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

QUALIFICATION THESIS

on the topic **Bioinformatics analysis and heterologous expression of RfbC gene in marine bacterium P. a. Hao2018**
First (Bachelor's) level of higher education
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Completed: student of group BEBT-20
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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

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Educational and professional program Biotechnology

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Scientific supervisor Ihor Hretskyi, Ph.D., As. prof

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I am familiar with the task:

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SUMMARY

Liu Zheng. Bioinformatic analysis and heterologous expression of RfbC genes in marine bacteria *Pseudoalteromonas agarivorans* Hao2018 – Manuscript.

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

There is a special kind of prokaryotic single-celled organisms in the ocean, which are called marine bacteria. Marine bacteria are a kind of microorganisms widely distributed in the marine environment. The high complexity of the marine environment enables marine bacteria to secrete macromolecular bioactive substances such as extracellular polysaccharide. As one of the main metabolites, the research on extracellular polysaccharide has been deepened in recent years, and the research and development and utilization of marine bacteria and extracellular polysaccharide have also developed rapidly. In this study, *Pseudoalteromonas agarivorans* Hao 2018, a marine bacterium, was taken as the research object to explore the cloning and expression of dTDP-4- dehydrorhamnose 3,5 epimerase gene in marine bacterium P.a.Hao.2018. Firstly, the amino acid sequence of Rfbc enzyme (dTDP-4-dehydrorhamnose 3,5 epimerase) was found from NCBI database, and the sequence alignment and phylogenetic tree were carried out. At the same time, the secondary structure and tertiary structure of Rfbc enzyme were predicted. In this experiment, the genome of marine bacteria *Pseudoalteromonas agarivorans* Hao 2018 was extracted, the dTDP-4- dehydrorhamnose 3,5 epimerase gene was cloned by PCR amplification, and then the cloned fragment sequence was connected with pET-16b (plasmid) to further construct an expression vector. It was transformed into the expression host Escherichia coli BL21(DE3). After the expression vector was verified, the target enzyme protein was finally obtained by IPTG induction, cell fragmentation and protein purification. Marine bacteria have important biological functions. The results of this study are expected to provide a theoretical basis for further exploring the

metabolic pathway of marine bacteria Hao 2018 and its application in the field of biotechnology.

Keywords: marine bacteria; RfbC gene; DTDP-4- dehydrorhamnose 3,5 epimerase; Bioinformatics analysis; Heterologous expression; Extracellular polysaccharide

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INTRODUCTION

Marine bacteria are a diverse group of microorganisms that play crucial roles in various ecological processes in the marine environment. One such bacterium is *Pseudoalteromonas agarivorans* Hao2018, which has been found to have unique metabolic capabilities and adaptations to its marine habitat. In recent years, there has been growing interest in studying the functional genes present in these bacteria, as they can provide valuable insights into their survival strategies and potential biotechnological applications.

One such gene of interest is RfbC, which encodes for the GDP-mannose pyrophosphorylase enzyme involved in the biosynthesis of bacterial cell surface polysaccharides. These polysaccharides have been shown to play important roles in various biological processes, including adhesion, biofilm formation, and pathogenesis. However, the functional characteristics and heterologous expression of RfbC genes in marine bacteria like *Pseudoalteromonas agarivorans* Hao2018 have not been well studied.

In this study, we aim to perform a comprehensive bioinformatic analysis of RfbC genes in *Pseudoalteromonas agarivorans* Hao2018 and investigate their heterologous expression in a model bacterial host.

The relevance of the topic is Bioinformatic analysis and heterologous expression of RfbC genes in marine bacteria *Pseudoalteromonas agarivorans* Hao2018, which has theoretical significance.

The purpose of the Identify and characterize the RfbC genes present in *Pseudoalteromonas agarivorans* Hao2018 using bioinformatics tools and databases. Analyze the phylogenetic relationships of these RfbC genes with other bacterial RfbC homologs to gain insights into their evolutionary history.

Predict the functional properties and structural characteristics of the RfbC proteins using bioinformatics predictions and modeling tools. Express the RfbC genes in a heterologous bacterial host and analyze their activity and function in vitro.

By performing these analyses, we hope to gain a better understanding of the role of RfbC genes in Pseudoalteromonas agarivorans Hao2018 and their potential applications in biotechnology. This study will provide valuable insights into the functional genomics of marine bacteria and contribute to the broader field of microbial biology.

The objectives of the length of nucleic acid sequence and the number of corresponding amino acid residues of the gene were determined by bioinformatics analysis, and the physical and chemical properties of its RfbC enzyme protein were deduced. Then the secondary structure and tertiary structure are predicted and analyzed to improve the understanding of the structural characteristics of RfbC enzyme. In order to reveal the evolutionary background of RfbC enzyme in a deeper level, the amino acid sequence of RfbC enzyme from *Pseudoalteromonas agarivorans* Hao 2018 was compared with the existing similar sequences with similar length in NCBI database, and a phylogenetic tree was constructed based on this data, in order to clearly describe the genetic relationship and differentiation path between them. The target gene and plasmid vector were extracted, cloned into Dh5α by PCR amplification, constructed into expression vector, and transformed into Escherichia coli BL21 for protein expression.

The object of the extracellular polysaccharide produced by marine bacterium *Pseudoalteromonas agarivorans* Hao 2018 . The RfbC enzyme related to rhamnose, namely dTDP-4- dehydrorhamnose 3,5 epimerase gene, was mainly explored.

CHAPTER 1
LITERATURE REVIEW

1.1 Overview of the marine bacteria *Pseudoalteromonas agarivorans*

Marine bacteria are prokaryotic monocellular organisms that live in the ocean and do not contain chlorophyll and phycocyanin. They are the most widely distributed and the largest number of organisms in Marine microorganisms, often in diameter below 1 micron, spherical, rod, spiral and branched filament microorganisms. No eukaryotic, cell wall tough. Swimming species move with flagella. Strictly speaking, marine bacteria are those that can only grow and reproduce in the ocean.

A marine bacterium was first isolated in the mid-19th century, one of which it was isolated in 1865. Since 1884, it have studied deep sea bacteria. In the early days, it only focused on classification, and after 1946, it entered a stage based on studying its physiology and ecology. Marine bacteria have different types of autotrophic and heterotrophic, light and chemical energy, aerobic and anaerobic, parasitic and saprophytic, and planktonic and attached. Gram-negative bacteria are dominant in seawater, Gram-positive bacteria in ocean floor sediment and Bacillus are most common in continental shelf sediment. Marine bacteria have a very wide distribution in the ocean, which can be found from the nearshore shallow sea to the bottom of the deep sea. Marine bacteria are large in number and are the most important component of Marine microbes. Their quantitative distribution characteristics are closely related to the Marine environment, and the bacterial density is usually higher in the offshore areas, especially in the inner bay and estuary areas. In addition, the number of Marine bacteria is also affected by the distribution of organic substances in the seawater. When the distribution of organic substances is not uniform, the number of bacteria often increases sharply.

The complexity of the Marine environment determines that Marine bacteria can be divided into multiple types according to their nutritional mode and metabolic

characteristics, including autotrophic and heterotrophic, light and chemical energy, aerobic and anaerobic, parasitic and saprophytic, and planktonic and attachment. In the ocean, Gram-negative bacilli are more common bacterial types, including pseudomonas, *Vibrio, Achromobacter, Flavobacteria*, *Micrococcus, Octoid, Bacillus, Corynebacterium, Clade, Nocardia* and *Streptomus* and other more than 10 genera.

Marine bacteria are always in the changing Marine environment, making the Marine bacteria living in them have unique characteristics, while Marine bacteria also have diverse cell forms [3]. Marine bacteria exopolysaccharide is [4] produced by Marine bacteria, which is the material produced by bacteria in order to cope with the changes in their external environment, and to improve their survival ability [5]. Changes in environmental factors can have some influence on the secretion of extracellular polysaccharide from marine bacteria. Pseudogalteromonas (Pseudoalteromonas) is one of the genera [5] that has been isolated to secrete exopolysaccharides. Marine bacterial exopolysaccharides act as biological macromolecules like proteins [3]. The discovered exopolysaccharides have important biological significance in promoting the formation of biological aggregates and adsorption of heavy metal ions [6]. Furthermore, a new bacterial exopolysaccharide was isolated and purified from *Pseudoalteromonas,* and the effect of this polysaccharide on the cellular immune response in mice was studied. The results showed that this polysaccharide could significantly promote the proliferation of lymphocytes, indicating that it has the potential to become a powerful immunomodulator, [21].

