

Enhancing Indigo Production by Over-Expression of the Styrene Monooxygenase in *Pseudomonas putida*

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Abstract As an important traditional blue dye, indigo has been used in food and textile industry for centuries, which can be produced via the styrene oxygenation pathway in *Pseudomonas putida*. Hence, the styrene monooxygenase gene *styAB* and oxide isomerase gene *styC* are over-expressed in *P. putida* to investigate their roles in indigo biosynthesis. RT-qPCR analysis indicated that transcriptions of *styA* and *styB* were increased by 2500- and 750-folds in the *styAB* over-expressed strain B4-01, compared with the wild-type strain B4, consequently significantly enhancing the indole monooxygenase activity. Transcription of *styC* was also increased by 100-folds in the *styC* over-expressed strain B4-02. Besides, *styAB* over-expression slightly up-regulated the transcription of *styC* in B4-01, while *styC* over-expression hardly exerted an effect on the transcriptional levels of *styA* and *styB* and indole monooxygenase activity in B4-02. Furthermore, shaking flask experiments showed that indigo production in B4-01

reached 52.13 mg L⁻¹ after 24 h, which was sevenfold higher than that in B4. But no obvious increase in indigo yield was observed in B4-02. Over-expression of *styAB* significantly enhanced the indigo production, revealing that the monooxygenase STYAB rather than oxide isomerase STYC probably acted as the key rate-limiting enzyme in the indigo biosynthesis pathway in *P. putida*. This work provided a new strategy for enhancing indigo production in *Pseudomonas*.

Introduction

Indigo has been used as an important dye in food and textile industry for a long history [4, 7]. For centuries, the main source of indigo was extracted from plants. Until the end of nineteenth century, chemical synthesis displaced the traditional preparation method because of its advantage in production cost and yield [1]. However, chemical synthesis of indigo may give rise to environmental pollution and health issues due to the toxicity of the substrates, catalysts, and intermediates [13]. Hence, nowadays the natural dyes have been favored by more and more customers.

As is known, microbial fermentation is an effective and environment friendly way to produce various metabolites including the natural dyes. Thus far, the biosynthesis of indigo has been reported in various microorganisms including *Pseudomonas* sp., *Acinetobacter* sp., *Bacillus megatreium*, and so on [2, 3, 6, 9, 11]. Previous studies suggested that some *Pseudomonas* strains could generate indigo from indole by monooxygenase or dioxygenase, which is closely related to the host's capacity of degrading aromatic hydrocarbon compounds [9, 14].

The pathway of indigo conversion from indole in *Pseudomonas* sp. has been previously proposed as shown

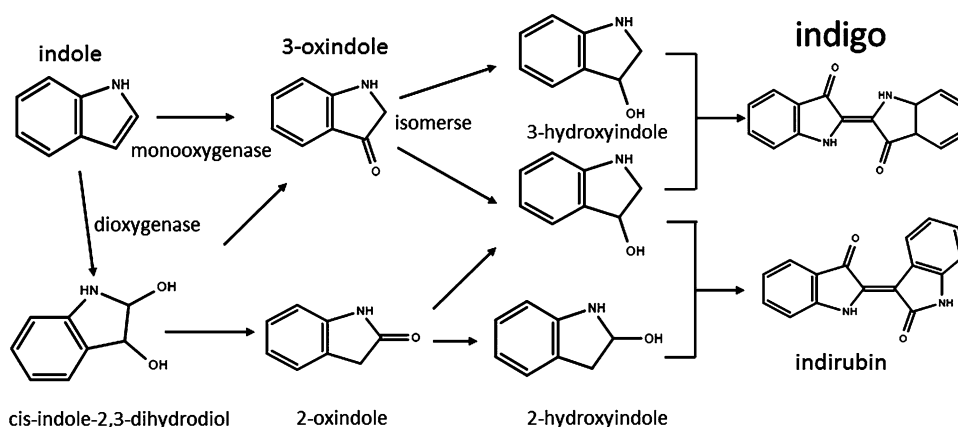
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Fig. 1 The putative pathway of indigo biosynthesis from indole in *Pseudomonas putida***Table 1** Strains and plasmids used in this work

