Studies on Microbial Conversion of Plant Essential Oil, Eugenol

植物精油オイゲノールの微生物変換に関する研究

Hirotaka Furukawa

June, 2003

ABBREVIATIONS

CAPS	3-Cyclohexylaminopropanesulfolic acid
DCIP	2,6-Dichlorophenolindophenol
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
e.e.	Enantiomeric excess
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC-MS	Gas chromatography-mass spectrometry
HEPES	*N*-2-Hydroxyethlpiperazine-*N*'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
K_m	Michaelis constant
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PAGE	Poly acrylamide gel electrophoresis
PMS	Phenazine methosulfate
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximum velocity

CONTENTS

INTRODUCTION

Microbial catalytic activity recognized as "fermentation-phenomenon" has been used for the production of various kinds of alcoholic beverages and traditional foods for a long time. In 1929, A. Fleming found "penicillin" and H. Florey, E. Chain and N. Heatley expanded on Fleming's work in 1938. They developed methods for growing, extracting and purifying enough penicillin to prove its value as a drug. In the course of the establishment of its industrial production, various significant and fundamental techniques such as the mutation and breeding to obtain an excellent strain, and the cultivation of a single cell on a large scale, under optimized conditions, *etc.* have been developed. Since then, the application of microorganisms has covered extensively the production of various useful compounds such as antibiotics, amino acids, nucleotides, organic acids, sugars, polysaccharides, vitamin, peptides, biopolymers, flavors and fragrance chemicals.

The conventional chemical synthesis has been carried out using organic solvent and heavy metal catalysts under strong alkaline or acid conditions. On the contrary, the biocatalyst works efficiently under mild conditions in the water. Thus, the bioprocess is energy saving and friendly to environment. The microbial process has begun to use applied for the commodity chemicals such as acrylamide and nicotinamide commercially (Nagasawa *et al.* 1989). In addition, the biocatalyst is characteristic of stereospecificity and regiospecificity. Nowadays various kinds of optically active pharmaceuticals and fine chemicals are produced using the biocatalyst.

Petrochemical industry was highly developed in the 20th century, however, petroleum resources are limited and probably will be exhausted within 21st century. In addition, the petrochemical industry brought about serious environmental pollution all over the world. The drastic increase of CO_2 content in the air has caused the green house effect on the earth. Chemical industry should be changed, improved and shifted energy saving and environmentally acceptable manner. The accomplishment of the green (clean) chemistry requires the clean energy instead of petroleum. Bioprocess should be introduced into the chemical industry furthermore in 21st centuries. At the same time, the renewable biomass produced by solar energy should be used more extensively in our life. In the present thesis, the author has focused on a renewable abundant resource, eugenol.

1. Eugenol and clove oil

Eugenol (4-allyl-2-methoxyphenol) is abundant in nature. It accounts for approximately 80% (w/w) of clove oil as a main component. Eugenol has been prepared by steam distillation of clove oil obtained from buds and leaves of *Syzygium aromaticum* (Fig. 1). Eugenol has been used as aromatherapy oil, sterilizer in mouth or painkiller (Rabenhorst 1996). *Syzygium aromaticum* originally comes from Moluccas (spice island) in Republic of Indonesia. It was introduced throughout European countries at the 8th century. Today, it has been cultivated in Ambon (Republic of Indonesia), Penang and Sumatra (Malaysia), and traded at relatively low price.

Fig. 1. ***Syzygium aromaticum*** **and its dried buds**

Sweet fragrance of *Clarkia breweri* (Fig. 2) also contains some phenylpropanoids, eugenol, isoeugenol, methyleugenol and isomethyleugenol. These phenylpropanoid units such as eugenol and isoeugenol are the building block of lignin, which consists xylogen of vascular plant. The chemical structures of eugenol and isoeugenol are shown in Fig. 3.

Fig. 2. **Flower of** ***Clarkia brewer***

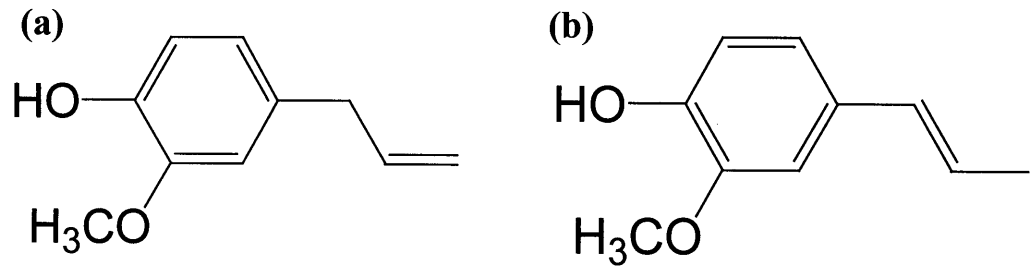

Fig. 3. **Chemical structures of eugenol (a) and isoeugenol (b)**

2. Biosynthesis of eugenol

There have been few reports regarding the biosynthesis of eugenol until now. However, as shown in Fig. 4, it is plausible that eugenol is considered as a secondary product derived from L-tyrosine. L-Tyrosine is metabolized into caffeic acid via *p*-coumaric acid or L-dihydroxyphenylalanine. Then *S*-adenosylmethionine-dependent *o*-methyltransferase might convert caffeic acid into ferulic acid. Eugenol is formed by the reduction of ferulic acid. The *S*-adenosylmethionine-dependent *o*-methyltransferase cDNA has 70% identity to caffeic acid *o*-methyltransferase. The protein encoded by the *S*-adenosylmethionine-dependent *o*-methyltransferase cDNA is able to use eugenol and isoeugenol as substrate, but not caffeic acid (Wang *et al.* 1997).

Enzymes involved in the biosynthesis of eugenol have not been elucidated until now. The biosynthetic pathway of isoeugenol is also unclear. As shown in Fig. 4, probably, isoeugenol is synthesized from ferulic acid, coniferyl alcohol or eugenol. *p*-Coumaric acid is known as a starting material for lignin biosynthesis. It is also closely related to the biosynthesis of eugenol and isoeugenol.

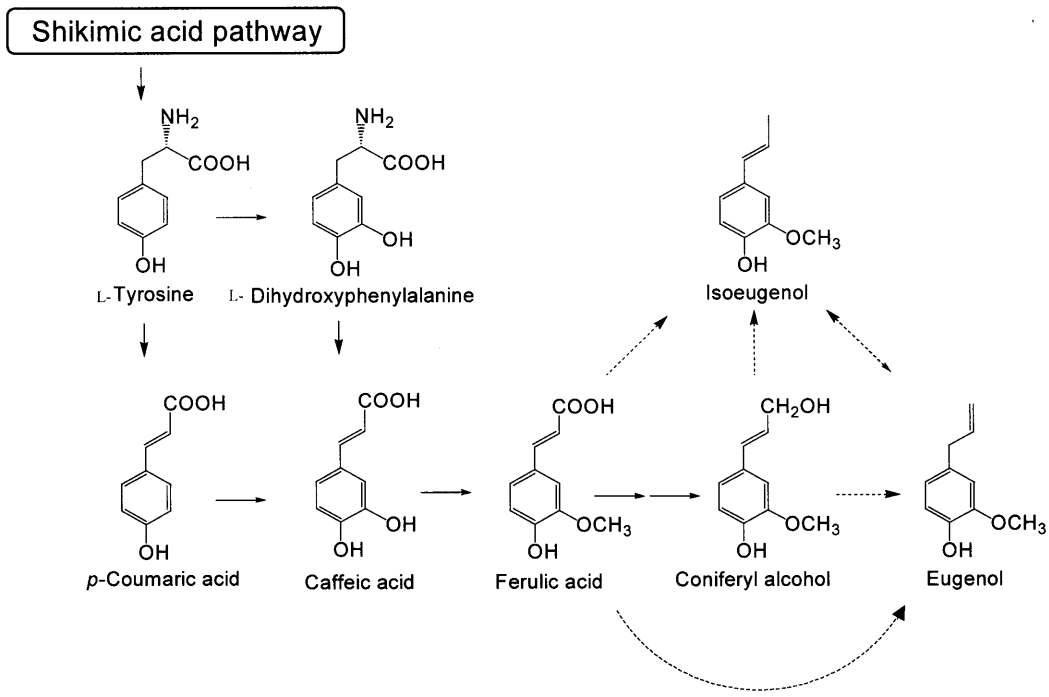

Fig. 4. **Proposed biosynthetic pathway of eugenol and isoeugenol**

3. Microbial degradation of eugenol

There are several reports regarding the microbial degradation of eugenol (Tadasa 1973, Tadasa and Kayahara 1983). *Pseudomonas* sp. and *Corynebacterium* sp. were found to metabolize eugenol to coniferyl alcohol, coniferyl aldehyde and ferulic acid (Fig. 5). It has been reported that two kinds of dehydrogenase are involved in the conversion of coniferyl alcohol into ferulic acid (Jaeger 1988, Achterholt *et al.* 1998). Ferulic acid is converted into vanillin (Huang *et al.* 1994, Rosazza *et al.* 1995) and then oxidized into vanillic acid (Bare *et al.* 2002). Protocatechuic acid is produced from vanillic acid (Buswell and Ribbons 1988). After the ring fission of protocatechuic acid, β-ketoadipic acid is generated (Dennis *et al.* 1973, Noda *et al.* 1990, Kuswandi and Roberts 1992). However, when the author started the present work, there had

been no reports regarding the enzyme to catalyze the initial step, the conversion of eugenol into coniferyl alcohol. The initial step is the double bond-transferring hydroxylation reaction.

Regarding isoeugenol, very recently, *Bacillus* sp. grown on isoeugenol as a sole carbon source was isolated (Shimoni *et al.* 2000). This strain degrades isoeugenol, however, the enzymatic studies on the degradation of isoeugenol has not been carried out yet. Thus, studies on the degradation pathway of isoeugenol are still untouched.

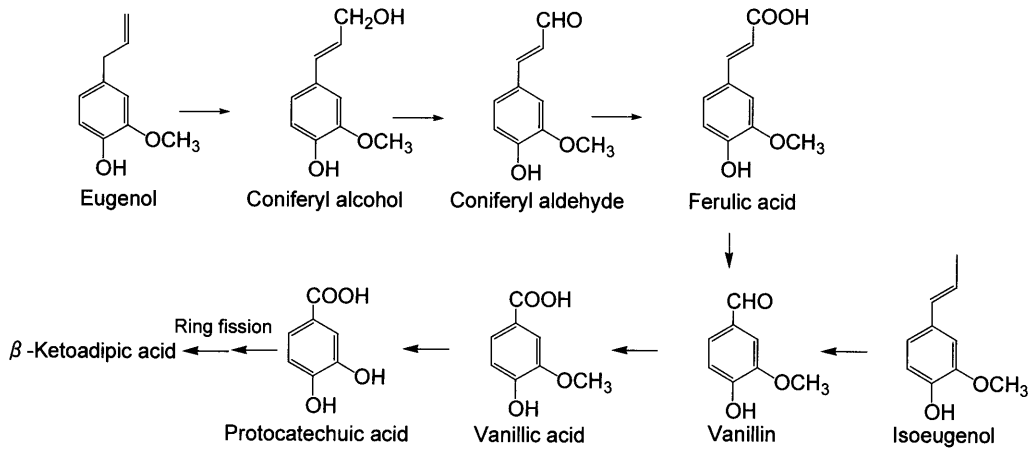

Fig. 5. **Microbial degradation pathway of eugenol and isoeugenol**

4. Useful chemicals involved in the degradation pathway of eugenol

Some useful compounds, vanillin, ferulic acid and coniferyl alcohol, are involved in the degradation pathway of eugenol, as shown in Fig. 5. They are characterized as follows,

Vanillin Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the characteristic aroma component of the vanilla pod. It is one of the most important aromatic flavor compounds used in the production of flavors for foods and fragrances for perfumes. More than 12,000 tons of synthetic vanillin is produced each year, mostly from petrochemicals such as guaiacol, and it has been also currently produced from lignin as a by-product of wood pulping (Clark 1990). Vanillin has been reported as a metabolic intermediate in the catabolism of phenylpropanoids such as eugenol and ferulic acid (Chen *et al.* 1982, Shiotsu *et al.* 1989, Tadasa 1977, Toms and Wood 1970).

Ferulic acid Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is potential due to its phenolic nucleus and an extended side chain conjugation. Ferulic acid readily forms a resonance stabilized phenoxy radical, which accounts for its potent antioxidant potential. Recently, it has been used as a food additive. In addition, due to the high degree of conjugated unsaturated bond, it is a strong UV absorber (Graf 1992).

Coniferyl alcohol Coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) is of interest for the foods, pharmaceuticals and cosmetics due to its widespread application as an antioxidant and flavor. It has been used for the studies of the synthesis of lignin (Pannala *et al.* 1998, Chan *et al.* 1995, Wu *et al.* 1998, Deigner *et al.* 1994).

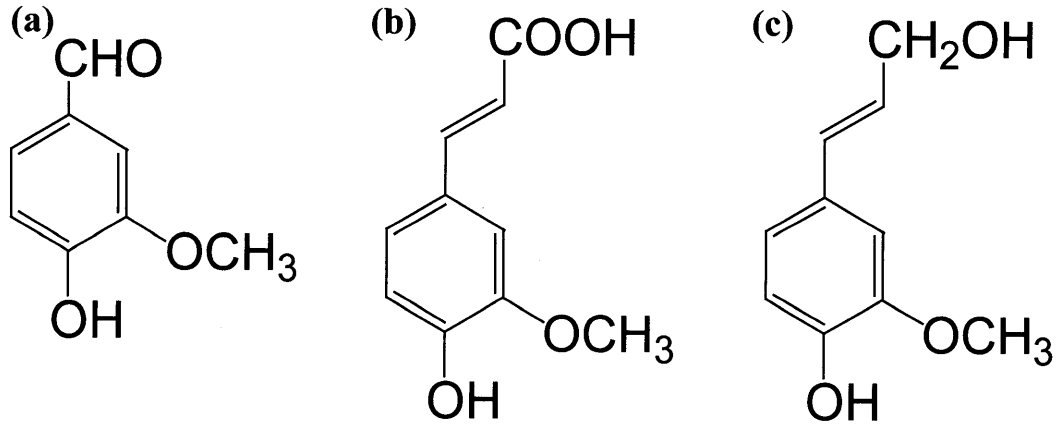

Fig. 6. **Chemical structures of vanillin (a), ferulic acid (b) and coniferyl alcohol (c)**

5. Purpose of this thesis

Compared to the conventional organic synthesis, bioprocess is energy saving and environmentally friendly. Enzymatic reactions proceed very efficiently under mild conditions. Moreover, the products through bioconversions are recognized as natural product legally (Lesage-Meessen *et al.* 1996). Therefore, the products synthesized through microbial reaction can have value added commercially. In addition, eugenol and isoeugenol are abundant cheap renewable resources. It is significant to convert eugenol and isoeugenol into more valuable compounds as described above. The author has attempted to carry out the enzymatic studies on the initial step of eugenol degradation. The author also has undertaken to throw light on the microbial degradation of isoeugenol. The author aims to establish new microbial production process of some valuable compounds from eugenol based on these fundamental knowledge attained through these studies.

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CHAPTER I

Studies on Eugenol Dehydrogenase Catalyzing Initial Step of Eugenol Degradation by *Pseudomonas fluorescens* E118

Section 1

Isolation of Eugenol-Degrading Bacterium, *Pseudomonas fluorescens* E118: Purification and Characterization of Eugenol Dehydrogenase

Eugenol is an abundant, lignin-related essential oil from clove tree *Syzigium aromaticum* (sy. *Eugenia cariophyllus*) used in perfume and aroma compositions due to its oriental and spicy odor (Rabenhorst 1996) and is of interest as renewable resource for the production of useful chemicals. An understanding of the biochemical mechanisms involved in the biotransformation of eugenol is required as a theoretical basis for the development of biocatalytic processes for the production of eugenol-derived aromatic chemicals. Eugenol is catabolized by *Pseudomonas* and *Corynebacterium* bacteria via coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, vanillic acid and protocatechuic acid (Tadasa and Kayahara 1983, Rosazza *et al.* 1995), the latter of which is the substrate of further ring cleavage and β-ketoadipate pathway. A few eugenol-converting enzymes have been reported, including vanillyl alcohol oxidase from *Penicillium simplicissimum* (De Jong *et al.* 1992) and 4-ethylphenol methylenehydroxylase from *Pseudomonas putida* DJ1, which acts mainly on 4-alkylphenols (Reeve *et al.* 1989). Similar enzymes that convert 4-alkylphenols are *p*-cresol methylhydroxylases from various sources (Rudolphi *et al.* 1991, Hopper *et al.*

1991, Kim *et al.* 1994). All the enzymes catalyze the oxidation of the side chain of benzyl compounds bearing a *para*-hydroxy group in common. By using eugenol as screening substrate, an effective eugenol-degrading microorganism has been obtained. In this section, the author purified and characterized eugenol dehydrogenase, the first enzyme of the eugenol degradation pathway in *Pseudomonas fluorescens* E118 and shows that versatile catalytic property of the dehydrogenase closely resembles those of 4-alkylphenol methylhydroxylases and vanillyl alcohol oxidase.

