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Co-expression of Prefoldin From Hyperthermophilic Archaea *Pyrococcus furiosus* in *Escherichia coli* Enhances The Catalytic Efficiency of Modified Cytochrome P450 BM3*

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Abstract P450 BM3 is derived from *Bacillus megaterium*, and its mutant (A74G, F87V, L188Q, D168H) could hydroxylate indole to indigo in *Escherichia coli* whereas the bioconversion yield is low under normal culture conditions (37℃, 250 r/min). Chaperones from hyperthermophilic archaea *Pyrococcus furiosus* were co-expressed with P450 BM3 mutant in *E. coli* to investigate whether they could improve the production of indigo, and results indicated that prefoldin could significantly increase the indigo production. Our research also revealed that enzyme activity was not the restrictive factor in the indigo bioconversion process. We also demonstrated that prefoldin could increase the intracellular nicotinamide adenine dinucleotide phosphate (NADPH)/$NADP^+$ ratio, and this ratio was probably related to the increase of the indigo bioconversion yield.

Key words cytochrome P450 BM3, *Escherichia coli*, indigo, prefoldin, *Pyrococcus furiosus*
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1 Introduction

Indigo is one of the oldest dyes still in use in the textile industry [1]. Increasingly, indigo and its derivatives are used for the treatment of bacterial infection and cancers [2], such as chronic myeloid leukemia [3]. Traditionally, indigo is extracted from plants, but this process is laborious and yields very low quantities[4]. Chemical synthesis of indigo has started at 1897, and gradually replaced the traditional method[5], but the materials used for these chemical reactions can have harmful health effects, while the wastewaters produced during this process can lead to severe environmental pollution. Therefore, bioconversion method for indigo synthesis represents a promising green process, which eliminates the side effects of the chemical production.

Cytochrome P450s catalyze the oxidation of a broad array of endogenous and exogenous organic substrates and exist in almost all organisms except *Escherichia coli* [6]. P450 BM3 was isolated from *Bacillus megaterium*, and its heme-domain is fused with a reductase domain, which allows an efficient electron transfer and a high catalytic turnover. Additionally, highly soluble P450 BM3 recombinant

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proteins, expressed in *E. coli*, require nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen only to function[7–8], and they have been widely used in mutagenesis studies. Li *et al*.[9] demonstrated that P450 BM3 molecules with mutations at A74G, F87V, D168H, and L188Q exhibit higher indole-hydroxylating activity and higher regioselectivity, which results in an increased indigo production.

Chaperone system found in hyperthermophilic archaea is simpler than in other organisms, which lacks of the large molecular mass chaperones such as heat shock protein (HSP)100, HSP90, and HSP70 that are thought to be indispensable in all other organisms[10–11]. *Pyrococcus furiosus* is a hyperthermophilic archaeon inhabiting extreme environments, which contains three main chaperones: prefoldin, hsp60, and small heat shock protein (shsp)[10]. Prefoldin exists in eukaryotes and archaea, but not in bacteria[12], and the archaeal prefoldin is a hexameric complex composed of two α-subunits and four β-subunits, which is able to prevent the aggregation of diverse heterogeneous proteins, such as lysozyme, citrate synthase, and rhodanese[13–15]. Zako *et al*.[16] demonstrated that prefoldin from hyperthermophilic archaea *P. horikoshii* can induce the refolding of denatured lysozyme. Overexpression of the prefoldin gene from *P. horikoshii* OT3 endowed *E. coli* with tolerance to organic solvents[17]. And Chen *et al*.[18] reported that over-expression of prefoldin from *P. furiosus* not only increased the survival rate of the *E. coli* cells in 50℃, but also rendered *E. coli* cells to keep on growth under the elevated temperature.

Here, we performed site-directed mutagenesis to obtain a P450 BM3 mutant (A74G, F87V, D168H, L188Q, abbreviated as P450 BM3-M), and to examine the efficiency of chaperones isolated from *P. furiosus* in the production of indigo, we co-expressed recombinant plasmids encoding *P. furiosus* chaperones with P450 BM3-M in *E. coli* cells. We also performed preliminary research on how hyperthermophilic chaperones increased the bioconversion of indigo.

