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Inhibitory Effect of the Selected Heavy Metals on the Growth of the Phosphorus Accumulating Microorganism, *Acinetobacter* sp.

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ABSTRACT: This study was initiated to evaluate the inhibitory effect of selected heavy metals on the growth of *Acinetobacter* sp. known as one of the phosphorus accumulating microorganisms (PAO) involved in the enhanced biological phosphorus removal (EBPR) process of the wastewater treatment plant. *Acinetobacter* sp. was initially selected as a starting model microorganism and was grown under aerobic condition for this experiment. The heavy metals selected and investigated in this study were cadmium (Cd), copper (Cu), mercury (Hg), nickel (Ni), and zinc (Zn). Median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations for Cd, Cu, Hg, Ni, and Zn were 2.95 and 1.45, 4.92 and 2.53, 0.03 and 0.02, 1.12 and 0.43, 14.84 and 5.46 mg L^{-1}, respectively. We demonstrated that most of heavy metals tested in the experiment inhibited the growth of *Acinetobacter* sp. in the range of predetermined concentrations. Based on the data obtained from the experiment, Hg was the most sensitive to *Acinetobacter* sp., then Ni, Cd, Cu, and Zn in order.

Key Words: Inhibition, growth, heavy metals, *Acinetobacter* sp., PAO

INTRODUCTION

Nutrients such as nitrogen (N) and phosphorus (P) released from point and non-point sources can cause eutrophication in water and wastewater, resulting in deterioration of the water quality and destruction of aquatic ecosystem[1)]. Phosphorus from the wastewater can be removed through the chemical precipitation and enhanced biological phosphorus removal (EBPR). In EBPR processes, phosphorus can be accumulated in the greater amount of P than that needed for their normal cell biosynthesis[2)]. As a result, it is wasted and removed in the form of sludge in EBPR process. The microorganisms involved in EBPR processes are called PAO (phosphorus accumulating organism). They include *Acinetobacter* sp., *Acinetobacter calcoaceticus*, *Pseudomonas* sp., *Pseudomonas fluorescens*, and *Bacillus megaterium*[3,4)]. However, the new strains of PAO from the *in situ* EBPR processes still need to be isolated and characterized by the conventional culturing method and relatively new molecular method to elucidate the mechanism of EBPR. Recently, the detection of the heavy metals such as arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) in the environments has frequently been reported[5,6)]. They may be contained in the wastewater from the industrial complex and the mining drainage and come to the river, ocean, and agricultural land. Therefore, those heavy metals may hinder the proper operation of the EBPR process of the wastewater treatment plant. Little information is available for the toxicity of heavy metals on the PAOs in the EBPR

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process. Therefore, the objective of this study is to evaluate the inhibitory effect of five heavy metals, such as Cd, Cu, Hg, Ni, and Zn, on the aerobic growth of *Acinetobacter* sp. well known as one of PAO through the comparison of the absorbances and specific growth rates measured at the respective concentrations of the heavy metals including control. Finally, the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations are calculated and compared for five heavy metals.

MATERIALS AND METHODS

Bacterial culturing

The freeze-dried bacteria (*Acinetobacter* sp.: KCTC 2702) were revived in flasks of 125 ㎖ of nutrient broth (Difco). The flasks were placed in a shaker and the *Acinetobacter* sp. allowed to grow in the medium for two days. Subsequently, the bacterial inoculum was transferred, using 10 ㎕ of sterile inoculating loops, onto the nutrient agar plate and incubated at 30℃ in a shaking incubator for two days. The media modified from Zafiri et al.[3] were used and their composition was as follows: 1.91 g of NH_4Cl, 0.003 g of $FeCl_3 \cdot 6H_2O$, 0.03 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of yeast extract, 1.25 g of $CH_3COONa \cdot 3H_2O$, 0.05 ㎖ of trace metal solution per 1L of deionized water. The trace metal solution included 0.5% (w/v) each of H_3BO_3, KI, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnSO_4$ H_2O, $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $NaB_4O_7 \cdot 10H_2O$, and $Na_2MoO_4 \cdot 2H_2O$. Phosphate in the form of K_2HPO_4 was added to give P the concentration of 20 mg L^{-1}.

