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Screening of *Exiguobacterium acetylicum* from Soil Samples Showing Enantioselective and Alkalotolerant Esterase Activity

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Abstract About 3,000 bacterial colonies with esterase activities were isolated from soil samples by enrichment culture and halo-size on Luria broth-tributyrin (LT) plates. The colonies were assayed for esterase activity in microtiter plates using enantiomerically pure (*R*)- and (*S*)-2-phenylbutyric acid resorufin ester (2PB-O-res) as substrates. Two enantioselective strains (JH2 and JH13) were selected by the ratio of initial rate of hydrolysis of enantiomerically pure (*R*)- and (*S*)-2-PB-O-res. When cell pellets were used, both strains showed high apparent enantioselectivity (E_{app}>100) for (*R*)-2PB-O-res and were identified as *Exiguobacterium acetylicum*. The JH13 strain showed high esterase activity on *p*-nitrophenyl acetate (*p*NPA), but showed low lipase activity on *p*-nitrophenyl palmitate (*p*NPP). The esterase was located in the soluble fraction of the cell extract. The crude intracellular enzyme preparation was stable at a pH range from 6.0 to 11.0.

Keywords: alkalotolerant, enantioselective, esterase, *Exiguobacterium acetylicum*, screening

INTRODUCTION

Many chemical intermediates of fine chemical industries, including drug and pesticide production, are ester compounds [1-3]. These compounds can be simply, efficiently, and sometimes enantioselectively produced by either lipases or esterases. The most notable differences between the two enzymes are substrate selectivity in relation to carboxylic moiety chain length and the enzyme activity at the oil-aqueous interface. While lipases show high activity for long-chain fatty acid esters and above critical micelle concentration (CMC), esterases typically show high activity for short-chain fatty acid esters and do not show any interfacial activation [4]. Therefore, unlike lipases, esterases are able to catalyze the hydrolysis of water-soluble substrates, but also they can also similarly catalyze the hydrolysis of water-insoluble substrates in organic phase [5]. Although lipase is commonly used in the chemical industry because of its high activity, high enantioselectivity, and broad substrate specificity, it has some limitations in applications involving water-soluble short-chain esters [6].

Compared to lipase, esterase is a member of a relatively under-investigated enzyme group. However, esterases have considerable potential for use as a catalyst in industrial processes, especially for a) resolution of racemic mixtures for the preparation of chiral building blocks [7-10], b) synthesis of flavor esters for the food industry [11], and c) modification of physicochemical properties of triglycerides for structured lipids [12]. Microbial esterases are more useful in industry than those obtained from mammalian sources due to their relatively easy cloning and large-scale production potential. Although a large number of microbial esterases have been studied and characterized [13-16], few kinetic resolution processes using microbial esterases have been developed. The major drawback to using microbial esterases is their low enantioselectivity. Therefore, it is important that screening of new esterases with high enantioselectivity be carried out in order to develop new industrial applications.

Chiral aryl carboxylic acids are valuable, but these synthetic intermediates, used to prepare a range of compounds of biological interest, are not easy to chemically obtain. For example, 2-arylpropionic acid derivatives are an important subgroup within the class of non-steroidal anti-inflammatory drugs (NSAIDs) [17]. Chiral β-arylcarboxylic acids have been used for the preparation of chiral 3-alkylindanones and the corresponding dihydrocoumarins [18]. Additionally, chiral aryl carboxylic acids can be further transformed into carboxylic acids with other functional groups such as chiral hydroxy or halogenated carboxylic acids [19]. Thus, we were interested in screening new esterases with high enantioselectivity for the aryl carboxyl esters. In this paper, the results of the screening of such an esterase from a soil-microorganism are introduced, and the enantioselectivity of one successful example toward various racemic carboxyl acid esters is reported.

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MATERIALS AND METHODS

Chemicals and Culture Medium

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA), and Aldrich (Milwaukee, WI, USA) at the highest available purity. Methyl carboxylates were synthesized *via* common procedures using methanol and H_2SO_4. Enantiomerically pure 2-phenylbutyric acid, resorufin, 1.1 equiv. of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and 0.1 equiv. of 4-pyrrolidinopyridine were mixed in anhydrous dichloromethane. The mixture was then stirred for 30 min at 0°C and for 24 h at room temperature. The resulting mixture was washed with brine solution, and the organic layer was dried with $MgSO_4$ and filtrated. The filtrate was concentrated and purified by chromatography to yield either (*R*)- or (*S*)-2-phenylbutyric acid resorufin ester ((*R*)- or (*S*)-2-PB-O-res). The mobile phase was a mixture of dichloromethane:ethyl ether (3:1, v/v). All other reagents and solvents used were of analytical grade.

