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Full Length Research Paper

Asymmetric biosynthesis of (1S, 2S)-ephedrine by *Morganella morganii* CMCC(B)49208

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(1S, 2S)-Ephedrine, one of the four ephedrine isomers, is a common pharmacological agent; however, it is difficult to synthesize in a stereospecific manner using traditional organic synthesis. In this paper, a novel enzymatic process was developed for the stereoselective synthesis of (1S, 2S)-ephedrine. *Morganella morganii* CMCC(B)49208 was found to asymmetrically reduce the prochiral carbonyl compound 1-phenyl-1-oxo-2-methylaminopropane (MAK) to optically pure (1S, 2S)-ephedrine which was measured with thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) technologies. In addition, a conversion process using air-dried *M. morganii* CMCC(B)49208 cells was developed to produce (1S, 2S)-ephedrine at a final concentration of 0.852 gL^{-1} (>99% ee) and 84.4% molar yield.

Key words: Ephedrine, stereoselective reduction, *Morganella morganii*, bioconversion.

INTRODUCTION

Biochemical reactions using microorganisms and enzymes have been extensively evaluated by synthetic organic chemists (Stephan et al., 2006; Schmid et al., 2002; Kaoru et al., 2003) for the preparation of pharmaceuticals, fine chemicals, food additives, and commodity chemicals. This synthetic technology is called microbial transformation, microbial conversion, biotransformation, bioconversion, or enzymation. It is a rapidly developing field of synthetic chemistry, which overlaps both organic chemistry and biochemistry. Many useful organic compounds, such as pharmaceuticals and food additives, have asymmetric carbon atoms and usually consist of a mixture of enantiomers. In most cases, only one enantiomer has the desired biological activity. The other enantiomer may cause side-effects or harmful effects. Therefore, a racemic mixture of such compounds, especially for pharmaceuticals, obtained through chemical synthesis can no longer be used directly. Optical resolution of the racemic mixture by conventional organic synthetic processes is often troublesome. Therefore, transformations using microbial cells or their enzymes that possess asymmetric synthesis abilities have been extensively investigated to give optically active substances (Yasohara, 2005).

Ephedrine (α-hydroxy-β-methylaminopropylbenzene) is a pharmacologically useful amino alcohol. It has four stereoisomers [(1R, 2R)-, (1R, 2S)-, (1S, 2R)-and (1S, 2S)-] derived from two asymmetric carbon atoms at the α- and β-positions (Figure 1). The naturally-occurring isomers (1R, 2S)- and (1S, 2S)-ephedrine, we usually call *l*- and *d*-ψ-ephedrine, are used as a bronchodilator and a decongestant, respectively. Each isomer is also useful as a resolving reagent for the synthesis of many other chiral compounds. However, it is difficult to stereospecifically synthesize only one isomer using conventional organic synthesis. Although there are some studies on biotransformation (Shin et al., 1996; Rogers et al., 1997) of benzaldehyde and pyruvic acid to L-Phenylacetylcarbinol (L-PAC) or fermentation process for L-PAC production (Tripathi et al., 1997), a key intermediate for L-ephedrine synthesis, the further steps of L-PAC extraction and L-ephedrine synthesis by chemical method are complicated. In this study, four strains of genus *Morganella* were screened and a process was developed for the stereoselective and asymmetric reduction of the prochiral carbonyl compound 1-phenyl-1-oxo-2-methylaminopropane (MAK) to (1S, 2S)-ephedrine.

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MATERIALS AND METHODS

Materials

dl-MAK was chemically synthesized from propiophenone by a bromination and amination processs (Sun and Shi, 2000). Mass-spectrometry (MS) and infrared spectroscopy were used to confirm the identity of MAK. Four stereoisomers of ephedrine were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Nicotinamide adenine dinucleotide (NAD^+) and glucose dehydrogenase (GDH) were purchased from Sigma. All other chemicals used in this study were of analytical grade and commercially available.

Microorganisms and cell culture

M. morganii CMCC(B)49203, 49208, 49209, and 49214 were purchased from the National Center for Medical Culture Collections (CMCC(B)) and preliminary results showed that they had carbonyl reductase activity. The bacterial cells were grown under aerobic conditions in a 15 L fermentor containing 9 L medium (25 gL^{-1} peptone, 5 gL^{-1} yeast extract, 30 gL^{-1} glucose, 3 gL^{-1} K_2HPO_4, 0.2 gL^{-1} $MgSO_4 \cdot 7H_2O$, 2 gL^{-1} NaCl; pH 7.0) with shaking at 150 r/min at 30℃ for 48 h. The air-dried cells were prepared as described previously (Shimizu et al., 1979).

