Master of Science

선택적 코발트 흡착능이 있는 펩타이드 기반의 폐수 중 코발트 회수 시스템

The Graduate School of University of Ulsan

School of Chemical Engineering

Jae Hoon Jeong

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Jae Hoon Jeong

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This certifies that the master's thesis

of Jae Hoon Jeong is approved.

Committee Chair Prof. Sung Gu Kang

Committee Member Prof. Ik Keun Yoo

Committee Member Prof. Soon Ho Hong

School of Chemical Engineering

University of Ulsan, Korea

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Abstract in Korean

본 연구에서는 세포 표면발현 시스템을 이용한 미생물 흡착 공정을 활용하였다. YiaT를 앵커링 모티프로 사용한 세포 표면발현 펩타이드 YiaT CP3를 대장균에 도입한 재조합 균주를 이용하여 흡착에 투여하는 세포의 농도 및 코발트 농도(1-10mM)에 따른 흡착 및 회수 실험을 진행하였다. 흡착된 코발트에 대한 정량분석은 ICP-OES를 이용하여 수행되었다. 더 나아가 산업적인 활용을 위하여 초음파를 이용해 파쇄한 세포의 흡착에 대한 영향과 동결건조한 세포의 코발트 흡착능을 비교하였다. YiaT CP3가 도입된 균주를 통한 코발트의 흡착은 야생종 균주에 의한 흡착에 비해 약 78.6% 높은 수치를 보였으며 Free cell의 경우 1 mM 코발트 농도에서 회수율이 가장 높은 것으로 측정되었으며 회수율은 77.46%를 보였다. 코발트의 농도가 높아짐에 따라 회수율이 감소되는 경향을 보였으며 3mM, 5mM, 10mM에서 각각 65.85, 65.4, 58.7%의 회수율을 보였다. 초음파를 이용해 파쇄한 세포의 흡착 실험에서는 회수율의 차이가 크지 않았으며 세포의 파괴가 흡착에 큰 영향을 끼치지 않는 것을 발견하였다. 동결건조한 세포의 경우 1, 3 mM 농도에서 78.46, 67.41%의 회수율을 보였고, 코발트의 농도가 높아짐에 따라 free cell에 비해 회수율이 감소하는 것을 확인하였다. 동결 건조한 세포를 통한 코발트의 생흡착은 보관, 운송, 낮은 농도의 코발트에서 유지되는 흡착능으로 코발트 회수에 유용한 전략이 될 수 있다.

Abstract in English

The cell surface display system was applied for the microbial adsorption process. The adsorption and recovery of cobalt was carried out using a recombinant *E.coli* in which the cell surface peptide YiaT CP3 using YiaT as an anchoring motif. Absorbed cobalt was evaluated with ICP-OES varying cell and cobalt concentration. Further, for industrial application adsorption capacity of the disrupted cell by sonication and freeze-dried cell was evaluated and compared with free cell. The surface displayed *E.coli* with CP3 peptide showed 78.6% higher adsorption than wild *E.coli*. The adsorption result with free cell was highest at 1 mM concentration of cobalt and its recovery was 77.46%. As the concentration of cobalt increases, the recovery tends to decrease. The result from the disrupted cell with sonication was not remarkable. Thus disruption of the cell does not affect adsorption. Freeze-dried cells had 78.46, and 67.41% of recovery at 1 mM and 3 mM concentration of cobalt, respectively. In 5 mM and 10 mM concentration of cobalt, freeze-dried *E.coli* showed lower recovery compared with free cell one. With the advantage of freeze-drying cells for storage, transportation, and usage, the recovery of cobalt from wastewater in low cobalt concentration with freeze-dried cell can be valuable strategy.

Keywords: *Bio-sorption, Cell surface display system, Bacterial freeze-drying, Cobalt*

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1. Introduction

1.1. Heavy metal pollution

The heavy metal refers to any metallic element that has a relatively high density and is toxic or poisonous even at low concentration [1]. Although there is no specific definition of a heavy metal, it is literally defined as a naturally occurring element having a high atomic weight and high density which is five times greater than that of water [2]. However, being a heavy metal has little relationship with density but concerns chemical properties. Heavy metals contamination is becoming a serious issue of concern all around the world with the growing demand of various human activities which is increasing rapidly as increasing of human population. Heavy metals such as lead (Pb), cadmium (Cd), Zinc (Zn), mercury (Hg), arsenic (As), silver (Ag), chromium (Cr), copper (Cu), iron (Fe), and the platinum group elements. Heavy metals are usually present in a trace amounts in natural waters. However, many of these heavy metals are toxic even at very low concentrations. Some heavy metals are either essential nutrients (typically iron, cobalt, and zinc) or relatively harmless (such as ruthenium, silver, and indium), but can be toxic in larger amounts or certain forms. Other heavy metals, such as cadmium, mercury and lead, are highly poisonous. Heavy metals become toxic when they are not consumed through metabolism by the body and accumulate in the soft tissues. They enter the human body by the uptake of food, water, air or absorption through the skin when they contact with humans in agriculture, manufacturing, pharmaceutical, industrial or residential settings [3,4]. Industrial exposure is the most common route of exposure for adults and ingestion is the most common of exposure in children. Natural and human activities are contaminating the environment and cause pollution more than what the environment can handle [3,5].

1.2. Sources of heavy metals

Heavy metals can be released into different environmental compartments (soil, water, air, and their interface) from both natural and anthropogenic processes.

1.2.1. Natural processes

Natural emissions of heavy metals occur with certain environmental conditions such as volcanic eruptions, sea-salt sprays, forest fires, rock weathering, biogenic sources, and wind-borne soil particles. These processes can lead to the release of metals from their original location to different environment compartments. Heavy metals can be found in the form of chemically-compound, such as hydroxides, oxides, sulphides, sulphates, phosphates, silicates and organic compounds [3].

1.2.2. Anthropogenic processes

1.2.2.1. Primary pollution

The release of pollutants like heavy metal to different environmental compartments was led by human activities such as industries, agriculture, wastewater, mining and metallurgical processes, and runoffs. Metals naturally emitted in wind-blown dusts from industrial areas. The sources which significantly contribute to the heavy metal contamination are include automobile exhaust which release lead, smelting which releases arsenic, copper and zinc, insecticides which release arsenic and burning of fossil fuels which release nickel, vanadium, mercury, selenium and tin. Human activities in industry contribute more portion of pollution by heavy metals more than natural processes due to the manufacturing of goods to meet the demands of the large population [4].

