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 **Effect of *Lactobacillus casei* Fermentation on the Content of Soybean Isoflavones in soybean milk**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Wang Yujie

1. Thesis topic **The effect of lactate dehydrogenase expression from different sources on lactate utilization in *Saccharomyces cerevisiae***
Scientific supervisor Ph.D., Assoc. Prof. Iryna Voloshyna

approved by the order of KNUTD "05" March 2025, № 50-уч

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

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WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
6	Draw up a bachelor's thesis (final version)	until 25 May 2025	
7	Submission of qualification work to the supervisor for feedback	until 27 May 2025	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	01 June 2025	Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	04 June 2025	

I am familiar with the task:

Student _____ Wang Yujie

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Wang Yujie. The effect of lactate dehydrogenase expression from different sources on lactate utilization in *Saccharomyces cerevisiae* – Manuscript.

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The excessive accumulation of lactic acid in corn pulp, a by-product of wet processing of corn starch, seriously restricts its resource utilization, especially in the field of feed, which is prone to cause digestive dysfunction in animals. This study aims to explore the expression characteristics of lactate dehydrogenase (LDH) from different sources in yeast and its influence on lactate metabolism, in order to optimize the biological fermentation process of corn slurry. This study aimed at the problem of insufficient natural lactic acid metabolism efficiency of *Saccharomyces cerevisiae*, systematically analyzed the microbial lactic acid catabolic network, and constructed highly efficient degradation strains through genetic engineering means. The heterologous expression strategy of multi-source lactate dehydrogenase (LDH) was adopted. Through genetic engineering technology, the yeast endogenous gene FCB2, LctO derived from Lactococcus lactis and LctD derived from Escherichia coli were introduced into Saccharomyces cerevisiae to construct three groups of engineered strains. Combined with CRISPR-Cas9 gene editing, yeast transformation and high performance liquid chromatography (HPLC) analysis, Systematically evaluate the effects of different LDH on the lactic acid utilization efficiency of Saccharomyces cerevisiae. Through fermentation tests on seven well-performing industrial strains of Saccharomyces cerevisiae preserved in the laboratory, a strain Y02 with the best fermentation effect of corn slurry was screened out. To further increase the lactic acid metabolism rate of strain Y02, lactate dehydrogenase genes from different sources were overexpressed in the form of chromosomal integration expression. In the YPL medium with lactic acid as the sole carbon source, the *FCB2* gene (cytotin c

REDOX enzyme) derived from yeast itself significantly increased the utilization rate of lactic acid. It is nearly twice as high as the original strain. However, continuing to increase the expression copy number of FCB2 instead led to a decrease in the lactic acid metabolism rate. It was found through the detection of intracellular pyruvate content that the growth of the strain was inhibited due to excessive accumulation of pyruvate. Therefore, moderate expression of the FCB2 gene can effectively enhance the lactic acid catabolism capacity of Saccharomyces cerevisiae.[46]

Key words: Lactate dehydrogenase, Saccharomyces cerevisiae, Metabolic engineering; Corn syrup; Electron transport chain

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INTRODUCTION

Corn slurry is a by-product of the corn starch production process. Its main components are lactic acid, protein, amino acids, sugars and minerals, etc. Lactic acid exists in large quantities in corn pulp, resulting in a relatively low pH, which is not conducive to storage and transportation. When used as a feed additive, it affects the feeding of animals. In addition, during the production process of corn pulp, anti-nutritional factors (such as phytic acid, tannin, etc.) and toxic substances (such as aflatoxin, sulfite, etc.) may be generated, which will limit its application in food, feed and other fields.

To address the issues of lactic acid, anti-nutritional factors and toxic substances in corn slurry, the biological fermentation method has become an effective solution. Through microbial fermentation, using lactic acid as the carbon source, lactic acid is decomposed and metabolized for the cultivation of bacterial cells, while anti-nutritional factors are degraded and the content of toxic substances is reduced. Lactate dehydrogenase (LDH) is a key enzyme in lactic acid metabolism, capable of catalyzing the interconversion between lactic acid and pyruvic acid, and plays an important role in the energy metabolism of microorganisms. Therefore, studying the expression of LDH from different sources in yeast and its influence on the utilization of lactic acid is of great significance for optimizing the biological fermentation process of corn slurry.

The main purpose of this study is to introduce three lactate dehydrogenase (LDH) genes (Fcb2, lctO, lctD) from different sources to explore the influence of their expression in yeast on the utilization efficiency of lactic acid, thereby providing theoretical basis and technical support for the biological fermentation process of corn pulp. The LDH genes derived from yeast itself (Fcb2), Lactococcus lactis (LctO), and Escherichia coli (LctD) were introduced into yeast cells to verify their expression in yeast and their influence on lactic acid metabolism. Through fermentation experiments, the differences in lactic acid utilization efficiency among three different sources of LDH were compared, and

the optimal LDH gene was screened out. Based on the experimental results, the biological fermentation process of corn pulp was optimized to improve the degradation efficiency of lactic acid and the application value of corn pulp.

Lactate dehydrogenase (LDH) is a key enzyme in lactate metabolism, and its expression and function vary in different organisms. This study compared the expression characteristics of LDH from three different sources in yeast to reveal its influence mechanism on lactic acid metabolism and further enrich the functional research of LDH. Yeast, as an important industrial microorganism, has broad application prospects in the research of metabolic engineering. In this study, both self-derived and exogenous LDH were expressed in yeast through gene editing technology, providing new ideas and methods for yeast metabolic engineering research. Corn slurry is a by-product in the production process of corn starch, rich in lactic acid. Meanwhile, the anti-nutritional factors and toxic substances it contains limit its application value. In this study, through the expression of LDH, the utilization efficiency of lactic acid by yeast was improved, providing a new biological fermentation strategy for the efficient utilization of corn pulp. This study adopted technical methods such as CRISPR gene editing, yeast transformation and high performance liquid chromatography to explore the role of LDH in lactic acid metabolism, providing theoretical support and technical reference for the development of biological fermentation technology. By enhancing the degradation efficiency of lactic acid, this study can provide safer and more efficient raw materials for the food and feed industries and promote the sustainable development of related industries.

Chapter I
LITERATURE REVIEW

1.1 The metabolic basis and regulation of lactic acid in corn slurry utilized by yeast

Saccharomyces cerevisiae exhibits unique biological advantages in lactic acid metabolism of corn slurry[6]. As a food safety-grade microorganism, its genome lacks the lactate dehydrogenase (LDH) encoding gene, avoiding the metabolic bypass that reduces pyruvate to lactic acid during the fermentation process. This characteristic makes it an ideal candidate strain for lactic acid clearance in corn pulp[3] - neither increasing lactic acid accumulation nor being able to tolerate a high-concentration lactic acid environment. The enrichment of lactic acid[4] in corn pulp mainly stems from two pathways: On the one hand, lactic acid needs to be added during the wet processing of corn starch to promote component separation; On the other hand, contamination by lactic acid bacteria during processing and storage or artificial introduction leads to anaerobic fermentation of sugars, further generating L-lactic acid. Although lactic acid bacteria metabolism can improve the quality and functionality of starch, the strongly acidic environment caused by excessive L-lactic acid can significantly reduce the application value of corn pulp. Especially in the field of animal feed, it is prone to cause gastrointestinal irritation and inhibit the growth performance of livestock and poultry. It is worth noting that Saccharomyces cerevisiae achieves the reverse metabolism of *L*-lactic acid through Cytochrome b2[6] (Cytochrome b2,CYB2, namely L-lactic acid cytochrome c oxidoreductase). This enzyme has L-lactate dehydrogenase activity and can convert L-lactate into pyruvic acid in oxidation reactions, endowing yeast[7] with the ability to utilize L-lactate as the sole carbon source. This unique metabolic pathway provides an entry point for engineering modification: By analyzing the CYB2 regulatory network and lactic acid transport mechanism, the lactic acid decomposition flux of yeast can be specifically enhanced. The current research

focuses on revealing the rate-limiting step of lactic acid metabolism in Saccharomyces cerevisiae, enhancing the activity of CYB2 enzyme through metabolic engineering methods (such as promoter optimization, cofactor balance, etc.), and thereby developing highly efficient lactic acid-degrading strains. Such renovations not only restore the neutral properties of corn pulp[28] but also simultaneously achieve the value-added of by-products (such as pyruvic acid synthesis), providing innovative solutions for the resource utilization of industrial by-products[44]. By understanding the metabolic pathway of L-lactic acid in Saccharomyces cerevisiae, Saccharomyces cerevisiae[8] can be better modified to acquire a stronger lactic acid catabolic capacity, so as to handle lactic acid in corn pulp[29] more effectively.[9]

