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Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

Bachelor's thesis

on the topic Analysis of the Effect of Histone H3K56Q Point Mutation on Acetic Acid Resistance of Saccharomyces cerevisiae

Completed: student of group BEBT-19
of specialty 162 «Biotechnology and Bioengineering»
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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

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APPROVE
Head of Department of Biotechnology,
Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA

«30» 05 2023

ASSIGNMENTS
FOR THE BACHELOR'S THESIS
Jinyao LOU
(Student's Full Name)

1. Thesis topic Analysis of the Effect of Histone H3K56Q Point Mutation on Acetic Acid Resistance of Saccharomyces cerevisiae

scientific supervisor Olga Shydlovska, PhD
(first name, last name, academic degree, academic title)

approved by the order of the higher educational institution on _____

2. Deadline for student submission of work 15.05.23

3. Initial data for work: Scientific data about influense of H3 mutations on xylose metabolism, experimental data of H3K56Q point mutation on acetic acid resistance of S. cerevisiae

4. Content of the thesis (list of questions to be developed): research background, materials and methods, experimental methods, results and analysis, conclusion

5. Date of issuance of the assignments 01.05.23

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ABSTRACT

The continuous development of petroleum resources makes mankind face two major challenges: resource depletion and environmental pollution. As a new renewable resource, energy fuels have broad prospects for development. The production of fuel ethanol can be achieved by utilizing lignocellulose in agricultural and forestry waste, thereby improving waste utilization and reducing dependence on fossil fuels. *Saccharomyces cerevisiae* is one of the preferred strains for producing fuel ethanol. Its high sugar content, high ethanol tolerance, and high ethanol fermentation performance make it an ideal microorganism for producing fuel ethanol. However, the complex chemical structure of lignocellulose determines that it cannot be directly used as a raw material for ethanol fermentation, and can only be used by microorganisms after being degraded into small molecule monosaccharides. In addition to producing fermentable monosaccharides, lignocellulose also produces small molecule compounds such as acetic acid, furfural, and phenolic substances during the degradation process. Among them, acetic acid, as one of the main small molecule inhibitors of lignocellulose hydrolysis, can escape the cell membrane through passive transport, inhibit the physiological metabolism of organisms, and cause oxidative damage to cells.

Histone is one of the main protein components of chromatin, and its free N-terminal lysine and serine residues can be modified in many ways, among which methylation and acetylation are the most common. Histone modification can affect gene expression directly or indirectly. In this experiment, we performed a point mutation on lysine (H3K56Q) at the 56th position in histone H3 of *Saccharomyces cerevisiae*. Glutamine residue can simulate the acetylation state of lysine, so as to detect the effect of H3K56 simulated acetylation state on acetic acid tolerance of *Saccharomyces cerevisiae*. The experimental results showed that H3K56 simulated acetylation reduced the xylose metabolism performance of brewing yeast.

The object of the work is brewing yeast *Saccharomyces cerevisiae*.

The subject of the work is influence of 56th position in histone H3 mutation on lysine to xylose metabolism of brewing yeast *Saccharomyces cerevisiae*.

The aim of the work is to investigate the role of H3K56 simulated acetylation to xylose metabolism performance of brewing yeast.

The tasks of the work is to provide 56th position in histone H3 mutation on lysine and study the influence of this mutation to xylose metabolism performance of brewing yeast.

Key words: Ethanol , Acetic acid tolerance , Lignocellulose , Saccharomyces cerevisiae, H3K56Q

CONTENT

INTRODUCTION

The degradation of cellulose by brewer's yeast to produce bioethanol is considered one of the most promising ways to alleviate oil scarcity, but the production of acetic acid as a by-product of lignin hydrolysis can severely inhibit normal nutrient uptake, energy metabolism, cellular value addition and even induce programmed death in brewer's yeast cells. Acetic acid is an inhibitor of hydrolysis products from cellulose in industrial processes such as brewing and bioethanol production. It is therefore crucial to understand the mechanisms by which yeast responds to acetic acid stress is used to improve acetic acid tolerance and ethanol production. Studying the mechanism of acetic acid stress on brewer's yeast cells is fundamental for modifying industrial strains to obtain strong tolerant, high yielding bioethanol, and for promoting the development of bioethanol as an alternative to traditional fossil fuels. Industrial microorganisms are inhibited by a variety of stress conditions, including products and substrates. Therefore, improvements in resistance to stress are of great importance for industrial microbial production. Acetic acid is a major inhibitor in cellulose hydrolysis products, which severely affects cell growth and metabolism in brewer's yeast. The molecular mechanisms underlying the adaptive response and the benefits of breeding brewer's yeast for more efficient production of robust strains of industrial yeast for acetic acid tolerance have been investigated. Therefore, improving the acetic acid resistance of brewer's yeast is of great importance to improve the efficiency of cellulosic ethanol fermentation.

The physiological and metabolic analysis of *Saccharomyces cerevisiae* was then carried out using DNA purification, PCR amplification systems, agarose gel electrophoresis, gum recovery, extraction of the yeast genome, yeast transformation, *E. coli* transformation, plasmid extraction, fermentation, Ura dropout, and the creation of a long curve. Specifically, the H3K56 mimetic acetylation state significantly inhibited xylose metabolism in *Saccharomyces cerevisiae*. This suggests that histone modifications play an important role in regulating the response

of brewer's yeast to acetic acid. In summary, this study reveals the important role of histone modifications in the regulation of acetic acid tolerance in *Saccharomyces cerevisiae* and provides new ideas for fuel ethanol production and sustainable energy development. In-depth studies on the effects of histone modifications on microbial metabolism will further promote research and applications in the field of bioengineering and facilitate the development and utilization of renewable energy.

The thesis consists of an abstract, an introduction, experimental equipment, experimental methods, results and analysis, a summary, references and acknowledgement.

CHAPTER I
RESEARCH BACKGROUND

1.1 Current status and prospects of fuel ethanol development

1.1.1 Advantages of fuel ethanol as a new energy source

Fuel ethanol has many advantages as a renewable biomass energy source. Firstly, the burning of ethanol emits less carbon dioxide than traditional petroleum fuels, helping to reduce air pollution and global warming[1.] Secondly, fuel ethanol can be blended with petroleum fuels, reducing dependence on traditional petroleum resources. In addition, fuel ethanol has good blendability and tunability, allowing it to be used well in different types of engines. The production of fuel ethanol can also contribute to the development of local agricultural industries and improve local economies. The use of fuel ethanol can have a positive impact on global energy and environmental issues. The use of fuel ethanol can reduce greenhouse gas emissions, reduce dependence on non-renewable resources and can support the economic development of rural areas. In addition, fuel ethanol can be used as an alternative to petroleum-based fuels, increasing energy diversity and security[2]. The use of fuel ethanol has good prospects for application in the automotive sector. A comprehensive assessment of the combustion, power, economic and environmental performance of fuel ethanol shows that fuel ethanol has a wide range of future applications in the automotive sector[3].

