Catalysis Communications 12 (2011) 781–784

Contents lists available at ScienceDirect

Catalysis Communications

journal homepage: www.elsevier.com/locate/catcom

Stereoselective hydroxylation of ethylbenzene to (R)-1-phenylethanol using mycelia of *Aspergillus niger* as catalyst

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ARTICLE INFO

Article history:
Received 4 December 2010
Received in revised form 20 January 2011
Accepted 21 January 2011
Available online 1 February 2011

Keywords:
Alkylbenzene
Aspergillus niger
Chiral alcohol
Benzylic hydroxylation
Oxygenase
Biocatalyst

ABSTRACT

The mycelia of *Aspergillus niger* MTCC-404 have been shown to act as a biocatalyst for the transformation of ethylbenzene to (R)-1-phenylethanol in 99% enantiomeric excess. 72% of the products are (R)-1-phenylethanol. The conversion yield is dependent on pH, temperature and mycelia concentration in suspension. *A. niger* MTCC-404 facilitates methylbenzene transformation to benzylalcohol, and propylbenzene to 1-phenylpropanol. Therefore, *A. niger* serves as a biocatalyst for hydroxylation of alkylbenzene's benzylic position.

1. Introduction

Bearing specific functional groups, chiral alcohols are important intermediates for the synthesis of many chiral medicines and are widely used in the preparation of hormones, flavors, fragrances, liquid crystals and chiral auxillaries [1]. β-Amino alcohols are widely used as building blocks in organic synthesis, and in medicinal chemistry. They have been used for the synthesis of 1-phenyl-2-[(2-phenyl-1-alkylethyl)amino]ethanol derivatives, a new important class of antidiabetic agents [2]. Racemic 1-phenylpropanol has been used as cholertic drug and its (R)-isomer has been used in the synthesis of optically active 1-chloro-1-phenylpropanol [3] and liquid crystals [4]. The (R)-1-phenylpropanol is also an auxiliary in the synthesis of terpines [5]. Chiral phenylalcohols are expensive and the traditional chemical routes of their preparations are difficult [6]. Though the hydroxylation of non activated centers in hydrocarbons is one of the most useful biotransformations, so far it has been studied mainly for the hydroxylation of steroids and terpeniods [6]. Nevertheless, the biotransformations of alkylbenzenes to their corresponding chiral phenylalcohols have rarely been studied [7–11]. *Bacillus megaterium* [7] has been used to transform propylbenzene to (R)-1-phenylpropanol with 74% enantiomeric excess and giving a conversion yield of 63%. The conversions of ethylbenzene and a number of its para-substituted derivatives to the corresponding optically active 1-phenylethanols with enantiomeric excesses varying between 5 and 40% using the fungus *Mortierella isabellina* have been reported [8]. The fungi *Cunninghamella echinulate* var elegans and *Heminthosporium* 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 [9]. Seventeen-fungi and two-yeast species which could hydroxylate ethylbenzene and propylbenzene to 1-phenylethanol and 1-phenylpropanol respectively have been reported [10]. One potent fungal strain identified as *Fusarium moniliforme* oxidizes ethylbenzene and propylbenzene to the corresponding benzylic alcohols with an enantiomeric excess of 98% in the (R)-(+) form. The involvement of cytochrome P450 in this transformation has been demonstrated [9]. Szaleniec et al. [12] have reported the oxidation of ethylbenzene to (S)-(−)-1-phenylethanol by the denitrifying bacterium Azoarcus sp. strain EbN1. A novel Mo–Fe–S enzyme anaerobic ethylbenzene dehydrogenase has been isolated and characterized [13]. Apart from these reports, there are no studies on the microbial transformations of alkylbenzenes to 1-phenylalcohols. Keeping in view the rare reported studies on the above transformations, we have initiated studies on the transformations of alkylbenzenes to 1-phenylalcohols by indigenous fungi. In this short communication, we report the biotransformation of ethylbenzene to (R)-1-phenylethanol [Scheme 1] in 99% enantiomeric excess using fungal mycelia of *Aspergillus niger* MTCC-404 biotechnological applications of which have not been reported in the literature so far. It is worth mentioning that due to inherent problems [14–18] associated with working with pure oxygenases; whole cell systems are preferred biocatalysts for applications in organic synthesis.

