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Total production of (*R*)-3,5-bistrifluoromethylphenyl ethanol by asymmetric reduction of 3,5-bis(trifluoromethyl)-acetophenone in the submerged culture of *Penicillium expansum* isolate

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ABSTRACT

A total of 82 fungal isolates were screened for their ability to reduce 3,5-bis(trifluoromethyl)-acetophenone **1**. *Penicillium expansum* was found to successfully reduce ketone **1** in the submerged culture. A second screening was performed on the active *P. expansum* strains identified by the first screening. A number of strains of *P. expansum* were found to produce (*R*)-3,5-bistrifluoromethylphenyl ethanol **2** with over 99% enantiomeric excess (ee) from ketone **1**. The most productive strain was identified as *P. expansum* EBK-9, and this strain was selected for further experiments. The total production of **2** was carried out by *P. expansum* EBK-9 in a laboratory-scale bioreactor employing optimized conditions as determined by our experiments. *P. expansum* EBK-9 gave **2** with ee >99% and 76% yield. On a large scale, a total of 3.35 g/L of **2** was produced from **1** after 56 h.

1. Introduction

Biocatalysts are used due to their high specific activities in the pharmaceutical industry. A significant number of pharmaceutical products are chiral and many have multiple stereogenic centers. The efficient syntheses of these stereogenic centers require enantioselective and/or regioselective catalysts. In recent years, enzymes have become the most efficient and most selective catalysts available.[1] It is well known that chiral alcohols are very important precursors for a large number of pharmaceuticals and (*R*)-3,5-bistrifluoromethylphenyl ethanol **2** is no exception. It is an important intermediate in the synthesis of therapeutic agents such as the NK-1 receptor antagonist used for the treatment of emesis.[2] A single approach for the synthesis of **2** is the asymmetric reduction of 3,5-bis(trifluoromethyl)-acetophenone **1**. There are various catalytic methods available such as direct enantioselective hydrogenation[3] or borane reduction[4] for the asymmetric reduction of **1**. However, they often require the utilization of hazardous reagents. In light of this, the asymmetric reduction of **1** was investigated using microbial resting cells and various enzymes. Gelo-Pujic et al. showed that the reduction of 1-[3,5-bis(trifluoromethyl)phenyl]ethanone with resting cells of the bacteria *Lactobacillus kefir* gave the corresponding (*R*)-alcohol with >99% ee.[5] On the other hand, the reduction by an alcohol dehydrogenase from bacteria *Rhodococcus erythropolis* gave the corresponding (*S*)-alcohol with 99% ee.[6] The (*S*)-alcohol is currently under clinical evaluation as an intermediate for antagonist. However, reactions catalyzed by enzymes demand the use of cofactors such as NADH, which are very expensive.

In recent years, there has been a growing interest in the isolation of microorganisms from the environment for the synthesis of organic materials. The screening of isolated microorganisms is an important way to obtain new microorganisms and compounds, or to improve the productivity of known compounds. In order to improve the yield and the enantioselectivity of a product, many synthetic chemists and biologists have adopted the methods of screening microorganisms,[7,8] which leads to constructing perfect gene engineering strains.[8] The addition of inhibitors to inhibit the enzymes, which convert the substrate to the undesirable configuration product[9] and the addition of co-substrates to increase the yield and control reaction stereoselectivity[10] have also been investigated and reported. In our laboratories, chiral alcohols have been prepared with various microorganisms using a similar reduction system.[11] However, there have been no reports regarding the production of **2** by the bioreduction of **1** in the submerged culture.

Herein, we report the asymmetric reduction of **1** to the corresponding chiral alcohol **2** in the submerged culture system with screening of microorganisms. The effects of co-substrates and reaction conditions on the yield and enantiomeric excess (ee) have also been investigated in detail.

