Journal of Molecular Catalysis B: Enzymatic 66 (2010) 234–240

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Bioconversion of aromatic compounds by *Escherichia coli* that expresses cytochrome P450 *CYP153A13a* gene isolated from an alkane-assimilating marine bacterium *Alcanivorax borkumensis*

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ARTICLE INFO

Article history:
Received 27 December 2009
Received in revised form 13 May 2010
Accepted 28 May 2010
Available online 8 June 2010

Keywords:
Alcanivorax borkumensis
Aromatic compound
Bioconversion
CYP153
Cytochrome P450
Escherichia coli

ABSTRACT

The cytochrome P450 CYP153 family has been isolated from alkane-assimilating bacteria. CYP153 has been shown to mediate terminal hydroxylations of linear alkanes or alkyl aromatics. We here performed the biotransformation of various aromatic compounds by *Escherichia coli* cells that expressed the *CYP153A13a* (P450balk) gene, which was isolated from an alkane-degading marine bacterium *Alcanivorax borkumensis*. Aromatic compounds including a short alkyl moiety or methyl ether moiety, and phenolic compounds were converted to their respective hydroxylated products, whose structures were determined by HRMS and NMR analyses. The present study revealed that the catalytic function of CYP153A13a is multifunctional, i.e., it can hydroxylate not only the terminal of short alkyl groups that attached to aromatic rings but also the *p*-position of phenolic compounds substituted with a halogen or the acetyl group. CYP153A13a was also shown to demethylate methylether-including aromatic compounds.

1. Introduction

Cytochromes P450 (P450s) compose the largest superfamily of heme-containing enzymes, and are ubiquitously distributed in organisms from bacteria to plants and mammals. P450s are involved in the activation of hormones or the metabolism of pharmaceuticals, agrichemicals and other xenobiotics in animals and plants, and mediate the biosyntheses of secondary metabolites such as antibiotics or the degradation of alkanes and aromatic compounds in microbes, while many of their functions remain unknown [1,2]. P450s catalyze mono-oxygenation reactions towards a vast variety of hydrophobic low-molecular-weight compounds such as alkanes, terpenes and aromatic compounds. These reactions contain not only hydroxylation reactions but also other redox reactions, e.g., epoxidation of alkenes, dealkylation of compounds including an *O*- or *N*-alkyl group, sulfoxylation, *N*-hydroxylation, oxidative cleavage of C–C bond, oxidative dehalogenation, oxidative aryl coupling, oxidative aryl rearrangement, Beayer–Villiger-type oxidation, and NO synthesis [1,2].

Cytochromes P450 belonging to CYP153 family have been isolated from bacteria such as *Acinetobacter calcoaceticus* EB104 and *Alcanivorax borkumensis* SK2, which utilize alkanes as the sole carbon source [3,4], amino acid sequence of which are found in a database (http://drnelson.utmem.edu/biblioE.html). *CYP153A13a* (called P450balk) that was isolated from marine bacterium *A. borkumensis* SK2 [4] is thought to mediate the first terminal hydroxylation reaction for the assimilation of an incorporated carbon source [5]. In order to act as monooxygenases, P450s must be coupled with one or two additional protein(s) to transfer two electrons from NAD(P)H to the heme domain of a P450 protein [6]. P450RhF (CYP116B2) derived from *Rhodococcus* sp. NCIMB 9784 was found as a novel self-sufficient P450 protein, in which the P450 domain is C-terminally fused to a reductase domain [7]. Nodate et al. [4] succeeded for the first time in functionally expressing a *CYP153* gene in *E. coli*, i.e., they expressed in *E. coli* the *CYP153A13a* gene with the functional expression vector pRED, which contained the DNA fragment encoding the P450RhF ferredoxin reductase (FMN) and ferredoxin (FeS) domains preceded with the linker sequence derived from P450RhF (called P450 RhF reductase domain), and showed that resultant recombinant *E. coli* cells hydroxylated the terminal regions of alkanes with carbon number

