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RESEARCH PAPER

Decolorization of Textile Dye by Spore Surface Displayed Small Laccase for the Enhanced Thermal Stability and Robust Repeated Reaction

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Abstract In this study, we tried to decolorize synthetic dyes using small laccase (SLAC) from *Streptomyces coelicolor*, which is resistant to pH, temperature change, and traditional inhibitors for the actual industrial applications using spore surface display system. We inserted SLAC-His6 tag at the C-terminal of CotE anchoring motif. The proper surface expression of CotE-SLAC fusion protein on the surface of *Bacillus subtilis* spore was verified with flow cytometry using FITC labeled anti-His6 tag antibody. After 6 h of reaction, more than 90% of Indigo carmine was decomposed using recombinant SLAC displaying *Bacillus* spore, whereas less than 10% of Indigo carmine was decomposed with wild type spore. Over 70% of laccase activity was retained with recombinant SLAC displaying spore, which was heat-treated for 3 h at 90ºC. For eight rounds of repeated decomposition of Indigo carmine, no significant decrease of enzymatic activity was observed. This showed the robust characteristics of spore display format for repeated and harsh condition reactions.

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1. Introduction

With the development of texture industry, dyeing has been an essential part of the related industry. Dyes and pigments are the most important colorants used to add a color or to change the color of something [1]. For long time dyeing was mainly dependent on natural dyes, but in 1856 it was replaced by synthetic dyes from the development of Perkin [2]. As the dyeing industry became active, dyeing wastewater treatment has become a very important problem. The dye wastewater composition is very complicated because various kinds of dyes and the dyeing method is different for each product. Therefore, several methods for purifying dye wastewater have been studied. Dye wastewaters are usually treated by the physical or chemical process [3]. However, these methods are usually inefficient in the decolorization, costly and difficult to adapt to a wide range of dye wastewater [3]. Biological process has attracted as a viable alternative to physical or chemical methods due to their low cost, availability, and environmental friendliness [4]. The numerous studies on biological decolorization have been focused on fungal strains [4]. But limitations of fungal decolorization for treating pollutants were high costs of production of microbial culture, slow process, and metabolic inhibition. Also, biological degradation of dyes included properties such as water solubility, large molecular weight, and fused aromatic ring structures, which inhibited permeation through biological cell membranes [4]. Therefore, it is necessary to develop new biological agents that overcome

these drawbacks.

The bacterial surface display system is based on expression of recombinant proteins fused to sorting signals that direct their incorporation on the cell surface [5,6]. Until recently various microbial cell surface display system such as yeast display, gram-positive bacteria display, gram negative bacteria display have been developed [7]. However, this system to be used in actual industrial processes, they must have heat resistance, alkaline tolerance, chemical tolerance, easy recovery, and reusability. The spore display system is the best choice to overcome these problems [8]. Spores are more stable than cells because the spores are covered by a thick peptidoglycan layer called the cortex [8,9]. For this reason, we focused on the spore surface display system and tried to express laccase, a protein capable of decolorizing dyes, making use of these stable spore characteristics.

Laccase (EC 1.10.3.2) are multi copper oxidase enzyme found in plants, fungi, insect, and microorganisms [10]. Laccase act on phenols and similar molecules, performing one-electron oxidations. The initial laccase studied was from *Rhus vernicifera* in 1883, a Japanese lacquer tree [11]. It is known that laccase obtains electrons necessary for redox reaction from hydrogen ions present in the surrounding, converts oxygen into water, oxidizes copper ions after converting copper divalent ions present in laccase into monovalent ions [12-15]. Sodium azide and EDTA known as laccase inhibitors are representative metal chelators [16,17].

Though fugal laccase has been the most actively studied because of its good activity, they are often active at acidic pH or low ionic strength, where the proteins are much less stable [18].

Therefore bacterial laccases are recently studied and reported to overcome those limitation and widen new application areas. First found in *Azospirillum lipoferum* in 1993 [19], bacterial laccases such as *Pseudomonas syringae* (*copA*) [20], *Xanthomonas campestris* (*copA*) [21], *Streptomyces griseus* (*epoA*) [22], *Escherichia coli* (*yacK*) [23,24], (*cueO*) [25], and *Bacillus subtilis* (*cotA*) [26] have been reported to have similar activities. Recently, several cases have been reported such as Indigo carmine decomposition with *Streptomyces coelicolor* [27], Congo red degradation with *B. subtilis* [28], Reactive Black 5 degradation with *Shewanella oneidensis* WL-7 [29], Reactive blue decomposition with *Pseudomonas* sp. [30] and Acid Green 25 and Acid Red 18 degradation using *Shigella dysenteriae* [31,32].

