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Biotransformation of medium-chain alkanes using recombinant P450 monooxygenase from *Alcanivorax borkumensis* SK2 expressed in *Escherichia coli*

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Abstract−A bioconversion system for medium-chain alkanes was constructed by using a recombinant *Escherichia coli* whole-cell biocatalyst expressing P450 monooxygenase genes, ferredoxin, and ferredoxin reductase cloned from *Alcanivorax borkumensis* as an operon. The recombinant *E. coli* harboring the P450 gene and two related expression component enzymes, ferredoxin and ferredoxin reductase, was constructed in a single vector pET21(a) and successfully expressed in *E. coli* BL21(DE3) as a soluble form, showing a molecular weight of 53 kDa on 10% SDS-PAGE. When the cell-free extract of *E. coli* BL21 expressing p450 monooxygenase was subjected to reduced CO difference spectral analysis, a soret band near 450 nm appeared indicating that the cloned P450 was expressed as a functionally active enzyme. The *E. coli* cells harboring the expressed P450 gene were able to convert *n*-octane and 1-decene, producing approximately 450 μg/ml of *n*-octanol and 290 μg/ml of 1,2-epoxydecane, respectively, at pH 7.0 and 30 °C. However, the recombinant *E. coli* cells were not able to convert the branched alkane, 2,6,10,14-tetramethylpentadecane (C19).

Key words: P450 Monooxygenase, Medium-chain Alkane, *Esheriachia coli*

INTRODUCTION

Cytochrome P450s (P450 or CYP) constitute the largest superfamilies of monooxygenase enzyme proteins, and are involved in many degradation pathways for a wide range of xenobiotic compounds (Phase I drugs). P450 enzymes, which are widely distributed in prokaryotes and in all eukaryotic organisms, are considered to be very important due to their extensive roles in the metabolism of sterols and other lipid derived hormones. Also, hydrophobic low-molecular-weight compounds such as alkanes and aromatics can be catalyzed by P450 monooxygenation reactions. Recently, focus has been placed on these enzymes for the construction of useful biotransformation processes due to their ability to hydroxylate the terminal, most unreactive carbon atom of aliphatic alkanes for the synthesis of pharmaceutical intermediates. Perillyl alcohol, known as a putative anticancer agent, was obtained by converting terpene limonene using recombinant P450 monooxygenase acquired from *P. putida* [1]. Five different CYP153 enzymes obtained from *Sphingomonas* sp. HXN-200 were also confirmed to convert piperidines, pyrrolidines, and azetidines to useful pharmaceutical intermediates [2,3].

Alcanivorax borkumensis SK2 is a γ-proteobacterium that has been isolated extensively in aquatic environments, especially in oil-contaminated seawater [4,5]. This strain rapidly predominates within such contaminated seawater and efficiently utilizes a broad range of aliphatic *n*-alkanes and the branched alkane, 2,6,10,14-tetramethylpentadecane (C19), as sources of carbon and energy [6]. In such alkane assimilation bacteria, membrane-anchoring non-heme iron type monooxygenases such as alkane hydroxylases (*alkB1*, *alkB2*), which need two electron transfer components, namely rubredoxin (Rd) and Rd reductase, for alkane hydroxylase activity, are thought to be mainly related to the first hydroxylation step of alkanes [7,8]. The oxygenized alcohols produced from alkane hydroxylase are further assimilated through several downstream enzyme complexes (*alkHJK*) as energy or carbon sources [9-11]. Previously, the *alkB1* and *alkB2* genes in *A. borkumensis* SK2 were cloned and disrupted in order to determine the function of these genes in alkane assimilation *in vivo* (Hara, A., S.-H. Baik, K. Syutsubo, N. Misawa, T. H. M. Smits, J. B. van Beilen, and S. Harayama. *Environ. Microbiol.* 6(3), 191-197. 2004.). Disruption analysis demonstrated that the *alkB1* gene was only responsible for the degradation of *n*-hexane (C6). However, the catalytic properties of the enzymes, which might be responsible for the transformation of other medium-chain alkanes, have not yet been confirmed.

