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ORIGINAL PAPER

Co-expression of P450 BM3 and glucose dehydrogenase by recombinant *Escherichia coli* and its application in an NADPH-dependent indigo production system

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Abstract P450 BM3 mutant can catalyze indole to indoxyl, and indoxyl can dimerize to form indigo. But the reaction catalyzed by P450 BM3 requires NADPH, as coenzyme regeneration is very important in this system. As we know, when glucose dehydrogenase oxidizes glucose to glucolactone, NADH or NADPH can be formed, which can contribute to NADPH regeneration in the reaction catalyzed by P450 BM3. In this paper, a recombinant *Escherichia coli* BL21 (pET28a (+)-P450 BM3-gdh0310) was constructed to co-express both P450 BM3 gene and glucose dehydrogenase (GDH) gene. To improve the expression level of P450 BM3 and GDH in *E. coli* and to avoid the complex and low-efficiency refolding operation in the purification procedure, the expression conditions were optimized. Under the optimized conditions, the maximum P450 BM3 and GDH activities amounted to 8173.13 and 0.045 U/mg protein, respectively. Then bioconversion of indole to indigo was carried out by adding indole and glucose to the culture after improved expression level was obtained under optimized conditions, and 2.9 mM (760.6 mg/L) indigo was formed with an initial indole concentration of 5 mM.

Keywords Coexpression · P450 BM3 · Glucose dehydrogenase · NADPH regeneration · Indigo

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Introduction

Indigo is the most important dye for textile dyeing. For many years, natural indigo has been replaced by synthetic indigo for use as dye. More recently, many researchers have reported some methods of synthesis of indigo by using bacterial systems [1, 2, 6, 17, 19].

P450 monooxygenases are found throughout nature from *Archebacteria* to humans and function primarily in the oxidation of a diverse range of chemicals [18]. The wild P450 BM3 from *Bacillus megaterium* can catalyze subterminal hydroxylation of saturated long-chain fatty acids [3, 16]. However, P450 BM3 with novel function of converting indole into indigo has been obtained by directed evolution [11, 12]. But as we know, NADH or NADPH is required as the electron donor in the reaction systems catalyzed by P450 monooxygenases [7], which has been a hindrance in P450 enzymes for industrial use. So cofactor regeneration is required for preparative applications. In recent years, existing regeneration methodologies have been improved and new approaches have been developed, such as regeneration of NAD(P)H with whole-cell catalysts [4, 8, 9]. And some efforts have been paid to construct a large size of plasmid pARGD with two target genes aldehyde reductase (AR) and glucose dehydrogenase (GDH) genes or one recombinant strain *Escherichia coli* JM109 with two different co-existing plasmids pKAR and pACGD [10].

Glucose dehydrogenase (GDH; EC 1.1.1.47), can oxidize glucose to glucolactone, at the same time, NADH or NADPH is formed. This feature of GDH might contribute to cofactor regeneration in NADH- or NADPH-dependent reactions. The reaction catalyzed by GDH can be employed as a cofactor regenerator

coupling with the bioconversion of indole to indigo. In the system of indigo production involving *E. coli* BL21 (pET28a (+)-P450 BM3) cells, commercially avaliable GDH should be added to the reaction mixture together with glucose and $NADP^+$ as a cofactor regenerator. In this study, the recombinant *E. coli* cell coexpressing both the P450 BM3 gene and the GDH gene from *B. megaterium* was constructed, which could be used as an NADPH regenerator instead of commercially avaliable GDH, and then the expression conditions of P450 BM3 and GDH were optimized, and the bioconversions from indole to indigo with *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) and *E. coli* BL21 (pET28a (+)-P450 BM3) would be compared.

Materials and methods

Enzymes and chemicals

Restriction enzymes and T4 DNA ligase were purchased from Takara biotech Co. Ltd. (Dalian, China). *Taq* Platinum DNA polymerase was purchased from Tianwei biotech Co. Ltd. (Beijing, China). NADPH and $NADP^+$ were obtained from Roche and Amresco, respectively. All other chemicals used were of analytical grade and commercially available.

