Bioorganic Chemistry 80 (2018) 174–179

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Efficient chemoenzymatic synthesis of (*S*)-α-amino-4-fluorobenzeneacetic acid using immobilized penicillin amidase

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A R T I C L E   I N F O

Keywords:
(*S*)-α-amino-4-fluorobenzeneacetic acid
Immobilized penicillin amidase
Kinetic resolution
Chemoenzymatic approach

A B S T R A C T

An efficient chemoenzymatic route was developed for synthesis of (*S*)-α-amino-4-fluorobenzeneacetic acid, a valuable chiral intermediate of Aprepitant, using immobilized penicillin amidase catalyzed kinetic resolution of racemic *N*-phenylacetyl-4-fluorophenylglycine. The optimum temperature, pH and agitation rate of the reaction were determined to be 40 °C, 9.5 and 300 rpm, respectively. Kinetic resolution of 80 g L^{-1} *N*-phenylacetyl-4-fluorophenylglycine by immobilized amidase 20 g L^{-1} resulted in 49.9% conversion and > 99.9% *e.e.* within 3 h. The unreacted *N*-phenylacetyl-4-fluorophenylglycine can be easily racemized and then recycled as substrate. The production of (*S*)-α-amino-4-fluorobenzeneacetic acid was further amplified in 1 L reaction system, affording excellent conversion (49.9%) and enantioselectivity (99.9%). This chemoenzymatic approach was demonstrated to be promising for industrial production of (*S*)-α-amino-4-fluorobenzeneacetic acid.

1. Introduction

Aprepitant is a potent and orally active antagonist of human neurokinin-1 (NK-1) receptor in the treatment of chemotherapy-induced emesis, and also has good effect to release severe major depression, pain and migraine [1–3]. The compound **6** is a critical precursor of Aprepitant (Fig. 1), and its synthesis has aroused great interest. Several synthetic routes of **6** have been reported [4–8] (Fig. 2), and most of them introduced the chiral building block via direct addition reaction, or by asymmetric synthetic methodologies. One of the most economically attractive route for synthesis of **6** starts from (*R*)-α-methylbenzylamine [1] (Scheme 1). However, this method involved a reduction step where hazardous reagents like DIBALH and $NaBH_4$ are used. Besides, the poor optical purity of intermediate compound oxazinone and the redundant procedure restricted its industrial applications. Other potential synthetic routes using *N*-benzyl ethanolamine, *p*-fluorobenzaldehyde, 3, 5-bis (trifluoromethyl)-1-vinylben or 4-fluorophenylacetic acid as raw material also have been reported [7,9–15] (Schemes 2–5). However, the dangerous reagents and precious metal catalyst used in the synthesis of key chiral block resulted in security risks and cost pressures. Comparatively, an additional approach (Scheme 6) based on the improvement of Scheme 5 with (*S*)-α-amino-4-fluorobenzeneacetic acid **4** as raw material not only reduced the procedure, but also avoided using environmental unfriendly reagents.

Asymmetric synthesis and crystallisation-induced resolution are two main types of routes reported for synthesis of **4** [11,16,17]. However, those traditional chemical processes exhibited a lengthy procedure and required the used of toxic organic reagents. By comparison, the enzymatic process has become an appealing approach for producing **4**, because of its shorter process, greener solvent, and environmental friendliness [18–23]. The transaminase mediated synthesis of **4** using prochiral keto-acid as substrate has been reported [24] (Scheme 7a). Although with excellent enantioselectivity (98% *e.e.*), substrate loading of the enzymatic reaction was low (2.3 g L^{-1}) and reaction time was long (71 h). In addition, the reversible enzymatic process requires pyridoxal 5-phosphate as co-factor and aspartate as amino donor, resulting in increased cost and incomplete conversion. Biocatalytic deracemization in the preparation of **4** from **1** using whole cells (*Nocardia Corallina* CGMCC 4.1037) containing *R*-amino acid oxidase and *S*-aminotransferase has been reported [25] (Scheme 7b). The low substrate concentration (10 mM) and relatively inferior catalytic efficiency (63% yield) restrict its industrial applications. Therefore, developing a robust biocatalysts and an efficient synthesis strategy for producing **4** is urgent. A penicillin amidase which exhibited a high hydrolytic activity and excellent enantioselectivity toward *N*-phenylacetyl-4-fluorophenylglycine, was employed as biocatalyst for enzymatic synthesis of

