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Using Recombinant *E. coli* Displaying Surface Heavy Metal Binding Proteins for Removal of Pb^{2+} from Contaminated Water

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Abstract

Water pollution remains a serious problem with economic and public health concerns worldwide. Lead (Pb^{2+}) is one of the dangerous metals related to chronic diseases and is responsible for many deaths around the world. Despite the advances in technologies for removal of heavy metals e.g., Pb^{2+} from water, all current techniques have shown some limitations that obstructed their application. Bearing in mind that there is a need to develop a novel technique for removal of heavy metals from water, we developed a quick, specific and efficient method for removal of Pb^{2+} from water using dead cells of recombinant *Escherichia coli*. Recombinant *E. coli* were engineered to display metallothionein (SmtB) and lead binding protein (PbrR) onto outer membrane. DNA fragments encoding these proteins were fused to DNA fragment encoding β- domain of antigen 43 (Ag43) for translocation of both heavy metal binding proteins. The resultant recombinant *E. coli* exhibited a capability to adsorb Pb^{2+} successfully from water samples containing 100 mg/L of Pb^{2+}, and concentrations of Pb^{2+} reached to undetectable level after 18 hours. Heat-inactivated *E. coli* displaying PbrR and SmtB on outer membrane showed comparable removal efficiencies to live *E. coli* cells. These observations suggest that our method can be used as a promising, specific and efficient approach for removal of Pb^{2+} from contaminated water.

Keywords: Heavy metal adsorption; Metallothionein; Lead binding protein; Ag43 display system

Introduction

Lead (Pb^{2+}) is an environmental pollutant that exists naturally and contaminates air, food and water. Excessive amounts of Pb^{2+} are released into the environment due to car exhausts, fuel burning and direct release from factories [1]. Lead can find its way into water sources via water pipes or discharging of Pb^{2+} contaminated wastes into rivers or lakes [2-4]. Humans may be exposed to Pb^{2+} via inhalation of polluted air or ingestion of contaminated food and/or water. World Health Organization (WHO) recognizes Pb^{2+} as one of the top-10 toxic chemicals causing major public health problems around the world [5]. In fact, the Institute for Health Metrics and Evaluation estimated approximately 853,000 deaths annually due to Pb^{2+} poisoning. Children are more susceptible to Pb^{2+} -poisoning because they absorb Pb^{2+} 5-times higher than adults [5]. Pb^{2+} accumulates in the body tissues including brain, liver, kidney, and skeletal system to reach toxic levels associated with organ dysfunction [6,7].

There are variety of methods for the removal of heavy metals from the contaminated water including chemical precipitation, membrane filtration, adsorption, electrolysis and photocatalysis [8]. Selection of the most suitable treatment should be considered according to some basic parameters such as the metal species, initial metal concentration, pH, environmental impact, as well as the operational costs [9]. Although chemical precipitation of Pb^{2+} is a broadly used method due to its low cost and simplicity, some deficiencies have been reported including sludge generation and poor efficiency with the low concentrations of Pb^{2+} [8,9]. Bioremediation has been proven to be beneficial for removal of metals from contaminated water through microbial uptake. This relies on accumulating the metal inside the living cells or adsorbing it by cell wall components [10]. However, bio-adsorption processes are more applicable than the bio-accumulative processes in large-scale systems because it doesn't require the addition of nutrients to maintain the microbial activity [11,12]. Factors including characteristics of the metal ion, biosorbent affinity and selectivity to the metal ions, pH, temperature, ionic strength, contact time and biomass concentration are known to influence the binding ability to heavy metals and efficiency of bio-sorption [2,13,14].

Numerous studies have documented improving the removal efficiency of Pb^{2+} by bacterial strains using recombinant protein technology. For instances, recombinant *E. coli* cells expressing the β-domain of IgA protease of N. gonorrhoeae with metallothionein (MT) from rats [15], mammalian metallothionein with LamB protein [16] and lpp-ompA-various sizes of peptides (EC20) have been used as bio-adsorbents with significant improvement of their efficiencies [17]. Heavy metal efflux (HME) family such as Cus, MerR and heavy metal binding domains like zinc finger and lead binding domains are used for the removal of target metals [13,14,18]. Lead specific operon (PbrR) from *C. metallidurans* CH34 (CmPbrR) is considered as the most specific polypeptide that binds Pb^{2+} [18-21]. MT (Synechoccocus SmtB) is a homodimeric transcriptional repressor belonging to the SmtB/ArsR family, with a winged helix DNA binding protein that represses the expression of SmtA operon in Cyanobacteria and is induced by specifically binding to Zn^{2+}. This protein usually harbors one or both of two structurally distinct metal-binding sites containing three to four conserved metal ligands which can bind to other heavy metals such as Pb^{2+}, Co^{2+} and Cu^{2+} [22,23].

Ag43 is an autotransporter protein exists in most of *E. coli* strains and has all requirements for membrane translocation and extracellular secretion [24,25]. It consists of 3 subunits, signal peptide (from 1st aa-52nd aa, for directing the secretion from cytoplasm to periplasm), α-domain (from 53rd aa to 552nd aa, the secretable or passenger protein, that translocate on the outer-membrane) and β-domain (from 553rd aa to 1038th aa, that form the β-barrel protein in outer-membrane for secreting passenger domain) [23]. Given the importance of surface-displaying proteins by *E. coli* for improving the removal efficiencies of Pb^{2+}; fused PbrR and SmtB were expressed as surface recombinant protein using Ag43 auto-translocator. The Pb^{2+} removal efficiency by alive and dead recombinant *E. coli* was investigated with optimized conditions for the best performance. This is the first study demonstrating the usefulness of usage of recombinant *E. coli* expressing fused two different metal binding proteins for the removal of Pb^{+2} from contaminated water.

Materials and Methods

Chemicals

DNA oligomers were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Restriction endonucleases, *BamHI* (20 U/ul), *BglII* (40 U/ul), *EcoRI* (20 U/ul), *SpeI* (10 U/ul), digestion buffers (3.1 buffer and CutSmart) and Antarctic Phosphatase (for dephosphorylation of 5' ends of DNA) were bought from New England Biolabs Japan Inc (Tokyo, Japan), DNA polymerase KOD plus Neo, KOD FX-Neo, and the solution for PCR were provided by TOYOBO Co Ltd (Osaka, Japan). Ligation kit, Mighty Mix, was obtained from TaKaRa BIO INC (Shiga, Japan). Kit for purification of DNA fragments, Wizard Sv Gel and PCR Clean-Up System, were supplied by Promega KK (Tokyo, Japan). BigDye Terminator v3.1 Cycle Sequencing Kit was purchased from Applied BiosystemsTM, USA. Luria-Bertani broth was purchased from Difco, MD (USA). KAPA Taq PCR Kit was obtained from KAPA biosystems (Massachusetts, USA). The Kit for preparation of plasmid DNA was bought from Nippon genetics (Tokyo, Japan). Arabinose was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). CBB staining, $Pb(NO_3)^2$, $CdCl_2$, $CuSO_4$ and $PbCl_2$ were purchased from Wako Pure Chemical industries (Osaka, Japan).

