



Highly chemo- and regio-selective hydroxylations of *o*- and *m*-substituted toluenes to benzyl alcohols with *Cellulosimicrobium cellulans* EB-8-4

Shiyao Dai^a, Jinchuan Wu^b, Zunsheng Wang^a, Yongzheng Chen^a, Zhi Li^{a,*}

^a Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117576

^b Institute of Chemical and Engineering Sciences, 1 Pesek Road, Jurong Island, Singapore 627833

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ABSTRACT

Highly chemo- and regio-selective benzylic hydroxylations of *o*- and *m*-substituted toluenes were achieved with the easily available and easy-to-handle resting cells of *Cellulosimicrobium cellulans* EB-8-4 as biocatalysts, giving the corresponding benzyl alcohols as single product. Benzyl alcohols were obtained in 78–94% yield, demonstrating the first green, clean, and simple method for the preparation of benzyl alcohols via hydroxylations. Biotransformation of 4-methylbenzyl chloride with the same strain gave 4-methylbenzyl alcohol in 67–81% yield, suggesting a novel dehalogenation activity of the cells and providing a novel, green, and efficient method for the preparation of 4-methylbenzyl alcohol as well as the application potential in biodegradation of chlorine-containing aromatics.

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1. Introduction

Chemo-, regio-, and stereoselective hydroxylations are important reactions for the preparation of alcohols. For instance, selective benzylic hydroxylation could give rise to the direct synthesis of benzyl alcohols that are useful fine chemicals and pharmaceutical intermediates.^{1–4} However, chemical benzylic hydroxylation suffers from low chemo- and regioselectivity and often results in a mixture of different products. As easily available industry feedstocks, xylenes are typical substrates for the preparation of benzyl alcohols and thus have been examined for the benzylic hydroxylations. Mn-complexes-catalyzed oxidation of *o*-xylene **1** gave 55% benzylic selectivity with the alcohol and aldehyde in 1:2.⁵ Mo-peroxo complex-catalyzed oxidation of *o*-xylene **1** afforded a mixture of the mono- and diol in 2:1 with 23% yield, and oxidation of *p*-xylene with the same system yielded 27% of the same product mixture in the same ratio.⁶ Similar results were reported by *N*-hydroxyphthalimide-catalyzed oxidation of *o*-, *m*-, and *p*-xylene, with 5.2–28% of the corresponding methylbenzyl alcohols and simultaneously produced aldehydes and acids.^{7,8} Therefore, the current industrial preparation of these methylbenzyl alcohols has to be achieved by other methods, such as the *non*-green one, which involves chlorination and hydrolysis with base at high temperature.^{9,10} Moreover, the preparation of other substituted benzyl alcohols, such as

2-ethylphenyl methanol, 2-propylphenyl methanol, is difficult, which involves the reduction of their corresponding aldehydes or acids^{11–13} or the reductive cleavage of phthalans.^{14,15}

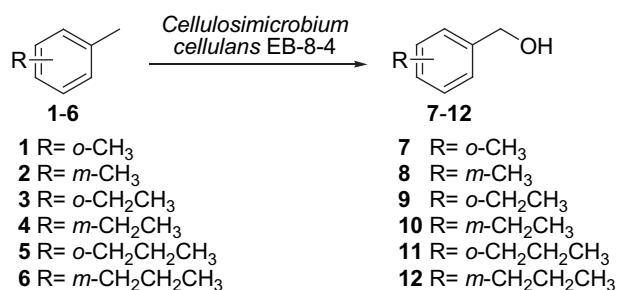
Enzyme-catalyzed hydroxylation is a green alternative for selective benzylic hydroxylation.^{16–18} Different enzymatic systems have been explored for the hydroxylation of xylenes,^{19–25} but the results are not satisfactory: Chloroperoxidase (CPO) catalyzed the oxidation of *o*-, *m*-, and *p*-xylene to give abundant tolualdehydes with only 0.2–0.4 mM methylbenzyl alcohols.¹⁹ Cytochrome P450-catalyzed hydroxylation of *m*- and *p*-xylene gave 80% alcohols with 20% aldehyde as by-product;²⁰ xylene monooxygenase-catalyzed hydroxylation of *m*- and *p*-xylene^{21,22} was known to be a multiple oxidation from xylene to its alcohol and then to the aldehyde.²³ A cloned *p*-cymene monooxygenase and a cytochrome P450 monooxygenase were also reported as potential enzymes for the selective biohydroxylation of *m*- and *p*-xylenes, but with no information on the selectivity and product purity.^{24,25} Recently, we discovered *Cellulosimicrobium cellulans* EB-8-4 for the regio- and stereo-selective allylic hydroxylation of *D*-limonene to (+)-*trans*-carveol.²⁶ Since the strain was isolated by growing on ethyl benzene, it may contain the enzyme that catalyzes the benzylic hydroxylation. Thus, we have investigated the hydroxylation of xylenes **1,2** and other substituted toluenes **3–6** and **13–15** with this strain. Here, we report the highly chemo- and regio-selective benzylic hydroxylations and the green and efficient syntheses of the corresponding benzyl alcohols. In addition, the novel enzymatic activity of dehalogenation of chlorine-containing aromatic substrate **13–15** with this strain is also reported.

