Summer 2020

Toward Development of a Paper-Based Microanalytical Device for Heavy Metal Ion Detection by Enzyme Inhibition

Adjoa Otubea Adams
Missouri State University, Adams001@live.missouristate.edu

TOWARD DEVELOPMENT OF A PAPER-BASED MICROANALYTICAL DEVICE FOR HEAVY METAL ION DETECTION BY ENZYME INHIBITION

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Chemistry

By

Adjoa Otubea Adams

August 2020

TOWARD DEVELOPMENT OF A PAPER-BASED MICROANALYTICAL DEVICE FOR HEAVY METAL ION DETECTION BY ENZYME INHIBITION

Chemistry

Missouri State University, August 2020

Master of Science

Adjoa Otubea Adams

ABSTRACT

In recent years, paper-based microanalytical devices have attracted great attention in broad application areas including the colorimetric detection of heavy metal ions. The simplicity, portability, and low cost of the devices offer unique advantages over conventional instrumental analysis. This project utilizes the inhibition of an enzyme-catalyzed reaction by metal ions to develop a paper-based analytical device for the detection of heavy metal contaminants such as mercury. I selected alkaline phosphatase for this study. This enzyme possesses Mg and Zn ions in its active site. Therefore, the competitive displacement of these metal ions and/or denaturation of the enzyme by heavy metal ions would lead to the inhibition of the enzyme's activity. I have investigated the effects of several metal ions at different concentrations on enzyme-catalyzed color development. To improve the sensitivity of the method, I envisioned to immobilize the enzyme onto the filter paper. An attempt to express alkaline phosphatase-cellulose binding domain fusion protein in *E. coli* did not yield successful results. However, I was able to express alkaline phosphatase fused with a pelB signal peptide (without cellulose-binding domain) in *E. coli*. This suggests that the pelB sequence may be contributing to the expression of this enzyme. This information provides an important insight for the preparation of alkaline phosphatase-cellulose binding domain fusion protein that can be applied to the development of a highly sensitive metal detection method in the future.

KEYWORDS: alkaline phosphatase, cellulose-binding domain, heavy metals, inhibition, plasmid, protein expression, pelB sequence

TOWARD DEVELOPMENT OF A PAPER-BASED MICROANALYTICAL DEVICE FOR HEAVY METAL ION DETECTION BY ENZYME INHIBITION

By

Adjoa O. Adams

A Master's Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Chemistry

August 2020

Approved:

Keiichi Yoshimatsu, Ph.D., Thesis Committee Chair

Adam K. Wanekaya, Ph.D., Committee Member

Cyren M. Rico, Ph.D., Committee Member

Kyoungtae Kim, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

ACKNOWLEDGEMENTS

I am very grateful to my research advisor, Dr. Keiichi Yoshimatsu, for his immense help and contributions throughout my research. Dr. Yoshimatsu has been a very good mentor who has imparted significant scientific knowledge to me and taught me many molecular biology techniques to equip me for the future. Your efforts are invaluable, Dr. Yoshimatsu.

Secondly, I would like to thank my thesis committee; Drs. Adam Wanekaya, Cyren Rico, and Kyoungtae Kim for their time in editing my thesis and giving helpful feedback on my work. I appreciate your contributions. Linda Allen deserves a special thanks for playing a key role in the Department of Chemistry by solving issues that we encounter and assisting with our travel plans to attend conferences to present our research works. I thank Jonathan Hardin as well, for assisting with the supply of reagents and lab consumables for research.

I am most grateful to my lovely and amazing parents, Mr. and Mrs. Opare Adams, as well as my dear siblings for supporting and encouraging me throughout my education.

I would like to acknowledge the Chemistry Department, Graduate College, College of Natural and Applied Science, Office of Research Administration at Missouri State University, and the ACS Ozark Local Section for their financial support. The pSANG14-3F plasmid (Addgene plasmid # 39265) was a gift from John McCafferty.

Finally, I thank my colleagues and friends especially Luckio Owuocha, Tyler Odom, Seth Amankrah, and Israel Bonsu who have been helpful during my master's program. Thank you to the Yoshimatsu lab members especially Shaylen Harris, for their assistance in the lab.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Heavy Metal Ion Detection

Traces of heavy metal ions such as mercury (Hg), cadmium (Cd), and lead (Pb) in drinking water and aquatic systems are great threats to humans, aquatic species, and other living organisms in the ecosystem that are directly or indirectly connected between them by the food chain.[1] The key reason behind the threat is that these heavy metals accumulate in biotic organisms due to their long biological half-lives and therefore become toxic, interfere with metabolic processes, and cause diseases. In severe cases, heavy metals can lead to the death of organisms including humans. [2, 3]

Due to this pertinent issue, analytical scientists have developed methods for the detection of heavy metals. The traditional methods known for metal ion detection especially at levels above permissible limits include atomic absorption spectroscopy (AAS) and the inductively coupled plasma mass spectroscopy (ICP-MS).[4] Although they provide very reliable results, they are not easy to transport, expensive to operate, require expertise, and have long response times. New methods are being developed to serve the same purpose more conveniently and reliably for users. They include electrochemical, fluorescent and colorimetric sensors, luminescent chemosensors, and many others.[5, 6]

Paper-based Microanalytical Device

The paper-based microanalytical device is an innovative tool that applies basic analytical chemistry principles for fast and inexpensive biochemical analysis, clinical and forensic diagnostics, heavy metal detection, and others.[7] It has garnered great attention due to its

simplicity and other numerous benefits including reliability, portability, and rapid response over the conventional instrumental analysis.[8] An example is a β-galactosidase-based paper sensor developed to detect heavy metals by colorimetric visualization of an enzyme's activity.[9] Enzymes such as urease, horseradish peroxidase, cholinesterase, and glucose oxidase may be used to develop this device.[10-13] Another example is an enzyme-based assay involving the use of acetylcholinesterase (AchE) and choline oxidase (ChOx) enzymes in the presence of TGA-capped CdTe quantum dots (QDs) for quantitative detection of insecticides by fluorescence intensity under black light.[14] Aside from enzymes, paper-based analytical devices for metal detection may be based on DNAzyme-based sensors,[15-17] whole-cell signaling,[18, 19] and carbon quantum dot-based nanoprobes.[20] The use of alkaline phosphatase for this study tackles the shortfalls of some metal detection systems by providing certain benefits such as simplicity, portability, sensitivity, reliability, and affordability.

Alkaline Phosphatase (ALP)

Alkaline phosphatase is typically an enzyme made up of two identical monomers consisting of α-helices and β-sheets.[21] The active sites of the enzyme contain two closely spaced zinc and a magnesium atom (**Figure 1**) indispensable for catalytic function.[22, 23] These metal co-factors contribute considerably to the conformation of the enzyme's monomer and indirectly regulate interactions between the subunits.[24] The enzyme has a broad specificity for substrates[25] such as 5-bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium (BCIP/NBT) and *para*-nitrophenylphosphate (pNPP)[23] and catalyzes dephosphorylation reactions to release inorganic phosphates. It is extremely stable in alkaline environments (pH 7.5–9.5) and works best at pH 8-10 depending on the substrate of interest, concentration, and ionic strength.[23]

Alkaline phosphatase is thermostable with a general optimum temperature of 37 °C.[21] In *E. coli* for instance, it is capable of effectively catalyzing reactions even at higher temperatures of 80 °C. Unlike other assay enzymes that get denatured at high temperatures, alkaline phosphatase has a unique advantage because it undergoes dimerization to preserve the secondary and tertiary structures.[26]

Alkaline Phosphatase Inhibition by Heavy Metals. The activity of the enzyme can be inhibited by metal ions such as Cu^{2+}, Pb^{2+}, and Co^{2+}. They do so by either binding at certain regions of the enzyme to induce conformational change or displacing the native Zn^{2+} ions at the active sites to render the entire enzyme inactive.[27, 28]

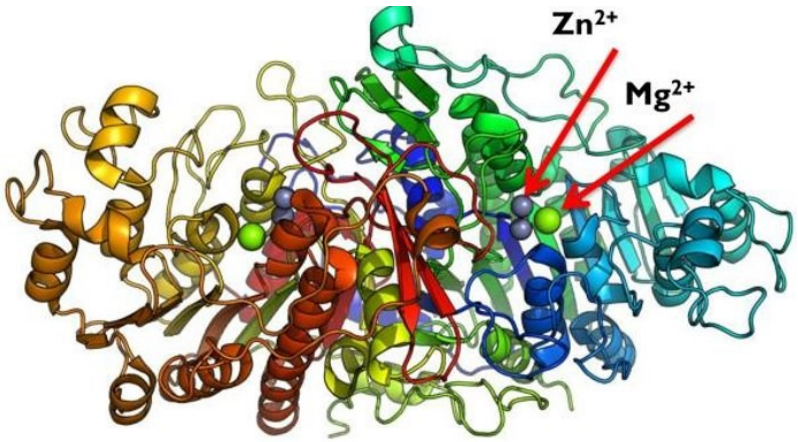


Figure 1. Structure of alkaline phosphatase. It comprises β-sheets and α-helices, and two zinc and a single magnesium ion on each protein monomer. Reprinted from reference 29.

BCIP/NBT Reaction. One of the most commonly used substrates for the detection of alkaline phosphatase activity is 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate. The hydrolyzed product, 5-bromo-4-chloro-3-indolol, reacts with nitroblue tetrazolium (NBT) and forms an intense purple-colored insoluble NBT diformazan product (**Figure 2**), which can be measured visually.[30] The colored product develops rapidly, and it is very stable and does not fade

in the presence of light.[30] When a sample containing a heavy metal ion species is introduced to the enzyme catalyzing this reaction, the presence or absence of the color development due to inhibition is used for metal detection. The ability to visualize the results without an instrumental readout makes the testing of heavy metals very convenient.

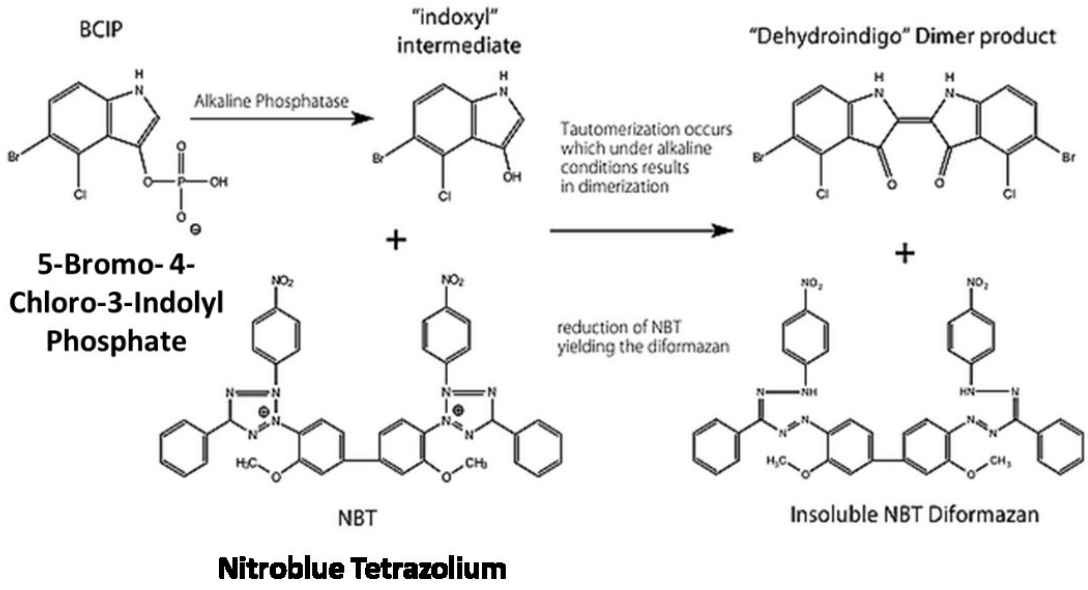


Figure 2. Colorimetric detection of alkaline phosphatase activity. This reaction occurs in the presence of BCIP substrate (bearing a phosphate group) and NBT to yield an insoluble purple-colored diformazan product. Reprinted from reference 30.

Recombinant DNA Technology

Herbert W. Boyer, Stanley N. Cohen, and other scientists made immense contributions to the invention of recombinant DNA technology in 1972. This made it possible to artificially connect DNA sequences from different species, to form a hybrid DNA that can be transformed into a host cell to replicate and express a target protein.[31, 32]

Expression of Proteins in *E.coli*. T7 promotors are one of the commonly used promotors for the expression of proteins in *E. coli*. The two popular methods used to induce the transcription of

the gene of interest to the corresponding messenger RNA (mRNA) under the T7 promotor are IPTG induction and Overnight Express™ autoinduction system.[33]

IPTG induction involves the use of a structural analog of lactose called isopropyl β-d-1-thiogalactopyranoside (IPTG) to trigger the expression of T7 RNA polymerase in *E. coli* strains that carry the lambda DE3 prophage. For this reason, in the absence of lactose or IPTG, the expression from the T7 promoter is suppressed. Also, IPTG binds to lac repressor protein (LacI) in an allosteric manner and releases LacI from the operator region, allowing for the transcription of the gene of interest (**Figure 3**). An advantage of IPTG over lactose is that it is non-hydrolyzable and cannot be used by the *E. coli* cell. Hence, the concentration that is used at the beginning is constant throughout the culturing process.[34, 35]

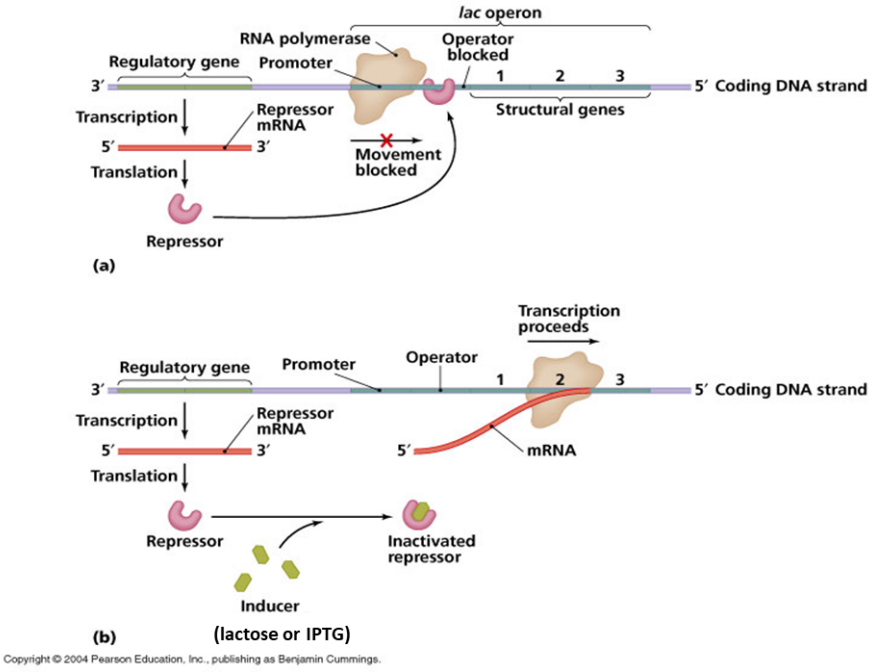


Figure 3. Protein suppression and expression in *E. coli*. The LacI repressor prevents protein expression by the lac operon (a). The inducer binds to the repressor for protein expression (b).

On the other hand, in the Overnight Express™ autoinduction system, *E. coli* cells metabolize glucose until reaching to a certain cell density.[33] Once glucose in the medium is depleted, the cells begin to express proteins for lactose metabolism including lactose transporter proteins. This triggers the uptake of lactose by *E. coli* cells and induces the transcription of the gene of interest encoded under T7 promotors. Typically, the media is supplemented with a few additional compounds to achieve a high cell density and protein yield.[33] The advantage of the Overnight Express™ autoinduction system is its capability to induce a spontaneous expression of proteins without monitoring the cells' density or using IPTG.

Alkaline Phosphatase from *E. coli*. There have been several reports of recombinant expression of *E. coli* alkaline phosphatase on plasmid vectors. For example, McCafferty and coworkers [36] previously reported the design of a pSANG14-3F vector for the expression of scFv-alkaline phosphatase fusion molecules for a convenient and sensitive way of determining the reactivity of recombinant antibody fragments in assays. Another example is the use of the promoter (*pho A*) of *E. coli* E5 gene for recombinant expression of alkaline phosphatase connected by four collagenase recognition sequences to the finger domain of a human tissue plasminogen activator.[37] The reason for the expression of this fusion protein was to optimize the regulated *E. coli pho A* expression system and increase protein production by considering factors such as phosphate concentrations, the stability of plasmid, and the synthesis of gene products in growth and production phases.[37]

Cellulose-Binding Domains (CBDs)

Cellulose-binding domains (CBDs) are protein domains present in many cellulolytic enzymes. Most cellulolytic enzymes consist of two domains, a catalytic and a CBD, connected by a linker.

