Fatty acids as biofuel precursors accumulation : an investigation by metabolic engineering approach

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**FATTY ACIDS AS BIOFUEL PRECURSORS
ACCUMULATION: AN INVESTIGATION BY
METABOLIC ENGINEERING APPROACH**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING
2015**

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METABOLIC ENGINEERING APPROACH**

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Table of Contents

Acknowledgements.....	I
Table of Contents.....	III
Summary.....	IX
List of Tables	XIII
List of Figures.....	XV
List of Abbreviations	XIX
1 Introduction.....	1
1.1 Background	1
1.2 Objective.....	3
2 Literature Review.....	7
2.1 Application of fatty acid.....	7
2.1.1 Fatty acid-derived biofuels	7
2.1.2 Research process in FFA production	8
2.2 Fatty acid metabolism	14
2.2.1 Fatty acid synthesis and regulation	14
2.2.2 Fatty acid degradation and regulation.....	25
2.3 Metabolic engineering.....	29

2.3.1	Application of metabolic engineering for biofuel production	29
2.3.2	Modification of fatty acid metabolism in <i>S. cerevisiae</i>	34
2.4	Proteomics and metabolomics.....	36
2.4.1	Proteomics.....	37
2.4.2	LC-MS/MS-based proteomics	40
2.4.3	iTRAQ™-based quantitative proteomics.....	41
2.4.4	Metabolomics.....	46
2.4.5	GC-MS-based metabolomics	49
3	Up-regulation of FFA production in <i>S. cerevisiae</i> through β -oxidation modification	51
3.1	Introduction	51
3.2	Materials and methods.....	54
3.2.1	Yeast strains and growth conditions	54
3.2.2	Construction of plasmids pVTU260-POX2 and pVTU260-POX2- CROT	54
3.2.3	Cell transformation and control cell development.....	57
3.2.4	Western blot analysis	58
3.2.5	Growth tests of control and engineered strains.....	59
3.2.6	Sample preparation for metabolite and fatty acid analysis	59

3.2.7	Fatty acid analysis of cells and culture supernatants	59
3.2.8	Metabolic profiling	61
3.3	Result.....	62
3.3.1	Gene modification of the β -oxidation pathway in <i>S. cerevisiae</i> .	62
3.3.2	Growth phenotype analysis.....	64
3.3.3	Analysis of fatty acid composition in cell extracts and culture supernatants.....	67
3.3.4	Metabolic profiling of control and engineered strains.....	71
3.4	Discussion	74
3.4.1	Impact of Aox2p and Crot on fatty acids composition in the engineered strains.....	74
3.4.2	Metabolic engineering of <i>S. cerevisiae</i> for MCFAs production .	76
3.5	Conclusion.....	76
4	Up-regulation of FFA production in <i>S. cerevisiae</i> by control of fatty acyl-CoA metabolism.....	79
4.1	Introduction:	79
4.2	Materials and methods.....	81
4.2.1	Strains and growth conditions.....	81
4.2.2	Plasmid and strain construction	83

4.2.3	Expression analysis of Acot5s	87
4.2.4	Sample preparation for metabolite and fatty acid analysis	88
4.2.5	Fatty acid analysis.....	89
4.2.6	Metabolic profiling	89
4.2.7	RT-PCR analysis of gene expression.....	89
4.3	Results	90
4.3.1	Constructing the genetically engineered <i>S. cerevisiae</i> strains	90
4.3.2	The effect of deletion gene <i>FAA4</i> or/and <i>FAA1</i> on fatty acid composition.....	91
4.3.3	The effect of cytosolic acyl-CoA thioesterase Acot5s on fatty acid composition.....	93
4.3.4	Intracellular metabolite analysis	95
4.3.5	Gene expression analysis	97
4.4	Discussion	98
4.4.1	Evaluation of FFA production and secretion in the strain <i>Δfaa1 Δfaa4</i>	99
4.4.2	FFA production was further enhanced in the strain <i>Δfaa1 Δfaa4 [Acot5s]</i>	101
4.5	Conclusion.....	103

5	Comparative proteomic analysis of engineered <i>S. cerevisiae</i> strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [<i>Acot5s</i>]	105
5.1	Introduction	105
5.2	Materials and methods.....	106
5.2.1	Strain growth and cell harvest.....	106
5.2.2	Protein Extraction, Digestion and Labelling.....	107
5.2.3	Online 2-D Nano-LC-MS/MS Analysis	108
5.2.4	LC-MS/MS Data Analysis and Interpretation	109
5.2.5	HPLC analysis of extracellular metabolites.....	109
5.2.6	ATP and NADPH assay.....	110
5.3	Results	110
5.3.1	ITRAQ analysis and protein identification.....	111
5.3.2	Relative changes in protein expression between the WT and engineered strains.....	115
5.3.3	Relative changes in protein expression between $\Delta faa1 \Delta faa4$ [<i>Acot5s</i>] and $\Delta faa1 \Delta faa4$	118
5.3.4	Physiological and metabolite analysis of the engineered and WT strains	119
5.4	Discussion	122

5.4.1	Comparative proteome analysis of the engineered strains as compared to WT	123
5.4.2	The impact of Acot5s expression on proteome changes.....	130
5.5	Conclusion.....	133
6	Conclusion and limitation	135
6.1	Background and Motivation.....	135
6.2	Conclusion.....	136
6.3	Limitation	139
7	Future direction.....	141
8	References.....	145
	List of publications	165
	Appendix.....	166

Summary

Production of biofuels derived from microbial fatty acids has attracted great attention in recent years owing to their potential to replace petroleum-derived fuels. To be cost competitive with current petroleum fuel, production of the direct precursor fatty acids need to be enhanced to approach high yields.

Herein, metabolic engineering approach was adopted to engineer *Saccharomyces cerevisiae* to accumulate more free fatty acids (FFA). In the third chapter, the β -oxidation pathway of *S. cerevisiae* was engineered to accumulate a higher ratio of medium chain fatty acids (MCFAs) when cells were grown on fatty acid-rich feedstock. For this purpose, the haploid deletion strain $\Delta pox1$ was obtained, in which the sole acyl-CoA oxidase encoded by *POX1* was deleted. Next, the *POX2* gene from *Yarrowia lipolytica*, which encodes an acyl-CoA oxidase with a preference for long chain acyl-CoAs, was expressed in the $\Delta pox1$ strain. The resulting $\Delta pox1$ [*pox2*⁺] strain exhibited a growth defect because the β -oxidation pathway was blocked in peroxisomes. To unblock the β -oxidation pathway, the gene *CROT*, which encodes carnitine O-octanoyltransferase, was expressed in the $\Delta pox1$ [*pox2*⁺] strain to transport the accumulated medium chain acyl-CoAs out of the peroxisomes. The obtained $\Delta pox1$ [*pox2*⁺, *crot*⁺] strain grew at a normal rate. The effect of these genetic modifications on fatty acid accumulation and profile was investigated when the strains were grown on oleic acids-containing medium. It was determined that the engineered strains $\Delta pox1$ [*pox2*⁺] and $\Delta pox1$ [*pox2*⁺, *crot*⁺] had increased fatty acid accumulation and an increased ratio of MCFAs. Compared to the wild-type (WT) strain, the total fatty acid production of the strains $\Delta pox1$

[*pox2*⁺] and Δ *pox1* [*pox2*⁺, *crot*⁺] were increased 29.5% and 15.6%, respectively. The intracellular level of MCFAs in Δ *pox1* [*pox2*⁺] and Δ *pox1* [*pox2*⁺, *crot*⁺] increased 2.26- and 1.87-fold compared to the WT strain, respectively. In addition, MCFAs in the culture medium increased 3.29-fold and 3.34-fold compared to the WT strain. These results suggested that fatty acids with an increased MCFAs ratio had accumulated in the engineered strains with a modified β -oxidation pathway. Our approach exhibits great potential for transforming low value fatty acid-rich feedstock into high value fatty acid-derived products.

In the fourth chapter, fatty acyl-CoA metabolism in *S. cerevisiae* was engineered to accumulate more FFA. For this purpose, haploid *S. cerevisiae* double deletion strain Δ *faa1* Δ *faa4* was constructed first, in which the genes *FAA1* and *FAA4* encoding two acyl-CoA synthetases were deleted. Then the truncated version of *Mus musculus* peroxisomal acyl-CoA thioesterase Acot5 (*Acot5s*) was expressed in the cytoplasm of the strain Δ *faa1* Δ *faa4*. The resulting strain Δ *faa1* Δ *faa4* [*Acot5s*] accumulated more extracellular FFA with higher unsaturated fatty acids (UFA) ratio as compared to the WT strain and double deletion strain Δ *faa1* Δ *faa4*. The extracellular total FFA in the strain Δ *faa1* Δ *faa4* [*Acot5s*] increased to 6.43-fold as compared to the WT strain during the stationary phase. A high level of FFA about 500 μ g/ml was achieved under shake-flask condition. UFA accounted for 42% of TFA in the strain Δ *faa1* Δ *faa4* [*Acot5s*], while no UFA was detected in the WT strain. In addition, the expression of *Acot5s* in Δ *faa1* Δ *faa4* restored the growth. The study presented here showed that through control of the acyl-CoA metabolism by deleting acyl-CoA synthetase and expressing thioesterase, more FFA could be produced in *S. cerevisiae*.

In the fifth chapter, comparative proteomic analysis were performed to get a global overview of metabolic regulation in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]. Over 500 proteins were identified, and 82 of those proteins were found to change significantly in the engineered strains. Proteins involved in glycolysis, acetate metabolism, fatty acid synthesis, TCA cycle, glyoxylate cycle, pentose phosphate pathway, respiration, transportation and stress response were found to be up-regulated in $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared to the WT strain. On the other hand, proteins involved in glycerol, ethanol, ergosterol and cell wall synthesis were down-regulated. Taken together with our metabolite analysis, our results showed that the disruption of Faa1 and Faa4 and expression of Acot5s in the engineered strain $\Delta faa1 \Delta faa4$ [*Acot5s*] not only relieved the feedback inhibition of fatty acyl-CoAs on fatty acid synthesis, but also caused a major metabolic rearrangement. The rearrangement redirected carbon flux towards the pathways which generate the essential substrates and cofactors for fatty acid synthesis, such as acetyl-CoA, ATP and NADPH. Therefore, our results help shed light on the mechanism for the increased production of fatty acids in the engineered strains, which is useful in providing information for future studies in biofuel production.

In short, our study demonstrated the great potential in improving metabolic efficiency of FFA production in *S. cerevisiae*, and implied that it could serve as a good platform for producing microbial fatty acid-derived biofuels. In addition, it added another excellent tool in guiding future biofuel production studies.

List of Tables

Table 3.1 Strains and plasmids list

Table 3.2 Primers used in this study

Table 3.3 Fatty acid production in the cell extract of the WT and the engineered strains

Table 3.4 Fatty acid composition changes in cell extract comparing the WT and the engineered strains in YNBD_{0.5}O₃ medium

Table 4.1 Plasmids and strains list

Table 4.2 Primers list

Table 4.3 Extracellular fatty acids content in *S. cerevisiae* WT and engineered strains

Table 5.1 List of differentially expressed proteins in the strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with the wild type strain.

Table 5.2 List of differentially expressed proteins in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with the strain $\Delta faa1 \Delta faa4$.

List of Figures

Figure 2.1 Reaction schemes of fatty acid synthesis and elongation

Figure 2.2 Fatty acid synthesis and elongation in yeast.

Figure 2.3 Schematic representation of central pathway and fatty acid metabolism in *S. cerevisiae*.

Figure 2.4 Schematic representation of fatty acid β -oxidation in yeast peroxisomes.

Figure 2.5 Production of fatty acid-derived biofuels through fatty acid synthesis pathway in *S. cerevisiae*.

Figure 2.6 The relationship between various omics

Figure 2.7 Schematic workflow for LC-MS/MS based proteomics experiments

Figure 2.8 Flowchart of iTRAQ-based quantitative proteomics experimental design

Figure 2.9 Working Mechanism of iTRAQ Reagents

Figure 2.10 Flowchart of iTRAQ-based quantitative proteomics experimental design

Figure 2.11 Schematic workflow for metabolomics experiments

Figure 2.12 The scheme of typical GC-MS

Figure 3.1 Genetic modification of the β -oxidation pathway in *S. cerevisiae*.

Figure 3.2 The restriction analysis results of plasmid pVTU260-POX2 from screening plate.

Figure 3.3 The restriction analysis results of plasmid pVTU260-POX2-CROT from screening plate.

Figure 3.4 Western for Aoxp2 and Crot.

Figure 3.5 Growth tests on the engineered strains and the WT strain in YNBO medium.

Figure 3.6 Growth curves of the engineered strains and the WT strain.

Figure 3.7 MCFAs analysis of the cell extracts.

Figure 3.8 MCFAs content in the culture supernatant of the engineered strains and the WT strain grown for 24 h in YNBD_{0.5}O₃ medium.

Figure 3.9 The representative GC-MS spectra for azelaic acid.

Figure 3.10 Differential expression levels of intracellular metabolites in the engineered strains compared to the WT strain.

Figure 4.1 Engineering *S. cerevisiae* for high levels of FFA accumulation.

Figure 4.2 The gene disruption procedure for generation of deletion strains.

Figure 4.3 Construction of expression plasmid pVTU260-Acot5s

Figure 4.4 PCR analysis to confirm correct gene deletion of *FAA1* and *FAA4*.

Figure 4.5 Western result for expression of *Acot5s* in *S. cerevisiae* WT, $\Delta faa1$, $\Delta faa4$ and $\Delta faa1 \Delta faa4$ deletion strains.

Figure 4.6 The effect of deleting both *FAA1* and *FAA4* on fatty acids production.

Figure 4.7 The effect of *Acot5s* expression on fatty acids production.

Figure 4.8 Total extracellular FFA accumulation in the WT strain and engineered strains in YNBD medium at 24h.

Figure 4.9 Growth curves of the WT and engineered strains in YNBD medium.

Figure 4.10 Metabolic profiling analysis of the WT and engineered strains.

Figure 4.11 Differential expression levels of fatty acid metabolism genes in the engineered strains as compared to the WT.

Figure 5.1 Functional classification of proteins identified.

Figure 5.2 Physiological features of engineered and WT strains.

Figure 5.3 ATP level in the engineered strains as compared with the WT strain. T

Figure 5.4 NADPH level in the engineered strains as compared with the WT strain.

Figure 5.5 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4$ as compared with WT.

Figure 5.6 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with WT.

Figure 5.7 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with $\Delta faa1 \Delta faa4$.

Figure 6.1 Free fatty acid accumulation in *S. cerevisiae* by modifying fatty acid metabolism.

Figure 6.2 Metabolic regulation in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*].

Figure 7.1 Engineering *S. cerevisiae* for high levels of FFA accumulation.

List of Abbreviations

ABC	ATP binding cassette
ACC1	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ACR	Fatty acyl-CoA reductase
ADC	Aldehyde decarbonylase
ADO	Aldehyde-deformylating oxygenase
AHR	Aldehyde reductase
APS	Ammonium persulfate
AT	Acetyl-transferase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAR	Carboxylic acid reductase
CoA	coenzyme A
CROT	carnitine octanoyltransferase

DES	Divinyl ether synthase
DH	Dehydratase
ECL	Enhanced chemi-luminescence
EI	Electron Impact Ionization
EPA	Environmental Protection Agency
EPR	Electron paramagnetic resonance
ER	Enoyl reductase
ESI	Electrospray ionization
EUROSCARF	European <i>S. cerevisiae</i> Archive for Functional analysis
FA	Fatty acid
FAR	Fatty acyl-CoA reductases
FAS	Fatty acid synthase
FFA	Free fatty acid
FID	Flame ionization detector
FT-ICR	Fourier transform ion cyclotron resonance

FT-SPK	Fischer-Tropsch Synthetic Paraffinic Kerosene
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ICAT	Isotope-coded affinity tags
iTRAQ	Isobaric tags for relative and absolute quantitation
KR	Ketoacyl reductase
KS	Ketoacyl synthase
LC	Liquid chromatography
LCFA	Long chain fatty acid
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MCFA	Medium chain fatty acid
MS	Mass spectrometry

MPT	malonyl-palmitoyl transferase
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PDH	pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween 20
PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
PT	Phosphopantetheine transferase
UFA	Unsaturated fatty acid
PVDF	Polyvinylidene fluoride
Q-TOF	Quadrupole time-of-flight
SCX	Strong cation-exchange
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFA	Saturated fatty acids
SIM	Selected ion monitoring
SE	Steryl esters
TAG	Triacylglycerol
TCA cycle	Tricarboxylic acid cycle
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Total fatty acids
TIC	Total ion current
TMB	Tetramethylbenzidine
VLCFA	Very long chain fatty acid

1 Introduction

1.1 Background

Energy and environment problems have stimulated interest in producing alternative biofuels from renewable sources for several decades. Great progress has been made in the first-generation biofuel: bioethanol has already been widely used as additive in petroleum-derived fuels in USA and Brazil; biodiesel has been used alone or blended with petro-diesel in standard diesel engines with certain modifications. However, there are many limitations. Firstly, they are mostly produced from edible food and oil, therefore competing with food supply and cause the rise of food price. Secondly, ethanol has low energy density and high hygroscopicity [1], which makes it not compatible with current engines, and also cannot be transported in pipeline like petroleum. Besides, there are also environmental side-effects. Biofuels produced from soy oil or waste grease result in more than 50% reduction in greenhouse gases compared to petroleum biofuels. However, the increasing need for biofuel feedstock such as oil palm, sugar cane, and soybeans resulted in tropical deforestation of rainforests in some countries such as Indonesia and Malaysia. The destruction of the climate ecosystems got a lot of criticism. The Environmental Protection Agency (EPA) concluded that biofuels made from palm oil don't belong to the nation's renewable fuels, due to the no climate-friendly data that was published in January 2012 (<http://en.wikipedia.org/wiki/Biodiesel>).

The second-generation biofuels have attracted great attention recently, such as cellulosic ethanol, long chain alcohols, isoprenoid-derived biofuels, fatty acid-

derived biofuels, algae fuel, biohydrogen, mixed alcohols, and wood diesel [2, 3]. They are all produced from sustainable feedstock, such as non-food part of current crops, non-food crops, or inedible waste products. Therefore they do not compete with food supply and are more environmental friendly. For woody or fibrous biomass, the first problem needs to address is to extract sugars from lignin, hemicellulose and cellulose, which usually need to be broken down by fungal enzyme catalyst [4]. After that, the extracted sugars are transferred to valuable biofuels products through microbes' cellular machinery. However, the microbes usually cannot produce biofuels, or can only produce low concentration of biofuel molecules in the cells. So modification of metabolic pathway is needed, such as introducing new biosynthesis reactions, increasing the accumulation of precursors, or preventing the degradation of the target products. Metabolic engineering have been widely used in engineering microbes to produce high energy contained biofuels [5-8]. Among them, fatty acid-derived biofuels such as fatty alcohols, alkanes, alkenes, and fatty esters (biodiesel) have got noticeable improvement in these years. There are several advantages of microbial synthesized fatty acid-derived biofuels. Firstly, free fatty acids (FFA) are easily transformed into many fuel molecules through biochemical or catalytic chemical methods [9-11]. Secondly, fatty acid-derived biofuels are similar to petroleum-based fuels, allowing us to continue using the existing transportation infrastructures. Thirdly, fatty acids are essential molecules in living organism and have been well studied from many aspects. Combining natural fatty acid metabolic pathway with some new biochemical reactions can divert cell metabolism towards fatty acid accumulation and apply to biofuels production.

Although this seems like a promising platform to build, we should notice the potential problems at the same time. Firstly, metabolic pathways of microorganisms are extremely complicated. An organism usually has responsive mechanism to environmental or genetic changes, several modifications should be made together in order to divert the metabolism to our interest products. Another problem is the toxicity of biofuels to producer cells. The engineered cells with altered metabolic pathways accumulate heterologous metabolites, these heterologous metabolites may cause metabolic imbalance in the meantime [12, 13]. Therefore, we hope to direct carbon flux towards FFA accumulation; meanwhile, we also want to study the metabolic response of engineered cells to guide our work.

1.2 Objective

The objective of this work is to enhance FFA accumulation through controlling fatty acid synthesis and degradation in the engineered *S. cerevisiae*, which can serve as a biofuel production platform. We wish to modify the biochemical reactions in metabolic pathways by deleting or introducing enzymes, therefore controlling the fatty acid metabolism and directing carbon flux toward FFA accumulation. By analyzing the engineered strains at proteome and metabolome level, we hope to gain a better understanding of the *in vivo* changes in global metabolic pathways, and get a systematic view of the cellular responses and adjustment to the metabolic perturbations.

Experimentally, metabolic engineering methods were used to modify the fatty acid metabolic pathways in *S. cerevisiae*. First, the studies were conducted to engineer fatty acid degradation pathway and fatty acid synthesis pathway. Then, we further

studied the engineered strains through proteomics and metabolomics approaches. By comparative proteomic analysis, we aim to achieve a better understanding of the cellular responses to the genetic modification and the mechanism of FFA accumulation. We also hope the platform developed in this study can be applied to valuable biochemical production, especially in biofuel production.

The rest of this dissertation is organized as follows:

In the second chapter, the background and existing knowledge of fatty acid synthesis and degradation, FFA accumulation, and fatty acid-derived biofuel production are presented. Liquid chromatography-mass spectrometry (LC-MS) based proteomics and gas chromatography-mass spectrometry (GC-MS) based metabolomics were also described. The purpose of this chapter is to elucidate the context and address the importance of the present work.

In the third chapter, the fatty acid degradation pathway of *S. cerevisiae* was studied and engineered. Firstly, the *S. cerevisiae* deletion strain $\Delta pox1$ was obtained in which the *POX1* gene encoding the only acyl-CoA oxidase was deleted. Then, acyl-CoA oxidase Aox2p (encoded by *POX2*) from *Y. lipolytica* which has a preference for long-chain acyl-CoA substrates was expressed. The replacement of *POX1* with *POX2* led to the blocking of β -oxidation pathway. To unblock the β -oxidation pathway in the Aox2p-expressing strain, peroxisomal carnitine octanoyltransferase (*CROT*) from *Mus musculus* was also expressed. The effect of β -oxidation cycle modification on fatty acid profile and accumulation was investigated and discussed.

In the fourth chapter, the fatty acyl-CoA metabolism was studied and modified.

Firstly, the genes *FAA1* and *FAA4* which encode the prominent acyl-CoA synthetases were first deleted to block the transformation of FFA to fatty acyl-CoAs. Secondly, *ACOT5* (*PTE-1c*) from *Mus musculus* was chosen to be expressed in the cytoplasm of *S. cerevisiae* to increase the production of FFA. Acot5 is a medium chain acyl-CoA thioesterase acting on C6-CoA to C20-CoA, with highest activity with C10-CoA. It was shown to produce FFA and also participate in the transportation system in *Mus musculus* peroxisomes. Then fatty acid and metabolite profile of the engineered strains were investigated and evaluated.

In the fifth chapter, proteomic analysis were used to investigate the alterations in the proteome and metabolome profile of the WT, $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$. Changes in proteins and metabolites in different metabolic pathways were detected and analyzed. The global metabolic regulation in both engineered strains was mapped and discussed. The purpose of this chapter is to help us understand the mechanism of FFA accumulation in engineered strains and provide directions for future work.

In the sixth chapter, the conclusions were drawn and limitations were pointed out.

In the seventh chapter, suggestions were posed for further work.

2 Literature Review

2.1 Application of fatty acid

2.1.1 Fatty acid-derived biofuels

In the second-generation biofuels, various efforts have been focused on the commercialization and sustainability requirements. Microbial fatty acid-derived biofuels, such as alkane, alkene, and diesel, meet this goal as they are accommodated to existing transportation infrastructure, and only take one or two catalytic steps away from fatty acid molecule. Moreover, fatty acids, being important molecules in biology system, have been well studied in many organisms, especially in model prokaryotic and eukaryotic cell.

There has been impressive progress in transforming microbial fatty acids to biofuels or biochemicals. On the one hand, fatty acids are overproduced in microbes and then converted to fuels or chemicals through chemical conversion [14] or bioconversion [8, 15]. In *E. coli*, FFA are overproduced and transesterified to alkanes through overexpressing plant medium chain acyl-ACP thioesterase, deleting fatty acyl-CoA synthetases, and overexpressing ACC [14]. Further overexpression of acyl-ACP reductase/carboxylic acid reductase (CAR) and aldehyde decarboxylase (ADC)/aldehyde reductase (AHR) can convert fatty acyl-ACP or fatty acid into fatty alkanes or fatty alcohols [16]. On the other hand, fatty acid-rich feedstocks are directly utilized as energy source to produce biofuels and chemicals through completely oxidizing fatty acids [17] or by using the intermediates of β -oxidation [18, 19]. As attractive alternative feedstocks besides lignocellulosic sugars, fatty acids have

several factors attracting us to exploit the microbial bioconversion platforms. Firstly, the availability of a broad range of sources: there are nonedible fatty acid-rich crops, oleaginous fungus/algae, and industrial by-products can be used as the cheap energy source. Secondly, they have product yield advantage over sugars when used as energy source due to the efficient metabolism process in β -oxidation [17], which has 100% carbon recovery.

2.1.2 Research process in FFA production

Studies about manipulation of fatty acid accumulation have been carried out for several decades [20]. There have been studies showing that overexpression of *ACCI* could increase fatty acid production [14, 21, 22]. By expressing different thioesterases, FFA chain length and yield in *E. coli* could be considerably altered [23, 24]. Production of fatty acid-derived chemicals can be increased by inactivating β -oxidation pathway through deletion of *fadD* and *fadE* genes. Recently, these approaches have been applied to biofuels production. After fatty acid profile and production were modified by methods mentioned above, extra enzymes were integrated in metabolic pathway to do *in vivo* transformation, or inorganic catalysts were used to do *in vitro* transformation to produce biofuels. The products include biodiesel, fatty alcohols [8, 25], and alkanes/alkenes [10, 15]. Beside the approach of optimizing fatty acid synthesis pathway, through utilizing fatty acid β -oxidation many biofuels can be produced; for example, genetically modified *E. coli* can convert fatty acids into various biofuels such as ethanol and butanol [17, 26].

There have been plenty good reviews summarizing the progress about utilizing fatty acid biosynthetic machinery to produce various valuable fuels [7, 27-30]. Among

various synthesis approaches, transferring FFA into high-value compounds via biochemical or *in vitro* catalytic pathways have attracted great attention because of the following advantages: “renewability, short production cycle, less labor requirement, less affection by venue, season and climate, and easier to scale up [31]”. In addition, for *in vitro* catalysis, fatty acids in cells usually need to be harvested and extracted by solvents. This process is energy intensive, accounting for 70-80% of the total cost [32]. If fatty acids or fatty acid-derived products could be secreted into the culture medium, the energy intensive separation steps could be skipped, thus largely decreasing the total cost of biofuel production. For these reasons, the microbial production of extracellular fatty acid has gained more and more attention recently. A key challenge is to increase the carbon flux toward FFA and increase the secretion efficiency. So in the following part, we will give a brief review in the viewpoint of FFA accumulation in microbes.

There have been plenty of studies about FFA accumulation. Some groups examined the effect of nutrition limitation on FFA yield. B Pflieger *et al* first studied the effect of carbon-limited conditions and indicated non-carbon limitations as a preferred media formulation for maximizing FFA yield. Then, they conducted further chemostat experiments to collect data under phosphate limitation. A yield of 0.1 g FFA/g glucose was obtained at a dilution rate of 0.1 h⁻¹. The highest biomass specific productivity of 0.068 g FFA/gDCW/h was obtained at a dilution rate of 0.25 h⁻¹, which indicated a ~45 % and ~300 % increase in yield and biomass specific productivity as compared with carbon-limited cultivation [33].

Many other groups used metabolic engineering strategies to improve yield of FFA

in *E. coli*. In one study, fatty acid synthetic ability of *E. coli* was enhanced by combining different genetic modifications: firstly, the *fadD* gene encoding the first enzyme in fatty acid degradation was deleted; secondly, endogenous *accABCD* gene was overexpressed; thirdly, an endogenous thioesterase was overexpressed; finally, a heterologous plant thioesterase with a substrate preference for shorter fatty acyl chains was overexpressed. The resulting strain accumulated 2.5 g/L/day fatty acids in a fed-batch fermentation [34]. After that, further improvement was made by putting the endogenous thioesterase and the plant thioesterase into one plasmid, the *E. coli* cell line was engineered to produce 4.5 g/L/day total fatty acid in a fed-batch fermentation [35]. In addition, a cell-free system was developed to quantitatively investigate fatty acid biosynthesis and regulation, it is identified that fatty acid synthesis is strongly dependent on malonyl-CoA supply, ACC activity, and an optimum concentration of thioesterase.

