Enantioselective Resolution of (R, S)-DMPM by a Adsorption-Covalent Crosslinked Esterase PAE07 from Pseudochrobactrum Asaccharolyticum WZZ003

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Abstract

(*R*)-N-(2,6-dimethylphenyl) alanine ((*R*)-MAP-acid) is an important chiral intermediate of the Fungicide (*R*)-Metalaxyl. In this study, ten kinds of immobilized resins(XAD1180N, H103, HAD7HP, D3520, NKA, D101 , DM11,850 JinKai, Primary amino resin and 850 synthetic resin) were used to adsorption-covalent crosslinked esterase PAE07 for splitting (*R, S*)-DMPM. The resin D3520 with porous structure and hydrophobic polystyrene was selected for immobilization as the carrier, after optimization of the immobilization conditions, the enzyme load is 20:1 (mg/g), the adsorption time is 4h, and the adsorption buffer pH is 7.0 . The K_m and V_{max} of the free esterases were 35.66 mM and 4.46 mM/mg·min, respectively, The K_m and V_{max} of the immobilized PAE07 were 19.05 mM and 2.84 mM/mg·min. The SEM analysis showed that the immobilized esterase PAE07 had higher thermal stability, pH stability and substrate specifity than those from the free esterase. Under the optimal conditions,the reaction was carried out at 35°C and 200 rpm for resolution of 350 mM substrate for 14 hours, the conversion rate reached 48%, and the $e.e._p$ was 99.5%.The repeatability of immobilized esterase PAE07 was evaluated by continuous catalytic resolution of (*R, S*)-DMPM. The results showed that after 15 times of repeated use, 86.2% of the relative enzyme activity was retained. These results proved that immobilized esterase PAE07 as a new catalyst had great potential for the application and industrial enzymatic resolution of (*R, S*)-DMPM to prepare (*R*)-metalaxyl.

1. Introduction

(*R*)-Metalaxyl is an excellent systemic fungicide [1]. It can effectively inhibited RNA synthesis in bacteria, and has effectual control over pathogenic fungi in hops, potatoes, liana and other crop [2,3]. The metalaxyl marketed is usually a racemic mixture, including (*R*)-DMPM and (*S*)-DMPM, and the (*R*)-enantiomer is found to play a major role in antifungal activity [4,5]. (*R*)-N-(2,6-dimethylphenyl)alanine ((*R*)-MAP-acid) is an important chiral intermediate of the (*R*)-Metalaxyl [6]. Traditionally, the method for preparing (*R*)-Metalaxyl is a chemical method, and requires toxic or chiral compounds, which pollute the environment and are not conducive to large-scale production [7]. The enzymatic resolution of (*R, S*)-DMPM has good enantioselectivity and is beneficial to environmental protection. Therefore, the advantages of enzymatic resolution (*R, S*)-DMPM are obvious, and more and more researches are being conducted.

Esterase (EC3.1.1.3) belongs to the α/β-hydrolase family, which catalyzes various reactions, such as esterification, ester hydrolysis, transesterification and acylation [8,9]. It can catalyze the breaking of ester bonds to form the corresponding alcohols and acids with water molecules [10]. Esterases are distributed in plants, animals, and microorganisms, and among them the microbial esterases are found to be more stable and favourable for production at a large scale [11-15]. Esterase is also used to resolve (*R, S*)-DMPM.A study reported that the enantiomeric excess of lipase MC 16-3 and lipase 99-2-1 resolution (*R, S*)-DMPM from *Burkholderia sp.*were 91.8% (conversion rate 25%) and 91%(conversion rate 30%). Commercial lipase PS from *Burkholderia cepacia* showed a relatively high conversion rate and excellent

enantiomeric excess (e.e.$_p$>98%) following immobilization in another study [16]. However, different limitations of the esterase for resolution, such as the low activity, poor thermal stability and high cost, restrict its wide industrial application. Therefore, it is necessary to develop an biocatalyst to catalyze the production of (*R*)-MAP-acid, so as to increase its industrialized potential and make the production of (*R*)-DMPM more efficient , stable and low cost.

Esterases are soluble in aqueous solutions, making it difficult to separate enzymes following completion of reaction, and the free enzymes are difficult to stored with easily denatured and inactivated under the inappropriate pH and temperature conditions. For these reasons, free enzymes cannot easily be reused impeding esterase's commercial and industrial utility [17-19]. Enzyme immobilization has been successfully applied during the last ten years to solve the problems of emzymatic instability and recover, and to reduce the cost of enzymatic industrial production [20]. The benefits of immobilized enzymes make immobilized technology more and more applications, including higher rigidity and flexibility, greater stability, greater specific surface area, more efficient operation and smaller diffusion limits. Esterase adsorption to a hydrophobic support is one of the most common methods for stabilizing the esterase. When the esterase is adsorbed onto a hydrophobic support, the enzymatic form open, exposing active centre and thereby opening the ester in the open form; the enzyme itself remains structurally stable [21]. This method of immobilization is simple and effective, but under severe conditions such as high temperature and high concentration of organic solvents and surface adsorbents, the enzyme detaches from the carrier [22]. Therefore, when choosing this immobilization, people can combine other methods such as chemical cross-linking to choose a hydrophobic support that can produce covalent bonds [23]. Therefore, other methods such as chemical cross-linking and modification are often chosen to be used together when choosing this immobilization method.

In our previous research, a strain capable of enantioselective resolution (*R, S*)-DMPM was successfully screened, and a genetically engineered strain containing PAE07 esterase was successfully constructed. Previous preliminary experiments proved that the adsorption covalent-crosslinking methods could validly immobilize PAE07 (Scheme 1). In the current work, PAE07 is subjected to enantioselective resolution (*R, S*)-DMPM following adsorption-covalent crosslinking. Immobilization conditions, immobilized esterase PAE07 separation (*R, S*)-DMPM with adsorption-covalent crosslinked, the reusability of immobilized esterase and the resolution reaction system after amplification are investigated

2. Materials And Methods

2.1. Materials

XAD1180N (Non-polar macroporous adsorption resin), H103 (Styrene-type weak polar interpolymer resin), HAD7HP (Styrene non-polar copolymer resin), D3520 (Macroporous acrylic anion resin), NKA (Styrene non-polar copolymer resin) and D101 (Styrene non-polar copolymer resin) were purchased from the Anhui Samsung Resin Technology Co., Ltd (Gu Zhen, China). DM11 (Non-polar macroporous adsorption resin) was acquired from the Zhengzhou Qinshi Technology Co., Ltd (Zheng Zhou, China). Polyethyleneimine

AR (50% in H_2O) was purchased from the Aladdin Industrial, Inc.(Shang Hai, country). (*R, S*)-DMPM was provided by Yifan Biotechnology Group (Wen Zhou, Country), confirmed with High performance liquid chromatography (HPLC)(Fig. S1) . Other chemical reagents used in this study were of analytical grade.

