

## Heavy Metals Biosorption by Copper Resistant Bacteria of *Acinetobacter* sp. IrC2

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Heavy metal pollution is a serious problem because it cannot be degraded by natural processes and persist in soil, water, and sediment. Many indigenous microorganisms isolated from heavy metal contaminated sites had tolerance to heavy metals toxicity and could be used for bioremediation agent because of its capability to biosorb heavy metals. The aim of this research was to study the potency of copper resistant bacteria *Acinetobacter* sp. IrC2 as a biosorbent of heavy metals. Biosorption was determined by measuring the heavy metals concentration on growing medium by using atomic absorption spectrophotometer. The research showed that *Acinetobacter* sp. IrC2 was capable of growing in medium containing each of 2 mM of copper, zinc, lead, cadmium, and the mixture of those heavy metals. The addition of copper and lead in the medium changed morphological appearance of colonies to green and brown, respectively, suggesting that the survival mechanism of the isolate was by biosorbing copper or lead inside the cells. The percentage of heavy metals biosorption efficiency using live cells of *Acinetobacter* sp. IrC2 were up to 64.31% of copper, 24.73% of zinc, 62.79% of lead, and 11.56% of cadmium. *Acinetobacter* sp. IrC2 also reduced copper, lead, and cadmium concentration up to 24.30, 75.93, and 16.38%, respectively, in medium supplemented with 1 mM of the mixture of these heavy metals. The findings of this study indicated that *Acinetobacter* sp. IrC2 was a promising bacterium for removal of heavy metals.

Key words: *Acinetobacter* sp. IrC2, biosorption, copper, heavy metals, resistant

Pencemaran logam berat merupakan permasalahan yang serius karena tidak dapat didegradasi secara alami sehingga tetap berada di tanah, air, maupun sedimen. Banyak mikroorganisme asli yang diisolasi dari daerah terkontaminasi logam berat memiliki toleransi terhadap toksisitas logam berat dan dapat digunakan sebagai agen bioremediasi karena kemampuannya dalam melakukan biosorpsi logam berat. Penelitian ini bertujuan untuk mempelajari potensi bakteri resisten tembaga *Acinetobacter* sp. IrC2 sebagai biosorben logam berat. Biosorpsi ditentukan dengan cara mengukur penurunan konsentrasi logam berat di dalam medium dengan menggunakan spektrofotometer serapan atom. Hasil penelitian menunjukkan bahwa *Acinetobacter* sp. IrC2 dapat tumbuh dalam medium yang masing-masing mengandung 2 mM tembaga, seng, timah, cadmium, dan campuran logam berat tersebut. Penambahan tembaga dan timah dalam medium mempengaruhi perubahan penampakan morfologi koloni menjadi hijau dan coklat sehingga diduga mekanisme pertahanan isolat tersebut adalah dengan cara melakukan biosorpsi tembaga dan timah di dalam sel. Efisiensi persentase biosorpsi logam berat dengan menggunakan sel hidup *Acinetobacter* sp. IrC2 adalah sebesar 64.31% tembaga, 24.73% seng, 62.79% timah, dan 11.56% kadmium. *Acinetobacter* sp. IrC2 juga dapat menurunkan hingga 24.30% tembaga, 75.93% timah, 16.38% seng, dan 47.62% kadmium di dalam medium yang mengandung 1 mM campuran logam berat tersebut. Penemuan ini menunjukkan bahwa *Acinetobacter* sp. IrC2 merupakan bakteri yang menjanjikan untuk memindahkan logam berat dari lingkungan.

Kata kunci: *Acinetobacter* sp. IrC2, biosorpsi, logam berat, resisten, tembaga

The presence of toxic heavy metal contaminants in aqueous stream, arising from the discharge of untreated metal containing effluents into water bodies, is one of the most important environmental issues (Rehman *et al.* 2008). The metals of most immediate concern include cadmium, copper, lead, nickel, mercury, and zinc. The presence of such metals in aquatic

environments cause damage to aquatic life and killing microorganisms during biological water purification process (Vinodhini and Narayan 2008).

