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Original Article

Cloning, Expression and Identification of the Enzyme P450-SCA12 from *Streptomyces cavourensis* YBQ59

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Abstract: The use of microbial cytochrome P450 as a biocatalyst offers significant potential for the development of natural compounds due to its ability to transform a wide range of structures with high regio- and stereoselectivity. In this study, a 1263 bp gene from *S. cavourensis* strain YBQ59, encoding the CYP154C subfamily enzyme P450-SCA12, was successfully expressed in *E. coli* CD43(DE3). The enzyme P450-SCA12 was produced in a soluble form when fused with thioredoxin (Trx) and 6xHis proteins from the pET32b(+) vector. After purification through ion affinity chromatography using a His-select® cobalt affinity gel column, the enzyme P450-SCA12 was obtained in an active form, with a molecular weight of approximately 63 kDa and a yield of around 1000 nmol/L from the Terrific Broth culture medium. Screening of substrates revealed that this enzyme specifically metabolizes three steroid compounds: testosterone, nandrolone, and 4-androstenedione. These findings highlight the potential of the P450-SCA12 enzyme from the actinomycete *S. cavourensis* YBQ59 in natural product development, particularly for metabolizing steroid compounds to create new steroid-based drugs.

Keywords: P450, steroids, CYP154C3, *Streptomyces cavourensis*, YBQ59.

1. Introduction

Cytochrome P450 (P450) is a superfamily of heme-thiolate proteins that function as monooxygenases, catalyzing the incorporation of an oxygen atom from an O_2 molecule into their substrates. These enzymes are known for their remarkable versatility as biocatalysts, capable of oxidizing a wide range of compounds, including steroids, alkaloids, terpenes, fatty acids, and fatty alcohols. They also catalyze various reactions, such as decarboxylation, S-, N-, and O-dealkylation, oxidative cyclization, nitrogen oxidation, sulfoxidation, epoxidation, decarbonylation, aryl–aryl coupling, and C–C bond cleavage [1, 2]. P450 enzymes are widely distributed

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across all domains of life, including viral genomes, with 2,252 families and over 300,000 sequences cataloged in the CYP450 database (https://drnelson.uthsc.edu/) [3]. The ability of P450 enzymes to selectively oxidize unactivated C–H bonds under mild conditions makes them highly valuable for natural product development, as this process is crucial but challenging in chemical synthesis [1, 4]. With these capabilities, P450 enzymes are promising biocatalysts in the pharmaceutical industry, meeting the increasing demand for novel therapeutics and enabling the discovery of more efficient synthetic pathways for existing drugs.

Among natural producers of P450 enzymes, the genus *Streptomyces* is considered one of the most promising candidates. Genome sequencing has revealed that *Streptomyces* species possess a significantly higher number of P450 genes compared to other organisms; for instance, *Streptomyces avermitilis* has 33, *Streptomyces scabies* has 25, and *Streptomyces coelicolor* contains 18 P450 genes in their genomes [5]. Furthermore, extensive research has shown that P450 enzymes in *Streptomyces* play a key role in catalyzing the late stages of biosynthetic pathways, thereby enhancing the bioactivity of secondary metabolites [6-8]. However, of the approximately 7,500 P450s identified in *Streptomyces*, only about 2.4% have had their functions characterized, and just 0.4% have had their structures resolved [9]. Therefore, investigating P450 enzymes in *Streptomyces* represents an emerging and promising research direction for enzyme applications across various industries, particularly in the pharmaceutical industry.

Streptomyces cavourensis YBQ59 is an actinomycete species isolated from the roots of cinnamon (*Cinnamomum cassia*) in Yen Bai, Vietnam. Genome sequencing results [10] revealed that *S. cavourensis* YBQ59 contained 21 putative P450 genes in its genome. In this study, we cloned, expressed, purified, and identified the enzyme P450-SCA12 from *S. cavourensis* YBQ59.