In another study [36], both strain improvements and cultivation process strategies were exploited to enhance FFA production. It was demonstrated that overexpressing native thioesterase 'Tesa without N-terminal signal sequence and deleting *fadL* encoding fatty acid transporter membrane protein in *E. coli* BL21 (DE3) improved FFA production. Moreover, by adjusting the temperature, adding ampicillin, and employing on-line extraction, FFA production was further increased. The *E. coli* engineered strain (pACY-'*tesA*- Δ *fadL*) produced 4.8 g/L FFA under optimal conditions, the specific productivity being 0.02 g/h/g dry cell mass.

To make microbial produced FFA more suitable for biodiesel production, free monounsaturated fatty acids were also produced in engineered *E. coli* by metabolic

engineering approaches [37]: first, fatty acyl-ACP thioesterase (AtFatA) and fatty acid desaturase (SSI2) from *Arabidopsis thaliana* were expressed in *E. coli* BL21 star (DE3) to specifically release free UFA and convert SFA to UFA. Second, the endogenous *fadD* gene was disrupted to block fatty acid β -oxidation. Next, the native *ACC*ase gene was overexpressed to increase the supply of malonyl-CoA. Finally, 82.6 mg/L FFA were produced by the engineered strain under shake-flask conditions. FFA yield on glucose reached about 3.3% of the theoretical yield. Palmitoleate and cis-vaccenate took up more than 75% of the total amount of FFA. In addition, production of 1.27 g/L FFA was achieved in fed-batch fermentation without affecting fatty acid compositions.

As stated above, several research groups increased the FFA yield by expressing various thioesterases. The overexpression of thioesterases not only increases the total amount of fatty acids produced, it also promotes the secretion of FFA therefore allows for more efficient separations [7].

Beside the method of expressing thioesterases, even and odd chain MCFAs with chain lengths from 4 to 13 carbons were produced in *E. coli* by changing both the thioesterase and fatty acid synthesis machinery itself. Induced degradation of an essential ketoacyl synthase in response to a chemical inducer slowed down acyl-ACP elongation and thus redirected carbon flux towards MCFA production [9].

In addition to *E. coli*, cyanobacteria were also exploited in FFA production. Cyanobacteria have a big advantage because they do not need carbon feed. Recombinant acyl-ACP thioesterase and acetyl-CoA carboxylase from the model green alga *Chlamydomonas reinhardtii* CC-503 were expressed in the model

cyanobacterium *Synechococcus elongates* PCC7942 to improve FFA production, however, FFA yield did not increase as expected [38]. In another work, six generations of *Synechocystis* sp. PCC6803 wild type (SD100)-derived strains were constructed for FFA secretion. In one engineered strain, through expressing codon-optimized thioesterase genes and weakening polar cell wall layers, FFA yield of about 197 mg/L was achieved at a cell density of 1.0×10^9 cells/mL [39].

Moreover, the efficiency of *E. coli* using different cheap carbon sources was also improved by different groups. The metabolic capability of *E. coli* BL21 to assimilate acetic acid to fatty acid was enhanced by overexpression of *acs* gene encoding AMP-forming acetyl CoA synthetase, *tesA* gene encoding endogenous thioesterase, and deletion of *fadE* gene involved in β -oxidation. This resulted in production of ~ 1 g/L fatty acids from acetic acid in a fed-batch fermentation, representing $\sim 20\%$ of the theoretical yield [40].

In another report, a metabolically engineered strain was used for FFA production from waste crude glycerol, which is from biodiesel production. Several genetic modifications were made to optimize the productivity: overexpression of native 3-hydroxyacyl-ACP dehydratase (*fabZ*) of *E. coli* and the acyl-ACP thioesterase from *Ricinus communis*; mutation of *sucC*, *ptsG* and *fabR* gene in host strain; overexpression of NAD kinase and transhydrogenases (UdhA and PntAB). When using pure glycerol as a carbon source, the engineered strain could produce 4.82 g/L FFAs with the yield of 29.55% (g FFAs/g glycerol), which was about 83% of the maximum theoretical yield. When crude glycerol was used as a carbon source, the FFA production was 3.53 g/L with a yield of 24.13%, which was slightly lower than

pure glycerol, while still was about 68% of the maximum theoretical yield [41].

With similar method, *E. coli* strain was engineered to produce FFA using lignocellulose hydrolysate, which mainly contains glucose and xylose. The engineered strains exhibited efficient fatty acid production, especially the strain ML190 (pXZ18Z), with mutation of *fadD* and *ptsG* and overexpression of native 3-hydroxyacyl-ACP dehydratase (*fabZ*) and the acyl-ACP thioesterase from *Ricinus communis*, produced 3.79 g/L FFA with a high yield of 21.42% when using hydrolysate from a commercial plant as the carbon source [42].

Although various methods have been used to produce a variety of fatty acids in *E. coli*, it has been shown that endogenously produced FFA, especially short and medium chain FFA have negative effects on membrane integrity and cell viability [31]. Approaches such as modulating membrane composition [43] or overexpressing efflux transporters [44] can improve FFA tolerance but only in moderate level. To directly address the toxic effects of incorporating FFA into bilayer membrane, Saken Sherkhanov *et al* studied the effect of deletion of *aas* gene which encodes 2-acyl-glycerophosphoethanolamine (2-acyl-GPE) acyltransferase/ acyl-ACP synthetase (Aas) [45]. Because Aas can directly incorporate MCFA into membrane phospholipids therefore altering the bilayer composition in MCFA producing cells. Deletion of the *aas* gene reduced the levels of MCFA incorporated into the membrane, thus restored membrane lipid composition, lowered the toxicity, and improved MCFA yield.

Although most studies on FFA production focus on *E. coli*, there is also report about fatty acid secretion genotype of *S. cerevisiae*. Scharnewski *et al* [46] found that the

yeast strain could secrete 220 $\mu\text{mol/L}$ of FFA by double deleting of two main acyl-CoA synthases, FAA1 and FAA4. Deletion of all four acyl-CoA synthases cannot further increase fatty acid secretion. It is proposed that the FFA in the deletion strain were derived from the lipid turnover processes, fatty acid secretion depends on cell growth phase, and a portion of those fatty acids are re-imported during the stationary phase.

2.2 Fatty acid metabolism

2.2.1 Fatty acid synthesis and regulation

A fatty acid is a carboxylic acid (with the structure $\text{RC}(=\text{O})\text{OH}$) with a long aliphatic chain. Fatty acids that have carbon-carbon double bonds are known as unsaturated. Fatty acids without double bonds are known as saturated. Fatty acids are usually contained in esterified form in triglycerides or phospholipids in cells, and they are known as free fatty acids (FFA) when not attached to other molecules [47]. Fatty acids are important compounds in biological systems, which have various biological functions such as maintaining membrane fluidity, taking part in signaling reactions, modifying protein, storing and supplying energy [48]. Thus fatty acids have been well studied from many aspects.

Fatty acid synthesis is the process of creation of fatty acids from acetyl-CoA by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [49]. The reaction mechanism is illustrated in Figure 2.1. In the initial step, ACC enzyme carboxylates acetyl-CoA into malonyl-CoA by adding one CO_2 unit. This carboxyl-transfer reaction needs biotin as essential cofactor and requires 1 molecule of ATP per

reaction:

Figure 2.1 Reaction schemes of fatty acid synthesis and elongation. This figure is modified based on Figure.1 in [48]. Malonyl-CoA produced by acetyl-CoA carboxylase serves as the elongation unit in the series of reactions. Fatty acid chain synthesis is initiated through loading the acetyl primer from CoA to ACP, and loading malonyl elongation substrate from malonyl-CoA to ACP. Acetyl and malonyl substrates are condensed to 3-ketoacetyl-ACP. The subsequent reaction steps, including ketoacyl reduction, dehydration and enoyl reduction, result in fully saturated acyl-ACP extended by two carbon atoms (acyl(n+2)-ACP).

The following elongation steps, including condensation, ketoacyl reduction, dehydration and enoyl reduction, are catalyzed by FAS. There are two kinds of FAS systems. Type I FAS system is used in mammals and yeast, in which fatty acid synthase consist of two subunits. While type II FAS system is used in most bacteria and mitochondria or chloroplasts of eukaryotic cells, in which fatty acid synthesis is catalyzed by sets of isolated enzymes. Generally, in each round of elongation, FAS system requires one molecule of acetyl-CoA, one molecule of malonyl-CoA and two molecules of NADPH. To synthesize one molecule of C16 fatty acyl chain, the reaction equation should be (ignoring H⁺ and water):

Taking into consideration of ATP-dependent synthesis of malonyl-CoA, the reaction equation should be:

The mechanism of fatty acid synthesis is well studied, especially in model organisms such as *E. coli* and *S. cerevisiae*. In *E. coli*, acetyl-CoA carboxylase is encoded by *accABCD* which contains four subunits [50]. Initially, acetyl-CoA carboxylase carboxylates acetyl-CoA into malonyl-CoA, then malonyl-CoA is transferred to acyl carrier protein (ACP) by malonyl-CoA: ACP transacylase (FabD), the subsequent condensation reaction cycle were catalyzed by β -ketoacyl-ACP synthase III (FabH), 3-oxoacyl-ACP synthase I (FabB), 3-oxoacyl-ACP synthase II (FabF), β -ketoacyl-ACP reductase (FabG), β -hydroxydecanoyl thioester dehydrase (FabA), (3R)-hydroxymyristoyl-ACP dehydratase (FabZ) and enoyl-ACP reductase (FabI) until

the formation of a palmitoyl-ACP [7].

Regulation of fatty acid biosynthesis exists at multiple levels in *E. coli* [51, 52], such as transcriptional regulation, feedback inhibition caused by end product acyl-ACPs, and growth condition regulation. Two repressors FabR and FadR mainly regulate fatty acid biosynthesis at transcriptional level. FabR represses fatty acid synthesis genes *FabB* and *FabA* [53]. FadR represses the translation of fad operon genes by binding to the upstream of the operators hence regulates fatty acid β -oxidation. It also has been shown that FadR is an activator of *FabA*, *FabB* and the operon *FabHGD* [54]. There are intricate interactions between FabR and FadR, which make it possible to not only regulate fatty acid concentration but also the ratio of UFA/SFA in *E. coli*. For example, the ratio of unsaturated to saturated fatty acid increased 2-fold by deleting the gene *FabR* [53]. Deficiency in FadR led to about 30% decrease in UFA due to lacking activation of the transcription of *FabA* and *FabB*. On the other hand, overexpression of FadR resulted in significantly enhanced fatty acid production [55].

Among the enzymes of FAS II system, accABCD, FabH and FabI are feedback inhibited by the end product acyl-ACPs. Currently, two methods have been used to affect the feedback inhibition. Firstly, periplasmic enzyme thioesterase I (TesA) had been found to relieve the product inhibition of fatty acid synthesis by cleaving the fatty acyl-ACP when expressed as a cytosolic enzyme. The resulting FFA were mostly released to culture medium [56]. Secondly, the expression of heterologous ACP has been shown to change fatty acid profile to facilitate the accumulation of UFA [57].

In addition, fatty acid biosynthesis is also regulated by growth condition. For example, transcriptional control of the genes involved in fatty acid synthesis or degradation are different under aerobic and anaerobic conditions, with or without fatty acid in the culture medium [52]. In a study of producing FFA in *E. coli* under phosphate-limited conditions, phosphate limitation increased the FFA yield by about 45% as compared to carbon-limited cultivations [33]. *E. coli* regulates fatty acid composition under different temperatures, and adjust membrane fluidity accordingly. The ratio of the UFA to SFA increased with decreasing temperature and decreased with increasing temperature. However, membrane fatty acid composition of *E. coli* becomes conditioned to a low-temperature physiology after several cycles of temperature change [58].

In *S. cerevisiae*, fatty acid can be synthesized both in cytoplasm and mitochondria. Because FAS system in mitochondria belongs to type II FAS system, the type I cytosolic FAS system will mainly be discussed below. Cytosolic acetyl-CoA carboxylase in *S. cerevisiae* is encoded by *ACCI* (*FAS3*) [59]. Acetyl-CoA carboxylase is a large multi-functional enzyme, including the biotin carboxylase domain, the biotin carboxyl carrier protein domain, and the carboxyltransferase domain. The reaction catalyzed by acetyl-CoA carboxylase has been demonstrated as the rate-limiting step in de novo fatty acid synthesis [60]. Similar to *E. coli*, acetyl-CoA carboxylase in *S. cerevisiae* needs ATP and biotin as cofactors for the carboxyl-transfer reaction. The product, malonyl-CoA, is the elongation unit of FAS.

The FAS [61-65] in yeast consists of Fas1 (β subunit) and Fas2 (α subunit), and is organized as a barrel-shaped $\alpha_6\beta_6$ complex of 2.5MDa. The α -subunit contains the

Palmitoyl-CoA(C₁₆)

Acetyl-CoA

Acetyl-ACP

AT

CoA

ACP

Malonyl-CoA

Malonyl-ACP

MPT

CoA

KS

β -ketoacyl-ACP

CO₂

ACCI

HCO₃⁻+ATP

ADP+Pi

NADPH + H⁺

NADP⁺

KR

β -hydroxyacyl-ACP

DH

H₂O

β -enoyl-ACP

ER

NADPH + H⁺

NADP⁺

Acyl(Cn+2)-ACP

FAS:

β -chain (230 kDa)

AT ER DH MPT

α -chain(210 kDa)

ACP KR KS PT

In the subsequent reaction steps, the β -carbon groups are processed by KR, DH, and

ER, which results in fully saturated acyl-ACP extended by two carbon atoms (acyl(n+2)-ACP). Acyl-ACP can serve directly as a primer for the next condensation reaction. The chain elongation step stops until C16- or C18-acyl-residues are back-transferred to CoA by MPT, yielding long-chain acyl-CoAs [48, 63, 66]. This is different from bacteria in which acyl-ACP was catalyzed directly to lipids [51], or mammalian cells in which chain-termination step was catalyzed by a thioesterase domain to release FFA from FAS [67]. VLCFA (C20-C26 carbon atoms) are synthesized by C16 or C18 saturated acyl-CoAs and malonyl-CoA in the endoplasmic reticulum. Enzymes Elo1, Elo2, and Elo3 catalyze the condensation or decarboxylation of acyl-CoA with malonyl-CoA [68, 69].

The regulation of fatty acid synthesis in yeast is also complicated and occurs at multiple levels, such as gene expression level, protein level, and growth condition regulation.

At the gene expression level, genes *ACCI*, *FAS1*, and *FAS2* are all subjected to coordinated control by lipid precursors inositol and choline, and the positive and negative transcription factors Ino2/Ino4 and Opi1, respectively [48]. The negative regulator Opi1p represses UAS_{INO}-containing genes (UAS_{INO} is a cis-acting inositol-sensitive upstream activating sequence), such as the highly regulated gene *INO1*, by inhibiting Ino2p and Ino4p transcription factors [70, 71]. Deletion of gene *OPI1* can de-repress the transcriptional regulation of *INO1*, which codes for the rate-limiting enzyme in inositol synthesis, thus leads to irrepressible overproduction of inositol. Because the genes *ACCI* [72], *FAS1*, and *FAS2* [73] also contain UAS_{INO} elements in their promoters, they are regulated by inositol through the same mechanism.

Inositol supplementation results in the repression of *ACC1*, *FAS1*, and *FAS2*, and is only less repressed than *INO1* [74, 75]. Expression of *ACC1* and *FAS1* are dependent on histone acetylation, and regulated by the SAGA and TFIID complexes. *ACC1* show cell cycle-dependent expression; the peak level appears at G1 phase. *FAS1* and *FAS2* reach the highest level of expression during the M/G1 transition [76], which indicates that there is coordination between the expressions of these genes. Besides, the transport and stability of mRNA might have significant effect on expression efficiency; still, specific information of mRNA stability of these genes is not studied well. There are several potential binding sites for general transcription factors, like Gcr1, Abf1, Grf1 (Rap1), and Reb1, in the promoter regions of the genes *FAS1*, *FAS2*, and *ACC1*. Removal of potential binding sites from the *FAS1* promoter led to a 40% reduction of expression of a reporter gene. Removal of Gcr1-binding site from the *FAS2* promoter results in a 90% reduction in expression. Likewise, deletion of Abf1, Reb1, and two Grf1 binding sites sequences from the *ACC1* promoter reduces expression of *ACC1* gene by 54% [77].

At the level of protein, Snf1 kinase in yeast phosphorylates and inactivates ACC1 at serine 1157. Acyl-CoAs, including malonyl-CoA and long chain acyl-CoAs, also inhibit the activity of ACC1, FAS1, and FAS2 [78]. For FAS holoenzyme, the amount is dependent on the level of FAS1. Over-expression of *FAS1* leads to concomitant expression of *FAS2* and substantially enhances FAS activity; on the other hand, *FAS1* expression cannot be regulated by *FAS2* gene dosage. A downstream repression site (DRS) responsible for *FAS1*-dependent expression of *FAS2* was identified within the coding sequence of *FAS2* [79]. Moreover, active FAS complex requires covalent modification of the ACP domain in the α subunit (Fas2)

by 4'-phosphopantetheine, which is catalyzed by an intrinsic phosphopantetheine: protein transferase (PPTase) activity in the C-terminal end of the α subunit [80].

Moreover, different growth conditions affect fatty acid synthesis; for example, the transcription of *ACC1*, *FAS1*, and *FAS2* are all decreased when there are fatty acids in the medium. There is study demonstrated that this is due to the long chain acyl-CoA feedback inhibition, which requires acyl-CoA synthase activity [73, 81]. Yeast can rapidly regulate ACC1 activity to adjust to different growth conditions, such as glucose limitation and salt stress. The changes are sensed and transduced to ACC1 by the Snf1 kinase [82]. Besides, there is study which showed that changing carbon source from ethanol to galactose may increase the level of FAS1 protein [83].

In de novo fatty acid synthesis [48], the constant supply of acetyl-CoA, malonyl-CoA, ATP and NADPH is important. In *S. cerevisiae*, there are several source of acetyl-CoA: it can be produced by the pyruvate dehydrogenase pathway in the mitochondria or by the pyruvate-acetaldehyde-acetate pathway in the cytosol. In the mitochondria, pyruvate dehydrogenase complex (PDH) is involved in catalyzing the overall conversion of pyruvate to acetyl-CoA [84]. In the cytoplasm, ACS enzymes Acs1p and Acs2p are involved in the reaction of catalyzing acetate to acetyl-CoA [85]. While in oleaginous microorganisms, there is an additional source of acetyl-CoA: the excess citrate exported from the TCA cycle out of the mitochondrion can be cleaved by ATP citrate lyase (ACL) in the cytosol. Overexpression of ACL can increase the acetyl-CoA support towards de novo lipid synthesis in non-oleaginous [86]. For malonyl-CoA supply, there are studies about overexpression of *ACC1* to enhance fatty acid synthesis [14, 21, 22]. ACC1 requires one molecule of ATP to

produce one molecule of malonyl-CoA. Oxidation of glucose in glycolysis, TCA cycle, and oxidative phosphorylation can generate ATP; among these pathway, oxidative phosphorylation release large amount of energy [87].

FAS requires two molecules of NADPH in the two reduction steps of the carbon chain elongation cycle. In the cytoplasm of yeast, NADPH is mainly produced by the pentose phosphate pathway through NADP⁺ dependent dehydrogenase, including glucose 6-phosphate dehydrogenase, decarboxylating P-gluconate dehydrogenase, and NADPH isocitrate dehydrogenase. NADP⁺ also can be generated by NAD kinase, which may play a role in establishing NAD/NADP ratios. Malic enzyme is showed to provide NADPH by catalyzing the reaction: Malate + NADP⁺ → pyruvate + NADPH. Malic enzyme has been found in most oleaginous microorganisms, and has been suggested as controlling activity for lipid production in *Mucor circinelloides* and *Aspergillus nidulans* [88, 89]. Sometimes there are several isoforms of malic enzyme in mitochondria and cytoplasm. Some isoform maybe prefer NADH instead of NADPH as substrate; some isoform may not be involved in the lipid-accumulation process although it supports NADPH formation. The malic enzyme linked to fatty acid synthesis belongs to cytoplasmic form in yeast. However, *S. cerevisiae* only has a single gene *MAE1* encoding a mitochondrial malic enzyme [90], which implies it should not supply NADPH for fatty acid synthesis. Besides, NADPH can also be provided by aldehyde dehydrogenase family [91, 92].

Monounsaturated fatty acids in *S. cerevisiae* are formed by the Δ-9 fatty acid desaturase (OLE1) in the endoplasmic reticulum membrane. Although it is a single enzyme system, the expression of gene *OLE1* is highly regulated and responds to a

number of environmental and nutritional stimuli; such as, carbon source, nutrient fatty acids, metal ions, and oxygen levels [93]. Because the double bond formation reaction catalyzed by OLE1 requires oxygen and reducing equivalents from NADH, OLE1 activity was observed to increase by several folds when yeast cells were shifted from fermentative to respiratory growth on glucose. Because mitochondria proliferate in respiratory growth phase, which maybe increase the demand of UFA precursors for membrane lipids synthesis [94]. Another study showed that *OLE1* mRNA levels increased 6-fold when yeast cells were cultured on non-fermentable carbon sources (glycerol or lactate) as compared with glucose, which is because mitochondrial genes are maximally expressed under the obligate respiratory conditions [93]. The content of UFA also exhibits an increase when *S. cerevisiae* adapts to low temperatures [95]; this is mediated by the mitochondria membrane proteins Spt23p and Mga2p. When UFA is added to the culture medium, *OLE1* mRNA levels decrease rapidly due to the transcription repression [96] and mRNA stability control [97]. Moreover, *OLE1* can be regulated by oxygen, certain metals, and irons [98, 99].

Another concept need to be noted, the process of fatty acid synthesis affects the lipid accumulation. There are several sources of fatty acids in cells, such as de novo synthesis, exogenous fatty acid uptake, protein turnover, and lipid turnover. An example of central pathway and fatty acid metabolism in *S. cerevisiae* was showed in Figure 2.3. De novo fatty acid synthesis provides precursors for fatty acid elongation, such as malonyl-CoA and the subsequent longer chain acyl-CoA (C14-C16). The fatty acyl-CoAs were then assembled to form triacylglycerols (TAG) and steryl esters (SE), which belongs to the lipid storage pathway. The neutral lipids are

stored inside the lipid body to serve as energy reserves. In the presence of external sources of fatty acids, yeast cells uptake these substrates, and do subsequent modification for storage and accumulation. It may involve incorporation and transportation of the extracellular fatty acids in the form of CoA-thioesters [100].

Figure 2.3 Schematic representation of central pathway and fatty acid metabolism in *S. cerevisiae*. This figure is drawn based on the information in [48, 101, 102]. See description on page 24-25.

2.2.2 Fatty acid degradation and regulation

Microorganisms are able to use fatty acids as carbon sources because they can take up and degrade fatty acids from the environment. In *E. coli*, the enzymes involved in fatty acid degradation are induced by long chain fatty acids (LCFAs) under both aerobic and anaerobic condition. The enzymes in aerobic degradation pathway are

encoded by the *fad* regulon, which is repressed by the control protein FadR. This regulon encodes proteins FadA, FadB, FadD, and FadE, which are responsible for transport, activate, and degrade fatty acids to shorter acyl-CoA and acetyl-CoA. In the absence of LCFAs, FadR binds to operator DNA sequences. In the presence of LCFAs, they are catalyzed into long chain acyl-CoAs and bind to FadR, which results in FadR releasing from the operator and allowing transcription of *fad* genes. There is another set of genes which encode enzymes YfcY, YfcX, and YdiD for an anaerobic fatty acid degradation pathway. *YfcYX* expression is repressed by FadR under aerobic condition, while it is not under FadR control under anaerobic growth on fatty acids [103].

In *S. cerevisiae*, fatty acid degradation occurs only in peroxisomes due to the metabolic compartmentalization [104, 105]; the breakdown route is shown in Figure 2.4. Fatty acids are converted to acyl-CoAs and shortened into acetyl-CoA by two carbons each cycle. Final product acetyl-CoA can be used as a building block in anabolic reactions, or catabolized in the TCA cycle. The process requires an array of β -oxidation enzymes including Pxa1p/Pxa2p, Pox1p/Fox1p, Mfe2p/Fox2p and Pot1p/Fox3p, and need various auxiliary enzymes to supply the proper substrates to the machinery.

Before the degradation steps, fatty acid released from triglycerides or imported from the environment are activated by fatty acid acyl-CoA synthetases [106]. There are six fatty acid acyl-CoA synthetases in *S. cerevisiae*, Faa1p-Faa4p, Fat1p and Fat2p. Faa1p and Faa4p are the main enzymes in cytoplasm. They are needed for activating LCFAs before they are transported into peroxisome by Pxa1p-Pxa2p. Faa2p and

Fat2p are located in peroxisome. It is suggested that Faa2p is involved in activating MCFAs. Fat1p activates VLCFA (>C20) before oxidation. There is no clear substrates preference for Faa3p. Besides the function of activating fatty acids, acyl-CoA synthetases also are needed in importing fatty acids from environment.

After LCFAs are activated to their CoA derivatives in the cytosol, they are transported into peroxisome by Pxa1p and Pxa2p transporter; while MCFAs are activated to medium chain acyl-CoA inside peroxisomes [107]. The first step, which is the rate-limiting step of β -oxidation, is catalyzed by acyl-CoA oxidase. *POXI* gene in *S. cerevisiae* encodes the only acyl-CoA oxidase, which can oxidize acyl-CoAs of various chain lengths. Disruption of the *POXI* gene makes yeast cells unable to survive on mediums with oleic acid as a sole carbon source [108]. While in *Y. lipolytica*, there are six acyl-CoA oxidases, each one has different chain length specificity [109, 110]. It has been demonstrated that modification of *POX* genotype can affect β -oxidation and further affect lipid accumulation in *Y. lipolytica* [111]. In the second step, Enoyl-CoA hydratases Mfe2p/Fox2p transform trans-2-enoyl-CoA into β -ketoacyl-CoA via (3R)-hydroxy intermediates. *S. cerevisiae* Mfe2p/Fox2p contain two dehydrogenase domains, domain A is most active with long chain and medium chain substrates, on the contrary, domain B prefers short chain substrates [104]. In the last step of β -oxidation, 3-ketoacyl-CoA thiolase Pot1p/Fox3p cleaves ketoacyl-CoA into acetyl-CoA and a C₂-shortened acyl-CoA, the latter is substrate for Pox1p/Fox1p. Acetyl-CoA is exported into mitochondria for further oxidation in TCA cycle or towards biosynthesis via the glyoxylate cycle [104].

Figure 2.4 Schematic representation of fatty acid β -oxidation in yeast peroxisomes. This figure is modified based on figure.1 in [104]. Protein Pxa1p-Pxa2p embedded in peroxisomal membrane translocate activated FAs into peroxisomes for degradations. The β -oxidation reactions are catalyzed by enzymes Pox1p/Fox1p, Mfe2p/Fox2p and Pot1p/Fox3p. See description on page 26-27.

The transcription of genes encoding enzymes in fatty acid β -oxidation can be induced dramatically by the fatty acids in the culture medium. The response of yeast cells is mediated by the Pip2p-Oaf1p transcription factors, which bind to ORE element in the promoters of these genes. It also causes the proliferation of peroxisome [104].

2.3 Metabolic engineering

2.3.1 Application of metabolic engineering for biofuel production

The development of microorganisms with high productivity and good metabolic performance is a prerequisite for efficient production of renewable biofuels. From the natural variant selection to the random mutagenesis and screening [112], industrial strains have been developed for several decades. However, there are some limitations in the traditional selection approaches, such as relative low efficiency, the unwanted unknown genotypic changes, and difficulty in further metabolic engineering of the strains. This leads to the seeking for more rational strategies to modify metabolic and cellular characteristics, for example, metabolic engineering [113] and enzyme engineering [114], in which molecular biological techniques such as recombinant DNA techniques [115] or gene disruption [116] are usually used. With the subsequent development of biotechnology: such as microarray; various omics including genome, transcriptome, proteome, metabolome, and fluxome [117]; high throughput phenotype screening; the construction of libraries of knock-out strains; multiplexed genome engineering; and genotyping methods [118], cell performance can be improved dramatically at the system level based on metabolic engineering approach [119-121]. In silico genome scale metabolic models were built, which greatly facilitate the modification of metabolic and regulatory networks of the cells [122-124]. The concept of systems metabolic engineering was also proposed as metabolic engineering integrated with systems biology and synthetic biology [29].

Until now, many promising biofuels have been produced in non-natural hosts through introducing synthetic metabolic pathways [28]. For example, *E. coli* KO11

was constructed by introduction of *pdc* and *adhB* from *Z. mobilis* and deletion of *frd* to prevent succinate production. *E. coli* KO11 produced ethanol at a yield of 95% in complex media [125]. In another example, metabolic engineering was performed in *E. coli* to create NADH driving forces by disrupting the *adhE*, *ldhA*, and *frd* genes, and to create acetyl-CoA driving forces by deleting the *pta* gene. Then, the driving force was coupled with the modified clostridial 1-butanol pathway, which provided an irreversible reaction catalyzed by trans-enoyl-CoA reductase (Ter). Finally, a high yield of 30 g/L 1-butanol was produced in the engineered strain [126].

