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Article

Efficient Bioproduction of Indigo and Indirubin by Optimizing a Novel Terpenoid Cyclase XiaI in *Escherichia coli*

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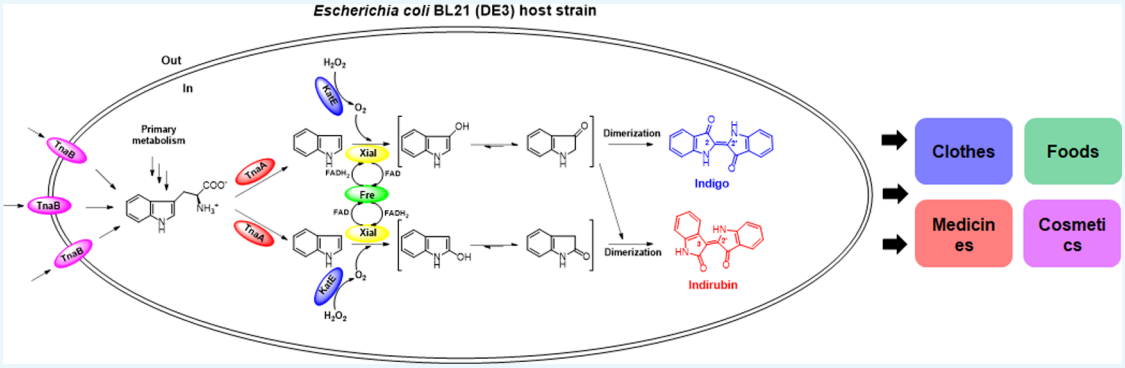

ABSTRACT: Blue indigo dye, an important natural colorant, is used for textiles and food additives worldwide, while another red isomer, indirubin, is the major active ingredient of a traditional Chinese medicine named "Danggui Longhui Wan" for treating various diseases including granulocytic leukemia, cancer, and Alzheimer's disease. In this work, we constructed a new and highly efficient indigoid production system by optimizing a novel terpenoid cyclase, XiaI, from the xiamycin biosynthetic pathway. Through introducing the flavin-reducing enzyme Fre, tryptophan-lysing and -importing enzymes TnaA and TnaB, and H_2O_2-degrading enzyme KatE and optimizing the fermentation parameters including temperature, the concentration of isopropyl-β-D-thiogalactopyranoside, and feeding of the L-tryptophan precursor, the final maximum productivity of indigoids by the recombinant strain *Escherichia coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) was apparently improved to 101.9 mg/L, an approximately 60-fold improvement to that of the starting strain *E. coli* BL21(DE3) (XiaI) (1.7 mg/L). In addition, when the fermentation system was enlarged to 1 L in the flask (feeding with 5 mM tryptophan and 10 mM 2-hydroxyindole), the indigoid productivity further increased to 276.7 mg/L at 48 h, including an indigo productivity of 26.0 mg/L and an indirubin productivity of 250.7 mg/L, which has been the highest productivity of indirubin so far. This work provided a basis for the commercial production of bio-indigo and the clinical drug indirubin in the future.

INTRODUCTION

Blue indigo dye is a well-known traditional pigment that has been used for textile dying since ancient times.[1] Currently, a huge amount of blue indigo is consumed every year for making clothes worldwide.[2] Indigo carmine, a synthetic indigo derivative, is also used as a colorant in the daily consumed foods, such as fruit juices, tomato ketchup, bread, dairy, and so on.[3,4] Compared to the blue indigo dye, another red isomer, indirubin, is known as a major ingredient of a traditional Chinese medicine named "Danggui Longhui Wan", which has been used for the treatment of chronic granulocytic leukemia for several decades.[5] Recently, indirubin was verified to be an effective inhibitor for two key targets, cyclin-dependent kinases and glycogen synthase kinase-3, under physiological conditions.[6−8] Therefore, indirubin or its derivatives were thought to be promising drugs against chronic granulocytic leukemia, cancer, and Alzheimer's disease.

Traditionally, indigo and indirubin are produced and extracted from several plant families including *Indigofera tinctoria*, *Lonchocarpus cyanescens*, *Polygonum tinctorium*, *Isatis tinctoria*, and so on.[9,10] However, their contents in these plants are not always very high,[9] and their production is always dependent on moderate-climate regions and seasons.[11] Currently, chemical methods for the synthesis of these

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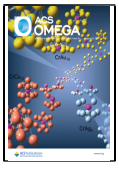