2.2. Preparation of esterase PAE07

The recombinant *E. coli* WZZ003 (CCTCC NO:M2014209) was inoculated into 250 mL of LB liquid medium containing ampicillin and cultured at 37^{o}C at 200 rpm. When the OD_{600} reached 0.6-0.8, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce the expression of the target gene, at 28 ^{o}C for 8 hours. After enrichment by centrifugation, the bacterial cells and 50 mM PB buffer solution(pH 7.0) were mixed at a ratio of 1 g : 20 mL, and then the crude enzyme solution was obtained following ultrasonication and centrifugation. Esterase PAE07 using affinity chromatography as previously reported [24]. The crude PAE07 solution was pass through a Ni-NTA column (1 ml. Bio Basic, Inc.), and the retained protein was gradually eluted at an increasing gradient of imidazole from 50 mM to 250 mM at a flow rate of 1 mL/min. PAE07 were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the target protein's size obtained was 31 Kda (Fig. S2). A Thermo Scientific Pierce BCA protein assay kit (Shanghai, China) was used to quantify the proteins, the specific activity of purified esterase is 5986 U/g and the purified esterase stored in a refrigerator at -20 °C for subsequent use.

2.3. The preparation of the immobilized esterase PAE07 by adsorption-covalent crosslinked

The immobilized esterase PAE07 was prepared by adsorption-covalent crosslinked method [25]. The dried resin was weighed following treatment, mixed with a corresponding amount of pure enzyme solution (1g:4 mL) in a 50 mL Erlenmeyer flask, and adsorption performed in a water bath shaker at 30 °C and 160 rpm for 4 hours. After the adsorption was completed, polyethyleneimine (PEI) was added to a final concentration of 0.25%, and the mixture shaken and mixed. The reaction was continued for 1 h at 30 °C and 160 rpm in a water bath shaker. After the immobilization, the unbound protein was washed with water, and finally dried at 45 °C under vacuum to obtain the immobilized enzyme. The immobilized enzyme was then stored in a 4 °C refrigerator for subsequent use.

2.4. Optimization of immobilization conditions for the esterase PAE07

In order to obtain the optimal resins and esterase and a higher stability, a variety of immobilized resins,such as XAD1180N, H103, HAD7HP, D3520, NKA, D101 , DM11,850 JinKai, Primary amino resin and 850 synthetic resin were screened.

In order to determine the optimal enzyme loading condition, the ratios of resin to enzyme protein, 5:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1 (mg/g) were set.

In order to optimize the time for adsorption of resin to enzyme protein, the time were set to 1 h, 2 h, 3 h, 4 h, 5 h and 6 h.

In order to optimize the pH for immobilization of esterase PAE07 preparation, the pH were set to 6.0, 6.5, 7.0, 7.5 and 8.0.

2.5. Kinetic constants for the free and immobilized PAE07

(*R, S*)-DMPM was prepared with a substrate concentration rang of 0-250 mM. The resolution reaction was carried out in 2 mL centrifuge tube containing 980 μl each of different concentrations (*R, S*)-DMPM solutions, and 0.02 g of immobilized PAE07 or 20 μl of PAE07 solution. High performance liquid chromatography was applied to detect the change of peak area over time of the substrate. The kinetic parameters, V_{max} and K_m, of the immobilized esterase PAE07 and free PAE07 were obtained by nonlinear fitting.

2.6. Hydrolysis of (*R, S*)-DMPM using the immobilized PAE07

In a 2 mL centrifuge tube, 0.02 g of immobilized PAE07, 990 μl of 0.2 M PB buffer (pH 7.0) and 50 mM of (*R, S)*-DMPM were mixed, and then reacted at 30 °C and 200 rpm for 30 min. Following the reaction, the pH was adjusted to 2 with 4 M HCl, and 1 mL of ethyl acetate was added thereto for extraction. After the solution was separated, 20 μl of the ethyl acetate layer was taken and evaporated to dryness used a rotary evaporator. This was dissolved with 1 mL at mobile phase to prepare a liquid phase detection sample. Substrate stereoselectivity, product enantiomeric excess ($e.e._p$) and enzyme activity were calculated.

2.7. Enzymatic activity assay

The hydrolysis activity of (*R, S*)-DMPM was determined according to the method

for producting (*R*)-MAP-acid as was previously described [16]. One unit of the immobilized esterase activity was defined as the amount of immobilized esterase able to catalyzed the production of 1 μmol of (*R*)-MAP-acid per minute under assay conditions. Esterase specific activity was defined as the enzyme activity per unit mass of immobilized esterase, expressed as U/g. The relative activity in the batch reaction was obtained by calculating the ratio of the enzyme activity of the N batches of immobilized esterase and the initial immobilized esterase.

High performance liquid chromatography(HPLC) was used to detect samples, and the enantiomeric excess of the substrate ($e.e._s$) was calculated based on the peak areas of (*R*)-DMPM (R_1) and (*S*)-DMPM(S_1). The peak areas of (*S*) -MAP-acid (S_2) and (*R*)-MAP-acid (R_2) were used to calculate the enantiomeric excess of the product ($e.e._p$). Conversion rate was calculated according to $e.e._p$ and $e.e._s$ by using the formula 3.Enantioselectivity (*E*) was calculated according to Eq. 4.

$$e.e._s = \frac{[S1]-[R1]}{[S1]+[R1]} \times 100\% \quad (1)$$

$$e.e._p = \frac{[R2]-[S2]}{[S2]+[R2]} \times 100\% \quad (2)$$

$$C = \frac{e.e_S}{e.e_S+e.e_p} \times 100\% \quad (3)$$

$$E = \frac{\ln[(1-C)(1-e.e._s)]}{\ln[(1-C)(1+e.e._s)]} \quad (4)$$

2.8. Statistical Analysis

All experiments were evaluated at least three times and the data were expressed as the mean ± SD (standard deviation). The data processing and charts in this article were completed by Origin software (Origin Lab Co., Pro. 9.1, Hang Zhou, China).

3. Results And Discussion

3.1. Selection of resin for immobilization

Compared with the low activity after immobilization of hydrophilic resin and the low specific activity after immobilization of ionic resin, hydrophobic resin often shows better immobilization effect. As shown in Table 1, the Resin D3520, representing the macroporous acrylic anion resin, showed the highest resolution activity for (*R, S*)-DMPM. Showing that based on the methyl acrylate matrix, the macroporous acrylic anion resin could adsorb to compounds with amino groups more easily [26]. The resean for that mat be that after the esterase was immobilized in D3520, it was activated in the hydrophobic phase and the active center was in an open form, thereby increasing the activity of the esterase. In addition, D3520 has a large internal surface area (480-520 m^2/g) and a small pore size (8.5-9.0 nm), which can adsorb more esterases in deep pores

[27,28]. This is consistent with the results showing that hydrophobic resins with an average pore size between 8.0 and 22 nm are more suitable for immobilizing lipase [25].Therefore, D3520 was selected as the immobilized resin of choice for subsequent experiment.

3.2. Optimized enzyme loading conditions

The initial concentration of enzyme protein has been significantly affected the enzyme activity of immobilized esterase [27]. Table 2 revealed that as the increase in the enzyme loading, the enzyme activity of the immobilized esterase also increased significantly: when the ratio of enzyme protein-to-resin increased to 20: 1 (mg/g), the highest enzyme activity was 26.2 U/g. Then, further increasing the loading concentration of enzyme protein would reduce the activity of immobilized esterase PAE07. Therefore, the enzyme activity of immobilized esterase PAE07 was inhibited under both low and high concentration of enzyme loading. This is because under a high concentration of enzyme loading, the resin will produce multi-layer adsorption of the enzyme, so that the resin does not have enough specific surface to bind with the enzyme, and it may also block the pores of the resin and affect the binding of the esterase and the substrate [29,30]. Conversely, at low concentrations of enzyme loading, the esterase will try to maximize contact with the resin surface, resulting in loss of conformation and inactivation [31]. Therefore, enzyme loading 20:1 (mg/g) was selected for subsequent experiment.

