Directed Evolution and Immobilization of a Novel Lipase LIP 906

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Title Page

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Directed Evolution and Immobilization of a Novel Lipase LIP 906

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Abstract

Background: The object of this experimental study is a new lipase screened from metagenomic libraries in the early stage of the laboratory and named it LIP 906. In order to improve the stability of the enzyme and develop and apply it as soon as possible, experiments use directed evolution and immobilization.

Results: A random mutation library was constructed by error-prone PCR technology, and finally a mutant lipase LIP 5-D with improved enzyme activity was screened out and then immobilized. Compared with the wild-type lipase LIP 906, the enzyme activity of the mutant enzyme LIP 5-D increased 4 times; the optimum reaction temperature was increased by 4 ℃ by mutation and 3℃ by immobilization; and the optimum reaction pH is changed from 7.8 to 7.5; temperature stability and pH stability has been improved. The mutant enzyme LIP 5-D can maintain a relative enzyme activity of about 70% at a temperature below 65 ℃ for 2 hours, and can also maintain a relative enzyme activity of about 60% at different pH 3 -10.

Conclusions: Error-prone PCR and immobilization improved the catalytic activity and stability of the enzyme, and promoted its development and application in many industries. The research on the properties and modification of the new lipase LIP906 provides a solid foundation for my next innovative research in application and environmental protection.

Key words: lipase; directed evolution; immobilization; enzymatic properties

Background

Lipase EC (3.1.1.3), also known as acylglycerol hydrolases, is widely found in prokaryotes (such as bacteria[1, 2]) and eukaryotes (such as mold[3], mammals and plants[4, 5]). The natural substrate of lipase is glycerides, which can hydrolyze esters and release monoglycerides, diglycerides, glycerol and free fatty acids [6-8]. A large number of studies have shown that in addition to catalyzing the hydrolysis of glycerides[9] and synthesis[10-12], lipases can also catalyze transesterification reactions [6], biosurfactant synthesis[13], peptide synthesis[8], polymer synthesis[9] and drug synthesis[14]etc., especially using the stereospecificity of certain lipases to catalyze the resolution of optical isomers and chiral drugs synthesis, it has become a new hotspot in the field of enzyme engineering. Therefore, lipase and its modified preparations are widely used in many fields such as food and nutrition, daily chemical industry, oleochemical industry, agrochemical industry, paper industry, detergent and biosurfactant synthesis, and pharmaceutical synthesis. However, since the production

cost of lipases is still relatively high compared to traditional chemical catalysts, in order to meet the requirements of industrial production, it is an urgent demand for industrialization to excavate and develop new microbial lipases with high catalytic activity and stability.

Enzymes, as a protein with natural catalytic activity, play a pivotal role in an individual's life. Although there are many types of natural enzymes, once they leave the native environment, they often show reduced catalytic activity and decreased stability, making it difficult to apply them to daily production and life. For this reason, the technology to modify the enzyme to make it adaptive has emerged, which is the directed evolution technology to be introduced[15]. Since the rise of error-prone PCR technology in the 1980s[16], directed evolution technology has flourished, such as DNA shuffling, Staggered Extension Process (StEP), and Random-priming in vitro recombination (RPR) and other emerging technologies, it provides convenience for people to obtain high-quality and efficient biocatalytic enzymes[17-19]. Directed evolution is mainly divided into the establishment of mutant libraries and targeted screening based on specific protein characteristics. Because the proportion of beneficial mutations in mutation libraries is very small, it often needs several or more times of directed evolution to get the specific enzymes we need. This is often accompanied by considerable workload, which also shows that there is still a lot of room for development and optimization for the directed evolution of enzymes.

As a natural biocatalyst, enzymes often have the characteristics of strong substrate specificity and high catalytic efficiency. They are widely used in modern industry, but free enzymes are often difficult to reuse and are easily affected by the reaction environment. These reasons have restricted the development and application of free enzymes[20, 21]. Therefore, the enzyme immobilization technology came into being[22]. This technology was formally proposed in 1973, which successfully restricted the enzyme to a certain range for the catalytic reaction, which allowed the enzyme to be used repeatedly many times, which greatly reduced the cost of industry, and the problem of easy residue of free enzyme were solved, and the subsequent separation and purification work was simplified. Compared with the enzyme in the free state, the immobilized enzyme tends to have certain changes in properties. This change is mainly reflected in three aspects: stability, reaction temperature, and reaction pH. In terms of stability, the immobilized enzyme usually makes it more resistant to high temperature, acid and alkali, and organic solvents. At the optimal reaction temperature, most of the immobilized enzymes will have an increase of 5-10 °C. The optimal reaction pH is usually affected by the nature of the carrier and the charged charge [23, 24]. At present, the more commonly used enzyme immobilization methods are: adsorption method[25], covalent binding method[26], embedding method[27] and cross-linking method[28]. The adsorption method can be further subdivided into ion-binding method and physical adsorption method, that is a method for forming immobilized enzyme by ion binding or physical adsorption between enzyme and carrier then carrying on the surface of the carrier. The covalent binding method uses immobilization by forming covalent bonds between amino acid residues on the surface of the enzyme and reactive groups on the surface of the carrier [29].Similarly,

the cross-linking method also forms a covalent bond between the enzyme and the carrier, but the cross-linking method usually uses a bifunctional or multifunctional reagent, so that a three-dimensional network structure can be formed after immobilization. The embedding method, as the name implies, is to load the enzyme into a gel or semi-permeable membrane to achieve immobilization. With the development of science and technology, more and more technologies such as nanotechnology immobilization、plasma immobilization and magnetic field immobilization have emerged, which has greatly developed the advantages of immobilized enzymes, making it useful in the food industry、medicine and environmental protection and other aspects have been more widely used[30-32].

Results

Cloning and Sequencing

The clones obtained after cloning were identified the quality of the mutant library met the library screening requirements by gel electrophoresis (Figure 1). The first round of random mutation library construction screened five mutants with improved enzyme activity, namely LIP5-1、LIP5-2、LIP5-3、LIP5-4 and LIP5-5，among them, the enzyme activity of LIP5-5 is more improved. It was used as a template for the second round of mutation, and 5 mutants with improved enzyme activity were screened, namely LIP5-A、LIP5-B、LIP5-C、LIP5-D、LIP5-E, where LIP5-D is the mutant with the highest enzyme activity increased by 4 times (Figure 2). LIP5-D sequencing results showed that two base mutations occurred, one was a synonym mutation: G831A; one was a missense mutation: A155G, and the corresponding amino acid changed to Q55R.

Enzymatic Properties

The substrate specificity results showed that the substrate specificity of the mutant enzyme did not change significantly, and the hydrolytic activity of p-nitrophenol myristate (C14) was still the highest (Figure 3). After mutation, the optimal temperature of the enzyme was increased from 56 °C to 60 °C (Figure 4); the optimal pH was changed from 7.8 to 7.5 (Figure 5). The mutant enzyme LIP5-D can maintain a relative enzyme activity of about 60% after 2 hours of incubation at different pH 3-10 (Figure 5), and can also maintain a relative enzyme activity of about 70% (Figure 4) when incubated for 2 hours at 65°C or lower. Compared with the wind-type lipase lIP906, the thermal stability and pH stability were improved. The mutant enzyme LIP5-D had no inhibitory effect on its activity when the concentration of various metal ions is 1 mM, among them, Ag^{+}, Ca^{2+}, and Zn^{2+} had a significant promotion effect on its activity. When it is 10 mM, except for Fe^{2+} and Ag^{+} which had obvious inhibitory effects on its enzymatic activities, the other high-concentration metal ions had a certain degree of promotion of enzymatic activity, especially Mg^{2+}, Cu^{2+}, Hg^{2+}, and Ni^{2+}(Figure 6). 30% isopropanol、methanol、ethanol and 10 mM EDTA can promote the activity of the mutant lipase LIP5-D to a certain extent, and its enzyme activity significantly increased, while other organic solvents at different concentrations have an effect which there was no significant inhibition or promotion

of activity on the mutant lipase LIP5-D (Figure 7).

Three-dimensional Structure Simulation of Mutant Enzyme LIP5-D

The three-dimensional structure of the mutant enzyme LIP5-D is derived from a hydrolase of Phospholiase a1-iigamma (the serial number is c2yijA, which contains 206 amino acids of the mutant enzyme, the similarity is 24% and the reliability is 100%). The three-dimensional structure shows that the mutant enzyme exists as a single subunit, and it consists of a 7-segment α-helix, a 9-segment β-sheet, and more random coils. The amino acid position of the mutation is not in the active center of the enzyme, but on the surface of the enzyme (Figure 8).

Immobilized

Optimization of various influencing factors in the immobilization, the results showed that the immobilization effect of LIP5-D crude enzyme solution was the best with 0.07g of chitosan carrier under the conditions of 40 ℃ and 12h adsorption time (Figure 9、10、11). The optimal temperature of LIP5-D after immobilization was increased from 60 ℃ to 63 ℃ (Figure 12); the optimal pH was still 7.5; after immobilization, it could maintain relative enzyme activity of about 80% after incubating at different pH 3-10 for 2 hours. Its acid-base stability was significantly improved; after heat treatment at 70 ℃ for 2h, about 65% of enzyme activity was still maintained (Figure 13), indicating the thermal stability and pH stability of the enzyme after immobilization were significantly improved. The sensitivity of the immobilized enzyme LIP5-D to various metal ions was increased, and its activity was severely inhibited, in addition to the Fe^{2+}concentration of 10 nM had a promoting effect(Figure 14); for methanol、ethanol、isopropanol、DMSO、EDTA、etc ,the resistance of organic solvents was also significantly reduced (Figure 15). After storage of the immobilized enzyme LIP5-D and the free enzyme at 4 ℃ and room temperature for one month, respectively, the storage activity of the immobilized enzyme was significantly better than it of the free enzyme (Table 1).

Discuss

Enzymes as a special protein, natural enzymes have many defects that prevent them form being directly used and developed. However, if we analyze the structure of each enzyme and then modify it, we will achieve the purpose we want. Obviously, this workload and time consumption are not allowed. Therefore, in vitro molecular directed evolution technology has rapidly risen in recent years, and it has become a new method for people to modify and modify the structure of proteins. In this study, a random error-prone PCR method was used to randomly mutate the wild-type lipase LIP906 based on laboratory conditions and experience. Error-prone PCR technology is one of the earliest methods used in molecular directed evolution in vitro, and it is currently the most mature method of molecular directed transformation in vitro. This method is generally aimed at proteins with small gene fragments. The principle is mainly to change the amount of various factors in the PCR process, so that the template in the PCR process occurs random base mismatches, thereby causing protein

mutation. In the error-prone PCR process, the mutation rate is a key factor of the mutation. It is only necessary to ensure that meaningful mutations are within a certain range. If the mutation rate is too high, too many meaningless mutations may be introduced, because significant mutations in the mutation process are only a few. The active center of the lipase is a fixed triplet structure, an excessively high mutation rate may actually damage its active center and affect its activity. However, if it is low, most of the libraries are still wild-type lipases and no meaningful mutations can be screened. According to statistics, the mutation rate in this study meets the ideal low mutation rate (mutations of 0 to 4 bases per 1kb gene fragment). It ensures that the subsequent screening work can proceed smoothly.

