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 bilirubin in *Escherichia coli***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
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_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Wang Xiaoyuan

1. Thesis topic **Synthesis of bilirubin in *Escherichia coli***

Scientific supervisor Dr. Sc., Prof. Tetiana SHCHERBATIUK

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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

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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	
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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	
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Student _____ Wang Xiaoyuan

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ABSTRACT

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Bilirubin is a bilirubin produced by the metabolic decomposition of heme. It is widely present in animals, especially in the liver, where it participates in the metabolism of bilirubin. It has various pharmacological activities and is also one of the main raw materials for preparing artificial bezoar. Bilirubin has significant application value in fields such as medicine, food and cosmetics.

Traditionally, bilirubin has mainly been extracted from animal tissues, but this method is costly, has a low yield and poses ethical issues. This article intends to utilize biocatalysis technology to catalyze the synthesis of bilirubin in the whole cells of *Escherichia coli* as the host. First of all, Construct several heme oxygenases (AtHO, TeHO) and biliverdin reductases from different sources The overexpression vectors pETDuet-1-*AtHO*、pETDuet-1-*TeHO*、pETDuet-1-*AtHO-RnBvR* and pETDuet-1-*TeHO-SsBvR* (RnBvR, SsRnBvR) *Escherichia coli* BL21 (DE3) and Nissle 1917 were transformed respectively. Later, whole-cell catalysis showed that in the case of adding heme co-solvents such as DMF, Nissle 1917 (pETDuet-1-*AtHO-RnBvR*) could more effectively sequentially catalyze the synthesis of bilirubin from substrate heme. It reflects the influence of different backgrounds of the host metabolic network on product synthesis. Further, the Nissle 1917 heme pump coding gene was cloned into the pRSFDuet-1 vector. After co-conversion with pETDuet-1-*AtHO-RnBvR* for BL21 (DE3), continuous whole-cell catalysis was carried out in a pH 8.0 0.2 mM Tris-HCl buffer for 20 hours. The amount of bilirubin synthesized from the substrate heme

conversion was 35% higher than that of Nissle 1917 (pETDuet-1-*AtHO-RnBvR*), reaching 21 mg/mL.

This study provides basic data for reference for the biosynthesis of bilirubin, especially for the subsequent development of synthetic biology techniques.

Key words *：Biosynthesis, Bilirubin, Rutin, Escherichia coli*

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INTRODUCTION

Bilirubin is a bile pigment with significant pharmacological activity and is widely used in medicine (such as artificial cow bile), food, and cosmetics. Traditional methods relying on the extraction of animal tissues have issues such as high cost, low yield, and ethical concerns. Therefore, developing efficient and sustainable biosynthetic methods is of great significance. This study utilized whole-cell biocatalysis with *Escherichia coli* to synthesize bilirubin, providing a new approach for industrial production.

This study developed a biosynthetic method based on whole-cell catalysis of *Escherichia coli* to address the high cost, low yield and ethical issues associated with traditional animal tissue extraction of bilirubin. By constructing expression vectors of heme oxygenase (AtHO, TeHO) and biliverdin reductase (RnBvR, SsBvR) from different sources and comparing the catalytic efficiency of BL21(DE3) and Nissle 1917 hosts, it was found that Nissle 1917 (pETDuet-1-*AtHO-RnBvR*) could efficiently catalyze the conversion of heme to bilirubin under the condition of adding DMF as a cosolvent. In further optimization, the heme pump gene of Nissle 1917 was introduced into BL21(DE3), increasing the bilirubin yield by 35% to 21 mg/mL. This study provides an efficient strategy for the biosynthesis of bilirubin and lays an important foundation for the subsequent development of synthetic biology technologies.

CHAPTER I

LITERATURE REVIEW

1.1 THE MAIN SYNTHETIC PATHWAYS AND FUNCTIONS OF BILIRUBIN

Bilirubin is an important tetrapyrrole compound. As the final product of heme metabolism, it is widely present in nature, especially participating in the metabolism of bilirubin in the liver. It has various pharmacological activities and is also one of the main raw materials for preparing artificial bezoar [1-3]. This orange-red pigment is mainly produced in the human body through the decomposition of aging red blood cells, with a daily production amount of approximately 300 mg. From a biochemical perspective, the molecular structure of bilirubin endows it with unique physiological activities and pharmacological properties, making it of significant value in the field of medicine[3]. Traditional Chinese medicine has long recognized the medicinal value of bilirubin and regards it as the main component of artificial bezoar, mainly used for treatments such as clearing heat and detoxifying, calming the mind and resolving phlegm [4].

Bilirubin is an important product in human metabolism, mainly generated by the decomposition of hemoglobin in red blood cells [5]. The synthesis process begins with the destruction of red blood cells. Hemoglobin is decomposed into heme and globulin in macrophages. Among them, globulin is hydrolyzed into amino acids, and heme is decomposed into bilirubin, carbon monoxide and iron ions through the action of heme oxygenase [6]. Bilirubin is reduced to bilirubin by bilirubin reductase. At this point, the bilirubin is unbound and lipid-soluble, and cannot be directly dissolved in water. Unconjugated bilirubin is transported to the liver through the blood. In the liver, bilirubin combines with glucuronic acid to form water-soluble conjugated bilirubin, which is then secreted into the intestine along with bile. In the intestine, bilirubin is reduced to urobilinogen by bacteria. Some of the urobilinogen is reabsorbed into the

blood and eventually excreted as urobilin through the kidneys, while the other part is excreted as fecalbilin through the intestine, making the stool brown.