Strains and plasmids

The *E. coli* strain DH5α (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyra*96 *thi-1 relA*1) was used as the host for gene manipulation. *E. coli* strain BL21(DE3) was used as the host for expression of proteins. Plasmid pET28a (+)-P450 BM3 encoding P450 BM3 gene was prepared as described previously [11, 12]. Plasmid pQE30-gdh0310, which was previously constructed to produce GDH [13], was used as template for the PCR to get the GDH gene.

Construction of expression plasmid

Plasmid containing both P450 BM3 and GDH genes in pET28a (+) was constructed as follows. The following PCR primers were employed for subclone the GDH gene: 5′-AAAAGAATTCATGTATACAGATTT AAAAGATAAAGTAGTAG-3′ and 5′-AAAAACT CGAGTTATTAACCTCTTCCCGCTT-3′. The 50 μl reaction system contained 1 ng plasmid pQE30-gdh0310 used as template, 10 nmol dNTP, 10 pmol each primer, 1.25 U *Taq* Platinum DNA polymerase and 5 μl buffer. Initial template denaturation was programmed for 8 min at 94°C. The profile (30 s at 94°C, 30 s at 54°C, and 1 min at 72°C) was repeated for 30 cycles, and the final 72°C extension step was increased to 10 min. The PCR-generated DNA fragment was purified and ligated into pMD18-T simple vector, and then transformed into *E. coli* DH5α. After ampicillin selection, several clones were picked up and plasmid DNA was examined by restriction analysis.

The GDH gene was digested with *Eco*RI and *Xho*I. The smaller fragment was recovered and ligated into pET28a (+)-P450 BM3 which was also digested by *Eco*RI and *Xho*I to obtain the plasmid pET28a (+)-P450 BM3-gdh0310, and then the plasmid was transformed into *E. coli* DH5α. After kanamycin selection, several clones were picked up and plasmid DNA was examined by restriction analysis, then plasmid pET28a (+)-P450 BM3-gdh0310 was transformed into *E. coli* BL21 (DE3) to co-express both P450 BM3 and GDH genes.

Co-expression of P450 BM3 and GDH and production of indigo

Recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) were picked with sterile toothpicks and transferred into a test tube containing 3 ml Luria–Bertani (LB) medium supplemented with 30 μg/ml kanamycin, followed by incubation at 37°C for 12 h with shaking at 180 r/min. Then the preculture was transferred to a 250 ml shaking flask containing 25 ml LB medium supplemented with 30 μg/ml kanamycin at the ratio of 2% (v/v) for co-expression of P450 BM3 and GDH at various cultivation conditions. Protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG). After further incubation for appropriate time, cells were harvested. While for production of indigo, indole and glucose were added to the medium after induction and the culture was incubated overnight at 30°C.

SDS-PAGE analysis

For SDS-PAGE analysis, cells were harvested by centrifugation at 5,000g for 10 min, resuspended in 20 mM Tris–HCl (pH 7.5) and disrupted ultrasonically. 1.5 ml suspension was centrifugated at 13,000*g* for 10 min, and the supernatant was the soluble fraction. The pellet was firstly washed with 50 mM Tris–HCl comprising 1% Triton and 1 mM EDTA, then washed with 20 mM Tris–HCl (pH 7.5), last dissolved in 250 μl urea (8 M) to obtain the insoluble fraction. Standard SDS-PAGE method (15% gel) was applied for target protein assay. The images of gels were scanned by GEL-DOC 2000 gel documentation system (Bio-Rad, USA).

Assay of P450 BM3 and GDH activity

For enzyme preparation, the cells were centrifuged at 5,000g for 10 min, resuspended in 100 mM potassium phosphate buffer (pH 7.4), and broken up under ice cooling by sonication (output level 200 W, three 2 min duty cycles of 20% each). After centrifugation (13,000*g* for 10 min), the supernatant was used as the cell-free extract and was used directly for the activity assay.