Microorganisms that are able to survive well in high concentrations of heavy metals are of great interest as bioremediation agents because they conduct bioaccumulation based on the incorporation of metals inside the living biomass or biosorption, in which metal ions are adsorbed at the cellular surface

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(Vijayaraghavan and Yun 2008). The surface of a cell has an important role in the relationship between the cell and its environment, as the surface is in direct contact with the ambient environment of the cell, and both essential and nonessential metal ions are transported across the surface into the cell. When heavy metals are deposited into an environment, they may bound on the surface of microorganisms, which is probably the initial step in the uptake of the metals by the microbes in toxic condition. The cell surface is important in microbial ecology, in the adhesion of microbes on surfaces, and in interactions between microorganisms and external environment (Lo *et al.* 1999).

Microbe-based technologies can serve as alternatives to conventional methods for pollution removal. In recent years, the biosorption process has been extensively studied using microbial biomass as biosorbents for heavy metal removal. The surface properties of bacteria enable them to adsorb different kinds of organic and inorganic pollutants from solutions (Aksu and Tunc 2005). Some reports have shown that indigenous microbes tolerate high heavy metals concentrations in different ways and may play a significant role in the restoration of contaminated soil (Ge *et al.* 2009). *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Citrobacteria*, *Klebsiella*, *Rhodococcus*, and *Acinetobacter* are organisms commonly used in bioremediation mechanism because of their ability for biosorbing heavy metal (Adeniji 2004).

*Acinetobacter* sp. IrC2 is a copper-resistant bacteria (accession number: JX 009133) isolated from activated sludge of an industrial wastewater treatment plant in Rungkut, Surabaya, Indonesia (Irawati *et al.* 2012). Previous study showed that *Acinetobacter* sp. IrC2 demonstrated multiple resistance to copper, lead, zinc, cadmium, and the mixture of these heavy metals with minimum inhibitory concentration of 10, 13, 9, 4, and 3 mM, respectively (Irawati *et al.* 2015). Copper resistance mechanism of *Acinetobacter* sp. IrC2 was facilitated through the bioaccumulation of copper inside the cell, particularly on the membrane fraction and inside the cytoplasm, albeit at a limited amount (Irawati *et al.* 2012). The aim of this research was to study the potency of *Acinetobacter* sp. IrC2 in biosorbing heavy metals such as copper, zinc, lead, and cadmium as a single heavy metal and as a mixture. Biosorption is defined as the amount of heavy metal removed from growth medium compared to the initial concentration.

## MATERIALS AND METHODS

**Bacterial Strain, Media and Growth.** Bacterial isolate was grown in Luria Bertani (LB) agar containing the following (per litre) : tryptone : 10 g, yeast extract 5 g, NaCl 10 g, glucose 0,1 g, and pure agar 0.15 g. Stock of 1 M CuSO<sub>4</sub> (copper); 1M Pb(NO<sub>3</sub>)<sub>2</sub> (lead); 1 M CdSO<sub>4</sub> (cadmium) ; 1M ZnSO<sub>4</sub> (zinc) were added to the autoclaved media (Irawati *et al.* 2014).

**The Effect of Heavy Metals on Bacterial Growth.** Cells were grown in LB broth supplemented with various concentration of copper, lead, cadmium, zinc, as individual heavy metal, and as a mixture of these heavy metals. In addition, a medium with no added heavy metal was also prepared to cultivate the cells. The cultures were incubated at 37°C on a shaker at 175 rpm speed. Growth was monitored by measuring optical density at 600 nm using spectrophotometer. The morphological changes of colony on medium containing heavy metal and without heavy metal were also observed.