In terms of industrial production, the main source of bilirubin has long relied on the extraction method from animal tissues. This method is usually used to separate and purify bilirubin from bovine bile. It is not only complex in process and high in cost, but also has limited output. According to statistics, only about 1 kg of bilirubin can be extracted from 1,000 kg of bovine bile. More importantly, with the enhancement of animal protection awareness and the change of livestock production models, this traditional production method is facing increasingly severe ethical challenges and the problem of unstable raw material supply. In addition, the organic solvents used in the extraction process also exert certain pressure on the environment. Biliverdin is the precursor of bilirubin, which is more abundant and cheaper than bilirubin, but it is not used in traditional Chinese medicine. Therefore, the biotransformation of biliverdin using biliverdin reductase (BvR) may be a practical method for the production of bilirubin [7].

Bilirubin is not only a product of hemoglobin metabolism in the body, but also has important physiological functions. As an antioxidant, bilirubin can eliminate free radicals in the body, slow down oxidative damage, and protect important molecules such as cell membranes and DNA, thereby playing a certain anti-aging role in the normal functions of cells and the aging process. In addition, the role of bilirubin in the immune system has also drawn attention. Its antioxidant properties help regulate the activity of immune cells and reduce inflammatory responses. Studies have shown that people with lower bilirubin levels are more prone to inflammatory diseases, which further demonstrates its important role in immune regulation. The antioxidant function and immunomodulatory effect of bilirubin can not only protect the body from environmental and endogenous factors, but also may play a positive role in the prevention and treatment of chronic diseases [5].

In conclusion, bilirubin is a product of the decomposition of hemoglobin and has important physiological functions, such as antioxidant effects, immunomodulatory effects, and a key role in liver metabolism. It is not only a biomarker reflecting liver

health, but also crucial for the normal operation of the nervous system and the immune system. Clinically, the detection of bilirubin is often used for the diagnosis of jaundice, liver diseases and other metabolic disorders, and helps doctors assess the severity of the condition. Therefore, an in-depth understanding of the synthetic pathway and physiological functions of bilirubin is of great significance for both basic medical research and clinical diagnosis.

1.2 RESEARCH STATUS OF BILIRUBIN SYNTHESIS AT HOME AND ABROAD

The synthetic pathway and metabolic mechanism of bilirubin have been studied in depth in mammals, especially the functions and action mechanisms of metabolic enzymes of bilirubin (such as heme oxidase, bilivermin reductase, etc.). In recent years, with the development of metabolic engineering technology, the focus has gradually shifted to the synthesis of bilirubin in microorganisms.

At present, some domestic and foreign research reports have shown that *Escherichia coli* can synthesize bilirubin by modifying its metabolic pathway. A foreign research team introduced the mammalian HO and BVR genes into *Escherichia coli* through genetic engineering methods, successfully achieving the synthesis of bilirubin. In the 1930s of this century, Fisher began the research on bilirubin synthesis. This orange-yellow bilirubin IXα crystal was artificially synthesized in 1942 [9]. In the 1970s, Pleninger and Thompson also conducted extensive research on bilirubin synthesis, and they synthesized bilirubin using different methods. In recent years, many researchers have engaged in this work and achieved gratifying achievements.

In the research on the microbial synthesis of bilirubin, although China started relatively late, it has also made many remarkable progress in recent years. Domestic research teams have constructed an efficient bilirubin synthesis pathway by using synthetic biology techniques. Specifically, research institutions such as Peking University and Fudan University have made significant progress in the study of bilirubin synthesis. For instance, a research team from Zhejiang University of Technology has disclosed a method for synthesizing bilirubin through

biotransformation. By using *Escherichia coli* (strain ZJUT-BVR) expressing bilivermin reductase to catalyze the conversion of bilivermin into bilirubin, the bilirubin content in the bacteria can reach 2.02%, which is 40 times more efficient than the traditional porcine bile extraction method [10-11]. The research team from Jiangnan University achieved in situ coenzyme regeneration by truncating the mutant enzyme protein, optimizing the coenzyme delivery pathway, and coupling formate dehydrogenase. Eventually, the bilirubin yield reached 415.5 mg/L, which is the highest level reported so far. Recently, the research team from Jiangnan University reviewed the research progress in the biosynthesis, acquisition, regulation and reuse of heme. They divided the heme synthesis gene into three modules (ALA synthesis, porphyrin ring assembly, and iron integration). After optimizing the expression, the fermentation yield in the flask reached 0.56 μmol/(L· OD), laying the foundation for the supply of bilirubin precursors. To provide support for improving the efficient production and application of hemoglobin and its derivatives [12].

In conclusion, significant progress has been made in the research of bilirubin synthesis both at home and abroad. However, foreign countries have a strong advantage in basic research and industrial application, while China has performed outstandingly in technological innovation and process optimization.

However, the current research still faces many challenges. Firstly, the bilirubin yield of engineered strains is generally low and still has a considerable gap from the requirements of industrialization. Secondly, the introduction of exogenous pathways often leads to an increase in the metabolic burden of the host and affects the normal growth of the bacteria. In addition, the instability of bilirubin also brings difficulties to the detection and purification of the product. The solution to these problems requires collaborative innovation of multi-disciplinary technologies, including protein engineering, systems metabolic engineering and optimization of fermentation processes, etc.

1.3 BIOTECHNOLOGY FOR THE SYNTHESIS OF BILIRUBIN USING *Escherichia coli*

With the rapid development of biotechnology, especially the breakthroughs in synthetic biology and metabolic engineering technologies, the production of high-value-added compounds using microbial cell factories has shown great potential[12]. *E. coli*, as one of the most commonly used model microorganisms, has the advantages of rapid growth, clear genetic background and simple operation, and is an ideal host for constructing the bilirubin synthesis pathway [14]. By introducing exogenous genes into *Escherichia coli* and reconstructing the heme metabolic pathway, de novo biosynthesis of bilirubin can theoretically be achieved.

