New Bacterial Surface Display System Development and Application Based on Bacillus subtilis YuaB Biofilm Component as an Anchoring Motif

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Abstract Bacterial surface display system has been adopted in various biotechnological applications. In the case of Bacillus subtilis, most of the studies have been developed using spore based surface display system utilizing the inherent rigidity of spore against heat, alkali, and shear stress. But, spore harvest, purification and separation need additional cost and labor. To eliminate this procedure and to use the gram-positive nature of B. subtilis, YuaB, which is one of the major B. subtilis biofilm components and locates in the cell wall, based cell surface display system, is developed. P43 promoter driven overexpression of YuaB-His$_6$ tag does not hamper bacterial cell growth and promoted biofilm formation of recombinant strain. Flow cytometry of recombinant strain and its protoplast using FITC-Anti His$_6$ antibody, verified that YuaB locate in plasma membrane and protrude to the outside of cell wall, which means YuaB can be used as very efficient anchoring motif. Using surface expressed YuaB-His$_6$ tag, removal of divalent metal ion, Cu$^{2+}$ and Ni$^{2+}$, was tried to test its possibility for the environmental application of developed system.

Keywords: YuaB, flow cytometry, Bacillus subtilis, bacterial surface display system, protoplast

1. Introduction

With increasing interest of global warming and environment, bioengineering has emerged as a promising alternative of chemical engineering industry. Application of bioengineering techniques can prevent formation of harmful byproducts, which is a main problem of chemical production process [1,2]. In bioengineering, many kinds of microorganisms have been widely used and studied in various field such as chemical [3,4], biopolymer [5,6], pharmaceutical [7,8], and nutraceutical [9]. Some of most popular microorganisms in biomaneufacturing are Escherichia Coli, Corynebacterium species, Bacillus species, Pseudomonas species, Clostridium Species, and Saccharomyces cerevisiae [10].

Among numerous species, Bacillus subtilis has been considered as attractive bacterium for industrial application. B. subtilis is a gram-positive bacterium which has distinct features such as good growth on cheap carbon source, clear inherited backgrounds, having genetic manipulation method, robustness in large-scale fermentation, spore formation and biofilm formation [11]. In addition, B. subtilis has been designated as generally recognized as safe (GRAS) according to the World Health Organization (WHO) [12]. A number of bio-products were already produced with dynamic strategies of engineering B. subtilis including metabolic optimization and spore display.

Bacterial surface display system has been applied in various biotechnological and industrial fields such as vaccine, biocatalyst, and biosensor development [13]. Bacterial surface display system was first introduced in 1986 by Roland Freudl et al. [14]. OmpA, which is one of the major outer membrane proteins of E. coli K-12 and exposed to external environment, was used as an anchoring motif for surface display system [14]. Recently, displaying laccase, CotA, of B. subtilis which has enzymatic activity decomposing
synthetic dye, on E. coli surface has been reported [15]. In the case of B. subtilis, most of studies have been focused on development of spore surface display system. B. subtilis spore is composed of peptidoglycan layer called the cortex, multilayer protein coat, and an outer coat layer [16]. Under harsh conditions such as heat, pH, ultraviolet radiation, and chemical stress, B. subtilis enters into the resting phase of sporulation and survive for a long time [12]. Due to its attributes, spore display system has been recognized as most promising engineering technology.

However, spore surface display system has some defects. In order to use spore of B. subtilis, bacterial cells have to be cultivated at least 24 h, because B. subtilis forms spores when they are exposed to environmental stress, like lack of nutrients. Moreover, after 24 h cultivation, we need additional process to isolate spore from cell. Commonly, spores have been separated by density difference between spore and cell with some chemicals that can provide density gradient such as PEG (poly ethylene glycol) [17] and Renografin (sodium diatrizoate hydrate) [18]. Spore separation process is complicate and highly expensive process, also difficult to get pure and large amounts of spores. Although spore display system has some drawbacks, cell surface display system of B. subtilis has not been studied as much as spore display system developed. Few studies have been reported by using sortase and sortase substrate [19,20]. Sortase makes covalent bond between B. subtilis cell wall and sortase substrate, but specific mechanism of B. subtilis sortase system is not certainly revealed yet.