P450 BM3 activity was determined spectrophotometrically as described previously [11, 12]. One unit of P450 BM3 activity was defined as the amount of producing 1 pmol indigo per min measured by the increase of absorption at 670 nm. GDH activity was determined at 25°C with the assay mixture containing 75 mM Tris–HCl (pH 8.0), 2 mM $NADP^+$ and 100 mM glucose, and the reactions were monitored as the increase in absorption at 340 nm. One unit of GDH was defined as the amount of producing 1 μmol NADPH per min. Bradford protein assay was used for quantitative analysis of proteins with bovine serum albumin as the standard [5].

Determination indigo concentration

The cells were collected by centrifugation at 13,000*g* for 10 min and washed with water. Appropriate volume of *N*,*N*-dimethylformamide (DMF) was added to the blue pellet to extract indigo, then the absorption was determined at 610 nm and the yield of indigo was estimated.

Data analysis

Each data point in the figures represents the mean of triplicate samples. The standard deviation is shown by an error bar. All optimization experiments and bioconversion experiments were performed in triplicate.

Results and discussion

Construction of expression plasmid

The GDH gene fragment was successfully ligated into pET28a (+)-P450 BM3 to obtain the expression plasmid pET28a (+)-P450 BM3-gdh0310, which was confirmed by agarose electrophoresis and DNA sequencing. The structure of the plasmid was as shown in Fig. 1. This expression vector was transformed into *E. coli* BL21 (DE3) to obtain the recombinant strain, *E. coli* BL21 (DE3) (pET28a (+)-P450 BM3-gdh0310), to produce target proteins.

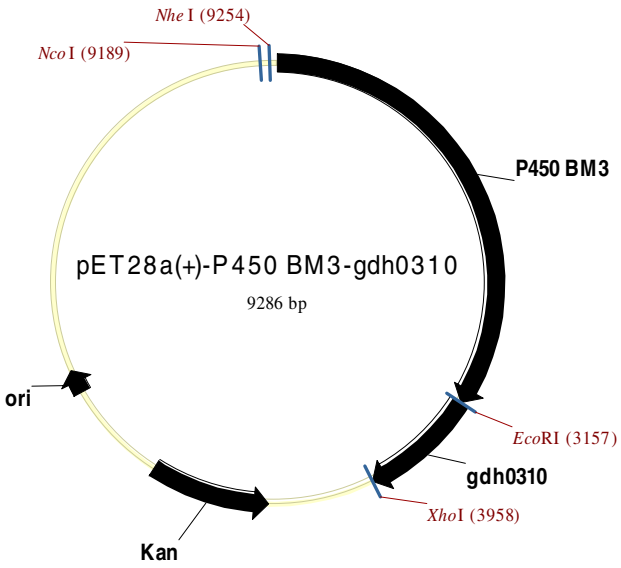

Fig. 1 Structure of plasmid pET28a (+)-P450 BM3-gdh0310

Expression of P450 BM3 and GDH genes

As we know, the molecular weights of P450 BM3 and GDH (one subunit) should be 119.0 and 28.2 kD, respectively. In SDS-PAGE analysis, compared to the negative control of *E. coli* BL21 (DE3), one of the target proteins P450 BM3 was expressed obviously in soluble or insoluble forms (Fig. 2a, b). However, when the induction temperature was 30°C, GDH was expressed in soluble form even in the negative control of BL21, as GDH is one of the constitutive enzymes expressed in *E. coli* BL21. This result was further confirmed by the enzyme activity assay (data not listed here). And it could not be concluded that GDH was expressed in *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310). When the induction temperature was increased up to 37°C, recombinant *E. coli* BL21 (pET28a (+)-P450 BM3) expressed P450 BM3 mainly in both soluble and insoluble forms, and the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) expressed P450 BM3 in soluble form and GDH in insoluble form. As the results showed, recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) could co-express P450 BM3 and GDH genes successfully. But from the results, it could be concluded that the expression conditions would have a great influence upon the forms in which the target proteins were expressed. To avoid the complex and low-efficiency refolding operation in the purification procedure and to increase the specific activities of P450 BM3 and GDH in soluble form, there was a need to optimize the co-expression conditions for high-level soluble enzymes expression.

