Directed evolution and immobilization of new lipase Lip 906

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Abstract. In this experimental study, a new lipase named Lip 906 was screened out from a metagenomic library in the laboratory. To improve the stability of the enzyme and develop and apply it as soon as possible, we adopted directed evolution and immobilization methods. A random mutation library was constructed by error-prone PCR and finally, a mutant lipase Lip 5-D with increased enzyme activity was screened out and immobilized. The activity of the mutant enzyme Lip 5-D was improved by 4 times compared with the wild-type lipase Lip 906. The optimal reaction temperature rose by 4 °C, and by 3 °C after immobilization. The optimal reaction pH increased from 7.8 to 7.5. Both temperature stability and pH stability were improved. The mutant enzyme Lip 5-D can maintain about 70% of the relative activity after incubation at 65 °C for 2 h, and it can keep 60% at pH 3-10. Error-prone PCR and immobilization improve the catalytic activity and stability of the enzyme, and promote its development and application in many industries.

1 Introduction

Lipase (*E.C. 3.1.1.3*), also known as acylglycerol hydrolases, is widely present in prokaryotes (e.g. bacteria[1,2]), eukaryotes (e.g. molds[3]), mammals, and plants[4,5]. Lipase can hydrolyze esters to release monoglycerides, diglycerides, glycerol and free fatty acids[6-8]. In addition to catalyzing the hydrolysis[9] and synthesis of glycerides[10-12], lipase can also catalyze the transesterification[6]and synthesis of biosurfactants[13], peptides[8], polymers[9] and drugs[14]. Especially, the stereospecificity of certain lipases can be adopted to catalyze the resolution of optical isomers and the synthesis of chiral drugs. Therefore, lipase and its modified preparations are used in many fields, such as food and nutrition, daily chemical, oleochemical and agrochemical industries, paper industry, detergent and biosurfactant synthesis, and drug synthesis. However, since the current production cost of lipases is still higher than traditional chemical catalysts and to meet the needs of industrial production and mining, developing new types of microbial lipases with high catalytic activity and stability is an urgent need for industrialization.

Since the rise of error-prone PCR in the 1980 s[15], directed evolution technology [16] has flourished, such as DNA shuffling, staggered extension process, and random-priming in vitro recombination, making it convenient to obtain high-quality and efficient biocatalytic enzymes[17-19]. Directed evolution is mainly divided into the establishment of mutation library and the directed screening with specific protein characteristics.

As natural biocatalysts, enzymes often have strong substrate specificity and high catalytic efficiency. They are widely used in modern industry. However, free enzymes are easily affected by the reaction environment and have poor stability, which restrict the development and application of free enzymes[20,21]. Therefore, the enzyme immobilization technology has emerged. This technology, formally proposed in 1973[22], can successfully restrict enzymes to a certain range for catalytic reactions, simplifying the subsequent separation and purification. Compared with the free-state enzymes, the immobilized enzymes have certain changes in properties, which mainly include stability, reaction temperature and reaction pH. At present, the more commonly-used enzyme immobilization methods are adsorption[23], covalent binding[24], embedding [25] and cross-linking[26]. Chitosan is a product from the deacetylation of chitin, which is widely present in the shells of crustaceans. Because chitosan has structural properties similar to glucan amine, it is used as a carrier for enzyme immobilization, which improves the preservation stability and operational stability of some enzymes. For example, the heat stability as well as acid and alkali resistance of marine microorganism *YS2071* lipase was significantly improved after immobilization with chitosan[27]. Li et al. [28] fixed pectinase with chitosan and clarified *maca juice* with high operational stability. Moreover, corn peptide fixed with chitosan and calcium alginate retained 88.9% of the enzyme activity even after storage after 35 days[29]. Cappannella et al.[30] used chitosan-immobilized lysozyme for lysis of lactic acid bacteria in beer production, and achieved good test results in a small reactor.

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In this experimental study, chitosan was used as a carrier to immobilize the mutant lipase Lip906 and to increase its stability and recyclability. It is expected to be applied into the food industry, medical field, and environmental protection[31-33].

