Master of Science

Development of valuable metal recovering recombinant *Escherichia coli* system using cell surface display

The Graduate School of University of Ulsan

School of Chemical Engineering

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Development of valuable metal recovering recombinant *Escherichia coli* system using cell surface display

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Development of valuable metal recovering recombinant *Escherichia coli* system using cell surface display

This certifies that the master's thesis

of So Won Chae is approved.

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Abstract in Korean

본 연구에서는 세포 표면발현 시스템을 이용한 미생물 흡착 공정을 활용하였다. *OmpC*를 앵커링 모티프로 사용한 세포 표면발현 펩타이드 *OmpC zraP*를 대장균에 도입한 재조합 균주를 이용하여 금속 흡착 이후 회수를 위해 처리하는 방법의 다양성 및 금속 흡착 이후 열분해로 생성되는 나노 파티클을 활용해 항생제로서의 역할을 확인하는 실험을 진행하였다. 흡착과 탈착이 된 아연에 대한 정량분석은 ICP-OES를 이용하여 수행되었다. 그리고 Langmuir, Freundlich 흡착 등온식을 통해 실험에서 얻은 데이터들을 확인해 보는 과정을 수행하였다. *OmpC zraP*가 도입된 균주에 아연을 투여하여 흡착시킨 후 활용한 탈착 방법에는 물리적 방법과 화학적 방법으로 나누어 진행하였다. 통상 미생물에 흡착된 금속을 탈착할 때는 화학적 방법인 EDTA를 이용하게 되는데 본 연구에서는 물리적인 방법으로도 금속 탈착 여부가 가능한지 확인하였다. 물리적 방법으로 금속을 탈착한 것과 화학적 방법으로 금속을 탈착한 후 그 양을 비교하니 물리적 방법이 화학적 방법의 비해 효율은 떨어졌으나 44.61 %의 효율을 보였기에 미비하다고 볼 수는 없다. 또한 미생물에 흡착된 금속 이온에 열분해를 가하여 아연 나노 파티클을 형성하였고, 이를 대장균 배양액에 투여하여 매 시간 성장 속도를 측정해 아연 나노 파티클이 항생제로서의 역할을 수행하는지 확인하였다. 배양액에 아연 나노 파티클의 양을 증가시킬수록 대장균의 성장 속도가 감소함을 확인할 수 있었다. 투여한 아연 나노 파티클의 양이 10 mg/mL 이상이었을 때부터 대장균의 성장 속도 감소를 관측할 수 있었고, 100 mg/mL 이상부터는 대장균이 2배가 되는 속도가 아연 나노 파티클을 투여 하지 않은 것에 비해 3, 4배 이상의 시간이 걸렸다.

Abstract in English

The cell surface display system was applied for the microbial adsorption process. The experiment to confirm was carried out that cell surface peptide *OmpC zraP* using *OmpC* as an anchoring motif is a recombinant strain introduced into *E. coli* to play a role as an antibiotic by utilizing zinc oxide nanoparticles (ZnO NPs) generated by thermal decomposition after metal adsorption and check the various methods of metal recovery after metal adsorption. Absorbed and desorbed zinc was evaluated with ICP-OES. In addition, a process was performed to verify the data obtained from this experiment through the Langmuir and Freundlich isotherm models. The desorption method used after zinc was administered and adsorbed to the strain into which *OmpC zraP* was introduced was divided into physical and chemical methods. In general, EDTA, which is a chemical method, is used to desorb metal adsorbed on microorganisms. In this study, it was confirmed whether metal desorption is possible with a physical method. When comparing the amount of metal desorbed by physical method and metal desorbed by chemical method, the efficiency of the physical method was not low even though the efficiency of physical method was inferior to that of the chemical method. In addition, thermal decomposition was applied to metal ions adsorbed by microorganisms to form zinc nanoparticles, which were administered to the *E. coli* culture medium to measure the growth rate every hour to confirm whether the zinc nanoparticles functioned as antibiotics. It was confirmed that the growth rate of *E. coli* decreased as the amount of zinc nanoparticles in the culture medium increased. When the amount of zinc nanoparticles administered was 10 mg/mL or more, a decrease in the growth rate of E. coli could be observed. It took more than four times the time.

Keywords: *Biosorption, Cell surface display system, Antibiotics, Zinc*

Contents

Figure list

Table list

1. Introduction

1.1. Toxicity of heavy metals

Heavy metals are defined as metallic elements that have a relatively high density compared to water [1]. These metals generally named heavy metals include: cobalt, copper, iron, lead, nickel, platinum, gold, silver and zinc. Some of these heavy metals are essential nutrients which are required for various biochemical and physiological functions in the body and may result to deficiency syndrome or diseases if not in adequate amounts but in large doses they may cause acute or chronic toxicities. In recent years, heavy metals are serious environmental pollutants and their toxicity is a problem of increasing significance for ecological and environmental reasons [2]. Heavy metals contamination is becoming a significant issue of concern all around the world with the growing demand of various human activities which is increasing rapidly as increasing of human population. However, many of these heavy metals are toxic or poisonous even at very low concentrations. And due to the discharge of large amounts of metal-contaminated wastewater, industries bearing heavy metals are the most hazardous among the chemical-intensive industries. Because of their high solubility in the aquatic environments, heavy metals can be absorbed by living organisms. If the heavy metals are digested over the permitted concentration, they can cause crucial health diseases. [3]. And then, industries about electrochemical products were produced from these industries 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 [4].

1.2. Methods for removal of heavy metals in wastewater

Heavy metals in wastewater can come from fertilizers or pesticides as well as from the plating, tanning, dyeing, textile, or electrochemical industries [5]. Thus it is necessary to treat metal-contaminated wastewater prior to its discharge to the environment. And the recovery of metals can reduce the environmental problems associated with it. Treatment of heavy metals in wastewater has been classified, and it is carried out in three stages [3]. They are:

(1) chemical methods

(2) physical methods

(3) biological methods

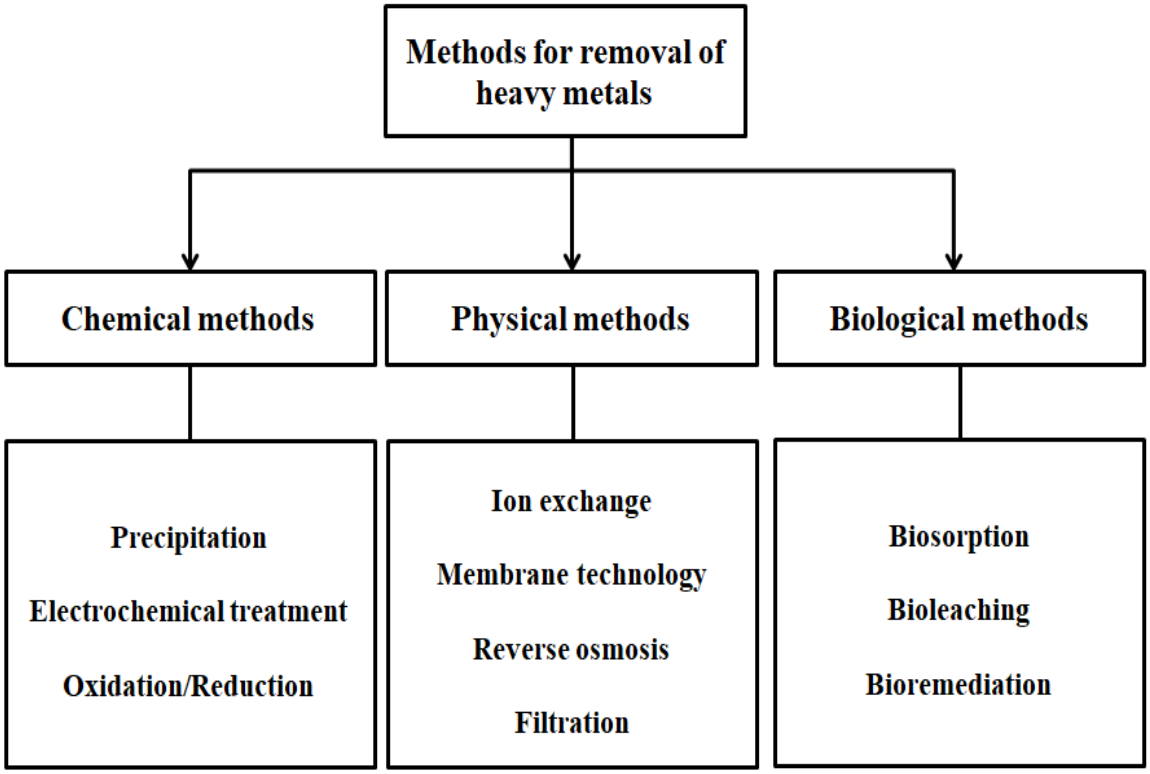

Fig.1. Schematic diagram showing the heavy metals removal methods in wastewater.

1.2.1. Chemical methods

The chemical methods for removal of heavy metals in wastewater are mainly hydrometallurgical methods involving chemical precipitation, electrochemical process, oxidation/reduction, acid or base leaching, solvent extraction or combination of the processes. Generally various agents were used for this purpose that includes complexing agents (EDTA, thiosulfate), mineral acids (HCl, HNO_3, H_2SO_4), organic acids (acetic acid, citric acid), etc. [6, 7].

1.2.1.1. Chemical precipitation

Chemical precipitation is a method that contaminants are separated from the solution as sediment, which can then be filtered, centrifuged, or otherwise separated from the liquid part [5]. Chemical precipitation processes, in conjunction with various physical operations, have been developed for the complete secondary treatment of untreated wastewaters, including the removal of either nitrogen or phosphorous, or both [8]. Chemical precipitation precipitates impurities from purified water via change of pH, electro-oxidizing potential or co-precipitation using precipitating agents such as ferrous or aluminum sulfates [9]. Common chemical reagents such as NaOH, CaO, $Ca(OH)_2$, $CaCO_3$, MgO, $Mg(OH)_2$, and NH_4OH precipitate metals as oxides and hydroxides; Na_2S, NaHS, H_2S, or FeS as sulfides; Na_2CO_3 or $CaCO_3$ as carbonates; and apatite or hydroxyapatite as phosphates [10]. Chemical precipitation is a highly efficient and simple process for removing metals from leachates with high metal concentrations; however, it is very pH sensitive and inefficient in leachates containing low concentrations of soluble metals and produces toxic sludge [9, 10].

1.2.1.2. Electrochemical treatment

Electrochemical treatment of heavy metals has been known as a very efficient method for the industrial wastewater treatment, especially for the removal of heavy metals ion [11]. Electrochemical technology provides solution for the recovery of heavy metals in their most valuable state. This technology includes electrocoagulation, electroflotation, electrodeposition, and electrosorption. But the issue in this field, little progress has been made toward electrode modification [12].

