

Ecofriendly one-pot biosynthesis of indigo derivative dyes using CYP102G4 and PrnA halogenase

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ABSTRACT

In this study, the biosynthesis of various indigoids with novel spectral features and antibacterial activities was investigated. First, 12 indole derivatives as substrates were biotransformed into functional indigoid dyes by *E. coli* cells expressing CYP102G4 hydroxylase. The indole derivatives included chloro (Cl-), nitro (NO₂-), hydroxy (HO-), methoxy (CH₃O-), methyl (CH₃-), carboxy (COOH-), amino (NH₃⁺), and cyano (CN-) indoles at the C4 to C7 positions. Interestingly, dramatic color shifts were observed from blue to red, green, purple, and even pink depending on the functional groups and their positions. Next, the biological and physical properties, antibacterial effects, and dying fastness of the prepared compounds were investigated and visually measured. Among the synthesized indigoid dyes, 6,6'-dichloroindigo and 5,5'-dichloroindigo showed the relatively higher cell growth inhibitory activity in the liquid phase. Finally, a one-pot producing strain which produced 7,7'-dichloroindigo from L-tryptophan using tryptophan-7-halogenase (PrnA) and CYP102G4 simultaneously was developed to overcome the disadvantages of uneconomical semi-synthesis through indole precursor feedstocks. The developed producing strain produced approximately 15.4 ± 1.4 mg/L of 7,7'-dichloroindigo in 24 h. To the best of our knowledge, this is the first report of the production of 7,7'-dichloroindigo in *E. coli* via a one-pot process.

1. Introduction

Indigo dye is one of the most widely used dye in the world and accordingly large markets exist for indigo dyes. They have diverse applications as synthetic and natural dyes whose demand continually increasing [1]. However, high demand has also caused problems of sustainability and environment. The chemical synthesis of indigo dyes starting with petrochemical resources such as aniline utilizes hazardous chemicals such as catalysts and reducing agents used for its synthesis and dyeing process must be addressed. For example, synthetic route starting from aniline developed by BASF in 1897 requires molar equivalent amount of formaldehyde and excess amount of NaNH₂ for synthetic indigo production. Approximately 50 ktons of synthetic indigo dyes were produced through this by 2011 [2–5]. Various studies have been conducted regarding the synthesis of indigo dyes from various sustainable bioresources to replace petrochemical resources

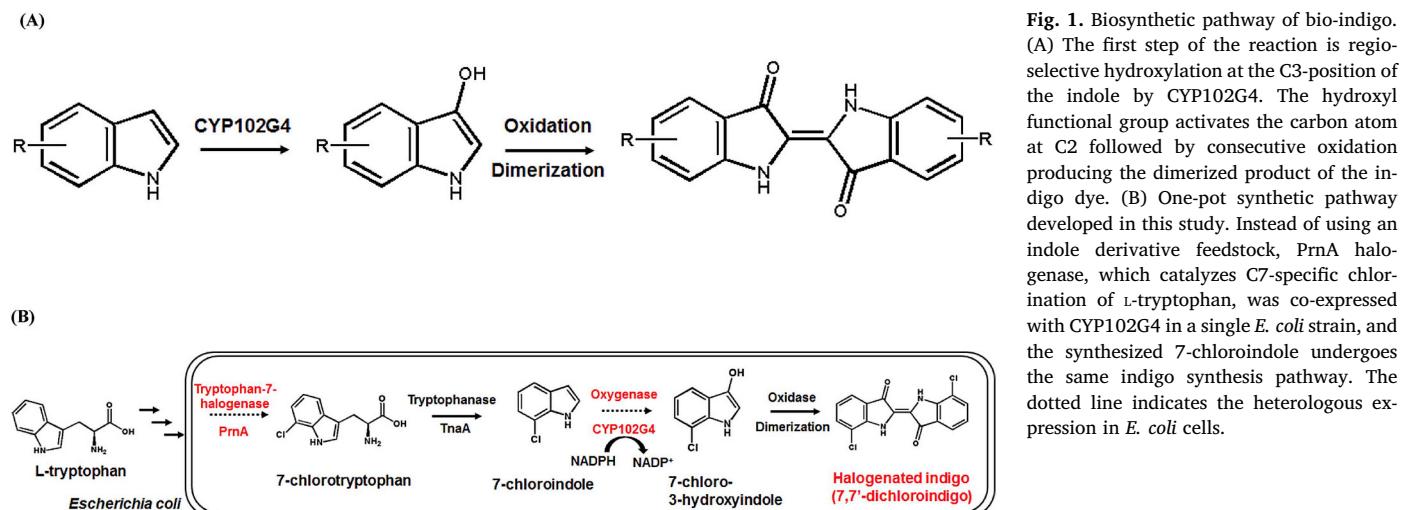
[6–12]. In particular, our lab engineered a cytochrome P450 (CYP102G4) enzyme to produce high concentrations of indigo through glucose and indole feedstocks, indicating that mass production of indigo through eco-friendly processes is possible [13,14]. Moreover, studies regarding the production of glucose-conjugated indican in recombinant *Escherichia coli* using glycosyltransferase have recently been reported, which avoided the use of sodium dithionite (SDT) as a reducing agent used for resolving the low solubility of indigo dye during dyeing process [3].

Along with abovementioned researches focusing on solving the sustainability and environmental issues associated with the production of indigo dyes, significant interest also exists about functional dyes. Attempts have been made to develop dyeing materials with anti-inflammatory, antibacterial, and antioxidant functions. For example, a violacein, which is a pigment produced by *Chromobacterium violaceum*, has been used as a dyeing material with antibacterial functionality

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[15–18]. Research to develop functional dyes from natural products and studies to improve their performance by introducing additional moieties have also become popular [12].

