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Efficient Synthesis of (*S*)-1-(2-chlorophenyl)ethanol in the Submerged Culture of *Alternaria alternata* Isolate

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Abstract: (*S*)-1-(2-chlorophenyl)ethanol is a key intermediate of L-cloprenaline used for relieving asthma symptoms. The asymmetric reduction of 2-chloroacetophenone **1** to (*S*)-1-(2-chlorophenyl)ethanol **2** in the submerged culture of *Alternaria alternata* isolates was studied. *A. alternata* EBK-8 isolate was the most effective biocatalyst. The bioactivity of the fungus could be significantly improved by the optimization of culture conditions. Parameters such as pH, temperature, agitation, and incubation time considerably influenced the substrate conversion and the optical purity of the product. The reaction was carried out in a culture medium at a substrate concentration of 30 mmol/L and produced the desired product with high conversion (100%) and isolated yield (80%) with an excellent enantiomeric excess (ee) of >99%. Under the optimum conditions, after 54 h reaction time, 24 mmol/L **2** from 30 mmol/L **1** could be produced. As a result, the submerged fermentation of *A. alternata* EBK-8 was found to be suitable for the asymmetric reduction of **1** to **2**.

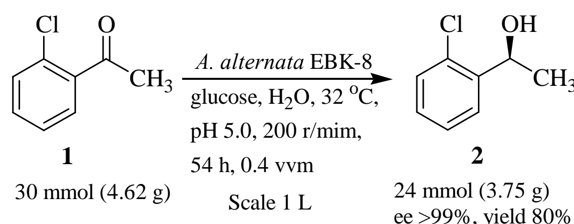
Key words: *Alternaria alternata*; bioconversion; biocatalysis; 2-chloroacetophenone; fermentation; optimization

Chiral alcohols play a pivotal role in the production of modern pharmaceuticals and agricultural chemicals. Chirality is a key factor in the efficiency of many drug products, and the production of single enantiomers of molecule has become increasingly important in the pharmaceutical industry. (*S*)-1-(2-chlorophenyl)ethanol is a phenylethanol derivative alcohol. Optically active phenylethanol and its derivatives are useful building blocks for the synthesis of complex molecules because the alcohol functionality can be easily transformed, without racemization, into other useful functional groups [1–5].

Biocatalytic asymmetric reduction of aromatic ketones to corresponding chiral alcohols has attracted more and more attention due to the high enantioselectivity, 100% theoretical yield, and mild reaction conditions. Transformations using isolated reductase as biocatalysts for these bioreductions can produce the desired yield and high enantiomeric excess values. However, these reactions generally require expensive cofactors such as NAD(P)H. The cofactors can be regenerated with whole cells as catalysts during the reduction processes [6–12].

(*S*)-1-(2-chlorophenyl)ethanol is particularly important in the drug industry. It is a key intermediate of L-cloprenaline for reducing asthma symptoms. Previously, *Candida pseudotropicalis* yeast that can catalyze asymmetric reduction of 2-chloroacetophenone to (*S*)-1-(2-chlorophenyl)ethanol has been reported [13].

Recently, a number of microbial strains performing asymmetric reduction of acetophenone have been reported and exploited for the enantioselective reduction of acetophenone derivatives. It is well known that the screening of a wide variety of microorganisms living in our environment is an efficient method to obtain the desired enantiomeric excess (ee) for a substrate. The preparative scale reductions of acetophenone or its derivatives by microorganisms found with these screening methods have been achieved with good yield and excellent enantioselectivity [14–19]. Herein, we report the asymmetric reduction on a preparative scale of 2-chloroacetophenone (**1**) to (*S*)-1-(2-chlorophenyl)ethanol (**2**) in the submerged culture of *Alternaria alternata* fungus without using any organic solvent or cofactor (Scheme 1). We also determined the reaction conditions and appropriate



Scheme 1. Asymmetric reduction of 2-chloroacetophenone (**1**) to (*S*)-1-(2-chlorophenyl)ethanol (**2**) by *A. alternata* EBK-8.

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microbial strain for high ee and conversion.

1 Experimental

1.1 Materials

In our previous work, *A. alternata* strains isolated from plant samples such as sunflower, grape, and apple collected from around Erzurum, Turkey were used for this study [15]. 2-Chloroacetophenone was obtained from Fluka. The other components of the culture media and the chemical reagents were obtained from Merck and Sigma in the highest purity available.