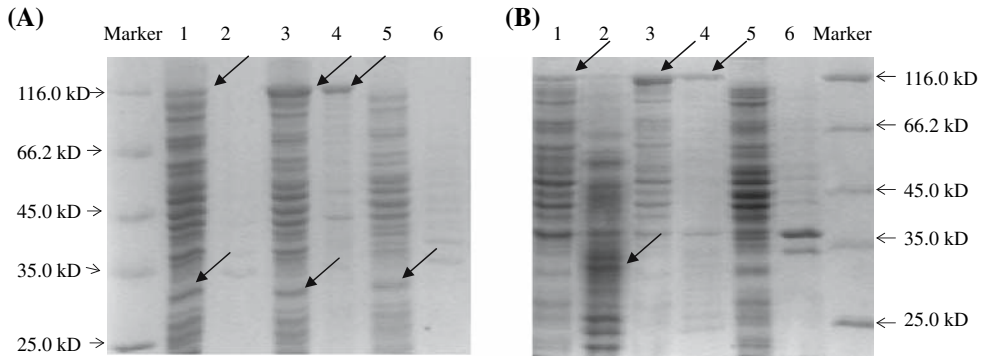

Fig. 2 P450 BM3 and GDH expression of strains. *Lanes 1*, *3*, and *5* are soluble proteins of BL21 (pET28a (+)-P450 BM3-gdh0310), BL21 (pET28a (+)-P450 BM3) and BL21 (serve as negative control). *Lanes 2*, *4*, and *6* are corresponding insoluble proteins, respectively. **a** The target proteins were induced at 30°C. **b** The target proteins were induced at 37°C

Optimization of co-expression conditions

The co-expression was employed in the shake flask scale. The co-expression ability was defined as the specific activities of P450 BM3 and GDH in soluble form.

Effect of medium volume

The medium volume is an important parameter reflecting the dissolved oxygen level in flask, and can influence the metabolic process of the cell and hence influence the target proteins expression. To study the effect of medium volume on the enzymes expression, cultivation was carried out in 250 ml flasks and the medium volume of 25, 50, 75 and 100 ml in each flask were evaluated, respectively. The specific activities of P450 BM3 and GDH were measured and calculated, and the results were shown in Fig. 3. The medium volume exhibited significant influence on P450 BM3 and GDH expression. It is obvious that when the medium volume was 25 ml, the specific activities of P450 BM3 and GDH in soluble form were the highest. The results indicated the sufficient dissolved oxygen is necessary for either cell growth or the expression of soluble proteins.

Effect of induction temperature

Temperature is an important factor in recombinant protein expression [20]. When the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) grew up to an OD_{600} value of 1.1 in LB medium, proteins expression was induced at 25, 30, 34, and 37°C, respectively. The results in Fig. 4 suggested that the temperature had great influence on the expression of proteins. At 25°C, the specific activity of GDH was highest, while the specific activity of P450 BM3 was lowest. When the temperature was 30°C, the specific activity of P450 BM3 was highest and the specific activity of GDH was lower

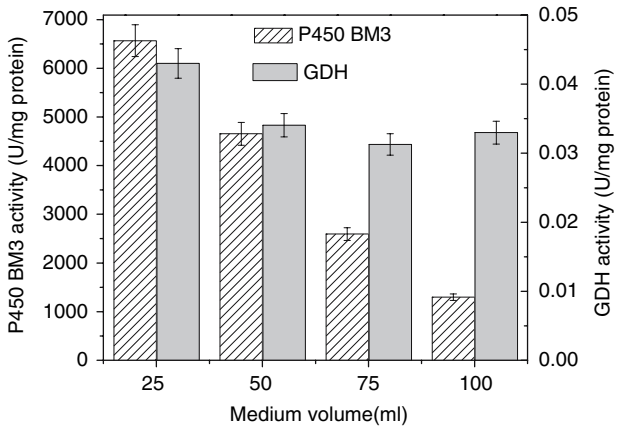

Fig. 3 Effect of medium volume on the enzyme activities. All tests were carried out in 250 ml flasks at 30°C, IPTG was added at OD_{600} of about 1.1 with a final concentration of 0.5 mM, and the post-induction expression time was 6 h

