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Efficient production of indigoidine in *Escherichia coli*

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Abstract Indigoidine is a bacterial natural product with antioxidant and antimicrobial activities. Its bright blue color resembles the industrial dye indigo, thus representing a new natural blue dye that may find uses in industry. In our previous study, an indigoidine synthetase Sc-IndC and an associated helper protein Sc-IndB were identified from *Streptomyces chromofuscus* ATCC 49982 and successfully expressed in *Escherichia coli* BAP1 to produce the blue pigment at 3.93 g/l. To further improve the production of indigoidine, in this work, the direct biosynthetic precursor L-glutamine was fed into the fermentation broth of the engineered *E. coli* strain harboring Sc-IndC and Sc-IndB. The highest titer of indigoidine reached 8.81 ± 0.21 g/l at 1.46 g/l L-glutamine. Given the relatively high price of L-glutamine, a metabolic engineering technique was used to directly enhance the in situ supply of this precursor. A glutamine synthetase gene (*glnA*) was amplified from *E. coli* and co-expressed with *Sc-indC* and *Sc-indB* in *E. coli* BAP1, leading to the production of indigoidine at 5.75 ± 0.09 g/l. Because a nitrogen source is required for amino acid biosynthesis, we then tested the effect of different nitrogen-containing salts on the supply of L-glutamine and subsequent indigoidine production. Among the four tested salts including $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2HPO_4$ and KNO_3, $(NH_4)_2HPO_4$ showed the best effect on improving the titer of indigoidine. Different concentrations of $(NH_4)_2HPO_4$ were added to the fermentation broths of *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA, and the titer reached the highest (7.08 ± 0.11 g/l) at 2.5 mM $(NH_4)_2HPO_4$. This work provides two efficient methods for the production of this promising blue pigment in *E. coli*.

Keywords Indigoidine · Glutamine synthetase · Metabolic engineering · *Escherichia coli* · *Streptomyces chromofuscus* · Substrate feeding

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Introduction

Nature provides a variety of molecules that have particular functions. Some compounds have significant health-benefiting properties such as antimicrobial, anticancer and anti-cholesterol activities. Many promising natural products have been developed into therapeutics for different diseases, such as vancomycin (antibacterial), paclitaxel (anticancer), artemisinin (anti-malarial) and lovastatin (anti-cholesterol). In addition to these therapeutic compounds, some other naturally occurring compounds have useful properties that may find uses in industry, such as natural pigments. Dyes are applicable in many fields and closely linked to our daily life. They are commonly used in the textile and food industries for desired colors. Dyes can be divided into two categories, natural dyes and synthetic dyes. The majority of natural dyes are from plants, fungi and lichens [12]. Indigo is a dark blue crystalline powder and one of the oldest blue dyes that has been known for more than 4000 years. It can be obtained from a variety of plant sources such as *Indigofera tinctoria* (Africa, Asia, East India, South America), *Polygonum tinctorium* (China, Korea) and *Isatistinctoria* (Europe) [15]. It is commonly used to dye cotton yarn for the production of denim cloth to make blue jeans [5, 18]. The precursor to indigo is indican which is a colorless natural product from plant leaves [20]. After extraction from plants, indican is hydrolyzed to

yield glucose and indoxyl. The latter is then converted to indigo through several steps. Also, the extraction needs a strong base such as lye and other substances that are harmful to the environment [15, 19]. Since the natural source for indigo is limited, chemical synthesis becomes a more economic method to produce this dye. However, chemical synthesis requires harsh conditions and use of a strong base [8]. Indigoidine, 5,5′-diamino-4,4′-dihydroxy-3,3′-diazadiphenoquinone-(2,2′), is a natural blue pigment from bacteria that was first isolated from *Erwinia* [16]. Because of the carbon–carbon double bonds conjugated with a carbonyl group in its structure, indigoidine is a powerful radical scavenger that enables phytopathogens to tolerate oxidative stress [13], thus representing a strong antioxidant agent. Recent studies have also shown that this blue pigment has antimicrobial activity. The redox activity and bright blue color make indigoidine a possible redox state sensor or industrial dye [3].