2 Materials and Methods

2.1 Strains and Plasmids

The lipase Lip906 gene was obtained by constructing a soil metagenomic library and ligating to the plasmid *pET-32a(+)* (Novagen) and was stored at -80 °C. The *E. coli* DH5α (clone strain) and *E.coli* BL21 (expression strain) were stored at -80 °C in our laboratory. The error-prone PCR kit was bought from Beijing Tianenze Company. The plasmid extraction kit (EZNA®Plasmid Mini Kit), plasmid gel extraction kit (EZNA® Gel Extraction Kit), and PCR product recovery kit (EZNA®.Cycle-Pure Kit) were purchased from OMEGA Company. Tool enzymes including restriction endonucleases *Hind III*, *EcoR I*, and quantitative DNA maker were offered by TaKaRa Biological Company. T4 DNA ligase was produced by Fermentas Company. Primers were synthesized by Novagen. Gene sequencing was completed by BGI. Antibiotics including ampicillin (AMP), isopropyl-β-D-thiogalactopyranoside (IPTG), and X-gal (5-bromo-4-chloro-3-indole-α-D-Glycoside) were purchased from Sigma.

2.2 Chemical reagents

Reagents used here included yeast extract, tryptone (Oxoid, UK); Gum Arabic (imported subpackage, Shanghai Shenggong); electrophoresis agarose (imported subpackage), tris (Beijing Probo Biotechnology); acetonitrile (Sigma); tributyrin, glutaraldehyde (Aladdin); acetone, sodium alginate, chitosan (Guangdong Onco Biotechnology). Moreover, ρ-nitrophenyl palmitate (C16), ρ-nitrophenyl myristate (C14), ρ-nitrophenyl laurate (C12), ρ-nitrophenyl laurate (C12), ρ-nitrophenyl decanoate (C10), ρ-nitrophenyl caprylate (C8), ρ-nitrophenyl caproate (C6), ρ-nitrophenyl butyrate (C4) and ρ-nitrophenyl acetate (C2) were purchased from Sigma. Other chemical reagents were analytical pure and produced in China.

2.3 Cloning, expression and purification

The primers were designed according to the gene of lipase Lip906. A pair of primers, including Lip906-F and Lip906-R, introduced *EcoR* I and *Hind* III restriction sites at both ends of the primers. The primer sequences were as follows:

Lip906-F:
5'CCGGAATTCATGACAACACCAGCAGCTAC CATCGAA GG 3' (the underlined part is *EcoR* I restriction site);

Lip906-R:
5'CCCAAGCTT TCAGGGGCAAACACCGGTGGG 3' (the underlined part is *Hind* III restriction site). With the plasmid containing pUC118-Lip906 as the template, and Lip906-F and Lip906-R as primers, PCR amplification was conducted on Prime STAR^{TM}Max Premix. The purified PCR product and vector *pET-32a(+)* with restriction fast endonuclease *Hind* III and *EcoR* I were double-enzyme digested respectively. After the digestion reaction, the digested product was subjected to agarose gel electrophoresis, and the gel was cut and recovered. Then they were connected under the action of T4 DNA Ligase. The product was converted by electric shock, and entered into calcium transcompetent cells to form clones. After that, the recombinant lipase Lip906 was purified using a Novagen's His.Bind®Resin kit, and the protein expression and purity were finally verified by sodium dodecyl sulfate - 12% polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Directed evolution

With recombinant lipase as the mutation template, each clone in the random mutation library was picked, spotted on the lipase screening plate (containing 100 μg/mL Amp and 0.1 mM IPTG), and cultivated in a constant temperature incubator at 37 °C for 2 to 3 days. After that, the mutant clone with the larger hydrolysis circle was taken, and sent to induced expression under optimal conditions. A crude enzyme solution was prepared by sonication. Reagents with different lengths of carbon chains were used as the substrates to accurately determine the enzyme activity. The wild-type lipase Lip906 was used as a control. Finally, the positive mutant clones with significantly improved enzyme activity were confirmed, and then sent to the gene company for sequencing. The location of the mutation site was determined by comparing the sequence and amino acid with the wild type.

2.5 Enzyme activity evaluation

Enzyme activity was evaluated by measuring the absorbance of ρ-nitrophenol at OD 405 nm. In detail, into a 400 μl reaction system, 10 μl of the crude enzyme solution, 10 μl of 1 mM substrate, 0.04 M Britton-Robinson buffer, and 1% acetonitrile were added, mixed well, and reacted at 45 °C for 15 min. Three parallel experiments and a blank control experiment were set up for the reaction. After the reaction, the reaction system was added to a 96-well plate, and the absorbance at OD405 nm was measured with a microplate reader. The enzyme activity was defined as the amount of the enzyme required to hydrolyze the substrate to produce 1 μmol ρ-nitrophenol per unit time.