* Corresponding author. Tel.: +65 6516 8416; fax: +65 6779 1936; e-mail address: chelz@nus.edu.sg (Z. Li).

2. Results

2.1. Biohydroxylation of xylenes 1–2 and other alkyl toluenes 3–6 to benzyl alcohols 7–12

To explore the chemo- and regio-selective benzylic biohydroxylation, *o*-xylene **1**, *m*-xylene **2**, and *p*-xylene were chosen as model substrates (Scheme 1). The hydroxylation was carried out by using the resting cells of *C. cellulans* EB-8-4 as an easy-to-handle catalyst, which was prepared on a large scale by simple growing procedures. With the cell density of 10 g cdw/L, different substrate concentrations of 12 mM, 24 mM and 50 mM were examined for the biohydroxylation of **1** and **2** at 30 °C and 200 rpm. The reactions were followed by taking samples at different time points for GC analysis. Calibration curves for **1**, **2**, **7**, and **8** were established with 1 mM *n*-hexadecane as internal standard to quantify the substrate and product concentrations. Highly regio-selective biohydroxylations of **1** and **2** were achieved. As shown in Figure 1, only the product peak of **7** at 12.6 min was detected in the GC spectra of the sample taken from the biohydroxylation of **1** at 2 h. When the reaction was further conducted to 8 h, no by-product such as benzylic aldehyde or benzylic acid was detected from the GC spectrum as we ll. Similarly, a clean reaction was also found in the biohydroxylation of **2** to **8**.



Scheme 1. Biohydroxylation of *o*- and *m*-alkyl toluenes **1–6** to their corresponding benzyl alcohols **7–12**.

The reaction courses of hydroxylation of **1** and **2** are shown in Figure 2, the activity calculated over the first 1 h reaction reached 3.3 U/g cdw (U=mmol/min; cdw=cell dry weight) and 8.7 U/g cdw for the biohydroxylation of **1** to **7** and **2** to **8**, respectively. The biohydroxylation of **1** to **7** was still active from 6–10 h and gave product concentration of 9.8 mM in 82% yield. On the other hand, the biohydroxylation of **2** to **8** was much faster and mostly completed at 6 h. 11.3 mM product in 94% yield was obtained. As a result, no further reaction was observed from 6–10 h, possibly due to the low substrate concentration in the reaction system. As shown in Table 1, the increase of substrate concentration from 12 to 24 mM in the biohydroxylation of **2** to **8** gave an improvement in product concentration to 15.6 mM with 65% yield. However, a similar increase of substrate concentration did not result in the increase of product concentration in the biohydroxylation of **1** to **7**. There was no reaction for the biotransformation of *p*-xylene for 10 h at different substrate concentrations under the same reaction conditions. This