Indigoidine is synthesized through the condensation of two molecules of L-glutamine under the catalysis of indigoidine synthetase (Fig. 1). Several indigoidine biosynthetic gene clusters have been reported from different bacterial strains such as *Erwinia* [13], *Streptomyces* [10, 17, 21] and *Photorhabdus* [2]. We have recently discovered an interesting indigoidine biosynthetic gene cluster from *Streptomyces chromofuscus* ATCC 49982 consisting of *Sc-indA*, *Sc-indB* and *Sc-indC*. Sc-IndC was identified as the indigoidine synthetase and it was successfully expressed in both *Escherichia coli* BAP1 and *Streptomyces coelicolor* CH999 to reconstitute indigoidine biosynthesis. The titer of indigoidine reached 2.78 g/l under the optimized fermentation conditions [21]. While the exact function of Sc-IndB is unclear, it is found that co-expression of this protein with Sc-IndC significantly increased the titer of indigoidine by 41.4 % in *E. coli* BAP1, suggesting that it acts as a helper in indigoidine biosynthesis [21]. Sc-IndA is a hypothetical protein. Its function is unknown and co-expression of this protein with Sc-IndC did not improve the production of indigoidine in *E. coli* [21]. In this work, we report the optimization of indigoidine production in *E. coli* by improving the availability of the biosynthetic precursor L-glutamine. Two approaches were used, including direct supplementation of L-glutamine in the fermentation broth and enhanced self-supply of L-glutamine by engineering a glutamine synthase (GS) into *E. coli* and adding extra nitrogen-containing salt. While the first approach allows the engineered *E. coli* strain to produce the blue pigment at 8.81 g/l, the second approach provides a more economical method to produce indigoidine and the titer reached 7.08 g/l.

Materials and methods

Vectors, strains and culture conditions

E. coli XL1-Blue (Agilent) and the pJET1.2 vector (Fermentas) were used for DNA cloning and sequencing. The expression vectors pET28a (Novagen) and pACYCDuet-1 (Novagen) were used to construct the expression plasmids. *E. coli* BAP1 was used as the host for protein expression. All *E. coli* strains were grown at 37 °C in liquid Luria–Bertani (LB) medium or LB agar supplemented with appropriate antibiotics at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 25 μg/ml. 200 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was used to induce gene expression.

Construction of plasmids

The plasmids pJV6 (pET28a-*Sc-indC*) and pDY53 (pACYCDuet-1-*Sc-indB*) were from our previous work [21]. The *glnA* gene (1284 bp) was amplified by the polymerase chain reaction (PCR) from the genome of *E. coli* BL21(DE3) with Phusion Hot Start High-Fidelity DNA Polymerase (New England Biolabs) using a pair of primers, 5′-aaCATATGTCCGCTGAACACGTACTGAC-3′ (the NdeI site are underlined) and 5′-aaCTCGAGTTAGACGCTGTAGTACAGCTC-3′ (the XhoI site are underlined). These primers were designed based on the reported *glnA* sequence [1] and synthesized by Sigma-Aldrich. The amplified *glnA* gene was ligated into the cloning vector pJET1.2 to yield pFC5 (Table 1).

After gene sequencing, the *glnA* gene was excised from pFC5 with NdeI and XhoI and ligated into pACYCDuet-1 or pDY53 between the same sites to generate pFC6 or pFC7, respectively (Table 1). *E. coli* BAP1 was co-transformed with pJV6 and each of these pACYCDuet-1-derived

Table 1 Plasmids used in this study

Plasmid	Description	References
pJV6	*Sc-indC* in pET28a	[21]
pDY53	*Sc-indB* in pACYCDuet-1	[21]
pFC5	*glnA* in pJET1.2	This work
pFC6	*glnA* in pACYCDuet-1	This work
pFC7	*glnA* and *Sc-indB* in pACYCDuet-1 under two separate T7 promoters	This work

