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

QUALIFICATION THESIS

on the topic **Analysis of the effect of TSA2 gene overexpression on acetic acid tolerance 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
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ASSIGNMENTS
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SUMMARY

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In order to protect the environment and maintain the sustainable development of ecology, the second-generation fuel ethanol has been widely developed and utilized. The raw material of the second generation of fuel ethanol is lignocellulose, but lignocellulose cannot be directly decomposed by microorganisms, and it needs to be pre-treated to make it fermentable monosaccharides before it can be used in the microbial fermentation process. During the pretreatment process, in addition to fermentable monosaccharides, many small molecule compounds are released, which can inhibit the growth and metabolism of microorganisms. Acetic acid is one of the most abundant small molecule compounds, and studies have found that excessive acetic acid can adversely affect the metabolism, signaling and cell growth of *Saccharomyces cerevisiae*, and even inhibit the production of ethanol.

In order to improve the acetic acid tolerance of the ethanol fermentation strain, a histone point mutant with significantly improved xylose utilization and ethanol yield was obtained. In order to further explore the regulatory mechanism, transcriptome sequencing was performed on this strain. Through analysis, it was found that the expression level of *TSA2* gene was significantly increased in this mutant. In order to verify the effect of *TSA2* gene on acetic acid tolerance of yeast, we carried out experiments related to this subject. The overexpression of *TSA2* gene in xylose-utilizing strains and the detection of acetic acid tolerance showed that the overexpression of *TSA2* gene could significantly improve the acetic acid tolerance of the strains. The experiment provided an effective element for the development of second-generation ethanol fermentation strains with high acetic acid tolerance.

Keywords: *ethanol; Saccharomyces cerevisiae; TSA2 gene; acetic acid stress*

CONTENTS

INTRODUCTION

In previous experiments, we obtained a histone point mutant with significantly improved xylose utilization and ethanol yield. In this study, we conducted transcriptome sequencing and found that the expression level of *TSA2* gene was significantly increased in strains with improved acetic acid resistance. Overexpression of *TSA2* gene was found to significantly improve acetic acid tolerance of *Saccharomyces cerevisiae*, which was conducive to the production of second-generation fuel ethanol. It plays an important role in protecting the environment and maintaining the sustainable development of ecology.

The relevance of the topic is ethanol; *Saccharomyces cerevisiae*; *TSA2* gene; acetic acid stress.

The purpose of the study is to further explore the regulatory mechanism of *TSA2* gene in histone point mutants on acetic acid tolerance in yeast, and to verify whether *TSA2* gene can improve acetic acid tolerance by overexpressing *TSA2* gene in xylose-utilizing strains, so as to provide effective components for developing second-generation ethanol fermentation strains with high acetic acid tolerance.

In order to achieve this goal, we use a variety of research methods. First, through transcriptome sequencing, we comprehensively analyzed the expression of *TSA2* gene in histone point mutants, providing basic data for understanding its role in acetic acid tolerance. Secondly, we used gene overexpression technology to overexpress *TSA2* gene in xylose-utilizing strains, and directly verified the effect of this gene on improving acetic acid tolerance through acetic acid tolerance detection. This approach allowed us to intuitively assess the role of the *TSA2* gene in enhancing acetic acid tolerance in yeast. We also combined molecular biology and genetics methods as well as bioinformatics analysis to study the mechanism of *TSA2* gene comprehensively and deeply in improving acetic acid tolerance. The comprehensive application of these methods not only provides support for experimental verification, but also provides an important reference for the

subsequent transformation and optimization of strains.

Through this research, we hope to improve the production efficiency and sustainability of second-generation fuel ethanol, reduce the inhibition of small molecule compounds such as acetic acid on microbial growth and metabolism, and further promote the development and utilization of biomass energy.

The objectives of the study Whether overexpression of *TSA2* gene can improve acetic acid tolerance of *Saccharomyces cerevisiae*.

The object of the study is *Saccharomyces cerevisiae* strains with histone point mutants with significantly improved xylose-utilizing capacity and ethanol yield, especially the effect of TSA2 gene on acetic acid tolerance.

The subject of the study is Acetic acid tolerance of *Saccharomyces cerevisiae*.

Research methods is transcriptome sequencing, gene overexpression, molecular biology and genetics, bioinformatics analysis and so on.

The scientific novelty is to reveal for the first time the central role of the *TSA2* gene in improving acetic acid tolerance in yeast, and to explore the regulatory mechanisms of the *TSA2* gene through interdisciplinary technical means, such as molecular biology, genetics, and bioinformatics. This breakthrough discovery not only provides a key element for the development of second-generation ethanol fermentation strains with high acetic acid tolerance, but also opens a new way for the development and utilization of biomass energy. By optimizing yeast strains, we can reduce the impact of inhibitory compounds such as acetic acid on microbial growth and metabolism, thereby improving the efficiency of biomass energy conversion and environmental sustainability. This innovative research not only demonstrates the power of interdisciplinary integration, but also lays a solid foundation for future advances in biomass energy technology.

The practical significance of the results obtained is dependent on its dual contribution to the biomass energy industry and environmental protection.

For the biomass energy industry, improving yeast acetic acid tolerance means

that the biological fermentation process can be optimized so that yeast cells can still efficiently perform metabolic activities in higher concentrations of acetic acid to produce fuel ethanol or other biological products. This can not only reduce production costs and improve production efficiency, but also promote the commercialization process of biomass energy technology to meet the growing energy demand.

From the perspective of environmental protection, the improvement of acetic acid tolerance of optimized yeast strains can help reduce waste and pollutants generated during biomass energy production. Acetic acid is a common by-product in the fermentation of biomass, which has inhibitory effect on the growth and metabolism of yeast cells. Improving the tolerance of yeast to acetic acid can reduce acetic acid accumulation, reduce its negative impact on the environment, and improve the sustainability of biomass energy.

Therefore, the result obtained not only provides new technical support for the biomass energy industry, but also makes a positive contribution to environmental protection and sustainable development.

CHAPTER 1
LITERATURE REVIEW

1.1 Importance of developing second-generation fuel ethanol

1.1.1 The development prospect of the second-generation fuel ethanol

Currently, fuel ethanol is the most widely used biofuel in the world. With the progress of the times and the continuous development of science and technology, the development of the fuel ethanol industry has ushered in new opportunities. According to the different raw materials and different stages of development of technology, biofuel ethanol is divided into 1st generation fuel ethanol, which is produced from food crops as raw materials. Such as the United States is the largest producer of fuel ethanol, mainly based on corn as raw material. By adding 10% ethanol to gasoline to formulate E10 ethanol gasoline, it has been successfully applied to the automobile industry and basically achieved territory-wide coverage[1] The first generation of fuel ethanol has been successfully used in the automobile industry and has almost achieved territory-wide coverage. The 1.5 generation of fuel ethanol is produced from cash crops such as sugar cane and cassava. For example, Brazil's biofuel ethanol is mainly made from sugarcane, and relevant policies have also been introduced to promote the development of E85 ethanol gasoline with higher ethanol content[2] .The second generation of fuel ethanol, which is produced from sugarcane, is the second generation of ethanol. The second generation of fuel ethanol is produced from lignocellulosic biomass such as corn stove. The third generation of fuel ethanol is produced from carbohydrates in microalgae[3]. The 3rd generation of fuel ethanol is produced from carbohydrates from microalgae. The main measure to achieve sustainable development of fuel ethanol is to realize the economic feasibility of producing second generation fuel ethanol from lignocellulosic feedstocks. The cost of the first generation of fuel is relatively low, but will be contradictory to human survival resources, there is competition, and with the rising global food prices and increasingly tight supply, we are facing a huge challenge; 1.5 generation of fuel ethanol production raw materials and

has a strong geographical; and lignocellulosic raw materials have a lower cost, abundant raw material reserves and renewable, the global annual production of 10 billion tons of estimated[4][5] The lignocellulosic raw material has the advantages of lower cost, rich and renewable raw material reserves, and the annual global production is estimated to be 10 billion tons, and does not compete with food for land, etc. The second generation fuel ethanol produced by it can alleviate the environmental pollution caused by straw burning and incomplete combustion of petroleum, and it can reduce greenhouse gas emissions by 30%-85% compared to the fossil fuels in the past and reduce the formation of atmospheric particulate matter, which shows the advantages of economy and environmental protection, and thus is the direction of the second-generation fuel ethanol to carry on the sustainable and large-scale development. It shows economic and environmental advantages and is therefore the direction for sustainable and large-scale development of second- generation fuel ethanol.

