Specific N-demethylation of verapamil by cytochrome P450 from *Streptomyces griseus* ATCC 13273

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Norverapamil, the N-demethylated derivative of verapamil, is a novel promising leading compound for attenuating multidrug resistance with less side effects compared with verapamil. However, the efficient synthetic method for norverapamil is absent. In this study, an innovative biotechnological method based on enzymatic catalysis was presented for the high-efficient production of norverapamil. CYP105D1, a cytochrome P450 from *Streptomyces griseus* ATCC 13273, was identified to carry out a one-step specific N-demethylation of verapamil along with putidaredoxin reductase (Pdr) and putidaredoxin (Pdx) as the redox partner. Docking calculations rationalized the specific N-demethylation observed in experiment and identified important amino acid residues for verapamil binding. Furthermore, a CYP105D1-based whole-cell system in *E. coli* BL21(DE3) was established and optimized for highly efficient N-demethylation of verapamil. The bioconversion rate of verapamil by the whole cell system came up to 60.16% within 24 hours under the optimized conditions. These results demonstrated the high potential of CYP105D1-based biocatalytic system for norverapamil production.

KEYWORDS

biocatalysis, cytochrome P450, N-demethylation, norverapamil, verapamil

1 | INTRODUCTION

Multidrug resistance (MDR) remains one of the most significant factors impeding the progress of cancer chemotherapy [1]. Verapamil, a phenylalkyl-amine calcium channel blocker, has been widely used as an antihypertensive for more than 30 years [2] that has also been shown to reverse MDR by inhibition of P-glycoprotein [3]. However, the side effects such as cardiotoxicity limit the clinical use of verapamil as MDR reversal agent [4]. Norverapamil is the N-demethylated metabolite of verapamil [5] and has been reported to be more effective than verapamil for reversing MDR while the cardiac activity of norverapamil is only about 20% of verapamil [6]. Therefore, norverapamil presents attractive alternative for MDR resistance that shows higher activity and less

cardiotoxicity than verapamil [6]. However, there are very few studies that have been reported on the synthesis of norverapamil, and it is highly imperative to explore new synthetic strategies for norverapamil.

One of the potential strategies for norverapamil synthesis is the N-demethylation of verapamil. But N-demethylation has proved to be a challenge in chemical synthesis, especially for the complex compound with multiple methyl groups. Bromide and chloroformate reagents were usually used as catalysts for the N-demethylation [7]. However, these methods usually suffer from high toxicity, poor selectivity (chemo and/or regio), or cost-ineffectiveness. The biocatalytic method is one of the alternative strategies for chemical N-demethylation and usually exhibited high selectivity, efficiency under mild conditions, and environmentally friendly [8].

Cytochromes P450s (P450s) are a large group superfamily of heme-dependent monooxygenases [9]. P450s can catalyze a wide variety of enzymatic reactions including hydroxylation, N-oxidations, sulfoxidations, peroxidations, N-oxide

Abbreviations: MDR, multidrug resistance; Pdr, putidaredoxin reductase; Pdx, putidaredoxin; PEG, polyethylene glycol

reductions, C–C bond cleavage, and so on [10]. To the best of our knowledge, there are only a few microsome cytochrome P450s investigated for demethylation of verapamil in pharmacokinetic studies. For instance, CYP1A2, CYP3A4, and CYP3A5 were reported to mediate N-demethylation of verapamil while CYP2C family can mediate O-demethylation of verapamil [5,11]. However, the low turnover rate, poor expression level in *Escherichia coli* cell system and lack selectivity are obstacles to the application of microsome P450s in biotransformation [12].

In this study, we aimed to develop an innovative biocatalytic method for norverapamil synthesis. CYP105D1, a bacterial P450 from *Streptomyces griseus* ATCC 13273, was firstly identified for selective N-demethylation of verapamil along with putidaredoxin reductase (Pdr) and putidaredoxin (Pdx) as redox partner [13]. And a CYP105D1-based whole cell system was established for high efficiency production of norverapamil. This was the first report concerning the N-demethylation of verapamil using prokaryotic cytochrome P450 and the CYP105D1-based catalytic system will be potential for highly efficient production of norverapamil in pharmaceutical industry.