2. Experimental

2.1. Materials

NADPH, acrylamide gel, phenylmethylsulfonyl fluoride (PMSF), isopropyl β-D-1-thiogalactopyranoside (IPTG), δ-aminolevulinic acid (δ-Ala), and the expression host *Escherichia coli* CD43(DE3) were purchased from Sigma-Aldrich (USA). Ethyl acetate, n-hexane, $FeSO_4$, and the expression plasmid pET32b(+) were purchased from Merck (Germany). EcoRI and HindIII were purchased from New England Biolabs (USA), and PCR master mix was purchased from Phusa Biochem (Vietnam). The DNA of *S. cavourensis* YBQ59 was provided by the VAST-Culture Collection of Microorganisms, Institute of Biotechnology, Vietnam Academy of Science and Technology. A library of natural compounds (Table 1) and plasmids containing redox proteins were provided by the Institute of Biochemistry II, Heinrich Heine University Düsseldorf, Germany.

2.2. Gene Cloning and Vector Construction

The gene encoding the putative P450-SCA12 was identified from the whole genome sequence of *S. cavourensis* YBQ59 (Accession number: JASEND000000000.1) and amplified using primers Sca12_F (CCAATgaattcCgtgaactgcccgcacgc) and Sca12_R (CCAATaagctttcagcccagcaggaccg), which were designed with EcoRI and HindIII restriction sites at their 5' ends, respectively. PCR cycling conditions were follows: Initial denaturation at 95 °C for 4 minutes; 35 cycles of 95 °C for 45 seconds, 58 °C for 45 seconds, and 72 °C for 1 minute 30 seconds; followed by a final extension at 72 °C for 10 minutes and a hold at 25 °C for 10 minutes. After amplification, the PCR product was purified, treated with EcoRI and HindIII, and inserted into the expression plasmid pET-32b(+), which had been linearized with the same enzymes, resulting in the expression vector pET32_P450-SCA12. This plasmid was then transformed into *E. coli* DH5α cells. Transformants carrying the recombinant plasmid were selected on LB agar containing 100 μg/mL ampicillin after overnight incubation at 37 °C.

2.3. Gene Expression of P450-SCA12

E. coli C43(DE3) cells transformed with the vector pET32_P450-SCA12 were inoculated into LB (Lysogeny Broth) medium containing 100 μg/mL ampicillin and cultured at 37 °C with vigorous shaking at 200 rpm. A 1% (v/v) aliquot of this LB culture was then transferred to TB (Terrific Broth) medium, supplemented with 100 μg/mL ampicillin, and grown under the same conditions until the optical density at 600 nm (OD_{600}) reached 0.6–1.0. Enzyme expression was induced by adding 0.375 μM IPTG, along with 0.5 μM δ-ALa and 0.1 μM $FeSO_4$ to facilitate heme synthesis. The culture was incubated at 25 °C for 24 hours with shaking at 200 rpm. Cells were harvested by centrifugation at 4,000×g for 15 minutes at 4 °C, washed with potassium phosphate buffer (20 mM, pH 7.4) containing 10% glycerol, and used directly for cell lysate preparation during enzyme purification. The expression of P450-SCA12 was checked by SDS-PAGE electrophoresis.

2.4. Purification of P450-SCA12

The cells were disrupted by sonication. The cell pellets were resuspended in lysis buffer (50 mM potassium phosphate, 100 mM NaCl, 0.1 mM PMSF, and 10% glycerol, pH 7.4) in a total volume of 30 mL and sonicated using a Sonics Vibra-Cell VCX130 (Thermo Fisher Scientific, USA) at 4 °C with 65% amplitude for 30 minutes (10 seconds on, 30 seconds off). The cell lysate was then centrifuged at 10,000×g for 20 minutes at 4 °C to remove cell debris. The P450-SCA12 enzyme, with a 6xHis tag, was purified using His-select® cobalt affinity gel (Thermo Fisher, USA). The affinity column was equilibrated with two resin-bed volumes of 50 mM potassium phosphate buffer, pH 7.4 containing 10 mM imidazole and 10% glycerol. The cell lysate was loaded onto the column, and non-specifically bound proteins and contaminants were washed away using a wash buffer (20 mM imidazole in 50 mM potassium phosphate buffer, pH 7.4 containing 10% glycerol). The bound enzyme was then eluted with an elution buffer (100 mM imidazole in 50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol). Enzyme-containing fractions were pooled, and potassium phosphate buffer (50 mM, pH 7.4) containing 10% glycerol was added. The mixture was concentrated using Amicon centrifugal filters (Millipore, USA) with a 30 kDa molecular weight cut-off at 4 °C and 4,000 x g for 30 minutes. The enzyme's purity was confirmed by SDS-PAGE electrophoresis.

