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 **The effect of ploidy on recombinant expression of brewing yeast**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Xu RUIQI

Scientific supervisor
Iryna VOLOSHYNA, Ph.D., As. prof.

Reviewer
Olena OKHMAT, Ph.D., As. 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
Xu Ruiqi

1. Thesis topic **The effect of ploidy on recombinant expression of brewing yeast**
Scientific supervisor Iryna Voloshyna, Ph.D., As. 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 _____ Xu RUIQI

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Ruiqi Xu. The effect of ploidy on recombinant expression of brewing yeast. – Manuscript.

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

This study investigated the effect of different ploidy on recombinant expression in *S. cerevisiae*. By comparing haploid, diploid, and polyploid *S. cerevisiae* differences in terms of recombinant protein expression levels, expression stability, and genetic stability, we found that ploidy has a significant effect on recombinant expression in S. cerevisiae. The experimental results show that polyploid *S. cerevisiae* usually have higher expression levels in recombinant proteins than haploid and diploid expression proteins, probably due to polyploids containing more genomes and transcription machines, resulting in improved transcription and translation efficiency. Moreover, polyploid *S. cerevisiae* also showed higher expression stability, in terms that the recombinant protein expression remained relatively stable during serial passages. Therefore, for recombinant expression using *S. cerevisiae,* the appropriate ploidy needs to be selected according to the specific application requirements. Polyploid *S. cerevisiae* may be a better option for applications requiring high expression and stability; haploid or diploid *S. cerevisiae* may be more appropriate. This study provides an important understanding of the impact of ploidy on recombination expression in S. cerevisiae and provides new ideas and methods for optimizing the recombinant expression system in S. cerevisiae.

Keywords: Saccharomyces cerevisiae, ploid, recombinant expression, recombinant protein

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INTRODUCTION

Ploidy, as a crucial concept in biology, precisely defines the number of chromosomes in the cells of an organism. In haploid cells, chromosome number is restricted to within an complete set of chromosomes. However, in diploid cells, the chromosome number was doubled and contained two complete sets of chromosomes. This difference in ploidy is not just a number game, it actually has profound effects on the basic genetic properties of the organism, cellular functions as well as the process of recombinant expression.

As a widely used model organism, S. cerevisiae plays an important role in the field of biotechnology and industrial production. The unicellular fungus is not only easy to culture, but also has a clear genetic background, allowing scientists to easily perform its genetic manipulation. Recently, with the rapid development of genomics and proteomics techniques, the relationship between ploidy and cellular recombinant expression in S. cerevisiae is increasingly studied.

The effect of ploidy on recombinant expression in S. cerevisiae cells is multifaceted. First, the change in ploidy can directly affect the genetic stability and expression efficiency of yeast cells. For example, polyploid yeast may exhibit higher stability and efficiency in the expression of some genes due to having more chromosomal copies. Secondly, the ploidy changes can also affect the metabolic pathways and physiological functions of yeast cells, thus affecting the ability of their cells to recombine their expression. For example, polyploid yeasts generally have higher metabolic activity and greater environmental adaptability, which allows them to produce more target products during recombinant expression in cells.

Further investigation of the relationship between ploidy and cellular recombinant expression of S. cerevisiae is important for deeply understanding the basic principles of biological recombination techniques and for optimizing industrial production. By revealing this relationship, we can better grasp the basic laws of cell recombinant expression, and thus develop more efficient and economical

bioengineering techniques. At the same time, it will also help us to improve the output and quality of the target products in industrial production, and to make a greater contribution to the sustainable development of human society.

The laboratory strain of X-insulin yeast has a low yield (~10mg), exploring the expression level of the gene dosage on the yeast ploidy network.

X-insulin mating genotype as MAT α by PCR with triple primers, The bacterial strain was named as I α; By transforming the sex-converted YCplac33-GHK plasmid to this host, And, after lactose induction, MATa type strain Ia; Both mating-type strains were then crossed in YPD medium, Single colonies were coated on plain plates, Diploid strain II was obtained by PCR; To the transplasmid YCplac33-HK, Two diploid II (MAT α / α) and II (MATa / a), Triploid III (MAT a / a / α) or III (MAT α / α / a) and tetraploid IV (MAT a / a / α / α) were obtained. Finally, the basic characterization of morphology, growth and recombinant protein yield of the engineered bacteria with different ploids was compared, and it was found that most haploid cells were round and small, while other ploid cells showed no obvious difference in appearance, and the tetraploid cells had the highest growth and the highest level of recombinant long-acting insulin. Moreover, polyploid S. cerevisiae also showed higher expression stability, in terms that the recombinant protein expression remained relatively stable during serial passages.

The relevance of the topic is ploidy of brewing yeast.

The purpose of the study is the understanding the Effect of Ploidy on Recombinant Expression of Brewing Yeast.

The objectives of the study is the understanding the Effect of Ploidy on Recombinant Expression of Brewing Yeast.

The object of the study are different ploids of S. cerevisiae.

The subject of the study are different ploids of S. cerevisiae.

Research methods Construction of HO plasmid and PCR validation of mating type.

The scientific novelty The ploidy changes in brewing yeast provide a unique perspective to study the mechanism of gene expression regulation.

The practical significance of the results obtained is significance of the results obtained is Different ploidy of brewing yeast have differences in morphology and insulin production.

CHAPTER 1
LITERATURE REVIEW

1.1 Topic selection purpose

Yeast are outstanding hosts for the production of functional recombinant proteins with industrial or medical applications, and S. cerevisiae is the most commonly used biological species in fermentation. Saccharomyces cerevisiae has the advantages of short growth cycle, strong fermentation ability, easy large-scale cultivation, and contains a variety of proteins, amino acids, vitamins, bioactive substances and other rich nutrients. It has always been the main object of basic and applied research, and has been widely used in food, medicine and other fields. The S. cerevisiae diploid strains are preferred by industry for their better fermentation efficiency in viability and endurance. In this paper, we aim to explore the role played by ploidy during recombinant expression in S. cerevisiae. To achieve this goal, we have constructed a specific plasmid that contains the HO gene and can be expressed under the induction of galactose. Subsequently, we used this plasmid to change the mating type of yeast cells, and then by yeast cell hybrid technology, successfully obtained polyploid cells. This series of experimental designs not only enables us to accurately manipulate the ploidy of yeast cells, but also provides us with an important basis for studying the effect of ploidy on recombinant expression in S. cerevisiae.