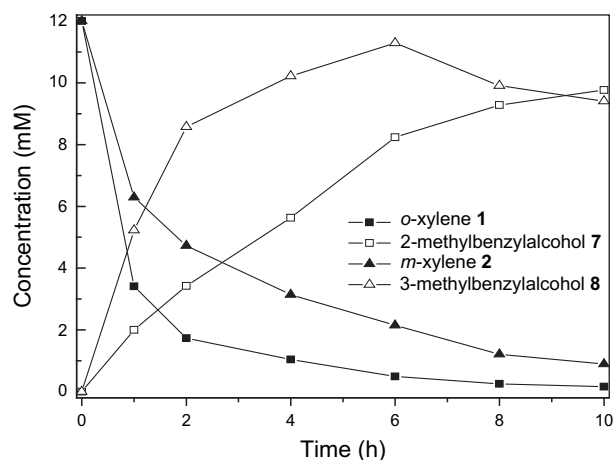


Figure 2. Reaction course of biohydroxylation of 12 mM xylenes **1–2** in 10 mL cell suspension (10 g cdw/L) in K-buffer (pH=7.0) at 30 °C and 200 rpm.

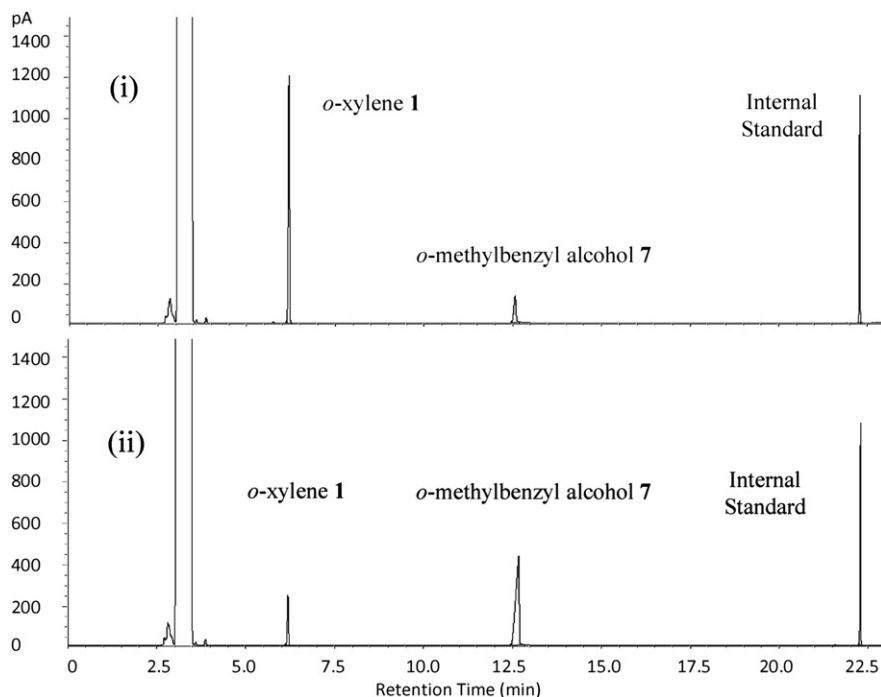


Figure 1. GC spectra of samples taken from biohydroxylation of *o*-xylene (12 mM) in 10 mL cell suspension (10 g cdw/L) in K-buffer (pH=7.0) at 30 °C and 200 rpm (i) at 2 h; (ii) at 8 h.

Table 1Chemo- and regio-selective biohydroxylation of *o*- and *m*-alkyl toluenes **1–6** to the corresponding benzyl alcohols **7–12**

Entry ^a	Substrate	Concn. (mM)	Time (h)	Product	Concn. (mM)	Yield (%)
1		1 12	10		7 9.8	81
2		1 24	10		7 6.2	26
3		2 12	6		8 11.3	78
4		2 24	10		8 15.6	65
5		3 10	5		9 9.4	94
6		3 15	9		9 11.6	77
7		3 20	9		9 8.8	44
8		3 40	9		9 9.2	23
9		4 10	7		10 5.2	52
10		4 15	10		10 6.6	44
11		4 20	7		10 4.7	23
12		4 40	9		10 5.5	14
13		5 10	9		11 8.5	85
14		5 20	9		11 10.1	51
15		6 10	9		12 1.6	16
16		6 20	9		12 3.0	4.3

^a Biohydroxylation of *o*- and *m*-alkyl toluenes **1–6** (10–40 mM) was carried out in 10 mL cell suspension (10 g cwt/L) in K-buffer (pH=7.0) at 30 °C and 200 rpm.

may due to the substrate specificity of the hydroxylation enzyme in the cells.