MATERIALS AND METHODS

Culture conditions

Cultures were incubated at 28°C with reciprocal shaking. A 4-ml overnight preculture, grown on 5 g polypeptone (Wako, Osaka, Japan), 5 g meat extract (Kyokuto, Tokyo, Japan), 0.5 g yeast extract (Oriental Yeast, Tokyo, Japan) and 5 g NaCl per liter (pH 7.0), was used to inoculate a 2-l flask with 400 ml medium consisting of 1 ml eugenol, 10 g sucrose, 1 g polypeptone, 3 g yeast extract, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 2 g K_2HPO_4 and metals in final concentrations of 0.2 mg $FeSO_4 \cdot 7\ H_2O$, 0.4 mg $CaCl_2 \cdot 2\ H_2O$, 0.3 mg H_3BO_3, 0.04 mg $CuSO_4 \cdot 5\ H_2O$, 0.1 mg KI, 0.4 mg $MnSO_4 \cdot 7\ H_2O$ and 0.2 mg $NaMoO_4 \cdot 2\ H_2O$ per liter (pH 7.0). After 24 h of incubation, cells were harvested by centrifugation at 8,000 × *g* at 4°C and washed with 50 mM potassium phosphate buffer, pH 7.0 (buffer A).

Enzyme assay

The standard activity was assayed at 30°C in a 1 ml shaken (160 strokes min^{-1}) reaction containing 10 µmol eugenol, 50 mg Plysurf A210G (Daiichi Kogyo, Osaka, Japan) for the solubility of eugenol, 10 µmol PMS and an appropriate amount of enzyme in buffer A. The reaction, shown to be linear in the first 10 min, was stopped after 10 min by adding 1 ml methanol, and the formation of coniferyl alcohol was determined by HPLC. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µmol coniferyl alcohol per min. The substrate specificity was determined using a photometric activity test in a 1-ml quartz cuvette under the same reaction conditions as above except that 150 µM PMS and additionally 75 µM DCIP were used, and the reaction was followed by measuring the reduction of DCIP at 600 nm. DCIP was reduced both by the enzyme and chemically by PMS. PMS was added as the better enzyme substrate compared to DCIP in order to support the reduction of DCIP. The activity of the complex spectrophotometric test, which could have been influenced by the ionic strength, was in good agreement with the activity determined in the stopped reaction test.

Enzyme purification

After each purification step at 4°C the enzyme was dialyzed and dissolved in the equilibration buffer of the next chromatographic step unless otherwise specified. Cells from 12 l culture (20 g wet weight) in 60 ml buffer A were disrupted 15 min by ultrasonication (19 kHz, Insonator 201M, Kubota, Tokyo, Japan) for 15 min and centrifuged at 20,000 × *g* for 30 min. After

fractionation of the crude extract with ammonium sulfate (30–50% saturation) and centrifugation as described above, the enzyme was loaded on a DEAE-Sephacel column (4 × 25 cm) previously equilibrated with buffer A, eluted by 1.2 l of buffer A containing 0.1 M KCl, and applied to a Phenyl-Sepharose CL-4B (2 × 15 cm) previously equilibrated with buffer A containing ammonium sulfate at 5% saturation. The enzyme was eluted by 300 ml of this buffer containing 30% (v/v) ethylene glycol, fractionated with ammonium sulfate (40–70% saturation) and loaded onto a Butyl-Toyopearl 650M (2 × 10 cm) equilibrated with buffer A containing ammonium sulfate at 15% saturation. Activity was eluted at 9% ammonium sulfate within a linear gradient of 15–0% (300 ml), followed by chromatography on DEAE Sephacel (2 × 10 cm) equilibrated and eluted (180 ml) as described. The purified enzyme was dialyzed against buffer A containing 50% (v/v) glycerol and stored at –20°C without activity loss for 6 months.

Enzyme characterization

The stability of the enzyme against pH and temperature was examined by preincubation for 30 min in 0.1 M buffers of various pH values at 30°C and for 10 min in buffer A at various temperatures, respectively. The buffers used for the determination of the pH influence on the enzyme activity were sodium acetate (pH 4–6), potassium phosphate (pH 6–8), HEPES (pH 6.5–8), Tris-HCl (pH 7.5–9) and CAPS (pH 9.5–11). The effects of pH and temperature on the enzyme activity were determined in the stopped test. The optimal temperature leading to the highest product formation in the 10 min standard reaction with buffer A was the resultant of the effect of temperature on the reaction rate and the stability of the

enzyme. Apparent K_m values were estimated from Lineweaver Burk plots with an accuracy of ± 6%, without performing a full two-substrate analysis. The K_m values of electron acceptors were determined in the stopped reaction test with fixed eugenol concentration of 10 mM and varying concentrations of electron acceptors in the range from 50 μM to 20 mM. The K_m values of substrates were determined by the photometric test using fixed concentrations of 150 μM PMS and 75 μM DCIP and a substrate concentration range from 5 μM to 10 mM. Inhibitors were tested by 15 min preincubation of the enzyme with 1 mM compound in the standard reaction mixture without substrate and started by the addition of eugenol. The incubation time for the inhibition of resting cells by KCN was 30 min. Products of the enzymatic conversion of different substrates were isolated with yields of 35–50% by ethylacetate extraction, evaporation, silica gel chromatography (Wakogel C300, Wako, Osaka, Japan) with benzene/methanol (95:5, v/v) as mobile phase and crystallization, and identified by ^{1}H-NMR and GC-MS using authentic compounds as references. The oxygen source of the hydroxylation was examined in a standard reaction containing 0.5 units enzyme and 50% (v/v) $H_2{}^{18}O$ followed by product isolation as described and GC-MS analysis.

Analytical methods

Coniferyl alcohol and eugenol were determined by HPLC on a Shimadzu LC-6A (Shimadzu, Kyoto, Japan) with an ODS C18 column (4.6 × 150 mm, M&S Instruments, Tokyo, Japan) and a methanol/H_2O/acetic acid (45:52:3, v/v) eluent at a flow rate of 1 ml/min monitored at 280 nm. Authentic compounds were used

for calibration. The retention times of coniferyl alcohol and eugenol were 3.2 and 15.4 min, respectively. UV/Vis spectra were recorded in a Shimadzu UV 1200 apparatus and NMR spectra in a Bruker WM-360 high field NMR spectrometer using methanol-D as solvent and tetramethylsilane as internal standard. GC-MS was performed with a Trio-1 mass spectrometer connected with a 5890 Hewlett-Packard gas chromatograph plus DB-1 capillary column (J&W Scientific, Tokyo, Japan) using helium as carrier gas and a temperature program with 1 min at 50°C and 50–250°C at 15°C/min. By matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) obtained from a Vision 2000 reflectron time-of-flight mass spectrometer (Finnigan MAT, Hemel Hempstead, England) using 2,5-dihydroxybenzoic acid as matrix, molecular masses in the range of 0.2 to 500 kDa could be determined. Proteins were separated by native and SDS-PAGE in 10% (mass/vol.) gels as described by Laemmli (1970), followed by Coomassie, heme (Thomas *et al.* 1976), or activity staining. For activity staining, the native gel was incubated in 10 mM eugenol, 1 mM PMS and 0.5 mM nitroblue tetrazolium in buffer A. Protein was quantified by the method of Bradford (1976) using BSA as standard. Purity and mass of the native enzyme were estimated by gel-permeation HPLC using a TSK G-3000SW column (0.75 × 60 cm, Toyo Soda, Tokyo, Japan) with buffer A plus 0.2 M NaCl as eluent at 0.7 ml/min. The molecular mass was calculated from a linear regression curve obtained from the mobilities of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa). For amino acid sequencing, enzyme subunits were separated by SDS-PAGE, electroblotted on a Pro-Blott PVDF membrane (Applied Biosystems, USA) in a

Sartoblot II-S semi-dry blotter (Sartorius, USA), and their N-terminal amino acid sequences were analyzed by automated Edman degradation in a 477A gas-phase amino acid sequencer and PTH amino acid analyzer 120A (Applied Biosystems).

Materials

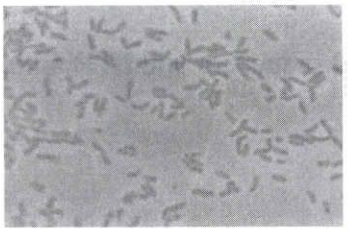
Eugenol, coniferyl alcohol and all tested electron acceptors were obtained from Aldrich (Milwaukee, USA), DEAE-Sephacel, Phenyl-Sepharose CL-4B and the low molecular mass marker for SDS-PAGE from Pharmacia (Uppsala, Sweden). Toyopearl was purchased from Tosoh (Tokyo, Japan), and molecular marker proteins for HPLC from Böhringer (Mannheim, Germany). Unless otherwise stated, all other chemicals were from Wako (Osaka, Japan).

RESULTS

Isolation and identification of eugenol-degrading bacterium

A gram-negative, motile, rod-shaped bacterium with the ability to form a fluorescent pigment was isolated from soil by a conventional screening using eugenol as enrichment substrate and sole source of carbon and energy. On the basis of further morphological and biochemical studies it was identified as *Pseudomonas fluorescens* (Table 1).

Table 1. **Taxonomical studies on strain E118**

Cell morphology	
Gram strain	−
Spore	−
Motility	+
Colony morphology	Circular, regular, entire, buff, semi-translucent, low convex, (3~4 mm in diameter)
Fluorescent pigment	+
Growth	30°C (+), 37°C (–), 41°C (–),
Catalase	+
Oxidase	+
Fermentative in glucose OF	−

Purification, activity, structure, and N-terminal amino acid sequence of the enzyme

Eugenol dehydrogenase, detected only after induction by eugenol, was purified 240-fold from a cell-free extract of *P. fluorescens* E118 (Table 2). Purified eugenol dehydrogenase was analyzed by native-PAGE (Fig. 1). The subunit molecular masses of the purified enzyme were determined to 10.4 kDa and 58.2 kDa (each ± 0.1 kDa) by MALDI-MS in consistence with the results obtained by SDS-PAGE (Fig. 2). The homogeneity of the enzyme was also shown by HPLC elution as a single symmetrical peak with a native molecular mass of 70

kDa, suggesting a heterodimeric enzyme structure. The purified enzyme catalyzed the stoichiometric conversion of eugenol to coniferyl alcohol with a V_{max} of 24.2 U/mg and an apparent K_m of 5 μM. The enzyme was stable between pH 5.0 and 9.0 and below 50°C. Above 50°C and pH 10.0 and below pH 5.0, the activity was rapidly lost. The pH-optimum for enzyme activity was 7.0. The temperature leading to the highest coniferyl alcohol formation in the stopped reaction was 50°C. The amino-terminal amino acid sequences of α and β subunit were ADNSTPDIYRPS and MDTTVILPEGV, respectively. Sequence similarities to vanillyl alcohol oxidase (Benen *et al.* 1998) and other sequences accessible in the protein databases SwissProt and PIR were not found. However, a short homologous LPXGV motif in the N-terminus of the β subunit of eugenol dehydrogenase and a *p*-cresol methylhydroxylase was observed (McIntire *et al.* 1986).

Table 2. **Purification of eugenol dehydrogenase from *P. fluorescens* E118**

Steps	Total activity (units)	Total protein[a] (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	584	5840	0.10	1.0	100
$(NH_4)_2SO_4$ (30-50%)	526	3550	0.15	1.5	90.1
DEAE-Sephacel	269	202	1.33	13.3	46.1
Phenyl-Sepharose CL-4B	239	21.1	11.3	113	40.9
$(NH_4)_2SO_4$ (40-70%)	180	14.1	12.8	128	30.8
Butyl-Toyopearl 650M	151	9.08	16.6	166	25.9
DEAE-Sephacel	131	5.42	24.2	242	22.4

[a] Protein was determined according to the method of Bradford (Bradford, 1976).

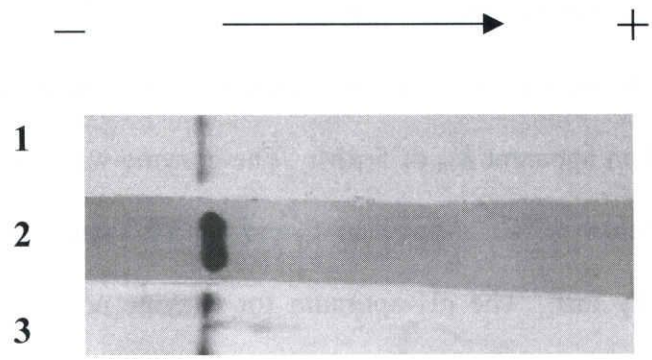


Fig. 1. **Native-PAGE of the purified eugenol dehydrogenase from *P. fluorescens* E118**
Lanes 1 Coomassie Blue staining, *Lanes 2* heme staining, *Lanes 3* active staining. In all lanes, 10 μg of purified eugenol dehydrogenase was loaded.

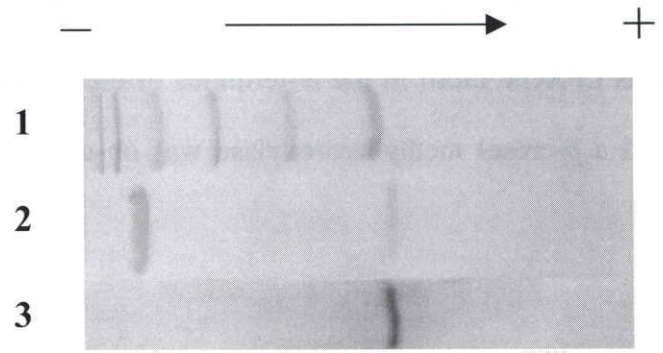


Fig. 2. **SDS-PAGE of the purified eugenol dehydrogenase from *P. fluorescens* E118**
Lanes 1 Low-molecular weight marker proteins with 94, 67, 43, 30, 20.1 and 14.4 kDa (from top to bottom); *2* Coomassie Blue staining; and *3* heme staining. In lanes 2 and 3, 6 μg of purified eugenol dehydrogenase was loaded.

Electron acceptor

Resting cells hydroxylated eugenol without external cofactor, but the activity of the purified enzyme depended on the addition of an electron acceptor such as PMS (K_m 0.42 mM) with the highest activity and V_{max} of 24.2 U/mg, followed by

DCIP (K_m 0.23 mM) with 87% relative activity, cytochrome *c* (K_m not determined) with 69% and $K_3Fe(CN)_6$ (K_m 0.83 mM) with 64% relative activity. These K_m values are significantly lower than those of vanillyl alcohol oxidase (De Jong *et al.* 1992) and *p*-alkylphenol methylhydroxylases (Reeve *et al.* 1989). Without electron acceptor or with 1 mM methylene blue, FAD, FMN, NAD^+, $NADP^+$, safranin O, nitroblue tetrazolium, menadione, or menatetranone, no activity was found. The electron transfer from the enzyme to PMS and further to nitroblue tetrazolium was used to characterize the enzyme by activity staining: a single, stained protein band in native PAGE with the same mobility as the Coomassie Blue stained enzyme was observed. The purified enzyme exhibited absorption maxima at 550, 522, and 417 nm after reduction by $Na_2S_2O_4$ or the substrate eugenol, however, after oxidation by a small amount of $KMnO_4$, the absorption maximum at 417 nm shifted to 410 nm and the other two peaks were replaced by a single broad peak (Fig. 3 A). The author separated α and β subunit by incubation of the purified enzyme in 10% (v/v) trichloroacetic acid (TCA) for 1 h and centrifugation at 48,000 × *g* for 45 min. Supernatant and precipitate contained high amounts of α and β subunit, respectively, as shown by SDS-PAGE. The reddish supernatant showed absorption maxima at 550, 522, and 417 nm under reducing conditions (Fig. 3 B). The absorption coefficients (in mM^{-1} cm^{-1}) of the reduced enzyme were 99.5 at 417 nm, 10.5 at 522 nm, and 18.35 at 550 nm, and in the oxidized enzyme 63.8 at 410 nm. The absorption coefficients (in mM^{-1} cm^{-1}) of the reduced α subunit were 62.7 at 417 nm, 7.13 at 522 nm, and 9.98 at 550 nm, and in the oxidized α subunit 44.0 at 410 nm. These coefficients are a factor 2.0–2.5 lower than the values determined in a *p*-cresol methylhydroxylase

(McIntire *et al.* 1985), which might be due to mistakes in the determination of protein caused by interference of the heme with the Bradford reagent. Using an α subunit protein concentration of 0.4 mg/ml suggested by the absorption at 300 nm of 0.4 (Fig. 3 B), almost the same absorption coefficients as in *p*-cresol methylhydroxylase were obtained ($\varepsilon_{410,\ ox.}$ 110 [123]; $\varepsilon_{417,\ red.}$ 157 [160]; $\varepsilon_{522,\ red.}$ 17.8 [18.1]; $\varepsilon_{552,\ red.}$ 25.0 [25.6]; all values in $mM^{-1}\ cm^{-1}$, absorption coefficients in brackets correspond to *p*-cresol methylhydroxylase). Absorption maxima, molecular mass, and the heme staining in SDS-PAGE (Fig. 2) indicate that the α subunit corresponds to cytochrome *c*550.

