubation, 3 mL samples were filtered through a 0.2 μm pore size membrane, and analyzed with Atomic Absorption Spectrometer. The removal ratios of Cu^{2+} and Cr^{6+} were determined by the following equation

$$\text{Removal ratio (\%)} = [\ (C_0 - C_e)/\ C_0] \times 100, \quad (10)$$

where C_0 and C_e are the initial and residual metal concentrations (mg^{-1}) (Li et al., 2010).

3.11 Scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS) analysis

SEM and EDS analyses were employed to confirm the presence and identity of metal ions adsorbed to the biomass. The biosorbent samples exposed to metal and the control samples (*e.g.*, cells not exposed to heavy metals) were prepared according to previously described procedure Mejias et al. (Mejias Carpio, 2012). Briefly, aliquots of 1.5 mL of controls and cells exposed to the heavy metals were fixed with 2% glutaraldehyde for 1 h and dehydrated by increasing concentrations of ethanol (25, 50, 75, 95, 100% v/v). The pre-treated samples were mixed to 10 µL of 1,1,1,3,3,3-hexamethyldisilazine (98%, Acros Organics) and then one drop of the solution was placed on a transmission electron microscopy (TEM) grid (150 mesh × 165 µm pitch, G1642, Sigma). After drying the samples overnight at room temperature, specimens were analyzed with SEM/EDS by Hitachi S-4800-II ultrahigh resolution scanning electron microscope at 15kV of accelerating voltage with an emitting current of 15 µA and 5000 times magnification.

3.12 Fourier-transform infrared spectroscopy (FT-IR) analysis

Infrared spectra of live and dead biomasses exposed or not to Cu^{2+} and Cr^{6+} were used to characterize the functional groups responsible for adsorbing the heavy metals. After exposing the samples to the heavy metals, 2 mL aliquots were filtered through 0.2 µm pore size membranes and dried at room temperature for 48 h. The dried samples were

analyzed with the Nicolet iS10 Mid Infrared FT-IR Spectrometer (Thermo Fisher Scientific) equipped with ZnSe crystal and the Omnic 8 Software (Thermo Fisher Scientific). Infrared spectra were recorded in the range of 4000-400 cm^{-1} with a resolution at 4 cm^{-1}. The background noise of atmospheric water and CO_2 was automatically subtracted from the sample spectra.

3.13 Statistical analysis

Each set of experiments were carried out in triplicate. For all results, average and standard deviations were calculated in excel. Further statistical analyses were performed using unpaired t-test to determine statistically the difference between the logarithmic phase and the control samples or the stationary phases and the controls. This was also done for the susceptibility assays and the competitive biosorption assays under different conditions (*i.e.*, pH and temperature).

4. Results

4.1 PCR identification of the *Ochrobactrum sp*. isolate

PCR analyses using specific primers for *O. intermedium* and *O. anthropic* were performed to determine the species of the *Ochrobactrum sp.* isolate. The result indicated that the new isolated was amplified by the *O. intermedium* specific primers (Figure 6)..

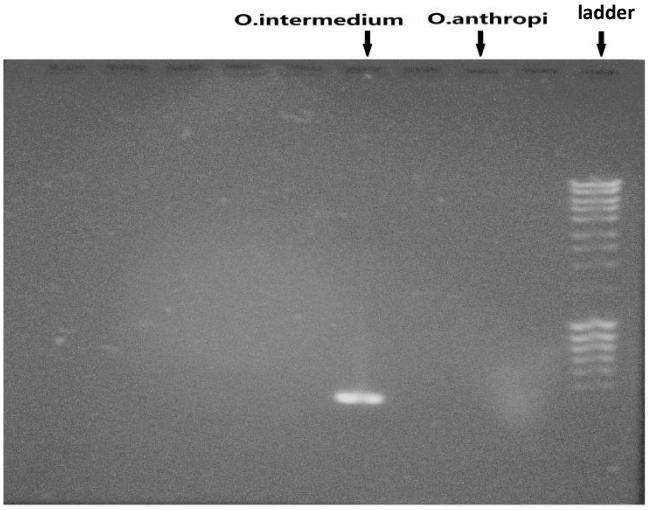

Figure 6: DNA amplification results of *Ochrobactrum sp*. isolate using specific primers for *O. anthropic* and *O. intermedium*. The results demonstrate that the isolate belongs to the *O. intermedium* species.

4.2 Minimum inhibitory concentration (MIC) of copper and chromium

The inhibitory effects of Cu^{2+} and Cr^{6+} on bacterial growth were investigated in minimal growth medium (TSM). The minimal growth medium was chosen because it minimizes the complexation and precipitation of heavy metals. The MIC of Cu^{2+} and Cr^{6+} for *C. metallidurans and O. intermedium* was evaluated every 60 min by OD_{600} measurements during cell exposure to heavy metal solutions for 60 h. As shown in Figure

7, the growth of both microorganisms decreased with increasing concentrations of heavy metals, while no growth was observed in the negative control (no bacterial inoculation). In fact, longer lag phases were observed as the concentrations of heavy metals increased in the growth medium. As expected, maximum growth rates were observed with the positive controls, which did not contain any heavy metal.

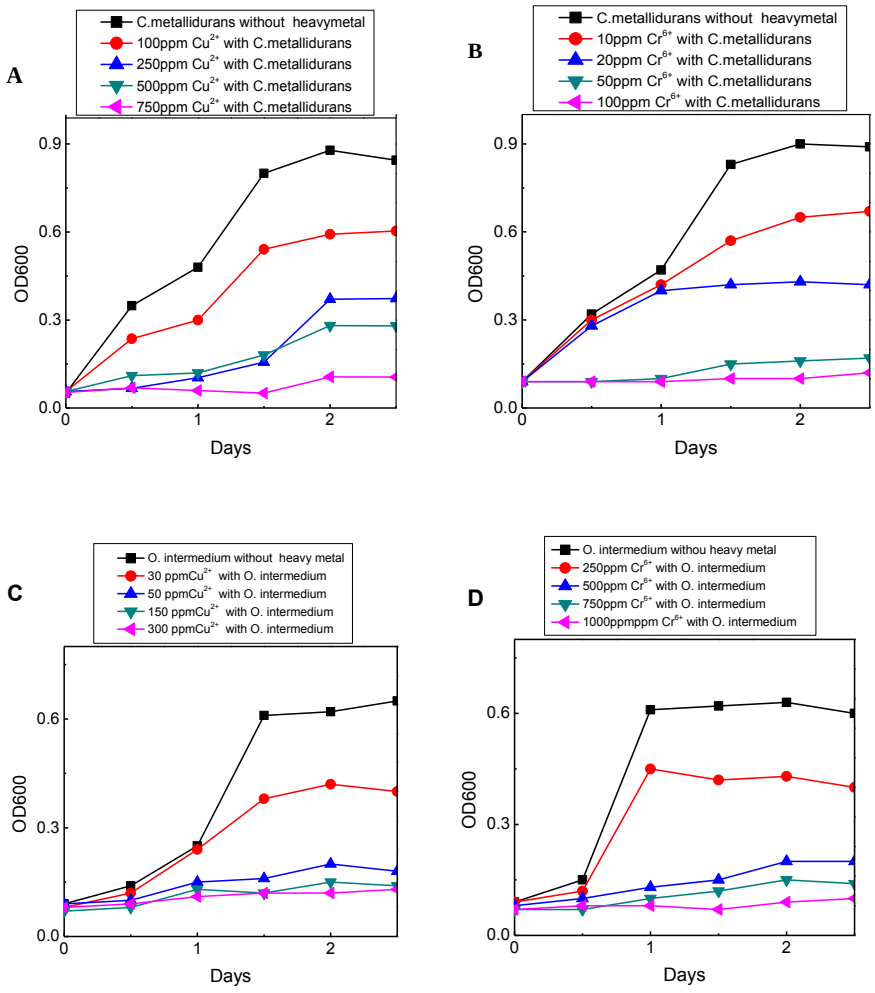

Figure 7: MIC growth curve (A) growth of *C. metallidurans* in the presence of Cu^{2+}; (B) growth of *C. metalliduran* in the presence of Cr^{6+}; (C) growth of *O. intermedium* in the presence of Cu^{2+}; (B) Growth of *O. intermedium* in the presence of Cr^{6+}.

The summary of the MIC results are also represented in Table 8. For *C. metallidurans,* the MIC for Cu^{2+} and Cr^{6+} were 750 and 100 ppm, respectively. For *O. intermedium,* the MIC of Cu^{2+} and Cr^{6+} were 300 and 1000 ppm, respectively. These values showed that *C. metallidurans* was more resistant to Cu^{2+} (750 ppm) than *O. intermedium* (300 ppm). In contrast, *O. intermedium* showed a MIC value of 900 ppm higher than *C. metallidurans* for Cr^{6+}.

