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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 18 (2007) 1529–1532

Highly enantioselective reduction of acetophenone by locally isolated *Alternaria alternata* using ram horn peptone

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Received 30 March 2007; accepted 14 June 2007

Abstract—Enantiomerically pure compounds are important building blocks in the synthesis of natural products. In this study, the reduction of acetophenone to the (*S*)-isomer of 1-phenylalcohol with a high enantiomeric excess (ee) by locally isolated *Alternaria alternata* using ram horn peptone (RHP) was investigated. Ten strains of *A. alternata* were isolated from different plant samples. These isolates were evaluated for the reduction of acetophenone (ACP) to 1-phenylethanol (PEA). Glucose, yeast extract and RHP in a shake flask and fermenter for growth of *A. alternata* cultures were used. *A. alternata* EBK-4 isolate was found to be an effective biocatalyst for the enantiomeric bioreduction of acetophenone. Conversions of up to 100% with excellent enantiomeric excesses (>99%) were obtained. Production of PEA was achieved via a fermenter. The yield was calculated as 86%. This is the first report on the enantioselective reduction of ACP by *A. alternata* using ram horn peptone from waste material.

1. Introduction

Chiral alcohols are useful intermediates for many pharmaceutical and chemical compounds. The need for optically active drugs has increased in pharmaceutical and agrochemical fields in the recent years and, therefore, chiral alcohols are under increasing demand. There have been many attempts to construct bioreduction systems for the industrial production of chiral alcohols.[1,2] There has also been much interest in the use of fungus or yeast for enantioselective reductions of aromatic ketones. There are many advantages to using microorganisms as biocatalyst instead of purified enzymes. Microorganisms are generally much less expensive, and in some cases, enzymes are 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 the addition of cofactors for their regeneration.[1–4] In addition, there is a need for alternative microorganisms for producing enantiomerically pure pharmaceuticals, because the racemic mixtures made today may not be allowed in the future. Synthetic chiral catalysts are being developed for this purpose, as well as new product separation techniques. Another possible option is to use biocatalysts, such as purified enzymes or microbial cells, since these can result in the production of mostly a single enantiomer.

On the other hand, biotechnology opens future prospects in the chemical field for the synthesis of complex compounds and combines inexpensive raw materials with environmentally friendly processes.[2,5,6]

Recently, ram horn peptone (RHP) has been utilized as a source of peptone for microbial growth media. One important option is the production of ram horn protein hydrolysate, which can be prepared by the hydrolysis of ram horns using proteolytic chemicals. The RHP also provides a rich source of nutrients for microbiological growth, and is excellent for microbial growth.[7]

Herein, we produced the highly enantiomerically enriched (*S*)-1-phenylethanol from acetophenone by locally isolated *Alternaria alternata* with a process and clarified the use of RHP from waste material for microbial reduction. We also optimized the reaction conditions for high enantiomeric excess (ee).

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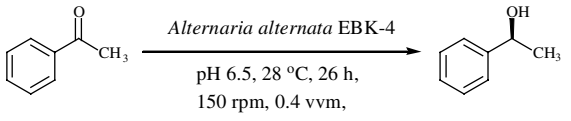
doi:10.1016/j.tetasy.2007.06.013



Acetophenone

(10 mmol) scale: 1L

(*S*)-1-Phenylethanol

ee = > 99%
Yield = 86%

2. Results and discussion

The ACP reduction was carried out in two stages. In the first stage, the reduction was studied in the flask culture in order to find the optimum reaction conditions; in the second stage, the total production of PEA under optimum conditions were performed in the fermenter. Ten different strains of *A. alternata* were isolated from plant samples and evaluated for the reduction of ACP to PEA, using glucose, yeast extract and RHP nutrients as substrate in shake flask and fermenter. These cultures were found to produce (*S*)-1-phenylethanol, ranging from 60% to 78% ee. The best results for the bioreduction were obtained when *A. alternata* EBK-4 was used. The ee and conversion were 78% and 62%, respectively (Table 1). The enantioselectivity observed for this isolate is in accordance with Prelog's rule. Acetophenone was also reduced with the other isolates in moderate conversion and enantioselectivity. Since the highest enantioselectivity was observed by using *A. alternata* EBK-4 as biocatalyst, it was selected for further studies.

Table 1. Screening of locally isolated *A. alternata* strains for the bioreduction of acetophenone

Isolates	Conversion	ee (%)—Config.
EBK-1	33	64 (*S*)
EBK-2	25	71 (*S*)
EBK-3	37	62 (*S*)
EBK-4	62	78 (*S*)
EBK-5	38	67 (*S*)
EBK-6	40	74 (*S*)
EBK-7	52	74 (*S*)
EBK-8	44	76 (*S*)
EBK-9	48	75 (*S*)
EBK-10	54	60 (*S*)

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

Different incubation times were chosen to monitor the progress of the bioreduction (Table 2). The best ee (87%) was observed after 24 h. When the ee of the PEA was observed after 72 and 96 h, the ee was lower. Contrary to these results, the conversion increased up to 90% for 96 h. On the basis of these observations, we suggested that *A. alternata* EBK-4 may express different ees and conversions depending on the growth conditions. The purpose of increasing the enantioselectivity of the PEA was studied with different pH. The best conditions found were used for further optimization of conversion and ee.

