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Short communication

Biocatalytic resolution of Boc-*DL*-alanine methyl ester by a newly isolated *Bacillus amyloliquefaciens* WZZ002

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ABSTRACT

A new esterase-producing strain (*Bacillus amyloliquefaciens* WZZ002) that exhibits high hydrolytic activity, excellent enantioselectivity, and high substrate tolerance on Boc-*DL*-Alanine methyl ester was isolated from soil samples. The reaction temperature, pH, and neutralizer optima of the cell-mediated biocatalysis were 35 °C, pH 8.0, and $NH_3 \cdot H_2O$, respectively. The optimal substrate concentration was 2 M, with a biocatalyst loading of 50 g/L. Results showed that the enantiomeric excess values of substrate and product were both greater than 99%. Thus, bioprocessing with the use of the isolated strain is a promising route for the commercial production of Boc-*D*-Ala-OMe.

1. Introduction

A total of 20 canonical, proteinogenic amino acids (except glycine) have chiral α-carbons. Nature has effectively selected *L*-amino acids (*L*-AAs) to serve as building blocks of ribosome-produced polypeptides and as key metabolic intermediaries among biological systems. *L*-AAs typically have higher abundance than their mirror-image counterparts. Nonetheless, a recent work has highlighted both the abundance and potential roles of *D*-amino acids (*D*-AAs) in nature [1]. *D*-AAs are abundant in fermented beverages and foods [2,3], as well as in extracts of gut, feces, and rumen of mammals [4,5]. Moreover, *D*-AAs are also important chiral building blocks in synthesizing antibiotics, chiral auxiliaries, pharmaceuticals, food additives, and agrochemicals [6–11].

D-Alanine (*D*-Ala) is a type of unnatural amino acid that is mainly used in chiral medicine, chiral additive, and chiral auxiliaries [12]. *D*-Ala has a strong sweet taste; it is one of the sweetest amino acids that are widely used as functional food sweeteners [13]. Moreover, *D*-Ala is an important raw material for synthesizing Alitame (dipeptide sweetener). The main production methods of *D*-Ala are biological fermentation, chemical asymmetric synthesis, and enzymatic preparation. On one hand, problems such as complex separation, pollution, and high cost [14,15] are encountered in fermentation method. On the other hand, chemical method [16–18] needs either pure chiral reagent or precious metal complex as catalyst. Aminoacylase-catalyzed resolution method is currently the most commonly used method for industrialized production of *D*-Ala [19]. However, aminoacylase is relatively expensive; thus, it is only used for small-scale industrial production.

In the present study, a new esterase-producing microorganism was isolated from soil samples. It was identified as *Bacillus amyloliquefaciens*. It exhibits both an excellent enantioselectivity and a strong tolerance towards Boc-*DL*-Alanine methyl ester (Boc-*DL*-Ala-OMe), as shown in Scheme 1. Therefore, we can use it to obtain *D*-Ala through hydrolysis and to remove t-butyloxycarboryl (Boc) using Boc-*D*-Ala-OMe.

2. Materials and methods

2.1. Chemicals and reagents

The *DL*-Ala and *L*-Ala samples were purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). Boc-*DL*-Ala and its related derivatives were synthesized in our lab, which were confirmed by gas chromatography–mass spectrometer (GC–MS). All other chemicals were reagent grade (unless otherwise stated) and were obtained from commercial sources.

2.2. Microorganisms and cultivation conditions

B. amyloliquefaciens WZZ002 has been isolated from soil samples and is currently deposited in China Center for Type Culture Collection (CCTCC M 2013366) Wuhan, China. The strain was identified as *B. amyloliquefaciens* through a phylogenetic dendrogram based on 16S rDNA sequence (Fig. S1).

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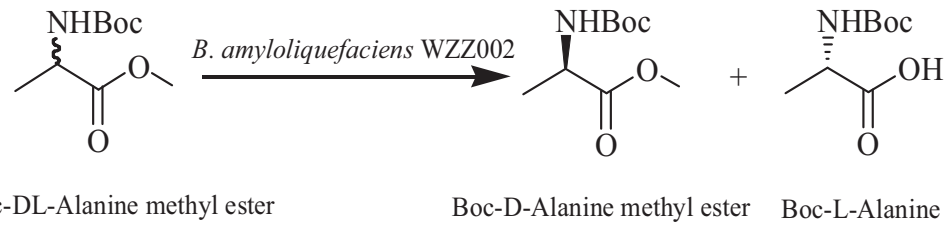

Scheme 1. The cells of *B. amyloliquefaciens* WZZ002 catalyzed resolution of Boc-DL-Alanine methyl ester.

