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

QUALIFICATION THESIS

on the topic **Study on Global Transcription Machine Engineering Breeding of *Escherichia coli* Producing Eketodoxin**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Scientific supervisor
Tetiana SHCHERBATIUK, Dr. Sc., Prof.

Reviewer
Olga ANDREYEVA, Dr. Sc., Prof.

Kyiv 2024

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APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Yanhui Zhang

1. Thesis topic **Study on Global Transcription Machine Engineering Breeding of *Escherichia coli* Producing Eketodoxin**
scientific supervisor Tetiana Shcherbatiuk, Dr. Sc., Prof.
approved by the order of KNUTD "__"_____2024, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____Yanhui ZHANG

Scientific supervisor _____Tetiana SHCHERBATIUK

SUMMARY

Yanhui Zhang. Study on Global Transcription Machine Engineering Breeding of Escherichia coli Producing Eketodoxin

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

Ectoin, whose chemical name is tetrahydromethylpyrimidine carboxylic acid, is a specific amino acid derivative, derived from the metabolic process of some halophilic bacteria, and ectoin has been widely used in many fields such as enzyme preparations, cosmetics, health care products and medical treatment because of its diverse physiological activities. In view of the fact that the accumulation of heterologous natural products in synthetic biology technology is often related to complex problems such as the adjustment of metabolic networks, the construction of heterologous pathways, and the matching of genes and hosts, it was decided to adopt global transcription machine engineering (gTME) technology, which is different from classical mutagenesis and pathway engineering.

We successfully cloned the endogenous global transcription factor *rpoD* by PCR, and constructed a mutant expression library based on pET28a using error-prone PCR. We used fluorescence-based biosensors as a preliminary high-throughput screening system, and further combined with 96 deep-well microculture technology for quantitative screening, and the yield of ectoin was used as the re-screening criterion. After this series of studies, we have successfully increased the yield of ectoin in E. coli engineered bacteria using gTME technology.

The successful implementation of this project will not only deepen our understanding of the biosynthetic mechanism of ectoin, but also lay a solid foundation for its future industrial application.

Key words: Ectoin; global transcription machine engineering; transcription factors; biosensors; high-throughput screening

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INTRODUCTION

In Europe and the United States and other developed countries, China has been applied in the field of cosmetics for more than 30 years. In contrast, China's market promotion and application are relatively slow, limited by patent rights, and China was officially introduced in 2019. With excellent moisturizing and repair performance, Eco has a promising application prospect in the field of cosmetics. Under the background of the explosion of appearance level economy, the application of Eco has gradually expanded to sunscreen, cleansing, makeup, eye care, hair care and other niche.[1]

Biological fermentation is the mainstream method of ectoine production. The bulk commercial production depends on the natural host fermentation, and the control process of "hypertonic-hypotonic cycle stimulation" is complicated. The resolution of metabolic pathways laid the foundation for the synthetic biology of ectodoin. By integrating related synthetic genes in chassis cells, cells can accumulate ectos to varying degrees, but the specific accumulation varies significantly due to the host (e. g., yeast or E. coli with different genetic backgrounds) and integration pathway components (e. g., promoter type or RBS sequences, etc.). Therefore, production dependent on engineered bacterial technology is still in its infancy [2].

In order to enhance the commercial competitiveness of engineering bacteria, the participation of microbial breeding research is often needed. Microbial methods mainly include mutation and in vitro and in vivo recombination techniques. Classical mutagenesis breeding is to use physical, chemical and other factors to induce organisms to produce mutations, and cultivate into new varieties of animals and microorganisms. Good varieties can be obtained in a short period of time and greatly change certain traits. However, the frequency of beneficial mutations is still low, and the direction and nature of the variation are still difficult to control. Gene engineering

is the genetic operation of genes at the molecular level, and genetic engineering breeding is not restricted by species and purposefully. For example, the acquisition of ectoin basic engineered bacteria is the integration of ectABC [3] derived from exohalophilus in the Escherichia coli host. In order to obtain the further improvement of ectoine, the aspartate kinase engineering bacteria against feedback inhibition were obtained through metabolic engineering means. Remodeling of the host metabolic flow in order to obtain more target products is also a commonly used strategy in metabolic engineering. For example, the knockdown or overexpression of genes on glucose intake and metabolic pathways can significantly increase the final accumulation of ectoine. However, due to the basis of synthetic biology technology, the accumulation of heterologous natural products often involves metabolic network remodeling, heterologous pathways, gene and host adaptation, and metabolic engineering or classical mutagenesis technology is often inefficient [4].

The global transcription machine engineering breeding method, developed by Alpher et al. [5], utilizes mutations in certain protein targets on the transcription machinery to cause perturbations to hundreds of gene transcription levels at a global scale, resulting in a transcriptome mutation library. Combined with an efficient screening strategy, it is expected to obtain mutant strains of cells with significantly elevated expected phenotypes. Global transcription machine engineering breeding is different from the specific gene target before genetic engineering or metabolic engineering method, not to some or some specific gene target, on the expression in the cell is not specific up or down regulation, but through the random introduction of gene transcription process mutation, produce large capacity mutation library, and then select the specific phenotype of strains. This technique is undoubtedly more suitable for the breeding of engineered bacteria produced by synthetic biology techniques.

The relevance of the topic is Global library of transcription factors and mutant genes.

The purpose of the study is it is proposed to increase the yield of E. coli engineering bacterium ectoin through gTME technology, and the successful implementation of the project will provide a foundation for its industrial application. **The objectives** of the study of the study study is the whole genome sequencing and analysis of *Leclercia peneumoniae* provided basic data support for further understanding and research on the diversity, environmental impact, material degradation, and infection prevention and control of Leclercia.

The object of the study colibacillus

Research methods Global transcription machine engineering techniques

The scientific novelty Globality

The practical Increase the yield of engineered bacteria.

CHAPTER 1

LITERATURE REVIEW

Breeding research from synthetic biology to global transcription machines:

Global transcription machine engineering breeding research is a breeding method based on synthetic biology principles. With the help of the research means and tools of synthetic biology, it is engineering the global transcription machine of organisms, in order to obtain new varieties with excellent traits. Global transcription machine is a key component of the transcription process in living organisms. Through its engineering modification, the precise regulation of biological traits can be realized, so as to cultivate new varieties that meet people's needs.

