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Bachelor's thesis

on the topic Effect of histone H4K16Q point mutation on xylose metabolism in Saccharomyces cerevisiae

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ABSTRACT

In today's world, facing the challenges of atmospheric environmental pollution and the depletion of fossil fuels, people begin to attach importance to renewable energy, among which fuel ethanol is considered to be the most promising substitute. Therefore, using lignocellulosic to prepare the second generation of fuel ethanol has become a hot research direction in the field of science. This initiative can not only improve the utilization rate of agricultural and forestry wastes, but also improve the utilization rate of agricultural and forestry wastes. Alleviating the problem of air pollution caused by straw burning can also reduce society's dependence on non-renewable resources such as fossil fuels.

Saccharomyces cerevisiae has high sugar and acetic acid tolerance and high ethanol fermentation ability, so it is the first choice for the production of second-generation fuel ethanol. In the production of second-generation fuel ethanol by *Saccharomyces cerevisiae* fermentation, lignocellulose can only be absorbed and utilized after hydrolysis to monosaccharides. However, *Saccharomyces cerevisiae* has a weak utilization capacity of xylose, which is one of the main monosaccharides generated by hydrolysis. Therefore, improving the utilization capacity of xylose has an important role in promoting the industrial production of second-generation fuel ethanol. As an important branch of epigenetics, covalent modification of histones is crucial for cell growth by directly affecting the structure of chromatin and the interaction between protein factors, thus affecting DNA

replication, DNA damage repair and gene expression. Common histone modifications include methylation, acetylation, ubiquitination, and fusinization, which mainly occur on lysine and serine residues. In previous studies, amino acids that can be covalently modified in histones are usually mutated into glutamine to simulate the acetylation state of histone amino acids.

In view of the fact that histone acetylation tends to loosen the chromatin structure by weakening the static interaction between histones and DNA, thus affecting gene expression, in this study, we mutated lysine (K) at the 16th position of the histone H4 in *Saccharomyces cerevisiae* to glutamine (Q), so as to explore the effect of histone H4K16Q point mutation on xylose metabolism in *Saccharomyces cerevisiae*. The results showed that the xylose utilization rate and ethanol yield of H4K16Q point mutant strain were lower than that of wild-type strain, which laid a certain theoretical foundation for the effect of protein point mutation on xylose metabolism of *Saccharomyces cerevisiae* in the next research group.

The object of the work is mutant strain of *Saccharomyces cerevisiae*.

The subject is influence of mutation in histone 4 to xylose utilization.

The aim of the work is to study xylose metabolism of mutant strain of *S. cerevisiae*. The tasks of the work are to obtain strain of *S. cerevisiae* with mutation in histone H4, to study influence of mutation to xylose utilization.

Key words: *Saccharomyces cerevisiae, Histone, Xylose, Fuel ethanol, H4K16Q*

CONTENT

INTRODUCTION

With the current problems of environmental pollution and shortage of oil resources, the preparation of secondary fuel ethanol from lignocellulosic raw materials has become a major research hotspot in the world of science. This process not only makes full use of agricultural waste, but also alleviates the environmental pollution caused by straw burning.This process not only makes full use of agricultural waste and alleviates the environmental pollution caused by straw burning, but also reduces the dependence of social development on non-renewable resources such as oil. This process not only makes full use of agricultural waste and reduces the environmental pollution caused by straw burning, but also reduces society's dependence on non-renewable resources such as oil and plays a pivotal role in sustainable development. Xylose is the second most abundant product of lignocellulose Xylose is the second most abundant sugar component after glucose, accounting for 30-40% of the hydrolysis product, but brewer's yeast can only make full use of glucose. However, brewer's yeast can only make full use of glucose, and the utilisation of xylose is very low. The free N-terminal amino acid residues of histones can be The amino acid residues at the free N-terminus of histones can be modified in a variety of ways, with methylation and acetylation being the most common types of modifications. Histone modifications not only regulate chromatin structure, but also affect DNA replication, DNA damage repair, RNA transcription and other processes. They also

affect processes such as DNA replication, DNA damage repair and RNA transcription.

In this study, we investigated the low utilisation of xylose in lignocellulosic hydrolysates in *Saccharomyces cerevisiae* by histone modification to explore the effect of histone H4K16Q point mutation on xylose metabolism in *Saccharomyces cerevisiae*, where lysine (K) on histone H4 is susceptible to modification, such as methylation and acetylation, by mutating lysine (K) at position 16 on histone H4 to glutamine (Q) This was used to mimic the acetylated state of histone amino acids. A DNA fragment carrying the H4K16Q point mutation was first obtained from background strain S288C and then transformed into strain BSPZ001, which was used as the background strain to transform into a plasmid containing xylose isomerase to construct a mutant strain capable of utilising xylose.

The to research methods used in this study were DNA purification, agarose gel electrophoresis, yeast chromosome extraction, yeast PCR, yeast transformation, *E. coli* transformation, 5-FOA screening for strains that lost Ura, *E. coli* plasmid extraction, and high performance liquid chromatography analysis.

The mutant strains were subjected to shake flask fermentation, and the xylose utilization and ethanol yield of the mutant and control strains were analyzed by high performance liquid chromatography. The results showed that the xylose utilization of the mutant strain was relatively low compared with that of the control strain, and the ethanol yield of the mutant strain was lower compared with that of the control strain, demonstrating that the histone H4K16Q point mutation had no

significant positive effect on the xylose utilization and ethanol yield of *Saccharomyces cerevisiae*, which provided a theoretical basis for the subsequent in-depth study on the effect of histone point mutation on xylose metabolism of *Saccharomyces cerevisiae*.

The structure of the paper includes an introduction, background introduction, introduction to the experimental material, experimental methods, analysis of the results, and references to the literature.