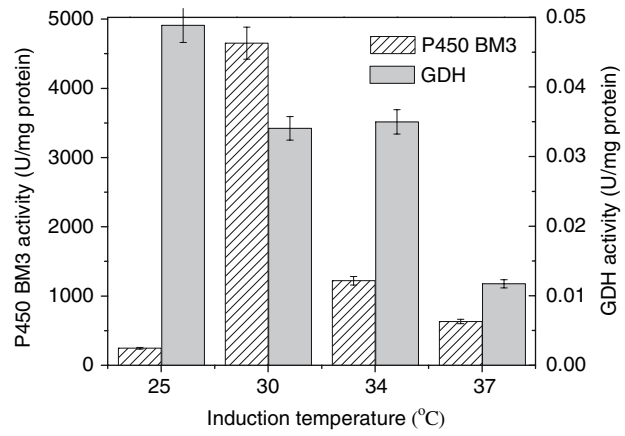

Fig. 4 Effect of induction temperature on the enzyme activities. Medium volume was 25 ml, IPTG was added at OD_{600} of about 1.1 with a final concentration of 0.5 mM, and the post-induction expression time was 6 h

than that at 25°C very little. However, at a higher temperature, both the specific activities of P450 BM3 and GDH decreased. Hence, the optimal temperature was 30°C.

Effect of IPTG concentration

For the expression plasmid pET28a (+)-P450 BM3-gdh0310, the proteins expression was induced by adding IPTG to the culture. Final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and potential harm to cell growth. In this work, IPTG concentration was examined from 0.2 to 1.0 mM and the expression of soluble product was analyzed in Fig. 5. The highest specific activities of P450 BM3 and GDH were obtained when induced with 0.5 mM IPTG so further studies were carried out under this condition.

Effect of induction timing

In the process of recombinant protein production in *E. coli*, IPTG induction is the turning point between cell growth and recombinant protein synthesis. The addition of IPTG means the beginning of the transcription of the foreign gene on the plasmid and can bring great changes to the metabolism of host cell by initiating the translation of heterologous protein. As shown in Fig. 6, the induction timing had a great influence on expression of P450 BM3 and GDH. When the OD_{600} values were 0.951 and 1.090, the higher specific activities of P450 BM3 and GDH were obtained. However, when IPTG was added at OD_{600} of 0.593 or after OD_{600} of 1.090, the expression level of both enzymes decreased rapidly. The results indicated that highest expression of

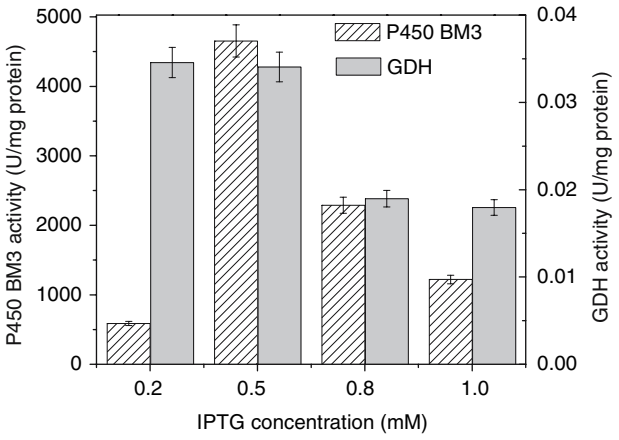

Fig. 5 Effect of IPTG concentration on the enzyme activities. All tests were carried out at 30°C, IPTG was added at OD_{600} of about 1.1, and the post-induction expression time was 6 h, the medium volume was 25 ml

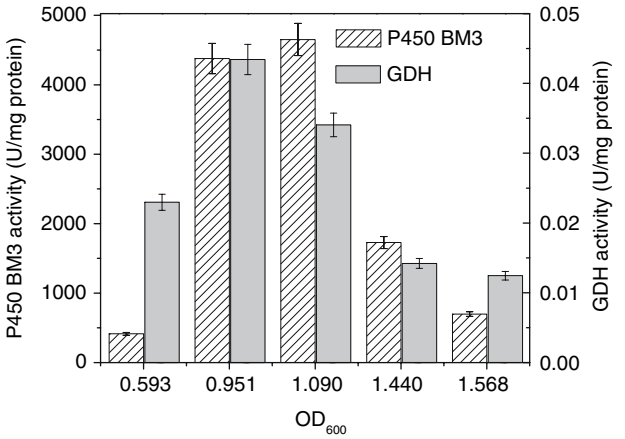

Fig. 6 Effect of induction timing on the enzyme activities. All tests were carried out at 30°C, IPTG was added with a final concentration of 0.5 mM at various stages of exponential growth phase, and the post-induction expression time was 6 h, the medium volume was 25 ml