1. Current status of breeding for global transcription machine engineering

Global transcription machine engineering breeding is a way to use gene transcription machine engineering techniques to improve biological traits. Recently, with the development of gene editing and synthetic biology, global transcription machine engineering breeding has been widely used in agriculture, medicine and industry. At present, the research of global transcription machine engineering breeding mainly focuses on the following aspects:

Improvement and optimization of transcription machines:

Gene transcription machines can be modified and optimized to improve the expression efficiency of genes, so as to obtain better trait performance. Specifically, the activity, stability and specificity of RNA polymerase are changed, so as to achieve fine control of gene expression levels, so that the purpose of gene expression can be improved and the purpose of improving crop traits can be achieved.

The so-called transcription machine is a molecular machine that can start gene transcription in response to cell signals, with the activity of RNA polymerase, which

can transcribe template gene sequences into complementary RNA chains, which plays a pivotal role in organisms, so that genes can be expressed at the right time and at the appropriate rate, so as to maintain the normal operation of life. Its transformation and optimization is of great significance, mainly in the following aspects: 1. Enhance the work efficiency of the transcription machine; 2. Improve the stability of the transcription machine; 3. Improve the adaptability of transcription machinery and cells; Fourth, the application range of transcription machines is expanded; Fifth, the stress resistance of the transcription machine is enhanced.

Improving transcription efficiency: Adapting transcription machines to optimize the activity of RNA polymerase is an effective means to improve transcriptional efficiency. This plays a pivotal role in the regulation of gene expression in organisms. Thus providing an important basis for maintaining the normal metabolism and physiological processes of life.

Precise regulation of gene expression: The modification and optimization of transcription machines, which play a key role in transcription machines, can enable us to regulate the expression of genes more precisely, so as to achieve precise intervention and treatment of specific genes in medical applications.

Promoting medical research: Translational transcription machines also play an important role in medical research, and the modification and optimization of these translational transcription machines can lead to a deeper understanding of the regulatory mechanisms of gene expression. In this way, it provides new ideas and means to promote medical research. Second, it is of great significance in medical research.

Promote the development of biotechnology: Transforming transcription machines is also one of the important directions of biotechnology development. In-depth modification and optimization of transcription machines can provide strong support for the development of high-performance gene editing and transcriptional regulation tools, which is of great benefit to the development and application of biotechnology.

Mining and utilization of transcription factors and promoters:

Transcription factors and promoters play a key role in gene expression. The exploration and utilization of new transcription factors and promoters can bring about new regulatory methods in gene expression, so as to achieve the purpose of obtaining richer trait expression.

Transcription factors can bind to specific sequences upstream of the 5' end of the gene, and form a transcription initiation complex with RNA polymerase II., which can jointly guide the beginning of the transcription process and ensure that the target gene is expressed at a specific intensity at a specific time and space. Transcription factors have a variety of functional regions, e.g., DNA-binding domains and effector domains. They can bind to regions that control transcription, such as promoters and enhancers on DNA, and control the transition of genetic information from DNA to RNA. This function can be done either alone or by forming complexes with other proteins.

A promoter is a sequence of DNA that RNA polymerase recognizes, binds, and guides the beginning of transcription. It contains conserved sequences required for specific binding to RNA polymerases and transcription initiation, and is essentially upstream of the transcription start of structural genes. Although the promoter itself is not involved in the transcription process, it plays a key role in the initiation of gene transcription. The properties of promoters were first identified by mutations, which can be regulated by the rate of gene transcription. In addition, there are special promoters such as the TRNA promoter located downstream of the transcription start point. These DNA sequences can also be transcribed. During transcription, trans factors bind specifically to the upstream activation sequence of cis elements, while transcription factors form a transcription initiation complex with RNA polymerase II and are involved in transcription initiation.

Combination of genome editing and transcription machine engineering breeding:

By combining with genome editing and transcription machine engineering breeding technology, more precise trait improvement can be obtained, so as to

achieve precise editing and regulation of the genome. For example, with CRISPR-Cas9 gene editing technology, key components of the transcription machine can be precisely knocked out or edited, thereby enabling the regulation of plant gene expression patterns [6].

1. Improving breeding efficiency [7]: Traditional breeding methods require a lot of time and resources to breed superior varieties through natural selection and artificial selection. Genome editing technology and transcription machine engineering breeding can accurately edit and regulate target genes, so as to quickly obtain new varieties with excellent traits, which greatly improves breeding efficiency.

2. Expand the scope of breeding: Traditional farming methods are often subject to reproductive isolation between species, making it difficult to combine the excellent characteristics of different species. Through the editing and regulation of target genes, the combination of excellent traits of different species can be realized, so as to expand the scope of breeding and provide more and better germplasm resources for agriculture, forestry, animal husbandry, etc., and genome editing technology and transcription machine engineering breeding can break through these limitations.

3. Improve crop quality: Through genome editing technology and transcription machine engineering breeding, we can meet consumers' demand for high-quality crops, such as improving the nutritional value of crops, improving the taste of crops, improving the appearance of crops, etc., and can accurately regulate the quality of crops.

4. Improve crop stress resistance: Using genome editing technology and transcription machine engineering breeding, genes related to stress tolerance can be edited and regulated, so as to improve crops' resistance to adversity (such as drought, salinity, pests and diseases, etc.), enhance crop adaptability, and improve yield and quality.

High-throughput screening and phenotypic identification:

Global transcription machine engineering breeding requires a large library of mutants and efficient screening strategies in order to quickly find mutants with

excellent traits. At present, high-throughput screening and phenotypic identification techniques have been widely used, such as the use of automated instruments and image recognition technology to achieve rapid screening and identification of a large number of mutants [8].

High throughput screening (HTS) technology is an experimental method based on the molecular and cellular level, using microplates as experimental tools, combined with automated operating systems, to detect tens of millions of samples quickly, sensitively and accurately. This technology uses microwell plates of different densities as experimental carriers and automation tools to operate the experimental process, and uses fast and sensitive detection equipment to measure the biological activity of a large number of samples, collect experimental data, and carry out digital analysis and processing. During the whole process, the corresponding information management software supports the normal operation of the system. The main characteristics of high-throughput screening technology include micro-volume, rapidity, sensitivity, and precision, which can obtain a large amount of information in a single experiment and screen valuable information from it.