3.3. Optimization of immobilization time

The effect of adsorption time on protein adsorption rate and immobilized esterase PAE07 activity is shown in Fig. 1. The resin adsorbed esterase in 4 hours and reached saturation, unable to adsorb more enzymes. This is because 4h is the time required for esterase to fully enter D3520, and other studies have also reached similar conclusions [25]. Therefore, adsorption time in 4 hours was selected for subsequent experiment.

3.4. Optimization of buffer pH on the immobilized PAE07

The pH of the buffer is an important factor affecting the efficiency of immobilization, and it will affect the molecular conformation changes of the enzyme [32]. The optimal pH of esterase PAE07 is 7.2, and it can maintain relatively good activity in the range of 5-9 pH. The adsorption effect of D3520 on pH changes basically does not change, so when immobilized adsorption, unsuitable pH will inhibit the activity of esterase, thereby affecting the activity of immobilized enzyme. As the Fig.2 showed when the pH of the esterase PAE07 immobilized solution was 7, the best catalytic activity is observed, showing significantly decline in the specific activity When the pH exceeded 7. Therefore, the pH of the adsorption buffer was selected to be 7 for subsequent experiments.

3.5. Kinetic parameter for free and immobilized esterase PAE07

The kinetics were tested at 30 °C following the resolution of (*R, S*)-DMPM and found to obey the Lineweaver–Burk type of rate equation, as shown in Fig. 3. The K_m and V_{max} of the free esterases were 35.66 mM and 4.46 mM/mg·min, respectively, The K_m and V_{max} of the immobilized PAE07 were 19.05 mM and 2.84 mM/mg·min. It is shown that the affinity of the immobilized esterase to the substrate is reduced, this is due to the porous structure of D3520 and the cross-linking effect of PEI, which increased the stiffness of the esterase and the steric hindrance of the carrier, and inhibited the substrate molecule from moving to the active center of the enzyme diffusion [33,34]. The increased of V_{max} after adsorption-

covalent crosslinking may be a adverse change in the conformation, similar results have been reported in other studies [35,36].

3.6. Optimum temperature and temperature stability of immobilized esterase PAE07

Biocatalysts have different biological activities at different temperatures, and their catalytic efficiency on substrates also different [37]. Enzymes also have such characteristics as biocatalysts, so the influence of temperature on enzyme-catalyzed reactions must be evaluated. Fig. 4a demonstrates compared to free enzyme , within the range of 25 °C-50 °C, immobilized enzyme all revealed good catalytic activity. Immobilization esterase PAE07 exhibited their greatest range of activity at 40 °C which is by 5 °C higher than that of the free PAE07. Fig.4b shows after 12 hours of incubation at 50°C, the relative activity of esterase PAE07 after immobilization was 86%, and the relative enzyme activity of free PAE07 was only 56%. This may be due to the larger internal surface area and porous structure of D3520, which can form a covalent connection under the action of PEI, thereby increasing the rigidity of the immobilized esterase PAE07, stabilizing the conformation of the esterase, and improving the esterase The thermal stability [38,39].

3.7. Optimum pH and pH stability of immobilized esterase PAE07

pH affects enzyme activity, stereoselectivity, and the spontaneous hydrolysis of the substrate during catalytic reactions [40]. The effect of a temperature of 35 °C and a 6.0 to 9.0 pH range on the enzyme activity of immobilized PAE07, and immobilized PAE07's and free PAE07's pH stability at 35 °C and pH 6.0 - 9.0 following 12 h of storage were studied.

Fig. 5a demonstrates relative to the optimal pH of free PAE07 is neutral, with the optimal pH of the immobilized enzyme being alkaline, increasing to 7.5. And the immobilized esterase PAE07 showed good catalytic activity between pH 6.0-9.0. The pH stability of immobilized and free PAE07's are shown in Fig. 5b, After remaining at pH 9.0 for 12 h, the remaining immobilized PAE07's esterase still had 90.2% of its activity, while the free PAE07 had only 72.1% activity, indicating that immobilization protects the enzyme and improves the pH stability of PAE07. This may be because the product (*R*)-MAP-acid forms a thin film on the outer surface of the free esterase, which causes external diffusion restriction, but has little effect on the immobilized esterase [41]. A study also reached a similar conclusion. After PPL lipase is immobilized on macroporous polystyrene, the suitable range of pH becomes wider and the stability becomes better [42].

3.8. Optimum of Rotation Speed Hydrolysis (*R, S*)-DMPM on Immobilized esterase PAE07

(*R, S*) -DMPM is an oily, low water-soluble substrate whose catalytic reaction is significantly affected by rotation speed. When the speed of the shaker was increased from 100 to 200 rpm, the enzyme activity of the immobilized esterases continued to increase. After the speed of the shaker exceeded 200 rpm, the rotation speed increased without any change in enzyme activity (Fig. S3). Many reports show that after

lipase is immobilized on the resin, the effect of rotation speed on the enzymatic reaction has the same trend result [43,44].

3.9. The influence of Substrate Concentration on Hydrolysis (*R, S*)-DMPM of Immobilized Enzyme PAE07

As the substrate concentration increased, the speed of the initial enzymatic reaction also rose, but substrate inhibition occurred with excessive substrate concentration, so it is necessary to explore the optimal substrate concentration for the immobilized enzyme-catalyzed reactions [45]. When the substrate concentration reached 350 mM (74.2 g/L), the maximum value was 2.58 mM/min. The product $e.e._p$ reached 99.5% and conversion rate reached 48%, corresponding to an E of 1393 when the reaction time was 15 h. Moreover, following immobilization, the substrate inhibition threshold value for the immobilized PAE07 increased by 94 mM as compared to that of the free PAE07. The increase in the optimal substrate concentration of the esterase after immobilization may be due to the immobilization of the esterase in the multi-empty carrier, which can partially protect the enzyme from the inhibitor's action and cause the loss of activity. The covalent linkage increases the rigidity of the enzyme and also makes esterase are more stable. In addition, if the diffusion of the substrate inside the D3520 particle is slower than its catalytic modification, the enzyme in D3520 will not obtain the same substrate concentration as the esterase near the resin surface, and may increase the optimal substrate concentration [27].

Zhang et al. reported that the optimal substrate concentration for the selective conversion of (*R, S*)-DMPM from *Achromobacter denitrificans* was 100 g/L, 80% $e.e._p$ [46]. The enzyme activity of the esterase of *Achromobacter denitrificans* was relatively low, and the low enantioselectivity lead to the low purity of (*R*)-metalaxyl. Lipase PS was immobilized on a polymer carrier, and then (*R*)-Metalaxyl was prepared on an enlarged scale, with 960 g of immobilized PS esterases and a substrate concentration of 400 g/L; the resulting product $e.e._p$ was 96% [47]. The lipase PS enzyme is expensive, low catalytic efficiency and low time-space conversion ratemaking it difficult for it to produce (*R*)-metalaxyl for industries. Compared with other enzymes, the immobilized esterase PAE07 has high catalytic efficiency, good stability, a low cost and good substrate tolerance.

