Screening of Microorganisms Producing Esterase for the Production of (R)-β-Acetylmercaptoisobutyric Acid from Methyl (R,S)-β-Acetylmercaptoisobutyrate

Boyapati Gokul[1], Je-Hyuk Lee[2], Ki-Bang Song[2], T. Panda[1], Sang-Ki Rhee[2], and Chul-Ho Kim*[2]

[1] Biotechnology Research Centre, Indian Institute of Technology, Madras, Chennai 600 036, India
[2] Microbial & Bioprocess Engineering Laboratory, Biotechnology Research Division, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea

Abstract (R)-β-acetylmercaptoisobutyric acid (RAM), a chiral compound, is an important intermediate for the chemical synthesis of various antihypertensive and congestive heart failure drugs. Microorganisms capable of converting (R,S)-β-acetylmercaptoisobutyric acid ((R,S)-ester) to RAM were screened from soil microorganisms. A strain of *Pseudomonas* sp. 1001 screened from a soil sample was selected to be the best. Cells showed an activity of 540 U/mL from culture broth and the enzyme was thermostable up to 70°C. This strain could produce RAM asymmetrically from (R,S)-ester.

Keywords: (R)-β-acetylmercaptoisobutyric acid, *Pseudomonas* sp., esterase, enzymatic resolution

Optically active α-alkylcarboxylic acids are important starting materials for the synthesis of pharmaceuticals. (R)-β-acetylmercaptoisobutyric acid (RAM) is one of the chiral compound which is used for the synthesis of angiotensin converting enzyme (ACE) inhibitor, such as Captopril [1] and Alacepril [2]. It has been reported that (R)-enantiomer of the mercaptoalkanoyl moiety of Captopril is about 100 times active than the (S)-enantiomer [3]. Regarding the production of RAM, many processes have been reported, like optical resolution of (R,S)-β-acetylmercaptoisobutyric acid ((R,S)-AM) by using various resolving agents [4-6]. Chemical synthesis of RAM using (R)-β-hydroxyisobutyric acid can be done, but it involves input of a large amount of energy and the reagents remain as impurities in the final product [7]. It has also been reported that chemical hydrolysis of (R,S)-β-acetylmercaptoisobutyrate ((R,S)-ester) decrease the optical purity of RAM resulting in poor yield [8]. The advantages of microbial or enzyme catalyzed reactions over chemical reactions are that the reactions are highly stereospecific, and the reactions are conducted at ambient temperature in aqueous solutions. In order to simplify the process, a biological process for the production of RAM, involving the enzymatic hydrolysis has been designed as shown in Fig. 1. In this study, we tried to isolate the microorganisms producing esterase to convert (R,S)-ester to RAM stereospecifically. (R,S)-β-acetylmercaptoisobutyric acid ((R,S)-ester) was prepared by reacting 1.0 mole of methyl methacrylate (MMA) with 1.6 mole of thioacetic acid (TA) at 80°C

for 6 hr and purified by distillation under reduced pressure [9]. All other reagents are of analytical grade and purchased from DIFCO laboratories (Detroit, MI, U.S.A.) and Sigma-Aldrich (U.S.A.). During the screening procedure, bacterial strains were cultivated on a media containing meat extract 10 g/L, peptone 10 g/L, and NaCl 5 g/L. The enzyme assay was done by adding 5 mL of the reaction mixture containing 10 g of (R,S)-ester in 1 L of 0.05 M phosphate buffer (pH 7.0) to the total cells obtained from 100 mL of culture broth, and was incubated at 37°C for 1 h with vigorous mixing and then the RAM was extracted with ethyl acetate. The enzyme unit is defined as the micromoles of product formed per unit volume of culture filtrate per unit time. The conversion of (R,S)-ester was performed by incubating the cells from 100 mL of culture broth in the same reaction mixture for a period of 24 h at 30°C. The hydrolyzed products and RAM production were determined by gas chromatography. The analysis was done using SUPELCOWAX-10 column, 30 m × 0.53 mm, 0.50 μm film (Supelco, U.S.A.). Analysis conditions for the GC were; oven temperature: 100°C; injector and detector temperature: 170°C and 180°C, respectively; the flow rate of the N_2 carrier gas: 20 m^3/min.

