Metabolic engineering for enhanced biofuel precursors production in *Saccharomyces cerevisiae*

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2015

Tang, X. (2015). Metabolic engineering for enhanced biofuel precursors production in *saccharomyces cerevisiae*. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/62229>

<https://doi.org/10.32657/10356/62229>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**Metabolic Engineering for Enhanced Biofuel Precursors
Production in *Saccharomyces cerevisiae***

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2015

**Metabolic Engineering for Enhanced Biofuel Precursors
Production in *Saccharomyces cerevisiae***

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A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

2015

Acknowledgement

Foremost, I would like to express my sincere gratitude to my supervisor, Prof. Chen Wei Ning, William, for providing me such a good environment for the research work. His valuable instructions and encouragement made the whole work go on wheels. I am truly and deeply indebted to him, who paid motivation and enthusiasm on my research project.

Secondly, I would like to express my great thanks to Nanyang Technological University, for providing me the experiment facilities and the opportunity for Ph.D. program with full research scholarship.

Thirdly, I would like to express my truly thanks to my lab colleagues: Dr. Zhang Jianhua, Mr. Tan Keeyang, Miss. Shi Jiahua, Miss Li Xiang, Miss. Chen Liwei, Miss Zhao Guili, Miss Lee Jie Lin and Miss Hui Toh Shi, for their constructive suggestions and friendship.

I also want to give my deep gratitude to Mr. Zheng Renchao, for his continuously encourage when I was depressed and strong help when I met challenges during my research work.

Last but not the least, I would like to express my heartwhole gratitude to my family: My beloved parents Tang Fugen and He Guifeng, my husband Yu Xinyan and my dear sister Tang Lingyan, for their strong support without a single word of complaint. My whole family devoted so much on me and with their continuous trust and sincerest love, I can become a woman confident and optimistic.

Table of contents

Acknowledgement	I
List of tables	VII
List of figures	IX
List of abbreviations	XV
Summary	XIX
1. Introduction	1
1.1 Microbial production of biofuels	1
1.2 Fatty acid-derived fuels produced from microorganisms	5
1.3 Fatty acid synthesis in yeast	9
1.3.1 Fatty acids in yeast	9
1.3.2 <i>De novo</i> biosynthesis of fatty acids in yeast cells	11
1.3.3 Substrates and cofactors for fatty acid synthesis	14
1.4 Regulation of fatty acid metabolism in yeasts	16
1.5 Effect of culture conditions on fatty acid production levels	20
1.6 Metabolomics	23
1.6.1 Principle of metabolomics analysis	23
1.6.2 Application of metabolomics in biofuel production	27
1.7 Proteomics	29
1.7.1 Principle of LC-MS based proteomic analysis	29
1.7.2 Application of proteomics in biofuel production	32
2. Materials and methods	36
2.1 Molecular cloning and expression of heterologous enzymes in <i>S. cerevisiae</i> ..	36
2.1.1 Strains, plasmids, genes and reagents	36
2.1.2 Competent cell preparation	37
2.1.3 Enzyme digestion, gene insertion and transformation	38
2.1.4 Verification of target gene by colony PCR	41
2.1.5 Protein extraction, SDS-PAGE and Western blot analysis	42
2.2 Gene disruption	44
2.2.1 Plasmid and primer design	44
2.2.2 Generation of disruption cassette and transformation	45
2.2.3 Verification of gene disruption by colony PCR	47
2.3 Culture of yeast cells	48
2.4 Metabolomics analysis	50
2.4.1 Cell lysis, extraction of soluble metabolite and lipid	50
2.4.2 Soluble metabolites derivatization	51
2.4.3 Fatty acid derivatization	52
2.4.4 GC-MS procedure	53
2.4.5 Data analysis	53
2.5 Proteomics	55
2.5.1 Protein extraction, digestion and labeling	55
2.5.2 On-line 2D Nano LC-MS/MS procedure	56
2.5.3 Protein identification and data analysis	58

2.5.4 Real-time PCR	58
3. Overexpression of a heterologous ACL in <i>Saccharomyces cerevisiae</i> and investigation of fatty acid production under nitrogen limited culture condition	61
3.1 Introduction	61
3.2 Experiment procedures	63
3.3 Results	64
3.3.1 Cloning and overexpression of <i>acl</i> in the <i>S. cerevisiae</i>	64
3.3.2 Effect of culture conditions with different carbon and nitrogen concentration on biomass	65
3.3.3 Investigation of fatty acids production levels in <i>S. cerevisiae</i> wild type strain with pVTU-260 vector (“we”) under different culture conditions	67
3.3.4 Investigation of fatty acids production levels in <i>S. cerevisiae</i> engineered strain expressing <i>acl</i> (“wa”) under different culture conditions	69
3.3.5 Comparison of fatty acids production levels between “we” strain and “wa” strain	72
3.3.6 Investigation of intracellular citrate level in <i>S. cerevisiae</i> “we” strain and “wa” strain	74
3.3.7 Transcriptomic response of <i>amd</i> and <i>idh</i> genes in <i>S. cerevisiae</i> to nitrogen limited condition	75
3.4 Discussion	77
3.5 Section conclusion	80
4. Investigation of citrate accumulation in the IDH gene disrupted strains of <i>Saccharomyces cerevisiae</i>	81
4.1 Introduction	81
4.2 Experiment procedures	83
4.3 Results	84
4.3.1 Gene disruption of <i>S. cerevisiae</i>	84
4.3.2 Growth rate description between the wild type strain and gene disrupted strains under YPD culture medium	85
4.3.3 Citrate level description between the wild type strain and the gene disrupted strains	86
4.3.4 ATP and NADPH level description between the wild type strain and gene disrupted strains	89
4.4 Discussion	92
4.5 Section conclusion	93
5. Overexpression of a heterologous ACL in the <i>S. cerevisiae</i> IDH gene disrupted strains for enhanced fatty acid synthesis	95
5.1 Introduction	95
5.2 Experiment procedures	96
5.3 Results	97
5.3.1 Cloning and expression of <i>acl</i> in the <i>S. cerevisiae</i> wild type strain and <i>idh1,2</i> disrupted strains	97
5.3.2 Growth rate description between the wild-type strain and engineered strains under SC culture medium	99

5.3.3 Investigation of fatty acids production levels in the <i>S. cerevisiae</i> wild type strain and engineered strains	100
5.3.4 Comparison of fatty acids production between the <i>S. cerevisiae</i> wild type strain and engineered strains	102
5.4 Discussion	105
5.5 Section conclusion.....	108
6. Comparative proteomics analysis of the engineered <i>Saccharomyces cerevisiae</i> with enhanced fatty acid synthesis	109
6.1 Introduction	109
6.2 Experiment procedures	110
6.3 Results	111
6.3.1 Differentially expressed proteins among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains	111
6.3.2 Real-time RT-PCR based mRNA levels investigation of differentially expressed proteins.....	115
6.3.3 Intracellular metabolites production levels among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains	117
6.4 Discussion	119
6.5 Section conclusion.....	125
7. Enhanced production of fatty acid derivatives in engineered <i>Saccharomyces cerevisiae</i>	126
7.1 Introduction	126
7.2 Experiment procedures	128
7.3 Results	129
7.3.1 Growth rate description	129
7.3.2 Effect of the engineered pathway on the lipid content	131
7.3.3 Enhanced production of intracellular fatty acid derivatives in the engineered <i>S. cerevisiae</i>	133
7.3.4 Investigation of fatty alcohols secretion in the engineered <i>S. cerevisiae</i>	135
7.4 Discussion	138
7.5 Section conclusion.....	141
8. Conclusions and future direction	142
1) Toward a better understanding of the fatty acid accumulation in the engineered <i>S. cerevisiae</i>	143
2) Toward the metabolic design of <i>S. cerevisiae</i> pathways for enhanced fatty acids production.....	144
3) The application of metabolomics and proteomics in the pathways analysis	145
4) Engineering the <i>S. cerevisiae</i> for enhanced production of fatty acid derivatives	146
8.2 Future direction	146
1) Effectively convert accumulated fatty acids to useful fuels and chemicals in <i>S. cerevisiae</i>	146

2) Overcoming the problems of ATP, NADPH deficiency resulted from the engineered pathway	148
3) The functional study of differentially expressed proteins in the engineered strains on the production of fatty acid/fatty acid derivatives	148
4) The study of industrial scale up of biofuel production by the engineered <i>S. cerevisiae</i>	149
References	151

List of tables

Table 1.1 Lipid accumulation and fatty acid profile of various oleaginous yeasts.

Table 3.1 Culture medium composition with different glucose and ammonium sulfate concentration.

Table 3.2 Residual glucose content in different culture medium of “we” and “wa” strains.

Table 3.3 Fold changes of specific fatty acid composition between the “we” strain and “wa” strain.

Table 4.1 Primers for the generation of gene disruption cassette in this study.

Table 4.2 NADPH/NADP⁺ ratio between the wild type strain and the gene disrupted strains.

Table 5.1 Fatty acids composition changes between the engineered *S. cerevisiae* and wild type strain.

Table 6.1 Relative changes in protein expression between *S. cerevisiae* wild type and engineered strains.

Table 6.2 Primer sequences for real-time RT-PCR.

Table 7.1 Fatty acid compositions included in the lipid content in the wild type strain and *dgal* gene disrupted strain.

Table 7.2 Fatty alcohol compositions in the wt-FAR strain and $\Delta dgal$ -FAR strain.

Table 7.3 Fold changes of fatty alcohol production levels in the $\Delta dgal$ -FAR

strain compared with that in the wt-FAR strain.

List of figures

Fig. 1.1 Adaptation of the *Clostridia* acetone-butanol-ethanol (ABE) fermentation pathway for the production of isopropanol and butanol in *E. coli*.

Fig. 1.2 The metabolic pathways from pyruvate to isobutanol in *S. cerevisiae*.

Fig. 1.3 The alkane/alkene synthesis pathway indicated in *E. coli* and *S.cerevisiae*.

Fig. 1.4. Schematic diagram of fatty acid synthesis and elongation.

Fig. 1.5 The metabolic pathway of TAG synthesis in yeast cells.

Fig. 1.6 The metabolic pathways for acetyl-CoA generation in yeasts.

Fig. 1.7 Working mechanism of iTRAQ labeling reagents.

Fig. 2.1 Schematic diagram of the pVTU260-*acl* construct.

Fig. 2.2 The pUG plasmids carrying loxP-flanked gene disruption cassettes.

Fig. 2.3 Outline of the one-step gene disruption approach for the generation of gene disrupted strains.

Fig. 3.1 Schematic diagram of fatty acid accumulation mechanism in oleaginous yeasts under nitrogen limited culture conditions.

Fig. 3.2 Colony PCR result of *acl* expression in the *S. cerevisiae*.

Fig. 3.3 Western blot result of *acl* expression in the *S. cerevisiae*.

Fig. 3.4 Biomass of the *S. cerevisiae* wild type strain and engineered strain under culture conditions with different carbon and nitrogen concentrations.

Fig. 3.5. Fatty acids production levels in the *S. cerevisiae* wild type strain with empty pVTU260 vector (“we”) under culture conditions with different carbon

and nitrogen concentrations.

Fig. 3.6 Fatty acids production levels in the *S. cerevisiae* engineered strain expressing *acl* (“wa”) under culture conditions with different carbon and nitrogen concentrations.

Fig. 3.7 The representative of GC-MS comparison spectra of the production levels of mono-unsaturated fatty acids C16:1 (A) and C18:1 (B) between the “we” strain and the “wa” strain under nitrogen limited culture condition and non-nitrogen limited culture condition.

Fig. 3.8 Intracellular citrate production levels between the *S. cerevisiae* wild type strain with empty pVTU260 vector (“we”) and engineered strain expressing *acl* (“wa”) under different culture conditions.

Fig. 3.9 The representative of GC-MS comparison spectra of the citrate levels between the “we” strain and the “wa” strain.

Fig. 3.10 Real-time PCR analysis of mRNA level of *amd* and *idh* gene in *S. cerevisiae* under nitrogen limited condition.

Fig. 4.1 The gene disruption results confirmed by the yeast colony PCR.

Fig. 4.2. Growth curve of the wild type strain and *idh1,2* gene disrupted strains.

Fig. 4.3 Differential production levels of cellular citrate between the *S. cerevisiae* wild type strain and the *idh1,2* gene disrupted strains.

Fig. 4.4 Differential production levels of extracellular citrate between the *S. cerevisiae* wild type strain and the *idh1,2* gene disrupted strains.

Fig. 4.5 The representative GC/MS spectrum of the citrate in the yeast

metabolites derived from total ion chromatograms.

Fig. 4.6 The representative of GC-MS comparison spectra of the citrate levels between the wild type strain and the gene disrupted strains.

Fig. 4.7 The NADPH production levels comparison between the *S. cerevisiae* wild type strain and *idh1,2* gene disrupted strains.

Fig. 4.8 The ATP production levels comparison between the *S. cerevisiae* wild type strain and *idh1,2* gene disrupted strains.

Fig. 5.1 The workflow of metabolic engineering pathway in *S. cerevisiae* connecting with citrate accumulation and cytosol acetyl-CoA generation via ATP-citrate lyase for fatty acid synthesis.

Fig. 5.2 Yeast colony PCR confirmation of the recombinant vector pVTU260-*acl* in *S. cerevisiae* wild type and $\Delta idh1,2$ strains.

Fig. 5.3 Western blot results of *acl* in *S. cerevisiae* wild type and $\Delta idh1,2$ strains.

Fig. 5.4 Growth curve and glucose consumption curve of the wild type strain and engineered strains.

Fig. 5.5 Total fatty acids production levels in the wild type strain and engineered strains.

Fig. 5.6 Fatty acids composition (% of total fatty acid) in the wild type strain and engineered strains.

Fig. 5.7 Differential production levels of individual types of fatty acids in the wild type strain and engineered strains.

Fig. 5.8 The representative of GC-MS comparison spectra of the production levels of mono-unsaturated fatty acids C16:1 (A) and C18:1 (B) between the wild type and engineered strains.

Fig. 6.1 Representative peptide fragmentation spectrum of triosephosphate isomerase: FALGQGVGVILCIGETLEEK.

Fig. 6.2 Real-time RT-PCR analysis of mRNA levels of selected differentially expressed proteins among wt-pvtu, *Δidh1/2-pvtu* and *Δidh1/2-acl* strains.

Fig. 6.3 Differential production levels of intracellular amino acids among wt-pvtu, *Δidh1/2-pvtu* and *Δidh1/2-acl* strains.

Fig. 6.4 Differential production levels of intracellular metabolites among wt-pvtu, *Δidh1/2-pvtu* and *Δidh1/2-acl* strains.

Fig. 6.5 Schematic diagram of metabolic pathway changes in the *S. cerevisiae* engineered strains.

Fig. 7.1 The workflow of the metabolic engineering pathway in the *S. cerevisiae* for the enhanced fatty acid derivatives production.

Fig. 7.2 Growth curves of the *S. cerevisiae* wild type strain and engineered strains.

Fig. 7.3 Fatty acid production levels between the wild type strain and the *dgal* gene disrupted strain.

Fig. 7.4 Intracellular fatty alcohol production levels between the wt-FAR strain and the *Δdgal*-FAR strain.

Fig. 7.5 Extracellular fatty alcohol production levels between the wt-FAR strain

and the $\Delta dga1$ -FAR strain.

Fig. 7.6 The representative of GC-MS comparison spectra of the production levels of fatty alcohols C14:0 (A), C16:0 (B) and C18:0 (C) in the wild type strain and engineered strains.

Fig. 8.1 Overall workflow of the research project and metabolic engineering pathways in *S. cerevisiae* for enhanced production of fatty acid and fatty acid derivatives.

List of abbreviations

ABE	Acetone-butanol-ethanol
ACC	Acetyl-CoA carboxylase
ACL	ATP-citrate lyase
ACP	Acyl carrier protein
ADP	Adenosine triphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BF ₃	Boron trifluoride
BSA	Bovine serum albumin
CoA	Coenzyme A
CTP	Citrate transport protein
2D	Two-dimensional
DAG	Diacylglycerol
DAGAT	Acyl-CoA: diacylglycerol acyltransferase
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid

ER	Endoplasmic reticulum
FAEE	Fatty acid ethyl ester
FAR	Fatty acyl-CoA reductase
FAS	Fatty acid synthetase
FCC	Frozen competent cell
FFA	Free fatty acid
G-3-P	Glycerol-3-phosphate
G418	Geneticin
GC	Gas chromatography
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IDH	Mitochondrial NADP ⁺ -isocitrate dehydrogenase
iTRAQ	Isobaric tag for relative and absolute quantitation
LB	Luria-Bertani
LC	Liquid chromatography
LiAc	Lithium acetate
LPA	Lysophosphatidic acid
ME	Malic enzyme
MS	Mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
NADPH	Nicotinamide adenine dinucleotide phosphate
NL	Nitrogen limited

NNL	Non-nitrogen limited
NMR	Nuclear magnetic resonance
PA	Phosphatidic acid
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene difluoride
RE	Restriction enzyme
RNA	Ribonucleic acid
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SCX	Strong cation exchange
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TAG	Triacylglycerol
TBST	Tris buffered saline-tween
TCA cycle	Citric acid cycle
TMCS	Trimethylchlorosilane
URA	Uracil
YNB	Yeast nitrogen base
YPD	Yeast extract-peptone-dextrose

Summary

Microbial production of biofuels has attracted a great deal of attention in modern society. The fatty acids are important precursors for the production of biofuels, such as aldehyde, alcohol and alkane/alkene. Its biosynthesis is initiated by converting acetyl-CoA to malonyl-CoA which requires acetyl-CoA as key substrate. To increase the starting materials for fatty acids synthesis could improve its production levels.

Among various microorganisms, the yeast *Saccharomyces cerevisiae* was selected to be metabolic modified for enhanced fatty acid production, due to its advantages of well-known genome information and metabolic pathways, good ability in anti-phage, organic solvent tolerance and high-concentration fermentation.

The oleaginous yeast has been found to accumulate lipid under nitrogen limited culture conditions and a well-known accumulation mechanism has been discovered, in which the activity of mitochondrial NAD^+ -dependent isocitrate dehydrogenase (IDH) was depressed due to the decrease of AMP concentration and more citrate was accumulated and cleaved by the cytosol ATP-citrate lyase (ACL) into acetyl-CoA for fatty acid synthesis. This gave clue that the depression of IDH is correlated with the accumulation of fatty acids and the ACL is a key enzyme to provide substrate for fatty acid biosynthesis.

To investigate the mechanism difference between the *S. cerevisiae* and oleaginous yeast in terms of fatty acid accumulation, the *S. cerevisiae* was firstly transformed with a heterologous ACL gene (*acl*) and cultured under different culture conditions. It was found that when the *S. cerevisiae* expressing the *acl* gene was performed under nitrogen limited culture condition, the production levels of fatty acids, mainly in terms of mono-unsaturated ones showed higher than that under non-nitrogen limited condition: about 20%-40% increase in both of C16:1 fatty acid and C18:1 fatty acid was observed. With the carbon concentration increased, the accumulation in mono-unsaturated fatty acids become more apparent, whereas in the wild type strain, no such correlation was found. Besides, the citrate level in both the *S. cerevisiae* original strain and engineered strain under nitrogen limited condition was found to be much higher than that under non-nitrogen limited condition, which indicated that the diminution of nitrogen could lead to citrate accumulation in the *S. cerevisiae*. However, compared with the wild type strain, the citrate level was much lower in the engineered strain, which indicated that parts of the citrate were consumed in the engineered strain since the heterologous ACL was expressed.

To accumulate the citrate directly in the *S. cerevisiae*, the *idh1* and *idh2* genes encoding IDH involved in the tricarboxylic acid cycle (TCA cycle) were knocked out and the gene disrupted strains $\Delta idh1$, $\Delta idh2$ and $\Delta idh1/2$ were

obtained. From the GC-MS results, the citrate production levels in the gene disrupted strains were found to be significantly increased, about 4- to 5-times as great as that in the wild type strain. In order to convert the accumulated citrate to cytosol acetyl-CoA, the heterologous ACL was successfully over-expressed in all the gene disrupted strains and their fatty acid production levels were investigated. It was found that the *idh1,2* disrupted strains expressing the *acl* mainly accumulated unsaturated fatty acids. Specifically in the $\Delta idh1$ strain expressing *acl*, 80% increase in C16:1 and 60% increase in C18:1 was observed. In the $\Delta idh2$ strain expressing *acl*, 60% increase in C16:1 and 45% increase in C18:1 was observed. In the $\Delta idh1/2$ strain expressing *acl*, there was 92% increase in C16:1 and 77% increase in C18:1 respectively. The increased fatty acids from this study were the potential substrates for the production of biofuels.

In order to have a better understanding of the proteomics changes in the engineered strains, the iTRAQ-coupled 2D LC-MS/MS based proteomics approach was applied to provide qualitative and quantitative analysis of the global proteins in the *S. cerevisiae* wild type strain, the engineered strain with *idh1,2* double disrupted genes and the engineered strain with *idh1,2* double disrupted genes and an over-expressed *acl*.

More than 300 proteins were identified. Among these proteins, 37 were

found differentially expressed in the engineered strains and they were classified into specific categories based on their enzyme functions. Most of the proteins involved in the glycolytic and pyruvate branch-point pathways were found to be up-regulated and the proteins involved in the respiration and glyoxylate pathway were however found to be down-regulated in the engineered strains. Moreover, the metabolic modification of the *S. cerevisiae* cells with enhanced fatty acids synthesis resulted in differentially expressed proteins involved in amino acid metabolism, protein biosynthesis pathways and a number of up-regulated proteins involved in stress response, including the heat shock protein, peroxiredoxin, glutaredoxin and thioredoxin.

To convert the fatty acids to the fatty acid derivatives, a more direct strategy was tried to accumulate cellular fatty acyl-CoAs in *Saccharomyces cerevisiae*, which were the activated forms of fatty acids and used as important precursors for various converting enzymes. The *dgal* gene was deleted to block the fatty acyl-CoAs dependent pathway of TAGs synthesis and a significant decrease in lipid content was observed. A fatty acyl-CoA reductase (FAR) was cloned and overexpressed in the wild type strain and the gene disrupted strain, to convert the fatty acyl-CoAs to the corresponding fatty acid derivatives. The metabolic engineered pathway resulted in the enhanced production of fatty alcohols. Compared with the wild type strain with overexpressed FAR gene, the yield of fatty alcohols in the $\Delta dgal$ strain with FAR dramatically increased: the

intracellular fatty alcohols increased to 1.7 times, from 26 mg/L to 45 mg/L, while the extracellular fatty alcohols increased to 1.9 times, from 2.2 mg/L to 4.3 mg/L. The results in this study gave a successful example of utilization of fatty acids for producing valuable chemicals and fuels. It also demonstrated the feasibility of the designed strategy for solving the bottleneck of utilization of TAGs in fatty acid derivatives production.

1. Introduction

1.1 Microbial production of biofuels

The rapid urbanization and industrialization result in an increasing need for fossil fuels. The supply of fossil fuels is unpredictable and the fossil fuel's price fluctuates erratically. Traditional fossil fuel also has a variety of problems. The efficiency associated with fossil fuel is low and there are serious environmental concerns on it [1]. Hence there is a need to look for an alternative renewable fuel source within high energy efficiency and cost-effective operation process. The biofuels is playing a more and more important role in modern society. It can be divided into the first-generation biofuels and second-generation biofuels. The first generation of biofuels is generated from the food crops, which is competitive to the land and water used for food and some of them produce only limited greenhouse gas emissions saving. While the second-generation of biofuels derives from the non-food biomass such as lignocellulosic biomass and they can help solve problems associated with the first-generation biofuels and supply fuels with environmental benefits and cost affordability. However, the extraction process for second-generation biofuels becomes harder and the technology needed to turn the cellulosic material into biofuel is still in the early stages of development.

A lot of research works focus on the biofuels production by microorganisms

since they are rich sources of natural products. The application of microbial produced biofuels can rectify the problems of large scale land needs and the potential environmental pollution [2, 3]. The cost of biofuels production from microorganisms could be significantly less expensive than the production of traditional fossil fuels. Besides, the biofuels could be produced from a wide range of materials, like waste water, food and other byproducts [4, 5]. Thus the source material problem could be solved. Moreover, the biofuels are more easily renewable, while it takes a long time to re-produce the fossil fuels. All of the advantages of biofuels production from microorganisms make it an ideal alternative to the fossil fuels in the near future.