Isolation of microorganisms was done using LT medium (LB medium + 0.5% tributyrin). A homogenizer from OMNI (Warrenton, VA, USA) was used for emulsification of tributyrin. This medium was used in the liquid form or as a solid with 1.5% agar.

Isolation of Bacterial Strains

Soil samples, obtained from land around chemical plants and composite sites, were added to 30 mL of LB medium in a shaking flask. After aerobic cultivation for 16 h at 30°C, 100 μL of culture broth were transferred to 10 mL LT medium. After culturing for 16 h at 30°C, 100 μL of broth were again diluted with 10 mL of fresh LT medium. These procedures were repeated in triplicate. Aliquots (100 μL) of the final culture broth were then spread onto 1.5% LT agar plates. After incubation for 16 h at 30°C, single colonies were isolated. Halo-forming colonies were picked up, inoculated into 3 mL LT medium, and incubated for 16 h at 30°C before plating. This cycle was repeated three times in order to obtain purified strains. The isolated strains were maintained at -78°C in 25% (w/v) glycerol.

Screening of Enantioselective Esterase

Enantioselective esterase was screened by rapid spectrophotometric measurements. Hydrolysis of pure enantiomers of (*R*)- and (*S*)-2PB-O-res liberates pink chromophores. The increase in absorbance at 572 nm was measured to calculate the initial rates of hydrolysis of each enantiomer. The apparent enantioselectivity (E_{app}) was then calculated by the ratio of these rates [20,21].

$$E_{app} = \frac{V_{max}/K_m(R)}{V_{max}/K_m(S)} \quad (1)$$

Enzyme solutions (10 μL) were mixed with 10 μL (*R*)- or (*S*)-2PB-O-res solution in 180 μL potassium phosphate buffer (50 mM, pH 7.0) and the amounts of liberated resorufin were determined at 572 nm and 30°C using a Hewlett-Packard 8453 UV-visible spectrophotometer (Palo Alto, CA, USA).

Enzyme Activity Assay

Esterase and lipase activities were spectrophotometrically determined in potassium phosphate buffer (50 mM, pH 7.0) using *p*-nitrophenyl acetate (*p*NPA, 10 mM dissolved in DMSO) and *p*-nitrophenyl palmitate (*p*NPP, 10 mM dissolved in DMSO) as substrate. After culturing for 16 h, cell pellets were harvested by centrifugation (12,000 rpm for 15 min at 4°C). Cell pellets were washed twice with phosphate buffer (50 mM, pH 7.0) and resuspended in 10 mL of the same buffer. The crude cell pellet solution (900 μL) was mixed with 100 μL *p*NPA or *p*NPP solution and the amount of liberated *p*-nitrophenol was determined at 410 nm ($\varepsilon = 14 \times 10^3\ M^{-1}cm^{-1}$) and 30°C using a Hewlett-Packard 8453 UV-visible spectrophotometer (Palo Alto, CA, USA). The esterase activity was calculated from the initial rate. The activity assay was carried out at 37°C at Ph 7.0 using the crude cell pellet solution. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol/min under the assay conditions.

The enzyme activity was determined within a pH range of 5.0 to 11.0. A reaction buffer containing 50 mM citric acid-sodium citrate (pH 5.0 to 8.0), 50 mM sodium phosphate (pH 8.0 to 10.0), and 50 mM boric acid-NaOH (pH 10.0~11.0) was used for the enzyme assay. The optimum temperature was determined in 50 mM phosphate buffer (pH 7.0) within a range of 4°C to 55°C.

Hydrolysis of Carboxylic Esters

All reactions were conducted in screw-capped glass vials in a shaking incubator (30°C, 250 rpm). The shaking speed was 250 rpm. In a typical experiment, 4 μmole of a carboxylic ester and 10 mg of whole cells were mixed in 2 mL of phosphate buffer (50 mM, pH 7.0). Aliquots were taken for HPLC or GC analysis at different time intervals. The hydrolysis was monitored by HPLC using a C18 column (Waters, Milford, MA, USA) at room temperature. The mobile phase was a mixture of water: acetonitrile: trifluoroacetic acid (90:10:0.1, v/v/v) at a flow rate of 0.8 mL/min. Substrates were detected at 210 nm. The enantiomeric excess of the substrate (ee_S) was determined by GC using a chiral column (Lipodex E, Macherey-Nagel, Duren, Germany) capable of separating (*R*)- and (*S*)-enantiomers of carboxylic esters without derivatization. *E*-values were calculated based on the enantiomeric excess of the substrate (ee_S) and the conversion (*c*), as reported by Chen and Sih [22].