The synthesis of bilirubin through microorganisms can reduce the negative impact on the environment and provide sustainability for the production process. Therefore, through genetic engineering methods, heme oxygenase (HO1) and bilirubin reductase (BvR) were overexpressed in *Escherichia coli*, an efficient biosynthetic pathway for BR was constructed, and an *Escherichia coli* strain for efficient synthesis of bilirubin was built, providing a new approach for the industrial production of bilirubin and making this research also have certain application value [15].

In conclusion, the biotechnology of synthesizing bilirubin using *Escherichia coli* has broad prospects in terms of market, technology, environment and social benefits. In the market, it can meet the growing demand for bilirubin in the pharmaceutical field at a low cost. Technically, through strain optimization, improvement of the cofactor regeneration system and integration with technologies such as AI, production efficiency can be continuously enhanced. In terms of the environment, green production and resource recycling comply with the requirements of sustainable development. In terms of social benefits, ensuring the stable supply of raw materials and promoting the innovative development of related industries such as synthetic biology are expected to become the mainstream direction of bilirubin production in the future.

Summary of the chapter I

The synthesis of bilirubin through microorganisms can reduce the negative impact on the environment and provide sustainability for the production process. Therefore, by means of genetic engineering, an *E. coli* strain that can efficiently synthesize bilirubin is

constructed, providing a new approach for the industrial production of bilirubin and making this research also have certain application value.

CHAPTER II
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 EXPERIMENTAL MATERIALs

2.1.1 PLASMIDS AND STRAINS

2.1.1.1 PLASMID

In the research on the synthesis of bilirubin using *E. coli*, choosing the pETDuet-1 plasmid as the expression vector has multiple advantages, mainly reflected in its multi-gene co-expression ability, flexibility, and the optimization suitable for biocatalysis systems.

(1) Multi-gene co-expression ability

pETDuet-1 contains two independent T7 promoters and can simultaneously express two exogenous genes, for example: heme oxygenase (HO) : catalyzes the degradation of heme to biliverdin. Biliverdin reductase (BVR) : Reduces biliverdin to bilirubin [17-18]. This design avoids the complexity of using multiple single-gene plasmids and simplifies the construction of metabolic pathways[19].

(2) It is applicable to the construction of coenzyme regeneration systems

In the biosynthesis process of bilirubin/biliverdin, NADPH is a key cofactor in the catalytic reaction of heme oxygenase (HO). pETDuet-1 allows for the simultaneous expression of HO and GdhA (glutamate dehydrogenase). The latter can regenerate NADPH through glutamate metabolism, reduce the addition of exogenous coenzymes, and improve catalytic efficiency.

The pETDuet-1 plasmid has become an ideal vector in the research of *E. coli* synthesis of bilirubin and related metabolites (such as bilirubin) due to its dual-gene co-expression ability, efficient protein expression level, suitability for the construction of

coenzyme regeneration systems, and flexible modularity. When optimizing the biocatalytic pathway, this plasmid can significantly increase the yield and conversion efficiency of the target product, providing a feasible technical solution for industrial production.

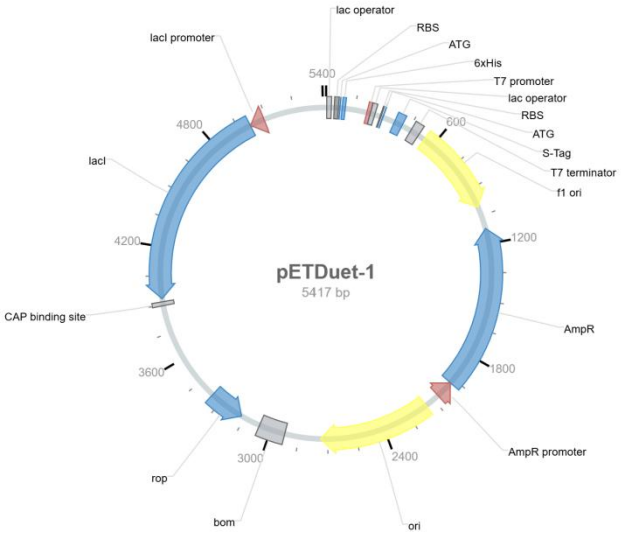

Figure 2.1 Carrier atlas of pETDuet-1

2.1.1.2 STRAIN

Table 2.1 Main Strains of the experiment

Name	genotype	source
Nissle1917	/	This laboratory keeps it
BL21(DE3)	F^- 、ompT、 $hsdS_e$ (rB^- mB^-)、 gal、 dcm、 (DE3)	This laboratory keeps it

2.1.2 CULTURE MEDIUM

(1) LB liquid medium

Peptone: 1%, yeast extract powder: 0.5%, NaCl: 1%, pH 7.5

(2) LB solid medium:

On the basis of LB liquid medium, add 15% AGAR powder to make it solidified

(3) LB-Amp (50 mg/mL) liquid medium:

Add the Amp stock solution in proportion (such as 100 mL LB plus 50 μL stock solution) and mix well

(4) LB-Kana (30 mg/mL) liquid medium:

Add the Kana stock solution in proportion (such as 100 mL LB plus 30 μL stock solution), and mix well

(5) LB-Amp-Kana liquid medium:

Add the Kana stock solution (such as 100 mL LB plus 30 μL stock solution) and the Amp stock solution (such as 100 mL LB plus 50 μL stock solution) respectively in proportion and mix well.

