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 the fusion expression of L-rhamnose-isomerase and D-psicose-3-epimerase**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor, Dr. Sc., Prof.
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Li Xiao

1. Thesis topic **Study on the fusion expression of L-rhamnose-isomerase and D-psicose-3-epimerase**
Scientific supervisor Dr. Sc., Prof. Olga Andreyeva

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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

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WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
6	Draw up a bachelor's thesis (final version)	until 25 May 2025	
7	Submission of qualification work to the supervisor for feedback	until 27 May 2025	
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I am familiar with the task:

Student _____ Li XIAO

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SUMMARY

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D-Allose, a rare cis-aldohexose with extensive physiological functions, has significant applications in food, healthcare, and pharmaceutical fields. Particularly due to its remarkable anticancer activity and adjuvant effects in cancer radiotherapy/chemotherapy, it has become a research hotspot. Current production methods primarily utilize D-psicose as substrate with L-rhamnose isomerase (L-RhI) as catalyst for direct synthesis. However, the process faces substantial economic constraints due to the market scarcity of D-psicose. This study addressed the high-cost issues in existing production systems by successfully achieving the co-expression of D-psicose 3-epimerase (DPEase) and L-rhamnose isomerase (L-RhI) in the E. coli BL21(DE3) host using the pETDuet-1 dual-expression vector. SDS-PAGE analysis confirmed the stable expression of two active proteins with molecular weights of 35 kDa (DPEase) and 45 kDa (L-RhI), respectively. This research establishes theoretical foundations for developing a bioconversion pathway to produce D-allose from the economical substrate D-fructose.

Key words: D-allose, D-psicose-3-epimerase, L-rhamnose-isomerase, Fusion expression

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INTRODUCTION

D-Allose is a liquid cis-aldohexose that performs many important physiological functions such as anticancer, cardiovascular protection and antioxidant effects, and has significant potential, especially in adjuvant tumor treatment. However, the low natural content of D-Allose causes shortages in food, medicine and health products.

In practice, D-allose is produced mainly by chemical synthesis and biological transformation methods. Among the latter, the most common is the biological enzyme method using a cascade catalytic system with a dual enzyme due to its high efficiency and environmental protection. Modern biological production methods mainly use D-psicose as a substrate with L-rhamnose isomerase (L-RhI) as a catalyst for direct synthesis. However, this process faces significant economic limitations due to the shortage of D-psicose in the market.

Taking into account the above, the aim and objectives of this study are formulated.

The aim of the study is to synthesize D-allosaccharide by expressing a fusion of two enzymes - DAEase and L-RhI using a cheap substrate D-fructose.

Research objectives:

1. To construct recombinant plasmids DPE and L-RhI;
2. To prepare competent states;
3. To analyze the expression level of the target protein.

The object of the study is the synthesis of D-allosaccharide using cheap D-fructose as a substrate.

The subject of the study is the expression of the fusion of L-rhamnose isomerase and D-psicose-3-epimerase.

Research methods: Gene and PCR amplification, construction of pETDuet-dpe, construction and validation of the recombinant plasmid L-RhI, obtaining a

competent state of Escherichia Coli BL21(DE3), analysis of the expression level of the target protein, protein electrophoresis, etc.

Scientific novelty of the research: A two-enzyme cascade catalytic system based on D-fructose has been scientifically substantiated; it has been experimentally confirmed that recombinant bacteria can be successfully expressed; the plasmid pETDuet-dpe-l-rhi has been constructed.

Practical significance of the research: Reducing the cost of production of D-allosaccharide, which will contribute to a better supply of food, medicines and health products.

Chapter I

LITERATURE REVIEW

1.1 Overview of Rare Sugars

In 2002, the International Society of Rare Sugars (ISRS) defined rare sugars as a class of monosaccharides and their derivatives that exist in nature but are present in very small amounts. Seeberger et al. found through the study of sugar components in bacteria and mammals that monosaccharides (such as D-glucose, D-fructose and D-galactose), which are widely present in large quantities in nature, are widely distributed in various animals and plants, while the distribution of rare sugars in nature is relatively limited, [1]but they play important physiological functions in fields such as diet, health care and medicine. [3] So far, scientists have discovered more than 50 kinds of rare sugars in nature. Common rare sugars and their applications are shown in Table 1.1.

Table 1.1 **Common Rare Sugars and Their Applications**

Name	Source/Preparation method	Apply
D-allulose	D-fructose enzymatic isomerization	Sugar-free foods and auxiliary treatment for diabetes
L-Rhamnose	Plant cell walls (such as citrus peels)	Drug carriers, cosmetic moisturizers
D-Alosaccharide	In the extracts of natural plants and bacterial metabolites	Cancer, immunosuppression, cryoprotection
D-Mannose	Yeast fermentation	Immunomodulatory and anti-inflammatory drugs
D-taggle sugar	Lactase conversion	Artificial sweeteners and prebiotics

1.2 Overview of D-Alosaccharide

1.2.1 Properties of D-Alosaccharide

D-allose is derived from the leaves of African shrubs and is the aldose isomer of D-allose and the heterotropic isomer at the C-3 position of D-glucose. It is a rare cis-hexalose with extensive physiological functions. D-alosaccharide (C6H12O, MW= 180.16) is a white crystalline powder with no special odor. Its melting point is 128°C and its optical rotation is [α]20+14°(c=1.00%, H2O). This monosaccharide is readily soluble in water but insoluble in organic solvents such as ethanol.

