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Biocatalytic Epoxidation of Cyclooctene to 1,2-Epoxycyclooctane by a Newly Immobilized *Aspergillus niger* Lipase

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Abstract: A newly immobilized *Aspergillus niger* lipase (ANL@ZnGlu-MNPs) was employed for the preparation of 1,2-epoxycyclooctane by oxidation of cyclooctene. The chosen variables, including substrate concentration, reaction temperature, immobilized enzyme dose, and H_2O_2 dose, were optimized in the reaction system of ethyl acetate. The yield and the enantiomeric excess of the product were achieved at 56.8% and 84.1%, respectively, under the following optimum reaction conditions: the concentration of substrate (cyclooctene) was 150 mM, the dosages of immobilized enzyme (ANL@ZnGlu-MNPs) and hydrogen peroxide were respectively 100 mg and 4.4 mmol, and the reaction was carried out in the system of 4 mL ethyl acetate at 40 °C. Further study on the operational stability of ANL@ZnGlu-MNPs showed that more than 51.6% of product yield was obtained after reusing for ten batches. A novel immobilized lipase was prepared and applied to synthesize 1,2-epoxycyclooctane from cyclooctene. Although ANL@ZnGlu-MNPs performs well in operational stability and the reaction can achieve high enantiomeric purity of the product, the yield of the catalytic reaction needs to be further improved.

Keywords: *Aspergillus niger* lipase; epoxidation reaction; cyclooctene; epoxides

1. Introduction

Epoxides are important functional intermediates, which play an active role in many fields such as pharmaceuticals [1,2], pesticides [3], cosmetics [2], and materials [4]. Phenyl glycidyl ether is widely used in the copolymerization with anhydride or lactone to synthesize linear polyesters, as well as ionic polymerization [5], and 1,2-epoxycyclooctane can be applied to prepare various derivatives of cyclooctane [6].

The use of biocatalysts in the synthesis of epoxides by olefins has received extensive attention due to mild reaction conditions, environmental friendliness, excellent selectivity, and sustainability [7]. Lipases show outstanding performance in many different kinds of reactions, for instance, hydrolysis, transesterification [8], and epoxidation [9]. Novoym 435 obtained by gene expression of *Aspergillus oryzae* has been widely used in various lipase-catalyzed epoxidation reactions due to its high efficiency [9–11]. Several olefins including cyclohexene, cyclooctene, and 1-octene can be epoxidized by lipase to achieve a high yield of corresponding product from 75% to 100% in ethyl acetate [12]. In addition, enzyme immobilization is necessary for the epoxidation of alkenes in systems containing strong oxidant and toxic organic solvent. With the application of nanotechnology used in enzyme immobilization, many delightful results have been achieved in the activity or the stability of biocatalyst. For example, *Candida antarctica* lipase B-inorganic crystal nanoflowers showed 25 times higher activity

than the native lipase in the epoxidation of fatty acid [13]. Furthermore, the novel immobilized enzyme has better operational stability than commercial Novozym 435, obtaining an over 90% conversion rate after 20 cycles of recycling [13]. Hybrid magnetic cross-linked *Candida antarctica* lipase B aggregates showed outstanding thermal stability compared to the free lipase, retaining 60% of its initial activity after incubation at 60 °C for 40 min [14]. Another enzyme, *Aspergillus niger* lipase (ANL) (EC. 3.1.1.3), has been used in the hydrolysis of fats to diglycerides, glycerol, free fatty acids, and monoglycerides [15,16]. Additionally, some studies also reported that the ANL was able to transform the olefins to epoxides with high catalytic rates, such as the biosynthesis of α-pinene oxide from α-pinene [17], and epoxidized methyl oleate from methyl oleate [18]. According to the above fact, this study chose ANL as a biocatalyst for the epoxidation of alkenes and discussed the catalytic performance of the immobilized ANL on the biosynthesis of epoxidation.