1.2.2. Physical methods

The physical processes generally include ion exchange, membrane technology, reverse osmosis, evaporation recovery, filtration, dissolution, manual or mechanical separation and pyrolysis [13].

1.2.2.1. Ion exchange

Ion exchange may be defined as the exchange of ions between the substrate and surrounding medium. The ion exchangers which contain cations or anions as counter-ions are called cation exchangers or anion exchangers, respectively. 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 requires high labor and limitation of certain concentration of metals in solution. The system also should be operated under specific temperature and pH [9, 13].

1.2.2.2. Membrane technology

Membrane technology processes include reverse osmosis, filtration, distillation, contactors etc. Basically, a membrane is a barrier which separates two phases from each other by restricting movement of components through it in a selective style [14]. Membrane technology is achieved without a phase change and use of expensive solvents. However, disadvantages of this technology are limited to thermal stability, limited about recycling number, membrane cleaning, durability, and membrane performance [15, 16].

1.2.2.2.1. Filtration

The filtration process is very simple. When the water contained heavy metals flows through the membrane, the functional active binding sites will combine with the target metal ions to remove them [17].

1.2.2.2.2. Reverse osmosis

Reverse osmosis is a separation process that uses pressure to force a solution through a membrane that retains the solute on one side and allows the pure solvent to pass to the other side. The membranes used for reverse osmosis have a dense barrier layer in the polymer matrix where most separation occurs. This process requires that a high pressure be exerted on the high concentration side of the membrane [16].

1.2.3. Biological methods

Conventional technologies to remove heavy metal in wastewater have drawbacks [18, 19]:

(1) chemical precipitation generates sludge waste.

(2) ion exchange resins are made from unsustainable non-renewable resources.

(3) membrane separation processes are quite promising for commercial applications.

(4) filtration has low rejection potential.

Biological methods have been suggested as an efficient strategy to remove heavy metals from wastewater. Also, cheapness and low sludge production are the main advantageous of biological treatment of heavy metals [13]. Moreover, using microbial biomass as the platform for heavy metals removal is an alternative method. Microorganisms have been found to be capable of efficiently accumulating heavy metals in both living and dead states [18]. Compared to ion exchange and adsorption resins, the use of dead biomass as biosorbent is more environmental friendly because of its easy disposal [20]. To enhance the biosorption properties of microbial cells to heavy metal ions, the cell surface display of various metal binding peptides/proteins have been performed using a cell surface engineering approach [21]. 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 [22].

1.2.3.1. Biosorption

The biosorption deals with surface adsorption by microbes [23]. Bacteria, yeasts, fungi, and algae species can absorb and accumulate heavy metals in their body [13]. Generally, biosorption is more suitable due to the simplicity, high efficiency and cost-effectiveness. Low cost and high abundance in nature caused widely use of clay minerals as the absorbents [6, 13].

1.2.3.1. Bioleaching

The bioleaching deals with extraction or solubilization of minerals. This method can be applied to recover metals from industrial residue [24]. As mentioned earlier, 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 chemical and physical technologies of heavy metals require handling of large amounts of sludge and it may possibly lead destroying of surrounding ecosystems and the cost is very expensive [25]. And chemical and physical methods commonly using chemical adsorbents for removal of heavy metals from industrial wastewater have several disadvantages including both economic and environmental aspect [26]. Hence it is essential to consider the cost-effective and eco-friendly method towards heavy metals recovery.

1.3. Mechanisms of biosorption

The process of heavy metal ion binding to bacterial cell wall can be metabolism dependent of independent. Biosorption through metabolically mediated (by the use of ATP) or spontaneous physicochemical pathways of uptake (not at the cost of ATP), or as a property of certain types of inactive, non-living microbial biomass which bind and concentrate heavy metals from even very dilute aqueous solutions [27]. Biosorption are often inspired from solutions for conventional sorption based methods:

extensive screening of microbial species with attractive adsorption properties, chemical modification of the anionic moieties on the outer membrane surface, and the genetic engineering of the extracellular space to have metal binding peptides/proteins [18].

1.3.1. Cell surface display

Microbial cell surface display allows peptides and proteins to be displayed on the surface of microbial cells by fusing them with the anchoring motifs, which are usually cell surface proteins or their fragments. [28, 29]. The peptides/proteins to be displayed – metal binding peptides/proteins in this study – can be fused to an anchoring motifs. Many different anchoring motifs, such as *OmpC*, *YiaT, OmpX*, *BcIA*, *PgsA*, *OmpA, OprF*, *OmpS*, *OprF*, *PhoE*, *InP*, etc., were developed for the cell surface display of proteins [20]. The anchoring motif, *OmpC*, was used to this work. Cell surface display of designer cellulosome complexes has attracted increased interest in recent years. Cellulosome complexes are intricate, multi-enzyme machines, produced by many cellulolytic microorganisms. These engineered microorganisms can efficiently degrade lignocellulosic biomass that represents an abundant resource for conversion into fermentable sugars, suitable for production of biofuels [30]. The microorganism used in this experiment was *E. coli*, which is a gram negative bacteria. The surface engineering of gram negative bacteria towards the biosorption of heavy metals are reviewed in this study. The gram negative bacteria possess a complex cell envelope that consists of a plasma membrane, a peptidoglycan cell wall, and an outer membrane [31]. Therefore, in order to make cell surface possible, the proteins should cross through the plasma membrane and the outer membrane before the display on the cell surface. Cell surface display, in which target proteins are expressed on the surface of host cells, is a powerful tool for biotechnological and industrial applications, including biocatalysis, bioremediation, and biosensors, and for high-throughput screening of protein libraries [32].

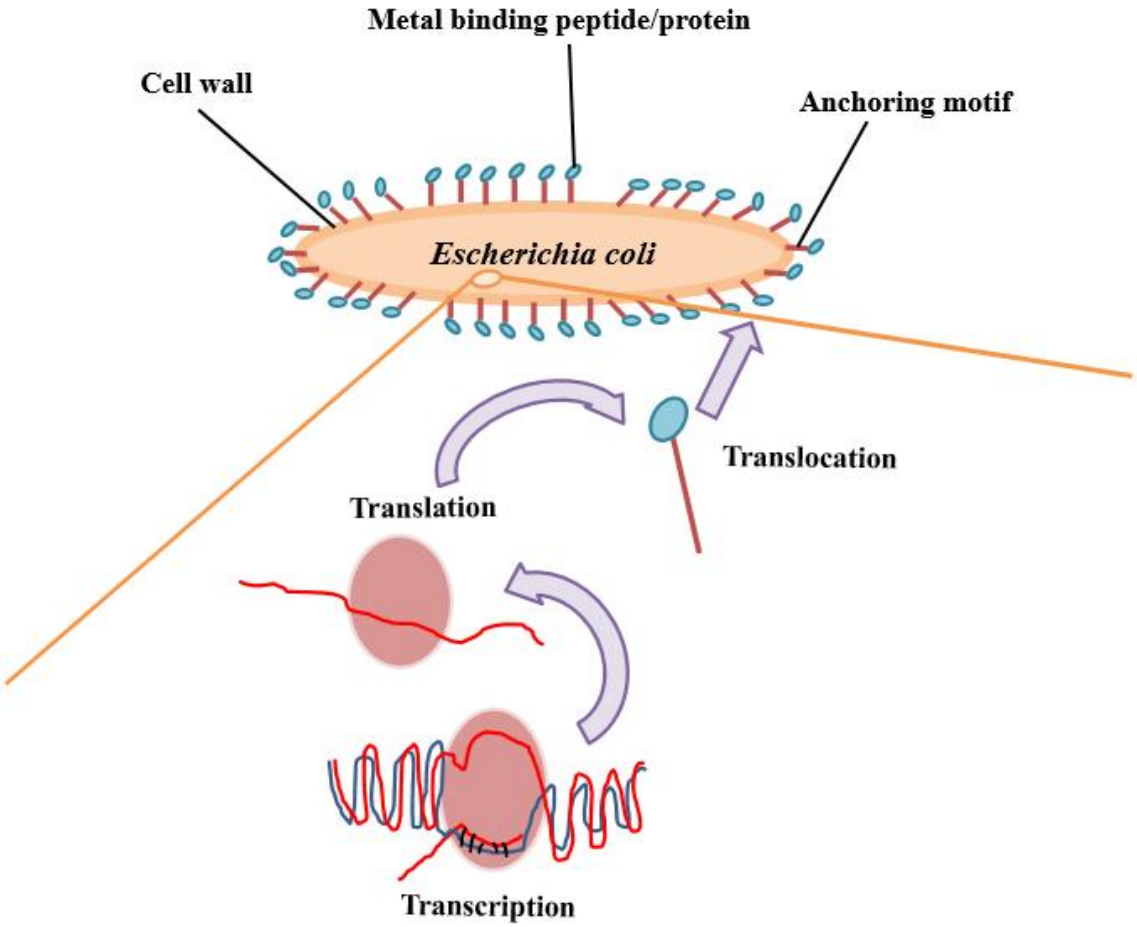

Fig.2. Schematic representation of cell surface display system.

1.3.2. Metal binding peptides

Many metal ions are essential but at higher concentrations they become toxic. Heavy metals are difficult to remove from the environment. And heavy metals are harmful for bacteria and organisms. Today, bacteria and higher organisms have developed resistance mechanisms to heavy metals to make them non-dangerous [20]. The metal binding peptides are selected according to the situation. It is to remove heavy metals by adsorbing it fusion of anchoring motifs and metal binding peptides. Considering the relative affinities of metal binding peptides and the cell wall to metal ion should be taken into account in the rational design of peptide sequences possessing specificity for a particular heavy metals. If well considered, metal binding peptides can offer a high affinity of metal binding capacity, sensitivity, specificity and selectivity for the desired metal ion [33]. These made it imperative for a cost-effective treatment method that is capable of removing heavy metals from aqueous effluents. Thus, microorganisms could be used to clean up to metal contamination by removing metals from contaminated water [34].

1.3.2.1. How to make metal binding peptides

There are three main steps to make the desired metal binding peptides.

(1) Transcription

(2) Translation

(3) Translocation

Transcription is the process by which the information in a strand of DNA is copied into a new molecule of messenger RNA (mRNA). The DNA of a gene serves as a template for complementary base-pairing, and enzyme called RNA polymerase catalyzes the formation of a pre-mRNA molecule, which is then processed to form mature mRNA. The resulting mRNA is a single-stranded copy of the gene, which next must be translated into a protein molecule [35].

Translation is the process in which ribosomes in the cytoplasm or endoplasmic reticulum synthesize proteins after the process of transcription of DNA to RNA in the cell's nucleus [35].

