Production of Indigo by Recombinant Escherichia coli with Expression of Monooxygenase, Tryptophanase, and Molecular Chaperone

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Abstract: Indigo is an important pigment widely used in industries of food, cosmetics, and textile. In this work, the styrene monooxygenase StyAB from Pseudomonas putida was co-expressed with the tryptophanase TnaA and the chaperone groES-groEL in Escherichia coli for indigo production. Over-expression of the gene styAB endowed the recombinant E. coli AB with the capacity of indigo biosynthesis from indole and tryptophan. Tryptophan fermentation in E. coli AB generated about five times more indigo than that from indole, and the maximum 530 mg/L of indigo was obtained from 1.2 mg/mL of tryptophan. The gene TnaA was then co-expressed with styAB, and the tryptophanase activity significantly increased in the recombinant E. coli ABT. However, TnaA expression led to a decrease in the activity of StyAB and indigo yield in E. coli ABT. Furthermore, the plasmid pGro7 harboring groES-groEL was introduced into E. coli ABP, which obviously promoted the activity of StyAB and accelerated indigo biosynthesis in the recombinant E. coli ABP. In addition, the maximum yield of indigo was further increased to 550 mg/L from 1.2 mg/mL of tryptophan in E. coli ABP. The genetic manipulation strategy proposed in this work could provide new insights into construction of indigo biosynthesis cell factory for industrial production.

Keywords: indigo; monooxygenase; tryptophanase; molecular chaperone; Escherichia coli

1. Introduction

Indigo is one of the oldest pigments used by human beings with a history of thousands of years, and it is still widely used in food, pharmaceuticals, cosmetics, and textile industries [1,2]. Indigo production is mainly achieved by extraction from plants and chemical synthesis [3]. However, indigo preparation from plants has obvious disadvantages in cost and yield, and chemical synthesis inevitably brings about harmfulness to human health and the environment due to toxic compounds from the reaction systems [4]. Previous studies reported indigo could be produced by various microorganisms such as Pseudomonas and Acinetobacter [5–10], which provides economic, effective, and eco-friendly approaches for indigo production. Various indigo biosynthesis pathways were found in different microbial species. Generally, indigo is generated via oxidation of the substrate by catalysis of oxygenase. In Pseudomonas species, multiple oxygenases, including xylene oxygenase, toluene-4-monoxygenase, toluene dioxygenase, 2-naphthoic acid oxygenase, naphthalene dioxygenase, and styrene monoxygenase, were proved to express the catalytic activities of indigo biosynthesis [8,11]. O’Connor et al. (1997) proposed the pathway of indigo biosynthesis from indole in Pseudomonas. Briefly, indole is oxidized into indole oxide by monoxygenase; indole oxide is then transformed to indoxyl by isomerase; and two
Indoxyl molecules form indigo by dimerization [12]. However, not only indigo but also the structurally similar by-product indirubin could be generated via the alternative branch pathway by catalysis of dioxygenase [13,14]. Therefore, indole conversion by catalysis of monoxygenase is a shortcut to obtain pure indigo.

In our previous work, over-expression of the styrene monoxygenase gene styAB was conducted in Pseudomonas putida, and it significantly enhanced indigo production from indole, revealing that the monoxygenase StyAB acted as the key rate-limiting enzyme for indigo biosynthesis [15]. In this work, the styrene monoxygenase gene styAB from P. putida was heterologously expressed in Escherichia coli for highly efficient indigo production, and co-expression of styAB with the tryptophanase gene TnaA and the molecular chaperone groES-groEL was further performed in E. coli to construct an indigo production system from tryptophan. The genetic manipulation strategy proposed in this work provided new insights into construction of indigo biosynthesis cell factory for industrial application.

2. Materials and Methods

2.1. Strains, Plasmids and Culture Conditions

Strains and plasmids used in this work are listed in Table 1. E. coli strains were grown in Luria–Bertani (LB) medium at 37 °C with vigorous shaking. P. putida strain was cultured at 30 °C in LB medium with vigorous shaking. When needed, kanamycin (50 µg/mL) and chloramphenicol (35 µg/mL) were added into LB medium for E. coli strain screening.

