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Asymmetric biocatalysis of S-3-amino-3-phenylpropionic acid with new isolated *Methylobacterium* Y1-6

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ABSTRACT

β-amino acids are widely used in drug research, and S-3-amino-3-phenylpropionic acid (S-APA) is an important pharmaceutical intermediate of S-dapoxetine, which has been approved for the treatment of premature ejaculation. Chiral catalysis is an excellent method for the preparation of enantiopure compounds. In this study, we used (±)-ethyl-3-amino-3-phenylpropanoate (EAP) as the sole carbon source. Three hundred thirty one microorganisms were isolated from 30 soil samples, and 17 strains could produce S-APA. After three rounds of cultivation and identification, the strain Y1-6 exhibiting the highest enantioselective activity of S-APA was identified as *Methylobacterium oryzae*. The optimal medium composition contained methanol (2.5 g/L), 1,2-propanediol (7.5 g/L), soluble starch (2.5 g/L), and peptone (10 g/L); it was shaken at 220 rpm for 4–5 days at 30 °C. The optimum condition for biotransformation of EAP involved cultivation at 37 °C for 48 h with 120 mg of wet cells and 0.64 mg of EAP in 1 ml of transfer solution. Under this condition, substrate ee was 92.1% and yield was 48.6%. We then attempted to use *Methylobacterium* Y1-6 to catalyze the hydrolytic reaction with substrates containing 3-amino-3-phenylpropanoate ester, N-substituted-β-ethyl-3-amino-3-phenyl-propanoate, and γ-lactam. It was found that 5 compounds with ester bonds could be stereoselectively hydrolyzed to S-acid, and 2 compounds with γ-lactam bonds could be stereoselectively hydrolyzed to (-)-γ-lactam.

1. Introduction

β-amino acids are widely used in drug research [1]. One β-amino acid, the chiral building block 3-amino-3-phenylpropionic acid (APA, β-phenylalanine), is present in several bioactive molecules that can be used as anticancer agents, such as Taxol [2]. The S-APA is the intermediate of S-dapoxetine, which has been approved for the treatment of premature ejaculation [3].

Enantiomerically pure APA can be obtained via several chemical reactions, such as Lewis base-catalyzed hydrosilylation [4], stereoselective reduction [5], and rhodium-catalyzed hydrogenation [6]. But the industrialization of these reactions are not viable because of their difficult operation, low enantiomeric excess (ee), and generation of by-products [7]. In contrast, biotransformation has many advantages, including eco-friendliness, fewer by-products, and high stereoselectivity [8]. Zhang et al. attempted the biocatalytic synthesis of ethyl R-2-hydroxy-4-phenylbutyrate by using *Candida krusei*, leading to the R-enantiomer with 99.7% ee and 95.1% yield [9]. In addition, Tasnadi et al. used commercial lipases such as Lipase PS and Lipase AK to selectively hydrolyze (±)-ethyl-3-amino-3-phenylpropanoate (EAP), but the yield was markedly insufficient [10].

In the current research, we aimed to screen a microbial strain with EAP as the sole carbon source that could stereoselectively hydrolyze EAP to S-APA with high conversion and good selectivity (Scheme 1).

2. Materials and methods

2.1. Preparation of 3-amino-3-arylpropanoate ester **2a–e**

EAP **(1)** was prepared using the one-pot synthesis method[11] from benzaldehyde (30 mmol) through condensation with malonic acid (1 equiv.) and ammonium acetate (2 equivs.) in EtOH at reflux for 8 h. The white solid was filtered off and washed with cold EtOH twice. 10 mmol **1** was dissolved in $SOCl_2$ (10 equivs.) and refluxed for 2 h [11]. The solvent was evaporated off and R-OH (30 equivs.) was added into the reaction, and then heated at reflux for 6–8 h. After removing the solvent, the yellow oil was washed with petroleum ether, and **2a–e**. HCl was crystallized (Scheme 2).

