Systems-level characterization and transporter engineering in *Saccharomyces cerevisiae* for improved hydrocarbon tolerance

Chen, Binbin

2014

Chen, B. (2014). Systems-level characterization and transporter engineering in *Saccharomyces cerevisiae* for improved hydrocarbon tolerance. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/61731>

<https://doi.org/10.32657/10356/61731>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**SYSTEMS-LEVEL CHARACTERIZATION AND
TRANSPORTER ENGINEERING IN *SACCHAROMYCES*
CEREVISIAE FOR
IMPROVED HYDROCARBON TOLERANCE**

BINBIN CHEN

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2014

**SYSTEMS-LEVEL CHARACTERIZATION AND
TRANSPORTER ENGINEERING IN *SACCHAROMYCES*
CEREVISIAE FOR
IMPROVED HYDROCARBON TOLERANCE**

BINBIN CHEN

School of Chemical and Biomedical Engineering

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

2014

ACKNOWLEDGEMENT

This thesis wouldn't be possible without my supervisor, Prof. Matthew Chang. I was lucky to join his lab, and I believe that I had many opportunities to learn and experience various researches under his supervision. He patiently guided me to design and troubleshoot my projects, write and present my data. He never felt disappointed about me but kept encouraging me throughout my PhD time here. His humorous, passion, and big picture view of projects have inspired me a lot and made me feel my work is valuable.

I would like to thank my senior - Aram Kang, not only because of her immense help in my experiments, but also her kind help in my life. Her encouragement made me motivated; her understanding made me feel accompanied; her calmness and suggestion made me look deep into myself and learn to face problems that I'm afraid of. I also want to express my great gratitude to my colleague - Dr Hua Ling for his kind and patient instruction on my research and life, and his wife - Ruirui Liu's care on my daily life in Singapore which made me feel at home always. I would like to give my thanks to Samuel Lo, who gave me a lot of encouragement, support and concerns during my PhD. I express my thanks to Simon Teo. His passion, curiosity and ways of sharing inspired me a lot. The help and support of my lab members Mui Hua Tan, Choon Kit Wong, Bahar RI, Kai Sheng Hee and Dr Lawrance Ho made my life here more enjoyable and memorable.

Finally, my gratitude goes out to my parents and my grandma. I always feel lucky to become a member of my family. Their support and love make me enjoy my life and give me courage to continue my research life in the future. Also I'm grateful to know my friends Shanshan Tong and Jiahua Shi, who accompany me, understand me, encourage me and help me, during which we have a lot of enjoyable moments and we grow together.

PUBLICATIONS

Journal papers

Chen B, Ling H, Chang MW: Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* 2013, 6(1):21.

Ling H, **Chen B**, Kang A, Lee J-M, Chang M: Transcriptome response to alkane biofuels in *Saccharomyces cerevisiae*: identification of efflux pumps involved in alkane tolerance. *Biotechnology for Biofuels* 2013, 6(1):95.

Lo T-M, Teo WS, Ling H, **Chen B**, Kang A, Chang MW: Microbial engineering strategies to improve cell viability for biochemical production. *Biotechnology Advances* 2013, 31(6):903-914.

Ling H, Teo WS, **Chen B**, Leong SSJ, Chang MW: Microbial tolerance engineering towards biochemical production: From lignocellulose to products. *Current Opinion in Biotechnology*

Patent

Inventors: 1) Matthew Wook Chang; 2) **Binbin Chen**. Improving Alkane Tolerance of *Saccharomyces cerevisiae* by Introducing Transporters of *Yarrowia lipolytica*

TABLE OF CONTENTS

ACKNOWLEDGEMENT.....	1
PUBLICATIONS.....	3
TABLE OF CONTENTS.....	4
LIST OF FIGURES.....	8
LIST OF TABLES.....	11
SUMMARY.....	12
CHAPTER 1 INTRODUCTION.....	14
1.1 Background.....	15
1.2 Aims and objectives.....	18
1.3 Thesis organization.....	18
CHAPTER 2 LITERATURE REVIEW.....	20
2.1 Microbial engineering for the production of biofuels.....	21
2.1.1 First-generation biofuels.....	21
2.1.2 Second-generation biofuels.....	21
2.1.2.1 Characteristics of second-generation biofuels.....	21
2.1.2.2 Alcohol-derived fuels.....	24
2.1.2.2.1 Butanol.....	24
2.1.2.2.2 Branched-chain alcohols	28
2.1.2.3 Isoprenoid-derived fuels.....	33
2.1.2.4 Fatty-acid-derived fuels.....	36
2.2 Microbial engineering strategies to improve cell viability for biochemical production.....	39

2.2.1	Introduction.....	40
2.2.2	Transcription factor engineering for improved stress response.....	42
2.2.3	Harnessing efflux pumps against product toxicity.....	45
2.2.4	Spatial engineering and compartmentalization against intermediate toxicity.....	50
2.2.5	Dynamic sensing-regulation of protein expression.....	52
2.2.6	Balancing co-factors in pathways and key metabolisms.....	58
2.2.7	Computational tools for design and prediction.....	63
2.2.8	Conclusion.....	69
CHAPTER 3 TRANSCRIPTOME RESPONSE TO ALKANE BIOFUELS IN <i>SACCHAROMYCES CEREVISIAE</i>.....		71
3.1	Introduction.....	72
3.2	Materials and methods.....	74
3.2.1	Strains and media.....	74
3.2.2	Cell viability, membrane integrity and reactive oxygen species (ROS).....	74
3.2.3	Alkane extraction and detection.....	75
3.2.4	RNA preparation, transcriptome analysis, and quantitative PCR.....	76
3.2.5	Network component analysis (NCA).....	77
3.3	Results and discussion.....	78
3.3.1	Toxicity and intracellular accumulation of alkanes.....	78
3.3.2	Genome-wide gene expression profiles upon exposure to biofuel alkanes.....	82

3.4	Conclusions.....	90
 CHAPTER 4 IDENTIFICATION OF TRANSPORTERS FROM SACCHAROMYCES CEREVISIAE INVOLVED IN ALKANE TOLERANCE.....92		
4.1	Introduction.....	93
4.2	Materials and methods.....	94
4.2.1	Strains, chemicals and plasmids.....	94
4.2.2	Growth conditions.....	94
4.2.3	Alkane extraction and detection.....	95
4.2.4	Yeast gene deletion.....	96
4.2.5	Plasmid construction and analyses of yeast strains overexpressing transporter genes.....	99
4.3	Results and discussion.....	100
4.3.1	Overexpression of transporters leads to improved tolerance to biofuel alkanes.....	100
4.3.2	Transporter SNQ2 and PDR5 contribute to alkane efflux pumping.....	103
4.4	Conclusions.....	107
 CHAPTER 5 IDENTIFICATION OF TRANSPORTERS FROM YARROWIA LIPOLYTICA INVOLVED IN ALKANE TOLERANCE.....108		
5.1	Introduction.....	109
5.2	Materials and methods.....	109
5.2.1	Strains and media.....	110
5.2.2	Plasmid construction.....	110
5.2.3	Quantitative RT-PCR.....	114

5.2.4	Western Blot Analysis.....	114
5.2.5	Fluorescence Microscopy.....	115
5.2.6	Alkane susceptibility assays.....	116
5.2.7	Determination of intracellular alkane levels.....	116
5.3	Results and discussion.....	117
5.3.1	Transcription activity of ABC transporters with alkanes.....	117
5.3.2	Expression and subcellular localization of ABC2 and ABC3.....	121
5.3.3	ABC2 and ABC3 contribute to increasing alkane tolerance....	122
5.3.4	ABC2 and ABC3-mediated efflux pumping of alkanes.....	127
5.3.5	Glutamate is required for energy-dependent efflux pumping of ABC2 and ABC3.....	128
5.4	Conclusions.....	131
CHAPTER 6 CONCLUSIONS AND FUTURE CONSIDERATIONS.....		133
6.1	Conclusions.....	134
6.2	Future considerations.....	137
BIBLIOGRAPHY.....		139

LIST OF FIGURES

Figure 2-1 Metabolic pathways used for the production of alcohol-based biofuels.....	25
Figure 2-2 Comparison of a high-yielding pathway for production of polyhydroxyalkanoates (PHAs) to the design of a chimeric pathway for fuel synthesis derived from three different organisms.....	27
Figure 2-3 Optimization of the synthetic Ter-dependent pathway for n-butanol production.....	28
Figure 2-4 Production of higher alcohols through the synthetic non-fermentative pathways.....	30
Figure 2-5 Summary of results for isobutanol and 1-butanol production in <i>E. coli</i>	32
Figure 2-6 Metabolic pathways used for the production of isoprenoid-based biofuels.....	34
Figure 2-7 Bisabolane from chemical hydrogenation of microbially produced bisabolene.....	36
Figure 2-8 Pathways for the production of fatty acid-based biofuels.....	39
Figure 2-9 An overview of the key approaches towards developing a microorganism platform for improved viability.....	41
Figure 2-10 Rational engineering of complex phenotypes by three approaches introduced in the text.....	45

Figure 2-11 Improvement of cell viability and pathway productivity by harnessing efflux pumps.....	50
Figure 2-12 Dynamic sensing-regulation of protein expression.....	56
Figure 2-13 Approaches towards balancing co-factors for boosting anaerobic growth under batch condition, supplying reducing power for reducing substrate to product and restoring NADPH/NADH balance.....	63
Figure 2-14 An in silico approach for designing the cell for bio-chemical production, but with host viability as key consideration.....	69
Figure 3-1 Viability and membrane permeability of <i>S. cerevisiae</i> cells under exposure to C9-C12.....	79
Figure 3-2 Cell morphology of <i>S. cerevisiae</i> BY4741 cells upon exposure to 2% C9.....	81
Figure 3-3 GC analyses of intracellular alkanes in <i>S. cerevisiae</i>	81
Figure 3-4 The predicted transcription regulatory network.....	88
Figure 3-5 Schematic representation of hypothesized mechanisms of cellular response to alkanes in <i>S. cerevisiae</i>	90
Figure 4-1 Growth of triple knock-out mutants expressing transporters under alkane exposure.....	102
Figure 4-2 Intracellular alkane levels of triple knock-out mutants expressing transporters under alkane exposure.....	104

Figure 4-3 GC chromatograms of C10 and C11 from <i>S. cerevisiae</i> BYL251K cells.....	106
Figure 5-1 mRNA transcript levels of ABC1–4 genes in <i>Y. lipolytica</i>	120
Figure 5-2 Expression and subcellular localization of ABC2 and ABC3...	122
Figure 5-3 Alkane susceptibility assay for <i>S. cerevisiae</i>	123
Figure 5-4 Alkane susceptibility assay on agar plates.....	124
Figure 5-5 Intracellular alkane levels of ABC2 and ABC3.....	128
Figure 5-6 Transporter sequence comparison and alkane susceptibility assay of ABC2 and ABC3 mutants.....	130
Figure 5-7 Topology model of ABC2 and ABC3.....	131

LIST OF TABLES

Table 2-1 Liquid transportation fuel properties and potential biofuels.....	23
Table 3-1 Validation of microarray data by qPCR upon exposure to C10 for 48h.....	82
Table 3-2 MIPS functional classification of genes differentially regulated by both C9 and C10.....	84
Table 3-3 Transcription factors with activity changes, predicted by Network Component Analysis (NCA).....	87
Table 3-4 ROS levels in <i>S. cerevisiae</i> upon exposure to C9 and C10 for 24h.....	90
Table 4-1 Primers used in this study.....	96
Table 4-2 Yeast strains and plasmids used in this study.....	99
Table 5-1 Primers used in this study.....	112
Table 5-2 Specific growth rates under C10 and C11 alkane treatments.....	125

SUMMARY

Hydrocarbon alkanes, components of major fossil fuels, are considered as next-generation biofuels because their biological production has recently been shown to be possible. However, high-yield alkane production requires robust host cells that are tolerant against alkanes, which exhibit cytotoxicity. To tackle this toxicity issue, it is essential to understand molecular mechanisms of interactions between alkanes and microbial hosts. Based on these mechanisms, we can further develop microbial host strains with improved tolerance against alkanes. Therefore, in this study, we aimed to improve the tolerance of *Saccharomyces cerevisiae*, a model eukaryotic host of industrial significance, to alkane biofuels by investigating and exploiting cellular mechanisms underlying alkane toxicity. To this end, we first investigated the mechanisms of cellular response to alkane biofuels at a system level through transcriptome analyses. Transcriptome analyses suggested that C9 and C10 induced a range of cellular mechanisms such as membrane transporters, membrane modification, radical detoxification and energy supply. Among these hypothesized mechanisms, we were interested in identifying plasma membrane transporters which possibly aid in alkane secretion, leading to improved tolerance. In support of this hypothesis, we then expressed the hypothesized native transporters and demonstrated that the expression of transporters - SNQ2 or PDR5 significantly improved cell tolerance against decane (C10) and undecane (C11) through maintaining lower intracellular alkane level. Other than native transporters, we further explored novel alkane transporters from oleaginous yeast *Yarrowia lipolytica* based on the observation that it

utilizes alkanes as a carbon source. Similarly, the expression of identified heterologous transporters – ABC2 or ABC3 resulted in improved cell tolerance against decane (C10) and undecane (C11). Here, we demonstrated that transporter engineering - identification and expression of native and heterologous transporters led to significantly improved tolerance against alkane biofuels in *S. cerevisiae*. We believe that the results here provide valuable insights into designing microbial engineering strategies to improve cellular tolerance for highly efficient alkane biofuel production.

