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 **Effect of Hog1p CD domain knockout on tolerance of complex inhibitors of *Saccharomyces cerevisiae***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Zihan Liu

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

Reviewer
Olga IUNGIN, 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
Zihan Liu

1. Thesis topic **Effect of Hog1p CD domain knockout on tolerance of complex inhibitors of *Saccharomyces cerevisiae***
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 _____ Zihan LIU

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Zihan Liu. Effect of Hog1p-CD domain knockout on tolerance of complex inhibitors of *Saccharomyces cerevisiae*. – Manuscript.

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

Abstract, the aim of this study was to improve the co-fermentation ability of *Saccharomyces cerevisiae* and its tolerance to inhibitors, so as to enhance the efficiency of producing second-generation fuel ethanol from *Saccharomyces cerevisiae*. It was found that there was an antagonism between the robustness of inhibition in hydrolysate and xylose metabolism of *Saccharomyces cerevisiae* using glucose/xylose. In this study, the 6M-15 yeast strain that has alleviated the robust antagonism of xylose-use and xylose-use was selected in the laboratory. The HOG1 knockout strains 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were constructed. The relationship between HOG1 gene and the robustness of yeast strains and the antagonism between xylose metabolic efficiency and robustness were discussed. Through a series of experiments, including strain construction, growth curve measurement, tolerance evaluation and xylose metabolizing ethanol production ability test, the results showed that the yeast strains with HOG1 gene knocked out had improved the tolerance of complex inhibitors, but decreased the xylose metabolizing ethanol production ability. In addition, a synergy index (SI) was established to characterize the degree of balance between xylose metabolizing ability to produce ethanol and robustness of the strains. The results showed that 6M-15-HOG1 $^{\Delta\ (303\text{-}316)Ww}$ strain had the highest degree of antagonistic balance between xylose-producing capacity and acetic acid tolerance, indicating that the strain had good application potential in the actual production process. This study not only provides a molecular mechanism for understanding the physiological response of *Saccharomyces*

cerevisiae in the face of environmental stress, but also provides an experimental basis for improving the production performance of yeast strains by means of genetic engineering, which is of great significance for promoting the industrialization of biomass energy.

Keywords: Second generation fuel ethanol, Robustness, Synergy index

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INTRODUCTION

With the intensification of the global energy crisis and the increasing problem of environmental pollution, it is particularly important to find renewable and clean alternative energy sources. Biomass energy, because of its renewability and environmental friendliness, has become a new focus of energy research. Especially in the field of fuel ethanol, the second-generation fuel ethanol produced from lignocellulosic biomass has attracted much attention because of its abundant raw materials and potential environmental benefits. However, inhibitors produced during lignocellulosic pretreatment have a significant impact on the fermentation process and the growth of yeast strains, which has become a major obstacle to improving the efficiency of fuel ethanol production.

In this context, the optimization of tolerance and metabolic efficiency of *Saccharomyces cerevisiae*, as an important microbial fermentation strain in industry, has become the key point of research. In particular, HOG gene, as a key signaling pathway for *Saccharomyces cerevisiae* to cope with high osmotic pressure environment, has attracted extensive research interest in its role in improving the robustness of yeast. The aim of this study was to explore the effects of Hog1p on the tolerance and xylose metabolism of saccharomyces cerevissae by eliminating the C-terminal domain (CD domain) of Hog1P, in order to solve the antagonistic phenomenon in the process of glucose/xylose co-fermentation and improve the production efficiency of fuel ethanol.

The research contents of this paper include: constructing HOG1 knockout strains, evaluating the complex inhibitor tolerance of strains, testing xylose metabolism to produce ethanol, and using the Synergy Index (SI) to characterize the performance of strains. Through these studies, we can not only deeply understand the physiological response mechanism of *Saccharomyces cerevisiae* in the face of environmental stress, but also provide experimental basis for improving the production performance of yeast strains through genetic

engineering. This has important theoretical and practical significance for promoting the industrialization of biomass energy.

The relevance of the topic is Second generation fuel ethanol with lignocellulose as raw material.

The purpose of the study is the development and application of the second generation fuel ethanol has a profound significance to ease energy shortage and environmental pollution.

The objectives of the study is to higher xylose fermentation efficiency of *Saccharomyces cerevisiae* was obtained in lignocellulosic hydrolysate.

The object of the study is C5/C6 co-fermentative strain, 6M-15.

The subject of the study is C5/C6 co-fermentative strain, 6M-15.

Research methods Hog1 CD Δ fragment was obtained by molecular biology related techniques (such as PCR, fusion PCR), and the marker gene NatMX and homologous arm were added. The target fragment was transformed into the 6M-15 genome using homologous recombination techniques, and the marker gene was knocked out using the Cre-LoxP recombinase system. After the integration of the two chromosomes, the fermentation performance was analyzed. Biomass and glucose metabolites were determined by microspectrophotometer and HPLC, respectively.

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The practical significance of the results obtained is the fermentation efficiency of *Saccharomyces cerevisiae* in industrial production was improved.

CHAPTER 1
LITERATURE REVIEW

1.1 Biomass resources

With the excessive consumption of fossil fuels, the global energy crisis has gradually intensified, and the problem of environmental pollution has become increasingly serious. As an environmentally friendly, renewable, clean and abundant form of energy, biomass energy has attracted wide attention [**Ошибка! Источник ссылки не найден.**]. Biomass refers to the organic matter formed by the conversion of solar energy by forest trees and other woody plants through photosynthesis. Biomass energy refers to the energy formed by the conversion of biomass stored in forest biomass, mainly refers to the energy formed by direct combustion or modern conversion technology that can be used for power generation and heating [1], including forestry resources and forest wood, agricultural resources and agricultural waste, aquatic plants, oil plants, urban and industrial organic waste and livestock manure. It has the characteristics of renewability, low pollution, wide distribution and total abundance [**Ошибка! Источник ссылки не найден.**]. China's biomass resources mainly come from agriculture and forestry industry, which has great potential for development. According to statistics, the amount of biomass resources available from forests in China is about 900 million tons per year, of which nearly 300 million tons can be used for energy development. In addition, there are about 2 billion mu of marginal land in China, which can be used as land for energy plants, and can produce 1 billion tons of biomass resources every year. As a large agricultural country, China has rich biomass resources, and the development of biomass energy is of great significance to adjust the energy structure, reduce environmental pollution and promote rural economic development [1].

As a rich renewable resource, lignocellulose is mainly derived from plant cell wall and is one of the main organic components of plants. Lignocellulose is

mainly composed of cellulose, hemicellulose and lignin three different polymers [3]. Cellulose, the main component, is a linear homopolymer composed of many D-glucopyranose units, which are connected by a β-1, 4-glucoside bond. Hemicellulose is a heteropolysaccharide composed of a variety of different monosaccharides, including pentose (mainly xylose and arabinose), hexose (glucose, galactose, mannose), and a number of other compounds. It is usually covalently bound to cellulose. Lignin is a complex aromatic polymer that provides cell wall rigidity and resistance to external pressures [4]. Due to its unique chemical structure and physical properties, lignocellulose has shown extensive application potential in many fields. Lignocellulose can be converted into biofuels, such as bioethanol and biodiesel, through pretreatment, enzymatic hydrolysis and fermentation processes [**Ошибка! Источник ссылки не найден.**].

