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Regio- and Stereoselective Allylic Hydroxylation of D-Limonene to (+)-*trans*-Carveol with *Cellulosimicrobium cellulans* EB-8-4

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Abstract: *Cellulosimicrobium cellulans* EB-8-4 was discovered by screening of microorganisms as a powerful catalyst for the regio- and stereoselective allylic hydroxylation of D-limonene to (+)-*trans*-carveol that is a useful and valuable fragrance and flavour compound. Cells of strain EB-8-4 were easily obtained, demonstrated more than 99% regio- and stereoselectivity, showed a specific hydroxylation activity of 4.0 U/g cdw (cell dry weight), and accepted 62 mM D-limonene without inhibition. The hydroxylation was possibly catalyzed by an nicotinamide adenine dinucleotide (NADH)-dependent oxygenase involved in the degradation of aromatic ring during cell growth. 13.4 mM of (+)-*trans*-carveol were obtained by biohydroxylation of D-limonene with resting cells of *C. cellulans* EB-8-4, thus being 11 times higher than that obtained with the best biocatalyst known thus far. High conversion and high yield were obtained in the biohydroxylation of 11.6 mM of D-limonene with the resting cells as catalyst in a closed shaking flask, giving 10 mM of (+)-*trans*-carveol, and 0.30 mM of carvone as the only by-product. Thus, a unique biocatalyst for the regio- and stereoselective allylic hydroxylation of D-limonene and an efficient synthesis of natural identical (+)-*trans*-carveol by biohydroxylation have been developed.

Keywords: biotransformations; *trans*-carveol; enzyme catalysis; hydroxylation; D-limonene; terpenoids

Introduction

Terpenes are very attractive renewable feedstock for sustainable chemical synthesis,[1] due to their easy availability, well-defined chirality, and high reactivity. The key challenge is the development of highly selective and productive transformations of terpenes. While chemical methods suffer from poor selectivity, biotransformation is a useful alternative and provides with additional advantage of producing natural identical aroma and flavour compounds. Over the years, biotransformations of terpenes have been intensively investigated.[2,3]

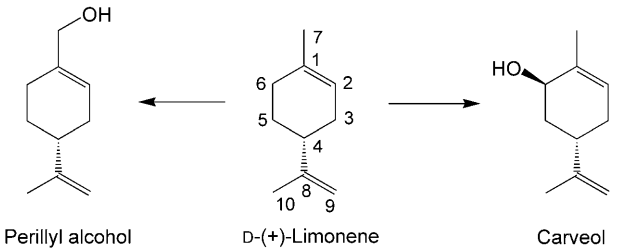
We are interested in developing regio- and stereoselective allylic hydroxylations for terpene transformations. The biohydroxylation of D-limonene to *trans*-carveol was selected as the target reaction, since a) D-limonene is an ideal renewable feedstock with an annual production of 50,000 tons and a price of 0.66–1.45 $ per kg in year 2000;[4] b) carveol is a useful and valuable fragrance and flavour additive[5] and exhibits chemoprevention of mammary carcinogenesis;[6] and c) carveol is prepared in the *cis*-form[7a,b] or as a mixture of *trans*- and *cis*-forms[7c] by reduction of (*S*)-(+)-carvone which is chemically produced from D-limonene,[5a,7d] and the biohydroxylation of D-limonene could provide with a direct access to and efficient synthesis of carveol in the pure *trans*-form. Many microorganisms have been examined for this hydroxylation,[8–11] but most of them gave mixtures of several products.[8b–h] Among the best, the Basidiomycete *Pleurotus sapidus* transformed D-limonene to give 0.39 mM of *cis*- and *trans*-carveol in a ratio of 2:3 and 0.26 mM of carvone after 12 days;[9] *Rhodococcus opacus* PWD4 and *Rhodococcus erythropolis* PWD 8 showed excellent regio- and stereoselectivity for the allylic hydroxylation of D-limonene to produce 1.21 mM of *trans*-carveol and 15 μM of carvone;[10] However, the product concentration is low and could not be increased by changing growth substrate, increasing substrate concentration, and applying two-liquid phase system.[11] Here we report the discovery of *Cellulosimicrobium cellulans* EB-8-4 as a highly selective and productive biocatalyst for the regio- and stereoselective allylic hydroxylation of D-limonene and the development of the biotransformation of D-limonene with resting cells of this strain to produce (+)-*trans*-carveol at 10–13.4 mM in a simple system.