2 Materials and methods

2.1 Chemicals, plasmid construction, and bacterial strains

Indole was purchased from Aladdin Chemistry (Shanghai, China). NADPH was purchased from Yeasen Co., Ltd (Shanghai, China), and DMSO from Genebase Gene-Tech Co., Ltd (Shanghai, China). Recombinant plasmids encoding *P. furiosus* chaperone genes were prepared as described previously[19]. *E. coli* DH5α and BL21(DE3) strains were used for plasmid preparation and protein expression, respectively.

2.2 Construction of recombinant plasmid encoding P450 BM3

Primers used to amplify P450 BM3 were as follows: sense, 5′ GGGGATCCATGACAATTAA-AGAAATGCCTCAG 3′ (*Bam*HⅠ restriction site, underlined); antisense, 5′ CTTGAATTCTTACCCA-GCCCACACGTCTTTTGCG 3′ (*Eco*RⅠ underlined). The indicated restriction enzymes were use for the double digestion of the amplified PCR fragments and pT7473 plasmids, which were then ligated by T4 ligase. DNA sequencing confirmed that the desired construct was obtained, and *E. coli* BL21(DE3) strains were used to express the recombinant proteins.

2.3 Multi site-directed mutagenesis

Multi site-directed mutagenesis (F87V, D168H, and L188Q sites) was performed using Agilent Quikchange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's instructions. Primers used for site-directed mutation were as follows: F87V, 5′ GCGTCCAGCTTGTAACTAACCCGTCTCCTG-CA 3′; D168H, 5′ AATAAATGGATGAGGCTGA-TGTCGGTAAAAGCTGTTAAAGC 3′; L188Q, 5′ CTGGATTTGCTCGCTGCTGCTTGTTCATTGC-TTCA 3′. Single site-directed mutagenesis was performed at A74G site, and the primers used were: sense, 5′ CTTAAGTCAAGGGCTTAAATTTGTAC-GTGATTTTGCAGGAG 3′, antisense, 5′ CAAATT-TAAGCCCTTGACTTAAGTTTTTATCAAAGCGT-GATTC 3′. The sequence of the obtained construct was confirmed by DNA sequencing and, afterward, *E. coli* BL21(DE3) cells were transformed using this plasmid.

2.4 Protein expression

Overnight *E. coli* cell culture expressing P450 BM3-M plasmids was inoculated at 1% into 50 ml of Luria-Bertani (LB) medium supplemented with the antibiotic required for plasmid maintenance. When the absorbance A_{600} of the culture reached 0.6–0.8, 1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration) was used to induce the expression of P450 BM3-M.

2.5 Strain cultivation

Overnight cultures of strains harboring recombinant plasmids were diluted 100-fold and

inoculated into fresh LB medium supplemented with the appropriate antibiotics. Cells were grown at 37℃ with shaking at 250 r/min. When A_{600} of the cell cultures reached 0.6–0.8, 1 mmol/L IPTG was applied for 3 h, in order to induce the expression of recombinant proteins. Following the induction, 0.5 mmol/L indole (final concentration) was added to the medium, for indigo production, and samples were obtained at 1 h, 2 h, 3 h, 4 h, and 18 h for indigo production determination. All experiments were repeated three times independently.

2.6 Cell sonification

Cell incubated with IPTG for 3 h were harvested and centrifuged at 3 000 *g* for 30 min. Afterward, they were resuspended in a lysis buffer (50 mmol/L phosphate-buffered saline (PBS) pH 7.4, 0.1 mmol/L PMSF, 1 mmol/L EDTA), and sonification was performed on ice with the following protocol: 2 s on, 2 s off; 99 cycles, 200 W. Debris and unbroken cells were removed by centrifugation at 12 800 *g* for 30 min at 4℃, and the supernatants were filtered through 0.22 μm membrane, and used in the subsequent experiments.

2.7 Determination of indigo concentration

Cells were harvested by centrifugation at 9 000 *g* at 4℃ for 10 min. Appropriate DMSO volumes were added to cell pellets to extract indigo, and the absorbance at 610 nm was determined and indigo yield was estimated.

2.8 P450 spectral assay and *in vitro* enzymatic activity assay

P450 BM3-M enzymes in the filtered supernatants were identified by a distinct absorption band at 450 nm, from the CO-bound ferrous form of the heme protein. Total P450 content was determined based on the method of Guengerich *et al*.[20].