1.1.2 Importance of second-generation ethanol as an alternative to fossil fuels

With the increasing environmental pollution and the depletion of fossil fuels, in order to protect the environment as well as to maintain ecological sustainable development, we have started to pay more and more attention to the development and utilization of renewable clean energy bio-second generation fuel ethanol. The basic process of second-generation fuel ethanol is divided into five stages: raw material pretreatment, enzymatic digestion, microbial ethanol fermentation, distillation, and dehydration. And many basic and applied research on the production of second-generation fuel ethanol, however, the conversion efficiency of microorganisms is still one of the challenging key technological bottlenecks, the main problem lies in the lignocellulosic hydrolysate of the whole sugar components of the conversion efficiency is low and produces a poor inhibitor resistance in the pretreatment process. Therefore, breaking through these dilemmas is conducive to the realization of economic production of second-generation fuel ethanol.

1.1.3 Advantages and significance of ethanol production from lignocellulosic biomass

There exists a huge number, various and extremely rich lignocellulose resources in nature[6] .The raw materials of lignocellulose are straw, wood, hay and other agricultural and forestry wastes, etc., which are widely distributed and inexpensive in our country and have great potential for application. According to relevant data from the Ministry of Agriculture, the amount of available straw resources in China is 600-700 million tons per year, in addition to feeding livestock and straw consumption, a large amount of excess straw material can be used to produce fuel ethanol and has a broad prospect[7]. In addition, lignocellulosic-based ethanol can be used to produce ethanol. In addition, lignocellulose as raw material for the second generation of fuel ethanol and food as raw material for the first generation of fuel ethanol compared, more conducive to mitigate the greenhouse effect. China's use of lignocellulose as raw material to produce second-generation fuel ethanol is more favorable, such as the annual production of more than 800 million tons of crop residues in China, with the potential to produce 200 million tons of ethanol, if it can be fully utilized to reduce emissions of 243million tons of carbon dioxide. Therefore, the production of second-generation fuel ethanol from crop straw is very suitable for China's national conditions, not only to reduce the degree of our country's dependence on foreign oil, but also by the conversion of crop straw into fuel belongs to the clean fuels, can promote environmental protection, and is of great significance to the realization of the goal of sustainable development.

1.2 Difficulties in producing second-generation fuel ethanol from lignocellulose

The second-generation fuel ethanol based on lignocellulosic feedstock has been strongly supported by the state, but the key to its widespread application is to have a cost-price advantage. Lignocellulosic raw materials contain a large number of fermentable sugars, however, such substances usually exist in the form of high

molecular weight cellulose and hemicellulose and other polysaccharides[7] In addition, it also contains a large amount of lignin (formed in the process of lignification at the final stage of cell differentiation, it permeates the skeleton and matrix substances in the cell wall, surrounded by microfibers, milli-fibers, etc., which is the main substance in the formation of the intercellular layer between the fibers and the fibers, but the cell wall is hard, and is said to be a crusting substance) and proteins, etc., which are the main components of chemical compositions and multilayers of lignocellulosic raw materials. These chemical components and multi-level supramolecular structure makes the plant body has a variety of anti-biodegradation properties, collectively referred to as "biomass degradation barrier". Lignocellulosic raw materials have a natural "anti-degradation barrier" makes its direct enzymatic hydrolysis efficiency is extremely low, so the production process of second- generation fuel ethanol is more complex than the first-generation ethanol, which is mainly based on grain raw materials, or the first generation of fuel ethanol, which is based on tapioca, sugarcane, sweet sorghum and other raw materials[7] .

Firstly, lignocellulosic feedstock needs to be pretreated to destroy its compact structure, and various pretreatment methods not only make the process more complicated, but also increase the production cost of second-generation fuel ethanol. Secondly, the enzymatic process after pretreatment requires efficient cellulase enzyme system to convert it into fermentable sugar, and the cellulase enzyme in this process occupies a high production cost. In addition, the hydrolysis of lignocellulose produces many fermentable monosaccharides, of which xylose cannot be utilized by traditional fermentation strains of brewer's yeast, which makes the competitiveness of second-generation fuel ethanol decrease.

1.3 Pretreatment of lignocellulose

Because crop straw is a multi-component material, to utilize it for cellulosic ethanol production, we first need to convert the cellulose and hemicellulose in the material into fermentable sugars through enzymatic hydrolysis[9].The material is a multi-component material. However, crop residues have a strong reticulated structure,

which makes it difficult for cellulose and hemicellulose to be degraded efficiently. Therefore, a pretreatment operation of crop residues is required prior to enzymatic hydrolysis. The purpose of the pretreatment is to change the structure of the crop straw, disrupt the connections between the cellulose internals, reduce the degree of polymerization, and thereby increase the conversion rate for enzymatic hydrolysis. There are many pretreatment methods, which we can roughly categorize into: physical method, chemical method, combined physical and chemical method and biological method. Physical method is mainly to use machinery under the action of shear force and grinding force, resulting in low polymerization of raw materials, and then to achieve the purpose of improving the conversion rate of enzyme hydrolysis sugar. However, the mechanical crushing method does not detach lignin from cellulose in crop straw, although it will improve the effect of enzymatic hydrolysis of crop straw, but the conversion rate of enzymatic hydrolysis of crop straw is not up to the ideal effect[10][12]. The biological method is to use the enzyme produced by microorganisms to produce the cellulose. Biological method is to use the enzyme produced by microorganisms to degrade cellulose and lignin, although it is conducive to the protection of the environment. However, there are problems such as high requirements for degradation conditions of bacterial strains and relatively low efficiency. In addition, part of the cellulose and hemicellulose will be consumed by microorganisms, resulting in unproductive loss of raw materials. Chemical pretreatment can substantially improve the enzymatic hydrolysis of crop straw and is also a pretreatment method often used in cellulosic ethanol production. Chemical pretreatment is divided into acidic, neutral, and alkaline according to the type of reagent. Acid pretreatment usually causes the degradation of hemicellulose in crop residues to monosaccharides such as xylose and arabinose, but the effect on the structure of lignin and hemicellulose is limited[13]. At present, there are many methods of lignocellulosic pretreatment, but each method has advantages and disadvantages, according to different kinds of raw materials to choose the appropriate treatment method.

1.4 Production of second-generation fuel ethanol with *Saccharomyces cerevisiae* strains

1.4.1 Characteristics of brewer's yeast and industrial applications

Saccharomyces cerevisiae is a yeast that has been defined as "generally recognize as safe", with the advantages of rapid growth, clear genetic background, high stress resistance, simple operation, high safety, etc., and has a wide range of applications in various fields, such as food, biopharmaceuticals, energy, and chemicals. It has been widely used in various fields such as food, biopharmaceutical, energy, and chemical industry.