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https://doi.org/10.1016/j.bioorg.2018.06.020
Received 26 April 2018; Received in revised form 11 June 2018; Accepted 11 June 2018
Available online 15 June 2018
0045-2068/

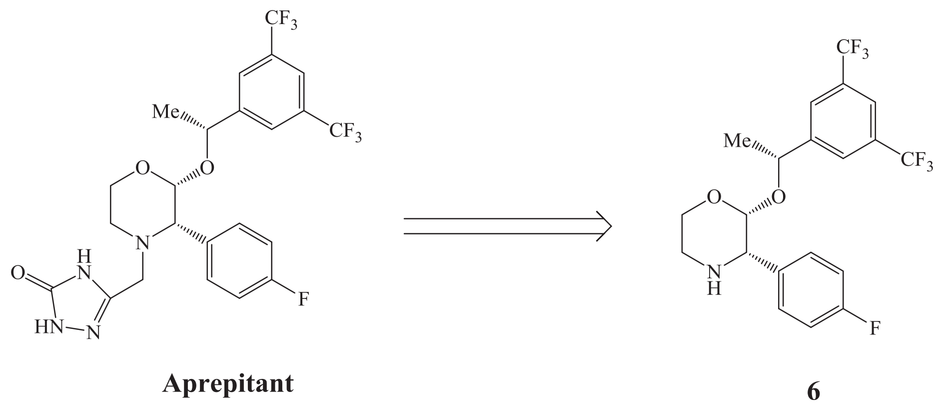


Fig. 1. Structures of Aprepitant and its chiral precursor **6**.

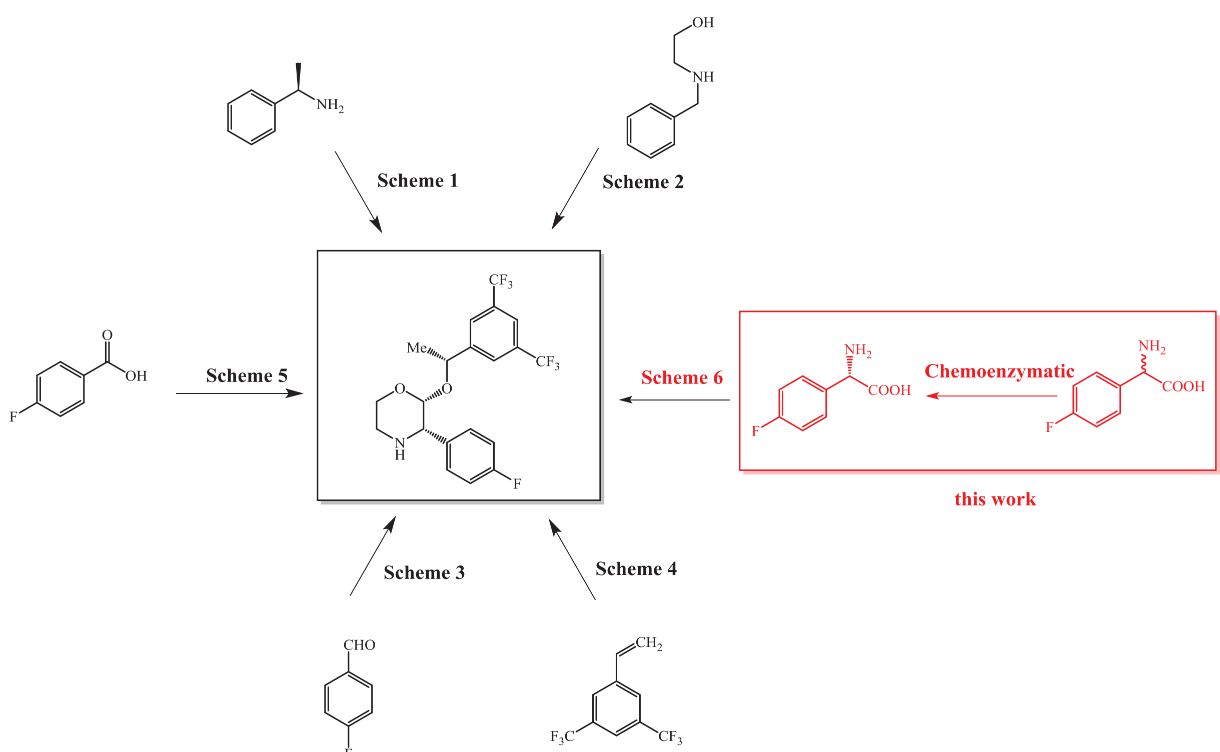
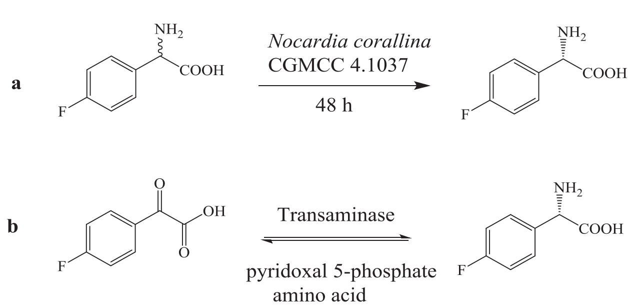