In the present study, a P450 monooxygenase gene was obtained from *A. borkumensis* SK2. We constructed an expression vector for P450 with the related expression enzymes, ferredoxin and ferredoxin reductase, in a single vector and expressed them in *E. coli*, and ultimately, investigated alkane oxygenation reactions with medium-chain *n*-alkanes such as *n*-octane (C8), 1-dodecene (C10), and 2,6,10,14-tetramethylpentadecane (C19).

EXPERIMENT

1. Strain, Plasmid, and Medium

A. borkumensis SK2 was obtained from DSMZ (DSM11573). A marine broth medium containing 1% pyruvic acid was used for the routine cultivation and preservation of the strain. The oligonucleotide primers used in this study are described in Table 1. All the restriction endonucleases were purchased from New England Biolabs.

2. Genetic Manipulation and Nucleotide Sequencing Analysis

General DNA manipulations such as plasmid preparation and subcloning were performed by following the method of Sambrook

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Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequences
AlcP450-1Nd	5′-AAAAA**CATATG**ATGGGAAAAATCACCTTTATTGAG-3′
AlcP450-2Nt	5′-AAAAA**GCGGCCGC**TCAGATCAAAGACTTTA-3′
Alcfer-Cfus	5′-AACTCGTTGACATTACATTTGAAATTCTGGC-3′
AlcP450-Nfus	5′-GCCAGAATTTCAAATGTAATGTCAACGAGTT-3′
AlcP450-Cfus	5′-ATGTTGTTTTTCGTTTTCCATTATTTTTTAGCCGACAACTTAACC-3′
Alcfered-Nfus	5′-GGTTAAGTTGTCGGCTAAAAAATAATGGAAAACGAAAAACAACAT-3′

et al., unless otherwise stated [13]. The nucleotide sequences were analyzed via a dye terminator cycle-sequencing reaction by following the supplier's instructions (Applied Biosystems). The sequencing products were detected with a 377 DNA sequencer (Applied Biosystems), and the resulting sequence data were analyzed by the GENETYX-MAC software package (Software Development, Tokyo, Japan).

3. Cloning of DNA Fragment Encoding P450s

The genes encoding P450 monooxygenase and related genes, such as ferredoxin and ferredoxin reductase, were amplified directly from *A. borukumensis* SK2 genomic DNA by means of the polymerase chain reaction (PCR). Two oligonucleotide primers with restriction sites of NdeI or NotI (forward primer AlcP450-1Nd and reverse primer AlcP450-2Nt) were prepared to anneal the N-terminal sequence of ferredoxin or the C-terminal sequence of ferredoxin reductase on the genomic DNA of *A. borkumensis* SK2. PCR was performed with a KOD plus PCR kit (Toyobo) for 29 cycles of: 94 °C for 60 s, 58 °C for 30 s, and 68 °C for 3 min. The PCR amplified fragment was approximately 4.5-kbp and directly inserted into the NdeI and NotI sites of the pET21(a) expression vector (p450E-1).

4. Construction of Expression Vectors

The obtained p450E-1 clone contained a putative alcohol dehydrogenase gene located between P450 and ferredoxin reductase. To reconstruct a P450 expression vector without the alcohol dehydrogenase gene, three rounds of PCR were performed to amplify each P450, ferredoxin, or ferredoxin reductase gene on p450E-1, as shown in Fig. 2(b), by using 6 oligonucleotide primers (Table 1). Since the amplified PCR products were designed for translational coupling, they overlapped each other at the N- and C-terminals, and we were able to obtain Fig. 2(d) by fusion PCR. The fusion PCR was performed using a KOD plus PCR kit (Toyobo) with two steps: 1) a self-annealing step for 8 cycles of: 94 °C for 60 s, 46 °C for 60 s, and 68 °C for 2 min; and 2) an amplification step with oligonucleotide primers containing the NdeI and NotI sites for 20 cycles of: 94 °C for 60 s, 58 °C for 30 s, and 68 °C for 3 min. The PCR amplified fragment of approximately 3-kbp was directly inserted into the NdeI and NotI sites of the pET21(a) expression vector (p450E-2) and transformed into *E. coli* BL21(DE3) competent cells. The recombinant proteins were incubated until OD_{612} reached 0.85 (approximately 3 h). Then, cultivation was continued for 9 hrs at 25 °C