2.5. Determination of Enzyme Concentration

The concentration of P450-SCA12 was determined using the method of Omura and Sato [11] with a double-beam spectrophotometer (UV-6300PC). The enzyme was diluted to a concentration of 2 μM in potassium phosphate buffer (50 mM, pH 7.4, containing 10% glycerol), and CO gas was bubbled through the solution for 30 seconds. The solution was then split between a sample cuvette and a reference cuvette. A few grains of dithionite were added to the sample cuvette and left for 1 minute at room temperature. The absorbance spectrum was recorded from 400 to 500 nm at room temperature and repeated several times to ensure complete enzyme reduction. Measurements were repeated three times, and the results were reported as means ± SD.

2.6. Substrate Screening

Substrate screening was performed based on the principle of inducing a type I spectral shift, where hit compounds bind to the P450 enzyme, generating UV-Vis spectra with a maximum around 390 nm and a minimum around 420 nm [12]. A library of 20 different compounds was screened following the protocol as described elsewhere [13].

2.7. In vitro Bioconversion

The experiment was conducted in a final volume of 500 μL at 30 °C. The reaction mixture contained 2 μM P450-SCA12 in potassium phosphate buffer (50 mM, pH 7.4, containing 10% glycerol), 200 μM of substrate (dissolved in DMSO), 25 μM of YkuN

(flavodoxin from *Bacillus subtilis*), 2.5 μM of FdR (flavodoxin reductase from *Escherichia coli*), and an NADPH regeneration system consisting of GDH (5 U/mL), glucose (20 mM), and NADPH (500 μM). NADPH was added last to initiate the reaction. After 1 hour of incubation, 500 μL of ethyl acetate was added to the reaction mixture to precipitate and remove the enzyme. The reaction mixture was extracted twice with ethyl acetate, and the solvent was evaporated using a vacuum dryer before analysis.

2.8. Thin Layer Chromatography Analysis

The products of the *in vitro* conversions were analyzed by thin-layer chromatography (TLC) using silica gel 60 F254 plates (Fluka, Germany) as the stationary phase and a mixture of ethyl acetate and n-hexane (4:1) as the mobile phase. The sample separations were visualized using a UV254 lamp.

3. Results and Discussion

3.1. Gene Cloning and Sequence Analysis

The 1263-bp gene encoding P450-SCA12 was amplified from the genome of *S. cavourensis* YBQ59 using specific primers. Electrophoresis confirmed that the amplified gene fragment matched the expected size (Figure 1). The deduced amino acid sequence of P450-SCA12, consisting of 420 amino acids (aa), was compared to the sequences of P450 enzymes available on the P450 homepage; showing 89% identity with CYP154C3 from *Streptomyces griseus* (WP_012378290.1). Therefore, this enzyme represents another isoform of CYP154C3.

3.2. Protein Purification and Spectrophotometric Characterization

The gene encoding P450-SCA12 was expressed in *E. coli* C43(DE3). After purification using a His-select® cobalt affinity gel column, the purified P450-SCA12 displayed a molecular mass of approximately 63 kDa on SDS-PAGE (Figure 2). As previously mentioned, the deduced amino acid sequence of P450-SCA12 consists of 417 amino acids and has a theoretical molecular mass of about 46 kDa. Therefore, the size of the purified protein is 17 kDa higher than the original protein due to the fusion with Trx-His-s-enterokinase when expressed using the pET32b(+) vector.

