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Decolorization of Acid Green 25 by Surface Display of CotA laccase on *Bacillus subtilis* Spores

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In this study, we expressed *cotA* laccase from *Bacillus subtilis* on the surface of *B. subtilis* spores for efficient decolorization of synthetic dyes. The *cotE*, *cotG*, and *cotY* genes were used as anchoring motifs for efficient spore surface display of *cotA* laccase. Moreover, a His_6 tag was inserted at the C-terminal end of *cotA* for the immunological detection of the expressed fusion protein. Appropriate expression of the CotE-CotA (74 kDa), CotG-CotA (76 kDa), and CotY-CotA (73 kDa) fusion proteins was confirmed by western blot. We verified the surface expression of each fusion protein on *B. subtilis* spore by flow cytometry. The decoloration rates of Acid Green 25 (anthraquinone dye) for the recombinant DB104 (pSDJH-EA), DB104 (pSDJH-GA), DB104 (pSDJH-YA), and the control DB104 spores were 48.75%, 16.12%, 21.10%, and 9.96%, respectively. DB104 (pSDJH-EA) showed the highest decolorization of Acid Green 25 and was subsequently tested on other synthetic dyes with different structures. The decolorization rates of the DB104 (pSDJH-EA) spore for Acid Red 18 (azo dye) and indigo carmine (indigo dye) were 18.58% and 43.20%, respectively. The optimum temperature for the decolorization of Acid Green 25 by the DB104 (pSDJH-EA) spore was found to be 50°C. Upon treatment with known laccase inhibitors, including EDTA, SDS, and NaN_3, the decolorization rate of Acid Green 25 by the DB104 (pSDJH-EA) spore decreased by 23%, 80%, and 36%, respectively.

Keywords: *Bacillus subtilis*, spore surface display, anchoring motif, decolorization, *cotA* laccase, synthetic dye

Introduction

Approximately 10,000 different dyes and pigments are used industrially, and over 7×10^5 tons of synthetic dyes are produced annually worldwide [1, 2]. Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetics, and other industries [3]. Approximately 128 tons of dye wastewater is discharged daily and high cost is incurred for its removal. Wastewater remediation is challenging because it contains a variety of pollutants and it is difficult to change the composition of the contaminants.

Physical, chemical, and biological methods have been widely used to decolorize dye wastewater [4]. The physical and chemical treatment methods are preferred over other methods; however, these methods require high operating cost and other accessory equipment. It would be desirable to develop a new decolorization method that is efficient and cost effective to reduce the overall cost of dye wastewater treatment.

Recently, many studies have been published on the decolorization of synthetic dyes by laccase [5, 6]. Laccase was first discovered in 1883 from *Rhus vernicifera*, the Japanese lacquer tree [7]. Laccases (benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2) are a family of multi-copper oxidases (MCOs) that require oxygen to oxidize organic compounds—particularly phenols and non-phenolic aromatic substrates—by one-electron transfer to form reactive radicals,

while reducing molecular oxygen to water [8, 9]. Laccases are widespread in nature and are found in plants and fungi [10]. However, bacterial laccase was first discovered in 1993 [11]. Since then, an increasing number of studies have shown that laccases are widespread among bacteria [12]. Laccase-like activity has been found in *Pseudomonas syringae* (*copA*) [13], *Xanthomonas campestris* (*copA*) [14], *Azospirillum lipoferum* [15], *Streptomyces griseus* (*epoA*) [16], *Escherichia coli* (*yacK*) [17], and *Bacillus subtilis* (*cotA*) [18]. Several studies have recently reported on the decolorization of synthetic dyes, including indigo carmine by *Streptomyces coelicolor* [19], congo red by *Bacillus* sp. [20], Reactive Black5 by *Shewanella oneidensis* WL-7 [21], and Reactive Blue by *Pseudomonas* sp. [22]. A recent study tested the efficacy of *cotA* on several synthetic dyes owing to its wide range of substrate specificity [23].