Fig. 2. Synthetic routes of **6**. The synthetic routes (Schemes 1–5) have been reported.



Scheme 7. Enzymatic synthesis of **4**.

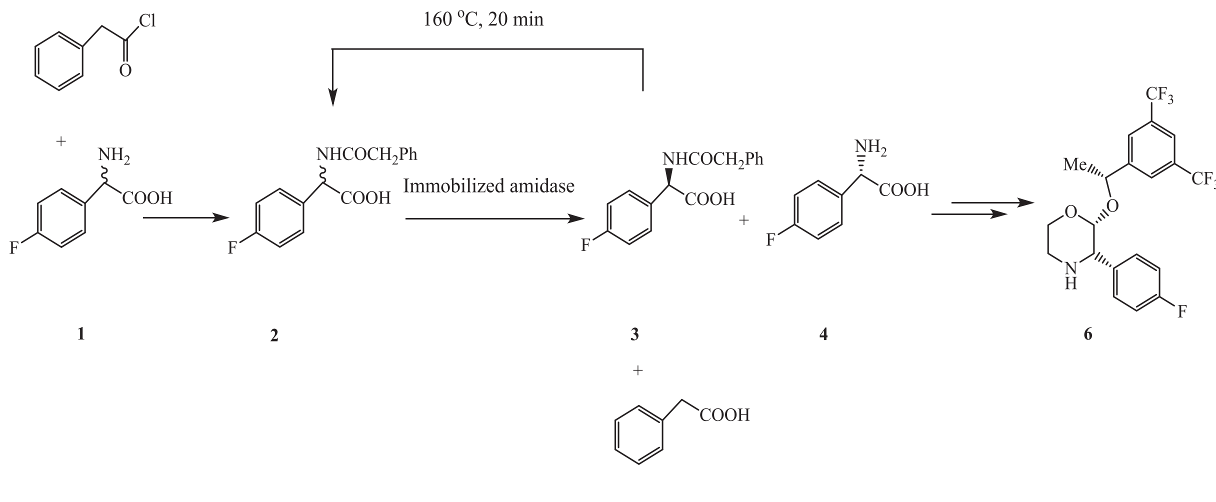
4. However, free enzyme has some drawbacks such as poor stability, difficulty in separation and recycling. Fortunately, these problems could be overcome by immobilized enzyme.

In this work, we aim to develop a practical chemoenzymatic approach for efficient synthesis of **4** via kinetic resolution combined with flexible chemical racemization (Scheme 8). The biocatalyst (immobilized amidase) was prepared by covalent immobilization of the recombinant penicillin amidase from *Bacillus megaterium* onto epoxy resin. After optimization of the reaction parameters, the chemoenzymatic reaction was carried out with high substrate concentration, high conversion and excellent enantioselectivity. This highly efficient chemoenzymatic route appears to be industrial promising in the production of (*S*)-α-amino-4-fluorobenzeneacetic acid.

2. Materials and methods

2.1. General

Recombinant penicillin amidase was constructed by our laboratory [26]. 4-Fluorophenylglycine was purchased from Shanghai Rongli Chemical Co. Ltd. (Shanghai, China). Phenylacetyl chloride was purchased from Shanghai Mayao Chemicals Co. Ltd. (Shanghai, China). Epoxy resin (LX-1000EP) was purchased from Xian Lanxiao Technology Co. Ltd. (Xian, China). Analytical reagent grade solvents were purchased from TEDIA, USA. All other reagents were obtained from



Scheme 8. Immobilized amidase mediated chemoenzymatic synthesis of **4.**

commercial sources and used without further purification.