Pseudoalteromonas agarivorans Is a bacterial [6] of the genus *Pseudogalteromonas* (*Pseudoalteromonas*). *Pseudogalteromonas* are Gram-negative bacteria, which can secrete a variety of macromolecular bioactive substances, which contain exopolysaccharides, which are widely distributed in the Marine environment, living in various locations of the ocean and attached to Marine organisms [2]. Its secreted exopolysaccharides have functions such as antioxidant, agar degradation and agar, which help the bacteria survive in the changing environment [7]. P.agarivorans

The mechanisms of adaptation to changes in the complex Marine environment are also the focus of current research. In this paper, we studied dTDP-4-dehydrorhamnose 3-, 5-isomerase in Pseudoalteromonas agarivorans Hao 2018 to complete the cloning and expression of [8] of dTDP-4-dehydrorhamnose 3,5, a key gene in the rhamnose precursor synthesis module.

1.2 Rhamnose for an overview

Rhamnose is also known as 6-deoxygen-L-mannose, is an organic compound, molecular formula for $C_6H_{12}O_5$, is a widely found in plant polysaccharide, glycosides, plant gum and bacterial polysaccharide in a substance, is a unique component of bacterial cell wall [9], is a widely exist in bacterial polysaccharide substance [3], microbial production will produce polysaccharide containing rhamnose, Marine bacteria can produce rhamnnose and lipid, produce rhamnnose fat is influenced by nitrogen source and pH [10]. The application of rhamnose is increasingly important in the food and drug industry, especially in the strong heart drugs. With the deepening of the research on rhamnose and rhamnose synthetic enzyme lines, there is an increasingly clear understanding of rhamnose, and its application prospects in medicine and food will continue to expand. Rfbc (dTDP-4-dehydrorhamnose 3,5 differential direction isomerase), one of the dTDP-rhamnose synthase lines involved in dTDP-rhamnose synthesis, is an important target for the development of new anti-tuberculosis drugs [8].

1.3 Study purpose and significance

1.3.1 Study Purpose

With the continuous development of research on pseudoganomonas aganophagy, the use of exopolysaccharides secreted by it is increasingly widespread. At the same time, the special instability in the Marine environment endows Marine bacteria with different characteristics [11], which has unique advantages in the study of exopolysaccharides [12]. The research of sugar substances has progressed slowly

in the past and has a higher heat in the future research development. This study aimed to explore the P. Cloning and expression of the dTDP-4-DDDNA isomerase gene in agarivorans Hao 2018. It is expected to lay the foundation for exploring the enzymatic regulation mechanism of dTDP-4-dehydrorhamnose 3,5 differential-direction isomerase in Hao 2018 in subsequent work.

1.3.2 Study Significance

This project starts with the bioinformatics analysis of Rfbc enzyme (dTDP-4-dehydrorhamnose 3,5 differential isomerase) and predicts the basic chemical parameters such as molecular weight and half-life of Rfbc enzyme protein. Thereafter, cloning and expression experiments of dTDP-4-dehydrorhamnose-3,5-isomerase gene were performed, and the gene expression vector of Rfbc enzyme gene construction in Hao 2018 was transferred into E. coli BL21 for expression.

Pseudoalteromonas agarivorans Is a kind of Marine bacteria that can secrete exopolysaccharide, exopolysaccharide has a variety of biological activity to ensure the host can survive in the environmental change, its biological characteristics including antioxidant and adsorption of heavy metal ions such as [13], exopolysaccharide application in the field of medicine showed good development prospects, such as anti-tumor, regulate the immune system such as [17]. EPS produced by marine strains Vibrio and Pseudomonas are known to have antiviral and immunostimulatory activity studies have implicated the mechanism of viral inhibition with the molecular weight of sulfated EPS, interactions between virus and target membranes [22]. By destroying the attachment of viral cells, the virus prevents the host cells and suppresses the tumor cell growth; this is also related to the presence of anionic groups in the molecules, which are cytotoxic to different human cancer cell lines and enhance the medical potential of EPS.

In this study, we started with dTDP-4-dehydrorhamnose 3,5 differential-oriented isomerase in *Pseudoalteromonas agarivorans* Hao 2018, starting with bioinformatics analysis and in vitro expression of [14]. To finally explore dTDP-4-

dehydrorhamnose 3,5 differential-directed isomerase in *P.agarivorans* Hao 2018 Role played in the synthesis module of the rhamnose precursor.to P.agarivorans Hao 2018 The mechanism of exopolysaccharide production is not clear yet for [7]. I hope that more information can be accumulated for further exploration in the future through this topic.

Summary of the chapter I

1.Rhamnose in extracellular polysaccharide of marine bacteria

2. that relationship between 2.Rfbc gene and rhamnose

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

This section on *P.agarivorans* Hao 2018 Source of dTDP-4-dehydrorhamnose 3,5, bioinformatics analysis of physicochemical parameters of the enzyme protein [16], secondary structure and tertiary structure prediction of Rfbc enzyme protein, and phylogenetic tree construction with Rfbc amino acid sequence of close material species published in NCBI, and multiple sequence alignment. Analyzed their bioinformatics analysis results.

2.1 Experimental materials

2.1.1 Sequence

Isolated *Pseudoalteromonas agarivorans* Hao 2018 from the surface of abalone seedlings. GenBank assembly accession:GCA_003668795.1.

Amino acid sequence of the *P. agarivorans* Hao 2018-derived Rfbc enzyme (dTDP-4-dehydrorhamnose 3,5-oriented isomerase)

>AYM85573.1 dTDP-4-dehydrorhamnose 3,5-epimerase [Pseudoalteromonas agarivorans]

MNFIETDIPDVKIIEPQVFGDERGFFMETFRTELFNQHCGERTFVQENHSKSAHGILRGLHYQTQNTQGKLVRVTQGEVFDVAVDMRKDSPTFGQWVGVLLSAENKRQLWVPEGFAHGFYVTTESAEFVYKCTDIYNPNAEVSIKWDDPTLNIDWPLVEGKLPQLSAKDEAGLAFTKAPTF

8 different species with the Rfbc amino acid sequence published in NCBI

(1)dTDP-4-dehydrorhamnose 3 5-epimerase [Enterococcus durans IPLA 655]

(2)dTDP-4-dehydrorhamnose 3 5-epimerase '[Rhodococcus sp.]

(3)dTDP-4-dehydrorhamnose 3 5-epimerase [Aeromonas schubertii]

(4)dTDP-4-dehydrorhamnose 3 5-epimerase [Northococcus DSM 17330]

(5)dTDP-4-dehydrorhamnose 3 5-epimerase '[Rhizobium Stenotrophomonas]

(6)dTDP-4-dehydrorhamnose 3 5-epimerase [Maris lysozyme]

(7)dTDP-4-dehydrorhamnose 3 5-epimerase [B. velezus]

(8)dTDP-4-dehydrorhamnose 3 5-epimerase [E. coli]

2.1.2 Experimental Tools

Amino acid sequence and nucleic acid sequence of dTDP-4-dehydrorhamnose 3,5-oriented isomerase from different species Source: NCBI database (https://www.ncbi.nlm.nih.gov/）

Bioinformatics analysis tools for protein physiochemical analysis:

ProtParam tool（https://www.expasy.org/）

Multiple sequence alignment analysis tools of amino acid sequences from different sources:

Clustal Omega（https://www.ebi.ac.uk/services）

Protein secondary structure prediction:

PRABI（http://www.prabi.fr/）

Protein tertiary structure prediction:

SWISS-MODEL（https://swissmodel.expasy.org/interactive）

Phylogenetic tree construction tool: M EGA 11[18]

2.2 Experimental materials

2.2.1 Strain and plasmid

Pseudoalteromonas agarivorans Hao 2018 The strain was derived from the microbial membrane on the surface of abalone seedlings and stored in A 217 laboratory in the Food Building of Qilu University of Technology

Plasmid: pET-16b

DH 5 α competent strain and B L21 competent strain (Nanjing Novizan Biotechnology Co., Ltd.)

2.2.2 Culture medium and experimental solution

LB liquid medium: 10 g, Na C l 10 g, 5 g of yeast extract, 1000 mL of distilled water, pH=7.

LB solid medium: 10 g of trypsin, N aCl10 g, 5 g of yeast extract, 1000 mL of distilled water, pH=7, and 15-20 g of agarose.

