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Biosensor Based on Heavy Metal-resistant Bacterial Cell Immobilized on Graphite Electrode

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Four heavy metal-resistant bacteria were isolated from sediment of Anyang-cheon and identified with API-kit. Cd-resistant, Cu-resistant, Pb- resistant and Zn-resistant bacteria were identified to be *Haeomophilus parainfluenza, Chromobacterium violaceium, Bacillus cereus* and *Pseudomonas fluoroscens*, respectively. The heavy metal-resistant bacteria were confirmed to produce fluoroscent compound on medium with heavy metal ions. The fluoroscent compound was thought to be a shield for protection of bacteria against toxic heavy metal ion. The fluoroscent compound was confirmed not to be electrochemical activity and to inhibit electrochemical reaction of bacterial cell with heavy metal ions, which may be a mechanism required to compose biosensor for heavy metal detection. The electrochemical reaction of heavy metal- resistant bacteria was inversely proportional to concentration of heavy metal ions added. The current signal produced in heavy metal biosensor composed with heavy metal-resistant bacteria was proportional to concentration of heavy metal ions.

Key words: API kit, Haeomophilus parainfluenza, Chromobacterium violaceium, Bacillus cereus, Pseudomonas fluoroscenss

1. Introduction

Generally, heavy metal ions which have been classified as metal elements of which some are essential for life but some have great potential for toxicity to most organism.[1-4] The heavy metal-resistant bacteria growing under contaminated soil or wastewater with high concentration of heavy metals have to have some special protection mechanism against toxic heavy metal.[5,6] *Bacillus subtillis* 168[7] was reported to block cellular uptake of cadmium by altering membrane transport system, *Pseudomonas putida*[8] was suggested to sequestrate cadmium ion by specific binding components such as metallothionein and *Stapohylococcus aureus*[9-12] was reported to pump out heavy metal ions by a highly specific efflux system which is dependent on ATP consumption. You and Park[13] reported that *Azomonas agilis* PY101 can grow under environment contaminated with Cd^{2+} by cadmium-binding pigment which is fluoroscent compound. The cadmium-binding pigment was reported to efflux cadmium ion from bacterial cell. O'Halloran[14-16] reported about genetic studies of various heavy metal-resistant bacteria and about heavy metal biosensor.[17]

Biosensor is one of bioelectrochemical device for dectection some special organic compounds or inorganic ions. Various organic materials such as hormones, enzymes and factors isolated from bacteria, fungi, algae, plants and animals or intact bacterial cell have been used for construction of biosensor.[18-21] O'Halloran used metal-containing enzyme or metal cheperon which can influence on gene regulation or enzyme activity for composition of biosensor.[22] If some special enzyme, hormone or factor can be applied for composition of heavy metal biosensor the detection system has to use negative reaction with heavy metal, which is a disadvantage for construction of heavy metal biosensor and a big difference from other biosensor such as glucose sensor, alcohol sensor, nitrate sensor or NAD^+ sensor from which positive signal is produced.[23-25] Heavy metal ions, in most case, can inhibit function of enzymes, hormones or factors by which the signal produced from

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sensor composing of enzymes, hormones or factors has to be decreased. In this research, we tried to isolate fluoroscent compound from heavy metal-resistant bacteria and tested electrochemical reaction of fluoroscent compound. The fluoroscent compounds, however, didn't have electrochemical activity and were electrochemically confirmed not to react with heavy metal. This is a reason why we tried to compose biosensor using intact cell of heavy metal-resistant bacteria which produce fluoroscent compound on only medium containing of heavy metal.

2. Experimental

2.1 Organisms

The heavy metal-resistant bacteria were isolated from sediment layered on bottom of Anyang-cheon (beside the Omok-kyo (bridge), Mok-dong, Yangcheon-gu, Seoul) and identified with APILAB PLUS identification kit (BioMérieux, France). The bacteria isolated were cultivated on modified LB medium (Peptone 5 g/L, Yeast extract 3 g/L and NaCl 5 g/L) containing Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}, respectively. The bacterial growth was spectrophotometrically measured by measurement of optical density at 660 nm.

2.2 Measurement of minimal inhibitory concentration (MIC)

Each heavy metal ion was added to medium which was gradually increased from 0 to 1000 mg/L, respectively, and MICs were roughly evaluated by using 100 mg/L increments and then finely evaluated by using 10 mg/L increments.[26] After the MIC of heavy metal ions to bacterial growth was determined, 95% heavy metal ions of MIC were added to growth medium.