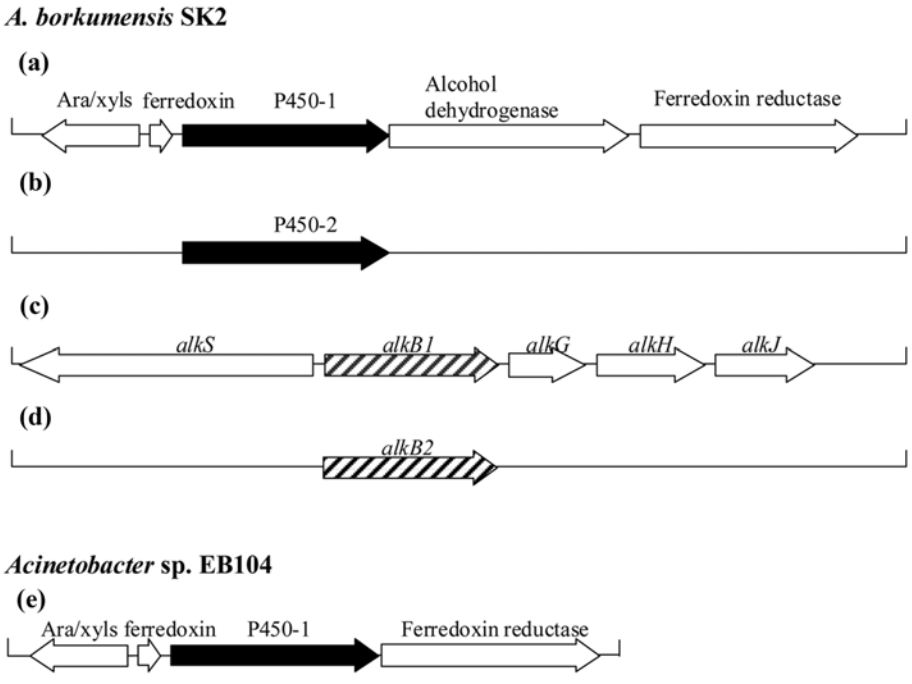

Fig. 1. Gene structure of P450 cluster from *A. borkumensis* SK2. (a) P450-1 gene cluster. (b) P450-2 single gene. (c) *alkB1* gene cluster. (e) *alkB2* gene. (e) *Acinetobacter* sp. EB104 gene cluster.