High-pressure liquid chromatography (HPLC) analysis was conducted on Model LC-20AT HPLC (Shimadu, Japan). ^{1}H and ^{13}C NMR were recorded on a Bruker AVANCE III (Bruker, Switzerland). High-resolution mass spectra (MS) were analyzed on an Agilent 6210 TOF LC/MS (Agilent, USA). Optical rotator dispersion was carried out by Rudolph Autopol IV polarimeter (Rudolph, USA). Concentrations of substrate and product were monitored on a C18 column (Welch Materials, Inc., Shanghai, China) by HPLC.

2.2. Preparation of 2

Rac-**1** (0.4 mol, 67.61 g) was dissolved in 4.0 M sodium hydroxide (500 mL) at ambient temperature with stirring for 20 min. Phenylacetyl chloride was added dropwise while the mixture was vigorously stirred at 0 °C. After 10 h, the reaction mixture was extracted using dichloromethane to remove small amount of phenylacetic acid. Subsequently, the aqueous layer was acidified to pH 2.0 with hydrochloric acid (6.0 M). The precipitate was filtered, washed with ultrapure water three times. The filtered precipitate dried at 70 °C for 12 h to afford the product **2**. Yield: 94.5%, 108.54 g white powder. ^{1}H NMR (500 MHz, D2O) δ 7.30 (dd, J = 7.2, 4.5 Hz, 2H), 7.10 – 7.00 (m, 2H), 4.28 (s, 1H); ^{13}C NMR (126 MHz, D2O) δ 181.00 (s), 162.87 (s), 160.94 (s), 138.15 (s), 128.61 (d, J = 8.4 Hz), 115.47 (s), 115.30 (s), 59.86 (s); MS (ESI): m/z = 285.7 $[M + H]^+$.

2.3. Activity assay of immobilized amidase

The immobilized amidase activity was assayed in a 10 mL reaction mixture containing 50 mM substrate **2** and 0.1 g immobilized amidase (the pH was adjusted to 9.5 with 5% anmmonia solution). The reaction was performed at 40 °C for 3 min and then terminated by centrifuging at 12,000*g* for 1 min. The concentration of **4** was determined by HPLC. One unit (U) of immobilized amidase activity was defined as the amount of immobilized amidase that produced 1.0 μmol **4** per minute under the described conditions.

2.4. Optimization of temperature and pH

The effect of temperature on immobilized amidase activity was investigated at different temperatures ranging from 30 to 50 °C under the assay conditions mentioned above, and the effect of pH on immobilized amidase activity was examined at 40 °C in buffers with different pHs (7.5–11.5), respectively.

2.5. Optimization of substrate concentration

The effects of substrate concentration on enzymatic reaction were evaluated at different concentrations of **2** (40–100 g L^{-1}). The enzymatic reactions were performed at 40 °C and pH 9.5 with 0.1 g of immobilized amidase in 10 mL reaction mixture.

2.6. Optimization of agitation rate

The effects of agitation rate on the enzymatic reaction were also investigated. The reaction was performed with different agitation rate (100–500 rpm) at 40 °C and pH 9.5 in 20 mL reaction mixture containing 80 g L^{-1} substrate and 0.3 g L^{-1} immobilized amidase. The sample was centrifuged at 12,000*g* for 1 min and diluted with ultrapure water (1:50) for HPLC analysis.

2.7. Optimization of immobilized amidase loading

The influence of immobilized amidase dose (5–30 g L^{-1}) on the enzymatic reaction was examined. The reaction was performed at 40 °C with loading 80 g L^{-1} substrate concentration for 3 h. The supernatant was diluted with ultrapure water (1:50) and analyzed by HPLC.

2.8. Kinetic resolution of 2 in 1L reaction system

The time course for catalyzed hydrolysis of **2** at the optimum reaction condition was evaluated. The reaction was carried out in a 1 L system with reaction mixture containing 80 g **2** dissolved in water (pH was adjusted to 9.5 with 5% anmmonia solution) and 20 g immobilized amidase at 40 °C. Samples were taken periodically and centrifuged, then assayed by HPLC.