CBDs can act as scaffolds on cellulose, to hold the catalytic domains (enzyme) near the cellulose substrate to increase the rate of reactions.[38, 39] There are over 200 different sequences of CBD from various species. One variant of particular interest is the family-3a CBD[40] from *Clostridium thermocellum* located at the C-terminal region to the catalytic domain. This domain has previously been expressed in *E. coli* and reported to bind to cellulose.[40] One of the important characteristics of this domain is its exceptional stability. The DNA sequence of the cellulose-binding domain (**Appendix B**) present in the cipB gene of *Clostridium thermocellum* can be retrieved from The UniProt Knowledgebase.[43] In **Figure 4**, the model of the family-3a bacterial cellulose-binding domain is shown. The aromatic residues of the domain are stack against the faces of sugar rings for protein-carbohydrate interactions. [41, 42] Interestingly, the spacing of the aromatic rings is a multiple of the pyranoside rings along a single cellulose chain and suggests the orientation of the CBD on the cellulose. Additional conserved polar residues located within hydrogen bonding distances from polar groups on neighboring chains provide more protein-cellulose contacts.

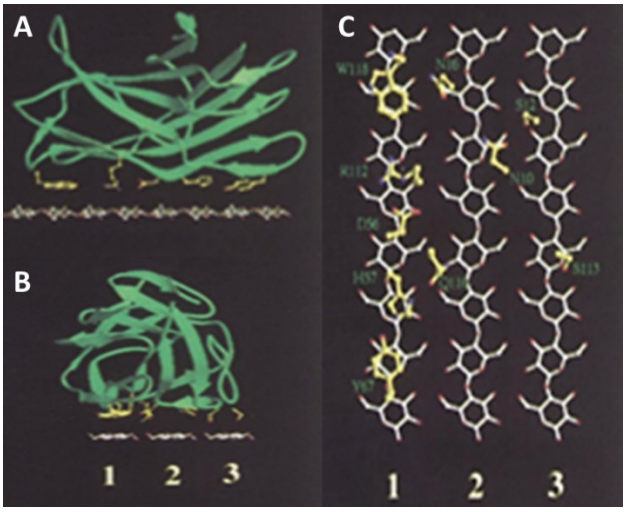


Figure 4. Cellulose-binding domain of family-3a. A) The ribbon structure of CBD positioned closely along a chain of cellulose. B) A 90°-rotated angle of CBD aligned on three separate chains of cellulose. C) Aerial perspective of the interaction between CBD and the three cellulose chains. Reprinted from reference 44 with permission from John Wiley & Sons, Inc.

Objectives

The objective of this work was to investigate the applicability of alkaline phosphatase, a metalloenzyme, in the colorimetric detection of heavy metal contaminants such as mercury(II) and copper(II) in water samples. The ultimate goal of the project is to develop a simple, portable, user-friendly, rapid, efficient, and reliable paper-based microanalytical device. Along these lines, for proof-of-concept, alkaline phosphatase-catalyzed reactions and inhibition of the enzyme by heavy metal ions were monitored on a filter paper and nitrocellulose membrane by using commercially available alkaline phosphatase from bovine intestine mucosa. In comparison to a previously reported system that uses β-galactosidase,[9] acetylcholinesterase (AchE),[14] and choline oxidase (ChOx),[14] this system holds the advantages in terms of the stability of the enzymes and availability of a broad spectrum of substrates that can be used to develop an inexpensive heavy metal detection system. In addition, to improve the sensitivity and reliability of the method by immobilizing alkaline phosphatase onto filter paper, I attempted to express alkaline phosphatase-cellulose binding domain fusion proteins in *E. coli*.

MATERIALS

The 1 kb DNA ladder and Gel Loading Dye Purple 6X were obtained from New England Biolabs® Inc. The 10X Fast Digest Buffer, ammonium sulfate (cat. # A702-500), calcium chloride (cat. # C-77), disodium phosphate (cat. # S-374), monopotassium phosphate (cat. # PX1565-1), sodium bicarbonate (cat. # S631-10), sodium chloride (cat. # BP358-10), sodium dodecyl sulfate (cat. # 28312), sodium hydroxide (cat. # SS255-1), hydrochloric acid (cat. # SA49), tris base (cat. # BP152-1), glycerol (cat. # BP229-1), isopropyl β-D-1-thiogalactopyranoside (IPTG) (cat. # FERR1171, 10 x 1.5mL (Aqueous Solution: 100mM)), HisPur™ Ni^{2+}-NTA resin (cat. # 88221), SYBR® Safe DNA gel stain (cat. # S33102), *Bam*HI (cat. # ER0051), and *Pst*I (cat. # ER0612) were supplied from Thermo Fisher Scientific. Bacteriological agar (product # A5306), bovine serum albumin (product # A3294), glucose (product # G8270), α-lactose (product # L3625), potassium acetate (cat. BDH9254-500G), and alkaline phosphatase from bovine intestinal mucosa ((product # P6774) were from Sigma-Aldrich Chemicals company. Protein Assay Dye Reagent Concentrate (cat. # 5000006), Mini-PROTEAN® TGX™ Gels 12% (cat. # 456-1046), Precision Plus Protein™ Unstained Standards (cat. # 161-0363), AP Conjugate Substrate Kit (cat. # 1706432), and Mini-PROTEAN Tetra Vertical Electrophoresis Cell (product # 1658004) were from Bio-Rad Laboratories, Inc. Chloramphenicol, 98% (cat. # 227920000), and Kanamycin sulfate (cat. # AC611290050) antibiotics were manufactured by ACROS Organics™. Coomassie® Brilliant Blue G-250 (cat. # ICN19034305) was from MP Biomedicals™. Imidazole (cat. # 50-491-514) and Magnesium sulfate (cat. # 50-503-765) were produced by Chem-Impex International, Inc.

METHODS

Part I: Metal-Ion Detection by the Inhibition of Commercially Available Alkaline Phosphatase

Reagent Preparations. A stock solution of alkaline phosphatase (ALP) from bovine intestinal mucosa (14 µg/µL = 14 mg/mL, 3507 units/mg protein) was diluted in 1X color development buffer [tris(hydroxymethyl)aminomethane] by 10-folds(to 1.4 µg/µL), 100-folds (to 0.14 µg/µL), 1000-folds (to 0.014 µg/µL), and 10000-folds (to 0.0014 µg/µL) at room temperature. The pH of the 1X color development buffer was 8.0.

The Bio-Rad alkaline phosphatase substrate kit comprising the color reagents A (NBT) and B (BCIP), as well as the color development buffer, was stored at -20 ℃ until use. Immediately before the alkaline phosphatase activity assay, the substrate mixture was prepared by mixing 1 µL each of both reagent solutions with 100 µL of 1X color development buffer in a single tube at room temperature.

Identification of Suitable Matrix for Alkaline Phosphatase Tests. The filter paper and nitrocellulose membrane were used as the matrix for the colorimetric detection of alkaline phosphatase activity. A half (0.5) µl of 1.4 µg/µL (10-fold diluted solution) of alkaline phosphatase was spotted (0.7 µg/spot) on the nitrocellulose membrane. Subsequently, the same volume of the substrate mixture was spotted over the same areas to detect the alkaline phosphatase activity on the matrix. This procedure was repeated with 0.14, 0.014, and 0.0014 µg/µL alkaline phosphatase solutions. An identical experiment was carried out on filter papers. In a control experiment, the 1X color development buffer was spotted instead of an alkaline

phosphatase solution. The proposed mechanism of reaction for the formation of a purple-color complex from NBT and BCIP in the presence of alkaline phosphatase was shown in **Figure 2**.

Duration of Purple Color Development. For each of the various concentrations of alkaline phosphatase (1 µL spotted on filter paper), an equal volume (1 µL) of the substrate mixture was spotted, and color development was observed over 15 minutes.

Inhibition of Alkaline Phosphatase by Metal Ions. In the first set of trial, onto the filter paper which had 1 µL of 1.4 µg/µL alkaline phosphatase solution (10-fold diluted solution) spotted on, an equal volume (1 µL) of 5 mM aqueous solution of $CuSO_4$, $Ni(C_2H_3O_2)_2$, $Ca(OAc)_2$ or $MgCl_2$ was spotted. Thereafter, 1 µL of substrate mixture was spotted onto the same spot and the development of the color was observed over 5 min.

In the second set of trial, onto the filter papers on which 1 µL of 0.14 and 0.014 and µg/µL alkaline phosphatase solution (100- and 1000- fold diluted solutions, respectively) were spotted, 1 µL of 5 mM aqueous solution of $HgCl_2$, $CuSO_4$, $Zn(CH_3CO_2)_2$, $Ni(C_2H_3O_2)_2$, $MgCl_2$, $Ca(OAc)_2$, or $CdCl_2$ was spotted. Thereafter, 1 µL of substrate mixture was spotted onto the same spot and the development of the color was observed over 5 min. The procedure for metal-ion detection was summarized in **Figure 5**.

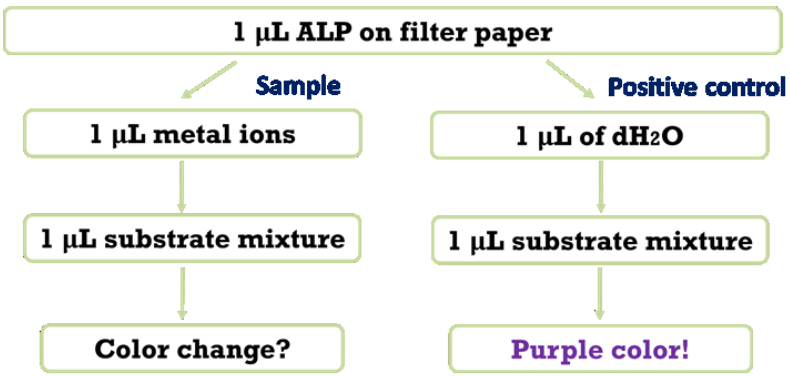


Figure 5. Procedure for metal-ion detection.

Alkaline Phosphatase Inhibition by Hg(II), Cu(II), and Cd(II). A series of solutions containing different concentrations of $HgCl_2$, $CuSO_4$, or $CdCl_2$ (0.01, 1.0, 2.0, 3.0, 4.0, and 5.0 mM) were prepared by dilution from their 5 mM aqueous stock solutions. These concentrations were selected for this preliminary screening to establish a rough idea on the dynamic range of the assay. The metal ion solutions (1 µL) were spotted onto the areas on the filter paper on which 1 µL of 1.4, 0.14 and 0.014 µg/µL alkaline phosphatase solution (10-, 100-, and 1000-fold diluted solutions) were spotted. Thereafter, 1 µL of substrate mixture was spotted onto the same spot and the development of the color was observed over 5 min. The ImageJ software was used to quantify the color intensities on each spot.

Part II: Design of Plasmid for the Expression of Alkaline Phosphatase-Cellulose Binding Domain Fusion Protein

Entry DNA (Eco-ALP_YS-S1-CBD) Design. The DNA sequence of the alkaline phosphatase-cellulose binding domain fusion protein (**Appendix C**) was a fusion of the sequence of *E.coli* alkaline phosphatase (**Appendix A**) and the *cipB* gene that encodes the family-3a cellulose-binding domain (CBD) from the cellulosome of *Clostridium thermocellum* (**Appendix B**).[44] The sequence of *E.coli* alkaline phosphatase on pSANG14-3F vector plasmid (plasmid number 39265)[36] were retrieved from the Addgene website. The sequence of *cipB* gene was retrieved from the UniProt database.[43] The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to confirm the DNA sequences of these proteins.

The DNA sequences of alkaline phosphatase and the cellulose-binding domain (CBD) were connected by a short linker sequence

(GGTAGCGGTAGCGCTTCGAGACCACACGAAGACAGCATGTAGCACCAACACCCCG), similar to the natural linker in cellulolytic enzymes. The codons of the linkers were selected by partly using the GenScript Codon Usage Frequency Chart. Customized flanking overhangs were inserted on several sites of the DNAs to allow Golden Gate cloning of the DNA fragments in the future work. A His6 tag sequence was inserted at the C-terminus. The process was represented in **Figure 6**.

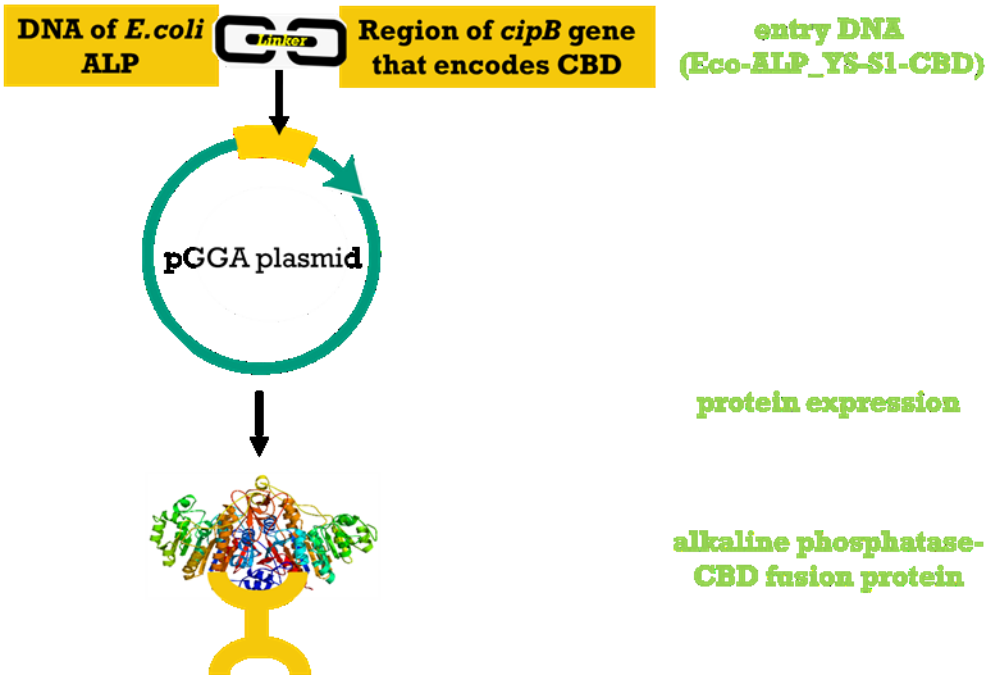


Figure 6. Entry DNA (Eco-ALP_YS-S1-CBD) inserted into plasmid for the expression of alkaline phosphatase-cellulose binding domain fusion protein.

The designed entry DNA was chemically synthesized and cloned into a pUC57 vector by GenScript Biotech Cooperation. The pUC57 plasmid that encodes the DNA sequence of the alkaline phosphatase-cellulose binding domain fusion protein was named pUC57_Eco-ALP_YS-S1-CBD plasmid. The entire sequence of the cloned DNA was confirmed by DNA sequencing.

The designed DNA sequence was subcloned into pGGA plasmid (New England Biolabs (NEB)) by GenScript Biotech Corporation. The pGGA plasmid that encodes the DNA sequence of the alkaline phosphatase-cellulose binding domain fusion protein was named pGGA_Eco-ALP_YS-S1-CBD plasmid.

General Procedure for Isolation of Plasmid DNAs

Preparation of LB Agar Plate. LB plates containing 50 μg/mL of antibiotics (chloramphenicol or kanamycin) were prepared by dissolving 2.50 g of pre-mixed LB powder in 100 mL of ddH_2O (~10 plates for 100 mL mixture). To this solution, 1.50 g of 1.5 % w/v of bacteriological agar was added and sterilized by autoclaving for 30 minutes at 120 °C. Before the obtained agar solution solidified, 100 μL of an appropriate antibiotic stock solution (50 mg/mL) was added to the mixture, swirled, and poured into sterile Petri dishes in about 10.0 mL aliquots. Once the agar was solidified, and the Petri dishes were sealed with Parafilm® and stored at 4 °C until use.

