Studies on Hydroxylation of Alkylbenzenes by *Fusarium moniliforme* Strain MS31

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Contents

Abstract

Oxidation of C–H bonds is a useful reaction in organic chemistry because desired oxyfunctionalized compounds can be obtained from common hydrocarbons. Much effort has been directed toward the development of efficient stereoselective oxidation of hydrocarbons, but resulted in only moderate enantioselectivity with most alkanes. Biocatalytic reactions are generally highly regio- and stereospecific. In this study, microorganisms from soil samples were firstly screened for conversion of alkylbenzenes to chiral compounds. Some fungi were found to convert of propylbenzene to (*R*)-1-phenylpropanol, which is useful for synthesis of optically active 1-chloro-1-phenylpropane and liquid crystals, and is an auxiliary used for preparation of terpenes. Optimization of conditions for the production, and elucidation of the reaction mechanism were then studied.

In Chapter 1, production of hydroxylated alkylbenzenes was described. The screening of microorganisms was preformed for the hydroxylation of alkylbenzenes in the section 1. Yeasts and molds that converted ethylbenzene and propylbenzene into their corresponding oxygenated products during incubation of cells were isolated. One particularly potent strain was identified as *Fusarium moniliforme*. The fungus oxidized the side chains of ethylbenzene and propylbenzene selectively at the benzylic position. The products were identified to be 1-phenylethanol and 1-phenylpropanol, respectively, by GC-MS and HPLC with a chiral column, with 100% enantiomeric excess of the (*R*)-(+)-form. To increase the specific activity of the hydroxylating enzyme in *F. moniliforme* strain MS31, the components of the medium and the reaction conditions were optimized in the section 2. Glucose and $NaNO_3$ were selected as carbon and nitrogen sources, respectively. The substrate, propylbenzene, inhibited the fungal growth and the enzyme activity. Acetoin added to the medium increased both the growth and the enzyme activity, and hydroxylation of propylbenzene increased by 1.4-fold. Maximum bioconversion of propylbenzene by resting cell system was at 25-30°C and pH 7.0 with cells at concentration of 40 mg (dry) per ml of reaction mixture. Conversion was accelerated as soon as propylbenzene was added; slowing 2 h later.

Finally, *F. moniliforme* strain MS31 produced (*R*)-1-phenylpropanol with an enantiomeric excess of 98% at the concentration of 16 mM (2.2 $mg \cdot ml^{-1}$). To elucidate the scope of substrate acceptability by the fungus, I used various aromatic compounds for the bioconversion in the section 3. The fungus hydroxylated various alkylbenzenes at the benzylic position to produce optically active alcohols. Butylbenzene was converted to nonbenzylic alcohols. In all cases, the *R* absolute configuration of products was more abundant. Aromatic compounds with linear side chains and (2-methylpropyl)benzene were converted to their corresponding alcohols with an enantiomeric excess of 94% to 100%. Further oxidation of the alcohols was detected, but it was weak.

In Chapter 2, the characterization of the hydroxylating enzyme of *F. moniliforme* strain MS31 was done. Hydroxylation of propylbenzene needed molecular oxygen, and NADPH as a coenzyme gave a higher yield than NADH. The hydroxylation increased when FAD and FMN were added, suggesting that the enzyme was a flavoprotein. Carbon monoxide inhibited the hydroxylation, as did other cytochrome P450 (P450) inhibitors such as SKF 525A and miconazole. These characteristics matched those of a microsomal P450 monooxygenase system that contained NADPH-cytochrome P450 reductase (CPR).

In Chapter 3, I tried to isolate a CPR gene and a P450 gene involving in the hydroxylation of propylbenzene. A cDNA encoding CPR was isolated by RT-PCR in the section 1. The deduced amino acid sequence showed a high similarity to the CPRs from other eukaryotes and the functional domains, NADPH-, FAD-, FMN-, and P450-binding, were highly conserved in these taxonomically diverse species. Expression in *Saccharomyces cerevisiae* under control GAL1 promoter resulted in 5-fold increase in NADPH-cytochrome *c* reductase activity in the yeast cell extract. Three cDNAs encoding P450 were isolated by RT-PCR in the section 2. One of them was a novel P450 and the deduced amino acid sequence matched 498 proteins in BLAST search with known protein database sequences, all belonging to the P450 family. The others were similar to P450-1 and P450-2 from *Gibberella fujikuroi*, which is sexual state of *F. moniliforme*, and the identities in the nucleotide sequence were 96 and 95%, respectively. The cells of *S. cerevisiae* harboring the cDNAs encoding P450 did not show the hydroxylation of propylbenzene.

General Introduction

The application of enzymes as catalyst is one of the fastest growing areas in the field of organic chemistry. Bioconversion is a method of harnessing the power of enzymes in organic synthesis. The ability of enzymes to perform reactions in a regio- and stereospecific manner, while functioning under mild conditions of temperature and pH, offers unsurpassed potential for the production of chiral molecules.

The production of optically pure compounds is increasing importance to the chemical and pharmaceutical industries (Stinson 1995). For example, the presence of the 'wrong' stereoisomer in a preparation of a chiral pharmaceutical compound can have severe adverse effects on patients or, in the case of a chiral agrochemical, may increase the burden on the environment. Most chiral compounds can be produced by chemical synthesis, by bioconversion, by a combination of both of these approaches, or by extraction from plant material. The bioconversion has several advantages. Biocatalysts operate under mild conditions, and they are also reaction-, regio-, and stereospecific, thus yielding fewer by-products than chemical synthesis. The useful enzymes in production of optically pure compounds are lipases, dehydrogenases, lyases, oxygenases, and so on. Lipases catalyses both the hydrolysis and the synthesis of esters to produce optically active compounds by the resolution of racemates (Jeager and Reetz 1998). Dehydrogenases catalyze the reduction of C=O bonds to produce optically active alcohols, hydroxy acids, and amino acids (Hummel 1999). The asymmetric reduction of C=O bonds catalyzed by baker's yeast are well studied (Eguchi and Mochida 1995). Lyases catalyze the addition of groups to C=C bonds or the formation of C–C bonds, resulting in the formation of optically active compounds (van der Werf *et al.* 1994). Oxygenases catalyze the addition of oxygen to C=C bonds and C–H bonds to produce optically active epoxides and alcohols (Holland 1992).

This study focuses on the microbial hydroxylation to produce optically active alcohols. Hydroxylation belongs to the most versatile reactions in organic chemistry, since potentially useful oxyfunctionalized synthons may be obtained from readily available hydrocarbons.

Metal-catalyzed asymmetric hydroxylations have been performed by employing chiral auxiliaries (Jacobsen *et al.* 1988) and optically active oxidants (Davis *et al.* 1984; Fuji *et al.* 1993; Hamada *et al.* 1998) to afford enantiomerically enriched products. Despite all these efforts, only a moderate enantioselectivity has been achieved. Microorganisms have been successfully applied to the selective oxygenation of unactivated C–H bonds in organic substrates. The major emphasis of such work has focused on the hydroxylation of steroids, terpenes, and other natural products (Holland 1999). The hydroxylation of xenobiotics is fewer than natural products. Xenobiotics are not readily introduced into cells for metabolism. Many monooxygenases, which are main catalysts of microbial hydroxylation, are highly substrate-specific, and therefore, their applications are restricted to closely related substrates. The purpose of this study was to search for microorganisms that are useful for the regio- and stereoselective hydroxylation with broad substrate specificity.

In this report, the regio- and stereoselective hydroxylation of alkylbenzenes by *Fusarium moniliforme* strain MS31 was investigated. In Chapter 1, microorganisms were screened for the hydroxylation of alkylbenzenes and the strain MS31 was used in the investigation of its optimal conditions for the hydroxylation and of possible substrates. For clarify the enzyme involved in the hydroxylation of propylbenzene, enzymatic properties were investigated in Chapter 2 and possible genes encoding the hydroxylation enzyme were cloned in Chapter 3.

Chapter 1

Production of hydroxylated alkylbenzenes

Microorganisms hydroxylate alkylbenzenes either at the aromatic ring or at the alkyl side chain. Some of hydroxylated products are useful for synthesis of drugs, flavors, liquid crystals, and so on. For example, 2-phenylethanol and racemic 1-phenylpropanol are commercially used as a rose flavor and a choleretic drug, respectively. (*R*)-1-Phenylethaol is used as chiral reagent for the determination of enetiomeric purity and for resolutions of acids (Takeuchi *et al.* 1987) and as an auxiliary for synthesis of a cholesterol-lowering agent, (+)-compactin (Rosen and Heathcock 1985). .

In this chapter, the hydroxylation of alkylbenzenes using whole cells was described. Microbial hydroxylation involves an enzyme of the oxidoreductase group, which contains oxygenases, oxidases, and dehydrogenases. These enzymes are generally unstable and expensive to prepare; many need expensive cofactors such as NAD(P)H. The use of whole cells rather than purified enzymes has the following advantages. Microbial cells are less expensive than purified enzymes, and the activity of the enzyme of interest may be more stable in the cell than when purified. The addition of purified cofactors is usually not necessary because generally they are already present within the cell, or can be generated there. The regeneration of cofactors outside the reaction system is unnecessary when whole cells are used.

The section 1 is concerned with screening for microorganisms that were useful for conversion of alkylbenzenes to chiral compounds. Some fungi converted ethylbenzene and propylbenzene into the corresponding oxygenated products and a fungal strain MS31 that showed highest yield of (*R*)-1-phenylpropanol was identified as *Fusarium moniliforme*. In the section 2, media for cultivation of *Fusarium moniliforme* strain MS31 and the conditions of the cell reaction were investigated to increase the yield of (*R*)-1-phenylpropanol. In the section 3, the substrate specificity was investigated.

Section 1
Screening of microorganisms for stereoselective oxidation of alkylbenzenes

Oxygenation is important in biodegradation as well as synthesis. Owing to the difficulty of adding oxygen atoms regio- and stereoselectively at nonactivated C–H bonds by chemical synthesis, biotransformation systems are often used in organic synthesis at present (Holland 1999). Some oxygenases participate in the degradation of organic compounds. Monooxygenases of methane- or alkane-utilizing bacteria oxidize a wide range of hydrocarbons. Methane monooxygenase is used in the production of alcohols, epoxides (Perry 1979; Higgins 1980), and hydroquinone (Yoshida *et al.* 1990). Alkene monooxygenase is used in the production of various epoxides (de Bont 1993). These oxygenases are induced by substrates other than alkenes and methane. The microbial pathways for the diterminal oxidation of alkane have secondary alcohols and methylethylketone (MEK) as intermediates (Markovetz 1971), all of which induce alkane monooxygenases (Yoshida *et al.* 1990; Onodera and Ogasawara 1990).

In this section, microorganisms that were used MEK as a sole carbon source were isolated from soil samples and were screened for stereoselective hydroxylation of alkylbenzenes. MEK is liquid at room temperature, miscible in water, and therefore more convenient to use in cultivation than methane and other gaseous alkanes for induction of oxygenases with broad substrate specificity.

MATERIALS AND METHODS

Chemicals All chemicals were purchased and used without further purification.

Isolation of microorganisms The basal medium (BM1) for isolation of assimilators of MEK contained, per 1 *l* of deionized water, 10 g of $NaNO_3$, 2 g of NH_4Cl, 2 g of KH_2PO_4, 3 g of K_2HPO_4, 2 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of yeast extract (dried;

type S; Nihon Pharmaceutical Co., Tokyo), and 2 ml of a metal mixture, pH 7.0. The metal mixture consisted of, per 100 ml of deionized water, 400 mg of $MnCl_2 \cdot 2H_2O$, 350 mg of $FeCl_2 \cdot 4H_2O$, 200 mg of $ZnCl_2$, 20 mg of $CoCl_2$, 20 mg of $CuCl_2 \cdot 2H_2O$, 10 mg of $Na_2MoO_4 \cdot 2H_2O$, 10 mg of $Na_2B_4O_7 \cdot 10H_2O$, and 2 ml of conc. HCl. MEK, unsterilized, was added to BM1 to a concentration of 1% (v/v). Small amounts of soil were added to 4 ml of the medium in 16.5 × 165-mm test tubes and the mixture was cultivated at 30°C on a reciprocal shaker at 300 rpm. When growth was observed, a 0.4-ml portion was transferred to 4 ml of fresh medium and cultivation was continued. Transfers were repeated for a total of four times for enrichment.

Cultivation All strains isolated were maintained on Bennett's agar, which was composed of 1% (w/v) glucose, 0.5% (w/v) peptone (Polypepton®; Nihon Pharmaceutical), 0.2% (w/v) yeast extract, 0.2% (w/v) Ehlrich's bonito extract (Riken Vitamin Co., Tokyo), and 1.5% (w/v) agar in tap water (pH 7.0). Microorganisms were cultured in 200 ml of BM1 containing 1% (v/v) MEK in a 2-*l* Erlenmeyer flask at 30°C on a rotary shaker at 150 rpm for 3 d. The mycelia or cells were collected by filtration or centrifugation, washed twice with 50 ml of 25 mM potassium phosphate buffer, pH 7.0 (KPB), and kept at –20°C until use.

Cell reaction Wet mycelia or cells weighing 0.1 g were suspended in 2 ml of 25 mM KPB in 16.5 × 165-mm test tubes, and 200 μmol of ethylbenzene (21 μl) or propylbenzene (24 μl) was added. The test tubes were sealed with butyl-rubber stoppers and incubated at 30°C on a reciprocal shaker at 300 rpm. After 24 h, the reaction mixture was acidified by the addition of 0.2 ml of 6 N HCl and extracted with 2 ml of *n*-hexane. The extracts were analyzed as follows.

GC GC was done with a gas chromatograph (model GC-14B; Shimadzu Corp., Kyoto) equipped with a flame ionization detector and an integrator (model 16A, Shimadzu). *n*-Hexane extract was injected into a DB-5 capillary column (30 m × 0.32 mm (i.d.); the coating was 0.25 μm thick; J & W Scientific Inc., Folsom, CA). GC was run initially at 80°C for 7 min and then with a temperature gradient of 10°C·min^{-1} to 120°C, with He gas at a flow rate of 2 ml·min^{-1} (split ratio, 20:1). The temperatures of the injector and the detector were 200 and 250°C, respectively.

GC-MS Products were analyzed by GC-MS with authentic samples and the Wiley mass spectrum database (McLafferty and Stauffer 1989) for reference. Mass spectra were obtained with a gas chromatograph (HP 5890 series II; Hewlett-Packard Co., Palo Alto, CA) coupled with a mass spectrometer (MS; MStation JMS-700; JEOL Ltd., Tokyo). GC conditions were the same as described above except that the splitless mode was used with He (5 ml·min^{-1}) as the mobile phase. The column was directly coupled to the MS ionization chamber, where the source was set at 200°C and the ionization energy and the trap current were at 70 eV and 300 μA, respectively. Ion detection and mass spectra were recorded and processed by MS-MP 7010 software (JEOL) on a 715/64 computer (Hewlett-Packard).

HPLC The HPLC system had L-7000 pump units, an L-4500 photodiode array detector, and a D-6500J system manager (Hitachi Ltd., Tokyo). A Chiralcel OD column (25 cm × 4.6 mm (i.d.), 5-μm particle size; Daicel Chemical Industries, Ltd., Osaka) was used for separation of the enantiomers of 1-phenylethanol or 1-phenylpropanol. The mobile phase was *n*-hexane–2-propanol (90:10, by volume) and the flow rate was 0.5 ml·min^{-1} for the separation of the enantiomers of 1-phenylethanol. 1-Phenylpropanol was separated with a 97:3 mixture of *n*-hexane and 2-propanol at a flow rate of 1 ml·min^{-1}. A 254-nm UV light was used for the examination of all substrates and products.

RESULTS

Screening of microorganisms that oxidized the side chains of ethylbenzene and propylbenzene Enrichment culture with 1% (v/v) MEK yielded 355 strains of bacteria, yeasts, and molds. Growth in BM1 containing 1% (v/v) MEK was poor, so strains were grown in BM1 containing 4% MEK in a secondary screening. Two bacteria, two yeasts, and 17 molds grew. In the cell reaction system, all yeasts and molds oxidized the side chains of ethylbenzene and propylbenzene to 1-phenylethanol and 1-phenylpropanol, respectively (Table 1.1.1), but the bacteria did not produce detectable products. Products of the five highest producers among the molds were analyzed and found to be in the (*R*)-(+)-form. No

strains grew in BM1 containing 0.1%, 0.5%, or 1% (v/v) ethylbenzene or propylbenzene as the sole carbon source. A fungus, strain MS31, was selected as being the most potent and was studied further.

TABLE 1.1.1. Oxidation of ethylbenzene and propylbenzene by a cell suspension of isolated microorganisms

Strain	Benzylic alcohol from:					
	Ethylbenzene			Propylbenzene		
	μmol	%[a]	% ee (*R*)	μmol	%[a]	% ee (*R*)
MH27 (mold)	2.4	1.2		2.4	1.2	
MS05 (yeast)	3.4	1.7		0.8	0.4	
MS27a (mold)	2.4	1.2		4.6	2.3	
MS27b (mold)	2.4	1.2		2.4	1.2	
MS29 (mold)	1.6	0.8		2.4	1.2	
MS30 (mold)	12.4	6.2	95	11.4	5.7	97
MS31 (mold)	13.4	7.2	98	16.2	8.1	98
MS33 (mold)	0.8	0.4		2.4	1.2	
MS37 (mold)	6.6	3.3	100	8.4	4.2	98
MS40a (mold)	3.4	1.7	100	9.2	4.6	99
MS40b (mold)	4.2	2.1		5.4	2.7	
MS43 (yeast)	2.4	1.2		2.4	1.2	
MS44 (mold)	2.4	1.2		6.2	3.1	
MS46a (mold)	1.6	0.8		1.6	0.8	
MS46b (mold)	0.8	0.4		0.8	0.4	
MS47 (mold)	5.0	2.5		4.6	2.3	
MS49 (mold)	9.2	4.6	98	6.2	3.1	98
MS50a (mold)	4.6	2.3		5.4	2.7	
MS50b (mold)	5.0	2.5		5.6	2.8	

The reaction procedure and identification of the products were as described in Materials and Methods.
[a]Percentage of μmoles of the product to μmoles of the starting substrate.

Identification of MS31 Strain MS31 was pale gray when growing on plates of Bennett's agar or Czapek-Dox agar. After 5 d of culture, the culture medium turned purple. Microscopic observation of the fungus on slides showed that it was filamentous, had septa, and its crescent-shaped conidia did not grow in chains but were borne on phialides. These

are characteristics of the genus *Fusarium*. The fungus was investigated further at the Centraalbureau voor Schimmelcultures (Baarn, the Netherlands) and it was identified as *Fusarium moniliforme* s.s. (=*Fusarium verticillioides* (Saccardo) Nirenberg).

Identification of reaction products Ethylbenzene and propylbenzene were incubated with cells of *F. moniliforme* strain MS31 for 24 h (Fig. 1.1.1). Two peaks appeared in both chromatograms, for ethylbenzene at 5.5 and 5.7 min (products 1 and 2), and for propylbenzene at 8.8 and 9.2 min (products 3 and 4). The ratios of the areas of major peaks (products 1 or 3) to those of minor peaks (products 2 or 4) were 39 and 40, respectively.

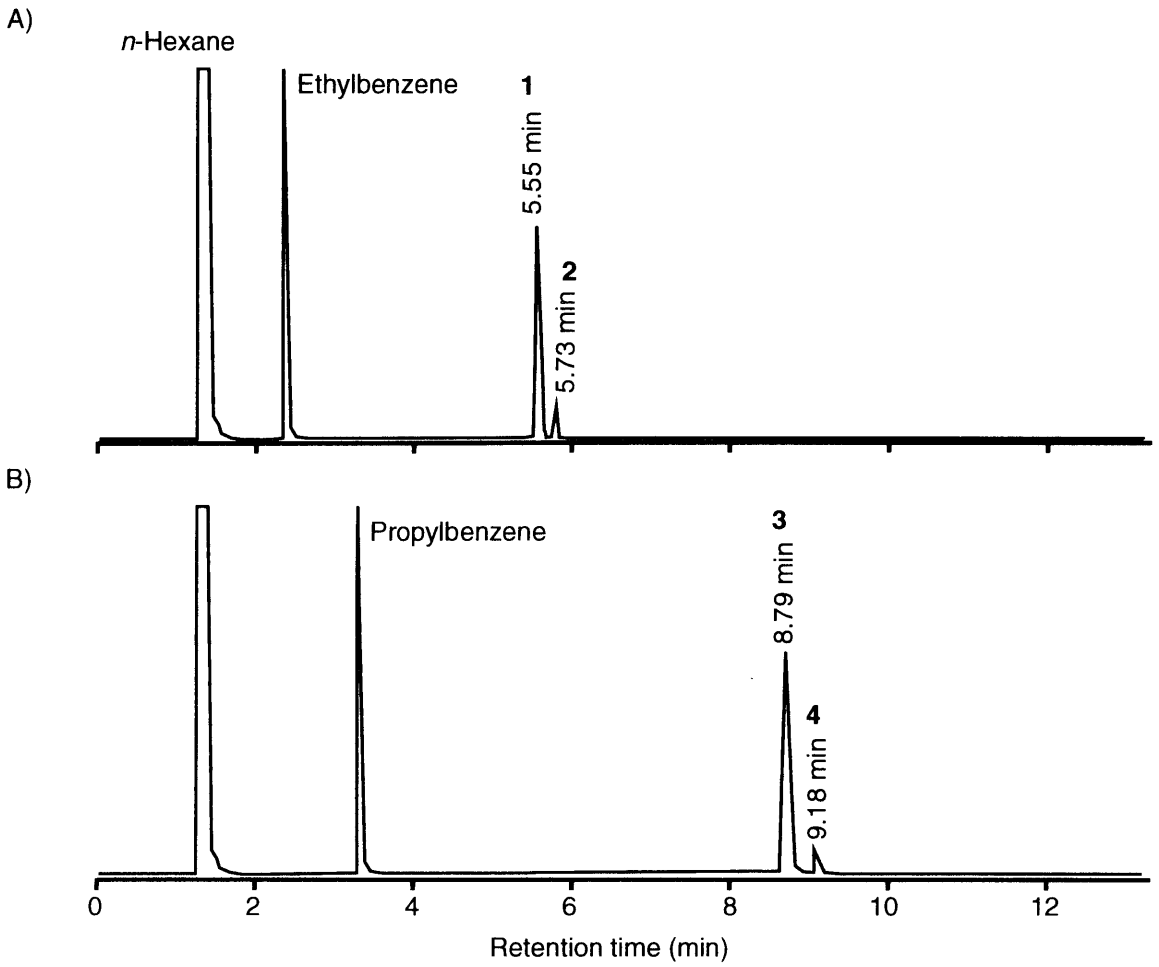


FIG. 1.1.1. Gas chromatograms of products from ethylbenzene (A) and propylbenzene (B) incubated with resting cells of *F. moniliforme* strain MS31. *n*-Hexane extracts were injected into a DB-5 capillary column connected with a GC equipped with a flame ionization detector. Conditions were as described in Materials and Methods. Product peaks are labeled with the retention time.