D-allose is a pentahydroxyaldehyde, existing in a linear and cyclic form. The cyclic form is further divided into two forms: furan type (formed by the condensation of the aldehyde group of D-allose with the hydroxyl group on C-4) and pyran type (formed by the condensation of the aldehyde group of D-allose with the hydroxyl group on C-5). Since the D-allose molecule contains both an aldehyde group and a hydroxyl group, it can react to form a hemiacetal, thereby forming a pair of diastereooptical isomers. Among natural compounds, D-alosaccharides mostly exist in the form of β -pyran, which is more stable. However, in aqueous solutions, D-alosaccharides contain four cyclic structures (β -D-pyran alosaccharides, α -D-pyran alosaccharides, β -D-furanalosaccharides and α -D-furanalosaccharides). [4]The acute and subchronic toxicity tests of D-alosaccharide indicate that D-alosaccharide is non-toxic. In the acute toxicity test, the LD50 value =20.5g/kg. In the subchronic toxicity test, there were no abnormalities in all blood biochemical indicators of the experimental animals. These results indicate that D-allose is non-toxic to rats.[5]

1.2.2 The Function of D-Alosaccharide

The diversified biological activities of the rare sugar D-alosaccharide have demonstrated significant application value in fields such as tumor adjuvant therapy and cardiovascular disease prevention, and have made it a research hotspot among rare sugars.

Studies have shown that D-alosin exerts its anti-cancer effects through the following pathways, [6]significantly inhibiting the proliferation of various epithelial tumors (such as prostate cancer, liver cancer, etc.) and hematological malignancies, by up-regulating the expression of the TXNIP tumor suppressor.

Effectively block the carcinogenesis process mediated by oxidative stress.

D-alosaccharide has a synergistic effect on radiotherapy and chemotherapy for cancer. In 2011, [7]Hoshikawa et al. confirmed that this monosaccharide had a significant radiosensitizing effect on head and neck squamous cell carcinoma (HSC-3 cell line), and the therapeutic effect of the combined treatment group was significantly better than that of the single therapy.

D-alosaccharide has a cardiovascular protective effect. Kimura [8]et al. studied two animal models of hypertension, namely salt-sensitive hypertensive DS rats and spontaneous hypertensive rats. After a 4% high-salt diet for 4 weeks, 2g/(kg· D) D-alosaccharide was given. The blood pressure of DS rats first increased, and then decreased after the addition of D-alosaccharide, significantly inhibiting the increase in blood pressure. Reduce the levels of aortic oxidative stress markers. However, no similar effect was observed in the spontaneous hypertension model.

D-alosaccharide also has antioxidant properties, enabling it to play corresponding anti-inflammatory and protective roles in related clinical applications.[9]D-alosaccharide also has other functions such as immunosuppression and cryoprotection.[101112]

1.3 The Preparation Method of D-allose

The distribution of D-alosaccharide in nature is limited, and its natural extraction amount is far from meeting the market demand. At present, the main methods for synthesizing D-allose include chemical synthesis and biotransformation.

Chemical synthesis methods based on organic synthesis chemistry usually adopt α -D-glucose as the starting material. Through complex multi-step reactions such as hydroxyl protection (such as isopropylation), REDOX reaction sequences, and finally hydrolysis to obtain the target product. Take the research of [13]Tianjin University as an example. Its synthetic route requires at least five steps of transformation, which not only limits the yield (usually <30%), but also generates various by-products, and involves the use of toxic reagents, with poor environmental compatibility. In the early stage of the biotransformation method, D-allulost was used as the substrate, which relied on the catalysis of L-rhamnose isomerase (L-RhI) and was limited by the availability of high-valent substrates. However, in 2006, a revolutionary technological breakthrough was achieved. The Izumoring strategy innovatively selected cheap D-fructose as the raw material and developed a dual-enzyme cascade catalytic system. DPE enzyme-catalyzed differential isomerization of C-3 (conversion efficiency 27.34%) and L-RHI-mediated secondary isomerization (conversion efficiency 34.64%) reduced the overall production cost by 40-50%. This method has many advantages such as less by-product generation, less environmental pollution, high productivity, good selectivity, and simple steps. It has become the main method for laboratory production of D-allose at the present stage.

1.3.1 Introduction to D-Allulose 3-Heteroisomerase

D-allulose 3-epimerase (DPEase) belongs to the hexose isomerase family and can specifically catalyze the epimisomerization reaction at position C-3 of the hexose molecule. This enzyme plays a key role in the biosynthesis of D-allulose and

can efficiently catalyze the conversion of D-fructose to D-allulose. It has the advantages of high reaction specificity, no by-product generation and high conversion efficiency.[22]

In 1993, the team of Izumori from Kagawa University in Japan first discovered a new type of enzyme from Pseudomonas sp.ST-24. This enzyme showed the highest catalytic activity for D-tagose and was thus named D-tagose 3-isoisomerase. In 2005, another novel isomerase was discovered in South Korea from Agrobacterium tumefaciens. It had the highest catalytic efficiency for D-allulose and was thus named D-allulose 3-anisotropic isomerase. Since then, with the development of biotechnology, multiple research teams have successively reported the discovery of a new type of DPEase. In 2008, Zhang Longtao's team discovered DPEase in Rhodobacter sphaeroides SK011. In 2012, Sun Yuanxia's team discovered Rummococcus sp. DPEase; Jiangnan university enami in Clostridtumcellulolyticum, Clostridnmbolteae and Clostridum scindens found in three kinds of clostridium DPEase. [23]These findings not only enrich the carbohydrate isomerase library, but also provide multiple enzyme source options for the industrial production of D-allulose. At present, researchers are modifying the existing DPEase through protein engineering techniques to obtain engineered enzymes with higher catalytic activity and stability.

It is reported that the enzymatic properties of D-allulose 3-heteroisomerase derived from microorganisms all have certain differences. Although there is consistency in the function of converting D-fructose into D-allulose, in terms of the amino acid sequence analysis of the enzymes, their homology is not high, approximately 20%-60%. However, there is a high degree of similarity in the amino acid residue sequences of some important regions in the active center, metal ion binding site and substrate binding site.