The introduction of halogen atoms to natural compounds has been studied with considerable interest in marine-derived actinobacteria [19–21]. For example, well-known halogenated macrolides, sporolides A or B were isolated from a marine actinomycete, *Salinispora tropica* and a recent study elucidated two groups of highly halogenated bisindole pyrroles, lynamicins and spiroindimicins and genomic clues of their synthesis were identified from a marine-bacteria *Streptomyces* sp. MP131-18 [22,23]. Interestingly, studies regarding the structure-physiological activity relationship of marine-derived natural products revealed that regio-selective halogenation of natural products increased their anti-bacterial activity [24,25]. For example, the introduction of chlorine to vancomycin, which is a popular antibiotic for the treatment of multiple-drug resistant *Staphylococcus aureus* infections, imparts increased antibacterial activity by controlling its clinically active conformation [26–28]. Many active compounds require this type of chlorine modification, such as salinoporamide A, rebeccamycin, neomangicol A and B [29–31]. In most cases, the halogenation reaction is largely site-specific, which maintain physiological activity although the understanding of the mechanism of action remains poor.

In addition to the halogenation of various natural products mentioned above, the introduction of moieties such as position-specific hydroxyl and nitrite groups to natural products alters their physical properties and physiological activity in a similar manner as halogenation. For example, the structural modification of isoflavone and thaxtomin phytotoxin through regio-selective hydroxylation and nitration using cytochrome P450 (CYP) monooxygenase enzymes increases its hydrophilicity and antioxidant/anti-inflammatory effects, respectively [32–34].

In this study, we investigated the functional modification indigo dye, and their antibacterial activity, dyeing performance were measured. Besides chloro-halogenation, interesting dyeing and antibacterial properties of the hydroxylated (-OH), carboxylated (-COOH), methylated (-CH₃), O-methylated (-OCH₃), and nitrated (-NO₂) indigo were investigated. Based on the results of the indole-produced indigo from *E. coli* expressing CYP102G4, the synthesis of various functional indigo molecules was achieved by feeding an indole derivative chemically substituted with functional residues to *E. coli* cells expressing CYP102G4. The most promising functional indigo was developed for a one-pot synthetic process using cells expressing CYP102G4 and halogenase simultaneously. Finally, we succeeded in developing a production strain applicable to consolidated bioprocessing through continuous enzymatic reaction in a single cell. This approach provides information for biosynthesis studies of various functional derivative compounds.

Fig. 1. Biosynthetic pathway of bio-indigo. (A) The first step of the reaction is regioselective hydroxylation at the C3-position of the indole by CYP102G4. The hydroxyl functional group activates the carbon atom at C2 followed by consecutive oxidation producing the dimerized product of the indigo dye. (B) One-pot synthetic pathway developed in this study. Instead of using an indole derivative feedstock, PrnA halogenase, which catalyzes C7-specific chlorination of L-tryptophan, was co-expressed with CYP102G4 in a single *E. coli* strain, and the synthesized 7-chloroindole undergoes the same indigo synthesis pathway. The dotted line indicates the heterologous expression in *E. coli* cells.

2. Materials and methods

2.1. Chemical reagents

Indigo, 12 indole derivatives including 2-carboxyindole, 4-chloroindole, 5-chloroindole, 6-chloroindole, 4-nitroindole, 5-nitroindole, 5-methylindole, 5-methoxyindole, 5-aminoindole, 5-bromoindole, 4-cyanoindole, and 5-hydroxyindole, glucose, δ-aminolevulinic acid, and L-tryptophan were purchased from Sigma-Aldrich Korea (Suwon, South Korea). All chemical reagents used in this study were of analytical grade or higher.

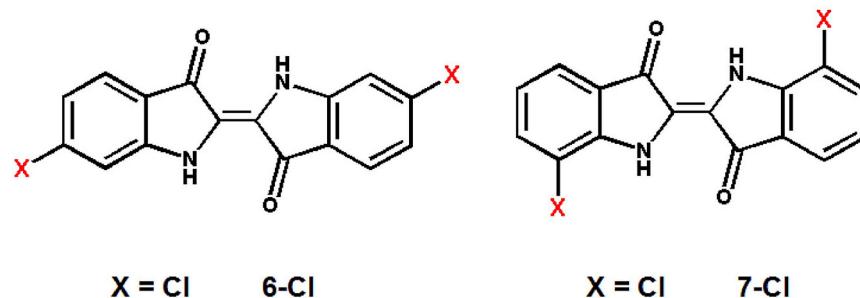
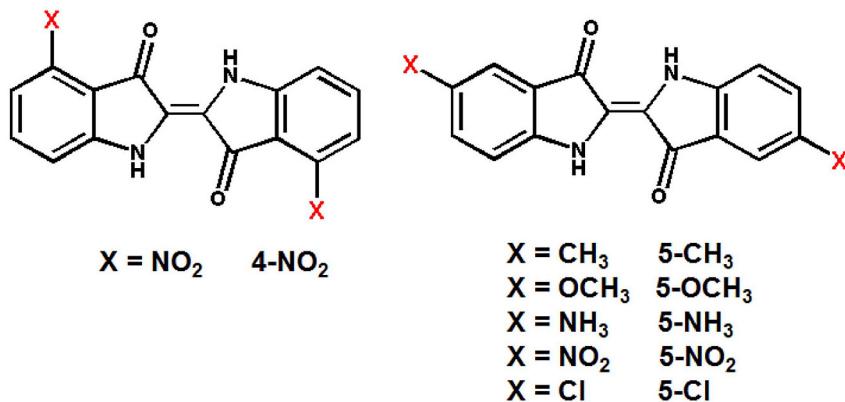
2.2. Expression of CYP102G4 and biotransformation of indole derivatives in *E. coli*

The *cyp102G4* gene sequences (Scat4838) were obtained from the *Streptomyces avermitilis* genome project homepage (<http://avermitilis.lskitasato-u.ac.jp/>) [14]. The gene was codon-optimized based on *E. coli* codon usage and synthesized by Bioneer (Daejeon, South Korea). The synthetic *cyp102G4* gene was cloned into a pET28a(+) vector and the plasmid was transformed into BL21(DE3) cells, after which the transformants were grown in 50 mL Terrific Broth (TB) medium containing 50 μg/mL kanamycin at 37 °C, until an OD₆₀₀ of 1.5 was reached. Subsequently, 12.5 μM of isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.25 mM δ-aminolevulinic acid (ALA) as a heme precursor were added with 1 mM of each indole derivative as biotransformation substrate. The cells were further incubated at 30 °C for 24 h in a high-speed incubator (200 rpm). Subsequently, the reaction was quenched by adding an equal volume of dimethylsulfoxide (DMSO), followed by vigorous vortexing. The mixtures were centrifuged at 13,000 rpm for 10 min and the supernatant was collected and concentrated under vacuum for overnight. The prepared samples were dissolved in 50 μL MeOH and prepared for TLC separation, HPLC, GC/MS, ¹H NMR analysis.