2.2. Preparation of N-substituted ethyl-3-amino-3-phenylpropanoate **4a–b**

N-(Phenylacetyl)-3-amino-3-phenylpropanoic acid **(3)** was prepared according to the method proposed by Giuliana Cardillo [12]; esterification from **3** to **4a** was the same as in Section 2.1. 5 mmol **2b** was added to 10 ml of THF, and then pH was adjusted between 8 and 9 with TFA; the temperature was kept under 0 °C [13]. To this solution, 5 ml TFA containing 5 mmol di-tert-butyl dicarbonate was added slowly. The mixture was stirred at room temperature overnight and extracted with ethyl acetate twice. Organic phase pH was adjusted to 6 with 10% citric acid, and

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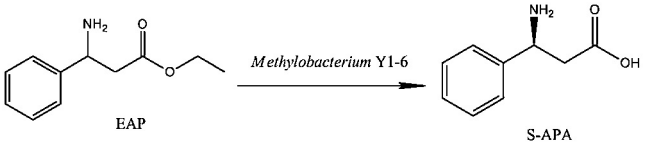


Scheme 1. *Methylobacterium* Y1-6 mediated asymmetric biotransformation of EAP to S-APA.

then the organic phase was washed with water and saturated brine. The solvent was evaporated off, and the resulting **4b** was crystallized as white acicular crystal (Scheme 2).

2.3. Soil samples and media

Soil samples used for screening were collected from different areas of several regions of China. The enrichment medium consisted of 1.6 g/L $NaH_2PO_4{\cdot}2H_2O$, 0.04 g/L $MgSO_4$, 1.24 g/L $K_2HPO_4{\cdot}3H_2O$, and 2 g/L NH_4Cl. Rich medium **1** consisted of 5 g/L olive oil, 10 g/L tryptone, 5 g/L yeast extract, 1 g/L NaCl, 3.2 mg/L $MgSO_4$, 20 mg/L $CaCl_2$, 2 g/L $(NH_4)_2SO_4$, and 2 g/L $KH_2PO_4{\cdot}3H_2O$. Rich medium **2** consisted of 7.5 g/L 1,2-propanediol, 2.5 g/L methanol, and 10 g/L tryptone.

2.4. Screening methods

In a 250 ml Erlenmeyer flask, 50 ml of MSM was inoculated with 0.1 g of soil sample, and EAP was added as the sole carbon source, giving a final substrate concentration of 1.6 g/L. After shaking at 200 rpm at 30 °C for 2–3 days, the cell supernatant was taken from the Erlenmeyer flask, diluted to 10^{-9}, and then plated onto MSM agar plates, which were further incubated at 30 °C for 4–5 days until colonies appeared. A single colony with different shapes was inoculated into MSM for the second round of screening. After 3–4 days, new cells were obtained. The isolated colonies were cultivated in rich medium **1** and shaken at 220 rpm at 30 °C for 48 h for conservation, and in rich medium **2** at 220 rpm at 30 °C for 4–5 days for cell collection. The cells were harvested and washed twice with 0.05 M phosphate buffer solution (PHS, pH 7) for biotransformation.

The reaction mixture, comprised of wet cells (100 mg) and a certain amount of EAP, was added into a 1.5 ml centrifuge tube and shaken at 220 rpm at 30 °C for 24 h. The reaction was stopped by centrifugation, and the supernatant was used for further analysis.

2.5. Analytical HPLC methods

The reaction from EAP to APA was monitored with an HPLC system (Shimadzu, Japan) equipped with a Diamonsil C18 column (0.46 mm × 250 mm × 5 μm). The HPLC conditions were as follows: methanol/0.05% TFA (1:2, v/v) as the mobile phase, a flow rate of 0.6 ml/min, and a UV detection wave at 210 nm.

The enantiomeric excess analysis was performed using an HPLC system (Shimadzu, Japan) equipped with a Shiseido chiral CD-Ph column (0.46 mm × 250 mm × 5 μm). 35 mmol KH_2PO_4: methanol (2:5, v/v) was used as the eluent at a flow rate of 0.525 ml/min, with UV detection at 210 nm. The concentration of each compound was calculated from the peak area.

The enantiomeric excess and yield were calculated as follows:

$$\text{ee}\,(\%) = \frac{|S_A - S_B|}{S_A + S_B} \times 100\%$$

where S_A and S_B are the peak areas of S-APA and R-APA, respectively, after the reaction; and

$$\text{yield}\,(\%) = \frac{S_{A0} - S_{A1}}{S_{A0} + S_{B0}} \times 100\%$$

where S_{A0} and S_{B0} are the peak areas of S-EAP and R-EAP, respectively, before the reaction, and S_{A1} is the peak area of S-EAP after the reaction.

2.6. Enzymatic activity assay

One unit (U) of enzyme activity is defined as the amount of wet cell required to hydrolyze 1 μmol of EAP in 24 h under the assay condition.

The peak area of EAP and APA in HPLC chromatogram was chosen to calculate the enzyme activity.