Screening of MAK-reducing strains

The reduction reaction was carried out using *M. morganii* CMCC(B)49203, 49208, 49209, or 49214 to evaluate the optimum strain to produce ephedrine. A reaction mixture containing *M. morganii* air-dried cells (0.5 g), MAK (10 mg), GDH (100 units), glucose (0.5 g), NAD^+ (9 µmol), and 0.2 M phosphate buffer (pH 7.0) in a final volume of 10 ml was stirred gently at 37℃ for 24 h before it was centrifuged for 15 min at 3,000 r/min. The supernatants (10 µL) of each reaction and standard solutions of (1R, 2S)-ephedrine and (1S, 2S)-ephedrine were spotted on a thin layer chromatography (TLC) plate with disposable calibrated micro-capillaries and the plate was developed as described below.

Effects of buffer system, initial pH and temperature on biotransformation

Basic biotransformatiom mixture: *M. morganii* CMCC(B)49208 air-dried cells (0.5 g), MAK (10 mg), GDH (100 units), glucose (0.5 g), NAD^+ (9 µmol) in 10 ml final volume.

Study on buffer system and initial pH: Sodium phosphate buffer, potassium phosphate buffer, Na_2PO_4-KH_2PO_4 buffer (defined as phosphate buffer in this paper), Tris buffer, acetic acid-sodium acetate buffer. All the buffers were prepared in the concentration of 0.2 M and were adjusted to 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively when used for making up reaction mixture. The experiments for buffer test were carried out at 30ºC.Study on reaction temperature: 20, 25, 30, 35, 40, 45, 50, 55 and 60ºC were tested in the first time, and 35, 37, 39, 41, 43 and 45ºC were examined to confirm relative the best factor. The reaction mixture was made up with phosphate buffer (pH 7.5).

Yield of (1S, 2S)-ephedrine in the end of reaction was analyzed by HPLC, and the results were evaluated by formation rate of (1S, 2S)-ephedrine (µmol/L·h).

Preparative-scale reaction

For preparative scale production of (1S, 2S)-ephedrine, a reaction mixture containing *M. morganii* CMCC(B)49208 air-dried cells (5 g), MAK (100 mg), GDH (1000 units), glucose (5 g), NAD^+ (90 µmol), and 0.2 M phosphate buffer (pH 7.5) in a final volume of 100 ml was prepared according to the optimized conditions mentioned before and was stirred gently at 39℃ for 24 h before it was sampled and centrifuged for 15 min at 3,000 r/min. The supernatant of the reaction mixture was analyzed by HPLC for the presence of MAK and ephedrine as described below.

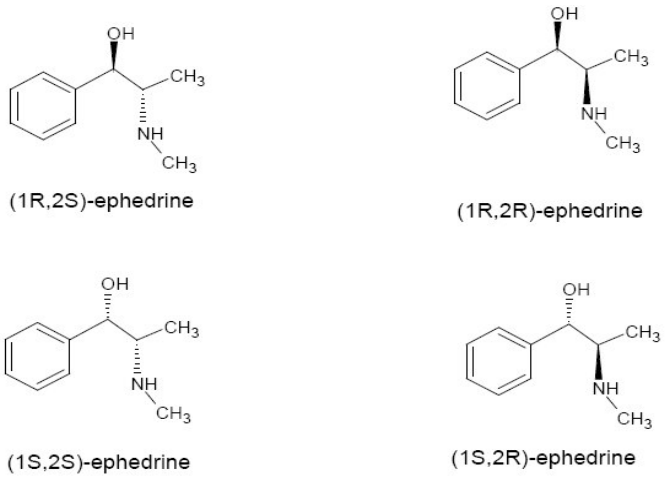


Figure 1. Chemical structures of the four stereoisomers of ephedrine.

Analysis of MAK and ephedrine by TLC

A thin-layer chromatography (TLC) plate was coated with Silica gel GF_{254} (60H) (15×15 cm, 0.25 mm layer thickness, glass support). The TLC plate was developed using a solvent system of methanol-25% ammonia (98:2 v/v) in a vertical development tank at room temperature. After development, the TLC plate was dried in the dark and was sprayed with ninhydrin (0.2% w/v in absolute acetone). Finally, the TLC plate was dried at 80℃ to visualize the permanent brown spots.