1.2.2.2. Secondary pollution

After products were produced from industry and used for its life time, discarded products can be also source of secondary pollution. The dumped industrial scrap can release metals and toxic materials through wastewater. With the advancement of technology, electronic device became one of the most essential parts in human life. In the study reported by International Solid Waste Association, 44.7 million metric tons of electronic wastes were generated in 2016. The amount of electronic waste is expected to increase to 52.2 million metric tons by 2021 [5]. (Fig. 1)

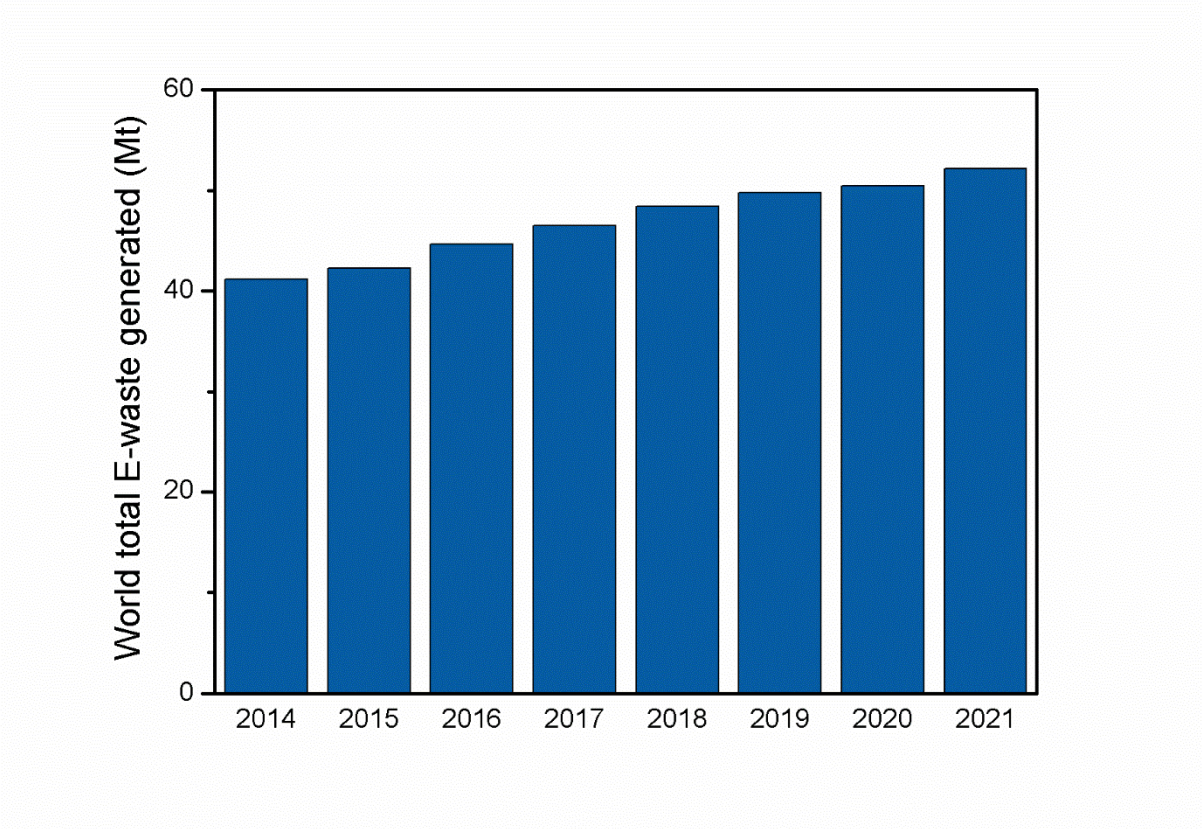

Fig.1. Global E-waste generated in annual year and forecast

1.3. Electronic waste (EW)

Electronic waste (EW) is generated with discarded electronic product after the end of its life. In recent times, electrical and electronic equipment are essential part of human living for everyday life. EW is caused with two major branches, includes infocomm technology (ICT) and home appliances. The former includes desktop, laptop, tablet computer, mobile phone and its battery. Accessories such as keyboards, modems, monitors, computer mice, docking stations and battery charges are also involved in ICT. The later includes such as TV, refrigerator, air conditioner, washing machine, and rice cooker, etc. Discarded EW can cause environmental pollution towards soil, water, and air. Additionally, the waste is rich in precious metals and valuable organic substance. Thus, recycling of EW is necessary.

1.4. Impact of EW towards environment

The metals from industrial scrap wastewater seeps into soil and groundwater and cause pollution. Once metals enter into the environment, it is hard to be destroyed. It react with other particles or absorb on soil particle or water sediments [6]. Approximately 40 million metric tons of EW are generated globally in each year and 13% of EW in weight is recycled mostly in developing countries [7]. Roughly 50% to 80% of EW is handled by shredding, burning and dismantling in developing countries' recycling market. Emissions from these recycling practices are damaging human health and the environment [8]. Pollution from improper disposed EW affects not only environment, but also indirectly and ultimately human and livestock. (Fig.2)

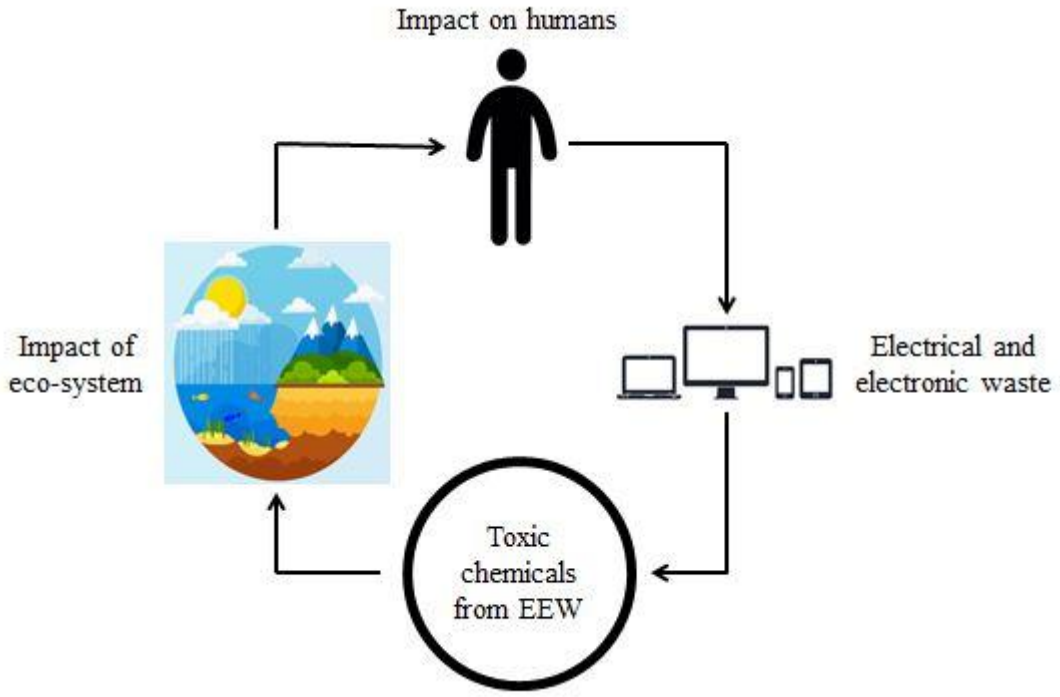

Fig.2. Improper disposal of electronic devices and its impacts on eco-system

1.4.1. Effect on soil

EW only accounts for 2% of the trash in landfills. However, it is cause for 70% of the toxic heavy metals [9]. Soil is affected by heavy metals from EW and contaminated in two ways, direct contact with contaminants from EW or byproducts of recycling and disposal; indirect irrigation from contaminated water. The presence of heavy metals in soils is a serious issue when it comes into food chains, thus make harmful effects on the entire ecosystem. As almost organic pollutants can be biodegradable, however with the presence of heavy metals in the environment, the biodegradation rate of organic pollutants is decreased and this leads the environmental pollution more serious. The danger is caused from improper disposal on the environment and makes impacts on human beings. The heavy metals came out from EW is suspected to cause serious health effects, for instance, birth defect (irreversible), brain, heart, liver, kidney and skeletal system damage [10]. EW is one of the fastest growing waste streams with the primary reason of increasing use of portable electronic devices [11-13]. Lithium-ion batteries are the most common type of used in portable electronic devices.