Results and Discussion

Isolation and Screening of Bacterial Strains for the Biohydroxylation of D-Limonene to *trans*-Carveol

We have previously discovered enzymes for the regio- and stereoselective hydroxylation of the *non*-activated carbon atom of alicyclic compounds by screening of *n*-alkane-degrading strains.[12] To find appropriate enzymes for the desired allylic hydroxylation, we started with the isolation of strains that degrade toluene, ethylbenzene, α-pinene, and *n*-octane. 40 samples from soil, sea water, and sewage sludge in Singapore were used for the enrichment in M9 medium containing 0.2% (v/v) of the individual compound mentioned above as the sole carbon source, respectively. After inoculation, growth, and separation of the obtained mixed strains on M9 agar plates, 65 pure bacterial strains were isolated. They are 4 ethylbenzene-, 5α-pinene-, 22 toluene-, and 34 *n*-octane-degrading strains.

To examine the desired hydroxylation activity, the isolates were grown in M9 medium containing the individual carbon source, the cells were harvested and resuspended into 1 mL KP-buffer (pH 7.0) to a cell density of 5.0 g cdw/L (cdw: cell dry weight), and D-limonene was added to 6.2 mM as substrate. The products after 2 h biotransformation were analyzed by GC. As shown in Table 1, 3 ethylbenzene-degrading strains and 3 toluene-degrading strains catalyzed the regio- and stereoselective allylic hydroxylation of D-limonene at position 6 to give *trans*-carveol. None of the 5α-pinene-degrading strains could transform D-limonene. On the other hand, 3 *n*-octane-degrading strains were able to hydroxylate D-limonene at allylic position 7 to afford perillyl alcohol (Scheme 1). This position is also a terminal position. These results further confirm our previous observation: the monooxygenases from *n*-octane-degrading strains prefer hydroxylation at the terminal position.[12]



Scheme 1. Allylic hydroxylation of D-(+)-limonene.

Among the 6 strains producing *trans*-carveol, the isolate EB-8-4 showed the highest productivity, giving 2.1 mM of *trans*-carveol. The isolate EB 2–1 produced 138 μM of *trans*-carveol, while other four strains generated only 20–56 μM of *trans*-carveol. The taxonomy of these strains was established based on partial 16S RNA sequencing and is given in Table 1. The best strain EB-8-4 is an ethylbenzene-degrading strain isolated from a topsoil sample taken from an electronic plant. This strain showed a 99.6% sequence identity with *Cellulosimicrobium cellulans* CCM 2813, thus being named as *Cellulosimicrobium cellulans* EB-8-4. In contrast, all other 5 strains belong to *Pseudomonas* species.

Growth and Biohydroxylation Activity of *C. cellulans* EB-8-4

With Ethylbenzene as Growth Substrate

Even during the initial screening experiments, biohydroxylation with *C. cellulans* EB-8-4 gave a higher concentration of *trans*-carveol than that produced with any other known biocatalyst. To further explore the potential of using this strain for the production of *trans*-carveol, growth conditions were optimized. As shown in Figure 1, *C. cellulans* EB-8-4 grew fast in M9 medium on vapour of ethylbenzene with a specific growth rate of 0.91 h^{-1}, and the cell density reached

Table 1. Isolation and screening of strains for biotransformation of D-limonene.

Growing substrates	No. of isolated strains	No. of strains producing *trans*-carveol	Name of strain	*trans*-Carveol produced [μM]	No. of strains producing perillyl alcohol
Ethylbenzene	4	3	*Cellulosimicrobium cellulans* EB-8-4	2142	0
			Pseudomonas monteilii EB-2-1	138	
			Pseudomonas monteilii EB-2-3	56	
Toluene	22	3	*Pseudomonas monteilii* TA-1	49	0
			Pseudomonas monteilii TB-3	45	
			Pseudomonas monteilii TC-1	20	
n-Octane	34	0			3
α-Pinene	5	0			0