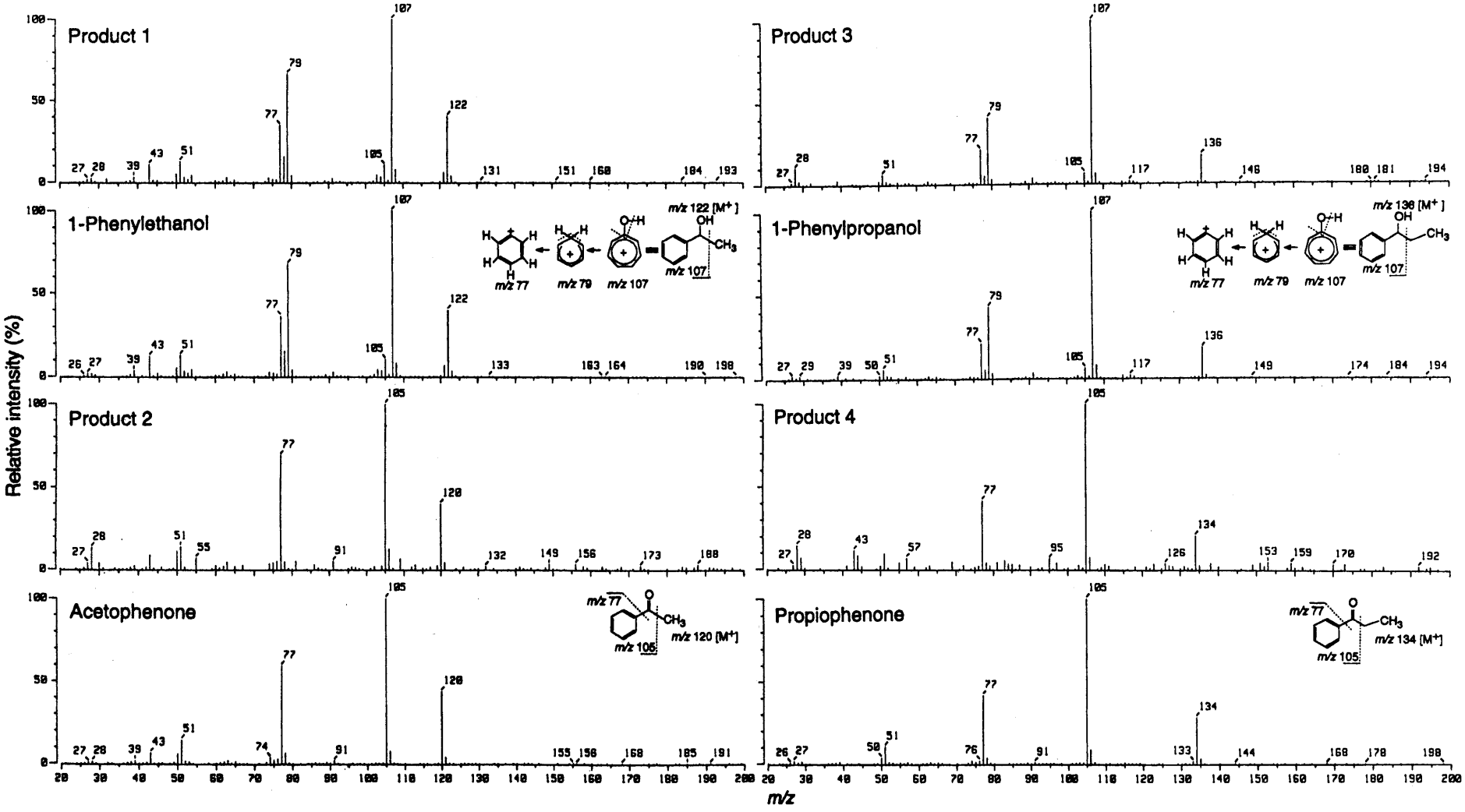


FIG. 1.1.2. Electron ionization-mass spectra of products from ethylbenzene (left) and propylbenzene (right) incubated with resting cells of *F. moniliforme* strain MS31. *n*-Hexane extracts were injected into a DB-5 capillary column connected with a GC-MS system. GC-MS was done as described in Materials and Methods. Product spectra are shown with those of the authentic chemicals below. General mass-spectral fragmentations are shown on the spectra of authentic chemicals.

These products were characterized by electron ionization GC-MS (Fig. 1.1.2). The molecular ion peaks of products 1 and 3 were at *m/z* 122 and 136 (M^+), respectively. Fragment peaks at *m/z* 77, 79, and 107 were characteristic of benzylic alcohols. Other fragment peaks matched those obtained from the corresponding authentic chemicals. The spectrum for product 1 matched that for 1-phenylethanol in the Wiley mass spectrum database (McLafferty and Stauffer 1989). Product 3 was identified in a similar way as being 1-phenylpropanol. The molecular ion peaks of products 2 and 4 were at *m/z* 120 and 134 (M^+), respectively. Products 2 and 4 were identified as acetophenone and propiophenone, respectively, by comparison of their spectra with those of authentic chemicals.

Stereochemistry Figure 1.1.3 shows results of HPLC of the reaction products on Chiralcel OD columns. 1-Phenylethanol and 1-phenylpropanol each were resolved into two peaks, both at 14 and 16 min. A comparison with enantiomerically pure samples of the

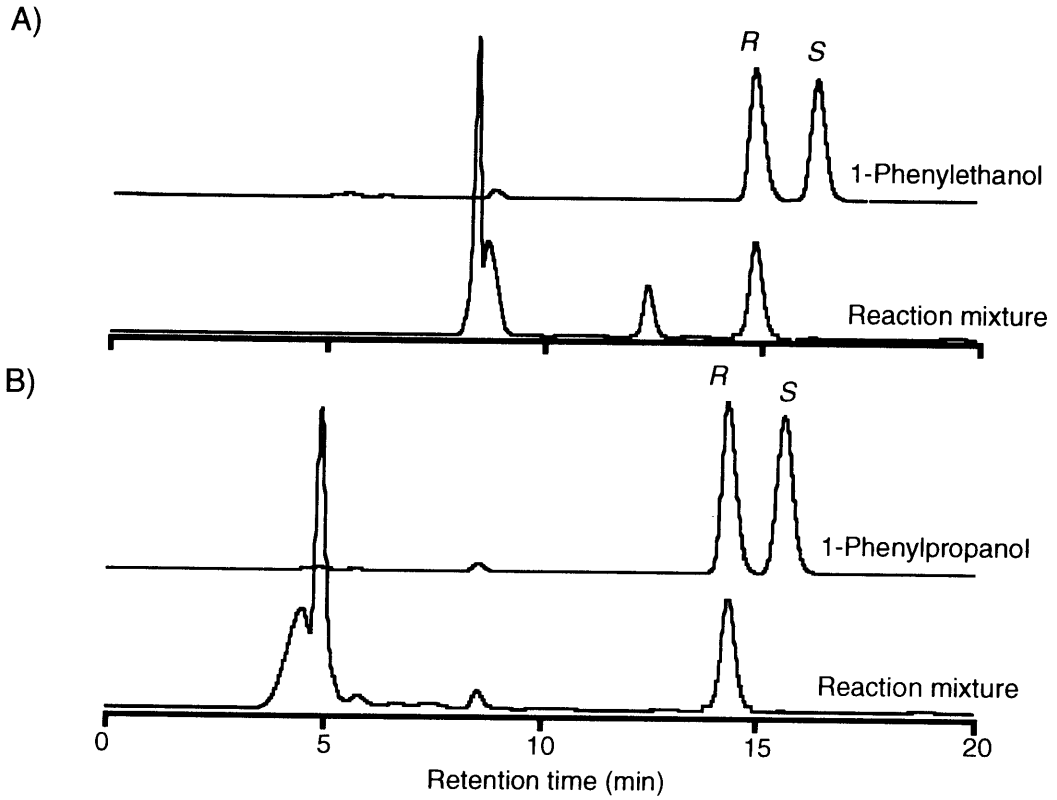
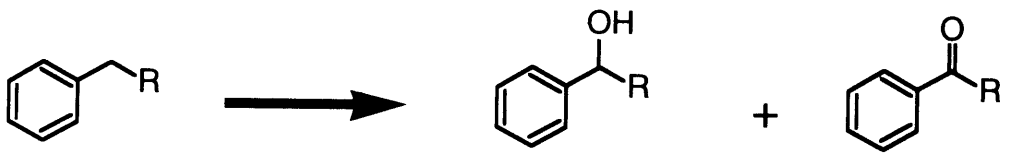


FIG. 1.1.3. HPLC of products from ethylbenzene (A) and propylbenzene (B) incubated with resting cells of *F. moniliforme* strain MS31. *n*-Hexane extracts were injected into a Chiralcel OD column. HPLC was done as described in Materials and Methods. Optical resolution chromatograms of authentic 1-phenylethanol and 1-phenylpropanol are shown over the chromatograms of the appropriate reaction mixture.

authentic alcohols showed that about 100% enantiomeric excess was the (*R*)-(+)-isomer. When either product was mixed with the (*S*)-(-)-isomer, the peak of the product was separated from that of the (*S*)-(-)-isomer. Addition of the (*R*)-(+)-isomer increased the intensity of the product peak.

DISCUSSION

Methane- or alkane-utilizing microorganisms so far reported were mostly bacteria and yeasts. Most of the assimilators of MEK isolated by Taguchi *et al.* (1995, 1996) as oxidizers of 2-methylnaphthalene were bacteria. The fungus I isolated, *F. moniliforme* strain MS31, oxidized ethylbenzene and propylbenzene to the corresponding benzylic alcohols with an enantiomeric excess of 98% in the (*R*)-(+)-form (Fig. 1.1.4). These products were not metabolites from these alkylbenzenes because the fungus did not grow on ethylbenzene or propylbenzene. Most of the microorganisms isolated as MEK assimilators in this study were filamentous fungi. Enzymes produced by these fungi might be different from the corresponding alkane monooxygenases from bacteria. All the four highest producers except strain MS31 were identified as *Fusarium solani* at NCIMB Japan Co., Ltd. (Shizuoka, data not shown). Filamentous fungi belonging to the genus *Fusarium* may convert alkylbenzenes to corresponding benzylic alcohols for (*R*)-form with high enantioselectivity.



$R=CH_3$	200 µmol	13.4 µmol 98% ee (*R*) (6.7%)	0.3 µmol (0.2%)
$R=C_2H_5$	200 µmol	16.2 µmol 98% ee (*R*) (8.1%)	0.4 µmol (0.2%)

FIG. 1.1.4. Transformation of ethylbenzene (R= CH_3) and propylbenzene (R=C_2H_5) by *F. moniliforme* strain MS31. Percentage is µmoles of the product to µmoles of the starting substrate.

The degradation of alkylbenzenes including ethylbenzene and propylbenzene by many bacteria has been reported. One *Nocardia* sp. oxidizes ethylbenzene and propylbenzene to phenylacetic acid and phenylacrylic (cinnamic) acid, respectively (Davis and Raymond 1961). *Pseudomonas putida* metabolizes ethylbenzene starting by oxygenation of C-2 and C-3 of the aromatic nucleus (Gibson *et al.* 1973). *Pseudomonas desmolytica* and *Pseudomonas convexa* grown on propylbenzene oxidize propylbenzene to 3-phenylpropionic acid and benzoic acid, respectively, through oxygenation of the terminal of the *n*-propyl side chain and then through β-oxidation (Jigami *et al.* 1979). The same strains oxidize propylbenzene to 3-(*n*-propyl)catechol through oxidation of C-2 and C-3 of the aromatic nucleus. An alkane monooxygenase from *Pseudomonas oleovorans* GPo1 converts ethylbenzene and propylbenzene into 2-phenylethanol and 3-phenylpropanol, respectively (van Beilen *et al.* 1994). These bacterial metabolic pathways for ethylbenzene and propylbenzene do not involve benzylic alcohols.

There has been only one report of the oxidation of alkylbenzenes by fungi. Certain fungi, *Mortierella isabellina* NRRL 1757, *Helminthosporium* sp. strain NRRL 4671, and *Cunninghamella echinulata* var. *elegans* ATCC 2629, metabolize alkylbenzenes including ethylbenzene and propylbenzene via benzylic alcohols (Holland *et al.* 1987). Among them, *M. isabellina* forms 1-phenylethanol and 1-phenylpropanol. However, the stereospecificity was low, with 33% and 25% enantiomeric excess of the (*R*)-(+)-forms, respectively.

Section 2
Optimization of production of (*R*)-1-phenylpropanol by *Fusarium moniliforme* strain MS31

Chiral secondary alcohols are used as chiral auxiliaries in organic chemistry. Racemic 1-phenylpropanol is used as a choleretic drug and the (*R*)-isomer of this chemical is useful for synthesis of optically active 1-chloro-1-phenylpropane (Stephenson *et al.* 1979) and liquid crystals (Gottarelli *et al.* 1981), and is an auxiliary used for preparation of terpenes (Zadel *et al.* 1991). Catalysts derived from chiral ligands catalyze enantioselective ethylation of benzaldehyde with diethylzinc. A zinc chelate derived from ethyl (*S*)-*N*-(ethoxycarbonyl)pyroglutamate catalyzes the reaction to form (*S*)-1-phenylpropanol with a 94% enantiomeric excess (Corey *et al.* 1990). Piperazine compounds with (2*S*,5*S*)-diphenyl groups catalyze the reaction to form enantiomers of 1-phenylpropanol in lower selectivity (Fuji *et al.* 1993).

Transformation with enzymes or intact cells of microorganisms takes place under moderate reaction conditions. A recombinant esterase from *Pseudomonas fluorescens* gives a high yield and high enantioselectivity in the acylation of 1-phenylethanol to 1-phenylethyl acetate with vinyl acetate in toluene, but 1-phenylpropanol is converted at a low yield with low enantioselectivity (Krebsf nger *et al.* 1998). With porcine pancreas lipase, resolution of racemic 1-phenylethanol and 1-phenylpropanol occurs by enantioselective esterification (Gerlach *et al.* 1989). Enantioselective oxidation of racemic alcohols by yeasts produces homochiral alcohols. The yeasts *Hansenula capsulata* IFO 0974 and *Pachysolen tannophilus* IFO 1007 oxidize the (*S*)-isomer of racemic 1-phenylethanol to acetophenone, leaving the (*R*)-isomer unchanged (Hasegawa *et al.* 1997). These yeasts are not used in the resolution of 1-phenylpropanol. The microbial production of homochiral alcohols through the asymmetric reduction of prochiral ketones has been widely investigated. Acetophenone has been converted to 1-phenylethanol by the asymmetric reduction by microorganisms such as *Saccharomyces cerevisiae* (Aragozzini *et al.* 1998), but there is no such report about

propiophenone. Alkylbenzenes may be oxidized to benzylic alcohols. *Mortierella isabellina* NRRL 1757 (Holland *et al.* 1987) and *Bacillus megaterium* (Adam *et al.* 2000) convert propylbenzene to (*R*)-1-phenylpropanol with enantiomeric excesses of 25% and 74% and chemical yields of 45% and 63%, respectively, in a resting cell reaction. *Fusarium moniliforme* strain MS31 converts propylbenzene to (*R*)-1-phenylpropanol with an enantiomeric excess of 98% and chemical yield of 8.1%. This fungus is useful in production of optically pure benzylic alcohol, but productivity is low. In this section, different conditions for cultivation and resting cell reaction were examined so that the yield could be increased.

MATERIALS AND METHODS

All experiments were done as already described unless otherwise noted.

Media The Df1 medium was composed of 5% (w/v) soluble starch (Wako Pure Chemical Industries, Osaka), 1.5% (w/v) soy bean meal (Showa Sangyo Co., Tokyo), 0.05% (w/v) KH_2PO_4 and 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ in deionized water (pH 7.0). LB was composed of 1% (w/v) peptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl. BM2 medium contained 1% (w/v) glucose (which was sterilized separately) in place of methylethylketone (MEK) in BM1 medium.

Cultivation The strain was cultured at 30°C in test tubes on a reciprocal shaker at 300 rpm or in Erlenmeyer flasks on a rotary shaker at 150 rpm. One loopful from a giant colony of *F. moniliforme* strain MS31 was used to inoculate 5 ml of Bennett's medium in 16.5 × 165-mm test tubes, and the mixture was cultivated. After 24 h, a 1.5-ml portion of the seed culture was transferred to 75 ml of fresh medium in a 500-ml Erlenmeyer flask, and the mixture was cultivated. After 24 h more, a 6-ml portion of the culture was used to inoculate 300 ml of a medium in 2-*l* Erlenmeyer flasks and the mixtures were incubated. Mycelia were collected from a single whole flask and assayed for their activity in the conversion of propylbenzene.

Measurement of dry cell weight (DCW) A portion of wet mycelia weighing 0.1 to 1 g was put in a Petri dish, and the dish was heated at 105°C in an oven until the weight was constant, cooled in a desiccator, and weighed to give the DCW. Typically, 1 g of wet mycelia corresponded to 0.24 g DCW.

Cell reaction Mycelia were collected from culture on filter paper (90 mm ϕ, No. 1; Toyo Roshi Kaisha, Tokyo) with reduced pressure. Cell reaction was done with propylbenzene as the substrate for 2 h. Amount of substrate and products in an *n*-hexane extract were measured by GC with butylbenzene as the internal standard. A unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of 1-phenylpropanol. The reactions were run in triplicate, and the mean is reported. Optical purity of 1-phenylpropanol was determined by HPLC with a model 996 photodiode array detector and a Millennium 2010J system manager (both from Nihon Waters K.K., Tokyo).

RESULTS

Effects of culture media on hydroxylating activity The different media used are listed in Table 1.2.1. After day 1, the cell yield was highest with the Df1 medium, but cells from this culture had low hydroxylating activity. Cells cultured with the BM2 medium had the highest hydroxylating activity at all times, and especially on days 4 and 6. The BM2 medium was used in the next set of experiments.

TABLE 1.2.1. Effects of the basal media on growth and hydroxylating activity

	1 d			2 d			4 d			6 d		
	Cell yield[a] ($g \cdot l^{-1}$)	pH	Activity ($U \cdot g^{-1}$)	Cell yield[a] ($g \cdot l^{-1}$)	pH	Activity ($U \cdot g^{-1}$)	Cell yield[a] ($g \cdot l^{-1}$)	pH	Activity ($U \cdot g^{-1}$)	Cell yield[a] ($g \cdot l^{-1}$)	pH	Activity ($U \cdot g^{-1}$)
Df1	11	7.0	6	26	7.0	20	38	7.4	16	37	7.5	7
Bennett's	12	6.4	8	12	6.4	4	8.7	7.8	10	5.0	8.2	5
LB	7.0	7.5	10	14	8.5	32	15	8.7	20	8.3	8.8	2
BM2	6.0	7.0	14	11	7.6	34	16	8.8	39	15	8.9	41

[a]Wet cell weight.

Effects of carbon sources on hydroxylating activity Glucose in the BM2 medium was replaced with other carbon sources and tested for their effects on growth and hydroxylating activity (Table 1.2.2). The fungus did not grow on 1-butanol or propylbenzene. Glucose gave the highest cell yield. Soluble starch, carboxymethyl cellulose, dextrin, 2-butanol, and acetone did not support much growth or give high specific activities. MEK delayed growth and the specific activity of the cells from the culture with MEK was same as that of the cells from the culture with glucose. Fructose, ethanol, and acetoin resulted in higher specific activity than the other carbon sources. Glucose was used at the concentration of 1% (w/v) in the BM2 medium in further experiments because it is inexpensive.

TABLE 1.2.2. Effects of carbon sources on growth and hydroxylating activity

Carbon source[a] (1%, w/v)	Time[b] (d)	pH	Cell yield[c] ($g \cdot l^{-1}$)	Specific activity ($U \cdot g^{-1}$)	Total activity ($U \cdot l^{-1}$)
Glucose	4	6.1	19	41	780
Fructose	5	5.6	11	49	540
Sucrose	4	5.9	15	40	600
Soluble starch	6	4.5	1.0	19	19
Carboxymethyl cellulose	6	6.7	0.1	10	1.0
Dextrin	4	4.0	4.0	15	60
Ethanol	4	6.0	17	47	800
1-Propanol	5	6.2	15	40	600
2-Propanol	4	6.1	15	20	300
2-Butanol	6	6.0	1.7	8	14
Acetone	6	6.1	1.7	8	14
MEK	6	6.0	13	39	510
Acetoin	4	6.0	16	51	820
Glycerol	6	5.9	8.3	22	180

Glucose in the BM2 medium was replaced with other carbon sources at the final concentration of 1% (w/v). A single flask of the 6 flasks cultured was tested every day.

[a] *F. moniliforme* strain MS31 did not grow with 1-butanol or propylbenzene.

[b] Time for the highest specific activity.

[c] Wet cell weight.

Effects of nitrogen sources on hydroxylating activity $NaNO_3$ and NH_4Cl in the BM2 medium were replaced with various nitrogenous compounds and tested for their effects on growth and hydroxylating activity (Table 1.2.3). Cell yields from organic nitrogenous compounds were generally higher than those from inorganic nitrogenous compounds. Cells from the culture with $NaNO_3$ had the highest specific activity. Specific activity of the cells from the culture with urea decreased after the first 2 d of cultivation, unlike the other cultures, which kept their specific activity longer (not shown). In further experiments, $NaNO_3$ was used as the sole nitrogen source at the concentration of 0.5% (w/v), in what was called the BM3 medium.

TABLE 1.2.3. Effects of nitrogen sources on growth and hydroxylating activity

Nitrogen source (0.5%, w/v)	Time[b] (d)	pH	Cell yield[c] ($g \cdot l^{-1}$)	Specific activity ($U \cdot g^{-1}$)	Total activity ($U \cdot l^{-1}$)
Control[a]	4	6.1	18	48	860
NH_4Cl	2	2.3	19	38	720
$(NH_4)_2SO_4$	2	2.5	20	36	720
NH_4NO_3	4	6.2	19	46	870
$NaNO_3$	4	9.0	19	61	1160
L-Asparagine	3	8.7	30	45	1350
Casamino acids	4	7.4	25	48	1200
Polypepton	4	7.2	30	48	1440
Yeast extract	3	7.4	27	39	1050
Meat extract	5	7.3	18	42	760
Soybean meal	6	7.2	20	36	720
Urea	2	8.7	23	44	1010

$NaNO_3$ and NH_4Cl in the BM2 medium (control) were replaced with various nitrogenous compounds at the final concentration of 0.5% (w/v). A single flask of the 6 flasks cultured was tested every day.

[a] 1% (w/v) $NaNO_3$ and 0.5% (w/v) NH_4Cl.

[b] Time for the highest specific activity.

[c] Wet cell weight.

Growth and hydroxylating activity in culture When the fungus was grown in the BM3 medium, cell growth was slow after 18 h of logarithmic phase (Fig. 1.2.1).

Hydroxylating activity was first detected at the mid-log phase, and continued until about 40 h. The specific activity did not decrease in longer cultivation. The enantiomeric excess of the (*R*)-1-phenylpropanol formed was more than 92% throughout fermentation. The pH of the culture broth was increased in the logarithmic phase of growth, after 18 h.

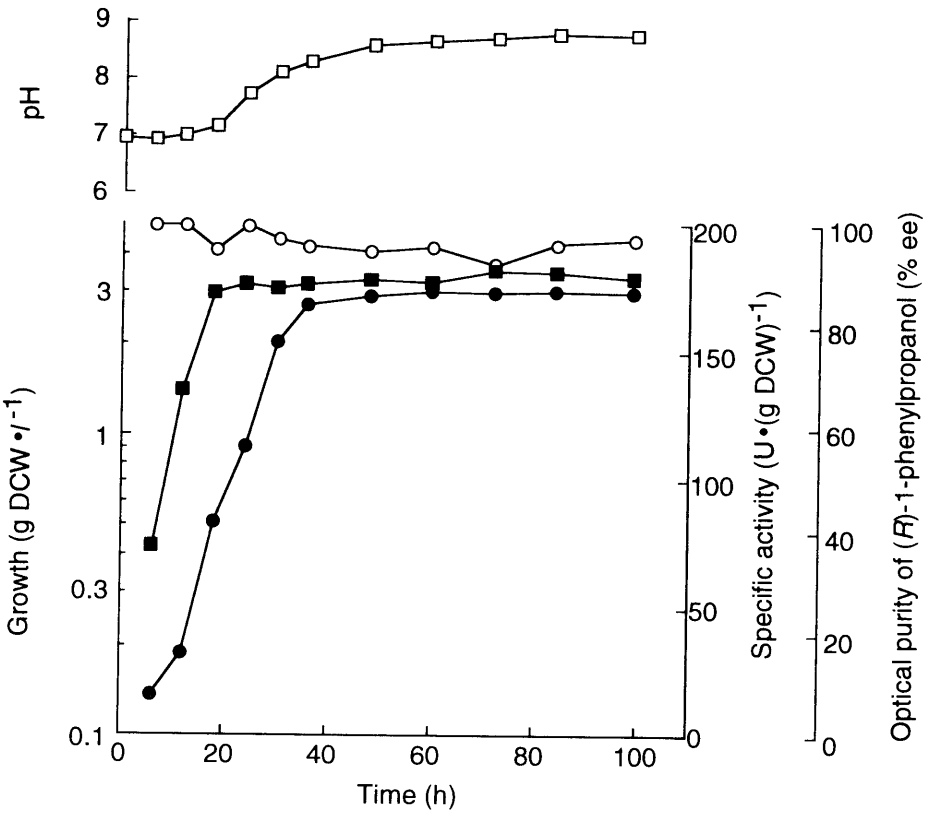


FIG. 1.2.1. Growth of *F. moniliforme* strain MS31 and hydroxylating activity in the BM3 medium. Sampling was at 6, 12, 18, 24, 30, 36, 48, 60, 72, 84, and 99 h. Symbols: open squares, pH; filled squares, growth; filled circles, specific activity; open circles, stereoselectivity as an enatiomeric excess for (*R*)-1-phenylpropanol.