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doi:10.1016/j.molcatb.2010.05.015

of 6–8. The *E. coli* cells were further revealed to convert cyclohexane, 4-phenyl-1-butene, *n*-butylbenzene, and 2-*n*-butylbenzofuran into cyclohexanol, 2-phenyl-oxirane, 4-phenyl-1-butanol, and 4-benzofuran-2-yl-butan-1-ol, respectively [8,9]. It was also shown that a gene cluster including another *CYP153A* gene and genes for a ferredoxin reductase (FAD) and a ferredoxin was isolated from soil bacterium *Acinetobacter* sp. OC4, and *E. coli* cells expressing this gene cluster converted *n*-ankanes with 6–12 carbons to corresponding 1-alkanols or α,ω-diols [10]. *Pseudomonas putida* that harbored *CYP153A6* gene from *Mycobacterium* sp. was reported to convert limonene to perillyl alcohol [11]. In this paper, we constructed a vector pUCRED to have higher expression levels than pRED, and showed that *E. coli* cells expressing *CYP153A13a* on this vector can achieve biotransformation towards a variety of aromatic compounds.

2. Experimental

2.1. Bacterial strains and genetic manipulations

E. coli DH5α (*ECOS* Competent *E. coli* DH5α; Nippon Gene; Tokyo, Japan) was used for DNA manipulations. BL21 (DE3) (Novagen; Madison, WI, USA) or *E. coli* BL21 (Takara Bio; Ohtsu, Japan) was used as the host for the functional expression of the *CYP153a13a* (P450balk) gene. The PCR amplifications were performed using KOD plus DNA polymerase version 2 (Toyobo; Osaka, Japan) and a thermal cycler (Applied Biosystems; Foster city, CA, USA). Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs (Beverly, CA, USA) or Takara Bio. Ligation-Convenience Kit (Nippon Gene) was also used. Plasmid DNA was prepared with a QIAprep Miniprep Kit (Qiagen; Hilden, Germany). Nucleotide sequences were confirmed with Bigdye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems) and a model 3730 DNA analyzer (Applied Biosystems). All recombinant DNA experiments were basically conducted according to Sambrook and Russell [12].

2.2. Construction of a plasmid

pRED vector [4] was digested with *Eco*RI and *Hin*dIII (Takara Bio), a 1.0-kb fragment containing the P450 RhF reductase domain was recovered from agarose gel, and inserted into the *Eco*RI and *Hin*dIII-double digested pUC18 (Takara Bio), yielding a desirable vector pUCRED. The P450balk (*CYP153A13a*) gene was isolated from a marine bacterium *A. borkumensis* SK2 (DSM 11573^T) that was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [4]. This gene was amplified by PCR using a primer set, Balk-MfeIF (5′-acatcaattggATGTCAACGAGTTCAAGTA-3′) and Balk-MfeIR (5′-cagaccaattgTTTTTTAGCCGTCAACTTA-3′) as follows: 12.5 μl of 2× PrimeSTAR MaxPremix, 1.0 μl of Balk-MfeIF (10 pmol/μl), 1.0 μl of Balk-MfeIR (10 pmol/μl), 0.5 μl (20 ng) of plasmid pBalk-Red including the P450balk gene [4,9], and 10.0 μl of water were mixed, and PCR reaction was carried out with 10 s at 98 °C, 5 s at 60 °C, 7 s at 72 °C (32 cycles). The amplified P450balk gene (1410 bp) was digested with *Mfe*I, and inserted into the *Eco*RI-digested pUCRED, yielding pUCRED-Balk.

2.3. Cultivation of bacteria

Individual plasmids, pBalk-Red and pUCRed-Balk, were introduced in *E. coli* BL21 (DE3) and *E. coli* BL21, respectively. Each recombinant *E. coli* was pre-cultured at 30 °C with shaking in an LB medium (**L**-broth; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 100 μg/ml of ampicillin (Ap). After 16 h of cultivation, 500 μl of the pre-culture was inoculated into 50 ml of an LB medium containing 100 μg/ml of Ap, 100 μg/l of $FeSO_4$–$7H_2O$, 80 μg/ml of 5-aminolevulinic acid hydrochloride (5-ALA), and solutions of the Overnight Express Autoinduction system 1 (1 ml of solution 1, 2.5 ml of solution 2 and 50 μl of solution 3; Novagen) in an Erlenmeyer flask, and cultivation was continued with rotary shaking (200 rpm) for 24 h at 28 °C. The cells were collected, and suspended in 5 ml of 50 mM phosphate buffer (pH 7.2) containing 5% glycerol. This cell suspension solution was called Mixture for Bioconversion (MFB).