CHAPTER 1

INTRODUCTION

1.1 Background

The development of biofuels helps to address energy security and climate change concerns. The first generation biofuels (conventional biofuels) such as bioethanol and biodiesel capture 90% of the current biofuel market. However, replacing petroleum fuels with the first generation biofuels would require crops for biofuel production, competing with world food supply. For example, bioethanol is fermented from corns, and biodiesel is esterified from edible vegetable oils or animal fats. In comparison with the first generation biofuels, the second generation biofuels (advanced biofuels) are produced from non-food biomass such as lignocellulose (e.g., wheat straw, forest waste, switchgrass, etc.), algal biomass and even green-house gas (e.g., carbon dioxide). Thus, the second-generation biofuels production from such non-food biomass does not compete with food supply, which is one of the key advantages that led to growing interests in advanced biofuels production.

Current advanced biofuels include butanol, terpene, fatty alcohol, fatty acid ethyl esters, alkane, etc. Among the aforementioned biofuels, hydrocarbon alkanes, components of major fuels, have drawn much attention due to their high-energy content and compatibility with existing transportation infrastructure. Recently, an alkane biosynthetic pathway was identified and characterized in cyanobacteria and re-constructed in *Escherichia coli* [1, 2]. In addition, plant alkane biosynthesis pathway was reconstituted in yeast [3]. These studies demonstrate a promising potential of microbial alkane production.

Despite this potential for alkane bio-synthesis, for industrial-scale production, alkane yields and titers are key consideration. Biochemical production, especially biofuel production usually affects cell viability, which could be a bottleneck for high product yields. Such low cell viability may come from 1) toxicity of substrate, intermediate and product, 2) protein metabolic burden and 3) imbalance of co-factors. To address these problems, metabolic engineering, and synthetic and systems biology approaches were widely used: first, the engineering of global transcription factors aided by systems biology approaches can improve the host cell's stress tolerance; second, through engineered efflux pumps, the accumulation of intracellular toxic products can be reduced, leading to increased cell viability; third, spatial organization and optimization of pathway enzymes improve cell viability via reduction of toxic intermediates accumulated; fourth, the engineering of dynamic sensing-regulation of protein expression enables improved metabolic balance, resulting in improved growth rates; fifth, the balancing of co-factor usage helps to maintain the cellular redox state for viable growth; finally, the use of computational tools assists in engineering optimized bio-catalysts.

Because alkanes were reported to be toxic towards cells, the toxicity of alkanes as a final product could be a key factor affecting cell viability and microbial alkane production. Alkane toxicity has been studied as an effort to improve the productivity of biochemical processes, such as whole-cell catalysed reactions performed in hydrophobic organic solvents, and to enhance the efficiency of hydrocarbon bioremediation [4, 5]. Those studies have so far suggested: first, hydrophobic organic solvents disrupt microbial

membranes by direct insertion, and the cytotoxicity of these chemicals shows a strong inverse correlation with the logarithm of their partition coefficients ($\log P_{ow}$) [6]; second, organic solvent tolerance might be related to lipopolysaccharides modification and induction of efflux systems [7]; and third, organic solvents affect the chain length and the composition of fatty acids, outer membrane compositions, and cell surface hydrophobicity [7]. Despite these findings, which are mostly focused on cell physiology and physicochemical properties of the cell membrane, there is a lack of understanding of cellular mechanisms underlying hydrocarbon response at the systems level. To precisely tackle this toxicity issue, it is essential to understand molecular mechanisms underlying the interactions between biofuels and microbial hosts, and thus based on these mechanisms, to develop cells with improved tolerance against alkanes.

To understand the underlying molecular mechanisms, a high-throughput biotechnology, which allowed researchers to investigate a number of genes, proteins and metabolites simultaneously in different experimental settings, is needed. Among the high-throughput technologies such as DNA microarray, comparative proteomic analysis and real competitive PCR [8-10], DNA microarray can readily be used to quantify transcriptomic level in order to study how living organisms develop dynamic profiles of transcripts in response to various environmental conditions. By compiling available information and prediction made by computational algorithms, certain targets can be identified. And genetic manipulations of the identified targets possibly lead to the desired phenotypes. Hence, in this study, we have exploited transcriptomic profiling to identify genes responsible for alkane

biofuel toxicity and further harnessed these mechanisms to improve cell tolerance against alkanes.

1.2 Aims and objectives

This dissertation aims to improve alkane tolerance in *Saccharomyces cerevisiae*, a key industrial microbial host. Specifically, the objectives are to:

- 1) Review recent scientific progress of biofuel synthesis including first- and second-generation biofuels;
- 2) Propose a range of key microbial engineering strategies, substantiated by recent advances, to improve cell viability for commercially sustainable production of biochemicals including biofuels;
- 3) Investigate molecular mechanisms underlying the interactions between alkane and *S. cerevisiae* through transcriptomic analyses;
- 4) Characterize and harness *S. cerevisiae* native transporters, as suggested by transcriptomic analyses, to improve alkane tolerance;
- 5) Identify and utilize heterologous transporters from oleaginous yeast *Yarrowia lipolytica* to improve alkane tolerance in *S. cerevisiae*.

1.3 Thesis organization

The present Introduction – **Chapter 1** lays down the background information of the thesis study, and leads to the Literature Review in

Chapter 2, where current research status relevant to the thesis objectives stated are presented, including scientific progress of biofuels synthesis and strategies to improve cell viability. In **Chapter 3**, DNA microarray was performed, transcriptomic level was analysed, and molecular mechanisms of alkane toxicity were hypothesized. To test the hypothesis proposed in **Chapter 3**, the hypothesized transporters were thoroughly characterized in **Chapter 4**. Furthermore, to develop microbial host strains with improved tolerance against alkanes, characterized transporters were harnessed in *S. cerevisiae*. Heterologous transporters from *Y. lipolytica* were identified and characterized in **Chapter 5**. Finally, **Chapter 6** summarizes the important findings, presents conclusions, and lays down recommendations for worthwhile future works.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial engineering for the production of biofuels

2.1.1 First-generation biofuels

First-generation biofuels (conventional biofuels) are made from sugar, starch or vegetable oil. Bioalcohols and biodiesel are the most widely used conventional biofuels. Bioalcohols are biologically produced alcohols, among which most commonly produced is ethanol, and less commonly are propanol and butanol. The use of ethanol is mainly due to its high level of natural production by microbes, through fermentation of cornflour (in the United States) or cane sugar (in Brazil). Biodiesel is produced by the transesterification of vegetable oil or animal fats with methanol. Despite their widespread applications, the first-generation biofuels show a couple of drawbacks. For example, bioethanol has only 70% of the energy content of gasoline, a high tendency to absorb water from the air leading to corrosion in engines and pipes, and energy intensive for its distillation from the fermentation broth; biodiesel has only 91% of the energy content of D2 diesel, and difficulties in transport with the current distribution infrastructure because wax may form at low temperature. Furthermore, because the first-generation biofuels were produced from food crops, their industry-scale production creates a link between food and fuel prices [11].

2.1.2 Second-generation biofuels

2.1.2.1 Characteristics of second-generation biofuels

Instead of using food crops as feedstock, more abundant and underused sustainable resources, including lignocellulose, algal biomass and greenhouse gases were used for second-generation biofuels (advanced biofuels) production. First, lignocellulose is the most abundant biomass on Earth, and releases about 70% sugars after thermal, chemical and biochemical processes. These released sugars can be used for microbial fermentation. Second, algal biomass is a compelling alternative since it can be produced in salt water rather than on arable land, even though its collection and dewatering is challenging. Third, carbon monoxide, which is released from the thermal conversion of lignocellulosic biomass and abundant in steel-mill flu gas, can also be utilized by microorganisms, while carbon dioxide can be transformed into biofuels through carbon fixation by engineered photosynthetic organisms.

Advanced biofuels produced by microorganisms showed similar properties to petroleum-based fuels, therefore can be used as “drop-in” fuels to the existing transportation infrastructure. For developing an organism to produce an advanced fuel, multiple factors including an engine type (spark or compression ignition), energy content, combustion quality or ignition delay, cloud point, volatility, lubricity, viscosity, stability, odour, toxicity, water miscibility and cost must be considered. Peralta et al. summarized the potential biofuels corresponding to different petroleum fuels in Table 2-1 [12].

Table 2-1 Liquid transportation fuel properties and potential biofuels [12]

Fuel type	Major components	Properties	Potential advanced biofuels
Gasoline	C ₄ –C ₁₂ hydrocarbons Linear, branched, cyclic aromatics	Octane number (87–91) Energy content	Butanol, isobutanol, short-chain alcohols, short branched-chain alkanes
Diesel	C ₉ –C ₂₃ hydrocarbons Linear, branched, cyclic aromatics	Cetane number (40–60) Good cold properties	Fatty alcohols, alkanes, linear or cyclic isoprenoids
Jet fuel	C ₈ –C ₁₆ hydrocarbons Linear, branched, cyclic aromatics	Heat density Very low freezing temperature	Branched alkanes, linear or cyclic isoprenoids

The advanced biofuels can be produced either from native microbial hosts or genetically well-established heterologous microbial hosts. In native hosts, endogenous regulation of biofuel-producing pathways limits the conversion of feedstocks into advanced biofuels with high yields. Reconstruction of advanced biofuel pathways in genetically well-established heterologous hosts, such as *E. coli* and *S. cerevisiae*, can solve this problem, but heterologous production presents its own challenges in balancing the enzyme activities and protein metabolic burden to maximize metabolic flux towards target biofuels. In order to overcome these challenges, the combination of metabolic engineering, synthetic biology and systems biology has been widely utilized for the engineering of microbes; and alternatively, developing microorganisms with high biofuel tolerance or with the ability to use non-sugar substrates are also of interest. In the following section, we discuss the microbial engineering of the pathways for the production of advanced biofuels.

2.1.2.2 Alcohol-derived fuels

2.1.2.2.1 Butanol

Short-chain or higher alcohols can be added to gasoline as oxygenates or to replace gasoline altogether. Compared to traditional biofuels such as ethanol, higher alcohols offer advantages as gasoline substitutes due to their higher energy density and lower hygroscopicity. For example, butanol has 84% of the energy content of gasoline, limited miscibility with water and complete miscibility with gasoline.

To produce butanol, a native *Clostridium* pathway has widely been engineered (Figure 2-1) [12]. In an effort to improve butanol production, species of the natural host *Clostridium* have been engineered to use different feedstocks, such as glucose, liquefied cornflour, glycerol and even syngas. However, butanol yields were limited by the host cells' slow growth rate and intolerance to butanol above 13 g/L. To overcome these limitations, the *Clostridium* butanol-production pathway has been widely reconstructed in organisms that grow faster or tolerate high concentrations of butanol, such as *S. cerevisiae* [13] and *E. coli* [14].

Figure 2-1 Metabolic pathways used for the production of alcohol-based biofuels. In blue, *Clostridium*'s butanol pathway converts acetyl-CoA into butanol. AAD, butyraldehyde dehydrogenase; BCD, butyryl-CoA dehydrogenase; BDH, butanol dehydrogenase; CRT, crotonase; HBD, 3-hydroxybutyryl-CoA dehydrogenase; THL, thiolase. In green, the 2-keto acid pathway produces isobutanol from pyruvic acid. ADH, alcohol dehydrogenase; ALS, acetolactate synthase; DHAD, dihydroxy-acid dehydratase; KAR, ketol-acid reductoisomerase; KDC, keto-acid decarboxylase [12].

Regardless of the host organisms, the production of butanol is also limited by reversibility and cofactor specificity of the enzymes in this pathway. This barrier to high butanol production was broken in *E. coli* by using a synthetic butanol pathway that combines enzymes from different organisms. Bond-Watts et al. [15] first constructed a synthetic chimeric pathway for butanol synthesis derived from three different organisms (Figure 2-2). To further improve butanol production, individual enzymes in this constructed pathway were analyzed and optimized. Rather than using *Clostridium*'s reversible, flavin-dependent, oxygen-sensitive enoyl-CoA reductase, the synthetic pathway used biosynthetic NADH-dependent trans-enoyl-CoA reductase - Ter from mitochondrial extracts of the photosynthetic flagellate *Euglena gracilis*. Further, PhaB was substituted with the NADH-dependent (S)-3-hydroxybutyryl-CoA dehydrogenase - Hbd from *Clostridium acetobutylicum*, which resulted in almost an order of magnitude increase of n-butanol titers at 2,950 mg/L (Figure 2-3a). At last, to increase the availability of acetyl-CoA and NADH, aceEF-lpd operon was overexpressed, which resulted in a 1.6-fold improvement of butanol to 4,650 mg/L compared to the empty control plasmid (Figure 2-3b) [15].

Figure 2-2 Comparison of a high-yielding pathway for production of polyhydroxyalkanoates (PHAs) to the design of a chimeric pathway for fuel synthesis derived from three different organisms. Blue, *Ralstonia eutrophus*; red, *Clostridium acetobutylicum*; black, *Scotophilus collinus*; *phaA*, acetoacetyl-CoA thiolase/synthase; *phaB*, 3-hydroxybutyryl-CoA dehydrogenase; *phaC*, pHa synthase; *crt*, crotonase; *ccr*, crotonyl-CoA reductase; *adhE2*, bifunctional butyraldehyde and butanol dehydrogenase [15].

Figure 2-3 Optimization of the synthetic Ter-dependent pathway for n-butanol production. (a) Stereochemical outcomes for different front-end pathways (Bu1–4). Proposed bottlenecks are indicated with a crossed arrow. Blue, *R. eutrophus*; red, *C. acetobutylicum*; black, *T. denticola*; light blue, *A. caviae*; hbd, 3-hydroxybutyryl-CoA dehydrogenase; phaJ, crotonyl-CoA hydratase. (b) Comparison of ethanol and n-butanol biosynthesis. Redox balance from glucose to product in terms NAD⁺ usage and recycling allows high yields of ethanol during fermentative growth. The production of advanced biofuels from acetyl-CoA rather than pyruvate requires an additional oxidation reaction to generate reducing equivalents for the assembly of saturated targets. Although several routes to acetyl-CoA are possible, the PDHc achieves redox balance with the synthetic n-butanol pathway. pdc, pyruvate decarboxylase; adh, alcohol dehydrogenase; aceEF.lpd, PDHc [15].