Seed liquid medium: 5 g of protein, 1 g of yeast paste, 35 g of sea salt, 1000 mL of distilled water, pH=7.6-7.8.

Ampicillin Amp (100 mg / mL): dissolve 1 g of Amp in sufficient water, dissolve to 10 mL, filter, and store in-20℃.

I PTG Solution (50 mg / mL): Mix as indicated and save at-20℃.

TGS buffer solution: add a bag of TGS instant particles (Jiangsu Yugong Life Science and Technology Co., LTD.) to 600 mL of distilled water. After complete dissolution, add distilled water to 1 L, and then keep at room temperature.

PBS solution: add a bag of 1 PBS instant particles to 600 mL of distilled water, after fully dissolved, add distilled water to 1 L, and then keep at room temperature.

TAE buffer: Take 50 TAE, dilute the buffer to 1 TAE buffer and store at room temperature.

50 ml of imidazole PBS: 0g of imidazole, 200 mL PBS.68

500 mM imidazole PBS: 6.8 g imidazole, 200 mL PBS

Protein staining solution: 250 mL of ethanol, 1 g of Coomassie brilliant blue, 100 mL of glacial acetic acid, mix well and add distilled water to 1 L.

Protein decolorization solution: take 200 mL of ethanol, 100 mL of glacial acetic acid, mix well and add distilled water to 1 L.

2.2.3 Rfbc primer

The whole genome sequence of *Pseudoalteromonas agarivorans* Hao 2018 in Genbank (GCA _ 003668795.1). The primers were synthesized by Shanghai Parxeno Biotechnology Co., Ltd., and the sequence information is shown in Table 2.1:

Table 2.1 - 2110 for primer

Primer	Order
CT -F	CGAGCTCGTGAATTTTATAGAAACCGATATTCCA
CF -R	ATGCGGCGCTTAAAACGTAGGTGCTTTTGTAAATG

2.2.4 Experimental reagent

Table 2.1 - **List of experimental reagents**

Reagent	Manufacturer
FastPure® Bacteria DNA Isolation Mini Kit Genomic extraction kit	Nanjing Novizan
Plasmid extraction kit	Nanjing Novizan
Product Purification (gel recovery) kit	Nanjing Novizan
DL 2000 Plus DNA Marker	Nanjing Novizan
DL 5000 Plus DNA Marker	Nanjing Novizan
RNase-free ddH_2O	Nanjing Novizan
2×Phanta Max Master mix（Dye Plus）	Nanjing Novizan
T4 DNA Ligase	
10×Ligase Buffer	Nanjing Novizan
2 × Taq Master Mix	Nanjing Novizan
LightNing™ NdeI	Nanjing Novizan
LightNing™ BamHI	Jiangsu Yugon
10×CutOne™ Color Buffer	Jiangsu Yugong
Color gel quick kit	Jiangsu Yugong
5 SDS-PAGE Loading Buffer protein loading buffer (reducing nature)	New Samme New Samme

Note: All other conventional reagents were stored in building A217

2.2.5 Experimental instruments

Table 2.2 - **List of the experimental instruments**

laboratory apparatus	**manufacturer**
Sterile ultra-clean work table	Su Jing group
A PCR amplicator	Hangzhou Bori Technology Co., Ltd
Constant-temperature biochemical incubator	Guangzhou Gredemuxue Technology
thermostat water bath	Development Company
Cell ultrasonic cruter	Shanghai Yiheng Technology
high-speed centrifuge	Jiangsu Tianli Instrument
electronic balance	Brocade E-commerce
pH count	Olebo Electronics
Nucleic acid electrophoresis instrument	Bangyi Precision Instrument
Temperature control shake bed	Beijing Junyi Oriental Electrophoresis
Autoclave steam sterilization cooker	Equipment Company
magnetic stirring apparatus	Shanghai Shanzhi Equipment Co., LTD
Low speed centrifuge	Aaibao Medical Technology Co., Ltd
Gel Imager	Gongyi City Yingyu Hongyuan instrument
ultra low temperature freezer	factory
Refrigerated refrigerator	Shanghai Keya Biotechnology Co., LTD
	Gene dirix, Inc
	Huitai Instrument & Equipment Co., Ltd
	Meiling refrigerator Co., Ltd

2.3 Experimental method

2.3.1 Bacterial reactivation

The *Pseudoalteromonas agarivorans* Hao 2018 plate was picked with 10 μ L tip and the colony tip was placed in 5 mL seed liquid medium for 37℃, 200 rpm overnight (8-16 h) for activation.

Select glycerol bacteria, add 100 μ L of glycerol solution to 5 mL LB liquid medium and activate at 37℃, 200 rpm overnight (8-16 h).

2.3.2 Genomic DNA extraction

Using the Vazyme genome extraction kit, there is no need for toxic reagents such as phenol chloroform, time-consuming alcohol precipitation, and maximum removal of RNA, miscellaneous proteins, lipids and other inhibitory impurities. The extracted genomic DNA has high purity and stable quality and can be used in downstream experiments such as enzyme digestion and PCR.

Buffer GA: Provide the sample enzymatic digestion environment;

Proteinase K: Enzymatic digestion of bacterial samples;

RNase A: RNA in the sample;

Buffer GB: Inactivation of Proteinase K, to provide the upper column environment;

Buffer PB: Remove the residual protein and RNA and other impurities in the DNA;

Buffer PW: Remove the residual salt ions in the DNA;

Elution Buffer: Elut the DNA on the binding column;

FastPure gDNA Mini Columns III: adsorption of genomic DNA;

Collection Tubes 2 ml: Filtrate collection tube.

1. Take 1-5 ml of the activated bacterial culture medium (less than 1.0109Bacteria), centrifuged at 10,000 rpm (11,500 g) for 1 min and discarded the culture medium.(The bacterial number can be measured by a spectrophotometer with 1 OD 600 of approximately 1.5 109A bacterium.）

2. Add 230 μl Buffer GA and shake until the bacteria are thoroughly suspended.

3. Add 20 μl Proteinase K and mix well with shaking.

4. Add 250 μl Buffer GB, mix well and 70℃ water bath for 10 min.(The addition of Buffer GB produces part of the white precipitation, which generally disappears at 70℃, and will not affect the subsequent experiments. If the solution is not clear, the cell lysis is not complete, which may result in small and impure amounts of extracted DNA.）

5. Add 4 μl RNase A to the digestive solution, shake it for 15 sec, and leave it at room temperature for 5-15 min.

6. Add 180 μ l of absolute ethanol and mix well with shaking, so that flocculent precipitation may occur, and then centrifuge briefly to collect the liquid on the inner wall of the tube cover.

7. Transfer the above mixture to a FastPure gDNA Mini Columns III adsorption column (the adsorption column has been placed into the collection tube), centrifuge at 12,000 rpm (13,400 g) for 1 min, and discard the filtrate.

8. Add 500 μl Buffer PB (absolute ethanol) added to the adsorption column, centrifuge at 12,000 rpm (13,400 g) for 1 min and discard the filtrate.

9. Add 600 μl Buffer PW (absolute ethanol) to the adsorption column, centrifuge at 12,000 rpm (13,400 g) for 1 min, and discard the filtrate.

10. Repeat the previous step

11. Place the adsorption column back into the collecting tube and centrifuge at 12,000 rpm (13,400 g) for 2 min.(After centrifugation of the empty column, the lid can be opened for 2-5min to completely evaporate the residual ethanol.）

12. Transfer the adsorption column to a new 1.5 ml centrifuge tube (self-supplied), add 50-100 μl Elution Buffer to the central part of the adsorption column membrane, place it at room temperature for 2-5 min, and centrifuge at 12,000 rpm (13,400 g) for 1 min.(The following steps can all help to increase the DNA production

(1) Preheat the Elution Buffer to 55℃ and then elute;

(2) To increase the concentration of DNA, the solution obtained from the first elution was added to the adsorption column for elution.）

13. The adsorption column was discarded, and the DNA product was stored at-30 to-15℃ to prevent degradation.

2.3.3 Plasmid extraction

The V azyme plasmid extraction kit was used

1. Add 1 mL of activated (12 h) overnight culture to a centrifuge tube and centrifuge at 10,000 rpm (11,500 g) for 1 min. Discard the medium, put the centrifuge tube upside down on the absorbent paper so that the residual liquid in the tube is completely discharged;