Through metabolic engineering, there have also been great progresses in higher alcohols production [127]. For example, higher branched-chain alcohols were synthesized by diverting the 2-keto acid pathway [12, 128]. By introducing exogenous pathways, Prenyl alcohols such as Farnesol [129] and Geranylgeraniol [130] were produced from pyrophosphate intermediates in isoprenoid pathway. Fatty alcohols were produced by controlling fatty acid synthesis and degradation. By the expression of thioesterases, fatty acyl-ACPs were cleaved to produce C8-C18 FFA; by further overexpression of acyl-CoA ligases and fatty acyl-CoA reductases, up to 60 mg/L C12-C16 fatty alcohols were produced in *E. coli* [8]. By adopting similar method with further optimization, specific chain lengths fatty alcohol were selectively produced in *E. coli*: 449.2 mg/L C12/C14 alcohol were produced via coexpressing thioesterase gene *BTE*, acyl-CoA synthase gene *fadD*, and acyl-CoA reductase gene *acrI*; 101.5 mg/L C16/C18 alcohol were produced by coexpressing thioesterases gene *tesA*, acyl-CoA synthase gene *fadD*, and acyl-CoA reductase gene *FAR* [25]. In another study, 1-dodecanol (C12) and 1-tetradecanol (C14) were produced by coexpressing *BTE*, *fadD*, and acyl-CoA/aldehyde reductase gene *maacr*.

Total fatty alcohol of 1.65 g/L was produced in a fed-batch cultivation by balancing expression levels of these three genes and adding an extracting solvent in the medium [131]. Besides synthesizing alcohol from fatty acyl-CoA, fatty alcohol also could be produced via reduction of FFA or fatty acyl-ACP. Carboxylic acid reductase (CAR) can convert FFA into fatty aldehyde. Through expressing a CAR from *Mycobacterium marinum* and combining an aldehyde reductase, more than 350 mg/L fatty alcohols in a range of C8-C16 were obtained in *E. coli* in minimal media [16]. By expressing a fatty acyl-ACP reductase from *Synechococcus elongates*, together with the activity of endogenous *E. coli* alcohol dehydrogenase AdhP, 0.75 g/L fatty alcohols were produced under 2.5 L fed-batch cultivation condition [132].

As the predominant constituents of gasoline, diesel, and jet fuels, alkane and alkene production have got great progresses recently. In one study, an acyl-ACP reductase and an aldehyde decarbonylase from cyanobacteria were introduced into *E. coli*, which can convert fatty acyl-ACP to alkane and alkene. Over 300 mg/L of C13 to C17 mixtures of alkanes and alkenes were produced [15]. In another method, different chain lengths and branched alkanes were synthesized by modification of FFA pools: this was done by the expression of a thioesterase to alter hydrocarbon chain length, and coexpression of the branched-chain α -keto acid dehydrogenase complex and β -keto acyl-acyl carrier protein synthase III to synthesize branched fatty acids. After that, the coexpression of the reductase (*luxC*), synthetase (*luxE*), and transferase (*luxD*) genes coding for the fatty acid reductase complex from the *Photobacterium luminescens* luciferase operon, coupled with aldehyde decarbonylase from *Nostoc punctiforme*, FFA were converted into aliphatic n- and iso-alkanes/alkenes of various chain lengths in engineered *E. coli* [10]. Short chain fatty

acids serving as important component of petrol also have been produced in *E. coli*. Similar with other methods, several steps were adopted. Firstly, the *fadE* gene was deleted to prevent the degradation of fatty acyl-CoAs. Secondly, the *fadR* gene was deleted to enhance the activity of 3-oxoacyl-ACP synthase (FabH) thereby promoting the initiation of fatty acid biosynthesis. Thirdly, modified thioesterase was expressed to convert short chain fatty acyl-ACPs to FFA. Next, the endogenous fatty acyl-CoA synthetase, fatty acyl-CoA reductase from *Clostridium acetobutylicum* and fatty aldehyde decarbonylase from *Arabidopsis thaliana* converted short chain FFA into short chain alkanes. Finally, up to 580.8 mg/L of short chain alkanes were produced in the engineered *E. coli* [11]. In addition, there have also been reports for introducing synthetic metabolic pathway for producing propane [133] and pentane [134]. Furthermore, because there are many highly active endogenous aldehyde reductases (ALRs) that can convert aldehydes to alcohols in *E. coli*, in one study, 13 known ALRs were collectively deleted to reduce endogenous ALR activity to improve alkane production [135].

Based on the important reaction catalyzed by aldehyde decarbonylase, there have been a lot of studies about this enzyme recently. To accurately describe the reaction it catalyzed, it is redesignated as aldehyde deformylating oxygenase (ADO) [136]. Its activity is found to be inhibited by hydrogen peroxide (H_2O_2). The inhibition can be prevented by fusing a catalase to its N-terminal, which can convert H_2O_2 to O_2 [137]. Moreover, there is study about improving the ligand specificity by site-mutation [138], and improving catalyzing efficiency by fusing to alternative electron transfer systems [139].

Biodiesel, such as fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs), are usually produced by transesterification of triacylglycerols with short chain alcohols. Recently, oleaginous microorganisms are used for substituting conventional oil in biodiesel production [140]. For example, lipid was first accumulated in oleaginous yeast *Rhodospiridium toruloides* using lignocellulosic biomass and then transformed into biodiesel by enzymatic transesterification [141]. Beside enzymatic transformation, lipase-displaying whole cells could also be used as biocatalysts due to their low preparation costs and simple recycling procedure. *Pichia pastoris* whole-cell biocatalysts with expression of *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase (RML) were used to produce biodiesel in co-solvent media [142]. Biodiesel could also be produced in microbes by introducing novel biochemical reaction [8]. By combining the fatty acid and ethanol overproduction pathways, 674 mg/L FAEEs were produced in the metabolically engineered *E. coli* [8]. By expressing lipase 2 from *Candida sp.* 99-125 in *S. cerevisiae*, transesterification is accomplished *in vivo*, combining with an addition of 4 % ethanol into culture broth, the yield of FAEEs in yeast reached 11.4 mg/g dry cell weight [143].

As stated above, recent advances in metabolic engineering have provided new tools to engineer the native and de novo pathways for the production of various biofuels [144]. While several key points should be considered when conducting pathway optimization: “Elimination of competing pathways, redox balance for necessary cofactors, optimal enzyme expression levels, forward driving force to best facilitate carbon flux to the desired end product, and finally, methods to collect the produced product which goes hand-in-hand with avoiding product toxicity to the strain [127].”

2.3.2 Modification of fatty acid metabolism in *S. cerevisiae*

Researchers like to use model organisms as platform due to their known genomes, metabolic pathway, and a lot of tool available for genetic modifications. The strategies of synthetic biology make it possible to build novel metabolic pathways in model organisms. To produce commercial competitive biofuels, further metabolic engineering is definitely required. Several microbes, such as *E. coli*, *C. acetobutylicum*, *S. cerevisiae*, oleaginous yeast, and some photosynthetic algae have been used to produce fuel molecules [27-29, 145-147]. All the hosts already have the capacity to produce fatty acids, as they are key molecules in native biological systems. Modifying the existing fatty acid metabolism pathways can improve fatty acid production. Creating new synthetic routes via synthetic biology methods can expand the product spectrum.

S. cerevisiae, as a model eukaryotic cell, has been widely explored in biochemical production [148]. Although the number of literature published on fatty acid production using *S. cerevisiae* is far more limited than *E. coli*, *S. cerevisiae* has its own advantages which make it an appealing host. It is a robust industrial organism that can grow under low pH and various harsh environmental conditions. It has a fully sequenced genome [149], availability of good genetic tools, and well characterized metabolic pathways [150]. Besides, it has better organic solvent tolerance and well-established industry fermentation techniques. Hence, in recent years, there is increased interest in developing *S. cerevisiae* as a cell factory for fatty acid-derived biofuel production. Production of biodiesel was engineered in *S. cerevisiae* by the expression of heterologous lipase 2, which can convert

triacylglycerol or FFA to FAEEs [143]. In another report, *S. cerevisiae* was transformed into an oleaginous yeast with high level of fatty acids production through overexpression of the active diacylglycerol acyltransferase Dga1p [151]. Intracellular long chain fatty acid accumulation was enhanced by disrupting the citrate turnover and overexpressing a heterologous ATP-citrate lyase, which led to a 1.92-fold increase in C16:1 and a 1.77-fold increase in C18:1 [152]. Moreover, short chain fatty acids were successfully produced in *S. cerevisiae* by using the heterologous Homo sapiens type I fatty acid synthase (hFAS) with the heterologous short chain thioesterases from *Cuphea palustris* (CpFatB1) or *Rattus orvegicus* (TEII) [153]. In addition, to study the toxicity of biofuel on *S. cerevisiae*, the role of efflux pumps Snq2p and Pdr5p on alkane exportation and tolerance were investigated at a transcriptome level in *S. cerevisiae* [154]. To show the potential of *S. cerevisiae* as a biofuel production platform, possible biofuels can be produced in *S. cerevisiae* was summarized in Figure 2.5.

In our project, we intend to construct an engineered *S. cerevisiae* with high FFA accumulation ability. Through the approach of metabolic engineering, we engineered the fatty acid synthetic and degradation pathway of *S. cerevisiae* by deletion of endogenous enzymes or introducing exogenous enzymes. GC-MS-based metabolomics and LC-MS-based proteomics were adopted to investigate the engineered strains and provide guidance in future study.

Figure 2.5 Production of fatty acid-derived biofuels through fatty acid metabolic pathway in *S. cerevisiae*. Dashed lines represent multiple reaction steps. PEP, phosphoenolpyruvate. ACC1: Acetyl-CoA carboxylase; ACP: acyl carrier protein; ACR: fatty acyl-CoA reductase (ACR1 [155] and ACR2 [156]); FAR: fatty acyl-CoA reductases [8] ; CAR: carboxylic acid reductase [16]; AHR: aldehyde reductase [16]; ADO: aldehyde deformylating oxygenase [135, 136]; Lip2: lipase [143]; AT: acyltransferase (ester synthase atfA) [8]. The fatty acid biosynthetic machinery could be utilized to produce these valuable biofuels: alcohols could be produced by reduction of fatty acyl-CoA or FFA to fatty alcohol, whereas alkanes could be produced by reduction of fatty acyl-CoA or FFA to the aldehyde followed by decarbonylation or by decarboxylation catalyst [14], and biodiesel could be produced by converting fatty acids to esters via esterification with small alcohols by ester synthase.

2.4 Proteomics and metabolomics

Proteomics and metabolomics, the universal detection and study of protein and metabolite changes in a specific biological sample, have drawn a significant amount of attention in recent years. As the downstream product of DNA and RNA (Figure 2.6), the holistic investigation of proteins and metabolites in cells could provide us more straightforward view of the regulation of biochemical networks in cells. The great potential they have made them attractive to apply not only on the understanding of normal physiological processes but also in biomarker discovery and disease

diagnosis [157-159]. They are also great tools in analyzing a perturbed metabolic network at a systemic level [160]. In the study of microbial biofuel production, they have been adopted to investigate lipid metabolisms [161] and accumulation mechanism [162].

Figure 2.6 The relationship between various omics. Genomics studies the complete set of genes in a cell, tissue, or organism, while transcriptomics investigates gene expression patterns. Proteomics looks into the expressed protein products and their interactions, while metabolomics aims to characterize the global metabolite profile in a cell, tissue, or organism.

2.4.1 Proteomics

Proteins perform an endless number of functions in the cell, including catalyzing biochemical reactions, replicating DNA, responding to environmental stress, and transporting molecules. The term of proteome refers to the complete set of proteins in a cell, tissue, or organism. The proteome is highly dynamic and it changes in different times and environments. Proteomics is the study of the proteome, including the study of protein structure and function, what they interact with, and how they contribute to biological function [163].

There are two major levels of scope for proteomics studies: global and targeted proteomics. Global proteomics aims to investigate all of the proteins in a cell or tissue at the same time. Targeted proteomics focuses on a well-defined subset of the proteome. The proteomic studies also can be classified into three types: i) Quantitative protein profile comparisons, such as profile comparisons between normal versus diseased cells and identification of the responses of cells to a stimulus at a certain time. ii) Protein composition analysis, such as comprehensive analysis of protein component in a cell or cellular compartment. Different from quantitative protein profiling, it does not involve comparisons of different samples or quantitation of protein levels. iii) Protein-protein interaction analysis, which aims at providing new insights into cellular pathways and developing of a protein-network map by determination of protein-protein interactions involved in different reactions [164]. Each type can have a global or targeted scope.

The key considerations in a proteomic experiment are the protein concentration, purification, digestion, and sample fractionation. There are different techniques used for sample separation, such as one and two dimensional gel electrophoresis, differential image gel electrophoresis, and liquid chromatography (LC). Compare to gel electrophoresis, LC is much quicker and more sensitive, especially the combination of LC with tandem mass spectrometry (MS) offers more accurate detection.

Numerous LC techniques have been developed, such as normal phase chromatography, reversed phase chromatography, flash chromatography, partition chromatography, liquid-solid chromatography, ion exchange chromatography, size-exclusion chromatography, affinity chromatography, and chiral chromatography. The improvement in LC makes it possible to detect a wide range of molecules from small

size metabolites to large size peptides and proteins.

As for protein identification in proteome projects, MS is the most common method [165, 166]. Figure 2.7 shows a general flow chart for MS. One mass spectrometer consists of an ion source, a mass analyzer, and a detector. Separated samples containing proteins or peptides are introduced into MS. An electrically heated cathode (filament) in the ion source emits a beam of electrons with high kinetic energy to collide and break molecules for creating charged fragments. Then, the charged molecular fragments are transported to the mass analyzer and separated according to their mass-to-charge (m/z) ratios. In the final step, the detector records a mass spectrum when the ion passes by or hits its surface. As shown in figure 2.7, different types of ion source and mass analyzer are currently in use. Different techniques offer different advantages and limitations in regard to instrumental sensitivity, resolution, mass accuracy, detection throughput, and detection range.

Figure 2.7 General flow chart for mass spectrometry. GC: Gas chromatography; LC: Liquid chromatography; CE: capillary electrophoresis; EI: electron impact ionization; CI: chemical ionization; APCI: atmospheric pressure chemical ionization. ESI: electrospray ionization, MALDI: matrix-assisted laser desorption/ionization. TOF: time of flight; FTICR: Fourier transform ion cyclotron resonance.

2.4.2 LC-MS/MS-based proteomics

Global protein analysis is challenging due to the highly diverse physicochemical properties of amino acids. MS-based proteomics has developed as an indispensable technique for comprehensively analyzing complex protein samples. Currently, there are two approaches used for characterizing proteins. The first approach is referred to as "top-down" proteomics, intact proteins are ionized by either ESI or MALDI, and then introduced into a mass analyzer. The second approach is called "bottom-up" proteomics, proteins are enzymatically digested into peptides, usually by trypsin. These peptides are subsequently introduced into the mass spectrometer and identified by peptide mass fingerprinting or tandem MS. The "bottom-up" approach is much more popular, because peptide MS is more sensitive than whole protein MS, cheaper instrument can be used in characterizing peptides, and sample preparation for smaller peptide fragments is much easier than whole protein analysis. A typical workflow for LC-MS/MS-based "bottom-up" proteomics experiment is showed in Figure 2.8.

In the first step of proteomics experiment, proteins needing analysis are extracted from cells or tissues. This step may involve biochemical fractionation or affinity separation. If sub-proteome needs to be analyzed, it often needs one-dimensional gel electrophoresis to pre-fractionate the proteins. In the second step, proteins of interest are digested enzymatically into peptides with C-terminally protonated amino acids, and then labeled with chemical or enzymatic methods. In the third step, the labeled peptides are separated by HPLC and then eluted into an ionization source where they are ionized into charged droplets. In the fourth step, the peptide precursor ions enter

the mass spectrometer and are scanned by MS. In the fifth step, single peptide precursor is introduced into tandem MS, the specific fragment ions are generated and spectra are recorded by MS/MS. Finally, proteins are identified by matching the MS and MS/MS spectra against known peptide sequences from protein sequence databases. The protein abundance are then interpreted based on the biological system under investigation.

Figure 2.8 Schematic workflow for LC-MS/MS based proteomics experiment. A typical LC-MS/MS based proteomics experiment includes: target protein extraction, protein digestion and labeling, peptide separation and ionization, protonated peptides scanned by MS, peptide fragmentation by MS/MS, and data analysis. See description on page 40-41.

2.4.3 iTRAQ™-based quantitative proteomics

Quantitative proteomics aims to determine the amount of proteins in a certain sample and providing information about differences between samples, such as comparing samples from healthy and diseased cells. For proteomic quantification, either label or label-free technique can be used.

For label-free proteomics, two or more samples were compared and quantified based on precursor signal intensity, the number of MS/MS spectra that identify the protein of interest (spectral counting), or the average of the total ion current (TIC) for all of the MS/MS spectra that identify a protein (spectral TIC) [167]. The first approach is useful when applied to high precision mass spectra, such as those signal extracted by high-resolution TOF, FTICR or Orbitrap mass analyzers. By comparison, the second spectral counting method just counts the number of spectra identified for a particular peptide in different samples. The third method is an extension to the spectral counting method whereby the unique abundance value is taken account of in every spectral count. The advantages of label-free method are: there is no cost for labeling reagents; there is no need for the time-consuming labeling steps; and it has higher dynamic range of quantification compared with stable isotope labeling method. However, the limitation is that it needs high reproducibility at each step because a small amount of sample differences will cause the variation of ionization rate and retention time of a given peptide [168].

Compared with label-free method, using stable isotope labels to label different peptides simultaneously allows for distinguishing identical proteins in different samples by the MS. The chemical reagents used to label the samples have difference on the isotopic composition therefore on their molecular weights. Separately labeled samples are mixed prior to LC-MS and analyzed simultaneously, the differences in the signal intensities of the isotope pairs indicate relative difference in the abundance of the given proteins. Thus the labeling strategies can increase the quantitative accuracy and throughput. Generally, there are three major types of labeling: metabolic labeling, chemical labeling and enzymatic labeling.

Metabolic labeling [169] is an approach for labeling protein *in vivo* by metabolic incorporation of stable isotopes (in the form of labeled amino acids) into protein. One of the examples is SILAC (Stable Isotope Labeling by Amino acids in Cell culture) method [170]. Usually isotope coded amino acids are leucine, arginine, tyrosine, or lysine. SILAC has high quantitative accuracy at the level of growing cells. However, the method is only applied to cell culture related experiments.

Chemical and enzymatic labeling methods are using chemical derivatization or enzymatic modification methods *in vitro* to label proteins or peptides after protein extraction and digestion. Because these two approaches are performed after harvesting of cells and purification of proteins, they have the advantage to label a variety of sample types. In chemical labeling method, the label is introduced into sulfhydryl groups or amine groups of proteins or peptides by a chemical reaction, or through acetylation or esterification of amino acid residues. On the other hand, the peptides can be labeled by the heavy stable isotope labels in an enzymatic reaction with heavy water (H_2^{18}O).

There are two kinds of tags used in the labeling methods: isotopic and isobaric tags. The former tags have different masses; the latter tags are the same in mass, but yield reporter ions of different masses during tandem MS. The typical example of isotopic labeling is isotope-coded affinity tags (ICAT) [171]. In an ICAT experiment, denatured and reduced proteins are first derivatized with the ‘heavy’ or ‘light’ ICAT reagent on the cysteine residues. Secondly, the labeled proteins are combined and cleaned up. Thirdly, they are digested with an appropriate protease. Next, the cysteine-containing peptides, labeling with ‘heavy’ and ‘light’ isotope tags, are

captured on an avidin column. Finally, Peptides are subsequently eluted from the column and analyzed by MS. The limitation of this method is that it is only applied to binary (2-plex) set of samples.

Multiplexing labeling can be achieved in the commercially available isobaric labeling method: tandem mass tags (TMT) [172] and isobaric tags for relative and absolute quantitation (iTRAQ) [173]. They have turned out to be useful methods for comparing multiple samples in the same experiment. Another advantage of isobaric tags is that the sensitivity in the MS mode is improved because the labeled peptides are co-eluted from the LC column, which means the MS signal is not split into different peaks, as with isotopic labeling. The disadvantages are that the isobaric labels can be cost-prohibitive and they are limited to MS which can efficiently detect ions that are present at a relatively low m/z .

In our project, the iTRAQ method was adopted. Currently, there are two main types of iTRAQ reagents available: 4-plex and 8-plex. Take the 4-plex as an example, as shown in Figure 2.9, the iTRAQ reagent uses differential isotopic enrichment with ^{13}C , ^{15}N , and ^{18}O atoms to maintain an overall mass of 145.1. It consists of a charged reporter group (114.1-117.1, based on N-methylpiperazine, unique to each of 4 reagents), a neutral balance group (31-28, carbonyl), and a peptide reactive group (NHS ester, labels all N-termini and lysine side-chains). All peptides from four different samples can be labeled simultaneously. In a general iTRAQ-based quantitative proteomics experiment, each sample is labeled with one member of the four iTRAQ Reagents and then pooled prior to LC-MS analysis, the mixture of four identical peptides appears as a single, unresolved precursor ion in MS (identical m/z).

In the following peptide fragmentation by MS/MS, the balance group is lost (neutral loss), while charge is retained by the reporter group fragment as distinct masses (114.1–117.1 Da).

Figure 2.9 Working Mechanism of iTRAQ Reagents [173]. (Permission from ref. 173 was obtained from publisher to use this figure). *A*: the components of the multiplexed isobaric tagging chemistry. The complete reagent is composed of a reporter group, a mass balance group, and a peptide-reactive group. *B*: The overall mass of reporter and balance group of the molecule remains constant (145.1 Da) using differential isotopic enrichment with ^{13}C , ^{15}N , and ^{18}O atoms. *C*: illustration of the isotopic tagging used to arrive at four isobaric combinations with four different reporter group masses.

iTRAQ™ reagents can provide researchers a much deeper understanding of biological samples, because they allow global peptide labeling, retain post-translational modification (PTM) information, can do multiplexing, and simplify workflows. A general flowchart of iTRAQ-based quantitative proteomics

experiment is showed in figure 2.10.

Figure 2.10 Flowchart of iTRAQ-based quantitative proteomics experimental design. Up to four samples can be analyzed in a single experiment using 4 plex iTRAQ® Reagents Applications Kit. In a typical workflow, total proteins are firstly extracted from control and samples separately, then they are reduced, digested and labelled with iTRAQ® reagents. After combining into one sample mixture, the mixture are analyzed by online 2D LC-MS/MS for protein identification and quantitation. Lastly, the identified proteins are analyzed by proteomics software, such as Spectrum Mill software.

2.4.4 Metabolomics

Metabolites are the intermediates and products of metabolism. They usually refer to small molecules and have various functions, including fuel, structure, signaling, and

interaction with enzymes. The metabolome refers to the complete set of metabolites in a cell, tissue, or organism. As final downstream product of gene transcription, the metabolome has a direct effect on the phenotype of the cell. Metabolomics aims at studying global metabolite profile under specific conditions [174].

Metabolomics can be used to determine phenotype differences between WT and genetically manipulated cells. It also can be used to predict the function of unknown genes via comparing the metabolic perturbations resulted from deletion/insertion of known genes. The pathologist or toxicologist can use metabolite profiling to detect the physiological changes caused by toxic attack of chemicals, which usually are related to specific syndromes [175]. Metabolomics can also be used to characterize the interactions of cells with their environments, which is useful in detecting the response of cells to environmental changes.

A typical workflow for metabolomics experiment is showed in Figure 2.11. The general analytical tools of metabolomics are NMR or MS. NMR is a rapid and non-invasive method to characterize the metabolite patterns. However, NMR has limited sensitivity and resolution, while MS methods in combination with GC or LC separation steps are preferred by lots of researchers. Although GC/LC-MS experiments (1-2 hour per sample) are slower than NMR or directed-injected MS experiments (minutes per sample), they have substantial increases in sensitivity and permit more analytes to be analyzed per run.

Gene modification or environmental influence usually affects the metabolic pathway perturbation. Elucidation of the regulation of metabolic pathways not only requires to examine the changes of proteome, but also needs to identify the changes of

physiological substrates of enzyme: metabolites. By integrating the metabolite profile with protein expression pattern, researchers hope to unravel the underlying mechanism of engineered microbial systems [176].

Figure 2.11 Schematic workflow for metabolomics experiments. A typical metabolomics experiment includes: metabolites extraction from target sample, metabolite separation by GC or LC, sample analysis by MS or NMR, metabolite identification and data analysis.

There has been great progress in applying proteomics and metabolomics analysis to investigate the engineered strains. For example, the metabolic responses to ethanol of *S. cerevisiae* were examined by GC-MS-based metabolomics approach [177]. Metabolic fluxes were predicted in *E. coli* via the metabolomics and proteomics analysis [178]. Metabolite profiling of fungi and yeast help researchers to identify new compounds and bioprocesses [179]. In our project, they were used to comparatively analyze the protein and metabolite changes between the engineered strains and WT, so as to investigate the metabolic network regulation in the engineered strains.

2.4.5 GC-MS-based metabolomics

GC coupled with MS with relative complete databases is a liable method in studying metabolomics. GC-MS has one of advantages is that identification of analytes is based on retention time and mass spectrum. Because a compound has reproducible MS fragmentation spectra when ionized by a fixed electron voltage (general $\sim 70\text{eV}$), the MS spectra gained in GC-MS are instrument independent. This makes it convenient to create public databases and have better compound identification capabilities as compared to other separation techniques. Furthermore, GC-MS allows for quantifying compounds. However, the drawback of GC-MS is that this method can only analyze volatile compounds or compounds that are volatile after derivatization, and derivatization usually need extensive sample treatment. But, if the analysis is focused on low molecular weight compounds, it is highly sensitive, efficient, and reproducible [180]. The detector should be well maintained including cleaning EI source and replacing the filament as necessary and in accordance with the manufacturer's guidelines to ensure the reproducibility and comparability. For accurate comparisons, it is important that control and experimental cell samples are processed and analyzed in parallel. Usually, GC-MS-based metabolomics is used to detect and analyze organic acids, amino acids, mono- and di-saccharides, sterols, and other compounds. A general GC-MS schematic is showed in Figure 2.12. A general GC-MS system consists of two main components: GC and MS. The GC usually uses a capillary column to separate different molecules based on their relative affinity for the stationary phase of the column. Different column dimensions (length, diameter and film thickness) and phase properties have different separation efficiency. Different compounds have different retention times which allows the MS

to capture the pure compounds, ionize them, and detect the ionized molecules separately using their mass to charge ratio.

Figure 2.12 The scheme of typical GC-MS. A typical GC-MS system consists of two main parts: the GC and the MS. After sample is injected into GC, the GC uses a capillary column to separate different molecules based on their relative affinity for the stationary phase. At different retention times, the MS captures these molecules and breaks each one into ionized fragments, and detects these fragments based on their mass to charge ratio.

3 Up-regulation of FFA production in *S. cerevisiae* through β -oxidation modification

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3.1 Introduction

The renewable synthesis of fatty acid-derived biofuels and chemicals has gained considerable attention in recent years. In several studies, fatty acids are overproduced in microbes and later converted to fuels or chemicals through chemical conversion [14] or bioconversion [8, 15]. Other studies directly utilize fatty acid-rich feedstock as a carbon source to produce biofuels and chemicals through the complete oxidation of fatty acids [17] or using the intermediates of β -oxidation [18, 19]. The availability of fatty acids from nonedible fatty acid-rich crops, oleaginous fungi/algae and industrial by-products encourage the use of fatty acid feedstock as a cheap carbon source. In addition, fatty acids have a product yield advantage over sugars when used as a carbon source due to the efficient β -oxidation metabolism process [17], which has 100% carbon recovery. In short, fatty acids can be exploited as an alternative feedstock to lignocellulosic sugars for microbial bioconversion platforms.

S. cerevisiae has been commonly used in industrial fermentation. It has better tolerance to organic solvent than *E. coli* and also owns a well-studied β -oxidation

pathway. Aiming to utilize fatty acid-rich feedstock to produce biofuels or biochemicals, we investigated fatty acid metabolism in *S. cerevisiae*. In this section, we intended to generate more medium chain fatty acids (MCFAs), which are of higher value compared to other fatty acids [5]. Because fatty acid-rich feedstock usually contains sugars and fatty acids in carbon length at C16 and C18 [140], we used oleic acid as a glucose co-substrate to test the bioconversion ability of engineered strains during culture.