2.1.3 EXPERIMENT REAGENT

Table 2.2 List of Experimental Reagents

Name	specification	manufacturer
Glucose	AR	Tianjin Damao Chemical Reagent Factory
Yeast extract	AR	Beijing Aobo Xing Biotechnology Co., LTD
NaCl	AR	Tianjin Hengxing Chemical Reagent Manufacturing Co., LTD
Peptone	AR	Beijing Aobo Xing Biotechnology Co., LTD
NaOH	AR	Laiyang Kangde Chemical Co., LTD
IPTG	AR	Maclean Reagent Co., LTD
Plasmid extraction kit	AR	Beijing Soleibao Technology Co., LTD

SDS-PAGE gel Preparation Kit	AR	Jinkelong (Beijing) Biotechnology Co., LTD
1× Tris-Glycine electrophoresis buffer	AR	Beijing Soleibao Technology Co., LTD
Ampicillin sodium	AR	Biyuntian Biotechnology Co., LTD
Kanamycin sulfate	AR	Biyuntian Biotechnology Co., LTD

2.2 EXPERIMENTAL METHODS

2.2.1 CONSTRUCTION OF EXPRESSION CARRIERS

2.2.1.1 TREATMENT OF BACTERIAL STRAINS

Take out the strain preserved in the glycerol tube, inoculate every 100 μL into 5 mL of LB medium corresponding to the resistance, and place it in a shaker for overnight culture at 37 ° C and 250 rpm.

2.2.1.2 EXTRACTION OF PLASMIDS

Extract the plasmid strictly in accordance with the steps specified in the plasmid extraction kit manual.

2.2.1.3 AGAROSE GEL ELECTROPHORESIS OF DNA

(1) Glue production: Weigh a certain amount of agarose, add 1×TAE buffer solution, heat and dissolve it to make its concentration 0.75%. When cooled to approximately 55℃, add 1 mL of 10 mg/mL EB solution for every 25 mL, mix well and pour it into the gel preparation plate inserted into the sampling comb. Let it stand at room temperature for 30 minutes to allow the gel to cure fully.

(2) Spotting: Pull out the spotting comb, transfer the gel to the electrophoresis tank with 1×TAE buffer, add Marker, and add 6 μL for quantification. After mixing the sample with the Loading Buffer, add it to the spotting hole with a spotting volume of 5μL.

(3) Agarose gel electrophoresis: Electrophoresis at a constant pressure of 100 V for approximately 30 minutes;

(4) Observation: Observe and analyze the size of the formed DNA bands on a gel imager.

2.2.2 INDUCED EXPRESSION OF IPTG

2.2.2.1 INDUCED EXPRESSION OF IPTG AT DIFFERENT CONCENTRATIONS

(1) Use a pipette to aspirate 100 μL of the activated and cultured strains, and resuspend them respectively to 5 mL of the corresponding resistant LB medium (such as 30 μg/mL Kana, 50 μg/mL Amp). Pay attention to aseptic operation and incubate them in a shaker at 37 ° C and 250 rpm for 4 hours;

(2) Then, use a pipette to draw 0.5 mL of the re-cultured strain and inocitate it respectively in 75 mL of the corresponding resistant medium and LB medium without any resistance. Pay attention to aseptic operation to form a control test for each group. Place it again in a shaker at 37 ° C and 250 rpm for 4 hours of culture;

(3) Take the culture medium out of the shaker, add IPTG solution of different concentrations to each medium, pay attention to aseptic operation, and place it overnight in a shaker at 24 ℃and 200 rpm for culture.

(4) After taking out all the culture media and performing operations such as cell disruption and wall breaking, SDS-PAGE protein electrophoresis was carried out.

2.2.2.2 COLLECTION AND DISRUPTION OF CELLS

Centrifuge (8000 rpm, 10 min, 4 ° C) to collect the bacteria. Wash twice with pre-cooled PBS or lysis buffer to remove the residual medium. Then resuspend in lysis buffer (containing protease inhibitor), and perform ice bath ultrasonic disruption (200 W, 5 s ultrasonic /5 s intermittent, 10 min) until the bacterial liquid is clear. If the target protein forms inclusion bodies, the subsequent processing methods need to be adjusted.

The crushed bacterial liquid was centrifuged at 12,000 rpm at 4 ° C for 30 minutes to separate the supernatant (containing soluble protein and bilirubin precursors) and the precipitate (containing cell fragments and possible inclusions).

2.2.2.3 COLLECTION AND DISRUPTION OF CELLS

Mix the centrifugal supernatant with an organic solvent (such as chloroform: methanol in a ratio of 2:1) in a 1:1 ratio, vortex shake for 5 minutes, centrifuge (8000 ×g, 10 minutes) to collect the organic phase, and repeat the extraction three times to increase the recovery rate. If bilirubin exists in the precipitate, it needs to be dissolved with urea or guanidine hydrochloride first before extraction. After combining the organic phase, crude bilirubin was obtained by rotary evaporation or nitrogen drying. If necessary, it was further purified by silica gel column chromatography or HPLC [20].

2.2.2.4 SDS-PAGE DETECTION SHOWED THE TARGET PROTEIN BAND

(1) The SDS-PAGE polyacrylamide gel is composed of two layers, the upper layer being the concentrated gel and the lower layer being the separation gel. Its formula is shown in Tables 2.3 and 2.4.