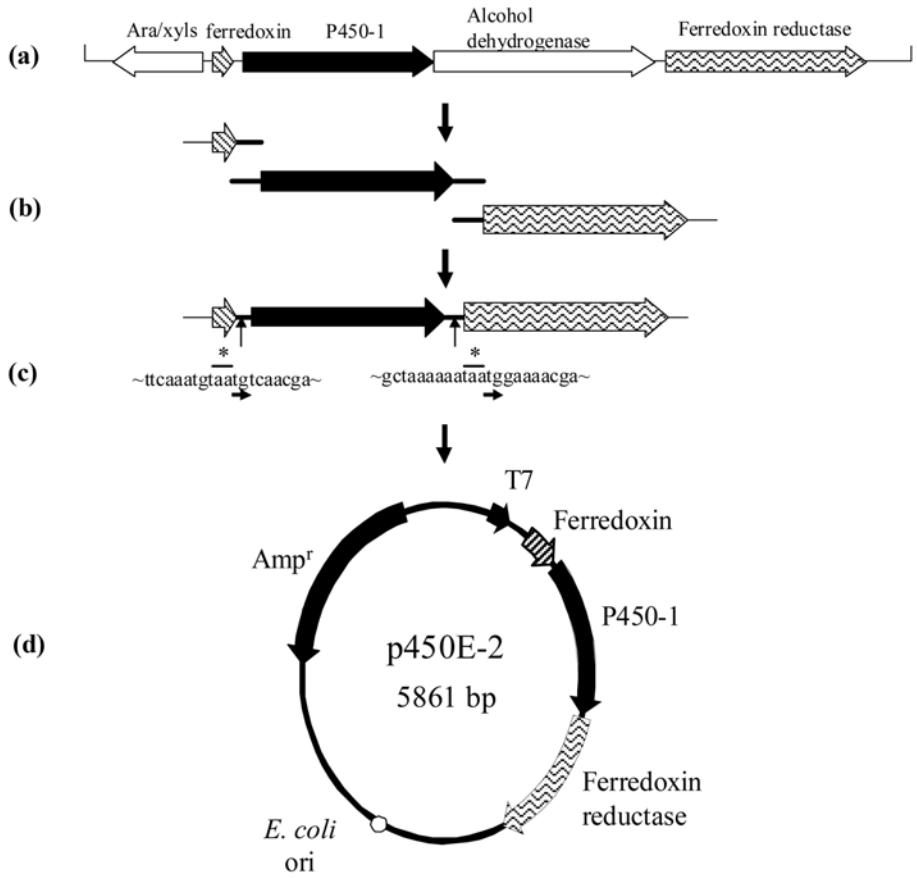

Fig. 2. Construction of P450 expression vector with ferredoxin and ferredoxin reductase from A. borkumensis SK2. (a) P450 gene cluster obtained from genomic DNA of *A. borkumensis* SK2. (b) Second PCR for amplification P450, ferredoxin and ferredoxin reductase gene only without alcohol dehydrogenase. (c) Fusion PCR for P450, ferredoxin and ferredoxin reductase gene with translation coupled gene structure. (d) Constructed P450, ferredoxin and ferredoxin reductase expression vector, P450E-2.

with reciprocal shaking. The cells were harvested by centrifugation (10,000 ×g, 10 min, 4 ℃), washed twice with 0.85% NaCl solution, and suspended in Tris-HCl buffer (10 mM, pH7.0).

5. Bioconversion of Medium-chain Alkanes

For the bioconversion examination of medium-chain alkanes, 250 µl of *n*-octane and *n*-decene were added and vigorously mixed with one milliliter of overnight culture and incubated with shaking for 24 h. The converted product was extracted with 200 µl of 1 N HCl and 250 ml of *n*-hexane and analyzed by gas chromatographic-mass spectrometry (GC-MS, QP5050A, Shimadzu). The temperature program for GC-MS was an initial temperature of 80 ℃ for 3 min, followed by increases to 105 ℃ (rate: 5 ℃ per min) and then 240 ℃ (rate: 20 ℃ per min). We also examined the effect of pH on the conversion of *n*-octane and *n*-decene at pH values of 4.0, 5.0, 6.0, 7.0 and 8.0 in the same tube for 24 h. To find the optimum temperature, we performed the conversion reaction at 20 ℃, 30 ℃, and 37 ℃ in 50 mM Tris/HCl buffer (pH 7.0) with time course reaction. All the reactions were carried out with same method as described above.

