Catal Lett (2017) 147:837–844
DOI 10.1007/s10562-017-1999-3

Bioproduction of L-2-Aminobutyric Acid by a Newly-Isolated Strain of *Aspergillus tamarii* ZJUT ZQ013

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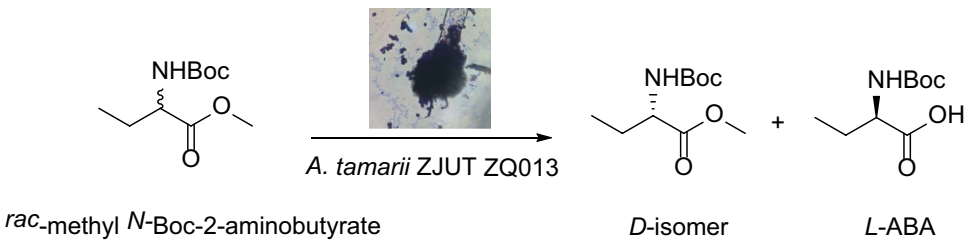
Received: 5 January 2017 / Accepted: 10 February 2017 / Published online: 28 February 2017

Abstract L-2-Aminobutyric acid (L-ABA), an unnatural amino acid, is a key intermediate of several important drugs. Although some methods have been developed to prepare pure chiral L-ABA, there are still many drawbacks, including low catalytic efficiency, cumbersome steps and high cost due to the addition of some expensive catalysts or coenzymes. Herein, with chemical and biological approaches together, we discovered a newly isolated *Aspergillus tamarii* ZJUT ZQ013 strain containing a microbial lipase which could be employed to resolve racemic methyl *N*-Boc-2-aminobutyrate to produce L-ABA with high enantioselectivity (e.e.$_s$ > 99.9%, E = 257). Moreover, the subsequent gram scale experiment confrimed that *A. tamarii* ZJUT ZQ013 could be an attractive biocatalyst for the efficient preparation of optically pure acid.

Graphical Abstract



Keywords L-2-Aminobutyric acid · Methyl *N*-Boc-2-aminobutyrate · *Aspergillus tamarii* · Enantioselective hydrolysis · Kinetic resolution

1 Introduction

L-2-Aminobutyric acid (L-ABA), an unnatural amino acid, has been used as a precursor for synthesis of many chiral drugs,such as anti-epileptic Levetiracetam, anti-tuberculotic Ethambutol and Brivaracetam [1–4]. For example, (*S*)-2-amino butanol, a key intermediate of ethambutol, can be synthesized by esterification and hydrogenation reduction starting from L-ABA [5]. Hence, due to the great market demand for L-ABA in both pharmaceutical and chemical

Electronic supplementary material The online version of this article (doi:10.1007/s10562-017-1999-3) contains supplementary material, which is available to authorized users.

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industries, the preparation of optically pure L-ABA with high efficiency has attracted much attention.

Up to now, the preparation of L-ABA is mainly achieved by chemical synthesis or enzymatic catalyst. Among the chemical methods, synthesis of L-ABA has been extensively reported including ammonolysis of α-Halogen acid [6], Reduction reaction [7], ammoniation hydrolysis reaction and butanone acid reduction [8]. But these chemical methods had many shortcomings, including poor selectivity, harsh reaction conditions, various byproducts, difficulty in separation and purification.

Compared with chemical synthesis, the biosynthetic methods aroused many interests due to their high selectivity and mild conditions. For example, L-ABA can be biochemically synthesized from the chemical L-threonine catalyzed by L-threonine deaminase and L-leucine dehydrogenase. But it needs formate as a co-substrate for NADH regeneration catalyzed by formate dehydrogenase [9]. Seo developed a fusion protein catalyst (VHb-DAAO) through binding to Vitreoscilla hemoglobin (VHb) on D-amino acid oxidase (DAAO), which can selectively catalyze D-ABA to ketoacid, while L-ABA was kept unchanged [10]. However these processes were complicated and required co-substrate or several enzymes. L-ABA could also be produced in a transamination reaction from L-threonine and L-aspartic acid catalyzed by threonine deaminase and aromatic aminotransferase. 2-Oxobutyric acid and benzylamine using ω-transaminase purified from Vibrio fluvialis JS17 was reported to produce L-ABA, but benzaldehyde, a harmful chemical to the microorganisms was also detected in the reaction [3]. Clearly, these methods have many weaknesses, such as low catalytic efficiency, cumbersome catalytic process, and the demand for expensive catalysts or coenzymes, which could not satisfy mass-production requirements [11].