2.4. CO difference spectral analysis

Two mM of dithiothreitol (DTT), 200 μl of 10× BugBuster (Novagen) and 1 μl of Benzonase Nuclease (Novagen) were added to 2 ml of each cell suspension (MFB). After 20 min of shaking at room temperature, cell debris and large part of the membranes were removed by centrifugation at 8000 rpm for 10 min. The soluble fraction was then used to measure CO-reduced CYP absorption with a spectrophotometer DU 640 (Beckman; Fullerton, CA, USA). The CO difference spectra were measured as described previously [13]. In order to calculate the amount of CYP functional form, an extinction coefficient of 91 mM^{-1} cM^{-1} was used [amount of CYP functional form (nM) = CO difference a peak at OD 450 nm (OD 450 nm–OD 490 nm)/91(mM^{-1} cM^{-1}) × 1000,000].

2.5. Bioconversion of 4-methylbiphenyl by the individual recombinant E. coli cells

Five mM of proline, 1 mM of disodium dihydrogen ethylenediamine tetraacetate (EDTA), and 0.2 mM of DTT were added to 1.5 ml of MFB in a test tube. Then, 4-methylbiphenyl as substrate dissolved in DMSO was added to final concentration of 1 mM. The reaction was performed at 28 °C for 24 h with rotary shaking (170 rpm).

2.6. Conversion experiments

E. coli BL21 harboring pUCRed-Balk was grown in an LB medium containing Ap (100 μg/ml) at 30 °C with rotary shaking for 16 h. *E. coli* BL21 harboring vector pUC18 was also used as a control to monitor any side reactions that may occur with endogenous enzymes in the *E. coli* BL21 cells. 2.5 ml of these cultures were inoculated into 250 ml of an LB medium including Ap (100 μg/ml) in an 1 l Erlenmeyer flask with baffle, and cultured at 30 °C for 5–7 h with rotary shaking (100 rpm), until the absorbance at OD 600 nm reached approximately 1. Then, 5-ALA (80 μg/ml), ammonium iron (II) sulfate (0.1 mM), and isopropyl β-D-thiogalactopyranoside (IPTG; 0.1 mM) were added to the culture, and cultivation was continued at 20 °C for further 19 h with rotary shaking (100 rpm). The cells were collected by centrifugation and resuspended in 25 ml of sodium phosphate buffer (50 mM, pH 7.2) containing glycerol (5%), proline (5 mM), EDTA (1 mM), and DTT (0.2 mM). The cell suspension was divided into 96-deep well plate with 0.5 ml each, and each substrate dissolved in DMSO was added at the final concentration of 1 mM to the cell suspension, and bioconversion was performed with cultivation at 28 °C for 24 h with vortex shaking.

The substrates used in this study were purchased from Sigma–Aldrich Co., Wako Pure Chemical Industries Co. (Osaka, Japan) or Tokyo Chemical Industry Co. (Tokyo, Japan), and ibuprofen methyl ester was prepared by esterification according to literature [14].

2.7. Extraction and HPLC analysis of the products

Five hundred μl of ethyl acetate was added to reaction mixture, and shaken to extract the converted compounds in the organic layer. After centrifugation, the organic phase was analyzed by high pressure liquid chromatography (HPLC). The organic phase (10 μl)

was applied to HPLC in an Xterra MS C_{18} column (4.6 mm × 100 mm, Waters), with a photodiode array detector: (model SPD-M20A, Shimadzu). Development was at a flow rate: 1 ml/min with solvent A (5% acetonitrile (CH_3CN) and 20 mM phosphoric acid) for 3 min, then by a linear gradient from solvent A to solvent B (95% acetonitrile (CH_3CN) and 20 mM phosphoric acid) for 25 min, and finally with solvent B for 15 min, the maximum absorbance being monitored in the range of 200–500 nm.