Analysis of MAK and ephedrine by HPLC

Analysis of MAK, (1R,2S)-ephedrine (E), and (1S,2S)-ephedrine (PE) was performed by HPLC using a ZORBAX SB C_{18} column (4.6 x 250 nm; 5 µm) at 40℃ and methanol-20 mM KH_2PO_4-acetic acid-triethyl amine (4:96:0.2:0.13, v/v/v/v) as the eluent at a flow rate of 1 ml/min. The absorbance was monitored at 210 nm. MAK, (1R, 2S)-ephedrine, and (1S, 2S)-ephedrine eluted at 32.2, 29.9, and 35.2 min, respectively. In the previous experiment, to be compared with C_{18} column, CHIRAL-AGP(50×4.0 mm, 5µm) at 30℃ and 10 mM K_3PO_4 buffer + 0.1 mM ammonium chloride as the eluent at a flow rate of 1 ml/min was used to analysis the sample and the absorbance was monitored at 213 nm. However, the CHIRAL-AGP column is not good (Dong, 2004).

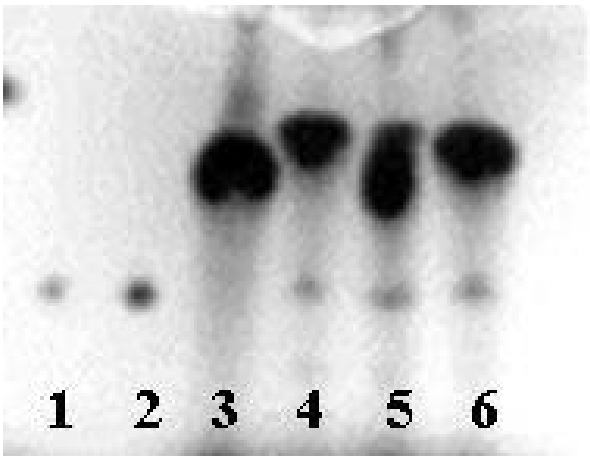
RESULTS

Screening of MAK-reducing strains

M. morganii CMCC(B) 49214, 49209, 49208, and 49203

Table 1. The effect of buffer systems and initial pH on biotransformation at 30ºC.

Buffer	Formation rate (μmol/L·h)					
	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5
Sodium phosphate buffer	0.2	3.2	12.6	20.3	26.1	24.9
Potassium phosphate buffer	0.3	4.1	15.4	29.3	26.6	26.5
Phosphate buffer	0.5	4.9	15.6	30.9	28.1	22.4
Tris buffer	0.1	2.3	11.9	16.0	6.7	1.3
Acetic acid-sodium acetate buffer	0.1	1.8	9.8	13.2	14.1	9.8



- **Figure 2**. Screening of *M. morganii* strains for the bioconversion of MAK to ephedrine by TLC. Lane 1. (1R,2S) -ephedrine; lane 2. (1S,2S) -ephedrine; lane 3. final reaction mixture using *M. morganii* CMCC(B)49214; lane 4. CMCC(B)49209; lane 5. CMCC(B)49208; and lane 6. CMCC(B)49203.

were investigated as catalysts for the bioconversion of MAK to ephedrine. As shown in Figure 2, only three strains (49209, 49208, and 49203) were found to possess MAK-reducing activity to produce ephedrine [(1S, 2S) or (1R, 2S)]. However, the optical purity of the product could not be deduced.

Screening of (1S, 2S)-ephedrine producing strains

To determine which strain could produce (1S, 2S)-ephedrine, reduction reactions were carried out with *M. morganii* CMCC(B)49203, 49208, and 49209 air-dried cells. The data of HPLC showed that all the strains can produce (1S, 2S)-ephedrine; however, (1R, 2S)-ephedrine also was detected in the reaction mixtures of *M. morganii* CMCC(B)49203 or 49209. Only *M. morganii* CMCC(B)49208 provided optically pure (1S, 2S)-ephedrine via the asymmetric reduction of MAK. Optimum buffer system, initial pH and temperature in biotransformation reactionFor the sake of the study of biotransformation of MAK to (1R, 2S)-ephedrine, the proper conditions for reaction has to be determined. Thus, Effect of buffer system, initial pH and reaction temperature on (1R, 2S)-ephedrine production was studied in this paper to better improve the reaction rate as well as the yield of product, which was the ultimate goal.