2.3. Separation and spectral features of the biosynthetically produced indigoids

The fractions collected from each biotransformation culture were separated by centrifugation and further taken for TLC, HPLC, and ¹H NMR. For TLC (silicagel matrix, Merk Millipore, Darmstadt, Germany) analysis, the mobile phase was composed of chloroform: hexane: methanol (5:4:1). The indigo derivatives were separated using a HPLC equipped with a C18 reverse phase column (Zorbax extend-C18 Waters, 250 mm × 4.6 mm, 3.5 μm, Agilent, USA) and eluted at 1.0 mL/min with acetonitrile (CAN)/water (50:50 v/v). The absorbance of the

(A)



5,5'-dibromo indigo	7,7'-dichloro Indigo	6,6'-dichloro indigo	5,5'-dichloro indigo
2,2'-dicarboxy indigo	5,5'-dihydroxy indigo	5,5'-dimethyl indigo	5,5'-dimethoxy indigo
5,5'-dinitro Indigo	4,4'-dinitro Indigo	5,5'-diamino Indigo	4,4'-dicyano Indigo

(B)

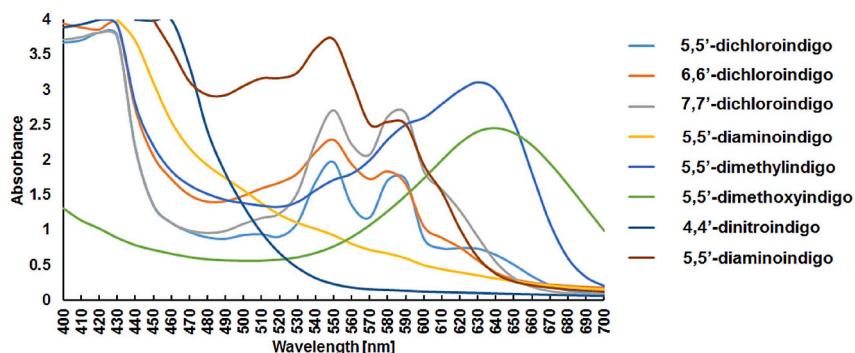


Fig. 2. The synthesized indigoids obtained by feeding indole derivatives to the CYP102G4 expressing cells. (A) Depending on functional group R, various indigoids were prepared and their color shifts were visualized in the plate. (B) Spectral features of synthesized indigoids. Spectral scanning was performed from 400 to 700 nm, revealing unique characteristics of the indigoids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

eluent was monitored at its most intense wavelength, as determined from spectral scanning results. The absorption spectra of the synthesized indigo derivative samples were measured by wavelength scanning from 400 to 700 nm via UV-vis spectrophotometry at 10 nm intervals. For ¹H NMR analysis, AVANCE II 400 (400 MHz, Bruker, MA, USA) was used (Fig. S2). Chemical shift values (δ) are given parts per million

using residual solvent protons ($\delta\text{H} = 2.49$ for DMSO- d_6).

2.4. Determination of dyeing fastness and performance of synthesized indigo derivatives

The dyeing fastness and performance were investigated using a

Table 1

List of indole derivatives used as substrates in this study and their R_f values in the TLC separation.

Substrate	R _f	Color
5-chloroindole	0.38	Brown
6-chloroindole	0.46	Pink
7-chloroindole	0.46	Purple
4-nitroindole	0.72	Yellow
5-nitroindole	0.51	Red
5-methylindole	0.55	Blue
5-methoxyindole	0.71	Pistachio
5-aminoindole	0.55	Brown

Table 2

Color differences of indigoid synthesized through biotransformation of indole derivatives.

Substrate	L*	a*	b*	dE
indigo	29.42	-1.62	-8.71	0.00
5,5'-dimethylindigo	39.44	9.10	-8.58	14.67
5,5'-dimethoxyindigo	39.20	-11.82	-9.17	14.14
5,5'-diaminoindigo	33.06	13.37	5.43	20.85
4,4'-dinitroindigo	28.54	-4.53	2.66	11.77
5,5'-dinitroindigo	30.61	15.76	0.54	19.72
5,5'-dichloroindigo	29.73	14.69	-1.33	17.90
6,6'-dichloroindigo	30.64	10.15	-2.46	13.38
7,7'-dichloroindigo	31.46	7.40	-5.57	9.77

L*: brightness, a*: the closer to +, the more red, the closer to -, the more green; b*: the closer to +, the more yellow, the closer to -, the more blue.

$$\Delta E_{ab}^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

general dyeing procedure of consecutive reduction-oxidation reactions in cotton. Cotton was selected as a dyeing material, as it is frequently used in fabric industry, and dyed using 8 of the synthesized indigoids and indigo as a control (indigo, 5,5'-dimethylindigo, 5,5'-dimethoxyindigo, 5,5'-diaminoindigo, 4,4'-dinitroindigo, 5,5'-dinitroindigo, 7,7'-dichloroindigo, 6,6'-dichloroindigo, and 5,5'-dichloroindigo). The same amount of each synthesized 5 mL of dyeing solution in methanol (1 mg/mL) was prepared, followed by dyeing the cotton fabric (2 cmx2 cm) for 2 min in dyestuff solution and was dried in air completely before washing. Subsequently, the stained cotton was collected for washing with distilled water. To compare dyeing fastness, three consecutive dyeing-washing processes were conducted.

2.5. Determination of color difference of synthesized indigoids

The color difference was measured using a Colorimeter JZ-600 instrument (Shenzhen Kingwell instrument Co., Ltd., Guangdong, China) and analyzed by Color Analysis Management Software.