Saccharomyces cerevisiae is an important model microorganism for biological research and has become an important host bacterium for the synthesis of many natural compounds, which has the advantages of high safety and acid resistance, and currently, *Saccharomyces cerevisiae* has a relatively complete means of genetic manipulation. In recent years, through the transformation of brewer's yeast, the efficient synthesis of many substances has been accomplished, and the transformation of scientific research results has been realized, creating high commercial value.

1.4.2 Advantages and drawbacks of ethanol production from brewer's yeast

There are many microorganisms in nature that can be used to produce fuel ethanol, such as brewer's yeast, which has high sugar and ethanol tolerance and high ethanol fermentation capacity[14]. It has high sugar and ethanol tolerance and high ethanol fermentation ability, and the production of second-generation fuel ethanol by brewer's yeast has high yield rate close to the theoretical value and has excellent production performance such as tolerance to ethanol and other inhibitors. It has become the most widely studied microorganism for second-generation fuel ethanol.

Saccharomyces cerevisiae in the production of ethanol, even when glucose is sufficient, usually first undergoes the production of pyruvate in the glycolytic pathway, followed by the decarboxylation of pyruvate to produce acetaldehyde, and finally the

reduction of ethanol to ethanol by NADH under the action of the inhibitory enzyme dehydrogenase, rather than the complete tricarboxylic acid cycle of respiration, a phenomenon known as the Crabtree effect[15].

Despite the relatively weak pentose metabolism of *Saccharomyces cerevisiae*, its strong ethanol synthesis pathway during fermentation offers the possibility of enhancing its ability process lignocellulose through metabolic engineering as it facilitates the utilization of cellulosic resources.

In conclusion, *Saccharomyces cerevisiae* is still the microorganism of choice for the production of second-generation fuel ethanol, however, *Saccharomyces cerevisiae* faces two major challenges in practical applications: first, how to effectively utilize pentoses and hexoses (two different sugars); and second, how to enhance its tolerance to inhibitors produced during lignocellulosic pretreatment[16].The solution of these two problems is considered to be the key to achieving efficient conversion of lignocellulose to ethanol production. This paper focuses on solving the latter problem.

1.5 The need to improve acetic acid tolerance

1.5.1 Mode of action of acetic acid hazards

During the fermentation process of brewer's yeast, it is often subjected to various stresses, such as osmotic pressure, high temperature, and acidity[17]. Especially organic acids, such as acetic acid, play an important role in regulating the second-generation ethanol fermentation in *Saccharomyces cerevisiae*. During fermentation, yeast produces small molecule inhibitors such as acetic acid. Excessive amounts of acetic acid can adversely affect the metabolism, signaling, and cell growth of *Saccharomyces cerevisiae*, and even inhibit ethanol production[18]. Studies have already shown that acetic acid enters yeast cells mainly by diffusion and then releases large amounts of hydrogen ions (H+) inside the cell, leading to cytoplasmic acidification and affecting metabolic activity[19]. In order to maintain a normal cellular growth environment, *Saccharomyces cerevisiae* will expel this H+ from the cell via the H+-ATPase, but this will consume a large amount of ATP and lead to a lack of

energy supply, or even lead to stagnation of cell growth or cell death[20][22]. The acidic stress may also lead to the stagnation of cell growth or cell death. In addition, acidic stress may also trigger oxidative stress, resulting in DNA and organelle damage. In summary, when facing acidic stress, *Saccharomyces cerevisiae* needs to cope with the changes in the intra- and extracellular environment and take measures to maintain normal metabolism and growth[23][24].

In industry, we usually treat lignocellulose with acids to improve its hydrolysis capacity as well as monosaccharide release. However, during pretreatment, the release of monosaccharides is accompanied by the production of toxic compounds such as acetic acid, which has a significant inhibitory effect on cell growth, metabolism, and ethanol production. Hydrolysis of lignocellulose produces weak acid inhibitors, mainly acetyl propionic acid, formic acid and acetic acid, with acetic acid being the most abundant. In addition, acetic acid is also a by-product of the fermentation process. Therefore, more studies on acetic acid have been conducted.

These inhibitors mainly induce the production of reactive oxygen species in the cell and inhibit cell growth thereby reducing the yield of yeast ethanol fermentation. When yeast cells utilize acetic acid as the sole carbon source, the anion enters the cell via the Jen1p or Ady2p monocarboxylate transporter proteins and is converted to acetyl-CoA for entry into the metabolic cycle of TCA or glyoxylate. Glucose impedes acetate transport and metabolism. At low pH (pKa = 4.76), in the presence of glucose, acetic acid enters the cell through the Fps1p water-glycerol protein channel, where the higher intracellular pH environment leads to its dissociation into acetate anion and hydrogen ion[25].As intracellular hydrogen ions increase, the proton pump Pma1p is specifically activated to transfer excess intracellular hydrogen ions to the extracellular compartment via the membrane ATPase. Acetic acid may activate Hog1p and the transcription factor Haa1p to adapt cells to different levels of acetic acid. On the other hand, lethal concentrations of acetic acid induce ROS accumulation, accompanied by an increase in cytochrome c release, impaired intracellular mitochondrial energy metabolism, a dramatic increase in cysteine asparaginase activity, and an imbalance in

intracellular homeostasis, which results in the structural denaturation of chromatin and nuclear DNA to the point of programmed cell death (AA-PCD)[26][28].Acetic acid leads to a decrease in metabolic enzyme activities in *Saccharomyces cerevisiae*, and enhancing the tolerance of fermenting microorganisms to acetic acid is a key issue in ethanol production from lignocellulose[29].

1.5.2 Means of improving acetic acid tolerance of *Saccharomyces cerevisiae*

Improving acetic acid tolerance of *Saccharomyces cerevisiae* is an important issue in brewing industry. There are several ways to improve acetic acid tolerance of *Saccharomyces cerevisiae*:

1) Selection of highly adaptable yeast strains: By screening and cultivating highly adaptable yeast strains, more acetic acid-resistant yeast strains can be obtained.

2) Genetic engineering: The use of genetic engineering technology can improve the tolerance of *Saccharomyces cerevisiae* to acetic acid by regulating the expression of related genes. For example, the rate of acetic acid degradation can be increased by increasing the amount of gene expression in the acetic acid metabolic pathway.

3) Gradual adaptation culture: By gradually increasing the acetic acid concentration in the medium, yeast can be induced to gradually adapt to the high acetic acid environment, thereby improving its acetic acid tolerance.

4) Optimization of fermentation conditions: Reasonable control of fermentation temperature, pH value, oxygen supply and other factors can improve the tolerance of *Saccharomyces cerevisiae* to acetic acid.

5) Global transcriptional control: Multiple stress response factors Msn2p/Msn4p in yeast cells are involved in the regulation of high temperature, high acetic acid, and oxidative stress. Msn2p/Msn4p enhances acetic acid tolerance of yeast cells by regulating genes related to stress response, such as CTT1 and GPXI[30].