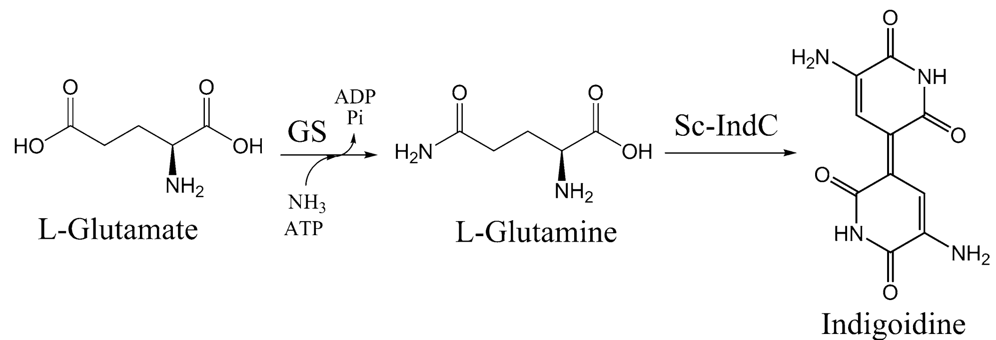

Fig. 1 Biosynthetic pathway of indigoidine. *GS* glutamine synthetase; *Sc-IndC* indigoidine synthetase; *ATP* adenosine triphosphate; *ADP* adenosine diphosphate; *NH_3* ammonia; *Pi* inorganic phosphate

plasmids (pDY53, pFC6 and pFC7) for indigoidine production.

Indigoidine production by engineered *E. coli* BAP1 with supplementation of L-glutamine

pJV6 was transferred into *E. coli* BAP1 and the correct transformant was grown in 250-ml Erlenmeyer flasks containing 50 ml of LB broth supplemented with 50 µg/ml kanamycin at 37 °C with shaking at 250 rpm. When the OD_{600} value reached 0.6, 200 µM IPTG was added to induce the expression of *Sc-indC*. After the induction, the fermentation broths were cultured at 18 °C and 250 rpm for 30 min and then L-glutamine powder was added at different concentrations (0, 0.73, 1.46, 4.38 and 11.68 g/l). The broths were maintained under the same conditions for an additional 28 h. The cultures were then harvested and the titers of indigoidine were determined according to the method described previously [21]. Briefly, 1 ml of fermentation broth was centrifuged at 21,000×*g* for 10 min. The supernatant was discarded and the pellet was successively washed with 1 ml of water and methanol and then dissolved in 1 ml of dimethyl sulfoxide (DMSO) by sonication. The DMSO-insoluble components and cell debris were removed by centrifugation (8509×*g*, 5 min). The absorption value of the DMSO solution at 600 nm was measured on a Thermo Scientific GENESYS 20 Visible Spectrophotometer. The titer of indigoidine was then calculated according to a standard curve of pure indigoidine. Similarly, pJV6 and pDY53 were co-transferred into *E. coli* BAP1 and the same procedure described above was applied to the resulting engineered *E. coli* strain except that two antibiotics were supplied in the cultures (kanamycin and chloramphenicol). The experiments were conducted in triplicate and the titers are shown as mean ± SD.

Co-expression of *glnA* with *Sc-indC* and/or *Sc-indB* in *E. coli* BAP1

Each of the pACYCDute-1 derived plasmids (pFC6 and pFC7) was co-transferred with pJV6 into *E. coli* BAP1. The resulting *E. coli* strains were grown in 250-ml Erlenmeyer flasks containing 50 ml of LB broth supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37 °C and 250 rpm. When the OD_{600} reached 0.6, 200 µM IPTG was added to induce gene expression and the induced broths were cultured at 18 °C with shaking at 250 rpm for an additional 28 h. The titers of indigoidine from each engineered strain were calculated and compared. The experiments were conducted in triplicate and the titers are shown as mean ± SD.

Supply of different nitrogen-containing salts for L-glutamine synthesis

The engineered *E. coli* strain (pJV6 + pFC7) expressing Sc-IndC, GlnA and Sc-IndB was cultured in 1-l Erlenmeyer flasks containing 250 ml of LB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37 °C with shaking at 250 rpm. When the OD_{600} reached 0.6, 200 µM IPTG was added to induce the expression of *Sc-indC*, *glnA* and *Sc-indB*. After the induction, the fermentation broth was cultured at 18 °C and 250 rpm for 30 min and then evenly divided into 5 flasks. Four nitrogen-containing salts including $(NH_4)_2HPO_4$ (10 mM), $(NH_4)_2SO_4$ (10 mM), NH_4Cl (20 mM) and KNO_3 (20 mM) were separately added into one of the flasks, with the extra one (without any exogenous salts) as control. The cultures were maintained under the same conditions for an additional 28 h. The experiments were conducted in triplicate and the titers are shown as mean ± SD.