1.2 Cultivation of fungi cells and inoculation

A. alternata strains were maintained at 4 ^{o}C on potato dextrose agar (PDA) slants. The cultures were transferred to new media at bimonthly intervals. These strains were pre-cultured on PDA medium for 10 days at 28 ^{o}C. The conidia from 10-day-old cultures were used for inoculation. The conidial suspension was prepared in 10 ml of sterilized physiological water by gently scratching conidia with a sterile wire loop, and then it was shaken vigorously for breaking the clumps of conidia. Ten or one milliliters of inocula were used for the next steps.

1.3 Submerged culture and reduction reactions

The per liter fermentation medium contained (g/L): glucose 30, yeast extract 3, and peptone 5. This medium was termed GYP. The initial pHs of the culture media were adjusted to an appropriate level with 1 mol/L HCl and 1 mol/L NaOH and sterilized at 121 ^{o}C for 15 min. The experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml of GYP. One milliliter of conidial suspension was added to each flask. The flasks were incubated on a reciprocal shaker at 150 r/min and 25 ^{o}C for 48 h. After the growth of the fungus, 2-chloroacetophenone **1** (1 mmol) was directly added to each culture. Then, incubation of the flasks was continued on a reciprocal shaker at 150 r/min and 25 ^{o}C for 48 h.

1.4 Preparative scale studies

Preparative scale studies in our lab were conducted in a 2-L fermenter (Biostat-M 880072/3, Germany) with a working volume of 1 L. Ten milliliters of the spore suspension was inoculated into the fermenter containing 1 L of sterile GYP. To prevent foam formation, sterilized silicone oil (0.01 mg/ml) was added twice at the beginning and after 24 h of fermentation. After 48 h incubation, substrate **1** (30 mmol) at log phase of the fungal growth cycle was directly added to the fermentation culture. Agitation, pH, aeration (0.4 vvm), and temperature were automatically controlled during the fermentation. The reaction time (6–72 h) was optimized for the reduction of **1** to **2** in a submerged system. At regular intervals (6 h) during fermentation, the conversion and the ee were determined.

1.5 Product purification and analytical methods

After reduction, the mycelium was separated by filtration, and the filtrate was saturated with sodium chloride and then extracted with ethyl acetate. The mycelia were also extracted with ethyl acetate. The ethyl acetate extracts were combined; the ethyl acetate was dried with $MgSO_4$ and evaporated. For analysis, a small fraction of the product was separated by preparative silica-gel TLC. The ee of the product was determined by HPLC with an OD column using eluent *n*-hexane-*i*-PrOH, (90:10), flow rate of 0.6 ml/min, detection performed at 220 nm. Retention times were 10.5 min for (*R*)-**2** and 10.7 min for (*S*)-**2**. The crude product was purified by silica gel column chromatography. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer in $CDCl_3$. Purified (*S*)-**2** was identified from spectral data (^{1}H and ^{13}C NMR). The purity of (*S*)-**2** produced via fermenter was also checked with HPLC analysis. Optical rotation was measured with a 589.3 nm spectropolarimeter as $[\alpha]_D = -61.3$ (c 0.675, $CHCl_3$). The absolute configuration of the compound was determined by comparing the sign of its specific rotation with that in the literature. The conversion for flask cultures was determined by ^{1}H NMR analysis with diphenylmethane as internal standard with an error ca. ± 5% of the stated values.

2 Results and discussion

2.1 Screening of *A. alternata* isolates

Ten different strains of *A. alternata* for the asymmetric reduction of acetophenone in our previous study were isolated, and *A. alternata* EBK-4 strain produced (*S*)-1-phenylethanol with high conversion (100%), a good yield (86%), and an excellent ee (> 99%) [15]. In this study, *A. alternata* strains were evaluated for the reduction of **1** to **2**. The costs of the fermenter studies are higher than those of flask cultures. Flask cultures are very useful in screening many media or strains for target product formation in relatively short periods. Therefore, first, the reduction of **1** to **2** was carried out in the flask culture in order to find the optimum reaction conditions and the most effective biocatalyst; second, the production on the preparative scale of **2** under optimum conditions was performed in the fermenter. *A.*

alternata isolates were used for the reduction of **1** to **2** in a medium containing peptone, yeast extract, and glucose. As shown in Table 1, among the ten positive isolates obtained during screening, five isolates, EBK-4, -6, -7, -8, and -10 gave excellent ee (> 99%). Among them, *A. alternata* EBK-8 gave the best conversion (90%). Moreover, **1** was reduced with the other isolates in moderate conversion and enantioselectivity. These results are not in agreement with our previous work [15]. In a previous study, the best isolate for the reduction of acetophenone to (*S*)-1-phenylethanol was *A. alternata* EBK-4. Furthermore, it did not reach 99% ee. The results showed that the conversion and optical purity are strongly dependent on the different strains of the same species according to the chemical test. On the basis of these results, we recommend a careful control of strain used as biocatalyst for optical purity in the asymmetric reduction. However, the good conversions and ee allow their use in future scaling-up processes. Because both the high ee and the high conversion were observed using *A. alternata* EBK-8 as the biocatalyst, it was selected for further studies.