**Heavy Metals Biosorption.** Cells were grown in LB broth containing various concentration of CuSO<sub>4</sub>; Pb(NO<sub>3</sub>)<sub>2</sub>; Cd(NO<sub>3</sub>)<sub>2</sub>; ZnSO<sub>4</sub> as a single and as a mixture solution. The cells were incubated at 37°C with shaking at 175 rpm. The cells were centrifuged at 6000 rpm for 10 min at 4°C. Supernatant was digested with HNO<sub>3</sub> at 100°C. The concentration of heavy metal remaining in supernatant was determined by an Atomic Absorption Spectrophotometer. Heavy metals biosorption were calculated as the difference in total heavy metals added to the medium and remaining total heavy metals in the medium after bacterial growth. Heavy metals Biosorption = Heavy metals added – Heavy metals total after growth (Andreazza *et al.* 2010).

## RESULTS

Growth pattern of *Acinetobacter* sp. IrC2 in medium containing 2 mM of heavy metals was shown in Figure 1. The growth of *Acinetobacter* sp. IrC2 declined with addition of each heavy metal and with the mixture of these heavy metals. There was no activity of bacterial growth in medium containing 2 mM CdSO<sub>4</sub>. The addition of heavy metals in the medium affected morphological appearance of *Acinetobacter* sp. IrC2. The isolate formed dark brown and green colonies in the presence of lead and copper indicated that bacterium accumulated heavy metals inside the

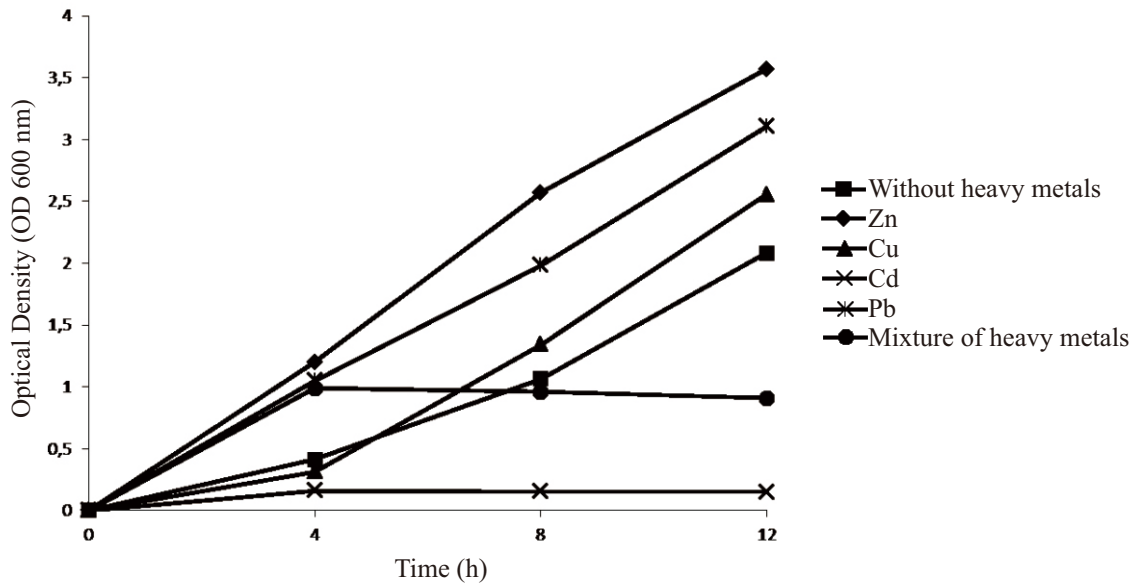


Fig 1 The growth of *Acinetobacter* sp. IrC2 in medium containing 2 mM of heavy metals.