To further study the regioselectivity of the benzylic biohydroxylation with resting cells of *C. cellulans* EB-8-4 and extend the substrate spectra for producing more useful benzyl alcohols, another five alkyl-toluenes were investigated for the biohydroxylation (Scheme 1). The high regioselectivity was also demonstrated for the biohydroxylation of 2-ethyltoluene **3**, 3-ethyltoluene **4**, 2-propyltoluene **5**, and 3-propyltoluene **6**. Single product of 2-ethylphenyl methanol **9**, 3-ethylphenyl methanol **10**, 2-propylphenyl methanol **11**, or 2-propylphenyl methanol **12** was detected from GC analysis, respectively. The results are summarized in Table 1 (entries 5–16). The reaction courses of hydroxylation of **3**, **4**, and **5** are shown in Figure 3. The activity over the first 1 h reaction was calculated as 6.2 U/g cdw, 5.4 U/g cdw, and 5.7 U/g cdw for the biohydroxylation of **3** to **9**, **4** to **10**, and **5** to **11**, respectively. After 10 h biohydroxylation, 9.4 mM **9** (94% yield), 5.2 mM **10** (52% yield), and 8.5 mM **11** (85% yield) were obtained, respectively.

As shown in Table 1, concentration of the product **9** in the biohydroxylation of **3** was increased to 11.6 mM (77% yield) by using 15 mM substrate. Further increase of substrate concentration did not

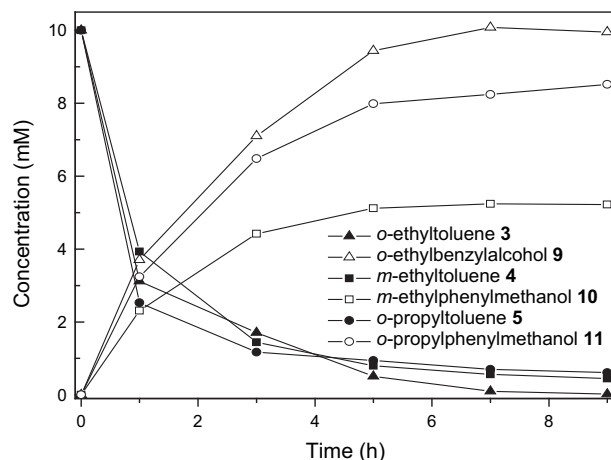


Figure 3. Reaction course of biohydroxylation of 10 mM alkyl toluenes **3–5** in 10 mL cell suspension (10 g cwt/L) in K-buffer (pH=7.0) at 30 °C and 200 rpm.

increase the product concentration. An increase of concentration of **11** from 8.5 to 10.1 mM was achieved for the hydroxylation of substrate **5** by increasing the substrate concentration from 10 mM to 20 mM. The biohydroxylation of **4** to **10** and **6** to **12** were relatively less active, but the concentration of product **10** still reached 5.2 mM in 52% yield, while the concentration of product **12** reached only 1.6 mM. The effect of substrate structure on the biohydroxylation rate is also shown in Table 1. The comparison between the biohydroxylation of **3** and **4** showed that *m*-substitution resulted in a much slower reaction rate than that of *o*-substitution. This trend was much clear in the comparison between the reactions of **5** and **6**. Under the same reaction conditions, hydroxylation of **5** with *o*-substituted propyl group produced 8.5 mM product, which is about five times higher than the product concentration obtained from biohydroxylation of **6** with *m*-substituted propyl group. However, both the biohydroxylation of **1** and **2** showed high reaction rate and high product concentration, although they have *o*- and *m*-substitution in their structures, respectively. A possible reason is that the methyl group at a different position may have an effect on the binding of substrate **1** or **2** to the catalytic pocket of the enzyme, but the freedom is still enough for them to reach the catalytic sites. So, the length of side chain may be an important factor that caused the difference in reaction rate, especially for *m*-substituted compounds. For the *o*-substituted compounds, substrate **5** showed comparable product concentration as substrate **3** and **1**. Therefore, the side-chain-length effect was less significant for *o*-substituted compounds in our study.

