Journal of Molecular Catalysis B: Enzymatic 94 (2013) 111–118

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Biocatalytic production of 5-hydroxy-2-adamantanone by P450cam coupled with NADH regeneration

Toshiki Furuya[a], Takaaki Kanno[a], Hiroaki Yamamoto[b], Norihiro Kimoto[b], Akinobu Matsuyama[b], Kuniki Kino[a,*]

[a] Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan
[b] Green Product Development Center, Daicel Corporation, Niigata 944-8550, Japan

ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 5 May 2013
Accepted 7 May 2013
Available online 27 May 2013

Keywords:
Glucose dehydrogenase
Monooxygenase
NADH regeneration
P450cam
Whole-cell catalyst

ABSTRACT

5-Hydroxy-2-adamantanone is a versatile starting material for the synthesis of various adamantane derivatives. In this study, we investigated the biocatalytic production of 5-hydroxy-2-adamantanone using P450cam monooxygenase coupled with NADH regeneration. We constructed *Escherichia coli* cells that expressed P450cam and its redox partners, putidaredoxin and putidaredoxin reductase, and cells that co-expressed this P450cam multicomponent system with a glucose dehydrogenase (Gdh) to regenerate NADH using glucose. Two types of cells – wet cells that did not receive any treatment after washing with glycerol-containing buffer, and freeze-dried cells that were lyophilized after the washing – were prepared as whole-cell catalysts. When wet cells were reacted with 2-adamantanone, *E. coli* cells expressing only the P450cam multicomponent system efficiently produced 5-hydroxy-2-adamantanone in the presence of glucose. However, the co-expression of this P450cam system with Gdh did not further enhance the amount of this product. These results indicate that enough amounts of NADH for P450cam catalysis would be supplied by endogenous glucose metabolism in the *E. coli* host. In contrast, when freeze-dried cells were used, only the cells co-expressing the P450cam multicomponent system with Gdh efficiently catalyzed the oxidation in the presence of glucose. These results suggest that the exogenous Gdh compensated loss of NADH regeneration by the endogenous glucose metabolism that would be damaged by the lyophilization process. Furthermore, we attempted to produce 5-hydroxy-2-adamantanone with repeated additions of the substrate using wet cells expressing only the P450cam multicomponent system and freeze-dried cells co-expressing this P450cam system with Gdh. These whole-cell catalysts attained high-yield production; the wet cells and the freeze-dried cells produced 36 mM (5.9 g/l) and 21 mM (3.5 g/l) of 5-hydroxy-2-adamantanone, respectively.

1. Introduction

Adamantane derivatives are important industrial chemicals that find wide use in production of pharmaceuticals and polymers [1–3]. 5-Hydroxy-2-adamantanone (5-hydroxyadamantan-2-one) is a versatile starting material for the synthesis of various 2,5- or 1,4-disubstituted adamantanes [4]. For example, *E*-2-amino-5-hydroxyadamantane, an intermediate for biologically active compounds, can be synthesized from 5-hydroxy-2-adamantanone [5]. Chemical methods for synthesizing 5-hydroxy-2-adamantanone include disproportionation of 2-hydroxyadamantane and oxidation of 2-adamantanone [4,6,7]. These chemical methods require harmful oxidants such as 60–75% sulfuric acid, 100% nitric acid, and chromic oxide. Furthermore, the procedures are elaborate and are often accompanied by contamination of several other oxygenated adamantanes, which leads to low yields of product.

Cytochrome P450 monooxygenases (P450s) are able to catalyze oxidation using molecular oxygen as an innocuous oxidant under mild reaction conditions. A heme moiety in the catalytic center of P450 activates molecular oxygen using electrons, which are transferred from NAD(P)H by reductase components. The resulting active oxidant, so-called Compound I, oxidizes substrates. P450s generally introduce one oxygen atom regio- and stereo-selectively into organic compounds. Hence, they are of considerable interest as oxidation biocatalysts for the synthesis of industrial chemicals and pharmaceuticals [8–13]. As mentioned above, P450s require NAD(P)H for their catalytic activity. Since NAD(P)H is an expensive reagent, NAD(P)H regeneration systems are essential for cost-effective biocatalytic oxidation processes [14,15].

$NAD(P)^+$-dependent dehydrogenases can function as a system for the regeneration of NAD(P)H using cheap hydrocarbons as an energy source [15–17]. These dehydrogenases catalyze the

* Corresponding author. Tel.: +81 3 5286 3211; fax: +81 3 3232 3889.
E-mail address: kkino@waseda.jp (K. Kino).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.05.003

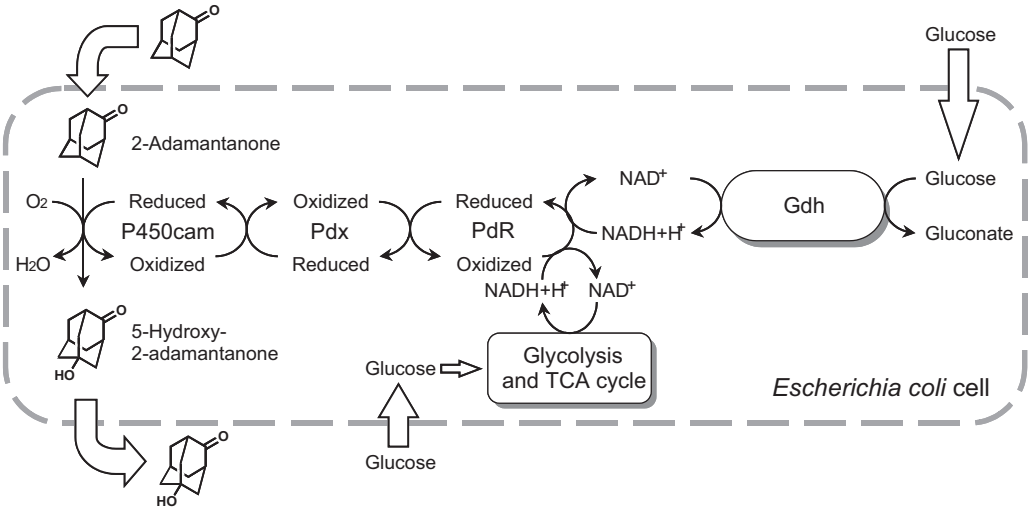

Fig. 1. Scheme of whole-cell catalyst expressing the P450cam multicomponent system coupled with NADH regeneration system for the regioselective oxidation of 2-adamantanone. Pdx, putidaredoxin; PdR, putidaredoxin reductase; Gdh, glucose dehydrogenase; TCA, tricarboxylic acid.

elimination of two hydrogen atoms from organic molecules such as glucose, glycerol, and alcohols, and consequently generate NAD(P)H from $NAD(P)^+$. Several studies have attempted to construct supplying system of NAD(P)H using these dehydrogenases in pure-enzyme or whole-cell P450 catalyses [15,18–23]. However, the effects of dehydrogenase co-expression on P450 whole-cell catalysts varied significantly among the reports [18–23].