The cell culture of *B. amyloliquefaciens* WZZ002 was placed in a 250 mL flask with 50 ml of sterile medium containing (g/L) maltose 5.0, beef extract 5.0, peptone 5.0, yeast extract 1.0, NaCl 0.5, and $MgSO_4$ 0.12 (pH 7.0). These cultivations were performed in cotton-stopped shake flasks at 30 °C, using an orbital shaker at 150 rpm, for 24 h. The cells were centrifuged at 9,000 rpm for 10 min at 4 °C. The cells were then washed with 0.85% NaCl. The microbial cells were then transferred to a 10 ml phosphate buffer (0.2 M, pH 7.0) and dried through a freeze-drying system (Christ, Osterode am Harz, Germany). The lyophilized cells of *B. amyloliquefaciens* WZZ002 were then used as biocatalysts.

2.3. Biotransformation

Enantioselective hydrolysis was performed on Boc-DL-Ala-OMe by adding both a substrate with a concentration range of 0.1 to 4.0 M and a 500 mg lyophilized cell of *B. amyloliquefaciens* WZZ002 in a 10 mL (50 mL flask) phosphate buffer solution (0.2 M, pH 6.0–12.0) at 20 °C to 60 °C. The solution was stirred at 400 rpm. The pH level was controlled through automatic titration using different alkali solutions (2 M). The samples were withdrawn at regular intervals and were immediately acidified with HCl (2 M) to stop the reaction and to enhance the extractability of Boc-DL-Ala. The sample was extracted using ethyl acetate, whereas the organic phase was isolated and dried using anhydrous Na_2SO_4 for gas chromatography (GC) analysis. All experiments were conducted in triplicate, unless specified.

The time course of enantioselective hydrolysis reaction was performed by adding 2 M of Boc-DL-Ala-OMe and 5 g of the lyophilized cell of *B. amyloliquefaciens* WZZ002 in 100 mL (250 mL flask) phosphate buffer solution (0.2 M, pH 8.0). The pH of the reaction was controlled through automatic titration using 6 M of $NH_3 \cdot H_2O$ to reduce the increasing amount of the neutralizer.

2.4. GC analysis

The enantiomeric excess values of substrate ($e.e._s$) and product ($e.e._p$) were determined through chiral GC (Agilent Technologies 6890N, USA) equipped with FID detector and chiral capillary column BGB 174 (BGB Analytik, Switzerland, 30.0 m × 250 μm × 0.25 μm film thickness) using nitrogen as a carrier gas, under the following conditions: oven temperature from 120 °C (maintained for 3 min) to 200 °C (maintained for 2 min) at 5 °C/min; injector temperature of 220 °C; and detector temperature of 220 °C. The retention times of Boc-D-Ala-OMe, Boc-L-Ala-OMe, Boc-D-Ala, and Boc-L-Ala were 14.3, 14.5, 23.3, and 23.6 min, respectively. The conversion (c) and enantiomeric ratio (E) of the substrate were calculated based on $e.e._s$ and $e.e._p$ through the method developed by Chen [20].

3. Results and discussion

3.1. Effect of pH on enantioselective hydrolysis of Boc-DL-Ala-OMe

pH is one of the most critical parameters of biotransformation [21]. Fig. 1 illustrates both the effect of pH on the conversion and the $e.e._p$ of enantioselective hydrolysis of Boc-DL-Ala-OMe, at 30 °C in the pH value range of 6.0 to 11.0. A spontaneous reaction occurred under harsh pH conditions (pH >8.0), which resulted in a remarkable decrease in $e.e._p$. As pH increased from 6.0 to 8.0, the conversion sharply increased from 19.7% to 48.3% ($e.e._s$ from 24.5% to 93.2%) and the $e.e._p$ value became greater than 99.8%. The conversion and $e.e._p$ decreased from 48.3% and 99.9% to 16.7% and 53.3%, respectively, as the pH value was increased from 8.0 to 11.0. Thus, serious spontaneous hydrolysis occurs in the reaction system under harsh pH conditions (pH > 8.0). Phosphate buffer solution (0.2 M, pH 8.0) caused the highest enantioselectivity and a higher reaction rate of *B. amyloliquefaciens* WZZ002 on hydrolytic reaction. Therefore, pH 8.0 was chosen as the favorable pH value.