Strains or plasmids	Relevant features	Reference or source
<i>Plasmids</i>		
pBBR1MCS-2	Broad-host-range cloning vector; lacZ α ; Km ^R	Laboratory collection
pBBR1MCS-2-styAB	<i>KpnI-HindIII</i> digested PCR product of <i>styAB</i> cloned in <i>P. putida</i> B4; Km ^R	This work
pBBR1MCS-2-styC	<i>KpnI-HindIII</i> digested PCR product of <i>styC</i> cloned in <i>P. putida</i> B4; Km ^R	This work
<i>Strains</i>		
<i>E. coli</i> DH5 α	Host for cloning	TIANGEN Inc, Beijing, CN
<i>P. putida</i> B4	Donor for <i>styAB</i> ; host for expression	Laboratory collection
<i>P. putida</i> B4-0	<i>P. putida</i> B4 harboring pBBR1MCS-2, Km ^R	This work
<i>P. putida</i> B4-01	<i>P. putida</i> B4 harboring pBBR1MCS-2-styAB, Km ^R	This work
<i>P. putida</i> B4-02	<i>P. putida</i> B4 harboring pBBR1MCS-2-styC, Km ^R	This work

in Fig. 1 [11]. The biosynthesis of indigo mainly consists of oxidation and dimerization reactions through two alternative branch pathways. On one hand, indole is first oxidized into indole oxide by monooxygenase; the oxide is then catalyzed into indoxyl by isomerase; two indoxyl molecules generates indigo by dimerization [10]. On the other hand, dioxygenase oxidizes indole into cis-indole-2, 3-dihydrodiol, which is then dehydrated into 3-oxindole and 2-oxindole; 3-oxindole and 2-oxindole turn to indoxyl and 2-hydroxyindole, correspondingly; indoxyl molecules generate indigo by homo-dimerization, and indoxyl plus 2-hydroxyindole could dimerize into indirubin at the same time [5, 15].

In this work, we isolated an indigo-producing *Pseudomonas putida* strain B4 according to the method described by Douky [3]. The strain B4 expresses high activities of monooxygenase and isomerase, which are encoded by the styrene monooxygenase (SMO) gene *styAB* and the styrene oxide isomerase (SOI) gene *styC* located in the styrene degradation gene cluster *sty*. Previous studies on the *sty* gene cluster mainly focus on its functionality for the styrene degradation [8, 12, 18]. The role of the *sty* cluster genes in indigo biosynthesis has rarely been further

investigated so far. Our work demonstrated that over-expression of *styAB* significantly enhanced indigo production in *P. putida* B4, which probably revealed a new insight into the indigo biosynthesis in *P. putida*.

Materials and Methods

Strains, Plasmid, and Culture Conditions

Strains and plasmids used in this research are listed in Table 1. *Escherichia coli* (*E. coli*) strains were grown at 37 °C in Luria-Bertani (LB) medium with vigorous shaking as the cloning host. *P. putida* was cultured at 30 °C in LB medium with shaking. The antibiotic kanamycin was added at the concentration of 50 $\mu\text{g mL}^{-1}$ when needed.

DNA Manipulation and Vector Construction

DNA manipulation procedure was performed as described by Sambrook and Russel [16]. Genomic DNA was isolated using TIANamp Bacteria DNA Kit (TIANGEN Biotech, CN) according to the manufacturer's instructions. Plasmids