In this study, we report a synthetic approach to produce 5-hydroxy-2-adamantanone using P450cam coupled with NADH regeneration as an oxidation biocatalyst. P450cam (CYP101A1) is a camphor monooxygenase [24] that has been shown to exhibit oxidation activity towards a variety of compounds including aliphatics and aromatics [25–27]. It was also confirmed that P450cam has catalytic activity in the oxidation of 2-adamantanone to 5-hydroxy-2-adamantanone using purified enzyme in biochemical studies [28]. In the application of P450s to biocatalysis, whole-cell catalysts have several advantages compared with purified enzyme. For example, enzymes in cells are protected from the external environment and often more stable than purified enzymes [29–31]. This stability provides longer lifetime for whole-cell catalysts. In addition, when enzymes are multicomponent, it is easier and less expensive to reconstitute their catalytic activity in vivo than in vitro [32]. Here, we constructed genetically engineered *Escherichia coli* cells expressing P450cam multicomponent system consisting of P450cam and its redox partners, putidaredoxin (Pdx) and putidaredoxin reductase (PdR) (Fig. 1). *E. coli* cells co-expressing this P450cam system with a glucose dehydrogenase (Gdh) was also constructed to regenerate NADH using glucose (Fig. 1). Furthermore, two types of whole cells – wet cells that did not receive any treatment after washing with glycerol-containing buffer, and freeze-dried cells that were lyophilized after the washing – were prepared. We examined the effects of Gdh co-expression on these P450cam whole-cell catalysts and investigated the application of these catalysts to a flask-scale production of 5-hydroxy-2-adamantanone.

2. Materials and methods

2.1. Bacterial strains, plasmids, cultivation media, and chemicals

The bacterial strains and plasmids that were used or constructed in this study are listed in Table 1. The bacteria were grown in Luria–Bertani (LB) medium, which contained (per liter) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) (pH 7.0). 2-Adamantanone and 5-hydroxy-2-adamantanone were purchased from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Construction of P450cam, pdx, pdR, and gdh expression plasmids

The plasmids used for expression of the *P450cam* and *pdx* genes in *E. coli* cells were constructed using the pETDuet-1 vector

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference or source
Strains		
E. coli JM109	Host used for cloning	Takara Bio
E. coli BL21 (DE3)	Host used for expression	Novagen
Plasmids		
pETDuet-1	Vector used for cloning, Ap^r	Novagen
pACYCDuet-1	Vector used for cloning, Cm^r	Novagen
pETDpdx	pETDuet-1 containing *pdx* in MCS-2 under the control of the T7 promoter	This study
pETDP450cam-pdx	pETDuet-1 containing *P450cam* in MCS-1 and *pdx* in MCS-2 under the control of the T7 promoters	This study
pACYCDpdR	pACYCDuet-1 containing *pdR* in MCS-1 under the control of the T7 promoter	This study
pACYCDpdR-gdh	pACYCDuet-1 containing *pdR* in MCS-1 and *gdh* in MCS-2 under the control of the T7 promoters	This study

Table 2
Oligonucleotide primers used in this study.

Primer	Sequence (5′–3′)[a]	Restriction site
pdx-F	TTC CAT **ATG** TCT AAA GTA GTG TAT GTG TCA	NdeI
pdx-R	GA AGA TCT **TTA** CCA TTG CCT ATC GGG AAC	BglII
P450cam-F	TTC TC**A** **TG**A CGA CTG AAA CCA TAC AAA GCA	BspHI
P450cam-R	CCG GAA TTC **TTA** TAC CGC TTT GGT AGT CGC	EcoRI
pdR-F	TTC TC**A** **TG**A ACG CAA ACG ACA ACG TGG TCA	BspHI
pdR-R	CGC GGA TCC **TCA** GGC ACT ACT CAG TTC AGC	BamHI
gdh-F	TTC CAT **ATG** TAT CCG GAT TTA AAA GGA AAA	NdeI
gdh-R	GA AGA TCT **TTA** ACC GCG GCC TGC CTG GAA	BglII

[a] Restriction sites are underlined and identified in the column on the right. Initiation and termination codons are indicated in bold.

(Table 1). Two oligonucleotide primers, pdx-F and pdx-R (Table 2), were designed to amplify the *pdx* gene (GenBank accession number, P00259). The region between the two oligonucleotide primers was amplified from the pET21a vector carrying the *pdx* gene [33] by PCR. PCR was performed with KOD Plus polymerase (Toyobo, Osaka, Japan) under the buffer conditions recommended by the manufacturer. The PCR mixture was heated at 94 °C for 2 min and then subjected to 30 cycles of amplification (94 °C for 15 s; 55 °C for 30 s; and 68 °C for 30 s). This amplified DNA fragment was digested with NdeI and BglII, and then inserted into the multi-cloning site (MCS)-2 of the pETDuet-1 vector that was digested with the same restriction enzymes. The resulting plasmid, pETDpdx, was amplified in *E. coli* JM109 cells. Next, two oligonucleotide primers, P450cam-F and P450cam-R (Table 2), were designed to amplify the *P450cam* gene (GenBank accession number, P00183). The region between the two oligonucleotide primers was amplified from genomic DNA of *Pseudomonas putida* JCM 6157 (ATCC 17453) by PCR. The PCR mixture was subjected to 30 cycles of amplification (94 °C for 15 s; 65 °C for 30 s; and 68 °C for 90 s). This amplified DNA fragment was digested with BspHI and EcoRI, and then inserted into MCS-1 of the pETDpdx plasmid that was digested with NcoI and EcoRI. The resulting plasmid, pETDP450cam-pdx, was amplified in *E. coli* JM109 cells.