1.1.2 Disadvantages of fuel ethanol as a new energy source

However, there are a number of limitations to the production of fuel ethanol. Firstly, the production of fuel ethanol is limited by the availability of biomass feedstocks, so fuel ethanol production may compete with agricultural needs such as food and feed production. Secondly, the process of producing fuel ethanol requires large amounts of water and energy, resources that may be scarce in some areas. In addition, the lower density and energy content of fuel ethanol requires greater

storage and transport space, which has implications for transport costs and ease of use. The cost of fuel ethanol production remains relatively high and therefore requires support and innovation in terms of policy and technology. In addition, fuel ethanol production may increase land use pressure and carbon emissions from agricultural production, and therefore requires attention to ecological conservation and sustainability in the production process[4]. There are also a number of technical and economic constraints to the use of fuel ethanol in the automotive sector. These include issues such as compatibility of fuel pumps and automotive components, durability of the fuel and cost. In addition, properties such as combustion performance and energy density of fuel ethanol need more research and optimisation in the automotive sector[5].

1.1.3. Prospects for fuel ethanol development

Fuel ethanol is a renewable energy source that is converted from biomass such as crops, waste and forest trees，Producing ethanol from starch is more costly and less economical than from sugar cane[6-11]. Compared to fossil fuels, fuel ethanol can reduce greenhouse gas emissions and reduce environmental pollution, while also reducing dependence on fossil fuels such as oil and diversifying energy sources. The use of fuel ethanol as a vehicle fuel also has the benefit of improving engine performance and efficiency, while reducing fuel costs. In some countries, governments have started to promote the use of fuel ethanol by setting up policies and regulations to facilitate its development. In addition, the process of producing fuel ethanol can also lead to the development of industries such as agriculture, forestry and waste treatment, creating more jobs. In conclusion, fuel ethanol is an important renewable energy source that can reduce greenhouse gas emissions, reduce environmental pollution, reduce dependence on fossil fuels and have multiple benefits in terms of economic development and job creation.

1.2 Scope for lignocellulosic ethanol

1.2.1 Sources and applications of lignocellulose

With the current increasing energy shortage, plant fibre resources have become one of the most promising raw materials for ethanol production, which are rich in cellulose and hemicellulose[12]. Of these resources, straw is one of the most abundant[13], with China producing around 630 million tonnes of straw each year, dominated by rice straw, maize straw, sorghum straw, sugar cane bagasse and cotton straw[14]. At present, only a small amount of straw is used in the paper, construction and textile industries, or as coarse feed and fuelwood (its thermal efficiency is only about 10%)[15], while most straw is underutilized (Table 1).

Table 1

Plant fibre resources for ethanol production

Fibre sources	Quantity available	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)
Grain shells	Available in large quantities	40-50	20-25	5-8	12-15
Cotton straw	Available in large quantities	65-75	10-20	2-5	7-10
Straw	Available in large quantities	35-45	20-30	2-5	10-15

Continuing table 1

Maize straw	Available in large quantities	40-50	25-30	2-4	6-8
Corn cobs	Available in large quantities	25-35	30-40	2-3	3-5
Peanut shells	Available in large quantities	36-42	15-20	4-5	3-4
Coconut shells	Available in large quantities	36-35	25-30	2-5	1-3
Bran	Moderate	35-45	20-25	10-15	5-7
Reeds	Larger	35-45	15-20	3-5	6-8

Therefore, the conversion of crop straw into ethanol through microbial fermentation has become an important research direction[16].

1.2.2 Advantages of using lignocellulose as a feedstock for the production of secondary ethanol

Lignocellulose is a carbon neutral bioenergy feedstock with huge reserves and is more sustainable than fossil fuels. Globally, around 200 billion tonnes of lignocellulosic pollutants are emitted each year, mostly in the form of agroforestry waste, horticultural waste and cash crop waste17. Making full use of these wastes can alleviate China's fossil energy shortage and effectively address the country's energy security18. China produces a total of 1.5 billion tonnes of agricultural and agroforestry waste each year, including 900 million tonnes of straw and various types of lignocellulosic waste. These wastes can be converted into energy with a potential of up to 100,000 tonnes of standard coal. Biorefinery technology can

convert lignocellulose into sugars and biomass fuels. Russia, which generates more than 287.5 million tonnes of lignocellulosic waste from wood each year, is another major contributor.

1.3 The need to improve the acetic acid tolerance of second-generation ethanol fermentation strains

1.3.1 Brewer's yeast in second generation ethanol production

Brewer's yeast is a microorganism that is widely used in the winemaking process. In the traditional winemaking process, brewer's yeast is used to ferment grape juice[19-21] or wort to produce alcohol and carbon dioxide. However, with advances in science and technology and the development of processes, the range of applications for brewer's yeast has expanded and is widely used in second-generation ethanol production. In second generation ethanol production, brewer's yeast is used to ferment other raw materials such as wort[22], starch and molasses to produce different types of alcoholic beverages. This type of production offers greater flexibility and versatility compared to traditional wine or beer production. Below we will present a few examples of the use of brewer's yeast in second-generation to pure production: Fruit wine production: Brewer's yeast can be used to ferment various fruit juices, such as apples, pears and grapes, in order to produce fruit wines. Fruit wines have a rich taste and aroma and are a popular beverage. By adjusting the yeast strains and fermentation conditions, fruit wines of different tastes and flavours can be obtained. Yellow wine production: Yellow wine is a traditional Chinese fermented wine, using rice[23] as the main raw material. Pure grain wine production: Pure grain wines are made using a variety of grains as raw materials for fermentation, such as wheat, corn and rice. Brewer's yeast can play an important role in the production of pure grain spirits. Yeast converts the starch in the grain into alcohol through fermentation and releases aromas and flavours, giving the wine a special taste and flavour. Foreign wine production: Brewer's yeast is also widely used in the production of foreign wines. The use of brewer's yeast in second-

generation Epsom production not only expands the range and flavour of alcoholic products, but also offers greater market potential and scope for innovation. By selecting different yeast strains, adjusting fermentation conditions and controlling yeast activity, producers can precisely tailor the taste, flavour and quality of their products to meet the diverse needs of consumers[24-25]. However, the use of brewer's yeast in secondary to pure production also faces a number of challenges, such as the selection and cultivation of yeast and the control of fermentation conditions. Therefore, it is necessary for alcoholic beverage producers to strengthen their research and technological innovation to continuously improve the application technology and fermentation process of yeast in order to enhance the quality and competitiveness of their products. In conclusion, brewer's yeast plays an important role in second generation to pure production, providing a key fermentation role in the production of various types of alcoholic products. By making rational use of yeast characteristics and regulating fermentation conditions, producers can produce alcoholic products with a rich and distinctive flavour to meet the diverse needs of consumers. The application of brewer's yeast will continue to drive the development and innovation of the alcohol industry.