2. Add 250 μL BufferP1 to the centrifuge tube, blow the pipete gun to resuspend the bacteria thoroughly; add 250 μL Buffer P2 to the centrifuge tube, mix gently 8-10 times to ensure the complete lysis; add 350 μL Buffer P3, immediately gently reverse 8-10 times to make the Buffer P2 thoroughly neutralized, and the white precipitate in the centrifuge tube. Centrifugation at 12,000 rpm (13,400 g) for 10 min;

3. Place the FastPure DNA Mini Columns adsorption column in a Collection Tube 2 mL collection tube. The step (2) supernatant was pipetted and 700 μ L was transferred to the adsorption column and centrifuged at 12,000 rpm (13,400 g) for 1 min. Remove the filtrate in the collection tube, and then place the adsorption column again in the collection tube;

4. Add 500 μL Buffer PW1 to the adsorption column. Centrifuge at 12,000 rpm (13,400 g) for 1 min. Remove the filtrate in the collection tube, and then place the adsorption column again in the collection tube;

5. Add 600 μL Buffer PW2 to the adsorption column and centrifuge at 12,000 rpm (13,400 g) for 1 min. Remove the filtrate in the collection tube, then rearrange the adsorption column in the collection tube, and repeat this step;

6. Put the adsorption column with the filtrate back into the collection tube.12,000 rpm (13,400 g) 1 min dry adsorption column, the purpose is to completely remove the residual rinse solution in the adsorption column;

7. Transfer to a sterile ultra-clean work table, and transfer the adsorption column to a new 1.5 mL centrifuge tube. 50 μL ddH_2O was added to the center of the membrane of the column adsorption column. Hold at room temperature for 2 min, and then centrifuge at 12,000 rpm (13,400 g) for 1 min to elute the plasmid DNA;

Note: Preheat ddH_2O to 55℃ and elute to improve elution efficiency;

Buffer P1 RNaseA (RNaseA stored in 4℃);

Buffer PW2 Yes, diluted with absolute ethanol.

8. After the centrifugation is completed, the adsorption column is discarded, the centrifuge tube is marked, and the product is stored at-20℃ to prevent degradation.

2.3.4 PCR of the Rfbc enzyme target genes

The PCR reaction system and reaction procedures are shown in Table 2.3 and Table 2.4. The configuration reaction system must be sterile and ultra-clean work table operated on ice. After the end of PCR, 25 μL PCR products were verified by agarose gel electrophoresis.

Table 2.3 - **The PCR reaction system**

Reaction components	Component volume
ddH_2O	10.0 μL
2 ×Phanta Max Master Mix(Dye Plus)	12.5 μL
DNA templet	0.5 μL
Upstream primers	1.0 μL
Downstream primers	1.0 μL

Table 2.4 - **PCR, reaction procedure**

Cycle steps	**temperature**	**time**	**recurring number**
Predenaturation	95℃	03：00	
denaturation	95℃		30
anneal	55℃	00：15	
extend	72℃	00：15	
Thoroughly extend	72℃	01：30	
		05：00	

2.3.5 Gel electrophoresis of Rfbc enzyme (gel recovery)

1. Allocation at 1% agarose gel for 20 mL

2 and agarose gel electrophoresis

Remove the fully solidified agarose gel and place it in the nucleic acid electrophoresis tank containing TAE buffer (the gel must be completely covered in TAE buffer), sample successively, and then electrophoresis gel for 30 min.

The addition order from left to right were PCR amplification products of the target gene (25 μ L), D L 2000P lus DNA M arker (7 μ L) and PCR amplification products of the target gene (25 μ L).

3. Gel recovery of the target fragments

（1） Use the Vazyme product purification (gel recovery) kit, according to the Buffer GW bottle label and stored at room temperature.

（2） After the electrophoresis, the gel was placed under the UV lamp irradiation, the gel containing the target DNA fragment was quickly cut off, the surface liquid of the gel was aspirated, and the excess surrounding gel was removed. The weight is 1.232 g and 1.156 g, respectively, the tube weight is 0.822 g, and the

gel weight is 0.410 g, 0 and 0.334 g, respectively. The 100mg gel is the same as 100 ul volume, as a gel volume.

(3) Add 300 μ L (equal volume) Buffer GDP, and take a 55℃ water bath for 7 min to ensure that the gel block is completely dissolved, and mix well twice during the water bath to accelerate the sol;

(3) The residual liquid on the tube wall and tube cover is collected by brief centrifugation. Place the FastPure DNA Mini Columns-G adsorption column in the Collection Tubes 2 mL collection tube, transfer the sol liquid to the adsorption column, and centrifuge at 12,000 rpm (13,400 g) for 1 min;

(4) discard, collect the filtrate in the tube, then place the adsorption column again in the collection tube, add 300 μL Buffer GDP to the adsorption column, and stand at room temperature for 1 min. Centrifuged at 12,000 rpm (13,400 g) for 1 min;

(5) Remove the filtrate in the collection tube, then rearrange the adsorption column in the collection tube, add 700 μL Bu f fer GW adsorption column, add Buffer GW, reverse and mix well 2-3 times to completely rinse the salt attached to the wall of the centrifuge tube, centrifuge at 12,000 rpm (13,400 g) for 1 min;

(6) Repeat step (5) (ensure that the salt attached to the wall of the centrifugal tube is completely rinsed clean);

(7) Remove the filtrate in the collection tube, and then place the adsorption column again in the collection tube at 12,000 rpm (13,400 g) for 2 min to ensure the complete removal of the liquid in the adsorption column;

(8) Transfer to a sterile superclean table, transfer the adsorption column to a new 1.5 mL centrifuge tube, add 30 μL ddH_2O to the center of the adsorption column for 2 min, 12,000 rpm (13,400 g) and centrifuge for 1 min. The adsorption column was discarded and the DNA was stored at-20℃.

Note: Preheat the ddH_2O to 55℃ in advance before elution to improve the elution efficiency;

Buffer GW absolute ethanol has been added proportionally.

2.3.6 Fragment and plasmid double digestion

1. Double digestion

The recovered products and plasmid of the target gene were double digested separately, and the recovered products and plasmid double digestion systems are shown in Table 2.5 and Table 2.65, respectively. The double digestion system must be sterile ultra-clean work table on ice. Complete the double digestion reaction system for 30 min; the plasmid double digestion reaction system shall complete the 37℃ water bath for 15 min.

Table 2.5 - **Double-enzyme digestion reaction system of the recovered products**

Reaction components	Component volume
LightNing™ N de I	1.0 μL
LightNing™ BamHI	1.0 μL
10×CutOne™ Color Buffer	3.0 μL
outcome DNA	25.0 μL

Table 2.6 - **Plasmid double-enzyme digestion reaction system**

Reaction components	Component volume
LightNing™ N de I	2.5 μL
LightNing™ BamHI	2.5 μL
10×CutOne™ Color Buffer	5.0 μL
plasmid DNA	40.0 μL

2. configure a 1% agarose gel for 20 mL

3 and agarose gel electrophoresis

(1) Remove the solidified agarose gel and place it in the nucleic acid electrophoresis tank containing TAE buffer (the gel should be completely covered in TAE buffer), sample successively, and then electrophoresis gel for 30 min.

(2) Double digestion of product is recovered, and the addition order from left to right is double digestion (30 μ L) and D L 2000P lus DNA M arker (7 μ L) double digestion (30 μ L).

(3) Plasmid was double digested, and the addition order from left to right was double product (30 μ L), D L 5000P lus DNA M arker (7 μ L) and double product (30 μ L).

4. Recof digested products

Use the Vazyme product purification (gel recovery) kit

(1) After DNA electrophoresis, place the gel under UV lamp irradiation, cut the gel containing the target DNA fragment, remove excess gel, standard number: 1 PCR, 2 PCR, 3 Pet-16b, 4 Pet-16b-1; weighing is 1g, 1g, 1g, 1g and 1 g, air tube weight is 0g, gel weight is 0g, 0g, 0g and 0g;.139 .107 .035 .054 .788 .351 .319 .247 .266

(2) Add 350 μ L (equal volume) Buffer GDP and take a 55℃ water bath for 7 min to ensure that the gel block is completely dissolved, reverse during the water bath and mix twice to accelerate the sol;

.2.5Experimental step (3) - (8) as step in section 3 (3) - (8).

Note: Preheat the ddH_2O to 55℃ before elution to improve the elution efficiency. Buffer GW absolute ethanol has been added proportionally

2.3.7 T4 ligation of the DNA fragment and the plasmid vector

The digested plasmid vector and the target gene fragment were ligated, and the T4 DNA ligase ligation system was configured at 16℃ overnight (12-16 h), and the ligation products were stored at-20℃. The connection system is shown in Table 2.7.

