MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Study on preparation technology of alorulose enzyme fixed resin material**

First (Bachelor's) level of higher education

Specialty 162 "Biotechnology and Bioengineering"

Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Kyiv 2024

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Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
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«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Qin Sihan

1. Thesis topic **Study on preparation technology of alorulose enzyme fixed resin material**
Scientific supervisor Olena Okhmat, Ph.D., Assoc. Prof.

approved by the order of KNUTD "____"_____2024, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

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SUMMARY

Sihan Qin. Study on preparation technology of alorulose enzyme fixed resin material – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

This study focused on the biosynthesis of D-allulose enzyme, the enzyme immobilization technology and its application in industrial production. Through screening and expression of D-allulose-3-epimerase (DPE enzyme) with high efficiency and immobilization technology, the production efficiency and stability of D-allulose-3-epimerase have been successfully improved. The optimized immobilized DPE enzyme showed better temperature and pH stability and reusability, which provided a research direction for the industrial production of D-allulose. In terms of research methods, a variety of immobilization techniques such as adsorption, cross-linking, embedding and carrier binding were used in this study, and the immobilization conditions were optimized through systematic experiments. The optimal reaction conditions of the immobilized DPE enzyme in the production of D-allulose were also investigated, which provided an important reference for its application in practical production.

In this study, the recombinant strain with high expression of DPE enzyme was screened, the optimal immobilization vector and conditions were determined, and the process parameters of D-allulose production were optimized. These results not only provide technical support for the industrial production of D-allulose, but also provide a new idea for the application of enzyme immobilization technology in the field of biotransformation. This study also proved that the immobilized enzyme technology had significant advantages in improving the production efficiency of D-allulose, which laid a solid foundation for the industrial production and wide application of D-allulose. D-allulose 3-epimerase has entered the domestic market, and the improvement in conversion rate, yield and production cost reduction has positive significance for the development of the industry.

Keywords: D-alorulose enzyme, Immobilized enzyme, Immobilization, resin.

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INTRODUCTION

D-psicose/D-allulose is a rare sugar, with 70% of the sweetness of sucrose, can occur Maillard reaction with amino acids or proteins in food, improve food color, and it provides less calories, only equivalent to 0.3% of the same quality of sucrose, will not bring additional digestive burden to the human body. Does not pose a health threat to humans. However, the chemical synthesis of D-aloxone is limited by the high energy consumption of the reaction, the complexity of the separation process, the generation of by-products and chemical waste, and so on, and the industrial applicability is low. In contrast, biosynthesis through enzymatic transformation has the advantages of mild reaction conditions, strong substrate specificity and high catalytic efficiency, and is more sustainable. The DAEase family of enzymes can efficiently catalyze the differential isomerization of C-3 position of D-fructose to produce D-alorulose, which is the core enzyme in the synthesis of D-alorulose by enzymatic conversion. However, the enzymatic conversion of D-alorulose is limited by the reaction balance and the enzyme utilization rate is low. The utilization efficiency can be improved and the cost of industrial production can be reduced by immobilizing D-alorulose enzyme. At present, the related research on the immobilization of D-alorulose enzyme is still in the preliminary stage, the preparation method is complicated and the recovery rate of enzyme activity is low, so it is urgent to explore a new carrier material and immobilization method for the immobilization of D-alorulose 3-heterotropy isomerase.

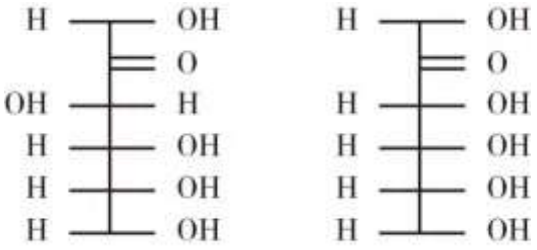
The main research **objects** are DPEase and resin materials. Research may include how to effectively fix the enzyme to the resin material to improve the stability, reusability and catalytic efficiency of the enzyme. The specific research may involve the selection of enzymes, the selection of resin materials, and the optimization of immobilization methods.

Research methods: preparation method of immobilized enzyme; methods for studying enzymatic properties.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction to D-allulose

D-allulose, also known as D-ribo-2-hexulose, is the differential isomer of D-fructose at the C-3 position 1, which can be obtained by the conversion of D-fructose to isomerase through the D-allulose 3-differential. The comparison of structural formula of D-fructose and D-allulose is shown in (Fig. 1.1).



D-fructose D-allulose

Figure 1.1 – D- Structural formulas for fructose and D-allulose

Because it was originally isolated from psicofuranine, it was originally named D-psicose 2. In 2014, Japan hosted the International Rare Sugar Congress, which officially corrected the conventional name D-psicose to D-allulose 3.

D-allulose appears as a white powder crystal, and its solubility is as follows: highly soluble in water, soluble in methanol and ethanol, insoluble in acetone. At room temperature, 100g of water can dissolve 291g of aloxulose. The molecular formula of D-allulose is $C_6H_{12}O_6$, the molecular weight is 180.156, the density is 1.589g/cm3, the surface tension is 92.6dyne/cm 4, and the melting point is 109℃. Due to its low melting point, D-allulose is not suitable for spray drying to produce powder products. The sweetness of D-allulose is 70% of sucrose, and the sweetness is mild. When consuming the same amount of aloxulose, the heat generated is only 0.3% of sucrose 5. D-allulose is very rare in nature, only distributed in a small number of plants and bacteria.

D-psicose/D-allulose is a rare sugar that was first discovered and reported by Itoh et al. 6 several decades ago. D-allulose has 70% of the sweetness of sucrose, and

it provides less calories, only equivalent to 0.3% of the same weight of sucrose 7, so it does not bring additional burden to the human digestive system, and D-allulose can react with amino acids or proteins in food to improve food color 7. In August 2011, the United States Food and Drug Administration (FAD) determined that D-allulose was generally recognized as safe food (GRAS) and could be used as a food or food additive 8. Several studies have shown that D-allulose has the effect of lowering blood sugar. D-allulose can also inhibit the rise of postprandial blood glucose levels, and can be used as an adjunct therapeutic agent for patients with borderline diabetes mellitus. No side effects or adverse reactions have been observed after 12 weeks of continuous intake of D-allulose 9. Therefore, the production of aloxulose becomes crucial.

According to the domestic research on D-allulose, the research process in China started relatively late, but in recent years, the biosynthesis of D-allulose has made great progress in China 10, and the selection of production bacteria is also more abundant. In the literature search, it was found that the literature related to D-allulose-3-differential isomerase had a high proportion of research progress published by Chinese scholars. Jiangnan University used sphaeroidoid bacteria in fish pond sludge to synthesize D-allulose 3-heterotropic isomerase, which was then used in biosynthesis to convert d-fructose into D-allulose, and the conversion rate of this enzyme was 6.54% 11. Tianjin Institute of Biotechnology extracted D-tagalose-3-differential isomerase gene from Clostridium cellulolytic and expressed it in Bacillus subtilis. D-fructose was used as substrate to catalyze the synthesis of D-allulose, with a conversion rate of 24.8% [12]. Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences cloned the D-allulose 3-aberrant isomerase gene obtained from rumen bacteria and expressed it into Escoli, converting D-fructose to synthesize D-allulose, and the thermal stability of this enzyme is higher than that of other D-allulose 3-aberrant isomerases 12. In Jiangnan University, D-allulose 3-differential isomerase produced by the fermentation of Clostridium cellulolytic was used to catalyze the synthesis of D-allulose from high fructose syrup, and the conversion rate reached 33.7% 1314. Cofco Biotechnology provides a new type of D-

allulose-3-differential isomerase from Ruminococcus (Ruminococcussp.CAG55). Using Bacillus subtilis as the host cell, after a series of operations including construction of recombinant plasmid, construction of expression strains, expression and purification of target proteins, The catalytic conversion rate of Rum55DPE crude enzyme solution was measured to be 29.75%, and it had high thermal stability and high biosafety, which could be directly used in industrial production 15. In addition, polyphosphate kinases have also been used in the synthesis of D-allulose. Wen Junting et al. coupling L-rhamnus gum kinase with D-allulose 3-differential isomerase can increase the yield of D-allulose to 50%16. At present, the conversion rate of D-allulose 3-aberrant isomerase is mostly between 28% and 33%. Using D-allulose 3-aberrant isomerase with high conversion rate to produce D-allulose can effectively improve the yield and reduce the production cost. At present, D-allulose-3-heterotropic isomerase has entered the domestic market, and the improvement in conversion rate, yield and production cost reduction has positive significance for industrial development 17.