Transformation of Competent *E. coli* BL21(DE3) Cells by Plasmid. Chemically ($CaCl_2$) competent *E. coli* BL21(DE3) cells stored at -80 ℃ were thawed on ice for 15 minutes. One microliter (1.0 μL) of solution containing plasmid DNAs was added to 50 μL each of the competent cell suspensions in 1.5 mL microcentrifuge tubes. The mixture was incubated on ice for 10 minutes and immediately immersed in a water bath preheated at 42 ℃ for 45 seconds ('heat shock'). The tubes were placed on an iced bath for 8 minutes. LB media (100 μL) was added to the mixture and incubated on an orbital shaker (Boekel Scientific Flask Dancer) at 200 rpm for 45 minutes at 37 ℃. The cell suspension (100 μL each) was transferred onto LB agar plates containing 50 μg/mL of an appropriate antibiotic (chloramphenicol or kanamycin) and

incubated overnight at 37 ℃ or for 3 days at room temperature. Once the formations of bacterial colonies on the LB agar plates were confirmed, the plates were sealed with Parafilm® and stored at 4 ℃.

Isolation of Plasmid DNAs. Aliquots of LB media (1.5 mL) were pipetted into 13 mm x 100 mm Pyrex® glass test tubes under aseptic conditions. An antibiotic stock solution (0.75 µL of chloramphenicol stock solution or 1.5 µL of kanamycin) was added into the LB media to obtain a final concentration of 25 µg chloramphenicol/mL medium or 50 µg kanamycin/mL medium. A sterile toothpick was used to pick a single colony from the plate, lightly spotted unto a clean LB agar plate for a replica of the colony, and finally dropped into the 1.5 mL LB media. The inoculated media was incubated at 37 °C on an orbital shaker shaking at 300 rpm (Boekel Scientific Flask Dancer) for about 24 hours. The inoculated plates were incubated overnight at 37 ℃. E.Z.N.A® Plasmid DNA Mini Kit I was used to isolate an estimated amount of 25 µg of plasmid DNA from 1.5 mL *E. coli* culture(s). The *E. coli* cells were collected by transferring the cell culture into a 1.5 mL sterile microcentrifuge tube(s) and centrifuged at 10,000 rpm on the Mikro 22R Hettich Zentrifuge with an aerosol-tight lid on the rotor, at 24 °C for 1 minute. After the supernatant(s) was discarded by decantation, the microcentrifuge tube was dabbed with a paper towel to absorb the remaining liquid in the microcentrifuge tube. The pellet(s) was suspended in 250 µL of the Mini-Prep Solution I containing RNase and flicked till it was well mixed. A volume of 250 µL of Mini-Prep Solution II (lysis buffer) was added and the microcentrifuge tube(s) was inverted several times to obtain a clear lysate. The mixture was then incubated for 2-3 minutes. Next, 350 µL of Mini-Prep Solution III (neutralization buffer) was added and inverted until a flocculent white precipitate was formed. The sample(s) was centrifuged at 10,000 rpm for 10 minutes. During the period of centrifugation, an HiBind® DNA

Mini Column(s) was inserted into a 2.0 mL collection tube and equilibrated with 100 µL of 3 M NaOH. The clear supernatant from the sample(s) was transferred into the equilibrated DNA Mini Column and centrifuged at 10,000 rpm for 60 seconds. The filtrate was discarded, and the collection tube was reused. A 500 µL portion of HBC buffer with isopropanol was added to the column(s) and centrifuged at 10,000 rpm for 60 seconds. The filtrate was discarded. The column(s) was washed twice with 700 µL of DNA wash buffer containing ethanol and span at 10,000 rpm for 30 seconds. The filtrate was discarded. Finally, the column(s) was centrifuged for 2 minutes at 10,000 rpm to dry. For elution, the column(s) was placed in a 1.5 mL nuclease-free microcentrifuge tube(s) and plasmid DNA was eluted with 30 µL of sterile deionized water at 10,000 rpm spin for 60 seconds. The eluted DNA was stored at -20 ℃ and the working area was decontaminated with 70 % ethanol. The outline of the procedure was summarized in **Figure 7**.

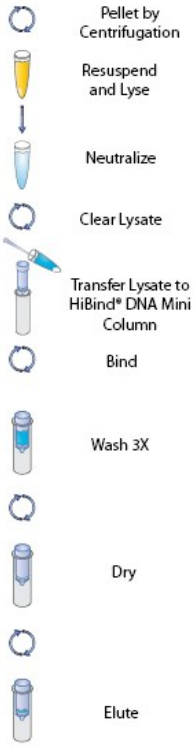


Figure 7. Steps for plasmid purification with E.Z.N.A® Plasmid DNA Mini Kit I. Reprinted from reference 45.

All solid and liquid wastes containing the recombinant DNAs as well as *E. coli cells* harboring the recombinant DNAs were carefully disposed of in properly labeled and sealed containers and decontaminated by autoclave before leaving the designated lab area for permanent disposal. Accidental spills were immediately cleaned with 0.53 % of a hypochlorite solution.

General Procedure for Confirming Plasmid Sizes by Agarose Gel Electrophoresis

Restriction Enzyme Digestion. The purified plasmid(s) was digested by mixing 7 µL sterile DI water, 1 µL of 10X Fast Digest Buffer, 1 µL of the plasmid sample, and 1 µL of restriction enzyme (*Bam*HI or *Pst*I) stock solution in a PCR tube. The solution(s) was incubated at 37 °C in the Applied Biosystems Veriti 96 Well Plate Thermal Cycler for 1 hour. The sample(s) was cooled at room temperature for electrophoretic analysis.

Agarose Gel Electrophoresis. The procedure below was followed to carry out the gel electrophoresis.

Agarose Gel Preparation (for 8-well agarose gels). The agarose powder (0.25 g) was weighed into a 125 mL Erlenmeyer flask and dissolved with 25 mL of TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.3) by heating in a microwave oven for 30 seconds. The solution was cooled and immediately poured into a casting tray. A comb was inserted into the tray to create wells. After about 30-50 minutes when the gel was solidified, the comb was gently removed from the gel and the casting tray containing the gel was placed in a Mupid®-2 plus Submarine electrophoresis system. The gel was positioned with the wells at the cathode. The electrophoresis chamber was filled with TAE buffer to a level slightly above the surface of the gel.

Electrophoresis. The 10 µL digested plasmid sample(s) was mixed with 2 µL of Gel Loading Dye, Purple (6X) to yield 1X dye concentration, and loaded into the wells. The DNA standard was a mixture of 1 µL of 1 kb DNA ladder (New England Biolabs® Inc.), 2 µL of Gel Loading Dye, Purple (6X), and 9 µL of sterile DI water. After loading all samples, the electrophoresis chamber was closed and run for about 30 minutes until the dye was seen at the opposite end of the gel. The gel was then immersed in a solution of SYBR® Safe DNA gel stain and incubated for about 30 minutes. The gel was viewed on a dark reader and the image was captured with a camera.

General Procedure for SDS-PAGE

Sample Preparation for Analysis of Whole Cell Lysate. The 0.5 µL protein-induced *E. coli* suspension (whole cell) in the 1.5 mL centrifuge tube was used for SDS-PAGE analysis. The suspension was span at 3, 000 rpm for 2 minutes, and the supernatant was discarded. The pellet was resuspended in 12.5 µL of 4X SDS sample loading buffer and 37.5 µL of dH_2O to obtain a final concentration of 1X SDS sample loading buffer. The total volume of the mixture was 50 µL.

Sample Preparation for Purified Protein Samples. The purified portions (the flow-through, wash buffer, and elution fractions; 7.5 µL each) were mixed with 2.5 µL of 4X SDS sample loading buffer to yield a total volume of 10.0 µL. All samples were heated in hot water at 90 ℃ for 10 minutes and centrifuged afterward, at 15, 000 rpm for 5 minutes.

SDS-PAGE. The procedure below was followed to carry out the analysis of proteins by SDS-PAGE gel electrophoresis.

Preparation of Polyacrylamide Gels (for a 15-well gel). For each gel, a large 1.0 mm spacer plate and a smaller glass plate were assembled in the casting frame with the former positioned at the rear end, and the latter at the front of the frame. The comb was placed between both plates and used to mark the maximum level for the resolving gel. The TGX™ FastCast™ Acrylamide Starter Kit was used. Ammonium persulfate (10 %) was prepared by dissolving 24.3 mg of the powder in 243 µL of distilled water. The resolving layer comprised 6.0 mL each of resolver A and B solutions, 60 µL of 10 % APS, and 6 µL of TEMED to promote polymerization. The mixture was immediately pipetted into the space between the two gel plates to the marked level. The stacking layer was a mixture of 2 mL each of stacker A and B solutions, 20 µL of 10 % APS, and 4 µL of TEMED. The solution was quickly transferred into the space between the two gel plates to the brim. The 15-well comb was inserted into the stacking gel and allowed to solidify for 30 minutes. The gels were stored in the cold room at around 4 ℃.

Electrophoresis. The precast or hand-casted gel was placed in the vertical gel electrophoresis apparatus and filled with 1X SDS running buffer until the bottom of the gel was in contact. Ten (10) µL of the supernatant of the centrifuged samples, as well as the Precision Plus Protein™ All Blue Prestained Protein Standards (BIO-RAD), were loaded into the wells of the SDS gel. The apparatus was run at a constant voltage of 100 V until the bromophenol blue electrophoretic marker in the wells was seen at the bottom of the gel. The gel was carefully removed for staining.

Staining and Destaining of polyacrylamide gels. The gel was submerged in a prestaining solution (20% methanol, 7.5% glacial acetic acid, 72.5% DI water) (v/v/v) for 15 minutes. It was then transferred into a staining solution (125 mL methanol, 12.5 mL glacial acetic acid, 112.5 mL DI water, and 0.625 g Coomassie Brilliant Blue R-250) for 30 minutes. Finally, it was

immersed in a destaining solution (12.5 mL methanol, 17.5 mL glacial acetic acid, and 220 mL DI water) and incubated overnight. The stained gel was placed on a white light transilluminator and an image of it was taken with a camera.

Attempt to Express Alkaline Phosphatase-Cellulose Binding Domain Fusion Protein without a Signal Peptide in *E. Coli* with pGGA_Eco-ALP_YS-S1-CBD Plasmid

Synthesis of Eco-ALP_YS-S1-CBD Gene and Subcloning into pUC57 and pGGA Plasmids. The structural gene of an alkaline phosphatase-cellulose binding domain fusion protein (Eco-ALP_YS-S1-CBD) was synthesized and subcloned into pUC57 and pGGA plasmids by GenScript Biotech Corporation. The sequence of the cloned DNA sequence was confirmed by GenScript Biotech Corporation. The synthesized plasmids were named as pUC57_Eco-ALP_YS-S1-CBD plasmid and pGGA_Eco-ALP_YS-S1-CBD plasmid, respectively. The plasmids were delivered as dry samples.

Transformation of *E. coli* BL21(DE3) Competent Cells by the pGGA_Eco-ALP_YS-S1-CBD plasmid. One microliter (1 µL) of the pUC57_Eco-ALP_YS-S1-CBD or pGGA_Eco-ALP_YS-S1-CBD plasmid solution was used to transform chemically ($CaCl_2$) competent *E. coli* BL21(DE3) cells. The plasmid DNAs were isolated from several *E.coli* colonies, digested with *Bam*HI, and analyzed by agarose gel electrophoresis as described in the previous section.

Protein Expression with IPTG induction. Pre-mixed LB powder (2.5 g) was dissolved in 100 mL of distilled water in a 500 mL Erlenmeyer flask and autoclaved. Five (5) mL of the sterile LB media was transferred into a glass test tube(s) and 5 µL of 50 mg/mL chloramphenicol was added to obtain a final antibiotic concentration of 50 µg/mL. The solution was inoculated with a colony of *E. coli* BL21(DE3) strain harboring pGGA_Eco-ALP_YS-S1-CBD plasmid and

incubated at 37 °C for 8 hours on an orbital shaker shaking set at 300 rpm (Boekel Scientific Flask Dancer). IPTG stock solution (100 mM) with volume 2.5 µL was added to the culture (final concentration of IPTG: 0.05 mM). The culture was then further incubated at room temperature overnight on an orbital shaker set at 300 rpm. The obtained cell culture was transferred into a 15 mL centrifuge tube and span at 3000 rpm for 15 minutes. The supernatant was decanted, and the pellets were washed with 6 mL of 0.9% NaCl by resuspension and centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded, and the pellets were stored in a -80 °C freezer unless used immediately.

Protein Expression by Overnight Express™ Autoinduction System. Sterile glucose-free LB media (5 mL) was pipetted into a glass test tube(s). Five (5.0) µL of 50 mg/mL chloramphenicol was added to the media to make a final antibiotic concentration of 50 µg/mL. To this medium, 108.0 µL of OnEx Solution 1 containing 0.5% glycerol (w/v), 0.05% glucose (w/v), and 0.2% α-lactose(w/v) as carbon sources, 269.0 µL of OnEx Solution 2 containing 50 mM Na_2HPO_4, 50 mM KH_2PO_4, and 25 mM $(NH_4)_2SO_4$ for metabolic acid production and as nitrogen source, and finally, 5.3 µL of OnEx Solution 3 containing 2 M $MgSO_4$ to improve maximum cell density were added. The prepared medium was inoculated with an *E. coli* colony harboring the pGGA_Eco-ALP_YS-S1-CBD plasmid and incubated on an orbital shaker shaking at 300 rpm overnight at 30 °C. An approximate volume of 1.5 mL of the *E. coli* culture was used for SDS-PAGE analysis. The remaining culture (about 3.5 mL) was transferred into a 15 mL centrifuge tube and span at 3000 rpm for 2 minutes at 4 ℃. The supernatant was discarded, and the pellets were stored in the -80° C freezer.

SDS-PAGE Analysis. The *E. coli* suspension (1.5 mL) was transferred into 1.5 mL centrifuge tube(s) from the larger protein-induced culture was used for SDS-PAGE analysis by

using a precast polyacrylamide gel. The details of the procedure in the subsequent steps of SDS-PAGE analysis are described in the previous section.

Expression of PelB-Alkaline Phosphatase

Isolation of DH5α/pSANG14-3F single clones. DH5α cells harboring pSANG14-3F plasmid was a gift from John McCafferty (Addgene plasmid # 39265; http://n2t.net/addgene:39265; RRID: Addgene_39265). This plasmid encodes the sequence of *E.coli* alkaline phosphatase including the N-terminus pelB signal peptide. The *E. coli* strain was delivered as an agar stab. To isolate single colonies, the *E.coli* cells on the agar stab was streaked on an LB agar plate containing 50 μg/mL of kanamycin. Once the formations of bacterial colonies on the LB agar plates were confirmed, the plates were sealed with Parafilm® and stored at 4 ℃.

Isolation of pSANG14-3F plasmid DNAs. Several single colonies of DH5α/pSANG14-3F cells were cultured in LB media (5.0 mL) containing 50 μg/mL of kanamycin on an orbital shaker shaking at 300 rpm (Boekel Scientific Flask Dancer) overnight at 37 ℃. E.Z.N.A® Plasmid DNA Mini Kit I was used to isolate the pSANG14-3F plasmid DNA from the 5.0 mL *E. coli* culture(s).

Analysis of pSANG14-3F Plasmid DNAs by Agarose Gel Electrophoresis. The purified plasmid(s) was digested by mixing 7 μL sterile DI water, 1 μL of 10X Fast Digest Buffer, 1 μL of the plasmid sample, and 1 μL of Fast Digest restriction enzyme (*Pst*I) in a PCR tube. The solution(s) was incubated at 37 °C in the Applied Biosystems Veriti 96 Well Plate Thermal Cycler for 10 minutes. The sample(s) was cooled at 25 ℃. The plasmid DNAs were analyzed by agarose gel electrophoresis as described in the previous section

Transformation of *E. coli* BL21(DE3) Competent Cells by pSANG14-3F plasmid. One microliter (1 µL) of the plasmid solution was added to 50 µL of chemically competent *E. coli* cells by following the procedure described for the transformation by pUC57_Eco-ALP_YS-S1-CBD and pGGA_Eco-ALP_YS-S1-CBD plasmids except for the use of LB agar plate containing 50 µg/mL of kanamycin.