1.4.2. Effect on water

The lead, barium, mercury, lithium, etc., are released from improperly disposed electronics and these metals seeps into soil and reach groundwater channels which eventually run to the surface as streams or small ponds of water [7]. Heavy metals can be found in trace amount in water sources and cause serious health problems to humans and other ecosystems through its high toxicity. The presence of toxic chemical and metals in water cause death of plants and animals. Further, humans and animals may consume the contaminated waters, be exposed to toxic effect of metals. The toxic heavy metals such as lead, mercury and cadmium (found in printed circuit boards and other electronics) impact the nervous and reproductive system [9]. Some of the heavy metals present in EW were considered to be carcinogenic. The heavy metals can be accumulated in organisms like fish and it lead to contaminate entire food chain, ultimately cause exposure through human.

1.4.3. Effect on air

EW can contaminate air primarily with improper recycling processes which was done from poorly regulated countries. In these countries the EW is often handled in dismantling, shredding and it release dust or large particulates into the immediate environment. The workers without proper protection equipment are damaged to their respiratory system chronically. Improper recycling of EW not only affects respiratory health of workers, but also can migrate to far miles away from the recycle site. As EW can be considered as valuable secondary raw materials, it is burnt to remove lower cost products and this cause release of dioxins into environment. Dioxins are considered as one of the several forms of cancer, as its fat soluble and hydrophobic characteristics to be

accumulated and remained in human body for a lifetime. Burning also releases fine particles leads chronic damage to respiratory health also increases the risk of wide range of chronic diseases including cancer. The high-cost precious metals such as gold, silver etc., are extracted with using acids, desoldreing and other chemical techniques further generating toxic fumes. Open burning can also generate the release of hydrocarbons into the air [9].

1.5. Lithium-ion battery (LIB)

Lithium-ion battery is the most common battery type used in portable electronic devices. In one research, forecasting for global lithium-ion battery market was continuous increasing from 2016 to 2024 with 10.6% of CAGR [5]. Lithium-ion battery (LIB) is most widely used battery type for powering portable electronic devices and electric vehicles [14-16]. It replaced nickel-cadmium and nickel metal hydride batteries and became the dominant energy supplier in portable electronic devices with its advantages of superior energy density and slow discharge in idle mode [17]. With the advent of fourth industrial revolution, lithium-ion batteries are getting more demands leading by electronic vehicles and IOT-based wearable devices. As lithium-ion battery is gaining more attention and demand, discarded battery EW is increasing with relatively shorter life span of rechargeable lithium-ion batteries and leading the substantial increase in hazardous EW [18,19]. Discarded LIBs after its lifetime are a new kind of waste that is different from other kinds of solid waste. The purpose of recycling spent LIBs is to reduce or eliminate potential environmental impacts. Further recycling spent LIB can also recover valuable metals involved in battery industry and promote the sustainable development of the LIB industry and industrial upgrading [7,20].

A LIB is composed with its each part, such as a cathode, an anode, electrolyte, a separator, etc [21-24]. The cathode materials of LIBs are mainly lithium intercalation oxides, such as $LiNiO_2$, $LiMnO_4$, $LiCoO_2$, $LiFePO_4$, $LiNi_xCo_yMn_{1-x-y}O_2$ and so on [25-27]. In cathode materials, toxic metals and organic chemicals such as lithium, cobalt, nickel, copper, lead, and electrolytes are used.

The compositions of the batteries were used to evaluate the value for one ton of waste batteries [28-30]. A study reported that the battery contained high quantities of aluminum, cobalt, copper, and lithium. (Table.1)

Table 1. Estimated value of major metals present in lithium based batteries [7]

Material	**Price ($AUD/ton)**	**$AUD Batteries**	**Available/ton**
Nickel	18684.00	803.40	
Aluminum	2464	135.55	
Copper	8168	735.10	
Steel	567	114.60	
Lithium cobalt oxide	36370	10001.75	

These metals accounted for 97.32% of total metals used in LIBs. LIBs have been considered to green and clean energy storage due to their high voltage, energy density, low self-discharge efficiency, and lower harmfulness to the environment. However, it still has harmfulness to the environment and to human health due to the presence of few hazardous materials used in LIBs [20]. Thus, recovery of metals such as lithium and cobalt from the cathode material in LIB will be profitable for economic aspect [7].

1.6. Cobalt

Cobalt is one of the essential metals with its versatile application. It is a key component of cobalamin, also known as vitamin B_{12}, essential for animal metabolism. It is naturally found in the earth's crust in chemically combined form. It has been used for jewelry and paints with its distinct blue color since ancient times [6-8,31]. In recent times cobalt is used for making alloys, batteries, and catalysts. Approximately 25% of the global demand for cobalt is for rechargeable batteries [32]. Lithium cobalt oxide ($LiCoO_2$) is widely used in lithium-ion battery cathode active material. Nickel-cadmium (NiCd) and nickel-metal hydride (NiMH) batteries also include cobalt to improve the oxidation of nickel in the battery [33]. The high demand for cobalt is one reason for the skepticism towards the widespread use of lithium-ion batteries in electric mobility [34]. The global demand for cobalt is expected to grow by 70% until 2020 [35]. In the forthcoming years, demand for refined cobalt is expected to increase rapidly [36]. Hence it is essential to recover the cobalt from the spent lithium-ion batteries. Several studies have been conducted to recycle and recover cobalt by chemical and physical therapies [37,38]. However, these methods failed to remove cobalt at lower concentration [39,40]. In the recent, the application of microbes to metal recovery is rising with its cost-effective and eco-friendly advantages.

1.7. Metal recovery

The recovery of metals can reduce the environmental problems associated with it. Further, the recovery of cobalt will improve the use of natural resources and help in reduce the production cost of batteries [41]. Several studies have been conducted towards recycle and recover cobalt from the spent LIB (Lithium-Ion Battery) by chemical precipitation, ion exchange, coagulation, flocculation, pyro metallurgy, and hydrometallurgy or bio-hydrometallurgy processes [37,38]. (Fig.3)

