Preparation of Enantiomerically Pure (*S*)-(−)-1-(1′-naphthyl)-ethanol by the Fungus *Alternaria alternata*

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ABSTRACT (*S*)-(−)-1-(1′-napthyl)-ethanol (*S*-NE) is an important intermediate for the preparation of mevinic acid analogs, which is used for the treatment of hyperlipidemia. The objectives of the study were to isolate a microorganism that could effectively reduce 1-acetonaphthone (1-ACN) to *S*-NE, to determine the influence that the physicochemical parameters would have on the reduction by the isolated microorganism, and to attempt large-scale studies with the microorganism. Over the years fungi have been considered a promising biocatalyst and it has been presumed that many fungal species have not been isolated and therefore the current study focused on possible isolation of these microorganisms. A total of 72 fungal isolates were screened for their ability to reduce 1-ACN to its corresponding alcohol. The isolate, EBK-62, identified as *Alternaria alternata,* was found to be the most successful at reducing the ketone to the corresponding alcohol in the submerged culture. The reaction conditions were systematically optimized for the reducing agent *A. alternata* EBK-62, which showed high stereospecificity and good conversion for the reduction. The preparative scale study was carried out in a 2 L bioreactor and a total of 4.9 g of *S*-NE in optically pure form (>99% enantiomeric excess) was produced in 48 h. *Chirality 28:669–673, 2016*.

KEY WORDS: asymmetric reduction; biotransformations; enantioselective; ketone; submerged culture

Chirality has become a grave concern in various industries including aroma/fragrance, agrochemical, fine chemical, and pharmaceutical. The growing interest within the pharmaceutical industry is fueled by regulatory agencies, who are demanding the use of single enantiomers in the production of drug formulations due to the vast differences in many enantiomer pairs, which may have different pharmacological activities and different pharmacokinetic and pharmacodynamic effects.[1,2] Over the past 20 years there has been an accelerated and continuous growth of single enantiomer drug sales. In light of this, it is not surprising that new enantioselective synthetic methodologies have been growing at a rapid rate.[3] One methodology that has been very successful and has become a common one for preparation of chiral compounds is biotransformation. An extensive collection of biocatalysts have been reported to assist biotransformations.[4–6] Biocatalysts have many advantages compared to chemical catalysts. Chemical catalysts produce toxic waste and a large range of by-products, whereas biocatalysts are biodegradable, and provide a clean and environment-friendly way to carry out chemical reactions under mild reaction conditions and great selectivity for the substrate.[7,8] Biocatalysis can be achieved by employing isolated enzymes or whole cells, with both having advantages and disadvantages.[9] Isolated enzymes catalyze specific reactions, eliminating by-product formation or product breakdown, and product purification is often much easier.[9] However, enzymes are very expensive and very unstable.[9] In addition, enzymes catalyze a single reaction, whereas a plethora of industrial products are afforded after a series of biochemical reactions using whole cells. Whole cells are sustainable, are much more stable and efficient, and more readily and cost-effectively prepared.[10] Hence, whole cells are preferred as biocatalysts in biotransformation processes. Fungi have been one of the most studied whole-cell systems for biotransformations.[11] However, it is believed that very few fungal species are known and have been isolated from the ecosystem and therefore can be considered as a promising source of new biocatalysts.[11]

Optically active alcohols are among the vast amount of chiral compounds that have become important building blocks in the pharmaceutical industry. A large number of optically active alcohols have been produced by means of biocatalytic processes.[12–16] Optically active phenylethanol and its derivatives have become key building blocks for the preparation of large complex molecules. This is attributable to the alcohol functional group, which can be easily transformed to other desired functional groups. (*S*)-(−)-1-(1′-napthyl) ethanol (*S*-NE) is an intermediate for the synthesis of mevinic acid analogs used for the treatment of hyperlipidemia, which acts as potent inhibitors of 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMGR).[17] Several methods for the preparation of optically active *S*-NE have been reported in the literature, including the asymmetric reduction of 1-acetonaphthone (1-ACN) catalyzed by *Rhodotorula glutinis*, Baker's yeast, *Geotrichum candidum*, *Candida viswanathii*, *Rhizopus arrhizus*, *Merulius tremellosus*

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ono991, and *Daucus carota*.[18–24] However, in light of the belief that not many fungal species are known, we set off on a search to find new fungal species for the biotransformation of 1-ACN. There are several advantages of mold utilization in a bioprocess. The principal advantage is the easy separation of the mold from the reaction mixture.