1.2 Development status of fuel ethanol

Since the development and research of fuel ethanol, it can be divided into the 1st generation fuel ethanol, the 1.5th generation fuel ethanol, the 2nd generation fuel ethanol and the 3rd generation fuel ethanol according to the different production raw materials and processing technology. The first-generation fuel ethanol uses sugar and starch as raw materials, that is, rice, wheat, rice and other food crops as raw materials [5]. 1.5 Fuel ethanol, also known as non-food ethanol, uses cash crops such as potato, sugarcane and sweet sorghum as raw materials, thus reducing the dependence of fuel ethanol on food crops to a certain extent [**Ошибка! Источник ссылки не найден.**]. The second generation of fuel ethanol is based on lignocellulosic biomass.China as an agricultural country, lignocellulosic biomass resources are very rich. There is a large amount of lignocellulose in straw, hay and various agricultural and forestry wastes, so the second-generation fuel ethanol using lignocellulosic biomass as raw material has high application value. However, using lignocellulosic biomass

as feedstock to produce fuel ethanol requires pretreatment and degradation. Therefore, its production technology cost is relatively high, which limits its industrialization [7][8]. The third-generation fuel ethanol uses microalgae as the raw material for ethanol production. Due to its potential environmental benefits, it is considered to be an important development direction for fuel ethanol in the future, but it is still in the research and development stage and the production process is not fully mature [9]. Natural lignocellulose has complex structure and biological stubbornness, so its biomass can not be fully utilized by microorganisms. It is necessary to separate lignin and/or hemicellulose from lignocellulose by pretreatment method [11]. Pretreatment generally includes the following purposes: 1. Dissolve the lignin and/or hemicellulose contained in natural lignocellulose; 2. Change the structure of lignin and reduce the crystallinity of cellulose; 3. Reduce the size of natural lignocellulose and increase the specific surface area, so that the enzymatic reaction can be carried out efficiently; 4. Reduce the degree of polymerization of cellulose and increase the efficiency of enzymatic hydrolysis [12]. Common pretreatment methods include physical method, physical chemical method, chemical method, biological method and so on. Common physical pretreatment methods include mechanical crushing, steam blasting and carbon dioxide blasting [13]. Chemical methods, the common chemical pretreatment methods mainly include acid treatment, alkali treatment and calculated reagent treatment. The destruction of the chemical bond between cellulose and hemicellulose reduces the crystallinity of cellulose, but compounds such as weak acids, furans and phenols will be produced during the pretreatment process to inhibit the growth of the fermentation strain [14]. Biological pretreatment means that microorganisms destroy the structure of wood cellulose through enzymatic reaction, so as to achieve the purpose of hydrolyzing hemicellulose [15]. Studies have found that during the pretreatment of lignocellulose, cellulose, hemicellulose and lignin in lignocellulose will inevitably decompose, thus producing a large number of inhibitors to inhibit the

growth and fermentation performance of fermentation strains [**Ошибка! Источник ссылки не найден.**]. Among them, inhibitors can be mainly divided into the following categories: weak acids: mainly including acetic acid, formic acid and levulinic acid. Acetic acid is produced by deacetylation of hemicellulose, formic acid and levulinic acid are degradation products of 5-Hydroxymethylfurfural (HMF), and formic acid can also be produced by the degradation of furfural in an acidic environment. Weak acid inhibitors affect the normal growth and metabolism of cells mainly by causing acidification of intracellular environment. Furan derivatives: mainly include furfural and 5-hydroxymethylfurfural (HMF). They are produced by the dehydration of pentose and hexose in acidic environment, respectively. Furan derivatives inhibit the growth and fermentation performance of the fermentation strain by inhibiting enzymes related to metabolic reaction of the fermentation strain, such as ethanol dehydrogenase and pyruvate dehydrogenase [16]. Phenolic compounds: Mainly represented by guaiacol, vanillin, etc., guaiacol family compounds, formed by the degradation of lignin, have a strong inhibitory effect on fermentation, especially low molecular weight phenolic compounds. Phenolic compounds may damage the integrity of cell membranes and affect their function [18].

Therefore, the existence of inhibitors is a major obstacle to the industrial production of biomass feedstock using lignocellulose as raw material. In order to reduce the inhibition of lignocellulosic pretreatment on enzymes and microorganisms, the detoxification methods of inhibitors can generally be divided into the following three categories: 1. Chemical detoxification, through the reaction of chemical reagents with inhibitors produced by lignocellulosic pretreatment to produce non-toxic or low-toxic substances, so as to achieve detoxification. Including alkali detoxification, reducing agent detoxification and amino acid detoxification; 2. Physical detoxification, through the semi-permeable membrane or resin adsorption method to achieve the separation of sugars and pretreatment inhibitors, so as to achieve the purpose of detoxification. Including

membrane detoxification, adsorbent detoxification, etc.; 3. Biological method refers to the reaction of enzymes produced by microorganisms with inhibitors to produce low-toxic or non-toxic substances or the detoxifying of microorganisms with high tolerance to inhibitors, so as to achieve the purpose of rapidly and effectively improving fermentation capacity. These methods include enzymatic detoxification and construction of strains with high tolerance to inhibitors [**Ошибка! Источник ссылки не найден.**]. In summary, the inhibitors produced during the pretreatment process have toxic effects on yeast cells through different mechanisms, but through genetic engineering and fermentation process optimization, the tolerance of yeast can be improved, thus enhancing the performance of the fermentation strain in the actual production and processing.

With the continuous development of modern biotechnology and metabolic engineering technology, saccharsacchara cerevisiae has realized efficient fermentation of glucose and xylose in the field of fuel ethanol, but inhibitors derived from lignocellulose interfere with sugar metabolism [20]. Therefore, improving the robustness of *Saccharomyces cerevisiae*, that is, the ability of fermentation strains to maintain stable physiological functions in the face of environmental stress, has become the focus of research. The robustness of *Saccharomyces cerevisiae* directly affects the efficiency of the fermentation process and the quality of the product, especially in the face of high osmotic pressure, extreme temperature, oxidative stress and other industrial conditions, it is particularly important to improve its tolerance. In recent years, with the development of synthetic biology and metabolic engineering, the research on improving the robustness of *Saccharomyces cerevisiae* has achieved a series of results. For example, the inhibition or overexpression of one or more genes related to inhibitor metabolism can be achieved through gene editing technology. Therefore, gene editing technology plays an important role in the construction of highly robust fermentation strains. The traditional gene editing technology uses the principle of homologous recombination to replace the original target gene

