Mycelia of *Aspergillus flavus* MTCC-9606 as a catalyst for stereoselective hydroxylations of ethylbenzene and propylbenzene

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**KEYWORDS**
Aspergillus flavus; Biocatalyst; Benzyl hydroxylation; (R)-1-phenylethanol (R)-1-phenylpropanol.

**ABSTRACT**
The biotransformation of ethylbenzene to (R)-1-phenylethanol in 97% enantiomeric excess and 67% yield using fungal mycelia of *Aspergillus flavus* MTCC-9609 has been reported. The mycelia also transform propylbenzene to (R)-1-phenylpropanol in 100% enantiomeric excess and 100% yield. Methylbenzene is converted to benzylalcohol in 97% yield. Thus the mycelia of *A. flavus* MTCC-9606 hydroxylate at least methylbenzene, ethylbenzene and propylbenzene at the benzylic positions hydroxylation of which are not convenient by chemical routes.

**INTRODUCTION**
Enantiomerically pure alcohols are particularly useful as building blocks for the synthesis of chiral medicines and are widely used in the preparation of hormones, flavors, fragrances, liquid crystals and chiral auxiliaries. β-amino alcohols have been used for the synthesis of 1-phenyl-2-[(2-phenyl-1-alkylethyl)amino] ethanol derivatives, a new important class of anti-diabetic agents. A large genus of mushroom flies have been reported to be attracted towards 1-phenylethanol in field tests. Thus secondary alcohols act as pheromones also. The chemical methods for their synthesis are not convenient and enantiomeric excesses are generally low. One the other hand, biocatalytic methods are convenient and enantiomeric excess are high.

Several biocatalytic methods to synthesize enantiopure secondary alcohols have been developed during the recent past due to the increasing demands of these valuable compounds. Stereoselective reduction of ketones and enantioselective oxidation of racemic secondary alcohols have been studies for the preparation of pure enantiomeric secondary alcohols. Though the hydroxylation of non activated centres in hydrocarbons is one of the most useful biotransformation, so far it has been studied mainly for the hydroxylation of steroids and terpenoids. The biotransformations of ethylbenzene to 1-phenylethanol have rarely been studied. The conversion of ethylbenzene and a number of its para-substituted derivatives to the corresponding optically active 1-phenylethanols with enantiomeric excess varying between 5 to 40 % using the fungus *mortierella isabellina* have been reported. The fungi *Cunninghamella echinulate varelegans* and *Hemintosporium* were also capable of performing some of these transformations. The hydroxylation of ethylbenzene almost exclusively at the secondary carbon atom giving 1-phenylethanols in the ratio 2:1 of the R and S isomers using cytochrome P450cam has been reported. Seventeen fungi and two yeast species which...
could hydroxylate ethylbenzene and propylbenzene to 1-phenylethanol and 1-phenylpropanol respectively have been reported\cite{12}. One potent strain *Fusarium moniliforme* oxidizes ethylbenzene and propylbenzene to the corresponding benzylic alcohols with an enantiomeric excess of 98% in the (R) (+) form \cite{13}. The involvement of cytochrome P450 in this transformation has been demonstrated\cite{13}. Szaleniec et al.\cite{14} have reported the oxidation of ethylbenzene to (S)-(−)-1-phenylethanol by the denitrifying bacterium *Azoarcus* species strain EbN1. A noble Mo-Fe-S enzyme anaerobic ethylbenzene dehydrogenase has been isolated and characterized\cite{15}. Keeping in view the rare studies reported on the biotransformation of ethylbenzene to optically pure isomers of 1-phenylethanol, we have initiated studies on search of the fungal strains which could do these useful transformations. In this article, we report another fungal strain *Aspergillus flavous* MTCC-9606 which transforms ethylbenzene to (R)-1-phenylethanol in 97% enantiomeric excess and propylbenzene to (R)-1-phenylpropanol in 100% enantiomeric excess.

**MATERIALS AND METHODS**

**Chemicals**

Methylbenzene, ethylbenzene, propylbenzene, ethylmethylketone, racemic (±)-1-phenylethanol, (R)-1-phenylethanol, racemic (±) 1-phenylpropanol and (R)-1-phenylpropanol were purchased from Sigma-Aldrich Chemicals Private limited, New Delhi, India. All other chemicals were purchased either from E. Merck (India) Ltd., Mumbai, India or s.d. fine-chem Ltd., Mumbai (India) or from Qualigens Chemicals, Mumbai (India) and were used without further purifications.