In this experiment, we used a small laccase (SLAC) from *S. coelicolor* [33]. The bacterial laccase is classified into three domain laccase and two-domain laccase according to their structural characteristics. According to previous studies, three domain laccases are *B. subtilis* laccase (CotA), *E. coli* laccase (CueO), and two domain laccases is *S. coelicolor* (SLAC). SLAC means small laccase, consist of only two domains with domain 2 of common laccases lacking [33].

In previous studies, we have studied the activity of laccase by surface expression of CotA using CotE as an anchor protein. In this paper, we tried surface display of structurally different SLAC laccase on the spore. Furthermore SLAC has excellent thermal stability, is not sensitive to changes in pH value, and has excellent resistance to specific inhibitors [27,33]. When these characteristics are combined with the stability of the spore surface display system, very robust environmental remediation format is expected for real situations.

2. Materials and Methods

2.1. Bacterial strains and vectors

E. 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. A two-protease deficient *B. subtilis* DB104 (*trpC2 nprE aprE*) was used as a host strain for the surface display of the SLAC on *B. subtilis* spore. Plasmid pSDJH100 was used for fusion protein expression.

2.2. Culture conditions

B. subtilis was grown in Schaeffer's medium containing 8% w/v nutrient broth, 0.25% $MgSO_4 \cdot 7H_2O$, and 1% KCl in 1 L water. After autoclaving, 1 mL of each filtered stock solution containing $FeSO_4 \cdot 7H_2O$ (1 mM), $MnCl_2 \cdot 4H_2O$ (10 mM), and $CaCl_2 \cdot 2H_2O$ (1 M) was added and supplemented with chloramphenicol (20 μM). Cultivation was carried out by shaking in a 250 mL flask containing 100 mL of Schaeffer's sporulation media at 180 rpm for 24 h at 37ºC. After harvesting all the cells, that was dissolved in 2 mL of sodium acetate buffer.

2.3. Construction of CotE-SLAC fusion protein expression vector and recombinant strain

The genomic DNA of *S. coelicolor* A3(2) was extracted by DNA purification kit (GeneAll Cell SV mini, Seoul, Korea) to amplify laccase gene. Polymerase chain reaction (PCR) amplification was carried out by a pair of primers that were designed to the small laccase gene of *S. coelicolor* A3(2). The structural gene was amplified from total DNA of *S. coelicolor* A3(2) by PCR using the following primers: 5-GGGGACCTGCAGATGGACACGCGAGGCTTTAAC-3 (F) and 5- AAAAACGTCGACTCAGTGGTGATGGTG GTGGTGAAGCTTGTGCTCGTGTTCGTGTGCGGC-3

(R). The forward primer contains the *Pst* I and reverse primer *Sal* I restriction enzyme recognition site to facilitate genetic manipulation. The amplified fragment was digested with *Pst* I and *Sal* I and inserted into pSDJH100 digested with the same enzymes to yield pCotE-SLAC. The PCR reaction was performed with Taq DNA polymerase (Takara, Shiga, Japan). The PCR program was started with an initial denaturation step of 10 min at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 66.7°C for 90 sec, extension at 72°C for 90 sec, and a final elongation step of 10 min at 72°C. The PCR product was purified using a PCR purification kit (Geneall, Seoul, Korea). The two-step (SP I, SP II) method was used for the transformation of the constructed vectors into competent cells of *B. subtilis* DB104 and chloramphenicol (20 μM) was used for the selection.

2.4. Spore purification and flow cytometry analysis

The cultured cell was harvested and purified using the Renografin (sodium diatrizoate, S4506; Sigma, Burlington, MA, United States) gradient method and confirmed under the microscope.

To confirm the surface localization of CotE-SLAC on spore by immunofluorescence staining, purified spores of recombinant strain were washed two times with phosphate-buffered saline (PBS) solution and then resuspended in 100 μL PBS solution containing FITC labeled anti-His6 tag antibody (1:200) (K0212220; Komabiotech, Seoul, Korea) for 1 h on ice. After, the spores were washed again two times with PBS solution. These spores were examined under flow cytometry (Beckman Coulter Epics XL, Brea, CA, USA). The host strain, *B. subtilis* DB104 spore (without the construction vector) was used as a negative control. Each 20,000 cells were counted for data collection.