$$ee_s = \frac{B-A}{B+A}, \quad E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \quad (2)$$

A: slow reacting enantiomer, B: fast reacting enantiomer

Table 1. Enzyme activity and enantioselectivity towards 2-PB-O-res of screened strains

Strain	Enzyme activity (U/mL)		Activity ratio of *p*NPA/ *p*NPP	2-PB-O-res E_{app}
	*p*NPA	*p*NPP		
JH2	42.1	1.8	23.4	>100 (*R*)
JH13	38.2	2.1	18.2	>100 (*R*)
JH23	73.2	23.6	3.1	4 (*R*)
JH29	64.1	4.3	14.9	3 (*R*)
JH35	34.5	7.8	4.4	4 (*S*)
JH52	8.4	23.3	0.36	2 (*R*)

Enzyme activity was assayed on the cell pellets.

Table 2. Localization of esterase activities in JH13

Fractions	Enzyme activity (U/mL)	
	*p*NPA	*p*NPP
Before cell breakage		
Cell pellets	38.2	2.1
Supernatant	23.4	0.3
After cell breakage*		
Precipitation	2.7	n.d.
Supernatant	13.3	0.1

* The cells were disrupted by French press.

RESULTS AND DISCUSSION

About 3,000 halo-forming colonies were picked up according to the procedure described in Materials and Methods, and incubated in 3 mL LT medium for 16 h at 30°C. The whole cells were then harvested by centrifugation, washed twice with PBS buffer (50 mM, pH 7.0), and resuspended in the same buffer. The whole cells were assayed in microtiter plates using enantiomerically pure (*R*)- and (*S*)-2-PB-O-res. Although most of the colonies showed low enantioselectivity, JH2 and JH13 showed high apparent enantioselectivity (E_{app}>100) towards (*R*)-2-PB-O-res (Table 1). By analyzing the cell fatty acid profile and partial 16S rRNA sequence from Korean Collection for Type Cultures (KCTC) (Taejon, Korea), we identified these colonies as *Exiguobacterium acetylicum*. The JH2 strain and the JH13 strain showed 97.8% and 99.2% similarities with *E. acetylicum*, respectively.

Activity of esterase is usually maximal on fatty acid esters of 2~4 carbon atoms, and lipase is more active on esters of 16~18 carbon atoms [23]. The activity of the screened enzymes on *p*NPA and *p*NPP was examined (Table 1). JH2, JH13, and JH29 showed high values (>10) of the ratio of the hydrolytic activity of *p*NPA to that of *p*NPP, indicating relatively high esterase activity. On the contrary, JH23, JH35, and JH52 showed the ratio values of 3.1, 4.4, and 0.36, respectively, suggesting lipase activity.

Activities on *p*NPA and *p*NPP were assayed for JH13 with and without cell lysis (Table 2). The activity of cell pellets on *p*NPA was higher than that of the supernatant prior to cell breakage, which suggests that the esterase is an intracellular enzyme. After cell breakout with sonicator or French press, the fraction of precipitation showed little activity on *p*NPA, sugesting that the esterase is located in the soluble fraction of the cell extract. As a means of producing the cell extract, a French press was preferable to a sonicator in terms of enzyme activity, suggesting that the esterase was thermally unstable (data not shown).

The initial rate of *p*NPA hydrolysis of the crude intra-

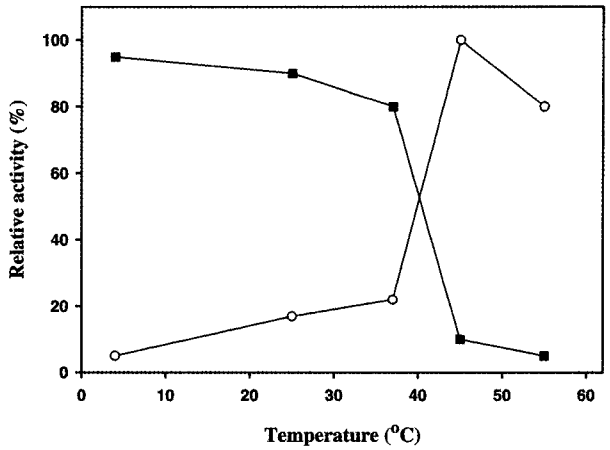

Fig. 1. Effect of temperature on activity (○) and stability (■) of the crude intracellular enzyme preparation from the screened strain (JH13). The temperature stability of the enzyme was examined by measurement of the residual activity after 5 h of incubation. Enzyme activity was measured with 1 mM *p*NPA as substrate.