2.6. Determination of antibacterial activity of the indigo derivatives

The antibacterial activity of the generated indigo derivatives (indigo, 5-CH₃, 5-OCH₃, 5-NH₃, 4-NO₂, 5-NO₂, 7,7'-dichloroindigo, 6,6'-dichloroindigo, and 5,5'-dichloroindigo) was determined by cell growth inhibition assay on solid plates (disk diffusion test) and liquid culture. *E. coli* BL21(DE3) and *Staphylococcus aureus* were used for the antibacterial tests. For the disk diffusion test, each synthesized indigo solution (0.1 mM in MeOH) was prepared and spotted on the solid plate (50 μL on each paper disk) containing spread cells. After 12 h of incubation, the hollow size was measured and compared between samples. Similarly, cell growth inhibition activity in liquid culture was investigated. After growing the cells in a test tube in the presence of 0.1 mM of indigoid dye, the final OD₆₀₀ was measured and compared.

2.7. Whole cell production of 7,7'-dichloroindigo by *E. coli* expressing both tryptophan halogenase (*PrnA*) and CYP102G4

The *prnA* gene sequences were obtained from the Database EMBL AAD46365.1, codon-optimized based on *E. coli* codon usage, and synthesized by Pioneer (Daejeon, South Korea). The synthesized *prnA* and *cyp102G4* genes were cloned into a pETduet-1 vector and the plasmid was transformed into *E. coli* BL21(DE3) cells. To verify the expression of PrnA and CYP102G4, *E. coli* cells harboring the plasmid (pETduet-1::*prnA*::*cyp102G4*) were cultured in 50 mL of LB-medium supplemented with 50 μg/mL of ampicillin at 37 °C, until reaching an OD₆₀₀ of 0.8. Subsequently, 12.5 μM of IPTG and 0.25 mM of ALA were added along with an additional 0.1 mM of L-tryptophan, after which the cells were further incubated at 30 °C for 24 h. For the production of 7,7'-dichloroindigo using the same *E. coli* strain, TB media was used and the same procedure was followed for the biotransformation of indole derivatives by *E. coli* cells expressing single CYP102G4, as described in Section 2. 2.

3. Results and discussion

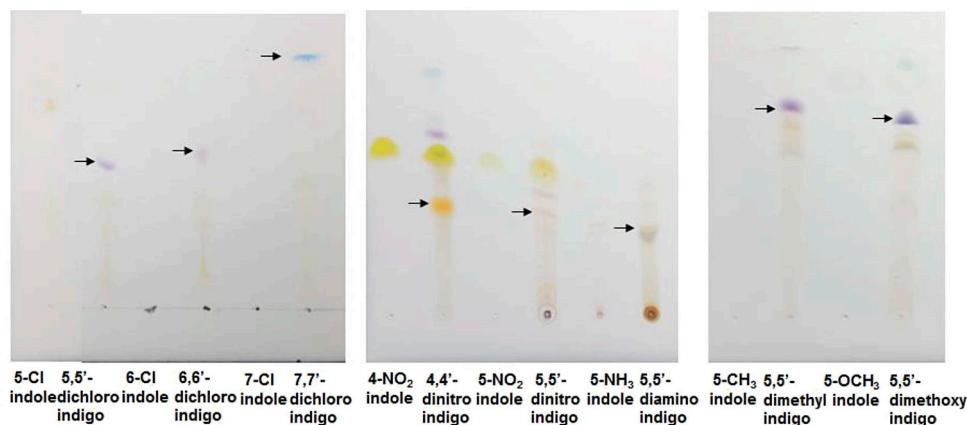
3.1. Biotransformation of the indole derivatives by *E. coli* cells expressing CYP102G4

Bio-indigo synthetic pathway was described in Fig. 1A. The first step of the reaction is regio-selective hydroxylation at C3-position of indole substrate. To date, several indole C3 hydroxylation reactions have been reported using a variety of enzymes such as naphthalene dioxygenase and toluene dioxygenases [35]. A self-sufficient cytochrome P450 monooxygenase of CYP102G4, which our group reported previously, was used in the indigoid synthesis for its superior regioselectivity and indigo production to other indigo-synthesizing enzymes [14]. The single expression of CYP102G4 in *E. coli* was able to produce a noticeable amount of indigo and these cells were used as a biotransformation platform for the synthesis of functional indigo derivatives (see Table 1).

The first step for the biotransformation of indole derivatives is to investigate the CYP102G4 substrate specificity against indole derivatives. As shown in Fig. 2A, various indole derivatives excluding structural modification at N1 to C3 of the pyrrole ring were examined for the production of functional indigo dye, as the positions seemed to exhibit steric hindrance or direct blocking towards the C3 hydroxylation reaction. This is consistent with the previously reported paper that indole derivatives with such modification could not dimerize to form indigoid structure [35]. The examined substrates included indoles with chloro-at positions 5 (5-Cl), 6 (6-Cl), and 7 (7-Cl), 5-bromo (5-Br), 2-carboxylic (2-COOH), 5-hydroxy (5-OH), 5-methyl (5-CH₃), 5-methoxy (5-OCH₃), 5-nitro (5-NO₂), 4-nitro (4-NO₂), 5-amino (5-NH₃), and 4-cyano (4-CN) derivatives. First, biotransformation of the indole derivatives by *E. coli* cells expressing CYP102G4 was examined to verify whether indole derivatives could be used as a substrate for C3-specific hydroxylation with CYP102G4. These substrates were fed to the cells and the production of the respective indigo derivative dyes was monitored. In results, indigo derivative compounds of various colors were synthesized via CYP102G4 dependent biotransformation of 9 different indole derivatives (Fig. 2A). In order to quantitatively compare the colorimetric results, color difference analysis software was used to analyze the measured color difference of three indicator indicators (L), red to green indicator (a), and yellow to blue indicator (b). Finally, the ΔE_{ab} index was quantitatively determined and compared in Table 2.