2.9. Racemization of 3

The unreacted (*R*)-enantiomer **3** (28.71 g, 0.1 mol) and phenylacetic acid (16.34 g, 0.12 mol) were added in three-necked flask. The reaction mixture was heated in an oil bath at 160 °C for 20 min, and added into 100 mL 5% anmmonia solution before cooling to room temperature. The mixture was extracted with dichloromethane to remove phenylacetic acid and the residue **2** was reused as substrate. Then, the mixture solution was used for optical rotation determination by polarmeter and concentration analysis by HPLC.

2.10. Analytical method

The concentrations of **2**, **4** and **5** were determined by HPLC (Shimadzu Co., Kyoto, Japan) with a C18 column (5 µm × 250 mm × 4.6 mm, Welch Materials, Inc., Shanghai, China) using acetonitrile-0.1% perchloric acid (50:50, v/v) as the mobile phase at a flow rate of 0.7 mL min^{-1}. The separated peaks were monitored using a UV detector (SPD-10A, Shimadzu Co., Japan) at 220 nm. The retention times of **2**, **4** and **5** were 8.20 min, 3.74 min and 6.56 min, respectively.

The *ee* value of **4** was determined on a chiral column (Chirobiotic™ R 250 × 4.6 mm, particle size 5 µm, Sigma, USA) using acetonitrile-0.5% acetic acid (80:20, v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}. The separated peaks were monitored using a UV detector at 220 nm. The retention times of (*S*)-**4** and (*R*)-**4** were 13.02 min and 14.84 min, respectively.

2.11. Synthesis of (S)-α-amino-4-fluorobenzeneacetic acid 4

Rac-**2** (80 g L^{-1}) was dissolved in water and the pH was adjusted to 9.5 with 5% anmmonia solution. Subsequently, the enzymatic reaction was carried out by adding 20 g L^{-1} immobilized amidase with 300 rpm at 40 °C in 100 mL reaction system. When the enzymatic reaction was finished after 3 h, the mixture was filtered to separate immobilized amidase. The supernatant was acidified to pH 4.0 with concentrated hydrochloric acid and then extracted with dichloromethane (3 × 100 mL) to remove by-product **5**. The aqueous layer was further acidified with concentrated hydrochloric acid to pH 2.0 at 0 °C with stirring. Then the unreacted **3** in the solution was slowly precipitated. The aqueous solution was collected and the pH was adjusted to 7.0. (*S*)-**4** was obtained via the evaporation of water, and then redissolved in hot methanol. The product (*S*)-α-amino-4-fluorobenzeneacetic acid was finally crystallized from methanol. $[\alpha]_D^{25} = +85.7°$ (c = 20 mg mL^{-1}, methanol); ^{1}H NMR (500 MHz, Acetone) δ 7.49 (dd, *J* = 8.5, 5.4 Hz, 2H), 7.34 (d, *J* = 7.3 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 2H), 7.22 (t, *J* = 7.2 Hz, 1H), 7.13 (t, *J* = 8.8 Hz, 2H), 5.57 (s, 1H), 3.65 (s, 2H); ^{13}C NMR (126 MHz, Acetone) δ 181.62 (s), 180.71 (d, *J* = 9.3 Hz), 173.97 (s), 172.03 (s), 146.50 (s), 144.17 (t, *J* = 3.5 Hz), 140.10 (s), 139.73 (s), 138.81 (s), 137.07 (s), 125.84 (d, *J* = 21.8 Hz), 66.26 (d, *J* = 11.6 Hz), 52.75 (d, *J* = 5.5 Hz); MS (ESI): *m/z* = 167.9 [M + H]+.

3. Results and discussion

3.1. Synthesis of substrate 2

Racemic **2** was prepared in one simple step by mixing 4-fluorophenylglycine **1** with phenylacetyl chloride under alkaline condition. To investigate the substrate scope of the immobilized amidase, we synthesized acetyl, benzoyl and *N*-phenylalanyl derivatives of the 4-fluorophenylglycine. Interestingly, only *N*-phenylacetyl-4-fluorophenylglycine **2** was able to be recognized as a substrate of the immobilized amidase. Meanwhile, only (*S*)-**2** was hydrolyzed by immobilized amidase, indicating strict stereoselectivity of the enzymatic process.