The data demonstrated that the effect of buffer on biotransformation of MAK to produce (1S, 2S)-ephedrine (Table 1). The analysis of formation rate of (1R, 2S)-ephedrine during the reaction showed that phosphate-containing buffers of pH7.5 - 8.0 were more suitable for (1R, 2S)-ephedrine accumulation. Potassium was the best; meanwhile, sodium may have the synergism to potassium phosphate. Therefore, phosphate buffer with initial pH 7.5 was selected. Tables 2 and 3 indicated the effect of different reaction temperatures on biotransformation of MAK to produce (1S, 2S)-ephedrine. In terms of formation rate, it was consequently illustrated that 39℃ should be selected as the reaction temperature for biotransformation of MAK to (1S, 2S)-ephedrine in this study.Accordingly, in regard to buffer system, initial pH and temperature, it was concluded that the optimum condition for transformation is: 0.2 M phosphate buffer with initial pH 7.5 at 39℃.

Preparative-scale conversion of MAK to (1S, 2S)-ephedrine

Based on the optimum condition, preparative-scale conversion of MAK to (1S, 2S)-ephedrine was carried out with *M. morganii* CMCC(B)49208 cells and the product was analyzed by HPLC. As shown in Figure 4, the concentration of (1S, 2S)-ephedrine in the reaction mixture reached 0.852 gL^{-1} (>99%) and no (1R, 2S)-ephedrine was detected after 24 h. The molar yield of (1S, 2S)-ephedrine in this conversion was 84.4%.

DISCUSSION

Dehydrogenase and reductase from whole cells are commonly used to catalyze the reduction of carbonyl groups. To exhibit catalytic activities, these enzymes require a coenzyme such as NADH or NAD(P)H from which a hydride is transferred to the substrate carbonyl carbon. For the regeneration of NAD(P)H, hydrogen sources such as glucose, ethanol, or 2-propanol are necessary to perform the reduction reaction. Therefore,

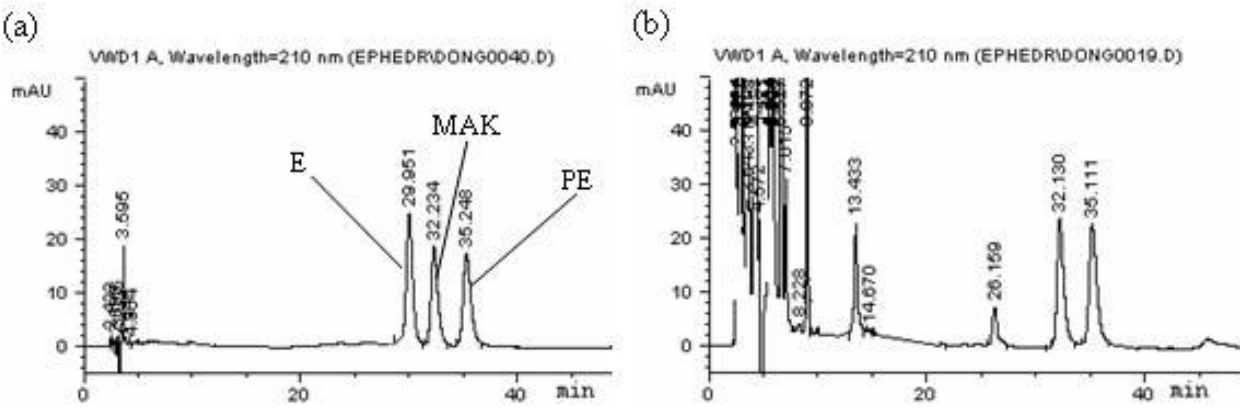


Figure 3. HPLC traces of standard samples and the reaction mixture using *M. morganii* CMCC(B) 49208 for the bioconversion of MAK to (1S, 2S) -ephedrine. (a) standard samples of (1R, 2S) -ephedrine (E), (1S, 2S) -ephedrine (PE), and MAK; (b) chemicals in the reaction mixture using *M. morganii* CMCC(B) 49208.

Table 2. The effect of reaction temperature on biotransformation (1).

Reaction temperature (ºC)	20	25	30	35	40	45	50	55	60
Formation rate (μmol/L·h)	18.1	22.1	34.9	38.2	45.0	40.8	36.3	20.0	18.4

Table 3. The effect of reaction temperature on biotransformation (2).

