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Biosynthesis of indigo in *Escherichia coli* expressing self-sufficient CYP102A from *Streptomyces cattleya*

Hee-Jung Kim [a], Seyoung Jang [a], Joonwon Kim [b], Yung-Hun Yang [c], Yun-Gon Kim [d], Byung-Gee Kim [b], Kwon-Young Choi [a, *]

[a] *Department of Environmental Engineering, College of Engineering, Ajou University, Suwon, Gyeonggi-do, South Korea*
[b] *Department of Chemical and Biological Engineering, Seoul National University, Seoul, South Korea*
[c] *Department of Microbial Engineering, College of Engineering, Konkuk University, Seoul, South Korea*
[d] *Department of Chemical Engineering, College of Engineering, Soongsil University, Seoul, South Korea*

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ABSTRACT

Cytochrome P450 monooxygenases (CYP) are a superfamily of heme-thiolate proteins which catalyze the incorporation of oxygen atoms into substrates. Here, a self-sufficient CYP102A from *Streptomyces cattleya* (CYP102A_scat) was cloned, produced recombinantly in *Escherichia coli* strain BL21 (DE3), and the characteristic features were investigated. However, unlike other self-sufficient CYP102A enzymes that have been reported, CYP102A_scat was found to be able to catalyze intracellular hydroxylation of indole molecules with 3-C specific regioselectivity. Consequently, *E. coli* strains producing CYP102A_scat could synthesize approximately 1.0 g/L of indigo in LB media. Optimization of indigo synthesis was investigated through additional feeding of indole precursors such as glucose, L-tryptophan, and indole. Indigo production reached up to 3.8 ± 0.1 g/L by adding 20 μM of extracellular indole and 0.2 mM of L-tryptophan to the LB media. To our knowledge, this is a record and the highest yield achieved so far.

1. Introduction

Indigo-blue, which has a deep dark-blue color, is one of the oldest and most popular dyes [1]. It is as a natural dye, originally extracted from plants such as *Indigofera tinctoria* [2]. Novel chemical and biological routes for the synthesis of indigo were sought as the demand for indigo started to increase. For example, Baeyer-Drewson synthesis is the most well-known organic reaction used for indigo production [3]. In that synthetic route, 2-nitrobenzaldehyde and acetone undergo an aldol condensation reaction, followed by cyclization and oxidative dimerization to produce indigo. This synthetic method was once one of the most useful routes to produce indigo. However, the Baeyer-Drewson synthesis was a relatively impractical process for the synthesis of indigo at the industrial-scale, and was eventually replaced by the preparation of indigo from aniline. Aniline is readily obtained and can be used to synthesize *N*-(2-carboxyphenyl)glycine, an indigo precursor [4]. The production of indigo from *N*-(2-carboxyphenyl) glycine provided a new and economically attractive route. Approximately 20 million kilograms of indigo are produced annually *via* this synthetic route, and that indigo is used mainly for dyeing blue jeans, and as a colorant in the food industry. Although indigo has a low oral toxicity, with an LD_{50} of 5.0 g/kg in mammals, large spills of blue cloth dyes, which include indigo, are nonetheless hazardous because they contain harmful chemical byproducts [5,6].

With the increasing need to develop eco-friendly processes and the growing interest in natural pigments, various attempts have been made to synthesize natural dyes and pigments biologically. One of the most promising of such processes is the use of microbial enzymes within engineered metabolic pathways for the synthesis of indigo, using any of several renewable biomass sources as a feedstock [7–10]. Compared to production routes based on synthetic chemistry, this biological route is relatively simple and fast. Given appropriate biologically-sourced starting-chemicals, biological conversions such as biotransformation and enzymatic modifications of structure can result in the effective synthesis of indigo dye with excellent production yields [11,12]. For example, several attempts for indigo production using flavin-containing monooxygenases (FMO) have been reported [13,14]. Recent research on

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* Corresponding author.
E-mail address: kychoi@ajou.ac.kr (K.-Y. Choi).

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FMO-dependent indigo production has shown that *Escherichia coli* (*E. coli*) that recombinantly produce FMO from *Corynebacterium glutamicum* can produce 685 mg/L of indigo and 103 mg/L of indirubin from 2.5 g/L of L-tryptophan [15].