1.3.2 Introduction to L-Rhamnose Isomerase

Ken Izumori from the Rare Sugar Research Center of Kagawa University in Japan has made foundational contributions in the field of rare sugar biotransformation. In 1997, this team successfully isolated and identified a new type of isomerase from Pseudomonas aershinii for the first time. Enzymatic characteristic analysis confirmed that it exhibited the highest catalytic activity for L-rhamnose, and thus it was officially named L-rhamnose isomerase (L-RhI). [12]However, this enzyme is not suitable for the production of D-allose because the by-product D-allose is generated, which increases the difficulty of product separation and purification. In addition, most L-RhI enzymes do not have the ability to catalyze the production of D-allose.[27]

Derived from Pseudomonas stutzeri 12, Bacillus pallidus 14, extreme thermophilic cellulose-degrading bacteria C. saccharolyticus 15, Bacillus subtilis B. subtilis 16 The L-RhI enzyme 17 of saccharolyticum, a thermophilic anaerobic bacterium, can be used for the biosynthesis of D-alosaccharide. The comparison of its enzymatic properties is shown in Table 1.2.

Bai et al. from Jiangnan University successfully expressed the l-rhi gene derived from B. subtilis in Escherichia coli and utilized metabolic engineering methods to achieve the fermentation synthesis of D-alosaccharide by recombinant bacteria. However, since the anisotropic isomerization reaction catalyzed by L-RhI is a reversible equilibrium reaction, when the reversible equilibrium reaction reaches equilibrium, the conversion rate is very low, and its protein structure and stereoisomerization selective catalytic mechanism are still unclear. Therefore, the improvement of the catalytic performance of L-RhI is limited, the yield level of D-

allose is still relatively low, and in addition, the cost of D-allose is relatively high. This makes it difficult to achieve industrial production of D-allose.

Table 1.2 **Comparison of enzymatic properties of L-RhIase derived from different bacteria**

Conditions Strains	Optimal Temperature/℃	Optimal pH	Heat resistance/h	With byproduct or not	Equilibrium ratio between D-allulose and D-allose
P. Stutzeri[28]	60	9.0	0.2 (50℃, 50%)	Yes	67:26（50℃）
B. Pallidus[29]	65	7.0	1.0 (60℃, 50%)	No	65:35（65℃）
C. Saccharolyticus[30]	90	7.0	6.0 (80℃, 90%)	No	67:33（90℃）
B. Subtilis[31]	70	9.0	4.0 (50℃, 65%)	No	67:33（70℃）
T. Saccharolyticum[32]	75	7.0	2.0 (70℃, 50%)	No	71:29（70℃）

Therefore, in recent years, most studies have mainly used D-fructose as the substrate and L-rhamnose isomerase and D-allulose 3-heteroisomerase as catalysts to synthesize D-allulose through a two-step reaction.

1.4 The Research Significance and Content of This Article

1.4.1 Research Significance

Since the 21st century, with the bioengineering technology industry gradually becoming one of the leading industries globally, significant progress has also been made in the research, development and application of rare sugars. With the continuous innovation and development of rare sugar production technology, the output of rare sugar has gradually increased and the production cost has gradually

decreased, thereby promoting the diversified development of rare sugar. Clinical studies have shown that D-alosaccharide has a wide range of pharmacological effects, mainly including excellent anti-cancer effects and auxiliary effects on cancer radiotherapy and chemotherapy, strong antioxidant effects, immunomodulatory effects and other functions. It is a rare sugar with very promising prospects and application value. At present, the output of D-alosaccharide is far from meeting the market demand.

The main production methods of D-allose include chemical synthesis and biocatalysis. Among them, the biocatalysis catalytic production of D-allose has received extensive attention due to its many advantages. D-allose can be isomerized to generate D-allose through sugar isomerases such as L-rhamnose isomerase (L-RhI), D-ribose-5-phosphate isomerase (RPI), and D-galactose-6-phosphate isomerase (GPI). Among them, the isomerization of D-allulose using L-rhamnose isomerase (L-RhI, EC 5.3.1.14) is the most direct method for the production of D-allulose, but it is not adopted due to the high price of the enzyme reaction substrate D-allulose. Therefore, using D-fructose as the substrate and catalyzing the two-step isomerization reaction of D-allulose 3-anisotropic isomerase (DPE) and L-rhamnose isomerase (L-RhI) is mostly common. [27]In this study, aiming at the high-cost problem of the existing production system, a dual-enzyme catalytic system based on D-fructose was innovatively constructed. Through molecular biological means, the fused expression of D-allulose-3-anisoisomerase (DAEase) and L-rhamnose isomerase (L-RhI) was achieved. It laid a theoretical foundation for establishing the biotransformation pathway of synthesizing D-alosaccharide from the cheap substrate D-fructose.

1.4.2 Research Content

(1) In this study, the pETDuet-1 dual expression vector fused with D-allulose 3-heteroisomerase and L-rhamnose isomerase was successfully constructed first.

(2) The co-expression of D-allulose 3-anisotropic isomerase and L-rhamnose isomerase was successfully achieved in the Escherichia coli BL21(DE3) host.

(3) Verified by SDS-PAGE, the recombinant strain could stably express two active proteins with molecular weights of 35 kDa and 45 kDa respectively.

Summary of the chapter I

1. The definition and significance of rare sugars: Rare sugars are monosaccharides and their derivatives that exist in nature but are present in very small amounts. They have important physiological functions and are widely used in the fields of food, medicine, etc. D-alosaccharide, as one of the representative rare sugars, has various biological activities such as anti-cancer, cardiovascular protection and antioxidation. However, its natural content is limited and the market demand is large.

2. The preparation methods of D-alosaccharide: Currently, it is mainly produced through chemical synthesis and biological transformation methods. Among them, the biological enzyme method (such as the double-enzyme cascade catalytic system) has become a research hotspot due to its advantages such as high efficiency and environmental protection. This method uses cheap D-fructose as the substrate and catalyzes the synthesis of D-alosaccharide in two steps using D-alosaccharide 3-anisotropic isomerase (DPEase) and L-rhamnoisomerase (L-RhI). However, the existing technology still has problems such as low conversion rate and high cost.