P450 BM3 and GDH in *E. coli* BL21 was obtained when the induction timing was set at the mid stage of the log phase. And obviously, induction timing was a significant factor influencing over the expression of P450 BM3 and GDH in *E. coli.*

Effect of post-induction time

After the addition of IPTG into the culture, the target proteins began to synthesize. The effect of post-induction time on the expression of proteins were observed by analyzing samples every 2 h up to 10 h after induction began. The results in Fig. 7 suggested that the specific activities of P450 BM3 and GDH reached the highest values simultaneously after 8 h of induction. However, overtime induction did not have influence over expression of enzymes.

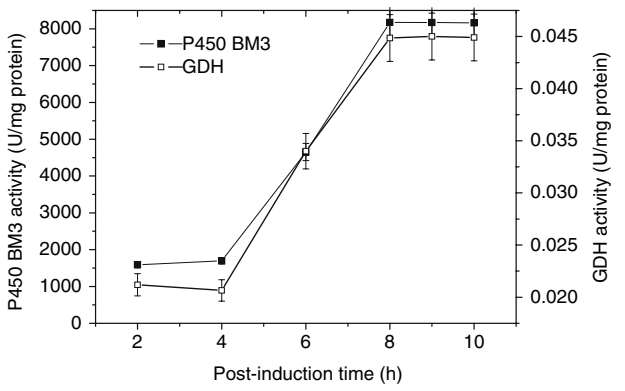

Fig. 7 Effect of post-induction time on the enzyme activities. All tests were carried out at 30°C, IPTG was added with a final concentration of 0.5 mM at an OD_{600} of about 1.1, and the post-induction expression time was 6 h, the medium volume was 25 ml

NADPH regeneration in biotransformation system

P450 BM3 and GDH expressed by the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) functioned corporately in the reaction. GDH was used to regenerate NADPH, and P450 BM3 catalyzed indole to indoxyl which dimerized to form indigo [14, 15] by utilizing intracellular NADPH. To explain the effect of coexpression of GDH, the extracellular NADPH concentrations over the time course of indigo formation in different systems were measured. The actual NADPH concentration was calculated from the absorption data using the value of the extinction coefficient ($6.2\ mM^{-1}\ cm^{-1}$). As shown in Fig. 8, without the addition of GDH in the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3) system, the profile of NADPH concentration was kept in low level and decreased. However, when GDH and $NADP^+$ were added to the system, the NADPH concentration increased obviously during 3 h. In the system catalyzed by the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310), the time course of NADPH concentration was similar to that in GDH and $NADP^+$ supplemented system catalyzed by BL21 (pET28a (+)-P450 BM3). It indicated that GDH performed a key role for NADPH regeneration. High intracellular GDH activity produced high concentration of NADPH. The performance of intracellular GDH could replace the commercial GDH. In the reaction system catalyzed by BL21 (pET28a (+)-P450 BM3-gdh0310), as high activity of recombinant GDH was obtained, NADPH could be regenerated and the biotransformation of indole to indigo could be efficiently completed with no exogenous GDH and $NADP^+$ addition.