1.2 Key enzymes in microbial L-lactic acid metabolism (NAD-dependent L-lactic acid dehydrogenase, NAD-independent L-lactic acid dehydrogenase)[30]

1.2.1 Bidirectional regulatory network of microbial lactic acid metabolism

The metabolic process of lactic acid by microorganisms shows significant dynamic equilibrium characteristics, mainly covering two key pathways: synthesis and decomposition. During anabolic processes, the regeneration mechanism of oxidized coenzyme I (NAD^+) is achieved by reducing pyruvate to lactic acid, which plays a core regulatory role in maintaining intracellular REDOX homeostasis. Catmetabolism, on the other hand, takes lactic acid assimilation and degradation as its core, converting it into pyruvic acid through oxidation reactions. Meanwhile, the reduced coenzyme (NADH) produced enters the electron transport chain to drive ATP synthesis, providing energy support for microbial growth. In response to the biological clearance requirements of L-lactic acid in corn slurry, an in-depth analysis of the catabolic network of Saccharomyces cerevisiae has significant application value.[11]

1.2.2 Classification and Functional Comparison of Key Enzyme Systems[12]

In order to consume the L-lactic acid in corn pulp completely through microbial fermentation, it is necessary to fully study the L-lactic acid catabolic pathways of microorganisms and Saccharomyces cerevisiae.[34] According to the differences in

cofactor requirements and electron transfer paths in catalytic reactions, the enzyme systems involved in the decomposition of L-lactic acid can be classified into two major categories[13]:

(1) NAD-dependent L-lactate dehydrogenase[14] (NAD-dependent L-lactate dehydrogenase,L-nLDH)[31]

L-nLDH is known as the fermentative L-lactate dehydrogenase, which plays an important role in maintaining the energy metabolism, material circulation and anaerobic survival of microorganisms. This type of enzyme uses NADH as a cofactor to convert pyruvate into L-lactic acid, while NADH is oxidized to NAD+. This reaction is shown in Equation 1-1[31] As a fermentative enzyme system, it mainly undertakes the reduction reaction of pyruvic acid to L-lactic acid, generating NAD^+ by consuming NADH. Its reaction equilibrium constant ($Keq=2\times10^{-12}$) is significantly biased towards the direction of lactic acid synthesis (Equation 1-1). Even under the condition of exogenous NAD^+ supplementation, the reverse reaction efficiency was still lower than 0.5%. Therefore, L-nLDH is not suitable for modifying Saccharomyces cerevisiae to metabolize lactic acid in corn pulp. This enzyme system has two obstacles in the engineering modification of Saccharomyces cerevisiae: the catalytic direction is contrary to the target metabolic requirements; The accumulation of NADH will activate the ethanol synthesis pathway, resulting in carbon flow loss.

$CH_3CHOHCOOH+NAD^+\rightleftharpoons CH_3COCOOH+NADH+H^+$ (Reaction Equation 1-1)

(2) NAD-independent lactate dehydrogenase (L-iLDH)

L-iLDH is called respiratory L-lactate dehydrogenase, enabling microorganisms to survive under conditions where L-lactic acid is the sole carbon source. The process of catalyzing lactic acid to pyruvic acid is irreversible, and the catalytic process does not require the participation of NAD+. According to the different receptors for electron transfer after the oxidation of L-lactic acid, L-iLDH can be roughly divided into L-Lactate oxidase (LOX) with molecular oxygenas the electron acceptor and L-Lactate monooxygenase (L-lactate monooxygenase). Two types of L-lactate dehydrogenases (LMO) with ubiquinone as the electron receptor,

Cytochrome b2(CYB2)[35] with cytochrome c as the monoreceptor, and some L-lactate dehydrogenases of unknown electron receptor types. This enzyme family achieves irreversible oxidation of lactic acid through diverse electron transport mechanisms and can be further classified into four subcategories based on receptor types[33]:

① Oxygen-dependent oxidase system, including L-lactate oxidase (LOX) and L-lactate monooxygenase (LMO), both of which take lutein cofactor (FMN/FAD) as the core catalytic unit. LOX catalytic system: generates pyruvic acid and hydrogen peroxide (reaction equations 1-2), mainly existing in the cytoplasm of aerobic microorganisms.

LMO catalytic system: The products are acetic acid, CO_2 and water(reaction equations 1-3), which are commonly found in specific actinomycetes genera

Both LOX and LMO use oxygen as the electron acceptor and FMN/FAD as the cofactor to specifically catalyze L-lactic acid as pyruvic acid, and both are free in the cytoplasm of microorganisms. Due to the different degrees of binding between the enzyme and the substrate, the final reaction products of the two are also different. LOX oxidizes L-lactic acid to generate pyruvic acid and hydrogen peroxide, while LMO converts L-lactic acid oxygen into acetic acid, CO2 and water.

$CH_3CHOHCOOH+O_2 \rightarrow CH_3COCOOH+H_2O_2$（reaction equations 1-2）

$CH_3CHOHCOOH+O_2 \rightarrow CH_3COOH+CO_2+H_2O$（reaction equations 1-3）

② Quinone-coupled dehydrogenase system

L-lactate dehydrogenase with ubiquinone as the electron acceptor is generally a composition-expressed membrane integration protein and directly linked to the respiratory chain. This type of enzyme uses FMN as a cofactor, transfers electrons to ubiquinone by oxidizing L-lactic acid, and directly transfers them to the electron transport chain via quinones. It is mainly distributed in the cell membranes of Gram-negative bacteria (such as Escherichia coli). The electrons generated from the oxidation of lactic acid are transferred to the ubiquinone reservoir (reaction

equations 1-4) through FMN-mediated electron transfer, forming a direct coupling with the respiratory chain.

$CH_3CHOHCOOH+FMN \rightarrow CH_3COCOOH+FMNH_2$ (Reaction Equations 1-4)

③ Cytochrome coupling system

Represented by cytochrome b2 (CYB2) [36]in the intermitochondrial space of Saccharomyces cerevisiae, this tetracer protein contains a dual cofactor of heme and FMN, and lactic acid oxidation is completed through electron transport mediated by cytochrome c (reaction equations 1-5). Studies have shown that the catalytic efficiency of CYB2 for L-lactic acid ($kcat/Km=1.8\times10^4$ $M^{-1}s^{-1}$) is significantly higher than that of the D-lactic acid isomer. Cytochrome b2, with cytochrome c as the electron acceptor, was found in yeast S.cerevisiae and Hansenula polymorpha, and it is a soluble protein existing in the intermembrane space of cell mitochondria. This enzyme is a bifunctional temeric protein, with each subunit approximately 55 kDa and two cogroups, heme and flavin (FMN), responsible for catalyzing the transfer of electrons from lactic acid to cytochrome c and the generation of pyruvic acid. The catalytic reaction equation is shown as 1-5.[37]

$CH_3CHOHCOOH \rightarrow CH_3COCOOH+2H^++2e^-$ (Reaction Equations 1-5)

④ Novel complex enzyme system

The LIdEFG complex discovered in Shewanella oneidensis[20] represents the latest research progress. This trimer enzyme contains iron-sulfur clusters and lutein cogroups, and its catalytic mechanism involves transmembrane electron transfer. Although the specific action path has not been fully analyzed yet, its strict substrate specificity provides a new target for directed evolutionary modification.