3. The content and innovation of this study: A fusion expression system of DPEase and L-RhI was constructed through molecular biological means, successfully achieving the co-expression of the two enzymes, providing a theoretical basis for reducing the production cost of D-alosaccharide and promoting industrial production. Specifically, it includes key steps such as the construction of dual expression vectors and the verification of protein expression of recombinant strains.

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental Materials

2.1.1 Strain and Plasmid

The expression strain was Escherichia coli *E.coli* BL21star (DE3), and the plasmid vector was pETDuet-1. As shown in Table 2.1 below.

Table 2.1 **Strains and Plasmids used**

Strain or plasmid	Source
Strain	
B.subtilis 168	Laboratory preservation
E.coli DH5α	All-style gold
E.coli BL21star(DE3)	Laboratory preservation
Plasmid	
pETDuet-1	Laboratory preservation
pUC18-dpe	Laboratory preservation
pUC18-l-rhi	Laboratory preservation

2.1.2 Main Culture Media and Reagents

(1) LB medium: 10g/L peptone, 5 g/L yeast extract, 10g/L sodium chloride, with 100mg /L ampicillin added.

(2) PBS buffer (pH 7.2-7.4, 500mL) :5.3mL NaH2PO4 (20 mm), 94.7mL Na2HPO4 (20 mm).

(3) 5× Sample addition buffer: 60mM Tris-HCl (pH 6.8), 25% glycerol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue.

(4) 5×Tris-Glycine electrophoresis buffer: Add 5g of sodium dodecyl sulfate, 72g of glycine, and 15.1g of chloroform to water and make up to 1000mL.

(5) Coomas Brilliant Blue staining solution: Dissolve 1.0g of Coomas Brilliant Blue R-250 in a mixed solution of 450mL of methanol and 450mL of

distilled water, stir thoroughly until completely dissolved, then add 100mL of glacial acetic acid and mix well.

(6) Decolorization solution: Mix 250mL of ethanol with 80mL of glacial acetic acid, then make up to 1 L with distilled water and mix thoroughly.

(7) Ampicillin stock solution (Amp): Filter and sterilize a 100 mg/mL aqueous solution, aliquot it and store it at -20 ℃.

(8) SDS-PAGE: Protein loading buffer, IPTG solution, agarose, MeilunGel PAGE white pre-prepared gel, DNA Maker, 10× loading buffer, Protein Maker, 5× protein loading buffer.

2.1.3 Main Instruments

Table 2.2 **Main Instruments**

Instrument name	Producer	Instrument model
Low-temperature centrifuge	Shanghai Lishen	Neofuge 23R
Constant temperature incubator	Weifang Medical devices	WMK-08
Induction cooker	Midea Electrical Appliances	C21-ST2118
Ultrasonic cleaner	Hangzhou Farant	KQ-50B
Electronic balance	Mettler Toledo	AL104
Circulating water multi-purpose vacuum pump	The Long March in Shanghai	SHB-III
Constant temperature water bath pot	Shanghai Jinghong	DK-S24
Gel imager	Bio-Rad Company	Gel Doc XR
PCR instrument	Eppendorf Company	22331
Combined full-temperature oscillator	Haocheng Experimental Instruments	HCY-123B
Nucleic acid quantifier	Eppendorf Company	Bio Photometer Plus
pH meter	Thermo orion	Thermo orion
Electrophoresis apparatus	Children's Day in Beijing	DYY-11B
Refrigerator	Zhongke Meiling	BCD-218ZM2D
Vacuum drying oven	Shanghai Senxin	DZG-6020

2.2 Experimental Methods

2.2.1 Gene and PCR Amplification

1. Primer design

Based on the vector pETDuet-1, the upstream and downstream primers of the genes *dpe* and *l-rhi* were designed in Table 2.1 below:

Table 2.1 **Primer design**

Plasmid	Primer naming and sequence（5'→3'）
pETDuet-1-*dpe*	Upstream primer: GGGAATTCCATATGATGAAACAAAAAGTG(The underlined part is the NdeI digestion site)
	Downstream primer: CCGCTCGAGTTACTTGCTGGTATC (The underlined part is the XhoI digestion site)
pETDuet-1-*l-rhi*	Upstream primer: CCGGAATTCATGACCATAAAAGCC (The underlined part is the EcoRI digestion site)
	Downstream primer:ATGCATCCAATGCATTGGTTCTGCAGTTAGACAAT CGGAGA(The underlined part is the PstI digestion site)

2. PCR amplification

Using the plasmids pUC18-dpe and pUC18-l-rhi preserved in the laboratory as templates, the PCR reaction system was prepared as shown in Table 2-3 below, and the dpe and l-rhi fragments were amplified respectively. PCR system for amplifying the target coding gene: 10 μL of 5×PrimeSTAR Buffer (containing Mg^{2+}), 4 μL of 2.5 mM dNTP mixture, 1 μL each of 10 μM upstream and downstream primers, 1 μL of B. Subtilis 168 genomic DNA template, 1 μL of DNA polymerase Finally, add sterile double-distilled water to make up to 50μL.

3. Processing of PCR amplification results

After the PCR reaction was completed, the PCR amplification results were verified by agarose gel electrophoresis (0.8%), and the amplification products of the target gene were recovered using the DNA purification and recovery kit in accordance with the operation manual of the kit. The DNA concentration of the

recovered products was detected by using a micro-nucleic acid analyzer. After completion, the remaining products were stored at -20 ℃ for future use.

2.2.2 Construction of pETDuet-dpe

1. Plasmid extraction

Take the DH5α Escherichia coli containing the target plasmid preserved in the laboratory and evenly spread it on the LB-Amp solid medium plate. Incubate upside down at 37℃ for 12 to 16 hours (overnight) until single colonies grow. Single colonies were picked up with a sterile pipette tip and inoculated into LB liquid medium containing ampicillin. Incubate overnight on a 37℃ constant temperature shaker at 200 rpm. The plasmids in the bacterial solution were extracted using the plasmid small extraction kit. Then, the plasmid concentration (ng/μL) was detected by a micronucleic acid analyzer. The purified plasmids were aliquot into sterile EP tubes and stored at -20℃.