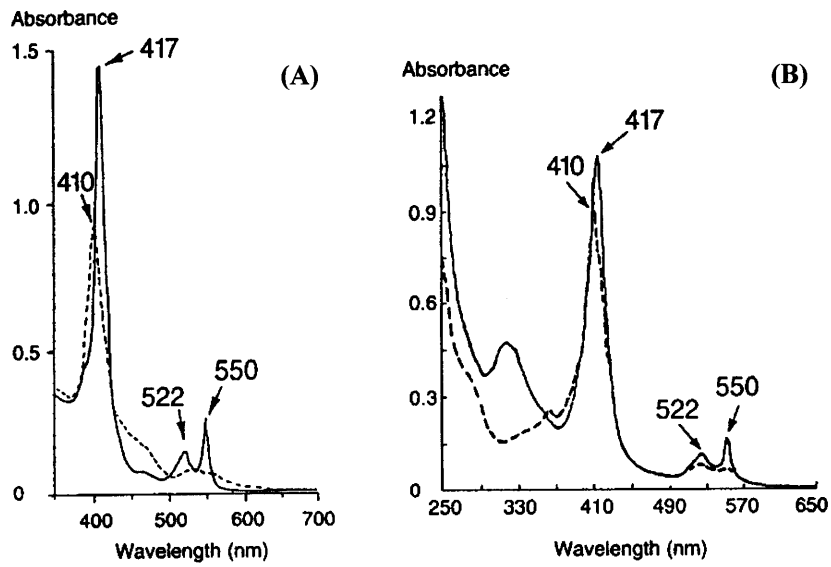


Fig. 3. **Absorption spectrum of (A) purified eugenol dehydrogenase (1 mg/ml) and (B) α subunit**

Plain lines indicate spectra under reducing conditions after addition of a small amount of $Na_2S_2O_4$, and dotted lines spectra under oxidizing conditions after addition of a small amount of $KMnO_4$.

Additionally, the enzyme showed an absorption shoulder at 460 nm under oxidizing but not under reducing conditions (Fig. 3 A). Furthermore, an absorption shoulder at 460 nm in the purified enzyme under oxidizing conditions

but not in the α subunit (Fig. 3 B) and a yellow color of the TCA precipitate suggested a flavin content in the β subunit. However, attempts to obtain a typical flavin spectrum with absorption maxima at 450 nm and 375 nm from the redissolved TCA precipitate, which had to be expected under oxidized conditions, failed. Only an unspecific absorption, which was enhanced under oxidized conditions, and a weak absorption shoulder at 460 nm were observed.

Substrate specificity

The enzyme catalyzed the dehydrogenation and hydration of eugenol, accompanied by a unique double-bond transfer, with the second highest activity and highest affinity, furthermore the same reaction sequence of 4-alkylphenols to the *p*-alkanols and the dehydrogenation of 4-hydroxybenzyl alcohols to the corresponding aldehydes (Table 3). Except for the conversion of *p*-cresol, no formation of alkenes or aldehydes in the conversion of 4-alkylphenols, was observed. The three different types of dehydrogenation catalyzed by the enzyme are shown in Fig. 4. 4-Allyl-2,6-dimethoxyphenol was not converted, indicating that an additional *ortho*-methoxy substituent introduces steric constraints. Also other 4-allylphenols such as isoeugenol, 3,4-dimethoxy-1-allylbenzene, 4-allyl-2,6-dimethoxyphenol, *o*-allylphenol, allylbenzene, 4-allylanisole, allyl phenyl ether, allylcyclopentane, (±)*o*-allylcyclohexanone, allylcyclohexylamine and furthermore 1-decene, 1,5,9-decatriene, myrcene, 4-ethylanisol, and 4-tert-butylphenol were not converted. The activity towards 4-alkylphenols decreased with the increasing length of the alkyl chain. 2- and 3-ethylphenol and

benzyl alcohol did not serve as substrates showing the requirement of the *para*-hydroxy group of substrates.

Table 3. **Substrate specificity of eugenol dehydrogenase from *P. fluorescens* E118**

Substrate	Activity (%)	K_m (μM)	Product
Eugenol	100	5	Coniferyl alcohol
p-Cresol	8	n.d.	4-Hydroxybenzaldehyde
4-Ethylphenol	78	142	1-(4-Hydroxyphenyl)ethanol
4-*n*-Propylphenol	73	40	1-(4-Hydroxyphenyl)propanol
4-Isopropylphenol	6	n.d.	2-(4-Hydroxyphenyl)propane-2-ol
4-*n*-Butylphenol	66	33	1-(4-Hydroxyphenyl)butanol
4-*sec*-Butylphenol	12	n.d.	2-(4-Hydroxyphenyl)butane-2-ol
4-*n*-Hexylphenol	38	265	1-(4-Hydroxyphenyl)hexanol
4-*n*-Octylphenol	14	n.d.	1-(4-Hydroxyphenyl)octanol
4-Hydroxybenzylalcohol	122	820	4-Hydroxybenzaldehyde
Vanillyl alcohol	90	398	Vanillin

Substrates (10 mM) were tested in a standard reaction with 0.1 units enzyme. Apparent K_m values were determined from Lineweaver-Burk plots (n.d. not determined). Activity with eugenol corresponds to the reduction of 47.3 nmol DCIP per min. Products were isolated by ethylacetate extraction and silica gel chromatography, and identified by ^{1}H-NMR and GC-MS.

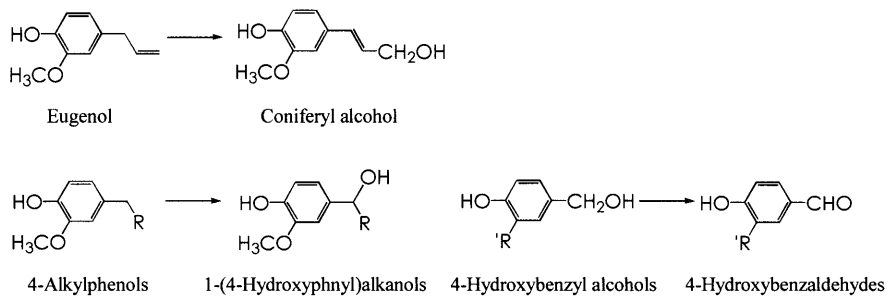


Fig. 4. **Reaction catalyzed by eugenol dehydrogenase from *P. fluorescens* E118**
R; Alkyl(C1-C7), R'; OCH_3 or H.

After performing an enzymatic conversion of eugenol in $H_2{}^{18}O/H_2{}^{16}O$ (50:50, v/v), the product coniferyl alcohol exhibited a 180 molecule ion peak and a

182 isotopic mass peak with the same intensities showing that the O incorporated into eugenol derived from H_2O (Fig.5).

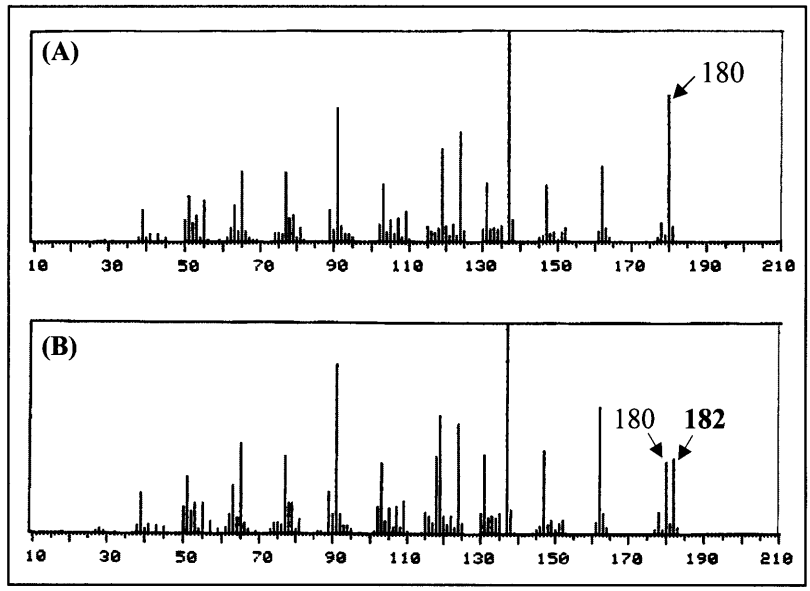


Fig. 5. **Incorporation of $H_2{}^{18}O$ into coniferyl alcohol**
(A) control, (B) in presence of 50% $H_2{}^{18}O$

Inhibitors

The enzyme was sensitive towards thiol-specific reagents such as $AgNO_3$ (0% remaining activity), $HgCl_2$ (7%), $CuCl_2$ (11%), *p*-chloromercuribenzoate (42%) and 5,5'-dithiobis(2-nitrobenzoic acid) (80%). Iron-chelating agents such as EDTA, 2,2'-bipyridyl, diethyldithiocarbamate, *o*-phenanthroline, 8-hydroxyquinoline and Tiron (4,5-dihydroxy-*m*-benzene disulfonate) were without influence. No significant effect was found with *N*-ethylmaleimide, iodoacetic acid, hydroxylamine, semicarbazide, cysteamine, D-cycloserine, cuprizione, NaN_3, D,L-penicillamine, dithiothreitol, 2-mercaptoethanol, $Na_2S_2O_4$, H_2O_2, $(NH_4)_2S_2O_8$ and the metal salts NaCl, $BaCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $PbCl_2$,

$ZnCl_2$, $CoCl_2$, $SnCl_2$, $NiCl_2$, $CdCl_2$ and $Al_2(SO_4)_3$. The influence of KCN on the purified enzyme was low (83% remaining activity). Resting cells were slightly activated (113% activity) by the addition of PMS (30 mM) compared to a reaction mixture without electron acceptor (100%). After preincubation of resting cells with 5 mM KCN, only 19% activity remained. If 5 mM KCN and 30 mM PMS were applied together, 75% activity remained indicating that PMS takes up the electrons before the KCN target.

DISCUSSION

P. fluorescens E118 was isolated from soil using eugenol as enrichment substrate and sole source of carbon and energy. The strain was additionally able to grow on coniferyl alcohol, ferulic acid, vanillin and vanillic acid, probably due to the eugenol degradation pathway via these compounds as proposed for another microorganism (Tadasa and Kayahara 1983). In this study, the first enzyme of the eugenol degradation was purified and classified as a dehydrogenase due to the requirement of an electron acceptor and the use of water as oxygen source during hydroxylation. Due to the bifunctional ability to dehydrogenate and hydroxylate eugenol and 4-alkylphenols and the unknown identity of the natural electron acceptor, the author suggests eugenol:(acceptor) oxidoreductase (hydroxylating) as systematic enzyme name. The enzyme was found to be a heterodimer consisting of a small cytochrome *c*550 subunit and a large subunit. The holoenzyme and the small subunit showed a typical cytochrome *c* spectrum identical to the spectra of

other flavocytochromes *c* (McIntire *et al.* 1985, Reeve *et al.* 1989, Hopper *et al.* 1991).

The author could not obtain clear evidence for a flavin in the larger subunit. However, the author assumes that the larger subunit contains flavin because the spectrum of the oxidized holoenzyme showed an enhancement of the absorption only at 460 nm, but lower absorption in all other spectral areas compared to the reduced spectrum (Fig. 3A). This might be due to the presence of flavin because only the oxidized flavin absorbs in the area of 460 nm. The lack of absorption at 460 nm in the oxidized cytochrome *c*550 subunit furthermore suggests a flavin content in the large subunit and was identically shown in the spectra of a flavocytochrome *c* from an *Achromobacter* species (Hopper *et al.* 1991). Furthermore, the yellow color of the TCA precipitate suggested a covalent linking of the flavin to the polypeptide chain, which was previously reported in the closely related flavoenzymes vanillyl alcohol oxidase with histidyl-FAD (van Berkel *et al.* 1994), *p*-cresol methylhydroxylase with tyrosyl-FAD (McIntire *et al.* 1981) and 4-ethylphenol methylenehydroxylase (Reeve *et al.* 1989). In flavocytochromes, the absorption of flavin is generally covered by the stronger cytochrome absorption, and some difficulties to detect flavin in the large subunit have been reported in these enzymes (Reeve *et al.* 1989, Hopper *et al.* 1991). However, after optimizing the isolation, the β subunits of these enzymes finally showed typical flavin spectra. Such clear evidence for a flavin could not be presented in this study, and further studies are intended to solve these difficulties.

Eugenol dehydrogenase has a wide substrate spectrum linking the catalytic properties of a vanillyl alcohol oxidase and *p*-alkylphenol methylhydroxylases

(Table 4). Vanillyl alcohol oxidase (EC 1.1.13.3), a fungal, homooctameric flavoenzyme, shares with eugenol dehydrogenase the conversion of 4-hydroxybenzyl alcohols and eugenol as the best substrates and the oxidation of the alkyl side chain of 4-alkylphenols (Drijfhout *et al.* 1998, Fraaije *et al.* 1998 b). For both enzymes, eugenol is the substrate with the highest affinity, and almost the same K_m values are observed. In both enzymes, the unique double bond transfer during eugenol dehydrogenation (Fig. 4) is proposed to be accomplished by an initial dehydrogenation of the side chain C_α leading to a *p*-quinone methide, followed by hydration at $C_{\beta\gamma}$ (Fraaije *et al.* 1995). However, vanillyl alcohol oxidase differs from eugenol dehydrogenase in the lack of a cytochrome *c* subunit, the use of O_2 as electron acceptor, the induction (Fraaije *et al.* 1997) and the catalysis of the oxidative deamination of 4-hydroxybenzylamines and oxidative demethylation of 4-(methoxymethyl)phenol (Fraaije and van Berkel 1997).

The hydroxylation of 4-alkylphenols links eugenol dehydrogenase additionally to 4-alkylphenol methylhydroxylases (EC 1.17.99.1) with flavocytochrome *c* structure from several *Pseudomonas* species (McIntire *et al.* 1985, Reeve *et al.* 1989, Hopper *et al.* 1991). In contrast to *p*-cresol methylhydroxylases, 4-ethylphenol methylenehydroxylase from *P. putida* DJ1 converts 4-hydroxybenzyl alcohol and eugenol, however, with low activity and unknown product identity (Reeve *et al.* 1989). Regarding substrate specificity and presumed flavocytochrome *c* structure, eugenol dehydrogenase is most closely related to 4-ethylphenol methylenehydroxylase. However, induction, subunit composition, affinity towards electron acceptors, and substrate preference distinguish these two enzymes.

Table 4. **Comparison of 4-hydroxybenzyl dehydrogenases**

Enzyme, source (reference)	Subunits × mass (kDa)	Prostethic group	Inducer	Substrates	Electron acceptors
Eugenol dehydrogenase, *Pseudomonas fluorescens* E118 (this work)	10, 58	Heme (Flavin)?	Eugenol	Eugenol 4-Hydroxybenzyl alcohols 4-Alkylphenols	PMS, DCIP, Cytochrome *c*, $K_3Fe(CN)_6$
4-Ethylphenol methylene-hydroxylase, *Pseudomonas putida* DJ1 (Reeve et al. 1989)	2 × 10, 2 × 50	Heme FAD	4-Ethylphenol, 4-Propylphenol	4-Alkylphenols 1-(4-Hydroxyphenyl)alcohols Eugenol 4-Hydroxybenzyl alcohol	Azurin, PMS, Cytochrome *c*, DCIP
p-Cresol methylhydroxylase, *Pseudomonas putida* NCIB 9869 (McIntire et al. 1985)	2 × 9, 2 × 49	Heme FAD	*p*-Cresol	4-Alkylphenols 1-(4-Hydroxyphenyl)alcohols	Azurin, PMS, Cytochrome *c*
Vanillyl alcohol oxidase, *Penicillium simplicissimum* (De Jong et al. 1992)	8 × 65	FAD	Veratryl alcohol Anisyl alcohol 4-(Methoxymeth-yl)phenol	Eugenol 4-Hydroxybenzyl alcohols 4-(Methoxymethyl)phenol 4-Hydroxybenzylamines 4-Alkylphenols	O_2, PMS

All these dehydrogenases including eugenol dehydrogenase use PMS as an artificial electron acceptor. O_2 (De Jong *et al.* 1992) and presumably azurin (Causer *et al.* 1984, Gorren *et al.* 1996) are the natural electron acceptors of vanillyl alcohol oxidase and 4-alkylphenol methylhydroxylases, respectively. The inhibition of eugenol dehydrogenase by KCN as cytochrome oxidase inhibitor and its reversion by PMS suggest the presence of an electron transfer from eugenol dehydrogenase to cytochrome oxidase. This was supported by the finding of high cytochrome oxidase activities in cell extracts of *P. fluorescens* E118. Assuming the presence of flavin in the large subunit and regarding the redox potentials of electron acceptors (Schlegel 1992), the author has proposed an *in vivo* electron transfer chain from the substrate to flavin, cytochrome, cytochrome oxidase and finally O_2 (Fig. 6).

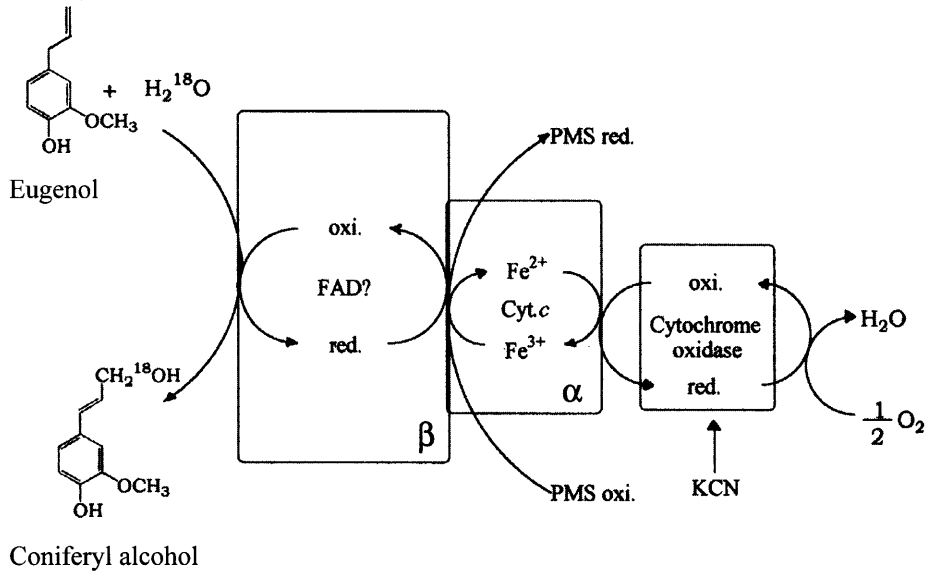


Fig. 6. **Proposed flow of electrons from eugenol dehydrogenase to cytochrome oxidase in *P. fluorescens* E118**

In vitro, using the isolated enzyme, PMS with a redox potential between flavin and cytochrome *c*550 would most likely take up the electrons from the assumed flavin. Compared to the intracellular vanillyl alcohol oxidase (Fraaije *et al.* 1998a) and periplasmic 4-alkylphenol methylhydroxylases (Hopper *et al.* 1985), the location of eugenol dehydrogenase is not known, but a proposed collaboration with cytochrome oxidase might suggest a membrane association. Recently, similarities in the structure of the flavoprotein subunits of vanillyl alcohol oxidase and 4-alkylphenol methylhydroxylase were described, suggesting their belonging to a novel family of structurally related flavoenzymes with covalently bound FAD (Kim *et al.* 1995, Mattevi *et al.* 1997, Fraaije *et al.* 1998c). Nevertheless, the N-terminal sequences of these flavoenzymes are not conserved. Further studies

on eugenol dehydrogenase from *P. fluorescens* E118 will reveal more information about a possible belonging to this novel class of oxidoreductases.