In recent years, a new type of L-iLDH has also been discovered. This enzyme may contain not only lutein cofactor (FMN/FAD), but also iron-sulfur clusters. Pinchuk et al. first studied this type of enzyme and the gene LIdEFG encoding it in Shewanella oneidensis. This enzyme is a three-protein complex encoded by three genes with unknown functions. The presence of any one of these genes will cause the enzyme to lose its activity. Such enzymes usually can only catalyze lactic acid

to pyruvic acid, but the mechanism of the enzymatic reaction catalyzed by this enzyme requires further research.

Since the reaction catalyzed by L-nLDH requires the participation of the coenzyme NADH, the reaction it catalyzes is usually reversible and mainly in the direction of producing lactic acid. The price of this coenzyme is relatively expensive, and the generated NADH will promote the production of ethanol in Saccharomyces cerevisiae. Therefore, this enzyme is not used for the modification of Saccharomyces cerevisiae strains in subsequent experiments. The coenzyme used in L-iLDH is FMN/FAD, and its binding with the enzyme is relatively firm. No additional coenzyme needs to be added. The catalytic reaction is unidirectional and only in the direction of generating pyruvic acid. Therefore, this type of enzyme is given priority for strain modification in subsequent experiments.[38]

Summary of the chapter I

1.Saccharomyces cerevicans, lacking lactate dehydrogenase (LDH), can irreversibly oxidize L-lactic acid to pyruvic acid by using cytochrome b2 (CYB2), avoiding the reverse synthesis of lactic acid, and thus becomes an ideal strain for eliminating lactic acid in corn pulp.

2. The enrichment of lactic acid in corn pulp is due to the addition during processing and contamination by lactic acid bacteria. Its strong acidity inhibits the application value of feed. It is necessary to restore neutrality through microbial metabolism and achieve the value-added of by-products.

3. Metabolic engineering enhances the lactate decomposition flux by optimizing the activity of CYB2 (such as promoter modification and cofactor balance), and simultaneously elucidates the lactate transport mechanism to improve the degradation efficiency of the strain.

4. Nad-independent L-iLDH (such as CYB2) is superior to NAD-dependent L-nLDH due to its characteristics of not requiring coenzymes and catalyzing irreversible reactions. It is more suitable for yeast modification to avoid carbon flow loss caused by NADH accumulation.

Chapter II
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Reagents, culture media and culture conditions

2.1.1 Reagents

Table 2.1 Main reagents used in this chapter

reagent	Manufacturer
Corn pulp	Provided by the cooperative company
Yeast extract,Y	OXOID
Tryptone,P	OXOID
AGAR powder	Beijing Soleibao Technology Co., LTD
0. 5 mm Acid wash glass beads	BioSpec
DNA extract	Beijing Dingguo Changsheng Biotechnology Co., LTD
Fish essence DNA	Beijing Soleibao Technology Co., LTD
DH5α Competent cells of E. coli	Self-made in the laboratory
Agarose	Biowest Agarose
Lithium dihydrate acetate	Shenggong Bioengineering (Shanghai) Co., LTD
Polyethylene glycol	Beijing Dingguo Changsheng Biotechnology Co., LTD
G418	Beijing Dingguo Changsheng Biotechnology Co., LTD
Kanamycin sulfate	Beijing Dingguo Changsheng Biotechnology Co., LTD
2×Multif Seamless Assembly Mix	ABclonal
FastDigest enzymes	Thermo Fisher Scientific
T4 DNA Ligase	Invitrogen
2×Phanta Max Master Mix	Novozyme Biotechnology Co., LTD
Phanta Max Super-Fidelity DNA Polymerase	Novozyme Biotechnology Co., LTD
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific

DNA Marker（DL5000）	Novozyme Bioengineering Co., LTD
E.Z.N.A.®Gel Extraction kit	Omega Company
E.Z.N.A.®Plasmid Mini Kit I	Omega Company
E.Z.N.A.®Cycle-Pure Kit	Omega Company

2.1.2 Culture Medium

1. LB medium preparation: Weigh 2 g of yeast powder, 4 g of peptone and 4 g of sodium chloride, add 400 mL of distilled water, and add an additional 8 g of AGAR powder to the solid medium. Sterilize in a pressure cooker at 115℃ for 30 minutes.

2. Preparation of kanamycin stock solution: Accurately weigh 2 g of kanamycin, make up to 10 mL volume with ultrapure water, filter and sterilize through a 0.22 μm microporous filter membrane, and store in a refrigerator at -20℃.

3.Kana+LB solid medium: Melt the LB solid medium and cool it to 60℃, then add Kana antibiotic with a final concentration of 60 ng/μL to it;

4. Preparation of G418 mother liquor: Accurately weigh 4 g of G418 antibiotic, make up to 10 mL volume with ultrapure water, filter and sterilize through a 0.22 μm microporous filter membrane, and store in a refrigerator at -20℃.

5. Preparation of G418+YPD solid medium: Melt the YPD solid medium and cool it to 60℃, then add G418 antibiotic with a final concentration of 200 ng/μL to it.

2.2 Strain, plasmid and Primer

The strains and plasmids used in this chapter are detailed in Table 2.2. The synthesis of primers, the optimization of gene codons and DNA sequencing were completed by Qingdao Weilan Biological Co., LTD. The information details of the primer sequences are shown in Table 2.3.

Table 2.2 Strains and plasmids used in this chapter

Strains/Plasmids	Genotype	Source
Strains	Diploid	
M8	Diploid	Commercial strain
Y02	Diploid	Commercial strain
Y04	Diploid	Commercial strain
RC212	Diploid	Commercial strain
BSIF	Diploid	Laboratory
6M	LF1, *pho13::*XI, *3δ::*XI, *gre3::*PPP, XK, AE-PCS, *N360S*, AE,MUT	Laboratory
A10-1	6M-15 AE	Laboratory
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Commercial strain
Y02-FCB2	Y02, *ARS416d::FCB2* ^{WW}	This study
Y02-LctD	Y02, *ARS416d::LctD*^{WW}	This study
Y02-LctO	Y02, *ARS416d::LctO*^{WW}	This study
Y02-FCB2-2	Y02-FCB2, *ARS416d::FCB2*^{WW}, *XⅡ-1 ::CYC1*^{Ww}	This study
Plasmids		
pTC-tR416d	Cas9, *TDH3p-tRNA-sgRNA(ARS416d) -TDH1t*	[21]
pTC-tR-XⅡ-1	Cas9, *TDH3p-tRNA-sgRNA(ARS911b)-TDH1 t*	[21]
pIYC04-KanMX	*2μ*, *KanMX, PCCW12-GFP-TPRM5*	Laboratory
pUC-GW-Ampr-LctD	*Ampr*, *LctD*	This study
pUC-GW-Ampr-LctO	*Ampr, LctO*	This study
pIYC04-KanMX-FCB2-416d	*2μ*, *KanMX*, *PCCW12-FCB2-TPRM5*	This study

pIYC04-KanMX-LctD-416d	*2μ*, *KanMX*, *PCCW12-LctD-TPRM5*	This study
pIYC04-KanMX-LctO-416d	*2μ*, *KanMX*, *PCCW12-LctO-TPRM5*	This study
pIYC04-KanMX-FCB2-XⅡ-1	*2μ*, *KanMX*, *PCCW12-FCB2-TPRM5*	This study

Note: Ww: One allele of the diploid Saccharomyces cerevisiae strain has been modified; ww: Two alleles of the diploid Saccharomyces cerevisiae strain have been modified.

