Highly Enantiomeric Reduction of Acetophenone and its Derivatives by Locally Isolated *Rhodotorula glutinis*

KANI ZILBEYAZ[1]* AND ESABI B. KURBANOGLU[2]

[1]*Science Faculty, Department of Chemistry, Ataturk University, Erzurum 25240, Turkey*
[2]*Science Faculty, Department of Biology, Ataturk University, Erzurum 25240, Turkey*

ABSTRACT Ninety isolates of microorganisms belonging to different taxonomical groups (30 bacteria, 20 yeast, and 40 fungi) were previously isolated from various samples. These isolates were screened as reducing agents for acetophenone **1a** to phenylethanol **2a**. It was found that the isolate EBK-10 was the most effective biocatalyst for the enantioselective bioreduction of acetophenone. This isolate was identified as *Rhodotorula glutinis* by the VITEK 2 Compact system. The various parameters (pH 6.5, temperature 32°C, and agitation 200 rpm) of the bioreduction reaction was optimized, which resulted in conversions up to 100% with >99% enantiomeric excesses (ee) of the *S*-configuration. The preparative scale bioreduction of acetophenone **1a** by *R. glutinis* EBK-10 gave (*S*)-1-phenylethanol **2a** in 79% yield, complete conversion, and >99% ee. In addition, *R. glutinis* EBK-10 successfully reduced various substituted acetophenones. *Chirality 22:849–854, 2010.*

KEY WORDS: acetophenone; asymmetric reduction; biotransformations; bioreduction; *Rhodotorula glutinis*

INTRODUCTION

Biocatalytic enantioselective reduction is an important research field in organic chemistry, and biocatalytic technology has become a vital tool for chemical synthesis. The past decade has seen the number of publications on biocatalytic synthesis of chiral compounds increases at a rapid rate. This is mainly attributed to the demand for new compounds and precursors and the decrease in productions costs in industry. One of the major advantages of biocatalysts is that they act under mild conditions of pH and temperature and they tend to be extremely selective, which is often difficult to achieve by the conventional chemical routes. It is well known that chiral alcohols are useful starting materials applicable to the synthesis of various compounds including pharmaceuticals. Global sales of single enantiomeric pharmaceutical products are growing at an alarming high rate every year. In light of this, tremendous efforts have been made in recent years to establish enantioselective routes to enantiomerically pure compounds. This is not only due to their importance in the pharmaceutical industry but also in the agricultural and food industries. The importance of these compounds have resulted in the dire need for new, efficient, and selective ways for making them.[1–3] Optically active phenylethanol and its derivatives are useful building blocks for the synthesis of complex molecules as the alcohol functionality can be easily transformed, without racemization, into other functional groups.[4] There has been much interest in the use of fungus or yeast for enantioselective reduction of aromatic ketones. They are frequently used as reducing agents because they are cheap and easily available. Essentially, there are many advantages of using microorganisms as biocatalysts instead of purified enzymes. Pure enzymes are often very expensive and not readily accessible, there is a limited availability of enzymes and they require cosubstrates such as cofactors. However, in some cases, enzymes are much more stable within the cell thus extending the life of the biocatalyst. The use of microbial cells is particularly advantageous for carrying out the desired reduction since they do not require addition of cofactors for their regeneration.[1–4]

Biotechnology opens future prospects in the chemical field for the synthesis of complex compounds and combines cheap raw materials with environment-friendly processes.[5] Fermentation medium can represent almost 30% of the cost for a microbial fermentation, with micronutrients representing the most significant cost of production. By-products can supply unique micronutrients to replace expensive peptone and yeast extracts. Consistency of ingredients used in commercial medium formulations and significant increase in product yield or cost reduction are critical for industrial fermentation utilization of any waste material.[6]

Ram horns, composed of fibrous proteins, are widely produced in the world. Recently, ram horn peptone (RHP) has been utilized as sources of peptone for microbial

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*Correspondence to: Kani Zilbeyaz, Science Faculty, Department of Chemistry, Ataturk University, Erzurum 25240, Turkey.
E-mail: kbeyaz25@yahoo.com

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growth media. In addition, RHP also provides a rich source of essential nutrients for microbial growth.[7–10] In this study, RHP was used as a supplement in the fermentation medium for the enantiomeric reduction of acetophenone and its analogous.