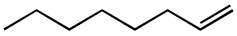
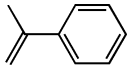
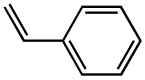
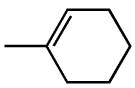
In this work, we firstly screened the optimal olefin substrates of the immobilized ANL for the production of epoxides. Moreover, the epoxidation reaction conditions of cyclooctene were assessed, including immobilized enzyme dose, reaction temperature, hydrogen peroxide dose, and substrate concentration. Furthermore, the operational stability of the immobilized ANL in the epoxidation reaction of cyclooctene was also evaluated.

2. Results and Discussion

2.1. The Catalytic Performance of ANL@ZnGlu-MNPs on the Epoxidation of Different Substrates

In order to determine the appropriate substrate, the epoxidation reactions of ANL@ZnGlu-MNPs were carried out with five kinds of olefins as the substrates, which were 2-phenyl-1-propene, 1-octene, 1-methyl-1-cyclohexene, styrene, and cyclooctene. As illustrated in Table 1, for the five substrates mentioned above, cyclooctene epoxidation obtained the maximum yield (47.2%). However, the yield of 1-methyl-1-cyclohexene was the worst (only 17.9%). In an aspect of enantiomeric excess, the epoxidation reactions of cyclooctene, styrene, and 1-methyl-1-cyclohexene showed the preferable optical purity of product at 80.4%, 85.8%, and 86.1%, respectively. Therefore, cyclooctene was selected for further study.

Table 1. Epoxidation of various olefins catalyzed by ANL@ZnGlu-MNPs.

Olefins	Structure	Yield (%)	e.e. (%)
1-octene		33.8	56.3 (*R*)
2-phenyl-1-propene		27.6	70.1 (*S*)
cyclooctene		47.2	80.4 (1*R*,2*R*)
styrene		23.4	85.8 (*S*)
1-methyl-1-cyclohexene		17.9	86.1 (1*R*,2*R*)

Reaction conditions: ethyl acetate 4 mL, various olefins 100 mM, ANL@ZnGlu-MNPs 75 mg, H_2O_2 4.4 mmol, 200 rpm, 40 °C.

The experimental data also suggested that the ANL can be used for the preparation of epoxides by the epoxidation of alkenes to achieve excellent optical purity of the product. In addition, this work indicated that an excellent enantiomeric excess (e.e.) could be obtained when the substrate contains significant steric hindrance of the group. However, the studied lipase showed a relatively low product yield compared to the typical lipase Novozym 435. For example, Novozym 435 can epoxidize a range of alkenes with the yield exceeding 75% [12]. Similarly, their results proved that excellent yields could be achieved when using cyclic olefins as substrate.

2.2. Effect of Substrate Concentrations on the Epoxidation of Cyclooctene by ANL@ZnGlu-MNPs

Figure 1 displays the effect of substrate concentrations within the range from 25 mM to 225 mM, on the oxidation of cyclooctene by ANL@ZnGlu-MNPs. Product e.e. declined slightly from 87.4% to 82.7%, while substrate concentration improved, indicating that the cyclooctene concentration examined had little impact on the stereoselectivity of ANL@ZnGlu-MNPs. Differently, as the substrate concentration increased from 25 mM to 150 mM, the initial rate of reaction was boosted from 10.7 mM/h to the maximum 15.6 mM/h; nevertheless, the initial rate of reaction remarkably decreased and the concentration of cyclooctene continually rose. This result indicated that substrate inhibition occurred noticeably when the concentration of cyclooctene exceeded 150 mM. Furthermore, the highest yield of product could be achieved at 150 mM of substrate concentration, while no significant change was found in the product yield with substrate concentration further increased. The results suggest that the reaction was mainly determined by the substrate inhibition but not product inhibition at the substrate concentrations examined. Consequently, 150 mM of cyclooctene concentration was selected for further study.