CHAPTER I

ACADEMIC BACKGROUND

1.1. The necessity of lignocellulosic ethanol production

1.1.1. The need for ethanol to replace fossil fuels

As a renewable energy source, fuel ethanol has many advantages. First of all, the second generation of fuel ethanol uses lignocellulosic materials and biomass resources such as waste crop residues, avoiding the harm of the first generation of fuel ethanol "competition for food" and reducing the threat to food security and land resources. However, currently fossil fuels are increasingly exhausted and cannot be renewable in a short time. The use of ethanol to replace fossil fuels is the future development trend. Secondly, fuel ethanol can be mixed into gasoline, which can help the complete combustion of gasoline, so as to reduce the emission of harmful substances to the atmosphere, so as to reduce environmental pollution. In addition, almost no toxic and harmful gases are produced in the combustion process of fuel ethanol, which is characterized by zero pollution [1·] . Compared to first-generation fuel ethanol, second-generation fuel ethanol is more environmentally friendly and sustainable because it is produced using resources such as waste and crop residues rather than food crops such as grain. This not only helps to reduce food waste, but also helps to protect the environment and the balance of the ecosystem. In the future, with the continuous development and

improvement of technology, the second-generation fuel ethanol will have a broader application prospect and make greater contribution to promoting energy revolution, protecting the environment and promoting sustainable economic development [2.] .

Nowadays, with the continuous consumption and large use of fossil fuels, a series of serious environmental pollution problems have been caused. Ethanol, a renewable energy source that can be produced by fermentation or chemical synthesis, is an alternative to fossil fuels. Reduce the dependence on fossil fuels, effectively reduce carbon emissions, fossil fuels are limited resources, and their combustion of carbon dioxide and other greenhouse gases are the main cause of climate change. Ethanol is a renewable energy with low carbon emissions in the production process, which can reduce the dependence on fossil fuels and reduce greenhouse gas emissions [3.] . The supply of fossil fuels carries political, economic and geopolitical risks in improving energy security. Ethanol can be produced domestically, reducing dependence on imported oil and improving the nation's basic energy security. At present, many countries and regions take the development of biofuel ethanol as a key project. Brazil and the United States rank the top two in fuel ethanol consumption and annual output in the world, and the domestic ethanol consumption is far lower than the output, and they are also ethanol exporters. As the third largest producer of fuel ethanol in the world, China has focused on the development of biofuel ethanol at present [5.] . Promote agriculture and rural economy. Ethanol production requires raw materials, such as corn, wheat, sugar beet, etc. The cultivation of these crops will promote the development of

agriculture and rural economy. Promote technological innovation. As an emerging energy source, ethanol needs various technical support for its production and use, which will promote technological innovation and development [4]. To improve urban air quality, ethanol fuel combustion produces less pollutants, using ethanol instead of fossil fuels can improve urban air quality.

1.1.2. The advantages of using lignocellulose to produce ethanol

The poduction of ethanol from lignocellulose has the advantage of abundant raw materials [6]. Wood cellulose is the most main component in plant cell wall, which is widely distributed in all kinds of plant such as forest, crop straw and other wastes. Our country is an agricultural country, some crop straw have not been effectively used, such as most crop straw is directly burned in fields, a waste of resources and pollution of the environment [5]. Compared with petroleum and other fossil energy sources, lignocellulose is a kind of renewable biomass resources. Using it to produce second-generation ethanol can reduce the dependence on fossil energy, which is sustainable and environmental protection. The process of producing ethanol from lignocellulose does not produce greenhouse gases such as carbon dioxide and other pollutants, and has relatively little impact on the environment [8]. As a waste or low-cost feedstock, lignocellulose is relatively inexpensive and can reduce the cost of producing ethanol. In the process of ethanol production, lignocellulosic products such as cellulase, sugar and acetic acid can be obtained through pretreatment, enzymatic hydrolysis and fermentation. Using

lignocellulosic to produce ethanol can reduce the dependence on traditional oil and help alleviate the shortage of oil resources [9]. In addition, as an additive to gasoline, ethanol can reduce the impact of exhaust emissions on the environment. The conversion of lignocellulosic and other renewable biomass into various bio-based chemicals and energy substances can effectively replace the traditional mode of chemical products based on petrochemical resources and has broad commercial application prospects [7].

1.2. *Saccharomyces cerevisiae* is the preferred strain for ethanol production

Saccharomyces cerevisiae is recognized as a food-grade safe microorganism and widely used in industrial production. Its strong tolerance to ethanol and fast fermentation rate under anaerobic conditions make *Saccharomyces cerevisiae* the preferred microorganism for ethanol fermentation [13]. Xylose accounted for 5.5%~22% of different agricultural wastes. However, natural yeast could not utilize xylose, which was second only to glucose in lignocellulosic pretreatment solution. The main reason was that *Saccharomyces cerevisiae* genome contained xylose metabolizing genes, but the expression level was too low to maintain xylose growth. Therefore, in order to achieve the economic applicability of second-generation fuel ethanol, the introduction of exogenous xylose metabolic pathway is the most basic metabolic engineering strategy [2].

1.2.1. Characteristics and application of *Saccharomyces cerevisiae* in industrial production

Saccharomyces cerevisiae is a single cell fungus, spherical or oval, large form, can convert glucose and other sugars into alcohol and carbon dioxide, is recognized as a food grade safe microorganism. *Saccharomyces cerevisiae* has strong adaptability and can adapt to low temperature, moderate temperature and high temperature environment, so it can survive and reproduce under various stress conditions. *Saccharomyces cerevisiae* can convert glucose into alcohol and carbon dioxide, and its fermentation efficiency is high and it can complete the fermentation process in a short time [19]. *Saccharomyces cerevisiae* has good stability and its fermentation process is not easily affected by environmental changes. In addition, *Saccharomyces cerevisiae* has a high demand for nutrients, such as nitrogen sources and minerals, in order to maintain its normal metabolism and growth. Different species of *Saccharomyces cerevisiae* have different tolerance to temperature, ethanol concentration and pH, so they are suitable for different fermentation conditions.

1.2.2. Advantages and disadvantages of using *Saccharomyces cerevisiae* to produce second-generation ethanol

There are many microorganisms that can ferments ethanol in nature, and the commonly used strains are *Saccharomyces cerevisiae*, Pichia stipitis, etc. Bacteria such as Clostridium Thermohydrophobic icum, Zymomonas mobilis, etc. [20], And

fungi such as Fusarium oxysporum and Neurospora crassa. Saccharobacteria cerevisiae is a traditional ethanol fermentation microorganism, which has excellent production performance such as rapid growth using hexose [21], strong fermentation ability, high ethanol yield, high tolerance to ethanol and other inhibitors, and food grade safety [22]. It is an ideal chassis cell for industrial production [23]. In addition, *Saccharomyces cerevisiae* has become the preferred industrial strain to produce second-generation fuel ethanol from lignocellulosic materials [24, 25] because of its yield from glucose to ethanol is close to the theoretical value, strong tolerance to various inhibitors in lignocellulosic hydrolysate, wide range of fermentation substrates, and high safety. In addition, *Saccharomyces cerevisiae* contains enzymes that can transform xylose, but its xylose utilization capacity is low due to low enzyme activity. *Saccharomyces cerevisiae*, for example, lacks an efficient way to convert xylose to xylose.