Next, UV/vis scanning (400–700 nm) was performed on the synthesized bio-dyes to identify characteristic spectral features (Fig. 2B). Most of the dyes exhibited very high absorbance near 450 nm. For the 5,5'-dinitroindigo, 7,7'-dichloroindigo, 6,6'-dichloroindigo, and 5,5'-dichloroindigo derivatives, double absorbance peaks were observed at 550 and 585 nm. The 5,5'-dimethylindigo and 5,5'-dimethoxyindigo

(A)



(B)

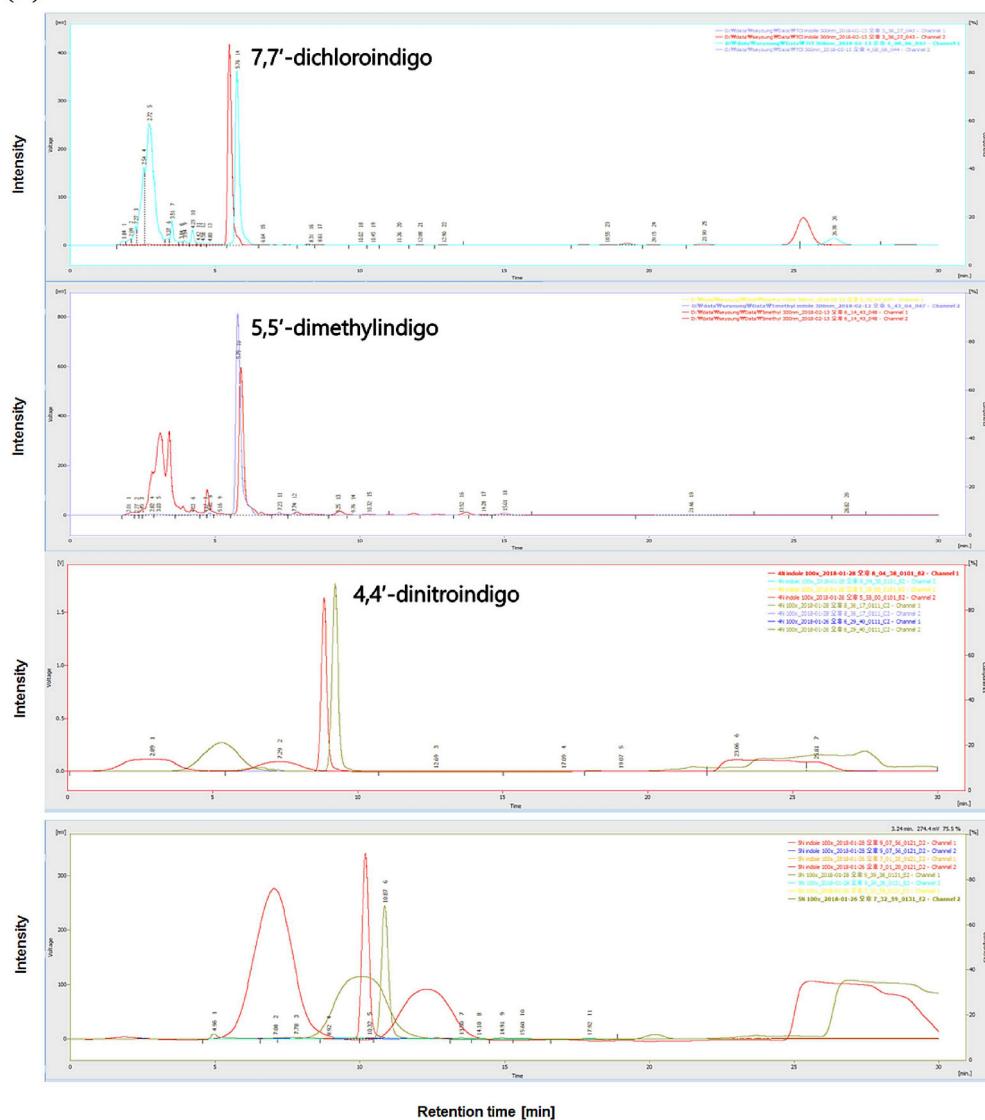


Fig. 3. Separation analysis of synthesized indigoids by TLC. (A) TLC separation conducted with optimized solvent composition. (B) HPLC analysis clearly separated the indole substrates and indigoid products as single products. The HPLC peaks were used for quantification of the synthesized indigoids. For further detailed structural analysis, ¹H NMR was performed and the results are shown in the Supplementary Information.

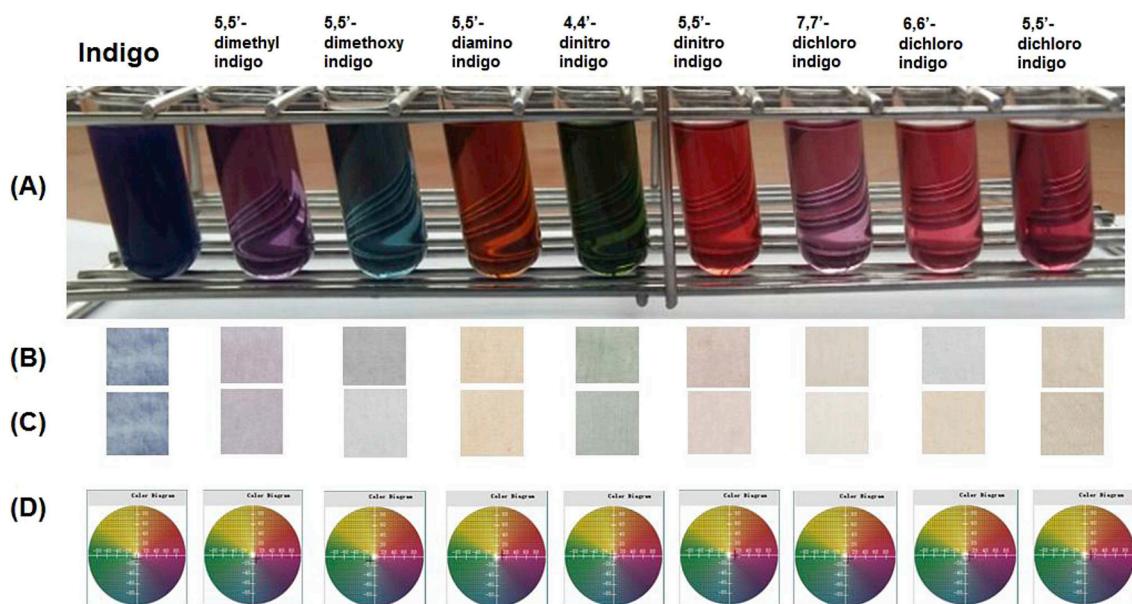


Fig. 4. The applicability of indigo derivatives synthesized as dyeing materials was investigated. (A) The 8 synthesized indole derivatives were examined for cotton dyeing, and each solvent extract after synthesis exhibited a unique color. Before washing (B) and after washing (C). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dyes exhibited a single peak at 630–640 nm. For the 5,5'-diaminoindigo and 4,4'-dinitroindigo derivatives, besides the broad spectrum at ≤ 500 nm, no specific absorption was observed at 500–700 nm.