E. coli and plasmid DNA

E. coli strain DH5α was used for all experiments in this study. Plasmid DNAs used as a templet in this study were derived from BioBrick registry (http://partsregistry.org/). BioBrick number of the plasmid DNA encoding wild type Ag43 gene (P_{BAD} -RBS-Ag43-T/SB1C3) is BBa_K759001, BioBrick number of the plasmid DNA encoding PbrR is BBa_K346004. Amino acid sequence of Cyanobacterial Metallothionein Repressor (SmtB) MMDB ID:27695 [23] has been obtained from Gene bank of NCBI website (https://www.ncbi.nlm.nih.gov/Structure/pdb/1R22) and synthesized by Integrated DNA Technologies (IDT, USA). All DNA oligomers used in this study (listed in Table 1) were purchased from Sigma-Aldrich, Japan.

Table 1: Nucleotide sequences of oligo DNAs used in this study.

Primer name	Primer sequence
Ag43-β-domain-F	5'-GGGAGATCTAGGAATGTCACTCTCGCCTC-3'
Ag43-SP-R	5'-CCCAGATCTAGCAGCCAGCACCGGGAG-3'
BglII-SmtB-F	5'-GAGATCTATGACCAAACCAGTATTGCAGGATGG-3'
BglII-SmtB-R	5'-GAGATCTGCGAGATTCCTGTAAATGGTCAAGTGC-3'
BamHI-PbrR-F	5'-CCCGGATCCATGCAGGATTGCGGTGAAGTC-3'
BglII-PbrR-R 2	5'-GGAGATCTCCCGCACGATTGGGCGGGCCTG-3'
SpeI-P*BAD*-R	5'- GGACTAGTGCTAGCCCAAAAAAACGGGTATGGAGA-3'
100 up-F	5'-AACCTATAAAAATAGGCGTATCAC-3'
P*BAD*-1	5'-ACGAAAGTAAACCCACTGGTG-3'
P*BAD*-2	5'-CGCAACTCTCTACTGTTTCTC-3'
Ag43-1	5'-ACCATCAATAAAAACGG-3'
Ag43-2	5'-GAATAACGGCGCCATAC-3'
Ag43-3	5'-GCCTTTAACTACTCCCTC-3'
Ag43-4	5'-GGCAGTGCACAACATG-3'
Ag43 200-β-domain-R	5'-CGGGCGTACAGGCAGGCTGATGGTGC-3'
200 dn-R	5'-TCCCCTGATTCTGTGGATAACCGT-3'

Preparation of PbrR bio-device

DNA encoding P_{BAD} Promoter was ampli ied by PCR using primer sets (100 up-F and *SpeI*-P*BAD*-R) and BioBrick (BBa_K759001) as a DNA templet. The resultant DNA fragment (1,319 bp) digested by *EcoRI* and *SpeI* enzymes was ligated with the *EcoRI*/XbaI PbrR/SB1C3 digested vector (BBa_K346004) to make plasmid expressing PbrR under control of P_{BAD} promoter.

Construction of translocator targeting outer membrane (AT)

For construction of translocator of fused polypeptide targeting outside of outer-membrane, α-domain (amino acids position from 53 a.a. to 552 a.a.) of wild type Ag43 gene (BioBrick number BBa_K759001) was replaced by *BglII* restriction site using PCR with Ag43 β-domain-F primer and Ag43-SP-R primer. The PCR product (5,054 pb) digested with *BglII* restriction enzyme was ligated and transformed into 20 µl of *E. coli* DH5α, then the sequence was confirmed.

Construction of SmtB-AT plasmid DNA

DNA fragment encoding SmtB (124 amino acid) truncated by *BglII* restriction sites was synthesized by IDT (USA), after optimizing the codon of DNA sequence to be expressed in *E. coli* cells. DNA fragment (372 bp) digested by *BglII* was inserted into *BglII* site of auto translocator to make plasmid expressing fusion protein of SmtB with β-domain of Ag43 in its C-terminal (SmtB-AT).

Construction of PbrR-AT plasmid DNA

DNA fragment encoding PbrR (109 amino acid) truncated by *BamHI* and *BglII* restriction sites was amplified by PCR using primer set (*BamHI*-PbrR-F and *BglII*-PbrR-R) and BioBrick (BBa_K346004) as DNA templet. The 340 bp amplified DNA fragment, containing the full-length ORF of PbrR gene flanked by *BamHI* and *BglII* respectively, was digested and inserted into *BglII* site of auto-translocator to make plasmid expressing PbrR fused to β-domain of Ag43 in its C-terminal (PbrR-AT).

Construction of PbrR-SmtB-AT plasmid DNA

DNA fragment encoding MT (372 bp) digested by *BglII* was inserted into *BglII* site of PbrR-AT plasmid DNA to make plasmid expressing fusion proteins of PbrR and SmtB with β domain of Ag43 in its C-terminal (PbrR-SmtB-AT).

Analysis of recombinant proteins by SDS-PAGE

Recombinant *E. coli* cells were cultured in LB containing 34 mg/mL Chloramphenicol at 37°C at 180 rpm agitation, final optical density of the cells at 600 nm was 0.5~0.6, L-arabinose was added (final concentration 1.3 mM) to the culture for induction of recombinant proteins, and cells were collected 4 hours after incubation at 30°C at 180 rpm.

Each culture (200 µl) was centrifuged at 13,500 × g for 2 min and the cell pellets were treated in SDS sample buffer (80 µl) at 98°C for 5 min. After centrifugation (13,500 × g, 1 min) of the samples, 8 µl of each sample was loaded onto SDS-PAGE (12% acrylamide) and electrophoresed at 100 V for 2 hours. Total proteins were visualized by staining the gel in Coomassie Brilliant Blue R-250. Images of proteins were recorded using Canon D550 DSLR camera.

Analysis of localization of outer membrane translocator

Condition of cell culture for analyses of localization of recombinant protein is as described before. Harvested cells were separated into two fractions (cytosolic and membrane fractions) as described below. *E. coli* cells were collected by centrifugation of culture medium (25 ml) at 4700 × g for 15 min at 4°C and rinsed with 10 mM Tris-HCl (pH 7.5) two times. After adding 0.5 ml of 10 mM Tris-HCl to the precipitate, all suspension was stored at-80°C for 12 hours.

