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Biotransformation of indole by whole cells of recombinant biphenyl dioxygenase and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase

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ABSTRACT

The introduction of hydroxyl groups into indole molecule by different mono- and dioxygenases leads to the production of indigo. As a well-known biocatalyst, biphenyl dioxygenase possessed the ability to transform indole to indigo. However, there has been little information about this enzymatic transformation process. In this study, the genes encoding biphenyl dioxygenase (BphA) and biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (BphB) were cloned from *Dyella ginsengisoli* LA-4 and heterologously expressed in *Escherichia coli* (DE3) (designated as AB_{IND}). The feasibility of indole transformation to indigo by strain $BphA_{LA-4}$ was predicted by molecular docking studies. The biotransformation ratios of indole (100 mg/L) reached the maximum (95%) when cells were induced at 15 °C with 0.25 mM IPTG in M9 medium. In addition, 44 mg/L indigo was produced from 200 mg/L indole when supplied with 0.28 g/L of biomass and 0.2% (w/v) glucose. HPLC–MS was used to identify the products, which showed that indigo was the major product. Meanwhile, indirubin and isatin were also identified during the transformation process. Furthermore, the pathway of indole transformation by strain AB_{IND} was also proposed.

1. Introduction

As a common N-heterocyclic compound, indole was reported to cause acute pulmonary edema, emphysema, lung diseases [1], hemoglobinuria and hemolysis to cattle and goats [2]. Previous studies have proved that indole can be bio-oxidized by lots of bacterial oxygenases [3,4]. One of the major products, indigo, is the earliest known dyestuff used worldwide [5], which is primarily produced by chemical synthesis. Besides, some biologically active compounds such as indirubin could also be produced during the biotransformation process, which can inhibit several cyclin-dependent kinases (CDKs) and induce apoptosis of cancer cells [6]. Therefore, there have been increasing studies focused on indole biotransformation catalyzed by various oxygenases.

As well known, a variety of genes encoding the corresponding enzymes have been cloned and heterologously expressed in *Escherichia coli* to produce indigo from indole, tryptophan or glucose [3,4]. The enzymes responsible for indigo formation are commonly divided into two types: monooxygenases and dioxygenases. Among these oxygenases, monooxygenases with strong hydroxylation ability are quite abundant, such as human cytochrome P450 enzymes [6–8], bacterial flavin-containing monooxygenase [9], toluene-4-monooxygenase [10], toluene *ortho*-monooxygenase [11] and 2-hydroxybiphenyl 3-monooxygenase [12]. Generally, indole is primarily monohydroxylated to indoxyl (3-hydroxyindole), 2-oxindole and isatin, and the subsequent non-enzymatic dimerization leads to the formation of indigo and indirubin. In parallel, it is reported that bacteria with the abilities of oxidizing the aromatic hydrocarbons to *cis*-dihydrodiols are able to oxidize indole to indigo [3]. Thus, it is implicated that some dioxygenases should be considered as the potential candidates for indigo production. However, dioxygenases which can also catalyze the biosynthesis of indigo and indirubin are relatively rarely concerned. Up to now, the most well studied dioxygenase is limited to naphthalene dioxygenase (NDO) [3].

Biphenyl dioxygenase (BPDO; BphA) is a NADH-dependent multicomponent enzyme, which consists of three components: large (α) and small (β) subunits of terminal dioxygenase (BphA1 and BphA2), ferredoxin (BphA3), and ferredoxin reductase (BphA4) [13,14]. As previously reported, cells carrying BphA were capable of transforming indole to *cis*-2,3-dihydroxy-2,3-dihydroindole, which was the key intermediate during the biosynthesis of indigo [3]. Biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (BphB) is able to further act on the diverse dioxygenation products derived from BphA catalysis. In our previous study, a novel biphenyl-degrading strain *Dyella ginsengisoli* LA-4 was isolated [15], and the

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Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Characteristics	Source
Strains		
Dyella ginsengisoli LA-4	Wild type, biphenyl degrader	[15]
E. coli JM109	Host strain for cloning of target genes	TaKaRa
E. coli BL21 (DE3)	Host strain for expression of the recombinant BphAB	TaKaRa
Plasmids		
pMD18-T	Vector used for cloning	TaKaRa
pBR322	Vector used for expressing	TaKaRa
pBR-bphAB	pBR322 derivative carrying the *bphAB* genes	This study

biphenyl-degrading gene cluster was analyzed, which harbored genes encoding BphA and BphB [16].

In this report, the genes encoding BphAB were cloned from *D. ginsengisoli* LA-4, and overexpressed in *E. coli*. Molecular docking was performed to examine the interactions between BphA and the substrates, through which the feasibility of indole transformation was investigated. Furthermore, the conditions for genes expression and indole biotransformation were both optimized, the main products derived from indole were also analyzed, and the transformation pathway was proposed.