2.1.2.2.2 Branched-chain alcohols

Compared with straight-chain alcohols, branched-chain alcohols were regarded as a better biofuel because of its relatively higher octane numbers compared with their straight-chain counterparts. A well-known route for the production of alcohols, especially branched-chain alcohol, is the Ehrlich or 2-keto acid pathway. As shown in Figure 2-4a [16], this pathway decarboxylates 2-keto acids, the intermediates in amino acid biosynthesis pathways, into aldehydes by a broad-substrate-range enzyme, 2-keto-acid decarboxylases (KDCs) followed by conversion into alcohols by alcohol dehydrogenases (ADHs). Atsumi et al. [16] demonstrated that heterologous expression of Kivd (KDC from *Lactococcus lactis*) and Adh2 (ADH from *S. cerevisiae*) in *E. coli*, produced six kinds of alcohols. As shown in Figure 2-4b, the isoleucine biosynthesis pathway generates 2-ketobutyrate and 2-keto-3-methyl-valerate, which can be further converted to 1-propanol and 2-methyl-1-butanol respectively. The valine biosynthesis pathway produces 2-keto-isovalerate which is the precursor for isobutanol. The leucine biosynthesis pathway generates 2-keto-4-methyl-pentanoate which is the substrate for 3-methyl-1-butanol production. The phenylalanine biosynthesis pathway produces phenylpyruvate which can further lead to 2-phenylethanol. The norvaline biosynthesis pathway, a side-reaction of the leucine biosynthesis, produces a substrate for 1-butanol production.

Figure 2-4 Production of higher alcohols through the synthetic non-fermentative pathways. a, Various 2-keto acid precursors lead to corresponding alcohols through 2-ketoacid decarboxylase and alcohol dehydrogenase. b, The synthetic networks for the non-fermentative alcohol production in engineered *E. coli*. Red arrows represent the 2-keto acid decarboxylation and reduction pathway. Blue enzyme names represent amino acid biosynthesis pathways. The double lines represent a side pathway leading to norvaline and 1-butanol biosynthesis [16].

Among the aforementioned alcohols, more work has recently focused on isobutanol production since it is the closest to industrial use considering its

better octane number and less cytotoxicity. To optimize isobutanol production level in *E. coli*, several strategies were utilized: overexpression of 2-ketoisovalerate biosynthetic genes *ilvHCD*, deletion of multiple genes (*adhE*, *ldhA*, *frdAB*, *fnr* and *pta*) competing for pyruvate consumption, and replacement of the endogenous acetolactate synthase enzyme *ilvH* with *alsS* from *Bacillus subtilis*, which has a higher specificity for pyruvate. As shown in Figure 2-5a, b [16], the final isobutanol titers reached 22 g/L after the optimization. In addition, to demonstrate the generality of this approach, the same strategy was also applied to 1-butanol production, which also showed significant improvement (Figure 2-5c, d) [16].

Figure 2-5 Summary of results for isobutanol and 1-butanol production in *E. coli*.

The cells were grown in M9 medium containing 36 g/L glucose in shake flasks at 30 °C with or without other nutrients indicated, and induced with 0.1 mM IPTG. Overexpressed genes and nutrient supplementation are indicated below the axis. Error bars indicate s.d. a, Left panel, isobutanol production; right panel, isobutanol yield per g of glucose. The theoretical maximum yield of isobutanol is 0.41 g/g. Knockout, Δadh , Δldh , Δfrd , Δfnr and Δpta . b, Isobutanol production with *B. subtilis* alsS and yeast extract (5 g/L) supplementation to increase cell density. The host is JCL260. c, d, Left panel, 1-butanol production; right panel, 1-propanol production in the same strain. The host strain is JCL16, with or without $\Delta ilvD$. l-Threonine, l-threonine (8 g/L) supplementation [16].

2.1.2.3 Isoprenoid-derived biofuels

Isoprenoids, a class of compounds widely used as flavours and pharmaceuticals, have the potential to serve as advanced biofuels because of the branches and rings found in their hydrocarbon chain. The branching allows hydrocarbon chains to have more tertiary carbon atoms to stabilize pressure-induced radicals, reducing premature ignition and increasing the octane number. Isoprenoid-based biofuels are produced from two C5 precursors, isopentenyl diphosphate and dimethylallyl diphosphate, generated from either the mevalonate or deoxyxylulose-5-phosphate pathways (Figure 2-6). Isoprenoid-derived biofuels include isopentanol, farnesane, bisabolane, pinene dimer, isopropyl-4-methylcyclohexane and C15 amorphadiene, which are summarized in Figure 2-6 [12].

Figure 2-6 Metabolic pathways used for the production of isoprenoid-based biofuels. In blue is the mevalonate pathway and in green is the deoxyxylulose-5-phosphate (DXP) pathway. AACT, acetyl-CoA transferase; BIS, bisabolene synthase; CMK, 4-(cytidine-5'-diphospho)-2-C-methylerythritol kinase; DXPS, DXP synthase; DXR, DXP reductoisomerase; FPPS, farnesyl diphosphate synthase; FS, farnesene synthase; GPPS, geranyl diphosphate synthase; HMGR, HMG-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; IPP, isopentenyl diphosphate; IPPS, IPP isomerase; LS, limonene synthase; MCT, 2-C-methylerythritol-4-phosphate cytidyltransferase; MECPS, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; MK, mevalonate kinase; PMD, phosphomevalonate decarboxylase; PMK, phosphomevalonate kinase; PS, pinene synthase [12].

Among the aforementioned biofuels, bisabolane was regarded as a novel biofuel compound, and we here elaborate more on bisabolane biosynthesis [17]. Native prenyltransferases assemble IPP and DMAPP into linear prenyl diphosphate precursors, such as farnesyl diphosphate (FPP, C15), which can be further converted into bisabolene by bisabolene synthase (Figure 2-7) [17]. To synthesize bisabolene in *E. coli*, five plant isoprenoid synthases (TPS12 and TPS13 from *Arabidopsis thaliana*, TPS-BIS from *Picea abies*, TPS3 from *Pseudotsuga meziessi*, and Ag1 from *Abies grandis*) were screened for efficient conversion of farnesyl diphosphate into bisabolene in *E. coli*. *A. grandis* Ag1 was found to produce the highest titers of bisabolene at 78 ± 14 mg/L and its codon optimized version further increased bisabolene production by 5-fold. In addition, by codon-optimizing heterologous genes in mevalonate pathway and further introducing an additional promoter to upregulate genes in this pathway, the titer of the new platform was boosted up to 912 ± 43 mg/L. Thus, via combination of enzyme screening and metabolic engineering, more than 10-fold increase in bisabolene titer was obtained in *E. coli*. Finally, biosynthetic bisabolene was chemically hydrogenated into bisabolane as a biosynthetic D2 diesel alternative.

Figure 2-7 Bisabolene from chemical hydrogenation of microbially produced bisabolene. The engineered microbe (yellow box) converts simple sugars into acetyl-CoA via primary metabolism. A combination of metabolic engineering of the heterologous mevalonate pathway to convert acetyl-CoA into FPP and enzyme screening to identify a terpene synthase to convert FPP into bisabolene (1) is used to produce bisabolene at high titers. Chemical hydrogenation of biosynthetic bisabolene leads to bisabolane (2), a biosynthetic alternative to D2 diesel [17].

2.1.2.4 Fatty-acid-derived biofuels

As a major component of cell membrane, fatty acids are synthesized in high flux and converted into phospholipids in all organisms. Fatty acid is ideally suited to providing biofuel precursors because of its high efficiency and high energy conservation. Although free fatty acids cannot be used as fuels directly, their derivatives, including fatty alcohols, fatty acid alkyl esters, fatty alkane and alkenes, are suitable biofuel targets because of their low water solubility, high energy density, and low toxicity to the production hosts. Pathways for fatty-acid-derived biofuels are summarized in Figure 2-8 [18].

Biodiesels, including fatty acid methyl, ethyl or propyl esters (FAME, FAEE and FAPE), are currently used in diesel engines with annual consumption of more than two billion gallons. Biodiesels have been produced in *E. coli* by introducing a wax-ester synthase gene (*atfA*) and an ethanol biosynthetic pathway that involves a pyruvate decarboxylase (*pdc*) and an alcohol dehydrogenase (*adhB*) [19]. Similarly, fatty alcohols were produced by overexpressing an acyl-CoA reductase from *Acinetobacter calcoaceticus* (*acr1*) [19].

Aliphatic hydrocarbons, such as alkanes and alkenes, are favorable targets for advanced cellulosic biofuels, as they are already predominant components of petroleum-based gasoline and diesel fuels, and thus would be compatible with existing engines and fuel distribution systems. Recently, two fatty alkane biosynthesis pathways have been devised and heterologously reconstituted in *E. coli*. First, Schirmer et al. demonstrated that alkane biosynthesis requires acyl-ACP reductase (AAR) which catalyzes the reduction of acyl-ACPs to form fatty aldehydes, and aldehyde decarbonylase (ADC) which decarbonylates the resultant aldehydes to alkanes [2]. Second, Akhtar et al. discovered that the fatty alkane biosynthesis may commence with free fatty acids, by direct conversion of fatty acid to aldehyde catalyzed by a carboxylic acid reductase (CAR) from *Mycobacterium marinum*. Aldehydes can be further converted into alkane (C7-C15) by aldehyde decarbonylase [20]. Furthermore, at least three pathways exist for the biosynthesis of terminal alkenes (olefins). First, a cytochrome P450 fatty acid decarboxylase, OleT_{JE}, from *Jeotgalicoccus* sp was characterized by Rude et al. which decarboxylated free fatty acids into

terminal alkenes [21]. Second, another gene was identified in the cyanobacterium *Synechococcus* sp. PCC 7002 which was suggested to convert fatty acyl-ACP to terminal alkene via an elongation decarboxylation mechanism [22]. Third, the production of long-chain alkenes (internal olefins, C24 - C31) by a head-to-head condensation of two acyl-CoA (or-ACP) thioesters followed by several reduction steps was reported in *Micrococcus luteus* [23] and *Shewanella oneidensis* [24, 25]. Several identified alkane/alkene synthesis pathways above have been reconstructed in *E. coli* for heterologous production of alkane/alkene biofuels [20, 21].

2.2.1 Introduction

During the microbial production of chemicals such as biofuels, pharmaceutical drugs, bio-polymers and other value-added compounds, bio-catalytic cells are often subjected to a variety of biotic and abiotic stresses that may threaten cell viability [26]. Diminished cell viability usually results in negative consequences, for example, lower productivity and sluggish fermentation [27]. In industrial production plants, process interruption due to sluggish fermentation or constant cell replacement may result in considerable economic losses. To ensure continuous bio-conversion with cell recycle for minimal process interruption and maximized bio-catalytic productivity, improving cell viability is crucial.

Cell viability is defined by various factors, namely stress tolerance towards toxic chemical compounds (substrates, intermediates, products), harsh external environments (pH, temperature, osmolarity, starvation, oxidation), and growth rates. Improving cell stress tolerance has been the main focus for many prior studies [28-32] because host cells with robust stress tolerance can grow well and accumulate biomass quickly. However, in the context of bio-catalysis, other factors such as heterologous protein expression [33] and metabolic flux balance can also greatly influence growth rates.

Towards the goal of enhancing bio-catalytic productivity, this review discusses various engineering strategies that can be harnessed for improvement of cell viability (Figure 2-9). First, the engineering of global transcription factors aided by systems biology approaches can improve the

host cell's stress tolerance. Second, through engineered efflux pumps, the accumulation of intracellular toxic products can be reduced in bio-catalytic cells, leading to increased cell viability. Third, spatial organization and optimization of pathway enzymes can potentially improve cell viability via reduction of toxic intermediates accumulated. Fourth, the engineering of dynamic sensing-regulation of protein expression enables improved metabolic balance and efficient usage of cellular resources, resulting in improved growth rates. Fifth, the balancing of co-factor usage and consumption in bio-catalytic pathways helps to maintain the cellular redox state for viable growth. Finally, to facilitate this multi-faceted scheme and balance conflicting objectives (e.g. enzyme expression vs. growth rates), this review also emphasizes the use of computational tools that assist in engineering optimized bio-catalysts.

Figure 2-9 An overview of the key approaches towards developing a microorganism platform for improved viability.

2.2.2 Transcription factor engineering for improved stress response

The development of bio-catalytic cells often requires genetic manipulation of microbial cells to carry heterogeneous genetic regulatory parts and metabolic pathways. For maximized productivity of the bio-catalysts, further optimization by engineering other physiological functions, such as growth rates, ancillary morphological changes (e.g. flagella or pilus development, biofilm formation) and tolerance to environment, is necessary [34]. These physiological functions are complex phenotypes which are determined by complicated interactions of multiple genetic components and environmental factors. As a result, it has been difficult to obtain a desired phenotype by a gene-by-gene modification approach [35]. Rather, strains that show desired phenotypes have been found by chance or by artificially inducing genetic mutations through a vast number of successive cultures. However, this approach has been limited to inflexible host choices, long time-frame and lack of modular host optimization processes. Therefore, there has been increasing interest in rationally engineering complex phenotypes while overcoming the above-mentioned limitations.