SUMMARY

Pseudomonas fluorescens E118 was isolated from soil as an effective eugenol-degrading organism by a screening using eugenol as enrichment substrate. The first enzyme involved in the degradation of eugenol in this organism, eugenol dehydrogenase, was purified after induction by eugenol, and the purity of the enzyme was shown by SDS-PAGE and gel-permeation HPLC. The enzyme is a heterodimer that consists of a 10 kDa cytochrome *c*550 and a 58 kDa subunit. The larger subunit presumably contains flavin, suggesting a flavocytochrome structure and an electron transfer via flavin and cytochrome *c*550 during dehydrogenation. The activity of the purified enzyme depended on the addition of a final electron acceptor such as PMS, DCIP, cytochrome (horse), or $K_3Fe(CN)_6$. The enzyme catalyzed the dehydrogenation of three different 4-hydroxybenzylic structures including the conversion of eugenol to coniferyl alcohol, 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols, and 4-hydroxybenzylalcohols to the corresponding aldehydes. The catalytic and structural properties of this enzyme were compared with a *Penicillium* vanillyl alcohol oxidase and 4-alkylphenol methylhydroxylase from several *Pseudomonas* species.

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Section 2

Synthesis of (*S*)-1-(4-Hydroxyphenyl)alcohols by Eugenol Dehydrogenase from *Pseudomonas fluorescens* E118

(*S*)-1-(4-Hydroxyphenyl)alcohols [(*S*)-4-(1-hydroxyalkyl)phenols] (Fig. 1B) are employed in the synthesis of anti-ulcer (Munson and Boswell 1992) and anti-inflammatory agents (Batt and Wright 1991) and liquid crystal materials (Azumai *et al.* 1991). The chemical access to enantiomerically enriched forms of 1-(4-hydroxyphenyl)alkanols is based on either a classical preferential crystallization of the phthalate ester salts after protection of the phenolic moiety as a formaldehyde acetal or the treatment of the *o*-protected 4-hydroxyphenyl alkyl ketones with the enantiomers of chlorodiisopinocampheylborane followed by deprotection (Everhart and Craig 1991). However, due to the necessity of chiral additives and auxiliaries, and a protection and deprotection step, enzymatic one-step preparations under mild reaction conditions have attracted increased interest. So far, two enzymatic syntheses of chiral 1-(4-hydroxyphenyl)alcohols (McIntire *et al.* 1984, McIntire and Bohmont 1987, Reeve 1990) using 4-alkylphenol methylhydroxylases from two *Pseudomonas putida* species have been described, however, these enzymes have the tendency to dehydrogenate the alcohol further to the corresponding ketones, and in one case 4-vinylphenol was additionally formed as a by-product, leading to the requirement of additional separation steps during product isolation. The author describes here a new enzymatic method for the synthesis of (*S*)-1-(4-hydroxyphenyl)alcohols using eugenol dehydrogenase from *Pseudomonas fluorescens* E118, which has been

purified and characterized (Section 1 in CHAPTER I). This enzyme catalyzes the dehydrogenation of a wide range of 4-hydroxybenzylic structures including the conversion of 4-alkylphenols (Fig. 1A) to the corresponding 1-(4-hydroxyphenyl)alcohols with the addition of an artificial electron acceptor such as PMS, $K_3Fe(CN)_6$, DCIP or cytochrome *c* as the enzyme cofactor. Since the hydroxylation of prochiral 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols introduces an asymmetric carbon in the alkyl side chain, the author has examined the (pro-)stereoselectivity of this enzyme.

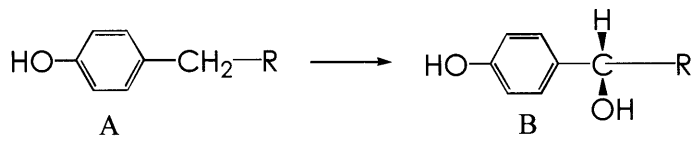

Fig. 1. **Enantioselective hydroxylation reaction by eugenol dehydrogenase from *P. fluorescens* E118**

MATERIALS AND METHODS

Preparation of eugenol dehydrogenase

Purified eugenol dehydrogenase from *P. fluorescens* E118 was prepared as described in Section 1 (CHAPTER I).

Analytical methods for chiral alcohols

(*S*) and (*R*)-1-(4-hydroxyphenyl)alcohols were separated and analyzed by HPLC on a Shimadzu LC-6A (Shimadzu, Kyoto, Japan) with an with a Chiracel OB column (4.6 × 250 mm, Daicel, Tokyo, Japan) and *n*-hexane:*n*-propanol (9:1,

v/v) as eluent at a flow rate of 0.2 ml/min and 30°C, monitored at 280 nm. Racemic 1-(4-hydroxyphenyl)alcohols were synthesized from corresponding aldehydes by reduction of $NaBH_4$ in methanol at room temperature.

Bioconversion of 4-alkylphenols

For each of the bioconversions of 40 mM and 75 mM 4-ethylphenol and 60 mM 4-propylphenol, 5 mg of the purified enzyme was used in a 3 h reaction in 20 ml 100 mM potassium phosphate buffer, pH 7.0, containing 10 mM PMS. Bioconversions were monitored by HPLC using an ODS C18 column (4.6 × 150, MS Instruments, Tokyo, Japan) and a methanol : H_2O : acetic acid (45:52:3, v/v/v) eluent at 1 ml/min, detected at 280 nm, and the *e.e.* was determined from the ratio of the peak areas obtained by the above-mentioned chiral stationary phase.

Purification and identification of (S)-1-(4-hydroxyphenyl) alcohols

1-(4-Hydroxyphenyl)ethanol and 1-(4-hydroxyphenyl)propanol were isolated by ethyl acetate extraction, evaporation and silica gel chromatography (Wakogel C300, Wako, Osaka, Japan) using benzene : methanol 95:5 (v/v) as eluent. The product identity was confirmed by GC-MS and ^{1}H-NMR. GC-MS spectra were recorded in a Trio-1 mass spectrometer (Raleigh, USA) connected with a 5890 Hewlett-Packard gas chromatograph (Palo Alto, USA) plus DB-1 capillary column (J&W Scientific, Tokyo, Japan) using helium as carrier gas and a temperature program of 1 min at 50°C and 50-250°C at 15°C/min. NMR spectra were obtained from a Bruker WM-360 high field NMR spectrometer (Billerica. USA) with methanol-d_4 as solvent and tetramethylsilane as internal standard.

The specific rotation $[\alpha]_D^{20}$ of the isolated products, measured in a Jasco DIP-1000 digital polarimeter (Easton, USA).

RESULTS AND DISCUSSION

Enantioselective hydroxylation reaction by eugenol dehydrogenase

Eugenol dehydrogenase from *P. fluorescens* E118 converted a number of 4-alkylphenols as described in Section 1 (CHAPTER I). Each product was purified by silica gel chromatography, and analyzed by HPLC. All products whose absolute configurations were (*S*). The retention times of 1-(4-hydroxyphenyl)ethanol were 23.7 min for the (*S*)-enantiomer and 27.0 min for the (*R*)-enantiomer, and the (*S*)- and (*R*)-forms of 1-(4-hydroxyphenyl)propanol eluted after 41.5 min and 45.9 min, respectively. Since the enzyme activity decreased with the increasing length of the alkyl side chain or the introduction of branched alkyl chains in the substrates (Section 1 in CHAPTER I), the author has confined our bioconversion studies here to the conversion of 4-ethylphenol and 4-*n*-propylphenol.

Bioconversion of 4-alkylphenol by eugenol dehydrogenase

4-Ethylphenol (40 mM) was converted quantitatively in a batch reaction to 40 mM (*S*)-1-(4-hydroxyphenyl)ethanol (Fig. 2A) with an *e.e.* of 96.6%. Due to a relatively high toxicity and inhibition effect on the enzyme, 4-propylphenol was added in three portionwise feeding steps of 20 mM each after 0, 1 and 2 h,

finally leading to 60 mM (*S*)-1-(4-hydroxyphenyl)propanol (Fig. 2B) with 95.2% *e.e.* Using a similar fed-batch approach for 4-ethylphenol added in three steps of 25 mM each, finally 74 mM (*S*)-1 -(4-hydroxyphenyl)ethanol were accumulated with 96.6% *e.e.* In all of these bioconversions, neither ketones nor 4-vinylphenol, the latter of which could have only been formed in the reaction of 4-ethylphenol, were detected as by-products.

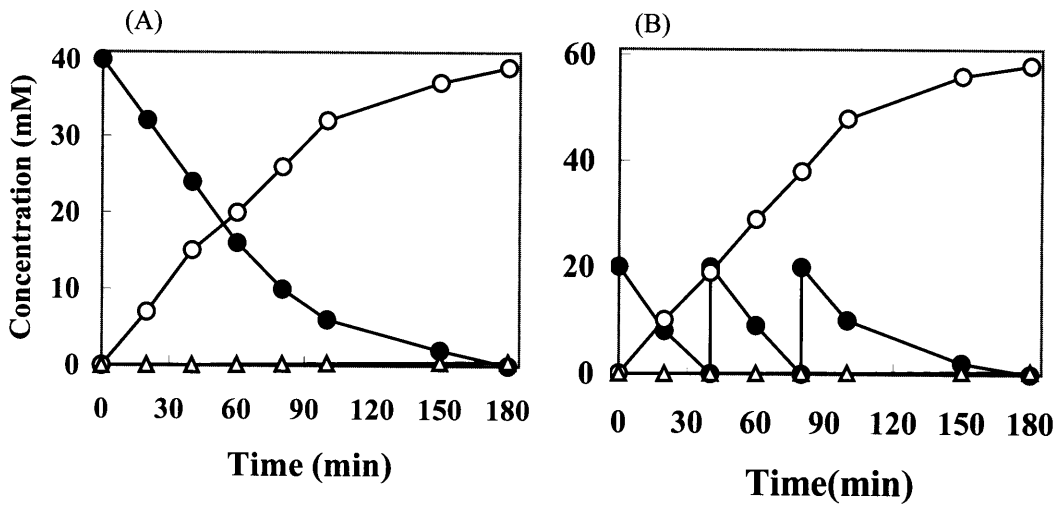

Fig. 2. **Time course of the conversion of 4-ethylphenol (A) and 4-propylphenol (B) by eugenol dehydrogenase from *P. fluorescens* E118**
The reactions were carried out at 30°C in 20 ml 100 mM potassium phosphate buffer, pH 7.0, containing 10 mM PMS and 5 mg of the purified enzyme, and followed by HPLC. 4-Ethylphenol was converted in a batch reaction, and 4-*n*-propylphenol in a fed-batch reaction. Symbols: 4-ethylphenol or 4- *n*-propylphenol (•), 1-(4-hydroxyphenyl)ethanol or 1-(4-hydroxyphenyl)propanol (○), 4-hydroxyacetophenone or 4-hydroxypropiophenone (Δ).

Identification of (S)-1-(4-hydroxyphenyl) alcohols

1-(4-Hydroxyphenyl)ethanol and 1-(4-hydroxyphenyl)propanol were isolated by ethyl acetate extraction, evaporation and silica gel chromatography with yields of 63% and 66%, respectively. The mass spectrum of the ethanol

product showed peaks at *m/z* 138 (M^+, 22.2% relative abundance), 123 (84.4%), 121 (16.7%), 120 (l00%), 95 (55.6%), 94 (15.5%), 91 (71.1%), 77 (48.9%), 65 (37.8%) and 63 (17.8%) consistent with the MS pattern of authentic 1-(4-hydroxyphenyl)ethanol. On the other hand, the *m/z* values of the (*S*)-propanol were 152 (M^+, 8.9%), 134 (33.3%), 133 (24.4%), 123 (100%), 107 (13.3%), 94 (44.4%), 91 (11.1%) and 77 (48.9%). The GC-MS data indicated the incorporation of an oxygen in the aliphatic carbon chain at the carbon adjacent to the aromatic ring. ^{1}H-NMR spectra of both products indicated a methine proton adjacent to the aromatic ring and methylene protons at the α position of the aliphatic carbon chain. The chemical shifts δ for the (*S*)-ethanol product were 1.39 (d, 3, C*H*$_3$), 4.72 (q, 1, C-*H*), 6.74 and 7.18 ppm (2 d, 4, Ar-*H*), and the protons of the product of 4-*n*-propylphenol appeared at chemical shifts δ of 0.85 (d, 3, C*H*$_3$), 1.76 and 1.81 (2 d, 2, C*H*$_2$), 4.41 (q, 1, C-*H*), 6.72 and 7.13 ppm (2 d, 4, Ar-*H*). Besides identical molecule ion masses, the MS and ^{1}H-NMR spectra of reference compounds bearing the alcohol group at the distal aliphatic carbon, 2-(4-hydroxyphenyl)-1-ethanol and 3-(4-hydroxyphenyl)-1-propanol, clearly differed from our bioconversion products, excluding the possibility of the hydroxylation of the distal aliphatic carbon. The specific rotation $[\alpha]_D^{20}$ of the isolated (*S*)-ethanol product, was -44.8 (*c* 1.74, ethanol) in agreement with the value of the almost enantiomerically pure (*S*)-form $[\alpha]_D^{20}$ -47.5 (*c* 4.98, ethanol). The specific rotation $[\alpha]_D^{20}$ of the isolated (*S*)-propanol was -36.7 (*c* 1.35, ethanol).

The enantiomeric excess of the products depended on the pH of the bioconversion mixture with the highest *e.e.* values reached at pH 7.0 (Fig. 3).

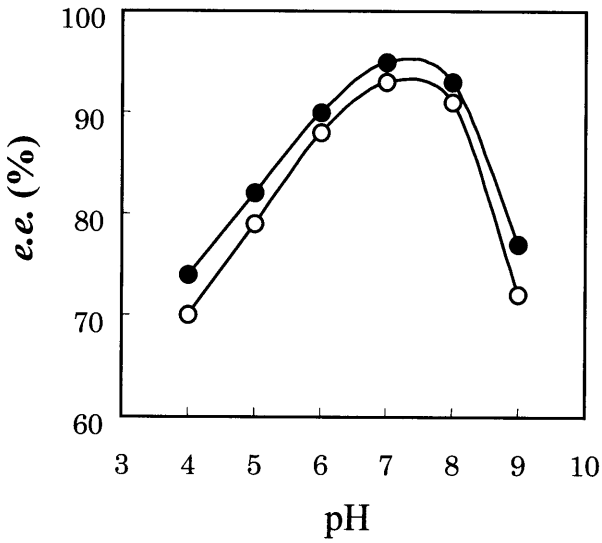

Fig. 3. **Effect of the pH on the enantioselectivity of eugenol dehydrogenase from *P. fluorescens* E118**
The reaction mixture was composed as described in the legend of Fig. 10 except for using potassium phosphate buffer of different pH. The reaction was stopped after 3 h, and *e.e.* values were determined by chiral HPLC. Symbols: 1-(4-hydroxyphenyl)ethanol (•), 1-(4-hydroxyphenyl) propanol (○).

This is likely due to the pH-dependent enantioselectivity of the enzyme, because the enantiomeric excesses of the products were not affected by acid and base treatment. A pH-dependent stereoselectivity has not been reported so far for similar 4-alkylphenol methylhydroxylases. The enantioselectivities of eugenol dehydrogenase were in the range of the analogous bioconversions catalyzed by 4-alkylphenol methylhydroxylases, which resulted in 31-94% *e.e.* for the (*S*)-alcohol and 92-98% *e.e.* for the (*R*)-alcohol, depending on the kind of alkyl rest. On the other hand, the 4-alkylphenol methylhydroxylases were only enantioselective with cytochrome *c*, but almost not with PMS, leading to a nearly racemic mixture of the alcohol products when PMS was used. This contrasts

with eugenol dehydrogenase, which was highly enantioselective with PMS, and the enantioselectivity of which did not depend on the kind of electron acceptor as tested with PMS, $K_3Fe(CN)_6$, DCIP-indophenol and cytochrome *c*. The asymmetric bioconversion described here has advantages compared to previous enzymatic preparations with regard to productivity, yield and absence of by-products.

SUMMARY

Eugenol dehydrogenase purified from *Pseumomonas fluoresecens* E118, catalyzed the enantioselective hydroxylation reaction of 4-alkylphenols. (*S*)-1-(4-Hydroxyphenyl)ethanol and (*S*)-1-(4-hydroxyphenyl)propanol were synthesized with enantiomeric excesses of 96.6% and 95.2%, respectively, from the corresponding 4-alkylphenols by eugenol dehydrogenase from *P. fluorescens* E118. The enantioselectivity of the hydroxylation reaction catalyzed by the enzyme was found to be pH-dependent.