Biosensors play a pivotal role in phenotypic identification. Phenotypic identification is a tool that can detect biomolecules or biological reactions, and it is the observation and description of the external characteristics and phenotypes of organisms. More accurate, rapid, and automated analysis can be performed by applying biosensors for phenotypic identification. A biosensor is a special detection device that converts biological processes into measurable signals. It is mainly composed of two parts: biometric component and signal conversion component.

Biometric elements are the core of biosensors, usually using biologically active substances such as enzymes, antibodies, nucleic acids, and cells as carriers. These substances have the ability to recognize and bind specific target molecules. For example, antibodies bind specifically to antigens, enzymes catalyze specific chemical reactions, and nucleic acids recognize specific target molecules by complementing them.

A series of reactions or changes are excited when the biometric element binds to the target molecule. These reactions or changes are then detected by signal conversion elements such as electrical signals, optical signals, etc., and converted into measurable signals. These signals can be further processed and interpreted to provide information to the target molecule.

Biosensors are characterized by high sensitivity, high selectivity, fast response, and reusability, so they have a wide range of application prospects and are used in many fields. In medical diagnosis, biosensors can be used for the rapid detection of disease markers, the monitoring of drug concentrations in living organisms, and the realization of personalized treatment. In addition, biosensors are also widely used in fields such as food safety, agriculture, and bioengineering[9].

2. Principles and research methods of global transcription machine engineering breeding

Global transcription machine engineering breeding is a method using RNA polymerases during gene transcription. This approach is based on the important role of RNA polymerase in gene transcription, which screens and uses its mutations in order to obtain cell mutant strains with significant expected phenotype.

In global transcription machine engineering breeding, mutations in RNA polymerase may lead to perturbation of the transcription levels of hundreds of genes at a global scale, thus generating the transcriptome mutation repertoire. Combined with an efficient screening strategy, mutants with excellent traits can be found from this mutation library for further breeding and improvement.

Specifically, the method of global transcription machine engineering breeding includes the following steps:

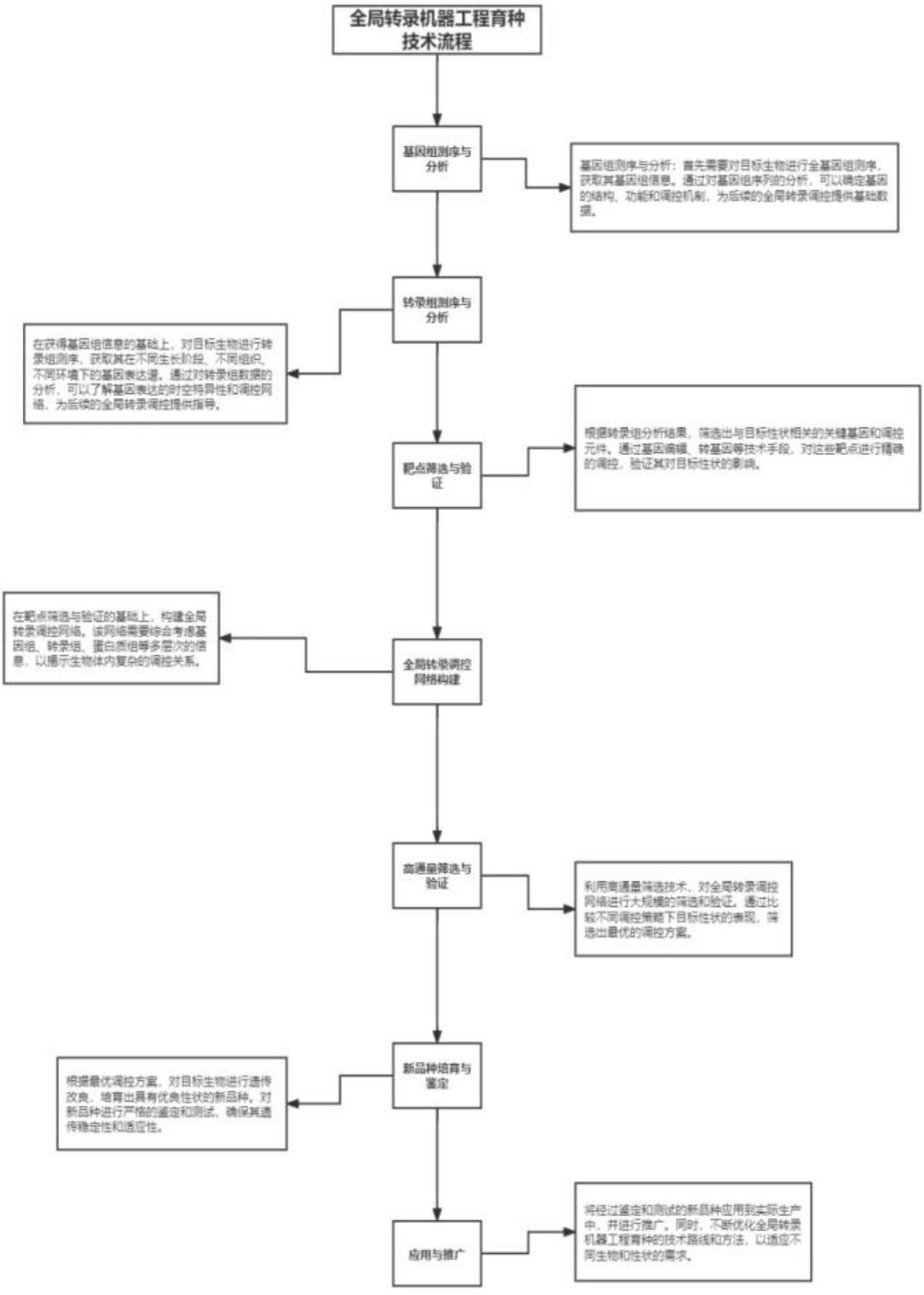
1. Mutagenesis: mutagenesis of the RNA polymerase of the target organism by physical, chemical or biological methods to produce a large number of mutants.

2. Screening: Use efficient screening strategies to find mutants with good traits from the mutant library. This can be achieved by comparing phenotypic differences

in growth, metabolism, and yield between mutant and wild-type organisms under specific conditions.