With the development of molecular system, synthetic biology and genetics and metabolic technologies, the biosynthetic pathways of microorganisms such as bacteria, yeast and micro-algae could be easily modified [6, 7]. The microbial production of advanced fuels such as alcohols, isoprenoid-derived fuels and fatty acid-derived fuels are on the road [7, 8]. Besides, the industrial fermentation and bioreactor technologies become more sophisticated and they help realize the scale up production of advanced biofuels. Last but not least, with the economically feasible of breakdown of cheap materials such as lignocellulosic, the microorganisms could generate biofuels from lignocellulosic biomass instead of from starchy agricultural products [9, 10].

Based on the wealth of genetic information available and the versatile tools designed to manipulate the metabolic pathways, the microbes *Escherichia coli* and *Saccharomyces cerevisiae* are always selected as ideal study models in improving the understanding of the metabolic mechanisms and to serve as host strains for the enhanced production of fuels and chemicals. Both of the *E. coli* and *S. cerevisiae* have advantages of ease cultivation, short generation time and high accessibility of molecular. Besides, they are regarded as potential candidates for biofuels production owing to their good ability in anti-phage, organic solvent tolerance and high-concentration fermentation [11, 12].

One successful application example in microbial production of biofuels was found in the engineered *E. coli* with a constructed acetone-butanol-ethanol (ABE) enzyme complex [13] (Fig.1.1). This metabolic pathway was responsible for the production of isopropanol. By overexpression of the acetyl-CoA acyltransferase (*thl*), acetoacetate decarboxylase (*adc*) genes from *Clostridia acetobutylicum*, acetoacetyl-CoA transferase (*atoAD*) gene from *E. coli* itself, together with the alcohol dehydrogenase (*adh*) gene from *Clostridia beijerinckii*, the engineered strain produced isopropanol with a level of 4.9 g/l. Moreover, by the strategy of separately expression of the genes under dedicated promoters, the isopropanol production level reached 13.6 g/l, which surpassed the highest reported isopropanol production level from original bio-synthesis pathways in *Clostridium* [14].

Fig. 1.1 Adaptation of the Clostridia acetone-butanol-ethanol (ABE) fermentation pathway for the production of isopropanol and butanol in *E. coli*. (Permission from ref.13 was obtained from publisher to use this figure).

The *S. cerevisiae* has also been successfully metabolic modified for the production of advanced fuels. One application of engineered *S. cerevisiae* in the field of biofuels production was found in isobutanol biosynthesis [15] (Fig.1.2). By expression of the genes *Ilv2*, *Ilv3* and *Ilv5* that are involved in the mitochondrial valine pathway, the isobutanol production increased from 0.16 mg/g to 0.97 mg/g using glucose as carbon source. It was a six-fold higher than that in the wild type strain. Later by co-expression of the *Bat2* gene, the production level was further improved by a two-fold.

Fig. 1.2 The metabolic pathways from pyruvate to isobutanol in *S. cerevisiae*. (Permission from ref.15 was obtained from publisher to use this figure).

The above examples indicated that the application of the genetic engineering and metabolic engineering tools enabled the modification of microbes for specific biofuels production. Through regulation or construction of the metabolic pathways in the microorganisms, the production of targeted components could be improved in both quality and quantity.

1.2 Fatty acid-derived fuels produced from microorganisms

In the last few decades, increasing research work has done on the microbial production of biofuels such as bio-ethanol and butanol [16, 17] and their industrial production led to significant success. However, the application of bio-ethanol or butanol has considerable limitations: their energy density is low

and the vapor pressure is relative high given the existing infrastructure. Besides, the corrosiveness prevents their widespread utilization [18]. Thus, the research focuses are gradually moving towards other advanced fuels such as fatty acid-derived biofuels, which are thought to be more valuable and suitable for the industrial use. The fatty acids are source materials for the biofuels production and they can be converted to the corresponding biodiesels, bioalcohols and alkanes/alkenes with different length of carbon chains [19]. By combining the natural fatty acids metabolic pathways with the synthetic pathways in microbes such as *E. coli* and *S. cerevisiae*, the diversion of fatty acids metabolism directly towards the interested chemicals and fuels are expected to be realized.

The biodiesels fatty acid methyl/ethyl esters could be made from the trans-esterification of triacylglycerols (TAGs) with methanol or ethanol. They exhibit attractive petroleum-like properties and positive ecological effects. As a result, they are always selected as the primary renewable alternatives to diesels [20]. The *E. coli* has been reported to be metabolic engineered for the production of biodiesels directly from glucose or ethanol [8]. The expression of genes that encode the wax-ester synthase (*arfA*), thioesterase (*tesA*), together with the *fadD* in *E. coli* resulted in the enhanced production fatty acid ethyl esters (FAEEs) and a production level of about 400 mg/l was observed after cultured in 2% of ethanol for 48 h. The compositions of FAEE ranged from C12

to C18. By further disrupting the *fadE* gene, the engineered strain markedly increased the FAEE production level. Compared with the traditional biodiesels production from plants, the drawbacks of geographical and seasonal restrictions, and the costly trans-esterification procedure could be overcome [21].

In addition to biodiesels, the fatty alcohols are also globally in great demand. The fatty alcohols could be produced by converting enzymes existed in the microorganisms. When an acyl-CoA reductase (*acrI*) was expressed in *E. coli* and took place of the ester synthase (*atfA*), the strain produced the medium chain fatty alcohols to a level of about 60 mg/l [8]. The enhancement of fatty alcohols production by engineered *E. coli* or *S. cerevisiae* has the potential to meet the global oil needs.

The bio-alkanes/alkenes could be produced from decarbonylation of fatty aldehydes with the “n-1” carbon chain length rules in microbes (Fig.1.3). Both of the *E. coli* and *S. cerevisiae* have been reported to be successfully metabolic modified to produce alkanes/alkenes from fatty acid activated forms: fatty acyl-acyl carrier protein (ACP) or fatty acyl-coenzyme A (CoA) [22, 23]. The cloning and expression of genes encoding fatty acyl-CoA or fatty acyl-ACP reductase in *E. coli* or *S. cerevisiae* successfully converted the fatty acyl-CoAs or fatty acyl-ACPs to their corresponding aldehydes. The generated fatty aldehydes could be further reduced to their corresponding alkanes or alkenes by

expression of the genes encoded aldehyde decarbonylase. Through construction of this alkanes/alkenes generation pathway in *E. coli*, the production of pentadecane and heptadecene has been reported to reach 30 mg/l and 40 mg/l respectively [22].

Fig. 1.3 The alkane/alkene synthesis pathway indicated in *E. coli* and *S.cerevisiae*. (Permission from ref.22 was obtained from publisher to use this figure). The acyl-CoA (or ACP) reductase is responsible for the conversion of fatty acyl-CoA (or ACP) to the corresponding fatty aldehyde. The aldehyde decarbonylase is responsible for the conversion of formed aldehyde to alkane or alkene.

In addition, the properties of chain length or saturation significantly affect the properties of fatty acid derivatives. For example, the biodiesels with higher ratio of unsaturated fatty acid methyl esters exhibit advantages of lower viscosity, pour points and cloud points, which could improve the biodiesel qualities with excellent cold flow property [24]. By modification of the pathways in microbes, the unsaturated could be expected to be increased and it

will lead to the production of fatty acids derivatives with higher quality.

1.3 Fatty acid synthesis in yeast

Fatty acids are not only essential compounds in microorganism cells, but also played an important role as biofuels precursors. The *de novo* fatty acids accumulation is an anabolic biochemical process and most of them are stored as cellular lipid components such as TAGs. The production levels and compositions of fatty acids in microbes are different from each other due to the specific accumulation mechanisms. The better understanding of fatty acid production and accumulation mechanism could help to further modify the metabolic pathways in microorganisms and improve the fatty acids production in quality and quantity.

1.3.1 Fatty acids in yeast

The mainly accumulated fatty acids in yeasts are long-chain fatty acids (C14, C16 and C18), including saturated and unsaturated ones: Myristic acid (C14:0), Palmitic acid (C16:0), Palmitoleic acid (C16:1, *n*-7), Stearic acid (C18:0), Oleic acid (C18:1 *n*-9), Linoleic acid (C18:2, *n*-6) and α -Linolenic acid (C18:3). In some species, the very long-chain C26 fatty acids, which plays as an essential role in composing sphingolipids and GPI-anchors are found [25, 26, 27]. Based on the different fatty acids production levels, the yeasts are generally classified into two catalogues, the oleaginous yeasts and non-oleaginous yeasts

[28]. In the oleaginous yeasts such as *Rhodotorula*, *Rhizopus*, *Candida*, *Cryptococcus*, *Lipomyces* and *Yarrowia*, the cellular lipids accumulation could reach to a level above 20% of their biomass on average. In some conditions, the maximum lipids could exceed 70% of their biomass. 80% to 90% of the lipids are stored as TAGs, whereas a small fraction of them are found in the form of steryl esters, which are called neutral lipids [29, 30]. However, in the non-oleaginous yeasts, such as *S. cerevisiae*, the lipid production level could only reach to less than 5% of their biomass.

Besides, the cellular lipid contents and spectrum differ among species (Table 1.1) [31]. When performed on glucose containing medium, a wide range of fatty acids in different species can be found. For instance, the *Cryptococcus curvatus* produces a high yield of palmitic acid (C16:0), and the *Cryptococcus albidus* prefers the production of oleic acid (C18:1). In different *Rhodotorula* species, the total lipid production levels vary significantly, but the fatty acid compositions remain similar.

Table 1.1 Lipid accumulation and fatty acid profile of various oleaginous yeasts (data adapted from ref.31 which was obtained from publisher to use this table)

Species	Lipid contents (%D.W.)	Major fatty acid residues (% w/w)					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
<i>Cryptococcus curvatus</i>	58	25	Trace	10	57	7	0
<i>Cryptococcus albidus</i>	65	12	1	3	73	12	0

<i>Candida sp 107</i>	42	44	5	8	31	9	1
<i>Lipomyces starkeyi</i>	63	34	6	5	51	3	0
<i>Rhodotorula glutinis</i>	72	37	1	3	47	8	0
<i>Rhodotorula graminis</i>	36	30	2	12	36	15	4
<i>Rhizopus arrhizus</i>	57	18	0	6	22	10	12
<i>Trichosporon pullulans</i>	65	15	0	2	57	24	1
<i>Yarrowia lipolytica</i>	36	11	6	1	28	51	1

1.3.2 *De novo* biosynthesis of fatty acids in yeast cells

Except for the fatty acids generation from the external feed and lipid turnover in yeast cells [32, 25], the *de novo* biosynthesis plays an important role in the supply of essential fatty acids. It starts from the precursors of acetyl-CoA and malonyl-CoA, and subsequently elongates to different intermediate levels (Fig.1.4).

Fig. 1.4. Schematic diagram of fatty acid synthesis and elongation.

A series of enzymes including acetyl-CoA carboxylase, fatty acid synthetase (FAS), fatty acid desaturase and fatty acyl elongase are responsible for the *de novo* fatty acid synthesis in yeast [33]. It started from acetyl-CoA, which is carboxylated to malonyl-CoA by the enzyme of acetyl-CoA carboxylase (ACC). The acetyl-CoA, together with the malonyl-CoA, is then destined to feed into the fatty acid synthesis pathway. The end fatty acid derivative products vary between different yeast species. The further elongation and desaturation are carried out in endoplasmic reticulum (ER) by the specific elongase and desaturase of yeast species. After cyclic series of elongation reactions, different carbon length contained fatty acyl-CoAs are formed, mainly varies among C14, C16 and C18 saturated ones. The various unsaturated ones are formed by the respective desaturases, which introduces the double bond into the saturated fatty acyl-CoA derivatives. The most common unsaturated fatty acyl-CoAs in yeasts are C16:1, C18:1, C18:2 and C18:3. They are regulated by different functional desaturases [34]: The Δ -9 fatty acid desaturase works on the formation of monounsaturated ones C16:1 and C18:1, whereas the Δ 12 and Δ 15 desaturases are responsible for introducing the additional double bonds into the location of Z12 and Z15 of the mono-unsaturated acyl chains, to form C18:2 and C18:3 fatty acyl-CoAs.

The fatty acyl-CoA precursors are then destined to the formation of lipids, such as TAGs and steryl esters [35] (Fig.1.5). The lipid storage pathway starts

from the generation of glycerol-3-phosphate (G-3-P), which is synthesized by glycerol-3-phosphate dehydrogenase (GPD1) from dihydroxyacetone phosphate (DHAP) or by acylation of glycerol via glycerol kinase (GUT1). The lysophosphatidic acid (LPA) is subsequently formed from the condensation of G-3-P and fatty acyl-CoA via the enzyme of G-3-P acyltransferase (GPAT). The acylation of LPA results in the formation of phosphatidic acid (PA) via the enzyme LPA acyltransferase (LPAT). Finally, after dephosphorylation of PA, the diacylglycerol (DAG) formed and it is catalyzed by DGA acyltransferase (DGAT) to generate triacylglycerol (TAG) [36]. The lipids are stored in the yeast cells as lipid particles or lipid bodies [37], which contribute more than 70% of the total lipid content. The stored lipids later serve as energy reserves if necessary.

Fig. 1.5 The metabolic pathway of TAG synthesis in yeast cells.

1.3.3 Substrates and cofactors for fatty acid synthesis

The fatty acid *de novo* synthesis requires substrates and cofactors. The acetyl-CoA is the most important precursor for fatty acid synthesis. The fatty acids synthesis is initiated by loading the acetyl-CoA onto the FAS complex and during the elongation process, the acetyl-CoA and malonyl-CoA need to be constantly supplied. In yeast, there are two different metabolic pathways for the generation of cytosol acetyl-CoA (Fig.1.6). One of them is the pyruvate-acetaldehyde-acetate pathway [38, 39]: In the yeast cells, the acetyl-CoA could be produced from the glycolysis pathway. The sugars first undergo fermentation process in yeasts cells to generate pyruvate. By the enzyme of pyruvate decarboxylase, the pyruvate is converted to acetaldehyde, and later by the enzymes of aldehyde dehydrogenase and acetyl-CoA synthetase, the acetaldehyde is converted to acetate, which is finally catalyzed to generate cytosol acetyl-CoA.

In the oleaginous yeasts, an additional cytosol acetyl-CoA generation pathway exists: The citrate generated from the citric acid cycle (TCA cycle) could be transported out of mitochondria to cytosol and then cleaved by the enzyme of ATP-citrate lyase (ACL), which exists widely in the oleaginous yeasts, but is absent in the non-oleaginous yeasts [40, 41]. The existence of ACL contributes to the oleaginous character of the oleaginous yeasts [42, 43].

Fig. 1.6 The metabolic pathways for acetyl-CoA generation in yeasts. (Permission from ref.39 was obtained from publisher to use this figure). The numbers represents the enzymes involved in the pathways. 1: acetyl-CoA synthetase; 2: ATP-citrate lyase; 3: aldehyde dehydrogenase; 4: pyruvate decarboxylase; 5: alcohol dehydrogenase; 6: pyruvate carboxylase; 7: pyruvate dehydrogenase complex; 8: citrate synthase; 9: cytoplasmic carnitine acetyltransferase; 10: acetyl-carnitine mitochondrial carrier; 11: Mitochondrial/peroxisomal carnitine acetyltransferase.

The NADPH is the most important cofactor for the fatty acids synthesis and in each step of the chain elongation, two molecules of NADPH are required by the FAS complex. In the oleaginous yeasts, the mainly parts of intracellular NADPH are generated by malic enzyme (ME) [44]. The malic enzyme is responsible for the conversion of malate into pyruvate and meanwhile regenerating NADPH from NADP^+ , to provide the vital cofactor for fatty acid synthesis. However, in the non-oleaginous yeasts such as *S. cerevisiae*, the NADPH is mainly generated by the pentose phosphate pathway in cytosol, which includes the enzyme activity of glucose-6-dehydrogenase,

6-phosphogluconate dehydrogenase, and NADPH-dependent isocitrate dehydrogenase.

Besides, the fatty acid bio-synthesis is a process depending on the condition of high energy load. The ATP is the main energy form and during the fatty acid synthesis, the ATP/AMP ratio has been found to be increased in the yeast cells.

On the one hand, the acetyl-CoA, NADPH and ATP are the necessary substrates and cofactors for the *de novo* fatty acid synthesis. On the other hand, the synthesis of fatty acids is also considered as an efficient strategy to keep balance of the intracellular acetyl-CoA and NADPH levels in yeast cells.

1.4 Regulation of fatty acid metabolism in yeasts

To regulate the fatty acid metabolic pathways in yeasts could either increase the fatty acid production levels or change its compositions.

One efficient way to increase the fatty acids production levels is to enhance the activity of participated enzymes. The ACC enzyme is responsible for generating malony-CoA by condensation of the acetyl-CoA unit with the bicarbonate anion. It is regarded as the crucial enzyme for *de novo* fatty acids synthesis. The over-expression of ACC in yeast resulted in significant increase of the fatty acids accumulation [45, 46].

In the oleaginous yeasts, except for the ACC activity, the existence of ME-FAS-ACL enzyme complex is to facilitate the fatty acids accumulation. The ACL is required for the cytosol acetyl-CoA production and only exists in oleaginous yeast [39]. In green plants, fungi, *Glaucophytes*, *Chlamydomonas* and prokaryotes species, the ACL enzyme is composed of two subunits: ACLA and ACLB, and requires an ammonium ion for its activation. In animal and human cells, this enzyme is homomeric, which presumably an evolutionary fusion of ACLA and ACLB genes. The sequences of ACL contain the conserved domains for ATP and CoA binding. There are researches focusing on the relationship between ACL activity and fatty acid accumulation. The results showed that a growth loss was observed in yeast cells with disrupted ACL gene under the culture condition without the external acetyl-CoA sources. Whereas, targeting a heterologous ATP citrate lyase into plastids of tobacco significantly led to fatty acid synthesis enhancement [47, 48]. These pieces of evidences strongly suggested that by regulating the ACL activity in specific microbes, the fatty acids production levels could be changed.

It is believed that ME activity could control the NADPH level and further affect the fatty acid production. There are research works focusing on the relationship of ME activity and lipid accumulation in oleaginous yeasts. Once the ME activity was inhibited or the enzyme was disrupted, a loss of lipid accumulation could be observed [49]. On the other hand, the enhancement of

NADPH supply was observed when the ME was over-expressed in microbes and this further led to the increase of lipid production [50, 51].

A second regulation strategy is to modulate the fatty acid degradation pathways. The β -oxidation that occurs in the peroxisome is mainly responsible for the fatty acids degradation and arrays of enzyme activities provide the β -oxidation machinery with specific precursors [52]. In the *S. cerevisiae*, the activated long-chain fatty acids are transported into peroxisome by the transporters Pxa1p and Pxa2p. Disruption of either the Pxa1p or Pxa2p resulted in the growth deficient in medium containing the long-chain fatty acids as the sole carbon source [53, 54]. The acyl-CoA oxidase (POX) in peroxisome is responsible for oxidation of the fatty acyl-CoAs to trans-2-enoyl-CoA, which is the core step involved in the β -oxidation degradation process. It has been reported that the POX genes mutants could alter the lipid profiles in *Yarrow. Lipolytica* [55]: In the POX2 gene disrupted strain, the utilization of long-chain fatty acids was blocked, whereas, in the POX3 gene disrupted strain, the degradation of short-chain fatty acids decreased. In the POX2, POX3 and POX5 gene disrupted strain, a “slim” phenotype was observed because of the feedback regulation of oxidation. On the other hand, over-expression of the POX2 gene resulted in an obese phenotype [55]. Although such kind of manipulation is not applicable in non-oleaginous yeast such as *S. cerevisiae*, it provides a good idea for the modification of yeast cells with enhanced biofuel

precursor production.

The fatty acid profile could be modified if the elongase and desaturase are regulated. The production of poly-unsaturated fatty acids in yeast attracts a lot of interest since these composites are on higher demand for biofuel production. In the *S. cerevisiae*, since the Δ^9 fatty acid desaturase (OLE1) exists as the sole desaturase, the main production types of fatty acids are saturated ones (C16:0, C18:0) and monounsaturated ones (C16:1, C18:1) [56]. Whereas, in some yeast species, other desaturases such as Δ^{12} and ω^3 fatty acid desaturases exist and they can convert the Δ^9 -monounsaturated fatty acids (C18:1) into linoleic acid (18:2) and α -linolenic acid (C18:3). Based on these protein information, the Δ^{12} and ω^3 fatty acid desaturases from *Kluyveromyces lactis* were cloned and over-expressed in the *S. cerevisiae* and this strategy made the *S. cerevisiae* successfully produce linoleic and α -linolenic acid [57].

Concerning the lipid synthesis pathway, the enzymes involved could be modified to change the lipid production levels. The glycerol-3-phosphate dehydrogenase (GPD1) of oleaginous yeast is responsible for the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate. Compared with the wild type strain, the deletion of GPD1 in *Y. lipolytica* resulted in 3-fold decrease in lipid accumulation [58]. On the contrary, the over-expression of the GPD1

successfully achieved a 4-fold increase in lipid accumulation [44]. The acyl-CoA: diacylglycerol acyltransferase (DGAT) is responsible for the terminal step of triacylglycerol formation. It has been reported that the ratio of TAG to FFA regulated the total lipid accumulation in yeast cells [31]. The over-expression of DGAT in the *S. cerevisiae* lead to an increase of total lipid accumulation [59], while overexpression of the DGAT gene in the *Y. lipolytica* under the POX2 constitutive promoter lead to a 78% increase of the TAG/FFA ratio when this engineered strain was cultured in oleic acid containing medium. Under this ratio, the overall lipid was 63% higher than that in wild-type [58].

All of the regulation strategies are powerful tools for the optimization of fatty acid production. Based on the well-known genetic information and metabolic engineering tools, the modification of yeast cells to produce competitive biofuels from fatty acids becomes possible.

1.5 Effect of culture conditions on fatty acid production levels

The culture conditions including temperature, pH, carbon source, nitrogen source, the ratio of carbon and nitrogen concentration, amino acid concentration and organic molecules all have great effects on the lipid yields and compositions in yeast cells [35, 60, 61].

The effects of nutrient feed conditions on fatty acids production levels in yeast strains are widely investigated. The existence of excess carbon source but

limited essential nutrient elements results in the limitation of cell proliferation, and this causes the shift of carbon usage directly to the lipid synthesis. This phenomenon is most obvious in oleaginous yeasts. When an excess carbon source exists, the depletion of any essential nutrient components, such as nitrogen, metal ion, or phosphorus can be effectively applied for setting on fatty acids accumulation and finally lead to the buildup of neutral lipid [62]. Among all of the nutrients components, the application of nitrogen limitation is the most common way to control the fatty acid production levels in the oleaginous yeasts [63, 64]. The mechanism of nitrogen nutrient regulation of lipid synthesis relies on the accumulated citrate from the TCA cycle [41]: Under nitrogen limitation condition, the activity of adenosine monophosphate (AMP) deaminase increases in order to break down the AMP to the supply of ammonium. This reaction definitely decreases the intracellular AMP concentration, which is the co-substrate for mitochondrial NADP^+ -isocitrate dehydrogenase (IDH). The IDH involved in the TCA cycle is responsible for the conversion of isocitrate to alpha-ketoglutarate. The inhibition of IDH activity results in the accumulation of citrate. The citrate can be transported to cytosol via the citrate/malate shuttle [65] and under the enzyme of ACL, the citrate can further be cleaved to acetyl-CoA and finally support for the fatty acid synthesis. This is the main mechanism for the fatty acid accumulation in the oleaginous yeasts under nitrogen limitation condition. However, in the non-oleaginous yeast, such as *S. cerevisiae*, no such correlation is observed.

When the non-oleaginous yeasts are performed under the same nitrogen limitation condition, the excess carbon is found to be directly used for trehalose or glycogen synthesis [66].

The culture parameters such as temperature, pH and oxygen also have significant effects on yeast lipid production. The adjustment of culture temperature could effectively change the fatty acid compositions. It has been reported that during the cultivation of *Candida utilis* at 30 °C, the proportion of α -linolenic acid (C18:3) decreased, whereas the proportion of oleic acid (C18:1) increased. When the cultivation of *Candida utilis* was carried out at an even lower temperature, the proportion of α -linolenic acid (C18:3) maintained a much higher level than that at 30 °C [67]. Similarly, a low fermentation temperature could increase the unsaturated fatty acid compositions in the *S. cerevisiae* [68]. Besides, when the *S. cerevisiae* was cultivated at low temperature, the medium-chain fatty acids and triacylglyceride content increased, whereas, the phosphatidic acid content and the ratio of phosphatidylcholine to phosphatidylethanolamine decreased [68]. These kinds of phenomena were regarded as an adjustment mechanism to maintain the membrane fluidity [69].