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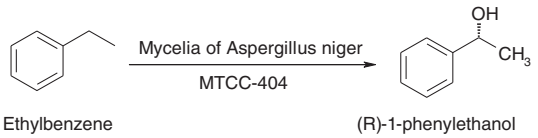
doi:10.1016/j.catcom.2011.01.020



Scheme 1. Bioconversion of ethylbenzene to (R)-1-phenylethanol.

2. Materials and methods

2.1. Chemicals

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

2.2. Preparation of mycelia

The fungal strain *A. niger* MTCC-404 was procured from the Microbial Type Culture Collection Centre and Gene Bank, Institute of Microbial Technology, Chandigarh and was maintained on Bennett's agar medium [10] which consisted of 1% (w/v) glucose, 0.5% peptone, 0.2% yeast extract, 0.2% Ehlrich's beef extract and 1.5% agar in tap water. The microorganism was cultivated in 100 ml of BM1 (Basal

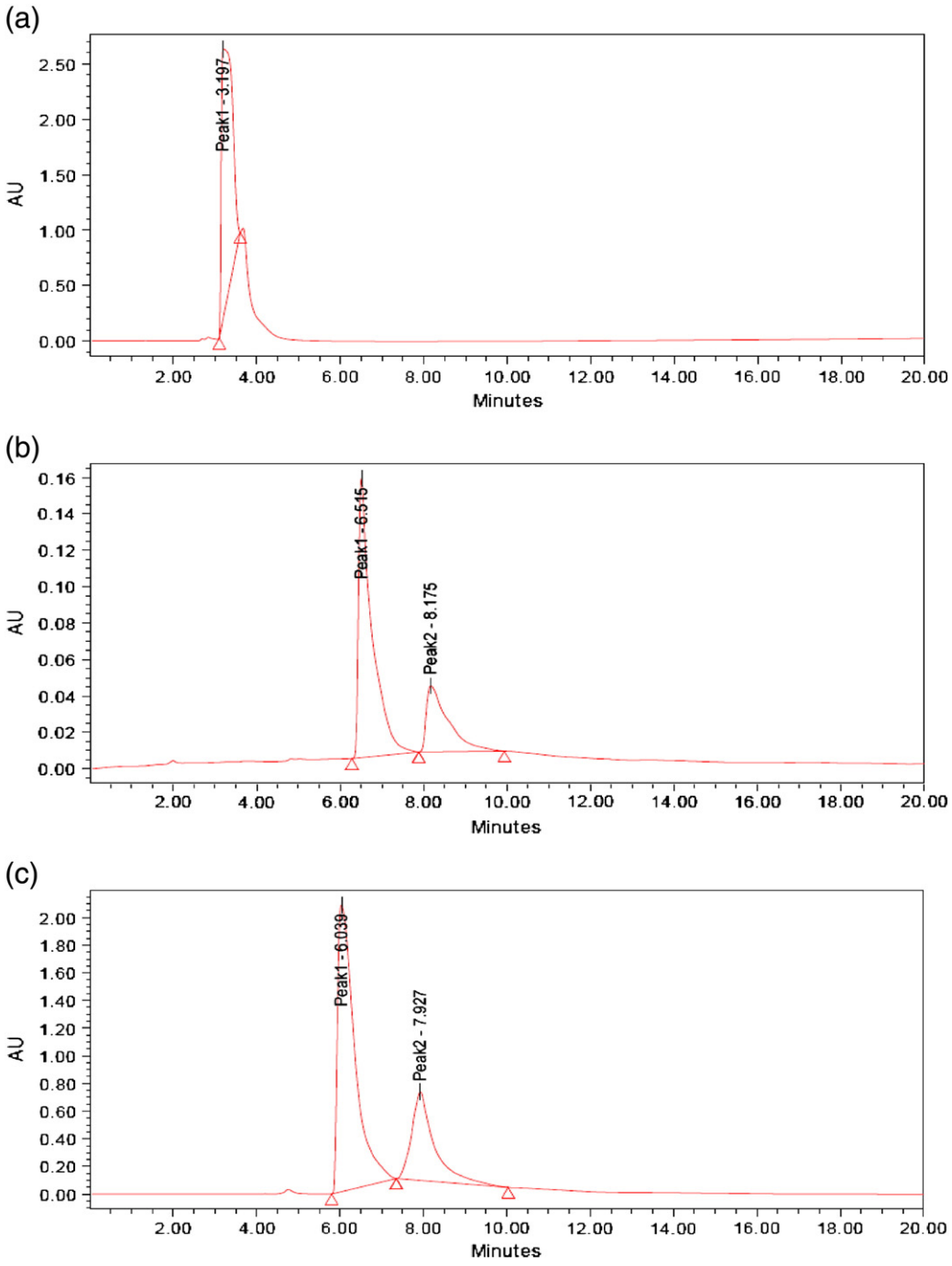


Fig. 1. HPLC analysis of (a) ethylbenzene, (b) bioconversion product 1-phenylethanol and (c) standard sample of (R)-1-phenylethanol.

medium) containing unsterilized 1% (v/v) ethylmethylketone 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 BM1 contained 10 g of $NaNO_3$, 2 g of NH_4Cl, 2 g of KH_2PO_4, 3 g of K_2HPO_4, 2 g of NaCl, 0.2 g of $MgSO_4.7H_2O$, 0.5 g of yeast extract and 2 ml of metal solution having pH 7.0 in 1 l of deionised water. The metal solution consisted of 400 mg of $MnCl_2.2H_2O$, 350 mg of $FeCl_2.4H_2O$, 200 mg of $ZnCl_2$, 20 mg of $CoCl_2$, 20 mg of $CuCl_2.H_2O$, 10 mg of $Na_2MoO_4.2H_2O$, 10 mg of $Na_2B_4O_7.10H_2O$, 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 and were used fresh.

2.3. Biotransformation reaction

The biotransformation reaction was performed using the reported method [8]. Wet mycelia 0.1 g were suspended in 2 ml of 25 mM potassium phosphate buffer in a test-tube of size 17 mm diameter and 150 mm height and 200 μmol of ethylbenzene (21 μl) was added. The test tube was closed with a stopper and incubated at 30 °C on a reciprocal shaker at 200 rpm. After 24 h, the reaction solution was acidified by addition of 0.2 ml of 6 N HCl. The products formed in the reaction solution were extracted thrice using 2 ml of n-hexane each time. The extract was analyzed for 1-phenylethanol by Waters HPLC Model 600E using spherisorb C_{18} 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 (20 μl) 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 ^{13}C 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 a flow rate of 0.5 ml/min.

3. Results and discussion

The results of HPLC analysis of the starting material ethylbenzene, biotransformation products of ethylbenzene and standard sample of (R)-1-phenylethanol are shown in Fig. 1(a), (b) and (c) respectively.