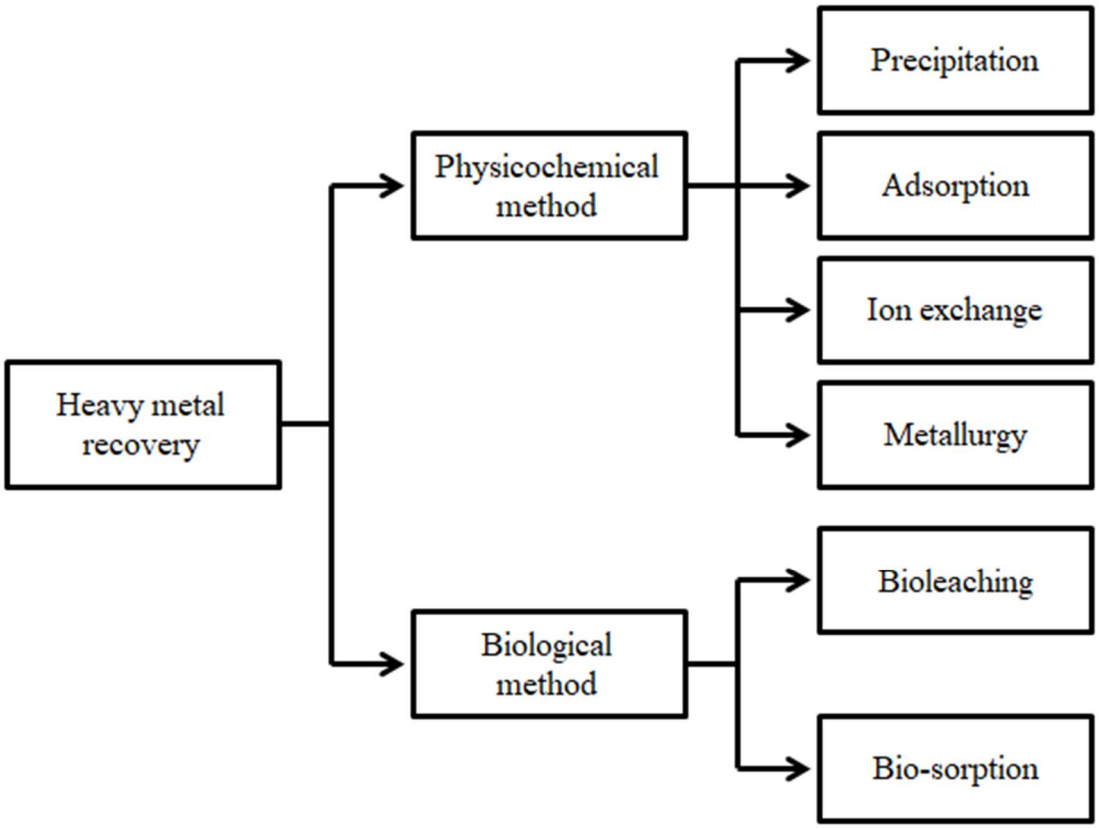

Fig.3. Mechanisms for the recovery of heavy metals

Pyro metallurgy have great capacity, and simple operation. However it has disadvantages such as requiring of high temperature, high energy consumption, and low metal recovery rate. Hydrometallurgy can recover metals with low energy consumption, high metal recovery rate, and high product purity. But it needs to take long recovery process, and high chemical reagents consumption [20]. Physicochemical methods commonly using chemical adsorbents for heavy metal removal from industrial wastes have several disadvantages including both economic and environmental aspect [42]. Additionally, above methods failed at removing cobalt particularly at lower concentration [40,43]. Hence it is essential to consider the cost-effective and eco-friendly method towards cobalt recovery.

1.7.1. Physicochemical process

The physical processes generally include dissolution, manual or mechanical separation and pyrolysis. The chemical processes are mainly hydrometallurgical methods involving acid or base leaching, solvent extraction, chemical precipitation, bioprocess and electrochemical process or combination of the processes [44]. In order to protect the human, plants, animals, soil and all the ecosystem, proper attention should be given to recovery technologies of heavy metals. Most physical and chemical recovery technologies of heavy metal require handling of large amounts of sludge and it may possibly lead destroying of surrounding ecosystems and the cost is very expensive [45].

1.7.1.1. Precipitation

The acid mine drainage (AMD) was neutralized with various alkaline chemical reagents for years in order to increase the pH and consequently precipitate and recover the metals. The most common alkaline reagents used for precipitation and recovery of metals resources from AMD are limestone ($CaCO_3$), caustic soda (NaOH), soda ash (Na_2CO_3), quicklime (CaO), slaked lime ($Ca(OH)_2$) and magnesium hydroxide ($Mg(OH)_2$) [46]. The process has recovered metals at varying pH regimes and synthesized commercially valuable metals such as pigments and magnetite [47]. Some metals are recovered and sold to metallurgical industries [45].

1.7.1.2. Adsorption

The adsorption process occurs when an adsorbate contacts and adheres to the surface of an adsorbent. With its reversibility and capable desorption, adsorption is considered the most effective and economical option for the recovery of metals from aqueous solution. The adsorption process is feasible for dilute solutions because in high concentrated solution, the adsorbent easily gets saturated with the adsorbate. Due to the concentration issue, in a large scale of metal recovery, adsorption is not applied for metal recovery [46].

1.7.1.3. Ion exchange

Ion exchange is occurred between two or more electrolyte solutions with exchanging of ions. For ion exchange, high cation exchange capacity clay and resins are used for recovery of metals from aqueous solutions. However, ion exchange has an issue that require high labour and have limitation of certain concentration of metals in solution. The system also should be operated under specific temperature and pH [45, 48].

1.7.2. Biological process

Microbial metal recovery is used in two ways, bioleaching and bio-sorption [49]. The bioleaching deals with extraction or solubilization of minerals. This method can be applied to recover metals from industrial residue [50]. However, bio-sorption deals with surface adsorption by

microbes [51]. Micro-organisms including bacteria, algae, fungi, and yeast are found to be capable of efficiently accumulating heavy metals [52-54]. The mechanism associated with metal removal by microorganisms are rather complex compared with those associated with chemical adsorbents and can be divided into three categories [55].

(1) bio-sorption of metal ions on the cell surface

(2) intracellular uptake of metal ions

(3) chemical transformation of metal ions by microorganisms

Non-living biomass is involved in removal mechanisms of metal ions through adsorption and ion exchange [56]. The recovery of metals with non-living biomass has strong affinity for metal ions due to the lack of protons produced during metabolism. The bio-sorption can be strengthened with Cell surface display (CSD) combining short metal-binding peptides on the cell's surface [7].

1.7.2.1. Cell surface display system

Microbial cell-surface display (CSD) system is carried out by expressing a heterologous peptide or protein of interest as a fusion protein with various anchoring motifs, which are usually cell-surface proteins or their fragments such as OmpA, OmpC, LamB, OprF, PhoE, OmpS, OmpX, InP, etc [57,58]. Microbial cell-surface display is one area of interest in synthetic biology, after the first report of this technology studied by Freud et al in 1986. The gram negative bacteria, which hold an inner cell membrane and the outer membrane, with a peptidoglycans cell wall in between. Therefore in order to make cell surface possible, the proteins should cross through the cytoplasmic and the outer membrane before the display on the cell surface. The anchoring motif was used to this work. (Fig.4) The cell-surface display can provide a means to circumvent separate expression, purification, and immobilization of binding proteins and enzymes [59]. With these advantages, CSD system can be applied to bio-sorbent or whole cell biocatalyst and also give possibility for immobilizing whole cell or enzyme.

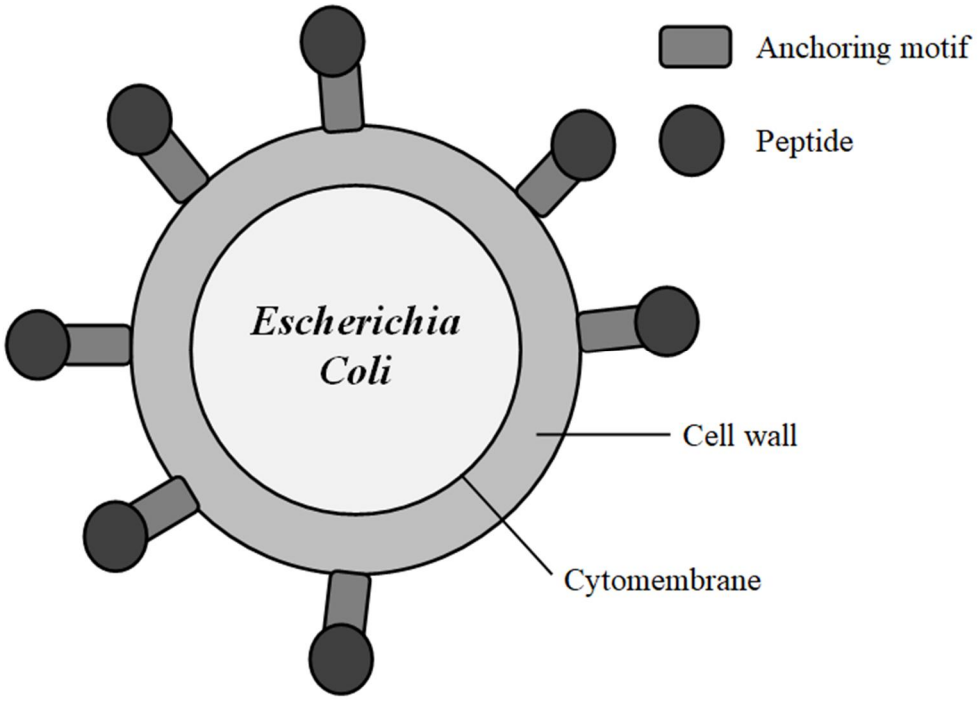

Fig.4. Schematic illustration of cell surface display (CSD) system

1.7.2.2. Metal binding peptides

Toxic metals are harmful for bacteria and organisms. The resistance mechanisms have been developed to make toxic metals harmless. The mechanism is using different systems, such as exclusion, compartmentalization, making complexes, and the synthesis of binding proteins such as metallothioneins (MT) or phytochelatins (PCs). Metal binding peptides (MBP) can offer a high affinity of metal binding capacity, specificity and selectivity for the target metal ion. These metal binding peptides can be designed de novo or selected by screening peptide libraries [60].

