CHIRALITY 22:543–547 (2010)

Production of (*R*)-1-phenylethanols Through Bioreduction of Acetophenones by a New Fungus Isolate *Trichothecium roseum*

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ABSTRACT A total of 120 fungal strains were isolated from soil samples and evaluated in the bioreduction of substituted acetophenones to the corresponding (*R*)-alcohols. Among these strains, isolate *Trichothecium roseum* EBK-18 was highly effective in the production of (*R*)-alcohols with excellent enantioselectivity (ee > 99%). Gram scale preparation of (*R*)-1-phenylethanol is reported. *Chirality 22:543–547, 2010.* © 2009 Wiley-Liss, Inc.

KEY WORDS: fungus; biocatalyst; enantioselective reduction; ketone; chiral alcohol

INTRODUCTION

Enantiomerically pure secondary chiral alcohols are important building blocks for the synthesis of bioactive compounds such as pharmaceuticals, pesticides, pheromones, flavors, fragrances, and natural products.[1–5] For example, enantiopure (*R*)-1-phenylethanol (**2a**) is an important chiral building block for pharmaceuticals, agrochemicals, and natural products.[6–10] For economic and environmental reasons, biocatalysis has recently gained increasing importance for the preparation of enantiopure alcohols from prochiral ketones. For this purpose, ketoreductases have been shown to be unique biocatalysts in the preparation of enantiopure alcohols from prochiral ketones.[11–19] For example, Mandal et al. reported that whole cells of *Trichothecium sp*. are an effective biocatalyst for the enantioselective bioreduction of acetophenone and its analogous compounds to their corresponding (*R*)-alcohols, e.g. (*R*)-**2a** (93.5 ee), (*R*)-**2k** (98.5 ee), and (*R*)-**2n** (90.5 ee).[20] Ou et al. reported the production of **2a** by chemoenzymatic route and the ee obtained was 97%.[21] A pure enzymatic method has also been applied for the production of **2a** with high ee; however, the reactions catalyzed by isolated enzymes require cofactors, which are often too expensive.[22] Recently, we found that ram horn peptone (RHP) could be utilized as a source of peptone for microbial growth media, as a supplement in fermentation medium for the asymmetric reduction of substituted acetophenones to the corresponding chiral alcohols.[23–29] In this work, we screened the submerged culture of *Trichothecium roseum* strain for the biocatalytic reduction of substituted acetophenone series to the corresponding chiral alcohols with *R*-configuration using RHP in fermentation medium. We found that 10 of the assayed 120 isolates of *Trichothecium roseum* enabled the formation of *R* enantiomer through the reduction of the substituted acetophenones. One isolate, namely EBK-18, yielded (*R*)-alcohols with excellent ee under optimized conditions and allowed gram scale preparation of (*R*)-1-phenylethanol (**2a**) from acetophenone.

EXPERIMENTAL

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 using the method described by Kurbanoglu and Kurbanoglu.[24]

Isolation of Microorganisms, Identification and Inoculation

The microorganisms used in this study were isolated from soil samples collected from the region around Erzurum, Turkey. The isolation process was performed by serial dilution of the samples according to standard techniques.[30] Filamentous fungi were taxonomically identified in-house using mature cultures on standard potato dextrose agar (PDA) to ensure good development of taxonomically relevant features, and following the identification keys provided by Von Arx and Domsch et al.[31,32] These cultures were maintained on PDA slants, incubated

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: The Scientific and Technological Research Council of Turkey (TUBITAK); Contract grant number: TBAG-107T670

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Received for publication 17 May 2009; Accepted 8 July 2009
DOI: 10.1002/chir.20775
Published online 9 September 2009 in Wiley InterScience (www.interscience.wiley.com).