Here, we demonstrate biosynthesis of indigo dye from glucose using a recombinant *E. coli* strain producing a cytochrome P450 monooxygenase (CYP). CYP enzymes are heme-thiolate proteins, which catalyze the introduction of one oxygen atom from molecular oxygen to a target substrate molecule in a regioselective and stereoselective manner [16–18]. A few examples of CYP-dependent production of indigo dye have been reported. Some CYP102A1 BM3 mutants were generated through rational design and site-saturation mutagenesis for indole hydroxylation activity [9]. A colorimetric colony method, based on the blue pigment generated from indigo, was utilized as a high-throughput screening assay to find active variants with enzymatic activity towards aromatic compounds [19]. Until now, indigo production using native CYP enzymes in recombinant microbial hosts has never been reported. A few attempts have been made using mutant CYPs [20,21]. In this report we demonstrate, for the first time, indigo production using a recombinant *E. coli* strain producing the wild-type CYP102A_scat enzyme isolated from *Streptomyces cattleya* (*S. cattleya*). Furthermore, increased indigo production was achieved by optimal feeding of indigo precursors such as glucose, indole, and L-tryptophan.

2. Materials and methods

2.1. Chemical reagents

All chemical reagents used in this study were of analytical grade or higher. Indigo, indole, glucose, and L-tryptophan were purchased from Sigma-Aldrich Korea (Suwon, South Korea).

2.2. Phylogenetic analyses

Amino acid sequences were aligned using the *ClustalW2* program *via* the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Alignments were visualized with *Bio-edit* software (Fig. 1).

2.3. Heterologous expression of the gene encoding CYP102A_scat in E. coli

The DNA sequence information for CYP102A_scat was obtained from the open-access website: (http://avermitilis.ls.kitasato-u.ac.jp/). The encoding gene was amplified from its genome and the PCR product was cloned into a pET-28a(+) expression vector (Novagen, Madison, WI). The pET-28a(+)-CYP102A_scat plasmid was subsequently transformed into *E. coli* BL21(DE3), after which, the transformants were grown in LB medium containing 50 μg/ml of kanamycin at 37 °C, until an OD_{600} of 0.8 was attained. At that point, isopropyl- β-D-thiogalactopyranoside (IPTG) was added to obtain a final concentration of 12.5 μM, along with 0.25 mM δ-aminolevulinic acid as a heme precursor, after which the cells were incubated at 30 °C for 12 h. After induction, 1 mL samples of every point were collected from the cell culture and prepared for indigo production analysis. At the same time the cells were subsequently harvested by centrifugation, washed twice with ice-cold PBS buffer, resuspended in 50 mM potassium phosphate buffer (pH 7.0) and used for subsequent CYP102A_scat protein preparation and in-vitro assay.

2.4. Spectral features of CYP102A_scat

Absorption spectra of CO-bound CYP102A_scat enzyme samples were measured following reduction by sodium dithionite, *via* UV/vis spectrophotometry (scanning wavelengths from 400 to 500 nm) [22]. Briefly, binding spectra were recorded following reduction of CYP102A_scat protein (oxidized form) by sodium dithionite (reduced form), followed in turn by the bubbling of carbon monoxide gas into the enzyme solution (CO-bound form). The protein concentration was subsequently estimated using reduced CO *versus* reduced difference spectra. Using an extinction coefficient of 91.9 mM^{-1} cm^{-1}, at a wavelength of 450 nm, the recombinantly produced protein content was determined [22,23].

2.5. Indigo production using E. coli expressing CYP102A_scat

Cells producing CYP102A_scat were cultured in LB media and the whole cell reactions were initiated by adding IPTG solution (final concentration: 12.5 μM) to the media, along with the heme precursor δ-aminolevulinic acid (final concentration: 0.25 mM). Whole cell production proceeded at 30 °C for 48 h in a high speed incubator (200 rpm), after which the reaction was quenched by the addition of an equal volume of DMSO, followed by vigorous vortexing. The mixtures were then centrifuged at 13000 rpm for 10 min, after which the blue-colored DMSO layer containing indigo was separated. The prepared samples were structurally and quantitatively analyzed using GC/MS, ^1H NMR spectroscopy, and other spectroscopic techniques.