1.2 Source and production of D-allulose

However, the chemical synthesis method of D-allulose is limited by high energy consumption, complex separation process, generation of by-products and chemical waste, and has low industrial applicability 18. In contrast, biosynthesis through enzymatic transformation has the advantages of mild reaction conditions, strong substrate specificity and high catalytic efficiency, and is more sustainable. The DAEase family of enzymes can efficiently catalyze the differential isomerization of C-3 position of D-fructose to produce D-allulose, which is the core enzyme in the synthesis of D-allulose by enzymatic conversion 19.

In 2006, Japanese scientists such as Oshima20 measured the content of D-allulose in a certain part of food, which showed that the content of D-allulose varied greatly in different foods. Due to the reversible conversion between D-fructose and D-allulose, once these foods are heated, the D-fructose in them will undergo non-enzymatic reaction and eventually become D-allulose. Therefore, if the raw material

of the food is already sugar-containing, there will be heat generated during the later processing. This can lead to a higher content of D-allulose in sauces and sweets 21.

Due to the very small content of D-allulose, it is difficult to extract and treat it from nature, and the purification difficulty of chemical synthesis method is high, and the reaction energy consumption of chemical synthesis method is large, the separation process is more complex and will produce a large number of by-products and chemical waste and other limitations, so the industrial applicability is relatively low. In contrast, biosynthesis through enzymatic transformation has the advantages of mild reaction conditions, strong substrate specificity, and high catalytic efficiency, and is more sustainable 19. The synthesis of D-allulose by enzymatic conversion can achieve very high product concentration, and the product extraction process is relatively simple, which is undoubtedly very promising.

Although pure D-allulose-3-heterotropy isomerase has its unique utility, its stability is relatively weak, and it is susceptible to environmental factors, such as pH value, temperature, etc., and even changes in other external conditions may cause its activity to be reduced or inactivated. The stability of pure D-allulose-3-aberrant isomerase is poor, and the utilization rate is very low. At the same time, the enzymatic conversion reaction of D-allulose is a balanced reaction, so the utilization rate of the enzyme is low, so the D-allulose enzyme can be immobilized to improve its utilization efficiency and achieve the result of improving the industrial production efficiency.

1.3 Immobilization of D-allulose enzyme

In the actual production process, the operation stability of free enzymes is poor, the reuse rate is low, the separation is difficult and the cost is high 19. In recent years, with the increasing application of enzyme catalytic process in chemical production, immobilized enzyme technology, which is the combination of materials and enzymes, has also shown a vigorous development momentum.

It was found that immobilized enzyme can overcome these limitations and make the enzyme more effective and widely used. Immobilized enzymes are defined

as enzymes that adhere to inert and insoluble materials while remaining active 22. The so-called immobilized enzyme, also known as solid phase enzyme, is an enzyme application technology developed along with enzyme engineering research, which is to fix the enzyme on a solid material by physical or chemical methods 23. The immobilization of the enzyme can change the catalytic properties of the enzyme, including substrate selectivity, enzyme activity, stability under extreme pH and temperature, and resistance to inhibitors. Moreover, immobilized enzyme not only has simple preparation process and low cost, but also has high catalytic activity, high stability and can be reused. Compared with free enzymes, immobilized enzymes in addition to retaining its high efficiency, specificity, mild reaction conditions, environmental protection and other performance characteristics, but also has the advantages of multiple reuse, can be separated from the reaction system by simple methods such as centrifugation or filtration, high stability, and easy transportation and storage, easy production of continuous and automated operation. At the same time, it also simplifies the post-treatment process of catalytic reactions. The technology of immobilizing enzymes in the carrier by means of chemical or physical means, which is spawned by the development of modern science24, not only allows the enzyme to maintain its catalytic properties in the past, but also reduces the defects of free enzymes themselves, and promotes the automatic and continuous reaction. Is a highly valuable technology. In the separation and purification process, the continuous separation and purification of D-allulose by special technology can obtain D-allulose with high purity and high yield, and at the same time realize the recovery and utilization of D-fructose, greatly reduce the production cost, reduce the environmental pollution in the production process, and promote the economic and social benefits of enterprises 22.

At present, the related research on the immobilization of D-allulose enzyme is still in the preliminary stage, and the preparation method is relatively complicated with low recovery rate, so it is urgent to explore a new carrier material and immobilization method for the immobilization of D-allulose 3-differential isomerase 19.

1.4 Main research contents of the subject

1. Find out suitable genetically engineered bacteria from published papers, obtain recombinant cells expressing DPE enzyme, and purify DPE enzyme.

2. The best carrier for fixing DPE enzyme was selected from a variety of resins, and the immobilization conditions were optimized through single-factor experiments, such as the selection of resin carrier, the optimization of the amount of enzyme added, the optimization of adsorption temperature and the optimization of adsorption time.

3, the free enzyme and the immobilized DPE enzyme were compared respectively, and the enzymatic properties of the immobilized enzyme were analyzed, such as the optimal reaction temperature and temperature stability, the optimal pH and pH stability, and the reusability.

Conclusions to chapter 1

Introduction to D-allulose;Source and production of D-allulose;Immobilization of D-allulose enzyme and main research contents of the subject.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

The main research topic is how to effectively fix DPEase to resin materials by immobilization technology. It includes selecting the appropriate resin material as the carrier of immobilized enzyme, exploring and optimizing the immobilization method of enzyme, and evaluating the influence of immobilization on the catalytic activity and stability of enzyme. At the core of the research theme is the development of an efficient and stable aloxone enzyme immobilized resin material to improve the production process of aloxone.

2.1 Experimental materials and reagents

2.1.1 Experimental equipment

High performance liquid chromatograph automatic sampler、 Parallax refraction detector （Shimadzu Co., LTD; Electronic balance （Nanjing Bonita scientific Instrument Co., LTD; Autoclave （Deqiang Purification Technology (Shandong) Co., LTD; Constant temperature water bath oscillator（Taicang City Qiangle experimental equipment Co., LTD）;Spectrophotometer（Shanghai Youke Instrument Co., LTD; Microcoder（Meigu molecular Instrument (Shanghai) Co., LTD; Bradford protein concentration assay kit（Beijing Coolaibo Technology Co., LTD; Syringe Filter （Tianjin Keyilong Experimental Equipment Co., LTD; Disposable sterile syringe（Henan Shuguang Hui Zhikang Biotechnology Co., LTD; 9-425Screw Thread Vial （ALWSCI Corporation; Pipette （Dalong Xingchuang Experimental Instrument (Beijing) Co., LTD; 1ml(1000ul) Suction head、 200ul Suction head、1.5ml imitation imported centrifugal tube、50ml screw round bottom centrifugal tube（Nantong Haizhixing Experimental Equipment Co., LTD.

2.1.2 Experimental materials

IR120（Beijing Qingke Biotechnology Co., LTD）;ZGA351、ZGA412、ZGA313、ZGA302、ZGA304、ZGD630、D319、D213、EP107F、EP109F（Ningbo Zhengguang Resin Co., LTD.

2.1.3 Experimental reagents

D-alor ketoenzyme(COFCO Group Co., LTD); D-fructose(Beijing Coolaibo Technology Co., LTD); Pure water(Changjiang Hutchison Co., LTD); PBS buffer(Beijing Coolaibo Technology Co., LTD); 95% ethanol (Jinan Tianyou Chemical Co., LTD).

2.2 Experimental methods

2.2.1 Preparation method of immobilized enzyme

The physical methods include physical adsorption method and embedding method. The advantage is that the enzyme is not directly involved in the chemical reaction, so its overall structure is kept intact, thus effectively preserving the catalytic activity of the enzyme. Chemical laws include two main categories: binding and crosslinking. The binding method can be subdivided into ionic binding method and covalent binding method. This method mainly connects enzymes to natural or synthetic polymer carriers through chemical bonding, and uses coupling agents to cross-link through groups on the enzyme surface to form immobilized enzymes with larger molecular weight and insolubility 25.

(1) Adsorption method

Adsorption method is a kind of separation and purification technology, especially in the field of bioengineering has a wide range of applications. The core principle of this method is the use of specific adsorbents, which usually have a high degree of specificity and affinity, by physical or chemical means, can effectively "trap" the enzyme or enzyme containing bacteria and firmly adsorb the enzyme or enzyme containing bacteria on its surface. Once these biomolecules are adsorbed to the surface of the adsorbent, they can be separated from the mixture by appropriate

elution conditions for purification or enrichment purposes. A significant advantage of this method is that it is easy to operate, relatively low cost, and has little effect on the activity of the target substance. Therefore, in the fields of bioengineering, pharmaceutical and biotechnology, adsorption has become an important means of separation and purification. Especially in the separation and purification process of enzymes, adsorption method because of its high efficiency and mild characteristics, in addition, by adjusting the type and nature of adsorbents, it can achieve selective adsorption of different types of enzymes or bacteria, so as to meet the diversified separation needs. This makes the adsorption method extremely flexible and applicable in practical applications.