Indigoidine production with the supplementation of different concentrations of $(NH_4)_2HPO_4$

The engineered *E. coli* strain expressing Sc-IndC, GlnA and Sc-IndB was cultured in 1-l Erlenmeyer flasks containing 300 ml of LB broth supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol at 37 °C with shaking at 250 rpm. When the OD_{600} value reached 0.6, 200 µM IPTG was added to induce the expression of the enzymes. After the induction, the fermentation broth was evenly distributed

into 6 flasks, which were cultured at 18 °C and 250 rpm for 30 min. $(NH_4)_2\ HPO_4$ was then added to the induced broths at different concentrations ranging from 0 to 20 mM. The cultures were maintained under the same conditions for an additional 28 h. The experiments were conducted in triplicate and the titers are shown as mean ± SD.

Results

Improved production of indigoidine by feeding L-glutamine

L-Glutamine is the direct biosynthetic precursor of indigoidine. It is a key amino acid in primary metabolism and thus naturally exists in *E. coli*. Expression of Sc-IndC in *E. coli* led to the production of indigoidine in our previous study [21]. We hypothesize that supply of exogenous L-glutamine can improve the production of indigoidine in *E. coli*. To test the hypothesis, different concentrations of L-glutamine were fed into the induced culture of *E. coli* BAP1/Sc-IndC or *E. coli* BAP1/Sc-IndC+Sc-IndB. As shown in Fig. 2, supplementation of L-glutamine at the four test concentrations (0.73, 1.46, 4.38 and 11.68 g/l) increased the titer of indigoidine significantly. The titer reached 7.22 ± 0.31 g/l when 1.46 g/l L-glutamine was supplied into the fermentation broth of *E. coli* BAP1-Sc-IndC. Further increase in the L-glutamine concentrations (4.38 and 11.68 g/l) led to the reduced production of the blue pigment (Fig. 2). The same pattern was observed for *E. coli* BAP1/Sc-IndC+Sc-IndB. 1.46 g/l L-Glutamine was found to be the best among the four test concentrations, at which the titer of indigoidine reached 8.81 ± 0.21 g/l. This represents a 124 % increase compared to the culture without supplementing L-glutamine.

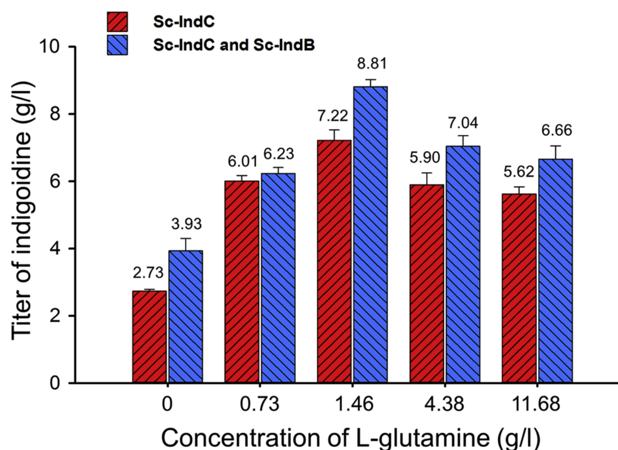

Fig. 2 Effect of L-glutamine concentrations on indigoidine production in *E. coli*