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2. Results and discussion

A total of 82 fungal isolates isolated from various soils from Erzurum, Turkey were tested for their ability to produce alcohol **2** from ketone **1**. Among the isolates, the initial screening determined three isolates that produced **2** with both a highest conversion (50–70%) and highest ee (88–99%). The three isolates were identified as *Penicillium expansum*. The 14 strains of the *P. expansum* determined by the first screening were then isolated from various samples and screened for their **2**-producing activity. Substrate tolerance of the biocatalyst is very important for its potential industrial application. Higher substrate tolerance of the biocatalyst can increase its production capability in industrial processes. In light of this, different *P. expansum* strains with the same substrate concentration (0.5 mmol substrate/100 mL culture) were tested for the determination of the catalytic capacities of the strains. It has been reported that reaction stereoselectivity and product yield are related to the kind of reductases in microorganisms and the molecular structure of the substrates.[12] The reduction results of compound **1** by 14 strains are listed in Table 1. It was found that all strains produced optically active **2** from **1**. Ten of the strains examined (EBK-2, EBK-3, EBK-4, EBK-5, EBK-6, EBK-9, EBK-11, EBK-12, EBK-13 and EBK-14) produced the desired product with high enantiomeric purity (>99% ee). The conversion rates of **1** from the 10 strains were compared and it was found that *P. expansum* EBK-9 showed the highest activity (90% conversion and >99% ee) in the second screening. The results showed that the conversion and enantiomeric purity are strongly dependent on the different strains of the same species. From these results, we suggest the careful control of the strain used as biocatalyst for enantiomeric purity and conversion in the asymmetric reduction. On the basis of these results, *P. expansum* EBK-9 was selected as the best **2**-producing strain and was used for further experiments. Gelo-Pujic et al.[5] reported that the bacteria *L. kefir* is an effective biocatalyst for the asymmetric bioreduction of **1** to its corresponding (*R*)-alcohol with 99% ee. They reported that their studies employed whole cells of *L. kefir*, whereas our current study used a submerged culture of *P. expansum* fungus. In order to improve the yield with high ee of **2**, the effects of fermentation conditions such as pH, temperature, reaction time and agitation were studied with *P. expansum* EBK-9 in the shake flasks. The aim was to achieve high conversion with high enantiomeric purity. In order to minimize the error in the experiments the substrate concentration (the concentration of **1** added to the submerged culture of *P. expansum* EBK-9) was increased from 0.5 mmol **1** to 1.0 mmol **1** for all subsequent experiments.

One of the prime requirements of a microbial bioconversion is the optimization of the reaction conditions. However, microbial production can be performed on a large scale so that it can be applied in an industrial process using an engineered microorganism and designed the reaction conditions.[13,14] Therefore, it was decided to determine the performance of the microorganism under optimum reaction conditions. The first parameter that was investigated was the effect of pH on the production of **2** from **1** in the submerged culture. Table 2 shows the results for the effect for the pH range from 3.0 to 8.0 on the product's ee and conversion of **1**. It was observed that the reduction of **1** was greatly affected by pH. The results indicate that ketone **1** could be reduced to alcohol **2** at pH 5.0–7.5. Above or below these values, no or weak growth was observed. At low pH values (5.0–6.0), low ee's were obtained and at higher pH values (6.5–7.5) ee's of >99% were obtained. The highest conversion (54%) with >99% ee was obtained at pH 6.5. At this pH the bioactivity of the EBK-9 strains was the best for the reduction of ketone **1**. All further studies were performed at pH 6.5.

The second parameter that was investigated was the effect of reaction temperature on the reduction of **1**. Table 3 shows the results that were obtained. The reaction temperature was varied from 22 to 30 °C and it was observed that maximum conversion (62%) was achieved at 26 °C. Temperatures below and above 26 °C saw a rapid decline in conversion. Interestingly, the enantiopurity of **2** remained at >99% at all the temperatures investigated. The optimum temperature for the reduction of ketone **1** to alcohol **2** was set at 26 °C and hence, all the subsequent reduction experiments were carried out at 26 °C.