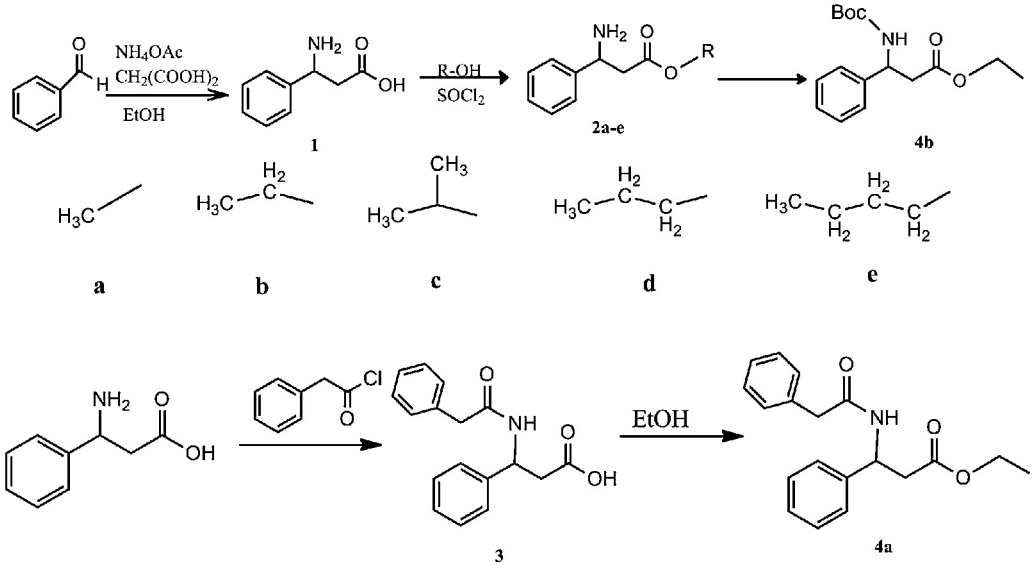
3. Results and discussion

3.1. Screening of microorganisms

In this study, more than 300 strains of microorganisms, including bacteria, yeasts, and molds, which were capable of using EAP as the sole carbon source were isolated from the MSM agar plates through two rounds of screening. Three-quarters of them could convert EAP to APA through a hydrolytic reaction. As shown in Fig. 1, 22 strains were found to be potentially useful for the preparation of S-APA, with enantioselectivity ranging from 20% to 70% (reaction time: 24 h) and improving with time. Among all the 22 strains, the strain Y1-6 showed relative stability after three-generation cultivation and gave the highest yield and higher enantioselectivity. Therefore, we chose this strain for further study.

3.2. Identification of strain Y1-6

Universal primers of bacteria (27F: 5′-AGAGTTTGATCCTGGCTCA-3′ and 1492R: 5′-AGAGTTTGATCCTGGCTCA-3′) were used to amplify the 16S rRNA gene sequence. DNA analysis was completed using BLAST network services provided by the National Center for Biotechnology Information (NCBI, United States). The sequence of Y1-6 was 99% identical with the 16SrRNA sequence of *Methylobacterium oryzae* CBMB20; therefore, the strain Y1-6 was identified



Scheme 2. Synthesis of β-amino acid ester.

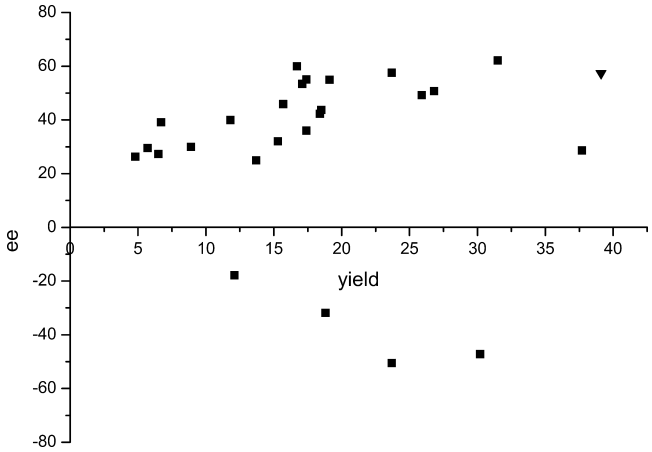


Fig. 1. The results of the second round of screening; 22 strains of microorganisms with obvious asymmetric hydrolytic activity for the production of EAP were found; ▼ represents Y1-6, and positive ee values represent S-APA absolute configuration.

