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Note

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

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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 *Byssochlamys fulva* V107.

Key words: flavors; fragrances; ligninolysis; coniferyl alcohol; fungal oxidoreductase

Coniferyl alcohol is of interest for the food and pharmaceutical industry due to its widespread application as an antioxidant and flavor in foods, beverages, and cosmetics, and as an antioxidant in anti-tumor agents,[1] and its requirement for studies of the structure and chemical synthesis of lignin.[2] It has been mainly prepared by chemical reduction of ferulic acid,[2] which for its part can be easily isolated from plant sources, for example from corn kernels.[3] However, the recent definition of 'natural flavors' as food additives requiring enzymatic or fermentative production by US and European legislation[4] has increased the demand for a biotechnological preparation of coniferyl alcohol.[5]

Coniferyl alcohol occurs as a lignin-related intermediate in the microbial degradation of eugenol. Eugenol is an abundant essential oil from the 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.[6] However, in *Byssochlamys fulva* V107, which was previously isolated from soil as an effectively vanillyl alcohol-using fungus,[7] eugenol was only metabolized to coniferyl alcohol and 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 a vanillyl-alcohol oxidase, which also oxidizes vanillyl alcohol to vanillin, as shown with the purified enzymes from *Byssochlamys fulva* V107[7] and *Penicillium simplicissimum*.[8] Vanillyl-alcohol oxidase from *Byssochlamys fulva* V107 was able to convert eugenol with an approximately 2-fold higher rate than vanillyl alcohol,[7] 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, only a small amount of coniferyl aldehyde was formed under the bioconversion conditions described here.

The whole cells biocatalyst was obtained by 50 h of cultivation of *Byssoclamys fulva* V107 at 28°C with

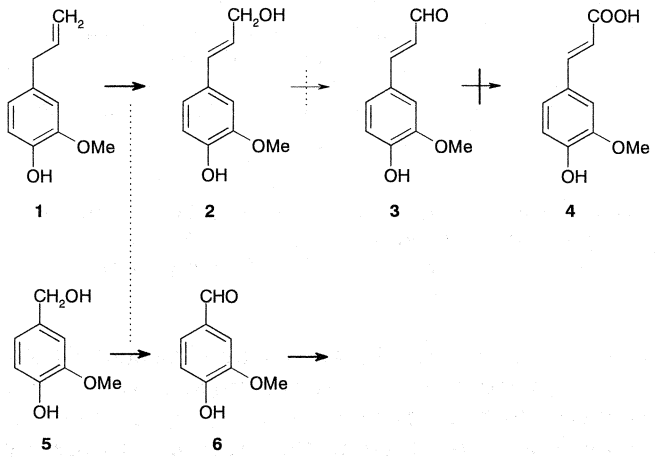

Fig. 1. Bioconversion of Eugenol (**1**) to Coniferyl Alcohol (**2**) and Small Amounts of Coniferyl Aldehyde (**3**) by *Byssochlamys fulva* V107.

The bioconversion is based on the enzyme vanillyl-alcohol oxidase, which has the additional ability to oxidize vanillyl alcohol (**5**), used as enzyme inducer, to vanillin (**6**). No enzyme activity for the conversion from coniferyl aldehyde to ferulic acid (**4**) was present in this organism, and only a minor oxidation of coniferyl alcohol to coniferyl aldehyde was observed under the conditions used here.

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 $MgSO_4 \cdot 7H_2O$ per liter and metals in final concentrations of 0.2 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $CaCl_2 \cdot 2H_2O$, 0.3 mg of H_3BO_3, 0.04 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of KI, 0.4 mg of $MnSO_4 \cdot 7H_2O$, and 0.2 mg of $Na_2MoO_4 \cdot 2H_2O$ per liter (pH 7.0), resulting in a dry cell yield of 1.9 g. 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. 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. After 36 h

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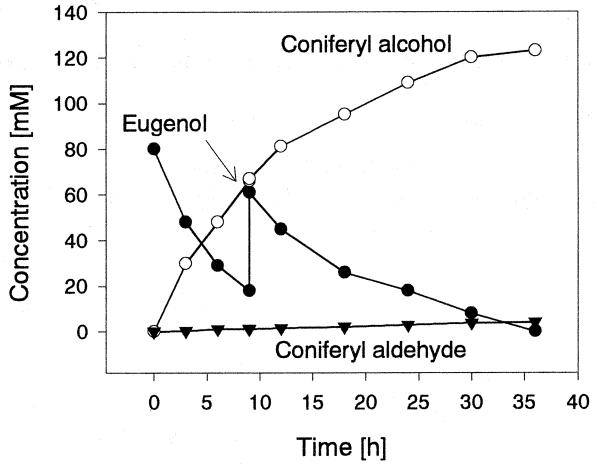

Fig. 2. Course of the Fed-batch Bioconversion of Eugenol to Coniferyl Alcohol by *Byssochlamys fulva* V107.
The arrow indicates the addition of 50 mM eugenol after 9 h.

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). Coniferyl alcohol was isolated from the reaction mixture by ethylacetate extraction, evaporation and silica gel chromatography (Wakogel C-300, Wako, Osaka, Japan) with benzene/methanol (95:5, v/v) as the mobile phase with an isolated yield of 48%. 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.

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.[9] 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,[10] the fungal biocatalyst has the advantage that it uses O_2 as a natural electron acceptor, as shown in previous studies of the purified enzyme,[7] without requiring a costly, external electron acceptor such as phenazine methosulfate.

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