3.10. Scale-up Hydrolysis (*R, S*)-DMPM of Immobilized Enzyme PAE07

We expanded the scale of the reaction system, packed 12.5 g immobilized PAE07 into a packed column (the heigh -to- diameter ratio was 15:1), and passed 200 g/L (*R, S*)-DMPM at 30 °C and a 0.5 mL/min flow rate. We took samples at intervals, acidified them with 4 M HCl and applied ethyl acetate extraction for HPLC detection. The conversion reaction was completed within 12 h (at a conversion efficiency of 48%), and the $e.e._p$ of the obtained product was greater than 98%. The space-time yield of (*R, S*)-DMPM in the packed bed reactor was 0.653g/L·h. The concentration of the catalytic substrate is significantly increased after amplification, and the reaction can be continuously cycled, which increases the reaction efficiency and saves costs, indicating that the immobilized PAE07 could be used for the industrial preparation of (*R*)-DMPM.

3.11. SEM analysis of Immobilized Esterase PAE07

The surface characteristics of Immobilized Esterase PAE07 and Carrier D3520 were analyzed by SEM. Fig. 8 is a SEM micrograph before and after immobilization at a magnification of 3×10^4. From the figure we can see the porous structure and small pore size of polystyrene resin D3520. The pores are significantly reduced after immobilization, and the surface becomes rougher, indicating that the esterase PAE07 has been successfully immobilized on the resin. Due to the porous structure of the immobilized esterase PAE07, it indicates that the improved stability of the esterase PAE07 after immobilization may be due to the restriction of the esterase PAE07 in the resin pores and the restriction of the stretching of the enzyme molecules.

3.12. Reusability of Immobilized Esterase PAE07

It has been previously reported that enzymes generally have improved stability after immobilization [48]. Operational stability and repeatability are also important criteria for industrial production to evaluate the performance of immobilized enzyme [49]. Under the optimal reaction conditions, batch reaction was carried out at a substrate concentration of 350 mM to study the stability.

After one reaction, the immobilized esterase was filtered out and used for the next cycle reaction.As shown in Fig. 9, immobilized PAE07 following 15 repeated cycles of catalytic reaction was still 86.2% relative to its initial activity. In the cyclic reaction, the decrease of enzyme catalytic ability is considered to be the leakage of enzyme protein and the structural change caused by long-term exposure to the substrate [42,43].

The reason for the improved operational stability and repeatability of the immobilized esterase PAE07 may be due to the porous structure and large internal surface area of the polystyrene resin, as well as the covalent cross-linking, which improves the rigidity and prevents the enzyme from leaking and inactivating. Liu et al. also obtained the repeated stability improvement of phospholipase after immobilization on polystyrene resin [50]. The immobilized esterase PAE07 has good operational stability, enzyme activity and enantioselectivity. It is better than other enzymes reported to Hydrolysis (*R, S*)-DMPM, and it is cheap and has the potential to be used in industrial production.

4. Conclusion

In short, the esterase PAE07 has been successfully adsorbed and covalently immobilized on the polystyrene resin D3520, and the immobilization conditions have been optimized, and the immobilized esterase PAE07 has also been characterized. Compared with the free esterase, the thermal stability and pH stability of the immobilized esterase PAE07 were significantly improved, and it could catalyze a higher substrate concentration. After the resolution of 15 repeated batches of (*R, S*)-DMPM, there was still had 86.2% relactive enzyme activity. The immobilized esterase PAE07 was a promising catalyst, showed excellent catalytic activity and satisfactory selectivity ($e.e._p$>99%), and the cost was relatively lower. This research provides an effective method for industrially enzymatic resolution of (*R, S*)-DMPM to produce (*R*)-metalaxyl.

Declarations

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Availability of data and material

The raw data used to support the findings of this study are available from the corresponding author upon request.

Code availability

The data processing and charts in this article were completed by Origin software (Origin Lab Co., Pro. 9.1, Hang Zhou, China).

Credit Author contributions

Zhou Mingpeng: Investigation, Data Curation, Writing - Original and Revised Manuscripts.

Xia Yuandan: Investigation, Data Curation.

Zhang Hongjun: Investigation, Formal analysis.

Yu Xinjun: Supervision, Writing - Review & Editing.

Zhang Yinjun: Conceptualization, Supervision, Writing- Review and Editing, Funding acquisition.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Participants have agreed to submit experimental data to the journal.

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Tables

Table 1 Selection of immobilized resin

Resin	Enzyme activity (U/g)
XAD1180N	5.2
H103	6.4
HAD7HP	9.3
850 JinKai	7.7
D3520	13.4
Primary amino resin	8.1
850 synthetic resin	6.3
HKA	9.5
D101	7.8
DM11	7.1

Table 2 Optimized enzyme loading conditions

Enzyme amount and resin ratio (mg/g)	Enzyme activity (U/g)
5:1	16.4
10:1	22.1
15:1	23.2
20:1	26.2
30:1	21.4
40:1	20.8
50:1	18.5

