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

QUALIFICATION THESIS

on the topic **Analysis of the effect of ATG34 gene overexpression on acetic acid tolerance of *Saccharomyces cerevisiae***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Ruyun FAN

Scientific supervisor
Iryna VOLOSHYNA, Ph.D., As. prof.

Reviewer
Igor GRETSKY, Ph.D., As. prof.

Kyiv 2024

KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

Faculty: Chemical and Biopharmaceutical Technologies
Department: Biotechnology, Leather and Fur
First (Bachelor's) level of higher education
Specialty: 162 Biotechnology and Bioengineering
Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology,
Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Ruyun Fan

1. Thesis topic **Analysis of the effect of ATG34 gene overexpression on acetic acid tolerance of *Saccharomyces cerevisiae***
Scientific supervisor Iryna Voloshyna, Ph.D., As. prof.

approved by the order of KNUTD "____" _____2024, №_____

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

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____ Ruyun FAN

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Ruyun Fan. Analysis of the effect of ATG34 gene overexpression on acetic acid tolerance of Saccharomyces cerevisiae – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

At present, global energy security continues to face threats, in order to ensure sustainable energy and improve energy efficiency, to alleviate global warming, the transformation from traditional fossil fuels to clean energy has been vigorously promoted. The second generation of fuel ethanol is derived from lignocellulosic biomass by microbial fermentation, which has the advantages of low cost, renewable and environmental protection. Lignocellulose is composed of hemicellulose, cellulose and lignin. Its complex structure can not be directly utilized by microorganisms, so it needs to undergo pretreatment and hydrolysis process to generate fermentable monosaccharides before they can be utilized by microorganisms. However, lignocellulose produces a large number of small molecule compounds during the pretreatment process, which inhibits the fermentation process of microorganisms. Among them, acetic acid is a common inhibitor in a variety of lignocellulosic biomass hydrolytic components, which inhibits the growth and metabolism of yeast cells during fermentation, leading to ATP starvation and oxidative stress, which in turn leads to poor production of lignocellulosic biomass fuels and chemicals. Therefore, the acetic acid tolerance of microorganisms will directly affect the production efficiency of second-generation ethanol from lignocellulose. Saccharomyces cerevisiae is the preferred strain for producing second-generation ethanol because of its high sugar, high ethanol and low pH tolerance. However, the bacteria had a weak tolerance to small molecule inhibitors produced during lignocellulosic pretreatment. A histone point mutant with high acetic acid tolerance was screened in the previous laboratory work.

Transcriptome sequencing analysis showed that the expression level of *ATG34* gene in the histone point mutant was significantly increased. In order to verify the effect of *ATG34* gene on acetic acid tolerance of strains, we overexpressed the *ATG34* gene of the parent strains, and found that the acetic acid tolerance of strains overexpressed by *ATG34* was significantly improved through the growth curve. The results provided an effective reference for the construction of the second generation ethanol production strains with high acetic acid tolerance.

Key words: Saccharomyces cerevisiae; Acetic acid tolerance; Histone modification; Second generation fuel ethanol; Transcriptome analysis

TABLE OF CONTENTS

SUMMARY 4
TABLE OF CONTENTS 6
INTRODUCTION 8
CHAPTER 1 8
LITRATURE REVVIEW 8
1.1 Industrial value of lignocellulose conversion to fuel ethanol 8
1.1.1 Defects of first generation fuel ethanol 10
1.1.2 Second Generation Fuel Ethanol 10
1.1.3 Bottlenecks faced by second-generation biofuel ethanol 11
1.1.4 Pretreatment of lignocellulose 12
1.1.5 Research status at home and abroad 13
1.2 Toxic side effects of acetic acid 14
1.3 Advantages and disadvantages of Saccharomyces cerevisiae yeast 15
1.4 Histone modification regulates gene overexpression 16
Conclusions to chapter 1 19
CHAPTER 2 21
OBJECT, PURPOSE AND METHODS OF THE STUDY 21
2.1 Materials and Instruments 21
2.1.1 Experimental Instruments 21
2.1.2 Object of the study 21
2.1.3 Upstream and downstream primers 22
2.1.4 Culture medium 22
2.2 Experimental procedure 22
2.2.1 Design primers and amplify PCR 22
2.2.2 Agarose gel electrophoresis 23
2.2.3 DNA purification 23
2.2.4 Plasmid construction 24
2.2.5 E. coli plasmid extraction 24

2.2.6 Yeast transformation 25
2.2.7 Yeast plasmid small lift 26
2.2.8 Yeast colony PCR operation 27
2.2.9 Growth curve analysis method 27
Conclusions to chapter 2 28
CHAPTER 3 29
EXPERIMENTAL PART 29
3.1 PCR verification of ATG34 gene 29
3.2 Screening of overexpressed ATG34 gene 29
3.3 Screening for overexpression of *Saccharomyces cerevisiae* ATG34 gene .. 30
3.4 Growth curve 31
3.5 Discussion 32
Conclusions to chapter 3 33
CONCLUSIONS 34
LIST REFERENCES 36
Acknowledgement 39
APPENDIX 41

INTRODUCTION

The relevance of the topic is at a time when global energy security continues to face threats, the transition from traditional fossil fuels to clean energy has been vigorously promoted in order to mitigate global warming, ensure energy sustainability and improve energy efficiency. Due to the limitations of natural geographical conditions, renewable energy is still in its infancy, and there are fluctuations and instability, which increases the supply pressure. Therefore, in the short and medium term, the world is still difficult to get rid of its dependence on fossil energy. In this context, the second-generation fuel ethanol as a new type of clean energy has been vigorously promoted.

The purpose of the study is the overexpression of ATG34 gene by gene editing to improve acetic acid tolerance of saccharomyces cerevisiae. Due to the pretreatment of lignocellulose, some inhibitory components are generally formed that hinder the development of microorganisms. Acetic acid is the main toxic by-product, which has a strong effect on inhibiting the growth and metabolism of yeast cells during lignocellulosic fermentation. Inhibiting the growth of saccharomyces cerevisiae leads to ATP starvation and oxidative stress, which leads to poor production of lignocellulosic biomass fuels and chemicals, as well as accumulation of ROS, which leads to oxidative damage. Therefore, to study the molecular mechanism of acetic acid tolerance of saccharomyces cerevisiae, it is helpful to improve the fermentation performance and save the energy required for fermentation through gene editing.

The objectives of the study is Analysis of the effect of *ATG34* gene overexpression on acetic acid tolerance of Saccharomyces cerevisiae.

The object of the study is Saccharomyces cerevisiae.Saccharomyces cerevisiae is the most frequently used and widely used strain in yeast. Guided by human activities and after long-term domestication and adaptation, Saccharomyces cerevisiae has obtained unique genetic characteristics related to alcohol fermentation,

and has been widely used in renewable biomass, especially in the production of second-generation fuel ethanol from lignocellulose.

The subject of the study is ATG34 gene and Saccharomyces cerevisiae

Research methods is 1. gene overexpression: The target gene is cloned into the corresponding plasmid or viral vector, and a large number of genes can be transcribed and translated under artificially controlled conditions by using regulatory elements constructed on the vector skeleton, so as to achieve the overexpression of the target gene.

2. Primer design principles:

1 Primers are specific in the conserved region of DNA sequence

2 The length of the primer is generally between 15-30 bases

3 The GC content is between 40%-60%, and the amplified fragment is preferably between 40%-60% GC content, which is easier to expand; Primer Tm value range is 55-65℃, upstream and downstream primer Tm value should not be too different, it is best not to exceed 5 degrees.

4 The 3 'end of the primer can not choose A, it is better to choose T

The base should be randomly distributed, especially the 3 'end should not exceed 3 consecutive G or C

There should be no complementary sequence between the primers themselves and primers to prevent the formation of hairpin structures

3. Drug resistance screening: Using the difference in cell sensitivity to drugs to screen fusion cells. In this experiment, the medium containing ampicillin was used to screen Escherichia coli to obtain the Escherichia coli successfully introduced by the plasmid.

The scientific novelty the tolerance of Saccharomyces cerevisiae to acetic acid is extremely urgent in industrial production

The practical significance of the results obtained is Overexpression of *ATG34* gene can improve the tolerance of Saccharomyces cerevisiae to acetic acid

CHAPTER 1
LITRATURE REVVIEW

1.1 Industrial value of lignocellulose conversion to fuel ethanol

Currently, global energy security continues to be under threat, and clean energy transformation as well as market development has been specifically introduced to ensure energy sustainability and increase energy utilisation to mitigate global warming. Although clean energy has grown rapidly, it is still unable to meet global energy demand. Fossil fuels still occupy the top spot in the global energy supply ranking, with oil being the most consumed. Renewable energy is still in its infancy and is subject to volatility and instability due to physical and geographic constraints, adding to the pressure on supply. Therefore, in the short to medium term, it is still difficult for the world to get rid of its dependence on fossil energy [1]. Against this background, ethanol, a very good new clean energy source, comes into view.