One approach to engineer desired complex phenotypes was to engineer global transcription machineries. Alper and co-workers have attempted to alter ethanol tolerance and lycopene production by combinatorial mutation of global transcription machineries such as sigma factors in *E. coli* [36] and a TATA-binding protein and other associated factors in the yeast *S. cerevisiae* [36, 37]. A similar approach has also been employed in *S. cerevisiae* to improve xylose fermentation [38]. In addition to global transcription machineries, a few known transcription factors have been

engineered to improve resistance to stress conditions. For example, cyclic AMP receptor protein (CRP) was fused with various types of zinc finger DNA-binding proteins in *E. coli* [39], and a randomly mutated CRP was expressed in *E. coli* [40]. In other studies, IrrE, a global regulator in *Deinococcus radiodurans*, was shown to confer osmotic pressure and ethanol tolerance in *E. coli* upon expression [41]. Further, it has been shown that an introduced exogenous regulator indeed significantly reconstituted transcriptome and proteome profiles [42].

The above-mentioned studies chose to modify genetic components which could lead to desired phenotypes by inducing global changes in a biological system. While these targets were not derived from systems-level understanding of physiological functions, there have been studies to identify targets by characterizing complex phenotypes and reconstituting genetic regulatory networks towards systems engineering. As mentioned earlier in this section, complex phenotypes are the outcome of interactions between multiple genetic components and environmental factors. In other words, it is the genetic regulatory networks that determine transcriptomic profiles that lead to desired phenotypes under a specific condition. It is worth to note that genetic regulatory networks consist of hierarchical, but highly inter-connected interactions of genetic components. In addition, genetic regulatory networks can be dynamically rewired due to activity of transcriptional regulators and degeneracy of transcription factors and transcription factor binding sites under different conditions [43]. These features of genetic regulations imply that different combinations of transcription factors can simultaneously bind to their regulon members in a

condition-dependent way. Therefore, it is important to identify global regulators that change their activities for a particular physiological function.

To this end, Brynildsen and co-workers identified 16 transcription factors of which activities were changed in response to isobutanol stress in *E. coli*. Network component analysis was used to predict activity changes of transcription factors from gene expression data and the essentiality of a few identified transcription factors was verified by gene deletion [44]. While this study was limited to inferring the activity changes of known transcription factors, a new algorithm was developed to reconstruct regulatory networks using the same transcriptome data used in the above study and predicted 7 new transcription factor activity changes. Although these studies identified transcriptional regulators and reconstructed regulatory networks for rational engineering of complex phenotypes, it was not addressed whether the engineering of the identified targets could rewire regulatory networks leading to desired phenotypes such as improved tolerance to stress conditions. Toward this goal, we have shown that rewiring of genetic regulatory networks can be achieved by combinatorial deletion of transcription factors identified from transcriptomic data [45]. We demonstrated that the rewired genetic regulatory networks resulted in increased tolerance to isooctane in *E. coli*. While iterative characterization and identification of regulatory networks can further improve isooctane tolerance, we established that modification of transcription factors could be a promising strategy to rationally engineer complex phenotypes towards improved bio-catalytic performance (Figure 2-10).

Figure 2-10 Rational engineering of complex phenotypes by three approaches introduced in the text. A schematic diagram presents steps of identification and reconstitution of regulatory networks towards improved cellular responses.

2.2.3 Harnessing efflux pumps against product toxicity

Product toxicity is often a major limitation to bio-catalytic conversion especially at industrial scales which require high titers and yield of end-products. The engineering of host cells with improved cell tolerance has led to significant boosts in productivity. For example, ethanol production in an engineered strain of *S. cerevisiae* was improved by 15% when its ethanol and glucose tolerance was improved through global transcriptional machinery engineering [36]. Commonly used engineering strategies for improving cell tolerance towards toxic end-products include mutagenesis, adaptive evolutionary engineering, genome shuffling and creation of genomic libraries [29, 36, 46, 47]. However, these classical tolerance-improving strategies are often time-consuming and laborious. To illustrate, more than 6 months was needed to generate ethanol-tolerant mutants via the adaptive evolutionary engineering method. If the genomic library

approach is to be used, huge libraries (>> 10,000 transformants) have to be obtained and screened. In contrast to the above approaches, harnessing efflux pumps to bio-catalytic cells provides a direct strategy for efficiently improving tolerance by expelling toxic compounds from cells.

Efflux pumps are membrane transporters involved in expulsion of toxic compounds from cells. There are two major classes of efflux pumps, ATP-binding cassette (ABC) transporters and secondary efflux pumps. ABC transporters are primary transporters that utilize ATP as energy source to catalyze the translocation of substrates. ABC transporters consist of two trans-membrane domains that bind the chemical compound and provide a translocation channel, and two cytoplasmic nucleotide-binding domains that hydrolyze ATP to drive transport [48]. On the other hand, secondary efflux pumps utilize proton or sodium gradients as a source of energy. They consist of three protein subunits: a cytoplasmic membrane export protein responsible for chemical compound recognition and proton exchange, a periplasmic linker, and an outer membrane channel [49].

The use of efflux pumps for active excretion of toxic compounds from cells has received much attention for its roles in alleviating toxicity and increasing productivity [50, 51]. Examples of characterized efflux pumps for reducing toxicity include *Pseudomonas aeruginosa* Mex pumps (MexA-MexB-oprM, MexC-MexD-OprJ, and MexE-MexF-OprN) for organic solvents such as n-hexane, and p-xylene [52, 53], *Pseudomonas putida* Ttg pumps (TtgABC, TtgDEF, and TtgGHI) for toluene [54], and *E. coli*

AcrAB-TolC pump for hexane, heptane, octane and nonane [55]. Recently, efflux pumps from the hydrophobe/amphiphile efflux (HAE1) family were heterogeneously expressed in *E. coli* to improve tolerance towards five representative biofuels (geranyl acetate, geraniol, α -pinene, limonene and farnesyl hexanoate) [56]. In addition, we discovered that the expression of heterologous ABC transporters conferred significant tolerance to hydrocarbons in yeast cells (unpublished data). Besides tolerance improvement, bio-catalyst productivity can also be enhanced by efflux pump-mediated export of toxic compounds. The yeast multidrug resistance ABC transporter Pdr18p plays an indirect role in ethanol efflux through decreasing ethanol-induced plasma membrane permeabilization and reducing the intracellular ethanol concentration, thereafter leading to 17% higher ethanol production yield at a toxic concentration [57]. In addition, the limonene-producing strain expressing an efflux pump demonstrates a 1.6 fold improvement in limonene yield [56]. However, while limonene is toxic to the *E. coli* host, the production yield is not yet at a toxic level. Hence, the efflux pump may have increased the productivity of the limonene bio-catalyst by relieving product inhibition of metabolic pathway enzymes. In another example of efflux pumps serving to relieve product inhibition of metabolic pathway enzymes for improved productivity, by overexpression of the ABC transporter AvtAB, avermectin productivity was improved by around 1.5 fold (from 3.3 to 4.8 g/L) in *Streptomyces avermitilis*. Moreover, the ratio of intracellular to extracellular accumulation of avermectin dropped from 6:1 to 4.5:1. It is highly likely that the AvtAB transporter exports

avermectin, and thus reduces the feedback inhibition of avermectin biosynthesis inside the cell [58].

While efflux pumps provide a direct mechanism for improving tolerance and productivity of bio-catalysts, there are a few barriers on harnessing efflux pumps. Firstly, pump overexpression can be toxic to host cells because excessive efflux pumps may overload membrane insertion machinery, and hamper cell division and protein homeostasis [59, 60]. To avoid toxicity from pump overexpression, inducible expression by exogenous inducers is more preferred than constitutive expression (Figure 2-11A). Further, several studies have contributed to resolving membrane protein toxicity by synthetic biology design (Figure 2-11B). For example, the expression of a family of ion pumps was increased 5- to 25-fold in *E. coli* by changing the frequency of transcription initiation under a “restrained expression” mode [61]. This suggests a mechanism of balancing the rates of protein production to the cell's capacity for membrane protein biogenesis. Furthermore, a model was developed for improving microbial productivity using a synthetic feedback loop that controls efflux pump levels to balance toxicity due to end-product and pump overexpression. As a result, final product yields were significantly improved [62]. Secondly, availability of efflux pumps with desirable substrate specificity is still limited. As shown in Figure 2-11C, directed evolution provides a strategy on altering the specificity of efflux pumps. According to Bokma et al. [63], the shuffled *E. coli* TolC variants adapted to the non-cognate *Pseudomonas* MexAB translocase, and conferred resistance to the efflux substrate novobiocin. As described by Huffer et al. [51], directed evolution of *E. coli* AcrB enhanced

the growth of *E. coli* by about 20% when short to medium chain alcohols were supplied exogenously in the medium. However, it is still challenging to develop desirable efflux pumps specific to biofuels especially to short chain alcohols such as ethanol, n-butanol, and isopentanol [56]. Thirdly, pump evaluation is limited due to shortage of producing strains. The functionality of efflux pumps towards chemicals added in growth medium might leave a gap between promise and practice.

Taken together, by harnessing efflux pumps in bio-catalytic cells, end-products can be recognized and expelled from the cells. This confers cells with tolerance towards the product and can greatly improve productivity. In consideration of current barriers on application of efflux pumps, we believe that the future direction would focus on (i) developing novel efflux systems with low pump toxicity and desirable substrate specificity towards improving tolerance and productivity; and (ii) evaluating novel efflux pumps in producing strains.

Figure 2-11 Improvement of cell viability and pathway productivity by harnessing efflux pumps. (A) Efflux pump expression is induced by an exogenous inducer. (B) Efflux pump expression is regulated by a transcription factor (TF), a synthetic promoter and end-product. (C) Efflux pumps are engineered with desired substrate specificity. P, exogenous inducer-responsive promoter; Ps, synthetic promoter responsive to TFs and end-product; purple ovals, efflux pumps; red stars, end-product synthesized in biocatalysts; blue stars, exogenous inducer; blue diamonds, chemicals or metabolites; circles, transcription factors. Blue and red arrows, transcriptional activation; end line, transcriptional repression.

2.2.4 Spatial engineering and compartmentalization against intermediate toxicity

In the microbial biosynthesis of chemicals, intermediates generated in the pathway may be toxic to the host cell [42, 64-67]. Reducing intermediate concentration for improved cell viability is undesirable as low intermediate concentrations limit the reaction rate. High intermediate concentrations without exposing the host cell to toxic compounds can be achieved by localizing the intermediates into compartments. In nature, organelles [68, 69] and large enzyme complexes [65] are commonly used as

compartments to segregate pathways and their metabolites. Metabolic engineers can take advantage of available compartments by tagging proteins with localization signals [69], or create synthetic compartments to spatially organize and optimize heterologous pathways. To this end, researchers have developed synthetic scaffolds using proteins [70], DNA [71] and RNA [72] as well as protein based shells [42, 66].

One demonstration of the effectiveness of synthetic scaffolds to reduce metabolic load and improve productivity involves the mevalonate biosynthetic pathway in *E. coli* [70]. This pathway involves three enzymes, one being acetoacetyl-CoA thiolase (AtoB), which is naturally present in the host, and two others, hydroxyl-methylglutaryl-CoA synthase (HMGS) and hydroxyl-methylglutaryl-CoA reductase (HMGR), which are heterologous to *E. coli*. The lower enzyme activity of HMGS relative to HMGR results in flux imbalance and accumulation of the toxic intermediate HMG-CoA. To overcome these challenges, the authors created protein scaffolds using three protein–protein interaction domains derived from metazoans. By varying a number of interaction domains on glycine–serine linkers, different ratios of pathway enzymes were co-localized on the scaffold. This enabled the engineering of a mevalonate production strain with a 77-fold increased product titer in addition to faster growth rates. At this point, the mechanism by which synthetic scaffolds enhance growth and productivity is unclear. It has been hypothesized that scaffold compartmentalization reduces accumulation of toxic intermediates outside the scaffold [66]. By co-localizing pathway enzymes at optimal stoichiometries, intermediates can be quickly converted into products.

Another potential strategy in compartmentalizing pathways involves protein based shells [42, 66]. By encapsulating pathway enzymes into protein shells, the shells act as physical barriers which can selectively regulate metabolite entry and exit. In this way, pathways which generate toxic intermediates can be sequestered to protect the host from the toxicity from the intermediates. Protein shells are available in nature, such as bacterial microcompartments [73], lumazine synthase complex [74] and encapsulins. These shells have been successfully assembled in heterologous hosts and tagged proteins were localized into the shells. However, the sequestration of synthetic metabolic pathways has yet to be demonstrated. Much work remains to be done, including finding a balance between shell complexity and the number of proteins that can be localized, and engineering metabolite selectivity of protein pores [66].

2.2.5 Dynamic sensing-regulation of protein expression

The engineering of synthetic pathways in bio-catalytic cells requires the heterologous expression of proteins. This creates a competition for cellular resources, resulting in an increased metabolic burden that adversely affects cell growth [75]. Furthermore, some proteins or metabolites could be toxic, resulting in lower cell viability and growth rates. In order to minimize undesired proteins and metabolites from being produced at the “wrong time” and hence maximize the cell's ability to proliferate, inducible promoters are often used. Inducible promoters enable the separation of growth phase from production phase; at low cell densities, cells proliferate

and accumulate biomass without the additional metabolic load of heterologous protein expression, whereas at higher cell densities, the inducers are added, so that the cells produce enzymes necessary for production of desired chemicals (Figure 2-12A). Even though inducible promoter systems are widely used in metabolic engineering for production of useful chemicals, the cost of inducers may prohibit their usage at industrial scales. On the other hand, inducible promoters regulated by native signals, such as nutrient starvation [76-78] and cell density [79-81], enable temporal control and inducer-free protein expression.