2 | MATERIALS AND METHODS

2.1 | Materials

Verapamil and norverapamil were purchased from Spring & Autumn Biological Engineering Co., Ltd. (Nanjing, China). Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Transgene (Beijing, China). 5-aminolevulinic acid (5-ALA) was purchased from Tianfeng Biocompany (Shanxi, Xi'an, China). All other chemicals and solvents were obtained from standard sources and were of the highest purity available.

Cloning experiments were carried out with *E. coli* DH5 α (Transgene, Beijing, China). Heterologous protein expressions were carried with *E. coli* BL21(DE3) (Transgene, Beijing, China). The primers were listed in Supporting Information Table 1 and other strains and plasmids used in this study were listed in Supporting Information Table 2.

2.2 | Construction the recombinant plasmids

The gene of CYP105D1 (GenBank: X63601.1) was amplified by PCR from the genomic DNA of *S. griseus* ATCC 13273 using primers F(cyp105D1) and R(cyp105D1). Thereby restriction sites *Nde*I and *Hind*III were introduced for subsequent cloning of *cyp105D1* into vector pET28a and pET22b. The resulting vector was named pHis-cyp105D1 and pcyp105D1, respectively. The gene of putidaredoxin (*pdx*, GenBank: J05406.1) was amplified by PCR from the genomic DNA of *Pseudomonas putida* using primers F(*pdx-Nde*I) and

PRACTICAL APPLICATION

A biocatalytic method is presented here for high-efficient production of norverapamil through specific N-demethylation of verapamil using cytochrome P450. Norverapamil is a promising leading compound used for reversing multidrug resistance with less side effects, while the development of an efficient synthetic technology giving access to norverapamil remained a challenge. The P450-based whole cell system established in this study exhibited high selectivity, under mild condition and cost-effectivity that provided an innovative, sustainable, and high-efficient strategy for norverapamil production and will be further applied in pharmaceutical industry. Moreover, PEG-200 was used for enhancing the substrate solubility and will be further developed as cosolvent for various substrates for biocatalytic industry. We also believe that the P450-based biocatalytic technique could greatly contribute to the pharmaceutical or chemical industry for the synthesis of fine chemicals with high added values in the future.

R(*pdx-Xho*I). Thereby restriction sites *Nde*I and *Xho*I were introduced for subsequent cloning of *pdx* into vector pET22b. The resulting vector was named pPdx-His. The gene of putidaredoxin reductase (*pdr*, GenBank: J05406.1) was amplified by PCR from the genomic DNA of *Pseudomonas putida* using primers F(*pdr-Nde*I) and R(*pdr-Xho*I). Thereby restriction sites *Nde*I and *Xho*I were introduced for subsequent cloning of *pdr* into vector pET22b. The resulting vector was named as pPdr-His. For preparation of plasmid for coexpression of *pdx* and *pdr*, the restriction sites *Nde*I and *Kpn*I and the restriction sites *Bam*HI and *Hind*III were respectively introduced for subsequent cloning of *pdx* and *pdr* into vector pACYCDuet-1. The resulting vector was named as pPdr-Pdx.

2.3 | Expression and purification of CYP105D1, Pdr, and Pdx

pHis-cyp105D1, pPdx-His, or pPdr-His was transformed to *E. coli* BL21(DE3) respectively named BL21-His-105D1, BL21-Pdx-His, and BL21-Pdr-His. For enzyme expression, a 50 mL TB medium supplemented with kanamycin (10 μ g/mL final concentration) or ampicillin (10 μ g/mL final concentration) was inoculated with 1% v/v of the overnight culture. At an optical density (OD₆₀₀) of 0.6–0.8 the system was induced with isopropyl- β -D-1-thiogalactopyranoside (IPTG, 0.1 mM final conc.). δ -aminolevulinic acid (84 μ g/mL final conc.) was added as a precursor for heme synthesis for CYP105D1 expression. After 24 hours of expression at 22°C

the cells were harvested by centrifugation ($5000 \times g$, 4°C for 15 min), washed with 50 mM pH 7.4 potassium phosphate buffer, centrifuged again and the pellets were used for protein purification.