In the current communication we report the asymmetric reduction of 1-ACN to the corresponding chiral alcohol *S*-NE in the submerged culture system by the fungus isolate *Alternaria alternata*. The effects of the reaction conditions such as pH, temperature, and agitation speed on the yield, conversion, and enantiomeric excess (ee) were also investigated in detail and reported.

MATERIALS AND METHODS

Chemicals

The components of the culture media and the chemical reagents were bought from Sigma-Aldrich (St. Louis, MO) in the highest purity available. 1-ACN and *S*-NE was bought from Fluka (Steinheim, Germany). Racemic 1-(1′-naphthyl) ethanol was prepared by $NaBH_4$ reduction of 1-ACN and its ^1H and ^{13}C NMR spectra were in agreement with those reported in the literature.[21,25]

Analysis

All analysis was performed on a Thermo Spectra Analysis HPLC System equipped with a UV detector using a chiral OD-H column (4.6 mm, 250 mm, 5 μm, Daicel, France). 1-ACN, *S*-NE, and racemic 1-(1′-naphthyl) ethanol were analyzed using *n*-hexane-i-PrOH (90:10) as the eluent, a flow rate of 0.6 ml/min, and the detection performed at a wavelength of 220 nm. The retention times of *S*-(−)- and *R*-(+)-1-(1′-naphthyl) ethanol were determined as 15.7 min and 23.3 min, respectively. Their ee was determined directly from their respective areas under the curve. ^1H and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA) 400 MHz spectrometer in $CDCl_3$. A polarimetric Chiralyser detector was used to assess the sign of configuration of the enantiomer formed.

Isolation, Identification, and Inoculation of Microorganism

The microorganisms used in the screening process were isolated from various soil, plant, and fruit samples that were collected from Erzurum, Turkey. Standard techniques were used for the isolation process, which involved serial dilution of the samples.[26] Cultures were prepared and identified as previously reported.[27–29]

Culture Media and Conditions

The culture medium contained (g/L): glucose 20, yeast extract 3, and peptone 4. The initial pH of the culture medium was adjusted to 6.0 with 1 N HCl and 1 N NaOH and autoclaved at 121 °C for 15 min.

Microbial Reduction of 1-Acetonaphthone

Screening of the microorganisms and optimization of the reaction parameters were done on an analytical scale, which was followed by the investigation of the reduction on a preparative scale. All experiments were done in duplicate and averaged values are presented in this study.

All isolated microorganisms were screened for their asymmetric reduction capability of 1-ACN to *S*-NE. The reactions for the screening process and the optimization of the reaction parameters were carried out in 250 ml Erlenmeyer flasks containing 100 mL of culture medium. One mL of spore suspension was added to each flask. For the screening process the flasks were incubated on a reciprocal shaker at standard conditions of 100 rpm and 30 °C for 48 h. After sufficient growth of the fungi in the flasks, ketone 1-ACN was directly added to each culture. The flasks were then incubated for 24 h on a reciprocal shaker at 100 rpm and 25 °C. The cells were then separated by filtration, the supernatant saturated with sodium chloride, and then extracted with ethyl acetate. The organic phase was dried over Na_2SO_4 and the solvent removed under reduced pressure. A small amount of sample was purified by preparative silica-gel thin-layer chromatography (TLC) for analysis by high-performance liquid chromatography (HPLC).

Optimization of the various parameters involved systematically changing the conditions for the reaction as outlined in the Results and Discussion section.