3.2. Effects of temperature and pH on activity of immobilized amidase

The effect of temperature on the kinetic resolution of **2** was studied at a temperature range from 25 to 65 °C. The results of the activity and enantioselectivity at different temperatures were shown in Fig. 3. The immobilized amidase activity increased with the increase of reaction temperature and the maximum activity was observed at 40 °C. However, with further increase of temperature, the activity decreased significantly, which might ascribe to the partial inactivation of the immobilized amidase by conformation change under high temperature [27]. Interestingly, temperature has less effect on the enantioselectivity

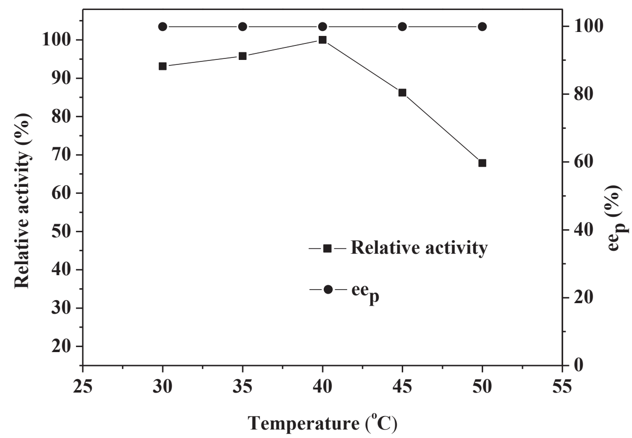


Fig. 3. Effect of temperature on the activity of immobilized amidase. The immobilized amidase activities were assayed at different temperatures (30–50 °C) in a 10 mL reaction mixture (pH 9.5) containing 50 mM substrate **2**, 0.1 g immobilized amidase.

in the temperature range from 25 to 65 °C. In the light of this, 40 °C was chosen as the optimal temperature in the subsequent reactions.

To evaluate the effect of pH on the immobilized amidase-catalyzed hydrolysis, we investigated different pH and found that the activity of immobilized amidase gradually increased with the increase of pH from 7.5 to 11.5 and the maximum activity was achieved at pH 9.5 (Fig. 4). However, the immobilized amidase activity decreased ramarkably with further increase of pH from 9.5 to 11.5. The enantioselectivity was not affected noticeably by pH in the enzymatic reaction. Generally, the amidase-catalyzed hydrolysis reaction were carried out in buffers or by adding extra alkaline solution to ensure the stable activity of biocatalyst [28,29]. Interestingly, the immobilized amidase mediated hydrolysis for the synthesis of **4** could work efficiently without pH regulation by additional alkali.

3.3. The effect of substrate concentration on activity of immobilized amidase

The effects of substrate concentration (40–100 g L^{-1}) on immobilized amidase-catalyzed hydrolysis were investigated. As shown in Fig. 5, the activity of immobilized amidase gradually increased with the increase of the substrate concentration (40–60 g L^{-1}) and then maintained high value in the range of 60–80 g L^{-1}. Mild inhibition was observed when the substrate concentration beyond 60 g L^{-1}. However, the activity sharply

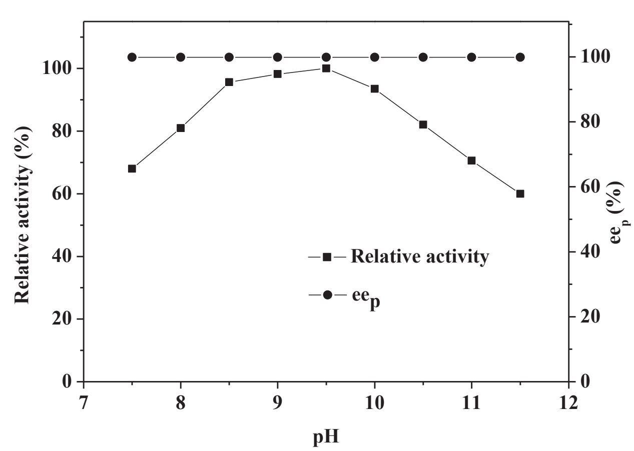


Fig. 4. Effect of pH on the activity of immobilized amidase. The reactions were carried out at 40 °C and the immobilized amidase activities were assayed with different pHs (7.5–11.5) in a 10 mL reaction mixture containing 50 mM substrate **2**, 0.1 g immobilized amidase.