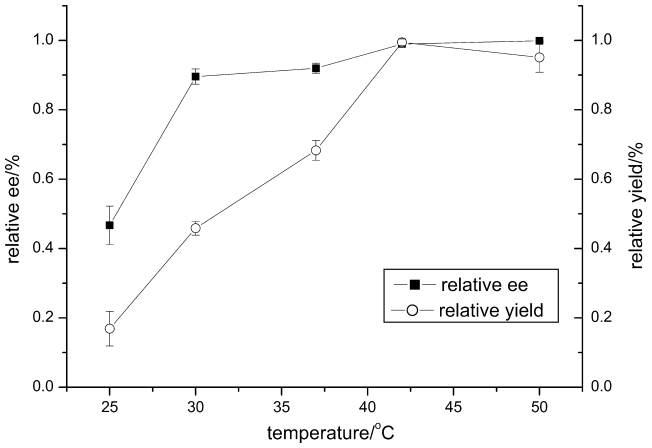


Fig. 3. The effect of temperature on the asymmetric resolution by *Methylobacterium* sp. Y1-6. Reaction condition: 100 mg wet cells, 0.64 g/L EAP in 1 ml transfer solution, shaken at 220 rpm for 48 h, with 37 °C chosen as the optimal temperature; ○ represents relative yield, and ■ represents relative ee.

as *Methylobacterium* sp. and subsequently marked as *Methylobacterium* sp. Y1-6. The colonies on the agar plate were pink, small, and smooth. A rod-shaped, Gram-negative, capsule-included bacterium based on observation via microscope, Y1-6 can use both methanol and 1,2-propanediol as carbon sources.

3.3. Optimization of reaction conditions of Y1-6

3.3.1. Optimization of reaction with time

Major parameters affecting the biotransformation mediated by *Methylobacterium* sp. Y1-6 were investigated, including reaction time, pH, and temperature. As shown in Fig. 2, the asymmetric hydrolase activity of the new isolated strain increased in parallel with the extension of reaction time, but after 50 h ee and yield of substrate were slightly decreased. Therefore, 48 h was chosen as the optimal reaction time.

3.3.2. Optimization of reaction with temperature

Generally, temperature can influence the rate and enantioselectivity of biotransformation. It can be seen from Fig. 3 that substrate ee and yield increased as temperature increased. The highest yield and substrate ee were obtained at 42 °C, but over-high temperature affected the stability of the cells, which released red pigment into the reaction buffer. Furthermore, over-high temperature led to a decrease of substrate ee and yield. Therefore, all subsequent bioreactions were carried out at 37 °C.

3.3.3. Optimization of reaction with pH

The enantioselectivity and activity of cells can be strongly influenced by initial pH and reaction temperature. The pH buffer containing 50 mM Tris, 50 mM boric acid, 33 mM citric acid, and 50 mM Na_2HPO_4 was adjusted to a pH value between 4 and 9 with either HCl or NaOH [14]. As shown in Fig. 4, under an alkaline environment (pH > 8), EAP spontaneously hydrolyzed, and the yield was insufficient. When pH was below 5, no product was obtained. Obviously, the optimal buffer pH for asymmetric resolution by *Methylobacterium* sp. Y1-6 should be 7.

3.3.4. Optimization of reaction with wet cell weight

Under the reaction condition of 100 mg of wet cells, substrate ee and yield were not higher than expected. Therefore, the impact of wet cell weight on the asymmetric resolution was tested, and the results are presented in Fig. 5. substrate ee and yield increased almost linearly with the increase of weight within the range of 60–120 mg. When the wet cell weight was higher than 120 mg, the substrate ee and yield were almost at the same level, indicating that cell weight inhibition had occurred. In the reaction system, when