RESULTS AND DISCUSSION

1. Cloning of the P450 Genes and Sequence Analysis of P450 with a Flanking Region

The P450 gene and related enzyme components in *A. borkumensis* SK2 were selected and cloned by PCR. The obtained PCR amplified products contained five open reading frames (394 a.a., 106 a.a., 469 a.a., 552 a.a., and 456 a.a.) which showed high similarity with a putative AraC/XylS regulator protein, P450 monooxygenase (CYP153), ferredoxin, and ferredoxin reductase, respectively, from *Acinetobacter* sp. EB104 at the amino acid level. The obtained P450 gene had a single open-reading frame of 1407 base pairs coding 469 amino acids. A comparison of the deduced amino acid sequence with other published sequences showed relatively low similarity with the already published P450 monooxygenase superfamily sequences of *Acinetobacter* sp. (66%) and *Mycobacterium* sp. (67%). However, it showed higher similarity with *Alcanivorax* sp. DG881 (83%), *Marinobacter aquaeolei* VT8 (80%), and *Arthrobacter* species (78%). It has been reported that most hydrocarbon-degrading strains possess enzymes related to CYP153A1 and CYP153A6, cytochrome P450 enzymes that were characterized as alkane hydroxylases [14]. In that study, it was also shown that the cloned P450s did not contain membrane binding amino acid sequences. In our experiments, *A. borkumensis* SK2 contained significantly conserved P450 common motifs that are typically shown in the P450 monooxygenase superfamily, *i.e.*, the HR2 conserved sequence motif: Phe410-X-X-Gly-X-Arg(His)-X-Cys-X-Gly419; and helix I sequence motif: Gly308-X-X-Thr401. Moreover, we could identify Arg156 and Arg358, which are usually found in the heme type P450 gene family as heme-

propionic acid conjugated amino acids [8,9]. However, no membrane binding amino acids sequences shown in the P450 of *Acinetobacter* sp. EB104 were observed.

2. Expression of P450 Genes in *E. coli*

The recombinant *E. coli* BL21 (DE3)-transformed p450E-2, under the control of a strong T7 promoter, overproduced soluble recombinant p450 after 9 hours of induction, as shown in Fig. 3(a). In this study, we constructed the expression vector as an operon with translation coupled start codon as shown in Fig. 2(c). The *E. coli* cells expressing p450E-2 changed color to white due to the overexpression of ferredoxin or ferredoxin reductase. A molecular weight band of approximately 53 kDa for p450, as estimated by SDS-PAGE, was observed [Fig. 3(a)]. Next, we examined the reduced CO difference spectra to determine whether or not the P450 enzyme was expressed with a correctly folded P450 heme domain. When the cell-free extract of *E. coli* BL21 was subjected to reduced CO difference spectral analysis, a soret band near 450 nm appeared, as shown in Fig. 3(b), indicating that the cloned P450 was expressed as a functionally active enzyme. The dysfunctional soret band that would usually appear near 420 nm in reduced CO difference spectral analysis was not observed from the *E. coli* cell-free extract. This result indicates that the cloned P450 was expressed with a correctly folded P450 heme domain.

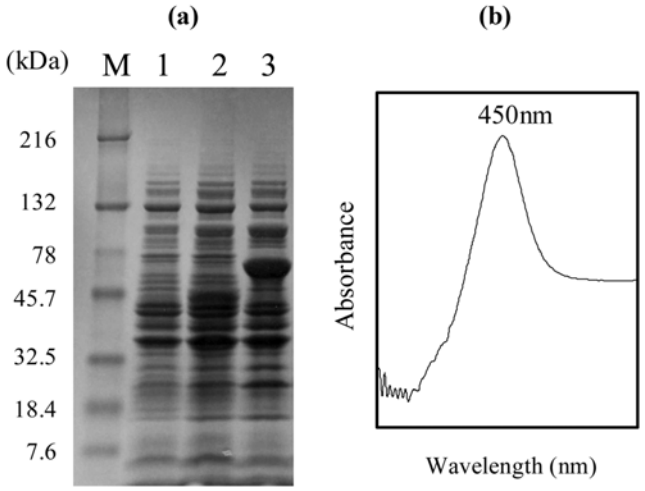

Fig. 3. Expression analysis of P450, ferredoxin and ferredoxin reductase from *A. borkumensis* SK2 in *E. coli* cells. (a) SDS-PAGE analysis (b) CO difference spectral analysis.