1.2 The meaning of the topic

As an important bioengineering tool, S. cerevisiae has been widely used for the production of a variety of valuable compounds, such as ethanol, biofuels, enzymes, drugs, etc. However, with the further study of S. cerevisiae, scientists have gradually discovered that the ploidy of yeast cells, that is, the number of their chromosomes, may have a significant impact on the performance of their recombinant expression system. The change in ploidy is not only directly related to the genetic stability of yeast cells, but also affects their metabolic pathways, growth rate and the yield of

target products. Therefore, exploring the effect of ploidy on recombinant expression in S. cerevisiae is crucial to improve industrial production efficiency and optimize expression systems.

With the rapid development of technology and the continuous maturity of genomics, transcriptomics and metabolomics, there is a deeper understanding of the genetic background of Saccharomyces cerevisiae. These technological advances provide a powerful means to study the physiological metabolism of yeast cells, allowing us to more comprehensively reveal the molecular mechanisms of yeast cells. Through the sequencing and analysis of the yeast genome, we can better understand its gene function, regulatory networks and metabolic pathways, providing theoretical support for optimizing the expression system and improving the yield.At the same time, the transcriptomics research also provides us with a valuable resource. By comparing the transcriptome data of yeast cells with different ploidy, we can discover which genes undergo significant expression changes after ploidy changes, which in turn reveal the role of these genes in the recombinant expression process of cells. Such transcriptomic data based analysis not only provides insight into the biology of S. cerevisiae, but may also may provide new ideas and methods for optimizing expression systems and increasing yield.

Therefore, this study has important theoretical significance and applied value. Through in-depth exploration of the effects of ploidy on recombinant expression in S. cerevisiae, we can better understand the biological mechanisms of yeast cells and provide more efficient and environmentally friendly bioengineering tools for industrial production. At the same time, the research is also multifaceted, not only involving biology, chemistry, engineering and other disciplines, but also will have a far-reaching impact on medicine, energy, environmental protection and other industries.

1.3 Research status, both at home and abroad

1.3.1 Current status of domestic research

The history of studying the ploidy of S. cerevisiae can date back to the end of the last century. With the rapid development of molecular biology technology, researchers have begun to deeply explore the chromosome composition, ploidy changes and their relationship with cell function in Saccharomyces cerevisiae. In recent years, with the development of high-throughput sequencing and ploidy in gene editing. In recent years, with the rapid development of gene editing technology, domestic researchers have made remarkable progress in the regulation and research of cell ploidy in S. cerevisiae1:

1. Research and development of improved varieties: A Chinese research team has successfully developed a variety of improved varieties of S. cerevisiae. These new varieties not only have higher fermentation efficiency, but also show stronger stress resistance and better nutritional adaptability. The development and development of these improved varieties mainly benefit from the extensive application of gene editing technology and metabolic engineering technology.

2. Survival in extreme environments: Chinese researchers pay particular attention to the viability of S. cerevisiae to survive in extreme environments. They have successfully developed Saccharomyces cerevisiae species that can survive and ferment under extreme conditions such as high salt and acid. This is of great application value to the food industry and the bioenergy industry.

3. Progress in genomics research: With the rapid development of genomics technology, the genomics research of S. cerevisiae in China has also made important breakthroughs. Researchers have been able to gain a deeper understanding of the genome structure of S. cerevisiae to reveal the key genes and regulatory networks associated with their cellular ploidy.

4. Improvement of fermentation efficiency: The Chinese scientific research team has significantly improved the fermentation efficiency of Saccharomyces cerevisiae by optimizing the fermentation process and improving the bacterial strains.

This will not only help to improve the production efficiency of the brewing industry, but also help to reduce the production costs.

5. Improvement of nutritional adaptability: Chinese researchers have successfully improved the nutritional adaptability of S. cerevisiae through genetic engineering and metabolic engineering. This allows S. cerevisiae to more efficiently utilize different carbon and nitrogen sources and thus adapt to more fermentation environments.

It is believed that with the increase of scientific research investment and the improvement of technical level, it is expected that the domestic research of S. cerevisiae will make more breakthroughs in the future and bring more benefits to human beings.

1.3.2 Current situation of overseas research

In foreign countries, S. cerevisiae has been widely concerned and studied. In the study of cell ploidy of S. cerevisiae, foreign researchers have made a series of important achievements. In ploidy research, Professor Botstein's team focused on the differences between haploids and diploids in S. cerevisiae and the effects of ploidy changes on yeast cells. By comparing the differences in the growth rate, metabolic activity, and fermentation ability of yeast cells in different ploidy states, they revealed the regulatory effect of ploidy on the biological characteristics of yeast. In addition, they have deeply investigated the effects of ploidy changes on the yeast cell cycle, DNA replication and repair processes, providing a new perspective into understanding yeast cell biology. It is worth mentioning that Professor Botstein's team also used modern biotechnology methods, such as gene editing, to accurately regulate the ploidy of S. cerevisiae to explore its potential in industrial fermentation, alcohol production and other fields. These studies not only demonstrate the broad application prospects of ploidy regulation in S. cerevisiae in the biotechnology field, but also provide theoretical support for optimizing the industrial fermentation process and improving alcohol yield and quality.

In addition, Professor Christiane Lehmann is also an outstanding representative in the field. Her research team focused on the effects of ploidy changes in S. cerevisiae on its cell cycle, DNA replication and repair processes. Professor Lehmann's research reveals a complex relationship between ploidy change and yeast cell growth and division, providing new insights into yeast cell biology. Professor Lehmann's study also addressed the effects of ploidy changes on genome stability in Saccharomyces cerevisiae2. She found that changes in ploidy may increase the mutation rate and recombination frequency of yeast cells, thus affecting the accuracy and stability of their genetic information. These studies not only contribute to understanding the genetic properties of yeast cells, but also provide a theoretical basis for the genetic improvement and variety breeding of S. cerevisiae.

1.4 Biological ploidy

Pidy refers to the number of chromosomes in the cells of an organism. In cell biology, ploidy is often used to describe the number of chromosomes in a cell or organism relative to a normal diploid state. For example, normal human somatic cells are diploid, that is, each cell contains 46 chromosomes and forming 23 pairs. The study of ploidy is important for the understanding of the genetics, development, evolution, and diseases of organisms. Based on the number of chromosome groups, biological ploidy can be classified into haploid, diploid, and polyploid. Coploidy has important effects on the inheritance of organisms. For example, haploid organisms can only reproduce by asexual because their cells have only one chromosome group to produce gametes by meiosis4. Diploid organisms can reproduce through sexual reproduction, producing haploid gametes (sperm and eggs), which then restore the diploid state through fertilization. Polyploid organisms may have more genetic diversity, but they may also face the challenges of fertility and survival. The change in ploidy can affect the phenotypic characteristics of the organisms. For example, some polyploid plants generally have larger size, more abundant nutritional value and greater adaptation to adversity than their diploid ancestors. In animals, polyploidy

may also cause phenotypic changes such as accelerated growth, increased body size and decreased reproduction. Ploidy has a significant effect on gene recombinant expression, specifically in the following aspects:

First, ploidy can influence the number of chromosomes, in turn the frequency and mode of recombination. Cells with different ploidy can pair and exchange chromosomes differently during meiosis, which may lead to changes in the pattern and frequency of genetic recombination. For example, polyploid cells may increase the number of pairing and exchange due to nonhomologous chromosomes, thus changing the pattern of gene recombination.