2. Double enzyme digestion of dpe and plasmid

The extracted plasmids and the target fragment dpe were subjected to double digestion using restriction endonucleases respectively. The double digestion reaction system is shown in Table 2.2 below:

Table 2.2 **Double Enzyme Digestion Reaction System**

Reagent	Dosage
Restriction endonuclease1	1 μL
Restriction endonuclease2	1 μL
10×buffer	2 μL
DNA	≤ 1 μg
ddH_2O	Make up for the volume20 μL

After approximately 6 hours of the double enzyme digestion reaction, the double enzyme digestion results of the plasmid were verified by 0.8% agarose gel electrophoresis. The plasmid fragments with sticky ends after digestion and the dpe gene were recovered respectively using the DNA purification and recovery kit. The concentrations of the plasmid fragments and gene fragments were detected by a micronucleic acid analyzer and stored at -20 ℃ for future use.

3. Ligation of the target fragment and the plasmid fragment

Take 2 μL of 10×T4 DNA ligase buffer as the reaction basis, add 0.5 μL of T4 DNA ligase as the catalytic component, and simultaneously add 80 ng of the dpe target fragment and 150 ng of the carrier fragment. Finally, the reaction system is supplemented to a final volume of 20 μL with sterile double-distilled water. The reaction takes place on ice. Finally, enzymes are added. After all the above components are mixed evenly, the reaction is carried out at 22 ℃ for 1.5 hours. The genes with sticky ends and plasmid fragments were ligated using the T4 ligation kit to form recombinant plasmid vectors.

4. Transformation of Escherichia coli DH5α

Take 50 L of DH5α competent cell suspension and mix it evenly with 10 L of ligating solution. React in an ice bath for 30 minutes to adsorb DNA. Take it out and thermally excite it in a 42 ° C water bath for 45 seconds to promote DNA internalization. Then immediately take an ice bath for 2 minutes. Add 500 L of non-resistant LB medium and incubate at 37 ℃ for 1 hour. Take 50 L of the transformation solution and evenly spread it on LB solid plates containing the corresponding antibiotic (ampicillin), and incubate at 37 ℃ for 12-16 hours.

5. Colony verification experiment

Approximately 10 single colonies were randomly picked from the LB-Amp plate and resuspended in 10 μL of sterile double-distilled water to prepare the bacterial suspension. Take 1 μL of the bacterial suspension as the PCR template and perform amplification through a 20 μL reaction system, which includes: 10 μL of

2×Taq enzyme premix, 1 μL each of the forward and reverse primers of 1, and 7 μL of sterile double-distilled water.

6. Double enzyme digestion verification

The positive colonies obtained through screening were inoculated into 10 mL of LB liquid medium containing the corresponding antibiotic (ampicillin), and incubated overnight at 200 rpm at 37 ° C. Subsequently, the recombinant plasmid was extracted for double restriction endonuctidase digestion analysis, and whether the digestion products met the expected size was verified by 0.8% agarose gel electrophoresis.

7. Sequencing of the target gene

The recombinant plasmid samples verified correctly by enzyme digestion were sent to Yingwei Jieji (Shanghai) Trading Co., Ltd. for DNA sequencing analysis. Through sequence alignment, the integrity of the reading frame and sequence accuracy of the target gene were confirmed to ensure the successful construction of the recombinant plasmid. The plasmid that was successfully sequenced was named pETDuet-dpe.

2.2.3 Construction and Validation of L-RhI Recombinant Plasmid

1. Double digestion of the target fragment and plasmid

The extracted plasmid pETDuet-dpe and the target fragment were subjected to double digestion using restriction endonucleases respectively. The double digestion reaction system is shown in Table 2.3.

After pETDuet-dpe and the target fragment underwent double digestion reaction for 6 hours, the double digestion results of the plasmid were verified by 0.8% agarose gel electrophoresis. After complete digestion, the plasmid fragments with sticky ends after digestion and the l-rhi gene were recovered respectively using the DNA

purification and recovery kit, and the concentration of the recovered fragments was determined by a micronucleic acid analyzer. Store at -20 ℃ for later use.

Table 2.3 **Double Enzyme Digestion Reaction System**

Reagent	Dosage
Restriction endonuclease1	1 μL
Restriction endonuclease2	1 μL
10×buffer	2 μL
DNA	≤ 1 μg
ddH_2O	Make up for the volume20 μL

2. Ligation of the target fragment and the plasmid fragment

Take 2 μL of 10×T4 DNA ligase buffer as the reaction basis, add 0.5 μL of T4 DNA ligase as the catalytic component, and simultaneously add 80 ng of L-RHI target fragment and 150 ng of carrier fragment. Finally, the reaction system is supplemented to a final volume of 20 μL with sterile double-distilled water. The reaction takes place on ice. Finally, enzymes are added. After all the above components are mixed evenly, the reaction is carried out at 22 ℃ for 1.5 hours. The genes with sticky ends were ligated with the pETDuet-dpe fragment using the T4 ligation kit to form the recombinant plasmid vector.

3. Transformation of Escherichia coli DH5α

Take 50 L of DH5α competent cell suspension and mix it evenly with 10 L of ligating solution. React in an ice bath for 30 minutes to adsorb DNA. Take it out and thermally excite it in a 42 ℃ water bath for 45 seconds to promote DNA internalization. Then immediately take an ice bath for 2 minutes. Add 500 L of non-resistant LB medium and incubate at 37 ℃ for 1 hour. Take 50 L of the transformation solution and evenly spread it on LB solid plates containing the corresponding antibiotic (ampicillin), and incubate at 37 ℃ for 12-16 hours.