Ethanol is a renewable energy source, not only an excellent fuel, but also an excellent fuel product additive. As an excellent property of fuel, ethanol is excellent in oxygenation and can be used as an oxygenator for gasoline to increase its internal oxygen content and promote fuller combustion, thus achieving the dual goals of energy saving and environmental protection. Ethanol can cost-effectively reduce aromatic and olefin content, thereby reducing refinery modification costs to comply with new gasoline standards. Bioethanol is ethanol obtained from biomass through biofermentation and other means. Bioethanol for automotive use can be manufactured by blending denatured bioethanol with gasoline in a certain ratio. Two of the second generation fuel ethanol production technologies, first generation and second generation, are more widely used [2].

In recent years, due to the volatility of oil prices, fuel ethanol consumption growth has accelerated. China's fuel ethanol industry started late, but it is developing rapidly, and fuel ethanol has broad prospects in China. With the further

increase of domestic oil demand, the energy supply diversification strategy represented by alternative energy such as ethanol has become a direction of China's energy policy. China has become the world's third largest biofuel ethanol producer and application country after Brazil and the United States. The National Development and Reform Commission issued the Guiding Opinions on Promoting the Healthy Development of the Corn Deep Processing Industry, requiring that no new fuel ethanol projects with corn as the main raw material should be built, and vigorously encouraging the development of fuel ethanol with non-food crops as raw materials. Fuel ethanol has moved towards the road of non-food ethanol development, and has been rapidly developed.

Fuel ethanol has clean, renewable and other characteristics, which can reduce carbon monoxide and hydrocarbon emissions in vehicle exhaust. In the future, the focus of China's fuel ethanol industry is to reduce production costs, reduce government subsidies, to develop biofuel ethanol production process consumption control standards, and product quality technical standards, unified fuel ethanol production consumption quota standards, including material consumption, water consumption, energy consumption, etc., is a powerful means to reduce costs and increase efficiency.

There are mainly two kinds of fuel ethanol production technology: the first generation and the second generation. The first generation of fuel ethanol technology is based on sugar and starch crops as raw materials to produce ethanol. The process is generally divided into five stages, namely liquefaction, saccharification, fermentation, distillation and dehydration. The second generation of fuel ethanol technology uses wood fiber quality as raw material to produce ethanol. Compared with the first generation technology, the second generation fuel ethanol technology first needs to be pre-treated, that is, to remove lignin, increase the porosity of raw materials to increase the contact between various enzymes and cellulose, and improve the efficiency of enzymes. After the raw material is

decomposed into fermentable sugars, it goes into fermentation, distillation and dehydration.

1.1.1 Defects of first generation fuel ethanol

Fermentation of first-generation bioethanol uses sugar or starch as feedstock, and the grains are first dry milled, i.e., they are ground and liquefied. This is followed by hydrolysis or saccharification, which breaks down the sugar (e.g. glucose) into monomers. Finally, the sugar monomers are converted into carbon dioxide and ethanol by yeast fermentation. Once fermentation is complete, the fermentation broth is distilled to purify the ethanol, which is then dehydrated to over 99.7% for use as a fuel. This process has been in industrial practice for decades and has low technical, feedstock and market risks. However, there is an ethical controversy arising from the use of potential human food as a feedstock, the so-called "food versus fuel" debate, with a wide divergence of views[3]. Therefore, although the first generation of bioethanol technology in the market has certain advantages but can not stop the rise of second-generation fuel ethanol.

1.1.2 Second Generation Fuel Ethanol

In response to the above problems as well as increasing global energy demand, greenhouse gas emissions and climate change, second generation biofuel ethanol production from lignocellulosic biomass in the form of organic residues has been introduced in search of sustainable, environmentally friendly, renewable and economically sustainable alternative energy sources [4]. On the other hand, large-scale production can apportion and reduce the unit cost, and given the limited global resources of starch and sucrose, it has become inevitable that the feedstock of fuel ethanol will expand from food to non-food feedstocks when scaling up the production. Second-generation ethanol based on lignocellulosic feedstock has made technological breakthroughs in pretreatment, enzymatic digestion and pentose/hexose co-fermentation, and has entered the industrial demonstration stage [5].

Second-generation fuel ethanol utilises lignocellulose - mostly biowaste such as straw and domestic waste. Lignocellulose, a major component of plant cell walls, is a natural polymer consisting of intertwined microfibres of three different polysaccharides [6] Lignocellulosic biomass material has the advantages of being widely available, abundant and conflict-free with food and arable land, making it one of the richest renewable resources on earth. Relying on the characteristics of its abundance and renewability, it is rapidly and widely utilised and has become the trend of the industry. Therefore, the improvement of its bio-application is crucial for the long-term development of society and sustainable biorefining.

1.1.3 Bottlenecks faced by second-generation biofuel ethanol

Lignocellulosic biofeedstock contains cellulose and hemicellulose however the available lignocellulose accounts for a relatively small proportion of the lignocellulose, due to the structural complexity and recalcitrance of lignocellulosic, its conversion into biofuels or other high value products requires special techniques and technologies and harsh conditions, and different sources of lignocellulosic feedstocks (e.g., agricultural wastes, forestry residues, etc.) have significant differences in their chemical composition and structure The chemical composition and structure of lignocellulosic raw materials from different sources (e.g., agricultural waste, forestry residues, etc.) vary significantly, making it difficult to unify pretreatment and hydrolysis conditions. The hydrolysis of lignocellulose to a variety of sugars accumulates non-sugar inhibitors (e.g., phenols, furfural, formic acid, acetic acid, etc.) in the hydrolysate that may interfere with the metabolic pathways of the yeast, resulting in a reduced ability to synthesise ethanol, which may also affect ethanol yield and purity. Secondly, the production cost of second-generation fuel ethanol is usually higher than that of fossil-fuel ethanol, mainly due to the high cost of processes such as feedstock pre-treatment, hydrolysis, fermentation and product separation and purification leading to the market competitiveness of second-generation bioethanol remaining limited. Finally, biofuel technology is still immature in some areas and still requires greater

investment in R&D to drive technological progress in the industry. Therefore, efficient fermentation of renewable sugars remains an important challenge for the economically viable production of biofuels and biochemicals [7].

1.1.4 Pretreatment of lignocellulose

The optimal pretreatment process should have low energy consumption and economy, minimise the destruction of active ingredients in the feedstock, while the pretreated product is easy to handle and free from substances that inhibit the decomposition of carbohydrates. Currently, a wide range of methods are used for the pretreatment of wood fibre biomass, including physical, chemical, biochemical and biological methods. Among the biological methods, microbial pretreatment, as an efficient and low-cost pretreatment technology, is widely used because of its gentle process, low energy consumption, low use of chemical additives, and high chemical utilisation.

Microbial pretreatment selectively degrades lignin in lignocellulose with the help of extracellular lignin-degrading oxidative enzymes such as cellobiose dehydrogenase, lignin peroxidase, laccase, and other enzymes secreted by microorganisms themselves [8]. Studies have shown that fungi play a dominant role in this process, and white-rot fungi are the microorganisms of choice for lignocellulosic biomass, relying on their strong lignin-degrading ability. However, some white-rot fungi can degrade both lignin and polysaccharides, which may lead to the loss of carbohydrates.

Numerous studies have now demonstrated the significant effect of combining biological pretreatment with chemical pretreatment to improve the degradation rate and sugar yield of lignocellulosic fibres. The chemical pretreatment method involves the hydrolysis of some of the chemical bonds of lignocellulose with the help of chemicals in order to accelerate the decomposition process. Although this method is effective, it produces moderately toxic intermediates and results in the loss of some active ingredients, and the pretreatment cost is relatively high. However, factors such as fungal species,

cultivation conditions, biomass type, degree of chemical pretreatment, and pretreatment sequence all have an impact on component content and sugar production[9]. Therefore, the cultivation of efficient and high-quality strains through genetic modification screening will become an important direction for the development of microbial pretreatment technology in the future.

1.1.5 Research status at home and abroad

1.1.5.1 International situation

Favorable regulations, policies, investments or funding provided by governments in North America and Europe have facilitated the development and commercialization of biofuels, while also driving the growth of biofuel sales. The global second-generation biofuels market is expected to grow at a compound annual growth rate of 49.4% between 2014 and 2020, and is expected to reach a total market value of $23.9 billion by 2020. Geographically, the second generation biofuels market is segmented into North America, Europe, Asia Pacific, and Latin America. Due to strong regulations and government financial support in developed countries - North America and Europe, these regions have historically had several market shares in 2013, but this situation will change by 2020. In order to pursue opportunities in the second generation bioethanol market, many companies are building plants.