3. Verification: Further verification of the selected mutants was conducted to confirm whether the excellent traits are caused by mutations in RNA polymerase. This can be done by analyzing the genome and transcriptome of the mutant, and by performing backcrossing experiments for the mutant.

4. Breeding: The validated excellent mutants are used for breeding and improvement. This can further improve the trait performance of the organism by crossing the mutants with the target organism to produce new varieties or strains.


全局转录机器工程育种技术流程
基因组测序与分析
基因组测序与分析：首先需要对目标生物进行全基因组测序，获取其基因组信息。通过对基因组序列的分析，可以确定基因的结构、功能和调控机制，为后续的全局转录调控提供基础数据。
转录组测序与分析
在获得基因组信息的基础上，对目标生物进行转录组测序，获取其在不同生长阶段、不同组织、不同环境下的基因表达谱。通过对转录组数据的分析，可以了解基因表达的时空特异性和调控网络，为后续的全局转录调控提供指导。
靶点筛选与验证
根据转录组分析结果，筛选出与目标性状相关的关键基因和调控元件。通过基因编辑、转基因等技术手段，对这些靶点进行精确的调控，验证其对目标性状的影响。
全局转录调控网络构建
在靶点筛选与验证的基础上，构建全局转录调控网络。该网络需要综合考虑基因组、转录组、蛋白质组等多层次的信息，以揭示生物体内复杂的调控关系。
高通量筛选与验证
利用高通量筛选技术，对全局转录调控网络进行大规模的筛选和验证。通过比较不同调控策略下目标性状的表现，筛选出最优的调控方案。
新品种培育与鉴定
根据最优调控方案，对目标生物进行遗传改良，培育出具有优良性状的新品种。对新品种进行严格的鉴定和测试，确保其遗传稳定性和适应性。
应用与推广
将经过鉴定和测试的新品种应用到实际生产中，并进行推广。同时，不断优化全局转录机器工程育种的技术路线和方法，以适应不同生物和性状的需求。

3. Application of global transcription machine engineering breeding in the microbial field

Global transcription machine engineering breeding is mainly used to engineer and optimize the metabolic pathways of microorganisms to improve their specific phenotypes. Here are some of its applications in microorganisms:

1. Improve the adaptability of microorganisms to the environment[8]: Through global transcription machine engineering breeding, researchers can engineer microbial RNA polymerases to transcribe genes more efficiently under specific environmental conditions. This can help microbes to better adapt to the environment, for example by enhancing it at high temperatures**Помилка! Джерело посилання не знайдено.**, low temperature[10], High salt[11]Or low oxygen[12]Viability in other extreme environments.

2. Optimize the metabolic pathways of microorganisms: Through global transcription machine engineering breeding, researchers can modify the metabolic pathways of microorganisms to produce specific metabolites more effectively. This can be used to produce biofuels, drugs, food additives, etc. For example, optimization of key biological processes can be achieved for crop photosynthesis and nutrient metabolism[13], Thus improving the yield and quality of crops.

3. Improve microbial stress resistance: Global transcription machine engineering breeding can also be used to improve the resistance of microorganisms to stresses such as biotin and toxic substances[15]. This helps to reduce the loss of microorganisms in industrial production processes and to improve product quality and safety.

Global transcription machine engineering breeding has broad applications in the microbial field, which can help researchers better understand and modify microbial metabolic pathways, improve their specific phenotypes, and provide new solutions for fields such as industrial production and environmental protection.

4.the application of global transcription machine engineering breeding in other fields

1. Medical field

Disease treatment: With gTME technology, scientists can design and implement personalized gene therapy regimens for specific diseases, such as inherited diseases or cancers[16]. Through the precise regulation of the transcription process of related genes in patients.

Drug development: The gTME technology can be used to discover new drug targets or to optimize drug mechanisms of action. By understanding gene transcriptional regulatory networks, researchers can predict the effects of drugs on organisms, thus accelerating the drug development process[17]。

2. Bioengineering field

Bioreactor optimization: With gTME technology, microorganisms or cell lines can be designed and engineered to produce the desired biological products more efficiently [18], Such as enzymes, antibodies, biofuels, etc.

Biomaterials research and development: using gTME technology, can improve the performance of biomaterials [19], Such as strength, flexibility, biocompatibility, etc., to meet the needs of different fields.

3. Environmental protection field:

Pollution bioremediation: Through gTME technology, microorganisms with the ability to efficiently degrade pollutants can be cultivated for the bioremediation of environmental pollution [20]。

Ecological balance maintenance: gTME technology can be used to design and cultivate organisms with specific ecological functions, such as promoting soil remediation and improving plant carbon sequestration capacity, so as to maintain the balance of the ecosystem **Помилка! Джерело посилання не знайдено.**。

4. Biotechnology field:

Biotech product improvement: Using gTME technology, the performance of biotech products can be optimized, such as improving enzyme stability and activity [21], To enhance the adaptability of microorganisms to the environment.

Synthetic biology: gTME technology provides new tools and methods for synthetic biology to help build more complex and powerful biological systems.

It should be noted that despite the broad application of gTME technology in other fields, its practical application still faces many challenges and limitations. For example, the feasibility, safety, and ethical issues of the technology all need to be thoroughly studied and evaluated. Therefore, various factors need to be careful and fully considered when promoting the application of gTME technology in other fields.

5.Research progress in engineering breeding for Escherichia coli

Recently, remarkable progress has been made in global transcription machine engineering breeding studies for ectoine-producing E. coli. The researchers have successfully improved the yield and quality of eckdoin by constructing efficient gene expression systems, optimizing transcriptional regulatory elements, and introducing exogenous regulatory factors. Meanwhile, meanwhile, high-throughput sequencing technology and bioinformatics analysis revealed the regulation mechanism of global transcription machine in the process of producing ectoin, which provided theoretical support for further engineering breeding.

With further research, scientists discovered the key role of global transcription machines in regulating the process of ectoin. The global transcription machine is a complex regulatory network composed of multiple transcription factors, promoters, enhancers, etc., which together regulate the expression of numerous genes in E. coli. In this network, several key transcription factors and regulatory elements play a decisive role.