The mono-hydroxylation selectivity was recognized in the biohydroxylation of **1** and **2** with *C. cellulans* EB-8-4 cells. Benzyl alcohols **7** and **8** were produced as the only products, respectively, without the formation of any diol. The highly selective hydroxylation of the benzylic methyl group was also demonstrated in the hydroxylation of **3**, **4**, **5**, and **6**. The biotransformation of 1,3-diethylbenzene with *C. cellulans* EB-8-4 cells was also examined, but no reaction occurred within 8 h.

2.2. Biohydroxylation of methylbenzyl chlorides **13–15** to benzylic alcohols

To explore the possibility of biohydroxylation of chloride-substituted aromatic compounds by *C. cellulans* EB-8-4, 4-methylbenzyl chloride **13**, 2-methylbenzylchloride **14** and 3-methylbenzyl chloride **15** were investigated. It was surprising to find that a different type of reaction happened in the biotransformation: 4-methylbenzyl alcohol **16** was formed. This demonstrated that the *C. cellulans* EB-8-4 cell was able to catalyze a dehalogenation reaction. As shown in Table 2, the biotransformation of 30 mM **13** gave 24.3 mM **16** in 81% yield, while the biotransformation of 50 mM **13** produced 33.4 mM **16** in 67% yield. The *C. cellulans* EB-8-4 cell was also found to survive at 100 mM **13** to produce 30.7 mM **16** without significant inhibition effect. Although the detailed mechanism of the dehalogenation reaction is not clear from this study, a high productivity for the biotransformation of **13** to **16** with 33.4 mM product, 67% yield, and good substrate resistance of 100 mM were successfully demonstrated. This provides a novel, green, and efficient method for the preparation of benzyl alcohol **16**, and it could also be a useful method for biodegradation of chlorine-containing aromatics.

The dehalogenation reaction was further confirmed by the biotransformation of substrates **14** and **15**. As shown in Table 2 (entry 4), a mixture of 3.0 mM **17**, 0.37 mM **7**, and 0.84 mM **18** were formed at 5 h. The products were isolated and characterized by GC–MS and ¹H NMR spectroscopy. Compound **7** from the mixture was produced by the dehalogenation reaction, while **17** was synthesized from the direct hydroxylation of the methyl group in **14**. When product concentrations between 1 h and 5 h (entry 4) were compared, the concentration of **18** increased from 0.06 mM to 0.84 mM. A control experiment by using **7** as substrate for

Table 2Chemo- and regio-selective biohydroxylation of methylbenzyl chlorides **13**–**15** to the corresponding benzyl alcohols

Entry ^a	Substrate	Concn. (mM)	Time (h)	Product	Concn. (mM)	Yield (%)
1		13	30	9		16 24.3 81
2		13	50	9		16 33.4 67
3		13	100	9		16 30.7 31
4		14	5	1		17 2.75 55
				1		7 0.24 4.8
		15		1		18 0.06 1.2
				5		17 3.01 60
				5		7 0.37 7.4
				5		18 0.84 17
				1		19 1.02 20
				1		8 0.17 3.4
				1		20 <0.01 0
				5		19 1.97 39
				5		8 0.47 9.4
				5		20 0.19 3.8

^a Biohydroxylation of methylbenzyl chlorides **13**–**15** (5–100 mM) was performed in 10 mL cell suspension (10 g cww/L) in K-buffer (pH=7.0) at 30 °C and 200 rpm.

biotransformation was carried out, and the result showed that <1% **18** was produced after 6 h. Thus, it could be deduced that compound **18** was formed from **17** via dehalogenation catalyzed by the resting cells. The activities for the production of **7**, **17**, and **18** were calculated over the first 1 h reaction as 0.4 U/g cdw, 4.6 U/g cdw, and 0.1 U/g cdw, respectively. The ratio of **7**, **17**, and **18** in the product mixture changed slightly during biotransformation. A similar phenomenon was observed in the biotransformation of **15** with the resting cells of *C. cellulans* EB-8-4, and product **19**, **8**, and **20** were obtained in a different ratio and lower yield. It is not clear whether the dehalogenation was catalyzed by the same enzyme for the hydroxylation or other enzymes in *C. cellulans* EB-8-4 cells.