Cells in the suspension were lysed in 5 intervals of 15 sec sonication at output level 7. The lysate was centrifuged at 6700 × g for 10 min at 4°C to remove cell debris. The supernatant (500 µl) was centrifuged at 108,000 × g for 10 min at 4°C to separate cytosolic fraction (supernatant) from membrane fraction (precipitate). After rinsing the precipitate with 500 µl of 10 mM Tris-HCl (pH 7.5) two times, the precipitate was suspended by 50 µl of 10 mM Tris-HCl and mixed with 50 µl of 2X SDS sample buffer for solubilization of the protein in membrane fraction.

Preparation of *E. coli* cells for bio-adsorption

Recombinant *E. coli* cells were cultured in LB medium (60 ml) containing 34 µg/ml chloramphenicol at 37°C with shaking at 180 rpm. L-Arabinose was added to the culture medium (final concentration 1.3 mM) for induction of recombinant proteins when optical density of the cells at 600 nm reached to 0.5. *E. coli* culture was shacked for 4 hours at 30°C then *E. coli* cells were collected by centrifugation at 4,700 × g for 15 min at 4°C. Cells were rinsed with 10 ml of 0.5 mM Tris-HCl (pH 7.0), followed by rinsing with 10 ml of 0.9% NaCl two times. Cells were then resuspended in 0.9% NaCl (final concentration 10 g (wet weight)/L) and stored at 4°C. Appropriate amount of Pb^{2+} (50, 100 or 200 mg/L) has been incubated with *E. coli* for measuring Pb^{2+} removal efficiency and cells adsorption capacity.

For inactivation of *E. coli* cells, the cells dissolved in 0.9% NaCl solution were heated at 60°C for 1 hour. Viability of inactivated *E. coli* was confirmed by culturing cells on LB agar plate containing 34 µg/ml chloramphenicol at 37°C for 24 hours.

Measurement of heavy metal concentrations using atomic absorption spectrophotometer (AAS)

E. coli cells incubated with heavy metals solutions were centrifuged at 6700 × g for 5 min at 4°C, then 2 ml from the supernatant was transferred into a plastic tube containing 7.9 ml of DW and 0.1 ml of 10 mM HNO_3 (final concentration of acid was 0.1 M), then the samples were kept at 4°C till measurement of heavy metal content (copper, cadmium and lead) using atomic absorption spectrophotometer (Hitachi A-2000, Hitachi instruments Co., Tokyo, Japan). Calibrations were performed using standards within a linear calibration range of 0-20 ppm and the correlation coefficients for the calibration curves were 0.98 or higher.

Statistical analysis

Statistical signi icance was evaluated using Tukey–Kramer honestly signi icant difference tests, with $p<0.05$ considered as signi icant (JMP program, SAS Institute, Cary, NC, USA).

Results

Successful expression of proteins, PbrR and PbrR-AT in *E. coli*

CmPbrR from *C. metallidurans* CH34, one of MerR-like proteins, that regulates transcription of Pb^{2+}-resistance genes through its binding to Pb^{2+} on operator in the promoter [20,21]. Since capability of Pb^{2+}-binding of PbrR in the absence of DNA has been described previously [18], the use of the protein as a tool for capturing Pb^{2+} using *E. coli* cells was tested.

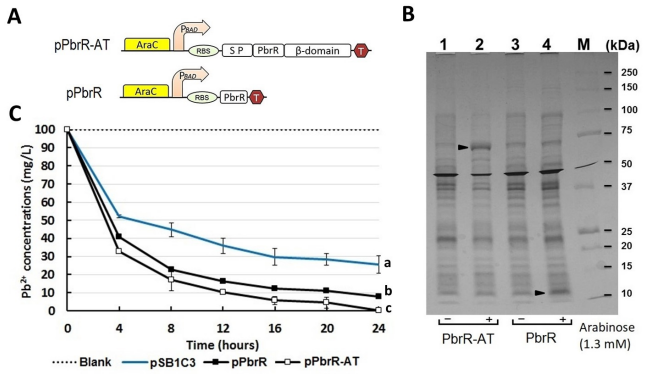

Figure 1: Constructs and expression of genes encoding recombinant lead binding protein (PbrR). (A) Schematic diagrams of genes for expression of PbrR (pPbrR) and PbrR fused to β-domain of Ag43 (pPbrR-AT) under control of P_{BAD} promoter. AraC: AraC activator expression unit under control of constitutive promoter (BBa_K808000). P_{BAD} : Inducible promoter in the presence of arabinose (BBa_I0500). RBS: Ribosome Binding Site (BBa_B0034), SP: signal peptide (position from 1st a.a. to 52nd a.a.) of Ag43, PbrR: coding region of lead binding operon (BBa_I721002), β-domain: translocation domain (position from 553rd a.a. to 1038th a.a.) of Ag43 which forms β-barrel structure in outer membrane, T: double terminator (BBa_B0015). (B) Analysis of induction of PbrR-AT and PbrR by addition of arabinose (1.3 mM) for 4 hours at 30°C. Protein extracted from equal amounts of *E. coli* cells grown in the absence or presence of arabinose were separated on SDS-PAGE (16% acrylamide) and visualized by CBB staining. Each black arrow head indicates the position of induced proteins (69.25 kDa for PbrR-AT and 11.71 kDa for PbrR respectively). (C) Time course of removal of Pb^{2+} from supernatant by incubating 5.0 g/L of *E. coli* cells in 0.9% NaCl solution containing 100 mg/L of Pb^{2+} (pH 6.0) at 37°C. *E. coli* cells containing pSB1C3 (empty vector control), pPbrR and pPbrR-AT were used as bio-sorbents of Pb^{2+}. Change of concentration of Pb^{2+} was monitored in the absence of *E. coli* cells under the same condition (Blank). Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are significantly different at $p<0.05$.

Furthermore, another construct of gene encoding PbrR fused to domains from Ag43 was made. Ag43 is a self-recognizing surface adhesion protein existing in most strains of *E. coli*. Expression of Ag43 confers aggregation and fluffing of cells, promotes biofilm formation and is often associated with enhanced resistance to antimicrobial agents [26,27]. Previous study revealed that presence of both N-terminal signal peptide and C-terminal ß-domain is sufficient for translocating intervening polypeptide to outer membrane [26].

Taking advantage of the characteristics of the translocator described above, domains required for the translocation were fused to both sides of PbrR as shown in Figure 1A. DNA fragments encoding PbrR and PbrR-AT (fusion protein containing signal peptide of Ag43 followed by PbrR and ß-domain of Ag43) were inserted downstream of P_{BAD} promoter, ligated with plasmid vector pSB1C3. Total proteins from equal amounts of bacterial cells were extracted and the induction of PbrR-AT (69.25 kDa) and PbrR (11.71 kDa) by arabinose was observed as shown in Figure 1B.