Table 2.3 5%SDS-PAGE concentrated gel

Gel	Volume of each component required (unit: mL)					
	Pure	30%Acr/Bis	1.0mol/L	10%SD	10% PAGE	PAGE glue

volume	water	(29:1)	Tris (pH6.8)	S	gelling agen	accelerator
5mL	3.42mL	0.83mL	0.625mL	50μL	75μL	7.5μL

Table 2.4 10%SDS-PAGE Separation gel

Gel concentration	Gel volume	Volume of each component required (unit: mL)					
		Pure water	30%Acr/Bis (29:1)	1.5mol/L Tris (pH 8.8)	10% SDS	10% PAGE gelling agen	PAGE glue accelerator
10%	10mL	4.0mL	3.3mL	2.5mL	100μL	100μL	10μL

(2) The separation gel prepared according to the formula in Table 2.4;

(3) Add 2 ml between two glass plates, and then seal the upper space with anhydrous ethanol. After the unadded separation gel has solidified, pour out the anhydrous ethanol between the glass plates, rinse with double-distilled water, and then suck clean with filter paper. Then add the prepared concentrated glue in the upper space and immediately insert it into the sample slot template. After the gel polymerizes (about 20 to 30 minutes), gently pull out the sample slot template. Add the electrophoresis buffer to the upper and lower electrophoresis tanks. Prepare the sample loading and start the electrophoresis[20].

2.2.3 CONSTRUCTION OF RECOMBINANT *ESCHERICHIA COLI*

2.2.3.1 COMPETENT CELLS WERE PREPARED BY THE CACL2 METHOD

Take out the *Escherichia coli* Nissle 1917 glycerol tube from the -20 ℃ refrigerator, draw 150 μL and inoculate it into 5 mL of LB liquid medium, and incubate at 37 ℃ for 15 hours. Then, 100 μL to 5 mL of LB liquid medium was aspirated and incubated at 37 ° C and 200 rpm for 3 hours until the logarithmic growth phase. Take

1.5 mL of the bacterial liquid and ice bath for 10 minutes. Centrifuge at 4 ° C and 4000 rpm for 10 minutes to collect the bacterial cells. Resuspend with 1mL of pre-cooled 0.1 mol/L $CaCl_2$ solution and ice bath for 30 minutes. Centrifuge again and discard the supernatant. Resuspend with 200 μL of pre-cooled 0.1 mol/L $CaCl_2$ solution containing 15% glycerol, aliquot into sterile centrifuge tubes, rapidly freeze in liquid nitrogen and store at -80 ° C [22].

2.2.3.2 CONSTRUCTION OF ENGINEERED STRAINS

Add 10 μL of the recombinant expression vector to the competent cell suspension, shake gently, and let it stand on ice for 30 minutes to ensure that the recombinant plasmid is fully adsorbed on the surface of the competent cells. The centrifuge tubes were heated in a 42 ° C water bath for 90 seconds, and then quickly cooled on ice for 5 minutes to restore the permeability of the cell membrane to normal and promote the entry of recombinant plasmids into the cells at the same time. Add 1 mL of LB liquid medium (without antibiotics) to the tube, mix well, and then shake and culture at 37 ° C and 200 r/min for 1 hour to restore the normal growth state of the bacteria and express the antibiotic resistance gene encoded by the plasmid.

2.2.4 EXTRACTION OF BILIRUBIN

The recombinant *Escherichia coli* fermentation broth after culture was centrifuged at 12,000 rpm for 10 minutes, and the cells were collected. Add an appropriate amount of 0.1 mol/L Tris-HCl buffer solution (pH 8.0) to the bacteria, resuspend the bacteria, and ultrasonically break the cells with a power of 300 W for 3 seconds, with an interval of 3 seconds, for a total of 3 minutes. After ultrasonic disruption, centrifuge at 12,000 rpm for 10 minutes and take the supernatant. Add an equal volume of chloroform to the supernatant, shake to mix well, and centrifuge at 12,000 rpm for 10 minutes. At this point, bilirubin will dissolve in the chloroform phase. Collect the chloroform phase, blow dry it with nitrogen, and obtain the crude bilirubin. The crude bilirubin was

dissolved in an appropriate amount of methanol and sieved through a 0.22 μm organic filter membrane for detection.

2.2.5 DETECTION OF BILIRUBIN

The bilirubin content was detected by high performance liquid chromatography (HPLC). The principle of HPLC is based on the different distribution coefficients of different substances between the stationary phase and the mobile phase, thereby achieving separation [23]. When detecting bilirubin, a C18 reversed-phase chromatography column was used, with methanol-water (85:15, v/v) as the mobile phase, a flow rate of 1.0 mL/min, and a detection wavelength of 450 nm[24].

The prepared bilirubin sample was injected into the HPLC injector with an injection volume of 20 μL. The content of bilirubin in the sample was calculated by comparing it with the retention time and peak area of the bilirubin standard. This detection method has the advantages of high separation efficiency, fast analysis speed and high sensitivity, and can accurately detect the content of bilirubin in the sample.

Summary of the chapter II

This chapter provides a detailed description of the materials and methods required for the experiment, including:

1. The pETDuet-1 and pRSFDuet-1 plasmids were used as expression vectors, which were respectively employed to construct the expression system for the recombinant enzyme.

2. The experimental strains included *Escherichia coli* BL21(DE3), Origami B(DE3), and Nissle 1917, which were used to compare the effects of different hosts on bilirubin synthesis.

The medium and reagent section lists the formula of LB medium and the chemical reagents required for the experiment.

3. Through gene cloning technology, expression vectors were constructed, including pETDuet-1-*AtHO*, pETDuet-1-*TeHO,* etc., and they were transformed into different hosts.

4. Induce the expression of the target protein using IPTG, and detect the protein expression level by SDS-PAGE.

5. The catalytic efficiency of different host-enzyme combinations was evaluated through whole-cell catalysis experiments. Bilirubin was extracted and quantitatively analyzed by HPLC.

CHAPTER III
EXPERIMENTAL PART

Studies have shown that heme oxidase and biliverdin reductase from various microorganisms, animals and plants in nature can catalyze the synthesis of corresponding products from heme or biliverdin, namely biliverdin or bilirubin. Physiological metabolism studies have indicated that human heme oxygenase is strongly inhibited by high-concentration substrates [25], while heme oxidase from Arabidopsis thaliana has better heme compatibility characteristics [26].