fragment by providing foreign gene fragment. Among them, the commonly used gene editing system in *Saccharomyces cerevisiae* is the Cre/LoxP system [22]. New gene-editing technologies, such as the CRISPR/Cas9 system, provide powerful tools for precisely modifying yeast genomes. Through this technology, it can be achieved, including gene knockout or integration, transcriptional activation or inactivation, so as to confer new or improve existing biological characteristics on *Saccharomyces cerevisiae* [23]. For example, through gene editing technology, researchers can knock out negative regulatory genes that affect yeast tolerance, or enhance the expression of specific stress-response genes to improve yeast adaptability to environmental stress. Metabolic engineering strategies improve the performance of yeast under industrial fermentation conditions by optimizing its metabolic pathway, enhancing its utilization efficiency of nutrients, and improving the supply and turnover of key cofactors in cells. In addition, synthetic biology approaches allow researchers to design and construct new metabolic pathways that can give yeast cells new biosynthetic capabilities or improve their tolerance to specific compounds [21]. Adaptive laboratory evolution is a technique that simulates the natural evolutionary process and promotes the rapid evolution of *Saccharomyces cerevisiae* to adapt to specific industrial fermentation conditions through artificial selection pressure. This method has been successfully used to improve yeast tolerance to high temperature, high osmotic pressure and high ethanol concentration [24]. Regulation of the in-protein equilibrium network is another strategy to improve the robustness of yeast. By optimizing the balance of protein synthesis, folding and degradation within cells, the response speed and adaptability of cells to environmental changes can be improved, thus enhancing their overall robustness [24]. The development of high-throughput genome editing technology allows researchers to edit multiple genes simultaneously, which opens up the possibility of modifying complex traits and building cell factories. In this way, the performance of *Saccharomyces*

cerevisiae can be systematically analyzed and improved to achieve fine regulation of cell functions [25].

1.3 Research progress on the relationship between HOG gene and robustness in *Saccharomyces cerevisiae*

The hyperosmotic glycerol signaling pathway of *Saccharomyces cerevisiae* plays a key role in the response of cells to osmotic stress and is closely related to the robustness of yeast. According to research findings, HOG gene of *Saccharomyces cerevisiae* has the following associations with robustness: 1. Activation of HOG signaling pathway. When *Saccharomyces cerevisiae* cells encounter high osmotic pressure environment, HOG signaling pathway will be activated. Activation of this pathway contributes to the accumulation of intracellular glycerol, thereby improving the osmotic pressure tolerance of cells. Among them, the key genes in HOG pathway include PBS2 and HOG1. PBS2 encodes a MAPKK (mitogen-activated protein kinase kinase), while HOG1 encodes a MAPK (mitogen-activated protein kinase). The mutation or deletion of these genes can affect the cell response to osmotic stress [26]. 2. Osmotic pressure tolerance and robustness. Activation of HOG pathway improves the adaptability of yeast to osmotic pressure changes, thus enhancing its robustness in food and industrial biotechnology applications [27]. 3. Environmental stress response: HOG pathway is not only affected by osmotic pressure stress, but also related to other types of environmental stress. For example, heat stress, oxidation stress and so on. Therefore, it can be shown that the activation of HOG pathway plays an important role in improving the comprehensive robustness of yeast cells [28]. 4. Cell cycle regulation, HOG pathway is also related to cell cycle regulation. The HOG pathway can regulate the cell cycle by influencing the expression of related genes. Therefore, the HOG pathway plays an important role in maintaining the stability of cell growth and division [29]. In conclusion, the activation of HOG signaling pathway in *Saccharomyces cerevisiae* plays a crucial

role in improving the adaptability and tolerance of yeast cells to various environmental stresses. Therefore, it enhances the robustness of yeast and has important application value in the food and biotechnology industries.

Conclusions to chapter 1

This chapter introduces the concept of biomass resources and their importance as renewable and clean energy sources. The development status of fuel ethanol is discussed in detail, including the raw materials and technology of the 1st to 3rd generation fuel ethanol production. In particular, it is pointed out that the second-generation fuel ethanol has high application value in the utilization of lignocellulosic biomass, but it also faces the challenge of high technical cost. In addition, this chapter also discussed the influence of inhibitors produced during lignocellulose pretreatment on fermentation process, and the importance of improving the robustness of *Saccharomyces cerevisiae* and related research progress.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 The research significance and research content of this thesis

With the development of technological society, the consumption of non-renewable energy sources (such as oil and coal) is very large, and brings serious environmental challenges. In order to ensure national energy security and maintain "green mountains", renewable clean energy ethanol has attracted global attention. Using lignocellulose as raw material to prepare hydrolysate and produce ethanol by microbial fermentation is called second-generation fuel ethanol. The development and application of the second generation fuel ethanol is of great significance to ease energy shortage and environmental pollution. Improving the ability of *Saccharomyces cerevisiae* to co-ferment glucose and xylose and its tolerance to inhibitors are the basic requirements for the production of second-generation fuel ethanol from *Saccharomyces cerevisiae*. It was found that there was an antagonism between the robustness of inhibition in hydrolysate and xylose metabolism of *Saccharomyces cerevisiae* using glucose/xylose. Through the study of the antagonistic mechanism, it was found that the overexpression of HOG1 promoted the robustness and decreased the xylose metabolic capacity. The elimination of *HOG1* decreased the robustness of the strain and promoted xylose metabolism. In this study, 6M-15, which was alleviated by xylose utilization and robust antagonism, was selected as the research object. By studying the relationship between *HOG1*CDΔ and the robustness of yeast strains, the antagonism between xylose metabolic efficiency and robustness of strains was further studied.

The second generation of fuel ethanol is characterized by lignocellulosic biomass as raw material, and the hydrolysate obtained by this method mainly contains glucose, followed by xylose, and contains complex inhibitors. Weak acids, furanaldehydes and phenols were the main inhibitors. Therefore, the special

fermentation strain of second-generation fuel ethanol should have high robustness and rapid glucose and xylose metabolism. As a traditional ethanol fermentation strain, *Saccharomyces cerevisiae* is one of the most potential strains for lignocellulosic hydrolysate fermentation. Wild yeast, on the other hand, use glucose as the first carbon source and hardly use xylose. After many years of research, *Saccharomyces cerevisiae* has been endowed with strong xylose metabolism ability. However, during the period, it was found that glucose/xylose co-utilizes *Saccharomyces cerevisiae* for the robustness of inhibitors in the hydrolysate There is antagonism between xylose and xylose metabolism. In the study of the antagonistic mechanism, it was found that the overexpression of HOG1 promoted the robustness and decreased the xylose metabolic capacity. The elimination of HOG1 decreased the robustness of the strain and promoted xylose metabolism. In order to obtain better xylose fermentation efficiency in lignocellulosic hydrolysate, balancing antagonism is one of the strategies that can be adopted at present.