3.2. TLC and HPLC separation and structural identification of the indigo derivatives by ^1H NMR

TLC separation of synthesized indigoids were performed. Indole derivatives except NO_2 -indole were not visually colored, so they were not visualized via TLC separation. However, it was confirmed that the synthesized indigoids were clearly separated on the TLC plate and noticeable by visual observation with their unique colors (Fig. 3A). From the TLC analysis, it was confirmed that 5,5'-dichloroindigo, 6,6'-dichloroindigo, 7,7'-dichloroindigo, and 5,5'-dimethylindigo were clearly separated as single reaction products. In the separation of the other biotransformation products of indole derivatives, however, a trace or relatively abundant amount of reaction by-product spots was detected on the TLC plate. Especially biotransformation of 4- NO_2 indole and 5- NO_2 indole resulted in 1 or 2 unidentified byproducts which were not separated or not detected in HPLC analysis. Similar byproducts, which were more hydrophobic than product and of bright yellow color, were also observed in 5- CH_3 and 5- OCH_3 indole biotransformation. Although the by-products separated by TLC analysis were not clearly characterized, they were presumed to be produced by other metabolisms of the indole derivative in the producing *E. coli* cell, and seemed insufficient to identify their chemical structures with these analytical data. After TLC separation, accurate structural analysis of the reaction product of the indole derivatives was performed by ^1H NMR, and it was confirmed that the expected products were obtained (Fig. 3B and Fig. S1).

3.3. Application of the indigo derivatives as dyeing materials with various functional groups

The applicability of the indigo derivatives as dyeing materials was investigated to evaluate the feasibility of developing a functional dyeing material with antibacterial effects. A total of 8 synthesized indole derivatives were examined for cotton dyeing. It has been previously reported that indigo dyeing of jeans often results in poor staining because of the poor interaction with cotton due to low

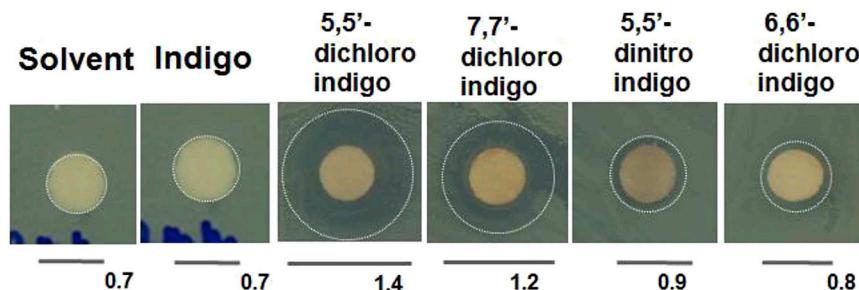
solubility. To overcome this challenge, treatment with a reducing agent such as SDT and repetitive oxidation during the dyeing process has been utilized. However, it was also reported that the increased solubility of indigo through chemical reduction causes environmental and human toxicity problems resulting from the residual reducing agents [2,3]. A recent study attempted to solve similar problems by synthesizing indican, which is water soluble, by adding glucose via glucose transferase enzymatic reactions. However, this process consumes one molecule of non-recyclable glucose per equivalent indigo molecule for appropriate dyeing, which is a non-economical way [3].

Regarding the solubility and dyeing relationship, it was expected that the introduction of hydrophilic moieties to the indigo structure would increase solubility and dyeing performance. However, as shown in Fig. 2A, 2,2'-dicarboxyindigo and 5,5'-dihydroxyindigo showed a very weak yellow color change which was not useful as a dyeing material. Regardless of hydrophilicity, interesting dyeing results were obtained for the other indole derivatives (Fig. 4). For the 5,5'-dimethylindigo, 4,4'-dinitroindigo, and 5,5'-dinitroindigo derivatives, dyeing of cotton was clearly achieved with the original color extracted from the liquid phase. However, the 5,5'-dimethoxyindigo and 5,5'-diaminoindigo derivatives showed color changes to black and yellow, respectively. In addition, not all textures treated with indigo molecules substituted with Cl were stained or their color remained unchanged. Thus, the dyeing performance order was as follows: 5,5'-dimethylindigo < 5,5'-dinitroindigo < 4,4'-dinitroindigo.

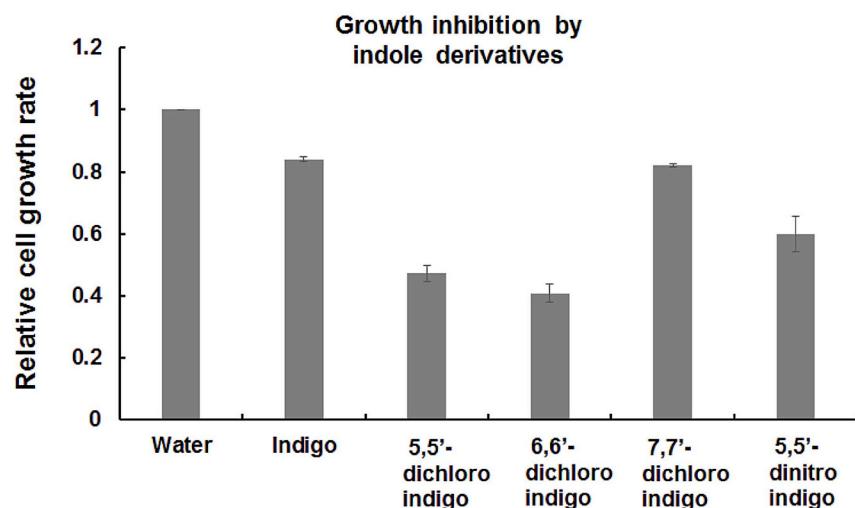
3.4. Characterization of the antibiotic activity of the indigo derivatives