Similarly, an activity test indicated that the biliverdin reductase activity derived from the liver of rats was the highest. Up to now, there are relatively few reports on the successful conversion of heme to bilirubin[27], Among them, the related enzymes derived from cyanobacteria were induced to be expressed in *Escherichia coli* in different forms such as fusion and independence. After endogenous gene editing, the host achieved a relatively high level of bilirubin accumulation (62.1 mg/L). Can the combination of related enzyme systems from different sources achieve a higher accumulation of bilirubin? Here, relevant research is carried out to answer this question.

3.1 COMPARISON OF CATALYTIC ACTIVITIES OF HO AND BVR FROM SEVERAL DIFFERENT SOURCES

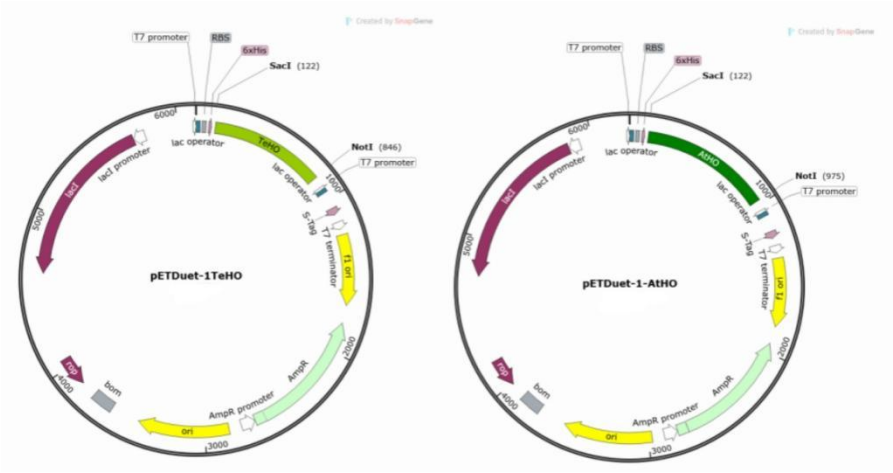

Figure 3.1 Schematic diagram of pETDuet-1-*AtHO* and pETDuet-1-*TeHO* expression vectors

The corresponding coding DNA was synthesized according to the amino acid sequences of heme oxidase derived from Arabidopsis thaliana and cyanobacteria, and cloned respectively into the two expression vectors pETDuet-1 to construct pETDuet-1-*AtHO* and pETDuet-1-*TeHO* (Figure 3.1). Transform *Escherichia coli* BL21 (DE3) respectively.

The results show that both can be soluble expressed in BL21 (DE3). Through optical density analysis, it can be preliminarily considered that the expression levels of the two are comparable and both are related to the concentration of IPTG. The expression levels were comparable when the IPTG concentrations were 0.5 mM, 0.75 mM and 1.0 mM, all significantly higher than those when the IPTG concentrations were 0.1 mM and 0.25 mM.

Carry out the catalytic reaction in accordance with the method described in 2.2.3. The variation of biliverdin synthesis over time is shown in Table 3.1 below.

Table 3.1 Changes in the amount of bilirubin catalyzed synthesis over time

Catalytic time	Bilirubin Synthesis (AtHO) (unit: mg/L)	Bilirubin Synthesis (TeHO) (unit: mg/L)
1 h	1.5	1.4
3 h	3.4	3.0
5 h	6.5	4.8
7 h	6.0	5.6
9 h	5.5	5.1

The results showed that when heme was used as the substrate, AtHO had the highest biliverdin synthesis at 5.0 h of catalysis, reaching 6.5 mg/L, while TeHO had the highest biliverdin synthesis at 7.0 h of catalysis, reaching 5.6 mg/ L. This implies that AtHO has better activity than TeHO. Subsequently, we used AtHO as the first enzyme for bilirubin synthesis.

The corresponding coding DNA was synthesized according to the amino acid sequences of biliverin reductase enzymes derived from rat liver and cyanobacteria, and cloned respectively to the *Nde Ⅰ/Xho Ⅰ* sites of pETDuet-1-*AtHO*. The expression vectors pETDuet-1-*AtHO-RnBVR* and pETDuet-1-*AtHO-SsBVR* were obtained (Figure 3.2), and transformed into BL21 (DE3). After culture and induction with 0.5 mM IPTG for 10 hours, the catalytic reaction was carried out according to the method described in 2.2.3. The variation of bilirubin accumulation over time is shown in Table 3.2.

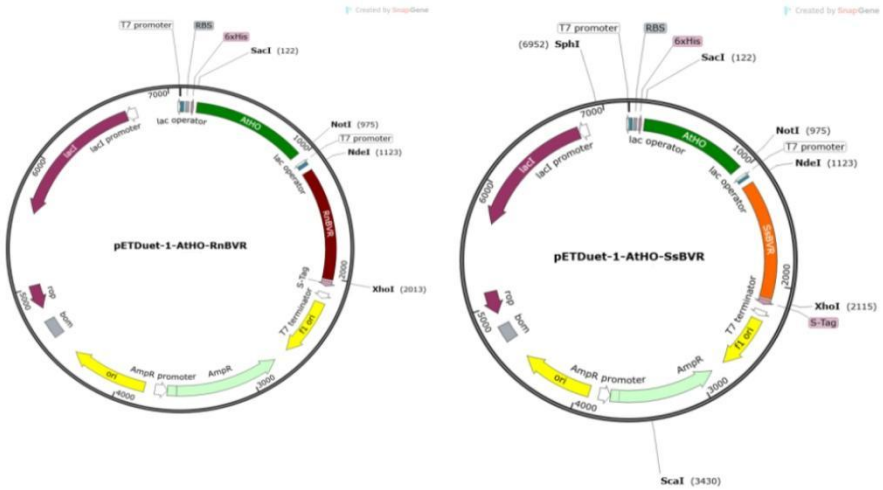

Figure 3.2 Schematic diagram of pETDuet-1-*AtHO-RnBVR* and pETDuet-1-*AtHO-SsBVR* expression vectors