The organic molecular could generate solvent stress for yeasts. When the yeast strains are performed under culture conditions containing organic solvent

such as alcohols, the cell membrane could be fluidized, which results in an uncontrolled decrease of proton flux across the membrane and the leakage of essential cofactors [70]. The ethanol and butanol are well known inhibitors to yeast growth and the existence of them in the culture medium could change the product compositions in the cells [71]. It has been found in the *S. cerevisiae* that when growing in medium with ethanol, the amount of mono-unsaturated fatty acids included in the intracellular lipid content significantly increased [72, 73]. The oleic acid (C18:1) has been demonstrated as the most efficacious unsaturated fatty acid in overcoming the toxic effects caused by ethanol [71]. The over-expression of the *Arabidopsis thaliana* Δ 12-fatty acid desaturase (FAD2) gene, together with the OLE1 gene in the *S. cerevisiae* resulted in the synthesis of dienoic fatty acids and increase of unsaturated fatty acid, which successfully engineered the *S. cerevisiae* strain with the highest viability in the presence of 15% (v/v) ethanol [74, 75]. These findings demonstrated that the existence of ethanol, butanol and other alcohols could increase the membrane fluidity and further cause the cofactor leakage and membrane potential loss. The reinforcement of the cell membrane and the adjustment of cellular lipid compositions in yeast cells could be an efficient response mechanism to such organic toxicity [70, 76].

1.6 Metabolomics

1.6.1 Principle of metabolomics analysis

The metabolome is defined as “ the complete set of small-molecule metabolites, such as intermediates, hormones, signaling molecules and secondary metabolites found in a biological sample” [77]. The small molecules are those with less than 1000Da molecular weight and involved in metabolic pathways of microorganisms. The metabolomics aims to the identification and quantification of metabolites within a biological system. The comprehensive metabolites profiling and quantitative and qualitative measuring of cellular metabolites in the organisms by multiple technologies are becoming more and more widely investigated [78, 79].

The metabolic profiling and quantitative information play an important role in identification of biomarkers and analysis of metabolic shifts in microorganisms. The metabolome identification and quantitative analysis is always achieved by two different approaches: the NMR-based and MS-based technologies [80, 81]. Compared with the NMR-based technology, the MS-based method has advantage of higher sensitive, which enables the detection of low-abundance metabolites, and thus provides an efficient way to analyze the metabolites in a much lower concentration range with steady results.

Currently, both of the LC-MS and GC-MS technologies are well applied on the metabolomics investigation [78, 82]. Compared with the GC-MS, the

LC-MS can be applied for the analysis of non-volatile metabolites without sample derivatization. The LC-MS system contains the liquid chromatography (LC) separation part and the mass spectrometer (MS) detection part. The MS part contains three basic modules: the ion source, mass analyzer and the detector. The samples are dissolved into a mobile phase composed of a liquid fluid and then go through a column packed with a stationary phase in a high pressure force. The stationary phase is always composed of small solid particles, a porous monolithic layer or a porous membrane. Based on the specific interaction strength with the stationary phase, different components in the sample solution can be eluted at different times, which are taken as retention times of the components. The compounds eluted from the LC are then injected into the MS and converted to neural fragments by the ion sources. By applying the electrical or magnetic field, the mass analyzer separates the ions according to the different m/z values. Finally, the detector module converts the abundances of ions into electrical signals and records the current or charge produced when an ion hits or passes through the detector, which enables the detection of hundreds to thousands of metabolites.

The GC-MS, in which the mobile phase is an inert gas such as helium, can be applied for the analysis of more volatile components. The working mechanism of GC-MS is quite similar to that of LC-MS. The metabolites extracted from species with different methods need to be derivatized [83]. The most widely

used derivatization method for GC-MS analysis is silylation. The N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% of trimethylchlorosilane (TMCS) is usually used for the silylation. During this operation, a silyl group ($-\text{Si}(\text{CH}_3)_3$) is added to a sample component and replaces an active hydrogen, which makes the sample component less polar and more volatile. The active hydrogen exists in a number of groups, such as hydroxyls, carboxylic acids, amides, thiols and imines. Thus most of metabolites with these functional groups can be derivatized and detected by the GC-MS. For enol forms, such as ketones and aldehydes, the operation of methoxymation is needed before silylation. The purpose of this step is to convert the functional groups to alkyloximes or oximes and the O-methoxylamine hydrochloride is widely used for the methoxymation. Based on this kind of derivative operation, large numbers of metabolites can be detected and analyzed efficiently.

The metabolite identification from hundreds of detected results is a big challenge in current researches. In contrast to the well-established databases for peptide and protein identification, the platforms for the metabolome identification in LC-MS system are not complete. The main search method is depended on the m/z value of an interest molecular. However, the discriminate isomers of one compound sometimes cannot be discriminated by the mass-based identification method. Besides, the extremely similar molecular

weights of some compounds lead to the non-sufficient identification in unambiguous metabolites [84]. Due to such loophole, the authentic compounds need to be applied. By comparison with the authentic compound, the identification of one interest metabolite can be further confirmed. Fortunately, the development of GC-MS system with relative complete databases enables the identification of 70-80% of metabolites from cell extracts and it is regarded as more liable in the study of metabolomics.

1.6.2 Application of metabolomics in biofuel production

The metabolomics is a useful tool enables a global analysis of complex samples in biological systems. It is widely used in the discovery of bio-markers and the study of metabolic pathways associated with drug treatment and diseases [85, 86]. In recent years, with the development of biofuel industry, the metabolomics becomes more and more important for identifying the metabolic changes occurring during the biofuel precursor production and biofuel production, which provides information in better understanding the metabolic shifts, exploring the relationships between different pathways and finding out the intermediates and cofactors needed for potential biofuel production [87, 88].

First of all, the regulation of metabolic pathways in microorganism could result in cellular intermediates changes. The metabolomics studies help analyze the metabolites production levels in the wild type strain and the modified strains,

and then reveal the distribution of the modified pathways to the specific compound production. Besides, by comparison with the branch-points by-products between the wild type and modified strain, the co-regulation of different metabolic pathways is expected to be explored.

Secondly, the culture conditions have great effects on biofuel precursor production. The application of metabolomics in study of the changes in cellular intermediates helps discover the relationship between the culture conditions and biofuel production. The nitrogen limited cultivation resulted in the increased fatty acids accumulation in oleaginous yeasts. The metabolite analysis was carried out under nitrogen limited condition between oleaginous and non-oleaginous yeasts and provided information to explain the fatty acids accumulation mechanism in oleaginous yeasts. The lactate, acetate, pyruvate, succinate, malate and citrate were found in the metabolomics analysis results and their production levels showed to be different in *S. cerevisiae* with original IDH activity and with that from oleaginous yeast *R. toruloides* IDH activity. The metabolites changes reflected that the pyruvate no longer flowed into the TCA cycle, but shifted to the branch-points pathway such as lactate production. The results confirmed the fact that the nitrogen limitation weakened the TCA cycle and therefore was beneficial for the fatty acid accumulation [89].

Last but not least, the metabolomic characteristics during biofuel precursor production can be obtained from the metabolomics approaches. The

intracellular metabolic profiling was carried out on the strain of *Schizochytrium* throughout the docosahexaenoic acid fermentation process. The potential bio-markers responsible for distinguishing different fermentation stages were identified as glucose, glycine, alanine, proline and glutamic acid. In addition, the glucose, galactose, ananine and glutamic acid were found to have great contribution to the fatty acid composition changes during normal and limited oxygen conditions [90]. Such kind of metabolomics approach provides meaningful information to discover the bio-characteristics of specific metabolic pathways.

1.7 Proteomics

1.7.1 Principle of LC-MS based proteomic analysis

The proteome is defined as the entire set of proteins, which is produced from an organism or system [91]. It reflected the expression situation, structure and function of the proteins. The proteomics is a study method for the large-scale of proteins and particularly focuses on the structures and functions. The LC-MS based proteomic analysis, dealing with a global protein profiling, functions as a bridge between the genomic information and complex cellular structures and behaviors. It is being widely studied to quantify the protein expression levels and reveal their up- or down- regulation mechanisms coordinated to the cellular response [92].

The LC-MS is a powerful analytical tool used for a series application with high sensitivity and selectivity [93]. Similar to the above description (Chapter 1.6.1), it combines the liquid chromatography (LC, sometimes HPLC) part together with the mass spectrometry (MS) part. The LC part is used for the components separation and the MS part is used for the components identification. Normally, for the proteomics analysis, the total LC-MS system consists of chromatography columns and can separate different peptide mixtures based on their physicochemical properties. Each of separation according to a physicochemical property forms a dimension in LC. The peptides are eluted with a gradient of organic solvent before entering MS. The MS is equipped with several parts: The ionization sources such as electrospray ionization or matrix-assisted laser desorption/ionization are used to build charges on eluted peptides for identification. The mass analyzer is used to separate the ions on the basis of m/z ratios (mass-to-charge ratio) and the detector is used to detect the relative abundance of ions at discrete m/z [94, 95].

There always exist the accurate detection problems in the application of LC-MS analysis system. In most cases, a design of tandem MS can overcome the ambiguous results limitation [96]. For the first MS step, the precursor ion, which represents the intact peptide, is detected and in the following step, the precursor ion is isolated from the other peptide ions and then dissociated into fragments. The mass of the peptides from the second step is then determined

and forms a MS/MS spectrum. By software analysis and comparison with the databases, the quantitative results can be obtained.

The established methods used in the quantitative analysis of proteins should undergo isotope labeling. The chemical reagents that have variations on the molecular weights of isotopic compositions are always selected for the labeling. The major three types of labeling include the protein level labeling, peptide level labeling and metabolic labeling. The relative quantification is carried out by comparing the obtained signal intensity. Commonly, the isobaric tag for the relative and absolute quantization (iTRAQ) is always used as one of the chemical labeling [97]. It belongs to the method of labeling on the peptide level and has the advantages of improving the quantitative measurement accuracy. The iTRAQ labeling reagents consist of reactive group, balance group and reporter group. During the operation, up to four protein samples can be analyzed simultaneously. According to the provided process, the proteins are digested to peptides and each peptide is combined to the active group and labeled by the reagents. They then appear at the same mass and the labeled sample will dissociate and release the reporter group as a singly charged masses ion, indicating as 114.1, 115.1, 116.1 and 117.1 (Fig.1.7). Finally, each sample is determined by relative peak area. The on-line database (sometimes off-line database) search will give a peptides or proteins list, showing data of the *p*-value and fold changes.

Fig.1.7 Working mechanism of iTRAQ labeling reagents. (Permission from ref. 97 was obtained from publisher to use this figure).

1.7.2 Application of proteomics in biofuel production

The LC-MS based proteomic is widely applied for the analysis of protein-protein interactions, protein post-translational modification and MS-based protein sequencing. With its high resolution, sensitivity and separation efficiency, a large number of proteins involved in different organisms and different cell lines can be identified. The protein functions explain the process of different biofuel production pathways and the mechanism of different impacts on the biofuel yields, such as nutrition stress, substrate specificity, *etc.* On the other hand, by qualifying and comparing the different protein expression levels during biofuel production, a better understanding of major enzymes' function taking part in specific pathways can be achieved. It

will provide valuable guidance for genetic modification of enzymes and pathways, to either increase the biofuel production efficiency or improve the productive properties.

The LC-MS based proteomic analysis in different species enabled the exploration of functional proteins in biofuel production and biomarker proteins in certain strains. The yeast *Xanthophyllomyces dendrorhous* was used for protein profiles study by *Martinez-Moya et al* [98]. In this study, 600 proteins were observed and 131 non-redundant protein were identified, and in which, 50 differentially expressed proteins were classified and their expression patterns were distinguished. An overview of potentially important proteins related to lipid and carbohydrate biosynthesis was provided according to the results. For the fatty acid metabolism, the expression levels of acetyl-CoA carboxylase, phosphoglucomutase, ATP-citrate synthase, ATP-citrate lyase and fatty acid synthase were indicated during lag-phase, exponential phase and stationary phase, specifically. All of these were key proteins involving in fatty acid synthesis. These kinds of proteomic data provided valuable information of the property and enzymatic marker in oleaginous microorganisms compared to non-oleaginous microorganisms.

The living microorganisms rely on outside nutrients intake to maintain their growth and metabolism. There were lots of interesting observations on nutrient

conditions related to biofuel production in microorganisms. The LC-MS based proteomic analysis study provided credible mechanism explanation on such relationship. *Kolkman et al* [61] studied the proteome of the *S. cerevisiae* response to various nutrient limitations by the method of Nanoflow-LC-MS/MS, together with the SDS-PAGE gel separation and digestion in-gel with trypsin. In this study, 928 proteins were identified. 51 proteins were found to be up-regulated in response to glucose limitation and 51 proteins were found to be up-regulated in response to ammonia limitation. Under glucose limitation condition, the fatty acid beta-oxidation related proteins displayed an increased protein level, while under nitrogen limitation condition, the genes ADH2, ACS1, MLS1, subjected to glucose repression, and SUC2, CYB2 subjected to sucrose and lactate degradation consistently displayed a decreased protein expression level. This kind of study gives a hint of how the fatty acid production is regulated under different nutrition conditions.

Besides, a better understanding of proteomic information on specific biofuel production pathways can contribute to better design strategies for culture medium, co-factor addition, side-effects removal and more importantly, elimination of substrate inhibition [99, 100].

It is concluded that the LC-MS based proteomic analytical approach is performing as a pivotal role in the research field of biofuel production, to

understand the specific biofuel production pathways, the protein functions and the up- and down-regulation mechanisms at the molecular levels. It is always carried out together with the metabolomics study and provides a biological information platform for the study of microbial production of biofuels.

2. Materials and methods

2.1 Molecular cloning and expression of heterologous enzymes in *S. cerevisiae*

2.1.1 Strains, plasmids, genes and reagents

The strains *Escherichia coli* (*E. coli*) Top10 and *S. cerevisiae* BY4741 (Δ MAT, his3 Δ , leu2 Δ , met15 Δ , ura3 Δ) were obtained from American Type Culture Collection (ATCC, USA) and used in this study as the gene transformation host. The plasmid used for expression of the heterologous gene in the *S. cerevisiae* was pVTU260 and obtained from European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF, Germany). The pVTU260 plasmid contained the ADH constitutive promoter/terminator and ampicillin and URA selection marker. The ACL gene from *Mus musculus* (accession BC056378.1) was synthesized by GENEART (USA) and the codon optimization was carried out according to the host strain *S. cerevisiae*. The suitable restriction enzyme (RE) sites were designed on the fragment. The primers for the colony PCR was designed and synthesized by 1st BASE Pte Ltd (Singapore).

The Taq DNA polymerase with standard Taq buffer, restriction endonuclease, T4 DNA ligase, and other used enzymes were obtained from New England Biolabs Inc. (NEB, Singapore). The plasmid DNA purification kit, PCR

purification kit and gel extraction kit were obtained from QIAGEN (Singapore). The reagent for the competent cell preparation, transformation and protein extraction were obtained from Sigma-Aldrich (Singapore). The Western ECL substrates were obtained from Bio-rad (Singapore). The anti-ACL antibody was obtained from Abcam (Singapore) and used to detect the gene expression in the *S. cerevisiae*. The cell culture medium was composed of all analytical reagents.

LB medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl (2% agar added for plate and 100 µg/ml ampicillin added for selection marker);

YPD medium: 10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose (2% agar added for plate);

Minimal glucose (YNB-URA) medium: 6.7 g/l yeast nitrogen base (YNB) w/o amino acids and with ammonium sulfate, 0.77 g/l URA drop-out mix, 20 g/l dextrose (2% agar added for plate).

Minimal glucose (YNB-HIS) medium: 6.7 g/l yeast nitrogen base (YNB) w/o amino acids and with ammonium sulfate, 0.65 g/l HIS drop-out mix, 20 g/l dextrose (2% agar added for plate).

2.1.2 Competent cell preparation

Preparation of *E. coli* competent cells

One single colony of *E. coli* was picked and cultivated in 5 ml LB medium at 37 °C, 200 rpm for overnight. 1 ml of the overnight culture was inoculated into

250 ml flask with 50 ml LB medium and cultivated at 37 °C, 200 rpm. Once it reached to $OD_{600} = 0.3-0.5$, the culture was placed on ice for 15 min. The pre-cool culture was centrifuged at 4 °C, 5000 rpm for 10 min and the medium was discarded. The cell pellet was re-suspended in 30 ml cold 0.1 M $CaCl_2$ and kept on ice for 30 min. After then, the cell pellet contained solution was centrifuged at 4 °C, 5000 rpm for 10 min again. The supernatant was discarded and 1 ml cold 0.1 M $CaCl_2$ solution with 15 % glycerol was added and mixed. 50 μ l of cell suspension was pipetted into a sterile 1.5 ml micro-centrifuge tubes. The prepared competent cells were frozen and kept at -80 °C freezer.

Preparation of *S. cerevisiae* competent cells

One single colony of the *S. cerevisiae* was picked and cultivated in 5 ml YPD at 30 °C, 250 rpm overnight. 1 ml of the overnight culture was inoculated into 250 ml flask with 50 ml YPD broth and cultivated at 30 °C, 250 rpm. Until the OD_{600} reached about 2.0, the culture medium was collected and centrifuged at 5000 rpm for 10 min. The medium was discarded and the cell pellet was washed twice with 25 ml distilled water. After then, 1 ml sterile frozen competent cell (FCC) solution (5% v/v glycerol, 10% v/v DMSO) was added. 50 μ l of cell suspension was pipetted into a sterile 1.5 ml micro-centrifuge tubes. The prepared competent cells were frozen and kept at -80 °C freezer.

2.1.3 Enzyme digestion, gene insertion and transformation

For the cloning and expression of the heterologous ACL, the synthesized gene *acl* hanged with *NheI* and *BamHI* restriction enzyme sites were digested by the *NheI* and *BamHI* restriction enzymes from the original plasmid, which was provided by the company. The digested fragments were run on the DNA gel and purified by the gel extraction kit. The purified products were then inserted into the pVTU260 plasmid, which was pre-digested by the same restriction enzymes, by T4 DNA ligase under suitable concentration (Fig.2.1).

Fig. 2.1 Schematic diagram of the pVTU260-*acl* construct. Restriction sites used to fuse *acl* gene to the promoter were displayed under the gene as *NheI* and *BamHI*, ADHp, ADH promoter; His, 6×His-tag; ADHt, ADH terminator. Arrows indicate the direction of transcription (5'-3').

The pVTU260 plasmid with the target gene insertion was first transformed into *E. coli* Top10 competent cells according to the following procedure: One tube of the competent cell was thrown on ice until it melted. 5 µl of insertion product was added into the competent cell tube and kept on ice for 30 min. After then, the product tube was kept at 42 °C for 90 s and then immediately put on ice again. The whole mix was added into 1 ml LB medium and cultured at 37 °C for 1 h. The culture was then sprayed onto the LB agar plate with 100 µg/ml ampicillin. The plates were incubated in 37 °C incubator until the colonies formed.

For the verification of the correct insertion result, the single colonies from the LB agar plate were picked and cultured into 5 ml LB medium with 100 µg/ml ampicilin. After 12-16 h, the cells were collected by centrifuge and the plasmids were extracted according to the protocol of the plasmid DNA purification kit. The purified plasmids were then digested by the *NheI* and *BamHI* restriction enzyme and run on the DNA gel, to verify if the insertion is correct or not.

The correct recombinant plasmid was then transformed into the *S. cerevisiae* competent cells according to the following procedure: One tube of the competent cell was thrown on ice until it melted. The cells were spined down by centrifuge at 10,000 rpm for 2 min and the supernatant liquid was removed. The transformation was carried out according to the PEG/LiAc method. The reaction mixes were added in the following order: 260 µl PEG (50% w/v polyethylene glycol), 36 µl 1 M lithium acetate, 5 µl purified plasmid and 59 µl sterile distilled water. The cells were mixed well with the transformation reagent and kept at 42 °C for 45 min. After heat shock, the transformation reaction mix was centrifuged and the supernatant was removed. 1 ml sterile distilled water was added and mixed well with the cell. Total of 100 µl cells were spread onto the plates of minimal glucose (YNB-URA) medium with URA dropout mix. The plates were then cultured at 30 °C incubator until single colonies generated.

For the cloning and expression of the heterologous fatty acyl-CoA reductase, the recombinant pYES-DEST52 plasmid with FAR gene from birds (accession JN638549) was obtained from Prof. *Frentzen*'s lab and transformed directly into the *S. cerevisiae* competent cells according to the above described method. The plates were similarly cultured at 30 °C incubator until single colonies generated.

2.1.4 Verification of target gene by colony PCR

The correct colonies in the *S. cerevisiae* were verified by the colony PCR. The designed primers for the confirmation of the cloned ACL were: 5' ATGTCAGCCAAGGCAATTCAGAGC3' for the forward strand and 5' TTACATGCTCATGTGTTCTGGAAGA3' for the reverse strand. The designed primers for the confirmation of the cloned fatty acyl-CoA reductase were designed as: 5' ATGGTTTCCATACCTGAATA 3' for the forward strand and 5' TCAGTATCTCATAGTACTGG 3' for the reverse strand. A volume of 20 µl reaction mix was composed of the following reagents: 7 µl RNase free water, 10 µl Taq 2X Master Mix, 1 µl 10% trixon-100, 1 µl forward primer (100 nM), 1 µl reverse primer (100 nM) and a single colony was dissolved into the mix. The PCR program was performed as following: 95 °C for 30 s, 55 °C for 45 s, 72 °C for 3 min for one cycle, total of 30 cycles were performed. The PCR products were detected by the DNA gel and a fragment of about 3300 bp should be obtained from the results of correct colonies.

2.1.5 Protein extraction, SDS-PAGE and Western blot analysis

The *S. cerevisiae* colonies with correct *acl* insertion plasmid and empty pVTU260 plasmid were cultured in 5 ml minimal glucose (YNB-URA) medium at 30 °C, 250 rpm, respectively. After reaching to the growth phase, 1 ml culture medium was inoculated into 250 ml flasks containing 50 ml YNB-URA medium and cultured at 30 °C, 250 rpm. The cells were collected from the stationary phase and washed twice with distilled water. It was then re-suspended in 500 µl yeast lysis buffer (50 mM HEPES, 1 mM DTT, 1 mM PMSF, 1 mM EDTA and 5 % glycerol). An equal volume of glass beads (425-600 µm) was added into the sample tubes. The mix was performed in the bead mill for cell breakage as following: speed: 4.0 m/s; vortex time: 20 s for one cycle, total of six cycles were carried out. After cell lysis, the samples were centrifuged at 12,000 rpm for 30 min at 4 °C and the supernatants were carefully collected. The protein quantification was determined following the protocol of 2D Quant Kit (GE Healthcare). A standard curve of BSA concentration was plotted using the same method and the sample protein concentration was calculated from the BSA standard curve.

Same amount of protein extracts from the *S. cerevisiae* with correct *acl* insertion and pVTU260 empty plasmid was loaded on a 10% SDS-PAGE and run at the current of 20 mA. Once the protein bands went crossed the sticky gel, the current was increased to 30 mA. After the proteins were totally separated,

the current was stopped and the SDS-PAGE was electrophoretically transferred to a PVDF membrane at 21V for 1h. The membrane was then blocked for 1h at room temperature with the blocking buffer: 5% fat-free milk in TBST buffer (25 mM Tris-HCl, 150 mM NaCl and 0.5% Tween-20 pH 7.4). After that, the blocking buffer was removed and the primary antibody contained solution was added, which contained 3% fat-free milk in TBST buffer and anti-ACL specific antibody in a dilution ratio of 1:1000. The membrane was incubated at 4 °C overnight. It was then washed 3 times with TBST buffer (15 min for one time) and later incubated with secondary antibody contained solution: 3% fat-free milk in TBST and anti-rabbit antibody in a dilution ratio of 1:5000. The membrane was incubated at room temperature for 1 h again. After combining with the secondary antibody, the membrane was washed with TBST buffer for 3 times, to minimize the signal background. The ECL Western blot working substrates were prepared by mixing 500 µl peroxide solution and 500 µl luminol enhancer solution and covered the membrane for 2 min at room temperature. The membrane was then removed from the substrate solution and placed in a plastic sheet protector. The covered membrane was then placed in a film cassette with protein side facing up. The following steps were operated in a dark room: the X-ray film was put on top of the membrane and exposed for 1 min. The film was then removed and incubated into enhancer solution for about 15 s, immediately following the operation of putting it into stable solution for 15 s. The signal band then appeared on the film.