Table 2.3 Primers used in this chapter

Primer name	Primer base sequence (5 '→3')
416-F	AAACTTTTATGATATTTTGCAATATTTTTTTTAAGCAGTG
416-R	TATTGATATAGTGTTTAAGCGAATGACAGAAGATTAAT
416-*LctD*-F	TCATTCGCTTAAACACTATATCAATAATGACTGAAGCTGCTTATGCTTATATTGC
416-*LctD*-R	ATATTGCAAAATATCATAAAAGTTTTTAATGTTCAGTTTCTTTATTTCTAATAAAACCTTGAGC
416-*LctO*-F	TCATTCGCTTAAACACTATATCAATAATGCACTTGTCTTCTACTGATGTCAAC
416-*LctO*-R	TATTGCAAAATATCATAAAAGTTTATTTAATCAATCAAAGAAGTATGTTTAATTTCTTCAATATTTTTAGTAC
416-FCB2-F	CATTCGCTTAAACACTATATCAATAATGCTAAAATACAAACCTTTACTAA
416-FCB2-R	ATTGCAAAATATCATAAAAGTTTATTCATGCATCCTCAAATTCTGTTAAA
416-KZ-F	TACACTTATTAACCGCTTTTACTATTATCTTC
416-KZ-R	GTCAATGTGGTAACAAAGGTGTTG
Kan-PIYC04-F	CTCAAAGGCGGTAATACGGTTATCC
Kan-PIYC04-R	ATTCATTAATGCAGCTGGATAAACGCCA
911-Up-F	GCGTTTATTCCAGCTGCATTAATGAATGCTTCTACCGCAACAGGTTGCTTAAATT
911-Up-R	GCGTCTATAAATAGCATGAAGTATCCGCCCTAATCCTATCGAACGATAGT
911-Down-F	TATTTATGTTATTTCCGTTGTCGTTATCGTTATGGAGACTTCCCGATACAT
911-Down-R	GGATAACCGTATTACCGCCTTTGAGCTCTACAGATGTCATATACATGTGGCAA
911-P-*FCB2*-F	ACTATCGTTCGATAGGATTAGGGCGGATACTTCATGCTATTTATAGACGCG

911-*FCB2*-T-R	ATGTATCGGGAAGTCTCCATAACGATAACGACAACGGAAATAACATAA ATAGC
911-KZ-F	GCTTCTACCGCAACAGGTTG
911-KZ-R	CTCTACAGATGTCATATACATGTGGCAA

2.3 Experimental instruments

Table 2.4 The main instruments used in this chapter

Instrument name	Instrument model	Manufacturer
Biosafety cabinet	BSC-03 Ⅱ A2	AIRTECH
Analytical balance	BSA124S-CW	Sartorius Scientific Instruments LTD
Ultra-low temperature refrigerator	DW-HL340	Zhongke Meiling Low Temperature Technology Co., LTD
Forced convection drying oven	DHG-9140A	Shanghai Jinghong Experimental Equipment Co., LTD
High-pressure steam sterilizer	LDZX-50KBS	Shanghai Shen 'an Medical Device Factory
Benchtop high-speed centrifuge	Fresco17	Thermo Fisher Scientific China Co., LTD
Constant temperature incubator shaker	ZWYR-D2403	Shanghai Zhicheng Analytical Instrument Manufacturing Co., LTD
BioPhotometer	D30	Eppendorf
High performance liquid chromatograph	Waters e2695	Waters Company
Liquid chromatography column	HPX-87H	Bio-Rad
Liquid chromatography column	WondaSil C18	GL Sciences
Large high-speed refrigerated centrifuge	CR22N	Gongji Holdings Co., LTD
Fully automatic reducing sugar analyzer	SGD-IV	Shandong Academy of Sciences (Institute of Biology)
Constant temperature water bath pot	DZKW-C	Shanghai Shuli Instrument & Meter Co., LTD
Ultraviolet-visible spectrophotometer	UV-8000S	Shanghai Yuanxi Instrument Co., LTD
Ultra-pure water system	Milli-Q Advantage A10	Merck Millippo, Germany
High-speed refrigerated centrifuge	Multifuge X1R	Thermo Fisher Scientific China Co., LTD

Ambient temperature plasma mutagenesis system	ARTP- Ⅱ	Tianmu Biotechnology Co., LTD
Gel imaging system	A1600	General Electric Company
PCR instrument	GT9611	Hangzhou Baiheng Technology Co., LTD
Microplate reader	EPOCH2TC	Burton Instruments Co., Ltd. of the United States
Constant-temperature metal bath	DTC-100	Hangzhou Mio Instrument Co., LTD
Gel electrophoresis apparatus	600	Beijing Junyi Company
Microwave oven	M1-211A	Beauty
Multi-functional sample homogenizer	PRECELLYS 24	BERTIN
Ph meter	PB-10	Sartorius Scientific Instruments LTD

2.4 Screening Method for Strains of corn Pulp Fermentation Chassis

Single colonies were picked from the YPD solid plates cultured with strains A10-1, M8, Y02, Y04, RC212, BSIF and 6M, inoculated into small flasks containing 5 mL of YPD liquid medium and activated for 12 hours. The activated bacterial liquid was then transferred to a 100 mL conical flask containing 20 mL of YPD liquid medium. Reactivated for 12 hours. The activated bacterial liquid was centrifuged respectively to collect the bacterial cells, and resuspended with a small amount of sterile ultrapure water. They were respectively inoculated into 40 mL of 50% (v/v) corn slurry (unsterilized, diluted twice with sterile water), with an inoculation volume of OD600=3.5. Samples were taken in the clean table once every 12 hours. Finally, the content of lactic acid was determined and the growth of the strain was detected.

2.5 Microbiology and Molecular Biology methods

(1) Methods for preserving bacterial strains

Escherichia coli culture solution: 50% (v/v) glycerol, sterilized at 115℃ for 30 minutes, and stored at room temperature.

Yeast culture solution: 50% (v/v) glycerol, 0.85% NaCl, sterilized at 115℃ for 30 minutes, stored in a 4℃ refrigerator.

Pick out individual colonies and cultivate them in the corresponding liquid medium for 12 hours. Take a small amount of the bacterial liquid and streak it on a solid plate. The plate can be stored in a 4℃ refrigerator for a short period of 7 to 10 days. Secondly, transfer the cultured bacterial liquid to a new liquid medium. When the strain grows to the logarithmic phase, collect the bacterial cells by centrifugation, resuspend them with the corresponding culture medium, and then transfer them to a culture tube for long-term storage at -80℃ in a refrigerator.

(2) Escherichia coli transformation and plasmid extraction

Take a sample of competent cells from the large intestine in an ice box and dissolve them into a semi-solid state. Add 10 μL of the recombinant plasmid (the total amount should not exceed 30 ng), gently tap with your fingers to mix well, and then place it in an ice bath on ice for 30 minutes.

Place the EP tube in a constant temperature water bath at 42℃ and heat it accurately for 90 seconds.

Quickly transfer the EP tube to the ice box for an ice bath for 3 minutes.

In the laminar flow hood, add 0.8 mL of LB liquid medium to the EP tube, place it in a constant temperature shaker, and incubate it slowly by shaking at 37℃ and 150 r/min for 45 minutes.

Centrifuge the EP tube at 5000 rpm for 3 minutes, remove the supernatant and add 1 mL of sterile water to resuspend the bacteria. Repeat the above steps, resuspend the bacteria in 200 m sterile water, take 100 μL of the bacterial solution and spread it on the LB solid plate with the required resistance, and then incubate in a constant temperature incubator at 37 ° C for 12 hours.

Select single colonies and place them in 5 mL of LB liquid medium containing the corresponding resistance, and incubate at 200 rpm and 37℃ for 8 to 16 hours.