The biological activities of the synthesized indigoids were investigated. Among them, the 5,5'-dichloroindigo, 6,6'-dichloroindigo, 7,7'-dichloroindigo, and 4,4'-dinitroindigo dyes showed interesting antibacterial activity. First, a disk diffusion test was performed on a solid plate against an *E. coli* strain. As shown in Fig. 5A, the four indigo derivatives have shown antibacterial activity against *E. coli* strains, whereas indigo and other indole derivatives did not show any detectable hollow at the plate. The order of the antibacterial effect on the solid plate was 5,5'-dichloroindigo > 7,7'-dichloroindigo > 5,5'-dinitroindigo > 6,6'-dichloroindigo, and the deviations of detected hollow diameters were all within 2 mm from three same experiments. As the

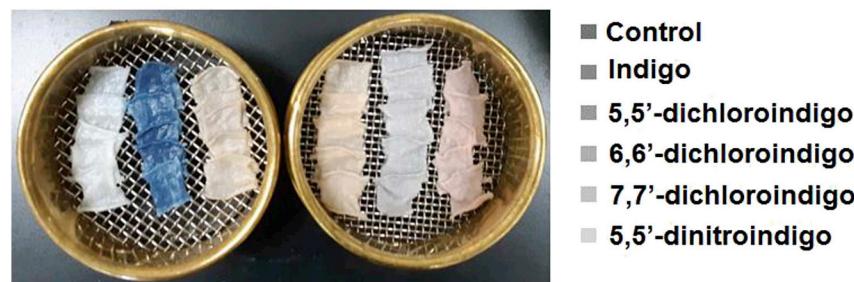
(A)



(B)



(C)



solid-state hollow tests include other variables in addition to anti-bacterial capacity itself, such as diffusivity and solubility inside the solid media, cell growth inhibition was measured in liquid culture as well. The same concentration of indigoid dye was added to the liquid LB medium and cell growth inhibition against *E. coli* was compared with a control (LB medium and same amount of solvent with indigoids). Contrary to the results obtained from disk diffusion test, the growth inhibitory activity of 6,6'-dichloroindigo in liquid phase was greatly increased to a level similar to the highest 5,5'-dichloroindigo. The observed activity followed the order of 6,6'-dichloroindigo ~ 5,5'-dichloroindigo > 5,5'-dinitroindigo > 7,7'-dichloroindigo (Fig. 5B).

3.5. Biosynthesis of 7,7'-dichloroindigo using *E. coli* cells expressing CYP102G4 and PrnA

The feeding of indigo derivatives to *E. coli* cells expressing CYP102G4 is a semi-synthetic method in terms of one-pot substrate conversion. In addition, extra substrate of indole derivatives must be obtained through chemical synthesis or commercial means. To overcome this limitation, the strategy of one-pot synthesis of indigo derivatives by feeding L-tryptophan instead of indole derivatives was developed. First, the target compound was selected to be 7,7'-dichloroindigo among the various indigo derivative compounds.

To replace the indole derivatives with L-tryptophan, as shown in Fig. 1B, two consecutive enzymatic reactions are required to convert L-tryptophan to 7-chloroindole. The first is a tryptophan halogenase

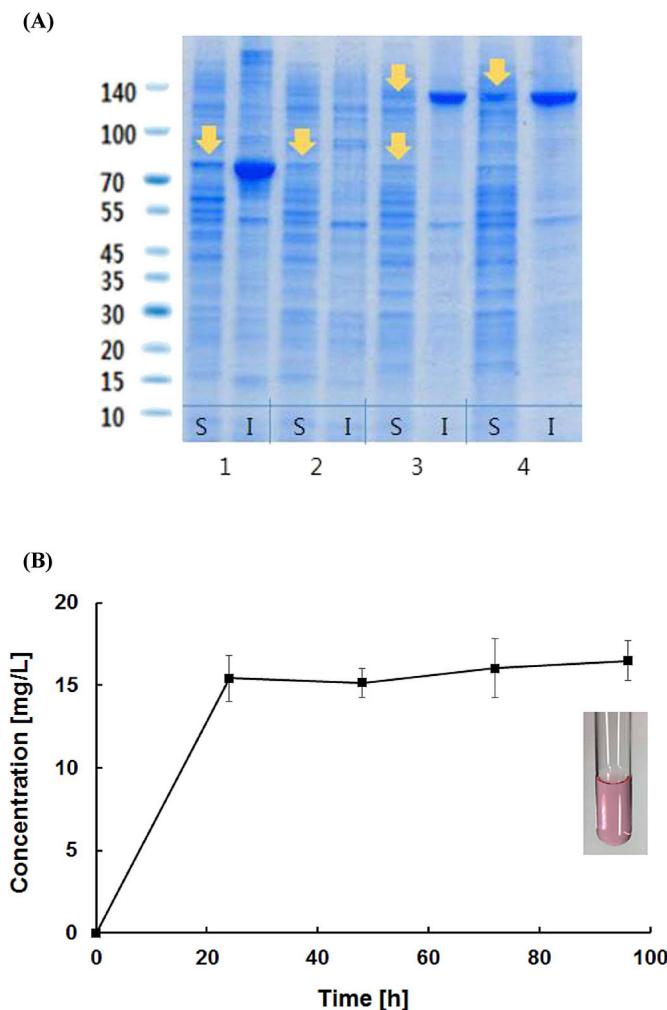


Fig. 6. Biosynthesis of 7,7'-dichloroindigo using *E. coli* cells expressing CYP102G4 and PrnA. (A) SDS-PAGE analysis of the soluble expression, lane 1; pET-28a::*prnA*, lane 2; pETduet::*prnA*, lane 3; pETduet::cyp102G4::*prnA*, lane 4; pET-28a::cyp102G4. (B) Time-dependent 7-Cl production profile. Error bars represent standard deviation ($n = 3$). When 0.6 mM of L-tryptophan was used as an initial substrate concentration, approximately 15.4 ± 1.4 mg/L of 7-Cl was produced within 24 h (■; 7-Cl).

