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 **Synthesis of billirubin in *Saccharomyces cerevisiae***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
Ji Xinping

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

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Ji Xinping

1. Thesis topic **Synthesis of billirubin in *Saccharomyces cerevisiae***

Scientific supervisor Ph.D., Asos. Prof. Olena OKHMAT

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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	
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)		Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)		

I am familiar with the task:

Student _____ Ji Xinping

Scientific supervisor _____ Olena OKHMAT

ABSTRACT

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Bilirubin, as a key product of heme metabolism, has potential value in the field of medicine for antioxidant, anti-inflammatory, and treatment of liver diseases. However, its traditional extraction methods have problems such as high cost and low efficiency, which limit its large-scale application. This article focuses on the biosynthesis of bilirubin and conducts preliminary explorations on the functional validation of *Arabidopsis* heme oxidase (*AtHO*) and *Rattus norvegicus* biliverdin reductase (*RnBVR*).

Key words*: bilirubin; Brewing yeast; CRISPR-Cas9;biosensor*

TABLE OF CONTENTS

INTRODUCTION

This study focuses on the synthetic biology-driven optimization of bilirubin biosynthesis, addressing the limitations of traditional extraction methods such as high costs and low efficiency, which hinder large-scale applications. Bilirubin, a critical metabolite of heme catabolism, holds significant therapeutic potential in antioxidant, anti-inflammatory, and hepatoprotective applications. Through functional validation of *Arabidopsis thaliana* heme oxygenase (AtHO) and *Rattus norvegicus* biliverdin reductase (RnBVR), combined with advanced genome-editing technologies, this research establishes novel microbial platforms for efficient bilirubin production. The relevance of the topic lies in advancing synthetic biology strategies to overcome industrial bottlenecks in bilirubin production and expanding its biomedical applications.

The purpose of the study is to develop a genetically engineered microbial system for high-efficiency bilirubin biosynthesis and elucidate regulatory mechanisms of heme-to-bilirubin conversion.

The objectives of the study include:

1. Functional validation of *AtHO* and *RnBVR* in diverse yeast chassis;

2. CRISPR-Cas9-mediated chassis optimization via *HMX1* knockout and multi-gene overexpression;

3. Comparative analysis of heme supply strategies (de novo synthesis vs. diffusion);

4. Biosensor-enabled metabolic network engineering for strain improvement.

The object of the study is the engineered Saccharomyces cerevisiae WAT11 strain with CRISPR-edited *HMX1* and dual-cistronic *AtHO-RnBVR-HEM4* expression.

The subject of the study encompasses the molecular mechanisms of heme-to-bilirubin conversion, chassis strain engineering, and biosensor-driven metabolic optimization.

Research methods integrate CRISPR-Cas9 genome editing, dual-cistronic expression systems, comparative chassis analysis (WAT11 vs. BY4741/CEN.PK2-1C), and biosensor construction for dynamic pathway monitoring.

The scientific novelty includes:

1. First application of dual-cistronic expression for AtHO-RnBVR co-regulation in yeast;

2. CRISPR-Cas9-mediated *HMX1* deletion to redirect heme flux toward bilirubin synthesis;

3. Pioneering use of bilirubin biosensors for metabolic network remodeling.

The practical significance of the results obtained is twofold:

1. Industrial potential via optimized microbial platforms for cost-effective bilirubin production;

2. Methodological advancements in biosensor-guided strain engineering, offering a blueprint for metabolic pathway optimization in synthetic biology.

These findings lay a robust foundation for further synthetic biology studies on bilirubin biosynthesis, bridging fundamental mechanistic insights with scalable biomanufacturing solutions.

CHAPTER I
LITERATURE REVIEW

1.1 OVERVIEW OF BILIRUBIN

1.1.1 STRUCTURE AND FUNCTION OF BILIRUBIN

Bilirubin is a breakdown metabolite of hemoglobin in the body, appearing as an orange to dark reddish brown powder or crystal. The biosynthesis of this molecule begins with the degradation of hemoglobin. In the reticuloendothelial system, protoporphyrin IX in hemoglobin undergoes specific oxidative cleavage of its alpha methylene bridge (located between pyrrole rings I and II) under the catalysis of heme oxygenase (HO-1), producing green biliverdin IX alpha. This reaction requires the participation of NADPH and molecular oxygen, and releases carbon monoxide (CO) and iron ions (Fe^{2+})[1]. Subsequently, biliverdin reductase utilizes NADPH to reduce the central methylene bridge to methylene, ultimately forming the bilirubin IX alpha isomer - the most predominant naturally occurring form in the human body[2] .

The molecular structure of bilirubin has unique chemical characteristics, and its basic skeleton is composed of four pyrrole rings (I, II, III, IV) connected by three carbon bridges (Figure 1.1), including: one methylene bridge (-CH_2-) connecting pyrrole rings I and II, and two methylene bridges (-CH=) connecting II-III and III-IV rings, respectively. Heme has four methylene bridges located at the α, β, and δ positions[3]. Therefore, the heme ring can be cleaved into four possible isomers of biliverdin: bilirubin IX alpha, biliverdin IX beta, biliverdin IX gamma, and biliverdin IX delta[4]. This special conjugated system endows bilirubin with a distinct orange yellow color and a characteristic absorption peak at wavelengths of 450-460 nm. As the main pigment in bile, bilirubin not only gives bile its characteristic yellow green color, but also plays important physiological functions in the body. Its unique molecular structure

gives it both lipophilic properties and the ability to transform into a water-soluble form through binding reactions, which is crucial for the metabolism and excretion of bilirubin.

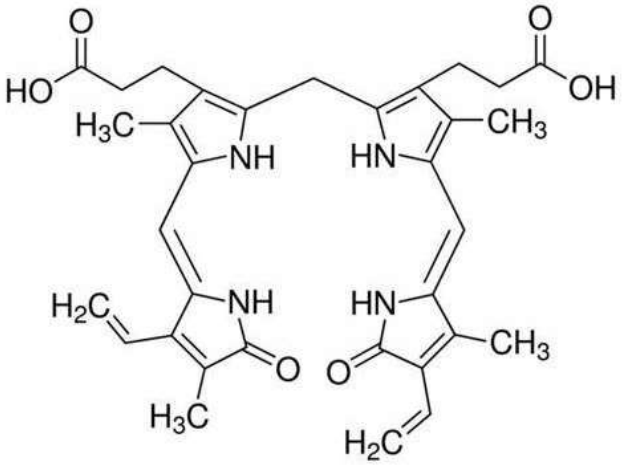

Figure 1.1 – Molecular Structure of Bilirubin

The total bilirubin (TB) in living organisms mainly includes two forms: unconjugated bilirubin (UCB) and conjugated bilirubin (CB)[5.6]. Unconjugated bilirubin is lipophilic and needs to be transported to the liver by binding with albumin; Through the catalysis of glucuronosyltransferase in the liver, it is converted into water-soluble conjugated bilirubin, which is then discharged into the intestine through bile[7]. After being discharged into the intestine, symbiotic bacteria in the intestine will further process bilirubin, producing urobilinogen. Most of the urobilinogen will be excreted from the body, and a small portion will be reabsorbed through the renal tubules, forming hepatic intestinal circulation. The level of serum bilirubin (BIL) is an important indicator for evaluating liver function and can be used for evaluating the treatment effect and prognosis of patients with cirrhosis[8]. Bilirubin is toxic and can cause irreversible damage to the brain and nervous system. But research has found that it also has significant antioxidant, anti-inflammatory, and neuroprotective effects. Foreign studies have shown that when the level of total bilirubin is low, its antioxidant effect is a protective measure for the body, which can clear oxygen free radicals; But when the total bilirubin level is high, it belongs to cytotoxic metabolites, which can lead to severe brain injury[9-11]. Moreover, when bilirubin metabolism is abnormal, it can lead to an

increase in blood concentration. Elevated bilirubin levels in infants and young children can cause neonatal jaundice, manifested as skin and sclera jaundice. According to the onset time, duration, and bilirubin levels, it can be divided into physiological jaundice and pathological jaundice[12]. Elevated bilirubin levels in adults can cause cardiovascular disease and pulmonary fibrosis[13.14].

Through research, it has been found that the metabolic conversion network of bilirubin also hides important physiological significance. The ten double bonds inside its molecule can capture twice the amount of free radicals equivalent to vitamin E, continuously clearing peroxidized lipids in the intestine during enterohepatic circulation. Moreover, unconjugated bilirubin can upregulate the expression of glutathione synthase by activating the nuclear receptor Nrf2, resulting in a cascade amplification effect that increases its actual antioxidant capacity to over 100 times its molecular equivalent. These findings not only explain why evolution has preserved this seemingly 'metabolic waste' substance, but also provide a theoretical basis for the development of neuroprotective agents based on bilirubin precursors - clinical trials have shown that trace amounts of unconjugated bilirubin (5-15 μmol/L) can reduce oxidative stress markers in the cerebrospinal fluid of Alzheimer's disease patients by 37%. The dual characteristics of bilirubin explain its important role in the metabolic system of life.

1.1.2 APPLICATION VALUE OF BILIRUBIN

Bilirubin, as an important bioactive substance, has shown extensive application value in various fields such as medicine, scientific research, and industry. Modern pharmacological research has revealed a wider range of biological activities of bilirubin. Its powerful antioxidant properties have attracted much attention in the field of neuroprotection. Studies have shown that bilirubin can effectively scavenge free radicals, reduce oxidative stress damage, and show promising prospects in the treatment of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In addition, experiments have shown that bilirubin has a significant inhibitory effect on W256 tumor cells, while also exhibiting multiple pharmacological activities such as

antipyretic, antihypertensive, and promoting red blood cell regeneration. These findings have opened up vast opportunities for the development of new drugs for bilirubin. In clinical diagnosis, bilirubin is a key indicator for liver function testing, and its serum concentration changes can effectively distinguish the types of jaundice (hemolytic, hepatocellular, and obstructive jaundice), providing important basis for the diagnosis and treatment of liver and gallbladder diseases. In the field of traditional Chinese medicine, bilirubin, as the main active ingredient of artificial bezoar (the content is up to 50% or more), is an indispensable raw material for Angong Niuhuang Pill, Niuhuang Qingxin Pill and other valuable traditional Chinese patent medicines and simple preparations. These drugs have significant effects in clearing heat, detoxifying, sedating and tranquilizing.

1.1.3 PRODUCTION METHOD OF BILIRUBIN

The market demand for bilirubin in China is huge, with an annual demand of over 100 tons for artificial bezoar alone[15]. At present, the production of bilirubin mainly relies on traditional extraction methods and chemical synthesis methods, but these processes have significant limitations. The animal bile extraction method uses bile from animals such as pigs, cows, and sheep as raw materials[16]. Although the process is relatively mature, it is limited by the amount of animals slaughtered, the supply of raw materials is unstable, and a large amount of organic solvents (such as chloroform, ethanol, etc.) are required during the extraction process. This not only incurs high costs, but also faces severe environmental pressure. The chemical synthesis method uses hemoglobin or protoporphyrin as precursors to prepare bilirubin through multi-step oxidation-reduction reactions. Although the product purity is high, the reaction conditions are harsh (such as strong acid, strong base, or high temperature and pressure), and there are many by-products with low yields (usually less than 30%). In addition, although the derived processes such as calcium salt method, ethanol method, resin method, and hydrolysis method[17-19] have been optimized in specific links, they are still difficult to break through bottlenecks such as strong dependence on raw materials, high

energy consumption, and difficult waste treatment, resulting in the existing production system being unable to meet the growing market demand.