Effect of propylbenzene in culture on hydroxylating activity Propylbenzene was added to portions of the BM3 medium and examined for the effects on hydroxylating activity. Growth was detected first at 48 h although growth was detected after 12 h in the control culture, without propylbenzene (Fig. 1.2.2). With propylbenzene present from the start, growth in terms of DCW was greatest, at 3.9 g·l^{-1}, when measured at 72 h. At that time and

thereafter, the specific activity was between 60% and 70% of that of the control culture. When the propylbenzene was added to the culture at 24 h, growth in terms of DCW decreased, as did the specific activity. Both growth and specific activity began to increase about 48 h after start, but they remained lower than in the other cultures. At 72 h and thereafter, growth was between about 70% and 80% of that of the control culture, and specific activity was between about 20% and 40%.

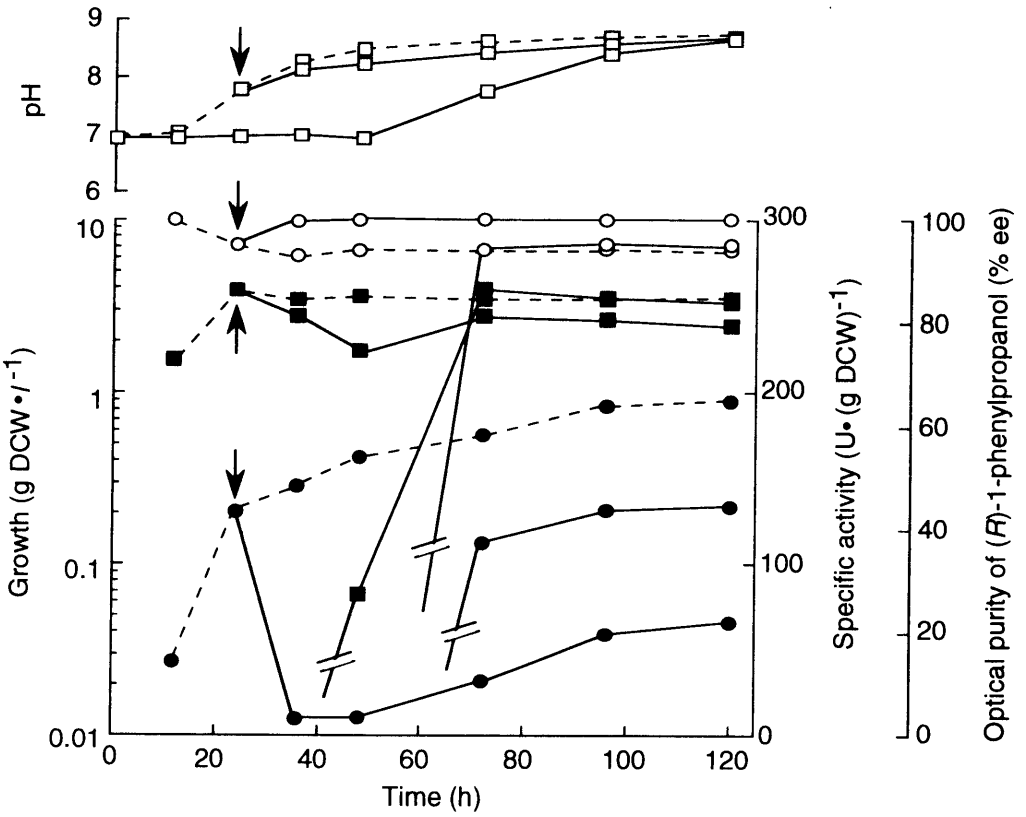


FIG. 1.2.2. Growth of strain MS31 and hydroxylating activity as affected by propylbenzene (a final concentration at 0.1% (v/v)) added to the BM3 medium before inoculation or at 24 h of culture. Sampling was at 12, 24, 36, 48, 72, 96, and 120 h. Symbols are the same as in Fig. 1.2.1. Solid lines, with propylbenzene added; broken lines, without propylbenzene. Arrows, addition of propylbenzene at 24 h.

Effects of acetoin in culture on hydroxylating activity Acetoin, which was used in culture and was effective for cells with high enzyme activity, was added to the BM3 medium. With all concentrations of acetoin, the specific activity increased until day 4 and

remained high to the end of the experiment on day 6. Table 1.2.4 shows the effects of glucose and acetoin at different concentrations as of day 4. The higher concentration of glucose increased growth but not the specific activity. Additions of acetoin increased both growth and the specific activity, with the highest (1.4-fold) specific activity in culture with 0.5% (w/v) acetoin. In further experiments, acetoin was used at this concentration.

TABLE 1.2.4. Effects of acetoin on growth and hydroxylating activity

Glucose (%, w/v)	Acetoin (%, w/v)	pH	Cell yield[a] ($g \cdot l^{-1}$)	Specific activity ($U \cdot g^{-1}$)	Total activity ($U \cdot l^{-1}$)
1	0	8.8	3.6	230	830
1.5	0	8.9	5.4	231	1250
1	0.01	8.8	3.6	237	850
1	0.05	8.8	3.8	242	920
1	0.1	8.8	4.0	268	1070
1	0.3	8.7	4.7	306	1440
1	0.5	8.7	5.1	317	1620
1	0.7	8.7	5.3	306	1620

Acetoin was added to the BM3 medium at final concentrations of 0.01% to 0.7% (w/v) just before inoculation.

[a]DCW.

Effects of sorbitan monooleate (Span 80) in culture on hydroxylating activity

In an earlier study, the addition of several nonionic surfactants to BM1 medium increased the specific activity slightly, with Span 80 causing the largest. Span 80 was added in the medium to determine the suitable concentration for high specific activity. For all concentrations, the specific activity was increased until day 5 and remained high to the end of the experiment on day 6. Table 1.2.5 shows the effects of Span 80 concentration on the activity at day 5. Specific activity increased with increasing Span 80 concentrations up to 0.1% (w/v). Span 80 is oil and could not be mixed with water before cultivation, but no oil was seen on the culture. From day 1, cells from the culture containing Span 80 at $\geq$ 0.01% (w/v) looked powdery.

Optimum conditions for the cell reaction Cells caused maximum bioconversion of propylbenzene to 1-phenylpropanol at between 25 and 30°C (Fig. 1.2.3). Above 30°C, bioconversion decreased. Bioconversion occurred at pHs from 6.0 to 12.5 (Fig. 1.2.4) with the highest activity was shown at pH 7.0. Increasing cell concentrations increased the yield

TABLE 1.2.5. Effects of Span 80 on growth and hydroxylating activity

Concentration (%, w/v)	pH	Cell yield[a] ($g \cdot l^{-1}$)	Specific activity ($U \cdot g^{-1}$)	Total activity ($U \cdot l^{-1}$)
0.0	8.8	5.0	320	1600
0.005	8.8	5.0	325	1630
0.01	8.8	5.0	334	1670
0.03	8.8	5.2	335	1740
0.07	8.8	5.4	343	1850
0.1	8.9	5.4	360	1940
0.2	8.9	6.6	344	2270

Span 80 was added to the BM3 medium to the final concentrations of 0.005% to 0.2% (w/v), the medium was autoclaved, and acetoin was added to the medium at the final concentration of 0.5% (w/v) just before the medium was inoculated.

[a]DCW.

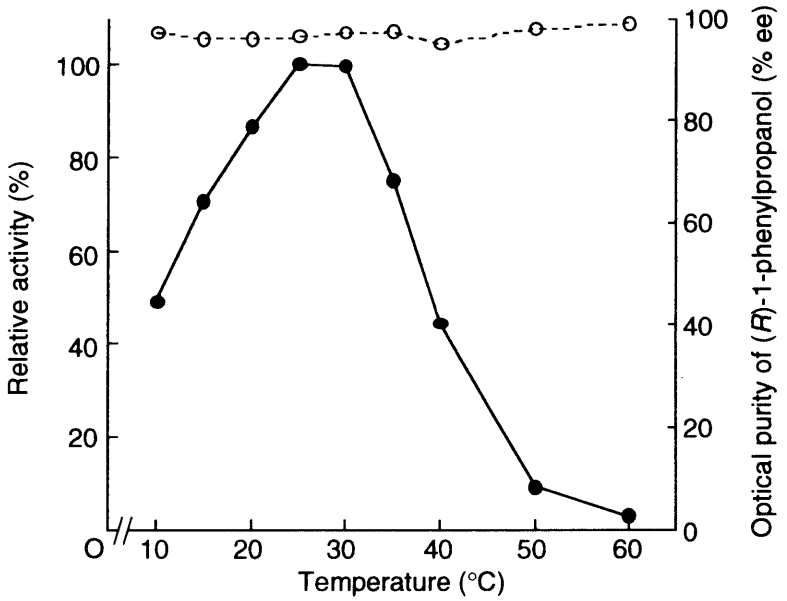


FIG. 1.2.3. Effects of temperature on hydroxylating activity with resting cells of strain MS31. Wet mycelia weighing 0.2 g, which were obtained from a 5-d culture in the BM3 medium containing 0.5% (w/v) acetoin, were suspended in 100 mM KPB (pH 7.0). In other ways, the reaction and assay were same as described in Materials and Methods except that the reaction temperature was 10, 15, 20, 25, 30, 35, 40, 50, or 60°C. Results are expressed as a percentage of the activity when incubation was at 30°C. Symbols: filled circles, activity relative to that at 30°C; open circles, optical purity of (*R*)-1-phenylpropanol.

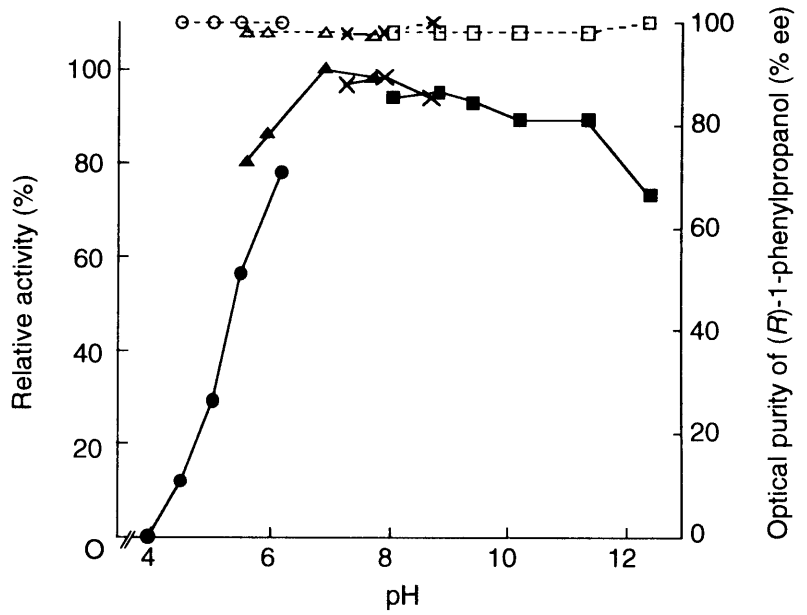


FIG. 1.2.4. Effects of pH on hydroxylating activity with resting cells of strain MS31. The reaction and assay were as the legend of Fig. 3 except for the buffers and used pHs. Results are expressed as a percentage of the activity at pH 7.0 with KPB. Acetate buffer (circles) was used for pHs 4.0, 4.5, 5.0, and 6.0; KPB (triangles) was used for pHs 5.6, 6.0, 7.0, and 7.8; Tris-HCl buffer (crosses) was used for pHs 7.3, 8.0, and 8.8; and glycine-NaOH buffer (squares) was used for pHs 8.1, 8.9, 9.5, 10.3, 11.4, and 12.4. Solid lines, relative activity; broken lines, optical purity of (*R*)-1-phenylpropanol.

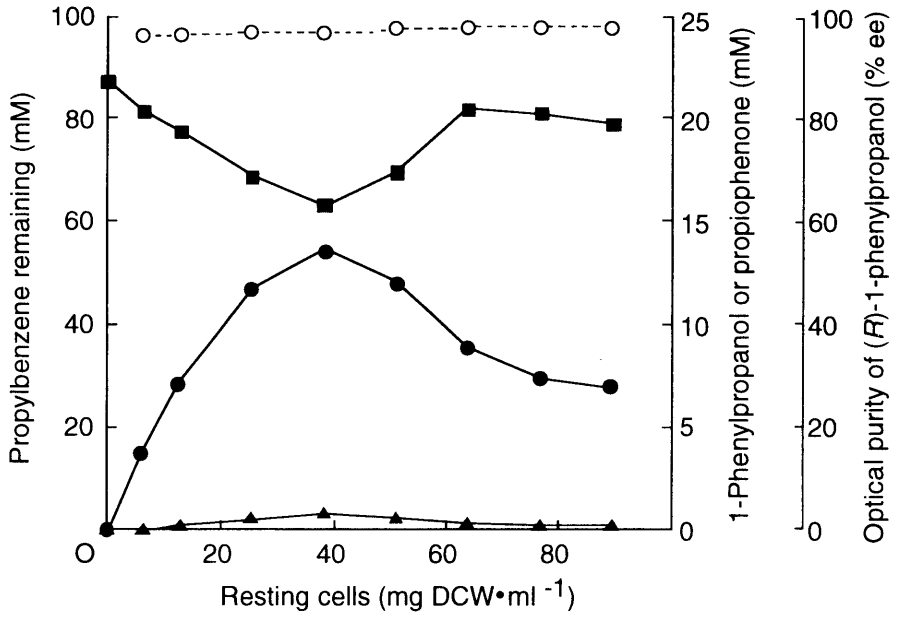


FIG. 1.2.5. Effects of cell concentration on hydroxylating activity with resting cells of strain MS31. The reaction and assay were same as in the legend of Fig. 1. 2. 3 except for the cell concentration in a reaction mixture, which was 6.4, 12.8, 25.6, 38.4, 51.2, 64.0, 76.8, or 89.6 mg DCW·ml^{-1}. Symbols: filled squares, propylbenzene remaining; filled circles, 1-phenylpropanol; triangles, propiophenone; open circles, optical purity of (*R*)-1-phenylpropanol.

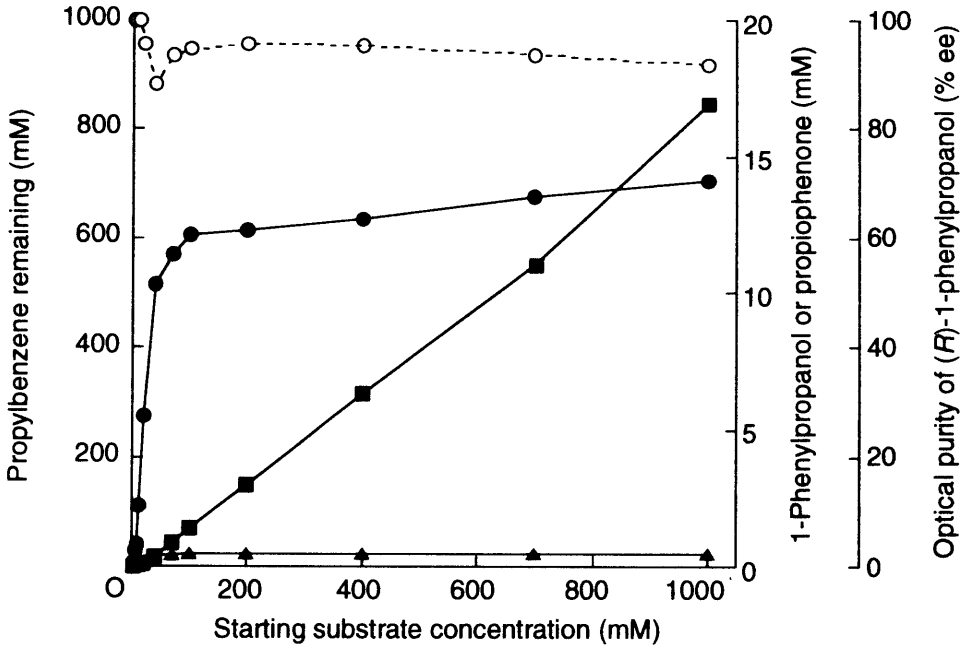


FIG. 1.2.6. Effect of substrate concentration on hydroxylating activity with resting cells of strain MS31. The reaction and assay were as the legend of Fig. 1.2.3 except for the starting substrate concentration, which was 2, 5, 7, 10, 20, 50, 70, 100, 200, 500, 700, or 1000 mM. Symbols are as in the legend of Fig. 1.2.5.

of products, which were 1-phenylpropanol and a small amount of propiophenone, up to about 40 mg DCW·ml^{-1}, after which both products decreased in amount (Fig. 1.2.5). Reaction mixtures containing high concentrations of cells were difficult to mix. With increasing starting concentrations of the substrate, the production of 1-phenylpropanol increased up to 100 mM, after which the increase slowed (Fig. 1.2.6). The conversion of propylbenzene began to slow after 2 h (Fig. 1.2.7). Propylbenzene decreased without cells and more than 20% of the starting concentration of propylbenzene was either converted or lost in 4 h. (*R*)-1-Phenylpropanol increased and the yield at 4 h was 16 μmol from 100 μmol of the substrate in 1 ml with an enantiomeric excess of 98%.

DISCUSSION

F. moniliforme strain MS31 converted propylbenzene to 1-phenylpropanol stereoselectively. A similar reaction was found in *Mortierella isabellina* NRRL 1757

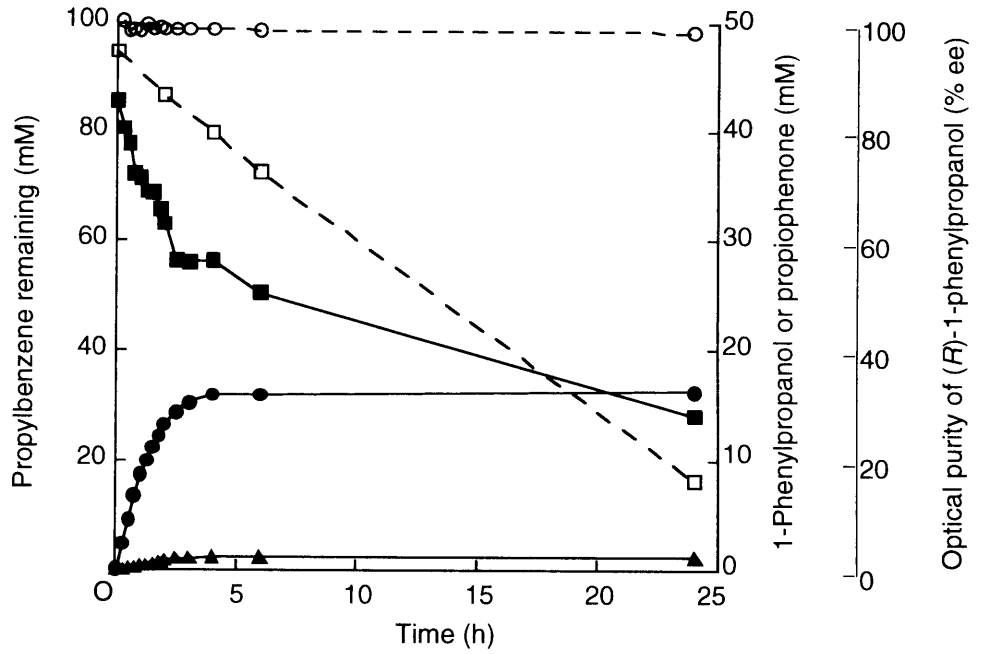


FIG. 1.2.7. Changes with time in amount of (*R*)-1-phenylpropanol produced with resting cells of strain MS31. The reaction and assay were as the legend of Fig. 1.2.3 except that the reaction temperature was 25°C. Sampling was at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, and 24 h. Symbols: as in the legend of Fig. 1.2.5, with the addition of open squares for propylbenzene remaining in the absence of cells.

(Holland *et al.* 1987) and *Bacillus megaterium* (Adam *et al.* 2000) by organic chemists, but information about biological conditions for the bioconversion was not included in the reports. I here identified suitable conditions for the cultivation of *F. moniliforme* strain MS31 and for the cell reaction. L-Asparagine is a nutrient needed for the growth of *Fusarium* species. *Fusarium equiseti* M41 grows better and has higher activity for hydroxylation of lithocholic acid when L-asparagine is used as the nitrogen source rather than casamino acids, peptone, tryptone, yeast extract, and meat extract (Kulprecha *et al.* 1985). For *F. moniliforme* strain MS31, L-asparagine increased cell yield, but the specific activity of the cells was almost same with any nitrogen sources mentioned above. Hydroxylation by *F. equiseti* M41 occurred during fermentation, but bioconversion by *F. moniliforme* strain MS31 used cells because of substrate toxicity for growth and enzyme activity. The differences in these reactions are a reason for the different effects of L-asparagine.

Propylbenzene did not increase the specific activity because it was toxic. Unexpectedly, MEK could not be used as inducer. Acetoin, a MEK derivative, was the best carbon source

for production of high specific activity of the desired enzyme, but it is too expensive to be used as the carbon source. Acetoin increased growth and specific activity increased only slightly. Certain inducible enzymes increase 5-fold or more by addition of their substrates or unspecific inducers (Adam *et al.* 2000; Hou *et al.* 1994; Tan *et al.* 1998). The hydroxylating enzyme from *F. moniliforme* strain MS31 may be produced constitutively.

Propylbenzene is an organic solvent that is not soluble in buffers. So that a hydrophobic substrate will come into contact with enzymes and cells, several surfactants can be used. Mycelia from the culture containing Span 80 at high concentrations looked powdery, might contain Span 80, and have enough hydrophobicity to react with propylbenzene.

Cells of *F. moniliforme* strain MS31 showed activity in a wide pH range. Similar pH profiles were found for the conversion of (*R*)-limonene by *Penicillium digitatum* NRRL 1202 (Tan *et al.* 1998). Cytochrome P450-dependent enzymes have been candidates for such fungal hydroxylation because of their versatility with respect to substrates and type of oxygenation. Some of their enzyme systems have two proteins, cytochrome b_5 and cytochrome b_5 reductase, that work with cytochrome P450 and NADPH-cytochrome P450 reductase (Takemori *et al.* 1993). Perhaps each component has a different optimum pH for its function; alternatively, the organization of the proteins depends upon pH.

Conversion of propylbenzene to (*R*)-1-phenylpropanol by *F. moniliforme* strain MS 31 ended within 4 h, and the maximum yield was 16% of the initial amount of the substrate. The conversion by *M. isabellina* NRRL 1757 (Holland *et al.* 1987) and *B. megaterium* (Adam *et al.* 2000) takes much longer (some 2 d). In this case, a short reaction time is important so that the substrate will not be lost (propylbenzene is volatile). Further improvement of *F. moniliforme* strain MS 31 to give higher specific activity might lead to more effective utilization of propylbenzene conversion to (*R*)-1-phenylpeopanol before the substrate evaporates.

Section 3
Conversion of various aromatic compounds by *Fusarium moniliforme* strain MS31

Oxidation of C–H bonds is a useful reaction in organic chemistry because desired oxyfunctionalized compounds can be obtained from common hydrocarbons. Much effort has been directed toward the development of efficient stereoselective oxidation of hydrocarbons, but resulted in only moderate enantioselectivity with most alkanes (Davis *et al.* 1984; Fuji *et al.* 1993; Jacobsen *et al.* 1988). Biocatalytic reactions are generally highly regio- and stereospecific. Activation of C–H bonds by biocatalytic oxidation was one of the first biotransformations to be exploited by chemical industries. Microbial hydroxylation is useful in the production of optically active compounds (Holland 1998; 1999; Sih *et al.* 1988).

Microbial hydroxylation involves an enzyme of the oxidoreductase group, which contains oxygenases, oxidases, and dehydrogenases. These enzymes are generally unstable and expensive to prepare; many need expensive cofactors such as NAD(P)H. The use of whole cells rather than purified enzymes has the following advantages. Microbial cells are less expensive than purified enzymes, and the activity of the enzyme of interest may be more stable in the cell than when purified. The addition of purified cofactors is usually not necessary because generally they are already present within the cell, or can be generated there. The regeneration of cofactors outside the reaction system is unnecessary when whole cells are used. A disadvantage of whole-cell biocatalysis is the possible formation of by-products because whole cells contain many other enzymes that can catalyze undesired reactions.