The bacterial surface display system was first introduced in 1986 [24]. Since then, this breakthrough technology has been generating promising opportunities for several applications, including bacterial vaccines, production of active enzymes and antibodies for cleanup of industrial and environmental pollution, screening of peptide libraries and use of peptides as biosensors and biocatalysts for bioconversion [25]. The bacterial surface display system can use bacterial surface proteins as surface anchoring motifs to express exogenous proteins [26, 27].

Several gram-positive and gram-negative bacteria expression systems have been developed until now. All bacterial surface display systems rely on the secretion of the fusion protein (anchoring motif-passenger protein) across the cell membrane or cell wall. However, protein transport across the cell membrane can be difficult to achieve if the passenger protein exists as a multimer, exhibits host cell toxicity upon expression, or has a high molecular weight.

Bacillus subtilis has developed a survival strategy to transfer genetic material to the next generation by forming endospores. The mother cell invaginates into a pre-spore and various sporulation proteins in the mother cell form spores by generating multiple outer coat layers [28]. Once the spores are sufficiently formed, the mother cell starts to decompose and release them to the external environment [29].

The spore-based surface display system can solve the problems associated with the bacterial surface display system. During sporulation, coat proteins are expressed in the mother cell and subsequently localize on the surface of the developing spore to form a multilayer coat. At the end of the sporulation process, the mother cell will lyse and the spore will be released into culture medium, with fusion protein embedded on it. This means spore will serve as a display vehicle of fusion protein, which does not need the secretion mechanism of the mother cell across the cellular membrane or cell wall structure.

The spores formed by these systems are generally stronger than the normal vegetative cells in terms of exhibiting thermal, chemical, and radiation resistance [30].

In this study, we attempted to decolorize synthetic dye by displaying *cotA* laccase on the surface of *B. subtilis* spores. *cotA* is a coat protein involved in the sporulation of *B. subtilis* and it displays similarities with multicopper oxidases.

Materials and Methods

Strains, Media, and General Procedures

The two protease-deficient *B. subtilis* DB104 strain (his, *nprR2*, *nprE18*, Δ*aprA3*) was used as the bacterial host for expression studies [31]. *Escherichia coli* strain JM109 [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15/* Δ*(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*] was used for genetic manipulation. Bacterial cultures were grown in Schaeffer's medium containing 8 g nutrient broth (Difco), 0.25 g $MgSO_4 \cdot 7H_2O$, and 1 g KCl/L. After autoclaving, the medium was supplemented with 1 ml of each filtered stock solution (10 mM $FeSO_4 \cdot 7H_2O$, 10 mM $MnCl_2 \cdot 4H_2O$, and 1 M $CaCl_2 \cdot 2H_2O$) and chloramphenicol (20 μM). Bacterial cultivation was carried out by shaking 100 ml of Schaeffer's medium in a 250 ml flask at 180 rpm for 24 h at 37°C.

The Hybrid-Q Plasmid Mini-prep Kit and the Expin PCR and Gel Purification Kit were purchased from GeneAll (Korea). Taq DNA polymerase was purchased from Bioneer (Korea). The restriction enzyme and T4 ligase were purchased from Takara (Japan). Anti-His_6tag-FITC conjugated antibody was purchased from Komabiotech (Korea) for flow cytometry analysis. Goat anti-mouse IgG-HRP for western blot was purchased from Santa Cruz Biotechnology (USA). Acid Green 25 and all other reagents were purchased from Sigma-Aldrich (USA).

Construction of Fusion Protein Expression Vector

The anchoring motif-containing expression vector was constructed with a PCR product using *B. subtilis* chromosome as a template, containing each promoter and structural gene of *cotE*, *cotG*, and *cotY* along with a C-terminal linker (Gly-Gly-Gly-Gly-Ser). The following primers were used for PCR:

cotE-5′: AACCGGATCCACCTGCTGAAAGGGGAAAC
cotE-3′: AGACCTGCAGGCTCCCTCCCCCCCCAGATCTGACGT CTTCTTCAGGATCTCCCAATA
cotG-5′: GCCTTTGGATCCAGTGTCCCTAGCTCCGAG
cotG-3′: CTATTGCTGCAGTGAACCCCCACCTCCTTTGTATTT CTTTTTGACTA,
cotY-5′: TCGTAGGGATCCTAGTTATCACTCTTGTCC
cotY-3′: GTTTGGCTGCAGACTCCCCCCTCCACCGGTACCTCC ATTGTGATGATG.