Lipase is a typical α / β-fold structure enzyme. Its active center is a catalytic triplet structure. The mutant amino acids are not on these structures, but are located on the random coils on the surface. However, the amino acid changes are still changes many of the enzymatic properties of wild-type lipase LIP906. This may be because the presence of a large number of random coils in the lipase protein to connect the α-helix or β-sheet, and plays an important role in maintaining the spatial conformation of the protein. While, the mutated amino acid is changed from acidic R-group-containing glutamine (Q) to basic R-group-containing arginine (R). Acidic glutamine plays an important role in the metabolism of various proteins in the baby. As a complex α-amino acid, arginine often appears at the reaction point of various enzymes. The properties of these two amino acids themselves are very different. The mutation of the site may change the original conformation and hydrophobicity of the protein surface, so that its optimum pH changes from 7.8 to 7.5. At the same time, the site also becomes a reaction site, therefore, the enzyme is more likely to contact the substrate for catalytic reaction under high temperature conditions, and the temperature stability and pH stability of the enzyme are also improved.

In order to improve the activity and stability of enzymes, the technology of enzyme immobilization has been developed. Immobilized enzyme has the advantages of improving enzyme activity、stability、reusability and low material cost, therefore, the enzyme immobilization technology was selected to modify the mutant lipase LIP5-D. By studying the enzymatic properties of the immobilized mutant lipase LIP5-D, the results showed that the optimal temperature was 63 °C, which was 3 °C higher than the free enzyme, and the optimal pH had not changed, which may be similar to the properties of chitosan. The solution of chitosan is weakly alkaline, but as an immobilized carrier, chitosan exists in solid form during the reaction, so it may not have any effect on its optimum pH. Thermal stability、storage stability and pH stability have been improved, which all show the advantages of enzyme immobilization. However, after immobilization, the enzyme's sensitivity to organic solvents and metal ions increased significantly, and the enzyme activity was severely inhibited. These may be because the chitosan carrier itself has a strong chemical reaction ability. There are many active groups in its structure, so when metal ions and organic solvents are present in the reaction system, the chitosan carrier itself first undergoes a chemical reaction to inhibit the enzyme activity.

Conclusions

LIP906, a new type of lipase gene was obtained through the construction of a Futian mangrove soil metagenomic library in Shenzhen in the early stage of the laboratory. This enzyme is a brand-new lipase, but due to its poor thermal stability, it is not sufficient for application industrial production. In this study, the molecular structure of the enzyme was modified by error-prone PCR method in vitro molecular directed evolution technology, and at the same time, the enzyme was immobilized to further modify the structure, in order to improve the thermal stability of the enzyme and catalytic activity. A random mutant library screened by error-prone PCR was used to obtain a mutant LIP5-D with a 4-fold increase in enzyme activity. Compared with the wild-type lipase LIP906, the thermal stability and pH stability are improved, in addition, the storage stability of the immobilized enzyme was also significantly better than free enzyme.

Since lipases are widely used in the production of medicine and food in industry, they are essential enzymes in our daily life and in industrial production. However, natural lipases derived from microorganisms、animals and plants often limit their applications because their activity and stability cannot meet the needs of industrial production. Therefore, various strategies are needed to improve the activity and stability of lipases. Because of the demand, it is currently necessary to modify the lipases. At present, new ideas and methods about protein modification have emerged endlessly. It is very important to choose the appropriate method to modify the target protein of your own research. In this experiment, the catalytic activity and stability of the new lipase LIP906 were greatly improved through PCR modification and immobilization, which promoted its application in industry and environmental protection.

Methods

Cloning, Expression and Purification

Primer design was based on the gene of lipase LIP906. A pair of primers were: LIP906-F and LIP906-R. *Eco*RI and *Hin*dIII restriction sites were introduced at the two ends of the primer. The primer sequence is as follows: LIP906-F: 5 ’CCGGAATTCATGACAACACCAGCAGCTAC CATCGAA GG 3’ (underlined part is *Eco*RI digestion site); LIP906-R: 5 ’CCCAAGCTTTCAGGGGCAAACACCGGTGGG 3’ (underlined part is *Hin*dIII digestion site).The plasmid containing pUC118-LIP906 was used as a template, LIP906-F and LIP906-R were used as primers, and the PCR was amplified using Prime STAR^{TM}Max Premix. The purified PCR product and vector pET-32a (+) were double-digested with the restriction fast endonuclease *Hin*dIII and *Eco*RI, respectively. After the digestion reaction was completed, the digested product was subjected to agarose gel electrophoresis and the gel was recovered, then the two were ligated under the action of T4 DNA Ligase. The clones were obtained in calcium-transformed competent cells and the recombinant lipase LIP906 was purified by using His.Bind®Resin kit from Novagen. Finally, the protein expression and purity were

verified by SDS-PAGE (12% polyacrylamide gel).

Directed Evolution

Recombinant lipase was used as a template for mutation, and in the random mutation library picked each clone, then plated on a lipase screening plate (containing 100 μg / mL Amp and 0.1 mM IPTG) and cultured 2-3 d at 37 °C in a constant temperature incubator. Taking the mutant clones with larger hydrolysis circle and inducing expression under the best conditions. Ultrasonic crushing to prepare the crude enzyme solution. Using reagents with different length carbon chains as the substrates, the enzyme activity was accurately measured according to the following method, and wild-type lipase LIP906 was used as a reference to compare enzyme activities. Finally, the positive mutant clones with obvious improvement in enzyme activity were sent to the gene company for sequencing, and we compared the sequences and amino acids with wild type to determine the position of the mutation site.

Enzyme Activity Determination

Enzyme activity was evaluated by measuring the absorbance of p-nitrophenol at OD405nm. In detail, in a 400 μl reaction system: add 10 μl of the crude enzyme solution to be tested、10 μl of 1 mM substrate、0.04 M Britton-Robinson buffer and 1% acetonitrile mix thoroughly, and react at 45 °C for 15 min. Three parallel experiments and one blank control experiment were set up. After the reaction was completed, the reaction system was added to a 96-well plate, and the absorbance at OD405nm was measured by a microplate reader. The amount of enzyme required to hydrolyze the substrate per unit time to produce 1 μmol p-nitrophenol is the enzyme activity.

Enzymatic Properties

The substrate specificity of lipase LIP906 was determined by the above method for the hydrolytic activity to different length carbon chains (C2-C16). The optimal reaction temperature was determined by measuring the enzyme activity at a temperature ranging from 30 °C to 70 °C (5 °C interval). The optimum reaction pH of the enzyme is determined in the pH range of 3-10. In order to determine the thermal stability of the enzyme, under the optimal pH conditions, the residual activity was measured at a temperature of 30℃ to 70℃ (5℃ interval) for two hours. In order to evaluate the pH stability of the enzyme, the residual activity was measured after 2h reaction at pH 3-10 at the optimal temperature. The effects of metal ions (Cu^{2+}, Ca^{2+}, Fe^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ag^{2+}, Zn^{2+}, Ni^{2+}) and the chelating agent EDTA were measured at a final concentration of 1 mM and 10 mM. The effects of organic solvents were determined at final concentrations of 1%, 15%, and 30%, respectively.

Prediction of Mutation 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 with wild-type lipase LIP906 in the NCBI (http://www.ncbi.nlm.nih.gov/) database to determine the position of the mutated base. Convert the base sequence into an amino acid sequence

in SMS (The Sequence Manipulation Suite), enter the amino acid sequence of the mutant protein in PHYRE2 (http://www.sbg.bio.ic.ac.uk/), predict its three-dimensional structure, and sent the result to the mailbox in PDB format. Open the 3D structure diagram in PDB format in Pymol software, enter the instruction to mark the position of the mutant amino acid in the 3D structure diagram, and combine the enzymatic properties of the mutant enzyme LIP5-D to analyze the effect changes caused by the mutation site.

Immobilized mutant lipase

The immobilized mutant lipase was prepared with chitosan as the carrier, and the optimal amount of chitosan was determined within the range of 0.01g-0.08g (0.01g interval). Measure the optimal adsorption temperature within the range of 10℃ -45℃ (5℃ interval). Other things being equal, the optimal adsorption time was determined by measuring the enzymatic recovery after 0.5h、2h、4h、6h、8h、10h and 12h respectively. Then, the enzymatic properties of the immobilized mutant lipase were evaluated according to the above method for measuring enzyme activity, including the optimal reaction temperature and temperature stability, the optimal reaction pH and stability, and the effects of metal ions and organic solvents. Finally, a certain amount of immobilized lipase chitosan pellets were stored at 4°C and room temperature respectively, and their storage stability was determined by the enzyme activity.

Amino Acid Accession Number

The protein sequence of LIP906 lipase has been stored in the National Information Technology Center (NCBI) of the United States under the accession number KM105171.

Abbreviations

E.coli: Escherichiacoli
EDTA: Ethylene Diamine Tetraacrtic Acid
g: gram
h: hour
mL: Milliliter
μl: Microliter
M: mol/L
OD: Optical Density
PCR: Polymerase Chain Reaction
pH: Potential Hydrogen
DMSO: Dimethyl Sulfoxide
SDS: Sodium Dodecyl Sulfate

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Availability of data and meterials
All data generated or analysed during this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
HL designed the experiments, analyzed the data, and drafted the manuscript. SD performed experiments, analyzed the data and drafted the manuscript. All authors read and approved the manuscript.