Comparison of the efficiency of the removal of Pb^{2+}

Recombinant *E. coli* cells containing PbrR and PbrR-AT could remove 60% and 69% of Pb^{2+}, respectively, at 4 hours after initiation of incubation (Figure 1C). While Pb^{2+} removal efficiencies were reached to 99% and 93% by PbrR-AT and PbrR strains, respectively after 24 hours (76% by strain containing empty vector used as negative control). These observations suggest that fusion of PbrR to the translocator had a slight effect in the elevation of removal efficiency of Pb^{2+} from supernatant.

Differential localization of PbrR and PbrR-AT in *E. coli* cells

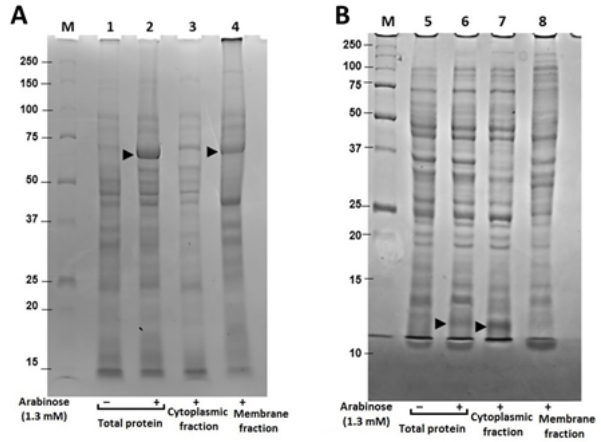

Figure 2: Differential localization of PbrR and PbrR-AT in *E. coli* cell. (A) Distribution of total, cytoplasmic and membrane proteins of *E. coli* containing pPbrR-AT after culturing cells in the absence or presence of arabinose (final concentration 1.3 mM) for 4 hours at 30°C. Lane M: polypeptide marker; lane 1: total protein without induction; lane 2: total protein after induction; lane 3: cytoplasmic proteins after induction; lane 4: membrane proteins after induction. Black arrow head indicates the position of the induced PbrR-AT (69.25 kDa). (B) Distribution of total, cytoplasmic and membrane proteins of *E. coli* containing pPbrR after culturing cells in the absence or presence of arabinose (final concentration 1.3 mM) for 4 hours at 30°C. Lane M: polypeptide marker; lane 1: total protein without induction; lane 2: total protein after induction; lane 3: cytoplasmic proteins after induction; lane 4: membrane proteins after induction. Black arrow head indicates the position of the induced PbrR (11.71 kDa).

The total protein from *E. coli* cells containing PbrR or PbrR-AT were separated into cytoplasmic protein and membrane protein fractions to analyze differential localization of these two different types of recombinant lead binding proteins. As shown in Figure 2, PbrR-AT (69.25 kDa) was successfully induced by arabinose (lane 2) and revealed to localize predominantly in membrane fraction (lane 4), while induced PbrR (11.71 kDa) was successfully induced (lane 6) and localizes in cytoplasmic fraction (lane 7). Theoretically, the signal peptide of Ag43 is known to play a role for secretion of fused polypeptide from cytoplasm to periplasm through the "sac complex" in inner membrane. After secretion of the whole protein to periplasmic space, the passenger domain is expected to localize outside of outer-membrane by passing through β-barrel structure of β-domain.

Comparison of the efficiency of removal of Pb^{2+} among *E. coli* strains expressing five different types of recombinant proteins

For constructing two more genes encoding fusion proteins, DNA fragment encoding α-domain of Ag43 was replaced by DNA fragment encoding SmtB from Cyanobacteria [23] or by DNA fragment encoding PbrR and SmtB. Expression and induction of all five recombinant genes (drawn in Figure 3A) were confirmed by SDS gel electrophoresis.