3. Conclusion

Highly chemo-, and regio-selective biohydroxylation of *o*- and *m*-alkyl toluenes **1**–**6** was achieved by using the easily available and easy-to-handle resting cells of *C. cellulans* EB-8-4 as biocatalyst. This synthetic route via biohydroxylation gave 100% regioselectivity, no by-product, and good to excellent yields, being much better than any other known oxidation methods for the preparation of pure benzyl alcohols without any aldehyde or acid by-products. A dehalogenation activity was also discovered in *C. cellulans* EB-8-4 cells, which provides with a novel, green, and efficient method for the preparation of 4-methylbenzyl alcohol **16** in 67–81% yield by biotransformation of 4-methylbenzyl chloride **13** with this strain. The novel enzymatic dehydrogenation could be useful for the biodegradation of chlorine-containing aromatics.

4. Experimental section

4.1. General

Chemicals: *o*-xylene **1** (anhydrous, 97%), *m*-xylene **2** (anhydrous, 99%), *p*-xylene (anhydrous, 99%), 2-ethyl toluene **3** (99%), 3-ethyl

toluene **4** (99%), 2-methylbenzylchloride **14** (99%), 3-methylbenzyl chloride **15** (98%), and 4-methylbenzylchloride **13** (98%) were purchased from Sigma–Aldrich. 2-Propyltoluene **5** (98.5%) and 3-propyltoluene **6** (99.7%) were obtained from TCI (Japan). 1,3-diethylbenzene (>99%) and ethyl benzene were ordered from Fluka. *n*-Hexadecane (>99%) was obtained from Merck. Ethyl acetate was bought from Fisher.

Analytical methods: Gas chromatography (GC) analysis was performed on an Agilent 7890A system 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. Inlet and detector temperature were 280 and 300 °C, respectively. 1 mM *n*-hexadecane was used as internal standard. The temperature was programmed to increase from 60 °C to 100 °C at a rate of 5 °C/min, increase from 100 °C to 116 °C at a rate of 2 °C/min, hold at 116 °C for 2 min, increase from 116 °C to 280 °C at a rate of 30 °C/min, and hold at 280 °C for 3 min. Retention time: 6.2 min for *o*-xylene **1**, 5.8 min for *m*-xylene **2**, 7.9 min for 2-ethyltoluene **3**, 7.5 min for 3-ethyltoluene **4**, 10.2 min for 2-propyltoluene **5**, 9.7 min for 3-propyltoluene **6**, 11.8 min for 2-methylbenzylchloride **14**, 11.7 min for 3-methylbenzyl chloride **15**, 11.9 min for 4-methylbenzyl chloride **13**, 12.6 min for 2-methylbenzyl alcohol **7**, 12.3 min for 3-methylbenzyl alcohol **8**, 12.2 min for 4-methylbenzyl alcohol **16**, 15.7 min for 2-ethylphenyl-methanol **9**, 15.8 min for 3-ethylphenyl methanol **10**, 19.2 min for 2-propylphenyl methanol **11**, 19.3 min for 3-propylphenyl methanol **12**, 20.2 min for 2-(chloromethyl)-phenyl methanol **17**, 20.6 min for 3-(chloromethyl)-phenyl methanol **19**, and 22.3 min for *n*-hexadecane.

GC–MS analysis was performed with an Agilent 7890A GC system coupled with a 5975C mass selective detector (EI) on the same HP5 column as described above. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Inlet and detector temperature were 280 and 300 °C, respectively. The temperature program was the same as the GC analysis mentioned above. The temperature was increased from 60 °C to 100 °C at a rate of 5 °C/min, increase from 100 °C to 116 °C at a rate of 2 °C/min, hold at 116 °C for 2 min, increase from 116 °C to 280 °C at a rate of 30 °C/min, and hold at 280 °C for 3 min.