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Figures

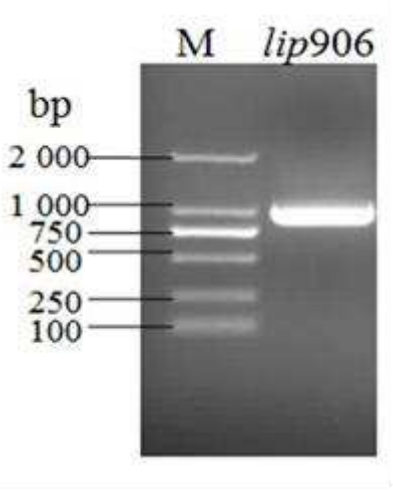

Fig.1 The electrophoresis analysis of error-prone PCR products of lipase LIP906

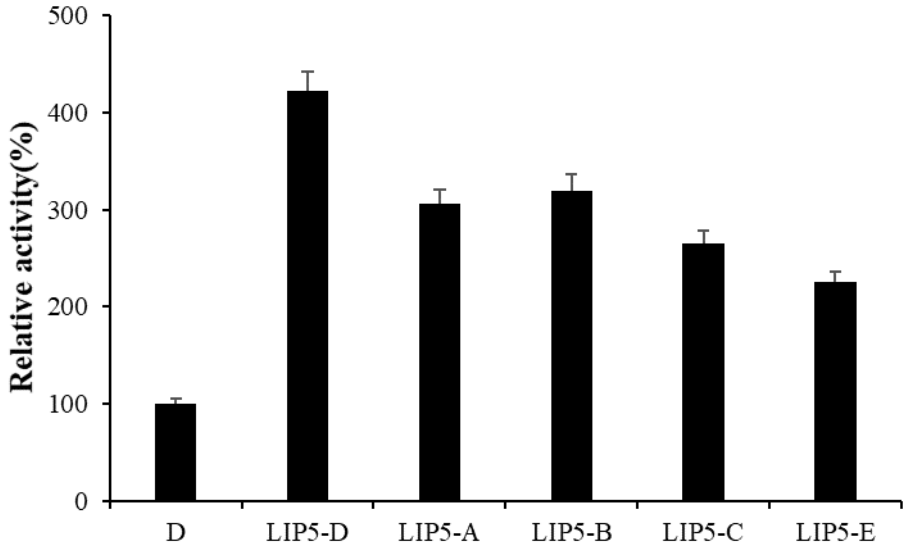

Fig.2 The secondary creening for transformants with high lipase activity

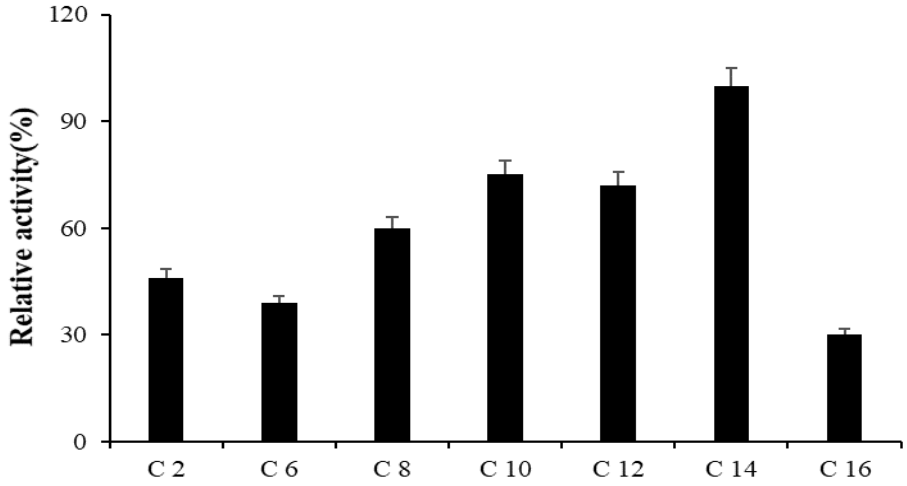

Fig.3 Substrate specifity of recombinant LIP5-D

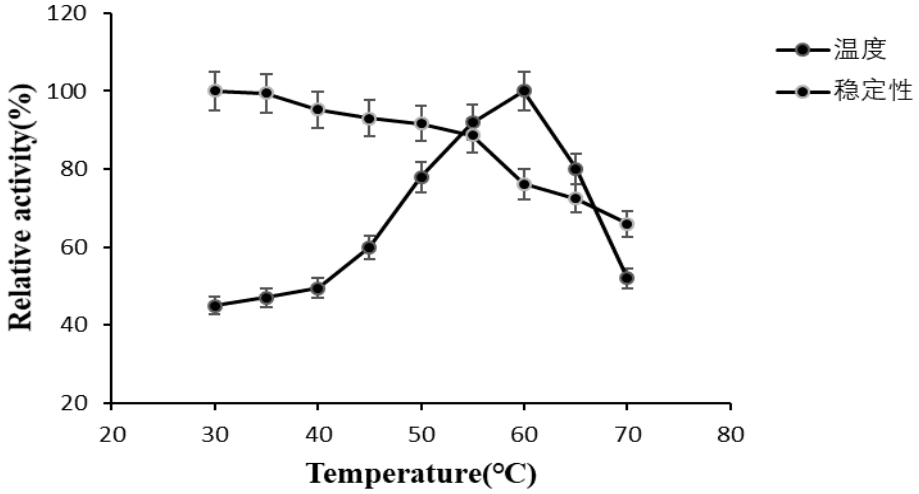

Fig.4 Effect of temperatures on the activities and stability of the mutant LIP5-D

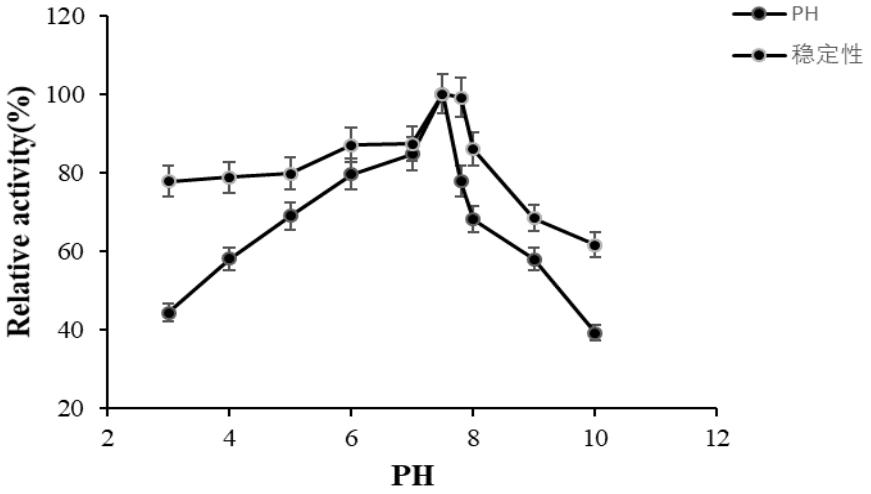

Fig.5 Effect of pH on the activities and stability of the mutant LIP5-D

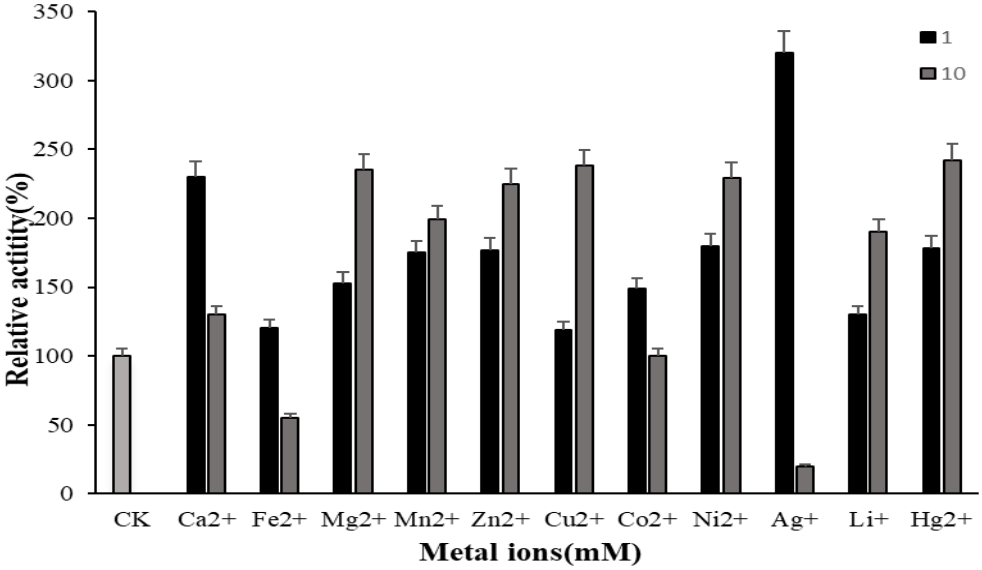

Fig.6 Effect of the various metal ions on the activities of the mutant LIP5-D

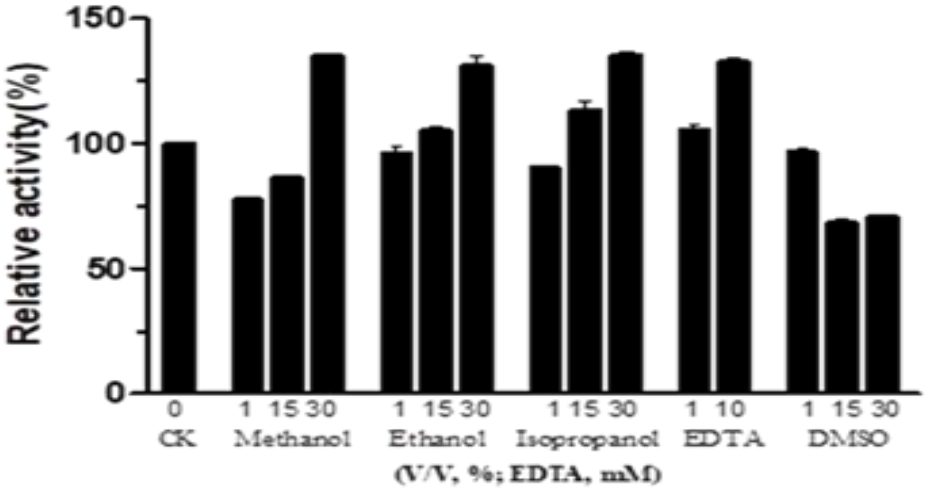

Fig.7 Effect of various organic solvents on the activities of the mutant LIP5-D

Fig.8 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.

