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Bachelor's thesis

on the topic   Effects of site-directed mutagenesis of HOG1 gene on xylose
metabolism and acetic acid tolerance in Saccharomyces cerevisiae

Completed: student of group BEBT-19
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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

Faculty Chemical and Biopharmaceutical Technologies
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Speciality 162 Biotechnology and Bioengineering
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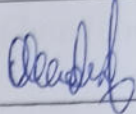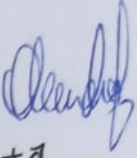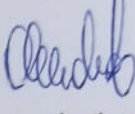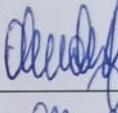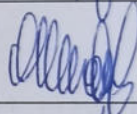
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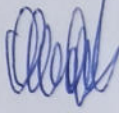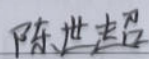
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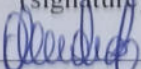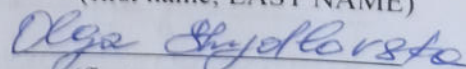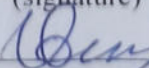
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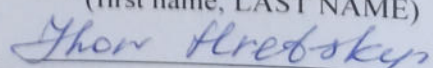
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ABSTRACT

In the future fuel ethanol development process, lignocellulosic is an important raw material，the use of lignocellulosic ethanol production ethanol can not only reduce environmental pollution. In addition, a variety of substances that inhibit the growth and fermentation of *Saccharomyces cerevisiae* were produced during the pretreatment of lignocellulosic raw materials，among which acetic acid was the main inhibitor component, and yeast was more sensitive to acetic acid during xylose fermentation than under glucose conditions. Therefore, improving the xylose metabolism capacity and acetic acid stress tolerance of *Saccharomyces cerevisiae* strains is the key to selecting and breeding efficient fermentation strains.

The object of the work is mutant strains of *S. cerevisiae* with *HOG1* gene.

The subject of the work is the effect of *HOG1* gene on xylose utilization and acetic acid tolerance of mutant *S. cerevisiae*.

This study aimed to investigate the effect of *HOG1* gene on xylose utilization and acetic acid tolerance. The *HOG1* gene was site-mutated by fusion PCR to convert the 144th aspartic acid of *HGO1* protein into alanine, and the *HOG1* mutant strains of *Saccharomyces cerevisiae* were constructed, and their growth and fermentation in the medium containing xylose-acetic acid were compared.

The tasks of the work to construct mutant strains of *S. cerevisiae* with *HOG1* gene, study their growth and fermentation in the xylose-acetic acid medium.

The results showed that the point mutations of *HOG1* gene had a significant effect on xylose utilization and acetic acid tolerance. This study provides a new metabolic engineering strategy and theoretical basis for improving the efficiency and economy of lignocellulosic biotransformation, and also provides a new perspective and method for revealing the molecular mechanism of stress response in *S. cerevisiae*.

Key words: HOG1 gene; *Saccharomyces cerevisiae*; lignocellulose; Ethanol; Tolerance to acetic acid

CONTENT

INTRODUCTION

Fuel ethanol is a high-purity ethanol with a volume content of more than 99.5%, which can improve the combustion efficiency and explosion resistance of gasoline[1] . Demand for fuel ethanol continues to grow, and technology and scale have huge room to improve under the guidance of the two-carbon policy[2] . At present, ethanol is mainly synthesized by biological methods and is fermented from biomass or agricultural products such as corn and wood[3] . Fuel ethanol can be used as a substitute for gasoline to improve the efficiency of fuel use in automobiles and help reduce exhaust emissions from automobiles. In order to expand the production and use of fuel ethanol, the state has issued a series of relevant policies, such as the Implementation Plan for Expanding the Production and Promoting the Use of Biofuel Ethanol Gasoline for Vehicles and the Notice on Establishing a Monthly Reporting System for Expanding the Production and Promoting the Use of Biofuel Ethanol Gasoline for Vehicles, which reflects the national attention to renewable energy sources such as fuel ethanol[4] . Fuel ethanol has the advantages of reducing environmental pollution, extensive sources, reusing agricultural wastes and energy substances, and reducing automotive exhaust emissions. The disadvantages of fuel ethanol include: high production cost and mixed with gasoline, which may lead to the performance decline and corrosion of automobile engines. Therefore, the development of fuel ethanol should fully consider the economic factors, environmental protection and other factors to find more effective and appropriate technology.

At present, fuel ethanol in China mainly refers to biomass fuel ethanol. With the vigorous promotion of biomass fuel ethanol in each province, the demand for biomass fuel ethanol is increasing, but the promotion of ethanol gasoline in China is not optimistic[5] . The popularization and application of fuel ethanol is of great strategic significance for ensuring energy security and environmental protection, and promoting the sustainable development of forestry and agriculture in China.

Fuel ethanol started later than other countries, but after 20 years of unremitting efforts by the Chinese people, the fuel ethanol level in China has reached a certain level. Fuel ethanol production capacity in China has been widely promoted in China. However, the fuel ethanol industry in China also faces many difficult problems, such as high production cost, unstable market demand, insufficient supply of raw materials and other problems.

CHAPTER I
INTRODUCTION TO THE STUDY OF *HOG1*

1.1 Composition and Characteristics of Lignocellulose

Lignocellulose is one of the most renewable resources on Earth and can be converted into high-value biochemicals through microbial ferment action[6] .Plant cell walls mainly contain lignocellulose, which is composed of cellulose, hemicellulose, and lignin. Lignocellulosic biomass is an important available energy source in addition to fossil energy sources (e.g., coal, oil, gas, etc.) and is regarded as the fourth largest energy source worldwide[7].

Lignocellulose has excellent physical and chemical properties such as high strength, high stiffness, high stability and high specific area, and can be used as a reinforcer or substrate for various composites. Lignocellulose has good biocompatibility and biodegradability and can be used as a functional material or carrier in the fields of medicine, food and cosmetics. The structure and properties of lignocellulose can be improved by chemical or physical methods to meet the needs of different applications, such as increasing water solubility, increasing reaction activity, and increasing conductivity. Lignocellulosic biomass contains a large amount of glucose and xylose after hydrolysis, so it is important to effectively utilize these two sugars[8].

1.1.1 Lignocellulose pretreatment

Lignocellulose production of ethanol mainly consists of the following steps: lignocellulose pretreatment enzymatic hydrolysis fermentation. Pretreatment of lignocellulose is an important process for ethanol production from lignocellulose and can convert lignocellulose into more valuable products[9] . Lignocellulose pretreatment is a process that alters the structure and properties of biomass to facilitate subsequent enzymatic hydrolysis or fermentation. There are many

pretreatment methods, and their classification is based on the mode of action and effect, mainly physical methods, chemical methods, biological methods and other solvent methods. The physical method is to use mechanical, thermal or electromagnetic forces to adjust the shape and structure of lignocellulose so that it is more easily hydrolyzed by enzymes. For example, milling can reduce the crystallinity and polymerization of cellulose and increase the specific surface area and porosity; extrusion can destroy the microfibril structure of cellulose and reduce cellulose resistance; microwave can generate local high temperature and pressure, resulting in hydrolysis and cleavage of cellulose; pyrolysis can convert lignocellulose into gas, liquid and solid three-phase products under anaerobic or hypoxic conditions[10].