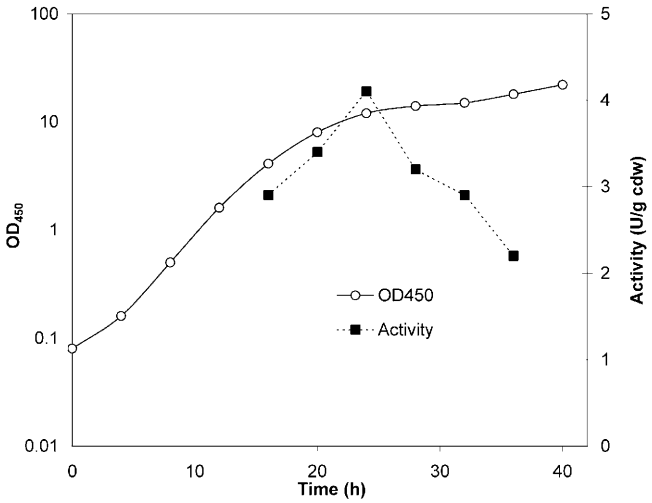


Figure 1. Growth of *C. cellulans* EB-8-4 on the vapour of ethylbenzene in M9 medium and the specific activity of hydroxylation of D-limonene with the resting cells.

2.9 g cdw/L at 24 h. The specific hydroxylation activity was determined by performing the biotransformation of D-limonene (12.4 mM) at 30 °C and 250 rpm for 30 min with cells harvested at different time points and resuspended (10 g cdw/L) in KP-buffer (pH 7.0). The cells produced at later exponential phase are quite active and reached the highest activity at 24 h: 4.1 U/g cdw. Thus, active cells of *C. cellulans* can be easily obtained in a large quantity within a short time. The cells in the stationary phase gradually lost the hydroxylation activity.

Hydroxylation of D-limonene to *trans*-carveol was also studied with the soluble cell-free extract (CFE) of *C. cellulans* EB-8-4. The enzyme responsible for the hydroxylation was found to be cofactor dependent. Nicotinamide adenine dinucleotide (NADH) is much more preferred than nicotinamide adenine dinucleotide phosphate (NADPH): the hydroxylation activity of CFE in the presence of NADH was 1.3 U/g protein, which was much higher than that in the presence of NADPH (lower than 0.1 U/g protein). No CO difference absorption at 450 nm was observed for the CFE, indicating that the hydroxylation enzyme is not a P450 enzyme. No hydroxylation happened with CFE in the presence of hydrogen peroxide, suggesting that the enzyme responsible for the hydroxylation is not a peroxygenase.

Further studies were carried out with the cells grown on ethylbenzene for bioconversion of benzene, toluene, and ethyl benzene. As listed in Table 2, the bioconversion of benzene with such cells gave phenol as the major product, which indicates that the enzyme responsible for the hydroxylation of D-limonene to *trans*-carveol might be an oxygenase involved in the degradation of aromatic ring.

With Other Growth Substrates

The growth of *C. cellulans* EB-8-4 was also examined using other aromatic compounds such as benzene, toluene, *o*-xylene, *m*-xylene, or *p*-xylene as carbon sources. As given in Table 2, the strain grew also quite fast on toluene, with a specific growth rate of 0.89 h^{-1}. Cells at the exponential phase showed a specific activity of 3.7 U/g cdw for the allylic hydroxylation of D-limonene. In contrast, very slow growth was observed with benzene and *o*-xylene as carbon sources, giving a specific growth rate of 0.09 h^{-1} and 0.02 h^{-1}, respectively. No significant growth was observed on *m*-xylene or *p*-xylene. Interestingly, the cells grown on benzene demonstrated also hydroxylation activity for D-limonene, giving a specific activity of 0.8 U/g cdw for the production of *trans*-carveol. This suggested again that the enzyme responsible for such a hydroxylation could be an oxygenase involved in the degradation of the aromatic ring.

Table 2. Growth and biotransformation with *C. cellulans* EB-8-4 on different substrates.

Growth substrate	Specific growth rate [h^{-1}]	Biotransformation substrate[a]	Biotransformation product	Activity [U/g cdw]
Benzene	0.09	D-limonene	*trans*-carveol	0.8
Toluene	0.89	D-limonene	*trans*-carveol	3.7
o-Xylene	0.02			
Ethylbenzene	0.91	D-limonene	*trans*-carveol	4.0
		benzene	phenol [2.3 μM]	
			catechol [0.7 μM]	
		toluene	benzyl alcohol [55 μM]	
		ethylbenzene	1-phenyl-1-ethanol [83 μM]	
			1-phenyl-1-ethanone [14 μM]	

[a] Biotransformation was performed with 12.4 mM substrate in 5 mL cell suspension (10 g cdw/L) in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.0) at 300 rpm and 30 °C for 30 min.