Preparation of Cd, Cu, Hg, Ni, and Zn

The stock solutions for the Cd, Cu, Hg, Ni, and Zn were properly made as 10,000 mg L^{-1} and added to give the predetermined concentrations in the respective growth experiments. The standard chemicals of Cd, Cu, Hg, Ni, and Zn were added in the form of $CdCl_2$, $CuSO_4 \cdot 5H_2O$, $HgCl_2$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, respectively.

Growth experiments

Precultured media in 250 ㎖ of flasks (100 ㎖ liquid volume) was inoculated from agar plates and stirred at a constant rate of 120 rpm on a rotary shaker for two days at 30℃. Approximately 10 ㎖ of culture volume was transferred to a fresh 90 ㎖ of liquid media and mixed to prevent any flocculation. This cell suspension was further transferred to the cell to measure the absorbance at a periodically predetermined time intervals. The flasks of batch reactor were periodically sampled for measurement of absorbance, pH and orthophosphate.

Analytical methods

Absorbance of the cell suspension was measured with ultraviolet spectrophotometer (Hitach U-2000, Japan) at 550 nm, with a path length of 1.25 cm. Samples for orthophosphate determination were prefiltered through 0.45 ㎛ pore size membrane filter with a 1.2 ㎛ pore size glass fiber filter (Whatman GF/C) as a pre-filter. Orthophosphate was determined using the UV ascorbic acid method. All analyses were in duplicate. Measurement of pH was done by a combination of electrode and analyzer (Istek-9801124P). The electrode was calibrated using the standard buffer solutions with pH of 7.00 and 10.0.

Calculation of specific growth rate and degree of inhibition

The specific growth rate of a culture was computed from the least-square linear regression of log absorbance versus time. Initial points falling within the lag period were discarded. Each value of specific growth rate was based on at least 5 data points, with a correlation coefficient (r) of 0.995 or higher. The degree of inhibition was computed from Inhibition (%) = $(\mu_{control} - \mu_{heavy\ metals})*100/\mu_{control}$, where $\mu_{control}$ is the specific growth rate of the control (zero concentrations of Cd, Cu, Hg, Ni, and Zn, respectively) and $\mu_{heavy\ metal}$ is the specific growth rate of the cells exposed to a specific heavy metal concentrations. Median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations were determined by fitting the inhibition data to the log gamma distribution[7] and calculating the concentration giving 50% and 10% inhibition from the fit, respectively.

RESULTS

The characteristics for the inhibitory effect of five heavy metals Cd, Cu, Hg, Ni, and Zn on the growth of *Acinetobacter* sp. were evaluated as a function of time as affected by the concentrations of heavy metals used. (Figures 1-10). The inhibitory effects of the heavy

metals on the growth of *Acinetobacter* sp. were different according to the kinds of heavy metals. The specific inhibition characteristcs for each heavy metal are explained as follows.

Figure 1 shows the growth of *Acinetobacter* sp. as a function of time in relation to Cd concentrations. The concentrations of Cd used in the experiment were 0, 1, 2, 3, 5, and 10 mg L^{-1}. As the concentrations of Cd were increased, the growth of *Acinetobacter* sp. was correspondingly decreased as indicated in the Figure 1. At the Cd concentrations of 0, 1, 2, 3, and 5 mg L^{-1}, during the initial two hours of incubation, its growth was not initiated, but, after two hours of incubation, its growth rate was increased, even though the growth rate was correspondingly decreased, as its concentrations were increased. However, at the maximum Cd concentration of 10 mg L^{-1}, the growth of *Acinetobacter* sp. was completely inhibited during the six hours of entire incubation period. Figure 2 shows the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Cd calculated from the Cd concentrations used and specific growth rate of *Acinetobacter* sp. IC_{50} and IC_{10} of *Acinetobacter* sp. for Cd were 2.95 and 1.45 mg L^{-1}, respectively.