During protein purification, all samples were kept at 4°C . The cell pellet was resuspended in Ni-NTA buffer A (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8) added with 0.1 mM EDTA, 20% glycerine and 0.1 mM PMSF and lysed by sonication. The lysate was clarified by centrifugation at $15\,000 \times g$ for 30 min, and the supernatant was loaded on a preequilibrated Ni-NTA column. After washing with two column volumes (cv) of Ni-NTA buffer A, CYP105D1 was eluted with two column volumes (cv) of Ni-NTA buffer B (50 mM NaH_2PO_4 , 300 mM NaCl, 50 mM imidazole, pH 8). Fractions were combined, and buffer exchanged with 50 mM pH 7.4 sodium phosphate buffer on a 10- or 30-kDa MWCO filter (EMD Millipore).

Functional CYP105D1 was quantified using CO-difference spectra analysis as described previously [14]. And the measurement of the concentration of Pdr and Pdx was performed as described previously [15].

2.4 | In vitro bioconversion and kinetic assays

The in vitro bioconversion was performed with a reconstituted system, containing CYP105D1, Pdx, and Pdr in a molar ratio of 1:20:2. For the electron supply, NADH was supplemented. The substrates concentration was 200 μM . All reactions were carried out in 250 μL total volume at 28°C in 50 mM potassium phosphate buffer (with 20% glycerol, pH 7.4) and quenched after 60 min by adding one reaction volume of ethyl acetate. The substrates were extracted twice with ethyl acetate. The organic phases were combined, evaporated to dryness and resuspended in acetonitrile for analysis.

The kinetic parameters were estimated with a substrate concentration ranging from 0 to 250 μM . The reactions were started by adding 0.2 mM NADH and quenched after 2 min by adding one reaction volume of ethyl acetate. The organic phases were combined, evaporated to dryness, and resuspended in menthol for analysis. The product amounts were determined from the conversion ratio using area under-peak of product from the HPLC chromatograms. The steady-state kinetic parameters K_m and k_{cat} were calculated by fitting the data to the Michaelis–Menten equation by nonlinear regression of a hyperbolic function using the OriginPro 8.5 software.

2.5 | Whole cell system bioconversion

Both of the constructed vector pcyp105D1 and pPdr-Pdx were cotransformed into *E. coli* BL21(DE3) for coexpressing CYP105D1 and redox partner Pdr and Pdx in one host named BL21-(pcyp105D1)-(pPdr-Pdx).

The BL21-(pcyp105D1)-(pPdr-Pdx) strains were cultured in LB liquid medium overnight for seed fermentation broth preparation. And inoculated in TB medium supplemented with ampicillin (10 $\mu\text{g}/\text{mL}$ final concentration) and chloramphenicol (10 $\mu\text{g}/\text{mL}$ final concentration). For enzyme expression, the media was inoculated at 37°C and 200 rpm for 3 h. At the optical density (OD600) of 0.4 the system was induced with IPTG (0.1 mM final conc.) and δ -ALA (84 $\mu\text{g}/\text{mL}$ final conc.) was added as a precursor for heme synthesis for CYP105D1 expression. After 8 hours of expression at 20°C and 200 rpm the cells were harvested by centrifugation ($5000 \times g$, 4°C for 10 min), washed with 50 mM potassium phosphate buffer pH 7.4, centrifuged again ($5000 \times g$, 4°C for 10 min) and the pellets were stored at -20°C .

In case of whole-cell conversion, frozen cells of BL21-(pcyp105D1)-(pPdr-Pdx) overexpressing Pdx, Pdr, and CYP105D1 were resuspended in 50 mM potassium phosphate buffer and cell wet weight was adjusted to 50 g/L. All bioconversions were carried out in 5 mL scale. The substrate was added to the reaction mixture with the final concentration of 100 mg/L. The temperature and the pH were optimized and set to the respective values.

A series of different organic cosolvents (menthol, ethanol, acetone, DMSO, DMF, PEG-200) at the concentration of 2.0 vol% were used to enhance the substrate solubility and further increase the bioconversion rate. And various permeabilization agents (Triton-X100 (3 $\mu\text{L}/\text{mL}$), CTAB(0.1%), Polymyxin B(20 $\mu\text{g}/\text{mL}$)) were tested for enhancing the bioconversion rate of whole cell system.