Translocation is a process by which proteins move between cellular compartments. Short amino-acid sequences within a protein, known as signal peptides or signal sequences, can direct its localization, although translocation also occurs in the absence of these signal sequences. Protein translocation can occur co-translationally of post-translationally [36]. Through these three steps, metal binding peptides is made, and heavy metals is adsorbed to the created metal binding peptides.

1.4. Factors affecting biosortion

Biosorption process is affected by following factors:

(1) pH

(2) Temperature

(3) Initial metal ion concentration

(4) Concentration of biomass

These factors could increase of decrease the amount of biosorption

1.4.1. pH

It affects solubility of metal ions and binding sites of biomass. At lower pH, the biosorption of metals is affected [37]. General range of pH for metal uptake is between 2.5–6. Above this limit, metal uptake ability of biosorbent gets compromised [38].

1.4.2. Temperature

For efficient removal of metal ions from environment, the optimum temperature needed to be investigated. It is generally assumed that biosorption is carried out between 20 ℃ and 35 ℃. High temperatures above 45 ℃ may results in damage to proteins which in turn affects metal uptake process [38].

1.4.3. Initial metal ion concentration

The initial concentration provides an important driving force to overcome all mass transfer resistance of metal between the aqueous and solid phases [39]. Increasing amount of metal adsorbed by the biomass will be increased with initial concentration of metals. Optimum percentage of metal removal can be taken at low initial metal concentration. Thus, at a given concentration of biomass, the metal uptake increases with in initial concentration [38].

1.4.4. Concentration of biomass

The concentration of biomass is directly proportional to the metal uptake [40]. It is reported that electrostatic interaction between the cells plays an important role in metal uptake. At a given equilibrium, the biomass adsorbs more metal ions at low cell densities than at high densities [41]. Metal uptake depends on binding sites. More biomass concentration or more metal ions restricts the access of metal ions to binding sites [38].

1.5. Zinc oxide nanoparticles

Nanotechnology deals with the manufacture and application of materials with size of up to 100 nm [42]. In general, nanoparticles used in the field of biotechnology range in particle size between 10 and 500 nm, seldom exceeding 700 nm [43]. Nanosize inorganic compounds, such as silver, gold, copper, titanium oxide, and zinc oxide, have shown remarkable antibacterial activity at very low concentration due to their high surface area to volume ratio and unique chemical and physical features. Zinc oxide (ZnO) nanoparticles (NPs) attract a lot of attention for fundamental studies and potential applications in different research areas: from physical chemistry to biomedical sciences. ZnO NPs represent a versatile functional material, and their superior properties find current and potential applications in catalysts, transducers, semiconductors, microelectronics, textile, cosmetics, water treatment, etc. [42]. Additionally, ZnO NPs have a broad spectrum of antibacterial activities against microorganisms including *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and the M13 bacteriophage. ZnO NPs have been studied as antibiotics to enhance antimicrobial activity against pathogenic bacteria and viruses with or without antibiotic resistance [44].

1.5.1. Antibiotics mechanism of ZnO NPs

ZnO exhibits significant antimicrobial activities when particle size is reduced to the nanometer range, then ZnO NPs can inhibit the growth of microorganisms by permeating into the cell membrane [42]. Under ultraviolet/visible light, the surface of ZnO NPs to generate Reactive Oxygen Species (ROS) with extreme chemical activity. The oxidative stress damages carbohydrates, proteins, lipids, and DNA [45]. Another mechanism that can kill bacteria is the release of Zn^{2+} ions. When ZnO NPs are in solution, partial dissolution results in the release of Zn^{2+} ions, which have antimicrobial activity. Therefore, the dissolution of ZnO NPs contributes to its antimicrobial activity by decreasing amino acid metabolism and perturbing the enzymatic system [46]. The other mechanism that can kill bacteria is ZnO NPs direct contact with cell membrane. This reaction may distort the membrane plasma structure and damage the bacterial cell integrity, resulting in the leakage of intracellular contents and ends with cell death [45]. The interactions between these unique materials and bacteria are mostly toxic, which have been exploited for antimicrobial applications such as in food industry [47].

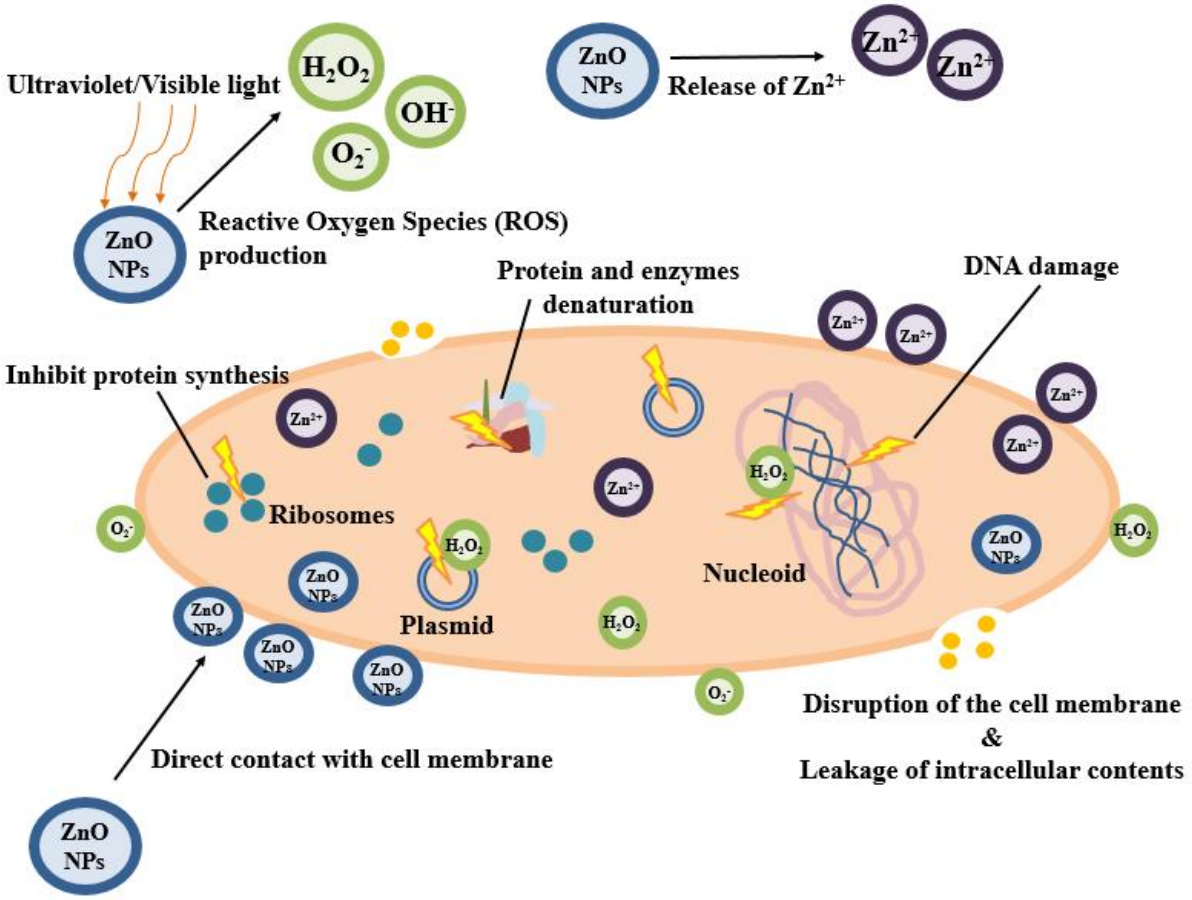

Fig.3 Schematic illustration of antibiotics mechanism of ZnO NPs.

1.5.2. ZnO NPs for applications

ZnO NPs are very much important due to their utilization in gas sensor, biosensors, cosmetics, drug-delivery systems, and so forth [48]. ZnO is a bio-safe material that possesses photo-oxidizing and photocatalysis impacts on chemical and biological species [47].

1.5.3. Synthesis of ZnO NPs

Two different methods were used for synthesizing ZnO NPs. In synthesis I (chemical method), zinc nitrate was dissolved in distilled water under constant stirring. While at room temperature, sodium hydroxide solution was added drop by drop. After completion of reaction, the solution was allowed to settle for overnight and the supernatant liquid was discarded. The white precipitate formed was washed thoroughly with double distilled water to remove all the ions and then centrifuged. The obtained precipitate was dried in a hot air oven. During drying, complete conversion of $Zn(OH)_2$ into ZnO took place. In synthesis II (biological method), a typical procedure was employed, where biosorbent broth were prepared with distilled water. Later, zinc nitrate was dissolved in the broth solution under constant stirring. After complete dissolution of the mixture, the solution was kept under vigorous stirring, allowed to cool at room temperature and the supernatant

was discarded. The pale white solid product was obtained by centrifugation [49]. In this study, a biological method was selected to make ZnO NPs, and the biomass used was the engineered *E. coli*.

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 OmpC *zrap* 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. Cell preparation

The transformed *E. coli* displaying the *zrap* 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 600 nm (OD600) reached 0.5-0.8. After 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) 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* cell pellets were collected in each amount with agitation for 10 minutes in the centrifuge at 10,000 rpm, 4 ℃.

2.3. Zinc oxide nanoparticles preparation

E. coli cell pellets from 100 mL of cultivated broth were collected in a 50 mL conical tube by centrifugation at 10,000 rpm and 4 ℃. *E. coli* cell pellets were washed with autoclaved double-distilled water. After the washing, *E. coli* cell pellets were re-suspended in 10 mL autoclaved double-distilled water, followed by dropwise addition of 1 mL zinc nitrate solution (Sigma-Aldrich). And then, the mixture was incubated at 30 ℃ with gentle shaking for 12 – 24 hours. After the metal adsorption, the mixture was transferred into water bath at 80 ℃ for 24 hours to allow mineralization. The products were isolated by agitation at 4 ℃ in the centrifuge at 10,000 rpm and washed with autoclaved double-distilled water. After that, the products were dried in a freeze-drying machine FDU-2200(EYELA). Finally, the products were annealed in a muffle furnace at 500 ℃ for 2 hours at a heating rate of 5 ℃ min^{-1} in air [50].

2.4. Zinc recovery and analysis

Samples were incubated in a solution containing zinc nitrate for 30 minutes in 30 ℃ with gentle shaking. After the adsorption, the recombinant strains were washed twice with 0.85 % (w/v) NaCl to remove physically adsorbed zinc. The Adsorbed zinc by the peptides was eluted by incubating with 5 mM EDTA for 30 minutes in ice. Samples were quantitatively measured with 10-fold dilution with water by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Agilent technologies 5110).