Table 1. Strains and plasmids used in this work.

| Strains or Plasmids | Relevant Features | Source |
|---------------------|------------------|--------|
| Plasmids            |                  |        |
| pBK-CMV             | Vector for gene expression in E. coli, Kan£ | Laboratory collection |
| pBK-AB              | styAB gene cloned in pBK-CMV, Kan£ | This work |
| pBK-ABT             | TnaA gene cloned in pBK-AB, Kan£ | This work |
| pGro7               | Chaperone plasmid harboring groES-groEL, Cm£ | Takara, Beijing, China |
| Strains             |                  |        |
| E. coli DH5α        | Host for gene expression | TIANGEN, Beijing, China |
| E. coli AB          | E. coli DH5α harboring pBK-AB | This work |
| E. coli ABT         | E. coli DH5α harboring pBK-ABT | This work |
| E. coli ABTP        | E. coli DH5α harboring pBK-ABT and pGro7 | This work |
| P. putida B4        | Donor of styAB gene | Laboratory collection |

2.2. DNA Manipulation Techniques

Standard DNA manipulation techniques were performed as described by Green and Sambrook [16]. Bacterial genomic DNA was prepared using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) following the manufacturer’s instructions. Plasmid DNA from E. coli was prepared using the High-purity Plasmid Miniprep Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. DNA amplification was performed using the Takara Primer SRTAR MAX DNA Polymerase following the manufacturer’s protocol (Takara, Beijing, China). Restriction endonuclease digestion and DNA ligation were conducted according to the manufacturer’s instructions (Takara, Beijing, China). Standard heat-shock transformation method was used to introduce plasmid DNA to E. coli [16]. Total mRNA from E. coli was prepared using the Trizol Extraction Kit according to the manufacturer’s instructions (BioTeke, Beijing, China). RNA was subject to reverse transcription to generate cDNA using FastKing RT Kit (TIANGEN, Beijing, China) following the manufacturer’s protocol. Quantitative Real-Time PCR (q RT-PCR) was performed using the SuperReal PreMix Plus (SYBR Green) Kit (TIANGEN, Beijing, China) in Light Cycler Nano System (Roche Diagnostics, Indianapolis, IN, USA) with the following cycling conditions: 95 °C for 10 s, followed by 45 cycles of 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 45 s. The 16S rRNA gene was used for transcript normalization. All reactions were performed in triplicate. Data were analyzed using the 2−∆∆Ct method corrected for primer efficiencies using the untreated group mean as the reference condition [17]. Primers used in this work were listed in Table 2.
Table 2. Primers used in this work.

| Gene   | Primer       | Sequence (5′–3′)             |
|--------|--------------|------------------------------|
| q RT PCR |              |                              |
| styA   | styA-F       | GGGCAGCTGATTTGAGATTC         |
|        | styA-R       | TTTTCCGTTATTGAGGCT           |
| styB   | styB-F       | AAAAGATGTGGTGGATTGA          |
|        | styB-R       | TGCTGAAGAATGCGCGAATT         |
| 16S rRNA | 16S-F       | CCACTGGAGACTGATACT           |
|        | 16S-R        | GCACCTGTCTCAATGT            |

2.3. Vectors Construction for Gene Expression in E. coli

The styAB gene was amplified by PCR from the genomic DNA of P. putida B4 using the specific primers styAB-F and styAB-R designed according to the sequence of styrene monooxygenase gene (GenBank accession no. DQ177365.1) from P. putida. The amplicon of styAB was inserted into the multiple cloning site (MCS) of the E. coli expression vector pBK-CMV to construct the recombinant vector pBK-AB (Figure S1), which was transformed into E. coli DH5α, and the recombinant strain E. coli AB was screened on LB agar plates containing kanamycin.

The TnaA gene was amplified by PCR from the genomic DNA of E. coli DH5α using the specific primers TnaA-F and TnaA-R designed according to the sequence of tryptophanase gene (GenBank accession no. NC_000913.3) from E. coli. The amplicon of TnaA was inserted into the upstream site of styAB in MCS of pBK-AB to construct the recombinant vector pBK-ABT (Figure S1). pBK-ABT was transformed into E. coli DH5α to construct the recombinant strain E. coli ABT.