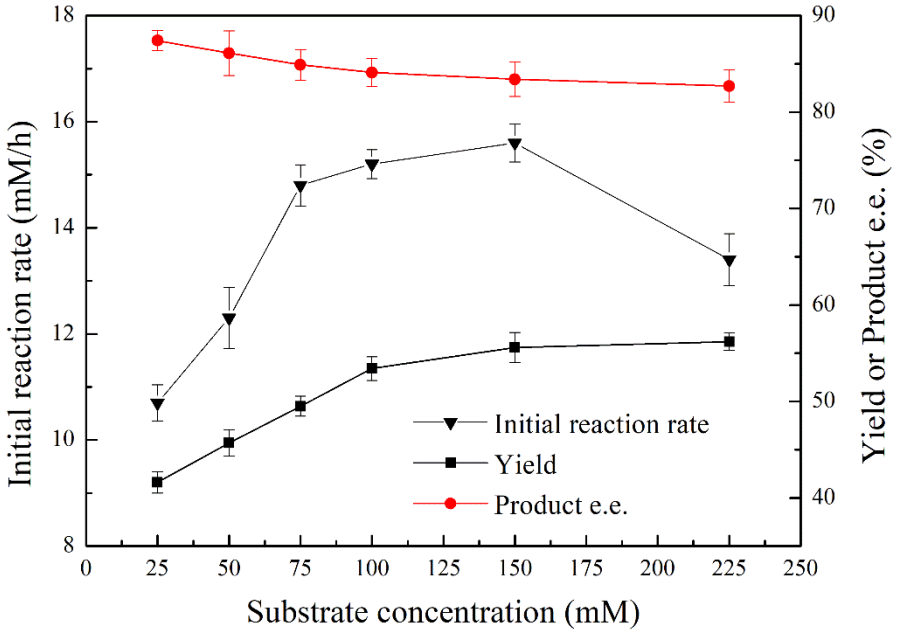


Figure 1. Effect of substrate concentration on epoxidation of cyclooctene catalyzed by ANL@ZnGlu-MNPs.

2.3. Effect of Reaction Temperatures on the Epoxidation of Cyclooctene by ANL@ZnGlu-MNPs

The impact of reaction temperature on the catalytic performance of ANL@ZnGlu-MNPs is shown in Figure 2. The tested reaction temperature affected the product e.e. values slightly but had a significantly impact on product yield and initial rate. The initial reaction rate gradually increased to the maximum as the temperature rose from 25 °C to 40 °C. However, when the reaction temperature rose further, the initial rate of the reaction decreased rapidly. The results indicated that more than 40 °C of reaction temperature would accelerate the inactivation of ANL@ZnGlu-MNPs. The maximum of initial reaction rate and product yield could be achieved simultaneously at 40 °C.

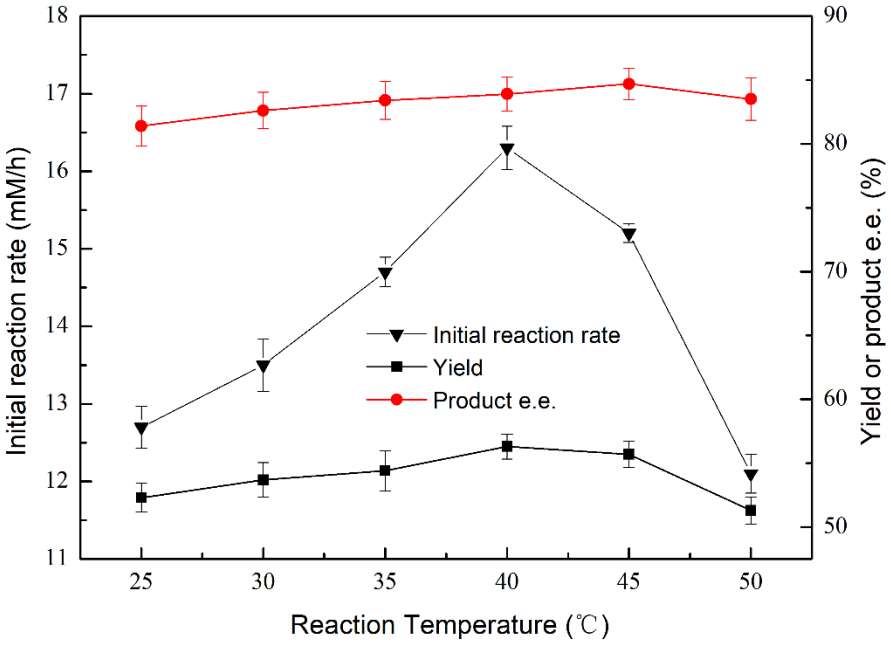


Figure 2. Effect of temperature on epoxidation of cyclooctene catalyzed by ANL@ZnGlu-MNPs.