Resting cells of *Fusarium moniliforme* strain MS31 hydroxylate ethylbenzene and propylbenzene to the corresponding alcohols with high regio- and stereospecificity. In this section, the substrate specificity of and by-product formation in the resting cell reaction with the fungus were investigated.

MATERIALS AND METHODS

Chemicals Chemicals used were commercial preparations and were used without further purification unless otherwise stated. (*S*)-(+)-1-Phenyl-2-butanol, $[\alpha]_D^{20}$ = +22 (c 1.5, diethyl ether) (Aleu *et al.* 1998), was synthesized by the reduction of 1-phenyl-2-butanone with baker's yeast as follows. Dried baker's yeast (Nisshin Flour Milling Co., Tokyo), weighing 40 g, was wet with and suspended in 400 ml of 100 mM Tris-HCl (pH 8.0) in a 2-*l* Erlenmeyer flask, and 6 ml (about 4 mmol) of 1-phenyl-2-butanone was added. The top of the flask was covered with aluminum foil and incubated at 30°C on a rotary shaker at 150 rpm. After 35 h, the reaction mixture was acidified by the addition of 40 ml of 6 N HCl and extracted twice, each time with 400 ml of diethyl ether. The extracts were combined and concentrated under reduced pressure in a rotary evaporator. The concentrate was pipetted in a straight line on a silica gel plate (silica gel 60 F_{254}; 20 × 20 cm, 2 mm thick; Merck KGaA, Darmstadt, Germany) and chromatographed with 1:1 *n*-hexane–diethyl ether as the solvent. Bands were inspected under UV light at 254 nm. The band identified as being that of 1-phenyl-2-butanol (with Rf 0.49) was separate from that of 1-phenyl-2-butanone (with Rf 0.62); the former band was scraped off the thin-layer plate and extracted with diethyl ether. The purified 1-phenyl-2-butanol had $[\alpha]_D^{23}$ = +14.7 (c 2.2, diethyl ether).

Cell reaction Wet mycelia were collected from a culture in BM3 medium containing 0.5% (w/v) acetoin, and were used in the cell reaction under the conditions described in section 2.

GC GC was done as described in section 1 with a TC-wax capillary column (30 m × 0.32 mm (i.d.); coating 0.25 μm thick; J&W Scientific Inc., Folsom, CA). GC was first at 100°C for 3 min and then with a temperature gradient increasing by 10°C·min^{-1} to 150°C.

GC-MS GC-MS was done as described in section 1 under the conditions for GC.

HPLC The HPLC system had L-7000 pump units (Hitachi Ltd., Tokyo), a photodiode array detector (model 996; Nihon Waters K.K., Tokyo), and a system manager for the photodiode array detector (Millennium 2010J; Nihon Waters). The same Chiralcel OD column as described in section 1 was used for separation of the enantiomers of the alcohols

that formed. The mobile phase was a mixture of *n*-hexane and 2-propanol at the proportions and the flow rates listed, together with retention times, in Table 1.3.1. The mixture of enantiomers of 1-phenylbutanol (**2eα**), 1-phenyl-2-butanol (**2eβ**), and 4-phenyl-2-butanol (**2eγ**) was not separated in a single step of chromatography. The concentration of (*R*)-**2eα** was calculated as that of racemic **2eα** (measured with the 97:3 solvent) minus that of (*S*)-**2eα** (measured with the 90:10 solvent).

TABLE 1.3.1. HPLC analysis of optically active alcohols by Chiralcel OD column

Alcohol		Elution conditions		Retention time (min)	
		Solvent *n*-Hexane–IPA[a]	Flow rate ($ml \cdot min^{-1}$)	Peak 1	Peak 2
1-Phenylethanol	(**2c**)	90:10	0.5	14.5 (*R*)	16.1 (*S*)
1-phenylpropanol	(**2d**)	97:3	1.0	14.3 (*R*)	16.0 (*S*)
1-Phenylbutanol	(**2eα**)	90:10	0.5	13.8 (*R*)	14.2 (*R*)
		97:3	1.0	12.8[b]	
1-Phenyl-2-butanol	(**2eβ**)	90:10	0.5	11.9 (*S*)	13.8 (*R*)
		97:3	1.0	9.1 (*S*)	11.9 (*R*)
4-Phenyl-2-butanol	(**2eγ**)	90:10	0.5	15.9 (*R*)	21.5 (*S*)
		97:3	1.0	16.6 (*R*)	25.3 (*S*)
2-Methyl-1-phenylpropanol	(**2j**)	90:10	0.5	12.8 (*R*)	14.5 (*S*)
1-Phenyl-1-hydroxy-2-propen	(**2m**)	90:10	0.5	15.5 (*R*)	17.6 (*S*)
1-Indanol	(**1n**)	90:10	0.5	15.2 (*S*)	16.3 (*R*)
1-Tetrarol	(**1o**)	90:10	0.5	14.2 (*S*)	14.8 (*R*)

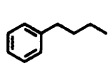
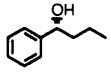
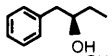
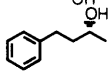
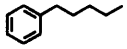
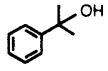
[a] 2-Propanol

[b] (*R*)- and (*S*)-enantiomers were not separated.

RESULTS

Conversion of benzene and alkylbenzenes To elucidate the scope of substrate acceptability in a reaction system with cells of *F. moniliforme* strain MS31, we used several hydrocarbons (Table 1.3.2). The fungus could hydroxylate the side chain of many aromatic compounds, most at the benzylic position. Many alcohols were formed and their optical purity was examined by chiral HPLC. Benzene (**1a**) and toluene (**1b**) were not oxidized. Substituted side chains at the benzylic position in (1-methylpropyl)benzene (**1h**),

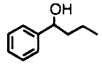
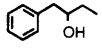
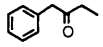
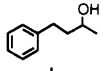
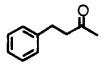
TABLE 1.3.2. Oxidation of aromatic compounds by resting cells of *F. moniliforme* strain MS31

Substrate		Products							
		Benzylic alcohol				Other(s)			
				mM	% ee (*R*)			mM	% ee (*R*)
	**(1a)**	Not detected				Not detected			
	**(1b)**	Not detected				Not detected			
	**(1c)**		**(2c)**	7.3	98		**(3c)**	0.7	
	**(1d)**		**(2d)**	12.1	98		**(3d)**	1.1	
	**(1e)**		**(2ea)**	0.8	100		**(2eb)**	3.1	94
							**(2eg)**	1.5	100
	**(1f)**	Three unknown compounds							
	**(1g)**		**(2g)**	0.5		Not detected			
	**(1h)**	Not detected				Not detected			
	**(1i)**	Not detected				Not detected			
	**(1j)**		**(2j)**	2.2	98	Not detected			
	**(1k)**	Not detected				Not detected			
	**(1l)**	Not detected				Not detected			
	**(1m)**		**(2m)**	0.7	63	Not detected			
	**(1n)**		**(2n)**	5.0	9		**(3n)**	0.2	
	**(1o)**		**(2o)**	6.1	4	Not detected			

(1,1-dimethylethyl)benzene (**1i**), ethenylbenzene (**1k**), and 1-propenylbenzene (**1l**) did not react. 1-Phenylethanol (**2c**) and acetophenone (**3c**) were formed from ethylbenzene (**1c**) with a conversion ratio of 7.3% and 0.7%, respectively. 1-Phenylpropanol (**2d**) and propiophenone (**3d**) were formed from propylbenzene (**1d**) with a conversion ratio of 12.1% and 1.1%, respectively. The product yield was highest with **2c**. From (1-methylethyl)benzene (**1g**) and (2-methylpropyl)benzene (**1j**), both of which have a nonlinear side chain, the hydroxylated products 2-phenyl-2-propanol (**2g**) and 2-methyl-1-phenylpropanol (**2j**), respectively, were formed. Butylbenzene (**1e**) gave all possible methylene-hydroxylation products, but no terminal oxidation of the side chain. The conversion ratio of these regioisomers, 1-phenylbutanol (**2e**α), 1-phenyl-2-butanol (**2e**β), and 4-phenyl-2-butanol (**2e**γ), were 0.8%, 3.1%, and 1.5%, respectively. All of the optically active alcohols from **2c** to **2j** were of an enantiomeric excess of 94% or higher for the (*R*)-form. The extract from the reaction mixture with pentylbenzene (**1f**) had three peaks on the gas chromatogram, the areas of which were smaller than the area of **2e**α. The mass spectra showed that these compounds each contained an aromatic ring and that the all had *m/z* of 164 (M^+), but the fragment patterns did not match any in the Wiley mass spectrum database (10). 2-Propenylbenzene (**1m**) was hydroxylated to the corresponding benzylic alcohol, 1-phenyl-1-hydroxy-2-propen (**2m**), with an enantiomeric excess of 63% for the (*R*)-form when its double bond was preserved. The bicyclic compound indane (**1n**) was oxidized to 1-indanol (**2n**) and indanone (**3n**), and tetralin (**1o**) was oxidized to 1-tetrarol (**2o**), respectively. The hydroxylation of these two compounds occurred in a highly regioselective way, but with low enantioselection.

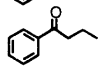
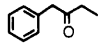
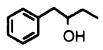
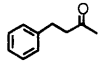
Conversion of benzylic alcohols and their regioisomers Oxidation of hydroxylated aromatics by the cell reaction was tested (Table 1.3.3). No hydroxylation of the alcohols tested was observed. None of primary alcohols tested (phenylmethanol, 2-phenylethanol, 3-phenylpropanol, or 4-phenylbutanol) were converted. Some compounds with secondary alcohol residues were oxidized to produce the corresponding ketones, but further conversion products were not found. Products from the hydroxylation of the alkylbenzenes, 1-phenylethanol (**2c**), 1-phenylpropanol (**2d**), 1-phenyl-2-butanol (**2e**β),

TABLE 1.3.3. Conversion of benzylic alcohols and their regioisomers by resting cells of *F. moniliforme* strain MS31

Substrate	Product	mM
 (**2c**)	 (**3c**)	9.1
(*R*)-form		2.8
(*S*)-form		13.7
 (**2d**)	 (**3d**)	0.9
(*R*)-form		1.7
(*S*)-form		1.8
 (**2dβ**)	Not detected	
 (**2eα**)	Not detected	
 (**2eβ**)	 (**3eβ**)	0.5
 (**2eγ**)	 (**3eγ**)	1.5
 (**2g**)	Not detected	
 (**2j**)	Not detected	
 (**2m**)	Not detected	
 (**2n**)	 (**3n**)	2.6
 (**2o**)	Not detected	

Phenylmethanol, 2-phenylethanol, 3-phenylpropanol, 2-phenyl-1-propanol, and 4-phenylbutanol were not converted.

TABLE 1.3.4. Conversion of phenylketones by resting cells of *F. moniliforme* strain MS31

Substrate	Product	mM
 (**3c**)	 (**2c**)	0.9
 (**3d**)	 (**2d**)	0.5
 (**3eα**)	Not detected	
 (**3eβ**)	 (**2eβ**)	0.5
 (**3eγ**)	Not detected	
 (**3n**)	Not detected	
 (**3o**)	Not detected	

4-phenyl-2-butanol (**2eγ**), and 1-indanol (**2n**), were converted to acetophenone (**3c**), propiophenone (**3d**), 1-phenyl-2-butanone (**3eβ**), 4-phenyl-2-butanone (**3eγ**), and 1-indanone (**3n**), respectively. Fungal cells had different degrees of activity against the two enantiomers of **2c**. The conversion rate for the (*S*)-enantiomer was about 5 times that of the (*R*)-enantiomer. However, the conversion of both enantiomers of **2d** was at the same rate.

Conversion of phenylketones Reduction activity of the fungal cells was tested with phenylketones (Table 1.3.4). Acetophenone (**3c**), propiophenone (**3d**), and 1-phenyl-2-butanone (**3eβ**) were reduced to produce the corresponding alcohols, but the amounts produced were too small for their optical configurations to be identified.

DISCUSSION

F. moniliforme strain MS31 caused selective insertions of aliphatic CH units without either epoxidation at the aryl position or hydroxylation at the benzene ring. The alkane hydroxylase system of *Pseudomonas oleovorans* GPo1 can hydroxylate aromatic compounds at the terminal of their side chain (van Beilen *et al.* 1994). The enzyme involved in the fungal hydroxylation is different from the bacterial alkane hydroxylase. The fungi *Mortierella isabellina* NRRL 1757, *Helminthosporium* sp. NRRL 4671, and *Cunninghamella echinulata* ATCC 26269 and the bacterium *Bacillus megaterium* also can hydroxylate the side chain of aromatic compounds. Hydroxylation of aromatic compounds with these three fungi occurs at the benzylic position in the same way as with *F. moniliforme* strain MS31, but the optical activity of all of the alcohols produced is low (Holland *et al.* 1987). The above bacterium gives a high yield from such transformation, and some optically active alcohols produced have a high enantiomeric excess for the (*R*)-form (Adam *et al.* 2000). Phenobarbital and salicylate, which are inducers of cytochrome P450's (P450), increase the hydroxylation in *B. megaterium*, which process involves at least two different P450. The bacterium produces two regioisomers from the hydroxylation of (2-methylpropyl)benzene (**1j**) and (1-methyl)propylbenzene (**1h**) and four regioisomers from the hydroxylation of

pentylbenzene. In contrast, *F. moniliforme* strain MS31 hydroxylated the alkylbenzenes tested other than butylbenzene (**1e**) only at the respective benzylic position with a high enantiomeric excess for the (*R*)-form. From butylbenzene (**1e**), three regioisomers of alcohols with (*R*)-configurations were produced. These results suggest that the fungus contained one or two enzymes that can act in the hydroxylation of these compounds.

Hydroxylation generally needs cofactors such as NAD(P)H, FAD, and FMN. A cell extract from *F. moniliforme* strain MS31 required cofactors for the hydroxylation of propylbenzene (data not shown). In such a case, whole cells can be used if by-products are not formed. The fungus oxidized the secondary alcohols 1-phenylethanol (**2c**), 1-phenylpropanol (**2d**), 1-phenyl-2-butanol (**2eβ**), 4-phenyl-2-butanol (**2eγ**), and 1-indanol. (*S*)-1-Phenylethanol was oxidized to acetophenone with a higher yield than the (*R*)-enantiomer. The conversion of ethylbenzene produced (*R*)-1-phenylethanol. Therefore, hydroxylation products from alkylbenzenes might accumulate in the reaction mixture without much loss. The reduction of acetophenone (**3c**), propiophenone (**3d**), and 1-phenyl-2-butanone (**3eβ**) was detected but the activity was very low. On the basis of these results, we propose a scheme for propylbenzene oxidation by cells of *F. moniliforme* strain MS31 (Fig. 1.3.1). Cells of the fungus were useful for the production of optically active benzylic alcohols without by-products and with satisfactory yields.

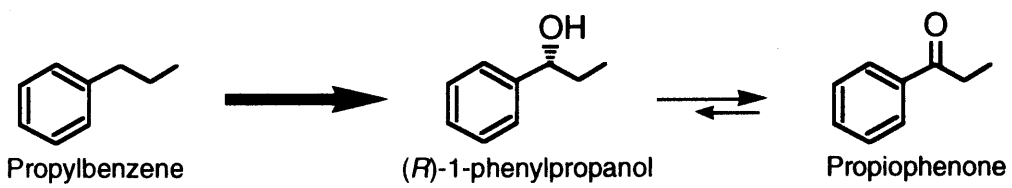


FIG. 1.3.1. Proposed conversion of propylbenzene by resting cells of *F. moniliforme* strain MS31.

Chapter 2

Involvement of Cytochrome P450 in Hydroxylation of Propylbenzene by *Fusarium moniliforme* Strain MS31

Enzymes dependent on cytochrome P450 (P450) are possible candidates for use in the reaction because of their presence in all categories of organisms and their versatility with respect to substrates and kinds of biooxygenation (Omura 1999). *Fusarium moniliforme* strain MS31 hydroxylates various alkylbenzenes at the benzylic position stereoselectively. In similar reactions with *Bacillus megaterium*, there are at least two hydroxylating enzymes for isobutylbenzene that bring about the hydroxylation caused by two inducers of P450, salicylate and phenobarbital (Adam *et al.* 2000). Mechanisms of hydroxylation in *Mortierella isabellina* NRRL 1757, *Helminthosporium* sp. strain NRRL 4671, and *Cunninghamella echinulata* var. *elegans* ATCC 2629 have been investigated with substrates labeled with deuterium, and were found to proceed by a P450-dependent reaction (Holland *et al.* 1988). The enzymes involved in these microbial hydroxylations were not reported.

Studies of P450 in fungi have been few because of problems in the isolation of active enzymes including their low concentration in some species. The enzymes of *F. moniliforme* strain MS31 are such enzymes. Involvement of P450 in the hydroxylation of aryl hydrocarbon by *Cunninghamella bainieri* (Ferris *et al.* 1976), in the hydroxylation of steroids by *Aspergillus ochraceus* (Madyastha *et al.* 1983; Samanta *et al.* 1987), or *Curularia lunata* (Suzuki *et al.* 1988), in the hydroxylation of benzo(a)pyrene by *Phanerochaete chrysosporium* (Masaphy *et al.* 1996), and in the transformation of phenothiazines by *Cunninghamella elegans* (Zhang *et al.* 1996) are verified using crude cell extract. This chapter describes the characterization of the hydroxylating enzyme of *F. moniliforme* strain MS31 using crude preparations.

MATERIALS AND METHODS

Chemicals All chemicals were purchased and used without further purification. Buffer used was 25 mM potassium phosphate (pH 7.0) (KPB) unless otherwise noted.

Preparation of cell extract *F. moniliforme* strain M31 was cultivated, and cells were collected and stored as described in the section 2 of Chapter 1. Wet mycelia, weighing 0.2 g, from frozen stock were suspended in 2 ml of KPB and mixed with 1 g of glass beads (0.5 mm ϕ; BioSpec Products Inc., Bartlesville, OK) in a 2-ml conical tube with a screw cap. The tube was set on a Mini-Beads beater (BioSpec) and was vibrated at 5,000 rpm for 20 s. The tube was put on ice for 40 s or more and then vibrated eight more times for 20 s each time to disrupt the fungal cells. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was called the cell extract and was used in further experiments. The cell extract was prepared on the day of use. The amount of protein was measured by the method of Bradford (1976) with a protein assay kit (Nippon Bio-Rad Laboratories K. K., Tokyo) and bovine serum albumin as the standard.

Assay of propylbenzene hydroxylation The reaction mixture contained 90 µl of the cell extract, 10 µl of a coenzyme mixture, and 10 µmol (1.2 µl) of propylbenzene in a 1.5-ml Eppendorf tube. Coenzyme mixtures contained, per 1 ml of KPB, 10 µmol each of NAD, NADH, NADP, NADPH, ATP, and ADP and 0.5 µmol each of FAD and FMN, in some combination (see Table 2.1); the standard mixture contained only NADPH and FAD. The reaction mixture was incubated at room temperature for 2 h on a vibration mixer (Twin 3-28; Asahi Techno Glass Co., Tokyo) at a speed of between 1 and 2 on the device. The reaction was stopped by the addition of 10 µl of 6 N HCl and the mixture was extracted with 100 µl of *n*-hexane. Extracts were analyzed by GC. One unit of enzymatic activity is defined as the amount of enzyme that produces 1 µmol of 1-phenylpropanol in 2 h. The reactions were run in triplicate. The cell extract was different in each experiment, so all experiments were done separately at least three times and the results are shown as the activity relative to that of the control. Relative activities from different experiments were almost the same and for that reason typical results are reported.

Gas test The procedure is shown in Fig. 2.1. A 1.8-ml portion of the cell extract and 0.2 ml of a coenzyme mixture were put into a 16.5 × 100-mm test tube. Nitrogen gas was supplied to the test tube for 2 min at the flow rate of 2 $l \cdot min^{-1}$ to remove oxygen and the test tube was sealed with a rubber double cap (W-15, Orion Inc., Kyoto). The septum closing off the connector (No. 1020-20104, GL Sciences Inc., Tokyo) for a disposable syringe (50 ml) on the top of a pressurized spray can containing a standard gas (O_2, N_2, or CO) was pierced with a needle (cave hole, 23 G × 4 cm long, GL Sciences) through which gas was collected into the syringe. A gas mixture was prepared in a syringe and injected into a test tube through the rubber double cap; at the same time, air was taken into another syringe. Injections of gas were done twice. Propylbenzene (200 μmol; 24 μl) was injected into the test tube with a microsyringe. Hydroxylation was assayed as described before.

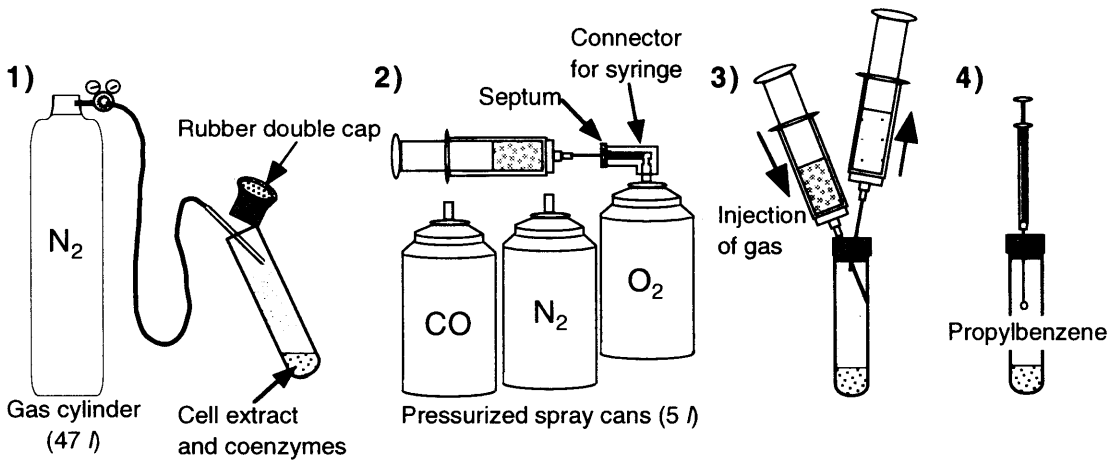


FIG. 2.1. Gas test. 1) N_2 gas from a gas cylinder was supplied to a test tube for 2 min . 2) The septum closing off the connector for a disposable syringe (50 ml) on the top of pressurized spray can was priced with a needle, through which gas was collected into the syringe. 3) The gas or gas mixture collected in the syringe was injected into a test tube through the rubber double cap; at the same time, air was taken into an empty syringe. 4) Propylbenzene was injected into the test tube.

Oxygen consumption Oxygen consumption during the reaction with the cell extract was monitored with a two-layer reaction system in the incubation chamber of a dissolved oxygen electrode (Digital Oxygen System model 10; Rank Brothers Ltd.,

Cambridge, UK). A 1-ml portion of the cell extract (1.5 U) was injected into the incubation chamber of the electrode at 30°C and then 300 µl of propylbenzene was added to the cell extract. The reaction was started by the addition of 100 µl of the coenzyme mixture containing 1 µmol of NADPH and 50 nmol of FAD.

Inhibitor test A 10-µl portion of the solution of an inhibitor in deionized water was mixed with 80 µl of the cell extract. As a control, 10 µl of deionized water was mixed with the cell extract. An inhibitor insoluble in water was dissolved in ethanol and 1 µl of the solution was mixed with 90 µl of the cell extract; the control had 1 µl of ethanol. The mixture was incubated for 5 min at room temperature, and then assayed for hydroxylation with the coenzyme mixture and propylbenzene added.

Induction test A 6-ml portion of a seed culture was added to 300 ml of BM3 medium in a 2-*l* Erlenmeyer flask. The mixture was cultivated at 30°C on a rotary shaker at 150 rpm. Test chemicals were added to 1- or 2-d cultures and cultivation was continued for 6 or 24 h more. Mycelia were collected and used in the cell reaction as described in the section 2 of Chapter 1.