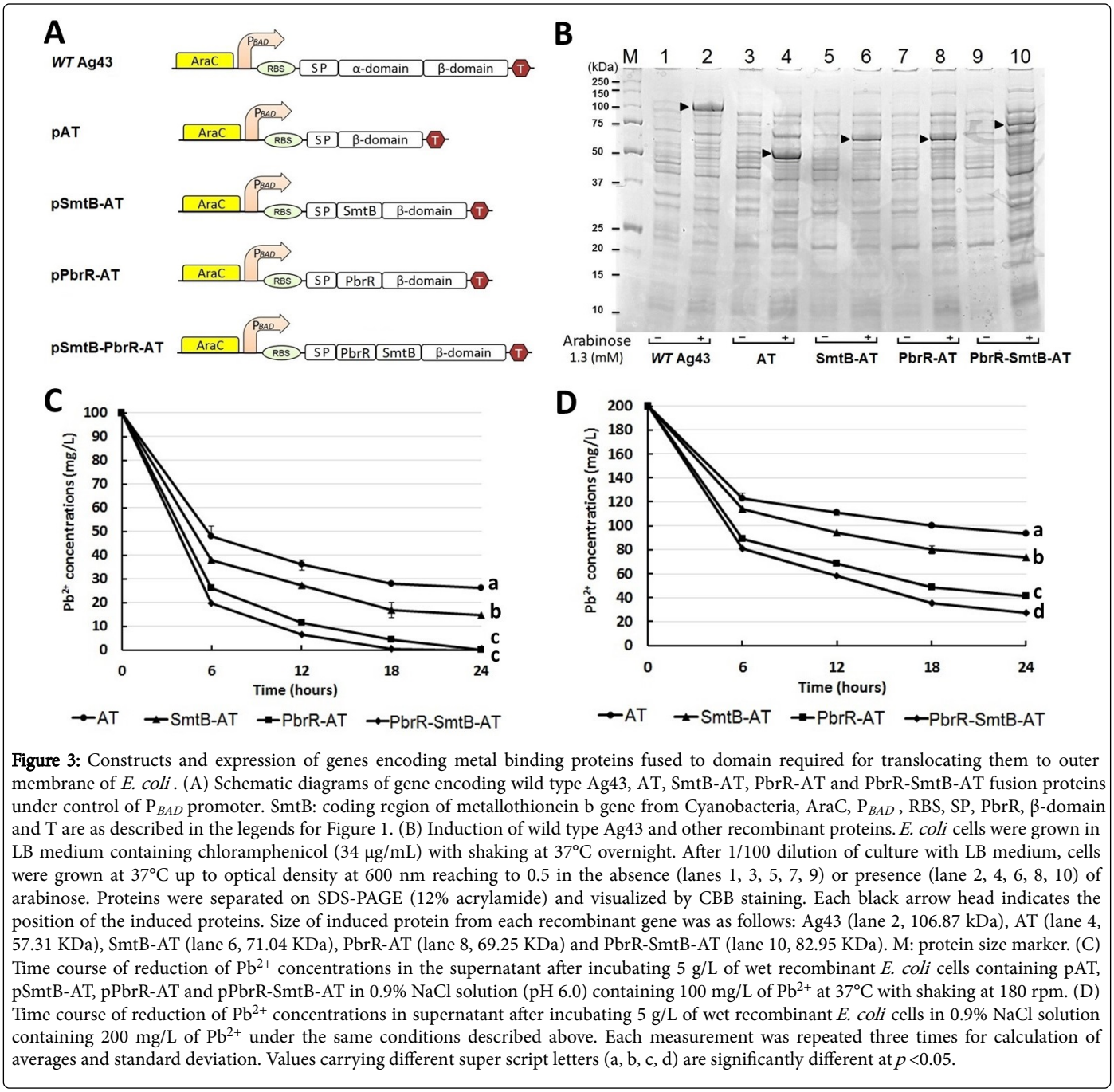

Figure 3: Constructs and expression of genes encoding metal binding proteins fused to domain required for translocating them to outer membrane of *E. coli* . (A) Schematic diagrams of gene encoding wild type Ag43, AT, SmtB-AT, PbrR-AT and PbrR-SmtB-AT fusion proteins under control of P_{BAD} promoter. SmtB: coding region of metallothionein b gene from Cyanobacteria, AraC, P_{BAD} , RBS, SP, PbrR, β-domain and T are as described in the legends for Figure 1. (B) Induction of wild type Ag43 and other recombinant proteins. *E. coli* cells were grown in LB medium containing chloramphenicol (34 μg/mL) with shaking at 37°C overnight. After 1/100 dilution of culture with LB medium, cells were grown at 37°C up to optical density at 600 nm reaching to 0.5 in the absence (lanes 1, 3, 5, 7, 9) or presence (lane 2, 4, 6, 8, 10) of arabinose. Proteins were separated on SDS-PAGE (12% acrylamide) and visualized by CBB staining. Each black arrow head indicates the position of the induced proteins. Size of induced protein from each recombinant gene was as follows: Ag43 (lane 2, 106.87 kDa), AT (lane 4, 57.31 KDa), SmtB-AT (lane 6, 71.04 KDa), PbrR-AT (lane 8, 69.25 KDa) and PbrR-SmtB-AT (lane 10, 82.95 KDa). M: protein size marker. (C) Time course of reduction of Pb^{2+} concentrations in the supernatant after incubating 5 g/L of wet recombinant *E. coli* cells containing pAT, pSmtB-AT, pPbrR-AT and pPbrR-SmtB-AT in 0.9% NaCl solution (pH 6.0) containing 100 mg/L of Pb^{2+} at 37°C with shaking at 180 rpm. (D) Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of wet recombinant *E. coli* cells in 0.9% NaCl solution containing 200 mg/L of Pb^{2+} under the same conditions described above. Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are significantly different at $p < 0.05$.

Each induced polypeptide corresponding to wild-type Ag43 (106.87 kDa), auto translocator (AT; 57.31 kDa), SmtB fused to translocator (SmtB-AT; 71.04 kDa), PbrR fused to translocator (PbrR-AT; 69.25 kDa) or PbrR-SmtB fused to translocator (PbrR-SmtB-AT; 82.95 kDa) was indicated by an arrow head in Figure 3B. Optimum expression of all ive recombinant proteins was observed at 4 hours after arabinose induction (1.3 mM) at 30°C. The results recorded in Figure 3C shows time course of the reductions of Pb^{2+} in supernatant at 6, 12, 18 and 24 hours after initiation of incubation. *E. coli* strain PbrR-SmtB-AT was shown to be the most efficient bio-adsorbent of Pb^{2+} among all strains, followed by PbrR-AT and SmtB-AT strains. In particular, Pb^{2+} concentration in supernatant was not detectable at 18 hours a ter initiation of the incubation with PbrR-SmtB-AT strain. We also compared the efficiency in the presence of higher concentration of Pb^{2+} (200 mg/l), and found that capability of each strain for removal of Pb^{2+} has been kept even in the presence of higher concentrations of Pb^{2+} (Figure 3D).

Selectivity of *E. coli* strains for adsorption of heavy metal ion species

To test selectivity of *E. coli* strains for adsorption of heavy metal ion species, mixture of heavy metal ions (Pb^{2+}, Cd^{2+} and Cu^{2+}) with equimolar concentration (480 μM) were mixed with each *E. coli* strain at 37°C for 24 hours. Concentrations of each heavy metal ion species were monitored every 3 hours after initiation of the incubation as shown in Figure 4.

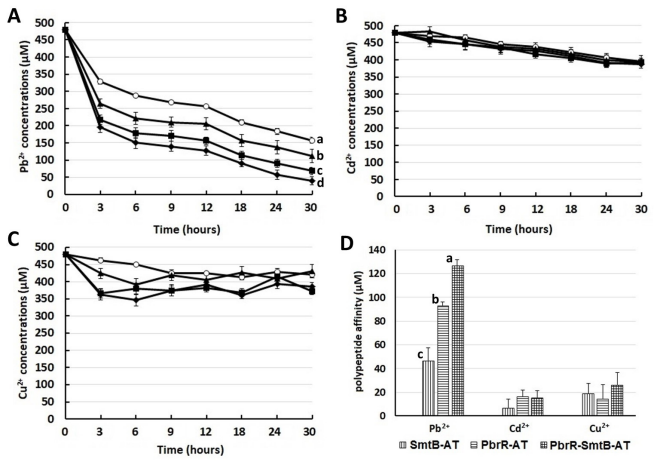

Figure 4: Selectivity of *E. coli* strains for adsorption of heavy metal ion species. Recombinant cells expressing AT (▼), SmtB-AT (▲), PbrR)-AT (■) or PbrR-SmtB-AT (◆) were incubated in 0.9% NaCl solution (pH 6.0) containing 480 μM of Pb^{2+}, Cd^{2+} and Cu^{2+}. Time course of removal of (A) Pb^{2+} from supernatant using three different types of recombinant *E. coli* cells, (B) Cd^{2+}, (C) Cu^{2+}. (D) comparison of different efficiencies of removal of Pb^{2+}, Cd^{2+} and Cu^{2+} from supernatant using different types of recombinant protein in each *E. coli* strain. Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are signi icantly different at $p<0.05$.

