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Production of (*S*)-(-)-1-(1'-Napthyl) Ethanol by *Rhodotorula glutinis* Isolate Using Ram Horn Peptone

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The bioreduction of 1-acetonaphthone **1** by locally isolated *Rhodotorula glutinis* strains using ram horn peptone (RHP) gave (*S*)-(-)-1-(1'-naphthyl) ethanol (**2**), an important pharmaceutical intermediate. *R. glutinis* strains were isolated from the water of fermented *Salix* leaves. Optimum fermentation conditions for the production of **2** were 200 rpm, 32 °C, and pH 6.5. The production of **2** with excellent enantiomeric excess (>99%), and good conversion (100%) and yield (78%) under optimum conditions was achieved up to preparative scale via a fermenter. Wide temperature, pH, time, and agitation ranges did not affect the enantiomeric excess (ee) of the bioreduction product. A simple process for the isolation of *R. glutinis* was also developed.

Key Words: Acetonapthone; bioreduction; microbial reduction; asymmetric reduction.

Introduction

Synthesis of chiral drugs within the pharmaceutical industry has become very important. Chirality is a key factor in the efficiency of many drug products, and the production of single enantiomers of molecules has become vital. There are different methods for preparing optically active compounds. These methods include resolution, starting from racemic mixtures, compounds originating from natural products, enzymatic and microbial transformations, asymmetric synthesis, asymmetric catalysis, and amplification of chirality.[1−3] It is well known that microorganisms are useful biocatalysts for asymmetric reduction of ketones. These biocatalysts catalyze the reactions under mild and economically viable conditions in an eco-friendly environment when compared to chemical reactions. Biocatalysis may be performed using whole cells or isolated

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enzymes. The use of isolated and purified enzymes is advantageous because undesirable by-product formation mediated by contaminating enzymes is minimized. However, isolation and purification of enzymes are expensive, and enzymes are frequently less stable under biocatalytic conditions. Thus, in many lab-scale and industrial biotransformation processes, biocatalysts are used in the form of whole cells, exhibiting the desired activity.[4−6] On the other hand, biotechnology opens future prospects in the chemical field for the synthesis of complex compounds and combines cheap raw materials with environmentally friendly processes.[7]

(S)-(-)-1-(1'-napthyl) ethanol (**2**), an intermediate for the synthesis of mevinic acid analogues, acts as a potential inhibitor of 3-hydroxy-3-methyl glutaryl co-enzyme A reductase (HMGR). Some previous papers report the microbial reduction of 1-acetonaphthone with different microorganisms such as *R. glutinis*, baker's yeast, *Geotrichum candidum*, and *Candida viswanathii*.[8−11]

We have recently shown that it is possible to use ram horn peptone (RHP) as a microbial growth medium. RHP provides a rich source of nutrients for microbial growth, making it an excellent medium for microbial growth.[12−18] This is the first report on the production of enantiomerically pure **2** by locally isolated *Rhodotorula glutinis* using RHP in the batch culture. The objectives of this study were to use RHP from abundant animal waste in fermentation medium for the production of **2**, isolate the *R. glutinis* strains with a simple process, and optimize the fermentation parameters for high enantiomeric excess (ee) and conversion (Figure 1).

Materials and Methods

Materials

Ram horns were obtained from a slaughterhouse in Erzurum, Turkey. The other components of the culture media and the chemical reagents were obtained from Merck and Sigma in the highest purity available. Production of RHP was carried out as described by Kurbanoglu and Kurbanoglu.[16] Racemic 1-(1'-naphthyl) ethanol was prepared by $NaBH_4$ reduction of **1**.

Isolation of microorganism and identification

Salix leaves were collected from the grounds of Atatürk University. They (50 g) were fermented for 20 days in a 1000-mL Erlenmeyer flask containing 300 mL of natural water. The isolation process was performed by serial dilution of the samples according to standard techniques.[19] The taxonomical identification of this strain was done by using the VITEK 2 compact device (BioMerieux Company, Marcy, France). The EBK-4 isolate was identified as *Rhodotorula glutinis* by VITEK 2 compact. This culture was maintained on PDA slants at 4 °C and recultured bimonthly.

Medium and inoculum

The per liter fermentation medium contained (g/L): glucose 20, yeast extract 3, and RHP 4. The pH values of the culture media were adjusted with 1 N HCl and 1 N NaOH and they were sterilized at 121 °C for 15 min. The first experiments were performed in 250-mL flasks containing 100 mL of the medium. Freshly grown *R. glutinis* on the plates for 48 h was used to inoculate the seed culture. The yeast strain grown in the 100 mL of broth medium on a reciprocal shaker at 200 rpm, 30 °C, and pH 6.5 for 48 h was used as the inoculum (6.8×10^8 cells/mL). The second experiments were on the production of **2** under optimum

conditions determined in the first experiments in a 2-L fermenter (Biostat-M 880072/3, Germany) with a working volume of 1 L.