1.8. Cell disruption with sonication

The cell disruption is a process of releasing the biological content from a cell or a process of obtaining intracellular fluid through breaking cell wall in various methods. The method of cell disruption divided into mechanical and non-mechanical method. Mechanical method of cell disruption is divided into solid shear methods and liquid shear methods. The major principle of mechanical disruption method is cells are being subjected to high stress via pressure, abrasion with rapid agitation with beads, or ultrasound. From mechanical disruption, heat is generated by dissipation of mechanical energy. Thus intensive cooling of suspension after treatment is required to remove generated heat during process. Disruption of cell with sonication is caused by ultrasonic vibrators that produce a high frequency sound with a wave. This method is the simplest way to

break cell walls for small volume of suspension of cultured or microbial cells. Since, considerable amount of heat is generated during the proccess, sample must be kept on ice flakes during treatment in order to remove the heat [61].

1.9. Freeze-drying

Freeze-drying, also known as lyophilisation or cryodesiccation, is a low temperature dehydration process that involves freezing the production, lowering pressure to vacuum, and removing the ice by sublimation. Freeze-drying results in a high quality product because of the low temperature used in processing. The original shape of the product is maintained during process [62, 63]. The primary applications of freeze-drying include biological, biomedical, food processing, and preservation. By removing the water from the material and sealing the material in a glass vial, the material can be easily stored, and shipped. The powder typed-bacteria can be produced by bulk freeze-drying of live microorganisms [64]. In this study, after *E.coli* has been freeze-dried, autoclaved double-distilled water was poured into powdered bacteria and exposed in various cobalt concentrations to recover cobalt.

2. Experimental methods

2.1. Bacterial strains and growth conditions

The bacterial strains and primer used in this study are listed in Table 2,3. The strains were transformed with YiaTCP3 and cultivated for overnight in LB plate (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, and 15 g/L bacto-agarose) containing 100 mg/L of ampicillin. After overnight cultivation, one single colony was picked into LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl) with supplement of 100 mg/L ampicillin and incubated overnight at 37 in shaking incubator at 250 rpm.

2.2. Free cell preparation

The transformed *E.coli* displaying the CBP3 was cultured overnight in LB medium at 37 , and 1 ml of cultures were inoculated into 100 ml in fresh LB medium and incubated until the optical density at 600nm (OD600) reached 0.5. After 0.5% arabinose was added to the culture broth to induce cells, the strains were further incubated at 30 for 5 hours till OD600 reached 1.5. The recombinant *E.coli* were collected in each amount with agitation for 5 minutes in the centrifuge at 5000 rpm.

2.3. Freeze-dried cell preparation

Cell pellets from 200 ml of cultivated broth were collected in a 50 mL conical tube by agitation at 4 in the centrifuge at 5000 rpm. Pellets were resuspended with 10 mL of autoclaved double-distilled water. Further, samples were poured into the petri dish and freeze-dried for 16 hours with freeze-drying machine FDU-2200(EYELA). After freeze-drying, the powder typed sample was divided by weight to make certain amount for experiment. The 10 mL of autoclaved double-distilled water was poured into powdered *E.coli* to make each sample.

2.4. Cell disruption with sonication

The arabinose-induced recombinant *E.coli* was collected with centrifuge for 5 minutes in 4 at 250 rpm. After cell pellets were prepared in each amount, it was resuspended with 10 mL of distilled water. The samples were sonicated for 120 seconds. Samples were placed under the ice flakes during sonication [61].

2.5. Cobalt recovery and analysis

Samples were incubated in a solution containing a varying concentration of cobalt chloride (1-10 mM) for 30 minutes. After the adsorption, the recombinant strains were washed twice with 0.85% (w/v) NaCl to remove physically adsorbed cobalt. The Adsorbed cobalt by the peptides was eluted by incubating with 1 mM EDTA for 30 minutes in ice. Samples were quantitatively measured with 5-fold dilution with water by ICP-OES(Agilent technologies 5110) [7].

2.6. Effect of adsorption time

Incubation was differed by various adsorption time (0 – 4 hours) to analyze effect of incubation time on the cobalt recovery. The adsorption was stopped after incubation time reached in certain point and washed twice immediately with 0.85% NaCl. The adsorbed cobalt was eluted with 1 mM EDTA incubating for 30minutes in ice.

Table 2. List of bacterial strains and plasmids used in this study [7]

Strain/Plasmid	Relevant genotype/ property	Source
***E. coli* strains**		
XB	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tetr)].*	Novagen
Plasmids		
pBAD30	Amp^R	NEB[a]
$pBADCp_3$	pBAD30 containing YiaT- CBP3	This work

Table 3. Primer used in this work [7]

Name	Sequence (5' to 3')
CP3_R	GGTACCGGTGGTGCTGCTGCCCAGCGGCAGGGTCGGATAATGACGATC AATCATCGGGCTGTCGGTAAT

3. Results and discussion

3.1. Cobalt bio-adsorption studies with surface displayed CP3

The bio-adsorption of cobalt with surface displayed CP3 was evaluated by comparing amount of recovered cobalt by wild type *E.coli* and the recombinant *E.coli*. The strain was cultivated in LB medium supplemented with ampicillin. The recombinant E.coli which contains YiaT without CP3 was used as a control. The peptides were overexpressed with the addition of 0.5% arabinose at 30 for 5 hours. After overexpression both recombinant *E.coli* samples were collected through centrifuge for 200 mL of cultured broth. The supernatants were discarded and 10 mL of double-distilled autoclaved water was poured and resuspended the pellets. After preparation, the recombinant *E.coli* was exposed to various concentration of cobalt (1-10 mM). The difference of recovery of cobalt between strain without CP3 and with CP3 was significant (Fig.5). The recombinant *E.coli* with surface displayed CP3 showed 78.6% higher amount of recovered cobalt than the strain without CP3 in 1 mM concentration of cobalt. Thus bio-sorption of *E.coli* was enhanced by surface display of CP3.