RESULTS

Effects of coenzymes on hydroxylation The effects of various coenzymes alone or in mixtures on the conversion of propylbenzene with the cell extract of *F. moniliforme* strain MS31 to 1-phenylpropanol are shown in Table 2.1. The activity when only NADPH was added was higher than that when only NADH was added. The addition of FAD, FMN, or both to the reaction mixture containing NADPH increased the activity. In further experiments, we used a coenzyme mixture that contained NADPH and FAD.

Effects of other cofactors on hydroxylation Some cofactors involved in the reactions of dehydrogenases and oxygenases were tested for their effects on hydroxylation, as were gas mixtures of O_2 and N_2 (Table 2.2). All electron acceptors for dehydrogenases

TABLE 2.1. Effects of coenzymes on hydroxylation

Coenzyme(s)	Relative activity (%)
None	Not detected
Basic coenzyme mixture[a]	100[b]
with NAD and NADH omitted	80
with NADP and NADPH omitted	20
with ATP and ADP omitted	100
with FAD and FMN omitted	78
NAD + NADH	17
NADP + NADPH	71
ATP + ADP	Not detected
FAD + FMN	Not detected
NADH	12
NADH + FAD	15
NADH + FMN	17
NADH + FAD + FMN	17
NADPH	61
NADPH + FAD	78
NADPH + FMN	65
NADPH + FAD + FMN	92

[a]A mixture of NAD, NADH, NADP, NADPH, ATP, ADP, FAD, and FMN was prepared in KPB at the concentrations given in Materials and Methods.

[b]Used as the control (100%). There was 5.2 mg of protein (2.4 U) per 1 ml of the reaction mixture.

(from $K_3Fe(CN)_6$ to cytochrome *c* in the table) gave less hydroxylation activity than their control. Hemin increased the hydroxylation by about 40%. Three cofactors for oxygenases, tetrahydrobiopterin, ascorbic acid, and catalase, did not increase hydroxylation activity, and hydrogen peroxide was not detected. Hydroxylation did not occur in the reaction mixture without oxygen. Hydroxylation occurred with 5% (v/v) oxygen and was greater with larger concentrations of oxygen to 50% (v/v). The concentration of dissolved oxygen in the reaction mixture was monitored with an oxygen electrode. In the chamber, 100 µmol of propylbenzene formed a ball and floated; 1-phenylpropanol was not detected later in a *n*-hexane extract of the reaction mixture. With a two-layer system, 1-phenylpropanol was detected, and the initial rate of decrease in dissolved oxygen in the reaction mixture was 0.26 $\mu mol \cdot ml^{-1} \cdot min^{-1}$. The oxygen consumption was proportional to the concentration of the cell extract (Fig. 2.2).

TABLE 2.2. Effects of cofactors or gaseous oxygen on hydroxylation

Compound	Concentration	Relative activity (%)[a]
$K_3Fe(CN)_6$	1 mM[d]	86[e]
Methylene blue	1 mM[d]	32[e]
DPIP[b]	1 mM[d]	58[e]
1-Methoxy-PMS[b]	1 mM[d]	21[e]
INT[b]	1 mM[d]	70[e]
MTT[b]	1 mM[d]	89[e]
Nitro blue tetrazolium	1 mM[d]	62[e]
Menadione	1 mM[d]	75[e]
Pyrroloquinoline quinone	1 mM[d]	11[e]
Cytochrome *c* (horse heart)	0.1 mM[d]	6[e]
Hemin	1 mM[d]	136[e]
Tetrahydrobiopterin	1 mM[d]	58[e]
Ascorbic acid	1 mM[d]	113[e]
Catalase (bovine liver)	5200 $U \cdot ml^{-1}$[d]	102[e]
Oxygen[c]	0%	0[f]
	5%	70[f]
	10%	85[f]
	20%	96[f]
	50%	111[f]
	80%	113[f]
	100%	109[f]

[a]The hydroxylation activity is expressed as a percentage of the activity of the control (100%).

[b]DPIP, 2,6-dichlorophenolindophenol; 1-methoxy-PMS, 1-methoxy-5-methylphenazinium methylsulfate; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide.

[c]Gas mixtures contained O_2 at the indicated concentrations in N_2 (v/v).

[d]Concentration in the reaction mixture.

[e]The control was the reaction without cofactors. There was 5.0 mg of protein (1.5 U) per 1 ml of the reaction mixture.

[f]The control was the reaction in an air atmosphere without replacement with N_2. There was 6.2 mg of protein (1.9 U) per 1 ml of the reaction mixture.

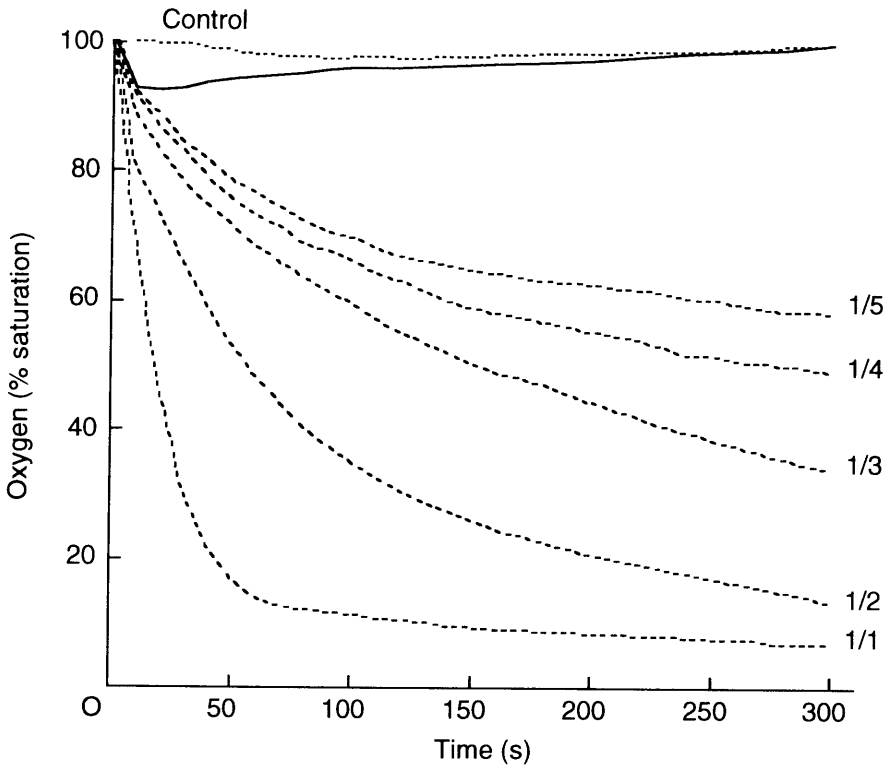


FIG. 2.2. Oxygen consumption during hydroxylation with various concentrations of the cell extract. Solid line, cell extract heated in a microwave oven at 600 W for 1 min to inactivate the enzyme; broken lines, a raw cell extract (1/1; there was 6.4 mg of protein (1.5 U) per 1 ml. 1/2, 1/3, 1/4, and 1/5; diluted solutions). Control; propylbenzene was not added.

Effect of inhibitors on hydroxylation Since enzymes dependent on P450 are possible candidates, effects of P450 inhibitors on hydroxylation were examined (Table 2.3). Carbon monoxide up to the concentration of 20% (v/v) allowed some hydroxylation, with no activity at the concentration of 50% (v/v). The other P450-specific inhibitors decreased hydroxylation, except for methoxsalen. When Cu^{2+}, Hg^{2+}, Ag^{+}, or Zn^{2+} was present, the residual activity was between 0% and 25% (Table 2.4). The activity in the presence of Ba^{2+}, Fe^{2+}, Mg^{2+}, or MoO_4^{2-} was the same as when the inorganic compound was absent. The effects of other potential inhibitors are shown in Table 2.5. When one of four thiol inhibitors, *p*-chloromercuribenzoate, 5,5'-dithiobis(2-nitrobenzoate), *N*-ethylmaleimide, or *N*-methylmaleimide, was present, the activity decreased to 35% or less. Inhibitors that bind with the heme site in the enzyme (KCN, NaN_3, or Na_2S) decreased the activity to about 30% or less. The metal-chelating reagents 2,2'-dipyridyl, EDTA, *o*-phenanthroline, 8-quinolinol,

TABLE 2.3. Effects of P450 inhibitors on hydroxylation

Inhibitor	Concentration	Residual activity (%)[a]
Carbon monoxide	0%[b]	103[f]
	20%[c]	7[f]
	50%[d]	0[f]
Metyrapone	1 mM[e]	43[g]
SKF 525A·HCl	1 mM[e]	49[g]
Methoxsalen	1 mM[e]	98[g]
Ketoconazole	1 mM[e]	44[g]
Miconazole	1 mM[e]	20[g]
Clotrimazole	1 mM[e]	29[g]
Menadione	1 mM[e]	42[g]
Piperonyl butoxide	1 mM[e]	48[g]

[a]The hydroxylation activity is expressed as a percentage of the activity of the control (100%).

[b]Gas mixtures used were 0:20:80, [c]20:20:60, or [d]50:20:30 CO–O_2–N_2 (by vol.).

[e]Concentration in the reaction mixture.

[f]The control was the reaction in an air atmosphere without replacement with N_2. There was 6.2 mg of protein (1.4 U) per 1 ml of the reaction mixture.

[g]Dissolved in ethanol; the control was ethanol mixed with the cell extract. There was 5.4 mg of protein (1.5 U) per 1 ml of the reaction mixture.

TABLE 2.4. Effects of inorganic compounds on hydroxylation

Compound (at 1 mM[a])	Residual activity (%)
None (deionized water)	100[b]
$AlCl_3$	75
$BaCl_2$	97
$CaCl_2$	62
$CoCl_2$	75
$CuCl_2$	19
$FeCl_3$	61
$FeSO_4$	98
$MgSO_4$	101
$MnCl_2$	57
$HgCl_2$	3
$NiCl_2$	74
$AgNO_3$	3
$ZnSO_4$	22
$Na_2B_4O_7$	84
Na_2MoO_4	96

Compounds were dissolved in deionized water.

[a]In the reaction mixture.

[b]Used as the control (100%). There was 5.2 mg of protein (2.6 U) per 1 ml of the reaction mixture.

and thiourea did not affect the activity. Of the other compounds tested, the residual activity with phenylhydrazine was lowest (at 4%), and those with diethyldithiocarbamate, hydroxylamine, and iodoacetate were between 50% and 70%.

TABLE 2.5. Effects of possible inhibitors of hydroxylation

Compound (at 1 mM[a])	Residual activity (%)
None (deionized water)	100[b]
None (1% (v/v)[a] ethanol)[c]	100
p-Chloromercuribenzoate	2
5,5'-Dithiobis(2-nitrobenzoate)	12
N-Ethylmaleimide	35
N-Methylmaleimide	32
KCN	31
NaN_3	24
Na_2S	12
2,2'-Dipyridyl[c]	100
EDTA	105
o-Phenanthroline[c]	92
8-Quinolinol[c]	88
Thiourea	101
L-Cysteine	87
Diethyldithiocarbamate	60
Hydrazine	90
Hydroxylamine	67
Iodoacetate	50
Phenylhydrazine	4
Semicarbazide	102

[a]In the reaction mixture.

[b]Used as the control (100%). There was 4.8 mg of protein (3.7 U) per 1 ml of the reaction mixture.

[c]2,2'-Dipyridyl, *o*-phenanthroline, or 8-quinolinol was dissolved in ethanol. There was 5.4 mg of protein per 1 ml of the reaction mixture. The residual activity was expressed as the activity per protein content and given as relative to that of the control with ethanol.

Effects of P450 inducers on hydroxylating activity Since most P450 monooxygenase systems are inducible by their substrates or by unspecific inducers, some of P450 inducers so far reported were tested with this strain (Table 2.6). With acetoin or

salicylic acid, the specific activity was higher than that of the control culture at all cultivation times tested. The specific activity per cell weight was highest (about 140% that of the control) when acetoin was added to a 2-d culture and the mixture was incubated for 24 h more.

TABLE 2.6. Effects of P450 inducers on hydroxylating activity

Inducer	Concentration[a]	Relative specific activity (%)			
		1-d culture		2-d culture	
		6-h induction	24-h induction	6-h induction	24-h induction
None	—	59[b]	97[c]	93[d]	100[e]
Acetoin	0.2% (w/v)	78	108	113	138
Clofibrate	0.2% (w/v)	47	28	35	13
Dexamethasone	2 mM	58	56	68	74
n-Hexane	0.2% (v/v)	67	78	100	95
Methylethylketone	0.2% (v/v)	71	85	93	134
β-Naphthoflavon	2 mM	55	101	68	109
Phenobarbital	10 mM	73	85	87	73
Pyridine	0.2% (v/v)	68	81	85	90
Salicylic acid	10 mM	73	115	117	120
Vegetable oil[f]	0.2% (v/v)	91	84	120	132

[a]Concentration in the medium during possible induction culture.
[b]Total culture time was 30, [c]48, or [d]54 h.
[e]Total culture (control) was for 72 h; 100%.
[f]Cooking oil (rapeseed, soybean, and corn oils; proportions in decreasing order).

DISCUSSION

In general, hydroxylation of organic compounds is catalyzed by an enzyme belonging to the oxidoreductase group of oxidases, oxygenases, and dehydrogenases. Propylbenzene was converted into (*R*)-1-phenylpropanol by the cell extract of *F. moniliforme* strain MS31 with NADPH or NADH. Propylbenzene hydroxylation did not require electron acceptors, and did not produce hydrogen peroxide, unlike an oxidase system (Sakai *et al.* 1995). These findings showed that the enzyme was not a dehydrogenase or an oxidase, respectively.

Hydroxylation required molecular oxygen and was increased most by the cofactor hemin. Such characteristics suggested that the enzyme hydroxylating propylbenzene was an oxygenase that contained heme.

Hydroxylation by the cell extract required NADPH or NADH and was increased by the addition of FAD and FMN. Fungal P450 monooxygenase systems contain an NADPH-P450 reductase, which has binding sites for FAD, FMN, and NADPH (van den Brink *et al.* 1998). The reductase transports an electron from NADPH to FAD to FMN and finally to P450 or other electron acceptors (Vermillion *et al.* 1981). Cytochrome *c*, DPIP, and ferricyanide are competitive electron acceptors of P450, and *p*-chloromercuribenzoate inhibits the electron transport chain of this reductase to P450 (Franklin and Estabrook 1971). Inhibition by these chemicals suggests the involvement of a protein like the reductase in propylbenzene hydroxylation.

Inhibition of hydroxylation by thiol inhibitors indicated the presence of a sulfhydryl group in the hydroxylase. Inhibition by Cu^{2+}, Hg^{2+}, and Ag^{+} also indicated that the enzyme was a hydroxylase with thiolate near the catalytic site. P450 has the characteristics of a heme-thiolate protein (Kitagawa 1993).

P450 inhibitors such as carbon monoxide, miconazole, clotrimazole, and cyanide inhibited the hydroxylation. I concluded that the hydroxylation was catalyzed by a P450 together with a P450 reductase (Fig. 2.3).

The specific activity was lower than expected if a P450 inducer was added to the culture. The hydroxylating enzyme may be produced constitutively. Constitutive P450 can be detected by CO difference spectra of whole cells of *Mortierella alpina* ATCC 8979 or *Cunninghamella blakesleeana* DSM 1906, which hydroxylate cycloalkylbenzoxazoles (Asperger *et al.* 1999), but P450 is not detected in their cell extracts or microsomal fractions (Asperger *et al.* 1999). CO difference spectra of the cell extract or the microsomal fractions from *F. moniliforme* strain MS31 did not show the presence of P450 (data not shown). P450 in strain MS31 may be similar to such fungal P450s.

P450 enzymes metabolize a wide range of man-made chemicals including drugs and environmental pollutants. P450 substrates also include natural products: steroids, fatty acids,

flavor components, flower pigments, and so on. Numerous P450 genes have been cloned from animals, plants, and microorganisms (Nelson *et al.* 1996; Genbank), but the P450 gene involved in the hydroxylation of propylbenzene or similar functions has not been reported yet.

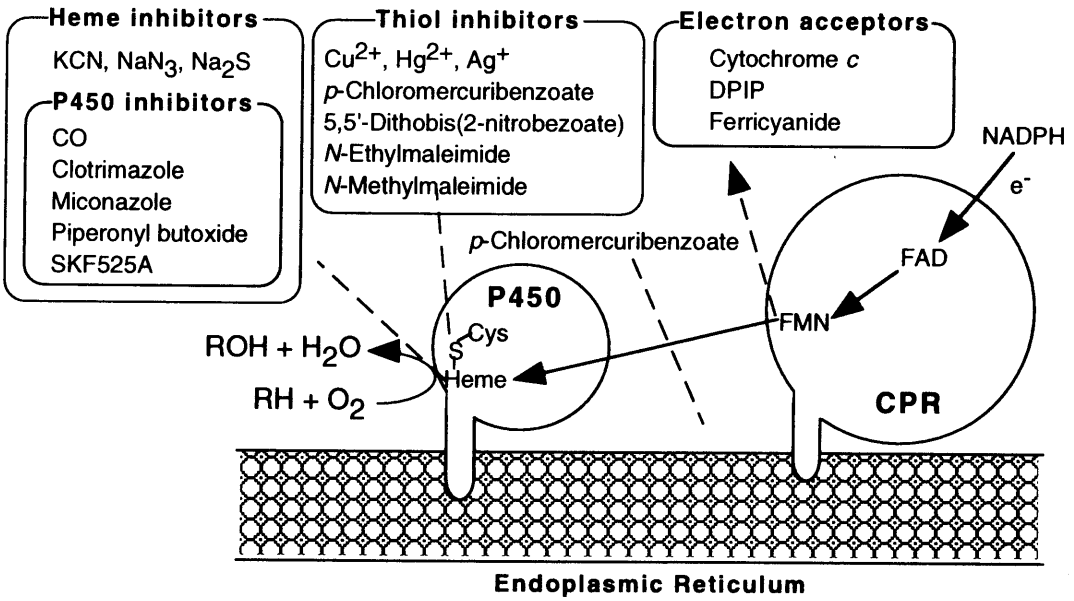


FIG. 2.3. Proposable mechanism of P450 and NADPH-cytochrome P450 reductase (CPR) involved in monooxygenation. Transfer of electron are shown with arrow. Broken lines are inhibition sites. RH, substrate; ROH, product

Chapter 3
Genetic characterization of cytochrome P450 in hydroxylation of propylbenzene

Cytochrome P450 (P450) enzymes are responsible for a very wide range of transformation including hydroxylation and demethylation. The process involves the addition of one atom of molecular oxygen to the substrate molecule, the other atom being converted to water. The electrons needed for the oxygen insertion in the substrate molecule are transferred from a separate electron-donating system. The system is either a two-protein system, adrenodoxin and adrenodoxin reductase, for mitochondrial and prokaryotic P450s or a single-protein system, NADPH-cytochrome P450 reductase (CPR), for P450s that are located in the membrane of the endoplasmic reticulum (Takemori *et al.* 1993). Most fungal P450s so identified are located in the endoplasmic reticulum (Yoshida 1993).

Fusarium moniliforme strain MS31 was useful for the production of optically active phenylalcohols but the yield was not enough for industrial use. In Chapter 2, involvement of P450 and CPR in the hydroxylation of propylbenzene was indicated. For increase of the activity of P450 enzymes, the interaction between P450 and CPR is important (Sakaki *et al.* 1994) and genetically engineered fused protein of these two enzymes is thought to be most useful (Yabusaki 1995). In this chapter, genes responsible for hydroxylation of propylbenzene were sought in *F. moniliforme* strain MS31. In the section 1, a gene encoding CPR was cloned in *Saccharomyces cerevisiae* and its function was determined by expression under control of GAL1 promoter. In the section 2, three genes encoding P450 were cloned in *S. cerevisiae*.

Section 1
Cloning of NADPH-cytochrome P450 Reductase Gene from *Fusarium moniliforme* Strain MS31

NADPH-cytochrome P450 reductase (CPR; EC 1.6.2.4) is an essential component of most eukaryotic cytochrome P450 monooxygenase systems. CPR is a flavoprotein and transfers electron from NADPH to P450 as required to activate molecular oxygen for the various P450-catalysed oxidation reactions. The flow of electrons is from NADPH to FAD to FMN to P450 or other electron acceptors (Vermilion *et al.* 1981). In contrast to the many different P450 genes that can be found in a single organism, only one CPR gene is found in most organisms (van den Brink *et al.* 1998). A CPR supports many different P450 enzyme activities (Porter 2002). The deduced amino acid sequence identities of CPR proteins between various fungal and yeast genera are 35-50%. The sequences for possible functional domains involved in binding cofactors FMN, FAD, and NADPH are well conserved in taxonomically diverse species (Miles 1992; van den Brink *et al.* 1995; Yadav and Loper 2000). In this section, cloning of a CPR gene from *Fusarium moniliforme* strain MS31 using RT-PCR is described.

MATERIALS AND METHODS

Microorganisms and culture media *F. moniliforme* strain MS31 was cultivated in the BM3 medium as described in the section 2 of Chapter 1. *Escherichia coli* JM109, which was the host cells for the construction of plasmids, were cultured in Luria-Bertani (LB) broth. The transformants were grown on LB containing 50 $\mu g \cdot ml^{-1}$ sodium ampicillin. *Saccharomyces cerevisiae* INV*Sc*1 (Invitrogen Corp., Carlsbad, CA) was the host cells for the expression of cDNA of *FMR* (encoding CPR of *F. moniliforme* strain MS31) in a vector pYES2.1 (Invitrogen). The yeast was maintained on YPG agar composed of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, and 1.5% (w/v) agar. SC medium was

composed of 2% (w/v) glucose (which was sterilized separately), 0.67% (w/v) yeast nitrogen base without amino acids with ammonium sulfate (Difco Laboratories, Detroit, MI), and 10% (v/v) amino acids solution, which consisted of, in 1 *l* of deionized water, 1 g each of adenine, L-arginine, L-cysteine, L-leucine, L-lysine, L-threonine, and L-tryptophan, 0.5 g each of L-aspartic acid, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-tyrosine, and L-valine, with addition of 1 g of uracil. The yeast transformants were selected on the plate of SC medium without uracil (SC–U medium). For induction, galactose (which was sterilized separately) was added to the SC or SC–U medium in place of glucose.

Preparation of genomic DNA *F. moniliforme* strain M31 was cultivated for 4 d, and cells were collected and stored as described in the section 2 of Chapter 1. Wet cells, weighing 1 g, were frozen with liquid N_2 in a mortar and were ground to be fine powder. The powdery cells were transferred to a 50-ml conical tube with a screw cap. For extraction of DNA, 8 ml of TE (composed of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and 8 ml of lysis buffer (composed of 50 mM EDTA and 0.5% (w/v) sodium dodecylsulfate) were added and the mixture was incubated at 37°C for 30 min. The mixture was centrifuged at 6,000 × *g* for 5 min at 4°C. The supernatant was treated with TE saturated phenol–chloroform–isoamylalcohol (25:24:1, by vol.) and RNase A (Nippon Gene Co., Ltd., Tokyo) by the standard protocol for purification of genomic DNA (Sambrook 2001). The isolated genomic DNA was 75 μg.