To overcome the drawbacks of chemical or enzymatic methods, herein, we report a simple method to obtain the optical pure L-ABA by combining both chemical and biological methods. First, methyl *rac*-Boc-2-aminobutyric ester was synthesized from *rac*-2-aminobutyric acid. Then, a microbe named *Aspergillus tamarii* ZJUT ZQ013 was successfully screened out among hundreds strains to resolve methyl *rac*-Boc-2-aminobutyric ester and (*S*)-isomer was obtained in a high yield. Its substrate spectrum of *rac*-N-Boc-2-aminobutyric esters was further determined.

2 Materials and Methods

2.1 Materials

DL-2-aminobutyric acid (purity 99%), Di-tert-butyl dicarbonate (98% purity) and other chemicals were purchased from Aladdin Industrial Corporation.

2.2 Synthesis of Methyl *rac*-N-Boc-2-aminobutyric Acid

Synthesis of *N*-Boc-2-aminobutyric acid [12]: 10.3 g (0.1 mol) DL-2-aminobutyric acid was dissolved in the solution of 95mL 1 M NaOH solution and 65mL of methanol, and 27.5mL Di-tert-butyl dicarbonate(0.12 mol) was added in ice bath. Then the solution was warmed to room temperature and stirred for 12 h. Solvent methanol was removed on a rotary evaporator. The pH of residue was adjusted to1–2 with 1 M hydrochloric acid. The product was extracted with ethyl acetate, and then washed with saturated sodium chloride solution (50mL×3), dried over anhydrous sodium sulfate and concentrated under vacuum to obtain the DL-2-aminobutyric acid (compound **a**).

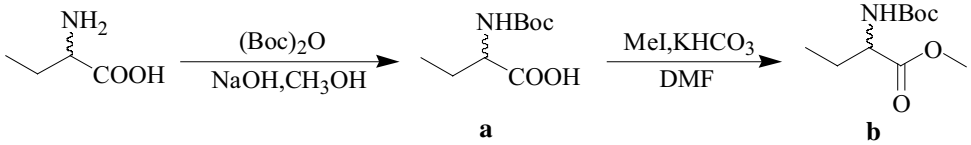
DL-*N*-Boc-2-aminobutyric acid was dissolved in DMF, and NH_4HCO_3 and CH_3I were added at room temperature. The mixture was stirred for 12 h in room temperature. The reaction solution (compound **b**) was extracted with ethyl acetate, and then washed with saturated sodium chloride solution (50mL×3), dried over anhydrous sodium sulfate and concentrated under vacuum [13] (Scheme 1).

a White solid (93.4% yield). 1H NMR (500 MHz, CDCl3) δ 4.31 (s, 1H), 1.91 (s, 1H), 1.73 (s, 1H), 1.46 (s, 9H), 0.99 (s, 3H). 13C NMR (126 MHz, CDCl3) ä 177.34, 155.64, 80.14, 54.46,27.81,25.63, 9.48.

b Light yellow liquid (84.3% yield). 1H NMR (500 MHz, CDCl3) δ4.28 (s, 1H), 3.75 (s, 3H), 1.65 (s, 2H), 1.45 (s, 9H), 0.92 (s, 3H). 13C NMR (126 MHz, CDCl3) ä 172.87, 155.09, 79.50, 54.19, 52.07, 27.81, 25.85, 9.54.