Table 8: MIC for *C. metallidurans* and *O. intermedium.*

Strain	MIC (ppm)	
	Cu^{2+}	Cr^{6+}
C. metallidurans	750	100
O. intermedium	300	1000

4.3 Microbial survival after exposure to high concentrations of Cu^{2+} and Cr^{6+}

In order to determine the relationship between cell tolerance to heavy metal and bacterial growth phases, cells in logarithmic and stationary phases were exposed to 100 ppm of Cu^{2+} and Cr^{6+} for a period below their doubling time, *i.e.* 3 h. The doubling times of *C. metallidurans* and *O. intermedium* were determined using growth curves of cells grown in TSM without heavy metals, which were 3.5 and 3.8 h, respectively.

The results of the susceptibility assay revealed that the control samples (no exposure to heavy metals) presented a maximum cell count of approximately 1.2×10^9 CFU/mL for *C. metallidurans* and 1.5×10^9 CFU/mL for *O. intermedium.* The results show a more than 2 log reduction in the bacterial counts after exposure to 100 ppm Cu^{2+}

and Cr^{6+} (Figure 3). Statistical analyses of the results revealed a significant difference in the viability of cells between the logarithmic and stationary phases (t-test, $p<0.005$) after Cu^{2+} and Cr^{6+} interaction. As can be seen in Figure 8, the Symbols * and + correspond to statistically different results between the control and other samples with a 95% confidence interval. These results showed that cells in stationary phase were less susceptible to heavy metals than the cells in the logarithmic phase, since the cells in logarithmic phase were at least 20% less viable than the ones in stationary phase.

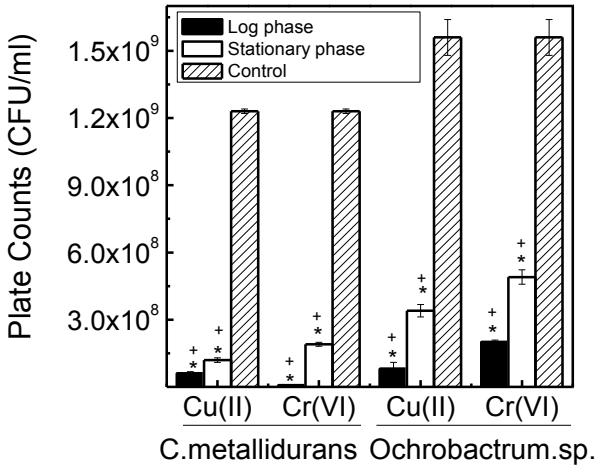

Figure 8: Survival of *C. metallidurans* and *O. intermedium* in different growth phases after exposure to 100 ppm of copper and chromium.

4.4 Time-course biosorption experiments of live biomass in stationary and logarithmic phases and dead biomass under environmental conditions

Batch biosorption studies were conducted to determine the time required to obtain the biosorption equilibrium, as well as the biosorption capacity in different growth phases.

In this investigation, the following environmental parameters were used: pH of 7.0, incubation temperature at 27^0C, agitation speed at 80 rpm, initial Cu^{2+} and Cr^{6+} concentrations of 100 ppm, and biosorbent concentrations of 0.3 g/L. Kinetic studies revealed that metal removal and sorption occurred mainly in the first 100-150 min (Figures 4 and 5) for all types of biomasses. However, after 50-100 min, a reduction on the growth rate and the heavy metal removal was observed. The maximum adsorption capacity was attained after 250 min for both microorganisms with a maximum removal rate of around 12% and 21% for Cu^{2+} and Cr^{6+}, respectively, for both microorganisms.

The percentage removal of Cu^{2+} and Cr^{6+} by *C. metallidurans* and *O. intermedium* obtained from heat killed biomass, logarithmic and stationary phases are shown in Figures 3 and 4. Cu^{2+} sorption by *C. metallidurans* was 9.1, 10.9, and 8.4 % (Figure 3 (A)) for heat killed, logarithmic and stationary phases, respectively. Note that higher adsorption capacity for Cu^{2+} was attained in the logarithmic phase for *C. metallidurans*. Similarly, the Cr^{6+} removal by *O. intermedium* cells harvested in logarithmic phase was 1.4 and 2.9 times higher than the Cr^{6+} removal by cells in stationary phase and heat killed, respectively (Figure 9 (B)). However, the biosorption of Cu^{2+} by the heat killed *O. intermedium* was approximately 10% and 60% higher than the cells in logarithmic and stationary phases, respectively. In addition, the *C. metallidurans* heat killed cells and the cells in logarithmic and stationary phases presented a biosorption capacity for Cr^{6+} of

20.8, 16.4, and 10.7 %, respectively (Figure 9 (A)). These results indicated that heat killed biomass can have a good heavy metal removal capacity.

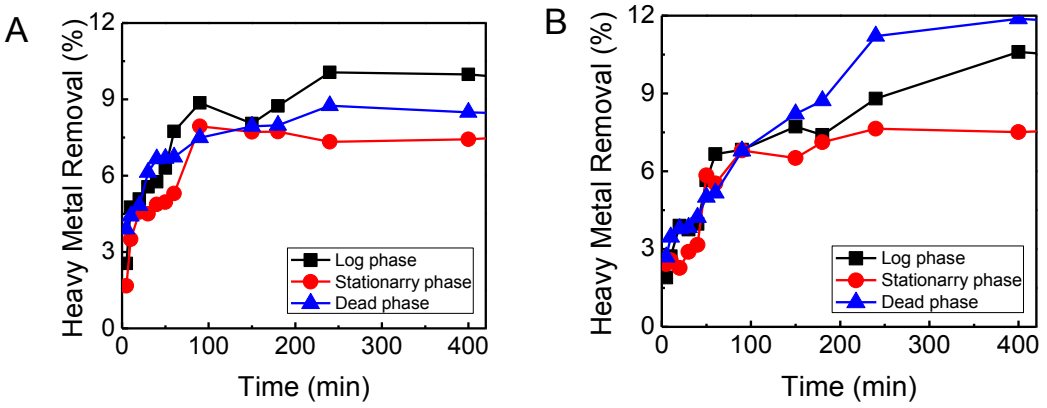

Figure 9: Equilibrium biosorption (A) *C. metallidurans* and (B) *O. intermedium* for 100 ppm Cu^{2+} in TSM at 27°C, 80 rpm for 400 min.

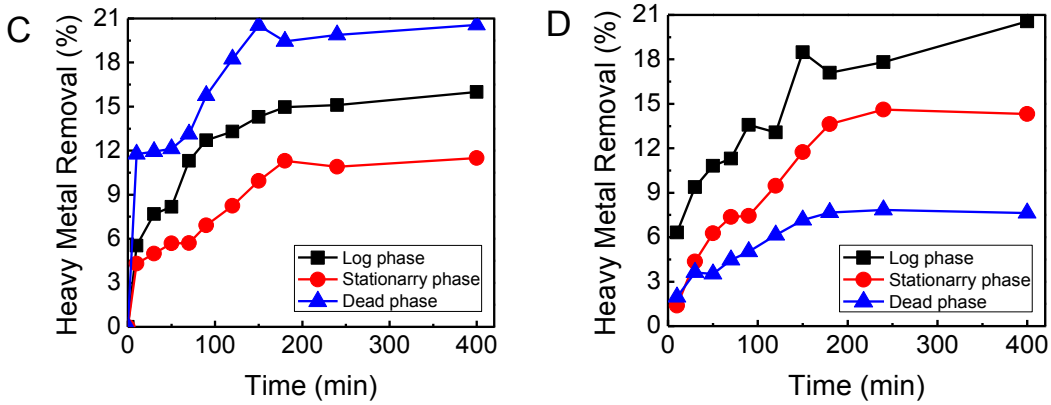

Figure 10: Equilibrium biosorption of (C) *C. metallidurans* and (D) *O. intermedium* for 100 ppm Cr^{6+} in TSM at 27°C, 80 rpm for 400 min.

4.5 Effects of pH

The different sorption profiles for various heavy metal ions exposed to different sorbents are generally affected by pH. In the present study, the effects of pH on Cu^{2+} and Cr^{6+} removal is shown in Figures 11 (A) and (B), respectively. The results indicate that

the Cu^{2+} sorption capacity of *C. metallidurans* and *O. intermedium* increased steadily with increase of pH and reached a peak removal at pH 6.0, followed by a drastic decrease from pH 6.0 to 8.0. The maximum Cu^{2+} removal capacities for *C. metallidurans* (14.5%) and *O. intermedium* (18.9%) were observed at pH 6.0.