Fatty acid β -oxidation is the main pathway for fatty acid degradation in yeast. It enables yeast cells to grow on medium containing fatty acids as the sole carbon and energy source. This process is carried out in the peroxisomes of *S. cerevisiae* [104, 105]. The first and rate-limiting step is catalyzed by acyl-CoA oxidase using acyl-CoA as the initial substrate. Before the degradation process begins, long chain fatty acids (LCFAs) are activated into long chain acyl-CoAs by acyl-CoA synthetases in the cytosol and then are transported into peroxisomes by Pxa1p/Pxa2p. In contrast to LCFAs, MCFAs can freely enter peroxisomes and become medium chain fatty acyl-CoAs with the help of acyl-CoA synthetases. In *S. cerevisiae*, the *POX1* gene encodes the only acyl-CoA oxidase, which can oxidize short to long chain acyl-CoAs. Disruption of *POX1* prevents yeast survival on medium using oleic acid as the sole carbon source [108]. In contrast to *S. cerevisiae*, the yeast *Y. lipolytica* has several acyl-CoA oxidases with different chain length specificities [109, 110]. It has been demonstrated that modification of *POX* genotype can affect β -oxidation and further influence lipid accumulation in *Y. lipolytica* [111], but there is no data about changes specifically to MCFAs.

To use fatty acid-rich feedstock to generate tailored fatty acids for biofuel production, we modified the β -oxidation pathway of *S. cerevisiae*, both to maintain LCFA oxidation and to prevent MCFA oxidation. The strain was engineered with the intention to accumulate more fatty acids with a higher ratio of MCFA when cells were grown on fatty acid-rich feedstock. To achieve this aim, the *S. cerevisiae* deletion strain $\Delta pox1$ was obtained in which the *POX1* gene, the sole acyl-CoA oxidase, was deleted. Then, acyl-CoA oxidase Aox2p (encoded by *POX2*) from *Y. lipolytica* [181], which has a preference for long-chain acyl-CoA substrates, was expressed. The replacement of *POX1* with *POX2* led to the blocking of β -oxidation pathway. To unblock the β -oxidation pathway in the Aox2p-expressing strain, peroxisomal carnitine octanoyltransferase (*CROT*) [182] from *Mus musculus* was also expressed. This transferase moves medium chain fatty acyl-CoAs out of peroxisomes by producing medium chain fatty acyl carnitine esters. The modified pathway is depicted in Figure 3.1. The effect of β -oxidation cycle modification on fatty acid profile and accumulation was investigated and discussed.

Figure 3.1 Genetic modification of the β -oxidation pathway in *S. cerevisiae*. The dashed line represents the original pathway; the solid line represents the modified

pathway. The only acyl-CoA oxidase (encoded by the gene POX1) in the S. cerevisiae genome was deleted, and the POX2 gene from Y. lipolytica, which encodes acyl-CoA oxidase with a preference for long chain acyl-CoAs, was expressed. To unblock the β -oxidation pathway, peroxisomal carnitine octanoyltransferase (CROT) from Mus musculus was also expressed to transport medium chain fatty acyl-CoAs out of peroxisomes.

3.2 Materials and methods

3.2.1 Yeast strains and growth conditions

The yeast strains (Table 3.1) used in this study were derived from *S. cerevisiae* BY4741. The strains were cultivated at 30 °C in YPD complete medium [1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose], supplemented with 200 μ g/ml G418 (Sigma, Singapore) when necessary. SC Minimal Medium (YNBD) was comprised of 0.67% yeast nitrogen base (without amino acids but with ammonium sulfate, Invitrogen), 2% dextrose and amino acid drop out (without URA, Clontech). YNBD_{0.5}O₃ (YNB supplemented with 0.5% dextrose and 3% oleic acid (from Fluka 75096)) was used for the optimum accumulation of fatty acids. Minimal oleic acid medium (YNBO) was used to test the fatty acid degradation ability of the engineered strains. YNBO was YNB supplemented with 1% oleic acid as the sole carbon source.

3.2.2 Construction of plasmids pVTU260-POX2 and pVTU260-POX2-CROT

Molecular cloning was performed according to standard procedures. The QIAprep Spin Miniprep Kit was used for plasmid preparation. The QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit (QIAGEN) were used for purification of gene fragments. Oligonucleotide primers (Table 3.2) were synthesized at 1st BASE

Pte Ltd (Singapore).

Table 3.1 Strains and plasmids list

Name	Description	Source
WT	<i>S. cerevisiae</i> wild type strain BY4741 (<i>Mat a</i> ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i>)	EUROSCARF
<i>Δpox1</i>	BY4741; <i>Mat a</i> ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i> ; YGL205w::kanMX4	EUROSCARF
WT[pVTU260]	Wild type strain transformed with pVTU260	Present study
WT[pox2⁺]	Wild type strain transformed with pVTU260-POX2	Present study
<i>Δpox1</i>[pVTU260]	<i>Δpox1</i> transformed with pVTU260	Present study
<i>Δpox1</i>[pox2⁺]	<i>Δpox1</i> transformed with pVTU260-POX2	Present study
<i>Δpox1</i>[pox2⁺crot⁺]	<i>Δpox1</i> transformed with pVTU260-POX2-CROT	Present study
pVTU260	Eukaryotic expression vector with ADH1 promoter and terminator, URA3 marker	EUROSCARF
pUG27	loxP-his5-loxP gene disruption cassette plasmid	[116]
pVTU260-POX2	The gene <i>POX2</i> encoding acyl-CoA oxidase 2 (Aox2p) with a His-tag sequence before the 3' termination codon, cloned into pVTU260	Present study
pVTU260-POX2-CROT	The <i>CROT</i> gene expression cassette containing the three PCR fragments was assembled into pVTU260-POX2	Present study

The gene *POX2* (GenBank: accession no. CR382132.1) encoding acyl-CoA oxidase 2 (Aox2p) was synthesized by Geneart (American) with optimized codons. The GenBank accession number for the codon-optimized sequence of *POX2* is KC912711. The primers used for PCR were POX2F-pvtu260 and POX2R-pvtu260. The forward primer introduced a unique *NheI* restriction site and a yeast consensus sequence (CAAA) before the 5' initiation codon. The reverse primer introduced a

His-tag sequence before the 3' termination codon and a unique *Bam*HI restriction site after the termination codon. The PCR products were digested with *Nhe*I and *Bam*HI and then ligated into the pVTU260 vector, which had also been digested with *Nhe*I and *Bam*HI. The ligation product was transformed into *E. coli* Top10. The resulting plasmid pVTU260-POX2 was verified by restriction analysis and sequencing.

The gene *CROT* (NM_023733.3) was also codon optimized and synthesized by Geneart. The GenBank accession number for the codon-optimized sequence of *CROT* is KC912712. Codon-optimized *CROT* was amplified using the primers TEFp-CROTF and CROTR-TEFt. A *Nhe*I restriction site, yeast consensus sequence and V5-tag was introduced before the 5' initiation codon. Because *Crot* contains a peroxisome-targeting signal peptide (AHL) at the C-terminal end, the V5-tag was placed at the N-terminal to prevent interference with the peroxisome-targeting signal peptide. The *Ashbya gossypii* *TEF2* promoter and terminator were PCR-amplified individually from pUG27 [116] using the primer pair TEFp-pUG27-F and TEFp-R and the primer pair TEFt-F and TEFt-R. The *CROT* gene expression cassette containing the three PCR fragments was assembled into pVTU260-POX2 digested with *Bst*EII and *Xma*I using the Cold Fusion Cloning Kit (SBI). The resulting plasmid pVTU260-POX2-CROT was verified through restriction analysis and sequencing.

Table 3.2 Primers used in this study

Primer name	Sequence(5'-3')
POX2F-pvtu260	CGGCTAGCCGCAAAATGAATCCAAACAATAC
POX2R-pvtu260	CGGGATCCGGTCAATGGTGATGGTGATGATGTTCTTCATCCAATT C
TEFp-CROTf	GTAGGAGGGCTTTTGTAGATGCTAGCCAAAGGTAAGCCTATCCC TAACCCTCTCCTCGGTCTCGATTCTACGATGGA
CROTf-TEFt	ACAACACTCCCTTCGTGCTTCCCCCGGGGGGTCACAAGTGGGC GGTATTCATC
TEFp-pUG27-F	GAAGATCGTCGTTTTGCCAGGTGACCGACAACCCTTAATATA
TEFp-R	GGCTTACCTTTGGCTAGCAT CTACAAAAGCCCTCCTAC
TEFt-F	CCGCCCCACTTGTGACCCCCCGGGGGGAAGCACGAAGGGAGTGT TGT
TEFt-R	TTAATTATATCAGTTATTACCCGGGTGAGCGAGGAAGCGGAAGA
Ps2	GGCTTCAAATCGTCCATC
POX2-F2	GCTGCTCATACTGCTAC
ADH-F	CCCCCGGGGGGTTTCGCCACCTCT
ADH-R	GGTGACCCTTGGATCATATCGC
crotF-2	TTAGAATCCCATCCC
crotR-2	CTCAAGTAACCGACCA

3.2.3 Cell transformation and control cell development

The plasmids pVTU260-POX2 and pVTU260-POX2-CROT were transformed separately into $\Delta pox1$ yeast competent cells using the lithium acetate method [183].

Control cells were created by transforming the empty vector pVTU260 into the WT and $\Delta pox1$ strain. To examine if expression of Aox2p caused growth defects, pVTU260-POX2 was also transformed into the WT strain as a control. Colonies on the screening plates were verified by colony PCR. Primer Ps2 and POX2-F2 are used to test if *POX2* gene is successfully transformed into yeast (PCR product 554bp). Primer ADH-F and ADH-R were used to test if the empty plasmid is successfully transformed into yeast. Primer crotF-2 and crotR-2 are used to test if CROT gene is successfully transformed into yeast (PCR product 1371bp).

3.2.4 Western blot analysis

After 24 h, 5 ml of cell culture was collected by centrifugation at 8000 rpm. After washing once with lysis buffer (50 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM EDTA), the pellet was resuspended in lysis buffer to an OD₆₀₀ of 100. The peroxisome proteins were isolated using a peroxisome isolation kit (Sigma). Total proteins were mixed with loading buffer and boiled at 95 °C for 5 min and separated on a 10% SDS%-PAGE (Bio-Rad). The separated proteins were transferred to a nitrocellulose (NC) membrane (GE Hybond, USA) at 21 V for 1 h 20 min using a semi-dry apparatus. After incubating the NC membrane in blocking buffer (PBST + 5% nonfat, dry milk, w/v) for 1 hour at room temperature, the membrane was probed with an anti-V5 antibody or an anti-His antibody (Sigma) at 4 °C overnight. Then, the membrane was incubated with an anti-mouse IgG HRP-conjugated secondary antibody (#31430, Pierce) at the dilution suggested by the manufacturer. After washing with PBST buffer, the signal was detected with the ECL Western blot reagent.

3.2.5 Growth tests of control and engineered strains

Yeast cells were collected after overnight culture in liquid YNBD medium at 30 °C. The cells were resuspended at a cell density of 5×10^3 cells/ μ l after washing twice in sterile distilled H₂O. Cells (5 μ l) were spotted onto YNBD or YNBO-agar plates. The first drop contained 2.5×10^4 cells, and each subsequent drop was diluted six fold more than the previous one.

3.2.6 Sample preparation for metabolite and fatty acid analysis

Five milliliters of liquid YNBD medium was inoculated with a single colony from agar plate and cultured overnight at 30 °C with shaking. Then, the overnight culture was used to inoculate 50 ml liquid YNBD or YNBD_{0.5}O₃ medium. The initial OD₆₀₀ was adjusted to approximately 0.3. The cells were cultured by shaking at 250 rpm and 30 °C. Same amounts of yeast cells (8×10^7 cells) were separately collected at different growth time points. The intracellular metabolites were extracted from control and engineered cells based on previously published work [184, 185]. In general, the culture sample was centrifuged for 5 min at 4 °C. The supernatant was collected for extracellular metabolite analysis. The cell pellet was washed twice with cold methanol (<-40 °C) and then collected.

3.2.7 Fatty acid analysis of cells and culture supernatants

The lipids were extracted from yeast cells using an adjusted chloroform-methanol 2:1 method [186]. The cell pellet was resuspended in 1000 μ l of 0.9% NaCl solution and then acidified with 200 μ l of acetic acid. As an internal standard (IS) to correct for metabolite loss during sample preparation, 10 μ l of 10 mg/ml heptadecanoic acid

and heptanoic acid dissolved in ethanol was added to the extraction solvent. After adding an equal volume of glass beads, the cells were disrupted in a FastPrep®-24 Instrument (6004-500, MP Biomedicals) for 30 s and cooled in ice for 30 s; this procedure was repeated 4 times. Then, 3 ml of a chloroform-methanol 2:1 mixture was added, and the samples were inverted several times, vortexed vigorously, and centrifuged at 10000 g for 10 min at 4 °C. The aqueous layer and cell debris were transferred to a new tube using aspiration. The chloroform layer (lower) was collected. An additional 3 ml of the chloroform-methanol 2:1 mixture was added to the aqueous layer to further extract lipids. Then, the chloroform layer was rotary evaporated to near dryness overnight using a TurboVapH LV Concentration Workstation.

For the fatty acids in the culture medium, the Solid-Phase Extraction (SPE) method [187] was adopted. First, the SPE column (Strata C18-E 200 mg/3 ml) was activated with 2.5 ml methanol and washed with 5 ml water. Then, 10 ml culture supernatant to which 0.5 ml 1 M HCl, 2 µl 10 mg/ml heptadecanoic acid and 2 µl 10 mg/ml heptanoic acid were added was passed through the SPE column. The SPE column was washed with 5 ml water and dried at room temperature. Next, 1.5 ml chloroform was used for fatty acid elution. The chloroform in elution products was also evaporated to near dryness.

Fatty acid analysis was performed according to previous work [187]. Fatty acid methyl esters (FAMES) mix C₈-C₂₄ (SUPELCO, 18918) was used as a standard. Three independent experiments were conducted. The dried lipid residue was redissolved in 500 µl BF₃-methanol 10% (FLUKA, 15716) and incubated in a sealed

vial in a 95 °C heater for 20 min. FAMES were extracted with 300 µl n-hexane after the addition of 300 µl saturated NaCl in water. All of the samples were analyzed using an Agilent Technologies 7890A-5975C GC-MS system equipped with a HP-5MS capillary column (30 m×0.250 mm i.d.; film thickness: 0.25 µm; Agilent). Samples of 1 µl were injected into the column by splitless mode using an autoinjector. Helium was used as a carrier gas at 1.1 mL/min. The inlets and MS source temperatures were maintained at 250 and 230 °C, respectively. For fatty acid analysis, the oven temperature was maintained at 80 °C for 1 min and ramped to 250 °C at a rate of 7 °C.min⁻¹, then held at 250 °C for 10 min. Data were acquired in a full scan from 35 to 600 m/z. The detected FAME peaks were integrated. Amounts were calculated with reference to the IS heptadecanoic acid, and the relative response factors were calculated.

3.2.8 Metabolic profiling

The sample for metabolic profiling was derivatized according to previous work [188]. For metabolic profiling, the same GC-MS system was used. The oven temperature was maintained at 75 °C for 4 min and raised at 4 °C.min⁻¹ to a final temperature of 280 °C and held for 2 min. Mass spectra were recorded from 35 to 600 m/z with a scan time of 0.2 s. Chromatogram acquisition and mass spectra identification were processed using the Agilent MSD Chemstation Data Analysis software. The detected metabolite peaks was identified by searching the NIST08 mass spectral library. The compounds were quantified from the peak area relative to the IS ribitol. No response factors were made.

3.3 Result

3.3.1 Gene modification of the β -oxidation pathway in *S. cerevisiae*

The modification of the β -oxidation pathway is based on the deletion of one gene and the expression of two proteins. The deletion strain $\Delta pox1$ was obtained from EUROSCARF and tested for growth on YNBO. The $\Delta pox1$ strain was unable to grow on medium in which oleic acid was the only carbon source. This is consistent with a previous report [108].

3.3.1.1 Plasmid construction of pVTU260-POX2 and pVTU260-POX2-CROT

Plasmid pVTU260-POX2 and pVTU260-POX2-CROT were successfully constructed in this section. The PCR product of gene *POX2* and vector pVTU260 were digested by *NheI* and *BamHI* and ligated to form plasmid pVTU260-POX2. The ligation product was transformed into *E. coli* top 10 competent cells, and spread on LB agar plate containing 100 μ g/ml ampicillin. Figure 3.2 shows the restriction analysis results of plasmids from screening plate. Clone 2 and 3 were positive clones with 6.9 kb and 2.1 kb fragments. They were also verified by sequencing.

For cloning gene *CROT*, TEF2 promoter and terminator were also separately amplified. They have 15-20 bp extension homologous to each other, TEF promoter and TEF terminator also have 15-20 bp extension homologous to vector pVTU260-POX2 ends digested by *BstEII*, *XmaI*. After cloned *CROT* gene expression cassette (TEF2 promoter-CROT-TEF2 terminator) into pVTU260-POX2 by Cold Fusion Cloning Kit, the positive clones were selected by screening on LB agar plate

containing 100 µg/ml ampicillin. Mini-prepared plasmids were digested by *EcoRI* and *PstI* separately (Figure 3.3). Positive clone has 3.5 kb and 8.2 kb fragments digested by *PstI*, and 2.8 kb and 8.8 kb digested by *EcoRI*. It was also verified by sequencing.

Figure 3.2 The restriction analysis results of plasmid *pVTU260-POX2* from screening plate. *M*: 1kb plus marker, lane 1-3: clone 1-3 digested by *NheI*, *BamHI*. Positive clones 2 and 3 have 6.9 kb and 2.1 kb fragments.

Figure 3.3 The restriction analysis results of plasmid *pVTU260-POX2-CROT* from screening plate. *M*: 1kb marker, lane 1: positive clone was digested by *EcoRI*, and the fragments are 2.8 kb and 8.8 kb. Lane 2: positive clone was digested by *PstI*, and the fragments are 3.5 kb and 8.2 kb.

3.3.1.2 Expression of Aox2p and Crot in POX1 deletion (Δ pox1) strain

To express acyl-CoA oxidase 2 (Aox2p), which has a preference for long chain substrates, the plasmid pVTU260-POX2 encoding the *POX2* gene under the control of the constitutive promoter *ADHI* was transformed into Δ pox1, resulting in the strain Δ pox1 [*pox2*+] . To express carnitine O-octanoyltransferase, the gene *CROT* was cloned into pVTU260-POX2 under the control of the constitutive promoter *TEF*. The resulting plasmid pVTU260-POX2-CROT was transformed into strain Δ pox1, creating strain Δ pox1 [*pox2*+, *crot*+] . These two enzymes were successfully expressed in the Δ pox1 strain. Western result was shown in Figure 3.4. Aox2p is approximately 78 kDa; Crot is approximately 72 kDa.

Figure 3.4 Western for Aoxp2 and Crot. A. Expression of Aoxp2 in WT [*pox2*+] , Δ pox1 [*pox2*+] and Δ pox1 [*pox2*+, *crot*+] . Lane 1: WT [*pvtu260*], lane 2: WT [*pox2*+] , lane 3: Δ pox1 [*pox2*+] , lane 4: Δ pox1 [*pox2*+, *crot*+] . B. Expression of Crot in Δ pox1 [*pox2*+, *crot*+] . Lane 1: WT [*pvtu260*], lane 2: Δ pox1 [*pox2*+, *crot*+] . WT [*pvtu260*] was used as negative control. Aoxp2 and Crot were successfully expressed in engineered strains with a molecular size of 78 kDa and 72 kDa.

3.3.2 Growth phenotype analysis

Growth tests were performed to evaluate the potential negative effects of the genetic modification on the growth of yeast cells. The Δ pox1 strain did not grow on the oleic

acid-based (YNBO) agar plate. The $\Delta pox1$ [$pox2+$] and WT [$pox2+$] strains showed a severe growth defect (Figure 3.5), whereas $\Delta pox1$ [$pox2+$, $crot+$] grew similar to the WT. When all the strains were cultured in glucose-based (YNBD) medium (as shown in Figure 3.6A), there was no severe growth defect observed in the engineered strains. It was observed that $\Delta pox1$ [$pox2+$] grew more slowly than the WT strain. $\Delta pox1$ [$pox2+$, $crot+$] showed a growth rate similar to the WT strain. However, in oleic acid-glucose-based (YNBD_{0.5O3}) medium (Figure 3.6B), $\Delta pox1$ showed reduced growth compared to the WT strain. The growth of $\Delta pox1$ [$pox2+$], expressing Aox2p, was more severely affected than the $\Delta pox1$ strain. The $\Delta pox1$ [$pox2+$, $crot+$] strain did not show a growth defect. The $\Delta pox1$ growth defect in oleic acid-containing medium was due to defective fatty acid degradation. Oleic acid could not be utilized by $\Delta pox1$ as a carbon source.

Figure 3.5 Growth tests on the engineered strains and the WT strain in YNBO medium. WT and $\Delta pox1$ transformed with the empty vector *pvtu260*, WT transformed with *pvtu260-pox2*, $\Delta pox1$ transformed with *pvtu260-pox2* and *pvtu260-pox2-crot* were all cultured overnight in liquid YNBD medium at 30 °C. Yeast pellets were collected and resuspended at a cell density of 5×10^3 cells/ μ l after

being washed twice in sterile distilled H₂O. To test growth, 5 μ l were spotted on YNBO-agar plates. The first drop contained 2.5×10^4 cells, and each subsequent drop was diluted six fold more than the previous one.

Figure 3.6 Growth curves of the engineered strains and the WT strain. A. Growth curves of the engineered strains and the WT strain in YNBD, B. Growth curves of the engineered strains and the WT strain in YNBD_{0.5}O₃. The results are the mean values of three independent experiments.

The growth defect in strain Δ pox1 [pox2+] indicated that β -oxidation was not fully rescued by Aox2p. Because Aox2p has very low activity towards acyl-CoAs with fewer than ten carbons [111], the over-accumulation of medium chain acyl-CoAs from LCFA degradation in the peroxisome prevented further fatty acid oxidation,

which restricted the carbon flux toward β -oxidation. The defective growth of the WT [*pox2+*] strain further supported this conclusion. In strain Δ *pox1* [*pox2+*, *crot+*], the medium chain acyl-CoAs accumulated were transferred to carnitine by Crot [189] and subsequently removed from peroxisomes, allowing unobstructed carbon flux.

3.3.3 Analysis of fatty acid composition in cell extracts and culture supernatants

To examine the effect of Aox2p and Crot on fatty acid accumulation and composition, the intracellular and extracellular fatty acids of cultured cells at the stationary phase were analyzed. When the engineered strains were cultured in YNBD medium, the total fatty acids in the engineered strains increased less than 10% compared with the WT strain. There was not an obvious change in the fatty acid profile and C8:0 and C10:0 were not detected in the culture supernatant of any of the engineered strains (data not shown).

When cells were cultured in YNBD_{0.5}O₃, the fatty acid accumulation and composition of the engineered strains were different from WT strain. For intracellular fatty acids from the cell extract at stationary phase (Table 3.3 and Table 3.4), the total fatty acids in strains Δ *pox1* [*pox2+*] and Δ *pox1* [*pox2+*, *crot+*] increased 29.5% and 15.6% compared to the WT strain, respectively. There is no increase of total fatty acids in the Δ *pox1* strain. The UFA ratio (unsaturated fatty acids compared to saturated fatty acids) increased to 0.083 (Δ *pox1*), 0.073 (Δ *pox1* [*pox2+*]) and 0.078 (Δ *pox1* [*pox2+*, *crot+*]) compared to 0.041 (WT). In terms of MCFAs, there was an increase in MCFA content (present only as lauric acid in the cell extract) in the strains Δ *pox1*, Δ *pox1* [*pox2+*] and Δ *pox1* [*pox2+*, *crot+*]

compared with the WT strain. Lauric acid (C12:0) content in $\Delta pox1$ (0.486 $\mu\text{g}/\text{OD}$), $\Delta pox1$ ($pox2+$) (0.77 $\mu\text{g}/\text{OD}$) and $\Delta pox1$ [$pox2+$, $crot+$] (0.64 $\mu\text{g}/\text{OD}$) were increased 1.42-, 2.26- and 1.87-fold compared to the WT strain (0.34 $\mu\text{g}/\text{OD}$) (Figure 3.7A). In the strains $\Delta pox1$, $\Delta pox1$ [$pox2+$] and $\Delta pox1$ [$pox2+$, $crot+$], lauric acid constituted 0.85%, 1.0% and 0.92% of the total fatty acids (Figure 3.7B) compared to 0.57% in the WT, which corresponds to a 1.47-, 1.75- and 1.61-fold increase. The data demonstrated that modification of the β -oxidation pathway changed the total fatty acids, UFA/SFA ratio and the MCFAs ratio in yeast cells.

Table 3.3 Fatty acid production in the cell extract of the WT and the engineered strains^a

Fatty acid type	WT	$\Delta pox1$	$\Delta pox1$ [$pox2+$]	$\Delta pox1$ [$pox2+$ $crot+$]
C12:0	0.341	0.486	0.772	0.637
C14:0	1.183	1.590	2.143	1.855
C16:1	0.881	1.384	1.878	1.788
C16:0	30.623	27.675	35.794	32.535
C18:1	1.449	2.977	3.334	3.170
C18:0	24.889	22.959	32.890	28.548
C20:0	0.120	0.143	0.202	0.227
Total fatty acids	59.486	57.214	77.014	68.760

^a Data represent fatty acid composition in $\mu\text{g}/\text{OD}$ cell when WT and engineered strains were cultured in YNBD_{0.5}O₃ medium. The values are the means from three experiments examining the cell extracts at 24 h. The standard deviations were <5% of the values.

Table 3.4 Fatty acid composition changes in cell extract comparing the WT and the engineered strains in YNBD_{0.5}O₃ medium ^b

Fold changes of fatty acids	<i>Δpox1</i>	<i>Δpox1 [pox2+]</i>	<i>Δpox1[pox2+crot+]</i>
C12:0	1.422	2.262	1.866
C14:0	1.344	1.812	1.568
C16:1	1.570	2.132	2.029
C16:0	0.904	1.169	1.062
C18:1	2.054	2.300	2.187
C18:0	0.923	1.322	1.147
C20:0	1.194	1.681	1.889
Total fatty acids	0.962	1.29	1.156

^b The fold change data for the engineered strains were analyzed compared to the WT strain. The amount of fatty acids in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 24 h. The standard deviations were <5% of the values.

Another observed change was an increase in the MCFAs content of the culture supernatants from engineered strains compared to the WT strain. MCFAs (chain length 8 to 12 carbons) are usually a minor constituent of total fatty acids present at a low density in the cell, and C8:0 and C10:0 are not generally detectable in culture supernatant. Because MCFAs can freely traverse the membrane of a cell, when MCFAs were produced, they were secreted into the culture medium. As shown in Figure 3.8, there was no detectable C8:0 or C10:0 in the WT strain, only C12:0. In contrast, C8:0, C10:0 and C12:0 were all detected in the engineered strains. MCFAs content in the three engineered strains increased 2.84-fold (*Δpox1*, 0.419 μg/ml),

3.29-fold ($\Delta pox1$ [$pox2^+$], 0.484 $\mu\text{g/ml}$) and 3.34-fold ($\Delta pox1$ [$pox2^+$, $crot^+$], 0.493 $\mu\text{g/ml}$) compared to the WT strain (0.147 $\mu\text{g/ml}$).

Figure 3.7 MCFAs analysis of the cell extracts. A. MCFAs content in the cell extracts from the engineered strains and the WT strain after 24 h when cultured in $YNBD_{0.5}O_3$ medium. B. Composition of MCFAs from the total fatty acids in the cell extracts from the engineered strains and the WT strain at 24 h when cultured in $YNBD_{0.5}O_3$ medium. Cells were collected after 24 h of growth in $YNBD_{0.5}O_3$ medium, and the cell pellet was separated by centrifugation. The total fatty acids were extracted and detected. There was C12:0 but not C8:0 and C10:0 in the cell extract, so the MCFAs were only C12:0 in this case.

Figure 3.8 MCFAs content in the culture supernatant of the engineered strains and the WT strain grown for 24 h in YNBD_{0.5}O₃ medium. Cells were collected after growth for 24 h in YNBD_{0.5}O₃ medium, and the culture supernatant was separated by centrifugation. MCFAs were detected with the SPE method (detailed information referred to in the fatty acids analysis section). Data are the mean values of three independent experiments. The error bar indicates the S.D. C8:0: open bar, C10:0: striped bars, C12:0: gridded bar, MCFAs: solid bar.

