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

QUALIFICATION THESIS

on the topic **Analysis of the effect of FAA2 gene overexpression on xylose metabolism in *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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Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Xiaomeng Huang

1. Thesis topic **Analysis of the effect of FAA2 gene overexpression on xylose metabolism in *Saccharomyces cerevisiae***
scientific supervisor Tetiana Shcherbatiuk, Dr. Sc., Prof.

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

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1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
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6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
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I am familiar with the task:

Student _____Xiaomeng HUANG

Scientific supervisor _____Tetiana SHCHERBATIUK

SUMMARY

Xiaomeng Huang. Analysis of the effect of FAA2 gene overexpression on xylose metabolism in *Saccharomyces cerevisiae*– Manuscript.

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The status quo of the global energy crisis is becoming more and more significant, the price of oil has risen sharply, leading to the economic production costs continue to increase, but also threaten our normal life. Therefore, the development of new energy sources has become an urgent event all over the world, and new energy sources have outstanding advantages such as low environmental pollution, wide range of raw materials or energy sources for production, and the ability to be utilized twice. The first generation of ethanol mainly uses food as raw material not only occupies a large amount of food land, which is not conducive to the rational use of food in the current global crisis, but also increases the dilemma of food crisis. The second generation of ethanol is based on lignocellulose as raw material, lignocellulose is the world's only resource that can be utilized twice on a large scale, and a variety of components can be metabolized by a large number of microorganisms and fermentation. It not only alleviates the problem of excessive food land area, but also eases the global food crisis. Saccharomyces cerevisiae lives in a high sugar, high ethanol environment and has a strong tolerance and high fermentation capacity, so Saccharomyces cerevisiae was chosen as the strain of choice for the production of second generation yeast.. The group has previously obtained mutant yeast with significant enhancement of xylose metabolism, in which the FAA2 gene is overexpressed. The present experiment is to investigate the effect of the FAA2 gene on xylose metabolism, and whether it can enhance the metabolism or inhibit the metabolism of xylose.

Keywords: Saccharomyces cerevisiae, xylose, second generation ethanol, FAA2.

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INTRODUCTION

The task of this study was to perform shake-flask fermentation of sugar and high performance liquid chromatography to determine the growth curve, ethanol production and xylose consumption of the mutant strains. The growth rate of the mutant strain of the control strain in glucose medium and that of the control group in xylose medium were compared to explore the effect of FAA2 gene on the efficiency of xylose conversion to ethanol of the strain.

The relevance of the topic is the widely known first-generation ethanol cannot be produced on a large scale due to its raw material being food crops or non-food starch, which is limited by food security and production scale. Now a new way has emerged, that is, to obtain lignocellulose as a raw material of ethanol at low cost and convenience, which greatly reduces the cost of ethanol production.

The purpose of the study is although the production of ethanol from lignocellulose has many advantages, the xylose in cellulose is difficult to be utilized by yeast, which is also an important factor causing the high production cost of second-generation ethanol. Although it has been reported that yeast engineered to metabolize xylose combined with directed evolution has been constructed, its utilization efficiency is low, and the mechanism of yeast metabolizing xylose to produce ethanol remains to be further analyzed. The production process of cellulosic ethanol includes pretreatment of physicochemical factors to change the complex structure of cellulosic biomass, degradation of cellulase to release monosaccharides (mainly glucose, xylose) and a small amount of oligosaccharides, and then microbial fermentation to complete the conversion of sugar alcohol and recovery of the product ethanol. Therefore, to achieve economically viable cellulosic ethanol production, it is necessary to reduce the cost of raw material collection, pretreatment, cellulase enzymolysis and other sections, and optimize the microorganisms themselves and their fermentation process to efficiently convert sugar alcohols.

Saccharomyces cerevisiae is a traditional ethanol producing strain with high productivity and tolerance. However, when the raw material of saccharomyces

cerevisiae is lignocellulose, it is almost difficult to use the xylose, which is the monosaccharide only second to glucose in lignocellulose. The mechanism of xylose metabolism in saccharomyces cerevisiae still needs further study. **The objectives** of the study probing the effect of the FAA2 gene on xylose metabolism in Saccharomyces cerevisiae.

The object of the study FAA2 gene and Saccharomyces cerevisiae.

The subject of the study effect of gene overexpression on xylose metabolism in brewer's mother wood.

Research methods gene overexpression.

The scientific novelty influence of genes on the fermentation of second-generation ethanol by Saccharomyces cerevisiae yeasts.

The practical significance of the results obtained is exploring the effect of brewer's yeast in metabolizing xylose, producing fermented second-generation ethanol, and thereby increasing fermentation rates.

CHAPTER 1
LITERATURE REVIEW

1.1 The need for second-generation ethanol production from lignocellulosic fuels

1.1.1 Limitations of fossil fuels

Fossil fuels are chemically synthesized from the earth's surface over millions of years, and although a large amount of energy can be released through a number of processes, such as combustion, the earth's resources are ultimately limited. Moreover, during the combustion process, fossil fuels, which are made up of a mixture of hydrocarbons or hydrocarbon derivatives, undergo a series of synthetic decomposition, releasing large quantities of carbon dioxide and numerous pollutants. Excessive carbon dioxide is not only harmful to the environment, but also affects people's health. The greenhouse effect and global warming caused by carbon dioxide have led to global climate anomalies, the melting of glaciers has destroyed the living environment of many species in the Antarctic region, and the sea level has increased so that coastal ecosystems have been tragically affected. Under the multiple pressures of environmental pollution, resource depletion and biodiversity reduction caused by fossil fuels, the development of new energy has become a global trend.

Along with the rapid social and economic development, energy demand is increasing, renewable energy has been widely noticed by virtue of reducing environmental pollution, alleviating the scarcity of resources and promoting the transformation of the energy structure, but the problem of traditional petroleum resources is becoming more and more prominent, and accelerating the substitution of traditional resources by renewable energy resources has become a trend that cannot be delayed. In the face of the rising demand for alternative biofuels, there is an urgent need to develop real biomass resources to avoid the potential risks and pressures associated with the use of food or biomass resources that necessitate drastic adjustments in land use patterns. Indeed, waste lignocellulosic biomass from

agriculture, the food industry, and forestry provides a rich source of fermentable sugars that are renewable and can provide a sufficient source of raw material for microbial production of bioethanol.