Regio- and Stereoselective Hydroxylation of D-Limonene with Resting Cells of *C. cellulans* EB-8-4

Substrate Concentration and Toxicity

The resting cells of *C. cellulans* EB-8-4 prepared by growing on ethylbenzene were used for the further development of an efficient allylic biohydroxylation of D-limonene to *trans*-carveol. The addition of glucose (100 mM) was found to improve the conversion rate, possibly through the regeneration of NADH during the metabolization of glucose by the cells.

D-Limonene is often a toxic substrate for microbial hydroxylations, thus resulting in low product concentrations.[10,11] To explore the potential of using high concentrations of D-limonene for the *C. cellulans* EB-8-4-catalyzed biohydroxylation, 6.2 mM to 154 mM D-limonene, calculated from the amount added and volume of buffer solution, were used for the biotransformation with resting cells (10 g cdw/L). Although D-limonene has very low solubility in aqueous systems (0.15 mM in water at 25 °C),[13] the conversion rate was found to be dependent on the amount of D-limonene added. As shown in Figure 2, there is a nearly linear increase of the conversion rate with the increase of substrate concentration from 6.2 to 62 mM. When the substrate concentration increased further from 62 mM to 93 mM, the conversion rate decreased dramatically from 1.1 to 0.2 mM/h. These results indicate strong inhibition at higher substrate concentrations. Nevertheless, it is possible to use substrate concentrations as high as 62 mM for efficient biotransformations.

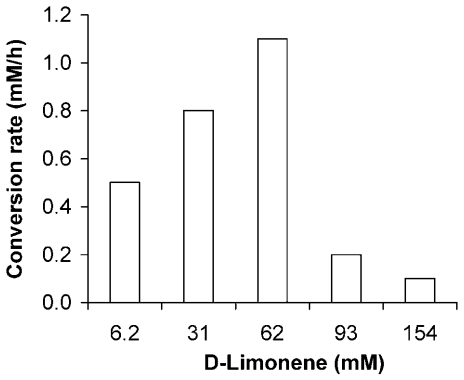


Figure 2. Effect of the concentration of D-limonene on the conversion rate of hydroxylation of D-limonene to *trans*-carveol with resting cells of *C. cellulans* EB-8-4 (10 g cdw/L). The conversion rate was calculated over the first three hours.

Biotransformation with Volatile Substrate

Like other terpenes, D-limonene is volatile, thus high yielding biotransformations should be performed with a system as closed as possible. On the other hand, the hydroxylation reaction needs oxygen as the oxidant, thus the availability of oxygen in the closed flask should have a significant influence on hydroxylation. To explore the best conditions for a high-yielding biotransformation, two experiments with the same concentration of D-limonene (40 mM) were designed and compared: System A, with 15 mL cell suspension (10 g cdw/L) in a 125-mL closed conical flask; and System B, with 28 mL suspension (10 g cdw/L) in a 125-mL closed conical flask. System A was proven to be much more efficient due to the larger availability of oxygen: specific hydroxylation activity within the first hour reached 4.0 U/g cdw, while only 1.3 U/g cdw was achieved for System B. The concentration of *trans*-carveol reached 13.4 mM at 11 h for System A, while only 4 mM product were produced at 11 h for System B. In both cases, the regio- and stereoselectivities were excellent, and only a small amount of carvone was formed as by-product. It was found that the substrate concentration decreased quickly and linearly during the biotransformation for both cases. This is possibly due to the evaporation of the volatile substrate during operation: the analytical samples were taken at several time points with a syringe through the rubber stopper.

The GC chromatograms of samples taken at 0 min, 1 h, and 11 h for biotransformation of System A are shown in Figure 3. The reaction gave *trans*-carveol as the major product, together with a very small amount of carvone which was produced from *trans*-carveol *via* the alcohol dehydrogenase in this strain. No detectable formation of alcohols and ketones at other positions and *cis*-carveol indicates more than 99% regio- and stereoselectivity of the allylic hydroxylation.