4. Colony verification experiment

Approximately 10 single colonies were randomly picked from the LB-Amp plate and resuspended in 10 μL of sterile double-distilled water to prepare the bacterial suspension. Take 1 μL of the bacterial suspension as the PCR template and perform amplification through a 20 μL reaction system, which includes: 10 μL of 2×Taq enzyme premix, 1 μL each of the forward and reverse primers of 1, and 7 μL of sterile double-distilled water.

5. Double enzyme digestion verification

The positive colonies obtained through screening were inoculated into 10 mL of LB liquid medium containing the corresponding antibiotic (ampicillin), and incubated overnight at 200 rpm at 37 ℃. Subsequently, the recombinant plasmid was extracted for double restriction endonuctidase digestion analysis, and whether the digestion products met the expected size was verified by 0.8% agarose gel electrophoresis.

6. Sequencing of the target gene

The recombinant plasmid samples verified correctly by enzyme digestion were sent to Yingwei Jieji (Shanghai) Trading Co., Ltd. for DNA sequencing analysis. Through sequence alignment, the integrity of the reading frame and sequence accuracy of the target gene were confirmed to ensure the successful construction of the recombinant plasmid. The plasmid with successful sequencing was named pETDuet-dpe-l-rhi. Further transform the plasmid pETDuet-dpe-l-rhi into Escherichia coli BL21(DE3).

2.2.4 Preparation of Escherichia Coli BL21(DE3) Competent State

Preparation of BL21(DE3) competent cells by CaCl2 method: Take 50 μL of the bacterial solution from the glycerol storage tube at -80℃, evenly spread it on the surface of LB solid medium without antibiotics, and incubate at a constant temperature of 37℃ for 12-16 hours to obtain single colonies. Single colonies were

picked and inoculated into 10 mL of LB liquid medium. The culture was oscillated at 200 rpm at 37 °C for 12-16 hours. Subsequently, the bacterial liquid was transferred to 50 mL LB medium at an inoculation volume of 5%. At the same temperature, it was oscillated at 180 rpm until the logarithmic growth phase (OD600≈0.8), and the bacteria were collected after centrifugation at 5000×g for 8 minutes. First, the cells were lightly suspended with 5 mL of pre-cooled Buffer A. After 10 minutes of ice bath, they were centrifuged at 5000 g for 8 minutes to collect the bacteria. Then, the cells were lightly suspended in approximately 1.5 mL of pre-cooled Buffer B and aliquot into sterilized 1.5 mL centrifuge tubes at 100 L per tube. Store at -80 ℃ for future use.

2.2.5 Analysis of the Expression Level of the Target Protein

1. Preparation of SDS-PAGE gel

Separation gel (12%) preparation: Take 2 mL of 30% acrylamide mixture solution, 1.3 mL of 1.5 M Tris-HCl buffer solution (pH 8.8), 50 μL of 10% SDS solution and 1.6 mL of deionized water. After thorough mixing, add 50 μL of 10% ammonium persulfate and 10 μL of TEMED. Carefully inject the mixed solution from both sides into the pre-assembled rubber sheet, then add ddH2O liquid seal. Let it stand for 30 minutes until it is completely polymerized, then pour out the water layer and suck up the remaining liquid with filter paper.

Preparation of concentrated gel (5%): After thoroughly mixing 0.67 mL of 30% acrylamide solution, 1.25 mL of 1.0 M Tris-HCl (pH 6.8), 50 μL of 10% SDS and 2.975 mL of deionized water, 50 μL of 10% ammonium persulfate and 10 μL of TEMED were added and rapidly mixed. Carefully inject the glue along both sides of the glue plate. After completion, immediately insert the sample comb and keep it vertical. After 30 minutes, slowly pull out the comb vertically to form the sample loading hole.

2. Preparation of protein samples

Take 1 mL of each fermentation broth under different conditions for 10,000 rpm2 min, discard the supernatant, and collect the bacteria. Resuspend the bacteria in 1 mL of PBS solution for 10,000 RPM ² min, discard the supernatant and collect the bacteria. Resuspend the bacteria with 500 L of PBS solution, and take 30 L of the sample for detecting the content of whole protein.

The remaining 470 L samples were crushed using a cell disruptor. After crushing was completed, they were placed in a centrifuge and centrifuged at 12,000 rpm at low temperature for 10 minutes. The supernatant was retained. 30 L of the sample was taken to detect the protein in the supernatant of the crushed liquid. The precipitate was resuspended with 200 L PBS buffer. 30 L of the sample was taken to detect the protein in the precipitate after crushing.

6 L of 5× loading buffer was added to the sample, mixed well, and then cleaved at 100 °C for 10 minutes for SDS-PAGE protein electrophoresis.

3. Protein electrophoresis

Spotting: Attach 20 μL of protein sample to each well;

Electrophoresis: Use 70 V for the concentrated gel. After the sample forms a straight line at the interface of the separation gel/concentrated gel, switch to a constant voltage of 135 V.

The reaction is terminated when the bromophenol blue band migrates to the bottom edge of the gel.

Staining: Peel off the protein gel, add an appropriate amount of Coomassie brilliant blue staining solution, heat in a microwave oven for 30 seconds, and shake on a shaker for staining for about 1 hour.

Decolorization: Initial decolorization: After recovering the dyeing solution, quickly rinse with distilled water, then add the decolorization solution and shake for decolorization for 2 hours. Final decolorization: Replace with fresh decolorization solution and decolorize overnight at room temperature until the background

becomes transparent. Result preservation: Images were collected using the Bio-Rad gel imaging system.

Summary of the chapter II

1. In this study, molecular cloning technology was adopted. The dpe and l-rhi gene fragments were amplified by PCR. The pETDuet-dpe-l-rhi dual-enzyme expression vector was constructed by restriction endonuclease digestion and T4 ligase ligation, and then transformed into Escherichia coli BL21(DE3) for expression.