1.3. Pretreatment of lignocellulose

Lignocellulosic pretreatment plays an important role in biomass conversion. Lignocellulose is a kind of complex polymer compound, which contains a lot of cellulose, hemicellulose and lignin components [14]. These components interweave with each other, making it difficult for lignocellulose to be directly degraded and utilized by microorganisms [13]. Pretreatment can damage the structure of lignocellulose and make it easier to be degraded into usable small molecule substances [11]. Therefore, pretreatment of lignocellulose is one of the important

steps in the biomass conversion process. Through pretreatment, the structure and composition of lignocellulose are changed [12], making it easier to undergo chemical or biological transformation. This not only improves conversion efficiency, but also helps reduce energy and time costs. In addition, by reducing the structural complexity and indegradability of lignocellulose, subsequent production processes and costs can be reduced [15].

The commonly used pretreatment methods include: physical method, chemical method, combined treatment method and biological method [10, 18]. The physical method mainly changes the form and structure of lignocellulose by mechanical method, which is conducive to the subsequent chemical and biological transformation. Common physical pretreatment methods include crushing, grinding, hot pressing, etc. Although this method has low threshold, it consumes a lot of energy and is not suitable for large-scale production [16]. Chemical method refers to the chemical process of changing the chemical structure and composition of lignocellulose in order to enhance its degradability and availability. Common chemical pretreatment methods include acid treatment, alkali treatment, oxidation treatment, ionic liquid method, organic solvent method, etc. [17, 18]. A single pretreatment technology often has its own limitations, which is easy to cause environmental pollution or fail to achieve the expected effect. Therefore, in recent years, there has been a combination of two or more methods to enhance or improve the pretreatment technology, collectively known as combined treatment. Including chemical-chemical method, physical-chemical method, bio-physical method and

bio-chemical method [18]. The biological process mainly uses enzymes produced by microorganisms to degrade hemicellulose and lignin. This method is environmentally friendly but inefficient. Biological method refers to the use of biological systems such as microorganisms or enzymes in living organisms to pretreat lignocellulose, so that it is easier to transform. Common biological pretreatment methods include enzymatic hydrolysis, microbial fermentation and so on. In addition, compared with physical and chemical pretreatment technologies, biological pretreatment technology has the advantages of low operation cost, high yield and fewer inhibitory by-products, but the low efficiency of biological pretreatment hinders its wide application [17]. At present, there are many pretreatment methods of lignocellulose, which can be selected according to the experimental needs and the technical advantages of each method to achieve the best treatment effect.

1.4. Pathway of xylose metabolism in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae strains can convert xylose to ethanol in two ways. The first pathway involves converting Xylose Reductase (XR) and Xylitol Dehydrogenase (XDH) to xylitol and then xylitol to xyketose. Then, Xylulokinase (XK) converts xylulose-5-phosphate to the pentose phosphate pathway (PPP) for fermentation to ethanol.

1.4.1. Xylose Reductase-Xylitol dehydrogenase Pathway (XR-XDH pathway)

The xylose-reductase gene (XYL1) and xylitol dehydrogenase gene (XYL2) derived from Pichia pastoris were first expressed in *Saccharomyces cerevisiae* and became active [1]. However, due to the imbalance of coenzyme factors required by XR and XDH, the accumulation of xylitol as a by-product and the restriction of ethanol production [2]. Specifically, XR requires both NADPH and NADH cofactors, but prefers NADPH, while XDH relies on NAD+. Since the two enzymes depend on different cofactors, this step leads to an imbalance in the REDOX state, which keeps the xylose conversion process in the xylitol stage and prevents the production of xylose. Therefore, solving REDOX imbalance has become the key point to optimize this pathway, and many researchers are exploring solutions [27].

1.4.2. Xylose isomerase pathway (XI pathway)

Xylose isomerase (XI), of bacterial origin, is able to catalyse the production of xylose in one step[29], which is then phosphorylated by xylulose kinase [28], converted to xylulose-5-phosphate and enters the pentose phosphate pathway, which is considered by many researchers to be the optimal pathway as compared to the XR-XDH pathway as it eliminates the intermediate step and avoids the problem of redox imbalance [2]. It is considered by many researchers to be the most optimal pathway [2]. When xylose isomerase is expressed in *Saccharomyces*

cerevisiae, there are factors such as protein folding errors, modification errors and environmental restrictions on the enzyme itself in yeast, which result in low xylose isomerase activity [42]. Therefore, the key to optimising the xylose isomerase pathway in yeast is to be able to correctly induce the expression of the enzyme or to modify it effectively as a means of increasing enzyme activity [27].

1.5. Epigenetics Overview

1.5.1. Concepts of epigenetics

Epigenetics is the study of the regulation of gene expression without alteration of the DNA sequence [34]. Within eukaryotic cells, DNA is not naked, but exists as chromatin. Chromatin is made up of DNA and protein complexes that are capable of compacting very long DNA into a tightly packed nucleus [35]. When gene products are required, the chromatin is selectively unwound and opened to allow transcription factors to enter (this is known as 'euchromatin'). This suggests that changes in the structure and accessibility of chromatin significantly affect the regulation of gene expression, both between different cells and over the lifetime of a single cell [44]. Histone modifications are an important branch of epigenetics, and these covalent modifications can affect processes such as gene transcription [45], RNA processing and translation, thereby influencing cellular function and phenotypic characteristics. The study of epigenetics includes DNA methylation, histone modifications, non-coding RNAs [46], and chromatin remodelling.

Epigenetic modification refers to the process of dynamic changes in genes through the action of specific enzymes on genes and their surrounding regions. These enzymes do not alter the DNA nucleotide sequence itself, but rather regulate it by altering the process of DNA transcription. These changes depend in part on epigenetics. Thus, the same genes can produce different phenotypes without altering the DNA sequence [30].