First, the researchers screened and modified key transcription factors for higher activity in ectoin production. These transcription factors can identify and bind to the promoter regions of target genes, thereby regulate gene expression. By optimizing the structure and activity of the transcription factors, the researchers successfully

increased the expression level of the target genes, and subsequently increased the production of eckdoin.

Second, promoters and enhancers in the global transcription machine. Promoters are the key elements that regulate the initiation of gene transcription, while enhancers can enhance the level of gene transcription. Through high-throughput sequencing technology and bioinformatics analysis, researchers have identified a number of promoters and enhancers with important roles in the process of Ikdoin production. By modifying these elements, the researchers successfully increased the transcription level of the target gene, further increasing the production of eckdoin.

Moreover, exogenous regulators in global transcription machines have also received the attention of researchers. By introducing exogenous regulators with specific functions, researchers can more precisely regulate the expression of their target genes. For example, some researchers have regulated the expression of key genes in the icdoin synthesis pathway by introducing inducible regulators, thus achieving the purpose of increasing ictoin production.

In the study of global transcription machine engineering breeding, Chinese scientists not only improved the yield and quality of Ikdoin, but also revealed the action mechanism of global transcription machine in the regulation process. These research results have provided strong technical support for the development of China's biological industry, and are expected to achieve higher yield and quality of Idoin production in the future. With the deepening of research, the application of global transcription machine in the field of biotechnology will be more and more extensive, making greater contributions to the development of food security and biological industry in China and even the world.

CHAPTER 2

EXPERIMENTAL PART

2.1 Experimental Materials:

2.1.1 Plasmids, strains, and primers:

Plasmids: YEplac195 (Ampr, URA 3), YEplac112 (Ampr, TRP 1), YEplac181 (Ampr, LEU 2)

Strains: Escherichia coli DH 5 α

primer:

Table 2-1 The primers used in this study

primer	Primer sequences
GAL1-P1	5′cccaagcttacggattagaagccgccg3′
GAL1-P2	5′aaaactgcagggtttttctccgacg3′
CYC1-T1	5′cgggatcccgatcatgtaattagttatg3′
CYC1-T2	5′cggaattcgcgcaaattaaagccttcgag3′
UP	5′gaactgcagatgcaattgttgagatg3′
SL	5′cgggatccttacaagtcttcgtgttcg3′
AK	5′cgggatccttagtctctcttagagaac3′

SGH	5′cgggatccttaagcgtagtctggaa c3′

2.1.2 Main experimental instruments:

Table 2-2 Main experimental instrumentation

Tab 2-2 Important apparatuses used in this work

Instrument name	Instrument model	producer
Automatic double pure water still	SZ-93	Shanghai Yarong Biochemical Instrument Factory
Fully automatic new biochemical incubator	ZXSD-A1090	Shanghai Zhicheng Analytical Instrument Manufacturing Co., LTD
ice box	BCD-590WTGVBP	Shandong Hisense Co., Ltd
microwave oven	WD800	Galanz Microwave Appliance Appliance Co., Ltd
ultra low temperature freezer	MDF-U53V	Sanyo Electric Corporation is made in Japan
Magnetic heating the stirrer	79-1	Guohua Electric Appliance Co., Ltd
thermostat water bath	KW-1000DC	Guohua Electric Appliance Co., Ltd
centrifuge	Centrifuge 5418	The German company eppendorf
microcentrifuge	B020197	American Andy Bio, Inc
Mini centrifuge	LX-200	Haimen Qiliinbel

		Instrument Manufacturing Co., LTD
Thermostatic metal bath	HB-100	Hangzhou Bori Technology Co., Ltd
Ultraviolet instrument	WD-9403C	Beijing Liuyi Instrument Factory
electronic balance	TP-A600	Fuzhou Huazhi Scientific Instrument Co., LTD
PCR appearance	6331CG703619	The German company eppendorf
Micropipette (in each range)	K13360B	The German company eppendorf
PH count	UB series	Fuzhou Huazhi Scientific Instrument Co., LTD
Multifunction microscope	H550S	Japan Nikon Inc
Electrophotometer power supply	DYV-6C	Beijing Liuyi Instrument Factory
Oscillation incubator	ZWY-211B	Shanghai Zhicheng analysis, instrument manufacturing
Automatic pressure steam sterilization pan	GI54DWS	Zhiwei (Xiamen) Instrument Co., LTD
Vortex oscillator		Haimen Qiliinbel Instrument Manufacturing Co., LTD
High power ice	HD-108	Shanghai Hongda Food

shredder		Machinery and Equipment Co., LTD
superclean bench	SW-CJ-2D	Suzhou Purification Co., Ltd
sequenator	FT2015	Dasqua Company

2.1.3 Main Reagents:

Table 2-3 Main experimental reagents

Tab 2-3 Important materials used in this work

Drug name	producer
Yeast Extraction substance (Yeast extract)	Beijing Aboxing Biotechnology Co., LTD
Trypsin (Tryptone)	OXOIDLTD .,BASINGSTOKE ,HAMPSHIRE,ENGLAND
Sodium hydroxide (NaOH)	Tianjin Damao Chemical Reagent Factory
Sodium chloride (NaCl)	Tianjin Xingxing Chemical Reagent Manufacturing Co., LTD
Protein (Peptone)	Beijing Aboxing Biotechnology Co., LTD
Agar powder (Agar Powder)	Beijing Aboxing Biotechnology Co., LTD
Glucose (Glucose)	Tianjin Chemical Reagent Factory No.3
All kinds of amino acids	Shanghai raw workers
The Global Transcription Machine engineering kit	Raw biological
The Plasmid extraction kit	Raw biological
DNA, polyase	Raw biological
DNA Marker	Raw biological
ampicillin	Raw biological
agarose	Beijing Aboxing Biotechnology Co., LTD

RNA, polyase	Raw biological
restriction enzyme	Raw biological
DNA, ligase	Raw biological
hydrochloric acid (HCl)	Tianjin Damao Chemical Reagent Factory

2.1.4 Culture Media:

LB liquid media (g / L):Trypsin: 1%, yeast extract: 0.5%, NaCl: 1%, pH 7.0

LB solid medium (g / L): Add 2% agar powder to the LB liquid medium.