Protein Expression by Overnight Express™ Autoinduction System. Sterile glucose-free LB media (5 mL) was pipetted into a glass test tube(s). Five (5.0) µL of 50 mg/mL kanamycin was added to the media to yield a final antibiotic concentration of 50 µg/mL. To this medium, OnEx Solution 1 (108.0 µL) containing 0.5% glycerol (w/v), 0.05% glucose (w/v), and 0.2% α-lactose(w/v), OnEx Solution 2 (269.0 µL) containing 50 mM Na_2HPO_4, 50 mM KH_2PO_4, and 25 mM $(NH_4)_2SO_4$, and OnEx Solution 3 (5.3 µL) containing 2 M $MgSO_4$ were added. The prepared medium was inoculated with an *E. coli* colony harboring the pSANG14-3F plasmid and incubated on an orbital shaker shaking at 300 rpm at 30 °C for 16 hours. An approximate volume of 0.1 mL of the *E. coli* culture was evenly aliquoted into three microcentrifuge tubes for activity test. Another portion (0.5 mL) was used for SDS-PAGE analysis. The remaining culture (about 4.2 mL) was transferred into a 15 mL centrifuge tube and span at 3000 rpm for 2 minutes at 4 ℃. The supernatant was discarded. The cells were washed with 5 mL of 0.9% NaCl and centrifuged at 3000 rpm for 2 minutes at 4 ℃. The supernatant was discarded. A part of the pellet from the 0.1 mL aliquot was suspended in alkaline phosphatase (1X) color development buffer (100 µl) for activity tests. One (1.0) microliter of the suspension was spotted unto a filter paper. An equal volume (1.0 µL) of alkaline phosphatase substrate mixture was spotted unto the enzyme and observed for color development.

Extraction of Proteins and Characterization of Crude Protein Samples

Protein Extraction. The harvested *E. coli* cell cultures were resuspended in an appropriate buffer in a 15 mL centrifuge tube. For BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD cells that were induced by IPTG and Overnight Express™ autoinduction system, PBS (2.5 mL) was used to resuspend the pellets. For BL21(DE3)/pSANG14-3F cells, 2.5 mL of equilibration buffer (25 mM sodium phosphate buffer, 500 mM NaCl, 10 mM imidazole, pH 7.0) was used to resuspend the cells. All cell suspensions were kept on ice to maintain a low temperature during sonication. The probe of the sonicator was inserted into the cell suspension and used to lyse the cells at an amplitude of 50. This was done in five sets; 30 seconds each with 1-minute pauses to allow the probe to cool. The lysed sample was then centrifuged at 15,000 rpm at 4 ℃ for 15 minutes. The supernatant was transferred into 2.0 mL microcentrifuge tubes for protein purification. The cell lysate (pellets) was stored at -20 ℃.

Alkaline Phosphatase Activity Test. One microliter of the supernatant from the cell lysate was spotted unto a filter paper. An equal volume (1 µL) of alkaline phosphatase substrate mixture was spotted unto the enzyme and observed for color development.

Bradford Assay. Protein concentrations in the cell lysates were determined by Bradford assay using a 96-well microplate on a microplate reader. Bovine Serum Albumin (BSA) standards were prepared from 0.8 mg/mL of stock BSA solution and diluted with potassium phosphate buffer to 0, 8.0, 20.0, 40.0, 60.0, and 80.0 µg/mL concentrations. The cell lysates were diluted by 10- and 100-folds by serial dilution. The standards and diluted cell lysates (160 µL) were added individually into the wells of a 96-well microplate. Forty microliters (40 µL) of Bio-Rad Protein Assay Dye Reagent Concentrate was added to each well and incubated for about 5 to

10 minutes. The absorbances of the standards and the two samples were read at 595 nm and the values were recorded.

Purification and Characterization of PelB-Alkaline Phosphatase

Protein Purification. PelB-alkaline phosphatase was further purified from the crude cell lysate of BL21(DE3)/pSANG14-3F. For this, 1 mL spin columns were packed with 150 µL of Ni^{2+}-NTA slurry, placed in 1.5 mL centrifuge tubes, and span by centrifugation at 1000 rpm for 1 minute to prepare columns with a resin bed of approximately 75 µL in gel volume. The equilibration buffer (300 µL) which was twice the volume of the Ni^{2+}-NTA resin, was run through the column and span by centrifugation at 1000 rpm for 1 minute. This procedure was repeated twice. The flow-through (supernatant from the protein extraction step) was loaded (500 µL) into the column and centrifuged four times at 1000 rpm for 1 minute. The weakly associated proteins were washed off with 300 µL of wash buffer (25 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.0) and span at 1000 rpm for 1 minute. One hundred (100) microliters of elution buffer (25 mM sodium phosphate buffer, 300 mM NaCl, 250 mM imidazole, pH 7.0) was pipetted into the column and centrifuged at the same speed, for 1minute. Each eluate was collected in a clean microcentrifuge tube. One microliter (1 µL) of the eluate was used for activity test with an equal volume of the alkaline phosphatase substrate mixture. The elution step was repeated until a fraction gave no purple color after the activity test. This step was repeated seven times in total until the final and the penultimate fractions showed no activity. Finally, 100 µL of 20 mM MES-NaOH buffer at pH 5.0 was pipetted into the column and centrifuged at 1000 rpm for a minute. A summary of the protein purification was summarized in **Figure 8**. The eluted fractions in the final step (7.5 µL) were pipetted into clean

microcentrifuge tubes for SDS-PAGE analysis by using a hand casted polyacrylamide gel. The remaining amounts were dialyzed using a 12-14 kD dialysis membrane (molecular weight of the protein was around 47 kD) in 300 mL of 10 mM Tris-HCl buffer, pH 8.0, at 1 ℃ and changed thrice after every 8 hours. All fractions from the purification steps, including the dialyzed fractions, were stored at 4 ℃.

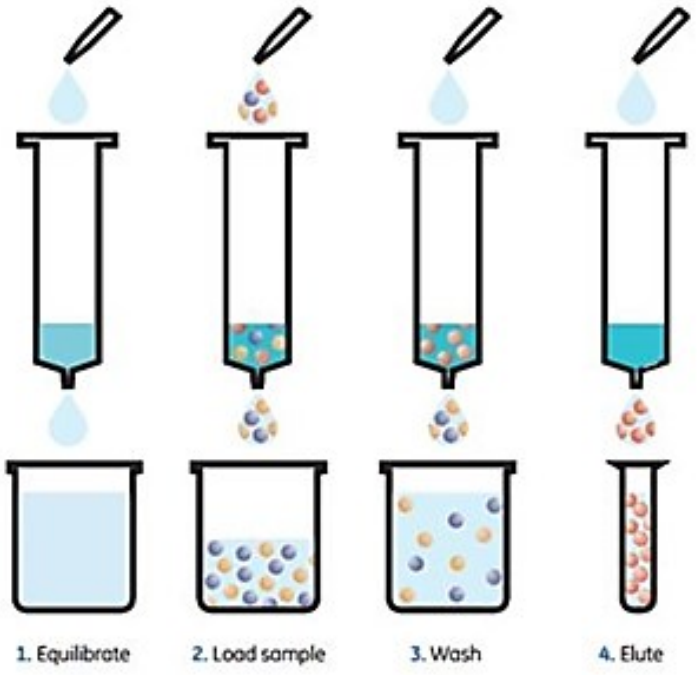


Figure 8. Scheme of purifying a His-tagged alkaline phosphatase using Ni2+-NTA. Reprinted from reference 46.

Alkaline Phosphatase Activity Test. The dialyzed fractions were tested for enzyme activity. One (1.0) microliter of each fraction was spotted unto a filter paper. An equal volume (1.0 µL) of the alkaline phosphatase substrate mixture was spotted unto the enzyme and observed for color development.

Bradford Assay. The protein concentrations of fractions from affinity column chromatography were determined by following the Bradford assay procedure described in the previous section. The procedure was mostly identical except for the concentration of bovine serum albumin (BSA) standards. In this study, the BSA standards were prepared by diluting a stock BSA solution (0.53 mg/mL) with potassium phosphate buffer to 0.0, 5.30, 13.25, 26.50, 39.75, and 53.0 μg/mL.

SDS-PAGE Analysis. The fractions from the Ni^{2+}-NTA affinity column chromatography were analyzed by SDS-PAGE with a precast polyacrylamide gel. The details of the procedure in the subsequent steps of SDS-PAGE analysis are described in the previous section.

RESULTS

Part I: Metal-Ion Detection by the Inhibition of Commercially Available Alkaline Phosphatase

Suitable matrix for alkaline phosphatase tests. The results of the colorimetric tests performed on the filter paper and nitrocellulose membrane are shown in **Figure 9.** On the filter paper, a purple color was uniformly spread in all areas which had the tested concentrations of alkaline phosphatase (14.0, 1.4, 0.14, 0.014, and 0.0014 μg/μL) spotted on. The purpose of the varied alkaline phosphatase concentrations was to enhance sensitivity and to yield reliable results with a minimum amount of enzyme. The purple color intensity decreased with increased dilution as expected. The negative control which had a 1X color development buffer in place of the enzyme did not yield a purple color in the presence of the substrate. This confirmed that the formation of the purple color with NBT and BCIP substrate mixture was due to the activity of alkaline phosphatase. For the nitrocellulose membrane, the purple color intensities which were observed by eyes were inconsistent with the concentrations of the enzyme and non-uniformly spread. Based on these results, subsequent tests were performed by using filter papers.

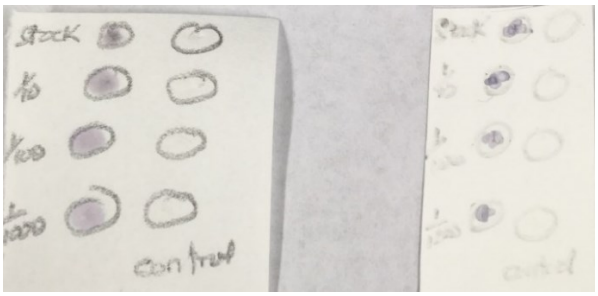


Figure 9. Colorimetric detection of alkaline phosphatase on filter paper (left) and nitrocellulose membrane (right). Amount of enzyme spotted within each circle: 1 μL of enzyme solution (stock: 14 μg/μL, 1/10: 1.4 μg/μL, 1/100: 0.14 μg/μL, and 1/1000: 0.014 μg/μL). No enzyme was spotted to control columns.

Duration of purple color development. For each of the concentrations of alkaline phosphatase, color development over time was visually observed. There was a slight increase in purple color intensity up to 5 minutes. After that, the color of each spot remained constant. A plausible explanation of this could be the evaporation of the aqueous solutions on the paper which stopped the reaction. The result was represented in **Figure 10.** The 0.0014 µg/µL alkaline phosphatase sample (10000-fold diluted enzyme solution) gave no purple color even after 15 minutes, implying that it was outside the appropriate concentration for this assay.

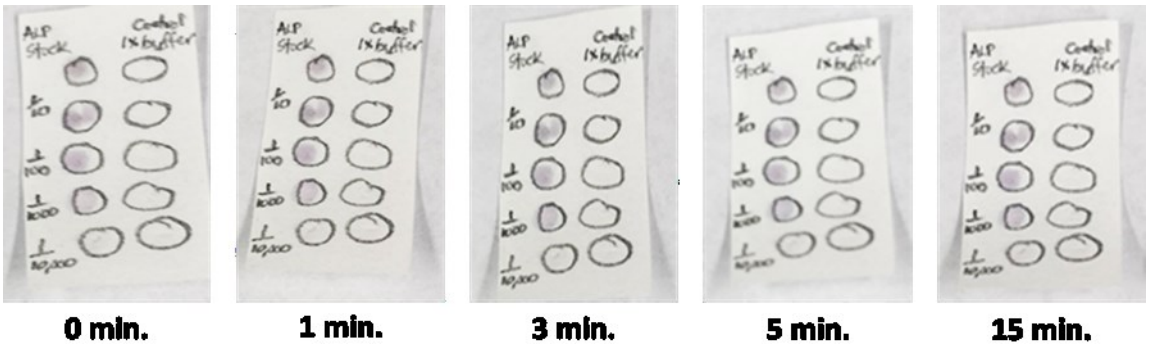


Figure 10. Purple color development with varied alkaline phosphatase concentrations. The stock concentration of the enzyme was 14 µg/µL. The enzyme concentration of 10-, 100-, 1000-, and 10000-fold diluted alkaline phosphatase samples were 1.4, 0.14, 0.014, and 0.0014 µg/µL, respectively. The amount of enzyme and metal ion solution spotted within each circle was 1 µL.

Inhibition of alkaline phosphatase by metal ions. In this experiment, 1.4, 0.14, and 0.014 µg/µL of alkaline phosphatase solution (10-, 100-, and 1000-fold diluted enzyme solutions) were spotted onto the filter paper. In our first trial, the inhibition of the enzyme (1.4 µg alkaline phosphatase per spot, 1 µL of 10-fold diluted enzyme solution) by aqueous solutions containing 5 mM $CuSO_4$, $Ni(C_2H_3O_2)_2$, $Ca(OAc)_2$, or $MgCl_2$ was investigated. The spot with the enzyme without metal ion was used as the positive control whereas the spot without the enzyme was the negative control. As can be seen in **Figure 11**, a decrease in the purple color intensities could be

visually observed by eyes on the spots with some of the metal ions, indicating the inhibition of ALP by metal ions. Cu(II) inhibited the 1.4 μg/μL alkaline phosphatase solution (10-fold diluted solution) and gave the lightest purple color, followed by Ni(II). Ca(II) and Mg(II) were found to be the least effective inhibitors.

The experiment with 0.14 μg/μL and 0.014 μg/μL alkaline phosphatase solution (100-fold and 1000-fold diluted solution) were performed on a different day by using enzyme samples that are diluted freshly from the stock solution. The positive controls in these trials developed a more intensive purple color than the corresponding control with a 10-fold diluted enzyme sample. We attributed the lower activity with 10-fold diluted enzyme sample to the inactivation of the diluted enzymes during the storage. For the areas with 0.14 μg/μL alkaline phosphatase solution (100-fold diluted solution), an aqueous solution of 5 mM $HgCl_2$, $CuSO_4$, $Zn(CH_3CO_2)_2$, $Ni(C_2H_3O_2)_2$, $MgCl_2$, $Ca(OAc)_2$, or $CdCl_2$ was spotted over the enzyme. It was observed that Hg(II) was the strongest inhibitor, followed by Cu(II), Zn, Ni, Mg, Ca, and Cd(II). Cd(II) was the least effective inhibitor (**Figure 11**). The areas with 0.014 μg/μL alkaline phosphatase solution (1000-fold diluted enzyme solution) were inhibited in the same manner as the 0.14 μg/μL alkaline phosphatase (100-fold diluted enzyme solution). Similarly, Hg(II) was the strongest inhibitor, followed by Cu(II), and Cd(II). The other metals gave inconclusive results. The results were also represented in **Figure 11**. As expected, the negative control had no purple color due to the absence of the enzyme in the presence of the substrate mixture.

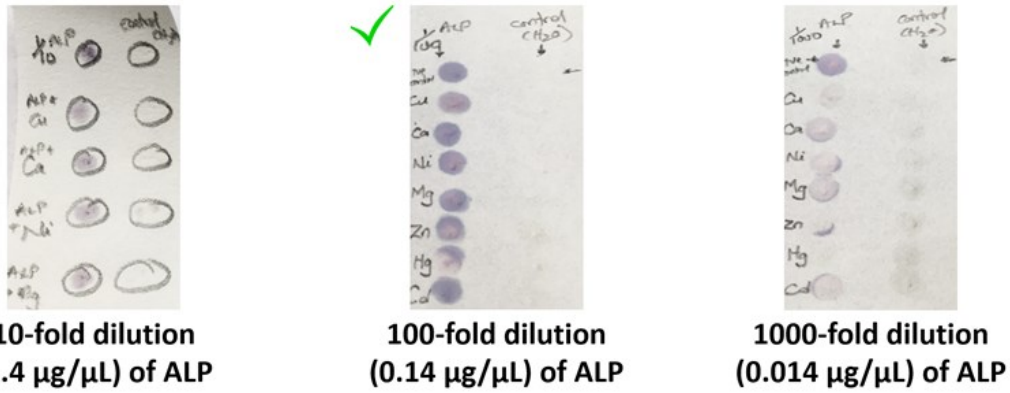


Figure 11. Inhibition of 10-fold (left), 100-fold (middle), and 1000-fold (right) diluted alkaline phosphatase (1.4, 0.14, and 0.014 µg/µL)ALP respectively by various metal ions. The amount of enzyme and metal ion solution spotted within each circle was 1 µL.

