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Kinetic resolution of N-acetyl-DL-alanine methyl ester using immobilized *Escherichia coli* cells bearing recombinant esterase from *Bacillus cereus*

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Abstract

D-alanine is widely used in medicine, food, additives, cosmetics, and other consumer items. Esterase derived from *Bacillus cereus* WZZ001 exhibits high hydrolytic activity and stereoselectivity. In this study, we expressed the esterase gene in *Escherichia coli* BL21 (DE3). We analyzed the biocatalytic resolution of N-acetyl-DL-alanine methyl ester by immobilized whole *E. coli* BL21 (DE3) cells, which were prepared through embedding and cross-linking.

We analyzed biocatalytic resolution under the optimal conditions of pH of 7.0, temperature of 40°C and substrate concentration of at 700 mM with an enantiomeric excess of 99.99% and $e.e._p$ of 99.50%. The immobilized recombinant *B. cereus* esterase *E. coli* BL21 (DE3) cells exhibited excellent reusability and retained 86.04% of their initial activity after 15 cycles of repeated reactions. The immobilized cells are efficient and stable biocatalysts for the preparation of N-acetyl-D-alanine methyl esters.

KEYWORDS

Bacillus cereus, enantioselectivity, esterase, reusability, whole-cell

1 | INTRODUCTION

D-alanine (D-Ala) is an essential amino acid that is mainly used in food, medicine, additives, and auxiliaries.[1-3] It is the sweetest amino acid and is widely used as a functional food sweetener, such as a dipeptide sweetener (2,2,4,4-tea-methylthietanylamine).[4] Moreover, D-Ala is the raw material for the production of vitamin $B6_{,}$ which has analgesic effects.[5] Recent studies have also shown that D-Ala exhibits the new physiological activities, such as such as inducing the ectopic expression of amino acid oxidase in the cytoplasm of tumor cells, preventing the oxidation of lipids in vivo.[6]

D-Ala is mainly prepared through the following processes: microbial fermentation, chemical synthesis, and enzymatic resolution.[7,8] However, microbial fermentation for D-Ala production presents several problems, such as low product concentration, high cost, and complicated separation. Two general chemical synthesis methods are used to produce D-Ala: One is direct asymmetric synthesis. The other invovles the chemical synthesis of DL-alanine compounds, which are then subjected to various optical resolution methods to yield D-Ala. The aminoacylase-catalyzed resolution method is the most commonly used method for the industrial production of D-Ala. Nevertheless, it is only used on a small-scale because of its high input requirements and low enantiomeric excess (e.e.) value.[9-11] Enzyme immobilization has been recently applied to produce D-Ala because this method is highly stable and reliable. Co-immobilized enzymes have been used to prepare optically pure D-Ala from sodium pyruvate.[12]

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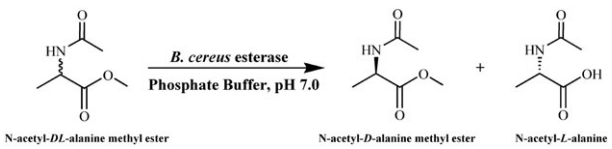
Esterase (EC 3.1.1.1) is a carboxylesterase that catalyzes ester hydrolysis. Specifically, esterases are lipases that hydrolyze lower fatty-acid esters.[13] Esterases have been applied in numerous fields and can be obtained from bacteria, such as *Streptomyces* sp., *Pseudomonas* sp., and *Aspergillus* sp., as well as from plants and animals.[14] Bacterial estersases have been derived from members of the *Bacillus* genus, including *Bacillus licheniformis* S-86, *Bacillus* sp. 4 and *Bacillus amyloliquefaciens* WZZ002.[15-17] The present work investigated the effects of different reaction parameters, such as pH, temperature, and substrate concentration, on the enzymatic-catalyzed enantio-selective hydrolysis of N-acetyl-DL-alanine methyl ester by immobilized recombinant *E. coli* BL21 (DE3) cells expressing *Bacillus cereus* esterase. Furthermore, the reusability of the immobilized recombinant cells as biocatalysts was evaluated. Results indicated that the whole recombinant *E. coli* BL21 (DE3) cells exhibit both high reusability and remarkable stereoselectivity toward N-acetyl-DL-alanine methyl ester, as shown in Scheme 1.