The highly permeable glycerol mitogen-activated protein kinase (HOG-MAPK) pathway of *Saccharomyces cerevisiae* is a highly conserved signal transduction pathway, similar in many respects to MAPK pathway of higher eukaryotes. This pathway controls signal transduction and gene expression under hypertonic stress and is essential for cell survival. The HOG-MAPK pathway consists of a tertiary kinase cascade system, MAPKKK→MAPKK (Pbs2) →MAPK (Hog1p), which activates Hog1p through cascade activation and participates in gene transcription regulation or other cellular regulation through transcription factors. Since functional interactions between Hog1p regulators require specific docking interactions, two adjacent Pbs2 binding sites for MAPK activators are defined in Hog1p, namely, the covalent binding domain (CD) and the Pbs2 binding domain (PBD-2). The study showed that the single knockout of CD domain in haploid *Saccharomyces cerevisiae* reduced Hog1p activity level and appropriately reduced strain robustness. In the laboratory, the engineering

Saccharomyces cerevisiae 66-15, which co-metabolizes glucose and xylose with high efficiency and certain robustness, was obtained through metabolic engineering, mutagenic and adaptive evolution technologies in the early stage (Wei et al., 2021). The antagonism degree of 6M-15 has been alleviated to some extent, but there is still room for optimization. It is important to further alleviate the antagonism with 6M-15 as the chassis for improving the fermentation efficiency of lignocellulosic hydrolysate.

The research work was mainly carried out in the following aspects: 1. Construction of fermentation strains: The target gene fragment was constructed by PCR, fusion PCR and other technologies, and one or two alleles in *Saccharomyces cerevisiae* 6M-15 were replaced by homologous recombination. Finally, strains 6M-15-*HOG1*$^{\Delta(303\text{-}316)Ww}$ with one allele integration and 6M-15-*HOG1*$^{\Delta(303\text{-}316)ww}$ with two allele integration were obtained. 2. The performance of the strains was evaluated using the co-index that characterized the balance between xylose utilization capacity and robustness. It includes ethanol fermentation experiment under YPX medium, growth curve under YPD medium and growth curve measurement experiment under YPD culture containing lignocellulosic derived inhibitor. Finally, the synergistic index was calculated to evaluate the effect of *HOG1*CD domain deletion on xylose utilization and robustness of strain 6M-15.

Using diploid saccharomycetes 6M-15 as chassis, the CD domain of transcription factor Hog1p was deleted and replaced at HOG1 site. Then fermentation was performed with synthetic medium containing glucose, xylose and complex inhibitors, and the change of fermentation performance was analyzed.

Specific experimental steps are as follows:

Preparation of target gene fragment:

The target fragment HOG1-CDΔ was amplified by PCR.

PCR products were detected by agarose gel electrophoresis.

The target DNA fragments were purified from the gel by gel recovery technique.

The marker gene NatMX and homologous arm were added by fusion PCR.

Construction of *Saccharomyces cerevisiae* strains:

The whole cell transformation method of *Saccharomyces cerevisiae* LiAc was used to transform the plasmid containing the target fragment into *Saccharomyces cerevisiae* 6M-15.

PCR was used to verify the successful integration of the added marker gene NatMX and the target fragment HOG1-CDΔ of the homologous arm.

The YP-CH plasmid was amplified by transformation of Escherichia coli, and the plasmid was extracted.

Construction of 66-15-Hog1δ(303-316) Ww and 66-15-Hog1δ(303-316) ww of *Saccharomyces cerevisiae*:

Single or double site mutant strains of *Saccharomyces cerevisiae* were obtained by the above transformation method.

Test of tolerance and xylose fermentation performance of *Saccharomyces cerevisiae*:

The growth curves of yeast strains in different media (YPD, YPX and medium containing complex inhibitors) were measured.

To evaluate the tolerance of yeast strains to complex inhibitors.

The ability of yeast to produce ethanol in xylose metabolism was tested.

Analysis of results:

The optical density OD600 was measured by spectrophotometer and the growth curve was drawn.

Xylose consumption and ethanol production were determined by high performance liquid chromatography (HPLC).

The Coordination index (SI) was calculated to assess the balance between xylose metabolizing ability to produce ethanol and robustness.

The experiment also involved detailed reagent preparation, instrument use and experimental operation steps, including but not limited to preparation of media, activation and inoculation of yeast, collection and treatment of bacteria, establishment of PCR amplification system, setting of electrophoresis conditions, recovery and purification of DNA fragments, screening and culture of inverters, etc. These steps together form the experimental methods part of this study, which aims to improve the production performance of yeast strains through molecular biology techniques to meet the needs of industrial fermentation.

2.2 Principal reagent

Peptone (OXOID Company), yeast powder (OXOID Company), AGAR (OXOID Company), YeastNitrogenBase (SunriseScience Company), glucose, xylose, glycerol and DNAMarker (Beijing Sanbo Polygonia Biotechnology Co., LTD.), DNA gel recovery kit (Beijing Sanbo Yuanzhi Biotechnology Co., LTD.), plasmid extraction kit (Beijing Sanbo Yuanzhi Biotechnology Co., LTD.).

2.3 Main experimental instrument

GT9611PCR instrument (Hangzhou Boheng Technology Co., LTD.), agarose level electrophoresis tank (Beijing Junyi Company), A1600 gel imaging system (General Electric Company), temperature control shaking table, super clean table (Shanghai Sujing Co., LTD.), high pressure steam sterilization pot (Shanghai Shen 'an Medical Equipment Factory), microwave oven (Midea), induction cooker (Midea), HPLC high efficiency Liquid chromatography analyzer (Waters USA), ultraviolet spectrophotometer (Ebender China Co., LTD.), constant temperature water bath (Shanghai Jinghong Experimental Equipment Co., LTD.).

2.4 Medium and culture condition

LB medium: Tryptone: 10g, YeastExtract: 5g, sodium chloride (NaCl) : 10g. Add the above ingredients to 1L deionized water, fully dissolve, adjust the pH value to 7.0-7.5, and then sterilize, using high pressure steam sterilization (121℃, 15-20min).

YPD medium: YeastExtract: 10g, Peptone: 20g, Dextrose: 20g. These ingredients are dissolved in 1L of deionized water, the pH is adjusted to 5.8-6.0, and then sterilized, usually by high-pressure steam sterilization (121 ° C, 15-20min). After sterilization and cooling to room temperature, YPD medium can be used for yeast culture.

YPX medium: YeastExtract: 10g, Peptone: 20g SolubleStarch: 20g. Add the above ingredients to 1L of deionized water and adjust the pH value to about 5.8-6.0 after it is fully dissolved. After sterilization, high-pressure steam sterilization (121℃, 15-20min) is generally used. After sterilization and cooling to room temperature, YPX medium can be used for yeast culture.