The objective of this study was to find the most successful microorganism isolate, in terms of percentage conversion and enantiomeric excess, for the asymmetric reduction of acetophenone and its derivatives. In addition, we report the use of the *R. glutinis* as a novel biocatalyst for submerged culture production of a chiral alcohol on a preparative scale.

EXPERIMENTAL

Materials

The utilized reagents and chemicals were purchased from Merck, Sigma, Oxoid, Fluka, and Difco. All chemicals were of the highest available purity. Ram horns were obtained from the Slaughterhouse of Erzurum, Turkey. Production of RHP was carried out by the method of Kurbanoglu and Kurbanoglu.[10]

Isolation and Identification of Microorganisms

The samples were collected from various sites in Erzurum, Turkey. One milliliter of diluted samples were incubated aerobically for 2 days at 30°C in a 1000-ml Erlenmayer flask containing 300 ml tryptic soy broth. Microorganisms belonging to different taxonomical groups were isolated from fermented broth according to standard techniques. The isolated pure strains were grown on tryptic soy agar. Initially, we investigated the effect of 90 isolates for the chiral alcohol's high enantiomeric purity. The best result obtained in terms of high enantiomeric excess was achieved from the EBK-10 isolate (these results are not reported). The taxonomical group to which the isolate EBK-10 belong was identified as *Rhodotorila glutinis*. This identification was made by means of the VITEK 2 compact (BioMerieux Company, Marcy, France). The isolate was protected in a medium containing (g/l) malt extract 3, glucose 20, yeast extract 3, peptone 3, and agar 15 at 4°C and recultured bimonthly. Fresh plate-grown (48 h) *R. glutinis* EBK-10 was used to inoculate the seed culture.

Medium and Inoculum

The per liter fermentation medium, termed ram horn medium (RHM), contained glucose (20 g), yeast extract (3.0 g), KH_2PO_4 (1.5 g), and RHP (4.0 g). The initial pH of the culture medium was adjusted to 6.5 with 1 N HCl and 1 N NaOH and sterilized at 121°C for 15 min. All the cultures were grown in 250-ml flasks containing 100 ml of medium. The yeast strain grown in the 100 ml of broth medium on a reciprocal shaker (200 rpm) at 32°C for 48 h and was used as the inoculum (6.8×10^8 cells/ml).

Reduction Reactions

The freshly prepared inoculum (1 ml) was added to each flask and incubated on a reciprocal shaker (200 rpm) at 32°C. After 40 h of fermentation, 1 mmol substrate (ketones) was directly added to each medium and incubated on a reciprocal shaker (200 rpm) at 32°C for 24 h. At the end of the incubation period, the cells were separated by centrifugation (5000 rpm) and the supernatant saturated with sodium chloride. The product was extracted into dichloromethane and the solvent removed in vacuo.

Production in the Preparative Scale of 2a

Preparative scale studies were conducted in a 2-L fermenter (Biostat-M 880072/3, Germany) with a working volume of one liter. The inoculum suspension (10 ml) was inoculated into a fermenter containing one liter of sterile RHM. To prevent foam formation, sterilized silicone oil (0.001%, w/v) was added twice to the mixture, one at onset and the other after 24 h of fermentation. After 40 h of incubation, at log phase of the yeast growth cycle, substrate **1a** (20 mmol) was added directly to the fermentation culture. Agitation, pH, aeration (0.4 v/v/m), and temperature were automatically controlled during the fermentation. The reaction time (6–54 h) was optimized for the reduction of **1a** to **2a** in a submerged system. The conversion and ee percentages were recorded at regular intervals of 6 h for up to 54 h. The results are illustrated in Figure 1.