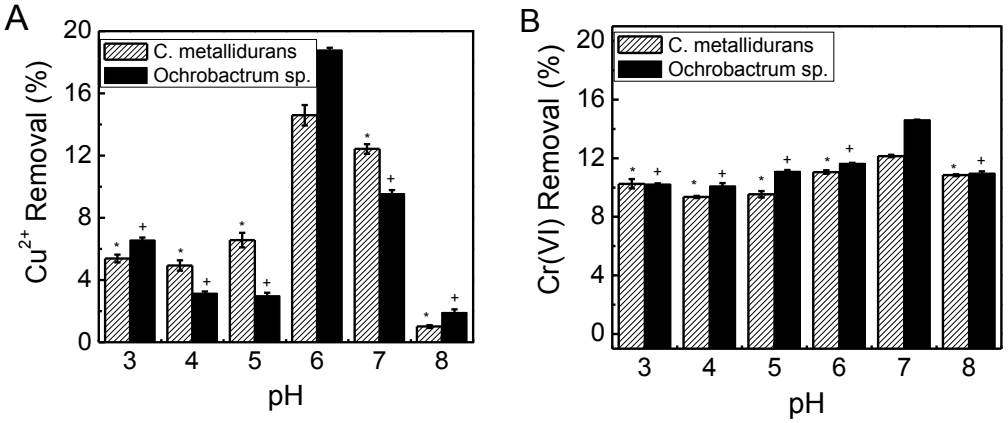

Figure 11: Effect of pH on biosorption**.** The symbols * and + correspond to results statistically different from the maximum (A) Cu^{2+} removal at pH=6 and (B) Cr^{6+} at pH=7 by *C. metallidurans* and *O. intermedium*, respectively.

In the case of Cr^{6+}, only a slight change was observed in the removal capacity by *O. intermedium* and *C. metallidurans* with increasing pH, with the maximum Cr^{6+} removal at pH 7. The maximum Cr^{6+} removal by *C. metallidurans and O. intermedium* were 12.3% and 15.1%, respectively.

4.6 Effects of temperature

Temperature plays an essential role in microorganisms' metabolism and cell death. Therefore, the effects of temperature on Cu^{2+} and Cr^{6+} removal were investigated between 20^0C and 40^0C. The results obtained for the removal of Cu^{2+} (Figure 12) showed

that the percentage of Cu^{2+} removal decreased from 15.2% to 12.4% for *C. metallidurans* with the increase in temperature from 22^0C to 32^0C. The maximum Cu^{2+} removal value was found to be 25.4% at 37^0C. On the other hand, the Cu^{2+} removal by *O. intermedium* showed no significant difference ($p>0.95$) at 22^0C, 27^0C and 37^0C. In the case of Cr^{6+}, the maximum removal of heavy metal by *O. intermedium* was obtained at 37^0C (25%). In the case of *C. metallidurans,* the best removal rate of Cr^{6+} occurred at 27^0C, with 15% removal. It is worth pointing out that removal of Cr^{6+} was significantly reduced at temperatures higher than 27^0C.

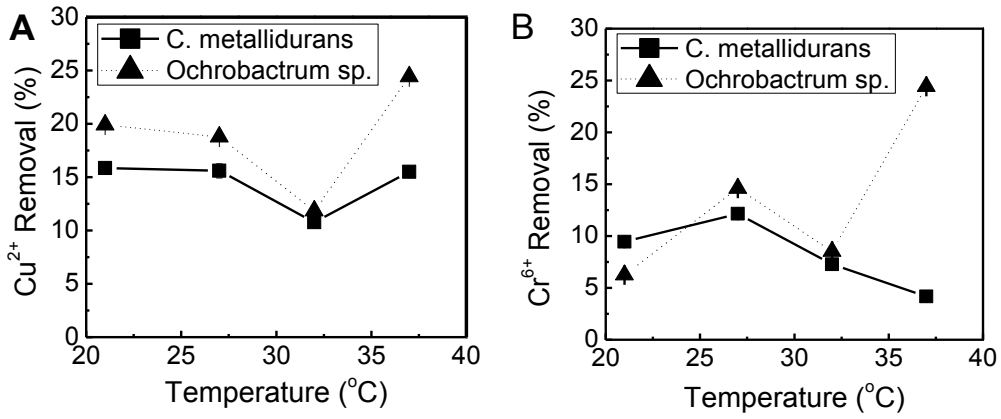

Figure 12: Temperature effect on biosorption. Percentage removal of Cu^{2+} (A) and Cr^{6+} (B) by *C. metallidurans* and *O. intermedium* in TSM media at pH 7, 80 rpm after 400 min incubation at different temperatures.

4.7 Langmuir isotherms of biosorption

The equilibrium adsorption isotherms help establish the ratio between equilibrium concentrations of the solute in the solution and the equilibrium concentration of the solute on the sorbent at a constant temperature. The Langmuir adsorption isotherm model,

which is a mono-layer adsorption model, was used to quantify and contrast the performance of the different biosorbents. Biosorption capacity results of Cu^{2+} and Cr^{6+} obtained with *C. metallidurans* and *O. intermedium* were fitted to the Langmuir isotherm model. The Q_{max} values and the Langmuir constant K_d were obtained from a nonlinear regression analysis and presented in Table 2. The results of the model show that the Cu^{2+} and Cr^{6+} biosorption data obtained by *C. metallidurans* and *O. intermedium* in different growth phases are fit well by the Langmuir isotherm model, with a high correlation coefficient (R^2>0.90).

As seen in Table 9, the Cu^{2+} removal capacity of *C. metallidurans* by dead cells and cells in log phase was 86.783 mg/g, which is about 1.1 times higher than in the cells in stationary phase. The Q_{max} value for Cr^{6+} biosorption of dead *C. metallidurans* was 47.794 mg/g, indicating a higher Cr^{6+} biosorption capacity compared to both cells in log (32.633 mg/g) and stationary (20.479 mg/g) phases. Furthermore, when the results obtained for Cu^{2+} and Cr^{6+} removal by the same strain were compared, the *C. metallidurans* always showed higher biosorption capacity for Cu^{2+} in a range of 1.1 to 6.8 times higher than that for Cr^{6+}.

O. intermedium also presented a similar adsorption trend to *C. metallidurans* for Cu^{2+}. The dead phase showed 30% and 50% higher removal capacity than the log and stationary phases, respectively. However, the adsorption capacity of Cr^{6+} for *O. intermedium* presented a Q_{max} of 57.956 mg/g and 23.611 mg/g for log and stationary

phases, respectively; while the dead biomass presented the lowest Q_{max} (10.938 mg/g). Additionally, the maximum biosorption capacities of *O. intermedium* for Cu^{2+} were always higher than that of Cr^{6+}.

Table 9: Langmuir model estimated parameters for *C. metallidurans* and *O. intermedium* in different bacterial growth phases.

Heavy metals	Bacterial growth phases	Bacterial strains as Absorbents	Estimated parameters for the Langmuir isotherm		
			K_d	Q_{max}(mg/g)	R^2
Cu^{2+}	log	*C. metallidurans*	0.0067	86.78	0.93
		O. intermedium	0.0062	57.68	0.95
	stationary	*C. metallidurans*	0.0025	38.72	0.97
		O. intermedium	0.0039	49.93	0.96
	dead	*C. metallidurans*	0.0080	86.78	0.97
		O. intermedium	0.0046	75.14	0.96
Cr^{6+}	log	*C. metallidurans*	0.0311	32.63	0.90
		O. intermedium	0.0116	51.96	0.96
	stationary	*C. metallidurans*	0.0229	20.48	0.99
		O. intermedium	0.0132	23.61	0.95
	dead	*C. metallidurans*	0.0288	47.79	0.96
		O. intermedium	0.0173	10.94	0.95

K_d: Langmuir constant; Q_{max}: maximum adsorption capacity; R^2: regression coefficient

4.8 Biosorption capacity of *C. metallidurans* and *O. intermedium* under different environmental conditions.

Microorganisms generally found in heavy metal contaminated environments are not necessarily within their optimum conditions for heavy metal removal. Therefore, it is necessary to compare their heavy metal removal efficiencies under optimum and environmental conditions. In the present study, the environmental conditions were defined as a temperature of 27^0C and pH 7. The optimum conditions for each microorganism in the presence of each heavy metal were determined in section of "Effect of pH and Effect of temperature." The results from the Langmuir isotherms showed that higher removal efficiencies were achieved under the optimum conditions for both microorganisms. The optimized heavy metal removal for *O. intermedium* was at 37^0C, while for *C. metallidurans* was at 27^0C. For both microorganisms, the optimum pH was 6 for Cu^{2+} removal, while the best pH for Cr^{6+} biosorption was 7. In most cases, the optimum conditions were different for each microorganism; with the exception of Cr^{6+} for *C. metallidurans*, which presented an optimal heavy removal under the same parameters as the environmental conditions.

Both live bacteria investigated in this study exhibited optimum heavy metal removal in the log phase. Therefore this phase was further investigated. The results for the Langmuir isotherm model parameters (Table 10) showed that the adsorption capacity of Cu^{2+} by *C. metallidurans* under optimum heavy metal removal conditions was 127.21

mg/g, which is 1.4 times higher than the maximum adsorption capacity observed under environmental conditions. For *O. intermedium*, the maximum capacity about Cu^{2+} removal efficiency under environmental conditions was about 48% of the value under the optimum laboratory conditions. In the case of Cr^{6+} removal, *O. intermedium* presented a 46% higher removal capacity under optimum conditions than the environmental condition.