PCR amplification of the CPR sequence from genomic DNA Degenerate primers were designed based on relatively conserved sequences in the FMN-binding domain of known CPR genes for forward primer and in the NADPH-binding domain for reverse primer (Fig. 3.1.1). The PCR was done using Ex Taq (Takara Shuzo Co., Kyoto) as DNA polymerase and the reaction mixture (25 μl) contained 25 ng of genomic DNA as template and 12.5 pmol each of forward and reverse primers. Amplification was preceded by denaturation at 94°C for 10 min, proceeded with repeating 30 cycles of denaturation at 94°C for 1 min, annealing (by gradient program) at between 40°C and 55°C for 1 min, and polymerization at 72°C for 2 min, and followed by extension at 72°C for 7 min in a thermal

Forward primers

FMN-1 domain

Consensus amino acid:		Q	T	G	T	A/G	E
Degenerate primer:	5'-	CAR	ACN	GGN	ACN	GCN	GAR

FMN-2 domain

Consensus amino acid:		G	E/D	P	T	D	N	A
Degenerate primer:	5'-	GGN	GAN	CCN	ACN	GAY	AAY	GC

FMN-3 domain

Consensus amino acid:		F	G	L	G	K
Degenerate primer:	5'-	TTY	GGN	YTN	GGN	AAY

Reverse primers

NADPH-2 domain

Consensus amino acid:	K	V	Y	V	Q	D/E	
Degenerate primer:	TTY	CAN	ATR	CAN	GTY	ST	-5'

NADPH-3 domain

Consensus amino acid:	Y	V	C	G	D	A	
Degenerate primer:	ATR	CAN	TGR	CCN	CTR	CG	-5'

FIG. 3.1.1. Degenerate primers used for the PCR-amplification of CPR gene from *F. moniliforme* strain M31. Primers were designed based on the conserved regions of CPR genes from fungal species including *Aspergillus niger*, *S. cerevisiae*, *S. pombe*, *Candida roseus* (van den Brink *et al.* 1995). Abbreviations are: N, A, C, G, or T; R, A or G; S, C or G; Y, C or T.

cycler incubation oven (iCycler; Nippon Bio-Rad Laboratories K. K., Tokyo). The annealing temperature was set automatically at 40, 41, 42.9, 45.4, 49.4, 52.1, 53.9, and 55°C. For determination of whole sequence of the CPR gene, gene walking was done using LA PCR in vitro cloning kit (Takara). A PCR product was isolated from the electrophoresis gels, purified, and ligated into a T-tailed pCR 2.1 vector (Fig. 3.1.2. A) in TOPO TA cloning kit (Invitrogen) and the ligation mixture was used to transform *E. coli* JM 109 for DNA sequencing.

Preparation of mRNA Cells of the strain MS31 were collected from 2-d culture, at which time the yield of total RNA was highest and the resting cells showed propylbenzene hydroxylation, and stored at -80°C until use. Wet mycelia from frozen stock, weighing 0.1 g, were suspended in 1 ml of an acid phenol-guanidinium thiocyanate reagent (Sepazol-RNA I; Nacalai Tesque, Inc., Kyoto) mixed with 0.5 g of glass beads (0.5 mm ϕ; BioSpec Products

Inc., Bartlesville, OK) in a 2-ml conical tube with a screw cap. The tube was set on a Mini-Beads beater (BioSpec) and was vibrated at 5,000 rpm for 30 s. The tube was put on ice for 1 min or more and then vibrated six more times for 30 s each time to disrupt the fungal cells. Purification was done according to the manufacture's instruction to obtain total RNA. Poly(A)$^+$ RNA was isolated using an oligo(dT) spin column (mRNA purification kit; Amersham Biosciences K. K., Tokyo) according to the manufacture's instruction but volume of the high salt buffer and the low salt buffer were increased to 2 ml in the washing steps. The mRNA was treated with DNase I (RNase-free; Takara) to remove DNA. Purified mRNA was 10 μg from 1 g of wet mycelia.

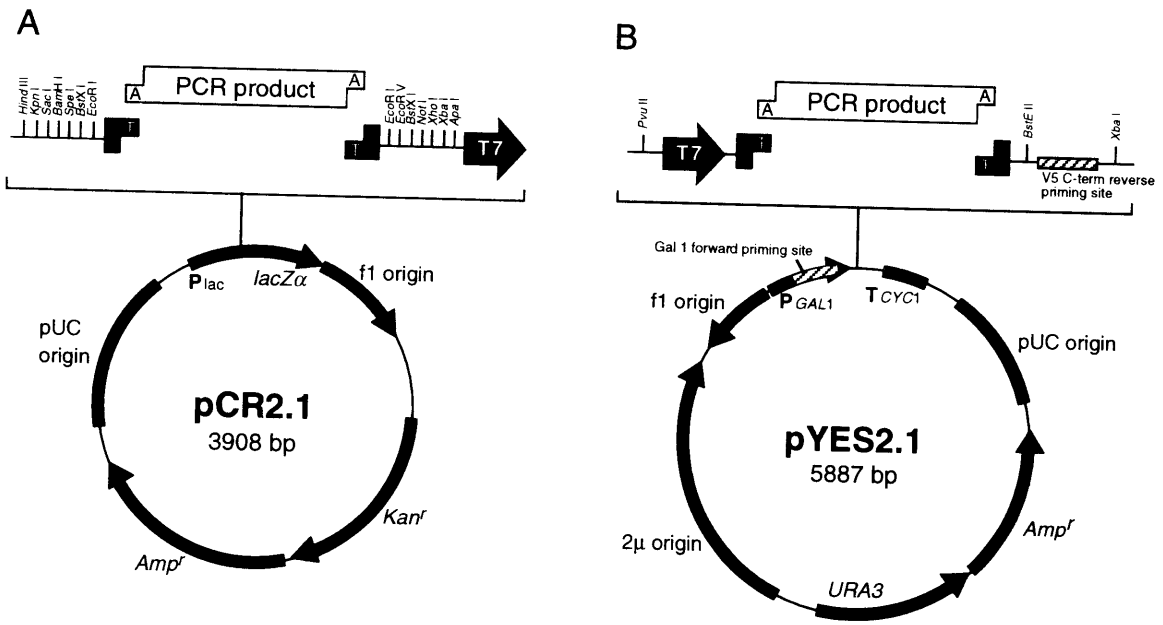


FIG. 3.1.2. Maps of pCR 2.1 vector (A) and pYES 2.1 vector (B). Plac, *lac Z* promoter; *Kan*^{r}, Kanamycin resistance ORF; *Amp*^{r}, Ampicillin resistance ORF; $P_{GAL\ 1}$, *GAL* 1 promoter; T_{CYC1}, *CYC* 1 transcription termination signal; *URA3*, *URA3* promoter and gene.

cDNA cloning of the CPR gene Library of single-strand cDNA was constructed using the First-strand cDNA synthesis kit (Takara). A 25-μl RT reaction mixture contained 1 μg of mRNA, 0.5 μg of oligo(dT) primers, 10 nmol of dithiothreitol, and 25 U of avian myeloblastosis virus reverse transcriptase. The mixture was incubated at 41°C for 1 h and

was heated to 95°C for 5 min to inactivate the transcriptase. Following PCR was done using Ex Taq (hot start version; Takara) and the reaction mixture (25 μl) contained 1 μl of the RT reaction mixture and 12.5 pmol each of primers. Primers were CPR-start (5'-GCAATGGCTGATATCGACATCATGA-3', forward) and CPR-end (5'-CACCTTATCTACCAGATGTCCTCCT-3', reverse). Amplification was preceded by denaturation at 94°C for 3 min, proceeded with repeating 30 cycles of denaturation at 94°C for 1 min, annealing (by gradient program) at between 55°C and 65°C for 1 min, and polymerization at 72°C for 2 min, and followed by extension at 72°C for 10 min in the thermal cycler. The annealing temperature was set automatically at 55, 55.8, 57, 58.7, 61.2, 63.1, 64.3, and 65°C. A PCR product was isolated from the gel, purified, and ligated into a T-tailed pYES 2.1 vector (Fig. 3.1.2. B) in the pYES 2.1 TOPO TA cloning kit (Invitrogen) to construct pYES-fmr. Plasmids were use to transform *E. coli* JM 109 for purification of the plasmid and DNA sequencing. Purified pYES-fmr was used to transform *S. cerevisiae* INVSc1 by lithium acetate method (Ito *et al.* 1983) with Alkali-Cation Yeast kit (Quantum Biotechnologies, Inc., Carlsbad, CA). The presence of pYES-fmr in the yeast was determined by colony PCR with a primer set of Gal 1 forward and V5 C-term reverse in the pYES 2.1 cloning kit.

DNA sequencing DNA sequencing of recombinant plasmid was done by the dideoxy chain-termination method (Sanger *et al.* 1977) with an automatic sequencer (PRISM 310 Genetic Analyzer; Applied Biosystems Japan Ltd., Tokyo).

Expression of CPR gene *S. cerevisiae* INV*Sc*1 and its transformants were cultured at 30°C in 500-ml Erlenmeyer flasks on a rotary shaker at 150 rpm. One loopful from the colony was used to inoculate 100 ml of SC or SC–U medium, and the mixture was cultivated. After 24 h, cells were collected by centrifugation at 1,500 × *g* for 5 min at 4°C and washed twice by 20 ml of the fresh SC–U medium without glucose. Cells were transferred to 100 ml of the induction medium to a final concentration 0.4 OD_{600} and the mixture was cultivated. Cells grown in 44-h induction were used to prepare total RNA in the same way as above. Total RNA was treated with DNase I (RNase-free; Takara) and then used for RT-PCR with primers CPR-start and CPR-end to amplify the expressed *FMR* in *S.*

cerevisiae INV*Sc*1.

Assay of CPR activity Cells were collected by centrifugation at 1,500 × *g* for 5 min at 4°C. Cells were suspended in 2 ml of 100 mM potassium phosphate buffer (pH 7.0; KPB) at a final concentration of 80 OD_{600} and mixed with 1 g of glass beads (0.5 mm ϕ; BioSpec) in a 2-ml conical tube with a screw cap. The tube was set on a Mini-Beads beater (BioSpec) and was vibrated at 3,600 rpm for 30 s. The tube was put on ice for 2 min or more and then vibrated six more times for 30 s each time to disrupt the cells. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C. CPR activity in the supernatant was measured by monitoring optically the reduction of cytochrome *c* in the assay mixture at room temperature in a spectrophotometer at 550 nm (ε=29) for 2 min. The reaction mixture contained, per 1 ml of KPB, 0.05 μmol cytochrome *c*, 1 μmol NADPH, and 0.2–0.5 mg of protein. A unit of enzymatic activity was defined as the amount of the enzymes that reduced 1 nmol of cytochrome *c* per 1 min. The amount of protein was measured by the method of Bradford (1976) with a protein assay kit (Nippon Bio-Rad) and bovine serum albumin as the standard.

Effect of NADPH and its generation systems on the hydroxylation of propylbenzene Wet mycelia of *F. moniliforme* strain MS31 from frozen stock at –80°C, weighing 0.1 g, were suspended in 1 ml of KPB. The reaction mixture contained 90 μl of the cell suspension, 10 μl of a cofactor solution, 10 μmol (1.2 μl) of propylbenzene, and 10 mg of glucose (if added) in a 1.5-ml Eppendorf tube. The cofactor solution contained, per 1 ml of KPB, 10 μmol of NADPH, 60 U of glucose dehydrogenase, and the cell extract from 44-h induction culture of *S. cerevisiae* INV*Sc*1 and INV*Sc*1/pYES-fmr, in some combination (see Table 3.1.1). Reaction and assay of the hydroxylation of propylbenzene were as described in Chapter 2.

RESULTS

DNA sequence of CPR gene PCR amplification was done on genomic DNA as template using all six combinations of degenerate primers. A first PCR using the primers

FMN-1 and NADPH-3 resulted in the formation of several faint bands on 1% (w/v) agarose gels (Fig. 3.1.3. A). A second PCR using the primers FMN-2 and NADPH-2 and 1 μl of the first PCR products (annealing at 45.4°C, 1:1000 dilution) as template resulted in the formation of a single band, the size of which was 1.5 kb (Fig. 3.1.3. B). The deduced amino

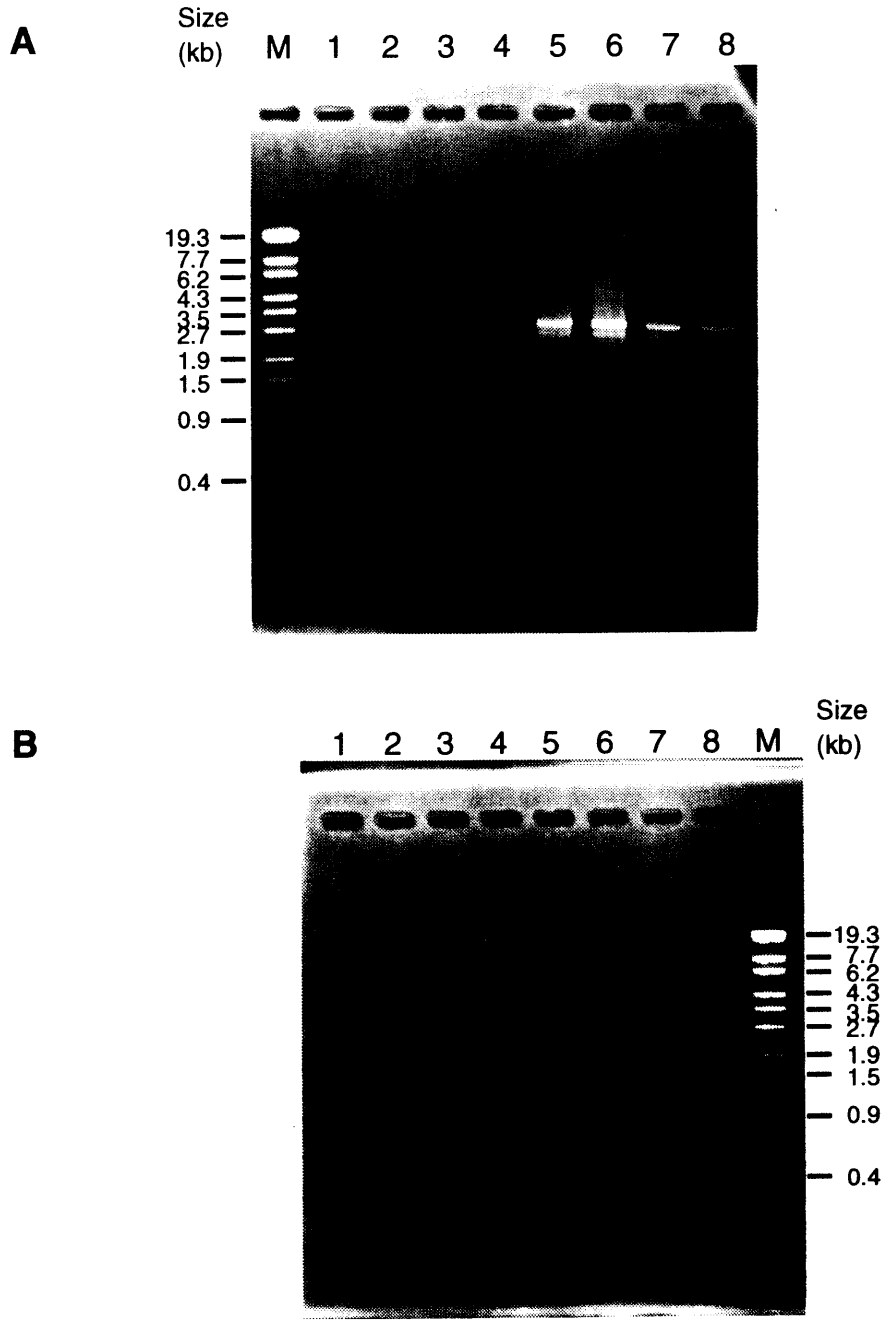


FIG. 3.1.3. PCR amplifications for CPR of *F. moniliforme* strain MS31. A, first PCR using genomic DNA and the primers FMN-1 and NADPH-3; B, second PCR using the first PCR mixture (annealing at 45.4°C, 1:1000 dilution) and the primers FMN-2 and NADPH-2. Lanes 1-8 are annealing temperature at 55, 53.9, 52.1, 49.4, 45.4, 42.9, 41, and 40°C, respectively. M, l-EcoT14 I digest.

acid sequence was similar to that of CPR in databases upon analysis by BLAST search program (GenomeNet 2002). Sequence the DNA containing whole CPR gene was determined by gene walking. A 4,106-bp sequence was generated by sequencing in both directions (Fig. 3.1.4).

Cloning of cDNA Three deduced amino acid sequences were obtained from the nucleotide sequence of the genomic DNA. Functional domains and other conserved regions were analyzed by comparison of deduced amino acid sequences for other fungal CPRs and a forward primer containing start codon and a reverse primer containing end codon (described in Materials and Methods) were designed. PCR with single-strand cDNA library as the template resulted in the formation of a single band of the size of 2.1 kb. The nucleotide sequence and its deduced amino acid sequence are shown in Fig. 3.1.4.

Gene sequence analysis The translation start codon was surrounded by the fungal consensus sequence TCA(C/A)(A/C)**ATG**(G/T)C (Ballance 1986) (Fig. 3.1.4). A comparison of the genomic DNA and cDNA sequences of the gene led to the identification of two introns. A base composition of the coding region was A (27.11%), T (24.39%), G (23.97%), and C (24.53%). NNPP (2002) analysis for eukaryotic promoter prediction indicated a putative transcription start site at the –353 nucleotide (nt) position on the (+) strand upstream of the assigned translation start codon. A CAAT box was at the –274 nt position and possible TATA box was at the –74 nt position. The polyadenylation signal AATAAA was not found.

Deduced protein The deduced amino acid sequence of CPR from *F. moniliforme* strain MS31, which protein is called FMR, was composed of 706 amino acid residues (Fig. 3.1.4) and its calculated molecular weight was 78,412. SOSUI (2002) analysis for prediction of transmembrane segments indicated that the FMR was soluble protein. BLAST search with peptide sequences of known proteins in databases indicated that the FMR was most related to CPR of the fungus *Beauveria bassiana* (identities 51%), which was followed by other fungi such as *Aspergillus niger* (50%), *Cunninghamella elegans* (43%), and *S. cerevisiae* (43%). Alignment of the deduced amino acid sequences of the FMR and representative CPRs from taxonomically diverse species are shown in Fig. 3.1.5. The

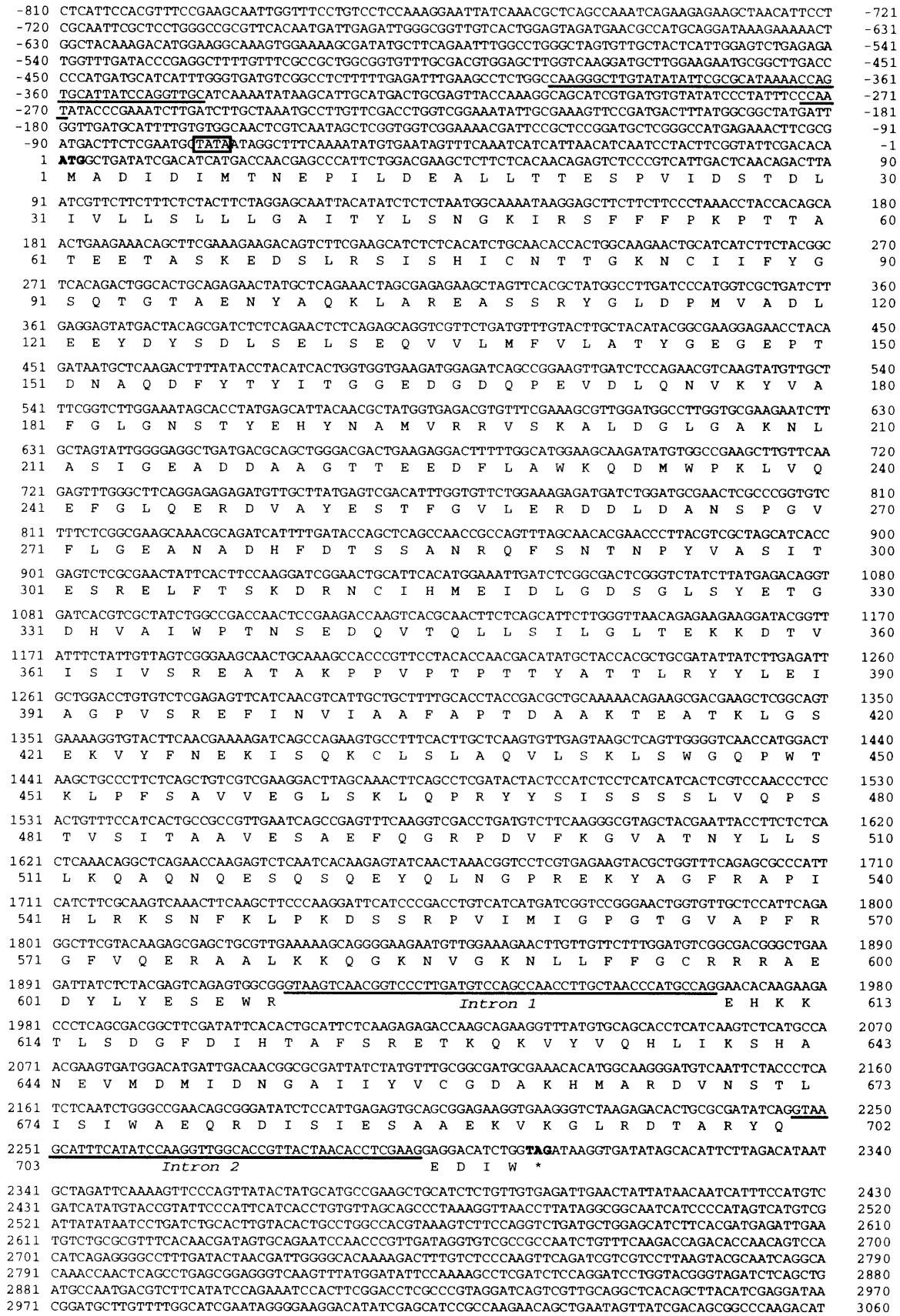


FIG. 3.1.4. Nucleotide and deduced amino acid sequences of *FMR* gene. Numbering for the sequence begins with the putative initial ATG codon. The start codon and the stop codon are shown in boldfaces. Introns are indicated with underlines. A CAAT box and a putative promoter resulting NNPP analysis are underlined. A possible TATA signal is in a box.

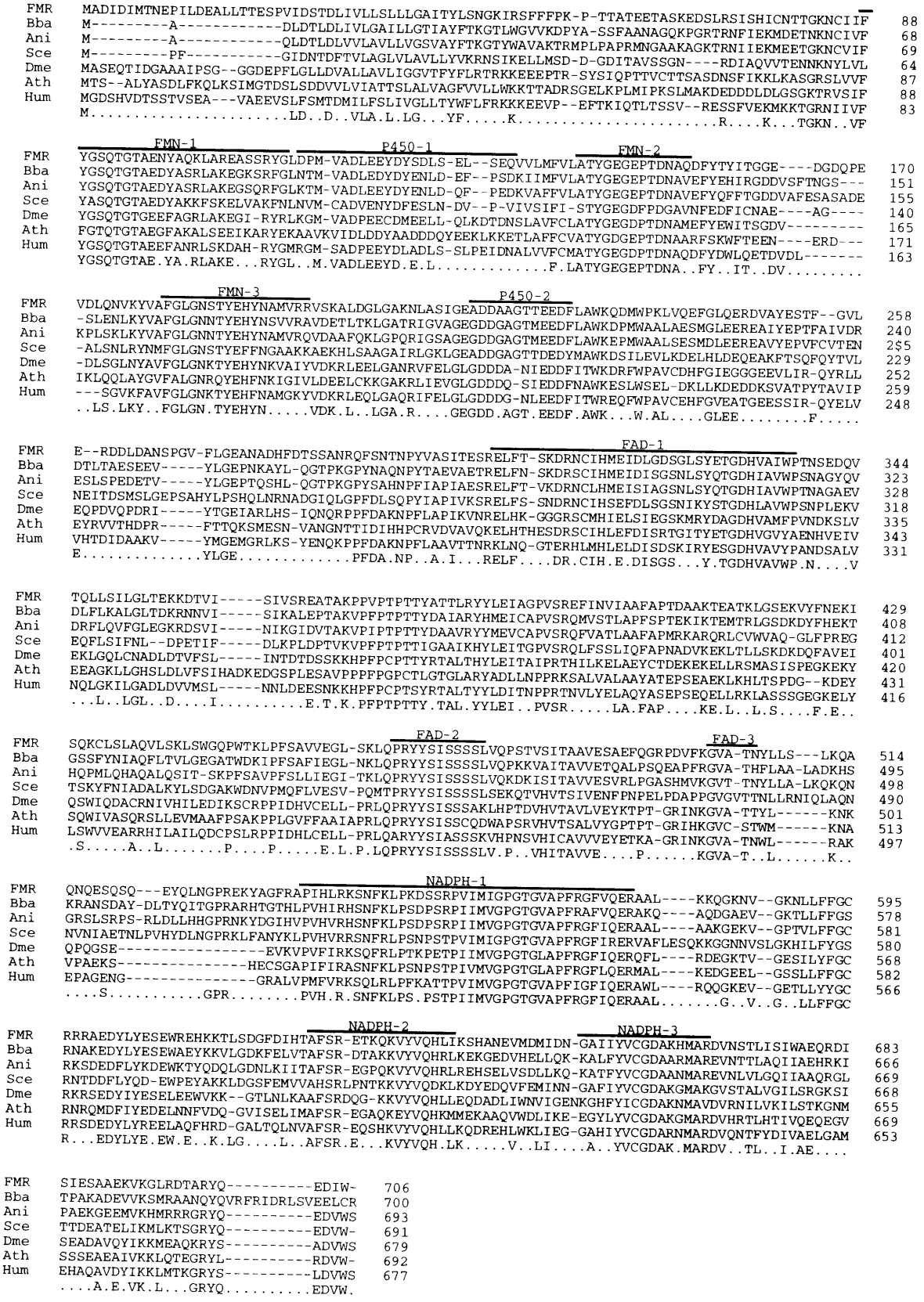


FIG. 3.1.5. Alignment of the deduced amino acid sequences of FMR and representative CPRs from taxonomically diverse species. Positions of the proposed functional domains are represented by the overlying lines. Consensus amino acid residues underlie the sequences. Bba, *Beauveria bassiana*; Ani, *Aspergillus niger*; Sce, *Sacchromyces cerevisiae*; Dme, *Drosophila melanogaster*; Ath, *Arabidopsis thaliana*; Hum, Human.

binding domains for FMN, FAD, and NADPH were all highly conserved. A domain for P450-binding, P450-1, was conserved in all organisms except a plant *Arabidopsis thaliana*.