2.2 Gene disruption

2.2.1 Plasmid and primer design

The pUG plasmid series carrying the gene disruption cassettes consisting of completely heterologous marker genes were efficient for the gene disruption in yeasts [101]. The cassettes pUG6 that carried the gene for resistance to the drug geneticin (G418/ kan^r), pUG66 that carried the gene for resistance to phleomycin (ble) and pUG27 that carried the gene for his^{5+} selection marker were selected for the single and double gene disruption in the *S. cerevisiae*. Each of the plasmid was flanked by loxP sites (Fig.2.2). The plasmids were obtained from the lab of *Dr. Johannes H. Hegemann* (Germany). The size of disruption cassette pUG6 was 1.7 kb and the size of disruption cassette pUG66 and pUG27 were 1.3 kb and 1.55 kb, respectively.

Fig. 2.2 The pUG plasmids carrying loxP-flanked gene disruption cassettes. (Permission from ref.101 was obtained from publisher to use this figure)

The genes of mitochondrial IDH (*idh1,2*) and diacylglycerol acyltransferase (*dgal*) were selected for the disruption primer design. The oligonucleotides generated from the pUG6, pUG66 and pUG27 plasmids were the same: the 3' nucleotides of the upstream P_{TEF} resp. of the T elements (OL5') is 5'CAGCTGAAGCTTCGTACGC3' and the 5' nucleotides of the downstream T_{TEF} resp. of the P elements (OL3') was 5'GCATAGGCCACTAGTGGATCTG3'. The sequences flanking the target gene in the genome were added to the 5' end of OL5' and OL3': 40-45 nucleotide stretches that were homologous to sequences upstream of the ATG start codon and downstream of the stop codon of targeted gene, respectively [101]. The primers to generate the disruption cassettes were full-length. The pUG6 disruption cassette was used for the gene disruption of *idh1*, the pUG66 disruption cassette was used for the *idh2* gene disruption, and the pUG27 disruption cassette was used for the *dgal* gene disruption.

2.2.2 Generation of disruption cassette and transformation

The PCR program was used to generate the gene disruption cassette. The pUG6, pUG66 and pUG27 plasmids were extracted and diluted to 10 ng/ µl as DNA template. The synthesized oligonucleotides from above description were diluted to 100 nM and taken as forward and reverse primers, respectively. The PCR program was carried out as following: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min, total of 30 cycles were performed.

The PCR products were run on the DNA gel and purified from the gel extraction kit. The purified fragments were transformed into the *S. cerevisiae* competent cells using the PEG/LiAc method and 50 µl pre-boiled carrier DNA (2 mg/ml) was added into the transformation mix. The reaction mix was cultured in 1 ml YPD medium at 30 °C for 3 h. The linear DNA fragments carrying the selectable marker gene with homology regions on either end were to integrate at the corresponding chromosomal locus in *S. cerevisiae* by homologous recombination and the targeted genes were then replaced by the marker gene [102]. The first gene disruption was carried out by pUG6 disruption cassette with G418 marker and the second gene disruption was carried out by pUG66 cassette with ble marker on the basis of the first gene disruption. For the selection of the gene disruption strain with G418 marker, 200 µl of the cultured transformation mix was spread on the YPD agar plate with 200 µg/ml G418. For the selection of the gene disruption strain with ble marker, 200 µl of the cultured transformation mix was spread on the YPD agar plate with 7.5 µg/ml Phleomycin. All the plates were kept at 30 °C for several days until the single colonies formed. The whole gene disruption strategy was shown in Fig.2.3.

Fig. 2.3 Outline of the one-step gene disruption approach for the generation of gene disrupted strains. (Permission from ref.101 was obtained from publisher to use this figure)

2.2.3 Verification of gene disruption by colony PCR

To verify if the colonies have integrated the disruption cassette correctly, the colony PCR was performed. The primers A, D, B-M and C-M were designed according to the schematic diagram in Fig.2.3. The A and D were the target disruption gene-specific primers and bound about 300 bp upstream and downstream of the integration cassette in the genome, respectively, whereas the B-M and C-M were the disruption cassette-specific primers. Three different sets of primers were added in different PCR reaction mixes: A/B-M, C/D-M and A/D. The PCR program was as performed as following: 95 °C for 30 s, 55 °C

for 30 s, 72 °C for 2min, total of 30 cycles were performed.

In this case, for the wild type strain, the PCR result with A/B-M and C/D-M primer sets should show no band on the DNA gel, while the PCR result with A/D set should show a band with the size of the target gene length plus the designed 300 bp upstream and 300 bp downstream of the target gene in the genome. For the gene disruption strain, the PCR result with A/B-M and C/D-M primer sets should show a band with the expected designed size and the PCR with A/D set should show a band with the size of the disruption cassette length plus the designed 300 bp upstream and 300 bp downstream of the target gene in the genome.

2.3 Culture of yeast cells

Generally, the *Δidh1* single gene disrupted strain was cultured in YPD medium with 200 μg/ml G418; the *Δidh2* gene disrupted strain was s cultured in YPD medium with 7.5 μg/ml Phleomycin; the *Δidh1/2* double gene disrupted strain was cultured in YPD medium with 200 μg/ml G418 and 7.5 μg/ml Phleomycin; and the *Δdga1* gene disrupted strain was cultured in YNB-HIS medium. The *S. cerevisiae* strains with heterologous expressed genes were cultured in YNB-URA medium. All the cultivations were performed at 30 °C, 250 rpm.

To investigate the effects of the culture conditions with different carbon and nitrogen concentration on the fatty acid production level of the *S. cerevisiae* wild type strain and the engineered strains, the cultivation was carried out as following: Single colony of both the wild type strain and the engineered strains were cultured in 5 ml YNB-URA medium and kept at 30 °C, 250 rpm. When the cells reached to growth phase, about 1×10^8 cells were injected into 250 ml flasks containing 50 ml fermentation medium: 1.7 g/l yeast nitrogen base without amino acids and ammonium sulfate, 0.77 g/l URA dropout amino acid mixture, different concentrations of dextrose and ammonium sulfate. All of the cultures were kept at 30 °C, 250 rpm.

The dextrose concentration was fixed at 20, 40, 60 g/l, respectively and the ammonium (NH_4^+) concentration was adjusted to 2 mM in nitrogen-limited (NL) medium and 40 mM in non-nitrogen limited (NNL) medium. The pH value was kept the same, about at pH 5.0 in all of the culture mediums.

The biomass was measured according to the value of OD_{600} by collecting the culture medium at specific growth stage. The growth curves were generated by plotting the OD_{600} value of the *S. cerevisiae* cultures every 2 hours until it reached to the stationary phase.

The detection of the glucose concentration in the culture medium was carried

out according to the previous reported method [103]. The medium supernatant was collected for the glucose analysis. 300 µl of it was reacted with 1.5 ml anthrone solution (0.2 g anthrone in 100 ml 98% sulfuric acid) in a glass tube in an ice-water bath. The reaction mixture was then placed into a 100 °C water bath for 10 min. After cooling to the room temperature, the UV/Viaible spectrophotometer was used to detect the absorbance of the reaction mixture at 620 nm. The standard curve was obtained by detecting the reaction absorbance of different glucose concentration at 0, 10, 20, 30, 40 60, 80 and 100 µg/ml, respectively. The glucose concentration in the supernatant was calculated according to the standard curve.

2.4 Metabolomics analysis

2.4.1 Cell lysis, extraction of soluble metabolite and lipid

Same amounts of cells from culture medium of *S. cerevisiae* wild type strain and engineered strains were collected from the specific growth phase and centrifuged at 12,000 rpm for 5 min. The supernatants were discarded and the cell pellets were washed twice with distilled water and then re-dissolved in 800 µl pure methanol. 10 µl ribitol (2 mg/ml) and 10 µl heptadecanoic acid ($C_{17}H_{34}O_2$) (2 mg/ml) were added into the sample tubes as internal control, to correct the metabolites concentration deviation caused during the cell lysis operation. The cell lysis was carried out in an ultrasonic processor. The ultra-sonication procedure was as carried out as following: the amplitude was

set at 50%; the time of one cycle was set at 0.3 s. A total of 60 cycles were performed on each sample. After that, the cell samples were kept in liquid nitrogen for ten minutes and then immediately took out to room temperature for five minutes. A second running of sonication was conducted on the cell samples, to make sure the complete breakage of the *S. cerevisiae* cells. 500 µl ddH₂O and 300 µl chloroform were added into the cell samples after sonication and the sample tubes were vibrated for 2 h at room temperature. After methanol/chloroform/ddH₂O extraction, the sample tubes were centrifuged for 10 min and a two-phase mix was formed. The chloroform phase was separated from the methanol/ddH₂O phase and collected. Afterwhich, 300 µl chloroform was added into the remaining mix again and vibrated for 1 h at room temperature. Finally, about the chloroform phase including the lipid contents and the methanol/ddH₂O phase including the soluble metabolites were collected, respectively and they were evaporated to complete dryness for the analysis of soluble metabolites and fatty acid.

2.4.2 Soluble metabolites derivatization

The methanol/ddH₂O phase containing intracellular soluble metabolites from above extraction method was collected and evaporated to complete dryness at 37 °C. The dried samples were then performed for derivatization: 50 µl of methoxyamine hydrochloride (2 mg/ml in pyridine) was added into the sample tubes at the first step, to convert the functional groups of enol forms to

alkyloximes or oximes. After incubated at 37 °C for 1 h, 100 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1 % of chlorotrimethylsilane (TMCS) was added into the sample tubes and kept at 70 °C for another 1 h. By this operation, the silyl group (-Si(CH₃)₃) was added to the metabolites at the active hydrogen position. After the reaction, the samples were shaken at room temperature for 2 h, and then transferred to GC-MS vials for analysis. All of the derivative samples were run on GC-MS within 24 h.

2.4.3 Fatty acid derivatization

The chloroform phase containing intracellular lipid contents from above extraction method was collected and evaporated to complete dryness at 37 °C. The trans-esterification was carried out as following: 500 µl boron trifluoride (BF₃) solution with 14% methanol was added into the dried lipid-contained tubes. The sample tubes were kept at 95 °C for 1 h, to carry out the trans-esterification reaction. After reaction, 50 µl of sodium chloride was added immediately. By this operation, the TAG included in the lipid was converted to form free fatty acids and all the fatty acids were esterified to fatty acid methyl esters (F.A.M.E.). After cooling to room temperature, the reaction sample tubes were added with 300 µl hexane and vortex for half an hour at room temperature. The F.A.M.E was extracted by hexane and the hexane phase was then transferred into GC-MS vials for fatty acid analysis.

2.4.4 GC-MS procedure

The prepared samples were performed on an Agilent 7890A and 5975C GC-MS system equipped with a DB-5 capillary column (30 m, 250 μm i.d. and 0.25 μm thickness, Agilent J&W Scientific, Folsom, CA, and USA). 1 μl of the derivative samples were injected into the GC-MS system by Agilent auto-sampler and separated by the DB-5 capillary column in a splitless mode. The helium was used as carrier gas and its flow rate was kept at 1.1 ml/min. The injection temperature was set at 280 $^{\circ}\text{C}$ and the ion source temperature at 200 $^{\circ}\text{C}$, respectively. For soluble metabolites analysis, the oven temperature was began with 100 $^{\circ}\text{C}$ and increased to 320 $^{\circ}\text{C}$ at a rate of 4 $^{\circ}\text{C}/\text{min}$, where it remained for 1.56 min. For fatty acid analysis, the oven temperature process was according to the following program: 100 $^{\circ}\text{C}$ for 4 min and then increased with a ramp of 8 $^{\circ}\text{C}/\text{min}$ to 280 $^{\circ}\text{C}$, where with a 3 min of solvent cutoff time. The MS detection was performed in an electron ionization mode. The mass spectrum was recorded from 35 to 300 m/z, with a 0.3 s of scan time. Before running of the samples, 1 μl of acetone was run in the system with the same running procedure, to avoid the cross-contamination.

2.4.5 Data analysis

The acquisition of chromatogram and identification of mass spectra were performed on the GC-MS solution software (Enhanced Chemstation, Agilent). The noise reduction and baseline correction were carried out prior to

the peak area integration. Each peak area of the added internal control was used to adjust and normalize the target components peak area.

For the fatty acids analysis, the F.A.M.E standard mix (C8-C24, Sigma) was run on the GC-MS system and taken as authentic reference for the identification and quantification of the F.A.M.E. derived from *S. cerevisiae* samples. Different concentrations (0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mg/ml) of F.A.M.E. were performed on GC-MS with the above described procedure and the standard curves of F.A.M.E. with different length of carbon chain were generated. The calculation of F.A.M.E. from yeast samples was according to the standard curve and the final fatty acid concentration of different yeast samples were obtained depending on the detected F.A.M.E. concentrations. The standard curve of internal control was generated from GC-MS result by injecting the derivative C17:0 fatty acid with different concentrations (2, 4, 6, 8, 10, 12, 14 mg/ml).

Different volumes (10, 20, 40, 60, 80, 100 µl) of citrate standard (1 mg/ml) were pipetted into tubes and evaporated to dryness. The dried standards were performed derivative reaction according to above soluble metabolites derivatization method and run on GC-MS to generate a standard curve. The citrate concentration in *S. cerevisiae* samples were calculated according to the citrate standard curve.

For identification of other metabolites, the peaks that with more than 2 seconds in width at half-height were selected and performed on Agilent metabolite mass spectra database, for the search of candidate compounds. The peaks with more than 70% of similarity index were selected to name the sample compounds. In some cases, if the standard of searched compounds was commercial available, it was then obtained to run in the GC-MS, which was taken as reference to further confirm the searched results.

2.5 Proteomics

2.5.1 Protein extraction, digestion and labeling

2×10^8 OD of the *S. cerevisiae* cells were collected from specific growth phase and centrifuged at 12,000 rpm at room temperature for 10 min, to remove the supernatant medium. The cell pellets were washed twice with distilled water and re-suspended in 500 μ l yeast lysis buffer, which contained 1 mM DTT, 50 mM HEPES, 1 mM EDTA, 1 mM PMSF, and 5 % glycerol. An equal volume of glass beads (425-600 μ m) was added into the sample mixes. The mix was performed in a bead mill for the cell lysis as following parameters: 4.0 m/s for vortex speed and the 20 s for one cycle for vortex time, after each cycle, the sample tubes were kept on ice for 5 min for cooling. Total of 5 cycles were performed on each sample. After cell lysis, the samples were centrifuged at 12,000 rpm, 4 °C for 10 min. The supernatants were then collected. The protein quantification was carried out following the protocol of 2-D Quant Kit (GE

Healthcare). A standard curve of Bovine Serum Albumin (BSA) was plotted according to the same method.

For each *S. cerevisiae* sample, 100 µg proteins were precipitated by 4 volumes of cold acetone at −20 °C for 2 hours, to generate iTRAQ labeled peptides. The precipitated samples were carefully centrifuged at 12,000 rpm for 5 min and the acetone was removed. To each sample tube, 20 µl dissolution buffer and 1µl denaturant contained in iTRAQ Reagent Multiplex Kit (Applied Biosystems, CA) were added and mixed, followed by adding 2 µl reducing reagent. The tubes were then incubated at 60 °C for 1 h. After incubation, 1 µl cysteine blocking reagent was added and the tubes were kept at room temperature for 10 min. After that, the samples were digested with 20 µl sequence grade modified trypsin (0.25 µg/µl) (Promega, US) at 37 °C overnight. The peptides from different *S. cerevisiae* samples were then labeled with iTRAQ tag 114, 115, 116 and 117, respectively. The labeled samples were then mixed together and transferred into vials for LC-MS/MS analysis.

2.5.2 On-line 2D Nano LC-MS/MS procedure

The labeled samples were performed on the online 2D Nano-LC-MS/MS system. An Agilent 1200 Series LC system, interfaced with a 6530 Q-TOF mass spectrometer was applied for the analysis.

In the first dimension, 4 μg of combined iTRAQ labeled peptides mixture was loaded onto the system and separated by a strong cation exchange (SCX) nano-column ($0.32 \times 50 \text{ mm}$, $5 \mu\text{m}$) (Agilent Technologies, CA). The peptides were eluted stepwise by injecting gradient ammonium formate solution with different concentrations of 10, 20, 40, 60, 80, 100, 200, 500 and 1000 mM. Total of 10 runs were carried out to finish the whole program.

In the second dimension, the peptides that sequentially eluted from the SCX column were trapped in a Zorbax C18 enrichment column ($0.3 \times 5 \text{ mm}$, $5 \mu\text{m}$) and washed isocratically with 97% solvent A (water with 0.1% formic acid) and 3% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of $4 \mu\text{l/min}$. The eluted peptides were then bound on a C18 reverse phase column integrated with a nano-LC-Chip and eluted with 95% solvent A (water with 0.1 % formic acid) and 5% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of $3 \mu\text{l/min}$ over 45 min. After then, the flow was kept at 90% B for 5 min and then brought to initial state in 10 min for reconditioning the column. Altogether 9 runs were performed to finish one round of detection.

A 6530 Q-TOF mass spectrometer, interfaced with a Nano-LC-Chip Cube nanospray source was applied for the electrospray analysis. The Agilent Masshunter Workstation Q-TOF was applied for obtaining MS data. Survey scans were acquired from m/z 450 to m/z 1500 at 3 Hz, with up to two

precursors selected for MS/MS from m/z 50-2000 at 2 Hz.

2.5.3 Protein identification and data analysis

The peptide identifications were performed using Spectrum Mill system (Agilent Technologist, CA). It was compared and analyzed through the UniprotKB/Swiss-Prot database (Geneva, Switzerland). Each MS/MS spectrum was searched for the specie of *S. cerevisiae*. The iTRAQ reagent labels were allowed by the database and the modification was fixed under the following situation: iTRAQ modification of free amine in the amino terminus; methylmethanethiosulfate-labeled cysteine, plus two missed cleavages. The mass tolerance for peptide identification was ± 2.5 Da for MS and ± 0.7 Da for MS/MS. The high confidence for searched proteins was carried out by auto-validation. The quantification of proteins in the case of iTRAQ was showed in peak area ratios of 115/114, 116/114 and 117/114. The overlapping isotopic contributions were considered as the correction of calculated peak area ratios, which was used to the estimation of relative abundances of a specific peptide. The standard deviation and confidence value for each average peak area ratio were calculated and the protein quantification data with confidence > 95 %, p value < 0.05 were selected for further analysis.

2.5.4 Real-time PCR

Total of 5×10^7 *S. cerevisiae* cells were collected from specific growth phase

and centrifuged at 12,000 rpm for 5 min. The liquid medium was discarded and the cell pellets were used for the total RNA extraction. The RNA of different samples was extracted according to yeast RNA extraction method chapter provided by the RNeasy Mini Kit (QIAGEN, USA). The obtained RNA was then transformed to cDNA by the QuantiTect Reverse Transcription Kit (QIAGEN, USA). The amount of cDNA was calculated by the NanoDrop 2000C system (Thermo Scientific, USA) and all of the cDNAs were diluted to a same concentration (10 ng/μl) and used as reaction templates. The primers for selected genes were designed and synthesized. The *S. cerevisiae* actin (ACT1) gene was used as endogenous control. The Real-time PCR reaction mix was prepared according to the methods of $2 \times \text{iQ}^{\text{TM}}$ SYBR Green supermix kit (Bio-Rad, USA): nuclease-free water: variable; forward primer: 300 nM; reverse primer: 300nM; $2 \times \text{iQ}^{\text{TM}}$ SYBR Green supermix: 10 μl, cDNA template: 10 ng; the final reaction volume was 20μl.

The Real-time RP-PCR cycling condition was carried out as following: 95 °C for 5 min and then 45 cycles as the following: 95 °C 10 s, 55 °C 30 s, 72 °C 30 s. The melt curve analysis was carried out at 55-95 °C (in 0.5°C increments), hold for 30 s.

The amplification analysis, melting curve analysis and threshold cycle numbers were obtained from the iQ5 optical system software (Bio-Rad). The mRNA change folds were calculated by the following formula:

$$\Delta \Delta Ct = \text{Sample } \Delta Ct - \text{Control } \Delta Ct;$$

$$\text{Sample } \Delta Ct = Ct_{\text{sample}} - Ct_{\text{actin}};$$

$$\text{Control } \Delta Ct = Ct_{\text{control}} - Ct_{\text{actin}};$$

$$\text{Fold changes of sample/control} = 2^{-\Delta \Delta Ct}.$$

3. Overexpression of a heterologous ACL in *Saccharomyces cerevisiae* and investigation of fatty acid production under nitrogen limited culture condition

3.1 Introduction

The fatty acids can be accumulated in the oleaginous yeasts under nitrogen limited culture condition. This phenomenon has been demonstrated to be linked to the inactivation of IDH, due to the decrease of cellular AMP concentration [89] (Fig. 3.1). In the non-oleaginous yeasts like *S. cerevisiae*, the IDH has been predicted to have no absolute dependency on mitochondrial AMP concentration [104, 35].

Fig. 3.1 Schematic diagram of fatty acid accumulation mechanism in oleaginous yeasts under nitrogen limited culture conditions. The red line indicated the main mechanism of the accumulation process. The upward and downward arrows represent activation or inhibition of enzymes and increase or decrease in intermediates level. *idh*:

NAD⁺-dependent isocitrate dehydrogenase; *acl*: ATP citrate lyase.

It was predicted that the depression of IDH activity contributed to the onset of lipid accumulation and the ACL was a key enzyme to provide substrate for fatty acid biosynthesis in the oleaginous yeasts [105, 106]. In the *S. cerevisiae*, the IDH existing in mitochondria is encoded by two distinct genes (*idh1* and *idh2*) and responsible for oxidative decarboxylation of isocitrate to alpha-ketoglutarate [107]. However, no original ACL gene (*acl*) is found in the *S. cerevisiae*. The acetyl-CoA for fatty acid synthesis in the *S. cerevisiae* is mainly generated from the pyruvate-acetaldehyde-acetate pathway in cytosol [39], but not from excess citrate cleavage pathway. The previous studies have indicated that if the non-oleaginous yeast, such as *S. cerevisiae*, was cultivated under the same nitrogen limited culture conditions as the oleaginous yeast, the cells would stop to proliferate, or they would accumulate polysaccharides such as glycogen, but no more lipids would be accumulated like in the oleaginous yeasts [108].

Herein, a heterologous *acl* is going to be cloned and expressed in the *S. cerevisiae* to construct a second acetyl-CoA production pathway. The culture conditions with different carbon and nitrogen concentrations will be performed on both the *S. cerevisiae* wild type strain and the engineered strain. The study will be carried out to investigate the fatty acid accumulation condition in the *S. cerevisiae* strains and by optimizing the culture condition; the engineered strain

is expected to improve fatty acids production in quantity and quality.

3.2 Experiment procedures

The *S. cerevisiae* wild type BY4741 was transformed with the pVTU260 empty vector to construct the wt-empty control strain (“we”) in this study. A heterologous *acl* PCR fragments from *Mus musculus* (accession BC056378.1) hanged with 5’ *NheI* and 3’ *BamHI* restriction enzyme (RE) sites was inserted into the pVTU260 vector at the same RE sites, under the ADH1 constitutive promoter and transformed into the *S. cerevisiae* wild type BY4741 to construct the wt-*acl* engineered strain (“wa”). The expression of *acl* in the *S. cerevisiae* was determined by the Western blot. The anti-ACL specific antibody was used as the first monoclonal antibody, to detect the expression fragment. The detail procedures were conducted according to the methods described in Section 2.1.

Both of the “we” and “wa” strains were cultivated under culture conditions with different carbon and nitrogen concentrations (Table 3.1). The cultivation was performed according to methods described in Section 2.3. Same amounts of cells from the stationary phase of “we” and “wa” strains were collected and the cellular metabolites and lipids were extracted according to methods described in Section 2.4.1. The intracellular citrate and fatty acids were derived for GC-MS analysis with the same methods from Section 2.4.2 to 2.4.4. The production levels of citrate and fatty acids between the “we” and “wa” strains were compared.