2 | MATERIALS AND METHODS

2.1 | Materials

N-acetyl-DL-alanine methyl ester was synthesized in our laboratory and confirmed through gas chromatography (GC)–mass spectrometery (Figure 1). N-acetyl-DL-alanine was purchased from Aladdin Chemistry Co Ltd (Shanghai, China). All other chemicals were of reagent grade (unless otherwise stated) and were obtained from commercial sources.



SCHEME 1 *Bacillus cereus* esterase catalyzed resolution of N-acetyl-DL-alanine methyl ester

2.2 | Microorganisms and cultivation conditions

B. cereus WZZ001 was isolated from soil and deposited in the China Center for Type Culture Collection (CCTCC M 2012403). The *B.cereus* esterase gene (GenBank accession number: MF111093) was ligated into pEASY-E1 vector and transformed into *E. coli* BL21 (DE3) by our laboratory. The transformed *E. coli* BL21 was cultured at 37°C in sterile medium containing 25 g/L glycerinum, 17.5 g/L peptone, 7.5 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, 11.4 g/L K_2HPO_4, 6.8 g/L KH_2PO_4, 2.0 g/L citric acid monohydrate, 3.7 g/L $MgSO_4$, and 100 mg/L ampicillin sodium. After 12 hours of incubation at 25°C when the optical density of fermentation solution at 600 nm (OD_{600}) had reached 0.6. Protein expression was induced by final concentration of 0.1 mM IPTG. Subsequently, cells were collected through centrifugation (10000 rpm for 10 min at 4°C).

2.3 | Immobilization of the whole cells of recombinant *E. coli* by embedding and cross-linking

Recombinant *E. coli* cells were firstly immobilized through embedding in carrageenan. Carrageenan 2.5% (*w*/*v*) was dissolved in distilled water at 60°C for 1 hour, then added 10% (w/v) of the cells *of E. coli* BL21 and mixed at 45°C for 5 minutes. The mixture was incubated in (3%, w/v) KCl solution at 4°C for 1 hour, and then cut into sections with dimensions of 4 mm × 4 mm × 4 mm. The sections were cross-linked with 1% (*w*/*v*) polyethylenepamine and 0.5% (w/v) glutaraldehyde for 1 hour. The immobilized cells were washed two times with distilled water. The immobilized cells were maintained in phosphate buffer (pH 7.0) at 4°Cand recovered via filtration for use as biocatalysts.