Reduction reactions

To each flask was added 1 mL of inoculum, followed by incubation on a reciprocal shaker. After 48 h of fermentation, 1 mmol substrate was directly added to each flask and incubation was performed again. On the other hand, 10 mL of the inoculum suspension for the fermenter studies was inoculated into a fermenter containing 1 L of sterile medium. After 48 h of fermentation, 20 mmol 1-acetonaphthone (**1**) was added to the fermentation medium. Agitation, pH, temperature, and aeration were automatically controlled during the fermentation. After addition of **1**, at regular intervals (6 h) of fermentation, the conversion and the ee were determined.

Purification of product and analytical processes

After the specified time, the cells were separated by centrifugation at 5000 rpm, and the supernatant saturated with sodium chloride and then extracted with ethyl acetate. The organic fraction was dried over Na_2SO_4 and the solvent removed in vacuum. Preparative silica-gel TLC was used to purify the product for analysis. The ee of the product was determined by HPLC on a chiral OD-H column using n-hexane–PrOH (90:10) as the eluent, at a flow rate of 0.6 mL/min, and the detection was performed at 220 nm. The crude product was purified by silica gel column chromatography and **2** was identified by ^{1}H- and ^{13}C-NMR spectra, which were recorded on a Varian 400 spectrometer in $CDCl_3$. The purity of **2** produced via fermenter was also checked via HPLC. The absolute configuration of the compound was determined and its specific rotation was compared with the literature value. All experiments were done in duplicate and averaged values are presented in this study.

Results and Discussion

Isolation and identification of microorganism

The strain used was isolated from the water of fermented *Salix* leaves. Liquid samples (1 mL) were diluted in sterile peptone water. Then 0.1 mL aliquots of the dilutions were inoculated over the surface of PDA plates, and all yeast colonies were isolated. This isolation process was repeated with fermentation of the *Salix* leaves at different times. The dominant species for all experiments was identified as *R. glutinis* by VITEK 2 according to its biochemical and morphological details. *R. glutinis* strains could easily be isolated on initial and subsequent plates due to extensive yeast growth. Thus, this study confirmed that the water of the fermented *Salix* leaves can be used for the isolation of *R. glutinis* and its strains. *R. glutinis* EBK-4 from 14 isolates obtained during screening gave the best ee and conversion (data not shown). Therefore, it was selected for further studies. *R. glutinis* EBK-4 isolate was then evaluated for reduction of **1** to **2** using glucose, yeast extract, and RHP nutrients as substrate in a shake flask and fermenter. The reduction of **1** by *R. glutinis* EBK-4 was carried out in 2 stages. Firstly, reduction was studied in the flask culture in order to optimize reaction conditions and later the production of **2** in the preparative scale under optimum conditions was performed in the fermenter. There are only a few studies on the synthesis of **2,** which is an industrially valuable compound. Asymmetric reduction of **1** to **2** was achieved by Bucciarelli et al.[8] using

baker's yeast and *R. glutinis*. Roy et al.[9] reported the multigram synthesis of **2** from 2 g of **1** with resting cells of *Geotrichum candidum* in phosphate buffer. Similarly, Kamble et al.[10] accomplished the synthesis of **2** from 2 g of **1** with 97% conversion and >99% ee by using the resting cells of a novel isolate of *Candida viswanathii*. This study is different from previous works. The method used for bioreduction is simpler than the previous ones. Namely, in this study, the substrate (**1**) was directly added to culture medium without using resting cells and other components (NADPH, buffer etc.). In addition, RHP from slaughterhouse waste was used in the culture medium for microbial growth. RHP is an inexpensive substrate for growth of microorganisms.[12−16] A biocatalyst capable of economically producing optically active alcohols under mild conditions is of great interest. The screening of the microbial species or strains is one of the most powerful tools for finding a biocatalytic reduction system that displays high activity and desired stereoselectivity.[4,7,20] Dahl and Madsen[21] indicated that the fermentation conditions of the yeast importantly influenced the ee in the reduction of 3-oxo esters by *Saccharomyces cerevisiae*. In order to increase the biocatalytic activity, further investigations will be on the effects of the fermentation parameters on the product ee and ketone conversion.