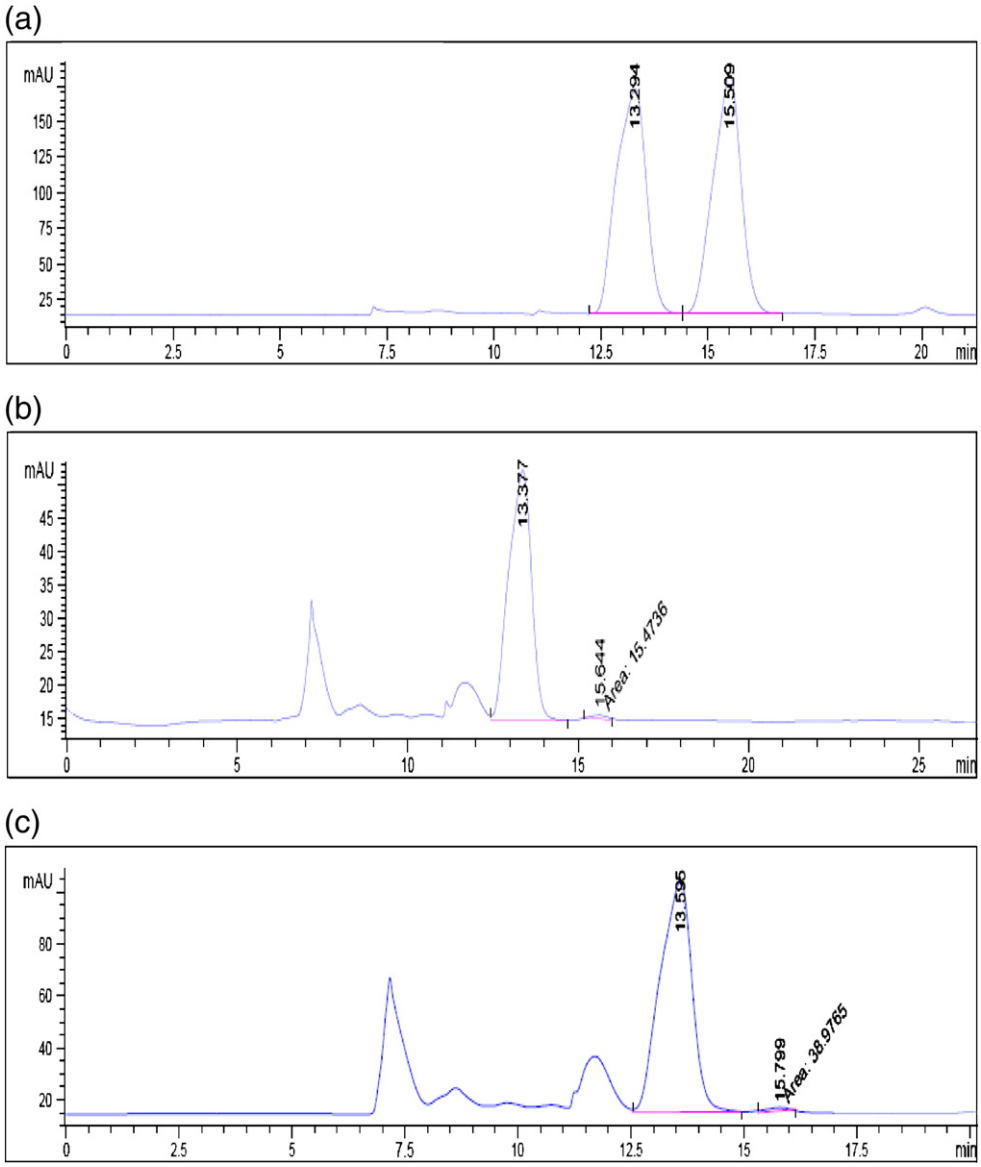


Fig. 2. HPLC analysis of enantiomeric excess of (a) recemic (±) mixture of 1-phenylethanol, (b) standard sample of (R)-1-phenylethanol and (c) bioconversion product 1-phenylethanol.

Table 1
Dependence of the yield of formation of (R)-1-phenylethanol from ethylbenzene by *Aspergillus niger* pH, temperature and concentration of mycelia in its suspension.

S.N.	pH	Percentage yield	Temperature (°C)	Percentage yield	Mycelia concentration (gm)	Percentage yield
1	5.0	30	25	81	0.2	35
2	7.0	72	30	72	0.5	72
3	8.0	53	35	93	1.0	87

Since (R)-1-phenylethanol gets converted to 1-phenylacetone even the standard sample of (R)-1-phenylethanol gives two peaks one due to (R)-1-phenylethanol (71%) at retention time 6.0 min and the other due to its oxidation products 1-phenylacetone (29%) at retention time 7.9 min. Like the standard sample of (R)-1-phenylethanol, the biotransformation product also gave two peaks one due to (R)-1-phenylethanol (72%) at a retention time 6.5 min and the other due to its oxidation product 1-phenylacetone (28%) with a retention time 8.2 min. The starting material ethylbenzene was eluted with a retention time 3.2 min. No peak corresponding to ethylbenzene in the HPLC chromatogram of the extract of the biotransformation product was detected indicating that all the ethylbenzene was converted to the product.

