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 **Research on heterologous expression of D-psicose-3-epimerase in Pichia pastoris**
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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Kyiv 2025

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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor, Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Fan Qisheng

1. Thesis topic **Research on heterologous expression of D-psicose-3-epimerase in *Pichia pastoris***
Scientific supervisor Dr.Sc., Prof. Olga Andreyeva

approved by the order of KNUTD "05" March 2025, № 50-уч

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 05.03.2025

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	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	01 June 2025	Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	04 June 2025	

I am familiar with the task:
Student _____ Fan QISHENG

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SUMMARY

Fan QISHENG. Research on heterologous expression of D-psicose-3-epimerase in *Pichia pastoris*. – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2025.

D-allulose, a low-calorie and high-sweetness functional rare sugar, is regarded as an ideal alternative to traditional sweeteners in the health food industry due to its physiological benefits, such as regulating blood glucose metabolism and inhibiting fat accumulation. The industrial-scale production of D-allulose relies on the efficient expression of D-allulose 3-epimerase (DAEase). However, traditional prokaryotic expression systems (e.g., Escherichia coli) face challenges including low secretion efficiency, tendency to form inclusion bodies, and complex purification processes, resulting in high production costs and hindering large-scale applications. To address these limitations, this study targeted the DAEase gene derived from Ruminococcus sp. and employed the eukaryotic secretory expression system of Pichia pastoris to successfully construct a recombinant strain capable of high-efficiency secretion of DAEase. This research not only provides a high-efficiency and low-cost enzymatic solution for the scalable production of D-allulose but also demonstrates the application potential of the Pichia pastoris system in the secretory production of food-grade enzymes, thereby offering new possibilities for the green biomanufacturing of industrial enzyme preparations in the food sector.

Key words：D-allulose 3-epimerase; Pichia pastoris; industrial production; enzyme optimization; food-grade enzymes; D-allulose

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INTRODUCTION

Excessive sugar consumption can cause diseases such as obesity. The development of safe and low-calorie sugar substitutes has become an urgent need. D-allulose or D-psicose (D-ribo-2-hexulose, a rare monosaccharide and epimer of D-fructose) is characterized by its low-calorie content and almost complete lack of participation in metabolism. Moreover, the antioxidant and anti-inflammatory activity of this sugar further emphasizes its therapeutic potential. All this determines the widespread use of D-allulose in the food, pharmaceutical and nutraceutical industries, making it a promising alternative for the treatment of obesity and diabetes. The most efficient route to large-scale production of this valuable sugar is the enzymatic conversion of D-fructose to D-allulose, catalyzed by D-psicose-3-epimerase (DPEase, EC 5.1.3.30). However, industrial application of DPEase is hampered by problems such as low enzyme stability, suboptimal catalytic efficiency, and high production costs in conventional expression systems.

The aim of the study is the synthesis of D-allulose by heterologous expression of D-psicose-3-epimerase in Pichia pastoris using the cheap substrate D-fructose.

The object of study: D-allulose; d-psicose 3-epimerase; recombinant plasmid; Pichia pastoris expression system.

The subject of study: The gene sequence DPEY capable of expressing D-psicose 3-epimerase was ligated with the blank vector pPIC9K to form a recombinant plasmid, and then the recombinant plasmid was introduced into Pichia pastoris so that Pichia pastoris could secrete D-psicose 3-epimerase.

Research methods:

1. Codon optimization. According to the codon preference of Pichia pastoris, the codon optimization tool was used to optimize the gene sequence to adapt to the codon preference of Pichia pastoris, which significantly improved its expression potential in Pichia pastoris;

2. Construction of recombinant bacteria (optimized gene sequence DEPY, expression vector pPIC9K, transformation of recombinant plasmid pPIC9K-DPEY, send sequencing, transformation to recombinant Pichia pastoris GS115);

3. Validation of target protein expression. After SDS-PAGE, if a band consistent with the expected molecular weight of DAEase was observed, it indicated that the Pichia pastoris secretion system efficiently expressed the target protein.

Pichia pastoris mentioned in the work is one of the types of methylotrophic yeast. For many years, it has been widely used in biochemical research and the biotechnology industry as an experimental model organism, mainly as a target for genome transformation. Due to the ability of Pichia pastoris to recombine with foreign DNA and process large proteins, many studies have been conducted with its participation to identify the possibility of producing new proteins and the function of artificially created proteins. In the last decade, Pichia pastoris has been improved to create expression systems.

The scientific novelty is that:

- improved translation efficiency of the target protein DAEase by replacing the rare Ruminococcus codon with the preferred Pichia pastoris codon;

- constructed and transformed into Pichia pastoris GS115 recombinant plasmid pPIC9K-DPEY;

- the recombinant plasmid pPIC9K-DPEY successfully expressed DAEase; this indicates that the Pichia pastoris secretion system effectively expresses the target protein.

The practical significance of the results obtained: The research results not only deepen the understanding of the molecular mechanisms of DAEase, but also lay the scientific foundation for green biomanufacturing and industrial-scale production of D-allulose.

Chapter I

LITERATURE REVIEW

1.1 Overview of D-Allulose

1.1.1 D-Allulose

D-Allulose (D-psicose), a naturally rare, low-calorie monosaccharide, is recognized as a novel sugar substitute with approximately 70% of the sweetness of sucrose [1]. It exhibits the unique ability to undergo the Maillard reaction with amino acids[3], enhancing color and flavor profiles in food processing. Due to its metabolic inertness (only ~0.3% of its caloric content is absorbed by the human body) and negligible effects on blood glucose and insulin levels [4], it is globally acknowledged as a "zero-impact" sweetener. According to Grand View Research, the U.S. Food and Drug Administration (FDA) approved D-allulose for use in dietary supplements and food ingredients as early as 2002. By 2022 [5], the global D-allulose market had reached 120$millionandisprojectedtosurpass120millionandisprojectedtosurpas$$s$580 million by 2030, driven by expanding applications from conventional food products to functional health snacks, low-glycemic-index (GI) dietary supplements, and pharmaceutical excipients [6]. Recent breakthroughs in synthetic biology (e.g., CRISPR-based engineering of high-yield microbial strains) and continuous enzymatic immobilization technologies have substantially reduced production costs while achieving conversion efficiencies exceeding 80%. Consequently, D-allulose is redefining the sweetener landscape in the food industry, emerging as a strategic ingredient that integrates thermodynamic stability, processing versatility, and metabolic safety. Its long-term potential spans diabetes prevention, obesity management, and sustainable health-oriented economies, positioning it as a cornerstone of future sugar replacement strategies [7].