Table 2.7. - **T4 DNA Ligase Connection system**

Reaction components	Component volume
10×ligase B uffer	1.0 μL
DNA part	4.0 μL
plasmid vector	4.5 μL
T 4 DNA L igase	0.5 μL

2.3.8 DH 5 α transformation

1. LB solid medium was inverted

Take the LB solid medium that has been sterilized, heat at high temperature for several minutes to melt completely (heating time is determined according to whether completely melted), cool to about 70℃, and add Amp (ampicillin) at the ratio of Amp: medium =1:1000.

After that, the plate was inverted, and an appropriate amount of LB medium was added to each plate. After the inverted plate, the plate was sealed with the sealing film. During the ultra-clean table should not be illuminated with ultraviolet.

2. Product conversion

(1) Remove the sensation from the-80℃ refrigerator and thaw the receptor on ice DH 5 α;

(2) 5 μ L of the ligation product was added to 50 μ L of the competent DH 5 α, gently mix well, and then stand on ice for 30 min;

(3) 42℃ water bath water bath heat shock 90 sec, after the heat shock, quickly transferred to the ice for cooling for 3 min;

(4) Shake 950 μ L of LB liquid medium with Amp (ampicillin) to the product of step (3) and press at 37℃ for 1 h (200 rpm);

(5) Take the first (4) product at 6000 rpm, centrifuge for 3 min, discard 850 μ L supernatant, blow and resuspend, take 150 μ L coating;

(6) The coated plate was sealed with a sealing membrane and then placed in a 37℃ inverted incubator for 12-16 h.

2.3.9 PCR validation of product-transformed colonies

Colony PCR amplification

Take out the plate, observe the growth of the colonies, pick up the single colony (take- -half colonies) from the ultra-clean workbench, pick up 6-12 colonies from a culture plate, make the serial number mark, and verify the subsequent experimental operation. After the completion of the picking, seal the sealing membrane at 4℃.

Concolony PCR to verify the reaction system. The colony PCR reaction systems and reaction procedures are shown in Table 9 and Table 10. After the end of the PCR amplification, 10 μL PCR of the products were verified by agarose gel electrophoresis.

Table 2.8 - **Colony PCR reaction system**

Re components	Component volume
ddH_2O	4.0. μL
Upstream primers	0.5 μL
Downstream primers	0.5 μL
2×Taq M aster M ix	50 μL

Table 2.9 - **Procedures of PCR reactions of the colonies**

2. Allocation concentration of 1% agarose gel was 20 mL

Cycle steps	Temperature	Time	Recurring number
Predenaturation	95℃	05：00	
denaturation	95℃	00：15	
anneal	55℃	00：15	30
extend	72℃	01：30	
Thoroughly extend	72℃	05：00	

3 and agarose gel electrophoresis

(1) Remove the solidified agarose gel and place it in the nucleic acid electrophoresis tank containing TAE buffer (the gel should be completely covered in TAE buffer), sample successively, and then electrophoresis gel for 30 min.

(2) Add sample: D L 2000P lus DNA M arker, PCR products

(3) Electrophoretic imaging verification, and the bands that meet the expected fragment length are marked.

2.3.10 PCR validation of the plasmid

Colonies that have been successfully verified in the colony PCR are selected and verified by plasmid PCR according to the labeled serial number

1. Find a single colony that has been dipped in half and verified successfully;

2. lip 10 μ L gun tip into the other half of the colony;

3. The head of the colony is placed in 5 mL LB liquid medium (5 μL A mp has been added);

4. Place the already inoculated LB liquid culture in a shaker for 37℃, 200 rpm overnight.

5. Overnight culture of bacterial solution for plasmid extraction, use V azyme plasmid extraction kit, make clear labeling;

6. Plasmid extraction (plasmid extraction step is the same as 3), and save the extracted recombinant plasmid at-20℃..3.3

7. configure plasmid PCR to verify the reaction system, the reaction system and reaction procedures are shown in Table 2.10 and Table 2.11. After the end of PCR amplification, 15 μL PCR products were verified by agarose gel electrophoresis.

Table 2.10 - **The plasmid PCR reaction system**

Reaction components	Component volume
ddH_2O	7.0 μL
Upstream primers	1.0. μL
Downstream primers	1.0 μL
2×Taq Master Mix	10 .0μL
plasmid DNA	1.0 μL

Table 2.11 - **The plasmid PCR reaction n procedures**

Cycle steps	temperature	time	recurring number
Predenaturation	95℃	03：00	30
denaturation	95℃	00：15	
anneal	55℃	00：15	
extend	72℃	01：30	
Thoroughly extend	72℃	05：00	

8. Arrange in a 1% agarose gel for 20mL

9 and by agarose gel electrophoresis

(1) Remove the solidified agarose gel and place it in the nucleic acid electrophoresis tank containing TAE buffer (the gel should be completely covered in TAE buffer), add the sample successively, and then run the electrophoresis gel for 30 min;

(2) addition: D L 2000P lus DNA M arker, plasmid PCR product;

(3) Electrophoretic imaging verification, and the bands that meet the expected fragment length are marked.

10 Plasmid PCR was successfully verified and maintained

After the verification of plasmid PCR, 500 μ L bacterial solution and 500 μ L 40% glycerol were stored in EP tubes.

Four branches of each culture medium were kept at-20℃ and-80℃, respectively.

2.3.11 Transformation of the BL 21 product

1. Remove the receptive from the-80℃ refrigerator and thaw the receptive B L21 (DE 3) on ice;

2. Add 10 μ L of plasmid DH 5 α to 50 μ L of competent DE 3, gently mix well, and then rest on ice for 30 min;

3.42℃ water bath pot water bath heat shock 90 sec;

4. Transfer to ice immediately after heat shock to cool 3 min ；

5. Add 950 μ L of LB liquid medium with Amp (ampicillin) to the step (4) product and shake 1 at 37℃ in the shaker h（200 rpm） ；

6. LB plate preheating (in advance);

7. Take 100 μ L of product for coating;

8. The coated dishes were sealed with a sealing membrane, and then placed in a thermostatic incubator with 37℃ inverted culture for 12-16 h;

9. Colony PCR verification (same as 3);.2.9

10 Liquid LB medium was overnight

Single colonies of the previous colony PCR verification were selected with 10 μ L gun tip dipped in 5 mL LB Overnight (8-16 h) culture in medium (5 μL A mp already added)

After the culture was completed, each tube was protected and stored at-20℃ and-80℃, respectively.

2.3.12 Verification of cell fragmentation

1.2% inoculum (overnight culture of bacterial solution in the previous step) was inoculated in 5 mL LB liquid medium at 37℃ for 1 h;

2. Add 2.5 μL IPTG inducer to the 1 step tube;

3.37℃ was induced for 24 h;

4. The overnight bacterial solution was transferred to a 10mL centrifuge tube for centrifugation, 8000 rpm, 5 min, and 200 μ L of the supernatant (this supernatant was extracellular);

5. Discard the supernatant and add 5 mL PBS for resuspension and mix;

6. Ice bath ultrasonic crushing for 5min

7. 200 μ L of whole cells were taken after fragmentation;

8. The remaining liquid was centrifuged at 8000 rpm for 5 min, and 200 μ L of the supernatant was removed (this supernatant was intracellular);

9. Add 50 μ L 5 SDS-PAGE L oading B uffer to the extracellular, whole cell and intracellular samples;

10. Configuration of the protein glue

Preparation by one-step glue filling using the ExpressCast PAGE color gel rapid kit:

(1) absorb 2.5 mL of the lower volume of glue solution and 2.5 mL of the lower glue buffer, and gently mix the lower layer;

(2) Take an equal volume of 0.75 mL of upper glue solution and 0.75 mL of upper glue buffer, respectively, and gently mix well;

(3) Draw 50 μ L of improved coagulant into the mixture of step 1, gently mix, and then pour into the rubber plate, so that the distance between the liquid level and the upper edge of the short glass plate is 0.5 cm longer than the distance between the comb tooth and the upper edge of the short glass plate;

(4) Add 15 μ L of improved coagulant into the mixture of step 2, gently mix well, without waiting for the lower adhesive to solidify, directly pour the mixed upper adhesive solution gently into the rubber board and inserted into the comb;

(5) After the glue is completely solidified (30 min), remove the comb and prepare the sample.