1.6 Function of *TSA2* gene

TSA2(Transcriptional Silencing Factor 2) is a transcription factor, which has the following functions in eukaryotes: (1) gene transcription regulation. The protein encoded by the *TSA2* gene plays an important role in the structure of chromatin by altering the tightness of chromatin, affecting gene accessibility and can create a state of chromatin silencing, inhibiting gene transcriptional activity, or opening certain gene regions to promote transcription. (2) DNA repair. The protein encoded by the *TSA2* gene is involved in the DNA repair process of cells. DNA repair is an important mechanism inside the cell to repair damage on DNA molecules and maintain the integrity of the genome. The protein encoded by the *TSA2* gene may interact with other proteins to participate in the DNA repair process. (3) Anti-oxidative stress. *TSA2* gene can effectively remove oxygen free radicals in the cell body, thereby reducing oxidative damage. When cells are subjected to oxidative stress, the expression of *TSA2* protein is increased to help cells maintain intracellular oxidative balance, which is the antioxidant function of the *TSA2* gene. (4) Cell cycle regulation. Researchers generally focus on studying gene expression patterns at different stages of the cell cycle and their effects on cell regulation[31]. *TSA2* gene can affect cell proliferation and differentiation by regulating the expression or activity of key cell cycle regulators. At different stages of the cell cycle, the expression level of *TSA2* gene can be dynamically regulated to meet the needs of cell growth and division. Further study of the mechanism of action of *TSA2* gene in cell regulation will help to better understand the control network of cell proliferation and differentiation and create new targets and strategies for the treatment of related diseases. Stress-induced cytoplasmic thioredoxin peroxidase can synergistically remove reactive oxygen species with *TSA1p*, using thioredoxin as a hydrogen donor for nitrogen and sulfur species.

1.7 Experimental purpose

Under normal conditions, acetic acid can inhibit the growth and fermentation of yeast. Taking this as the starting point, a histone point mutant with significantly

improved xyloses utilization capacity and ethanol yield was obtained in the laboratory. Through experiments, it was found that histone point mutation significantly improved the acetic acid tolerance of the strain. In order to further explore the regulatory mechanism, transcriptome sequencing was performed on this strain. It was found that the expression level of *TSA2* gene was significantly increased in the histone mutant whose xylose utilization capacity and ethanol yield were significantly increased. To verify the effect of *TSA2* gene on acetic acid tolerance of yeast, we carried out experiments related to this subject. Through overexpression of *TSA2* gene in xylose-utilizing strains and detection of acetic acid tolerance, it was found that overexpression of *TSA2* gene could significantly improve the acetic acid tolerance of strains [6], and this experiment provided an effective element for the development of second-generation ethanol fermentation strains with high acetic acid tolerance.

Conclusions to chapter 1

After an in-depth discussion of the second-generation fuel ethanol, we come to the following conclusions, First, as a renewable and environmentally friendly energy substitute, second-generation fuel ethanol has broad development prospects and is of great significance for reducing dependence on fossil fuels, reducing greenhouse gas emissions, and promoting sustainable development. The use of lignocellulose to produce second-generation fuel ethanol can not only effectively use non-food crop resources, improve the added value of agricultural waste, but also provide a new development direction for the combination of agriculture and energy industry. Second, lignocellulose is an ideal raw material to produce second-generation fuel ethanol, but its complex structure and difficult degradation bring challenges to the production process. Therefore, the development of efficient pretreatment technology becomes the key to solve this problem. Future research should focus on exploring new pretreatment methods and optimizing existing technologies to improve the conversion and production efficiency of lignocellulose. Third, *Saccharomyces cerevisiae* is the main fermentation strain to produce second-generation fuel ethanol, and its acetic acid

tolerance is an important factor affecting the production of ethanol. Improving acetic acid tolerance of *Saccharomyces cerevisiae* will help to increase ethanol yield and reduce production cost. Future studies could explore genetically engineering *Saccharomyces cerevisiae* to improve its acetic acid tolerance and optimize fermentation conditions to further increase ethanol production. Fourth, *TSA2* gene may play an important role in improving acetic acid tolerance of strains, which provides a new direction for follow-up research. By further studying the function and regulatory mechanism of *TSA2* gene, we can further understand the mechanism of acetic acid tolerance of *Saccharomyces cerevisiae* and provide theoretical support for improving acetic acid tolerance of strains through genetic engineering.

In summary, the development of second-generation fuel ethanol needs to consider many factors such as raw material selection, pretreatment technology, fermentation strains and genetic engineering. Through continuous research and technological innovation, we can optimize the production process of second-generation fuel ethanol, increase production, and reduce costs, and contribute to promoting the development and application of renewable energy technologies.

CHAPTER 2

OBJECT, PURPOSE, AND METHODS OF THE STUDY

The object of the study is *Saccharomyces cerevisiae* strains with histone point mutants with significantly improved xylose-utilizing capacity and ethanol yield, especially the effect of TSA2 gene on acetic acid tolerance. The subject of the study is Acetic acid tolerance of *Saccharomyces cerevisiae*.

2.1 Experimental apparatus

Ultra-clean bench, temperature-controlled shaker, thermostatic water bath, microwave oven, miniature palm centrifuge, vortex mixer, bench-top high-speed centrifuge, ice machine, gene amplifier, three-well thermostatic water bath, multi-purpose electrophoresis instrument, biological safety cabinet, biological spectrophotometer, gel imager.

2.2 Experimental reagents

Salmonid DNA, D(+)-glucose, ammonium sulfate, anhydrous ethanol, agar powder, polyethylene glycol, DMSO，dNTP Mix, Phanta Max Super-Fidelity DNA Polymerase, DL15000, DNA Marker, Loading buffer, 2×Rapid Taq Master Mix, etc.

2.3 Strain BSPZ001 Plasmid PJFE3-XIH

Primers are short, single-stranded DNA fragments used to recognize and bind to the target DNA sequence in the polymerase chain reaction (PCR). Primer design is a very critical step in PCR experiments, and designing appropriate primers can ensure the accuracy and efficiency of the PCR reaction. The basic principles of primer design include:

1) Primer lengths are usually between 18-25 base pairs, preferably around 20.
2) The GC content of the primers should be between 40-60% to ensure the stability of the primers.

3) To avoid dimer formation between primers, Tm values between primers should be similar.
4) Primers cannot bind to each other with non-target DNA sequences.
5) The ends of the primers should be specific to ensure that the primers bind only to the target DNA sequence.

***TSA2* Upstream primer :**

TTTAATTACAAAGGATCCATGGTAGCAGAAGTTCAAAAAC

***TSA2* Downstream primer:**

TCAATCCTGCAGGTCGACTTAATTATTGGCATTTTTGAAATACTCC

2.4 Experimental method

2.4.1 DNA amplification

1) Preparation of reaction mixture; 32.5 μL sterile water was first added into the microcentrifuge tube, and water was the basis of the reaction solution. This is followed by a 5 μL loading buffer, which is used to incorporate the sample into the gel more efficiently. 2 μL dNTP Mix, this step is to provide four nucleotide monomers. Then 3 μL $MgSO_4$ is added, which acts as an ion buffer for the reaction. A 1.5 μL primer A and a 1.5 μL primer B were then added to amplify the target DNA. Then 1 μL Phanta Max Super-Fidelity DNA Polymerase,1 μL KOD Plus Neo,2.5 μL DMSO were added in sequence.

2）Setting of reaction procedure: the first line is pre-denaturation. The PCR reaction procedure sets the temperature to 95^{o}C and the time to 3 min until the DNA template is completely desaturated. Next comes denaturation, where the temperature is set to 95 ° C and the time is set to 15 min, a step that can separate the DNA double strands. This is followed by annealing, where the temperature is set to 55 ° C and the time is set to 15 s, the purpose of which is to bind the primer to the target DNA. This is followed by extension, where the temperature is set to 72 ° C and the time is set to 4 min, for the DNA polymerase to synthesize new DNA links at this temperature condition. Then a general cycle (95 ^{o}C,15 s; 55 ^{o}C,15 s; 72 ° C,4

min) about 35 times, this step is intended to amplify the target DNA. Finally, the extension is carried out, and the temperature is set to 72 ° C and the time is set to 5 min until all the DNA strands that have not completed the extension are fully extended. Then the reaction is stored at 4 ° C.