2.6. Structural and quantitative analysis of biosynthetically produced indigo

The fractions collected from the indigo producing cell culture were separated by centrifugation and further taken for TLC, HPLC, and ^1H NMR. For TLC analysis, the mobile phase was composed of chloroform: hexane: methanol (5:4:1). The indigo standard solution was prepared by dissolving 262 mg of synthetic indigo in 1 mL of DMSO and diluted up to several μM ranges using methanol. Indigo products were also separated with 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 ACN/Water (50:50 v/v). The absorbance of the eluent was monitored at 540 nm.

For quantitative analysis of the reaction products, the absorption intensity of the extracted solution was monitored at 610 nm by UV/vis spectrometry. The production yield of indigo was further determined using a standard calibration curve obtained using the same quantification methods with commercially available synthetic indigo. In addition, the collected indigo product was analyzed by ^1H NMR spectroscopy, and the resulting chemical shifts were compared to that of synthetic indigo.

3. Results and discussion

3.1. Self-sufficient CYP102A_scat and their sequence analysis

Cytochrome P450 (CYP) enzymes are generally classified into several family, depending on their protein sequence identities. One of the most studied CYP family is CYP102A family, such as CYP102A1 BM3, CYP102A2, CYP102A3, CYP102A5, and CYP102A7, which are reported as long-chain fatty acid hydroxylase [24–28]. Likewise, the novel CYP that we are demonstrating in this manuscript, CYP102A_scat, also belongs to the CYP102A family and has very high sequence identities with CYP102A enzymes. As was expected, CYP102A_scat has several unique motifs, such as a heme binding domain, a dioxygen binding domain, and a substrate binding domain (Fig. 1). Among the CYP102A family, CYP102A_scat has highest sequence identity with CYP102A1 BM3 (41.3%), and