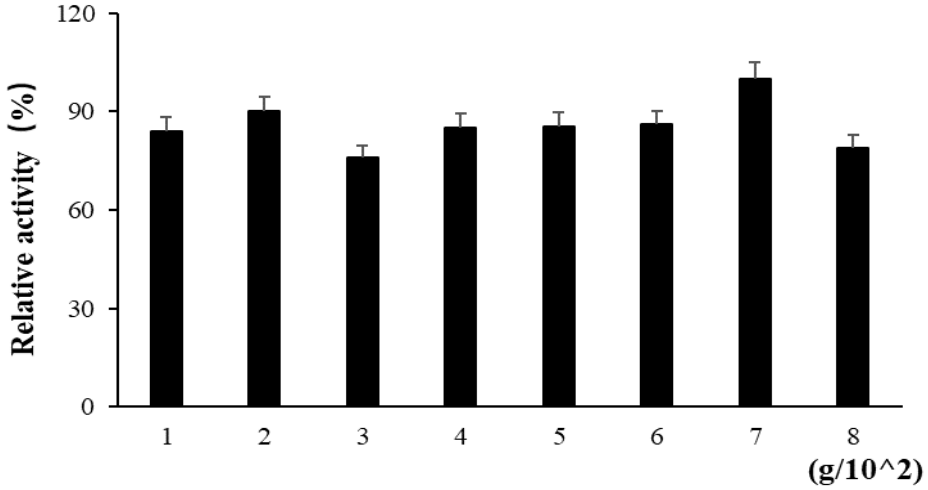

Fig.9 The concertration of Chitosanon on the activity of the immobilized mutant LIP5-D

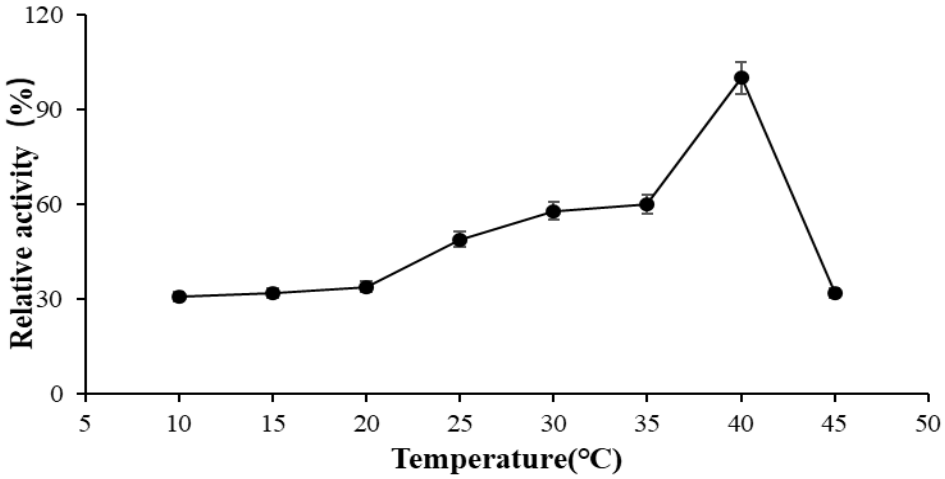

Fig.10 Effect of temperature on the activity of the immobilized mutant LIP5-D

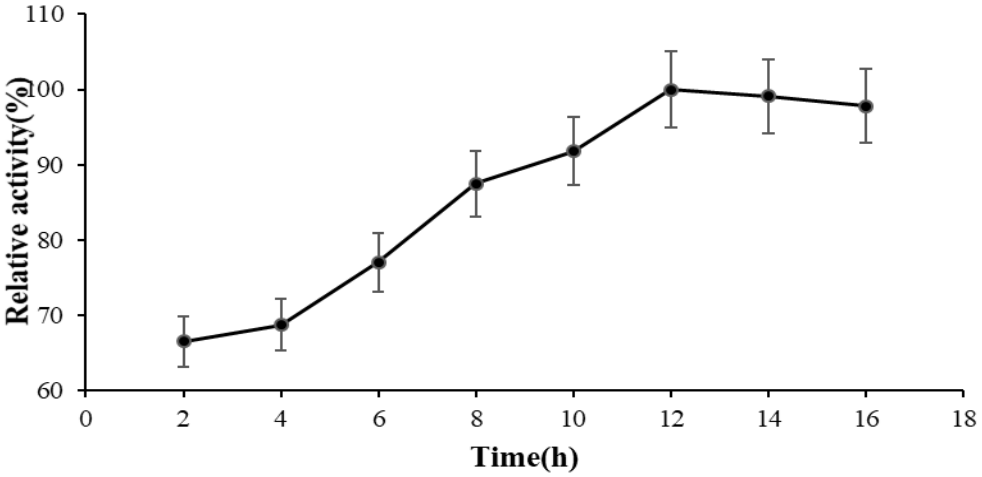

Fig.11 Effect of time on the activity of the immobilized mutant LIP5-D

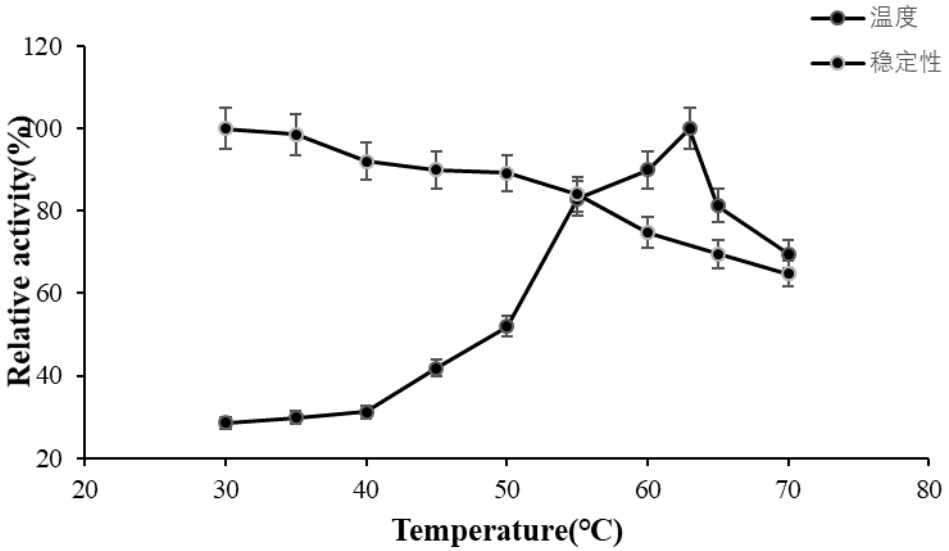

Fig.12 Effect of temperatures on the activities and stability of the immobilized mutant

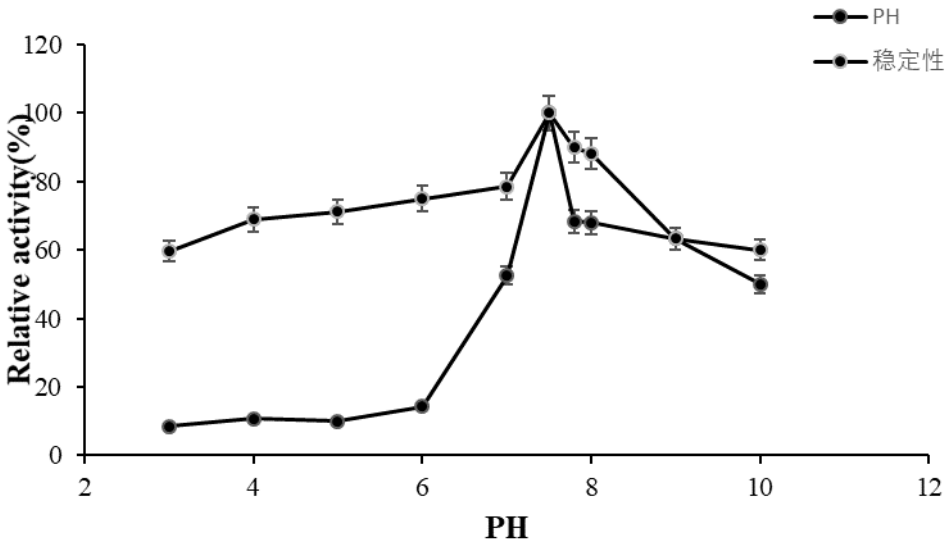

Fig.13 Effect of pH on the activities and stability of the immobilized mutant LIP5-D

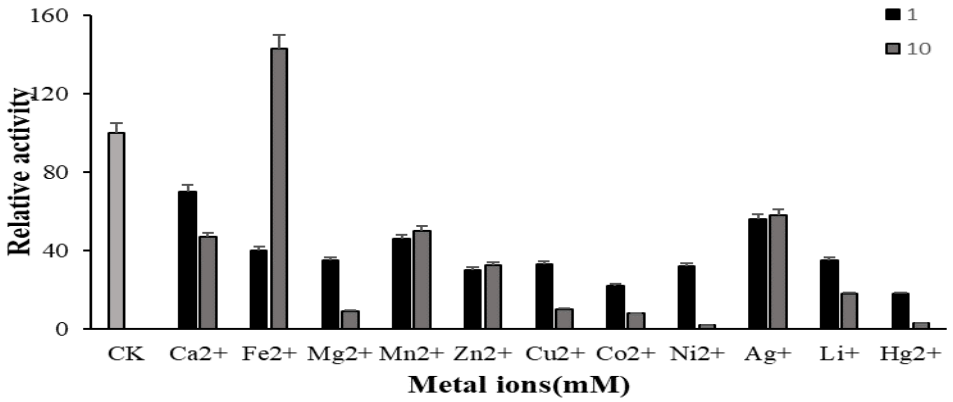

Fig.14 Effect of the various metal ions on the activities of the immobilized mutant LIP5-D

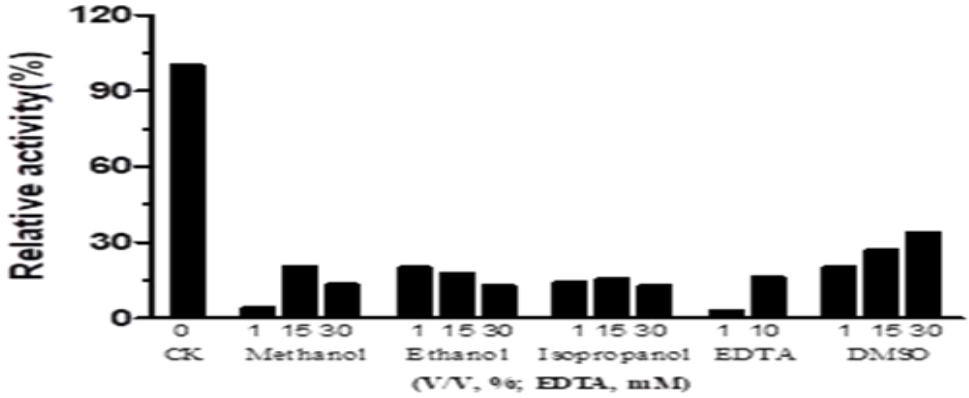

Fig.15 Effect of various organic solvents on the activities of the mutant immobilized LIP5-D

Table 1 The storage stability of immobilized and free mutant LIP5-D

Storage time（d）	Residual enzyme activity of immobilized mutant lipase LIP5-D（%）		Free mutant lipase 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