Chemical methods Decompose or dissolve components of lignocellulose with chemicals such as acids, bases, or solvents to make it easier to separate and utilize. For example, dilute acid can effectively hydrolyze hemicellulose and produce fermentable monosaccharides; alkalization can effectively remove lignin and increase cellulose accessibility; ammonia fiber blasting can treat lignocellulose with ammonia at high temperature and high pressure to expand and dehydrate it; co-solvents can dissolve lignocellulose using some organic solvents or mixed solvents to form a homogeneous solution [9].Biological methods use microorganisms or enzymes to digest lignocellulosic components and convert them into valuable substances. For example, white rot bacteria can secrete a variety of enzymes to degrade lignin and hemicellulose and retain cellulose; brown rot bacteria can secrete low-molecular-weight oxidants to degrade lignin and expose cellulose; cellulase can catalyze the hydrolysis of cellulose into glucose and improve the sugar conversion rate[11,11]. Equal solvent method is a method to degrade lignocellulosic biomass by a combination of organic solvents and water to enhance its transformability and reduce its crystallinity. This approach allows efficient recovery of lignin, extraction of cellulose and hemicellulose, and generation of highly purified lignin as a valuable byproduct[12]。

1.1.2 Enzymatic hydrolysis of lignocellulose

Lignocellulose hydrolysis is a process that hydrolyzes lignocellulose into soluble sugars and other products using lignocellulose[13] . This process is widely used in industrial, agricultural and environmental fields, such as biofuels, textiles, paper making, feed and food production. For example, agricultural wastes such as straw, corn cob, and sugarcane bagasse can be converted into biofuels such as ethanol and butanol by lignocellulose hydrolysis, thereby reducing dependence on fossil energy sources and greenhouse gas emissions. The efficiency and efficiency of enzymatic hydrolysis of lignocellulose are restricted by many factors, such as the type, activity, stability and ratio of enzymes, as well as the structure, composition, water content and particle size of substrates.

Different enzymes have different mechanisms of action and optimized conditions, while the structure and composition of substrates can also affect the contact and hydrolysis of cellulose by enzymes. In order to improve the efficiency and efficiency of lignocellulose hydrolysis, it is necessary to optimize and regulate these factors, and also to develop new enzymes and substrates, as well as improve the conditions and equipment of enzymatic hydrolysis reactions. For example, the activity and stability of enzymes can be improved by genetic engineering or directed evolution, or the synergistic effect can be enhanced by mixing enzymes from different sources or functions; the substrates can be pretreated by physical or chemical methods to reduce their crystallinity and lignin content and increase their specific surface area and porosity; and the hydrolysis rate and yield can be increased by controlling parameters such as reaction temperature, pH, and time. The benefit of lignocellulose hydrolysis is that it can be performed at lower temperatures and pressures, reducing the production of harmful substances, and also increasing the sugar conversion rate and fermentation efficiency. The disadvantage is that it needs to consume a large number of enzymes to crack the

resistance of lignocellulose, and the price of enzymes is high, which needs to be recycled to reduce costs[14] .

1.2 Problems in ethanol production from lignocellulose

Ethanol production from lignocellulose is a renewable clean energy technology, but there are also many problems and challenges. The use of lignocellulose to produce ethanol is a method to turn the stalk of plants into oil that can be used by automobiles, but this method has many imperfect problems, such as the stalk of plants is not easy to collect and preserve, and there are many types, and different stems may require different treatment methods, secondly, the stalk of plants is very hard, to soften it in order to allow the enzyme to decompose it into sugar, but the softening process will consume a lot of energy and water, but also produce some toxic things, affecting the following steps. Enzyme is a biocatalyst that can decompose stalks into sugars, but the enzyme is very expensive and inefficient, and many enzymes need to be used to decompose stalks very clean. Sugar is a substance that can be fermented into ethanol by microorganisms, but there are two different sugars in the stalk, and there is no microorganism that can turn both sugars into ethanol at the same time. And there are things in the fermentation broth that hinder microbial fermentation and need to be removed or diluted first.

In the process of ethanol production from lignocellulose, the difficulty and cost of pretreatment technology should be considered. Pretreatment technology is a method to reduce the structure and crystallinity of lignocellulose and increase its sensitivity to hydrolytic enzymes and reaction rate. But pretreatment technology needs to consume a lot of energy, produce harmful substances and wastewater, and adversely affect the fermentation step.

The utilization rate of enzymatic hydrolysis is low and the yield effect is poor. Enzymatic hydrolysis of lignocellulose requires the synergistic action of multiple different enzymes, such as cellulase, hemicellulase, and lignase. The

performance and efficacy of these enzymes can be constrained by a variety of conditions, such as temperature, pH, etc. Moreover, the solid state characteristics of lignocellulose can also hamper the diffusion and adsorption of enzyme molecules, making the enzymatic hydrolysis efficiency decreased.

In order to solve these problems, it is necessary to improve from the following aspects: first of all, to select stalks with high cellulose content, low lignin content, easy to deal with and decompose, such as sugarcane bagasse, corn cob and so on. Establish a stable supply chain of stalks, improve the utilization rate and homogeneity of stalks, and reduce the storage and transportation costs of stalks. Secondly, develop low energy consumption, low pollution, high efficiency softening methods, such as steam blasting, biological methods. Genetic engineering or directed evolution is used to improve the performance of enzymes and improve their stability, catalytic activity and tolerance. Develop multifunctional or complex enzyme preparations to achieve simultaneous or continuous decomposition of cellulose and hemicellulose. Reduce the production cost and use cost of enzymes, and improve the decomposition rate and yield. Finally, microorganisms that can ferment mixed sugars are cultivated by genetic recombination techniques, or integrated hydrolytic fermentation is performed using immobilized enzymes or immobilized microorganisms.

1.3 Saccharomyces cerevisiae

Saccharomyces cerevisiae is a eukaryotic model organism and a good cell factory[15]. *Saccharomyces cerevisiae* is the most commonly used yeast in humans[16] and is widely used in fermented beverages such as beer, glucose, and sake. *Saccharomyces cerevisiae* has high glycolytic capacity and excellent industrial performance. However, *S. cerevisiae* cannot naturally utilize xylose from lignocellulose, which is an important renewable carbon source and can produce a variety of biofuels and chemicals. Therefore, modification of *S. cerevisiae* is the key to improve lignocellulose efficiency and reduce production costs. The US

Food and Drug Administration believes that *S. cerevisiae* is generally safe, and because its rapid growth rate is easy to be widely cultured on a large scale, *S. cerevisiae* can metabolize glucose well for ethanol production, with the advantages of strong stress resistance and good fermentation performance[17] .

1.4 HOG1 gene function

Protein Kinases *HOG1* Found 20 years ago, it was revealed to be a central signaling mediator of cell osmoregulation in budding yeast, and its cognate protein is ubiquitous in eukaryotes and essential for cells to cope with various external stresses and stimuli[18] . *HOG1* Genes regulate cellular regulation of external osmotic pressure changes by controlling changes in cellular metabolism and gene expression[19] . *HOG1* It is also able to detect different stress stimuli, such as increased osmolality, decreased pH, increased temperature, partial pressure of oxygen, etc., and affect the expression levels of stress response genes through phosphorylation modification of multiple transcription factors and chromatin structure[20]. In addition, *HOG1* Genes can also respond to other types of environmental stresses, such as oxidative stress, heat shock, and heavy metal toxicity. *HOG1* Genes have relatively few functional studies in other organisms, but there are also some reports indicating *HOG1* Genes are also involved in signaling under osmotic stress and other stresses in plants and animals.

The plasma membrane activity of HOG1 affects cells more rapidly than its transcriptional action, and it rapidly adjusts the transmembrane trafficking of Na^+ and K^+ by phosphorylating some ion transporters in permeating cells[21] . In *S. cerevisiae*, changes in external osmolality trigger activation of stress-activated protein kinase *HOG1* , which regulates gene expression, metabolism, and cell cycle progression[22] . *The effect of HOG1* gene on xylose metabolism may be related to its regulation of cellular metabolism and signal transduction, for example, it can promote xylose absorption and utilization through phosphorylation and activation of xylose transporters and xylose isomerases[23].