2.3 Fluoroscent compound production

The heavy metal resistant-bacteria produced the fluoroscent compound of which concentration was proportional to the concentration of heavy metal added to the culture. The concentration of fluoroscent compound was spectrophotometrically measured by scanning methode. However, the fluoroscent compound productivity was not presented as a datum for this article because fluoroscent compound was not final goal in this research.

2.4 Bacterial growth

The bacterial growth was measured on medium without heavy metal, with half MIC of heavy metal and with around 98% MIC of heavy metal, respectively. The growth was spectrophotometrically measured using optical density at 660 nm.

2.5 Cyclovoltammetry

The cyclic voltammograms was obtained using graphite working electrode modified with heavy metal-resistant bacteria which were modified with neutral red, platinum wire counter electrode and an Ag/AgCl reference electrode in 50 mM phosphate buffer (pH 7.0). The scanning rate used was 25 mVs^{-1} over the range of +0.6 volt to -1.2 volt. For cyclovoltammetry of fluoroscent compounds, normal graphite working electrode was used instead of the modified electrode with bacterial cell. The scanning rate used was 25 mVs^{-1} over the range of +1.0 volt to -1.0 volt. Cyclic voltammetry was performed using a cyclic voltammetric potentiotat (model CV50W, BAS, USA) linked to an IBM personal computer data acquisition system. Prior to use, the electrodes were cleaned using ultrasonic cleaner.[27]

2.6 Construction of biosensor

The biosensor was constructed with intact cells of heavy metal-resitant bacteria (5.0 OD_{660}) as shown in Fig. 1. The bacterial suspension was changed to paste by mixed with graphite powder (10% W/V, Sigma, 1 μM diameter) which functions as an electron conductor from bacterial cell to electrode. The ferric ion functions as an electron acceptor and bacterial cell functions as an electron donor. The ferrous ion reduced from ferric ion must be re-oxidized to ferric ion by atmospheric oxygen.

2.7 Immobilization of bacteria on graphite electrode

The bacterial cell was immobilized by using modified method that Bae *et al.* used.[28] Generally, proteins such

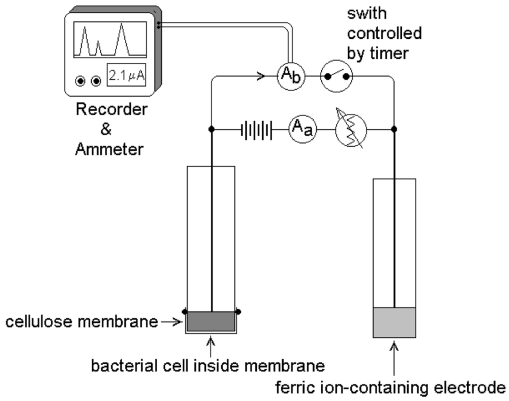

Fig. 1. Schematical structure of bacterial sensor for detection of heavy metal ion. Aa is amperemeter for adjustment of base ampere value between two electrodes under zero heavy metal concentration. The base ampere can be controlled with variable resistance and was tunned to 0.01 to 0.1 μA in heavy metal-free water. Ab is amperemeter for calibration and measurement of heavy metal ions. The viable cell number inside cellulose membrane was over 5x106 and diameter of both electrode tip is 5 mm. The potential between two electrodes was adjusted to 0.8-1.2 volt. The ferric ion-containing electrode was made from graphite power, kaolin and ferric ion by baking at 1200 °C.

enzyme, hormone, antibody or factor have been immobilized by covalent bond, coagulation, cross-liking or entrapment which, however, can't use for immobilization of whole cell or intact cell on graphite or metal electrode because activity of bacterial cell can be influenced by chemicals used for immobilization of proteins.

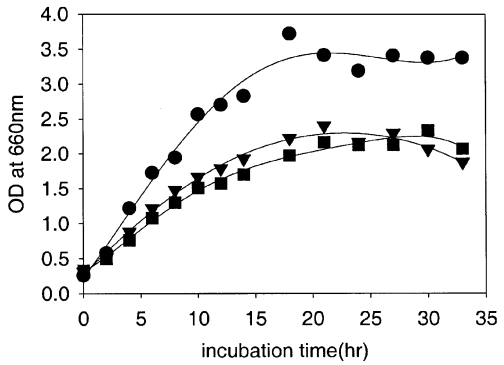

Fig. 2. Growth of Cd-resistant bacterium Haemophilus parainfluenza on LB-medoum without Cd ((●), with 100 mg/L of Cd ((■) and 200 mg/L of Cd ((▼). The minimal inhibitory concentration of Cd to growth of Haemophilus parainfluenza is 210 mg/L.