High-Yielding Biotransformation

To avoid the loss of substrate and achieve high conversion, the biohydroxylation of D-limonene (11.6 mM) was performed with 15 mL cell suspension (10 g/L) in a 125-mL conical flask with a glass stopper sealed by parafilm without taking samples during the reaction (System C). The same transformations were carried out with 8 different flasks, and the whole mixture in the individual flask was worked up for analysis at 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 10 h, respectively. As shown in Figure 4, the total concentrations of product and substrate were balanced at each of the 8 time points. Moreover, 8 control experiments with the same mixture but no cells were also carried out and worked up for analysis at the 8 different time points. In these experiments, no disappearance of substrate was observed during 10 h. Thus, such systems can effectively avoid the loss of D-limonene during biotransformation, thus allowing for high conversion

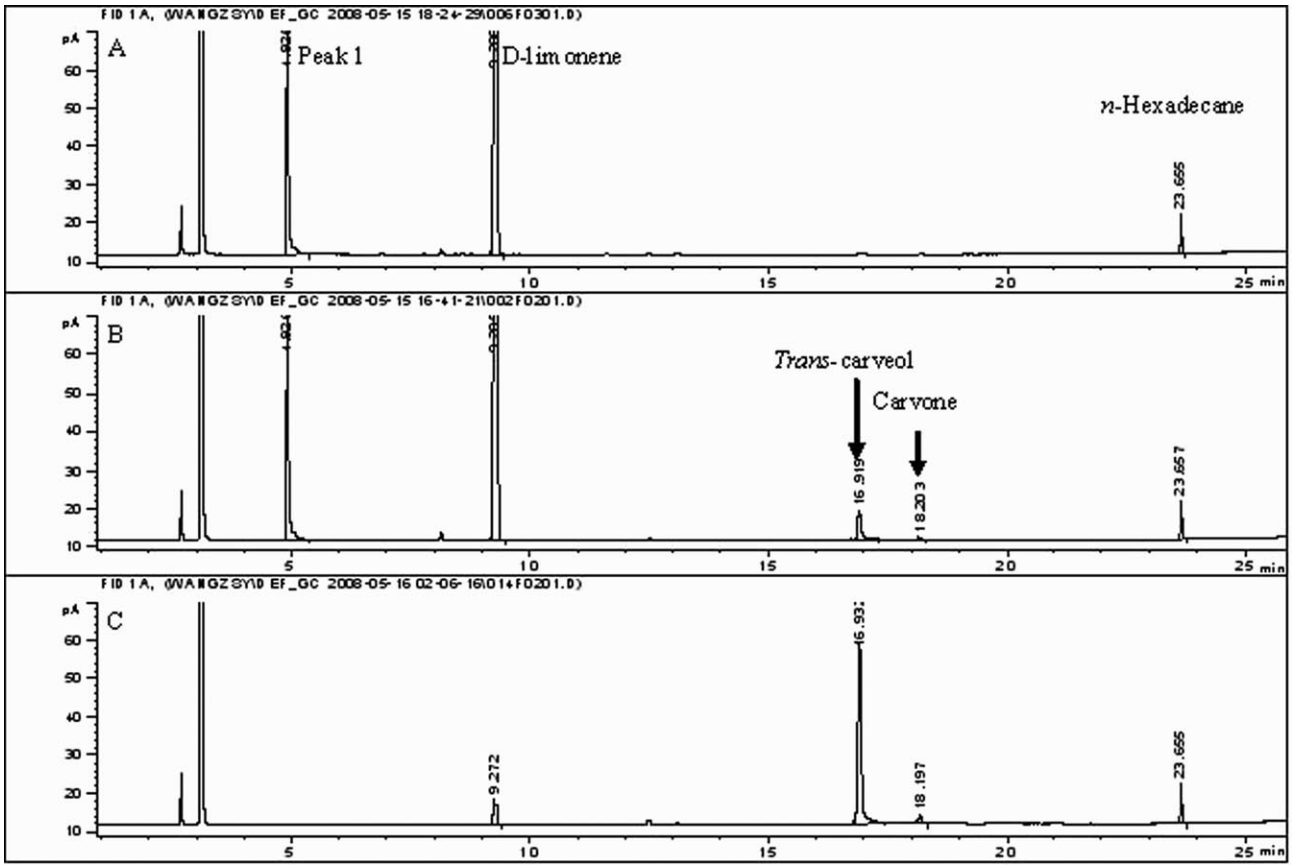


Figure 3. The GC chromatograms of samples taken from biotransformation of D-limonene (42 mM) in 15 mL cell suspension in a 125-mL conical flask with rubber stopper; **A**: at 0 min, peak 1, impurity from substrate; *n*-hexadecane as internal standard; **B**: at 1 h; **C**: at 11 h.