2.6 Enzymatic properties

The substrate specificity of lipase Lip906 was detected to measure the hydrolysis activity of different length carbon chains (C2-C16) by the above method. The optimal reaction temperature was determined by measuring the enzyme activity from 30 to 70 °C (5 °C interval). The optimal reaction pH of the enzyme was identified from pH 3 to 10. To determine the thermal stability of the enzyme,

we measured the residual activity after the reaction at 30 to 70 °C (5 °C interval) for 2 hours under the optimal pH. To evaluate the pH stability of the enzyme, we measured the residual activity after 2 hours of reaction at pH 3-10 at the optimum temperature. The metal ions at the final concentration of 1 mM, 10 mM (Cu^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ag^{2+}, Zn^{2+}, Ni^{2+}) and chelating agent EDTA were detected. The role of organic solvents was clarified when the final concentration was 1%, 15%, and 30%.

2.7 Prediction of mutant enzyme structure and analysis of mutation site

The genome of the mutant was extracted and submitted to BGI to determine its mutant base sequence. The sequencing results were sequenced in the NCBI (http://www.ncbi.nlm.nih.gov/) database with the wild-type lipase Lip906 as a control to determine the specific location of the mutant base. The base sequence was converted into an amino acid sequence in the Sequence Manipulation Suite (SMS). The amino acid sequence of the mutant protein was submitted in PHYRE2 (http://www.sbg.bio.ic.ac.uk/) to predict its three-dimensional structure and the forecast result was automatically sent to the mailbox in PDB format. The three-dimensional structure diagram was opened in PDB format in Pymol, and the instructions were entered to mark the position of the mutant amino acid in the three-dimensional structure diagram. Together with the enzymatic property study of the mutant enzyme Lip5-D, we analyzed the effect changes caused by the mutation site.

2.8 Preparation of immobilized mutant lipase

Chitosan, sodium alginate and eggshell were used as carriers, and each carrier material was cross-linked with glutaraldehyde to prepare an immobilized mutant lipase. The enzyme activity recovery rate was used as an index to select the most suitable carrier material.

2.9 Optimization of preparation conditions of immobilized mutant lipase and study on enzymatic properties

Chitosan was used as a carrier to prepare immobilized mutant lipases, and the optimal dosage of chitosan within 0.01 -0.08 g (0.01 g interval) was determined. The best adsorption temperature within 10 -45 °C (5 °C interval) was identified. Under the same other conditions, after adsorption for 0.5, 2, 4, 6, 8, 10, 12 h, the recovery rate of enzyme activity was measured to determine the best adsorption time. Then the enzymatic properties of the immobilized mutant lipase were evaluated according to the above method, including the optimal temperature and temperature stability, the optimal pH and stability, and the influence of metal ions and organic solvents. Finally, a certain amount of immobilized lipase chitosan pellets were stored at 4 °C and room temperature, and the storage stability was determined by the enzyme activity.

2.10 Amino acid registration number

The protein sequence of Lip906 lipase has been deposited in the National Center for Biotechnology Information (NCBI) under the accession number KM105171.

3 Results

3.1 Cloning and sequencing

The clones were identified by gel electrophoresis (Figure 1) and the quality of the mutant library met the library screening requirements. In the first round of random mutation library construction and screening, five mutants with increased enzyme activity were obtained, namely Lip5-1, Lip5-2, Lip5-3, Lip5-4, and Lip5-5. Among them, Lip5-5 had a higher enzyme activity and was used as a template in the second round of mutations. Then it was screened to form 5 mutants with increased enzyme activity, namely Lip5-A, Lip5-B, Lip5-C, Lip5-D, Lip5-E. Among them, Lip5-D has the highest enzyme activity, with an increase by 4 times (Figure 2). The sequencing results of Lip5-D show the presence of two base mutations, including a synonymous mutation G831A, and a missense mutation A155G. The corresponding amino acid is changed to Q55R.