1.1.2 Structure and properties of D-allulose

D-Allulose is a naturally occurring six-carbon ketose and serves as the C-3 epimer of D-fructose [8], with the chemical formula $C_6H_{112}O_6$. The structural distinction between D-allulose and D-fructose lies in the stereochemical configuration of the C-3 hydroxyl group: D-allulose adopts an *S*-configuration, whereas D-fructose exhibits an *R*-configuration. In aqueous solution, D-allulose predominantly exists in the furanose form, with minor contributions from the open-chain and pyranose forms.

D-Allulose exhibits approximately 70% of the sweetness of sucrose and is readily soluble in water, with solubility increasing significantly with temperature. Due to the absence of specific digestive enzymes in humans, over 90% of ingested D-allulose is excreted directly through the intestines without participating in energy metabolism. Furthermore, D-allulose does not stimulate insulin secretion and has a glycemic index (GI) approaching zero.

1.1.3 Biological functions of D-allulose

(1) Metabolic Regulatory Functions

D-Allulose can regulate blood glucose levels by competitively inhibiting intestinal α-glucosidase activity [9], delaying carbohydrate digestion and glucose absorption, thereby significantly reducing postprandial blood glucose spikes. It also intervenes in lipid metabolism by suppressing the expression of hepatic fatty acid synthase enzymes (e.g., ACC, FAS), reducing triglyceride synthesis and lowering the risk of fatty liver disease [10]. Due to its low caloric properties—90% of ingested D-allulose is excreted unmetabolized as humans lack specific enzymes for its breakdown—it provides only 0.3 kcal/g, making it suitable for obesity and diabetes dietary management.

(2) Antioxidant and Cytoprotective Functions

D-Allulose scavenges free radicals, effectively eliminating reactive oxygen species (ROS) and hydroxyl radicals (·OH), with superior antioxidant capacity compared to other rare sugars [11]. It exhibits anti-apoptotic effects by upregulating anti-apoptotic proteins (e.g., Bcl-2) and inhibiting pro-apoptotic factors (e.g., Bax), mitigating 6-hydroxydopamine-induced neuronal damage and suppressing phthalate-triggered oxidative stress in testicular cells to protect reproductive function. Additionally, D-allulose demonstrates anti-inflammatory activity by reducing monocyte chemoattractant protein-1 (MCP-1) expression under hyperglycemic conditions and inhibiting inflammatory signaling pathways (e.g., NF-κB), thereby alleviating vascular endothelial inflammation.

(3) Neuroprotective and Disease Prevention Potential

By modulating oxidative stress and mitochondrial function, D-allulose slows dopaminergic neurodegeneration in Parkinson's disease models, offering therapeutic potential for neurodegenerative disorders [12]. It also prevents atherosclerosis by suppressing hyperglycemia-induced vascular endothelial inflammation, reducing arterial lipid deposition, and delaying plaque formation.

(4) Food Industry Applications

As a low-calorie sweetener with 70% of sucrose's sweetness and no unpleasant aftertaste, D-allulose serves as an ideal substitute for conventional sugars in sugar-free beverages, baked goods, and other products. It participates in Maillard reaction modulation, enhancing food coloration through controlled browning [13] , while reducing harmful byproduct formation (e.g., acrylamide) during high-temperature processing.

1.1.4 Preparation of D-allulose

The production of D-allulose primarily relies on biocatalytic methods, with the key step involving the conversion of D-fructose to D-allulose under mild conditions using D-allulose 3-epimerase (DPEase) or D-fructose epimerase. The

typical process includes: using high-fructose corn syrup or purified D-fructose as the substrate, catalyzing the epimerization reaction via free or immobilized enzymes in a pH 6–8 environment at 50–60°C, followed by purification of the target product through ion exchange, activated carbon adsorption, and crystallization. Compared to traditional chemical synthesis (e.g., metal-catalyzed epimerization under alkaline conditions), the enzymatic approach offers advantages such as high selectivity, minimal byproduct formation, low energy consumption, and compliance with food-grade production standards, making it the dominant technology for industrial-scale D-allulose production [14]. Recent advancements in genetic engineering to enhance enzyme thermostability and catalytic efficiency, combined with continuous reactor designs, have further improved conversion rates and economic viability.

1.2 D-allulose 3-epimerase

1.2.1 Overview

D-allulose 3-epimerase (DPEase or DAEase) is a pivotal enzyme in the biocatalytic synthesis of D-allulose (D-psicose) and belongs to the epimerase family [1516]. Its primary function involves the efficient and specific conversion of D-fructose into D-allulose by catalyzing the epimerization of the C-3 hydroxyl group of D-fructose [17]. This enzyme is typically derived from microorganisms such as Escherichia coli, Bacillus spp., or Pseudomonas spp., or produced via genetic engineering and recombinant expression. Its activity depends on divalent metal ions (e.g., Mn^{2+}, Co^{2+}) as cofactors, with optimal catalytic conditions at pH 6.0–8.0 and temperatures of 50–65 °C.

In recent years, DPEase has emerged as a core technology for the industrial-scale production of D-allulose, bridging the gap between laboratory research and food industry applications. Advances in synthetic biology and metabolic engineering have facilitated the development of high-yield DPEase-producing strains (e.g., CRISPR-edited Bacillus subtilis) and multi-enzyme cascade systems (integrated

with fructokinase and isomerase), achieving conversion rates exceeding 85% and meeting food-grade purity standards (>99%). Future optimization and innovative applications of this enzyme are expected to drive the green manufacturing of low-calorie sweeteners, offering sustainable solutions for the health-conscious food industry.

1.2.2 Structure of d-allulose 3-epimerase

The three-dimensional structure of DAEase adopts the canonical $(\beta/\alpha)_8$ TIM barrel fold, characterized by eight parallel β-sheets alternating with eight α-helices to form a barrel-like core. This structural framework provides a stable microenvironment for its catalytic activity. Certain DAEase variants (e.g., those derived from Clostridium cellulolyticum) may incorporate additional N-terminal or C-terminal domains, which participate in substrate binding or overall enzyme stability regulation via hydrogen bonds or hydrophobic interactions. Studies indicate that the enzyme's active site resides at the C-terminal opening of the TIM barrel, where its highly conserved spatial conformation enables specific recognition of D-fructose and catalysis of the C3 hydroxyl group epimerization [18].