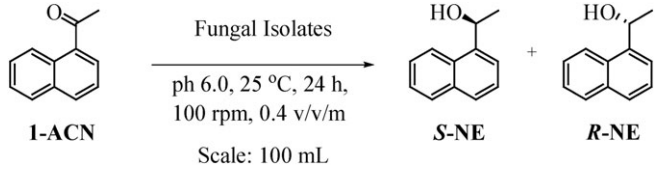
The scale-up for the production of *S*-NE was carried out in a 2 L bioreactor (Biostat-M 880072/3, Germany) with a working volume of 1 L under the optimized conditions obtained through process optimization. The medium as described before was sterilized at 121 °C for 15 min and inoculated with 10 mL of the spore suspension selected in the screening process. In order to prevent foam formation, sterilized silicone oil (0.001%, *w/v*) was added to the reaction at two different times, once at onset and once after 24 h of fermentation. After a 48-h incubation period, 1-ACN (40 mmol/L for the initial reaction and 35 mmol/L for subsequent reactions) was added directly to the fermentation culture. Agitation, pH, and temperature were set to the optimum values and were automatically controlled during the fermentation. The aeration was set to 0.4 vol/vol/min. The reaction was carried out for 60 h to determine the optimum time for the reduction of substrate 1-ACN to product *S*-NE in a submerged system. Samples were withdrawn periodically at 4-h intervals, purified, and analyzed by HPLC. The conversion of the substrate and the ee of the product were determined and the yields calculated. After 60 h the product was extracted as described before and purified by silica gel column chromatography. *S*-NE was identified by its ^1H and ^{13}C NMR spectra. In addition, the purity of *S*-NE produced via the fermenter was checked by HPLC. The absolute configuration and specific rotation of the product was determined and compared with that of an authentic sample as well as the literature value, which were all in agreement.

RESULTS AND DISCUSSION

Analytical Scale Bioreduction and Screening of Microorganism

The screening of the fungal microorganisms for the production of *S*-NE from 1-ACN found that isolate EBK-62, isolated from soil, was the most productive isolate, with considerably high activity and enantioselectivity. The isolate was identified as *Alternaria alternata*. Scheme 1 outlines the reaction conditions for selection of the best active biocatalyst.

A good biocatalyst should be highly active and stable for continuous use. In our previous study, we investigated the biocatalytic activity and stability of *A. alternata* EBK-4 and we showed that *A. alternata* possessed these required qualities. (*S*)-1-phenylethanol was obtained up to gram scale with this microorganism from acetophenone using a bioreactor.[29] In light of this, it was not surprising that of the large number of isolates screened *A. alternata* EBK-62 gave the best ee and conversion (>99 ee and 50% conversion; data for the screening experiments are not shown in this article). All the other isolates screened were able to reduce the substrate but gave low ee's (8–86%). Screening a diverse collection of microorganisms is a great way to obtain the desired ee and conversion for a substrate.[11,30,31] However, the literature has described the qualities of a good biocatalyst and this could aid researchers in the production of superior intermediates and products.[32] Cheetham reported that a good active biocatalyst can be developed from an isolated



Scheme 1. Screening of fungal isolates for the asymmetric reduction of 1-ACN to *S*- or *R*-NE in flask cultures containing 100 ml medium.

microorganism with the needed enzyme activities.[32] This may be achieved by using methods such as fermentation optimization, gene cloning, or mutagenesis.[32] The current biocatalysts known are still limited and therefore it is necessary and of vital importance to discover new biocatalysts with novel and improved activities. Not many publications on the biocatalytic activity of *A. alternata* have been reported and we believe that it is a good biocatalyst, since it has good reaction activity and high selectivity.

Optimization of Physicochemical Parameters

To address the second objective of the study the reduction of 1-ACN was carried out with *A. alternata* EBK-62 at various pH values, temperatures, and agitation speeds to determine the optimum reaction conditions for the reduction. The first parameter investigated was the pH. The reactions were carried out for 24 h using 2 mmol/100 ml 1-ACN in shake flasks. pH values ranging from 4.5 to 7.5 were chosen. pH .0, 6.5, and 7.0 were found to be optimum for enantioselectivity (ee >99%) and pH .5 was optimum for conversion (38%). The results are shown in Table 1. pH .5 was thus used for all further optimization studies.

The second parameter studied was the reaction temperature. Various temperatures ranging from 24–36 °C were investigated. The best results for cellular growth, conversion, and enantioselectivity were observed at 30 °C. From Table 1 it can be seen that the conversion of the substrate increased steadily from 24–30 °C, with a maximum conversion of 48% at 30 °C and then decreased once again from 32–36 °C. Lower and higher temperatures showed a drastic reduction in conversion of the substrate. The ee of the product formed with cells grown at higher temperatures of 32, 34, and 36 °C was 96, 72, and 40%, respectively, while at lower temperatures 24, 26, 28, and 30 °C the ee of the product was >99%. Taking into account the temperature that gave the best conversion and best ee, subsequent reactions were carried out at 30 °C.