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CHAPTER II

Studies on Vanillyl Alcohol Oxidase from *Byssochlamys fulva* V107 Acting on Eugenol

Section 1

Isolation of Vanillyl Alcohol-Degrading Bacterium, *Byssochlamys fulva* V107: Purification and Characterization of Vanillyl Alcohol Oxidase

The degradation of lignin, the most abundant biopolymer, leads to the formation of a broad spectrum of aromatic molecules that can be used by various fungi as sole sources of carbon. One of the key intermediates in the degradation of lignin is ferulic acid, which is metabolized to vanillin, vanillic acid and protocatechuic acid (Rosazza *et al.* 1995), the latter of which is the substrate of further ring cleavage and the β-ketoadipate pathway. Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) is connected to this degradation route through dehydrogenation to vanillin (4-hydroxy-3-methoxybenzaldehyde) by vanillyl alcohol oxidase. So far, a vanillyl alcohol oxidase containing covalently bound FAD has been studied in detail using *Penicillium simplicissimum* (De Jong *et al.* 1992, Van Berkel *et al.* 1994, Fraaije *et al.* 1995, Fraaije and Berkel 1997, Benen *et al.* 1998). Related fungal oxidases involved in lignin degradation of white-rot fungi are extracellular aryl-alcohol oxidases with loosely bound FAD (Guillén *et al.* 1992, Marzullo *et al.* 1995). All these flavoenzymes generate H_2O_2, which is required for the ligninolytic process.

Related enzymes converting 4-hydroxybenzyl alcohols to the corresponding aldehydes are eugenol dehydrogenase from *Pseudomonas fluorescens* E118 catalyzing the side chain oxidation of a wide range of 4-hydroxybenzylic compounds (CHAPTER I) and 4-ethylphenol methylenehydroxylase from *Pseudomonas putida* DJ1 that mainly acts on 4-alkylphenols (Reeve *et al.* 1989). These dehydrogenases are of biotechnological interest due to the enantioselective synthesis of 1-(4-hydroxyphenyl)alcohols from 4-alkylphenols; however, they require the addition of an electron acceptor such as PMS. Therefore, the author attempted to find an enzyme catalyzing the same reaction with O_2 as an electron acceptor. By a screening with vanillyl alcohol as an enrichment substrate and eugenol as an enzyme substrate, the author obtained an effective vanillyl alcohol-degrading and eugenol-converting microorganism that was additionally able to oxidize 4-alkylphenols. Here, the author describes the purification and characterization of the enzyme responsible for these conversions and the enzymatic synthesis of (*S*)-1-(4-hydroxyphenyl)alcohols.

MATERIALS AND METHODS

Materials

Vanillyl alcohol, eugenol, and 4-alkylphenols were obtained from Aldrich. Phenyl- and Octyl-Sepharose CL-4B and the low molecular mass marker for SDS–PAGE were from Pharmacia. Toyopearl was purchased from Toyosoda,

and molecular marker proteins for HPLC were from Boehringer Mannheim. Unless otherwise stated, all other chemicals were obtained from Wako, Japan.

Screening and culture conditions

Cultivation was carried out at 28°C with reciprocal shaking. Enrichment culture for vanillyl alcohol degrading microorganisms was performed with soil samples in minimal medium consisting of 1 g vanillyl alcohol, 2 g NH_4Cl, 2 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g yeast extract, and metals in final concentrations of 0.2 mg $FeSO_4 \cdot 7\ H_2O$, 0.4 mg $CaCl_2 \cdot 2\ H_2O$, 0.3 mg H_3BO_3, 0.04 mg $CuSO_4 \cdot 5\ H_2O$, 0.1 mg KI, 0.4 mg $MnSO_4 \cdot 7\ H_2O$, and 0.2 mg $Na_2MoO_4 \cdot 2\ H_2O$ per liter. The pH was adjusted to 4.5 for the enrichment of fungi, which are known to bear oxidases (De Jong *et al.* 1992, Guillén *et al.* 1992) without an external cofactor requirement, in contrast to bacteria, which usually produce dehydrogenases demanding the addition of electron acceptors (Reeve *et al.* 1989). A 4-ml overnight preculture of the isolated strain, grown on 5 g polypepton (Wako, Osaka, Japan), 5 g meat extract (Kyokuto, Tokyo, Japan), 0.5 g yeast extract (Oriental Yeast, Tokyo, Japan) and 2 g NaCl per liter (pH 7.0), was used to inoculate a 2-l shaking flask with 400 ml medium consisting of 3 g vanillyl alcohol, 15 g glycerol, 2.5 g $(NH_4)_2SO_4$, 1 g yeast extract, 2 g K_2HPO_4, and 0.5 g $MgSO_4 \cdot 7\ H_2O$ per liter and metals as described above (pH 7.0). After 50-h cultivation, cells were harvested by centrifugation at 8,000 × *g* at 4°C and washed with 50 mM potassium phosphate buffer, pH 7.0 (buffer A).

Enzyme assay

The standard activity was assayed at 30°C in a shaken (160 strokes min^{-1}) reaction mixture containing 10 mM eugenol and an appropriate amount of enzyme in 1 ml buffer A. The reaction was stopped after 10 min by adding 1 ml methanol, and the formation of coniferyl alcohol was determined by HPLC. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol coniferyl alcohol per min.

Enzyme purification

After each purification step at 4°C, the enzyme was dialyzed and dissolved in the equilibration buffer of the next chromatographic step unless otherwise specified. Centrifugation was carried out for 30 min at 20,000 × *g*. Cells from 3 l culture (5 g wet weight) in 15 ml buffer A were disrupted for 30 min by ultrasonication at 19 kHz (Insonator 201M; Kubota, Tokyo). After fractionation of the crude extract with ammonium sulfate (50–80% saturation), the enzyme was loaded onto a Phenyl-Sepharose CL-4B column (2 × 15 cm) previously equilibrated with buffer A containing 15% ammonium sulfate, eluted at 10% within a linear gradient from 15 to 0%, and applied onto a Butyl-Toyopearl 650M column (2 × 10 cm) previously equilibrated with buffer A containing ammonium sulfate at 40% saturation. The enzyme was eluted at 20% in a linear 40–0% gradient, and loaded onto an Octyl-Sepharose CL-4B column (2 × 10 cm) equilibrated and eluted as described for the previous step, followed by chromatography on hydroxyapatite (2 × 3 cm) equilibrated with buffer A and eluted at 20% saturating ammonium sulfate within a linear gradient of 0–40%.

The purified enzyme was dialyzed against buffer A containing 50% (v/v) glycerol and stored at –20°C without activity loss for 6 months.

Enzyme characterization

The stability of the enzyme against pH and temperature was examined by preincubation for 30 min in 0.1 M buffers of various pH values at 30°C and for 10 min in buffer A at various temperatures, respectively. K_m values were estimated from Lineweaver–Burk plots. Products were isolated by ethylacetate extraction, evaporation, silica gel chromatography (Wakogel C-300; Wako, Osaka, Japan) with benzene/methanol (95:5, v/v) as a mobile phase, and crystallization, and identified by ^{1}H-NMR and GC–MS. The oxygen source of enzymatic hydroxylation was examined in a standard reaction mixture containing 50 mU enzyme and 0.5 ml $H_2{}^{18}O$ followed by product isolation as described and GC–MS analysis.

Analytical methods

Eugenol, coniferyl alcohol, and other substrates and products were determined by HPLC (Shimadzu LC-6A) with an ODS C18 column (4.6 × 150 mm; M & S Instruments) and a methanol/H_2O/acetic acid (45:52:3, v/v/v) eluent at a flow rate of 1 ml/min monitored at 280 nm using authentic compounds for calibration. Chiral HPLC analysis was carried out on a Chiracel OB column (4.6 × 250 mm; Daicel) using *n*-hexane/*n*-propanol (9:1, v/v) as an eluent at 0.2 ml/min and 30°C, monitored at 280 nm. The retention time of 1-(4-hydroxyphenyl)ethanol was 23.7 min (*S*-form) and 27.0 min (*R*-form), and

for 1-(4-hydroxyphenyl)propanol 41.5 min (*S*) and 45.9 min (*R*). UV/Vis and NMR spectra were respectively recorded using a Shimadzu UV 1200 instrument and a Bruker WM-360 high field NMR spectrometer with methanol-D as a solvent and tetramethylsilane as an internal standard. For GC–MS a Trio-1 mass spectrometer was connected to a 5890 Hewlett-Packard gas chromatograph plus a DB-1 capillary column (J & W Scientific, Tokyo, Japan) using helium as the carrier gas and a temperature program with 1 min at 50°C and 50–250°C at 15°C/min. SDS–PAGE was performed in 10% (w/v) gels (SchŠgge and van Jagow 1987), followed by Coomassie blue staining. Protein was quantified by the method of Bradford (Bradford 1976) using BSA as a standard. The purity and mass of the native enzyme were estimated by gel-permeation HPLC using a TSK G-3000SW column (0.75 × 60 cm; Toyo Soda, Tokyo) with buffer A plus 0.2 M NaCl as an eluent at 0.7 ml/min. The molecular mass was calculated from a linear regression curve obtained from the mobilities of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa). For colorimetric H_2O_2 determinations, 10-µl samples were withdrawn from a standard reaction mixture containing 50 mU purified enzyme, added to 1 ml of a mixture consisting of 7 mM phenol, 3 mM 4-aminoantipyrine, and 60 U/ml peroxidase, and measured photometrically at 500 nm.

RESULTS AND DISCUSSION

Microorganism isolation and identification

Among 32 vanillyl alcohol-degrading fungi isolated from soil by screening using vanillyl alcohol as an enrichment substrate and sole source of carbon and energy, 5 were additionally able to convert eugenol. The fungus showing the highest eugenol and vanillyl alcohol converting activity was selected. The strain, which bore heat-resistant ascospores, was identified as *Byssochlamys fulva*; its anamorph was shown to be *Paecilomyces fulvus*, closely related to *Peacilomyces varioti*, from which it differs in the shape of the conidia (Samson and Reenen-Hoekstra 1988).

Enzyme purification and structure

Vanillyl alcohol oxidase, detected only after induction by vanillyl alcohol, was purified 73-fold from a cell-free extract of *B. fulva* V107 (Table 1). The purified enzyme appeared as a single band in SDS–PAGE with a subunit molecular mass of 58 kDa (Fig. 1). The homogeneity of the enzyme was confirmed by HPLC-elution as a single symmetrical peak with a native molecular mass of 110 kDa, suggesting a homodimeric enzyme structure. For comparison, the vanillyl alcohol oxidase from *Penicillium simplicissimum* was initially found to be a homooctamer of 65 kDa subunits (De Jong *et al.* 1992), but further studies additionally revealed the occurrence of active dimers (Van Berkel *et al.* 1994). Other aryl-alcohol oxidases are monomeric (Guillén *et al.* 1992, Marzullo *et al.* 1995) and *p*-alkylphenol methylhydroxylases are heteromeric flavocytochromes.

Table 1. **Purification of vanillyl alcohol oxidase from *B. fulva* V107**

Steps	Total protein[a] (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	621	75.2	0.12	1.0	100
$(NH_4)_2SO_4$ (30–50%)	174	63.7	0.37	3.0	84.7
Phenyl-Sepharose CL-4B	48.7	38.9	0.80	6.6	51.7
Butyl-Toyopearl 650M	46.0	38.2	0.83	6.9	50.8
Octyl-Sepharose CL-4B	13.5	37.2	2.76	22.8	49.5
Hydroxyapatite	3.77	33.4	8.86	73.2	44.4

[a] Protein was determined according to the method of Bradford (Bradford, 1976).

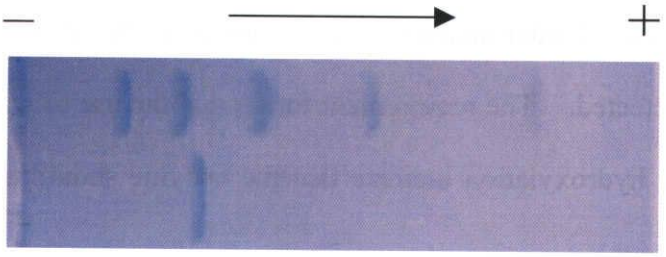

Fig. 1. **SDS–PAGE of the purified vanillyl alcohol oxidase from *B. fulva* V107**
Upper lane: low molecular weight marker proteins of 94, 67, 43, 30, and 20.1 kDa (from left to right); Lower lane: 6 μg of the purified enzyme.

Stoichiometry, enzyme activity and stability, and H_2O_2 production

The purified enzyme catalyzed the stoichiometric conversion of eugenol to coniferyl alcohol with a V_{max} of 8.86 U/mg and a K_m of 6 μM. The enzyme was stable between pH 4.5 and 9.0 and below 55°C. Above 55°C and pH 9.0, the activity was rapidly lost. The pH- and temperature optima were 7.5 and 55°C, respectively. During dehydrogenation of vanillyl alcohol, stoichiometric amounts

of H_2O_2 were formed, indicating O_2 as a final electron acceptor. All aryl-alcohol oxidases so far described share the generation of H_2O_2, which is supposed to be further used as a substrate in the ligninolytic process by laccases (phenol oxidases) and peroxidases for the formation of phenoxy radicals and aryl cation radicals, respectively (Palmieri *et al.* 1997, Martinez *et al.* 1996). In order to obtain data on the source of oxygen during hydroxylation of eugenol, a standard reaction was performed in the presence of 50 vol. % $H_2{}^{18}O$ followed by GC–MS of the enzyme product, coniferyl alcohol. The GC–MS spectrum of the product revealed both a 180 molecule ion peak and a 182 isotopic mass peak with almost the same intensities, showing that water is the source of the oxygen atom incorporated into coniferyl alcohol. Under anaerobic conditions of an N_2 atmosphere, no enzyme activity was detected. The requirement for O_2 and the use of water as a source of oxygen during hydroxylation indicate that the enzyme should be classified as an oxidase.

Prosthetic group

When untreated or oxidized by a small amount of $KMnO_4$, the enzyme exhibited a yellow color and a characteristic FAD spectrum with absorption maxima at 362 and 432 nm and shoulders at 378 and 453 nm (Fig. 2). The hypsochromic shift compared to the absorption maxima of free FAD at 370 and 450 nm and the shoulder pattern resemble those of the vanillyl alcohol oxidase from *Penicillium simplicissimum* (De Jong *et al.* 1992) and 4-ethylphenyl methylenehydroxylase from *Pseudomonas putida* DJ1 (Reeve *et al.* 1989), and are indicative of flavin covalently bound to protein. The A_{280}/A_{432} ratio of 12.2 is

similar to the A_{280}/A_{439} value of 13.4 of the *Penicillium* enzyme with 8α-(N^3-histidyl)-FAD as the covalently bound prosthetic group. Under $Na_2S_2O_4$ reducing conditions, the characteristic absorption disappeared. FAD was tightly bound and was not removed by 48-h dialysis against buffer A. Other extracellular aryl-alcohol oxidases have FAD loosely bound (Guillén *et al.* 1992, Marzullo *et al.* 1995).

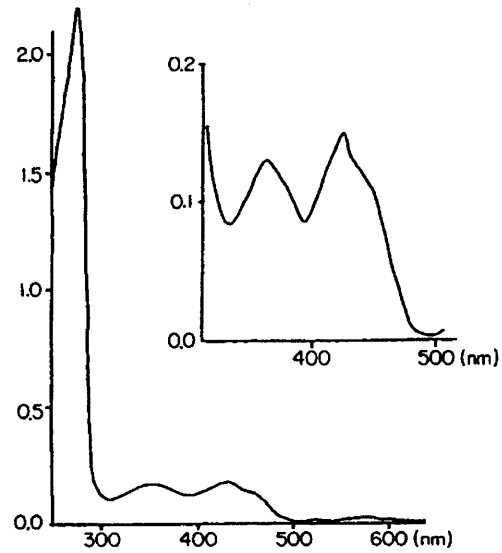

Fig. 2. **Absorption spectrum of 2 mg/ml purified vanillyl alcohol oxidase from *B. fulva* V107**

Inhibitors

After incubation of 25 mU enzyme with 1 mM compound for 15 min at 30°C, the remaining activity was tested under standard reaction conditions. The enzyme was totally inhibited by the thiol-specific reagents $AgNO_3$, $HgCl_2$, $CuCl_2$, and *p*-chloromercuribenzoate and to a lesser extent by phenylhydrazine (14% remaining activity), NaN_3 (72%), and iodoacetic acid (75%). These results might suggest the involvement of cysteine residues at the catalytic enzyme site. The

iron-chelating agents EDTA, 2,2'-bipyridyl, diethyldithiocarbamate, *o*-phenanthroline, 8-hydroxyquinoline, and Tiron (4,5-dihydroxy-*m*-benzene disulfonate) were without influence. No significant effect was found with 5,5'-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, hydroxylamine, semicarbazide, D-cycloserine, D- and L-penicillamine, L-ascorbate, $Na_2S_2O_4$, and KCN, nor with the metal salts $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $FeCl_2$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CdCl_2$, and $PbCl_2$.