2.3 Screening of Strains and Enantioselective Hydrolysis to Methyl *rac*-*N*-Boc-2-aminobutyrate

The soil samples were collected from various locations in China, and then the strains were screened by utilizing *N*-Boc-2-aminobutyrate as a sole carbon source and selective plate with bromocresol purple indicator. Isolated strains were transferred to 150 mL fermentation medium and cultivated at 200 rpm, 30 °C for 48 h. After the cells were harvested,



Scheme 1 Synthesis of *rac*-*N*-Boc-2-aminobutyric acid and methyl *rac*-*N*-Boc-2-aminobutyric acid

1 g wet cell were washed and suspended in 5 mL 0.1 M, pH 7.0 potassium phosphate buffer (KPB). Cell suspensions were mixed with 50 μL substrate solution (*N*-Boc-2-aminobutyrate:acetone = 1:4,V/V) and kept at 200 rpm, 30 °C for 6 h reaction, the enantioselective hydrolysis of the substrate was determined by GC-MS. The screening medium contained (per liter, pH 7) 0.5 g KCl, 0.5 g $MgSO_4$•$7H_2O$, 1 g K_2HPO_4•$3H_2O$, 3 g $NaNO_3$, 0.01 g $FeSO_4$, 0.25 g Bromocresol purple, 20 g agar. Fermentation medium contained (per liter, pH 7) 0.5 g KCl, 0.5 g $MgSO_4$•$7H_2O$, 1 g K_2HPO_4•$3H_2O$, 3 g $NaNO_3$, 0.01 g $FeSO_4$, 20 g sucrose.

2.4 Identification of the *Aspergillus tamarii* ZJUT ZQ013

The isolated strain was preliminarily identified by morphological and microscopic observation. Furthermore, its 18 S-internal transcribed spacer (ITS) regions of the rDNA were obtained through gene sequencing. The 18 S-ITS region sequence was deposited in the GenBank database. Related sequences were obtained from GenBank database (National Center for Biotechnology Information) using the BLAST system. The 18 S-ITS regions determined were aligned with the reference sequences obtained from GenBank databases using ClustalW ver.1.81 [14]. MEGA ver.5.1 was applied for the calculation of evolutionary distance and finally a phylogenetic tree was constructed using the neighbor-joining method [15, 16].

2.5 Preparation of Derivatives of Methyl *rac*-*N*-Boc-2-aminobutyrate

As described in synthesis of methyl *rac*-*N*-Boc-2-aminobutyric acid, compounds **1, 6, 7, 8** were synthesized. Compounds **9**–**12** are purchased from Aladdin. The structure of compounds **1**–**12** were showed in Table 1.

The synthesis of compounds **2**–**5** was shown in Scheme 2. 5 mmol (1 eq.) *N*-Boc-2-amino butyric acid was dissolved in 30 mL dry dichloromethane, adding 0.5 mmol 4-DMAP (0.1 eq.), 6 mmol alcohols (R–OH, 1.2 eq.) and 6 mol DCC (1.2 eq.) at 0 °C. Then the reaction was allowed to warm slowly to room temperature and stirred overnight. The precipitate was removed by filtration, then washed with saturated sodium chloride solution (30mL × 2), dried over anhydrous sodium sulfate and concentrated under vacuum to get the product. Products have been purified by thin layer chromatography.

Compound **1**: Light yellow solid (78.7% yield),1 H NMR (500 MHz, $CDCl_3$) δ 5.06 (d, J = 6.9 Hz, 1 H), 4.26 (dd, J = 13.3, 7.2 Hz, 1 H), 3.73 (s, 3 H), 1.83 (q, J = 6.6 Hz, 1 H), 1.67 (dt, J = 14.2, 7.3 Hz, 1 H), 1.44 (s, 9 H), 0.92 (t, J = 7.5 Hz, 3 H).

Compound **2**: Light yellow liquid (67.1% yield),1 H NMR (500 MHz, $CDCl_3$) δ 5.06 (d, J = 7.0 Hz, 1 H), 4.27–4.19 (m, 2 H), 1.94–1.79 (m, 1 H), 1.70 (dd, J = 14.2, 7.1 Hz, 1 H), 1.28 (dd, J = 13.8, 6.6 Hz, 10 H), 0.98–0.81 (m, 6 H).