Table 3.2 Changes in the catalytic synthesis amount of bilirubinase over time

Catalytic time	Bilirubin Synthesis (RnBVR combined with AtHO) (unit: mg/L)	Bilirubin Synthesis (SsBVR combined with AtHO) (unit: mg/L)
3 h	3.4	8.0
5 h	5.5	10.6
9 h	6.0	16.0
10 h	5.4	15.1

The results showed that when heme was used as the substrate, RnBVR combined with AtHO had the highest bilirubin synthesis at 10.0 h of catalysis, reaching 10.5 mg/ L. While SsBVR combined with AtHO had the highest bilirubin synthesis at 9.0 h of

catalysis, reaching 16.0 mg/ L. This means that RnBVR has better activity than SsBVR. Subsequently, we used AtHO combined with RnBVR as the bilirubin synthase.

3.2 WHOLE-CELL CATALYTIC SCREENING OF *Escherichia coli* HOSTS

In the biocatalysis process, to increase the enzyme activity maintenance time of the reaction system, reduce the production of by-products and lower costs, the whole-cell catalytic strategy is often adopted. Here, the constructed expression vector pETDuet-1-*AtHO-RnBVR* was respectively transformed into competent cells of *Escherichia coli* BL21 (DE3), Origami B (DE3), and Nissle $1917_{pAR1219}$, and different host-mediated whole-cell catalysis was tested.

According to Method 2.2.4, different recombinant *Escherichia coli* after culture were treated. After whole-cell catalysis, bilirubin was extracted and the bilirubin content was detected by HPLC. The results are shown in Table 3.3

Table 3.3 Maximum Synthetic Bilirubin levels (mg/L) of Different recombinant *Escherichia coli*

Host	Biliverdin content	Bilirubin content
BL21（DE3）	0.4	2.2
Nissle $1917_{pAR1219}$	0.7	3.2
Origami B (DE3)	0.8	1.9

The synthesis amount of bilirubin varies significantly among different hosts, among which the Nissle $1917_{pAR1219}$ host is the most. This kind of difference exists in different studies of product-engineered bacteria, mostly indicating that it is caused by genetic background differences [28], Nissle 1917 is a classic probiotic and is widely present in the human body. Studies have shown that Nissle 1917 has a better heme

absorption function [29], which is driven by ATP and mediated by ChuA, and its expression is less dependent on the intracellular and extracellular heme concentration levels than that of other hosts. Although the $1917_{pAR1219}$ host has an advantage in bilirubin synthesis, its culture rate is slower than that of conventional industrial strains, and there are fewer studies on metabolic control. Therefore, transplanting the heme absorption capacity into conventional commercial strains such as BL21 (DE3) is a better choice.

3.3 THE EFFECT OF CHUA GENE OVEREXPRESSION ON BL21 (DE3) BILIRUBIN SYNTHESIS

The corresponding coding DNA was synthesized according to the amino acid sequence of ChuA derived from *Escherichia coli*, cloned into the expression vector pRSFDuet-1, and pRSFDuet-1-*ChuA* (Figure 3.3) was co-transformed with pETDuet-1-*AtHO-RnBVR* into BL21 (DE3). According to methods 2.2.4 and 2.2.5, different recombinant *Escherichia coli* after culture were treated to extract bilirubin. HPLC detection revealed that compared with the unoverexpressed ChuA gene, the cumulative amount of bilirubin increased by 90% to 4.2 mg/L, but it was still relatively low.

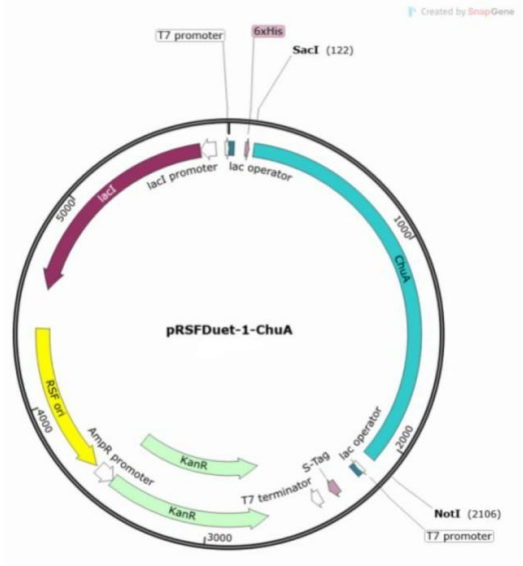

Figure 3.3 Schematic diagram of the pRSFDuet-1-*ChuA* expression vector

The initial step of the biosynthesis process of heme precursor porphyrin is catalyzed by Porphobilinogen synthase (PBGS), which is also a key enzyme. Therefore, here we have attempted to overexpress this gene in the hope of increasing the supply of endogenous heme and thereby enhancing the synthesis of bilirubin.