B. subtilis forms rugose pellicles called biofilm, which is the group of bacterial societies by sticking cells each other [21]. Biofilm of B. subtilis is composed of self-produced extracellular matrix including exopolysaccharide (EPS), proteins, and some nucleic acid [22,23]. Development of biofilm is strictly regulated by gene expression of extracellular matrix components. Under certain conditions, for instance, nutrient depletion, impaired respiration or surface adherence [24-26], B. subtilis switches from a planktonic to a sessile state and start to stimulate genetic expression regarding production of extracellular components. After expansion and maturation of biofilm, it continues development by secretion of self-produced extracellular matrix. As a result, extensive wrinkles are created at the surface of biofilm matrix. Ruggedness of biofilm provides several advantages for the bacterial society. By increasing surface-to-volume ratio, bacteria within the biofilm have a greater access to oxygen [24,27]. In addition, biofilm has well-defined channels, which can transport liquid through extracellular matrix [28]. Liquid flux is crucial for providing nutrients and disposal of wastes. Moreover, genetic differentiation and sporulation of B. subtilis depend on the location inside the biofilm matrix [29]. It means that biofilm brings heterogeneity of cellular phase of B. subtilis.

EPS and proteins comprise of structural biofilm [30]. EPS, one of the major molecules of biofilm, is controlled by the espA-O gene operon (eps) [29-31]. However, the specific structure of EPS has not been fully understood. A study regarding the role of eps has been reported as deficiency of resulted in formation of weak and fragile pellicles. Although, other matrix components support deficiency of EPS and EPS mutant can keep growing in extracellular matrix [30]. TasA and TapA are two important proteins, which are the products of three-gene operon tapA-sipW-tasA (tapA operon) [32]. Long intercellular amyloid fibers, which provide structural stability of biofilm, are formed by self-assembly of TasA protein [30,33]. This amyloid fibers anchor to the cell wall with the help of TapA [33]. Additionally, YuaB (or BslA) is essential protein for the maturation of B. subtilis biofilm. Deletion of yuaB presents reduced complexity of the biofilm architecture and cannot be compensated by overexpression of other extracellular components. Importantly, YuaB interact with the TasA amyloid fiber and the EPS polymer as synergistic manner to maturate biofilm. However, YuaB does not affect production of EPS and TasA [34]. In the early secretome study of B. subtilis, YuaB has been presumed as an extracellular protein [35]. However, recently, it has been discovered that YuaB is an cell-associated protein, which locates in the cell wall [34]. YuaB has putative signal peptide sequence, 28 amino-acid at its N-terminus, and transmembrane region. The signal sequence removed YuaB neither have wild type function nor be detected in the cytoplasm [34]. Signal peptide of YuaB is vital for secretion and function of YuaB.

In this study, YuaB was used as an anchoring motif for development of a new B. subtilis cell surface display system. This is the first attempt to display target protein on the B. subtilis cell surface by using a biofilm component, YuaB. We fused His	ag at the C-terminus of YuaB to analyze the possibility as anchoring motif in surface display. p43 promoter, which is overlapping promoters transcribed by B. subtilis σ55 and σ37 [36], is also fused at N-terminus of YuaB to stimulate production of YuaB fusion protein. We confirmed that YuaB can be used for B. subtilis cell surface display system by analyzing recombinant cells with flow cytometry. Furthermore, a new surface display system has been applied heavy metal adsorption and showed positive potential of its industrial utilization.

2. Materials and Methods

2.1. Bacteria and growth condition
Two protease deficient B. subtilis DB104 (trpC2 nprE
**New Bacterial Surface Display System Development and Application Based on Bacillus subtilis**

2.2. Construction vector and strain

pSDJH100 [38] was used for pDE100-Y1 vector construction. Chromosome of DB104 was used for PCR template. p43 promoter was amplified with primers p43-5’ (5’- tcgag ctgctag cag gccgaaac atc t-3’) and p43-3’ (5’- tctgca ggttctctctca ctctctcct cttacct-3’) by PCR. pSDJH 100 and p43 PCR fragment were digested with restriction enzyme BamI and PstI, which is included in vector and PCR fragment, and ligated with T4 ligase. pSDJH100 containing p43 promoter was named as pDE100. **yuaB** was amplified with primers **YuaB-5’** (5’-ctcgag gtcgac atgaaacgcaaattattatct-3’) and **YuaB-3’** (5’-ctcgag gtcgac gcctgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
His$_6$ tag at the C-terminal end of anchoring motif.

Vector pDE100 and PCR fragment of yuaB- His$_6$ tag were digested with restriction enzyme, Pst I and Xho I, and ligated with T4 ligase to construct expression vector pDE100-Y1.

The constructed expression vector was extracted using plasmid mini prep kit (plasmid SV mini, Geneall, Korea) and the correct insertion of gene into the plasmids was verified by sequencing analysis. The construction of pDE100-Y1 is shown in Fig. 1. The recombinant plasmid was transformed into strain DB104 followed by selection on LB plates containing chloramphenicol. Constructed strain was named as DB104(pDE100-Y1).