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, $CDCl_3$)

$$\delta = 7.20\text{–}7.29(\mathrm{m}, 5\mathrm{H}, \mathrm{Harom}),\quad 4.74(\mathrm{q}, 1\mathrm{H}, \mathrm{J} = 6.3\ \mathrm{Hz}, \mathrm{CHOH}),\quad 2.92(\mathrm{brS}, 1\mathrm{H}, \mathrm{OH}),\quad 1.40\ (\mathrm{d}, 3\mathrm{H}, \mathrm{J} = 6.3\ \mathrm{Hz}, \mathrm{CH_3}).$$

^{13}C NMR (75 MHz, $CDCl_3$):

$$\delta = 145.7, 128.2, 127.1, 125.3, 70.0, \text{ and } 25.0$$

IR (Film):

$$3309, 3110, 2907, 1608, 1160, 764, \text{ and } 710\ \mathrm{cm}^{-1}$$

GC-MS: showed the characteristic fragments of 1-phenylethanol with m/z values of 107, 79 and 77 along with the molecular ion peak of 122.

The results of determination of enantiomeric excess of the biotransformation product (R)-1-phenylethanol are shown in Fig. 2, in which Fig. 2(a) is the chromatogram of racemic (±)-1-phenylethanol obtained using chiralcel OD column (4.6×250 mm) indicating clearly 50:50 percent peak areas of (R) and (S) forms of the racemic mixture (±) 1-phenylethanol. Fig. 2(b) shows the chromatogram of the standard sample of (R)-1-phenylethanol showing 99% enantiomeric excess of the (R) form. Fig. 2(c) shows the chromatogram of the biotransformation product indicating that it is 99% (R) form.

The experiments were performed to see the effect of pH, temperature and mycelia concentration on the percentage conversion of ethylbenzene to 1-phenylethanol. The results are summarized in Table 1. It becomes evident from the data listed in Table 1 that the percent yield of the product 1-phenylethanol is dependent on the experimental conditions. Due to our limitations for determination of enantiomeric excess of the product under different experimental conditions, enantiomeric excess of (R)-1-phenylethanol could not be determined under all the experimental condition studied.

In order to test the applicability of this transformation at a larger scale, 1.0 g fungal mycelia were suspended in 20 ml of 25 mM potassium phosphate buffer pH 7.0 and 300 µl of ethylbenzene was added. The reaction mixture was placed on the rotatory shaker at 180 rpm at 30 °C for 24 h. The product was extracted with 20 ml of n-hexane. No ethylbenzene was detected in the extract by HPLC indicating that it has been fully converted to the products namely 72% to (R)-1-phenylethanol and 28% to 1-phenylacetone.

In order to resolve the question whether the mycelia of the same fungal strain could be used for benzylic hydroxylation of other alkylbenzenes, the biotransformations of methylbenzene and propylbenzene were also tested. The biotransformation of methylbenzene gave 56% benzylalcohol and 40% benzaldehyde. Propylbenzene gave only 47% of 1-phenylpropanol leaving 38% propylbenzene unreacted. These results showed that other alkylbenzenes could also be converted to their corresponding alcohols at benzylic positions though the conversion yields need to be optimized by changing the experimental conditions.

Though we have not attempted to identify the enzyme involved in the above conversions, cytochrome P450 mono-oxygenases is the most likely enzyme. There are reports [9,11] also that cytochrome P450 mono-oxygenase is involved in such conversions.

4. Conclusions

This short communication reports the biotransformation of ethylbenzene to (R)-1-phenylethanol in 99% enantiomeric excess with 72% yield using the fungal mycelia of *A. niger* MTCC-404. Methylbenzene and propylbenzene were also converted to benzylalcohol and 1-phenylpropanol respectively, but the conversion yields were low and needed optimization of the experimental conditions to achieve better yields. Such studies are in progress.

Acknowledgments

The financial support of CST, U.P., Lucknow through project No. CST/SERPD/D-2895, dated 01.02. 2008 under which this research work has been done is thankfully acknowledged. The scientific helps rendered by Prof. V. K. Yadav, Department of Chemistry, Indian Institute of Technology, Kanpur, U.P., India, in the determination of enantiomeric excess of the product and by Prof. R. Gurunath of the same department in GC-MS analysis of the product, are thankfully acknowledged.

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