1.1.5.2 The domestic situation

The key to the development of cellulosic ethanol, in addition to production technology, is to have sufficient raw material supply. With the rapid and healthy development of agricultural and forestry production in China, it is expected that the amount of crop straw available for use will continue to increase every year. This is a large amount of biomass raw material resources, in addition to the development of cellulosic ethanol, we should also develop biomass jet fuel and biomass diesel. China has not made fundamental breakthroughs in the production technology of cellulase and the construction of pentose fermentation strains, etc. At present, the raw material consumption of each cellulosic ethanol in the pilot study of various

units is more than 6 tons, and the production cost is estimated to be more than 5000-6500 yuan/ton of ethanol, which is not suitable for industrial production. It is reasonable to estimate that the large-scale production of cellulosic ethanol in China will take at least 3-4 years. At present, for cellulose ethanol should be more scientific research efforts rather than industrial development efforts.

1.2 Toxic side effects of acetic acid

Acetic acid, also called acetic acid, is an organic compound, chemical formu la CH3COOH, is an organic monic acid, the main component of vinegar. Pure anh ydrous acetic acid (glacial acetic acid) is a colorless hygroscopic liquid with a freez ing point of 16.6 ° C (62 ° F). After solidification, it becomes a colorless crystal, w hich is weakly acidic and corrosive in its aqueous solution. It is strongly corrosive t o metals, and the steam is irritating to the eyes and nose.Pretreatment of lignocellul ose generally results in the formation of some inhibitory components that can hind er the development of microorganisms. Since lignin and hemicellulose are highly a cetylated, they will produce acetic acid during pretreatment and appear in the hydr olysis products, so acetic acid tolerance is very important in industrial yeast strains used for second-generation bioethanol production [10], and in first-generation starch hydrolysates, acetic acid inhibits the growth and metabolism of yeast cells very str ongly during lignocellulosic fermentation, and it also leads to intracellular Acidific ation. Inhibition of the growth of Saccharomyces cerevisiae leads to ATP starvatio n and oxidative stress, which results in poor production of lignocellulosic biomass fuels and chemicals[11], and also leads to accumulation of ROS (reactive oxygen sp ecies), which can lead to oxidative damage[12]. Therefore, investigating the molecul ar mechanisms of acetic acid tolerance in Saccharomyces cerevisiae could help in t he selection of superior strains and could contribute to the improvement of ferment ation performance and the saving of energy required for fermentation. Although se veral genes for acetic acid tolerance have been identified in laboratory yeast strains and are included in the database (http://www.yeastgenome.org/observable/resistan ce_to_acetic_acid/overview), the genes that are naturally present in some strains of

Saccharomyces cerevisiae are also known to be responsible for high acetic acid tol erance. The genetic basis of high acetic acid tolerance remains unknown. Previous experiments have demonstrated that acetic acid tolerance in yeast strains used for s econd-generation bioethanol production can be improved by precise genome editin g, thereby minimising the risk of negative effects on other yeast properties[13] .

1.3 Advantages and disadvantages of Saccharomyces cerevisiae yeast

Saccharomyces cerevisiae (Saccharomyces cerevisiae) is the most frequently used and versatile strain of yeast [14], guided by human activities, and after a long period of domestication and adaptation, Saccharomyces cerevisiae has acquired unique genetic properties associated with winemaking and is widely used in the production of biofuels and biochemicals from renewable biomass, especially lignocellulosic feedstocks. As a Gram-staining positive bacterium, it has a short growth cycle, rapid reproduction, easy to be cultivated on a large scale, and has significant economic benefits. Saccharomyces cerevisiae is a parthenogenetic anaerobic microorganism that can survive in low or anaerobic environments, *and* can produce ATP through glycolysis and the tricarboxylic acid cycle under aerobic conditions, as well as alcohol and carbon dioxide through glycolysis under anaerobic or anoxic conditions. In addition, Saccharomyces cerevisiae is a typical eukaryotic model organism with complete genome sequencing and mature molecular genetic manipulation technology. In industrial production, Saccharomyces cerevisiae has a highly efficient sugar conversion ability, producing second-generation fuel ethanol so that the yield rate is close to the theoretical value, and also has a strong environmental adaptability to adapt to a variety of stressful conditions during the fermentation process, such as high sugar, high ethanol, low pH, anoxic conditions, etc., and the nutritional requirements are relatively low, requiring only glucose, ethanol, and a small amount of nitrogen source to carry out fermentation, which helps to reduce the cost of raw materials.

The role of Saccharomyces cerevisiae in alcoholic fermentation was analysed in depth through the biological properties, fermentation kinetics, and genetic engineering modification of Saccharomyces cerevisiae. Under aerobic or anaerobic conditions, Saccharomyces cerevisiae breaks down glucose into ethanol and carbon dioxide through glycolysis. Specifically, glucose is first converted to pyruvate, and then pyruvate loses a carbon atom catalysed by decarboxylase to produce acetaldehyde. Acetaldehyde is reduced to ethanol by the enzyme alcohol dehydrogenase. This process produces two ATP molecules for every glucose molecule broken down, providing energy for the growth and reproduction of brewer's yeast.

With global warming, the growth of some brewer's yeasts has been affected. Therefore, it has become particularly important to develop strains of Saccharomyces cerevisiae that can work effectively at higher or lower temperatures. With advances in molecular biology and genetics, our knowledge of Saccharomyces cerevisiae will become deeper and deeper, allowing us to better understand its metabolic pathways and gene regulatory mechanisms, and thus develop more efficient and distinctive Saccharomyces cerevisiae strains. In addition, studies on the ecology and evolution of Saccharomyces cerevisiae will also help us to better manage and utilise this valuable natural resource [15].

1.4 Histone modification regulates gene overexpression

The metabolic pathway of Saccharomyces cerevisiae is very complex and involves the interaction of many genes and enzymes. Through genetic engineering techniques, we can make targeted modifications to the metabolic pathways of Saccharomyces cerevisiae to improve its ability to ferment specific substrates, increase the production of by-products, or reduce the production of by-products.

Histone modification is an important aspect of epigenetics, which focuses on covalent modifications that occur on histones in chromatin that can affect the structure of chromatin and thus regulate gene transcription and expression. The structural proteins of chromosomes include histones and non-histone proteins,

which bind to DNA to form nucleosomes, which are further folded and compressed to form chromatin [16]. The amino-terminal tails of histones protrude from the nucleosome and are targets for a variety of enzymes that can undergo a number of different covalent modifications, including complex regulatory networks that can act individually or in concert with each other and that can affect chromatin structure and function, such as acetylation, methylation, glycosylation, phosphorylation, and so on. They are involved in a variety of biological processes, such as development, differentiation, and dealing with environmental changes. These modifications can act individually or collaborate with each other to form a complex regulatory network that can affect chromatin structure and function, thereby regulating gene expression and participating in a variety of biological processes such as development, differentiation, and dealing with environmental changes. The Saccharomyces cerevisiae used in the experiment is a modification of histone H3, i.e., simulating continuous acetylation to change the 23rd lysine (K) mutation A of histone H3 to arginine (R), acetylation is usually associated with gene overexpression, and transcriptome analysis was used to identify the differences in the expression of genes between the histone-modified strains and the normal strains under the same conditions, which led to the acquisition of several overexpressed genes. The transcriptome analysis was used to identify the differences in gene expression between histone-modified strains and normal strains under the same conditions, resulting in several overexpressed genes. Since epigenetic regulation, such as histone acetylation, is highly conserved among different yeasts, it was hypothesised that overexpression of the *ATG34* gene might disrupt its epigenetic regulation, which in turn affects the ability to tolerate acetic acid. In this thesis, engineered yeast was applied to construct strain *BSPZ001-Nat-pJFE3-XIH-(pJFE3-Kan-ATG34)-1*, and impact analysis was performed to assess the acetic acid tolerance, which verified the expected speculative results.

Posttranslational modification of histones has great influence on histone expression level, chromatin structure, gene expression, DNA replication and DNA

damage repair. Histone methylation is the methylation of n-terminal lysine or arginine of histones H3 and H4 under the action of histone methyltransferase. Histone methylation plays an important role in promoting DNA methylation, regulating gene expression and maintaining genome stability. Histone acetylation can reduce the positive charge of histone, weaken its ability to bind DNA, and cause nucleosome depolymerization, thereby regulating the initiation and extension of chromatin transcription. Histone H3 is the most modified histone, and modification plays a role in balancing transcriptional output. By studying the specificity of epigenetic marks and the dynamic changes of histone modification, it is helpful to analyze the metabolism-related changes and stress responses of organisms in response to environmental signals. Saccharomyces cerevisiae is a typical model organism. The study of its response to environmental stress is an important part of eukaryotic cell biology, and transcriptional activation plays an important role in this process. Therefore, using Saccharomyces cerevisiae as a model organism, it is of great significance to study the role of epigenetic modification in environmental stress.

In order to regulate acetic acid tolerance of saccharomyces cerevisiae more effectively, it is necessary to further reveal the regulatory mechanism of yeast acetic acid. The acetic acid production of Saccharomyces cerevisiae is a quantitative trait controlled by multiple genes, which is the result of the interaction between genes and genes, and between genes and environment. Each gene has a complex expression regulatory network, which poses a challenge to the analysis of the molecular mechanism of low acetic acid production. Therefore, it is more convenient and feasible to improve acetic acid tolerance of saccharomyces cerevisiae.