HOG1 gene can also influence xylose fermentation by modulating ethanol and acetic acid production, as well as resistance to oxidative stress. In addition, *HOG1* gene can also affect the effect of xylose on cell growth, differentiation and tolerance, for example, it can adapt to the presence of xylose by regulating processes such as cell cycle, apoptosis and autophagy[23] . *HOG1* is able to sense the presence of acetic acid, as acetic acid can alter the osmotic balance inside and outside the cell and activate *HOG1* by activating upstream Sln1 and Sho1 sensors. *HOG1* reduces the entry of acetic acid into cells by phosphorylating the aquaporin Fps1 on the plasma membrane, promoting its ubiquitination, endocytosis, and degradation. This mechanism protects cells from toxic damage by acetic acid[25] . *HOG1* also regulates the expression of the catalase gene *CTT1* , improving cellular resistance to acetic acid-induced oxidative stress. Catalase decomposes hydrogen peroxide produced in cells, thereby mitigating oxidative damage. *HOG1* also inhibits acetic acid-induced programmed cell death and requires the involvement of *RTG2* gene. RTG2 is a positive regulator of the mitochondrial reverse signal transduction (RTG) pathway, which impacts cellular metabolism and gene expression to accommodate mitochondrial dysfunction[26] .

Summary of the chapter I

Vegetable lignocellulosic raw materials contain high xylose content, while some inhibitors, such as sugar degradation products, residual lignin, oxidative stress, etc. are generated during pretreatment, which can seriously affect the fermentation efficiency and product yield of yeast. Therefore, improving xylose metabolism ability of *S. cerevisiae* to robustness inhibitors is a basic requirement for fuel ethanol. Because *S. cerevisiae* does not naturally utilize xylose, it must be engineered or artificially modified to utilize xylose[28]. At present, the xylose metabolic pathway has been successfully constructed in *S. cerevisiae* by metabolic engineering and adaptive evolution, and the metabolic efficiency of xylose has been improved[29.].

However, there are still problems of acetic acid stress in xylose metabolism, acetic acid is one of the main inhibitors of lignocellulosic hydrolysate, which can reduce the growth and fermentation of *S. cerevisiae*[30]. Especially when xylose is used as carbon source, the sensitivity of *S. cerevisiae* to acetic acid increases because xylose metabolism requires more energy consumption and reducing power, resulting in decreased intracellular ATP and NADH levels, thus reducing cellular resistance to acetic acid stress. According to the literatures, *HOG1* gene has an effect on both acetic acid and xylose, we speculated that a certain amino acid mutation in *HOG1* gene may have an effect on xylose metabolism and acetic acid tolerance in *S. cerevisiae*[31].

Therefore, this paper used site-directed mutagenesis to point mutate *HOG1* gene to obtain a mutant of *HOG1* gene, and then oxygen-limited shake flask fermentation was performed under xylose conditions to assess the xylose metabolism ability of the strains before and after mutation, and coated plates in acetic acid were used to assess the changes in acetic acid tolerance of the strains before and after mutation[32]. The relevant experimental results can expand the understanding of *HOG1* gene function and also provide new perspectives and methods for revealing the molecular mechanisms of stress response in *S. cerevisiae*, and provide technical support for the construction of industrial strains of *S. cerevisiae* with high xylose metabolism and high acetic acid tolerance[33].

CHAPTER II
MATERIALS AND METHODS

1.2 Strain

The strains used in this paper are shown in Table 1 (Appendix A).

1.3 Reagents

Yeast extract and Tryptone were purchased from OXOID; acetic acid, glucose, xylose were purchased from Sinopharm Chemical Reagent Co., Ltd.; agar was purchased from Beijing Laibao Technology Co., Ltd.; Yeast Nitrogen Base (YNB) was purchased from Sunrise Science Co., Ltd.; phanta Max buffer was purchased from Vazyme Co., Ltd.

1.4 Main instruments used in the experiment

The main instruments used in this paper are shown in Table 2 (Appendix B).

1.5 Primer sequence

The primer sequences used in this paper are shown in Table 3 (Appendix C).

Table 3 Primers used in this paper and their sequences.

1.6 Primer Design

LB medium: 1% peptone, 0.5% yeast extract, 1% sodium chloride, distilled water (solid medium plus 2% agar powder), sterilized at 115 ° C for 30 min.

YEPD medium: yeast powder 10 g/l, glucose 20 g/l, peptone 20 g/l, solid medium supplemented with 20 g/l, agar powder, sterilized at 115 ° C for 30 min. When necessary, 400-800 mg/l G418 was added for recombinant strain selection and culture[34,35]

SC-Ura auxotrophic medium: 1.7 g/l Yeast Nitrogen Base (YNB), 5 g/l ammonium sulfate, 0.77 g/l CSM-Ura (essential amino acid mixture for uracil-

deficient), 20 g/l glucose; solid medium was supplemented with 2% agar and adjusted to pH 6.0 – 6.5 with NaOH solution, and sterilized at 115 ° C for 30 min[36,37].

2.6 Culture of yeasts

Yeasts were isolated from the culture medium, and then a single colony was selected to activate the culture twice by shaking on a shaker at 30 ° C, and the time of each activation was 12 – 24 h according to different circumstances of bacterial growth[38]. The activated bacteria were removed and inoculated into an appropriate amount of fresh medium with 0.2 as the initial OD $_{600}$ for shake flask fermentation experiments to measure the OD $_{600}$ of *S. cerevisiae* broth (appropriately diluted with distilled water to make its OD $_{600}$ within the range of 0.05-0.3) and plot the growth curve of the strains[39].

2.6.1 Culture preservation

Culture preservation is divided into short-term preservation and long-term preservation.

Short term storage: Spread the cultured yeasts on solid plates and allow them to remain at 4 ° C for several weeks after incubation.

Long-term storage: Select a single colony to inoculate into 3 ml liquid medium, incubate at 200 rpm at 30 ° C for 24 h, activate the strains twice, centrifuge for 3 min to take out the strains, resuspend in 1 ml seed preservation solution (25% glycerol, 0.85% NaCl), load into the seed preservation tube and place in -80 ° C refrigerator, and store for more than 5 years[40].

2.6.2 Fusion PCR technique

Saccharomyces cerevisiae transformation technology refers to the introduction of exogenous DNA into *S. cerevisiae* cells for the production of biofuels or chemicals[41]. Yeast transformation techniques include protoplast sphere

fusion, PEG/LiAc, and electroporation. In this paper, we mainly use PEG/L iAc method, which uses polymer PEG and lithium acetate LiAc to improve the uptake ability of exogenous DNA in yeast cells. First, the activated bacteria were transferred into a 40 ml collection tube, and the adjusted bacterial concentration OD was about 0 .2, 3 shaken and cultured at 0 ° C to reach OD 0. 6-0.8. This typically requires incubation 3 -4 h[42]. Then, the bacterial fluid was collected and centrifuged at 5000 rpm for 5 min, the supernatant was discarded, sterile water was added, the cells were reset, centrifuged at 5000 rpm for 15 s, and the supernatant was discarded.

Then 1 ml, 0.1 mol L iAc was added, the cells were resuspended, transferred to a sterile E P tube, centrifuged 8000 rpm for 15 s, and 240 microliters of the supernatant was discarded, 5 0% of P EG3350, Mix slightly with the tip, 36 μl 1 mol L iAc, 5 μl protamine DNA, 10 μl PCR fusion fragment, 360 μl dd -H_2O Then place them in 30 ° C incubator for culture 30 min, heat shock in 42 ° C water bath 25 min, 8000 rpm centrifuge and discard the supernatant, resuspend the cells, place them in incubator for incubation for 4 h, centrifuge the culture medium, add 50 μL sterile water to wash the bacteria, and finally, suck the bacterial solution and hit the plate, Spread evenly with a spreader stick and incubate in the incubator[43].