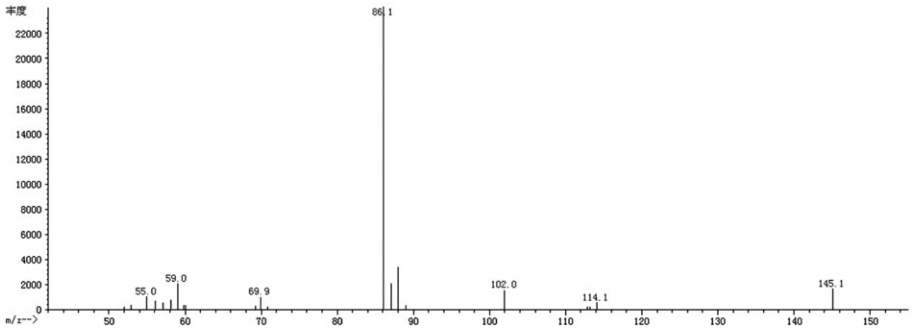


FIGURE 1 GC-MS spectrum of N-acetyl-DL-alanine methyl ester

2.4 | Biocatalytic resolution of N-acetyl-DL-alanine methyl ester

Biocatalysis was performed by adding 0.07 to 1.0 M substrate and 0.1 g immobilized cells in 10 mL (50 mL flask) of phosphate buffer solution (0.2 M, pH 7.0-9.5) at 35°C to 60°C. The solution was stirred at 400 rpm. pH was controlled through automatic titration using NaOH (1 M). Samples were withdrawn at regular intervals and immediately acidified with HCl (2 M) to stop hydrolysis. The sample was extracted using ethyl acetate. The organic phase was separated and dried using anhydrous Na_2SO_4 for GC analysis. All experiments were conducted in triplicate, unless specified.

2.5 | GC analysis

The e.e. of N-acetyl-DL-alanine methyl ester ($e.e._s$) and conversion (c) were determined through GC (Agilent 7890A) using a chiral capillary column BGB-174 (30 m × 0.25 mm × 0.25 μm). Highly pure N_2 was adopted as carrier gas and applied at a pressure of 75 kPa. Inlet port and FID detector temperatures were both 250°C, and air flow and makeup flow were 300 and 25 mL/min, respectively. The column temperature was increased from 100 (maintained for 3 min) to 200°C (maintained for 5 min). The split ratio was 50:1, and the injection volume was 1 μL. The retention times of N-acetyl-D-alanine methyl ester and N-acetyl-L-alanine methyl ester were 14.3 and 14.7 minutes, respectively, as shown by the results of GC (Figure 2). The enantiomeric ratio (*E*) was calculated on basis of the conversion and $e.e._s$.[18]

3 | RESULTS AND DISCUSSION

3.1 | Effect of buffer pH on the biocatalytic resolution of N-acetyl-DL-alanine methyl ester

pH is a key parameters of enzymatic activity because it can affect the properties of amino acid residues and the structure of the enzyme.[19] Figure 3 illustrates the effect of pH on the conversion of N-acetyl-DL-alanine methyl ester under 40°C and pH of 6.5 to 9.0. Intensive and spontaneous hydrolysis occurred in the reaction system under alkaline pH conditions (pH > 7.0). As pH increased from 6.0 to 7.0, the conversion increased from 36.8% to 46.9%. Therefore, pH 7.0 was selected as the optimal pH for the reaction.

3.2 | Effect of temperature on the biocatalytic resolution of N-acetyl-DL-alanine methyl ester

Temperature is an important factor of enzyme-catalyzed reactions in industrial application.[20,21] The appropriate values of conversion was observed at 40°C (Figure 4). Biocatalytic activity improved when temperature was increased from 35°C to 40°C. Gradually, increasing temperature to beyond 40°C mildly decreased conversion.

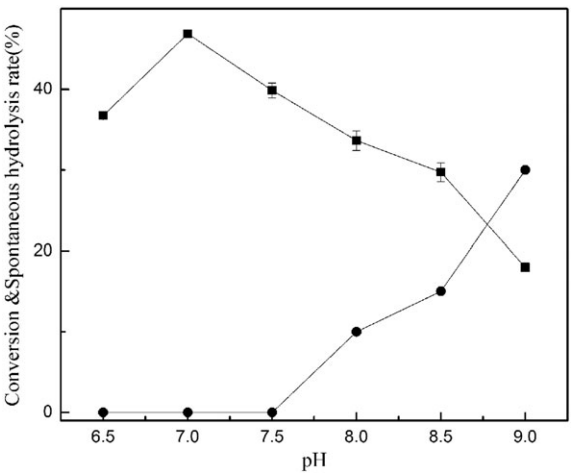


FIGURE 3 Effect of pH on enantioselective hydrolysis of N-acetyl-DL-alanine methyl ester. Reaction conditions 0.1 g immobilized cells, 70 mM N-acetyl-DL-alanine methyl ester in 10 mL buffer solution, 400 rpm, 45°C, for 10 min, and titrating with1 M NaOH. Phosphate buffer solution: pH 7.0~8.5; Tris-HCl buffer solution: pH 9.0. Symbols: spontaneous hydrolysis rate (●); enzymatic conversion (■)