Transfer the bacterial solution to a 15 mL centrifuge tube and centrifuge at 5000 rpm for 5 minutes. Extract the plasmid according to the plasmid extraction

kit. Determine the concentration of the extracted plasmid using an enzyme-linked immunosorbent assay (ELISA) reader and store it in a refrigerator at -20 ° C.

(3) Chemical transformation method of Brewer's yeast

Select single colonies and inoculate them into a small amount of YPD liquid medium. Incubate at 200 rpm and 30℃ for 12 hours. Then transfer the bacterial liquid to 5 mL of YPD liquid medium, with an inoculation volume of OD600=0.2.

The strain was placed in a constant temperature shaker at 200 rpm and 30℃ for cultivation until the logarithmic growth phase of the strain, that is, OD600=0.6-0.9, and the cultivation time was approximately 3 hours.

Transfer the bacterial solution to a 15 mL centrifuge tube and centrifuge at 4500 rpm for 5 minutes. Remove the supernatant, collect the bacteria, and transfer them to a 1.5 mL sterile EP tube.

Resuspend the bacteria with 1 mL of 100 mM lithium acetate, centrifuge at 8000 rpm for 15 seconds, remove the supernatant, and repeat this step once.

Resuspend and mix 240 μL of 50% (w/v) polyethylene glycol in a laminar flow table. Add 36 μL of 1 M lithium acetate, 10 μL of single-stranded fish essence DNA (boil in advance for 10 minutes and place on ice), 1.5 μg of plasmid and an appropriate amount of DNA fragments (add 1 μg per kb of fragment). Replenish the system with water to 360 μL and thoroughly mix by blowing and suction.

Incubation and transformation were carried out in a constant temperature metal bath according to the procedures of 30℃ for 30 min, 42℃ for 45 min and 16℃ for 8 min.

Centrifuge the reaction system at 8000 rpm for 15 seconds, remove the supernatant, add 1 mL of YPD liquid medium, and place it in a constant temperature shaker at 30 ° C and 150 rpm for slow shaking and incubation for 2 hours.

Centrifuge the EP tube at 8000 rpm for 15 seconds, remove the supernatant and add 1 mL of sterile water to resuspend the bacteria. Repeat the above steps, resuspend the bacteria in 600 μL of sterile water, draw 70 μL of the bacterial

solution and spread it on the YPD solid plate with the required resistance, and then incubate it in a constant temperature incubator at 30 ° C for 3 to 5 days.

(4) Method for extracting the Saccharomyces cerevisiae genome

Add 200 μ l of DNA extract I to the EP tube, pick out a small amount of bacterial sludge and add it, then mix thoroughly.

Add 0.4 g of acid-washed glass beads to the EP tube and then add 200 μL of DNA extract II.

Vortex and oscillate the EP tube for 1 minute, centrifuge at 12,000 rpm for 10 minutes, and let it stand to wait for stratification.

Take the supernatant, add 1 mL of anhydrous ethanol, centrifuge at 12,000 rpm for 10 minutes, and remove the supernatant.

After inversion and drying, add 50 μL of ultrapure water, vortex and shake, and store in a refrigerator at -20℃.

(5) Amplification of gene fragments and verification by agarose gel electrophoresis

Using plasmids or Saccharomyces cerevisiae genomes as templates, the target gene fragments were amplified using the high-fidelity enzyme Phanta Max Super-Fidelity DNA Polymerase. The reaction system was as follows: DNA template: 1 μL, F: 2 μL, R: 2 μL, dNTP: 1 μL, DNA Polymerase: 1 μL, 2×Phanta Max Master Mix: 25 μL, ddH20 supplemented to 50 μL. The reaction procedure is as follows: 95℃ for 3 minutes, 95℃ for 30 seconds, Tm-5℃ for 15 seconds, 72℃ for every 30-40 seconds /kb, with a total of 35 cycles. If a plasmid is used as the template, 1 μL of Dpn Ⅰ is required to digest the template plasmid in the PCR product. Verification was conducted using 1% agarose gel electrophoresis. The DNA electrophoresis products were recovered using a gel recovery and purification kit, and the recovered concentration was detected using an enzyme-linked immunosorbent assay (ELISA) reader. They were stored in a refrigerator at -20℃.

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(6) Construction of recombinant vectors and acquisition of yeast transformation fragments

The vector fragments with promoter PCCW12, terminator TPRM5 and upstream and downstream homologous arms (416-Up, 416-Down) were amplified from the 416-GFP plasmid by reverse PCR technology, and the target gene fragments were amplified from the vector plasmids or genomes with the target gene. The vector fragment was seamlessly ligated with the target gene fragment using Gibson Assembly technology[22], and then the 416 Up-PCCW12-Gene-TPRM5-416-Down transfer-in fragment was obtained by PCR with primers 416-KZ-F and 416-KZ-R. For details, see Figure 2.1.

(7) Integration and verification of the diploid Saccharomyces cerevisiae gene[23]

The CRISPR Cas9 gene editing system[24] was adopted to co-transform the constructed recombinant plasmids carrying gRNA (such as pTC-tR416d) with the integrated DNA fragments containing the target genes into Saccharomyces cerevisiae strains. The transformation products were coated on G418-resistant plates. After the formation of single colonies, some of the cells were selected for

conservation, and the remaining cells were used for genome extraction. Using genomic DNA as a template, PCR amplification was performed on the insertion region of the target gene using specific primers (416-KZ-F and 416-KZ-R). The amplification products were separated by 1% agarose gel electrophoresis and observed and analyzed by the gel imaging system.

The criteria for determining the electrophoresis results are as follows: If a single bright band only appears at the expected insertion length position of the target gene, it indicates that both chromosomes of the diploid strain have successfully integrated the target gene; If bands appear simultaneously near 500 bp and at the expected length of the target gene, it indicates that only one chromosome has completed integration. If a band is observed only at the 500 bp position, it indicates that the target gene has not been successfully integrated.

For the integrated strains identified as positive, passage culture was carried out using YPD liquid medium, aiming to promote the loss of plasmids carrying the screening markers, thereby ensuring the stable existence of the integrated genes and building a reliable genetic basis for subsequent multiple rounds of gene editing. The bacterial liquid after subculture was evenly spread on the surface of YPD solid medium. Single colonies were picked and inoculated respectively on YPD plates with G418 resistance and without antibiotics. The screening criteria are set as follows: Select colonies that can grow normally on non-resistant plates but cannot on resistant plates, and then repeat the PCR verification process on them. Only strains whose target genes have been confirmed to be stably present will be preserved in accordance with standard operating procedures.