2.1.5 Main solution:

(1) NaOH solution: 1.0 mol / L;

(2) Ampicillin: put ampicillin into 100 mg/mL of storage solution with-20℃ for storage;

(3) NP-40 cell lysis buffer: 50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4,1% counterstain, 0.02% NaN 3.

(4) 1 SDS gel sample buffer:

50 mmol/L	Tris·Cl (pH 6.8)
100 mmol/L	DTT
2%	SDS (electrophoresis grade)
0.1%	bromophenol blue
10%	glycerol

(5) Fluorescence reagent: 100 mg fluorescein dissolved in 100 mL ethanol; bromine reagent: 5% carbon tetrachloride solution of bromine;

(6) Ecdoin solution: 60g / L and each gradient concentration;

(7) NaAc solution: 3.0 mol/L, adjust the pH value to 7.0 with glacial acetic acid;

(8) TE buffer: 10 mmol / L Tris-HCl (pH 8.0), 1 mmol / L EDTA (pH 8.0);

(9) Tris · HCl solution: 50 mmol/L, hydrochloric acid adjusted pH to 8.0;

(10) 50 TAE Electrophoresis Buffer (1 L): Tris 242 g, chilled acetic acid 57.1 mL, 0.5 mol/L EDTA 100 mL (pH 8.0)

(11) phenol chloroform solution: 1:1 (v / v)

(12) NaCl solution: 1.2 mol / L;

(13) EDTA solution: 0.5 mol/L, NaOH adjusted pH value to 8.0;

(14) CTAB solution: 5%;

(15) STET solution: 50 mmol/L EDTA; 0.1% (v / v) TritonX-100; 8% (w / v) sucrose; 50 mmol/L Tris · HCl (pH 8.0).

2.2 Experimental method:

2.2.1 Culture of Escherichia coli:

Remove a small amount of broth into a dish or tube containing LB medium into a temperature aker or incubator at 37°C.

2.2.2 Heat shock transformation method of Escherichia coli:

1. Put E. coli DH 5 α competent cells on ice, and after their melting, add an appropriate amount of foreign DNA (such as plasmids, ligation products, etc.).

2. Place on ice for 30 min to fully contact foreign DNA with competent cells.

3. The mixture was transferred to a 42℃ water bath for 90s and then quickly transferred to ice for cooling for 2 min.

4. Under sterile conditions, add an appropriate amount of LB liquid medium (generally 200 \ ~600 μ l) to the mixture, and then transfer to a 37℃ shaker at low speed for 1 hour to resuscitate the cells and express resistance genes.

5. Apply on LB solid medium containing ampicillin, after air drying, seal plate and incubated in 37℃ incubator for at least 14 hours.

6 Individual colonies were picked for identification and subsequent experiments.

2.2.3 (CTAB method) Small-scale extraction of E. coli plasmid method

1. Place the bacterial solution in the EP tube, centrifuged, and discard the supernatant.

2. Add the 1.5m bacterial solution again, and repeat the step once.

3. Add Eco lysate and 3 μ l of leukase K solution (20 mg/ml), mix well, and incubate at 37℃ for 1h.

4. The CTAB precipitate was added, mixed well, and incubated at 65℃ for 10min.

5. Add 780 protein clearing solution, mix well, and centrifuge at 12,000 g for 5-10 min.

6. Transfer the supernatant to a new EP tube, add 2 x volume of Eco rinse, mix well, and leave 20℃ for 30min or overnight.

7 The above mixture was centrifuged again at 12,000g for 5 – 10 min.

8. Transfer supernatant to a new EP tube, add 0.6 x volume of isopropanol and mix well until DNA precipitated.

9. Place the EP tube in-20℃ refrigerator and stand for 30 minutes to 1 hour.

10 The mixture was centrifuged at 12,000g for 10 min and the supernatant was discarded.

111 ml of 70% ethanol was added and the DNA precipitate was then gently washed, followed by centrifugation at 12,000g for 5 min.

12. Discard the supernatant and reverse the EP tube on the absorbent paper to evaporate the residual ethanol.

13. Add an appropriate amount of TE buffer (or sterile water) to dissolve the DNA precipitation.

14 DNA was fully dissolved in a 37℃ water bath.

15. Mix the appropriate amount of DNA sample with sample buffer and add to the wells of agarose gel. Electrophoresis was performed, and then the DNA bands were visualized under a UV lamp.

2.2.4 Restriction-dependent enzyme digestion of the DNA

1. Restriction enzyme digestion of sample DNA: first, add a certain amount (such as 2 μ g) of sample DNA solution to the Eppendorf tube, and then add appropriate restriction enzyme buffer, corresponding restriction enzyme and disinfection double steaming water to make the total volume reach a certain volume

(e. g., 20 μ l). Next, at the appropriate temperature (e. g., 37℃) for a certain time (e. g., 2 hours). This process can be single or double digested using one or more different endonucleases.

2. Termination reaction: After the enzymatic reaction is completed, the reaction can be terminated by heating (e. g. 65℃ for 5 minutes) or by adding an appropriate amount of EDTA (e. g. 0.5 mol/L EDTANa2).

3. Electrophoretic separation of DNA fragments: take a certain amount of DNA products, add loading buffer (containing bromophenol blue indicator and glycerol), and then perform horizontal electrophoresis on agarose gel (e. g. 0.8% agarose, containing 0.5 μ g / ml ethidium bromide). The conditions (such as the voltage) shall be adjusted according to the specific circumstances.

4. Radiolabeling and autoradiography: The DNA fragments that need to be radiolabeled can be labeled using the appropriate labeling methods (e. g., 32P end-labeling). Then, the products were separated by gel electrophoresis and those DNA fragments labeled only were purified by electrophoresis. Next, the isolated DNA fragments were partially digested with the limiting enzyme cut at relatively high frequency by serial dilution enzyme, and again separated by electrophoresis. Finally, autoradiographic exposure was performed to observe and analyze the length and number of DNA fragments.

2.2.5 Agarose gel electrophoresis of the DNA

1. Gluing: weigh 0.15-0.20g agarose, and add 20 mL TAE buffer, heat dissolved for 1 min, to cool to about 60℃, add a small amount of EB solution, insert the point sample comb, mix and pour into the rubber plate, placed at room temperature for 10 min;

2. Point sample: after the gel is fully fixed, pull out the comb, move the glue to the electrophoresis tank, add 5 μL Marker to the first hole, and then mix 5 μ L sample for 1 μL Loading Buffer before adding the sample hole with a pipete gun;

3, agarose gel electrophoresis: 100 V, 100 mA electrophoresis to the third band (about 30 min);

4, the DNA bands were visualized on a gel imager.