Table 3.1 Culture medium composition with different glucose and ammonium sulfate concentration

Culture condition	YNB w/o amino acids and ammonium sulfate (g/l)	Ura Do supplement (g/l)	Glucose concentration (g/l)	Ammonium concentration (mM)
NL-C20	1.7	0.77	20	2
NNL-C20				40
NL-C40	1.7	0.77	40	2
NNL-C40				40
NL-C60	1.7	0.77	60	2
NNL-C60				40

Notes: NL, nitrogen limited condition; NNL, non-nitrogen limited condition; C20, 20 g/l glucose; C40, 40 g/l glucose; C60, 60 g/l glucose.

3.3 Results

3.3.1 Cloning and overexpression of *acl* in the *S. cerevisiae*

The *acl* encoding the ACL was successfully cloned and expressed in the *S. cerevisiae* wild type strain. Both of the colony PCR and Western blot methods were applied to confirm the results. The wild type strain with the empty pVTU260 plasmid was used as control. The PCR fragment size of the recombinant plasmid expressed in the *S. cerevisiae* was about 3.3 kb and no band was observed from the control strains (Fig.3.2). The protein size from Western blot result was about 120 kDa; and similarly, there were no protein bands from the control strains (Fig.3.3).

Fig. 3.2 Colony PCR result of *acl* expression in the *S. cerevisiae*. Lane 1: the *S. cerevisiae* with empty pVTU260 vector (“we”); lane 2: the *S. cerevisiae* with *acl* gene (“wa”).

Fig. 3.3 Western blot result of *acl* expression in the *S. cerevisiae*. Lane 1: the *S. cerevisiae* with empty pVTU260 vector (“we”); lane 2: the *S. cerevisiae* with *acl* gene (“wa”).

3.3.2 Effect of culture conditions with different carbon and nitrogen concentration on biomass

The carbon and nitrogen source were the most important culture elements for yeast. Their metabolism provided essential energy, redox balance and intermediates for cell proliferation, fermentation activities and protein and nucleic synthesis. The cultures with different carbon and nitrogen concentrations have been studied in terms of their effects on biomass of the *S. cerevisiae* wild type strain and the engineered strain. The glucose was selected

as carbon source and fixed at 20, 40, 60 g/l respectively, while the ammonium was taken as nitrogen source and kept at 2 mM to form nitrogen limited (NL) condition and 40 mM to form non-nitrogen limited (NNL) condition. The optical density value at wavelength of 600 nm (OD_{600}) of yeast culture samples obtained from stationary phase was taken to characterize the biomass.

It was found that under culture conditions with same glucose concentration, but different nitrogen concentration, the biomass did not show significant differences between the “we” strain and “wa” strain. However, with the increase of glucose concentration in culture medium, the biomass of all these strains showed a slight increase accordingly. Meanwhile, there were no considerable biomass difference between “we” and “wa” strains under various culture conditions (Fig. 3.4).

Fig. 3.4 Biomass of the *S. cerevisiae* wild type strain and engineered strain under culture conditions with different carbon and nitrogen concentrations. The value of OD_{600} was taken to characterize the cell mass and yeast culture samples were selected

from the stationary phase. NL: nitrogen limited; NNL: non-nitrogen limited.

The residual glucose contents were detected from different culture broth of “we” strain and “wa” strain, and they showed no significant differences. Almost all of the initial add-in glucose in the medium was finally taken in by the yeast strains (Table 3.2). It was concluded that the nitrogen limited culture condition did not affect the biomass of *S. cerevisiae* wild type strain and the engineered strain significantly, but the increased glucose concentration was somehow beneficial for cell growth.

Table 3.2 Residual glucose content in different culture medium of “we” and “wa” strains

Culture condition	NL-C2	NNL-C2	NL-C4	NNL-C4	NL-C6	NNL-C6
	0	0	0	0	0	0
“we” Residual	0.497	0.468	0.492	0.489	0.777	0.621
” glucose (g/l)	0.379	0.331	0.535	0.576	0.985	0.668

Notes: The residual glucose content was detected from the culture broth of the stationary phase; the collect time was consistent with that of the biomass detection time.

3.3.3 Investigation of fatty acids production levels in *S. cerevisiae* wild type strain with pVTU-260 vector (“we”) under different culture conditions

The fatty acid started to be bio-synthesized in yeast cells when they entered into stationary phase and mainly existed in the form of TAG included in lipid. The oleaginous yeasts have been reported to accumulate more lipids when they were exposed to culture medium with excess carbon source and limited nitrogen source. Herein, the *S. cerevisiae* wild type strain with empty pVTU260

vector (“we”) were cultured under different concentrations of carbon source and nitrogen source. Their fatty acids production levels were investigated.

The mainly detected fatty acids were palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0). When the “we” strain was exposed to culture medium with same carbon concentration but different nitrogen concentration, neither the saturated nor the unsaturated fatty acids showed obvious differences in fatty acid production levels (Fig. 3.5). As the glucose concentration increased, the increase in fatty acids production was observed under both NL and NNL conditions. Under the culture condition with 20 g/l glucose, the unsaturated fatty acid palmitoleic acid yield was about 20 µg/ml and the oleic acid yield was about 6 µg/ml. When the glucose increased to 60 g/l, a 35 % higher yield in palmitoleic acid and a 70 % higher yield in oleic acid were observed (Fig. 3.5 A). For the saturated fatty acid palmitic acid and stearic acid, the production was about 20-30 % higher under 60 g/l than that under 20 g/l (Fig. 3.5 B).

Fig. 3.5. Fatty acids production levels in the *S. cerevisiae* wild type strain with empty pVTU260 vector (“we”) under culture conditions with different carbon and nitrogen concentrations. The production levels of mono-unsaturated fatty acids (C16:1, C18:1) (A) and saturated fatty acids (C16:0, C18:0) (B) in cells were reflected. The standard deviation was calculated from three independent experimental results.

3.3.4 Investigation of fatty acids production levels in *S. cerevisiae* engineered strain expressing *acl* (“wa”) under different culture conditions

In this study, the *acl* was cloned and expressed in the *S. cerevisiae*. The engineered strain (“wa”) was cultured under different concentrations of carbon source and nitrogen source and the fatty acids production levels were investigated.

When the “wa” strain was exposed to culture medium with same carbon concentration, more fatty acids were accumulated under NL conditions. Interestingly, the mainly accumulated fatty acids were mono-unsaturated ones (C16:1 and C18:1) (Fig. 3.6A). The amount of palmitoleic acid under NL was at

about 1.4 times to that under NNL conditions, regardless of the glucose concentration. The amount of oleic acid under NL condition was about 1.2 times to that under NNL condition when the glucose concentration was fixed at 20 g/l. This kind of enhancement increased to 1.4 and 1.6 times respectively when the glucose concentration was fixed at 40 g/l and 60 g/l. However, the production of saturated fatty acids (C16:0 and C18:0) showed no such significant differences (Fig. 3.6B).

Fig. 3.6 Fatty acids production levels in the *S. cerevisiae* engineered strain expressing *acl* (“wa”) under culture conditions with different carbon and nitrogen concentrations. The production levels of mono-unsaturated fatty acids (C16:1, C18:1) (A) and saturated fatty acids (C16:0, C18:0) (B) in cells were reflected. The standard deviation was calculated from three independent experimental results.

Similar to the “we” strain, the production levels of fatty acids in “wa” strain enhanced with the increase of glucose concentration, both under NL condition and NNL culture conditions. The yield of palmitoleic acid in “wa” cells was 28 $\mu\text{g/ml}$ under NL condition and 20 $\mu\text{g/ml}$ under NNL condition respectively when the glucose was fixed at 20 g/l. The accumulation of palmitoleic acid

reached up to 36 $\mu\text{g/ml}$ under NL condition and 25 $\mu\text{g/ml}$ under NNL condition when the glucose concentration increased to 40 g/l. The amount further increased to 41 $\mu\text{g/ml}$ under NL condition and 28 $\mu\text{g/ml}$ under NNL condition when the glucose concentration increased to 60 g/l. Besides, the production level of oleic acid was about 9 $\mu\text{g/ml}$ under NL condition and 7 $\mu\text{g/ml}$ under NNL condition when the glucose was fixed at 20 g/l, and the values increased to 14, 19 $\mu\text{g/ml}$ under NL condition and 10, 12 $\mu\text{g/ml}$ under NNL condition respectively when the glucose concentration later increased to 40 and 60 g/l. The saturated fatty acids production levels also increased when the glucose concentration increased, however, compared with the unsaturated fatty acid, their increase didn't exhibit obviously significant.

The representative of the GC-MS spectra for the mono-unsaturated fatty acid C16:1 and C18:1 derived from the total ion chromatograms were showed in Fig. 3.7, which confirmed the increase of the mono-unsaturated fatty acids in the “wa” strain under nitrogen limited condition.

Fig. 3.7 The representative of GC-MS comparison spectra of the production levels of mono-unsaturated fatty acids C16:1 (A) and C18:1 (B) between the “we” strain and the “wa” strain under nitrogen limited culture condition and non-nitrogen limited culture condition. The black line and blue line represented the fatty acid production level in the “wa” strain under NL and NNL condition respectively; the red line and green line represented the fatty acid production level in the “we” strain under NL and NNL condition respectively.

3.3.5 Comparison of fatty acids production levels between “we” strain and “wa” strain

Compared with the “we” strain, the production levels of mono-unsaturated fatty acids in “wa” strain showed about 40% to 60% higher under NL culture conditions and this observation was statistical significant. However, under NNL culture condition, this kind of increase was not significant. Besides, the yield of saturated fatty acids production between “wa” strain and “we” strain also

showed no significant differences. The saturated fatty acids amount in “wa” strain was about 10-20% higher than that in “we” strain both under NL and NNL culture conditions (Table 3.3).

Table 3.3 Fold changes of specific fatty acid composition between the “we” strain and “wa” strain

Culture condition	Palmitoleic acid	Palmitic acid	Oleic acid	Stearic acid
NL-C20	1.38±0.171*	1.15±0.041	1.40±0.025*	1.08±0.013
NNL-C20	1.04±0.012	1.13±0.018	1.13±0.045	1.04±0.032
NL-C40	1.39±0.060**	1.06±0.020	1.42±0.121**	1.05±0.025
NNL-C40	1.07±0.016	1.18±0.011	1.11±0.090	1.17±0.018*
NL-C60	1.48±0.131**	1.03±0.012	1.62±0.211***	1.07±0.009
NNL-C60	1.02±0.027	1.12±0.009*	1.03±0.068	1.08±0.024

Notes: The fatty acid production levels of the “we” strain were taken as controls (100%), and the production levels in the “wa” strain were being ratio to that in the “we” strain. The standard deviation was calculated from three independent experiments. The “asterisk” indicated the statistical significance of the fatty acid changes between the “we” strain and “wa” strain: *, p<0.05; **, p<0.01; ***, p<0.001.

The results reflected that the nitrogen concentration in culture medium had no significant effect on the fatty acids production levels in “we” strain, but significantly affected that in “wa” strains. Specifically, under nitrogen limited culture conditions, the mono-unsaturated fatty acids production in “wa” strain increased to a higher level than that under non-nitrogen limited culture conditions. This phenomenon was not observed in the “we” strain. Besides, the increase of glucose concentration was beneficial for fatty acids production in both “we” and “wa” strains, regardless of the nitrogen concentration.

3.3.6 Investigation of intracellular citrate level in *S. cerevisiae* “we” strain and “wa” strain

The intracellular citrate level in *S. cerevisiae* “we” strain and “wa” strain was investigated. In accordance with our expectation, the citrate level in both “we” strain and “wa” strain showed much higher under NL culture condition than that under NNL condition (Fig. 3.8). When exposed to NNL condition, the citrate amount was about 10-15 $\mu\text{g/ml}$ in “we” strain and 8-10 $\mu\text{g/ml}$ in “wa” strain. However, it increased to 30-40 $\mu\text{g/ml}$ and 20-30 $\mu\text{g/ml}$ respectively under NL condition. The results showed that the citrate production level was much lower in “wa” strain than that in “we” strain, which indicated that the citrate was consumed partially in “wa” strain since the heterologous *acl* was expressed.

Fig. 3.8 Intracellular citrate production levels between the *S. cerevisiae* wild type strain with empty pVTU260 vector (“we”) and engineered strain expressing *acl* (“wa”) under different culture conditions. The standard deviation was calculated from two independent experimental results.

One set of the comparison spectra of the citrate levels between the “we” strain and the “wa” strain under nitrogen limited and non-nitrogen limited conditions were showed in Fig.3.9

Fig. 3.9 The representative of GC-MS comparison spectra of the citrate levels between the “we” strain and the “wa” strain. The blue line and green line represented the citrate level in the “we” strain under NL and NNL condition, respectively; the black line and the red line represented the citrate level in the “wa” strain under NL and NNL condition, respectively.

3.3.7 Transcriptomic response of *amd* and *idh* genes in *S. cerevisiae* to nitrogen limited condition

The AMP deaminase gene (*amd1*) and two subunits of mitochondrial IDH

genes (*idh1* and *idh2*) were investigated on their mRNA expression levels. The *S. cerevisiae* actin gene (*act1*) was selected as the endogenous control. The length of end products was controlled between 200-250 bp (See primer design in Table 3.4). The fold changes of mRNA levels of the selected genes in *S. cerevisiae* under non-nitrogen limited condition to that under nitrogen limited were shown in Fig.3.10.

Table 3.4 Primer sequences for Real-time PCR

Gene name	Primers	Product (bp)
amd1	Forward: 5'TTGGAGCAACCATCGCATTC3'	245
	Reverse: 5'TGCTGTGAAGCATCATCAGC3'	
idh1	Forward: 5' CTATGGCACACTCCTGCTGA3'	207
	Reverse: 5'CACTACACCAGGGACGGATT3'	
idh2	Forward: 5'CTGGTTGCACTAAAAGGTCC3'	221
	Reverse: 5'ACAACGCCAGGGCAAACCTAT3'	
act1	Forward: 5' AACTTTCAACGTTCCAGCCT3'	216
	Reverse: 5' CCACGTTCACTCAAGATCTT3'	

From the Real-time PCR results, the mRNA level of AMP deaminase showed higher under nitrogen limited condition than that under non-nitrogen limited condition. The fold change was apparent, which indirectly confirmed the increase in AMP deaminase activity under NL condition. However, there was no significant change in mRNA level of IDH under NL condition. The *idh2* gene showed almost the same in mRNA level between>NNL and NL condition, whereas the *idh1* gene showed even a little higher in mRNA level under NL condition. This phenomenon was consistent with the previous study in oleaginous yeast *Yarrowia lipolytica* during the fatty acid accumulation process

[109]. The inconsistency of the IDH mRNA level with the expected decrease in IDH activity reflected that the IDH activity was controlled by the cellular AMP concentration rather than the transcriptional level.

Fig.3.10 Real-time PCR analysis of mRNA level of *amd* and *idh* gene in *S. cerevisiae* under nitrogen limited condition. The mRNA level in the yeast cells under non-nitrogen limited (NNL) condition was taken as control (100%) and mRNA level in the yeast cells under nitrogen limited (NL) condition was being ratio to that under NNL condition. The standard deviation was calculated from three independent experimental results.

3.4 Discussion

The comparative study of fatty acid production levels between the *S. cerevisiae* wild type strain and engineered strain reflected that the mono-unsaturated fatty acid could be accumulated under nitrogen limited culture condition when a heterologous *acl* was overexpressed.

In the oleaginous yeasts, when exposed to nitrogen limited conditions, the

cells kept on assimilating the carbon source but stopped proliferating. The metabolic flux of excess carbon was then diverted towards fatty acid synthesis [110]. This kind of fatty acid accumulation can be explained by the decreased activity of IDH, which was caused by the decrease of AMP concentration under NL condition. However, in the *S. cerevisiae*, the IDH activity was predicted to have no such dependency on mitochondrial AMP concentration [104, 106]. This was regarded as the main reason for the infeasibility of fatty acid accumulation in the non-oleaginous yeasts under NL condition [89]. There were studies focused on replacement of original mitochondrial IDH of *S. cerevisiae* with IDH from the oleaginous yeast, such as *Rhodospiridium toruloides* [89]. The results showed that the lipid content in *S. cerevisiae* Δidh carrying the oleaginous yeast *idh* increased under NL condition, while the wild type strain showed no such correlation. Interestingly, the study herein showed that the expression of ACL made the *S. cerevisiae* accumulate more mono-unsaturated fatty acids under NL culture condition.

Both of the *S. cerevisiae* “we” and “wa” strains were cultivated under culture conditions with different carbon and nitrogen concentrations. It was found that the intracellular citrate level in *S. cerevisiae* was much higher under NL condition than that under>NNL condition. Although the activity of IDH in *S. cerevisiae* was demonstrated to be non-regulated by the cellular AMP concentration [41], the results herein reflected that there should be some

relationship between nitrogen depletion and citrate accumulation. In *S. cerevisiae* “we” strain, there was no ACL activity and the accumulated citrate could not be cleaved to acetyl-CoA for fatty acid synthesis. Once the heterologous ACL was expressed, the citrate could be consumed. This was why the citrate level in “wa” strain was much lower than that in “we” strain.

With the increase of carbon/nitrogen concentration ratio (C/N), the enhancement of mono-unsaturated fatty acids in the *S. cerevisiae* with expressed ACL showed more obviously. From the above results, the intracellular citrate in *S. cerevisiae* was accumulated more under higher C/N ratio, and the increased fatty acids yield in the engineered strain could be benefited from this accumulated citrate, which could be cleaved to acetyl-CoA for fatty acid synthesis. Interestingly, the main accumulated fatty acids in “wa” strain under nitrogen limited condition were mono-unsaturated ones. The fatty acid composition in yeasts could be modified by environmental adjustment, such as changes of temperature, pH, nitrogen source or carbon source [111, 112]. The mono-unsaturated fatty acid accumulated in yeast was regarded as an important role in physiological functions, such as to keep membrane integrity and increase the resistance of yeast to elevated ethanol concentrations [34]. In another important aspect, the biodiesel fuels with higher ratio of mono-unsaturated fatty acid methyl esters derived from mono-unsaturated fatty acids exhibited advantages of lower viscosity, lower pour points and cloud

points, which could improve the biodiesel qualities with excellent cold flow property [24]. Thus, the engineered strain with capacity in enhancement of mono-unsaturated fatty acids could be an important potential candidate for biofuel production, to realize the feasibility of biodiesel production in quantity and quality.

3.5 Section conclusion

In summary, the results obtained in this study demonstrated that the fatty acid could be accumulated under nitrogen limited condition in the *S. cerevisiae* engineered strain expressing a heterologous ACL, while the wild type strain, no such accumulation was observed. By modifying the metabolic pathway and optimizing the culture medium, the production of fatty acids, especially the long chain mono-unsaturated fatty acids in the *S. cerevisiae* was found significantly enhanced and this could better serve the biofuel production.

4. Investigation of citrate accumulation in the IDH gene disrupted strains of *Saccharomyces cerevisiae*

4.1 Introduction

Citrate produced by the yeast strains is an important intermediate that serves as donor for cytosol acetyl-CoA, the key starting material of fatty acid synthesis. It is formed by the citrate synthase in the TCA cycle and processed by the TCA-related enzymes to generate FADH₂ and NADH, leading to form ATP ultimately. As described in Chapter 3, when the yeast strains were performed under certain culture conditions, such as nitrogen limited culture condition, the citrate can be accumulated and transported to cytosol through citrate transport protein (CTP) [65, 113], where the citrate can be further broken down to form acetyl-CoA by the ACL. Since the acetyl-CoA cannot be directly transported out of mitochondrial matrix to cytosol, to accumulate citrate in the TCA cycle and then transport it out to generate cytosol acetyl-CoA by applying the ACL can be a promising pathway to provide fatty acid synthesis donor in the *S. cerevisiae* cells [114].

Two genetic strategies can be enrolled to directly accumulate citrate in the *S. cerevisiae*: either by increasing the activity of citrate synthesis related enzymes or by decreasing that of citrate catabolism related enzymes. During the TCA cycle of eukaryotes cells, the phosphoenolpyruvate carboxylate (PEPC:

responsible for catalyzing the addition of bicarbonate to phosphoenolpyruvate to form oxaloacetate), citrate synthase (CS: responsible for catalyzing the condensation reaction of acetyl-CoA with oxaloacetate to form citrate), and malate dehydrogenase (MDH: responsible for catalyzing the conversion of malate into oxaloacetate) are the up-regulated enzymes of the citrate synthesis [115, 116]. On the other hand, the aconitase (ACO1: responsible for catalyzing the stereo-specific isomerization of citrate to isocitrate) and NAD⁺-isocitrate dehydrogenase (IDH: responsible for catalyzing the oxidative decarboxylation of isocitrate to form alpha-ketoglutarate) are known as down-regulated enzymes of the citrate catabolism [115]. Citrate consumption could be reduced by decreasing these enzymes' activities.

The study here focuses efforts on examining the citrate accumulation by disruption of citrate catabolism related genes in the *S. cerevisiae*. Based on the citrate accumulation model in the oleaginous yeasts described in Section 1.5, the inactivation of IDH has been demonstrated to be linked to the accumulation of citrate and the onset of lipid storage. So herein, the *idh* genes in the *S. cerevisiae* is selected to be directly disrupted and the citrate production level between the wild type strain and the gene disrupted strains are going to be compared. The citrate yield in the *idh* gene disrupted strains is expected to be enhanced.

4.2 Experiment procedures

The disruption of *idh1* and *idh2* genes, which encoded the two subunits of IDH was carried out according to the methods described in Section 2.2. The *idh1* disruption cassette containing the G418 selection marker was transformed into the *S. cerevisiae* wild type strain, to generate the $\Delta idh1$ strain; the *idh2* disruption cassette containing the ble selection marker was transformed into the *S. cerevisiae* wild type strain, to generate the $\Delta idh2$ strain; and the *idh2* disruption cassette containing the ble selection marker was further transformed into the $\Delta idh1$ single gene disrupted strain, to generate the $\Delta idh1/2$ double gene disrupted strain.

Together with the wild type strain, the two single disrupted strains and one double disrupted strain were cultured in the YPD medium on a rotary shaker at 250 rpm and 30 °C. The corresponding antibiotics were added in an appropriate concentration.

The intracellular metabolites containing the citrate product were extracted according to the method described in Section 2.4.1. For extracellular citrate analysis, the supernatants of culture broth were collected and passed through a 0.45 μm filter and washed with methanol. All of these were evaporated to complete dryness. The derivatization and analysis of citrate was performed according to the methods described in Section 2.4.2, 2.4.4 and 2.4.5.

4.3 Results

4.3.1 Gene disruption of *S. cerevisiae*

The primers designed for the *idh1,2* gene disruption cassette were described in Table 4.1. According to the gene disruption strategy, two single mutant strains $\Delta idh1$, $\Delta idh2$ and one double mutant strain $\Delta idh1/2$ were obtained.

Table 4.1 Primers for the generation of gene disruption cassette in this study

Name	Description
<i>Primers for gene disruption</i>	
<i>idh1</i> -F	5'CTCATTCTTCTCCCTTTTCCTCCATAATTGTAAGAG AAAACAGCTGAAGCT TCG TAC GC3'
<i>idh1</i> -R	5'CACTTAAGTTGCAGAACAAAAAAGGGGAATT GTTTTCAGCATAGGCCACTAGTGGATCTG3'
<i>idh2</i> -F	5'ACAGATCGGGAACGAACAACAATTATAATATTTTT TAATACAGCTGAAGCTTCGTACGC3'
<i>Idh2</i> -R	5'CAAGGCGTGAAAAAACCTGAGAGGGGAAAAGAAT AGGACTTGCATAGGCCACTAGTGGATTG3'

The confirmation of the correct gene disrupted colonies was carried out by the colony PCR (Fig.4.1). The test primers for the colony PCR were designed according to the method described in 2.2.3. It was found that in the wild type strain, there were no bands for the *idh2* A-B and *idh1* A-B PCR fragments found in the DNA gel. However, in the *idh1,2* gene disrupted strains, the bands were found and showed in the correct size according to the designed test primers. The correct mutants were then collected and cultured for the further studies.

Fig. 4.1 The gene disruption results confirmed by the yeast colony PCR. The ladder used here was 1kb standard ladder (Fermentas). The tested band was at the location of about 500 bp size, which was consistent with the designed primers. The bands from left to right in (A) represented: marker; lane1: *idh2* A-B for the wild type strain; lane 2: *idh1* A-B for the wild type strain . The bands from left to right in (B) represented: marker; lane 1: *idh2* A-B for $\Delta idh2$ strain; lane2: *idh2* C-D for $\Delta idh2$ strain; lane 3: *idh2* A-B for $\Delta idh1/2$ strain; lane 4: *idh2* C-D for $\Delta idh1/2$ strain; lane 5: *idh1* A-B for $\Delta idh1$ strain; lane 6: *idh1* C-D for $\Delta idh1$ strain.