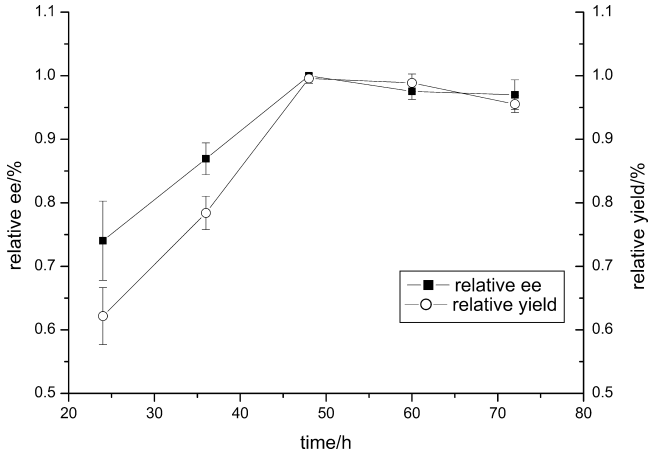


Fig. 2. The effect of reaction time on the asymmetric resolution by *Methylobacterium* sp. Y1-6. Reaction condition: 100 mg wet cells, 0.64 g/L EAP in 1 ml transfer solution, shaken at 220 rpm at 30 °C, with 48 h chosen as the optimal time; ○ represents relative yield, and ■ represents relative ee.

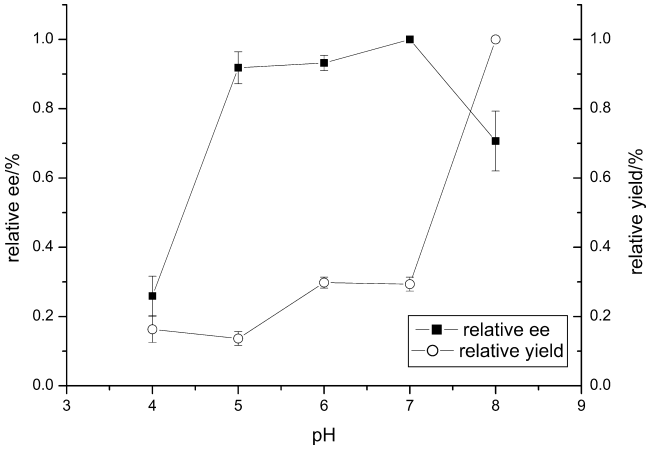
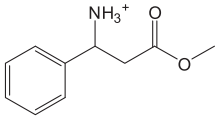
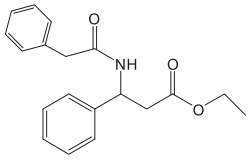
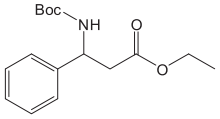
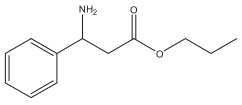
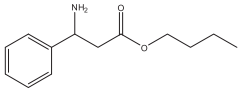
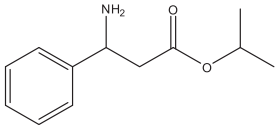
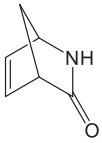
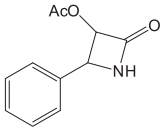
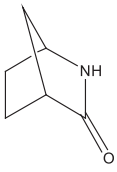
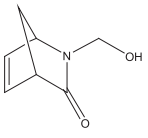


Fig. 4. The effect of pH on the asymmetric resolution by *Methylobacterium* sp. Y1-6. Reaction condition: 100 mg wet cells, 0.64 g/L **2b** in 1 ml transfer solution, shaken at 220 rpm at 37 °C for 48 h, with the pH of deionized water chosen as the optimal pH; ○ represents relative yield, and ■ represents relative ee.

Table 1
Biotransformation of different substrates (0.64 g/L) by *Methylobacterium* sp. Y1-6.

Enter	Substrate	Structure	Time	ee[a]	Yield[b]
1	Methyl-3-amino-3-phenylpropanoate		24 h	19%(S)	47%
2	Ethyl-3-(brnxoylamino)-3-phenylpropanoate		16 h	31%(S)	39%
3	Benzenepropanoic acid-β-[[(1,1-dimethylethoxy)carbonyl]amino]-ethyl ester		8 h	11.5%(S)	28%
4	Propyl-3-amino-3-phenylpropanoate		48 h	45.4%(S)	35.2%
5	Butyl-3-amino-3-phenylpropanoate		48 h	34.7%(S)	25.9%
6	Isopropyl-3-amino-3-phenylpropanoate		24 h	–	–
7	γ-Lactam		24 h	76%(+)	19%
8	3-(Acetyloxy)-4-phenyl-2-Azetidinone		12 h	65.2%	38%
9	2-Azabicyclo[2.2.1]heptan-3-one		24 h	–	–
10	2-(Hydroxymethyl)-2-Azabicyclo[2.2.1]hept-5-en-3-one		24 h	–	–

[a] ee were determined by chiral HPLC, reaction condition: 30 °C, 100 mg wet cell, 220 rpm, substrates concentration, 0.6 g/L, reaction volume, 1 ml.
[b] The yield were determined by HPLC.