Secondly, ploidy may also affect the expression level and regulation mechanisms of genes. Changes in chromosome number may cause changes in the expression of some genes, or affect genetic interactions and regulatory networks. These changes may further affect the expression and effect of gene recombination.

Moreover, ploidy may also affect the fitness and evolutionary potential of living organisms. Organisms with different ploidy may differ in the expression and effect of genetic recombination when responding to environmental changes and adapting to new environments. For example, certain polyploid organisms may be more resilient and adaptable because their genomes may contain more advantageous genes and variants. The effects of ploidy on regene reexpression are complex and diverse, involving many aspects such as chromosome number, gene expression regulation, and biological adaptability.These effects contribute to a deeper understanding of the genetic mechanisms and evolutionary processes of organisms. However, further research and exploration are still needed on the specific mechanism and effects of ploidy on gene recombination expression.

For example, diploid and tetraploid organisms were used to explore the effect of ploidy on the recombinant expression of the gene. First, consider the diploid organisms. During normal sexual reproduction, diploid cells undergo meiosis to form haploid gametes, which return to become diploid upon binding. In this process, homologous chromosomes link during the prophase of the minus first division3.

1.5 HO plasmid conversion sex

In S. cerevisiae, the HO gene plays a key role. This gene is unique in that it encodes a special class of enzymes called endonucleases, which are extremely precise and able to recognize and cleave specific DNA sequences. This process of recognition and cutting is actually the key to the sex conversion process of S accharomyces cerevisiae.

After introducing a plasmid carrying the HO gene into S. cerevisiae cells, the plasmid began to play its magical role. First, it is able to self-replicate within S. cerevisiae cells, ensuring a steady increase in its own number. This plasmid is then cleverly integrated into the nucleoplasmic DNA of S. cerevisiae cells to achieve a stable presence in S. cerevisiae. Once the HO plasmid is successfully integrated and expressed, the HO gene is subsequently activated, thus triggering the sex-switching process in S. cerevisiae.

As a single-celled fungus, Saccharomyces cerevisiae has two main haploid cell types: type a and type α. The two cell types are genetically and physiologically distinct, yet they are able to form a third cell type — a / α diploid by means of mating. At the heart of this mating process is the mating type (MAT) site in Saccharomyces cerevisiae cells. The information encoded by this locus determines the mating type of cells, i. e., it determines whether the cell is type a, α, or a / α diploid.

Specifically, haploid S. cerevisiae cells would carry one of the MAT a or MAT α alleles. When these cells mate, if they each carry different alleles (i. e., one carries MAT a and the other carries MAT α), then they will form a / α diploid cells. These two MAT alleles are together in diploid cells and encode a series of master regulatory genes. The intracellular expression of these master regulatory genes will affect the expression patterns of many other genes, thus leading to cell-type-specific differentiation and functional differences5.

Thus, the HO gene and its encoded endonuclease play key roles in the sex switch of S. cerevisiae, while the MAT locus determines the type of cell mating and the subsequent direction of cell differentiation. These complex regulatory mechanisms make Saccharomyces cerevisiae an ideal model organism for the study of eukaryotic genetics and molecular biology.

1.6 Effect of ploidy on recombinant expression in Saccharomyces cerevisiae

As an important bioreactor, S. cerevisiae has wide applications in the field of recombinant protein expression. However, yeast ploidy, the number of its chromosomes, is one of the key factors affecting the efficiency of its recombinant expression. We review the recent progress on the effect of ploidy on recombination expression in S. cerevisiae.

First, we will understand the ploidy type of S. cerevisiae. Saccharomyces cerevisiae has two main haploid (haploid) and diploid (diploid). Haploid yeasts contain only a complete set of chromosomes, while diploid yeasts contain two sets. This difference in ploidy has significant effects on recombinant expression in S. cerevisiae. In terms of recombinant expression, ploidy may affect the expression level of foreign genes in S. cerevisiae. Moreover, ploidy may also affect processes such as folding, modification and secretion of recombinant proteins in S. cerevisiae. These processes are essential for the proper expression and activity of the foreign protein in yeast. The ploidy changes may lead to changes in the mechanism of protein synthesis and modification in yeast cells, thereby affecting the quality and yield of foreign proteins5.

The differences in gene expression of S. cerevisiae with different ploidy are mainly due to the quantitative changes of their chromosomal groups, which then affect gene transcription, translation and regulation. At the transcriptional level, S. cerevisiae with different ploidy may have different transcriptional efficiencies. As polyploid yeast contains more chromosomes and therefore may have more

transcription factor binding sites, which may lead to higher transcriptional activity than haploid or diploids. Moreover, some genes may have multiple copies in polyploid yeast, thus increasing the chances of their transcription and expression.

In terms of translation and regulation, S. cerevisiae with different ploidy also has different mechanisms. They may have unique transcription factors and regulatory elements that recognize and bind to specific DNA sequences to regulate gene expression in a specific manner. Moreover, ploidy changes may also affect epigenetic modifications in S. cerevisiae, such as DNA methylation and histone modifications, which can affect gene accessibility and transcriptional activity.