1.3.2 Pretreatment of lignocellulosic hydrolysate

The conversion from lignocellulosic biomass to fuel ethanol is a very complex process very complex process, which mainly consists of raw material pretreatment, cellulose lignocellulosic biomass to fuel ethanol is a very complex process that involves pre-treatment of the feedstock, hydrolysis of the cellulose, fermentation and distillation[26].Pretreatment of lignocellulose is one of the key steps in the biomass saccharification process, aiming to break the crystalline structure of the cellulose and make it amenable to subsequent saccharification and fermentation. Common pretreatment techniques include physical, chemical and biological methods such as mechanical grinding, acid-base treatment, hot water treatment, biomass decomposition and ionic liquids[27]. Among these, acid-base treatment is a common method to promote degradation and depolymerisation of lignocellulose by adjusting

the pH of the hydrolysate[28]. In addition, hot water treatment is an economical and practical pretreatment method that can break the crystal structure of lignocellulose by means of high temperature and pressure, making it easier to degrade[29].

1.3.3 Strategies to improve the tolerance of brewer's yeast to acetic acid

The aggregative nature of yeast cells can be induced by specific environmental factors. Aggregation is widely used in the recycling of cells, and aggregation properties also contribute to strain survival under stress conditions[30-31]. The addition of zinc ions can alter the global transcription of genes, thus affecting fermentability under acetic acid stress conditions[32-34]. The method for improving acetic acid resistance in *Saccharomyces cerevisiae* by point mutation involves several steps: Selecting the target genes: Genes related to acetic acid metabolism are selected as mutation targets, for example ADH1 and ADH2, which play a key role in the acetic acid metabolism pathway[35]. Designing mutation sites: Mutation sites are identified by comparing gene sequences to identify conserved regions related to acetic acid metabolism. Mutation prediction software, such as PolyPhen-2 and SIFT, is often used to predict the effect of mutations on protein structure and function[36]. Selecting mutation methods: Mutations can be performed using chemical mutagens, gene editing techniques and genetic engineering, with gene editing techniques most commonly used, such as CRISPR/Cas9 technology[37]. Screening for mutants: Selection pressures such as high concentrations of acetic acid are used to screen for mutants. For multiple mutants, functional analysis is performed to screen for mutants with significant acetic acid resistance[38] .

1.4 A brief introduction to epigenetics

1.4.1 Concepts of epigenetics

Epigenetics is the study of genetic changes in the transmission of genetic information that do not involve alterations in DNA sequence. It is concerned with the mechanisms that affect gene expression and cellular function through genetic modifications such as DNA methylation, histone modifications and non-coding RNA regulation. Epigenetic studies reveal how environmental factors and lifestyles interact with genes to influence individual health, disease susceptibility and developmental processes[39-41].

Epigenetics is studied in a wide range of fields, including cell differentiation, tumourigenesis, environmental adaptation, and behavioural expression. The main studies in epigenetics include the types, distribution and functions of epigenetic modifications, and the role of epigenetic modifications in the development, progression and treatment of disease. Common epigenetic modifications include DNA methylation, histone modifications, and non-coding RNAs, which can affect cellular function and physiological states by regulating gene expression. The study of epigenetics is important for understanding the occurrence, development and treatment of diseases, as well as for further development of the fields of biology and medicine.

1.4.2 The role of histone modifications in the regulation of gene expression

In eukaryotic cells, the basic unit of chromatin is the nucleosome. In the 1990s, histones were generally considered to be envelope proteins for DNA and had no role in the regulation of gene expression. Now, however, with the development of epigenetics, histones play an important role not only in the regulation of gene expression but also in DNA damage repair, replication and recombination. Histone modifications include acetylation and deacetylation, methylation and demethylation, phosphorylation and dephosphorylation, and deubiquitination, and are critical steps in the process of DNA replication, transcription and repair. Histone modification is

an epigenetic regulatory mechanism that can affect gene expression by altering the structure and state of chromatin. Histone modifications include acetylation, methylation, phosphorylation and ubiquitination[42,] of which acetylation and methylation are the most common. Acetylation[43] is the addition of acetylated groups to histones by histone acetyltransferases (HATs), while histone deacetylases (HDACs) remove acetylated groups. Methylation[44] is regulated by histone methyltransferases and demethylases, which can add methyl groups to lysine and arginine residues.

Histone modifications can affect gene expression levels by a number of mechanisms including:

(1) altering chromatin accessibility: acetylation of histones can promote chromatin relaxation and transcription factor binding, thereby increasing gene transcription levels; whereas histone methylation is usually associated with chromatin compacting and gene silencing.

(2) Recruitment of transcription factors: Histone modifications can recruit transcription factors to bind to chromatin, thereby regulating gene expression.

(3) Activation or inhibition of transcriptional complex activity: Histone modifications can promote or inhibit the activity of transcriptional complexes, which in turn regulate gene expression.

In conclusion, histone modification is an important epigenetic regulatory mechanism that plays an important role in the regulation of gene expression [45].

1.5 Purpose of the experiment

The degradation of cellulose by brewer's yeast to produce bioethanol is considered one of the most promising ways to alleviate oil scarcity, but the production of acetic acid as a by-product of lignin hydrolysis can severely inhibit normal nutrient uptake, energy metabolism, cellular value addition and even induce programmed death in brewer's yeast cells.

Acetic acid is an inhibitor of hydrolysis products from cellulose in industrial processes such as brewing and bioethanol production[46]. Therefore, understanding

the mechanisms by which yeast respond to acetic acid stress is essential for improving acetic acid tolerance and ethanol production. Studying the mechanisms of acetic acid stress on brewer's yeast cells is fundamental for modifying industrial strains to obtain strongly tolerant, high-yielding bioethanol, and for advancing the development of bioethanol as an alternative to traditional fossil fuels. Industrial microorganisms are inhibited by a variety of stress conditions, including products and substrates. Therefore, improvements in stress tolerance are of great importance for industrial microbial production.