(2) Cross-linking method

By means of bifunctional group reagents, cross-linking between enzyme molecules can be realized, and a network structure can be formed. This bifunctional group reagent plays a key role in enzyme immobilization technology. The principle is to use the two active groups in the reagent to react with the specific groups on the two enzyme molecules, so as to connect the two enzyme molecules together. When multiple enzyme molecules are connected in this way, a network structure is formed, which makes the bond between the enzyme molecules even stronger. Importantly, this cross-linked agglutination reaction not only enhances the stability and mechanical strength of the enzyme, but also renders the overall structure of the enzyme insoluble in water. In addition, this cross-linking can significantly improve the thermal stability and acid and alkali resistance of the enzyme, allowing it to maintain highly efficient catalytic activity under a wider range of environmental conditions. This also means that the enzymes fixed by this technology can show a longer service life and higher catalytic efficiency in practical applications.

(3) Embedding method

Embedding method is an effective enzyme immobilization technology. One method is to wrap the enzyme in a tiny lattice of gel. The other method is to surround the enzyme with a semi-permeable polymer membrane, so as to realize the fixation and protection of the enzyme. In the process, the enzyme solution is mixed with a

specific gel or polymer material, which then forms tiny lattice or membrane structures that tightly enclose the enzyme molecules. It provides a stable and conducive environment for enzyme molecules to play their role, which can effectively prevent external interference and damage. At the same time, this embedding structure also allows the substrate and products to pass through, but limits the flow of enzyme molecules, thus ensuring the continuous action of enzymes in a fixed position. After immobilization, the enzyme has a greater tolerance to changes in temperature and pH. Although its optimal pH may be slightly shifted, its substrate specificity does not change in any way. More importantly, this immobilization technology significantly improves the actual use efficiency of the enzyme, sometimes even tens of times as much. In addition, the embedding method has the advantages of simple operation, little influence on enzyme activity and high immobilization efficiency. This technique is not only applicable to a single enzyme, but also can be used to fix a variety of enzymes to form a multi-enzyme system, so as to achieve multiple enzymatic reactions in the same reaction system. By embedding method, we can fix the enzyme on a specific carrier, which not only improves the stability and reusability of the enzyme, but also facilitates the control and optimization of the enzyme reaction. This technology has a wide application prospect in many fields such as bioengineering, biopharmaceutical, food processing and so on.

(4) Carrier binding method

Bifunctional group reagents play an important role in enzyme immobilization technology. This class of reagents has two or more reaction groups and can chemically react with specific groups on the surface of the enzyme molecule, thereby promoting cross-linking between the enzyme molecules. When bifodal reagent is added to the enzyme solution, these reagents will quickly find and lock the reaction site on the enzyme molecule, such as amino, carboxyl, etc. Once locked on to the target, the bifunctional reagent uses the reaction groups at its ends to react with both enzyme molecules simultaneously, binding them tightly together. This process continues until a complex network of enzyme molecules forms. The formation of this network structure is essential for the immobilization of enzymes. It not only enhances

the stability of the enzyme molecule, so that it can maintain activity in the face of the challenges of the external environment, but also improves the reusability of the enzyme. In addition, the network structure also makes it easier for the enzyme to contact the substrate during the reaction, thus improving the catalytic efficiency. The selection and use conditions of bifunctional group reagent have significant influence on the immobilization effect of enzyme. Different types of bifocal reagents may lead to different crosslinking degree and network structure, which may affect the activity and stability of enzymes. Therefore, in practical applications, it is necessary to select the appropriate bifunctional group reagent according to the specific enzyme types and reaction conditions.

2.2.2 Research methods of enzymatic properties

(1) Optimum reaction temperature and temperature stability

Temperature has two sides to the action of enzymes. On the one hand, it can promote the rate of enzyme reaction; On the other hand, since enzymes are proteins in nature, high temperature will accelerate the denaturation process of enzyme proteins. When the enzyme reaction rate reaches its peak, the corresponding temperature is called the optimal temperature of the enzyme reaction.

A series of experiments were conducted to investigate the effect of temperature on enzyme activity while other reaction conditions were kept constant. Enzyme activity was measured under different temperature conditions, ranging from low to high, in order to get a complete picture of how temperature affects the catalytic performance of enzymes. In order to ensure the accuracy and reliability of the experiment, it is necessary to strictly control all other factors that may affect enzyme activity, such as substrate concentration, pH value and enzyme concentration, so that they remain consistent throughout the experiment, so that it can be assured that any observed changes in enzyme activity are caused by temperature changes. At each set temperature point, we carefully measured enzyme activity and recorded the data. This data is crucial for us to understand how enzymes behave at different temperatures. In order to visually display the experimental results, a detailed chart was drawn with

temperature as the horizontal coordinate and enzyme activity as the vertical coordinate. By analyzing these charts, it is possible to observe the trend of enzyme activity with temperature. In the lower temperature range, the activity of the enzyme gradually increases with the increase of temperature, which indicates that the appropriate heating can improve the catalytic efficiency of the enzyme. However, when the temperature exceeds a certain threshold, the activity of the enzyme begins to decline, which may be due to the denaturation of the enzyme protein caused by high temperature. This experiment can not only understand the effect of temperature on enzyme activity, but also provide an important basis for optimizing the conditions of enzyme-catalyzed reaction. In practical applications, the reaction temperature can be adjusted according to these experimental results to achieve the best efficiency of enzyme-catalyzed reactions.

Thermal stability test is to evaluate the thermal stability of enzyme samples by exposing them to heat treatment at high temperatures and observing changes in their catalytic activity: this test method can help us understand the biochemical properties of enzymes at high temperatures and provide an important reference for the industrial application of enzymes. In the thermal stability test of enzymes, it is necessary to prepare enzyme samples first. The extracted enzyme sample needs to be purified and concentrated to obtain a high purity and appropriate sample. The enzyme sample then needs to be heated to a specific temperature. Different enzymes exhibit different thermal stability at different temperatures. Therefore, a suitable temperature range needs to be determined prior to testing to ensure that the decomposition and inactivation of the enzyme sample is observed. During the heating process, the catalytic activity of the enzyme sample will gradually decrease with the increase of temperature. This is because high temperatures can cause structural changes in enzymes that damage or inactivate their active centers. By measuring the activity of enzyme samples, a temperature dependent activity curve can be obtained. To illustrate the thermal stability of a chemical enzyme, it is usually shown as a graph of enzyme activity relative to temperature. This graph shows the activity of enzyme samples at different temperatures. For example, it can be seen from the figure that the

activity of the enzyme sample decreases rapidly with increasing temperature, and when the temperature exceeds a certain critical point, the enzyme sample is almost completely deactivated. In addition to the activity curve, the thermal stability of enzymes can also be assessed using the semi-inactivation temperature (T50). T50 refers to the temperature required to reduce the enzyme sample activity to 50% of the original activity under high temperature conditions. A higher T50 value indicates that the enzyme has good thermal stability and is able to maintain high activity over a higher temperature range.

(2) Optimum pH and pH stability

Enzymes are special proteins in living organisms that catalyze chemical reactions under certain conditions. The catalytic activity of enzymes is closely related to environmental factors. Among many factors, pH value is one of the key factors that affect enzyme activity. The pH value at the peak of enzyme activity is the optimum pH of enzyme, which is of great significance for the research and application of enzyme. In general, the activity of enzymes shows different trends at different pH values. The enzyme activity may be inhibited or reduced under the conditions below the optimal pH value. At conditions above the optimal pH value, enzyme activity may also be inhibited or reduced. By measuring enzyme activity at different pH values, the dependence of enzyme activity on pH can be understood. According to the optimal pH of the enzyme, the appropriate pH conditions can be selected to optimize the catalytic reaction of the enzyme. The optimal pH of an enzyme is determined by measuring the activity of the enzyme at different pH values. Therefore, by measuring the activity of the enzyme at different pH values and plotting the curve between the activity and pH value, the optimal pH of the enzyme can be determined.