Figures

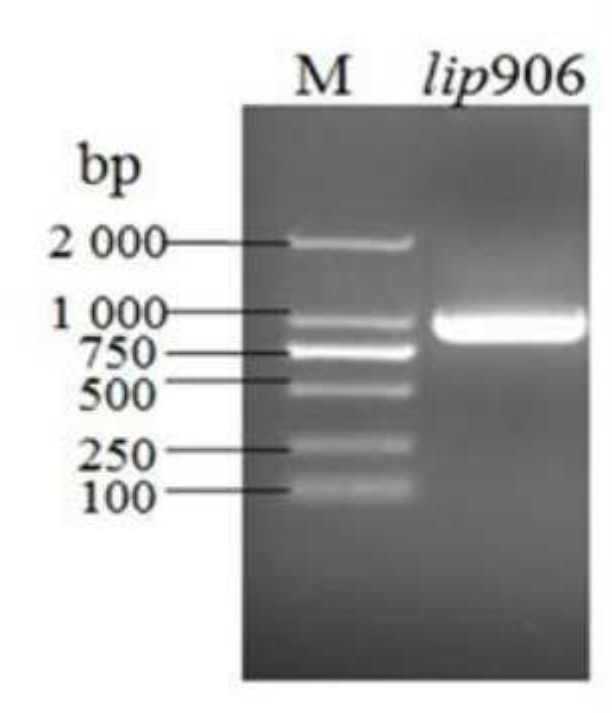

Figure 1

The electrophoresis analysis of error prone PCR products o f lipase LIP906

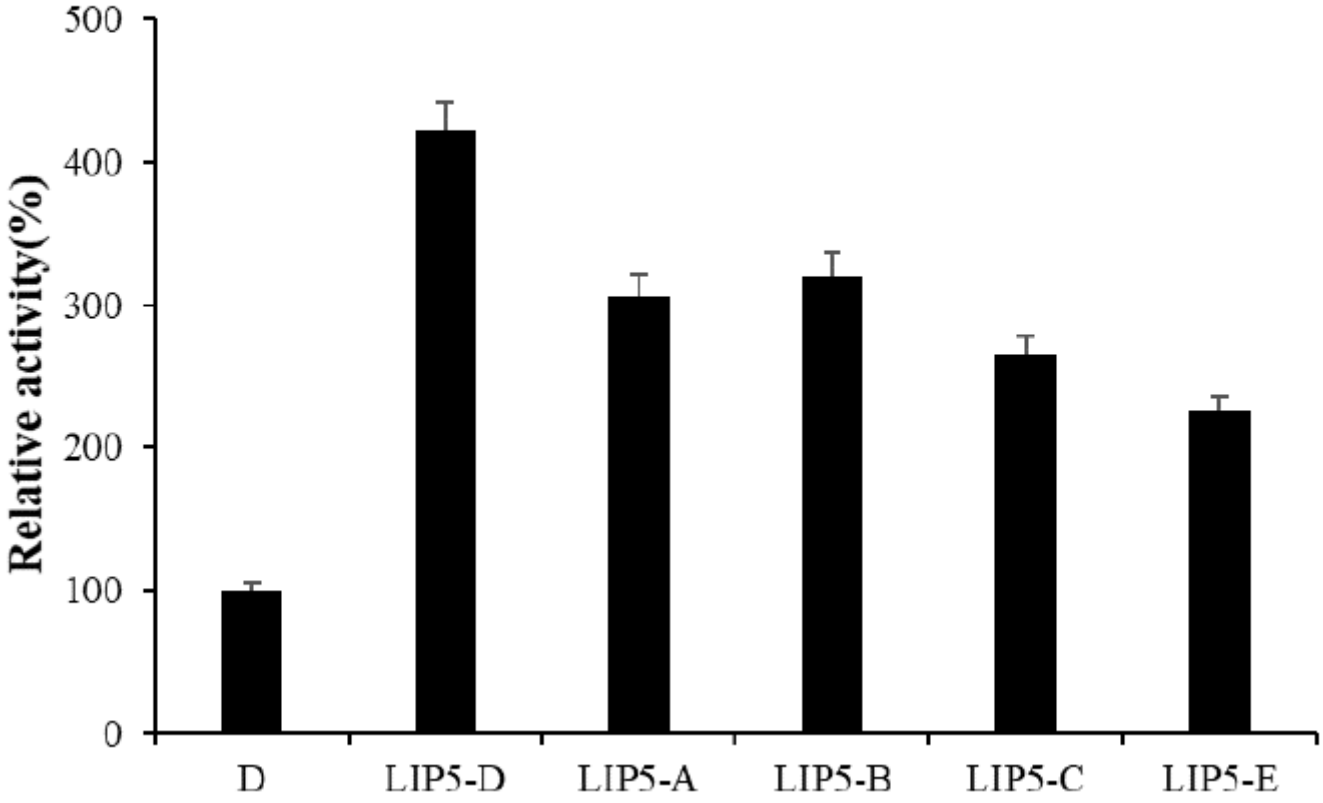

Figure 2

The secondary creening for transformants with high lipase activity

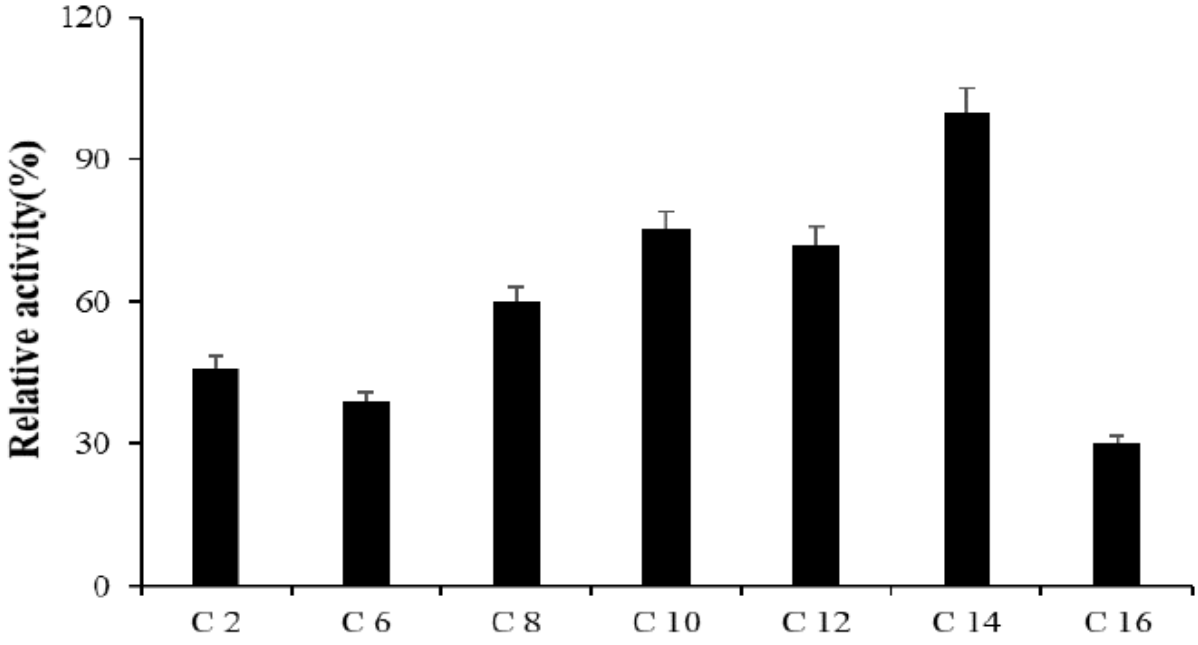

Figure 3

Substrate specifity of recombinant LIP5-D

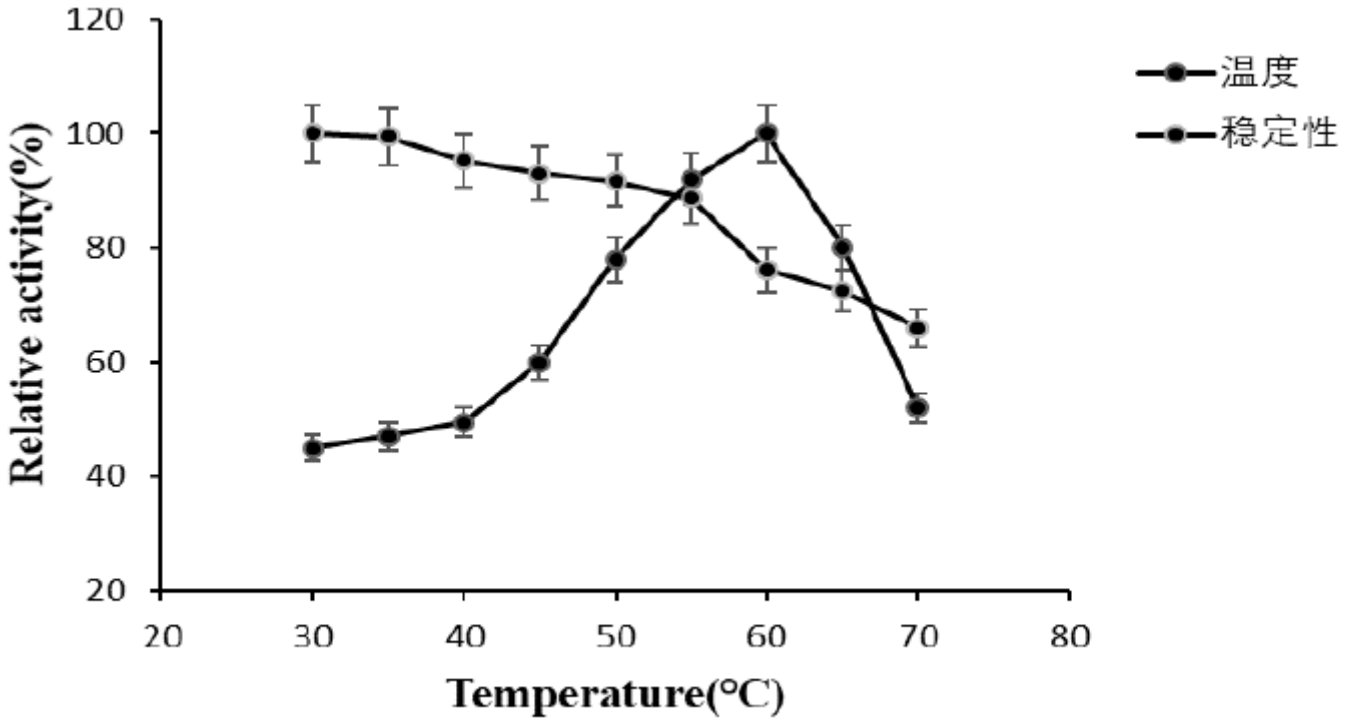

Figure 4

Effect of temperatures on the activities and stability of the mutant LIP5-D

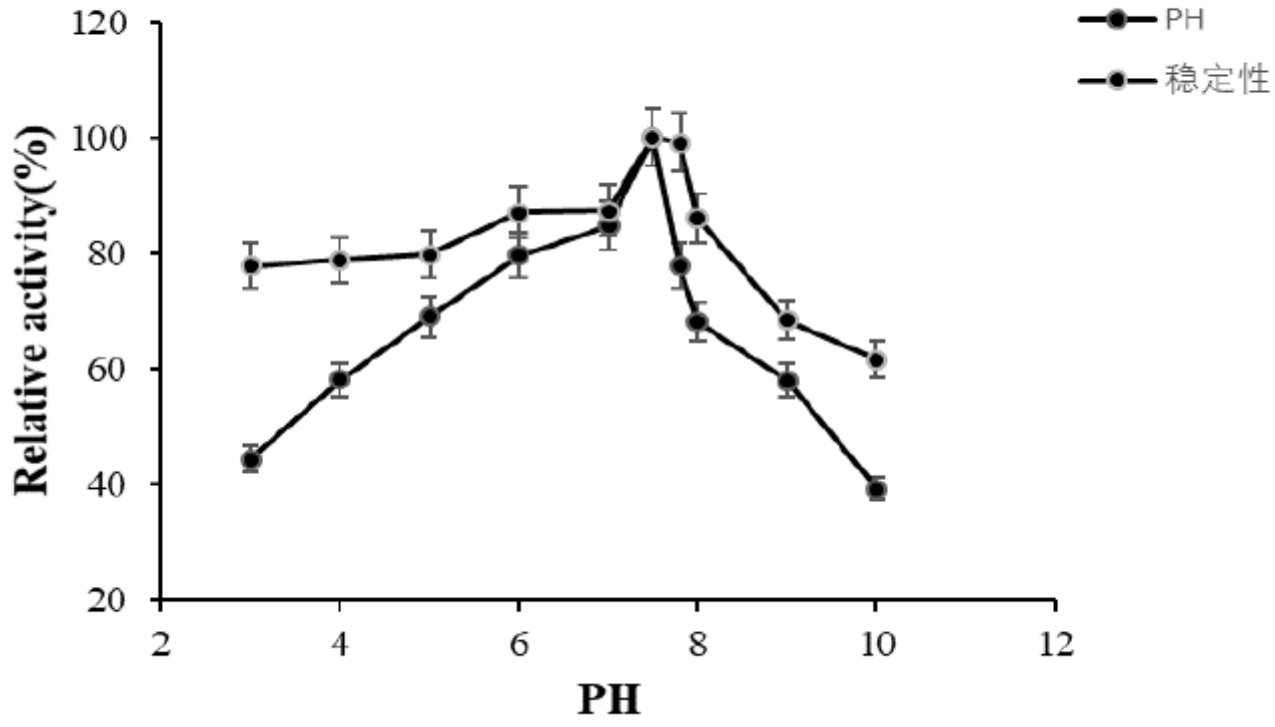

Figure 5