Each PCR product was digested with BamHI and PstI and ligated into the same enzyme-digested pCSK1 to yield pSDJH100 (pSDJH-CotE), pSDJH200 (pSDJH-CotG), pSDJH300 (pSDJH-CotY). The pCSK1 vector was derived from pHP59, which is a low copy *E. coli/B. subtilis* shuttle vector described by [32].

The thermal cycling conditions for PCR were as follows: an initial denaturation step at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Two primers were used for PCR amplification of *cotA* laccase (*cotA*-PstI-5′: GGACAGCTGCAGATGACACTTGAAAAATTTGTGG and *cotA*-HindIII-his$_6$ tag-SalI-3′: AAAAACGTCGACTCAGTG GTGATGGTGGTGGTGAAGCTTATGGGGATCAGTTATATCCAT CGGTCTC). The PCR product was digested with PstI and SalI, and ligated into the same enzyme-digested pSDJH100, pSDJH200, and pSDJH300 to yield pSDJH-EA, pSDJH-GA, and pSDJH-YA, respectively. The two-step (SP I, SP II) method was used for the transformation of each plasmid into *B. subtilis* DB104, and chloramphenicol (5 μM) was used for the selection.

Spore Purification Method

Bacillus subtilis DB104 strain and recombinant cells were grown in Schaeffer's media for 24 h at 37°C and harvested by centrifugation (7,224 ×*g*, 10 min, and 4°C). Vegetative cells, sporulating cells, and spores were harvested and purified using the Renografin (sodium diatrizoate, S4506, Sigma) gradient method. Purified spores were confirmed under the microscope.

Western Blot Analysis

Purified spores were suspended in distilled water and heated to 100°C for 8 min with 5× sample buffer to extract the outer coat layer of *B. subtilis* spores. Following centrifugation at 12,225 ×*g* for 10 min, the extracted coat protein in the supernatant fraction was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA).

The membrane was blocked with skim milk for 10 min, washed and incubated with a 1:1,000 diluted anti-His$_6$ tag antibody monoclonal antibody (H1029, Sigma) for 30 min, and washed three times with TBST (Tris-buffered saline, 0.1% Tween 20) buffer for 5 min. The membrane was subsequently incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2031, Santa Cruz) at a dilution of 1:2,000 for 20 min, washed three times with TBST buffer, and visualized using ECL western blotting detection reagents. Specific bands were visualized on a film (LAS 3000, Fuji Film).

Flow Cytometry Analysis

Purified spores were washed three times with phosphate-buffered saline (PBS) solution for immunofluorescence staining. The washed spores were resuspended in 1 ml PBS solution containing anti-His$_6$ tag-FITC labeled antibody (1:500) (Komabiotech, Korea) and incubated for 1 h on ice. Thereafter, they were washed three times with PBS, resuspended in 500 μl PBS [32], and examined under a flow cytometer (Beckman Coulter Epics, UK).

Decolorization Assay

Synthetic dye was prepared in sterilized 0.1 M sodium acetate buffer [pH 5.0]. The reaction was initiated with 3 mg of each purified spore in a 20 ml volume and incubated at 37°C with shaking at 180 rpm. The concentration of Acid Green 25 was 100 μM and its absorbance was measured by UV-210PC spectrometer (Dong-il, Japan) at 640 nm. Decolorization activity was calculated as follows: decolorization (%) = [(initial absorbance)−(observed absorbance)/(initial absorbance)]×100 [33].

For the inhibition study of laccase, 0.25 M EDTA (ethylenediamine tetraacetic acid), 50 mM SDS (sodium dodecyl sulfate), and 50 mM NaN_3 (sodium azide) were used.