2. Materials and methods

2.1. Molecular docking studies

D. ginsengisoli LA-4 has been deposited as a patent bacterium in China General Microorganism Culture Center with the accession number CGMCC 2723. Due to the absence of three-dimensional structure of $BphA1_{LA-4}$, homology modeling was constructed based on sequence similarity using SWISS-MODEL server [17]. Crystal structure of BphA1 from *Comamonas testosteroni* B-356 (PDB number: 3gzxA) was identified as a template structure for $BphA1_{LA-4}$, and the amino acid sequence homology between the two proteins was 75.9% according to the sequence-based blast in protein data bank. Molecular docking was carried out by Autodock 4.2 [18]. Ligand structure was generated by PRODRG server [19]. The GA_r number of individuals in the population was set up to 100. Molegro Molecular Viewer was used for visualizing the docking result and displaying the interactions between protein and ligands.

2.2. Cloning and expression of bphAB genes

The bacterial strains and plasmids used in this study were listed in Table 1. The *bphAB* genes were amplified from the genomic DNA of *D. ginsengisoli* LA-4 extracted using a Bacterial Genomic DNA Extraction Kit (TaKaRa). The specific primers used were as follows: bphA1-F, 5′-GAATTCATGAAGAATGCAGTAGAGAAAGC-3′ (*Eco*RI site underlined); bphB-R, 5′-AAGCTTTCATTTCAATGCTCCCAGATCA-3′ (*Hin*dIII site underlined). The amplification program was as follows: 94 °C for 2 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 5 s, 72 °C for 1 min, and the final elongation step was 72 °C for 5 min. The amplified fragment containing the *bphAB* genes was then subcloned into the vector pBR322 by *Eco*RI and *Hin*dIII yielding the recombinant plasmid pBR-bphAB. Subsequently, the resulting plasmid (pBR-bphAB) was introduced into *E. coli* BL21 (DE3) for overexpression, and the recombinant *E. coli* was designated as AB_{IND}.

2.3. Chemicals and culture conditions

All chemicals used in this study were of analytically grade or above, which were purchased from TaKaRa, Sigma, or Amresco.

Strain AB_{IND} was routinely cultured in Luria–Bertani (LB) or M9 medium [20] with 30 μg/mL chloromycetin aerobically until the OD_{600} reached about 0.4, and then the culture was induced by isopropyl-β-thiogalactopyranoside (IPTG). When the OD_{600} reached about 1.0, cells were harvested by centrifugation at 9820 × *g* for 5 min (20 °C). After washed twice with 20 mM sterile sodium phosphate buffer (PBS, pH 7.4), the cell pellets were used for further analysis.

2.4. SDS-PAGE analysis

For SDS-PAGE analysis, cells were resuspended in PBS (10 mL), sonicated at 4 °C for 30 min (225 W, Ultrasonic processor CPX 750, USA), and gathered by centrifugation at 58,000 × *g* for 20 min (4 °C). The supernatant contained the soluble fraction and the pellet was dissolved in PBS (10 mL) to obtain the insoluble fraction. Standard SDS-PAGE method (30% gel) was applied for target protein assay. A protein marker from 97.2 kDa to 14.3 kDa was used as the standard to estimate the molecular weight of the enzyme sample.

2.5. Biotransformation of indole by recombinant strain AB_{IND}

All transformations were conducted in 250-mL Erlenmeyer flasks with a 100-mL working volume at 30 °C and 150 r/min. The reaction mixtures consisted of 0.56 g (cell dry weight, CDW) of strain AB_{IND} resuspended in 100 mL sterile PBS, 1% glucose (w/v), and 100 mg/L of indole dissolved in acetone as the cosolvent. The biotransformation process was monitored by sampling at 2 h intervals.

To study the effects of different inducing conditions on the expression of $BphAB_{LA-4}$, transformation process of indole was investigated with different inducing temperature (15, 20, 37 °C), IPTG concentrations (0.05, 0.1, 0.25, 0.5, 1.0 mM) and culture media (LB, M9), using the non-induced *E. coli* as control. To obtain the optimal conditions for indigo production from indole, the transformations were performed with different concentrations of biomass (0.14, 0.28, 0.56, 1.2 g/L), indole (50–400 mg/L) and glucose (0, 0.2, 1.0% (w/v)).

2.6. Analytical methods

To determine the concentrations of indole and indigo, the culture broth was centrifuged at 9820 × *g* for 5 min (20 °C). The cell-free culture was extracted with an equal volume of chloroform and the chloroform layer was collected. The cell pellets were mixed with an equal volume of dimethylsulfoxide (DMSO) and centrifuged at 9820 × *g* for 5 min (20 °C) to obtain the blue supernatant. The chloroform layer and the blue DMSO supernatant were subsequently analyzed by high performance liquid chromatography (HPLC) (SHMADZU SIL-20A, Japan), respectively. HPLC, equipped with a C18 column (5 μm, 250 mm × 4.6 mm), was performed at a flow rate of 1 mL/min and monitored at 265 nm and 280 nm for indole and indigo, respectively. The compounds were eluted using a linear gradient from 60/40 to 75/25 (v/v) (methanol/water) over 20 min.

To identify the biotransformation products, the mixed sample was analyzed by the high performance liquid chromatography and mass spectrometry (HPLC–MS) (Hewlett Packard 1100 MSD, America). MS analysis was performed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer equipped with a standard API-1 atmospheric pressure chemical ionization (APCI) source in the positive or negative ion mode. N_2 was used as a sheath gas (50 p.s.i.) and vaporizer temperature was set to 500 °C.