The effect of agitation speed on the reaction rate was the third parameter investigated and the results are given in Table 4. Agitation speeds from 100 rpm to 300 rpm were chosen for the experiments. The results obtained from the experiments showed that the conversion of **1** was at a maximum at high agitation speeds (more than 200 rpm). The enantiopurity, however, was recorded at >99%-(*R*) for most agitation speeds but decreased significantly to 79% at 300 rpm. This decrease might be due to the effect of shear stress on the fungus cells as well as on the enzyme structure. The best yield of alcohol **2** with high ee was obtained at an agitation rate of 250 rpm. In light of this, an agitation speed of 250 rpm was used for subsequent studies. We also added 1.5 mmol of **1** instead of 1 mmol of **1** to the reaction culture medium due to the 100% conversion.

The effects of co-substrates and their concentrations on the conversion and enantioselectivity for preparative scale were also examined. In an attempt to improve the conversion of **1** to **2** by *P. expansum* EBK-9, three different co-substrates, 2-propanol, glycerol and ethanol, were assayed and compared in this study. Employing the optimum conditions, the co-substrates at various %/v concentrations and ketone **1** directly were added to the submerged culture medium after the growth of the fungus for 48 h. The results for these experiments are presented in Table 5. The enantiopurity of the desired product was not affected by the addi-

Table 1
Screening of *P. expansum* strains for the bioreduction of **1**

Isolates	Conversions (%)	ee (%)-(*R*)-configuration
EBK-1	15	88
EBK-2	11	>99
EBK-3	38	>99
EBK-4	75	>99
EBK-5	12	>99
EBK-6	48	>99
EBK-7	42	79
EBK-8	46	85
EBK-9	90	99
EBK-10	55	80
EBK-11	80	>99
EBK-12	54	>99
EBK-13	34	>99
EBK-14	22	>99

Reaction conditions: substrate 0.5 mmol, temperature 30 °C, reaction time 24 h, pH 7.0, agitation 150 rpm.

Table 2
Effects of different pHs on the reduction of **1** by *P. expansum* EBK-9

pH	Conversions (%)	ee (%)-(*R*)-configuration
3.0	No growth	—
4.0	Weak growth	—
5.0	10	60
5.5	25	79
6.0	42	97
6.5	54	>99
7.0	47	>99
7.5	27	—
8	Weak growth	—

Reaction conditions: substrate 1 mmol, temperature 30 °C, reaction time 24 h, agitation 150 rpm

Table 3
Effects of temperature on the bioreduction of **1** by *P. expansum* EBK-9

Temperature (°C)	Conversion (%)	ee (%)-(*R*)-configuration
22	42	>99
24	54	>99
26	62	>99
28	52	>99
30	46	>99

Reaction conditions: substrate 1 mmol, pH 6.5, time 24 h, agitation 150 rpm.

Table 4
Effects of agitation speed on the bioreduction of **1** by *P. expansum* EBK-9

Agitation (rpm)	Conversion (%)	ee (%)-(*R*)-configuration
100	57	>99
150	62	>99
200	94	>99
250	100	>99
300	100	79

Reaction conditions: substrate 1 mmol, temperature 26 °C, pH 6.5, time 24 h.

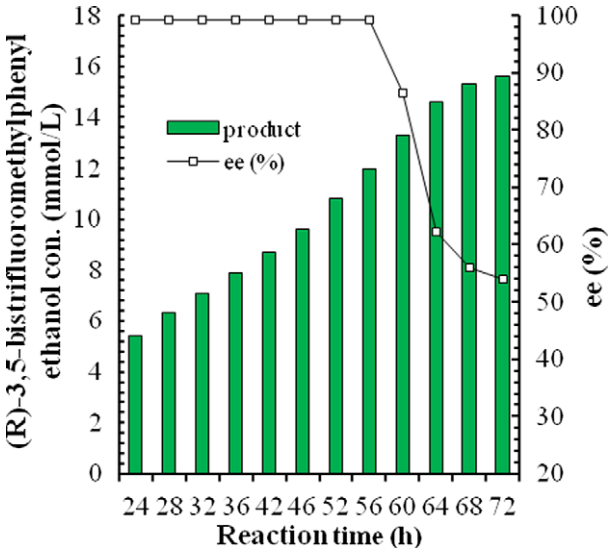


Figure 1. Time course of the production on a preparative scale of (*R*)-3,5-bistrifluoromethylphenyl ethanol by *P. expansum* EBK-9. Substrate concentration is 20 mmol/L.