Table 1 Screening of *A. alternata* isolates for the asymmetric reduction of **1**

Isolate	Conversion (%)	ee(*S*)/%
EBK-1	38	76
EBK-2	56	82
EBK-3	44	96
EBK-4	69	99
EBK-5	52	87
EBK-6	66	99
EBK-7	30	99
EBK-8	90	99
EBK-9	36	92
EBK-10	48	99

Reaction conditions: substrate 1 mmol, temperature 25 °C, time 48 h, pH 7, agitation 150 r/min.

2.2 Effect of pH

Table 2 shows the effect of pH on the substrate conversion and product ee. Microbial production can be performed on a large scale so that it can be applied in an industrial process using an engineered microorganism and designing the reaction conditions [20–22]. Therefore, we decided to determine the performance of microorganisms under the optimum reaction conditions. The aim of optimizing the reaction conditions was to increase the substrate conversion with maximum ee (> 99%). Thus, we added 3 mmol instead of 1 mmol **1** to the submerged culture of *A. alternata*. The organism adapted to four different pH values (4–7). The highest conversion (48%) with optimum ee was obtained from pH 5.0. This pH indicated higher bioactivity of the *A. alternata* isolate for the reduction of **1**. All further studies were carried out at pH 5.0.

Table 2 Effect of pH on the reduction of **1** by *A. alternata* EBK-8

pH	Conversion (%)	ee(*S*)/%
3	weak growth	—
4	36	> 88
5	48	> 99
6	42	> 99
7	30	> 99
8	weak growth	—

Reaction conditions: substrate 3 mmol, temperature 25 °C, time 48 h, agitation 150 r/min.

2.3 Effect of temperature

The effect of reaction temperature on the conversion and ee of **1** bioreduction is shown in Table 3. The reaction was carried out at different temperatures ranging from 28 to 40 ºC. Conversion declined appreciably at 38 and 40 ºC, while at other temperatures it remained almost the same. Temperature variation considerably influenced the conversion but had no significant effect on the optical purity of the product. The increase (32 °C) in the reaction temperature resulted in an increase in the conversion of **1**. Hence, all the subsequent reduction experiments were carried out at 32 ºC.

Table 3 Effect of temperature on the reduction of **1** by *A. alternata* EBK-8

Temperature (°C)	Conversion (%)	ee(*S*)/%
28	66	> 99
30	73	> 99
32	74	> 99
34	72	> 99
36	68	> 99
38	40	> 99
40	18	> 99

Reaction conditions: substrate 3 mmol, pH 5.0, time 48 h, agitation 150 r/min.

2.4 Effect of agitation

To further optimize the reaction conditions, the reduction experiments were carried out under different agitation conditions. The results are shown in Table 4. As can be seen from Table 4, agitation exerted a strong influence on the conversion and ee. Shaking speed influences the diffusion and partition of the substrate and the product [20]. A significant increase in conversion of **1** was observed at 250 r/min (94%) as compared to 100 and 350 r/min (40% and

10%, respectively). A progressive increase in conversion was observed with the increase in agitation rate up to 250 r/min, and thereafter, a decrease in conversion was recorded. Agitation rate not only affects the conversion, but it also exerts an influence on the enantioselectivity of the product. For example, the optical purity of the product decreased at a shaking speed above 200 r/min. This decreasing ee is due to the effect of shear stress on fungus cells as well as on the enzyme structure. Considering the high product selectivity, the best result was 200 r/min (conversion 86%, ee > 99%).

Table 4 Effect of different agitation on the reduction of **1** by *A. alternata* EBK-8

Agitation (r/min)	Conversion (%)	ee(*S*)/%
100	40	>99
150	73	>99
200	86	>99
250	94	>86
300	60	>53
350	10	>20

Reaction conditions: substrate 3 mmol, pH 5.0, time 48 h, temperature 32 °C.