2.5. Reaction condition for the measurement of laccase activity

Reaction buffers for dye decomposition were 50 mM sodium acetate buffer at acidic pH and 50 mM Tris-HCl buffer at alkaline pH. Total reaction volume was 10 mL and 5 mg of purified spore was added for enzymatic decomposition of synthetic dye. Indigo carmine (200 μM) was used for the enzymatic reaction. Reaction was performed at 37ºC.

After reaction, the absorbance difference was measured with an EMC-11D-V spectrophotometer (EMCLAB, Duisburg, Germany). Each dye was measured at its maximum absorption wavelength, and the absorption wavelength for Indigo carmine and Alizarin was 610 nm and 575 nm, respectively.

2.6. Repeated usage of SLAC displayed spore

For the repeated continuous decomposition of Indigo carmine, simple centrifugation of used recombinant DB104 (pCotE-SLAC) spore and washing with PBS buffer and resuspension into newly prepared Indigo carmine containing reaction buffer were executed between each round.

3. Results and Discussions

3.1. Construction of CotE-SLAC fusion protein expression vector

For the proper spore surface expression of target protein, selection of appropriate surface anchoring motif is most important. The most important property of anchoring motif is its location and its abundance in the whole spore structure. The location of each anchoring motif (generally spore coat protein needed for the spore formation) is determined by protein-protein interaction in the spore formation process, and its abundance is determined by specific sigma factor related RNA polymerase and translation process. Though we can overexpress specific coat protein with strong promoter and other regulations, amount of spore embeddable coat protein is determined by spore formation mechanism.

For the last several years, our group tried many spore coat proteins as efficient anchoring motives for the spore surface expression of target proteins. Selected anchoring motives were yuaB, cotE, cotG, and cotY [5,34-41].

Among these anchoring motives, cotE was most efficient in various applications because of its abundance and proper location in spore structure [36,38-40]. CotE with a size of 20.8 kDa is needed for the assembly of the outer coat protein.

SLAC is a small laccase from protein composed of 343 amino acids (37 kDa). SLAC is enzymatically active in dimeric form, which is a good target substrate for spore surface display, which overcome the translocation of expressed fusion protein across cell membrane or cell wall. In normal bacterial surface display system, multimeric protein can't be transported across cell membrane. SLAC was chosen because of its pH optimum in the alkaline region, its resistance to known inhibitors, and its thermal stability in high temperature, which will be highly preferred in real decomposition of synthetic dyes.

For the construction of SLAC expression vector, SLAC was connected to C-terminal of the anchoring motif CotE, and His6 tag is connected to the C-terminal of the SLAC. Flexible linker (Gly-Gly-Gly-Gly-Ser) was inserted between CotE and SLAC, to enhance the flexibility of fusion protein. The construction of the SLAC expression vector is shown in Fig. 1. This expression vector was named as pCotE-SLAC. Constructed expression vector was transformed into host bacteria DB104 with Chloramphenicol selection to yield recombinant DB104 (pCotE-SLAC).