ALP inhibition by Hg(II), Cu(II), and Cd(II). After observing that 5 mM of Hg(II) and Cu(II) significantly inhibit the enzyme, inhibition of the enzyme by 0.01, 1.0, 2.0, 3.0, 4.0, and 5.0 mM Hg (II) and Cu(II) was studied. The purple color intensities were quantified with ImageJ software using the grayscale method where the mean gray values of the pixels in the selected regions were quantified. A highly inhibited region gives an 8-bit integer value closer to white (255) while a less inhibited region is darker in color with value lesser than 255. The results for mercury, copper, and cadmium are shown in **Figures 12**, **13**, and **14**, respectively. In most of the tested combinations, the differences between the color intensities of the spots for positive controls, and the spots with metal ions were not statistically significant ($p > 0.05$). Exceptions to this include the cases of the inhibition of the 1000-fold diluted alkaline phosphatase (0.014 µg/µL) by 4 mM and 5 mM mercury ions, where p-values were 0.022 and 0.026, respectively (**Table 1**). This result was consistent with the result presented in Figure 11. The p-values for the inhibition of 10-fold diluted alkaline phosphatase (1.4 µg/µL) by 2 mM cadmium and 1000-fold diluted alkaline phosphatase (0.014 µg/µL) by 4 mM cadmium were also < 0.05. However, the higher concentration of cadmium did not show statistically significant differences from positive

controls. This made the conclusion somewhat ambiguous. Further experiments must be carried out to conclude if cadmium would inhibit the diluted alkaline phosphatase.

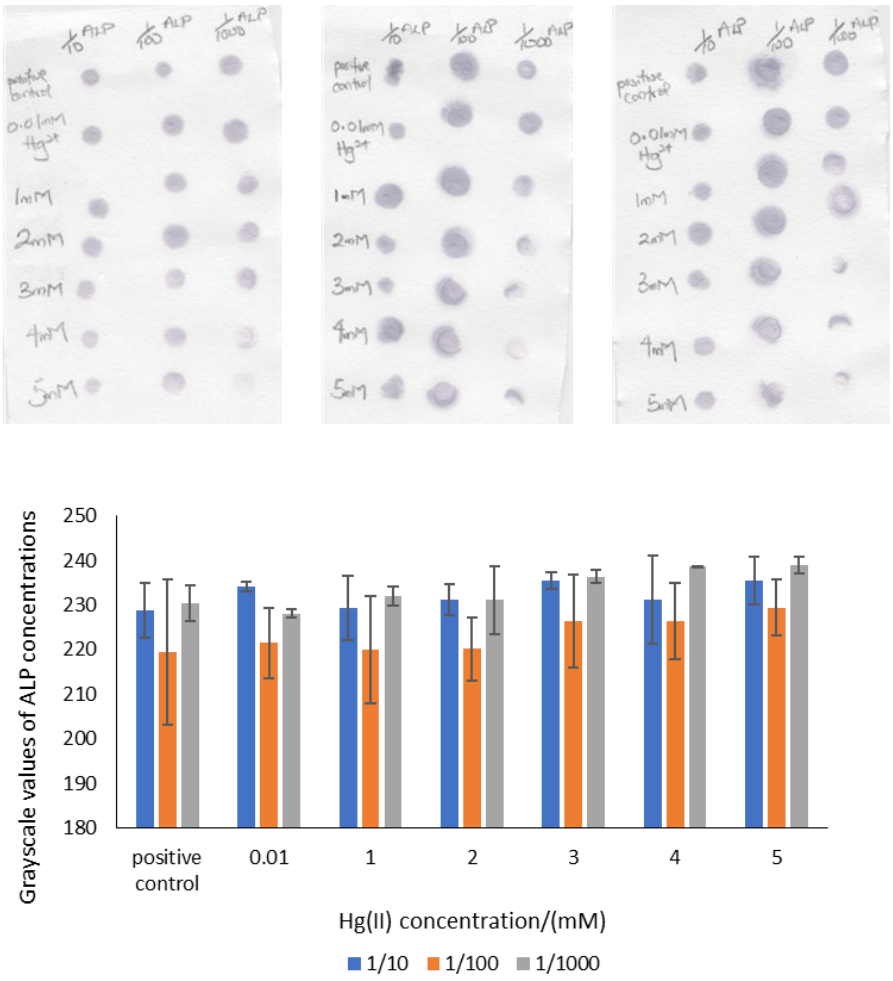


Figure 12. Inhibition of 1.4, 0.14 and 0.014 µg/µL alkaline phosphatase (10-, 100-, and 1000-fold diluted alkaline phosphatase solutions) by 0.01, 1.0, 2.0, 3.0, 4.0, and 5.0 mM of Hg (II) on filter paper (top) and graphical representation of metal inhibition by measurement of color intensity (bottom). The amount of enzyme and metal ion solution spotted within each circle was 1 µL.

Table 1. T-test for alkaline phosphatase inhibition by mercury (II).

[Hg^{2+}], mM	Grayscale values			p-value (vs positive control, t-test, single tailed, equal variance)
0 (positive control)	233.17	225.92	232.19	N.A.
0.01	227.56	229.15	227.45	0.367
1	234.08	229.76	232.30	0.566
2	237.25	222.63	233.55	0.892
3	236.13	237.92	235.04	0.070
4	238.46	238.80	238.66	0.022
5	238.26	237.74	241.18	0.026

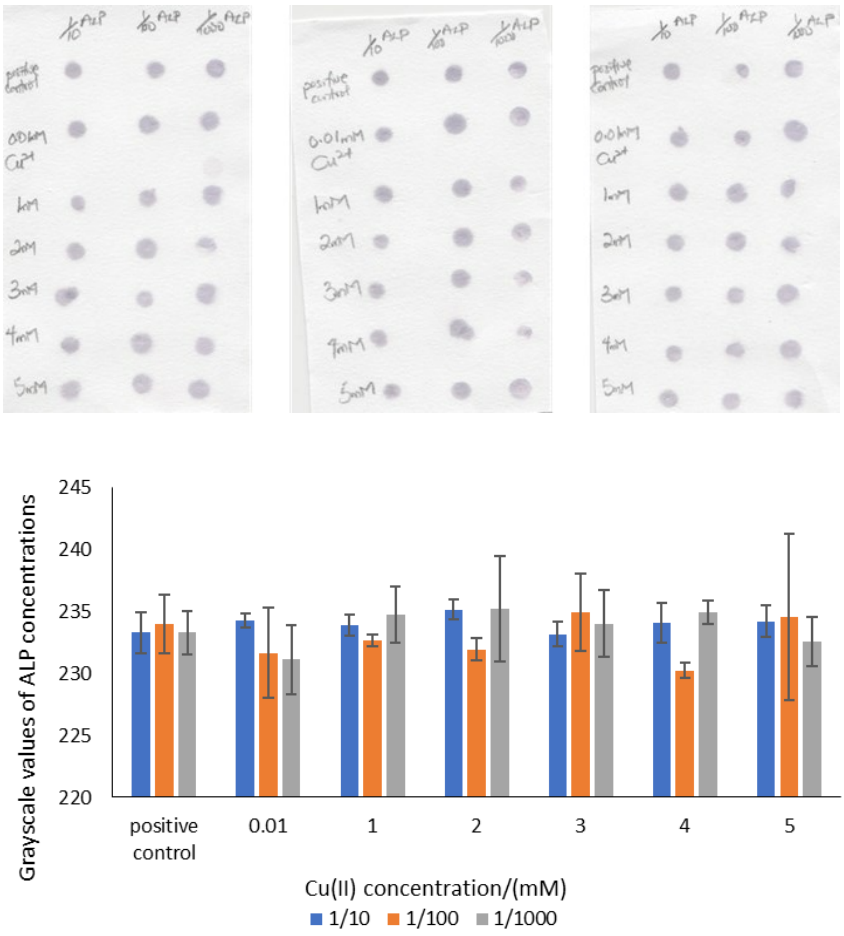
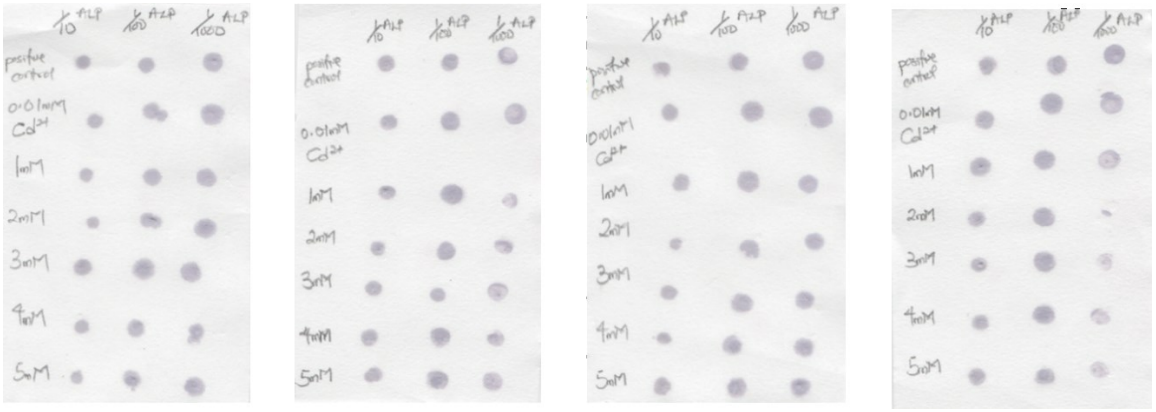


Figure 13. Inhibition of 1.4, 0.14 and 0.014 µg/µL (10-, 100-, and 1000-fold dilutions) of ALP by 0.01, 1.0, 2.0, 3.0, 4.0, and 5.0 mM of Cu (II) on filter paper (top) and graphical representation of metal inhibition by measurement of color intensity (bottom). The amount of enzyme and metal ion solution spotted within each circle was 1 µL.

Cd(II) at the same concentrations as Hg(II) and Cu(II), was used to inhibit 1.4, 0.14, and 0.014 µg/µL alkaline phosphatase (10-, 100-, and 1000-fold diluted) solutions and the result was represented in **Figure 14.** Quadruplicate measurements were performed, and the trend was comparable to Hg(II) and Cu(II). There was no statistically significant difference between the metal-inhibited enzymes and the positive control. When a t-test was performed, the 2- and 4-mM cadmium ions showed significant inhibition of the 10- and 1000-fold (0.14 and 0.014 µg/µL) dilutions respectively.



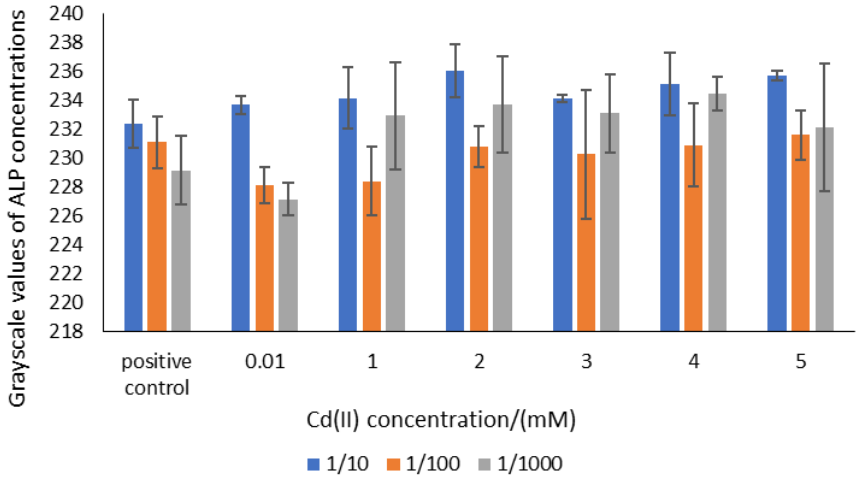
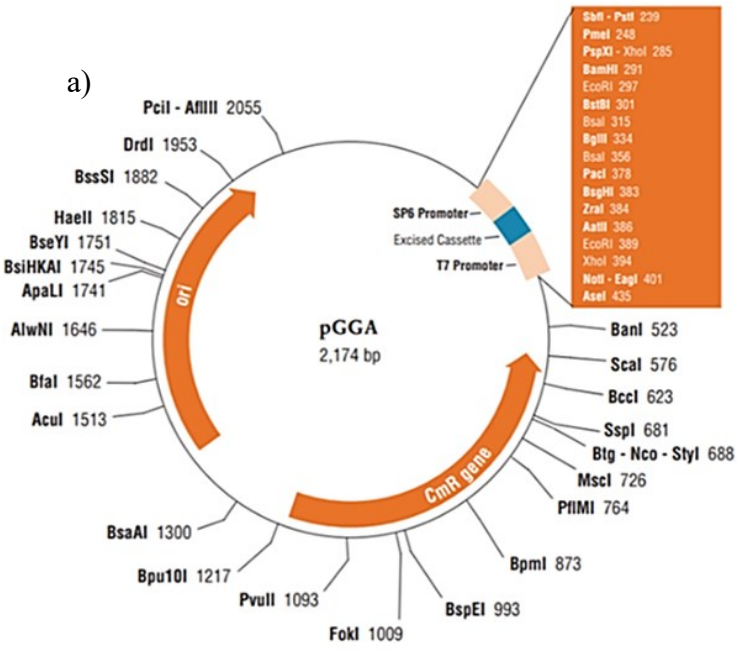


Figure 14. Inhibition of 1.4, 0.14 and 0.014 µg/µL (10-, 100-, and 1000-fold dilutions) of ALP by 0.01, 1.0, 2.0, 3.0, 4.0, and 5.0 mM of Cd (II) on filter paper (left) and graphical representation of metal inhibition by change in color intensity (right). The amount of enzyme and metal ion solution spotted within each circle was 1 µL.

Part II: Design of Plasmid for the Expression of Alkaline Phosphatase-Cellulose Binding Domain Fusion Protein. Based on the promising results observed with commercially available alkaline phosphatase, we set out to develop an engineered alkaline phosphatase. This alkaline phosphatase was to be expressed with a cellulose-binding domain that would act as a scaffold to immobilize the enzyme onto the filter paper of the microanalytical device so that the enzyme does not get displaced by the water sample to be tested. The engineering of the alkaline phosphatase-cellulose binding domain fusion protein involved the design of an entry DNA (Eco-ALP_YS-S1-CBD) which was inserted into the protein expression vector, pGGA (map represented in **Figure 15 a**) to yield a new plasmid called pGGA_Eco-ALP_YS-S1-CBD (map represented in **Figure 15 b**). The pGGA_Eco-ALP_YS-S1-CBD plasmid encodes the designed entry DNA sequence for the expression of the fusion protein.



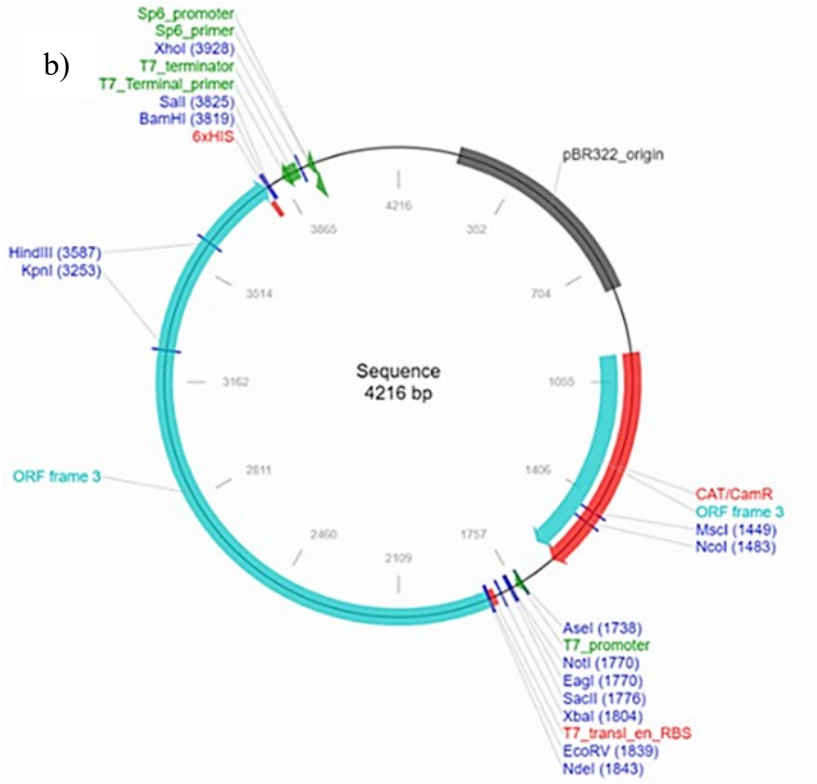
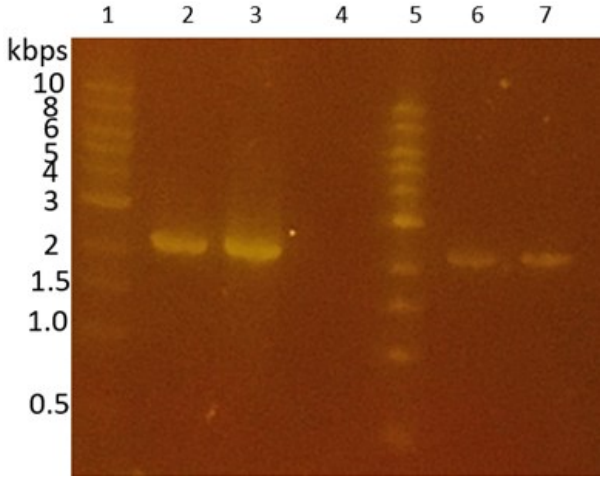


Figure 15. The maps of pGGA (a) reprinted from reference 47 and pGGA_Eco-ALP_YS-S1-CBD (b) plasmid.