Table 1. Strains and Plasmids Used in This Study

strains/plasmids	characteristic	source
E. coli DH5α	used for plasmid construction and maintenance	Invitrogen
E. coli BL21(DE3)	protein expression host strain induced by IPTG	Invitrogen
E. coli BL21(DE3) (pETduet-1)	the plasmid pETduet-1 was transformed into *E. coli* BL21(DE3), used as a control	this study
E. coli BL21(DE3) (XiaI)	the plasmid P1 was transformed into *E. coli* BL21(DE3)	this study
E. coli BL21(DE3) (XiaI-Fre)	the plasmid P2 was transformed into *E. coli* BL21(DE3)	this study
E. coli BL21(DE3) (XiaI-Fre-TnaA)	the plasmid P3 was transformed into *E. coli* BL21(DE3)	this study
E. coli BL21(DE3) (XiaI-Fre-TnaB)	the plasmid P4 was transformed into *E. coli* BL21(DE3)	this study
E. coli BL21(DE3) (XiaI-Fre-TnaAB)	the plasmid P5 was transformed into *E. coli* BL21(DE3)	this study
E. coli BL21(DE3) (XiaI-Fre-TnaAB-KatE)	the plasmid P6 was transformed into *E. coli* BL21(DE3)	this study
pETduet-1	the IPTG-induced protein expression vector with two MCS; ampicillin resistance	Novagen
pACYCduet-1	the IPTG-induced protein expression vector with two MCS; chloramphenicol resistance	Novagen
P1	*xiaI* gene was cloned into the *BamH*I site of the plasmid pETduet-1	this study
P2	*fre* gene was cloned into the *Nde*I and *Kpn*I sites of the plasmid P1	this study
P3	*tnaA* gene was cloned into the *Nde*I and *Kpn*I sites of the plasmid pACYCduet-1	this study
P4	*tnaB* gene was cloned into the *Nde*I and *Kpn*I sites of the plasmid pACYCduet-1	this study
P5	*tnaA* and *tnaB* genes were simultaneously cloned into the *Nde*I and *Kpn*I sites of the plasmid pACYCduet-1	this study
P6	*katE* gene was cloned into the *Nco*I and *BamH*I sites of the plasmid P5	this study

Table 2. Primers Used in This Study

primers	characteristic (5′ to 3′)	source
fre-PF	cagatctcaattggatatcgATGACAACCTTAAGCTGTAAA	this study
fre-PR	tcagcgatcgcgtggccggcTCAGATAAATGCAAACGCATCG	this study
tnaA-PF	gttaagtataagaaggagatatacatATGGAAAACTTTAAACATC	this study
tnaA-PR	agcagcggtttctttaccagactcgagTTAAACTTCTTTCAGTTTTG	this study
tnaB-PF	gttaagtataagaaggagatatacatATGACTGATCAAGCTGAA	this study
tnaB-PR	agcggtttctttaccagactcgagTTAGCCAAATTTAGGTAAC	this study
tnaAB-PF	gttaagtataagaaggagatatacatATGGAAAACTTTAAACATC	this study
tnaAB-PR	agcggtttctttaccagactcgagTTAGCCAAATTTAGGTAAC	this study
katE-PF	gtttaactttaataaggagatataccATGTCGCAACATAACGAA	this study
katE-PR	cgccgagctcgaattcggatccTCAGGCAGGAATTTTGTC	this study

compounds, especially for indigo at the ton scale, have been established and have replaced the previous plant-based methods.[11] The production through chemical synthesis does not start from renewable plant tissues but instead relies on cheaper fossil chemicals, such as aniline. However, the chemical synthetic processes are known to be not environment-friendly, producing a lot of problems for late-stage treatment of dye wastes.[11] In addition, the production process involves high temperature, high pressure, and strong acids and alkalis, which is very strict, hazardous, and dangerous.[12]

Microbial synthesis of indigo and indirubin is a sustainable, green, and promising strategy, which neither depends on petrochemical precursors like the chemical synthesis does nor depends on seasons like the plant-based production does.[11] This method relies on the indole oxidative enzymes, which give microorganisms the powerful ability to produce indigo and indirubin. Currently, mining and discovering new indole oxidases having high catalytic efficiency are the key and limiting factor in promoting the large-scale bioproduction and application of indigo and indirubin in the future. Naphthalene dioxygenase from *Pseudomonas putida* PpG7 was first reported to be expressed in *Escherichia coli* to produce bio-indigo dye in 1983.[13] Subsequently, many types of indole oxidases have been discovered one after another, including non-heme iron oxygenases,[14,15] cytochrome P450 monooxygenases,[16−19] unspecific peroxidases,[20,21] and flavin-containing monooxygenases.[22−25]

Herein, we adopted a novel terpenoid cyclase XiaI from the xiamycin biosynthetic gene cluster in *Streptomyces* sp. SCSIO 02999 and carefully optimized an artificial biotransformation process of the tryptophan precursor to the final products blue indigo dye and indirubin, resulting in the efficient production of these two compounds compared to that with the starting recombinant strain *E. coli* BL21(DE3) (XiaI), especially producing the highest fermentation titers (250.7 mg/L) for the clinical drug indirubin so far. This work provided a basis for further metabolic engineering and commercial production of bio-indigo and high-value indirubin in the future.

MATERIAL AND METHODS

Chemicals. Indole, L-tryptophan, 2-hydroxyindole, indigo, indirubin, dimethyl sulfoxide (DMSO), and isopropyl-β-D-thiogalactopyranoside (IPTG) were bought from Aladdin (China) and Sigma-Aldrich (USA). Other reagents were bought from Sinopharm (China).

Bacterial Strains and Plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used for plasmid construction and maintenance. *E. coli* BL21(DE3) was used for protein expression and fermentation.