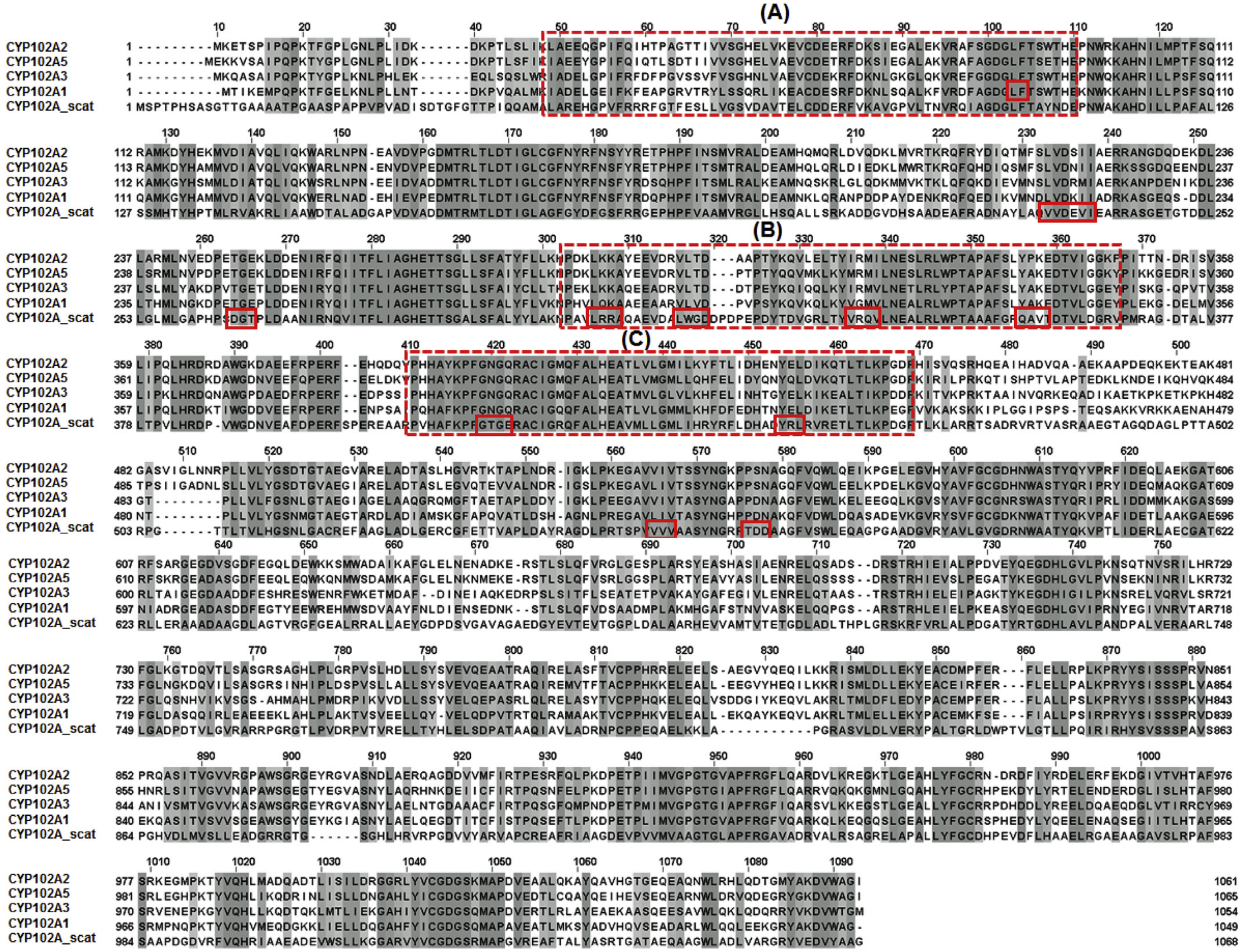

Fig. 1. Multiple sequence alignment of heme domain and reductase domain of the self-sufficient CYP, CYP102A_scat, with other CYP102A family members. (A) Key amino acid residues in the CYP102A_scat substrate binding pocket and that affect the opening of the substrate access channel; (B) the mechanistically important I-helix region; and (C) the heme-binding region. Distinctive differences in the active sites are indicated with red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lowest identity with CYP102A5 (39.1%). Since CYP102A enzymes exhibit very narrow substrate specificities for long-chain fatty acids, CYP102A_scat was also expected to have fatty acid hydroxylation activity. Consequently, an attempt was made to characterize the enzymatic activity of CYP102A_scat following its heterologous production in *E. coli* BL21(DE3).

3.2. *Preparation of recombinant E. coli BL21(DE3) producing a self-sufficient CYP102A_scat*

Recombinant *E. coli* cells capable of expressing the gene encoding CYP102A_scat were prepared as demonstrated in materials and method section. There was a good yield of protein. Recombinant CYP102A_scat has an apparent mass of 101.2 kDa, according to an SDS-PAGE analysis (Fig. 2A). The presence of an activated CYP fraction was confirmed by a CO (99.99%) binding assay; CYP102A_scat was reduced by sodium dithionite, and after that, the sample had CO gas bubbled through it. Scanning the absorption wavelength from 400 to 550 nm in a UV/vis spectrophotometer revealed that the highest absorbance peak occurred at 450 nm, which is a characteristic feature of CYP enzymes (Fig. 2C, dotted line). In the initial stages of CYP102A_scat enzyme characterization, the media changed color, becoming blue when CYP102A_scat was produced (Fig. 2B). At this stage, we did not realize that the color arose from indigo dye. After several repeated rounds of careful CYP102A_scat production, ensuring definite autoclave sterilization, it was finally proved that the blue pigment arose from the heterologous CYP102A_scat production, not from any contamination source. The unknown pigment was finally identified upon extraction of the blue pigments from the growth media, followed by structural analyses using ^{1}H NMR spectroscopy.