Analytical Procedures

For analysis purposes, a small fraction of the product was separated by preparative silica-gel TLC. The enantiomeric excess (ee) of the product was determined by HPLC with Chiralcel OD, OB, and OB-H columns using *n*-hexane-*i*-PrOH (90:10) as eluent, a flow rate of 0.6 ml/min, and the UV detection was performed at 220 nm. ^1H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer in $CDCl_3$.[7–9] All experiments were done in duplicate and averaged values are presented in this study. Racemic alcohols were prepared by $NaBH_4$ reduction of the ketones in the study and used as standards for the determination of the (*R*) or (*S*)-enantiomers by HPLC.

RESULTS AND DISCUSSION

Various microorganisms were screened as bioreduction agents of **1a**, and after screening 90 isolates, it was found that many yeast, fungus, and bacterium strains produced optically active **2a** from **1a**. The enantiomeric value of each **2a** obtained from the various bioreduction reactions revealed that out of the 90 isolates screened, the strain EBK-10, gave the best ee and conversion. The strain, which was identified by the VITEK 2 compact device as *Rhodotorula glutinis*, successfully reduced **1a** to **2a** with >99% ee. The isolate was thus selected for further studies, which involved reduction of acetophenone derivatives by *R. glutinis.* This strain was isolated from the water of fermented (20 days in a 1000-ml Erlenmeyer flask containing 300 ml of natural water) *Salix* leaves (50 g), which were collected from the grounds of Ataturk University (Erzurum, Turkey).[9] The reduction of **1a** by *R. glutinis* was carried out by two different methods. First, reduction was studied to optimize the reaction conditions and second on a preparative scale in a fermenter.

Initially, we investigated the effects of reaction conditions on the product's enantioselectivity and substrate con-