Medium and Culture Conditions. For gene cloning and routine plasmid transformation, the medium for *E. coli* was a Luria−Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L) containing appropriate antibiotics, such as 50 μg/mL ampicillin and 50 μg/mL chloramphenicol. For

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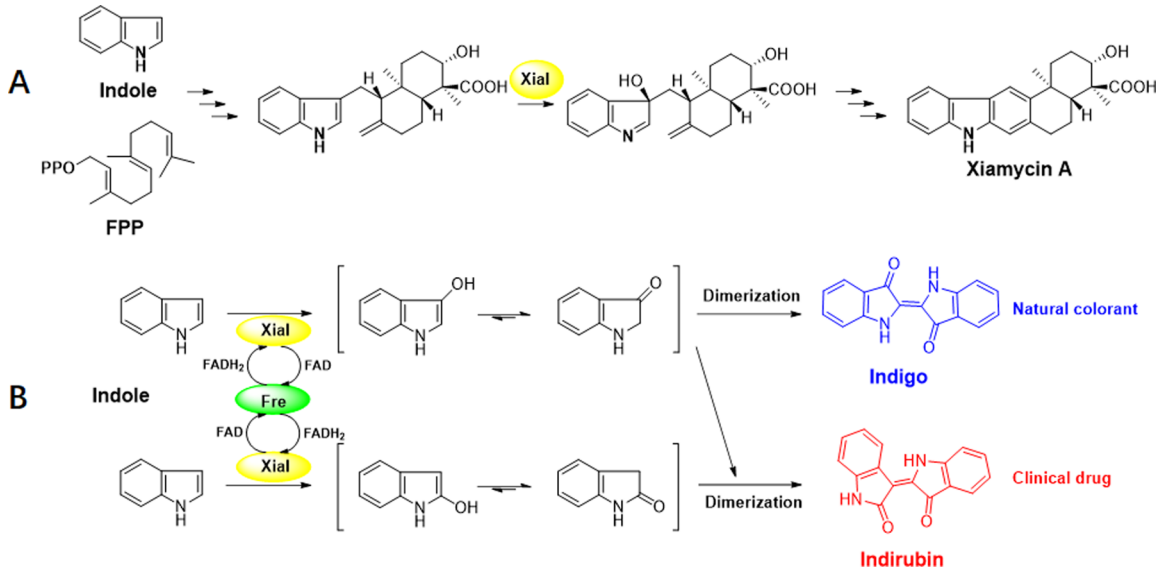

Figure 1. Functions of XiaI, a novel terpenoid cyclase, in the biosynthesis of xiamycin A previously (A) and the bioproduction of blue indigo dye and the clinical drug indirubin in this work (B).