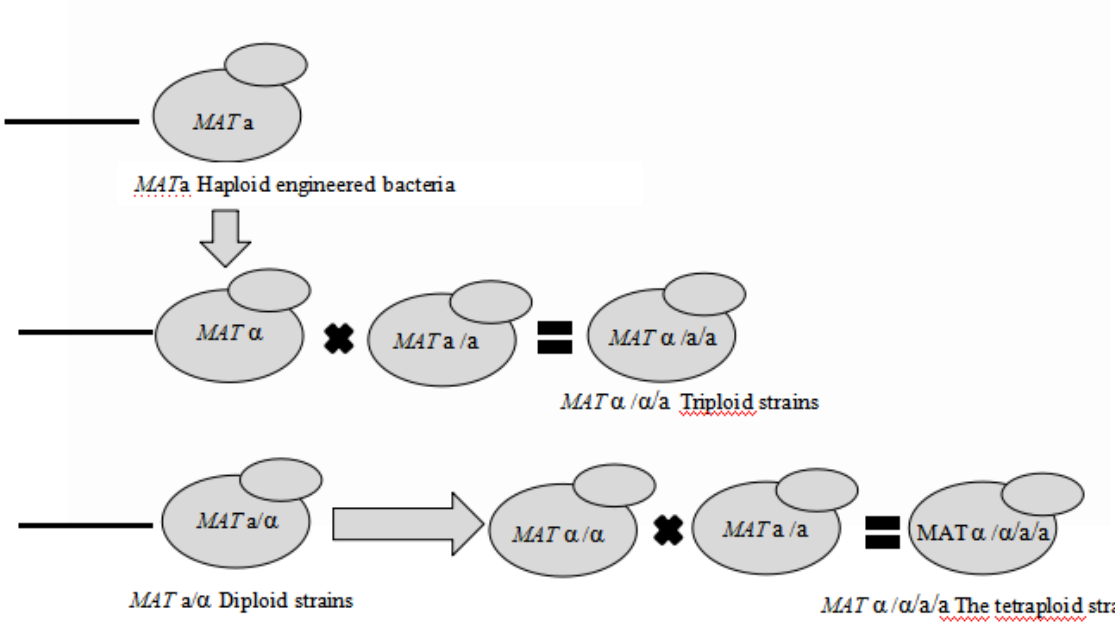
From the perspective of genetic recombination, S. cerevisiae has different patterns of chromosome pairing and exchange at meiosis, which may lead to some change in the frequency and pattern of genetic recombination. These changes not only affect genomic stability and diversity, but may also potentially have profound effects on the whole metabolic pathways and cellular functions, by affecting the expression of certain key genes6.

S. cerevisiae with different ploidy also differ in environmental fitness and metabolic pathways. They may have different mechanisms of response to environmental changes that involve the expression changes of a range of genes to adapt to new growth conditions. At the same time, ploidy changes may also affect the metabolic pathways and enzymatic activities in S. cerevisiae, thus affecting the yield and quality of specific metabolites.

It should be noted that these differences are not isolated but intertwined and influential. Meanwhile, these differences may also be influenced by various factors, such as the genetic background, growth conditions, and culture medium components of S. cerevisiae. Therefore, when deeply studying the gene recombinant expression differences in different ploidy S. cerevisiae, we need to consider these factors comprehensively and make in-depth analysis and comparison with the help of modern molecular biology techniques.

Overall, the differences in gene expression of S. cerevisiae with different ploidy are multifaceted, and these differences provide us important clues into the biological characterization of S. cerevisiae and optimization for industrial applications. Through further research, we can better utilize these differences and improve the performance and application value of S. cerevisiae.

1.7 technology roadmap



Conclusions to chapter 1

In this paper, we aim to explore the role played by ploidy during recombinant expression in S. cerevisiae. To achieve this goal, we have constructed a specific plasmid that contains the HO gene and can be expressed under the induction of galactose. Subsequently, we used this plasmid to change the mating type of yeast cells, and then by yeast cell hybrid technology, successfully obtained polyploid cells.

Understand knowledge about biological ploidy, HO plasmid sex transfer, and the impact of ploidy on recombinant expression in brewing yeast.

CHAPTER 2

OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Strains and plasmids

Strain:EBY100-W（MATa leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100）

rDNA: insulin ; DH5α(Genotype slightly)

2.2 Culture medium

(1) YPD liquid medium: yeast extract: 1%, protein: 2%, glucose: 2%

(2) YPD solid medium: 2% agar powder was added to the YPD liquid medium

(3) LB liquid media: trypsin: 1%, yeast extract: 0.5%, NaCl: 1%, pH 7.0

2.3 Main reagents and solutions

2.3.1The main reagent

Table 2.1 – **Important materials used in this work**

№	Name	Manufacturer
1	Yeast extract	OXOID LTD., BASINGSTOKE, HAMPSHIRE, ENGLAND
2	Tryptone	OXOID LTD., BASINGSTOKE, HAMPSHIRE, ENGLAND
3	NAOH	Laiyang Kant Chemical Co., Ltd
4	HCL	Laiyang Kant Chemical Co., Ltd
5	NACL	Sinopharm Group Chemical Reagent Co., LTD
6	amylaceum	Sinopharm Group Chemical Reagent Co., LTD
7	powdered agar	JAPAN
8	peptone	Beijing Aboxing Biotechnology Co., LTD
9	galactolipin	Beijing raw workers
10	uracil	Beijing raw workers
11	Yeast nitrogen source YNB	Beijing raw workers

2.3.2 The main solution

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

(2) Tris HCl Solution: 50 mmol/L, hydrochloric acid adjusted pH to 8.0;

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

(4) 50 TAE electrophoresis buffer (1 L): Tris 242g, 57.1 mL, 0.5 mol/L EDTA 100 mL (pH 8.0);

(5) NaCl Solution: 1.2 mol / L;

(6) NaOH Solution: 2.0 mol / L;

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

(8) CTAB solution: 5%;

(9) Ampicillin: 100 mg / mL storage solution in sterile ultrapure water, stored at-20℃;

(10) Breaking buffer: Triton X-100 2% (v / v), SDS 1% (w / v), NaCl 100 mmol/L, EDTA 1 mmol/L, Tris HCl (pH 8.0) 10 mmol / L;

(11) Phenol chloroform solution: 1:1 (v / v);

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

2.4 Main instrument

Table 2.2 – **Important apparatuses used in this work**

Instrument name	Instrument model	producer
microwave oven	WD 5241	Galanz Microwave Appliance Appliance Co., Ltd
Fully automatic new biochemical incubator	ZX A D -V 1890	Shanghai Yihua Analytical Instrument Manufacturing Co., LTD
ice box	FHS -206BIA	Qingdao Haier Co., Ltd
Automatic double pure	S Q -93	Shanghai Yarong Biochemical

water still		Instrument Factory
ultra low temperature freezer	VF F -U 66V	Sanyo Electric Corporation is made in Japan
Magnetic heating the stirrer	79-1	Guohua Electric Appliance Co., Ltd
thermostat water bath	KW -1000JB	Guohua Electric Appliance Co., Ltd
centrifuge	Centrifuge 5418	The German company eppendorf
Ultraviolet instrument	JY -9403C	Beijing Liuyi Instrument Factory
electronic balance	KA -I 820	Fuzhou Huazhi Scientific Instrument Co., LTD
Micropipette (in each range)	J 13360Q	The German company eppendorf
PCR appearance	63SGVF5503619	The German company eppendorf
Multifunction microscope	H550K	Japan Nikon Inc
Electrophotometer power supply	DYV -6C	Beijing Liuyi Instrument Factory
table	ZWY -211B	Shanghai Zhicheng analytical instrument manufacturing
Automatic pressure steam sterilization pan	GI54NJD	Zhiwei (Xiamen) Instrument Co., LTD

2.5 In Experimental Procedures

2.5.1 Yeast culture and the growth curves were plotted

First, single colonies were picked from S. cerevisiae plates kept at 4℃, and seeded as underlined on YPD plates. Subsequently, incubation at 28℃lasted for 24 to 48 h to ensure full colony growth. Next, from the activated YPD solid medium, single colonies were further selected and transferred to a container containing 15mL YPD

liquid medium. Incubation was shaken in a shaker at 28℃, 200 r/min for about 48h for subsequent experimental use or hourly sampling to determine OD_{600} to draw the growth curve7.