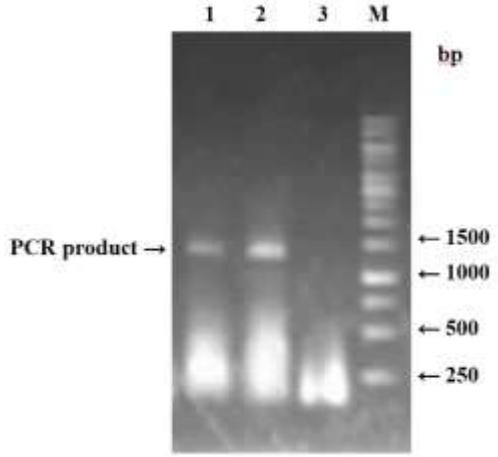

Figure 1. PCR product of gene encoding P450-SCA12. Lane 1, 2: PCR products of P450-SCA12 gene; Lane 3: negative control without DNA template; Lane M: marker DNA (1 kb- Fermentas).

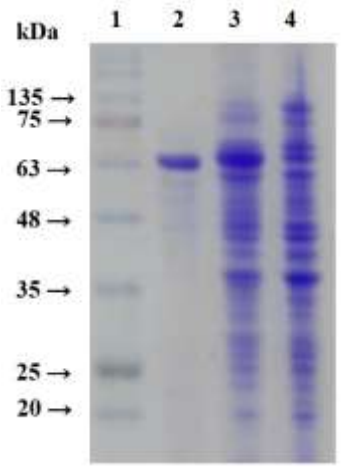

Figure 2. SDS-PAGE of recombinant P450-SCA12 expressed in *E. coli* C43(DE3) system. Lane 1: Protein marker (GangNam STAIN); Lane 2: Purified P450-SCA12; Lane 3: Crude extract of *E.coli* expressing P450-SCA12; Lane 4: Crude extract of *E. coli* cells habouring pET32b(+) as negative control.

It is known that the Fe^{3+} ion in the heme structure gives P450 a maximum absorption spectrum at 420 nm. In the presence of CO and a reducing agent (sodium dithionite), the heme moiety binds to CO, and the heme iron is reduced to Fe^{2+}, which causes an absorption maximum at 450 nm. As illustrated in Figure 3, the absorption spectrum of purified P450-SCA12 showed a typical maximum peak at 449 nm and a slight absorption peak at 420 nm, confirming that the protein was successfully expressed in its active form. The yield of recombinant P450-SCA12 was calculated using the formula of Omura and Sato [11] to be around 1000 nmol per liter of TB culture medium (corresponding to approximately 60 mg per liter of culture).

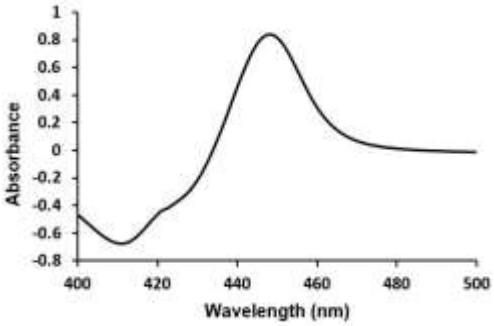

Figure 3. Absorbance spectrum of purified P450-SCA12 in the presence of CO.

The P450-SCA12 from *S. cavourensis* YBQ59 was expressed in its active form when fused with Trx and the 6xHis tag of pET32b(+) in *E. coli* C43(DE3). This result agrees with Subedi et al. [14], who expressed CYP154C3-1 and CYP154C3-2 from *Streptomyces* sp. in *E. coli* C41(DE3) with the pET32a plasmid. These P450s were also expressed as fusions with Trx-6xHis-enterokinase at the N-terminus and displayed a CO spectrum at 449 nm [14]. This suggests that the Trx and 6xHis tag at the N-terminus does not interfere with the activity of the CYPs. TrxA significantly enhances the high-level production of soluble fusion proteins in the *E. coli* cytoplasm. In many cases, heterologous proteins expressed as TrxA fusion proteins are correctly folded and exhibit biological activity [15].