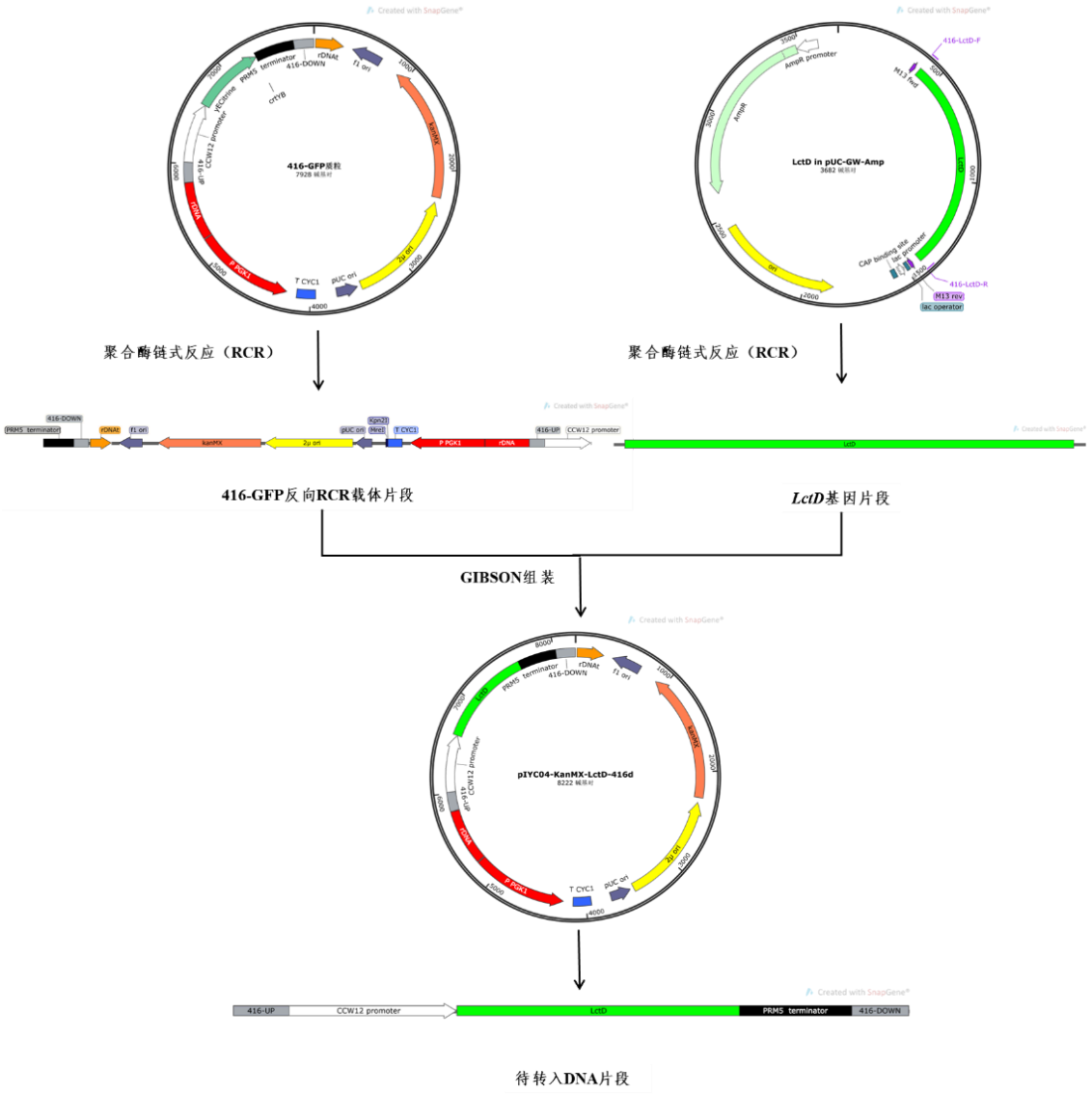

Figure2.1 Construction of Recombinant Plasmid and Process Flow Diagram for Acquiring Transformed DNA Fragments

2.6 Test of L-lactic acid metabolic capacity of modified strains and Detection methods of metabolites

(1) Analysis of L-lactic acid metabolic capacity of L-iLDH expression strains from different sources

The engineered strans Y02-FCB2, Y02-LCTO, Y02-LCTD and the original strain Y02 were respectively inoculated into the YPD liquid medium and subjected to second-generation activation culture under the conditions of 30℃ and 200 r/min. Take the culture and transfer it to a 50 mL centrifuge tube. Centrifuge at 5000 r/min for 5 minutes to collect the bacteria. Resuspend with sterile water and inoculate it into a conical flask containing 40 mL of YPL medium at an inoculation

volume of OD600=3.5. Place it on a shaker at 30 ° C and 200 r/min for aerobic fermentation. During the fermentation process, samples were taken every 12 hours to monitor the concentration of lactic acid in the culture medium and the growth of the strain simultaneously (characterized by the OD600 value).

(2) Evaluation of the effect of FCB2 gene dose on lactic acid metabolism

For the strains Y02-FCB2, Y02-FCB2-2 and the original strain Y02, the lactic acid metabolic capacity test was carried out under the same culture conditions (YPL medium, inoculation amount, fermentation parameters) as mentioned above. By comparing the lactic acid consumption rates of different strains and the growth curves of the cells, the effects of FCB2 gene expression levels on the lactic acid metabolic efficiency and growth performance of Saccharomyces cerevisiae Y02 strain were analyzed.

(3) Metabolomics detection of pyruvate accumulation [25]

During the peak period of lactic acid metabolism rate of each strain, 1 mL of the fermentation broth was taken for cell disruption treatment, and the total amount of intracellular and extracellular pyruvate was quantitatively analyzed by high performance liquid chromatography (HPLC) technology. By monitoring the dynamic changes of pyruvate during the fermentation process, exploring whether its accumulation has an inhibitory effect on the growth and metabolism of the strain, and thereby clarifying the correlation between pyruvate accumulation and the physiological state of the strain.

Analysis of L-lactic acid metabolic capacity of L-iLDH expression strains from different sources

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fermentation. During the fermentation process, samples were taken every 12 hours to monitor the concentration of lactic acid in the culture medium and the growth of the strain simultaneously (characterized by the OD600 value).

(2) Evaluation of the effect of FCB2 gene dose on lactic acid metabolism

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Summary of chapter II

1. Reagent selection

2 Culture medium preparation and antibiotic treatment

3. Strain and plasmid

4. Primer and gene processing

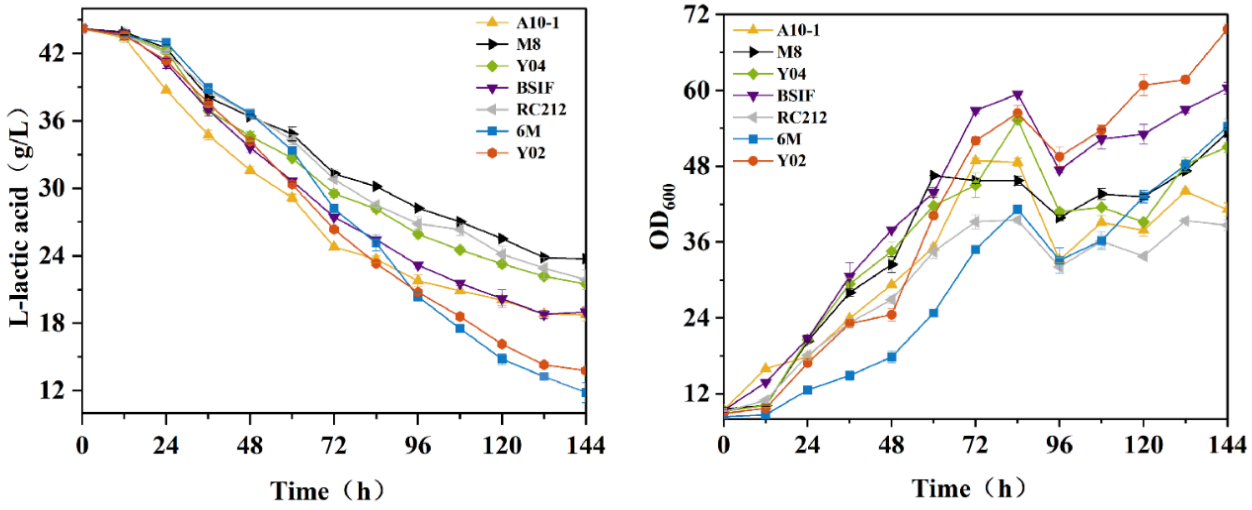
Chapter III
EXPERIMENTAL PART

3.1 Screening of *Saccharomyces cerevisiae* strains using lactic acid in corn pulp as the fermentation carbon source

This study systematically analyzed the differences in metabolic characteristics among different Saccharomyces cerevisiae strains and their adaptability to corn pulp. Through the functional evaluation of 7 strains of industrial yeast preserved in the laboratory (including characteristics such as high fermentation efficiency, strong resistance to environmental stress, tolerance to high osmotic pressure, tolerance to high temperature, and adaptation to compound inhibitors), it was found that each strain showed significant differences in lactic acid metabolism efficiency and growth characteristics. In the specific experiment, the activated strains were transferred to the medium containing 50% (v/v) corn pulp at the same inoculation amount, and their growth kinetics and lactic acid degradation kinetics were monitored (Figure 2.3).