Effect of the initial pH of the used growth medium on the reduction of ACP is shown in Table 3. A significant amount of work has been carried out concerning the effect of pH on enzymatic reactions in an aqueous phase.[8] The

Table 2. Effect of incubation period on the reduction of acetophenone by *A. alternata* EBK-4

Time (h)	Conversion	ee (%)—Config.
24	44	87 (*S*)
48	62	78 (*S*)
72	70	56 (*S*)
96	90	30 (*S*)

Reaction conditions: substrate 1 mmol, temperature 25 °C, pH 7, agitation 150.

results, shown in Table 3, indicate that the fermentation pH clearly has a significant effect on conversion and ee. The conversion and ee increased when the pH of the growth medium was increased from pH 4.5 to pH 6.5 and then decreased. The optimum pH for the reduction activity was found to be 6.5. When pH 6.5 was used, the conversion and ee were 90% and 93%, respectively. The conversion and ee were lower when the pH was over 7.0. The *A. alternata* was not able to grow at the initial pH of 8.5. Under the optimum conditions (pH 6.5, 24 h), the effect of different incubation temperatures was investigated in order to determine whether it could increase the conversion and ee of acetophenone reduction. The results are shown in Table 4.

Table 3. Effects of different pHs on the reduction of acetophenone by *A. alternata* EBK-4

pH	Conversion (%)	ee (%)—Config.
4.5	20	20 (*S*)
5.5	80	84 (*S*)
6.5	90	93 (*S*)
7.5	57	30 (*S*)
8.0	Weak growth	—
8.5	No growth	—

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

As can be seen from Table 4, a variation in temperature resulted in different conversion and ee levels on the reduction of ACP. Notably, the temperature has a significant effect on the ee and conversion. The highest ee (99%) was obtained from 28 °C with 100% conversion. Temperatures over 30 °C had an inhibitory effect on ee. For example, the lowest ee (60) was obtained at 36 °C. These results suggest that an increase in temperature had a negative effect on the ee. Conversely, the high temperature did not have a

Table 4. Effect of temperature on the reduction of acetophenone by *A. alternata*

Temperature (°C)	Conversion (%)	ee (%)—Config.
26	96	96 (*S*)
28	100	99 (*S*)
30	100	93 (*S*)
32	100	86 (*S*)
34	100	70 (*S*)
36	100	60 (*S*)

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

negative effect on the conversion of ACP. The conversion of ACP for 36 °C was 100%, although the ee was rather low. The optimum conditions for the high enantiomeric excess of PEA with temperature studies were found. As a result, other fermentation parameters such as agitation and initial substrate concentration were not studied. These fermentation conditions are time 24 h, pH 6.5, temperature 28 °C and agitation 150 rpm. The ee and conversion under these conditions were at their maximum. Therefore, we continued the research with optimum fermentation conditions for further studies.

The bioreduction of ACP for the total production of PEA by *A. alternata* EBK-4 was performed in a fermenter. These results are summarized in Figure 1. The ee for all incubation times was >99%. The best results for the bioreduction were obtained after 26 h incubation. The reaction occurred with good conversion (100%) and excellent ee (>99%). In addition, the amount of PEA produced with the observed enantioselectivity was 8.6 mmol/L. (*S*)-1-Phenylethanol was produced by *A. alternate* via a fermenter under optimum growth conditions. This fungus was firstly used for the production of a chiral alcohol. Therefore, this microorganism must be useful for biotechnological purposes such as microbial reductions. Many papers report with different microorganisms the microbial reduction of acetophenone and its derivatives.[2,4,9–11] They are used as a reducing agent as they are easily available and inexpensive. The 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 extract. The consistency of the ingredients used in the commercial medium formulations and significant increase in product yield or cost reduction are critical for the industrial fermentation utilization of any byproduct.[12] Therefore, an inexpensive substrate, such as ram horn waste for microbial growth in this study was successfully used. The RHP for microbial growth in the previous study was compared with some standard peptones. It was found that the RHP could be utilized instead of some standard peptones.[7]