Furthermore, the expression yield of P450-SCA12 was quite favorable compared to previous studies on P450s in the CYP154 family and other P450s from *Streptomyces* sp. in *E. coli* hosts. The CYP154C3 from *S. griseus*, which is fused at the C-terminus with the P450 reductase domain (RED) of a P450 from *Rhodococcus* sp., was expressed in its active form (11.5 ± 2.2 nmol per liter of culture) in *E. coli* BLR(DE3) [16]. Similarly, CYP105D1 from *S. griseus* was produced in its active form in *E. coli*, *yielding amounts more than 1000 nmol per liter of culture* under the control of the tac and phoA promoters, respectively [17, 18].

3.3. Substrate Screening

Iron in the heme cofactor of P450 can exist in both a low-spin state (six-ligand binding) and a high-spin state (five-ligand binding). In solution, P450 primarily exists in a low-spin state, with the sixth axis ligand being water. When the substrate is present, the bond with water is broken, resulting in a shift in the spin state that leads to a type I spectral change [19]. Type I spectral changes are characterized by an absorption spectrum featuring a peak around 385 nm and a trough near 420 nm [20]. Compounds that induce this change are considered potential substrates for the P450 enzyme. The ligand bond between water and iron can be replaced by the direct binding of a free electron pair donor to the iron ion. This transformation causes a type II spectral change, which is marked by a broad absorption trough between 390 and 420 nm and a peak between 425 and 435 nm [20]. Substances that produce this type of spectral change are typically enzyme inhibitors.

In this study, 20 natural compounds from five different groups were used for substrate screening (Table 1). The substrate screening revealed that all three steroids (testosterone, nandrolone, and 4-androstenedione) induced type I spectra with P450-SCA12, while the other compounds did not (Figure 4). This finding suggests that the enzyme is capable of metabolizing steroids.

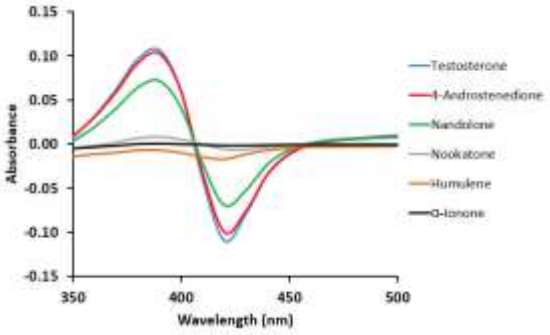

Figure 4. Type I spectra of 6 screened compounds (testosterone, 4-androstenedione, nootkatone, humulene, α-ionone, nandrolone) with P450-SCA12.

3.4. In vitro Conversion

To confirm the ability of P450-SCA12 to convert steroids, we tested the in vitro conversion of three substrates: 4-androstene-3,17-dione, nandrolone, and testosterone. The results presented in Figure 5 indicate that P450-SCA12 completely converted all three steroids into more hydrophilic derivatives, which migrated more slowly than their parent substrates on TLC plates. Their conversion products were specific, with only one compound formed for each substrate.

Table 1. List of screened compounds

Number	Compound	Number	Compound
Monoterpenoids		*Fatty acids*	
1	Limonene	11	Hexanoic acid
2	∝- Pinene	12	Octanoic acid
3	Carvone	13	Nonanoic acid
4	Camphor	14	Decanoic acid
Sesquiterpenoids		15	Dodecanoic acid
5	∝-Ionone	*Phenolic compounds*	
6	Nootkatone	16	p-Coumaric acid
7	Humulene	17	Eugenol
Steroids		18	Catechol
8	Testosterone	19	Cafeic acid
9	Nandrolone		
10	4-Androstenedione	20	Ferrulic acid