To improve the tolerance of Saccharomyces cerevisiae, acetic acid-tolerant mutant strains could be obtained under the long-term domestication of acetic acid. Transcriptome sequencing was performed on the mutant strains and the transcriptome was compared with that of wild type strains. Genes with different

gene expression were screened out, and the recombinant strains were constructed by gene editing, i.e. knockout or overexpression. The increased acetic acid tolerance in saccharomyces cerevisiae was verified by growth curve measurements. Amino acid metabolism is also closely related to acetic acid tolerance of saccharomyces cerevisiae. Some studies have shown that the absorption of lysine by saccharomyces cerevisiae is related to the resistance to oxidative stress. Because amino acid metabolism and protein translation are closely related, specific tRNA modifications can be used to improve acetic acid tolerance of saccharomyces cerevisiae.

Conclusions to chapter 1

Hemicellulose, cellulose and lignin are the remaining lignocellulosic biomass in agriculture and forestry production. Acetic acid will inevitably be produced in the hydrolysis process of glucose and xylose. These toxic by-products have a toxic effect on the fermentation production and growth of saccharsacchara cerevisiae, and the second-generation fuel ethanol cannot be produced without wood cellulose biomass as raw material. High efficiency and stress are required for tolerant yeast in industrial production. Point mutation was performed on histone H3K23 before the experiment, that is, the 23rd lysine of histone H3 was mutated to arginine (R) by simulated continuous acetylation, and it was found that acetic acid tolerance of saccharsaccharhia cerevisiae was positively correlated with H3K23 histone modification. Transcriptome analysis showed that genes ATG34, ATO2, TSA2 and DSF1 were overexpressed compared with the control group. Therefore, the aim of this study was to overexpress the ATG34 gene in the parent strain of BSPZ001-Nat-pJFE3-XIH, The growth rate of SC-URA strain BSPZ001-Nat-pJFE3-XIH-(pJFE3-Kan)-1 was several times higher than that of BSPZ001-NAT-PJFE3-XIH -(PJFE3-KAN)-1 in the medium containing acetic acid with a certain concentration of acetic acid, which was the main weak acid released during the pretreatment of wood fiber. These findings provide a feasible way to improve the

acetic acid tolerance of BSPZ001-NAT-PJFE3-XIH by overexpression of ATG34 gene in order to stably and efficiently produce the second generation bioethanol from lignocellulosic biomass.

CHAPTER 2

OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Materials and Instruments

2.1.1 Experimental Instruments

Ultra-clean bench, Bio-Gener TECHOLOGY PCR instrument, microwave oven, agarose gel electrophoresis instrument, high-performance liquid chromatography (HPLC), OD instrument, gel imager, temperature-controlled shaking table, electrothermal constant-temperature incubator, benchtop high-speed centrifuge, high pressure steam sterilisation kettle LDZX-50KBS, blast drying oven, constant-temperature incubator, water bath, enzyme labelling instrument, electromagnetic furnace, Pipette gunt.

2.1.2 Object of the study

Strain BSPZ001-pJFE3-KIH (laboratory storage)

Plasmid pJFE3-Kan

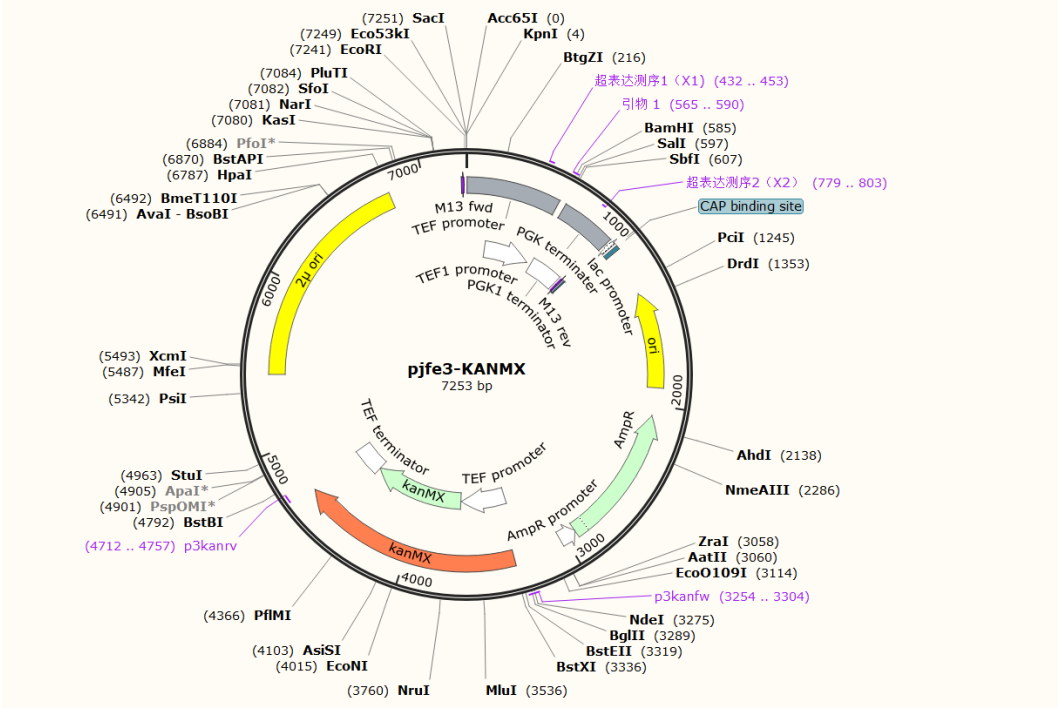

Figure 2.1 – Distribution of plasmid cleavage sites

2.1.3 Upstream and downstream primers

TTTAATTACAAAGGATCCATGAAAATTGCGGTAGAAACG

TCAATCCTGCAGGTCGACTTATATTTCTTCCCAAGTAAATGGCC

2.1.4 Culture medium

LB medium: the prepared system is configured in the ratio of 1% peptone, 1% NaCl and 0.5% yeast powder, if configuring solid medium add 2% agar powder. For example, in 400ml of the system need to add 4g of peptone and sodium chloride and 2g of yeast powder configuration of liquid LB medium;

YEPD medium: the system is configured according to the ratio of 1% yeast, 2% glucose, 2% peptone, plus 2% agar powder for the configuration of solid medium. For example, 4g of yeast, 8g of glucose and 8g of peptone should be added to 400ml system for liquid YPD medium;

SC-Ura medium (uracil-deficient complete synthesis medium): add 0.77g/L CSM-Ura, 1.7g/LYNB, 5g/L (NH4)2SO4 and 20g/L glucose, and then add 8g of gelatin powder to make a suitable pH between 6.0-6.5;

SC (Yeast Complete Synthesis Medium): This formula includes 0.77g/L CSM-Ura, 1.7g/L LYNB, 5g/L (NH4)2SO4, 100g/L uracil, 20g/L glucose and 20g/L agar powder, which needs to be fully dissolved with 180mL deionised water.

2.2 Experimental procedure

2.2.1 Design primers and amplify PCR

The principles of primer design are: end with G\C, keep the content of G and C as high as possible, and keep the Tm value of the upstream and downstream fragments in the similar value range as much as possible.

PCR amplification of the target gene fragments to make them carry homologous sequences, the configuration of 50 μL of the system, including Buffer 5 Ml, MgSO43 μL, designed upstream and downstream primers each 1.5 μL, KOD Plus Neo 1 μL, dimethyl sulfoxide 2.5 μL, water to make up the full. Magnesium ions to improve the efficiency of the PCR reaction, reduce the activation energy of

the enzymatic reaction, improve the activity of taqDNA polymerase and stabilise the nucleotides; DMSO (PCR grade) used in the PCR reaction is conducive to the high efficiency of GC-rich templates amplification, reduce the denaturation temperature of the base dependence can also improve the stability of Taq DNA polymerase.Buffer: in the PCR reaction to ensure the stability of PCR reaction. Buffer: It acts as a buffer in the PCR reaction to ensure the stability of the PCR reaction.

PCR reaction according to the denaturation, annealing and extension of the three steps, according to the different enzymes used to select different running procedures, extension time based on the length of the PCR fragments set, because 15s extension 1kb, so the experiment amplification of the *ATG34* gene and the primers on both sides of a total of 1739bp set to 26s.

2.2.2 Agarose gel electrophoresis

The target gene carrying homologous ammonia sequence after PCR was subjected to gel electrophoresis, the configuration of medium-sized gel needs to be 0.4g agarose and 50ml TAE shaking well in the microwave oven to be heated, slightly cooled down after adding 5 microlitres of gel dye (used up and put it back to the refrigerator), shaking quickly and evenly in a corner of the pouring into the mould, and then inserted into the recycling comb, pay attention to the presence of air bubbles. After it solidified to determine the positive and negative poles correctly put into the electrophoresis tank, TAE did not exceed the gel plate, gel electrophoresis for about 15min.