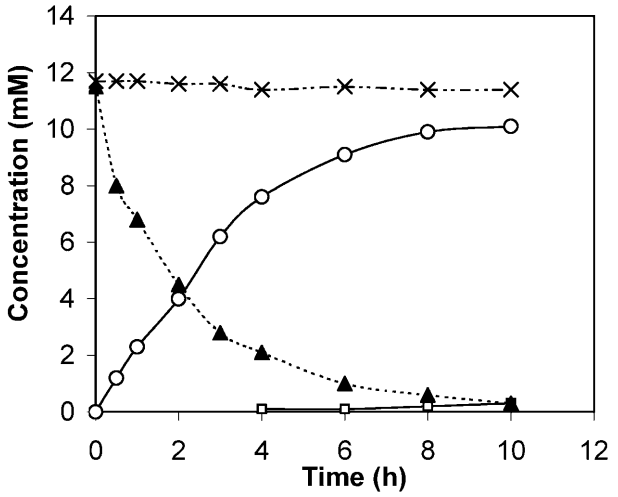


Figure 4. Biohydroxylation of D-limonene with resting cells of *C. cellulans* EB-8-4 in a 125 mL conical flask with glass stopper sealed with Parafilm. 15 mL cell suspension: (□) for carvone, (▲) for D-limonene, (○) for *trans*-carveol; (×) for D-limonene in a control experiment omitting *C. cellulans* EB-8-4.

of the substrate to the product. As shown in Figure 4, *trans*-carveol was formed at 10.0 mM in 86% conversion, in addition to the formation of 0.3 mM carvone as the only by-product.

Preparation of (+)-*trans*-Carveol by Regio- and Stereoselective Allylic Hydroxylation of D-Limonene with *C. cellulans* EB-8-4

The preparative biotransformation was performed by scaling the above described system to 60 mL cell suspension in 500-mL conical flask with 115 μL D-limonene (93.7 mg, 0.69 mol, purity 97%). After 12 h of incubation, the conversion of D-limonene was more than 90%. Extraction of the product with organic solvent followed by evaporation gave 95.8 mg of crude product. Further purification by chromatography on silica gel afforded 70.8 mg (+)-*trans*-carveol in 69% yield. The purity was determined by GC as >99%. The structure was confirmed by ^{1}H NMR and GC-MS. The specific optical rotation of the isolated product was measured to be $[\alpha]_D^{20}$: +220 (*c* 0.87, $CHCl_3$),

which is comparable to the reported value of +210° (*c* 2.0, $CHCl_3$).[14]

Conclusions

The ethylbenzene-degrader *Cellulosimicrobium cellulans* EB-8-4 was discovered by screening of microorganisms as a powerful biocatalyst for allylic hydroxylation of D-limonene to (+)-*trans*-carveol with high regio- and stereoselectivity. The cells of this strain can be easily prepared and used for the biotransformation of D-limonene to produce *trans*-(+)-carveol at 13.4 mM, an 11-fold increase of the product concentration in comparison with the best biocatalyst known thus far. High conversion of substrate and high yield of product were achieved with a simple shaking flask system.

Experimental Section

Chemicals

(−)-Carveol [97%; mixture of 58% (−)-*trans*-carveol and 42% (−) *cis*-carveol; the only commercial available carveol with high purity], (+)-(*S*)-carvone (>97%), perillyl alcohol (>98%), perillic acid (>98%), D-limonene (>97%), α-pinene (>95%), phenol (>98%), benzyl alocohol (>97%), 1-phenyl-1-ethanol (>98%), 1-phenyl-1-ethanone (>97%), catechol (>98%), *o*-xylene (>99%), *m*-xylene (>99%), *p*-xylene (>99%), nicotinamide adenine dinucleotide (NADH) (>99%), and nicotinamide adenine dinucleotide phosphate (NADPH) (>99%) were purchased from Sigma–Aldrich. Benzene (>99%), toluene (>99%), and ethylbenzene (>99%) were obtained from Fluka. *n*-Octane (>97%) was bought from Acros Organics. *n*-Hexadecane (>99%) and silica gel 60 (200–300 mesh) were obtained from Merck.