The change of hydrogen ion concentration is not only closely related to the optimal pH value of the enzymatic reaction, but also involves the important influence of pH on the stability of the enzyme, which is essentially different. Because of the difference of amino acid composition and conformation, especially the conformation of its action center and the uniqueness of its microenvironment, each enzyme has a

specific function and catalytic activity. This specificity stems from the folding pattern and spatial structure of the enzyme molecule, which in turn is determined by the amino acid sequence. In addition, the tertiary structure of the enzyme molecule, stabilized by non-covalent forces between amino acid residues (such as hydrogen bonds, ionic bonds, hydrophobic interactions and van der Waals forces), plays a key role in the catalytic efficiency and specificity of the enzyme. Therefore, the different amino acid composition and conformation, especially the uniqueness of the action center, give various enzymes different catalytic properties and biological functions. Therefore, this PH-induced denaturation can be reversed, and by adjusting the pH value, the activity of the enzyme can be fully restored.

(3) Reusability

Immobilized enzymes have the advantage of being reusable. In the process of substrate conversion of the immobilized enzyme, the substrate can penetrate through the fixed carrier to the reaction site of the fixed enzyme and react at the site. In this way, after the reaction is over, the enzymes on the fixed carrier that are still attached to the fixed carrier can be easily separated and recovered. Since the fixed enzyme is reusable, production costs can be reduced and efficient production can be achieved.

2.3 Experimental Procedure

2.3.1 Screening resin for fixing DPE enzyme

From the published articles, the types of resins with better fixing effect were screened. IR120 is a cation exchange resin, ZGA351 is a large porous strong alkaline negative resin, ZGA412 is a weak alkaline acrylic negative resin, ZGA313 is a strong acrylic negative resin, ZGA302 is a gel strong alkaline negative resin, ZGA304 is a strong basic anionic resin, ZGD630 is a large porous strong alkaline negative resin, and D319 is a large porous strong negative resin Alkaline negative resin, D213 are large pore strong alkaline negative resin, EP107F, EP109F.

2.3.2 Resin pretreatment

95% ethanol soak: Weigh 5g of each resin in a 50ml centrifuge tube and label it. Soak the mixture with 95% ethanol for about 20h. Stir the mixture during the soaking process, which can achieve better results in the pretreatment process of the resin.

Blank control: Weigh 5g of each resin into 50ml centrifuge tube, wash it with deionized water twice, and then wash it with PBS twice.

2.3.3 Cross-linking adsorption of resin and enzyme

The resin was mixed with the enzyme solution, and 30ml of the enzyme solution was added to each centrifuge tube after cleaning the resin. The resin and enzyme system was put into a shaking table at 200rpm at 30℃ for cross-linking, and 0.5ml was taken after 1h, 2h, 3h and 4h, respectively.

2.3.4 Determination of adsorption efficiency of resin-fixed DPE enzyme

With the Bradford Protein concentration Assay Kit, we can quickly, stably and accurately determine the protein concentration of the sample taken. The kit is designed based on the Bradford method, and its features include fast detection speed, high sensitivity and good stability. In particular, 10 to 20 samples can be tested in less than 10 minutes. At the same time, the detection limit can reach 25ug/mL, and the minimum detectable protein amount is as low as 0.5ug. The kit is suitable for sample volumes ranging from 1 to 20L and has excellent linear relationships over concentrations ranging from 50 to 1000ug/mL. It is worth mentioning that the kit showed good stability within 1 hour, especially in the period of 5 to 20 minutes, stability is best. In addition, the Bradford method has a strong tolerance to most of the chemicals in the sample when determining protein concentrations. However, it is important to note that this method may be affected by slightly high concentrations of detergent. Therefore, when using, you should ensure that the concentration of SDS does not exceed 0.05%, the concentration of TritonX-100 does not exceed 0.05%, and the concentration of Tween20, Tween60 or Tween80 does not exceed 0.02%. For

samples containing detergent, the BCA protein concentration assay kit (SK1070) from Coolaber is recommended.

The specific steps are as follows:

1. Take a certain amount of BSA protein standard solution, and then dilute it with PBS diluent until its final concentration reaches 0.5mg/mL, so that the required BSA protein working solution can be obtained.

2. Take an appropriate amount of G-250 dyeing solution (concentration is 5×) and dilute it by adding distilled water in a ratio of 1:4 to prepare 1× concentration of G-250 dyeing solution. Please note that this dye solution needs to be used on the spot to ensure its effectiveness.

3. Add the BSA protein standard sample and the appropriately diluted protein sample to be measured into the 96-well plate in the preset order, and ensure that the standard sample is also added in the prescribed order for subsequent determination and analysis. The standard sample should be filled with PBS diluent to 20μL. Add 1×G-250 dyeing solution 200μL to each well and leave for 3-5min at room temperature (Tab. 2.1).

Table 2.1 – **Adding method of Bradford protein concentration assay kit**

Adding sequence	Standard sample number								Sample number	
Mark number	1	2	3	4	5	6	7	8	9	10
BSA protein standard	0	1μL	2μL	4μL	8μL	12μL	16μL	20μL	20μL sample	…
PBS diluent	20μL	19μL	18μL	16μL	12μL	8μL	4μL	0		
1*G-250 dyeing solution	200μL									

Note: BSA standard sample concentrations (ug/mL) are 0, 25, 50, 100, 200, 300, 400, 500, respectively.

4. Use an enzyme-labeled instrument to measure the absorbance in each hole at 595nm, or choose any wavelength between 560-610nm for measurement. According to the absorbance data of BSA protein standard samples, the standard curve was

drawn. Finally, the concentration of the protein sample to be measured can be calculated by the standard curve.

Note:

1.G-250 dyeing solution (5×) Please reverse 3-5 times before use and mix well.

2. Please mix the BSA protein standard solution after it is completely dissolved and then dilute it into the BSA protein standard sample.

Protein immobilization rate (%)=(A1-A2)/A1

Note: A1: Initial total protein (mg) before immobilization; A2: Immobilized liquid protein (mg)

2.3.5 Immobilized enzyme treatment

The cross-linked resin was washed twice with PBS (PH=8) to remove the surface free enzymes.

Setting of blank group: Free enzyme solution with similar concentration was prepared according to adsorption efficiency.

2.3.6 Determination of conversion rate of resin-immobilized DPE enzyme

(1) Preparation of substrate. To prepare 55% fructose by volume: weigh and dissolve 550g fructose in 1L deionized water, stir until the fructose is completely melted, and obtain a clear and transparent fructose solution. Sample 1ml as a control.

(2) Resin treatment. After rinsing the resin with pure water for 2-3 times, rinse it with PBS buffer for 2-3 times, and remove all the liquid in the centrifuge tube.

(3) Conversion reaction. 30ml fructose was added to each bottle of resin, and the reaction was carried out at 150rpm at 60℃ in a water bath shaker. 1h later, 0.5ml was sampled. The reacted solution in the bottle is filtered using filter paper to minimize resin loss.

(4) Take samples for 7h and number them successively.

(5) Take the sample to sample: pure water 1:9 dilution into 1ml solution.

(6) After the diluted solution is filtered through a filter membrane, it is carefully packed into a threaded sealed bottle for subsequent liquid chromatography operations.

First check whether the column is installed correctly, whether it is clean, and check whether the preparation of the mobile phase is accurate. Then the prepared sample to be measured, that is, the screw bottle, is put aside to be measured. Then turn on the power, turn on the power switch of the instrument, wait for the instrument to initialize, and ensure that all parts of the instrument are working normally. Then set the parameters required for the analysis, including detector type, detection wavelength, flow rate, temperature, etc. Then start the HPLC system and wait for the system to stabilize. The column is then calibrated to ensure the accuracy and reliability of the results. Finally, the sample was injected into the chromatographic column through the syringe, and the test was started, and the results were analyzed.

2.3.7 Determination of reutilization times of resin-immobilized DPE enzyme

The reusability of immobilized DAE was assessed by measuring enzyme activity over twelve consecutive reuse cycles. The immobilized DAE was recovered by centrifugation (8000xg, 5 min, 4C) at the end of each reaction cycle. The recovered enzymes were then washed twice with a 50mmol/LPBS buffer (pH7.5) and then re-suspended in a fresh fructose solution for the next reaction cycle. The initial activity of immobilized DAE was considered to be 100% in the first cycle, and the residual activity after each cycle was compared with that in the first cycle, and the conversion rate of enzymes was used as an index to reflect the repeatable utilization rate of enzymes.

The active recovery rate is calculated as follows:

Recovery rate (%) =C1/C2×100

Note: C1: enzyme activity of immobilized enzyme (U); C2: Initial enzyme activity before free enzyme fixation (U)

Conclusions to chapter 2

Experimental materials and reagents; Experimental methods include preparation method of immobilized enzyme and research methods of enzymatic properties. Experimental Procedure include screening resin for fixing DPE enzyme; Resin pretreatment; Cross-linking adsorption of resin and enzyme; Determination of adsorption efficiency of resin-fixed DPE enzyme; Immobilized enzyme treatment; Determination of conversion rate of resin-immobilized DPE enzyme and Determination of reutilization times of resin-immobilized DPE enzyme.