enzymatic reaction that transfers chlorine locally to the C7-position of L-tryptophan, followed by a tryptophanase enzymatic reaction that converts 7-chlorotryptophan to 7-chloroindole [36]. Since tryptophanase is already present in the *E. coli* genome (*tnaA*) [37], a production strain that co-expresses 7-specific L-tryptophan halogenase enzymes, PrnA and CYP102G4, was constructed. Two genes encoding PrnA and CYP102G4 were separately cloned in pET-28a vectors and expressed in *E. coli* BL21(DE3). The co-expression system in pETduet exhibited good rate of target protein accumulation in the soluble fraction, although the expression level was relatively lower than that observed in a single-gene system (Fig. 6A). Finally, 7,7'-dichloroindigo production from L-tryptophan was investigated in 24 h intervals using the engineered *E. coli* BL21(DE3) pETduet::*prnA*::cyp102G4 strain. The developed strain could produce 15.4 ± 1.4 mg/L of 7,7'-dichloroindigo in 24 h (Fig. 6B). In terms of yield, 15.4 ± 1.4 mg/L of 7,7'-dichloroindigo (46.5 μ M) from 0.5 mM of L-tryptophan corresponds a yield of approximately 17.8% when the amount of residual L-tryptophan in the TB medium was ignored. When only CYP102G4 was expressed in *E. coli* BL21(DE3), approximately 0.8 ± 0.1 g/L of indigo was produced. The low yield was likely caused by several factors including the low activity of tryptophanase and CYP102G4 enzymes towards 7-chlorotryptophan

or 7-chloroindole and the low activity of the flavin-dependent PrnA halogenase [38,39]. First, tryptophan halogenase (PrnA) catalyze chlorination of L-trp to produce chloro-tryptophan. This enzyme was reported to have 17.1 ± 4.4 μ M of K_m and 43 ± 4 pmol/min of V_{max} [38]. The next reaction is decarboxylation of chloro-tryptophan by tryptophanase enzyme (TnaA). The kinetics of TnaA were reported as 0.3–0.6 mM of K_m and 14 μ mol/min of initial velocity [40]. The last reaction is oxidative modification of 7-chloroindole by CYP102G4 monooxygenase enzyme. For several years, our group has studied this enzyme for various applications. The kinetics regarding indole hydroxylation of CYP102G4 were reported to have 3.43 ± 0.19 mM of K_m and 21.05 ± 1.14 min⁻¹ of k_{cat} with 35.3 \pm 1.6 of coupling efficiency [13].

Besides, enzyme activity several bypassing pathways could limit the 7-Cl production. The L-trp has another possible pathway into indole by tryptophanase. In such case, the related final product could be indigo which could limit 7-Cl production. Furthermore, PrnA was reported to have indole-C2 and -C3 chlorination activity along with L-trp C7 chlorination activity, which resulted in 2-chloroindole and 2,3-dichloroindole byproduct formation [35,38]. However, HPLC analysis of generated products revealed that no bypassing indole based products were not observed suggesting that PrnA enzyme takes more L-trp substrate than TnaA tryptophanase enzyme.

In summary, it seems that limiting factor is obviously CYP102G4 dependent hydroxylation reaction. In order to increase the production of 7-Cl in the engineered *E. coli* strain, several basic researches were attempted. First, our research group is doing directed evolution of CYP102G4 enzyme in order to increase catalytic activity against 7-Cl indole hydroxylation activity with higher affinity at the same time. However, CYP enzymes have considerably lower turnover rate intrinsically, so a study to increase conversion would require considerable research and effort. Research is currently underway to apply halogenase enzymes such as PyrH, Stth and RebH, which are respectively known as C5, C6, and C7-specific L-tryptophan halogenases to this system, and to increase the yield through enzyme engineering [41–43].

4. Conclusion

In this study, the biosynthesis of indigo derivative compounds through the C3-hydroxylation reaction and continuous oxidation via CYP102G4 was achieved. Various functional moieties were considered in the selection of indole derivatives for use as antibacterial dyes. It was confirmed that various colors could be exhibited depending on the substituted functional groups, and the position of the functional groups also affected the color. However, no general trend was observed for functional groups and site specificity on the indigoid colors. The antimicrobial effect of the indigo pigments in the solid and liquid states was determined to evaluate its application as a functional dyeing material. The indole derivative substituted with halogens exhibited relatively higher antimicrobial effects, especially the 5,5'-dichloroindigo showed good activity at both solid and liquid phase test. In addition, 5,5'-dinitroindigo showed similar antimicrobial effects to the halogen-substituted indigo molecules.

The greatest limitation of this study is the indirect synthesis of the indole precursor feedstock, as it is considered to be less economical for mass production. Thus, a one-pot biosynthetic process was developed with a system that coexpresses the halogenase (i.e. PrnA) and CYP102G4 enzymes. We succeeded in developing a strain capable of producing 7,7'-chloroindigo from L-tryptophan to 15.4 ± 1.4 mg/L in a one-pot system through simultaneous expression of the two enzymes in *E. coli*. We are currently investigating a one-pot biosynthesis of 5,5'-dichloro- and 6,6'-dichloroindigo by simultaneous expression of a site-specific halogenase enzyme other than PrnA. Besides, the synthesis of indigo substituted with various halogen atoms of fluorine and bromine by using haloperoxidase enzyme are under investigation. Finally, the physiological activity of various indigo derivative compounds and

various applications using them will be further investigated.

Competing interests

The authors declare no conflict of interest.

Consent for publication

Not applicable.

Ethical approval and consent to participate

This article does not contain any studies with human participants performed by any of the authors.

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Appendix A. Supplementary data

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