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# Removal of Cadmium and Zinc from Soil using Immobilized Cell of Biosurfactant Producing Bacteria

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**Abstract**

Immobilized biosurfactant producing bacteria (*Bacillus subtilis TP8 and Pseudomonas fluorescens* G7) were assessed for survival in heavy metal contaminated soil and for their ability to remove cadmium and zinc from contaminated soil. *P. fluorescens* G7 was considered to be a good candidate for bioremediation of heavy metals because of its high minimum inhibitory concentrations (MIC) for each heavy metal and because of the obviously increased numbers of cell surviving after incubation in the heavy metal contaminated soil up to 4 weeks. The results of soil remediation showed that approximately 19% of Zn and 16.7% of Cd could be removed by this immobilized biosurfactant producing bacteria after incubation for 2 weeks. The results confirm the potential applicability of the immobilized biosurfactant producing bacteria for heavy metal bioremediation.

***Keywords:*** cadmium; zinc; immobilized cell; biosurfactant

## 1. Introduction

Pollution of soil by heavy metals is a worldwide problem and is of growing concern because of health risks for both people and animals. Although substantial progress has been made in reducing industrial releases, toxic metal pollution still occurs from industrial and metallurgical processes. Cadmium, copper, lead, mercury, nickel and zinc are considered the most hazardous and are included on the US Environmental Protection Agency's (EPA) list of priority pollutants (Cameron, 1992). A number of remediation technologies such as excavation and landfill, thermal treatment and stabilization and solidification have been developed (Mulligan *et al*., 2001a). However, due to the cost or land space requirements, the application of these methods has been limited. Moreover, traditional treatment technologies for cadmium contaminated soils have several inherent disadvantages because they cannot completely remove, but only stabilize it in the contaminated medium. Thus, an ideal complexing agent to mobilize metals is one that is soluble in water, chemically stable under environmental conditions, not strongly bound to soil particles, and has a high affinity for complexing metal (Chang and Broadbent, 1981). Removal of metal such as cadmium, copper, lanthanum, lead and zinc using biological complexing agents (exopolymers, biosurfactants) has been reported (Chang and Broadbent, 1981; Ochoa-Loza, 1998; Mulligan *et al*., 2001b). Biosurfactants are produced by a wide variety of bacteria, yeast, and fungi. They include peptides, glycolipids, glycopeptides, fatty acids and phospholipids (Banat, 2000). This potential of the biomolecules as soil washing agents is due to their ability to solubilize metal within their micellae. They can be potentially as effective with some distinct advantages over the highly used synthetic surfactants, including high specificity, biodegradability, biocompatibility and low toxicity (Copper, 1986). Nevertheless, biosurfactant production by bacteria in contaminated soil could be limited by several soil factors such as toxic chemicals, temperature, pH, salinity and other environmental conditions. In this aim, immobilization of living cells in porous support offers enormous advantages in production of biosurfactant and cell protection. It is an efficient way to reduce or avoid the factors that caused low product yields. Entrapment in soluble calcium alginate is recognized as a rapid, nontoxic, inexpensive, versatile method, which was successfully used for biosurfactant production (Hye-Sung *et al*., 2004; Siemann and Wagner, 1993). The objective of this study was therefore to examine the survival of immobilized biosurfactant producing bacteria in heavy metal contaminated soil and to determine their feasibility on the removal of cadmium and zinc from soil.

## 2. Material and Methods

### *2.1. Screening and selection of heavy metal resistant/ tolerant bacteria for bioremediation*

#### *2.1.1. Bacterial strains and culture conditions*

The biosurfactant producing strains, *Bacillus subtilis* TP8 and *Pseudomonas fluorescens* G7 were isolated from oil contaminated soil by the method described by Bodour *et al*. (2003). They were formerly examined and showed promising ability for the removal of cadmium and zinc from soil by using their partial purified biosurfactant foam (Bunchai, 2009). They were grown in nutrient broth (NB, Merck) and maintained in an NB containing 10% glycerol solution and stored below 0°C until use. From frozen stock, bacteria were streaked on nutrient agar (NA, Merk) plates and incubated at 30°C for further culturing. To prepare subcultures, the NA was inoculated with a colony from the plate and incubated overnight at 30°C.

#### *2.1.2. Minimum inhibitory concentrations (MIC) determination*

MIC was determined in 100 µl of NB with various concentrations of metal salt solutions ($CdCl_2$:$2.5H_2O$ and $ZnSO_4$:$7H_2O$) and 100 µl of bacterial suspension (set to McFarland Standard No.0.5) in round-bottomed 96-well microplates. After incubation at 30°C for 24 h, the tested microplates were assayed by tetrazolium salt (Singh, 2008). To each well, 50 µl of 2,3,5-triphenyltetrazolium chloride (0.5% w/v) was added. Cultures were then incubated at 30°C for 24 h. A change in color from yellow to red indicated growth of bacteria and the MIC was interpreted visually. The MIC was estimated as the first dilution which completely inhibits bacterial growth in NB medium.