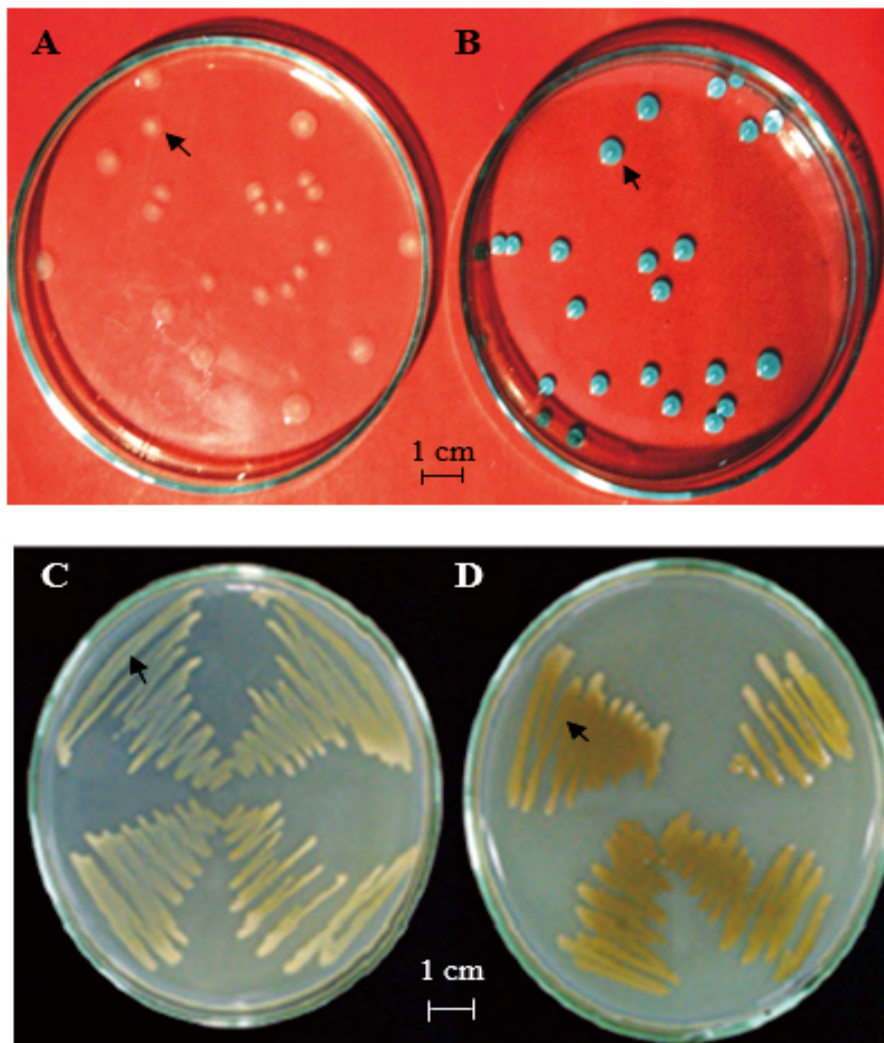


Fig 2 The growth of *Acinetobacter* sp. IrC2 in medium containing (A) without  $\text{CuSO}_4$ , (B) 5 mM  $\text{CuSO}_4$ , (C) 8 mM  $\text{Pb}(\text{NO}_3)_2$ , and (D) 10 mM  $\text{Pb}(\text{NO}_3)_2$ . Arrows indicates the changes of colonies color to green and brown in medium containing copper and lead.