In the construction of synthetic pathways for production of chemicals, often multiple genes are required. However, enzymes at different steps in the pathway may have varying expression levels (or activities). This results in bottlenecks, leading to accumulation of intermediates that may be toxic to the host. This also indicates a waste of cellular resources in the overproduction of enzymes for non-rate limiting steps. Hence, coordination of protein expression levels would greatly enhance growth and maximize productivity. To this end, researchers have developed strategies for tuning gene expression, including promoter strength [82], ribosome binding sites [83], copy number [84] and enzyme degradation [85]. Indeed, these strategies have been found to be effective in improving pathway productivity and cell growth. However, when disturbances in bioreactor conditions result in sub-optimal conditions, for which these static controllers have not been designed, the system may not respond accordingly, and productivity is not maximized. A more stable system can be created by the use of feedback mechanisms, i.e. dynamic regulation of protein levels [86].

Dynamic regulation enables real-time adjustments of metabolic flux upon variation in the host or environmental conditions. It also facilitates efficient utilization of cellular resources, where expression of pathway enzymes is controlled to an optimal level for conversion of substrates into products, and intermediates are converted into downstream products immediately after synthesis. These advantageous properties of dynamic regulation lead to robust cell growth and enhanced pathway productivity.

Dynamic regulation is commonly used in cells, with one example being catabolite repression. The cell's ability to sense glucose enables metabolism of preferred carbon sources, minimizing expression of enzymes for utilization of alternative carbon sources [87]. To synthetically engineer such a feedback mechanism, a sensor-regulator system for the metabolite of interest needs to be identified (Figure 2-12B). Most basic sensor-regulator systems involve endogenous sensing systems, where one would only need to identify and use promoters or native riboswitches [88] involved in the regulation mechanism. However, many metabolites and desired products in metabolic engineering endeavors are not naturally found in the host cell. Therefore, introduction of heterologous proteins or synthetic riboswitches is often necessary. A recent review by Michener et al. has described the possible protein and RNA sensors that can potentially be applied in the construction of sensor-regulator systems [89].

The first demonstration of dynamic sensing-regulation of a heterologous pathway for improved growth and productivity was by Liao and co-workers (Figure 2-12C) [90]. In this study, a global regulatory system in *E. coli*, the Ntr regulon was engineered to regulate a synthetic lycopene biosynthesis

pathway. The deletion of the transcription factor nitrogen regulator II (NRII) allowed NRI to become a sensor for acetyl phosphate (ACP). The sensing of intracellular ACP levels, which were suggested to be a glucose availability indicator [91], together with the *glnAp2* promoter, enables regulation of two rate limiting enzymes in lycopene biosynthesis. Hence, excess glucose flux can be efficiently utilized for the desired lycopene biosynthesis pathway and at the same time, reduce formation of the toxic product acetate. This dynamic regulation control loop was found to significantly enhance lycopene production and reduce growth retarding metabolic imbalance.

Recently, a dynamic sensor-regulator system (DSRS) was developed by Keasling and co-workers for the production of the biodiesel fatty acid ethyl ester (FAEE) (Figure 2-12D) [40]. Instead of employing indirect indicators (as used in the lycopene biosynthesis example), the direct sensing of critical pathway intermediates would serve to create a better regulation system, where production and consumption of intermediates can be controlled to maintain optimal concentrations and maximize pathway productivity. In this work, fatty acyl-CoA was identified to be a key intermediate for regulation. Since *E. coli*'s native FadR transcription factor is capable of responding to fatty acyl-CoA, the DNA-binding sequence for FadR was integrated into phage promoters along with DNA-binding sequence for LacI, a repressor that can be antagonized by isopropyl β -D-1-thiogalactopyranoside (IPTG). The creation and adoption of this hybrid promoter responsive to both IPTG, which eliminates leaky expression and enables synthetic exogenous induction, and fatty acyl-CoA, which controls

expression levels of key enzymes led to a superior FAEE producing strain. FAEE yield increased three-fold, and metabolic balance was improved, while the engineered strain had greater genetic stability.

Figure 2-12 Dynamic sensing-regulation of protein expression. (A) Inducible promoters enable the separation of growth and production phase, resulting in higher growth rates and overall productivity. (B) Dynamic sensing-regulation facilitates metabolic balance. When the concentration of key intermediates rises, downstream genes that utilize the intermediates can be up-regulated whereas upstream genes that increase the formation of the key intermediates can be down-regulated. (C) Sensing of ACP levels, an indirect indicator of glucose availability, allows the regulation of two rate-limiting enzymes (Idi and Pps), improving production of lycopene and at the same time reducing formation of waste by-products. (D) The direct sensing of the critical pathway intermediate fatty acyl-CoA (FA-CoA) enables regulation of multiple enzymes (FadD, AtfA, Pdc, AdhB) involved in the biosynthesis of FAEE. The application of this DSRs increased FAEE yield by three-fold, improved metabolic balance and enhanced genetic stability.

These examples have experimentally proven the importance and effectiveness of dynamic regulation for the engineering of microbial cell factories with improved productivity and growth. Besides the direct sensing and regulation via critical pathway intermediates, further improvement of the system would exclude the need for any exogenous inducer, and at the same time, maintain minimal basal expression at early growth phases. Our group has recently developed an engineered *E. coli* strain for production of lignin-derived chemicals. In this work, a sensor-regulator for the substrate lignin constituents was engineered to enable dynamic regulation of producing enzymes. This was coupled with a time-delay element, allowing the cells to auto-induce pathway enzymes at higher cell densities. We found the growth rate and productivity of the engineered strain to be significantly improved (unpublished data). Other research groups have established the importance and utility of dynamic regulation in silico [62, 92, 93]. The role of modeling in the engineering of production strains with robust growth and improved productivity will be addressed in Section 7.

Even though sensor-regulator systems were found to be effective in improving host growth and productivity, suitable sensor-regulator systems for metabolic engineering purposes are limited. Metabolite biosensors that use auxotrophic strains, or protein and RNA sensors with small dynamic ranges can only be used as screening tools instead of pathway regulation [94]. Besides identifying sensors for a metabolic pathway of interest, much engineering effort is required to create regulators (e.g. hybrid promoters) that possess appropriate strength and dynamic range [40]. Signal to noise ratios as well as tightness of regulation are also crucial factors that

determine the utility of a sensor-regulator system in pathway regulation applications [95].

2.2.6 Balancing co-factors in pathways and key metabolisms

Redirecting metabolic fluxes by altering the level of co-factors such as NADH, NADPH and CoA is one of the promising metabolic engineering approaches to boost productivity in bio-catalysts. Co-factor manipulation allows overcoming bottleneck of co-factor availability in co-factor dependent bio-synthesis pathways. Furthermore, co-factors influence a broad range of host cellular functions such as cellular respiration, metabolite synthesis and membrane transport [96]. As such, utilization of co-factors during bio-chemical synthesis may re-shape the metabolic landscape of the host, resulting in diminished host viability. One example is metabolic engineering for succinate production in *E. coli* [97], where gene knockouts (Δ ldhA; Δ pflB) were performed to redirect the metabolic flux towards succinate production, but resulted in a 2:1 NADH/NAD⁺ imbalance. The unusually high intracellular NADH/NAD⁺ ratio led to the growth impairment of the engineered strain. Hence, in recognition of the importance of maintaining the intracellular co-factor balance for host viability, this section is devoted towards strategies for limiting co-factor imbalances, which often result from metabolic engineering and novel pathway introduction.

One key co-factor imbalance that needs to be addressed promptly is the NADH/NAD⁺ ratio (Figure 2-13). NADH/NAD⁺ ratio may be regarded as

the indicator of the intracellular redox state [98], and it plays a major role in microbial metabolism by overseeing over 300 redox reactions [99], notably, in glycolysis. During glycolysis, glucose is oxidized using NAD⁺ and the reducing equivalent in the form of NADH is produced. For continued cell growth, the reduced NADH needs to be re-oxidized to NAD⁺ to support glucose oxidation. One approach to regenerate NAD⁺ is to recycle intracellular NAD breakdown products and scavenge pyridine compounds such as nicotinic acid from the environment. To do so, the enzyme nicotinic acid phosphoribosyltransferase (NAPRTase) that converts pyridine compounds such as nicotinic acid into a direct precursor of NAD⁺ can be expressed. NARTases such as PncB from *Salmonella typhimurium* were expressed in *E. coli* [99, 100], and PncB1 and PncB2 from *Mycobacterium tuberculosis* were studied for their role in NAD synthesis [101]. It was observed that overexpression of PncB in *E. coli* had greater effects on the final distribution of metabolites during anaerobic batch cultivation than in anaerobic chemostat conditions [100]. Under anaerobic batch cultivation, increase in total level of NAD increases the rate of NADH-dependent pathways. As such, cultivation conditions became one of the key considerations when the NAD⁺ level is increased. In addition to NAPRTase expression, alternative approaches to boost NAD⁺ availability include expression of water-forming NADH oxidase, which was successfully attempted in *Lactococcus lactis* NZ9800 [102] by using the *nox-2* gene from *Streptococcus mutans*, and in *S. cerevisiae* [103] by using the *noxE* gene from *L. lactis*, which reduced cytosolic NADH accumulation.

While increased NAD⁺ availability aids in maintaining NADH/NAD⁺ intracellular pool, in situations where NADH is needed to reduce metabolic intermediates for bio-chemical synthesis, NADH availability becomes the primary focus (Figure 2-13). To regenerate NADH from NAD⁺, Berrios-Rivera et al. [100] expressed NAD⁺-dependent formate dehydrogenase (FDH) from *Candida boidinii* in *E. coli*, which doubled the yield of NADH from 2 to 4 mol NADH per mol glucose consumed at the end of anaerobic fermentation. The increase in availability of NADH even induced a shift to fermentation in *E. coli* during aerobic growth [100]. Other FDHs from organisms such as *Mycobacterium vaccae* N10 and *S. cerevisiae* were expressed to improve D-mannitol productivity [104] and to increase glycerol yield on glucose in *S. cerevisiae* [105], respectively. Another approach for improving NADH regeneration is the use of methanol oxidation pathways. Methanol, rather than commonly used glucose, serves as an auxiliary substrate for NADH regeneration in terms of 2 molecules of NADH produced per molecule of methanol. The oxidation of methanol to CO₂ is spontaneous as it is driven by a favorable thermodynamic force [106]. Additionally, this pathway exists naturally in methylotrophic yeasts such as *Pichia pastoris*. Schroer et al. [106] demonstrated that by overexpressing formaldehyde dehydrogenase (FLD), one of the key enzymes in the methanol utilization pathway, endogenously in *P. pastoris*, higher theoretical NADH formation rate was achieved, and butanediol production rate was significantly improved for whole-cell bio-transformation.

Though the NADH/NAD⁺ ratio can be maintained through enzymatic expression that oversupplies NADH, compartmentalization of NADH

production in eukaryotic hosts was shown to have important metabolic consequences. For example, Hou et al. [107] evaluated the effect of increasing NADH in *S. cerevisiae*'s mitochondria and cytosol. Increasing mitochondrial NADH in *S. cerevisiae* triggers ethanol production while increasing NADH in cytosol results in glycerol production. This finding suggests possible manipulation of carbon flux in the central carbon metabolism for rational engineering of eukaryotic hosts through compartmentalizing NADH production.

In addition to the NADH/NAD⁺ ratio, the NADH/NADPH ratio is also of importance (Figure 2-13). While NADH is the chief redox product of catabolism, NADPH plays a major role in anabolism where it serves as a reducing agent for amino acid, lipid and nucleotide biosynthesis [108]. Introduction of bio-synthesis pathways such as α -santalene formation pathway leads to a net production of NADH and consumption of NADPH. To restore NADH/NADPH balance, Scalcinati and co-workers reshaped the host co-factor landscape by deleting the NADPH consuming reaction of glutamate dehydrogenase (GDH1) and coupling it with overexpression of NAD⁺-dependent glutamate dehydrogenase (GDH2) that resulted in NADH consumption. Such a strategy may require the use of genome-scale modeling of hosts to predict the global network effects of cofactor balancing, as demonstrated by Ghosh et al. in their attempt to engineer *S. cerevisiae* towards effective utilization of pentose sugars. In addition to metabolic engineering for co-factor balancing, enzymatic specificity towards NADH versus NADPH can be adjusted for desired outcome. Hasegawa et al. [109] changed co-factor requirements for L-valine synthesis from NADPH to

NADH by modifying the co-factor specificity of acetohydroxy acid isomeroreductase (AHAIR) through site-directed mutagenesis, where NADP-specific positively charged arginine was replaced with NAD-specific negatively charged glutamate.

Another important co-factor is CoA and its derivative acetyl-CoA. Acetyl-CoA is a key intermediate in various metabolic pathways for energy production, and is involved in the synthesis of esters and lipid molecules [99]. In the work by Lee et al. [110] where *E. coli* was engineered to convert ferulic acid into vanillin, the introduced bio-synthesis pathway led to the entrapment of CoA as acetyl-CoA, a by-product of vanillin synthesis. This entrapment caused a shortage of CoA for the first step in the vanillin production pathway which requires ferulic acid to be converted to feruloyl-CoA. To overcome this problem, Lee and co-workers overexpressed the citrate synthase *gltA* which catalyzes the first reaction of the TCA cycle so that acetyl-CoA (by-product) could be utilized to synthesize citrate for CoA release. The *gltA* amplification led to a 2-fold increase in vanillin production.

In general, the redox state of the engineered cells should be carefully monitored with balancing NADH/NAD⁺ ratio as an emphasis. Restoring cellular redox balance through co-factor manipulation may improve the physiological state of the cells. Besides affecting host viability, co-factor perturbation also carries a huge impact on metabolic flux distribution which can cause substantial deviation from engineered behavior. To restore co-factor balance, metabolic engineering to increase the pool of co-factor consumed and/or enzyme engineering to modify co-factor specificity can

be performed. Use of computational tools such as genome-scale modeling may aid in predicting the global network effects of these actions.