Analysis of Plasmids and Outcomes of Protein Expression

Confirmation of the Size of Purified pGGA Plasmid. Purified pGGA plasmids from two clones were digested with *Bam*HI restriction enzyme at the 5'-GGATCC-3' recognition sites, to give single, clean cuts. The digested plasmids were run on agarose gel electrophoresis. In the presence of an electric field, the plasmid fragments which have a negative charge due to the negatively charged phosphate groups in the DNA backbone, migrate through the gel from the negative to the positive electrode. They are separated based on their size and charge.[48] The results were represented in lanes 2 and 3 (**Figure 16**). The samples were diluted by 4-folds and run through lanes 6 and 7 (**Figure 16**). When compared with the 1 kb DNA Ladder (New England Biolabs Inc.), the size of pGGA in both clones was approximately 2.1-2.2 kbp. This

agreed with the size of the pGGA plasmid reported by the supplier (2174 bp) (**Figure 15 a**). After confirming the size of pGGA, the purified plasmid was sent to GenScript Biotech Corporation for subcloning of the designed alkaline phosphatase-cellulose binding domain fusion protein gene.

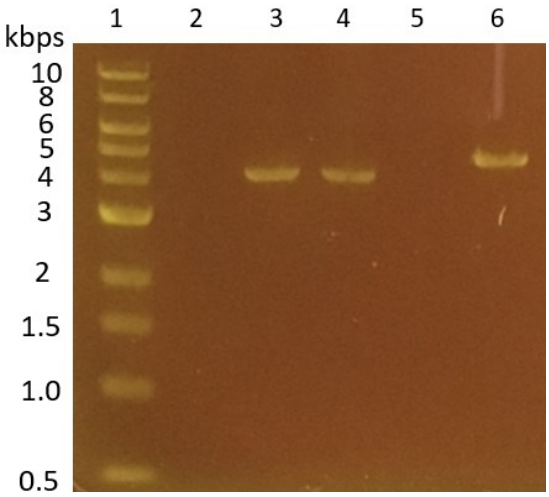


Plasmid Digest with *Bam*HI - pGGA
Lane 1: 1 kb ladder (NEB)
Lane 2 and 3: pGGA, concentrated for clones 1 and 2 respectively
Lane 4: empty lane
Lane 5: 1 kb ladder (NEB)
Lane 6 and 7: pGGA, diluted from clones 1 and 2 respectively

Figure 16. Electrophoresis of concentrated and diluted pGGA on 1% agarose gel. Lane 1: 1 kb DNA Ladder (New England Biolabs® Inc.®). Lanes 2 and 3: pGGA concentrated (2174 bp) for two clones and Lanes 6 and 7: pGGA diluted (2174 bp). All samples were digested with *Bam*HI.

Confirmation of the Size of Purified pGGA_Eco-ALP_YS-S1-CBD Plasmid. Before the subcloning of the designed entry DNA sequence into the confirmed pGGA plasmid, the entry DNA sequence was chemically synthesized and cloned into a pUC57 vector by GenScript Biotech Corporation. It was named pUC57_Eco-ALP_YS-S1-CBD plasmid and had a reported size of 4875 bp. Subsequently, the synthesized entry DNA was subcloned into pGGA plasmid to encode the alkaline phosphatase-cellulose binding domain fusion protein. The cloned pGGA plasmid was named pGGA_Eco-ALP_YS-S1-CBD. After the transformation of *E.coli* BL21(DE3) and plasmid purification, both plasmids were digested with *Bam*HI and analyzed by agarose gel electrophoresis (**Figure 17**). When compared with the 1 kb DNA Ladder (New

England Biolabs Inc.), the size of the pGGA_Eco-ALP_YS-S1-CBD plasmid was about 4.1-4.2 kbp, confirming its reported size on the map in **Figure 15 b**. Similarly, the size of the pUC57_Eco-ALP_YS-S1-CBD plasmid was about 4.8-4.9 kbp, also confirming its size. One of the clones (clone a), however, did not show any band in its lane (lane 5). The plasmid in this clone may not have been successfully isolated.



Plasmid Digest with *Bam*HI - pGGA_Eco-ALP_YS-S1-CBD and pUC57_Eco-ALP_YS-S1-CBD
Lane 1: 1 kb ladder (NEB)
Lane 2: empty lane
Lanes 3 and 4: pGGA_Eco-ALP_YS-S1-CBD for clones 1 and 2 respectively
Lanes 5 and 6: pUC57_Eco-ALP_YS-S1-CBD for clones a and b respectively

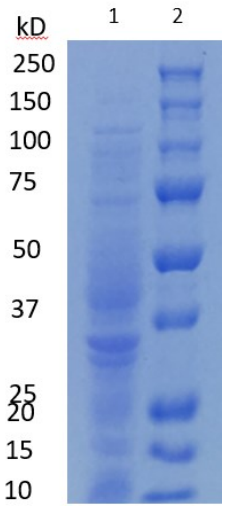
Figure 17. Electrophoresis of pGGA_Eco-ALP_YS-S1-CBD and pUC57_Eco-ALP_YS-S1-CBD on 1% agarose gel. Lane 1: 1 kb DNA Ladder (New England Biolabs® Inc.®). Lanes 3 and 4: pGGA_Eco-ALP_YS-S1-CBD (4216 bp) and Lanes 5 and 6: pUC57_Eco-ALP_YS-S1-CBD. All samples were digested with *Bam*HI.

Analysis of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD – IPTG-Induced Protein Expression

BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD strain was cultured at a 5 mL scale and the addition of IPTG was employed to induce the expression of the alkaline phosphatase-cellulose binding domain fusion protein.

SDS-PAGE. A part of the obtained cell culture (0.5 mL) was analyzed by SDS-PAGE to investigate the expression level of the alkaline phosphatase-cellulose binding domain fusion protein (**Figure 18**). The calculated molecular weight (Mw) of the alkaline phosphatase-cellulose

binding domain fusion protein using the ExPASy Mw tool was 67,338.73 D (~ 67 kD). However, no intense band was observed around 67 kD for BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD (Lane 1, **Figure 18**), indicating the low expression level of the fusion protein.



SDS-PAGE of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD (IPTG induction)

Lane 1: BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD
Lane 2: Precision Plus Mw marker (Bio-Rad)

Figure 18. SDS-PAGE of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD with IPTG induction. Lane 1: BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD. Lane 2: Precision plus molecular weight marker (Bio-Rad).

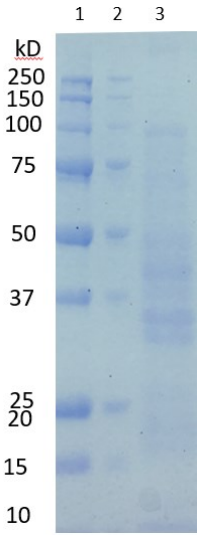
Bradford Assay on Cell Lysate. The protein concentration in the cell lysate was determined by Bradford assay. The 100-fold diluted cell lysate had a protein concentration of 14.1 µg/mL. Hence, the protein concentration in the undiluted cell lysate was estimated to be 1.41 mg/mL.

Alkaline Phosphatase Activity Test of Cell Lysate. The expression level of the alkaline phosphatase-cellulose-binding domain fusion protein was further investigated by enzyme activity measurements with the cell lysate. Since no purple color was developed, little to no alkaline phosphatase-cellulose binding domain fusion protein was expressed in the obtained *E. coli* BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD cells.

Analysis of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD – Overnight Express™ Autoinduction System

Since little to no protein expression was observed with IPTG-induction, we attempted to express the proteins using the Overnight Express™ autoinduction system at a 5 mL scale. This was performed at 30 ℃ overnight with shaking at 300 rpm.

SDS-PAGE. A part of the obtained cell culture (0.5 mL) was analyzed by SDS-PAGE. The result was shown in **Figure 19**.



SDS-PAGE of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD (Overnight Express™ autoinduction system)

Lane 1 and 2: Precision Plus Mw marker (Bio-Rad)
Lane 3: BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD

Figure 19. SDS-PAGE of BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD (Overnight Express™ autoinduction system). Lane 1 and 2 (spillover): Precision plus molecular weight marker (Bio-Rad). Lane 3: BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD with Overnight Express™ autoinduction system.

There was no strong band observed for BL21(DE3)/pGGA_Eco-ALP_YS-S1-CBD in lane 3, at the 67 kD region. This alternative protein expression approach confirmed that the alkaline phosphatase-cellulose binding domain fusion protein was either not expressed or minimally expressed in the *E. coli* BL21(DE3) suspension.

Alkaline Phosphatase Activity Test of Cell Lysate. An alkaline-phosphatase activity test was carried out on the cell lysate and there was no purple color development. This concludes that there was no or minimal expression of the enzyme.

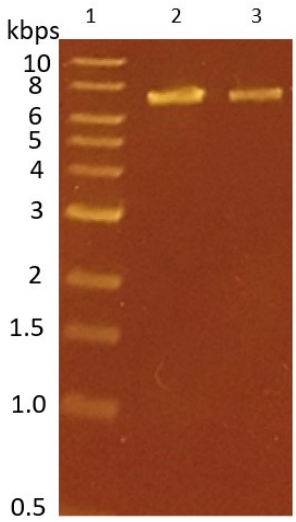
Bradford Assay on Cell Lysate. The protein concentration of the 100-fold diluted cell lysate was 17.33 µg/mL. Hence, the undiluted cell lysate had an estimated protein concentration of 1.73 mg/mL.

Expression of PelB-Alkaline Phosphatase

The lack or low expression of the alkaline phosphatase-cellulose binding domain fusion protein after performing the IPTG-Induced and Overnight Express™ autoinduction systems made us hypothesize that this is due to the absence of pelB signal peptide that promotes the translocation of alkaline phosphatase to the periplasmic region of *E. coli*. [36]

Confirmation of the Size of Purified pSANG14-3F Plasmid. The pSANG14-3F plasmid which encodes the sequence of *E.coli* alkaline phosphatase is known to express an N-terminus pelB signal peptide. The plasmid was digested with *Pst*I to confirm the size by gel electrophoresis. It yielded clean, single cuts for the two clones (**Figure 20**) as expected.

The reported size of the pSANG14-3F plasmid was 6791 bp. After digestion with *Pst*I, single bands were seen around 6.7-6.8 kbp for both clones in lanes 2 and 3. This confirmed the presence of the plasmid in the *E. coli* BL21(DE3) cells.

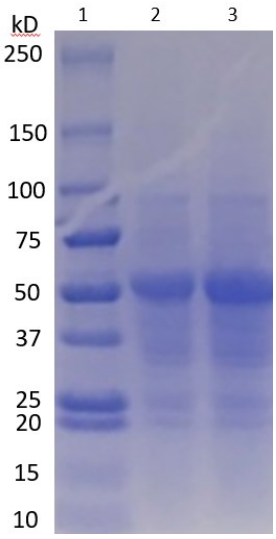


Plasmid Digest with *Pst*I – pSANG14-3F
Lane 1: 1 kb ladder (NEB)
Lanes 2 and 3: pSANG14-3F for clones A1 and B1 respectively

Figure 20. Electrophoresis of BL21(DE3)/pSANG14-3F on 1% agarose gel. Lane 1: 1 kb DNA Ladder (New England Biolabs® Inc.®). Lanes 2 and 3: pSANG14-3F (6791 bp) for clones A1 and B1 respectively, digested with *PstI*.

Analysis of BL21(DE3)/pSANG14-3F – Overnight Express™ Autoinduction System

SDS-PAGE. BL21(DE3)/pSANG14-3F strain was cultured at a 5 mL scale by the Overnight Express™ autoinduction system to induce the expression of pelB alkaline phosphatase. A part of the suspension (0.5 mL) was used for SDS-PAGE analysis. The calculated molecular weight was 47,541.19 D (~48 kD). The result (**Figure 21**) showed strong bands around the 48-52 kD region for the two clones in lanes 2 and 3. These appear to indicate overexpression of pelB alkaline phosphatase.



SDS-PAGE of BL21(DE3)/pSANG14-3F (Overnight Express™ autoinduction system)

Lane 1: Precision Plus Mw marker (Bio-Rad)
Lanes 2 and 3: BL21(DE3)/pSANG14-3F for clones A1 and B1 respectively

Figure 21. SDS-PAGE of BL21(DE3)/pSANG14-3F (Overnight Express™ autoinduction system). Lane 1: Precision plus molecular weight marker (Bio-Rad). Lanes 2 and 3: BL21(DE3)/pSANG14-3F for clones A1 and B1 respectively.

Alkaline Phosphatase Activity Test of Cell Lysate. An alkaline-phosphatase activity test was performed on the cell lysates. A purple color (**Figure 22**) developed after 10-15 seconds, confirming the expression of pelB-alkaline phosphatase in BL21(DE3). This suggests that the fusion of the pelB signal peptide sequence may be contributing to the overexpression of alkaline phosphatase in *E. coli*.

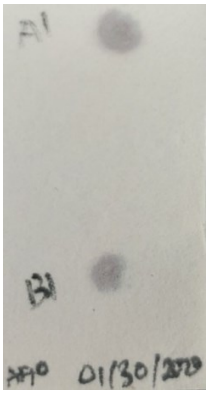


Figure 22. Alkaline phosphatase test on cell lysates of BL21(DE3)/pSANG14-3F. Both clones (A1 and B1) gave a purple color as expected.

Protein Purification. The pelB alkaline phosphatase in the cell lysate of clone A1 was purified by Ni^{2+}-NTA column chromatography. The fractions collected in the elution step as well as the fraction from the final step with the 20 mM MES-NaOH buffer, were used for alkaline phosphatase activity tests (**Figure 23**). For the fractions from the elution step, all but the last two gave a purple color indicating a successful protein purification. The last two fractions and that of the final step had no purple color, confirming the completion of the purification process. The first fraction gave a faint purple color because the protein was beginning to be eluted from the column.

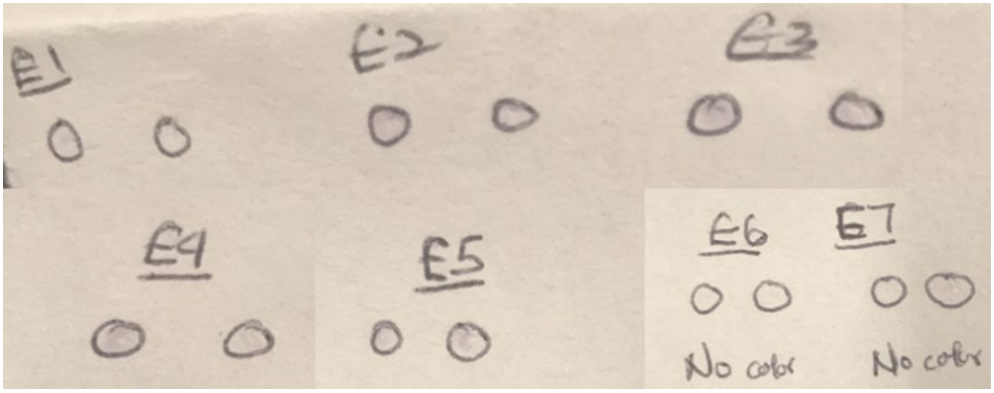
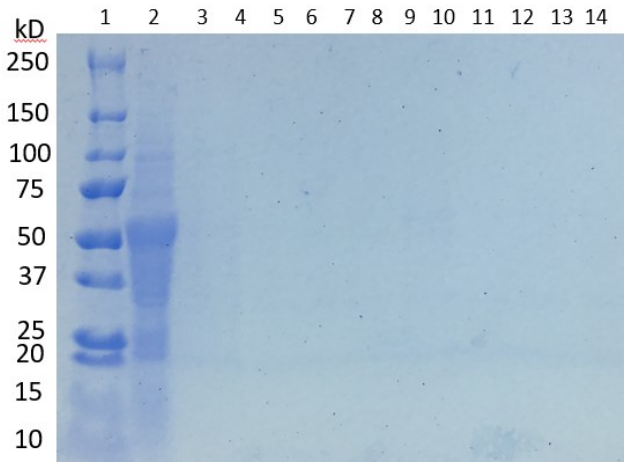


Figure 23. Alkaline phosphatase activity test with purified fractions. The seven fractions from the elution step were labeled Elution fractions 1 to 7 (E1-E7), respectively. The activity tests were repeated for each fraction.