Table 10: Parameters of the Langmuir isotherm model for *C. metallidurans* and *O. intermedium* under different growth conditions.

		Optimum condition			Environmental Condition		
		K_d	Q_{max}(mg/g)	R^2	K_d	Q_{max}(mg/g)	R^2
Cu^{2+}	*C. metallidurans*	0.01	127.21	0.90	0.006	86.68	0.93
	O. intermedium	0.11	107.04	0.91	0.062	51.62	0.94
Cr^{6+}	*C. metallidurans*	0.031	32.63	0.90	0.031	32.63	0.90
	O. intermedium	0.023	125.22	0.97	0.011	57.95	0.96

4.9 Cu^{2+} and Cr^{6+} adsorption competition assays under environmental and optimum conditions

After investigating the single metal biosorption, the next step was to understand the heavy metal binding preference by each microorganism. In this assay, a solution containing different ratios of Cu^{2+} and Cr^{6+} were exposed to each microorganism. These competitive biosorption experiments were conducted under environmental and optimum conditions to determine any confluent interaction among the heavy metals.

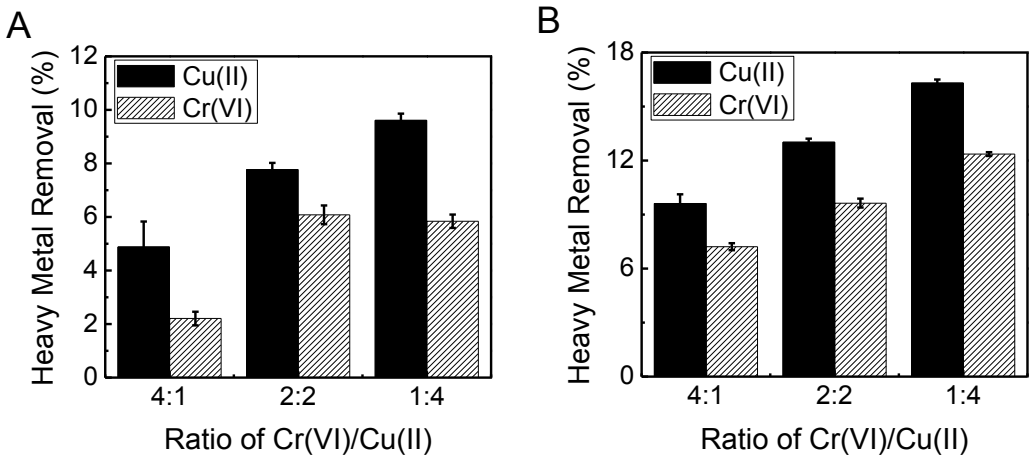

Figure 13: Heavy metal removal in the presence of different ratios of Cu^{2+} and Cr^{6+}. Result for heavy metal competition for *O. intermedium* under (A) environmental conditions and (B) optimum heavy metal removal conditions.

The results of the competitive biosorption assays with *O. intermedium* indicated that both Cu^{2+} and Cr^{6+} removal increased with the decreasing ratio of Cr^{6+}/Cu^{2+} (Figure 13). It is worth noting that the percentage of Cu^{2+} removal was always 1-2 times higher than the removal of Cr^{6+} under both environmental and optimum conditions in different ratios of Cr^{6+}/Cu^{2+}. Moreover, the optimum condition was an important parameter which led to 30% higher removal of Cu^{2+} and Cr^{6+} than the environmental condition.

When comparing the results of the environmental conditions of a single metal with a mixture of Cu^{2+} and Cr^{6+}, the removal of Cu^{2+} decreased by 10% with the lowest concentration of Cu^{2+} in the presence of Cr^{6+}, the competing metal ion. A much lower removal rate was achieved with a higher ratio of Cr^{6+}. The Cr^{6+} removal efficiency decreased from 25% to 7.4% in a mixture of Cr^{6+} and Cu^{2+} (4:1 v/v) compared to Cr^{6+} alone under the optimum removal conditions. Similar results were observed for *C.*

metallidurans, which presented a lower removal capacity of Cu^{2+} and Cr^{6+} in the heavy metal mixture than in a solution with a single metal.

4.10 SEM and EDS

In order to confirm that the metal ions are binding to the cell biomass, SEM and EDS were employed. The EDS analysis showed the percentage of Cr^{6+} found in the cell biomass of *O. intermedium* after exposure to Cr^{6+}. In the control sample, as expected, no Cr^{6+} was detected, as opposed to 1.6% found in the biomass exposed to the heavy metals (Fig. 15). In the figure, (A) represented an *O. intermedium* control sample without heavy metal exposure, (B) represented *O. intermedium* biomass exposed to Cu^{2+}, (C) represented *O. intermedium* biomass exposed to Cr^{6+}. For *C. metallidurans*, (D) represented the control sample without heavy metal exposure, (E) represented *C. metallidurans* biomass exposed to Cu^{2+}, (F) represented *C. metallidurans* biomass exposed to Cr^{6+}. Similarly, the Cu^{2+} percentage in the bacterial biomass also increased from 2.94% to 24.54% after exposure to Cu^{2+}. In the case of *C. metallidurans*, the percentage of Cu^{2+} in the biomass increased from 5.34% to 29.76% after exposure to Cu^{2+}, while the Cr^{6+} went from 0 to 0.19%. These results confirmed that the biomass is indeed adsorbing the heavy metals.

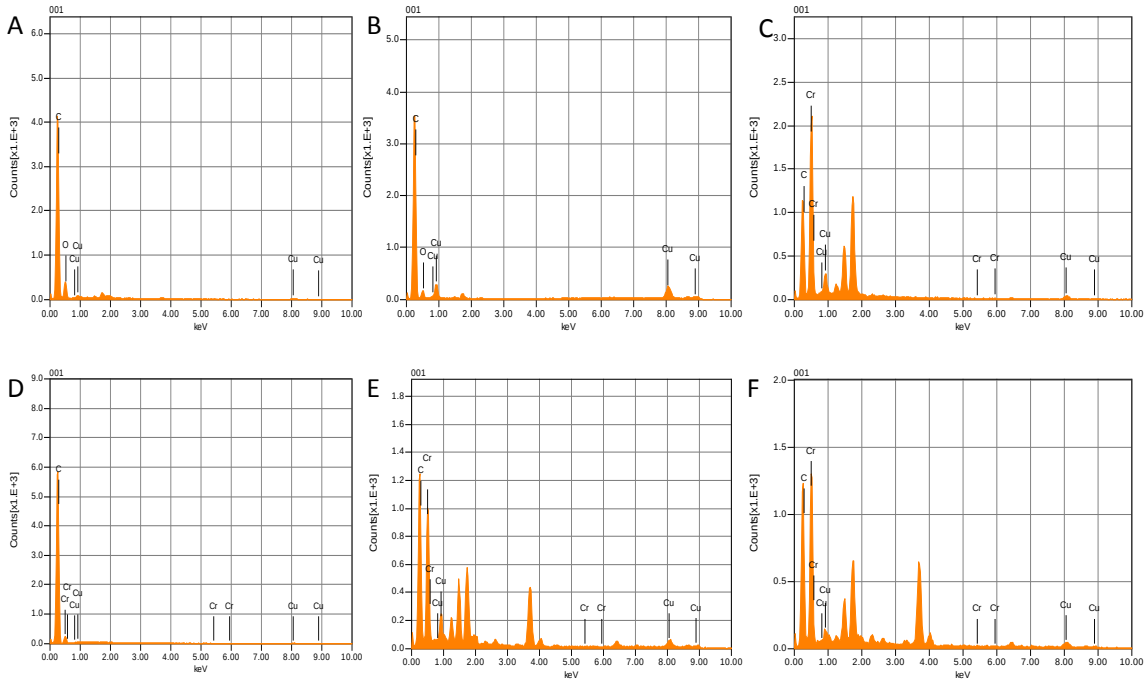

Figure 14: EDS analyses of *O. intermedium* and *C. metallidurans* in the presence of heavy metals

4.11 FT-IR spectral analysis

The bacterial cell membrane contains different functional groups responsible for the heavy metal biosorption. FT-IR spectroscopy analyses of the control biomasses, the heavy metal exposed biomasses at log and stationary phases, and heat killed biomass were carried out to obtain information about the functional groups on the bacterial surface that could be involved in the adsorption process. The results are shown in Figures 14 (A) and (B), and a summary of the possible metal binding sites is presented in Table 11. The results of the FT-IR spectra of the live biomass without interaction with heavy metal showed five distinct peaks. The broad stretching adsorption band at 3327 cm^{-1} corresponds to both–NH_2 and –OH groups. The intense peak observed at 1710 cm^{-1} and

1651 cm^{-1} is attributed to C=O group. The band peaks corresponding to C-H groups were evident at 2940 cm^{-1} and 1440 cm^{-1}. By comparing the spectra of log and stationary phases to the peaks of dead biomass, a shift was observed from 1710 cm^{-1} and 1651 cm^{-1} to 1703 cm^{-1} and 1620 cm^{-1}, respectively. An extra peak at 1728 cm^{-1} was also observed for the heat killed biomass.