Reaction temperature, as one of the essential factors for enzyme catalysis, not only had a dramatic impact on the number of active substrate molecules but also influenced the catalytic activity of the enzyme remarkably. Our results showed that the catalytic activity of lipase would be damaged if the reaction temperature exceeded 40 °C. The yield of the product did not change much when the temperature ranged from 25 °C to 45 °C. However, when the reaction temperature reached 50 °C, the yield of the product decreased significantly. This result may be explained in this way: the simultaneous action of hydrogen peroxide and high temperature led to the damage of the enzyme and the reduction of enzyme activity [19].

2.4. Effect of ANL@ZnGlu-MNPs Doses on the Epoxidation of Cyclooctene

Figure 3 reveals the effect of different doses of ANL@ZnGlu-MNPs on the epoxidation of cyclooctene. There was no significant change in e.e. value of the product at different immobilized enzyme doses. With the increase of enzyme dosage, the initial rate of the reaction gradually increased to the maximum. When the addition of immobilized enzyme exceeded 100 mg, the product yield would only change slightly. However, the product yield was notably lower with an enzyme dose less than 100 mg, demonstrating that the reaction was affected not only by the reaction time but also by the catalyst dose. The similar result suggests that the yield of ethyl (*R*)-3-hydroxybutyrate is also affected by the immobilized cell dose [20]. Other researchers, however, have found that there was no significant change in the yield of HMF at different biocatalyst doses [21]. Finally, we chose the concentration of 100 mg as the best dosage of ANL@ZnGlu-MNPs.

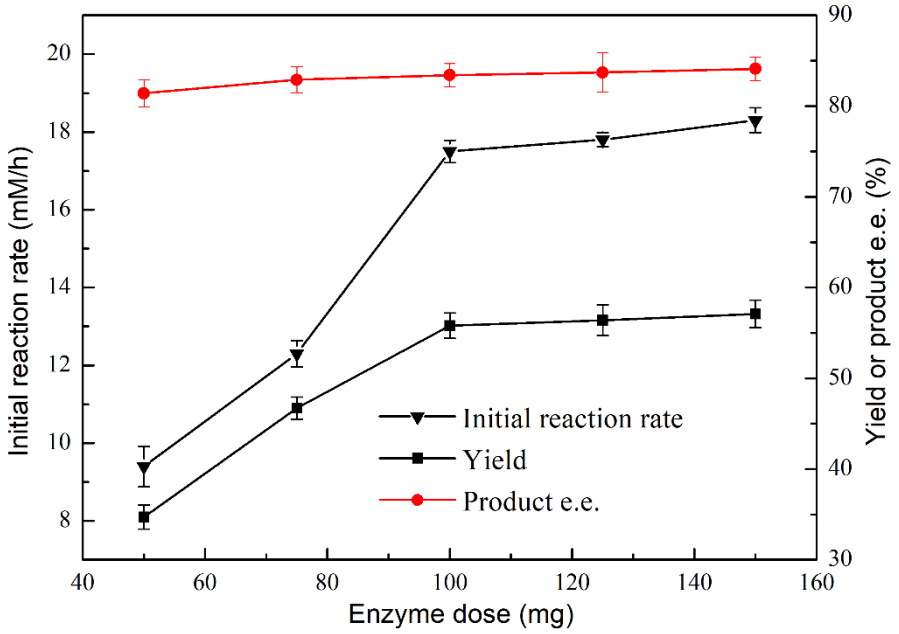


Figure 3. Effect of ANL@ZnGlu-MNPs dosage on epoxidation of cyclooctene catalyzed by ANL@ZnGlu-MNPs.