Figure 2-13 Approaches towards balancing co-factors for boosting anaerobic growth under batch condition, supplying reducing power for reducing substrate to product and restoring NADPH/NADH balance.

2.2.7 Computational tools for design and prediction

Towards balancing multi-faceted objectives such as cell growth, flux optimization, thermodynamics, economic costs and other factors in biocatalytic cells, computational methods are extremely useful in handling these enormous data and providing some very handy predictions for the in silico design. Current computational methods on designing new enzymatic

pathways have been developed based on characterized pathways and theoretical thermodynamic feasibility, and re-adjustment of metabolic routes (gene knock-out or amplification), which are useful for tuning the host cell and constructing genetic parts required for the assembly of genetic circuits. In this section, we relate the use of these tools to optimizing host viability (Figure 2-14).

The first stage of engineering bio-catalysts is the construction of synthetic pathways that lead to the synthesis of target compounds. To aid in designing bio-synthetic routes, useful databases such as the Kyoto Encyclopedia of Genes and Genome (KEGG) [111] and MetaCyc [112] can be referred to. However, information on biochemical pathways and metabolic networks from those databases is frequently insufficient in consideration of energetic costs of new pathway introduced. Energetic cost is the number of moles of NADPH/ATP equivalents consumed in the production of one mole of product. Different synthetic pathways, which ultimately perform the same metabolic conversion, might consume resources to a different degree, thereby unequally affecting the growth of the organism [113, 114]. In addition, since the metabolic load of heterologous enzyme expression directly affects cell viability, pathway distance should be considered carefully. However, pathway distance can only be an applicable criterion if a host is selected a priori, since a host cell may contain one or more enzymes from the designed pathway in its inherent metabolic network. This issue can be alleviated by using computational strategies such as the one employed in the Biochemical Network Integrated Computational Explorer (BNICE) framework [115],

which can be useful for prioritizing novel biosynthetic routes based on pathway length, thermodynamic feasibility, maximum achievable yield and maximum achievable enzymatic activity. Henry et al. applied the BNICE framework to generate novel shortest pathways for synthesis of 3-hydroxypropanoate from glucose. In addition, another prediction system DESHARKY [116] included transcription, translation and metabolic load into its calculation, which can be particularly useful for optimizing cell viability because it provides information on the impact of designed metabolic pathways together in a cellular context.

Once an enzymatic pathway has been selected for introduction into a host organism, its impact on host cells can be predicted using genome-scale modeling. Genome-scale modeling utilizes the reconstructed map of genome-scale metabolic bio-chemical reactions which are defined by their reaction stoichiometry. In this manner, model predictions can be made based on the constraints imposed on the reaction fluxes and a defined objective function, such as cell biomass growth, for which the fluxes are optimized. Users can refer to the Biochemically, Genetically and Genomically structured (BiGG) knowledgebase for reconstructed genome-scale metabolic maps. These maps are designed to work with analytic tools such as the Constraint Based Reconstruction and Analysis (COBRA) [117] Toolbox for quantitative prediction of cellular metabolism. COBRA Toolbox has been frequently used for constraint-based analyses on genome-scale models [118]. Our group utilized COBRA Toolbox on BiGG maps such as *E. coli* iAF1260 for predicting the impact of the designed bio-synthesis pathways on steady-state metabolic flux distribution and growth rate. This

was done by adding stoichiometric coefficients and novel compounds from the designed reactions into the BiGG map, and then, new steady-state flux distribution was predicted using the constraint-based flux balance analysis (FBA) [119], a function available in COBRA Toolbox. Besides FBA, COBRA Toolbox v2.0 [120] can also be used to direct metabolic flux towards the production of targeted compounds. This may frequently require gene deletions which can have a considerable impact on host viability. Therefore, to examine whether the suggested gene deletion set would have minimal impact on host growth, the OptKnock [121] function in COBRA Toolbox v2.0 can be used. The OptKnock algorithm identifies gene deletion strategies that allow production of targeted compounds to become an obligatory byproduct of cellular growth. Further, the OptKnock-suggested gene-deletion sets can be completed by the minimization of metabolic adjustment (MOMA) [122] function in COBRA Toolbox. MOMA works based on the hypothesis that the knock-out metabolic fluxes would undergo a minimal redistribution with respect to the flux configuration of the wild-type. Brochado et al. improved vanillin production by 2-fold by using MOMA as the biological objective function for their in silico simulations [123].

After the COBRA Toolbox has been used for in silico prediction and thus, the whole genome-scale metabolic landscape has been reshaped, genetic part construction frequently starts. Genetic parts such as promoters, ribosome binding sites (RBS) and coding sequences (CDS) play a significant role in allocation of metabolic resources, which in turn affects host growth. For example, strong constitutive promoters and RBS initiate

high level of gene expression which may consume considerable amount of metabolic resources. The diversion of cellular resources from essential metabolic activities may subsequently lead to growth retardation and deviation from engineered behaviors [124], both of which can decrease yield and productivity. To avoid such situations, the minimalist approach of moderate expression of enzymes can be employed in bio-catalytic cells. The first step towards this approach is the codon optimization of CDS. Codon optimization circumvents rate-limiting translations, a consequence due to codon bias in host organisms. By identifying rate-limiting codon sets and replacing them with synonymous codons that are more recognizable by abundant tRNA species in the host, codon elongation rate can be higher [125]. One handy web-based tool Optimizer [126] can be utilized for codon optimization process for over 150 prokaryotic species. For advanced users who prefer visual computer-aided design (CAD) tool, Gene Designer 2.0 can be helpful [127]. In addition to codon optimization, the intrinsic properties of mRNA sequence should also be examined, including mRNA stability and secondary structure. Having a loop structure at the 5' -end of the mRNA may impede translation. To both minimize the 5' structure and match codon bias, the backtranslation in Gene Designer 2.0 can be used, which first minimizes the 5' structure, and then designs remaining gene sequences for desired codon usage. An optimized CDS enables a higher level of translation per mRNA transcript, therefore could allow the use of weaker synthetic promoters to reduce over-transcription and save on precious metabolic resources.

Further, the optimized CDS can be coupled with a suitable RBS for tuning expression towards optimality. Enzymes with different kinetic efficiencies should be expressed according to their levels of activity. For instance, highly active enzymes can be expressed on a weaker RBS to prevent overexpression and results in a more efficient use of metabolic resources. To tune the expression towards the desired level, synthetic RBSs designed using RBS Calculator [83] can be used. Users can decide on the level of translation by simply inputting the CDS and the preferred translation initiation rate which ranges from 0 to 100,000-fold into the RBS Calculator, and the synthetic RBS sequences to deliver the desired outcome would be generated. While this approach enables greater control of protein expression levels, the optimal translation rate has to be obtained through trial-and-error.

Taken together, the use of computational methods can greatly benefit the design and construction of bio-synthesis pathways that lead to robust host cell viability. As bio-catalytic productivity is directly linked to host viability, there is a strong need to utilize such methods for efficient optimization of metabolic load of heterologous enzyme expression, co-factor balance and metabolic resource competition. Computational methods can be further improved by factoring in substrate/intermediate/product toxicity on the host as well as the impact of gene amplification.

Figure 2-14 An in silico approach for designing the cell for bio-chemical production, but with host viability as key consideration. After determining bio-synthesis pathways, both the top-down and the bottom-up approaches can be carried out concurrently. The top-down approach refers to host cell engineering which requires the prediction of the impact on the global flux distributions by metabolic engineering and novel pathway introduction. The bottom-up approach involves optimizing DNA sequences and adjusting genetic circuit layout for minimal metabolic requirements, but yet achieving maximal bio-catalytic performances.

2.2.8 Conclusion

Biological production of chemicals is gaining importance and could possibly define the future of chemical synthesis. However, commercially feasible bioprocesses for the production of biofuels, pharmaceuticals, chemicals, and other valuable compounds demand high yields and titers, and at the same time, low costs. Given that bio-catalytic performances are frequently affected by toxicity arising from chemical production and stresses resulting from metabolic imbalances, engineering host cells to sustain robust viability during bioconversion processes, which can increase biomass accumulation

and stability in bio-catalytic performances, is certainly one of the most critical steps for the successful development of commercially feasible bio-catalytic cells. Consequently, it is imperative to design engineering strategies to improve microorganisms' viability against the aforementioned stresses and limit their impact.

Herein, we reviewed various strategies to engineer an improved microorganism platform with improved response towards cellular stresses generated by introduced novel bio-synthesis pathways and by metabolic engineering which often threatens host viability. These strategies can be implemented separately or concurrently. We believe that this review provides valuable insights into designing strain optimization strategies to improve host viability for enhanced bio-productivity.

CHAPTER 3

TRANSCRIPTOME RESPONSE TO ALKANE

BIOFUELS IN *SACCHAROMYCES*

CEREVISIAE

3.1 Introduction

Next-generation biofuels, such as long-chain alcohols, and fatty-acid- and isoprenoid-derived biofuels, offer advantages of high energy density, low freezing point, and compatibility with the existing fuel storage and distribution infrastructure [11, 56, 128, 129]. Recently, hydrocarbon alkanes, main components of fossil fuels, have been considered as important next-generation biofuels because their microbial production was demonstrated. Schirmer et al. converted intermediates of fatty acid metabolism to alkanes in *E. coli* by introducing two enzymes involved in an alkane biosynthesis pathway from cyanobacteria [2]. Bernard et al. identified Arabidopsis alkane synthesis enzymatic components and reconstituted plant alkane biosynthesis in yeast [3].

Despite these successes in microbial alkane production, the toxicity of alkanes to microbial hosts can eventually be a bottleneck for high productivity. To overcome this toxicity issue, it is imperative to develop engineering strategies to improve microbial tolerance against biofuel alkanes, which requires a clear understanding of molecular mechanisms of interaction between microbial hosts and alkanes. Physiologically, hydrocarbon accumulation in cell membrane causes loss of membrane integrity and function, which ultimately leads to cell death [130]. In response, cells protect themselves against the toxicity of hydrocarbons by ordering the lipid bilayer to modify lipopolysaccharide (LPS) and cell wall/S-layer hydrophobicity, as well as activating the excretion by energy-consuming transport systems. Despite the aforementioned studies, which primarily focused on physiological or cytological effects, there is lack of

understanding of the molecular mechanisms of interactions between microbes and alkanes especially at a systems level.

Toxicogenomics, which combines genomics and toxicology, is useful for identification of toxicants and their putative mechanisms of action at a systems level. Recently, toxicogenomics has been applied to elucidate mechanisms underlying environment stresses and chemical toxicity to microorganisms [131, 132]. For instance, Chang et al. studied the toxicogenomic response of pathogens to antimicrobials by using microarray-based transcriptome analyses [133]. Carvalho et al. studied transcriptomic response in marine diatom *Thalassiosira pseudonana* exposed to benzo[a]pyrene. Notably, toxicogenomics has also offered an effective means to study the molecular mechanisms of cell response to organic solvents. For example, based on genome-wide microarray analyses, our group previously identified and reconstituted genetic regulatory networks to improve the tolerance of *E. coli* against isooctane [45]. In *S. cerevisiae* strain KK-211, genes involved in tolerance to organic solvents were successfully identified based on transcriptome analyses [134, 135]. Note that *S. cerevisiae*, an established and widely used eukaryotic model for molecular and cellular biology studies, has been used as an experimental model in toxicogenomics [136, 137]. In this light, this study aimed to investigate molecular mechanisms underlying cellular response to alkanes at the systems level by transcriptome analyses in *S. cerevisiae*, a model eukaryotic host of industrial significance. Here, we showed that nonane (C9) and decane (C10) induced cellular mechanisms associated

with efflux pumps, membrane modification, radical detoxification and energy supply.

3.2 Materials and methods

3.2.1 Strains and media

S. cerevisiae BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) was obtained from ATCC (American Type Culture Collection). Yeast extract and peptone were obtained from BD (NJ, USA). Alkanes and other chemicals were obtained from Sigma-Aldrich unless specifically mentioned.

S. cerevisiae strains were grown in YPD medium (Yeast extract 10 g/L, Peptone 20 g/L, and D-Glucose 20 g/L) and minimum medium (Yeast Nitrogen Base 6.7 g/L, D-Glucose 20 g/L, L-Leucine 0.2 g/L, Uracil 0.1 g/L, 2% n-alkanes), at appropriate temperatures. *E. coli* cells were grown in LB broth at 37°C.

3.2.2 Cell viability, membrane integrity and reactive oxygen species (ROS)

S. cerevisiae BY4741 cells were grown overnight in YPD, diluted into minimal medium with amino acids which contains each of 2% alkanes (C9-C12) with an initial OD600 of 0.2, and followed by shaking incubation at 28°C, and viable cells measurement by CFU (Colony Forming Unit) counting. To investigate whether these alkanes resulted in membrane

damage, membrane permeability was analyzed by cell staining. After exposure to alkanes, cells were stained with fluorescent nucleic acid stains SYTO 9 and propidium iodide (PI) (Invitrogen) that only penetrates cells with damaged membrane. Finally, signal intensity in cells was measured using Synergy HT multi-mode microplate reader (Biotek, VT, USA) at wavelength of 485/20 nm (excitation), 528/20 nm (SYTO 9 emission), and 645/40 nm (PI emission). To investigate reactive oxygen species (ROS) generation, alkane-treated cells were collected and stained by CellROX® Green Reagent (Life Technologies). Fluorescence signals were measured by TECAN Infinite 200 microplate reader at wavelength of 485 nm (excitation)/535 nm (emission) and normalized to cell density (OD600).