Table 2 Primers used in this work

Primer	Sequence (5'–3')	Restriction sites
F-AB	CGGGGTACCAGCAACCCACAACAAAAACAAG	<i>KpnI</i>
R-AB	CCCAAGCTTGTGATCGGCACAGAAAAGG	<i>HindIII</i>
F-C	CGGGGTACCATGCTTCATGCCTTCCAAC	<i>KpnI</i>
R-C	CCCAAGCTTTAGGCTCCTTGTCATTCCGC	<i>HindIII</i>
StyA-F	GCTCCTGAATACCGTTGCT	None
StyA-R	AGATCATACTGCTCCGACA	None
StyB-F	AAAAGATGTGGTGGTGGAT	None
StyB-R	TGCTGAAGAATGCCGATAA	None
StyC-F	TGGTGTGGCCTTTGGATG	None
StyC-R	CGGTTGGGAGAGAAGTTGG	None
16S-F	ACCACCTGGACTGATACT	None
16S-R	AGCACCTGTCTCAATGTT	None

was prepared using TIANprep Rapid Mini Plasmid Kit (TIANGEN Biotech, CN) following the manufacturer's instructions. Restriction endonuclease digestion and DNA ligation were operated according to suppliers' instructions (TaKaRa, CN). Introducing plasmid DNA to *E. coli* DH5 α followed the procedures of standard heat shock transformation method [16]. Plasmid DNA was transformed into *Pseudomonas* by electroporation using an ice-cold electroporation cuvette with a 2-mm electrode gap, and the electric pulse was delivered by the Gene Pulser XcellTM electroporation system (Bio-Rad, Richmond, CA) using the following parameter settings: 1.5 kV, 400 Ω , and 25 μ F.

For vector construction, the *styAB* and *styC* genes were amplified with primers F-AB, R-AB, F-C, and R-C shown in Table 2 based on the styrene monooxygenase gene and the styrene oxide isomerase gene (GenBank accession no. DQ177365.1). PCR amplification procedures were performed using TaKaRa Ex *Taq* DNA polymerase following the suppliers' recommendations (TaKaRa, CN). The PCR product was inserted into the multiple cloning site of the vector pBBR1MCS-2 to construct the recombinant vectors pBBR1MCS-2-*styAB* and pBBR1MCS-2-*styC*. The vectors pBBR1MCS-2, pBBR1MCS-2-*styAB*, and pBBR1MCS-2-*styC* were then transformed into *P. putida* B4 by electroporation, respectively.

Shaking Flask Fermentation

Pseudomonas putida strains were grown in LB media at 200 rpm at 30 °C for overnight. The fresh cultures were then inoculated into the fermentation media as described by Qu et al. [14], which was composed of 1.71 g Na₂HPO₄·12 H₂O, 0.3 g KH₂PO₄, 0.1 g NH₄Cl, 0.05 g NaCl, 0.01 g MgSO₄, and 0.1 g yeast extract in 100 mL sterile water. The fermentation for indigo production was conducted at 30 °C at 200 rpm. Samples were collected at 4, 8, 12, 24, 36, and 48 h for measurement of indigo concentration.

RT-qPCR Analysis

For real-time quantitative PCR (RT-qPCR) analysis, total RNA was isolated from cells grown in the fermentation media using RNeasy Mini Kit (Qiagen, CN). Reverse transcription was carried out with primers (StyA-F&R, StyB-F&R, styC-F&R, and 16S-F&R) listed in Table 2 using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, CN) according to the manufactures instructions. qPCR was performed using SuperReal PreMix Plus (TIANGEN Biotech, CN) with following conditions: 95 °C for 5 min; 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. Relative quantification of gene expression was calculated using the 16s rDNA as the internal control gene.

Enzyme Assays

Indole Monooxygenase activity (IMO) was measured via the indole consumption. Cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer containing 50 mM indole. For enzymatic reaction, 20 mL of suspension was transferred into a 50 mL tube and incubated in shaking water bath at 30 °C at 150 rpm for 30 min, and the indole depletion was monitored every 5 min. Samples were directly analyzed after filtration. 1 μ mol of indole depletion in 1 min was defined as 1 unit (U) of IMO activity.

Measurement of Indole and Indigo Concentration

For indigo detection, 50 mL of the fermentation broth was centrifuged for 10 min at 9500 rpm to harvest blue pellets, which were collected and washed with water. Dimethyl formamide (DMF) was added to suspend indigo. The suspension was subjected to sonication for 5 min repeatedly and filtrated for determination.