2.8. Preparation experiments

E. coli BL21 harboring pUCRED-Balk was grown in an LB medium containing Ap (100 μg/ml) at 30 °C with rotary shaking for 16 h. Five ml of this culture was inoculated into 500 ml of an LB medium including Ap (100 μg/ml) in 2 l Erlenmeyer flasks with baffle (×4 times), and cultured at 30 °C for 5–7 h with rotary shaking (100 rpm), until the absorbance at OD 600 nm reached approximately 1. Then, 5-ALA (80 μg/ml), ammonium iron (II) sulfate (0.1 mM), and IPTG (0.1 mM) were added to the culture, and cultivation was continued at 20 °C for further 19 h with rotary shaking (100 rpm). The cells were collected by centrifugation, and resuspended in 400 ml of sodium phosphate buffer (50 mM, pH 7.2) containing glycerol (5%), proline (5 mM), EDTA (1 mM), and DTT (0.2 mM) in an 2 l Erlenmeyer flask with baffle. Each substrate dissolved in DMSO was added at the final concentration of 1 mM to the cell suspension, and bioconversion was performed with cultivation at 28 °C for 24 h with rotary shaking (160 rpm).

2.9. Purification and identification of the products

The reaction mixture (400 ml) was extracted with ethyl acetate (EtOAc; 400 ml × 2 times). The organic layer was concentrated in vacuo, and analyzed by thin-layer chromatography (TLC) on silica gel [0.25 mm E. Merk silicagel plates (60F-254)]. The converted compounds were purified by silica gel column chromatography [12 mm × 250 mm, Silica Gel 60 (Merk)]. Their structures were analyzed by mass spectrometry (MS) [HRMS (EI), Jeol AX-505W instrument or HRMS (ESI or APCI), Jeol JMS-T100LP] and nuclear magnetic resonance (NMR) (400 MHz, Bruker AMX400 instrument or 500 MHz, Varian INOVA-500AS instrument).

2.10. Chromatographic and spectroscopic data of the products

Chromatographic and spectroscopic data of the individual products (**1**–**18**) are described in Supplemental Material.

3. Results

3.1. Measurement of the functional forms of CYP153A13a proteins synthesized and conversion ratio of 4-methylbiphenyl in the individual recombinant E. coli cells

In order to measure amounts of the active form of CYP153A13a proteins synthesized in *E. coli* strains carrying the respective plasmids, pBalk-Red and pUCRED-Balk, we performed reduced CO difference spectral analysis on each cultivated cell suspension, the results being shown in Table 1. The functional proteins in *E. coli* cells harboring pUCRED-Balk were 1.6 times larger than those in *E. coli* cells harboring pBalk-Red. Accordingly, the conversion ratio of 4-methylbiphenyl to 4-hydroxymethybiphenyl with *E. coli* (pUCRED-Balk) was higher than *E. coli* (pBalk-Red).

Table 1
Expression levels of CYP153A13a by the CO difference spectrum method and conversion ratio of 4-methylbiphenyl to 4-hydroxymethylbiphenyl.

	Expression level of CYP153A13a	Conversion ratio
pRed-Balk/BL21	71 nM	28.8%
pUCRed-Balk/BL21	116 nM	62.3%

3.2. Bioconversion of various aromatic compounds

Bioconversion experiments of various aromatic compounds were performed with the *E. coli* BL21 harboring pUCRED-Balk. The conversion ratio was analyzed by HPLC, the results being shown in Table 2 . Aromatic compounds including short alkyl moieties such a 4-methylbiphenyl, phenolic compounds such a 2-chlorophenol, and aromatic compounds including methyl ether moieties such as 4-methoxybiphenyl were selected as substrates as shown in Table 2. Compounds converted from 2,6-dimethyl naphthalene, ibuprofen methyl ether, and 3-bromopehnol generated a small peak by HPLC analyses, which is likely to be a minor product, in addition to each major peak, while compounds converted from the others generated one peak with HPLC. Products (only major products in the case of 2,6-dimethyl naphthalene, ibuprofen methyl ether and 3-bromopehnol) were purified by large-scale biotransformation, and identified by spectroscopic methods, the results also being shown in Table 2.

3.2.1. Bioconversion of aromatic compounds including short alkyl moieties

3.2.1.1. Product converted from 4-methylbiphenyl. The molecular formula of product **1** converted from 4-methylbiphenyl was determined to be $C_{13}H_{12}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **1** showed that the methyl group in 4-methylbiphenyl (substrate) was oxidized to the corresponding primary alcohol. Thus, **1** was determined to be 4-hydroxymethybiphenyl (Table 2).