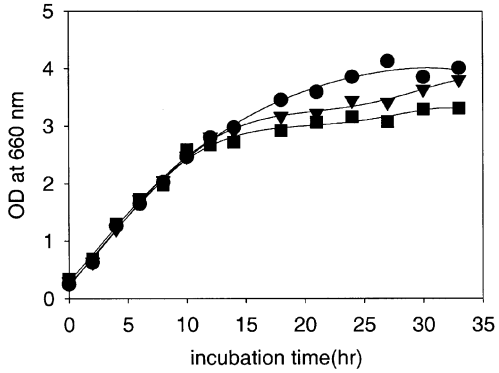

Fig. 3. Growth of Cu-resistant bacterium Chromobacterium violaceium on LB-medoum without Cu ((●), with 55 mg/L of Cu ((■) and 115mg/L of Cu ((▼). The minimal inhibitory concentration of Cu to growth of Chromobacterium violaceium is 120 mg/L.

3. Results and Discussion

The growth of heavy metal-resistant bacteria and bacterial production of fluoroscent compounds on medium with heavy metal ions can be a key factor for determination whether bacteria can be a sensor or not for detection of heavy metal ion in aquatic environment. The growth of Cu ion and Pb ion-resistant bacteria was not influenced by concentration of Cu^{2+} and Pb^{2+} added to culture as shown in Fig. 3 and 4, respectively, but growth of Cd ion and Zn ion-resistant bacteria was partially inhibited in proportion to concentration of Cd^{2+} and Zn^{2+} added to culture as shown in Fig. 2 and 5,

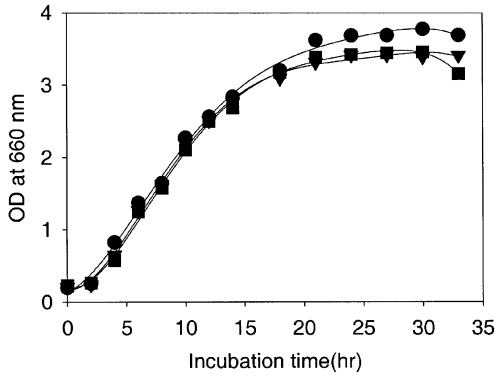

Fig. 4. Growth of Pb-resistant bacterium on LB-medoum without Pb ((●), with 360 mg/L of Pb ((■) and 720 mg/L of Pb ((▼). The minimal inhibitory concentration of Pb to growth of Bacillus cereus is 730 mg/L.

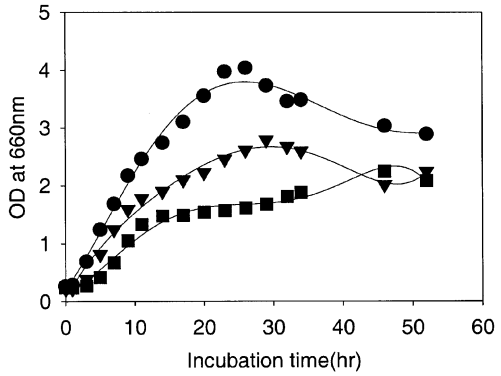

Fig. 5. Growth of Zn-resistant bacterium Pseudomonas fluoroscens on LB-medoum without Zn ((●), with 200 mg/L of Zn ((■) and 400mg/L of Zn ((▼). The minimal inhibitory concentration of Zn to growth of Pseudomonas fluoroscens is 410 mg/L.

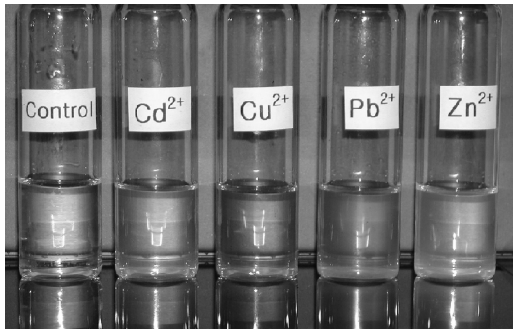

Fig. 6. Pictures of fluoroscent compounds secreted by heavy metal-resistant bacteria when grown on medium with 100 mg/L of Cd^{2+}, 55mg/L of Cu^{2+}, 360 mg/L of Pb^{2+} and 200 mg/L of Zn^{2+}, respectively. The higher concentration of fluoroscent compound was produced on culture with higher concentration of heavy metal ions.