2.5 Effect of incubation time

In order to scale up the bioprocess, the effect of incubation time on the conversion and enantioselectivity was studied. These results are summarized in Table 5. The optimum incubation time was found to be 72 h after addition of **1**. The ee at all fermentation times was > 99%. With increasing incubation time conversion was increased. This is a satisfactory result. There was no inhibitor effect on the organism activity of the product from substrate up to 30 mmol/L concentration. Based on this reaction concept, we carried out the reaction with 40 mmol/L substrate concentration, but after a reaction time of 96 or 120 h the desired conversion was not achieved. However, the resulting conversion was somewhat high (data not shown). Therefore, the optimum substrate concentration was 30 mmol/L. The best reaction conditions (pH 5.0, agitation 200 r/min, temperature 32 °C, and substrate 30 mmol/L) found so far were used for the production of 2 on a preparative scale using a fermenter. The bioreduction of **1** for the gram scale production of **2** by *A. alternata* EBK-8 under optimum reaction conditions was achieved via a fermenter. These results are shown in Fig. 1.

Table 5 Effect of incubation time on the reduction of **1** by *A. alternata* EBK-8

Time (h)	Conversion (%)	ee(*S*)/%
24	24	>99
48	73	>99
72	100	>99
96	100	>99
120	100	>99
144	100	>99

Reaction conditions: substrate 3 mmol, pH 5.0, agitation 200 r/min, temperature 32 °C.

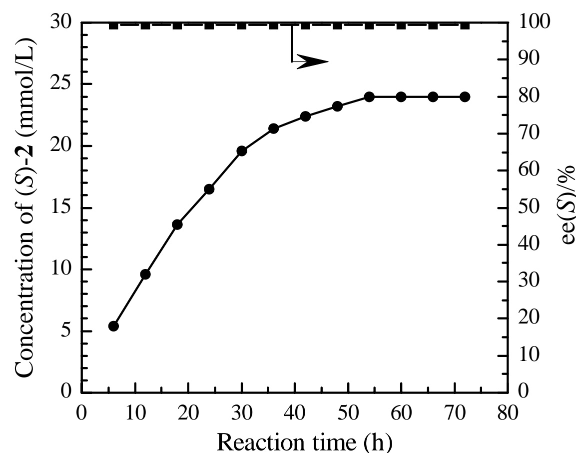

Fig. 1. Production of **2** in a fermenter by *A. alternata* EBK-8 on a preparative scale.

2.6 Preparative scale production of 2

A significant decrease in fermentation time in the reduction of **1** to **2** was observed in the fermenter as compared to the reduction at a shake flask level. The reaction time was reduced from 72 to 54 h. All parameters optimized at the flask level were used in fermenter studies. In addition, air was supplied at 0.4 vvm rate to the fungus culture. This increase could be due to oxygen, uniform distribution of medium components, and automatic pH regulation. As shown in Fig. 1, the cultivation period of *A. alternata* EBK-8 did not affect the product ee. The product ee for all cultivation times was > 99%. After 54 h reaction period, the concentration of **2** obtained from complete conversion of **1** was 24 mmol/L. The yield of **2** was calculated as 80% after 54 h when the initial concentration of **1** was 30 mmol/L. The conversion of **1** increased when the fermentation time increased up to 30 h, remained almost constant between 36 and 48 h, and then was complete (54 h).

A longer fermentation time resulted in a decrease in substrate conversion speed. The decline in conversion speed must be due to the inhibition of the enzyme responsible for the bioreduction by the product concentration formed in fermentation culture. However, in many cases, the asymmetric reduction results obtained from microbial reactions are affected by the product concentration. A change in product concentration results in a change in the apparent activity of each enzyme. On the other hand, although **2** is an industrially valuable compound, there are few microbial

studies on the total synthesis of **2**. For example, recently, the reduction of **1** to **2** with whole cells of *Candida pseudotropicalis* yeast in potassium phosphate buffer was achieved [13]. Our study is different from that work, namely, in our study, the method used for bioreduction was different because the substrate was directly added to the culture medium without using resting cells or buffer. In addition, a fungus was used instead of yeast as the microorganism. There is a need for alternative microorganisms for the production of enantiomerically pure pharmaceuticals because the racemic mixtures made today may not be allowed in the future. Unsurprisingly, given that the human body functions using chiral catalysis, the trend for new chiral pharmaceutical reagents is continuing [23]. Similarly, we successfully produced (*S*)-1-phenylethanol from acetophenone using *A. alternata* EBK-4 isolate [15]. The results of batch fermentation from this study demonstrated that *A. alternata* EBK-8 would be an excellent (*S*)-1-(2-chlorophenyl)ethanol producer.