1.1.2 Advantages of second-generation ethanol over first-generation ethanol

At present, many countries and regions around the world are committed to researching the preparation and utilization of biofuel ethanol, expecting to fully explore its potential advantages. Ethanol is a high-octane oxygenated compound in the form of a low-carbon alcohol, with multiple advantages such as easy preparation, high oxygen content and renewability. In addition, ethanol can also be used as a combustion aid to promote the full combustion of gasoline, thereby effectively reducing carbon dioxide and incomplete combustion of harmful gases in the exhaust of automobiles. At the same time, ethanol is expected to replace fossil energy as an environmentally friendly and efficient bio-liquid fuel, further improving air quality and mitigating the greenhouse effect. However, since the raw materials of the first generation of ethanol are mainly food crops or non-food starch, there are limitations in food security and scale of production, making it difficult to realize large-scale production, which poses a lot of difficulties for the promotion of ethanol. However, a new solution has emerged today to provide a new breakthrough to the plight of generation one ethanol by using lignocellulose, which is inexpensive and easy to obtain, as the feedstock for ethanol. First, second-generation fuel ethanol uses crop residues as feedstock, avoiding the risk of competing with people for food and with food for land. Secondly, using lignocellulose from crop residues as raw material gives full play to the value of agricultural products and effectively alleviates the environmental pollution and greenhouse effect caused by the burning of waste straw. Lignocellulose is extremely rich in content, and its hydrolysis products can be utilized by a large number of microorganisms. Therefore, the second-generation ethanol using lignocellulose as raw material is of great significance for both ecological environment and economic production.

1.2 Current status of research on second-generation ethanol

Biomass is considered the most predictable raw material for the transition of the global matrix from fossil to sustainable processes, and their energy potential is closely related to the lignocellulosic structure of biomass[1]. Brazil produces large amounts of biomass every year, especially derivatives of sugarcane processing (bagasse and straw), with a considerable potential to contribute to the world's primary energy demand and to the development of biorefineries [2]. Second-generation ethanol processes, which are now online at demonstration and full commercial scale, are mainly based on the fermentation of lignocellulosic biomass hydrolysis products by engineered strains of Saccharomyces cerevisiae.

The development of biofuel ethanol is now regarded as an important project in many countries and regions, with Brazil and the United States ranking firmly among the top two countries in the world in terms of fuel ethanol consumption and annual production, and with China being the third largest producer of fuel ethanol, the country has now made the development of the development of biofuel ethanol a key area of research**Помилка! Джерело посилання не знайдено.**. The outbreak of New Coronavirus has drawn worldwide attention to hygiene as the first line of defense in infection control, the World Health Organization (WHO) advises the public to sanitize their hands on a regular basis and recommends the use of hand sanitizers containing alcohol, ethanol is the most common and popular alcohol that is effective in eliminating a wide range of pathogens and is easy to use and produce[3]. The Government of India announced a brand new program in March 2019 to provide financial assistance totaling about $3.72 billion to sugar mills and about $750 million to molasses ethanol distilleries with the aim of boosting ethanol production. In view of the dependence on food crops and their by-products, the inadequate supply of first generation ethanol and in order to meet the daily needs of a growing population, extensive and intensive research is being carried out on the production of second generation ethanol from lignocellulosic biomass[5] .

1.3 Advantages and disadvantages of using Saccharomyces cerevisiae as a strain for ethanol production

Saccharomyces cerevisiae, as an exemplary model organism for the study of microorganisms, possesses numerous superior characteristics such as extremely high multiplication rate, complete disclosure of gene sequences, excellent fermentation efficacy, and short developmental cycles, as well as ease of cultivation and manipulation. As a result, Saccharomyces cerevisiae is recognized as the strain of choice for ethanol production by fermentation, and it has demonstrated strong tolerance to high sugar concentrations, low pH environments, and high ethanol concentrations during fermentation. By hydrolyzing lignocellulosic biomass, a variety of sugars including glucose, xylose and arabinose are released, which the microorganisms are able to use as feedstock for fermentation and synthesis of various compounds. Although Saccharomyces cerevisiae has excelled in first-generation bioethanol production and is highly adaptable to genetic modification, it is unable to effectively hydrolyze cellulose or hemicellulose.

The main reason for the inability of Saccharomyces cerevisiae to utilize xylose is that it lacks the appropriate xylose metabolism pathway and can only ferment with the help of xylulose-xylulose isomers. If the direct metabolism of xylose by yeast can be enhanced, the efficiency of ethanol fermentation will undoubtedly be greatly improved, thus reducing the cost of industrialized production. Therefore, improving xylose utilization and realizing the co-fermentation of glucose and xylose have become the key strategies to improve the fermentation performance of brewer's yeast. In Saccharomyces cerevisiae, some genes closely related to xylose metabolism do exist, such as the GRE3 gene encoding aldose reductase and the XYL2 gene encoding xylitol dehydrogenase and other protein genes, however, regrettably, the expression levels of these genes are often too low to efficiently maintain the normal growth of Saccharomyces cerevisiae strains in a xylose environment. Xylose is the most abundant pentose sugar in the hemicellulose fraction of lignocellulosic biomass and the second most abundant plant sugar after glucose. Most lignocellulosic biomass consists of 40-60% cellulose and 10-25% lignin [6]. Hemicellulose is rich in natural

plant fibers, a mixture of lignin and cellulose, forming a complex and solid structure that makes it difficult for cellulases to invade and thus hinders cellulose degradation. Therefore, it is usually necessary to break down the solid structure and decompose the polysaccharide components of lignocellulose by pretreating it in order to improve the efficiency of ethanol fermentation.

1.4 Influence of pretreatment of lignocellulosic raw materials

There are various treatments for lignocellulose, such as acid hydrolysis, hydrothermal treatment, alkaline treatment, and biological treatment. Acid hydrolysis is usually carried out using inorganic acids, and dilute sulfuric acid pretreatment has been widely studied and applied to various lignocellulosic biomasses[7]. This method improves the recovery of hemicellulosic sugars and solid cellulose fractions from the pretreatment solution and enhances enzymatic conversion. However, acid pretreatment has some shortcomings, such as high material costs and the possibility of inhibitory by-products after sulfuric acid treatment[8]. Hydrothermal treatment is a method of pretreating lignocellulosic biomass using liquid or vapor phase water. This method is relatively gentle, does not require a catalyst, and does not cause significant corrosion problems. Under high pressure, water molecules penetrate into the interior of the biomass to hydrate the cellulose and remove most of the hemicellulose and a small portion of the lignin [9]. Alkaline treatment, on the other hand, is mainly used for the removal of lignin, thus improving the digestive utilization of cellulose. The mild alkaline pretreatment leads to reduced hemicellulose solubilization and reduced production of inhibitory compounds compared to acid and hydrothermal processes, and it operates at lower temperatures, but its cost becomes a major constraint [8]. Given the high impact of pretreatment on downstream technologies, the environmental characteristics of biological pretreatment are particularly impressive from an overall economic point of view.

In contrast, biological methods require less energy and do not produce inhibitors during the treatment process because they are carried out under mild conditions and there is an abundance of microorganisms in nature that can be used for

biological pretreatment. In lignocellulosic treatment technology, effective pretreatment should meet the criteria of preparation for subsequent hydrolysis, avoiding the formation of by-products that inhibit enzymatic saccharification, economic feasibility, and reduction of environmental pollution and resource waste. However, almost no microorganisms can meet all of the above requirements, so it is necessary to genetically or metabolically modify them so that the target microorganisms can meet the pretreatment requirements as much as possible to improve the fermentation efficiency.