3.2. Effect of temperature on enantioselective hydrolysis of Boc-DL-Ala-OMe

The thermal stability of biocatalysts is usually regarded as a major criterion for industrial applications. Moreover, temperature obviously affects the activity of biocatalyst and the thermodynamic equilibrium of the reaction [22]. The appropriate values of both the conversion and the $e.e._p$ were commonly observed at 35 °C (Fig. 2). The activity of biocatalyst improved when the temperature increased from 20 °C to 35 °C. Moreover, he maximum of both the conversion (48.9%) and the $e.e._s$ (95.6%) was reached at 35 °C. A mild rise in temperature beyond 35 °C can tardily reduce both the $e.e._s$ and the conversion. A spontaneous hydrolysis reaction occurred at high temperature conditions (T > 45 °C), which resulted in a remarkable decrease of $e.e._p$. Thus, 35 °C was established as the optimum temperature. Under optimized conditions,

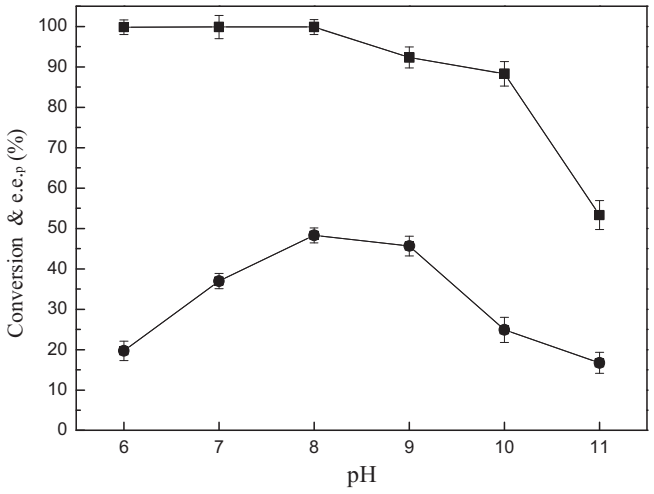

Fig. 1. Effect of pH on enantioselective hydrolysis of Boc-DL-Ala-OMe. Reaction conditions: 100 mg lyophilized cell of *B. amyloliquefaciens* WZZ002, 0.5 mmol Boc-DL-Ala-OMe in 10 mL buffer solution, 400 rpm, 30 °C, 10 min, titrated with 2 M NaOH. Phosphate buffer solution: pH 6.0–8.0; Tris–HCl buffer solution: pH 9.0; glycine–sodium hydroxide buffer solution: pH 10.0–12.0. Symbols: conversion (●); $e.e._p$ (■).

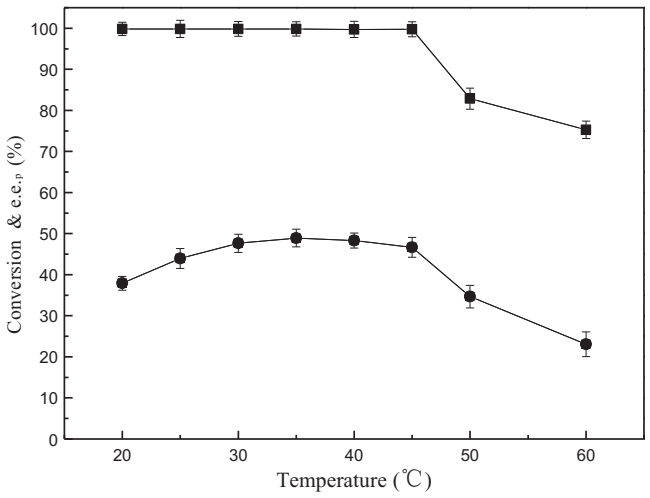

Fig. 2. Effect of temperature on enantioselective hydrolysis of Boc-*DL*-Ala-OMe. Reaction conditions: 100 mg lyophilized cell of *B. amyloliquefaciens* WZZ002, 0.5 mmol Boc-*DL*-Ala-OMe in 10 mL phosphate buffer solution (0.2 M, pH 8.0), 400 rpm, 20–60 °C, 10 min, titrated with 2 M NaOH. Symbols: conversion (●); *e.e.*$_p$ (■).

the reaction rate was increased and the enantiomeric excess of the product was maintained at a relatively high level. Moreover, the spontaneous hydrolysis of the substrate was kept at a very low level. In addition, a high *e.e.*$_p$ (>99.7%) was obtained between 20 °C and 45 °C, which demonstrates that *B. amyloliquefaciens* WZZ002 shows good enantioselectivity in a relatively broad temperature range.