Effect of pH on the activities and stability of the mutant LIP5-D

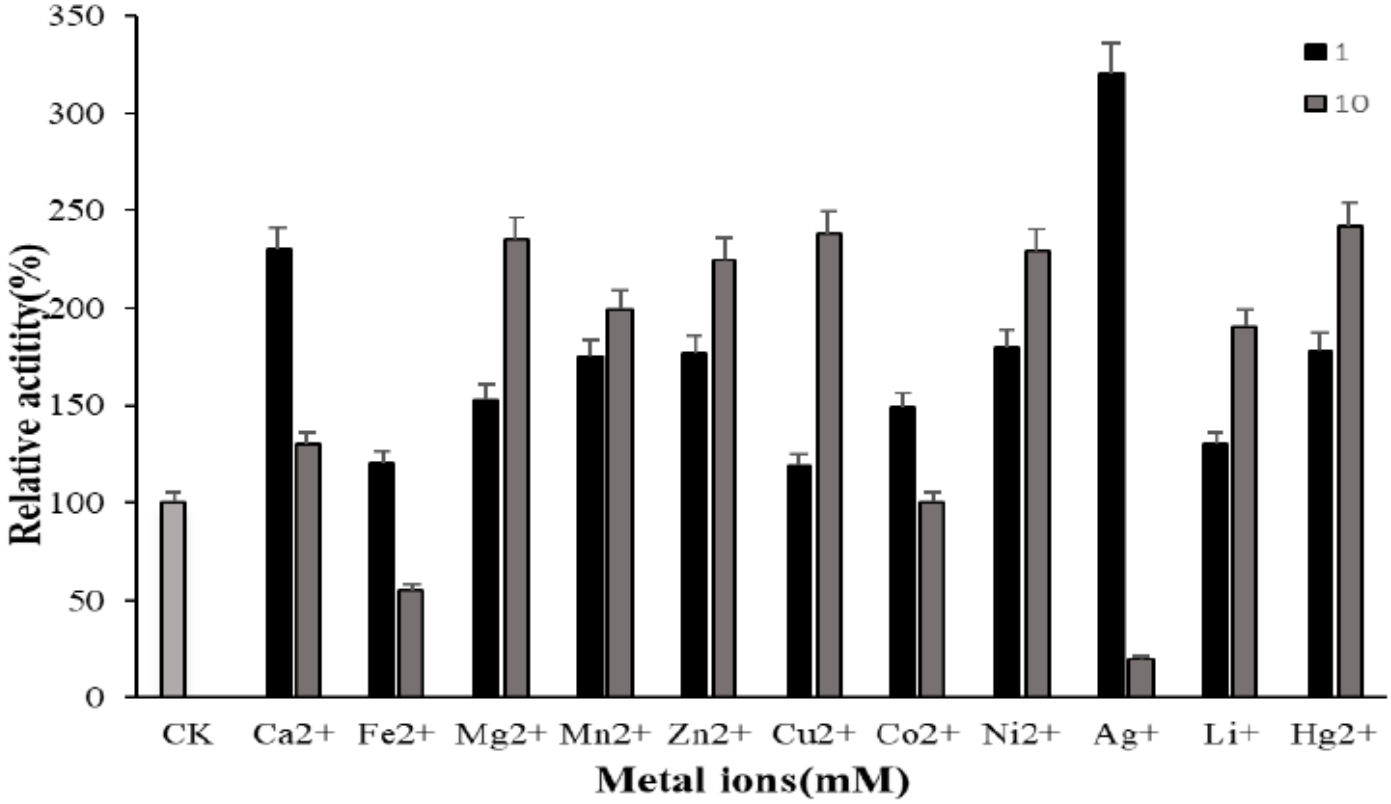

Figure 6

Effect of the various metal ions on the activities of the mutant LIP5-D

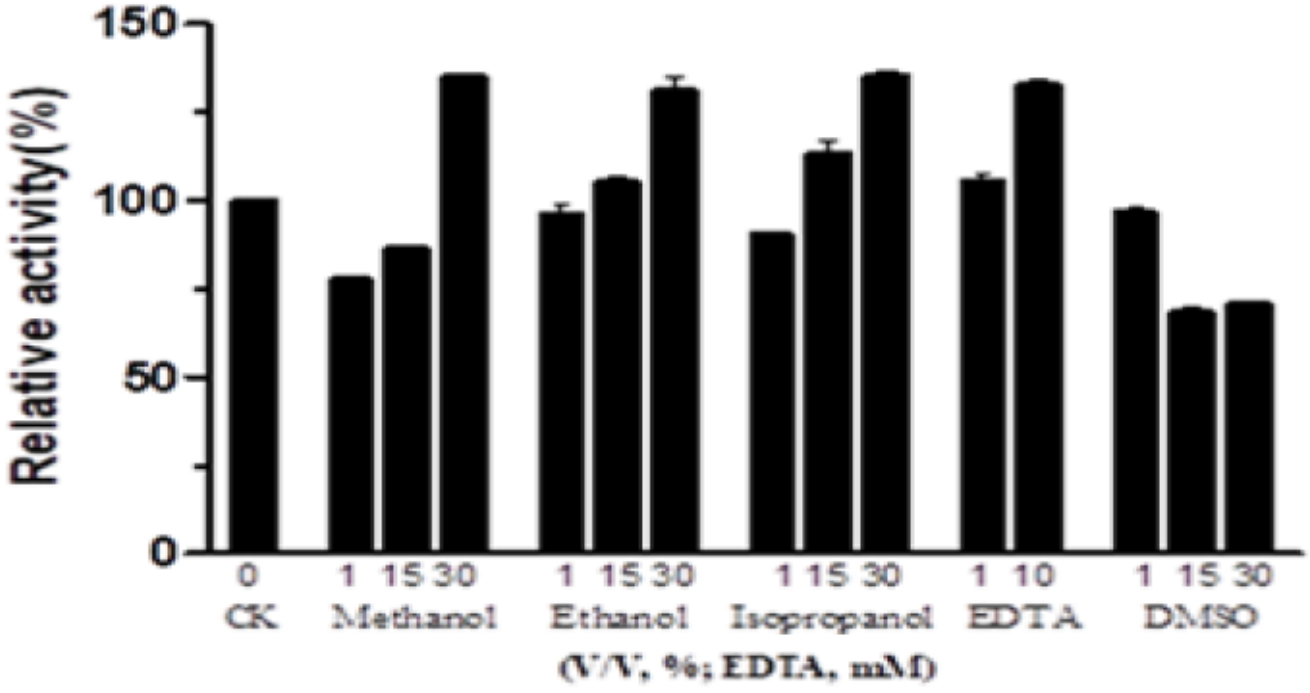

Figure 7

Effect of various organic solvents on the activities of the mutant LIP5-D

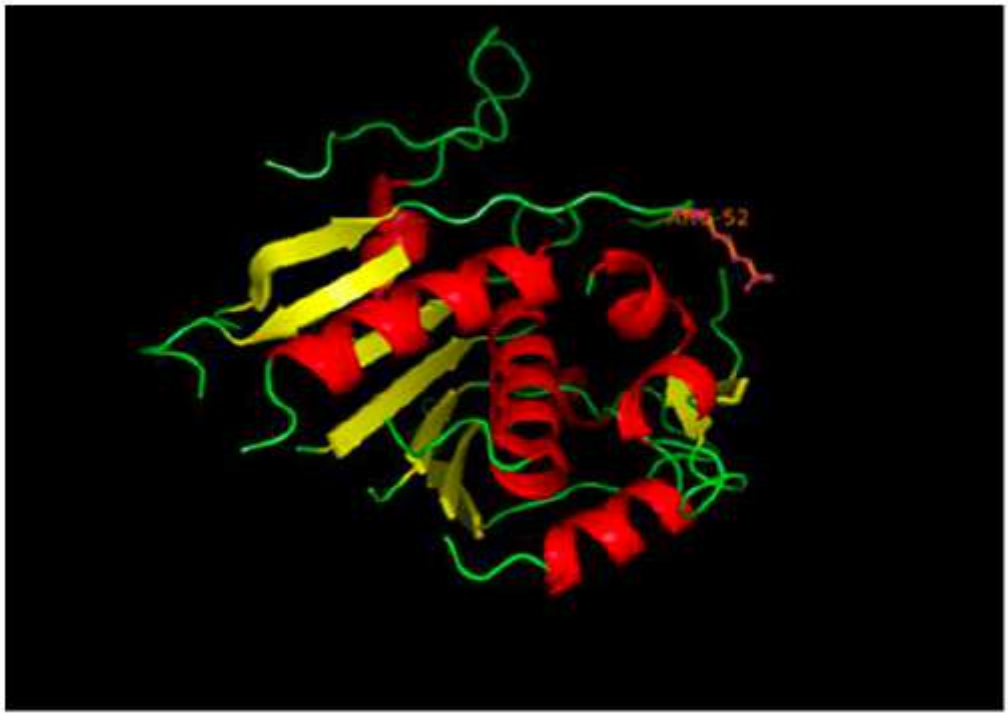

Figure 8

Overall topology and folding of the proposed 3D homology models of LIP5-D. α-Helixes (red), β-sheets (yellow), loops (green). Mutation site(Q52R) is displayed purple.