respectively. Concentration of fluoroscent compound produced from heavy metal-resistant bacteria was higher on culture with higher concentration of heavy metal ions. This is shown a possibility that the fluoroscent compound may be functioning as a shield for protection of bacterial growth against heavy metal ions. What the fluoroscent compounds began to be produced around 3% of MIC is a clue that the fuoroscent compounds act as an shield to protect bacterial cell against heavy metal ions. The fluoroscent compounds produced from heavy metal-resistant bacteria were emitting fluoroscence on UV illuminator as shown in Fig. 6.

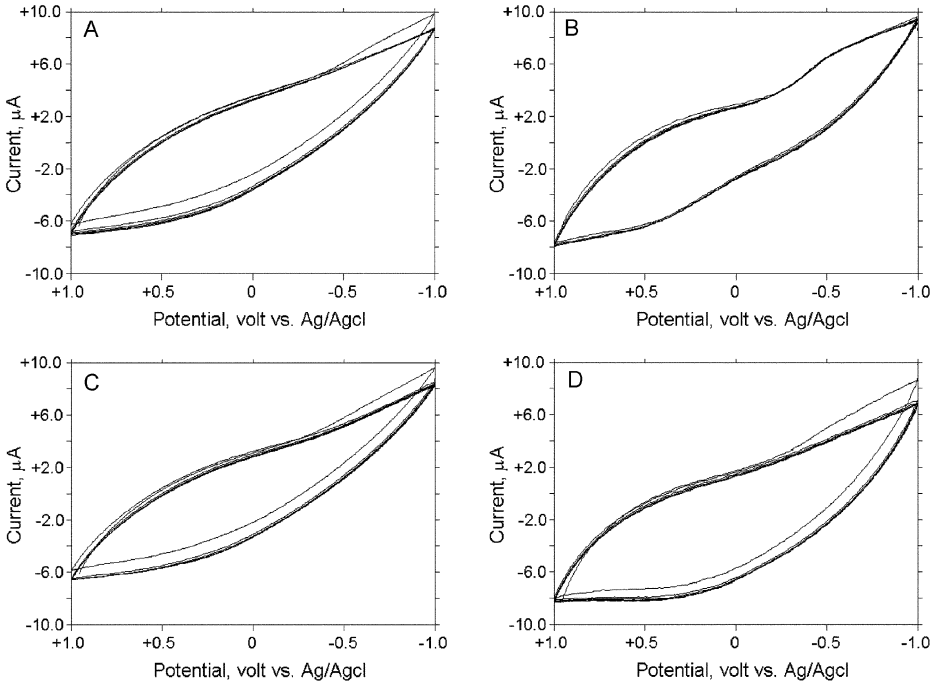

Fig. 7. Cyclovoltammogram of fluoroscent compound produced from Cd-resistant bacterium (A), Cu-resistant bacterium (B), Pb-resistant bacterium (C) and Zn-resistant bacterium (D), respectively. Any electrochemical reaction and current variation were not observed in each cyclovoltammogram.

Electrochemical reaction of fluoroscent compounds, however, measured with cyclovoltammetry was not observed and any current variation was not observed in cyclovoltammetry measured with fluoroscent compounds as shown in Fig. 7. This is the reason that fluoroscent compounds can't be a sensor for electrochemical detection of heavy metal but intact cell being responsible to heavy metal ion can be a sensor for detection of heavy meal ion. As shown in Fig. 8A, 9A, 10A and 11A current peaks in cyclovoltammogram of heavy metal-resistant bacterial cell modified with neutral red were decreased in proportion to concentration of heavy metal ion added. These results show that heavy metal ions added to biosensor device induces bacteria to produce fluoroscent compound. The fluoroscent compounds can't be electrochemically react with carbon electrode but can interfere electron transfer from bacterial cell to electrode through neutral red immobilzed on