Although nonspecific adsorptions of Pb^{2+} or Cu^{2+} were observed when these heavy metals were incubated with *E. coli* strain AT as negative control, three *E. coli* strains showed efficient adsorption of Pb^{2+} (Figure 4A), while they showed inefficient adsorption of Cu^{2+} (Figure 4C). The most efficient adsorption of Pb^{2+} was observed when PbrR-SmtB-AT strain was mixed with the heavy metal mixture and followed by PbrR-AT and SmtB-AT (Figure 4A). On the other hand, efficient adsorption of Cd^{2+} by all of these *E. coli* strains has not been observed (Figure 4B). Relative efficiency of removal of each heavy metal ion was summarized in Figure 4D. These observations suggest that adsorption of Pb^{2+} by *E. coli* strains PbrR-AT or PbrR-SmtB-AT can be used as bacterial adsorbent for selective removal of Pb^{2+}.

Optimal condition of removal of Pb^{2+} by bacterial adsorbent, PbrR-SmtB-AT

To optimize conditions for removal of Pb^{2+} by *E. coli* strains PbrR-SmtB-AT, the bacterial cells were incubated with 100 mg/L of Pb^{2+} in different pH or different temperatures. The highest efficiency of the adsorption was observed at pH 6.0 at 37°C as shown in Figures 5A and 5B. Higher pH than 6.0 was not tested for the evaluation, because Pb^{2+} tends to be precipitated chemically in the higher pH. We also tested effects of increase or decrease of wet-weight of PbrR-SmtB-AT (2.5 g/L, 3.75 g/L or 5.0 g/L) added to the mixture containing 100 mg/L of Pb^{2+} at 37°C as shown in Figure 5C.

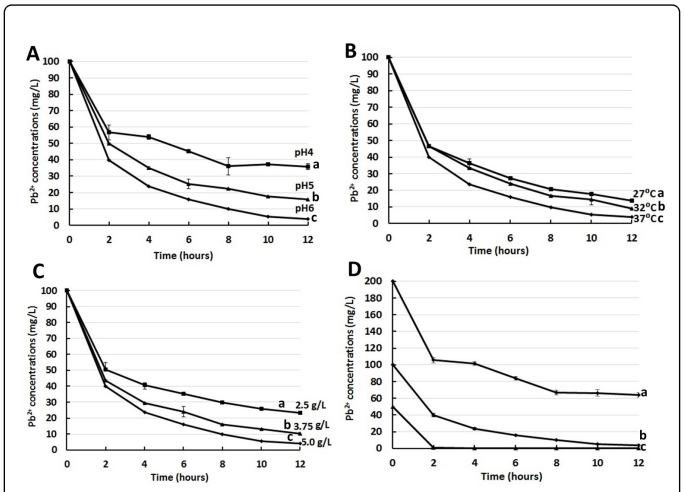

Figure 5: Effects of pH, temperature and amounts of *E. coli* cells on removal of Pb^{+2} (A) Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution containing 100 mg/L of Pb^{2+} at different pH 4.0, 5.0 or 6.0 at 37°C. (B) Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L *E. coli* cells (PbrR-SmtB-AT) at different temperatures (27°C, 32°C or 37°C) at pH 6.0. (C) Time course of reduction of Pb^{2+} concentrations after incubating 2.5 g/L, 3.75 g/L or 5.0 g/L of *E. coli* cells (LBP/MT-AT) at pH 6.0 and 37°C. (D) Time course of reduction of Pb^{2+} concentrations after incubating 5.0 g/L of *E. coli* cells (PbrR-SmtB-AT) with 50, 100 or 200 mg/L of Pb^{2+} at pH 6.0 and 37°C. Each measurement was repeated three times for calculation of averages and standard deviation. Values carrying different super script letters (a, b, c, d) are signi icantly different at $p<0.05$.

Efficiency of the removal of Pb^{2+} increased depending on the increase of the bacterial adsorbent added to the mixture. The 2.5 g/L of PbrR-SmtB-AT has the capability of removal of 77 mg/L of Pb^{2+} from 100 mg/L of Pb^{2+} in 12 hours.

Retaining of the activity as a bacterial adsorbent after heat treatment of PbrR-SmtB-AT

The efficiencies of the removal of Pb^{2+} between PbrR-SmtB-AT and heat-treated PbrR-SmtB-AT were tested and it was found that there is no much difference in capability to adsorb Pb^{2+} between live and dead bacterial cells as shown in Figure 6A. *E. coli* strain PbrR-SmtB-AT was incubated at 60°C for 1 hour with shaking, then the cells were cooled down to 37°C before adding Pb^{2+}. The removal of live cells was confirmed by measuring number of colonies on LB agar plates containing 34 µg/ml chloramphenicol (data are not shown). Interestingly, intact and heat-treated bacterial adsorbents did not show any significant differences in their removal efficiency even in solution containing higher concentration of Pb^{2+} (200 mg/L) as shown in Figure 6B. The current observations suggest that the bacterial adsorbent which was generated can play a significant role for the removal of Pb^{2+} even after heat treatment.

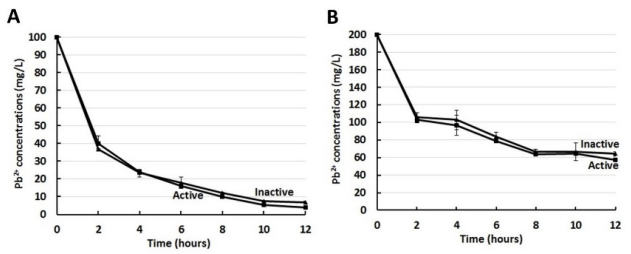

Figure 6: Effect of heat-treatment of *E. coli* cells (inactivation) on the efficiency of removal of Pb^{2+}. (A) Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of heat-treated or untreated *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution containing 100 mg/L of Pb^{2+} (pH 6.0) at 37°C/180 rpm. (B) Time course of reduction of Pb^{2+} concentrations in supernatant after incubating 5 g/L of heat-treated or untreated *E. coli* cells (PbrR-SmtB-AT) with 0.9% NaCl solution containing 200 mg/L of Pb^{2+} (pH 6.0) at 37°C/180 rpm. For heat treatment process, the cells which dissolved in 0.9% NaCl solution were incubated at 60°C for 1 hour. Each measurement was repeated three times for calculation of averages and standard deviation.

Discussion

Removal of heavy metals by bacteria depending on their biosorption activity and ability of binding metal ions is an environmentally friendly method [12]. Accumulating evidences showed the advantage of metal binding proteins in the removal of heavy metals such as Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{+}, Ni^{2+} and Zn^{2+}. Studying of the removal of Pb^{2+} by bacteria is controversy and that might be due to the nature of Pb^{2+} and its tendency to precipitate in most of bacterial cultures, buffer solutions, neutral and alkaline medium [13,28-32].