4.3.2 Growth rate description between the wild type strain and gene disrupted strains under YPD culture medium

The growth curves of the wild type strain and the gene disrupted strains were tested in the YPD medium (Fig.4.2). All of these strains entered stationary phase after about 16 h in cultivation. The OD_{600} value of the single gene disrupted strains $\Delta idh1$ and $\Delta idh2$ showed about 5% lower than that of the wild type strain, while the double gene disrupted strain $\Delta idh1/2$ showed about 10% lower than that of the wild type strain. From the results, it showed that the blockage of the TCA cycle in the *S. cerevisiae* didn't significantly cause the growth deficient under culture medium with sufficient nutrients and with the glucose as the carbon source.

Fig. 4.2. Growth curve of the wild type strain and *idh1,2* gene disrupted strains. The strains were grown in 250 ml flasks, with 50 ml YPD medium supplemented with appropriate antibiotics. The cultures were grown in a rotary shaker at 250 rpm, 30 °C.

4.3.3 Citrate level description between the wild type strain and the gene disrupted strains

The citrate level was expected to be higher in the $\Delta idh1,2$ strains compared to that in the wild type strain since the *idh1,2* genes responsible for the down regulation of citrate metabolism were deleted. From the results, it could be seen easily that both the single and double gene disrupted strains led to a significant increase in the intracellular citrate levels, with 4- to 5-folds. The double gene disrupted strain $\Delta idh1/2$ showed more citrate accumulation compared with the single gene disrupted strains and the maximum concentration of intracellular citrate reached about 170 $\mu\text{g/ml}$ (Fig.4.3).

Fig. 4.3 Differential production levels of cellular citrate between the *S. cerevisiae* wild type strain and the *idh1,2* gene disrupted strains. The concentration of citrate was calculated from citrate standard curve of GC-MS results. The standard deviation was calculated according to three independent experiments.

Fig. 4.4 Differential production levels of extracellular citrate between the *S. cerevisiae* wild type strain and the *idh1,2* gene disrupted strains. The concentration of citrate was calculated from citrate standard curve of GC-MS results. The standard deviation was calculated according to three independent experiments.

The increased citrate pool in the $\Delta idh1,2$ strains also led to increase citrate

secretion. The results of the citrate concentration in the culture supernatant showed that the citrate levels in the $\Delta idh1,2$ strains were about 2-3 times higher than that in the wild type strain (Fig.4.4). Since our focus was about cellular citrate metabolism, more attention was placed on the changes of intracellular citrate in the *S. cerevisiae* strains.

One representative of the GC-MS spectra for the citrate derived from the total ion chromatograms were showed in Fig.4.5 and one set of the comparison spectra of the citrate levels between the wild type strain and the gene disrupted strains were showed in Fig.4.6, which confirm the results of the citrate accumulation in the *idh1,2* gene disrupted strains.

Fig. 4.5 The representative GC/MS spectrum of the citrate in the yeast metabolites derived from total ion chromatograms.

Fig. 4.6 The representative of GC-MS comparison spectra of the citrate levels between the wild type strain and the gene disrupted strains. The red line represented the citrate level in the wild type strain; the black line represented the citrate level in the $\Delta idh1$ strain, the blue line represented the citrate level in the $\Delta idh2$ strain and the green line represented the citrate level in the $\Delta idh1/2$ strain.

4.3.4 ATP and NADPH level description between the wild type strain and gene disrupted strains

The detection of ATP and NADPH in the *S. cerevisiae* cells were performed according to ATP colorimetric/fluorometric assay kit and NADP⁺/NADPH quantification kit (both provided by BioVision, USA). Since the specific amounts of these components were hard to control in different culture batches, herein, the comparative ratio was used to reflect the changes between the wild type strain and the gene disrupted strains.

NADPH is the key source of reducing equivalents for fatty acids synthesis. Since the genes disrupted here encode the enzyme of IDH which is partially

responsible for NADPH production in yeast mitochondria, there are concerns about that the accumulation of citrate is at the cost of NADPH reduction. In this study, the intracellular NADPH level (Fig. 4.7) and the NADPH/NADP⁺ ratio (Table 4.2) of the wild type strain and the $\Delta idh1,2$ strains were examined. The NADPH/NADP⁺ ratio and NADPH production levels in all strains were much higher in the growth phase than that of the stationary phase. This could be regarded as a result of glucose depletion in the stationary phase which disturbed the redox balance. However, there were no significant decreases of NADPH levels in the $\Delta idh1,2$ strains compared to that in the wild type strain.

Fig. 4.7 The NADPH production levels comparison between the *S. cerevisiae* wild type strain and *idh1,2* gene disrupted strains. The cell samples were collected at the same culture time of exponential phase and stationary phase. The NADPH level of wild type strain in exponential was taken as control (100%) and others were a ration to the control. The standard deviation was calculated according to three independent experiments.

Table 4.2 NADPH/NADP⁺ ratio between the wild type strain and the gene disrupted strains

	wild type strain	$\Delta idh1$ strain	$\Delta idh2$ strain	$\Delta idh1/2$ strain
exponential phase	9.45±1.06	8.95±1.12	8.95±1.31	8.57±1.24
stationary phase	3.16±0.48	2.18±0.32	2.02±0.47	2.05±0.41

Notes: The standard deviation was calculated according to three independent experiments.

ATP is the important cellular energy transfer component which transports chemical energy within cells of cellular metabolism. Together with the acetyl-CoA and NADPH, these three comprises the substrates and cofactors required for fatty acids synthesis. Herein, the ATP level of the wild type strain and the $\Delta idh1,2$ strains were examined in both growth phase and stationary phase (Fig.4.8). At the exponential phase, the ATP level was much lower in the $\Delta idh1,2$ strains, only about 50-60% of that in the wild type strain and the reduction was significant ($p<0.05$). The result was in accordance with the situation: The blockage of TCA cycle to accumulate citrate was at the expense of ATP production to a certain extent. Once the yeast strains entered stationary phase, lots of ATP has been consumed for the cellular metabolism and its level reduced to only about 20% of that in wild type strain in the growth phase.

Fig. 4.8 The ATP production levels comparison between the *S. cerevisiae* wild type strain and *idh1,2* gene disrupted strains. The cell samples were collected at the same culture time of exponential phase and stationary phase. The ATP level of wild type strain in exponential was taken as control (100%) and others were a ration to the control. The standard deviation was calculated according to three independent experiments. The “asterisk” indicated the statistical significance of ATP reduction in the engineered strain: *, $p < 0.05$.

4.4 Discussion

The citrate metabolism is important to provide cells with biosynthesis precursors and energy. The control of citrate metabolism by modifying *idh* activity can make a greater flux from carbon source to lipid. Upon regulating enzymes in the TCA cycle, the citrate can be successfully accumulated. In this study, all of the $\Delta idh1$, $\Delta idh2$ and $\Delta idh1/2$ mutant strains showed obviously higher production levels of intracellular and extracellular citrate. The accumulated citrate was benefit to the fatty-acid biosynthetic pathways.

The source of NADPH for fatty acid synthesis in *S. cerevisiae* is mainly from:

(1) pentose-phosphate pathway (PPP), under the enzymes of glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase; (2) the reaction catalyzed by IDH; (3) the reaction catalyzed by the NADP⁺-dependent acetaldehyde dehydrogenase and (4) the reaction catalyzed by malic enzyme [117, 118]. The PPP pathway is responsible for the main source of NADPH production in the *S. cerevisiae*. Under the activity of glucose-6-phosphate dehydrogenase, 12 mol of NADPH can be produced from 1 mol of glucose-6-phosphate (G-6-P), whereas, in the TCA cycle, only 2 mol of NADPH can be produced from 1 mol of G-6-P under the activity of NADP⁺-linked dehydrogenase [119]. The little effect on NADPH production for fatty acid synthesis by the *S. cerevisiae* engineered cells here no need to be taken into consideration so much. The detection of NADPH in our study matched this point well: there were no significant NADPH changes in gene disrupted strains compared to wild type strain, which ensured the supply of NADPH cofactor for fatty acid synthesis.

However, the blockage of TCA cycle caused the reduction of ATP, which was the important energy source for the synthesis of various products. It was inevitable in the gene disrupted strains and if this problem could be successfully solved, the fatty acid accumulation pathway was expected to be more smoothly.

4.5 Section conclusion

Through the gene disruption strategies, the genes encoding the IDH were knocked out and the TCA cycle was blocked. The citrate was successfully accumulated in the *S. cerevisiae* cells. It gives hope that more important biosynthesis precursor acetyl-CoA can be obtained from the accumulated citrate. In the further study, a heterogonous ACL will be cloned and expressed into the gene disrupted strains and more fatty acids were expected to be accumulated by this modified pathway.

5. Overexpression of a heterologous ACL in the *S. cerevisiae* IDH gene disrupted strains for enhanced fatty acid synthesis

5.1 Introduction

As described in Chapter 4, the citrate successfully accumulated in the *idh1,2* disrupted strains. The accumulated citrate in the TCA cycle could be transported out to cytosol and the function of ACL is required for the production of cytosolic acetyl-CoA from citrate. Since there is no original ACL activity in the *S. cerevisiae*, a heterologous ACL gene is designed to be cloned and expressed in the *S. cerevisiae* gene disrupted strains.

The whole work flow in this study is to increase the amount of biofuel precursor by modifying the normal yeast cellular machinery. Together with the gene disruption strategy, the gene cloning and expression method will be used to introduce the enzyme of ACL to catalyze the accumulated citrate to acetyl-CoA in cytosol for fatty acid synthesis (Fig.5.1).

By modifying the metabolic pathway in the *S. cerevisiae* strain, the cellular fatty acid production levels in the engineered strains were expected to be enhanced.

Fig. 5.1 The workflow of metabolic engineering pathway in *S. cerevisiae* connecting with citrate accumulation and cytosol acetyl-CoA generation via ACL for fatty acid synthesis.

5.2 Experiment procedures

The pVTU260 empty plasmid was transformed into the *S. cerevisiae* wild type BY4741, to construct the control strain wt-empty in this study. The construction of the recombinant pVTU260 plasmid with the *acl* gene was described in Chapter 3. It was transformed into the *S. cerevisiae* wild type strain, to form the wt-*acl* strain and transformed into the $\Delta idh1$, $\Delta idh2$ and $\Delta idh1/2$ gene disrupted strains, to form the $\Delta idh1$ -*acl*, $\Delta idh2$ -*acl* and $\Delta idh1/2$ -*acl* engineered strains. The expression of *acl* in the *S. cerevisiae* engineered strains was determined by the Western blot. The anti-ACL specific antibody was used as the first monoclonal antibody. The detail procedures were conducted according to the methods in Section 2.1.

Both of the control strain and the engineered strains were cultivated in minimal glucose (YNB-URA) medium (see the detail components in Section 2.1.1) with appropriate antibiotics. The cultivation was performed according to methods described in Section 2.3. Same amounts of cells from the stationary phase of the wild type strain and engineered strains were collected and the cellular lipids were extracted according to the methods described in Section 2.4.1. The intracellular fatty acids were derived for GC-MS analysis with the same methods from Section 2.4.2 to 2.4.4. The production levels of fatty acids between the wild type strain and the engineered strains were compared.

5.3 Results

5.3.1 Cloning and expression of *acl* in the *S. cerevisiae* wild type strain and *idh1,2* disrupted strains

The cloning result of the *acl* in the *S. cerevisiae* wild type strain and $\Delta idh1,2$ strains was detected by the colony PCR method. The wild type strain with empty pVTU260 plasmid was added as control. The results showed that the *acl* encoding the ACL was successfully cloned into the *S. cerevisiae* strains. The PCR fragment size of recombinant strains colony PCR on DNA gel was about 3.3 kb and no band was observed from the control strains (Fig.5.2).

Fig. 5.2 Yeast colony PCR confirmation of the recombinant vector pVTU260-*acl* in *S. cerevisiae* wild type and $\Delta idh1,2$ strains. Lane 1: wild type with empty vector; lane 2: wild type with *acl* gene; lane 3: $\Delta idh1$ with *acl* gene; lane 4: $\Delta idh2$ with *acl* gene; lane 5: $\Delta idh1/2$ with *acl* gene.

The expression of *acl* in the *S. cerevisiae* wild type strain and $\Delta idh1,2$ strains was examined using protein samples of yeast cells collected from stationary phase in shake flasks with 50 ml YNB-URA medium supplemented with antibiotics. The expression results were confirmed by the Western blot methods. The protein size from Western blot result was about 120 kDa; similarly, there were no protein bands from the control strains (Fig.5.3). The protein concentration of each sample was analyzed by 2D Quant Kit and calculated from the BSA standard curve, same amount of protein samples was loaded on the SDS-page gel. The Western blot results reflected the fact that the expression level of *acl* was almost equal in the $\Delta idh1,2$ strains compared to the wild type strain.

These results matched the real situation that the *acl* PCR fragment from *mus musculus* (accession BC056378.1) was 3342 bp and the protein molecular weight was about 119.7 kDa. Hence, the *acl* gene was successfully cloned in the

S. cerevisiae strains and could be well expressed.

Fig. 5.3 Western blot results of *acl* in *S. cerevisiae* wild type and $\Delta idh1,2$ strains. Lane 1: wild type with empty vector; lane 2: wild type with *acl* gene; lane 3: $\Delta idh1$ with *acl* gene; lane 4: $\Delta idh2$ with *acl* gene; lane 5: $\Delta idh1/2$ with *acl* gene.

5.3.2 Growth rate description between the wild-type strain and engineered strains under SC culture medium

The expression plasmid here was the pVTU260 which contains constitutive promoter ADH1, and no need to be induced for the expression. Therefore, the culture of the engineered strains could be performed in medium without inducible reagent. However, compared with the wild type strain cultured in YNB-URA medium, the gene disrupted strains with *acl* gene grew a little slower. The growth curve and glucose consumption details were shown in Fig.5.4. The results showed that the growth rate of the $\Delta idh1/2$ -*acl* strain showed slower than that of the wild type strain. When entered into the stationary phase, all of gene disrupted strains with *acl* showed lower cell density compared to the wild type strain. The $\Delta idh1/2$ -*acl* strain reached about $OD_{600} = 8$, 10% less than that of $\Delta idh1$ -*acl* strain and $\Delta idh2$ -*acl* strain, and 30% less than that of the wt-*acl* strain. Meanwhile, the glucose consumption

situations of different strains were consisted with the growth curve. The $\Delta idh1/2-acl$ strain consumed glucose a little slower than the wild type strain and the $\Delta idh1-acl$ and $\Delta idh2-acl$ strains. After about 24 hours, the glucose was consumed out and all the cells entered into the stationary phase. Compared with the growth curve from Chapter 4, it was found that the $\Delta idh1/2$ gene disrupted strain cultured under YNB medium showed much more growth defect than cultured under YPD medium.

Fig. 5.4 Growth curve and glucose consumption curve of the wild type strain and engineered strains. The strains were grown in 250 ml flasks, with 50 ml YNBD-URA medium supplemented with appropriate antibiotics. The cultures were grown in a rotary shaker at 250 rpm, 30 °C.

5.3.3 Investigation of fatty acids production levels in the *S. cerevisiae* wild type strain and engineered strains

The designed metabolic engineered pathway of *S. cerevisiae* strains in this project aims at providing more precursor acetyl-CoA for fatty acids synthesis. Herein, GC-MS detection system was applied for metabolites analysis in yeast strains.

The total fatty acids levels from lipid were shown in Fig.5.5. Compared with the growth phase, the maximum production amount was observed in the stationary phase, which reached to 120-150 µg/ml. The strains with *acl* gene expression showed a 10%-20% increase compared to the wild type strain: 10.6% increase in *wt-acl* strain, 15.5% increase in Δidh -*acl* strain, 15.4% increase in $\Delta idh2$ -*acl* strain and 21.1% increase in increased in $\Delta idh1/2$ -*acl* strain. However, in the growth phase, no such significant increase was observed.

Fig. 5.5 Total fatty acids production levels in the wild type strain and engineered strains. The cell samples were collected at same culture time of exponential phase and stationary phase. The concentration of fatty acids was calculated from fatty acid methyl ester standard curves of GC-MS results. The standard deviation was calculated according

to three independent experiments.

In the *S. cerevisiae* strains studied here, the major detected compositions of fatty acid were Palmitic acid (C16:0), Palmitoleic acid (C16:1), Stearic acid (C18:0) and Oleic acid (C18:1) (Fig.5.6). Calculated from the standard curve, it was found that the unsaturated fatty acids C16:1 and C18:1 accounted for about 10-20% and 3%-5% individually and others were saturated fatty acids. No C16:2 and C18:2 fatty acids were detected.

Fig. 5.6 Fatty acids composition (% of total fatty acid) in the wild type strain and engineered strains. The cell samples were collected at the same culture time of stationary phase. The concentration of different fatty acid types were calculated from fatty acid methyl ester standard curves of GC-MS results. The standard deviation was calculated according to three independent experiments.

5.3.4 Comparison of fatty acids production between the *S. cerevisiae* wild type strain and engineered strains

Compared with the wild type strain with empty plasmid, the fatty acids

production levels changed in the engineered strains (Fig.5.7).

Fig. 5.7 Differential production levels of individual types of fatty acids in the wild type strain and engineered strains. The cell samples were collected at the same culture time of stationary phase. The concentration of different fatty acid types were calculated from fatty acid methyl ester standard curves of GC-MS results. The standard deviation was calculated according to three independent experiments.

The *wt-acl* strain showed higher level of saturated fatty acids: 14% increase in C16:0 and 17% increase in C18:0, but no significant changes were observed in C16:1 and C18:1. On the contrary, the gene disrupted strains with *acl* gene showed much higher level in mono-unsaturated fatty acids: For $\Delta idh1$ -*acl*, 80% increase in C16:1 and 60% increase in C18:1; for $\Delta idh2$ -*acl*, 60% increase in C16:1 and 45% increase in C18:1; for $\Delta idh1/2$ -*acl*, 92% increase in C16:1 and 77% increase in C18:1. However, no significant changes were observed in saturated fatty acids (Table 5.1).

Table 5.1 Fatty acids composition changes between the engineered *S. cerevisiae* and wild type strain

	Palmitic acid (C16:1) (fold changes)	Palmitoleic acid (C16:0) (fold changes)	Oleic acid (C18:1) (fold changes)	Stearic acid (C18:0) (fold changes)
wild type with <i>acl</i> gene	1.09±0.0090	1.14±0.0300	1.04±0.0009	1.17±0.0359*
$\Delta idh1$ strain with <i>acl</i> gene	1.80±0.2322**	1.07±0.0247	1.59±0.1116***	0.96±0.0252
$\Delta idh2$ strain with <i>acl</i> gene	1.60±0.2079**	1.03±0.0088	1.45±0.1645*	1.06±0.0159
$\Delta idh1/2$ strain with <i>acl</i> gene	1.92±0.1384**	1.04±0.0135	1.77±0.2541***	1.25±0.0941

Notes: The data of fold changes for engineered strains herein were based on the comparison results with wild type strain with empty plasmid. The fatty acid amount of wild type strain was taken as control (100%), and others are a ratio to the control. The standard deviation was calculated according to three independent experiments. The “asterisk” indicated the statistical significance of the changes of fatty acid changes between the wild type strain and engineered strain: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

One representative of the GC-MS spectra for the mono-unsaturated fatty acid C16:1 and C18:1 derived from the total ion chromatograms were showed in Fig. 5.8, which confirmed the increase of the mono-unsaturated fatty acids in the wild type strain and the engineered strains.

Fig. 5.8 The representative of GC-MS comparison spectra of the production levels of mono-unsaturated fatty acids C16:1 (A) and C18:1 (B) between the wild type strain and engineered strains. The black line represented the fatty acid production level in the wt-empty strain; the blue line and green line represented the fatty acid production level in the $\Delta idh1-acl$ and $\Delta idh2-acl$ strains respectively; the red line represented the fatty acid production level in the $\Delta idh1/2-acl$ strain.

5.4 Discussion

In this study, the citrate were accumulated, transported to cytosol and cleaved by the ACL to acetyl-CoA for fatty acids synthesis. The ACL, which exists in most oleaginous yeast cells but not in *S. cerevisiae* has been proved to have a correlation of lipid accumulation [120, 121, 47]: (1) its activity closely relates to the fatty acid synthesis rate; (2) the citrate, as the substrate for ACL, physically accumulates in yeast cytosol and then in culture media during lipogenesis; (3) the ACC activity by citrate has been proved to ensure that the acetyl-CoA generated in the ACL reaction is directly utilized for fatty acid synthesis by a “feed-forward” form. These facts give the clue to modify the *S. cerevisiae* cell in which the *idh* encoding genes were directly deleted and a heterologous ACL was expressed.

The fatty acids in various cells were detected and different engineered *S. cerevisiae* cells showed increased amounts in different fatty acid types. The Palmitic acid and Stearic acid are the main fatty acids synthesized by most yeast fatty acid synthase [122]. There was an interesting phenomenon found in the fatty acid compositions changes in this study: For the *wt-acl* strain, the increased levels of fatty acids mainly performed in terms of saturated fatty acids: C16:0 and C18:0. However, for the $\Delta idh1,2$ -*acl* strains, the increased levels of fatty acids mainly performed in terms of mono-unsaturated fatty acids: C16:1 and C18:1. The increased fatty acids in engineered strain with *acl* demonstrated the activity of ACL on fatty acid enhancement.

In the *S. cerevisiae*, the unsaturated fatty acid compositions consist almost exclusively of the mono-unsaturated fatty acids formed by the Δ -9 fatty acid desaturase, which introduces a double bond between carbons 9 and 10 of palmitoyl (C16:0)- or stearoyl (C18:0)-CoA to form palmitoleic (C16:1) or oleic (C18:1) acid. The mono-unsaturated fatty acids are essential for membrane expansion in growing cells and are major components of storage lipids in stationary phase cells [123]. So most of the additional lipids studied in this paper were speculated to be stored in additional membrane. There were a lot of researches focus on the relationship between fatty acid compositions and environmental conditions [73, 124]. It has been found that the *S. cerevisiae* cell grown in the presence of ethanol appears to increase the amount

of mono-unsaturated fatty acids in cellular lipid [124, 71]. The authors suggested that the increased proportions of mono-unsaturated fatty acids counteract the membrane-fluidizing effects of ethanol and confer ethanol tolerance by maintaining functional mitochondria during ethanol stress. Herein, the $\Delta idh1,2$ strains led to the blockage of TCA cycle; hence, the reasons for accumulated mono-unsaturated fatty acids in our study were boldly assumed: compared with the wild type strain, more pyruvate from glucose-6-phosphate did not enter into TCA cycle but were used for ethanol production, the increased ethanol was accompanied by the increased amount of mono-unsaturated fatty acids to reduce the ethanol stress on membrane-fluidizing effect. Meanwhile, the saturated fatty acids would be partially consumed for desaturation to form these mono-unsaturated fatty acids. This was why the saturated fatty acids level in $\Delta idh1,2\text{-}acl$ strains showed relative lower than that of $wt\text{-}acl$ strain. However, more evidence needs to be discovered to support this explanation and later studies on proteomic profiling analysis and metabolic profiling analysis, will be performed to understand deep metabolic mechanism of the engineered strains.

The fatty acids are important precursors for biodiesel production and one of major problems associated with the application of biodiesel is poor cold flow properties, which was indicated by parameters such as pour points and cloud points [24]. Compared with the saturated fatty acid methyl esters, the

mono-unsaturated fatty acid methyl esters have significantly lower melting points and thus, biodiesel fuels derived from fatty acids with increased mono-unsaturated fatty acids will display lower pour points and cloud points. Besides, the biodiesel fuels from the mono-unsaturated fatty compounds giving a much lower viscosity than that from saturated fatty compounds. All of these effects can improve the cold flow property of biodiesel.

5.5 Section conclusion

By the molecular cloning and expression strategy, the accumulated citrate in the gene disrupted strains described in Chapter 4 were successfully cleaved to cytosol acetyl-CoA for fatty acid synthesis. The different types of metabolic engineered yeast cells showed enhancement in different fatty acids compositions. The increased fatty acids are potential candidates for biofuel production such as biodiesel and bio-alkane. A global metabolomics and proteomic system for the engineered strains will be later established for better understanding of the metabolic mechanism, for further improvement of yield of intracellular biofuel precursors.