2. The correctness of the recombinant plasmid was confirmed through colony PCR, double enzyme digestion verification and DNA sequencing in the experiment. The successful expressions of two proteins, DPEase (35 kDa) and L-RhI (45 kDa), were verified by SDS-PAGE electrophoresis analysis.

3. The entire experimental process includes key steps such as gene cloning, plasmid construction, protein expression and detection, laying the foundation for the subsequent research on the biocatalytic synthesis of D-alosaccharides.

Chapter III
EXPERIMENTAL PART

3.1 Genome Extraction and Amplification of the Target Gene

In this study, the plasmids pUC18-dpe and pUC18-l-rhi preserved in the laboratory were respectively used as templates to amplify the dpe and l-rhi genes. As shown in Figure 3.1 (A and B), it can be seen that there is a target band approximately between 750 bp and 1000 bp DNA Makers in Figure 3-1A, which is consistent with the size of the target gene fragment of 891 bp. There is a bright amplification band approximately at 1300bp in Figure 3-1B, which is basically consistent with the expected molecular weight of 1275bp.

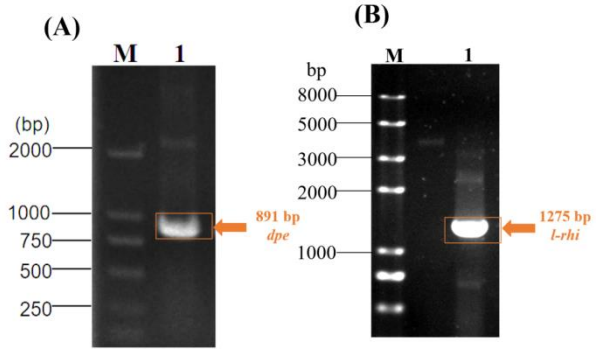

Figure 3.1 PCR electrophoresis diagram

3.2 Construction of Recombinant Plasmids

Operate in accordance with the instructions of the small plasmid extraction kit to purify the plasmid. After extraction, draw approximately 2μL of the plasmid solution into the tube and determine its concentration. Double digestion was performed using restriction endonucleases. After digestion was completed, the digestion products were stored in a freezer at -20 ℃.

The purification of nucleic acids was carried out by using the EC adsorption column enrichment method. The plasmid and the enzyme digestion products of the target gene were taken out and left to melt at room temperature. Both were respectively poured into the EC adsorption column for enrichment. During elution, 50μL of ddH2O was also used, which could improve the elution effect and facilitate the subsequent recombination steps. The construction gene of the DPE recombinant plasmid was linked to the vector pETDuet-1 by recombination to obtain the recombinant double-chain cyclic vector pETDuet-1-dpe. Then, the construction gene of the l-rhi recombinant plasmid was linked to the loaded PetDuet-1 dpe. Finally, the recombinant double-chain ring-shaped carrier pETDuet-dpe-l-rhi was obtained. The construction method is shown in Figure 3.2.

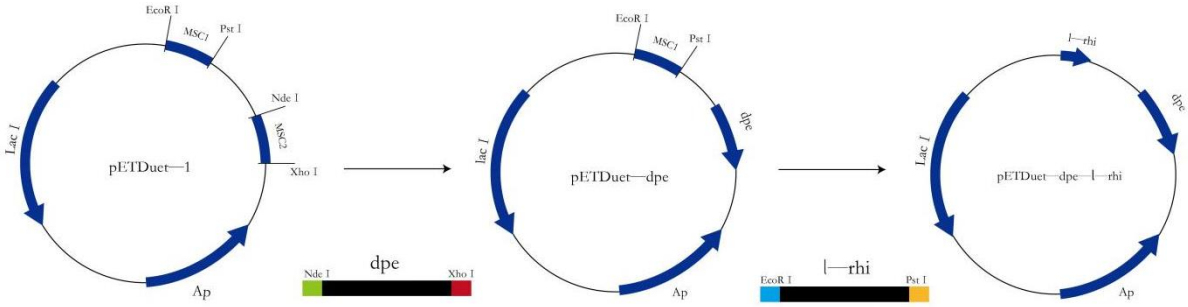

Figure 3.2. Schematic diagram of the construction process of recombinant plasmids 3-2

3.3 Construction and Verification of Engineered Strains

The recombinant plasmid was used to transform the Escherichia coli clone vector DH5α, and the LB-Amp resistant solid medium was used for screening. The next day, single colonies were picked, and positive colonies with better culture conditions were picked. The single colonies were transferred to the LB-Amp liquid

medium and cultured overnight in a constant temperature shaker at 37 °C and 200r/min.

The plasmids pETDuet-1, PetDuet-1 dpe and pETDuet-dpe-l-rhi were extracted according to the instructions of the plasmid small extraction kit. Gel electrophoresis was directly performed after extraction. Among them, the plasmids PetDuet-1 dpe and pETDuet-dpe-l-rhi were verified by gel running after double enzyme digestion respectively. The results are shown in Figures 3.3 and 3.4, indicating the successful construction of the carrier. It can be known from Figure 3-3A that after pETDuet-dpe double enzymatic digestion (Nde I and Xho I), two bands can be obtained, namely the plasmid fragment and the dpe gene. It can be known from Figure 3.3B that after pETDuet-dpe-l-rhi double enzymatic digestion (EcoR I and Pst I), two bands can be obtained, namely the plasmid fragment and the l-rhi gene. The results in Figure 3.4 further confirm that the genes dpe and l-rhi have been successfully attached to the vector.