tion of any co-substrate. However, the conversion of **1** was found to be strongly dependent on the structure and concentration of the co-substrate. The conversion rate of **1** was highest when the less bulky ethanol was used as a co-substrate compared to the bulky glycerol. The conversion did not significantly increase when using 2-propanol, however, when using ethanol the conversion increased to 100%. It should be noted that the concentration of the co-substrate also had an affect on the conversion. When ethanol or 2-propanol was employed the conversion increased from concentration 0.2 to 0.6%/v and then rapidly decreased as the concentration was increased above 0.6%/v to 0.8 and 1.0%/v. The reason for such a low conversion must be related to the inhibition of the enzyme involved in the bioreduction. Using ethanol at a concentration of 0.6%/v gave 100% conversion of ketone **1** to alcohol **2**; this was the highest conversion obtained for the various experiments and thus ethanol at a concentration of 0.6%/v was selected as the co-substrate and used for preparative scale production.

The preparative scale production is one of the most vital issues that needs to be addressed in asymmetric reductions by biocatalysts. In light of this, we undertook a study of the preparative scale productions of **2** which were carried out on a 1 L scale using a fermenter under the optimized conditions (pH 6.5, agitation 250 rpm, temperature 26 °C substrate 20 mmol/L and ethanol as co-substrate 6 mL/L). Figure 1 illustrates the product **2** concentration and percentage ee over a 72 h incubation period. The product concentrations as reported were obtained after purification via column chromatography. Initial experiments were carried out using 20 mmol/L of **1** and incubated for up to 72 h. After 72 h, complete conversion was achieved and 15.6 mmol/L of **2** was produced. However, the ee of the desired alcohol was only 54% ee. The unsatisfactory ee result obtained is due to the degradation of the microorganism cells as a result of the long incubation period. It is well known that degradation products, which are often different enzymes, cause low ee's. It was observed that the enantioselectivity of *P. expansum* EBK-9 was dependent on the incubation time. Although 100% conversion was achieved after 72 h, the enantioselectivity was of greater importance and therefore, the optimum incubation time for this bioprocess was selected as 56 h and was thus used for further studies.

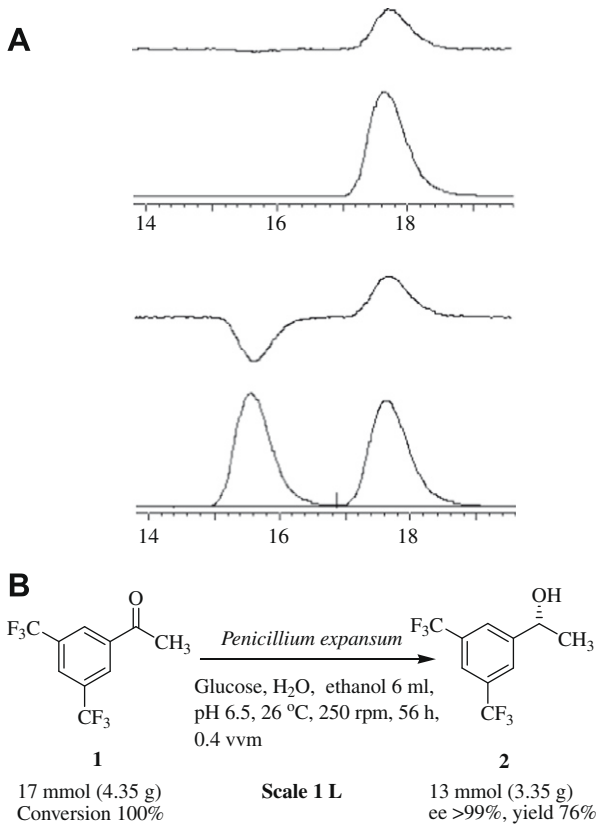
It is well known that in biocatalytic reactions, substrate concentration is a very important parameter, since it either influences the full utilization of enzyme activity or may result in substrate inhibition. The bioreduction was thus further studied by employing the above-determined operational conditions and 17 mmol/L **1** instead of 20 mmol/L **1**. The results for the reaction are shown in Scheme 1. The reaction was stopped after 56 h, to give a final concentration of 13 mmol/L of **2** in very high purity (>99% ee). The product, (*R*)-3,5-bistrifluoromethylphenyl ethanol **2** was obtained in 76% yield (3.35 g/L) from 4.35 g/L **1**; $[\alpha]_D^{20} = +16.4$ (*c* 0.85, $CHCl_3$); >99% ee as determined by HPLC on a OD-H chiral column; retention times were 15.6 min for (−) (*S*) and 17.6 min for (+) (*R*) (Scheme 1). *P. expansum* EBK-9 has been shown to be a potent biocatalyst for the bioconversion of 3,5-bis(trifluoromethyl)-acetophenone to the corresponding (*R*)-enantiomer with >99% ee, however, there has been no report regarding the production of **2** by this organism in a laboratory-scale bioreactor. This is thus the first report for the enantioselective synthesis of **2** by this microorganism.