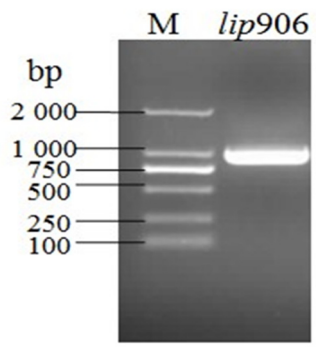

Fig.1. The electrophoresis analysis of error-prone PCR products of lipase Lip906

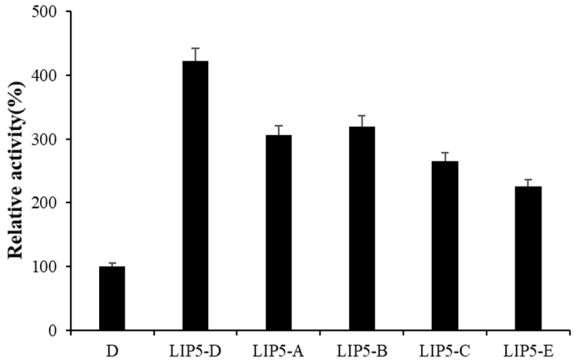

Fig.2. The secondary creening for transformants with high lipase activity

3.2 Enzymatic properties

The substrate specificity of the mutant enzyme did not change significantly, and it still had the highest hydrolysis activity on ρ-nitrophenol myristate (C14) (Figure 3a).

After the mutation, the optimal temperature of the enzyme rose from 56 to 60 °C (Figure 3b), and the optimal pH changed from 7.8 to 7.5 (Figure 3c). Lip5-D maintained about 60% of the relative enzyme activity after incubation at pH 3-10 for 2 hours (Figure 3c), and reserved about 70% of the relative enzyme activity after 2 hours of culture at 65 °C (Figure 3b). Compared with wild-type lipase Lip906, its thermal stability and pH stability were improved. When Lip5-D was added with 1 mM of various metal ions, almost no metal ions had inhibitory effect on its activity. Among them, Ag^{+}, Ca^{2+}, and Zn^{2+} had a significant promotion effect. When the concentration of metal ions was 10 mM, Fe^{2+} and Ag^{+} significantly inhibited the enzyme activity, and other high concentrations of metal ions had a certain promotion effect on the enzyme activity, especially Mg^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} (Figure 3d). The 30% isopropanol, methanol, ethanol and 10 mM EDTA promoted the activity of Lip5-D to a certain extent, and significantly increased the enzyme activity, while other organic solvents at different concentrations had no significant inhibition or promotion (Figure 3e).