CHAPTER 3
EXPERIMENTAL PART

3.1 Optimization of resin immobilized DPE enzyme conditions

3.1.1 Selection of resin carrier

The choice of resin carrier depends on the specific application needs and the properties of the resin. Here are some factors to consider when choosing a resin carrier:

The physical properties of the resin: the resin carrier should have a certain mechanical strength and should be stable in the chemical reaction. At the same time, in order to ensure the smooth operation of the experiment, it is essential to accommodate the growing peptide chain and facilitate the smooth entry of the reactants into the carrier, and polystyrene resins with a concentration of 1%-2% crosslinking degree are usually chosen. This concentration choice is carefully considered to provide an environment that is both suitable for the growth of the peptide chain and easy for the reactants to penetrate into the interior of the carrier, resulting in more accurate and reliable results. However, when the cross-linking is too low, the internal structure of the material is relatively loose, and the connection between the molecular chains is not tight enough. In this case, the material may exhibit excessive swelling. Due to the large void between molecules, the material is easy to absorb and retain a large amount of solvent or water, resulting in a significant expansion of its volume. When the swelling is too large, the material will become viscous and difficult to filter through conventional methods, which undoubtedly increases the difficulty of processing. Too low crosslinking will also have an adverse effect on the mechanical properties of the material. In the case of insufficient crosslinking, the interaction between polymer chains is weak, which makes it difficult to effectively transfer and disperse stress when the material is subjected to external forces. Therefore, such materials often show poor mechanical properties, such as tensile strength, compressive strength, etc., will be significantly reduced. Moreover, due to the instability of the internal structure, these materials are easily broken when

they are impacted or squeezed by external forces, thus limiting their use in various application scenarios.

Linking sites and reaction groups: Resin carriers are a key component in polymer chemistry, especially in biochemical processes such as peptide chain synthesis. In order to ensure that the peptide chain can be effectively attached to the resin and successfully cut off from the resin in the following steps, the resin carrier should be specially designed and embedded with a number of binding sites for the capture and fixation of the peptide chain. Each site is carefully designed to ensure that it binds tightly to a specific part of the peptide chain while also being released when needed. This design not only ensures the stability and controllability of the peptide chain in the synthesis process, but also greatly improves the efficiency and success rate of synthesis. When the peptide chains are attached to the resin carrier through these specific sites, they can undergo subsequent chemical reactions or modifications in a stable environment. This process avoids uncontrolled polymerization or other side reactions caused by the peptide chains floating freely in solution. Once the synthesis of the peptide chain or other desired reaction is completed, these specific sites can play a key role. By specific chemical methods, such as the use of specific cutting reagents or changing environmental conditions, peptide chains can be cut from these sites, resulting in a pure, structurally complete peptide chain product. In addition, a reaction group (joint) needs to be introduced into the benzene ring of the resin in order to attach the first amino acid. The commonly used reaction group is chloromethyl, but due to the potential carcinogenicity of chloromethylated chloromethyl ether, and the chloromethylation of PS may be an uncertain complex reaction, careful selection is required.

Attachment capacity of the carrier: The carrier should provide enough attachment points so that each unit volume of the carrier can be attached to the peptide. At the same time, the interaction between the carrier and the peptide chain should be minimized to avoid interference with the experimental results.

3.1.2 Optimization of adding enzyme quantity

In order to determine the optimal amount of enzyme solution, 0.5g resin with excellent performance was selected, and different volumes of purified enzyme solution (enzyme activity was 150U/mL) were added for test. Specifically, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5mL of enzyme liquid were added to the resin, and then PBS buffer was added to form a 1mL enzyme system, which was placed in a 4mL centrifuge tube and immobilized and adsorbed on a water bath table at 30℃ for 4h, so as to observe the influence of different enzyme liquid addition levels on the reaction effect. It is expected to find the optimal amount of enzyme solution to match the resin, so as to achieve the best reaction efficiency and effect. After the adsorption was completed, the immobilized enzyme was obtained by rinsing with PBS buffer three times. The immobilized enzyme was converted and the enzyme activity was measured. The optimal amount of added enzyme was selected based on the recovery rate of enzyme activity.

3.1.3 Optimization of adsorption temperature

In order to determine the optimal adsorption temperature, 0.5g resin with excellent performance was selected, the pure enzyme solution was diluted to 15U/mL, 1mL was added to the resin, the mixture was placed in a 4mL centrifuge tube, and 6 different temperature conditions were set at 10℃ as the temperature gradient (10, 20... 60℃) were respectively adsorbed in a water bath shaker for 4 hours. After the adsorption was completed, the immobilized enzyme was obtained by rinsing with PBS buffer three times. The immobilized enzyme was converted and the enzyme activity was determined. The optimal adsorption temperature was selected based on the enzyme activity recovery rate. In this way, the influence of temperature on the reaction between resin and enzyme solution is explored, and the most appropriate reaction temperature is found to achieve the best reaction effect. This step is of great significance for optimizing the enzymatic reaction process and improving the product quality.

3.1.4 Optimization of adsorption time

In order to determine the best adsorption time, 0.5g resin with excellent performance was selected, and the pure enzyme solution was diluted to 15U/mL, then 1mL was added to the resin, the mixture was placed in a 4mL centrifuge tube, and adsorbed for 1, 2, 3, 4, 5, and 6h on a water bath shaking table at 30℃, respectively. After the adsorption was completed, the immobilized enzyme was rinsed 3 times with PBS buffer solution. The immobilized enzyme was converted and the enzyme activity was determined. The optimal adsorption time was selected with the recovery rate of enzyme activity as the index.

3.1.5 Study on enzymatic properties and stability of resin-immobilized DPE enzyme

(1) Optimum temperature and temperature stability

Under the optimal reaction conditions, the immobilization of DPE enzyme was carried out. In order to study the optimum temperature of immobilized enzyme in detail, 0.5g of immobilized enzyme was first taken and placed into eight 4mL centrifuge tubes. Set 8 different temperatures with 10℃ as the temperature gradient (20, 30... 90℃), 400μL100g/ LD-fructose was added under different temperature gradients, homogenized, and reacted under water bath conditions for 5 min. At the same time, the centrifuge tubes containing immobilized enzymes were observed in detail. Finally, samples were collected and the activity of free enzymes was determined. The purpose of these operations is to further explore the effect of temperature on the activity and stability of immobilized DPE enzymes, so as to ensure the optimal catalytic performance of immobilized DPE enzymes in practical applications. At the same time, the same free enzyme was taken and repeated to determine the activity of free enzyme.

Under the optimal reaction conditions that have been determined, the immobilized DPE enzyme, first take 0.5g of immobilized enzyme, respectively into 9 4mL centrifuge tubes, add 1mLPBS buffer, and then set 9 different temperatures (30, 35... After standing in a water bath at 70℃ for 1 hour, the sample was taken out and

washed with deionized water for 3 times to determine the enzyme activity of the immobilized enzyme. At the same time, 100μL of free enzyme was taken into 1.5mL centrifuge tube, and the above operation was repeated to determine the enzyme activity of free enzyme.

(2) Optimum pH and pH stability

Under the optimal reaction conditions that have been determined, the DPE enzyme is immobilized. First, 0.5g of the immobilized enzyme is put into 6 4mL centrifuge tubes, and 6 different pH values are set with pH 1 as the gradient (5, 6... 10) Each buffer was added into a 700μL centrifuge tube, then 300μL400g/ Ld-fructose was added, and the enzyme activity was measured after reaction at 55℃ for 10min. At the same time, the same free enzyme was taken and repeated to determine the activity of free enzyme.

Under the optimal reaction conditions that have been determined, the immobilized DPE enzyme, first take 0.5g of immobilized enzyme, respectively, into 9 4mL centrifuge tubes, pH 0.5 as the gradient set 9 different pH (5.5, 6.0... 9.5) Add 1mL of buffer into a centrifuge tube, place it at 4℃ for 1h, rinse it with deionized water for three times, add D-fructose solution for reaction, and finally take immobilized enzyme to measure its enzyme activity. At the same time, the above experiment was repeated with 100μL free enzyme, and after standing for 1h at 4℃, 600μL200g/ LD-fructose reaction was added, and then the enzyme activity was determined.