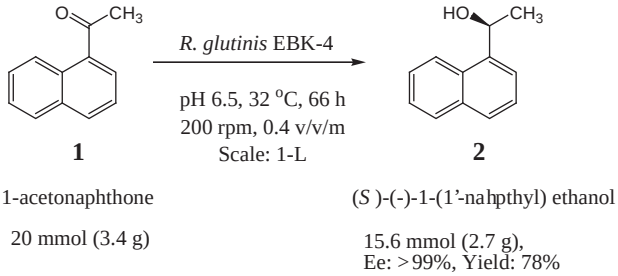

Figure 1. Scheme of the asymmetric reduction of **1** to **2** by *R. glutinis* EBK-4 using RHP.

Effect of initial pH

As shown in Table 1, the optimum pH 6.5 gave 58% conversion of **1**. At lower and higher pH ranges, the conversion drastically decreased while the ee's of the product formed remained constant. Similar results were found by Bhattacharyya and Banerjee[11] for reduction of **1** using carbonyl reductase enzyme from *Geotrichium candidum*.

Table 1. Effects of pH on the reduction of **1** by *R. glutinis* EBK-4.

pH	Conversion (%)	ee (%)-Config.
4.5	20	$> 99 - S$
5.5	50	$> 99 - S$
6.5	58	$> 99 - S$
7.5	44	$> 99 - S$
8.5	10	$> 99 - S$

Reaction conditions: substrate 1 mmol, temperature 25 °C; reaction time 24 h, agitation 150 rpm

Effect of temperature

Table 2 shows the temperature effects on the bioreduction. The greatest conversion was obtained at 32 °C with no change in the ee values. The aim of changing the fermentation parameters was to determine the

best conditions for the best conversion of **1** to **2** and the highest stereoselectivity of the product.[21]

Table 2. Effects of temperature on the reduction of **1** by *R. glutinis* EBK-4.

Temperature (°C)	Conversion (%)	ee (%)-Config.
24	54	$> 99 - S$
26	60	$> 99 - S$
28	66	$> 99 - S$
30	74	$> 99 - S$
32	78	$> 99 - S$
34	70	$> 99 - S$
36	60	$> 99 - S$
38	40	$> 99 - S$

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

Effect of fermentation agitation

The optimum agitation parameter, which was 200 rmp, gave 100% conversion for the reduction from **1** to **2** (Table 3). This indicates that agitation has a significant effect on the conversion. Agitation higher than 200 rpm had a negative effect on the conversion. The ee results were constant as before, proving that a change in the different parameter conditions has no effect on the stereoselectivity of the reduction. Fermentation conditions are essential in successful production of a chiral alcohol, and optimization of parameters such as pH, temperature, and agitation are important in developing the production process. The good conversion and ee allow their use in future scaling up processes; however, to avoid the lack of efficiency, we recommend a careful control of fermentation parameters, since the bioreductions are strongly dependent on the reaction conditions. From the results obtained the optimum conditions for high conversion of **1** to **2** are pH 6.5, 32 °C, and an agitation value of 200 rpm. These results were used in further studies.

Table 3. Effects of agitation on the reduction of **2** *R. glutinis* EBK-4.

Agitation (rpm)	Conversion (%)	ee (%)-Config.
100	46	$> 99 - S$
150	78	$> 99 - S$
200	100	$> 99 - S$
250	80	$> 99 - S$
300	60	$> 99 - S$

Reaction conditions: substrate 1 mmol, pH 6.5; reaction time 24 h, temperature 32 °C

Production in the preparative scale of 2

Under optimized conditions, the bioreduction of **1** for the production of **2** in preparative scale was performed in the fermenter. These results are summarized in Figures 1 and 2. Interestingly the ee was not sensitive to the fermentation parameters. Although there was a regular decrease in conversion rate with the increase in incubation time, the enantioselectivity of the product remained the same throughout the reaction. The