The chaperone plasmid pGro7 was transformed into E. coli AB and E. coli ABT, respectively, and the recombinant strains E. coli ABP and E. coli ABTP were screened on LB agar plates containing kanamycin and chloramphenicol. The recombinant vectors were verified by sequencing and alignment analysis using DNAMAN software package and BLAST Program at NCBI against the GenBank database, and the recombinant strains were verified by plasmid profile and sequencing.

2.4. Indigo Production by E. coli Fermentation

For indigo production, fresh overnight culture of E. coli was inoculated (1%) in the fermentation medium (17 g/L Na₂HPO₄ • 12 H₂O, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 0.1 g/L MgSO₄, and 3 g/L yeast extract) containing indole or tryptophan as the substrates, and fermentation was conducted at 30 °C with vigorous shaking at 200 rpm for 24–48 h. For fermentation of the E. coli strain harboring the chaperone plasmid pGro7, fresh overnight culture of E. coli was inoculated (1%) in LB medium and cultured at 37 °C with vigorous shaking at 200 rpm. When cells density reached 0.2 (OD 600 nm), 150 µg/mL of arabinose was added in LB medium, and cells were cultured at 30 °C with vigorous shaking at 200 rpm until OD 600 nm reached 0.8. The fresh culture was then inoculated (1%) in the fermentation medium, and fermentation was conducted as described above. The fermentation data were representative of three independent experiments performed in triplicate.

2.5. Enzymatic Activity Assay

Monooxygenase activity was measured by indole consumption as described previously [15]. Cells were harvested from cultures by centrifugation at 10,000×g for 10 min,
washed with potassium phosphate buffer (50 mM, pH 7.0), and resuspended in the same buffer containing 5 mM indole. Cell suspension was incubated in shaking water bath at 30 °C at 150 rpm for 30 min. Indole depletion was determined by HPLC, and 1 unit (U) of monooxygenase activity was defined as 1 μM indole depletion in 30 min.

Tryptophanase activity was assayed by indole production from tryptophan. Cells were harvested from cultures by centrifugation at 10,000 × g for 10 min, washed with phosphate buffer (100 mM, pH 7.0), and resuspended in the same buffer. Cell resuspension solution was subject to sonication in ice-bath, and the supernatant was collected by centrifugation at 10,000 × g for 5 min. Cell supernatant was mixed with glutathione (5 mM) and tryptophan (5.0 mg/mL) and incubated in shaking water bath at 37 °C at 150 rpm for 10 min. Indole production was determined by HPLC, and 1 unit (U) of tryptophanase activity was defined as 0.01 μm indole production in 10 min. The results were representative of three independent experiments performed in triplicate. Significant differences between different strains were identified by the unpaired Student’s t-test or ANOVA analysis.

2.6. Measurement of Indole, Indigo, and Tryptophan

For indigo determination, fermentation culture was centrifuged at 10,000 × g for 10 min to collect blue indigo pellets, which were washed with water and resuspended in dimethyl formamide (DMF). The indigo suspension was subject to sonication for 5 min repeatedly and filtrated with 0.22 μm millipore for HPLC analysis. Indigo and indole were measured by a HPLC system (Agilent 1290, Agilent, Santa Clara, CA, USA) equipped with an Agilent Eclipse plus C18 RRHD column (1.8 μm, 2.1 mm × 50 mm) and diode array detector [15]. The mobile phase was water/methanol (10: 90, v/v), and the operating conditions were as follows: detection at 610 nm and flow rate of 0.2 mL/min.

For tryptophan determination, fermentation culture was centrifuged at 10,000 × g for 5 min to collect supernatant. The supernatant was filtrated with 0.22 μm millipore and used for tryptophan determination by a HPLC system (Agilent 1200, Agilent, Santa Clara, CA, USA) equipped with an Agilent C18 column (5.0 μm, 150 mm × 4.6 mm) and diode array detector. The mobile phase was 0.03% KH₂PO₄ solution/methanol (90:10, v/v), and the operating conditions were as follows: detection at 278 nm and flow rate of 1.0 mL/min. The results were representative of three independent experiments performed in triplicate. Significant differences between different strains were identified by the unpaired Student’s t-test.