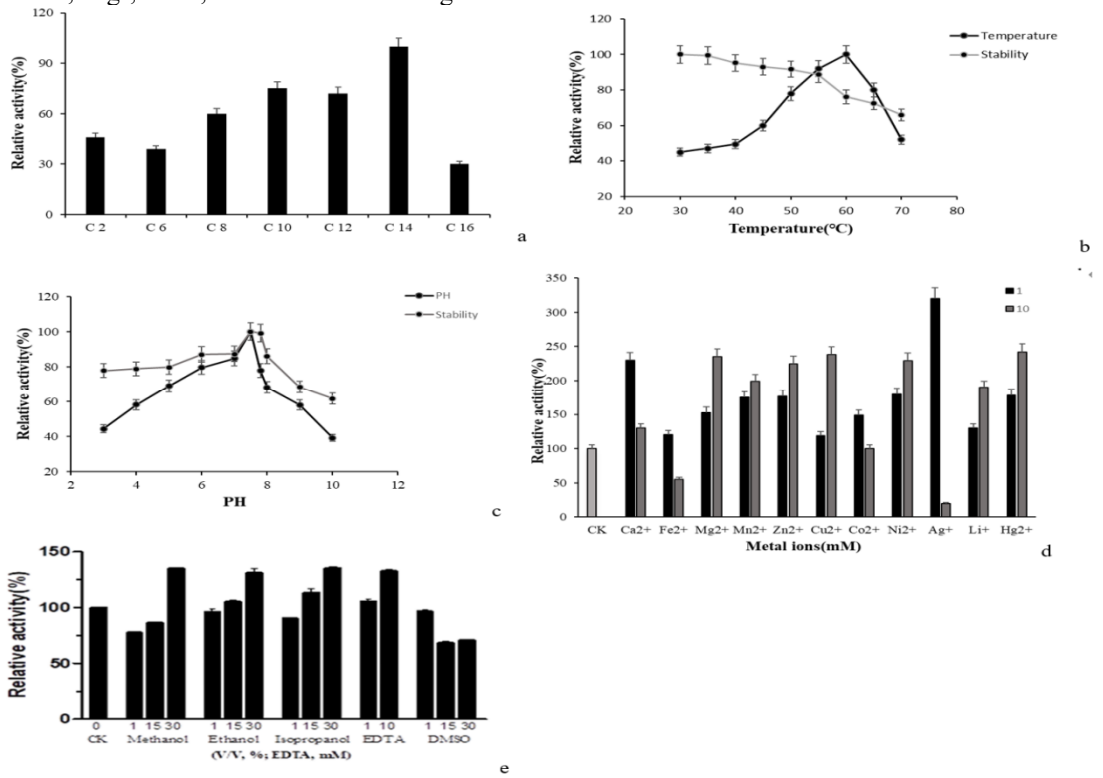

Fig.3. a. Substrate specifity of recombinant Lip5-D; b. Effect of temperatures on the activities and stability of the mutant Lip5-D; c. Effect of pH on the activities and stability of the mutant Lip5-D; d. Effect of the various metal ions on the activities of the mutant Lip5-D; e. Effect of various organic solvents on the activities of the mutant Lip5-D

3.3 Three-dimensional structure simulation of Lip5-D

The simulated three-dimensional structure diagram of the mutant enzyme was derived from a hydrolase of phospholipase (Phospholiase a1-iigamma). Its serial number is c2yijA, which contains the 206 amino acids of the mutant enzyme (24% similarity, 100% accuracy). In the three-dimensional structure, the mutant enzyme exists as a single subunit, and its structure is composed of 7-segment α helix, 9-segment β-sheet, and more random coils. The amino acid position of the mutation is on the surface, rather than the active center, of the enzyme (Figure 4).

Fig.4. Overall topology and folding of the proposed 3-D homology models of Lip5-D. α-Helixes (red), β-sheets (yellow), loops (green). Mutation site(Q52R) is displayed purple

3.4 Selection of immobilized carrier materials

The enzyme activity recovery rate was determined. Results showed the chitosan pellet carrier had the best immobilization effect on Lip5-D, and the recovery rate reached 92%. In comparison, the mutant enzyme recovery rates with sodium alginate and egg shells as the carriers were 15% and 60% respectively. The recovery rates of chitosan and egg shells cross-linked by glutaraldehyde were 35% and 41% respectively. Finally, chitosan was selected as the immobilization material (Table 1).

3.5 Optimization of preparation conditions of immobilized mutant lipase and study on enzymatic properties

Various influencing factors on immobilization were optimized. Results showed that 0.07 g of chitosan as the carrier had the best immobilization effect on the Lip5-D crude enzyme solution after adsorption at 40 °C for 12 h (Figure 5a, 5b, 5c). The optimal temperature of the immobilized Lip5-D rose from 60 to 63 °C (Figure 6a), the optimal pH was still 7.5, and it maintained about 80% at pH 3-10 for 2 hours. The acid-base stability was obviously improved. After heat treatment at 70 °C for 2h, it maintained about 65% of the enzyme activity (Figure 6b), indicating that the thermal stability and pH stability of the enzyme were both significantly improved after immobilization. The sensitivity of the immobilized Lip5-D to various metal ions was increased, and its activity was severely inhibited, except for 10 mM Fe^{2+} (Figure 6c). The resistance of organic solvents (methanol, ethanol, isopropanol, DMSO, EDTA) was also significantly reduced (Figure 6d). After the immobilized Lip5-D and the free enzyme were stored at 4 °C or room temperature for one month, the storage stability of the immobilized enzyme was significantly higher from the aspect of enzyme activity (Table 2).

Table 1 The recovery of immobilized mutant Lip5-D by different material

Immobilized material	Enzyme activity recovery rate（%）
Chitosan	92
Glutaraldehyde cross-linked chitosan	35
Egg shell	60
Glutaraldehyde cross-linked egg shell	41
Sodium Alginate	15