2.5.2 Agarose-gel electrophoresis of the DNA

Agarose gel preparation: First, 0.15 to 0.2 g of agarose was precisely weighed and 20 ml of TAE buffer was added. Subsequently, the agarose was completely dissolved by heating for about 1 min. When cooled to about 60℃, a small amount of EB solution was added and the dot comb was quickly inserted. Next, the mixture was poured evenly into the rubber plate and left at room temperature for 10 minutes to ensure adequate solidification**Ошибка! Источник ссылки не найден.**.

Preparation and electrophoresis preparation: when the gel is completely fixed, gently pull out the comb. Next, the prepared gel was carefully moved into the electrophoresis tank. First, 5 microliters of Marker was added to the first well as a reference. Then, 5 microliters of the sample was fully mixed with 1 microliter of Loading Buffer, and the mixture was added to the spot-sample wells using a pipetting gun.

Conduct agarose gel electrophoresis: Set the electrophoresis instrument parameters to 100 V, 100 mA to perform electrophoresis until a third DNA band is observed (approximately 30 minutes).

Analysis of electrophoresis results: carefully observe the DNA bands on the gel imager, record and analyze the electrophoresis results.

2.5.3 DNA glue recovery

1. First, the 1.5 mL centrifuge tube was weighed. Protective from the UV light source, the agarose gel block containing the target DNA fragment was precisely cut using a clean surgical blade, then placed into a weighed 1.5 mL centrifuge tube and weighed again to determine the exact weight of the gel block.

2. next, calculate the volume of the required Buffer B2 to be added. The appropriate amount of Buffer B2 was calculated precisely at the ratio of 300 μL Buffer B2 corresponding to each 100mg of agarose gel block.

3. Place the centrifuge tube equipped with the gel block and Buffer B2 in a 50℃ water bath, and heat it continuously until the gel block is completely dissolved to form a uniform mixture.

4. the dissolved mixture was transferred to the adsorption column and centrifuged at 5500 g for 30 seconds. Remove the liquid from the collection tube and put the adsorption column back into the same collection tube.

5. add 300 μL of Buffer B2 to the adsorption column and subsequently centrifuged again at 7000 g for 30 seconds. Remove the liquid from the collection tube and keep the adsorption column in the same collection tube.

6. ensured thorough cleaning, added 500 μL of Wash Solution to the adsorption column and centrifuged at 7000 g for 30 seconds. Remove the liquid from the collection tube and return the adsorption column back to the same collection tube.

7. Repeat the cleaning process of Step 6 once to ensure that the impurities in the adsorption column are completely removed.

8. place the empty adsorption column and collecting tube into the centrifuge and centrifuge at 7000 g for 1 minute to remove any residual liquid.

9. 35 μL of Elution Buffer was added to the center of the adsorption membrane, allowed to stand at room temperature for 2 minutes, and subsequently centrifuged at 7000 g for 1 minute. The recovered DNA solution was transferred to-20℃ conditions for subsequent experiments**Ошибка! Источник ссылки не найден.**.

2.5.4 Methods for yeast genomic DNA isolation

1. Saccharomyces cerevisiae EBY100-W or commercially available fermented powder yeast was inoculated in 5 mL of liquid YPD and incubated overnight in a thermostatic incubator at 30℃ and 200 r/min with shaking;

2. bacteria were collected by centrifugation at 2.14500 g and washed in pure water.

2.5.5 Yeast cells in a hybrid with different mating types

1, plasmids carrying different screening markers were introduced into two yeast cell strains with different mating types.

2 Subsequently, the two freshly prepared yeast cells carrying the screening marker plasmid were seeded into the same bottle of YPD medium and incubated for two days at 30°C9.

3. After the incubation, we diluted the bacterial solution appropriately and coated it evenly on the plates containing both screening conditions.

4. After a single colony grew on the plate, we used the DNA content determination method of yeast cells to verify whether the yeast hybrid was successful10.

Conclusions to chapter 2

Clear information on how S. cerevisiae cells are cultured.

List the experimental equipment and main reagent that will be used in the experiment.

The experimental methods will be used in the experiment, such as DNA glue recovery,agarose-gel electrophoresis of the DNA,yeast genomic DNA isolation,and how to culture yeast cells of different mating types.

CHAPTER 3
EXPERIMENTAL PART

The utilization of insulin-producing engineered bacteria in this study capitalizes on the efficient post-translational protein processing mechanisms inherent in these organisms. This natural processing pathway results in the production of active insulin directly, eliminating the need for additional denaturation, renaturation, and enzymatic digestion steps. However, despite this advantage, the overall yield of insulin remains low. Moreover, attempts to enhance production through heterologous secretory expression pathways have proven to be limited by the reliance on a few specific pathways or the rational modification of physiological and biochemical processes, which often results in inefficiencies. To address these challenges and improve insulin production, it is imperative to shift the focus towards a more comprehensive overhaul of the entire cellular metabolic network. Given the unique reproductive characteristics of Saccharomyces cerevisiae, which exhibits both haploid and diploid vegetative reproduction as well as a sexual hybrid life cycle, it becomes feasible to engineer cell systems of varying ploidy levels using these mechanisms. By leveraging this capability, it becomes possible to manipulate the entire metabolic network by modulating gene dosage at the genomic level, thereby achieving a more systematic and effective approach to insulin production optimization.

3.1 Verification of the mating type of insulin-producing engineered bacteria

Based on the structural characteristics of the mating type control gene in S. cerevisiae, three primers were designed to quickly identify the mating type of yeast by colony PCR10. X-insulin was coated on YPD dishes and incubated for 24 to 48 h before colony PCR and detection by agarose gel electrophoresis. The results are

shown in Figure3.1 and are shown as smaller fragments, meaning that the engineered bacterium X-insulin is MAT α and denoted as I MATα.