Figures

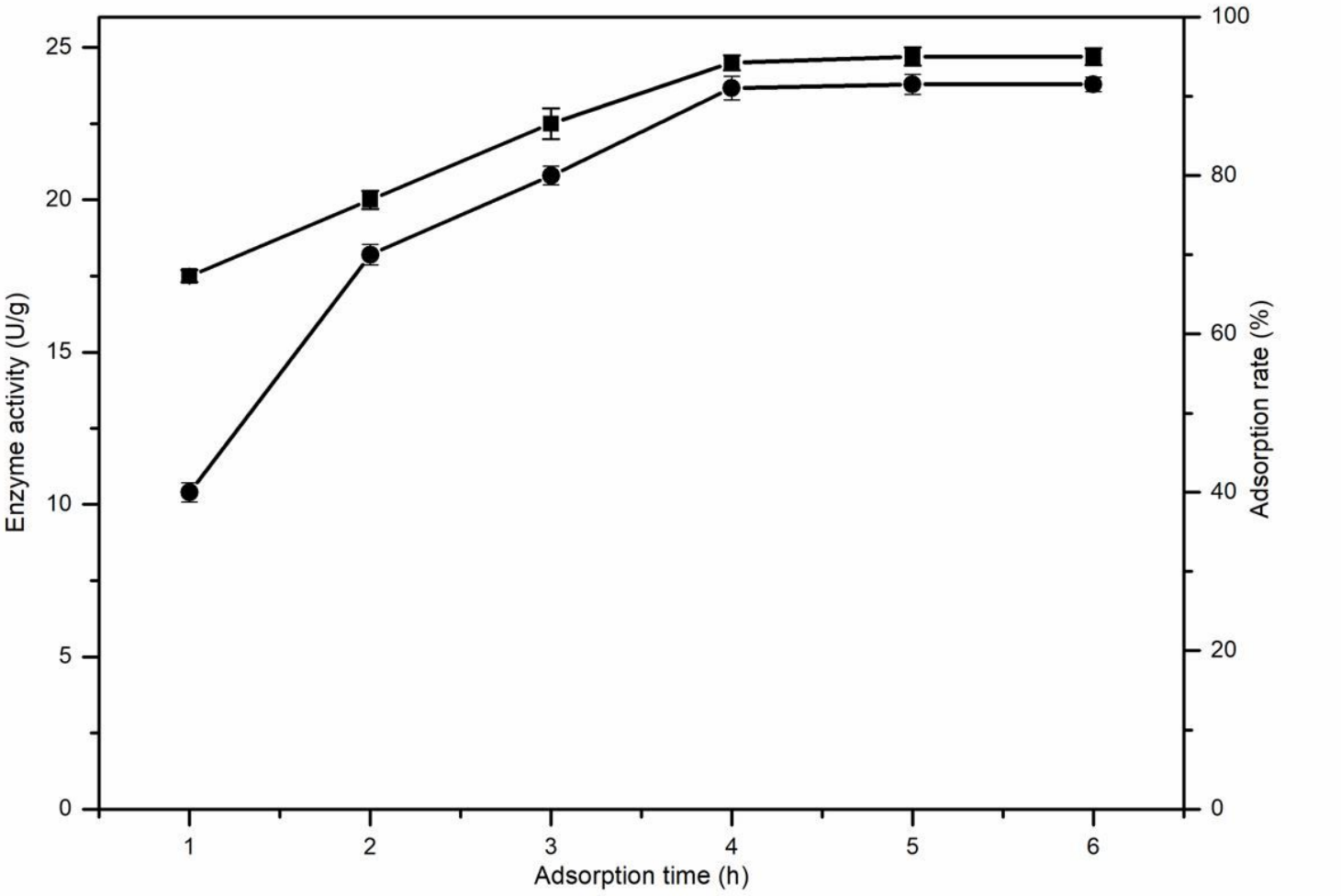

Figure 1

The effect of adsorption time on the immobilized enzyme. Symbols: "■,"enzyme activity; "●,"adsorption rate.

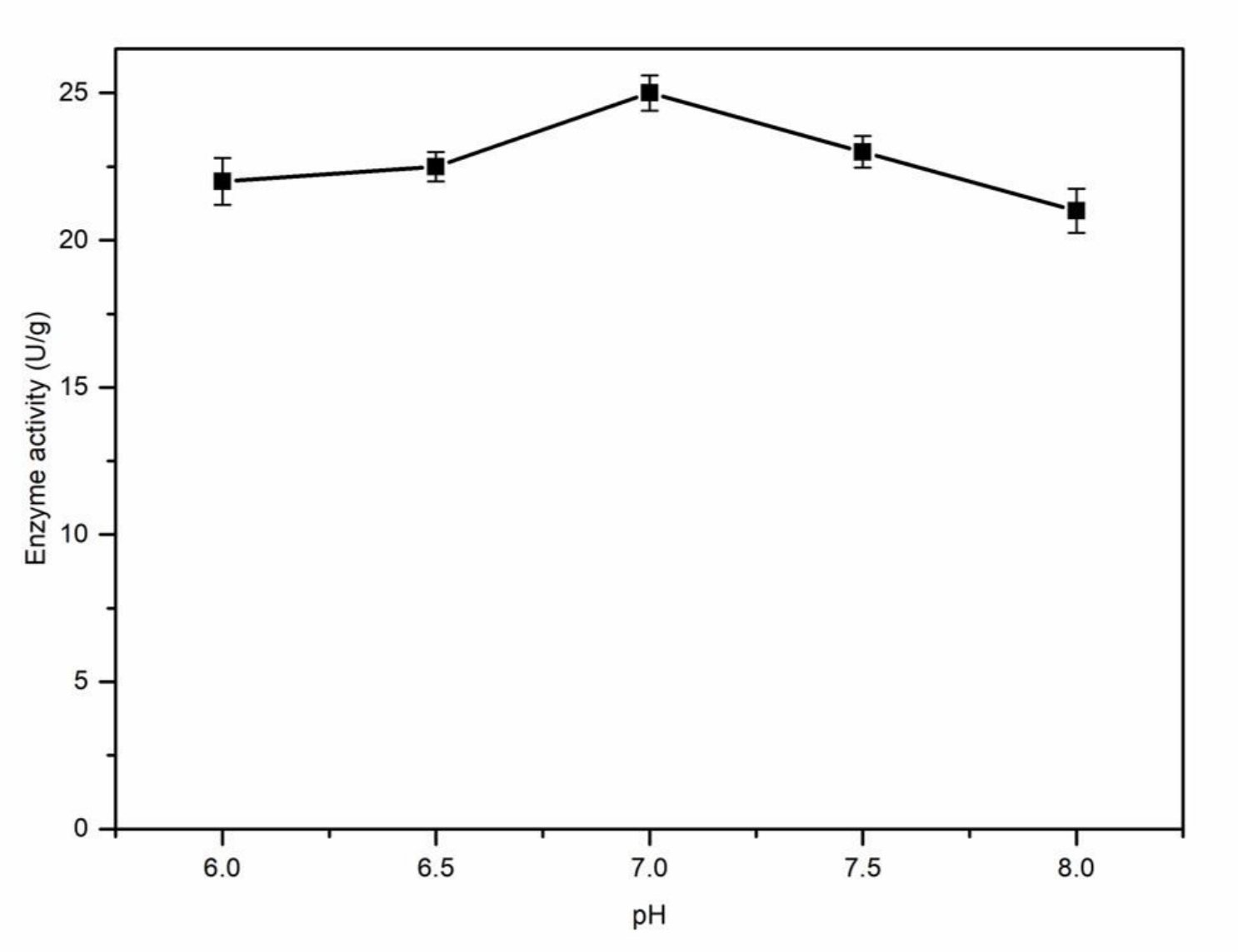

Figure 2

The effect of bufeer pH on the immobilized enzyme.