3.3. *Structural identification of the unknown blue pigment*

The unknown blue pigment was extracted from 1 mL of growth medium using an equal volume of DMSO. After 10 min of centrifugation, the deep blue supernatant was separated and prepared for analysis by TLC, HPLC, and ^{1}H NMR spectroscopy. A verified sample of synthetic indigo was purchased from Sigma-Aldrich Korea and analyzed alongside the unknown pigment, to aid in the precise analysis of its structure. The product separated by TLC reveled a single indigo product while indigo standard has two spots

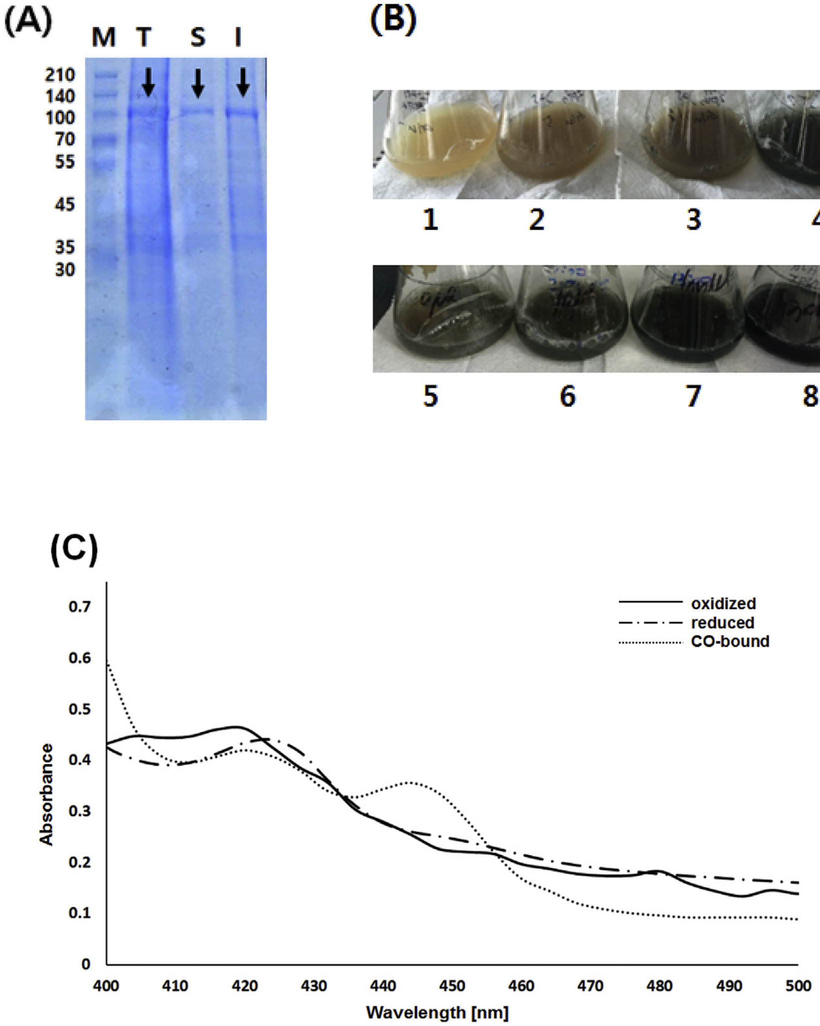

Fig. 2. Production of functional CYP102A_scat. (A) SDS-PAGE results; M: marker, T: total proteins, S: proteins in the soluble fraction, I: proteins in the insoluble fraction. (B) Formation of indigo dye, following production of CYP102A_scat, adding various concentrations of certain intermediates involved in tryptophan synthesis. (C) Oxidized, reduced, and CO-bound spectral features of CYP102A_scat.

of indigo and indirubin in Fig. 4A. The single spot exactly matched with the blue indigo spot separated from indigo standard. Also, HPLC separation of produced blue pigment was attempted and resulted in a single peak with same retention time of corresponding standard indigo as well (Fig. 4A). ^{1}H NMR spectroscopic analysis was attempted to further the detailed identification. The assignment of ^{1}H NMR spectrum for blue pigment is shown in Fig. 4B and the ^{1}H NMR spectrum gave peaks at δ 6.551 ppm, δ 6.990 ppm, and δ 7.496 ppm corresponding to aromatic ring protons, while at δ 10.465 ppm corresponding to aromatic C-NH. The chemical shifts observed were well-matched with those of the reference, synthetic indigo. In GC/MS analysis, a 262 m/z reading for the isolated blue compound demonstrated that its molecular weight matched that of the verified, synthetic indigo (data not shown). Using these structural techniques, the blue pigment generated by heterologous production of CYP102A_scat was finally identified as indigo dye.