**Preparation of mycelia**

The fungal strain *A. Flvous* MTCC-9606 was isolated in our laboratory, was got identified and deposited at the Microbial Type Culture Collection Centre and Gene Bank, Institute of Microbial Technology, Chandigarh (India). It was maintained on Bennett’s agar medium\cite{12} which consisted of 1.5% (w/v) glucose, 0.5% peptone, 0.2% yeast extract, 0.2% Ehrlich’s beef extract and 1.5% agar in tap water. The microorganism was cultivated in 100 ml of BM I (Basal medium) containing unsterilized ethylmethylketone 1 ml (v/v) in per 100 ml of solution in a 250 ml Erlenmeyer flask at 30 °C on a rotary shaker at 150 rotations per minutes (rpm) for three days. The culture medium BM I contained 10g of NaNO₃, 2g of NH₄Cl, 2g of KH₂PO₄, 3g of K₂HPO₄, 2g of NaCl, 0.2 g of MgSO₄ ·7H₂O, 0.5 g of yeast extract and 2 ml of metal solution having pH 7.0 in one liter of deionised water. The metals solution consisted of 400 mg of MnCl₂ ·2H₂O, 350 mg of FeCl₂ ·4H₂O, 200 mg of ZnCl₂, 20 mg of CoCl₂, 20 mg of CuCl₂ ·2H₂O, 10 mg of Na₂MoO₄ ·2H₂O, 10 mg of Na₂B₄O₄ ·7H₂O, and 2 ml of concentrated HCl in 100 ml of deionised water. The mycelia were collected by filtration on ordinary filter paper, washed twice with 30 ml of 25 mM potassium phosphate buffer (KPB) pH 7.0 and were used fresh.

**Biotransformation reaction**

The biotransformation reaction was performed using the reported method\cite{12}. Wet mycelia 0.1 g was suspended in 2 ml of 25 mM potassium phosphate buffer pH 7.0 in a test–tube of size 17mm diameter and 150mm height and 200 μmol of ethylbenzene (21μl), was added. The test tube was closed with a stopper and incubated at 30 °C on a reciprocal shaker at 200 rpm. After 24 hrs, the reaction solution was acidified by addition of 0.2 ml of 6N HCl. The products formed in the reaction solution were extracted thrice using 2ml of n-hexane each time. The extract was analyzed for 1-phenylethanol, 1-phenylpropanol and benzylalcohol by Waters HPLC Model 600E using spherisorb C₁₈, 5 UV, 4.5 mm × 250 mm column. The eluent phase was methanol water mixture in ratio 1:1 (v/v) at 1 ml/min. The n-hexane extract of the product (20μl) was injected and the detection was made using Waters UV detector model 2487 at 254 nm. For the biotransformations of methylbenzene and propylbenzene, similar procedures were adopted. The identifications of the biotransformation products were determined by IR, ¹H and ¹³C NMR and GC-MS. The enantiomeric excess was determined using chiralcel OD column (4.6 × 250 mm) manufactured by Daicel Chiral Technologies Pvt Ltd (Japan) using 90: 10 (v/v) mixture of n-hexane and isopropylalcohol as the eluent phase at the flow rate of 0.5 ml/min.
RESULTS AND DISCUSSIONS

The results of HPLC analysis of the starting material ethylbenzene, biotransformation products of ethylbenzene and standard sample of (R)-1-phenylethanol are shown in Figure 1 (a), (b) and (c) respectively. The starting material ethylbenzene was eluted with retention time 3.4 minutes [Figure 1(a)] and no peak corresponding to this retention time is present in the chromatogram of the biotransformation products [Figure 1(b)] indicating that all the ethylbenzene has been converted to the products. The chromatogram of the biotransformation products [Figure 1(b)] contained two peaks: one due to 1-phenylethanol with retention time 6.0 minutes with peak area 67% and the other due to phenylacetone with retention time 7.5 minutes with peak area 33%. The chromatogram of the biotransformation products [Figure 1(b)] was similar to the chromatogram of the standard sample of (R)-1-phenylethanol [Figure 1(c)] in which 1-phenylethanol was eluted with retention time 6.0 minutes with peak area 71% and phenylacetone was eluted with retention time 7.9 minutes with peak area 29%. Since even the pure (R)-1-phenylethanol contains 29% phenylacetone, the biotransformation product was 1-phenylethanol some of which got converted to phenylacetone.

The results of $^1$H NMR, $^{13}$C NMR, IR and GC-MS analyses of the biotransformation product clearly confirmed the presence of 1-phenylethanol.