2.6.3 Growth Curve Determination

Select a single yeast colony and shake twice on a shaker at 3 0 ° C to activate the culture. According to different conditions of bacterial growth, the time for each activation is 1 2-24 h. The activated bacteria were removed and inoculated into an appropriate amount of fresh medium with 0 .2 as the initial O D $_{600}$, and an oxygen-limited shake flask fermentation experiment was performed to measure the O D $_{600}$ of *S. cerevisiae* solution (appropriately diluted with distilled water to make its O D $_{600}$ within the range of 0. 05-0.3) and draw the growth curve of the strains[44,45].

2.6.4 Drip Plates

First, sterile culture dishes, culture media, inoculation loops, inoculation needles, alcohol lamps and other equipment should be prepared. Next to the sterile room or alcohol lamps, wipe the upper cover and bottom of the culture dishes with alcohol balls, then open the culture dishes, pour the culture media into the culture dishes, about 10 ml – 20 ml, cover the covers, gently shake well, wait for the culture medium to condense, separate yeasts by plate streaking method, pick out strains from single colonies, culture twice at 30 ° C, take strains at the late logarithmic growth stage, centrifuge to collect bacteria, wash with sterile water three times, then suspend in 1 ml sterile water, incubate in an incubator at 30 ° C for 9 h, allow the bacteria to consume endogenous nutrients, and prepare resting cells. Adjust the concentration of resting cells so that OD_{600} of suspension is about 1.0, dilute three gradients (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}) according to 10-fold gradient, spot 4 μL on acetic acid solid plate containing 3 g/l respectively, incubate at 30 ° C for 3-5 days, observe the colony growth on different plates, take photos and record the results.

2.6.5 Oxygen-limited shake flask fermentation technique

A single colony of *Saccharomyces cerevisiae* was selected from the YPD plate, activated and cultured twice in YPD medium to prepare the seed culture medium, centrifuged at 4500 rpm to obtain the bacteria, washed with sterile water, and inoculated into YPD medium, YPD containing 3 g/l acetic acid, and YPD medium containing 20 g/l xylose, respectively, with an initial inoculation OD_{600} of 0.2 and a fermentation volume of 30 ml, and cultured in a shaker at 30 ° C and 200 rpm. Bottles were closed with rubber stoppers, oxygen limited conditions were maintained, and CO_2 was released by inserting a needle over the stopper. Samples were collected periodically to measure OD_{600}, and the supernatant was collected by centrifugation, stored at -20 ° C, and analyzed by high performance liquid chromatography.

2.7 High performance liquid chromatography

High performance liquid chromatography is a chromatographic method in which a column containing stationary phase, such as different solvents and buffers, is pumped at high pressure using a liquid as the mobile phase to separate the sample from the column and then measured with a detector. The operation procedure was as follows: 1 ml fermentation broth was centrifuged at 13,000 rpm for 2 min, the supernatant 0.22 μm filter membrane was taken and loaded into a liquid chromatographic flask, and the contents of glucose, xylose, and ethanol in the culture medium were determined by Waters e2695 high performance liquid chromatograph and Aminex HPX-87Hion exchange residence, with a mobile phase of 5 mM H_2SO_4, a flow rate of 0.6 ml/min, a column temperature of 45 °C, and a detector as a refractive index.

Summary of chapter II

All experiments of *HOG1* gene on xyloss metabolism and acetic acid tolerance of *Saccharomyces cerevisiae* can be made through the experimental instruments, materials and methods described above. The experimental procedures and materials of each step have been introduced in detail[46].

CHAPTER III
RESULTS AND DISCUSSION

3.1 Construction of point mutant strains of *HOG1* gene

The *HOG1* upstream homology arm was amplified using primers og*mera*- F and *144* m*ar*- R using the *S. cerevisiae* BSPZ001 genome *D*NA as a template; *HOG1* downstream homology arms were amplified using primers 1*44* m*ar - F and* o*gmera* R; The fragment containing the URA marker was amplified with primers URA-F and URA-R and verified by agarose gel electrophoresis, and the results were shown in Figure (a), with Marker of 5000 bp as a control, i.e. The fragment band containing URA marker in Chongdao 1 was 1214 bp, the downstream homology arm band in Chongdao 2 was 1388 bp and the upstream homology arm band in Chongdao 3 was 743 bp, indicating that the amplified fragment containing URA marker and the upstream homology arm, and the downstream homology arm were successfully amplified. The successfully amplified upstream homology arm, downstream homology arm and fragment containing URA marker were fused PCR using URA-1-F and URA-1-R as primers to form a fusion PCR fragment and verified by agarose gel electrophoresis, As shown in the figure (b), with Maker at 5 000 bp as a control, the band of fragment formed by fusion PCR in Chongdao 1 was 3585 bp. These results indicate that the fusion PCR fragment has been successfully fused[47,48].

The fragment formed by the fusion PCR was transformed into the parent strain B SPZ001, and the URA contained in the fragment was removed using a plate spread containing 5 -fluoroorotic acid to extract the genome DNA[49], i.e. P CR amplification validation was performed using primers URA-2-F and URA-2-R as shown in Figure (c), using Marker at 5000 bp as a marker, and the results of Chongdao 1 showed that the amplified band of the fusion PCR fragment was 3585 bp, indicating that the fragment formed by the fusion PCR had been successfully transformed into the starting strain, i.e. A Hog1^{D144A} strain was constructed.

3.2 *HOG1* site-directed mutant strains in acetic acid

The results of spreading *hog1* Δ, Hog1[D144A], and wild-type strains on SC-URA plates containing 3 g/l acetic acid are shown in Figure 3.2 (Appendix E). Wild type grew best in plates, and *hog1Δ* strain grew worst compared with wild type strain, indicating a significant decrease in *hog1Δ* tolerance to acetic acid; Hog1 [D144A] strain also grew worse than wild type strain, but better than *hog1Δ* .

3.3 Growth of site-directed mutant strains of *HOG1* gene in xylose

Strains BSPZ001, hog1Δ strain and Hog1 [D144A] strain were inoculated into shake flasks containing 20g/l xylose for oxygen-limited shake flask fermentation and growth curves were determined as shown in Figure 3.3 (a) (Appendix F) and compared to BSPZ001, *The OD $_{600}$* values of hog1Δ and Hog1 [D144A] were greater than the OD $_{600}$ values of BSPZ001 strain, indicating that both *hog1Δ* and Hog1 [D144A] had improved xylose metabolism ability, i.e. *Hog1 [D144A]* strain had the highest OD $_{600}$ value, indicating that this strain had the highest improved xylose metabolism ability[50].

The xylose consumption and ethanol production of *hog 1Δ* , Hog 1 [D144A] and wild-type strains were analyzed by high performance liquid chromatography (HPLC), and the results of HPLC analysis are shown in Figure 3.3 (b) (Appendix F), where the rate of xylose consumption by *hog1Δ* was almost comparable to that by the wild-type strain, i.e[51,52]. However, the ethanol production rate *hog1Δ* was significantly higher than that of the wild-type strain; the xylose consumption rate of the Hog 1 [D144A] strain was almost the same as that of the wild-type strain, and the ethanol production rate was also higher than that of the wild-type strain, but lower than that of the *hog1Δ* strain, indicating that the *hog1Δ* and Hog 1 [D144A] strains' s ability to produce ethanol from xylose was improved[53].

Summary of chapter III

1. Amplification of HOG1 upstream homology arm: The HOG1 upstream homology arm was amplified using primers ogmera-F and 144mar-R with *S. cerevisiae* BSPZ001 genomic DNA as the template.

2. Amplification of HOG1 downstream homology arm: The HOG1 downstream homology arm was amplified using primers 144mar-F and ogmera-R.

3. Amplification of the URA marker fragment: The fragment containing the URA marker was amplified using URA-F and URA-R primers and confirmed through agarose gel electrophoresis.