3.4. Optimization of indigo production by adding synthetic precursors of indigo

In order to quantify the production of indigo by the cells producing CYP102A_scat, the time-dependence of indigo production was monitored in LB medium. The highest indigo production (~1.0 g/L) was recorded around 21 h following the induction by IPTG (Fig. 5). The glucose concentration-dependence of indigo production was investigated, in order to examine the effects of providing an additional carbon source. Media containing three different concentrations of glucose were examined for indigo production. Unanticipated production profiles resulted. The higher the concentration of glucose added, the lower was the production of indigo (Fig. 5). The increased flux from glucose did not improve indigo production at all, rather it led to a decreased production.

E. coli cells synthesize the amino acid tryptophan through the shikimate pathway. Glucose is converted into shikimate, then indole, and finally into tryptophan, by the action of several enzymes (Fig. 3). The most important intermediate in the synthesis of tryptophan is indole, which is an intercellular signaling molecule. Indole has been reported to regulate certain physiological processes of cells, such as sporulation, biofilm formation, plasmid stability, and virulence. In order to investigate the dependency of indigo production on the extracellular feeding of indole, different concentrations of indole were added to the growth media and the resulting indigo production was monitored over time. Interestingly, the higher the indole concentration in the media, the higher the indigo production that was achieved. With the provision of 20 μM of additional indole, the indigo production reached 2.0 ± 0.1 g/L,

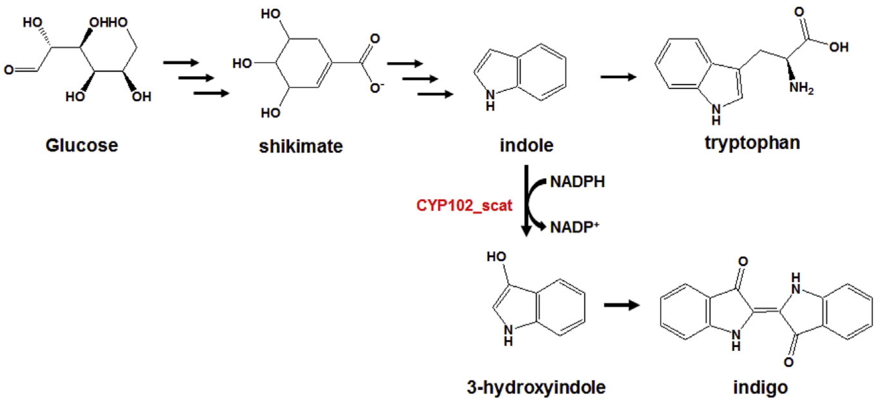

Fig. 3. Synthetic pathways of indigo production in *E. coli* cells. Cells utilize the shikimate pathway for indole and tryptophan synthesis. Intracellular indole becomes intercepted by heterologously produced CYP102A_scat, which catalyzes C-5 specific hydroxylation of indole, resulting in indigo production.