2.5. Effect of H_2O_2 Doses on the Epoxidation of Cyclooctene by ANL@ZnGlu-MNPs

Figure 4 shows the effect of H_2O_2 doses on the synthesis of R-cyclooctene oxide. The dosage of hydrogen peroxide examined had only a marginal impact on the product optical purity. Our results showed that the amount of H_2O_2 had a remarkable effect on the initial reaction rate and product yield. The initial reaction rate and product e.e. value increased to the maximum with the increase in the amount of hydrogen peroxide from 1.1 mmol to 4.4 mmol. Moreover, the further increase in the amount of H_2O_2 resulted in a decline of both of the two values mentioned above. The result indicates that adding more than 4.4 mmol of hydrogen peroxide will destroy the activity of ANL@ZnGlu-MNPs.

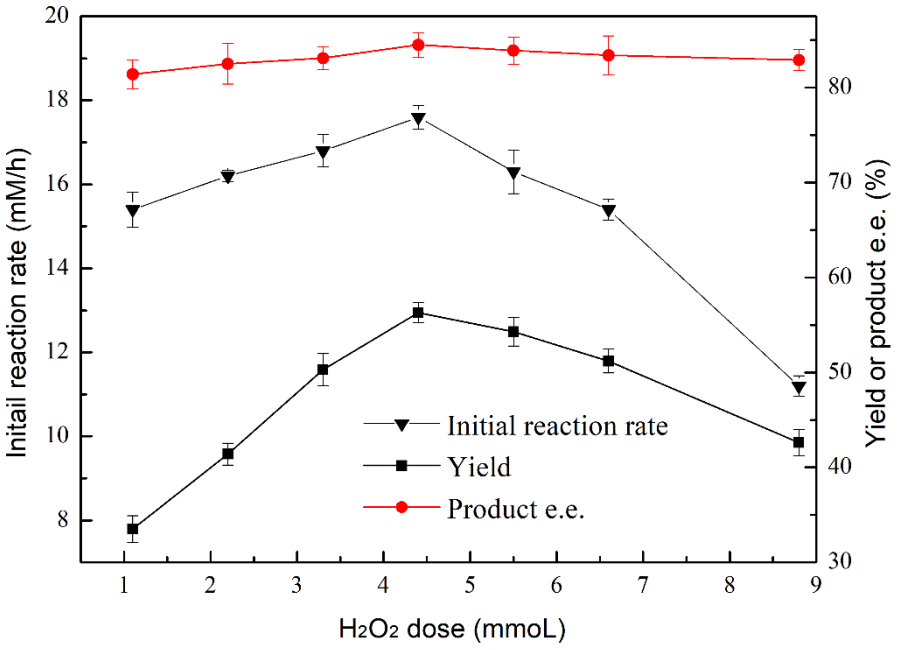


Figure 4. Effect of H_2O_2 dosage on epoxidation of cyclooctene catalyzed by ANL@ZnGlu-MNPs.

H_2O_2, as an essential factor in the epoxidation reaction, can provide oxygen to start the reaction. However, excessive hydrogen peroxide can lead to inactivation of biocatalysts because of its strong oxidation capacity [22]. In addition, many side effects can be caused by the excessive addition of hydrogen peroxide. Finally, we chose to add the oxygenant in four batches to avoid the shortcomings mentioned above. The results showed that the initial rate and product yield of the reaction decreased significantly when the amount of hydrogen peroxide added to the reaction system exceeded 1.1 mmol. This result meant that the lipase used might be inactivated by adding more than 1.1 mmol hydrogen peroxide in one step, resulting in a lower yield of the product. Moreover, when the total amount of hydrogen peroxide was more than 4.4 mmol, the product e.e. values decreased slightly with the increase of oxygenant, indicating that the side effects occurred. According to these results, we tended to inject hydrogen peroxide multiple times. Although these results differed from some published studies, which suggested that there was no difference in the product yield when hydrogen peroxide was added into the reaction system in one step or multiple steps, even higher initial reaction rates could be observed with one step of complete addition [23].