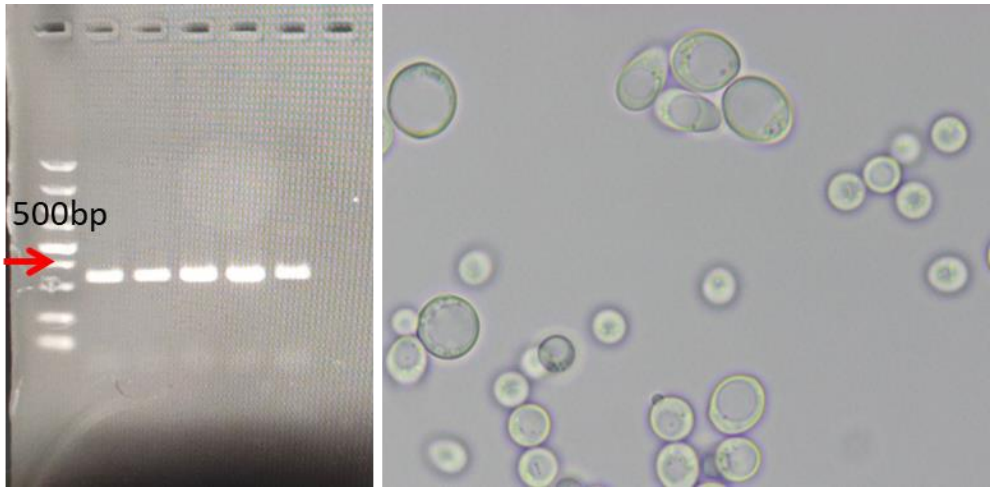


Figure 3.1 – X-insulins validation of ploidy and mating type

In the course of our experimental investigation, we successfully obtained an electrophoresis band of less than 500 base pairs (bp) through the application of polymerase chain reaction (PCR) with three distinct primer methodologies. This specific band was of particular interest due to its potential significance in identifying the genetic makeup of the target strain. Upon further analysis, we observed that this band exhibited a distinct pattern that was distinct from other bands obtained in the PCR process.

To gain a more comprehensive understanding of the characteristics of this band, we employed microscopic observation techniques. Through this approach, we were able to visualize the morphology of the strain in question. Our observations revealed that the strain exhibited a spherical shape, which is a common feature among yeast cells. However, it was notable that the size of this strain was significantly smaller compared to the diploid strain, specifically the Angel yeast strain, which served as a reference point in our analysis.

Based on these combined observations, we were able to make a preliminary judgment regarding the identity of the engineered starting strain. Specifically, we concluded that it was likely a MAT α strain. This conclusion was supported by the fact that MAT α strains are known to exhibit smaller cell sizes compared to diploid

strains, which is consistent with our microscopic observations. Additionally, the presence of the specific electrophoresis band obtained through PCR further corroborated our assessment, as it is indicative of the genetic makeup associated with MAT α strains.In summary, our experimental approach, which combined PCR analysis and microscopic observation, allowed us to identify a unique strain that exhibited characteristics consistent with MAT α strains.11.

3.2 Construction of diploid engineering bacteria

Ho controls the yeast mating type conversion, genetically transformed MAT α of X-insulin, and PCR. Thereafter, diploid strain II was intercrossed by YPD liquid co-culture(As shown in Figure 3.2)13.

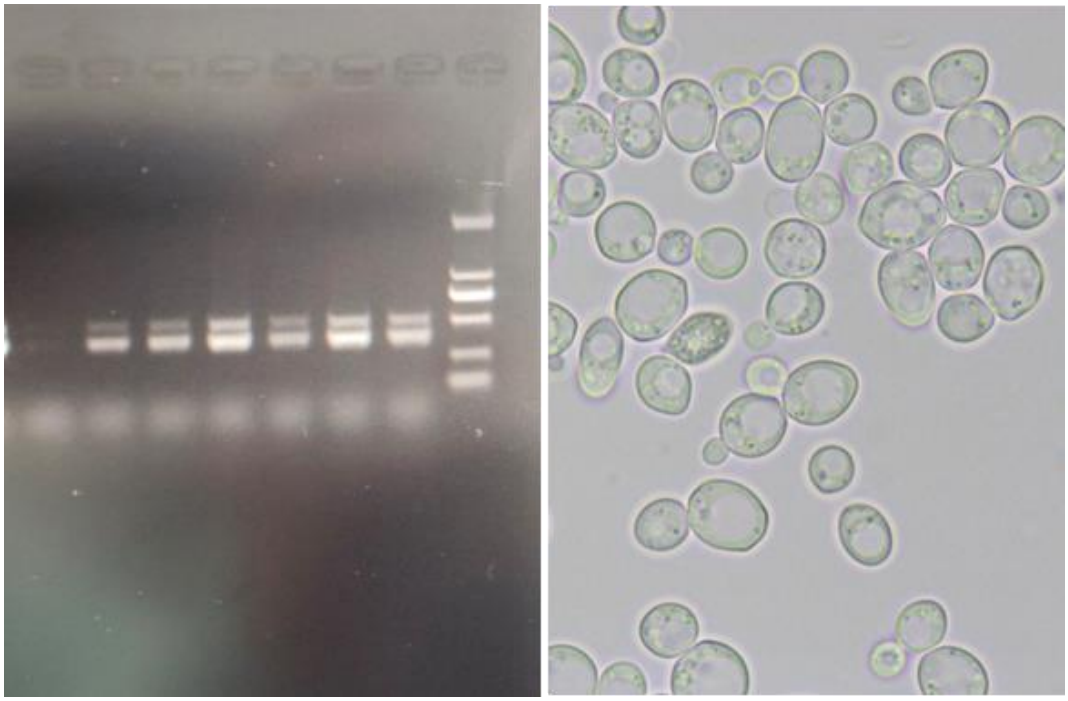


Figure 3.2 –PCR validation and microscopy of diploid engineered bacteria

In our experimental protocol, we followed a systematic approach to genetically transform and cross-breed yeast strains in order to obtain specific mating genotypes. Specifically, we initiated the process by genetically transforming the plasmid pYClac33-GHK into the diploid strain II. This transformation was crucial in ensuring

that the strain possessed the necessary genetic material to undergo the subsequent crossing experiments.

After successful transformation, we selected the diploid strain II MATa/a and MATα/α, which were homozygous for their respective mating genotypes. This homogeneity was essential for ensuring consistent genetic outcomes in the subsequent crossing experiments.

Next, we performed a controlled crossing experiment between the diploid strain II MATa/a and the starting strain X-insulin MATα. The objective of this crossing was to obtain a triploid strain III MATa/a/α. This triploid strain was of particular interest due to its unique genetic makeup, which offered the potential for studying the effects of multiple gene copies on yeast phenotypes.

In addition, we also crossed the diploid strain II MATa/a with the MATα/α strain to obtain a tetraploid strain IV MATa/a/α/α. The tetraploid strain was significant in our study as it allowed us to investigate the effects of increased ploidy on yeast growth, metabolism, and other phenotypic traits.