3. Results

3.1. Expression of Styrene Monooxygenase Gene StyAB Generated Indigo Biosynthesis in E. coli

The 1815 bp styAB gene was cloned from P. putida B4, and sequencing analysis showed that the DNA fragment shared a 100% homology with the styrene monooxygenase gene (GenBank accession no. DQ177365.1). The 6.3 kb recombinant expression vector pBK-AB was then constructed by inserting the styAB gene into pBK-CMV and transformed into E. coli DH5α, generating the recombinant strain E. coli AB. The fermentation results indicated that E. coli AB obtained the ability of indigo biosynthesis from indole, and its indigo production yield was quite higher than that of P. putida B4 (Figure 1). It was observed that indigo production in E. coli AB was indole dose-dependent. The highest yield of indigo (70 mg/L) in E. coli AB was produced from indole at 160 μg/mL, but higher concentrations of indole generated cytotoxicity and consequently led to a sharp decrease in indigo production (Figure 1).

In order to avoid the cytotoxicity of indole, tryptophan was used as the substrate of indigo biosynthesis. As shown in Figure 2, much more indigo was produced from tryptophan than that from indole in E. coli AB. The maximum yield of indigo from 1.0 mg/mL of tryptophan in E. coli AB was determined to be about 380 mg/L after 24 h of fermentation (Figure 3), which was about 5.4-fold higher than the highest yield (70 mg/L) from 160 μg/mL of indole, revealing that tryptophan was more suitable than indole as the substrate for indigo production by E. coli fermentation.
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**Figure 1.** Indigo biosynthesis from different concentrations of indole in fermentation of *P. putida* B4 and *E. coli* AB. Bars with asterisk (*) are significantly different (*p* < 0.05).

**Figure 2.** Indigo production from indole and tryptophan in *E. coli* AB fermentation. CK, *E. coli* DH5α; 1, *E. coli* AB fermentation with 160 μg/mL of indole; 2, *E. coli* AB fermentation with 1.0 mg/mL of tryptophan.

**Figure 3.** Indigo production from 1.0 mg/mL of tryptophan in *E. coli* AB fermentation.
Furthermore, the influence of different concentrations of tryptophan on indigo production was investigated in *E. coli* AB fermentation. The results (Figure 4) indicated that low concentrations of tryptophan (<0.8 mg/mL) could be almost completely transformed to indigo, but indigo yield was limited to some extent due to low substrate concentration. When the concentration of tryptophan increased to 0.8–1.2 mg/mL, though the conversion rate of tryptophan decreased to 75%, the maximum indigo yield was 530 mg/L from 1.2 mg/mL of tryptophan (Figure 4). However, as the concentration of tryptophan rose (>1.2 mg/mL), the conversion rate of tryptophan and indigo yield both fell rapidly. It suggested that excessive tryptophan possibly led to the inadequate catalytic capacity of tryptophanase for subsequent indigo biosynthesis.

![Figure 4. Indigo biosynthesis from different concentrations of tryptophan in *E. coli* AB fermentation.](image)

**3.2. Co-Expression of Monoxygenase Gene StyAB and Tryptophanase Gene TnaA for Indigo Biosynthesis in *E. coli***

In order to enhance the utilization rate of tryptophan and improve indigo biosynthesis, the tryptophanase gene *TnaA* was over-expressed in *E. coli* AB. The 1416 bp *TnaA* gene was cloned from *E. coli* DH5α, which shared a 100% homology with the *E. coli* tryptophanase gene (GenBank accession no. K00032.1). The 7.7 kb recombinant expression vector pBK-ABT was constructed by inserting the *TnaA* gene into pBK-AB and transformed into *E. coli* DH5α, generating the recombinant strain *E. coli* ABT. Tryptophanase activity assay indicated that the recombinant strain *E. coli* ABT with expression of *TnaA* exhibited much higher tryptophanase activity than *E. coli* AB in response to high concentrations (0.8–2.0 mg/mL) of tryptophan added in fermentation (Figure 5), demonstrating that the *TnaA* gene was successfully expressed in *E. coli* ABT.