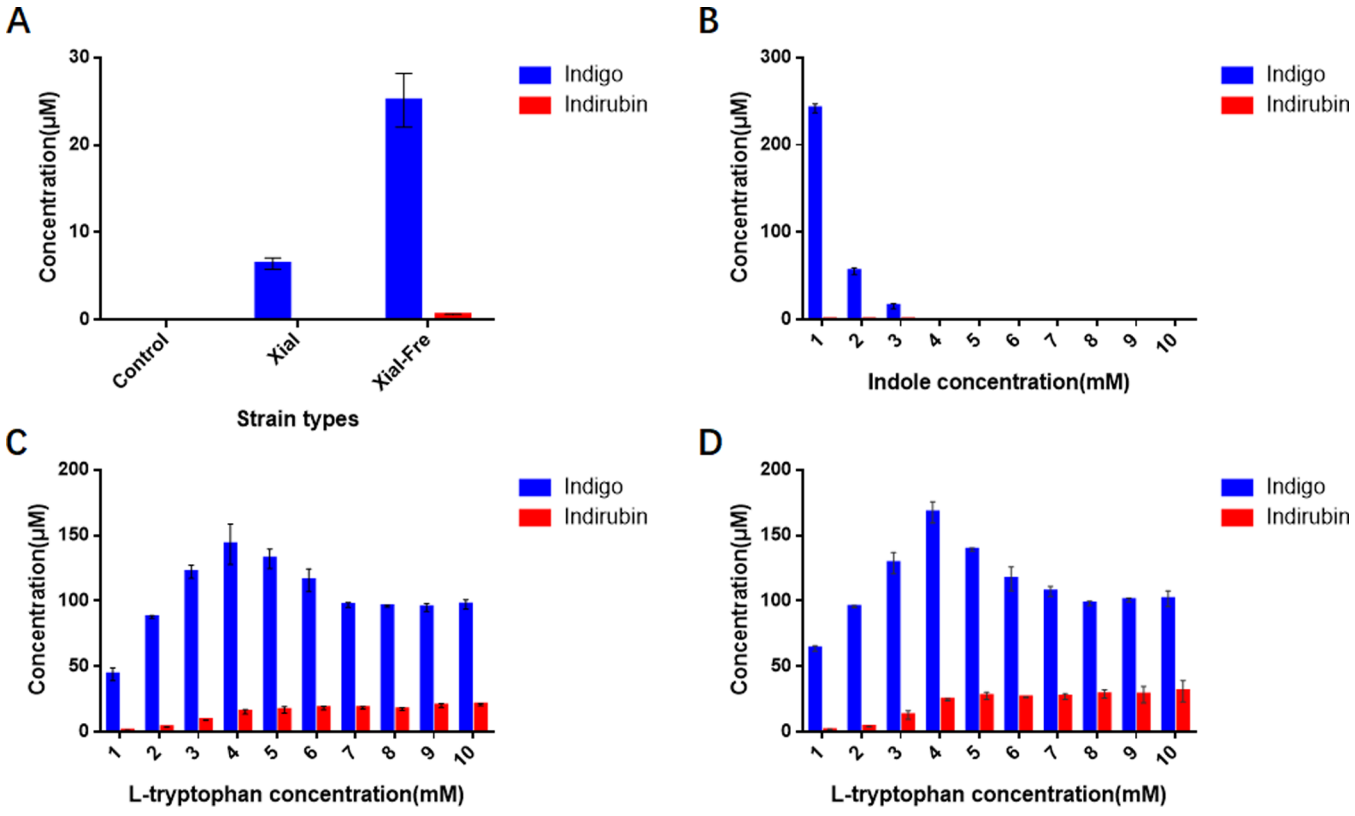

Figure 2. Optimizing the producing potential of XiaI by introducing the flavin-reducing enzyme Fre and feeding precursors. (A) Productivities of three recombinant strains: *E. coli* BL21(DE3) (pETduet-1) as a control, *E. coli* BL21(DE3) (XiaI), and *E. coli* BL21(DE3) (XiaI-Fre). (B) Productivity of *E. coli* BL21(DE3) (XiaI-Fre) fed with different concentrations of indole for 24 h. (C) Productivity of *E. coli* BL21(DE3) (XiaI-Fre) fed with different concentrations of L-tryptophan for 72 h. (D) Productivity of *E. coli* BL21(DE3) (XiaI-Fre) fed with different concentrations of L-tryptophan for 7 d.

test-tube fermentation, the *E. coli* strain was cultured in a 12 mL testtube with 3 mL of LB medium and incubated at 37 °C with shaking at 220 rpm until the OD_{600nm} reached 0.8 (detection at a nanodrop 2000). The temperature was changed to appropriate values such as 20, 25, and 30 °C, and at the same time, IPTG was added at 220 rpm for 24 h. For enlarged flask fermentation, the *E. coli* strain was cultured in a 2 L flask with 1 L of LB medium. 10 mL of the seed culture after incubation at 37 °C overnight was transferred into the flask and then incubated at 37 °C, 220 rpm until the OD_{600nm} reached 0.8 (detection at a nanodrop 2000). The temperature was changed to 25 °C, and at the same time, 0.02 mM IPTG

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was added at 220 rpm for 48 h. Three 200 μL aliquots of samples were collected at different times for quantification.

DNA Manipulation and Plasmid Construction. The primers used in this work are listed in Table 2. The gene *xiaI* was synthesized and ligated into the *BamH*I site of pETduet-1 by Sangon Biotech (China) according to the reported accession sequence AFK78075.1 in NCBI. The codon usage was optimized based on *E. coli*. The gene *fre* was cloned from the genome of *E. coli* BL21(DE3) by polymerase chain reaction (PCR) and ligated into the *Nde*I and *Kpn*I sites of plasmid *xiaI* + pETduet-1. The genes *tnaA*, *tnaB*, and co-transcription *tnaAB* were cloned from the genome of *E. coli* BL21(DE3) by PCR and respectively ligated into the *Nde*I and *Kpn*I sites of plasmid pACYCduet-1. The gene *katE* was cloned from the genome of *E. coli* BL21(DE3) by PCR and ligated into the *Nco*I and *BamH*I sites.

Quantification and Analysis of Indigoids in the Fermentation. The indigoids could not dissolve into the medium but were bound tightly with the strain debris. Therefore, the sample processing method is as follows. First, 200 μL of the culture was collected and centrifuged for 5 min at 12,000 rpm. Second, the supernatant was removed, but the strain debris was washed with 200 μL of DMSO using a vortex mixer. Then, centrifugation was performed at 12,000 rpm for another 5 min, and 20 μL of the supernatant was injected into a high-performance liquid chromatography (HPLC) system. The HPLC method was the same as that in our previous report.[36] The flow rate is 0.6 mL/min. A ZORBAX Eclipse XDB-C18 (250 × 4.6 mm, 5 μm, Agilent, USA) was used. The detection was carried out at 540 nm (for indirubin) and 610 nm (for indigo). The standard curves for indigo and indirubin were obtained by HPLC and are listed in Figure S1.

RESULTS AND DISCUSSION

Initial *In Vivo* Study of the Terpenoid Cyclase XiaI from the Xiamycin Biosynthetic Pathway. Xiamycins including xiamycin A and xiamycin B and indosespene are types of indolosesquiterpenes showing selective anti-HIV bioactivity, which were produced by several endophytic Streptomyces strains.[26,27] The biosynthetic routes of these bioactive compounds involved a special pentacyclic ring formation, which was catalyzed by XiaI, a flavin-dependent terpenoid cyclase, in 2012[28] (Figure 1A). Recently, another research group identified that a XiaI homologue named XiaF from another xiamycin-producing Streptomyces strain unexpectedly could catalyze the formation of indigo and indirubin.[29] Therefore, this work further investigates whether XiaI could be optimized to be an efficient catalyst in the production of these valuable indigoids (Figure 1B).