Substrate specificity

The vanillyl alcohol oxidase of *B. fulva* converted a number of 4-hydroxybenzylic structures encompassing three main dehydrogenation types (Fig. 3). It dehydrogenated and hydrated 4-hydroxybenzylalcohols, including vanillyl alcohol, to the corresponding aldehydes (Table 2), thus resembling the analogous enzyme from *Penicillium simplicissimum* (Fraaije *et al.* 1995). Additionally, it shares with the *Penicillium* vanillyl alcohol oxidase the highest activity and affinity towards eugenol as a substrate, with the same K_m value. 4-Allyl-2,6-dimethoxyphenol was not converted, indicating that an additional *ortho*-methoxy substituent introduces steric constraints, and nor were other 4-allylphenols, isoeugenol, 3,4-dimethoxy-1-allylbenzene, 2-allylphenol, and allylbenzene. Furthermore, the enzyme did not convert 4-*n*-octylphenol, 4-ethylanisol, 4-tert-butylphenol, veratryl alcohol, or coniferyl alcohol. Eugenol conversion was accompanied by a unique double-bond transfer, which has been previously explained for the analogous *Penicillium* enzyme by assuming an initial *p*-quinone methide (Fraaije *et al.* 1995). On the other hand, the *Penicillium*

enzyme additionally oxidized 4-hydroxybenzylamines (Fraaije *et al.* 1995) and 4-(methoxymethyl)phenol (Fraaije and Berkel 1997), which was not observed in our enzyme.

The *B. fulva* enzyme additionally hydroxylated 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols, linking it to 4-alkylphenol methylhydroxylases from several *Pseudomonas* species (Reeve *et al.* 1989, McIntire *et al.* 1985, Hopper *et al.* 1991, Kim *et al.* 1994). In this reaction, the activity towards 4-alkylphenols decreased with longer alkyl chains (Table 2).

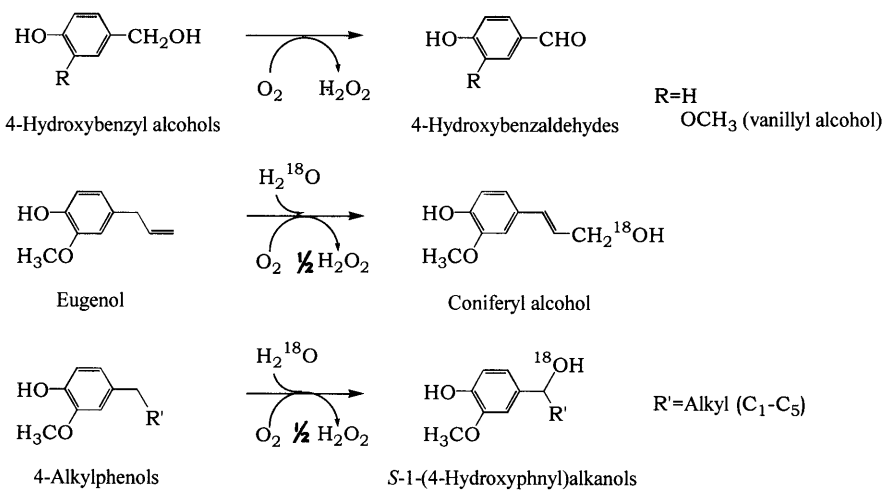

Fig. 3. **Main dehydrogenation types catalyzed by vanillyl alcohol oxidase from *B. fulva* V107**

However, *p*-cresol and 2-methoxy-4-methylphenol were oxidized in two steps to 4-hydroxybenzaldehyde. This might be due to the instability of the initially formed *p*-quinone methide, whereas longer alkyl side chains might stabilize the *p*-quinone methide. Benzyl alcohol and 2- and 3-ethylphenol did not

serve as substrates, showing the requirement for the *para*-hydroxy group in substrates. Among the *Pseudomonas* 4-alkylphenol methylhydroxylases, which mainly act on 4-alkylphenols, only the 4-ethylphenol methylenehydroxylase from *Pseudomonas putida* DJ1 has been reported to convert 4-hydroxybenzyl alcohol and eugenol, though with low activity and unknown product identity and without vanillyl alcohol being mentioned as a substrate (Reeve *et al.* 1989). Furthermore, several features – electron acceptors, induction, the enzyme structure with regard to the cytochrome *c*550 content, subunit composition, and substrate preference – distinguish the *B. fulva* vanillyl alcohol oxidase from 4-alkylphenol methylhydroxylases.

Table 2. **Substrate specificity of vanillyl alcohol oxidase from *B. fulva* V107**

Substrate	Activity (%)	K_m (μM)	Product
Eugenol	100	6.0	Coniferyl alcohol
Vanillyl alcohol	53.6	213	Vanillin
4-Hydroxybenzyl alcohol	38.8	3010	4-Hydroxybenzaldehyde
p-Cresol	0.16	n.d.	4-Hydroxybenzaldehyde
2-Methoxy-4-methylphenol	0.49	n.d.	Vanillin
4-Ethylphenol	80.7	78	1-(4-Hydroxyphenyl)ethanol
4-*n*-Propylphenol	82.8	77	1-(4-Hydroxyphenyl)propanol
4-Isopropylphenol	15.2	n.d.	2-(4-Hydroxyphenyl)propane-2-ol
4-*n*-Butylphenol	17.6	n.d.	1-(4-Hydroxyphenyl)butanol
4-*sec*-Butylphenol	11.7	n.d.	n.d.
4-*n*-Hexylphenol	10.6	n.d.	n.d.

Substrates (10 mM) were tested under the standard conditions using 0.1 U enzyme. K_m values were obtained from Lineweaver-Burk plots. Activity was determined as substrate consumption or product formation by HPLC. Activity with eugenol corresponds to 8.86 μmol min^{-1} (mg protein)$^{-1}$. Products were isolated by ethylacetate extraction and silica gel chromatography and identified by ^{1}H-NMR and GC-MS. n.d., Not determined.

Since vanillyl alcohol was the only enzyme inducer and growth substrate, the enzyme was named vanillyl alcohol oxidase. However, vanillyl alcohol was

converted with much lower affinity and only 50% activity compared to eugenol as the best enzyme substrate. Therefore, the standard activity test was performed with eugenol as the enzyme substrate.

Asymmetric synthesis of 1-(4-hydroxyphenyl)alcohols

Since the hydroxylation of prochiral 4-alkylphenols introduces an asymmetric carbon in the side chain of the 1-(4-hydroxyphenyl)alcohol products and due to the potential of derivatives of these chiral alcohols as anti-ulcer and anti-inflammatory agents and liquid crystal materials (Märki *et al.* 1988), the atuthor investigated the stereospecificity of these enzyme conversions by chiral HPLC.

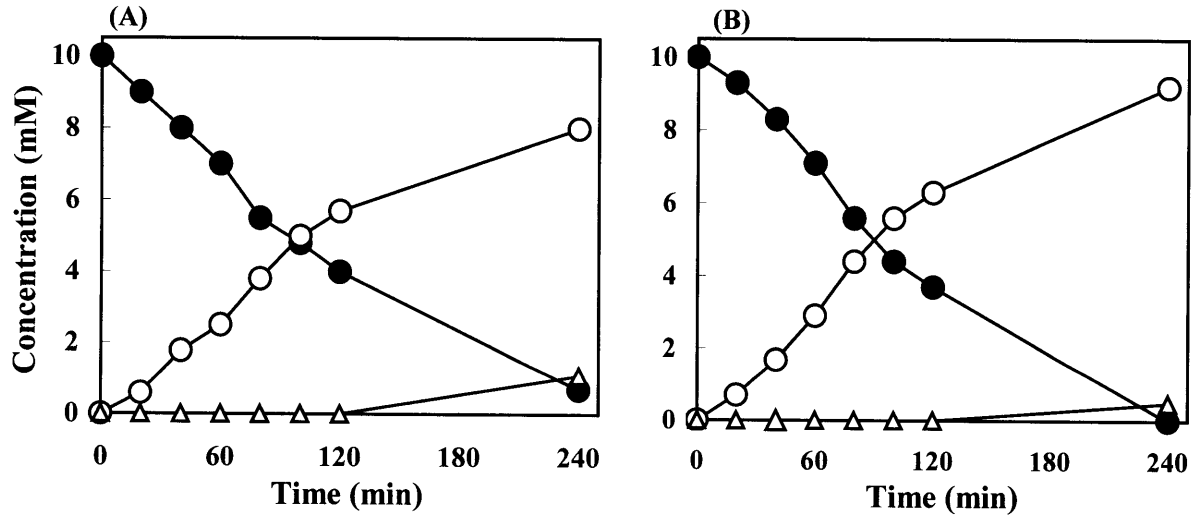

Fig. 4. **Stereospecific conversion of 4-ethylphenol (A) and 4-*n*-propylphenol (B) by vanillyl alcohol oxidase from *B. fulva* V107**
The reactions were carried out at 30°C in 2 ml buffer A containing 10 mM 4-alkylphenol and 25 mU vanillyl alcohol oxidase. Enantiomeric excesses were determined using a chiral HPLC column. Symbols: 4-ethylphenol or 4-*n*-propylphenol (•), 1-(4-hydroxyphenyl)ethanol or 1-(4-hydroxyphenyl)propanol (○), 4-hydroxyacetophenone or 4-hydroxypropiophenone (Δ).

Table 3. **pH-dependent enantioselectivity of vanillyl alcohol oxidase during conversion of 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols**

pH	(*S*)-1-(4-Hydroxy-phenyl)ethanol (% *e.e.*)	(*S*)-1-(4-Hydroxy-phenyl)propanol (% *e.e.*)
4.0	67.6	66.0
5.0	71.4	68.4
6.0	75.8	75.2
7.0	81.9	86.0
8.0	79.6	72.0
9.0	73.6	42.8

Buffer A was used at pH 6.0 and 7.0, acetate/sodium acetate at pH 4.0 and 5.0, Tris/HCl at pH 8.0 and glycine/NaOH at pH 9.0 (each 50 mM). Each reaction was carried out until the substrate was completely consumed, and the product chirality was analyzed by chiral HPLC.

When 25 mU enzyme were used, (*S*)-1-(4-hydroxyphenyl)ethanol with 81.9% *e.e.* and (*S*)-1-(4-hydroxyphenyl)propanol with 86% *e.e.* together with small amounts of the corresponding ketones as further dehydrogenation by–products were formed (Fig. 4). However, using 250 mU enzyme, more than 80% of both alcohols were further converted to the respective ketones. The enantioselectivity of the enzyme depended on the pH (Table 3), a feature not described so far for similar enzymes. The author assumes a pH-induced conformational change at the catalytic enzyme site rather than pH-influence on the product, because the enantiomeric excesses of the products were not changed by acid or alkali treatment. Other reported bioconversions for the synthesis of chiral 1-(4-hydroxyphenyl)ethanol using different *Pseudomonas* enzymes depended on the addition of an electron acceptor and resulted in the production of the (*R*)-enantiomer by 4-ethylphenol methylenehydroxylase with 92–98% *e.e.* (Reeve *et al.* 1990) and the (*S*)-enantiomer by *p*-cresol methylhydroxylase with 31–39% *e.e.* (McIntire *et al.* 1984). 4-Alkylphenol methylhydroxylases are distinguished

from vanillyl alcohol oxidase by the tendency to further dehydrogenate the alcohols to the corresponding ketones, and the additional formation of 4-vinylphenol as a by-product in the case of *p*-cresol methylhydroxylase, which was not observed in our enzyme. An organic chemical method for the synthesis of enantiomeric 1-(4-hydroxyphenyl)alcohols using the asymmetric resolution of a protected phenylalcohol derivative has also been reported; however the theoretical yield is limited to 50% (Everhart and Craig 1991). The enzymatic synthesis described here has the advantages of a theoretical yield of 100%, a one-step procedure, and mild reaction conditions.

SUMMARY

Vanillyl alcohol oxidase from *Byssochlamys fulva* V107 was purified to apparent homogeneity as shown by SDS–PAGE and gel-permeation HPLC. The enzyme is a homodimeric flavoenzyme consisting of two 58 kDa subunits. It catalyzes the dehydrogenation of different 4-hydroxybenzylic structures, including the conversion of 4-hydroxybenzylalcohols such as vanillyl alcohol to the corresponding aldehydes, eugenol to coniferyl alcohol, and 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols. The latter reaction was (*S*)-stereospecific and was used for the synthesis of (*S*)-1-(4-hydroxyphenyl)ethanol and -propanol with enantiomeric excesses of 81.9 and 86.0%, respectively. The catalytic and structural similarities to *Penicillium* vanillyl alcohol oxidase and *Pseudomonas* 4-alkylphenol methylhydroxylases were discussed.

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Section 2

Microbial Synthesis of Coniferyl Alcohol by the Fungus *Byssochlamys fulva* V107

Coniferyl alcohol is of interest for food and pharmaceutical industries due to its widespread application as an antioxidant and flavor in foods, beverages and cosmetics, and as an antioxidant in anti-tumor agents (Pannala *et al.* 1998, Chan *et al.* 1995, Wu *et al.* 1998, Deigner *et al.* 1994). Studies of the structure and chemical synthesis of lignin are also reported (Quideau and Ralph 1992). Coniferyl alcohol has been mainly prepared by chemical reduction of ferulic acid, which for its part can be easily isolated from plant sources, for example, from corn kernels (Antrim and Harris 1977). However, the recent definition of 'natural flavors' as food additives requiring enzymatic or fermentative production by US and European legislation (Code of Federal regularions, 1993) has increased the demand for a biotechnological preparation of coniferyl alcohol (Krings and Berger 1998).

Coniferyl alcohol occurs as a lignin-related intermediate in the microbial degradation of eugenol. Eugenol is an abundant essential oil from clove tree *Syzigium aromaticum* (sy. *Eugenia cariophyllus*), and is catabolized by several microorganisms via coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, vanillinate, protocatechuate, ring cleavage and the β-ketoadipate pathway (Tadasa 1977, Rosazza *et al.* 1995). However, in *Byssochlamys fulva* V107, which was isolated from soil as an effectively vanillyl alcohol-using fungus as described in Section 1(CHAPTER II), eugenol was only metabolized to coniferyl alcohol; a

small amount of coniferyl aldehyde without further degradation, due to the lack of a coniferyl aldehyde oxidizing enzyme. The conversion of eugenol to coniferyl alcohol is catalyzed by vanillyl alcohol oxidase, which also oxidizes vanillyl alcohol to vanillin, as shown with the purified enzymes from *Byssochlamys fulva* V107 and *Penicillium simplicissimum* (Fraaije *et al.* 1995). Vanillyl alcohol oxidase from *Byssochlamys fulva* V107 was able to convert eugenol with an approximately 2-fold higher rate than vanillyl alcohol (Section 1, CHAPTER II), which was used here for the accumulation of coniferyl alcohol. Due to a low enzyme activity for the second dehydrogenation step from coniferyl alcohol to coniferyl aldehyde, it was expected that only a small amount of coniferyl aldehyde was formed under the bioconversion conditions by the resting cells of *B. fulva* V107 as shown in Fig. 1.

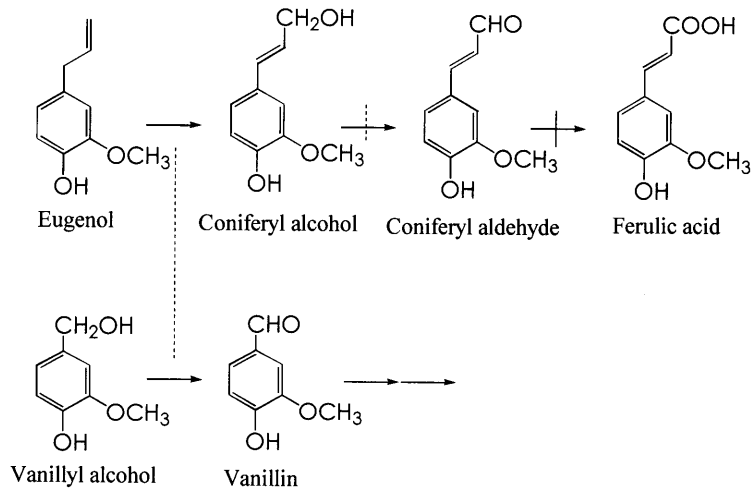

Fig. 1. **Bioconversion of eugenol to coniferyl alcohol and small amounts of coniferyl aldehyde by *B. fulva* V107**
The bioconversion is based on the enzyme vanillyl alcohol oxidase, which has the additional ability to oxidize vanillyl alcohol, used as enzyme inducer, to vanillin. No enzyme activity for the conversion from coniferyl aldehyde to ferulic acid was present in this organism, and only a minor oxidation of coniferyl alcohol to coniferyl aldehyde was observed under the conditions used here.

In this section, the author investigated microbial synthesis of coniferyl alcohol by resting cells of *B. fulva* V107 containing vanillyl alcohol oxidase catalyzing the hydroxylation of eugenol.

MATERIALS AND METHODS

Preparation of resting cells of Byssochlamys fulva V107

The whole cells biocatalyst was obtained by 50 h of cultivation of *Byssochlamys fulva* V107 at 28°C with reciprocal shaking in a 2-l flask with 400 ml of medium consisting of 3 g of vanillyl alcohol as enzyme inducer, 15 g of glycerol, 2.5 g of $(NH_4)_2SO_4$, 1 g of yeast extract (Oriental Yeast), 2 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7\ H_2O$ per liter and metals in final concentrations of 0.2 mg of $FeSO_4 \cdot 7\ H_2O$, 0.4 mg of $CaCl_2 \cdot 2\ H_2O$, 0.3 mg of H_3BO_3, 0.04 mg of $CuSO_4 \cdot 5\ H_2O$, 0.1 mg of KI, 0.4 mg of $MnSO_4 \cdot 7\ H_2O$, and 0.2 mg of $Na_2MoO_4 \cdot 2\ H_2O$ per liter (pH 7.0), resulting in a dry cell yield of 1.9 g.