4. Fusion PCR: The successfully amplified upstream homology arm, downstream homology arm, and URA marker fragment were fused using URA-1-F and URA-1-R primers, generating a fusion PCR fragment, which was verified by agarose gel electrophoresis.

5. Transformation and URA marker removal: The fusion PCR fragment was transformed into the parent strain BSPZ001. The URA marker contained in the fragment was subsequently removed by culturing on a plate spread with 5-fluoroorotic acid. The genomic DNA was extracted from the resulting colonies[62].

6. PCR amplification validation: PCR amplification was performed using URA-2-F and URA-2-R primers to validate the successful transformation of the fusion PCR fragment. The amplified band of the fusion PCR fragment was confirmed through agarose gel electrophoresis[63].

The resulting gel electropherogram (Figure 3.1, Appendix D) showed the successful amplification and transformation of the HOG1 gene with the desired point mutation.

CONCLUSION

In this paper, the mutation of aspartic acid to alanine at position 144 of *HOG1* gene by point mutation has a great impact on xylose ethanol production ability and acetic acid tolerance, indicating that this site plays an important role in *S. cerevisiae* coping with acetic acid stress[60]. In the next step, saturation mutation will be performed at position 144 of *HOG1* gene to determine what amino acids are mutated into what amino acids, and a new metabolic engineering strategy and theoretical basis will be provided to improve the efficiency and economy of lignocellulose biotransformation, as well as a new perspective and method to reveal the molecular mechanism of stress response in *S. cerevisiae*[54].

The results showed that *HOG1* gene point mutation had significant effects on xylose utilization and acetic acid tolerance[56]. This study provided a new metabolic engineering strategy and theoretical basis for improving the efficiency and economy of lignocellulosic transformation, and also provided a new perspective and method for revealing the molecular mechanism of stress response in *Saccharomyces cerevisiae*[58].

REFERENCE

1. Name, Yang Jing, Guo Xin, Han Jianghua, Wang Wei, Liu Zelong, Liu Yingrong, Wang Naixin. Progress in Fuel Ethanol Source Identification Methods [J/OL]. Journal of Petroleum Processing.

2. Xia Miao, Hu Meng, Huang Yiping, Cai Xinlei, Fan Xichao, Ji Yu Fan. Progress in Modification Methods of Biofuel Ethanol Dehydration Pervaporation Membrane [J]. Contemporary Chemical Research, 2022 (13): 43-45.

3. Tang Zhaoping, Sun Jianping. Development of Ethanol Fuel and Its Application in Engines [J]. Tractors and Agricultural Transport Vehicles, 2008 (01): 12-13 + 16.

4. Yin Zhimin, Bai Xiaoyu, Zhao Yawei, Chen Jue. Development Status and Promotion Suggestions of Fuel Ethanol Industry [J]. Shanxi Chemical Industry, 2020, 40 (02) :15-19.DOI:10.16525/j.cnki .cn14-1109/tq.2020.02.06.

5. Jiang Xiangxiang, Lu Yun, Fengxia, Xiao Qidong, Gou Jinsheng, Peishuang, Fu Zongying. Progress in lignocellulose-based packaging materials [J]. Wood Science and Technology, 2022, 36 (06): 13-23 + 40.

6. A Ze-lin, LIU Jia-heng, HUANG Xu, CAIYIN Qing-gele, ZHU Hong-ji. Research Progress on Utilization of Lignocellulosic Biomass by Microorganisms. China Biotechnology, 2017, 37 (6): 124-133.

7. Zhang Zichen, Song Yuanda. Progress in Biotechnology for Comprehensive Utilization of Lignocellulose Materials [J]. Guangdong Agricultural Science, 2021, 48 (01) :150-159.DOI:10.16768/j.issn .1004-874X.2021.01.019.

8. Sun Tao, Yu Yizi, Wang Lexin, Qi Yichun, Xu Tian, Wang Zhe, Lin Lu, LedesmaAmaro Rodrigo, Ji XiaoJun. Combination of a Push-Pull-Block with a Synthetic Heterologous Xylose Assimilation Pathway Production from Lignocellulose in Yarrowia lipolytica. [J]. ACS Strategy, 2023.

9. Mankar Akshay R., Pandey Ashish, Modak Arindam, Pant K.K. Pretreatment of lignocellulosic biomass: A review on recent advances [J]. Bioresource Technology, 2021, 334.

10. Nauman Aftab, Muhammad, et al. 'Different Pretreatment Methods of Lignocellulosic Biomass for Use in Biofuel Production'. Biomass for Bioenergy - Recent Trends and Future Challenges, Intechopenes, 18 Dec. 2019. Crossref, doi: 10.5772/intechopen.84995.

11. Kumar Adepu Kiran, Sharma Shaishav. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. [J]. Bioresources and bioprocessing, 2017, 4 (1).

12. Meng Xianzhi, Zhang Shuyang, Scheidemantle Brent, Wang Yun-yan, Pu Yunqiao, Wyman Charles E., Cai Charles M., Ragauskas Arthur J. Preparation and characterization of aminated co-enhanced nococyanosic urethane fractionation lignin as a solvent synthesis block for the renewable of non-renewable polyurethanes [J]. Industrial Crops ampampampampampampampampampampampampampampampampa mpampampampampampampampane; Products, 2022, 178.

13. Lan, T., Jiang, Y., Zheng, W. *et al.* Comprehensively Understanding Enzymatic Hydrolysis of Lignocellulose and Cellulase – Lignocellulose Adsorption by Analyzing Substrates' Physicochemical Properties. *Bioenerg. Res.* 13, 1108 – 1120 (2020).

14. Gabriela Piccolo Maitan-Alfenas, Evan Michael Visser, Valéria Monteze Guimarães, Enzymatic hydrolysis of lignocellulosic biomass products: food conversion waste in valuable biomass products, Current Opinion in Food Science, 2015, 1.

15. Zheng Huihui. Study on the key influencing factors of xylose utilization in *Saccharomyces cerevisiae* and optimization of rDNA mediated gene integration and expression methods [D]. Shandong University, 2022.DOI:10.27272/d.cnki .gshdu.2022.000347.

16. Du Chenglong, Wang Mian, Ma Jintong, Qin Liming, He Hongkui, Cao Runjie, Li Anjun, Zhou Penglei. Optimization of Screening and Culture Conditions of *Saccharomyces cerevisiae* Strains [J]. *Saccharomyces cerevisiae*, 2023, 50 (02): 89-92.

17. Li Hongbiao, Liang Xiaolin, Zhou Jingwen. Progress in Gene Editing Technology of *Saccharomyces cerevisiae* [J]. Journal of Bioengineering, 2021, 37 (03) :950-965.DOI:10.13345/j.cjb .200542.

18. Jay L. Brewster, Michael C. Gustin. *Hog1*:20 years of discovery and impact [J]. Sci. Signal., 2014, 7 (343).

19. Ruiz-Roig Clà udia, Noriega Núria, Duch Alba, Posas Francesc, de Nadal Ulà lia. The *Hog1* SAPK multiple regulatory activities of the Rtg1/Rtg3 transcriptional biology complex controls by molecular mechanisms. [J]. Molecular biology controls of the cell, 2012, 23 (21).

20. Seo-Young Kim, Young-Joon, Ko, Kwang-Woo Jung, Anna Strain, Kirsten Nielsen, Yong-Sun Bahn Published: April 13, 2011

21. Mollapour Mehdi, Piper Peter W. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. [J]. Molecular and cellular biology, 2007, 27 (18).

22. Tognetti Silvia, Jiménez Javier, Vigan ò Matteo, Duch Alba, Queralt Ethel, de Nadal Eulà lia, Posas Francesc. Hog1 mitotic activation via phosphorylation of Net1. [J]. Proceedings of the National Academy of Sciences of the United States of America, 2020, 117 (16).