3 Conclusions

A. alternata EBK-8 has potential for the preparation of (*S*)-1-(2-chlorophenyl)ethanol as a pharmaceutical intermediate. This fungus formed the corresponding **2** with an excellent enantiomeric excess (ee > 99%) from **1**. In our work, thorough screening of ten *A. alternata* isolates for their asymmetric reduction activity revealed a new fungus strain *A. alternata* EBK-8 that can catalyze asymmetric reduction of 2-chloroacetophenone with high conversion and ee in submerged culture. Under optimal conditions such as pH 5.0, 32 °C, and 200 r/min, the strain EBK-8 showed conversion up to 100% with a high enantioselectivity (ee > 99%). As a result, this work showed the production of 24 mmol/L **2** from 30 mmol/L **1** with submerged fungus culture as catalyst on a preparative scale. It is well known that the production of a pharmaceutical intermediate is important in organic synthesis. Therefore, a detailed study on the production of another intermediate has already been carried out in our laboratory.

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References

1 Comasseto J V, Andrade L H, Omori A T, Assis L F, Porto A L M. *J Mol Catal B*, 2004, **29**: 55
2 Griffin D R, Yang F, Carta G, Gainer J L. *Biotechnol Prog*, 1998, **14**: 588
3 Knox T, Clifee K R. *Process Biochem*, 1984, **19**: 188
4 Mandal D, Ahmad A, Khan M I, Kumar R. *J Mol Catal B*, 2004, **27**: 61
5 Gao K L, Song Q X, Wei D Z. *Appl Microbiol Biotechnol*, 2006, **71**: 819
6 Kataoka M, Kita K, Wada M, Yasohara Y, Hasegawa J, Shimizu S. *Appl Microbiol Biotechnol*, 2003, **62**: 437
7 Murzin D Y, Maki-Arvela P, Toukoniitty E, Salmi T. *Catal Rev-Sci Eng*, 2005, **47**: 175
8 Drepper T, Eggert T, Hummel W, Leggewie C, Pohl M, Rosenau F, Jaeger K-E. *Chem Ing Tech*, 2006, **78**: 239
9 Nie Y, Xu Y, Mu X Q. *Org Process Res Dev*, 2004, **8**: 246
10 Patel R N, Goswami A, Chu L, Donovan M J, Nanduri V, Goldberg S, Johnston R, Siva P J, Nielsen B, Fan J, He W, Shi Z, Wang K Y, Eiring R, Cazzulino D, Singh A, Mueller R. *Tetrahedron: Asymmetry*, 2004, **15**: 1247
11 Bradshaw C W, Hummel W, Wong C H. *J Org Chem*, 1992, **57**: 1532
12 Kataoka M, Rohani L P S, Yamamoto K, Wada M, Kawabata H, Kita K, Yarase H, Shimizu S. *Appl Microbiol Biotechnol*, 1997, **48**: 699
13 Xie Q, Wu J, Xu G, Yang L. *Biotechnol Prog*, 2006, **22**: 1301
14 Kurbanoglu E B, Zilbeyaz K, Kurbanoglu N I, Kilic H. *Tetrahedron: Asymmetry*, 2007, **18**: 1159
15 Kurbanoglu E B, Zilbeyaz K, Kurbanoglu N I, Taskin M. *Tetrahedron: Asymmetry*, 2007, **18**: 1529
16 Roy A, Bhattacharyya M S, Kumar L R, Chawla H P S, Banerjee U C. *Enzym Microb Technol*, 2003, **33**: 576
17 Zhao G G, Wang J J, Ma K, Yang L, Wu S, Liu Y, Sun W. *Biotechnol Lett*, 2004, **26**: 1255
18 Kamble A L, Soni P, Banerjee U C. *J Mol Catal B*, 2005, **35**: 1
19 Findrik Z, Vasic-Racki D, Lutz S, Daußmann T, Wandrey C. *Biotechnol Lett*, 2005, **27**: 1087
20 He J Y, Sun Z H, Ruan W Q, Xu Y. *Process Biochem*, 2006, **41**: 244
21 Singh R S, Sooch B S, Puri M. *Bioresour Technol*, 2007, **98**: 2518
22 Houng J Y, Liao J H, Wu J Y, Shen S C, Hsu H F. *Process Biochem*, 2007, **42**: 1
23 Pollard D J, Woodley J M. *Trends Biotechnol*, 2007, **25**: 66