Microorganisms were screened from soil samples collected near the oil industries of Korea and India. The soil samples (1.0 g) were suspended in distilled water (9.0 mL), and different diluted suspensions were poured on nutrient agar plates to obtain single colonies. Small portion of cells grown on the agar plates were added to 0.2 mL of water containing 1% (w/v) of (R,S)-ester and 0.1% (w/v) of bromocresol purple as a pH indicator and incubated at 30°C for 4 hr. Strains capable of producing the enzyme hydrolyzing (R,S)-ester produce the car-

* Corresponding author
Tel: +82-42-860-4452   Fax: +82-42-860-4594
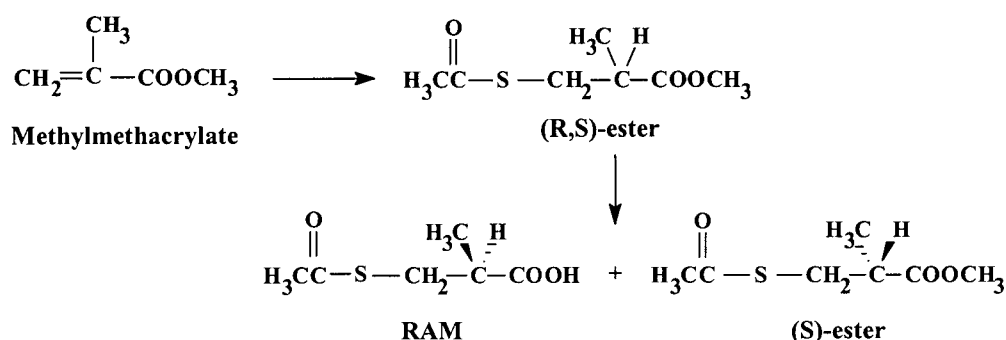e-mail: kim3641@kribb4680.kribb.re.kr

Fig. 1. Scheme for the bioconversion of (R,S)-β-acetylmercaptoisobutyrate ((R,S)-ester) to (R)-β-acetylmercaptoisobutyric acid (RAM).

boxylic acid, which changed the color of bromocresol purple from blue to yellow, due to a decrease of pH. Many of isolates could change the color of the reaction mixture and were selected as (R,S)-ester consumers [9]. However, most of these (R,S)-ester consumers not only hydrolyze the ester bonding but also the thioester bonding of the (R,S)-ester resulting in several hydrolyzed products. From the results of analysis of RAM-production from (R,S)-ester by the isolates, the colony named at IS 1001 was selected as the best RAM-producer among colonies obtained from the screening. IS 1001 has been selected after considering the fact that the thioester bonding is not hydrolyzed and further study has been done.

The strains from Korean Collection for Type Cultures (KCTC) and Agricultural Research Service (ARS Collection in United States Department of Agriculture), also referred to as the NRRL Culture Collection, were tested for the comparison of RAM-production with IS 1001. The identification of IS 1001 was been done by Korean Collection for Type Cultures (KCTC). A comparison of amount of RAM produced by IS 1001 was made with other strains with α-alkylcarboxylic acid and alkyl ester-hydrolyzing enzyme [10,11] and is given in Table 1. IS1001 strain had produced 0.14% of RAM from (R,S)-ester, but *Pseudomonas putida, Pseudomonas aeuruginosa, Pseudomonas fluorescens,* and *Acinetobactor* produced a little amounts of RAM or unable to produce RAM from (R,S)-ester.

IS 1001 strain was a rod shaped and gram-negative. Also in the chemical taxonomic analysis of the cell wall (particularly quinone is an important component in chemotaxonomic analysis), the cell was observed to contain ubiquinone-9. And in the fatty acid analysis, $C_{18:1}$ (oleic acid) and $C_{16:0}$ (palmitic acid) were found to be the major components. In addition, cell fatty acids also contain 3-hydroxy $C_{10:0}$ (capric acid) and $C_{12:0}$ (lauric acid), specially. From the above mentioned results, the strain IS 1001 was identified as a typical *Pseudomonas* sp.