23. Li Xiaowei, Wang Yanyan, Li Gang, Liu Quanli, Pereira Rui, Chen Yun, Nielsen Jens. Metabolic network remodelling enhances fitness on xylose using aerobic glycolysis [J]. Nature Catalysis, 2021, 4 (9).

24. Ellen R Wagner, Kevin S Myers, Nicholas M Riley, Joshua J Coon, Audrey P Gasch. PKA and HOG signaling separable roles contribute to anaerobic

xylose fermentation in yeast engineered for biofuel production. [J]. PLoS ONE, 2019, 14 (5).

25. Nicoletta Guaragnella, Mariarita Stirpe, Domenico Marzulli, Cristina Mazzoni, Sergio Giannattasio. Acid Stress Triggers Resistance to Acetic Acid-Induced Regulated Cell Death Through Hog1 Which Requires RTG2 in Yeast [J]. Oxidative Medicine and Cellular Longevity, 2019, 2019.

26. Eun Joong Oh, Na Wei, Surun Kwak, Heejin Kim, Yong-Su Jin. Overexpression of RCK1 improves acetic acid tolerance in Saccharomyces yang [J]. Journal of Biotechnology, 2018, 292.

27. Zhang Mei, Gao Zhi-Chao, Chi Zhe, Liu Guang-Lei, Hu Zhong, Chi Zhen-Ming. cAMP-PKA and HOG1 signaling pathways differentially regulate via the transcriptional pathway Msn2 in Aureobasidium melanogenum [J]. Enzyme and Microbial Technology, 2021, 143.

28. Nicoletta Guaragnella, Mariarita Stirpe, Domenico Marzulli, Cristina Mazzoni, Sergio Giannattasio. Acid Stress Triggers Resistance to Acetic Acid-Induced Regulated Cell Death Through Hog1 Which Requires RTG2 in Yeast [J]. Oxidative Medicine and Cellular Longevity, 2019, 2019.

29. Eun Joong Oh, Na Wei, Surun Kwak, Heejin Kim, Yong-Su Jin. Overexpression of RCK1 improves acetic acid tolerance in Saccharomyces yang [J]. Journal of Biotechnology, 2018, 292.

30. Sugar Discuss; Researchers at Suzhou University of Science and Technology Discuss Findings in Sugar AlcoholEfficis (Construction of an Alcoholent Mutant Strain of Trichosporonoides oedocephalis with HOG1 Gene Deletion for Production of Erythritol) [J]. Biotech Week, 2017.

31. Alicia Rodríguez, Ángel Medina, Juan J. Córdoba, Naresh Magan. Development of a HOGe-based real-time PCR method to detect stress response changes in mycotoxigenic moulds [J]. Food Microbiology, 2016, 57.

32. Li Liangzhi, Yang Tianyi, Guo Weiqiang, Ju Xin, Hu Cuiying, Tang Bingyu, Fu Jiaolong, Gu Jingsheng, Zhang Haiyang. Construction of an Efficient

Mutant Strain of Trichosporonoides oedocephalis with HOG1 Gene Deletion for Production of Erythritol. [J]. Journal of microbiology and biotechnology, 2016, 26 (4).

33. Mariona Nadal-Ribelles, Carme Solé, Zhenyu Xu, Lars M. Steinmetz, Ulàlia de Nadal, Francesc Posas. Control of Cdc28 CDK1 by a Stress-Induced lncRNA [J]. Molecular Cell, 2014, 53 (4).

34. S.X.Xiao, C.M.Tian, Y.L.Wang. Hog1-MAPK gene VdHOG1 regulates microsotial formation and stress response in smoke-tree vascular wilt Verticillium dahliae [C]//Chinese Society of Plant Pathology.Abstracts of ICPP 2013 International Congress of Plant Pathology. Chinese Society of Plant Pathology , 2013:569.

35. Cao Xiao-Xong, Meng Meng, Wang Yuan-Yuan, Wang Chun-Ling, Hou Li-Hua. Identification of salt-tolerant gene letters HOG1 in Torulopsis versatilis. [J]. Biotechnology letters, 2011, 33 (7).

36. Solé Carme, Nadal-Ribelles Mariona, Kraft Claudine, Peter Matthias, Posas Francesc, de Nadal Ulàlia. Control of Ubosmop3 ubiquitin protease activity modulated by the Hog1 SAPK transcription upon stress. [J]. The EMBO journal, 2011, 30 (16).

37. Serge Pelet, Fabian Rudolf, Mariona Nadal-Ribelles, Ulàlia de Nadal, Francesc Posas, Matthias Peter. Transient Activation of the HOG MAPK Pathway Regulates Bimodal Gene Expression [J]. Science, 2011, 332 (6030).

38. Qian Jiangchao, Qin Xiulin, Yin Qin, Chu Ju, Wang Yonghong. Cloning and characterization of Kluyveromyces marxianus Hog1 gene. [J]. Biotechnology, 2011, 33 (3).

39. Eraso Pilar, Mazón María J, Posas Francesc, Portillo Francisco. Gene expression profiling of yeasts overexpressing wild type or misfolded Pma1 reveals activation of the Hog1 MAPK pathway. [J]. Molecular microbiology, 2011, 79 (5).

40. Zhang Yongjun, Zhao Jianhua, Fang Weiguo, Zhang Jianqing, Luo Zhibing, Zhang Mi, Fan Yanhua, Pei Yan. Mitogen-activated protein kinase hog1 in

the entomopathogenic Beauveria bassiana environmental stress responses and virulence to insects. [J]. Applied environmental and microbiology, 2009, 75 (11).

41. Godge Mandar R, Kumar Dhinoth, Kumar Prakash P. Arabidopsis HOG1 gene and its petunia homolog PETCBP act as key regulators of yield parameters. [J]. Plant cell reports, 2008, 27 (9).

42. Migdal Iwona, Ilina Yulia, Tamás Markus J, Wysocki Robert. Mitogen-activated protein kinase checkpoint Hog1 mediates adaptation to G1 arrest during arsenite and hyperosmotic stress. [J]. Eukaryotic cell, 2008, 7 (8).

43. Moriwaki Akihiro, Kubo Emiko, Arase Sakae, Kihara Junichi. Disruption letters of SRM1, a mitogen - activated protein kinase gene, affects sensitivity to osmotic and stressors in the phytopathogenic fungus Bipolaris oryzae [J]. FEMS microbiology, 2 006, 257 (2).

44. Taline Elmayan, Florence Proux, Hervé Vaucheret. Arabidopsis RPA2: A Genetic Link among Transcriptional Gene Silencing, DNA Repair, and DNA Replication [J]. Current Biology, 2005, 15 (21).

45. Rocha Pedro S C F, Sheikh Mazhar, Melchiorre Rosalba, Fagard Mathilde, Boutet Stéphanie, Loach Rebecca, Moffatt Barbara, Wagner Conrad, Vaucheret Hervé, Furner Ian. The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING1 gene silencing codes for an S-hydroosyl-L-homocysteine silencing codes required for DNA methylation-dependent gene. [J]. The Plant Cell, 2005, 17 (2).

46. Yaakov Gilad, Bell Michal, Hohmann Stefan, Engelberg David. Combination of two activating mutations in one HOG1 gene forms hyperactive enzymes that induce cellular growth arrest. [J]. Molecular and cellular biology, 2003, 23 (14).

47. Turk Martina, Plemenitas Ana. The HOG pathway in the halophilic black yeast Hortaea werneckii: isolation of the HOG1 homolog gene and activation of HwHog1p [J]. FEMS microbiology letters, 2002, 216 (2).

48. Iwaki Tomoko, Tamai Youichi, Watanabe Yasuo. Two putative MAP kinase genes, ZrHOG1 and ZrHOG2, cloned from the salt-tolerant yeast Zygosaccharomyces rouxii are homologous to the *Saccharomyces cerevisiae* HOG1 gene. [J]. Microbiology (Reading, England), 1999, 145 (Pt 1) (1).