Therefore, conducting research on bilirubin biosynthesis and developing efficient and low-cost preparation processes not only has important scientific significance, but also can create significant economic value. With the development of synthetic biology technology, the production of bilirubin through microbial fermentation is expected to break through the existing production bottleneck and provide a sustainable solution to meet the growing market demand.

1.2 RESEARCH ON THE SYNTHESIS BIOLOGY OF BILIRUBIN

Synthetic biology is an interdisciplinary field that integrates theories and technologies from multiple disciplines such as biology, engineering[20], chemistry, and computer science[21]. Its aim is to design and construct new biological components, devices, and systems to achieve precise regulation and directed modification of biological functions, or to create biological systems with specific functions. At present, in the research of bilirubin synthesis biology, genetic engineering technology has been used to construct Escherichia coli, which simultaneously expresses heme oxygenase (HO) and biliverdin reductase (BVR) and synthesizes bilirubin using its own heme[22]; Some modifications have also been made to Pichia pastoris to construct recombinant Pichia pastoris expressing heme metabolizing enzymes, which convert heme into bilirubin or its precursor biliverdin[23]. However, prokaryotic Escherichia coli lacks the protein modification system of eukaryotic cells, which affects the activity of heme metabolism enzymes and results in lower yields; However, due to the unclear regulatory network of metabolic pathways, the yield of Pichia pastoris is limited.

In contrast, brewing yeast, as a classic eukaryotic model organism, exhibits unique and significant advantages in synthetic biology research.

In the field of biotechnology applications, brewing yeast has become one of the most important protein expression platforms. Modern genetic engineering technology has realized the large-scale production of a variety of high-value recombinant protein

drugs using this microorganism, including: human insulin[24], hepatitis B vaccine[25], human serum albumin[26], etc. With the development of synthetic biology technology, brewing yeast has been successfully modified for the production of complex plant natural products such as artemisinin and cannabinoids[27], as well as important pharmaceutical intermediates such as β - lactam antibiotics. Compared with Escherichia coli and Pichia pastoris, brewing yeast has a complete endoplasmic reticulum Golgi protein processing system, which can precisely fold and glycosylation key metabolic enzymes such as heme oxygenase (HO) and biliverdin reductase (BVR) derived from mammals or other eukaryotes, ensuring that enzyme proteins have a complete spatial structure and high activity state, thus overcoming the problem of limited enzyme function caused by the lack of such systems in prokaryotes such as Escherichia coli. In terms of metabolic pathway adaptation, brewing yeast has an endogenous heme synthesis and degradation pathway similar to that of mammals, starting from the de novo synthesis pathway of glycine and succinyl CoA, as well as mitochondria as the natural site for heme synthesis[28]. This makes it more suitable for bilirubin synthesis in terms of metabolic environment and substrate supply. Compared with the metabolic regulatory network that has not been fully resolved in Pichia pastoris, brewing yeast can more clearly streamline and optimize metabolic flow.

From the perspective of industrial production, brewing yeast has a long history of industrial application, and its fermentation process is mature. Under high-density cultivation and batch feeding strategies, it can adapt to harsh conditions such as shear force, pH, and dissolved oxygen in industrial grade bioreactors. Compared with E. coli, which is prone to plasmid loss and protein inclusion bodies in large-scale cultivation of Pichia pastoris, it exhibits stronger adaptability to large-scale production[29]. Moreover, as a GRAS (generally considered safe) strain[30], brewing yeast has no risk of endotoxin contamination during production. Its cell wall structure makes cell breakage easier, greatly reducing the technical difficulty and cost of bilirubin extraction and purification, and providing convenience for downstream process optimization.

Therefore, using brewing yeast as a carrier, cross species metabolic pathway reconstruction is carried out to screen the combined expression of dominant heme

metabolism enzyme genes from different species in the future; Implement dynamic metabolic regulation strategies, utilizing promoters responsive to heme concentration to achieve dynamic coupling between key enzyme expression and heme synthesis. Not only is it expected to break through the yield bottleneck of existing hosts in bilirubin synthesis, but it can also provide a universal technical paradigm that can be referenced and promoted for the biosynthesis of complex natural products, promoting the deep development and application of synthetic biology in multiple fields such as biomedicine and chemical raw material production.

1.3 GENOME EDITING IN *SACCHAROMYCES CEREVISIAE*

Genome editing technology is a key technology for modifying cell metabolism, improving the productivity and yield of target products[31], and is also a core tool for modifying the metabolic pathway of brewing yeast and enhancing the synthesis efficiency of target products. This technology has been developing since the late 20th century. The zinc finger nuclease (ZFN) system, which was introduced in the 1990s, was the first to combine artificially designed zinc finger proteins with FokI nucleases, achieving precise editing of specific DNA sequences. This technology can theoretically locate specific sites of 18 bp by concatenating 3-4 zinc finger modules (each module recognizing a 3 bp sequence) in the DNA binding domain. Subsequently, TALEN technology with higher recognition accuracy emerged, pushing DNA recognition accuracy to the single base level. Its core lies in the highly modular 34 amino acid repeat units in the TALE protein, each unit specifically recognizing a nucleotide through the "repeat variable dual residue" (RVD) at the 12th and 13th residues. Both of these techniques require designing specific protein structures for each target, which is complex and costly to operate.

With the continuous innovation of genome editing technology and the development of more precise editing methods, the CRISPR-Cas9 system is the most breakthrough technology in the field of genome editing in recent years. The system uses RNA molecules to guide nucleases for targeted cleavage, greatly simplifying the

experimental process and reducing costs, making it quickly become a routine tool in the laboratory. Its core consists of two parts: Cas9 nuclease and guide RNA (gRNA). Cas9 is a DNA cutting enzyme derived from the bacterial immune system, while gRNA is a short RNA sequence designed to guide Cas9 protein to specific genomic sites. After gRNA recognizes the target DNA sequence through base pairing, Cas9 will cut the DNA double strand at a specific position, and then the cell uses its own repair mechanism to complete gene editing (Figure 1.2).

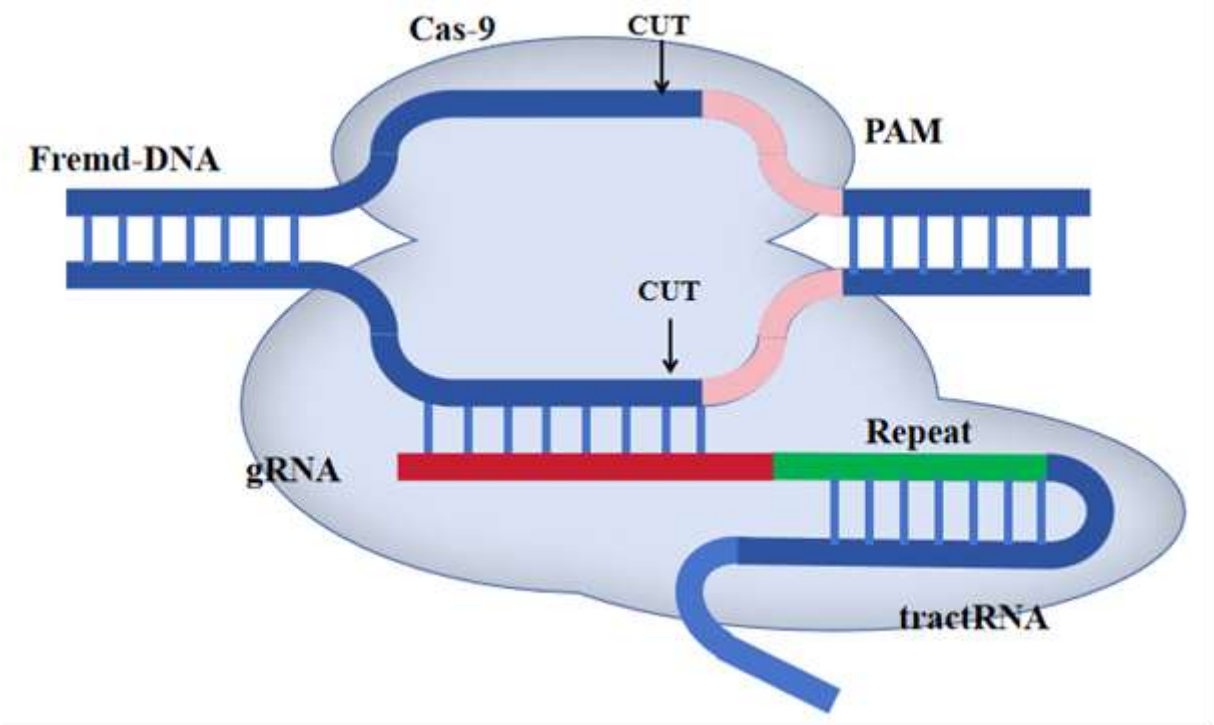

Figure 1.2 – CRISPR-Cas9 system

Compared with traditional gene editing methods such as zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs), CRISPR-Cas9 has higher efficiency and lower cost[32]. Combined with a multi cis expression strategy, it significantly enhances the precision and scalability potential of metabolic engineering in brewing yeast.

In recent years, the emergence of internal ribosome entry sites (IRES)[33], self cleaving peptides 2A[34], HACKing[35], and other technologies have provided new methods for genome editing in yeast.

The internal ribosome entry site (IRES) can guide the localization of the 40S subunit of ribosomes to the translation initiation region of mRNA through the mediation of specific trans acting factors, breaking through the limitations of traditional 5-terminal cap structures and enabling ribosomes to initiate protein synthesis from the internal site of mRNA. This feature enables the synergistic expression of multiple gene products under a single promoter regulatory system, but its size typically exceeds 500 nt, with vector capacity limitations and extremely imbalanced upstream and downstream protein expression levels.

The cleavage site of self cleaving peptide 2A is often between glycine and proline at its C-terminus. After self cleavage, the N-terminal amino acid residue of peptide 2A is connected to the upstream protein, while the proline residue remains connected to the downstream protein. So, the tail of the previous protein will leave a peptide of over 20 amino acids, while the N-terminus of the following protein will leave an excess proline. The function of the cleaved protein may be affected by these more than 20 amino acids.

HACKing system uses 9 bp IGG6 sequence to mediate multi cis trans expression. Through CRISPR-Cas9, the target gene is coupled with host endogenous high expression genes. For example, the fluorescence intensity of GFP in *TDH3: IGG6-GFP* is 12-130 times higher than that in IRES system. It can also build a four cis trans system to synthesize β - carotene, etc. Combining with GTR-CRISPR technology, rapid integration of 13 genes is achieved, and the yield in squalene and Arhat fruit glycoside synthesis is significantly increased.