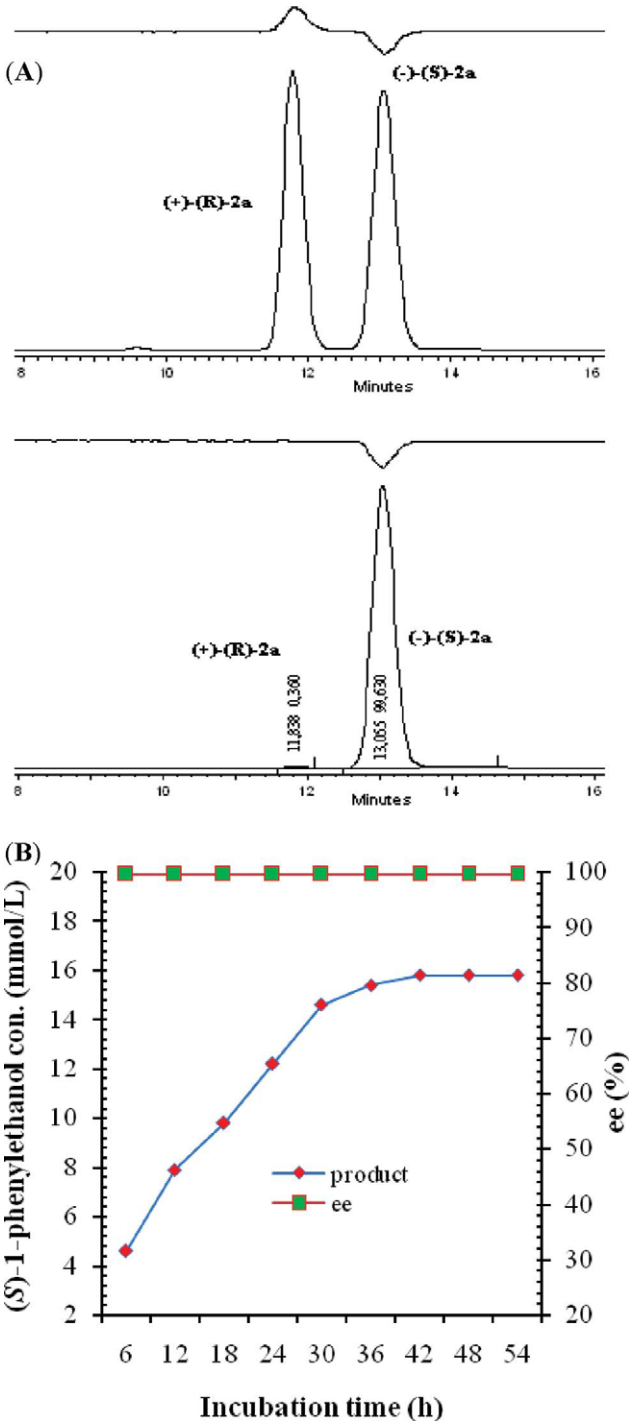


Fig. 1. **A**: Chiral analysis of the product by chiral HPLC. **B**: Production of (*S*)-1-phenylethanol by *R. glutinis* EBK-10 using laboratory scale bioreactor. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

version (Table 1). Cultivation conditions are essential in the successful production of a chiral alcohol and optimization of parameters such as pH, temperature, and media composition is important in developing the cultivation process.[11] It was observed that the pH of the media had a great effect on the conversion and a slight effect on the ee of the product alcohol. At low (5.0–6.0) and high (7.0–7.5) pH values, the percentage conversion was below 70%. It was also observed that the ee's were low at pH 5.0, 5.5, and pH 7.5. Under suitable culture conditions, the effects of different culture temperatures (24–34°C) were also examined. *R. glutinis* was able to show sufficient catalytic activity through a broad temperature range. However, relatively higher reaction activity was obtained at 32°C. Temperature did not have an effect on the enantioselectivity of the product. Different agitation speeds were chosen to monitor the progress of the bioreduction. However, complete conversion of **1a** was only observed at 200 rpm. Agitation speed did not make a difference on the ee's of the alcohols. In summary, for the first method, the optimum conditions were found as pH 6.5, temperature 32°C, and agitation 200 rpm (Table 1). These conditions were satisfactory for both ee (>99%) and conversion (100%). The second method utilized the optimum conditions for the production of **2a** in the preparative scale in a fermenter. It should be mentioned that the reaction conditions such as reaction time, fermentation agitation, temperature, and pH were similar to the bioreduction of 1-acetonaphthone to (*S*)-(−)-1-(1′-nahpthyl)-ethanol by another strain of *R. glutinis*.[9]

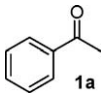
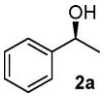
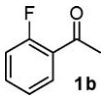
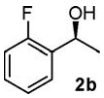
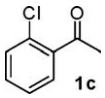
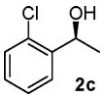
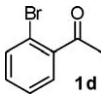
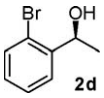
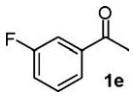
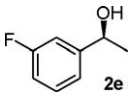
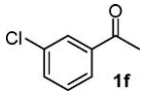
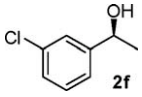
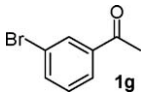
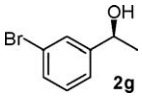
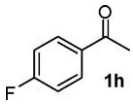
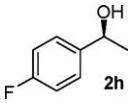
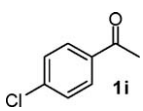
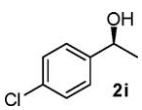
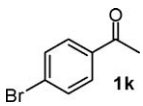
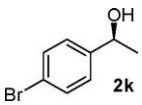
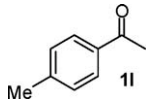
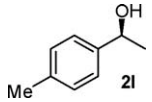
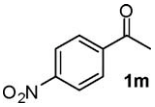
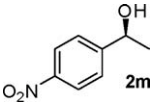
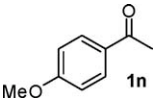
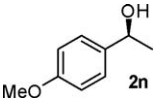
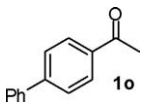
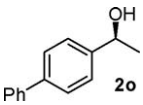
Under the optimized conditions, the biocatalytic potential of *R. glutinis* EBK-10 was studied using a series of acetophenone derivatives (Table 2). White biotechnology uses microorganisms and enzymes to manufacture a large variety of chemical products. Hence, the demand for new and useful biocatalysts is steadily and rapidly increasing. The successful application of a biocatalyst in a biotechnological process requires the identification of a microorganism suitable for over expression.[12] On the other hand, although a large amount of data have been published on the synthesis of the pharmaceutically valuable substituted phenylethanols **2a–o**, very few examples have been reported for the reduction with high ees.[13,14] This study demonstrates that the isolated yeast *R. glutinis* successfully reduces a range of acetophenone derivatives **1a–o** to its corresponding chiral alcohols **2a–o** with maximum ee (>99%), Table 2. The absolute configuration of all the