3.2.1.2. Product converted from 3-methylbiphenyl. The molecular formula of product **2** converted from 3-methylbiphenyl was determined to be $C_{13}H_{12}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **2** showed that the methyl group in 3-methylbiphenyl (substrate) was oxidized to the corresponding primary alcohol. Thus, **2** was determined to be 3-hydroxymethybiphenyl (Table 2).

3.2.1.3. Product converted from 4-ethylbiphenyl. The molecular formula of product **3** converted from 4-ethylbiphenyl was determined to be $C_{14}H_{14}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **3** showed that the methyl group in 4-ethylbiphenyl (substrate) was oxidized to the corresponding primary alcohol. Thus, **3** was determined to be 4-(2-Hydroxyethyl)biphenyl (Table 2).

3.2.1.4. Product converted from 4,4′-diethylbiphenyl. The molecular formula of product **4** converted from 4,4′-diethylbiphenyl was determined to be $C_{16}H_{18}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **4** showed that one of the methyl groups in 4,4′-diethylbiphenyl (substrate) was oxidized to the corresponding primary alcohol. Thus, **4** was determined to be 2-(4′-ethylbiphenyl-4-yl)ethanol (Table 2).

3.2.1.5. Product converted from 4-isopropylbiphenyl. The molecular formula of product **5** converted from 4-isopropylbiphenyl was determined to be $C_{15}H_{16}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **5** showed that one of methyl groups in 4-isopropylbiphenyl (substrate) was oxidized to the corresponding primary alcohol. Thus, **5** was determined to be 4-(1-Hydroxy-2-propyl)biphenyl (Table 2).

3.2.1.6. Product converted from 2-(p-tolyl)-pyridine. The molecular formula of product **6** converted from 2-(*p*-tolyl)-pyridine was determined to be $C_{12}H_{11}NO$ by HRMS (ESI). The ^1H and ^{13}C NMR spectra of **6** showed that the methyl group in 2-(*p*-tolyl)-pyridine

Table 2
Bioconversion of various aromatic compounds by *Escherichia coli* expressing cytochrome P450 *CYP153A13a* genes.

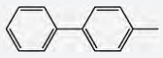
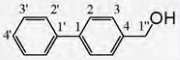
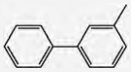
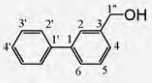
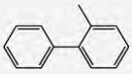
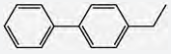
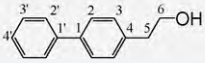
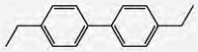
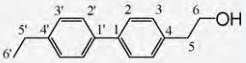
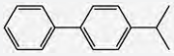
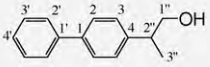
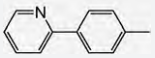
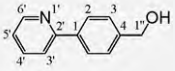
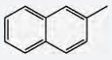
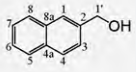
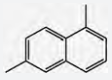
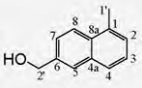
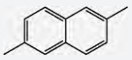
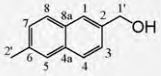
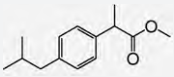
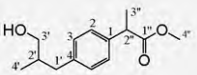
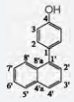
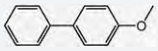
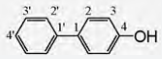
Substrate		Product		Conversion ratio
	4-Methylbiphenyl		1	90.2%
	3-Methylbiphenyl		2	73.2%
	2-Methylbiphenyl	–		Not converted
	4-Ethylbiphenyl		3	90.8%
	4,4′-Diethylbiphenyl		4	28.8%
	4-Isopropylbiphenyl		5	40.1%
	2-(*p*-tolyl)-Pyridine		6	90.1%
	2-Methylnaphthalene		7	61.6%
	1,6-Dimethylnaphthalene		8	34.5%
	2,6-Dimethylnaphthalene		9	16.3%
	Ibuprofen methyl ester		10	74.3%
	2-Chlorophenol		11	56.1%
	2-Bromophenol		12	37.4%
	3-Bromophenol		13	36.5%
	2-Iodophenol		14	48.8%
	2-Acetylphenol		15	28.6%

Table 2 (*Continued*)

Substrate		Product		Conversion ratio
	1-Phenylnaphtharene		16	15.4%
	4-Methoxybiphenyl		17	34.0%
	1-Methoxynaphthalene		18	14.0%

(substrate) was oxidized to the corresponding primary alcohol. Thus, **6** was determined to be (4-(pyridine-2-yl)phenyl)methanol (Table 2).