Thermal stability of the enzyme in the whole cells was determined by measuring the residual enzyme activity of cells after incubation for 1 h at the desired temperature. The cells were washed twice with distilled

Table 1. Comparison of RAM production ability of various strains

Strain	RAM(%)
IS1001(this work)	0.14
Pseudomonas putida (NRRL B-955)	0.0035
Pseudomonas putida (NRRL B-727)	0.0211
Pseudomonas aeruginosa (NRRL B-14678)	0.0173
Pseudomonas putida (KCTC 1644)	0.0268
Pseudomonas putida (KCTC 2401)	0.0097
Pseudomonas putida (KCTC 2408)	0.0048
Pseudomonas aeruginosa (NRRL B-23)	-[a]
Pseudomonas putida (NRRL B-28)	-
Pseudomonas fluorescens (NRRL B-2641)	-
Pseudomonas aeruginosa (NRRL B-14781)	-
Pseudomonas ovalis (KCTC 1133)	-
Pseudomonas fluorescens (KCTC 1645)	-
Pseudomonas aeruginosa (KCTC 1750)	-
Pseudomonas putida (KCTC 2349)	-
Acinetobacter (KCTC 2702)	-

[a] No RAM production.

water and suspended in 10 mL of 0.05 M phosphate buffer (pH 7.0). The pH was adjusted to 7.0 by addition of 0.1 N NaOH and the reaction was carried out for 1 hr at the desired temperature with constant agitation. Results have shown (Fig. 2) that the enzyme was found to be thermostable up to 70°C, and maintained the same activity for a period of 1 h. It was also observed that the enzyme is highly active at 50°C and in alkaline pH.

Fermentation for the RAM-production by *Pseudomonas* sp. 1001 was carried out in a 5-L jar fermentor (Bioflo III, New Brunswick Scientific Inc., U.S.A.) with a working volume of 3.5 L and the following procedure has been used. A loop of microbial cells are inoculated in the seed medium containing 5 g/L of meat extract, 7.5 g/L of peptone, 5 g/L of glucose, 1.5 g/L of yeast extract, 1.5 g/L of malt extract, and 2.5 g/L of NaCl in a 500-mL Erlenmeyer flask containing 100 mL of medium. Ten percent of inoculum is transferred to another 100 mL seed medium having same composition as mentioned above. After the second transfer 10% inoculum is added
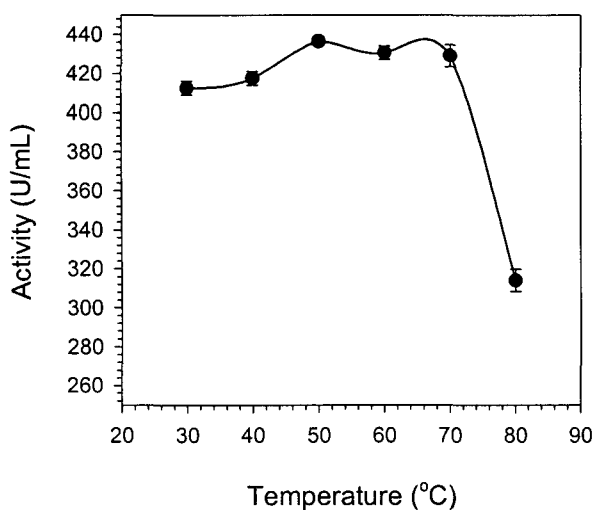
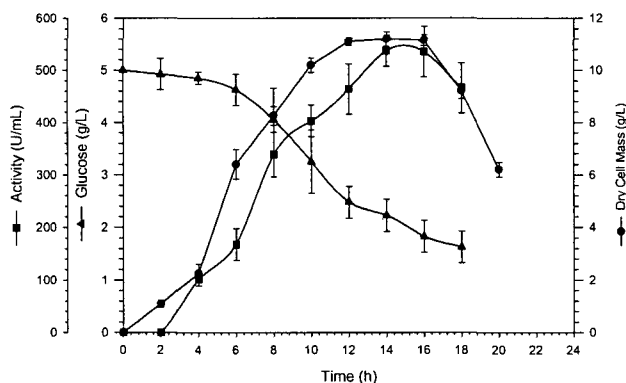Fig. 2. Thermal stability of esterase in the whole cell of *Pseudomonas* sp. 1001.