3.3. Effect of neutralizer on enantioselective hydrolysis of Boc-*DL*-Ala-OMe

The pH value of the enantioselective hydrolysis reaction was controlled through automatic titration with different neutralizers. However, the use of different neutralizers will likely affect both the biocatalytic rate and enantioselectivity hydrolysis of Boc-*DL*-Ala-OMe. The *e.e.*$_p$ of Boc-*DL*-Ala-OMe was always greater than 99.7% in each neutralizer. We can also observe the maximum of *e.e.*$_p$ (99.9%) and conversion (49.6%) by using $NH_3 \cdot H_2O$ as the neutralizer (Fig. S2). A strong base (e.g., NaOH) causes inactivated biocatalyst, and either reduces the reaction rate or exacerbate the spontaneous hydrolysis reaction, as well as decreases *e.e.*$_p$. As a result, $NH_3 \cdot H_2O$ was established as the optimum neutralizer.

3.4. Optimization of the substrate concentration on enantioselective hydrolysis of Boc-*DL*-Ala-OMe

Substrate concentration is a crucial factor that needs careful investigation because it may either affect the activity of the biocatalyst or even result in substrate inhibition [23]. The time course of product concentration is described in Fig. 3a. Moreover, *e.e.*$_p$ was always 99.8% in the enantioselective hydrolysis of Boc-*DL*-Ala-OMe. The *e.e.*$_s$ continuously increased with time and was greater than 99% by the end of the enantioselective hydrolysis reaction. The initial reaction rate improved when the substrate concentration was increased from 0.1 M to 2 M and reached the maximum of 27.1 mM/min at 2 M (Fig. 3b). The initial reaction rate decreased from 27.1 mM/min to 7.2 mM/min as the substrate concentration was increased to 2 M. The optimal substrate concentration of the enantioselective hydrolysis reaction was 2 M, with a reaction of 220 min. Therefore, 2 M is the optimum substrate concentration for the biocatalyst loading of 50 g/L, which indicates that a high concentration of *B. amyloliquefaciens* WZZ002 has a potential industrial value for biocatalytic resolution of racemic substrate. Boc-*DL*-Ala-OMe is slightly soluble in water (solubility of 8.7 g/L at 25 °C, pH 8.0) (data from