2.2.3 DNA purification

1) Cut off the bright stripes observed in the correct position under the gel imager into an EP tube, tare and weigh it, then add Binding Buffer to a concentration of 0.1mg/μL;

2) Melt the adhesive strip by heating in a water bath at 60°C and transfer it into the adsorption column with a pipette gun;

3) Strictly levelled and centrifuged at 12000rpm for 1min, discard the fee solution;

4) Add 600 μL Wash Buffer, centrifuge at 12000rpm for 1min, discard the waste solution, add 600 μL Wash Buffer again, centrifuge at 12000rpm for 1min, discard the waste solution;

5) Centrifuge the empty tube at 12000rpm for 2min;

7) Transfer the adsorption column to a new EP tube and leave to dry;

8) Add 45μLddH2O suspension added to the filter membrane, let it stand for 2min and then centrifuged at 12000rpm for 1min to obtain the purified DNA, and the concentration of the target gene was measured as 23.696ng/ul using an OD meter.

2.2.4 Plasmid construction

In this study, the plasmid was constructed mainly by using Yeast Tool Kit, which was mainly constructed by Golden Gate reaction with the help of restriction endonucleases BamH I and Bal I [17]. The constructed plasmids were transformed and amplified in JM109. Preparation of E. coli receptor cells and transformation of plasmids by chemotaxis [18].

2.2.5 E. coli plasmid extraction

1) Pick the single colony that has been verified successfully in 5mLLB+Amp medium to complete single colony activation;

2) Centrifuge at 4000rpm for 5min and discard the clear liquid;

3) Add 250μL SolutionI solution (add RNaseI in advance and store at 4°C) into the centrifuge tube and resuspend the bacterial solution;

4) Add 250μL SolutionII, gently up and down for about 7 times and let it stand for 4min;

5) Add 350 μLSolutionIII, gently up and down for about 7 times to make it mixed into a flocculent, centrifuged at 12000rpm for 10min;

6) Take the supernatant in the adsorption column AC, centrifuge at 12000rpm for one minute, discard the waste liquid;

7) Add 500μLBufferHBC in the adsorption column AC, centrifuge at 12000rpm for 1min, discard the clear liquid;

8) Add 600μLWashbuffer (add ethanol in advance) in the adsorption column AC, centrifuge at 12000rpm for 30s, discard the clear liquid;

9)Repeat the operation of (8);

10) Centrifuge the empty tube at 12000rpm for two minutes to remove as much rinsate as possible;

11) Open the tube cap and leave it for 5 min waiting for the ethanol to evaporate completely;

12) Place the filtered residence in a sterile EP centrifuge tube, add about 60 μL of ddH2O (warm bath in a water bath at about 70°C in advance), and centrifuge at 12000 rpm for 1 min after 2 min of standing;

13) Detect the concentration and PCR verification.

2.2.6 Yeast transformation

1) Inoculate the strain and cultivate it under suitable conditions and then activate the transfer. Dispense the obtained bacterial liquid into 40mL and adjust its optical density (OD) value between 0.2-0.25;

2) Transfer the above bacterial solution to a shaker at 30°C and oscillate at 200 rpm for 4 hours until the OD value reaches about 1.0 when it is ready for use;

3) Transfer the bacterial solution into a sterile centrifuge tube and centrifuge at 4500 rpm for 1 min to remove the supernatant. Subsequently, the organisms were gently suspended in appropriate sterile water, centrifuged again to remove the supernatant and washed with water 1-2 times;

4) Add 1 mL of LiAC solution at a concentration of 0.1 mL to the centrifuge tube, gently suspend the organisms and mix them well by gentle and gentle inversion. This mixture was then transferred to an EP tube and centrifuged for 1 min also at 4500 rpm to remove the supernatant;

5) Add 240 μL of PEG solution at a concentration of 50% to the EP tube, mix thoroughly, and then sequentially add 36 μL of LiAc solution at a

concentration of 1 mmol/L, 30 μL of fish sperm DNA (2 mM) (used within 30 min of boiling for 5 min and then immediately placing on ice to cool for 30 min), 4 μL of deionised water (53 μL if it is a plasmid), and 50 μ L of DNA fragment (1 μL in case of plasmid) and mix well;

6) Place the EP tubes in a 30°C incubator for 30 min, after which they were removed and placed on ice for 5 min, and finally placed in a 42°C water bath for 25 min for heat-excited treatment;

7) Centrifuge again at 4000 rpm for 1.5 min to remove the supernatant. Subsequently, 400 μL of deionised water was added for suspension and gently blown with a pipette, and 200 μL of it was taken out for coating the YPD plate, while the remaining portion was used for coating the SC-URA-Nat plate; for plasmids, 50 μL was to be taken out for coating the SC-URA-Nat plate;

8) Invert the medium into a 30℃incubator to cultivate colony growth.

2.2.7 Yeast plasmid small lift

After the saccharomyces cerevisiae strains were cultured to a stable stage, 5-10 mL of bacterial liquid was put into a 15 mL centrifuge tube.Centrifuge at 4000 rpm for 5 min and drain the supernatant. Add 250 μL YP1 (add RNAase A before use).After resuspension, the cells were transferred to the cell breaking tube containing 0.6 g pickling glass beads for vortex shock breaking.Then add 250 μL YP2, upside down 8 times to thoroughly mix the liquid, and let it sit at room temperature for 5-10 min You can see the liquid clear; Next, add 350 μL YP3 and immediately flip it up and down 6-8 times, paying attention to the movement gently, at this point a white flocculent will appear. Then put it into the centrifuge and centrifuge at 12000 rpm for 20 min.The supernatant was transferred to the adsorption column CP2 placed in the collection tube and centrifuged at 12000 rpm for 30 s.Drain the waste liquid from the collection pipe and put it back into the collection pipe; Add 500μL of PD Buffer and put it in Centrifuge at 12000 rpm for 30s, wash off the residual protein in the adsorption column, and pour away the waste liquid; Add another 600 μL.PW Buffer with anhydrous ethanol added was

centrifuged at 12000 rpm for 1 min, and the waste liquid was poured away.Repeat the above steps once; Next, centrifuge an empty column at 12000 rpm for 2 min to remove the remaining liquid in the tube; The most then the centrifuged adsorption column was placed in a 1.5mL EP centrifuge tube, dried for 10 min with the lid open, and 50μL was added.ddH2O was placed in the adsorption column, stood for 3 min, and centrifuged at 12000 rpm for 2 min to obtain a plasmid solution Store at -20 ℃ for later use.

2.2.8 Yeast colony PCR operation

1) Add 50 μL of NaOH solution at a concentration of 20 mmol/L into a non-inflamed EP tube and pick out a single colony for thorough mixing;

2) Heat treat the mixture in step 1 in boiling water for 10 min;

3) After cooling to room temperature, centrifuge the mixture at 6000 rpm for 1 minute, and then aspirate the supernatant and set aside;

4) Take out 1 μL in the supernatant and add it to the prepared PCR reaction system (including 5 μL of buffer, 0.2 μLP1, 0.2 μLP2, and 3.6 μL of deionised water), followed by PCR reaction.

2.2.9 Growth curve analysis method

1) Five millilitres of fermentation cultures were inoculated onto a shaker at a temperature of 30°C and incubated continuously at 200 rpm for 12 hours;

2) The culture was activated and incubated in the same manner for another 12 hours at constant temperature and speed until its absorbance (OD) reached 0.2;

3) Remove 35 ml of seed culture from the activated fermentation broth and inoculate it into glucose-acetic acid fermented conical flasks with antibiotic G418 200 mg/ml per 400 ml of medium plus 80 microlitres, forty per cent concentration of dextrose, 20 ml of 380 ml of medium plus 20 ml, and acetic acid concentration of 3 g/L. Adjust the concentration of the bacterial broth so that the original absorbance value (OD sensitivity of 600 nm)at about 0.2;

4) Place the inoculated fermentation culture at a constant 30°C as well as 200 rpm for shock incubation;

5) 1 ml of culture solution was taken from the culture bottle every 1 hour and added to a 1 cm colorimetric cup to monitor the fermentation process by measuring its absorbance value (OD = 600 nm), three repetitions of the experiment were required for each sample and the average value was calculated; if the bacterial solution was found to be too thick, appropriate dilution operation was required;

6) According to the relationship between incubation time t and absorbance, the growth curve of yeast was plotted graphically.

Conclusions to chapter 2

In order to determine the effect of ATG34 overexpressed Saccharomyces cerevisiae strains on acetic acid tolerance, we first used PCR amplification technology to link ATG34 gene amplification with the enzymatically cut plasmid and complete the transformation of the large intestine. After activation of a single colony of Escherichia coli, a single colony was selected for PCR amplification and gel electrophoresis, and the success of gene overexpression was verified by electrophoretic gel map. The plasmid in Escherichia coli was extracted, then the transformation of saccharomyces cerevisiae was completed, the yeast was activated, and the yeast was grown to A certain concentration of bacterial liquid and cultured in the medium of SC-URA+G and SC-URA+A, respectively, and the concentration of bacterial liquid was measured every few hours, thus the growth curve was drawn. The concentration of saccharomyces cerevisiae BSPZ001 with the same initial concentration was compared, and it was confirmed that the overexpression of ATG34 gene improved the acetic acid tolerance of saccharomyces cerevisiae.