1.5 Xylose metabolic pathways in Saccharomyces cerevisiae

1.5.1 Xylose reductase-xylitol dehydrogenase pathway

Xylose is initially reduced to xylitol by Xysugar Reductase (XR) with NAD(P)H as a coenzyme, and then oxidized to xylulose by Xylitol Dehydrogenase (XDH) with NAD+ as a coenzyme. Xylulose undergoes phosphorylation to xylulose 5-phosphate, which is further metabolized via the pentose-phosphate pathway and the glycolytic pathway to produce ethanol[10]. In the xylose reductase-xylulose ethanol dehydrogenase metabolic pathway, the two cofactors need to be kept in dynamic balance, and excessive increase or decrease of either one will lead to a decrease in the efficiency of ethanol fermentation. Common causes of cofactor imbalance include disruption of the ZWF1 gene encoding glucose 6-phosphate dehydrogenase, overproduction of cytoplasmic transhydrogenase, and catalytic conversion of NADP and NADPH to NADH and NADPP[11].

1.5.2 Xylose isomerase pathway

The xylose isomerase pathway differs from the xylose reductase-xylitol dehydrogenase pathway in that the conversion of xylose to xylulose does not require coenzymes and cofactors but is accomplished directly with the help of Xylose Isomerase (XI) [12], and this product is then catalyzed by xylulose kinase to produce xylulose 5-phosphate [13], which then enters the pentose phosphate pathway (PPP). It is worth noting that the XI pathway does not require the participation of cofactors,

which is a significant feature that effectively circumvents the problem of reduced fermentation efficiency caused by the imbalance of cofactors during the reaction process, and thus greatly improves the fermentation efficiency. In addition to this, xylitol has been shown to be an inhibitor of XI, so removing the GRE3 gene in XI strains would help prevent the production of this compound, thus positively affecting xylose fermentation [14]. However, according to Maurizio Bettiga, an Italian biologist, although the xylose reductase-xylosyl ethanol dehydrogenase pathway is superior in terms of pentose uptake and total ethanol production compared to the xylose isomerase pathway, each pathway has its own unique characteristics, and the selection of the appropriate metabolic pathway should be based on the actual needs [14].

1.6 Effects of other factors on xylose metabolism

Xylose metabolism by recombinant Saccharomyces cerevisiae from the fungal pathway carrying xylose reductase and xylitol dehydrogenase requires NADH and NADPH and is prone to cofactor imbalances during xylose growth. Overexpression of NADH kinase in the cytoplasm redirects carbon flow from carbon dioxide to ethanol during aerobic growth of glucose and to ethanol and acetate during anaerobic growth of glucose. However, cytoplasmic NADH kinase has the opposite role during anaerobic metabolism of xylose consumption by directing carbon from ethanol to xylitol. In contrast, overexpression of NADH kinase in mitochondria does not affect physiology to any great extent[15]. Overall, NADH kinase can provide an important source of NADPH in yeast, which plays an important role in manipulating the dynamic balance between the two coenzymes and thus indirectly controlling xylose metabolism.

Gene overexpression is to transform the target gene into the corresponding plasmid or viral vector, using the regulatory elements constructed on the vector backbone, so that the gene can realize a large number of transcription and translation under human-controlled conditions, thus realizing the overexpression of the target gene, and gene overexpression can make regulatory genes or protein genes over-

replication and thus enhance the expression of the gene. Therefore, genes controlling xylose metabolism can be overexpressed or inhibited from replication, thus enhancing or inhibiting the role of xylose metabolism genes. According to relevant data, overexpression of XYL1 and XYL2 genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) was associated with increased xylose utilization[16], which may be synergistically regulated by the four key metabolic genes (i.e., XYL1, XYL2, XKS1, and TAL1), and the changes in the expression of PPPs improved the glucose growth of the yeast during the fermentation process, and it was inferred that the that the strain is designed to increase ethanol yield and productivity by rapidly consuming glucose to alleviate glucose inhibition and then effectively consuming xylose[17]. In order to improve the defect of brewer's yeast that cannot metabolize xylose, the group has previously obtained yeast mutants with improved xylose metabolism through research, and now it is also possible to transgenic the corresponding overexpressed target genes to yeast for overexpression. By transcriptome sequencing, one of the FAA2 genes has been overexpressed, and this thesis is also to investigate whether this gene can enhance the metabolism of xylose in yeast.

1.7 Mechanism of histones in xylose metabolism in Saccharomyces cerevisiae

1.7.1 Effect of Histones on Epigenetic Phenomena

In addition to the effects of gene overexpression on xylose metabolism, histones are also able to participate in and regulate xylose metabolism processes. The effect of histones on epigenetic inheritance is mainly manifested in irreversible changes that stabilize heredity without the need to change base sequences and species. Common epigenetic aspects are DNA methylation, histone modification, chromatin remodeling and non-coding RNA regulation. Nucleosomes are the basic structural units of chromosomes, which form chromosomes in tandem and play an essential role in the regulation of gene expression. Each nucleosome consists of a histone octamer (containing four proteins, H2A, H2B, H3, and H4), a linker protein

(H1), and a segment of linker DNA. The core histone is a spherical three-dimensional structure consisting of two H2A and H2B proteins and one H3 and H4, known as the histone octamer, which tightly wraps the DNA strand around it, allowing for precise control of DNA replication and transcription. The N-terminal tails of histones undergo a variety of chemical modifications that provide binding targets for effectors such as histone methyltransferases and acetyltransferases[18]. Depending on the type and location of the modifications, they may have different effects on chromatin structure and gene transcription, which in turn can have profound effects on xylose metabolism and even other biological traits[19]. Common types of histone modifications include methylation, histone acetylation, histone phosphorylation, histone crotonylation, and histone β-hydroxybutyrylation[20].

1.7.2 The effect of histones on gene expression

Chromosomes are composed of histone nucleosomes connected in tandem, so the alteration of histone proteins significantly affects the chromosome structure, which indirectly modulates gene expression through the chromosomes, including replication, transcription, and damage repair. Histone methylation modification is based on the methylation of the N atom at the end of lysine (Lys, K) or arginine (Arg, R) residues, and it is one of the most intensively researched and widely used forms of histone modification at present[21], and different degrees of methylation modification have made the modification of histone methylation and its modulation of gene expression more complex. It has been demonstrated that histone methylation modification plays a key role in transcriptional regulation of genes, maintenance of genome stability, and epigenetic modification, and that there is a close correlation between methylation modification of specific histone lysine or arginine residues and gene activation or repression **Помилка! Джерело посилання не знайдено.**.

Histone acetylation mainly occurs in the promoter region and 5' end of transcribed genes, and according to related studies, it is revealed that histone acetylation is closely related to the transcription process, suggesting that there is a causal relationship between the two. Histone acetylation can act as both a trigger and

a product of transcription; in addition, it has been further pointed out that histone acetylation is actually the result of RNA polymerase II (RNAPII) promoting the recruitment and activity of histone acetyltransferases [22]. In summary, histone acetylation provides an important pathway for regulating and maintaining chromatin structure to facilitate or repress transcription processes. Histone ubiquitination modifications, on the other hand, are mainly used to repair DNA damage, especially one of the most severe damages, DNA double-strand breaks, as the main mode of repair. There are two main categories of double-strand break repair: homologous recombination repair and non-homologous end linkage[23]. According to relevant research results, it is understood that both repair modes are closely linked to histone ubiquitination, which manipulates chromosome structure by regulating chromosome polymerization and dissociation, and then finely regulates gene expression[24].