2.6 | Analytical methods

The identification of the products was carried out using Agilent High-performance liquid chromatography 1260 (Agilent Technologies, Palo Alto, CA, USA). The products and substrates were separated on a reversed phase C18 column (4.6×250 mm, 5 μm ; Shim-pack VP-ODS; Japan) at a flow rate of 1 mL/min. The mobile phase A was deionized water containing 0.1% v/v formic acid and 1 mM ammonium formate, the mobile phase B was acetonitrile. The ratio of phase A and phase B equilibrated at 75:25 for analysis biotransformation profile of verapamil and detention time was 30 min, the column oven temperature was 30°C , and the UV absorbance was 278 nm.

An Agilent 6530 Q-TOF mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source was used to perform the MS analysis. The acquisition parameters were as follows: drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature, 350°C ; nebulizer, 35 psig; capillary, 4000 V; OCT RFV, 750 V; and fragmentor voltage, 120 V. The mass range was recorded from m/z 100–1500 in negative modes with collision energy (CE) from

10 eV–50 eV. Peaks were detected by positive ionization mode of MS and MS/MS detection.

For nuclear magnetic resonance (NMR) analysis of the purified product, compounds were dissolved in CD₃OD solution and subjected to Bruker AV-300 spectrometer for one-dimensional ¹H-NMR, ¹³C-NMR.

NMR data for norverapamil

¹H NMR (500 MHz, MeOD): δ = 7.04 (ddd, J = 19.0, 9.5, 2.2 Hz, 3H), 6.94 (d, J = 8.2 Hz, 1H), 6.88 (d, J = 2.0 Hz, 1H), 6.81 (dd, J = 8.2, 2.0 Hz, 1H), 3.86 (dd, J = 15.4, 8.4 Hz, 12H), 3.18 (td, J = 7.5, 2.1 Hz, 2H), 3.08 (ddd, J = 12.2, 10.7, 5.6 Hz, 1H), 3.02–2.85 (m, 3H), 2.28 (td, J = 11.8, 4.8 Hz, 2H), 2.14–2.00 (m, 1H), 1.80–1.69 (m, 1H), 1.39 (ddd, J = 17.5, 12.2, 5.4 Hz, 1H), 1.26 (d, J = 6.6 Hz, 3H), 0.82 (d, J = 6.7 Hz, 3H) ppm.

¹³C NMR (126 MHz, MeOD): δ = 151.08(C17), 150.89(C4), 150.42(C5), 149.89(C18), 131.49(C14), 130.23(C1), 122.14(C15), 122.05(C28), 120.50(C2), 113.66(C3), 113.48(C6), 113.02(C16), 111.08(C19), 56.75(C23), 56.54(C21), 56.54(C31), 56.52(C33), 54.70(C13), 50.08(C10), 48.40(C8), 38.69(C25), 35.62(C12), 32.84(C7), 24.13(C11), 19.34(C27), 18.88(C26) ppm.

2.7 | Molecular docking

CYP105D1 has been simulated in our previous study [16]. The default parameters of MOE-Dock program were used for the molecular docking of the ligands. The parameters used were the default values.

3 | RESULTS

3.1 | In vitro conversion of verapamil by CYP105D1

Soluble CYP105D1 (calculated MW 42 kDa) was obtained from *E. coli* BL21(DE3) and purified with Ni-NTA to apparent homogeneity as judged by SDS-PAGE (Figure 1A). The CO-reduced difference spectrum of purified CYP105D1 showed that CYP105D1 exhibited absorption maximum at around 450 nm, indicating that CYP105D1 was primarily in an active form. The Pdx and Pdr were cloned into the expression vector pET22b respectively for fusing Hig 6-tag in the C-terminal. The SDS-PAGE analysis of soluble fraction of proteins showed a protein band of corresponding molecular weight of Pdx and Pdr, respectively (Figure 1B, C).