11 From left to right, the order is two-color protein Marker, extracellular, whole cell, and intracellular; two-color protein Marker, extracellular, whole cell, and intracellular.

In addition, 5 μ L of M arker and 30 μ L of extracellular, extracellular, whole cell and intracellular.

12. In Tris-Glycine buffer system;

13. After the electrophoresis, remove the protein glue and put it into the protein staining solution for staining (horizontal shaker for 3 h);

14. After the staining, put the protein discoloration solution to color (horizontal shaking table for several hours);

15 Subsequent experiments were performed after successful validation.

2.3.13 IPTG was induced by the optimized expression conditions

1. Activated BL21 (DE 3) seed bacterial solution

Add 100 μ L of salvage solution to 5 mL LB liquid medium (5 μL A mp added) for 37℃, 200 rpm overnight culture (8-12 h)

2. Take 2 mL of activated seeds (previous product) and add 200 mL LB of liquid medium (200 μL A mp already added)

3. Place LB liquid medium (200 mL) in a shaker for 37℃, 200 rpm and grow for 1 h

4. 100 μL IPTG of inducer was added to the conical flask and incubated at 16℃ at 200 rpm for 24 h

5. Cell breakage (all operations on ice)

(1) 200 mL of bacterial solution (16℃, I PTG induction for 24 h) was completely centrifuged using 50 mL centrifugal tubes (4 tubes) for 4℃, 8000 rpm, and 10 min

(2) The supernatant was discarded and resuspended in 50 mM imidazole PBS, and resuspended for 25 min after cell fragmentation

(3) In cell fragmentation, 200 μ L (whole cell) was removed, the residual liquid was centrifuged at 8000 rpm and 5 min, and all the supernatant (200 μ L was intracellular) was left over 0.22 μ m filter membrane

6. Protein purification (Ni column purification)

(1) Remove the protective liquid in the Ni column (leave 0cm) to prevent total drying.5

(2) Add 5 mL of sterile water (over 0.22 μ m filter membrane)

(3) When the water discharge is finished, add 5 mL 50 m M imidazole PBS to the Ni column (over 0.22 μ m filter membrane)

(4) When the discharge is finished, add the cell crushing fluid (over 0.22 μ m filter membrane), collect the filtrate, and leave 200 μ L sample filtrate

(5) Add 5 mL 50 mM imidazole PBS (over 0.22 μ m filter membrane)

(6) When the discharge is finished, 5 mL 500 m M imidazole PBS is added to collect the filtrate. When the discharge is finished, the collection ate is added to the column again and then collected. The filtrate collected at this time was the target protein (200 μ L retention sample filtrate)

(7) When completion, 10 mL 500 m M imidazole PBS was added to the column (over 0.22 μ m filter)

(8) Add 10 mL 50 m M imidazole PBS to the column (over 0.22 μ m membrane)

(9) Add 20 mL of sterile water to the column (over 0.22 μ m filter film), and leave half of the water in the column.

(10) Use the sealing membrane to seal the Ni column and save the lid.

7. Activate the dialysis bag in the ddH_2O for 30 min

Put the protease solution (target protein) into an activated dialysis bag and soak it in PBS solution to remove imidazole, 4℃, and soak overnight.

The extracellular, whole cell, intracellular, filtrate, target protein and pure protease were added to 50 μ L 5 SDS-PAGE L oading B uffer. Heat was boiled for 2min and centrifuged at 10000r for 5min

9. Configure the protein glue

One-step glue-filling preparation using the ExpressCast PAGE Color Gel Quick kit

One-step glue preparation: 10% separation glue, glue thickness of 1.0mm as a column

(1). Take an equal volume of 2.5ml of lower glue solution and 2.5 ml of lower glue buffer, respectively. Gentle mix.

(2). Take equal volume of 0.75ml of upper glue solution and 0.75ml of upper glue buffer, and mix gently (shake well before use).

（3). To the mixing solution of step 1, add 50ul of improved procoagulant and gently mix into the rubber plate, so that the distance between the liquid level and the upper edge of the short glass plate is 0.5cm longer than the comb tooth

（4). To the mixture of step 2, add 15ul of improved coagulant, gently mix, without waiting for the lower glue to solidify, directly pour the mixed solution gently into the rubber board and insert into the comb.(Avoid flushing the upper glue solution into the lower glue solution).

（5). After solidification (about 20-30min), remove the comb in the Tris-Glycine electrophoresis buffer system at 150V and electrophoresis for 40-60 minutes.(Note: after the glue is solidified, the flatness of the upper and lower adhesive boundary is weaker than that of the conventional two-step method, but it has no effect on the subsequent electrophoresis).

10. From left to right, the top sample order is two-color prestained protein Marker, whole cell, intracellular, filtrate, target protein, pure protease, pure protease, and two-color prestained protein Marker.

Among them, 5 μ L of double-color predye protein Marker was added, and 30 μ L of whole cell, intracellular, filtrate, target protein and pure protease were added.

11. In the Tris-Glycine electrophoresis buffer system, electroresis for 60 min;

12. After the electrophoresis, remove the protein glue and place it in the protein staining solution for staining (horizontal shaker for 3 h);

13. After the staining, put in the protein discoloration solution for coloring observation (horizontal shaking table for several hours);

14 Observe the SDS-PAGE analysis images and judge the results.

2.4 Experimental method

2.4.1 Bioinformatics analysis of physicochemical parameters of Rfbc enzyme proteins

Using the ProtParam tool (https: / / www.expasy. Og /) Prediction P.agarivorans Theoretical molecular weight, isoelectric point and instability

coefficient (Instability index, II) of the source-derived dTDP-4-dehydrorhamnose 3,5 isomerase protein.

2.4.2 Secondary structure and tertiary structure prediction of the Rfbc enzyme protein

Utilizing the PRABI (http: / / www.prabi. Secondary structure prediction of Rfbc enzyme protein by the process (HNN) of Hierarchical Neural Network in fr /);

Using the SWISS-MODEL (https: / / swissmodel.expasy. Org / interactive) for homology modeling prediction of the tertiary structure of the Rfbc enzyme protein.

2.4.3 Phylogenetic tree construction

support P. Phylogenetic tree for construction of the amino acid sequence of agarivorans Hao 2018-derived Rfbc with the Rfbc of 17 different species published in NCBI.

2.4.4 multiple sequence alignment

The amino acid sequences of Rfbc enzyme proteins from the seven species closest to the Pseudomonas agarivorans branch were selected for multiple sequence alignment using Clustal Omega (https: / / www.ebi.ac.uk/services.

Summary of chapter II

1. Bioinformatics analysis method of RFBC gene
2. Construction of Escherichia coli heterologous expression system

CHAPTER 3
EXPERIMENTAL PART

This chapter is the main experimental link of this topic. In this experiment, the *Pseudoalteromonas agarivorans* Hao 2018 genome was extracted and the dTDP-4-dehydrorhamnose 3,5 isomerase gene was amplified by PCR; the cloned fragment sequence was connected with pET-16b (plasmid), and the expression vector (BL21) [15] was constructed. After verifying the expression vector, the protein expression condition [14] was optimized, and then the enzymatic study was conducted.

3.1 Bioinformatics analysis of physicochemical parameters of Rfbc enzyme proteins

The nucleic acid sequence of the agarivorans-derived Rfbc enzyme contains 545 base pairs and encodes 181 amino acid residues. The theoretical molecular weight (MW) is 20.64 kDa and the theoretical isoelectric point (Theoretical pI) is 5.00. The number of negatively charged residues (Glu + Asp) is 26, and the number of positively charged residues (Lys + Arg) is 17. The instability coefficient (Instability index, II) is 33.95, indicating that for Rfbc, the enzyme protein is relatively stable. Meanwhile, the Rfbc enzyme protein aliphatic (Aliphatic index, AI, amino acid index) was 72.65, indicating that Rfbc has better thermal stability. Furthermore, the Rfbc enzyme protein has a predicted half-life of 30 h in mammalian reticulocytes cultured in vitro, over 20 h in yeast and 10 h in *Escherichia coli*.

3.1.1 Secondary structure and tertiary structure prediction of the Rfbc enzyme protein

The secondary structure prediction results showed that α helix (Alpha helix) accounted for 14.36%, 32.60% extended backbone (Extended strand) and 53.04% uncoiled (Random coil), and no other secondary structure (Figure 3.1). Homology

modeling prediction of the tertiary structure of the Rfbc enzyme protein was then performed using S WISS-MODEL (Figure 3.2).