Figure 3 demonstrates the growth of *Acinetobacter* sp. as a function of time in relation to Cu concentrations. The concentrations of Cu used in the experiment were 0, 4, 8, 12, 16, and 20 mg L^{-1}. As the concentrations of Cu were increased, the growth rate of *Acinetobacter* sp. was correspondingly decreased as indicated in the Figure 3. At the Cu concentrations of 0 and 4 mg L^{-1}, during the initial one hour of incubation, its growth was not initiated, but, after one hour of incubation, its growth rate was increased, even though the growth rate was correspondingly decreased, as its concentration was increased. It is noticeable that after one hour of incubation, the growth rate of *Acinetobacter* sp. was much slower at the Cu concentration of 4 mg L^{-1} than

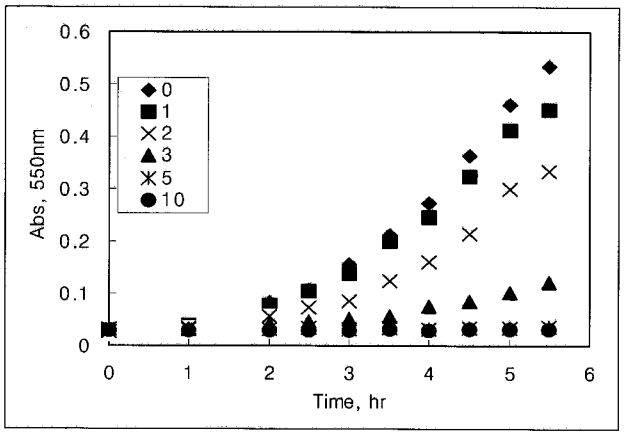

Fig. 1. Growth of *Acinetobacter* sp. as a function of time in relation to Cd concentrations.

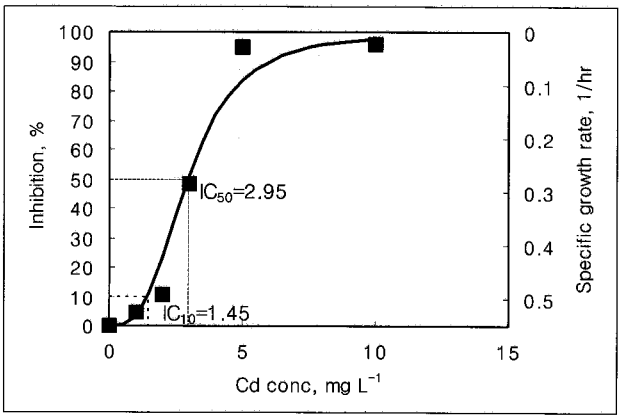

Fig. 2. Estimation of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Cd.

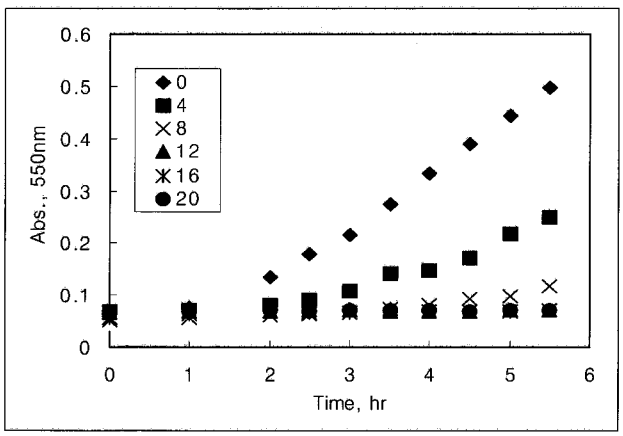

Fig. 3. Growth of *Acinetobacter* sp. as a function of time in relation to Cu concentrations.