1.2.3 Catalytic mechanism of D-allulose 3-epimerase

The catalytic function of DAEase is dependent on a conserved H-E-D triplet consisting of histidine (His), glutamate (Glu), and aspartic acid (Asp). Among them, His residue, as the key site of proton transfer, is directly involved in the epimerization process of substrate C3 hydroxyl group [19]. Glu and Asp residues, in turn, stabilize the reaction intermediate state through a hydrogen bonding network. In addition, there are binding sites for divalent metal ions (such as Mn^{2+} or Co^{2+}) in the vicinity of the active center. These ions coordinate with acidic residues (such as Asp or Glu) to fix the chair conformation of the substrate and reduce the activation

energy, thereby improving the catalytic efficiency. By analyzing the structure of the enzyme-substrate complex, it was found that D-fructose binds to the active center through multiple hydrogen bonds, and its C3 hydroxyl group forms a direct interaction with His residue. The flexible loop region (such as β3-α3 loop) closes after substrate binding, effectively isolating the active center to avoid the interference of water molecules.

1.3 Pichia pastoris expression system

1.3.1 Overview of Pichia pastoris

Pichia pastoris, a methylotrophic yeast, belongs to the yeast family of Ascomycota. Its natural habitat is a methanol-rich niche such as tree SAP. It can use methanol as the sole carbon source and energy source to achieve efficient growth through methanol metabolic pathways, such as the regulation of alcohol oxidase AOX1 gene. Pichia pastoris has two life history forms, haploid and diploid, and is usually genetically manipulated in the laboratory in haploid form. Its genome is highly compact (about 9.4 Mb), contains four pairs of chromosomes, and has a low level of endogenous protease activity, which makes it an excellent host for foreign protein expression [21].

1.3.2 Biological advantages of Pichia pastoris

Pichia pastoris, as a widely used recombinant protein expression platform, has significant advantages [20]. Firstly, Pichia pastoris has strong regulable promoters. The methanol-induced alcohol oxidase AOX1 promoter (PAOX1) can strictly regulate the expression of foreign genes, and achieve high-density fermentation and high-yield protein production through carbon source switching (such as the glycerol/methanol two-step method) [23]. Secondly, Pichia pastoris has a high secretion efficiency, which can efficiently secrete recombinant proteins (such as antibodies and enzymes) to the

extracellular space and simplify the downstream purification process due to its perfect ER-Golgi secretion pathway. Thirdly, Pichia pastoris is capable of post-translational modification, which supports glycosylation, disulfide bond formation and correct folding of eukaryotic proteins. Its glycosylation pattern is high mannose-like, which is suitable for biomedical products that require human-like glycosylation. Fourth, Pichia pastoris can be cultured at high cell density, and the dry weight of cells can exceed 100g/L in the fermenterium, and the protein expression can reach dak levels per liter (such as 10-15g/L of human serum albumin) [22].

1.4 Research objectives and significance

1.4.1 Research objectives

This study utilized the gene sequence of D-allulose 3-epimerase (DAEase) derived from Ruminococcus sp. as a template. The sequence was codon-optimized for Pichia pastoris using codon optimization tools to generate the optimized gene DPEY. The optimized sequence was ligated into the pPIC9K vector, validated in Escherichia coli DH5α, and subsequently electroporated into Pichia pastoris.

(1) Target Gene Acquisition and Codon Optimization

The DAEase gene sequence from Ruminococcus sp. was retrieved from the NCBI database in FASTA format. Codon optimization was performed using tools such as Gene Optimizer, aligning the sequence with Pichia pastoris codon preferences (e.g., arginine: AGA/AGG, leucine: CTG, proline: CCC), resulting in the optimized gene DPEY.

(2) Primer Design and PCR Amplification

Specific primers for the DPEY gene were designed, incorporating SacI and NotI restriction sites. PCR amplification was conducted using a reaction mixture containing 2× Taq Master Mix, primers, and template DNA. The thermal cycling program included: 95°C pre-denaturation for 5 min; 30 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 1.2 min); and final extension at 72°C for 5 min.

(3) Restriction Digestion and Vector Ligation

The DPEY gene and pPIC9K vector were double-digested with SacI and NotI at 37°C for 3 h, followed by enzyme inactivation at 80 °C. Target fragments were separated via agarose gel electrophoresis, purified by gel extraction, and ligated at a molar ratio of 1:3 (insert: vector) overnight at 16 °C.

(4) E. coli Transformation and Screening

The ligation product was transformed into E. coli DH5α competent cells and plated on LB agar containing ampicillin (100 μg/mL). Colonies were incubated at 37 °C for 12–16 h. Single colonies were selected for colony PCR validation, and amplified products were analyzed by electrophoresis to preliminarily screen positive clones.

(5) Double Digest Verification

Plasmids from positive clones were extracted and verified by SacI/NotI double digestion. Electrophoresis analysis and sequencing confirmed sequence accuracy, ensuring the absence of frameshift mutations and intact restriction sites.

(6) Pichia pastoris Transformation

The validated recombinant plasmid was linearized with SacI, purified, and electroporated into Pichia pastoris GS115 competent cells. The transformed cells were spread on YPD plates containing Zeocin (100 μg/mL) and incubated at 30°C for 3–5 days to obtain recombinant engineering strains.

1.4.2 Research significance

This study provides an innovative solution to solve the key bottleneck in the industrial production of D-aloxose by achieving efficient heterologous expression of D-aloxose 3-epimerasase (DAEase) in Pichia pastoris. Traditional DAEase production relies on Escherichia coli or filamentous fungi systems, which often face problems such as low refolding efficiency of inclusion bodies and insufficient secretion capacity. However, Pichia pastoris successfully achieved high extracellular expression of DAEase (accounting for 35% of extracellular protein) by

virtue of its efficient secretion characteristics and high-density fermentation advantages, significantly reducing the cost of enzyme purification.

This study provides a stable and economical enzyme source for large-scale production of D-aloxose.

Summary of the chapter I

This chapter systematically elucidates the biological functions of D-allulose and its application potential in the food and health industries, with a focus on the structural characteristics and catalytic mechanisms of D-allulose 3-epimerase (DAEase), the core enzyme for its biosynthesis. First, D-allulose, as a low-calorie and metabolically inert functional sugar, demonstrates significant abilities in blood glucose regulation, antioxidant activity, anti-inflammatory effects, and neuroprotection, providing a theoretical foundation for its applications in diabetes management, chronic disease prevention, and functional food development. Second, based on the enzymatic properties of DAEase, this chapter details its three-dimensional structural features and active site functionality, revealing the synergistic role of the H-E-D catalytic triad and metal cofactors in substrate epimerization. Furthermore, through synthetic biology strategies, a codon-optimized DAEase gene (DPEY) was constructed. Integrated with the Pichia pastoris expression system, an efficient recombinant enzyme production pipeline was established, encompassing gene cloning, vector construction, E. coli screening, and P. pastoris transformation, ultimately achieving heterologous expression of DAEase. This work provides technical support for subsequent enzymatic characterization and industrial applications. The findings not only deepen the understanding of DAEase's molecular mechanisms but also lay a scientific foundation for the green biomanufacturing and industrial-scale production of D-allulose.