To evaluate the decolorization possibility of other synthetic dyes, Acid Red 18 (azo dye) and indigo carmine (indigo dye) were used as representative dyes with different structures from Acid Green 25 (anthraquinone dye). The absorbances of Acid Red 18 and indigo carmine were measured at 510 nm and 610 nm, respectively. The starting concentration for the reactions of both synthetic dyes was 50 μM.

Effect of Temperature on Laccase Activity

The thermal stability of spore surface-displayed *cotA* was determined in 0.1 M sodium acetate buffer [pH 5.0] following incubation for 5 h at 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C. The decolorization rate was calculated for absorbance reduction.

Results and Discussion

Construction of Expression Vector and Recombinant *B. subtilis* Strain

For the surface display of *cotA* laccase on *B. subtilis* spores, *cotE*, *cotG*, and *cotY* were selected as anchoring motifs, not only because of their localization on *B. subtilis* spores, but also due to their relative abundance over other spore coat proteins [34].

CotE is a major coat protein involved in spore formation and is needed for the assembly of the outer coat protein. CotE is a 20.8 kDa protein that guides the assembly of a major subset of coat proteins [35]. Owing to its abundance in the spore coat layer, it has been used as an efficient anchoring motif for displaying bacterial beta-galactosidase and bacterial tyrosinase [36, 37].

CotG is another major morphogenetic coat protein, located in the outer coat layer of *B. subtilis* spore. CotG is a 24.0 kDa protein [38] that recruits a small subset of proteins to the coat [39]. CotG was selected as an anchor protein for this study because it has been studied extensively [34]. The

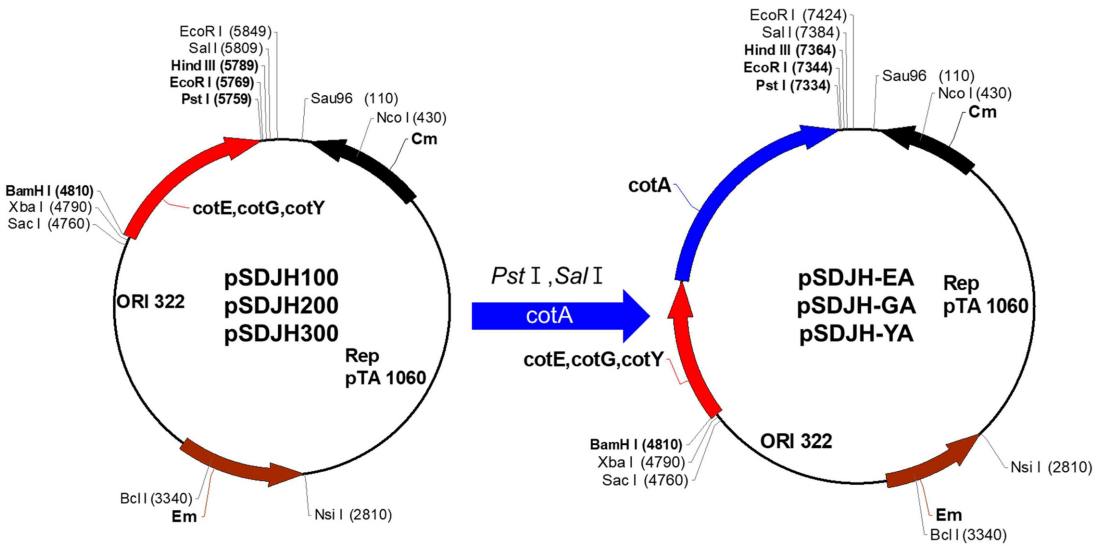

Fig. 1. Construction of pSDJH-EA, pSDJH-GA, pSDJH-YA expression vector.
Each fusion protein is expressed under original *cotE*, *cotG* and *cotY* promotor. And flexible linker (Gly-Gly-Gly-Gly-Ser) was inserted between each anchoring motif and *cotA* laccase. And His_6 tag sequence was inserted at the C-terminal of *cotA* laccase for the western blot and flow cytometry.

proteins expressed using CotG as an anchoring motif include streptavidin [29], ω-transaminase [40], chitinase [41], and L-arabinose isomerase [42].