The plasmids used for expression of the *pdR* and *gdh* genes in *E. coli* cells were constructed using the pACYCDuet-1 vector (Table 1). Two oligonucleotide primers, pdR-F and pdR-R (Table 2), were designed to amplify the *pdR* gene (GenBank accession number, P16640). The region between the two oligonucleotide primers was amplified from the pET21a vector carrying the *pdR* gene [33] by PCR. The PCR mixture was subjected to 30 cycles of amplification (94 °C for 15 s; 60 °C for 30 s; and 68 °C for 90 s). This amplified DNA fragment was digested with BspHI and BamHI, and then inserted into MCS-1 of the pACYCDuet-1 vector that was digested with NcoI and BamHI. The resulting plasmid, pACYCDpdR, was amplified in *E. coli* JM109 cells. Next, two oligonucleotide primers, gdh-F and gdh-R (Table 2), were designed to amplify the *gdh* gene (GenBank accession number, NP_388275). The region between the two oligonucleotide primers was amplified from genomic DNA of *Bacillus subtilis* strain 168 (ATCC 23857) by PCR. The PCR mixture was subjected to 30 cycles of amplification (94 °C for 15 s; 60 °C for 30 s; and 68 °C for 60 s). This amplified DNA fragment was digested with NdeI and BglII, and then inserted into MCS-2 of the pACYCDpdR plasmid that was digested with the same restriction enzymes. The resulting plasmid, pACYCDpdR-gdh, was amplified in *E. coli* JM109 cells.

After the plasmid constructs were confirmed by sequencing, these plasmids were introduced into *E. coli* BL21 (DE3) cells by electroporation.

2.3. Preparation of whole cells

The transformed *E. coli* BL21 (DE3) cells carrying pETDP450cam-pdx and pACYCDpdR, and the cells carrying pETDP450cam-pdx and pACYCDpdR-gdh were cultivated at 30 °C in LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (100 μg/ml). After cultivation for 6 h (OD_{600} = 0.8–1.0), isopropyl-β-D-thiogalactopyranoside (1 mM), 5-aminolevulinic acid (0.5 mM), and $FeSO_4$ (0.5 mM) were added to the medium, and cultivation was continued for an additional 15 h at 25 °C. Cells were harvested by centrifugation and washed with potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10%, v/v). These cells were used as wet cells. After treatment with lyophilizer (FDU-1000, EYELA, Tokyo, Japan), the resulting cells were used as freeze-dried cells.

2.4. Protein and P450 analyses

When cell-free extracts were prepared, cells were suspended in potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10%, v/v) and were disrupted by sonication. After centrifugation at 15,000 × *g* for 30 min at 4 °C, the resulting supernatant was used as the cell-free extract. The cell-free extracts were used to analyze protein levels and P450 expression. Protein concentration was measured using a Coomassie protein assay kit (Pierce, Rockford, IL, USA) with a bovine serum albumin standard [34]. The expression levels of P450cam, Pdx, PdR, and Gdh were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. Active P450cam was quantified based on the reduced CO difference spectrum characteristic of thiolate-heme enzymes using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm [35]. Before analysis of these spectra, the protein concentration of the cell-free extracts was adjusted to 2 g/l.

2.5. Reactions using whole cells

The reaction mixture (50 ml) contained cells of the transformed *E. coli* strain (15 g dry cell weight (DCW)/l), 2-adamantanone (20 mM), dimethylsulfoxide (DMSO) (10%, v/v), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10%, v/v). The reaction mixture was supplemented with glucose (50 mM) when required. Fifteen grams of DCW corresponded to 50 g of wet cell weight. The reactions were performed at 30 °C with rotary shaking at a speed of 120 rpm.

2.6. Production of 5-hydroxy-2-adamantanone with repeated additions of the substrate

The reaction was performed in a 500-ml flask that contained cells of the transformed *E. coli* strain (15 g DCW/l), 2-adamantanone (20 mM), glucose (50 mM), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10%, v/v) in a volume of 50 ml. After 4-h incubation, 2-adamantanone (20 mM) and glucose (50 mM) were again added to the reaction mixture. The reactions were carried out at 30 °C with rotary shaking at a speed of 120 rpm.

2.7. Product analysis and glucose measurement

Gas chromatography (GC) analysis was performed using a GC-2010 system (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 30-m type DB-5 column (J&W Scientific, Folsom, CA, USA). A portion of the reaction mixture was acidified by the addition of HCl (pH 2–3) and was extracted with ethyl acetate. The resulting extract was injected into the GC system. The carrier gas was helium and the flow rate was 1.0 ml/min. The injection temperature was maintained at 250 °C. The oven temperature was programmed as follows: start temperature of 120 °C, increased to 165 °C at a rate of 15 °C/min, further increased to 180 °C at a rate of 5 °C/min, and held for 4 min at finish temperature