2.2.6 DNA Rubber recovery

1. Cut an agarose gel containing the target DNA under a UV lamp.

2. Weigh the gel weight and convert the gel volume in 1mg=1 μ l. Three gel volumes of nj buffer were then added. After uniform suspension, heat at 55℃ -65℃ for 7min, and shake every 2-3min until the gel block is completely melted.

3. Transfer the melted gel solution to a DNA preparation tube and place it in a 2-ml centrifuge tube.

4. Was centrifuged at 6000rpm for 1 minute and the filtrate was discarded.

5. Preparation and tubes were reback into 2ml centrifuge tubes, adding 500 μ l of wash solution W1 and centrifuged at 6000rpm for 30 seconds and the filtrate was discarded.

6. The prepared and tubes were returned into 2ml centrifuge tubes, adding 700 μ l of wash solution W2 (the indicated volume of absolute ethanol) and centrifuged at 6000rpm for 30 seconds and the filtrate was discarded.

7 were washed once again with 700 μ l of wash solution W2 in the same manner and the filtrate was discarded.

8. Prepare and tubes back into 2ml centrifuge and centrifuged at maximum speed for 1 minute.

9. Transfer the preparation tube into a new 1.5ml centrifuge tube, add 25-30 μ l of eluate EB (or sterile water) to the center of the preparation tube membrane, and stand at room temperature for 1 minute. The samples were then centrifuged at maximum speed for 1 min to elute the DNA.

2.2.7 Ligation reaction of the DNA

1. In a 1.5 mL centrifuge tube, according to the characteristics of the gene and the vector, a 25 μ L connection system is established according to the following components:

Table 2-6 The DNA connection system

reagent	volume
5× T4 ligase Buffer	2 μL
pET28a The vector plasmid	4 μL
rpoD gen	13μL
T4 ligase	1 μL

2. Mix the system well and warm the system in 16℃ environment for overnight.

3. After DNA ligation, enzyme digestion or PCR analysis was performed.

2.2.8 PCR amplification reactions

1. Prepare a 50 μ L reaction system;

The PCR amplification system

double distilled water	11.0μL
PCR Buffer	2.5μL
dNTP	3.0μL
DNA templet	2.0μL
primer	1.0μL
Taq	1.0μL

2. Set the temperature and time of PCR amplification according to the gene size;

Fusion fragment system setting:

预热温度	95℃	2min		
正式变性	95℃	30s	}	×30
退火温度	54℃	30s		
正式延伸	72℃	1min		
最终延伸	72℃	6min		
保温温度	10 ℃			

2.2.9 Purification of the DNA (PCR) product

1. dissolve the precipitate in 7 ml ddH2O and be used.

2. Transfer the PCR reaction liquid and water into a 1.5 ml eppendorf tube.

3. Add 100 ml of direct extraction buffer and mix well.

4. Add 1 ml PCR DNA of purified resin and mix it 3 times in 1 minute.

5. Take the disposable syringe, remove the plug, and connect the syringe with the Wizard micro column, add the above mixture to the injection cylinder with a pipette gun, and gently with the plug to make the mixture into the micro column.

6. Separate the syringe from the micro column, remove the injection plug, and then connect the injection cylinder to the micro column. Add 2 ml of 80% isopropyl alcohol to wash the micro column.

7. The mini column was removed and placed in a eppendorf tube and centrifuged at 12000 g for 20 seconds to remove the lotion in the micro column.

8 The microcolumn was placed in a new eppendorf tube with 50 μl TE or water, stationary for 1 min and centrifuged at 12000 g for 20 seconds.

9. The microcolumn is discarded, and the solution in the eppendorf tube is purified DNA and stored at 4℃ or-20℃.

2.2.10 to test the transformation results of the *rpoD* gene

If the *rpoD* gene is successfully amplified by the PCR reaction, its sequence can be further verified by gene sequencing. Insertion of the *rpoD* gene were detected using Sanger sequencing. To obtain the pET28a-R vector.

2.2.11 Mumutation expression library was constructed

Prepare the template DNA: use the pET28a-R vector as described above as a template.

2. Design mutation sites: Design the mutation sites that you want to introduce in the pET28a-R vector by using genome editing tools (such as CRISPR-Cas 9).

3. Synthetic mutant DNA: Replace the designed mutation site with the correct gene sequence, and then amplify these mutant DNA using PCR.

4. Purified mutated DNA: The amplified mutated DNA was purified into high concentrations of fragments using centrifugation, filtration and purification steps.

5. Construction of mutant expression library: connect the purified mutant DNA and the fragments with pET28a-R vector to form a mutant expression library.

6. Sequencing and analysis: Mutant expression libraries are sequenced using high-throughput sequencing technologies (such as Illumina NextSeq or Illumina HiSeq). The sequencing results were then aligned and analyzed using bioinformatics tools (e. g., BLAST, Clustal Omega, or MAFFT) to determine the location and type of mutation sites.

2.2.12 Screening of the desired clones

Culture of the mutant library bacteria

After plasmid extraction, ectoine engineered bacteria were transformed and entered into screening.

The fluorescent biosensor constructed by the research group was used as the preliminary high-throughput screening system, and then the 96-deep hole microculture technology was used to obtain the desired cloning.

CHAPTER 3

RESULTS

The engineering bacteria constructed on the basis of synthetic biology technology have the problem of heterologous pathway gene and host compatibility, and increasing the accumulation of target products not only involves the modification of known genes, but also involves the adjustment of many unknown genes. Therefore, the breeding process not only has rational metabolic engineering transformation, but also irrational metabolic network remodeling. Global transcription machine engineering is a global and efficient breeding method developed for the latter. The purpose of this study was to use global transcription machine engineering breeding to remodel the transcriptome of E. coli genes, so as to improve the yield of host ectoin.[23]

3.1 Construction of the overexpression vector for the global *rpoD* transcription factor

The purpose of this study was to influence the transcriptome by regulating the activity of the transcription factor *rpoD*. After designing appropriate primers according to the *rpoD* gene sequence, the coding region of *rpoD* gene was amplified by PCR, and then the amplified coding region of *rpoD* gene was inserted into the selected expression vector pETDuet-1, and then the constructed overexpression vector pETDuet-1-*rpoD* was transformed into DH5α of recipient cells.Figure 3.2-1 Construction framework for the global transcription factor *rpoD* overexpression vector.