CHAPTER 3

EXPERIMENTAL PART

3.1 PCR verification of ATG34 gene

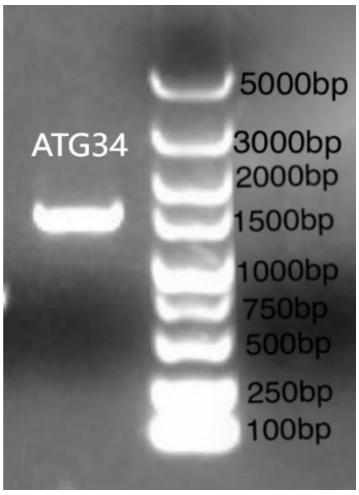

Figure 3.1 – Amplification of ATG34 gene fragments

3.2 Screening of overexpressed ATG34 gene

In order to screen out the *ATG34* gene overexpression *E. coli*, we firstly, introduced the plasmid carrying ampicillin resistance into *E. coli* to make it overexpress the *ATG34* gene, and spread the bacterial fluid evenly on the plate containing ampicillin. Only strains carrying the ampicillin gene and expressing ampicillin resistance could survive on the petri dish, that is, *E. coli* with successful plasmid introduction could survive on this plate. Through this screening, we succeeded in obtaining *E. coli* with successful plasmid introduction. These plasmid-carrying E. coli can grow into single colonies at a faster rate, preparing for the subsequent plasmid extraction and verification of gel electrophoresis after PCR amplification, as well as overexpression of *ATG34* gene in Saccharomyces cerevisiae.

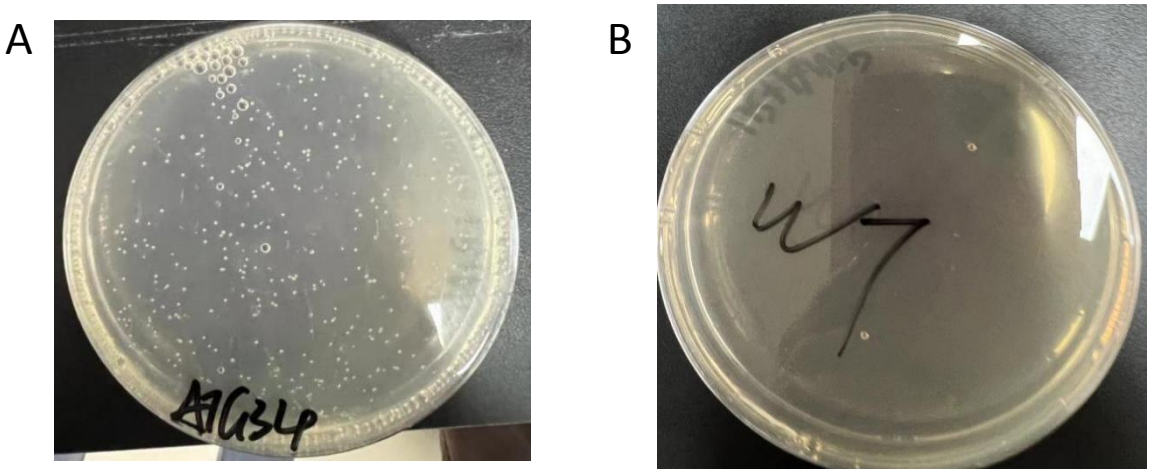

Figure 3.2 – Screening for ampicillin-resistant E. coli

The A figure is the E. coli carrying plasmid, and the B figure is the blank control group

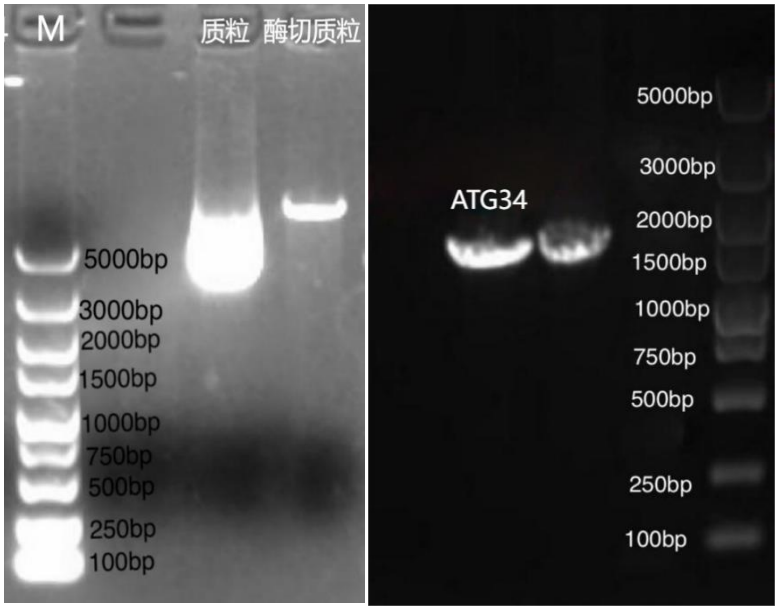

Figure 3.3 – Verification of plasmid digestion and ligation

3.3 Screening for overexpression of *Saccharomyces cerevisiae* ATG34 gene

By DNA recombination technology, we transferred the recombinant plasmid pJFE3-Kan with *ATG34* gene into the parental *Saccharomyces cerevisiae* strain.

This recombinant plasmid with URA4 gene was used as a screening marker and was screened using SC-URA plates. Finally, we successfully obtained strains overexpressing the *ATG34* gene. The acquisition of these strains laid the foundation for subsequent studies on the effect of *ATG34* gene overexpression on acetic acid tolerance in Saccharomyces cerevisiae.

A B

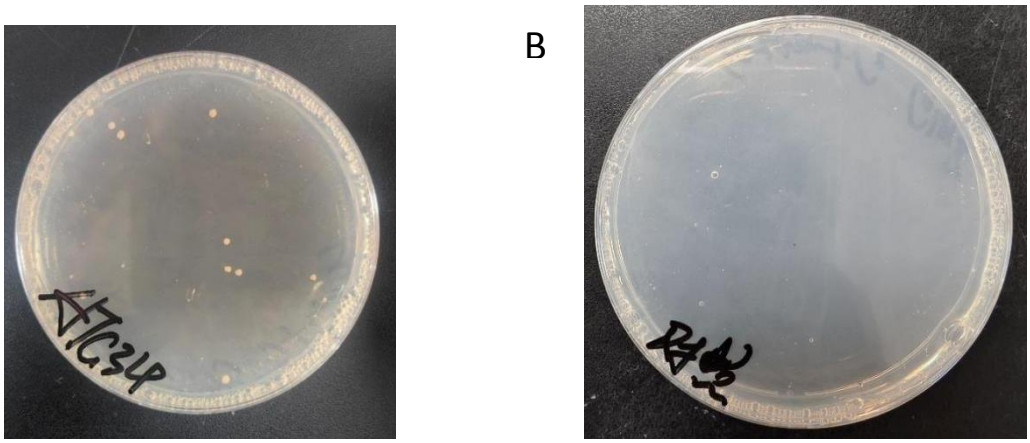

Figure 3.4 – Screening with SC-URA medium

A: *Saccharomyces cerevisiae* with *ATG34* overexpression

B: Blank control group.

3.4 Growth curve

In the experiment, the concentration of *Saccharomyces cerevisiae* in different media was monitored in real time, and the growth curve of Saccharomyces cerevisiae was plotted. The experimental results showed that in the first 6 hours the yeast was in the adjustment phase, 6-20 hours in the logarithmic phase, and after 20 hours it entered the stabilisation phase. In SC-URA+G medium (left panel), the growth rate of the *ATG34* gene overexpression strain was not significantly different from that of the wild-type strain, and even the growth trend slightly decreased after 20 hours. Similarly, in SC-URA+A medium (right panel), the growth rate of *ATG34* gene overexpression strains showed a higher increase compared to the wild-type strain, indicating a significant increase in acetic acid tolerance. This indicated that *ATG34* gene overexpression had little effect on the

normal growth rate of *Saccharomyces cerevisiae*, but the tolerance to acetic acid was significantly improved. In summary, *ATG34* gene overexpression significantly increased the tolerance to acetic acid in *Saccharomyces cerevisiae*. This provides an important clue for further research on the role of *ATG34* overexpression on acetic acid tolerance in Saccharomyces cerevisiae.