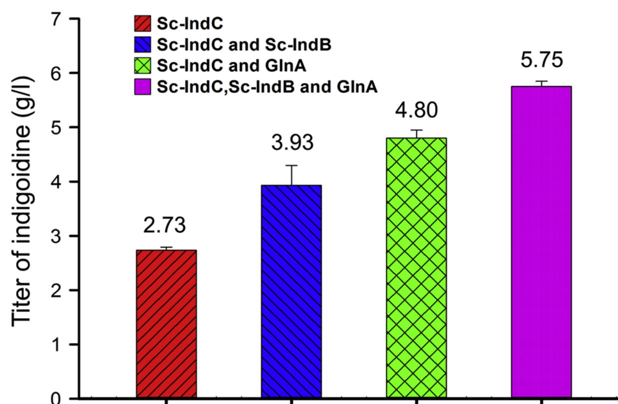

Fig. 3 Improved production of indigoidine in *E. coli* through the overexpression of a glutamine synthetase

Engineering of a glutamine synthetase into *E. coli* for enhanced supply of L-glutamine and production of indigoidine

While supplementation of L-glutamine significantly increased the titer of indigoidine, its relatively high price will increase the production costs of the blue pigment. An alternative approach is to enhance the ability of *E. coli* to generate L-glutamine in situ using metabolic engineering techniques. Glutamine synthetase (GS) synthesizes L-glutamine from L-glutamate (Fig. 1). The GS in *E. coli* is GlnA. We thus amplified the corresponding gene from *E. coli* BL21(DE3) and co-expressed it with the indigoidine biosynthetic genes. As shown in Fig. 3, the titer of indigoidine in *E. coli* BAP1/Sc-IndC+GlnA was 4.80 ± 0.15 g/l, which is 76 % higher than *E. coli* BAP1/Sc-IndC. Similarly, *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA produced the blue compound at 5.75 ± 0.09 g/l, representing a 46.3 % increase compared to *E. coli* BAP1/Sc-IndC+Sc-IndB. Thus, it is apparent that overexpression of GlnA in *E. coli* BAP1 can greatly increase the production of indigoidine. This provides another approach to improve the production of the blue pigment in *E. coli*.

Effect of different nitrogen-containing salts on indigoidine production

GS plays a key role in the amino acid metabolism through the synthesis of L-glutamine from L-glutamate and ammonia. We next tested the effect of different nitrogen-containing salts on L-glutamine biosynthesis and subsequent indigoidine production. Four salts were used, including $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2HPO_4$ and KNO_3. $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were supplemented into the induced broth of *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA at 10 mM,

while NH_4Cl and KNO_3 were supplied at 20 mM so that equivalent amounts of nitrogen were provided for the strain. As shown in Fig. 4a, all the three ammonium salts were found to increase the titer of indigoidine to certain extent, among which $(NH_4)_2HPO_4$ has the best effect on increasing indigoidine production (Fig. 4a), with a titer of 6.27 ± 0.10 g/l. By contrast, when the nitrate salt KNO_3 was supplied, the production of indigoidine was decreased and the titer was only 5.08 ± 0.09 g/l (Fig. 4a). The pH values of the cultures were measured right after the addition of the salts and at the end of the fermentation. As shown in Table 2, the pH values of the fermentation broths supplied with NH_4Cl, $(NH_4)_2SO_4$, and KNO_3 were similar to those of the control in which no salt was added, suggesting that the supply of NH_4Cl and $(NH_4)_2SO_4$ increased the production of indigoidine without causing pH changes. In contrast, the pH values of the cultures with $(NH_4)_2HPO_4$ were 7.22 ± 0.02 (0 h) and 7.37 ± 0.06 (28 h), which are slightly basic and higher than those supplemented with other salts. At higher pH values, more ammonia will be generated from the ammonium ion for L-glutamine biosynthesis. This may explain why the titer of indigoidine with the supply of $(NH_4)_2HPO_4$ was higher than those with NH_4Cl and $(NH_4)_2SO_4$ even though equivalent amounts of ammonium were present in the cultures.