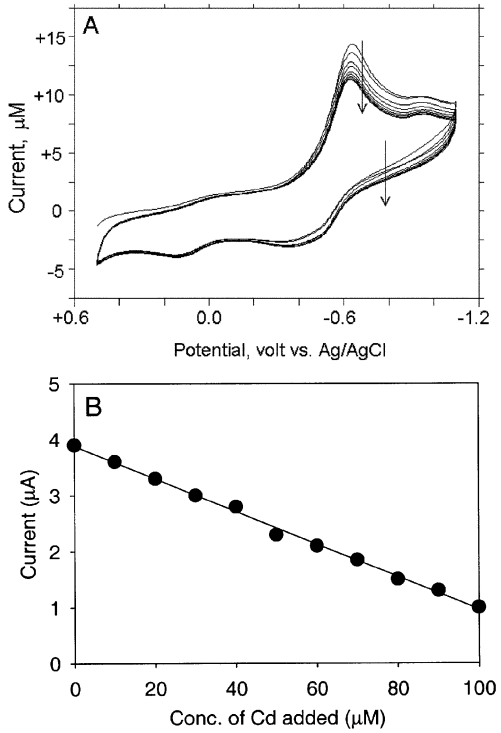

Fig. 8. Cyclovoltammogram (A) of Cd-resistant bacteria modified with Neutral red in which the current was decreased by addition of Cd-ions, and amperometric response (B) of the whole-cell biosensor constructed with Cd-resistant bacteria to Cd ion.

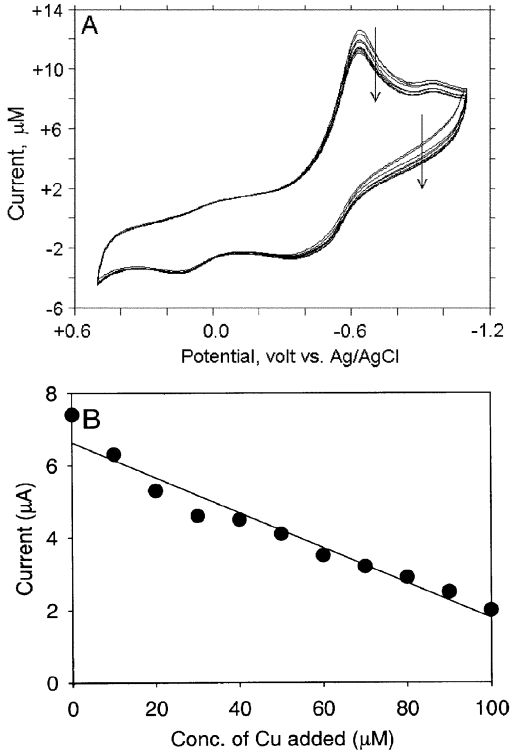

Fig. 9. Cyclovoltammogram (A) of Cu-resistant bacteria modified with Neutral red in which the current was decreased by addition of Cu-ions, and amperometric response (B) of the whole-cell biosensor constructed with Cu-resistant bacteria to Cu ion.

bacterial membrane.[30,31] Naturally, the neutral red immobilized on bacterial membrane can mediate electron transfer from bacterial cell to electrode as show in Fig. 12A, but fluoroscent compound may interfere electron transfer from bacterial cell to electrode as shown in Fig. 12B. The electrons transferred from bcterial cell to anode can move through circuit line to cathode which is current. As shown in Fig. 8B, 9B, 10B and 11B, the current produced from heavy metal-resistant bacteria was decreased in proportion to the concentraion of heavy metal ion added to biosensor device. From which we would like to propose the mechanism that bacterial cell inside working electrode of biosensor (Fig. 1) may produce the fluoroscent compound by stimulation of heavy metal ion and the fluoroscent compound may inhibit electron transfer from bacterial cell to electrode as shown in Fig. 12B.

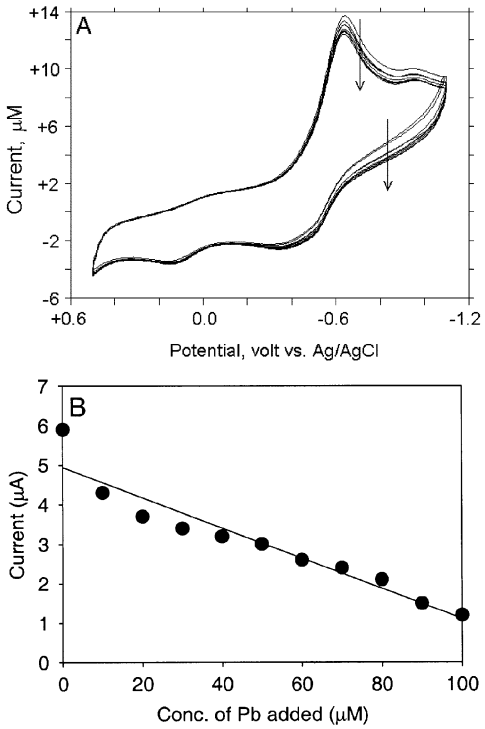

Fig. 10. Cyclovoltammogram (A) of Pb-resistant bacteria modified with Neutral red in which the current was decreased by addition of Pb-ions, and amperometric response (B) of the whole-cell biosensor constructed with Pb-resistant bacteria to Pb ion.