### *2.2. Bacterial cell immobilization*

Heavy metal resistant bacterial cells were obtained from culture grown in 250 ml of mineral salt medium (MSM) containing 2% of glucose (Bodour *et al*., 2003) and incubated with shaking (200 rpm) at 30°C for 24 h. The cells were then harvested by centrifugation at 9,000g for 15 min at 4°C and washed thoroughly with 0.85% sterile saline solution. The pellets were re-suspended in 3.5 ml sterile peptone water (1%w/v). The cells were encapsulated by mixing of one part culture concentration with one part Na-alginate solution (4%w/v). This mixture was added drop-wise into 100 ml of a sterile solution of calcium chloride (0.05 M) through pipette tips with an inner diameter of 0.5 mm and stirred. To ensure complete gelling, the resulting 0.3-0.4 mm beads were stirred for 1 h in this solution and were finally washed with sterile distilled water and conserved at 4°C in a buffer solution prior to use.

### *2.3. Determination of cell concentrations in alginate bead*

For determination of internal cell concentrations, samples of beads were re-suspended in 0.1 M sterile phosphate buffer solution (pH 6.8) followed by gently shaking for 45 min or until liquefiction. The samples were then serially diluted with 0.85% sterile saline solution and plate counts were conducted in triplicate at each dilution on NA. Plates were then incubated at 30°C for 24 h.

### *2.4. Determination of immobilized biosurfactant producing bacteria survival in heavy metal contaminated soil*

#### *2.4.1. Soil inoculation*

The soil sample, obtained from an agricultural site near the university, was crushed with a mortar and pestle and sieved at 2.0 mm after air drying for 48-72 h. The soil was dried again in an oven at 105°C for 2 h. It was then contaminated artificially in the laboratory. Metal salts ($CdCl_2$:$2.5H_2O$ and $ZnSO_4$:$7H_2O$) were dissolved in distilled water to give concentrations of 150 mg/L of Cd and 400 mg/Lof Zn. The solutions were then added together into the soil sample (without adjusting the pH value). The soil suspension was shaken on a wrist action shaker at 200 rpm for 48 h at 30°C and subsequently left for settling for 24 h. The supernatant was discarded and the contaminated soil was air dried for at least for 48 h and sieved at 2.0 mm. A hundred grams of sterilized soil with metal salt of cadmium and zinc and without (as control) were inoculated with 1.5 g of bacterial alginate bead in 250 ml flask. The soil moisture was controlled at 30% (w/v) by adding the MSM containing 2% of glucose. Soil microcosms were incubated at room temperature for 4 weeks.

#### *2.4.2. Enumeration of bacteria*

At the end of incubation time (1, 2, 3 and 4 weeks), ten grams of soil samples were collected for the tested flask and serially diluted with 0.85% sterile saline solution. Plate counts were conducted in triplicate at each dilution on NA. Plates were then incubated at 30°C for 24 h. The residual soil suspension samples were collected for further evaluating of metal removal.

### *2.5. Determination of the feasibility on removal of heavy metals from soil*

The soil suspension samples of each week trials

were centrifuged at 9,000g for 15 min at 4°C. The supernatant was discarded and the contaminated soil was collected. To determine the total cadmium and zinc concentrations in the soil, the soil samples were first digested with aqua regia method. The extract was then filtered and diluted before measurement. The metal concentrations were measured by AA analysis with an Atomic Absorption Spectrophotometer (GBC, Avanta PM)

## 3. Results and Discussion

### *3.1. Screening and selection of heavy metal resistant/ tolerant bacteria for bioremediation*

Both biosurfactant producing strains, *B. subtilis* TP8 and *P. fluorescens* G7 were tested for Cd and Zn MIC estimation. *Pseudomonas fluorescens* G7 was chosen due to the highest MIC for each heavy metal. Results of MIC estimation are shown in Table 1. These results are consistent with the screening for multiresistant bacteria performed in previous studies (Vullo, *et al*., 2005; 2008), where isolates with Cd resistance were resistant to Zn, belonging to culturable microorganisms found in the soil assayed. Two factors can be considered to account for Cd and Zn toxicity: (1) the Cd metal is not an essential trace element for organisms while the Zn metal is an essential trace element and (2) the low complexing capacity of the culture medium used in this assay may leave cadmium almost completely bioavailable (Cretti *et al*., 2006).

### *3.2. Determination of cell concentrations in alginate bead*

The cell concentrations in alginate bead were estimated after immobilization. The obtained results were about $2x10^9$ CFU/bead of *P. fluorescens* G7. High cell concentrations of biosurfactant producing bacteria, *Pseudomonas fluorescens*, have been reported to be immobilized in alginate beads (Abouseoud *et al*., 2008). The encapsulation of the cell in capsules offers space for cell growth and good diffusion properties with a typical pore size distribution of 5 to 200 nm (Smidsrød and Skjåk-Bræk, 1990) for the bioremediation experiment.