3.3.4 Metabolic profiling of control and engineered strains

S. cerevisiae was modified to maintain the ability to degrade LCFAs but limited ability to degrade MCFAs. To analyze the difference between the engineered strains in the oxidation of MCFAs, GC-MS-based metabolic profiling (Figure 3.9) was performed. The metabolic responses of the engineered strains were useful in understanding the changes to the fatty acid degradation pathway after gene modification. It was found that there were obvious differences in the cellular metabolite levels between engineered strains and the WT strain, as shown in Figure 3.10. Fewer dicarboxylic acids were present in the engineered strains compared with the WT strain when cultured in YNBD_{0.5}O₃ medium. This observation is consistent with a previous study [190] reporting that dicarboxylic acids (hexanedioic acid, heptanedioic acid, octanedioic acid, azelaic acid and sebacic acid) are formed when

fatty acid oxidation is increased or inhibited. Because the formation of dicarboxylic acids could facilitate the degradation process, there are higher levels of dicarboxylic acids when fatty acid oxidation is increased than when it is inhibited. In the WT strain, dicarboxylic acids were at the highest level, which was due to increased β -oxidation compared to the engineered strains. There were much lower levels of dicarboxylic acids in the $\Delta pox1$, $\Delta pox1 [pox2+]$ and $\Delta pox1 [pox2+, crot+]$ strains, which had negligible β -oxidation of MCFAs. This difference was consistent with the fatty acid metabolism of several strains.

Figure 3.9 The representative GC-MS spectra for azelaic acid. (A): the total GC-MS spectra for metabolite profiling. (B): the enlarged GC-MS spectra between retention time 29.20-29.80. Intracellular metabolites were extracted from the engineered strains and the WT strain, and metabolic profiling was conducted. WT (black), $\Delta pox1$ (blue), $\Delta pox1 [pox2+]$ (red), and $\Delta pox1 [pox2+, crot+]$ (green).

With regard to L-proline, glutamine, and L-ornithine, these changes might be related to alterations in the metabolism of the tricarboxylic acid cycle (TCA) cycle. These amino acids are synthesized from intermediates of the TCA. When a strain has a growth defect, the carbon flow toward the TCA cycle becomes restricted, further decreasing the concentration of amino acids related to the TCA cycle. In the strain $\Delta pox1$ [*pox2*⁺, *crot*⁺], there was no observed growth defect, and the amino acid levels were upregulated relative to normal levels. As for the increase of ornithine in $\Delta pox1$ [*pox2*⁺], this may be due to the very low growth rate. Thus, there was a relative excess of nitrogen driving ornithine synthesis.

Figure 3.10 Differential expression levels of intracellular metabolites in the engineered strains compared to the WT strain. Differential expression levels of

intracellular metabolites were analyzed in the engineered strains as compared to the WT. The amount of metabolites in the WT strain was set as 1. The values are the means from three experiments.

3.4 Discussion

3.4.1 Impact of Aox2p and Crot on fatty acids composition in the engineered strains

Our study showed that when oleic acid was used as a co-substrate of glucose, the total fatty acids were increased in engineered strains compared to the WT strain. As shown in Table 3.4, the total fatty acids from strains $\Delta pox1$ [*pox2+*] and $\Delta pox1$ [*pox2+*, *crot+*] were increased compared to the WT strain. In contrast, the total fatty acids of strains $\Delta pox1$ were not increased. This indicated that the β -oxidation defect had a side effect on fatty acid accumulation other than fatty acid uptake. Modification of the β -oxidation pathway not only affected the total fatty acids content but also changed the composition. There was MCFAs (C8:0, C10:0 and C12:0) accumulation, and C8:0 and C10:0 were secreted into the medium. For the initial gene modification step, we obtained the deletion strain $\Delta pox1$. It was observed that there was an increase in C8:0 and C10:0 in $\Delta pox1$. From a previous report [191], it had been shown that medium chain acyl-CoAs can be released from the fatty acid synthetase (FAS) complex due to the accumulation of saturated LCFAs during the fermentation conditions. Medium chain acyl-CoAs are hydrolyzed to recycle CoA-SH. MCFAs can diffuse passively through the cytoplasmic membrane. Our findings therefore suggested that medium chain acyl-CoAs might be released from the FAS complex in the $\Delta pox1$ strain when cultured in oleic acid-containing medium, even under respiratory conditions. In contrast, C8:0 and C10:0 did not

accumulate in the WT strain. In $\Delta pox1$ [*pox2*+], the increase of MCFAs was partly due to the effect of *POX1* deletion and partly due to the expression of *POX2* from *Y. lipolytica*. In the peroxisomes of $\Delta pox1$ [*pox2*+], only long-chain acyl-CoAs were degraded to medium chain acyl-CoAs and acetyl-CoA. The degradation product acetyl-CoA was activated to acetyl carnitine and transported out of the peroxisome by carnitine acetyltransferase (Cat2p) [104]. However, there is no transferase in *S. cerevisiae* that is involved in transporting medium chain acyl-CoAs out of the peroxisome and recycling CoA-SH. Although there is a peroxisomal acyl-CoA thioesterase (PTE1) [192] in *S. cerevisiae* to hydrolyze acyl-CoAs of all chain lengths to generate free CoA-SH, this enzyme does not appear to possess sufficient activity to hydrolyze all of the medium chain acyl-CoAs accumulated in peroxisomes in the strain $\Delta pox1$ [*pox2*+] . Owing to the expression of Aox2p and thioesterase in the peroxisome, there was an increase of MCFAs in $\Delta pox1$ [*pox2*+] . However, the accumulated medium chain acyl-CoAs led to depletion of free CoA-SH and eventually the termination of the β -oxidation pathway, causing the growth defect. When Crot was expressed in the strain $\Delta pox1$ [*pox2*+, *crot*+] , the carbon flux through β -oxidation became unobstructed because the accumulated medium chain acyl-CoAs could be transported out of the peroxisome, balancing the CoA concentration in peroxisomes. Therefore, the growth rate was recovered to the levels of the WT strain. However, it should be noted that there was not a continuous increase of MCFAs in the strain $\Delta pox1$ [*pox2*+, *crot*+] . It was assumed that there was an increase of cytosolic medium chain acyl-CoAs in the engineered strains. In future studies, we will quantify medium chain acyl-CoAs, as well [193].

3.4.2 Metabolic engineering of *S. cerevisiae* for MCFAs production

It has been shown that MCFAs accumulation is related to fermentative metabolism in *S. cerevisiae* [191, 194]. During fermentation, fatty acid synthesis is inhibited by the lack of oxygen. As a result, medium chain acyl-CoAs are released from the FAS and hydrolyzed to recycle CoA-SH. Cell growth ceases due to the inhibition of fatty acid synthesis. During our investigation of the role of Aox2p and Crot in fatty acid profile and MCFAs accumulation, we expressed Aox2p and Crot in the $\Delta pox1$ strain. As expected, the total fatty acids and MCFAs were increased in the engineered strains compared to the WT strain. We showed that in aerobic conditions, MCFAs could be produced in *S. cerevisiae* through modification of the β -oxidation pathway. The metabolic responses in different strains helped us to further investigate the impact of Aox2p and Crot. In the WT strain, there was a high concentration of medium chain dicarboxylic acids detected, which facilitated β -oxidation in the cells [190]. In contrast, the MCFAs oxidation ability decreased dramatically in the engineered strains. A much lower concentration of medium chain dicarboxylic acids were detected. On the contrary, MCFAs were secreted into the culture medium.

3.5 Conclusion

To sum up, we observed that the production of both intracellular and extracellular MCFAs increased in the engineered strains compared to the WT strain when grown in YNBD_{0.5}O₃. It was demonstrated that modification of β -oxidation was capable of turning low value fatty acid feedstock into higher value fatty acids containing more MCFAs. However, MCFAs remained a minor component of the total fatty acids. The observed increase in total fatty acids indicated that most of the oleic acid in the

medium went into the storage pathway instead of degradation pathway. Thus, further investigation is needed to improve the oxidation of LCFAs and the formation of MCFAs. Future studies will aim at the expression of other β -oxidation related genes and medium chain acyl-CoA hydrolases, such as Acot5 (medium chain acyl-CoA thioesterase) [195]. Meanwhile, endogenously produced lipase can be used [196] to increase the uptake of fatty acid-rich feedstock, such as triacylglycerols (TAG), in the engineered strains. The current work terminated the β -oxidation of yeast in peroxisomes at medium chain acyl-CoAs. It was demonstrated that heterologous expression of β -oxidation enzymes in *S. cerevisiae* system is a promising tool to exploit bioconversion platforms.

4 Up-regulation of FFA production in *S. cerevisiae* by control of fatty acyl-CoA metabolism

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4.1 Introduction:

Renewable synthesis of microbial fatty acid-derived biofuels and chemicals is a promising approach to cope with the current energy crisis. It has been shown that the FFA profile can be efficiently modified, for application in biofuel production, through overexpressing thioesterase with different acyl-ACPs chain length preference in *E. coli* [9, 10, 25].

In *E. coli*, fatty acids are synthesized by a dissociated type II fatty acid synthase (FAS) system, with several of its enzymes feedback inhibited by long-chain fatty acyl-ACPs [51]. It has been shown that overexpression of acyl-ACP thioesterases can relieve the feedback inhibition [24], and modify the composition of fatty acid [23], and lead to an overproduction of significant levels of FFA [34]. On the other hand, *S. cerevisiae* uses type I FAS system and only fatty acyl-CoAs can be released from the FAS complex. It has been proposed that the MPT domain of FAS is able to transfer C6–C18 substrates with comparable efficiencies. The chain length distribution of fungal FAS products is regulated by the equilibrium of substrate and product concentrations. However, C16/C18 fatty acyl-CoAs are preferentially

synthesized under physiological conditions [62]. Acyl-CoA esters have been proposed as key regulators of fatty acid synthesis since 1963 [197]. It has been shown that long chain acyl-CoAs regulate several enzymes involved in lipid synthesis [81] by feedback inhibition of *ACCI* [198], *FAS* [199] and *OLE1* [96]. Studies showed that dimyristoyl-lecithin dispersions, *E. coli* plasma membranes and BSA relieved the inhibition of acyl-CoAs on yeast FAS [199]. It is believed that the low intracellular concentration of acyl-CoAs (0.1-200 nM) is tightly controlled by the feedback inhibitory mechanism, buffered by acyl-CoA binding proteins, and regulated by acyl-CoA synthetases, carnitine acyltransferase and acyl-CoA hydrolase [81]. The studies concerning acyl-CoA synthetases and carnitine acyltransferase have been well reviewed [200, 201]. However, knowledge of the acyl-CoA hydrolase in yeast is sparse. The only well characterized acyl-CoA hydrolase in *S. cerevisiae* is peroxisomal acyl-CoA thioesterase PTE1, which is involved in fatty acid degradation, rather than fatty acid synthesis [192, 202]. In this regard, the role of cytosolic thioesterase is not well illustrated in *S. cerevisiae*. So far, it is unclear if the acyl-CoA thioesterase can alleviate the feedback inhibition of long chain acyl-CoAs in a similar fashion as in *E. coli*.

Assuming that regulating the metabolism of fatty acyl-CoAs can in turn regulate the fatty acid accumulation, herein we engineered a strain of *S. cerevisiae* defective in acyl-CoA synthetases, and also carrying a heterologous acyl-CoA thioesterase Acot5s which encodes a short version of *Mus musculus* peroxisomal acyl-CoA thioesterase 5. Previously, acyl-CoA synthetases have been shown to regulate the fatty acid metabolism [201]. In our study, the *FAA1* and *FAA4* genes which encode the prominent acyl-CoA synthetases were firstly deleted to block the transformation

of FFA to fatty acyl-CoAs. Secondly, *ACOT5* (*PTE-1c*) from *Mus musculus* was chosen to be expressed in the cytoplasm of *S. cerevisiae* to increase the production of FFA (Figure. 4.1). Acot5 is a medium chain acyl-CoA thioesterase acting on C6-CoA to C20-CoA, with highest activity with C10-CoA. It was shown to produce FFA and also participate in the transportation system in *Mus musculus* peroxisomes [203]. Then fatty acid and metabolite profile were investigated to evaluate its potential in biofuel production.

Figure. 4.1 Engineering *S. cerevisiae* for high levels of FFA accumulation. Double deletion of *FAA1* & *FAA4* and expression of cytosolic acyl-CoA thioesterase encoded by *Acot5s* enhanced the flux to FFA, released the repression of fatty acyl-CoAs on fatty acid synthetase, and accumulated more FFA.

4.2 Materials and methods

4.2.1 Strains and growth conditions

All yeast strains and plasmids used in this study were listed in Table 4.1. YPD complete medium [1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose] was used to cultivate the WT strain. Drop-out Medium (YNBD) comprised of 0.67% yeast nitrogen base (without amino acids but with ammonium sulfate, Invitrogen), 2% dextrose and amino acid drop out (without His or Ura, Clonetechn) was used to

cultivate the modified strains. 200 µg/ml geneticin was added when necessary.

Table 4.1 Plasmids and strains list

Name	Description	Source
WT	<i>S. cerevisiae</i> wild type strain BY4741 (<i>Mat a</i> ; <i>his3</i> Δ1; <i>leu2</i> Δ0; <i>met15</i> Δ0; <i>ura3</i> Δ0)	EUROSCARF
Δ<i>faa1</i>	BY4741; <i>Mat a</i> ; <i>his3</i> Δ1; <i>leu2</i> Δ0; <i>met15</i> Δ0; <i>ura3</i> Δ0; <i>FAA1::His5</i>	Present study
Δ<i>faa4</i>	BY4741; <i>Mat a</i> ; <i>his3</i> Δ1; <i>leu2</i> Δ0; <i>met15</i> Δ0; <i>ura3</i> Δ0; <i>FAA4::kanMX4</i>	Present study
Δ<i>faa1</i>Δ<i>faa4</i>	BY4741; <i>Mat a</i> ; <i>his3</i> Δ1; <i>leu2</i> Δ0; <i>met15</i> Δ0; <i>ura3</i> Δ0; <i>FAA4::kanMX4</i> ; <i>FAA1::His5</i>	Present study
WT[pVTU260]	Wild type strain transformed with pVTU260	Present study
Δ<i>faa1</i>[pVTU260]	Δ <i>faa1</i> transformed with pVTU260	Present study
Δ<i>faa4</i>[pVTU260]	Δ <i>faa4</i> transformed with pVTU260	Present study
Δ<i>faa1</i>Δ<i>faa4</i>[pVTU260]	Δ <i>faa1</i> Δ <i>faa4</i> transformed with pVTU260	Present study
WT[Acot5s]	Wild type strain transformed with pVTU260-Acot5s	Present study
Δ<i>faa1</i>[Acot5s]	Δ <i>faa1</i> transformed with pVTU260-Acot5s	Present study
Δ<i>faa4</i>[Acot5s]	Δ <i>faa4</i> transformed with pVTU260-Acot5s	Present study
Δ<i>faa1</i>Δ<i>faa4</i>[Acot5s]	Δ <i>faa1</i> Δ <i>faa4</i> transformed with pVTU260-Acot5s	Present study
pUG6	loxP-kanMX-loxP gene disruption cassette plasmid	[116]
pUG27	loxP-his5-loxP gene disruption cassette plasmid	[116]
pVTU260	Eukaryotic expression vector with ADH1 promoter and terminator, URA3 marker	EUROSCARF
pVTU260-Acot5s	The mouse <i>Acot5</i> gene without the nucleotide sequence encoding C-terminal peroxisomal targeting tripeptide of -AKL, cloned into pVTU260	Present study

4.2.2 Plasmid and strain construction

Molecular cloning was performed according to standard procedures. Q5[®] High-Fidelity DNA Polymerase and restriction enzymes were purchased from New England Biolabs (Singapore). Cold fusion cloning kit was purchased from SBI (Singapore). Oligonucleotide primers (Table 4.2) were synthesized at 1st BASE Pte Ltd (Singapore).

To delete gene *FAA1* and *FAA4*, loxP-marker gene-loxP gene disruption cassettes were generated by PCR as described in Figure 4.2 [116]. After purification, loxP-His5-loxP and loxP-KanMX-loxP gene disruption cassettes were transformed into WT competent cells, respectively. After screening on YNBD-His agar plate and YPD agar plate containing 200 µg/ml geneticin separately, the deletion strains $\Delta faa1$ and $\Delta faa4$ were obtained and verified by colony PCR. Then, loxP-His5-loxP gene disruption cassette for deleting the gene *FAA1* was transformed into $\Delta faa4$ competent cells and screened on YNB-His agar plate containing 200 µg/ml geneticin. The deletion strain $\Delta faa1 \Delta faa4$ was obtained and verified by colony PCR.

Table 4.2 Primers list

Name	Description	Sequence (5'→3')
FAA1-F	Forward deletion primer containing 45bp of homology upstream of the FAA1 gene	CAATAAAACTAGAACAAACACAAA AGACAAAAAAGACAACAATcagctgaa gcttcgtacgc
FAA1-R	Reverse deletion primer containing 45bp of homology downstream of the FAA1 gene	TGCTTTAGTATGATGAGGCTTTCCTAT CATGGAAATGTTGATCCAgcataggccact agtggatctg
FAA4-F	Forward deletion primer containing 45bp of homology	TCTGTTCTTCACTATTTCTTGAAAAA CTAAGAAGTACGCATCAAAcagctgaagc

	upstream of the FAA4 gene	ttcgtacgc
FAA4-R	Reverse deletion primer containing 45bp of homology downstream of the FAA4 gene	GTGTTTATGAAGGGCAGGGGGGAAA GTAAAAAACTATGTCTTCCTgcataggcc actagtggatctg
FAA1-A	Verification primer for <i>FAA1</i> gene	TAGAACAAACACAAAAGACA
FAA1-B	Verification primer for <i>FAA1</i> gene	TTTGGCTCACCTGTAGAA
FAA1-C	Verification primer for <i>FAA1</i> gene	ATCTGCCCTATGCTTATT
FAA1-D	Verification primer for <i>FAA1</i> gene	CTTTAGTATGATGAGGCTTT
FAA4-A	Verification primer for <i>FAA4</i> gene	ACTAAGAAGTACGCATCAA
FAA4-B	Verification primer for <i>FAA4</i> gene	ATCAACCCACTCAGCAAG
FAA4-C	Verification primer for <i>FAA4</i> gene	CTTTACCGATGATGGCT
FAA4-D	Verification primer for <i>FAA4</i> gene	TGTTTATGAAGGGCAGGG
<i>kan^r /his5⁺-S</i>	Verification primer for disruption cassette	GCCACTGAGGTTCTTCTTT
<i>kan^r /his5⁺-A</i>	Verification primer for disruption cassette	GACCAGCATTACATACGAT
ACOT5s-F	Forward primer for cloning <i>Acot5s</i> into pVTU260	tgcaccatcaccatcaccATGCTAGCCAAAGG TAAGCC
ACOT5s-R	Reverse primer for cloning <i>Acot5s</i> into pVTU260	cctgaaaataagattctcgtagTCAAGGACTAG GTCTCTTGTCACC
ACOT5s-C-F	Forward colony PCR primer	TGGTCCCTACTGTCTCAT
ACOT5s-C-R	Reverse colony PCR primer	ACCCAAACCTGGCAAT
ADHp	Forward sequencing primer	CGACAAAGACAGCACCAA
ADHt	Reverse sequencing primer	TGCTGCCACTCCTCAA
ACT1-F	Forward RT-PCR primer	ATGGTCGGTATGGGTCA
ACT1-R	Reverse RT-PCR prime	GATTTAGGGTTCATTGGAG
ACC1-F	Forward RT-PCR primer	TTATGAACGCTTCCTTGC
ACC1-R	Reverse RT-PCR prime	AGAACGTGCAACTAACTC
FAS1-F	Forward RT-PCR primer	CCCCAGACAAGGACTA
FAS1-R	Reverse RT-PCR prime	TACGGAGACGAAGAAGGAT
FAS2-F	Forward RT-PCR primer	GACTTCTACAAGAGGGACC

FAS2-R	Reverse RT-PCR prime	CCAATGACGGGATTACC
OLE1-F	Forward RT-PCR primer	AACCGCTTTTCGTCATTCC
OLE1-R	Reverse RT-PCR prime	ATGTAATGAGCCAAGGAG
POX1-F	Forward RT-PCR primer	AGTTATACAACGCTTCCT
POX1-R	Reverse RT-PCR prime	CCACCCAGTCGTCATAG
FAA2-F	Forward RT-PCR primer	TTACATAAACCAGACCCA
FAA2-R	Reverse RT-PCR prime	CCACCTCTTGCGGTAAAT

Figure 4.2 The gene disruption procedure for generation of deletion strains. This figure is modified based on Figure 1 in [116]. The disruption cassette consists of a marker gene flanked by two 34 bp of *loxP* sequences and 45 bp of sequences flanking the target ORF to be deleted. The disruption cassette is synthesized by PCR using primers FAA1-F and FAA1-R, or primers FAA4-F and FAA4-R. After transforming the disruption cassette into competent cells, positive transformants are verified by PCR. Primers A, B, C and D consist of sequences complementary to sequences

within the target gene. Primers *kan^r /his5⁺-A* and *kan^r /his5⁺-S* consists of sequences complementary to the disruption cassette.

Figure 4.3 Construction of expression plasmid pVTU260-Acot5s. *ACOT5s* was cloned into pVTU260 using cold fusion cloning kit (SBI). Primer ACOT5s-F contains a *NheI* restriction site, and ACOT5s-R contains a *BamHI* restriction site. Clones were verified by digestion with *NheI* and *BamHI*. Positive clones have 6.9 kb and 1.3 kb fragments.

The gene *ACOT5* (GenBank: accession no. NM_145444.3) which encodes the peroxisomal acyl-CoA thioesterase 5 from *Mus musculus* was synthesized by Genescript (Singapore), with codon optimization. The GenBank accession number for the codon-optimized sequence of *ACOT5* is KF573587. In the synthesized *ACOT5* gene, yeast consensus sequence and V5-tag were introduced before the start codon. To construct the expression plasmid pVTU260-Acot5s, a truncated version of the *ACOT5* gene, which does not contain the nucleotides encoding the C-terminal

peroxisomal targeting peptide AKL, was generated by PCR using primers ACOT5s-F and ACOT5s-R. The purified PCR product of *ACOT5s* was mixed with linearized pVTU260 vector. Then cold fusion reaction and transformation were carried out according to the manual. The resulting plasmid pVTU260-Acot5s was verified by restriction analysis and sequencing (the procedure is illustrated in Figure 4.3).

4.2.3 Expression analysis of Acot5s

Cell pellet was collected from 5 ml cell culture by centrifugation at 8000 rpm. After washing, the cell pellet was resuspended in lysis buffer containing 50 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM PMSF and 1 mM EDTA to a final optical density of 50~100 at 600 nm (OD₆₀₀). Then 300 µl acid-washed glass beads were added into the resuspended cells, and the cells were broken using a FastPrep®-24 Instrument (6004-500, MP Biomedicals) for 30 s, and cooled on ice for 30 s, and repeated 4 times. Next the cells were centrifuged at 12000 rpm for 10 minutes at 4 °C. 20 µl cell lysate was combined with SDS-PAGE loading buffer and the sample was boiled at 95 °C for 5 min, and separated on a 10% SDS-PAGE gel in a Bio-Rad Mini-PROTEAN Tetra Cell. The separated proteins were then transferred to a Turbo PVDF membrane by using a Trans-Blot Turbo™ Transfer System (Bio-Rad, Singapore). The primary antibody used was a V5 mouse monoclonal antibody (Invitrogen Life Science Technologies). The secondary antibody was an anti-mouse IgG HRP-conjugated antibody (#31430, Pierce). All dilutions used were as suggested by manufacturer. The signal was detected with Tetramethylbenzidine (TMB) (Sigma T0565).

4.2.4 Sample preparation for metabolite and fatty acid analysis

A single colony of the engineered yeast strain or WT strain was inoculated from a fresh agar plate into 5 mL fresh liquid YNBD medium, and cultured overnight at 30 °C with shaking. Then the overnight culture was inoculated into a 50 mL liquid YNBD medium with an initial OD₆₀₀ of 0.2, and incubated by shaking at 250 rpm at 30 °C. Yeast culture samples were collected at different growth time points, and centrifuged for 10 min at 10000 rpm. The supernatant and cell pellet were collected separately for further fatty acid or metabolite analysis.

The lipids were extracted from yeast cells using adjusted chloroform-methanol 2:1 method [186, 204]. Cell pellet with an OD of 10 was re-suspended in 1000 µl of 0.9% NaCl solution, and then acidified with 200 µl of acetic acid. Next, 5 µl of 10 mg/ml heptadecanoic acid and heptanoic acid dissolved in ethanol and 10 µl of 2 mg/ml ribitol dissolved in water were added into the extraction solvent as internal standard (IS) to correct for metabolites losses during sample preparation. The cell was disrupted in an ice bath using an ultrasonicator for 120 times. Then 3 ml of chloroform-methanol in a ratio of 2:1 was added, the samples were inverted several times, vortexed vigorously, and centrifuged at 10000 rpm for 10 min. The lower chloroform layer was then collected, while additional 2 ml chloroform was added to the upper aqueous layer to further extract the remaining lipids. Then the chloroform layers were combined and rotary evaporated to near dryness overnight. For metabolite profiling, 1 ml of the aqueous phase layer was collected and evaporated to dryness too.

To extract FFA in the culture medium, 4 mL of collected culture supernatant was

centrifuged for another 10 min at 10000 rpm to remove the remaining suspended cells. Next, 500 µl 10% (wt/vol) NaCl, 500 µl glacial acetic acid, 5 µl of 10 mg/ml heptadecanoic acid and heptanoic acid and 2 ml ethyl acetate was added to the supernatant. After vortexed for 3 min and rotated for 1 hour at room temperature, the mixture was centrifuged at 10000 rpm for 10 min. Then the ethyl acetate layer was collected. Two milliliters ethyl acetate was added to aqueous layer to extract the remaining FFA for another time [9]. The ethyl acetate layer extracts were then combined and rotary evaporated overnight to dryness.

4.2.5 Fatty acid analysis

Fatty acid analysis was carried out according to previous work [187, 204]. Fatty acid methyl esters (FAMES) mix C₈-C₂₄ (SUPELCO, 18918) was used as the standard. The dried lipid residue and FFA were each re-dissolved in 600 µl BF₃-methanol (FLUKA, 15716), and incubated in sealed vials at 95 °C oven for 30 min. 600 µl saturated NaCl was added to stop the reaction and the FAMES were extracted with 600 µl of hexane. Then the samples were analyzed using the same GC-MS system and setup as described in section 3.2.7

4.2.6 Metabolic profiling

Sample preparation and GC-MS analysis for metabolic profiling was conducted as described in section 3.2.8

4.2.7 RT-PCR analysis of gene expression

RNeasy Mini kit (QIAGEN) was used to extract the RNA from the WT and

engineered strains. The amount of RNA was measured using NanoDrop 2000C system (Thermo scientific). Real-time RT-PCR was performed using the iScript one-step RT-PCR kit (Bio-Rad). RT-PCR reaction was carried out and analyzed using the IQ5 Real-time PCR detection system (Bio-Rad). The relative gene expression analysis was conducted by the $2^{-\Delta\Delta C_T}$ method [205].

4.3 Results

4.3.1 Constructing the genetically engineered *S. cerevisiae* strains

The engineered fatty acid pathway is based on the deletion of two genes and the expression of one protein. The single and double deletion strains $\Delta faa1$, $\Delta faa4$ and $\Delta faa1 \Delta faa4$ were obtained and tested by colony PCR (Figure 4.4) using primers described in Table 4.2 and Figure 4.2. To express cytosolic acyl-CoA thioesterase, a short version of *ACOT5* (*Acot5s*), without peptide AKL, was expressed in the cytoplasm under the control of the constitutive promoter ADH1. Western blotting results showed the protein Acot5s with V5-tag is about 48.4 kDa (Figure 4.5).

Figure 4.4 PCR analysis to confirm correct gene deletion of FAA1 and FAA4. PCR was carried out on transformants obtained in gene deletion experiments, using verification primers specific for target gene and disruption cassette (*kan^r* /*his5⁺*-S and A), as described in Figure 4.2 and Table 4.2. The marker was 1kb DNA ladder

from Fermentas. Primers A and B were used in lane 1, primers C and D were used in lane 2, primers A and kan^r /his5⁺-A were used in lane 3, primers kan^r /his5⁺-S and D were used in lane 4.

Figure 4.5 Western result for expression of *Acot5s* in *S. cerevisiae* WT, Δ *faa1*, Δ *faa4* and Δ *faa1* Δ *faa4* deletion strains. Lane 1: WT(*Acot5s*), lane 2: Δ *faa1*(*Acot5s*), lane 3: Δ *faa4*(*Acot5s*), lane 4: Δ *faa1* Δ *faa4*(*Acot5s*), lane 5: WT(*pVTU260*). Strain WT (*pVTU260*) was used as control. *Acot5s* was showed to be successfully expressed in the engineered strains WT(*Acot5s*), Δ *faa1*(*Acot5s*), Δ *faa4*(*Acot5s*) and Δ *faa1* Δ *faa4*(*Acot5s*) with a molecular size of 48.4 kDa.