Experimental data show that all strains have the ability to utilize lactic acid and maintain normal growth, but there are significant differences in metabolic rates and patterns. The lactic acid degradation efficiency of strains Y02 and 6M is the best. After 144 hours of fermentation, the residual concentrations decreased to 13.78 g/L and 11.83 g/L respectively. It is notable that the metabolic activity of strain A10-1 was most prominent within the first 72 hours, but the metabolic rate dropped sharply in the later period and basically stagnated at 132 hours. Strain BSIF also shows a similar pattern. It is speculated that this phenomenon is related to its prominent inhibitor tolerance characteristics: Strong environmental adaptability enables it to rapidly enter the logarithmic growth stage, but in the later stage, insufficient metabolic dynamics may occur due to the accumulation of metabolic by-products or energy distribution strategies.

Through comparison, it was found that at 60 hours before fermentation, the lactic acid metabolic capacity of Y04 and M8 was significantly weaker than that of other strains.[41] Further analysis indicates that the sugar metabolism superiority of these two strains may have an inhibitory effect on carbon metabolism, suppressing the biosynthesis of cytochrome b2 (a key enzyme that specifically catalyzes the oxidation of lactic acid), thereby delaying the decomposition of lactic acid. Observed from the growth curve, the biomass of all strains showed a brief stagnation or decrease at 96 h, which might be related to the temporary accumulation of the metabolic intermediate product pyruvic acid. As pyruvate is gradually converted into substances needed for growth, bacteria exhibit the characteristics of secondary proliferation.[43] Among them, the biomass accumulation of Y02 was the most significant (with an OD value reaching 72), and it had advantages in terms of comprehensive metabolic efficiency and growth performance. Finally, this strain was selected as the starting strain for the subsequent study.[42]



(a) Lactic acid metabolism (b) Diagram of the growth

Figure 2.3 Lactic Acid Metabolism and Growth of *S.cerevisiae* Strains in Corn Steep Liquor Medium

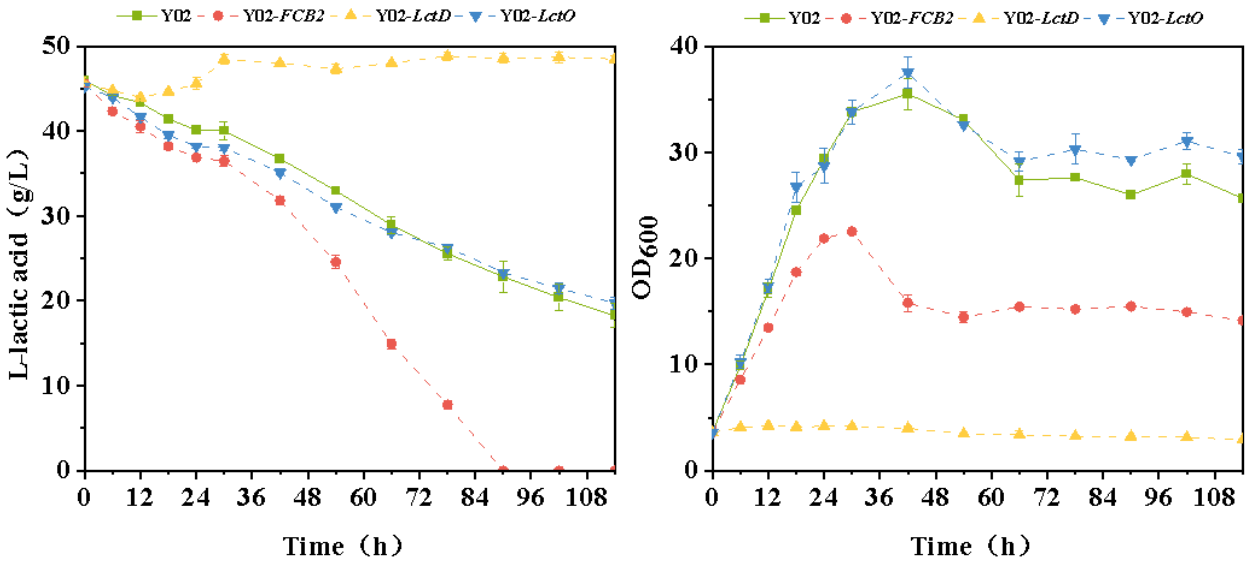
3.2 Expressing L-iLDH from different sources improves the lactic acid utilization ability of Saccharomyces cerevisiae

To improve the degradation efficiency of lactic acid by the target strain Y02, in this study, its metabolic network was reasonably modified, with a focus on strengthening the key nodes of lactic acid oxidation. The recombinant strains Y02-FCB2, Y02-LctO and Y02-LctD were obtained by heterointegrating three non-NAD dependent L-lactate dehydrogenase genes of Escherichia coli LctD, Lactococcus lactis IL1403 LctO and endogenous FCB2/CYB2 of Saccharomyces cerevisiae. To minimize the interference of complex substrates, the YPL medium with lactic acid as the sole carbon source was used for phenotypic analysis.

The experimental results (Figure 2.6) show that due to the expression of exogenous enzymes, the metabolic traits have changed significantly: Y02-LctD has completely lost the ability to degrade lactic acid, while there is no statistically significant difference in the metabolic efficiency of Y02-LctO compared with the parental strain. It is worth noting that the strain overexpressing endogenous cytochrome c oxidorereductase (encoded by FCB2) showed a significant advantage, completely consuming 45 g/L lactic acid within 90 hours, and the degradation rate was 100% higher than that of the original strain. However, its biomass accumulation decreased by approximately 30%, indicating a trade-off effect between growth and catabolic flux redistribution.

The dynamic monitoring results indicated that the biomass of the recombinant strain briefly decreased at 48 hours, which was consistent with the result of the fermentation of the corn soaking solution. The intracellular pyruvate concentration at the end of fermentation (Figure 2.7) indicated that Y02-FCB2 accumulated 4.97 mg/g DCW, which was significantly higher than that of other strains, verifying the hypothesis that excessive intermediate accumulation inhibited cell growth. Importantly, although Y02-LctD lost the utilization of lactic acid in YPL, its normal growth in the nutrient-rich YPD medium ruled out the possibility of necessary gene destruction during the genetic integration process. Further analysis indicates that the high expression of LctD may lead to an imbalance in pyruvate metabolism, which may be confirmed by regulating its expression through promoter engineering.

To sum up, although the growth is inhibited, the excellent lactic acid degradation efficiency of Y02-FCB2 makes it the best candidate for subsequent studies. These findings emphasize the necessity of systematically balancing the flux of target pathways and the physiological state of cells in metabolic engineering.