TABLE 1. Optimization of reaction conditions for the asymmetric reduction of acetophenone (1a) by *Rhodotorula glutinis*[a]

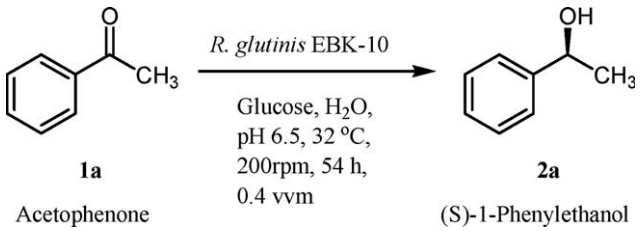
pH			Temperature			Agitation speed		
	Con. (%)[b]	ee (%)[c,d]	°C	Con. (%)[b]	ee (%)[c,d]	rpm	Con. (%)[b]	ee (%)[c,d]
5.0	20	90	24	60	>99	100	78	>99
5.5	35	96	26	65	>99	150	86	>99
6.0	65	>99	28	73	>99	200	100	>99
6.5	75	>99	30	75	>99	250	92	>99
7.0	60	>99	32	86	>99			
7.5	45	98	34	72	>99			

[a]Substrate 1 mM, time 24 h.
[b]Conversion was determined by ^{1}H NMR analysis with diphenylmethane as an internal standard; error about 65% of the stated values.
[c]It was determined by HPLC using Chiralcel OD column.
[d]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.

TABLE 2. Enantioselective reduction of substituted acetophenones 1a–o by *R. glutinis*[a]

Substrate	Product	ee (%)[b,c]	Conversion (%)[d]	Yield (%)
 1a	 2a	>99 (*S*)	100	79
 1b	 2b	>99 (*S*)	100	77
 1c	 2c	>99 (*S*)	100	79
 1d	 2d	>99 (*S*)	100	80
 1e	 2e	>99 (*S*)	100	76
 1f	 2f	>99 (*S*)	100	79
 1g	 2g	>99 (*S*)	100	75
 1h	 2h	>99 (*S*)	100	74
 1i	 2i	>99 (*S*)	100	78
 1k	 2k	>99 (*S*)	100	74
 1l	 2l	>99 (*S*)	100	81
 1m	 2m	>99 (*S*)	100	78
 1n	 2n	>99 (*S*)	44	35
 1o	 2o	>99 (*S*)	23	16

[a]Reaction conditions—substrate: 1 mmol; temperature: 32°C, time: 24 h, pH: 6.5, agitation: 200 rpm.
[b]It was determined by HPLC using chiralcel OD, OB, and OB-H columns.
[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the literature values.
[d]Conversion was determined by ^{1}H NMR analysis with diphenylmethane as an internal standard; error about ±5% of the stated values.



Scheme 1. Bioreduction (one liter scale) of acetophenone (20.0 mmol, 2.4 g) to (S)-phenylethanol (15.8 mmol, 1.9 g, 79% yield, ee >99%). The yield was calculated based on the following: Yield (%) = 100 × PC/ISC, PC, and ISC are the concentrations of product and initial substrate, respectively.

alcohol products **2a–o** had the (*S*)-enantiomer, which is in agreement with Prelog's rule.[15] The bioreduction of the ortho-, meta-, and para-substituted chloro, bromo, and fluoro acetophenones, as well as the para- methyl and nitro acetophenones were transformed to the corresponding alcohols with complete conversion. However, in the presence of an electron donating group at the para position on the aromatic ring, the conversion was lower, with 44% conversion for the methoxy substituent **1n** and 23% conversion for the phenyl substituent **1o**. Furthermore, in these cases, the reduction of the methoxy **1n** and phenyl **1o** substrates proceeded with poor yields, 35% and 16%, respectively. Nevertheless, excellent ees (>99%) were obtained. It should be noted that the outcome of the ees of the desired products were not affected by steric or electronic factors of the various substituents.