3.2 Resin screening results

After several experiments, two resins, D213 and ZGA351, were selected from the existing resins, and the two resins were treated with ethanol in the control group. The four resins were fixed with DPE enzyme at the same time, while the two enzymes, 213A and GD, were fixed with only two enzymes treated with ethanol, respectively labeled as follows:

	D213(Ethanol treatment)	ZGA351(ethanol treatment)	D213(untreated)	ZGA351(untreated)
DPE	A	B	C	D
213A	E	F		
GD	G	H		

Each immobilized enzyme was placed under the optimal conditions for reaction, and samples were taken once every hour for four times, labeled as A1, A2, A3, A4, B1, B1, B3, B4... H3, H4. As can be seen from (Fig. 3.1), The adsorption rate of A, B, C and D is obviously lower than that of E, F, G and H. In E, F, G and H, the adsorption rate of G is relatively good and reaches a higher value at 4 hours. Therefore, better results can be obtained by fixing GD and D213 resin after ethanol treatment.

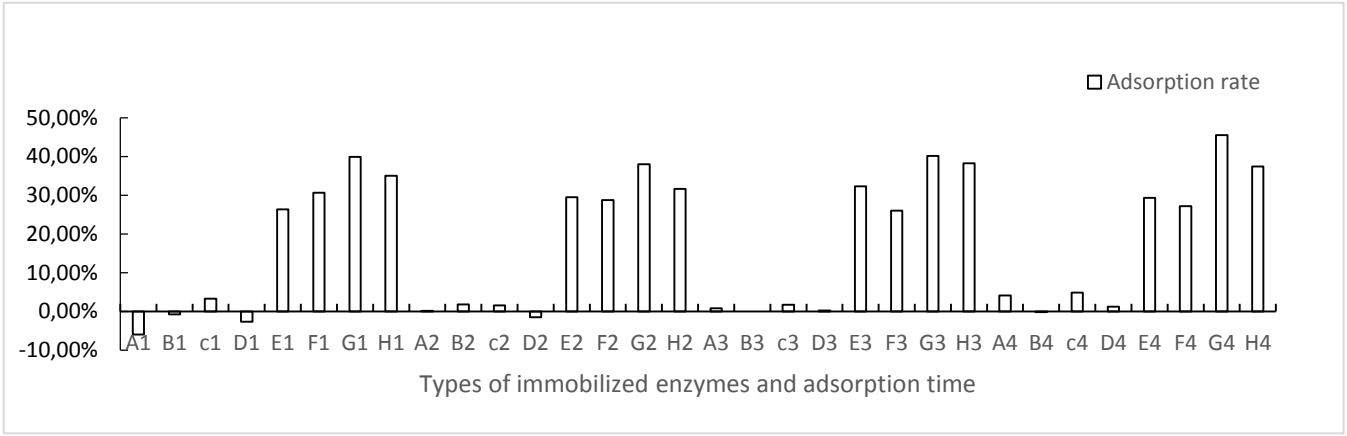


Figure 3.1 – Types and adsorption time of immobilized enzymes

As the resin of D213 and ZGA351 treated with ethanol has a good adsorption rate, the ethanol-treated D213 and ZGA351 resins were selected to fix the free enzymes, washed with deionized water for 2-3 times, and then washed with PBS

buffer for 2-3 times. The treated immobilized enzymes were put into a centrifuge tube and D-fructose was added for conversion. According to (Fig. 3.2), it can be clearly seen that the conversion rate of D213 model resin after immobilization of free enzymes is higher, and the conversion rate can reach about 30% after 7 hours. Therefore, the resin screening result is D213 type resin.

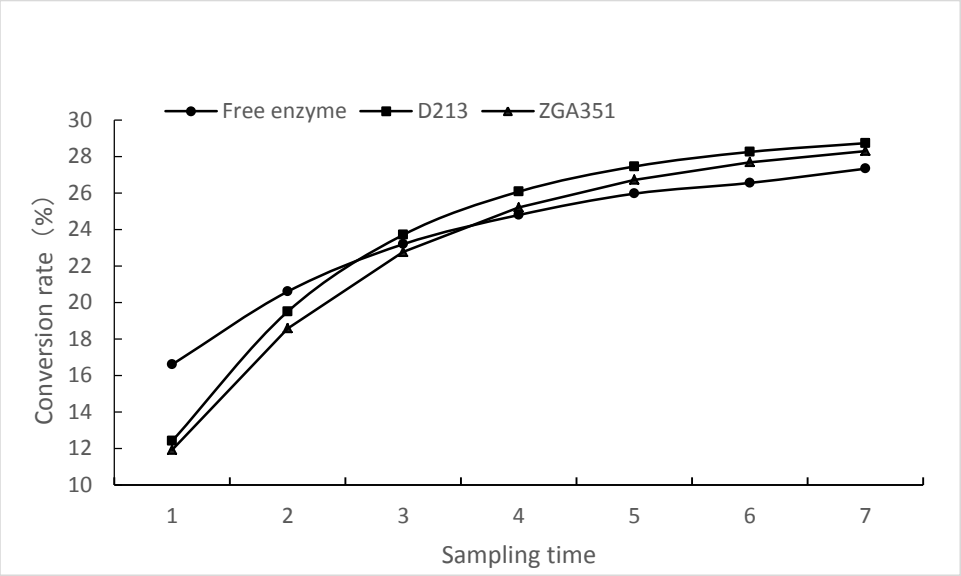


Figure 3.2– Types and conversion rates of immobilized enzymes

3.3 Optimization result of adding enzyme quantity

There is a close relationship between the number of carrier adsorptive enzymes and the recovery rate of enzyme activity, as shown in (Fig 3.3). In a certain range, the activity of immobilized enzymes will also increase with the increase of the amount of enzyme added. However, when the amount of enzyme added exceeds a certain limit, the recovery rate of enzyme may decrease. This may be due to the limited capacity of the carrier to carry protease, and the addition of too many enzymes will cause the enzyme molecules to aggregate into clusters, covering some of the active centers of the enzyme molecules, thus reducing the catalytic activity of the enzyme. Therefore, according to (Fig. 3.3), it can be seen that 50U/g resin should be selected as the best amount of enzyme.

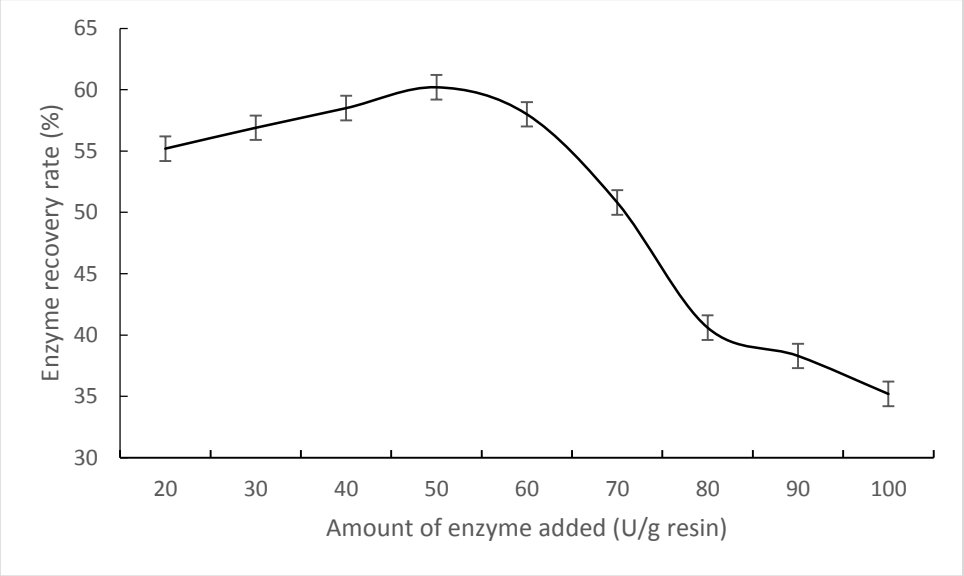


Figure 3.3 - Influence of enzyme dosage on immobilization effect

3.4 Optimization results of adsorption temperature

Under the optimal conditions (adding 50U/g resin with enzyme, 4h adsorption time and 4h cross-linking time), the temperature gradients of 10℃, 20℃, 30℃, 40℃, 50℃, 60℃ and 70℃ were selected to study the adsorption temperature, and the labels were carried out. As can be seen from (Fig. 3.4), 25℃-35℃ is the most suitable temperature. Because in the case of low temperature, it will lead to low efficiency of resin adsorption; When the temperature is too high, the enzyme will lose its activity because of the high temperature. Therefore, the adsorption temperature is selected at about 30℃.