3.2. Bacterial cell growth and biofilm formation of constructed strain

The N-terminus of YuaB is located at cytoplasmic membrane of B. subtilis. Then it penetrates through the cell wall and, the C-terminus of YuaB is located at the extracellular region. As YuaB is a composition of cell wall, the growth curve of B. subtilis has been drawn to check whether the overexpressed YuaB-His$_6$ tag affect cell growth or cell viability. Wild type, DB104 and recombinant cell, DB104 (pDE100-Y1), was inoculated in 50 mL of Schaeffer’s media with the same O.D. Both cells were grown under the condition of 37°C at 180 rpm. Every 1 h, optical density was measured. Fig. 2 shows that the overexpression of YuaB-His$_6$ tag does not affect cell growth and viability in planktonic growth B. subtilis.

YuaB plays a major role in biofilm formation. YuaB deleted mutant strain does not form biofilm. In other words, when other proteins, which involve in biofilm formation, such as DegU, TasA, lose their function, YuaB can compensate their absence [34]. pDE100-Y1 vector is designed to overexpress YuaB-His$_6$ tag under the control of p43 promoter. Therefore, capability of biofilm formation of DB104 and DB104(pDE100-Y1) has been compared. Both strains were inoculated into sterile flask with 50 mL of Schaeffer’s media with initial same O.D. Chloramphenicol (20 µm/mL) was added in the medium. Both culture were sustained for 72 h at 30°C without shaking.

![Fig. 2. Bacterial cell growth of DB104 (●) and DB104(pDE100-Y1) (▲). Both strains were grown under the condition at 37°C, 180 rpm in LB media. Chloramphenicol (20 µm/mL) was used as a selective marker for DB104(pDE100-Y1). Optical density of both cultures was measured every hour.](image)

![Fig. 3. Biofilm formation between wild type DB104 and DB104(pDE100-Y1), which overexpresses YuaB-His$_6$ tag. Both strains were inoculated into sterile flask with 50 mL of Schaeffer’s media with initial same O.D. In the case of DB104(pDE100-Y1), chloramphenicol (20 µL/mL) was added in the medium. Both culture were sustained for 72 h at 30°C without shaking.](image)
Scaefler’s media with initial same O.D. In the case of DB104(pDE100-Y1), chloramphenicol (20 µL/mL) was added in the culture medium. Both culture were sustained for 72 h at 30°C without shaking. Both strains formed biofilm at the liquid-air interface. DB104(pDE100-Y1) builds more thick and wrinkled biofilm than wild type DB104 (Fig. 3). This result indicates that DB104(pDE100-Y1) overexpressed functional p43 YuaB-His₆ tag, and this overexpressed YuaB-His₆ tag contributed the formation of wrinkled biofilm at the liquid-air interface well functions for biofilm formation even with the addition of His₆ tag.

3.3. Verification of surface display of YuaB-His₆ tag
To identify that YuaB can be used as an anchoring motif for a new *B. subtilis* cell surface display system, YuaB-His₆ tag was overexpressed in DB104. Vegetative cell of DB104 and DB104(pDE100-Y1) were both reacted with (FITC)-labeled anti-rabbit His₆ for 1 h and analyzed by flow cytometry. Flow cytometric histogram of DB104(pDE100-Y1) shows increased fluorescence intensity compared that of control strain DB104 (Fig. 4A). This result demonstrates that YuaB-His₆ tag was successfully expressed and located on the *B. subtilis* cell surface. In the preceding study, YuaB was detected in three different cell fractions including the cytoplasm, membrane and non-covalently bounded membrane proteins [34]. Based on this result, we hypothesized that YuaB is located not only in the cell wall but also in the cytoplasmic membrane. To confirm the location of YuaB, protoplast, of which peptidoglycan layer is removed from vegetative cell, was prepared. YuaB-His₆ tag was observed at protoplast, DB104(pDE100-Y1)-proto (Fig. 4B). This result explains that YuaB of *B. subtilis* located at plasma membrane. Comprehensively, our study illustrates that YuaB, which is a biofilm component, can be used as an anchoring motif for a new *B. subtilis* cell surface display system.

3.4. Ni²⁺ and Cu²⁺ adsorption capacity
In this study, His₆ tag was fused at the C-terminus of YuaB for the flow cytometric analysis of surface display. Poly-

![Fig. 4. Flow cytometric analysis of YuaB-His₆ tag overexpressing strain. (A) Flow cytometric histogram of DB104 (left) and DB104(pDE100-Y1) (right). DB104 is used as negative control and surface display of YuaB-His₆ tag was observed by shifted histogram of DB104(pDE100-Y1). (B) Flow cytometric histogram of protoplast analysis. DB104/proto indicates protoplast of *Bacillus subtilis* DB104. DB104(pDE100-Y1)-proto represents protoplast of DB104(pDE100-Y1).]
histidine can adsorb divalent metal ion by forming metal-polyhistidine complex [40,41]. Accordingly, recombinant B. subtilis, DB104(pDE100-Y1), which has His6 tag at C-terminus of YuaB, was utilized for heavy metal adsorption for their functionality. Cu$^{2+}$ and Ni$^{2+}$ were selected as the representative heavy metal for His6 tag, which is immobilized on the B. subtilis cell surface using YuaB. Standard means a standard control of heavy metal solutions for adsorption analysis.