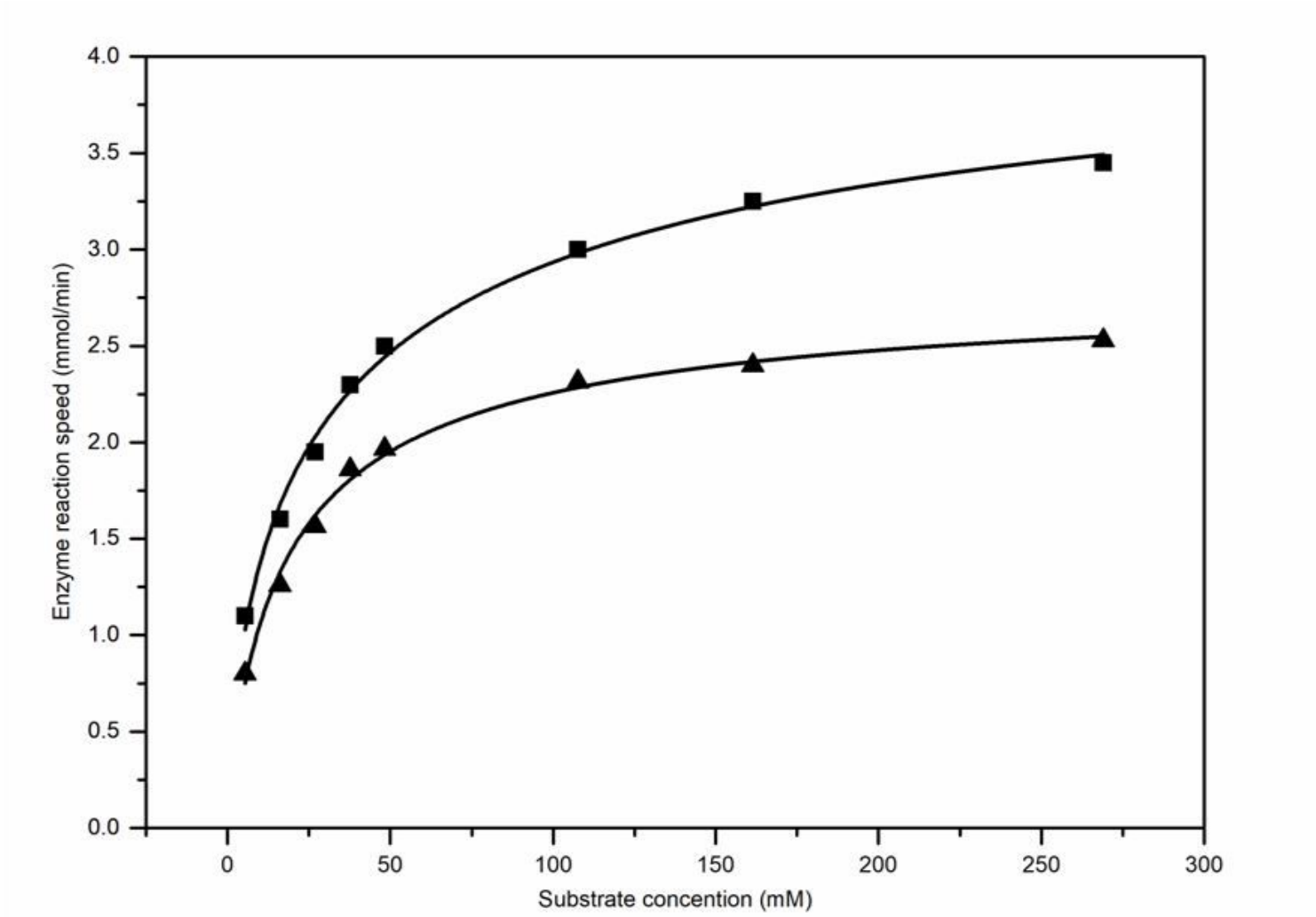

Figure 3

The relationship between enzyme reaction speed and substrate concentration. Symbols: "■," free eaterase; "▲," immobilized esterase PAE07

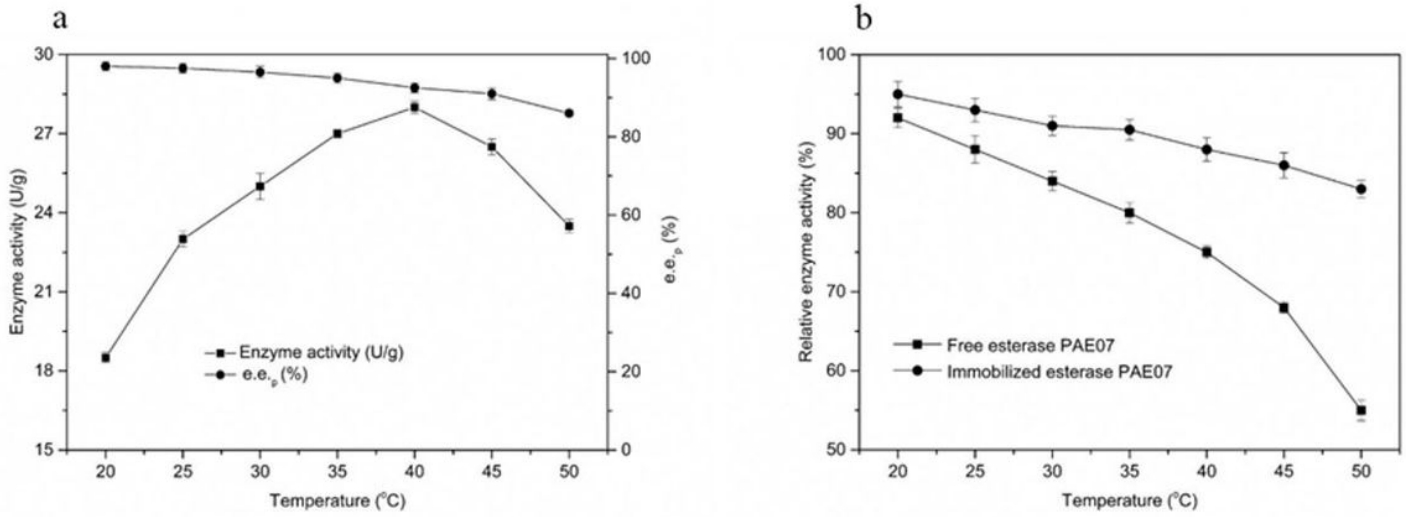

Figure 4

(a) The effect of temperature on the biocatalytic resolution of (R, S)-DMPM. Reaction conditions: 0.02 g immobilized enzymes and 10 μl (R, S)-DMPM in 1 mL 50 mM PH 7.0 PB buffer, 200 rpm, 20–50 °C, for 30 min. (b) Thermal stability of immobilized and free esterases. Reaction conditions: 0.02 g of immobilized enzymes after 12 h incubation at 20-50 °C and 50 mM (R, S)-DMPM in 1 mL 50mM PH 7.0 PB buffer, 200 rpm, 20–50 °C, for 30 min, and titrated with 4 M HCl.

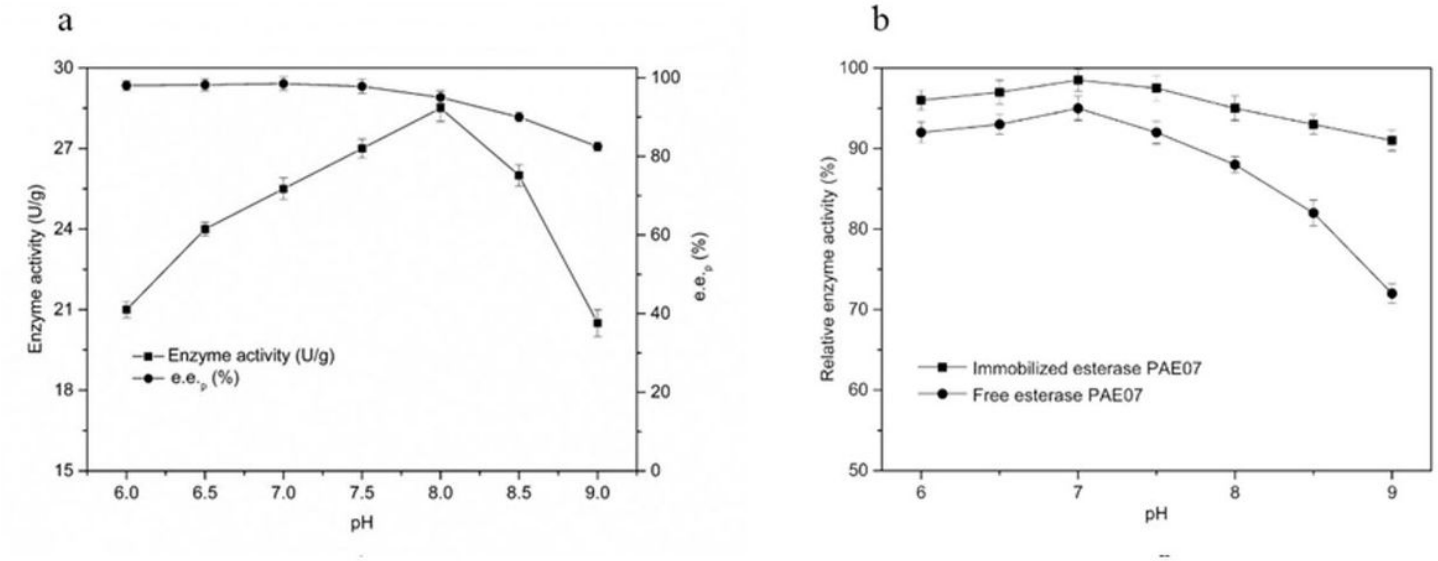

Figure 5

(A) The effect of pH on the biocatalytic resolution of (R, S)-DMPM. Reaction conditions: 0.02 g immobilized esterases and 50 mM (R, S)-DMPM in 1 mL 50mM pH 6.0-9.0 PB buffer, 200 rpm, 20–50 °C, for 30 min. (B) pH stability of immobilized and free esterases. Reaction conditions: 0.02 g of immobilized esterases after 12 h incubation at 35 °C, 50 mM (R, S)-DMPM in 1 mL 50 mM pH 7.0-9.0 PB buffer, 200 rpm, 35 °C, for 30 min, and titrating with 4 M HCl.