2.5. Affecting factors of zinc uptake in biosorption

2.5.1. Effect of pH in biosorption

Incubation was differed by broad range of pH (pH 1-11) to investigate effect of pH in biosorption on the zinc recovery. After the biosorption of zinc, the bacterial cell pellets were washed twice with 0.85 % (w/v) NaCl. Samples were quantitatively measured with 20-fold dilution with water by ICP-OES.

2.5.2. Effect of temperature in biosorption

Incubation was differed by assorted temperature (20-35 ℃) to analyze effect of temperature in biosorption on the zinc recovery. After the biosorption of zinc, the *E. coli* cell pellets were washed twice with 0.85 % (w/v) NaCl. Samples were quantitatively measured with 20-fold dilution with water by ICP-OES. Finally, the zinc uptake, q (mg zinc ion/g DCW), was determined as follows:

$$q = \frac{(C_0 - C_t)V}{m} \quad \text{(Eq. 1)}$$

where C_0 and C_t are the initial and final zinc ion concentrations (in mg/L), respectively, V is the volume of the solution (in mL), and m is the dry cell weight of *E. coli* (in g DCW). The pseudo-first-order and pseudo-second-order models were used to describe the kinetic behavior of the biosorption of Zn (II) by the recombinant *E. coli*.

2.5.3. Isotherms at different temperatures

The biosorption experiments were carried out at different temperatures: 20 ℃, 25 ℃, 30 ℃, and 35 ℃. Langmuir and Freundlich models were used to obtain the equilibrium parameters, such as the maximum adsorption capacity of the recombinant *E. coli* for Zn (II) under the experimental conditions of this work.

Table 1. List of bacterial strains, peptides, and plasmids used in this study [51].

Strain/Plasmid	Relevant genotype/ property	Source
***E. coli* strains**		
BL21(DE3)	*F– dcm ompT hsdS (rB- mB-) gal λ(DE3)*	Enzynomics
Plasmids		
pUC19	Amp^R	NEB[a]
pZZ1056	pUC19(237 bp *zraP*-hydH intergenic region), *zraP'* - *ompC*$_t$ transcriptional fusion vector, containing *zraP* promoter region, Amp^r	This work

Table 2. Primer used in this study [51].

Name	Sequence
*zraP*_FBamH	5'-GGATCCGGTTAATCCTCCAGTGGTTGTC-3'
*zraP*_RSaI	5'-GTCGACCTTCTTCTTTGCCTGCTCATCCC-3'

3. Results and discussion

3.1. Zinc biosorption by engineered bacteria

The results presented that the genetically engineered bacterial cells show a dynamic response on exposure to an exogenous Zn^{2+} concentration. In order to evaluate metal biosorption by this engineered strain, the strain was grown in LB medium in the presence of concentrations of 1.0, 2.0, 3.0 and 4.0 mM $Zn(NO_3)_2$.

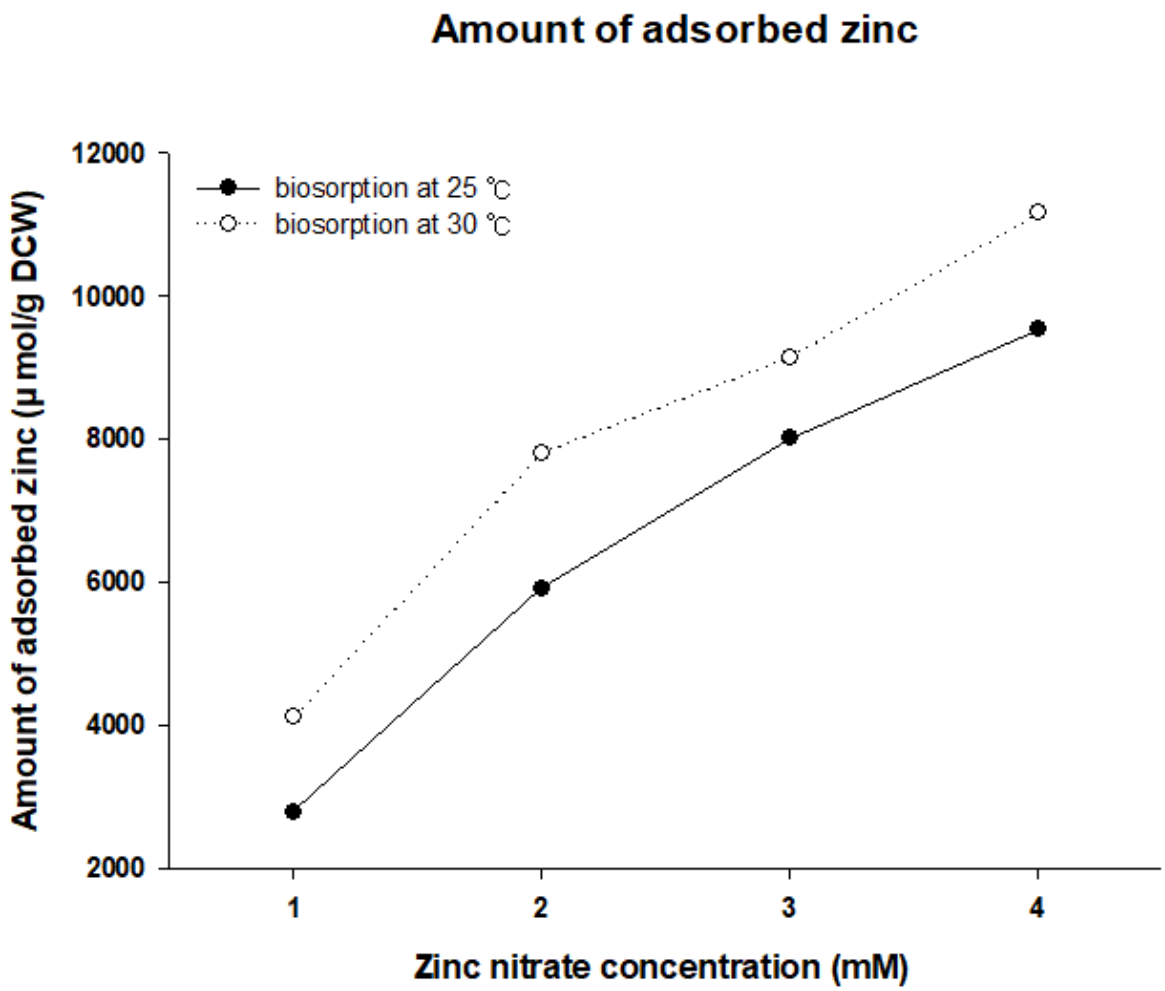

Fig.4 Graph of amount of adsorbed zinc.

The recombinant *E. coli* adsorbed 2784.52, 5904.83, 8010.46, 9532.52 μmol per gram DCW when zinc nitrate solutions were added 1, 2, 3, 4 mM at 25 ℃, respectively. And the recombinant *E. coli* adsorbed 4115.20, 7806.14, 9142.15, 11156.40 μmol per gram DCW (Dry Cell Weight of *E. coli*) when zinc nitrate solutions were added 1, 2, 3, 4 mM at 30 ℃.

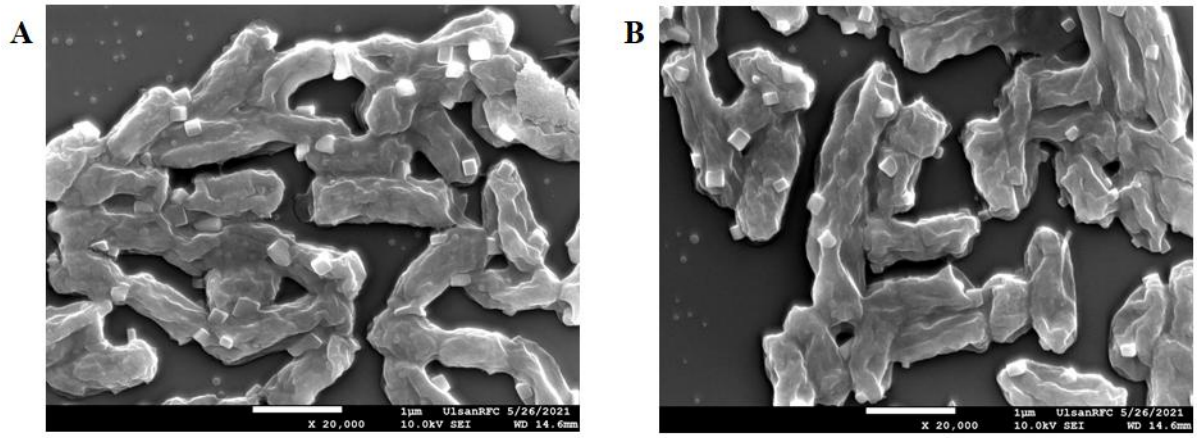

Fig.5. FE-SEM images recombinant *E. coli* with (A) 1 mM of zinc nitrate, (B) 5 mM of zinc nitrate.

In Fig.4., it can be intuitively seen that the recombinant *E. coli* adsorbed zinc

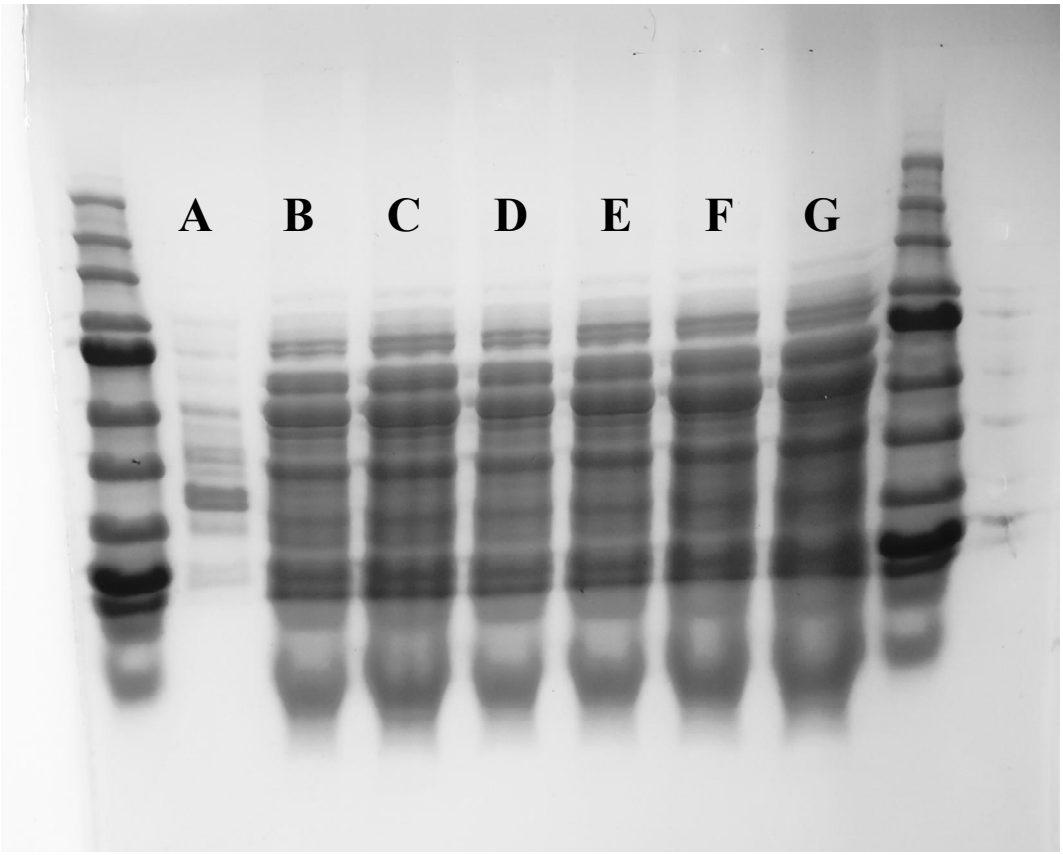

Fig.6. SDS-PAGE images with IPTG induction of different concentration of BL21, PZZ1056 (A) BL21, (B) 0.01 mM, (C) 0.05 mM, (D) 0.1 mM, (E) 0.5 mM, (F) 1 mM of PZZ1056.