Compound **3**: Light yellow liquid (73.4% yield),1 H NMR (500 MHz, $CDCl_3$) δ 5.09 (d, J = 7.6 Hz, 1 H), 4.23 (d, J = 13.1 Hz, 1 H), 4.11–4.01 (m, 3 H), 2.23 (s, 1 H), 1.81 (dd, J = 17.2, 10.1 Hz, 2 H), 1.65 (dt, J = 14.2, 7.0 Hz, 4 H), 1.42 (s, 9 H), 1.22 (s, 2 H).

Compound **4**: Light yellow liquid (71.8% yield),1 H NMR (500 MHz, $CDCl_3$) δ5.10 (d, J = 7.7 Hz, 1 H), 4.14–4.09 (m, 2 H), 3.62 (t, J = 6.7 Hz, 2 H), 2.99 (s, 3 H), 2.18 (d, J = 6.0 Hz, 2 H), 1.64 (dq, J = 13.8, 6.8 Hz, 2 H), 1.55 (d, J = 4.3 Hz, 2 H), 1.44 (s, 9 H), 1.34 (d, J = 3.3 Hz, 3 H).

Compound **5**: Light yellow liquid (64.2% yield),1 H NMR (500 MHz, $CDCl_3$) δ 4.13–4.08 (m, 1 H), 3.61 (t, J = 6.7 Hz, 3 H), 2.03 (d, J = 6.4 Hz, 2 H), 1.63 (t, J = 11.0 Hz, 1 H), 1.58–1.50 (m, 3 H), 1.43 (s, 4 H), 1.37–1.33 (m, 1 H), 1.28 (d, J = 6.9 Hz, 9 H), 0.98–0.78 (m, 7 H).

Compound **6**: Light yellow liquid (76.8% yield),1 H NMR (500 MHz, CDCl3) ä 5.10 (s, 1 H), 4.38–4.25 (m, 1 H), 3.73 (s, 3 H), 1.43 (s, 9 H), 1.37 (d, J = 7.2 Hz, 3 H).

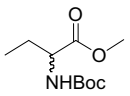
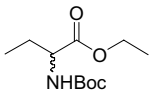
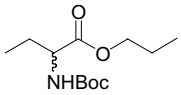
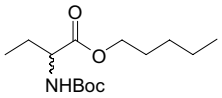
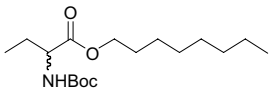
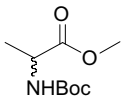
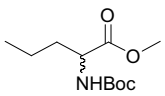
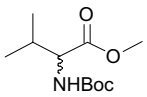
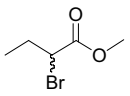
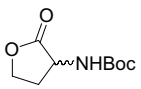
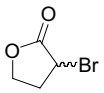
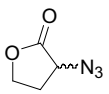
Compound **7**: Light yellow liquid (74.2% yield),1 H NMR (500 MHz, CDCl3) ä 5.03 (d, J = 7.5 Hz, 1 H), 4.29 (d, J = 5.5 Hz, 1 H), 4.11 (q, J = 7.1 Hz, 1 H), 3.73 (m, 4 H), 3.70 (s, 3 H), 1.44 (s, 9 H), 0.93 (d, J = 7.3 Hz, 3 H).

Compound **8**: Light yellow liquid (79.1% yield),1 H NMR (500 MHz, CDCl3) ä 5.04 (d, J = 8.3 Hz, 1 H), 4.21 (dd, J = 9.0, 4.8 Hz, 1 H), 3.73 (s, 3 H), 2.12 (dd, J = 12.2, 6.6 Hz, 1 H), 1.44 (s, 9 H), 0.94 (s, 3 H), 0.88 (d, J = 6.9 Hz, 3 H).