Microbial synthesis of coniferyl alcohol

The bioconversion was done with 1.9 g cells (dry weight) in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, complemented with 5% (v/v) *n*-nonane in order to increase the solubility of hydrophobic eugenol. *n*-Nonane has been found to be the most suitable additive when tested against Tween 20, Plysurf A210G, and *n*-hexadecene (each tested at 1, 5, and 10%, v/v). Eugenol was added in two steps, initially using 80 mM followed by a second addition of 50 mM after 9 h.

Analytical methods for production of coniferyl alcohol

The bioconversion was followed by HPLC using a Shimadzu LC-6A (Kyoto, Japan) with an ODS C18 column (4.6 × 150 mm, M&S Instruments, Tokyo, Japan) and a methanol/H_2O/acetic acid (45:52:3, v/v/v) eluent at a flow rate of 1 ml/min monitored at 280 nm.

Purification and identification of coniferyl alcohol

Synthesized coniferyl alcohol was isolated from the reaction mixture by ethylacetate extraction, evaporation, silica gel chromatography (Wakogel C-300, Wako, Osaka, Japan) with benzene/methanol (95:5, v/v) as the mobile phase and crystallization. The identity and purity of the product was confirmed by ^{1}H-NMR in a Bruker WM-360 spectrometer (Billerica, USA) with methanol-D as solvent, and GC-MS in a Trio-1 mass spectrometer (Raleigh, USA) connected with a 5890 Hewlett-Packard gas chromatograph (Palo Alto, USA) plus DB-1 capillary column (J&W Scientific, Tokyo, Japan) with helium as the carrier gas and a temperature program of 1 min at 50°C and 50–250°C at 15°C/min, using authentic coniferyl alcohol (Aldrich, Milwaukee, USA) as a reference.

RESULTS AND DISCUSSION

Purification and identification of synthesized coniferyl alcohol

Synthesized coniferyl alcohol by microbial conversion was isolated from the reaction mixture by ethylacetate extraction, evaporation, silica gel chromatography with an isolated yield of 48%. The identity and purity of the product was confirmed by ^{1}H-NMR and GC-MS, using authentic coniferyl alcohol as a reference.

Microbial synthesis of coniferyl alcohol

After 36 h of reaction, 123 mM (21.9 g/l) coniferyl alcohol (94.6% of the theoretical yield) were accumulated with 4.0 mM coniferyl aldehyde as a by-product (Fig. 2).

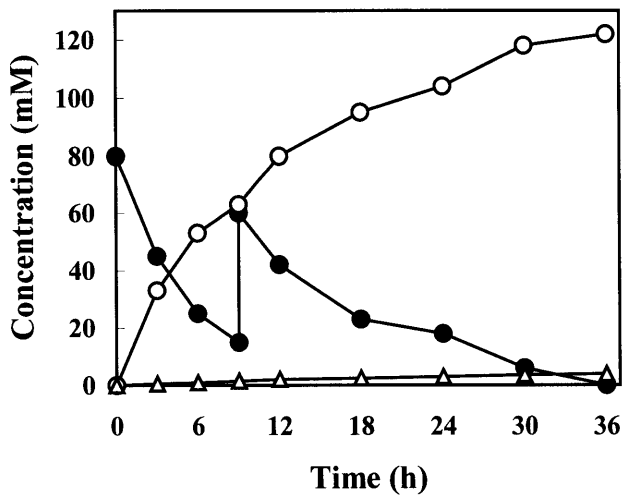

Fig. 2. **Course of the Fed-batch Bioconversion of Eugenol to Coniferyl Alcohol by *B. fulva* V107**
The arrow indicates the addition of 50 mM eugenol after 9 h. Symbols: eugenol (●), coniferyl alcohol (○), coniferyl aldehyde (Δ).

A similar biotransformation of eugenol catalyzed by a *Pseudomonas* species gave a mixture of 17.9 mM (3.2 g/l) coniferyl alcohol (43.5% of the theoretical yield), 23.5% ferulic acid, 7.3% unreacted eugenol, and traces of coniferyl aldehyde and vanillic acid after 48 h (Rabenhorst 1996). Compared with this bioconversion, the fed-batch reaction described here has advantages with regard to productivity and yield of coniferyl alcohol and a reduced amount of by-products. Furthermore, compared with the same reaction catalyzed by a bacterial eugenol dehydrogenase characterized in CHAPTER I, the fungal biocatalyst has the advantage that it uses O_2 as a natural electron acceptor, as shown in the studies of the purified enzyme (Section 1, CHAPTER II), without requiring a costly, external electron acceptor such as PMS.

SUMMARY

In this section, the author investigated microbial production of coniferyl alcohol by resting cells of *Byssochlamys fulva* V107. Coniferyl alcohol (123 mM = 21.9 g/l) was synthesized from eugenol with a yield of 94.6% in a 36 h fed-batch bioconversion using resting cells of the fungus exhibiting vanillyl alcohol oxidase activity. Since the resting cells contain few activities of coniferyl alcohol oxidase and no activity of coniferyl aldehyde oxidase, the conversion is most efficient as far as the author knows.

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APPENDIX

Section 1

Ferulic Acid Production from Eugenol and Clove Oil by *Pseudomonas fluorescens* E118

Clove oil is essential oil isolated from buds and leaves of *Syzygium aromaticum* by steam distillation. Eugenol accounts for approximately 80% (w/w) of clove oil as a main component and has been used as aromatherapy oil, sterilizer in mouth or painkiller (Rabenhorst 1996). The author previously reported that the initial step of eugenol degradation by *Pseudomonas fluorescens* E118 was catalyzed by eugenol dehydrogenase to produce coniferyl alcohol (CHAPTER I). Coniferyl aldehyde and ferulic acid are consecutively formed as the intermediates of eugenol degradation (Tadasa and Kayahara 1983). The following step, the decarboxylation of ferulic acid, results in the formation of vanillin, an essence in great demand. The formed vanillin is further degraded β-ketoadipic acid via vanillic acid and protocatechuic acid. Since useful chemicals are involved in the degradation pathway of eugenol, its microbial transformation has attracted considerable attention (Washisu *et al.* 1993, Hopp and Rabenhorst 1994). Although microbial production of natural aroma chemicals from eugenol has been reported (Rabenhorst 1996, Overhage *et al.* 1999), there has been no report on the production of useful chemicals from clove oil as the raw material. Since clove oil is much cheaper than eugenol, the author focused it as the starting material. In this section, the author describes the microbial

production of ferulic acid, a useful antioxidant (Graf 1992) and a material with a natural aroma (Muhein and Lerch 1999, Oddou *et al.* 1999, Li and Rosazza 2000), from eugenol and clove oil.

P. fluorescens E118 was isolated in CHAPTER I. In addition, some eugenol-degrading bacteria were isolated using medium containing 0.1 g/l eugenol as a sole carbon source. Clove oil contains some antibacterial impurities such as β-caryophyllene and α-humulene in addition to eugenol (Muchalal and Crouzet 1985, Simic *et al.* 2002, Shafi *et al.* 2002). Therefore, the author tested the growth of the isolated strains on nutrient medium containing 2 g/l clove oil. The nutrient medium consisted of 5 g of peptone, 0.5 g of yeast extract, 5 g of meat extract and 2 g of NaCl in 1 l of tap water (pH 7.0). Except for *P. fluorescens* E118, which was used in our previous study on eugenol dehydrogenase, the tested eugenol-degrading miccroorganisms could not grow in the presence of 2 g/l clove oil.

The basal medium for ferulic acid production contained 4 g of K_2HPO_4, 1 g of Na_2HPO_4, 0.5 g of Polypepton, 0.2 g of NaCl and 0.2 g of $MgSO_4$ in 1 l of tap water (pH 7.0). Subculture was carried out in 200 ml of nutrient medium and transferred into a jar fermentor (model TS-F; Takasugi, Tokyo) containing 2 l of the basal medium. Cultivation was carried out at 30°C with an agitation (200 rpm) and an aeration (0.2, v/v/m), in which eugenol (Tokyo Kasei Kogyo, Tokyo) or clove oil (Wako Pure Cheimical Industries, Osaka) was added. Eugenol, ferulic acid, coniferyl alcohol and vanillic acid were determined by HPLC as described in CHAPTER I.

In the conversion of eugenol into ferulic acid by *P. florescens* E118,

eugenol concentration in the culture medium was kept below 1.0 g/l. After 28-h cultivation, 3.5 g/l ferulic acid accumulated in the culture medium, with a molar conversion yield of 51% from eugenol (Fig. 1). Prolonged cultivation caused the accumulation of coniferyl alcohol in the culture medium.

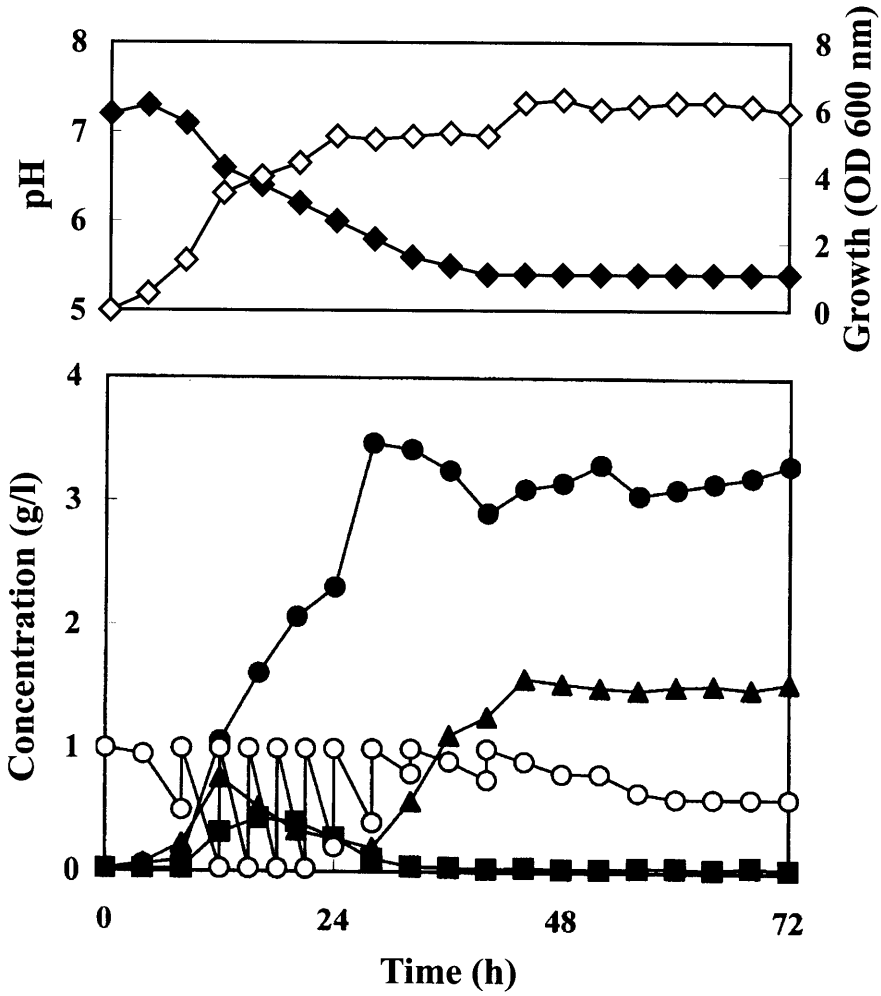

Fig. 1. **Formation of ferulic acid from eugenol**
The basal medium initially contained 1.0 g/l eugenol. According to the consumption of eugenol, eugenol was fed to give a final concentration of 1.0 g/l. Symbols: pH (♦), growth (◊), eugenol (○), ferulic acid (●), coniferyl alcohol (▲), vanillic acid (■).

As shown in Fig. 1, the decrease in pH of culture broth affected ferulic acid production. Therefore, the effect of pH on ferulic acid production was

examined. When the pH of culture medium was controlled at 5.0, 6.0, 7.0 or 8.0, ferulic acid production was observed in the cultivation at pH 7.0 and 8.0. The optimal pH was 7.0; 3.7 g/l ferulic acid accumulated for 40-h cultivation. The cultivation at pH 8.0 for 40 h resulted in almost half of ferulic acid production (1.8 g/l). In the case of pH 5.0 and 6.0, no formation of ferulic acid was observed.

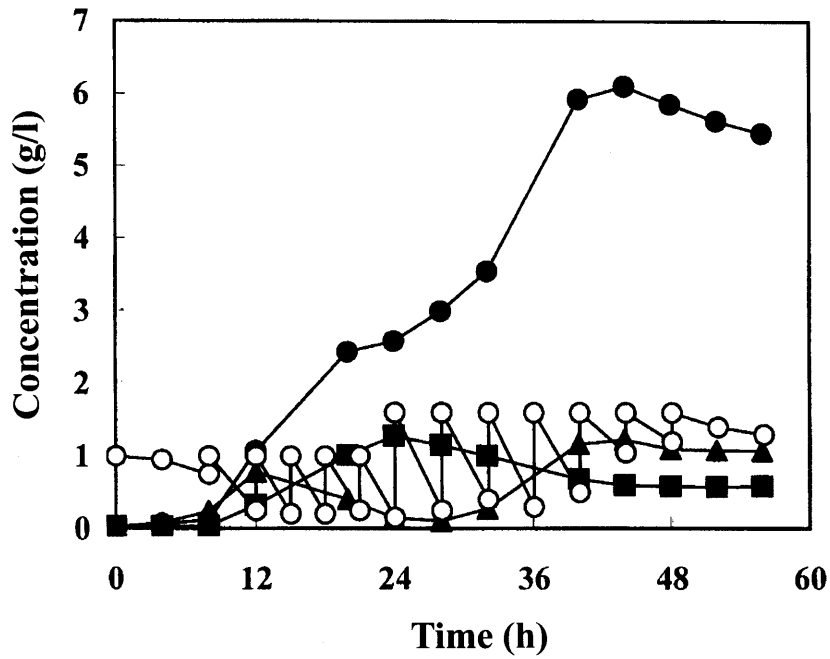

Fig. 2. **Effect of eugenol concentration on ferulic acid formation** The basal medium initially contained 1.0 g/l eugenol. During the initial 24-h cultivation, eugenol concentration was kept below 1.0 g/l. After24-h cultivation, eugenol was fed to give a final concentration of 1.6 g/l. Symbols: eugenol (○), ferulic acid (●), coniferyl alcohol (▲), vanillic acid (■).

Although *P. fluorescens* E118 grew slowly in the basal medium supplemented with eugenol at a concentration of more than 1.0 g/l, the conversion of eugenol into ferulic acid was not inhibited. Therefore, after the bacterium had grown well, eugenol concentration was raised up to 1.6 g/l (Fig. 2). Under pH control at 7.0, the accumulation of ferulic acid in the culture medium increased to

6.1 g/l by 44-h incubation, with a molar conversion yield of 54%. In the fermentation of ferulic acid from eugenol, the accumulation of coniferyl alcohol and the further degradation of ferulic acid into vanillic acid were observed. However, the productivity of ferulic acid by *P. fluorescens* E118 is superior to that by *Pseudomonas* sp., which produced 5.8 g/l ferulic acid by 75 h-cultivation (Rabenhorst 1996).

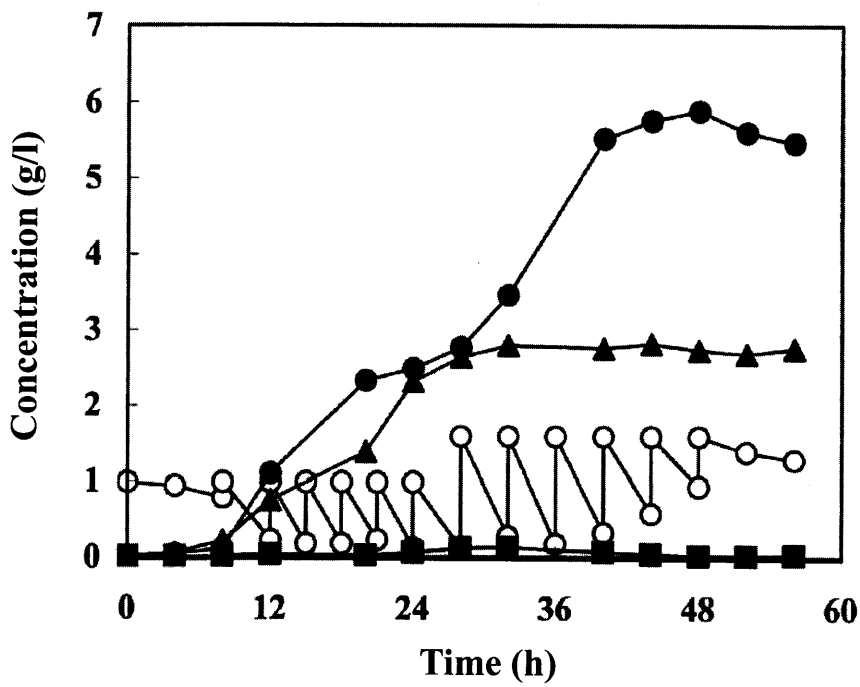

Fig. 3. **Formation of ferulic acid from clove oil**
The basal medium initially contained 1.25 g/l clove oil. The clove oil concentration was kept below 1.25 g/l during the initial 32-h cultivation. After the bacterial growth by 32-h cultivation, clove oil was fed to give a final concentration of 2.0 g/l. Symbols: eugenol (○), ferulic acid (●), coniferyl alcohol (▲), vanillic acid (■).