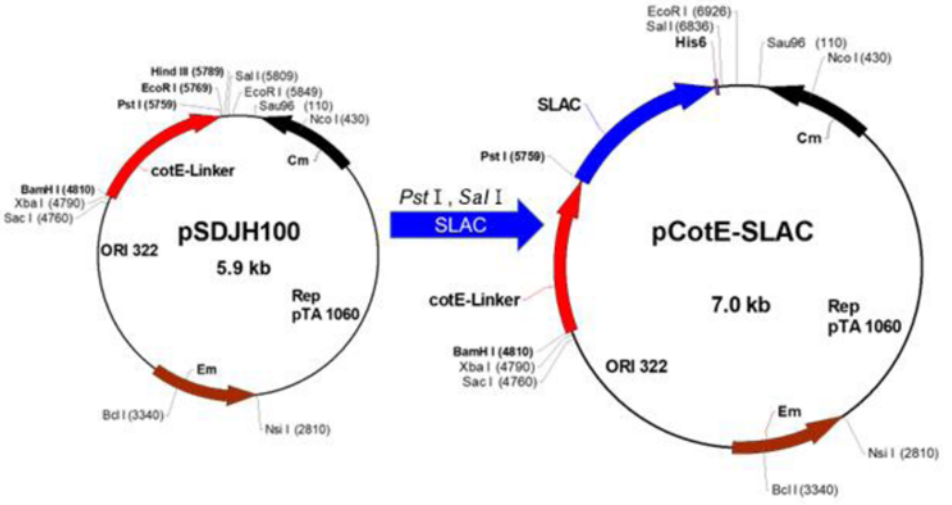

Fig. 1. Construction of pCotE-SLAC expression vector. The amplified fragment was digested with *Pst* I and *Sal* I and inserted into pSDJH100 digested with same enzymes. SLAC: small laccase.

3.2. Verification of surface display of SLAC on *B. subtilis* spore

Surface localization of SLAC on the spores was confirmed by flow cytometry. Flow cytometry can quickly detect each particle or cell which has different characteristics such as fluorescent emission. Purified spores of DB104 (pCotE-SLAC) and wild type DB104 (negative control) were reacted with FITC labeled anti-His6 tag antibody.

FITC labeled anti-His6 tag antibody can only access His6 tag motif when it is expressed on the surface of spore. Fluorescence intensity of the spores of DB104 (pCotE-SLAC) expressing CotE-SLAC fusion protein considerably increased in comparison with that of the negative control DB104 (Fig. 2). This result showed that CotE-SLAC fusion protein was expressed correctly on the outer spore surface which can be detectable by anti-His6 tag antibody.

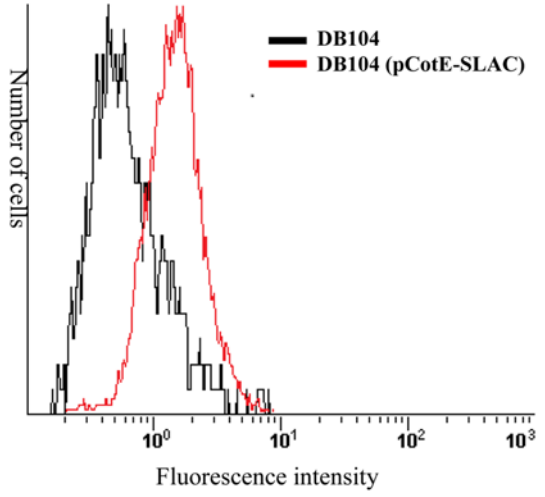

Fig. 2. Flow cytometry of spores of DB104 (black line) and recombinant DB104 (pCotE-SLAC) (red line) using FITC labeled anti-His6 tag antibody which detect the C-terminal fused His6 tag. Fluorescence intensity of the spores of DB104 (pCotE-SLAC) expressing CotE-SLAC fusion protein considerably increased in comparison with that of the negative control DB104. SLAC: small laccase.

3.3. Measurement of the enzymatic activity of surface displayed SLAC against Indigo carmine

After the construction of recombinant DB104 (pCotE-SLAC) spore, enzymatic activity of surface displayed SLAC was confirmed with ABTS assay (data not shown).

Indigo carmine was selected as a model synthetic dye. Though SLAC showed enzymatic activity using ABTS and DMP in a wide range of pH (pH 4-pH 11) [27], Indigo

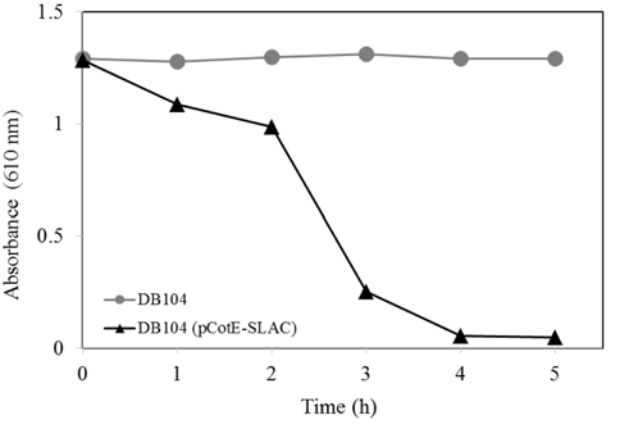

Fig. 3. Decolorization of Indigo carmine by spore surface expressed SLAC. Recombinant DB104 (pCotE-SLAC) spore (closed triangle) expressing SLAC decomposed more than 90% of Indigo carmine within 5 h of reaction, whereas DB104 spore (closed circle), which was used as a negative control, decomposed less than 10% of Indigo carmine in the same experiment. SLAC: small laccase.

carmine was adsorbed on the surface of spore in a lower pH range, therefore the decolorization experiment was executed in alkaline condition (pH 9).