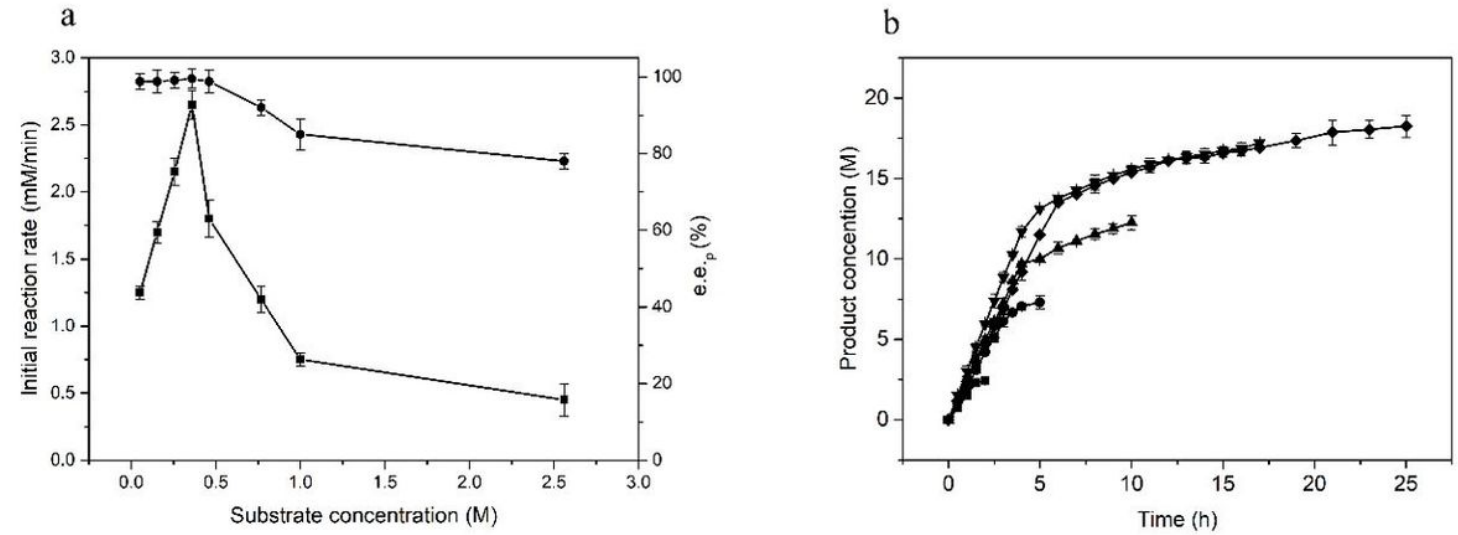

Figure 6

(a) The initial rate of enzyme reactions at different substrate concentrations. Symbols: “■,” initial reaction rate; “●,” e.e.p (b) Time course of product concentration at different substrate concentrations. Symbols: “■,” 0.05 M; “●,” 0.15 M; “▲,” 0.25 M; “□,” 0.35 M; “▼,” 0.45 M.