In Fig.5., it can be observed that the band becomes darker as the IPTG concentration increases. The band is strongest at 1 mM, indicating that the protein expression is best when the IPTG concentration is 1 mM.

3.2. Zinc ( ) uptake

3.2.1. Adsorption pH effect on the amount of adsorbed zinc

The recombinant *E. coli* was induced with 1 mM of IPTG for 5 hours and collected by centrifuge for 10 minutes at 4 ℃, 10,000 rpm. Pellets were resuspended with 10 mL of autoclaved double-distilled water. The mixtures were exposed in wide range of pH for 25, 30 ℃ in shaking incubator, 30 minutes. The residual zinc was analyzed by ICP-OES as described below. The recombinant *E. coli* was showed that pH affects biosorption (Fig.6.).

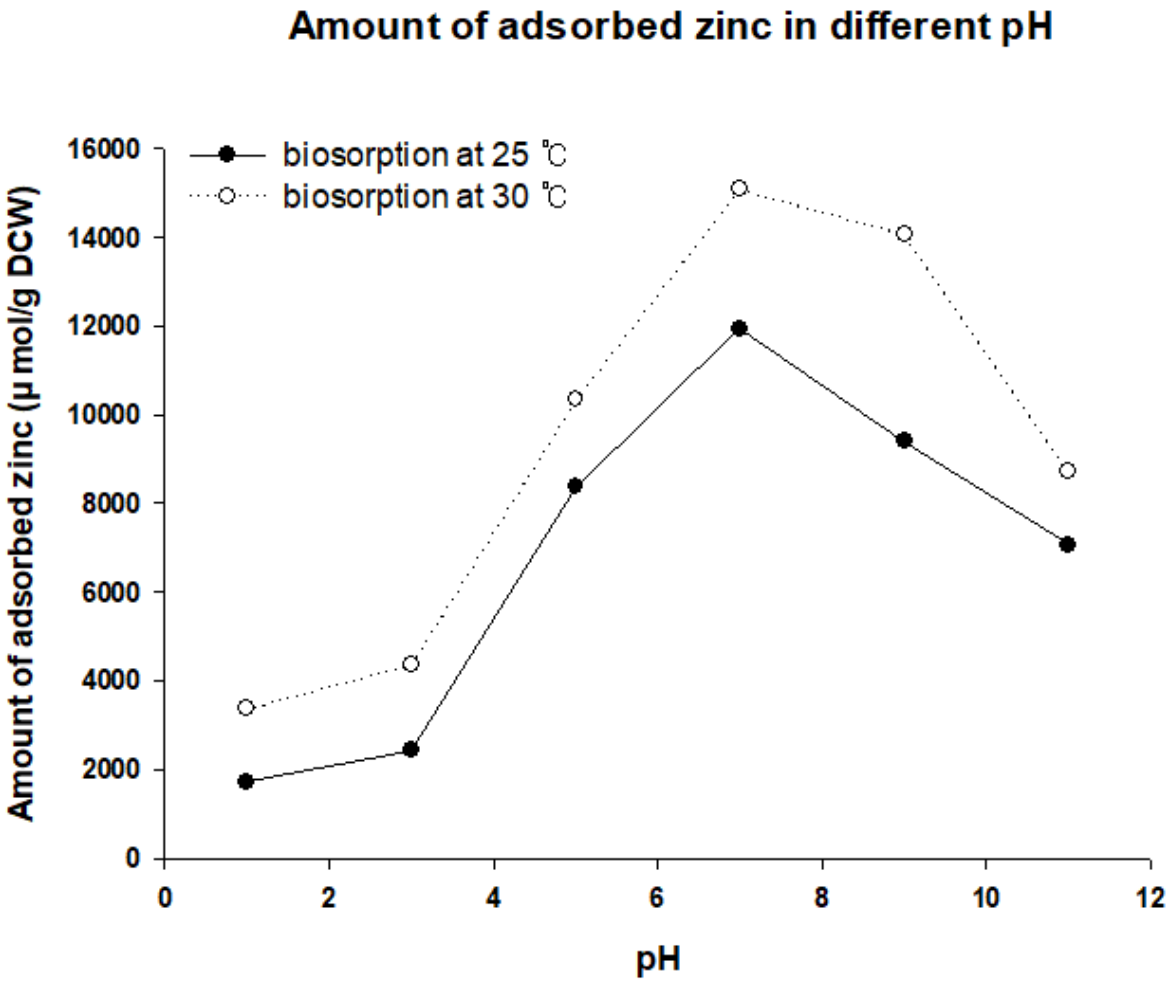

Fig.7. Effect of adsorption pH on the biosorption of zinc in 100 mL of cultivated cell amount.

At pH 1, almost no biosorption occurred, and the amount of adsorbed zinc increased as the pH increased. The adsorbed zinc amount was highest at pH 7, and the amount of adsorbed zinc decreased at a higher than pH 7. According to several authors, it is expected that the biosorption of metals decreases at low pH values because of competition for binding sites between cations and the products of acid hydrolysis [52]. Protons and oxonium ions (H_3O^+) concentrations are relatively high at low pH and compete with metals for ion exchange [53]. Therefore, as in this experiment, it

seems that the biosorption is hardly performed of the efficiency is lowered at high or low pH, which is not neutral (pH = 7). Results of the process occurred in this work, with the maximum biosorption of zinc (86 %) at the neutral pH of 7.

3.2.2. Adsorption temperature effect on the amount of adsorbed zinc

The recombinant *E. coli* cell pellets were collected for 100 mL volume of cultivated broth with centrifuge for 10 minutes, at 4 ℃, 10,000 rpm. Autoclaved double-distilled water was added to make a 10 mL volume of sample. The pH of the solution was initially adjusted to 7. Samples were exposed to 20-35 ℃ of various temperatures with the supplement of zinc nitrate solution. The zinc ion concentration in the remaining solutions was measured with ICP-OES. The effect of adsorption temperature was analyzed by differing adsorption temperature (Fig.7.).

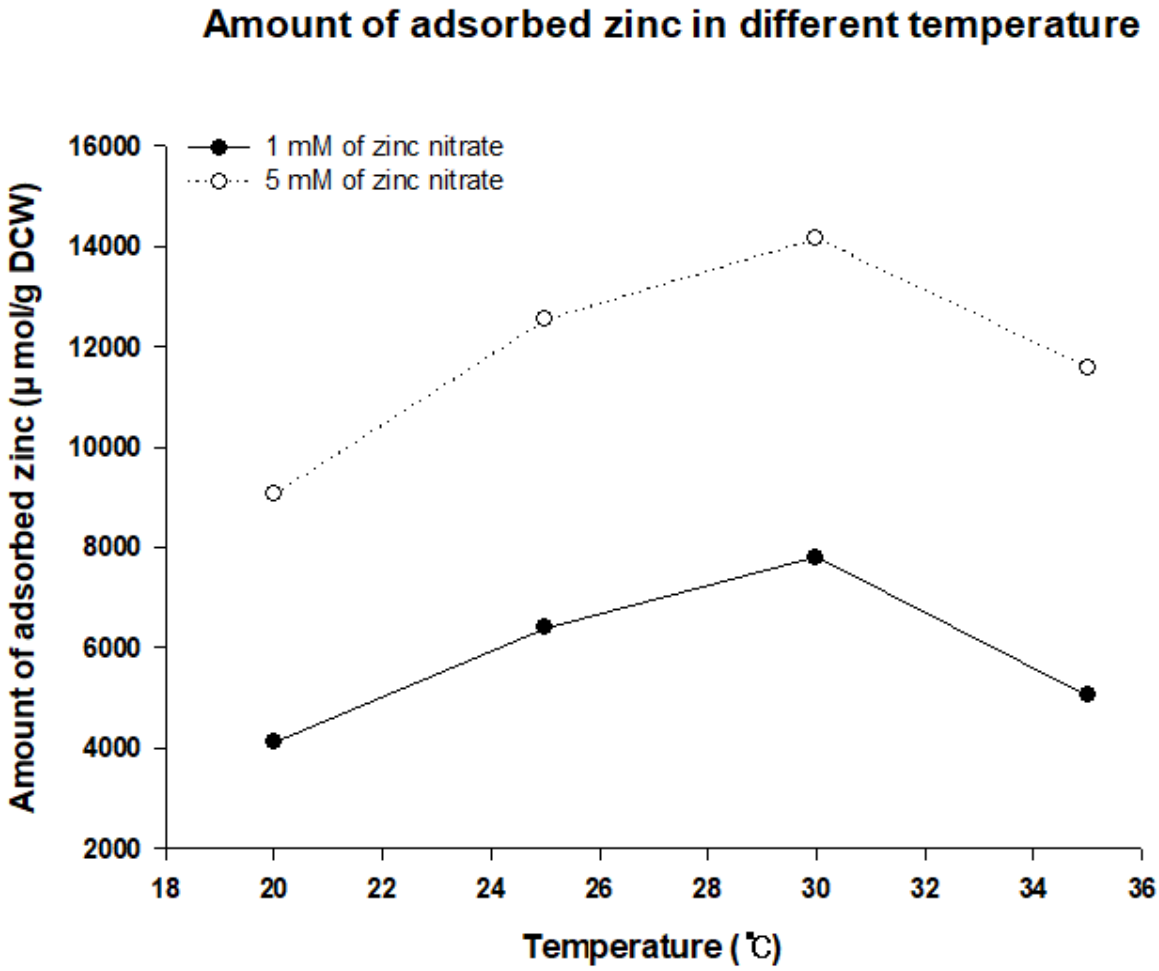

Fig.8. Effect of adsorption temperature on the biosorption rate of zinc in 100 mL of cultivated cell amount.