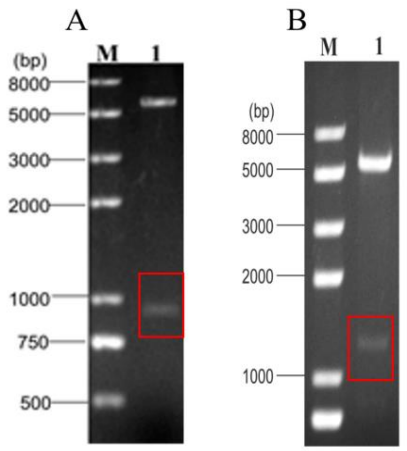

Figure 3.3 Verification images of double enzyme digestion of *pETDuet-dpe* and *PETDuet-dpe-rhi* plasmids

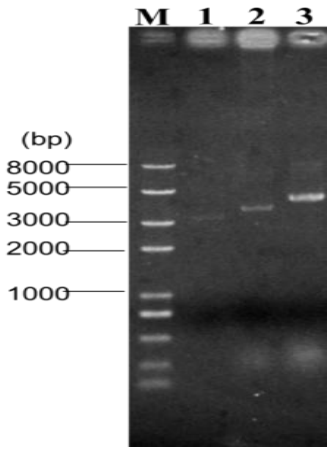

Figure 3.4 pETDuet-1, pETDuet-*dpe* and pETDuet-*dpe-l-rhi* Plasmid validation image（M：Trans 2K PlusII® DNA Marker；Line1: pETDuet-1 Line2:pETDuet *dpe*；Line3: pETDuet-*dpe-l-rhi*）

The recombinant plasmids verified as positive and sequenced correctly were transferred into the Escherichia coli BL21 (DE3) expression strain. After screening the positive clones, resuscitation, transformation and induction of IPTG were carried out. After the induction and crushing were completed, the whole protein, the crushed supernatant and the bacterial precipitate were respectively taken for SDS-PAGE to detect the protein expression. The results are shown in Figure 3.5. It can be seen from the figure that the engineered bacteria successfully expressed the DPE and L-RhI proteins.

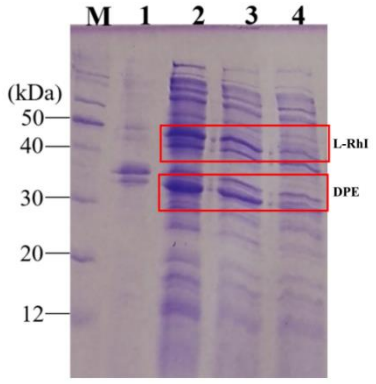

Figure 3.5 Co-expression SDS-PAGE analysis of DPE and L-RhI (Lane 1: Control; Lane 2: Induction of whole protein at 16℃; The supernatant is induced at 3:16 ℃ in the swimming lane. Lane 3:16 ℃ induced precipitation

Summary of the chapter III

1. The target gene was successfully amplified and the recombinant plasmid was constructed： The target gene fragments of 891 bp (dpe) and 1275 bp (l-rhi) were amplified from the pUC18-dpe and pUC18-l-rhi plasmids by PCR, respectively (Figure 3.1). The recombinant plasmids pETDuet-dpe and PetDuet-DPE-L-RHI were successfully constructed by using the double enzyme digestion and ligation reaction, and their correctness was confirmed by enzyme digestion verification (Figure 3.3) and sequencing.

2. Construction and verification of engineered strains： The recombinant plasmids were transformed into Escherichia coli DH5α and BL21(DE3). After resistance screening and colony PCR verification, the plasmids were extracted and double-enzyme digestion analysis was performed (Figure 3.4), confirming that the dpe and l-rhi genes had been accurately inserted into the vectors. Finally, the engineered strains that could stably express DPEase and L-RhI were obtained.

3. Verification of dual-enzyme co-expression： The SDS-PAGE analysis (Figure 3.5) showed that under the induction condition of 16 °C, the engineered bacteria successfully expressed the target proteins of approximately 35 kDa (DPEase) and 45 kDa (L-RhI), and the proteins were mainly present in the supernatant, indicating good soluble expression and laying the foundation for the subsequent biocatalysis synthesis of D-alosaccharides.

CONCLUSIONS

In this study, a dual-enzyme co-expression system was successfully constructed through gene recombination technology. The main achievements include:

The coding sequences of D-allulose 3-isoisomerase (DPEase) and L-rhamnose isomerase (L-RhI) were specifically amplified from the genome, primers were designed, and after amplification, the recombinant expression vector pETDuet-1-dpe-l-rhi was successfully constructed by enzymatic digestion and ligation. And it was transformed into competent cells of Escherichia coli BL21(DE3) by the thermal shock method;

The co-expression of DPEase and L-RhI in Escherichia coli was achieved for the first time. SDS-PAGE confirmed that the recombinant bacteria could stably express two proteins with molecular weights of 35 kDa (DPEase) and 45 kDa (L-RhI), respectively.

It provides key technical support for establishing a dual-enzyme cascade catalytic system based on D-fructose and has important theoretical value for reducing the production cost of D-alosaccharide.

Although this study preliminarily verified the feasibility of the dual-enzyme co-expression system, the following in-depth research still needs to be carried out in terms of industrial application:

Determination of enzyme properties and conversion rates: Although two engineered bacteria expressing enzymes were obtained, further research is needed on the enzymatic properties and conversion rates of the enzymes.

Catalytic system optimization: The effects of parameters such as pH (5.0-8.0), temperature (25-45 ℃), Mn^{2+} concentration (0-5 mM), and substrate concentration (50-200 g/L) on the yield of D-alosaccharide were systematically investigated. The response surface method was further adopted to establish a multi-factor interaction model and determine the optimal combination of response conditions.

Process scale-up research: Conduct pilot-scale verification of engineered strains, verify the scalability of the system using a 5-L fermenter, focus on the effects of dissolved oxygen control and feeding strategies on cell density and enzyme activity, and simultaneously develop efficient downstream separation and purification processes.

1. In this study, an engineered Escherichia coli strain with co-expression of DPEase and L-RhI dual enzymes was successfully constructed. The co-expression of the two functional proteins (35 kDa and 45 kDa) was achieved through gene recombination technology, laying the foundation for the establishment of an efficient D-alosaccharide biocatalytic system.

2. In the future, it is necessary to further optimize the enzymatic properties, reaction conditions and carry out pilot-scale amplification studies to promote the industrial application of this technology.

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