In vitro enzymatic activity assay was performed using 0.5 mmol/L indole dissolved in DMSO, PBS buffer (pH 7.4, 20 mmol/L), and supernatant aliquots with a final volume of 2.5 ml. Following the pre-incubation for 3 min at 37℃, the reaction was initiated by the addition of 0.2 mmol/L NADPH. Samples were obtained every 3 min, and indigo production was determined from the absorbance at 610 nm.

2.9 Determination of intracellular NADPH/ $NADP^+$ ratio

Intracellular NADPH and $NADP^+$ concentrations were determined using EnzyChrom $NADP^+$/NADPH kit (BioAssay System) according to the manufacturer's instructions, with minor modifications: cells were resuspended in 100 μl extraction buffer and sonicated for 10 s on ice before heat extraction.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Total supernatant and pellet proteins were collected after the sonification. SDS-PAGE was performed using 4% (*w*/*v*) stacking and 10% (*w*/*v*) separating gels in a vertical slab gel apparatus. Subsequently, gels were stained with 0.05% Coomassie Brilliant Blue R-250 for 4 h and destained in solution containing acetic acid, methanol, and water (1:3:6, *v*/*v*/*v*).

3 Results and discussion

3.1 P450 BM3 gene expression

P450 BM3 mutant P450 BM3-M was cloned into pT7473 plasmid and expressed in *E. coli* BL21(DE3) cells. 1 mmol/L IPTG was used to induce the expression of P450 BM3-M and then the indigo bioconversion yield was measured after the addition of indole (0 h, 1 h, 2 h, 3 h, 4 h, and 18 h). Results of Figure 1a showed that the recombinant P450 BM3-M was around 119.0 ku, with relatively less fraction expressed as soluble protein. The indigo final bioconversion yield and the productivity of the recombinant strains were both low, with the final indigo production lower than 40 μmol/L after 18 h cultivation(Figure 1b). As shown in Figure 1a, although P450 BM3-M performed soluble expression to some extent, most of them were expressed as insoluble inclusion bodies. We assumed that the low soluble expression of P450 BM3 mutant and the limited enzymatic activity might be the reason for the low indigo bioconversion. Based on our previous study, chaperones from *P. furiosus* (especially prefoldin) could significantly improve the soluble expression level and enzymatic activity of exogenous protein expressed in *E. coli* [19]. We next tried to co-express *P. furiosus* chaperones (alone or in pairs) with P450 BM3-M in *E. coli* to see whether hyperthermophilic chaperones could improve the soluble expression and the enzymatic activity of cytochrome P450 BM3 mutant, and finally the indigo production as well.

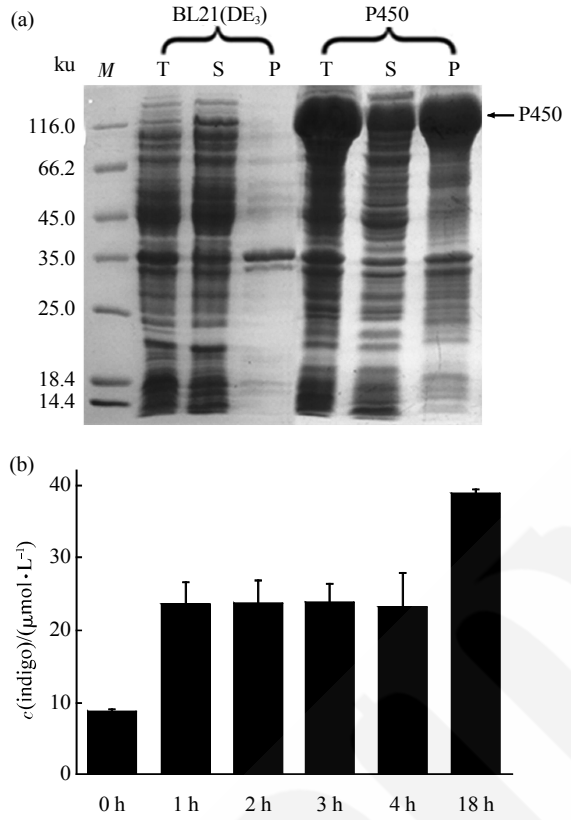

Fig. 1 The expression of P450 BM3-M in E. coli

(a) SDS-PAGE analysis of P450 BM3-M expression. *M*: Protein molecular mass marker; P450: *E. coli* cells overexpressing P450 BM3-M protein (designated as P450); *T*: Total cells, *S*: Supernatant, *P*: Pellet. (b) Indigo yield of P450 strains after the addition of indole for different time: 0 h, 1 h, 2 h, 3 h, 4 h and 18 h. The values are representative results obtained from at least three independent experiments and data are given as mean ± SEM.