1.5.2. The role of histone modifications

The nucleosome histone is composed of two heterodimers of H2A and H2B, and a tetramer of H3 and H4 [31]. Histones are highly conserved basic proteins found in the nucleus and are primarily responsible for wrapping DNA into tight chromatin structures, thus allowing for stable storage and delivery of DNA in the nucleus. Histones have highly variable histidine residues at their N termini, and chemical modification of these residues can affect the function and structure of histones [46], and thus the state of chromatin and gene expression. Histone modification refers to the modification of histones by chemical modifications that can include phosphorylation, methylation, ubiquitination [47], acetylation and many other [32] modifications. These modifications can affect the structure and affinity of histones, and thus the readability of DNA and gene expression [48]. Different histone modifications can comprise different patterns of histone modifications, which in turn determine gene expression status and cell fate.

As an important class of chemical modifications in epigenetics [33], histone

modifications can be recognised by a range of enzymes or binding proteins [49], which in turn affect chromatin structure and DNA metabolism, including transcription, DNA replication, DNA splicing, DNA damage repair and other processes. In addition, histone modifications also have a role in regulating the cell cycle and signal transduction [36]. Histone modifications can affect the transcriptional activity and expression of genes by directly or indirectly regulating chromatin organisation [38]. Among these, histone acetylation plays an important role in the regulation of transcriptional levels [39]. The mechanism by which histone acetylation regulates gene transcription is largely dependent on histone acetyltransferase (HAT) and histone deacetylase (HDAC). Levels of histone acetylation increase the repulsion of histones from positively charged DNA [43], which in turn promotes the binding of transcription factors and thus activates gene transcription. Conversely, histone deacetylation weakens the repulsion between negatively charged DNA and histones [40], leading to chromatin condensation and inhibiting the binding of transcription factors, thereby inhibiting gene transcription [37]. Histone methylation modifications occur mainly at amino acid residues of histone H3 and H4. Unlike histone acetylation [50], the promotive or inhibitory effect of methylation modifications on gene transcription depends on the location [51], type and extent of methylation [41].

Summary of the chapter I

This section provides background on the feasibility and urgency of replacing fossil fuels with second-generation ethanol, its advantages and disadvantages, the low xylose utilisation of lignocellulose hydrolysates by brewer's yeast, the need to construct upstream pathways, the reduction of the structural complexity and difficulty of degradation of lignocellulose through pretreatment of lignocellulose, and the reduction of subsequent production processes and costs. The paper also introduces the XR-XDH and XI pathways of xylose metabolism in *Saccharomyces cerevisiae*. The role of histone modifications is introduced.

1. The urgency and feasibility of second-generation ethanol replacing fossil fuels.

2. *Saccharomyces cerevisiae* can utilize xylose in lignocellulosic hydrolysate, but the utilization rate is low.

3. The pretreatment of lignocellulosic hydrolysate can make it better used and reduce the complexity of structure and the difficulty of degradation.

4. Xylose can be metabolized in two ways, through histone modification to improve xylose utilization and ethanol production.

CHAPTER II

EXPERIMENTAL MATERIALSExperimental apparatus

Ultra Clean Bench, Benchtop Centrifuge, PCR, Vortex Mixer, Vortexer, Induction Oven, Constant Temperature Water Bath, Microwave Oven, Pipette Gun, Agarose Gel Electrophoresis, Gel Imager, Blue Light Cutter, Biological Spectrophotometer, Autoclave, Constant Temperature Shaker, Constant Temperature Incubator.

2.2. Experimental reagents

DNA extract, lithium acetate (0.1 mmol/L PH 7.6), polyethylene glycol (PEG), ssDNA, agarose, 1 x TAE buffer, nucleic acid dye, dimethyl sulfoxide (DMSO), magnesium sulphate, anhydrous ethanol, dNTPs, NaOH solution, Loding buffer, Binding buffer (XP), Washing buffer, peptone, yeast powder, glucose, yeast basic nitrogen source (YNB), ammonium sulphate, pentafluoroorotic acid (5-FOA).

2.3. Experimental strains, primers

1) Strain: BSPZ001

2) Primer

In the Table 2.1 presented primers used in the work.

Table 2.1

Primers used in the work

Name	Primes sequence
P3	CTTGGTACTAATTCCGGAAG
P4	GTGGTGGATTTTGGAAGG
Ura	CTTGACTGATTTTTCCATGG
Ura-2	CCCTTCCCTTTGCAAATAG
P6	CTGGAGTAATTTTGAGATTGCGC
P8	GGGGAGATATACCGTAGCAG

2.4. Culture medium

1) LB liquid medium: 1% peptone, 0.5% yeast powder, 1% sodium chloride. 2% agar (solid medium)

2) YPD liquid medium: 2% peptone, 1% yeast powder, 2% glucose (last addition). 2% agar (solid medium)

3) Complete synthesis medium for uracil deficiency (SC-Ura): yeast basic nitrogen source (YNB) 1.7 g/L, ammonium sulphate 5 g/L, CSM-Ura 0.77 g/L with 2% agar and pH adjusted to 6.0-6.5 with sodium hydroxide.

4) 5-FOA medium: CSM-Ura 0.77 g/L, YNB 1.7 g/L, (NH4)2SO4 5 g/L, uracil 100 g/L, glucose 20 g/L, agar powder 20 g/L, dissolve with 180 mL of deionised water.

Summary of the chapter II

This section describes the experimental equipment, reagents, strains and primers used in the study, and the media used.

1. Introduction of background strains, template strains and primers.

2. The specific steps of medium configuration and the substances required to be added.

3. Description of laboratory instruments and materials.

EXPERIMENTAL METHODSDNA purification

1) Weigh the empty centrifuge tube, place the gel block after agarose gel electrophoresis on a blue light cutter, cut off the block showing brightness into an EP tube, then weigh using an electronic balance and add equal proportions of Binding Buffer to a concentration of 0.1 mg/μL according to the weight of the block.

2) Place the EP tube containing the target gel block in a 60°C water bath, turn the tube up and down and shake it slightly every two minutes until the gel block is completely dissolved, then add the entire solution to the adsorption column.

3) Place in a centrifuge and centrifuge at 11,000 rpm/min for 1 min. After centrifugation, aspirate the liquid from the collection tube into the adsorption column and centrifuge again at 11,000 rpm/min for 1 min to remove the waste solution.

4) Add 300 μL of Binding Buffer, centrifuge at 11000 rpm for 1 min and discard the supernatant waste.

5) Add 700 μL of Wash Buffer, centrifuge at 11,000 rpm for 1 min and repeat the operation once.

6) Discard the waste solution and leave the empty centrifuge tube empty at 12,000 rpm for 1 min, discard the waste solution.