4.3.2 The effect of deletion gene *FAA4* or/and *FAA1* on fatty acid composition

It has been demonstrated that fatty acyl-CoA synthetases Faa1p and Faa4p take part in activation exogenously supplied fatty acids and intracellular acyl-CoA metabolism, and a deficiency in one or two of them will result in an altered endogenous acyl-CoA pools, but will have no significant effect on intracellular fatty acid levels [206]. In our work, both intracellular fatty acids and extracellular fatty acids were investigated to demonstrate the changes between the WT strain and deletion strains. When the engineered strains were cultured in YNBD medium for 24h, the intracellular TFA level of all engineered strains did not show a great change (data not shown), which was consistent with previous report. Similarly, for extracellular fatty acids as shown in Figure 4.6, the single deletion strain Δ *faa1* and Δ *faa4* did not demonstrate evident changes, as compared with the WT strain. They

accumulated mainly saturated fatty acid (SFA) C14:0, C16:0 and C18:0 in the culture medium, like the WT strain. For the double deletion strain $\Delta faa1 \Delta faa4$, there was a clear difference in the fatty acid profile, and a significant increase in TFA, including all types of fatty acid. As shown in Table 4.3 and Figure 4.6, the strain $\Delta faa1 \Delta faa4$ had increased levels of medium chain fatty acids (MCFA) (C8:0, C10:0 and C12:0) to 8.26 $\mu\text{g/ml}$, as compared to trace amounts in the WT. SFA increased to 193.74 $\mu\text{g/ml}$, which is about 2.5-fold as compared to the WT strain. In addition, UFA increased to 124.74 $\mu\text{g/ml}$, whereas it could not be detected in the WT strain and single deletion strains.

Table 4.3 Extracellular fatty acids content in WT and engineered strains

Fatty acid type	WT	$\Delta faa1 \Delta faa4$	$\Delta faa1 \Delta faa4$ [<i>Acot5s</i>]
MCFA	0.129±0.0346	8.258±3.360	4.903±0.941
UFA	0	124.735 ±30.007	208.224 ±16.938
SFA	76.374±11.620	193.741±20.703	279.130±19.455

Cells were grown in 50 ml of YNBD medium at 30 °C for 24h. Total extracellular fatty acids were extracted according to described methods. Data represented are expressed in $\mu\text{g/ml}$. they are the means from at least three independent experiments. MCFA: C8:0, C10:0 and C12:0. UFA: C16:1 and C18:1. SFA: C14:0, C16:0 and C18:0. WT: wild type.

Figure 4.6 The effect of deleting both *FAA1* and *FAA4* on fatty acids production. Differential extracellular fatty acid levels in the WT, deletion strains $\Delta faa1$, $\Delta faa4$ and $\Delta faa1 \Delta faa4$ were presented. Cells were collected after 24 h of growth in YNBD medium, and the culture medium were separated by centrifugation. The total extracellular fatty acids were extracted and analyzed. Data are the mean values of at least three independent experiments performed in duplicate. The error bar indicates the S.D.

4.3.3 The effect of cytosolic acyl-CoA thioesterase *Acot5s* on fatty acid composition

It has been proven that acyl-ACP thioesterase can be overexpressed in *E. coli* to modify the fatty acid profile and relieve the feedback inhibition [23]. However, no similar work has been conducted in yeast cells to illustrate the presence of a similar regulatory mechanism until now. To evaluate if the expression of cytosolic acyl-CoA thioesterase could likewise enhance fatty acids production in yeast cells, we expressed cytosolic acyl-CoA thioesterase in the WT strain and deletion strains $\Delta faa1$, $\Delta faa4$ and $\Delta faa1 \Delta faa4$. As expected, only the strain $\Delta faa1 \Delta faa4$ containing *Acot5s* showed additional significant increase of FFA, while the WT or single

deletion strain containing *Acot5s* did not show significant increase of FFA. As shown in Figure 4.7 and Table 4.3, in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*], C14:0, C16:1, C16:0, C18:1, C18:0, UFA and SFA increased by about 20%, 54%, 42%, 100%, 55%, 66% and 44%. TFA reached about 500 $\mu\text{g/ml}$ at 24h, it is 6.43-fold increase as compared to the WT strain, and 1.5-fold increase as compared to the strain $\Delta faa1 \Delta faa4$ (Figure 4.8). By comparison, as shown in Figure 4.8, extracellular FFA in WT [*Acot5s*] did not increase as compared with the WT strain. Similar results were found in $\Delta faa1$ [*Acot5s*] (no more than 30% increase) and $\Delta faa4$ [*Acot5s*] (no increase observed). This indicated that the expression of *Acot5s* in WT or single deletion strain did not result in significant effects on the fatty acid level. The simultaneous deletion of *FAA1* and *FAA4* was required for *Acot5s* to further improve the fatty acid level.

Figure 4.7 The effect of *Acot5s* expression on fatty acids production. Differential extracellular fatty acid levels in WT, WT [*Acot5s*], $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] were presented. Cells were collected after 24 h of growth in YNBD medium, and the culture medium were separated by centrifugation. The total extracellular fatty acids were extracted and detected. Data are the mean values of at least three independent experiments performed in duplicate. The error bar indicates the S.D.

Figure 4.8 Total extracellular FFA accumulation in the WT strain and engineered strains in YNBD medium at 24h. Cells were collected after 24 h of growth in YNBD medium, and the culture medium were separated by centrifugation. The total extracellular fatty acids were extracted and detected as described in methods. Data are the mean values of at least three independent experiments performed in duplicate. The error bar indicates the S.D.

4.3.4 Intracellular metabolite analysis

The data presented above illustrated that modification of acyl-CoA metabolism enhanced FFA accumulation. In addition, there were also differences in growth phenotypes between WT, $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [Acot5s] strains. In agreement with previous results, the strain $\Delta faa1 \Delta faa4$ showed slower exponential growth rates and reduced final cell concentrations as compared with the WT strain (Figure 4.9). It is believed that the FFA-producing strain should be growth defective, because high concentrations of FFA are toxic to the cells, due to its detergent-like character [207]. To our surprise, the better FFA-producing strain $\Delta faa1 \Delta faa4$ [Acot5s] did not show defect in growth, but displayed a similar growth phenotype

as the WT strain, with a higher final cell concentrations than the WT strain. The normal growth phenotype of $\Delta faa1 \Delta faa4$ [*Acot5s*] indicated that FFA was not toxic to cells. Therefore the observed growth defective in FFA accumulation strain may be due to a metabolism disorder, but not fatty acids inhibition. As discussed in other reports [208, 209], FFA could enter and leave cells freely by diffusion. This mechanism supports our hypothesis that FFA can be increased greatly without inhibiting cell growth, as long as the metabolic pathway is unobstructed.

Figure 4.9 Growth curves of the WT and engineered strains in YNBD medium. The WT and engineered strains were cultured in shake flask with YNB medium containing 20 g/L glucose as the carbon source. OD_{600} was measured at different time points. The results are the mean values of three independent experiments.

To further understand the changes in the metabolic pathway in the engineered strains, metabolic profiling was conducted to analyze the intracellular metabolites (Figure 4.10). The data presented demonstrated some amino acids were differentially produced in the strain $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with the WT strain. Among these amino acids, L-Lysine, L-Ornithine, L-Proline, Glycine and L-Alanine showed a decreased level in strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$

[*Acot5s*]. In both engineered strains, butanoic acid showed up-regulation, whereas glycerol exhibited a down-regulation.

Figure 4.10 Metabolic profiling analysis of the WT and engineered strains. Intracellular metabolites were extracted from the WT and engineered strains, and metabolic profiling was conducted. Differential expression levels of intracellular metabolites were analyzed in the engineered strains as compared to the WT. The amount of metabolites in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 24 h.

4.3.5 Gene expression analysis

As suggested by several reports [96, 201, 206, 210], fatty acyl-CoAs regulate gene expression by combining with gene regulatory elements. The fatty acid synthesis genes including *ACC1*, *FAS1*, *FAS2* and *OLE1* were up-regulated as a result of the decrease in levels of acyl-CoA esters in the cell. In our study, the main acyl-CoA

synthetases were deleted and acyl-CoA esters were hydrolyzed by thioesterase. Hence, a decrease in the availability of acyl-CoA esters in the cytoplasm and an up-regulation in the expression of *ACC1*, *FAS1*, *FAS2* and *OLE1* was logically expected. Since the strain $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] were observed to have increased extracellular fatty acid levels, we predicted that there should be an increase in the expression of *ACC1*, *FAS1*, *FAS2* and *OLE1* in the strain $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*]. To support this hypothesis, RT-PCR was carried out to analyze the relative gene expression of these genes. In addition, the gene *POX1* which encodes peroxisomal acyl-CoA oxidase and the gene *FAA2* which encodes peroxisomal medium chain acyl-CoA synthetase, both of which are related to fatty acid metabolism, were also analyzed. As presented in Figure 4.11, the expression of gene *ACC1*, *FAS1*, *FAS2* and *OLE1* were all increased, especially *OLE1*. The expression levels increased about 16 times in the strain $\Delta faa1 \Delta faa4$, and 23 times in $\Delta faa1 \Delta faa4$ [*Acot5s*]. The difference in the expression levels of the genes *POX1* and *FAA2* were not so obvious, the expression level of gene *POX1* increased slightly, while slight decrease was observed in the expression level of *FAA2*. These data are consistent with the conclusion that the expressions of fatty acid synthesis genes are increased in the strain $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*]. The large increase in *OLE1* expression levels reflected the significant increase in UFA levels.

4.4 Discussion

Fatty acid metabolism is tightly regulated at a delicate systemic level in yeast. In addition to the enzymes involved in both fatty acid synthesis and degradation pathway, we propose that the accumulation of FFA is directly regulated by both

intracellular acyl-CoA synthetase and acyl-CoA thioesterase (Figure 4.1). In our strategy, we first disrupted acyl-CoA synthetases *FAA1* and *FAA4* to block the flux from FFA to acyl-CoAs. In our next step, we further expressed acyl-CoA thioesterase *Acot5s* to reinforce the flux from acyl-CoAs to FFA. The mechanism of FFA accumulation is discussed below.

Figure 4.11 Differential expression levels of fatty acid metabolism genes in the engineered strains as compared to the WT. Real-time RT-PCR analysis of mRNA levels of fatty acid synthesis and degradation proteins were carried out. Three independent experiments were conducted with β -actin as internal standard.

4.4.1 Evaluation of FFA production and secretion in the strain

$\Delta faa1 \Delta faa4$

There are four acyl-CoA synthetases in *S. cerevisiae* [201, 211-213], in which Faa1p and Faa4p are essential enzymes in activating exogenous fatty acids and maintaining endogenous long chain acyl-CoA concentration. Thorough studies have been carried out to show how they affect the acyl-CoA concentration using cells cultured in medium supplied with fatty acids [206, 214]. In our study, the strains containing

both *FAA1* and *FAA4* deletions had extracellular FFA level increased to nearly 4.27-fold as compared to the WT strain, whereas a deletion of *FAA1* or *FAA4* had no obvious increase in extracellular FFA (Figure 4.8). This is similar to another report, which showed that the combined deletion of Faa1p and Faa4p is responsible for the secretion of FFA into the culture medium [46]. As opposed to the observation from Scharnewski [46], in this study we detected FFA in the culture medium of all strains while they could not detect FFA in the medium of their single deletion strain. The discrepancy may be due to the fact that deletion strains were constructed based on different yeast strains, and different extraction and esterification methods were used.

The first question raised about the increased extracellular FFA is the metabolic source. Scharnewski *et al* proposed that the FFA in the $\Delta faa1 \Delta faa4$ strain were derived from the lipid turn over processes [46]. Another report about reduced formation of triacylglycerol (TAG) in the $\Delta faa1 \Delta faa4$ strain support this view [215]. However, it was also shown in the same report that acyl-CoA dependent TAG synthesis accounted for only 30% of total cellular TAG [215]. In our work, no evident changes of intracellular TFA was observed but extracellular TFA was significantly increased. If TAG is the metabolic origin of FFA, when extracellular FFA is increased, intracellular TFA should decrease, and the intracellular glycerol level should increase due to lipid hydrolysis process. However in our study, the analysis of metabolites demonstrated that the strain $\Delta faa1 \Delta faa4$ had lower intracellular glycerol than the WT strain, and similar intracellular TFA levels. This implied that $\Delta faa1 \Delta faa4$ accumulated lower TAG levels than WT strain as a result of the defective acyl-CoA synthetases, but it could not be considered as the reason of the accumulation of FFA. As illustrated in Figure 4.1, the disruption of acyl-CoA

synthetases led to less available intracellular acyl-CoA, which resulted in higher levels of FFA and lower level TAG in the same time.

Thus, as TAG is not the metabolic source, we attempted to understand the underlying mechanism. After reviewing previous studies regarding the role of acyl-CoAs in gene expression regulation, it was observed that fatty acyl-CoAs instead of fatty acids repress the expression of acetyl-CoA carboxylase or Δ^9 -desaturase in yeast [81]. The disruption of both *FAA1* and *FAA4* completely blocked the repression of *OLE1* by UFA [93, 96]. The significant increase of UFA in strains deficient in *FAA1* and *FAA4* is presumably due to the de-repression of acetyl-CoA carboxylase and Δ^9 -desaturase by fatty acyl-CoAs. Additionally, it is known that fatty acid overproduction is associated with induction of fatty acid degradation, while *FAA1* or *FAA4* were required for the transcriptional regulation of the genes *POX1* and *FAA2*. For degradation of exogenously supplied fatty acids, β -oxidation level in the strain $\Delta faa1 \Delta faa4$ was reduced to 25% of WT strain [206]. Supported by the result of RT-PCR (Figure 4.11), the expression of gene *ACCI*, *FAS1*, *FAS2* and *OLE1* were all increased in the strain $\Delta faa1 \Delta faa4$, whereas the expression of gene *POX1* and *FAA2* were not significantly increased. These data demonstrated that the deletion of *FAA1* and *FAA4* not only relieved the feedback inhibition of acyl-CoA on fatty acid synthesis, but also weakened the link between overproduction of fatty acids and the up-regulation of genes in the β -oxidation pathway.

4.4.2 FFA production was further enhanced in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]

As stated above, FFA production was enhanced by the disruption of acyl-CoA

synthetases Faa1p and Faa4p. Hence we are interested to further exploit the strain $\Delta faa1 \Delta faa4$ in FFA production. To clarify the role of cytosolic acyl-CoA thioesterase in fatty acid metabolism in *S. cerevisiae*, we expressed cytosolic acyl-CoA thioesterase Acot5s in the double deletion strain $\Delta faa1 \Delta faa4$. As expected, it turned out to lead to more FFA accumulation. A 50% increase in extracellular total FFA was observed in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with the strain $\Delta faa1 \Delta faa4$. In addition, a higher ratio of UFA was achieved, which is preferred in biodiesel production due to the greater monounsaturated content giving a better quality of biodiesel [216]. The expression of Acot5s undoubtedly promoted the generation of product FFA and reinforced the flux towards the FFA. On the other hand, due to less cellular acyl-CoAs, the feedback inhibition on fatty acid synthesis genes was further relieved. As demonstrated in the RT-PCR result (Figure 4.11), the expression of gene *FAS1*, *FAS2* and *OLE1* was further up-regulated in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*].

On the basis of previous report by others and our present data, we conclude that the increase of FFA levels in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] is achieved by the control of acyl-CoA metabolism and de-repression of fatty acid synthesis. We chose to express acyl-CoA thioesterase Acot5s in the cytoplasm of *S. cerevisiae* as it has broad substrate activity (acting on C6 to C20-CoA) and highest activity with C10-CoA. We expected to increase the production of FFA, and investigate the possibility to modify the fatty acid profile, especially MCFA. However, the obtained strain $\Delta faa1 \Delta faa4$ [*Acot5s*] only accumulated more long chain fatty acids as compared with the strain $\Delta faa1 \Delta faa4$ (Figure 4.7). This might be due to the fact that the MCFA production rate is related to the cellular concentration of medium chain acyl-CoAs,

which is similar to *E. coli* [9]. Hence the low levels of MCFA accumulation were probably due to the low cellular concentration of medium chain fatty acyl-CoAs in yeast, since long chain acyl-CoAs are mainly released from FAS.

The yield of extracellular FFA (500 µg/ml=500 mg/L =1.88 mmol/L) is pretty high compared to other available reports under shake-flask condition. Scharnewski *et al* [46] found that double deletion of genes *FAA1* and *FAA4* in yeast strain could secrete 220 µmol/L FFA. Besides *FAA1* and *FAA4* disruption, overexpression of the FAS, acetyl-CoA carboxylase, and *E. coli* acyl-ACP thioesterase yielded 400 mg/L FFA in *S. cerevisiae* [217]. In another report, through disruption of the β-oxidation pathway and the acyl-CoA synthetases, overexpression of different thioesterases and increasing of the supply of acetyl-CoA, more than 120 mg/L FFAs was produced [218].

4.5 Conclusion

In short, the present study exploited the role of acyl-CoA thioesterase Acot5s in the engineered *S. cerevisiae* strain deficient in acyl-CoA synthetases *FAA1* and *FAA4*. The extracellular FFA in constructed strain increased 6.43-fold as compared to the WT. We succeeded in achieving high yield of FFA to ~500 µg/ml under shake-flask condition. The UFA took up 42% of the total FFA. This could have great potential in biofuel production. Future work will aim to increase the levels of fatty acids production by increasing the level of the precursor acetyl-CoA. In addition, to identify the detailed metabolic network changes and how the gene modification affects the de novo fatty acid synthesis and FFA secretion, LC-MS based proteomics analysis and GC-MS based metabolic flux analysis will be employed to study the

engineered strains.

5 Comparative proteomic analysis of engineered *S. cerevisiae* strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$

5.1 Introduction

In previous chapter, we successfully increased the accumulation of FFA by engineering the acyl-CoA metabolism of *S. cerevisiae*. This was done by double deletion of acyl-CoA synthetases Faa1 and Faa4, and cytosolic expression of truncated version of acyl-CoA thioesterase Acot5 (Acot5s) from *Mus musculus* [219]. We found a significant increase in extracellular fatty acids in the engineered strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$, as compared to the wild type (WT) strain. This showed that the enzymes acyl-CoA synthetases Faa1, Faa4 and thioesterase Acot5s had a direct effect on FFA accumulation. In addition, our RT-PCR results showed the genes involved in fatty acid synthesis were de-repressed in the strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$, although it should be taken into account that mRNA levels do not always correlate with protein expression [83, 160]. Therefore, to understand the mechanism for the increase of FFA in the engineered strains, proteomic analysis are needed.

The regulation of the gene, protein and metabolic network are closely connected in *S. cerevisiae* [179]. The enzymes involved in fatty acid synthesis and elongation are regulated on both the gene expression and protein level. In addition, compounds needed in the fatty acid synthesis process such as acetyl-CoA, ATP and NADPH are competed for by many other cellular reactions and metabolic pathways [48].

Previous study on acyl-CoA metabolism in engineered cells has provided important insights into the regulation of fatty acid synthesis. The use of proteomic analysis will further aid the understanding of the effects of gene modification, and also enable a global overview of metabolic regulation. Hence, we conduct comparative proteomic analysis between the WT, $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ strains in this study. The changes in proteins involved in different metabolic pathways were identified and analyzed. Furthermore, the global metabolic changes in the engineered strains were mapped.

5.2 Materials and methods

5.2.1 Strain growth and cell harvest

All strains used in this study were constructed based on *S. cerevisiae* WT strain BY4741 (*MATa*; *his3* $\Delta 1$; *leu2* $\Delta 0$; *met15* $\Delta 0$; *ura3* $\Delta 0$, EUROSCARF). As described in last chapter [219], the control strain WT [pVTU260] was constructed by transforming the WT strain with plasmid pVTU260 (also shown as WT). The double deletion strain $\Delta faa1 \Delta faa4$ was transformed with the plasmid pVTU260 to construct the strain $\Delta faa1 \Delta faa4$ [pVTU260] (also shown as $\Delta faa1 \Delta faa4$). The strain $\Delta faa1 \Delta faa4 [Acot5s]$ was constructed by transforming $\Delta faa1 \Delta faa4$ with the plasmid pVTU260-*Acot5s*. All strains were streaked on YNBD-Ura agar plates (0.67% yeast nitrogen base (with no amino acids but with ammonium sulfate, Invitrogen), 2% dextrose, amino acid drop out (without Ura, Clonotech), and 2% agar), and incubated at 30 °C for 2-3 days. Five mL of YNBD-Ura liquid medium were inoculated with a single colony and incubated overnight at 30 °C, 250 rpm.

This pre-culture was then used to inoculate 50 mL YNBD-Ura liquid medium with an initial OD₆₀₀ (Optical density at 600 nm) of 0.2. OD₆₀₀ was measured by Nano Drop 2000 (Thermo Scientific) to monitor growth status. Samples were collected at different time points. The culture supernatant and cell pellet were collected separately by centrifugation at 10000 rpm for 10 min at 4 °C.

5.2.2 Protein Extraction, Digestion and Labelling

Cells with an OD₆₀₀ of 20 from each sample were collected, washed with PBS buffer, and re-suspended in 500 µL NP40 cell lysis buffer (Invitrogen) supplemented with 1 mM PMSF and protease inhibitor cocktail. Then, 300 µL acid-washed glass beads were added and the cells were lysed using a FastPrep®-24 Instrument (6004-500, MP Biomedicals) for 20 s, and chilled on ice for 30 s. This was repeated 6 times. Next, the cells were centrifuged at 12000 rpm for 10 minutes at 4 °C. The clear lysate was then transferred to new sterile microcentrifuge tubes which were pre-chilled on ice.

Protein concentration was determined by 2-D Quant Kit (GE Healthcare). Then, 100 µg of protein from each sample was cleaned up using acetone precipitation. Four volumes of ice cold acetone was added to each sample and the protein was precipitated at -20 °C for at least 2 hours. After centrifugation at 12000 rpm for 10 min, the acetone was removed. Then the sample was digested and labelled using the iTRAQ Reagent Multiplex Kit (AB SCIEX) according to manufacturer's instructions. First, each protein sample was re-dissolved in 20 µL dissolution buffer and 1 µL denaturant, followed by adding 2 µL reducing reagent and incubated at 60 °C for 1 h. Then 1 µL cysteine blocking reagent was added into each tube and

incubated at room temperature for 10 minutes. Next, 20 μL of 0.25 $\mu\text{g}/\mu\text{L}$ sequence grade modified trypsin (Promega) was added into each sample. The digestion reaction was carried out at 37 $^{\circ}\text{C}$ for 12-16 hours. Samples from three strains were labeled with a different iTRAQ tag respectively. The labelled peptides were mixed together accordingly and vacuum evaporated to proper concentration.

5.2.3 Online 2-D Nano-LC-MS/MS Analysis

The labelled samples were analyzed using an Agilent 1200 series nanoflow LC interfaced with 6530 Q-TOF MS [220]. A HPLC-Chip Cube MS Interface (Agilent Technologies) coupled with the columns PolySulfoethyl A SCX column (0.32 x 50 mm, 5 μm) and HPLC-Chip (Zorbax 300SB C18 enrichment column (0.3 mm x 5 mm, 5 μm) and Zorbax 300SB C18 reversed-phase column (75 μm x 50 mm, 3.5 μm) was used for two dimensional analyses.

In the first dimension, 4 μL of the combined peptides mixture was loaded onto the SCX column and eluted stepwise by 8 μL of ammonium formate solutions in a series of concentrations: 20, 40, 60, 80, 100, 300, 500 and 1000 mM. In the second dimension, the peptides were eluted from SCX column and trapped on the Zorbax 300SB C18 enrichment column using buffer A (5% acetonitrile and 0.1% formic acid in water) at a flow rate of 4 $\mu\text{L}/\text{min}$. The peptides which were trapped on the enrichment column were then eluted and separated by the Zorbax 300SB C18 reversed-phase column. The elution buffers used were Buffer B (0.1% formic acid in water) and Buffer C (a nanoflow gradient of 5- 80% acetonitrile + 0.1% formic acid in water) at a flow rate of 300 nL/min. The total run time was 62 min for each round running. In all 9 runs were carried out for each sample due to the gradient

elution. The effluent was analyzed by the MS. Survey scans were obtained from m/z 300 to 2000 at acquisition rate of 4 spectra per second, with up to two precursors selected for MS/MS analysis from m/z 50 to 2000 [220].

5.2.4 LC-MS/MS Data Analysis and Interpretation

Protein identification and quantification were performed using Spectrum Mill software (Agilent Technologies). The MS/MS spectra acquired were searched against the database UniProtKB/Swiss-Prot with the following criteria: two maximum missed cleavages, 20 ppm precursor mass tolerance, 50ppm product mass tolerance, methylmethanethiosulfate-labeled cysteine as the fixed modification and iTRAQ modification of free amine in the amino terminus. For further statistical analysis, the following criteria were used: two or more identified unique peptides, protein MS/MS search score greater than 11, peptide score greater than 6. Three independent experiments were carried out to calculate the standard deviation and confidence value. Those with more than 95 % confidence were selected for further analysis [220].

5.2.5 HPLC analysis of extracellular metabolites

Metabolites in the filtered culture supernatant such as glucose, ethanol, glycerol, and acetate were quantified using an Agilent 1200 HPLC system (Singapore) equipped with a Rezex ROA-organic acid H⁺ (8%) column (300x7.8mm, OmegaScientific Phenomenex, Singapore). 5 mM H₂SO₄ was used as the mobile phase with a flow rate of 0.6 mL/min. The oven was set at 40°C, and a refractive index detector was used. The samples were quantitated using standards.

5.2.6 ATP and NADPH assay

Intracellular ATP was detected using ATP Colorimetric/Fluorometric Assay Kit (BioVision). NADPH was detected using NADP/NADPH Quantitation Kit (BioVision). Because they are labile during sampling, we used comparative ratio to indicate the changes between the engineered cells and the WT.

5.3 Results

In our previous work, it was shown that FFA production was enhanced in engineered strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$. By comparing the fatty acids levels in the WT $[Acot5s]$, $\Delta faa1$, $\Delta faa1 [Acot5s]$, $\Delta faa4$, $\Delta faa4 [Acot5s]$, $\Delta faa1 \Delta faa4$, $\Delta faa1 \Delta faa4 [Acot5s]$ and WT strains, we observed a significant increase in extracellular FFA in the engineered strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$, while no significant differences were observed in WT $[Acot5s]$, $\Delta faa1$, $\Delta faa1 [Acot5s]$, $\Delta faa4$, and $\Delta faa4 [Acot5s]$. The removal of genes *FAA1* and *FAA4* which encode for acyl-CoA synthetases led to the accumulation of a more than 4-fold extracellular fatty acid (326.97 $\mu\text{g/mL}$) as compared to the WT strain (76.5 $\mu\text{g/mL}$). The expression of cytosolic acyl-CoA thioesterase *Acot5s* in $\Delta faa1 \Delta faa4 [Acot5s]$ enhanced the total FFA production to about 500 $\mu\text{g/mL}$, which is more than 6-fold as compared to WT, and 1.5-fold increase as compared to the strain $\Delta faa1 \Delta faa4$. In addition, we also detected a high proportion of unsaturated extracellular fatty acids (UFA: C16:1 and C18:1) in $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$, whereas these could not be detected in the WT or the single deletion strains. Acyl-CoAs act as feedback inhibitors on fatty acid biosynthesis. The modification of acyl-CoA

metabolism reduced the accumulation of acyl-CoAs, therefore relieving the inhibition and enhanced FFA production [219]. The ability of *S. cerevisiae* to secrete FFA into medium also provided a driving force for fatty acid production, similar to what was reported before [217]. To obtain a global overview of the *in vivo* proteome changes in the engineered strains, a comparative proteomic analysis was performed.

5.3.1 ITRAQ analysis and protein identification

iTRAQ labelling was used to compare and analyze the *in vivo* protein changes between the WT, $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ strains. Proteins were extracted from samples harvested at 24 h, and labelled using the iTRAQ Reagent Multiplex Kit (AB SCIEX). Proteomic analysis was performed at least 3 times for statistical analysis. In total, over 500 proteins were detected. Among them, 82 proteins were found to be significantly altered in the engineered strains as compared to the WT as listed in Table 5.1 with their relative fold changes. 30 proteins were found to be significantly altered in $\Delta faa1 \Delta faa4 [Acot5s]$ as compared to $\Delta faa1 \Delta faa4$ as listed in Table 5.2 with their relative fold change.

Table 5.1 List of differentially expressed proteins in the strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ as compared with the wild type strain.