3.2.1.7. Product converted from 2-methylnaphthalene. The molecular formula of product **7** converted from 2-methylnaphthalene was determined to be $C_{11}H_{10}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **7** showed that the methyl group in 2-methylnaphthalene (substrate) was oxidized to the corresponding primary alcohol. Thus, **7** was determined to be 2-hyrdoxymetylnaphthalene (Table 2).

3.2.1.8. Product converted from 1,6-dimethylnaphthalene. The molecular formula of product **8** converted from 1,6-dimethylnaphthalene was determined to be $C_{12}H_{12}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **8** showed that one of methyl groups in 1,6-dimethylnaphthalene (substrate) was oxidized to its primary alcohol. An analysis of the gDQF COSY and gHMBC spectra of **8** suggested the attachment of one OH group at C-2′. The position of this OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-5 (δ 7.82) to C-2′ (δ 65.5) and from H-7 (δ 7.53) to C-2′ (δ 65.5), and a vicinal spin network from H-7 to H-8. These findings enabled product **8** to determine as (1-methylnaphthalene-6-yl)methanol (Table 2).

3.2.1.9. Product converted from 2,6-dimethylnaphthalene. The molecular formula of product **9** converted from 2,6-dimethylnaphthalene was determined to be $C_{12}H_{12}O$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **9** showed that one of methyl groups in 2,6-dimethylnaphthalene (substrate) was oxidized to the corresponding primary alcohol. Thus, **9** was determined to be (6-methylnaphthalene-2-yl)methanol (Table 2).

3.2.1.10. Product converted from ibuprofen methyl ester. The molecular formula of product **10** converted from ibuprofen methyl ester was determined to be $C_{14}H_{20}O_3$ by HRMS (EI). The ^1H and ^{13}C NMR spectra of **10** showed that one of methyl groups in ibuprofen methyl ester (substrate) was oxidized to its primary alcohol. An analysis of the gDQF COSY and gHMBC spectra of product **10** suggested the attachment of one OH group at C-3′. The positions of this OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-1′ (δ 2.40, 2.59) to C-3′ (δ 67.6) and from H-4′ (δ 0.92) to C-3′ (δ 67.6), and a vicinal spin network from H-1′ to H-4′. These findings enabled product **10** to determine as methyl 2-(4-(3-hydroxy-2-methylpropyl)phenyl)propanoate (Table 2).

3.2.2. Bioconversion of phenolic compounds

3.2.2.1. Product converted from 2-chlorophenol. The molecular formula of product **11** converted from 2-chlorophenol was determined to be $C_6H_5ClO_2$ by HRMS (APCI). Consistent with its molecular formula, the replacement of one phenolic OH group in the aromatic ring is proposed. An analysis of the gDQF COSY and gHMBC spectra of product **11** suggested the attachment of one phenolic group at C-4. The position of this phenolic OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-6 (δ 6.89) to C-4 (δ 149.4), and a vicinal spin network from H-5 to H-6. These findings enabled product **11** to determine as 2-chlorobenzene-1,4-diol (Table 2).

3.2.2.2. Product converted from 2-bromophenol. The molecular formula of product **12** converted from 2-bromophenol was determined to be $C_6H_5BrO_2$ by HRMS (APCI). Consistent with its molecular formula, the replacement of one phenolic OH group in the aromatic ring is proposed. An analysis of the DQF COSY and HMBC spectra of product **12** suggested the attachment of one phenolic group at C-4. The position of this phenolic OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-6 (δ 6.73) to C-4 (δ 150.9), and a vicinal spin network from H-5 to H-6. These findings enabled product **12** to determine as 2-bromobenzene-1,4-diol (Table 2).