Conclusions to chapter 2

This chapter describes in detail the main reagents, experimental instruments, media and culture conditions required for the experiment.Hog1CDΔ fragment was obtained by molecular biology related techniques (such as PCR, fusion PCR), and the marker gene NatMX and homologous arm were added. The target fragment was transformed into the 6M-15 genome using homologous recombination techniques, and the marker gene was knocked out using the Cre-LoxP recombinase system. After the integration of the two chromosomes, the fermentation performance was analyzed. Biomass and glucose metabolites were determined by microspectrophotometer and HPLC, respectively.This paper focuses on the effect of Hog1p-CD domain knockout in *Saccharomyces cerevisiae* on its tolerance in lignocellulosic hydrolysates. The background of the research is the global demand for renewable clean energy, in particular ethanol,

which can be produced from lignocellulosic biomass by microbial fermentation. *Saccharomyces cerevisiae* is the main microsubstance for the production of second-generation fuel ethanol, but it requires high robustness and the ability to rapidly metabolize glucose and xylose. Studies have found that HOG1 gene overexpression or knockout can affect yeast tolerance to inhibitors and xylose metabolism, so balancing these two capabilities is key to improving fermentation efficiency. The study involved the use of molecular biology techniques to knock out the CD domain of Hog1p in *Saccharomyces cerevisiae* 6M-15 and replace it at the site of HOG1, and then analyze its performance changes by fermentation. The research methods involved PCR, fusion PCR, homologous recombination and Cre-LoxP recombinant enzyme system.

CHAPTER 3
EXPERIMENTAL PART

3.1 Experimental process and procedure

3.1.1 Preparation of target gene fragment

(1) The target fragment HOG1-CDΔ was amplified by PCR

Table 3.1 – **PCR system**

Constituent	Volume
ddH2O	18 μL
dNTP	1 μL
Primer 1	2 μL
Primer 2	2 μL
DNA template	1 μL
Buffer	25 μL
Taq Enzyme	1 μL
Final Volume	50 μL

Table 3.2 – **PCR reaction parameters**

Procedure	Temperature	Time
Predegeneration	94 ℃	5 min
Denaturation	94 ℃	30 s
Anneal	61 ℃	40 s
Extend	72 ℃	40 s
Keep Warm	72 ℃	5 min
Save	4 ℃	∞

(2) agarose gel electrophoresis

1. To prepare 0.7% or 2% agarose gel: Weigh 0.14g(0.4g) agarose and place in a conical bottle, add 20mL1×TAE, and turn the mouth of the bottle upside down into a small beakers. Microwave and boil until agarose is completely melted. Shake well to create the desired concentration of agarose gel.

2. Preparation of rubber plate: wash the inner tank of plexiglass in the electrophoresis tank, dry it, and put it into the rubber glass plate. Take transparent tape to seal the edges of the glass plate and the inner groove to form a mold. Place the inner slot in a horizontal position and place the comb in a fixed position. Add a trace amount of ethyl bromide into the agarose gel solution, mix the agarose gel solution cooled to about 65℃, and carefully pour into the inner tank glass plate, so that the gel solution slowly spread until the entire surface of the glass plate formed a uniform glue layer. Let stand at room temperature until the gel is fully set, then gently pull the comb vertically, place the gel and inner tank into the electrophoresis tank, and add 1 x TAE electrophoresis buffer until it is 1-2mm below the rubber plate.

3. Add sample: Mix the target DNA sample and the required loading buffer on the sample plate, and the dilution ratio of loading buffer should not be less than 1×. Use 10μL micro pipette gun to add the sample into the sample small tank of the rubber plate, add one sample in each tank, and replace the gun head every time the sample is added to prevent contamination. The gel surface around the sample hole should not be damaged when adding the sample. (Note: Remember the order of adding samples when adding samples).

4. Electrophoresis: After sample addition, the gel plate should be electrified immediately for electrophoresis, the voltage is 60-100V, the sample moves from the negative electrode (black) to the positive electrode (red), the voltage increases, the effective separation range of agarose gel will be reduced. When bromophenol blue moved to about 1cm away from the lower edge of the rubber plate, electrophoresis was stopped. 5.After electrophoresis, remove the gel, stain

with 1×TAE solution containing 0.5ug/ml Ethidium bromide for about 20min, and then rinse with water for 10min. 6.Observation and photography: After electrophoresis, remove the gel, observe it under ultraviolet lamp, DNA will show red fluorescent bands, and finally take photos and save.

(3) Glue recovery

Use a clean scalpel to cut as many pieces of glue containing the target DNA as possible and put them into a 1.5mL centrifuge tube. Note the change in the weight of the centrifuge tube before and after putting in the glue block. According to the weight of the glue blocks, Binding Buffer was added in proportion according to the glue recovery kit, and the mixture was kept in a metal bath at 60℃ for 10min. When the agarose gel was completely dissolved, the mixture was absorbed into BL balance column at 12000rpm and centrifuged for 1min. Discard the supernatant, add 600μL SPW Buffer at 12000rpm, centrifuge for 1min, discard the supernatant, and repeat. Centrifuge at 12000rpm for 2min, suspend BL in balance on EP centrifuge tube, place at room temperature for 2~3min, and dry. Add 30μL sterile redistilled water, 12000rpm, centrifuge for 2min. The solution was re-absorbed into BL balance column at 12000rpm and centrifuged for 2min. Measure the concentration. The resulting DNA solution was stored at 4 °C or used for subsequent experiments.

(4) Fusion PCR added the marker gene NatMX and the target fragment HOG1-CDΔ of the homologous arm

Table 3.3 – **PCR system**

Constituent	Volume
ddH2O	17 μL
dNTP	1 μL
Primer 1	2 μL

Primer 2	2 μL
DNA template 1	1 μL
DNA template 2	1 μL
Buffer	25 μL
Taq Enzyme	1 μL
Final Volume	50 μL

Table 3.4 – **PCR reaction parameters**

Procedure	Temperature	Time
Predegeneration	94 ℃	5 min
Denaturation	94 ℃	30 s
Anneal	61 ℃	40 s
Extend	72 ℃	40 s
Keep Warm	72 ℃	5 min
Save	4 ℃	∞

The DNA mixture samples amplified by fusion PCR were subjected to agarose gel electrophoresis, and the gel was recovered and the concentration was measured. Finally, the target fragment HOG1$^{\Delta(303\text{-}316)}$ with the addition of marker gene NatMX and homologous arm was obtained. The resulting DNA solution was stored at 4 °C or used for subsequent experiments.

3.1.2 Construction of *Saccharomyces cerevisiae* 6M-15-HOG1 $^{\Delta(303\text{-}316)Ww}$

(1) Complete cell transformation method of *Saccharomyces cerevisiae* LiAc

A single colony was selected on a plate, activated in YPD liquid medium overnight (about 12h), and repeated. Then, appropriate amount of activated bacterial solution was inoculated into 20mL fresh YPD liquid medium, so that the initial OD_{600} value of bacterial suspension was about 0.2, and cultured in a shaking table at 30℃ until OD_{600} reached 0.7-1.0 (about 3-4h). The bacterial solution was transferred to a sterile centrifuge tube at 4000rpm for 5min, and the supernatant was removed to collect the bacterial body. The bacterial body weight was suspended in 1mL 100mM LiAc, mixed, and transferred to a sterile 1.5mL centrifuge tube at 8000rpm for 15s. After the supernatant was removed, the cells were suspended in 1mL100mMLiAc, mixed well, centrifuged at 8000rpm for 15s. Remove the supernatant, add 240μL 50% PEG successively, mix well, then add 30μL 1M LiAc, 10μL 10mg/mL single-stranded fish essence DNA (boiled at 100℃ in advance, quickly placed on ice after 10min for use), 60μL sterile resteam water and 10μL DNA mixture, mix well. The mixture was kept warm in a metal bath at 30℃ for 30min, heat shock in a metal bath at 42℃ for 25min, 8000rpm, centrifuged for 15s, and supernatant was removed. The cells were suspended in 1mL YPD liquid medium, mixed well, cultured in an incubator at 30℃ for 4h, centrifuged at 8000rpm for 15s, supernatant was removed, and 1mL aseptic redistilled water was added. Centrifuge 8000rpm for 15s, remove supernatant, add 100μL sterile redistilled water, mix well. The mixed medium was coated with a selective plate and cultured in an incubator at 30℃ for 2~3 days to obtain a single colony of transformed seed.