In order to improve the defects of brewer's yeast that cannot metabolize xylose, this thesis, through the transcriptome sequencing of the yeast mutant with improved xylose metabolism obtained from the research group, overexpressed the FAA2 gene in the yeast BSP2001-Pjfe3-XIH, so as to investigate whether this gene can enhance the metabolism of xylose in yeast, thus further improving the productivity of second-generation fuel ethanol.

Conclusions to chapter 1

1. The problem of traditional oil resources has become increasingly prominent. The development of renewable energy can reduce environmental pollution, alleviate resource scarcity and promote the transformation of energy structure. Therefore, the replacement of traditional resources by renewable energy has become an urgent trend.
2. The second generation ethanol from lignocellulose is of great significance. The second-generation fuel ethanol uses crop residue as raw material, avoiding the risk of competing with people for food and food for land. Secondly, lignocellulose in crop residue is used as raw material, which gives full play to the value of agricultural products and effectively alleviates

the environmental pollution and greenhouse effect caused by the burning of waste straw. The content of lignocellulose is very rich, and its hydrolyzed products can be used by a large number of microorganisms.

3. The main reason why saccharomyces cerevisiae cannot use xylose directly is the lack of corresponding xylose metabolic pathways. If the ability of yeast to metabolize xylose can be improved, the efficiency of ethanol fermentation will undoubtedly be greatly improved.
4. There are xylose reductase-xylitol dehydrogenase pathway and xylose isomerase pathway for xylose metabolism, both of which have their advantages and disadvantages.
5. Histone modifications also affect yeast's ability to metabolize xylose through mutation.

CHAPTER 2
EXPERIMENTAL PART

2.1 Laboratory Instruments

Bio-Rad PCR instrument, vortex mixer, drug balance, refrigerator, agarose gel electrophoresis, thermostatic mixer, microwave oven, electrically heated thermostatic incubator, ultra-clean workbench, electronic balance, precision balance, bio-spectrophotometer, acidimeter, gas-bath thermostatic shaker shaking bed, microscope, autoclave, blue light cutter, electrophoresis bath, gel imager, thermostatic shaker, multifunctional intelligent Triple stack, concentration detector, ultra-low temperature refrigerator, water bath, induction cooker, autoclave, liquid chromatograph, bench-top high-speed centrifuge, micro-palm centrifuge, biological safety cabinets, automatic snowflake ice machine, three-well constant temperature water bath

2.2 Reagents

dNTPs, MgSO4, KOD Plus Neo Buf, KOD Plus Neo, Dimethyl Sulfoxide Dimethyl Sulfoxide (DMSO), marker, looding buffer, XP2 Binding Buffer, 10×Buffer, ddH_2O, SPw Buffer. BAMH I, SalI, BAMHI, Pjfe-Kan, 2×MultiF Seamless, Agarose, TAE Buffer, LiAc Lithium Acetate, PEG, Salmon Extract, Tryptone

2.3 Experimental Methods

2.3.1 Extraction of target gene

1) Add 200 μl of cell extract to the EP tube and pick a small amount of the desired strain colony with the tip of a gun and dissolve it in the EP tube.

2) Add 0.4 g of acid-washed filter beads, add 200 μl of DNA extract 25:24:1 and mix and shake for 1 min.

3) Centrifuge at 12000 rpm for 10 min, then let it rest and wait for stratification, and remove the supernatant.

4) Add 1 ml of anhydrous ethanol to a new EP tube and mix well, then let stand for 10 min.

5) Centrifuge at 1200 rpm for 10 min, then discard the supernatant, dry, and add 35 μl of external ddH_2O.

2.3.2 Amplification of target gene

1) Add 2 μl dNTPs, 3 μl MgSO4, 5 μl KOD Plus Neo Buf, 1 μl KOD Plis Neo, 1.5 μl each of upstream and downstream primers, 2.5 μl Dimethyl Sulfoxide (DMSO), 1 μl DNA template and 32.5 μl ddH_2O.

2) Perform PCR amplification, pre-denaturation at 94℃ for 5 min; denaturation at 94℃ for 15 min; annealing at 55℃ for 15 s; extension at 72℃ for 15 s; and extension at 72℃ for 10 min.15 s; extension 72℃, 4 min; cycle 35 times.

2.3.3 Electrophoresis verification

1) Gel preparation: Add 0.4 g of Agarose, 50 ml of TAE buffer, put it into the microwave oven and heat it to boiling, if it is still not homogeneous, then continue to heat it until it is homogeneous, and then add 5 μl of nucleic acid dye, shake it well, then pour it into the cartridge after it cools down a little bit, and then wait for it to solidify into a gel in 13 minutes. When picking up the gel, the comb should be picked up slowly, otherwise the gel is easy to crack. When the gel is put into the electrophoresis tank, the bottom of the gel and the residue on this side should be cleaned up, otherwise it will affect the result of gel running.

2) Sample addition: add marker to one well for reference, and add reagent to each well after mixing withlooding buffer. The tip of the gun can not be inserted too deep when adding samples, otherwise it will damage the gel: it can not be not extended into the gel, otherwise the reagent can not be added to the gel holes.

3) Run the gel: connect the positive and negative electrophoresis tanks correctly, set the voltage and time, and run the gel.

4) Observation: put the finished gel into the blue light cutter or gel imager for observation, compare the distance between the fragment and the marker run to verify whether the target gene is correct.

2.3.4 Recovery of target gene

1) Put the verified gel on the blue light cutter, and use the blade to accurately cut off the fragment where the target gene is located.

2) Weigh the empty EP tube on an electronic balance, tare it, weigh the EP tube with the fragment again, and add the solvent XP2 Binding Buffer at the ratio of 1:100 according to the weight.

3) Heat the target gene at 60℃ for 10 min until the pellet is completely dissolved.

4) Add into the adsorption column, centrifuge at 12000 rpm for 1 min and discard the supernatant. Repeat this step again until the sample is completely adsorbed into the column, discard the supernatant.

5) Add 600 μl of SPW Buffer, centrifuge at 12000 rpm for 1 min and repeat the procedure again.

6) Transfer the column to a new EP tube and allow to air dry for 2 min.

7) Add 50 μl of ddH_2O to the center of the membrane and leave it at room temperature for 2 min, then centrifuge at 2000 rpm for 1 min to retain the liquid.

8) Measure the concentration: the concentration of the target gene was 38.124 ng/μl.

2.3.5 Construction of gene expression vector

1) Run the plasmid for gel verification, and then perform gel recovery.

2) Add 5 μl of enzyme (Assembly Mix) 2×MultiF Seamless, 2 μl of target gene, 1 μl of vector, and 2 μl of H_2O to the EP tube.

3) Heat the system at 50℃ for 15 min on a shaker with a gas bath thermostat.