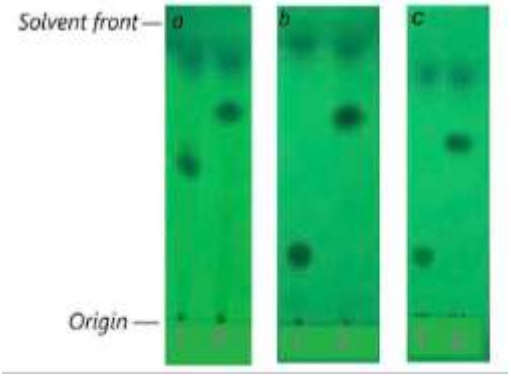

Figure 5. Conversion of steroids by P450-SCA12.

a) Lane 1: Extract from the *in vitro* conversion of 4-androstenedione by SCA12, Lane 2: Control 4-androstenedione sample; b) Lane 1: Extract from the *in vitro* conversion of testosterone by SCA12, Lane 2: Control testosterone sample; c) Lane 1: Extract from the *in vitro* conversion of nandrolone by SCA12, Lane 2: Control nandrolone sample.

Members of the CYP154C subfamily are known to metabolize a wide variety of compounds with diverse chemical structures, sizes, and molecular weights, and most can convert steroids [21-24]. Notably, CYP154C3 has been reported to transform various steroids. The CYP154C3 enzyme from *S. griseus* IFO13350 specifically metabolized at the 16α-hydroxy position of various steroids, including progesterone, testosterone, adrenosterone, dehydroepiandrosterone, deoxycorticosterone, 1,4-androstadiene-3,17-dione, and 4-pregnane-3,11,20-trione [16]. Additionally, two enzymes, CYP153C3-1 and CYP153C3-2 from *Streptomyces* sp. W2061, also converted steroids such as adrenosterone, cortisone, and prednisone [14]. The screening revealed that P450-SCA12 can efficiently transform three steroids: testosterone, 4-androstene-3,17-dione, and nandrolone demonstrating high conversion rates and regioselectivity. This suggests that the enzyme has potential for cost-effective and environmentally friendly applications in the production of pharmaceutical steroid derivatives. Such advancements could significantly contribute to the field of steroid biotransformation, which has been studied since around 1950 [25].

4. Conclusion

In this study, we cloned, expressed, and purified cytochrome P450-SCA12, a CYP154C3 enzyme from *S. cavourensis* YBQ59, an actinomycete species isolated from cinnamon roots in Yen Bai, Vietnam. This enzyme effectively metabolized steroids such as testosterone, nandrolone, and 4-androstene-3,17-dione into more hydrophilic derivatives with high conversion rates. This result demonstrates the potential of P450-SCA12 for use in developing novel steroid-based drugs.

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References

[1] G. D. Nardo, G. Gilardi, Natural Compounds as Pharmaceuticals: The Key Role of Cytochromes P450 Reactivity, Trends Biochem. Sci., Vol. 45, 2020, pp. 511-525, https://doi.org/10.1016/j.tibs.2020.03.004.

[2] J. A. McIntosh, C. C. Farwell, F. H. Arnold, Expanding P450 Catalytic Reaction Space through Evolution and Engineering, Curr. Opin. Chem. Biol., Vol. 19, 2014, pp. 126-134, https://doi.org/10.1016/j.cbpa.2014.02.001.

[3] D. R. Nelson, Cytochrome P450 Diversity in the Tree of Life, Biochim, Biophys, Acta Proteins Proteomics, Vol. 1866, 2018, pp. 141-154, https://doi.org/10.1016/j.bbapap.2017.05.003.

[4] M. Moir, J. J. Danon, T. A. Reekie, M. Kassiou, An Overview of Late-Stage Functionalization in Today's Drug Discovery, Expert Opin, Drug Discov., Vol. 14, 2019, pp. 1137-1149, https://doi.org/10.1080/17460441.2019.1653850.