As aforementioned, Gelo-Pujic et al.[5] also studied the enantioselective bioreduction of **1** but they instead carried out the reactions with whole cells of *L. kefir* and obtained the (*R*)-enantiomer with 99% ee. On the other hand, Pollard et al.[6] produced the (*S*)-enantio-

Table 5
Effects of co-substrates and their concentrations on the bioreduction of **1** to **2** by *P. expansum* EBK-9

Co-substrate concn (%/v)	Glycerol		2-Propanol		Ethanol	
	Conversion (%)	ee (%)-(*R*)	Conversion (%)	ee (%)-(*R*)	Conversion (%)	ee (%)-(*R*)
0.2	68	>99	72	>99	88	>99
0.4	67	>99	78	>99	96	>99
0.6	67	>99	82	>99	100	>99
0.8	68	>99	74	>99	97	>99
1.0	68	>99	68	>99	74	>99

Reaction conditions: substrate 1.5 mmol, temperature 26 °C, pH 6.5, agitation 250 rpm, time 24 h.



Scheme 1. (A) Chiral analysis of the product by HPLC. (B) Total production of the **2** in a laboratory-scale bioreactor. Yield (%) = 100 × **2**/**1**.

mer with 99% ee using the enzyme obtained from *R. erythropolis*. However, the reaction was catalyzed using cofactors, which are very expensive. Herein, 3,5-bis(trifluoromethyl)-acetophenone **1** was reduced to the corresponding (*R*)-enantiomer with >99% ee using a submerged culture of the *P. expansum* EBK-9. Active biocatalysts have been obtained by screening a broad variety of fungi isolates. This fungus was firstly used for the production of a chiral alcohol. This biotransformation was successfully carried out on a preparative level as well. The results indicate that *P. expansum* fungus is an excellent source of production of **2**, which suggests that the fungus has the potential to undergo genetic engineering in order to improve its production level. Furthermore, the isolation of a new microorganism from the environment has sparked the interest of researchers because it is estimated that only very few existing microbial species are actually known. A new microorganism can lead to the discovery of new enzymes able to catalyze various types of reactions.

3. Conclusions

In the first set of experiments, submerged cultures of 82 fungus isolates were screened for the bioreduction of **1** to optically active **2**. Using a two-step screening strategy, *P. expansum* EBK-9 was identified as an effective biocatalyst with high enantioselectivity and a remarkable conversion value. In the second set of experiments, the reaction parameters for the conversion of ketone **1** with maximum ee by *P. expansum* EBK-9 were optimized. The optimum reaction parameters (pH 6.5, temperature 26 °C, time 56 h, agitation 250 rpm, co-substrate 6 mL/L) led to high conversion of **1** and with excellent ee (>99%) of **2**. In the last set of experiments, the production of gram amounts of alcohol **2** on a preparative scale was investigated. The preparative scale bioreduction of **1** by *P. expansum* EBK-9 gave **2** in 76% yield, complete conversion and >99% ee. From the fermentative studies 13 mmol/L (3.35 g) of alcohol **2** was obtained from 17 mmol/L (4.35 g) of ketone **1**. This is the first report on the mass production of **2** with *P. expansum* as a biocatalyst on a laboratory-scale bioreactor, which can be directly applied for the industrialization of **2**. Furthermore, this study demonstrates the first example of the effective synthesis of (*R*)-3,5-bistrifluoromethylphenyl ethanol by the fungus *P. expansum*.