¹H NMR (500 MHz) spectra were recorded in CDCl₃ at room temperature with a Bruker AMX 500 NMR instrument. Chemical shifts were referred to TMS at 0 ppm.

Optical density of cell cultures was measured at 450 nm (OD₄₅₀) with a Hitachi U-1900 spectrophotometer. A cell density of 1 g cdw/L corresponds to OD₄₅₀ of 0.26.

Growth of *C. cellulans* EB-8-4: *C. cellulans* EB-8-4 was cultured according to previous report.²⁶ The strain 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 ethyl benzene. The culture was incubated at 30 °C and 250 rpm. Cells were harvested at early stationary phase at 24 h with a cell density of 2.9 g cdw/L.

General procedures for biohydroxylation of alkyl toluenes **1**–**6** and methylbenzyl chlorides **13**–**15** to the corresponding benzyl alcohols with resting cells of *C. cellulans* EB-8-4: Cells of *C. cellulans* EB-8-4 was resuspended to 10 g cdw/L in 10 mL K-buffer (pH=7.0) containing 100 mM glucose in a 125-mL conical flask with glass stopper sealed with parafilm. Alkylbenzene **1**–**6** (10–40 mM) or methylbenzyl chlorides **13**–**15** (5–100 mM) were added to the cell suspension and the biotransformation was performed at 30 °C and 200 rpm. At regular time intervals, a 0.2 mL sample was taken and mixed with 0.4 mL ethyl acetate containing 1 mM *n*-hexadecane. After extraction, centrifugation and separation, the organic phase was analyzed by GC.

Product identification: Benzyl alcohols **9**, **10**, **11** and **12** produced from biohydroxylation of substrate **3**, **4**, **5**, and **6** were isolated by column chromatography on silica gel with ethyl acetate: *n*-hexane

(1:4) as eluent. Benzyl alcohols **19** from substrate **15** were isolated by column chromatography on silica gel with ethyl acetate: *n*-hexane (1:1) as eluent. The *R_f* value for all alcohols was in the range of 0.2–0.3. The isolated benzyl alcohols had a purity of >99% (GC). Their ¹H NMR and Mass (from GC–MS) data were given as follows: **9**¹¹: ¹H NMR (CDCl₃, 500 MHz): δ=7.221–7.286 (m, 4H, Ph), 4.732 (s, 2H, CH₂OH), 2.705–2.750 (m, 2H, CH₂Me), 1.236–1.266 (m, 3H, CH₃), *m/z*=136 (M⁺). Compound **10**¹³: ¹H NMR (CDCl₃, 500 MHz): δ=7.133–7.299 (m, 4H, Ph), 4.679 (s, 2H, CH₂OH), 2.658–2.688 (m, 2H, CH₂Me), 1.235–1.261 (m, 3H, CH₃), *m/z*=136 (M⁺). Compound **11**¹⁴: ¹H NMR (CDCl₃, 500 MHz): δ=7.222–7.383 (m, 4H, Ph), 4.728 (s, 2H, CH₂OH), 2.647–2.678 (m, 2H, CH₂CH₂Me), 1.587–1.688 (m, 2H, CH₂Me), 0.976–1.005 (m, 3H, CH₃), *m/z*=150 (M⁺). Compound **12**²⁷: ¹H NMR (CDCl₃, 500 MHz): δ=7.111–7.291 (m, 4H, Ph), 4.669 (s, 2H, CH₂OH), 2.586–2.617 (m, 2H, CH₂CH₂Me), 1.621–1.696 (m, 2H, CH₂Me), 0.938–0.994 (m, 3H, CH₃), *m/z*=150 (M⁺). Compound **17**²⁸: *m/z*=156 (M⁺). Compound **19**²⁹: ¹H NMR (CDCl₃, 500 MHz): δ=7.261–7.407 (m, 4H, Ph), 4.708 (s, 2H, CH₂Cl), 4.596 (s, 2H, CH₂OH), *m/z*=156 (M⁺).

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Supplementary data

The Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.06.039.

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