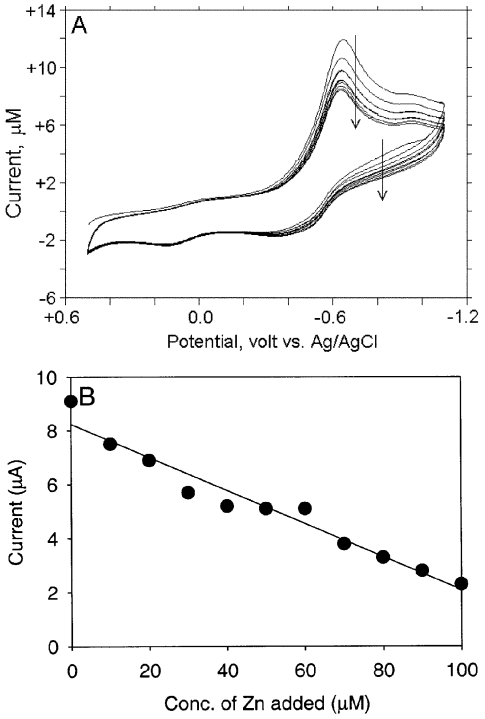

Fig. 11. Cyclovoltammogram (A) of Zn-resistant bacteria modified with Neutral red in which the current was decreased by addition of Zn-ions, and amperometric response (B) of the whole-cell biosensor constructed with Zn-resistant bacteria to Zn ion.

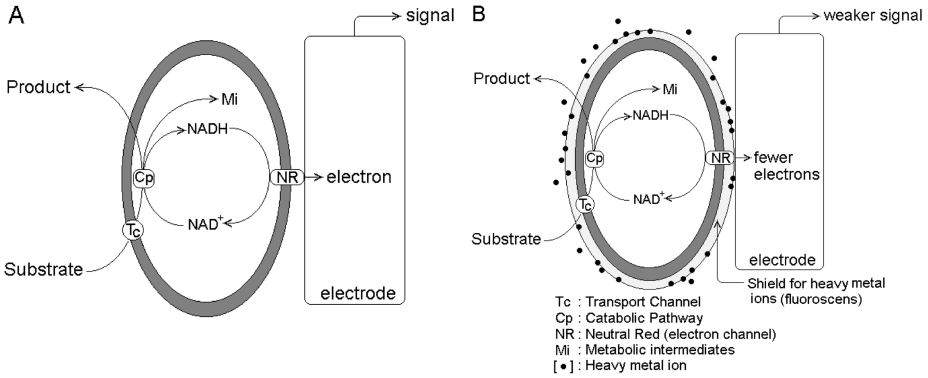

Fig. 12. Schematic diagram of heavy metal-resistant bacterial cell responding with electrode. The electron flow from bacterial cell to electrode may be not inhibited in wastewater without heavy metal ion (A) but may be inhibited by fluoroscence compounds secreted from haeavy metal-resistant bacteria when wastewater was contaminated with heavy metal ions (B). Any organic compounds contained in wastewater can be substrate for bacterial cell.

Conclusion

The biosnsor was constructed with intact cell of heavy metal-resistnat bacteria but couldn't compose the biosensor with fluoroscent compound because the fluoroscent compound was not electrochemically active. In this research, we can get a possibility that the bacteria inside working electrode of the electrochemical biosensor may produce fluoroscent compound by responding to heavy metal ion and then the fluoroscent

compound may interfere electron transfer from bacterial cell to electrode. The current produced from bacterial cell was decreased in proportion to concentration of heavy metal ion added to the culture and sample for measuring. By which we can reach a conclusion that the biosensor for detection of heavy metal ion may be composed with intact cell being responsible to heavy metal ion but for construction of electrochemical biosensor with fluoroscent compound produced heavy metal-resistant bacteria we have to produce a special fluroscent compound or other compound with electrochemical activity produced from heavy metal-resistnat bacteria or we have to change electrochemical biosensor device to spectrophotometrical biosensor device which can detect fluoroscent compound produced from heavy metal-resistnat bacteria being responded with heavy metal ion.

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