Indigo and indole were quantified by a high-performance liquid chromatography (HPLC) system (Agilent 1290, USA) equipped with a Agilent Eclipse Plus C18 RRHD column (1.8 μm , 2.1 \times 50 mm) and Diode array detector (DAD) at 610 nm. 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⁻¹. All samples were analyzed in triplicate.

Statistical Analysis

The numerical data were expressed as mean \pm standard deviation (SD). A One-Way ANOVA statistical difference was accepted as significant if $0.01 < p < 0.05$ and extremely significant if $p < 0.01$ in this study.

Results and Discussion

Construction of Recombinant Over-Expression Vectors and Strains

The 1815-bp *styAB* and 510-bp *styC* genes were cloned from *P. putida* B4. Sequence analysis indicated that they shared a 100 % identity with the styrene monooxygenase and the styrene oxide isomerase genes (GenBank accession no. DQ177365.1) in *P. putida*. For over-expression of *styAB* and *styC*, the two amplicons were digested by *Kpn*I and *Hind* III and inserted into pBBR1MCS-2, respectively, generating the 6.9-kb pBBR1MCS-2-*styAB* and 5.6-kb pBBR1MCS-2-*styC*. Then pBBR1MCS-2-*styAB*, pBBR1MCS-2-*styC*, and the empty vector pBBR1MCS-2 were introduced into the wild-type strain *P. putida* B4, respectively, generating recombinant strains B4-01, B4-02, and the control strain B4-0.

RT-qPCR Analysis of *styAB* and *styC* Genes

To investigate the transcriptional levels of *styA*, *styB*, and *styC* genes at different growth phases during indigo fermentation in *P. putida* strains, the cell growth was monitored as shown in Fig. 2. The growth curves indicated that the wild-type strain, control strain and recombinant strains shared a highly similar growth tendency during indigo fermentation under the same fermentation conditions. It revealed that introduction of the expression vectors hardly exerted a negative impact on cells growth in the indigo fermentation system.

The transcriptional levels of *styA*, *styB*, and *styC* genes in logarithmic and stationary phase's cells were evaluated by RT-qPCR analysis. As shown in Figs. 3a, b, over-expression of *styAB* led to a 2500- and 750-folds higher transcriptional levels of *styA* and *styB* in strain B4-01,

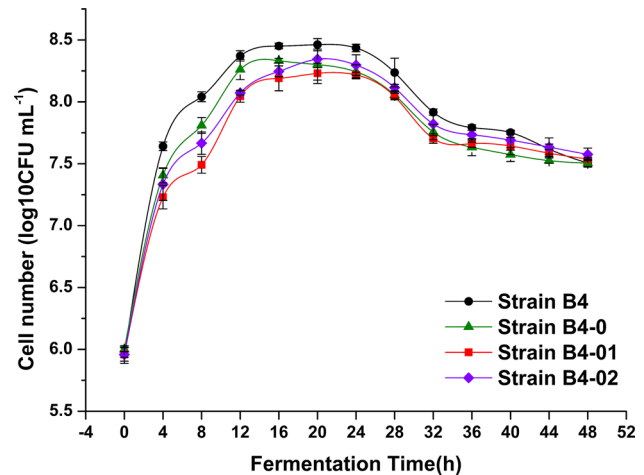


Fig. 2 Growth curve of *Pseudomonas putida* strains in fermentation media. B4, wild-type strain; B4-0, recombinant strain harboring the empty vector pBBR1MCS; B4-01, recombinant strain harboring pBBR1MCS-2-*styAB*; B4-02, recombinant strain harboring pBBR1MCS-2-*styC*

respectively. Besides, it is found that the transcriptional level of *styA* was obviously higher than that of *styB* when the two genes were co-over-expressed at the same time. The possible reason was that *styA* encoded an oxygenase while *styB* encoded a NADH-dependent flavin oxidoreductase subunit [17], which was obliged to bind to NADH and flavin sequentially [12]. Hence, the expression of *styB* was relevant and limited to the levels of NADH and flavin in cells. When *styA* and *styB* were co-over-expressed, the transcription level of *styB* might exhibit lower than that of *styA*. Additionally, the transcriptional levels of *styC* encoding styrene oxide isomerase was significantly up-regulated by about 100-folds in strain B4-02 (Fig. 3c), suggesting that *styC* was successfully over-expressed.