This experiment aims to achieve precise modification of the metabolic network of brewing yeast by integrating the HACking system with CRISPR-Cas9 gene editing technology. CRISPR-Cas9 mediated genome editing is used to target the knockout of the heme degradation gene *HMX1* to block competitive metabolic pathways. At the same time, the multi cis co expression mechanism of the HACking system is utilized to couple the heme synthase gene *HEM4* with host endogenous high expression driver genes (such as *TDH3*), achieving stable overexpression of *HEM4* and constructing an efficient gene expression vector. At the level of metabolic network remodeling, *HMX1* knockout is used to reduce heme loss, enhance precursor supply through *HEM4*

overexpression, and optimize the expression level of heme transmembrane transporter Hrg-4p, systematically increasing the metabolic flux of bilirubin synthesis pathway and ultimately increasing bilirubin production.

Summary of the chapter I

1.Core Applications of Bilirubin

2.Critical Bottlenecks in Current Production Systems

3.Synthetic Biology-Driven Technological Breakthroughs

4.Industrialization Prospects and Strategic Significance

CHAPTER II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 EXPERIMENTAL MATERIALS

2.1.1 STRAIN AND PLASMID

The experimental strains used in this experiment are detailed in Table 2-1.

Table 2-1 Strains used in this experiment

Bacterial Strain	Genotype
BY4741	MATα his3Δ1 leu2 met15Δ ura3-52
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0
CEN.PK2-1C	MATα ura3-52,trp1-289,leu2-3,112,his3Δ1;MAL2-8c; SUC2
WAT11	MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1his3-11,15
WAT11-1	Originating from WAT11, *HMX1 Δ:: AtHO IGG RnBVR*
WAT11-2	Originating from WAT11-1, *TDH3:: IGG-HEM4*

The plasmids used in this experiment are detailed in Table 2-2.

Table 2-2 Plasmids used in this experiment

Plasmid	Genetic Marker	Carrying Key Genes	Source
pUC19-*HMX1*	Amp	*AtHO1/RnBVR*	This Experiment
pUC19-*HEM4*	Amp	*HEM4*	This Experiment
pUC57-*HEM1*	Amp	*HEM1*	This Experiment
pETDuet-1-*AtHO*	Amp	*AtHO*	This Experiment
pETDuet-1-*AtHO-RnBVR*	Amp	*AtHO*、*RnBVR*	This Experiment
pESC-LEU-*UnaG*	Amp	*UnaG*	This Experiment
pESC-URA-*hrg-4*	Amp	*hrg-4*	This Experiment
Sc-Cas9-gRNA-Ura	Kana	Cas9、gRNA	Laboratory Preservation
Sc-Cas9-*HEM4*	Kana	*HEM4*-gRNA	This Experiment

Sc-Cas9-*HMX1*	Kana	*HMX1*-gRNA	This Experiment

2.1.2 EXPERIMENTAL REAGENTS

The experimental reagents used in this experiment are detailed in Table 2-3.

Table 2-3 List of Experimental Reagents

Drug Name	Manufacturer
Glucose	Tianjin Damao Chemical Reagent Factory
Sodium Chloride	Tianjin Hengxing Chemical Reagent Manufacturing Co., Ltd
Sodium Hydroxide	Laiyang Kangde Chemical Co., Ltd
Peptone	AOBOX Biotechnology
Agar Powder	AOBOX Biotechnology
Yeast Extract	AOBOX Biotechnology
95% Ethanol	Hebei Ruikang Pharmaceutical Technology Co., Ltd
Anhydrous Ethanol	Tianjin Fuyu Fine Chemical Co., Ltd
Agarose Gel	Gene (Hong Kong) Limited
Glycerol	China National Pharmaceutical Group Chemical Reagent Co., Ltd
0.9% Sodium Chloride	Shandong Kelun Pharmaceutical Co., Ltd
Tris Acetic Acid Electrophoresis Buffer	Fuzhou Weibokang Biotechnology Co., Ltd
Yeast Conversion Of Peg/Liac Mixture	Aleshan (Guangzhou) Biotechnology Co., Ltd
Salmon Essence Dna Solution	JinClone (Beijing) Biotechnology Co., Ltd
Yeast Colony Pcr Kit	Biyun Tian Biotechnology Co., Ltd
Md 5000 Dna Marker	Beijing Cryptography Biotechnology Co., Ltd
Fast Pure Gel Dna Extraction Mini Kit	Nanjing Nuoweizan Biotechnology Co., Ltd
Sma Ⅰ Endonuclease	Shenggong Bioengineering (Shanghai) Co., Ltd
Sph Ⅰ Endonuclease	Shenggong Bioengineering (Shanghai) Co., Ltd
Goldview Nucleic Acid Dye	Lanjieke Technology Co., Ltd
Super Red Nucleic Acid Dye	Lanjieke Technology Co., Ltd
Plasmid Small Amount Extraction Kit	Beijing Solaibao Technology Co., Ltd
Yeast Sensitivity Preparation And Conversion Kit	Biyun Tian Biotechnology Co., Ltd
Sample Buffer Solution	Biyun Tian Biotechnology Co., Ltd
Antibiotics (Amp, Kana)	Biyun Tian Biotechnology Co., Ltd

2.1.3 EXPERIMENTAL EQUIPMENT

The experimental equipment used in this experiment is detailed in Table 2-4.

Table 2-4 List of Experimental Equipment

Name	Model	Manufacturer
Constant Temperature Incubator	BXP-6	Shanghai Lichen Bangxi Instrument Technology Co., Ltd
Electronic Balance	FA-2004B	Shanghai Youke Instrument Co., Ltd
Vertical High-Pressure Steam Sterilization Pot	GMJ-80L	Shanghai Shen'an Medical Equipment Factory
High Speed Tabletop Centrifuge	5418	Abend (Shanghai) International Trade Co., Ltd
Microwave Oven	M1-L202B	Guangdong Midea Electrical Appliance Manufacturing Co., Ltd
Ph Meter	PHS-3C	Hangzhou Qiwei Instrument Co., Ltd
Table	ZQPZ-228A	Tianjin Laibotrui Instrument Equipment Co., Ltd
Water Bath	BHS-1	Qun'an Scientific Instruments (Zhejiang) Co., Ltd
Pcr Instrument	TC-96/G/H(b)C	Hangzhou Borui Technology Co., Ltd
Gel Imager	Gel-SMART	Dalong Xingchuang Experimental Instrument Co., Ltd
Electrophoresis Apparatus	JY600C	Beijing Junyi Oriental Electrophoresis Equipment Co., Ltd
Ice Maker	ZB-9Y16F	Hefei Rongshida Electronic Technology Co., Ltd

2.1.4 CULTURE MEDIUM

LB liquid culture medium (per 100 mL): 1 g of peptone, 0.5 g of yeast extract, 1 g of NaCl, adjusted to pH around 7.0, 121 ℃, sterilized with high-pressure steam for 20 minutes, and added antibiotics such as ampicillin and kanamycin depending on the subsequent strain cultivation.

LB solid culture medium (per 100 mL): 1 g of peptone, 0.5 g of yeast extract, 1 g of NaCl, 1.5 g of agar powder. Adjust the pH to around 7.0, sterilize with high-pressure steam at 121 ℃ for 20 minutes, cool to around 55 ℃, and add antibiotics such as

ampicillin and kanamycin depending on the subsequent strain cultivation situation before pouring onto a plate.

YPD liquid culture medium (per 100 mL): 1 g yeast extract, 2 g peptone, natural pH, 121 ℃, 20 min high-pressure steam sterilization, then add 5 mL of 40% glucose solution (115 ℃, 20 min, high-pressure steam sterilization).

YPD solid culture medium (per 100 mL): 1 g yeast extract, 2 g peptone, 1.5 g agar powder, natural pH, 121 ℃ for 20 min high-pressure steam sterilization, then add 5 mL of 40% glucose solution (115 ℃, 20 min high-pressure steam sterilization), cool to around 55 ℃, and pour onto a plate.

SC-URA medium (per 100 mL): Add 0.02739 g of SD URA premix powder, pH natural, 121 ℃, and sterilize with high-pressure steam for 20 min.

SC-URA solid culture medium (per 100 mL): Add 0.02739 g of SD Ura premix powder, 1.5 g of agar powder, natural pH, 121 ℃, high-pressure steam sterilization for 20 min, cool to around 55 ℃, and then pour onto a plate.

SC-URA-LEU medium (per 100 mL): Add 0.02739 g of SD Ura Leu premix powder, pH natural, 121 ℃, and sterilize with high-pressure steam for 20 min.

SC-URA-LEU solid culture medium (per 100 mL): Add 0.02739 g of SD Ura Leu premix powder, pH natural, 121 ℃, high-pressure steam sterilization for 20 min, cool to around 55 ℃, and then pour onto a plate.

2.2 EXPERIMENTAL METHODS

2.2.1 ACTIVATION OF BACTERIAL STRAINS

Take out the yeast strains WAT11, CEN.PK2-1C, BY4741, and BY4742 stored in frozen glycerol, inoculate them into 5 mL of liquid YPD medium, shake at 30 ° C for 15 hours, then re inoculate and place them on a shaker at 250 rpm, 30 ° C for 5 hours.

Take out the Escherichia coli carrying the vector, inoculate it into 5 mL of corresponding resistant culture medium according to its genetic markers, shake the shaker at 250 rpm, 37 ℃, and culture for 12-15 hours.

2.2.2 PLASMID EXTRACTION

This experiment used a low-dose plasmid extraction kit to extract plasmids, with slight modifications. The steps are as follows:

1. Transfer 5 mL of overnight cultured bacterial solution into a 1.5 mL centrifuge tube, centrifuge at 12000 rpm for 1 minute, discard the supernatant, and retain the precipitate.

2. Take 250 μL of solution I that has been pre added with RNase A, add it to the collected precipitate, and use a pipette to resuspend the cell precipitate.

3. Take 250 μL of solution II and add it to the resuspended cell solution. Gently flip the centrifuge tube up and down until the solution is clear and viscous, allowing the bacterial cells to fully lyse.

4. Take 350 μL of solution III, quickly add it to a clear viscous solution, immediately invert it up and down to mix thoroughly, and observe the formation of a large amount of white flocculent precipitate. After centrifuging at 12000 rpm for 10 minutes, use a pipette to aspirate the supernatant and avoid inhaling the precipitate as much as possible. Transfer the supernatant to another clean centrifuge tube.

5. Take the supernatant from the previous step, add 0.35 times the volume of anhydrous ethanol, and mix it evenly.

6. Take an adsorption column, transfer the mixed solution obtained in step 5 to the adsorption column, let it stand for 2 minutes, then put it into a centrifuge, centrifuge at 12000 rpm for 1 minute, discard the liquid obtained by centrifugation in the collection tube, and put the adsorption column back into the recovery collection tube.

7. Take 750 μL of rinsing solution with anhydrous ethanol added in advance, add it to the adsorption column in step 6, centrifuge at 12000 rpm for 1 minute, discard the filtrate in the collection tube, and place the adsorption column back into the recovery collection tube.

8. Take 700 μL of rinsing solution that has been added with anhydrous ethanol again, add it to the adsorption column, centrifuge again at 12000 rpm for 1 minute, discard the liquid in the collection tube, and place the adsorption column again in the recovery collection tube.