We got the *xiaI* gene by gene synthesis and then optimized it according to the codon usage of *E. coli*. In order to maximize the highly expressed target protein and further increase the yield of target metabolites, the optimized *xiaI* gene was cloned into pETduet-1, a high-copy-number plasmid in *E. coli*, used for protein expression. As expected, the strain *E. coli* BL21(DE3) (XiaI) apparently gained the ability to produce blue indigo in the liquid culture at 25 °C when 0.1 mM IPTG was added (Figures 2A and S2). However, the productivity was only 6.4 μM (about 1.7 mg/mL), which was very low. Previously, XiaI was *in vitro* biochemically verified as a flavin-dependent enzyme that needed $FADH_2$ for catalyzing the oxygenation reaction.[28,29] Therefore, we speculated that the low yield of blue indigo might be caused by the insufficient supply of reduced flavin in the recombinant strain *E. coli* BL21(DE3) (XiaI), although it contained a copy of the flavin-reductase-encoding gene *fre* in the genome. To accelerate the cofactor recycling by NAD(P)H: flavin oxidoreductase *in vivo*, the *fre* gene was cloned and ligated into another multiple cloning site (MCS) of the high-copy-number plasmids pETduet-1, which already contained the *xiaI* gene. The co-expression of *xiaI* and *fre* in the new strain *E. coli* BL21(DE3) (XiaI and Fre) apparently resulted in a higher productivity of blue indigo of about 25.1 μM, which was 3.9 times that of *E. coli* BL21(DE3) (XiaI) (Figures 2A and S2). At the same time, the production of indirubin of about 0.6 μM was also detected, which was proposed to be spontaneously formed by the dimerization of 3-hydroxyindole and 2-hydroxyindole (Figures 1B and 2A).

Because the direct precursor for XiaI was indole, we investigated whether adding this compound would further improve the titers of indigoids. Thus, different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM) of indole were fed into the fermentation broth of *E. coli* BL21(DE3) (XiaI and Fre) (Figure 2B). The results showed that indole at lower concentrations (1−2 mM) could efficiently increase the productivity. Especially, a high yield of about 241.9 μM was obtained at 1 mM indole. This result suggested that the producing potential of the XiaI-Fre system was limited by the insufficient supply of the substrate. However, we also found that adding high concentrations of indole would significantly inhibit the growth of bacteria, causing an apparent decrease in the production of indigoids (Figures 2B and S3A). In the previous report, indole was reported as a signaling molecule for regulating diverse microbial behaviors, such as bacterial motility, antibiotic resistance, and biofilm formation.[30] High concentrations of indole may reduce the electrochemical potential of the cytoplasmic membrane when it is transported inside the cell.[30]

Removing the Speed-Limiting Steps in the Conversion Process to Indigoids. Because indole showed toxicity to cell growth, we next tested another precursor L-tryptophan, which is a primary metabolite and a protein building block. Several decades of development in the areas of metabolic engineering have manufactured a lot of tryptophan-hyper-producing strains, resulting in tryptophan being an easily obtained and very cheap renewable material.[31,32] Tryptophan in the medium could be imported into the cell by several transporters including TnaB, AroP, and Mtr and then converted into indole, pyruvate, and ammonia by the PLP-dependent lyase TnaA.[30] To test the effect of tryptophan on the productivity of indigoids, different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM) were fed along with the fermentation of *E. coli* BL21(DE3) (XiaI-Fre) (Figure 2C). The results showed that the highest yield of about 143.3 μM was obtained at 4 mM tryptophan for 72 h. However, even if the fermentation time was extended to 7 d, the final yields had not been significantly improved (Figure 2D). Furthermore, in the case of feeding high concentrations of tryptophan precursors (7−10 mM), a large amount of the tryptophan precursors was not converted into products but remained in the fermentation broth (Figure S4). However, tryptophan at high concentrations did not affect the cell growth (Figure S3B). Consequently, we speculated that the importation of tryptophan and then conversion into the direct precursor indole might be the new speed-limiting steps in producing indigoids.