2.4.2 DNA purification

1) Preparation, weighing and dissolution of agarose gel: First, the weight of the empty centrifuge tube was weighed, and the target DNA band after agarose gel electrophoresis was cut off and weighed in the Ep tube. Combined with the weight of the gel, the amount of Binding Buffer required was calculated. The step was to make the concentration of Binding Buffer reach 0.1 mg /μL. Then the Ep tube was heated in a 60 ° C water bath and shaken every 2 min. All agarose was completely melted and poured into a DNA adsorption column.
2) DNA adsorption and washing: Set the rotation speed of 12000 rpm for centrifugation for 1 min, the liquid in the collection tube was sucked into the adsorption column, repeated once, discarded the waste liquid, and then added 700 μL Wash Buffer to the adsorption column, centrifuged at 12000 rpm for 1 min, discarded the waste liquid, and repeated.
3) Removal of residual waste liquid: After discarding all the liquid, the empty adsorption column was placed in a centrifuge tube and centrifuged at 12,000 rpm for 2 min to discard the waste liquid. Continuing the operation, 700 μL Wash Buffer was added to the adsorption column, centrifuged at 12000 rpm/min for 1 min, discarded the waste solution, and repeated once.
4) DNA purification: The adsorption column was placed on the side of the new Ep tube and dried for 5 min. 45 μL of sterile water was added to the center of the filter membrane of the tube, centrifuged at 12000 rpm for 1 min, and then the liquid in the tube was sucked again, and the purified DNA was obtained by repeated centrifugation.

2.4.3 Agarose gel electrophoresis

1) Preparation, dissolution, and coagulation of agarose gel electrophoresis ; according to the number of DNA molecules to be separated, an appropriate amount of agarose was weighed in a conical flask, and an appropriate amount of 1 × Tae buffer was added for stirring and shaking. The prepared agarose solution was placed in a microwave oven, and the corresponding time and temperature were set for high temperature dissolution. After it was boiled three times without impurities, it was taken out, and then the nucleic acid dye was added to mix well. The comb was fixed on the rubber plate, and the mixed agarose solution was poured along the rubber plate.

2) Preparation of electrophoresis tank and sample addition : When the gel is completely solidified, carefully pull out the comb, put the gel and the gel into the electrophoresis tank, add 1 × Tae buffer, and then mix the appropriate amount of 10 × DNA Loading buffer with the DNA sample to be tested, and add the mixture to the sample hole.

3) Electrophoresis: Connect the power supply, electrophoresis. After that, the agarose gel was placed in a gel imager for observation and analysis.

2.4.4 *E. coli* transformation

1) Preparation of transformation mixture: 10 μL of recombinant product and 100 μL of competent cells were mixed in a micro-centrifugal tube and cooled on ice for 30 min.

2) Heat shock conversion and stop: The mixture of recombinant product and *E.coli* cells was placed in a water bath at 42 °C for a short heat shock conversion, usually 45 s, and then the mixture was immediately cooled in ice for 3 min. This step can effectively protect DNA and improve the conversion efficiency.

3) Culture and screening: The transformation mixture was transferred to the medium plate. 900 μL LB liquid was transferred into the culture medium and evenly smeared using a pipette gun. After that, the screening plate (LB + 100μg / mL Ampicillin) was coated and incubated at 37 °C overnight to promote the growth of

transformed *E.coli* cells.

4) Analysis and verification: A single colony was selected from a medium plate containing appropriate antibiotics for culture and PCR verification to confirm the presence of target DNA fragments in the colony and ensure successful transformation.

2.4.5 *E. coli* plasmid extraction

1) Single colonies were picked for culture: The single colonies containing the above-mentioned transformed *E.coli* were placed on a plate, and the single colonies were coated with 5 mL LB medium and antibiotic medium using a sterile bacterial ring. The temperature was set to 37 ° C, and the rotation speed was 200 rpm / min.

2) Preparation of bacterial liquid and washing bacteria : After removing the culture, the bacterial liquid was transferred to the centrifuge tube, and centrifuged at a speed of 12000 rpm / min for 1 min. At the same time, the supernatant was poured out, and the bacteria were washed with appropriate sterile water to ensure the removal of residues in the culture medium.

3) Cell lysis and DNA release: 250μL Solution I was added to the cell precipitate obtained above, and the cells were suspended by shaking. Then 250 μL Solution II was added for dissolution, and the solution was shaken evenly up and down for about 10 times until the solution was clear and transparent. Then 350 μL Solution III was added for precipitation, and the above operation was repeated to form flocculent precipitation, which was centrifuged for 5 min. The speed was also set to 12000 rpm / min.

4) The supernatant obtained by the above steps was transferred to a collection tube with an adsorption plasmid and centrifuged at a speed of 12000 rpm / min for 1 min again to discard the supernatant in the collection tube.

5) Cleaning plasmid: the same operation, then add 500 μL Buffer HBC to the collection tube, and centrifuge again at a speed of 12000 rpm / min for 1 min to discard the supernatant. Subsequently, 600 μL DNA Wash buffer was added, and the step was repeated once to complete the plasmid cleaning.

6) Use the speed of 12000 rpm / min for idle for 2 min. The lid was opened at room

temperature and dried for 5 min, until the ethanol was completely volatilized. Add 600 μL sterile water to the bottom of the plasmid and place it on a sterile bench for 2 minutes to ensure that the plasmid is not contaminated. Finally, the plasmid concentration was detected, and PCR verification was performed to confirm the purity of the target gene.

2.4.6 Configuration of the medium

1) LB liquid medium needs to be transformed into LB solid medium. The LB liquid medium needs to be mixed with 1 % peptone, 0.5 % yeast powder and 1 % ammonium chloride. After mixing, an appropriate amount of agar is added. The new mixture is heated to agar dissolution, and the obtained solution is poured into a petri dish for cooling and solidification to obtain a colony. YEPD liquid medium consists of 1% yeast paste, 2% glucose and 2% peptone, while YEPD solid medium can be made by adding agar to YEPD liquid medium.
2) The conversion of YEPD liquid medium to YEPD solid medium is the same as the above process, but the mixture of 1 % yeast extract, 2 % peptone and 2 % glucose needs to be added. After the addition is completed, repeat the process in step 1), and the colony growth and reproduction can be carried out
3) The composition of the yeast basic medium includes 0.1% yeast paste, 0.025% ammonium sulfate, 0.025% magnesium sulfate, 2% dextrose and 0.05% dipotassium hydrogen phosphate.
4) The yeast complete medium contains 2.5% sucrose, 0.05% ferrous sulfate, 0.05% magnesium sulfate, 0.5% zinc sulfate, 0.05% dipotassium hydrogen phosphate and 0.3% ammonium sulfate.

2.4.7 Yeast transformation

1) Initial culture: The obtained bacteria were inoculated and then contained in 5ml YPD medium. Under the working environment of 30 ° C, the rotation speed of the shaker was set to 200rpm / min, and the overnight culture was carried out for up to 12h to make the bacteria grow. Then the capacity in the medium was added

to 40ml, and the culture was continued to the optical density under the working environment of 30 ° C, that is, the OD value was adjusted to the level of 0.2, and then cultured at the same speed for about 5h.