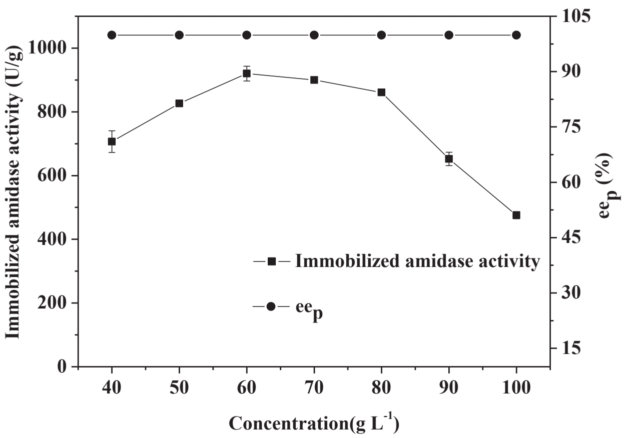


Fig. 5. Effect of substrate concentration on the activity of immobilized amidase. The reactions were performed at 40 °C in reaction mixtures (pH 9.5) consisted of 0.1 g immobilized amidase with and various substrate concentration (40–100 g L^{-1}).

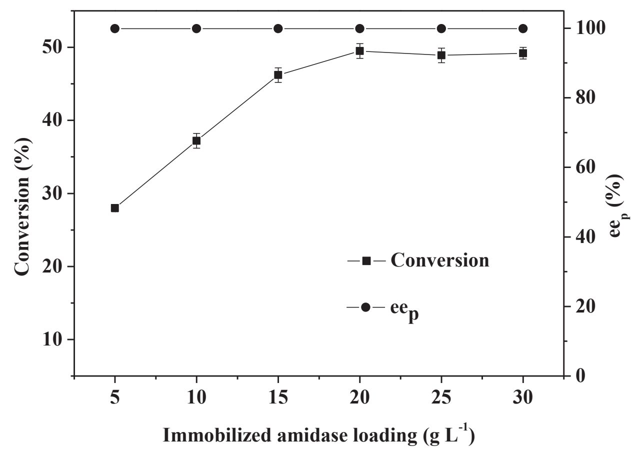


Fig. 7. Effect of immobilized amidase loading on the kinetic resolution of **2**. The reactions were performed at different immobilized amidase loading (5–30 g L^{-1}) with 300 rpm agitation rate in reaction mixture containing 80g L^{-1} substrate.

decreased with further increase of substrate concentration (80–100 g L^{-1}). The increase of substrate concentration can accelerate the reaction rate from the perspective of chemical equilibrium. However, higher substrate concentration could cause inhibition of enzyme activity. Considering the practical industrial application, 80 g L^{-1} was chosen as the optimal substrate concentration in further study.

3.4. Effect of agitation rate

It was observed that the reaction mixture gradually became viscous because of precipitation of compound **4** during the reaction course at a substrate loading of 80 g L^{-1}. Thus, the effect of the agitation rate on the enzymatic reaction was investigated. As shown in Fig. 6, it was found that the conversion was significantly improved with the increase of the agitation rate (100–300 rpm), and then was basically flat with the agitation rate increased (300–500 rpm), indicating that it was sufficient to afford satisfied mass transfer with 300 rpm in this reaction. Thus, 300 rpm was chosen as the optimal agitation rate in further study.

3.5. Effect of immobilized amidase loading

The dosage of biocatalyst was an importance factor in terms of industrial application. We evaluated the effect of different doses of immobilized amidase (5–30 g L^{-1}) on the enzymatic reaction and observed that the conversion steadily increased with the increase of immobilized amidase loading from 5 to 20 g L^{-1} and leveled out at values for further increasing the amount of immobilized amidase (Fig. 7). Thus, 20 g L^{-1} was chosen as the optimal dosage of immobilized amidase in further study.

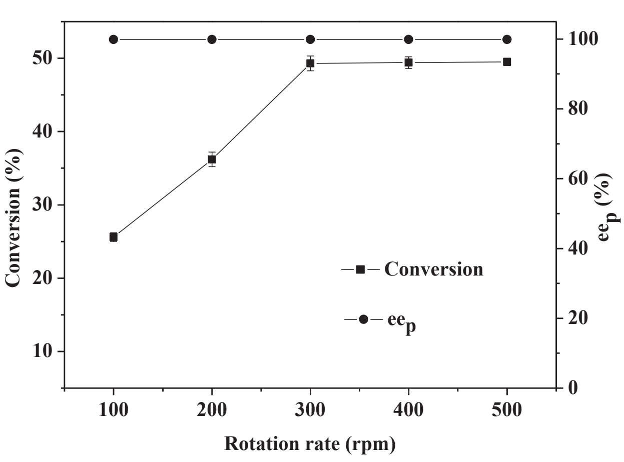


Fig. 6. Effect of agitation rate on the kinetic resolution of **2** using the immobilized amidase. The conversions of the reactions were investigated at 40 °C with different agitation rates (100–500 rpm). The reaction mixture (pH 9.5) was composed of 80 g L^{-1}substrate **2** and 0.3 g immobilized amidase.