Analytical Methods

GC analysis was performed by using an Agilent 6890N instrument with a flame ionization detector on a HP-5 capillary column (30.0 m × 322 μm × 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Inlet and detector temperatures were 260 and 280 °C, respectively. The temperature was programmed to increase from 60 °C to 100 °C at a rate of 5 °C min^{-1}, hold at 100 °C for 2 min, increase from 100 °C to 116 °C at a rate of 2 °C min^{-1}, increase from 116 °C to 280 °C at a rate of 30 °C min^{-1}, and hold at 280 °C for 3 min. Retention times: 16.93, 17.68, 18.20, 19.86, 22.15, 9.29, 7.00, and 23.25 min for *trans*-carveol, *cis*-carveol, carvone, perillyl alcohol, perillic acid, D-limonene, ethyl benzene, and *n*-hexadecane, respectively; 7,74, 9.14, 9.92, 10.14, and 13.35 min for phenol, benzyl alocohol, 1-phenyl-1-ethanol, 1-phenyl-1-ethanone, and catechol, respectively. 1 mM *n*-hexadecane was used as internal standard.

GC-MS analysis was performed with a Hewlett Packard HP6890 GC system coupled with a 5973 mass selective detector (EI) on the same HP-5 column as described above. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. Inlet and detector temperatures were 260 and 280 °C, respectively. The temperature was programmed to increase from 60 °C to 280 °C at a rate of 10 °C min^{-1} and then held at 280 °C for 3 min. Retention times: 11.25, 11.80, and 6.38 min for *trans*-carveol, carvone, and D-limonene, respectively.

The ^{1}H NMR spectrum was recorded in $CDCl_3$ with TMS as internal standard at 500 MHz on a Bruker machine. The specific optical rotation was measured on a Jasco™ Spectropolarimeter. The optical density of cell cultures was measured at 450 nm (OD_{450}) with a Hitachi U-1900 spectrophotometer. A cell density of 1 g cdw/L corresponds to OD_{450} of 0.26.

Isolation of Microorganisms with Enrichment Culture Technology

40 Samples of soil (30), sea water (5), and sewage sludge (5) were collected from different places in Singapore. 1 g solid sample or 1 mL liquid sample was added into 10 mL M9 mineral medium[15] containing trace element[16] and 0.2% (v/v) carbon source (α-pinene, *n*-octane, toluene, or ethylbenzene) in a 28-mL glass bottle with a metal screw cap. After incubation at 24 °C and 200 rpm for 2 weeks, the enrichment culture was diluted 10^3–10^5 times, and 0.1 mL of the diluted culture was dispensed onto M9 agar plates. The resulting plates were incubated in desiccators with the vapour of α-pinene, *n*-octane, toluene, or ethylbenzene as carbon source. Each single colony was picked up and then inoculated once again in M9 agar plate with the corresponding carbon source. A total of 65 strains were isolated: 4, 5, 22, and 34 strains degrading ethylbenzene, α-pinene, toluene, and *n*-octane, respectively. The isolates were stored in glycerol at −80 °C.

Screening of Microorganisms for Allylic Hydroxylation of D-Limonene

Each isolate was inoculated from M9 agar plate into 5 mL Luria-Bertani (LB) broth medium and incubated at 30 °C and 250 rpm for 14–16 h. 3 mL LB seed culture were transformed into 100 mL M9 medium in a 250-mL conical shaking flask containing a tube with 1 mL *n*-octane, α-pinene, toluene, or ethylbenzene. The culture was shaken at 250 rpm and 30 °C for 26–30 h, and the growth was monitored by measuring OD at 450 nm. Cells were harvested at early stationary phase by centrifugation at 12,000 *g* and 4 °C for 20 min, washed twice with K_2HPO_4-KH_2PO_4 buffer (50 mM; pH 7.0) (K-Buffer), and re-suspended to 5 g cdw/L in 1 mL K-buffer containing 100 mM glucose and 6.2 mM D-limonene in a 2-mL culture tube. The mixture was shaken at 1000 rpm and 30 °C for 2 h. 1 mL sample was taken, mixed with 0.4 mL chloroform containing 1 mM *n*-hexadecane, and centrifuged to remove the cells. The organic phase was separated, dried over $MgSO_4$, and analyzed by GC.

Growth and Hydroxylation Activity of *C. cellulans* EB-8-4

C. cellulans EB-8-4 was grown in 5 mL LB medium at 30 °C and 250 rpm for 14–16 h, and 3 mL LB culture were then transformed into 100 mL M9 medium in a 250-mL shaking flask with a tube containing 1 mL ethylbenzene. The culture

was incubated at 30 °C and 250 rpm. At different time points, samples were taken for the OD measurement at 450 nm and activity tests. To determine the hydroxylation activity, cells were suspended to 10 g cdw/L in K-buffer containing 12.4 mM D-limonene, the biotransformation was performed for 30 min at 300 rpm and 30 °C, and the product formation was determined by GC analysis. Typical growth and activity curves are shown in Figure 1. Cells were harvested at early stationary phase at 24 h with a cell density of 2.9 g cdw/L and a specific activity of 4.0 U/g cdw.