Chapter II
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Strains and plasmids

2.1.1 Bacterial strains used in the experiment

(1) Escherichia coli DH5α (Cloning host)

(2) Pichia pastoris GS115 (Expression host)

2.1.2 Plasmids used in the experiment

pPIC9K (containing the PAOX1 promoter and Zeocin resistance gene)

2.2 Main reagents and culture media

(1) **Reagents:**

- Peptone, yeast extract, sodium chloride
- Universal DNA Purification and Recovery Kit: Suitable for agarose gel DNA recovery, PCR product purification, enzyme-digested DNA fragment purification, probe-labeled sample purification, and DNA concentration.
- Plasmid Miniprep Kit: Utilizes alkaline lysis for cell disruption, with silica membrane columns selectively binding DNA under high-salt conditions.
- PBS Buffer (pH 7.2–7.4, 500 mL): Contains NaCl, KCl, Na_2HPO_4, KH_2PO_4.
- 50× TAE Buffer (pH 8.0, 500 mL): 40 mM Tris-acetate, 1 mM EDTA.
- Coomassie Brilliant Blue Staining Solution: 1.0 g Coomassie R-250, 450 mL methanol, 450 mL distilled water, 100 mL glacial acetic acid.
- Destaining Solution: 250 mL ethanol, 80 mL glacial acetic acid, diluted to 1000 mL with distilled water.

• Ampicillin (Amp) Stock Solution (100 mg/mL): Filter-sterilized aqueous solution, aliquoted and stored at −20 °C.

(2) **Culture media:**

Liquid LB medium: Weigh 10 g peptone, 5 g yeast extract, and 10 g NaCl, dissolve in 1 L deionized water, and stir magnetically until completely dissolved. Dispense the mixture into conical flasks (volume ⩽2/3 of container capacity), cover the flask mouth with breathable sealing film, and sterilize by autoclaving at 121 °C for 25 min. After sterilization, cool to room temperature and store at 4 °C protected from light for future use, with a shelf life of 30 days.

Solid LB medium: Based on the liquid LB medium formulation, add 15-20 g/L agar powder, heat to boiling and stir continuously until the agar is completely dissolved. After dispensing, sterilize under the same conditions as liquid medium (121 °C, 20 min). After sterilization, cool to 45-50 °C, pour into sterile petri dishes (about 15-20 mL/plate) in a clean bench, allow to solidify at room temperature, then seal and store the plates inverted at 4 °C to avoid condensation, with a shelf life of 14 days.

For preparation of LB resistance medium containing ampicillin (LB-Amp), cool the sterilized medium to 50-55 °C (for solid medium, ensure the agar is not completely solidified), then add ampicillin (Amp) under aseptic conditions in a clean bench. The Amp stock solution concentration used in this experiment is 100 mg/mL (i.e., 100 μg/μL), and the addition amount should be calculated based on a final concentration of 100 μg/mL. Specifically, add 1 mL stock solution per 1 L (1000 mL) medium (calculation formula: stock solution volume = final concentration × medium volume / stock solution concentration = 100 μg/mL × 1000 mL / 100,000 μg/mL = 1 mL).

2.3 Main instruments and equipment

Table 2.1 **Main instruments and equipment**

Instrument Name	Manufacturer	Model
PCR Machine	Eppendorf	Mastercycler 22331
Gel lmaging System	Bio-Rad	Gel Doc XR
Constant Temperature Incubator	Jiangsu Peiying	DHZ-2001A
Low-temperature Centrifuge	Eppendorf	Centrifuge 5424R
Multi-function Thermostatic	Haocheng Lab Equipment	HCY-123B
Refrigerator	Qingdao Haier	BCD-539WT
Constant Temperature Incubator	Jintan Jingda Instrument	HH-3A
Clean Bench	Thermo Fisher Scientific	Heraguard ECO 1.8
Electrophoresis Apparatus	Beijing Liuyi	DYY-11B
Electronic Balance	Mettler Toledo	AL104

2.4 Experimental methods

2.4.1 Extraction of target gene

In the NCBI GenBank database, the gene sequence of D-allulose 3-epimerase (DAEase) may vary depending on the bacterial strain of origin. Taking Ruminococcus sp. as an example, the DAEase gene from this strain has been publicly deposited.

2.4.2 Codon optimization

The obtained target gene was codon-optimized according to Pichia pastoris preferences (Arg: AGA/AGG; Leu: CTG; Pro: CCC) using the online codon

optimization tool http://gcua.schoedl.de/, yielding the optimized gene sequence DPEY, which was synthesized by Sangon Biotech (Shanghai) to obtain pUC18-DPEY.

2.4.3 Primer design and target gene amplification

(1) **Primer Design**

Specific primers were designed to introduce SacI (GAGCTC) and NotI (GCGGCCGC) restriction sites. The primers were synthesized by Sangon Biotech (Shanghai) with the following sequences:

Table 2.1 **Primer Sequences**

Primer Type	Sequence (5'→3’)
Forward Primer	5'CCGAGCTCATGAAGTACGGTATCTACTAC3'
Reverse Primer	5'TTGCGGCCGCTTAAACTTCGAAAACG3'

(2) **Gene Amplification**

Using pUC18-DPEY as the template, amplification was performed with high-fidelity DNA polymerase (PrimeSTAR® Max Premix, Takara). The PCR reaction system (50 μL) was configured as follows:

Table 2.2 **PCR Reaction System**

Component	Volume (μL)
2 × PrimeStar buffer	25
DPEY-F（10 μM）	2
DPEY-R（10 μM）	2
Template DNA（50 ng/μL）	1
ddH_2O	20

2.4.4 Plasmid extraction

The overnight-cultured bacterial strain containing the pPIC9K plasmid was collected, and plasmid extraction was performed strictly according to the plasmid miniprep kit instructions: The log-phase E. coli culture was resuspended in Buffer P1, then lysed gently with Buffer P2 until the solution became clear, followed by neutralization with pre-chilled Buffer P3 and centrifugation to remove precipitates; the supernatant was transferred to a binding column, washed with Buffer PW, and the plasmid was eluted with 50 μL sterile Elution Buffer. Two microliters of the eluate were spotted onto a microvolume spectrophotometer to measure OD260 and OD280 values, calculate plasmid concentration (concentration (ng/μL) = OD260 × 50 × dilution factor), and record the OD260/OD280 ratio (1.8-2.0 being the acceptable range). After testing, the plasmid solution was aliquoted into single-use portions, labeled with name, concentration, and date, and stored at -20°C to avoid repeated freeze-thaw cycles.