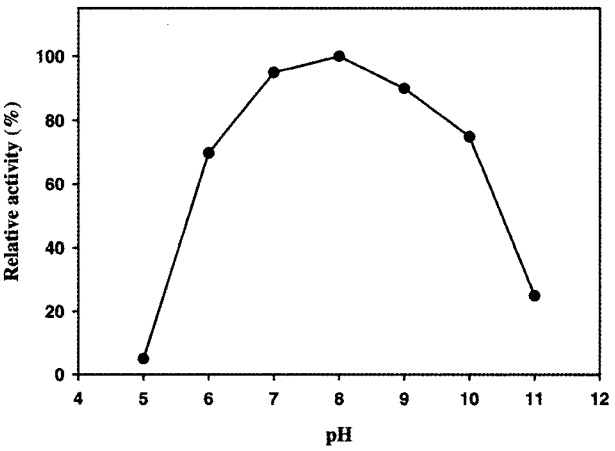

Fig. 2. Effect of pH on activity of the crude intracellular enzyme preparation from the screened strain (JH13). The buffers used were: pH 5.0~8.0, 50 mM citric acid-sodium citrate buffer; pH 8.0~10.0, 50 mM sodium phosphate buffer; pH 10.0~11.0, 50 mM boric acid-NaOH buffer. Enzyme activity was measured with 1 mM *p*NPA as substrate.

Table 3. Kinetic resolutions for various carboxylic esters by JH13 in phosphate buffer (50 mM, pH 7.0)

Substrate	Conversion (%)	ee_S (%)	E
Resorufin 2-phenylpropionate	34.0	27	4.1 (*R*)
Resorufin 2-phenylbutyrate	15.7	16	15.0 (*R*)
Resorufin 3-phenylbutyrate	17.2	4	1.5 (*R*)
Methyl 3-hydroxy butyrate	44.1	52.4	8.2 (*R*)
Methyl lactate	30.3	27.6	5.8 (*R*)
Ibuprofen methyl ester	57.8	33.4	2.2 (*R*)

Enzyme activity was assayed on the cell pellets.

cellular enzyme preparation was measured at temperatures ranging from 4 to 55°C (Fig. 1). The optimum activity was observed at approximately 45°C. However, the crude intracellular enzyme preparation is rapidly inactivated around this temperature. While the esterase activity of the crude intracellular enzyme preparation was retained for at least 5 h at pH 7.0 and 37°C, the sample incubated for 5 h at 45°C retained only 9.3% of its original activity (Fig. 1). When incubated for 30 min at 45°C, 23.1% of the original activity of the sample was retained (data not shown).

Using pNPA as a substrate, we investigated the effect of pH on the activity of the crude intracellular enzyme preparation (Fig. 2). The activity profile followed a typical bell-shaped curve with a maximum at pH 8.0. At pH 11.0 the activity was 60% of the maximum, and at pH 5.0 there was almost no activity. The crude intracellular enzyme preparation was relatively stable in alkaline solution. The pH effect on the enzyme half-life in crude cell extract was also investigated. The crude intracellular enzyme preparation for 5 h at pH 9.0 and 37°C retained 94% of its original activity. However, the sample incubated for 5 h at pH 5.0 retained only 36% of its original activity. When incubated for 5 h at pH 11.0, 82% of the original activity of the sample was retained (data not shown).

Kinetic resolutions of racemic 2-phenylpropionic acid, 2-phenylbutyric acid, 3-phenylbutyric acid, 3-hydroxybutyric acid, lactic acid, and ibuprofen esters were examined using JH13 (Table 3). JH13 showed fairly good enantioselectivity towards (*R*)-2-PB-O-res (E=15). When it is predicated that the E_{app} of JH13 towards (*R*)-2-PB-O-res is over 100, the large difference in E might come from our neglect for the competitive binding of the two enantiomers to the enzyme [20]. JH13 showed moderate enantioselectivity (E=8.2) towards methyl (*R*)-3-hydroxybutyrate. However, JH13 showed low enantioselectivities towards the other tested substrates.

In conclusion, we screened a microorganism with enantioselective esterase activity that may be interesting for the organic synthesis of chiral short-chain carboxylic acids. JH13, which was identified as *Exiguobacterium acetylicum*, is enantioselective to the (*R*) forms of chiral acids, such as (*R*)-2-PB-O-res (E=15) and methyl 3-hydroxybutyrate (E=8.2). The esterase was mainly located in the soluble fraction of the cell extract. The crude intracellular enzyme preparation was stable under alkaline conditions, but easily lost its activity at temperatures above 40°C. The alkalotolerant property is very useful for various processes such as waste treatment and textile industries which require high enzymatic activity under basic reaction conditions.

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