Expression of CPR gene in *S. cerevisiae* *S. cerevisiae* INV*Sc*1 showed NADPH-cytochrome *c* reductase activity (Fig. 3.1.6). The activity of INV*Sc*1/pYES2.1/*lacZ*, which was in pYES2.1 TOPO TA cloning kit as expression control plasmid, was the same as the activity of the strain INV*Sc*1. The activity of the strain INV*Sc*1/pYES-fmr was higher than activities of other strains and the specific activity was the highest at 44 h after induction. RT-PCR with total RNA of the strain INV*Sc*1/pYES-fmr produced a band at the size of 2.1 kb (Fig. 3.1.7).

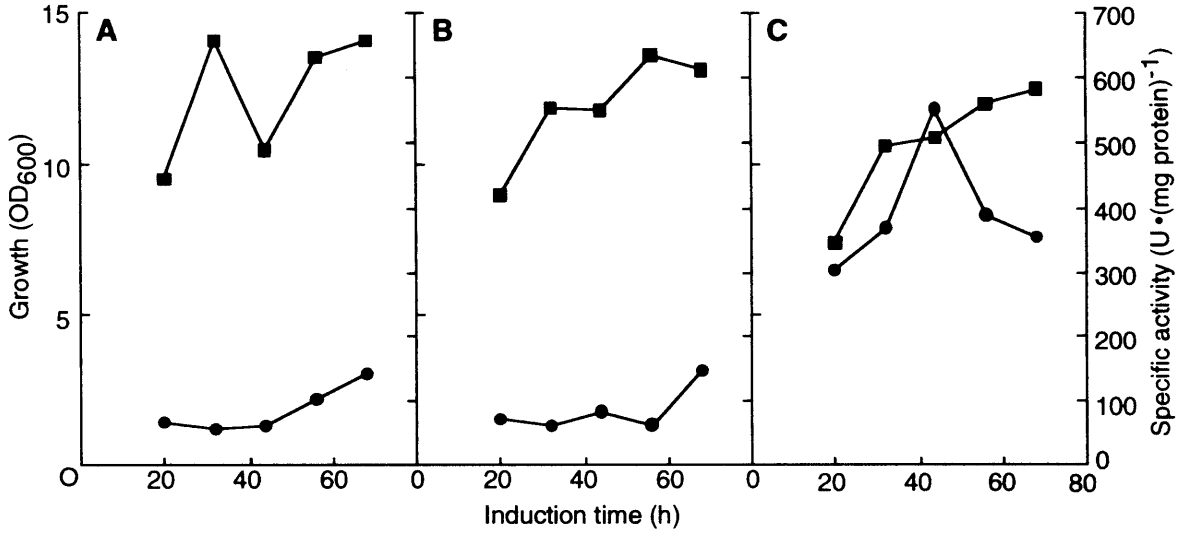


FIG. 3.1.6. Growth and CPR activity of *S. cerevisiae* INV*Sc*1 (A), INV*Sc*1/pYES2.1/*lacZ* (B), and INV*Sc*1/pYES-fmr (C) in the induction cultures. Sampling was at 20, 32, 44, 56, and 68 h after cells grown in SC or SC–U medium were transferred to induction medium. Symbols: squares, growth; circles, specific activity of CPR.

Effect of NADPH-generating systems on propylbenzene hydroxylation Since addition of coenzymes and its generating system in the reaction increased the yield, NADPH and its generating systems were added to the reaction mixture with cells of *F. moniliforme* strain MS31. Hydroxylations of propylbenzene were decreased in reaction mixtures

containing glucose (Table 3.1.1). Addition of NADPH only or with CPR did not increase the hydroxylation.

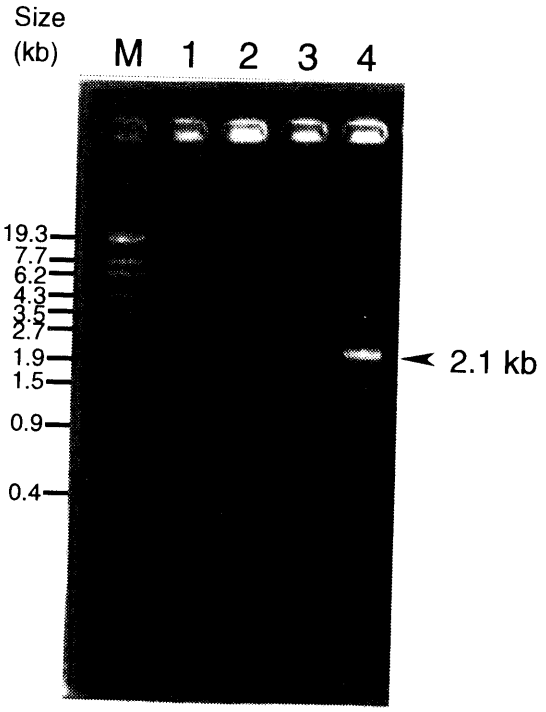


FIG. 3.1.7. RT-PCR to amplify the expressed FMR gene in *S. cerevisiae* INV*Sc*1. Lanes, M, λ-EcoT14 I digest; 1, INV*Sc*1 without RT; 2, INV*Sc*1 with RT; 3, INVSc1/pYES-fmr without RT; 4, INV*Sc*1/pYES-fmr with RT.

TABLE 3.1.1. Effects of NADPH and its generating systems on hydroxylation of propylbenzene by cells of *F. moniliforme* strain MS31.

Condition	Relative activity (%)
None	100[d]
NADPH	95
Glucose	84
NADPH + glucose + GDH[a]	76
NADPH + CPR_{yeast}[b]	101
NADPH + CPR_{fmr}[c]	102

[a]Glucose dehydrogenase.

[b]The cell extract from 44-h induction culture of *S. cerevisiae* INV*Sc*1. There was 21 U of CPR per 1 ml of the reaction mixture.

[c]The cell extract from 44-h induction culture of INV*Sc*1/pYES-fmr. There was 266 U of CPR per 1 ml of the reaction mixture.

[d]Used as the control (100%). There was 0.1 g of cells (11 U) of *F. moniliforme* strain MS31 per 1 ml of the reaction mixture.

DISCUSSION

Using degenerate primers on genomic DNA of *F. moniliforme* strain MS31, a fragment was generated and the partially deduced amino acid sequence matched to other CPR proteins. Similar primers were used in the cloning of CPR genes from *Aspergillus niger* (van den Brink *et al.* 1995) and *Phanerochaete chrysosporium* (Yadav and Loper 2000). In these reports, the produced fragments were used as probe for screening the cDNA libraries. cDNA library was not constructed in this study but cDNA encoding FMR of *F. moniliforme* strain MS31 was isolated by RT-PCR. Despite the low sequence identity with whole CPRs, the functional domains such as FAD-, FMN-, and NADPH-binding are highly conserved in all species. In fungal genome, introns are usually shorter than those of other eukaryotic species and many CPRs are registered in databases such as GenBank (2002) and SwissProt (2002). The techniques used in this study were useful as the shortcut in isolating fungal CPR genes.

Several lines of heterologous expression systems have been developed to produce functional P450 enzymes in *E. coli*, *S. cerevisiae,* mammalian and insect cultured cell lines, and plant cells. Many human P450 genes were cloned in *S. cerevisiae* and the recombinant P450s catalyzed the oxidation of their substrate with the yeast CPR (Yabusaki 1995). The P450-binding domain of FMR was identical with that of *S. cerevisiae* and the identity of FMR to the yeast CPR was higher than the identity of human CPR to the yeast CPR. If P450 genes of *F. moniliforme* strain MS31 is expressed in yeast, the recombinants may be able to show their own catalytic activities with the aid of yeast CPR.

In the coenzyme-dependent reactions, generation of coenzymes is useful to increase the product yield. A generation system, which is composed NADPH, glucose dehydrogenase, and glucose was added to the reaction mixture with the resting cells of *Nocardia fusca* and the production of (*R*)-3-pentyn-2-ol was increased (Xie *et al.* 1999). In this bioconversion process, even if addition of glucose only increased the yield of (*R*)-3-pentyn-2-ol because glucose acts as energy source for coenzymes *in vivo*. In the hydroxylation of propylbenzene by *F. moniliforme* strain MS31, presence of glucose decreased the yield of 1-phenylpropanol. CPR is the electron-donating protein for several known oxygenases (Fig. 3.1.8) found on the

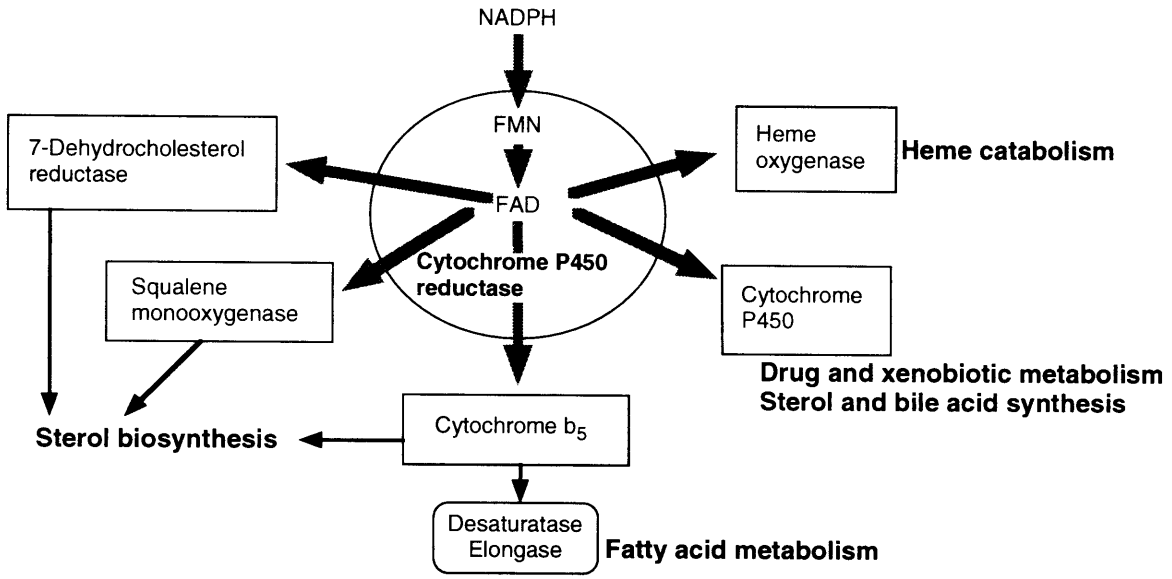


FIG. 3.1.8. The electron donating system of CPR (Porter 2002).

endoplasmic reticulum of most eukaryotic cells. The rate-limiting step of P450 reactions is the first in electron transfer from CPR to P450 (Sakaki *et al.* 1994). Introduction of CPR genes into P450-overproducing *S. cerevisiae* resulted in a considerable increase P450 enzyme activity (Imaishi *et al.* 2000; Murakami *et al.* 1990), suggesting its importance for P450 enzyme activities. Construction of P450-CPR fusion protein also was proved to be a valuable tool for increasing P450 enzyme activities in *S. cerevisiae* (Hayashi *et al.* 2000; Murakami *et al.* 1987), *Aspergillus oryzae* (Sakuradani *et al.* 1999), and transgenic tobacco plants (Shiota *et al.* 2000). Thus, generation of coenzyme might not be effective in the P450 enzyme reaction.

S. cerevisiae INV*Sc*1/pYES-fmr showed increasing NADPH-cytochrome *c* reductase activity but the activity was not detected in the cell extract of *F. moniliforme* strain MS31. Since cDNA of *FMR* has been cloned, *FMR* was certainly expressed in the strain MS31 grown in BM3 medium but its concentration was very low. In *A. niger*, the CPR gene was introduced in multiple copies in itself and a transformant showed a 14-fold increase in CPR activity (van den Brink *et al.* 1995). Such approach, if transformation system of *F. moniliforme* is established, may be useful for improvement of *F. moniliforme* strain MS31 in increasing the yield of (*R*)-1-phenylpropanol.

Section 2
Cloning of Cytochrome P450 Genes from *Fusarium moniliforme* Strain MS31

Cytochromes P450 exhibit a spectrophotometric absorption peak at or near 450 nm when carbon monoxide is bound to the reduced form of the enzyme molecules (Omura and Sato 1964). These proteins are not 'cytochromes' in the true sense of the word. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) prefers the term 'heme-thiolate protein' instead of 'cytochrome' for P450.

Many fungal transformations have been shown to be depending on P450. Studies of these fungal enzymes have been limited due to low specific content and sensitivity to homogenization and solubilization. With molecular techniques such as the differential screening and the gene disruption, some inducible P450 genes involving in metabolisms of endogenous and xenobiotic compounds are isolated and characterized (van den Brink *et al.* 1996; Mingot *et al.* 1999). Genome sequencing provides many different P450 genes (Nelson *et al.* 1996; Omura 1999; Cytochrome P450 web matrix 2002). Many of them have been expressed in yeast for identification of their functions (Del Carratore *et al.* 2000; Haudenschild *et al.* 2000; Imaishi *et al.* 2000; Le Bouquin *et al.* 1999; Oeda *et al.* 1985; Urban *et al.* 1990). In fungal genome sequencing, three different P450 genes were found in *Saccharomyces cerevisiae* complete genome. *Gibberella fujikuroi*, sexual state of *Fusarium moniliforme*, was found to have four different P450 genes in the gibberellin biosynthesis cluster (Tudzynski and H lter 1998; Tudzynski *et al.* 2001).

In this section, I isolated three cDNAs that encode P450 by RT-PCR from *F. moniliforme* strain MS31 and the cDNAs were cloned in a yeast expression system. Since the enzyme involving in the hydroxylation of propylbenzene was constitutive and the strain MS31 could not assimilate propylbenzene, degenerate primers designed from consensus region of various origins were used to isolate P450 genes.

MATERIALS AND METHODS

All experiments were as described in the section 1 unless otherwise noted.

Degenerate primers P450F (5'-GSIGGIVRISAIACIACIGC-3', forward) and P450R (5'-CCIADRCAIIIIYKIVBICCIMVIYYRAA-3', reverse), were designed based on the information in a report of Kullman and Matsumura (1997). HB-Probe (5'-GCRWAGAAMCKYCCWGGRCAKGMRT-3', reverse) was designed based on the heme-binding region of four P450s from *G. fujikuroi* (accession numbers; Y15277, Y15278, Y15279, and Y17243; Fig. 3.2.1). Another series of degenerate primer set was designed

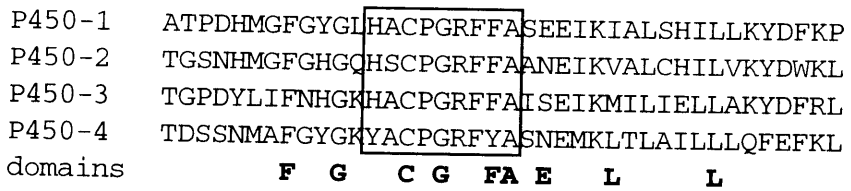


FIG. 3.2.1. Comparison of the heme-binding motifs of the four P450s from *G. fujikuroi*. Accession numbers of P450-1, P450-2, P450-3, and P450-4 are Y15277, Y15278, Y15279, and Y17243, respectively. The motifs conserved in all P450-type enzymes are designated in bold letters. A degenerate primer, HB-Probe, was designed from the highly consensus sequence; H/Y A/S C P G R F F/Y A, shown in a box.

using CODEHOP program in the World Wide Web (Rose *et al.* 1998). In designing the primers, amino acid sequences of four P450s from *G. fujikuroi* and a P450 from *Aspergillus terreus* (accession number; AAD34552) were used to design the degenerate primers. CYPF1 (5'-CAGCGCCCCGAGTACATHGARCCNYTA-3') and CYPF2 (5'-CCTGGGCCAGCACCCNGARYWNAT-3') were designed as the forward primer. CYPR1 (5'-GCAGGCGTGCTTGCCRTRNYYRAA-3'), CYPR2 (5'-TCATCTCGTTGGTGGCGWARAANCKNCC-3'), and CYPR3 (5'-CGTTGGTGGCGAAGAACCKNCCNGGRCA-3') were designed based on heme-binding domain of P450s as the reverse primer. Abbreviations are: B, C, G or T; D, A, G or T; K, G

or T; H, A, C or T; M, A or C; N, A, C, G or T; R, A or G; S, C or G; V, A, C or G; W, A or T; Y, C or T; I, hypoxanthin.

PCR PCR was done using Ex Taq (Takara Shuzo Co., Kyoto) as DNA polymerase and the reaction mixture (25 μl) contained 25 ng of genomic DNA as the template and 12.5 pmol each of forward and reverse primers. Amplification was proceeded by denaturation at 94°C for 10 min, and proceeded by repeating 30 cycles of denaturation at 94°C for 1 min, annealing between 40°C and 65°C for 1 min, and polymerization at 72°C for 2 min, and then extension at 72 °C for 10 min in a thermal cycler incubation oven (iCycler; Nippon Bio-Rad Laboratories K. K., Tokyo). The annealing temperature was in gradient mode and set at 40-55°C or 55-65°C for melting temperature of a pair of primers. For cDNA, 1 μl of RT-reaction mixture was used as the template in a 25-μl PCR reaction mixture, DNA polymerase was Ex Taq-hot start version (Takara), and the first denaturation was for 3 min.

Transformation PCR products were isolated from electrophoresis gels, purified, and ligated into a T-tailed pCR 2.1 vector in the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) or a T-tailed pYES 2.1 vector in the pYES 2.1 TOPO TA cloning kit (Invitrogen). The ligation mixture was used to transform *E. coli* JM 109 for purification of the plasmid and DNA sequencing. The whole-length cDNA of P450 genes in pYES 2.1 vector were used to transform *S. cerevisiae* INV*Sc*1.

Expression of P450 genes *S. cerevisiae* INV*Sc*1 and its transformants were cultivated as described in the section 1. Cells were collected and used in the cell reaction with propylbenzene as substrate.

Cell reaction Wet cells (dry weight , 10 mg) were suspended in 0.1 ml of 100 mM potassium phosphate buffer (pH 7.0; KPB) and mixed with 10 μmol (1.2 μl) of propylbenzene in a 1.5-ml Eppendorf tube. The reaction and assay of the activity were as described in Chapter 2.

RESULTS

Cloning of a novel P450 gene RT-PCR was done with total RNA in the reaction mixture for PCR amplification with a pair of forward and reverse degenerate primers. Using a combination of P450F and P450R, the product was not detected in agarose gels. Another pair, P450F and HB-Probe, was used in PCR and two products were detected in agarose gels at 1.2 kbp and 0.9 kbp and extracted from the gels. One of the three deduced amino acid sequences derived from the 1.2 kbp nucleotide sequence was found to match a region of P450 proteins by BLAST search (GenomeNet 2002). The DNA sequence containing whole the gene was determined by gene walking procedure. A 4,400-bp sequence was generated through sequencing the genomic DNA in both directions. Three deduced amino acid sequences were obtained from the nucleotide sequence of the genomic DNA. By analysis of the conserved heme-binding region and the putative promoter regions, a forward primer was designed to contain the putative start codon sequence (CYP-start; 5'-GATATGGCTCGTGGATGGTATAAGTA-3') and a reverse primer was designed to contain the putative end codon sequence (CYP-end; 5'-GGTGAGACTTAGTATACAAATAACCCC-3'). PCR with the primer pair for the single-strand cDNA library resulted in the formation of a single band at 1.6 kbp. This fragment was isolated from gels, purified, and ligated into a T-tailed pYES 2.1 vector to construct pYES-fmp5. The nucleotide sequence and its deduced amino acid sequence are shown in Fig. 3.2.2 with genomic sequence. The translation-starting codons were surrounded by the fungal consensus sequence TCAMM**ATG**KC (Ballance 1986). A comparison of the genomic and cDNA sequence of the gene led to the identification of four introns. A CCAAT box beginning at the –1180 nucleotide position was found. The polyadenylation signal AATAAA is not found.

Deduced protein The deduced amino acid sequence derived from *FMP5* was generated Fmp5, which was composed of 537 amino acid residues (Fig. 3.2.2) and its calculated molecular weight was 61,716. A possible membrane anchor domain was found between 40 and 61 amino acid position by SOSUI (2002) analysis for prediction of

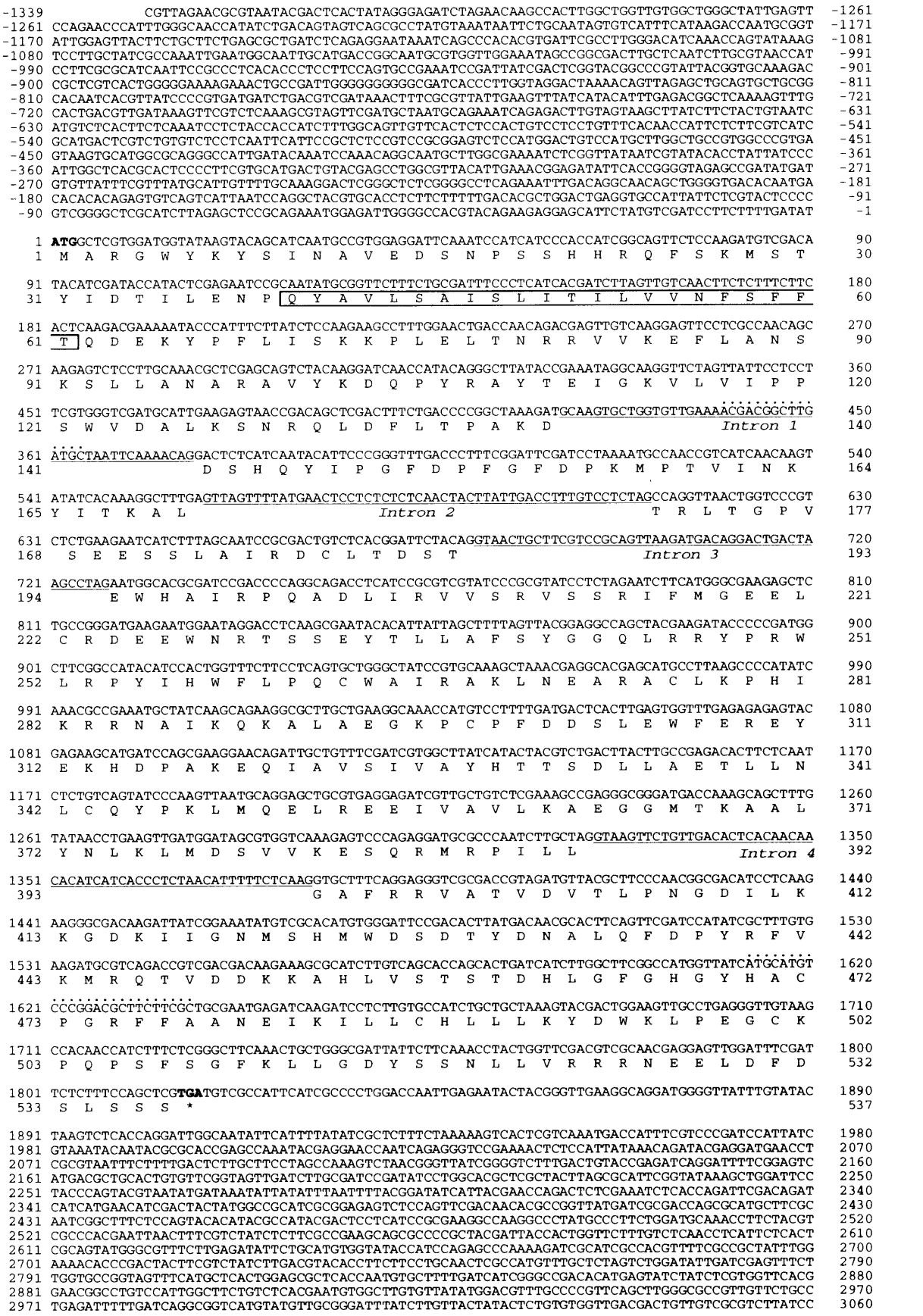


FIG.3.2.2. Nucleotide and deduced amino acid sequences of *FMP5* gene. Numbering for the sequence begins with the putative initial ATG codon. The start codon and the stop codon are shown in bold letters. Introns are indicated with underlines. A putative membrane-anchor domain is in a box. The mucleotide that annealed with degenerate primers, P450F and HB-Probe, is marked by an overlying dot.