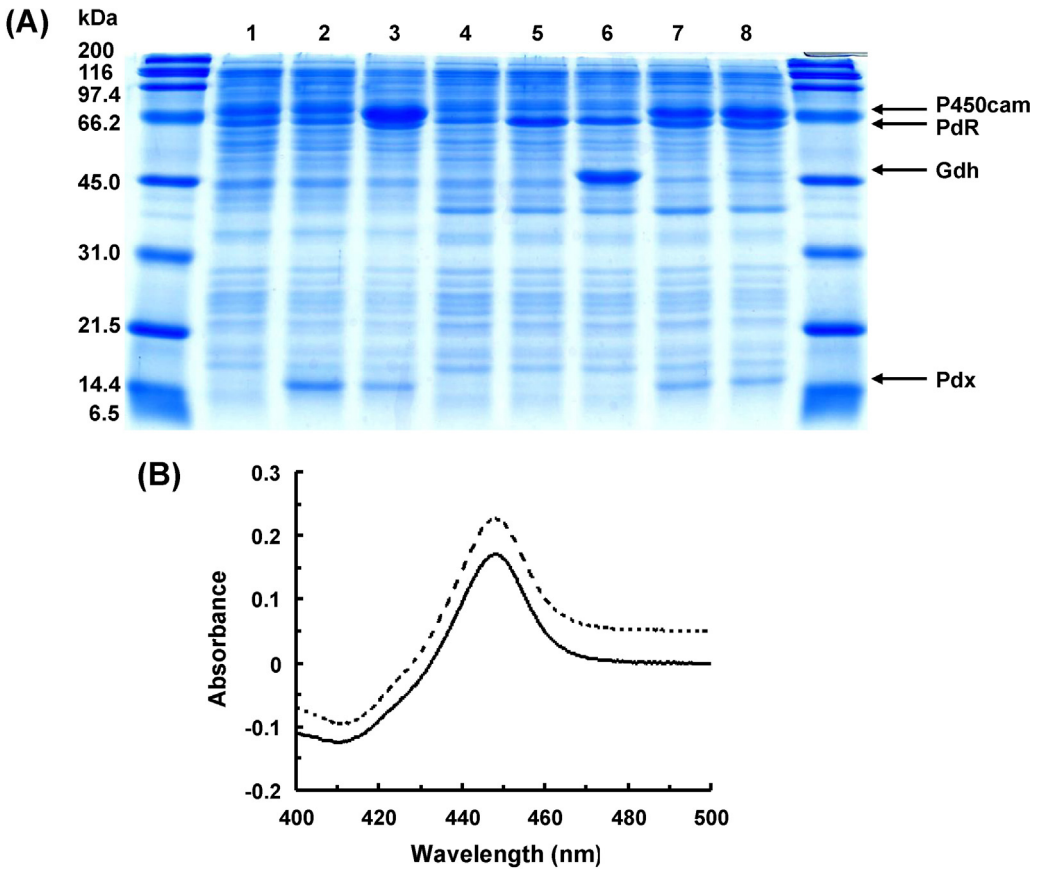

Fig. 2. Expression analysis of P450cam, Pdx, PdR, and Gdh in *E. coli*. (A) SDS-PAGE analysis. Samples (5 μg of cell lysate) from the soluble fractions of the transformed *E. coli* cells were loaded onto a polyacrylamide gel (20%). Lane 1, cells carrying pETDuet-1; lane 2, pETDpdx; lane 3, pETDP450cam-pdx; lane 4, pACYCDuet-1; lane 5, pACYCDpdR; lane 6, pACYCDpdR-gdh; lane 7, pETDP450cam-pdx and pACYCDpdR; lane 8, pETDP450cam-pdx and pACYCDpdR-gdh. The molecular weights corresponding to P450cam, Pdx, PdR, and Gdh are indicated by arrows. (B) CO-reduced difference spectra. Samples (2 mg protein/ml) from the soluble fractions of the transformed *E. coli* cells were analyzed. Solid line, cells carrying pETDP450cam-pdx and pACYCDpdR; dotted line, cells carrying pETDP450cam-pdx and pACYCDpdR-gdh.

of 180 °C. The amounts of 2-adamantanone and 5-hydroxy-2-adamantanone were calculated from standard calibration curves that were made using these compounds purchased from Tokyo Kasei. Gas chromatography–mass spectrometry (GC–MS) analysis was performed using a JMS-GCmateII system (JEOL, Tokyo, Japan) with a DB-5 column. Ions were generated using an electron ionization source, and the mass detector was tuned using 2-admantanone to optimize the instrumental parameters. Reaction products were identified based on GC–MS spectra.

Glucose concentration was measured using a Glucose CII-test kit (Wako, Osaka, Japan) according to the instruction manual.

3. Results and discussion

3.1. Preparation of P450cam whole-cell catalysts

We constructed *E. coli* cells that expressed P450cam and its redox partners, Pdx and PdR, and cells that co-expressed this P450cam multicomponent system with Gdh. The *P450cam* and *pdx* genes were cloned into the pETDuet-1 vector, and the *pdR* and *gdh* genes were cloned into the pACYCDuet-1 vector. The expression of these genes in *E. coli* BL21 (DE3) cells was induced by isopropyl-β-D-thiogalactopyranoside under the control of their respective T7 promoters. SDS-PAGE analysis showed the major bands corresponding to P450cam, Pdx, PdR, and Gdh in the soluble fractions of the transformed *E. coli* cells (Fig. 2A). The expression levels of P450cam were also determined based on the reduced CO difference spectrum (Fig. 2B); *E. coli* cells carrying the *P450cam*, *pdx*, and *pdR* genes produced 0.56 μmol P450cam per liter of culture (0.093 μmol P450cam per g of wet cells), and *E. coli* cells carrying the *P450cam*, *pdx*, *pdR*, and *gdh* genes produced 0.57 μmol P450cam per liter of culture (0.097 μmol P450cam per g of wet cells).

3.2. Biocatalytic oxidation of 2-adamantanone using wet E. coli cells expressing P450cam

We explored the catalytic potential of P450cam for the oxidation of 2-adamantanone. Wet cells that did not receive any treatment after washing with glycerol-containing buffer were first used for whole-cell reactions (Fig. 3). *E. coli* cells that carried the *P450cam*, *pdx*, and *pdR* genes, but not the *gdh* gene, were reacted with 20 mM 2-adamantanone. The reaction mixture was supplemented with 50 mM glucose. GC analysis of the reactions with 2-adamantanone showed a major peak (retention time, 6.5 min) in addition to the substrate peak (4.6 min) (see Fig. 4A). The retention time and the GC–MS spectrum of this product coincided with those of an authentic sample of 5-hydroxy-2-adamantanone. Based on these observations, this product was identified as 5-hydroxy-2-adamantanone, as reported previously using purified enzyme [28]. The transformed *E. coli* cells oxidized 2-adamantanone regioselectively at the C-5 position and produced 5.4 mM 5-hydroxy-2-adamantanone within 8 h in the absence of glucose (Fig. 3A). Furthermore, addition of glucose to the reaction mixture strongly enhanced the amount of product; the whole-cell catalyst produced 19 mM 5-hydroxy-2-adamantanone within 8 h in the presence of glucose (Fig. 3B). These results suggest that NADH