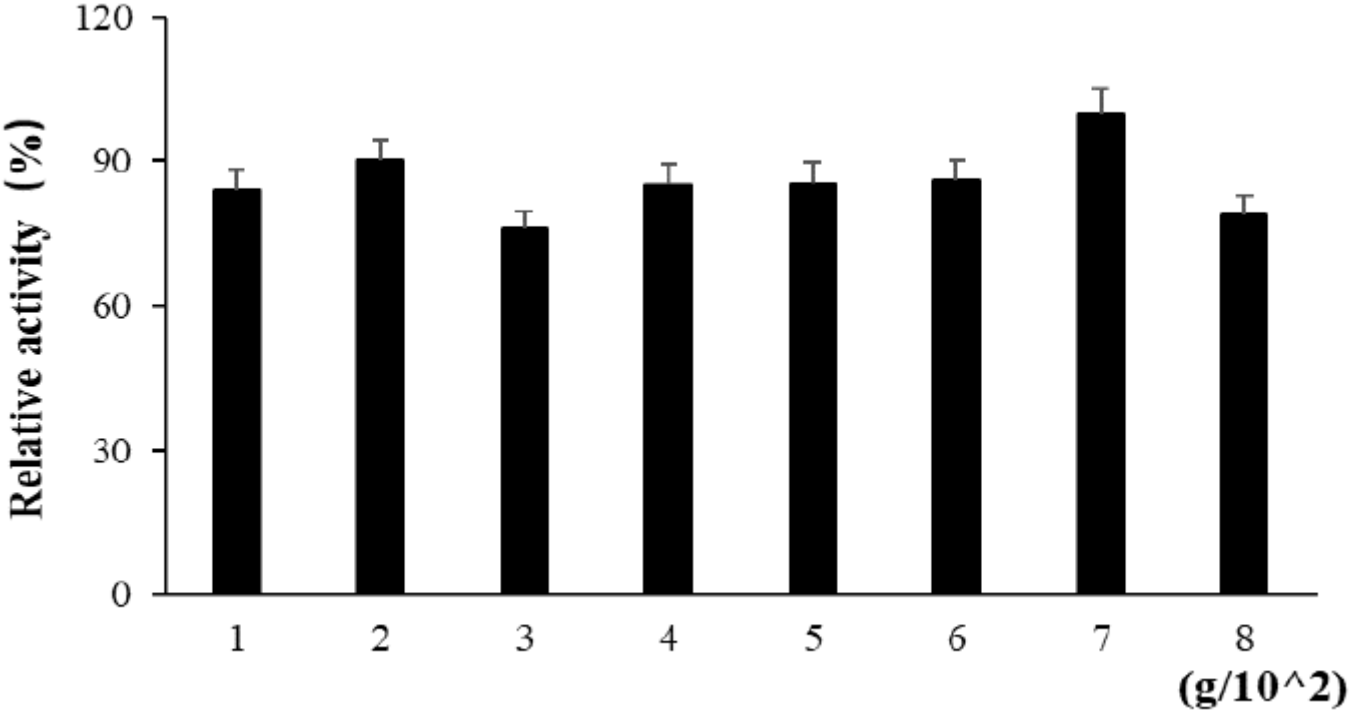

Figure 9

The concertration of Chitosanon on the activity of the immobilized mutant LIP5-D.

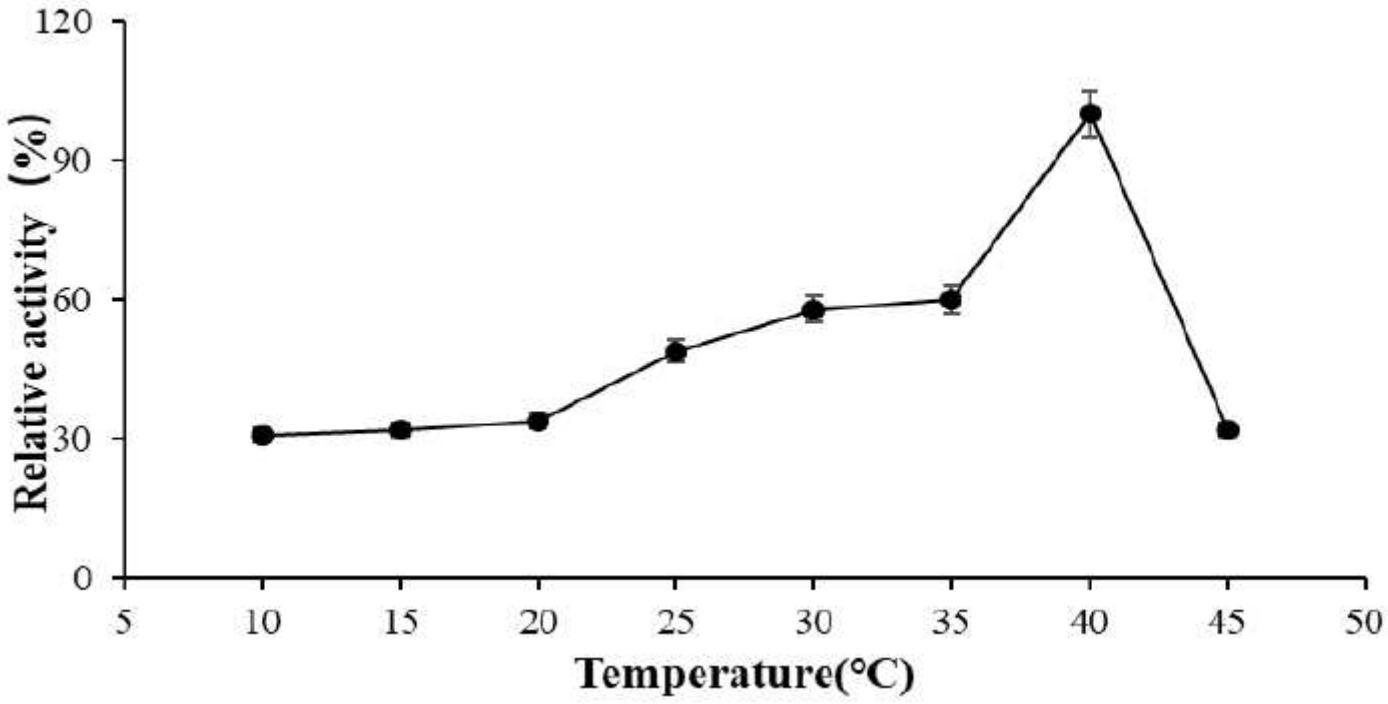

Figure 10

Effect of temperature on the activity of the immobilized mutant LIP5-D.

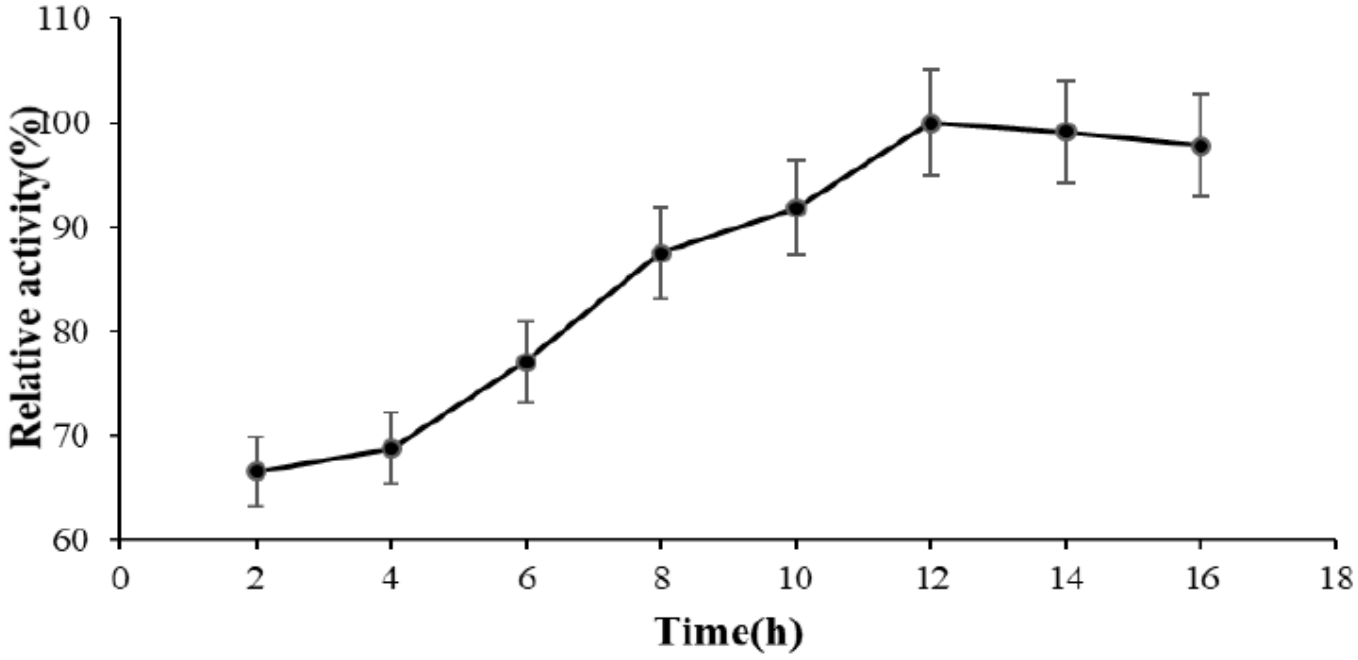

Figure 11

Effect of time on the activity of the immobilized mutant LIP5-D.