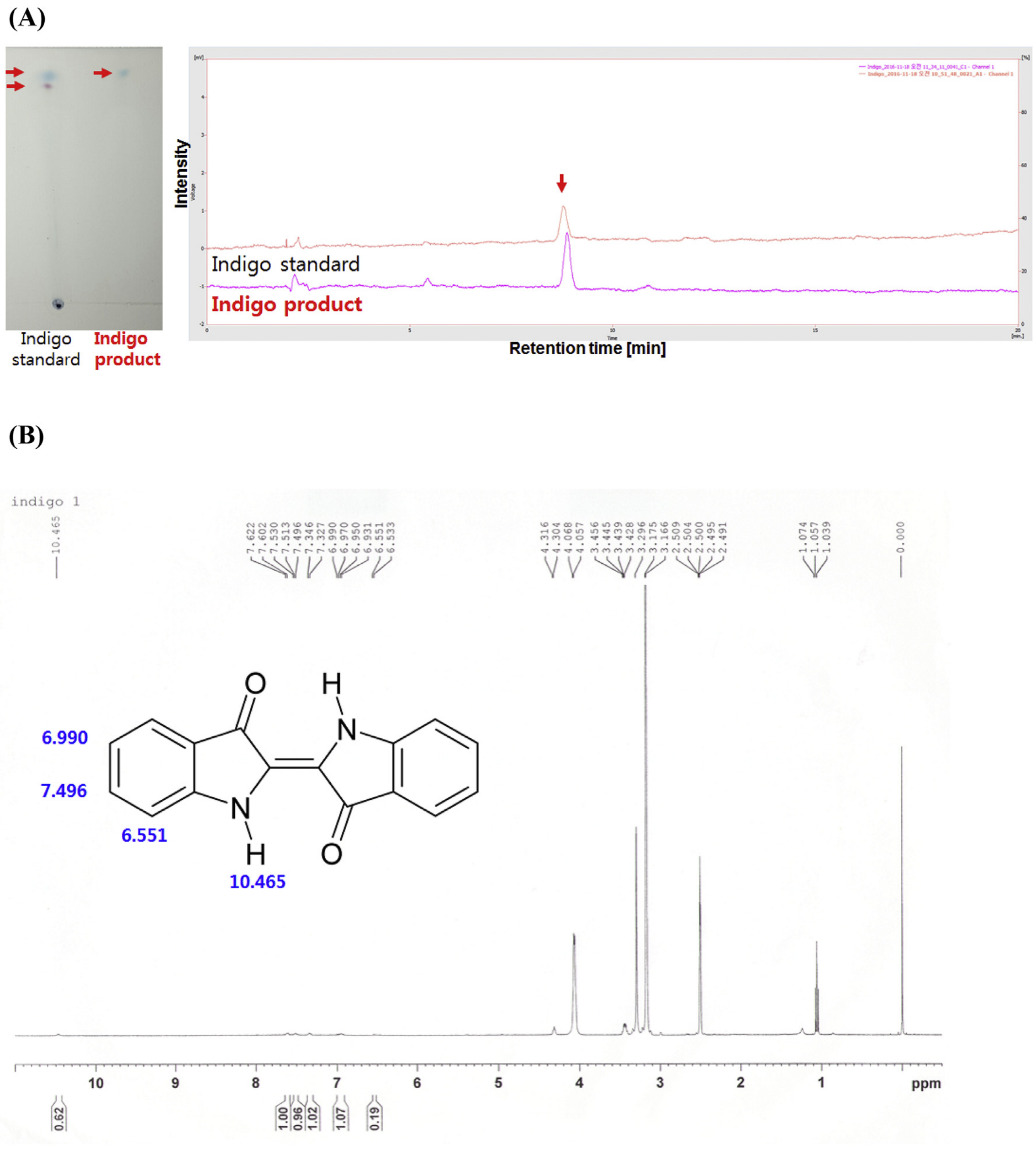

Fig. 4. Separation and structural analysis of produced indigo. (A) Separation of generated indigo by TLC and HPLC. (B) Structural analysis of generated indigo by ^{1}H NMR analysis.

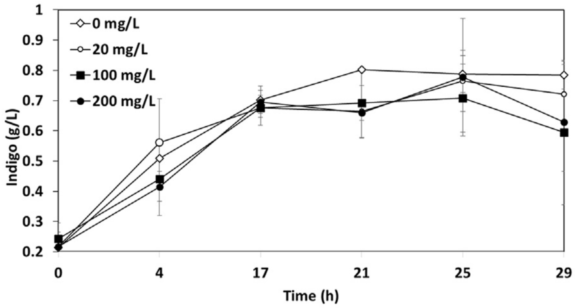

Fig. 5. Indigo production over 29 h in LB medium (◊line, without additional glucose) and the effect on indigo production of adding additional glucose to the LB medium (○; 20 mg/L, ■; 100 mg/L, ●; 200 mg/L).

which amounts to a doubling of the production yield (Fig. 6A). Along with indole supplementation, the effect of additional L-tryptophan in the LB media on the production of indigo was also investigated and resulted in increased indigo production up to 1.6 ± 0.3 g/L with the addition of 0.2 mM of L-tryptophan (Fig. 6B).