Acetic acid is a major inhibitor in cellulose hydrolysis products, which severely affects cell growth and metabolism in brewer's yeast[47]. The construction of acetic acid tolerant yeast, which is the chassis microorganism for the industrial production of ethanol, can accelerate the enzymatic hydrolysis and shorten the production time of ethanol, making the production process economical and addressing the high production cost, which is an important factor limiting the development of the bioethanol industry[48-50].

To investigate the molecular mechanisms underlying the adaptive response and the benefits of breeding brewer's yeast for more efficient production of robust strains of industrial yeast with acetic acid tolerance. The aim of this experiment was to perform a point mutation of lysine (H3K56Q) at position 56 in brewer's yeast histone H3 in order to obtain brewer's yeast that are highly tolerant to acetic acid and to improve the efficiency of lignocellulose utilization by brewer's yeast.

Summary of the chapter I

The degradation of cellulose by brewer's yeast to produce bioethanol is considered one of the most promising ways to alleviate oil scarcity. The first chapter focuses on the importance of lignocellulose utilization by brewer's yeast, as well as the prospects for fuel ethanol development, sources and applications of lignocellulose, and the need to improve acetic acid tolerance in second-generation ethanol fermentation strains, and the role of histone modifications in regulating gene expression.

1.Current status and prospects of fuel ethanol development.

2. Scope for the development of lignocellulosic ethanol .

3.The need to improve the acetic acid tolerance of second-generation ethanol fermentation strains.

4. A brief introduction to epigenetics.

5. Purpose of the experiment

CHAPTER II
MATERIALS AND METHODS

2.1. Experimental apparatus

Ultra-clean bench, microwave oven, agarose gel electrophoresis, gel imager, temperature-controlled shaker, tabletop high speed centrifuge Fresco1, high pressure steam extinguisher, bacterial pot LDZX-50KBS, constant temperature incubator, water bath, induction cooker, vortex oscillator, high performance liquid chromatography (HPLC), ice machine, gene amplification instrument, three-well thermostatic water bath, micro palm centrifuge, spectrophotometer.

2.2. Culture media and reagents

2.2.1. Method of preparing the culture medium

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

2) YEPD liquid medium: 2% peptone, 1% yeast powder, 2% glucose plus 2% agar (solid medium)

3) Yeast complete synthesis medium (SC): Yeast basic nitrogen source (YNB) 1.7 g/L, ammonium sulphate 5 g/L, CSM-URA 0.77 g/L, supplemented with uracil to a final concentration of 20 mg/L.

4) Complete synthesis medium for uracil deficiency (SC-URA): yeast base (YNB) 1.7 g/L, ammonium sulphate 5 g/L, CSM-URA. nitrogen source (YNB) 0.77 g/L. Add 2% agar and adjust pH to 6.0-6.5 with NaOH (solid medium). Note: Glucose is usually additionally prepared as a master batch at 40% concentration, 44 g/100 mL, and then diluted to 2%.Sterilisation: 115°C, 30min.

2.2.2. Reagents

Anhydrous ethanol, agar powder, salmon sperm DNA, agarose, tryptone, pentafluoroorotic acid, magnesium sulphate, topical dd water, KOD buffer, DMSO solution, yeast nitrogen source base, nucleic acid dye, yeast paste, polyethylene glycol, DL5000 DNA Marker, DNA Loading buffer, Rapid Taq Master Mix, dNTPs, glucose.

2.3. Experimental strains and primers

2.3.1. Experimental strains

Strain:BSPZ2001

Template :S288C

2.3.2. Primers

In the work we used primers, listed in Table 2.

Table 2

Primers used un the study

Primer name	Primer sequence number
P1	GGAGCCATTTGTTAATATACCG
P3	CTTGGTACTAATTCCGGAAG
P4	GTGGTGGATTTTGGAAGG
P6	CTGGAGTAATTTTGAGATTGCGC
P8	GGGGAGATATACCGTAGCAG
Ura	CTTGACTGATTTTTCCATGG
Ura-2	CCCTTCCCTTTGCAAATAG

Summary of the chapter II

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

CHAPTER III
EXPERIMENTAL METHODS

3.1. DNA purification

Place the cut pieces of gel containing the target segment in an EP tube, weigh using a balance and add BindingBuffer to a concentration of 0.1 mg/μL.

2) Place in a water bath at 60°C and turn the tube upside down every 2 min to accelerate the dissolution of the gel until it is completely dissolved and the solution is added to the column.

3) Centrifuge at 10,000 rpm for 1 min and discard the supernatant, then again at 10,000 rpm for 1 min and discard the supernatant.

4) Add 300 μL of Binding Buffer, centrifuge at 10,000 rpm for 1 min and discard the clear solution.

5) Add 700 μL of Wash buffer and centrifuge at 10,000 rpm for 1 min.

6) Repeat, again at 10,000 rpm for 1 min.

7) Transfer the column to a new EP tube and leave for 2 min.

8) Add 35 μL of ddH2O to the centre band, centrifuge at 12,000 rpm for 1 min, discard the clear solution and centrifuge again. Purified DNA was obtained.

3.2. PCR amplification system

1) Reagents: KOD buffer, 5μL, external dd water: 31μL, dNTPs: 2μL, MgSO4: 2.5μL, DMSO: 2.5μL, primers: 1μL, template: 1μL.

2) Reaction procedure: pre-denaturation 95 ℃, 3 min; denaturation 95 ℃, 15 min; annealing 55 ℃, 15 s; extension 72 ℃, 4 min; 35 cycles (95 ℃ 15 s, 55 ℃ 15 s, 72 ℃ 4 min); post-extension 72 ℃, 5 min; storage 4 ℃.

3.3. Agarose gel electrophoresis

Weigh 0.25 g of agarose into a conical flask, add 25 mL of 1 x Tae buffer (1 g of agarose per 100 mL of 1 x Tae buffer, depending on the number of samples), shake and mix well.

2) Dissolve in a microwave oven on high and boil three times until colourless, transparent and homogeneous without impurities.

3) Add 2.5 μL of nucleic acid dye (at a concentration of 0.1 μg/mL) and mix wel.

4) Place and fix the comb on the glue-making board and pour the glue along the board in one go.