TABLE 1. Optimization of parameters for the bioreduction of acetophenone (1a) by *Trichothecium roseum*[a]

pH			Temperature			Incubation period			Agitation speed		
	Convn (%)[b]	ee (%)[c,d]	°C	Convn (%)[b]	ee (%)[c,d]	h	Convn (%)[b]	ee (%)[c,d]	rpm	Convn (%)[b]	ee (%)[c,d]
5.0	30	65-(*R*)	26	68	90-(*R*)	24	80	90-(*R*)	100	20	70-(*R*)
5.5	50	80-(*R*)	28	75	95-(*R*)	48	92	95-(*R*)	150	80	95-(*R*)
6.0	60	95-(*R*)	30	80	95-(*R*)	72	100	77-(*R*)	200	100	99-(*R*)
6.5	50	87-(*R*)	32	74	87-(*R*)	96	100	77-(*R*)	250	100	60-(*R*)
7.0[e]	0		34	30	60-(*R*)				300	100	40-(*R*)

[a]Substrate 1 mM.
[b]Conversion was determined by ^{1}H NMR analysis with diphenylmethane as an internal standard; error ca. ±5% of the stated values.
[c]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.
[e]Weak growth.

at 25°C, and stored at 4°C. The conidia from 8-day-old cultures were used for inoculation. The conidial suspension was prepared in 10 ml sterilized and distilled water by gently scratching conidia with a sterile wire loop and then it was shaken vigorously to break up the clumps of conidia.

Medium, Culture Conditions and Screening for Acetophenone Reduction

The fermentation medium per liter contained (g/l): glucose 20, yeast extract 3, KH_2PO_4 1.5, and RHP 4. The initial pH of the culture medium was adjusted to 7.0 with 1 M HCl and 1 M NaOH and sterilized at 121°C for 15 min. All the cultures were grown in 250 ml flasks containing 100 ml of medium. Then 1 ml of conidial suspensions was added to each flask. The flasks were incubated on a reciprocal shaker at 150 rpm and 25°C for 72 h. After the growth of the fungal strains, acetophenone (**1a**) (1 mmol) was added directly to each medium and then the incubation continued on a reciprocal shaker at 150 rpm and 25°C for 24 h. A total of 120 fungal strains were screened to produce (*R*)-**2a** from **1a** with RHP as nitrogen and mineral sources. Among them, ten fungal strains reduced **1a** to (*R*)-**2a**. The most productive strain (EBK-18) was identified as *Trichothecium roseum*. This strain was selected for further research.

*Production of (*R*)-2a by* T. roseum *EBK-18 in a Laboratory-Scale Bioreactor*

All production experiments under optimum fermentation conditions were performed in a 2 l fermenter (Biostat-M 880072/3, Germany) with a working volume of 1 liter. Ten milliliters of the spore suspension were inoculated into the fermenter containing 1 l of sterile medium. Agitation, pH, aeration (vol/vol/min), and temperature were automatically controlled during fermentation. At regular intervals (6 h) during fermentation, the conversion, yield, and ee were determined.

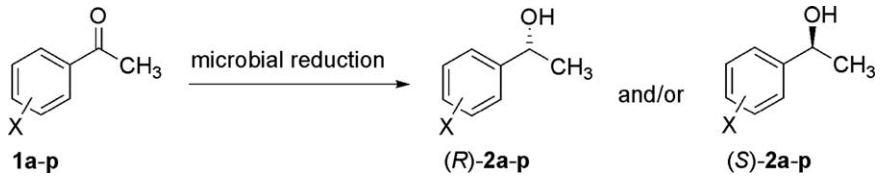
Analytical Procedures

At the end of the incubation period, mycelium was separated by filtration, and the filtrate was saturated with sodium chloride and then extracted with ethyl acetate. The mycelia were also washed with ethyl acetate. Ethyl acetate extracts were combined and dried over Na_2SO_4. The conversion was determined by ^{1}H NMR analysis with diphenylmethane as internal standard; error ca. ±5% of the stated values. After removal of the solvent, the crude products were purified by short silica gel column chromatography and identified by NMR analysis. The absolute configuration was determined by the sign of the specific rotation and comparison with the literature.[33,34] The ee of the alcohols was then determined by HPLC analysis using Chiralcel OD and OB columns. The purity of (*R*)-1-phenylethanol (**2a**) produced *via* a fermenter was checked by HPLC analysis. The specific rotation was measured with a polarimeter at 589.3 nm. (*R*)-1-phenylethanol **2a**:[35–40] 76% yield (1.85 g, 15.1 mmol); $[\alpha]_{20}^{D}$ +52.8 (c, 0.85, $CHCl_3$); >99% ee determined by HPLC on a OD chiral column; retention times were 11.8 min for (+)-(*R*) and 13.0 min for (−)-(*S*).