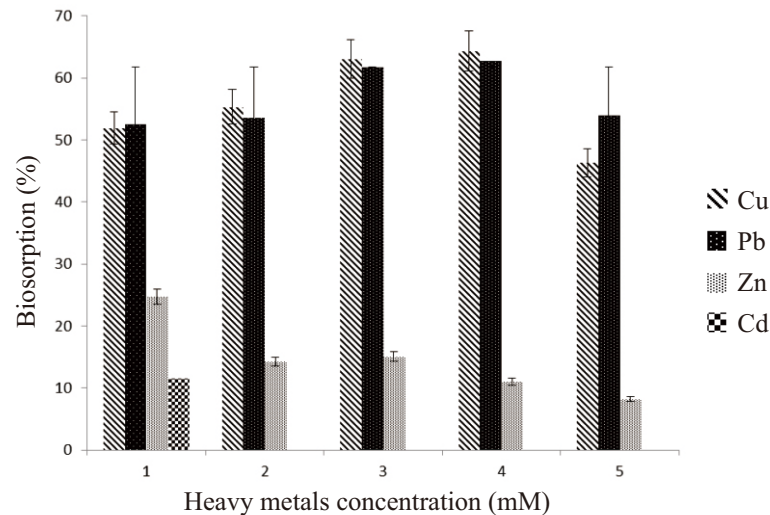


Fig 3 Biosorption capability of *Acinetobacter* sp. IrC2 in medium containing various concentration of heavy metals. Bars represent standard deviations (error bars) from three independent experiments.

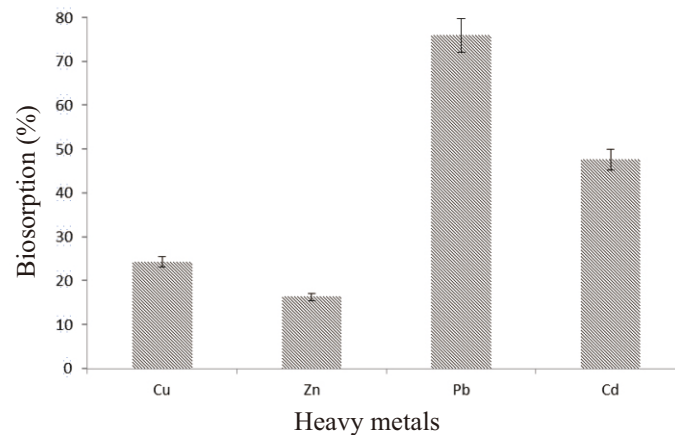


Fig 4 Biosorption capability of *Acinetobacter* sp. IrC2 in medium containing the mixture of 1 mM of copper (Cu), zinc (Zn), lead (Pb), and cadmium (Cd). Bars represent standard deviations (error bars) from three independent experiments.

cell. Figure 3 showed the percentage of copper biosorption efficiency using live cells of *Acinetobacter* sp. IrC2 in medium containing various concentration of heavy metals. It is clearly shown that *Acinetobacter* sp. IrC2 had the capability to biosorb copper, zinc, lead, and cadmium up to 64.31, 24.73, 62.79, and 11.56%, respectively.

Figure 4 showed the ability of *Acinetobacter* sp. IrC2 to biosorb each of heavy metal when the isolate grown in medium containing the mixture of 1 mM of copper, zinc, lead, and cadmium. The bacteria demonstrated growth in the medium containing the mixture of 1 mM copper, zinc, lead, cadmium and reduced the concentration of  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{Pb}(\text{NO}_3)_2$ , and  $\text{CdSO}_4$ , up to 24.30, 16.38, 75.93, and 47.62%, respectively.

## DISCUSSION

*Acinetobacter* sp. IrC2 was isolated from activated sludge of an industrial wastewater treatment plant. Such contaminated environment made this bacterium developed resistance mechanism to heavy metals. In this study, it was observed that the presence of lead, copper, and zinc in the medium affected the growth of *Acinetobacter* sp. IrC2 by reducing the growth rate, while the presence of 2 mM of cadmium in the medium stopped the bacterial growth. Cadmium is known to be extremely toxic at low concentrations (El-Sersy and El-Sharouny 2007). It has deleterious impact on microbial communities and their functional activities in soil. However, microorganisms exposed to higher concentrations of toxic metals may develop resistance against elevated levels of the metal (Habi and Daba 2009).