2.6 Analytical Methods

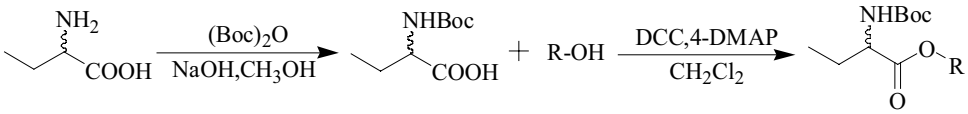
N-Boc-2-aminobutyric acid and its ester were detected and separated by GC (6890 N of Agilent) with chiral capillary column BGB-175. The initial temperature of oven was 120 °C for 2 min, 4 °C/min to 195 °C for 2 min, and the flow rate was 1mL/min. The value of $e.e._s$ is expressed as enantiomeric excess of the remaining ester, which was calculated using the following equation: $e.e._s = ([R]-[S])/([R]+[S])$. E value was enantiomeric ratios of esters, which was calculated using the following equation: $E = \ln[(1-c)(1-e.e._s)]/\ln[(1-c)(1+e.e._s)]$ (c conversion, $e.e._s$ ee of the remaining epoxide) [17].

Table 1 Enantioselective hydrolysis of methyl *rac*-N-Boc-2-aminobutyrate and its analogues

No	Substrate	Time (h)	Configuration	e.e.$_s$ (%)	e.e.$_p$ (%)	*c* (%)	E
1		6	S	99.98	99.99	50.10	>200
2		6	S	96.90	99.96	52.24	68.59
3		6	S	53.25	99.96	38.36	21.82
4		6	S	48.75	99.93	37.37	16.04
5		6	S	10.43	N.P.	19.97	2.7
6		6	S	85.91	N.P.	51.65	25.17
7		6	S	64.46	N.P.	42.64	27.24
8		6	S	19.55	99.98	25.72	4.33
9		6	S	57.11	N.P.	69.61	2.76
10		6	No reaction	0.21	N.P.	23.44	1.02
11		6	No reaction	1.90	N.P.	45.36	1.06
12		6	No reaction	1.74	N.P.	34.28	1.09

Reaction conditions 0.1 g Fungus powder, 50 μL methyl N-Boc-2-aminobutyrate, 200 μL DMF, 5 mL 0.1 M KPB (pH 7.2), 200 rpm, 30 ºC, 6 h

Scheme 2 Synthesis of derivatives of methyl *rac-N*-boc-2-aminobutyric acid



3 Results and Discussion

3.1 Screening and Identification of a Strain Producing L-ABA

We have successfully isolated more than 60 strains from soil samples using methyl *N*-Boc-2-aminobutyrate as the sole carbon source. The entioselectivity of the isolated microorgansims towards methyl *N*-Boc-2-aminobutyrate-were checked by chiral GC-MS. More than 30 strains showed (*S*)-configuration, whereas, 14 of them showed moderate and good enantioselectivity towards *R* configuration. After the D7 strain was inoculated at 30 °C for 3 days on PDA agar plate, the colonies were flat, velvety, spore dense and violet-black sclerotia in the incubator. In the process, the white colony turned to yellowish green gradually till a deep kelly or brown, with a light brown in the reverse simultaneously. The conidiophores showed a spherical shape, and spore stems are separated under the microscopic observation (Fig. 1).

The ITS sequence analysis of the ZJUT ZQ013 was further carried out [18]. The sequence data had been submitted to GenBank under the accession NO. KJ470706. A phylogenetic tree (Fig. 2) was further constructed, and this strain was closely clustered with *Aspergillus tamari* (GenBankaccession No. D63701.1), having 99% sequence