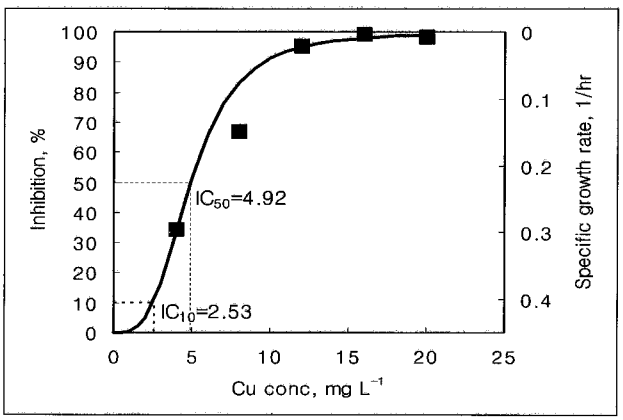

Fig. 4. Estimation of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Cu.

zero mg L^{-1} used as the control. However, at the Cu concentration of 8, 12, 16, and 20 mg L^{-1}, the growth of *Acinetobacter* sp. was almost or completely inhibited during the six hours of entire incubation period. Figure 4 indicates the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Cu calculated from the Cu concentrations used and specific growth rate of *Acinetobacter* sp. IC_{50} and IC_{10} of *Acinetobacter* sp. for Cu were 4.92 and 2.53 mg L^{-1}, respectively.

Figure 5 presents the growth of *Acinetobacter* sp. as a function of time in relation to Hg concentrations. The concentrations of Hg used in the experiment were 0, 0.005, 0.01, 0.05, 0.1, and 0.5 mg L^{-1}, respectively. At the first lower range of Hg concentrations at 0, 0.005, and 0.01 mg L^{-1}, as the concentration of Hg was increased, the growth rate of *Acinetobacter* sp. was not remarkably and correspondingly decreased as indicated in the Figure 5. However, at the second higher range of Hg concentrations at 0.05, 0.1, and

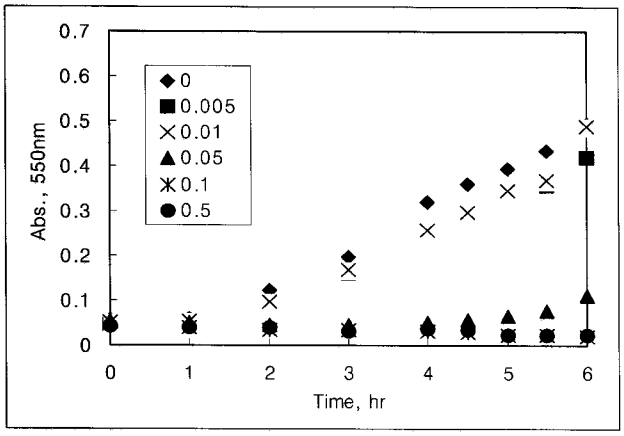

Fig. 5. Growth of *Acinetobacter* sp. as a function of time in relation to Hg concentrations.

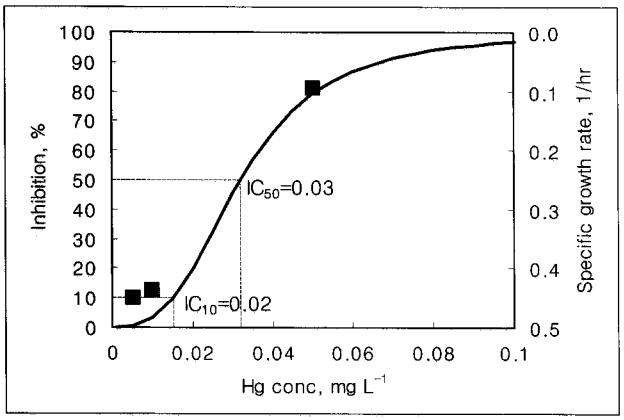

Fig. 6. Estimation of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Hg.

0.5 mg L^{-1}, its growth rate was correspondingly and remarkably decreased, as the Hg concentration was increased, compared to the first lower range of Hg concentrations of 0, 0.005, and 0.01 mg L^{-1}. At first lower range of Hg concentrations at 0, 0.005, and 0.01 mg L^{-1} used, during the initial one hour of incubation, its growth was not initiated, but, after one hour of incubation, its growth rate was increased. At the higher concentration range of Hg of 0.05, 0.1, and 0.5 mg L^{-1}, the growth of *Acinetobacter* sp. was almost and completely inhibited during the six hours of entire incubation period. Figure 6 indicates the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Hg calculated from the Hg concentrations used and specific growth rate of *Acinetobacter* sp. IC_{50} and IC_{10} of *Acinetobacter* sp. for Hg were 0.03 and 0.02 mg L^{-1}, respectively.