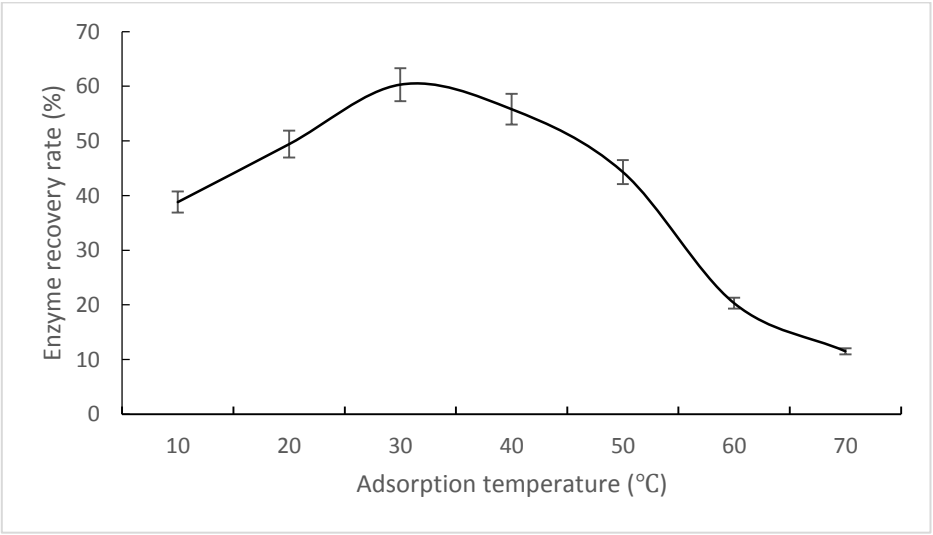


Figure 3.4 – Effect of adsorption temperature on recovery rate of enzyme activity

3.5 Optimization result of adsorption time

When all conditions were optimal (adding 50U/g resin with enzyme, adsorption temperature 30℃, crosslinking time 4h), different time gradients of 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h were set to study the adsorption time. As can be seen from （Fig. 3.5）, the recovery rate of enzyme activity showed a decreasing trend after the time was greater than 4h. The results indicated that the resin could keep the enzyme in a relatively stable and balanced state after 4h adsorption. With the extension of adsorption time, the enzyme protein began to leak, which led to the decrease of the enzyme recovery rate. Therefore, 4h is the best adsorption time.

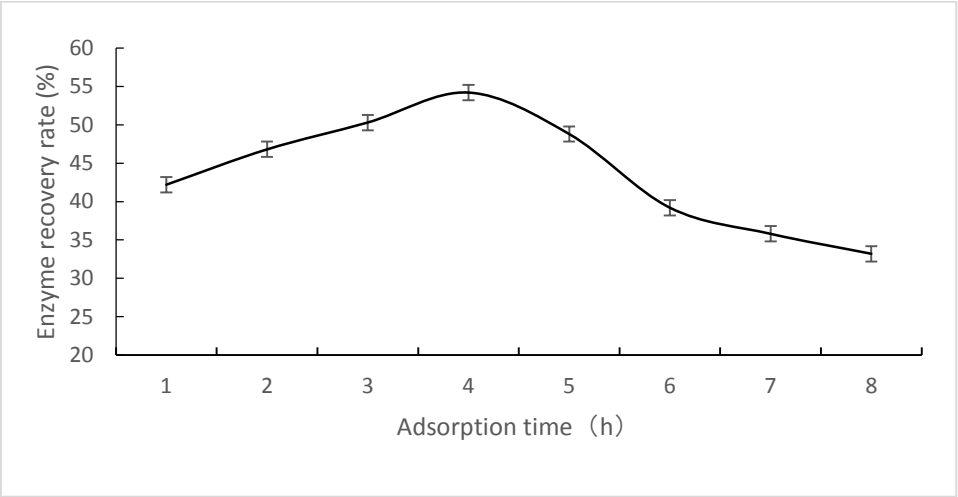


Figure 3.5 – Influence of adsorption time on the recovery rate of enzyme activity

3.6 Study on enzymatic properties and stability of resin-immobilized DPE enzyme

(1) Optimum temperature and temperature stability

Appropriate amount of free enzyme and immobilized enzyme were taken for reaction under the temperature gradient of 20℃-90℃. As shown in (Fig. 3.6), the optimum temperature range for immobilized enzymes is 65℃-75℃, while the optimum temperature for free enzymes is 55℃-65℃. The above data indicated that the immobilized carrier played a role in protecting enzyme activity to a certain extent.

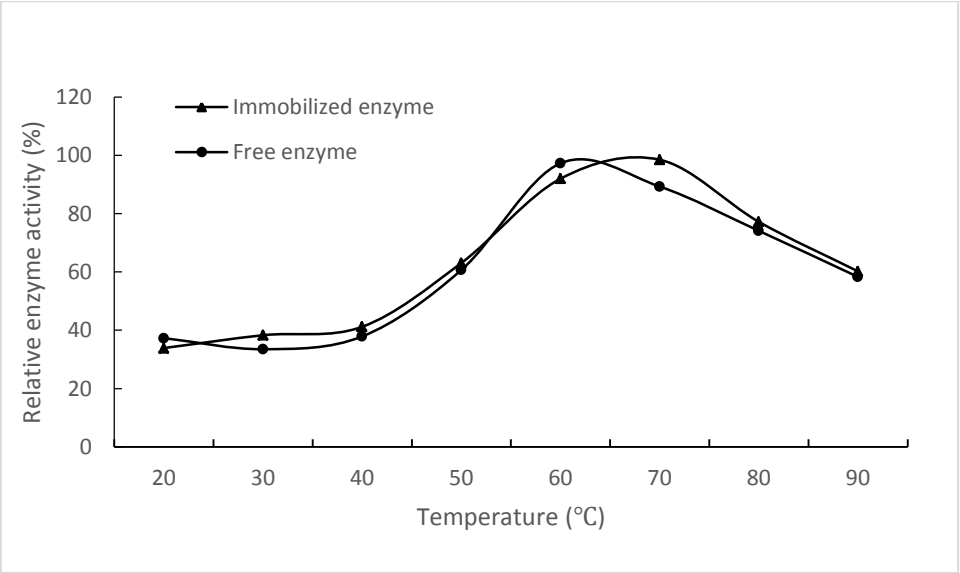


Figure 3.6 – Effect of temperature on immobilized and free enzymes

Appropriate amount of immobilized enzyme and free enzyme were taken, and the two were kept at 30℃~70℃ for 1 hour to detect enzyme activity. As shown in （Fig. 3.7）, it can be seen that the enzyme protein had certain temperature stability after being immobileized. The main reason is that the immobilized enzyme reduces the freedom of movement of the enzyme molecule. In the free state, the enzyme molecule can move freely, which leads to its inactivation at high temperature. However, immobilized enzymes limit the freedom of movement of the enzyme molecules, thus reducing the possibility of such conformational changes. Moreover, the immobilized carrier provides a more stable microenvironment for the enzyme molecules, which helps to protect the enzyme from high temperature damage. Therefore, the immobilized enzyme protein has a certain temperature stability.

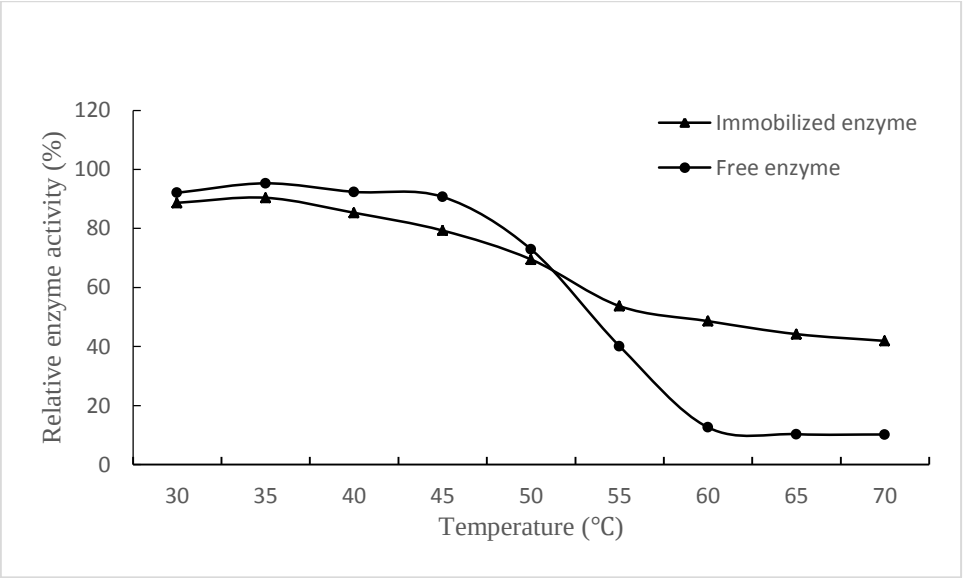


Figure 3.7 – Temperature stability of immobilized and free enzymes

(2) Optimum pH and pH stability

The immobilized enzyme and free enzyme react under different pH conditions. Results As shown in （Fig. 3.8）, the overall enzyme activity of the immobilized enzyme was relatively higher than that of the free enzyme, and the optimal pH of the immobilized enzyme was 8, showing a weak alkalinity. The reason may be related to the alkaline nature of the resin.