9. Place the collection tube into a centrifuge, centrifuge at 12000 rpm for 2 minutes, and then leave the adsorption column open in a 50 ℃ incubator to allow residual alcohol to evaporate as much as possible and remove any residual rinse solution from the adsorption column.

10. Take a clean centrifuge tube that has been sterilized at high temperature and place the adsorption column in the centrifuge tube. Take 50-200 μL of eluent preheated in a 65 ℃ water bath, suspend and drop it onto the central filter membrane of the adsorption membrane, let it stand at room temperature for 2 minutes, and then centrifuge at 12000 rpm for 1 minute.

11. Re suspend and drop the plasmid extract obtained by centrifugation onto the central filter membrane of the adsorption column, let it stand at room temperature for 2 minutes, and centrifuge at 12000 rpm for 1 minute. This step is to increase the recovery rate of plasmids.

2.2.3 CONSTRUCTION OF DUAL ENZYME DIGESTION SYSTEM

In a 200 μL PCR tube, establish an enzyme digestion system with a total volume of 50 μL according to the following components, as shown in Table 2-5:

Table 2-5 Enzyme digestion system construction

Reagent	Volume
Enzyme Digestion Buffer	5 μL
Plasmid	41 μL
Rapid Type Restriction Endonuclease I	2 μL
Rapid Type Restriction Endonuclease Ii	2 μL
Total Volume	50 μL

Mix the above reagents according to the proportions in the table and incubate at 37 ℃ for 30 minutes. The product can be analyzed by agarose gel electrophoresis according to the band size.

2.2.4 DNA AGAROSE GEL ELECTROPHORESIS

1. Gelatin preparation: prepare 0.1% agarose gel. Weigh 0.3 g of agarose on an electronic scale and place it in a 25 mL conical flask. Pour in 30 mL of 1 × TAE buffer,

microwave for about 40 seconds to ensure complete dissolution of agarose, let the solution cool to about 55°C, add 3 μL SuperRed nucleic acid dye with a pipette, gently shake and mix, immediately pour into a 6cm × 6cm gel plate, insert the sampling comb vertically, and ensure that there are no bubbles near the sampling comb. Let it stand and cool to make the gel completely solidify;

2. Sampling: Pull out the sampling comb vertically without damaging the sampling hole. Put the gel into the electrophoresis tank filled with 1 × TAE buffer solution, and ensure that the buffer solution level is higher than the gel surface. Add 6 μL of Marker (5000 bp) to the first sampling well. Mix 5 μL of sample with 1 μL of Loading Buffer and add the remaining sample wells in sequence;

3. Electrophoresis: Set the electrophoresis parameters to a constant voltage of 80 V and electrophorese for about 20 minutes until the bands are completely separated;

4. Observation: Place the gel in the UV gel imager, observe and analyze the developed DNA zone.

2.2.5 RECOVERY OF DNA FROM AGAROSE GELS

1. Use a protective cover to block UV light to avoid burns. Cut agarose gel containing DNA fragments quickly on the UV light source gel imager, as accurate as possible, without redundant edges. Weigh and cut the weight of gel, add Buffer GDP with a volume ratio of 1:3 (1 mg: 300 μL) of sol solution, and take a water bath at 50~55 ℃ for 7-10 minutes. During this period, turn it upside down twice to mix it evenly.

2. Centrifuge briefly for a few seconds to collect the droplets on the centrifuge tube wall at the bottom of the tube. Place the FastPure DNAMini Columns-G adsorption column into a Collection Tubes 2 mL collection tube, transfer the collected sol mixture to the adsorption column, and centrifuge at 12000 rpm for 30-60 seconds.

3. Discard the liquid in the collection tube and place the adsorption column back into the collection tube. Add 300 μL Buffer GDP to the adsorption column. Let it stand for 1 minute. Centrifuge at 12000 rpm for 30-60 seconds.

4. Discard the filtrate from the collection tube and place the adsorption column back into the collection tube. Add 700 μL Buffer GW (with anhydrous ethanol added) along the wall of the adsorption column, mix it upside down, and centrifuge at 12000 rpm for 30-60 seconds.

5. Repeat step 4.

6. Discard the liquid in the collection tube and place the adsorption column back into the recovery collection tube. Centrifuge at 12000 rpm for 2 minutes.

7. Place the adsorption column in a 1.5 mL centrifuge tube sterilized with high-pressure steam, take 20-30 μL Elution Buffer and add it dropwise to the center of the adsorption column. After standing at room temperature for 2 minutes, centrifuge at 12000 rpm for 1 minute. Discard the adsorption column and retain the liquid in the centrifuge tube. The recovery rate of the recovered DNA was verified by agarose gel electrophoresis, and it was frozen at -20 ℃.

2.2.6 PREPARATION OF YEAST COMPETENT CELLS

This experiment was conducted using a yeast receptive state preparation and transformation kit, with slight modifications. The steps are as follows:

1. Re culture the activated yeast strain and collect the bacterial cells when the OD reaches 0.5-0.8. Centrifuge at 4000 rpm for 5 minutes and discard the supernatant.

2. Resuspend the precipitate with 1 mL of Y1 solution, centrifuge at 4000 rpm for 5 minutes, and discard the supernatant.

3. Take 100 μL of Y2 solution, resuspend the precipitate obtained in the previous step, and divide it into 1.5 mL sterile cryovials, with 50 μL per vial. The prepared competent cells can be directly used for transformation.

2.2.7 TRANSFORMATION OF YEAST CELLS

1. Add 276 μL of yeast transformed PEG/LiAC mixture, 50 μL of salmon sperm DNA, 40 μL of recovered plasmid DNA, and 10 μL of sgRNA to the prepared competent cells, and mix well.

Water bath at 2.30 ℃ for 30 minutes.

Heat shock in a 3.42 ℃ water bath for 25 minutes.

4.8000 rpm, Centrifuge for 1 minute and collect yeast cells.

5. Resuspend the collected bacterial cells in 1 mL YPD liquid medium, place them in a shaker at 30 ℃ and 185 rpm, and incubate.

After 1-4 hours of cultivation, wash twice with 1 mL of 0.9% sodium chloride, then add 200 μL of 0.9% sodium chloride and resuspend for plate coating.

Cultivate for 2-4 days and perform colony PCR validation on the growing colonies.

2.2.8 YEAST COLONY PCR VALIDATION

This experiment was conducted using a yeast colony PCR kit with slight modifications. The steps are as follows:

1. Cracking of yeast colonies

a. Transfer the colonies grown on the plate onto another plate and culture for 2-4 days.

b. Add 10 uL Yeast Lysis Buffer to each PCR tube.

c. Use a sterile pipette to pick up 0.2-1 mm yeast clones and transfer them into a PCR tube containing 10 uL Yeast Lysis Buffer. Blow and mix well, centrifuge at low speed for a few seconds to allow the liquid to accumulate at the bottom of the tube. Simultaneously label the colony or inoculate it onto liquid culture medium or a new plate.

d. Heat the PCR instrument at 95 ℃ for 5 minutes to fully release yeast DNA.

e. Add 10 μL of Neutralization Buffer, mix well, and then use it as a DNA template for PCR detection.

2. Setting up yeast colony PCR reaction system

a. Set up the following PCR reaction system on an ice bath (Table 2-6), and the primers used in the experiment are detailed in Appendix A

Table 2-6 PCR Reaction System

Reagent	Volume
Ultrapure Water	7.4 μL
Primer Mix (5μm Each)	1.6 μL
Yeast Colony Pcr Mix (Green, 2X)	10 μL
Total Volume	19 μL

Transfer 1 μL of the prepared DNA template from step l to the prepared 19 μL PCR reaction system, gently blow and mix well, centrifuge at low speed for a few seconds at room temperature to allow the liquid to accumulate at the bottom of the tube. Place the set PCR reaction tube on the PCR machine and start the PCR reaction.

b. Setting PCR reaction parameters

STEP1 (Initial denaturation): 94 ℃ for 3 minutes

STEP2 (denaturation): 94 ℃ 30 seconds

STEP3 (Annealing): 55 ℃ for 30 seconds

STEP4 (Extension): 72 ℃ 1 min/kb

STEP5 (Loop): Go to STEP2 for 30 cycles

STEP6 (Final Extension): 72 ℃ for 10 minutes

STEP7 (temporary storage): 4 ℃

3. Result detection

After PCR, there is no need to add sample buffer. Take 5-10 μL directly for electrophoresis detection.

2.2.9 YEAST CULTIVATION METHODS FOR EXPERIMENTAL USE

WAT11: Inoculate in 5 mL YPD liquid medium and culture on a shaker at 30 ℃ and 250 rpm for 12-16 hours until the logarithmic growth stage (OD_{600}=0.6-0.8). Then, take 200 mL of bacterial solution and transfer it to 5 mL YPD liquid medium for further experimental operation.

BY4741, BY4742: Inoculate in 5 mL YPD liquid medium and culture on a shaker at 30 ℃ and 250 rpm for 12-16 hours until the logarithmic growth stage (OD $_{600}$=0.6-0.8), then transfer 200 mL of bacterial solution to 5 mL YPD liquid medium and culture for 5 hours for further experimental operations.

CEN.PK2-1C: Inoculate in 5 mL YPD liquid medium and culture on a shaker at 30 ℃ and 250 rpm for 12-16 hours until the logarithmic growth stage (OD $_{600}$=0.6-0.8), then transfer 200 mL of bacterial solution to 5 mL YPD liquid medium and culture for 5 hours for further experimental operations.

WAT11-1 (knocking out HMX1): Inoculate in 5 mL SC-URA YPD liquid medium and culture on a shaker at 30 ℃ and 250 rpm for 12-16 hours until logarithmic growth stage (OD $_{600}$=0.6-0.8), then transfer 200 mL of bacterial solution to 5 mL SC-URA YPD liquid medium and culture for 5 hours for further experimental operations.

WAT11-2 (overexpressing HEM4): Inoculate in 5 mL of SC-URA YPD liquid medium and culture on a shaker at 30 °C and 250 rpm for 12-16 hours until the logarithmic growth phase (OD $_{600}$=0.6-0.8), then transfer 200 mL of bacterial solution into 5 mL of SC-URA YPD liquid medium and culture for 5 hours for further experimental operations.