7) Rack the adsorption column in a new EP tube and leave it to stand for 5 min.

8) Add 35 μL of sterile water to the centre of the membrane, perform centrifugation at 12,000 rpm for 1 min, aspirate the liquid from the collection tube and repeat the centrifugation once to obtain purified DNA.

3.2. Agarose gel electrophoresis

1) Depending on the number of DNA molecules in the sample, weigh the agarose powder and the volume of 1 x TAE buffer added (1 g of agarose per 100 mL of 1 x TAE buffer) and shake well.

2) Place in a microwave oven to dissolve, observe the state of dissolution, remove after boiling and shake several times until the bottom is completely dissolved without fine particles.

3) Add nucleic acid dye (at a concentration of 0.1 mg/μL) to this and mix well.

4) Place the comb and fix it on the glue making board, place it horizontally and then slowly pour the glue into the board in one go.

5) After the gel has completely solidified, gently and slowly pull up the comb and transfer the gel bath to the electrophoresis bath, with the addition of 1 x TAE buffer near the cathode on one side of the sample hole, until the gel surface is submerged.

6) Use a pipette gun to aspirate the DNA sample to be tested into the glue well and add the sample by placing the tip of the pipette gun along the edge of the glue well to avoid damaging the glue block and to add the sample quickly.

7) After the sample has been added, connect the electrophoresis tank and electrophoresis instrument correctly and turn on the power, set the voltage and time of electrophoresis and start electrophoresis.

8) After electrophoresis, the gel block is carefully removed (gloves are required when removing the gel block) and placed horizontally in the gel imager to observe the results.

3.3. Extraction of yeast chromosomes

1) Take the strain and apply on SC-Ura+Nat medium and incubate for 24 hours in a constant temperature incubator (30°C).

2) Take a clean, sterile EP tube (1.5 ml centrifuge tube) and add 200 μL of DNA Extraction Solution I to it.

3) Scrape the bacteria from (1) into an EP tube on the ultra clean table.

4) Add 0.4 g to 0.8 g of glass beads.

5) Remove DNA Extract II from the refrigerator at 4°C (store away from light) and add 200 μL of DNA Extract II to the EP tube in the fume hood (aspirate its liquid phase below the aqueous phase, which is about 5/1 of the total volume).

6) Shake for 90 s using a vortex mixer and centrifuge for 10 min at 13,000 rpm.

7) Take a new EP tube and add 1 ml of anhydrous ethanol to it.

8) Aspirate (6) the supernatant of the EP tube after centrifugation (observe whether it is stratified: aqueous phase in the upper layer, protein in the middle and

phenol chloroform lipid in the lower layer) into a new EP tube with anhydrous ethanol, (taking care not to aspirate the protein layer in the middle), gently reversing the top and bottom.

9) Leave the EP tube to stand for 10 min, centrifuge at 13000 rpm for 15 min, remove the supernatant and wait for it to dry and precipitate.

10) Add 35 μL ddH2O and mix thoroughly, store at 4°C.

3.4. Yeast conversion

1) The bacteria were first inoculated in 5 ml of YPD medium and incubated overnight at 30°C for 12 h in a 200 rpm shaker.

2) The activation was transferred to 40 ml of YPD medium, the OD was adjusted to between 0.2-0.25 and incubated at a constant temperature (30°C) for 4-6 hours on a shaker at 200 rpm.

3) When the OD value is around 1.0, transfer the bacterial solution to a sterile centrifuge tube in the ultra clean bench and centrifuge at 3000 rpm for 5 min, discard the supernatant waste.

4) Shake and mix with an appropriate amount of sterile water, centrifuge at 3000 rpm for 1 min, discard the supernatant and repeat the operation once.

5) Add 1 ml of 0.1 mmol/L LiAc, suspend and add to the EP tube, gently invert and mix, centrifuge at 4500 rpm for 1 min and discard the supernatant.

6) Fish essence DNA was removed from the ice box, placed in boiling water at 100°C for 5min and then quickly inserted into the ice box.

7) ①If transferring DNA fragments: Add 240 μL 50% PEG (inhale slowly and mix well after addition), 5 μL sterile water, 36 μL 1mmoL/L LiAc, 49 μL fragment DNA (need to be added below the liquid level), 30 μL fish essence DNA (remove from ice box and use) to (4) centrifuge tube in order and mix well. (ii) If transferring plasmid: Add 240 μL 50% PEG, 36 μL 1mmoL/L LiAc, 5 μL sterile water, 1 μL plasmid, 30 μL fish essence DNA to (4) centrifuge tube in that order and mix well.

8) The EP tubes were incubated in a constant temperature (30°C) incubator for 30 min, then placed on ice for 5 min and then placed in a water bath at 42°C for 40 min of heat excitation.

9) Centrifuge at 4500 rpm for 1 min, discard the supernatant and add 400 μL of sterile water suspension by aspiration.

10) ①If transferring fragments: aspirate about 200 μL, apply to a YPD plate, place in a constant temperature (30°C) incubator and incubate overnight, then photocopy the bacterial solution onto a plate of SC-Ura+N. (ii) If transferring plasmid: aspirate 50 μL, apply to a SC-Ura+N plate, place in a constant temperature (30°C) incubator and incubate overnight.

3.5. Yeast PCR

1) Take a sterile EP tube, add 50 μL of 20 mmol/L NaOH to it and pick single colonies into it and mix well.

2) Insert the EP tube into the float and boil for 10 min.

3) Cool to room temperature, centrifuge at 6000 rpm for 1 min and aspirate the supernatant.

4) Aspirate 1 μL of supernatant and add to the PCR reaction system (KOD Buffer 5 μL, sterile water 31 μL, dNTPs 2 μL, Mg2SO4 2.5 μL, enzyme KOD 1 μL, primers 1 μL, DMSO 2.5 μL) and run the reaction.

3.6. 5-FoA screening for strains that have lost Ura

1) Yeast is inoculated in YPD medium and incubated in a constant temperature (30°C) shaker at 200 rpm , 12 hours.

2) The bacterial fluid was collected, washed twice with dd H2O, centrifuged at 4500 rpm for 1 min and the supernatant removed.

3) Add 500 μL of dd H2O to resuspend the cells and aspirate 100-200 μL of the bacterial solution onto 5-fluoroorotic acid plates.

4) Leave to incubate and wait for it to grow single colonies to screen out the target strains.