Bearing in mind these facts, we constructed recombinant *E. coli* cells expressing lead-binding proteins (PbrR) from *C. metallidurans* CH34, and metallothionein (SmtB) from Synechoccocus as membrane-associated proteins. The resultant recombinant *E. coli* expressing PbrR-AT as membrane-associated exhibited higher efficiency in removal of Pb^{2+} (99%) than cells expressing same protein (PbrR) in the cytoplasm (93%). In a related study, displaying of PbrR in *E. coli* cell surface constructed with two different translocators showed that the adsorption efficiency depends on the type of translocator and the initial metal concentration [21,33]. On the other hand, localization of metal binding protein influences the removal efficiencies of bacteria as noted that removal efficiency of cells displaying different kinds of MT is slightly higher (1.6-3.5%) than cells expressing MT in the cytoplasm [33].

Therefore, we constructed different recombinant *E. coli* encoding AT, SmtB-AT, and PbrR-AT and PbrR/SmtB-AT for expressing PbrR and SmtB on their surface membranes, and to examine their removal efficiency. The adsorbate capacity of *E. coli* to 40 mg Pb^{2+}/g cells (21.30, 25.29, and 31.78 mg Pb^{2+}/g cells, respectively) seemed to be comparable to the earlier studies used surface displayed recombinant *E. coli* [21,28,33]. These results revealed the successful expression of functional SmtB-AT and PbrR-AT recombinant proteins in *E. coli*. Furthermore, we speculated that improvement of the removal efficiency of *E. coli* might be achieved by fusing PbrR with SmtB-AT. The constructed *E. coli* expressing PbrR/SmtB-AT exhibited remarkable increases in the adsorbate capacities of *E. coli* as compared to other recombinant *E. coli*. Lipopolysaccharide (LPS) and extracellular polymers (EPs), which are the major components of the outer membrane are responsible for binding cations of toxic metals and play a role in Pb^{2+} adsorption. Generally, EP is known to have high content of uronic acids (28.29%), which play an important role in specific binding to Pb^{2+} [20]. However, the capacity of Pb^{2+} adsorption can be increased by expressing the recombinant heavy metal binding proteins on the cell wall [20,21,28].

In our study, recombinant *E. coli* demonstrated highly specific affinity to Pb^{2+} and relatively lower affinity to other metals such as Cd^{2+}, and Cu^{2+}. The maximum adsorption of Pb^{2+} had observed with PbrR/SmtB-AT *E. coli* cells, which has PbrR that shows specific response to Pb^{2+} 1000-fold over other metals such as Hg^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cr^{3+} and Ag^{2+} [21,34]. On the other hand, expressing SmtB in recombinant *E. coli* exhibited higher adsorption capacity than wild type due to the nature of MT containing three to four conserved metal ligands, which are capable of binding heavy metals including Zn^{2+}, Pb^{2+} and Cu^{2+} [35-37]. Another important finding in our study that incubation of the recombinant *E. coli* at low temperature resulted in a slight reduction in adsorption capacity. These results suggest the efficiencies of our *E. coli* (PbrR/SmtB-AT) in removal Pb^{2+} at broad range of temperatures.

Giving the importance of pH for the capability of recombinant *E. coli* in removal of Pb^{2+}, different conditions of pH were studied. Our data showed that the removal efficiency of recombinant *E. coli* was pH-dependent and noted to be highest at pH 6. These seem to be in agreement with earlier studies reporting the dependency of Pb^{2+} uptake adsorption on pH conditions [20,38-40]. The mechanism of uptake of Pb^{2+} at different pH conditions can be explained by the presence of functional groups of several macromolecules that involved in binding to Pb^{2+}. However, in low pH the H^{+} ions compete with Pb^{2+} for the adsorption sites of negative groups like OH^{-}, S^{--} and PO_4^{---} [40,41].

The present study revealed that *E. coli* cells have the characteristics to remove Pb^{2+} from acidic solution, which is a difficult issue because of the high solubility of Pb^{2+} compounds in acidic condition. Unlike

the most common methods for removal of Pb^{2+} from wastewater (chemical precipitation in alkaline solution), this method could be considered for removal of Pb^{2+} at acidic solution. It is believed that removal of heavy metals by bacteria depending on their specific binding activity to metal ions has a major concern toward releasing of recombinant organisms into the environment [12]. However, it is noteworthy to mention that heat-treatment (at 60°C for 60 min) is enough to remove viability of recombinant *E. coli* strains without losing its activity to bind Pb^{2+}. which is an important finding for the possibility of application of this system.

Conclusions

The current study has succeeded to develop a useful bio-device, PbrR-SmtB-AT *E. coli* strain, which has the ability to remove Pb^{2+} selectively under acidic pH, at room temperature, and with minimum requirements for safety consideration.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Data Availability

All DNA plasmids and bacterial strains used in this study are available from the corresponding author upon request.