3. Bioconversion of *E. coli* Whole-cells Expressing P450 Genes

Thus, we attempted bioconversion experiments using *E. coli* whole-cells to investigate catalytic functions on several medium chain *n*-alkanes. As shown in Fig. 4, the *E. coli* cells harboring the P450 gene were able to convert *n*-octane and *n*-decene. However, the recombinant *E. coli* cells were not able to convert the branched alkane, 2,6,10,14-tetramethylpentadecane (C19). This result shows that the P450-1 gene from *A. borkumensis* SK2 can be successfully expressed

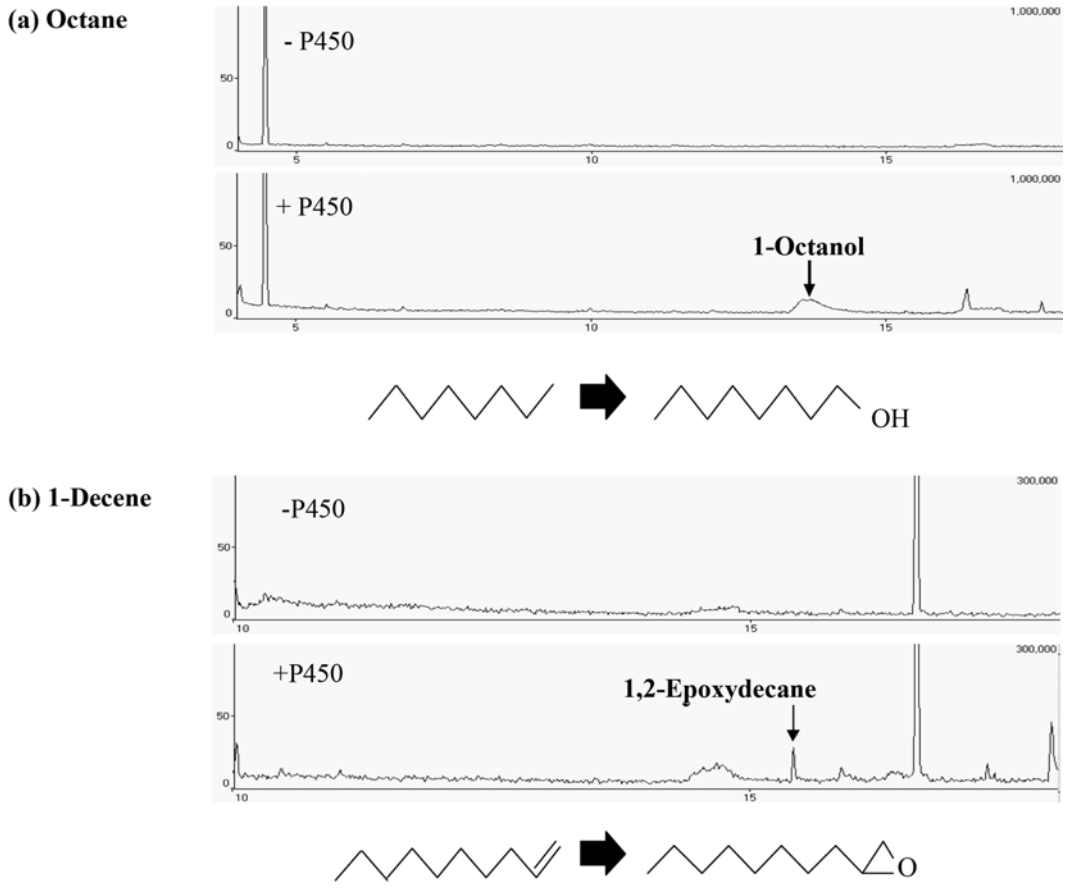

Fig. 4. GC-MS analysis.