2.6. Operational Stability of ANL@ZnGlu-MNPs

As shown in Figure 5, the relative yield of ANL@ZnGlu-MNPs was still over 85% when it was reused continuously for five batches, while with the increase of reuse times, the catalytic properties of the immobilized enzyme decreased significantly. For instance, when the immobilized enzyme was reused ten times, the relative product yield of the immobilized enzyme was approximately 52%. Additionally, no significant change in the product e.e. was observed with the increase of recycling number, within the range from 82.5% to 84.5%.

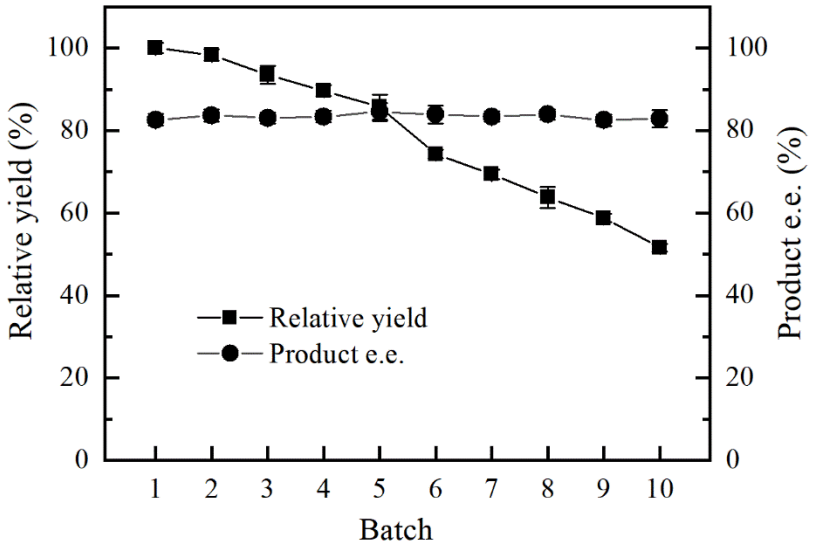


Figure 5. Operational stability of ANL@ZnGlu-MNPs in the epoxidation of cyclooctene.

The main advantages of enzyme immobilization are easy separation and stronger stability. The improvement of operational stability of an enzyme is a benefit for reducing the cost of the catalytic reaction. Comparison of our findings with those of other studies confirms that our immobilized lipase had better operational stability. For example, the catalytic yield of *Candida antarctica* lipase B immobilized in hydroxyl-propyl-methyl cellulose microemulsion-based organogels preserved only 25% of the initial yield when it was reused ten times [10], while the yield of ANL@ZnGlu-MNPs retained more than 50% of the initial yield after the same number of recycling times, as mentioned above. This result indicated that the catalytic activity of ANL was still partly affected by the components of the reaction system, although the ANL was immobilized. However, it also emphasized the necessity of ANL immobilization in industrial applications.

3. Material and Methods

3.1. Material

Aspergillus niger lipase was purchased from Shenzhen Lvweikang Bio-Engineering Co. Ltd. (Shenzhen, China). The preparation of immobilized lipase (ANL@ZnGlu-MNPs) was referenced by our previous work [24]. Ethyl acetate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Hydrogen peroxide and acetic acid were purchased from Chinasun Specialty Products Co., Ltd. (Jiangsu, China), and 2-phenyl-1-propene, 1-octene, 1-methyl-1-cyclohexene, styrene, and cyclooctene were purchased from Shaen Chemical Technology (Shanghai) Co., Ltd. (Shanghai, China). All reagents used in the study were from commercial sources and of analytical grade.

3.2. Screening of Substrates

The appropriate substrate for the epoxidation of ANL was sought from five olefins as follows: 2-phenyl-1-propene, 1-octene, 1-methyl-1-cyclohexene, styrene, and cyclooctene. Briefly, 100 mg of ANL@ZnGlu-MNPs and 8.8 mmol of acetic acid were mixed with 4 mL of ethyl acetate, and one of the above substrates (0.6 mmol) was injected. The reactions were subsequently conducted in a constant temperature shaker at 40 °C and 200 rpm. During the reaction, hydrogen peroxide solution (4.4 mmol in total, 30%, *w*/*w*) was added four times within one hour to drive the reaction. No special annotation of hydrogen peroxide solution means that its concentration is 30% (*w*/*w*) and its amount is in total. Samples (20 uL) were periodically withdrawn from the reaction mixture and extracted with the equal volume of ethyl acetate. *n*-Dodecane was used as the internal standard. After adequate extraction, the supernatants obtained by centrifugation at 13,000 rpm for 3 min were used for the analysis of product yields and enantiomeric excess (e.e.) values by gas chromatography.