2.3.6 E. coli transformation

1) Thaw the cloned receptor cells on ice .

2) Take 10 μl of recombinant product and add it to 100 μl of receptor cells, flick the wall of the tube to mix well, and leave it on ice for 30 min.

3) Heat-excite at 42℃ for 45 sec, then cool on ice for 2~3 min.

4) Add 900 μl of Soc or LB medium and shake the bacteria for 1 h at 37℃.

5) Preheat the corresponding resistant LB solid medium plate at 37℃.

6) Centrifuge the plate at 5000 rpm (2400 xg) for 5 min and discard 900 μl of supernatant, resuspend the bacteria with the remaining medium and spread on the plate with the correct resistance.

7) Invert the incubator at 37℃ for 12~16 h.

8) Configure 8 EP tubes, add 0.2 μl each of upstream and downstream primers, 5 μl of enzyme and 4.6 μl of ddH_2O to each EP tube.

9) Draw 8 grids on the empty plate, select the appropriate E. coli to pick single colonies on the aseptic manipulator, and apply them in the empty plate grids.

10) And transfer the remaining colonies to the EP tube containing the system according to the serial number and resuspend it evenly.

11) The colonies of E. coli were subjected to PCR amplification with an extension time of 41 s. Gel electrophoresis was performed to verify the completion of amplification.

2.3.7 Yeast transformation

1) Take 5 ml of bacteria in YDD medium, adjust to OD at 0.8~1.7.

2) Transfer the bacteria in the bottle to EP tube, centrifuge at 5000rpm for 1min, discard the supernatant.

3) Add ddH_2O, centrifuge at 5000 rpm for 1 min, discard the supernatant.

4) Add 0.1 mM LiAc lithium acetate, centrifuge at 5000rpm for 1min, discard the supernatant.

5) Add 240 μl PEG, 36 μl 1Mm LiAc lithium acetate, 53 μl H_2O, 2 μl plasmid, and then resuspend.

6) Add 35 μl frozen salmon sperm DNA, heat in boiling water for 5 min, then let stand on ice for up to 30 min, and mix in a shaker.

7) Heat 30℃ for 30 min and 42℃ for 25 min sequentially, centrifuge at 5000 rpm for 1min and resuspend.

8) Heat 30℃ for 2 h, centrifuge at 5000 rpm for 1 min, discard the supernatant, add 400 μl H_2O and resuspend.

9) Take the appropriate amount of bacterial solution for coating the plate, the amount of bacteria less coated 60 μl, the amount of more coated 50 μl, inverted in 30 ℃ incubator culture.

2.3.8 5-Foa plate screening for strains that lost ura

1) Take 5 ml of yeast liquid in YPD medium, then put it in 30℃ shaker for 12 h.

2) Add ddH_2O, centrifuge at 5000 rpm for 1 min, discard the supernatant, and repeat this step twice.

3) Add 400 μl ddH_2O, resuspend, take 100-200 μl and apply it on the plate of 5-FOA.

4) Invert the plate and place it in 30℃ incubator for incubation, and sieve out URA3 colonies.

2.3.9 Determination of growth curve

1) Take 5 μl of yeast liquid in culture medium and put it in 30℃ shaker for 12 h.

2) Transfer the activated yeast to 40 ml culture flask, adjust the OD to 0.2, and incubate at 30℃ in shaker for 12 h.

3) Transfer the yeast culture to 35 ml fermentation medium, adjust the initial OD to 0.2.

4) Cultivate the yeast culture in shaking bed at 30℃ for 12 h, take 100 μl samples every 12 h, measure the OD, and centrifuge at 12000 rpm for 6 min, and make a graph.

2.3.10 Determination of xylose utilization by chromatography

1) Configure the mobile phase and ultrasonic treatment for 30 min to eliminate air bubbles.

2) Filter the supernatant of the sample with filter membrane.

3) Turn on the liquid chromatography and computer power, set the chromatographic parameters and flow rate.

4) Record the data at the end of the analysis and save it to your own file. Turn off the pump, detector, bench and finally the computer in order.

Conclusions to chapter 2

1. The mutant strains were analyzed by shake-flask fermentation and high performance liquid chromatography. The growth curve, ethanol yield and xylose consumption were measured.
2. The growth rate of the mutant strain of the control strain in glucose medium and that of the control group in xylose medium were compared to explore the effect of FAA2 gene on the efficiency of xylose conversion to ethanol of the strain.
3. The effects of FAA2 gene on xylose metabolism were investigated through primer design, Saccharomyces cerevisiae, DNA purification, DNA amplification, agarose gel electrophoresis, yeast chromosome extraction, yeast transformation, gene knockout, Escherichia coli transformation, Escherichia coli plasmid extraction, 5-Foa Diura, growth curve determination, and liquid phase determination of xylose utilization.

CHAPTER 3 CONCLUSIONS

3.1 Amplification of FAA2 Gene Fragment to Target Gene Linkage Plasmid

Because wild Saccharomyces cerevisiae is unable to efficiently utilize xylose for fermentation, this situation results in a failure to maximize the utilization of raw materials. If the uptake of xylose, the sugar that occupies the second largest proportion of lignocellulosic hydrolysis products, can be improved, then the fermentation efficiency will be significantly enhanced. The group previously developed a mutant strain through histone mutagenesis that significantly enhanced the ability of yeast to metabolize xylose, in which the gene fragment FAA2 (Figure 4-1, Figure 4-2) was overexpressed. This thesis is to verify the effect of this gene on the xylose metabolizing ability of yeast by transferring this gene into yeast BSP2001-pJFE3-XIH.

After obtaining the gene, it was verified by agarose gel electrophoresis (Figure 4-3), and after comparing with the corresponding marker to ensure that it was correct, it was then cut off from the gel using a blue light gel cutter and processed for gel recycling, so as to enable subsequent experimental operations to be carried out smoothly.

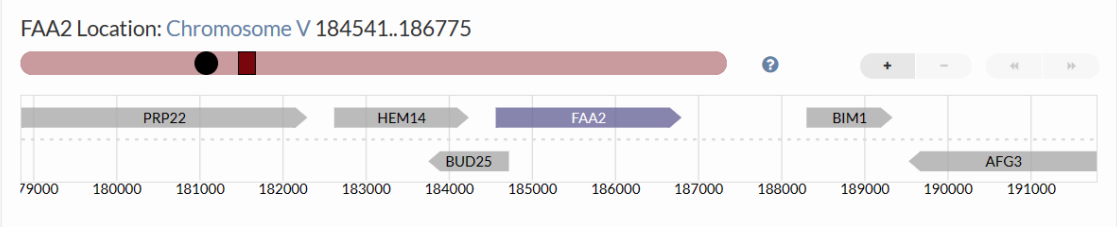

Figure 4-1 Location of the FAA2 gene in the Saccharomyces cerevisiae chromosome

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Figure 4-2 FAA2 DNA sequence