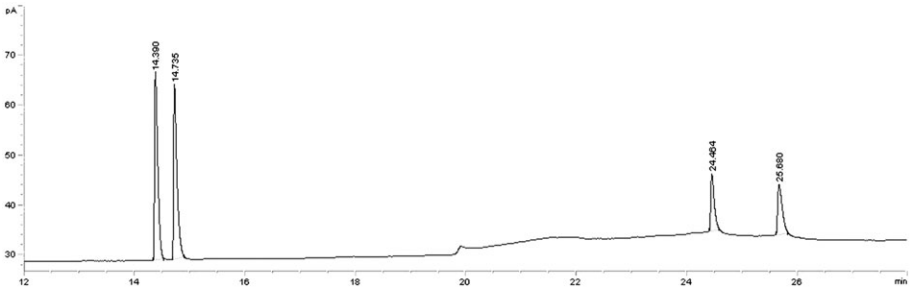


FIGURE 2 GC analysis of N-acetyl-DL-alanine methyl ester and N-acetyl-DL-alanine

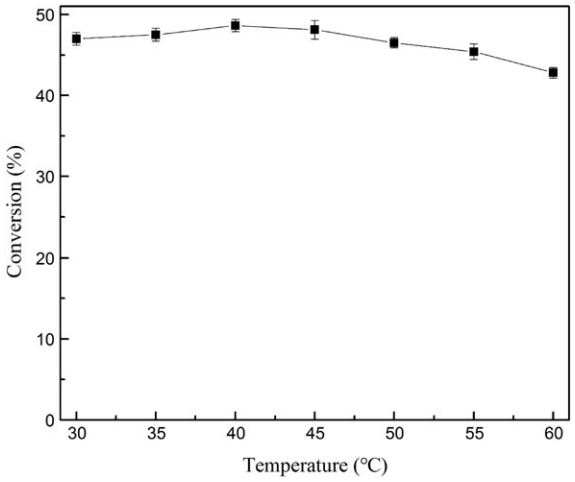


FIGURE 4 Effect of temperature on enantioselective hydrolysis of N-acetyl-DL-alanine methyl ester. Reaction conditions 0.1 g immobilized cells, 100 mM N-acetyl-DL-alanine methyl ester in 10 mL phosphate buffer solution (0.2 M, pH 7.0), 400 rpm, 35~60°C, for 10 min, and titrating with 1 M NaOH. Symbols: conversion (■)

Under optimized conditions, the reaction rate increased, and conversion rates were maintained at a relatively high level. Moreover, the spontaneous hydrolysis of the substrate was maintained at a low level.

3.3 | Effect of substrate concentration on the biocatalytic resolution of N-acetyl-DL-alanine methyl ester

Substrate concentration is an essential factor of biocatalytic reaction because it may result in substrate inhibition, which can decrease low substrate concentration and production efficiency. Moreover, the tolerance of the substrate is a crucial index of an enzyme's potential for the industrial application.[22,23] The time course of product conversion by the immobilized cells is shown in Figure 5A. When substrate concentration decreased to less than 700 mM, the enzymatic hydrolysis of N-acetyl-DL-alanine methyl ester was completed within 1 hour, with conversion rates of exceeded 40% and an $e.e._s$ value of 99%. When the substrate concentration was 700 mM, $e.e._s$ and conversion were 99.99% and 49.95% after 1 hour of reaction, respectively. The initial reaction rate increased when substrate concentration was increased from 70 mM to 700 mM and reached the maximum value of 22.75 mM/min at the substrate concentration of 700 mM (Figure 5B). The initial reaction rate decreased from 22.75 mM/min to 17.75 mM/min as substrate concentration increased from 700 mM to 1 M. Therefore, 700 mM is the most suitable substrate concentration for the biocatalyst