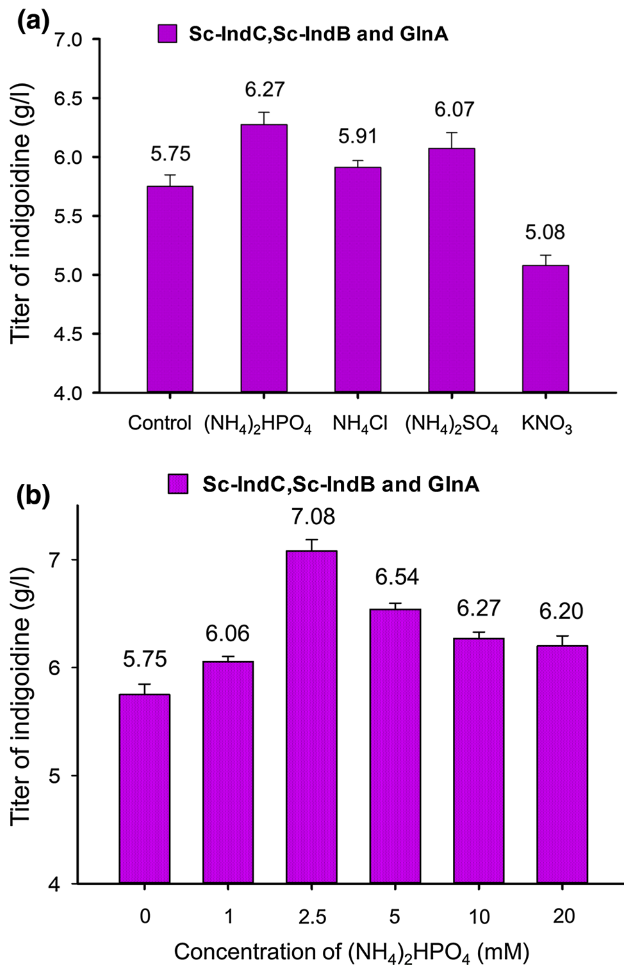

Fig. 4 Improved production of indigoidine in *E. coli* through the supplementation of nitrogen-containing salts. **a** Effect of different nitrogen-containing salts on indigoidine biosynthesis in *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA. **b** Effect of the concentrations of $(NH_4)_2HPO_4$ on indigoidine biosynthesis in *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA

Effect of the concentrations of $(NH_4)_2HPO_4$ on indigoidine production

To determine the optimal concentration of $(NH_4)_2HPO_4$ for indigoidine production, this salt was added to the IPTG-induced broths of *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA at 1, 2.5, 5, 10 and 20 mM, respectively. As shown in Fig. 4b, compared to the control in which no exogenous salt was added, all the samples with $(NH_4)_2HPO_4$ showed higher titers, further confirming that supplementation of this ammonium salt has a positive impact on the production of the blue pigment. When $(NH_4)_2HPO_4$ was fed at 1 mM, the titer of indigoidine was 6.06 ± 0.05 g/l. When the salt concentration was increased to 2.5 mM, the titer of the target product reached 7.08 g/l. Higher concentrations of the salt did not yield more indigoidine. Instead, the titers of this pigment slightly decreased. The blue compound was produced at 6.54 ± 0.06, 6.27 ± 0.10, and 6.20 ± 0.09 g/l at 5, 10 and 20 mM $(NH_4)_2HPO_4$, respectively. Thus, 2.5 mM was found to be the best for indigoidine production among the five test concentrations.

Discussion

Natural pigments represent promising colorants for foods and drinks. Blue compounds have been found from various

Table 2 The pH values of the fermentation broths of *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA in the presence of different nitrogen-containing salts

Time[a] (h)	pH				
	Control	$(NH_4)_2HPO_4$	NH_4Cl	$(NH_4)_2SO_4$	KNO_3
0	6.76 ± 0.01	7.22 ± 0.02	6.74 ± 0.01	6.78 ± 0.02	6.75 ± 0.01
28	6.92 ± 0.16	7.37 ± 0.06	6.88 ± 0.06	7.01 ± 0.03	6.91 ± 0.01