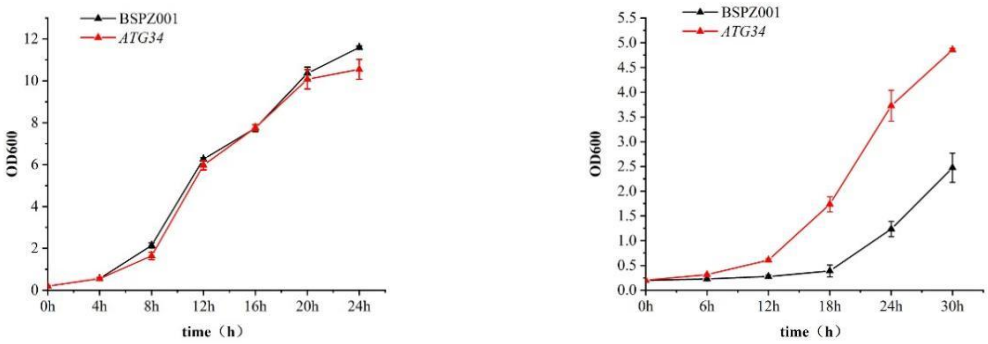

Figure 3.5 – Growth curve of SC-URA+G medium Growth curve of SC-URA+A medium

(Left) Growth curve of the strain in glucose-sugar medium (Right) Growth curve of the strain in acetic acid medium

3.5 Discussion

In this experiment, we found empty plasmids in the agarose gel electrophoresis of the amplified plasmids, which was presumed to be the problem of plasmid digestion, leading to the obstacle of the target gene introduction into the plasmid, or the problem of the target gene that we amplified, in order to address these two problems, we carried out the plasmid re-linking and PCR amplification and gel electrophoresis of the plasmid with other gene fragments of similar length to the target gene fragment, and found that it still failed to be ligated. After several adjustments of the size of the digestion system and the ratio of enzyme and primer, we finally got a breakthrough and locked the 15 μL system as the most efficient plasmid digestion and target gene ligation system, which provided a large number of experimental bases for the ligation of target genes of similar lengths.

In this study, the *ATG34* gene was overexpressed by PCR, and then linked to the plasmid and transformed into E. coli for early validation, and the plasmid extracted from E. coli was transferred into the genome of BSPZ001 strain by Holle transformation, and the ATG34 gene was successfully overexpressed in *BSPZ001-Nat-pJFE3-XIH-(pJFE3-Kan-ATG34). -Kan-ATG34)-1* strain. The strain was then cultured in conical flasks containing either acetic acid or glucose medium and the concentration of the sap was measured in real time. The growth rate of the *ATG34* strain in acetic acid was much higher than that of the wild type, but the growth rate in glucose medium did not differ much from that of the wild type, and even declined slightly in the later stages of overexpression, suggesting that the editing of the *ATG34* gene had a significant impact on the growth of the wild type. This suggests that the editing of *ATG34* gene has an important role in the enhancement of acetic acid tolerance in Brewer's yeast Holes. This provides an experimental basis for the in-depth study of the mechanism of the effect of gene editing on the tolerance of Breast Acetic Acid.

Conclusions to chapter 3

The *ATG34* gene overexpression strain was successfully constructed by using Saccharomyces cerevisiae laboratory strain *BSPZ001-Nat-pJFE3-XIH* as the parental strain through a series of molecular biology experimental operations. Specifically, the steps included DNA amplification, purification and sequencing, yeast transformation, yeast plasmid extraction, and shake flask culture. By measuring the growth curve of the overexpression strain, the effect of *ATG34* gene overexpression on the growth of the strain in acetic acid was thoroughly investigated, and finally, it was verified that the acetic acid tolerance of the second-generation fuel ethanol was significantly improved.

CONCLUSIONS

Through the above experiments, it was concluded that overexpression of the *ATG34* gene in Saccharomyces cerevisiae is beneficial to the improvement of its acetic acid tolerance. However, since it was found that the xylose metabolism efficiency of strains with high acetic acid tolerance was generally low at this stage of the study, it is possible to carry out additional gene editing on the basis of the *BSPZ001-Nat-pJFE3-XIH-(pJFE3-Kan-ATG34)-1* strain in the following further study. strain can be further edited with other genes to obtain a high xylose metabolism efficiency strain with acetic acid tolerance. Since the *ATG34* gene in *Saccharomyces cerevisiae* plays a crucial role in the selective autophagy process. Therefore, it was hypothesised that gene editing of it would potentially have other effects on Saccharomyces cerevisiae. The gene encodes the Atg34 protein, which has a specific function and contains a substrate-binding domain (ABD) similar to that of Atg19 that specifically recognises the substrate α-mannosidase. Given this recognition ability and the importance of the autophagy process, the Atg34 protein works synergistically with Atg19 to mediate the α-mannosidase autophagy pathway under starvation [19]. In addition, the *ATG34* and ATG19 genes may work together to regulate the selective autophagic degradation of α-mannosidase to ensure that α-mannosidase is efficiently degraded when necessary. Overall, the *ATG34* gene bears the burden of specifically recognising α-mannosidase in Saccharomyces cerevisiae and participates in the selective autophagy process together with Atg19[20]. If the editing of the *ATG34* gene results in the loss or weakening of the Atg34 protein function, Saccharomyces cerevisiae may not be able to properly initiate or complete the autophagic degradation process of α-mannosidase under starvation or other stress conditions. This would lead to intracellular accumulation of α-mannosidase, which may trigger metabolic abnormalities or other cellular dysfunctions. Therefore, subsequent experiments need to conduct more in-depth and comprehensive testing of the *BSPZ001-Nat-pJFE3-XIH-(pJFE3-Kan-ATG34)-1* strain and cautious editing of the

Saccharomyces cerevisiae genes with a full understanding of the gene's function and regulatory mechanism, in order to anticipate and avoid potential adverse effects.

Currently, the production of second-generation fuel ethanol is still facing many bottlenecks, such as cumbersome processing steps, high production costs, unstable sources of raw materials and other issues, and the use of fossil fuels is still dominated by the use of renewable energy is not popular enough, in order to overcome the above problems, it is necessary to increase the research and development efforts to promote technological innovation and the industrialisation process, in order to achieve the sustainable production of second-generation bioethanol and its wide application. In order to overcome the above problems, it is still necessary to increase R&D efforts and promote technological innovation and industrialisation to achieve sustainable production and wide application of second-generation bioethanol.

LIST REFERENCES

1. Li Xinlei. (2022). Germany's multiple dilemmas and path selection to maintaining energy security. The People's Forum, *23*, 111–115.

2. Wei Guoxiang Xiang. (2020). Research on the best way of ethanol production by mixed bacteria fermentation [Master, South China Agricultural University].
https://kns.cnki.net/kcms2/article/abstract?v=1ya23wS0yuBl6A5XyesidZMXfjbK4xhdPZM_ZbIraEol4Bh1ti6B_7wvQdH7qJJVGUgh2_RPx27v0gCjXUOCVkEsQuQaeC03sDrzuxWNNJ-bQVzijaJsxw==&uniplatform=NZKPT&language=gb

3. Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology*, *165*, 3–8. https://doi.org/10.1016/j.biortech.2014.01.127

4. Xu Xuewen, Li Penghui, & Tong Guolin. (2023). Progress in the second-generation bioethanol preparation. Chinese paper-making, *42*(2), 94–101.

5. Wu Guoqing, Xue Xiaozhou, Min Jian, & Lin Hailong. (2024). The status quo and prospect of biological liquid fuel industry under the global energy low-carbon transformation. The Chinese Journal of Bioengineering, *44*(1), 88–97. https://doi.org/10.13523/j.cb.2312101

6. Zhao Jian, & Qu YinBo. (2014). Research progress in biological refining technology for lignocellulosic resources. life sciences,*26*(5), 489–496. https://doi.org/10.13376/j.cbls/2014072

7. Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Institute of Fermentation Technology and Microbiology, Department of Spirit and Yeast Technology Wolczanska 171/173, PL-90- 924 Lodz, Poland, Robak, K., Balcerek, M., & Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Institute of Fermentation Technology and Microbiology, Department of Spirit and Yeast Technology Wolczanska 171/173, PL-90- 924 Lodz, Poland. (2018). Review of Second-Generation Bioethanol

Production from Residual Biomass. *Food Technology and Biotechnology*, *56*(2). https://doi.org/10.17113/ftb.56.02.18.5428

8. Zhu Jianwei, Gong Dehong, MAO Jiahua, & Wei Qi. (2022). Research progress of lignofibrous biomass pretreatment technology. Progress in new energy sources, *10*(4), 383–392.