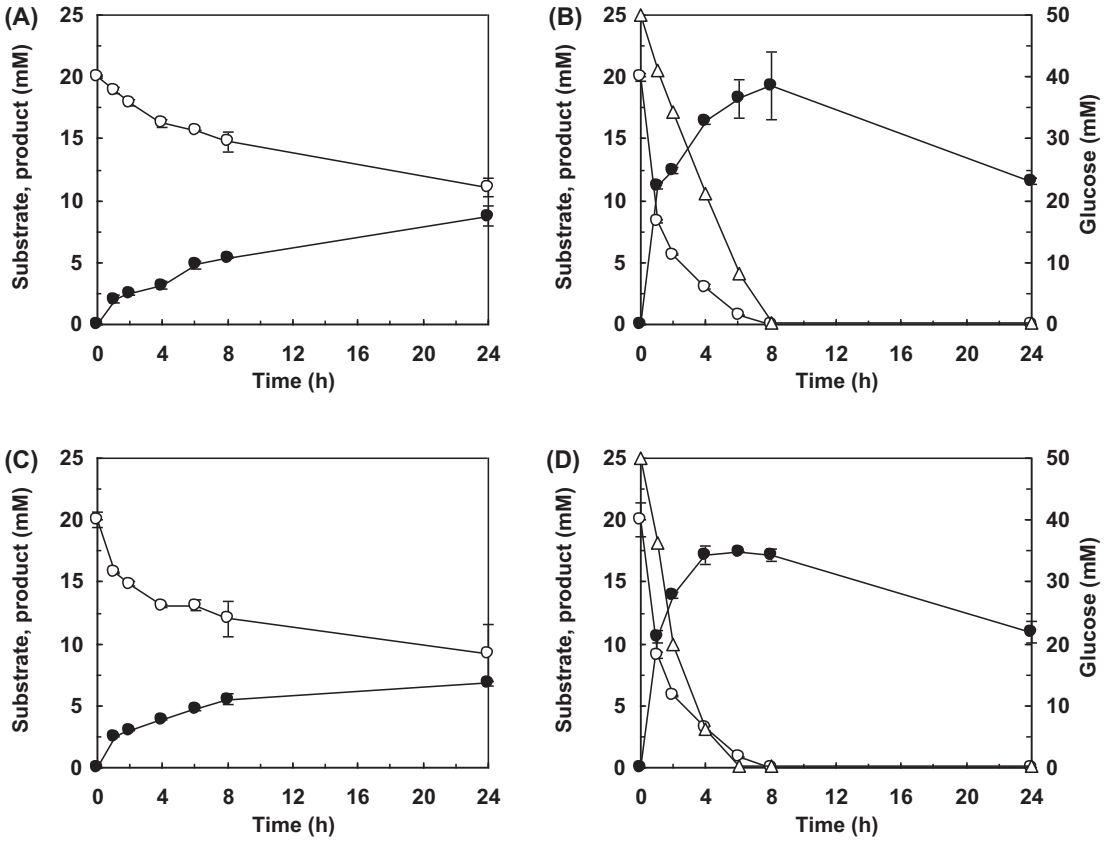

Fig. 3. Biocatalytic oxidation of 2-adamantanone using wet whole-cell catalysts. *E. coli* cells expressing only the P450cam multicomponent system (A and B) and *E. coli* cells co-expressing this P450cam system with Gdh (C and D) were reacted with 2-adamantanone in the absence (A and C) or the presence (B and D) of glucose. The time courses of 2-adamantanone consumption (open circles) and of 5-hydroxy-2-adamantanone production (closed circles) are shown. Plots indicate the average values of determinations performed in double, and error bars indicate standard deviation from the mean. In (B) and (D), glucose consumption (triangles) is also shown.

was effectively regenerated through endogenous glucose metabolic pathways such as glycolysis and the tricarboxylic acid cycle in the *E. coli* host.

This product, however, gradually decreased after 8 h in the presence of glucose (Fig. 3B). By GC analysis, a small peak (retention time, 7.0 min) and a trace of peak (8.6 min) were newly detected after 8 h (Fig. 4A). We confirmed that these two peaks were also detected after the reaction using 5-hydroxy-2-adamantanone as a substrate (data not shown). By GC–MS analysis, the *m*/*z* value of parent ion of the product at 7.0 min was found to be 168 (Fig. 4B). This value corresponds to the compound formed from 5-hydroxy-2-adamantanone (molecular weight, 166) through two-hydrogen addition. Furthermore, the GC–MS spectrum of this product coincided with that of 1,4-adamantanediol that had been previously determined [36]. These observations strongly suggest that the product at 7.0 min would be 1,4-adamantandiol. We also confirmed that *E. coli* cells without the *P450cam* gene were able to convert 5-hydroxy-2-adamantanone to 1,4-adamantanediol (data not shown). These results indicate that 5-hydroxy-2-adamantanone was reduced to 1,4-adamantanediol (the compound corresponding to 2,5-adamantanediol) by an endogenous enzyme in the *E. coli* host. On the other hand, the parent ion of the product at 8.6 min was *m*/*z* 182 (Fig. 4C). This value corresponds to the compound formed from 5-hydroxy-2-adamantanone through one-oxygen addition. Furthermore, the fragment ions at *m*/*z* 95 and *m*/*z* 111, which are typical for dihydroxylated adamantanes [36], were also detected. Based on these observations, the product at 8.6 min was tentatively identified as dihydroxy-2-adamantanone. This product was not detected when *E. coli* cells without the *P450cam* gene were reacted with 5-hydroxy-2-adamantanone (data not shown). These results suggest that 5-hydroxy-2-adamantanone was oxidized to dihydroxy-2-adamantanone by P450cam, although elucidation of this reaction needs detailed characterization using purified enzyme.

Whole-cell reactions were also performed using wet *E. coli* cells co-expressing the P450cam multicomponent system with Gdh. The transformed *E. coli* cells produced 17 mM 5-hydroxy-2-adamantanone within 8 h in the presence of glucose (Fig. 3D). *E. coli* cells co-expressing Gdh consumed glucose more rapidly than cells without the *gdh* gene (Fig. 3B and D). The rate of 5-hydroxy-2-adamantanone production, however, was almost the same between these two transformed *E. coli* strains (Fig. 3B and D). These results indicate that enough amounts of NADH for P450cam catalysis would be supplied by endogenous glucose metabolism in the *E. coli* host.

3.3. Biocatalytic oxidation of 2-adamantanone using freeze-dried cells expressing P450cam

Freeze-dried cells that were lyophilized after washing with glycerol-containing buffer were next used for whole-cell reactions (Fig. 5). *E. coli* cells carrying the *P450cam*, *pdx*, and *pdR* genes, but not the *gdh* gene, were reacted with 20 mM 2-adamantanone. The reaction mixture was supplemented with 50 mM glucose. However, the transformed *E. coli* cells produced hardly any 5-hydroxy-2-adamantanone even in the presence of glucose (Fig. 5B).