3.2 Effects of chaperones from P. furiosus on the indigo production by bioconversion

We cloned the coding genes of chaperones from *P. furiosus* (alone or in pairs) into pACYCDuet-1 plasmid and transferred them with pT7473-P450 BM3-M plasmid into *E. coli* BL21(DE3). Strains with pT7473-P450 BM3-M plasmid (designated as P450) and strain with both empty pACYCDuet-1 and pT7473-P450 BM3-M plasmids (designated as pACYC + P450) were used as controls. Indigo concentrations in all tested strains at different treatment time (0 h, 1 h, 2 h, 3 h, 4 h, and 18 h) were determined. As indicated in Figure 2, co-expression of prefoldin from *P. furiosus* significantly increased the P450-catalyzed indigo production (pACYA-prefoldin+ P450, pACYA-prefoldin-hsp60 +P450, and pACYA-prefoldin-shsp +P450). Hyperthermophilic chaperones hsp60 and shsp could improve indigo yield to some extent, but not as effective as prefoldin. Besides, co-expression of prefoldin greatly enhanced the recombinant *E. coli* bioconversion rate from indole to indigo. As shown in Figure 2, the indigo yield in pACYA-prefoldin +P450 strain quickly reached to ~150 μmol/L after the addition of indole for 1 h, which was nearly 15-fold higher than the control. Taken together, these results indicated that co-expression of prefoldin from *P. furiosus* could effectively improve both the final indigo yield and the productivity of the recombinant *E. coli*.

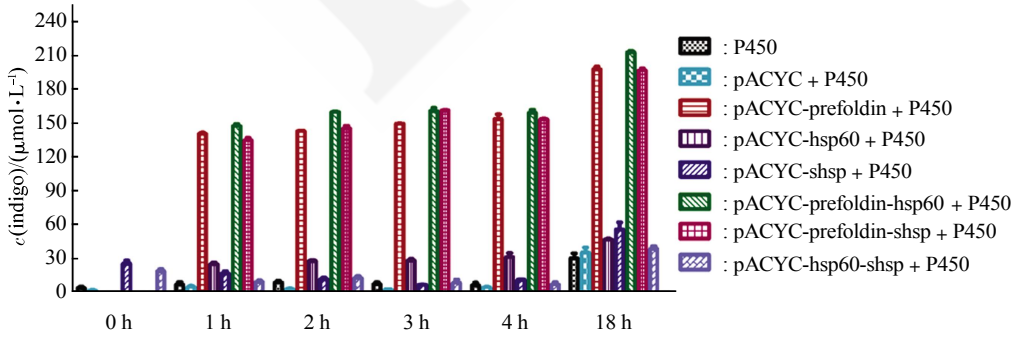

Fig. 2 Prefoldin originating from Pyrococcus furiosus increased the indigo yield produced by bioconversion

Indigo production of strains overexpressing *P. furiosus* chaperones. Strains overexpressing pT7473-P450 BM3-M (P450 strain) and strains harboring empty pACYCDuet-1 and pT7473-P450 BM3-M plasmids (pACYC + P450 strain) were used as controls. The values are representative results obtained from at least three independent experiments and data are given as mean ± SEM.

3.3 Effect of hyperthermophilic prefoldin on P450 BM3-M enzyme levels and activity

The above results revealed that prefoldin could significantly increase the yield of indole bioconversion to indigo, we guessed that this would be because the presence of prefoldin enhanced the soluble expression

of P450 BM3-M and thereby increased its enzyme activity. SDS-PAGE analysis indicated that prefoldin is co-expressed with P450 BM3-M in *E. coli* (pACYA-prefoldin +P450) (Figure 3a). Inconsistent with our expectation, co-expression of prefoldin cannot improve the soluble expression level of recombinant P450 BM3-M (Figure 3a), we then measured the enzyme content and *in vitro* enzymatic activity of P450 BM3-M. The enzyme content was measured by CO-spectral assay. The enzyme content in pACYC-prefoldin+P450 strains was not higher (and even lower) than that in P450 strain (Figure 3b). Additionally, *in vitro* enzyme activity analysis result indicated that pACYC-prefoldin+P450 strain exhibited lower enzymatic activity than P450 strain as well (Figure 3b). Taken together, these results indicated that co-expression of prefoldin could not increase the *in vivo* P450 BM3-M enzyme levels or activity, and P450 BM3-M enzyme activity was not the restrictive factor in the indigo production through bioconversion.