To analyze the potential catalytic ability of CYP105D1 for biotransformation of verapamil, and to confirm the region of the selectivity, the in vitro experiment was performed. The biocatalytic activity of P450s generally consists of many steps associated with redox partners, which transfer electrons from NAD(P)H to the heme active center. In this study, Pdx/Pdr

was used as the universal redox partner system in vitro experiment. The reaction mixture containing the CYP105D1 and Pdx/Pdr was used for the biotransformation of verapamil. The reaction mixture only containing Pdx/Pdr without CYP105D1 was used as a negative control. The substrate was examined at the concentration of 0.2 mM. After in vitro biocatalysis of verapamil within 60 min, the formation of the product was detected by High performance liquid chromatography with diode array detector (HPLC-DAD). The products can be detected only in the reaction mixture containing CYP105D1 indicating that CYP105D1 could biotransform verapamil.

Following, crude extract of the reaction mixture was analyzed by HPLC and HPLC-qTOF-MS. HPLC analysis suggested that verapamil could be biotransformed by CYP105D1, producing only one product in the presence of the redox partner Pdx/Pdr (Figure 2D). The retention time of the product was 22.357 min that was identical to that of the authentic norverapamil sample (Figure 2A–D). HPLC-qTOF-MS analyses of biocatalytic profile of verapamil revealed the accumulation of demethylated product with m/z 441 [M+H]⁺. The qTOF-MS/MS spectral was used to further analyse product. The major fragment obtained from the product gave the [M+H]⁺ at m/z 398, 289, 260, 247, and 218, which matched well with the authentic norverapamil (Figure 2E, F). The fragment at 398.2236 and 289.1918 of the product suggested that the N-methyl of verapamil was demethylated (Figure 2G). These results demonstrated that CYP105D1 catalyzed verapamil N-demethylation to produce norverapamil (Figure 3).

The apparent steady-state kinetic parameters were determined by directly monitoring the accumulation of products using HPLC with the electron-transport redox partner Pdx and Pdr. The K_m and k_{cat} values for verapamil were $50.94 \pm 8.49 \mu\text{M}$ and $34.76 \pm 1.74 \text{ min}^{-1}$, respectively (Figure 2H). The catalytic efficiency (k_{cat}/K_m) for verapamil was $0.68 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$.

3.2 | Whole cell biotransformation of verapamil

The conversion of verapamil using a coexpression system of CYP105D1 and the redox partner Pdx and Pdr (BL21-(pcyp105D1)-(pPdr-Pdx)) was conducted for preparative synthesis of norverapamil (Figure 4A). Matched well with the in vitro bioconversions, only one product was detected during the whole cell biotransformation and the retention time of the product (22.36 min) was identical to that of the authentic norverapamil. The *E. coli* BL21 strain containing the pPdr-Pdx vector, which was used as a negative control for the CYP105D1-dependent conversions, showed no activity toward verapamil. The conversion rate of verapamil by the whole cell system was 13.21% during 24 h in the initial tests (Figure 4B).

FIGURE 1 Expression and purification of CYP105D1, Pdx, and Pdr. (A) SDS-PAGE of expression and purification of CYP105D1. Lane 1, prestained protein marker. Lane 2, cell lysate before induction with IPTG. Lane 3, cell lysate after 24 h of expression. Lane 4 purified CYP105D1. (B) SDS-PAGE of expression and purification samples of Pdx. Lane 1, prestained protein marker (BioLab). Lane 2, cell lysate before induction with IPTG. Lane 3, cell lysate after expression for 24 hours. Lane 4 purified Pdx. (C) SDS-PAGE of expression and purification of Pdr. Lane 1, prestained protein marker (BioLab). Lane 2, cell lysate before induction with IPTG. Lane 3, cell lysate after expression for 24 hours. Lane 4, purified Pdr.

To further test, the product was identified by nuclear magnetic resonance (NMR) spectroscopy. In order to obtain sufficient amounts of verapamil metabolites for further characterization by NMR spectroscopy, whole-cell conversions with a total culture volume of 1 L were performed. The product was obtained with sufficient purity and amounts (5 mg) for structural characterization via NMR spectroscopy (Supporting Information Fig. 1). The ^{13}C -NMR spectrum of the product showed the lack of signals at δ 42.9 ppm and other signals were matched with verapamil, which indicated that the N-methyl group of verapamil was eliminated by the biotransformation. NMR analysis revealed that CYP105D1 is able to N-demethylate verapamil resulting in the formation of norverapamil.