(2) Extraction of *Saccharomyces cerevisiae* genome

A single colony was selected on a plate, activated in YPD liquid medium overnight (about 12h), and repeated. Centrifuge at 12000rpm for 1min, discard the supernatant and collect the bacteria. Add 100μL yeast cracking buffer (0.2M LiAc, 1% SDS) and mix well. Heat the mixture in a metal bath at 75℃ for 10min. Add 300μL anhydrous ethanol and swirl for 5min, discard the supernatant. The suspension cells were mixed in 500 μl 70% ethanol. 13000rpm, 2min, discard

supernatant. The suspension cells were mixed in 500 μl 70% ethanol. 13000rpm, 1min, discard supernatant. Leave at room temperature for 2 to 3 minutes to dry. Add 30μL distilled water, swirl and mix well. Centrifuge at 13000rpm for 1min. The supernatant was used as the PCR template.

(3) PCR verified the addition of marker gene NatMX and the target fragment HOG1-CDΔ of homologous arm

Table 3.5 – **PCR system**

Constituent	Volume
ddH2O	18 μL
dNTP	1 μL
Primer 1	2 μL
Primer 2	2 μL
DNA template	1 μL
Buffer	25 μL
Taq Enzyme	1 μL
Final Volume	50 μL

Table 3.6 – **PCR reaction parameters**

Procedure	Temperature	Time
Predegeneration	94 ℃	5 min
Denaturation	94 ℃	30 s
Anneal	61 ℃	40 s
Extend	72 ℃	40 s
Keep Warm	72 ℃	5 min
Save	4 ℃	∞

(4) Transformation amplification of the YP-CH plasmid of Escherichia coli

Take 1μL plasmid and add it to the receptive state of DH-5α Escherichia coli melted on ice at 50μL, leave it on ice for 30min, then heat shock at 42℃ for 90s, place it on ice for 5min, add 1mLLB liquid medium on a super-clean table, incubate it at 37℃ for 40 to 60min, then centrifuge at 8000rpm for 15s, and wash it with water. After centrifugation at 8000rpm for 15s, the supernatant was removed, and the bacteria were uniformly coated on a screening plate (LB+100μg/mL ampicillin) after suspension with sterile water. The bacteria were cultured in a 37℃ temperature box for about 12h. After the transformed seeds grew out, single colonies were picked up for coliform colony PCR verification.

(5) Plasmid extraction from Escherichia coli

The E. coli was shaken and cultured, and the cloned bacteria solution with correct identification and sequencing was applied to the LB solid plate. Put in a constant temperature incubator at 37℃, culture for 12-17h, until the colony grows. The bacteria were harvested and cracked, and the amplified bacterial solution was centrifuged. The bacterial solution was re-suspended according to the instructions and transferred into 1.5ml centrifuge tube. The plasmid was extracted according to the instructions with a small amount of plasmid extraction kit. The bacteria were centrifuged at high speed for 1min and the supernatant was completely removed. Adding 250μlRB solution, the oscillator fully suspended bacteria. Add 250ulLB solution. Immediately shake up and down once to crack the bacteria, at room temperature, leave for 2min. Add 250ulNB solution. Immediately turn it upside down 10 times to fully neutralize, at room temperature, and leave for 2min. At room temperature, 1500rpm, centrifuge at high speed for 15min. The adsorption column was placed in the collection tube, and the supernatant obtained by centrifugation was transferred to the adsorption column at room temperature, 15000rpm, and centrifuged at high speed for 30s. Discard the waste liquid, put the adsorption column into the collection tube, add 700ulWB solution into the adsorption column, and centrifuge at 15000rpm for 30s at high speed. Repeat the

previous operation. The adsorption column was put into a clean 1.5ml centrifuge tube with 30-50ul of preheated Elution Buffer at room temperature for 2min and centrifuged at high speed for 1min. The sample was detected by electrophoresis: 1% agarose gel electrophoresis, the loading amount was 2ul, and the brightest band was superhelix form, which could indicate the purity of the extracted plasmid to a certain extent. For the purification of plasmids, the extracted plasmids were put into 1.5ml centrifuge tube and 3mol/L sterile sodium acetate solution of 1/10 volume was added. Add 2 times the volume of anhydrous ethanol and place it at -20℃ to precipitate for 4-6h or overnight. Centrifuge at 14000rpm at 4℃ for 20min. Discard the supernatant and wash it with 70% ethanol twice. The air is dry. After drying with 200ul sterile aqueous solution, the precipitation obtained is the plasmid solution.

(6) Complete cell transformation method of *Saccharomyces cerevisiae* LiAc

Using the above complete cell transformation method of *Saccharomyces cerevisiae* LiAc, the YP-CH plasmid amplified by *E. coli* was transformed into *Saccharomyces cerevisiae*. The mixed medium was coated with a selective plate and cultured in an incubator at 30℃ for 2 to 3 days, and the plate medium containing mesocin was screened. The yeast *Saccharomyces cerevisiae* 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ was obtained.

3.1.3 Construction of *Saccharomyces cerevisiae* 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$

The YP-CH plasmid amplified by enterobacter was transformed by *Saccharomyces cerevisiae* and screened by selective plate. The experimental strain was *Saccharomyces cerevisiae* 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ obtained from the above process. The yeast *Saccharomyces cerevisiae* 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ was obtained.

3.1.4 Testing of complex inhibitor tolerance and xylose fermentation performance of *Saccharomyces cerevisiae* 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$

(1) Determination of activation and growth curve of *Saccharomyces cerevisiae*

The isolated yeast colonies were selected and inoculated into YPD liquid medium, and then oscillated and activated for two times in a shaking table at 30℃, each activation time was 12h. The activated bacteria were collected and inoculated into a proper volume of fresh medium with an initial OD_{600} of 0.2, and the shaking flask fermentation experiment was carried out. After fermentation experiment for 3, 6, 9 and 12h, an appropriate amount of *Saccharomyces cerevisiae* liquid in culture was taken, and the optical density OD_{600} of the liquid was determined by spectrophotometer. The growth curve of the strain was obtained after time mapping.