CotY is located in the outermost layer of the spore, which is a newly defined crust layer [43]. CotY is a 17.7 kDa protein that plays a morphogenetic role in crust assembly around the spore [44]. To our knowledge, only one report exists on CotY for surface display of β-galactosidase [45].

The endospore coat component of *B. subtilis*, known as *cotA*, is the most studied bacterial laccase. The *cotA* gene encodes an approximately 65 kDa monomeric protein that localizes in the outer spore coat. The high proline content of CotA contributes to the thermostability of laccase [46]. CotA participates in the biosynthesis of the characteristic brown spore pigment, which protects from UV light [47].

In this study, CotA was used as the target protein to decolorize synthetic dye due to its thermostability under harsh environmental conditions; moreover, dye decomposition occurs in these conditions. The construction of vectors is shown in Fig. 1. These expression vectors were named pSDJH-EA, pSDJH-GA, and pSDJH-YA.

Verification of Surface Expression of Fusion Protein

Surface expression of the fusion protein on *B. subtilis* spore was verified by flow cytometry (Fig. 2). Purified spores of recombinant DB104 (pSDJH-EA), DB104 (pSDJH-GA), DB104 (pSDJH-YA), and DB104 were incubated with the anti-His_6tag-FITC antibody. Although all recombinant spores showed significantly improved fluorescence intensity over the control spore of DB104, CotY served as the most effective anchoring motif for the display of *cotA* laccase. This is because CotY is located in the crust layer, which is the

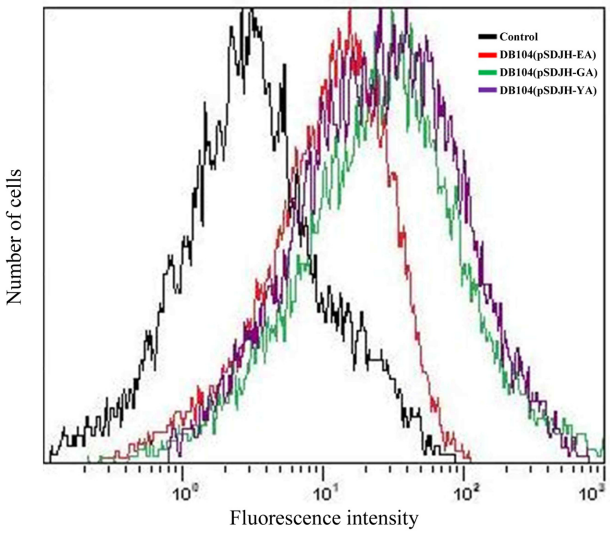

Fig. 2. Flow cytometry of purified spores of *B. subtilis* DB104 and each recombinant spore. DB104 spore and DB104 recombinant spore were stained with FITC-labeled his_6 tag antibody and examined under flow cytometry.

outermost part of the spore coat; this result compared to CotE and CotG, also corresponds to the previous result [43].

The appropriate expression of fusion proteins under their native promoters and their incorporation into *B. subtilis* spores was confirmed by western blot with the extracted fraction of each recombinant spore. We confirmed the expected sizes of CotE-CotA (74 kDa), CotG-CotA (76 kDa), and CotY-CotA (73 kDa). No similar band was detected for the DB104 spore (data not shown). These results suggest that each fusion protein is appropriately expressed and localized in the outer coat layer of the spore, and can be extracted using a simple extraction method, as described in this study.

Laccase Activity

Synthetic dyes have been classified according to their chemical structure—such as anthraquinone, azo, indigo, nitro, phthalein, triphenyl methyl, and nitrated dyes—or their industrial application [48].

Acid Green 25 [49], an anthraquinone dye, was used to evaluate the decolorization activity of laccase localized on the spore surface. After a 5 h reaction, the decolorization extent of Acid Green 25 by DB104 (pSDJH-EA), DB104 (pSDJH-GA), DB104 (pSDJH-YA), and DB104 spores were 48.7%, 16.12%, 21.1%, and 9.9%, respectively (Fig. 3A). The degree of decolorization of Acid Green 25 was also visualized (Fig. 3B). The reason for decolorization of Acid Green 25 by DB104 spore is the inherent *cotA* of the host strain, which is expressed by *B. subtilis* chromosomes. However, the three recombinant spores have higher decolorization activity than the control DB104 spore because of the relative abundance of surface displayed CotA protein.