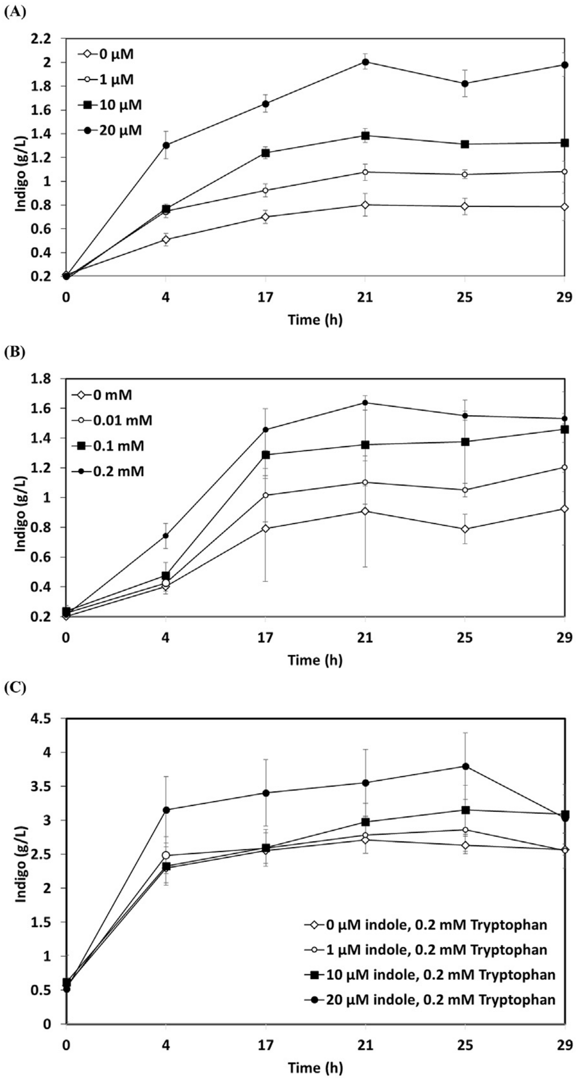

Fig. 6. (A) Indigo production with various concentrations of supplemental indole in the LB medium. (◊; 0 μM, ○; 1 μM, ■; 10 μM, ●; 20 μM), (B) Indigo production with various concentration of supplemental L-tryptophan in the LB medium (◊; 0 mM, ○; 0.01 mM, ■; 0.1 mM, ●; 0.2 mM), (C) Indigo production with various concentration of supplemental indole and 0.2 mM of L-tryptophan in the LB medium (◊; 0 mM, ○; 0.01 mM, ■; 0.1 mM, ●; 0.2 mM of indole).

Finally, the combinatorial effects from both of indole and L-tryptophan supplementation was investigated by varying concentration of indole while the L-tryptophan concentration being constant for 30 h. The optimum Indigo production reached up to 3.8 ± 0.1 g/L by adding 20 μM of extracellular indole and 0.2 mM of L-tryptophan to the LB media at 25 h after IPTG induction (Fig. 6C). And the production rate of indigo was measured as 0.15 g/L/hr with the same optimized condition. To our knowledge, this is a record and the highest yield achieved so far.

4. Conclusions

Here we have isolated a unique self-sufficient monooxygenase from *S. cattleya*, termed 'CYP102A_scat'. The gene encoding CYP102A_scat was cloned, and then expressed in *E. coli* BL21(DE3), in order to investigate its characteristic features. The amino acid sequence of CYP102A_scat is highly similar with other CYP102A family members. Unlike other CYP102A species, CYP102A_scat uniquely produces a blue-dye following IPTG induction in LB medium. The isolated blue pigment (1.0 g/L) was eventually identified as indigo using TLC, HPLC separation, and ^{1}H NMR spectroscopic analysis. Additional feeding of glucose did not increase the productive yield of indigo. The highest productive yield of indigo was achieved by adding both 20 μM of indole and 0.2 mM of L-tryptophan to the culture medium, which boosted indigo production to 3.8 g/L. This novel, self-sufficient CYP102A enzyme, is the first self-sufficient CYP that exhibits indole hydroxylation activity, resulting in indigo production—without having to mutate the wild-type enzyme. Further investigation of a CYP102A_scat structural model, together with indole docking simulations extends our understanding of the indole hydroxylation activity, and these insights could be applied to other self-sufficient CYP enzymes in the future.

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