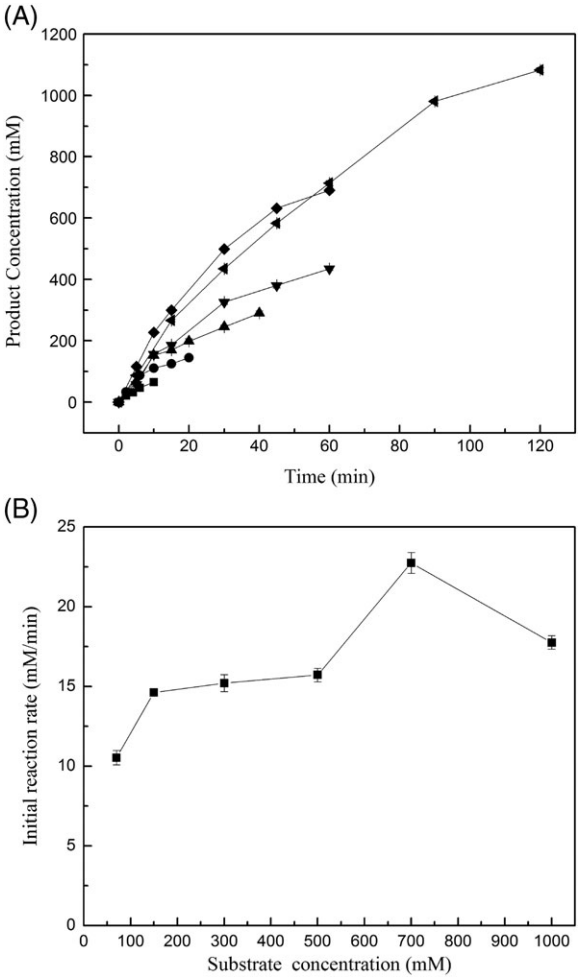


FIGURE 5 Effect of substrate concentration on enantioselective hydrolysis of N-acetyl-DL-alanine methyl ester. A, The time course of product concentration at different substrate concentration. B, The initial reaction rate of different substrate concentration. Reaction conditions 0.1 g immobilized cells, 70~1000 mM N-acetyl-DL-alanine methyl ester in 10 mL phosphate buffer solution (0.2 M, pH 7.0), 400 rpm, 40°C, and titrating with 1 M NaOH. Symbols: 70 mM (■); 150 mM (●);300 mM (▲); 500 mM (▼); 700 mM (◆); 1000 mM (◀)

loading of 10 g/L. This result indicated that the immobilized recombinant esterase has potential applications in the industrial biocatalytic resolution of N-acetyl-DL-alanine methyl ester.

3.4 | Time course of biocatalytic resolution

The time course of the enantio-selective hydrolysis of N-acetyl-DL-alanine methyl ester catalyzed by immobilized cells is shown in Figure 6. pH was controlled through automatic titration using 1 M NaOH as the neutralizer. The reaction was conducted in a 100 mL reaction system under the optimal process

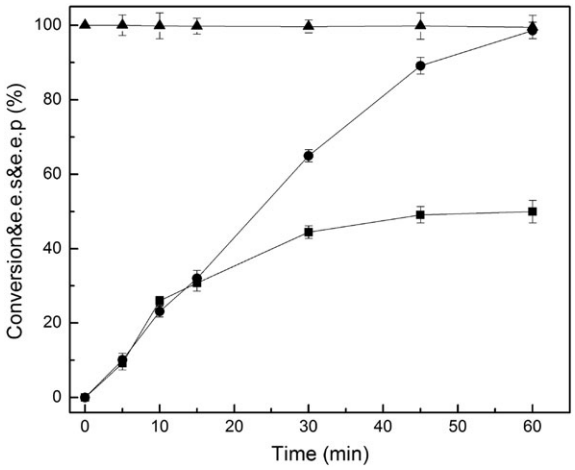


FIGURE 6 Time course of enantioselective hydrolysis of N-acetyl-DL-alanine methyl ester. Reaction conditions 1 g immobilized cells, 700 mM N-acetyl-DL-alanine methyl ester in 100 mL phosphate buffer solution (0.2 M, pH 7.0), 400 rpm, 40°C, and titrating with 1 M NaOH. Symbols: conversion (■); $e.e._s$ (●); $e.e._p$ (▲)