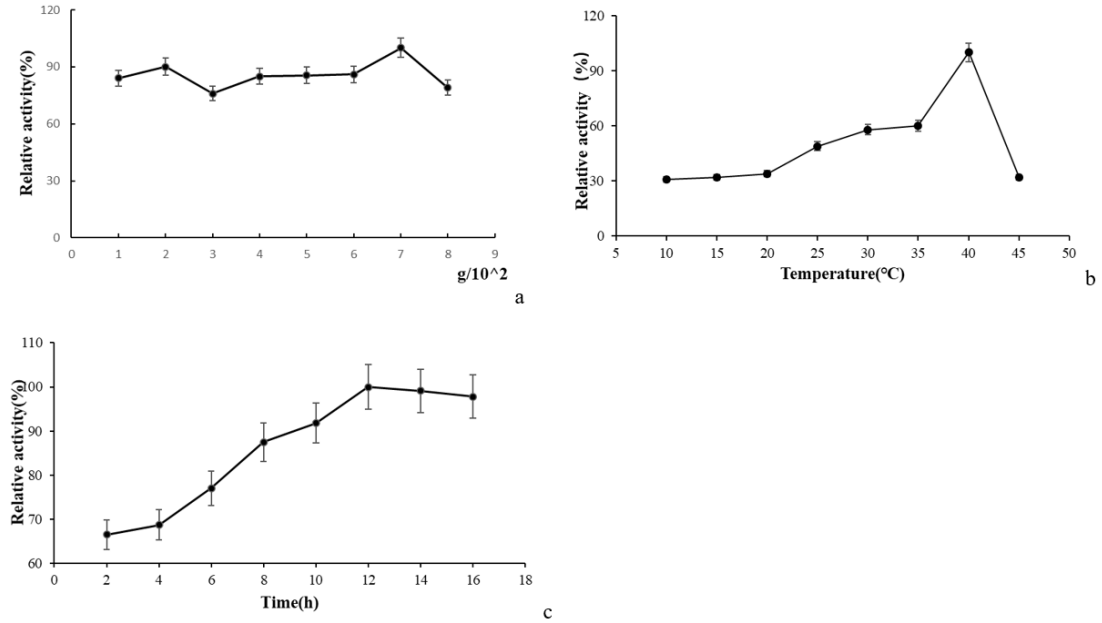

Fig.5. a. The concertration of *Chitosanon* on the activity of the immobilized mutant Lip5-D b. Effect of temperature on the activity of the immobilized mutant Lip5-D c. Effect of time on the activity of the immobilized mutant Lip5-D

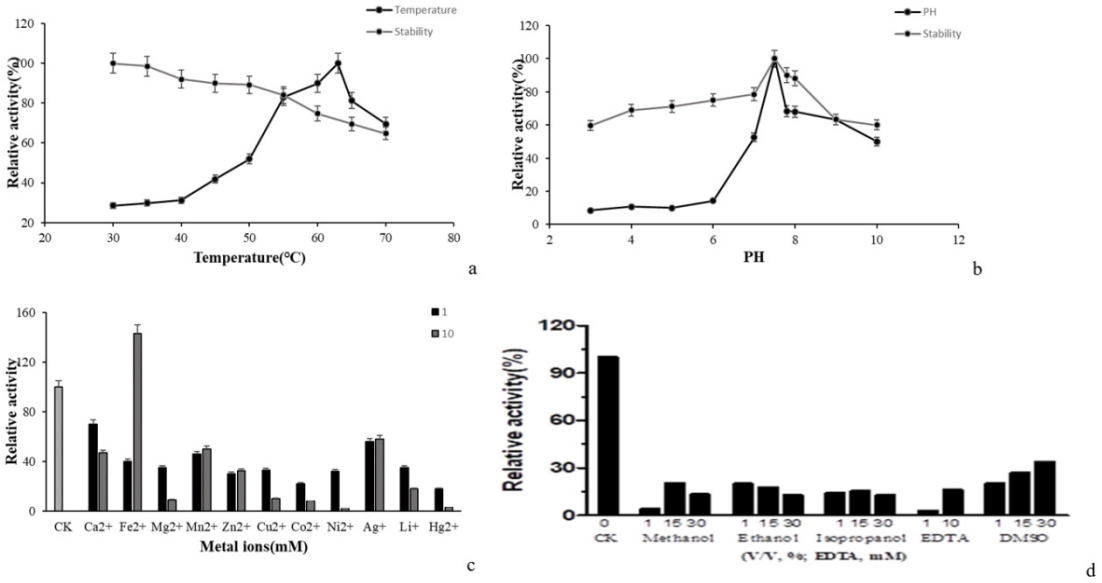

Fig.6. a. Effect of temperatures on the activities and stability of the immobilized mutant;
b. Effect of pH on the activities and stability of the immobilized mutant Lip5-D;
c. Effect of the various metal ions on the activities of the immobilized mutant Lip5-D
d. Effect of various organic solvents on the activities of the mutant immobilized Lip5-D