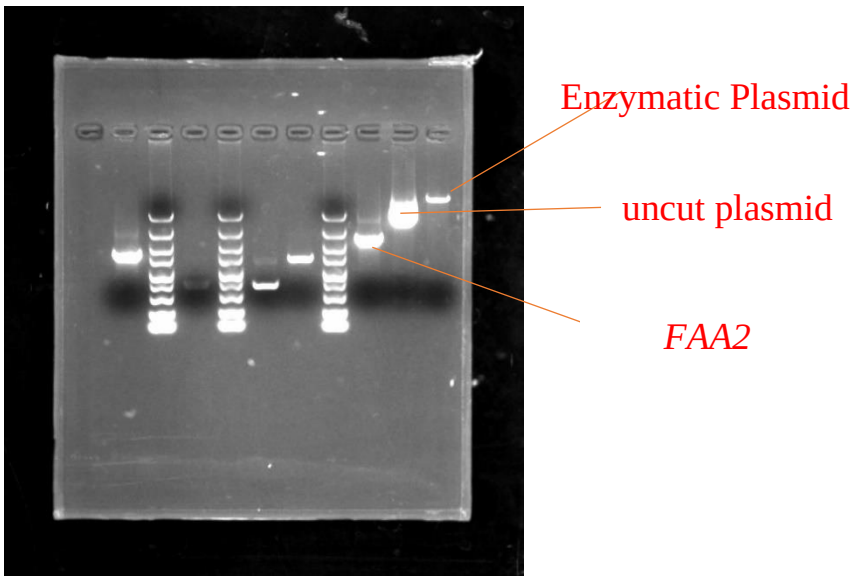

Figure 4-3 Gel electrophoresis validation of FAA2 gene fragments

3.2 Target gene ligation plasmid

According to the gene and pJFE3-K plasmid sequences, we need to carefully design efficient and reliable upstream and downstream primers (Figure 4-4), as well as select appropriate enzymes to complete the ligation reaction (Figure 4-5 Figure 4-6 Figure 4-7) and PCR amplification. When selecting a plasmid, make sure that it has several different types of restriction endonuclease recognition sites to choose from, and that these recognition sites must be located between the promoter and the terminator, so as to ensure that the target gene can be transcriptionally expressed in its entirety. The plasmid also needs to have marker genes for subsequent screening, and the marker genes must not be recognition sites for the restriction endonucleases used in the ligation process.

上游引物：TTTAATTACAAAGGATCCATGGCCGCTCCAGATTATG↵
下游引物 TCAATCCTGCAGGTCGACCTAAAGCTTTTCTGTCTTGACTAGTG↵
↵

Figure 4-4 Upstream and downstream primer sequences

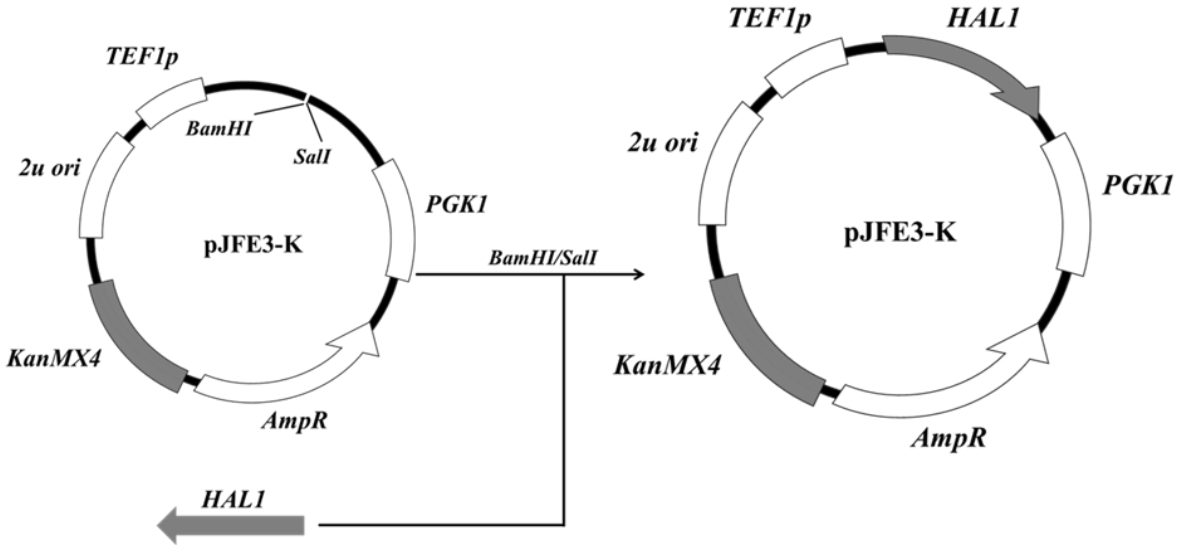

Figure 4-5 Target gene ligated into pJFE3-K plasmid by enzymatic cleavage.