2.2.10 KEY PRODUCT DETECTION METHODS

Determination of Heme by High Performance Liquid Chromatography

a. Instrument parameter settings:

Chromatographic column: C18 reverse phase column (4.6 × 250 mm, 5 μM)

Mobile phase: methanol: acetonitrile: water: acetic acid=(40:40:19:1): water=(90:10) Equimolar elution

Flow rate: 1.0 mL/min

Column temperature: 35 ℃

Detection wavelength: 405 nm (characteristic absorption peak of hemoglobin)

Injection volume: 10 μL

Duration: 20 minutes

Determination of biliverdin by high-performance liquid chromatography

a. Instrument parameter settings:

Chromatographic column: C18 reverse phase column (4.6 × 250 mm, 5 μM)

Mobile phase: methanol: acetonitrile: water: acetic acid=(40:40:19:1): water=(90:10) Equimolar elution

Flow rate: 1.0 mL/min

Column temperature: 35 ℃

Detection wavelength: 370 nm (characteristic absorption peak of biliverdin)

Injection volume: 10 μL

Duration: 20 minutes

3. Determination of bilirubin by high-performance liquid chromatography

a. Instrument parameter settings:

Chromatographic column: C18 reverse phase column (4.6 × 250 mm, 5 μM)

Mobile phase: 1% glacial acetic acid aqueous solution: acetonitrile=(5:95) isocratic elution

Flow rate: 1.0 mL/min

Column temperature: 30 ℃

Detection wavelength: 450 nm (characteristic absorption peak of biliverdin)

Injection volume: 10 μL

Duration: 20 minutes

Conclusions to chapter II

1. Experimental System Construction

Comprehensive listing of yeast strains (WAT11, BY4741, etc.) and plasmids (e.g., pUC19-*HMX1*, pESC-LEU-*AtHO*-*RnBVR*), with detailed genotypes and functional annotations (Tables 2-1 to 2-4).

Complete inventory of reagents and equipment, covering critical steps such as medium preparation, plasmid extraction, and PCR validation.

2.Core Methodologies

CRISPR-Cas9 genome editing combined with the HACking system enabled targeted knockout of endogenous HMX1 and overexpression of HEM4, generating recombinant strains WAT11-1 and WAT11-2.

Precision validation via double restriction enzyme digestion, plasmid transformation, yeast competent cell preparation (Section 2.2.7), and colony PCR screening (Section 2.2.8).

HPLC-based quantification of heme, biliverdin, and bilirubin levels (Section 2.2.10), establishing robust analytical protocols.

3.Medium Optimization and Cultivation

Customized selective media (e.g., SC-URA, YPD) tailored for strains with distinct genetic backgrounds (Table 2-4).

Defined protocols for strain activation, amplification, and induction (e.g., 30°C, 250 rpm shaking culture).

CHAPTER III
EXPERIMENTAL PART

3.1 PREFACE

In the 1970s, scientists discovered that timely diagnosis and monitoring of neonatal jaundice were crucial for preventing serious complications[36], and neonatal jaundice was directly related to abnormal bilirubin metabolism. This seemingly single metabolic pathway has become the key to unlocking the mysteries of life. As the "terminal station" of heme decomposition, the metabolic mechanism of bilirubin has long been focused on liver detoxification and pathological monitoring. However, the development of modern biomedical science has made us realize that the starting point of this metabolic chain - the regulation of heme synthesis - is the central switch that truly affects the life system.

Heme is a porphyrin derivative composed of Fe^{2+} and protoporphyrin[37]. Heme is not only the core of hemoglobin, but also a key cofactor for over 400 proteins such as cytochromes and catalase. It is an essential component of hemoglobin found in almost all prokaryotic and eukaryotic cells, playing an important role in oxygen transport, reactive oxygen species elimination, and electron transfer[38]. Traditional research is mostly limited to pathological phenomena caused by heme decomposition, such as skin jaundice caused by bilirubin accumulation, but neglects the precise regulatory network of the synthesis process. The intervention of synthetic biology has enabled scientists to reverse analyze the heme synthesis network like dismantling a precision clock, and unexpectedly discovered that there is a "molecular buffer pool" hidden in its metabolic pathway for cells to cope with oxidative stress: when the activity of ferrochelase is inhibited, free porphyrin precursors can spontaneously clear free radicals. This evolutionarily preserved emergency mechanism provides a new target for the development of anti-aging therapies. Even more revolutionary is that by reconstructing the heme synthesis module of blue-green algae, researchers have successfully cultivated engineering strains that can directly synthesize human hemoglobin using carbon dioxide. This technological breakthrough not only frees the industrial production of blood products from dependence on biological donors, but also suggests that in the future,

"living materials" with autonomous oxygen supply function may be designed for building artificial ecosystems in deep space exploration. From decoding metabolic codes to rewriting life programs, heme synthesis biology is blurring the boundary between natural evolution and artificial design. This chapter will conduct a systematic analysis of the experimental results related to heme synthesis biology, verify the functions of key enzymes, explore the effects of different expression systems and gene editing strategies on bilirubin synthesis, and provide theoretical basis for constructing an efficient bilirubin synthesis system.

3.2 FUNCTIONAL VALIDATION OF HEME OXIDASE AND BILIVERDIN REDUCTASE

The key enzymes for catalyzing the synthesis of bilirubin using heme as a substrate are heme oxidase and biliverdin reductase, which are widely distributed in nature. To verify the catalytic activity of heme oxidase (encoding gene *AtHO*) derived from Arabidopsis *thaliana* and biliverdin reductase (encoding gene RnBVR) derived from rat liver, relevant coding sequences were synthesized (see Appendix B.1 and B.2), and expression vectors pETDuet-1-*AtHO* and pETDuet-1-AtHO-RnBVR were constructed (Figures 3.1-3.2). After converting BL21 (DE3), engineered bacteria were obtained for whole cell catalysis.

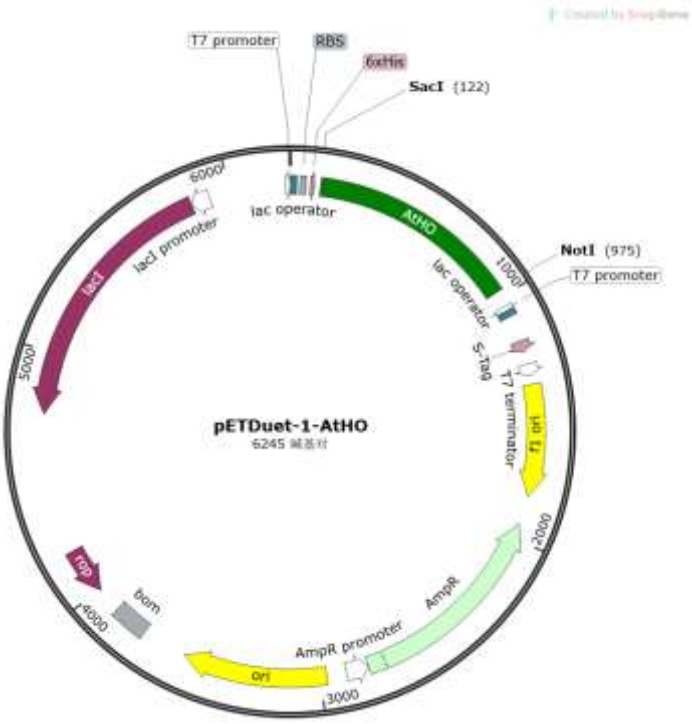

Figure 3.1 – Schematic diagram of the main components of pETDuet-1-*AtHO* expression vector

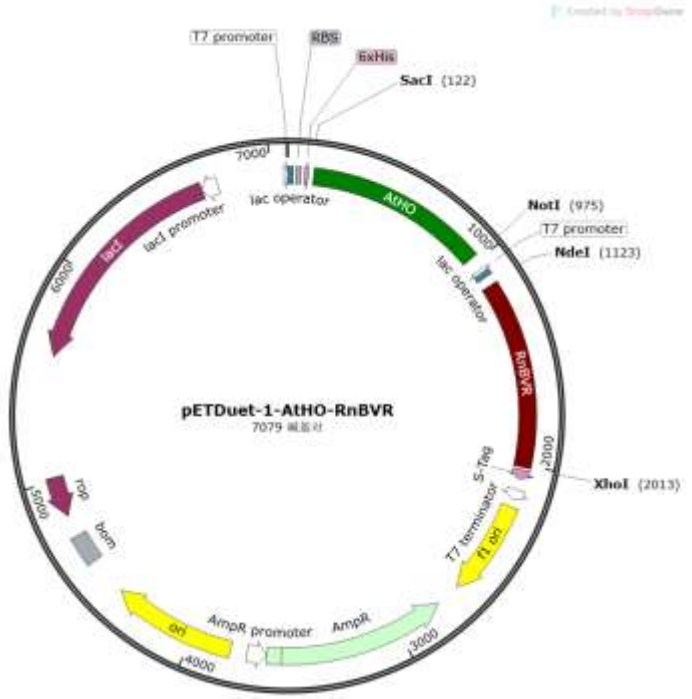

Figure 3.2 – Schematic diagram of the main components of pETDuet-1-*AtHO-RnBVR* expression vector

Further utilizing the whole cell catalytic system, the recombinant bacteria were incubated with heme substrate at 37 ℃ and 250 rpm. Through HPLC detection (Figure 3.3-3.4), it was found that characteristic peaks of biliverdin and bilirubin appeared in the system after 3 hours of reaction. The control group (without inducing recombinant bacteria) did not detect the target product, confirming that HO can catalyze the generation of biliverdin from hemoglobin, and BVR can further reduce biliverdin to bilirubin. The results showed that, similar to the heme oxidase and biliverdin reductase reported in the literature from *Thermosynechococcus elongatus* and *Synechocystis sp*, *AtHO* and *RnBVR* can effectively synthesize bilirubin through sequential catalysis using heme as a substrate.

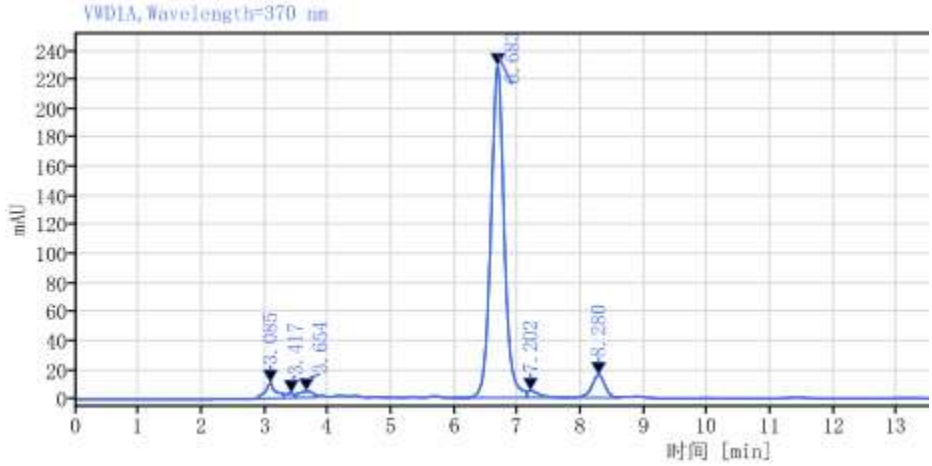

Figure 3.3 – Detection of biliverdin using HPLC

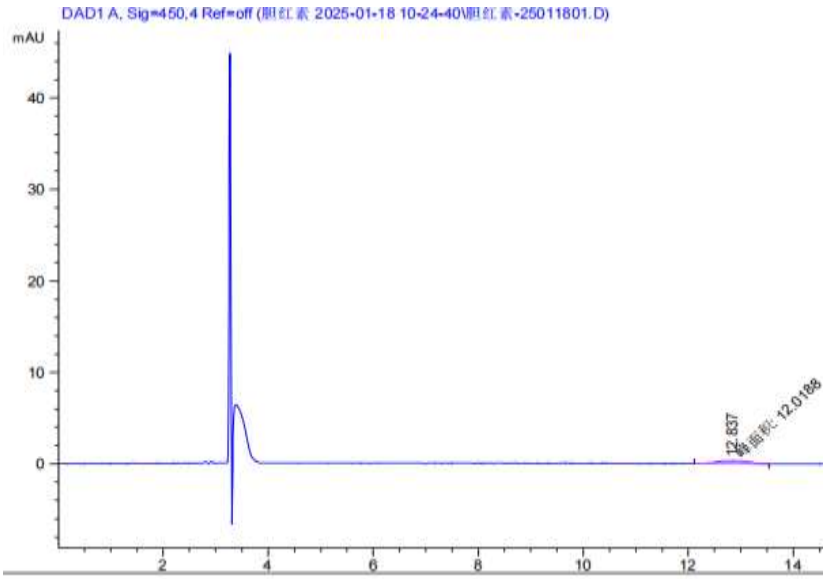

Figure 3.4 – Detection of bilirubin using HPLC

Considering the biological basis of heme absorption and synthesis in brewing yeast, we plan to use brewing yeast as the chassis cell, integrate *AtHO* and *RnBVR* genes, and investigate bilirubin synthesis.