3.4 Effect of prefoldin from P. furiosus on the intracellular NADPH/NADP$^+$ ratio

P450 BM3 is a self-sufficient enzyme containing both the heme and a diflavin reductase domain, and it accepts electrons directly from NADPH[6]. Because of the importance of NADPH in the bioconversion process, we tried to determine whether prefoldin expression could influence the *in vivo* NADPH level in *E. coli*. We analyzed the intracellular NADPH and NADP$^+$ in *E. coli* expressing P450 BM3-M with or without co-expression of prefoldin(pACYC-prefoldin+ P450 or P450). As shown in Table 1, after IPTG induction for 3 h, the intracellular NADPH/NADP$^+$ ratio measured in pACYC-prefoldin +P450 was 2.86 times more than that in the P450 cells, indicating that NADPH/NADP$^+$ ratio considerably increased with the presence of prefoldin.

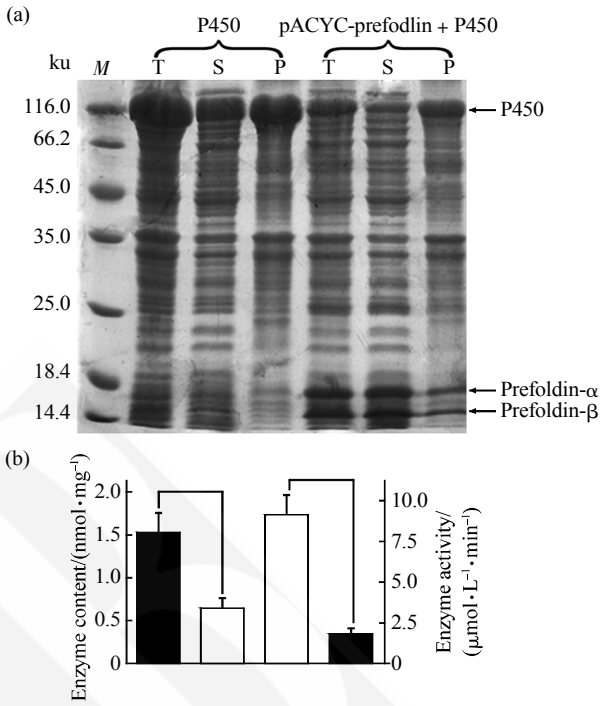

Fig. 3 Prefoldin from hyperthermophilic archaea did not affect P450 BM3-M content and enzymatic activity

(a) SDS-PAGE analysis of P450 BM3-M expression level in the cells overexpressing P450 BM3-M (P450) or co-expressing prefoldin and P450 BM3-M (pACYC-prefodlin + P450). *M*: Protein molecular mass marker, *T*: Total cells, *S*: Supernatant, P: Pellet. (b) P450 BM3-M content (analyzed by cospectral assay) and *in vitro* enzymatic activity determined in the cells overexpressing P450 BM3-M (P450) or co-expressing prefoldin and P450 BM3-M (pACYC-prefodlin + P450). The values are representative results obtained from at least three independent experiments and data are given as mean ± SEM. ■: P450; □: pACYC-prefodlin + P450.

Table 1 NADPH and NADP$^+$ levels and their ratio in recombinant E. coli strains expressing P450 BM3-M and/or archaeal prefoldin[1)]

	Construct and treatment time	Cofactor level/(nmol·mg^{-1})		Cofactor ratio
		NADPH	NADP$^+$	NADPH/NADP$^+$
P450	Before induction	1.60 ± 0.02	1.47 ± 0.04	1.09 ± 0.02
	Induction for 3 h	0.55 ± 0.01	7.54 ± 0.03	0.07 ± 0.01
	Indole addition for 2 h	0.62 ± 0.01	5.80 ± 0.02	0.11 ± 0.03
pACYC-prefoldin+P450	Before induction	1.79 ± 0.06	1.64 ± 0.05	1.09 ± 0.07
	Induction for 3 h	0.68 ± 0.01	3.48 ± 0.02	0.20 ± 0.01
	Indole addition for 2 h	0.61 ± 0.01	3.97 ± 0.12	0.15 ± 0.01

[1)] Data are representative of three independent experiments and presented as the average and standard deviation of measurements from two independent cultures.