Whole-cell reactions were also performed using freeze-dried *E. coli* cells co-expressing the P450cam multicomponent system with Gdh. The transformed *E. coli* cells produced hardly any 5-hydroxy-2-adamantanone in the absence of glucose (Fig. 5C). In contrast,

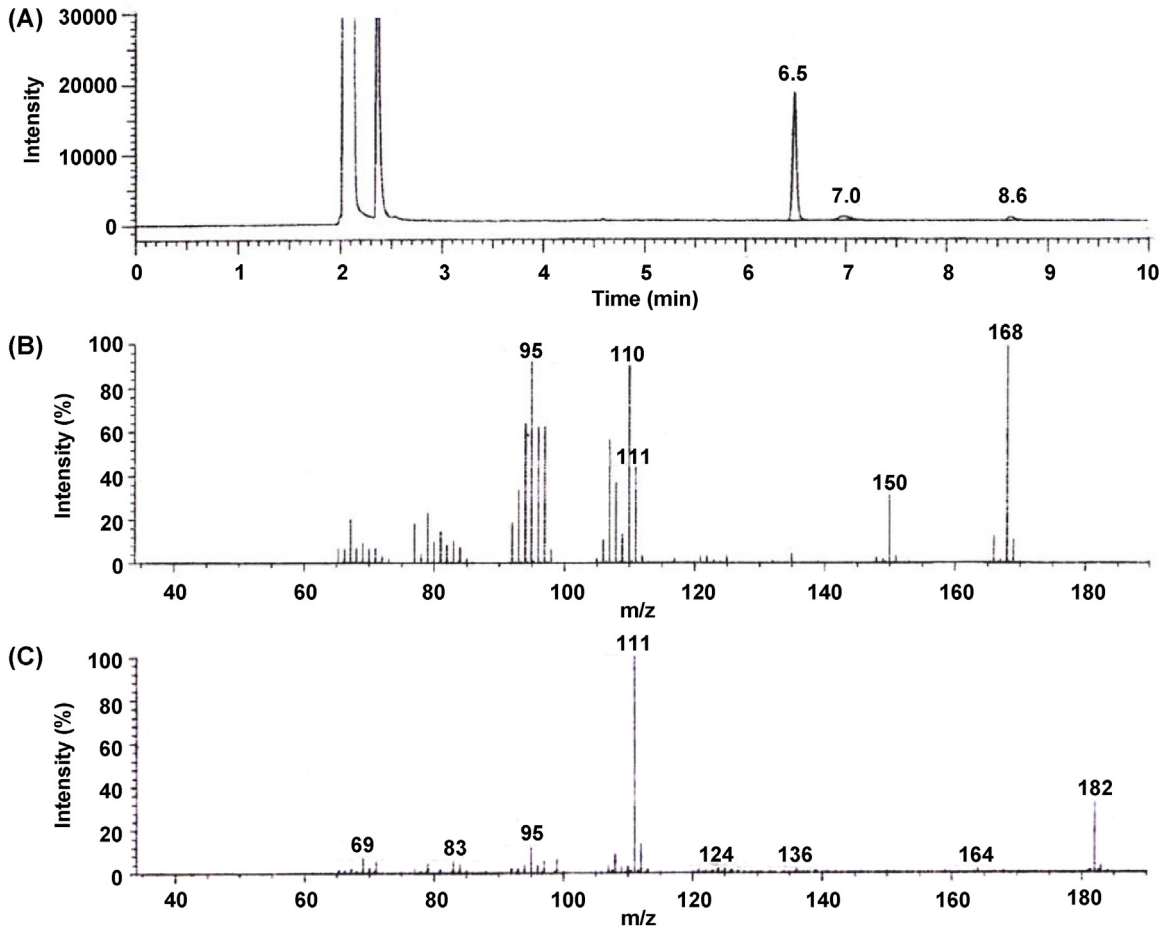

Fig. 4. GC–MS analysis of by-products generated by the P450cam whole-cell reaction. Wet *E. coli* cells that carried the *P450cam*, *pdx*, and *pdR* genes were reacted with 20 mM 2-adamantanone in the presence of glucose for 24 h. (A) GC chromatogram. The peak at 6.5 min was found to correspond to 5-hydroxy-2-adamantanone. (B) GC–MS spectrum for the peak at 7.0 min in chromatogram (A). (C) GC–MS spectrum for the peak at 8.6 min in chromatogram (A).

these cells rapidly oxidized the substrate in the presence of glucose; 16 mM 5-hydroxy-2-adamantanone was produced within 8 h, and this production was accompanied by rapid consumption of glucose (Fig. 5D). These results suggest that endogenous glucose metabolism in the *E. coli* host would be damaged by the lyophilization process, and thus the freeze-dried cells required co-expression of Gdh for the oxidation activity.

Interestingly, 5-hydroxy-2-adamantanone was not further transformed by the freeze-dried cells after 8 h (Fig. 5D). This result was different from that observed for the reaction using wet cells (Fig. 3B and D), suggesting that an *E. coli* enzyme that catalyzed the reduction of 5-hydroxy-2-adamantanone to 1,4-adamantanediol might be deactivated by the lyophilization process. This method might be widely applicable to whole-cell reactions accompanied by undesired side reactions derived from host cells.

3.4. Production of 5-hydroxy-2-adamantanone with repeated additions of the substrate

The results presented above demonstrate that *E. coli* cells carrying the *P450cam*, *pdx*, and *pdR* genes might be an efficient biocatalyst for the oxidation of 2-adamantanone. We thus first attempted to produce 5-hydroxy-2-adamantanone using these wet cells in a 500-ml flask containing 50 ml of the reaction mixture. The substrate 2-adamantanone (20 mM) and glucose (50 mM) were initially added to the reaction mixture and, after 4-h incubation, the same amounts of 2-adamantanone and glucose were again added. Although 2-adamantanone was added as a DMSO solution in the experiments presented above, the solid substrate was directly added to the reaction mixture to avoid the toxicity of the organic solvent DMSO in this process. As shown in Fig. 6A, the whole-cell catalyst completely transformed 20 mM 2-adamantanone into 5-hydroxy-2-adamantanone within 4 h. The initial rate of 5-hydroxy-2-adamantanone production, estimated for the first 1 h of the reaction, was 52 mol (mol P450)$^{-1}$ min^{-1} (16 μmol g-DCW^{-1} min^{-1}). This rate was approximately 1.3 times higher compared with that of 5-hydroxy-2-adamantanone production in the presence of DMSO (Fig. 3B). Furthermore, after the second addition of substrate, the production of 5-hydroxy-2-adamantanone continued to increase. Within 24 h of reaction, the amount of this product reached 36 mM (5.9 g/l), with a molar conversion yield of 90%.

Using a similar technique, we also attempted to produce 5-hydroxy-2-adamantanone with freeze-dried *E. coli* cells co-expressing the P450cam multicomponent system with Gdh (Fig. 6B). This whole-cell catalyst produced 16 mM 5-hydroxy-2-adamantanone within 4 h. The initial rate of 5-hydroxy-2-adamantanone production was estimated to be 49 mol (mol P450)$^{-1}$ min^{-1} (16 μmol g-DCW^{-1} min^{-1}). Furthermore, after the second addition of substrate, the amount of this product reached 21 mM (3.5 g/l).