(2) Determination of the growth curve of *Saccharomyces cerevisiae* in medium containing complex inhibitors

The complex inhibitor tolerance of saccharyeasts 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ was tested. The isolated yeast colonies were selected and inoculated into YPD liquid medium, and then oscillated and activated for two times in a shaking table at 30℃, each activation time was 12h. After collecting activated bacteria, the initial OD_{600} was 0.2 and inoculated into an appropriate volume of medium containing complex inhibitors, and the shaking flask fermentation experiment was carried out. After 6, 12, 18 and 24h of fermentation experiment, a proper amount of *Saccharomyces cerevisiae* liquid in culture was taken, and the optical density OD_{600} of the liquid was determined by spectrophotometer.

(3) Fermentation performance test of *Saccharomyces cerevisiae* 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$

A single colony of Saccharomyces cervetica was selected from YPD plate and activated twice in YPD medium to prepare seed medium. The bacteria were collected by centrifugation at 4500rpm, then the superliquant was discarded and washed with sterile water. The bacteria were inoculated into YPD, YPD containing 3g/L acetic acid and YPX medium containing 40g/L xylose, respectively, with an initial inoculation OD_{600} of 0.2. The fermentation volume was 30mL and cultured in a shaking bed at 30℃ and 200rpm. The oxygen-restricted condition is maintained with a rubber plug inserted with a syringe needle to release the CO2 produced during the culture. The OD_{600} value was determined by regular sampling, and the supernatant was obtained by centrifugation and stored at -20℃ for HPLC analysis.

3.1.5 Growth curve of *Saccharomyces cerevisiae* 6M-15 after deletion of Hog1pCD domain in glucose medium.

Saccharyeasts 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were inoculated into YPD liquid medium for oxygen limited shaker fermentation, and their growth was monitored. As shown in Figure 4-1, the growth of 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were similar. OD_{600} at 12 h can reach about 23.

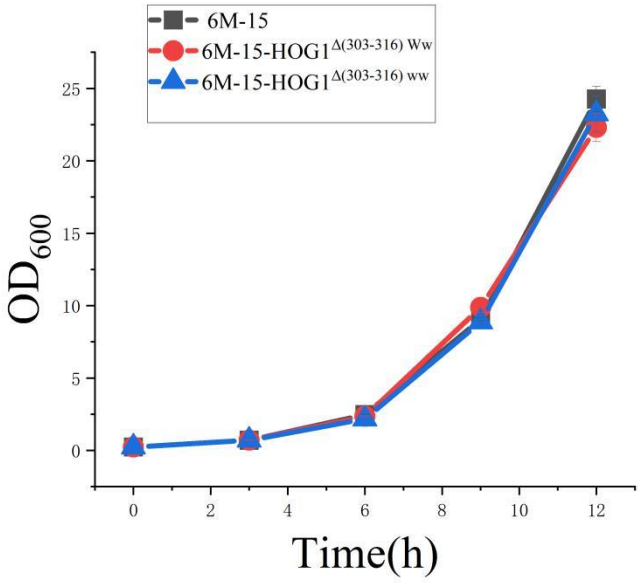

Figure 3-1. Growth curves of 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ in YPD medium

3.1.6 Growth curve of Saccharomycetes cerevisiae 6M-15 after deletion of Hog1pCD domain in medium containing complex inhibitors.

Yeast saccharyeasts 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were inoculated into YPD liquid medium containing complex inhibitors for oxygen-restricted shak-flask fermentation, and their growth was monitored. As shown in Figure 4-2, *Saccharomyces cerevisiae* 6M-15 had the slowest growth rate and the highest degree of inhibition. In 24h growth OD_{600} can reach about 3.5. Secondly, the growth of saccharyeasts 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ was similar, and the growth OD_{600} could reach about 14 at 12h, which was about 3 times higher than 6M-15.

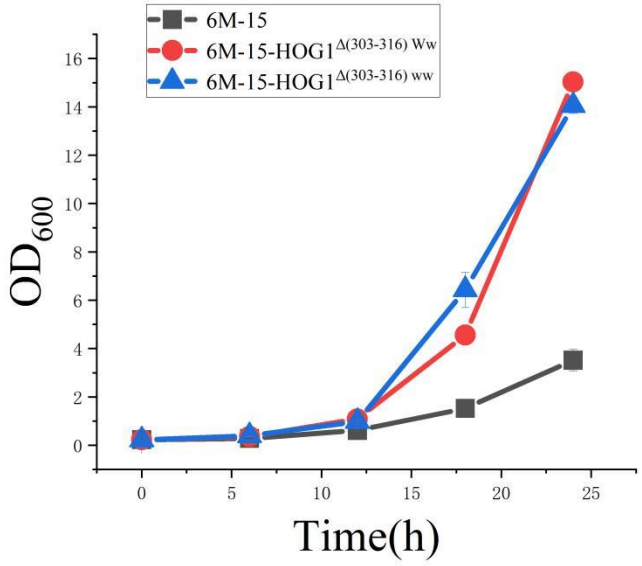

Figure 3-2. Growth curves of 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ in media containing complex inhibitors

3.1.7 Ethanol production from xylose metabolism of *Saccharomyces cerevisiae* 6M-15 after deletion of Hog1pCD domain

Previously, we collected growth assessment data to investigate the relationship between HOG1CDΔ and the robustness of *Saccharomyces cerevisiae* in the context of 6M-15, and evaluated the tolerance of these strains to complex inhibitors. That is, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were the best inhibitors to saccharomyces saccharomyces, and 6M-15 were the worst inhibitors. We further evaluated the xylose metabolizing capacity of these three strains to produce ethanol. The C5/C6 co-fermentation strains 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were inoculated into YPX containing 40g/L xylose for oxygen limited shaker fermentation, and xylose metabolism and ethanol production during fermentation were monitored. Among the three strains, *Saccharomyces cerevisiae* 6M-15 had the strongest xylose metabolism and ethanol production capacity in YPX, consuming 37.068g/L xylose and producing 16.221g/L ethanol in 24h, and the sugar alcohol conversion rate was 0.434g/g, reaching 85% of the theoretical value. Secondly, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ xylose metabolism and ethanol production capacity is not as good as 6M-15, which consumes 35.543g/L xylose in 24h, ethanol production is 15.415g/L, and sugar alcohol conversion is 0.408g/g, reaching 80% of the theoretical value. 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ xylose had the weakest ability to produce ethanol, consuming 33.187g/L xylose in 24h, producing 14.264g/L ethanol, and converting sugar alcohol to 0.377g/g, which reached 74% of the theoretical value.

In summary, the tolerance to complex inhibitors and the ability of xylose metabolism to produce ethanol were evaluated. The tolerance of 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ to complex inhibitors was stronger than 6M-15, indicating that the deletion of CD domain enhanced the robustness of strains. However, the xylose ethanol production capacity of 6M-15 strain was stronger than 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$, indicating that the deletion of CD domain reduced the xylose ethanol production capacity of the strain.