Gene name	Protein name	Fold change*	
		$\Delta faa1 \Delta faa4 /$ WT	$\Delta faa1 \Delta faa4$ [<i>Acot5s</i>]/WT
Glycolysis			
<i>GLK1</i>	Glucokinase-1	1.35±0.12	1.29±0.14
<i>PYK1</i>	Pyruvate kinase 1	1.35±0.07	--
<i>TPI1</i>	Triosephosphate isomerase	1.29±0.03	--
<i>PGI1</i>	Glucose-6-phosphate isomerase	1.29±0.11	--
<i>HXK1</i>	Hexokinase-1	1.51±0.11	1.42±0.06
<i>HXK2</i>	Hexokinase-2	1.15±0.07	--

<i>GPM2</i>	Phosphoglycerate mutase 2	--	1.45±0.12
<i>ENO2</i>	Enolase 2	1.17±0.08	1.18±0.18
<i>PDB1</i>	Pyruvate dehydrogenase E1 component	1.35±0.17	1.17±0.08
Pyruvate branchpoint			
<i>ADH1</i>	Alcohol dehydrogenase 1	2.01±0.08	--
Acetate metabolism			
<i>ALD6</i>	Cytosolic aldehyde dehydrogenase	1.25±0.05	1.15±0.02
<i>ALD4</i>	Mitochondrial aldehyde dehydrogenase	1.26±0.06	1.43±0.21
<i>ACS2</i>	Acetyl-CoA synthetase 2	1.15±0.07	1.16±0.07
<i>ACH1</i>	Acetate: succinyl-CoA transferase	1.19±0.11	2.22±0.18
Fatty acid metabolism			
<i>ACCI</i>	Acetyl-CoA carboxylase	1.17±0.06	1.16±0.2
<i>ACB1</i>	Acyl-CoA-binding protein	2.45±0.5	2.42±0.43
Glycerol metabolism			
<i>GPP1</i>	(DL)-glycerol-3-phosphatase 1	0.79±0.11	0.53±0.04
<i>GUT2</i>	Glycerol-3-phosphate dehydrogenase, mitochondrial	1.38±0.30	1.32±0.29
Glyoxylate cycle			
<i>ICL1</i>	Isocitrate lyase	1.35±0.12	1.71±0.14
Pentose phosphate pathway			
<i>GND1</i>	6-phosphogluconate dehydrogenase (decarboxylating)	1.57±0.10	1.55±0.11
<i>TKL1</i>	Transketolase 1	1.27±0.08	--
<i>TAL1</i>	Transaldolase	1.22±0.07	1.10±0.11
<i>NQM1</i>	Transaldolase NQM1	1.53±0.17	--
TCA cycle			
<i>CIT1</i>	Citrate synthase, mitochondrial	1.59±0.08	1.53±0.17
<i>IDH1</i>	Isocitrate dehydrogenase [NAD] subunit 1	1.52±0.08	1.23±0.35
<i>IDH2</i>	Isocitrate dehydrogenase [NAD] subunit 2	1.40±0.08	1.40±0.17
<i>KGD1</i>	2-oxoglutarate dehydrogenase, mitochondrial	1.13±0.04	1.35±0.05
<i>FUM1</i>	Fumarate hydratase, mitochondrial	1.30±0.13	1.21±0.29
<i>MDH1</i>	Malate dehydrogenase, mitochondrial	1.19±0.13	1.80±0.36
Carbohydrate biosynthesis; gluconeogenesis			
<i>PGM2</i>	Phosphoglucomutase-2	1.51±0.14	1.26±0.15
<i>ERG13</i>	Hydroxymethylglutaryl-CoA synthase	1.12±0.07	0.64±0.11
Protein involved in pathways other than carbon metabolism			
Amino acid biosynthesis			
<i>ARG4</i>	Argininosuccinate lyase	0.61±0.12	0.51±0.05
<i>ARG1</i>	Argininosuccinate synthase	0.65±0.05	0.56±0.04
<i>SAH1</i>	Adenosylhomocysteinase	0.83±0.04	0.77±0.01
<i>CYS3</i>	Cystathionine gamma-lyase	1.55±0.2	2.29±0.32
<i>SER3</i>	D-3-phosphoglycerate dehydrogenase 1	1.56±0.11	0.89±0.10
<i>SHM2</i>	Serine hydroxymethyltransferase, cytosolic	0.68±0.04	0.75±0.07
<i>LYS1</i>	Saccharopine dehydrogenase [L-lysine-forming]	0.80±0.06	0.76±0.13
<i>LYS12</i>	Homoisocitrate dehydrogenase, mitochondrial	0.84±0.05	0.62±0.12

<i>LYS21</i>	Homocitrate synthase, mitochondrial	0.86±0.07	0.66±0.06
<i>GDH1</i>	NADP-specific glutamate dehydrogenase 1	1.36±0.06	0.51±0.01
<i>UGA1</i>	4-aminobutyrate aminotransferase	1.23±0.1	--
<i>HIS4</i>	Histidine biosynthesis trifunctional protein	0.55±0.03	0.62±0.10
Nucleotide-sugar biosynthesis, Cell wall synthesis			
<i>GAS1</i>	1,3-beta-glucanosyltransferase GAS1	0.74±0.08	0.60±0.03
<i>MPG1</i>	Mannose-1-phosphate guanyltransferase	0.89±0.02	0.70±0.06
<i>PMI40</i>	Mannose-6-phosphate isomerase	0.86±0.06	0.63±0.06
Protein biosynthesis			
<i>PDI1</i>	Protein disulfide-isomerase	1.18±0.12	1.10±0.09
<i>ILS1</i>	Isoleucyl-tRNA synthetase	1.19±0.08	--
<i>FPR3</i>	Peptidyl-prolyl cis-trans isomerase	1.31±0.12	1.37±0.12
<i>STM1</i>	Suppressor protein STM1	1.28±0.12	--
<i>RPS2</i>	40s ribosomal protein S2	--	1.36±0.10
Respiration			
<i>OYE2</i>	NADPH dehydrogenase 2	1.25±0.04	--
<i>ATP1</i>	ATP synthase subunit alpha, mitochondrial	1.20±0.06	1.62±0.16
<i>ATP2</i>	ATP synthase subunit beta, mitochondrial	1.24±0.05	1.31±0.07
<i>ATP3</i>	ATP synthase subunit gamma, mitochondrial	1.18±0.11	1.33±0.11
<i>ATP5</i>	ATP synthase subunit 5, mitochondrial	1.18±0.07	1.26±0.08
<i>AAC2</i>	ADP,ATP carrier protein 2	1.13±0.05	1.32±0.05
Transport			
<i>HXT6</i>	High-affinity hexose transporter HXT6	3.06±0.3	3.07±0.3
<i>PMA1</i>	Plasma membrane ATPase 1	1.32±0.05	1.19±0.06
<i>TRX2</i>	Thioredoxin-2	1.31±0.10	1.44±0.12
<i>POR1</i>	Mitochondrial outer membrane protein porin 1	1.26±0.12	1.27±0.08
<i>CHC1</i>	Clathrin heavy chain	1.42±0.13	--
<i>GRX1</i>	Glutaredoxin-1	1.20±0.13	1.27±0.08
Stress response			
<i>GRE2</i>	NADPH-dependent methylglyoxal reductase GRE2	1.79±0.22	1.56±0.21.
<i>MCR1</i>	NADH-cytochrome b5 reductase 2	1.39±0.06	1.53±0.15
<i>TPS1</i>	Trehalose-6-phosphate synthase	1.24±0.02	--
<i>TPS2</i>	Trehalose-6-phosphate Phosphatase	1.15±0.07	--
<i>DCS1</i>	Scavenger mRNA-decapping enzyme DcpS	1.34±0.17	1.28±0.20
<i>YNK1</i>	Nucleoside diphosphate kinase	1.41±0.09	--
<i>PRB1</i>	Cerevisin	1.36±0.07	--
<i>GLR1</i>	Glutathione reductase	1.20±0.07	--
<i>HSP26</i>	Heat shock protein 26	1.57±0.08	1.65±0.11
<i>HSP30</i>	30 KDa heat shock protein	--	1.40±0.07
<i>HSP42</i>	Heat shock protein 42	1.43±0.19	--
<i>HSP104</i>	Heat shock protein 104	1.45±0.12	1.13±0.02
<i>ARA1</i>	D-arabinose dehydrogenase	1.26±0.04	--
Antioxidants			

<i>AHP1</i>	Alkylhydroperoxide reductase	1.22±0.10	--
<i>SOD1</i>	Superoxide dismutase [Cu-Zn]	1.43±0.10	1.41±0.11
<i>SOD2</i>	Superoxide dismutase [Mn], mitochondrial	1.29±0.08	--
Others			
<i>RIB4</i>	6,7-dimethyl-8-ribityllumazine synthase	3.05±0.35	1.14±0.08
<i>INO1</i>	Inositol-3-phosphate synthase	1.25±0.06	1.15±0.04
<i>SAC1</i>	Phosphoinositide phosphatase SAC1	1.26±0.13	1.44±0.05

*Data represented mean±S.D. based on three independent replicate experiments. "--" indicated the protein was not shown significant change.

Table 5.2 List of differentially expressed proteins in the strain *Δfaa1 Δfaa4* [*Acot5s*] as compared with the strain *Δfaa1 Δfaa4*.

Pathway	Gene name	Protein name	Fold change*
Glycolysis	<i>PKY1</i>	Pyruvate kinase 1	0.73±0.021
	<i>PGI1</i>	Glucose-6-phosphate isomerase	0.66±0.040
	<i>HXK2</i>	Hexokinase-2	0.76±0.068
Pyruvate branchpoint	<i>ADH1</i>	Alcohol dehydrogenase 1	0.49±0.052
Acetate metabolism	<i>ACH1</i>	Acetyl-CoA hydrolase	1.90±0.340
Glycerol biosynthesis	<i>GPP1</i>	(DL)-glycerol-3-phosphatase 1	0.70±0.151
Glyoxylate cycle	<i>ICL1</i>	Isocitrate lyase	1.27±0.012
TCA cycle	<i>KGD1</i>	2-oxoglutarate dehydrogenase, mitochondrial	1.19±0.049
	<i>MDH1</i>	Malate dehydrogenase, mitochondrial	1.31±0.053
Ergosterol biosynthesis	<i>ERG13</i>	Hydroxymethylglutaryl-CoA synthase	0.58±0.133
Amino acid biosynthesis	<i>CYS3</i>	Cystathionine gamma-lyase	1.49±0.261
	<i>SER3</i>	D-3-phosphoglycerate dehydrogenase 1	0.58±0.061
	<i>LYS21</i>	Homocitrate synthase, mitochondrial	0.77±0.078
	<i>GDH1</i>	NADP-specific glutamate dehydrogenase 1	0.37±0.017
	<i>UGA1</i>	4-aminobutyrate aminotransferase	0.73±0.088
Cell wall synthesis	<i>GAS1</i>	1,3-beta-glucanosyltransferase GAS1	0.82±0.051
	<i>PMI40</i>	Mannose-6-phosphate isomerase	0.74±0.076
Respiration	<i>ATP1</i>	ATP synthase subunit alpha, mitochondrial	1.54±0.351
	<i>ATP3</i>	ATP synthase subunit gamma, mitochondrial	1.22±0.119
	<i>AAC2</i>	ADP,ATP carrier protein 2	1.27±0.159
Stress response	<i>TPS1</i>	Trehalose-6-phosphate synthase	0.58±0.077
	<i>TPS2</i>	Trehalose-6-phosphate Phosphatase	0.61±0.074
	<i>GLR1</i>	Glutathione reductase	0.81±0.08
	<i>HSP42</i>	Heat shock protein 42	0.55±0.132

	<i>HSP104</i>	Heat shock protein 104	0.76±0.07
	<i>ARA1</i>	D-arabinose dehydrogenase	0.76±0.111
Antioxidants	<i>AHP1</i>	Alkylhydroperoxide reductase	0.80±0.057
	<i>SOD2</i>	Superoxide dismutase [Mn], mitochondrial	0.75±0.102
Others	<i>RIB4</i>	6,7-dimethyl-8-ribityllumazine synthase	0.38±0.045
	<i>SAC1</i>	Phosphoinositide phosphatase SAC1	1.15±0.127

*Data represented mean±S.D. based on three independent replicate experiments. Fold change refers to the average ratio of protein expression level in the strain *Δfaa1 Δfaa4 [Acot5s]* over that in the strain *Δfaa1 Δfaa4*.

5.3.2 Relative changes in protein expression between the WT and engineered strains

The identified proteins which were shown to have significant changes could be classified into different biological process categories according to their functions as annotated in the UniProtKB database (<http://www.uniprot.org/>) (Figure 5.1).

Figure 5.1 Functional classification of proteins identified. Proteins were classified into eight groups based on their functions annotated in the UniProtKB database (<http://www.uniprot.org/>). The percentage of proteins of each function group was exhibited.

These functions included carbon metabolism, amino acid and nucleotide metabolism,

protein biosynthesis, respiration, transportation, stress response, antioxidants and others. The majority of proteins belonged to the category of carbon metabolism, which accounted for 37.80% of all proteins (Figure 5.1), showing that a major metabolic rearrangement occurred in the engineered cells as a result of the genetic modifications.

Among these proteins involved in carbon metabolism (Table 5.1), 8 proteins (Glk1, Pyk1, Tpi1, Pgi1, Hxk1, Hxk2, Eno2 and Pdb1) which are involved in the glycolysis were up-regulated in the strain $\Delta faa1 \Delta faa4$, whereas five proteins (Glk1, Hxk1, Gpm2, Eno2 and Pdb1) were up-regulated in the strain $\Delta faa1 \Delta faa4 [Acot5s]$. Enzyme Adh1 which is responsible for ethanol production showed up-regulation in the strain $\Delta faa1 \Delta faa4$, whereas it remained unchanged in the strain $\Delta faa1 \Delta faa4 [Acot5s]$. Four enzymes (Ald6, Ald4, Acs2 and Ach1) involved in acetate metabolism were up-regulated in both $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ strains. Apart from acetate biosynthetic process [221], Ald6 and Ald4 are also involved in NADPH regeneration [92]. Acs2 is involved in acetyl-CoA synthesis from acetate in pyruvate-acetaldehyde-acetate pathway [222]. Ach1 is involved in shuttling mitochondrial acetyl-CoAs for cytosolic C2 provision [223] and acetate detoxification [224]. The enzyme Gpp1 [225] which takes part in the glycerol biosynthetic process was down-regulated in both $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ strains, whereas the enzyme Gut2 [226] involved in glycerol catabolic process was up-regulated in both $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$ strains. Moreover, two enzymes (Acc1 and Acb1) which participate in fatty acid metabolism, one enzyme (Icl1) involved in glyoxylate cycle [227], and six enzymes (Cit1, Idh1, Idh2, Kgd1, Fum1 and Mdh1) involved in the TCA cycle were up-regulated in both

Δfaa1 Δfaa4 and *Δfaa1 Δfaa4 [Acot5s]* strains. Four enzymes (Gnd1, Tk11, Tal1 and Nqm1) involved in pentose phosphate pathway were up-regulated in the strain *Δfaa1 Δfaa4*, whereas two enzymes (Gnd1 and Tal1) were up-regulated in the strain *Δfaa1 Δfaa4 [Acot5s]*. For enzymes involved in carbohydrate biosynthesis, the major phosphoglucomutase isoform Pgm2 [228] responsible for hexose metabolism showed up-regulation in both *Δfaa1 Δfaa4* and *Δfaa1 Δfaa4 [Acot5s]* strains. Another study showed that protein abundance of Pmg2 also increased in response to various stresses [229]. For enzymes involved in ergosterol biosynthetic process, Erg13 [230] was up-regulated in *Δfaa1 Δfaa4*, whereas it was down-regulated in *Δfaa1 Δfaa4 [Acot5s]*.

Three enzymes (Gas1, Mpg1 and Pmi40) involved in cell wall synthesis were down-regulated in both *Δfaa1 Δfaa4* and *Δfaa1 Δfaa4 [Acot5s]* strains. Six enzymes (Oye2, Atp1, Atp2, Atp3, Atp5 and Aac2) involved in respiration were up-regulated in *Δfaa1 Δfaa4*, while five enzymes (Atp1, Atp2, Atp3, Atp5 and Aac2) were detected up-regulated in *Δfaa1 Δfaa4 [Acot5s]*. For proteins involved in amino acid biosynthesis pathway, it was detected that 8 enzymes (Arg1, Arg4, Sah1, Shm2, Lys1, Lys12, Lys21 and His4) were down-regulated and 4 enzymes (Cys3, Ser3, Gdh1 and Uga1) were up-regulated in *Δfaa1 Δfaa4*. Ten enzymes (Arg1, Arg4, Sah1, Ser3, Shm2, Lys1, Lys12, Lys21, Gdh1 and His4) were down-regulated and one enzyme Cys3 was up-regulated in *Δfaa1 Δfaa4 [Acot5s]*. For proteins involved in protein biosynthesis pathway, 4 enzymes (Pdi1, Ils1, Fpr3 and Stm1) were up-regulated in the strain *Δfaa1 Δfaa4*, and 3 enzymes (Pdi1, Fpr3 and Rps2) were up-regulated in the strain *Δfaa1 Δfaa4 [Acot5s]*.

For proteins involved in sugar, protein and other ion transport process, 6 proteins (Hxt6, Pma1, Trx2, Por1, Chc1 and Grx1) were up-regulated in the strain $\Delta faa1 \Delta faa4$. Among these enzymes, all other 5 proteins except Chc1 showed similar increase in the strain $\Delta faa1 \Delta faa4$ [Acot5s]. 12 proteins (Gre2, Mcr1, Tps1, Tps2, Dcs1, Ynk1, Prb1, Glr1, Hsp26, Hsp42, Hsp104 and Ara1) involved in stress response showed increase in their expression levels in the strain $\Delta faa1 \Delta faa4$. Comparatively, 6 proteins (Gre2, Mcr1, Dcs1, Hsp26, Hsp30 and Hsp104) involved in stress response showed increase in the strain $\Delta faa1 \Delta faa4$ [Acot5s].

The level of antioxidant proteins Ahp1 [231], Sod1 [232] and Sod2 [233] were increased in the strain $\Delta faa1 \Delta faa4$, while only Sod1 was increased in the strain $\Delta faa1 \Delta faa4$ [Acot5s]. Moreover, Rib4 involved in cofactor riboflavin biosynthesis [234], inositol-3-phosphate synthase (Ino1) [78] involved in inositol synthesis, and phosphoinositide phosphatase Sac1 [235] involved in the coordination of the activities of the secretory pathway showed up-regulation in both strains.

5.3.3 Relative changes in protein expression between $\Delta faa1 \Delta faa4$ [Acot5s] and $\Delta faa1 \Delta faa4$

To investigate the influence of Acot5s expression on proteome changes, a direct comparison of protein changes between $\Delta faa1 \Delta faa4$ [Acot5s] and $\Delta faa1 \Delta faa4$ was conducted. As shown in Table 5.2, 10 proteins which play a role in carbon metabolism were found to be markedly changed in $\Delta faa1 \Delta faa4$ [Acot5s] as compared to $\Delta faa1 \Delta faa4$. Among these proteins, three enzymes (Pyk1, Pgi1, and Hxk2) involved in glycolysis, one enzyme (Adh1) involved in ethanol production, one enzyme (Gpp1) involved in glycerol biosynthetic process, and one enzyme

(Erg13) involved in ergosterol biosynthesis pathway were down-regulated in the strain *Δfaa1Δfaa4 [Acot5s]*. One enzyme (Ach1) that is involved in acetate metabolism, two enzymes (Kgd1 and Mdh1) involved in the TCA cycle, and one enzyme (Icl1) involved in glyoxylate cycle were up-regulated.

Moreover, three proteins (Atp1, Atp3 and Aac2) involved in respiration and one phosphoinositide phosphatase Sac1 were up-regulated in *Δfaa1Δfaa4 [Acot5s]*. Six proteins involved in stress response (Tps1, Tps2, Glr1, Hsp42, Hsp104 and Ara1) and two antioxidant proteins (Ahp1 and Sod2) were found down-regulated. Two enzymes (Gas1 and Pmi40) involved in cell wall synthesis and one enzyme (Rib4) involved in cofactor biosynthesis were also down-regulated in *Δfaa1Δfaa4 [Acot5s]*. Besides, five enzymes involved in amino acid biosynthesis were found differentially expressed.

5.3.4 Physiological and metabolite analysis of the engineered and WT strains

The WT and engineered strains were cultured in shake flask with YNB medium containing 20 g/L glucose as the carbon source. Growth curve and the concentration of several extracellular metabolites were studied. As shown in Figure 5.2 (A), strain *Δfaa1Δfaa4* had a defective growth and slower exponential growth rate, while strain *Δfaa1Δfaa4 [Acot5s]* displayed a higher growth rate as compared with the WT strain. This was also seen in our previous study [219]. As showed in Figure 5.2 (B), glucose consumption was delayed in the strain *Δfaa1Δfaa4* as compared with WT and *Δfaa1Δfaa4 [Acot5s]*, the delayed glucose consumption in strain *Δfaa1Δfaa4* was consistent with its slow growth rate. As for glycerol accumulation (Figure 5.2 (C)),

Figure 5.2 Physiological features of engineered and WT strains. WT strain and engineered strains were cultured in YNBD medium. Cell growth (A), extracellular concentration of glucose (B), glycerol (C), acetate (D) and ethanol (E) were measured at different time points as indicated in method. Data are the mean values of three independent experiments. The error bar indicates the S.D.

$\Delta faa1 \Delta faa4$ [*Acot5s*] had the lowest glycerol concentration, whereas WT strain had the highest glycerol concentration. For acetate accumulation (Figure 5.2 (D)), $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] had much lower final acetate concentration as compared with the WT strain. Regarding ethanol accumulation (Figure 5.2 (E)), the three strains had similar ethanol concentration curve. The strain $\Delta faa1 \Delta faa4$ [*Acot5s*] gradually accumulated lower level of ethanol after 24 h than WT and $\Delta faa1 \Delta faa4$ strains. In addition, intracellular ATP and NADPH level of three strain were examined. As expected, the strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] had higher ATP and NADPH level as compared to the WT strain in both exponential phase and stationary phase (Figure 5.3 and Figure 5.4).

Figure 5.3 ATP level in the engineered strains as compared with the WT strain. The amount of ATP in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 8 h and 24 h. The error bar indicates the S.D

Figure 5.4 NADPH level in the engineered strains as compared with the WT strain. The amount of NADPH in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 8 h and 24 h. The error bar indicates the S.D

5.4 Discussion

In our previous study, it was shown that FFA production was enhanced by disrupting both acyl-CoA synthetases Faa1 and Faa4, and expressing acyl-CoA thioesterase Acot5s in *S. cerevisiae*. This modification restrained FFA activation, relieved the feedback inhibition of acyl-CoAs on fatty acid synthesis, and directed carbon flux towards FFA accumulation [219]. However, how the cells sensed the changes in fatty acid metabolism and responded accordingly was unknown. Therefore, we carried out proteomic analysis in this study, and mapped the changes in proteins in relation to the biochemical pathways to give a global overview of the pathway regulations (Figure 5.5 and Figure 5.6).

5.4.1 Comparative proteome analysis of the engineered strains as compared to WT

There were many changes in enzymes involved in crucial biological pathways, due to the double deletion of genes *FAA1* and *FAA4* and expression of *Acot5s*. As shown in Table 5.1, several enzymes involved in glycolysis, a fundamental pathway in glucose metabolism to provide energy in yeast [236], were up-regulated in both engineered strains. The average increment of these enzymes was 30.75% in $\Delta faa1 \Delta faa4$ and 30.2% in $\Delta faa1 \Delta faa4$ [*Acot5s*]. In the glycerol metabolism pathway, the abundance of the enzyme Gpp1 [225], which is involved in glycerol biosynthetic process, was found to decrease to 79% and 53% in the strain $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4$ [*Acot5s*] respectively, as compared with the WT strain. The relative abundance of the enzyme Gut2 [226], which is involved in the glycerol catabolic process, increased more than 30% in both engineered strains. This was consistent with the data that both engineered strains accumulated less glycerol than the WT strain (Figure 5.4 (C)). In addition, decrease in intracellular glycerol level were also seen in both engineered strains in our previous metabolite profiling data [219]. The decrease in glycerol synthesis probably provided extra pyruvate for other pathways. In the pyruvate-ethanol pathway, the relative abundance of protein Adh1, which is the main alcohol dehydrogenase that catalyzes the conversion of acetaldehyde to ethanol [237], increased 2-fold in the strain $\Delta faa1 \Delta faa4$, while it remained unchanged in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]. Because several proteins in pyruvate-acetaldehyde-acetate pathway were up-regulated in $\Delta faa1 \Delta faa4$ [*Acot5s*], it is not surprised that the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] had lower concentration of ethanol than the WT and $\Delta faa1 \Delta faa4$ after 24 h (Figure 5.2 (E)). However, we did

not find significant increase of ethanol concentration in the strain $\Delta faa1 \Delta faa4$. This may be due to having slower growth rate and lower biomass accumulation than the WT strain, and several proteins in its pyruvate-acetaldehyde-acetate pathway were also up-regulated. There were 3 enzymes (Erg10, Erg13 and Erg20) detected in the ergosterol biosynthesis pathway. Erg13 were up-regulated to 112% in $\Delta faa1 \Delta faa4$ and down-regulated to 64% in $\Delta faa1 \Delta faa4 [Acot5s]$ as compared to WT. There were no significant changes in the level of Erg10 and Erg20 (data not shown). This might indicate that there was decreased carbon flux towards ergosterol production in the strain $\Delta faa1 \Delta faa4 [Acot5s]$. Further experiment to estimate the regulation of ergosterol pathway in $\Delta faa1 \Delta faa4$ would be required.

Besides the above mentioned carbon metabolic regulation, multiple levels of regulation in fatty acid metabolism of *S. cerevisiae* exist. Fatty acid synthesis is a highly energy-demanding process, which requires large amounts of acetyl-CoA, ATP and NADPH [48]. The supply of these compounds has significant effect on fatty acid synthesis. Acetyl-CoA in *S. cerevisiae* can be produced from both the mitochondrial pyruvate dehydrogenase pathway and the cytosolic pyruvate-acetaldehyde-acetate pathway [102, 238]. As shown in Table 5.1, enzyme Pdb1 [84], which is part of the pyruvate dehydrogenase complex and involved in catalyzing the overall conversion of pyruvate to acetyl-CoA in the mitochondria, was up-regulated in both strains. The levels of several enzymes in pyruvate-acetaldehyde-acetate pathway were also increased. Cytosolic aldehyde dehydrogenase (Ald6) which plays a major role in acetate formation and a compensative mitochondrial aldehyde dehydrogenase (Ald4) [221] were up-regulated in both engineered strains. Acetyl-CoA synthetase 2 (Acs2) [239, 240], which generates acetyl-CoA by direct

activation of acetate in the cytoplasm, was also up-regulated by about 15% in both strains. This was consistent with the observation that both engineered strains accumulated less acetate than the WT strain (Figure 5.2(D)). In addition, the enzyme Icl1 [227], which catalyzes the formation of succinate and glyoxylate from isocitrate, a key step in the glyoxylate cycle, was also up-regulated. Glyoxylate cycle is one known route for transporting acetyl-CoA from the peroxisomes/cytosol into the cytosol/mitochondria through converting acetyl-CoA into glyoxylate cycle intermediates [241]. The up-regulation of Icl1 in glyoxylate cycle implied there may be increased need for acetyl-CoA transportation. Furthermore, the up-regulation of Ach1 may imply the same increased need, since mitochondrial Ach1 can transport acetyl-CoA produced in the mitochondria to the cytosol via conversion of acetyl-CoA into acetate, which can cross the mitochondrial membrane freely [223]. Although we did not observe significant increase in cellular acetyl-CoA level in the engineered strains (data not shown), the significant increase of FFA production implied that there should be increased supply of acetyl-CoA.

ATP can be generated through oxidation of glucose in glycolysis, the TCA cycle, and oxidative phosphorylation. The amount of energy released by oxidative phosphorylation is much higher as compared to glycolysis [87]. In addition to the up-regulation of enzymes in glycolysis, six enzymes involved in the TCA cycle, and several subunits of ATP synthase [242] in aerobic respiration were observed to be significantly up-regulated in both engineered strains. In the strain $\Delta faa1 \Delta faa4$, enzymes in the TCA cycle were up-regulated from 1.13 to 1.59-fold, and subunits of ATP synthase were up-regulated from 1.18 to 1.24-fold as compared to the WT strain. In the strain $\Delta faa1 \Delta faa4$ [*Acot5s*], enzymes in the TCA cycle were up-

regulated from 1.21 to 1.80-fold, and subunits of ATP synthase were up-regulated from 1.26 to 1.62-fold as compared to the WT strain. Besides, the major ADP/ATP carrier of the mitochondrial inner membrane Aac2 was also up-regulated in both engineered strains. Aac2 imports cytosolic ADP into mitochondria, where it can be phosphorylated by ATP synthase, and exports ATP into the cytosol [243, 244]. So the up-regulation of Aac2 was consistent with the up-regulation of ATP synthase. The analysis of ATP level in three strains demonstrated that there were higher levels of ATP in two engineered strains as compared to WT, which further demonstrated the up-regulation of ATP synthesis.