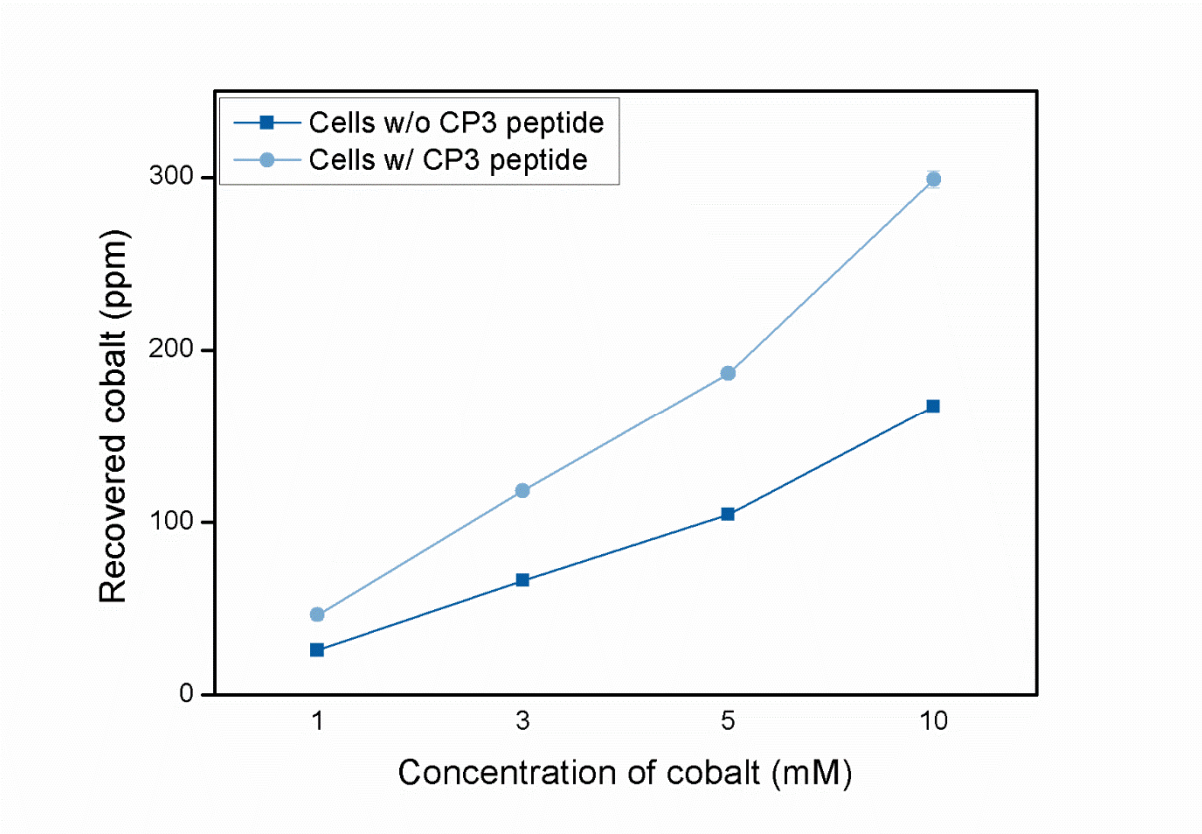

Fig.5. Cobalt bio-adsorption studies by the recombinant *E.coli* with surface displayed CP3 peptide in various concentration of cobalt (1-10 mM).

3.2. Recovery rate affected by the concentration of cobalt

The recovery of the recombinant bacteria was conducted by incubating samples under various concentration of cobalt (1 – 10 mM) in 25 for 30 minutes. The recovery rate of the recombinant *E.coli* in each cell amount and cobalt concentration is given in Table 4. In 1 mM concentration of cobalt, the recovery rate by the recombinant bacteria showed 77.46% in 200 mL of broth sample. The recovery rate in 10 mM concentration of cobalt showed lower value and it showed only 58.77% of cobalt was recovered. As the concentration of cobalt increased, recovery rate of cobalt was decreased. (Fig.6)

Table 4. Recovery rate of cobalt by the recombinant E.coli with cell surface displayed YiaTCP3 in various amount of cells and cobalt concentration.

Recovery rate of cobalt (%)				
Volume of cultivated broth (mL)	Concentration of cobalt (mM)			
	1	3	5	10
25	47.3	37.1	40.6	34.2
50	63.6	48	49.7	37.4
75	66	51.8	54.4	39.2
100	68.7	60.6	57.7	48.6
200	77.5	66.1	65.9	58.7

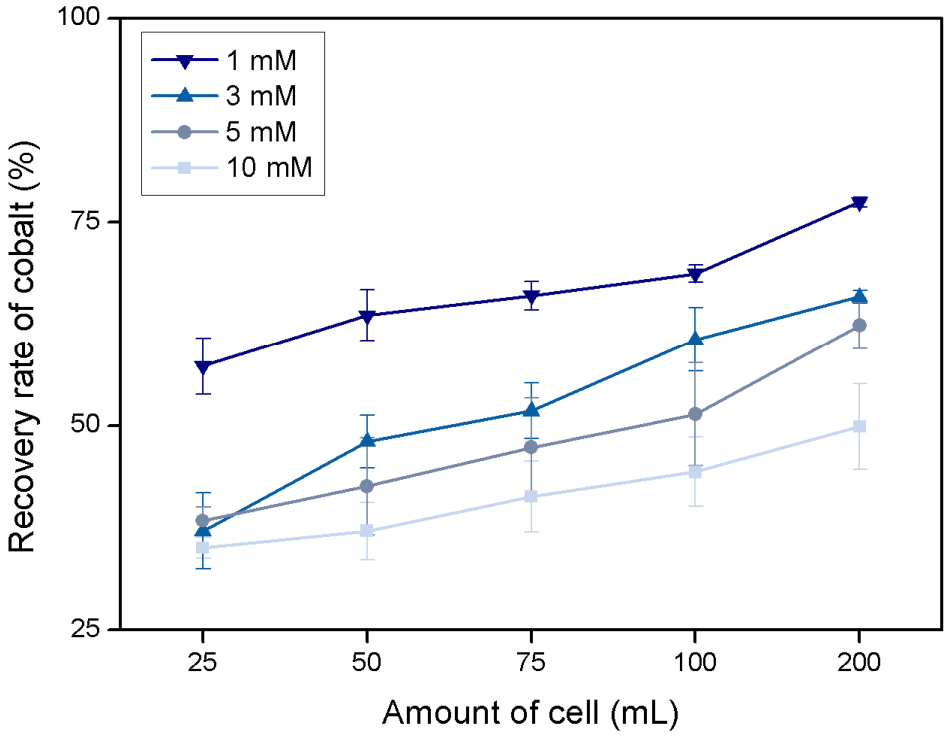

Fig.6. The recovery rate of cobalt differs by cell amount in various concentration of cobalt (1 – 10 mM)

3.3. Recovery of cobalt with freeze-dried *E.coli*

The arabinose-induced recombinant *E.coli* was collected in 200 mL of broth with the centrifuge at 4 , 250 rpm for 5 minutes. Autoclaved double-distilled water was added to make a 10 mL volume of sample. After preparation, freeze-drying was done with FDU-2200(EYELA) for 16 hours. The freeze-dried *E.coli* with YiaTCP3 was exposed in each concentration of cobalt by supplement of cobalt chloride solution. The recovery rate of freeze-dried *E.coli* showed lower value in small amount of cell. (Table. 5) However, the recovery rate of 200 mL of cultivated broth sample showed higher value compared with free cell in 1 and 3 mM of cobalt concentration. In 5, and 10 mM of cobalt concentration, the recovery rate of freeze-dried *E.coli* only recovered 57 and 47.4 % of cobalt lower than free cell's value. (Fig.7) With this result, freeze-drying on the recombinant *E.coli* does not affect to cobalt recovery in low concentration of cobalt (1 – 3 mM). As the concentration of cobalt was increased, the recovery rate of freeze-dried recombinant *E.coli* was decreased.

Table 5. Recovery rate of cobalt by freeze-dried recombinant *E.coli* with cell surface displayed YiaTCP3 in various amount of cells and cobalt concentration.