5) When the gel is completely solidified, gently pull out the comb and transfer the gel bath with the gel to the electrophoresis bath, adding 1×Tae buffer until the gel plate is submerged.

6) Take 2 μL of 10×DNA Loading buffer and the DNA sample to be tested, mix well and add the tip of the pipette along the edge of the wells to avoid damaging the wells, and add the sample quickly.

7) Connect the electrophoresis tank and electrophoresis instrument correctly, switch on the power, set the voltage and time, and carry out electrophoresis.

8) At the end of electrophoresis, transfer the gel block to the gel imager and observe the results.

3.4. Glue recycling

1) Add 5μL of loading buffer.

2) Mix well.

3) Dispense all 50μL of PCR in the glue wells.

4) Running Gum.

5) dd an equal proportion of the loinding buffer to the cut EP tube.

6) 60 degrees water bath for 10min until the gel is completely melted, transfer to the adsorption column, 1000r, 1min to suck the lower part of the liquid back to the adsorption column and centrifuge again.

7) Remove supernatant, add 600μL washing bufler and repeat twice.

8) Empty clutch, 2000r, 2min.

9) Set up the adsorption column on a new EP tube and allow to dry.

10) Add 35 μL dd water.

3.5. Extraction of the yeast genome

Add 200 μL of DNA extraction solution.

2) Planting the yeast into the DNA extract.

3) Mix the yeast and DNA extracts well.

4) Place the glass beads in the EP tube, 0.4g.

5) Open the air cupboard.

6) Take DNA Extract 2 (avoid light) Aqueous phase above, liquid phase below.

7) Lift the following DNA extract to an EP tube, 200μL, capping the EP tube.

8) Using a vortex mixer, put symmetrically and mix evenly, vortex for 90 seconds.

9） Centrifugation: 13000r, 10min

10) Take ten new EP tubes and add 1 ml of anhydrous ethanol.

11) After centrifugation is complete, the bottom layer of glass beads, with the broken cells in the middle in white.

12) Whisk the supernatant into anhydrous ethanol.

13) Turn top and bottom upside down and leave for 10min net.

14) Centrifugation: 13000r, 10min.

15) After centrifugation, pour off the liquid and control the supernatant dry.

16) After natural drying, add 35μL dd water.

3.6. yeast transformation

Take bacteria 056-Nat and incubate in 5 mL of YPD medium at 30 °C for 12 h in a 200 rpm shaker.

2) Place on a shaker at 30°C for 4 h at 200 rpm until the OD is around 1.0.

3) Transfer the bacterial solution into a sterile isolation tube, centrifuge at 4500 rpm for 1 min, remove the supernatant, suspend in suitable sterile water, centrifuge the supernatant and wash with water 1-2 times.

4) Add 1 mL of 0.1 mol/L LiAC, suspend the organism, mix gently and invert, transfer to an EP tube, centrifuge at 4500 rpm for 1 min and remove the supernatant.

5) Add 240 μL of 50% PEG, mix well, then add 36 μL of 1 mmol/L LiAc, 30 μL of single salmon salmon dextrin DNA (boil at 100°C for 5 min and place on ice quickly), 4 μL of ddH2O and 50 μL of DNA and mix well.

6) The EP tubes were placed in an incubator at 30°C for 30 min, then on ice for 5 min, and then thermally stimulated in a water bath at 42°C for 40 min.

7) Centrifuge at 4500 rpm for 1 min, remove the supernatant, add 400 μL of ddH2O to the suspension and aspirate, apply 200 μL of DNA to a YPD plate and photocopy onto a SC-URA-Nat plate on the second day; apply 50 μL of plasmid to a SC-URA-Nat plate.8) Place upside down, incubate at 30°C and wait for single colonies to emerge.

3.7. *E. coli* transformation

1) Add 1 μL of plasmid to 50-100 μL of frozen-thawed receptor cells and place on ice for 30 min.

2) Heat in a 42 °C water bath for 90 s and place on ice for 3 min.

3) Add 1 mL of LB medium and place in a 37°C incubator for 1 h.

4) Apply 100 μL-200 μL of bacterial solution to a screening plate (LB+100 μg/mL Ampicillin), place in a 37°C incubator for 12 h, and then select single colonies for PCR validation.

3.8. plasmid extraction

1) Extract single colonies and apply to 5 mL of LB+Amp medium, incubate at 37 °C, 200 rpm/min for 12h.

2) Take the culture solution into an Ep tube, centrifuge at 12000 rpm/min for 1 min, collect the organisms, centrifuge and discard the supernatant until all the organisms are collected. Add 250 μL of Solution I (pre-filled with RNase I, stored in a refrigerator at 4 ℃) and shake to suspend the organism.

4) Add 250 μL of Solution II and gently invert up and down 8-10 times until the liquid is clear.

5) Add 350 μL of Solution III, gently invert up and down until a flocculent precipitate appears and centrifuge at 12000 rpm/min for 5 min.

6) Centrifuge the supernatant into a collection tube with the adsorbed plasmid, centrifuge at 12000 rpm/min for 1 min and discard the clear solution from the collection tube.

7) Add 500 μL of Buffer HBC, centrifuge at 12000 rpm/min for 1 min and discard the clear solution from the collection tube.

8) Add 600 μL of DNA Wash buffer, centrifuge at 12000 rpm/min for 1 min, discard the clear solution from the collection tube and repeat the procedure once.

9) Centrifuge at 12000 rpm/min and idle for 2 min.

10) Leave to dry at room temperature for 5 min with the lid open and allow the ethanol to evaporate completely.

11) Add 600 μL of sterile water and leave for 2 min, centrifuge at 12000 rpm/min for 1 min, add sterile water to the bottom of the isolated plasmid, then aspirate and add to the membrane, centrifuge again at 12000 rpm/min for 1 min.

3.9. Fermentation

1) The configured yeast solution is placed on a greenhouse shaker to be shaken and tightly sealed to prevent contamination by bacteria and other microorganisms. By adding medium and microorganisms to the shaker and then shaking the flask at a constant temperature and humidity, the growth of microbial cells and metabolic reactions can be promoted.

3.10 Throwing out the Ura

1) In a microwave oven at about 50 degrees, heat the pentafluoroorotic acid to a liquid.

2) Pentafluoroorotic acid, which has become an aqueous solution, is placed onto the ultra-clean table.

3) Make the medium by pumping and filtering and adding sugar.

4) After YPD inoculation, shake the bacteria for 15h.