NADPH can be provided by several sources such as malic enzyme, the pentose phosphate pathway, NADPH isocitrate dehydrogenase, NAD kinase [48] and the aldehyde dehydrogenase family [91, 92]. In the present analysis, we found that several enzymes involved in NADPH generation were up-regulated. For example, the level of Gnd1 (6-phosphogluconate dehydrogenase), which catalyzes the NADPH generation step in the pentose phosphate pathway, increased by about 55% in both strains. Ald6 and Ald4, which utilize NADP⁺ as the preferred coenzyme when performing the conversion of acetaldehyde to acetate and play important roles in NADPH regeneration [92], were up-regulated by 25% and 26% in $\Delta faa1 \Delta faa4$, 15% and 43% in $\Delta faa1 \Delta faa4 [Acot5s]$. The increased intracellular NADPH level in both engineered strains further proved there were increased NADPH synthesis in both engineered strains.

Figure 5.5 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4$ as compared with WT. The primary pathway are described according to the changes in protein expression as listed in Table 5.1. Red boxes and red text indicate up-regulated pathways and proteins. Blue boxes and blue text indicate down-regulated pathways and proteins

Furthermore, the enzymes involved in fatty acid synthesis were also altered, although the results were different from the transcriptional level analysis [219]; that is, the expression of genes related to fatty acid synthesis which are repressed by fatty acyl-CoAs were increased significantly in the engineered strains $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [Acot5s]$, especially *OLE1* which encodes a Δ -9 fatty acid desaturase. However, the protein Acc1 was increased by only about 15%, while the RT-PCR result showed several fold increase. The increase in Acc1 protein level was smaller than its increase in gene expression level. There was no observed significant increase in the level of Fas1 and Fas2. Δ -9 fatty acid desaturase was not detected, which may be due to its location in the endoplasmic reticulum membrane [245]. Also, regulation

of these proteins are complex and occur at multiple levels, including histone acetylation, mRNA stability, protein stability and coordination with other genes expression [48]. The increase in mRNA level with no subsequent increase in protein expression level is understandable and also supported by other studies [83, 160]. Although Acc1 affects fatty acid synthesis directly and overexpression of Acc1 could enhance fatty acid synthesis [14, 21, 22], the study about overexpression of Acc1 in eukaryotic organism generally results in small improvement of fatty acid production [217, 218]. Further study is needed to explore the role of up-regulation of Acc1 in the increased fatty acid production, which probably due to the possibility that the cellular level of enzyme Acc1 cannot meet the increased need of malonyl-CoA in fatty acid synthesis. Moreover, the level of acyl-CoA binding protein Acb1, which facilitates the cellular transportation of acyl-CoA and lessens the inhibition effect of acyl-CoA on fatty acid synthesis [246, 247], was increased 2.45-fold and 2.43-fold respectively in $\Delta faa1 \Delta faa4$ and $\Delta faa1 \Delta faa4 [\Delta cot5s]$.

Several proteins involved in transportation were found to be up-regulated in both engineered strains, especially the high-affinity hexose transporter Hxt6, which plays an important role in glucose uptake and utilization [248]. It was up-regulated more than 3-fold in both strains. This was consistent with the up-regulation of glycolysis in both strains. Previous study showed that overproduction of fatty acids in *E. coli* resulted in the change of membrane properties because of fatty acid intercalation in the membrane, fatty acid accumulation in the periplasm, and alteration of membrane lipids; these changes then led to membrane stress and compromised membrane integrity [249]. In our engineered strains, the down-regulation of enzymes (Gas1, Mpg1 and Pmi40) involved in cell wall synthesis [250-252] and up-regulation of

enzyme Sac1 (Phosphoinositide phosphatase) involved in secretory pathway [235] should also be the response of fatty acid overproduction, such regulation may facilitate FFA secretion outside the cells.

In cells, amino acid biosynthesis pathways are not only essential process for protein synthesis, they are also critical for yeast cells to achieve coordinated regulation between nitrogen source utilization and nucleotide biosynthetic pathways [253]. In our observation, many enzymes involved in amino acid biosynthesis (Table 5.1) were differentially expressed in two engineered strains as compared to the WT. Among these enzymes, Shm2 [254] is the major enzyme responsible for converting serine to glycine. Lys1, Lys12 and Lys21 [255] are responsible for L-lysine biosynthesis. The down-regulation of these enzymes were consistent with the observed decrease in L-lysine and glycine levels in previous metabolic profiling result (Figure 4.10). For other enzymes involved in amino acid synthesis, there was no significant changes detected in the relevant amino acids levels in our previous study. Since cells have natural ability to reduce biosynthetic cost to optimize growth and reproduction, the regulation of amino acid, protein and nucleotide biosynthesis should be under the need of metabolic cost optimization [256].

Also, DNA microarray experiment showed that environmental stresses, including temperature shock and oxidative stress, greatly stimulate up-regulation of the transcription of stress responsive genes in yeast cells [257]. In our study, we observed that more enzymes involved in stress response and antioxidants were up-regulated in the strain $\Delta faa1 \Delta faa4$ as compared to the strain $\Delta faa1 \Delta faa4 [Acot5s]$. For example, D-arabinose dehydrogenase Ara1 [258, 259], which was re-annotated as α , β -dicarbonyl reductase and found involved in reducing a variety of toxic

metabolic by-products such as methylglyoxal, diacetyl and pentanedione produced during stress, was only up-regulated in $\Delta faa1 \Delta faa4$. This was consistent with the growth defect of the strain $\Delta faa1 \Delta faa4$ and recovered growth of the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] (Figure 5.2 (A)), which indicated that the stress caused by removing Faa1 and Faa4 was relieved in a certain degree by introducing *Acot5s*.

Figure 5.6 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared with WT. The primary pathway are described according to the changes in protein expression as listed in Table 5.1. Red boxes and red text indicate up-regulated pathways and proteins. Blue boxes and blue text indicate down-regulated pathways and proteins

5.4.2 The impact of *Acot5s* expression on proteome changes

As stated above, the growth phenotype of the strain $\Delta faa1 \Delta faa4$ is more severe than the strain $\Delta faa1 \Delta faa4$ [*Acot5s*], thus it is not surprising that there are more changes

about proteins involved in central metabolism in $\Delta faa1 \Delta faa4$ as opposed to $\Delta faa1 \Delta faa4$ [*Acot5s*]. We wonder how the $\Delta faa1 \Delta faa4$ [*Acot5s*] cells responded to the driving force provided by *Acot5s* and had higher FFA production and growth rate compared to $\Delta faa1 \Delta faa4$ and WT strains. Through comparatively analyzing protein expression levels between $\Delta faa1 \Delta faa4$ [*Acot5s*] and $\Delta faa1 \Delta faa4$, the affected proteins that take part in crucial metabolic pathways were mapped in Figure 5.7. Among them, the level of alcohol dehydrogenase *Adh1*, which is involved in ethanol production, decreased to 49% in $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared to $\Delta faa1 \Delta faa4$. The level of enzyme *Gpp1* involved in glycerol biosynthetic process was down-regulated to 70% in $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared to $\Delta faa1 \Delta faa4$. This indicated that the added driving force provided by *Acot5s* made cells direct part of their carbon flux from ethanol and glycerol production to acetyl-CoA synthesis. Although we did not observe additional increase in protein level involved in pyruvate-acetaldehyde-acetate pathway, we did find decrease in ethanol and glycerol production and increase of FFA production in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]. In addition, *Erg13* involved in ergosterol biosynthesis pathway were down-regulated to 58% of that in $\Delta faa1 \Delta faa4$, which may imply decreased carbon flux toward ergosterol.

Protein *Ach1*, which is involved in acetyl-CoA transportation and acetate detoxification [223], was up-regulated 1.9 fold in $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared to $\Delta faa1 \Delta faa4$. In addition, the reported study [223] also showed that overexpression of *ACH1* in the $\Delta ach1$ strain not only rescued growth on glucose but also led to slightly faster growth than the $\Delta ach1$ strain. The higher expression level of *Ach1* may explain the higher growth rate of $\Delta faa1 \Delta faa4$ [*Acot5s*]. Furthermore,

the down-regulation of stress response and antioxidants proteins also showed the *Δfaa1 Δfaa4 [Acot5s]* cells adapted to the new balanced state built by *Acot5s*. Moreover, the up-regulation of protein *Kgd1* and *Mdh1* (1.19 and 1.31 fold) involved in the TCA cycle, and *Atp1*, *Atp3* and *Aac2* (1.54, 1.22 and 1.27 fold) involved in respiration were consistent with the higher ATP level.

The direct effect of removing acyl-CoA synthetases *Faa1* and *Faa4* was restraining FFA activation, and led to FFA secretion into the culture medium as demonstrated before [46]. Although the mechanism of fatty acid secretion has not been unraveled completely yet, it was reported that the fatty acid transport could be driven by the concentration gradient between extracellular and intracellular fatty acid and need the activities of fatty acyl-CoA synthetase (*Faa1* and *Faa4*) and fatty acid transport protein 1 (*Fat1*). The deletion of *FAA1* and *FAA4* increases the concentration of FFA in the cytosol which leads to the secretion of FFA [46], it also decreases the concentration of intracellular acyl-CoA which results in changes in intracellular signaling to fatty acid-responsive genes [201, 206]. In our study, the expression of thioesterase reinforced the effect, thus the modification provided a higher driving force towards higher fatty acid production. Compared with other recent work [217, 218, 260], besides using different background strains, the main difference between these studies is the driving force, which is provided by different thioesterases [217, 218] or lipid body forming enzymes with lipase [260]. Our study showed that *Acot5s* is a pretty efficient enzyme to transform acyl-CoA to FFA. The expression of *Acot5s* affected the regulation of proteins involved in crucial metabolic pathways, which led to a major metabolic rearrangement while not harming growth.

Figure 5.7 Changes of metabolic pathways in the strain $\Delta faa1 \Delta faa4 [Acot5s]$ as compared with $\Delta faa1 \Delta faa4$. The primary pathways are described according to the changes in protein expression as listed in Table 5.2. Red boxes and red text indicate up-regulated pathways and proteins. Blue boxes and blue text indicate down-regulated pathways and proteins.

5.5 Conclusion

In summary, to gain insights into the underlying metabolic regulation associated with the genetic modification in the engineered strains, we set out to investigate the proteomic profile changes between WT and engineered strains. It was found that the disruption of genes *FAA1* and *FAA4* and expression of *Acot5s* in *S. cerevisiae* contributed to elevated carbon flux towards FFA accumulation. It was identified that proteins involved in glycolysis, pentose phosphate pathway, TCA cycle, fatty acid synthesis, ATP synthesis and transportation were up-regulated, while proteins involved in glycerol synthesis, pyruvate-ethanol synthesis, ergosterol synthesis and

cell wall synthesis were down-regulated in the strain *Δfaa1 Δfaa4 [Acot5s]*. Our detected changes in metabolites supported the finding regarding the changes in protein expression in the different metabolic pathways. Previous study demonstrated that the genetic modification relieved the feedback inhibition of fatty acyl-CoAs. The global metabolic regulation found in this study indicated that the cells up-regulated certain pathways to facilitate fatty acid synthesis and substrates supply, and efficiently utilize the carbon flux by down-regulating some pathways and channeling the carbon flux towards FFA accumulation. This information is of great help to unravel the mechanism for the increased production of FFA in the engineered strain. It also shows the great potential of the engineered strain in the metabolic adjustment, and it will certainly aid future work in rational engineering of the yeast system for biofuel production. For example, the flux toward alcohol biosynthesis could be further decreased in the engineered strain, and enzymes in pyruvate-acetaldehyde-acetate pathway or Ach1p could be overexpressed to reinforce the carbon flux toward cytosolic acetyl-CoA supply. Several genes in β -oxidation could be deleted to diminish cellular fatty acid degradation. More importantly, the driving force could be enhanced to further promote FFA production.

6 Conclusion and limitation

6.1 Background and Motivation

Fatty acids and their derivatives have gained lots of attention as excellent candidates of renewable energy. Not only biofuels synthesized by fatty acids via biochemical and *in vitro* catalytic pathway are under investigation by many companies and academic organizations, other chemicals traditionally produced from petroleum can also be made from fatty acids, such as R-3-hydroxybutyric acid [261, 262], polyhydroxyalkanoates [19, 263], and flavonoid [264]. Given the structural similarity of fatty acids to petrochemical products, there is great potential in optimizing microbial fatty acid metabolism pathway serving as an industrial biosynthesis platform for many valuable compounds. Through metabolic engineering of fatty acid synthesis and degradation pathway, fatty acid-derived biofuels and biochemicals can be accumulated in microbes, such as alkanes [10, 11, 15], alcohol [8, 25], and biodiesel [8, 34]. And, to be competitive with current petrochemicals, flux in fatty acid metabolism must be optimized to reach high yields.

Enhancing FFA production in *S. cerevisiae* has several advantages: the fatty acid synthesis and degradation pathway is well-studied; there is mature technique using *S. cerevisiae* in industrial fermentation; it's tolerance to organic solvent is better than *E. coli*. While, the disadvantage is that it needs more specific control because the subcellular compartmentalization. For example, de novo fatty acid synthesis takes place in both cytoplasm and mitochondrion; fatty acid is elongated in endoplasmic reticulum; fatty acid degradation happens in peroxisome. It has been shown that

modification of acyl-CoA oxidase can influence lipid accumulation in *Y. lipolytica* by affecting β -oxidation. There is also study about modified fatty acid profile through overexpressing thioesterase with different acyl-ACP chain length preferences in *E. coli* (for detail description see chapter 2 literature review). Based on the current knowledge and great potential of FFA, we would like to explore *S. cerevisiae* as a FFA production platform by modifying fatty acid degradation and synthesis pathway.

6.2 Conclusion

In our study, we aimed to engineer *S. cerevisiae* into a useful platform for accumulating high yield of FFA. Based on our work described in chapter three to chapter five, we drew several conclusions, as shown in Figure 6.1 and Figure 6.2:

Firstly, we constructed a strain $\Delta pox1$ [*pox2+*, *crot+*], in which gene *POX1* was deleted, the *POX2* gene from *Y. lipolytica* encoding an acyl-CoA oxidase with a preference for long chain acyl-CoAs was co-expressed with the gene *CROT* from *Mus musculus* encoding carnitine O-octanoyltransferase. It was observed that the concentration of both intracellular and extracellular MCFAs increased in the strain $\Delta pox1$ [*pox2+*, *crot+*] compared to the WT strain when grown in YNBD_{0.5}O₃. It was demonstrated that the β -oxidation of yeast in peroxisomes is terminated at medium chain acyl-CoAs, and the genetic modification was capable of transforming low value fatty acid feedstock into higher value fatty acids containing more MCFAs.

Secondly, in another part of the study, we firstly constructed an engineered strain $\Delta faa1 \Delta faa4$, which is deficient in two main acyl-CoA synthetases Faa1 and Faa4.

Then cytosolic acyl-CoA thioesterase *Acot5s* was further expressed in the strain $\Delta faa1 \Delta faa4$, resulting the strain $\Delta faa1 \Delta faa4 [Acot5s]$. The extracellular FFA in the strain $\Delta faa1 \Delta faa4 [Acot5s]$ significantly increased as compared to the WT and $\Delta faa1 \Delta faa4$ strain. A high yield about 500 $\mu\text{g/mLk}$ was achieved under shake-flask conditions. The UFA was improved to 42% of TFA. Because acyl-CoAs are feedback inhibitor of fatty acid biosynthesis, the genetic modification we made reduced the accumulation of cellular acyl-CoAs thus relieved the inhibition. The ability of yeast to secrete FFA outside of the cells and the expression of *Acot5s* provided the driving force for the enhanced FFA production.

Figure 6.1 Free fatty acid accumulation in *S. cerevisiae* by modifying fatty acid metabolism.

Furthermore, to investigate the metabolic regulation associated with the genetic modification, we examined the proteomic profile changes between the WT and engineered strains. It was found that several metabolic pathways were affected by

altering the acyl-CoA metabolism. Compared to wild type, enzymes involved in glycolysis, pentose phosphate pathway, TCA cycle, fatty acid synthesis, ATP synthesis and transportation were up-regulated; while enzymes involved in glycerol synthesis, pyruvate-ethanol synthesis, ergosterol synthesis and cell wall synthesis were down-regulated in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]. The global metabolic regulation implied that the cells up-regulated several pathways to facilitate fatty acid synthesis and substrates supply, and down regulated certain pathways to efficiently utilize the carbon flux and channel it towards FFA production. This information is helpful to unravel the mechanism for the increased production of FFA in the engineered strain.

Figure 6.2 Metabolic regulation in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*]. The main changes of metabolic pathways is summarized based on proteomic and metabolite analysis of the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] as compared to the wild type strain.

In conclusion, the results presented in this study provide new insights into utilization of *S. cerevisiae* as a microbial platform for FFA production. The construction of strain $\Delta pox1$ [*pox2+*, *crot+*] is a novel study in producing MCFA in *S. cerevisiae*. The expression of *Acot5s* can efficiently transform acyl-CoA to FFA. The final yield of FFA in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] is pretty high as compared to currently reported yields to our knowledge. The global metabolic regulation observed in $\Delta faa1 \Delta faa4$ [*Acot5s*] offers extensive information to help us understand the underlying mechanism. Our study shows the great potential of the engineered yeast strains, it has direct influence on future rationally engineering yeast into a biofuel production strain.

6.3 Limitation

The results listed above demonstrated that it is feasible to accumulate high yield of FFA in engineered *S. cerevisiae*. Our findings provided insights in understanding the regulation mechanism in the engineered strains using comparative proteomic analysis. However, some limitations in our study need to be taken into account.

For the modification of fatty acid degradation pathway, the engineered strain $\Delta pox1$ [*pox2+*, *crot+*] can be used to transform long chain fatty acid-rich feedstock to higher value medium chain fatty acids. In our observation, MCFA were increased in strain $\Delta pox1$ [*pox2+*, *crot+*], however they are still the minor components of total fatty acids, so further improvement of the engineered strain is needed. Overexpression of another gene *Acot5*, which encodes peroxisome thioesterase was carried out (data not shown). However, the efficiency of utilizing fatty acid as feedstock was not

improved greatly in the newly engineered strain. Further modification is still needed to improve the MCFA production in *S. cerevisiae*.

Regarding the control of fatty acyl-CoA metabolism, the cytosolic acyl-CoA thioesterase Acot5s we used has broad substrate activity, acting on C6 to C20-CoA and has highest activity with C10-CoA. It is expected that total FFA would be increased, especially MCFA. However, there is no further increase of MCFA in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*] compare to $\Delta faa1 \Delta faa4$ (see detail in part 4.4.2). The possible reason is that the cellular concentration of medium chain acyl-CoAs is low thus affecting the MCFA production rate. In future work, the degradation of MCFA is planned to be blocked to increase the MCFA accumulation. So it is an option to combine two parts of work together.

Besides, GC-MS based metabolic profiling is only applicable to volatile compounds or compounds that are volatile after derivatization, which means it is not enough to give a complete picture of metabolome. The complementary analytical technologies, such as LC-MS based metabolomics, also could be used to investigate the metabolome in future work to give a complete overview.

7 Future direction

Our study has preliminarily established a microbial platform for FFA production in *S. cerevisiae*, in which the metabolic efficiency of FFA production was greatly improved. In addition, intracellular protein profiling revealed the changes of enzymes involved in metabolic pathways in the engineered cells, while intracellular and extracellular metabolite analysis gave us more evidence about metabolic regulation of the engineered strains. It is expected that this platform would be used to synthesize more FFA and fatty acid-derived biofuels. Such study is also expected to provide more information about *S. cerevisiae* and its industrial application.

In future work, we will continue to investigate various methods for further optimizing the engineered strains to give increased production of FFA or fatty acid-derived products. Several possible studies are listed below:

Firstly, given the important role of cytosolic acetyl-CoA in fatty acid synthesis, it is hypothesized that increase of the PDH bypass flux and decrease of the flux toward alcohol biosynthesis could increase the concentration of cytosolic acetyl-CoA thus enhance fatty acid production. It has been shown that engineering PDH bypass can enhance a wide range of acetyl-CoA-derived products by overproducing acetyl-CoA. Isoprenoids production in yeast was increased by overexpression of acetaldehyde dehydrogenase (ALD6) and a *Salmonella enterica* acetyl-CoA synthetase variant (ACS_{SE}^{L641P}) [265]. The production of α-santalene in *S. cerevisiae* was improved 4-fold by introducing a plasmid pIYC08 containing genes *ADH2* encoding alcohol dehydrogenase, *ALD6* encoding acetaldehyde dehydrogenase, ACS_{SE}^{L641P} encoding

acetyl-CoA synthetase, and *ERG10* encoding acetyl-CoA acetyltransferase [266]. Furthermore, the production of polymer polyhydroxybutyrate (PHB) was improved more than 16-fold by co-transformation of a plasmid containing the bacterial PHB pathway genes together with the plasmid pIYC08 [261]. Similarly, butanol was improved 6.5-fold by co-expressing the butanol pathway with pIYC08 in the *MLS1* or *CIT2* deletion strains [267]. In future work, co-expression of *ADH2*, *ALD6*, *ACS_{SE}^{L641P}* and *Acot5s* is expected to further enhance FFA accumulation (Figure 7.1).

Figure 7.1 Engineering *S. cerevisiae* for high levels of FFA accumulation. Genes encode for native *S. cerevisiae* alcohol dehydrogenase (*ADH2*), acetaldehyde dehydrogenase (*ALD6*), cytosol acyl-CoA thioesterase (*Acot5s*), and a *Salmonella enterica* acetyl-CoA synthetase variant (*ACS_{SE}^{L641P}*) will be expressed in double deletion strain $\Delta faa1 \Delta faa4$ to enhance the flux to FFA.

Secondly, in this study, FFA synthesis and degradation pathway were modified separately, and there are limitations in both projects as described in section 6.3. If two modified pathways can be combined together, for example, blocking the degradation of MCFA in the strain $\Delta faa1 \Delta faa4$ [*Acot5s*], which has high FFA accumulation, maybe can increase the amount of MCFA to a certain degree.

Thirdly, fatty acid accumulation is affected by growth conditions, such as O₂ concentration, temperature, pH, and culture medium condition (carbon, nitrogen, or phosphate concentration). Growth conditions would be examined and optimized to enhance FFA production. Besides, the engineered strains were cultured in shake-flask level currently. In order to further investigate the feasibility for larger-scale production of FFA, the engineered strains could be cultured in a larger scale laboratory fermenter to study the effect of various conditions on FFA accumulation.

Fourthly, given the important role of thioesterases in alleviating feedback inhibition of fatty acyl-CoAs on fatty acid synthesis and FFA accumulation, a lot of factors need to be taken into account when expressing thioesterases for fatty acid production. There has been study suggested that not only codon optimization, the copy number of carrier plasmid also affects fatty acid levels. Highest fatty acid yield was obtained in an engineered *E. coli* strain by over-expressing a low copy plasmid carrying thioesterase [7]. Thus, further study would include the optimization of fatty acid level by using other carrier plasmids containing different copy numbers and promoters.

Furthermore, efficiently transforming FFA to valuable biofuel products will also increase the cellular FFA production, because it will reduce the feedback inhibition

of FFA, and push the equilibrium toward FFA accumulation. Therefore, there are a lot of work can be done on the established platform. For example, expressing lipase in the engineered strain to synthesize biodiesel. Expressing enzyme CAR and AHR to synthesize fatty alcohol (Figure 2.5). Co-expressing CAR and AOD in the engineered strain to synthesize alkane. In addition, the ability of the engineered strain to use other carbon sources can also be exploited; for example, combining *Scheffersomyces stipitis* xylose utilization pathway in the engineered *S. cerevisiae* [268] to use xylose to synthesize FFA.

8 References

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List of publications

1. Chen LW, Lee J, Zhang JH, and Chen W*. (2015) Comparative proteomic analysis of engineered *Saccharomyces cerevisiae* with enhanced free fatty acid accumulation. In press
2. Chen LW, and Chen W*. (2014). Metabolite and Fatty Acid Analysis of Yeast Cells and Culture Supernatants. Bio-protocol 4(17): e1219. <http://www.bio-protocol.org/e1219>.
3. Chen LW, Zhang JH, and Chen W*. Engineering the *Saccharomyces cerevisiae* β -Oxidation Pathway to Increase Medium Chain Fatty Acid Production as Potential Biofuel. PLoS ONE,2014. 9(1):e84853.
4. Chen LW, Zhang JH, Lee JH and Chen W*. Enhancement of free fatty Acids production in *Saccharomyces cerevisiae* by control of fatty acyl-CoA metabolism. Appl Microbiol Biotechnol., 2014. 98(15):6739-5.
5. Lee J, Chen LW, Shi JH, Trzcinski A, and Chen W*. Metabolomic profiling of *Rhodospiridium toruloides* grown on glycerol for carotenoids production during different growth phases. J. Agr. Food Chem. 2014. 62(41):10203-9

Appendix

Figure S1. The representative GC-MS spectra for a *S. cerevisiae* fatty acid profile.

Fatty acid composition and concentration may differ from those shown here as a result of altered cell culturing conditions and/or alternative genetic backgrounds of the yeast cells employed

Figure S2. Representative FAME standard curves. A: Caprylic acid methyl ester ($\text{CH}_3(\text{CH}_2)_6\text{COOCH}_3$) standard curve; B: Capric acid methyl ester ($\text{CH}_3(\text{CH}_2)_8\text{COOCH}_3$) standard curve; C: Lauric acid methyl ester ($\text{CH}_3(\text{CH}_2)_{10}\text{COOCH}_3$) standard curve; D: Myristic acid methyl ester ($\text{CH}_3(\text{CH}_2)_{12}\text{COOCH}_3$) standard curve; E: Palmitoleic acid methyl ester ($\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOCH}_3$) standard curve; F: Palmitic acid methyl ester

($\text{CH}_3(\text{CH}_2)_{14}\text{COOCH}_3$) standard curve; G: Heptadecanoic acid methyl ester ($\text{CH}_3(\text{CH}_2)_{15}\text{COOCH}_3$) standard curve; H: Oleic acid methyl ester ($\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{CH}_3$) standard curve; I: Stearic acid methyl ester ($\text{CH}_3(\text{CH}_2)_{16}\text{COOCH}_3$) standard curve. Different concentrations (0.125-0.75 mg/ml) of FAMES standard (Sigma-Aldrich, Supelco 18918) and Heptadecanoic acid methyl ester were used for GC-MS analysis. Standards or samples of 1 μl were injected into the column by splitless mode using an autoinjector. To make individual FAME standard curves, the concentration of FAME was determined by multiplying its percent concentration (as indicated on the certificate of analysis from the supplier) by the prepared concentrations (0.125-0.75 mg/ml).

Figure S3. Representative glucose standard curves. Different concentrations (2.5-40

g/L) of glucose standard were used for HPLC analysis. Standards or samples of 10 µl were injected into the column using an autoinjector. The glucose levels in samples were calculated based on this standard curve.

Figure S4. Representative glycerol standard curves. Different concentrations (3.125-50 g/L) of glycerol standard were used for HPLC analysis. Standards or samples of 10 µl were injected into the column using an autoinjector. The glycerol levels in samples were calculated based on this standard curve.

Figure S5. Representative acetate standard curves. Different concentrations (2.5-40 g/L) of acetate standard were used for HPLC analysis. Standards or samples of 10 μ l were injected into the column using an autoinjector. The acetate levels in samples were calculated based on this standard curve.

Figure S6. Representative ethanol standard curves. Different concentrations (7.89-78.9 g/L) of ethanol standard were used for HPLC analysis. Standards or samples of 10 μ l were injected into the column using an autoinjector. The ethanol levels in samples were calculated based on this standard curve.

Figure S7. A representative GC-MS spectra for extracellular fatty acid profile (as presented in figure 4.6).

Figure S8. A representative GC-MS spectra for extracellular fatty acid profile (as presented in figure 4.7).

Figure S9. Representative ATP standard curve. 0, 2, 4, 6, 8, 10 μ l of 1 mM ATP standard were added into a series of wells and adjust volume to 50 μ l/well with ATP assay buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of ATP standard. 50 μ l of reaction mix was added to each well containing the ATP standard and samples. After mixed well and incubated at room temperature for 30 min, absorbance at OD₅₇₀ was

measured for colorimetric assay in a micro-plate reader. The sample ATP concentrations were calculated based on this standard curve.

Figure S10. Representative NADPH standard curve. 0, 2, 4, 6, 8, 10 μ l of 10 pmol/ μ l of NADPH standard were added into a series of wells to generate 0, 20, 40, 60, 80, 100 pmol/well standard and adjust volume to 50 μ l/well with NADP/NADPH extraction buffer. 100 μ l of NADP cycling mix was added into each well and the plate was incubated at room temperature for 5 min. Then 10 μ l NADPH developer was added into each well and the reaction was developed for 1-4 hours. The plate was read at OD₄₅₀ nm.