Besides, it was noteworthy that over-expression of *styA* and *styB* led to slight up-regulation of *styC* expression in B4-01, but over-expression of *styC* hardly exerted an effect on the transcriptional levels of *styA* and *styB* in strain B4-02. The results revealed the transcription of *styC* was probably regulated by *styAB*. As *styAB* was expressed at a high level, the consequent strong IMO activity caused a rapid accumulation of intermediate styrene oxides, further leading to an increase of *styC* transcription.

Indole Mono Oxygenase Activity Assay

According to the growth curves, cells in the logarithmic and stationary phases of indigo fermentation were selected for indole monooxygenase activity determination, respectively. As shown in Fig. 4, in accord with the expression of *styAB* genes, a similar changing pattern of monooxygenase activity was observed in all four strains. And the

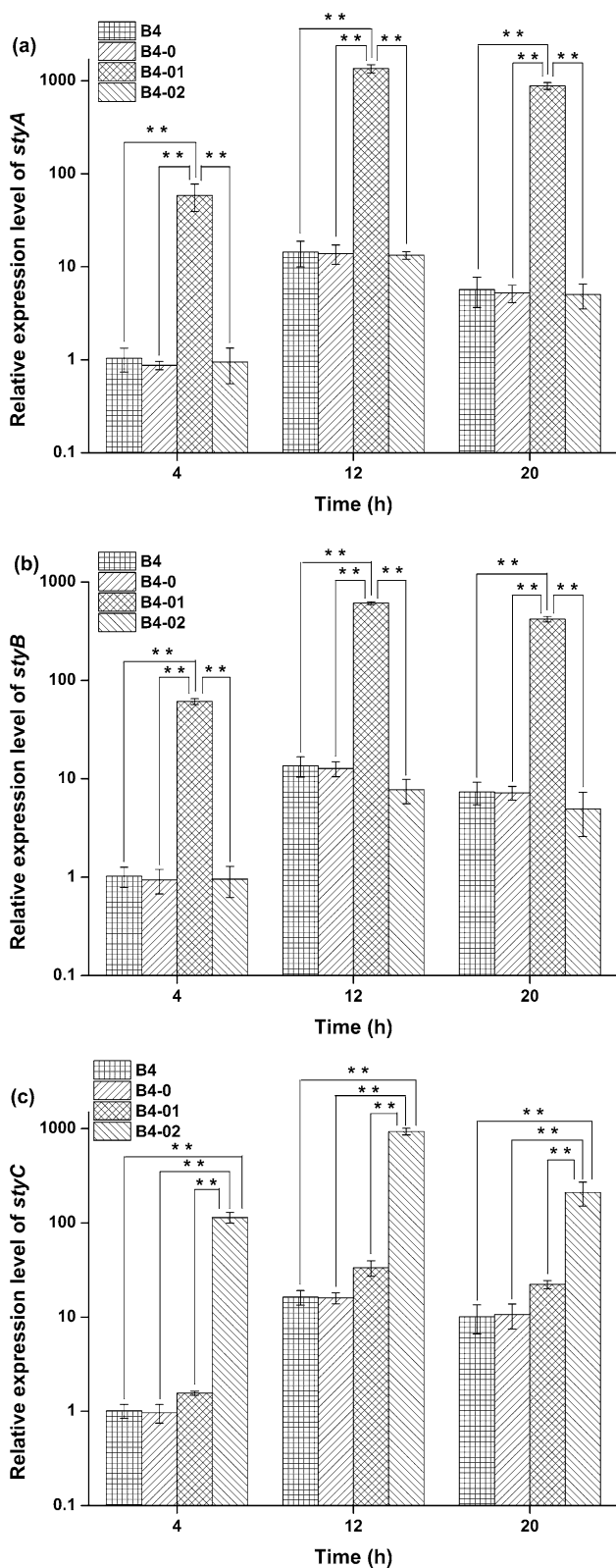


Fig. 3 RT-qPCR analysis of mRNA expressions of *styA*, *styB* and *styC* in B4, B4-0, B4-01 and B4-02 strains. **a** The expression level of *styA*. **b** The expression level of *styB*. **c** The expression level of *styC*. B4, wild-type strain; B4-0, recombinant strain harboring the empty vector pBBR1MCS; B4-01, recombinant strain harboring pBBR1MCS-2-*styAB*; B4-02, recombinant strain harboring pBBR1MCS-2-*styC*. ** $P < 0.01$