3.3 RECOMBINANT EXPRESSION OF HEME OXIDASE AND BILIVERDIN REDUCTASE IN BREWING YEAST

Considering the low natural supply of heme in brewing yeast cells, we increased the supply of precursor heme by overexpressing *hrg-4* 38.LI Y T, ZENG W Z, TANG L. Fermentation optimization for enhancing heme synthesis in Pichia pastoris[J]. Food and Fermentation Industries, 2024, 50(22): 60-66. DOI: 10.13995/j.

39.Abbhirami R, UAR, Julio A, et al.Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins.[J].Nature,2008,453(7198):1127-31.. Construct pESC-URA-*hrg*-4 vector (Figure 3.5). Transform hosts WAT11, CEN.PK2-1C, and BY4741 separately. According to Method 1 of 2.2.10, the intracellular hemoglobin content was measured to be 6.7 ± 0.6 mg/L, 4.8 ± 0.5 mg/L, and 3.4 ± 0.8 mg/L, respectively, all of which showed significant increases compared to the blank control, indicating that *hrg-4* was functionally expressed in different hosts.

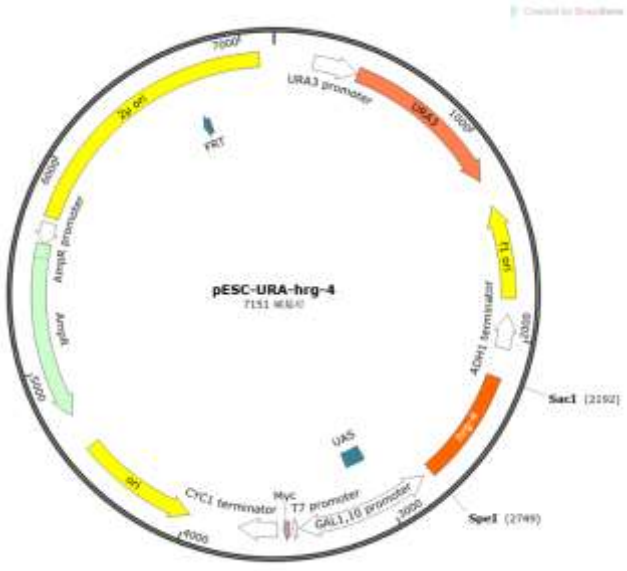

Figure 3.5 – Schematic diagram of the main components of pESC-URA-*hrg*-4 expression vector

Based on pESC-LEU, expression vectors pESC-LEU-*AtHO-RnBVR* encoding AtHO and RnBVR genes were constructed (Figure 3.6), and transformed into brewing

yeast strains WAT11 (pESC-URA-*hrg*-4), CEN.PK2-1C (pESC URA-*hrg-4*), and BY4741 (pESC-URA-hrg-4), respectively. After 48 hours of night SC Ura Leu culture and induction culture, the intracellular bilirubin content was quantitatively analyzed by HPLC. The bilirubin accumulation of WAT11 engineering bacteria (pESC-URA-*hrg-4*, pESC-LEU-AtHO *RnBVR*, later the same) reached 32.6 ± 1.8 mg/L. Correspondingly, the accumulation of bilirubin in CEN.PK2-1C (pESC-LEU-*AtHO-RnBVR*) and BY4741 (pESC-LEU-*AtHO-RnBVR*) hosts reached 15.7 ± 1.2 mg/L and 8.3 ± 0.9 mg/L, respectively. The bilirubin accumulation of strain WAT11 is 2.1 times and 3.9 times higher than that of CEN.PK2-1C and BY4741, respectively. This result indicates that the genetic background of the host strain significantly affects bilirubin synthesis ability in the basic culture medium, and the WAT11 strain can be used as the preferred host for subsequent research. At present, the use of free plasmids without screening markers for the expression of recombinant proteins in lower cost complete culture media still poses problems such as strain growth being affected and low expression levels. Considering the issues of large-scale production and bilirubin synthesis after adaptation, as well as multi gene control and influence in the host's metabolic network, it is currently a better choice to construct strains with better genetic stability by integrating and editing relevant genes at the genome level. Therefore, it is necessary for us to carry out genome editing work on brewing yeast, in order to reshape certain metabolic characteristics of the host based on the integration of *AtHO* and *RnBVR* encoding genes.

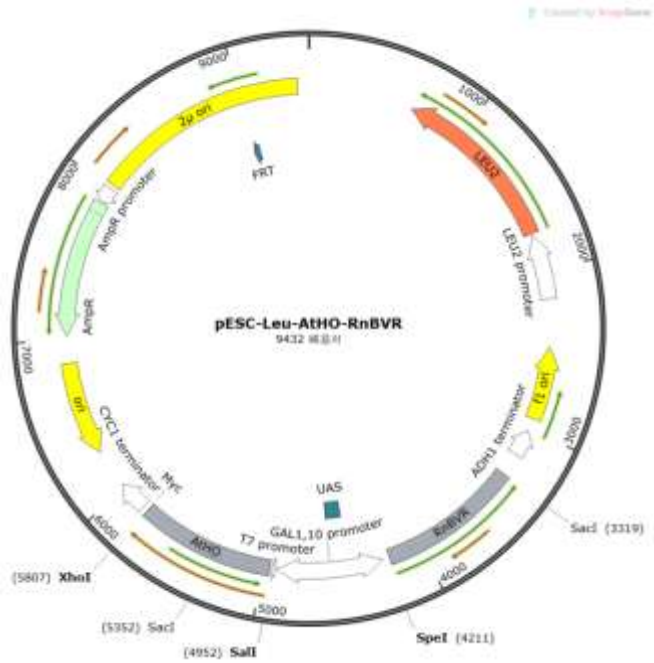

Figure 3.6 – Schematic diagram of the main components of pESC-LEU-*AtHO-RNBVR* expression vector

3.4 CONSTRUCTION OF BILIRUBIN SYNTHESIS PATHWAY THROUGH GENOME EDITING OF BREWING YEAST

Through literature analysis and data retrieval, a comprehensive analysis of the heme metabolism pathway in brewing yeast was conducted, and multiple potential regulatory targets were screened. Taking into account gene expression levels, metabolic flux, and functional redundancy, the gene loci containing *HMX1*, *HXT12*, *TDH3*, and other genes were ultimately selected as editing targets.

Here, the genome editing work is carried out using the constitutive expression Cas9 protein and the Sc-Cas9-gRNA-Ura vector, which can insert DNA encoding different gRNAs.

Based on the selected target, a series of gRNAs were obtained through online design evaluation (and) (Table 3-1). Based on the integration of genes and editing site upstream and downstream sequences, cloning vectors such as pUC19-*HMX1*, pUC19-*HEM4*, and *pUC57-HEM1* were designed and constructed (Figure 3.7-3.9).

Table 3-1 Editing Targets and gRNAs

gRNAs	Edit target	Sequence
sgRNA-HMX1	*HMX1*	CGAATTTGTACAGAAGACTG
sgRNA-*HXT12*	*HXT12*	CAGCTGGTACAATTGGACCA
sgRNA-HEM4	*TDH3*	AAGTAAATTCACTTAAGCCT

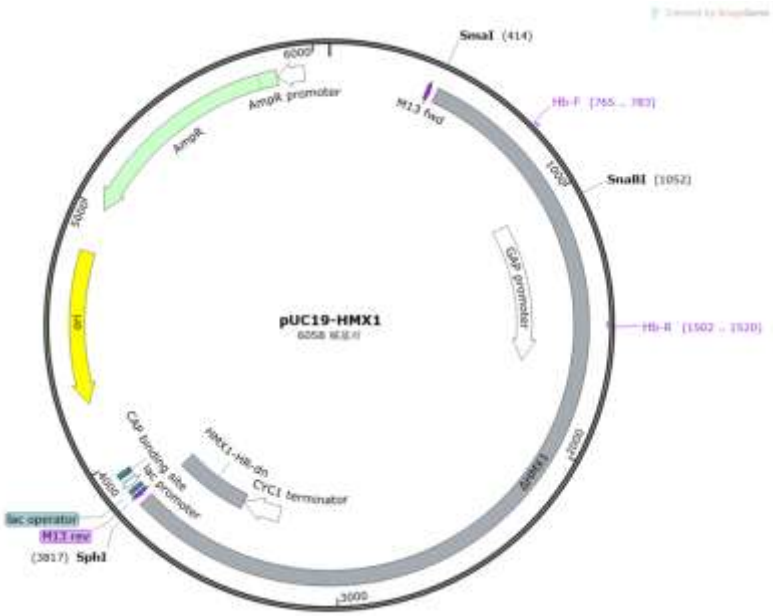

Figure 3.7 – Schematic diagram of the main components of pUC19-*HMX1* expression vector

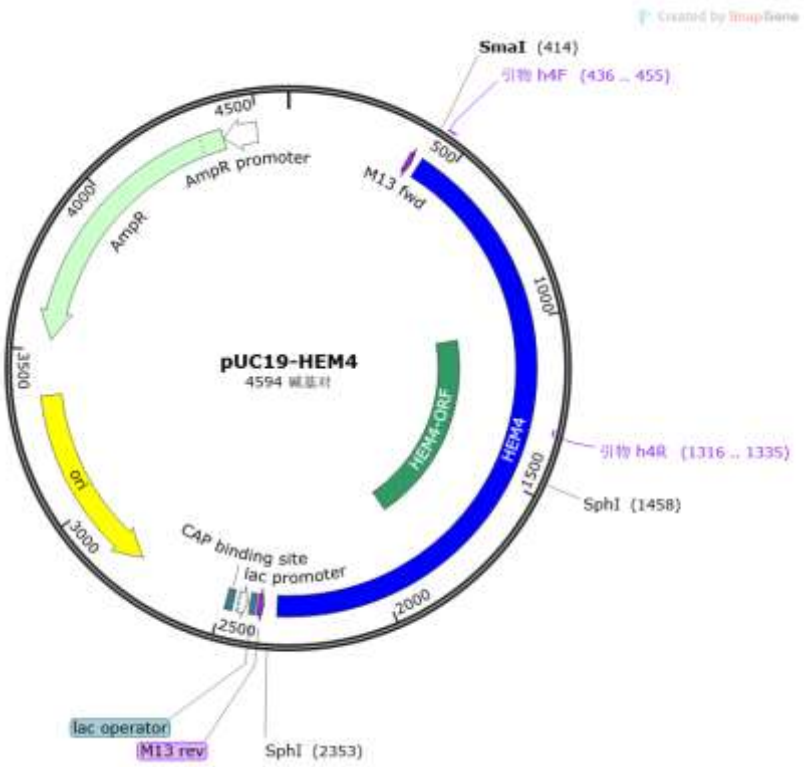

Figure 3.8 – Schematic diagram of the main components of pUC19-*HEM4* expression vector