49. Tomoko Iwaki, Youichi Tamai, Yasuo Watanabe. Two putative MAP kinase genes, ZrHOG1 and ZrHOG12, cloned from the salt-tolerant yeast Zygosaccharomyces rouxii are homologous to the *Saccharomyces cerevisiae* HOG1 gene [J]. Microbiology, 1999, 145 (1).

50. San José C, Monge R A, Pérez-Díaz R, Pla J, Nombela C. The mitogen-activated protein kinase homolog HOG1 gene controls accumulation in the pathogenic fungus Candida albicans. [J]. Journal of bacteriology, 1996, 178 (19).

51. Yang Li,Ameair Irqeba,Mahmoud Panahi,Ming Zhu,Yuqi Wang. Regulation global sumoylation by a MAP kinase Hog1 and its potential role in osmo- tolerance in yeast (802.17)[J]. The FASEB Journal,2014,28.

52. Charlotte Hamngren Blomqvist,Peter Dinér,Morten Grøtli,Mattias Goksör,Caroline B. Adiels. A Single-Cell Study of a Highly Effective Hog1 Inhibitor for in Situ Yeast Cell Manipulation[J]. Micromachines,2014,5(1).

53. Lee Jongmin,Reiter Wolfgang,Dohnal Ilse,Gregori Christa,Beese-Sims Sara,Kuchler Karl,Ammerer Gustav,Levin David E. MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators.[J]. Genes & development,2013,27(23).

54. Wei Yonghua,Wang Cong,Wang Meng,Cao Xiaohong,Hou Lihua. Comparative analysis of salt-tolerant gene HOG1 in a Zygosaccharomyces rouxii mutant strain and its parent strain.[J]. Journal of the science of food and agriculture,2013,93(11).

55. Babazadeh Roja,Furukawa Takako,Hohmann Stefan,Furukawa Kentaro. Rewiring yeast osmostress signalling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation.[J]. Scientific reports,2014,4(1).

56. Dinér Peter,Veide Vilg Jenny,Kjellén Jimmy,Migdal Iwona,Andersson Terese,Gebbia Marinella,Giaever Guri,Nislow Corey,Hohmann Stefan,Wysocki Robert,Tamás Markus J,Grøtli Morten. Design, synthesis, and characterization of a highly effective Hog1 inhibitor: a powerful tool for analyzing MAP kinase signaling in yeast.[J]. PloS one,2011,6(5).

57. Solé Carme,Nadal-Ribelles Mariona,Kraft Claudine,Peter Matthias,Posas Francesc,de Nadal Eulàlia. Control of Ubp3 ubiquitin protease activity by the Hog1 SAPK modulates transcription upon osmostress.[J]. The EMBO journal,2011,30(16).

58. Eraso Pilar,Mazón María J,Posas Francesc,Portillo Francisco. Gene expression profiling of yeasts overexpressing wild type or misfolded Pma1 variants reveals activation of the Hog1 MAPK pathway.[J]. Molecular microbiology,2011,79(5).

59. Gónzalez-Párraga Pilar,Alonso-Monge Rebeca,Plá Jesús,Argüelles Juan Carlos. Adaptive tolerance to oxidative stress and the induction of antioxidant enzymatic activities in Candida albicans are independent of the Hog1 and Cap1-mediated pathways.[J]. FEMS yeast research,2010,10(6).

60. Torres-Quiroz Francisco,García-Marqués Sara,Coria Roberto,Randez-Gil Francisca,Prieto Jose A. The activity of yeast Hog1 MAPK is required during endoplasmic reticulum stress induced by tunicamycin exposure.[J]. The Journal of biological chemistry,2010,285(26).

61. Ching-Hsuan Lin,Kuang-Ren Chung. Specialized and shared functions of the histidine kinase- and HOG1 MAP kinase-mediated signaling pathways in Alternaria alternata , a filamentous fungal pathogen of citrus[J]. Fungal Genetics and Biology,2010,47(10).

62. Yin Zhikang,Stead David,Walker Jan,Selway Laura,Smith Deborah A,Brown Alistair J P,Quinn Janet. A proteomic analysis of the salt, cadmium and peroxide stress responses in Candida albicans and the role of the Hog1 stress-

activated MAPK in regulating the stress-induced proteome.[J]. Proteomics,2009,9(20).

63. Zhang Yongjun,Zhao Jianhua,Fang Weiguo,Zhang Jianqing,Luo Zhibing,Zhang Mi,Fan Yanhua,Pei Yan. Mitogen-activated protein kinase hog1 in the entomopathogenic fungus Beauveria bassiana regulates environmental stress responses and virulence to insects.[J]. Applied and environmental microbiology,2009,75(11).

Table 1 Strains used in this paper

Strain	Genotype	Origin
BSPZ001	CEN.PK113-5D derivative; *MATa; ura3-52, (-194, -1) XKS1: loxP-TEF1p, (-241, + 338) gre3: TPI1p-RKI1-RKI1t-PGK1p-TAL1-TAL1t-FBA1p-TKL1-TKL1-TKL1-TKL1t-ADH1p-RPE1-RPE1t-XIH/(XK, gre3 : PPP, Ru - XylA)*	Maintained in this laboratory
Hog1 Δ	BSPZ001 derivative; hog1: *KanMX*	Maintained in this laboratory
D114A	BSPZ001 derivative; *HOG1* D114A	Construction of this paper

Appendix B

Table 2 | Main instruments used in this paper

Instrument Name	Instrument Model	Manufacturer	
Autoclave	LDZX-50KBS	Shanghai Shen'an Medical Device Factory	
Desktop high speed centrifuge	Fresco17	Thermo Fisher Scientific China	
Ultrapure Water System	Milli-Q Advantage A10	Merck Millipore, Germany	
Analytical Balance	BSA123S-CW	Sartorius Scientific Instruments Co., Ltd.	
Blowing oven	DHG-9140A	Shanghai Jinghong Laboratory Instrument Co., Ltd.	
PCR Amplifier	Gene-Explorer Touch	Hangzhou Baiheng Technology Co., Ltd.	
Biosafety Cabinet	BSC-1300IIA2	Suzhou Antai Air Technology Co., Ltd.	
Thermomixer	MTC-100	Hangzhou Miou Instrument Co., Ltd.	
Electronic Constant Temperature Loading Station	ECT-4100	Mona Biotechnology Co., Ltd.	
Thermostatic Water Bath	DK-8B	Shanghai Jinghong Laboratory Instrument Co., Ltd.	
Ultrasonic Cleaner	KH-300SPV	Kunshan Hechuang Ultrasonic Instrument Co., Ltd.	

The primer sequences used in this paper are shown in Table 3.