2) Cell preparation and treatment: Observe the OD value carefully. Once the OD value reaches more than 0.8, the culture medium is transferred to a 50 mL sterile centrifuge tube, centrifuged at 5000 rpm / min for 5 minutes on an ultra-clean bench, and the supernatant is removed. The obtained solution was added with sterile water, mixed with an oscillator, and centrifuged again at a speed of 5000 rpm / min for 1 min to remove the supernatant. This step was repeated once, and the cells were thoroughly washed.

3) DNA preparation: The colonies were resuspended in an Ep tube and centrifuged at 5000rpm / min for 1 min to remove the supernatant. Salmon sperm DNA is then boiled at 100 ° C for 5 min and quickly placed in an icebox for a cooling time of 30 min to ensure preparation before the next step.

4) If the DNA fragment was transferred: 50 % PEG and sterile water were added to the cells and the salmon sperm DNA and LiAc obtained in the above experimental steps were mixed evenly to obtain the desired DNA fragment.

5) Put the transformed mixture in the incubator at 30 °C for 30 min, then take it out for 5 min of ice bath, and then put the solution in the water bath at 42 °C for 25 min to ensure the transformation of DNA.

6) Centrifuge the centrifuge at a speed of 85000 rpm / min for 1 min, remove the conversion solution, and then resuspended the cells in 400 μL sterile water. The obtained bacteria were coated on YPD plate or SC-Ura + N plate according to the needs, and cultured in static culture and overnight culture respectively, and then the experiment was selected.

2.4.8 Growth curve determination

1) Pre-culture: 5 ml of bacteria were placed in an incubator with a temperature of 30 °C, and the speed of the shaker was set to 200 rpm / min for 12 h culture, so that the bacteria were cultured in a good environment.
2) Activation transfer: The culture medium was transferred to 40 ml of medium, and the shaker was continued at 30 °C for 12 h, until the optical density (OD value) reached 0.2.
3) Strain culture: The activated yeast seeds were transferred to a new medium until the optical density reached 0.2. Continue to culture in a shaker at 30 °C.
4) Growth detection and growth curve drawing: Every 4 h and 6 h, 10 μL of culture medium was taken out from the culture flask with a pipette that met the range and added to the colorimetric cup, and diluted with 900 μL of water. The OD_{600} value was measured to detect the growth of bacteria and record the OD600 value at the corresponding time. According to the culture time t as the abscissa, the absorbance as the coordinate, the growth curve of *Saccharomyces cerevisiae* strain was drawn, and the growth trend was observed and analyzed.

Conclusions to chapter 2

After detailed execution and result analysis of the experimental materials and methods described in Chapter 2, the following conclusions are drawn. The experimental instruments used by us have reached the accuracy and stability required by the experiment, ensuring the accuracy and reliability of the experimental results. The reagents used in the experiment are all from reliable suppliers and stored and used in accordance with standard operating procedures to ensure the smooth conduct of the experiment.

The plasmids, strains and primers selected in the experiment have been verified and are suitable for the purpose and requirements of this experiment. The activity of the strain was good, the copy number and stability of the plasmid met the experimental requirements, the specificity of the primers was high, and the

amplification efficiency was high.

The DNA amplification experiment successfully amplified the target DNA fragment, and the amplified product was clear and specific. The DNA purification experiment effectively removed the impurities in the amplified products and improved the purity and quality of DNA. Agarose gel electrophoresis successfully separated DNA fragments of different sizes and accurately judged the quality and quantity of DNA. The transformation experiment of Escherichia coli successfully introduced foreign DNA into Escherichia coli cells and achieved stable inheritance. Plasmid DNA was successfully extracted from Escherichia coli cells, and the extracted plasmid was of high purity and good quality. The experiment of culture medium configuration ensured that the composition and proportion of culture medium were accurate and provided good conditions for the subsequent yeast transformation and growth curve measurement experiments. The yeast transformation experiments successfully introduced exogenous DNA into yeast cells and realized its expression in yeast. The growth curve test accurately reflected the growth of yeast under different conditions and provided an important basis for the subsequent study of gene expression and metabolism.

To sum up, the experimental materials and methods described in this chapter have been strictly verified and tested to ensure the accuracy and reliability of the experiment. The data and results obtained in the experiment provide a strong support for the follow-up research and provide a reliable reference for the follow-up experiment.

CHAPTER 3

EXPERIMENTAL PART

3.1 Enzyme digestion of PJFE3-XIH plasmid

When reading a plasmid atlas (Fig. 3.1), first, check the position of Ori in the plasmid atlas.

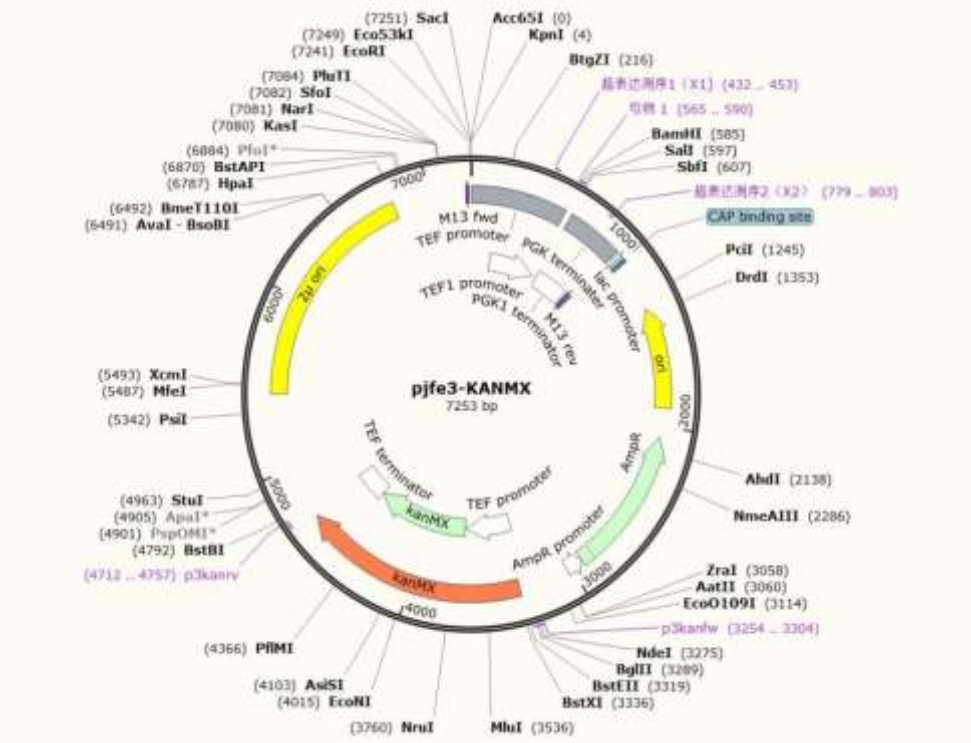

Figure 3.1 – Schematic diagram of pJFE3-XIH plasmid

Ori is the starting point of plasmid replication, and its position determines how the plasmid replicates and how fast it replicates. By knowing the position of Ori, one can understand the replication ability of the plasmid and its stability in the host cell. Secondly, attention needs to be paid to the screening markers in the plasmid map, such as resistance genes. These screening markers can help us to select appropriate screening methods and antibiotics to screen cells for successful transformation. Third, it is necessary to check the plasmid mapping for multiple cloning sites (MCS).MCS is a region in the plasmid with multiple restriction enzyme cutting sites for insertion of exogenous DNA fragments. The presence of MCS allows the plasmid to conveniently

insert exogenous DNA fragments of different sizes and types. Fourth, the size of the exogenous DNA insert fragment also needs to be determined. Usually, plasmids can only accommodate exogenous DNA fragments smaller than 10 Kb. The longer the exogenous DNA fragment is, the more difficult it is to insert, the stability of the plasmid may be reduced, and the transformation efficiency may be affected. The plasmid profile should also be checked for the presence of expression system components such as promoters, ribosome binding sites (RBS), cloning sites and transcription termination signals. These elements are necessary to enable transcription and translation of exogenous genes. With the incorporation of these elements in the plasmid, the plasmid can be used as an expression vector. Finally, the extracted plasmid needs to be cut using an appropriate restriction endonuclease. When constructing gene expression vectors, it is usually necessary to cut the plasmid and the exogenous DNA fragments using appropriate restriction endonucleases to ensure that they can be properly ligated. Selection of the correct restriction enzyme cleavage site is essential to ensure the stability and function of the vector. In this experiment, *SalI* and *BamHI* enzymes were used for enzyme digestion, which could achieve efficient cutting on most DNA molecules.