3.4 ANALYSIS OF THE GROWTH CHARACTERISTICS OF BILIRUBIN SYNTHESIZED BY *Escherichia coli* ENGINEERED BACTERIA

The corresponding coding DNA was synthesized according to the amino acid sequence derived from *Escherichia coli* PBGS, cloned into the expression vector pRSFDuet-1, and obtained PRSFDuet-1-*PBGS* (Figure 3.6). Co-transform BL21 (DE3) with pETDuet-1-*AtHO-RnBVR*. According to methods 2.2.4 and 2.2.5, different recombinant *Escherichia coli* after culture were treated to extract bilirubin. HPLC detection revealed that compared with the control (BL21 (DE3) -pRSFDuet-1-*PBGS*), the cumulative amount of bilirubin slightly increased to 2.9 mg/L. It was significantly lower than that of the engineered strains overexpressing ChuA bilirubin synthesis, indicating that the de anew synthetic biology research of bilirubin still faces the problem of overall metabolic network remodeling.

Figure 3.6 Schematic diagram of the pRSFDuet-1-PBGS expression vector

Summary of the chapter III

This chapter presents the main results of the experiment and their analysis:

1. AtHO (hemoglobin oxidase from Arabidopsis thaliana) exhibited higher activity (6.5 mg/L) in catalyzing the formation of biliverdin from hemoglobin, outperforming TeHO (enzyme from cyanobacteria).

2. RnBVR (biliverdin reductase from rat liver) combined with AtHO performed the best in bilirubin synthesis (10.5 mg/L).

3. The Nissle 1917 host exhibited the best performance in bilirubin synthesis (3.2 mg/L), which is speculated to be related to its efficient ability to absorb heme.

4. Overexpression of the ChuA gene (a heme pump gene) increased the bilirubin production of BL21(DE3) by 90% (4.2 mg/L).

5. Overexpression of the PBGS gene (a key enzyme in the heme synthesis pathway) has a limited effect on increasing yield (2.9 mg/L), indicating that the metabolic network needs to be further optimized.

CONCLUSION

This study focused on the catalytic activities of heme oxidase (HO) and bilirubin reductase (BVR) from different sources and the host engineering modification, optimizing the efficiency of bilirubin synthesis by *Escherichia coli*. The main conclusions are as follows:

(1) Enzyme activity screening and combination optimization

By comparing the catalytic performance of Arabidopsis thalian-derived HO (AtHO) and cyanobacteria-derived HO (TeHO), it was found that the maximum amount of biliverbin catalyzed by AtHO within 5 hours was 6.5 mg/L, and its activity was significantly better than that of TeHO (5.6 mg/L). Therefore, AtHO was selected as the first-step enzyme for bilirubin synthesis.

Further comparison of the combined activities of hepato-derived BVR (RnBVR), cyanobacterial-derived BVR (SsBVR) and AtHO in rats revealed that AtHO combined with RnBVR could accumulate 10.5 mg/L bilirubin within 10 hours, which was superior to AtHO combined with SsBVR. There might be data errors. Finally, AtHO-RnBVR was determined as the optimal enzyme combination.

(2) Whole-cell catalytic host screening

Among the three hosts, BL21 (DE3), Origami B (DE3), and Nissle $1917_{pAR1219}$, the bilirubin synthesis level of Nissle $1917_{pAR1219}$ was the highest (3.2 mg/L). It is speculated that its advantages stem from genetic background differences and efficient heme absorption capacity (driven by ATP and mediated by ChuA protein). However, this host still has the disadvantages of slow culture speed and insufficient research on metabolic regulation.

(3) Host metabolic engineering modification

Overexpression of the ChuA gene can increase the bilirubin accumulation of BL21 (DE3) by 90% (up to 4.2 mg/L), confirming that heme absorption capacity is one of the rate-limiting factors.

Overexpression of the PBGS gene, a key enzyme for heme synthesis, only slightly increased the bilirubin level to 2.9 mg/L, indicating that the metabolic network of the de vivo synthesis pathway needs to be further optimized.

Although this study initially improved the efficiency of bilirubin synthesis through enzyme screening and host modification, the overall yield is still relatively low. Further exploration can be conducted in the following directions: ①Enzyme engineering optimization, modifying AtHO and RnBVR through directed evolution/rational design to enhance catalytic performance, and exploring multi-enzyme co-immobilization to reduce the loss of intermediate products; ②Reconstruction of the host metabolic network: By using gene editing to knock out competitive pathways, overexpressing rate-limiting enzymes and introducing transport proteins, an efficient metabolic module is constructed; ③Application of new synthetic biology technologies, adopting cell-free systems/cell surface display techniques to enhance enzyme utilization, and developing inducible promoters to balance growth and synthesis; ④ Industrial adaptability optimization: Optimize the culture conditions of Nissle $1917_{pAR1219}$ or transplant its heme absorption element into industrial strains to enhance the anti-interference ability and adaptability; ⑤Expand application scenarios, explore the direct application of bilirubin in the medical field and in vivo biosynthesis therapy, and establish a real-time monitoring system to optimize the fermentation process.

This study provides basic data on enzyme combinations and host modifications for the synthesis of bilirubin by microorganisms. Subsequently, it is necessary to break through the yield bottleneck through the integration of multi-disciplinary technologies (enzyme engineering, metabolic engineering, synthetic biology) to promote its transformation from laboratory to industrial production. In future research, global metabolic optimization guided by systems biology and the design of novel bioreactors will be key breakthrough points.

Summary of the chapter IV

This chapter summarizes the main conclusions of the research and proposes future directions:

AtHO-RnBVR is the optimal enzyme combination, and Nissle 1917 is the best host.

2. Metabolic engineering can be employed to modify (such as overexpressing the ChuA gene) to significantly increase the yield of bilirubin.

3. Enzyme engineering optimization: Enhance enzyme activity through directed evolution or rational design.

4. Metabolic network reconfiguration: Knock out competing pathways or introduce transport proteins to optimize the metabolic flow.

5. Industrial adaptability optimization: Transplant the heme absorption component of Nissle 1917 into industrial strains.

6. Application Expansion: Explore the direct application of bilirubin in the medical field or in in vivo biosynthesis therapies.

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