RESULTS AND DISCUSSION

To establish the optimal reaction conditions for the asymmetric reduction with *Trichothecium roseum*, pH, temperature, incubation period, and agitation speed were investigated in the reduction of acetophenone (**1a**). The results of these optimizations are given in Table 1. Different pH ranges (5.0, 5.5, 6.0, 6.5, and 7.0) were chosen to monitor the progress of the bioreduction. The highest conversion (60%) and ee (95%) were achieved when the medium pH was controlled at 6.0. Under suitable culture conditions, the effects of different culture temperatures were examined by carrying out the fermentation processes within different temperature ranges (26–34°C). The highest conversion (80%) was obtained at 30°C with 95% ee. Temperatures over 30°C, both ee and conversion dropped substantially. For example, the lowest ee (60%) and conversion (30%) were obtained at 34°C. These results suggest that an increase in temperature had a negative effect on the ee and conversion. Therefore, we continued the research with 30°C Different incubation times were chosen to monitor the progress of the bioreduction. The complete conversion of **1a** was observed after 72 and 96 h, but the ee (77%) of **2a** decreased. In contrast to these

TABLE 2. Enantioselectivities for the microbial reduction of substituted acetophenones by *Trichothecium roseum*, *Aspergillus niger*, and *Alternaria alternate*



1a-p (*R*)-**2a-p** (*S*)-**2a-p**

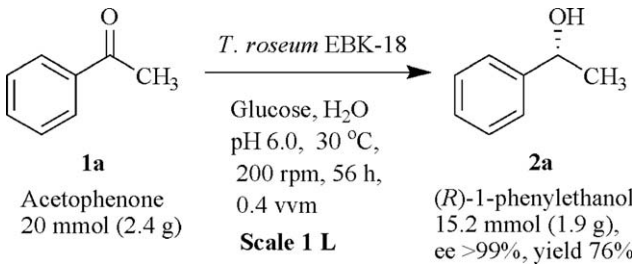
Entry	Substrate	Product	Convn. (%)[a]	Yield (%)[a]	*ee* (%)-Config.[b,c] *Trichothecium roseum*[a]	*Aspergillus niger*[d]	*Alternaria alternata*[e]
1	H (**1a**)	H (**2a**)	100	75	> 99-(*R*)	87-(*R*)	99-(*S*)
2	*o*-F (**1b**)	*o*-F (**2b**)	...[f]	–	–	...[g]	99-(*S*)
3	*o*-Cl (**1c**)	*o*-Cl (**2c**)	...[f]	–	–	99-(*S*)	99-(*S*)
4	*o*-Br (**1d**)	*o*-Br (**2d**)	...[f]	–	–	96-(*S*)	99-(*S*)
5	*m*-F (**1e**)	*m*-F (**2e**)	90	66	> 99-(*R*)	...[g]	99-(*S*)
6	*m*-Cl (**1f**)	*m*-Cl (**2f**)	82	58	> 99-(*R*)	57-(*S*)	99-(*S*)
7	*m*-Br (**1g**)	*m*-Br (**2g**)	77	56	> 99-(*R*)	66-(*S*)	99-(*S*)
8	*m*-NO_2 (**1h**)	*m*-NO_2 (**2h**)	100	79	> 99-(*R*)	...[g]	...[g]
9	*p*-F (**1i**)	*p*-F (**2i**)	97	75	> 99-(*R*)	...[g]	...[g]
10	*p*-Cl (**1k**)	*p*-Cl (**2k**)	86	63	> 99-(*R*)	99-(*R*)	29-(*R*)
11	*p*-Br (**1l**)	*p*-Br (**2l**)	81	55	> 99-(*R*)	99-(*R*)	49-(*R*)
12	*p*-NO_2 (**1m**)	*p*-NO_2 (**2m**)	100	78	> 99-(*R*)	...[g]	20-(*S*)
13	*p*-Me (**1n**)	*p*-Me (**2n**)	96	74	> 99-(*R*)	95-(*R*)	...[g]
14	*p*-MeO (**1o**)	*p*-MeO (**2o**)	...[f]	–	–	90-(*R*)	49-(*R*)
15	*p*-Ph (**1p**)	*p*-Ph (**2p**)	...[f]	–	–	65-(*R*)	90-(*S*)