140 or 160 mg of wet cells catalyzed 0.64 g/L of substrate, we can't find obvious increase of ee and yield. Therefore, the optimal wet cell weight was chosen to be 120 mg. We chose the peak area of EAP and APA in HPLC chromatogram to calculate the enzyme activity of Y1-6. The enzyme activity of Y1-6 was 22.7 U/L.

3.3.5. Effect of two-phase system and metallic ions

In addition, the asymmetric resolutions in an aqueous two-phase system and in a metallic ion solution were also tested using *Methylobacterium* sp. Y1-6. Several aqueous/organic (4:1, v/v) reaction systems were investigated, such as n-hexane, cyclohexane, methyl tert-butyl ether (MTBE), isopropyl ether, ethyl acetate, toluene and dichloromethane. The results showed that two-phase system had negative effect on yield and ee. Usually, metallic ions such as Ca^{2+} and Mg^{2+} may influence the rate of enzymatic reaction. We chose $CaCl_2$, $MgSO_4$, KCl, NaCl, $FeCl_2$ and $CuCl_2$ to prepare 10 mmol/L buffer as the reaction condition, but no significant increases in reaction rate were observed. Therefore, we

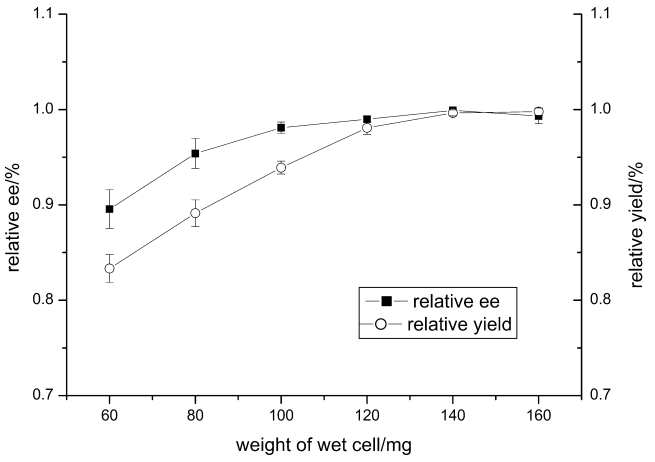


Fig. 5. The effect of wet cell weight on the asymmetric resolution by *Methylobacterium* sp. Y1-6. Reaction condition: 0.64 g/L **2b** in 1 ml transfer solution, shaken at 220 rpm at 37 °C for 48 h, with 120 mg chosen as the optimal dry cell weight; ○ represents relative yield, and ■ represents relative ee.

did not use either a two-phase or a metallic ion buffer as reaction systems.

After several tests, the optimal condition of biotransformation was determined to be as follows: aqueous solution of 0.64 g/L EAP containing 120 mg wet cells in 1 ml transfer solution at 37 °C for 48 h. Under this condition, substrate ee was 92.1% and yield was 48.6%.

3.4. Biotransformation of different substrates

To explore the application of *Methylobacterium* sp. Y1-6, 10 other substrates were examined under the same condition (Table 1). Although *Methylobacterium* sp. Y1-6 was not as efficient with other substrates containing ester bonds as it was with EAP, 5 compounds with ester bonds (Enter **1–6**) also could be stereoselectively hydrolyzed. With an increase of steric hindrance (the number of carbon atoms and the molecular weight of the N-substituted group), the yield decreased. Moreover, instability of ester bonds led to decrease of substrate ee because of spontaneous hydrolysis. Surprisingly, the strain Y1-6 contained the activity of not only esterase but also lactamase (Enter **7–10**), which can selectively degrade (+)-γ-lactam (substrate ee: 76%, yield: 19%), although the yield was insufficiently high to do so.

4. Conclusions

We have succeeded in isolating a new bacterial strain with EAP as a sole carbon source, which has high activity and ideal stereoselectivity for preparing S-APA from soil samples. This strain was identified as *Methylobacterium* sp. Y1-6. The conditions for bioreduction catalyzed by Y1-6 were optimized. Under the optimal condition (48 h, 37 °C, substrate concentration of 0.64 g/L with 120 mg of wet cells in 1 ml of transfer solution), 48.6% yield and 92.1% substrate ee were obtained. This strain can also catalyze the enantioselective hydrolysis of chiral ester and γ-lactam.

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