(a) Lactic acid metabolism (b) growth curve

Figure 2.6 Lactic acid metabolism and growth curve of the modified strain and control strain in YPL medium

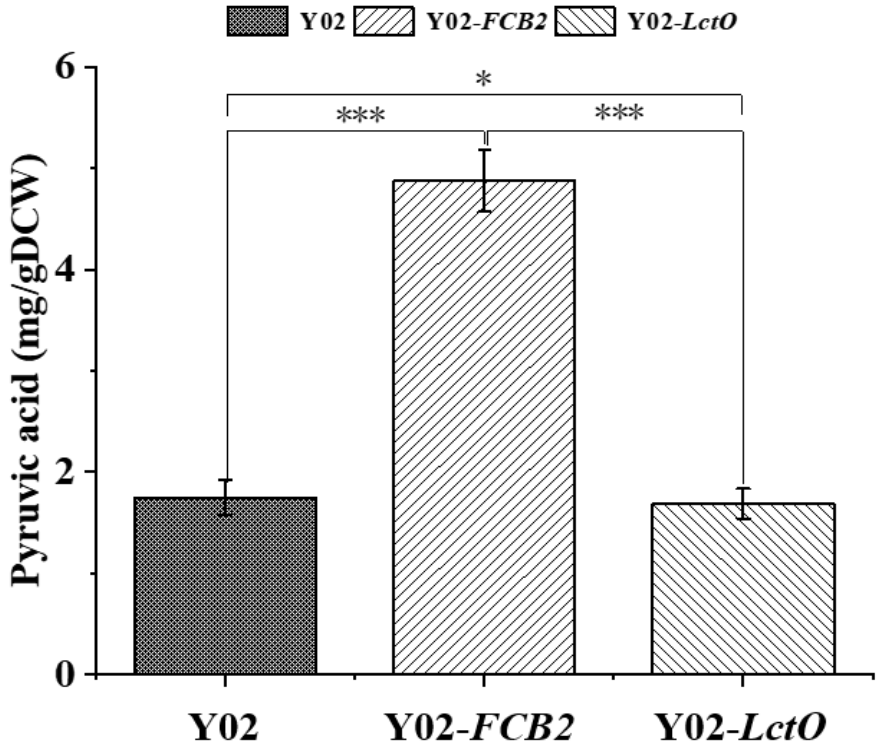
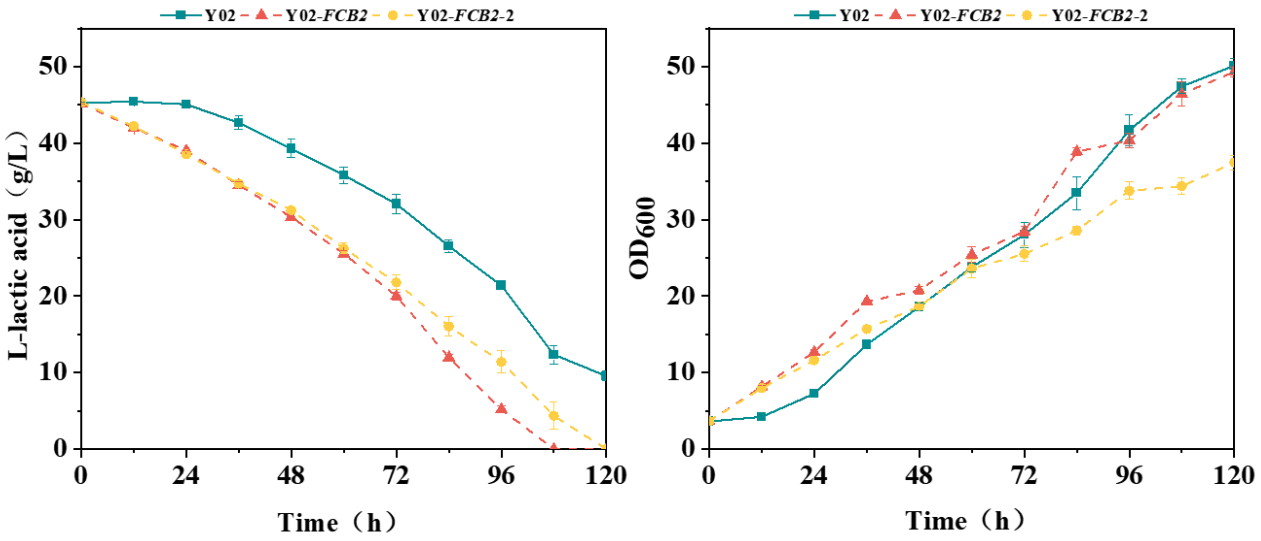


Figure 2.7 Pyruvic acid content in strains fermented in YPL medium for 96 hours

3.3 Utilization of Lactic acid in Saccharomyces cerevisiae by overexpression of the FCB2 gene

To explore the methods for enhancing the lactic acid metabolism efficiency of the strain, the research team carried out the copy number amplification experiment of the FCB2 gene. However, the data show that with the increase of the copy number of FCB2 gene expression, the consumption rate of lactic acid by the strain shows a decreasing trend, and the growth of the bacteria is also significantly inhibited (as shown in Figure 2.8). Through metabolic pathway analysis, it is speculated that this might be due to the excessive accumulation of intracellular pyruvate exerting a feedback inhibitory effect on the catalytic activity of cytochrome b2, thereby leading to a decrease in the reaction rate of the lactic acid metabolic pathway. The above experimental results indicate that the expression level of the FCB2 gene has become a key factor in coordinating the lactic acid metabolic efficiency and growth status of the strain. Based on this, subsequent

studies can focus on constructing a precise expression regulation system of the FCB2 gene, and achieve a balanced improvement of the lactic acid utilization ability and growth performance of the strain by optimizing its expression level.



(a) Lactic acid metabolism (b) Growth condition

Figure 2.8 Effects of Increasing *FCB2* Expression Copy Number on Strain Lactic Acid Metabolism and Growth

Summary of chapter III

1. The screening results of the strains showed that Y02 and 6M performed the best, and Y02 was selected.

2 After genetic modification, the degradation rate of Y02-FCB2 increased, but the biomass decreased.

The intracellular accumulation of pyruvate in the Y02-FCB2 strain significantly inhibited its growth

4. The conclusion emphasizes the importance of balance in metabolic engineering.

CONCLUSION

In this study, through multiple rounds of fermentation performance evaluations, strain Y02 was screened out from seven industrial diploid Saccharomyces cerevisiae strains with excellent characteristics as the base chassis. To prevent contamination by miscellaneous bacteria, a sterilization process based on a low-temperature water bath (65℃, 30 min) was established. This condition can effectively inactivate common contaminating bacteria such as Lactobacillus acidophilus (survival rate <0.1%). The exogenous genes LctD (lactate transconductance enzyme), LctO (lactate oxidase) and endogenous FCB2 (cytochrome b2) were integrated into the Y02 genome respectively by CRISPR/Cas9 technology, and the Y02-LCTD, Y02-LCTO and Y02-FCB2 engineered bacteria were successfully constructed. The fermentation test of YPL medium showed that the lactic acid degradation rate of Y02-FCB2 was 2.1 times higher than that of the original strain ($p<0.05$), so it was selected as the subsequent research object. It is notable that increasing the dose of the FCB2 gene through the multi-copy integration strategy did not produce a synergistic effect ($p>0.1$), suggesting the existence of post-translational regulatory limitations. Through the experimental design of three-factor and three-level response surfaces, the effects of the initial pH (4.5-6.0), substrate concentration (30-50%), and the addition amount of H2O2 (0.1-0.3 mM) on the fermentation performance were optimized. The final determined optimal conditions were: pH5.2, corn slurry concentration of 45%, and 0.2 mM H2O2. This combination increased the lactic acid degradation efficiency by 37% ($\Delta AUC=15.8$). To overcome the key limiting factors in the metabolic process of Y02-FCB2, this study innovatively combined the atmospheric pressure room-temperature plasma (ARTP) mutagenesis technology with the progressive adaptive evolution method. Based on the colony morphological characteristics and growth kinetics data as the screening basis, through the systematic analysis of the mutant strain library, the engineered strain YF3 with significant metabolic advantages was finally successfully obtained.

This strain demonstrated significantly enhanced metabolic activity in a 50% corn pulp system The lactic acid degradation amount reached 32.7 g/L within a 156h fermentation cycle, which was 28% higher than that of the parent strain. The yield of the synchronously synthesized bacterial protein was 32.6 g/L (19.8 g/L in the Y02 control group), and the nitrogen source conversion efficiency increased by 64.6%. This improvement significantly enhanced the nutritional value of corn slurry (with a 21.3% increase in crude protein content) and its market application potential. The technological breakthrough of this study is to successfully construct an industrial yeast strain with both efficient lactic acid metabolism and environmental adaptability by integrating genetic engineering and evolutionary domestication strategies, providing an innovative solution for the value-added transformation of biomass waste.

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