Table 3 Primers used in this paper and their sequences

| Primers	Primer Sequence (5 '→ 3')	
ogmera -F	TACGGGAGGATCTTCGAAGGGA	
ogmera -R	CCATAAGTGACGGTTCTTGGAGTCTT	
144 mar -R	CATTAATCAGAATGTTGCTCGGTTTCAATGCTCTATGAATGACGCCCGCGGA	
144 mar -F	TCCGCGGGCGTCATTCATAGAGCATTGAAACCGAGCAACATTCTGATTAATG	
URA- 1-F	AAGACTCCAAGAACCGTCACTTATGG	
URA- 1-R	TCGACGCTAGCATATAAAGCGGGAAGTGGTAAGTATACGCTTGTCTGGGTG	
URA-F	CCCGCTTTATATATGCTAGCGTCGA	
URA-R	GGACATCCGCGGATAACTTCGT	
URA- 2-F	CTGCTCTCATTCATTAATTCTAACGGAGC	
URA- 2-R	CTTGAGGACTACTCGTAGGTTATTCTGT	
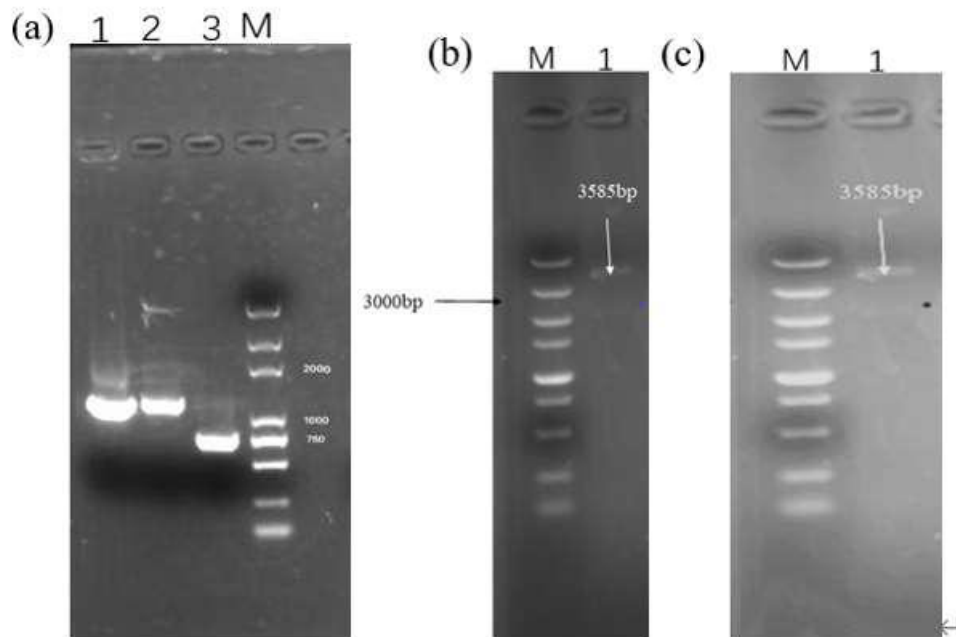
Figure 3.1 PCR Validation electropherogram of *HOG1* gene with point mutation. (a) *Up- and downstream homology arms of HOG1* gene and fragment containing U RA marker P CR amplification product; M: 5000 bp Marker; 1: URA verified fragment; 2: homology arm upstream of *H OG1* gene; 3: homology arm downstream *H OG1* gene; (b) Up- and downstream homology arms and fusion P CR products containing U RA marker fragments; M : 5 000 bp M arker; 1: fusion P CR fragments; (c) The fusion P CR fragment was transformed into the parent strain B SPZ001 product; M: 5000 bp M arker; 1: fusion P CR fragment amplification product.
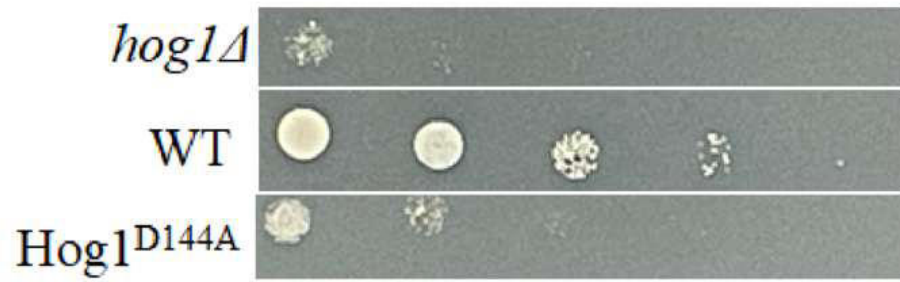
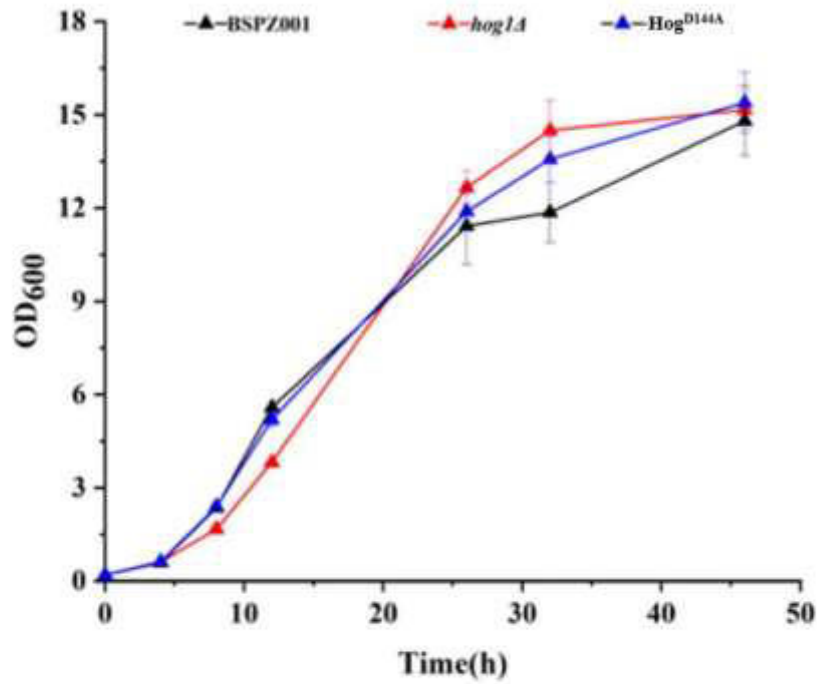Figure 3.2 *Growth of hog1Δ*, Hog1^{D114A}, and wild-type strains at 3g/l acetic acid

Figure 3.3 (a) Growth of BSPZ001, *hog1* Δ, and HOG1 [D114A] strains in wood
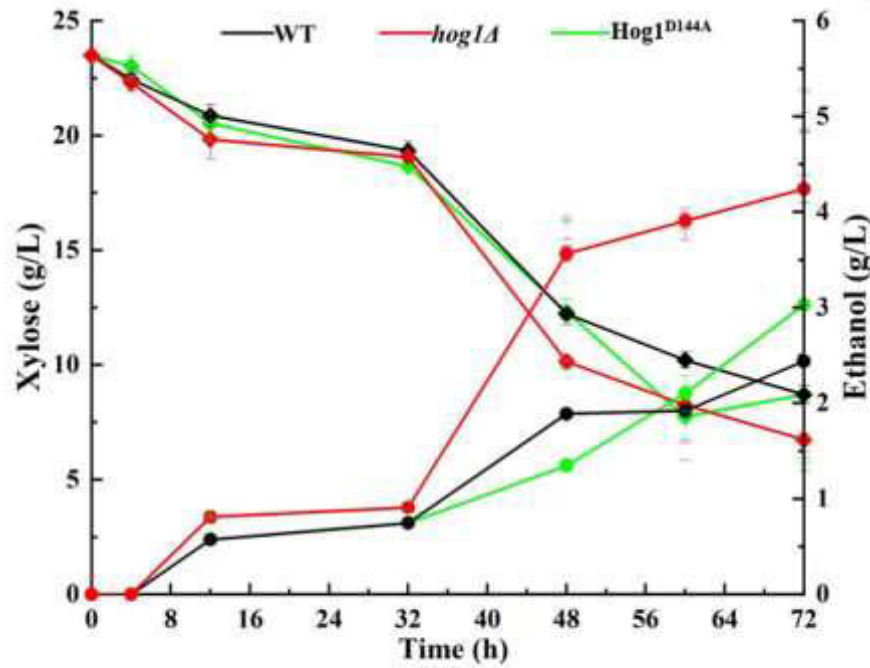pond solution containing 2 0 g/l

Figure 3 .3 (b) Ethanol production from xylose by wild type, *hog 1 Δ* and Hog 1