Figure 7 shows the growth of *Acinetobacter* sp. as a function of time in relation to Ni concentrations. The concentrations of Ni used in the experiment were 0, 0.1, 0.3, 0.5, 0.7, 1 mg L^{-1}, respectively. At the first lower range of Ni concentrations of 0 and 0.1 mg L^{-1}, as the concentration of Ni was increased, the growth rate of *Acinetobacter* sp. was not correspondingly decreased as indicated in the Figure 7. However, at the second middle range of Ni concentrations of 0.3 and 0.5 mg L^{-1}, its growth rate was correspondingly and remarkably decreased, as the Ni concentration was increased, compared to the first lower range of 0 and 0.1 mg L^{-1}. Finally, at the third higher range of Ni concentrations of 0.7 and 1 mg L^{-1}, its growth rate was further decreased, as the Ni concentration was increased, compared to the first lower and second middle range of Ni concentration. At all the concentrations used, during the initial two hours of incubation, its growth was not initiated, but, after two hours of incubation, its growth rate was increased. Even at the maximum Ni concentration of 1 mg L^{-1} used, the growth of *Acinetobacter* sp. was not completely inhibited during the six hours of entire incubation period, even though low growth rate of *Acinetobacter* sp. was observed. Figure 8 shows the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Ni calculated from the Ni concentrations used and specific growth rate of *Acinetobacter* sp. IC_{50} and IC_{10} of *Acinetobacter* sp. for Ni were 1.12 and 0.43 mg L^{-1}, respectively.

Figure 9 shows the growth of *Acinetobacter* sp. as a function of time in relation to Zn concentrations. The

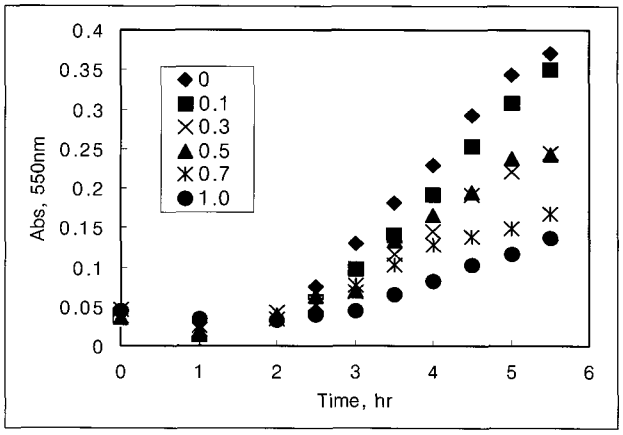

Fig. 7. Growth of *Acinetobacter* sp. as a function of time in relation to Ni concentrations.

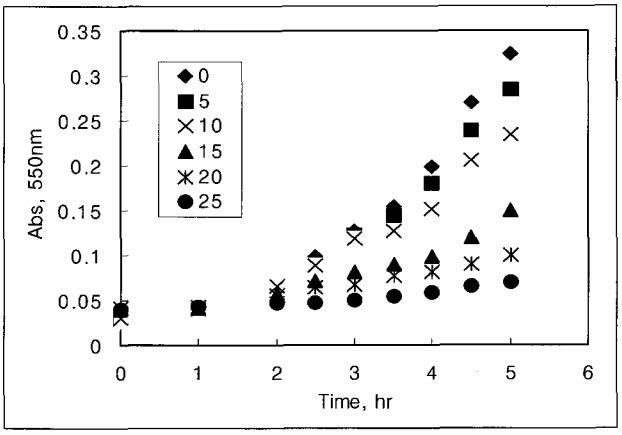

Fig. 9. Growth of *Acinetobacter* sp. as a function of time in relation to Zn concentrations.