9. Wu, D., Wei, Z., Mohamed, T. A., Zheng, G., Qu, F., Wang, F., Zhao, Y., & Song, C. (2022). Lignocellulose biomass bioconversion during composting: Mechanism of action of lignocellulase, pretreatment methods and future perspectives. *Chemosphere*, *286*, 131635. https://doi.org/10.1016/j.chemosphere.2021.131635

10. Stojiljkovic, M., Foulquié-Moreno, M. R., & Thevelein, J. M. (2020). Polygenic analysis of very high acetic acid tolerance in the yeast Saccharomyces cerevisiae reveals a complex genetic background and several new causative alleles. *Biotechnology for Biofuels*, *13*(1), 126. https://doi.org/10.1186/s13068-020-01761-5

11. Meijnen, J.-P., Randazzo, P., Foulquié-Moreno, M. R., van den Brink, J., Vandecruys, P., Stojiljkovic, M., Dumortier, F., Zalar, P., Boekhout, T., Gunde-Cimerman, N., Kokošar, J., Štajdohar, M., Curk, T., Petrovič, U., & Thevelein, J. M. (2016). Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast Saccharomyces cerevisiae. *Biotechnology for Biofuels*, *9*(1), 5. https://doi.org/10.1186/s13068-015-0421-x

12. Zhang, M.-M., Xiong, L., Tang, Y.-J., Mehmood, M. A., Zhao, Z. K., Bai, F.-W., & Zhao, X.-Q. (2019). Enhanced acetic acid stress tolerance and ethanol production in Saccharomyces cerevisiae by modulating expression of the de novo purine biosynthesis genes. *Biotechnology for Biofuels*, *12*(1), 116. https://doi.org/10.1186/s13068-019-1456-1

13. Mukherjee, V., Lind, U., St. Onge, R. P., Blomberg, A., & Nygård, Y. (2021). A CRISPR Interference Screen of Essential Genes Reveals that Proteasome

Regulation Dictates Acetic Acid Tolerance in Saccharomyces cerevisiae. *mSystems*, *6*(4), e00418-21. https://doi.org/10.1128/mSystems.00418-21

14. Li Xiao, Dong Shengsheng, Fu Xiaomeng, Wang Pengfei, & Dong Jian. (2018). Effects of overexpression of the transcription factor MSN2 gene on tolerance in Saccharomyces cerevisiae. Modern food technology, *34*(9), 95-101+87. https://doi.org/10.13982/j.mfst.1673-9078.2018.9.015

15. Eldarov, M. A., Kishkovskaia, S. A., Tanaschuk, T. N., & Mardanov, A. V. (2016). Genomics and biochemistry of Saccharomyces cerevisiae wine yeast strains. *Biochemistry (Moscow)*, *81*(13), 1650–1668. https://doi.org/10.1134/S0006297916130046

16. Xu Shouming, Hong Xianzhen, & Wang Deli. (2007). Regulatory mechanism and progression of transcription repression in eukaryotic genes. Science and technology information in China, *12*, 181+189.

17. Lee, M. E., DeLoache, W. C., Cervantes, B., & Dueber, J. E. (2015). A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS Synthetic Biology*, *4*(9), 975–986. https://doi.org/10.1021/sb500366v

18. Chan, W.-T., Verma, C. S., Lane, D. P., & Gan, S. K.-E. (2013). A comparison and optimization of methods and factors affecting the transformation of *Escherichia coli*. *Bioscience Reports*, *33*(6), e00086. https://doi.org/10.1042/BSR20130098

19. Suzuki, K., Kondo, C., Morimoto, M., & Ohsumi, Y. (2010). Selective Transport of α-Mannosidase by Autophagic Pathways. *Journal of Biological Chemistry*, *285*(39), 30019–30025. https://doi.org/10.1074/jbc.M110.143511

20. Watanabe, Y., Noda, N. N., Kumeta, H., Suzuki, K., Ohsumi, Y., & Inagaki, F. (2010). Selective Transport of α-Mannosidase by Autophagic Pathways. *Journal of Biological Chemistry*, *285*(39), 30026–30033. https://doi.org/10.1074/jbc.M110.143545

Acknowledgement

As time passes by, it is the graduation season, at this moment I have mixed feelings, not only for the past time reluctant to part with, but also for the future years full of longing, but most of the heart is still grateful.

First of all, I would like to thank my alma mater - Qilu University of Technology (Shandong Academy of Sciences) for their careful cultivation of me, to give us the allocation of high-quality educational resources and experimental facilities, and I will always remember our school motto: "virtue and motivation, practical and still able". No matter how far I can go in my future study, no matter whether I will do more in-depth research in this direction in the future or not, I will be full of reverence for academics, full of love for my profession, and constantly pursue progress and seek truth from facts.

Meanwhile, I would like to express my heartfelt thanks to my supervisor, Professor He Deyun. She has always treated me with patience and tolerance. I thank you for your encouragement and trust, and for your valuable advice and guidance during the thesis writing process. I am honoured and sincerely grateful to be taught by you. I wish you all the success in your endeavours, and I wish you all the happiness in the world.

In addition, I would like to thank Xuerui Zhang for his help in the experiments. He taught me the use of various experimental instruments and standard operation, patiently explained the experimental content and the principle analysis, and pushed forward the whole experiment, so that I benefited a lot from it. I wish my senior brother can have results every day and publish more papers.

Finally, I would like to thank my parents and my best friends. I would like to thank my parents for their support on my journey of study, always accompanying me when I was frustrated and giving me selfless love and support. Thank you for your open-mindedness and respect, so that I can grow up healthily under your shelter. At the same time, I thank all my classmates who did experiments together, we stayed up all night to measure the data of zone growth curves, and analysed the

reasons for each other's failures or successes in the lab together, which made my university life better, and I also thank the rice hitchhiker and my boyfriend who encouraged and accompanied me during my examination and supported me to finish the long road to the examination. May we all have a bright and brilliant future.

That summer, I entered QIT with ignorance and expectation, and this summer, I will step into the next academic hall with determination and expectation.

APPENDIX

FTB Food Technology & Biotechnology

review
ISSN 1330-9862
doi: 10.17113/ftb.56.02.18.5428

Review of Second Generation Bioethanol Production from Residual Biomass

Katarzyna Rabak* and Maria Balcerek

Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Institute of Fermentation Technology and Microbiology, Department of Spirit and Yeast Technology, Wolczanska 171/173, PL 90-924 Lodz, Poland

Received: 18 July 2017
Accepted: 11 January 2018

SUMMARY

In the context of climate change and the depletion of fossil fuels, there is a great need for alternatives to petroleum in the transport sector. This review provides an overview of the production of second generation bioethanol, which is distinguished from the first generation and subsequent generations of biofuels by its use of lignocellulosic biomass as raw material. The structural components of the lignocellulosic biomass such as cellulose, hemicellulose and lignin, are presented along with technological unit steps including pretreatment, enzymatic hydrolysis, fermentation, distillation and dehydration. The purpose of the pretreatment step is to increase the surface area of carbohydrate available for enzymatic saccharification, while minimizing the content of inhibitors. Performing the enzymatic hydrolysis releases fermentable sugars, which are converted by microbial catalysts into ethanol. The hydrolysates obtained after the pretreatment and enzymatic hydrolysis contain a wide spectrum of sugars, predominantly glucose and xylose. Genetically engineered microorganisms are therefore needed to carry out co-fermentation. The excess of harmful inhibitors in the hydrolysate, such as weak organic acids, furan derivatives and phenol components, can be removed by detoxification before fermentation. Effective saccharification further requires using exogenous hemicellulases and cellulolytic enzymes. Conventional species of distiller's yeast are unable to ferment pentoses into ethanol, and only a very few natural microorganisms, including yeast species like *Candida shehatae*, *Pichia* (*Scheffersomyces*) *stipitis*, and *Pachysolen tannophilus*, metabolize xylose to ethanol. Enzymatic hydrolysis and fermentation can be performed in a number of ways: by separate saccharification and fermentation, simultaneous saccharification and fermentation or consolidated bioprocessing. Pentose-fermenting microorganisms can be obtained through genetic engineering, by introducing xylose-encoding genes into metabolism of a selected microorganism to optimize its use of xylose accumulated in the hydrolysate.

Key words: second generation bioethanol, biofuel, lignocellulosic biomass, biomass pretreatment, enzymatic hydrolysis, co-fermentation

INTRODUCTION

Chief among the many challenges facing the modern world are the interconnected issues of global warming, reliance on fossil fuels, and food and energy security. Population growth and increasing industrial development lead to greater demand for energy, but conventional fossil fuels, including petroleum, are a both a finite resources and emit greenhouse gasses (GHG) when combusted. Sustainable and environmentally friendly energy sources are required in order to meet the world's future energy needs (*1,2*). Biofuels, namely cellulosic bioethanol, butanol and biodiesel, are therefore of considerable interest to researchers, industrial partners and governments (*3,4*). In particular, bioethanol is considered a promising drop-in fuel, which could provide an alternative to petrol in the transport sector.

The use of ethanol in gasoline in 2016 reduced CO_2-equivalent GHG emissions from transportation by 43.5 million metric tonnes — the equivalent of removing 9.3 million

*Corresponding author:
Phone: +48426813477;
E-mail: katarzyna.rabak@edu.p.lodz.pl

ORCID IDs: 0000-0002-8765-0055 (Rabak), 0000-0003-3731-8680 (Balcerek)