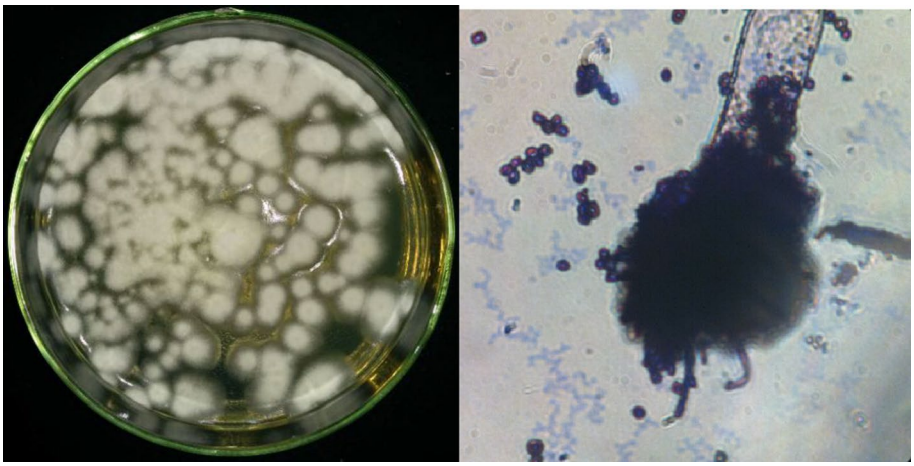


Fig. 1 The colony morphology of Strain D7 (*left*) and Microscopic observation of strain ZJUTZQ013 cultured on PDA agar plate for 48 h at 30 °C (*right*)

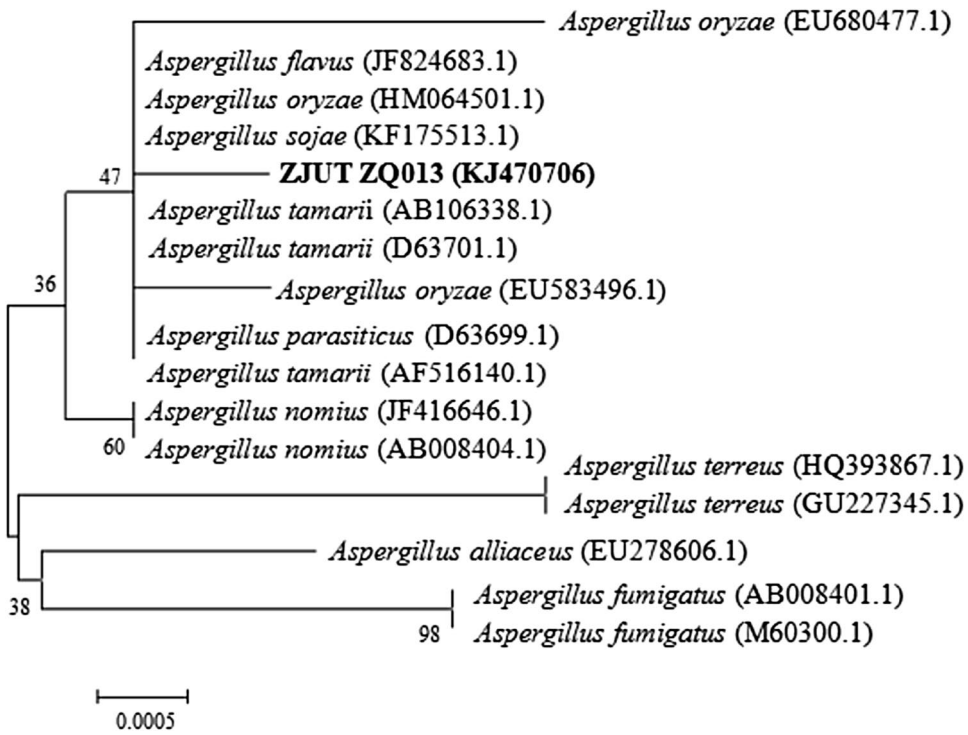


Fig. 2 The phylogenetic tree based on 18 S-ITS rDNA sequences, constructed by the neighbor-joining method, showing the relationship between strain ZJUTZQ013 and representatives of some related taxa

identity. Based on the results of phylogenetic analysis and phenotypic tests, the isolated strain ZJUT ZQ013 was designated as *Aspergillus tamarii* ZJUT ZQ013 and deposited in the China Center for Type Culture Collection (CCTCC M 2,014,046).