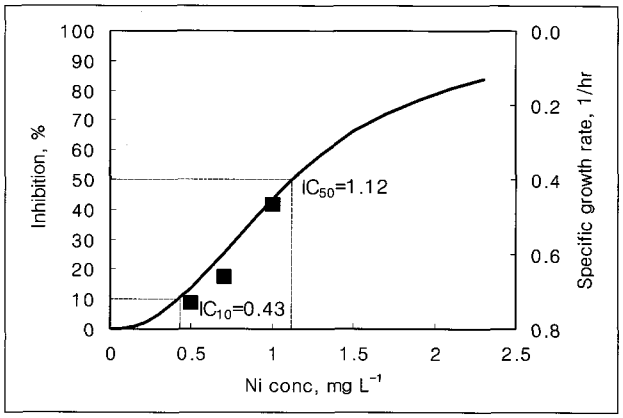

Fig. 8. Estimation of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Ni.

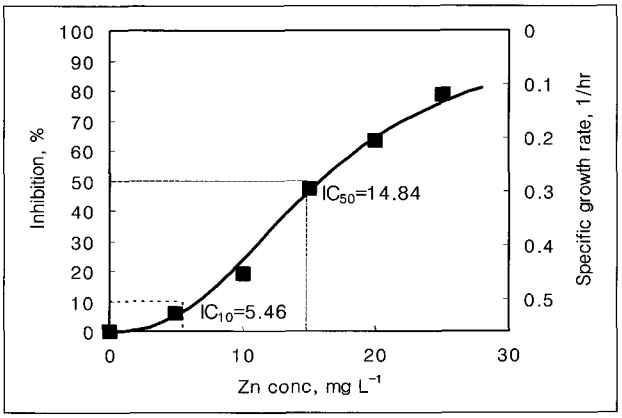

Fig. 10. Estimation of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Zn.

concentrations of Zn used in the experiment were 0, 5, 10, 15, 20, 25 mg L^{-1}, respectively. At the first lower range of Zn concentrations of 0, 5, and 10 mg L^{-1}, as the concentration of Zn was increased, the growth rate of *Acinetobacter* sp. was not remarkably and correspondingly decreased as indicated in the Figure 9. However, at the second higher range of Zn concentrations of 15, 20, and 25 mg L^{-1}, its growth rate was correspondingly and remarkably decreased, as the Zn concentration was increased, compared to the first lower range of Zn concentrations of 0, 5, and 10 mg L^{-1}. At all the concentrations used, during the initial two hours of incubation, its growth was not initiated, but, after two hours of incubation, its growth rate was increased. At the maximum Zn concentration of 25 mg L^{-1}, the growth of *Acinetobacter* sp. was almost and not completely inhibited during the six hours of entire incubation period. Figure 10 shows the median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Zn calculated from the Zn concentrations used and specific growth rate of *Acinetobacter* sp. IC_{50} and IC_{10} of *Acinetobacter* sp. for Zn were 14. 84 and 5.46 mg L^{-1}, respectively.

Table 1. Summary of median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations of Cd, Cu, Hg, Ni, and Zn

Toxic substance	Inhibition concentration, mg L^{-1}	
	IC_{50}	IC_{10}
Cd	2.95	1.45
Cu	4.92	2.53
Hg	0.03	0.02
Ni	1.12	0.43
Zn	14.84	5.46

Table 1 summarizes the IC_{50} and IC_{10} values for five toxic heavy metals Cd, Cu, Hg, Ni, and Zn. IC_{50} indicates the concentrations of heavy metals to give 50% inhibition of the growth of *Acinetobacter* sp. IC_{10} indicates the concentration of heavy metals to give

10% inhibition of the growth of *Acinetobacter* sp.

DISCUSSION

Toxicity tests through use of the various living organisms, fish, water flea, and bacteria are currently available. Among other things, toxicity test through the use of bacteria is advantageous in that it is simple, save the time and expense, easy to interpret the data, compared to other tests[8]. Microplate using the luminescent bacteria, *Vibrio fischeri*, is one of the widely used tests commercially available[9-11].