*E. coli* ABT was then used in fermentation with addition of different concentrations of tryptophan for indigo biosynthesis. Surprisingly, the fermentation results indicated that both the indigo yield and the conversion rate of tryptophan in *E. coli* ABT were significantly lower than that in *E. coli* AB at each concentration of tryptophan (Figure 6), revealing that expression of the *TnaA* gene hardly contributed to more indigo biosynthesis. Besides, more indole accumulated in *E. coli* ABT than that in *E. coli* AB, which was in accordance with the lower production yield of indigo (Figure 6). Though the tryptophanase activity was substantially enhanced, and tryptophan could be efficiently utilized, the monoxygenase activity must be strong enough for transformation of indole derived from tryptophan into indigo in *E. coli* ABT. It suggested that co-expression of the genes *TnaA* and *styAB* led to the deficiency in the catalytic activity of the monoxygenase StyAB because of some specific reasons, and it revealed that a delicate balance between the activities of tryptophanase and monoxygenase was essential for highly effective indigo biosynthesis from tryptophan in *E. coli*. 
3.3. Introduction of Molecular Chaperone Enhanced the Activity of Monoxygenase StyAB and Indigo Biosynthesis in E. coli

In order to further enhance the activity of the monoxygenase StyAB, the molecular chaperone plasmid pGro7 was introduced into E. coli AB and E. coli ABT, respectively, generating the recombinant strain E. coli ABP and E. coli ABTP. Monoxygenase activity assay indicated that activities of the monoxygenase StyAB were significantly higher in the strain harboring pGro7 (E. coli ABP or E. coli ABTP) than in its corresponding strain without pGro7 (E. coli AB or E. coli ABT) during fermentation (Figure 7), which demonstrated that the presence of the molecular chaperone plasmid pGro7 contributed to improving the enzymatic activity in E. coli.

Moreover, it was observed that the strain with over-expression of TnaA (E. coli ABT or E. coli ABTP) expressed a significantly lower monoxygenase activity than its corresponding strain without TnaA expression (E. coli AB or E. coli ABP) when indigo was produced in quantity during fermentation (6 h and 18 h) (Figure 7), suggesting that expression of the styAB gene was reduced when it was co-expressed with the TnaA gene in the same vector.

Fermentation results showed that indigo accumulated faster in the first 24 h of fermentation in strains E. coli ABP and E. coli ABTP than in E. coli AB and E. coli ABT (Figure 8). Though a significant difference in the maximum yield of indigo was hardly detected between strains with and without pGro7, the introduction of the molecular chaperone benefited the catalytic activity of StyAB and consequently led to more indigo production (Figure 8). Besides, in comparison with E. coli ABT and E. coli ABTP, about 2-fold higher maximum yield of indigo was achieved in E. coli AB and E. coli ABP after 20 h of fermen-
tation (Figure 8), which demonstrated that co-expression of TnaA with styAB exerted a negative effect on indigo biosynthesis due to a great reduction in the monooxygenase StyAB activity.

Furthermore, the fermentation with different concentrations of tryptophan showed that the conversion rate of tryptophan declined as the concentration of tryptophan rose, and the maximum yield of indigo was detected to be 550 mg/L from 1.2 mg/mL of tryptophan in fermentation of E. coli ABP (Figure 9).
4. Discussion

Different strategies of genetic manipulations were conducted to enhance indigo biosynthesis in *E. coli* in this study. The styrene monooxygenase gene *styAB* from *P. putida* was successfully expressed in *E. coli*, and it fulfilled a large number of indigo biosynthesis, demonstrating that the monooxygenase StyAB was the key enzyme for indigo biosynthesis. In *P. putida*, both styrene monooxygenase and styrene oxide isomerase are indispensable for transformation of indole to indigo [9]. The conversion of indole to indigo in *P. putida* is the result of a two-step biotransformation including formation of indole oxide from indole by styrene monooxygenase and generation of 3-oxindole from indole oxide by styrene oxide isomerase, and indigo is finally synthesized by dimerization of 3-oxindole [18]. This study verified that indigo production from indole could be simply achieved by expression of solo styrene monooxygenase in *E. coli*, which is an economic pathway for industrial production. Further, it suggested that there was a possible alternative isoenzyme of styrene oxide isomerase that could catalyze indole oxide to 3-oxindole in *E. coli*.