References

1. Tong S, Schirnding YEV, Prapamontol T (2000) Environmental lead exposure: a public health problem of global dimensions. Bull World Health Organ 78: 1068-1077.
2. Mao Q, Huang G, Ma K, Sun Z (2014) Variations of soil lead in different land uses along the urbanization gradient in the Beijing metropolitan area. Int J Environ Res Public Health 11: 3199-3214.
3. Renner R (2009) Out of plumb: when water treatment causes lead contamination. Environ Health Perspect 117: A542.
4. Zhang N, Zhang J, Li Z, Chen J, Zhang Z, et al. (2018) Resistance strategies of Phragmites australis (common reed) to Pb pollution in flood and drought conditions. PeerJ 6: e4188.
5. Christopher JL, Murray EA (2015) Institute for Health Metrics and Evaluation (IHME). University of Washington, GBD Compare, Seattle, USA.
6. Canfield RL, Jusko TA, Kordas K (2005) Environmental lead exposure and children's cognitive function. Riv Ital Pediatr 31: 293-300.
7. Mohod CV, Dhote J (2013) Review of heavy metals in drinking water and their effect on human health. International Journal of Innovative Research in Science, Engineering and Technology 2: 2992-2996.
8. Barakat MA (2011) New trends in removing heavy metals from industrial wastewater. Arabian Journal of Chemistry 4: 361-377.
9. Kurniawan TA, Chan GY, Lo WH, Babel S (2006) Physico–chemical treatment techniques for wastewater laden with heavy metals. Chem Eng J 118: 83-98.
10. Gavrilescu M (2004) Removal of heavy metals from the environment by biosorption. Eng Life Sci 4: 219-232.
11. Akpor OB, Muchie M (2010) Remediation of heavy metals in drinking water and wastewater treatment systems: Processes and applications. International Journal of Physical Sciences 5: 1807-1817.
12. Perpetuo EA, Souza CB, Nascimento CAO (2011) Engineering bacteria for bioremediation. In: Progress in Molecular and Environmental Bioengineering. Analysis and Modeling to Technology Applications InTech 16: 25.
13. Sarı A, Tuzen M (2008) Biosorption of Pb (II) and Cd (II) from aqueous solution using green alga (Ulva lactuca) biomass. J Hazard Mater 152: 302-308.
14. Wang L, Wang N, Zhu L, Yu H, Tang H (2008) Photocatalytic reduction of Cr (VI) over different TiO2 photocatalysts and the effects of dissolved organic species. J Hazard Mater 152: 93-99.
15. Valls M, Atrian S, de Lorenzo V, Fernández LA (2000) Engineering a mouse metallothionein on the cell surface of Ralstonia eutropha CH34 for immobilization of heavy metals in soil. Nat Biotechnol 18: 661-665.
16. Kotrba P, Pospisil P, de Lorenzo V, Ruml T (1999) Enhanced metallosorption of Escherichia coli cells due to surface display of β-and α-domains of mammalian metallothionein as a fusion to LamB protein. J Recept Signal Transduct Res 19: 703-715.
17. Bae W, Chen W, Mulchandani A, Mehra RK (2000) Enhanced bioaccumulation of heavy metals by bacterial cells displaying synthetic phytochelatins. Biotechnol Bioeng 70: 518-524.
18. Brown NL, Stoyanov JV, Kidd SP, Hobman JL (2003) The MerR family of transcriptional regulators. FEMS Microbiol Rev 27: 145-163.
19. Chen PR, He C (2008) Selective recognition of metal ions by metalloregulatory proteins. Curr Opin Chem Biol 12: 214-221.
20. Jarosławiecka A, Piotrowska-Seget Z (2014) Lead resistance in micro-organisms. Microbiol 160: 12-25.
21. Wei W, Liu X, Sun P, Wang X, Zhu H, et al. (2014) Simple whole-cell biodetection and bioremediation of heavy metals based on an engineered lead-specific operon. Environ Sci Technol 48: 3363-3371.
22. Busenlehner LS, Weng TC, Penner-Hahn JE, Giedroc DP (2002) Elucidation of primary (α3N) and vestigial (α5) heavy metal-binding sites in Staphylococcus aureus pI258 CadC: evolutionary implications for metal ion selectivity of ArsR/SmtB metal sensor proteins. J Mol Biol 319: 685-701.
23. Eicken C, Pennella MA, Chen X, Koshlap KM, VanZile ML, et al. (2003) A metal–ligand-mediated intersubunit allosteric switch in related SmtB/ArsR zinc sensor proteins. J Mol Biol 333: 683-695.
24. Klemm P, Hjerrild L, Gjermansen M, Schembri MA (2004) Structure-function analysis of the self-recognizing antigen 43 autotransporter protein from Escherichia coli. Mol Microbiol 51: 283-296.
25. Ramesh B, Sendra VG, Cirino PC, Varadarajan N (2012) Single-cell characterization of autotransporter-mediated Escherichia coli surface display of disulfide bond-containing proteins. J Biol Chem 287: 38580-38589.
26. Van der Woude MW, Henderson IR (2008) Regulation and function of Ag43 (flu). Annu Rev Microbiol 62: 153-169.
27. Muñoz-Gutiérrez I, Moss-Acosta C, Trujillo-Martinez B, Gosset G, Martinez A (2014) Ag43-mediated display of a thermostable β-glucosidase in Escherichia coli and its use for simultaneous saccharification and fermentation at high temperatures. Microb Cell Fact 13: 106.
28. Almaguer-Cantú V, Morales-Ramos LH, Balderas-Rentería I (2011) Biosorption of lead (II) and cadmium (II) using Escherichia coli genetically engineered with mice metallothionein I. Water Sci Technol 63: 1607-1613.
29. Niu H, Xu XS, Wang JH, Volesky B (1993) Removal of lead from aqueous solutions by Penicillium biomass. Biotechnol Bioeng 42: 785-787.
30. Puranik PR, Paknikar KM (1997) Biosorption of lead and zinc from solutions using Streptoverticillium cinnamoneum waste biomass. J Biotechnol 55: 113-124.

31. Selatnia A, Boukazoula A, Kechid N, Bakhti MZ, Chergui A, et al. (2004) Biosorption of lead (II) from aqueous solution by a bacterial dead Streptomyces rimosus biomass. Biochem Eng J 19: 127-135.
32. Tunali S, Akar T, Özcan AS, Kiran I, Özcan A (2006) Equilibrium and kinetics of biosorption of lead (II) from aqueous solutions by Cephalosporium aphidicola. Sep Purif Technol 47: 105-112.
33. Kao WC, Chiu YP, Chang CC, Chang JS (2006) Localization effect on the metal biosorption capability of recombinant mammalian and fish metallothioneins in Escherichia coli. Biotechnol Prog 22: 1256-1264.
34. Hynninen A, Touzé T, Pitkänen L, Mengin-Lecreulx D, Virta M (2009) An efflux transporter PbrA and a phosphatase PbrB cooperate in a lead-resistance mechanism in bacteria. Mol Microbiol 74: 384-394.
35. Blindauer CA (2011) Bacterial metallothioneins: past, present, and questions for the future. J Biol Inorg Chem 16: 1011.
36. Mejáre M, Bülow L (2001) Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol 19: 67-73.
37. Busenlehner LS, Pennella MA, Giedroc DP (2003) The SmtB/ArsR family of metalloregulatory transcriptional repressors: structural insights into prokaryotic metal resistance. FEMS Microbiol Rev 27: 131-143.
38. Farghali AA, Bahgat M, Allah AE, Khedr MH (2013) Adsorption of Pb (II) ions from aqueous solutions using copper oxide nanostructures. Beni-Suef University Journal of Basic and Applied Sciences 2: 61-71.
39. Glatstein DA, Francisca FM (2015) Influence of pH and ionic strength on Cd, Cu and Pb removal from water by adsorption in Na-bentonite. Applied Clay Science 118: 61-67.
40. Karimi H (2017) Effect of pH and Initial pb (II) Concentration on The Lead Removal Efficiency from Industrial Wastewater Using Ca(OH)2. Int J Water Wastewater Treat vol: 3.
41. Cangelosi V, Ruckthong L, Pecoraro VL (2017) Lead (II) Binding in Natural and Artificial Proteins. Met Ions Life Sci.