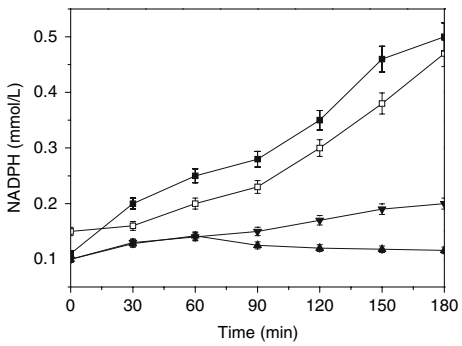

Fig. 8 Comparison of NADPH concentration in the different reaction systems. System (*filled squares*), *E. coli* BL21(pET28a(+)-P450BM3-gdh0310), 100 mM glucose, 2 mM indole; system (*open squares*), *E. coli* BL21(pET28a(+)-P450BM3), 100 mM glucose, 2 mM indole, 0.2 mM $NADP^+$, and 10 U/ml GDH; system (*filled inverted triangles*), *E. coli* BL21(pET28a(+)-P450BM3-gdh0310), 100 mM glucose,; system (*filled triangles*), *E. coli* BL21(pET28a(+)-P450BM3), 100 mM glucose, 2 mM indole

Production of indigo with *E. coli* cells coexpressing the P450 BM3 and GDH genes

The efficiency of conversion of indole to indigo was investigated. Using the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3), 0.104 mM indigo was formed after 10 h reaction (Fig. 9b) with an initial substrate concentration of 0.5 mM, and only 0.001 mM indigo could be formed with an initial substrate concentration of 5 mM. When the recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) was used, the better results were obtained. The highest yield was obtained after 4 h reaction when the initial indole concentrations were 0.5 and 2 mM. And after 8 h incubation, indigo formed amounted to 2.9 mM when the indole concentration was 5 mM. The results here indicate that recombinant *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) might find application in the commercial production of indigo for the dye market. The yield of indigo formed in P450 2A6 cultures and in naphthalene dioxygenase cultures were about 0.038 and 0.095 mM, respectively [21, 22].

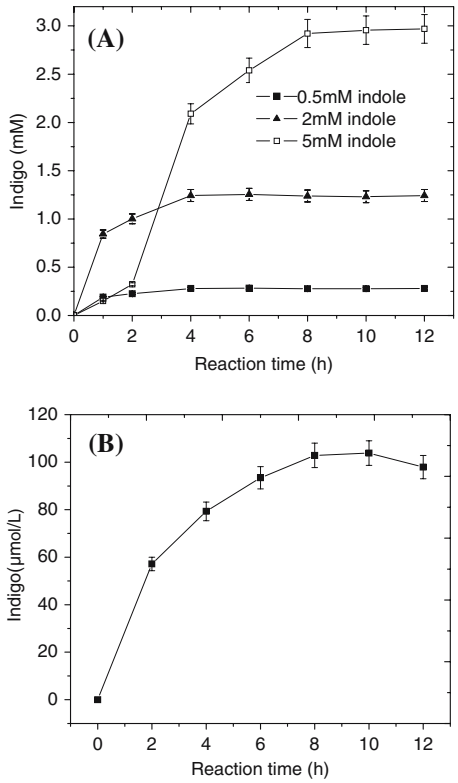

Fig. 9 Production of indigo by *E. coli* BL21 cells. Reactions were carried out by adding indole and 100 mM glucose to the cultures, after optimal expression level was obtained under optimized conditions, and the flask was incubated overnight at 30°C. **a** Reactions were carried out with BL21 (pET28a (+)-P450 BM3-gdh0310) and **b** BL21 (pET28a(+)-P450) with the indole concentration of 0.5 mM

In the reaction system with *E. coli* BL21 (pET28a (+)-P450 BM3-gdh0310) cells, NADPH was regenerated to satisfy the requirement. Apparently, this was contributed by the introducing of GDH gene to the same plasmid with P450 BM3, which could provide GDH and regenerate NADPH efficiently. The work of Kataoka et al. [10] offers the successful demonstration of the use of recombinant strain co-expressing two enzymes for cofactor regeneration. And the results in our work also indicated that the new strategy was a promising process.

Conclusion

Cofactor regeneration has been a barrier in the reactions catalyzed by P450 enzymes for long time. In our study, the recombinant *E. coli* BL21 (DE3) harboring pET28a (+)-P450 BM3-gdh0310 was constructed, which could co-express P450 BM3 and GDH, and could serve as an NADPH regenerator instead of commercially available GDH. In the reaction system of conversion of indole to indigo, NADPH necessary might be supplied sufficiently, since GDH could regenerate NADPH efficiently. The results also showed the strategy had the potential to be employed in biotransformation with cofactor regeneration.