Recombinant DB104 (pCotE-SLAC) spore expressing SLAC decomposed more than 90% of Indigo carmine within 5 h of reaction, whereas DB104 spore, which was used as a negative control, decomposed less than 10% of Indigo carmine in the same experiment (Fig. 3).

Electron mediator, Syringaldehyde is frequently added to promote electron transfer in redox reaction. To confirm the electron mediator effect in our system, same experiment was done with 3 mM Syringaldehyde presence. With Syringaldehyde, recombinant DB104 (pCotE-SLAC) spore decomposed more than 90% of Indigo carmine with 1 h of reaction, whereas less than 50% of Indigo carmine was decomposed without Syringaldehyde. This result is consistent with those of Dubé *et al*. [27].

3.4. Thermal stability of spore surface displayed SLAC

Spore surface display format provide additional advantage to displayed protein such as thermal stability in elevated temperature [42,43]. Because Indigo carmine is self-decomposed at a higher temperature over 60°C (data not shown), recombinant DB104 (pCotE-SLAC) spore was pre-heated in a water incubator for 3 h with a different temperature range from 70°C to 100°C. Prepared SLAC displaying spore was used for the decomposition of Indigo carmine. Spore which was incubated in the same condition at 25°C was used as positive control. After 5 h of reaction, the relative amount of decomposed indigo carmine is represented (Fig. 4).

Though the remaining laccase activity of spore displayed SLAC is lowered with increasing incubation temperature from 25°C to 100°C, over 70% of laccase activity was retained with SLAC displaying spore, which was heat-treated for 3 h at 90°C. Even after incubation at 100°C for 3 h, 26% of laccase activity was retained in the same experiment. Though the experimental condition is a little bit different, thermal stability of our experiments is consistent with the result of previous reports [27,44]. Spore display format might endowed SLAC with additional thermal stability in our experiment.

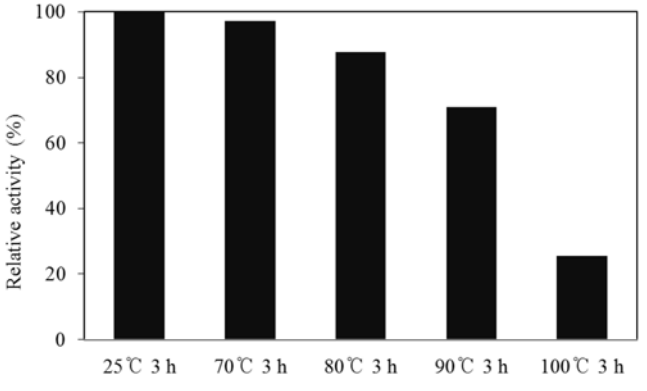

Fig. 4. Thermal stability of spore surface expressed SLAC. Recombinant DB104 (pCotE-SLAC) spore was pre-heated in a water incubator for 3 h with a different temperature range from 25°C to 100°C. Prepared SLAC displaying spore was used for the decomposition of Indigo carmine. SLAC: small laccase.

According to previous studies, the optimum pH range for dye decolorization of SLAC protein is 4-5 or 9-10 [27,45]. However, in our experiment, at pH 4 the substrate Indigo carmine was adsorbed on the spore surface (data not shown).

At alkaline pH range, Indigo carmine was efficiently decomposed using SLAC expressing spore, and highest decomposition was observed around pH 9 and pH 10. These results were comparable to those of previous study by Sherif *et al*. [44].

At pH 6, the lowest enzymatic decomposition of Indio carmine was observed with less than 20% of the values obtained at pH 10.

3.5. Decomposition of other synthetic dyes

After successful decomposition Indigo carmine, we tried decomposition of other synthetic dyes such as Remazol brilliant blue R, Brilliant Green, Congo Red, New Coccine and Alizarin using recombinant DB104 (pCotE-SLAC) spore.