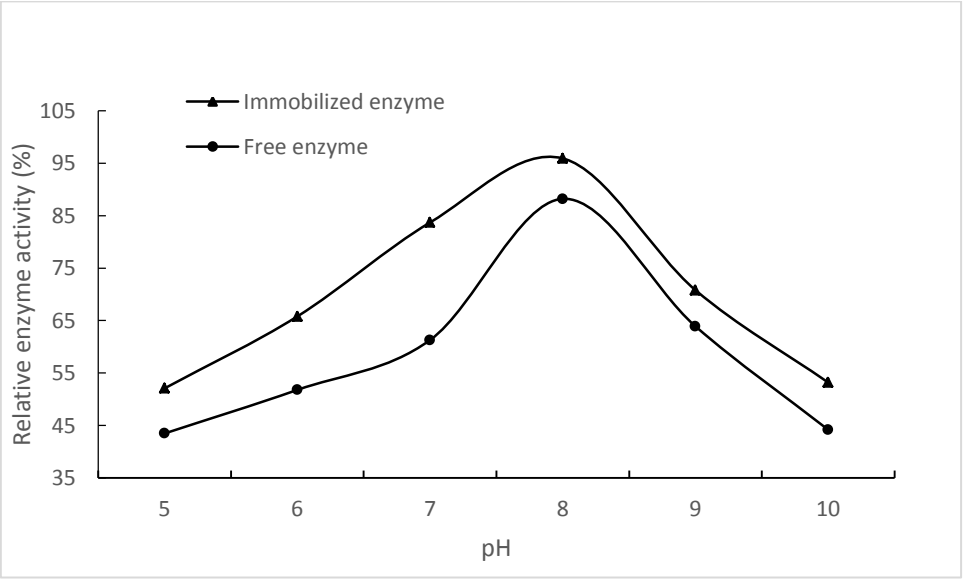


Figure 3.8 – Optimum pH of immobilized and free enzymes

Under different pH solutions, the relative activities of free enzyme and immobilized enzyme were measured 1h later, as shown in （Fig. 3.9. The pH stability of immobilized enzyme was better than that of free enzyme. The immobilized enzyme molecules are connected to the carrier at multiple points, which can prevent the extension and deformation of the enzyme molecules. At the same time, the immobilized carrier also changes the microenvironment in which the enzyme acts, thus increasing the stability of the enzyme at different pH. This stability is essential for biocatalytic and biochemical processes in industrial applications, as it allows the enzyme to maintain efficient and stable catalytic activity under a wider range of conditions.

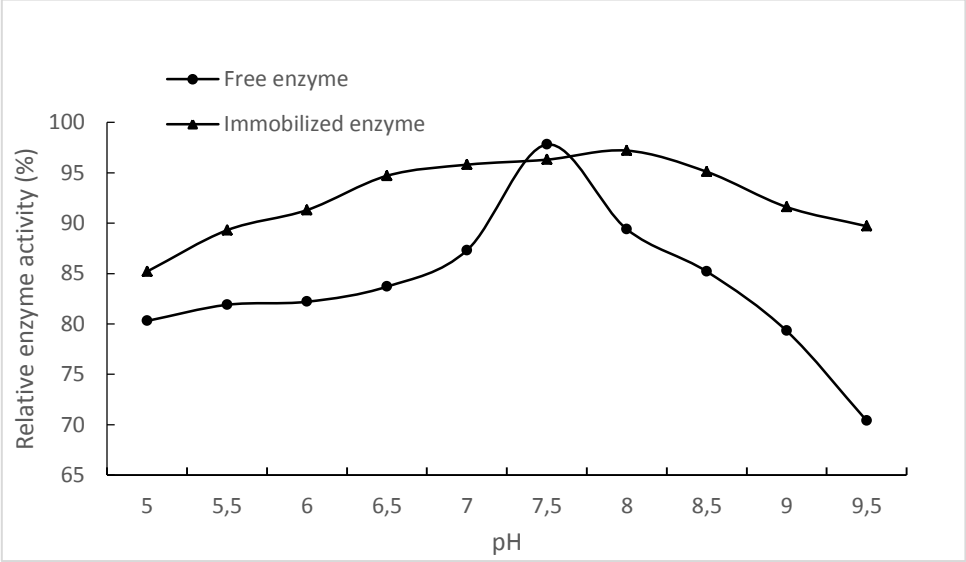


Figure 3.9 – pH stability of immobilized and free enzymes

3.7 Results of the number of times the resin can be reused

Reusability is one of the important indexes to measure the technical performance of solidified enzymes. In the experiment, a certain immobilized enzyme was selected and reused for 8 times under the optimal conditions, and the remaining enzyme activity after each immobilized enzyme reaction was measured (the activity of the first immobilized enzyme measured was 100%). As shown in （Fig 3.10） the immobilized enzyme can be reused many times, and its enzyme activity still maintains a high state even after 7 times of use. It shows that the enzyme molecule of immobilized enzyme is more stable than that of free enzyme molecule, and the

enzyme after immobilization is not easily affected by the external environment, so it can achieve multiple reuse. Compared with free enzyme, its economic benefits are very obvious.

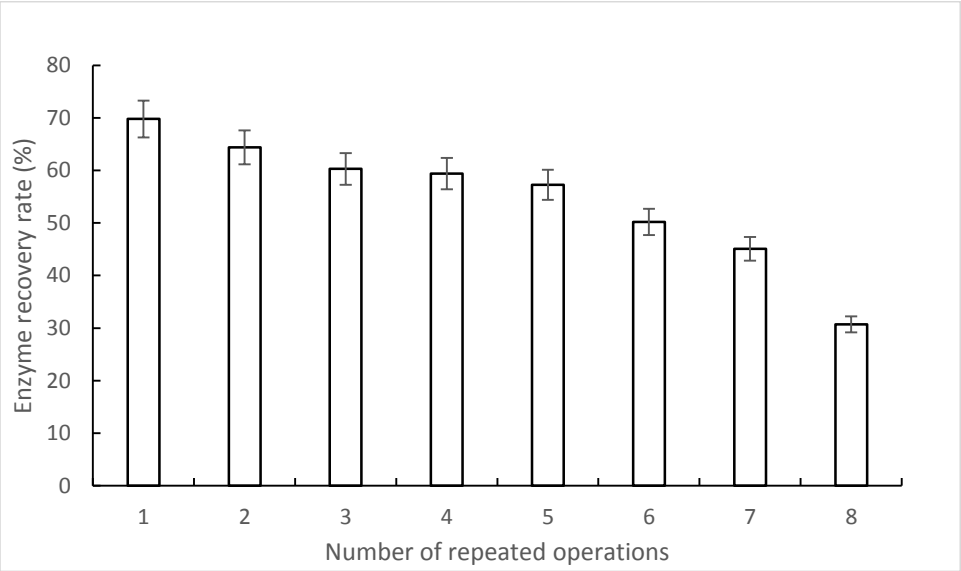


Figure 3.10 – Effect of repeated operation times on the recovery rate of enzyme activity

Conclusions to chapter 3

Experimental part include optimization of resin immobilized DPE enzyme conditions (Selection of resin carrier; Optimization of adding enzyme quantity; Optimization of adsorption temperature and time; Study on enzymatic properties and stability of resin-immobilized DPE enzyme); Resin screening results; Optimization result of adding enzyme quantity; Optimization results of adsorption temperature and time; Study on enzymatic properties and stability of resin-immobilized DPE enzyme and results of the number of times the resin can be reused.

CONCLUSIONS

1. In this experiment, the efficient conversion resin was screened, the best immobilized carrier conditions were determined, and the technological parameters of D-allulose production were optimized. These results not only provide technical support for the industrial production of D-allulose, but also provide a new idea for the application of enzyme immobilization technology in the field of biotransformation.
2. This experiment proved that the immobilized enzyme had greater advantages than the free enzyme, and the immobilized enzyme technology had a remarkable effect on improving the production efficiency of D-allulose, which laid a solid foundation for the industrial production and wide application of D-allulose. This study will continue to explore the application value of D-allulose in food, medicine and other fields, and further optimize the production process to promote the sustainable development of D-allulose industry.

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