Biohydroxylation of D-Limonene to *trans*-Carveol with Resting Cells of *C. cellulans* EB-8-4

Cells of *C. cellulans* EB-8-4 prepared above were resuspended to 10 g cdw/L in K-buffer containing 100 mM glucose and 42 mM D-limonene. Biotransformation was performed with 15 mL cell suspensions (System A) or 28 mL suspensions (System B) in a 125-mL conical flask with a rubber stopper sealed by parafilm. The mixtures were shaken at 30 °C and 250 rpm. At regular time intervals, a 1 mL sample was taken using a syringe through the rubber stopper and mixed with 0.4 mL chloroform containing 1 mM *n*-hexadecane. The organic phase was separated, dried over $MgSO_4$, and analyzed by GC. GC chromatograms of samples taken from System A are given in Figure 3.

In System C, biotransformations were performed in eight 150-mL conical flasks with glass stoppers sealed with parafilm. To each flask were added 8 mL cell suspension (10 g cdw/L) in K-buffer containing 100 mM glucose and 11.6 mM D-limonene, and the mixture was shaken at 30 °C and 250 rpm. At each time point (30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 10 h), one of the eight flasks was removed from the shaker, put into ice for 20 min, opened, and mixed with 8 mL chloroform containing 1 mM *n*-hexadecane. The flask was kept on ice for another 10 min, shaken by hand, and the organic phase was separated, dried over $MgSO_4$, and analyzed by GC. Similarly, 8 control experiments were performed under the same conditions without cells. The results are given in Figure 4.

Biohydroxylation of D-Limonene to *trans*-Carveol with Cell-Free Extract of *C. cellulans* EB-8-4

The cells of *C. cellulans* EB-8-4 were resuspended into 60 mL K-buffer to 10 g cdw/L, and the suspension was passed through a homogenizer three times. The cell debris was removed by centrifugation at 120,000 *g* for 30 min. The resulting cell-free extract contained 3.1 g/L protein, determined by using the Bradford assay.[17] Biotransformations were carried out with 5 mL cell-free extract containing 2 mM NADH or NADPH, 1.2 mM D-limonene, and 100 mM glucose in a 50-mL shaking flask. The mixture was shaken at 30 °C and 250 rpm for 30 min, followed by mixing with 2 mL chloroform containing 1 mM *n*-hexadecane, the organic phase was separated, dried, and analyzed by GC analysis.

Preparation of (+)-*trans*-Carveol by Biohydroxylation of D-Limonene with Resting Cells of *C. cellulans* EB-8-4

To a 60 mL of cell suspension (10 g cdw/L) of *C. cellulans* EB-8-4 in a 500-mL conical flask were added 115 μL D-limonene (93.0 mg, 11.4 mM, 97% purity). The flask was closed with a glass stopper, sealed with parafilm and shaken at 30 °C and 250 rpm for 12 h. After cooling at 4 °C for 20 min, cells were separated from the supernatant by centrifugation and washed twice with a total of 40 mL water. The combined supernatants were extracted three times with chloroform. The organic phase was separated, washed with 5% sodium carbonate aqueous solution, and dried with $MgSO_4$ followed by filtration. The solvent was removed by evaporation at reduced pressure and at 4 °C, giving 95.8 mg crude product containing 90% *trans*-carveol as analyzed by GC. Purification by chromatography with a short column on silica gel using *n*-hexane/ethyl acetate 30/1 (v/v) as eluent gave 70.8 mg *trans*-carveol ($R_f = 0.4$) with an isolated yield of 69%. GC-MS: retention time, 11.25 min; MW, 152; ^{1}H NMR ($CDCl_3$): $\delta = 5.61$ (m, 1H), 4.77 (m, 1H), 4.75 (s, 1H), 4.04 (s, 1H), 2.33 (m, 1H), 2.16 (m, 1H), 1.95 (m, 1H), 1.89 (m, 1H), 1.82 (m, 3H), 1.76 (s, 3H), 1.62 (m, 1H); $[\alpha]_D^{25}$: + 220 (*c* 0.87, $CHCl_3$) [lit.[14]: $[\alpha]_D^{25}$: + 210, (*c* 2.0, $CHCl_3$).

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