5) Apply 100-200 μL of bacterial solution to a 5-FOA plate.

6) Leave to incubate and allow single colonies to grow.

7) Expansion of training.

3.11 Determination of the growth curve

1) Incubate 5 ml of bacteria in a shaker at 30°C for 12 h at 200 rpm.

2) Then transfer 40 ml to a shaker at 30°C for 12 h at 200 rpm to an OD of 0.2 for use.

3) Transfer the seed culture of the activated yeast into a 100 mL tri-oxide flask filled with 35 mL of fermentation medium, so that the initial OD600=0.23.

4) Incubate at 30°C and 200 rpm/min with shaking.

5) Aspirate the culture solution from the bottle every 6 hours and monitor the value at OD600, take the average value for each sample 3 times, if the solution is too thick, it should be diluted appropriately.

6) Plot the length of the yeast growth curve using the incubation time t as the horizontal coordinate and the absorbance as the vertical coordinate.

Summary of the chapter III

Chapter 3 focuses on the experimental methods and the specific procedures to be followed, step by step.The following sections are included:

1. DNA purification.
2. PCR amplification system.
3. Agarose gel electrophoresis.
4. Gum recovery.
5. Extraction of the yeast genome.
6. Yeast transformation
7. Transformation of *E. coli*.
8. Plasmid extraction.
9. Fermentation.
10. Dropping out the Ura
11. Estimating the temperature curve。

CHAPTER IV
RESULTS AND ANALYSIS

4.1 H3K56Q histone point mutant construction process

Natural brewer's yeast has a low tolerance to acetic acid in lignocellulose. However, histone modifications can regulate chromatin structure and DNA metabolism, including biological processes such as RNA transcription, DNA replication, DNA recombination and damage repair, and also have functions in regulating the cell cycle and signal transduction. Among them, histone lysine (K) modification is one of the most common forms of modification in chromatin. The effect of H3K56 on acetate tolerance in *Saccharomyces cerevisiae* was examined by point mutating lysine at position 56 (H3K56Q) in *Saccharomyces cerevisiae* histone H3, where the glutamine residue mimics the acetylated state of lysine. The final strain was obtained by PCR amplification in template strain S228C and cross-validation of the amplification into strain BSPZ2001 with the loss of Ura, and the H3K56Q histone point mutant was constructed as shown in (Figure 4.1, Appendix A):

4.2 Construction process of the H3K56Q histone point mutant

4.2.1 Amplification and transformation of DNA fragments

The genome of strain S288C was firstly amplified by PCR using the Ura-tagged histone point mutation S288C as a template and P1 and P4 as primers, respectively, to obtain DNA fragments carrying a point mutation from lysine (K) to glutamine (Q) at position 56 on histone H3 (Figure4.2, Appendix A).

4.2.2 PCR identification of positive clones

The extracted HHT2 gene containing the histone point mutation was transferred into the BSPZ2001-Nat strain using the PEG lithium acetate transformation method and positive clones were screened by SC-Ura+Nat plates

(Figure.4.4, Appendix B). Single colonies grown on plates of SC-Ura+Nat were selected. Positive clones grown on the plates were delineated on SC-Ura+Nat plates (Figure.4.3, Appendix B). DNA from the delineated strains was extracted and cross-validated by PCR using upstream:P8-Ura-2 and downstream:P6-Ura3 as primers.

4.2.3 Screening for URA gene deletion in histone white point mutant strains

The Ura gene was then applied to a plate containing 5-F-lactic acid (5-FOA) for screening. Since 5-FOA kills strains capable of synthesising uracil, the 5-FOA plate screening resulted in a histone white mutant strain with the Ura gene missing (Figure 4.5, Appendix C). To further validate the results, DNA was extracted from the obtained strains using P6 and P8 as primers and PCR amplification was performed. The PCR products were subjected to agarose gel electrophoresis and the electrophoretic bands were observed under UV light. The amplified bands, which were consistent with the predicted open reading frame length, were lower than before the loss of Ura, indicating that the mutant strain had lost Ura (Figure 4.6, Appendix C).

4.3 Acetic acid tolerance assay for the H3K56Q point mutant

4.3.1 Growth curve to detect acetic acid tolerance of the strain

In this experiment, the experimental results of brewer's yeast showed that: brewer's yeast was in the growth adjustment period in the first 4h of normal glucose medium, in the logarithmic period from 4 to 15h, and in the stable period after 15h. As shown by the yeast growth curves: the growth of the histone H3K56Q mutant strain was basically not very different from the control, as shown in (Figure4.7, Appendix D); in the glucose medium with acetic acid the brewer's yeast was in the growth adjustment period for the first 10h, in the logarithmic period from 10 to 50h, and in the stable period after 50h. As shown by the yeast growth curves: the growth

of the histone H3K56Q mutant strain was significantly lower than the control after 10h, as shown in (Figure 4.8, Appendix D)

Summary of the chapter IV

This chapter describes the H3K56Q histone point mutant construction process and construction procedures, as well as the analysis of experimental results and the depiction of growth curves including the following:

1.H3K56Q histone point mutant construction process.

2. Construction of the H3K56Q histone point mutant.

3. Acetic acid tolerance assay of H3K56Q point mutant.

CONCLUSION

1.In this experiment, we obtained a DNA fragment carrying H3K56Q from strain S228C by PCR and then inserted it into the genome of strain BSPZ2001-NAT using a homologous recombination process to successfully obtain a point mutant of H3K56Q in the background of BSPZ2001.

2.Through physiological experiments and metabolic analysis of *Saccharomyces cerevisiae*, we found that H3K56 mimics the acetylated state and reduces the tolerance to acetic acid in *Saccharomyces cerevisiae*. Specifically, the H3K56 mimetic acetylation state significantly inhibited xylose metabolism in *Saccharomyces cerevisiae*. This suggests that histone modifications play an important role in regulating the response of brewer's yeast to acetic acid.

3. In summary, this study reveals the important role of histone modifications in the regulation of acetic acid tolerance in *Saccharomyces cerevisiae* and provides new ideas for fuel ethanol production and sustainable energy development. In-depth studies on the effects of histone modifications on microbial metabolism will further promote research and applications in the field of bioengineering and facilitate the development and utilization of renewable energy.