Table 1. Cd and Zn minimum inhibitory concentrations (MIC) in NB media of the isolates

| Strain | MIC (ppm) | |
|---|---|---|
| | Cd | Zn |
| *Bacillus subtilis* TP8 | 31.25 | 62.5 |
| *Pseudomonas fluorescens* G7 | 125 | 250 |

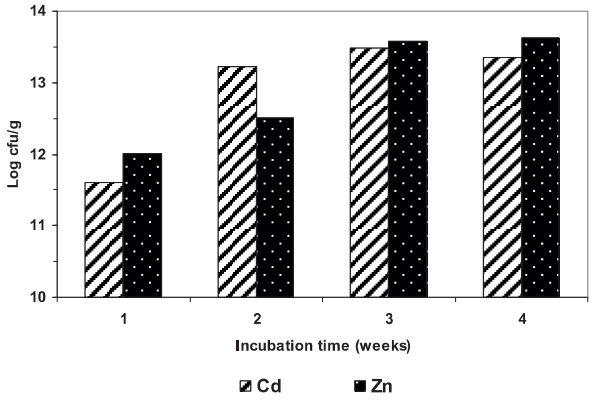


Figure 1. Cell concentration of *P. fluorescens* G7 in the heavy metal contaminated soil at difference incubation times

This also provides a gentle, simple and cheap method for immobilizing bacteria.

### *3.3. Determination of immobilized biosurfactant producing bacteria survival in heavy metal contaminated soil*

Cell release from beads and cell growth in soil during consecutive bioremediation was monitored at the end of each incubation time. The total numbers of cells in Zn contaminated soil trials increased during the consecutive bioremediation while the viability of immobilized cells in Cd contaminated soil trials increased in the first few weeks and slightly decreased in the end of incubation time (Fig. 1). There were large differences of the total numbers of cells in Zn soil compared with Cd soil. This could be explained by the bioavailability and less toxicity of Zn metal. However, the cell concentration in the soil after releasing from beads was not reduced with the presence of Cd and Zn in soil. It can be concluded that the cell encapsulated in alginate beads gave the possibility to keep viability of cells and to protect cells in heavy metal contaminated soil as already shown by Jézéquel *et al*., (2005), and that this transitional stage may be useful for the inoculated bacteria to adapt to their new environment.

### *3.4. Determination of the immobilized biosurfactant producing bacteria feasibility on removal of heavy metals from soil*

The pattern of Cd and Zn removal from contaminated soil using the immobilized biosurfactant producing bacteria is presented in Fig. 2. The results show that rhamnolipid biosurfactant (data not shown) produced *P. fluorescens* G7 readily mobilizes Zn in comparison to Cd after incubation for 2 weeks. The Zn is removed amounts of 19% of the total Zn present in the contaminated soil, while The Cd is removed amounts of 16.7%

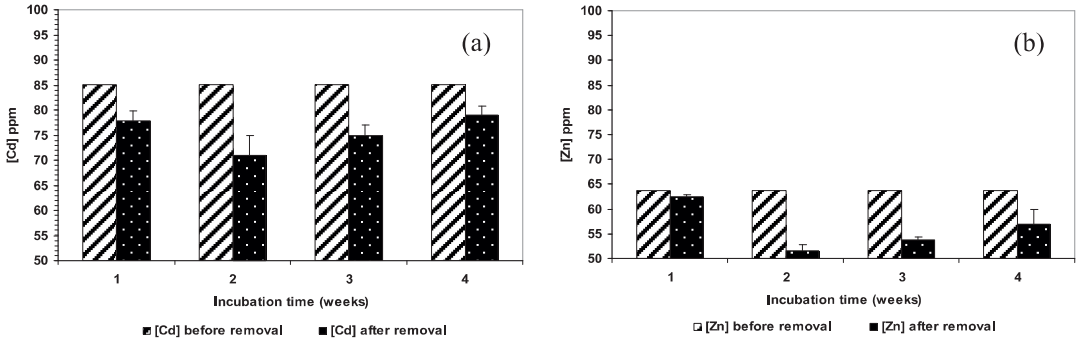


Figure 2. Cd (a) and Zn (b) removal from heavy metal spiked soil using the immobilized biosurfactant producing *P. fluorescens* G7 at difference incubation times (*N=5*)

of the total Cd present This could be explained by the high affinity of Cd ions for organic chelating agents (Stevenson, 1975) as well as with the clay minerals and organic materials in soil (John, 1971; Anderson and Nilsson, 1974). The low efficiency biosurfactant-facilitated removal of soil-bound metal can be attributed to the effect of biosurfactant sorption to soil (Torrens *et al*., 1998), bacterial cell sorption or bacterial electric properties (Vullo *et al*., 2008) and degradation of biosurfactant when the incubation time was longer than 2 weeks. However, this experimentation of Cd and Zn from contaminated soil by the immobilized biosurfactant producing bacteria revealed a positive indication for its use in bioremediation of these contaminated soil. Although this study was carried out under idealized laboratory conditions, the results illustrate some general considerations that are important for the use of the immobilized biosurfactant producing bacteria for soil bioremediation during the field application.

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