3.7. *E. coli* transformation

1) Add 1 μL of plasmid to the receptor cells and place on ice for 3 min, followed by an ice bath for 30 min.

2) Place in 42°C water for 90s, followed by an ice bath for 5 min.

3) Add 1 mL of LB medium to the ultra-clean bench, invert and mix well, and incubate in a constant temperature incubator at 37°C for 45min.

4) Coat screening plates (LB+100 μg/mL Ampicillin) with 100 μL-200 μL of bacterial solution.

5) Incubate in a constant temperature (37°C) incubator for 12 h. Wait for single colonies to form and then pick a number of colonies for PCR validation.

3.8. *E. coli* Plasmid Extraction

1) Single colonies were picked and coated in 5 mL of LB+Amp medium and incubated at 37°C for 12 h in a constant temperature incubator at 200 rpm.

2) Add the culture to a sterile EP tube, centrifuge for 1 min at 12000 rpm, discard the supernatant and collect the bacteria from the bottom.

3) Add 250 μL of SolutionI (pre-spiked with RNaseI, stored at 4°C in the refrigerator) and shake to suspend the bacterium.

4) Add 250 μL of Solution II and mix well with a quick up-and-down inversion.

5) Add 350 μL of Solution III, mix gently and quickly by inverting up and

down to a flocculent state and centrifuge at 12,000 rpm for 5 min.

6) Take a sterile collection tube and place it in an EP tube.

7) Centrifuge the resulting supernatant into a collection tube and centrifuge at 12000 rpm for 1 min, discard the clear solution from the collection tube.

8) Add 500 μL of Buffer HB to the collection tube and centrifuge at 12000 rpm for 1 min, discard the clear solution.

9) Add 600 μL Wash buffer to the collection tube, centrifuge at 12,000 rpm for 1 min, discard the clear solution and repeat.

10) Perform a vacuo, 12,000 rpm, 2 min.

11) Allow to cool to room temperature, uncap the tube and leave for 5 min for the ethanol to evaporate completely.

12) Add 600 μL of sterile water and leave for 2 min, centrifuge at 12,000 rpm for 1 min, add sterile water to the bottom of the isolated plasmid, re-aspirate onto the membrane and centrifuge at 12,000 rpm for 1 min.

13) Detection of concentrations, PCR validation.

3.9. Xylose utilisation measured by high performance liquid chromatography

1) A 1 ml sample of this xylose fermentation was taken at 12 h intervals for analysis.

2) Firstly, the chromatographic conditions need to be determined, the type

of mobile phase and its ratio, the flow rate, the column temperature, the injection volume and the detection wavelength.

3) Carry out the configuration of the sample solution.

4) The mobile phase is sonically degassed for 30 min before use to ensure that the air bubbles are removed.

5) Check and connect the testing system and mobile phase piping.

6) Adjusting various parameters to pre-designed data such as flow rate, wavelength, acquisition time, column temperature etc.

7) First the exhaust is carried out and then equilibrated for 30 min after completion, followed by the start of the analysis.

8) Once the analysis is complete, it needs to be cleaned and stored according to the different requirements of each column, followed by switching off the lights and pumps, closing the test program, shutting down the computer and finally switching off the power supply.

9) Finally the data is processed by data processing equipment or the chromatogram is recorded by a recorder.

Summary of the chapter III

This chapter describes the experimental methods used during the study, detailing the specific operations and attention to detail for each step, including DNA purification, agarose gel electrophoresis, yeast chromosome extraction, yeast transformation, yeast PCR, 5-FOA screening for Ura-losing strains, *E. coli*

transformation, *E. coli* plasmid extraction, and high performance liquid chromatography for xylose utilisation.

1. DNA purification related detailed steps and precautions.
2. Agarose gel electrophoresis related detailed steps and precautions
3. Yeast chromosome extraction related detailed steps and precautions.
4. Yeast transformation and extraction related detailed steps and precautions.
5. Yeast PCR related detailed specific steps and precautions.
6. 5-FOA screening of missing Ura strain related detailed steps and precautions.
7. Escherichia coli transformation related detailed steps and precautions.
8. Escherichia coli plasmid extraction related detailed steps and precautions.
9. High performance liquid chromatography related detailed steps and precautions.

CHAPTER IV

RESULTS AND ANALYSIS

4.1. Construction of H4K16Q point mutant strain with BSPZ001 as background

4.1.1. Scheme for the overall construction of the H4K16Q point mutant

As an important branch of epigenetics, histone modification can regulate nucleic acid metabolic processes including RNA transcription, DNA replication, DNA damage repair, as well as basic biological processes such as protein folding, regulation of cell cycle and signal transduction. In this study, we mutated the lysine (K) at position 16 of brewer's yeast histone H4 to glutamine (Q), in which the glutamine R group can mimic the acetylated state of amino acids, in order to examine the effect of histone H4K16Q mimicking acetylation on brewer's yeast The effect of histone H4K16Q on xylose metabolism was examined. The specific protocol for the construction of the H4K16Q histone point mutant using strain BSPZ001 as a background is shown in Figure 4.1 (Appendix A).

4.1.2. Amplification and transformation of DNA fragments carrying the H4K16Q point mutation

The DNA carrying the Ura screening marker histone H4K16Q point mutant was first extracted, and the DNA fragment with the point mutation of amino acid 16 on histone H4 to glutamine (Q) was obtained by PCR amplification using the genome of histone point mutant S288C strain with markers P1 and P4 as primers, respectively. The products were subjected to agarose gel electrophoresis and the electrophoretic bands were observed. The target DNA fragments were cut on a UV gel cutter and stored at 4°C. The DNA fragment carrying the histone point mutation was transferred to the laboratory strain BSPZ001 by lithium acetate transformation. Positive clones were screened using plates from Sc-Ura (Figure 4.2, Appendix A).

4.1.3. Identification of positive clones using PCR

Transformants were screened using SC-Ura+Nat plates, multiple transformants were randomly selected from each mutant, single colonies growing on SC-Ura+Nat plates were picked (Figure 4.3.A, Appendix B), lines were drawn on SC-Ura+Nat plates (Figure 4.3.B, Appendix B), genomic DNA of the crossed strain was extracted, and then PCR amplification was performed and validated using primers P6/Ura2 and P8/Ura3 using the genomic DNA of the transformants

as templates.

The results of agarose gel electrophoresis indicated that the mutant strain was successfully constructed.