3.2.3 Alkane extraction and detection

Alkanes were extracted by chloroform-methanol extraction method [138] with modifications. Firstly, yeast cells were collected and washed with 50 mM Tris.Cl (pH 7.4) then resuspended in 0.4 ml chloroform/methanol mixture (v/v: 2/1). Subsequently, 0.3 g acid-washed glass beads (425-600 μm , Sigma-Aldrich) were added into each sample and cells were lysed using FastPrep-24 (MP Biomedicals, Solon, OH, USA). Then, after the lysate was centrifuged at 4°C at maximum speed for 10 min, an appropriate volume of chloroform and 50 mM Tris.Cl were added to the supernatant for extraction. Finally, the chloroform phase with alkanes was transferred into glass vials for Gas Chromatography (GC) analysis. This was performed using Agilent GC 7890A system (Agilent Technologies,

Santa Clara, CA, USA) under the following conditions. With Agilent HP-5 GC column, oven temperature was started at 40°C for 30 sec, and was ramped from 40°C to 220°C at 40°C/min, and FID detector temperature remained at 275°C. A mixture of n-alkanes (10 ppm each) was used as the standard for GC analysis.

3.2.4 RNA preparation, transcriptome analysis, and quantitative PCR

Total RNA was extracted with lyticase for cell wall disruption using RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA concentration and integrity were measured using NanoDrop (Thermo Scientific, Wilmington, DE, USA) and Agilent Bioanalyzer 2100, respectively. Subsequently, 200 ng of the qualified total RNA was transcribed and labeled using Agilent RNA Spike-in Kit and Low Input Quick Amp Labeling Kit (two colors). The Cy3- or Cy5-labeled cRNA was hybridized onto Yeast (V2) Gene Expression Microarray (8 × 15 K) at 65°C for 17 h, using Agilent GE Hybridization Kit. Thereafter, the microarray slide was scanned using Agilent Microarray Scanner G2505B. The two-color scanning data were extracted using Agilent Feature Extraction software (v10.5) and analyzed using GeneSpring GX 11. From two biological replicates, differentially expressed genes were selected based on their p values (<0.05, t-test) and fold changes (FC) in n-alkane-treated cells in comparison to control cells (≥ 2.0).

To validate the microarray data, quantitative PCR (qPCR) was performed with primers targeting the selected genes and SsoFast™ EvaGreen® Supermix using iQ5 real-time PCR detection system (Bio-Rad). The qPCR data analysis was conducted with ACT1 as a reference gene using Bio-Rad iQ5 optical system software.

3.2.5 Network component analysis (NCA)

The Network Component Analysis is an algorithm that calculates relative activities of transcription factors from gene expression data [45]. In brief, a matrix of gene expression data ([E]), which contain genes in rows and experimental conditions in columns, are prepared for NCA analysis. Subsequently, the [E] matrix is decomposed into two matrices, a connectivity strength (CS) matrix ([A]) with transcription factors (columns) and genes (rows) and a transcription factor activity (TFA) matrix ([P]) with transcription factors (rows) and experimental conditions (columns). In our study, to perform NCA analysis, first, genes in the gene expression data matrix ([E]) were built from 147 genes (fold changes ≥ 2.0 , and p values < 0.05), up- or down-regulated under the three conditions (C10 vs. control at 24 h and 48 h, and C9 vs. control at 48 h); second, the connectivity strength (CS) matrix [A] from Lee and coworkers was used to primarily define connectivity relations between the genes and the transcription factors; third, the final TFA matrix [P] was deduced by NCA.

3.3 Results and discussion

3.3.1 Toxicity and intracellular accumulation of alkanes

We first examined the toxicity of nonane (C9), decane (C10), undecane (C11), and dodecane (C12). In this work, we primarily focused on C9-C12 because our preliminary studies had indicated low cytotoxicity of C13 and longer alkanes. *S. cerevisiae* BY4741 cells were incubated in the medium containing each alkane (2% [v/v]). Figure 3-1A shows that cell viability was decreased upon exposure to C9-C12. In particular, treatment with C9-C11 decreased cell viability by about 50%. Based on this result, we hypothesized that C9-C12 might affect cell membrane permeability or integrity. Figure 3-1B shows that the intensity of red fluorescent signal of propidium iodide (PI) and the ratio of the fluorescent signals (PI/SYTO9) were increased upon exposure to C9-C12. If more PI molecules penetrate into cell wall/membrane and stain nucleic acids, PI intensities and PI/SYTO9 ratios become higher. Therefore, this result suggests that C9-C11 increased cell membrane permeability. Among these alkanes, C9 and C10 resulted in the highest PI intensities and PI/SYTO9 ratios, which indicates the highest membrane permeability.

Figure 3-1 Viability and membrane permeability of *S. cerevisiae* cells under exposure to C9-C12. The viability (A) and membrane permeability (B) were measured upon exposure to alkanes (2% each) for 48 h. RFU, relative fluorescence unit. Error bars represent standard deviations from four biological replicates.

To determine whether this increased membrane permeability was contributed by damage on cell surface, cell surface was examined using Field Emission Scanning Electron Microscopy (FESEM). As shown in Figure 3-2, damage was not apparent on cell surface upon exposure to 2% C9, which is in agreement with de Smet and coworkers' study [139]. It was noted that there are some potential drawbacks of SEM-based characterization: possible artifacts that arise from SEM sample preparation, some incubation times for the membranes to recover, and the metal coating procedure that eliminates opportunities for fine examination. However, based on the result here, it is reasonable that we hypothesized

that alkane toxicity might be attributed at least partly to intracellular accumulation. GC analyses show that a peak corresponding to each alkane was detected from alkanes-treated cells, while control cells without alkanes exhibited a negative signal (not shown). To measure intracellular alkane amounts, we normalized GC peak areas to the internal standard and the total protein amount. Among C9 to C12, C10 showed the highest intracellular level, followed by C9 and C11, and C12 level was the lowest which was 90% lower than C10 level (Figure 3-3). These results indicate that alkanes were accumulated in cells, which likely contributed to alkane toxicity. According to Inoue and Kawamoto [6, 140], toxicity of a compound is related to coefficient of octanol-water partition ($\log P_{ow}$). Gill and coworkers reported that higher aliphatic chain alkanes tend to show lower toxicity [141]. In our study, C12 ($\log P_{ow}$ 7.0) showed lower toxicity than C9 to C11 ($\log P_{ow}$ <6.0), which could be partially due to low accumulation of C12 in *S. cerevisiae*.

Figure 3-2 Cell morphology of *S. cerevisiae* BY4741 cells upon exposure to 2% C9. C9-treated cells were collected for FESEM (Field Emission Scanning Electron Microscopy) analysis after exposure to C9 for 48 h. For sample preparation, cells were washed with Tris.Cl (pH 7.4) immediately after alkane treatment, and then fixed in 2% glutaraldehyde at 4°C for overnight followed by 1% osmium tetroxide for 10 min. Cell samples were dehydrated through ethanol: 2 min in each of 30%, 50%, 70%, 95% (twice) and 100% (three times). Thereafter, cells were loaded onto silicon slides and dried. Then, samples were coated with platinum and observed using FESEM JSM-6700 F (JEOL). Control, *S. cerevisiae* BY4741 cells in alkane-free medium.

Figure 3-3 GC analyses of intracellular alkanes in *S. cerevisiae*. Cells were exposed to 2% of each n-alkane for 48 h. Intracellular alkanes were measured by GC and normalized to the total protein amount and the internal standard n-tridecane. A.U., arbitrary unit. Error bars represent standard deviations from three biological replicates.

3.3.2 Genome-wide gene expression profiles upon exposure to biofuel alkanes

To understand cellular mechanisms underlying *S. cerevisiae* response to C9-12, transcriptome profiles were generated using whole-genome *S. cerevisiae* microarrays. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE38653. In addition, quantitative PCR (qPCR) was carried out to validate the microarray data (Table 3-1).

Table 3-1 Validation of microarray data by qPCR upon exposure to C10 for 48h

Gene	Fold change (microarray)	Fold change (qPCR)
<i>GRE1</i>	9.35	8.61±0.04
<i>HSP12</i>	4.4	3.01±0.70
<i>HXT5</i>	3.78	5.20±0.62
<i>ICT1</i>	3.32	2.28±0.12
<i>PDR5</i>	3.5	5.76±0.45
<i>YHB1</i>	2.33	3.02±0.52

Our transcriptome analysis indicated that 526 (C10 for 24 h), 692 (C9 for 48 h), 428 (C10 for 48 h), and 119 (C11 for 48 h) genes were differentially regulated, while 5 (24 h) and 8 (48 h) genes were regulated by C12. Functional analysis of those genes suggested that C11 primarily regulated genes related to heat shock response and sugar transport, whereas C9 and C10 induced a range of similar groups of genes. Therefore, to look into

cellular mechanisms commonly stimulated by alkanes, we analyzed the genes regulated by both C9 and C10. 147 genes were significantly regulated by both C9 and C10. Among the 147 genes, 105 genes were induced by both C9 and C10, whereas 42 genes were repressed by both C9 and C10 (except for COS12 gene which was down-regulated by C10 but up-regulated by C9). To further understand gene functions, we functionally classified the aforementioned regulated genes using MIPS tools (<http://funspec.med.utoronto.ca/> website). Primary functional classes of the regulated genes were (i) efflux pumps, (ii) stress response, (iii) fatty acid, lipid, and derivatives biosynthesis, and (iv) hexose transport, ergosterol biosynthesis, and thiamine biosynthesis (Table 3-2). The genes in these classes were mostly induced by C9 and C10.

Table 3-2 MIPS functional classification of genes differentially regulated by both C9 and C10

Functional category	k	f
<u>Up-regulated</u>		
fatty acid metabolism	5	24
phospholipid metabolism	7	68
oxidative stress response	6	55
tetracyclic and pentacyclic triterpenes	5	36
cellular import	7	90
lipid/fatty acid transport	5	44
ABC transporters	4	28
lipid, fatty acid and isoprenoid metabolism	8	133
degradation of glutamate	2	4
metabolism of amino acid-derived secondary products	2	4
NAD/NADP binding	4	36
C-compound and carbohydrate metabolism	10	223
heat shock response	3	20
chemical agent resistance	3	22
S-adenosyl-methionine-homocysteine cycle	2	7
pH stress response	2	8
oxidation of fatty acids	2	9
transport ATPases	4	53

Down-regulated

biosynthesis of thiamine	6	110
transport facilities	5	87
degradation of lysine	2	5
degradation of glycine	2	6
transport routes	2	11
metabolism of thiamine	3	43
tetrahydrofolate-dependent C-1-transfer	2	13

Note: functional classification was generated by the online program FunSpec (<http://funspec.med.utoronto.ca/webcite>) with p-value cut-off of 0.01. k, number of genes from the input (105 up-regulated genes, and 42 down-regulated genes) in the given category, f, number of genes total in the given category.

First of all, notably, four plasma membrane efflux pump genes YOR1, SNQ2, PDR5, and PDR15 were significantly induced by alkane stress. Plasma membrane efflux pumps recognize and extrude a large spectrum of functionally and structurally unrelated drugs such as oligomycin, mycotoxins, and anticancer drugs [142-144]. That is, when cytotoxic compounds cross the plasma membrane to enter cells, a number of efflux pumps help to protect cells from unwanted or damaging xenobiotics. According to Ernst and coworkers [145], efflux pumps serve as a defense line mediating pleiotropic drug resistance (PDR). Therefore, the result that efflux pump genes were significantly induced here suggested that they might also provide protection to the cells against alkanes.

Other than efflux pump genes, genes involved in stress response for radical detoxification, fatty acid and lipid synthesis, and hexose transport were also induced by alkanes. For example, GRE genes associated with multiple stresses and heat shock protein gene HSP12 were induced up to 16-fold, and fatty acid synthase genes FAS1 and FAS2 were induced over 4-fold. Acetyl-CoA carboxylase gene ACC1 was up-regulated about 6-fold. Acc1p is a biotin-containing enzyme that participates in fatty acid synthesis by converting acetyl-CoA to malonyl-CoA, which is a rate-limiting step in the fatty acid synthesis pathway [146]. In addition, INO1 gene was induced over 50-fold, which is associated with synthesis of phosphatidylinositol by using glucose-6-P and CDP-DAG as precursors [147].

Upon alkanes exposure, six transcription factor genes, ASH1, GAL4, NRG2, MGA2, YPR015C, and THI2, were differentially regulated. Because changes in gene expression levels of transcription factors frequently do not correlate with changes in their regulatory activities, to further identify transcription factors with perturbed regulatory activities under alkane exposure, we analyzed our transcriptome data using Network Component Analysis (NCA). NCA is an algorithm that calculates relative activities of transcription factors from gene expression data [45]. NCA results suggested that regulatory activities of nine transcription factors were changed upon exposure to C9 (48 h) and C10 (24 h, and 48 h) (Table 3-3). Among the nine transcription factor genes identified from NCA, the activities of six transcription factors were increased, and the activities of three were decreased. To further understand regulatory relationships between transcription factors and the genes involved in C9 and C10

response, a regulation matrix was generated by YEASTRACT (<http://www.yeasttract.com/> website) [148]. In the generated transcription networks (Figure 3-4), INO2 showed regulation of genes associated with efflux pumps, stress response, lipid metabolism and ergosterol biosynthesis. Hereby, we predicted that INO2 would most likely be one of the key transcription factors that regulate gene networks in response to alkanes exposure in *S. cerevisiae*. Further, the three repressed transcription factors (i.e. CIN5, FHL1, and HAP1) likely up-regulate their target genes such as GRE2, GND2, and ERG6 (Figure 3-4). Therefore, there is a possibility that the aforementioned transcription factors induce genes associated with efflux pumps, stress response, and lipid metabolism in *S. cerevisiae* upon exposure to both C9 and C10.