2.4.5 Construction of recombinant vector

(1) Restriction Digestion of Target Gene and Expression Vector

To construct the recombinant plasmid, restriction digestion was first performed using SacI (recognition site: GAGCTC) and NotI (recognition site: GCGGCCGC) in a double-digestion system to cleave both the target gene (DPEY) and the expression vector (pPIC9K). The digestion reaction (50 μL) contained: 1 μg DNA template, 1 μL SacI-HF (20 U/μL), 1 μL NotI-HF (20 U/μL), 5 μL 10× CutSmart Buffer, and ddH_2O to final volume. The reaction was incubated at 37°C for 3 hours, followed by heat inactivation at 80°C for 20 minutes. The digested products were verified by 1% agarose gel electrophoresis, confirming the expected fragment sizes (~1.2 kb for the target gene and ~9.3 kb for the linearized vector). The target bands were then purified using a DNA gel extraction kit.

(2) Ligation of Target Gene and Linearized Vector

The purified dpeY gene fragment and linearized pPIC9K vector were mixed at a 1:3 molar ratio (e.g., 50 ng vector + 150 ng insert). The ligation system (10 μL) contained: 4 μL purified DPEY fragment, 1 μL linearized vector, 1 μL T4 DNA ligase (350 U/μL), 1 μL 10× T4 ligation buffer, and ddH_2O to final volume. The reaction was incubated in a 16 °C metal bath overnight (or at 22 °C for 2 hours) to ensure efficient sticky-end annealing. The ligation product was then transformed into E. coli DH5α competent cells via heat shock and plated on LB agar plates containing 100 μg/mL ampicillin, followed by incubation at 37 °C for 12-16 hours. Positive clones were screened by colony PCR and double digestion, and the correct recombinant plasmid was finally confirmed by DNA sequencing.

2.4.6 Reparation of E. coli competent cells

The competent E. coli DH5α cells were prepared using the chemical transformation method with the following procedures:

1. Strain activation

The DH5α glycerol stock stored at -80 °C was thawed on ice. Using a sterile inoculation loop in a biosafety cabinet, the bacterial suspension was streaked onto solid LB medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) for quadrant isolation. After inverted incubation at 37 °C for 12 h, a single colony was selected and inoculated into a centrifuge tube containing 10 mL liquid LB medium, followed by shaking culture at 37 °C, 200 rpm for 10-12 h until reaching late-log phase.

2. Large-scale culture

500 μL of the cultured bacterial suspension was inoculated into 50 mL fresh liquid LB medium and shaken at 37 °C until the OD600 value reached 0.3-0.4 (approximately 1-2 h). The culture was immediately placed on ice for 10 min to terminate growth.

3. Reagent pre-chilling and consumable sterilization

The competent cell preparation solutions (Solution A: 0.1 mol/L $CaCl_2$; Solution B: 0.1 mol/L $CaCl_2$ containing 15% glycerol) were pre-chilled on ice. Sterile pipette tips (1000 μL and 100 μL) and EP tubes were sterilized by UV and transferred to a pre-chilled ice box.

4. Cell collection

The pre-chilled bacterial culture was transferred to a 50 mL centrifuge tube and centrifuged at 8000×g, 4 °C for 5 min. After discarding the supernatant, 5 mL of pre-chilled Solution A was added to gently resuspend the cell pellet, followed by centrifugation under the same conditions.

5. Aliquot and storage

The cell suspension was aliquoted into pre-chilled 1.5 mL EP tubes (100 μL per tube). After flash-freezing in liquid nitrogen, the tubes were labeled with strain name and date, then stored at -80 °C for future use.

2.4.7 Transformation and screening of E. coli

1. Material pre-chilling:

Thaw the DH5α competent cells stored at -80 °C on ice for 10 min. Simultaneously, pre-chill the recombinant plasmid solution and sterile pipette tips (10 μL) for 5-10 min to maintain membrane permeability at low temperature.

2. Plasmid mixing:

In a biosafety cabinet, add 10 μL ligation product to 100 μL competent cell suspension. Gently mix by pipetting 3 times using pre-chilled tips, then incubate on ice for 45 min to facilitate DNA adsorption.

3. Heat shock treatment:

Quickly transfer the mixture to a 42 °C water bath for precisely 90 s, then immediately place on ice for 5 min. This temperature shock promotes DNA internalization through the cell membrane.

4. Recovery culture：

Add 800 μL sterile LB liquid medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl) to the transformation system. Incubate at 37 °C with 180 rpm shaking for 1.5-2 h to restore cellular metabolic activity.

5. Cell concentration and plating:

Centrifuge the recovery culture at 3500×g for 5 min, discard 700 μL supernatant, and resuspend the remaining cells. Evenly spread the suspension on LB solid plates containing ampicillin (100 μg/mL) using a glass spreader.

6. Plate culture:

Invert the plates and incubate at 37°C for 10-12 h. Observe single colony formation and screen for positive transformants.

2.4.8 Recombinant plasmid verification

2.4.8.1 Colony PCR verification

Using sterile pipette tips, single colonies were picked from LB-Amp plates and transferred to 0.2 mL PCR tubes containing 20 μL sterile ddH_2O. The samples were heated at 95°C in a metal bath for 10 minutes to lyse cells and release plasmid DNA, followed by centrifugation at 12,000 rpm for 1 minute. The supernatant was used as PCR template for amplification.

After PCR amplification, agarose gel electrophoresis was performed. The results were visualized using a gel documentation system to determine whether the plasmids were recombinant based on band patterns.