Unfortunately, most of them have some difficulties in solubilization and reaction process. Some of them were not soluble in our reaction conditions at pH 4 and pH 9, and most of them were adsorbed on the surface of recombinant DB104 (pCotE-SLAC) spore. Therefore it was very hard to follow the proper decomposition reaction of each dye (Table 1). The adsorption of synthetic dyes is attributed to

Table 1. Decomposition of various synthetic dyes using recombinant DB104 (pCotE-SLAC) spore

Synthetic dye	pH 4	pH 9
Indigo carmine	Adsorption	**Decomposition**
Remazol brilliant blue R (RBBR)	Adsorption	Adsorption
Alizarin Red S	Adsorption	**Decomposition**
Brilliant Green	Adsorption	Precipitation
New Coccine	No reaction	Adsorption
Congo Red	Precipitation	Adsorption

Precipitation means that specific dye was not soluble, but precipitated in the reaction buffer.
Adsorption means that dye was soluble in the reaction buffer, but dye was mainly adsorbed onto the spore surface. A little amount of dye can be decomposed by recombinant DB104 (pCotE-SLAC) spore, but measurement of the exact amount of decomposition was very hard.
No reaction means that dye was soluble in the reaction buffer and the dye was not adsorbed onto the spore, but there was no enzymatic decomposition of dye.
Decomposition means that dye was soluble in the reaction buffer, dye was not adsorbed onto the spore, and dye was successfully decomposed in a limited reaction time.

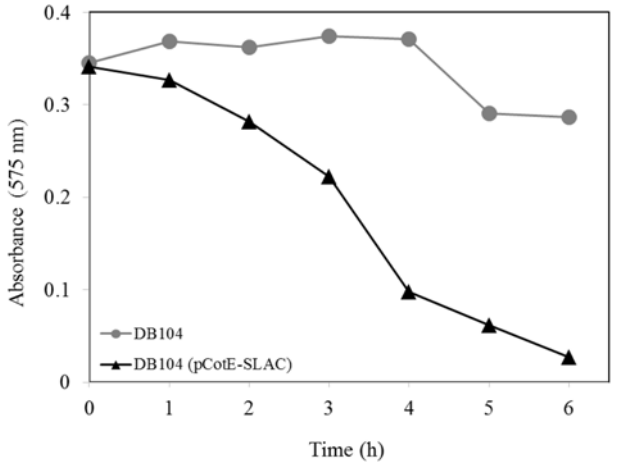

Fig. 5. Decolorization of Alizarin by spore surface expressed SLAC. Recombinant DB104 (pCotE-SLAC) spore (closed triangle) expressing SLAC decomposed more than 90% of Alizarin within 6 h of reaction, whereas DB104 spore (closed circle), which was used as a negative control, decomposed less than 10% of Alizarin in the same experiment. SLAC: small laccase.

the surface charge and interaction forces between spore and each dye.

Beside Indigo carmine at pH 9, only Alizarin was properly decomposed at pH 9 and showed significant decomposition rate using recombinant DB104 (pCotE-SLAC) spore (Fig. 5). From the structure of Alizarin, 1,2-dihydroxyanthraquinone, this result is fairly understood. SLAC is known to have highest enzymatic activity on large phenolic compound at alkaline pH because of the predicted influence of pH on the redox potential of phenolic substrates [44].

3.6. Repeated usage of SLAC displayed spore

Repeated usage of whole cell or immobilized enzyme system is always appreciated in practical application of biotechnological process [46,47]. To exploit the advantage of spore surface display system, repeated usage of recombinant SLAC displayed spore was done to decompose Indigo carmine. Surface display format has another advantage over soluble expression of target protein. We can easily harvest the surface displayed enzyme just by centrifugation and reuse it for another round of reaction.

For 14 h of reaction, eight rounds of decomposition of Indigo carmine were repeated using recombinant DB104 (pCotE-SLAC) spore (Fig. 6). Between each round, simple centrifugation of used SLAC displayed spore and resuspension into newly prepared Indigo carmine containing reaction buffer were executed and the decomposition of Indigo carmine was monitored with appropriate intervals. For eight rounds, no significant decrease of enzyme activity was observed. To shorten reaction time, syringaldehyde was added into reaction mixture.