After interaction of *O. intermedium* at log and stationary phases with Cu^{2+} and Cr^{6+}, the band at 1710 cm^{-1} shifted to 1708 cm^{-1}, while the band at 1651 cm^{-1} shifted to 1638 cm^{-1}. The intensity of the bands at 1440cm^{-1}, 2940cm^{-1} and 3327cm^{-1} decreased significantly and almost disappeared after *O. intermedium* was exposed to 100 ppm Cu^{2+} and Cr^{6+}. Moreover, substantial changes at 1511 cm^{-1}, including the appearance of multiple peaks in between 1511 cm^{-1} and 1650 cm^{-1} were also observed after *C. metallidurans* in the logarithmic phase interacted with Cr^{6+} and Cu^{2+}.

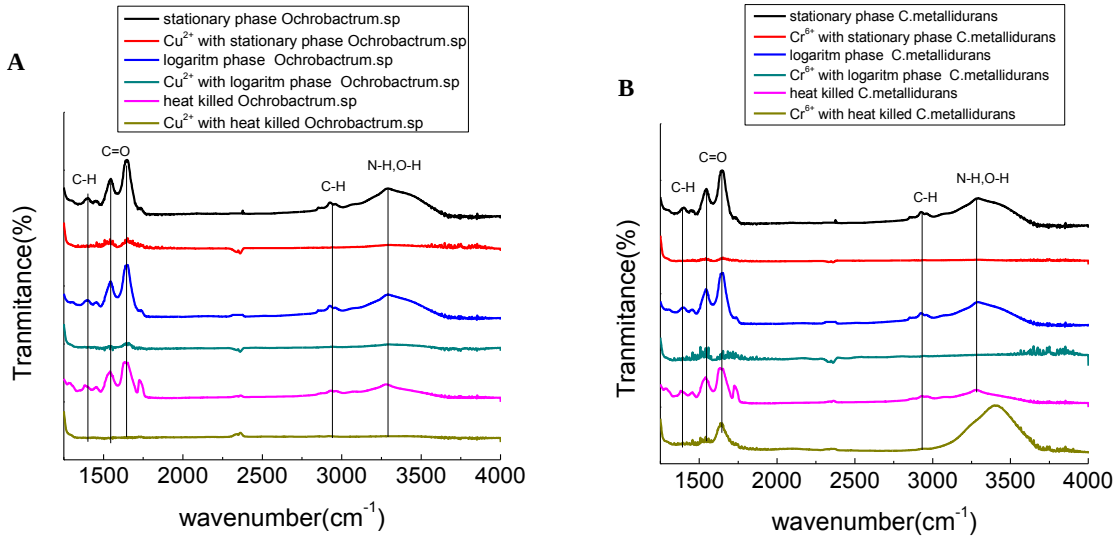

Figure 15: FTIR spectra of (A) log and stationary phases, and heat killed cells of *O. intermedium* exposed to $Cu^{2+}$and their respective controls; (B) log and stationary phases, and heat killed *C. metallidurans* exposed to Cr^{6+} and their respective controls.

The spectrum for the non-exposed heat killed *O. intermedium* cells to heavy metal was compared to that of dead cells exposed to Cu^{2+} and Cr^{6+}, all the peaks at 1440 cm^{-1}, 1620 cm^{-1}, 1703 cm^{-1}, 1728 cm^{-1}, 2940 cm^{-1} and 3327 cm^{-1} decreased significantly after exposure to the heavy metals. Similar observation was found in the biosorption of Cu^{2+} for *C. metallidurans*. However, the biosorption of Cr^{6+} for *C. metallidurans* was slightly different. As observed in Figure 14 (B), bands at the wavelength of 3327 cm^{-1} shifted to 3416 cm^{-1}, and bands at 1708 cm^{-1} shifted to 1709 cm^{-1}. Besides, 1440 cm^{-1}, 1620 cm^{-1} and 2940cm^{-1} peaks in heat killed biomass drastically decreased in the presence of Cr^{6+}.

Table 11: The FTIR band assignments and the possible metal binding sites in both bacteria.

Wave number (cm^{-1})			Assignment	Probable sites for Cu^{2+} and Cr^{6+} interaction with live *O. intermedium* and *C. metallidurans*	Probable sites for Cu^{2+} and Cr^{6+} interaction with dead *O. intermedium* and *C. metallidurans*
Log phase	Stationary phase	Heat killed			
1440	1440	1440	C-H	Binding to lipoprotein	Binding to lipoprotein
1651	1651	1620	C=O	Interaction with protein	Interaction with protein
1710	1710	1703	C=O	Interaction with protein	Interaction with protein
-	-	1728	C=O and COOH	-	Interaction with protein
2940	2940	2940	C-H	-	-
3327	3327	3327	N-H_2 and O-H	Interaction with Polysaccharide and proteins	Interaction with Polysaccharide and proteins

5. Discussion

5.1 *O. intermedium* and *C. metallidurans* presents high tolerance to Cu^{2+} and Cr^{6+}

C. metallidurans presented a MIC for Cu^{2+} and Cr^{6+} of 750 ppm and 100 ppm, respectively, while *O. intermedium* presented a MIC of 300 and 1,000 ppm for Cu^{2+} and Cr^{6+}, respectively. These results strongly suggested that different microorganisms have different levels of tolerance to Cu^{2+} and Cr^{6+}. In a previous study with another strain of *O. intermedium,* the MIC for Cu^{2+} was 2.3 times lower (Faisal & Hasnain, 2004) than our results. On the other hand, our MIC result for Cr^{6+} was consistent with a previous study (Thacker et al., 2007).

The variation on the MIC results of Cu^{2+} for *O. intermedium* from one research group to another can be explained by the use of different growth medium. In our study, TSM was used; however, the other group used nutrient broth to determine the MIC of Cu^{2+} for *O. intermedium*. The later medium is fundamentally different from ours in the content of sugar and amino acids, which have chelating properties (Ramamoorthy & Kushner, 1975). Studies on complexation between the metal cations and components of the growth medium showed that MIC values obtained in rich media are usually two to five times higher than in salt-based minimum media, such as the TSM (Zakaria et al., 2007). Similarly, another study with rich media reported that the MIC of *C. metallidurans* for Cu^{2+} and Cr^{6+} to be 950 and 175 ppm, respectively (Rojas et al., 2011), which were 16%

to 26% higher than our results. While Monsieurs (Monsieurs et al., 2011) obtained very similar MIC values for *C. metallidurans* in the presence of Cu^{2+} and Cr^{6+} because they used a minimum medium.

It is worthwhile to point out that both microorganisms investigated in our study showed higher MIC to Cu^{2+} than other well-known microorganisms, such as *Escherichia coli* (140 ppm) (Ruparelia et al., 2008), *Bacillus subtilis* (40 ppm) (Ruparelia et al., 2008), *Staphylococcus aureus* (140 ppm) (Ruparelia et al., 2008), *Pseudomonas sp.* (50 ppm) (Malik & Jaiswal, 2000) and *Ochrobactrum anthopi* (125 ppm) (G. Ozdemir et al., 2003). In the case of Cr^{6+}, the MIC results obtained with the *O. intermedium* isolate was considerably higher than the *C. metallidurans* in this study and other reported microorganisms, such as bacteria (Munoz et al., 2012; G. Ozdemir et al., 2004; G. Ozdemir et al., 2003; Zafar et al., 2007) and fungi (Ahmad et al., 2006; Zafar et al., 2007). These results show that this new isolate is well adapted to survive in environments polluted with high levels of Cu^{2+} and Cr^{6+}.

5.2 Microorganisms in stationary phase are less susceptible to heavy metal exposure

It has been established that microorganisms in different growth phases have different membrane lipid compositions and metabolic activities (Engel & Alexander, 1958; Tweeddale et al., 1998). Previous studies have established that microorganisms in logarithmic phase are more susceptible to toxic chemicals and heavy metals (Chandran &

Love, 2008; Yang et al., 2007). For instance, in the study of Teitzel (Teitzel & Parsek, 2003), logarithmically grown *Pseudomonas aeruginosa* showed higher resistance to lead and copper than cells in stationary phase.