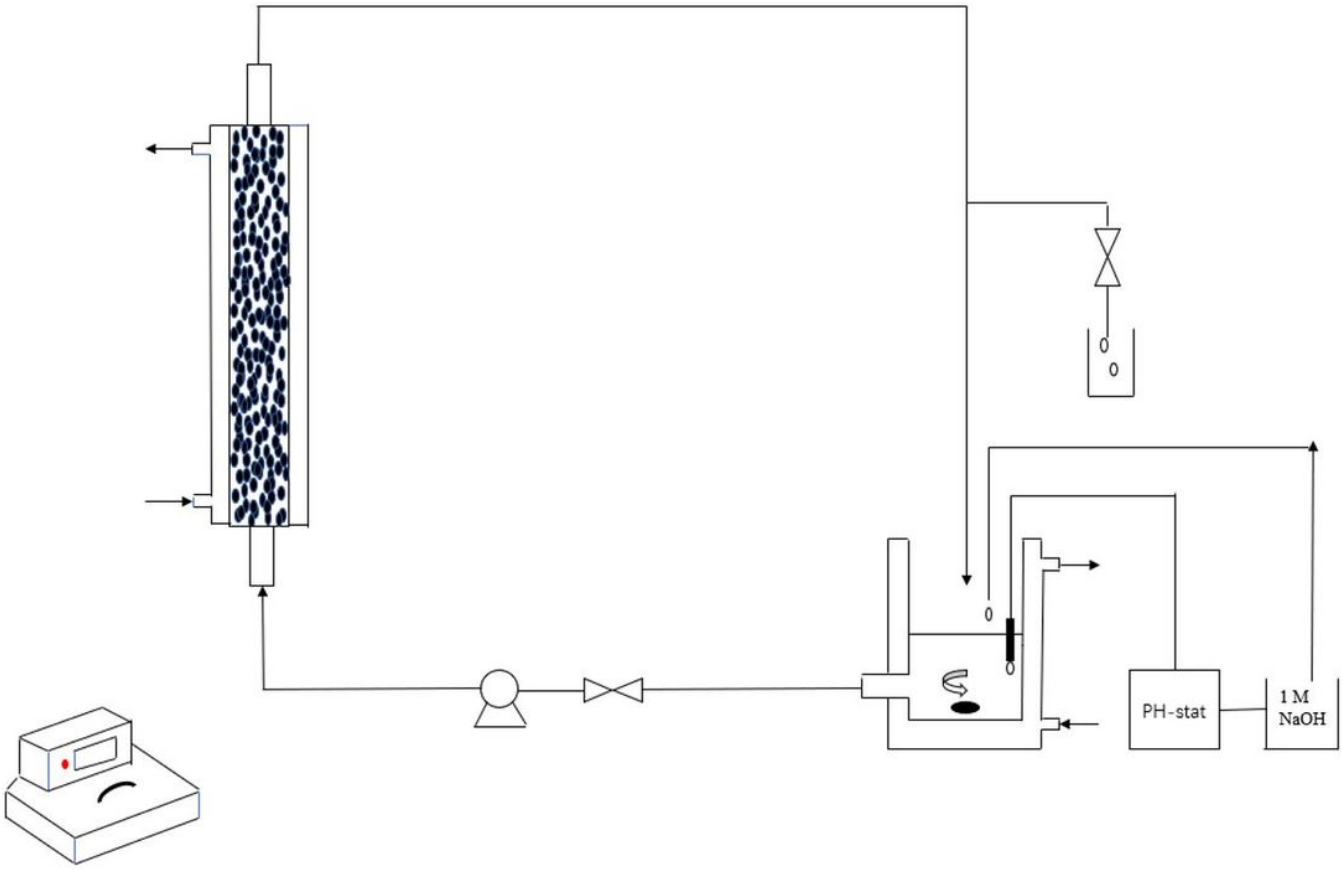

Figure 7

Preparation process and amplification reaction of immobilized esterase PAE07

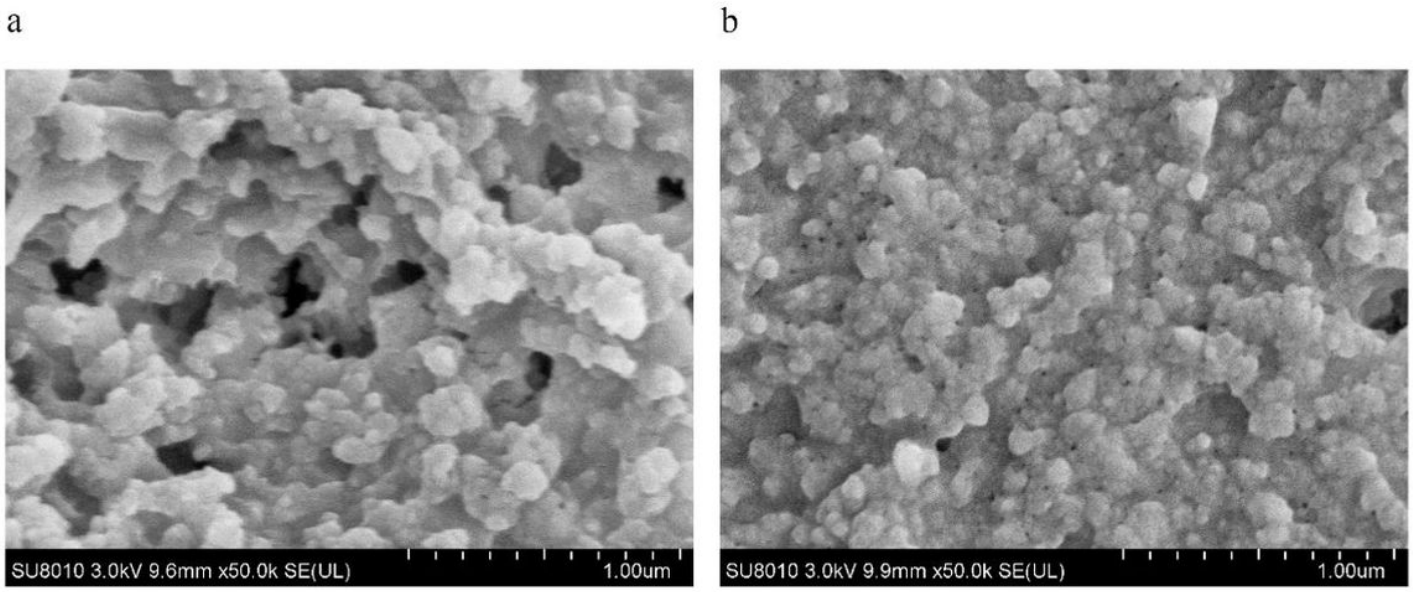

Figure 8

The surface characteristics of Carrier D3520 (a) and immobilized esterase PAE07 (b).

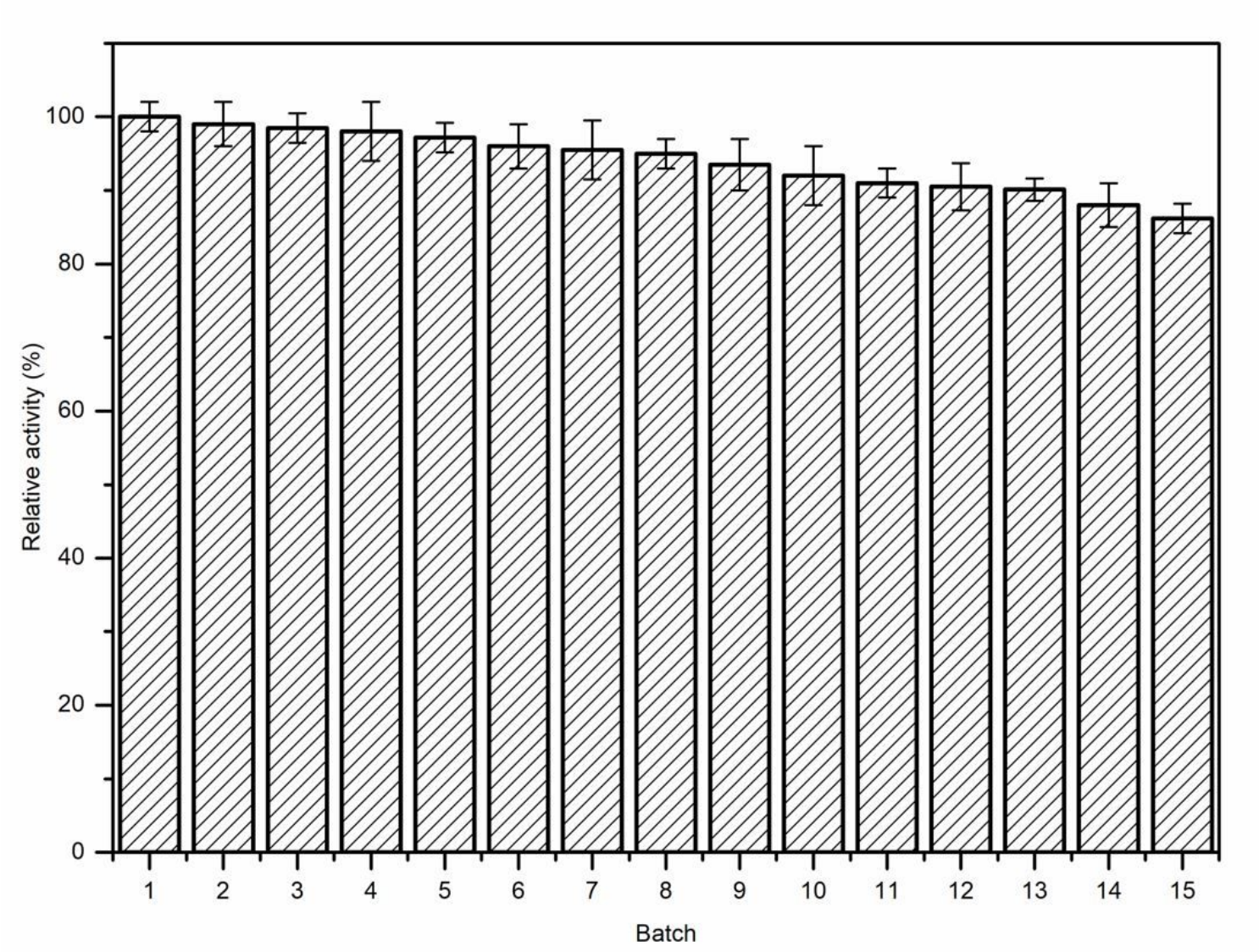

Figure 9

Reusability of immobilized esterase PAE07 on enantioselective reaction.Reaction conditions:1.0 g immobilized esterases, 350 mM (R, S)-DMPM in 50 mL 50 mM pH 7.5 PB buffer, 200 rpm, 35 °C, for 12 h,and titrated with 4 M HCl.

Supplementary Files

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