3.2 Amplification and enzyme digestion of *TSA2* gene fragment

The sequence information of the *TSA2* gene fragment was first known and the *TSA2* gene fragment was amplified from the sample using PCR. Ensure that the primers used in the PCR reaction match the target sequence and optimize the PCR conditions to obtain high specificity and high yield of the amplified product and analyze the PCR amplified product by agarose gel electrophoresis. The success of PCR amplification was confirmed by observing whether bands of DNA of the expected size (e.g., Fig. 3.2) appeared, verifying that such gene fragments were consistent with the expected results, and checking for nonspecific amplification products or contamination.

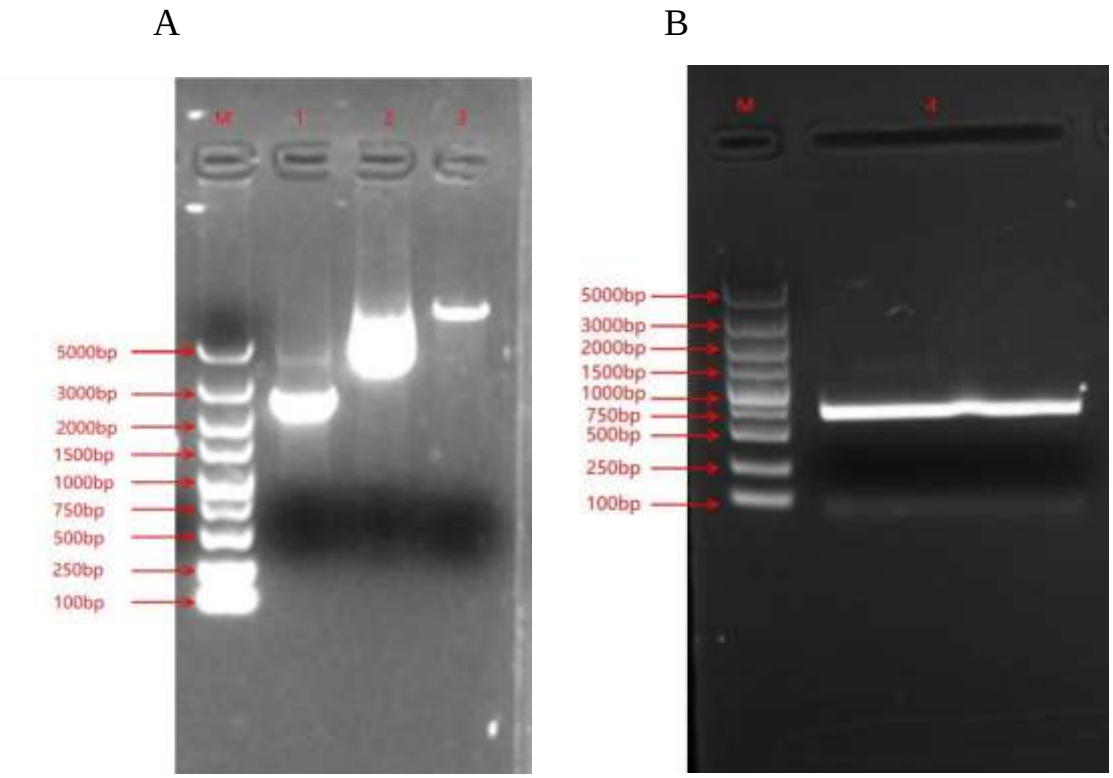

Figure 3.2 – *TSA2* fragment vs. vector map

In the above figure, PCR gelling was performed using DL5000,DNA Marker. In Figure 2A, 2 is the uncut plasmid, which refers to the plasmid DNA that has not been subjected to restriction enzyme digestion in the PCR reaction, and 3 is the enzyme cut plasmid, which refers to the plasmid DNA that has been subjected to enzyme digestion, usually to insert foreign DNA fragments into it. 4 In Figure 2B, the TSA2 fragment is between 500bp and 750bp in size, which is consistent with the expected size of about 591bp.

3.3 Linking and validation of pjfe3-XIH-*TSA2* plasmid

The process of validating a plasmid is usually aimed at confirming that the plasmid was successfully constructed and contains the correct insertion sequence. For the validation of plasmid PJFE3-XIH, the plasmid extracted from the screened positive clone was sequenced to verify the integrity and correctness of the plasmid (Fig. 3.3).

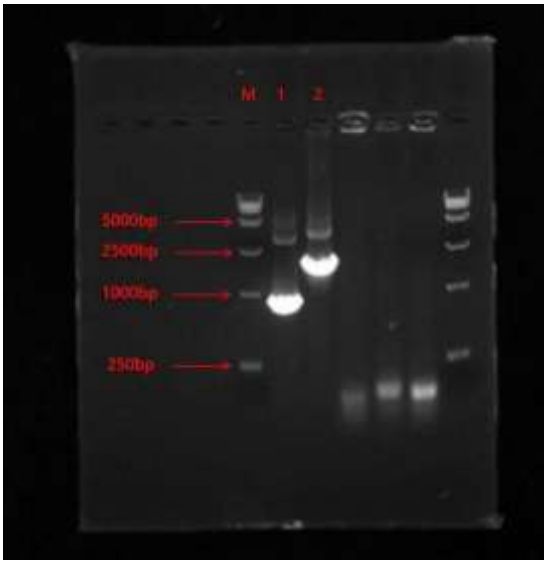

Figure 3 – Linking and validation of pjfe3-XIH-*TSA2* plasmid.

Figure 3 uses DL15000, DNA Marker, and 1 is the plasmid verification of *TSA2*. Since it is the outboard product after the connection, the length is longer than the fragment length, which is also in line with the expected effect.

3.4 Transformation of *Saccharomyces cerevisiae* by plasmid pJFE3-XIH-*TSA2*

The main purpose of this experiment was to construct histone mutant strains capable of improving acetic acid tolerance, using PEG lithium acetate transformation method, the plasmid was transferred into BSPZ001 strain containing histone mutation, which contained Ura screening markers, and positive clones were screened with SC-Ura+Nat medium, and BSPZ001 strain containing pJFE3-XIH plasmid was amplified by streaking the positive clones on SC- Ura+Nat plates were streaked (Fig. 3.4), thus amplifying the positive clone strain.

Figure 3.4 – SC-Ura+Nat medium plate monoclonal

3.5 Determination of growth curve of *Saccharomyces cerevisiae*

The research group first measured the growth status of *TSA2* gene in glucose, the antibiotic g418 used for glucose acetic acid fermentation, 200mg/ml, 80 μL per 400 ml medium, glucose 40% concentration, 380 ml medium, 20 ml, acetic acid concentration 3 g/L.