Considering that the recombinant DB104 (pSDJH-EA) spore showed the highest decolorization of Acid Green 25, the decolorization of a representative azo dye, Acid Red 18 [50], and a representative indigo dye, indigo carmine [51], was measured. The decolorization rates of the DB104 (pSDJH-EA) spore for Acid Green 25, Acid Red 18, and indigo carmine were 48.7%, 18.5%, and 43.2%, respectively (Fig. 4)

Several dyes including indigo carmine and orange G were decomposable with extracellularly expressed *cotA* laccase [52]. Congo red, Coomassie Brilliant Blue R, and crystal violet were decolorized by intracellularly expressed *cotA* laccase [53]. Malachite Green, Acid Blue 62, and methyl orange were also decolorized by surface-displayed *cotA* laccase using *E. coli*. [23] Until now, we could not find any tendency of the decolorizing activity toward structurally different synthetic dyes, which could be attributed to the broad enzyme specificity of *cotA* laccase [54].

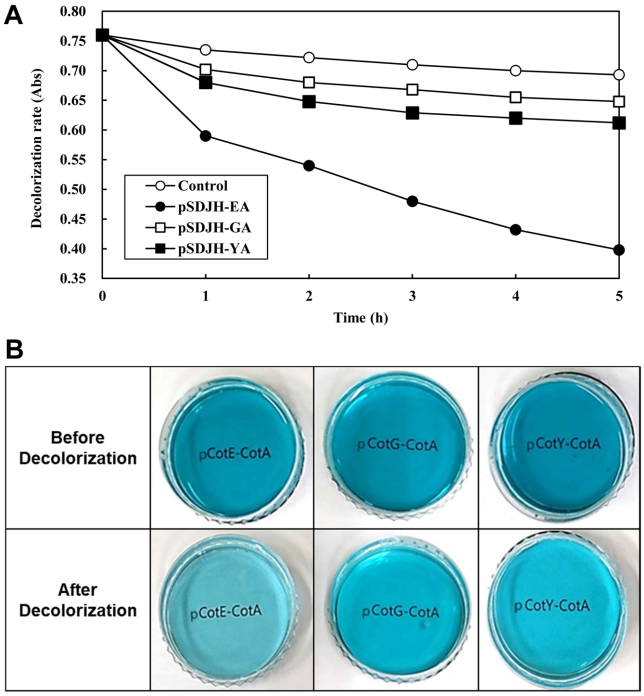

Fig. 3. (**A**) Comparison of laccase activity of DB104 and each recombinant spore on Acid Green 25. The decolorization of Acid Green 25 by DB104 spore was 9.9%, decolorization by recombinant spore was 48.7% by DB104 (pSDJH-EA), 16.1% by DB104 (pSDJH-GA) and 21.1% by DB104 (pSDJH-YA) after 5 h. (**B**) The top photo is before decolorization and the bottom is after decolorization.

Effect of Inhibitors on Laccase Activity

Laccase contains four copper ions that accept and transfer electrons to reduce molecular oxygen; therefore, laccase is susceptible to agents that chelate or reduce copper ions [55].

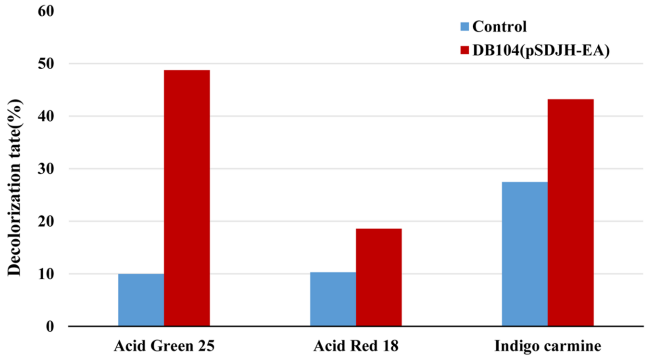

Fig. 4. The decolorization of Acid Red 18 and indigo carmine by DB104 (pSDJH-EA) spore.