This study showed that the isolate could not only grow in medium containing copper, lead, and zinc individually but also in the mixture of those heavy metals. Previous study showed that the isolate had multiple resistance to five kinds of heavy metals, such as copper, zinc, lead, cadmium, and mercury (Irawati *et al.* 2015). According to Rajbanshi (2008) about 52.23 % of bacteria had multiple resistance to three and more heavy metals. A previous study has revealed that 60 % of bacterial isolates from a wastewater treatment plant had multiple resistance to heavy metals. Bacterium which has multiple resistance to many heavy metals can be used as a biosorbent efficiently as metal polluted-environments is usually contaminated by more than one kind of heavy metals. The resistance of *Acinetobacter* sp. IrC2 to some heavy metals might be due to the selective pressure imposed by the pollution of industrial wastewater at Rungkut, the location where the bacterium was isolated (Irawati *et al.* 2012a). Since heavy metals are increasingly found in microbial habitats as the result of natural and environmental processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals (Adarsh *et al.* 2007).

The addition of copper and lead in the medium affected morphological appearance to green and brown, respectively. It indicated that the isolate developed resistance mechanism by biosorbing copper and lead inside the cell. Previous study showed that *Acinetobacter* sp. IrC2 had the capability to accumulate copper and lead up to 138.96 mg and 327.15 mg per gram dry weight of cells, respectively. This result was similar to what has been demonstrated in heavy metals resistant bacteria *Brevundimonas* sp. HgP1. This isolate formed darker color colonies in the presence of mercury, such as in *Escherichia coli*, mercury-resistant transgenic bacteria (Irawati *et al.* 2012b). Bacteria expressing metallothionein and polyphosphate kinase demonstrated cell aggregation, precipitation and darker color when the cells were grown in high mercury concentrations. The effects may depend on high mercury resistance and accumulation by transgenic bacteria due to the presence of metallothionein. Metallothionein protects bacteria against harmful effects of mercury and provides mercury bioremediation capabilities. The clumping and precipitation characteristics of the bacteria can be applied to the development of a simple sifting mechanism to recover cells that have accumulated high mercury concentrations (Ruiz *et al.* 2011).

According to Abdel-Ghani *et al.* (2009) the

bacterium with dark colonies might have the ability to accumulate and biosorb heavy metals. The most copper-resistant bacteria were selected due to previous knowledge that higher resistance infers a better capacity to retard metal diffusion to the inside of the cell by biosorbing heavy metals onto the cell surface (Nies 1999). Biosorption is a process which represents a biotechnological innovation as well as a cost effective excellent tool for removing heavy metals from aqueous solutions (Das *et al.* 2008). The percentage of biosorption efficiency by *Acinetobacter* sp. IrC2 increased with increasing concentration of heavy metals in the medium up to appropriate concentration and decreased beyond this level. The decrease might be due an increase in electrostatic interactions between cells at higher concentrations, causing cells to agglomerate, which contributed to decrease in the amount of binding sites available (Cho *et al.* 2010). According to Gabr *et al.* (2008) and Tunali *et al.* (2006) the biosorption capacity of the biomass decreased by increasing the initial concentration of heavy metals. At lower concentrations, all metal ions presented in the solution could interact with the binding sites and thus the biosorption percentage was higher than those at higher ion concentrations. At higher concentrations, lower adsorption yield was due to the saturation of adsorption sites.

Andreazza *et al.* (2010) reported that bacterial growth rate, Cu (II) removal and Cu (II) bioreduction were directly related to Cu (II) concentration. Increasing the concentration of heavy metals lowered biosorption percentage of bacteria due to its toxic effects. Nies (1999) reported that inside the cell, heavy-metal cations tend to bind to SH groups. By binding to SH groups, the metals may inhibit the activity of sensitive enzymes. Other heavy-metal cations may interact with physiological ions thereby inhibiting the function of the respective physiological cation. Many mechanisms of resistance are known in bacteria for maintaining intracellular homeostasis of metal ions.