Similarly, in this study, it was observed that the cells grown in stationary phase were more resistant to the heavy metal exposure than cells in log phase (Figure 8). Heavy metal effects on microorganisms involve interference in the normal processes of cell wall synthesis and cell division (Brown et al., 1990; Brown & Williams, 1985; Gilbert et al., 1990). The decreased susceptibility of cells in stationary phase to the heavy metals can be explained by the reducing growth rates and uptake of nutrients and heavy metals under conditions of dwindling energy resources (Chandran & Love, 2008). The reduced growth rates of microorganisms under nutrient starvation have been widely reported to influence microbial susceptibility to antimicrobial agents (Brown et al., 1990; Brown & Williams, 1985; Delarosa et al., 1982; Tuomanen et al., 1986) for a wide range of microorganisms (Brown & Williams, 1985; Gilbert & Brown, 1980; Holme, 1972; Meers & Tempest, 1970).

Additionally, microorganisms in different growth phases can have different membrane lipid compositions, cell metabolism (Kjelleberg et al., 1987), and cell wall compositions (Falkinham et al., 2003), which would directly affect their susceptibility to toxic chemicals (Falkinham et al., 2003). Therefore, the lower inactivation of *C. metallidurans* and *O. intermedium* in the stationary-phase after exposure to the heavy

metals is probably due to changes in cell metabolism, membrane lipids and cell wall compositions. Further studies, however, should investigate these cellular changes in the different growth phases to better understand the specific mechanisms of resistance of these microorganisms to these heavy metals.

5.3 Biosorption of logarithmic and stationary phases and heat killed bacteria over time

In addition to heavy metal susceptibility at different microbial growth phases, results reveal that the growth phase can also affect the bacterial biosorption capacities of heavy metals, as observed in figures 9 and 10. The results show that cells in exponential phase exposed to heavy metals exhibited higher metal removal than the cells in stationary phase. This phenomenon was well explained by Daughney (Daughney et al., 2001). They reported that the effect of growth phase on proton adsorption is likely related to changes in the structure and composition of the cell walls and cell membranes, which are directly affected by the changing chemistry of the growth medium. As a result, the high abundance of nutrients in the log-phase available to cells stimulates the synthesis of cell walls and membranes containing large number of molecules with binding sites for these nutrients. These molecules, which generally are made of proteins, can also absorb cations such as heavy metals (Daughney et al., 2001; Mclean et al., 1990). Another study by Simmons and Singleton (Simmons & Singleton, 1996) indicated that intracellular components can also play an important role in the heavy metal binding capacity.

According to their study, the metal uptake capacity of cells growing exponentially is generally higher than the ones in stationary phase. Other reports have confirmed this finding (Daughney et al., 2001; Kiran et al., 2005; Macaskie & Dean, 1984).

Although log and stationary growth phases presented different removal efficiencies of heavy metals for both *O. intermedium* and *C. metallidurans*, both growth phases displayed two-stage sorption behavior. The first stage corresponded to a rapid initial biosorption of Cu^{2+} and Cr^{6+}, followed by a slow stage after one to two hours. This similar trend was previously reported for Cu^{2+} removal by *C. metallidurans* (W. M. Chen et al., 2008). In this study and our study, the initial rapid biosorption was the dominating mechanism behind removal of the heavy metal. This fast initial heavy metal removal indicated that the adsorption mechanism on the cell surface was probably the main mechanism for the heavy metal removal, which seems to be similar to processes like ion-exchange, complexation, precipitation on and within the microfibrillar cell wall (Blackwell et al., 1995; Das et al., 2007).

Following this initial fast heavy metal removal, there is a slow stage where the binding sites on the biomass are becoming saturated and the sorption become less efficient (Saeed et al., 2005). The slow stage may continue for several hours to a day, while the rapid stage last for several minutes to a few hours (Saeed et al., 2005). In our study, the rapid stage happened in the first 150 min and the slow stage occurred for 250 min. The first stage has been described as being independent of cell metabolic activity,

and referred to as biosorption or passive uptake (Yetis et al., 2000). It usually involves the rapid binding of metal ions to the cell biomass. The second stage involves slow intracellular diffusion represented by the uptake of metal ions across the cell membrane (Isik, 2008; Yetis et al., 2000).

In addition to live cells, the heavy metal removal properties of heat killed bacteria were also investigated. Most of the results showed that dead biomass presented higher biosorption capacity than live cells in both phases (see figures 9(B) and 10(A)). This biosorption capacity enhancement of heat killed biomass could be attributed to an increase release and exposure of intracellular binding sites caused by the heat treatment (Gabr et al., 2008). Additionally, Gabr reported that increase of organic compounds per unit gram of biomass, and the absence of competing H^+ ions, commonly produced by live cells, could explain the higher removal capacity of dead cells. A zeta potential study (Huang et al., 2013) showed that dead cells have a more negatively charged surface and exhibit greater biosorption capacity than live cells. According to these reports, dead cells should have a better removal capacity than live ones. However, according to the data showed in Figure 10 (B), a statistically significant decrease in the Cr^{6+} sorption capacity was observed in the heat killed *O. intermedium* with respect to the microorganisms' log and stationary phases. Similar observation was reported by other studies using heat killed mixed culture of bacteria (Isik, 2008; Paul et al., 2012). Another study (D. Park et al., 2005) also demonstrated that the chromium biosorption efficiency of thermally treated

cells of *Electonia sp*. was less than that observed for the control cells. They suggested that the physical and chemical properties of the cells may have been altered by the thermal treatment. In this case, it is possible that the binding sites with high affinity for this heavy metal have been denatured, destroyed or modified by the heat treatment. These findings suggest that different heavy metals can have different binding sites in the biomass and that some of these binding sites can be heat sensitive. However, more work is necessary to better understand the biosorption behavior by dead and live biomass of different microorganisms due to the complexity of the biosorption phenomenon.

5.4 Effects of Environmental factors on heavy metal biosorption: pH and temperature

Heavy metal removal capacity can be affected not only by different growth phases of the biosorbents as mentioned above, but also by certain environmental parameters, like pH and temperature. The pH controls the surface properties of sorbents, functional groups and ionic state of metal species (Kiran et al., 2006; Kiran et al., 2005). In order to investigate the pH effect on *O. intermedium* and *C. metallidurans,* batch adsorption experiments at pH ranging from 3.0 to 8.0 were prepared with biomass in log phase, which showed the highest removal capacity. The pHs below 3.0 were not investigated because these microorganisms do not grow in highly acidic conditions,as discovered in preliminary experiments. The maximum pH of 8 was chosen because at higher pHs, the metals precipitate and interfere with the analyses (Kiran et al., 2005; Tunali & Akar,

2006).

The main reason for investigating the effect of pH in heavy metal removal is because in general, at low pHs (pH<4), heavy metal removal efficiency is reduced. This phenomenon is caused by hydronium ions (H_3O^+) competing with the heavy metals for their binding sites (Karthikeyan et al., 2007). Another study also reported that functional groups become negatively charged as the pH increases (Kiran et al., 2006). This could explain the increasing removal capacity observed by both microorganisms from pH 4 to 6. At higher pH (pH>6), the metal hydroxide, which is copper hydroxide in this case, will form due to hydrolysis of metal ions. Hence, the removal of copper cannot be fully attributed to adsorption, but also to precipitation (Demirbas, 2009; Lisal et al., 2004; Musico et al., 2013).

It is worth noting that the increasing Cu^{2+} biosorption by *C. metallidurans* with the increasing pH agrees with previously reported studies (Mohan et al., 2006). The study of Chen also determined that the optimum pH values for Cu^{2+} removal by *C. metallidurans* is 6 (W. M. Chen et al., 2008). This study demonstrated for the first time that *O. intermedium* has an optimum pH of 6 for the Cu^{2+} removal, which is similar to other microorganisms previously investigated (Kapoor et al., 1999; Mohan et al., 2006).

In the case of Cr^{6+}, the maximum removal was observed to be at pH 7.0 for both *C. metallidurans* and *O. intermedium*. The removal of Cr^{6+} has been described to involve multiple phenomena, such as chemical reduction of Cr^{6+} to Cr^{3+}, adsorption of chromium

ions, and protonation of the cell wall functional groups depending on the pH solution. The increase in pH leads to deprotonation of the metal binding sites, which increase the negative charges on the cell surface , which in return attracts Cr^{3+} (Quintelas et al., 2011). The optimum pH obtained for the removal of Cr^{6+} (pH 7.0) by our new isolate, *O. intermedium*, is similar to another isolate of the same species, *Ochrobactrum intermedium* strain SDCr-5 (Sultan & Hasnain, 2007). In addition, Wang et al. (Y. T. Wang & Xiao, 1995) and Bopp et al. (Bopp & Ehrlich, 1988) reported that *Bacillus sp*. and *Pseudomonas fluorescens* requires a pH of 7 for an optimum biosorption of Cr^{6+}. Therefore, the results show that, for some heavy metals, such as Cu^{2+} and Cr^{6+}, the removal seems to be dependent more on the pH rather than on the biosorbents.