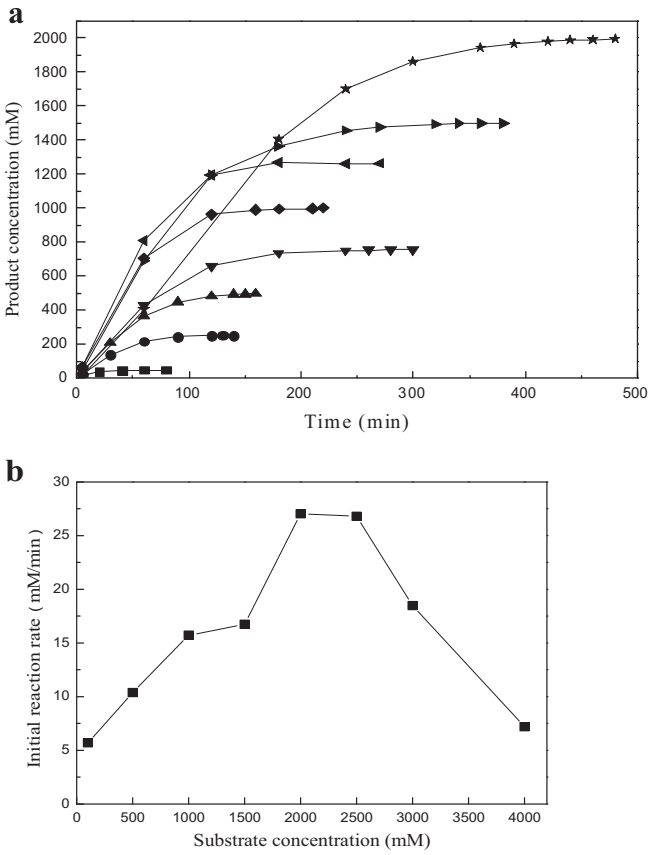

Fig. 3. Effect of substrate concentration on enantioselective hydrolysis of Boc-*DL*-Ala-OMe. *a* The time course of product concentration at different substrate concentrations. *b* The initial reaction rate of different substrate concentration. Reaction conditions: 500 mg lyophilized cell of *B. amyloliquefaciens* WZZ002, 1–40 mmol Boc-*DL*-Ala-OMe in 10 mL phosphate buffer solution (0.2 M, pH 8.0), 400 rpm, 35 °C, titrated with 2 M $NH_3 \cdot H_2O$. Symbols: 100 mM (■); 500 mM (●); 1000 mM (▲); 1500 mM (▼); 2000 mM (♦); 2500 mM (◄); 3000 mM (►); 4000 mM (★).

Scifinder Scholar). When the substrate concentration is 2 M, the biocatalytic reaction is an oil–water two phase system.

3.5. Optimized reaction course of the cells of B. amyloliquefaciens WZZ002 catalyzed biotransformation

The optimized reaction course of enantioselective hydrolysis of the Boc-*DL*-Ala-OMe in the lyophilized cells of *B. amyloliquefaciens* WZZ002 is shown in Fig. 4. The pH value was controlled through automatic titration using 6 M of $NH_3 \cdot H_2O$ to reduce the increasing amount of the neutralizer. The reaction (100 mL reaction system) was conducted under the optimal conditions of 0.2 M phosphate buffer solution (pH 8.0) at 35 °C. The substrate concentration and the increased amount of *B. amyloliquefaciens* WZZ002 were 2 M and 50 g/L, respectively. After 10 h of reaction, the conversion reached 50.1%, *e.e.*$_s$ at 99.9%, and *e.e.*$_p$ at 99.3%. Moreover, the *E* value was greater than 500. Thus, *B. amyloliquefaciens* WZZ002 has a great potential in industrial catalysis.

4. Conclusions

In the present work, *B. amyloliquefaciens* WZZ002-catalyzed enantioselective hydrolysis of Boc-*DL*-Ala-OMe was investigated. We also studied the effects of the reaction conditions (such as pH, temperature, neutralizer, and substrate concentration) on reaction activity and enantioselectivity through the enantioselective hydrolysis of

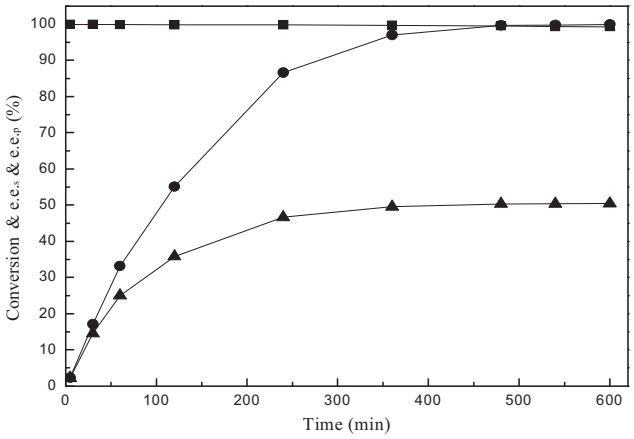

Fig. 4. Optimized reaction course of enantioselective hydrolysis of Boc-*DL*-Ala-OMe. Reaction conditions: 5 g lyophilized cell of *B. amyloliquefaciens* WZZ002, 2 M Boc-*DL*-Ala-OMe in 100 mL phosphate buffer solution (0.2 M, pH 8.0), 400 rpm, 35 °C, titrated with 6 M $NH_3 \cdot H_2O$. Symbols: conversion (▲); $e.e._s$ (●); $e.e._p$ (■).

Boc-*DL*-Ala-OMe. In our experiment, the optimal reaction conditions were pH 8.0, 35 °C, and $NH_3 \cdot H_2O$. With regard to substrate concentration, 2 M was the optimal substrate concentration for the biocatalyst loading of 50 g/L, at which the initial reaction rate reached its maximum value of 27.1 mM/min. We also investigated the time course of enantioselective hydrolysis reaction under the optimal reaction conditions. The enantiomeric excess ($e.e._s$ and $e.e._p$) values were greater than 99% at approximately 50% conversion at 2 M substrate concentration after approximately 10 h of reaction. Therefore, *B. amyloliquefaciens* WZZ002 has a potential industrial value to the resolution of Boc-*DL*-Ala-OMe with a high substrate concentration.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.catcom.2014.10.017.

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