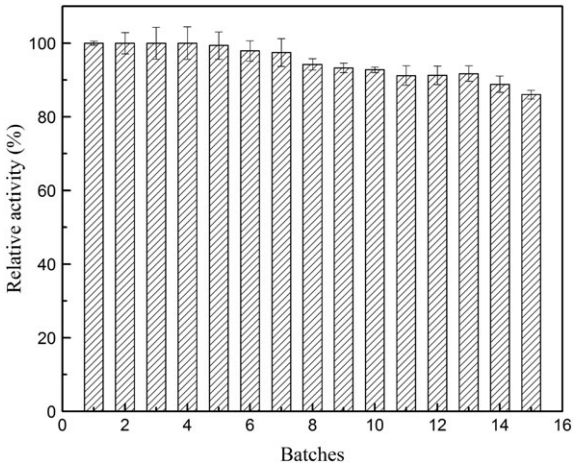


FIGURE 7 Reusability of immobilized cells on enantioselective hydrolysis reaction. Reaction conditions 1 g immobilized recombinant esterase, 700 mM N-acetyl-DL-alanine methyl ester in 100 mL phosphate buffer solution (0.2 M, pH 7.0), 400 rpm, 40°C, and titrating with 1 M NaOH

conditions of 0.2 M phosphate buffer solution at 40°C and pH 7.0. The substrate concentration and immobilized cell loading were 700 mM and10 g/L, respectively. The conversion rate and $e.e._s$ increased sharply during the first 1 minute of reaction. The conversion rate and $e.e._s$ gradually increased when the reaction duration exceeded 10 minutes. After 1 hour of reaction, the conversion rate reached 49.95% with $e.e._s$ of 99.99%, $e.e._p$ of 99.50%, and *E* ratio greater than 700. During the reaction, the $e.e._p$ always exceeded 99.50%. Thus, the immobilized recombinant esterase has potential industrial applications.

3.5 | Reusability of immobilized whole *E. coli* cells

Reusability is one of the most crucial criteria for evaluating the technological and economic feasibility production of immobilized enzyme.[24] The reusability of immobilized *E. coli* cells was studied under optimized conditions to validate the stability and recyclability of the enzyme (Figure 7). After each cycle of biocatalytic resolution, the immobilized cells were recovered from the reaction mixture through filtration and directly used in the next cycle. The residual activity of immobilized cells maintained 92.76% of its initial activity after 10 cycles of reaction, and 86.04% after 15 cycles of reaction. These results suggest that the immobilized cells can be reused for more than 10 cycles.

4 | CONCLUSION

We investigated the enzyme-catalyzed enantio-selective hydrolysis of N-acetyl-DL-alanine methyl ester by immobilized recombinant *E. coli* BL21 (DE3) cells expressing *B. cereus* esterase. Moreover, we investigated the effects of reaction conditions, specifically, pH, temperature, and substrate concentration, on the reaction. We found that the optimal reaction conditions were pH 7.0 and 40°C. The optimum substrate concentration at which the initial reaction rate reached its maximum value of 22.75 mM/min was 700 mM. Under the substrate concentration of 700 mM, the $e.e._s$ of the biocatalytic reaction exceeded 99.99%, and 49.95%conversion was achieved after approximately 1 hour of enzymatic reaction. We also investigated the reusability of immobilized recombinant esterase *E. coli* cells, which retained 86.04% of their initial activity after 15 cycles. Therefore, recombinant *B. cereus* esterase, which has high catalytic activity and enantio-selectivity, has potential industrial applications in the resolution of N-acetyl-DL-alanine methyl ester.

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