Recovery rate of cobalt (%)				
Volume of cultivated broth (mL)	Concentration of cobalt (mM)			
	1	3	5	10
25	34.4	26.4	23.8	16.6
50	63.8	47.9	33.5	30
75	66.6	50.4	44.6	30.2
100	68.2	50.5	47.8	35.3
200	78.5	67.4	57	47.4

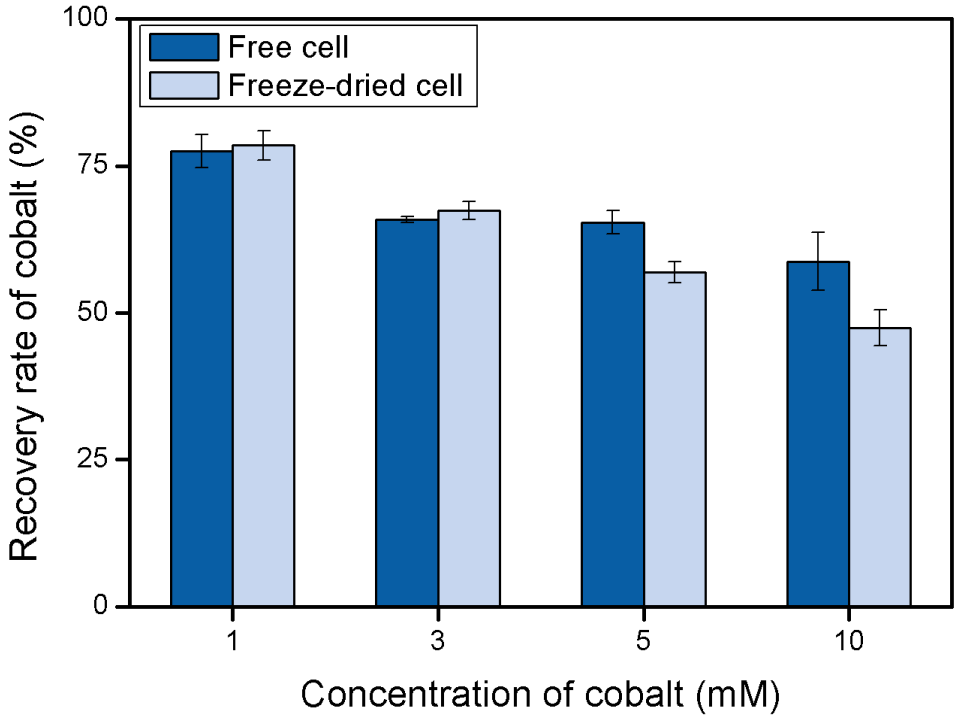

Fig.7. The recovery rate of cobalt from free the cell and freeze-dried recombinant *E.coli* in 200 mL of cultivated cell amount

3.4. Recovery of cobalt with disrupted cell by sonication

3.4.1 Effect by sonication to free cell

The recombinant *E.coli* was induced with 0.5% of arabinose for 5 hours and collected by centrifuge for 5 minutes at 4 , 250 rpm. Pellets were resuspended with 10 mL of autoclaved double-distilled water. Sonication was done for 120 seconds with samples under ice flakes. Disrupted cell with each volume was exposed in various cobalt concentration for 30 minutes. The recovery rate of cobalt from free cell and disrupted cell using 200 mL of cell amount is summarized in Table 6. The recombinant *E.coli* disrupted by sonication showed 76.29, 66.05, 63.9, 57.7% of recovery rate from 1, 3, 5, and 10 mM concentration of cobalt, respectively. The recovery rate of the disrupted cells by sonication showed no remarkable difference compared with the free cell. (Fig.8)

3.4.2. Effect by sonication to freeze-dried cell

The sonication was done for the arabinose-induced recombinant *E.coli* before freeze-drying for 120 seconds with samples under ice flakes. After sonication, samples were freeze-dried for 16hours with freeze-drying device, FDU-2200(EYELA). The comparison of recovery rate by freeze-dried *E.coli* and disrupted *E.coli* by sonication before freeze-drying with 200 mL of cultivated cell amount is summarized in Table 7. For 2oo mL of cultivated cell amount samples, the recovery rate of cobalt was 74, 69.3, 59.8, and 44.2 for 1, 3, 5, and 10 mM of cobalt concentration, respectively. The difference in the recovery rate between freeze-dried cell with sonication and without sonication was not significant in 1 – 10 mM concentration of cobalt. (Fig.9) From the result of the recovery rate of disrupted cell by sonication, both free cell and freeze-dried cell was not affected by cell disruption.

Table 6. The difference in recovery rate between free cell and disrupted cell by sonication in 200 mL of cultivated cell amount

Recovery rate of cobalt (%)				
Free cell	Concentration of cobalt (mM)			
	1	3	5	10
w/o sonication	77.5	66.1	65.9	58.7
w/ sonication	76.3	66	63.9	57.7

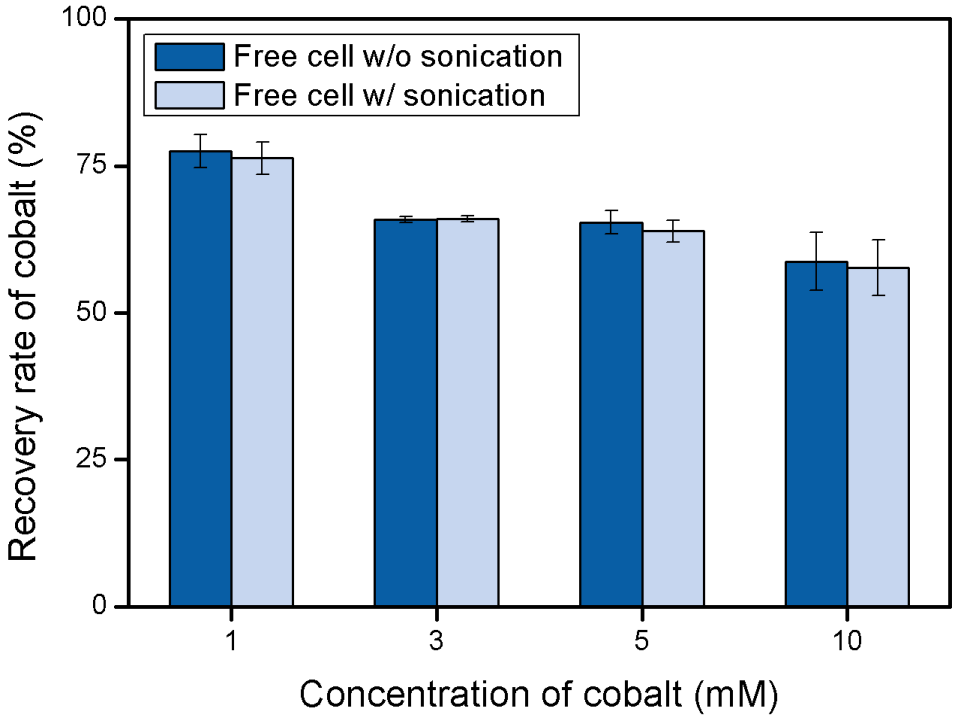

Fig.8. Effect of cell disruption for free cell by sonication to the recovery of cobalt in 2oo mL of cultivated cell amount

Table 7. The difference in recovery rate between freeze-dried cell and disrupted cell by sonication before freeze-drying in 200 mL of cultivated cell amount