3.3. Reaction Parameter of ANL@ZnGlu-MNPs

In the typical experiment, the reaction solvent consisting of ethyl acetate (4 mL) and acetic acid (8.8 mmol) was contained in a 10-mL Erlenmeyer flask capped with a septum. The amount of immobilized ANL arranged from 50 mg to 150 mg was added. The above mixture was previously incubated in a constant temperature shaker at different temperatures (25–50 °C) and 200 rpm for 15 min. Then, the reaction was triggered by the addition of substrate at various amounts (25 mM to 225 mM), and 1.1–8.8 mmol of hydrogen peroxide was added four times within one hour during the reaction. Samples (20 uL) were periodically withdrawn from the reaction mixture and extracted with an equal volume of ethyl acetate. N-dodecane was used as the internal standard. After adequate extraction, the supernatants obtained by centrifuging at 13,000 rpm for 3 min were used for the analysis of product yields and product e.e. values by gas chromatography.

3.4. Operational Stability of ANL@ZnGlu-MNPs

The reuse batch of the immobilized lipase in the epoxidation of cyclooctene was used to evaluate the operational stability. The evaluative reaction system comprised 4 mL of ethyl acetate, 8.8 mmol of acetic acid, 0.6 mmol of cyclooctene, 100 mg of ANL@ZnGlu-MNPs, and 4.4 mmol of hydrogen peroxide. The epoxidation reaction was conducted at 40 °C and 200 rpm for 6 h. In the meantime, 4.4 mmol of hydrogen peroxide was added four times within one hour. After each batch, ANL@ZnGlu-MNPs was separated from the reaction medium by a magnet, washed thrice with ethyl acetate, and dried at 40 °C for 30 min. The dried immobilized enzyme was added to a fresh batch of reaction medium again. The product yield of each batch was analyzed, and the relative product yield of the first batch was defined as 100%.

3.5. Analytical Methods

The product was assayed for the e.e. values and yield using a Shimadzu 2010 gas chromatograph (Japan) equipped with a flame ionization detector and an HP-Chiral-10B (30 m × 0.25 mm, Agilent, Santa Clara, CA, USA) chiral column. The carrier gas in GC analysis was nitrogen. The initial reaction rates were calculated according to the generated amount of product within a 30 min reaction. All experiments were repeated at least twice. The relative standard deviation was to be not be greater than 1%, and the data were expressed as mean ± standard deviation.

4. Conclusions

In this investigation, ANL@ZnGlu-MNPs was developed as an efficient and selective biocatalytic approach for the epoxidation reaction of cyclooctene and applied to synthesize chiral 1,2-epoxycyclooctene. ANL@ZnGlu-MNPs exerted excellent enantioselectivity and good operational stability for the epoxidation of cyclooctene. The enantiomeric excess rate of the product could reach more than 88% when it was reused for five batches. However, it probably still has enormous potential for increasing the product yield. Further study should be concentrated on the advancement of epoxide yield and the discovery of enzymes with highly efficient catalytic performance.

Author Contributions: Q.C., M.Z., and W.L. conceived and designed the experiments; Q.C. and F.P. performed the experiments with partial participation by Q.C. and G.X.; Q.C. analyzed the data and wrote the manuscript; F.L. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

e.e.	enantiomeric excess
ANL	*Aspergillus niger* lipase
ZnGlu	Zn-glutamate
MNPs	magnetic iron oxide nanoparticles
ZnGlu-MNPs	ZnGlu-coated MNPs
ANL@ZnGlu-MNPs	ANL immobilized on ZnGlu-MNPs

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