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References

1. Alemayehu D, Gordon LM, O'Mahony MM, O'Leary ND, Dobson ADW (2004) Cloning and functional analysis by gene disruption of a novel gene involved in indigo production and luoranthene metabolism in Pseudomonas alcaligenes PA-10. FEMS Microbiol Lett 239:285–293
2. Bhushan B, Samanta SK, Jain RK (2000) Indigo production by naphthalene-degrading bacteria. Lett Appl Mcrobiol 31:5–9
3. Boddupalli SS**,** Pramanik BC, Slaughter CA, Estabrook RW, Peterson JA (1992) Fatty acid monooxygenati on by P450B M-3: product identification and proposed mechanisms for the sequential hydroxylation reactions. Arch Biochem Biophys 292(1):20–28
4. Boonstra B, Rathbone DA, French CE, Walker EH, Bruce NC (2000) Cofactor regeneration by a soluble pyridine nucleotide transhydrogenase for biological production of hydromorphone. Appl Environ Microbiol 66:5161–5166
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. Choi HS, Kim JK, Cho EH, Kim YC, Kim JI, Kim SW (2003) A novel flavin-containing monooxygenase from *Methylophaga* sp. Strain SK1 and its indigo synthesis in *Escherichia coli.* Biochem Biophys Res Commun 306:930–936
7. Coon MJ (2003) Multiple oxidants and multiple mechanisms in cytochrome P450 catalysis. Biochem Biophys Res Commun 312:163–168
8. Endo T, Koizumi S (2001) Microbial conversion with cofactor regeneration using genetically engineered bacteria. Adv Synth Cat 343:521–526
9. Itoh N, Matsuda M, Mabuchi M, Dairi T, Wang J (2002) Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled with in situ regeneration of NADH. Eur J Biochem 269:2394–2402
10. Kataoka M, Yamamoto K, Kawabata H, Wada M, Kita K, Yanase H, Shimizu S (1999) Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes. Appl Microbiol Biotechnol 51:486–490
11. Li HM, Mei LH, Urlacher V, Schmid RD (2005) Cytochrome P450 BM-3 mutants with improved catalytic properties hydroxylating indole to indigo by error-prone PCR (in Chinese). Prog Biochem Biophys 32:1–6
12. Li QS, Schwaneberg U, Fische P, Schmi RD (2000) Directed evolution of the fatty-acid hydroxylase P450 BM-3 into an indole-hydroxylating catalyst. Chem Eur J 6:1531–1536
13. Liu Y, Xu ZN, Jing KJ, Jiang XX, Lin JP, Wang F, Cen PL (2005) Asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to ethyl (*R*)4-chloro-3-hydroxybutanoate with two coexisting, recombinant *Escherichia coli* strains. Biotechnol Lett 27:119–125
14. Mermod N, Harayama S, Timmis KN (1986) New route to bacterial production of indigo. Biotechnology 4:321–324
15. Murdock D, Ensley BD, Serdar C, Thalen M (1993) Construction of metabolic operons catalyzing the de novo synthesis of indigo in Escherichia coli. Biotechnology 11:381–386
16. Narih LO, Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000-Dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus Megaterium*. J Biol Chem 261:7160–7169
17. O'Connor KE, Dobson ADW, Hartmans S (1997) Indigo formation by microorganisms expressing styrene monooxygenase activity. Appl Environ Microbial 63:4287–4291
18. Ortiz de Montellano PR (1995) Cytochrome P450: Structure, mechanism, and biochemistry, second edn. Plenum, New York
19. Royoa JL, Moreno-Ruiza E, Cebollab A, Santero E (2005) Stable long-term indigo production by overexpression of dioxygenase genes using a chromosomal integrated cascade expression circuit. J Biotechnol 116:113–124
20. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor, New York
21. Gillam EMJ, Notley LM, Cai H, Voss JJD, Guengerich FP (2000) Oxidation of indole by cytochrome p450 enzymes. Biochemistry 39:13817–13824
22. Ensley BD, Ratzkin BJ, Ossulund TD, Simon MJ, Wackett LP, Gibson DT (1983) Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science 22:167–169