To verify the success of our crossing experiments, we conducted microscopic examinations of the resulting strains. These examinations revealed that the appearance and morphology of the triploid and tetraploid strains were similar to that of the original diploid strain. This similarity was reassuring, indicating that the crossing experiments had not adversely affected the fundamental characteristics of the yeast cells.

Overall, our experimental protocol demonstrated a rigorous approach to genetically transforming and crossing yeast strains to obtain specific mating genotypes.

3.3 Characterization of the growth characteristics of the engineered bacteria and the insulin production characteristics

3.3.1 Growth feature

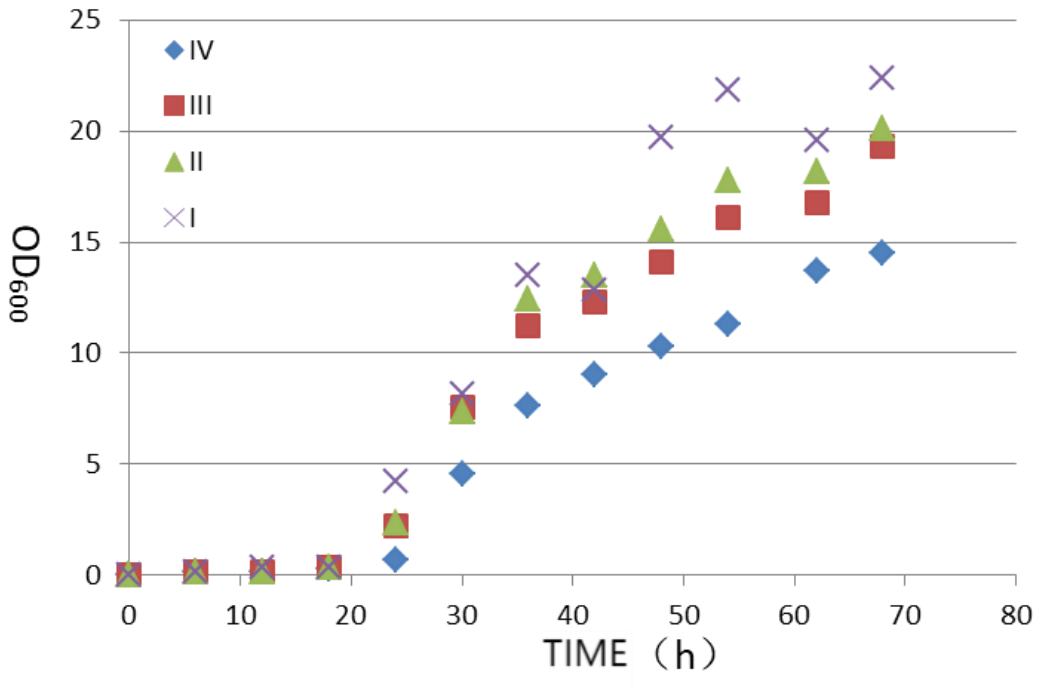


Figure 3.3 –Growth curves of yeast strains with different ploidy

The growth status of diploid Saccharomyces cerevisiae (S. cerevisiae) cultivated in liquid yeast extract, peptone, and dextrose (YPD) medium is depicted in Figure 3.3. This figure serves as a crucial data point in our investigation of how variations in ploidy impact the growth patterns of yeast cells in YPD.

As evident from Figure 3.3, there is a clear correlation between ploidy and the growth rate of S. cerevisiae. Specifically, as the ploidy increases, the growth rate of the strain decelerates. Haploid strains, which possess a single set of chromosomes, exhibit the most rapid growth and ultimately achieve the highest cell density. This trend suggests that the increased complexity of genetic material in cells with higher ploidy levels may hinder their ability to proliferate efficiently.

One possible explanation for this observation is that ploidy changes influence the asexual reproduction process within yeast cells. Cellular events such as mitosis, which is responsible for the duplication of genetic material and the division of the

cell nucleus, are profoundly affected by the number of chromosomes present. As ploidy increases, the complexity of these cellular events also increases, potentially leading to slower growth rates.

In Figure 3.3, a distinct phase of logarithmic growth is observed between 20 hours and 45 hours. This phase is characterized by an exponential increase in cell numbers as the yeast cells proliferate rapidly. However, as the graph illustrates, the duration and rate of this logarithmic growth phase vary significantly depending on the ploidy of the strain. These differences highlight the importance of considering ploidy as a critical factor that determines the growth patterns of S. cerevisiae in liquid YPD medium.

In summary, Figure 3.3 provides valuable insights into how variations in ploidy impact the growth of S. cerevisiae in YPD. Our findings indicate that ploidy changes not only affect the overall growth rate of yeast cells but also the specific cellular events that govern their reproduction.

3.3.2 Insulin production

In the pursuit of optimizing the production of insulin by diploid yeast engineered bacteria, a rigorous experimental protocol was implemented. Specifically, the yeast strains were cultivated in YPD medium, a nutrient-rich medium commonly used for yeast growth, for a duration of 25 to 30 hours. This cultivation period was chosen to ensure that the yeast cells reached an optimal state for the subsequent steps of the experiment.

After the growth phase, the yeast cells were collected using centrifugation, a process that separates the cells from the liquid medium based on differences in density. This step was crucial to isolate the yeast cells for further manipulation.

Subsequently, the collected yeast cells were resuspended in a volume of YPG medium that was 1/5 of the original YPD medium volume. This specific ratio of YPG medium was chosen to induce the expression and secretion of insulin. YPG medium,

which contains glycerol as a carbon source, is known to trigger the expression of certain genes in yeast, including those encoding for insulin production.

Once the yeast cells were resuspended in YPG medium, they were allowed to incubate for a sufficient period to induce insulin expression and secretion. During this phase, the yeast cells utilize the glycerol in the YPG medium as an energy source, which triggers the expression of the insulin-encoding genes. The resulting insulin protein is then secreted into the surrounding medium.

After the induction phase, the supernatant containing the secreted insulin was separated from the yeast cells using centrifugation. This supernatant was then analyzed using SDS-PAGE gel electrophoresis, a technique that separates proteins based on their size and charge. The resulting SDS-PAGE bands (depicted in Figure 3.4) provided a visual representation of the insulin protein, allowing for qualitative assessment of insulin production.

To obtain quantitative data on insulin secretion, the bands corresponding to insulin on the SDS-PAGE gel were analyzed. The relative intensity of these bands was used to estimate the amount of insulin secreted by each strain. This data was then compiled in Table 3.1, providing a comprehensive overview of the insulin secretion capabilities of the various yeast strains.13.