3.2 Optimization of Biotransformation Conditions

To obtain the optimum reaction conditions for asymmetric hydrolysis of methyl *N*-Boc-2-aminobutyrate by *Aspergillus tamarii* ZJUT ZQ013, different factors, such as buffer solution, pH, reaction time, reaction temperature, were investigated [19]. It is well known that buffer solutions and pH values can influence both of structures of enzymes and substrates, resulting in the alternation of the complex between substrates and the active site of enzymes [20–22]. As shown in Fig. 3, the rate of reaction was increased marginally with the increscent of pH when pH was below 5.6, at the cost of the activity and stereo selectivity of enzymes. Interestingly, even at the same pH, the selective catalytic activity in KPB buffer system was significantly better than Tris–HCl. Therefore, 0.1 M KPB (pH 7.2) was selected as the best enzyme catalytic reaction meidum.

The optimization of temperature was analyzed from 15 to 50 °C. As shown in Fig. 4, the e.e.$_s$ of methyl *N*-Boc-2-aminobutyrate reached the maximum 99.999% at 30 °C, and the yield of L-*N*-Boc −2-amino butyric acid was up to 45.4%.

Under the above optimized condition, the catalytic reaction time was further studied. According to Fig. 5, the e.e.$_s$ of methyl *N*-Boc-2-aminobutyrate gradually increased, it reached higher than 99% at 5.5 h. After 1 h, optical pure *N*-Boc-*L*-2- amino butyric acid was detected (e.e.$_p$ > 99.9%), and the yield was gradually increased, reaching a maximum of 45.7% at 6 h.

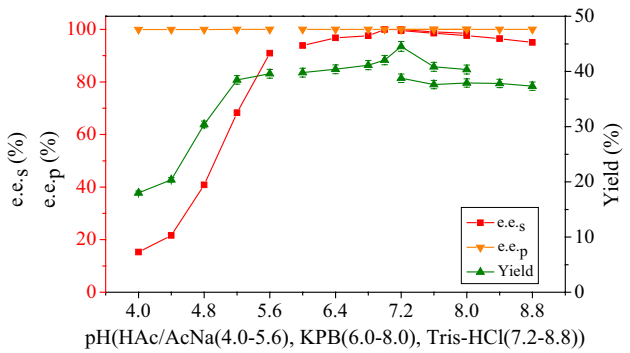


Fig. 3 Effects of buffer and pH on the catalytic reaction. Reaction conditions: 5 mL different buffer solutions (pH 4.0–5.6 HAc–NaAc, pH 6.0–8.0 Tris–HCl, pH 7.2–8.8 KPB), 0.1 g Fungus powder, 50 µL methyl N-Boc-2-aminobutyrate, 200 µL DMF, 5 mL 0.1 M KPB (pH 7.2), substrate/acetone = 1:8, 200 rpm, 6 h

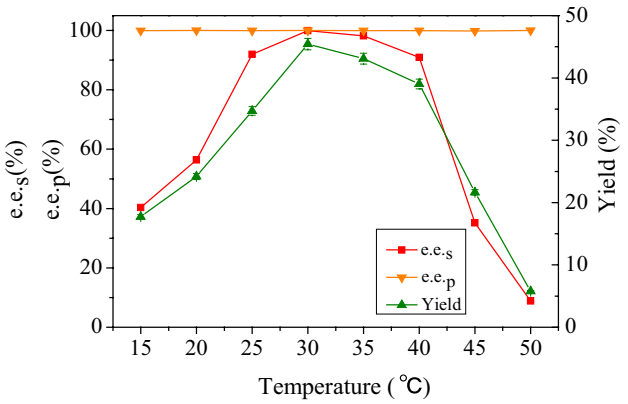


Fig. 4 Effects of temperature on the catalytic reaction. Temperature varying from 15 to 50 °C; *Reaction conditions* 0.1 g Fungus powder, 50 µL methyl N-Boc-2-aminobutyrate, 200 µL DMF, 5 mL 0.1 M KPB (pH 7.2), substrate/acetone = 1:8, 200 rpm, 6 h