As shown in Figure 3-3, the *rpoD*-overexpressing strain and the control strain were inoculated in LB medium for 3.5 h and then 0.5 mM IPTG was added to continue to be cultured for 20 h (the average data of the three groups of culture and

induction tests were taken). It was found that the overall growth of *rpoD*-overexpressing strains was similar to that of the control strain, but the maximum growth of *rpoD*-overexpressing strains was slightly lower than that of the control strain. However, the accumulation of tetrahydropyrimidine in *rpoD* overexpressing strains was 36% higher than that of the control bacteria, reaching 3.83 g/L.

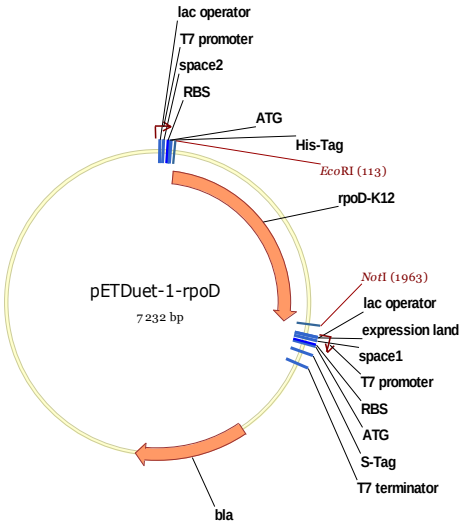


Figure 3.1-2 Eco l electrophoresis of double digested agarose gel with Notl

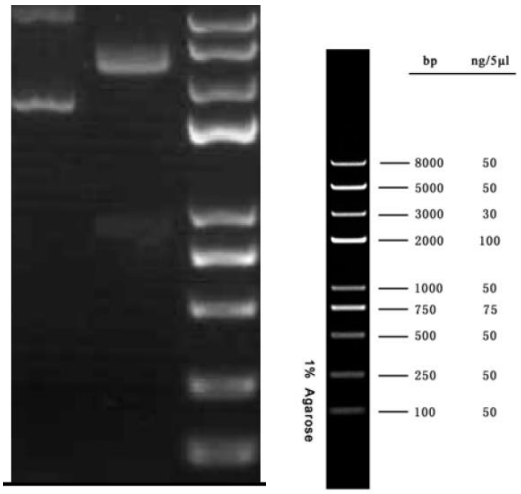
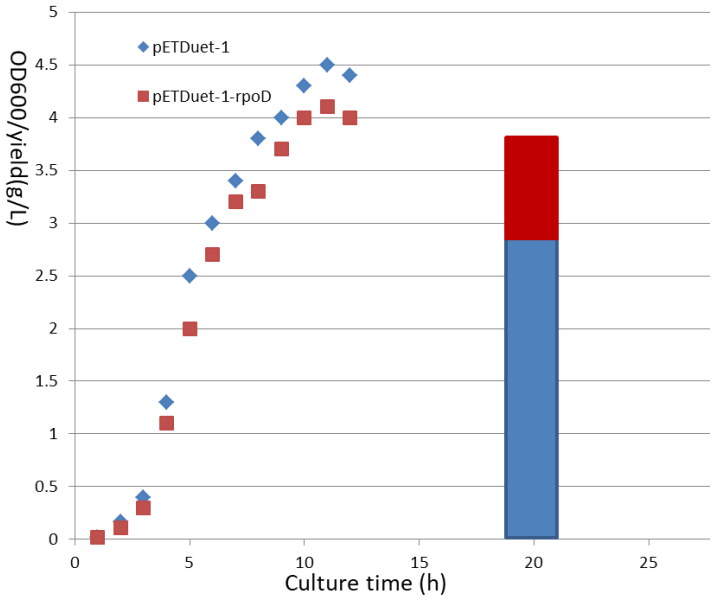


Figure 3.2 Construction and screening of a random mutation library for the global transcription factor *rpoD*

Using pETDuet-1-*rpoD* as a template, the *rpoD* random mutation PCR products with low mutation frequency and high mutation frequency were obtained after different cycle amplification according to the random mutation kit, which were

purified and set aside. pETDuet-1-*rpoD* was digested by EcoRl and Notl, separated by electrophoresis, and the large fragments of the vector were recovered by gel and linked with the above two PCR products, respectively, and DH5α was efficiently transformed to obtain *rpoD* random mutation libraries with two mutation frequencies (I and II). Through random sampling and sequencing, it was found that the base mutation rates of the two libraries were 0.32% and 0.75%, respectively, and the corresponding protein amino acid mutation rates were 0.29% and 0.71%.

We extracted two mutation rate library plasmids and transformed the host BL21 (ΔaraC: LysC*-asd, pRSFDet-1-ectABC), which was temporarily unable to be screened due to unsuccessful biosensor construction. We randomly selected 10 clones for downstream testing. No strains were found to improve the product, but the yield of tetrahydropyrimidine in one strain decreased significantly (1.3 g/L), and the host was re-transformed after plasmid extraction, and the yield traits were stable, and it was preserved as a seed resource.



CONCLUSIONS

As a global breeding method, gTME has been widely used since its development. Here, we cloned the endogenous global transcription factor *rpoD* of *E.coli* by PCR, and randomly mutated it to construct an *rpoD* mutation library. From this experiment, we can draw the following conclusions:

1. A library of *rpoD* random mutations with two specifications of mutation rates was established to provide more biodiversity for the future E. coli host breeding process.

2. High-throughput screening is still the biggest limiting factor for irrational global breeding.

Due to the failure to construct a suitable tetrahydropyrimidine biosensor and the failure to give full play to the breeding potential of gTME, the project can be extended in the following aspects in the later stage:

1. A negative mutant strain was obtained in this experiment, which can provide a data source for subsequent comparative transcriptomics studies and reverse genetic breeding of strains.

2. Build a biosensor with sensitivity and linear response range as soon as possible to achieve efficient breeding.

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