3.3 | Determination of temperature and pH

The effect of different temperature ranges on the biotransformation of verapamil by BL21-(pcyp105D1)-(pPdr-Pdx) has been shown in Figure 5A. The optimum temperature for biotransformation of verapamil by resting cells of BL21-(pcyp105D1)-(pPdr-Pdx) was 25°C. When the reaction temperature was higher than 30°C or lower than 20°C, the product formation began to decrease significantly.

The effect of pH was evaluated on norverapamil yield and the results have been shown in Figure 5B. It was evident from the data that the optimal pH for verapamil biotransformation by BL21-(pcyp105D1)-(pPdr-Pdx) was 7.5. At lower pH (6–7), product formation was dramatically decreased.

3.4 | Effect of the cosolvent and cell permeabilization on bioconversion rate

By adding the cosolvents, the solubility of the substrate can be improved which further increased the bio-catalytic

efficiency. In this study, we tested the effect of various cosolvents (methanol, ethanol, acetone, DMF, DMSO, PEG-200) on whole cell system. As shown in Figure 6A, the highest bioconversion rate could be obtained when PEG-200 was used as the cosolvent for the biotransformation of verapamil compared with the other cosolvents. In the presence of 2.0 vol% PEG-200, the turnover rate of verapamil reached to $40.61 \pm 5.28\%$.

The hindered transport of the substrate over the outer membrane of the Gram-negative *E. coli* cells is one of the limitations of whole cell biotransformation. To overcome this problem, we tested the influences of different permeabilizations on whole cell biotransformation. In this study, the effects of CTAB, Triton-X100, and the peptide antibiotic polymyxin B at an appropriate concentration were tested. The results are presented in Figure 6B. The highest bioconversion rate could be observed with polymyxin B as permeabilizing agent together with PEG-200 as cosolvent.

For further characterization, we analyzed the time course of product formation during the whole-cell conversion. As shown in Figure 7, the maximum turnover rate of verapamil reached to $60.16 \pm 3.42\%$ within 24 hours.

3.5 | Molecular docking of substrates to CYP105D1

The crystal structure of CYP105D1 simulated in our lab previously (data will be published) was used to investigate the substrate-enzyme interaction and observed regioselectivity of N-demethylation for verapamil, by docking of the substances into the active site of CYP105D1. Both substrates appeared in docking positions allowing demethylation at experimentally identified positions as nitrogen (distance ~ 6.33 Å) for verapamil. Nine amino acid residues including Ile402, Thr401, Asp400, Leu188, Gln302, Phe92

FIGURE 2 Reverse-phase HPLC profile and Mass spectra of verapamil and its metabolite by CYP105D1. The HPLC analysis of authentic standard of verapamil (A) and norverapamil (B), the HPLC analysis of verapamil biotransformation by CYP105D1 (D) or without the enzyme as blank (C). The mass spectra of the authentic standard of norverapamil (E) and the biotransformed product (F). The chemical structures of norverapamil and its mainly MS fragmentation scheme (putative structures) were shown in section G. Determination of the kinetic parameters for verapamil catalyzed by CYP105D1. The kinetic studies were performed with a reconstituted system containing CYP105D1, Pdx, and Pdr. The reaction velocities were plotted against increasing substrate concentrations (H)

Leu103, Leu187, and Leu301 were predicted to interact with the substrate (Figure 8). The results predicted that the verapamil was bound by van der Waals forces and hydrophobic interactions.

4 | DISCUSSION

Norverapamil is a promising leading compound for reversing MDR with less side effects [6,17]. Herein, an efficiently

FIGURE 3 Schematic representation of the N-demethylation of verapamil by CYP105D1

FIGURE 4 (A) Schematic representation of the regioselective N-demethylation of verapamil for synthesis of norverapamil by CYP105D1 with Pdx/Pdr in the *E. coli* BL21 system. (B) SDS-PAGE showing the coexpressed proteins of CYP105D1 and Pdx/Pdr. Lane 1, protein marker; lane 2, before induction form of CYP105D1 and Pdx/Pdr; lane 3 and 4, total form of CYP105D1 and Pdx/Pdr; lane 5, soluble form of CYP105D1 and Pdx/Pdr. (C) Reverse-phase HPLC profile of verapamil and its metabolite norverapamil by CYP105D1-based whole cell system