3.3 Substrate Specificity of Chiral Resolution by *Aspergillus tamarii* ZJUT ZQ013

Enzymes are known to have strict selection towards their substrates [23, 24]. To explore the scopes of substrates and to better understand the interaction between the enzyme and substrate, the substrate spectrum was determined by checking the length of carbon chain on ester and types of amino acids etc. As shown in Table 1, with the extension of the carbon chain of the alcohols (**1**–**5**), the entioselectivity of *Aspergillus tamarii* ZJUT ZQ013 was gradually decreased. The bulky groups on carbon chain of the amino acids also have great impact on the catalytic activity and stereoselectivity of the organism. When a longer and branched carbon chain (**7** and **8** respectively) was introduced into the substrate, the selective catalytic ability of *Aspergillus tamarii* ZJUT ZQ013 was cut down sharply. When the Boc group

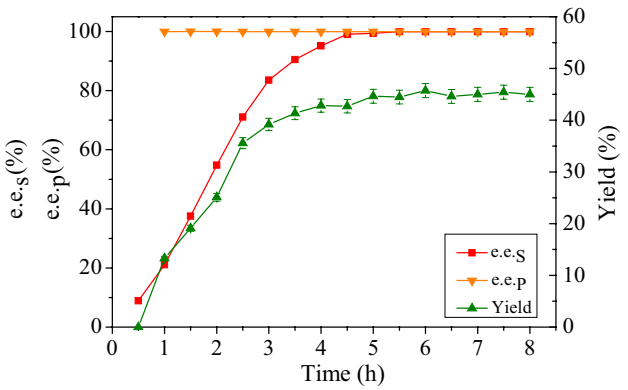


Fig. 5 Effects of reaction time on the catalytic reaction. Time varying from 0 to 8 h; *Reaction conditions* 0.1 g Fungus powder, 50 µL methyl N-Boc-2-aminobutyrate, 200 µL DMF, 5 mL 0.1 M KPB (pH 7.2), substrate/acetone = 1:8, 200 rpm, 6 h

was replaced with bromide (**9**), the enantioselectivity of *Aspergillus tamari* ZJUT ZQ013 decreased significantly (high conversion rate, low E value). The results also show that there is no enantioselectivity for γ-butyrolactone compounds (**10**–**12**), indicating that entioselectivity of *Aspergillus tamarii* ZJUT ZQ013 has strict substrate selectivity.

3.4 Preparative Scale Production of L-2-Aminobutyric Acid

To further evaluate the practical application of *Aspergillus tamarii* ZJUT ZQ013 in industry, a preparative scale resolution of rac-*N*-Boc -2-amino butyric acid was carried out in gram scale. Under the optimized resolution conditions, the enantioselective hydrolysis reaction was successfully performed at a concentration of 50 mM of rac-*N*-Boc -2-amino butyrate. Up to 2.5 g of optically pure *N*-Boc-L-2-amino butyric acid (99.9% $e.e._p$) was successfully obtained, and the protecting group Boc was removed in 20% TFA solution affording L-2-aminobutyric acid in 42% total yield. This result indicates that *Aspergillus tamarii* ZJUT ZQ013 is a powerful biocatalyst which could be employed for the efficient preparation of optically pure L-ABA.

4 Conclusion

L-ABA was an important chiral material and pharmaceutical intermediates. In this work, a newly isolated strain, *Aspergillus tamarii* ZJUT ZQ013 capable of resolving methyl *rac-N*-Boc-2-aminobutyrate to L-ABA with high enantioselectivity was reported herein. By single-factor experiment, the conditions of the kinetic resolution, such as pH, reaction time, reaction temperature were optimized. The results showed that *Aspergillus tamarii* ZJUT ZQ013 could afford an excellent enantio pure L-ABA with $e.e._s > 99.9\%$, conversion rate of 45.7%, and E value of 257, which are, to our best knowledge, the highest values. It was demonstrated that *Aspergillus tamarii* ZJUT ZQ013 was an efficient biocatalyst and has promising potential for industry application.

Acknowledgements This work was supported by National Science Foundation of China (Nos. 21272212, 21472172) and Zhejiang Natural Science Fund (Nos. LY17B060009, LY12B02018), Project of Science Technology Department of Zhejiang Province (2014C33141), and Project of Department of Science Technology Jinhua City (2013-3-003).

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