6. Comparative proteomics analysis of the engineered *Saccharomyces cerevisiae* with enhanced fatty acid synthesis

6.1 Introduction

In Chapter 4 and Chapter 5, the yeast *Saccharomyces cerevisiae* was metabolically modified for the enhanced fatty acid production by knocking out genes encoding the mitochondrial IDH and over-expression of a heterologous ACL. The analysis of proteomic changes between the *S. cerevisiae* wild type strain and the engineered strains could help us better understand the protein functions involved in specific metabolic pathways and the adjustment mechanism of *S. cerevisiae* cells to counteract with growth stress.

The 2D LC-MS/MS based proteomics approach provides qualitative and quantitative analysis of global proteins in microorganism cells [93]. It is a common and useful tool for the systematic research. The application of the proteomics based systematic research could provide extensive information in identifying potential protein-protein interactions, signal pathways and in turn the possible mechanism of changes in biofuel production levels [125].

The present study focuses on the comparative proteomics analysis between *S. cerevisiae* wild type strain and engineered strains with enhanced fatty acid synthesis. The iTRAQ-coupled 2D LC-MS/MS analysis approach is applied to

identify and quantify the proteins. This study not only offers a global overview of ubiquitous protein expression changes in *S. cerevisiae* modified cells but also provides meaningful information about the regulation mechanism of *S. cerevisiae* metabolic pathways.

6.2 Experiment procedures

The construction of the *S. cerevisiae* wild type strain with empty pVTU260 plasmid (wt-pvtu), $\Delta idh1/2$ strain with empty pVTU260 plasmid ($\Delta idh1/2$ -pvtu) and $\Delta idh1/2$ strain with *acl*-pVTU260 recombinant plasmid ($\Delta idh1/2$ -*acl*) were described in Chapter 5. The strains were cultured in the minimal glucose (YNB-URA) medium and kept at 30 °C with a centrifugation speed of 250 rpm.

Same amounts of the yeast cells were collected and centrifuged at 12,000 rpm for 5 min, to remove the supernatant medium. The protein extraction, digestion and labeling were carried out according to the method described in Section 2.5.1. The labeled samples were performed on the online 2D Nano-LC-MS/MS system, according to the method described in Section 2.5.2. The peptide identification was carried out by the Spectrum Mill system and they were further compared and analyzed by the UniprotKB/Swiss-Prot database under the species of *S. cerevisiae*. The analysis parameters were set according to the strategy described in Section 2.5.3.

The proteomics difference among the wt-pvtu, $\Delta idh1/2$ -pvtu, and $\Delta idh1/2$ -acl strains were analyzed and the Real-time RT-PCR was applied to partially confirm the protein expression results.

The intracellular metabolites among these strains were extracted, derived and analyzed by the GC-MS system according to the methods described in Section 2.4. Their production levels between the wild type strain and engineered strains were compared to support the proteomics results.

6.3 Results

6.3.1 Differentially expressed proteins among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains

The On-line 2D LC-MS/MS system was applied to analyze the proteome differences among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The Spectrum Mill system was used for the peptide identifications. One example of the peptide fragmentation spectrum of the protein triosephosphate isomerase was shown in Fig.6.1.

Fig. 6.1 Representative peptide fragmentation spectrum of triosephosphate isomerase: FALGQGVGVLICIGETLEEK.

Based on the analysis method, a total of more than 300 proteins were detected. The protein identification levels were based on ProtScore with an Unused protein score more than 2.0 and with a confidence value of > 95 %, whereas, the proteins with ProtScore less than 2.0 were not selected for the analysis.

Among these detected proteins, 37 differentially expressed ones were found and classified into specific categories based on their enzyme functions (Table 6.1). It was noteworthy that five proteins (PGI1, TPI1, TDH2, GPM1, PYK1) playing a role in the glycolytic pathway, and three proteins (PDC1, ADH1 and PYC2) that were responsible for pyruvate branch-point pathways were up-regulated in both the *Δidh1/2-pvtu* and *Δidh1/2-acl* strains. The proteins PDA1 and ACO1 involved in the TCA cycle showed up-regulation in both *Δidh1/2-pvtu* and *Δidh1/2-acl* strains, whereas the protein SDH1, which was not detected in *Δidh1/2-pvtu*, showed down-regulation in *Δidh1/2-acl* strain. The proteins ICL1 and MLS1 involved in glyoxylate cycle showed down-regulation in *Δidh1/2-pvtu* and *Δidh1/2-acl* strains.

Five proteins involved in stress response were also found differentially expressed in *Δidh1/2-acl* strain. These proteins included two heat shock proteins (SSA1, HSP26), one peroxiredoxin (AHP1), one glutaredoxin (GRX2) and one thioredoxin (TRX2). Compared with that in wt-pvtu strain, most of the

stress response related proteins showed more than 1.5- fold increase in *Δidh1/2-acl* strain. However, the heat shock proteins SSA1, HSP26 and peroxiredoxin AHP1 in *Δidh1/2-pvtu* strain showed no expression difference.

Three proteins (MCR1, GPD1, and AAC2) involved in respiration pathway were identified as down-regulation proteins in *Δidh1/2-pvtu* and *Δidh1/2-acl* strains. Moreover, various proteins involved in protein biosynthesis and amino acid metabolism were also found differentially expressed between wild type and engineered strains.

Table 6.1 Relative changes in protein expression between *S. cerevisiae* wild type and engineered strains

Protein	Description	No. of peptides	Average of A/C	Average of B/C
Glycolysis				
PGI1	Glucose 6-phosphate isomerase	19	1.44±0.014	1.46±0.205
TPI1	Triosephosphate isomerase	15	1.80±0.021	2.50±0.460
TDH3	Glyceraldehydes-3-phosphate dehydrogenase 3	24	1.43±0.036	1.60±0.071
GPM1	Phosphoglycerate mutase	19	1.45±0.010	1.48±0.216
PYK1	Pyruvate kinase	35	1.34±0.022	1.45±0.004
Pyruvate branch-point				
PDC1	Pyruvate decarboxylase isozyme	26	1.35±0.002	1.65±0.057
ADH1	Alcohol dehydrogenase 1	15	1.57±0.345	1.60±0.004
PYC2	Pyruvate carboxylase 2	2	1.21±0.107	1.30±0.176
TCA cycle				
PDA1	Pyruvate dehydrogenase E1 component subunit	3	1.39±0.300	1.54±0.106
ACO1	Aconitate hydratase	19	1.75±0.132	2.48±0.113
SDH1	Succinate dehydrogenase	2	--	0.55±0.190
Glyoxylate cycle				
ICL1	Isocitrate lyase	6	0.68±0.019	0.77±0.028
MLS1	Malate synthase	6	0.62±0.105	0.53±0.007

Glycogenesis					
UGP1	UTP—glucose-1-phosphate uridylyltransferase	2	1.77±0.058	1.84±0.064	
Respiration					
MCR1	NADH-cytochrome b5 reductase 2	4	0.55±0.026	0.58±0.035	
GPD1	Glycerol-3-phosphate dehydrogenase 1	3	0.43±0.019	0.27±0.011	
AAC2	ADP, ATP carrier protein 2	2	0.79±0.113	0.73±0.060	
Antioxidants					
TRX2	Thioredoxin-2	6	1.57±0.024	1.83±0.148	
GRX2	Glutaredoxin 2, mitochondrial	5	--	1.46±0.032	
AHP1	Peroxiredoxin type 2	12	1.04±0.018	1.76±0.265	
Amino-acid metabolism					
ASN1	Asparagine synthetase	5	1.99±0.488	2.86±0.672	
HOM2	Asparatate-semialdehyde dehydrogenase	3	1.28±0.045	1.33±0.138	
AAT2	Aspartate aminotransferase	2	--	1.55±0.074	
HOM6	Homoserine dehydrogenase	10	1.31±0.010	1.26±0.014	
SHM2	Serine hydroxymethyltransferase	9	0.51±0.150	0.57±0.042	
ARG1	Argininosuccinate synthase	3	--	1.84±0.251	
Protein biosynthesis					
YEF3	Elongation factor 3A	6	1.21±0.004	1.71±0.042	
RPL3	60s ribosomal protein L3	4	1.35±0.128	1.49±0.057	
RPL8B	60s ribosomal proteion L8-B	3	1.6±0.007	1.84±0.360	
RPS0A	40s ribosomal protein S0-A	3	1.12±0.031	1.52±0.219	
RPS5	40s ribosomalprotein S5	2	1.42±0.111	1.55±0.060	
Heat shock proteins					
SSA1	Heat shock protein SSA1	32	1.02±0.001	1.55±0.085	
HSP26	Heat shock protein 26	12	0.99±0.005	1.60±0.039	
Others					
MPG1	Mannose-1-phosphate guanyltransferase	3	1.43±0.104	1.54±0.173	
GPP1	(DL)-glycerol-3-phosphatase 1	6	0.99±0.011	1.36±0.095	
ADE13	Adenylosuccinate lyase	3	1.31±0.034	1.76±0.085	
INO1	Inositol-3-phosphate synthase	5	0.77±0.015	0.75±0.028	

“Average of A/C” refers to the average ratio of protein expression level in *Δidh1/2*-pvtu strain over that in wt-pvtu strain and “Average of B/C” refers to the average ratio of protein expression level in *Δidh1/2-acl* strain over that in wt-pvtu strain. Herein, each protein expression level in wt-pvtu was taken 100% and the deviation was calculated from three independently LC-MS/MS analysis results. Several proteins that were not detected in *Δidh1/2*-pvtu strain was indicated by “--”.

6.3.2 Real-time RT-PCR based mRNA levels investigation of differentially expressed proteins

The mRNA played an important role as protein precursor and its cellular level could be a reflector of protein expression result. In order to examine the mRNA levels of differentially expressed proteins from LC-MS/MS results among wt-pvtu, *Δidh1/2*-pvtu and *Δidh1/2-acl* strains, the Real-time RT-PCR procedure was performed on 9 selected proteins (Table 6.2). The *S. cerevisiae* actin (ACT1) was selected as the endogenous control. The final products length was controlled between 100-250 bp.

Table 6.2 Primer sequences for real-time RT-PCR

Protein name	Sense primer	Antisense primer	PCR product (bp)
TDH3	5'ACGATGACAAGCACAT CATC3'	5'ATGTGCTTTTGAGCAG TGTC3'	151
ACO1	5'AGCAACTGGTCGTGGT AAGA3'	5'TGACATCCAATGGCCA GTTA3'	216
TRX2	5'ATACGACAGTGCTTTAG CAT3'	5'TAGGCATGGAAGAAA CTTCA3'	194
ADH1	5'CACGAAGGTGCCGGTG TCGT3'	5'AGAACCGTCGTGGGT GTAAC3'	177
PYK1	5'TGGACGACGGGATTCTC TCT3'	5'GACTCCGAATTGCAA GTCCT3'	176
MLS1	5'CCAAAGATGGAGCACC ACTT3'	5'AGTCCCAACGTCCGCA ATTC3'	196
SHM2	5'TGTTTACTCCGCTATCA TGA3'	5'TTGGCCAATACTTGCA AGTT3'	198
GPD1	5'AACATTGCCACCGAAG TCGC3'	5'CAACAACGTTCTTCAA AGCA3'	199
SDH1	5'CTTTGCCCTCGATCTGT TGA3'	5'ATGCTCTACCATAGCC ACCA3'	131
ACT1	5'AACTTTCAACGTTCCAG CCT3'	5'CCACGTTCACTCAAGA TCTT3'	216

In each strain, the total mRNA was extracted from three independent culture batches of cells and used as templates at the same concentration. The mRNA levels of proteins in wt-pvtu strain were taken as controls (100%), and the mRNA levels of the same proteins in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains were being ratio to that in the wt-pvtu strain.

The results were shown in Fig.6.2. The results showed that for most of the proteins, the changing trends exhibited in the mRNA levels were consistent with the changing trends in protein expression levels. In both of the $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains, the mRNA levels of TDH3, ACO1, TRX2 and PYK1 showed similar up-regulation and the mRNA levels of SHM2, GPD1, SDH1 showed similar down-regulation as protein levels from the LC-MS/MS result. However, the changing trends which showed inconsistency between Real-time RT-PCR and LC-MS/MS result were found in proteins MLS1 and ADH1. For MLS1, it showed up-regulation in the mRNA level, but was demonstrated as down-regulation protein, whereas for ADH1, it showed down-regulation in the mRNA level, but was demonstrated as up-regulation protein in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. Such kind of variances could be explained by the phenomenon of post-translational modification in eukaryotic cells, such as acetylation, amidation, methylation and phosphorylation.

Fig. 6.2 Real-time RT-PCR analysis of mRNA levels of selected differentially expressed proteins among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The mRNA levels in wt-pvtu strain were taken as controls (100%) and mRNA levels in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains were being ratio to that in the wt-pvtu strain. The standard deviation was calculated according to three independent experiments.

6.3.3 Intracellular metabolites production levels among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains

The GC-MS analysis system was performed for intracellular metabolites identification and quantification among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. A number of metabolites were identified from the NIST library and SHIMADZU GC/MS Metabolite Mass Spectra Database. Herein, the production levels of metabolites in wt-pvtu strain were taken as controls, and the production levels of the same components in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains were calculated accordingly.

Among the detected metabolites, various amino acids were found differentially produced between *S. cerevisiae* wild type and engineered strains. These amino acids included L-valine, L-proline, L-lysine, L-glutamine, Asparagine, Aspartic acid and L-tyrosine (Fig.6.3). Except for the amino acid Aspartic acid, others showed down-regulation in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains.

Fig. 6.3 Differential production levels of intracellular amino acids among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The amino acid levels in wt-pvtu strain were taken as controls and the amino acid levels in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains were calculated accordingly. The standard deviation was calculated according to three independent experiments.

Moreover, the metabolites lactic acid, acetic acid, d-mannose showed up-regulation in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strain, whereas, the metabolites myo-inositol and malic acid showed down-regulation in both of these two engineered strains (Fig.6.4).

Fig. 6.4 Differential production levels of intracellular metabolites among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The metabolites production levels in wt-pvtu strain were taken as controls and the metabolites production levels in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains were calculated accordingly. The standard deviation was calculated according to three independent experiments.

6.4 Discussion

The LC-MS/MS approach enabled the analysis of relative protein expression levels among the wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The metabolic engineering of *S. cerevisiae* resulted in significant changes in protein levels that were involved in crucial biological pathways (Fig.6.5).

Fig. 6.5 Schematic diagram of metabolic pathway changes in the *S. cerevisiae* engineered strains. The engineered pathway in yeast with enhanced fatty acid synthesis was indicated by red dashed box. Both of the up-regulated and down regulated metabolic pathways in $\Delta idh1/2$ -*acl* strain were indicated by the different colored arrows. The proteins involved in other pathways that haven't been detected from LC-MS/MS analysis were not shown in this diagram.

During the fermentative growth of *S. cerevisiae*, the glycolytic pathway plays an important role in energy and intermediates provision [126]. The results from Table 6.1 showed that the expression levels of five proteins (PGI1, TPI1, TDH3, GPM1 and PYK1) involved in glycolytic pathway were significantly increased in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -*acl* strains. These proteins were responsible for converting glucose to pyruvate. Moreover, the PDC1 and ADH1 were two crucial enzymes involved in pyruvate-ethanol metabolic pathway and the PYC2 was involved in pyruvate-oxaloacetate metabolic pathway. Their expression levels also showed up-regulation in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -*acl* strains. It could be explained that since the genes *idh1* and *idh2* were knocked out, the

TCA cycle was definitely blocked. As a result, during its growth process, the $\Delta idh1/2$ strain would have preferred the fermentation process to the respiration process. Parts of the glucose were shifted from the TCA cycle to alcoholic fermentation and other by-pass pyruvate metabolic process. The study here was consistent with the previous report that during alcoholic fermentation process, enzymes involved in the glycolytic pathway were much more efficiently expressed in the yeast cells [127, 128]. Besides, the higher amounts of acetic acid and lactic acid found in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains indirectly verified this phenomenon. The glycolysis was a critical energy pathway and the enhanced glycolytic pathway in $\Delta idh1/2$ could provide energy compensation for the energy loss from the TCA cycle [129]. The comparison of protein expression levels between *S. cerevisiae* wild type and engineered strains provided useful information to regulate the sugar metabolism in the yeast cells.

Associated with the blockage of the TCA cycle, the proteins involved in the respiration pathway were found down-regulated in the $\Delta idh1/2$ -pvtu strain and $\Delta idh1/2$ -acl strain. The expression levels of MCR1, which was involved in the electron transfer chain and GPD1, a major contributor of electrons to the electron transport chain in mitochondria showed to be much lower in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl. The expression level of AAC2, which was taken as ADP, ATP carrier protein, also decreased in $\Delta idh1/2$ strains. The result was consistent with previous result that ATP level was much lower in $\Delta idh1/2$

strains than that in wild type strain. These protein expression differences reflected the energy loss problem due to the blockage of the TCA cycle in *Δidh1/2* strains. Since the fatty acid biosynthesis was an ATP-dependent process and restricted to the conditions of high energy load of the yeast cells, the biofuel precursor production was expected to be enhanced much more if the energy loss problem could be solved in the *Δidh1/2-acl* strain.

Interestingly, influenced by the modified pathways in *S. cerevisiae*, the proteins involved in glyoxylate cycle showed expression decrease in *Δidh1/2-pvtu* and *Δidh1/2-acl* strains. The ICL1 and MLS1 were two major enzymes involved in glyoxylate cycle and both of these two enzymes could be depressed by glucose [130]. There were research works which focused on coordinate regulation of the TCA cycle and glyoxylate cycle. The studies revealed that the enzymes in the TCA cycle had considerable effects on enzymes involved in glyoxylate pathways, regardless of their intracellular localization [131, 132]. The enzymes ACO1, *IDH1*, and FUM1 involved in the TCA cycle were reported to be essential for acetate utilization and deleting one of these enzymes caused significant defect of the glyoxylate cycle in *S. cerevisiae*. This was again consistent with our results. Besides, the intracellular metabolites analysis results showed that the production level of malic acid was much lower in both of *Δidh1/2-pvtu* and *Δidh1/2-acl* strains. The malic acid could be produced from both the TCA cycle and glyoxylate pathway. Herein,

the decreased amount of malic acid in $\Delta idh1/2$ cells was ideally correlated to the blockage of the TCA cycle and glyoxylate cycle.

A number of proteins related to stress response were up-regulated in $\Delta idh1/2$ -*acl* strain. These proteins included two heat shock proteins, one peroxiredoxin (AHP1), one glutaredoxin (GRX2) and one thioredoxin (TRX2). The accumulation of biofuels such as fatty acids-derived bioaldehyde, bioalkane or bioalcohols could generate solvent stress to yeast cells [133]. They could interfere with membrane protein function and result in an increased membrane permeability and membrane fluidity while diminishing the energy transduction [134, 135]. The solvent stress from short-chain alcohols has been reported to have many parallels with heat shock in microorganisms [136] and one efficient way to solve the problem was to increase the expression levels of heat shock proteins. The up-regulation of heat shock proteins in $\Delta idh1/2$ -*acl* was a response to such a kind of solve stress. The proteomic results matched the previous explanation of why mono-unsaturated fatty acids accumulated in $\Delta idh1/2$ -*acl* strain: the shift of pyruvate metabolism from respiration to alcoholic fermentation resulted in increased production of ethanol and fatty acids, and this could generate solvent stress on *S. cerevisiae* cells. The mono-unsaturated fatty acids were essential components for membrane expansion in yeast cells [71]. The accumulation of mono-unsaturated fatty acids and up-regulation of heat shock proteins in $\Delta idh1/2$ -*acl* could be taken as an

adjustment mechanism to counteract the membrane-fluidizing effects caused by solvent stress.

The thioredoxin TRX2 was involved in many cellular processes, including oxidatively damaged proteins repair, protein folding, sulfur metabolism and redox homeostasis [137]. The peroxiredoxin AHP1 was identified as a key peroxidase involved in osmotic stress resistance and detoxification [138] and the glutaredoxin GRX2 was involved in reducing cytosolic protein- and non-protein-disulfides [139]. The up-regulation of these antioxidants in *Δidh1/2-acl* could be beneficial for *S. cerevisiae* to counteract with the organic solvent stress, oxidative stress and repair oxidatively damaged proteins caused by the modified pathway.

The amino acid metabolism was important for synthesis of unique proteins, peptides and other nitrogen-containing substances. They could be indirectly metabolized to release energy for the biological process [140]. The possible reason of difference in amino acids production level in *Δidh1/2-pvtu* and *Δidh1/2-acl* strains was to keep them in balance in intracellular metabolism pathways. In addition, the protein GPP1 in yeast cells played an important role in osmo-adaptation [141]. The up-regulation of GPP1 in *Δidh1/2-acl* but not in *Δidh1/2-pvtu* could be due to the fact that the fatty acids accumulated in *Δidh1/2-acl* strain but not in *Δidh1/2-pvtu* strain. The MPG1 was involved in

cell wall synthesis and it catalyzed alpha-D-mannose 1-phosphate to generate GDP-mannose [142]. The production level of D-mannose was higher in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains, which was consistent with the increased protein expression level of MPG1. The protein INO1 was involved in myo-inositol biosynthesis process and the myo-inositol served as an important component in structural lipids synthesis in yeast [143]. The protein level of INO1 was down-regulated in $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strain and this was consistent with the result of lower production level in cellular myo-inositol. The mechanism details of such expression and production changes in *S. cerevisiae* engineered strains however need to be further investigated.

6.5 Section conclusion

The comparative proteomics identification and quantification was performed among wt-pvtu, $\Delta idh1/2$ -pvtu and $\Delta idh1/2$ -acl strains. The proteins involved in various metabolic pathways were found to be differentially expressed and their functions were analyzed. The LC-MS/MS based proteomics analysis, together with GC-MS based metabolites analysis, not only offered an overview of ubiquitous cellular protein expression changes in *S. cerevisiae* engineered strains, but also provided a meaningful information platform which was beneficial for further modification of *S. cerevisiae* cells in terms of improving biofuel production.

7. Enhanced production of fatty acid derivatives in engineered *Saccharomyces cerevisiae*

7.1 Introduction

In eukaryotic cells, most of fatty acids are stored in the forms of cellular lipid components such as triacylglycerols (TAGs) and sterol esters, and they are required for the membrane biosynthesis [44, 59]. However, the TAGs cannot be directly derived to most of fatty acid derivatives. The conversions of fatty acids to the corresponding chemicals in microbes are always initiated from their activated forms: fatty acyl-CoAs or fatty acyl-ACP [144, 145]. Thus, to increase the cellular levels of these activated intermediates offers a promising strategy for the microbial production of fatty acid derivatives.

The metabolism of neutral lipid in yeast has been reported to play a central role in regulating the fatty acyl-CoAs [146, 37]. In *S. cerevisiae*, the acyl-CoA: diacylglycerol acyltransferase (DAGAT), encoded by *dgal*, has been demonstrated to be one of the essential enzymes in TAGs synthesis [147, 59]. It is responsible for the terminal step of TAGs formation by condensation of acyl-CoAs and diacylglycerol. Since the TAGs synthesis by DAGAT is acyl-CoA-dependent, the cellular acyl-CoAs production levels are expected to be altered through the regulation of DAGAT activity. The product of *S. cerevisiae* FAS is already in the activated form of fatty acyl-CoAs, therefore a modified pathway to reduce the turnover of acyl-CoAs to lipid becomes an

attractive approach to provide increased amount of donors for producing the fatty acid derivatives.

A series of enzymes have been found to have the ability to convert the fatty acyl-CoAs to useful chemicals, such as fatty aldehydes, fatty alcohols and further to alkanes/alkenes [22, 148, 149]. The fatty acyl-CoA reductase (FAR) performs as one of the most studied enzymes. It can be divided into aldehyde-forming and alcohol-forming classes [148]. The aldehyde-forming FAR catalyzes a two-electron reduction of the active forms of fatty acids to fatty aldehydes, while the alcohol-forming FAR catalyzes a four-electron reduction of the active forms of fatty acids to fatty alcohols.