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Appendix A

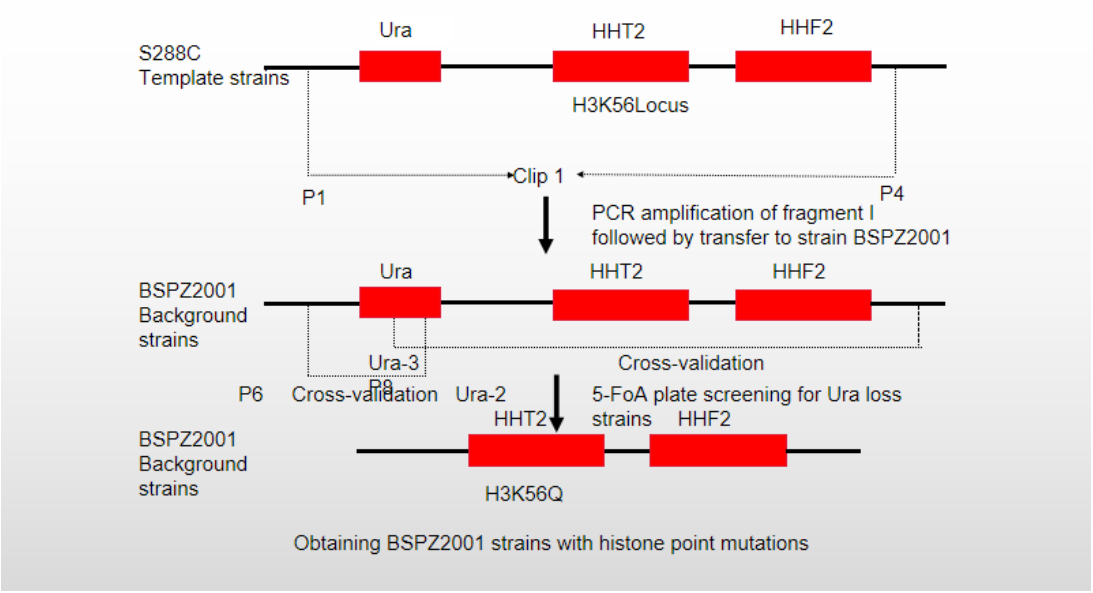

Figure 4.1. Design of primers

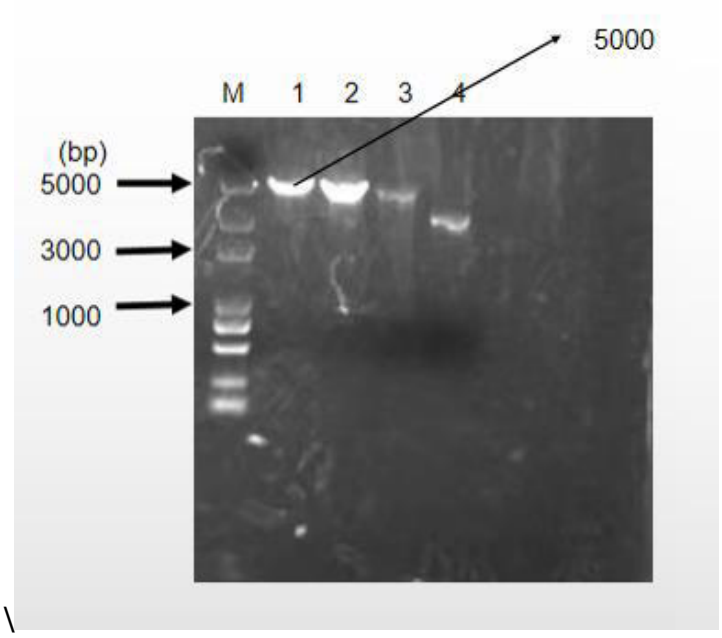

Figure 4.2. Amplification of DNA fragments carrying the H3K56 point mutation

M:DNA marker; 1:H3K56Q point mutation fragment

Appendix B

Figure 4.3. SC-Ura+Nat medium paddle monoclonal

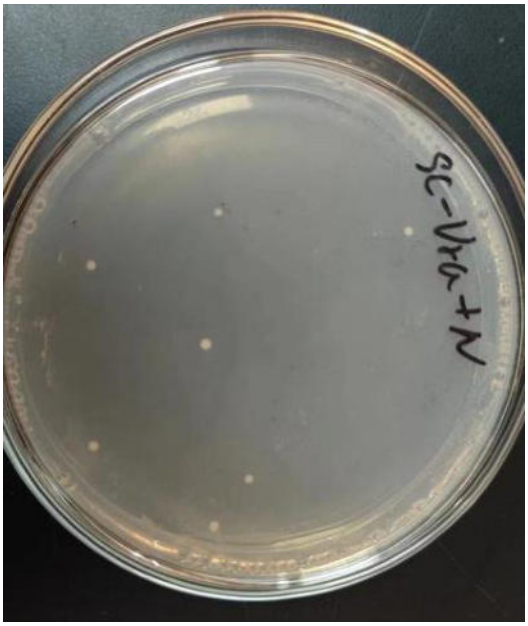

Figure 4.4. Positive clones delineated on SC-URA-NAT plates