The effect of agitation speed on the reduction was the last parameter investigated. It was observed that agitation exerted a strong influence on both the ee and conversion. A progressive increase in conversion was observed with an increase of the agitation rate (from 100–250 rpm) with a maximum conversion of 86% obtained at a speed of 250 rpm. However, at this speed a low ee of 60% was obtained. This decrease can be attributed to the effect of shear stress on the *A. alternata* cells at high agitation speeds, which alters the cell internal structure and in turn lowers their activity. Good ee's were obtained at lower speeds and we thus compromised conversion for maximum ee. Optimum agitation speed for the reduction was set at 150 rpm.

The optimum conditions for the asymmetric bioreduction of 1-ACN to *S*-NE were pH .5, temperature 30 °C, and agitation speed 150 rpm.

Preparative Bioreduction of 1-ACN TO S-NE

The final objective of the study was to attempt large-scale production of *S*-NE using the determined optimum reaction conditions. The bioreduction of 1-ACN for the production of *S*-NE by *A. alternata* EBK-62 in the bioreactor is outlined in Figure 1. The conversion of the substrate to product increased steadily with time. After 60 h a 100% conversion was observed and gave an 85% product yield. The ee remained consistent (ee >99%) up to 48 h; however, thereafter it decreased drastically. By the end of the 60 h reaction time an ee of 58% was observed. Enantiomeric excess cannot be compromised for conversion, and thus 48 h was selected as the optimal reaction time for the bioreduction. The concentration of *S*-NE after 48 h was 27.3 mmol/L, which corresponds to a 68% product yield. It has been shown that a change in substrate concentration has an effect on the conversion and outcome of the product yield, higher concentrations decrease the conversion, which has been

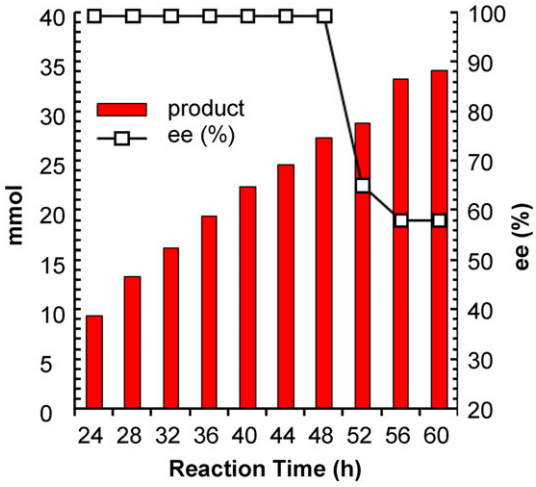


Fig. 1. Time course of the production in gram scale of (*S*)-(−)-1-(1′-napthyl)-ethanol by *Alternaria alternata* EBK-62 (ee (%) and mmol) by means of a bioreactor over a period of 60 h (substrate concentration: 40 mmol/L).

TABLE 1. Optimization of reaction conditions for the asymmetric reduction of 1-ACN by *Alternaria alternata*[a]

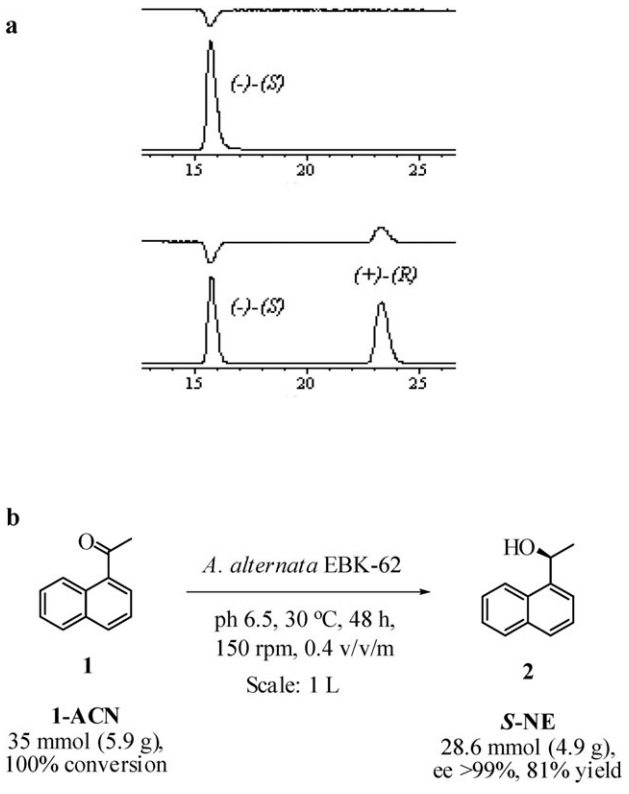
pH			Temperature			Agitation speed		
pH	Conv. (%)[b]	ee (%)[c,d]	°C	Conv. (%)[b]	ee (%)[c,d]	rpm	Conv. (%)[b]	ee (%)[c,d]
4.5	12	55	24	20	>99	100	48	>99
5.0	18	70	26	32	>99	150	68	>99
5.5	22	86	28	36	>99	200	77	92
6.0	25	>99	30	48	>99	250	86	60
6.5	38	>99	32	35	96			
7.0	36	>99	34	24	72			
7.5	25	96	36	18	40			