[a]From this work with *Trichothecium roseum*. Isolated yields after column chromatography on silica gel.
[b]Determined by HPLC using Chiralcel OD and OB columns.
[c]Absolute configurations were assigned by comparison of the sign of optical rotations relative to the values in the literature.
[d]See Ref. 25.
[e]See Ref. 26.
[f]No conversion was observed.
[g]Data not available.

results, the conversion increased up to 92% with 95% ee for 48 h. Although the ee of **2a** remained steady after 48 h, the conversion rate was obviously different. The best condition (48 h) obtained was used for further optimization of conversion and ee. The highest values for both ee (>99%) and conversion (100%) were obtained at 200 rpm and thus this agitation speed was determined as optimum for fermentation.

Under the optimum conditions (pH 6.0, temperature 30°C, time 48 h, and agitation 200 rpm) asymmetric bioreductions of the other derivatives of **1a** by *T. roseum* EBK-18 were investigated in the shake flask scale. The results are shown in Table 2. All resulting alcohols had an *R*-configuration with >99% ee. In the first set of experiments, we studied the influence of the ketone structure. The prominent trend displayed in Table 2 is that the conversion of the substrates decreases with the degree of electron donating-withdrawing groups at the aromatic ring. Clearly, the electron-deficient substrates show higher reactivity. When para-substituted acetophenones were reduced, electron-donating groups provided no conversions (Table 2, entries 13–14) except for para-methyl derivative **1n** (entry 13), while electron-withdrawing substituents afforded conversions in the range of 77–100%. Moreover, ketones with a strong electron-withdrawing group at the para or meta position such as nitroacetophenones **1h** and **1m** furnished quantitative conversions. The reduction of the meta- or para-substituted acetophenone was more favorable as compared to ortho-substituted acetophenone. There was no reaction for any ortho-substituted acetophenones. Presumably, steric repulsion between the catalytically active site and the ortho-substituents hinders the transfer of the hydrogen atom.

After successful determination of the reaction parameters, we decided to conduct the transformation of **1a** to (*R*)-**2a** on a gram scale to demonstrate industrial feasibility.



1a
Acetophenone
20 mmol (2.4 g)

2a
(*R*)-1-phenylethanol
15.2 mmol (1.9 g),
ee >99%, yield 76%

Scheme 1. A gram scale production of (*R*)-1-phenylethanol (**2a**)

Preparative scale production of **2a** was performed on a 1l scale in a 2 l fermenter (Scheme 1). Bioreduction of **1a** (3.0 g, 25 mmol) after 62 h resulted in complete conversion, but the ee of the desired product was rather low (60%). It was noted that the enantioselectivity of *T. roseum* EBK-18 depended on the incubation time used for cultivation and substrate concentration. Therefore, **1a** (2.4 g, 20 mmol) was directly added to the fermentation medium. Complete conversion of **1a** was achieved after 56 h of incubation, and then the mixture was extracted with EtOAc (3 X 25 ml) and dried over Na_2SO_4. After evaporation of the solvent the product **2a** was purified on a silica gel column.

Recently, we reported the bioreduction of acetophenones by *Aspergillus niger* and *Alternaria alternata*.[25–27] In comparison, *Trichothecium roseum* is sensitive to the position and electronic effect of the substituent. Thus, the derivatives **1b-d** and **1o-p** did not afford the corresponding alcohols 2 (Table 2, entries 2–4, and 13–14); however, in contrast to this observation, *Aspergillus niger* and *Alternaria alternata* are not substrate structure-dependent reducers. While *Trichothecium roseum* exhibits *R* selectivity in all cases, *Aspergillus niger* and *Alternaria alternata* do not show any preference in enantioselectivity. Thus, depending on the substrates, they produce either (*R*)- or (*S*)-alcohols **2**.

In the present study, acetophenone (**1a**) and its derivatives were reduced to the corresponding (*R*)-enantiomer with >99% ee using submerged culture of *T. roseum* EBK-18. We have demonstrated a novel microbial system to obtain enantiopure *sec*-alcohols that possess several advantages: conversion and enantioselectivity are controlled by the substituent position and electronic effect, and the process can be scaled up. This is a convenient system that exhibits excellent enantioselectivity and can be applied for the clean synthesis of valuable enantiopure alcohols.

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