decrease in conversion with increased incubation time may be due to cell toxicity or product inhibition. More than 79% of the product (12.4 mmol/L) was obtained after a 36-h incubation period. After incubation of 66 h, the conversion of substrate was 100%. The amount of **2** produced with maximum conversion within 66 h was 15.6 mmol/L. As a result, 15.6 mmol (2.7 g) **2** from 20 mmol (3.4 g) **1** could be produced. According to this result, the yield was calculated as 78%. In previous studies, we reported the production and synthesis of some chiral alcohols with the biotransformation of the acetophenone and its analogues microbially using RHP from ram horn waste.[17,18] In this way, the RHP could be successfully utilized for production of **2** in this work as well. The *R. glutinis* isolate catalyzed the reactions in an eco-friendly environment when compared to chemical reactions. Moreover, the RHP from waste material as a cheap substrate for microbial growth was used. Meat industry wastes are an important environmental contamination source. This research is of great importance because of the formation of little waste, use of acceptable solvents, transformation of waste materials into valuable products, and highly asymmetric synthesis of the desired products. According to Tao et al.,[4] the successful implementation of biotransformation on an industrial scale requires the strategic use of medium screening, substrate modulation, reaction engineering, enzymology, biocatalyst discovery, and evolution. Biotransformation is uniquely suited for the development of cost-effective and environmentally friendly solutions for drug manufacture. Earlier, production of *S*(−)-1-(1'-naphthyl) ethanol was reported through a similar microbial reduction with *R. glutinis* and baker's yeast.[8,22] The selectivity is reported to be excellent with *R. glutinis*. Baker's yeast led to poor yield. In this study, we report a practical method for producing *S*(−)-1-(1'-naphthyl) ethanol with high yields and excellent selectivity (>99%). An industrial scale for the synthesis of *S*-(-)-1-(1'-naphthyl) ethanol at 2.7 g is also described.

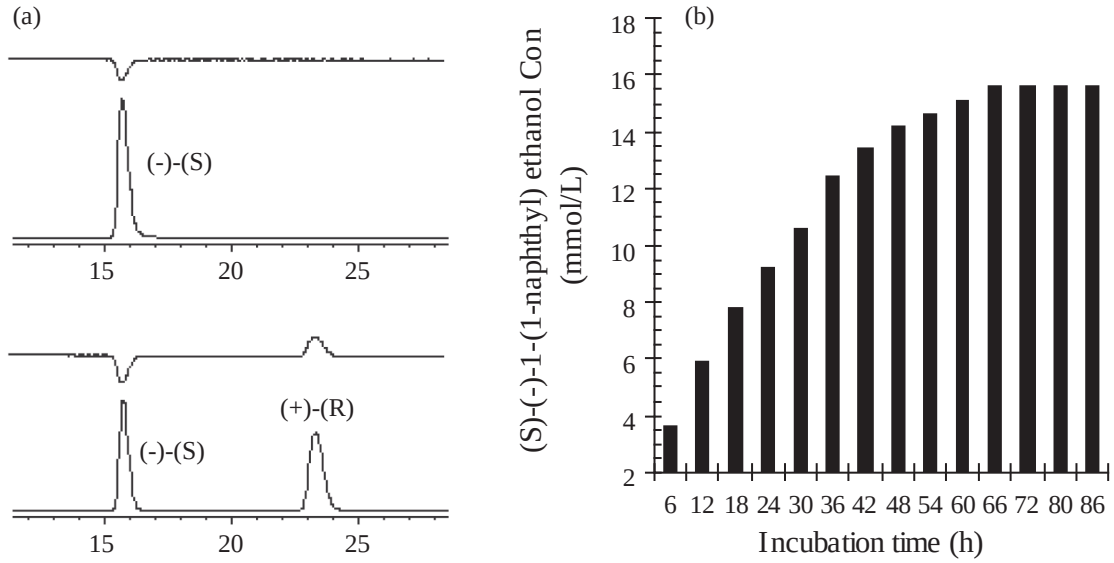

Figure 2. Chiral analysis of product by HPLC (a): Retention time (min): (-)-(S), 15.7; (+)-(R), 23.3; Production in preparative scale of (S)- (-)-1-(1'-naphthyl) ethanol via fermenter by *R. glutinis* EBK-4 (b): Conversion, yield, and polarimetric value after incubation of 66 h. The yield and conversion were calculated using the following equations: Yield (%) = 100 × **2**/**1**, Conversion (%) = **2**/**2** + **1** × 100, **1** and **2** are the concentrations of initial substrate and product, respectively. Ee = >99%, Conversion = 100%, Yield = 78% and $[\alpha]_D$ = -76 (*c* 0.615, CH_3OH).

Conclusions

This study investigated the reduction of **1** to **2** by *R. glutinis* EBK-4 isolate to yield good conversion with high ee. The microorganism was initially isolated, and the bioreduction was then optimized at analytical scale. The RHP was utilized as microbial substrate for the production of **2**. We have developed an efficient biocatalytic process for the preparation of **2,** because this valuable product was successfully produced in preparative scale. It was found that *R. glutinis* EBK-4 could produce **2** with good conversion (100%) and excellent ee (>99%) in medium containing RHP. The ee of product was not affected by parameters such as pH, temperature, or agitation, but the conversion was improved with parameters. Due to the importance of chirality in the drug industry further studies are being carried out at Atatürk University, which will be published at a later stage.

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