Recovery rate of cobalt (%)				
Freeze-dried cell	Concentration of cobalt (mM)			
	1	3	5	10
w/o sonication	78.5	67.4	56.9	47.4
w/ sonication	74	69.3	59.8	44.2

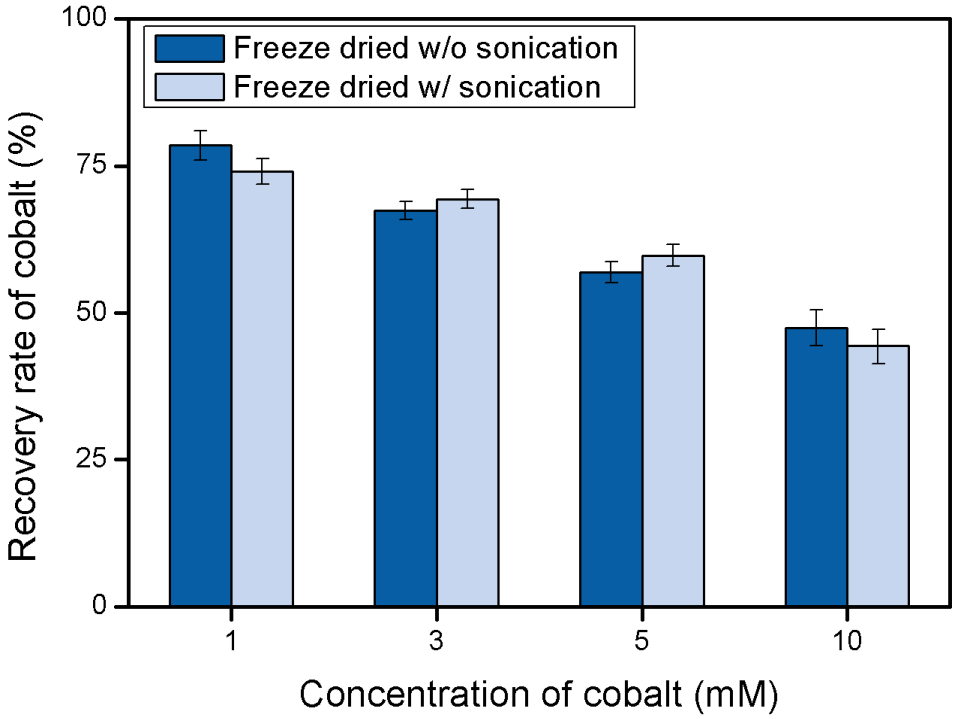

Fig.9. Effect of cell disruption by sonication to freeze-dried recombinant *E.coli* for recovering cobalt in 200 mL of cultivated cell amount

3.5. Adsorption time effect on the recovery rate of cobalt

3.5.1. The effect in free cell

The recombinant *E.coli* was collected for 50 mL volume of cultivated broth with centrifuge for 5 minutes, at 4 , 250 rpm. Autoclaved double-distilled water was added to make a 10 mL volume of sample. Samples were exposed to 1 and 5 mM of cobalt concentration with the supplement of cobalt chloride solution. The effect of adsorption time was analyzed by differing adsorption time (0 – 4 hours). The recovery rate was low until adsorption was done for 20 minutes. After 30 minutes, recovered cobalt was certain as incubation time for 4 hours. (Fig. 10 A)

3.5.2. The effect in freeze-dried cell

The freeze-dried recombinant *E.coli* was liquefied with 10 mL of autoclaved double-distilled water. Cobalt chloride solution was added to make 1 and 5 mM of cobalt concentration. The samples were incubated for various time (0 – 4 hours). In the freeze-dried *E.coli*, adsorption was done more rapidly compared with free cell. Before adsorption time was reached to 30 minutes, The recovery of cobalt with freeze-dried cell showed the remarkable difference compared with free cell. (Fig.10 B) After 30 minutes, the recovery rate of freeze-dried *E.coli* showed certain value.

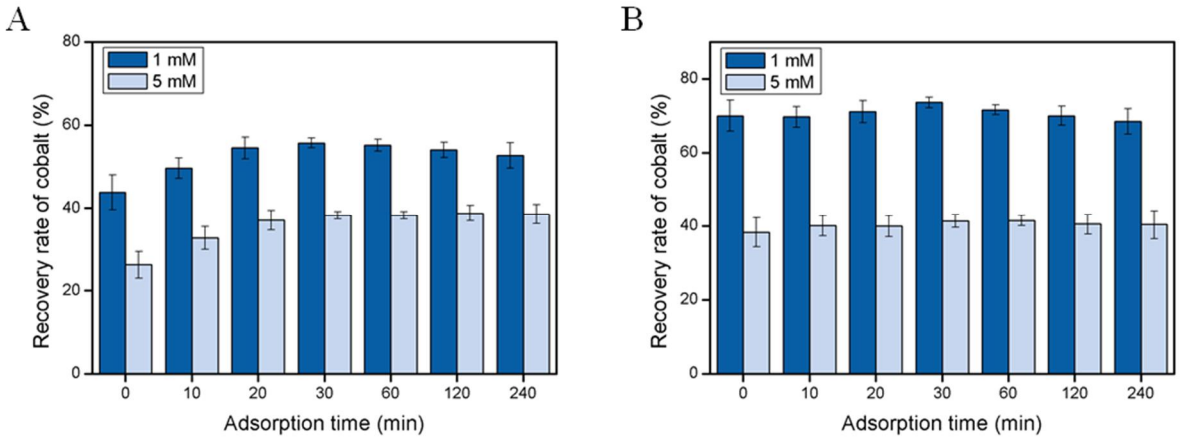

Fig.10. Effect of adsorption time on the recovery rate of cobalt in 50 mL of cultivated cell amount (A) Free cell YiaT CP3 containing recombinant *E.coli* (B) Freeze-dried YiaT CP3 containing recombinant *E.coli*

4. Conclusion

Cobalt is considered one of the most essential metals in the lithium ion battery industry. With the advent of the fourth industrial revolution and the leading industry of the electronic vehicle, the importance of cobalt is increasing further. In this study, the recovery of cobalt with the recombinant *E.coli* was conducted with cell surface-displayed YiaCP3 peptides. The peptide-based highly cobalt recovery system was developed with CSD of cobalt binding peptide. The peptide CP3 was displayed on the cell surface with YiaT as an anchoring motif. The recombinant *E.coli* was found to recover 77.46% in 200 mL volume of the cultivated broth for 1 mM cobalt concentration. The recovery rate decreased as the concentration of cobalt is increasing and only showed 58.77% of recovery rate in 10 mM. Further, the freeze-drying was performed to arabinose-induced *E.coli* and its recovery rate of cobalt showed the comparable result with free cell in the range of 1 – 3 mM of cobalt concentration. After the concentration of cobalt reached 5 mM, freeze-dried *E.coli* showed a lower recovery rate compared with free cells. Effect by sonication for cell disruption was conducted with sonication for 120 seconds to sample under the ice flakes. With the results in Fig.4 and Fig.5, cell disruption with sonication gives not remarkable effect on the recovery of cobalt both free cell and freeze-dried cell. Additionally, time effects on recovery of cobalt were analyzed by differing adsorption time. For free cell samples, the recovery rate showed increasing until 30 minutes. After 30 minutes, the recovery rate showed a certain value. The freeze-dried cell showed rapid adsorption before 30 minutes compared with free cell. In 30 minutes, the recovery rate of freeze-dried cell showed maximum value and after 30 minutes, the recovery rate showed certain value. In recovery of cobalt, freeze-drying can be valuable strategy with its effectiveness in storage, transport, and remained ability for recovering cobalt in low concentration.

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