With standard metal solution, DB104, which is used as negative control, also went through same procedure as DB104(pDE100-Y1) to rule out technical variables. Each sample was reacted at 37°C for 1 h to optimize cell condition during the reaction. The graphs show relative remaining amount of each metal ion, Ni$^{2+}$ and Cu$^{2+}$, after treatment of standard metal solution with DB104 and DB104(pDE100-Y1).

In the case of Ni$^{2+}$ adsorption, DB104(pDE100-Y1) adsorbed 10% more Ni$^{2+}$ of compared to DB104, which is shown as a small decrease in the relative remaining amount of metal (Fig. 5A). In the case of Cu$^{2+}$, DB104(pDE100-Y1) adsorbs 30% more of Cu$^{2+}$ DB104, which is shown as a significant decrease in the relative remaining amount of metal (Fig. 5B). These data mean that YuaB-His$_6$ tag has different metal adsorption ability according to the nature of heavy metal ion.

YuaB served successfully for the adsorption of metal ions, Cu$^{2+}$ and Ni$^{2+}$, and could be used as motif for a new B. subtilis cell surface display system for the other protein with various biotechnological application.

4. Discussion

B. subtilis spore display system has been well established and already applied in various biotechnological fields. Contrastively, B. subtilis cell surface display was hardly studied. Herein, we attempted to develop a new B. subtilis cell surface display system using B. subtilis biofilm component, YuaB. Recently, biofilm phenomena draw attention to exploit the benefits of biofilm formation [42]. Biofilm is highly structural and characteristic.

YuaB is one of the major proteins, which composes extracellular matrix of B. subtilis biofilm. Previously, it has been revealed that YuaB locates in the B. subtilis cell wall [34]. Based on this finding, YuaB has been chosen for an anchoring motif for a new B. subtilis cell surface display system in this study. We confirmed that YuaB was successfully overexpressed by p43 promoter and displayed on the B. subtilis cell surface and its surface localization is verified by flow cytometry analysis.

Moreover, from protoplast analysis, YuaB was observed in cytoplasmic membrane of B. subtilis. This result provides two possible hypotheses. First, YuaB locates only in the cytoplasmic membrane and penetrate through peptidoglycan layer. Second, YuaB places not only in the plasma membrane but also in the cell wall. The exact position of YuaB needs to be studied in the further studies.

Though we attached His$_6$ tag for the verification of surface display of YuaB, the attached His$_6$ tag can be used for the adsorption and removal of divalent heavy metal for the bioremediation. Several research groups used engineered microorganisms displaying multiple histidine residues for the adsorption of heavy metal using bacterial surface display technology. We would like to focus and compare those results which were used for the adsorption of Ni$^{2+}$ among various heavy metal adsorption researches. Several kinds of His tag, with varying length of repeating unit of His$_6$, have been displayed on the surface of E. coli cells and B. subtilis spore.

In the case of E. coli, using CS3 pili as an anchoring motif, 276.5 nmol of Ni$^{2+}$ was removed per mg cell, which is very high value among reported cases [43]. B. subtilis spore, having higher thermos-stability and resistance to the low pH environment, lytic enzymes and resistance to various antibacterial agents.
Table 1. Comparison of bioaccumulation of Ni\textsuperscript{2+} and Cu\textsuperscript{2+} with the surface displayed polyhistidine

| Metal ion | Anchoring motif | Displayed peptide | Reaction condition | Bioaccumulation (nmole/mg) | Reference |
|-----------|----------------|-------------------|--------------------|---------------------------|-----------|
| Ni\textsuperscript{2+} | YuaB (Bacillus subtilis) | His\textsubscript{6} | 37°C | 4.68 | This work |
| | Cot B (B. subtilis) | His\textsubscript{18} | 25°C, pH7 | 24.79 | [44] |
| | Cot E (B. subtilis) | His\textsubscript{12} | 37°C, pH7 | 18.24 | [45] |
| | CS3 (Escherichia coli) | His\textsubscript{6} | 37°C | 11.66 | This work |
| Cu\textsuperscript{2+} | YuaB (B. subtilis) | His\textsubscript{6} | 37°C, pH17.8 | 0.11 ± 0.03 | [46] |

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The authors declare no conflict of interest.

Neither ethical approval nor informed consent was required for this study.

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