The author attempted, for the first time, to produce ferulic acid from clove oil as the starting material. The clove oil concentration was kept below 1.25 g/l during the initial 24-h cultivation, and then raised up to around 2 g/l until

48-h cultivation at pH 7.0. From a total amount of 26.6 g of clove oil, 11.6 g of ferulic acid accumulated in 2 l of culture broth (Fig. 3). Clove oil is a cheap and renewable resource, and further studies on its application are under way.

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Section 2

Degradation of Isoeugenol by *Pseudomonas putida* I58

Lignin-related phenylpropanoides such as eugenol and isoeugenol have attracted attention as natural renewable resources for the production of useful chemicals (Rabenhorst 1996). Especially, eugenol has great potential as a starting material for the synthesis of aromatic flavorings and aromas such as vanillin. *Pseudomonas* and *Corynebacterium* strains degrade eugenol into vanillin via coniferyl alcohol, coniferyl aldehyde and ferulic acid as shown in Fig. 1 (Tadasa 1977, Tadasa and Kayahara 1983). In CHAPTER I, the author has elucidated that the initial step of eugenol degradation is the double bond-transferring hydroxylation catalyzed by eugenol dehydrogenase. The gene loci *ehyA* and *ehyB* of *Pseudomonas* sp. HR199 were identified as the structural genes of eugenol hydroxylase (dehydrogenase) (Priefert *et al.* 1999). The biotransformation of eugenol to ferulic acid was reported, in which *Ralstonia eutropha* H16 carrying *ehyAB*, *calA* and *calB* genes, encoding eugenol hydroxylase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase, respectively, was used (Overhage *et al.* 2002). With respect to isogugenol, there have been few studies on its degradation pathway and efficient bioconversion system. *Bacillus* sp. transforming isoeugenol to vanillin was recently reported (Shimoni *et al.* 2000). In this section, the author isolated a novel isoeugenol-degrading bacterium and attempted biocatalytic conversion of isoeugenol, since isoeugenol is cheap and abundant in nature.

A basal medium contained 1 g of yeast extract (Oriental Yeast, Tokyo), 4

g of NH_4Cl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 g of K_2HPO_4 and 1 ml of metal mixture in 1 l of tap water, pH 7.0. The metal mixture consisted of 0.2 g of $FeSO_4 \cdot 7H_2O$, 0.4 g of $CaCl_2 \cdot 2H_2O$, 0.3 g of H_3BO_3, 0.04 g of $CuSO_4 \cdot 5H_2O$, 0.1 g of KI, 0.4 g of $MnSO_4 \cdot 7H_2O$ and 0.2 g of $NaMoO_4 \cdot 2H_2O$ in 1 l of distilled water. Enrichment culture was carried out at 28°C in a 500-ml shaking flask containing 50 ml of the basal medium supplemented with 5 g/l isoeugenol. Among the isoeugenol-degrading microorganisms isolated from soils, a bacterial strain I58 rapidly grew well and was used for the following experiments. The stain I58 was identified as *Pseudomonas putida* by NCIMB (National Collections of Industrial Food and Marine Bacteria).

Table 1. **Utilization of eugenol-related compounds as a carbon source**

Carbon sources	Growth
Isoeugenol	+
Eugenol	–
Coniferyl alcohol	–
Coniferyl aldehyde	–
Ferulic acid	–
Vanillin	+
Vanillic acid	+

P. putida I58 was inoculated in the basal medium containing the indicated carbon source at 1 g/l. After 48-h cultivation at 28°C with shaking at 120 strokes/min, bacterial growth was checked.

P. putida I58 utilized isoeugenol, vanillin and vanillic acid as a sole carbon source in the basal medium, whereas eugenol, coniferyl alcohol, coniferyl aldehyde and ferulic acid, which are intermediates in eugenol degradation, were not utilized (Table 1). This suggests that isoeugenol is directly degraded to

vanillin without the formation of ferulic acid as shown in Fig. 1. The subsequent degradation pathway of isoeugenol is probably similar to that of eugenol in *P. fluorescens* E118 (CHAPTER I).

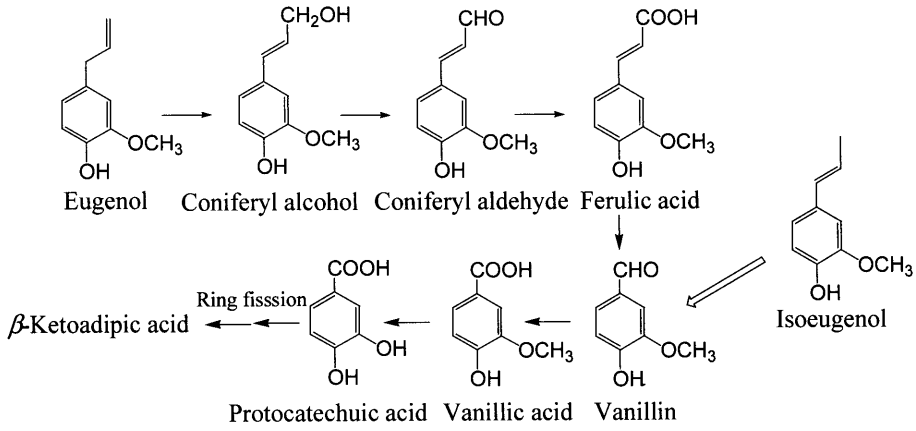

Fig. 1. **Degradation pathway of eugenol and isoeugenol**

Culture medium for the improvement of isoeugenol-degrading activity was optimized. The cultivation was carried out at 28°C for 24 h with shaking at 120 strokes/min in a 500-ml flask containing 50 ml of medium. Isoeugenol was determined by the method described previously in CHAPTER I. When meat extract (Kyokuto, Tokyo) was added at 10 g/l to the basal medium containing 5 g/l isoeugenol, the isoeugenol added was completely consumed within 24 h. In the growth experiment using *Bacillus* sp. (Shimoni *et al.* 2000), it takes over 12 days to degrade 1.6 g/l isoeugenol completely in the presence of glucose and casamino acids. *P. putida* I58 cells grown on the optimized medium were harvested by centrifugation at 16000 × *g* for 10 min, and then washed with 100 mM potassium phosphate buffer (pH 7.0). The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.0) and used as resting cells. A standard

mixture for resting cells reaction contained 20 mg of cells (as dry matter), 10 mM isoeugenol and 100 mM potassium phosphate buffer (pH 7.0) in a total volume of 2 ml. The reaction was carried out at 30°C with shaking at 160 strokes/min. The resting cells rapidly converted isoeugenol into vanillic acid with a molar conversion yield of 98% (Fig. 2a). In the course of the reaction, a small amount of vanillin was formed. When vanillin was used as a substrate instead of isoeugenol, vanillin was completely converted into vanillic acid (Fig. 2b). Under anaerobic conditions, no formation of vanillin and vanillic acid was observed, indicating the involvement of oxidative reactions in the conversion of eugenol into vanillic acid. The cells grown on the basal medium containing 10 g/l meat extract and 5 g/l eugenol did not exhibit isoeugenol-degrading activity. Thus, the enzymes catalyzing isoeugenol degradation are specifically induced by isoeugenol in *P. putida* I58 cells.

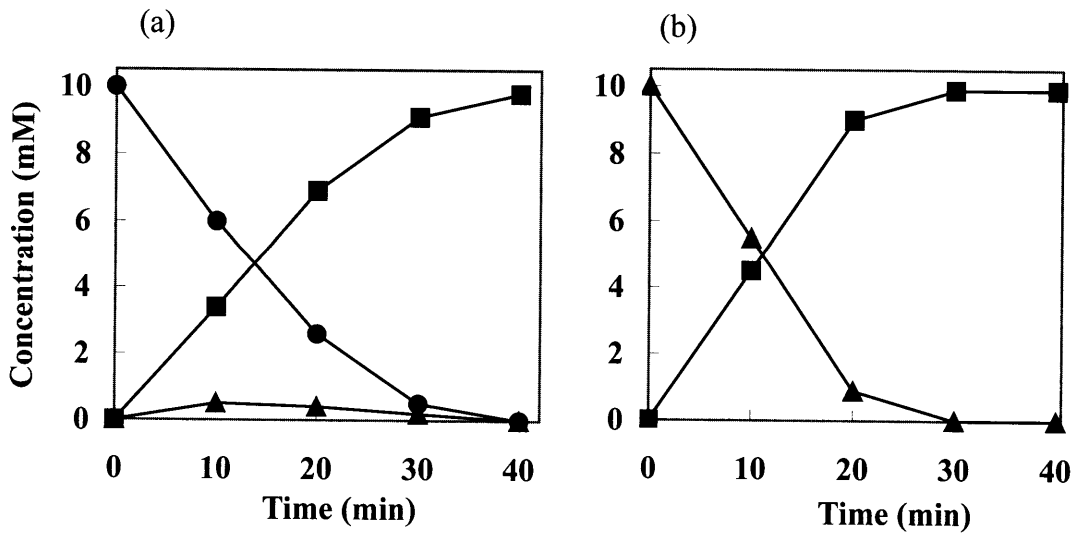

Fig. 2. **Conversion of isoeugenol (a) and vanillin (b) by resting cells of *P. putida* I58**
Symbols: isoeugenol (●), vanillin (▲), vanillic acid (■).

This is the first report regarding isoeugenol degradation by a Gram-negative bacterium, pseudomonad. Recently, the degradation pathway of *trans*-anethole, an analogue of isoeugenol, has been elucidated using *Arthrobacter* sp. (Shimoni *et al.* 2002). As for isoeugenol degradation, *Bacillus* sp. capable of transforming isoeugenol to vanillin has been reported (Shimoni *et al.* 2000). Although the enzymes involved in the degradation pathways have not been characterized at all, the initial step of isoeugenol degradation is of interest because of the oxidative cleavage of the double bond of isoeugenol. Cell-free extract prepared from *Bacillus* sp. cells converts isoeugenol into vanillin with a conversion yield of 14%, and further degradation to vanillic acid is observed (Shimoni *et al.* 2000). The degradation activity of isoeugenol of *P. putida* I58 cells is extremely higher than that of *Bacillus* sp. If the author could inhibit the degradation of vanillin into vanillic acid, *P. putida* I58 would be a promising biocatalyst for the industrial production of vainillin, natural flavor, from isoeugenol.

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CONCLUSION

In this thesis, the author has studied on the microbial conversion of plant essential oils, eugenol and isoeugenol. The author has isolated an eugenol-degrading bacterium, *Pseudomonas fluorescens* E118, and purified and characterized eugenol dehydrogenase from this strain, which catalyzed novel double bond-transferring hydroxylation reaction. The author has also isolated *Byssochlamys fulva* V107, of which vanillyl alcohol oxidase was purified and characterized. The enzyme converts eugenol to form coniferyl alcohol; the same reaction is catalyzed by eugenol dehydrogenase. The author attempted to establish the microbial production process of useful chemicals by using the above two kinds of microbial enzymes. In addition, the author has isolated an isoeugenol-degrading bacterium, *Pseudomonas putida*. The author attempted to throw the light on the outline of the microbial degradation of isoeugenol. The occurrence of a novel enzyme to cleavage the double bond of isoeugenol was estimated.

The findings obtained in each chapter are summarized as follows:

CHAPTER I

Section 1

A eugenol-degrading bacterium, *Pseudomonas fluorescens* E118, was isolated from soil through the conventional enrichment technique using eugenol as a sole carbon source. The strain had strong ability to degrade eugenol. The enzyme involved in the initial step of the degradation of eugenol, eugenol

dehydrogenase, was purified from the eugenol-induced cells. The dehydrogenase was purified to homogeneity. The enzyme consists of a 10-kDa-cytochrome c_{550} and a 58-kDa subunit with flavin. The activity of the purified enzyme was dependent on an electron acceptor such as PMS, DCIP, cytochrome *c* or $K_3Fe(CN)_6$. The large and small subunits contained flavin and heme, respectively. The enzyme belongs to the group of a flavo-cytochrome protein, and the hydroxylation reaction proceeds in accordance with the electron transfer to cytochrome via flavin in the course of reaction. The author confirmed that the oxygen atom of $H_2{}^{18}O$ was incorporated into the hydroxyl of coniferyl alcohol. The enzyme catalyzed the following three kinds of dehydrogenation reaction: (1) conversion of eugenol to coniferyl alcohol, (2) 4-alkylphenols to 1-(4-hydroxyphenyl) alcohols and (3) 4-hydroxybenzylalcohols to the corresponding aldehyde. The catalytic property was very similar to *Penicillium* vanillyl alcohol oxidase.

Section 2

The enantioselectivity of the hydroxylation of 4-alkylphenols catalyzed by eugenol dehydrogenase was studied. The reaction products were isolated and identified, and then their absolute configurations have been examined. It was revealed that the hydroxylation reaction proceeded enantioselectively. (*S*)-1-(4-Hydroxyphenyl)ethanol and (*S*)-1-(4-hydroxyphenyl)propanol were synthesized with enantiomeric excesses of 96.6% and 95.2%, respectively, from the corresponding 4-alkylphenols. The author also found that the enantioselectivity was pH-dependent.

CHAPTER II

Section 1

A vanillyl alcohol-degrading mold (strain V107) was isolated and identified as *Byssochlamys fulva*. The vanillyl alcohol oxidase was purified from *Byssochlamys fulva* V107 to homogeneity. The purified enzyme catalyzed the same reactions as eugenol dehydrogenase and its substrate specificity was similar to that of eugenol dehydrogenase. The enzyme was a homodimeric flavoenzyme consisting of two 58 kDa subunits. It catalyzed the following three kinds of reaction, the conversion of 4-hydroxybenzylalcohols such as vanillyl alcohol to the corresponding aldehyde, eugenol to coniferyl alcohol, and 4-alkylphenols to 1-(4-hydroxyphenyl)alcohols. The latter reaction was *S*-stereospecific and was used for the synthesis of *S*-1-(4-hydroxyphenyl) ethanol and *S*-1-(4-hydroxyphenyl) ethanol-propanol with enantiomeric excesses of 81.9 and 86.0%, respectively.

Section 2

Vanillyl alcohol oxidase and eugenol dehydrogenase catalyze the conversion of eugenol into coniferyl alcohol. Vanillyl alcohol oxidase is more advantageous than eugenol dehydrogenase for the enzymatic production of coniferyl alcohol, because it requires the addition of no any electron acceptor. Thus, the author examined the production of coniferyl alcohol using vanilly alcohol oxidase of *Byssochlamys fulva* V107. Coniferyl alcohol (123 mM = 21.9 g/l) was efficiently produced from eugenol with a yield of 94.6% in a 36 h fed-batch bioconversion using *B. fulva* V107 cells. The accumulation of

coniferyl alcohol with high conversion yield has been attained using *B. fulva* V107 vanillyl alcohol oxidase.

APPENDIX

Section 1

The production of ferulic acid, a useful antioxidant, from eugenol and clove oil was investigated using degradaion pathway of *Pseudomonas fluorescens* E118. When *Pseudomonas fluorescens* E118 was cultivated with intermittent addition of eugenol, 6.1 g/l ferulic acid accumulated under the optimized culture conditions, with a molar conversion yield of 54% by 44-h cultivation. This strain also produced 5.8 g/l ferulic acid with intermittent addition of clove oil. Since clove oil is much cheaper than eugenol, the ferulic acid production from clove oil by the bacterium is promising for industrial production of ferulic acid, a useful antioxidant.

Section 2

Pseudomonas putida I58 was isolated from soil through enrichment culture using isoeugenol as a sole carbon source. The strain utilized isoeugenol, vainillin and vanillic acid as a carbon source. On the other hand, the intermediates of eugenol-degrading pathway, eugenol, coniferyl alcohol, coniferyl aldehyde and ferulic acid, were not utilized by this strain. The resting cells of *P. putida* I58 rapidly converted isoeugenol into vanillic acid via vanillin with a conversion yield of 98% by 40 min incubation. The occurrence of the enzymes to catalyze the direct cleavage of double bond of isoeugenol was estimated.

The author has obtained some significant results and extended his knowledge from the studies on the microbial metabolisms of eugenol and isoeugenol through the present thesis. Based on the valuable information and knowledge, the author intends to establish new production process of useful compounds from renewable resource, eugenol, isoeugenol and clove oil. At the same time, the author also realizes that further studies are necessary to accomplish his purpose.

ACKNOWLEDGEMENT

The author would like to express his gratitude to Professor Toru Nagasawa, of the Department of Biomolecular Science, Gifu University, for his continuous guidance and encouragement throughout this work.

The author is also grateful to Associate Professor Toyokazu Yoshida, of the Department of Biomolecular Science, Gifu University, for his thoughtful advice and instruction throughout this work.

The author is greatly indebted to Professor Tsuyoshi Sugio, Okayama University for his valuable discussion and technical support through this work.

The author appreciates Dr. Marco Wieser for his cooperation, who worked as JSPS fellow at Gifu University.

The author wishes to express his thanks to General Manager Yutaka Kakuyama, of Fine chemical Division, General Manager Masaharu Seki, of Yokohama Research Center, Dr. Masato Harada, Dr. Hiroshi Morita and Dr. Jun Hiraki, of Chisso Corporation for their supports and encouragement in this work.

The author is deeply grateful to my colleagues of the Department of Biomolecular Science, Gifu University and of the Department of Biological Function and Genetic Resources Science, Okayama University, the laboratory members of Chisso Corporation Yokohama Research Center.

Finally, the author expresses his gratitude to his parents and his wife, Ayako, for their incessant understanding and encouragement.

PUBLICATIONS

CHAPTER I

Section 1
Furukawa H., Wieser M., Morita H.,Sugio T. and Nagasawa T. (1998)
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CHAPTER II

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J. Biosci. Bioeng. in press