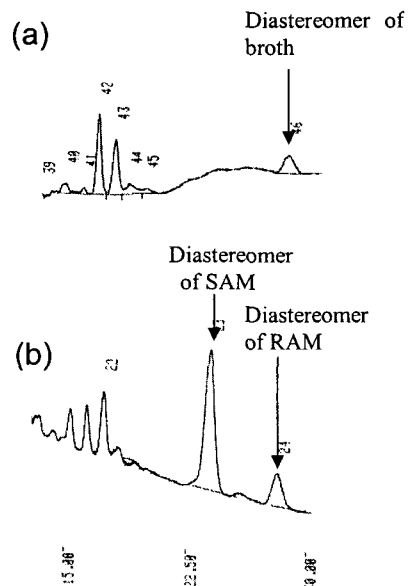Fig. 3. Esterase activity and growth curve of *Pseudomonas* sp. 1001.

Fig. 4. Gas chromatogram showing the hydrolyzed products of (R,S)-ester. (a) Diastereomer of RAM produced by the IS 1001, (b) Diastereomers of the authentic standard of SAM and RAM.

to the production medium. In case of the production medium, various media were tested as problems arose during downstream processing of the product, due to the color intensity of the final product. Therefore the medium contained 20 g/L of yeast extract, 5 g/L of casamino acids, 10 g/L of ammonium sulfate, 40 g/L of glucose, and 10 g/L of potassium phosphate. The process conditions of the fermentor were: agitation speed 500 rpm; aeration rate 4 vvm; temperature 30°C; pH controlled to 7.0 ± 0.5. The whole cells of *Pseudomonas* sp. show maximum activity after 14-16 h of cultivation as shown in Fig. 3. Traces of enzyme activity in the culture filtrate have been detected after 20 h of cultivation in fermentor. The enzyme activity of whole cells was increased after the addition of 0.5% glycine after 12 h and it gradually decreased after 20 h of cultivation. The activity of cells obtained from 1 mL of culture filtrate was found to be 540 U.

For the certification of stereomeric conformation of RAM produced by *Pseudomonas* sp. 1001, the diaste-

reomers derived from RAM have been prepared by the following method using achrial reagents. One mL of reaction mixture filtrate was evaporated to remove solvent. One mL of 10% thionyl chloride solution in 25 mL of *n*-hexane containing 2.5 μL of dimethylforamide was added to the vial, mixed thoroughly on a vortex mixer and incubated at room temperature for at least 30 min. The reagent was subsequently evaporated under a gentle stream of nitrogen at 50°C. *d*-2-Octanol (0.3 mL) was added to the dried residue, mixed thoroughly on a vortex mixer, and incubated at 60°C for 30 min in a heating block. The reactants were evaporated under a stream of nitrogen at 50°C and the residue was dissolved in 0.5 mL of methylene chloride [8]. It was determined that the strain is producing only RAM and not SAM, which was supported by GC analysis. The hydrolytic reaction of (R,S)-ester was done by the whole cells of *Pseudomonas* sp. The esterase in the whole cells of *Pseudomonas* sp. catalyzed asymmetrical hydrolysis of the ester bond of (R)-form of (R,S)-ester and converted it into RAM (Fig. 4).

The optical rotation of RAM produced by *Pseudomonas* sp. has been measured by polarimeter (POLARTRONIC, Schmidt + Haensch, Int. Co., U.S.A.). The optical rotation was found to be -44.4 ($[\alpha]^{25}_D$(C=2.00, CHCl$_2$) and the color of the product was slightly brown, presumably due to the presence of the media components. During the bioconversion of (R,S)-ester to RAM, methyl (S)-β-acetylmercaptoisobutyrate (S-ester) was also formed as shown in Fig. 1. The ester bond of (S)-ester decreased the optical purity of RAM.

From the results shown above, we could conclude that enzyme has asymmetrical hydrolytic activity towards the ester bonding of the (R)-form of (R,S)-ester

and having no activity towards the thioester bonding. The asymmetric hydrolysis of α-alkylcarboxylic acid alkyl ester using enzymes and microorganisms was reported previously [11] and only a few reports exist on the asymmetric hydrolysis of (R,S)-ester, which is also α-alkylcarboxylic acid alkyl ester [12].

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