[5] M. A. Cho, S. Han, Y. R. Lim, V. Kim, H. Kim, D. Kim, Streptomyces Cytochrome P450 Enzymes and Their Roles in the Biosynthesis of Macrolide Therapeutic Agents, Biomol. Ther., Vol. 27, 2019, pp. 127-133, https://doi.org/10.4062%2Fbiomolther.2018.183.

[6] D. C. Lamb, F. P. Guengerich, S. L. Kelly, M. R. Waterman, Exploiting Streptomyces Coelicolor A3(2) P450s as a Model for Application in Drug Discovery, Expert Opin. Drug Metab. Toxicol., Vol. 2, 2006, pp. 27-40, https://doi.org/10.1517/17425255.2.1.27.

[7] J. D. Rudolf, C. Y. Chang, M. Ma, B. Shen, Cytochromes P450 for Natural Product Biosynthesis in Streptomyces: Sequence, Structure, and Function, Nat. Prod. Rep., Vol. 34, 2017, pp. 1141-1172, https://doi.org/10.1039/c7np00034k.

[8] A. Worsch, F. K. Eggimann, M. Girhard, C. J. von Bühler, F. Tieves, R. Czaja, A. Vogel, C. Grumaz, K. Sohn, S. Lütz, M. Kittelmann, V. B. Urlacher, A Novel Cytochrome P450 Mono-Oxygenase from Streptomyces Platensis Resembles Activities of Human Drug Metabolizing P450s, Biotechnol. Bioeng., Vol. 115, 2018, pp. 2156-2166, https://doi.org/10.1002/bit.26781.

[9] N. Kanoh, A. K. Asano, K. Suzuki, Y. Takahashi, T. Miyazawa, T. Nakamura, T. Moriya, H. Hirano, H. Osada, Y. Iwabuchi, S. Takahashi, An Integrated Screening System for the Selection of Exemplary Substrates for Natural and Engineered Cytochrome P450s, Sci. Rep., Vol. 9, 2019, pp. 18023, https://doi.org/10.1038/s41598-019-54473-8.

[10] H. N. T. Vu, D. T. Nguyen, H. Q. Nguyen, H. H. Chu, S. K. Chu, M. Van Chau, Q. T. Phi, Antimicrobial and Cytotoxic Properties of Bioactive Metabolites Produced by Streptomyces cavourensis YBQ59 Isolated from Cinnamomum cassia Prels in Yen Bai Province of Vietnam, Curr. Microbiol., Vol. 75, 2018, pp. 1247-1255, https://doi.org/10.1007/s00284-018-1517-x.

[11] T. Omura, R. Sato, The Carbon Monoxide-Binding Pigment of Liver Microsomes, J. Biol. Chem., Vol. 239, 1964, pp. 2370-2378, https://doi.org/10.1016/S0021-9258(20)82244-3.

[12] K. C. Leibman, A. G. Hildebrandt, R. M. Estabrook, Spectrophotometric Studies of Interactions Between Various Substrates in Their Binding to Microsomal Cytochrome P-450, Biochem. Biophys. Res. Commun., Vol. 36, 1969, pp. 789-794, https://doi.org/10.1016/0006-291X(69)90678-0.

[13] T. T. B. Ly, Y. Khatri, J. Zapp, M. C. Hutter, R. Bernhardt, CYP264B1 from Sorangium cellulosum So ce56: A Fascinating Norisoprenoid and Sesquiterpene Hydroxylase, Appl. Microbiol. Biotechnol., Vol. 95, 2012, pp. 123-133, https://doi.org/10.1007/s00253-011-3727-z.

[14] P. Subedi, K. H. Kim, Y. S. Hong, J. H. Lee, T. J. Oh, Enzymatic Characterization and Comparison of Two Steroid Hydroxylases CYP154C3-1 and CYP154C3-2 from Streptomyces Species, J. Microbiol. Biotechnol., Vol. 31, 2021, pp. 464-474, https://doi.org/10.4014%2Fjmb.2010.10020.