Bradford Assay and SDS-PAGE Analysis. The absorbances of the 10- and 100-fold diluted fractions from the elution step were determined by Bradford assay and used to determine their protein concentrations. The values obtained were 0.0704, 0.165, 0.00676, 0.0168, and 0.00500 mg/L for Elution 1, 2, 3, 4, and 5 respectively.

The fractions from the Ni^{2+}-NTA affinity column chromatography were analyzed by SDS-PAGE (**Figure 24**). The bands for the purified fractions were not clear on the gels. Except for the

lane for the whole cell lysate (lane 2), no band could be observed. Therefore, the purity of the pelB-alkaline phosphatase fractions could not be confirmed.



SDS-PAGE of Ni^{2+}-NTA purified fractions from of BL21(DE3)/pSANG14-3F cell lysate

Lane 1: Precision Plus Mw marker (Bio-Rad)
Lane 2: BL21(DE3)/pSANG14-3F (whole cell)
Lane 3: Flow-through (supernatant of cell lysate)
Lane 4: Empty lane
Lane 5: Wash buffer
Lane 6 to 13: Fractions from the elution step (Elution 1 to 7 respectively)
Lane 14: Final fraction with 20 mM MES-

Figure 24. SDS-PAGE of Ni2+-NTA purified fractions of BL21(DE3)/pSANG14-3F cell lysate. Lane 1: Precision Plus Molecular Weight Marker (Bio-Rad). Lane 2: BL21(DE3)/pSANG14-3F (whole cell lysate). Lane 3: Flow-through (supernatant of cell lysate). Lane 4: Empty lane. Lane 5: Wash buffer. Lane 6 to 13: Fractions from elution step (Elution 1 to 7 respectively) but with Elution 6 repeated in two lanes. Lane 14: Final fraction with 20 mM MES-NaOH buffer.

Alkaline Phosphatase Activity Test after Dialysis. The purified fractions were dialyzed in 10 mM Tris-HCl buffer, pH 8.0 to keep the enzyme in a right buffer system and pH environment. The Elution 1 and 5 fractions dried up a couple of days after dialysis. As expected, Elution 6 and 7 fractions showed no purple color because they did not contain the enzyme. Out of the Elution 2, 3, and 4 fractions, only Elution 3 had a purple color development. The other two fractions (Elution 2 and 4) showed no purple color after the substrate mixture was introduced to the spots.

DISCUSSION

Part I: Metal-Ion Detection by the Inhibition of Commercially Available Alkaline Phosphatase

The inconsistent purple color intensities with respect to the concentrations of the enzyme, as well as the nonuniform spread of the purple color on the nitrocellulose membrane, is presumably due to the uneven immobilization and strong affinity and hydrophobic interaction of the alkaline phosphatase with the nitrocellulose membrane.[49] This interference would have it difficult to quantify the color intensity. In contrast, the purple color was developed more evenly on filter papers. Based on these observations, filter papers appear to offer advantages as a matrix for the enzyme inhibition-based metal ion detection.

The observed inhibition of alkaline phosphatase by some of the tested metal ions may be due to the displacement of the inherent Zn and Mg cofactors at the active site of the enzyme.[22] These metals are critical for dimer formation and catalytic activity of the enzyme. The substrate binds to Zn1 which stabilizes it in the active site for the second step. A hydroxyl bound to Zn2 hydrolyzes the phosphoseryl intermediate. At alkaline pH, the rate-limiting step in the mechanism involves the release of phosphate from the noncovalent enzyme–phosphate complex.[22] On the other hand, magnesium is not close enough to directly partake in the hydrolysis mechanism in the active site. However, it contributes to the shape of the electrostatic potential around the active center.[22] Overall, the displacement of these metal ions could lead to enzyme inhibition by the heavy metal ions.

The subsequent experiments supported that the presence of 4 mM or higher concentrations of mercury can be detected by monitoring the activity of alkaline phosphatase(**Figures 12**). On the

other hand, our experiments could not conclusively show the inhibition of the enzyme by other heavy metal ions.

Part II: Design of Plasmid for the Expression of Alkaline Phosphatase-Cellulose Binding Domain Fusion Protein

The engineering of an alkaline phosphatase fused with a cellulose-binding domain for immobilization onto the filter paper was performed by the insertion of a designed entry DNA (Eco-ALP_YS-S1-CBD) into the protein expression vector, pGGA which had a T7 promoter. The reason for the choice of the pGGA plasmid was its compatibility to construct new vectors via golden gate cloning. Also, due to the presence of this T7 promoter, the new plasmid, pGGA_Eco-ALP_YS-S1-CBD, could produce high levels of the fusion protein in the BL21(DE3) T7 expression *E. coli* strain.

The little to no expression of the alkaline phosphatase-CBD fusion protein was observed upon the induction of protein expression by IPTG or Overnight Express™ autoinduction system. Meanwhile, the *E.coli* alkaline phosphatase with an N-terminus pelB signal peptide could be expressed in BL21(DE3) with pSANG14-3F plasmid. Even though it was necessary to scale-up the volume of cell culture to obtain more proteins, the results appear to suggest the importance of pelB leader sequence in the expression of alkaline phosphatase in *E. coli*. The pelB sequence can promote the translocation of the fusion protein to the periplasmic space of *E. coli,* where it can fold into its native conformation by intramolecular disulfide bonds and be enzymatically active to catalyze reactions.[36, 50] Since pelB-alkaline phosphatase could be expressed in *E. coli*, the lack of expression of alkaline phosphatase-CBD fusion proteins might be attributed to the truncation of the pelB signal sequence. Based on this information, it would be interesting to construct a

plasmid vector for the expression of alkaline phosphatase-CBD fusion proteins that are fused with the N-terminus pelB signal sequence.

CONCLUSION

In this work, the potential applicability of a heavy metal ion detection system involving the use of alkaline phosphatase to catalyze the BCIP/NBT reaction has been investigated. The results showed the promises of the approach for the development of a simple, user-friendly, reliable, portable, and efficient paper-based microanalytical device with the immobilization of the enzyme on a filter paper. Thus far, we should confirm the inhibition of alkaline phosphatase by 4 mM or higher concentration of mercury whereas further investigation must be carried out to conclude the effect of other metal ions. In terms of the expression of engineered alkaline phosphatase, the result suggested that the pelB signal sequence may play an important role in the overexpression of the alkaline phosphatase-cellulose binding domain (CBD) fusion protein. This information would hopefully be useful for the expression of the fusion protein and development of a highly sensitive metal detection system in the future.

REFERENCES

(1) Malik, L.; Bashir, A.; Qureashi, A.; Pandith, A. Detection and removal of heavy metal ions: a review. *Environ. Chem. Lett*. **2019**, *17,* 1-3.

(2) Barbier, O.; Jacquillet, G.; Tauc, M.; Poujeol, P.; Cougnon, M. Acute study of interaction among cadmium, calcium, and zinc transport along the rat nephron in vivo. *Am. J. Physiol. Renal Physiol.* **2004**, *287*, F1067-75.

(3) Barbier, O.; Jacquillet, G.; Tauc, M.; Cougnon, M.; Poujeol, P. Effect of heavy metals on, and handling by, the kidney. *Nephron Physiol.* **2005**, *99*, 105-110.

(4) Zhou, G. Real-Time, Selective Detection of Heavy Metal Ions in Water Using 2d Nanomaterials-based Field-effect Transistors. Ph.D. Theses and Dissertations, University of Wisconsin, Milwaukee, **2017**.

(5) Bargossi, C.; Fiorini, M. C.; Montalti, M.; Prodi, L.; Zaccheroni, N. Recent developments in transition metal ion detection by luminescent chemosensors. *Coord. Chem. Rev.* **2000**, *208*, 17-32.

(6) Bonacchi, S.; Genovese, D.; Juris, R.; Montalti, M.; Prodi, L.; Rampazzo, E.; Sgarzi, M.; Zaccheroni, N. Luminescent Chemosensors Based on Silica Nanoparticles. In *Luminescence Applied in Sensor Science*, Prodi, L.; Montalti, M.; Zaccheroni, N. Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, **2011**, *300,* 93-138.

(7) Lisowski, P.; Zarzycki, P. K. Microfluidic Paper-Based Analytical Devices (μPADs) and Micro Total Analysis Systems (μTAS): Development, Applications and Future Trends. *Chromatographia* **2013**, *76*, 1-2.

(8) Ghaderinezhad, F.; Amin, R.; Temirel, M.; Yenilmez, B.; Wentworth, A.; Tasoglu, S. High-throughput rapid-prototyping of low-cost paper-based microfluidics. *Sci. Rep.* **2017**, *7*, 3553.

(9) Hossain, S. M. Z.; Brennan, J. D. β-Galactosidase-Based Colorimetric Paper Sensor for Determination of Heavy Metals. *Anal. Chem.* **2011**, *83*, 8772-8778.

(10) Senillou, A.; Jaffrezic-Renault, N.; Martelet, C.; Cosnier, S. A miniaturized urea sensor based on the integration of both ammonium based urea enzyme field effect transistor and a reference field effect transistor in a single chip. *Talanta* **1999**, *50*, 219-226.

(11) Han, S.; Zhu, M.; Yuan, Z.; Li, X. A methylene blue-mediated enzyme electrode for the determination of trace mercury(II), mercury(I), methylmercury, and mercury-glutathione complex. *Biosens. Bioelectron.* **2001**, *16*, 9-16.

(12) Krawczyński vel Krawczyk, T.; Moszczyńska, M.; Trojanowicz, M. Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. *Biosens. Bioelectron.* **2000**, *15*, 681-691.

(13) Starodub, N. F.; Kanjuk, N. I.; Kukla, A. L.; Shirshov, Y. M. Multi-enzymatic electrochemical sensor: field measurements and their optimisation. *Anal. Chim. Acta.* **1999**, *385*, 461-466.

(14) Apilux, A.; Siangproh, W.; Insin, N.; Chailapakul, O.; Prachayasittikul, V. Paper-based thioglycolic acid (TGA)-capped CdTe QD device for rapid screening of organophosphorus and carbamate insecticides. *J. Anal. Methods Chem.* **2017**, *9*, 519-527.

(15) Zhang, X. B.; Kong, R. M.; Lu, Y. Metal ion sensors based on DNAzymes and related DNA molecules. *Annu. Rev. Anal. Chem.* **2011**, *4*, 105-28.

(16) Torabi, S.-F.; Lu, Y. Small-molecule diagnostics based on functional DNA nanotechnology: a dipstick test for mercury. *Faraday discuss.* **2011**, *149*, 125-157.

(17) Shen, Y.; Mackey, G.; Rupcich, N.; Gloster, D.; Chiuman, W.; Li, Y.; Brennan, J. D. Entrapment of Fluorescence Signaling DNA Enzymes in Sol−Gel-Derived Materials for Metal Ion Sensing. *Anal. Chem.* **2007**, *79*, 3494-3503.

(18) Lehmann, M.; Riedel, K.; Adler, K.; Kunze, G. Amperometric measurement of copper ions with a deputy substrate using a novel Saccharomyces cerevisiae sensor. *Biosens. Bioelectron.* **2000**, *15*, 211-9.

(19) Tauriainen, S.; Karp, M.; Chang, W.; Virta, M. Luminescent bacterial sensor for cadmium and lead. *Biosens. Bioelectron.* **1998**, *13*, 931-938.

(20) Gao, X.; Du, C.; Zhuang, Z.; Chen, W. Carbon quantum dot-based nanoprobes for metal ion detection. *J. Mater. Chem. C.* **2016**, *4*, 6927-6945.

(21) Lustig, V.; Kellen, J. A. Temperature optimum of alkaline phosphatases in some homeothermic and poikilothermic species. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1971**, *39*, 311-315.

(22) Coleman, J. E. Structure and mechanism of alkaline phosphatase. *Annu Rev Biophys Biomol Struct.* **1992**, *21*, 441-83.

(23) Millán, J. L. Alkaline Phosphatases: Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signal.* **2006**, *2*, 335-341.

(24) Hoylaerts, M. F.; Manes, T.; Millán, J. L. Mammalian alkaline phosphatases are allosteric enzymes. *J. Biol. Chem.* **1997**, *272*, 22781-22787.

(25) Reid, T. W.; Wilson, I. B. E. coli Alkaline Phosphatase. In *The Enzymes*, Boyer, P. D., Ed. Academic Press. **1971**, *4,* 373-415.

(26) Schlesinger, M. J.; Barrett, K. The reversible dissociation of the alkaline phosphatase of *Escherichia coli.* I. Formation and reactivation of subunits. *J. Biol. Chem.* **1965**, *240*, 4284-92.

(27) Marcos, J.; Townshend, A. Studies on the inhibition of immobilised alkaline phosphatase by metal ions and EDTA in a flow-injection system. *Anal. Chim. Acta* **1994**, *299*, 129-136.

(28) Plocke, D. J.; Vallee, B. L. Interaction of Alkaline Phosphatase of E. coli with Metal Ions and Chelating Agents. *Biochemistry* **1962**, *1*, 1039-1043.

(29) Fan, S.; Lopez, S.; Pusec, C.; Shi, J. Chelating Agents and Temperature Effects on Alkaline Phosphatase Activity.PowerPoint Presentation. **2020** https://www.slideserve.com/azize/chelating-agents-and-temperature-effects-on-alkaline-phosphatase-activity. (accessed July 15, **2020**).

(30) MilliporeSigma. Immunodetection Using BCIP/NBT Substrate. https://www.sigmaaldrich.com/technical-documents/protocols/biology/bcip-nbt-immunodetection.html. (accessed June 5, **2020**).

(31) Cohen, S. N.; Chang, A. C.; Boyer, H. W.; Helling, R. B. Construction of biologically functional bacterial plasmids in vitro. *Proc. Natl. Acad. Sci.* **1973**, *70*, 3240-3244.

(32) Jackson, D. A.; Symons, R. H.; Berg, P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2904-2909.

(33) Grabski, A.; Mehler, M.; Drott, D. The Overnight Express Autoinduction System: High-density cell growth and protein expression while you sleep. *Nat. Methods* **2005**, *2*, 233-235.

(34) Blommel, P. G.; Becker, K. J.; Duvnjak, P.; Fox, B. G. Enhanced Bacterial Protein Expression During Auto-Induction Obtained by Alteration of Lac Repressor Dosage and Medium Composition. *Biotechnol. Prog.* **2007**, *23*, 585-598.

(35) Rosano, G. L.; Ceccarelli, E. A. Recombinant protein expression in Escherichia coli: advances and challenges. *Front. Microbiol.* **2014,** *5*, 172.

(36) Martin, C. D.; Rojas, G.; Mitchell, J. N.; Vincent, K. J.; Wu, J.; McCafferty, J.; Schofield, D. J. A simple vector system to improve performance and utilisation of recombinant antibodies. *BMC Biotechnol.* **2006,** *6*, 46.

(37) Lübke, C.; Boidol, W.; Petri, T. Analysis and optimization of recombinant protein production in Escherichia coli using the inducible pho A promoter of the E. coli alkaline phosphatase. *Enzyme Microb. Technol.* **1995,** *17*, 923-928.

(38) Linder, M.; Salovuori, I.; Ruohonen, L.; Teeri, T. T. Characterization of a double cellulose-binding domain. Synergistic high affinity binding to crystalline cellulose. *J. Biol. Chem.* **1996,** *271*, 21268-21272.

(39) Arola, S.; Linder, M. B. Binding of cellulose binding modules reveal differences between cellulose substrates. *Sci. Rep.* **2016,** *6*, 35358.

(40) Ofir, K.; Berdichevsky, Y.; Benhar, I.; Azriel-Rosenfeld, R.; Lamed, R.; Barak, Y.; Bayer, E. A.; Morag, E. Versatile protein microarray based on carbohydrate-binding modules. *Proteomics* **2005,** *5*, 1806-1814.

(41) Quiocho, F. A. Probing the atomic interactions between proteins and carbohydrates. *Biochem. Soc. Trans.* **1993,** *21*, 442-448.

(42) Toone, E. J. Structure and energetics of protein-carbohydrate complexes. *Curr. Opin. Struct. Biol.* **1994,** *4*, 719-728.