Appendix C

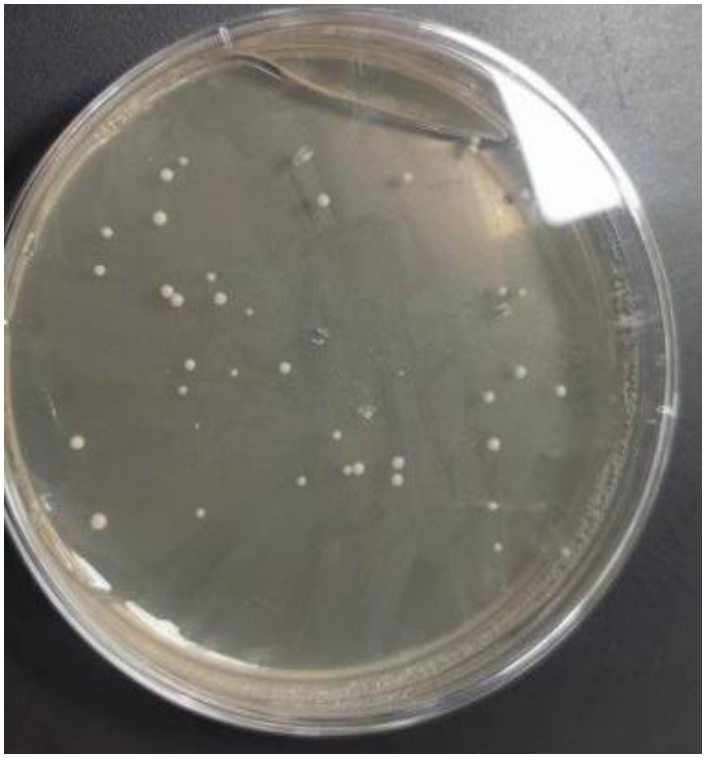

Figure 4.5. H3K56Q point mutant strain

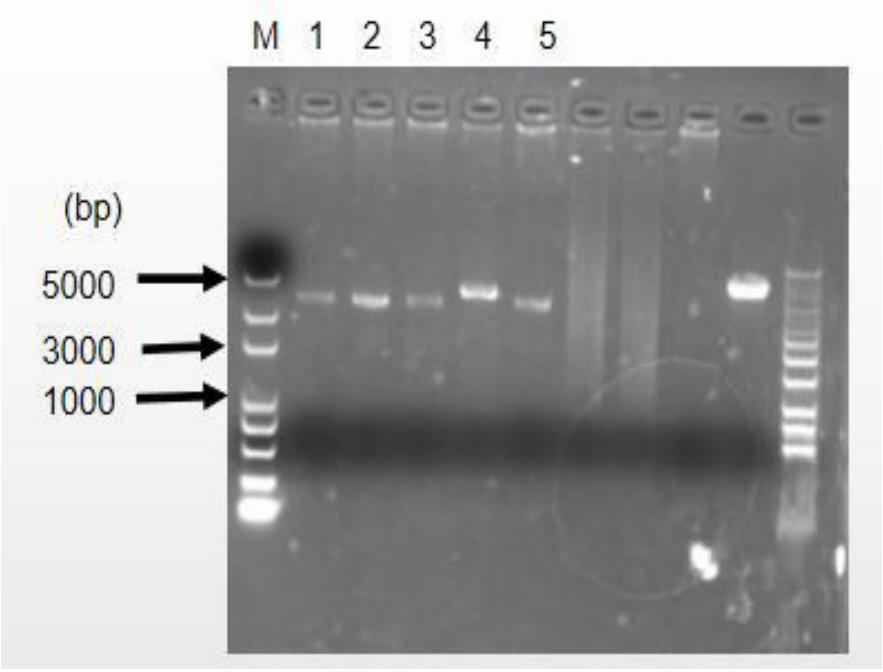

Figure 4.6. PCR amplification of the H3K56 mutant with deletion of the Ura gene.

Appendix D

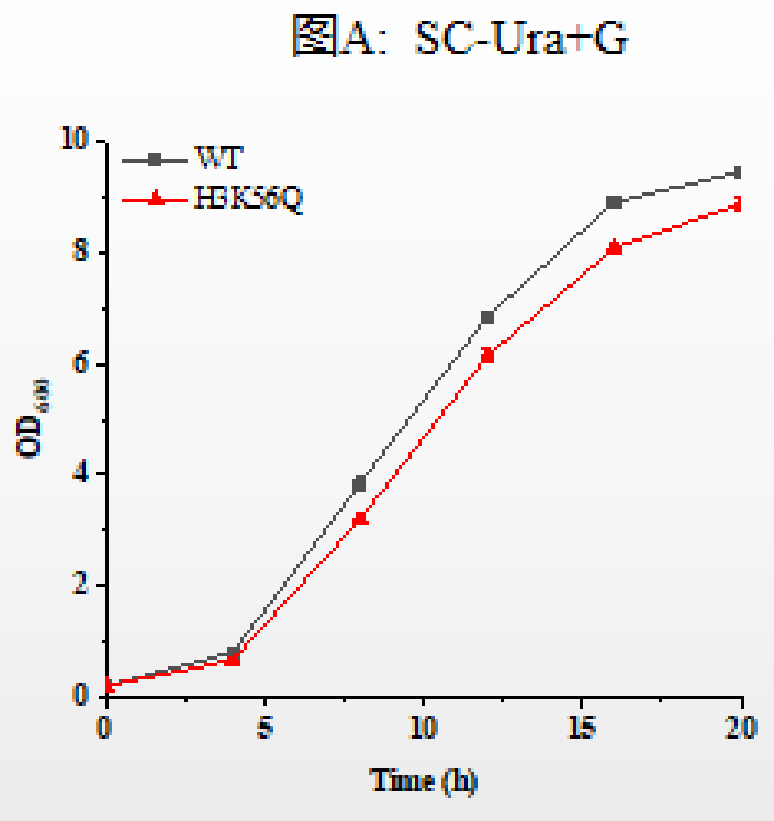

Figure 4.7. Growth curve of the strain in glucose medium。

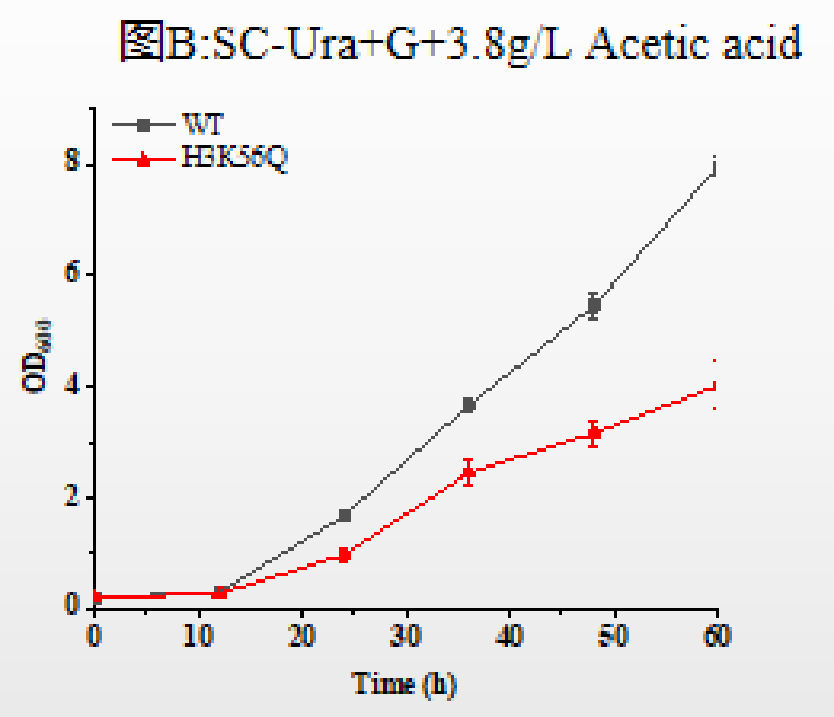

Figure 4.8. Growth curve of the strain in acetic acid and glucose medium