4.1.4. Screening for histone point mutant strains without Ura markers

The medium containing the 5-FOA reagent is capable of producing toxic effects on strains capable of synthesizing uracil, so it can be used to screen for histone point mutant strains that have lost the Ura marker.

The results showed that some of the mutant strains could grow normally on the 5-FOA medium, but not on the SC-Ura+Nat medium, indicating that these strains had lost the Ura marker.

To further verify the experimental results, DNA was extracted from the obtained strains and PCR amplification was performed.

The PCR amplification products were later subjected to agarose gel electrophoresis, and the results are shown in Figure 5, indicating that the mutant strains have lost the Ura.

4.2. Transfer of the XI xylose metabolic pathway to the H4K16Q point mutant

The yeast transformation was used to construct a brewer's yeast point mutant strain capable of efficiently utilising xylose.

The group transferred an additional plasmid containing xylose isomerase to a background strain containing the H4K16Q point mutation, which contained a Ura marker, and used SC-Ura+Nat medium plates to screen the transformants and eventually obtained a control strain of BSPZ001 expressing XI as well as a histone mutant strain (Fig. 4.5 and 4.6, Appendix C).

4.3. Assay of xylose fermentation performance of H4K16Q point mutant

By using high performance liquid chromatography to detect the fermentation performance of the control strain and the constructed mutant strain, the consumption of xylose of the histone H4K16Q point mutant strain was lower than that of the control strain within 120 h as time increased (Figure 4.4.A, Appendix B). The histone HK16Q point mutant strain produced less ethanol than the control strain within 120 h (Figure 4.4.B, Appendix B). The results showed that the mutant strain of histone H4K16Q had no positive effect on the utilization of xylose and ethanol yield.

Summary of the chapter IV

This section is an analysis of the relevant experimental results during the study, the construction process of the H4K16Q point mutant strain in the context of strain BSPZ001, the overall construction scheme of the H4K16Q point mutant, the amplification and transformation of DNA fragments carrying the H4K16Q point

mutation, the identification of positive clones by PCR, the screening of histone point mutant strains without Ura markers, the transfer of the XI xylose metabolic pathway into the H4K16Q point mutant, the testing of the xylose fermentation performance of the H4K16Q point mutant, and finally the corresponding conclusions.

1. Detailed flow chart of construction of H4K16Q point mutant strain.

2. Amplification and transformation of DNA fragments carrying H4K16Q point mutants.

3. PCR was used to isolate positive clones, and the transformants were extracted and marked on 5-FOA medium to screen the strains without Ura label.

4. Transfer into the mutant using the XI pathway.

5. The xylose fermentation performance of the mutant was tested and there were no positive results for xylose utilization and ethanol production in the mutant strain relative to the control.

CONCLUSION

1. A DNA fragment carrying the H4K16Q point mutation was obtained from background strain S288C and then transformed into strain BSPZ001, which was used as the background strain to transform into a plasmid containing xylose isomerase to construct a mutant strain capable of utilizing xylose.

2. The mutant strains were subjected to shake flask fermentation, and the xylose utilization and ethanol yield of the mutant and control strains were analyzed by high performance liquid chromatography. The results showed that the xylose utilisation of the mutant strain was relatively low compared to the control strain, and the ethanol yield of the mutant strain was lower compared to the control strain.

3. This study demonstrates that histone H4K16Q point mutations have no significant positive effect on xylose utilization and ethanol production in *Saccharomyces cerevisiae*, providing a theoretical basis for subsequent in-depth studies on the effects of histone point mutations on xylose metabolism in *Saccharomyces cerevisiae*.