FIGURE 5 (A) Effect of temperature on the production of norverapamil from verapamil by CYP105D1-based whole biotransformation. The reactions were conducted at pH 7.5 for 24 h by varying temperature from 20 to 35°C. (B) Effect of pH on the production of norverapamil from verapamil by CYP105D1-based whole biotransformation. The reactions were conducted at 25°C for 24 h by pH value from 6.0 to 8.0. Data represent the means of three experiments and error bars represent the standard deviation

biocatalytic method for norverapamil synthesis was successfully developed. Under the optimized conditions, the bioconversion rate of verapamil reached to 61.73% using the CYP105D1-based whole cell biocatalytic system with high specific. To the best of our knowledge, this is the

first report about the biocatalytic synthesis of norverapamil using prokaryotic cytochrome P450 that is a promising method for synthesis of norverapamil in pharmaceutical industry. Compared with the chemical synthesis of norverapamil [18], CYP105D1-based bioconversion system is under

FIGURE 6 (A) Effects of different cosolvents (at 2.0% concentration) on the bioconversion ratio of verapamil by CYP105D1-based whole cell system. The reactions were conducted at 25°C for 24 h by pH value of 7.5. (B) Effects of different permeabilization (CTAB (0.1% w/v), Triton X-100 (3 μ L/mL), Polymyxin B (20 μ g/mL)) on the bioconversion ratio of verapamil by CYP105D1-based whole cell system. The reactions were conducted at 25°C for 24 h by pH value of 7.5 using PEG-200 (2.0% v/v) as cosolvent

FIGURE 7 Time-dependent norverapamil formation by CYP105D1 based whole-cell system

mild conditions, environmentally friendly and easier to scale-up [19].

It was reported that microsome P450s (such as CYP1A2 and CYP3A4) can mediated the biotransformation of verapamil [5]. While their low turnover rates and poor expression levels in *Escherichia coli* cell system were obstacles for application of the microsome P450s. It was also reported that *Cunninghamella blakesleeana* can mediate the biotransformation of verapamil [20]. Although norverapamil is one of the metabolisms of verapamil by *Cunninghamella blakesleeana*, the selectivity of the biotransformation by *Cunninghamella blakesleeana* is very poor (23 metabolisms can be produced), which is an obstacle for the purification and also not suitable for norverapamil production. Compared with the microsome P450s and *Cunninghamella blakesleeana*, CYP105D1 could be easily expressed in *E. coli* system [21] and showed relatively high efficiency and selectivity that exhibited promising properties for norverapamil synthesis.

Interestingly, the verapamil molecular has multiple methyl groups including four different oxy-methyl groups and one

FIGURE 8 Docking model of verapamil as a substrate for the active site of CYP105D1: the interaction between CYP105D1 active site with verapamil (A); the distance between the heme iron of CYP105D1 with the N-methyl of verapamil (B)

nitrogen-methyl group, all of which would be the active reaction sites of cytochrome P450s [22]. Our results exhibited that CYP105D1 showed high specificity to the nitrogen-methyl group and no other demethylated derivatives were detected in the biotransformation profiles. Docking analysis was conducted to explain the specificity of the biotransformation. And it was shown that the N-methyl of verapamil was faced with the heme-iron, which might reveal the mechanism of the specific N-demethylation.

Verapamil exhibits relatively low aqueous solubility in the alkaline buffer. The low aqueous solubility led to poor availability of the substrate to whole-cell biocatalysts. In this study, PEG-200 was selected as the optimal cosolvent for verapamil. Polyethylene glycols (PEGs) have been frequently used as pharmaceutical cosolvents in the preparation of liquid dosage forms [23]. PEGs provide several preferred properties such as nontoxic to strains, environment-friendly, wide application and cost effectively than organic solvents and surfactants.

In summary, we were able to demonstrate the specific N-demethylation of verapamil catalyzed by CYP105D1 both in vitro or in whole cell system. The constructed CYP105D1-based whole cell biocatalytic system has the potential for norverapamil production with high efficiency in industrial scale. The N-demethylated product norverapamil represent pharmaceutically interesting for further development.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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