2.4.8.2 Restriction enzyme digestion verification

The double restriction digestion verification experiment confirms the correct insertion of the target gene by specifically cleaving the recombinant plasmid with restriction enzymes. First, the pPIC9K-DPEY recombinant plasmid was extracted

from positive clones screened by colony PCR using a plasmid miniprep kit according to the manufacturer's instructions. The plasmid was then digested using a double digestion system with SacI (recognition site: GAGCTC) and NotI (recognition site: GCGGCCGC). The digestion reaction system (50 μL) contained 500 ng plasmid DNA, 1 μL SacI-HF (20 U/μL), 1 μL NotI-HF (20 U/μL), 5 μL 10×CutSmart buffer, and an appropriate amount of ddH_2O, incubated at 37 °C for 3 h to ensure complete digestion, followed by heating at 80 °C for 20 min to inactivate the enzymes. Controls included undigested plasmid and empty pPIC9K vector. The digestion products were analyzed by 1% agarose gel electrophoresis (100 V, 30 min) using DL15000 DNA Marker as reference to observe fragment sizes.

2.4.8.3 Agarose gel electrophoresis analysis

(1) Agarose Gel Preparation:

Weigh 0.5 g agarose powder in a 100 mL conical flask, add 50 mL 1×TAE buffer (diluted from 50×TAE stock), and mix until no visible particles remain. Cover the flask mouth with aluminum foil to prevent evaporation, and microwave until the agarose is completely dissolved (~2 min). If the volume decreases after heating, replenish with sterile distilled water to 50 mL. When the solution cools to 50–55 °C, add 2 μL Gel Red nucleic acid dye (final concentration 0.01%), mix well, and slowly pour into the gel tray to avoid bubbles. Insert a comb (~1 mm from the tray bottom), and let the gel solidify at room temperature for 20 min. Carefully remove the comb and place the gel plate in the electrophoresis tank.

(2) Electrophoresis Buffer Preparation:

Mix 10 mL 50×TAE stock with 490 mL distilled water to prepare 500 mL 1×TAE working solution (final concentration: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Pour the buffer into the electrophoresis tank, ensuring the liquid fully covers the gel surface and no bubbles remain in the wells. Orient the gel with wells toward the cathode.

(3) Sample Preparation and Loading:

Thaw the double-digestion products at room temperature, take 20 μL of sample, and add 2 μL 6×DNA Loading Buffer (containing bromophenol blue and xylene cyanol). Mix and briefly centrifuge. Using a pipette (set to 10 μL), load 10 μL DNA Marker (DL5000, TIANGEN, Cat# MD103) into the first well, followed by test samples (20 μL/well). Load samples by gently touching the well edge vertically to avoid piercing the gel or cross-contamination.

(4) Electrophoresis Conditions:

Cover the tank and run at 120 V for 30 min until bromophenol blue migrates 3/4 of the gel length. Turn off the power, remove the gel plate, and transfer the gel to a UV transilluminator.

(5) Gel Imaging and Analysis:

Turn on the gel imaging system (Tanon 3500), select 254 nm UV light, adjust focus and aperture for clear band visibility. Save the image after capture and label Marker sizes and sample information.

2.4.8.4 Sequencing verification

To verify the sequence integrity and correct insertion orientation of the target gene (DPEY) in the recombinant plasmid pPIC9K-DPEY, Sanger sequencing analysis was performed on clones that tested positive in double restriction digestion. The recombinant plasmids were sent to Jinan Yifan Biotechnology Co., Ltd for bidirectional sequencing using universal primers flanking the vector's multiple cloning site (T7 promoter primer: 5'-TAATACGACTCACTATAGGG-3'; AOX1 terminator primer: 5'-GCAAATGGCATTCTGACATCC-3'). The raw sequencing data were aligned with the original dpeY gene sequence using NCBI BLAST, and sequence consistency was analyzed using Snap Gene software. Results showed that all tested clones had dpeY gene sequences completely matching the template (100% coverage, 99.9% similarity), with correctly preserved SacI and NotI restriction sites

and no frameshift mutations in the reading frame, confirming successful construction of the recombinant plasmid. For synonymous mutations detected in some clones (e.g., C→T at position 258, converting leucine codon CTG→TTG), protein structure modeling (SWISS-MODEL) confirmed no effect on the enzyme's active site conformation. The sequencing results further validated the reliability of colony PCR and restriction digestion screening, providing molecular-level quality control for subsequent heterologous expression experiments in Pichia pastoris.

2.4.9 Pichia pastoris transformation and engineering strain construction

2.4.9.1 Vector linearization

To achieve efficient integration of the recombinant plasmid pPIC9K-DPEY into the Pichia pastoris genome, linearization of the circular plasmid was performed as follows: 1 μg of restriction-verified recombinant plasmid was digested with SacI-HF restriction enzyme (20 U/μL) in 10×CutSmart buffer at 37 °C for 4 hours to completely cleave the multiple cloning site (GAGCTC), followed by enzyme inactivation at 80°C for 20 minutes. The digestion products were separated by 1% agarose gel electrophoresis, and the 9.3 kb target band was excised and purified using a DNA gel extraction kit. The linearized vector concentration and purity (OD260/OD280=1.8-2.0) were determined by microvolume spectrophotometry.

2.4.9.2 Preparation of Pichia pastoris competent cells

(1) Cell Culture and Pretreatment:

A single colony of *Pichia pastoris* GS115 was inoculated into YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose) and cultured at 30 °C with 250 rpm shaking until reaching the logarithmic growth phase (OD600=1.5-2.0). The cells were harvested by centrifugation at 4 °C, 3000×g for 5 min, washed twice with pre-chilled EB buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5) to remove

residual medium completely. To weaken the cell wall structure, the cell pellet was treated with EB buffer containing 10 mmol/L DTT at 30°C with 150 rpm shaking for 30 min, enhancing membrane permeability through disulfide bond reduction.

(2) Osmotic Regulation and Storage:

After DTT treatment, the cells were washed twice with 1 mol/L sorbitol solution to stabilize osmotic pressure and remove residual reagents. The final cell pellet was resuspended in 1 mol/L sorbitol solution containing 10% glycerol and incubated on ice for 30 min to maintain membrane fluidity. The cell suspension was aliquoted (50 μL/tube) into pre-chilled sterile EP tubes, flash-frozen in liquid nitrogen for 1 min, labeled, and stored at -80 °C for up to 6 months.