In order to investigate whether prefoldin could regulate the intracellular NADPH/NADP$^+$ ratio, we subsequently measured this ratio in *E. coli* harbouring pACYC-prefoldin plasmid, and *E. coli* with pACYC empty plasmid was used as control. We demonstrated that the intracellular NADPH/NADP$^+$ ratio in *E. coli* overexpressing prefoldin was 2.1 times as that in the control after 3 h of IPTG induction (Table 2), indicating that prefoldin improved the intracellular ratio of NADPH/NADP$^+$ efficiently. Taken together, our results demonstrated that NADPH/NADP$^+$ ratio was probably related to the indigo bioconversion process and prefoldin may increase the indigo yield by increasing intracellular NADPH/NADP$^+$ ratio.

Table 2 NADPH and NADP$^+$ levels and their ratio in the recombinant E. coli strains[1)]

Construct and treatment time		Cofactor level/(nmol·mg^{-1})		Cofactor ratio
		NADPH	NADP$^+$	NADPH/NADP$^+$
pACYC	Before induction	2.10 ± 0.13	1.65 ± 0.01	1.28 ± 0.09
	Induction for 3 h	1.51 ± 0.01	4.84 ± 0.01	0.31 ± 0.01
pACYC-prefoldin	Before induction	2.32 ± 0.06	2.16 ± 0.06	1.07 ± 0.01
	Induction for 3 h	1.26 ± 0.03	1.92 ± 0.01	0.66 ± 0.01

[1)] Data are representative of three independent experiments and presented as the average and standard deviation of measurements from two independent cultures.

In conclusion, our study demonstrated that prefoldin from *P. furiosus* notably improves indigo production in *E. coli*, which is expected to have applications in the textile as well pharmaceutical industries. P450 BM3 mutant (A74G, F87V, D168H, L188Q) could catalyze indole to indigo, while the yield is low under normal culture conditions (37℃, 250 r/min). By co-expressing hyperthermophilic chaperones and P450 BM3 mutant in *E. coli*, we found that prefoldin originated from *P. furiosus* could improve the indigo production significantly. We also found that co-expression of prefoldin from *P. furiosus* could not increase (even decrease) the content and enzymatic activity of cytochrome P450 in *E. coli*, implying that P450 BM3 mutant enzyme content and its activity was not the factor which restricted the indigo bioconversion yield. By measuring the intracellular NADPH and NADP$^+$ concentrations, we found that prefoldin increased the intracellular NADPH/NADP$^+$ ratio significantly, which is probably related to the increased indigo bioconversion production, given that NADPH is an important cofactor in the indigo biosynthesis process.

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共表达极端菌伴侣蛋白 prefoldin 提高 P450 BM3 突变体催化效率 *

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摘要 P450 BM3 来源于巨大芽孢杆菌，其突变体(A74G、F87V、L188Q、D168H)能够在大肠杆菌细胞内催化吲哚合成靛蓝．然而在常规的培养条件下(37℃，250 r/min)，大肠杆菌细胞内靛蓝的生物转化量极低．本文将极端嗜热古菌 *Pyrococcus furiosus* 的分子伴侣蛋白与 P450 BM3 突变体在大肠杆菌进行共表达，以研究分子伴侣蛋白是否能够提高靛蓝的生物转化量．实验结果表明，极端嗜热古菌的分子伴侣蛋白 prefoldin 能够显著提高靛蓝的产量．同时，实验结果发现 prefoldin 能够明显提高大肠杆菌细胞内的 NADPH/$NADP^+$ 比率．鉴于 NADPH 是参与靛蓝生物转化过程的重要因素，靛蓝生物转化量的显著增加可能与该比率的提高有关．

关键词 细胞色素 P450 BM3，大肠杆菌，靛蓝，prefoldin，*Pyrococcus furiosus*

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