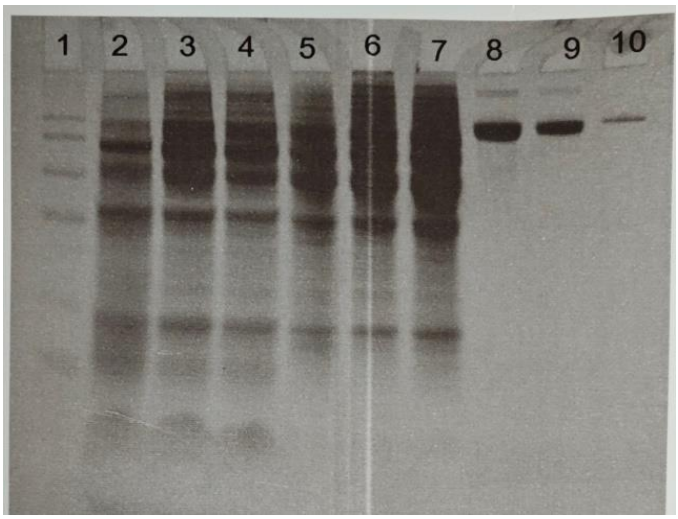


Figure 3.4 –SDS-PAGE analysis of insulin production in different strains

Table 3.1 Insulin production in diploid S. cerevisiae

Saccharomyces cerevisiae strains	Insulin production(mg/L)
X-insulin *MATα*	10
II *MATa/α*	17
III *MATa/a/α*	28
IV *MATa/a/α/α*	16
H	15
H*	51

In a comprehensive analysis of insulin production in engineered bacteria with varying ploidy levels, we observed distinct differences in the efficiency and stability of insulin secretion. Specifically, when comparing the insulin production among haploid, diploid, and triploid strains, the haploid strain exhibited the lowest level of insulin production. This finding suggests that the haploid state, with its single set of chromosomes, may not provide the optimal genetic environment for the efficient expression and secretion of insulin.

In contrast, the triploid strain displayed the highest insulin production, with a constant output of 30 ± 4 mg/L. This consistency in insulin secretion indicates that the triploid strain possesses a genetic stability that is conducive to the reliable and sustained production of insulin. The triploid state, with its three sets of chromosomes, may provide a more robust genetic background that supports the expression of insulin-related genes and the subsequent secretion of the hormone.

The diploid strain, with its intermediate level of insulin production, may reflect a compromise between the haploid's lower production and the triploid's higher stability. However, given the superior performance of the triploid strain, it emerges as the preferred candidate for subsequent breeding efforts aimed at further optimizing insulin production.

In summary, our findings demonstrate that the ploidy level of engineered bacteria has a significant impact on insulin production. The triploid strain, with its

superior insulin secretion and genetic stability, holds promise as a starting strain for future breeding programs focused on enhancing insulin yield and reliability.

Conclusions to chapter 3

Specific analysis on how to construct HO plasmids and different ploidy engineering bacteria.

Mating type validation of insulin producing engineering bacteria.

Based on the experimental results, analyze the growth characteristics and insulin production characteristics of engineering bacteria.

CONCLUSIONS

In the current study, the focus lies on insulin-producing engineered bacteria that leverage the advantageous post-translational protein processing capabilities to generate naturally active insulin. This approach circumvents the need for subsequent processing steps such as denaturation, renaturation, and enzymatic digestion, thus simplifying the overall production process. However, despite these benefits, a significant limitation remains: the yield of insulin produced by these engineered bacteria is comparatively low.The reason for this low yield often stems from the reliance on a limited number of secretory pathways or the inefficiency of rational modifications to physiological and biochemical processes. To address this challenge, it is imperative to shift our approach from merely targeting individual pathways or processes to a more comprehensive and global remodeling of the entire cellular metabolic network.

One promising strategy is to harness the unique characteristics of Saccharomyces cerevisiae, a yeast species that possesses both haploid and diploid vegetative reproduction as well as a sexual hybrid life cycle. Through this mechanism, we can construct cell systems with varying ploidy levels, which allows us to regulate the entire metabolic network at the genomic level by altering the gene dosage.

By systematically manipulating the gene dosage in Saccharomyces cerevisiae, we can fine-tune the expression levels of key enzymes and proteins involved in insulin production. This approach not only addresses the issue of low yield but also opens up new possibilities for optimizing the metabolic pathways and cellular processes that contribute to insulin synthesis and secretion.

In the vast field of bioengineering, S. cerevisiae plays a pivotal role. Among them, the ploidy change in S. cerevisiae, the state transition from haploid, diploid to polyploid, has a direct and profound effect on its gene expression pattern. This diversity of ploidy not only reveals the complexity of life activities in yeast, but also

provides us with potential ways to optimize the performance of their industrial applications.

When we talk about the ploidy change in S. cerevisiae, we are actually exploring the diversity of its chromosomal components. Haploid yeasts contain only a complete set of chromosomes, while diploids have two sets and polyploids contain more. This change in the number of chromosome directly affects the gene expression pattern in yeast cells. These yeast cells with different ploidy may display distinct transcriptional activity during recombinant expression. Specifically, some genes may be in a low expression state in haploid yeast and may be highly active in polyploid yeast. This differential expression pattern of yeast cells not only affects the physiological function of yeast cells, but also is directly related to the yield and nature of the target proteins that we care about.

The ploidy change in S. cerevisiae also had significant effects on its metabolic pathways. Polyploid yeasts generally display higher metabolic activity and greater environmental adaptability. This enhanced metabolic activity, not only allows yeast cells to grow and reproduce faster, but also provides the possibility to produce more target proteins in recombinant expression. For example, during insulin production, polyploid yeast are able to achieve higher insulin production with their superior metabolic capacity and environmental adaptability.

Undoubtedly, the in-depth exploration of the ploidy changes in Saccharomyces cerevisiae (S. cerevisiae) holds immense significance in our comprehension of the intricate laws governing yeast's life activities. Not only does it provide us with a deeper understanding of the fundamental biological processes within this yeast species, but it also presents us with novel avenues to optimize its performance in various industrial applications.

Furthermore, the study of ploidy changes in S. cerevisiae also holds promise in the field of bioenergy. As a robust microbial cell factory, yeast has the potential to convert renewable biomass into valuable biofuels and bioproducts. By optimizing its metabolic pathways through ploidy manipulation, we can enhance the efficiency of

this conversion process, contributing to the development of sustainable energy sources.

In conclusion, the thorough investigation of ploidy changes in S. cerevisiae offers us a powerful tool to expand our knowledge of yeast biology and to enhance its performance in diverse industrial applications. This research not only advances our understanding of fundamental biological processes but also paves the way for the development of innovative technologies that will shape the future of biotechnology.

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