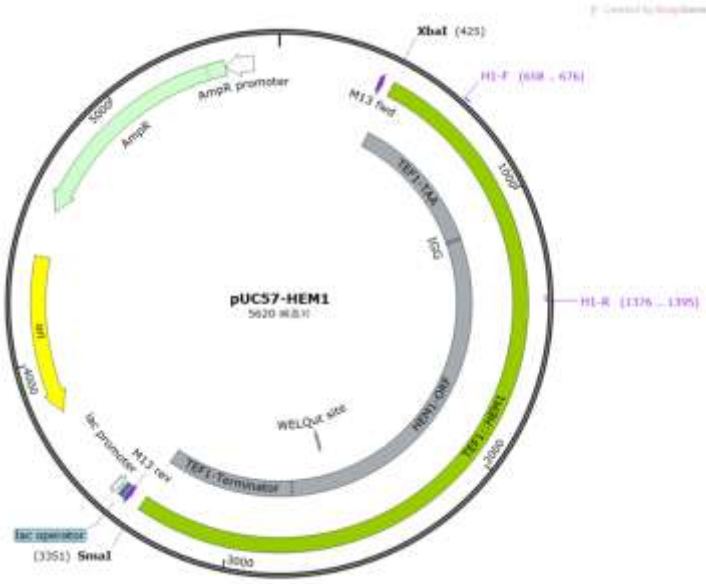

Figure 3.9 – Schematic diagram of the main components of pUC19-*HEM1* expression vector

ΔHMX:AtHO-RNBVR genome editing. PUC19-*HMX1* was digested with *SmaI*/*SphI* double enzymes, and the fragments were recovered and co transformed with Sc-Cas9-*HMX1* into WAT11 host. After colony PCR validation (Figure 3.10) and

sequencing (Figure 3.11), it was confirmed to be consistent with the design. BLAST detection did not detect any mutations. The partial DNA sequence of the *HMX1* locus in the positive clone was successfully replaced by the target DNA sequence, and the resulting gene edited strain was named WAT11-1. According to HPLC detection, the bilirubin production was 5.2 ± 0.3 mg/L. The results indicate that genome editing technology can reconstruct the bilirubin synthesis pathway in yeast. Although the *HMX1* gene related to heme oxidation has been deleted, the supply of precursor heme remains the main limiting factor. To this end, we further strengthen the endogenous pathway of heme.

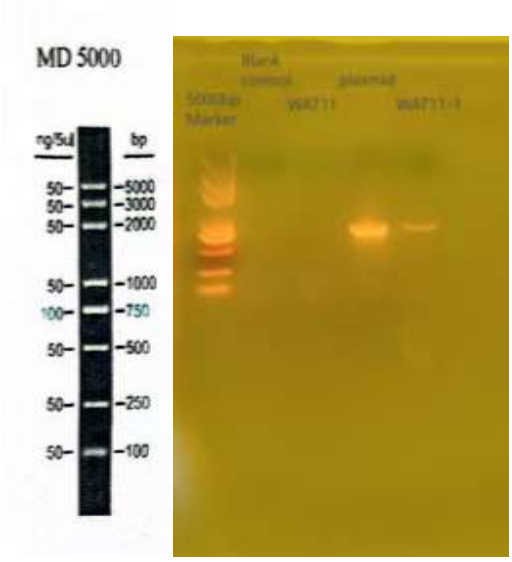

Figure 3.10 – Colony PCR Validation of WAT11-1

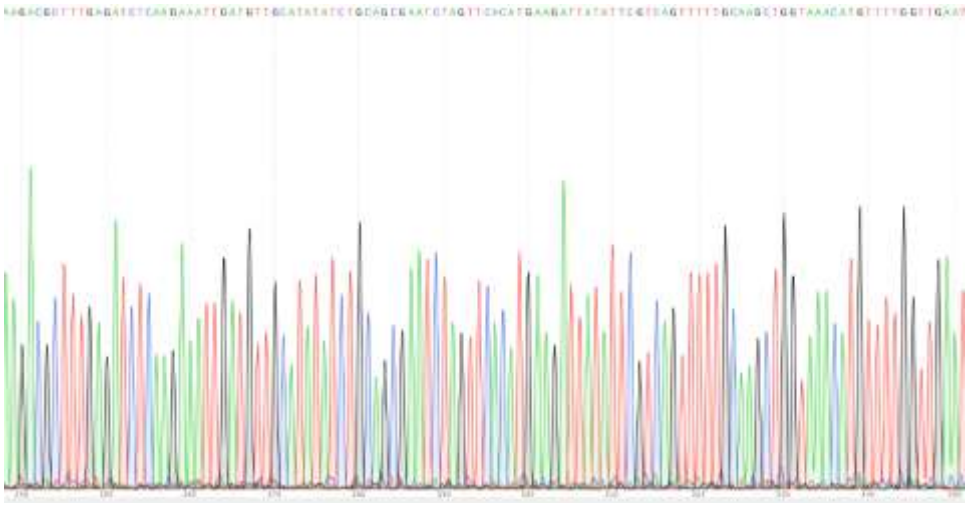

Figure 3.11 – Sequencing of WAT11-1

TDH3-HEM4 genome editing. The pUC19-*HEM4* fragment was recovered by *SmaI*/*XbaI* double enzyme digestion and co transformed with Sc-Cas9-*HEM4* into WAT11-1 host. After colony PCR validation (Figure 3.12) and sequencing (Figure 3.13), it was confirmed to be consistent with the design. BLAST detection did not detect any mutations. The *TDH3* gene of the positive clone was correctly fused with the *HEM4* encoding DNA sequence after opening the reading frame TAA, and the resulting gene edited strain was named WAT11-2. According to HPLC detection, the bilirubin production was 8.8 ± 0.5 mg/L. The results showed *that* the *TDH3* driven *HEM4* co expression mediated by the interval sequence significantly increased the accumulation of bilirubin. Compared with the construction of WAT11 (pESC-URA-*hrg*-4, pESC-LEU-*AtHO-RnBVR*) in 3.2, the accumulation of the target product was significantly reduced without obtaining the differentiated diffusion protein Hrg-4p.

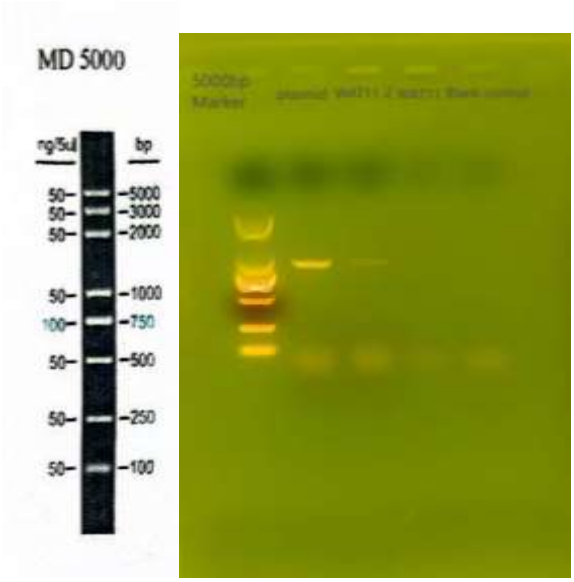

Figure 3.12 – Colony PCR Validation of WAT11-2

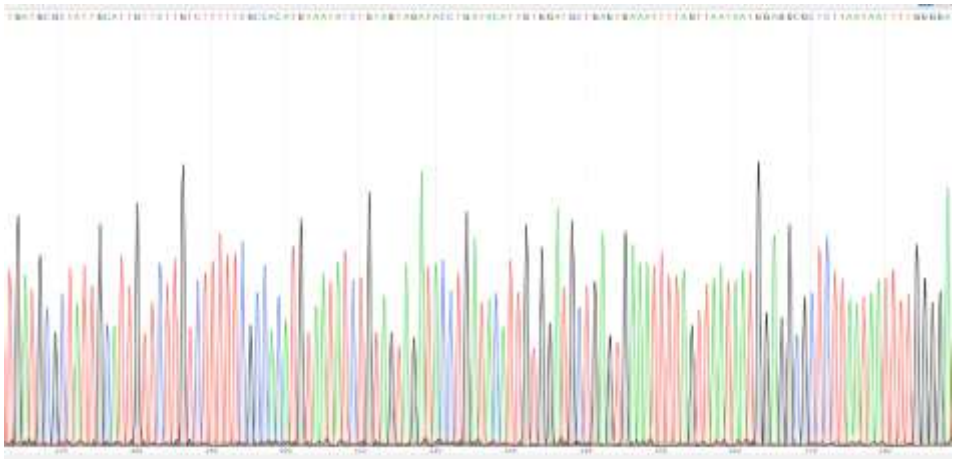

Figure 3.13 – WAT11-2 sequencing

3.5 EFFECT OF HEME FACILITATING DIFFUSION PROTEIN ON BILIRUBIN ACCUMULATION

Transform the pESC-*URA*-*hrg*-4 expression vector constructed in 3.2 into the brewing yeast strain WAT11-2. According to HPLC analysis, the bilirubin accumulation of the strain was 21.3 ± 2.1 mg/L, which is about four times that of the WAT11-2 strain, but still significantly lower than the bilirubin accumulation of WAT11 (pESC URA-hrg-4, pESC-LEU-*AtHO*-*RnBVR*). The results indicate that the location and mode of genome integration, as well as copy number, may affect the product accumulation of the final engineered bacteria. At the same time, it indicates that although overexpression of this protein significantly increases the accumulation of products to a certain extent, Hrg-4p protein belongs to the category of alienated diffusion proteins, is non ATP dependent, and has low efficiency in transporting precursor heme. Relying solely on exogenous heme intracellular transport is difficult to meet the needs of large-scale production. In the future, de novo synthesis pathway optimization or multi gene synergistic regulation strategies need to be combined to further improve bilirubin synthesis efficiency; Meanwhile, through genome editing such as deletion of *HMX1* and overexpression of *HEM4*, we obtained engineered bacteria capable of de novo synthesis and accumulation of bilirubin, but their yield was relatively low. To achieve multi gene metabolic network reshaping breeding in the future, a high-throughput screening platform is needed, and constructing an intracellular bilirubin biosensor is a good solution to this problem.