[a] The pH values were measured at two time points: 0 h (right after adding the salts) and 28 h (the end of the fermentation)

sources including plants, animals and microorganisms. Several major types of blue pigments have been identified, such as azulenes, flavonoids, quinones and quinoids, linear tetrapyrrole alkaloids, phenazine alkaloids, indole alkaloids, pyridine alkaloids, as well as organometallics and metalloproteins [9]. Development of a new colorant requires several important factors, such as color intensity, stability and efficient method to produce in large scale. Indigoidine belongs to the family of nonribosomal peptide natural products. It is synthesized by a modular nonribosomal peptide synthetase (NRPS) which condenses two units of L-glutamine to form the blue pigment. We have previously identified a gene cluster from *S. chromofuscus* that is involved in indigoidine biosynthesis [21]. By co-expressing the indigoidine synthetase Sc-IndC and the helper protein Sc-IndB, indigoidine biosynthesis can be reconstituted in *E. coli* BAP1 that contains a chromosomal copy of the phosphopantetheinyl transferase Sfp to activate the thiolation domain of Sc-IndC [11]. Biosynthesis of natural products in a heterologous host can be limited by the availability of the precursors. L-Glutamine is one of the 20 proteinogenic amino acids. It also acts as an energy source and plays a central role in cell metabolism and function. The heterologous Sc-IndC can pull L-glutamine from the natural metabolic pathways in *E. coli* to generate indigoidine. However, the availability of this indigoidine biosynthetic precursor relies on the ability of the host to synthesize this amino acid, and the best titer achieved in *E. coli* BAP1 was 3.93 g/l [21]. In this work, we demonstrated that supplementation of L-glutamine into the fermentation broth can effectively increase the production of indigoidine. Feeding of exogenous L-glutamine provides extra substrate for Sc-IndC to synthesize indigoidine, leading to a titer of 8.81 g/l, which represents an efficient production process for this pigment.

E. coli can recruit free amino acids including L-glutamine from tryptone and yeast extract in the LB medium using its oligopeptide permeases and peptidases. However, the amount of L-glutamine from the medium is limited. A previous study showed that the concentration of L-glutamine in LB medium after acidic hydrolysis was 0.6 mM (0.088 g/l) [14]. To get sufficient L-glutamine for primary and secondary metabolism, *E. coli* uses its own biosynthetic machinery to generate this key amino acid. GS is an enzyme that plays an essential role in catalyzing the condensation of glutamate and ammonia to form glutamine [7]. When performing its reaction, the active site of GS binds to glutamate, ammonia, and ATP that powers the reaction [4]. This enzyme is a central control point in nitrogen metabolism. L-Glutamine acts as a storage form of ammonia and the amino group donor in the biosynthesis of many metabolites. Although *E. coli* contains the GS gene *glnA*, overexpression of the same gene can increase the titer of indigoidine from 3.93 to 5.75 g/l, indicating that the elevated expression of this enzyme enhanced the in situ supply of L-glutamine in the cells for the biosynthesis of this blue compound. This provides an alternative approach to improving the production of indigoidine. L-Glutamate and ammonia are the two substrates for L-glutamine biosynthesis. Supplementation of ammonium salts into the culture of the engineered *E. coli* strain increased the production of indigoidine, suggesting that these exogenous salts provided more ammonia for the formation of L-glutamine. At 2.5 mM $(NH_4)_2HPO_4$, indigoidine was produced at 7.08 g/l by *E. coli* BAP1/Sc-IndC+Sc-IndB+GlnA. Although the titer from this approach is slightly lower than direct feeding of L-glutamine, this process can avoid using L-glutamine that is more costly than common ammonium salts. Additionally, previous studies have reported various metabolic engineering approaches for overproduction of L-glutamine in *E. coli*. For example, a novel mechanism for L-glutamine overproduction was found in an *E. coli* K-12-derived strain by random mutagenesis and genome-wide mutation analysis. Reduction of chromosomal DNA supercoils in the mutant caused an increase in L-glutamine accumulation in the cells. L-Glutamine was produced at 500 mM using DNA gyrase mutations in *E. coli* [6]. These studies provide useful approaches for further improvement of indigoidine production in *E. coli*.

In summary, this work demonstrates two different approaches for efficient production of indigoidine in *E. coli*. Direct supply of exogenous L-glutamine at 1.46 g/l in the fermentation broth leads to the production of this blue pigment at 8.81 g/l. Engineering of a GS into the *E. coli* host followed by the addition of the ammonium salt $(NH_4)_2HPO_4$ at 2.5 mM led to the production of indigoidine at 7.08 g/l.

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