4. Experimental

4.1. Materials

3,5-Bis(trifluoromethyl)-acetophenone **1** was bought from Aldrich Organics (St. Louis, USA). The racemic 3,5-bistrifluoromethylphenyl ethanol was produced via reductions of **1** catalyzed with sodium borohydride. The other components of the culture media and the chemical reagents were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, USA) in the highest purity available.

4.2. Isolation of microorganisms, identification and inoculation

Microorganisms used in the screening were isolated from various soil samples collected from around Erzurum, Turkey. The isolation process was performed by serial dilution of the samples according to standard techniques.[15] Taxonomic identification of filamentous fungi was identified in-house by using mature cultures on a standard potato dextrose agar (PDA) in order to ensure a good development of taxonomically relevant features, and following the identification keys provided by Von Arx[16] and Domsch et al.[17] These cultures were maintained on PDA slants, incubated at 30 °C and stored at 4 °C. The conidia from 7-days-old cultures were used for inoculation. The conidial suspension was prepared in 10 mL of sterilized distilled water by gently scratching conidia with a sterile wire loop and then shaking vigorously to break the clumps of conidia.

4.3. Growth medium and reduction reactions

The fermentation medium contained: glucose (30 g/L), yeast extract (3 g/L), KH_2PO_4 (2 g/L) and peptone (4 g/L). The initial pHs of the culture media were adjusted to the appropriate level with 1 M HCl and 1 M NaOH and the media sterilized at 121 °C for 15 min. The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of culture medium. Then, 1 mL of conidial suspension was added to each flask. The flasks were incubated on a reciprocal shaker at 150 rpm and 30 °C for 48 h. After the growth of the fungus, **1** (0.5 mmol) was directly added to each culture. Then, incubation of the flasks continued on a reciprocal shaker at 150 rpm, 25 °C for 24 h.

4.4. Preparative scale production

Preparative scale studies were conducted in a 2-L fermenter (Biostat-M 880 072/3, Germany) with a working volume of 1 L. Ten milliliters of the spore suspension was inoculated into the fermenter containing 1 L of sterile culture medium. To prevent foam formation, sterilized silicone oil (0.001%, w/v) was added twice, at the beginning and after 24 h fermentation. After a 48 h incubation period, **1** (20 or 17 mmol/L) was directly added to the fermentation culture. Agitation, pH, aeration (0.4 vol vol min) and temperature were automatically controlled during the fermentation. The reaction time (24–72 h) was optimized for the reduction of substrates **1** to product **2** in a submerged system. At regular intervals of 4 h during fermen-

tation, the conversion and the ee were determined and the yields were calculated (Fig. 1 and Scheme 1).

4.5. Product purification and analytical procedures

After reduction, the mycelium was separated by filtration, the filtrate saturated with sodium chloride and then extracted with ethyl acetate. The mycelia were separately extracted with ethyl acetate. The ethyl acetate extracts were combined; dried with $MgSO_4$ and evaporated. For HPLC analysis, a small fraction of the product was separated by preparative silica-gel TLC. The ee of the product was determined by high performance liquid chromatography (HPLC) with an OD-H column (Daicel) using *n*-hexane/*i*-PrOH (98/2) as eluent, a flow rate of 0.5 mL min^{-1} and the detection was performed at 220 nm. The crude product was purified by silica gel column chromatography. ^1H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer in $CDCl_3$ and used as the identification tool for purified **2**. In addition the purity of the (*R*)-isomer of **2** produced via the fermenter was also checked via HPLC analysis. The absolute configuration of the compound was determined by comparing the sign of its specific rotation with that in the literature.[18] The conversion for the flask cultures was determined by ^1H NMR analysis with diphenylmethane as internal standard; error ca. ±5% of the stated values. All experiments were replicated twice and averaged values are presented in this study.

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