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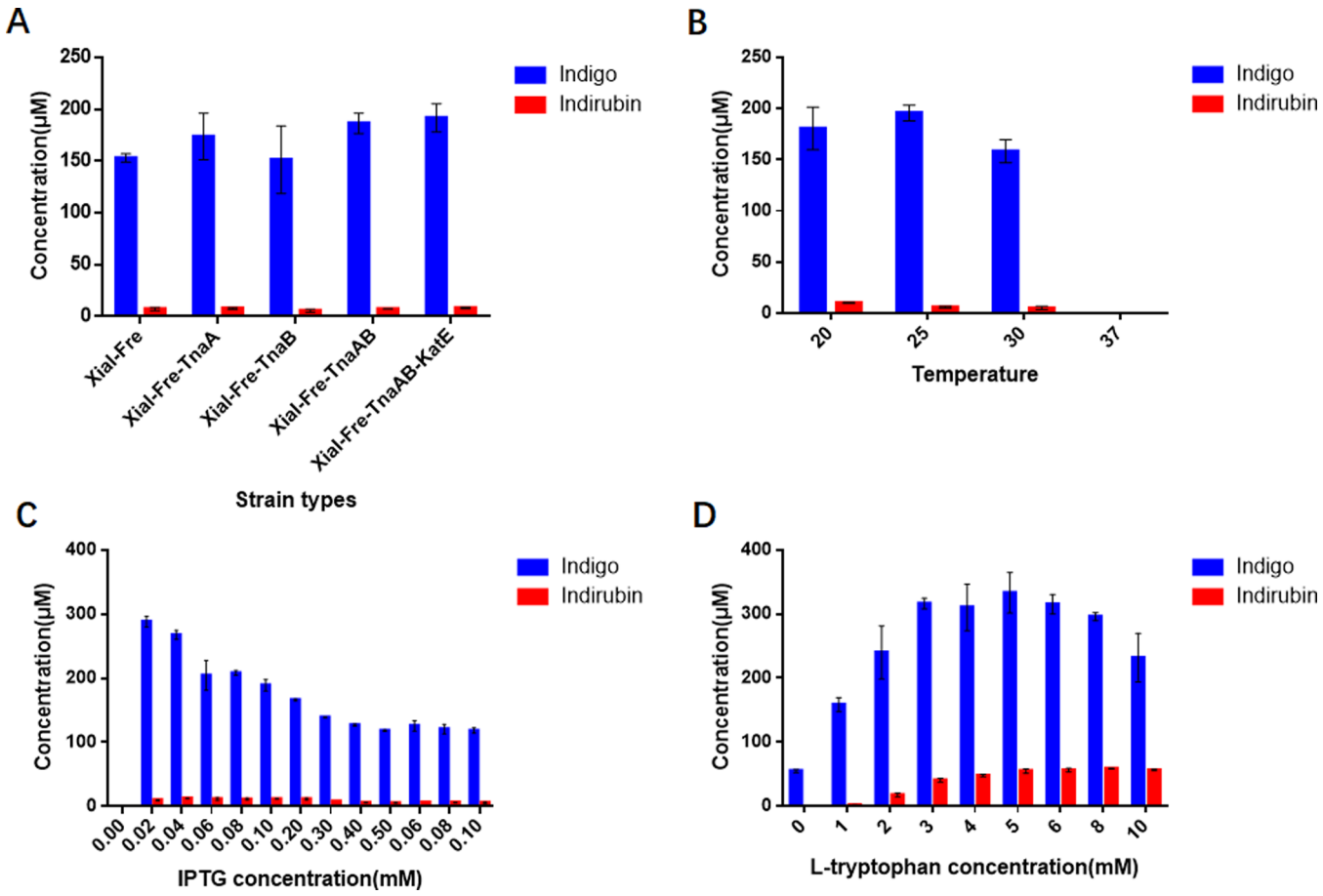

Figure 3. Optimizing the producing potential of the XiaI-Fre system. (A) Productivities of strains *E. coli* BL21(DE3) (XiaI-Fre-TnaA), *E. coli* BL21(DE3) (XiaI-Fre-TnaB), *E. coli* BL21(DE3) (XiaI-Fre-TnaAB), and *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) compared with that of the basic strain *E. coli* BL21(DE3) (XiaI-Fre). (B) Optimizing the temperatures of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE). (C) Optimizing the IPTG concentrations of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE). (D) Productivity of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) fed with different concentrations of L-tryptophan for 48 h.

Next, we constructed three versions of modules to increase the transportation of tryptophan and supply of the direct precursor, indole. The *tnaA* gene, *tnaB* gene, and co-transcription *tnaAB* gene from *E. coli* were, respectively, cloned into pACYCduet-1, a plasmid with a low copy number, and then co-expressed with the XiaI-Fre system in *E. coli* BL21(DE3). The reason for choosing a low-copy-number plasmid is to prevent the negative effect on the expression of genes for indigoid biosynthesis. The following fermentation process was carried out with the addition of a 2 mM tryptophan precursor. Detected results showed that the overexpression of the *tnaA* gene would apparently increase the production (approximately 173.8 μM) of indigo (Figure 3A). This indicated that the expression of endogenous tryptophan lyase TnaA is insufficient, causing the step of tryptophan to indole to be limiting. Although the expression of tryptophan-importing transport protein TnaB alone did not significantly promote the production (approximately 151.6 μM) of indigo, the co-expression of TnaA and TnaB further increased the titers (approximately 186.6 μM) of indigo, suggesting that the transportation step of tryptophan was also important (Figure 3A).

Dioxygen (O_2) was also a key substrate for the recycling of the XiaI-Fre system, and therefore, the supply of dioxygen in the cell should also be important for the titer improvement of indigoids. There should be two main routes for the supply of oxygen to the cells. One is oxygen from the air that is dissolved into the fermentation broth and then imported into the cells and another may be hydrogen peroxide (H_2O_2) produced from the central metabolism of the cell. H_2O_2 was decomposed by the catalase such as KatE or KatG to produce dioxygen.[33] In addition, excessive accumulation of hydrogen peroxide would increase the oxidative pressure inside the cell, which is detrimental to cell growth and the normal metabolism, especially at the late stage of fermentation.[34,35] Therefore, to know whether overexpression of the catalase would further improve the production of indigoids in this work, gene *katE* was cloned and ligated into another MCS of pACYCduet-1 together with *tnaAB*. The production of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) was approximately 191.9 μM (for indigo), slightly higher than that of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB) (Figure 3A).