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Appendix A

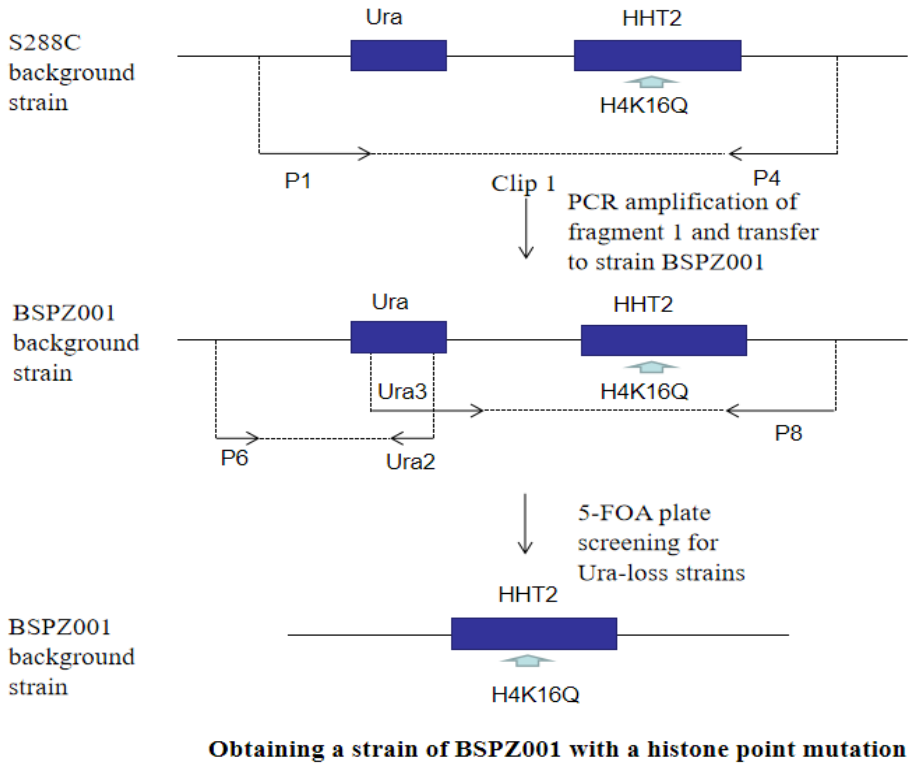

Figure 4.1 Construction of BSZP001 strain with histone point mutation

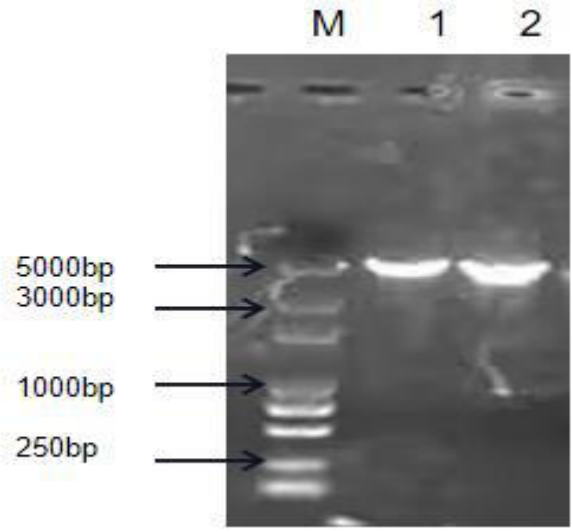

Figure 4.2 Electrophoresis of PCR amplification of DNA fragments

Appendix B

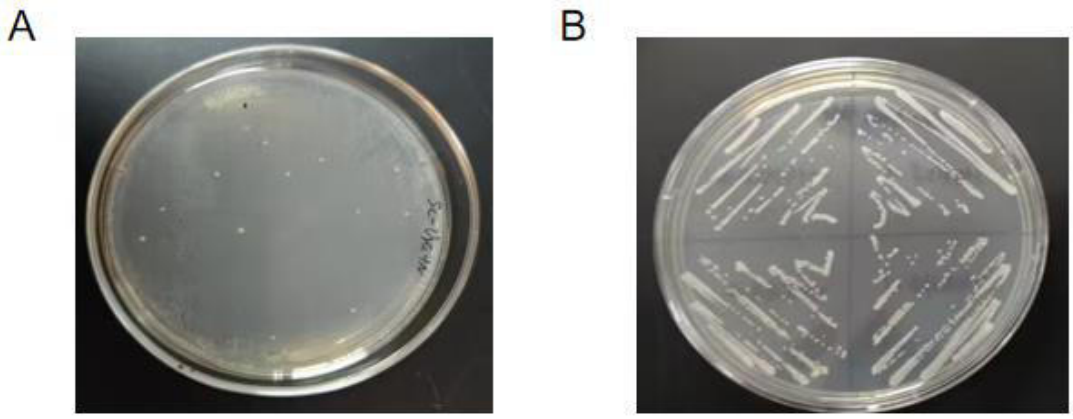

Figure 4.3. A – SC-Ura screening for positive clones; B – H4K16Q point mutation monoclonal

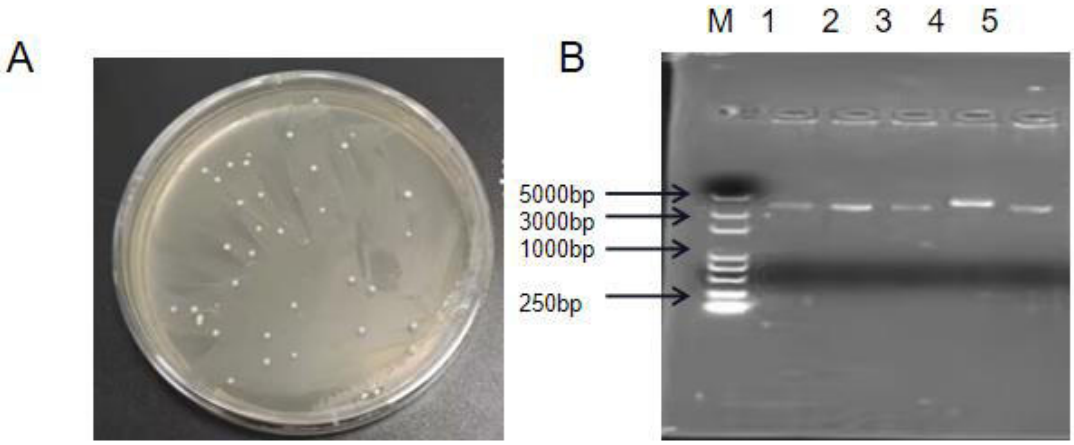

Figure 4.4. A – 5-FOA plate screening for Ura-loss histone point mutant strains; B – PCR validation of transformants with H4K16Q point mutant DNA fragments

Appendix C

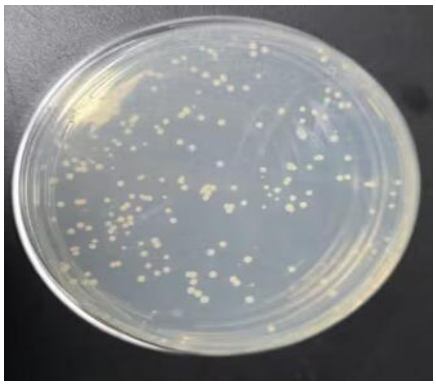

Figure 4.5 SC-Ura+Nat plate screening for strains containing an additional type of plasmid for xylose isomerase XI

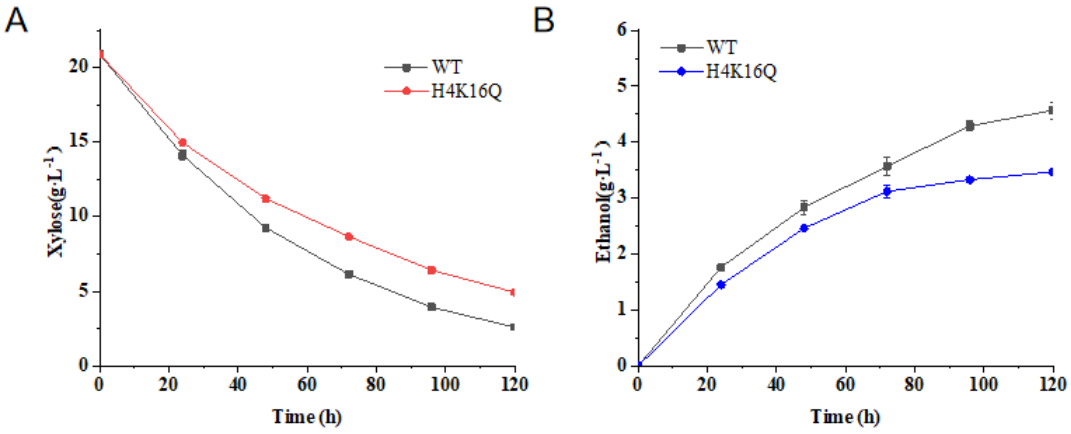

Figure 4.6 Xylose consumption (4.6-A) and ethanol production (4.6-B) of the H4K16Q mutant strain and the control strain