The result was showed that temperature affects biosorption. The adsorbed zinc amount showed a tendency to increase from 20 ℃ to 30 ℃, but to decrease as the temperature exceeded 30 ℃. It can therefore be concluded that along with the increase in temperature (from 20 to 30 ℃), the biosorption grows and remains positive on the temperature augmentation. However, with the further increase in temperature producing a negative temperature effect on the biosorption. The two most

frequently isotherm equations used in the literature to reach that objective are the Langmuir and Freundlich models [54]. The biosorption data from different initial metal concentrations were analyzed in terms of both Langmuir and Freundlich equations at pH 7 and a variable temperature of 20 ℃, 25 ℃, 30 ℃, and 35 ℃, and the values obtained for the respective constants are shown in Table.3. The biosorption experimental data were fit with Langmuir and Freundlich isotherm models to explain the behavior of the Zn (II) adsorption by the engineered *E. coli*. The Langmuir isotherm model assumes a monolayer adsorption onto a surface containing a finite number of identical sites, and the Freundlich isotherm is used for modeling the adsorption on heterogeneous surfaces [55]. For the Langmuir model, the following equation was used:

$$q_e = \frac{K_L q_{max} C_e}{1+K_L C_e} \quad \text{(Eq. 2)}$$

where,

q_e Concentration of metal ion adsorbed at equilibrium, (in mg/g DCW).

C_e Equilibrium solution concentration of metal ion (in mg/L).

q_{max} Maximum adsorption capacity of adsorbent (in mg/g DCW).

K_L Langmuir constant (in mg/L)

Freundlich isotherm constants were calculated with the equation:

$$q_e = K_F C_e^{1/n} \quad \text{(Eq. 3)}$$

q_e Concentration of metal ion adsorbed at equilibrium, (in mg/g DCW).

C_e Equilibrium solution concentration of metal ion (in mg/L).

K_F Freundlich constant related to adsorption capacity.

$1/n$ Freundlich heterogeneity coefficient.

Table 3. Parameters from the Langmuir and Freundlich isotherm models applied to the biosorption of Zn (II) by zinc nitrate of *E. coli* at different temperatures (pH = 7).

Zn ( )						
Temperature (℃)	Langmuir			Freundlich		
	q_{max} (mg/g DCW)	K_L (mg/L)	R^2	K_F (L/g DCW)	$1/n$	R^2
20	94.96	0.0002236	0.7444	0.07238	0.79	0.9980
25	95.72	0.0002501	0.7116	0.04757	0.88	0.9979
30	103.28	0.0002452	0.6763	0.02641	0.99	0.9998
35	103.69	0.0002136	0.8093	0.05014	0.86	0.9915

Freundlich isotherm equation (R^2 = 0.9915-0.9998) seemed to describe better the biosorption process of Zn (II) by the recombinant *E. coli* than the Langmuir equation (R^2 = 0.6763-0.8093) for the tested temperatures. (Table 3.) In all cases, the experimental data were better fit by the Freundlich isotherm model. The Langmuir constant (K_L) decreased with increasing temperature except for 20 ℃. The Freundlich constant (K_F) decreased with increasing temperature except for 35 ℃. The temperature of the biosorption medium could be important for energy dependent mechanisms in metal binding peptides process [52]. In this work, an increase of temperature (except for 30 ℃ to 35 ℃) resulted in a higher maximum biosorption of zinc, with the highest one at 30 ℃. For Zn (II), the Freundlich $1/n$ values varied from 0.79 to 0.99, almost increasing when the temperature was increased. According to Acar and Malkoc [56], the adsorption of an adsorbate on the anion exchanger is favorable when $0.1 < 1/n < 1.0$. Our findings indicate that the biomass of the recombinant *E. coli* can be used effectively for the removal of zinc from aqueous solutions.

3.3. Biosynthesis of zinc oxide nanoparticles

The recombinant *E. coli* cell pellets from cultivated broth were collected by centrifugation. Cell pellets were washed with autoclaved double-distilled water. After the washing, Cell pellets were re-suspended in autoclaved double-distilled water, followed by dropwise addition of zinc nitrate solution. The mixture was incubated at 30 ℃ with gentle shaking for overnight. After the metal adsorption, the mixture was transferred into water bath at 80 ℃ for 24 hours to allow mineralization. The products were isolated by centrifugation and washed with autoclaved double-distilled water. And then, the products were dried in a lyophilizer. After that, the powder type sample were collected and the products were annealed in a muffle furnace. Finally, the biosynthesized ZnO NPs were obtained (Fig.9.).

Fig.9. Zinc oxide nanoparticle synthesized after calcination.

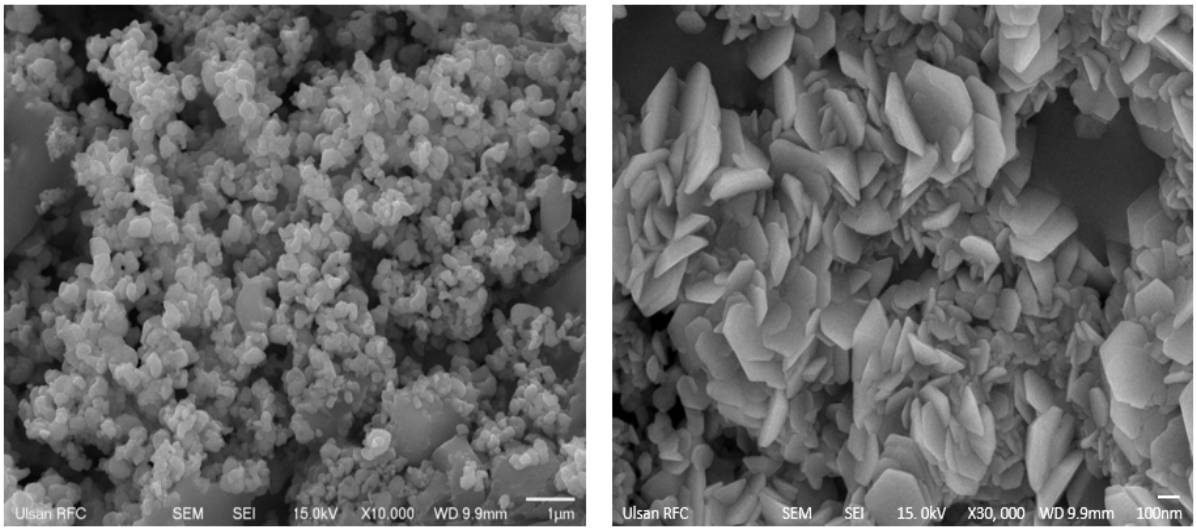

Fig.10. FE-SEM images of biosynthesis ZnO NPs.

Fig.10. is the FE-SEM images of ZnO NPs. Several different structures of ZnO NPs created in this experiment, such as hexagonal round granules, etc., are shown.

For comparison, the structure and size of the experimentally made ZnO NPs and that of other biosynthesis were compared (Fig.11.) [57, 58].

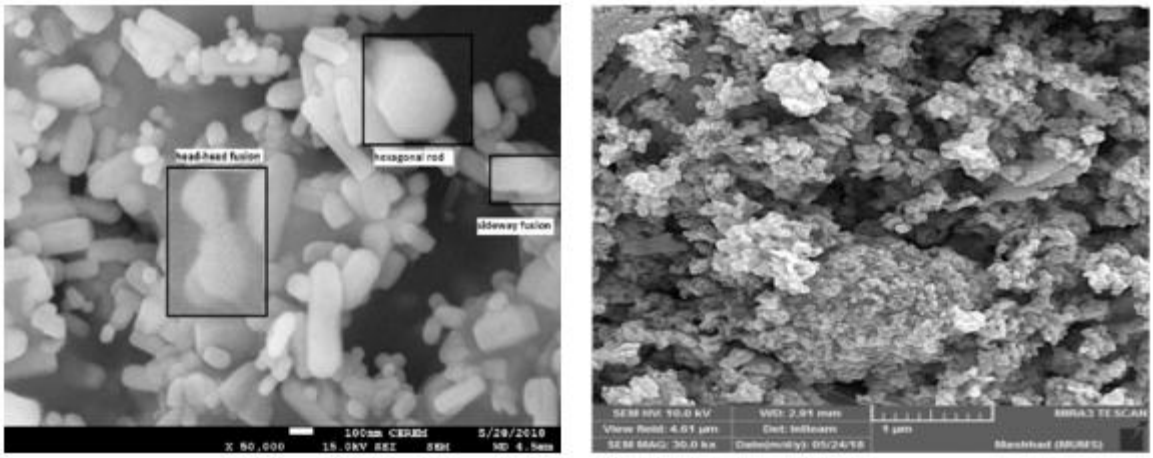

Fig.11. FE-SEM images of other biosynthesis ZnO NPs.

In ZnO NPs produced from other biosynthesis, head-head fusion, hexagonal rod, sideway fusion, or small round shapes are seen. The ZnO NPs created in this work also appears to have a hexagonal rod or small round shape and is somewhat similar in size. So, it can be considered that ZnO NPs has been made.

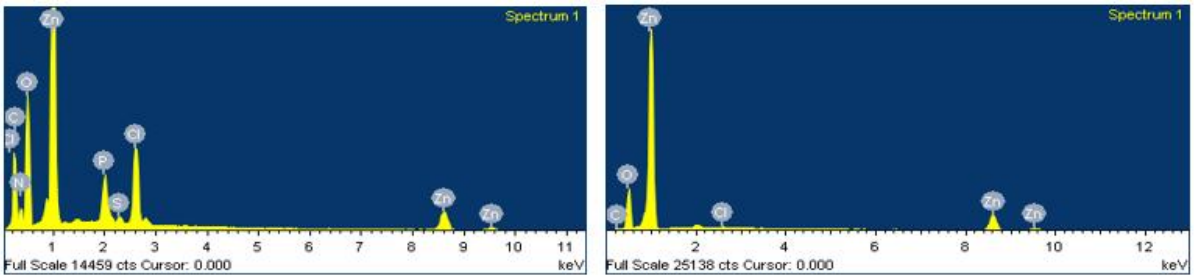

Fig.12. EDS images of biosynthesis ZnO NPs.

Fig.12. is the analysis of ZnO NPs made in this work with EDS (Energy Dispersive X-ray Spectroscopy). In this EDS analysis, zinc and oxygen were observed in a similar ratio, indicating that ZnO NPs were formed. In addition to zinc and oxygen, carbon, nitrogen, sulfate, and others are also observed, which is thought to be observed because *E. coli* burn less. So, if we want to obtain a higher purity ZnO NPs in this experiment, we will have to react at a temperature above the *E. coli* burn enough.