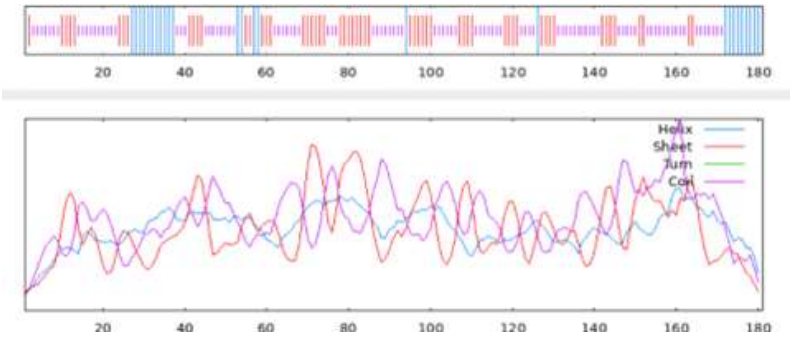

Figure 3.1. - Secondary structure prediction of the *P. agarivorans*-derived Rfbc protease

Note: The blue color represents the helical structure, the positive red color represents the extended structure, and the rose red color represents the coiled structure

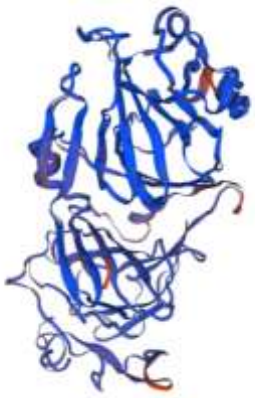

Figure 3.2. - Tertiary structure homology modeling prediction results of the Rfbc enzyme protein by SWISS-MODEL

3.1.2 Phylogenetic tree construction

The amino acid sequence of *P. agarivorans* Hao 2018-derived Rfbc was subjected to multiple sequence alignment with Rfbc amino acid sequences from 10

different species published in NCBI and a phylogenetic tree was constructed[18]. The phylogenetic tree alignment showed that the 10 species involved in the analysis formed two relatively independent clades (Figure 3.3A).

3.1.3 Multiple sequence alignment

Rfbc from a total of 10 species closest to this clade were selected for multiple sequence alignment (Figure 3.3B). The alignment results indicate that *P. agarivorans*-derived Rfbc has a similar folding manner, active site and catalytic mechanism [20] as other sources Rfbc have been reported.

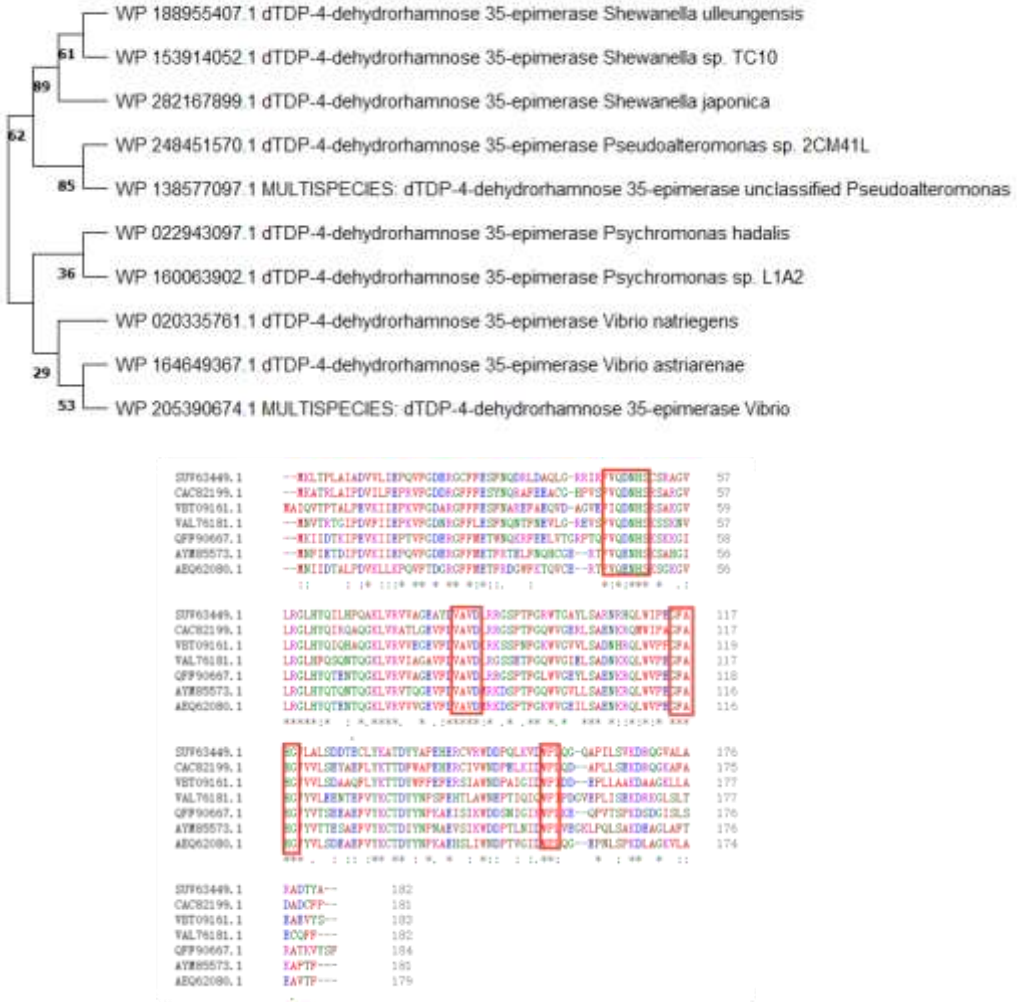

Figure 3.3 (A) phylogenetic relationships of amino acid sequences of Rfbc from different species sources; (B) multiple sequence alignment of Rfbc from different species sources

After bioinformatics analysis of Rfbc enzyme (dTDP-4-dehydrorhamnose 3,5 differential-directed isomerase), the basic physicochemical parameters of Rfbc enzyme protein were obtained, which was grounds for the cloning and expression of dTDP-4-dehydrorhamnose 3-5-directed isomerase. The category of the Rfbc enzyme is predicted by its secondary structure prediction. Finally, by constructing a phylogenetic tree and multiple sequence alignment, the *P. agarivorans*-derived Rfbc has a similar folding mode, active site and catalytic mechanism to other reported sources, Rfbc. It lays the theoretical basis for future exploration of the restriction site, the catalytic mechanism during rhamnose synthesis.

3.2 Results and Discussion

3.2.1 PCR amplified electrophoresis images of Hao 2018 target fragments

The cloned Rfbc enzyme gene was verified by electrophoresis. As shown in Figure 3.4, the nucleic acid sequence of Rfbc enzyme gene was 545 bp, and the target fragment showed that the cloning of Rfbc enzyme gene was successfully completed.

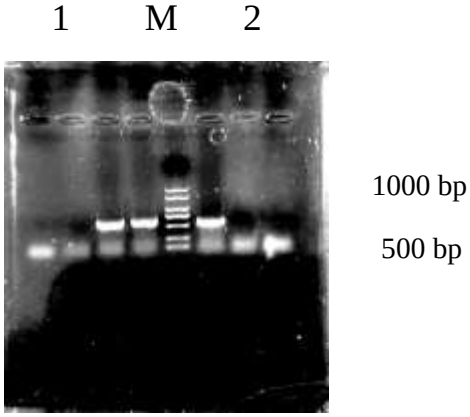

Figure 3.4 - Target fragment by PCR electrophoresis

(1,2: cloned Rfbc enzyme M： DL 2000 Plus DNA Marker）

3.2.2 Double digestion electrophoreimage of Rfbc fragment and plasmid

The Rfbc enzyme gene cloned by PCR and the extracted empty plasmid were double digested, and the electrophoresis verification of the double digestion of the

Rfbc enzyme gene was shown in Figure 3.5 (A), and the double digestion of the plasmid.

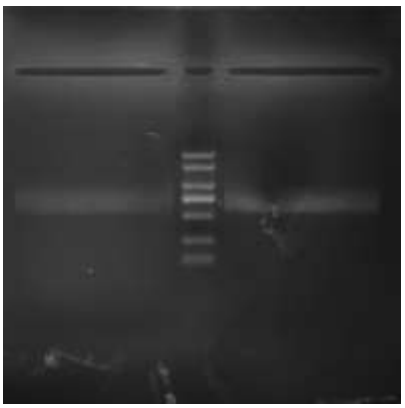

Figure 3.5 (A)

(1,2: Rfbc enzyme gene

M：DL 2000 Plus DNA Marker）

Figure 5 (B) Double plasof double digestion

(1,2: digested plasmid Pet-16b

M:DL 5000 Plus DNA

Image, Figure 5 (B), shows that the digested Rfbc enzyme gene and the plasmid pET-16b were successfully obtained.