3.2.2.3. Product converted from 3-bromophenol. The molecular formula of product **13** converted from 3-bromophenol was determined to be $C_6H_5BrO_2$ by HRMS (APCI). Consistent with its molecular formula, the replacement of one phenolic OH group in the aromatic ring is proposed. An analysis of the gDQF COSY and gHMBC spectra of product **13** suggested the attachment of one phenolic group at C-4. The position of this phenolic OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-6 (δ 6.89) to C-4 (δ 149.5), and a vicinal spin network from H-5 to H-6. These findings enabled product **13** to determine as 2-bromobenzene-1,4-diol (Table 2).

3.2.2.4. Product converted from 2-iodophenol. The molecular formula of product **14** converted from 2-iodephenol was determined to be $C_6H_5IO_2$ by HRMS (APCI). Consistent with its molecular formula, the replacement of one phenolic OH group in the aromatic ring is proposed. An analysis of the gDQF COSY and gHMBC spectra of product **14** suggested the attachment of one phenolic group at C-4. The position of this phenolic OH group was confirmed by the observation of ^1H–^{13}C long-range coupling from H-6 (δ 6.67) to C-4 (δ 150.9), and a vicinal spin network from H-5 to H-6. These findings enabled product **14** to determine as 2-iodobenzene-1,4-diol (Table 2).

3.2.2.5. Product converted from 2-acetylphenol. The molecular formula of product **15** converted from 2-acetylphenol was determined to be $C_8H_8O_3$ by HRMS (APCI). An analysis of the gDQF COSY and gHMBC spectra of product **15** suggested the attachment of one phenolic group at C-5. The position of this phenolic OH group was

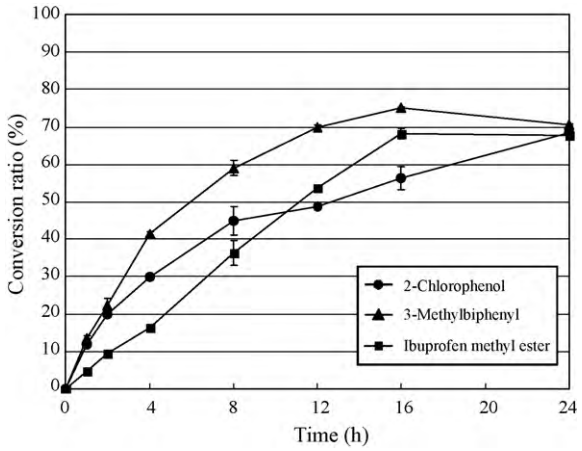


Fig. 1. Time course of bioconversions on 3-methylbipehnyl, ibuprofen methyl ether, and 2-chlorophenol. Each value is shown as mean ± SE ($n = 3$).

confirmed by the observation of ^{1}H–^{13}C long-range coupling from H-3 (δ 6.78) to C-5 (δ 149.5), and a vicinal spin network from H-3 to H-4. These findings enabled product **15** to determine as 1-(2,5-dihydroxyphenyl)ethanone (Table 2).

3.2.2.6. Product converted from 1-phenylnaphthalene. The molecular formula of product **16** converted from 1-phenylnaphthalene was determined to be $C_{16}H_{12}O$ by HRMS (APCI). An analysis of the gDQF COSY and gHMBC spectra of product **16** suggested the attachment of one phenolic group at C-4. The position of this phenolic OH group was confirmed by the observation of ^{1}H–^{13}C long-range coupling from H-2 (δ 7.38) to C-4 (δ 154.9), and a vicinal spin network from H-2 to H-3. These findings enabled product **16** to determine as 4-(naphthalene-1-yl)phenol (Table 2).

3.2.3. Bioconversion of aromatic compounds including methyl ether moieties

3.2.3.1. Product converted from 4-methoxybiphenyl. The molecular formula of product **17** converted from 4-methoxybiphenyl was determined to be $C_{12}H_{10}O$ by HRMS (APCI). The ^{1}H and ^{13}C NMR spectra of **17** showed that the methyl group in 4-methoxybiphenyl (substrate) was cleaved to the corresponding alcohol. Thus, **17** was determined to be 4-hydroxybiphenyl (Table 2).

3.2.3.2. Product converted from 1-methoxynaphthalene. The molecular formula of product **18** converted from 1-methoxynaphthalene was determined to be $C_{10}H_8O$ by HRMS (ESI). The ^{1}H and ^{13}C NMR spectra of 18 showed that the methyl group in 1-methoxynaphtol (substrate) was cleaved to the corresponding alcohol. Thus, **18** was determined to be 1-naphthol (Table 2).