[a]Substrate: 2 mmol/100 mL, time: 24 h.
[b]Conversion was determined by ^{1}H NMR analysis with diphenylmethane as an internal standard; error ~ 5% of the stated values.
[c]Determined by HPLC using a Chiralcel OD-H column.
[d]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values of the authentic samples and those in the literature.

ascribed to cell toxicity and substrate/product inhibition.[19] In light of this, the substrate concentration was slightly varied and investigated. Higher concentrations (45 and 50 mmol/L) decreased the product concentration. The best results were obtained with a substrate concentration of 35 mmol/L (Scheme 2). Complete conversion and an ee of >99% was observed. The product concentration after the 48 h reduction was 28.6 mmol/L, indicating a product yield increase from 68% to 82%. These results show that *A. alternate* can be used on a large scale in the synthesis of important building blocks.

Biocatalytic multigram studies on the total production of *S*-NE have been explored by several groups and reported in many articles. Roy et al. reported the multigram synthesis of *S*-NE (0.93 g, ee >99%, 84% conversion) from 2 g 1-ACN with resting cells of *Geotrichum candidum* in phosphate buffer.[19] Similarly, Kamble et al. accomplished the synthesis of *S*-NE from 2 g 1-ACN with 97% conversion and >99% ee with resting cells of *Candida viswanathii*.[20] We also previously reported the reduction of 3.4 g 1-ACN to *S*-NE (2.7 g, 78% product yield, ee >99%) by *Rhodotorula glutinis*. Many more have been reported but most of these processes either gave relatively lower conversion, product yield, or low substrate tolerance.

The present study offers advantages of higher substrate tolerance (5.9 g/L), use of the fungus isolate without using an expensive cofactor such as NADPH, complete conversion, and >99% ee. The various reaction parameters and conditions for the bioreduction were optimized and the reaction was successfully carried out on a preparative scale. As illustrated in Scheme 2, the fermentative studies allowed the production of 28.6 mmol (4.9 g) *S*-NE from 35 mmol (5.9 g) 1-ACN with an 81% product yield. This is the first report on the asymmetric reduction of ketone 1-ACN with *A. alternata* as a biocatalyst using a submerged culture system. In addition, optically pure *S*-NE was obtained with excellent selectivity.



Scheme 2. (**a**) Chiral analysis of (*S*)-(−)-1-(1′-napthyl)-ethanol by HPLC. Retention time (min): (−)-(*S*), 15.7; (+)-(*R*), 23.3. (−)-(*S*), $[\alpha]_D^{20} = -76$ (*c* 0.530, CH_3OH). (**b**) The asymmetric bioreduction of **1** to **2** with a laboratory-scale bioreactor.

CONCLUSION

In summary, this study presented the fungus *A. alternata* as a biocatalyst for the production of optically active (*S*)-(−)-1-(1′-napthyl)-ethanol. The fungus shows promise as a highly attractive candidate for the biocatalytic preparation of other chiral aryl alcohols. It was demonstrated that the asymmetric reduction of 1-ACN can be performed at high substrate concentration by submerged culture of *A. alternata* EBK-62 fungus. Vital parameters such as pH, temperature, incubation time, and agitation speed were optimized for maximum product yield with excellent enantioselectivity. Enantiomerically pure (*S*)-(−)-1-(1′-napthyl)-ethanol was produced in gram scale with excellent ee (up to >99%) and good yield (up to 82%). A biocatalyst with high selectivity and stereospecificity is vital and indispensable for the development of a biocatalytic process, a green process that offers higher yields and purer products.

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