The monooxygenase StyAB directly catalyzes indole into indigo, but a high concentration of indole was toxic to *E. coli* cells [19,20], so there are inevitable limitations for indigo production using indole as the substrate. As *E. coli* could transform tryptophan to indole by catalysis of tryptophanase [21], tryptophan was used as the alternative substrate in fermentation for indigo production. The tryptophanase gene *TnaA* was successfully co-expressed with the monooxygenase gene *styAB* in *E. coli*, which obviously enhanced the tryptophanase activity. However, expression of *TnaA* unexpectedly resulted in a decrease in the monooxygenase activity, and consequently, co-expression of *TnaA* with *styAB* failed to promote indigo biosynthesis from tryptophan. In order to figure out the reason for StyAB activity decrease, the transcriptional level assay of the genes *styA* and *styB* was performed in *E. coli* AB and *E. coli* ABT in fermentation with different concentrations of tryptophan. As shown in Figure 10, the relative expression levels of both *styA* and *styB* in *E. coli* AB were significantly higher than that in *E. coli* ABT, which demonstrated that the StyAB activity decrease resulted from the low expression of *styAB* in *E. coli* ABT. For construction of the co-expression vector pBK-ABT, the gene *TnaA* was inserted into the upstream region of *styAB*. *TnaA* was adjacent to the promoter and consequently kept *styAB* distant from the promoter region. As the two genes were under the control of the same promoter, the transcription of *styAB* was probably attenuated due to the longer distance from the promoter [22]. Royo et al. [23] reported that co-expression of tryptophanase from *E. coli* and dioxygenase from *Sphingomonas macrosporitabida* did not improve the rate of indigo production from tryptophan in *E. coli*, and the reason probably was that tryptophanase production is not a limiting factor. Indigo biosynthesis from tryptophan in *E. coli* is a cascade reaction involved with sequential catalysis of tryptophanase and monooxygenase, so it is reasonable to assume that an appropriate balance in activities of the two key enzymes is likely essential for achievement of highly efficient indigo production. Though the genes
TnaA and styAB were co-expressed in E. coli, their expressions were not under a tightly regulatory control. Hence, it was possible that the irrational ratio of expression levels of TnaA and StyAB led to indole accumulation and indigo biosynthesis decline.

![Graph](https://www.mdpi.com/article/10.3390/foods11142117/s1, Figure S1: Construction of the expression vector pBK-AB harboring StyAB and pBK-ABT harboring TnaA and styAB)

Figure 10. Transcriptional level assay of the genes styA and styB in E. coli AB and E. coli ABT in fermentation with different concentrations of tryptophan. Bars with asterisk (*) or pound sign (#) are significantly different ($p < 0.05$).

The plasmid pGro7 harboring the chaperone protein groES-groEL could help the recombinant protein fold correctly in E. coli [24]. Therefore, introduction of pGro7 significantly enhanced the activity of StyAB and consequently sped up the rate of indigo biosynthesis from tryptophan. However, the effect of the molecular chaperone on the production yield of indigo was very finite. It suggested that other limiting factors might exit in the indigo biosynthesis system. Efficient cofactor regeneration is critical to oxidation-reduction reactions. Since the epoxidation of indole requires the activity of the flavin-dependent monooxygenase StyAB, enough supply of NADH or FAD is essential for the reaction system and productivity of indigo biosynthesis [25]. It is expectable that introduction of a NADH or FAD regeneration system will further promote indigo production by catalysis of the monooxygenase StyAB in E. coli.

5. Conclusions

The styrene monooxygenase gene styAB from P. putida was heterologously over-expressed in E. coli, and it fulfilled an economic pathway for indigo production from indole and tryptophan. Introduction of the chaperone protein groES-groEL significantly enhanced the catalytic activity of StyAB and consequently sped up the rate of indigo biosynthesis from tryptophan.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11142117/s1, Figure S1: Construction of the expression vector pBK-AB harboring styAB and pBK-ABT harboring TnaA and styAB.

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