Growth inhibition of bacteria was widely used as one of toxicity tests[12-19]. In this study, the inhibitory effect of the selected heavy metals on the growth of the *Acinetobacter* sp. well known as the phosphorus accumulating bacteria in the wastewater treatment was used to qualititively or quantitively evaluate their toxic effects in the environment. Based on the results obtained from this study, we demonstrated that the toxicity effect of five selected heavy metals on the growth of *Acinetobacter* sp. was different according to the heavy metals. *Acinetobacter* sp. was the most sensitive to Hg, Ni, Cd, Cu, and Zn. in order. Recently, Cho et al.[20] reported the effect of heavy metals on the growth of *E. coli*. In their study, *E. coli* was the most sensitive to Hg, then Cr, Cu, Cd, and Zn in order. Mercury and Zn was the most and least sensitive to both *Acinetobacter* sp. and *E. Coli*, respectively. Whereas, the toxicity of Cd and Cu to *Acinetobacter* sp. was reverse to *E. Coli*. It was reported that the different responses of the bacteria to the toxicity of heavy metals may be due to the kinds and amount of heavy metals adsorbed to extracellular polymer produced by bacteria[21]. Considering the inhibitory effect of selected heavy metals on the growth of *Acinetobacter* sp. and *E. coli*, the growth of other bacteria belonging to PAOs in EBPR process may be inhibited by the heavy metals. The informations obtained from this research can be used in the design and operation of the advanced EBPR process, especially for the industrial wastewater treatment.

The microorganism is also known to have the tolerance to heavy metals and unique detoxification mechanism. They can resist to the flux of the heavy metals into the cells by the combining with the extracellular polysaccharide and immobilizing with the protein metallothionein. They can reduce the concentrations of heavy metals by volatilizing and precipitating. In addition, they can pump the heavy metals out of the cells and stop accumulating them inside the cells[21]. The microorganism have a variety of mechanisms detoxifying the heavy metals and toxic effects. In addition, the toxic effects of the heavy metals in the same microorganism depends on the environmental factors, such as pH, temperature, the concentrations of inorganic and organic matters complexing with the heavy metals[21]. Therefore, IC_{50} indicating the sensitivity to heavy metals can be different, depending on the toxicity tests used[22]. The heavy metals can inhibit the growth of microorganism by the complexation with major cellular components, such as proteins and nucleic acids and the decrease of enzyme activity, the denaturation of proteins, the decrease of cell division. In addition, the metal portions of the biomolecules can be replaced with the heavy metals, resulting in the decreased biochemical metabolism and impairment of DNA and can cause genotoxicity inhibiting transcription and translation[21]. Also, a possible mechanism of the growth inhibition of *Acinetobacter* sp. by the heavy metals is that they are transported across the bacterial cell membrane decreases intracellular pH, thus reducing the pH gradient needed to generate proton motive force and, hence, ATP synthesis. Since the regulation of the poly P synthesis pathway is via ATP/ADP ration, inhibition of ATP synthesis by the heavy metals, leading to low ATP/ADP ratio, would consequently decrease poly P synthesis and phosphorus uptake[4].

Therefore, the further research is needed for the toxic effect of heavy metals on the other bacteria belonging to PAO in EBPR process. In addition, the molecular and proteomic analysis may be needed to elucidate the possible mechanism for the growth inhibition of *Acinetobacter* sp. by heavy metals which is one of PAOs involved in the EBPR.

CONCLUSIONS

Specific growth rates of *Acinetobacter* sp. grown under aerobic condition were correspondingly decreased as the concentrations of Cd, Cu, Hg, Ni, and Zn were gradually increased, respectively. The median (IC_{50}) and threshold (IC_{10}) inhibitory concentrations for Cd, Cu, Hg, Ni, and Zn were 2.95 and 1.45, 4.92 and 2.53, 0.03 and 0.02, 1.12 and 0.43, 14.84 and 5.46 mg L^{-1},

respectively. We demonstrated that all the five heavy metals tested in the experiment inhibited the growth of *Acinetobacter* sp. in the reasonable range of predetermined concentrations. Based on the data obtained from the experiment, Hg was the most sensitive to *Acinetobacter* sp., then Ni, Cd, Cu, and Zn in order. Therefore, it appears that the heavy metals used in the study may hinder EBPR processes, especially for the industrial wastewater treatment.

ACKNOWLEDGEMENT

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