We have recently shown the beneficial effects of the presence of RHP in growth medium of microorganisms. Compounds **2a**,[7]**2i**,[16] and **2k**[17] were isolated in gram amounts from fermentation medium in the preparative scale productions by different microorganisms using RHP. The different microbial mediated reductions of acetophenone and its derivatives were previously reported by us[8,16] and the importance of RHP in microbial media was discussed in our previous studies.[9,10] Since the results of a microbial transformation often depend on various factors such as fermentation conditions, culture medium composition, and different microbial strains, it was decided to use the same successful substrates as in our previous studies[8,16] for this study. In this study, although all acetophenones afforded excellent enantioselectivities for the reduction on a small scale, we decided to conduct the transformation of **1a** to **2a** on a large scale to demonstrate the viability of the present system as industrially feasible.

The bioreduction of **1a** for the production of **2a** in preparative scale was performed in a fermenter containing one liter of sterile ram horn medium. These results are summarized in Scheme 1 and Figure 1. For this method, we utilized the optimum conditions from the first method for the production of **2a** in the preparative scale in a fermenter resulting in a 79% yield, complete conversion, and >99% ee. Although there was a regular decrease in conversion speed with the increase of incubation time, enantioselectivity of the product remained the same throughout the reaction. The reason for the decrease of conversion speed with increased incubation time may be due to cell toxicity or product inhibition. After a 30 h incubation period, 92% conversion of **1a** was obtained with a product yield of 73% (14.6 mmol/l), whereas after a 42 h incubation period, the conversion of **1a** completed as 100% with 15.8 mmol/l of **2a** produced from 20 mmol (2.4 g) **1a**. The yield for the conversion was calculated as 79%, which is a good result for preparative scale. This result demonstrates and confirms that the system is feasible for industrial purposes.

There will always be a need for alternative microorganisms for the production of enantiomerically pure pharmaceuticals. Unsurprisingly, given that the human body functions using chiral catalysis, the trend for new chiral pharmaceutical reagents is continuing.[4] Biocatalytic asymmetric reduction of aromatic ketones to corresponding chiral alcohols has attracted 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 NADPH. The cofactors can, however, be regenerated with whole cells as catalysts during the reduction processes.[14,18–20] In this study, the method used for the bioreduction is different, because the substrate was directly added to the culture medium without using resting cells, cofactors, and buffers. In addition, the *R. glutinis* EBK-10 isolated could effectively be used for the bioreduction of acetophenone and its derivatives. All products resulted had satisfactory optical purities.

Consequently, ram horns are an organic waste that is highly rich in protein content and other basic nutrients that could support microbial growth. A simple medium containing RHP proved suitable for growth of *R. glutinis* for the asymmetric reduction of substituted acetophenones to chiral alcohols. Moreover, a medium with RHP is economically more viable than other peptone sources. The preparative scale bioreduction of **1a** by *R. glutinis* EBK-10 gave **2a** in 79% yield, complete conversion, and >99% ee. This is the first report on the asymmetric reduction of substituted acetophenones with *R. glutinis* as a biocatalyst using a submerged culture system. In addition, all the chiral phenylethanols obtained from the submerged culture system were produced with the maximum ee.