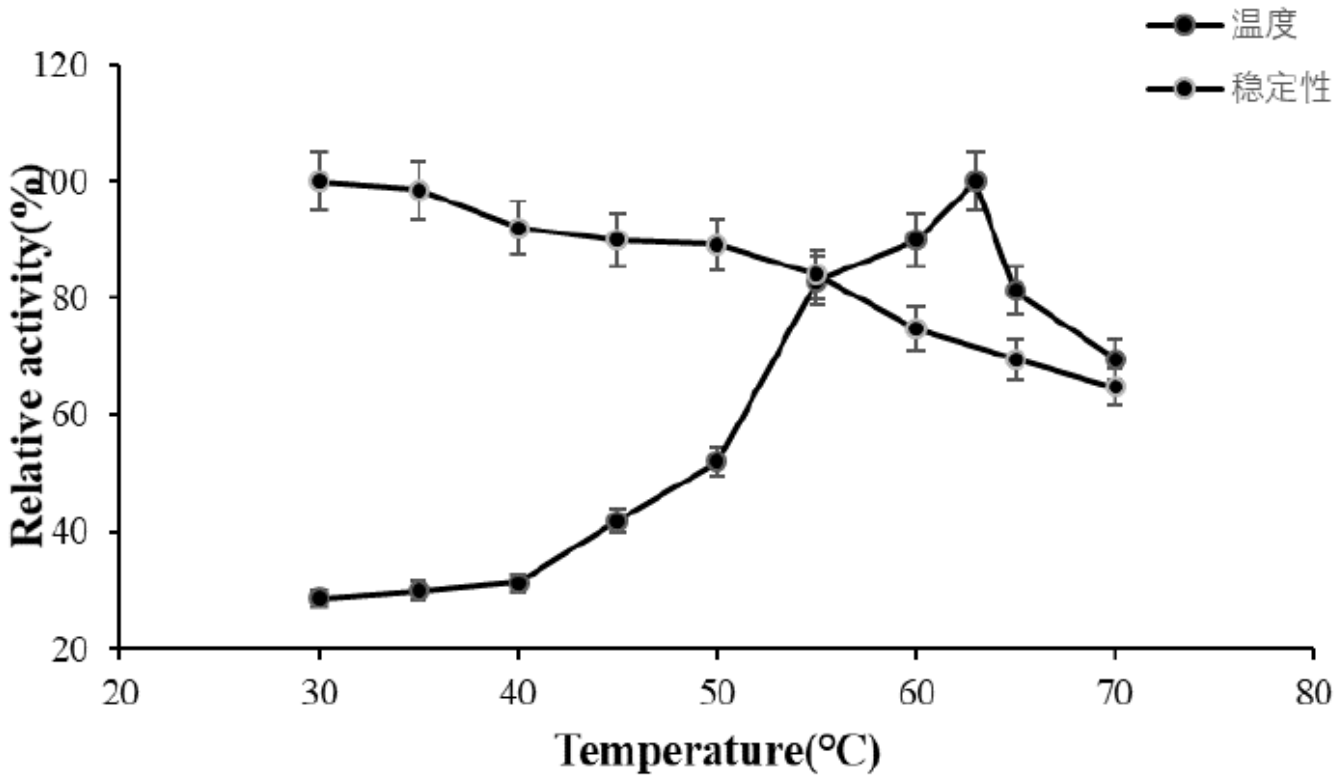

Figure 12

Effect of temperatures on the activities and stability of the immobilized mutant.

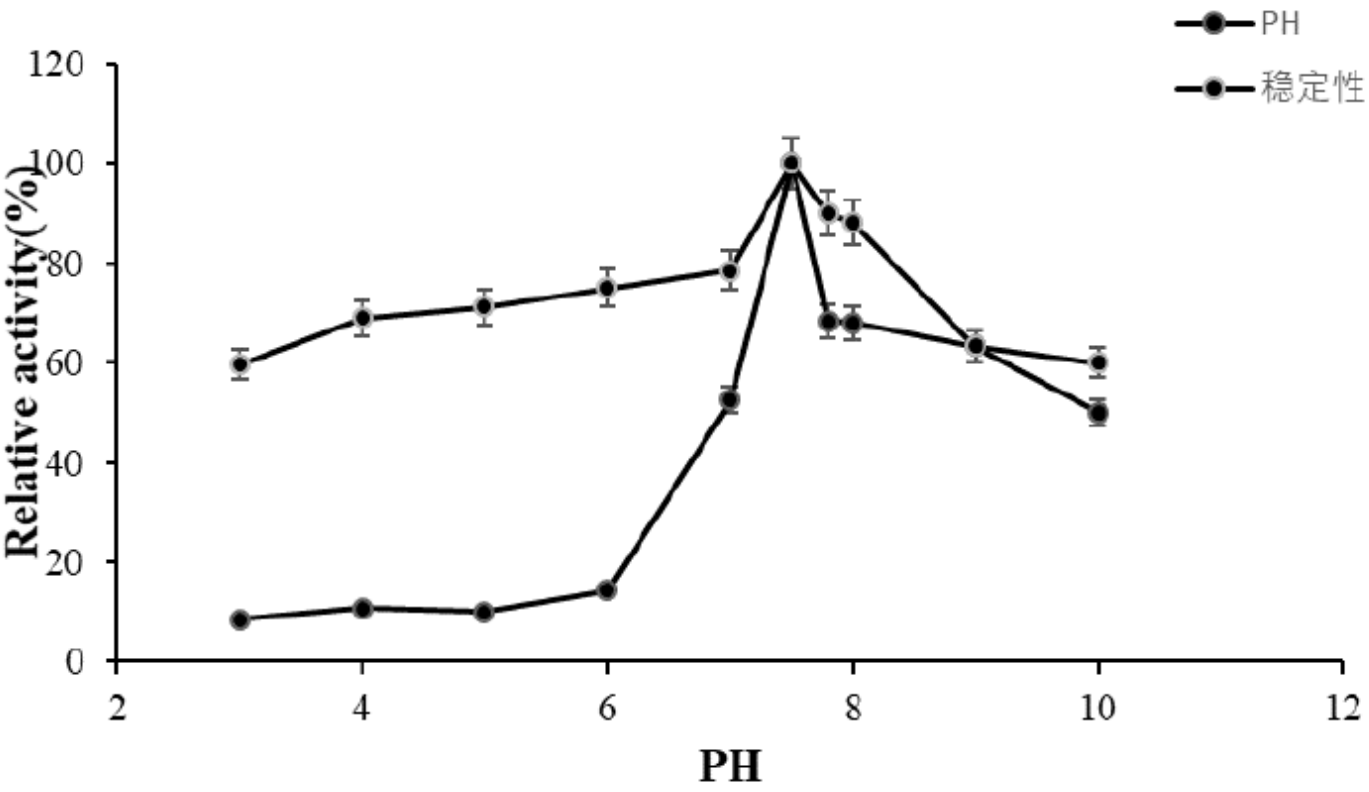

Figure 13

Effect of pH on the activities and stability of the immobilized mutant LIP5-D.

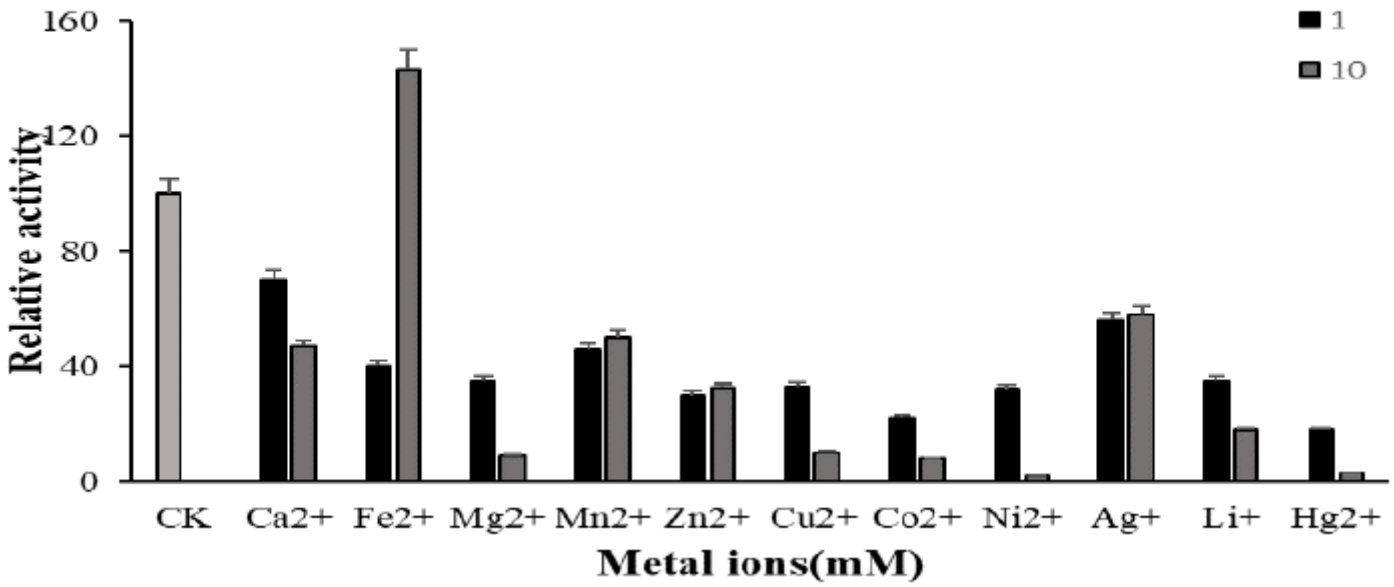

Figure 14

Effect of the various metal ions on t he activities of the immobilized mutant LIP5-D.

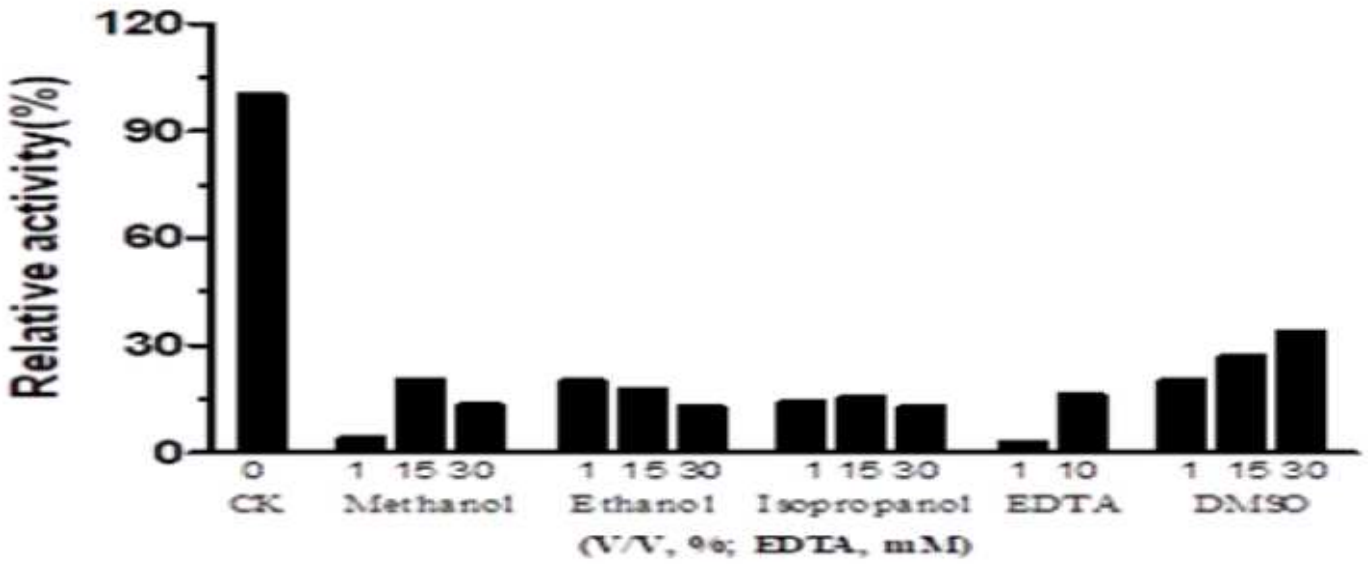

Figure 15

Effect of various organic solvents on the activities of the mutant immobilized LIP5-D.