monooxygenase showed highest enzymatic activity when cell growth reached the stationary phase after 12 h of fermentation. Besides, it is notable that strain B4-01 expressed the highest activity of indole monooxygenase at every detected growth phase, more than fivefold higher compared to the wild-type strain B4, the control strain B4-0, and the recombinant strain B4-02. Meanwhile, no obvious difference of enzyme activity was found among the strains B4, B4-0, and B4-02, suggesting that neither introduction of the empty vector pBBR1MCS-2 nor over-expression of *styC* exerted an effect on indole monooxygenase expression. In all, the results demonstrated that over-expression of *styAB* via pBBR1MCS-2-*styAB* led to a significant enhancement of the indole monooxygenase activity in host cells.

Indigo Production in Recombinant Strains

To investigate the effect of the *styAB* and *styC* genes on indigo biosynthesis, the wild-type strain B4, the control strain B4-0, and the recombinant strains B4-01 and B4-02

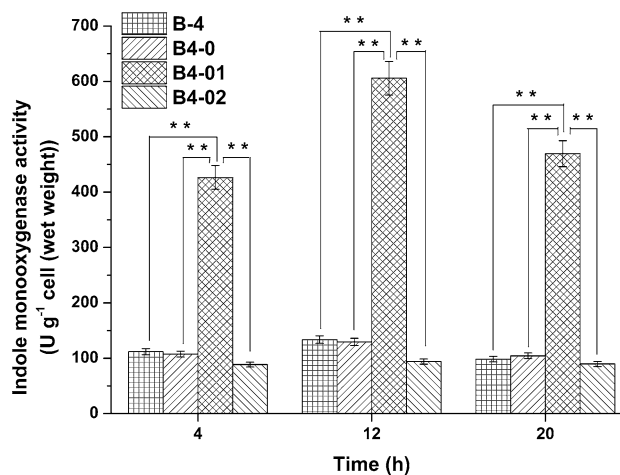


Fig. 4 Detection of indole monooxygenase (IMO) activity in B4, B4-0, B4-01, and B4-02 strains. B4, wild-type strain; B4-0, recombinant strain harboring the empty vector pBBR1MCS; B4-01, recombinant strain harboring pBBR1MCS-2-*styAB*; B4-02, recombinant strain harboring pBBR1MCS-2-*styC*. ** $P < 0.01$