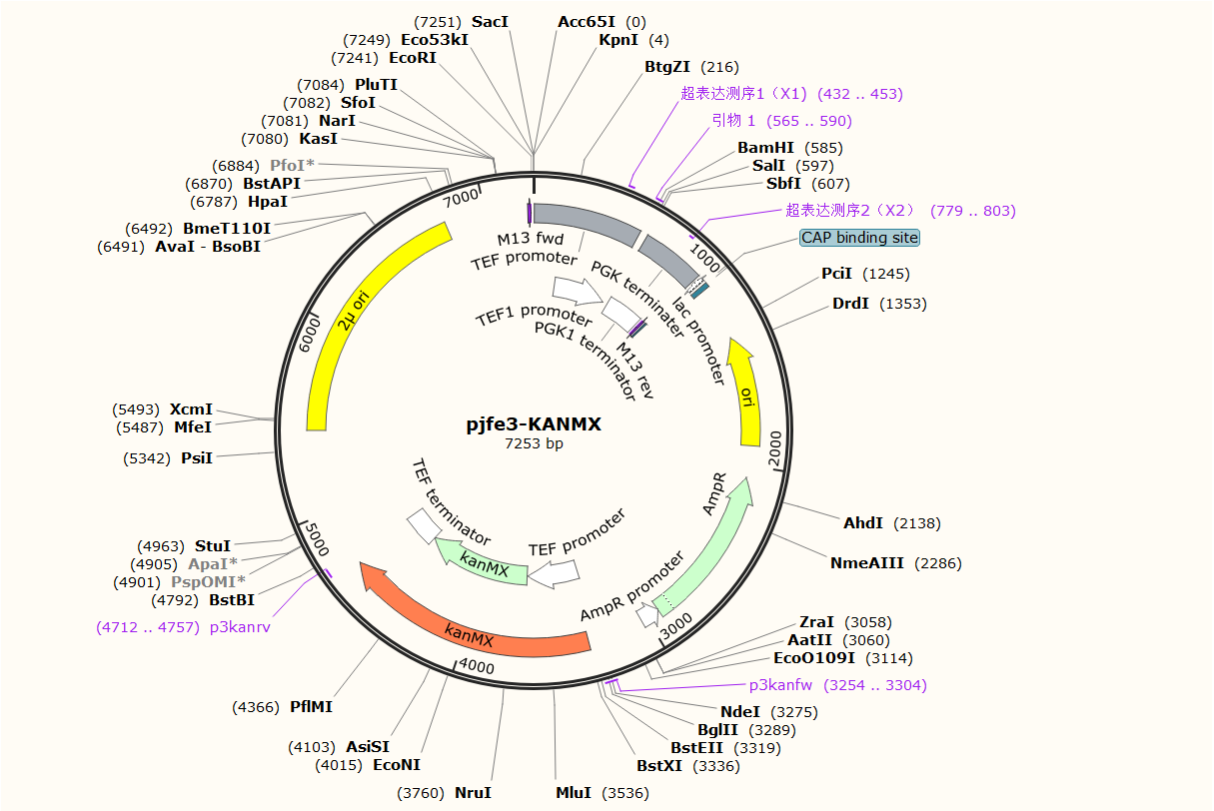

Figure 4-6 pJFE3-K plasmid digest site

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Figure 4-7 Plasmid Sequence

3.3 Plasmid validation

In the process of constructing the recombinant plasmid, although we have used PCR technology for amplification, its yield still cannot meet the demand for transformation of a large number of yeast cells, so we must first transform it into E. coli and use it as the basis for the subsequent amplification step. In this step, in order to achieve efficient plasmid introduction, we chose to induce E. coli into a receptive state using thermal stimulation. It was found that the plasmid binding to the surface of E. coli could be effectively promoted by placing the tubes containing the plasmid at a low temperature (0 ℃) for 30 min, and the subsequent thermal stimulation (45 sec) could help the plasmid to invade into the E. coli smoothly. After completing the above steps, we need to incubate E. coli for 12 hours, and then carry out picking, PCR and verification. Choose the right size colony when picking bacteria, then draw the plate and inject the PCR system, and finally run the gel (Figure 4-8) to verify whether the target gene is inserted in the plasmid or not. the FAA2 DNA sequence is 2200, and when we successfully ligated it to the plasmid vector, the total DNA length should be about 2500.

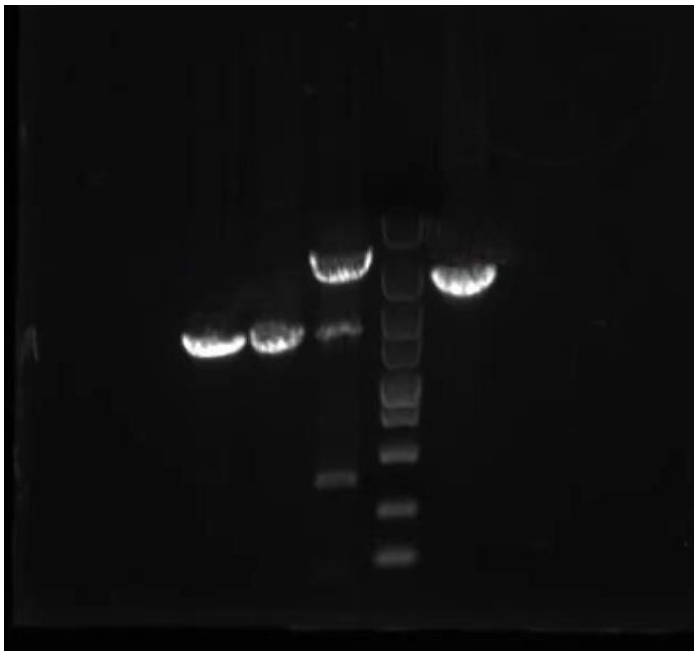

Figure 4-8 Recombinant plasmid validation of FAA2 gene

3.4 Construction of transformed yeast strains

We designed an experimental group and a control group. Specifically, the control group (Figure 4-9 A) is a blank medium plate without any treatment, and the experimental group (Figure 4-9 B) is a recombinant pJFE3-K plasmid carrying the FAA2 gene introduced into the yeast BSP2001-pJFE3-XIH with appropriate coating operations.

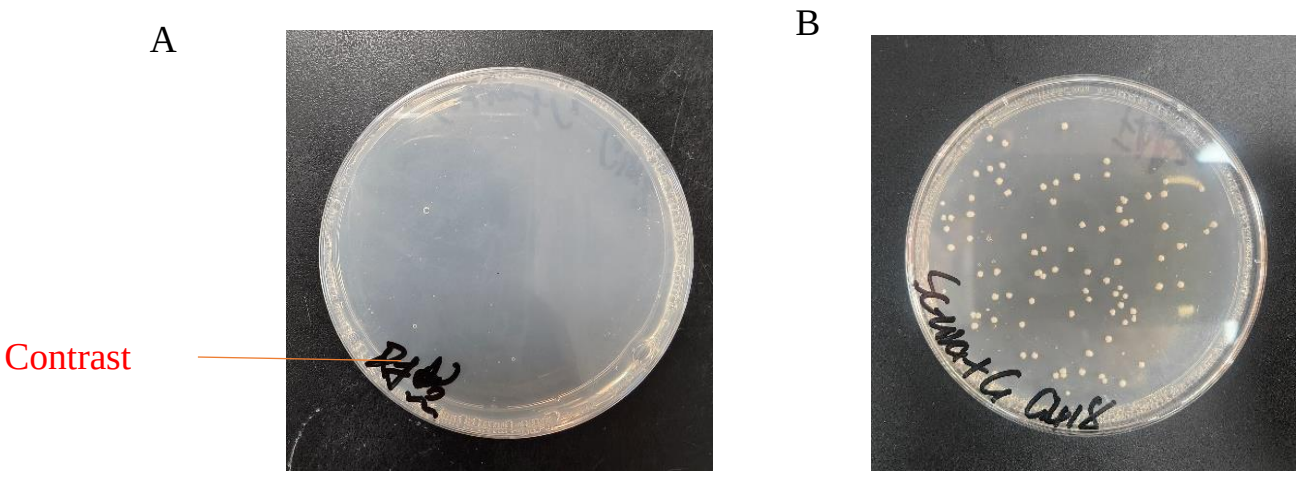

Figure 4-9 Recombinant yeast cultured on plate medium

(A) Control group of yeast strain culture;

(B) Experimental group of yeast strain culture

3.5 Determination of Growth Curve

The fermentation of recombinant yeast in culture medium (Figure 4-10) showed that the yeast was in the adjustment period in the first 6 h. From 6 to 24 h, it was in the logarithmic period, and its proliferation rate was significantly increased, and the growth rate did not show significant difference compared with that of the control group. 24 h later, it entered into the stabilization period, and the growth rate of the experimental group of yeast cells was increased in this period compared with that of the control group, which indicated that the FAA2 gene had the ability to This indicates that FAA2 gene has the ability to accelerate the growth and reproduction rate of yeast cells in xylose environment.