LITERATURE CITED

1. Comasseto JV, Andrade LH, Omori AT, Assis LF, Porto ALM. Deracernization of aryl ethanols and reduction of acetophenones by whole fungal cells of *Aspergillus terreus* CCT 4083, *A. terreus* CCT 3320 and *Rhizopus oryzae* CCT 4964. J Mol Catal B Enzym 2004;29:55–61.
2. Patel RN, Goswami A, Chu L, Donovan MJ, Nanduri V, Goldberg S, Johnston R, Siva PJ, Nielsen B, Fan J, He W, Shi Z, Wang KY, Eiring R, Cazzulino D, Singh A, Mueller R. Enantioselective microbial reduction of substituted acetophenones. Tetrahedron: Asymmetry 2004;15: 1247–1258.
3. Murzin DY, Maki-Arvela P, Toukoniitty E, Salmi T. Asymmetric heterogeneous catalysis: science and engineering. Catal Rev 2005;47:175–256.
4. Pollard DJ, Woodley JM. Biocatalysis for pharmaceutical intermediates: the future is now. Trends Biotechnol 2006;25:66–73.
5. Gao K, Song Q, Wei D. Coupling of enantioselective biooxidation of DL-1,2-propanediol and bioreduction of pinacolone via regeneration cycle of coenzyme. Appl Microbiol Biotechnol 2006;71:819–823.

6. Lee B, Pometto AL, Demirci A, Hinz PN. Media evaluation for the production of microbial enzymes. J Agric Food Chem 1998;46:4775–4778.
7. Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Taskin M. Highly enantioselective reduction of acetophenone by locally isolated *Alternaria alternata* using ram horn peptone. Tetrahedron: Asymmetry 2007;18: 1529–1532.
8. Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Kilic H. Asymmetric reduction of acetophenone analogues by *Alternaria alternata* using ram horn peptone. Tetrahedron: Asymmetry 2007;18:2332–2335.
9. Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Taskin M, Kilic H. Production of (S)-(−)-1-(1′-Napthyl) ethanol by *Rhodotorula glutinis* isolate using ram horn peptone. Turk J Chem 2008;32:685–692.
10. Kurbanoglu EB, Kurbanoglu NI. Ram horn peptone as a source of citric acid production by *Aspergillus niger*, with a process. J Ind Microbiol Biotechnol 2004;131:289–294.
11. Pereira RS. The use of baker's yeast in the generation of asymmetric centers to produce chiral drugs and other compounds. Crit Rev Biotechnol 1998;18:25–64.
12. Drepper T, Eggert T, Hummel W, Leggewie C, Pohl M, Rosenau F, Wilhelm S, Jaeger KE. Novel biocatalysts for white biotechnology. Biotechnol J 2006;1:777–786.
13. Valadez-Blanco R, Livingston AG. Enantioselective whole-cell biotransformation of acetophenone to S-phenylethanol by *Rhodotorula glutinis*. Part I. Product formation kinetics and feeding strategies in aqueous media. Biochem Eng J 2009;46:44–53.
14. Nakamura K, Matsuda T. Asymmetric reduction of ketones by the acetone powder of *Geotrichum candidum*. J Org Chem 1998;63:8957–8964.
15. Prelog V. Specification of the stereospecificity of some oxido-reductases by diamond lattice sections. Pure Appl Chem 1964;9:119–130.
16. Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Kilic H. Enantioselective reduction of substituted acetophenones by *Aspergillus niger*. Tetrahedron: Asymmetry 2007;18:1159–1162.
17. Zilbeyaz K, Kurbanoglu EB. Production of (R)-1-(4-bromo-phenyl)-ethanol by locally isolated *Aspergillus niger* using ram horn peptone. Bioresour Technol 2008;99:1549–1552.
18. Bradshaw CW, Hummel W, Wong CH. *Lactobacillus kefir* alcohol dehydrogenase: a useful catalyst for synthesis. J Org Chem 1992;57: 1532–1536.
19. Kataoka M, Rohani LP, Yamamoto K, Wada M, Kawabata H, Kita K, Yanase H, Shimizu S. Enzymatic production of ethyl (R)-4-chloro-3-hydroxybutanoate: asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by an *Escherichia coli* transformant expressing the aldehyde reductase gene from yeast. Appl Microbiol Biotechnol 1997;48:699–703.
20. Chen Q, Hu Y, Zhao W, Zhu C, Zhu B. Cloning, expression, and characterization of a novel (S)-specific alcohol dehydrogenase from *Lactobacillus kefir*. Appl Biochem Biotechnol 2010;160:19–29.