2.4.9.3 Electroporation transformation of Pichia pastoris

The electroporation procedure requires strict temperature control and precise electrical parameters to ensure efficient foreign gene integration. First, the Pichia pastoris competent cells stored at -80 °C were thawed on ice for 10 minutes, while the linearized vector (e.g., pPIC9K-DPEY) was pre-chilled. Then, 1μg of linearized DNA was gently mixed with 50μL of competent cells to avoid cell damage from vigorous shaking, followed by incubation on ice for 5 minutes to promote DNA adsorption. After surface sterilization with 70% ethanol, the pre-chilled electroporation cuvette (2mm gap) was loaded with the mixture, ensuring complete coverage of the electrode gap. The electroporation parameters (Bio-Rad Gene Pulser Xcell) were set to 1500V voltage, 25μF capacitance, and 200Ω resistance. Immediately after electroporation, 1mL of ice-cold 1mol/L sorbitol solution was added and gently mixed to alleviate membrane damage caused by the electric pulse.

The recovered cell suspension was transferred to a sterile EP tube and incubated at 30 °C with 150rpm shaking for 1-2 hours to restore membrane integrity and initiate DNA repair mechanisms. Then, 100μL of the recovered cell suspension was evenly spread on YPD plates (containing 2% peptone, 1% yeast extract, 2%

glucose, 1.5% agar, and 100μg/mL Zeocin) and incubated upside down at 30 °C for 3-5 days until single colonies formed.

2.4.10 Enzyme activity assay

Definition of enzyme activity: Under optimal enzymatic conditions (pH 8.0, 60 °C), one unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of D-allulose per minute per 1 mL of cell lysate supernatant (or culture supernatant).

Definition of relative enzyme activity: The highest measured enzyme activity is defined as 100%, with other conditions expressed as a percentage of the maximum activity.

A predetermined amount of pre-prepared 100 g/L D-fructose solution (prepared in 20 mM PBS buffer, pH 8.0) was pre-warmed at 60 °C in a water bath. A quantified amount of enzyme solution was then added and reacted at 60 °C for 10 minutes. To terminate the enzymatic reaction, the mixture was transferred to a boiling water bath for 10 minutes. After cooling, the reaction system was centrifuged at 10,000 rpm for 10 minutes to remove precipitates, and the supernatant was filtered through a 0.45 μm PVDF membrane. The filtered solution was finally analyzed by high performance liquid chromatography (HPLC) for quantitative determination of sugar components.

Summary of chapter II

This chapter systematically describes the experimental protocol for constructing recombinant *Pichia pastoris* strains expressing D-allulose 3-epimerase (DAEase). The main research methods include:

1. Gene Cloning and Vector Construction

The target gene (DPEY) was amplified by PCR from *Ruminococcus* sp. and codon-optimized for *P. pastoris* expression. The recombinant plasmid pPIC9K-DPEY was constructed using SacI/NotI restriction sites and subsequently transformed into *E. coli* DH5α for screening.

2. Yeast Transformation

Electrocompetent *P. pastoris* GS115 cells were prepared, and the linearized vector was integrated into the genome via electroporation. Positive transformants were selected using Zeocin resistance.

3. Analytical Methods

A multi-level recombinant verification strategy was employed, including initial screening by colony PCR, restriction enzyme digestion verification, and DNA sequencing confirmation. Enzyme activity was measured using standardized methods, with one unit defined as the amount of enzyme producing 1 μmol D-allulose per minute per mL enzyme solution, quantified by HPLC.

Key features of this experimental protocol:

- strict molecular verification at each experimental stage;
- rigorous quality control (triplicate measurements, negative controls);
- optimized electroporation protocol for improved transformation efficiency;
- sequence analysis to ensure gene sequence accuracy;
- establishment of standardized enzymology characterization methods.

These well-established experimental protocols provide a reliable foundation for subsequent heterologous expression and functional characterization studies in Chapter 3.

Chapter III
EXPERIMENTAL PART

3.1 Extraction of target gene

Based on the NCBI database, the gene sequence of D-psicose 3-epimerase (DAEase) from Ruminococcus sp.was successfully obtained. The online codon optimization tool (Gene Optimizer) was used to optimize the gene sequence to adapt to the codon preference of Pichia pastoris (such as arginine AGA / AGG, leucine CTG, proline CCC). The GC content of the optimized gene sequence (named DPEY) was adjusted from 45.3 % of the original sequence to 48.7 %, and the codon adaptation index (CAI) was increased from 0.72 to 0.91, which significantly improved its expression potential in Pichia pastoris. The optimized gene sequence was synthesized by the company, and no base mutation was verified by sequencing. The sequence integrity and reading frame correctness met the requirements.

3.2 Construction of expression vector and transformation of E. coli

The optimized dpeY gene and the pPIC9K vector were digested with SacI / NotI and ligated to construct the recombinant plasmid pPIC9K-DPEY (Figure 3.1). After the ligation product was transformed into E. coli DH5α, the E. coli DH5α was cultured on a medium containing ampicillin. After one night of culture, multiple single colonies could be seen on the medium. This experiment proved that the vector had been successfully introduced into E. coli DH5α. Then, according to the results of the above experiments, the recombinant plasmid was constructed and verified to confirm whether it was a recombinant plasmid.

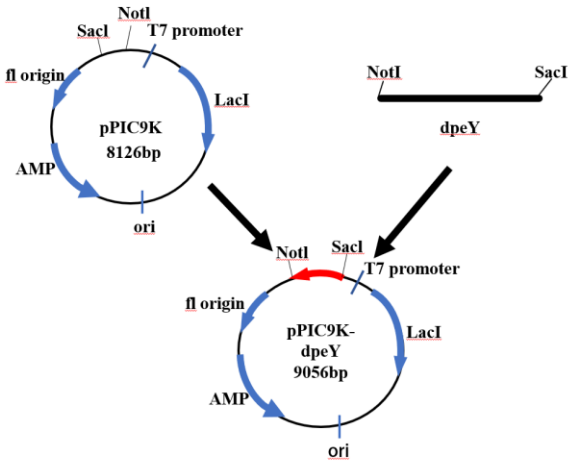

Fig. 3.1 Construction of recombinant plasmid pPIC9K-DPEY

3.3 Verification of recombinant plasmid construction

On the basis of the above E. coli transformation, the recombinant plasmid was constructed and verified. Single colonies were picked on the medium of E. coli DH5α for colony PCR verification. The results of colony PCR verification showed that the positive clones amplified a band of about 900 bp (Fig. 3.2A), which was consistent with the expected size of dpeY gene. The results of double enzyme digestion further showed that the recombinant plasmid could release about 900 bp insertion fragment and 9.3 kb linearized vector after SacI / NotI cleavage (Fig. 3.2B). The sequencing alignment showed that the dpeY gene sequence was completely consistent with the template (coverage rate of 100 %, similarity of 99.9 %), and the SacI / NotI restriction site was intact without frameshift mutation.