The production rate decreased after 6 h in the wet-cell reaction and after 5 h in the freeze-dried-cell reaction (Fig. 6). When the pH of the reaction mixture dropped below 6.5, the production rate

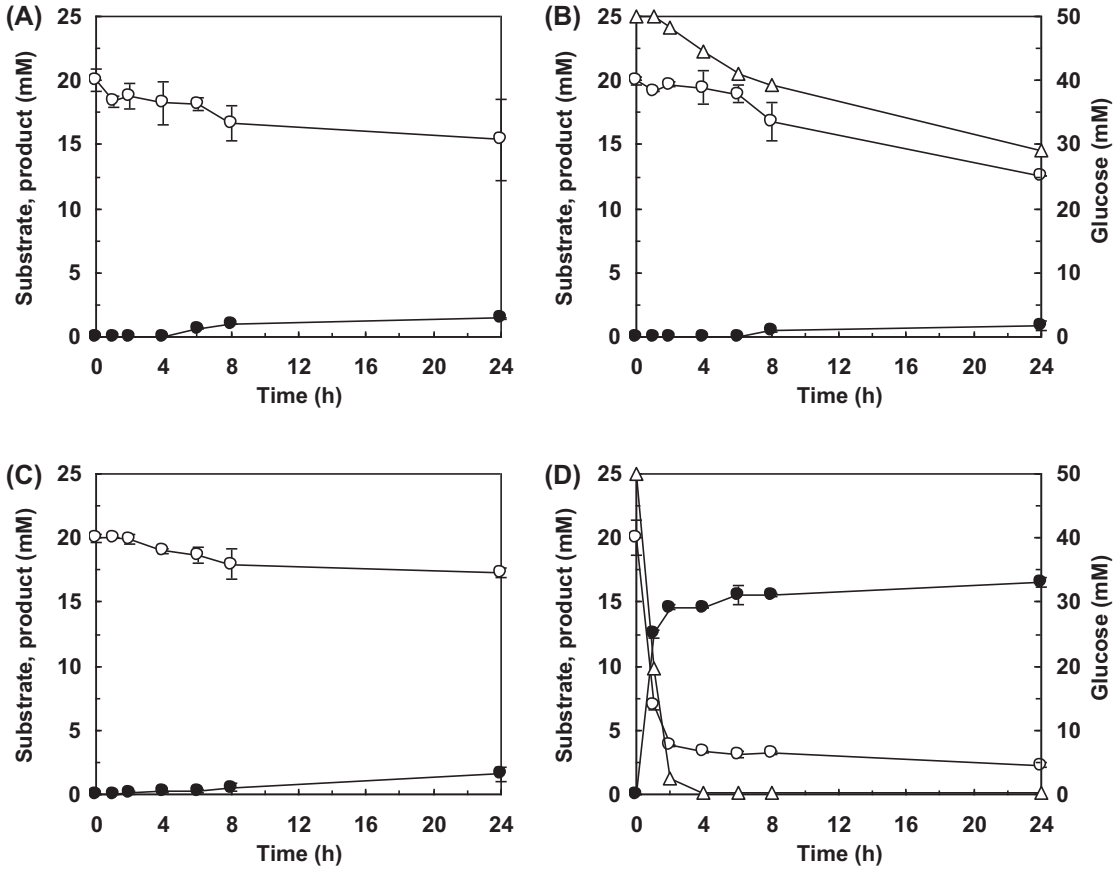

Fig. 5. Biocatalytic oxidation of 2-adamantanone using freeze-dried whole-cell catalysts. *E. coli* cells that expressing only the P450cam multicomponent system (A and B) and *E. coli* cells co-expressing this P450cam system with Gdh (C and D) were reacted with 2-adamantanone in the absence (A and C) or the presence (B and D) of glucose. The time courses of 2-adamantanone consumption (open circles) and of 5-hydroxy-2-adamantanone production (closed circles) are shown. Plots indicate the average values of determinations performed in double, and error bars indicate standard deviation from the mean. In (B) and (D), glucose consumption (triangles) is also shown.

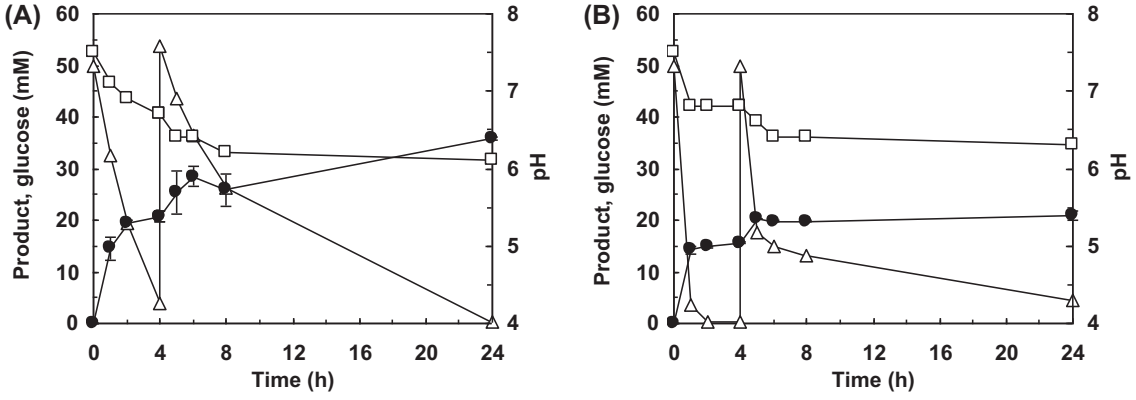

Fig. 6. Production of 5-hydroxy-2-adamantanone with repeated additions of the substrate. Wet *E. coli* cells expressing only the P450cam multicomponent system (A) and freeze-dried *E. coli* cells co-expressing this P450cam system with Gdh (B) were reacted with the substrate 2-adamantanone in the presence of glucose. The time courses of 5-hydroxy-2-adamantanone production (closed circles), glucose consumption (triangles), and pH (squares) are shown. 2-Adamantanone (20 mM) and glucose (50 mM) were initially added to the reaction mixture and, after 4-h incubation, the same amounts of 2-adamantanone and glucose were again added.

decreased in both wet cells and freeze-dried cells (Fig. 6). It was reported that P450cam exhibits low oxidation activity under acidic conditions lower than pH 6.5 [37]. These results suggest that the decrease of production rate might be caused by the decrease of pH. However, although the pH decreased more rapidly in wet cells than in freeze-dried cells, the production after the second addition of substrate was higher in wet cells (Fig. 6), suggesting that other factors also affected the production. Detailed characterization will be necessary for further enhancement of productivity.