(43) Poole, D. M.; Morag, E.; Lamed, R.; Bayer, E. A.; Hazlewood, G. P.; Gilbert, H. J. Identification of the cellulose-binding domain of the cellulosome subunit S1 from Clostridium thermocellum YS. *FEMS Microbiol. Lett.* **1992,** *78*, 181-186.

(44) Tormo, J.; Lamed, R.; Chirino, A. J.; Morag, E.; Bayer, E. A.; Shoham, Y.; Steitz, T. A. Crystal structure of a bacterial family-III cellulose-binding domain: a general mechanism for attachment to cellulose. *EMBO J.* **1996,** *15*, 5739-5751.

(45) Celgen-Bio.tech. E.Z.N.A.® Plasmid Mini Kit II, Q(capless) Spin. http://www.celgen-bio.co.kr/gnb/bbs/board.php?bo_table=sub02_01&wr_id=10&sst=wr_datetime&sod=asc&sop=and&page=1&device=pc (accessed July 10, **2020**).

(46) Merck KGaA, D., Germany and/or its affiliates. Gravity-flow Purification using His GraviTrap™ and His GraviTrap™ Kit. https://www.sigmaaldrich.com/technical-documents/protocols/biology/affinity-chromatography-tagged-proteins/gravity-flow-purification-using-his-gravitrap-and-kit.html (accessed June 6, **2020**).

(47) New England Biolabs. DNA Sequences and Maps Tool. https://www.neb.com/tools-and-resources/interactive-tools/dna-sequences-and-maps-tool (accessed June 6, **2020**).

(48) Maffeo, C.; Yoo, J.; Comer, J.; Wells, D. B.; Luan, B.; Aksimentiev, A. Close encounters with DNA. *J. Phys. : Condens. Matter* **2014,** *26*, 413101-413101.

(49) Tovey, E. R.; Baldo, B. A. Protein binding to nitrocellulose, nylon and PVDF membranes in immunoassays and electroblotting. *J. Biochem. Biophys. Methods* **1989,** *19*, 169-183.

(50) Derman, A. I.; Beckwith, J. Escherichia coli alkaline phosphatase localized to the cytoplasm slowly acquires enzymatic activity in cells whose growth has been suspended: a caution for gene fusion studies. *J. Bacteriol.* **1995,** *177*, 3764-3770.

APPENDICES

Appendix A. pelB-alkaline phosphatase. The DNA sequence of pelB-alkaline phosphatase.

GGCCGCAACCCCGGAAATGCCTGTTCTGGAAAACCGGGCTGCTCAGGGCGATATTACTACACCCGGCGGTGCTCGCC
GTTTAACGGGTGATCAGACTGCCGCTCTGCGTGATTCTCTTAGCGATAAACCTGCAAAAAATATTATTTTGCTGATT
GGCGATGGGATGGGGGACTCGGAAATTACTGCCGCACGTAATTATGCCGAAGGTGCGGGCGGCTTTTTTAAAGGTAT
AGATGCCTTACCGCTTACCGGGCAATACACTCACTATGCGCTGAATAAAAAAACCGGCAAACCGGACTACGTCACCG
ACTCGGCTGCATCAGCAACCGCCTGGTCAACCGGTGTCAAAACCTATAACGGCGCGCTGGGCGTCGATATTCACGAA
AAAGATCACCCAACGATTCTGGAAATGGCAAAAGCCGCAGGTCTGGCGACCGGTAACGTTTCTACCGCAGAGTTGCA
GGGTGCCACGCCCGCTGCGCTGGTGGCACATGTGACCTCGCGCAAATGCTACGGTCCGAGCGCGACCAGTGAAAAAT
GTCCGGGTAACGCTCTGGAAAAAGGCGGAAAAGGATCGATTACCGAACAGCTGCTTAACGCTCGTGCCGACGTTACG
CTTGGCGGCGGCGCAAAAACCTTTGCTGAAACGGCAACCGCTGGTGAATGGCAGGGAAAAACGCTGCGTGAACAGGC
ACAGACGCGTGGCTATCAGTTGGTGAGCGATGCTGCCTCACTGAACTCGGTGACGGAAGCGAATCAGCAAAAACCCC
TGCTTGGTCTGTTTGCTGACGGCAATATGCCAGTGCGCTGGCTAGGACCGAAAGCAACGTACCACGGCAATATCGAT
AAGCCCGCAGTCACCTGTACGCCAAATCCGCAACGTAATGACAGTGTACCAACCCTGGCGCAGATGACCGACAAAGC
CATTGAATTGTTGAGTAAAAATGAGAAAGGCTTTTTCCTGCAAGTTGAAGGTGCGTCAATCGATAAACAGAATCATG
CTGCGAATCCTTGTGGGCAAATTGGCGAGACGGTCGATCTCGATGAAGCCGTACAACGGGCGCTGGAATTCGCTAAA
AAGGAGGGTAACACGCTGGTCATAGTCACCGCTGATCACGCCCACGCCAGCCAGATTGTTGCGCCGGATACCAAAGC
TCCGGGCCTCACCCAGGCGCTAAATACCAAAGATGGCGCAGTGATGGTGATGAGTTACGGGAACTCCGAAGAGGATT
CACAAGAACATACCGGCAGTCAGTTGCGTATTGCGGCGTATGGCCCGCATGCCGCCAATGTTGTTGGACTGACCGAC
CAGACCGATCTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAAGTACCGTCTGCA

Appendix B. Cellulose-Binding Domain. The DNA sequence of the cellulose-binding domain of family-3a.

GCAAATACACCGGTATCAGGCAATTTGAAGGTTGAATTCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAA
TCCTCAGTTCAAGGTTACTAATACCGGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAG
TAGACGGACAGAAAGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATT
ACTTCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTTGAAATAAGCTT
TACAGGCGGAACTCTTGAACCGGGTGCACATGTTCAGATACAAGGTAGATTTGCAAAGAATGACTGGAGTAACTAT
ACACAGTCAAATGACTACTCATTCAAGTCTCGTTCACAGTTTGTTGAATGGGATCAGGTAACAGCATACTTGAACGG
TGTTCTTGTATGGGGTAAAGAACCCGGTGGCAGTGTAGTA

Appendix C. pGGA_Eco-ALP_YS-S1-CBD Plasmid. DNA sequence of pGGA_Eco-ALP_YS-S1-CBD plasmid.

GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTA
AAAAGGCCGCATTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAG
AGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCC
GACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTA
GGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGC
GCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAA

CAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAA
GAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAA
CAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGA
TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGACAACTT
TTGGCGAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCG
TATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTT
GATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGT
TCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTC
TTGCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTT
CACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCG
GCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTG
AGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAAC
TTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTCAGGT
TCATCATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGG
GCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTGCTACTCATGAGCGGATACATATTTGA
ATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCCCGCGAAATTAATACGACTCACTATAGGGAGACGCACATGCG
GCCGCGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATC
ATATGGGTCTCCAGGTGGCCGCAACCCCGGAAATGCCTGTTCTGGAAAACCGGGCTGCTCAGGGCGAT
ATTACTACACCCGGCGGTGCTCGCCGTTTAACGGGTGATCAGACTGCCGCTCTGCGTGATTCTCTTA
GCGATAAACCTGCAAAAAATATTATTTTGCTGATTGGCGATGGGATGGGGGACTCGGAAATTACTGC
CGCACGTAATTATGCCGAAGGTGCGGGCGGCTTTTTTAAAGGTATAGATGCCTTACCGCTTACCGGG
CAATACACTCACTATGCGCTGAATAAAAAAACCGGCAAACCGGACTACGTCACCGACTCGGCTGCAT
CAGCAACCGCCTGGTCAACCGGTGTCAAAACCTATAACGGCGCGCTGGGCGTCGATATTCACGAAAA
AGATCACCCAACGATTCTGGAAATGGCAAAAGCCGCAGGTCTGGCGACCGGTAACGTTTCTACCGCA
GAGTTGCAGGGTGCCACGCCCGCTGCGCTGGTGGCACATGTGACCTCGCGCAAATGCTACGGTCCGA
GCGCGACCAGTGAAAAATGTCCGGGTAACGCTCTGGAAAAAGGCGGAAAAGGATCGATTACCGAACA
GCTGCTTAACGCTCGTGCCGACGTTACGCTTGGCGGCGGCGCAAAAACCTTTGCTGAAACGGCAACC
GCTGGTGAATGGCAGGGAAAAACGCTGCGTGAACAGGCACAGACGCGTGGCTATCAGTTGGTGAGCG
ATGCTGCCTCACTGAACTCGGTGACGGAAGCGAATCAGCAAAAACCCCTGCTTGGTCTGTTTGCTGA
CGGCAATATGCCAGTGCGCTGGCTAGGACCGAAAGCAACGTACCACGGCAATATCGATAAGCCCGCA
GTCACCTGTACGCCAAATCCGCAACGTAATGACAGTGTACCAACCCTGGCGCAGATGACCGACAAAG
CCATTGAATTGTTGAGTAAAAATGAGAAAGGCTTTTTCCTGCAAGTTGAAGGTGCGTCAATCGATAA
ACAGAATCATGCTGCGAATCCTTGTGGGCAAATTGGCGAGACGGTCGATCTCGATGAAGCCGTACAA
CGGGCGCTGGAATTCGCTAAAAAGGAGGGTAACACGCTGGTCATAGTCACCGCTGATCACGCCCACG
CCAGCCAGATTGTTGCGCCGGATACCAAAGCTCCGGGCCTCACCCAGGCGCTAAATACCAAAGATGG
CGCAGTGATGGTGATGAGTTACGGGAACTCCGAAGAGGATTCACAAGAACATACCGGCAGTCAGTTG
CGTATTGCGGCGTATGGCCCGCATGCCGCCAATGTTGTTGGACTGACCGACCAGACCGATCTCTTCT
ACACCATGAAAGCCGCTCTGGGGCTGAAAGTACCGTCTGCAGGTAGCGGTAGCGCTTCGAGACCAGG
TACCCACGAAGACAGCATGAGCACCAACACCCCGGCAAATACACCGGTATCAGGCAATTTGAAGGTT
GAATTCTACAACAGCAATCCTTCAGATACTACTAACTCAATCAATCCTCAGTTCAAGGTTACTAATA
CCGGAAGCAGTGCAATTGATTTGTCCAAACTCACATTGAGATATTATTATACAGTAGACGGACAGAA
AGATCAGACCTTCTGGTGTGACCATGCTGCAATAATCGGCAGTAACGGCAGCTACAACGGAATTACT
TCAAATGTAAAAGGAACATTTGTAAAAATGAGTTCCTCAACAAATAACGCAGACACCTACCTTGAAA
TAAGCTTTACAGGCGGAACTCTTGAACCGGGTGCACATGTTCAGATACAAGGTAGATTTGCAAAGAA
TGACTGGAGTAACTATACACAGTCAAATGACTACTCATTCAAGTCTCGTTCACAGTTTGTTGAATGG
GATCAGGTAACAGCATACTTGAACGGTGTTCTTGTATGGGGTAAAGAACCCGGTGGCAGTGTAGTAG
GAAGCGTCTTCCATCATCATCATCATCATTAAGGATCCGTCGACCAAAGCCCGAAAGGAAGCTGAGTT
GGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTT

TTGCTCGAGCGATACACACTTCTATAGTGTCACCTAAATGCGTTTAAACCTTCCTGCAGGTGACGATTACCTAACAA
TCGGTCGATTCGTTTGATGTTATGTTTTGTTCTCGCTTTGGTTGGCAGGTTACGGCCAAGTTCGGTAAGAGTGAGAG
TTTTACAGTCAAGTAATGCGTGGCAAGCCAACGTTAAGCTGTTGAGTCGTTTTAAGTGTAATTCGGGGCAGAATTGG
TAAAGAGAGTCGTGTAAAATATCGAGTTCGCACATCTTGTTGTCTGATTATTGATTTTTCG

KEY for the pGGA_Eco-ALP_YS-S1-CBD DNA sequence

1. T7 Promoter
2. Start codon
3. XhoI and NotI sequences present in pGGA
4. Stop codon
5. Lac operator
6. T7 Terminator
7. Ribosome binding site

Appendix D. List of Chemicals and Reagents

1 kb DNA Ladder (New England Biolabs® Inc.)
10X Fast Digest Buffer (Thermo Scientific)
Agarose (VWR Life Science), 9012-36-6
Ammonium sulfate (Fischer Scientific), 7783-20-2
Bacteriological Agar (Sigma Aldrich), 9012-18-0
Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD) 5000006
Bovine Serum Albumin (Sigma)
Calcium chloride (Fischer Scientific), 10043-52-4
Chloramphenicol (Acros Organics)
Coomassie® Brilliant Blue G-250 (MP Biomedicals), 6104-58-1
Disodium phosphate (Fischer Scientific Company), 7558-79-4
Ethanol (Ultra-Pure), 64-17-5
Gel Loading Dye Purple 6X, supplied as a component of 1 kb DNA Ladder (New England Biolabs® Inc.)
Glucose (Sigma Aldrich), 50-99-7
Glycerol (Fischer Scientific), 56-81-5
HisPur™ Ni^{2+}-NTA Resin (Thermo Scientific) 88221
Hydrochloric acid (Fischer Scientific), 7647-01-0
Imidazole (CHEM-IMPEX INT'L INC.), 288-32-4

Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific)
Kanamycin (Acros Organics), 25389-94-0
α-Lactose (Sigma Aldrich), 5989-81-1
LB Media (Fischer Scientific)
Magnesium sulfate (CHEM-IMPEX INT'L INC.), 7487-88-9
Mini-PROTEAN TGXTM Gels 12% (Bio-Rad Laboratories, Inc.) 456-1046
Monopotassium phosphate (Fischer Scientific), 7778-77-0
Potassium acetate (Sigma Aldrich), 127-08-2
Precision Plus Protein ™ Unstained Standards (Bio-Rad Laboratories, Inc.) 161-0363
Sodium bicarbonate (Fischer Chemical), 144-55-8
Sodium chloride (Fischer Scientific), 7647-14-5
Sodium dodecyl sulfate (Fischer Scientific), 151-21-3
Sodium hydroxide (Fischer Scientific), 1310-73-2
SYBR® Safe DNA gel Stain (Invitrogen)
Tris-Base (Fischer Scientific), 77-86-1

Kits
Bio-Rad AP Conjugate Substrate Kit
E.Z.N.A® Plasmid DNA MiniKit I (Omega bio-tek)
TGX™ FastCast™ Acrylamide Starter Kit

Plasmids
pGGA (New England Biolabs® Inc.)
pGGA_Eco-ALP_YS-S1-CBD (Constructed by GenScript Biotech Cooperation)
pUC57_Eco-ALP_YS-S1-CBD (Constructed by GenScript Biotech Cooperation)
pSANG14-3F (Addgene plasmid # 39265)

Enzymes
*Bam*HI (Thermo Scientific)
Phosphatase, Alkaline from bovine intestinal mucosa (Sigma Aldrich)
*Pst*I (Thermo Scientific)
RNase A (Omega bio-tek)

Bacteria
E. coli BL21(DE3) strain

Instrumentation
Cary 60 UV-Vis Spectrophotometer (Agilent Technologies)
Veriti 96 Well Plate Thermal Cycler (Applied Biosystems)
Autoclave (Primus Sterilizer Co)
Boekel Scientific Flask Dancer

Incubator
Mikro 22R Hettich Zentrifuge
Qsonica Sonicator Model CL-188
Sartorius 0.1 µL- 3 µL pippetor
Sartorius 0.5 µL- 10 µL pippetor
Sartorius 10 µL – 100 µL pippetor
Sartorius 100 µL- 1000 µL pippetor
Sorvall Legend X1R Centrifuge (Thermal Scientific)
SpectraMax M5 (Molecular Devices Inc.)

Other Equipment
1 L Erlenmeyer flask
1 L Media Bottles
1.5 mL Micro Centrifuge Tubes
100 mL beaker
100 mL Media Bottles
15 mL Centrifuge Tubes
-20 °C Freezer
250 mL Erlenmeyer flask
3.5 kD MWCO Dialysis Membrane (Spectrum Laboratories, Inc.)
50 mL Centrifuge Tubes
50 mL Media Bottles
500 mL Media Bottles
-80 °C Freezer
96-well plate (greiner bio-one)
Clare Chemical Research Dark Reader (Clare Chemical Research, Inc.)
Ethanol Lamp
Flint Striker
Hot Water Bath
Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, Inc.)
Mupid®-2plus Submarine electrophoresis system (Takara Bio USA, Inc.)
Nephlo culture flask with side arm
PCR Tubes
Petri Dishes
Thermometer