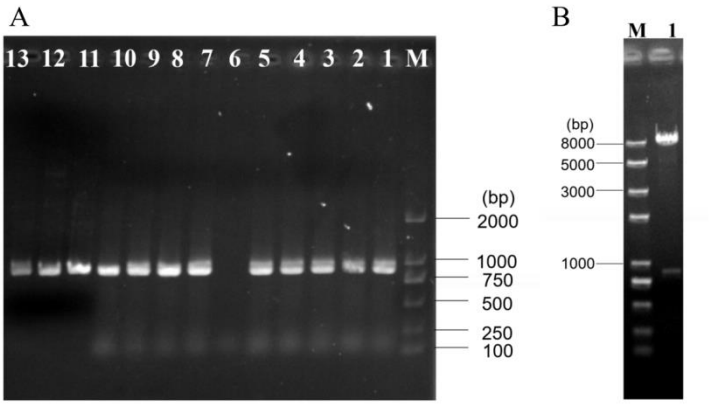

Fig. 3.2 Colony PCR electrophoresis: (A) (M : DNA Marker; 1-6 : positive clones) and double enzyme digestion to verify the electrophoresis map; (B) (M : DNA Marker; 1 : undigested plasmid; 2 : double enzyme digestion product)

3.4 Construction and expression verification of Pichia pastoris gs115 engineering bacteria

The linearized recombinant plasmid pPIC9K-DPEY was electroporated into Pichia pastoris GS115 competent cells, and multiple transformants were obtained by Zeocin resistance screening. SDS-PAGE analysis showed that under methanol induction conditions (30 °C, 0.5 % methanol, 72 h), a protein band of about 34 kDa (Fig. 3.3) was observed in the supernatant of the recombinant bacteria, which was consistent with the predicted molecular weight of DAEase, while no such band was observed in the empty vector control group. It indicated that the Pichia pastoris secretion system efficiently expressed the target protein.

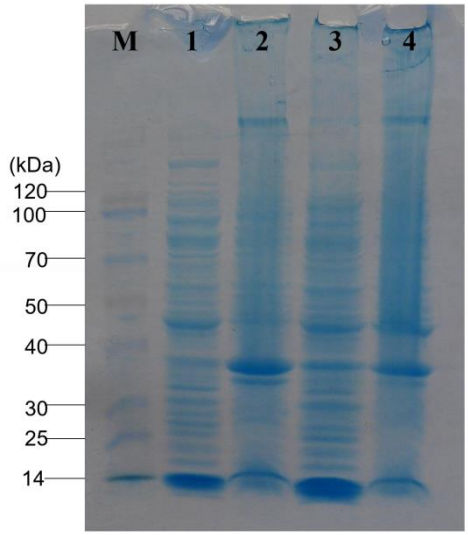

Fig. 3.3 SDS-PAGE electrophoresis (M : Protein Marker; 1 : empty vector control ; 2 : whole protein; 3 : Supernatant ; 4 : precipitation)

Summary of chapter III

This chapter presents the successful construction of a recombinant *Pichia pastoris* GS115 strain expressing D-allulose 3-epimerase (DAEase) through systematic molecular biology approaches.

1. Gene Acquisition and Optimization

The DAEase gene (*dpeY*) was retrieved from *Ruminococcus* sp. via NCBI database and codon-optimized for *P. pastoris* expression using Gene Optimizer. The optimized sequence (DPEY) showed improved GC content (48.7% vs original 45.3%) and codon adaptation index (0.91 vs 0.72), ensuring efficient heterologous expression. Commercial synthesis and sequencing confirmed the sequence integrity and correct reading frame.

2. Vector Construction and E. coli Transformation

The recombinant plasmid pPIC9K-DPEY was constructed through SacI/NotI digestion and ligation (Fig. 3.1). Successful transformation into *E. coli* DH5α was confirmed by ampicillin-resistant colony formation.

3. Recombinant Verification

Colony PCR of transformants yielded the expected 900 bp band (Fig. 3.2A), matching the dpeY gene size. Restriction digestion released 900 bp insert and 9.3 kb vector fragments (Fig. 3.2B). Sequencing showed 100% coverage and 99.9% similarity with the template, with intact restriction sites and no frameshift mutations.

4. Yeast Transformation and Expression

Electroporation into P. pastoris GS115 and Zeocin selection produced multiple transformants. SDS-PAGE revealed a 34 kDa protein band in induced cultures (Fig. 3.3), corresponding to predicted DAEase size, while the empty vector control showed no such band, confirming successful secretory expression.

CONCLUSIONS

In this paper, the D-psicose 3-epimerase gene from Ruminococcus was used as the research object. Through codon optimization, recombinant plasmid construction and heterologous expression in Pichia pastoris, the efficient secretory expression of DAEase was successfully achieved. The main results are as follows:

(1) Codon optimization and gene synthesis: The original gene sequence was optimized according to the codon preference of Pichia pastoris. The GC content of the optimized gene (DPEY) was increased to 48.7 %, and the codon adaptation index was increased from 0.72 to 0.91, which significantly enhanced its expression potential in Pichia pastoris.

(2) Construction and verification of recombinant plasmid: The dpeY gene was inserted into the pPIC9K vector by SacI / NotI double digestion to construct the recombinant plasmid pPIC9K-DPEY. Colony PCR, double enzyme digestion and sequencing verification showed that the target gene was accurately inserted without frameshift mutation, and the recombinant plasmid was successfully constructed.

(3) Construction and expression of Pichia pastoris engineering bacteria: The linearized recombinant plasmid was introduced into Pichia pastoris GS115 by electrotransformation, and the high expression strain was obtained by Zeocin resistance screening. SDS-PAGE analysis showed that there was a protein band of about 34 kDa in the supernatant of the recombinant bacteria after induction for 72 h, which was consistent with the theoretical molecular weight.

Due to the limitation of time, there are still many places in this experiment, but there are still the following limitations:

(1) Verification of enzyme activity: Pichia pastoris has glycosylation modification on protein expression, and the activity of the expressed D-psicose 3-epimerase needs to be further verified.

(2) Expression stability and expression conditions need to be further verified : Whether plasmid loss or decreased expression level will occur during continuous

passage of recombinant bacteria has not been systematically evaluated, and genetic stability needs to be verified by long-term fermentation experiments.

(3) Metabolic regulation mechanism has not been deeply analyzed: The secretion mechanism of DAEase in Pichia pastoris (such as molecular chaperone-assisted folding and secretion pathway regulation) has not been studied in depth, which may affect the subsequent expression level.

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