Further Optimizing the Fermentation Conditions. To further increase the productivity of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE), the fermentation conditions including temperature and the concentration of IPTG were optimized. Temperature is one of the most critical factors which not only affects the growth rate of bacteria but also affects the correct folding of targeted proteins.[36] In this study, four temperatures (20, 25, 30, and 37 °C) were used (Figure 3B). Higher temperatures increased the growth of bacteria, resulting in a higher biomass (Figure S5A). However, the detection

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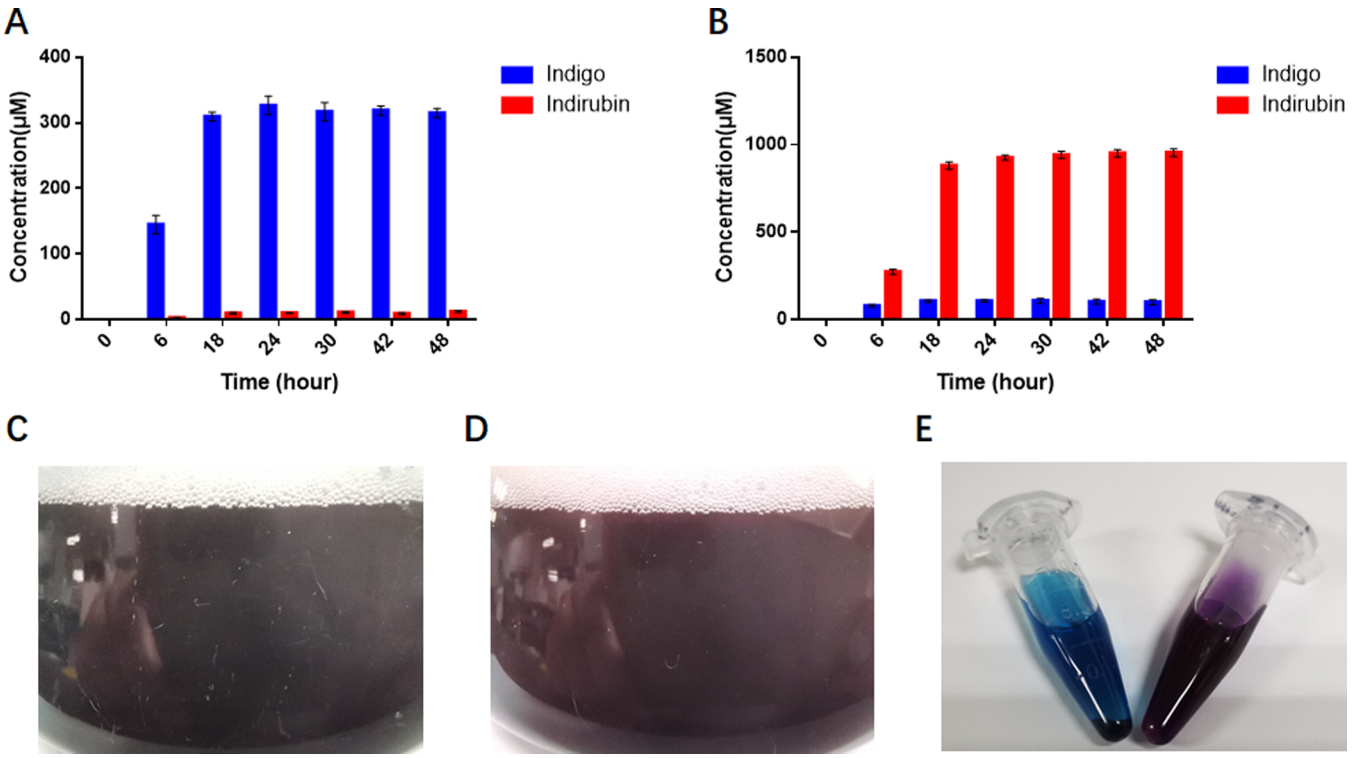

Figure 4. Productivities of blue indigo dye and the clinical drug indirubin by strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) in an enlarged fermentation system. (A) Yields of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) fed with 5 mM tryptophan in 1 L of LB medium across 48 h. (B) Yields of *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) fed with 5 mM tryptophan together with 10 mM 2-hydroxyindole in 1 L of LB medium across 48 h. (C) Phenotype of the culture in Figure 4A at 48 h. (D) Phenotype of the culture in Figure 4B at 48 h. (E) Phenotypes of products extracted by DMSO in 4C (left) and 4D (right).

indicated that a high temperature of 37 °C was not conductive to the production (0 μM) of indigo and indirubin, while a lower temperature of 25 °C was the most helpful, and the yield was about 195.6 μM for indigo, which was consistent with the above results. At the temperature of 25 °C, the concentration of IPTG was further optimized. The amount of IPTG used affects the expression speed of the target protein, and high concentrations of IPTG inhibit the growth of bacteria.[36] Thirteen different concentrations (0.00, 0.02, 0.04, 0.06, 0.08, 0.10, 0.20, 0.40, 0.50, 0.60, 0.80, and 1.00 mM) of IPTG were used in this study. Although the biomass samples were similar, the fermentation results showed that higher concentrations of IPTG obviously affect the indigoid production (Figures 3C and S5B). We speculated that at high concentrations of IPTG, the expressions of the five proteins, XiaI, Fre, TnaA, TnaB, and KatE, may be not at a good balance. The lower concentration (0.02 mM) was the most helpful for the productivity (293.3 μM for indigo).