3.6. Time course of enzymatic kinetic resolution process

The enzymatic reaction was scaled up to further evaluate the feasibility of the route. The reaction was performed with 80 g substrate loading in a 1 L thermostated stirred tank reactor under the optimized reaction parameters. As shown in Fig. 8, the desired **4** was obtained with high conversion (49.9%) and excellent enantioselectivity (99.9% *e.e.*) after 3 h.

*3.7. Recycling of **3** by racemization*

The recycling of remaining enantiomer **3** was explored by chemical racemization after the enzymatic reaction was accomplished. Compound **3** was completely racemized to obtain racemic **2** with 95.3% yield. The racemization makes the enzymatic resolution process overcome the limitation of the theoretical yield of 50%.

To assess the feasibility of the chemoenzymatic strategy, enzymatic

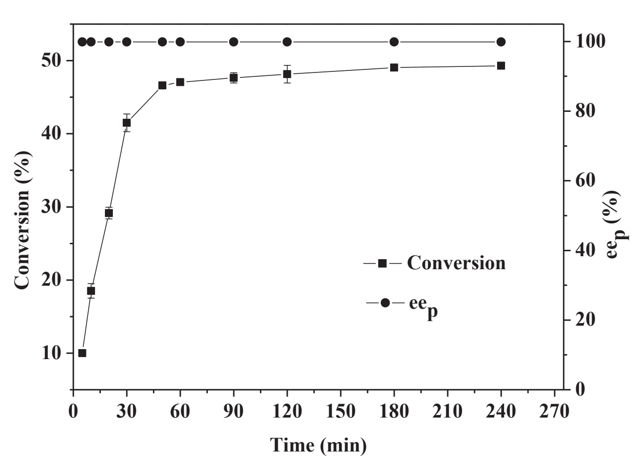


Fig. 8. Time course of kinetic resolution of **2** using immobilized amidase. The reaction was performed at 40 °C with 300 rpm agitation rate in reaction mixture (1 L, pH 9.5) consisted of 80 g L^{-1} substrate and 20 g L^{-1} immobilized amidase.

resolution and the subsequent separation steps were carried out, affording **4** in 46.2% isolated yield. The unreacted enantiomer of the substrate was completely racemized to produce substrate **2**, which was again subjected to the enzymatic resolution, giving **4** in 21.5% yield. Thus, the total yield of 67.7% was achieved after one recycle of (*R*)-**2**. The space time yield of the bioprocess reached $10.6\ g\ L^{-1}\ h^{-1}$, which was 963.6 and 240.9 times higher than that reported by Cameron et al. ($1.1 \times 10^{-2}\ g\ L^{-1}\ h^{-1}$) [24] and Xia et al. ($4.4 \times 10^{-2}\ g\ L^{-1}\ h^{-1}$) [25], respectively. Thus, this chemoenzymatic approach using immobilized amidase as the biocatalyst exhibited higher efficiency and feasibility compared with other enzymatic method ever reported.

4. Conclusions

In summary, we have developed a simple, high efficient chemoenzymatic route for the synthesis of (*S*)-α-amino-4-fluorobenzeneacetic acid using immobilized amidase as biocatalyst. The enzymatic reaction was carried out using water as reaction medium without pH regulation during the reaction process. The reaction parameters such as pH, temperature effect, catalyst loading, and agitation rate were optimized. The hydrolysis reaction was demonstrated to be a robust catalytic process for preparation of (*S*)-α-amino-4-fluorobenzeneacetic acid with a high efficiency (49.9% conversion) and excellent enantioselectivity (99.9% *e.e.*) on a scale of $80\ g\ L^{-1}$ substrate concentration in 1 L reaction system. Moreover, the unreacted enantiomer of substrate could be easily racemized and reused. The overall process has an excellent industrial potential, and further study on recycle of immobilized amidase and pilot scale are in progress.

Acknowledgement

This work was financially supported by the National High Technology Research and Development Program of China (No. 2012AA022201).

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