4. Conclusions

We succeeded in producing 36 mM (5.9 g/l) 5-hydroxy-2-adamantanone using wet cells expressing only the P450cam multicomponent system (Fig. 6A) and 21 mM (3.5 g/l) of the product using freeze-dried cells co-expressing this P450cam system with Gdh (Fig. 6B). The oxidation activities of the whole cells towards 2-adamantanone were almost the same as that of the purified P450cam system previously reported (52 mol (mol P450)$^{-1}$ min^{-1})

[28], whereas our experimental setup using whole cells achieved gram-per-liter-scale production of 5-hydroxy-2-adamantanone. Through the experiments, we found that wet cells were able to effectively oxidize 2-adamantanone without co-expression of Gdh, whereas freeze-dried cells required co-expression of Gdh for the oxidation activity (Figs. 3 and 5). In other words, these results demonstrate that the condition of the host cells markedly affects the NADH regeneration system used in whole-cell oxidation catalysis (Fig. 1). Wet cells, which attained higher-level production than freeze-dried cells, are more desirable for use immediately after the cell preparation. On the other hand, freeze-dried cells repressed undesired side reactions derived from host cells, and may be advantageous in the preservation of the whole-cell catalyst. These efficient whole-cell catalysts might pave the way for industrial biocatalytic production of 5-hydroxy-2-adamantanone.

References

[1] G. Lamoureux, G. Artavia, Curr. Med. Chem. 17 (2010) 2967–2978.
[2] I.A. Novakov, B.S. Orlinson, Polym. Sci. Ser. C 47 (2005) 50–73.
[3] A.A. Spasov, T.V. Khamidova, L.I. Bugaeva, I.S. Morozov, Pharm. Chem. J. 34 (2000) 1–7.
[4] H.W. Geluk, Synthesis (1972) 374–375.
[5] L. Jaroskova, L. Van der Veken, P. de Belser, G. Diels, A. de Groot, J.T.M. Linders, Tetrahedron Lett. 47 (2006) 8063–8067.
[6] H.W. Geluk, J.L.M.A. Schlatmann, Tetrahedron 24 (1968) 5369–5377.
[7] S. Srivastava, W. Le Noble, Synth. Commun. 14 (1984) 65–68.
[8] R. Bernhardt, J. Biotechnol. 124 (2006) 128–145.
[9] T. Furuya, Y. Arai, K. Kino, Appl. Environ. Microbiol. 78 (2012) 6087–6094.
[10] T. Furuya, K. Kino, Appl. Microbiol. Biotechnol. 86 (2010) 991–1002.
[11] G. Grogan, Curr. Opin. Chem. Biol. 15 (2011) 241–248.
[12] P.R. Ortiz de Montellano, Chem. Rev. 110 (2010) 932–948.
[13] V.B. Urlacher, M. Girhard, Trends Biotechnol. 30 (2012) 26–36.
[14] E. O'Reilly, V. Köhler, S.L. Flitsch, N.J. Turner, Chem. Commun. (Camb.) 47 (2011) 2490–2501.
[15] A. Chefson, K. Auclair, Mol. Biosyst. 2 (2006) 462–469.
[16] M. Kataoka, K. Yamamoto, H. Kawabata, M. Wada, K. Kita, H. Yanase, S. Shimizu, Appl. Microbiol. Biotechnol. 51 (1999) 486–490.
[17] H. Yamamoto, K. Mitsuhashi, N. Kimoto, A. Matsuyama, N. Esaki, Y. Kobayashi, Biosci. Biotechnol. Biochem. 68 (2004) 638–649.
[18] Y. Lu, L. Mei, J. Ind. Microbiol. Biotechnol. 34 (2007) 247–253.
[19] T. Mouri, J. Michizoe, H. Ichinose, N. Kamiya, M. Goto, Appl. Microbiol. Biotechnol. 72 (2006) 514–520.
[20] S.Q. Pham, P. Gao, Z. Li, Biotechnol. Bioeng. 110 (2013) 363–373.
[21] H. Schewe, D. Holtmann, J. Schrader, Appl. Microbiol. Biotechnol. 83 (2009) 849–857.
[22] A. Siriphongphaew, P. Pisnupong, J. Wongkongkatep, P. Inprakhon, A.S. Vangnai, K. Honda, H. Ohtake, J. Kato, J. Ogawa, S. Shimizu, V.B. Urlacher, R.D. Schmid, T. Pongtharangkul, Appl. Microbiol. Biotechnol. 95 (2012) 357–367.
[23] J.D. Zhang, A.T. Li, H.L. Yu, T. Imanaka, J.H. Xu, J. Ind. Microbiol. Biotechnol. 38 (2011) 633–641.
[24] M. Katagiri, B.N. Ganguli, I.C. Gunsalus, J. Biol. Chem. 243 (1968) 3543–3546.
[25] S.G. Bell, X. Chen, R.J. Sowden, F. Xu, J.N. Williams, L.L. Wong, Z. Rao, J. Am. Chem. Soc. 125 (2003) 705–714.
[26] V.B. Urlacher, R.D. Schmid, Curr. Opin. Biotechnol. 13 (2002) 557–564.
[27] F. Xu, S.G. Bell, Z. Rao, L.L. Wong, Protein Eng. Des. Sel. 20 (2007) 473–480.
[28] R.E. White, M.B. McCarthy, K.D. Egeberg, S.G. Sligar, Arch. Biochem. Biophys. 228 (1984) 493–502.
[29] C.C. de Carvalho, Biotechnol. Adv. 29 (2011) 75–83.
[30] W.A. Duetz, J.B. van Beilen, B. Witholt, Curr. Opin. Biotechnol. 12 (2001) 419–425.
[31] T. Ishige, K. Honda, S. Shimizu, Curr. Opin. Chem. Biol. 9 (2005) 174–180.
[32] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Nature 409 (2001) 258–268.
[33] T. Furuya, T. Nishi, D. Shibata, H. Suzuki, D. Ohta, K. Kino, Chem. Biol. 15 (2008) 563–572.
[34] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[35] T. Omura, R. Sato, J. Biol. Chem. 239 (1964) 2370–2378.
[36] K. Mitsukura, H. Sakamoto, H. Kubo, T. Yoshida, T. Nagasawa, J. Biosci. Bioeng. 109 (2010) 550–553.
[37] T. Mouri, N. Kamiya, M. Goto, Biotechnol. Lett. 28 (2006) 1509–1513.