Another environmental parameter significantly affecting heavy metal biosorption is temperature (Congeevaram et al., 2007). Temperature affects biosorption by changing (a) the stability of metal ion species initially placed in the solution; (b) the stability of microorganism-metal complexes depending on the biosorption sites; (c) the microorganism cell wall configuration; (d) the ionization of chemical moieties on the cell wall (Sag & Kutsal, 2000). These factors may simultaneously or individually affect the binding sites present in the bacterial biomass. Our results also confirmed that temperature played a major role in the adsorption of heavy metal, since *C. metallidurans* and *O. intermedium* showed different optimum temperatures for the different heavy metal removals (Fig 12 (A) and (B)). A previous study with another strain of *O. intermedium*

showed that the optimum temperature for Cr^{6+} biosorption was 37^0C, which is consistent with our results (Faisal & Hasnain, 2004). For *C. metallidurans*, the temperature of 37^0C was determined by our study, for the first time, to be also the optimum temperature for Cr^{6+} biosorption. It is important to point out that different organisms can have similar optimum temperatures for the same type of heavy metal removal. It is possible that the removal of certain heavy metals is not necessarily linked to the type of microorganism used, but rather to the combination of environmental factors, such as pH and temperature.

It is worth note that in the case of Cu^{2+} removal for *C. metallidurans,* the temperature did not play a major role in the heavy metal removal. A similar lack of relationship was observed by other researchers with copper over a temperature range of 25^0C to 55^0C (Antunes et al., 2003; Dang et al., 2009). The lack of temperature effect on adsorption can be explained by the type of molecules interacting with the heavy metal in question. Another study explained further that depending on the isolation site, similar species of microorganisms could generate different biosorption efficiencies for the same heavy metal (Murphy et al., 2007).

5.5 Maximum biosorption capacity of different biosorbents under environmental and optimum pH and temperature conditions.

After determining the effects of temperature and pH in the adsorption of Cu^{2+} and Cr^{6+}, adsorption capacities were modeled using the Langmuir model. Essentially, the Langmuir isotherm determines the maximum adsorption capacity (Q_{max}), which normally

cannot be determined through experimental conditions (H. Li et al., 2010). This model is commonly used in studies related to heavy metal removal using biomass. The Langmuir model assumes that the sorption has a monolayer mechanism and that all the active sites on the sorbent surface have the same affinity for the sorbate. Furthermore, the Langmuir isotherm model assumes that the adsorbent surface has uniform energies of adsorption (Colak et al., 2009; H. Li et al., 2010).

In the Langmuir model, a high value of K_d, represented by a steep initial slope in the isotherm, indicates high sorbent affinity. Thus, high Q_{max} and a steep initial isotherm slope (high K_d) are desirable for a good biosorbent (Vijayaraghavan et al., 2006). This study demonstrates that both *C. metallidurans* and *O. intermedium* possess great ability to adsorb Cu^{2+} and Cr^{6+}. In terms of the weight-based maximum adsorption capacity (Q_{max}), *C. metallidurans* and *O. intermedium* had much better capacity for Cu^{2+} adsorption under environmental and optimum conditions when compared to other effective Cu^{2+} biosorbents, such as *Myriophyllum spicatum* (G. X. Li et al., 2010), *Sphaerotilus natans* (Pagnanelli et al., 2003). Additionally, *O. intermedium* had a particularly high affinity for Cr^{6+} removal under its optimum condition as opposed to *C. taiwanensis* (W. M. Chen et al., 2008) and *O. antropi* (G. Ozdemir et al., 2003). These results suggest that *C. metallidurans* and *O. intermedium* could be good candidates for cleaning up environments contaminated with both Cu^{2+} and Cr^{6+} (W. M. Chen et al., 2008).

Furthermore, the results (Table 9, 10) of this model showed that the maximum

uptake capacities for Cu^{2+} were always higher than for Cr^{6+}. Furthermore the higher biomass adsorption for Cu^{2+} was independent from the type of microorganism used, the growth phase, and the environmental factors (i.e., environmental condition or optimum pHs and temperatures). These results suggested that Cu^{2+} has greater affinity to the binding sites presented on the surface of *C. metallidurans* and *O. intermedium* than Cr^{6+}. Previous reports also suggested that metal sorption increased with increasing valence and atomic number (Holan & Volesky, 1994; Ozer & Ozer, 2003). Therefore, as expected, in this study, the sorption capacity for $^{29}Cu^{2+}$ was higher than for $^{24}Cr^{6+}$, which is in agreement with previous reports that also demonstrated this sorption preference (Nasernejad et al., 2005; Schmuhl et al., 2001).

In the Langmuir model, a monolayer sorption mechanism is observed when the sorbent surface reaches its full saturation (Cruz et al., 2004). Previous studies with heat killed biomass also suggested that even when sufficient biosorption sites are available, a monolayer sorption is highly probable (Huang et al., 2013; H. Li et al., 2010; Paul et al., 2012). In this study, the dead biomass presented a R^2 value higher than the log and stationary phases for the biosorption of Cu^{2+}. This indicates that the biosorption of Cu^{2+} onto dead cells of *C. metallidurans* and *O. intermedium* were likely to be of a monolayer type of sorption. Similar results were reported by a number of studies on adsorption of heavy metals by the dead cells (H. Li et al., 2010; Sari & Tuzen, 2008; Vilar et al., 2007). Further studies suggested that because dead bacteria can have higher biosorption

capability than live cells, they could potentially be used as biosorbents for heavy metal remediation (Selatnia et al., 2004; H. Li et al., 2010).

The time-course biosorption assays and Langmuir isotherm models for dead *C. metallidurans* also presented higher Cu^{2+} and Cr^{6+} removal capacity. Additionally, dead *O. intermedium* was also capable of up taking higher amounts of Cu^{2+} than living biomass. However, in one special case, dead *O. intermedium* was not as effective as live biomass in the removal of Cr^{6+}. Therefore, not all dead biomass from microorganisms present a good biosorption capacity and requires further investigation to better understand the mechanisms of adsorption.

In this study, the Langmuir isotherm models for heavy metal removal under environmental conditions were also compared with the optimum temperature and pH adsorption capacity conditions for the different microorganisms at the log phase. The only exception for this comparison was Cr^{6+} removal by *C. metallidurans*, which presented an optimal heavy removal in the environmental condition tested in this study. According to the preliminary experiment, log phase biomass showed higher removal capacity than stationary phase. Therefore, log phase biomass was selected for the comparison of heavy metal removal capacity under environmental and optimum conditions. The results indicated that Cu^{2+} and Cr^{6+} removal capacity by *O. intermedium* decreased from 50% to 300%, when the pH and temperature changed from optimum to environmental conditions. At the same time, the biosorption of Cu^{2+} by *C. metallidurans*

under optimum conditions showed 1.4 times higher adsorption capacity than in the environmental condition. Therefore, the results indicated that *C. metallidurans* and *O. intermedium* always performs better under their optimum temperature and pH on single metal biosorption. This comparison is useful for real bioremediation applications, for the selection of the proper microorganism for a certain environment, and to allow maximization of heavy metal biosorption in different contaminated sites. Therefore, these results not only demonstrated the importance of the environmental parameters like pH and temperature, but also yielded information for predicting final removals under optimum and non-optimum conditions.

Under these same pH and temperature conditions, binary metal solutions with different ratios of Cu^{2+}/Cr^{6+} were also investigated. The mechanism of multi-metal ions uptake by microorganisms is quite complex. Competition for the surface binding sties among the different metal-ions will occur and will partially depend on the ionic characteristics (Aksu & Donmez, 2006; Pavasant et al., 2006; Tunali & Akar, 2006). There are also possible interactive effects of different species in solution and potential interactions with the surface (Aksu & Donmez, 2006). In general, a mixture of metals can produce three possible types of behavior: synergism, antagonism, and non-interaction (Aksu & Donmez, 2006; Mohan et al., 2006; Ziagova et al., 2007). In this study, the removal of Cu^{2+} and Cr^{6+} decreased when both metals were present in the solution. For example, for *O. intermedium* the removal of Cu^{2+} decreased by more than 10% in the

presence of the competing metal ion Cr^{6+}. The value of Cr^{6+} removal efficiency by *C. metallidurans* also decreased from 25% to 7.4% in a mixture of Cr^{6+} and Cu^{2+} (4:1 v/v), when compared to Cr^{6+} alone under the optimum removal conditions. A 16%-33% decrease in Cr^{6+} removal in the presence of Cu^{2+} was also reported in *E. coli* (Shen & Wang, 1994). Li (H. Li et al., 2010), Yan et al. (Yan & Viraraghavan, 2003) and Aksu (Aksu et al., 2002) reported that this antagonistic action is due to the competition for the same adsorption sites on the cells. Another widely reported explanation for this antagonistic effect is the screening effect (Sag & Kutsal, 1996). A screening effect may arise as the concentration of the biomass increases, leading to the decrease in attraction between electron and nucleus, and then rendering some binding sites partially hindered to metal ions. Hence, the specific metal uptake capacity will decrease (Sheng et al., 2008) .