The *TSA2* gene underwent shake-flask fermentation in a liquid medium containing glucose. The results of the growth curve showed (Fig. 3.5) that the growth of the strain was in the adjustment period in the first 4h. During 4-20h, the growth of the strain was in the logarithmic phase, and the growth rate of *TSA2* gene was not different from that of the control group. The growth of the strain was stable after 20h. The overall growth of *TSA2* gene in glucose medium was worse than that in control group.

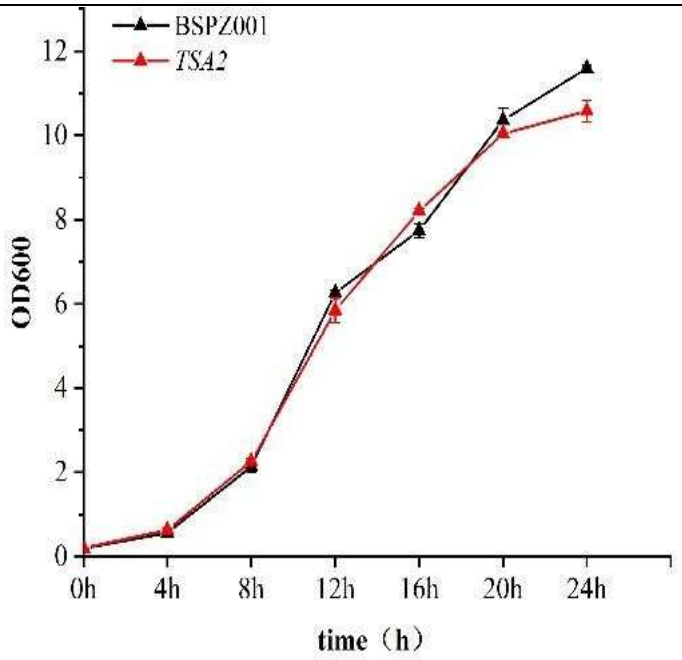

Figure 3.5 – OD_{600} values of BSPZ001 background strain and *TSA2* grown in liquid medium containing glucose.

The *TSA2* gene was then subjected to shake-flask fermentation in a liquid medium containing acetic acid. The results of the growth curve showed (Fig. 3.6) that the growth of the strain was in the adjustment period in the first 12h, and in the logarithmic period from 12-30h, when the growth rate of *TSA2* gene was higher than that of the control group. The overall growth of *TSA2* gene in acetic acid medium was slightly higher than that in control group. By comparing the growth curves of the control strains and *TSA2* gene, *TSA2* gene can improve the growth of yeast in acetic acid, and the effect is very significant.

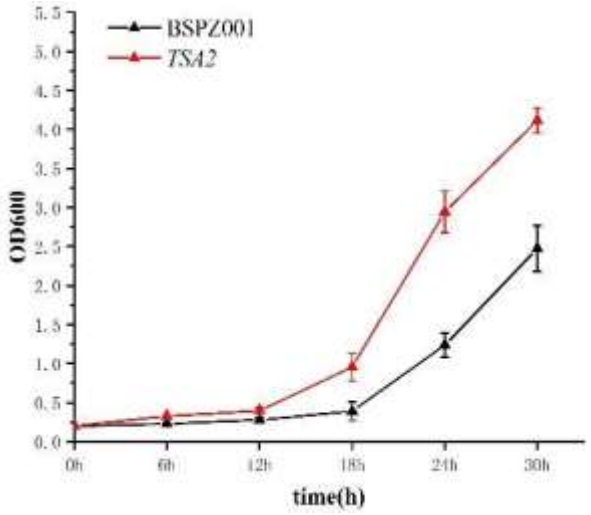

Figure 3.6 – OD_{600} values of BSPZ001 background strain and *TSA2* grown in liquid medium containing acetic acid

Conclusions to chapter 3

In this study, we successfully completed the following key steps and drew the corresponding conclusions. Enzyme digestion of pJFE3-XIH plasmid. The enzyme digestion experiment was successful, and the expected enzyme digestion product was obtained, which provided the basic plasmid material for the subsequent experiment. Amplification and enzyme digestion of *TSA2* gene fragment. The *TSA2* gene fragment was successfully amplified, and the target size DNA fragment was obtained by enzyme digestion, which provided important genetic material for the subsequent plasmid construction. Construction and verification of pJFE3-XIH-*TSA2* plasmid. pJFE3-XIH-*TSA2* plasmid containing *TSA2* gene was constructed successfully, and its correctness was confirmed by verification experiment. This provided a key vector for the subsequent expression of *TSA2* gene in *Saccharomyces cerevisiae*. Transformation of *Saccharomyces cerevisiae* by plasmid pJFE3-XIH-*TSA2*. Plasmid pJFE3-XIH-*TSA2* was successfully transformed into *Saccharomyces cerevisiae*, and positive transformers were obtained through screening. This provided a foundation for further study on the function of *TSA2* gene in *Saccharomyces cerevisiae*. Measurement of growth curve of *Saccharomyces cerevisiae*. The growth curve of *Saccharomyces*

cerevisiae transformed with pJFE3-XIH-*TSA2* plasmid was determined successfully, which provided important data for analyzing the effect of *TSA2* gene on the growth of *Saccharomyces cerevisiae*.

In summary, the experimental operation in Chapter 3 successfully completed the key steps from plasmid construction to *Saccharomyces cerevisiae* transformation and obtained the expected experimental results. These results provide important experimental basis for further study on the function and application of *TSA2* gene in *Saccharomyces cerevisiae*.

CONCLUSIONS

1. During the utilization of glucose in lignocellulose by wild type *Saccharomyces cerevisiae*, acetic acid inhibitor is produced, which will affect the production of ethanol. In this study, histone mutants with significantly improved xylose-utilization ability and ethanol yield were transferred to the genome of strain BSPZ001, and the TSA2 gene in the genome of strain BSPZ001 was overexpressed, and the plasmid pjfe3-XIH-TSA2 was transformed into *Saccharomyces cerevisiae*.

2. TSA2 gene was fermented with glucose and acetic acid in shake bottle, ethanol production was recorded, and the growth curve was measured. The results showed that the overall growth of TSA2 gene in glucose medium was worse than that in control group. The overall growth of TSA2 gene in acetic acid medium was slightly higher than that in control group. It was proved that overexpression of TSA2 gene has acetic acid tolerance, and TSA2 gene can improve the production efficiency of ethanol. The experiment provided an effective element for developing the second-generation ethanol fermentation strains with high acetic acid tolerance.

3. Due to the relatively short time of the study and the complexity of the *TSA2* gene, there are still many problems to be solved. Through this experiment, we found that the gene overexpression is to improve the acetic acid tolerance, and we are going to do related experiments about whether *TSA2* gene overexpression has any effect on other genes, to further explore the effect of *TSA2* gene overexpression on other genes, and to understand the molecular mechanism of the regulation of acetic acid tolerance in a more in-depth way. It may be found that *TSA2* gene overexpression not only affects genes related to acetic acid metabolism, but also other genes of cell survival, metabolism or signaling pathways.

4. This may reveal the important role of *TSA2* in regulating the entire cellular physiological state. It is expected to discover new key regulators or signaling pathways that may play important roles in the regulation of acetic acid tolerance. This will provide new research directions and breakthroughs for future studies. Finally, it will

provide new insights for biotechnological applications in related fields, such as genetic engineering or metabolic engineering, which will improve the efficiency and yield of microbial applications in biofuel production, biodegradation, or biopharmaceutical production.

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