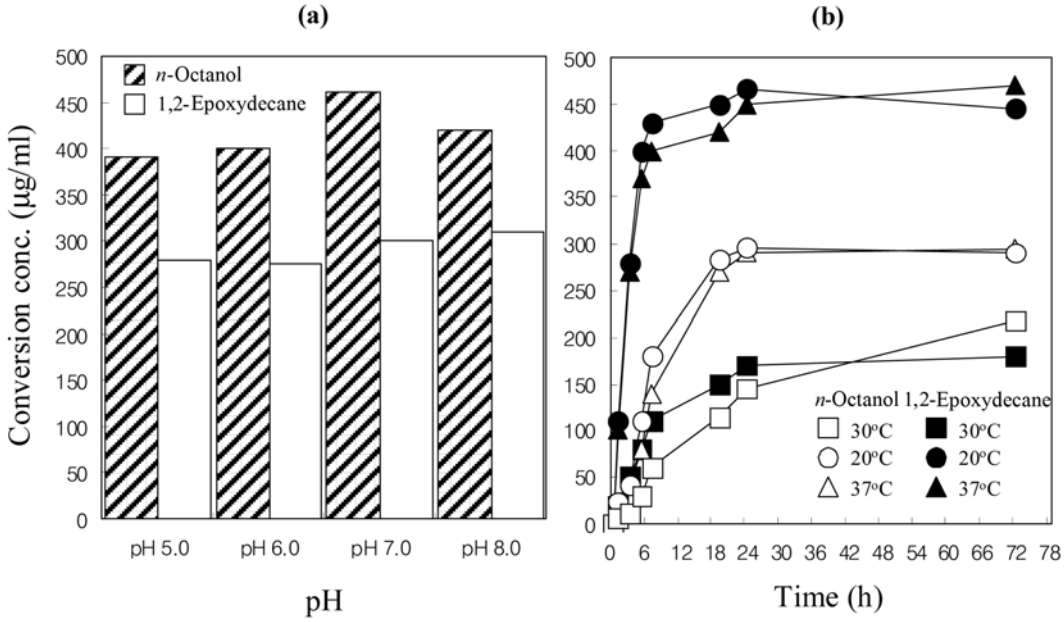

Fig. 5. Optimal condition for the bioconversion of *n*-octane and 1-decene by whole-cells of recombinant *E. coli* harboring the P450, ferredoxin and ferredoxin reductase gene from *A. borkumensis* SK2. All measurements were conducted in triplicate and are represented by the means of the each measurement. (a) Effect of pH for *n*-octane and 1-decene bioconversion. (b) Effect of temperature.

in *E. coli* and was able to hydroxylate the terminal carbon atom of *n*-medium chain alkanes, but could not hydroxylate the branched alkane, 2,6,10,14-tetramethylpentadecane (C19). The optimal pH for bioconversion of both *n*-octane and *n*-decene is 7.0 as shown in Fig. 5(a). We also examined the effect of temperature for bioconversion; a lowering the temperature of 20 °C was found to not stimulate significantly the production of *n*-octanol, resulting in the lowest conversion yield (Fig. 5(b)). Under optimized reaction conditions as described above by means of whole-cell biocatalyst of recombinant *E. coli* harboring the P450, ferredoxin and ferredoxin reductase gene from *A. borkumensis* SK2, were able to convert *n*-octane and 1-decene, producing approximately 450 μg/ml of *n*-octanol and 290 μg/ml of 1,2-epoxydecane, respectively at pH 7.0 and 30 °C.

CONCLUSION

We reported on the cloning of a P450 gene from *A. borkumensis* SK2 along with the expression analysis of the cloned P450 in *E. coli* cells. Our results clearly indicate that the P450 gene was able to function successfully in *E. coli* cells by way of ferredoxin and ferredoxin reductase, and was responsible for the terminal hydroxylation of the medium-chain alkanes, *n*-octane and *n*-decene.

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