The strategy herein aims to supply sufficient activated forms of fatty acids for enhanced production of fatty acid derivatives. The *dgal* gene responsible for the rate limiting step of fatty acyl-CoA dependent pathway of TAGs synthesis is going to be deleted, to release more fatty acyl-CoAs from TAGs. The FAR gene will be cloned and overexpressed in the gene disrupted strain, to convert the accumulated fatty acyl-CoAs to the corresponding fatty acid derivatives (Fig.7.1). The optimization in culture medium will be carried out in the engineered strain for the further enhancement of fatty acid derivatives production.

Fig.7.1 The workflow of the metabolic engineering pathway in the *S. cerevisiae* for enhanced fatty acid derivatives production. FAS: fatty acid synthase; GPAT: glycerol-3-phosphate acyltransferase; LPAT: lysophosphatidate acyltransferase; PAP: phosphatidate phosphatase; DGAT: diglyceride acyltransferase; FAR: fatty acid reductase. The designed pathway was indicated by red colour.

7.2 Experiment procedures

The disruption of *dgal* gene, which encoded the acyl-CoA: diacylglycerol acyltransferase was carried out according to the methods described in Section 2.2. The primers designed for the generation of disruption cassette were as following:

5'ATACATAAGGAAACGCAGAGGCATACAGTTTGAACAGTCACATAAC
AGCTGAAGCTTCGTACGC3' for the forward strand and
 5'CCTTATTTATTCTAACATATTTTGTGTTTTCCAATGAATTCATTAGGCAT

AGGCCACTAGTGGATCTG3' for the reverse strand. The *dga1* disruption cassette containing the HIS⁺ selection marker was then transformed into the *S. cerevisiae* wild type strain, to generate the $\Delta dga1$ strain. The pYES-DEST52 expression vector contain the FAR gene was transformed into the *S. cerevisiae* wild type strain, to construct the “wt-FAR” and into the Δdga disrupted strain to construct the “ Δdga -FAR”. Meanwhile, the empty pYES vector was transformed into the *S. cerevisiae* wild type strain to construct the “wt-E” and into the Δdga disrupted strain to construct the “ Δdga -E”.

All the recombinant strains were cultivated in the in the minimal selective medium with ura⁺ marker (0.77 g/l) and induced by 2% galactose at 30 °C, 250 rpm. The cellular metabolites and lipids were extracted according to methods described in Section 2.4.1. The derivatization of fatty alcohols was carried out according to the methods for the soluble metabolites derivatization, described in Section 2.4.2 and the derivatization of fatty acids were carried according to the method described in Section 2.4.3. The derived samples were then injected into the GC-MS system for the analysis with the same methods in Section 2.4.4.

7.3 Results

7.3.1 Growth rate description

Through the gene disruption and overexpression strategies, the wild type strain with overexpressed FAR gene and $\Delta dga1$ strain with FAR were obtained.

The optical density value at wavelength of 600 nm (OD_{600}) was used to determine the cell concentration. The FAR gene was inserted into the pYES vector under the GAL1 promoter and cultivated under the minimal ura^+ selective medium containing 1% raffinose as carbon source and 2% galactose as inducer. The growth curves of all the four strains wt-E, wt-FAR, Δdga -E and Δdga -FAR were reflected in Fig.7.2. The results showed that there was no significant growth difference between the wild type strain and the engineered strains. All of the strains entered into stationary phase after about 40 h and the gene disruption strain exhibited a slight higher cell concentration in the stationary phase than the wild type strain.

Fig. 7.2 Growth curves of the *S. cerevisiae* wild type strain and engineered strains. The strains were grown in 250 ml flasks, with 50 ml YNB medium supplemented with raffinose and galactose as carbon source. The value of OD_{600} was taken to characterize the cell concentration.

7.3.2 Effect of the engineered pathway on the lipid content

The deletion of the *dgal* gene blocked the TAGs synthesis pathway in the *S. cerevisiae* and the TAGs content was supposed to be reduced. Herein, the lipid contents in the wild type strain and the gene disrupted strain were investigated. When the strains were performed under culture medium with raffinose and galactose as carbon source, the mainly detected fatty acids included in the lipids were myristic acid (C14:0), palmitoleic acid (C16:1), palmitic acid (C16:0), oleic acid (C18:1) and stearic acid (C18:0). The palmitoleic acid was found in the maximum yield in both of the wild type strain and gene disrupted strain, represented about 60% of the total fatty acids. The production of palmitic acid, oleic acid and stearic acid accounted for about 10-15% of the total fatty acids, respectively and the myristic acid constituted only 1-2% of that (Table 7.1).

Table 7.1 Fatty acid compositions included in the lipid content in the wild type strain and *dgal* gene disrupted strain

Strain type	Total fatty acid	Compositions				
	(mg/L)	C14-0	C16-1	C16-0	C18-1	C18-0
wt	116	1.3%	61.2%	11.7%	13.0%	12.8%
<i>Δdgal</i>	77	1.7%	58.9%	12.1%	11.0%	16.3%

The lipid contents in the *Δdga* strain were dramatically decreased compared with that in the wild type strain (Fig.7.3). The total fatty acids reduced from 116 mg/L to 77 mg/L ($p<0.01$). Among the fatty acid compositions, the production

level of myristic acid decreased to 87% of that in the wild type strain, from 1.5 mg/L to 1.3 mg/L ($p<0.05$); the palmitoleic acid decreased to 64%, from 71 mg/L to 45 mg/L ($p<0.005$); the palmitic acid decreased to 68%, from 13.5 mg/L to 9.3 mg/L ($p<0.05$); the oleic acid decreased to 56%, from 15.0 mg/L to 8.0 mg/L ($p<0.005$); and the stearic acid decreased to 85%, from 14.8 mg/L to 12.6 mg/L ($p<0.05$). The obtained results reflected that the disruption of *dga1* gene resulted in the reduction of lipid contents, which was regarded as a consequence of the blocked TAGs synthesis pathway depending on the fatty acyl-CoAs as substrates. The gene disrupted strain was then used for the enhanced production of fatty acid derivatives, in which the fatty acyl-CoA reductase was overexpressed.

Fig. 7.3 Fatty acid production levels between the wild type strain and the *dga1* gene disrupted strain. The standard deviation was calculated from three independent experimental results. The “asterisk” indicated the statistical significance of the fatty acid changes between the wild type strain and the gene disrupted strain: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

7.3.3 Enhanced production of intracellular fatty acid derivatives in the engineered *S. cerevisiae*

The designed metabolic pathway in the *S. cerevisiae* herein aimed to convert the activated form of fatty acid into fatty acid derivatives. By overexpressing the FAR gene in the *S. cerevisiae* strains, the fatty alcohols were observed to be produced (Fig.7.4), while no fatty aldehydes were detected. In both of the wt-FAR and $\Delta dga1$ -FAR strains, the detected fatty alcohols in the cells were myristyl alcohol (C14:0), cetyl alcohol (C16:0), palmitoleyl alcohol (C16:1) and stearyl alcohol (C18:0).

Fig.7.4 Intracellular fatty alcohol production levels between the wt-FAR strain and the $\Delta dga1$ -FAR strain. The standard deviation was calculated from three independent experimental results. The “asterisk” indicated the statistical significance of the fatty alcohols changes between the wt-FAR strain and the $\Delta dga1$ -FAR strain: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

The cetyl alcohol was found in the maximum amount, represented more than 80% of the total fatty alcohols and the stearyl alcohol represented about 10% of the total fatty alcohols. The myristyl alcohol and palmitoleyl alcohol only accounted for a small percentage, about 2-3% and 0.4%, respectively (Table 7.2). The wt-E and Δdga -E strains did not produce any detectable levels of fatty alcohols.

Table 7.2 Fatty alcohol compositions in the wt-FAR strain and $\Delta dga1$ -FAR strain

	Strain type	Total fatty alcohols (mg/L)	Compositions			
			C14-0	C16-1	C16-0	C18-0
intracellular	wt-FAR	26	2.0%	0.4%	85.7%	11.9%
	$\Delta dga1$ -FAR	45	3.4%	0.4%	84.7%	11.5%
extracellular	wt-FAR	2.2	-	-	78.7%	21.3%
	$\Delta dga1$ -FAR	4.3	-	-	79.5%	20.5%

Due to the blockage of fatty acyl-CoAs dependent TAGs synthesis pathway, the fatty alcohols produced in the Δdga -FAR cells increased drastically (Table 7.3). The total fatty alcohols increased to about 1.7 times of that in wt-FAR ($p<0.05$) and the maximum production level reached 45 mg/L, compared with 26 mg/L in the wt-FAR strain. The production level of myristyl alcohol was increased to 3 times ($p<0.005$), from 0.5 mg/L to 1.5 mg/L; the palmitoleyl alcohol yield was increased to about 2 times ($p<0.05$), from 0.1 mg/L to 0.2 mg/L; while the cetyl alcohol and stearyl alcohol production levels were both

increased to about 1.7 times, from 22 mg/L to 38 mg/L ($p<0.005$) and 3.0 mg/L to 5.1 mg/L ($p<0.01$) respectively. The results were consistent with the designed pathway for the enhanced production of fatty acid derivatives, in which a more direct strategy was applied to reduce the turnover from fatty acyl-CoAs to TAGs and then convert the accumulated cellular fatty acyl-CoAs straight to fatty alcohols by the converting enzyme FAR.

Table 7.3 Fold changes of fatty alcohol production levels in the $\Delta dga1$ -FAR strain compared with that in the wt-FAR strain

	Compositions				
	C14-0	16-1	C16-0	C18-0	total
intracellular	3.0±0.214***	2±0.201*	1.7±0.096***	1.7±0.109**	1.74±0.066*
extracellular	-	-	1.9±0.253***	1.8±0.388*	1.90±0.098*

Notes: The fatty alcohol production levels in the wt-FAR strain were taken as controls (100%), and the production levels in the $\Delta dga1$ -FAR strain were being ratio to that in the wt-FAR strain. The standard deviation was calculated according to three independent experiments. The “asterisk” indicated the statistical significance of the changes of fatty alcohol yield between the wt-FAR strain and $\Delta dga1$ -FAR strain: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

7.3.4 Investigation of fatty alcohols secretion in the engineered *S. cerevisiae*

The fatty alcohols produced in the engineered *S. cerevisiae* were supposed to be partially exported into the medium. The culture broth collected from the stationary was extracted by the ethyl acetate. The obtained fatty acid derivatives were derived by the same BSTFA+ TMCS method and analyzed by the GC-MS. The results showed that the fatty alcohol contents secreted in the culture medium was much less than that in the cells. The total fatty alcohols yield was

only 10% of the intracellular ones (Fig.7.5). The detectable fatty alcohols were cetyl alcohol (C16:0) and stearyl alcohol (C18:0). The cetyl alcohol represented about 80% of the total fatty alcohols in the supernatant and the stearyl alcohol represented about 20% (Table 7.2).

Fig.7.5 Extracellular fatty alcohol production levels between the wt-FAR strain and the $\Delta dga1$ -FAR strain. The standard deviation was calculated from three independent experimental results. The “asterisk” indicated the statistical significance of the fatty alcohols changes between the wt-FAR strain and the $\Delta dga1$ -FAR strain: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Similar to the comparison results of the intracellular fatty alcohols production between the wt-FAR strain and Δdga -FAR strain, the yield of extracellular fatty alcohols in the Δdga -FAR strain also increased significantly. The production of the extracellular cetyl alcohol in the Δdga -FAR strain increased to 1.9 times compared with that in the wt-FAR strain ($p < 0.005$), from 1.8 mg/L to 3.4 mg/L;

while the production of the extracellular steary alcohol by the Δdga -FAR strain increased to 1.8 times ($p<0.05$), from 0.5 mg/L to 0.9 mg/L (Table 7.3). The results reflected that the yields of both the intracellular and extracellular fatty alcohols were improved by applying the engineered pathway in the *S. cerevisiae*.

One representative of the GC-MS spectra for the fatty alcohols C14:0, C16:0 and C18:0 derived from the total ion chromatograms were showed in Fig.7.6, which confirmed the increase of the fatty alcohols in the engineered strains.

Fig. 7.6 The representative of GC-MS comparison spectra of the production levels of fatty alcohols C14:0 (A), C16:0 (B) and C18:0 (C) between the wild type strain and engineered strains. The red line represented the fatty alcohol production level in the wild type strain with empty vector (wt-pyes); the black line represented the fatty alcohol production level in the wild type strain with FAR gene (wt-TaFar) and the blue line represented the fatty alcohol production level in the *dga1* gene disrupted strain with FAR gene (dga-TaFar).

7.4 Discussion

The TAGs are the most important storage forms for energy and fatty acids in the yeast cells, and they are always regarded as potential fuel targets [151]. In the last few decades, a lot of research work focused on the investigation of TAGs synthesis in microorganisms. Three different pathways were discovered to contribute to the TAGs biosynthesis in *S. cerevisiae*: The acyl-CoA: diacylglycerol acyltransferase encoded by *dga1* gene was responsible for the rate-limiting step of TAGs formation by condensation of acyl-CoAs and diacylglycerol [59, 152]; the phospholipid: diacylglycerol acyltransferase encoded by the *lro1* gene took part in the TAGs synthesis from the conversion

of phospholipid and diacylglycerol [152, 153]. Besides, the TAGs could be formed using free fatty acids as substrates and diacylglycerol as acceptor directly [59, 154]. Among these three pathways, the *dgal* and *lro1* played a predominant role in the synthesis of total cellular TAGs and the *dgal*-regulated pathway was the only one that directly depends on the fatty acyl-CoAs as substrates.

While the TAGs are valuable, they cannot be used as fuels or for other applications directly. The conversion of TAGs to fatty acid derived aldehydes, alcohols or alkanes/alkenes suffers from a practical standpoint. In the yeast cells, the more activated starting materials derived from fatty acids are fatty acyl-CoAs. The fatty acyl-CoAs released from the FAS complex are either used for the synthesis of steryl ester by the enzyme of acyl-CoA: sterol acyltransferase, which is encoded by the *are1* and *are 2* genes [155, 156]; or destined for the TAGs storage pathway. In this study, the fatty acyl-CoA dependent pathway of TAGs synthesis was modified to be blocked. The metabolic engineering of the conversion process from fatty acyl-CoAs to lipids has been demonstrated as one of the efficient means to accumulate precursors for fatty acid derivatives synthesis. The deletion of *dgal* resulted in the distinct decrease of total fatty acids included in the lipid content, which demonstrated the blockage of the TAGs synthesis pathway depending on the fatty acyl-CoAs. The turnover of fatty acyl-CoAs to the lipid was therefore regarded to be

reduced. It was verified by the increased production levels of fatty alcohols in the engineered strains with disrupted *dgal* and overexpressed FAR. Compared with that in wt-FAR strain, the fatty alcohols yield in the $\Delta dgal$ -FAR strain increased to more than 1.5 times, reflected in both the intracellular contents and extracellular contents.

The FAR activity herein exhibited in the alcohol-forming type and no fatty aldehydes were detected. The increased production levels were found in the long-chain fatty alcohols (C14-C18) and the saturated ones predominated in the total fatty alcohols. This was determined by the released types of fatty acyl-CoAs (C14-C18) in the *S. cerevisiae*. Various converting enzymes have been reported to have the ability to convert fatty acyl-CoAs to the corresponding fuels in the *S. cerevisiae* cells: The FAEEs (fatty acid ethyl esters) could be produced by the acyl-CoA: alcohol transferase, which uses fatty acyl-CoAs and ethanol as substrates [151,157]; the fatty aldehydes could be generated by the aldehyde-forming FAR [149] and it could be further transformed to alkanes/alkenes by the aldehyde decarbonylase [22]. These kinds of tool-enzymes make the production of valuable chemicals from fatty acids go on wheels. The metabolic engineering of the TAGs synthesis pathway studied here is expected to play a crucial role in providing precursors for all of these bio-reactions, to realize the idea of enhanced production of fatty acid derived chemicals.

7.5 Section conclusion

In conclusion, this study not only successfully improved the production of fatty alcohols in the *S. cerevisiae* cells, which are used in a wide range of products and demanded by an increasing large market, but also provided a more direct strategy to transform the fatty acids included in the lipid into useful fuels and chemicals, to solve the bottleneck of the utilization of TAGs as precursors.

8. Conclusions and future direction

Overall, the whole workflow of this research work was designed and carried out as described in Fig.8.1

Fig.8.1 Overall workflow of the research project and metabolic engineering pathways in *S. cerevisiae* for enhanced production of fatty acid and fatty acid derivatives.

The existed fatty acid accumulation mechanism under nitrogen limited culture condition found in the oleaginous yeasts revealed the specific relationships between the citrate accumulation and fatty acid enhancement. Based on the information of the ACL function in the oleaginous yeasts, the *S. cerevisiae* was transformed with a heterologous *acl* and its fatty acid production

under certain culture conditions was studied. After confirming that the expression of *acl* could lead to the enhancement of fatty acid production based on the accumulated citrate, the genes involved in the citrate turnover pathway were directly knocked out. The *acl* was further expressed in the gene disrupted strains and through this strategy, the fatty acid production levels was successfully increased in the engineered strains. The accumulated fatty acids are then going to be regulated for the fatty acid derivatives production.

1) Toward a better understanding of the fatty acid accumulation in the engineered *S. cerevisiae*

The fatty acid production levels and compositions in yeast strains differ from each other. Different enzyme functions and pathways have been distinguished between the oleaginous and non-oleaginous yeasts. Based on the knowledge of fatty acid accumulation in the oleaginous yeasts, the *S. cerevisiae* was targeted to be modified towards the enhanced fatty acid production.

In this study, the *S. cerevisiae* was expressed with a heterologous ACL and cultured under different culture conditions. Similar to the oleaginous yeasts, the engineered *S. cerevisiae* was found to accumulate more fatty acids, while the wild type *S. cerevisiae* was found to accumulate much more intracellular citrate when performed under nitrogen limited condition. It revealed that the increased citrate level played a crucial role in the fatty acid synthesis in the engineered

strain.

A similar fatty acid accumulation mechanism found in oleaginous yeasts was applied in the engineered *S. cerevisiae*. It gave a better understanding of the relationship between the citrate accumulation and fatty acid enhancement in the yeast cells, in which the ACL activity was responsible for the conversion of citrate into acetyl-CoA, to provide enough substrate for the fatty acids synthesis.

2) Toward the metabolic design of *S. cerevisiae* pathways for enhanced fatty acids production

Based on the understanding of fatty acid accumulation condition in the yeast cells, especially the well-known relationship between the increase of the citrate level and the enhancement of the fatty acid production, the *S. cerevisiae* was easily designed and modified to produce the biofuel precursors.

The gene disruption strategy and gene cloning and expression strategy were combined to successfully re-construct the *S. cerevisiae* pathways: the disruption of the *idh1,2* genes in the TCA cycle aimed to the accumulation of intracellular citrate and the expression of *acl* in the *idh1,2* gene disrupted strain aimed to convert the citrate into acetyl-CoA for fatty acid synthesis. Within our expectation, the final production of the fatty acids, especially in terms of the

mono-unsaturated ones, increased to a much higher level. The increased mono-unsaturated fatty acids could better serve the biofuel production and improve its qualities, such as to ameliorate the cold flow properties.

3) The application of metabolomics and proteomics in the pathways analysis

The proteomics by the iTRAQ-coupled 2D LC-MS/MS method was well applied in the analysis of protein relationships and their changes in specific pathways in the *S. cerevisiae* cells.

The up-regulated proteins involved in the glycolytic and pyruvate branch-point pathways in the *idh1,2* gene disrupted strain reflected the glucose utilization shift and the energy compensation result; the down-regulated proteins involved in the respiration and glyoxlate pathways were found to be consistent with the blockage of the TCA cycle. Besides, the up-regulated proteins involved in the stress response in the engineered strains provided a better understanding of the adjustment mechanism of the *S. cerevisiae* cells to counteract with the growth stress generated from the modified pathways.

The metabolomics results reflected the complementary information to the proteomics data. Together with the proteomics analysis, the application of these two strategies in the different yeast cells offered an important information

platform for the discovery of new protein functions and analysis of specific metabolic pathways after the cell modification.

4) Engineering the *S. cerevisiae* for enhanced production of fatty acid derivatives

In the yeast cells, the main storage forms of fatty acids are TAGs. However, the conversion of TAGs into fatty acid derivatives suffers from a practical standpoint. The study herein provided an effective strategy to convert the fatty acid into the corresponding fuels. The TAGs synthesis pathway was blocked by deleting the *dgal* gene and the accumulated fatty acyl-CoAs were converted to fatty acid derivatives by expressing the fatty acyl-CoA reductase gene in the gene disrupted strain. The metabolic engineered pathway resulted in the enhanced production of fatty alcohols. It showed a successful example to manipulate the fatty acid metabolic process towards the synthesis of valuable chemicals.

8.2 Future direction

1) Effectively convert accumulated fatty acids to useful fuels and chemicals in *S. cerevisiae*

The accumulated fatty acids from our results could be used as precursors for biofuel production. However, the *S. cerevisiae* itself cannot efficiently transform the fatty acids to useful fuels. The strategies to produce biofuels from

these precursors need to be carried out in the *S. cerevisiae* cells.

Inspired from our research work, the destination of fatty acids in the yeast cells can be regulated. The designed pathway to turn the TAGs storage forms to the fatty acyl-CoAs activated forms will be combined with the enhanced fatty acid production pathways, to efficiently convert the fatty acids into the fatty alcohols. In the future work, other enzymes involved in the TAGs biosynthesis will be co-deleted to further increase the fatty acyl-CoAs levels and various converting enzymes will be expressed in the engineered strain, to produce series fatty acid derivatives. These enzymes include acyl-CoA: alcohol transferase, which uses fatty acyl-CoAs and ethanol as substrates to generate FAEEs, aldehyde-forming FAR responsible for the production of fatty aldehydes and aldehyde decarbonylase responsible for the alkane/alkene biosynthesis from fatty aldehydes.

Besides, the carboxylic acid reductase has been discovered as a versatile enzyme, which can directly convert the fatty acids into the corresponding fatty aldehydes [158]. Together with the enzymes of aldehyde reductase or aldehyde decarbonylase, the intermediates can be finally converted to fatty alcohols or alkanes. Such kinds of enzyme systems are powerful and they will be constructed in the yeast cells to serve the biofuel production field.

2) Overcoming the problems of ATP, NADPH deficiency resulted from the engineered pathway

The blockage of the TCA cycle in this research work resulted in about 40% decrease of intracellular ATP level during the growth phase in *S. cerevisiae*. The ATP is the molecular unit of the intracellular energy transfer and it is important in the biosynthesis of various chemicals. Hence, the problem of ATP deficiency needs to be overcome in order to smooth the pathway of fatty acids synthesis and their conversion to valuable fuels in the *S. cerevisiae* cells.

The NADPH serves as the electron donor for the fatty acid synthesis. In the *S. cerevisiae*, the pentose phosphate pathway (PPP) is responsible for the generation of the majority NADPH. To regulate the PPP pathway is expected to increase the NADPH level. Besides, in the oleaginous yeasts, the malic enzyme is demonstrated to be linked to the NADPH production and lipid accumulation [49]. It has been reported that the manipulation the malic enzyme in the *S. cerevisiae* also resulted in the increase the NADPH production [118]. Hence, by manipulating or co-expression of the malic enzyme, together with the APT-citrate lyase, an enzyme complex which is beneficial for fatty acids synthesis can be constructed and it can provide more precursors for biofuel production.

3) The functional study of differentially expressed proteins in the

engineered strains on the production of fatty acid/fatty acid derivatives

Based on the iTRAQ-coupled 2D LC-MS/MS proteomics analysis, 37 differentially expressed proteins were found in the engineered strains for the enhanced production of fatty acids. These proteins were roughly classified into specific categories based on their described functions, but hadn't been particularly studied for their roles in the production of fatty acids or fatty acid derivatives. It would be meaningful to further discover their specific functions in the metabolic pathway of fatty acid metabolism and other bypasses.

4) The study of industrial scale up of biofuel production by the engineered *S. cerevisiae*

The ultimate goal of the research project is to realize the industrial microbial production of biofuels. Hence, the effects of culture condition such as the nutrient concentration, temperature, pH and oxygen content on the scale up fermentation capacity and productivity of the engineered *S. cerevisiae* need to be studied. The yields of the industrial production of biofuels are expected to be increased by achieving the optimal production conditions.

Besides, the downstream separation and purification process also act as a dominant role in the industrial production of chemicals and fuels. The studies to find out efficient strategies for the separation and purification of biofuels from the industrial fermentation process need to be carried out. The way to realize

the production and application of biofuels will be shorted based on persevere research works.

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