C. metallidurans and *O. intermedium* have a preference for Cu^{2+} ions. Reports with other microorganisms confirmed the preferential removal of Cu^{2+} over Cr^{6+} (Sag & Kutsal, 1996). This selectivity also reconfirmed one of the conclusions from our time-course biosorption assays and the Langmuir isotherms that heavy metals with higher atomic numbers were preferentially biosorbed.

Another important parameter that determines preferential heavy metal species removal is pH. As showed in the section of "Effects of Environmental factors on heavy metal biosorption," the optimum pH for Cu^{2+} biosorption was determined to be 6, while the optimum pH for Cr^{6+} removal was 7. In order to minimize the pH effect on the

preferential biosorption, the Cu^{2+} and Cr^{6+} mixture was adjusted to pH 6.5. Surprisingly, at this pH, Cu^{2+} was still the preferred metal to be biosorbed. It is possible that the affinity of the binding sites for Cr^{6+}, at the pH 6.5, were reduced and; therefore, Cu^{2+} was preferentially biosorbed (Aksu et al., 2002; Sag & Kutsal, 1996). Since, in this study the effect of this pH in the individual adsorption of these metals were not investigated, there is no experimental evidence to prove that the biosorption of these two metals were equal at pH 6.5.

Additionally, as depicted in Figure 7, the uptake percentage of Cu^{2+} increased with the increasing of Cu^{2+} concentration. This result suggests that the Cu^{2+} binding sites are more abundant or have preferential binding sites for Cu^{2+} rather than Cr^{6+} on the cell surface, which enhanced the uptake of Cu^{2+} metal ions (Pagnanelli et al., 2001). Another explanation might be the toxicity levels of Cr^{6+}. The higher concentrations of Cr^{6+} may damage the main structural components of the cells. Therefore, in mixtures with higher Cu^{2+} concentrations will have lower Cr^{6+} concentration, which lead to a less toxic mixture (Sag & Kutsal, 1996). Therefore, the removal capacity of both heavy metals will increase.

Furthermore, the comparison of heavy metal competition between the environmental conditions and optimum conditions was also conducted. The results reconfirmed that the optimum conditions of pH and temperature were important parameters which led to more than 30% higher removal of Cu^{2+} and Cr^{6+} than the

biosorption under environmental conditions.

5.6 *C. metallidurans* and *O. intermedium* heavy metal binding sites

After evaluating the biosorption capacity, the following step was to determine the biosorption mechanisms. The initial investigation of the adsorption of heavy metal by *C. metallidurans* and *O. intermedium* was performed by EDS. In these preliminary results, the presence of heavy metal on the cell surface was measured. Mechanisms involved in metal biosorption are complicated since they depend on the biomass employed and the types of metals. Among various proposed removal mechanisms, ion exchange was thought to be one of the most important process for heavy metal removal by bacterial biomass (Bueno et al., 2008). For instance, Tunali and collaborators reported that ion exchange was involved in the Pb^{2+} and Cu^{2+} removal by *Bacillus sp.* (Tunali, Cabuk, et al., 2006). Moreover, Srivastava et al. also revealed that Cr^{3+} was adsorbed and later up taken inside the cells through TEM and EDS analyses (Srivastava & Thakur, 2006).

Furthermore, heavy metal removal by surface complexation with the cell membrane and cell wall functional groups is also widely reported to be an important mechanism for biosorption (Han et al., 2006; Huang et al., 2013; Murphy et al., 2007; Quintelas, Femandes, et al., 2008). Therefore, FTIR was investigated to assess the contribution of the functional groups in the cell membrane. The spectrum of cells in log and stationary phases, and heat killed cells not exposed to the heavy metals showed a broad band at 3327 cm^{-1}, which has been attributed to the overlapping of N-H and O-H

stretching vibrations from polysaccharides and proteins (L. Deng et al., 2007; Paul et al., 2012). Other peaks were also observed in different wavelengths between 1500 cm^{-1} and 1800 cm^{-1}, which usually belong to amide I and amide II functional groups. These functional groups are related to proteins and peptides sites (Vander Mei et al., 1996). The peaks at 1440 cm^{-1} and 2940 cm^{-1} usually correspond to C-H groups, usually found in lipoproteins and fatty acid components, respectively (L. Chen & Lu, 2006; Paul et al., 2012; Vander Mei et al., 1996).

When comparing the heat-treated biomass with the live biomass, multiple peaks of low intensity for the C=O functional groups coming from proteins between 1500 cm^{-1} and 1800 cm^{-1} were observed for heat-treated biomass, as opposed to one to two peaks of high intensity for the live biomass. This suggested that more binding sites became available after the thermal treatment and that the cellular membranes were no longer intact, which increased the biosorption capacity of the dead cells, as previously observed in our earlier results (Du et al., 2012; H. Li et al., 2010).

The FTIR spectra for the biomasses exposed to Cu^{2+} and Cr^{6+} showed substantially reduced intensity in some of the peaks found in the control biomass. These results suggest that these functional groups were probably binding to Cu^{2+} and Cr^{6+}. As depicted in Figure 8, the shifts of the C=O after interaction with Cu^{2+} and Cr^{6+} was typical for the complexation of the carboxyl groups by coordination with metal ions (Andreazza et al., 2010; Aravindhan et al., 2004; G. X. Li et al., 2010).

The changes in the corresponding functional groups for Cu^{2+} and Cr^{6+} biosorption observed in our results are identical to previous studies with other microorganisms (Guibal et al., 1995; Kiran et al., 2005). They concluded that the main function groups responsible for biosorption of heavy metals in bacteria are carboxylic, hydroxyl and amino groups (Akar & Tunali, 2005; Tunali & Akar, 2006; Tunali, Akar, et al., 2006). These results implied not only the involvement of the related functional groups in biosorption, but also the possibility that there is biosorption through an ion exchange mechanism (Kiran et al., 2005). In addition, the difference between the live and dead biomass biosorption showed that dead biosorbents incurred more changes than the live ones, indicating that more biological functional groups can be involved in the Cu^{2+} and Cr^{6+} biosorption process of dead biosorbents (Huang et al., 2013; H. Li et al., 2010).

6. Conclusions

C. metallidurans is known to resist and biodegrade organic compounds (*e.g*., phenolics or chlorinated aromatic/aliphatic compounds) and inorganic pollutants (*e.g*., heavy metals), and hence this microorganism is highly valuable for bioremediation applications. In this study, a new isolate, *O. intermedium,* isolated from a waste highly contaminated with heavy metal, was compared to *C. metallidurans*.

Through a detailed comparison, the results showed that both *C. metallidurans* and *O. intermedium* have a very good tolerance to Cu^{2+}, while *O. intermedium* presented a much higher tolerance to Cr^{6+}. In addition, all cells harvested in stationary phase showed better survivability than the ones in log phase after exposure to high concentrations of heavy metals. The results also showed that the adsorption of Cu^{2+} and Cr^{6+} microorganisms are largely dependent on growth phase. In this study, *C. metallidurans* and *O. intermedium* had better adsorption capacity in the logarithmic phase. Heavy metal removal by heat killed biomasses, on the other hand, depended largely on the type of heavy metals and microorganism. Environmental factors, such as pH and temperature also affected the adsorption capacity significantly. For instance, the equilibrium biosorption capacity of *O. intermedium* increased about 107% and 116% for Cu^{2+} and Cr^{6+}, respectively, when the pH and temperature were set to their respective optimum conditions. The Langmuir model was found to be a good fit for the experimental data. The maximum adsorption capacities of *C. metallidurans* for Cu^{2+} and Cr^{6+} was

determined to be 107.04 mg/g and 125.22 mg/g, respectively, while for *O. intermedium* the sorption capacities for Cu^{2+} and Cr^{6+} were 127.21 mg/g and 32.63 mg/g, respectively. These results suggest that both *O. intermedium* and *C. metallidurans* are promising biosorbents for the removal of Cr^{6+} and Cu^{2+}. Additionally, mixtures of heavy metal at different concentrations affected the adsorption efficiency of heavy metals. The Cu^{2+} was preferentially adsorbed by both microorganisms. The removals of the heavy metals were directly linked to the presence of amide, carboxyl, and phosphate groups on the biomass surface. Dead biomass presented additional functional groups capable to adsorb heavy metals, leading to higher heavy metal removal than the live biomass.

These microorganisms performed differently under different environmental conditions. They always performed better under their optimum temperature and pH. It is worth emphasizing that their optimum conditions were different for the different heavy metals; therefore they could be used in different environments where the pH and temperature is closer to their optimum conditions. Further studies should be done with different heavy metals, as well as on their performance under multi-metal conditions.

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