Table 1. Comparison of thermostability and effect of inhibitor on CotA activity at previous study.

Fusion protein	Temp. stability	Effect of inhibitor on CotA decolorization activity		
		EDTA (mM)	SDS (mM)	NaN_3 (mM)
CotE-CotA[a]	70°C, 50%	77% (25)	20% (5)	63% (5)
PgsA-CotA[b]	70°C, 90% 90°C, 40%	13.3 ± 3.2% (10)	75.3 ± 1.3% (1)	5.5 ± 2.35% (1)

[a]Our research

[b]2018 Yue Zhang *et al.*

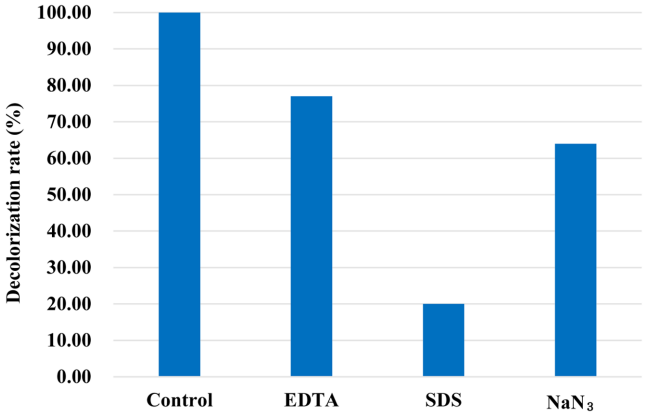

Fig. 5. Effect of inhibitors on the decolorization activity of recombinant DB104 (pSDJH-EA) spore.

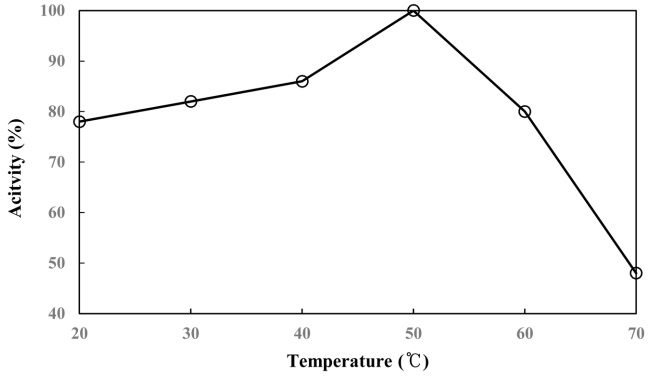

Fig. 6. Effect of temperature on the decolorization of Acid Green 25 using DB104 (pSDJH-EA) spore.

EDTA and NaN_3 are representative metal chelators, while SDS is a representative protein denaturation agent.

The decolorization activity of the recombinant spore decreased by 23% (EDTA), 80% (SDS), and 36% (NaN_3) compared to the control DB104 spore without any inhibitor treatment. The inhibitory effect of EDTA was low even though it was used in abundance. SDS showed the highest inhibitory effect (Fig. 5)

Table 1 shows a comparison between our results and those of the previous studies performed with ABTS [23]. Despite the high concentration of EDTA and NaN_3, the activity of spore surface-displayed *cotA* laccase was higher than that shown in a previous study. This could be attributed to the rigid structure of the spore.

Effect of Temperature on Laccase Stability

The decolorization rate increased with an increase in the temperature from 20°C to 50°C. The decolorization activity at 50°C was the highest at 100%. It decreased after 50°C and was 50% at 70°C (Fig. 6). One study has reported the thermal stability of surface-displayed *cotA* laccase using *pgsA* in *E. coli* [23]. However, contrary to the expectations, the decolorization activity of cell surface-displayed *cotA* was maintained at 40% at 90°C, whereas that of our recombinant spore was reduced to 50% at 70°C (Table 1).

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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