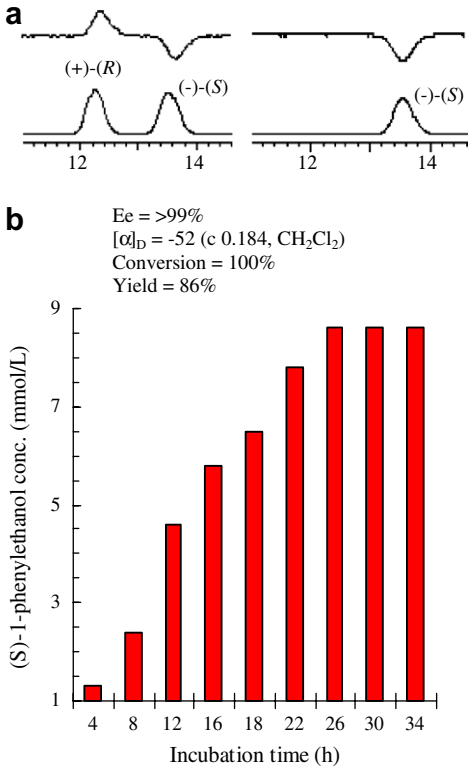


Figure 1. Chiral analysis of the product: (a) Production on a preparative scale of (*S*)-1-phenylethanol via fermenter by *A. alternata* EBK-4. (b) Conversion, yield and polarimetric value after 26 h incubation. Reaction conditions: pH 6.5, temperature 28 °C, agitation 150 rpm, substrate 10 mmol, aeration 1.0 v/v/m. The yield was calculated based on the following: Yield (%) = 100 × PC/ISC, PC and ISC are the concentrations of product and initial substrate, respectively.

3. Conclusion

This report has dealt with the first microbial reduction of acetophenone by *A. alternata* on a preparative scale. It seems more adequate to use raw materials such as waste material from the food industry as the basis of the microbial culture media. We believe that selectivity is valuable in organic synthesis. Therefore, a detailed mechanism for the high selectivity of the reduction of acetophenone derivatives by altering the reaction conditions and microorganisms is under investigation in our laboratory.

4. Experimental

4.1. Materials

Ram horns were obtained from the slaughterhouse of 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 ram horn peptone was carried out with the method of Kurbanoglu and Kurbanoglu.[7]

4.2. Isolation of microorganisms, identification and inoculation

The microorganisms used in this study were isolated from plant samples, such as sunflower, grapes and apple collected from the region around Erzurum, Turkey. The isolation process was performed by serial dilution of the samples according to standard techniques.[13] Taxonomic identification of filamentous fungi was identified in-house by using mature cultures on a standard potato dextrose agar (PDA) in order to ensure a good development of taxonomically relevant features, and following the identification keys provided by Von Arx[14] and Domsch et al.[15] These cultures were maintained on PDA slants, incubated at 25 °C and stored at 4 °C. The conidia from 10 days old cultures were used for inoculation. The conidial suspension was prepared in sterilized 10 mL distilled water by gently scratching conidia with a sterile wire loop and then it was shaken vigorously for breaking the clumps of conidia.

4.3. Culture conditions and reduction of acetophenone

The fermentation medium per liter contained (g/L): glucose 20, yeast extract 3 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. 1 mL of conidial suspension was added to each flask. Flasks were incubated on a reciprocal shaker at 150 rpm, 25 °C for 48 h. After the growth of the fungus, acetophenone (1 mmol) was added directly to each medium and then the incubation continued on a reciprocal shaker at 150 rpm, 25 °C for 48 h. The total production experiments were performed in a 2-L fermenter (Biostat-M 880072/3, Germany) with a working volume of 1 L. Ten milliliter of the spore suspension was inoculated into the fermenter containing 1 L of sterile medium. Agitation, pH and temperature were automatically controlled during the fermentation. At regular intervals (4 h) of fermentation, the conversion and the ee were determined.

4.4. Purification of metabolic products and analytical processes

After the specified time, the mycelium was separated by filtration, and the filtrate saturated with sodium chloride and then extracted with diethyl ether. The mycelia were also extracted with diethyl ether. The diethyl ether extracts were combined; ether was dried over N_2SO_4, and evaporated to dryness. For analysis, a small fraction of the product was separated by a preparative silica-gel TLC. The ee of the product was determined by HPLC with OD column using eluent *n*-hexane–*i*-PrOH, 90:10, flow rate of 0.6 mL/min, detection performed at 220 nm. Retention time (min): (*R*), 12.3; (*S*), 13.5. The crude product was purified by silica gel column chromatography. ^1H and ^{13}C NMR spectra were recorded on a Varian 400 spectrometer in $CDCl_3$. Purified 1-phenylethanol was identified with spectral data (^1H and ^{13}C NMR). The purity of (*S*)-1-phenylethanol produced via a fermenter was also checked with HPLC analysis (Fig. 1). The specific rotation was measured with a 589.3 nm spectropolarimeter as $[\alpha]_D = -52$ (*c* 0.184, CH_2Cl_2). The absolute configuration of the compound was determined by comparing the sign of its specific rotation with that in the literature. The conversion was determined by ^1H NMR analysis with diphenylmethane as internal standard; error ca. ±5% of the stated values.

Acknowledgements

This study was supported by a grant from the Research Funds Appropriated to Ataturk University, Erzurum, Turkey. The authors thank Professor Ismet Hasenekoglu for his help in identification of fungus.

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