Reaction temperature (ºC)	35	37	39	41	43	45
Formation rate (μmol/L·h)	37.8	42.2	46.0	43.0	31.8	40.4

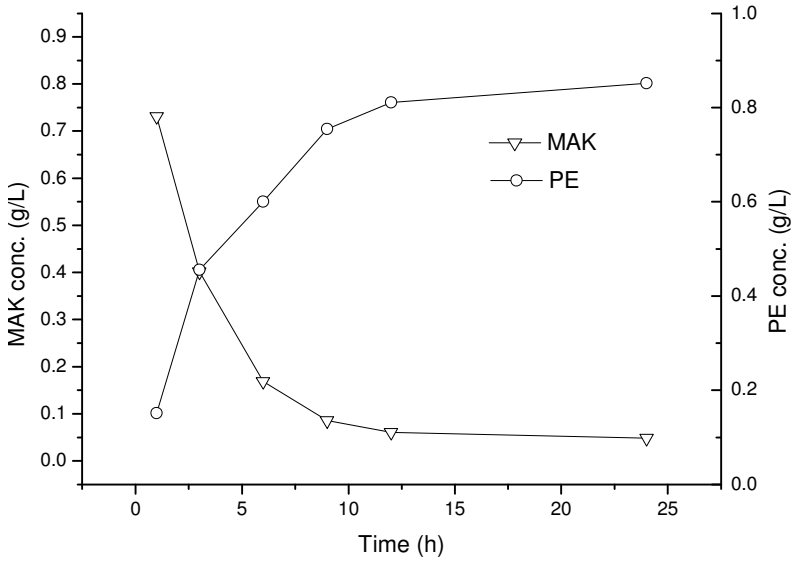


Figure 4. Preparative-scale conversion of MAK to (1S, 2S)-ephedrine using *M. morganii* CMCC(B) 49208 cells.

GDH, NADH, and glucose were added to the reaction mixture with the *M. morganii* cells for the biotransformation of MAK to ephedrine in our study. The mechanism of the biotransformation reaction is illustrated in Figure 5.

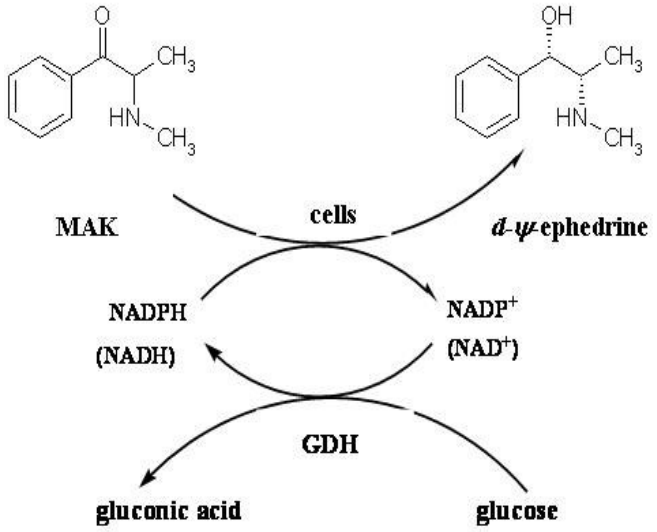


Figure 5. Biotransformation of MAK to (1S, 2S) -ephedrine by *M. morganii* CMCC(B) 49208 coupled with regeneration of NADH.

In this study, *M. morganii* CMCC(B)49208 was selected as the best producer of the enzyme that catalyzes the stereoselective reduction of MAK to (1S, 2S) -ephedrine, which is the most desirable isomer. The key enzyme of *M. morganii* CMCC(B)49208 which catalyzes the reduction of MAK seems to be a novel NAD(P)H-dependent carbonyl reductase, although the characteristics of this enzyme are still unclear. The preparative-scale reaction using *M. morganii* CMCC(B)49208 cells showed quite low molar yield of (1S, 2S)-ephedrine. The asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to ethyl (*R*)-4-chloro-3-hydroxybutanoate using *Escherichia coli* cells, which express the aldehyde reductase gene as a catalyst, produced a higher molar yield of the product with a shorter reaction time (Kataoka et al., 2006; 2008). Therefore, cloning of the gene encoding MAK reductase and overexpression of the enzyme in *E. coli* cells could make industrial enzymatic production of (1S, 2S)-ephedrine possible.

ACKNOWLEDGEMENTS

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