[15] J. McCoy, E. LaVallie, Expression and Purification of Thioredoxin Fusion Proteins, Curr. Protoc. Mol. Biol., Vol. 28, 1994, pp. 16-8, https://doi.org/10.1002/0471140864.ps0607s10.

[16] T. Makino, Y. Katsuyama, T. Otomatsu, N. Misawa, Y. Ohnishi, Regio- and Stereospecific Hydroxylation of Various Steroids at the 16α Position of the D Ring by the Streptomyces Griseus Cytochrome P450 CYP154C3, Appl, Environ. Microbiol., Vol. 80, 2013, pp. 1371-1379, https://doi.org/10.1128/aem.03504-13.

[17] M. A. Kaderbhai, C. C. Ugochukwu, S. L. Kelly, D. C. Lamb, Export of Cytochrome P450 105D1 to the Periplasmic Space of Escherichia Coli, J. Appl. Environ. Microbiol., Vol. 67, 2001, pp. 2136-2138, https://doi.org/10.1128/aem.67.5.2136-2138.200.

[18] M. Taylor, D. C. Lamb, R. Cannell, M. Dawson, S. L. Kelly, Cytochrome P450105D1 (CYP105D1) from Streptomyces Griseus; Heterologous Expression, Activity and Activation Effects of Multiple Xenobiotics, Biochem. Biophys. Res. Commun., Vol. 263, 1999, pp. 838-842, https://doi.org/10.1006/bbrc.1999.1427.

[19] K. P. Conner, C. M. Woods, W. M. Atkins, Interactions of Cytochrome P450s with Their Ligands, Arch. Biochem. Biophys., Vol. 507, 2011, pp. 56-65, https://doi.org/10.1016/j.abb.2010.10.00.

[20] C. Jefcoate, Measurement of Substrate and Inhibitor Binding to Microsomal Cytochrome P-450 by Optical-Difference Spectroscopy, Methods Enzymol., Vol. 1978, Elsevier, pp. 258-279, https://doi.org/10.1016/s0076-6879(78)52029-6 .

[21] B. Dangi, K. H. Kim, S. H. Kang, T. J. Oh, Tracking Down a New Steroid-Hydroxylating Promiscuous Cytochrome P450: CYP154C8 from Streptomyces sp. W2233-SM, Chembiochem, Vol. 19, 2018, pp. 1066-1077, https://doi.org/10.1002/cbic.201800018.

[22] B. Dangi, C. W. Lee, K. H. Kim, S. H. Park, E. J. Yu, C. S. Jeong, H. Park, J. H. Lee, T. J. Oh, Characterization of Two Steroid Hydroxylases from Different Streptomyces spp. and Their Ligand-Bound and -Unbound Crystal Structures, FEBS J., Vol. 286, 2019, pp. 1683-1699, https://doi.org/10.1111/febs.14729.

[23] L. M. Podust, Y. Kim, M. Arase, B. A. Neely, B. J. Beck, H. Bach, D. H. Sherman, D. C. Lamb, S. L. Kelly, M. R. Waterman, The 1.92-Å Structure of Streptomyces coelicolor A3(2)

CYP154C1: A New Monooxygenase That Functionalizes Macrolide Ring Systems, J. Biol. Chem., Vol. 278, 2003, pp. 12214-12221, https://doi.org/10.1074/jbc.M212210200.

[24] J. Zhu, C. Shen, W. Zhao, X. Liu, J. Liu, B. Yu, Regio- and Stereoselective Hydroxylation of Testosterone by Cytochrome P450 from Streptomyces griseus ATCC 13273, Biocatal, Biotransformation, Vol. 39, 2021, pp. 130-137, https://doi.org/10.1080/10242422.2020.1799990.

[25] W. Y. Tong, X. Dong, Microbial Biotransformation: Recent Developments on Steroid Drugs, Recent Pat. Biotechnol., Vol. 3, 2009, pp. 141-153, https://doi.org/10.2174/187220809788700157.