3.3. Time course of bioconversions

Time course experiments of bioconversions on 3-methylbipehnyl, ibuprofen methyl ether, and 2-chlorophenol were further performed with the *E. coli* BL21 harboring pUCRED-Balk, the results being shown in Fig. 1. In the log phase (2–8 h), the order of bioconversion efficiency on these substrates was as follows: 3-methylbipehnyl > 2-chlorophenol > ibuprofen methyl ether. However, in 24-h cultivation, their conversion ratios reached similar levels (70–74%). This result suggests that 24 h is enough for bioconversions with the recombinant *E. coli*.

4. Discussion

We here constructed a new vector pUCRED for the functional expression of a bacterial P450 gene using an *E. coli* vector pUC18. In pUCRED, an inserted P450 gene is designed to utilize the *lac* promoter and *lacZ* translational signal that includes the *lacZ* leader sequence, i.e., ATG ACC ATG ATT ACG AAT TCG ATG (the last ATG is the start codon of an inserted gene, and the *Eco*RI site is underlined). The *CYP153A13a* (P450balk) gene was shown to exhibit better expression level with this vector pUCRED (plasmid PUCRED-Balk) rather than with vector pRED that utilizes the T7 promoter derived from pET21a (plasmid pBalk-Red) [4]. Such trials to utilize the transcriptional and translational signals derived from pUC vectors such as pUC18 were found to be successful for the functional expression of carotenoid biosynthesis genes such as *crtW* and *crtZ* in *E. coli* [15–17]. The pUC series may be one of promising vectors for the functional expression of genes involved in chemical conversions.

Cytochrome P450 CYP153 family, particularly CYP153A subfamily, is known as bacterial alkane terminal hydroxylase [4,8,10]. Recently, we revealed that CYP153A can achieve the terminal hydroxylation on aromatic compounds including butyl moieties such as *n*-butylbenzene and 2-*n*-butylbenzofuran [9]. *A. borkumensis*, which the *CYP153A13a* gene was isolated from [4], was found as a predominant bacterium among bacterial populations on oil-contaminated marine environments [18]. Thus in the present study, we checked bioconversion ability towards a variety of aromatic compounds using recombinant *E. coli* cells that expressed *CYP153A13a*, and found that this P450 fused to the P450RhF reductase domain performed such bioconversions as a multifunctional enzyme. CYP153A13a hydroxylated not only the terminals of short alkyl groups that attached to aromatic rings but also the *p*-positions of phenolic compounds substituted with a halogen or the acetyl group, as shown in Table 2. The terminal hydroxylation reactions were performed towards a series of biphenyl derivatives that include methyl group, ethyl group(s) or isopropyl group with the exception of 2-methylbiphenyl. CYP153A13a also hydroxylated the methyl group of naphthalene derivatives including methyl or dimethyl group (Table 2), while it was not able to convert 1-methylnaphthalene (data not shown). A drug ibuprofen methylether was also shown to have the terminal hydroxylation reaction with the P450 with high conversion efficiency.

Cytochrome P450BM-3 that originated from *Bacillus megaterium* has the structure that a P450 portion is C- terminally fused to a reductase domain composed of a mammalian-like diflavin NADPH-P450 reductase [19]. P450BM-3 catalyzed the hydroxylation and epoxidation reactions of long-chain unsaturated fatty acids [19–21]. Its Phe87Val mutant P450BM-3 (F87V) was shown to hydroxylate the *p*-position of various phenolic compounds such as 2-bromophenol to yield hydroquinone [22]. In this study, we revealed that CYP153A13a can also hydroxylates the *p*-position of such phenolic compounds. Moreover, CYP153A13a was able to convert a phenolic compound with a bulky substituent, 1-phenyl naphthalene, into the product hydroxylated at the *p*-position. We also showed that it catalyzed demethylation reactions of aromatic compounds with a methoxy group (Table 2). The broad substrate affinity (specificity) of CYP153A13a (P450balk) should carry over to the synthesis of various low-molecular-weight compounds including drug-like compounds, which are difficult to synthesize chemically.

Acknowledgement

This work was in part supported by New Energy and Industrial Technology Development Organization (NEDO).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.05.015.

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