Table 2 The storage stability of immobilized and free mutant Lip5-D

Storage time （d）	Residual enzyme activity of immobilized mutant lipase enzyme Lip5-D （%）		Free mutant lipase enzyme Lip5-D （%）	
	4 ℃	25 ℃	4 ℃	25 ℃
0	100	100	100	100
2	100.5	101.2	100.1	98.4
4	101.3	100.6	99.8	92.3
6	100.8	99.2	94.6	85.6
8	99.8	92.4	85.2	81.4
10	98.2	85.6	80.6	72.6
15	97.4	84.5	70.4	65.2
20	95.5	75.7	60.6	52.4

4 Discussion

Enzymes are special proteins. Natural enzymes have many defects and thus cannot be directly used and developed. However, if we analyze its structure clearly, we can modify any enzyme as expected. The workload and time consumption of such task are obviously not allowed. Therefore, in vitro molecular directed evolution has rapidly emerged in recent years and become a new method to transform and modify protein structures. In this study, based on laboratory conditions and experience, error-prone PCR was selected to randomly mutate the wild-type lipase Lip906. Error-prone PCR is one of the earliest methods that were used in in vitro molecular directed evolution, and is the most mature in vitro molecular directed transformation method[16]. This method generally targets at proteins with small gene fragments. The principle is to change the amounts of various factors in PCR, so that the template will have random base mismatch during the PCR, which will cause protein mutations. In error-prone PCR, the mutation rate is a key factor influencing the success of mutations. It is only necessary to ensure that meaningful mutations are within a certain range. A too high mutation rate may introduce too many meaningless mutations, because there are only a few meaningful mutations in the mutation. A high mutation rate may damage the active center, which is a fixed triple structure, and affect its activity. If the mutation rate is too low, most of the library is still wild-type lipase, and no meaningful mutation can be screened. According to statistics, the mutation rate in this study meets the ideal low mutation rate (0~4 bases are mutated per 1 kb gene fragment)[34,35], which ensures that the subsequent screening work can be proceeded smoothly. Lipase is a typical α/β sheet structure enzyme, and its active center is a catalytic triad structure. The mutant amino acids are not on this structure, but on the surface irregular coils. Many enzymatic properties of wild-type lipase Lip906 are changed. This is because in the lipase protein, a large number of random coils are connected to α-helices or β-sheets[36], which are important in maintaining the spatial conformation of the protein. The mutation site changes the original conformation and hydrophobicity of the protein surface, making the optimal pH change from 7.8 to 7.5. At the same time, the site becomes a reaction point, making it is easier to catalyze the reaction in contact with the substrate under high temperature, and improving the

temperature and pH stability of the enzyme.

Enzyme immobilization has been developed to improve the activity and stability of enzymes. Immobilized enzymes have the improved enzyme activity, stability, reusability and low material price, so enzyme immobilization was selected to modify the mutant lipase Lip5-D. Experiments show it is better to choose chitosan directly as the carrier of immobilized enzymes. The reason is that chitosan is a heat-resistant and porous network natural polymer powder material, and the amino and hydroxyl groups in the molecule can be formed on the active interface, which has obvious affinity for proteins and can adsorb enzymes through ionic bonds, hydrogen bonds, and van der Waals forces to bind to the carrier[37]. As its amount increases, the chitosan carrier will compete for the adsorption of enzyme molecules, and the spatial structure of the enzyme will be destroyed during the whole process. As a result, the enzyme activity will be greatly reduced even if the enzyme is adsorbed on the carrier. By studying the enzymatic properties of immobilized Lip5-D, we find its optimum temperature (63 °C) is 3 °C higher than that of the free enzyme, and its optimum pH is not changed. The chitosan solution is weakly alkaline, but as an immobilized carrier, chitosan exists in the solid form during the reaction, so its optimal pH is not affected. After immobilization, the thermal, storage and pH stabilities of the enzyme are all improved, which reflect the advantages of enzyme immobilization. However, after immobilization, the enzyme sensitivity to organic solvents and metal ions is increased significantly, and only 10 mM Fe^{2+} significantly promotes enzyme activity. This is because the chitosan molecules are rich in -NH_2 and -OH, so when metal ions and organic solvents exist in the reaction system, they cooperate with the lone pair of electrons on the chitosan carrier, which change the nature of the chemical bond in the polymer as well as the interaction between its atoms.

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