$^1$H NMR (300 MHz, CDCl$_3$)

$\delta = 7.36 – 7.25$ppm (m, 5H, H arom), 4.1 ppm (q, 1H, J=6.4Hz, CHO), 2.1 ppm (br s, 1H, OH), 1.40 ppm (d, 3H, J=6.6 Hz, CH$_3$).

$^{13}$C NMR (500MHz, CDCl$_3$)

$\delta = 145.8$ppm, 128.9 ppm, 127.8 ppm, 125.5 ppm, 77.0 ppm, 25.3 ppm.

IR (KBr)

3440, 2968, 2936, 2880, 1639, 1150, 675 cm$^{-1}$.

GC-MS

Showed the characteristic fragments of 1-phenylethanol with m/z values of 107 and 79, 77 along with the molecular ion peak of 122 (M$^+$) as shown in figure 2.

![Figure 2: GC-MS spectra of fragmentation of bioconversion products 1-phenylethanol and 1-phenylpropanol](image_url)

GC-MS showed the characteristic fragments of 1-phenylethanol with m/z values of 107 and 79, 77 along with the molecular ion peak of 122 (M$^+$) as shown in figure 2.
phenylethanol which has retention time of 13.4 minutes with percentage area of 98%. Figure 3(c) is the chromatogram of the biotransformation product which has a peak with retention time 13.5 minutes and having peak area of 97%. These results clearly show that the product 1-phenylethanol has been formed in 97% (R)-enantiomeric excess.

The spectral analysis given below confirmed the identity of 1-phenylpropanol.

1H-NMR (300 MHz, CDCl₃, Me₄Si)

δ=7.21-7.30ppm (m, 5H, H arom), δ= 4.6ppm (t, 1H, CH-OH J=6.6 Hz), δ=3.5ppm (S, OH) δ=1.91-1.69 (m, 2H), 0.92 (t, 3H, J =7.5 Hz).

13C NMR (500MHz, CDCl₃)

δ=146.7ppm, δ=129.2ppm, δ=127.8ppm, δ=126.5ppm, δ=70.0ppm, δ=26.3ppm and δ=15.3ppm.

IR (KBr.)

3360 cm⁻¹ for stretching of –OH cm⁻¹, 2963 cm⁻¹, 1492 cm⁻¹, 1377 cm⁻¹, 1200 cm⁻¹, 1097 cm⁻¹, 974 cm⁻¹, 670 cm⁻¹.

GC-MS

The results of GC-MS analysis of the biotransform product 1-phenylpropanol gave peaks corresponding to fragments with m/z values of 107 and 79, 77 along
with molecular ion peaks m/z=136 [M]+ shown in figure 2.

The results of the determination of the enantiomeric excess of biotransformation product (R)-1-phenylpropanol are shown in figure 5. The figure 5 (a) is the chromatogram of racemic (+)-1-phenylpropanol obtained using chiralcel OD column (4.6 x 250 mm) indicating clearly 50:50 percent peak areas of (R) and (S) forms of the racemic mixture (+)-1-phenylpropanol with retention time 13.3 minutes and 14.3 minutes. Figure 5(b) shows the chromatogram of the standard sample of (R)-1-phenylpropanol having 100% enantiomeric excess of the (R) form with retention time 13.3 minutes. The chromatogram of the biotransformation product 1-phenylpropanol as shown in figure 5(c) contains one peak at 13.5 minutes which corresponds to (R)-1-phenylpropanol. The enantiomeric excess of the biotransformation product was 100%.

The biotransformation of methylbenzene gave 97% benzylalcohol and 3% benzoic acid (results not shown here). Though we have not attempted to identify the enzyme which is responsible for the above transformations, cytochrome P450 monooxygenase is the most likely enzyme involved in these transformations. There are reports[11,13] also that cytochrome P450 monooxygenase is involved in such conversions. It is worth mentioning that due to inherent problems[16-20] associated with working with pure oxygenases, whole cell systems are the preferred biocatalysts for applications in biotransformations in organic syntheses.

CONCLUSIONS

Thus mycelia of A. flavus MTCC-9606 transform ethylbenzene to (R)-1-phenylethanol in 97% enantiomeric excess and propylbenzene to (R)-1-phenylpropanol in 100% enantiomeric excess. The same fungal mycelia also transform methylbenzene to 96% benzylalcohol and hence is a good biocatalyst for these biotransformations. By using increased amounts of mycelia we have achieved (R)-1-phenylethanol and (R)-1-phenylpropanol up to 1ml scales.

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