3.3.1. Effect of synthesis temperature in calcination

The calcination temperature affects ZnO NPs synthesis. If the calcination temperature is above 500 ℃, the color of the generated sample is white. And when the calcination temperature is below 300 ℃, the generated sample is black or brown. Generally, the color of ZnO NPs is known as white.

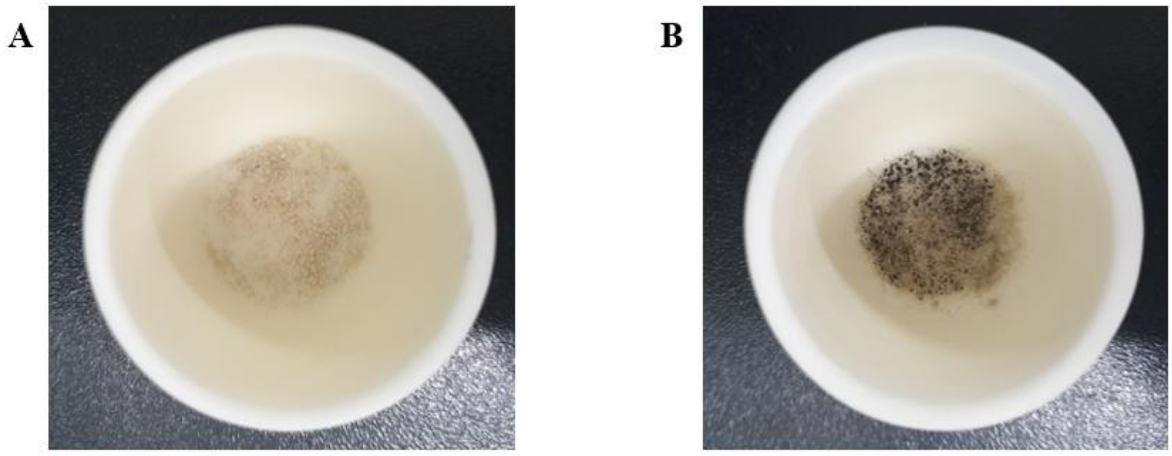

Fig.13. (A) Samples (white color) with a calcination temperature at 500 ℃, (B) Samples (black/brown color) with a calcination temperature at 200 ℃.

Although the color is not white, it may be ZnO NPs, so samples were analyzed by FE-SEM (Fig. 14).

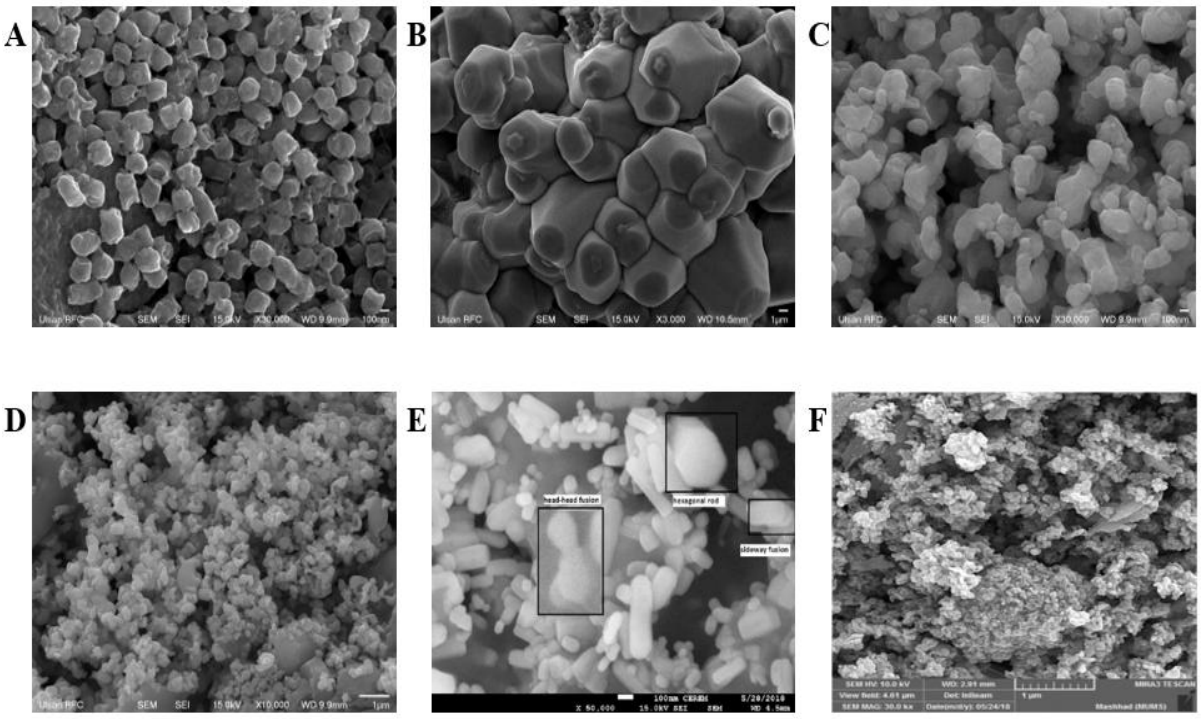

Fig.14. FE-SEM images of effect of temperature (A) 200 ℃, (B) 350 ℃, (C) 450 ℃, (D) 500 ℃ in

calcination, and (E), (F) is to compare the ZnO NPs made in other study.

When the calcination temperature is 200 ℃, it can be seen that *E. coli* remains. It was observed that some crystals were formed when the calcination temperature exceeded 300 ℃. As the calcination temperature increased, the size of the nanoparticles also increased. Fig.14. (E), (F) are control images to compare the ZnO NPs made through this experiment [57, 58]. Fig.14. (E), the biosynthesized ZnO NPs structure exhibits head-head fusion, hexagonal rod, and sideway fusion. The structure of the ZnO NPs produced in this experiment was mainly observed as a head-head fusion, hexagonal rod structure in Fig.14. (E), or a structure similar to the image in Fig.14. (F). And even at the same temperature, there were cases where other structures were present together as in Fig.14. (E). (Fig.15.)

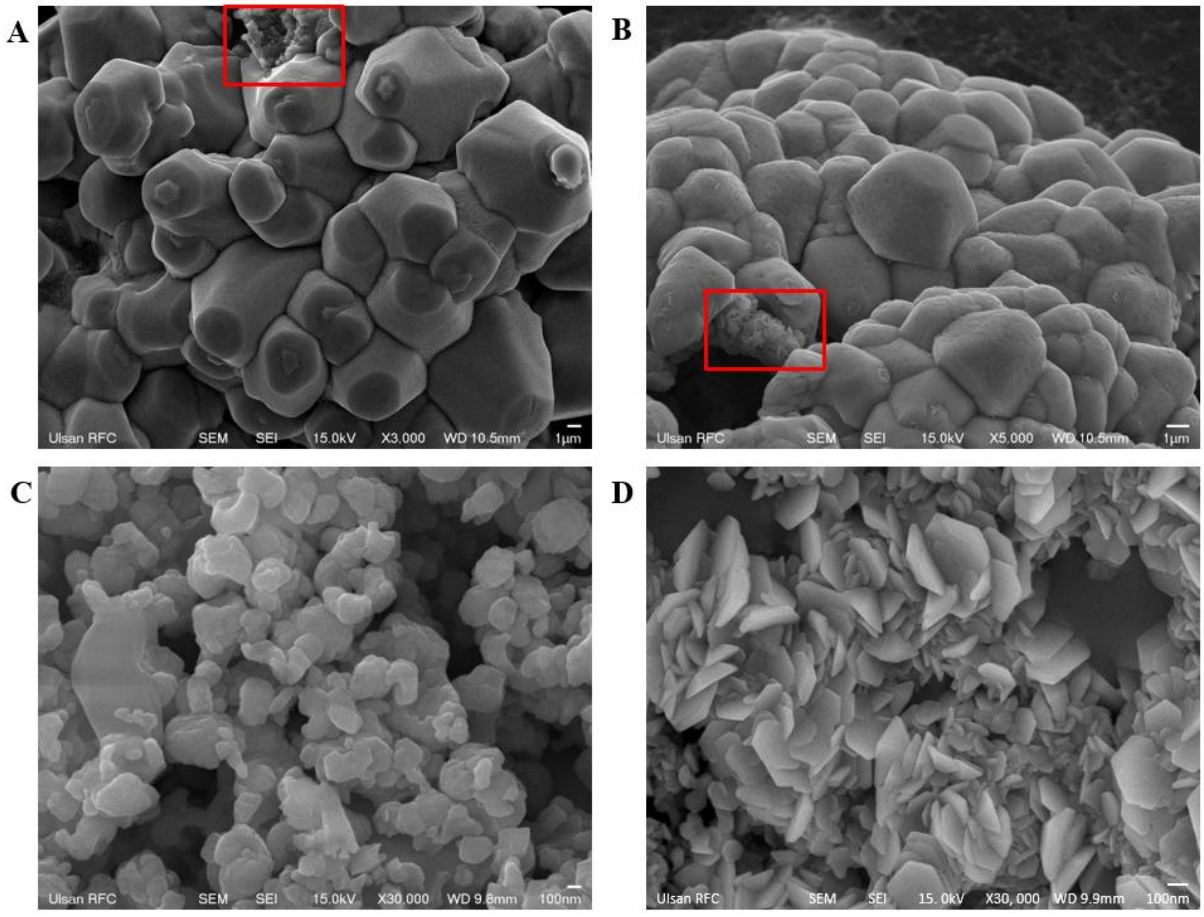

Fig.15. FE-SEM images of different structures at the same temperature (A) 350 ℃, (B) 350 ℃, (C) is an enlarged image of (A)'s red box (D) is an enlarged image of (B)'s red box.

Even below 500 ℃, it is not impossible to observe the ZnO NPs structure. Hexagonal rod and other structures could be observed.

3.4. Antibiotics of zinc oxide nanoparticles

To confirm the antibiotics properties of ZnO NPs, the synthesized ZnO NPs was added to the 100 mL of cultivated broth and the optical density was observed every hour.