The result of this research showed that *Acinetobacter* sp. IrC2 was able to remove copper, zinc, lead, and cadmium in the medium up to 52.98, 21.65, 72.01, and 11.56%, respectively. Lead biosorption as a single solution was the highest, followed by copper, zinc, and cadmium. *Acinetobacter* sp. IrC2 could also remove copper, zinc, lead, and cadmium in the medium supplemented with 1 mM the mixture of those heavy metals up to 24.30, 16.38, 75.93, and 47.62%, respectively. The capability of *Acinetobacter* sp. IrC2 to biosorb copper, lead, and

cadmium was higher than other heavy metal resistant microorganisms. *Stenotrophomonas maltophilia* was able to remove 22% of copper, 24% of cadmium, and 42.75% of lead as the single metal, and was also capable of removing up to 16% of copper, 8% of cadmium, and 35% of lead as mixture solutions (Parungao *et al.* 2007). Yeast strain ES10.4 isolated from industrial sewage in Surabaya was able to remove copper and lead up to 30% and 29%, respectively (Christian 2013). *Candida tropicalis* isolated from industrial effluents was capable to remove 68% of lead (Rehman *et al.* 2017), bacterial strain isolated from secondary effluents was able to remove 61.9% of lead (Adel *et al.* 2014), meanwhile *Pseudomonas aeruginosa* was able to remove 65% of lead from medium containing 100 mg/l of lead (Raja *et al.* 2006).

Biosorption was found to be higher when the metals were present individually in medium than metals which were present collectively. This is because in medium mixture, more metals ions were in competition against one another for metal-binding sites in bacterium. Lead biosorption in mixed metals was the highest, followed by copper, zinc, and cadmium. Such results has also been reported by Parungao *et al.* (2007) in *Stenotrophomonas maltophilia*. The study demonstrated that Pb ions strongly inhibited the sorption of Cu and Cd due to ion exchange effect. Lead, being a larger and heavier ion of atomic radius 180 pm and 207.2 g/mol atomic mass, was likely to replace the smaller and lighter Cu, and Cd ions of 135 pm and 155 pm atomic radius, respectively and 63.5 g mol<sup>-1</sup> and 112.4 g mol<sup>-1</sup> atomic mass, respectively. It means that Pb ions are bigger than Cu and Cd ions that support the explanation as to why Pb selectively replaces the other two ions in binding sites during equilibrium in solution.

Microorganisms have a high affinity for metals and can accumulate heavy metals by developing variety of mechanisms (Rehman *et al.* 2008). The binding characteristics of the lead ions involved the carboxyl, hydroxyl, and amino groups in the biomass (Parungao *et al.* 2007). The negatively-charged groups like carboxyl and hydroxyl of bacterial cell wall adsorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or combination of such processes (Chojnacka *et al.* 2005). Mechanism of biosorption includes mainly ionic interactions and formation of complexes between metals cations and acidic sites in the cell wall of bacterium (Oh *et al.* 2009). *Acinetobacter* sp. IrC2 was a Gram negative bacteria (Irawati *et al.* 2012). The outer membrane of Gram negative bacteria, which

consists of lipopolysaccharides, lipoproteins, and phospholipids carries a strong negative charge, because the cell surface of bacteria carries a net negative charge due to the presence of carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups. It can adsorb appreciable quantities of positively charged cationic metals (Tortora *et al.* 2005).

The response of microorganisms towards toxic heavy metals is of importance in view of the interest in the reclamation of polluted sites (Shankar *et al.* 2007). The findings of the study indicated that *Acinetobacter* sp. IrC2 is a promising bacteria for removal of heavy metals especially copper, lead, zinc, cadmium, and the mixture of these heavy metals. The capability of *Acinetobacter* sp. IrC2 to grow and remove multiple heavy metals may be explored as a potential bioremediation agent. However, further studies is needed to employ *Acinetobacter* sp. IrC2 as a biosorbent agent for heavy metals removal in wastewater treatment plant.

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