Based on the above optimized fermentation conditions for the recombinant strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE), we investigated whether once again improving the supply of tryptophan would increase the titers of indigoids. Nine concentrations (0, 1, 2, 3, 4, 5, 6, 8, and 10 mM) of tryptophan were fed along with the fermentation process (Figure 3D). As expected, the productivity was effectively improved to a high level. The highest yields including 333.2 μM indigo and 54.7 μM indirubin were obtained at 5 mM tryptophan.

Scaling Up the Fermentation System. As mentioned above, to facilitate the optimization of fermentation conditions, all the experiments were carried out in a 12 mL tube and the fermentation system volume was only 3 mL. Thus, in order to further explore the fermentation performance of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) during large-volume fermentation, this study expanded the fermentation system to the scale of 1 L in a 2 L flask. The fermentation was first carried out at 37 °C until the OD_{600nm} reached 0.8, and then, the temperature was changed to 25 °C. At the same time, 0.02 mM IPTG and 5 mM tryptophan were fed into the fermentation broth. The fermentation process time was 48 h. We detected that at 24 h, the production of indigo reached the highest value, about 326.6 μM indigo (Figure 4A,C,E). With the extension of the fermentation time, the production of indigo declined slightly (314.8 μM at 48 h). However, only a small amount of indirubin was produced (about 12.2 μM at 48 h) (Figures 4A and S6). Thus, the productivity of indigoids in the 1 L flask system was approximate with that in the 3 mL tube system (Figures 4A, 3D, and S7).

At present, indirubin is a high-value-added first-line drug, and its price on the official website of Sigma-Aldrich is 423 USD/25 mg. The production of indirubin is proposed as the spontaneous condensation of 3-hydroxyindole and 2-hydroxyindole.[37] In order to explore the ability of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) to produce indirubin, this study fed 10 mM 2-hydroxylindole based on the above study of indigo production (Figure 4B,D,E). To our surprise, the highest total titers of indigoids were greatly increased to 1053.6 μM (about 276.7 mg/L) at 48 h, including an indigo productivity of 26.0 mg/L and an indirubin productivity of 250.7 mg/L, which has been the highest productivity of indirubin so far.[24,37,38] Due to the addition of 10 mM 2-hydroxyindole, the total productivity (1053.6 μM) of *E. coli*

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BL21(DE3) (XiaI-Fre-TnaAB-KatE) was about 3.2 times higher (327.0 μM) compared to that when 2-hydroxyindole was not added (Figures S6 and S7). We speculated that the 3-hydroxyindole produced by the XiaI-Fre system may be unstable or toxic to the cell *in vivo* and its production could be speeded up by quick condensation with the added 2-hydroxyindole to form more stable and nontoxic indirubin. Thus, based on this interesting discovery, we will explore other ways to further increase the production of indirubin in the future.

In summary, a novel flavin-dependent terpenoid cyclase, XiaI, was deeply studied *in vivo*, and a new and highly efficient indigoid bioproduction system was artificially constructed and optimized in this work. The final maximum productivity of indigoids by the recombinant strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) was apparently improved to 101.9 mg/L, an approximately 60-fold improvement to that of the starting strain *E. coli* BL21(DE3) (XiaI) (1.7 mg/L). When the fermentation system was enlarged to 1 L by feeding with 5 mM tryptophan and 10 mM 2-hydroxyindole, the indigoid productivity further reached 276.7 mg/L at 48 h, including an indigo productivity of 26.0 mg/L and an indirubin productivity of 250.7 mg/L, which has been the highest productivity of indirubin so far. This work provided a basis for the commercial production of bio-indigo and the high-value-added clinical drug indirubin in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02679.

Data and standard curves for indigo and indirubin; SDS-PAGE for the detection of XiaI and Fre; biomass of strain *E. coli* BL21(DE3) (XiaI-Fre) with different concentrations of indole and L-tryptophan; consumption of L-tryptophan by strain *E. coli* BL21(DE3) (XiaI-Fre) fed with different concentrations of L-tryptophan; biomass of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) at different temperatures and different concentrations of IPTG; detection at 540 nm for the products of strain *E. coli* BL21(DE3) (XiaI-Fre-TnaAB-KatE) with or without the feeding 2-hydroxyindole; and phenotypes of the enlarged fermentation system with or without feeding 2-hydroxyindole (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONSPARA

CDKs	cyclin-dependent kinases
CDW	Cell dry weight
DMSO	dimethyl sulfoxide
GSK-3	glycogen synthase kinase-3
HPLC	high-performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria−Bertani
PCR	polymerase chain reaction
MCS	multiple clone sites
SDS−PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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