FIG. 3.2.3. Alignment of deduced amino acid sequences of P450s. Positions of the proposed function domain is represented by the overlying lines. Consensus amino acid residues are shown in lines "Cons" under the sequences. Ncl, *Neurospora crassa lov*A, the accession number is T49413; GFP1–4, P450s in gibberellin biosynthesis cluster of *G. fujikuroi.*

transmembrane segment. BLAST search with known protein sequences in databases found 498 matches, all belonging to the P450 family. The most similar protein, P450 (*lov*A) from *Neurospora crassa* and four P450s from *G. fujikuroi* are aligned in Fig. 3.2.3. Three characteristic domains for P450 proteins, which are helix-K, aromatic, and heme-binding, are conserved in all P450s. The deduced amino acid sequence of FMP5 shares 38, 36, 35, 22, and 26% identity with *lov*A, P450-1, P450-2, P450-3, and P450-4, respectively.

Cloning of cDNA encoding P450-1 and P450-2 All six combinations of degenerate primers designed by CODEHOP program were used in the PCR with genomic DNA or single-strand cDNA as the template. The PCR products of about 500 bp were purified and cloned. In deduced amino acid sequences of genomic DNA clones, 3 and 2 clones matched *P450-2* and *P450-4*, respectively. In cDNA clones, 23 clones matched *P450-2*. The identities in the nucleotide sequence of all 28 clones were 98% or more. Other clones did not match P450. *G. fujikuroi* has four P450 genes in the gibberellin biosynthesis gene cluster. To determine the presence of the same genes in *F. moniliforme*, a primer pair of each P450 in *G. fujikuroi* (Fig. 3.2.4) was used to PCR. When genomic DNA was used as template, the PCR gives rise to the expected DNA fragments of 1,736 bp, 1,903 bp, 1,456 bp, and 1,727 bp for *P450-1*, *P450-2*, *P450-3*, and *P450-4*, respectively (Fig. 3.2.5).

P450-1:	GFP1-start	5'-**G**AAATGGCGAACCATTCTTCTTCTTAC
	GFP1-end	5'-CAGCAATCAAATCGCAATCTCCTCC
P450-2:	GFP2-start	5'-GAAATG**G**GCATCTTCAATATGATCACCAGCT
	GFP2-end	5'-TCAAATTGCTTCCAAATCCAACTCAACTGAC
P450-3:	GFP3-start	5'-**G**TCATG**G**AATACACAACATGCCAAATG
	GFP3-end	5'-TCATCTCCTTCGCATCTCAAGACCA
P450-4:	GFP4-start	5'-**G**GAATG**G**GTAAGTCCAACAGCATGAA
	GFP4-end	5'-TCATTCATCTCTCAGTGATCGCTTCC

FIG. 3.2.4. Primers to be used for P450s found in gibberellin biosynthesis cluster of *G. fujikuroi*. The start codon and the compliment of end codon are indicated with a single underline and double underlines, respectively. Nucleotides shown with boldfaces were changed for the desired expression in yeast cells so that the sequences match the genomic DNAs with highly identity considering most common codon usage in yeast.

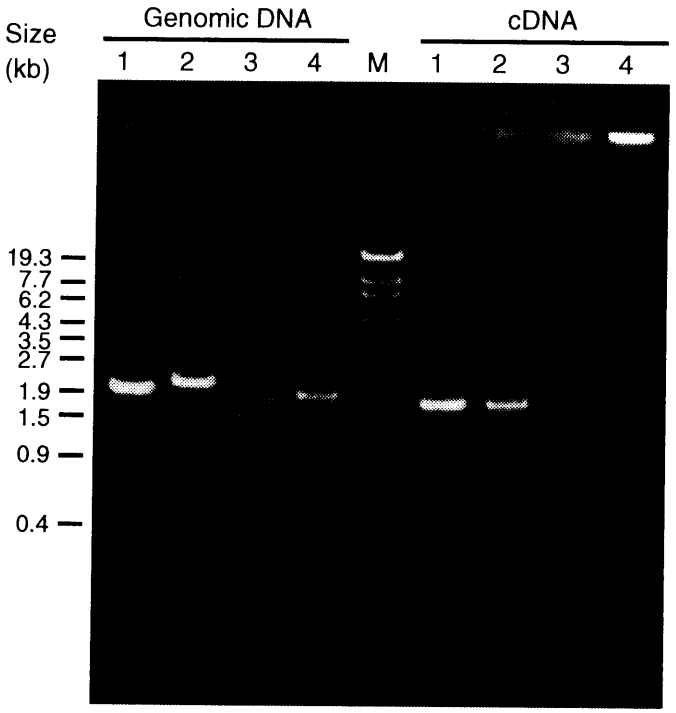


FIG. 3.2.5. PCR analysis of P450 genes in the gibberellin biosynthesis cluster of *G. fujikuroi*. The template of PCR was genomic DNA or single-strand cDNA of *F. moniliforme* strain MS31, the annealing temperature was 60°C. PCR amplification products (5 µl from 25 µl of reaction mixture) were electrophoresed in 1% agarose gels. 1, *P450-1*; 2, *P450-2*; 3, *P450-3*; 4, *P450-4*; M, λ-EcoT14 I digest.

The partial nucleotide sequence of each DNA fragment matched its corresponding gene of *G. fujikuroi*, and the identity was 98% or more. When single-strand cDNA was used as the template, *P450-1* and *P450-2* gave products but *P450-3* and *P450-4* did not (Fig. 3.2.5). The whole cDNA of *P450-1* and *P450-2* from the strain MS31 were ligated into a T-tailed pYES 2.1 vector to construct pYES-fmp1 and pYES-fmp2, respectively. Whole nucleotide sequences of *FMP1* and *FMP2* matched cDNAs of *P450-1* and *P450-2* and the identities were 96% and 95%, respectively. The deduced amino acid sequence of FMP1 was compared with that of P450-1 (Fig. 3.2.6). Ten amino acid residues surrounding the possible membrane-anchor domain were different from those in *G. fujikuroi*. In the deduced amino acid sequence of FMP2, all eight amino acid residues were different (Fig. 3.2.7).

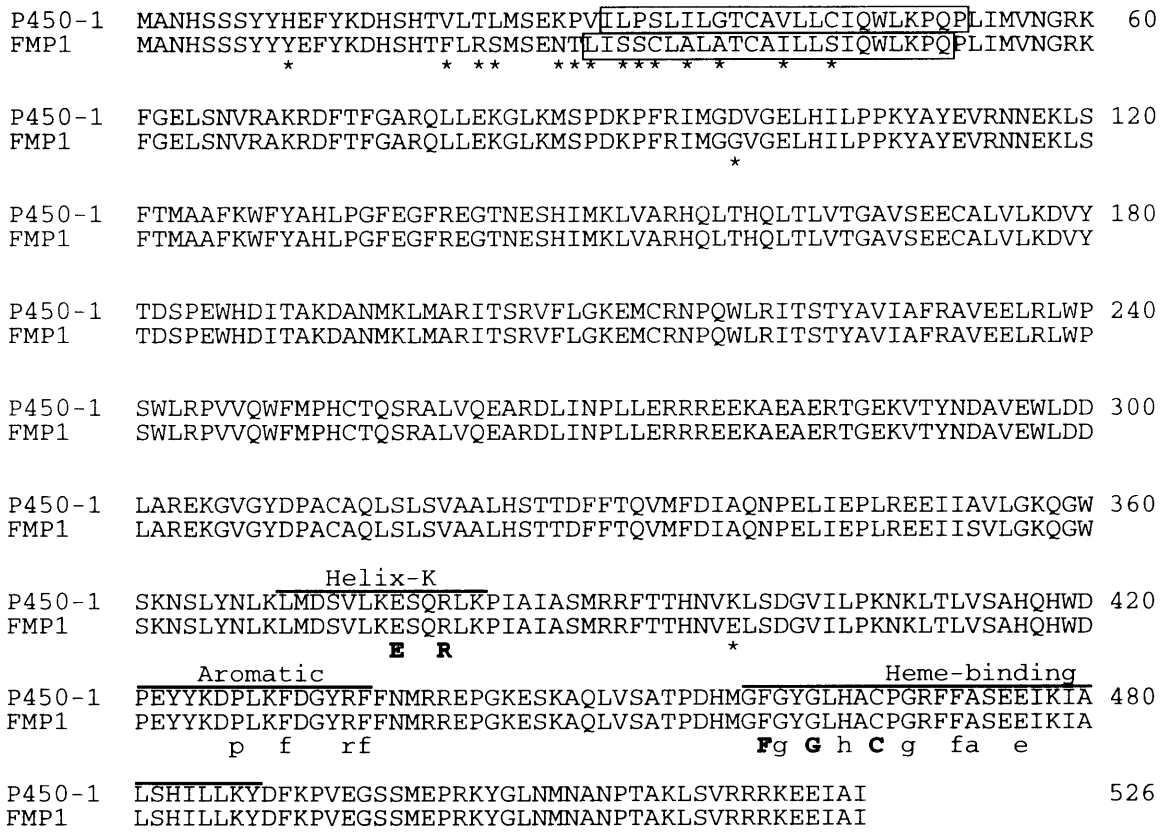


FIG. 3.2.6. Comparison of the deduced amino acid sequence of P450-1 from *G. fujikuroi* and FMP1 from *F. moniliforme* strain MS31. Position of the consensus sequence for the domains is represented by overlying lines and highly and relatively conserved amino acid residues are shown as bold and lower-case symbols underlying the lines of sequences. Asterisks indicate positions of different amino acids. Putative membrane-anchor domains are in boxes.

Expression of P450 genes in yeast To see whether or not the recombinant genes are expressed in *S. cerevisiae* INV*Sc*1, RT-PCR was done with total RNA of the strain INV*Sc*1 and its transformants as the template. Products corresponding to *FMP1*, *FMP2*, and *FMP5* were detected in the PCR mixtures with a cDNA library of INV*Sc*1/pYES-fmp1, INV*Sc*1/pYES-fmp2, and INV*Sc*1/pYES-fmp5, respectively (Fig. 3.2.8). Hydroxylation of propylbenzene was not detected in reaction mixtures with cells of the strain INV*Sc*1 or its transformants.

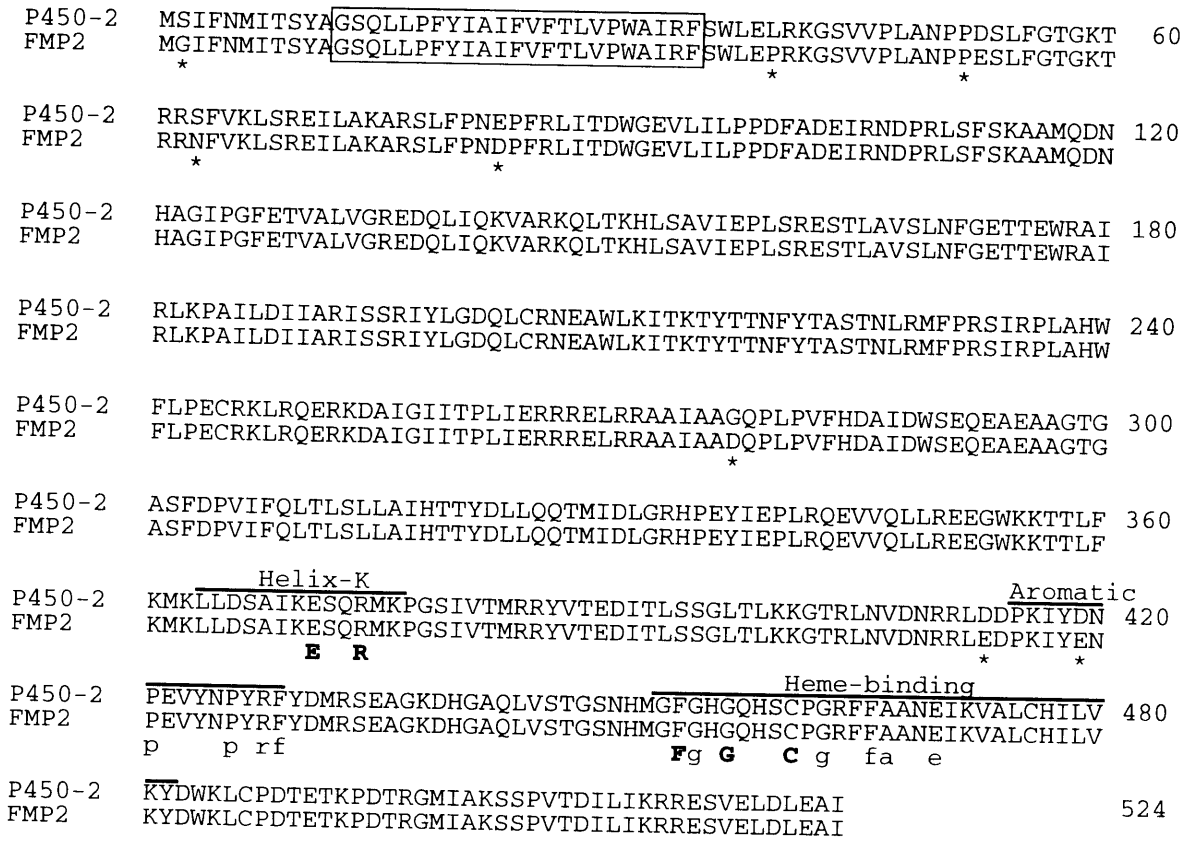


FIG. 3.2.7. Comparison of the deduced amino acid sequence of P450-2 from *G. fujikuroi* and the FMP2 from *F. moniliforme* strain MS31. Position of the consensus sequence for the domains is represented by overlying lines and highly and relatively conserved amino acid residues are shown as bold and lower-case symbols underlying the lines of sequences. Asterisks indicate positions of different amino acids. A putative membrane anchor domain is in a box.

DISCUSSION

The enzymic characteristics of the cell extract of *F. moniliforme* strain MS31 suggest that the hydroxylation of propylbenzene is involved in P450 monooxygenase system. In Chapter 2, CO difference spectra of the cell extract did not show the presence of P450 in the cells. Three cDNA encoding P450 were cloned in this section, which fact clearly indicate the presence of P450 enzymes in the cells.

P450s, which exhibit more than 40% amino acid sequence identity are classfied as belonging to the same gene family (Nelson *et al.* 1996; Nelson 1999; Cytochrome P450 web matrix 2002). From this result, FMP5 seems distinct from others in the CYP families.

The most similar protein, *lov*A from *N. crassa* is named CYP68D1. Although identity between P450-1 and P450-2 is only 37%, the Nomenclature Committee rule allows the classification of them in a single family, CYP68 (Tudzynski and H lter 1998). Since identities between FMP5 and *lov*A, between FMP5 and P450-1, and between FMP5 and P450-2 were the same as that P450-1 and P450-2, FMP5 seems to be in the family CYP68. The function of P450-1 have been clarified by the specific disruption of this gene and by inserting this gene into a mutant of *G. fujikuroi* that lacks the entire gibberellin biosynthesis cluster (Rojas *et al.* 2001). P450-1 converts *ent*-kaurenoic acid to GA_{14} and catalyzes four steps in the gibberellin biosynthetic pathway in this manner. Functions of *lov*A and P450-2 are not clear yet.

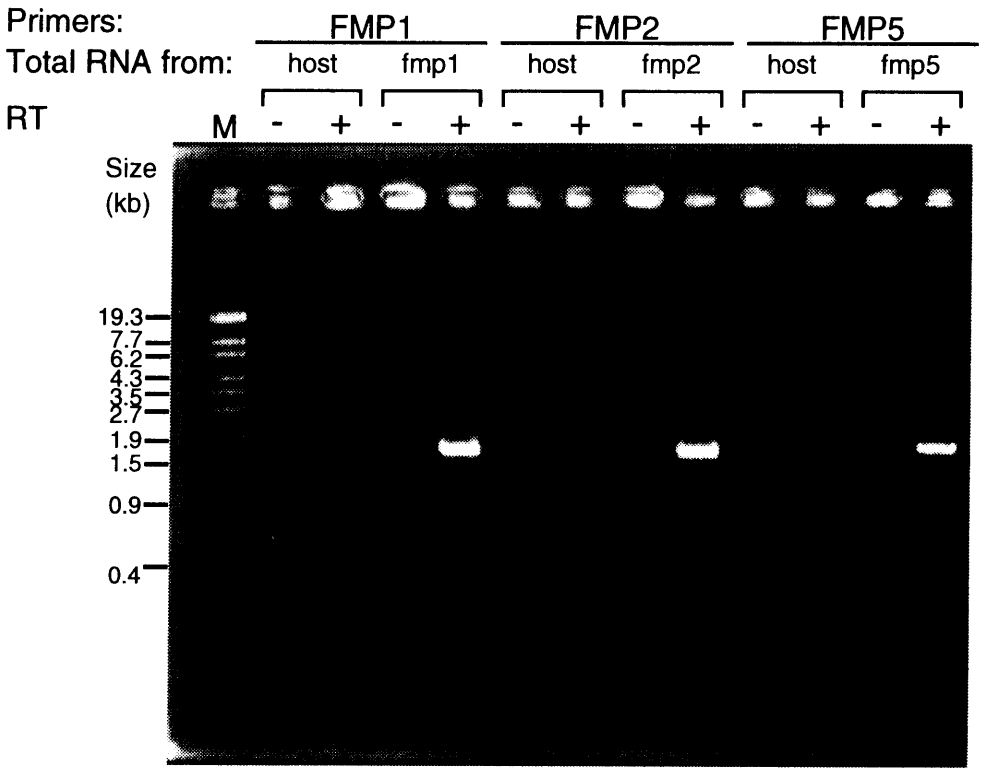


FIG. 3.2.8. RT-PCR to amplify the expressed P450 genes in *S. cerevisiae* INV*Sc*1. Total RNA was isolated from *S. cerevisiae* INV*Sc*1 (host), INV*Sc*1/pYES-fmp1 (fmp1), INV*Sc*1/pYES-fmp2 (fmp2), and INV*Sc*1/pYES-fmp5 (fmp5) as described in Materials and Methods. Primers were pairs of GFP1-start and GFP1-end (for FMP1), GFP2-start and GFP2-end (for FMP2), and CYP-start and CYP-end (for FMP5). Total RNA with RT (RT +), without RT (RT -). PCR conditions were for cDNA as described in Materials and Methods and the annealing temperature was 60°C.

The yeast cells expressing recombinant P450s did not show hydroxylation of propylbenzene. P450 is one of the most structurally diverse and functionally versatile proteins. Estimates from current genome projects imply that the number of P450 genes exceeds 50 or 70 in mammals, 80 in *Drosophila melanogaster* and *Caenorhabditis elegans*, and 400 in *Arabidopsis thaliana*. At the start of this study, fungal P450 genes are thought to be relatively few because the yeast genome yielded only three P450 genes. Some fungal genome projects are in progress and 38 different P450 genes and 163 different P450 fragments are found in *N. crassa* and *Phanerochaete chrysosporium*, respectively (Nelson 2002). Since involvement of P450 enzyme in fungal transformation has often been reported (Holland 1999; Holland and Weber 2000), a fungus may have many different P450 genes. In conversions of dehydroepiandrosterone by *F. moniliforme* occurs with the inducible P450 in the microsomes (Cotillon *et al.* 1997). For isolation of the P450 gene to be involved in the hydroxylation of propylbenzene, screening for many P450 genes is important step.

Conclusions

For screening of microorganisms useful for hydroxylation of alkylbenzenes, I isolated from soil samples microorganisms, which used methylethylketone as a carbon source. Some fungi converted ethylbenzene and propylbenzene into the corresponding oxygenated products. The most potent strain MS31 was identified as *Fusarium moniliforme*. The products were 1-phenylethanol and 1-phenylpropanol, respectively, with 100% enantiomeric excess of the (*R*)-form (section 1 of Chapter 1). To increase the hydroxylation of propylbenzene by *F. moniliforme* strain MS31, glucose and sodium nitrate were selected as carbon and nitrogen sources, respectively. Acetoin added to the medium increased activity of the enzyme by 1.4-fold. Maximum conversion of propylbenzene by cells of the fungus was at 25–30°C and pH 7.0 with cells at the concentration of 40 mg (dry) per 1 ml of reaction mixture. To the end, the fungus produced 16 mM (*R*)-1-phenylpropanol (2.2 $mg \cdot ml^{-1}$) with enantiomeric excess of 98% (section 2 of Chapter 1). The fungal cells hydroxylated various alkylbenzenes at the benzylic position to produce optically active alcohols. Butylbenzene was converted to nonbenzylic alcohols. In all cases, the *R* absolute configuration of products was more abundant. Most aromatic compounds with linear side chains and (2-methylpropyl)benzene were converted to their corresponding alcohols with an enantiomeric excess of 94% to 100% (section 3 of Chapter 1).

In the reaction mixture containing the cell extract of *F. moniliforme* strain MS31, hydroxylation of propylbenzene needed molecular oxygen and NADPH as a coenzyme. The hydroxylation proceeded further if or when FAD and FMN were added, suggesting that the enzyme was a flavoprotein. Carbon monoxide inhibited the hydroxylation, as did other cytochrome P450 (P450) inhibitors such as SKF 525A and miconazole. These characteristics matched those of microsomal P450 monooxygenase systems that contained NADPH-cytochrome P450 reductase (CPR) (Chapter 2).

A cDNA encoding CPR was isolated from *F. moniliforme* strain MS31 by RT-PCR. The deduced amino acid sequence showed a high similarity to the CPRs from other

eukaryotes and probable NADPH-, FAD-, FMN-, and P450-binding domains of the CPR were among those, which are highly conserved in taxonomically diverse species. Expression in *Saccharomyces cerevisiae* resulted in 5-fold increase in NADPH-cytochrome *c* reductase activity in the cell extract (section 1 of Chapter 3). Three cDNAs encoding P450 were isolated from the fungus. One of them was a novel P450 and its deduced amino acid sequence matched proteins, which belong to the P450 family, as studied by BLAST search. The others were similar to P450-1 and P450-2 from *Gibberella fujikuroi* and their identities in the nucleotide sequence were 96 and 95%, respectively. The cells of *S. cerevisiae* harboring the cDNAs encoding P450 did not show the hydroxylation of propylbenzene (section 2 of Chapter 3).

F. moniliforme strain MS31 was useful to produce optically active phenylalcohols with high enantiomeric excess. The activity for hydroxylation of propylbenzene was increased by conventional techniques but the yield was not enough for industrial use. Involvement of cytochrome P450 monooxygenase system in the hydroxylation of propylbenzene was suggested based on nonconclusive experiments. To increase the activity of P450 enzymes, the interaction between the electron-donating CPR and the catalytic P450 is important and the fusion product from these two proteins by genetic engineering is thought to be most useful. The CPR gene of *F. moniliforme* strain MS31 was cloned and expressed in *S. cerevisiae* functionally but the P450 gene catalyzing the hydroxylation of propylbenzene has not yet been localized. Genome sequencing reveals numerous different P450 genes and the functions of many mammalian P450 genes are clear. Some fungal genome projects are in progress and the functions of fungal P450 genes also will become easy to understand several years after.

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