3.2.3 DH 5 α transformation validation

The build Rfbc enzyme protein expression vector into competent DH5α, colony PCR verification and plasmid PCR verification, electrophoresis validation images are Figure 3.6 (A) and Figure 3.6 (B), colony PCR validation images in left 1 and left 2, left 1, left 3 and left 4 in plasmid PCR validation, validation results show that the constructed expression vector can be expressed in *E. coli.*

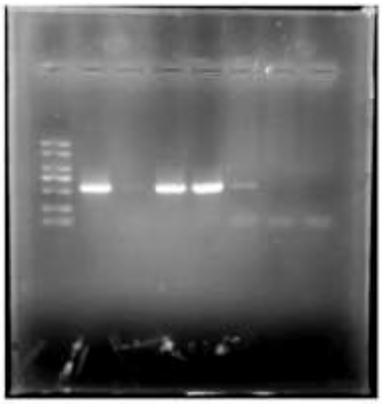

Figure 3.6 - Colony PCR validation

(1-6: Verification of PCR of expression vector colonies

M :DL 2000 Plus DNA Marker）

3.2.4 PCR validation of BL 21 product-transformed colonies

The validated expression vector was transformed into B L21 E. coli, which showed obvious single colony growth, as shown in Figure 3.7 (B), followed by colony PCR. The validation images are shown in Figure 3.7 (A), and the electrophoretic bands were relatively clear. The results showed that the construction of Hao 2018-derived Rfbc enzyme protein expression vector could complete heterologous expression.

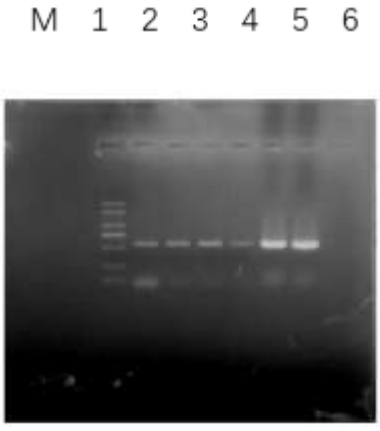

Figure 3.7 (A) Colony PCR validation

(1-6: PCR validation of BL21 colonies

Figure 3.7 (B) A BL21 product transformation dish

3.2.5 IPTG-induced protein purification

The constructed Rfbc enzyme protein expression vector successfully expressed the target enzyme protein in E. coli, which was purified by Ni column affinity chromatography, and the SDS-PAGE analysis results are shown in Figure 8. The results proved that the target enzyme protein with a molecular weight of about 24.95 kDa and good purity was successfully obtained.

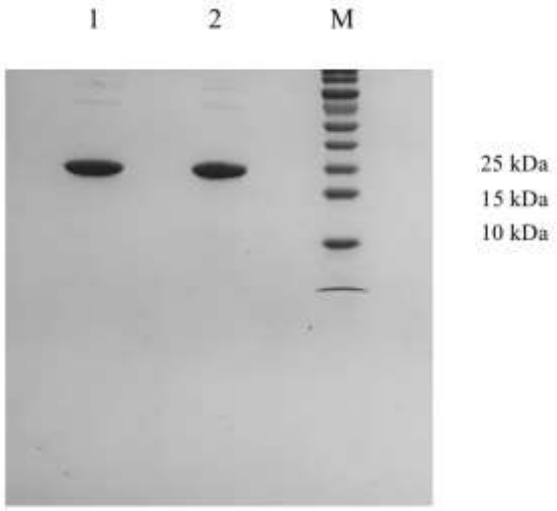

Fig. 8 protein gel image of protein purification (1: whole cell, 2: purified protein

3.3 Discussion

This study on *P. agarivorans* Hao 2018, the dTDP-4-dehydrorhamnose 3,5 differential-directed isomerase gene was cloned and expressed, the Rfbc enzyme protein expression vector from Hao 2018 was successfully constructed, and the enzyme protein was successfully expressed in E. coli. After isolation and purification by Ni column, SDS-PAGE analysis showed that the target enzyme protein with good purity and correct size was obtained. The cloning and expression of Rfbc enzyme gene are analyzed in detail, but the study of dTDP-4-rhamnose 3-5 differential-oriented isomerase in *Pseudoalteromonas agarivorans* Hao 2018 still needs further depth, the study of key modules of exopolysaccharide synthesis is still lacking, and the enzymatic properties of Rfbc enzyme still need to be explored.

Summary of chapter III

1.Extraction experiment of target gene and plasmid.

2.PCR of the Rfbc enzyme target genes，T4 ligation of the DNA fragment and the plasmid vector，PCR validation of BL 21 product-transformed colonies，IPTG was induced by the optimized expression conditions

CONCLUSIONS

This project starts from the protein sequence analysis of Rfbc enzyme (dTDP-4-dehydrorhamnose 3,5 isomerase) from Hao 2018 (NCBI database), first obtains the length of coding amino acid residues and predicts the basic physicochemical parameters of Rfbc enzyme protein; then predicts the secondary structure of Rfbc enzyme, and finally P. The amino acid sequence of agarivorans Hao 2018-derived Rfbc was subjected to multiple sequence alignment with Rfbc amino acid sequences from different species published at NCBI and phylogenetic tree construction.

1. At the experimental phase of this subject, First, by extracting the Pseudoalteromonas agarivorans Hao 2018 genome, After adding upstream and downstream primers to the gene PCR system, The Rfbc enzyme (dTDP-4-dehydrorhamnose 3, 5-isomerase) gene, Later, the PCR amplified cloned genes were verified by agarose gel electrophoresis and gel recovery; After the plasmid extraction of the activated completed empty plasmid pET-16, After extraction, the target gene fragments and plasmids recovered were double digested and subjected to agarose gel electrophoresis and gel recovery. Then, the cloned fragment sequence was connected with pET-16b, and the expression vector was constructed. After the expression vector construction, the colony PCR verification was performed. After the verification, plasmid PCR was performed and the plasmid was extracted. After that, the plasmid expression vector was further used to construct BL21 expression vector with the extracted recombinant plasmid and verified by colony PCR. After the validation of the expression vector, the protein expression conditions were optimized, cell fragmentation and protein purification were completed, and the SDS-PAGE analysis was performed. So far, the cloning and heterologous expression of the key gene, dTDP-4-dehydrorhamnose 3,5, in the synthesis module of Pseudoalteromonas agarivorans Hao 2018-derived rhamnose sugar precursor, was completed. In this study, the cloning and expression of the Rfbc enzyme gene were analyzed in more detail. Underlaid the foundation for studying the enzymatic regulatory mechanism of

dTDP-4-dehydrorhamnose 3,5 differential orientation isomerase in Pseudoalteromonas agarivorans Hao 2018.

2. Marine bacteria are able to produce various bioactive species such as exopolysaccharides during adaptation to a highly dynamically changing Marine environment, given that P. The exopolysaccharides secreted by agarivorans Hao 2018 have been proved to be safe and non-toxic and have good production-promoting effects on degradation products, and future studies should further explore their biological activity. This includes studying the potential of extracellular polysaccharides in cellular immune regulation, heavy metal ion adsorption, etc.

3. With the deepening of scientific research, the application prospect of rhamnose in the field of medicine and food is more and more broad, However, for P. The study of the activity of exopolysaccharides secreted by the agarivorans Hao 2018 strain, especially the activity and mechanism of the key enzyme behind it, —— dTDP-4-dehydrorhamnose 3,5 differential-directed isomerase (Rfbc), still need to be further explored and verified.

4. This study focused on the cloning and expression of the Rfbc enzyme gene, and has successfully completed the cloning and construction of the expression vector for the Rfbc enzyme gene to achieve its expression in host cells. However, further research and optimization are still needed for the precise determination method of Rfbc enzyme activity. For example, a more accurate and efficient enzyme activity measurement method should be developed to comprehensively analyze the enzymatic reaction kinetic characteristics of Rfbc enzyme, determine the optimal pH and reaction temperature of the enzymatic reaction, reveal the cofactors and activators necessary for the enzyme activity, and determine the kinetic parameters of the enzymatic reaction.

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