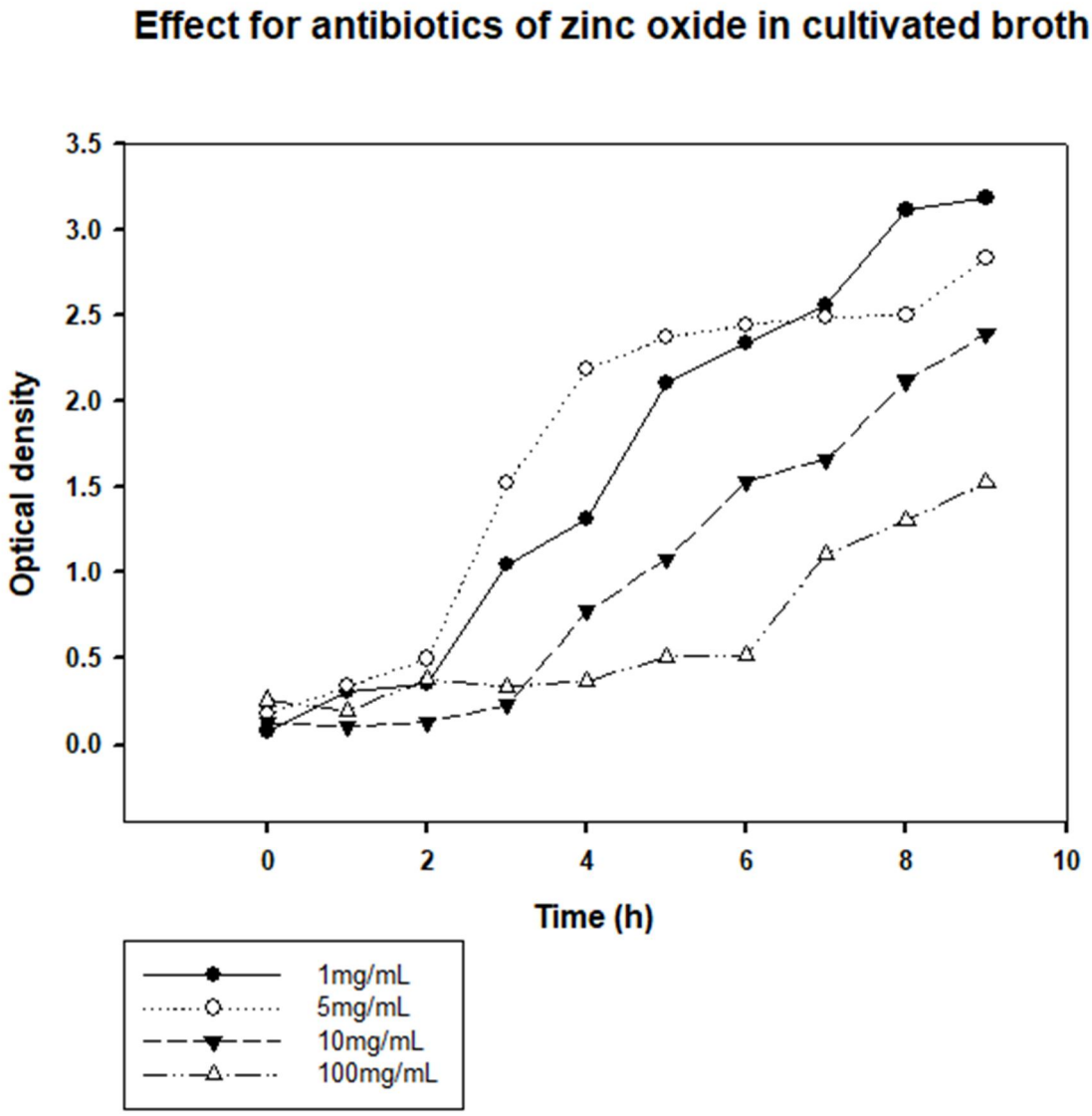

Fig.16. Effect for antibiotics of zinc oxide nanoparticles with ZnO NPs in cultivated broth.

When the cultivated broth was added up to 5 mg/mL of ZnO NPs, the optical density didn't decrease significantly, but from 10 mg/mL of ZnO NPs, the optical density decreased considerably. Optical density didn't exceed 2.0 when 100 mg/mL or more was administered. And the antibiotic properties of ZnO NPs were also confirmed using the plate.

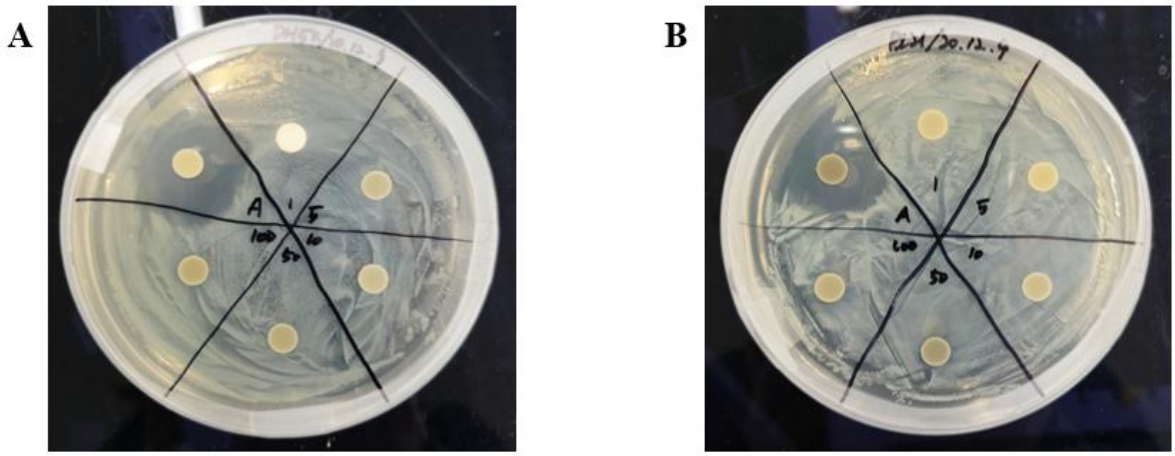

Fig.17. Antibiotics of ZnO NPs in plate using wild type of *E. coli* (A) DH5α, (B) BL21.

The ZnO NPs antibiotics properties were confirmed by dividing the area in the plate. A solution was made with the synthesized ZnO NPs, and 1, 5, 10, 50, 100, 2000 μL each was dropped on the filter and cultured in 37 ℃ stationary incubator to see if *E. coli* grew. When O/N cultured plate was observed, a circle without *E. coli* was formed in the area dropped by 2000 μL on the filter.

3.5. Compare the methods of zinc desorption

E. coli cell pellets from 100 mL of cultivated broth were collected in a 50 mL conical tube by centrifugation at 10,000 rpm and 4 ℃. *E. coli* cell pellets were washed with autoclaved double-distilled water. After the washing, *E. coli* cell pellets were re-suspended in 10 mL autoclaved double-distilled water, addition of zinc nitrate solution. And then, the mixture was incubated at 30 ℃ with gentle shaking. After the adsorption, different types of desorption were carried out to determine how removable they could be.

Table 4. The desorption rate between different desorption methods.

Desorption method	Amount of zinc absorbed (μmol/g DCW)	Amount of zinc after the desorption (μmol/g DCW)	Amount of zinc desorbed (μmol/g DCW)	Desorption rate of zinc (%)
30 ℃ with movement	6784.005	6162.395	621.61	9.16
80 ℃ with movement	6997.54	3876.14	3121.4	44.61
80 ℃ without movement	6842.78	4975.59	1867.19	27.29
EDTA	6555.89	819.75	5736.14	87.49

The desorption method used after zinc was administered and adsorbed to the strain into which OmpC *zraP* was introduced was divided into physical and chemical methods. The physical desorption method started with the idea that the recombinant bacterial strain used in the experiment could be reused if it could be physically desorbed during the mineralization process at 80 ℃. In general, EDTA, which is a chemical method, is used to desorb metal adsorbed on microorganisms. EDTA desorbs metals ion by using the property of EDTA to substitute instead of attached metal ions. EDTA substituted for metal ions is difficult to drop, so there is a problem that *E. coli* used for biosorption can't be reused. In this study, it was confirmed whether metal desorption is possible with a physical method. If the metal ions can be dropped by a physical method, the used *E. coli* could be reused. When comparing the amount of metal desorbed by physical method and metal desorbed by chemical method, the efficiency of the physical method was not low even though the efficiency of physical method was inferior to that of the chemical method.

4. Conclusion

In this study, the process of adsorbing zinc to the recombinant *E. coli* and changes in pH and temperature during biosorption conditions were confirmed to have an effect on the biosorption amount. And the process of biosynthesis of zinc adsorbed to the engineered strain to form zinc oxide nanoparticles (ZnO NPs) was performed. The reason for adsorbing zinc to the recombinant bacterial strain is not only to make ZnO NPs but also to use it to remove zinc dissolved in wastewater. Some of metals such as zinc, cobalt, nickel, platinum are essential nutrients which are required for various biochemical and physiological functions but in large doses they may cause acute or chronic toxicities. Thus it is necessary to treat metal-contaminated wastewater prior to its discharge to the environment. In our laboratory use the recombinant *E. coli* to remove these heavy metals. In this experiment, in particular, zinc was adsorbed to the engineered bacterial strain using the cell display system in biosorption to find a way to utilize it. And then biosorption process is affected by some factors, such as pH, temperature, initial metal ion concentration, biomass concentration, etc.. In this work, it was confirmed how much pH or temperature affects biosorption. It seems that the biosorption is hardly performed of the efficiency is lowered at high or low pH, which is not neutral (pH = 7). The temperature of cultivated medium affects the biosorption process of the engineered *E. coli*. It was confirmed by the Langmuir and Freundlich isotherm models that this process was affected by temperature. Freundlich isotherm equation seemed to describe better the biosorption process of zinc by the recombinant *E. coli* than the Langmuir equation for the tested temperatures. This recombinant bacterial strain effectively adsorbs the zinc from aqueous solutions. This work was performed to confirm that ZnO NPs generated by zinc adsorption with the recombinant *E. coli* play a role as antibiotics. It was confirmed that the growth rate of *E. coli* decreased as the amount of ZnO NPs increased in the medium. Moreover, the size and structure of the ZnO NPs were changed according to the calcination temperature. In addition, check the various methods of metal recovery after metal adsorption. Desorption methods used after zinc was adsorbed to the *E. coli* was introduced was divided into physical and chemical methods. In general, a chemical method, especially EDTA, is used to desorb metal adsorbed on microorganisms. EDTA substituted for metal ions is difficult to drop, so there is a problem that *E. coli* used for biosorption can't be reused. In this study, it was confirmed whether metal desorption is possible with a physical method. When comparing the amount of metal desorbed by physical method and chemical method, the efficiency of the physical method was not low even though the efficiency of physical method was inferior to that of the chemical method. However, since it does not show high efficiency, it is necessary to find better conditions by changing the

reaction temperature, time or something else. This experiment was carried out in a solution containing only zinc. But, in the future, it would be good to conduct the experiment to confirm the trend of the recombinant *E. coli* when incubated with other heavy metals. And in order to find out the properties of ZnO NPs antibiotics, there will be a way to use it for other microorganisms instead of *E. coli*.

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