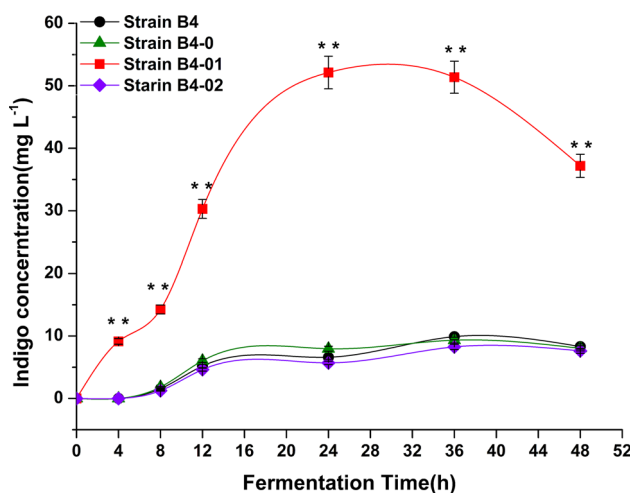


Fig. 5 Measurement of indigo production in B4, B4-0, B4-01, and B4-02 strains. B4, wild-type strain; B4-0, recombinant strain harboring the empty vector pBBR1MCS; B4-01, recombinant strain harboring pBBR1MCS-2-styAB; B4-02, recombinant strain harboring pBBR1MCS-2-styC. ** $P < 0.01$

were continuously cultivated in fermentation media for 48 h, respectively. Indigo detection results (Fig. 5) showed that indigo accumulated rapidly as cell proliferated at the beginning in B4-01, reached the peak around 30 h of fermentation and then declined slowly. 52.13 mg L⁻¹ of indigo was obtained in strain B4-01 after 24 h of fermentation, which was approximately sevenfold higher than that in strains B4, B4-0, and B4-02. In sharp contrast, the indigo yield in the recombinant strain B4-02 varied quite moderately and hardly exceeded 10 mg L⁻¹ during the whole fermentation procedure, showing no significant difference compared with the wild-type strain B4 and the control strain B4-0. Obviously, under the same fermentation condition, the strain B4-01 not only produced a remarkable indigo yield but also presented a sharply higher indigo synthesis rate, which definitely resulted from enhanced monooxygenase activity by over-expression of the *styAB* gene. Besides, the yield of indigo in the recombinant strain B4-01 was significantly increased comparing to previous studies on indigo production in *Pseudomonas*, which was about 23.60 mg L⁻¹ [11] and 3.54 mg L⁻¹ [2], respectively. Although the styrene oxide isomerase encoded by *styC* is in charge of catalyzing the intermediate indole oxide into indigo, it is demonstrated that over-expression of *styC* hardly exerted an influence on the synthesis rate and yield of indigo. Therefore, it concluded that the monooxygenase STYAB rather than the oxide isomerase STYC probably acted as the key rate-limiting enzyme in the indigo biosynthesis pathway in *P. putida* B4.

Previous work revealed that *styAB* and *styC* encoded styrene monooxygenase and oxide isomerase and played a key role in the degradation of styrene through oxidation

reaction. The present work demonstrated that the styrene monooxygenase STYAB can also catalyze oxidation of indole efficiently and consequently promoted indigo biosynthesis, revealing STYAB could be the key rate-limiting enzyme in the indigo biosynthesis pathway in *P. putida*. The reason probably due to that the substrate indole for indigo synthesis shares a highly similar molecular structure with styrene, which could be recognized correctly and catalyzed by monooxygenase.

Indole can also be oxidized by dioxygenase and converted into indigo and indirubin at the same time [5, 12]. However, the presence of indirubin mixed with indigo in the final metabolic products would certainly give rise to extra difficulty in the separation and purification of indigo. Therefore, the gene *styAB* encoding monooxygenase was selected for over-expression. The genetic engineering strain has exhibited strong indole monooxygenase activity and high synthesis rate of sole indigo compound, which provided a new strategy for the bio-indigo production enhancement in *Pseudomonas*.

Conclusions

In this work, the *styAB* and *styC* genes located in the styrene degradation gene cluster were cloned and successfully over-expressed in *P. putida* for indigo production investigation. Over-expression of *styAB* significantly enhanced indole monooxygenase activity and consequently led to sevenfold higher indigo yield in the recombinant strain. But *styC* over-expression hardly exerted an effect on indigo biosynthesis. Our study revealed that the styrene monooxygenase encoded by *styAB* gene probably was the key rate-limiting enzyme for indigo production in *P. putida*. Taken together, based on the results of present study, *styAB* was a vital gene in bio-indigo synthesis process, which could be one of the applications to the industrial strains and fermentation systems in the future.

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