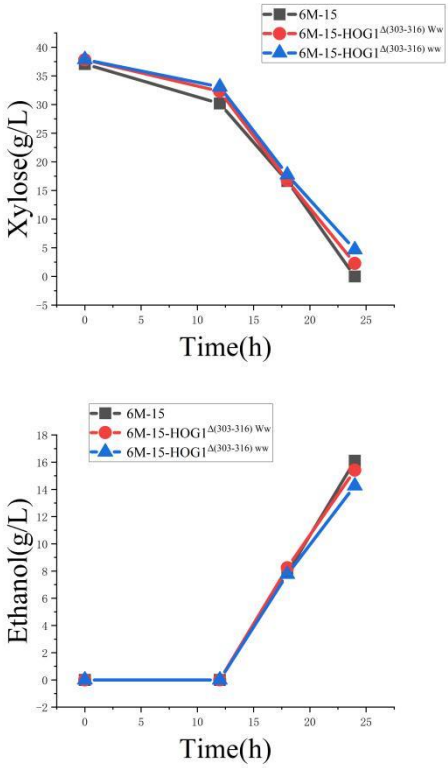

Figure 3-3. Fermentation of 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ in a limited oxygen shaker in YPX

(a) Xylose consumption; (b) Ethanol production

3.1.8 Strain performance was evaluated by an index that characterized the degree of antagonistic balance between high xylose metabolism and high robustness

Using the ratio of ethanol yield or RYe/xT (ratio of ethanol yield from xylose to theoretical value) to reflect the strain of xylose metabolic capacity of ethanol, the higher the numerical, shows that using the stronger the ability of xylose to produce ethanol. The specific calculation method is shown in formula (4-1), which is the ratio of the actual ethanol yield of C5/C6 co-fermentation strain under xylose conditions to the theoretical ethanol yield, where the theoretical ethanol yield value is 0.511g/g (total xylose).

$$\mathrm{RYe/xT} = \frac{\text{ethanol yield}}{0.511\ \text{g/g (total xylose)}} \qquad (4\text{-}1)$$

Survival rate (survival rate under stress) was used to reflect the robustness of C5/C6 co-fermentative strains. The higher the survivalrateunderstress value, the higher the survivalrate and the robustness of C5/C6 co-fermentative strains under the condition of inhibitors. The specific calculation method is shown in formula (4-2), which is the ratio of the maximum specific growth rate (μ_{max}) of C5/C6 co-fermentative strain under inhibitor conditions to the maximum specific growth rate without inhibitor with glucose as the only carbon source.

$$SRS = \frac{\mu max\ (\text{in inhibitors})}{\mu max\ (\text{in glucose})} \qquad (4\text{-}2)$$

As shown in formula (4-3), the product of RYe/xT and SRS is called the cooperation index, which is expressed by SI.

$$\text{Synergy Index(SI)} = RYe / xT \times SR \qquad (4\text{-}3)$$

The calculated results are shown in Table 3-1. The theoretical value of ethanol yield in xylose medium is 6M-15 > 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ > 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$; The SRS values of specific survival rate were 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ > 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ > 6M-15. The SI values of the coordination index are 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ > 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ > 6M-15. In summary, among the three *Saccharomyces cerevisiae* 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$, The highest degree of antagonistic balance between xylose metabolism to ethanol and acetic acid tolerance was found in 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$. That is, truncating the CD domain of Hog1p can alleviate the antagonism, which has been confirmed in industrial strains.

Table 3-7. Calculation of synergistic indices of *Saccharomyces cerevisiae* strains 6M-15, 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$

Strains	YPX			YPD	YPD +inhibitors	RYe/xT	SRS	SI
	Xylose conversion (%) a	Ethanol Accumulation (g/L) b	Ethanol Yield (g/g) c	μ_{max} (/h) d				
6M-15	100 ±0.00	16.1 ±0.01	0.434 ±0.001	0.397 ±0.005	0.140 ±0.006	0.852 ±0.002	0.353 ±0.011	0.301 ±0.009
6M-15-*HOG1*$^{\Delta(303\text{-}316)Ww}$	93.97 ±0.76	15.41 ±0.09	0.408 ±0.005	0.393 ±0.005	0.100 ±0.000	0.799 ±0.1	0.536 ±0.007	0.428 ±0.000
6M-15-*HOG1*$^{\Delta(303\text{-}316)ww}$	87.65 ±1.28	14.26 ±0.16	0.377 ±0.004	0.397 ±0.005	0.210 ±0.001	0.737 ±0.008	0.529 ±0.008	0.391 ±0.010

a: Xylose utilization rate, initial xylose concentration minus remaining xylose concentration in 24h, divided by initial xylose concentration;

b: 24h ethanol production;

c: Ethanol yield, 24h ethanol yield divided by initial xylose concentration;

d: maximum specific growth rate;

WW: The chromosome is not modified; Ww: One allele is modified; ww: One allele was modified.

Conclusions to chapter 3

In order to assess the effect of the deletion of the Hog1pCD domain of the industrial strain 6M-15 on xylose metabolism and robustness, the performance of the strain was evaluated by an index (synergy index) that characterizes the degree of antagonistic balance between high xylose metabolism and high robustness. Yeast saccharsaccharae 6M-15, a modified allele strain 6M-15-HOG1$^{\Delta(303\text{-}316)Ww}$ and two modified allele strains 6M-15-HOG1$^{\Delta(303\text{-}316)ww}$ were inoculated into YPX medium, YPD medium and YPD medium with lignocellulosic derived inhibitors, respectively. It was found that the deletion of Hog1pCD domain significantly increased the robustness of the strain, slightly decreased the xylose metabolic capacity, and finally significantly increased the synergistic index value. That is, deletion of Hog1pCD domain can alleviate the antagonism between high xylose metabolism and high robustness, which has been confirmed in industrial strains.

CONCLUSIONS

In this study, HOG1CDΔ fragment was obtained by molecular biology related techniques (such as PCR, fusion PCR), and the marker gene NatMX and homologous arm were added. The target fragment was transformed into 6M-15 gene by homologous recombination technique, and the marker gene was knocked out by the Cre-LoxP recombinase system. 6M-15-HOG1Δ(303-316) Ww and 6M-15-HOG1Δ(303-316) ww were obtained to complete the construction of fermentation strains. The growth curve, tolerance to complex inhibitors of *Saccharomyces cerevisiae* and xylose metabolizing ability to produce ethanol were tested and evaluated. The results showed that the yeast strains with truncated HOG1CD domain improved their tolerance to complex inhibitors, but decreased their xylose metabolizing ability to produce ethanol. Finally, the balance between the xylose metabolizing ability to produce ethanol and the robustness of the fermentation strain was characterized by using the synergistic index (SI). The results of cooperation index calculation showed that the truncated Hog1pCD domain significantly increased the robustness of the strain, slightly decreased the xylose metabolic capacity, and finally significantly increased the cooperation index value. That is, truncating Hog1pCD domain can alleviate the antagonistic relationship between high xylose metabolism and high robustness.

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