3.6 CONSTRUCTION OF BILIRUBIN BIOSENSOR

Unlike transcription regulatory systems such as TetR or AraC, a protein was isolated from Japanese eel, UnaG, When combined with bilirubin, it produces fluorescence[40-42]

Based on this, a pESC-LEU-*UnaG* expression vector was constructed (Figure 3.14), bilirubin was detected, and the vector was transformed into brewing yeast strains WAT11-1 and WAT11-2 to detect their green fluorescence expression (Figure 3.15).

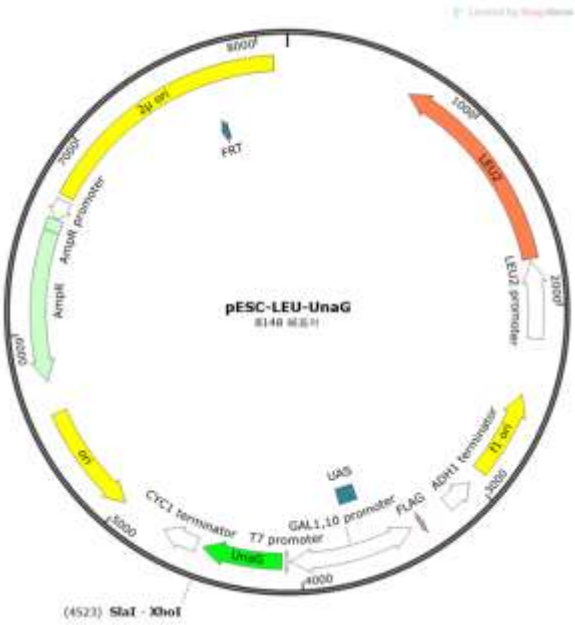

Figure 3.14 – Schematic diagram of the main components of pESC-LEU-*UnaG* expression vector

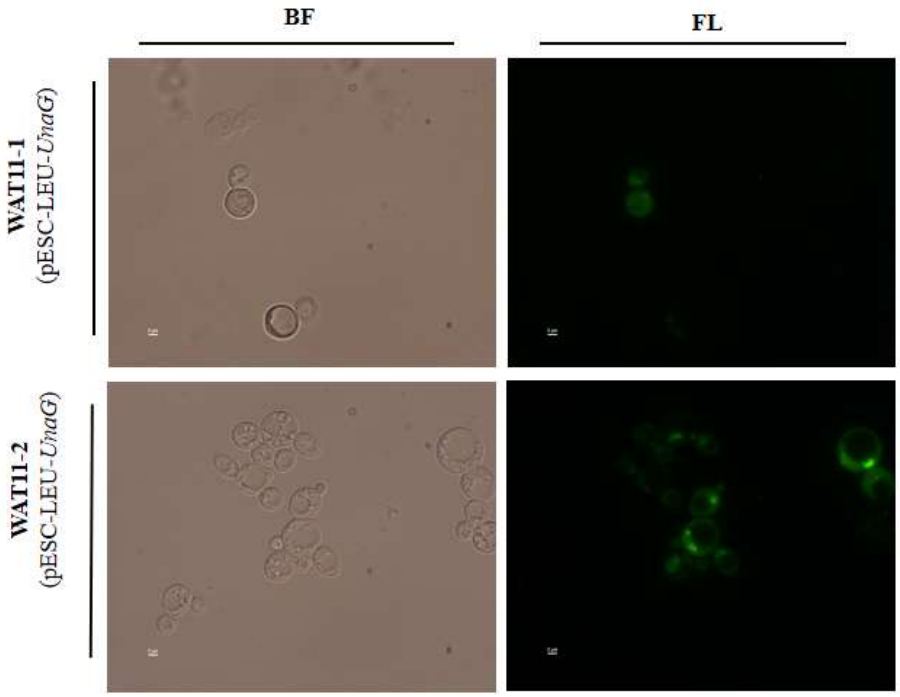

Figure 3.15 – Fluorescence expression in brewing yeast

3.7 GROWTH CHARACTERISTICS OF BILIRUBIN SYNTHESIS BREWING YEAST ENGINEERING STRAIN

The growth curves of wild-type and engineered strains were determined by shake flask fermentation (Figure 3.16), and the results showed that the wild-type strain entered the logarithmic growth phase after 12 hours of cultivation, reaching the maximum OD value (8.2 ± 0.3) at 24 hours; The bilirubin synthesis engineering bacterium WAT11-1 reached its maximum OD value after about 40 hours and the maximum OD value decreased to 4.3 ± 0.2; WAT11-2 reached its maximum OD value in approximately 36 hours and the maximum OD value decreased to 5.8 ± 0.2.

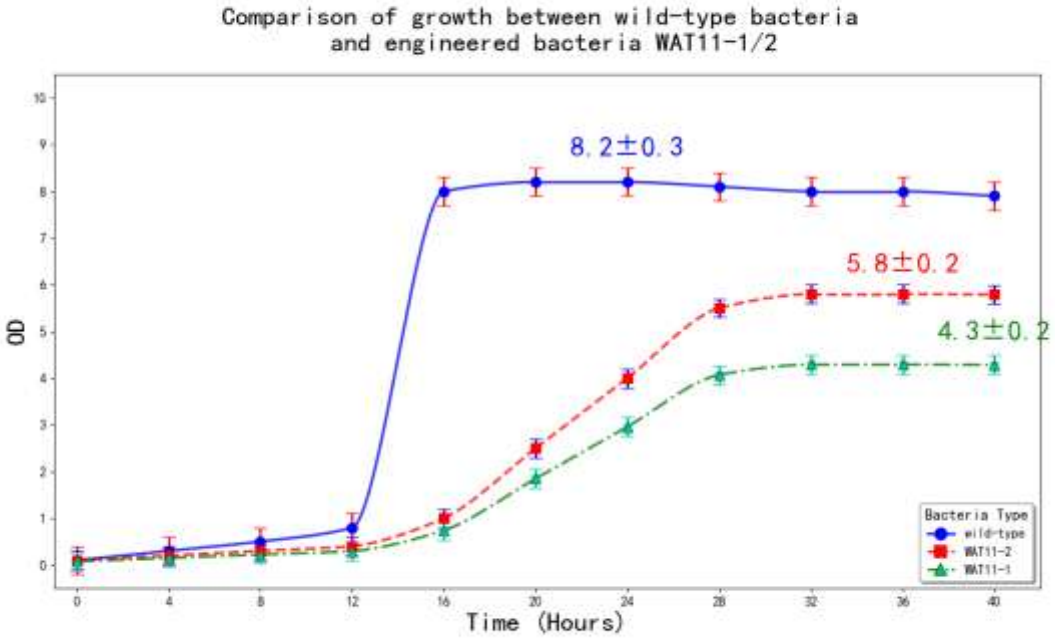

Figure 3.16 – Growth curves of wild-type and engineered bacteria measured by shake flask fermentation

Similarly, Dilution analysis (Figure 3.17) showed that the growth of the engineered bacteria was significantly inhibited compared to the wild type. At the same time, the inhibition of WAT11-2 was weakened compared to WAT11-1, which may be related to overexpression of heme synthase HEM4. That is, the enhancement of heme biosynthesis to some extent strengthened the respiratory energy metabolism process.

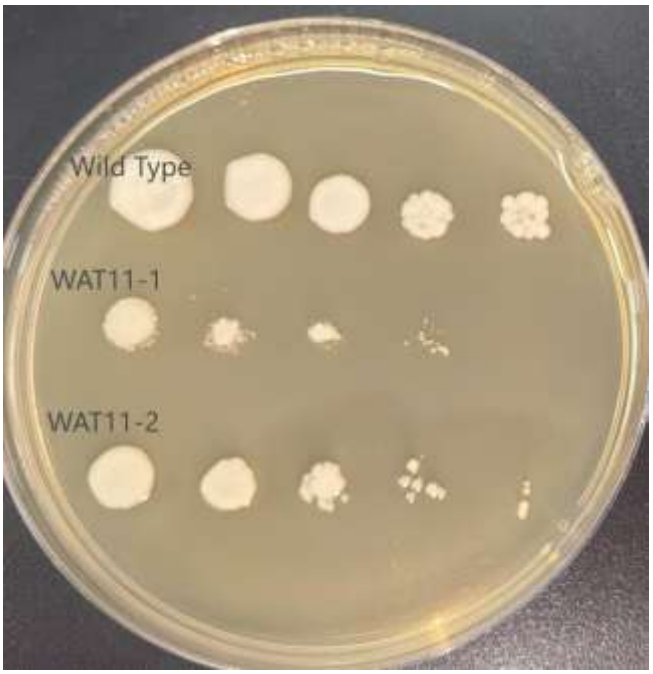

Figure 3.17 –Dilution Analysis Diagram

It is speculated that the reasons for growth inhibition may be: (1) Heme synthesis and bilirubin metabolism cause cells to consume a large amount of precursor substances and energy; (2) The accumulated bilirubin inside the cell can have toxic effects on the cell membrane or organelles. Therefore, in the future, it is necessary to use metabolic flow optimization, detoxification strategies (building efflux systems) and other means to lift growth restrictions, achieve a balance between yield and cell activity, and lay the foundation for industrial fermentation.

Conclusions to chapter III

1.Key Enzyme Functional Validation

Successful confirmation of sequential catalysis by AtHO and RnBVR. HPLC analysis revealed bilirubin accumulation up to 32.6 mg/L in the WAT11 host.

2. Metabolic Pathway Remodeling via Genome Editing

HMX1 knockout blocked competing heme degradation, while *TDH3*-driven *HEM4* overexpression enhanced heme supply, elevating bilirubin production to 8.8 mg/L in WAT11-2.

Sequencing and PCR confirmed precise genomic integration in edited strains (Figures 3.10–3.13).

3.Biosensor Implementation and Physiological Profiling

Construction of a UnaG-based bilirubin biosensor enabled real-time fluorescence monitoring of intracellular metabolites (Figure 3.15).

Growth curve analysis indicated metabolic burden: WAT11-2 showed reduced maximum OD (5.8 vs. 8.2 in wild type), mitigated partially by enhanced heme synthesis (Figure 3.16).

4.Critical Limitations and Insights

Heterologous heme transporter Hrg-4p improved bilirubin yield (21.3 mg/L), yet underscored the necessity of optimizing endogenous de novo synthesis pathways.

Physiological stress (e.g., growth retardation) highlights future directions for metabolic flux balancing and detoxification strategies.

CONCLUSION

1. The co expression of AtHO-RnBVR mediated by pESC-LEU vector leads to more effective bilirubin accumulation in WAT11 compared to BY4741 and CEN.PK2-1C;

2.CRISPR-Cas9 genome editing combined with interval sequence bicistronic expression technology, using WAT11 as the chassis cell, deleted endogenous HMX1 while overexpressing AtHO, RnBVR, and HEM4 genes;

3. Heme substrate differentiation diffusion protein cannot maintain high levels of bilirubin accumulation, and de novo synthesis is a better choice;

4. The successful construction of bilirubin biosensors provides a research foundation for irrational breeding and reshaping the metabolic network related to chassis cells;

5. Heterologous expression of the bilirubin synthesis pathway leads to significant physiological stress in the host of WAT11.

The above conclusion will lay the foundation for in-depth research on the synthetic biology of bilirubin.

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