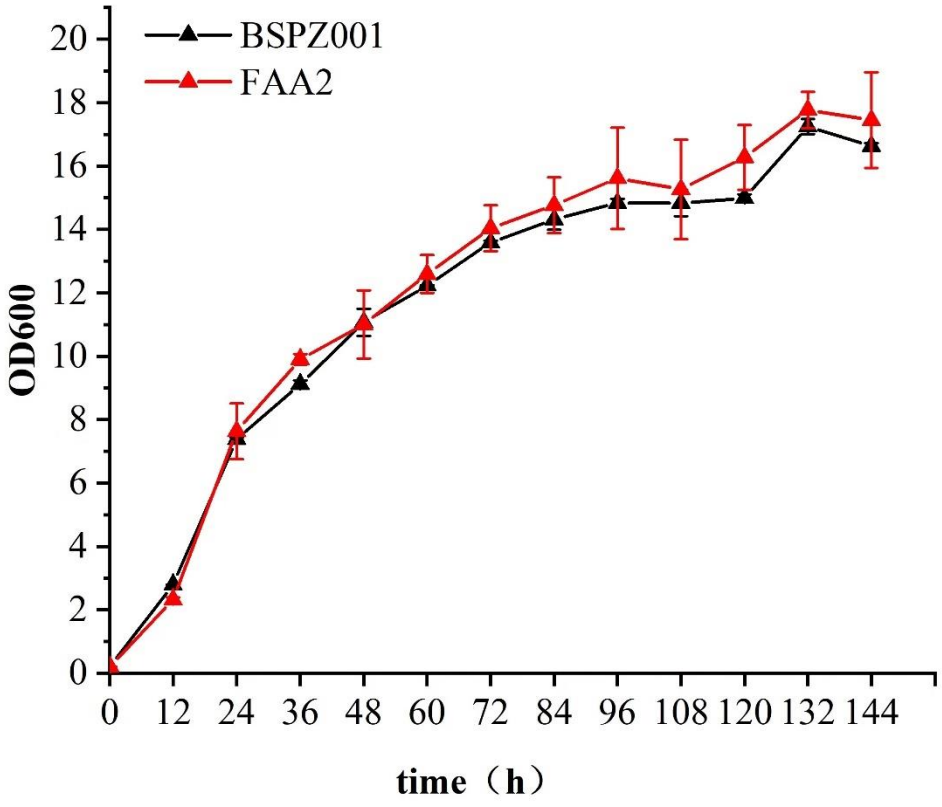

Figure 4-10 Determination of growth curves of recombinant yeast versus controls

3.6 Analysis of yeast fermentation metabolism xylose

According to the results of high performance liquid chromatography (HPLC) analysis (Figure 4-11), after comparing the differences in xylose metabolism and ethanol production between the experimental group and the control group, it was clearly found that the xylose metabolism and ethanol production of the brewer's yeast cells that had been transferred with the FAA2 gene were lower than those of the control cells that had not been genetically modified, which was a strong proof that the FAA2 gene had a certain degree of inhibitory effect on the process of ethanol fermentation. This strongly demonstrated that the FAA2 gene inhibited the ethanol fermentation process to a certain extent.

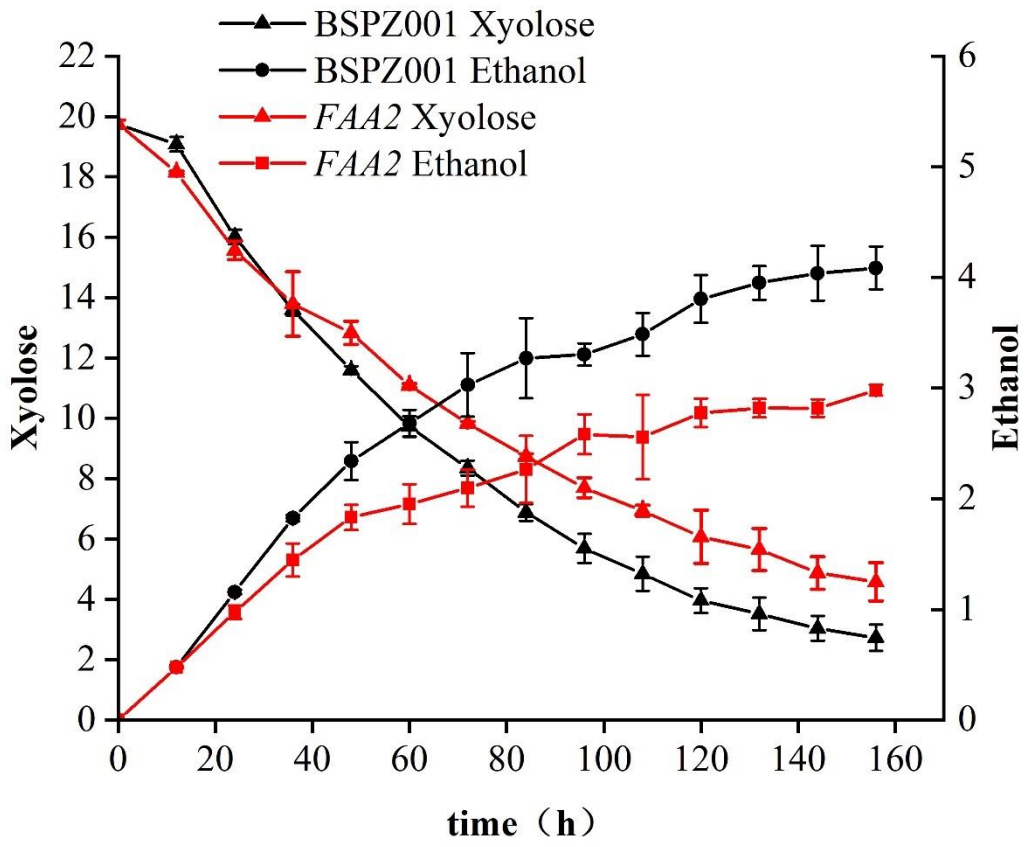

Figure 4-11 Xylose metabolism and ethanol production in control and recombinant yeasts

Conclusions to chapter 3

1. FAA2 gene can inhibit the ethanol fermentation process to some extent.
2. The fermentation of recombinant yeast in culture medium showed that FAA2 gene had the ability to accelerate the growth and reproduction rate of yeast cells in xylose environment.
3. This indicates that this gene is of great value in studying how yeast metabolizes xylose effectively and ferters acetic acid.

Summary

Since the xylose metabolizing ability of wild-type yeast is weak, if we can deeply explore the influence and mechanism of action of related genes on xylose metabolism during yeast fermentation, then this will have a profound and significant impact on improving the xylose metabolizing ability of yeast. In this study, we chose a mutant strain with enhanced xylose metabolism and overexpressed the FAA2 gene, ligated it with the pJFE3-K plasmid and amplified it, and then carried out the yeast transformation operation. By observing the growth curve of yeast, we found that the FAA2 gene could indeed significantly improve the growth and reproduction efficiency of yeast in xylose environment; at the same time, by analyzing the xylose metabolism ability and ethanol production of recombinant yeast through high-performance liquid chromatography (HPLC), we found that the FAA2 gene had a certain degree of inhibitory effect on xylose metabolism and ethanol fermentation, which indicated that this gene was useful for the study of how the yeast This indicates that this gene is of great value for studying how yeast metabolizes xylose and ferments acetic acid effectively.

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