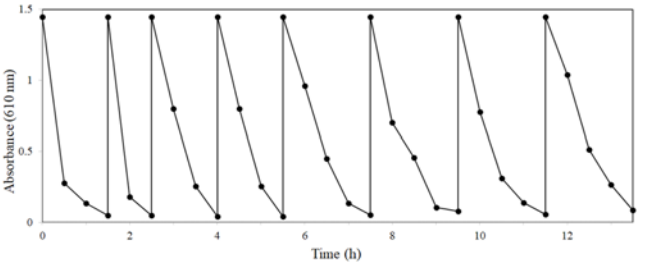

Fig. 6. Repeated usage of recombinant DB104 (pCotE-SLAC) spore for the decomposition of Indigo carmine. For 14 h of reaction, eight rounds of decomposition of Indigo carmine were repeated. Between each round, simple centrifugation of used SLAC displayed spore and resuspension into newly prepared Indigo carmine containing reaction buffer were executed and the decomposition of Indigo carmine was monitored with appropriate intervals. For eight rounds, no significant decrease of enzyme activity was observed. To shorten reaction time, syringaldehyde (3 mM) was added into reaction mixture. SLAC: small laccase.

4. Conclusions

Laccases are very important enzymes for the removal of synthetic dye especially in the field of textile industry. For the practical application, enhancement of enzyme stability in the point of harsh condition such as alkaline condition, high temperature reaction, and repeated usage of prepared enzyme.

In this report, we combined SLAC, which is known to have enzymatic activity in a wide pH range against synthetic dye, high thermal stability, resistance to known laccase inhibitor, with spore surface display platform to exploit its advantage for thermal stability enhancement of target protein and its robustness in repeated reaction.

For thermal stability enhancement of target protein using bacterial surface display platform, there are several reports till now. Several spore coat anchoring motif were compared to compare the activity of enhanced green fluorescent protein (eGFP) under extreme conditions (heat, pH, and protease challenges). Compared to the control groups, the activities of ΔCotB-eGFP and CotB-eGFP were maintained at 56% and 41% at 80°C and 88% and 55% at pH 10, respectively [43]. Laccase CotA from *B. subtilis* 168 was successfully displayed on the membrane of *E. coli* cells using poly-γ-glutamate synthetase A protein (PgsA) from *B. subtilis* as an anchoring matrix. Further analyses demonstrated that the fusion protein PgsA/CotA efficiently translocates to the cell surface of *E. coli* with an enzymatic activity of 65 U/10 cells. Surface-displayed CotA was shown to possess improved enzymatic properties compared with those of the wild-type CotA, including higher thermal stability (above 90% activity at 70°C and nearly 40% activity at 90°C after 5-h incubation) [42].

In our experiment, though the remaining laccase activity of

spore displayed SLAC is lowered with increasing incubation temperature from 70°C to 100°C, over 70% of laccase activity was retained with SLAC displaying spore, which was heat-treated for 3 h at 90°C. Even after incubation at 100°C for 3 h, 26% of laccase activity was retained in the same experiment.

For repeated usage of target proteins, there are also several reports using bacterial surface display of laccase. A bacterial laccase (WlacD) was engineered onto the cell surface of the solvent-tolerant bacterium *Pseudomonas putida*. The engineered system exhibited a comparably high activity compared with those of separate dyes in a continuous three-round shake flask decolorization of AG25/AR18 mixed dye (each 1 g/L). No significant decline in decolorization efficacy was noted during first two-rounds but reaction equilibriums were elongated, and the residual laccase activity eventually decreased to low levels. However, the decolorizing capacity of the system was easily retrieved via a subsequent 4-h cell culturing [32]. A triplicate volcanic rock matrix–*Bacillus thuringiensis*–laccase WlacD (VRMs–Bt–WlacD) dye decolorization system was developed. WlacD was displayed on the *B. thuringiensis* MB174 cell surface to prepare a whole-cell laccase biocatalyst by using two repeat N-terminal domains of autolysin Mbg (Mbgn)2 as the anchoring motif. Repeated decolorization-activation operations showed the high decolorization capacity of VRMs-Bt-WlacD and have the potential for large-scale or continuous operations [48].

In our case, practically no reduction in enzymatic activity for 8 rounds of repeated usage over 14 h of reaction time, was observed. This is much better result compared to the reported cases.

Overall, the excellent performance of our CotE-SLAC on spore display platform in thermal stability and repeated usage for the decomposition of synthetic dye, Indigo carmine and alizarin will provide new insight in the real field remediation of industrial dye contaminated water.

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Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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