Table 3-3 Transcription factors with activity changes, predicted by Network Component Analysis (NCA)

Transcription factors		Regulatory activity		
		C10-24h	C10-48h	C9-48h
YKL112W	<i>ABF1</i>	+3	+3	+3
YKR099W	<i>BAS1</i>	+3	+3	+4
YDR123C	<i>INO2</i>	+12	+18	+19
YOL108C	<i>INO4</i>	+3	+4	+3
YGL162W	<i>SUT1</i>	+4	+5	+5
YDR207C	<i>UME6</i>	+6	+4	+4
YOR028C	<i>CIN5</i>	-2	-3	-10
YPR104C	<i>FHL1</i>	-3	-4	-8
YLR256W	<i>HAP1</i>	-3	-3	-7

Figure 3-4 The predicted transcription regulatory network. Regulation matrices were generated by YEASTRACT (<http://www.yeasttract.com/> website) based on the documented regulations, with our transcription factors as regulator candidates and regulated genes as target candidates. Transcription factors are shown in a grey box, and target genes are in an open box. Genes regulated by the same transcription factor in each group are shown with arrows in the same color.

Based on our transcriptome data, and the functions and physiological roles of the regulated genes, we hypothesized that *S. cerevisiae* responded to alkanes stresses through integrative mechanisms (depicted in Figure 3-5). First, efflux pump genes (YOR1, SNQ2, PDR5, and PDR15) are induced to export intracellular alkanes to reduce alkane toxicity. Second, alkanes exposure generates radicals and induces stress-responsive genes towards radical detoxification. Alkane molecules penetrate cell wall, disperse into cytoplasmic membrane, and generate radicals (e.g. reactive oxygen species (ROS), and NO), while stress-responsive genes (GRE1, GRE2,

CTT1, HSP12, TSA2, and YHB1) are induced to detoxify radicals including ROS. Notably, in line with this hypothesis, we observed that ROS levels were increased by 110% and 78% in *S. cerevisiae* upon exposure to C9 and C10, respectively (Table 3-4). Such increase in ROS levels is most likely correlated with alkanes toxicity. Third, more energy sources are generated through acceleration of glucose and fatty acid metabolism. Hexose transport genes (HXT5, HXT6, and HXT7) are induced to increase glucose import. Thereafter, energy sources (FADH, NADH, NADPH, and ATP) are enriched through enhanced glucose metabolism involving pentose phosphate pathway (GND2), β -oxidation of fatty acids (FAA4, and POT1), and ALD3-involved pathways. ATP may be used for alkanes export by efflux pumps. Fourth, the increase in glucose import and consumption aids in modifying membrane components (i.e. lipid, and ergosterol), thereby helping cells to adapt to alkanes exposure. Enriched glucose inside the cells may lead to the synthesis of more fatty acids, lipids, and derivatives (i.e. phospholipid, glycerophospholipid, phosphatidylinositol, and phosphatidylcholine), contributed to by such induced genes as FAS1, FAS2, ACC1, ICT1, OLE1, CHO1, PSD1, INO1, ITR1, and OPI3. Further, ergosterol biosynthesis is enhanced by the four induced genes HMG1, EGR3, ERG6, and ERG25. Such a change in the expression of genes involved in lipid and ergosterols metabolism may lead to membrane modification, possibly facilitating adaptation to alkanes exposure.

Figure 3-5 Schematic representation of hypothesized mechanisms of cellular response to alkanes in *S. cerevisiae*. The mechanisms include alkane efflux (blue), detoxification of radicals (green), energy supply (red), and membrane modification (purple).

Table 3-4 ROS levels in *S. cerevisiae* upon exposure to C9 and C10 for 24h

Control	C9	C10
1.00±0.27	2.10±0.3	1.78±0.00

Note: ROS levels in control cells without alkanes were set as 1. Three biological replicates were performed.

3.4 Conclusions

To investigate molecular mechanisms underlying cellular response to alkanes, in this study, we first confirmed that C9, C10, and C11 were significantly toxic and accumulated in *S. cerevisiae*. Secondly,

transcriptome analyses were performed and suggested that C9 and C10 induced a range of cellular mechanisms such as efflux pumps, membrane modification, radical detoxification and energy supply. We believe that the mechanisms presented here provide valuable insights into further designing microbial engineering strategies in order to improve cellular tolerance. In **Chapter 4**, we will further discuss the strategy to improve cellular tolerance toward alkanes based on these hypothesized mechanisms.

CHAPTER 4

IDENTIFICATION OF TRANSPORTERS FROM
***SACCHAROMYCES CEREVISIAE* INVOLVED**
IN ALKANE TOLERANCE

4.1 Introduction

Recently, hydrocarbon alkanes, main components of fossil fuels, have been considered as important next-generation biofuels because their microbial production was demonstrated. Despite these successes in microbial alkane production, the toxicity of alkanes to microbial hosts can eventually be a bottleneck for high productivity. To overcome this toxicity issue, in the **Chapter 3**, genes involved in cell tolerance toward medium chain alkanes were successfully identified based on transcriptome analyses in *S. cerevisiae*. We demonstrated that nonane (C9) and decane (C10) induced cellular mechanisms associated with efflux pumps, membrane modification, radical detoxification and energy supply.

Among the aforementioned mechanisms, we were interested in identifying potential efflux pumps involved in alkane tolerance, since efflux pumps were utilized to alleviate product toxicity and increase final productivity recently, due to their capability to pump out target products from cells [56, 57]. Since transporters could possibly aid in alkane export, thereby reducing the cytotoxicity, we formed the hypothesis that those induced transporters could contribute to alkane tolerance. There are two major classes of efflux pumps, ATP-binding cassette (ABC) transporters and secondary efflux pumps. ABC transporters, which utilize the energy of ATP hydrolysis to carry out efflux pumping, are the main efflux pumps in yeast cells. Hence, in this study, we focused on i) identifying native ABC transporters that potentially transport alkane biofuels according to our previous DNA microarray data and ii) further harnessing those transporters as a direct mechanism for increasing tolerance through efflux pumping of

alkanes from cells. According to the microarray data, four potential transporters - YOR1, SNQ2, PDR5, and PDR15 were tested for alkane tolerance. Among them, we demonstrated the roles of the transporters Snq2p and Pdr5p in reducing the intracellular levels of C10 and C11, and enhancing the tolerance levels against C10 and C11. This result provided the evidence that Snq2p and Pdr5p were associated with alkane export and tolerance in *S. cerevisiae*. We envision that the engineering strategy established in this study can readily be extended to develop robust microbial hosts for alkane biofuels production.

4.2 Materials and methods

4.2.1 Strains, chemicals and plasmids

S. cerevisiae BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) was obtained from ATCC (American Type Culture Collection), and gene deletion mutants were constructed in this study. Yeast extract and peptone were obtained from BD (NJ, USA). Alkanes and other chemicals were obtained from Sigma-Aldrich unless specifically mentioned. pUG6 and pSH47 were used for gene deletion and marker rescue in yeast cells. Plasmid pYES2 (Invitrogen, Grand Island, NY, USA) was used for gene expression in yeast cells. *E. coli* Top10 was used for gene cloning.

4.2.2 Growth conditions

S. cerevisiae strains were grown in YPD medium (Yeast extract 10 g/L, Peptone 20 g/L, and D-Glucose 20 g/L), minimum medium (Yeast Nitrogen Base 6.7 g/L, D-Glucose 20 g/L, Yeast Synthetic Drop-out Medium Supplements-Ura- 1.92 g/L), or induction medium (Yeast Nitrogen Base 6.7 g/L, Raffinose 10 g/L, Galactose 20 g/L, and Yeast Synthetic Drop-out Medium Supplements-Ura- 1.92 g/L) at appropriate temperatures. *E. coli* cells were grown in LB broth at 37°C. Geneticin G418 (PAA Laboratories GmbH, AT) or Ampicillin was used at 200 µg/mL and 100 µg/mL, respectively.

4.2.3 Alkane extraction and detection

Alkanes were extracted by chloroform-methanol extraction method [138] with modifications. Firstly, yeast cells were collected and washed with 50 mM Tris.Cl (pH 7.4) then resuspended in 0.4 ml chloroform/methanol mixture (v/v: 2/1). Subsequently, 0.3 g acid-washed glass beads (425-600 µm, Sigma-Aldrich) were added into each sample and cells were lysed using FastPrep-24 (MP Biomedicals, Solon, OH, USA). Then, after the lysate was centrifuged at 4°C at maximum speed for 10 min, an appropriate volume of chloroform and 50 mM Tris.Cl were added to the supernatant for extraction. Finally, the chloroform phase with alkanes was transferred into glass vials for Gas Chromatography (GC) analysis. This was performed using Agilent GC 7890A system (Agilent Technologies, Santa Clara, CA, USA) under the following conditions. With Agilent HP-5 GC column, oven temperature was started at 40°C for 30 sec, and was

ramped from 40°C to 220°C at 40°C/min, and FID detector temperature remained at 275°C. A mixture of n-alkanes (10 ppm each) was used as the standard for GC analysis.

4.2.4 Yeast gene deletion

Gene deletion was carried out as previously described [149]. Briefly, loxP-Kan-loxP gene disruption cassettes were amplified using PCR with primers as shown in Table 4-1 and pUG6 as the template, which were then purified using QIAquick Gel Extraction Kit (Qiagen). The purified cassettes were used for yeast transformation as previously described. The colonies were screened on YPD plates containing 200 µg/mL G418 and confirmed by PCR. To perform marker rescue, pSH47 was transformed into mutants, selected on minimal medium (Ura-) plates, and followed by Cre/loxP-mediated marker removal in induction medium. Finally, selection for the loss of marker was performed in YPD containing 1 mg/mL 5-fluoro-orotic acid (Thermo Scientific).

Table 4-1 Primers used in this study

Primer names	Sequence (5'→3')
<u>Gene deletion</u>	
YOR1-delF	CTGTTTTTATATTCAAAAAGAGTAAAGCCGTTGCTATATACGAATCA GCTGAAGCTTCGTACGC
YOR1-delR	ATATAAATAAATAAAAGAGAAAAATCATGCAACAAATAATATAAAGC ATAGGCCACTAGTGGATCTG

YOR1UF1	TTTCCTACAAATAGATCTGCTGTCC
YOR1UR1	GTCCACAATATATTCACCTGTAGGC
SNQ2-delF	AGTGGATAGAATAACACAGCTACCAAATACGTAAAGAGAATTCAC AGCTGAAGCTTCGTACGC
SNQ2-delR	AAAGGCAGATGAATGCACAAAATGTTAAGTTATCTGAAGCCCACA GCATAGGCCACTAGTGGATCTG
SNQ2UF1	CACCACTTTTTATGCTTGTATATGCT
SNQ2UR1	GTCCTGTCAGCATTTTTGTTAGTTT
PDR5-delF	CTTTTAAGTTTTTCGTATCCGCTCGTTCGAAAGACTTTAGACAAAAC AGCTGAAGCTTCGTACGC
PDR5-delR	GTCCATCTTGTAAGTTTCTTTTCTTAACCAAATTCAAAATTCTAGC ATAGGCCACTAGTGGATCTG
PDR5UF1	TTGAACGTAATCTGAGCAATACAAA

PDR5UR1	TACCTAAAACGACTAGCAATTCACC
PDR15-delF	ACACACACACACACAAGCAAACACACTTATAATTATCAAAAACCTC AGCTGAAGCTTCGTACGC
PDR15-delR	TATAATAAAAAGATAATATAACTAAAAAAAAGGAAAATAACGTCAGC ATAGGCCACTAGTGGATCTG
PDR15UF1	GAGGGAAAAGAATACTGCTACTGCT
PDR15UR1	GATGTTAGCGAAAGTTGACTGGTAT
KanD	GCGTTTCCCTGCTCGCAGGT

qPCR

GRE1F1	AAGTGCCACCACCAGTGCCG
GRE1R1	CCTCAACGCCAGCAGCCACA
HSP12F1	AAGGTGTCCACGACTCTGCCGA
HSP12R1	ACTTGGAAGTTGGCGGCTCCCA
HXT5F1	ACCTCCGATAGCGCAGCCGAT

HXT5R1	AGCACCCGTGCAAACGGGAC
ICTF1	TGCAATGCTGGCGCCTCTGA
ICTR1	GCTGCCTCGTCGATGGCGTT
PDR5F1	AGGGAACGCCCCCTCAAGGACT
PDR5R1	CCAGCTGCAGACGCGTTGGA
YHB1F1	GGCGCACAACCAAATGCC TTG G
YHB1R1	AGGCACGATGCTTGTGGCCA
ACT1F	ACCTTGGTGTCTTGGTCTACCGACG
ACT1R	TGTGTAAAGCCGGTTTTGCCGGT

Overexpression

YOR1F2	GGTACCGAGCTCTAAACAATGTCCATTACCGTGGGGGATGCAGTT
YOR1R2	TGCATGCTCGAGCTAATGGTGATGGTGATGATGACTTCTGTTCTCGA
	AATCATTTTCC
SNQ2F2	GGTACCGAGCTCTAAACAATGTCCAATATCAAAAGCACGCAAGAT
SNQ2R2	TGCATGCTCGAGCTAATGGTGATGGTGATGATGCTGCTTCTTTTTCCT
	TATGTTTT
PDR5F2	GGTACCGAGCTCTAAACAATGTCCGAGGCCAAGCTTAACAATAA
PDR5R2	TGCATGCTCGAGCTAATGGTGATGGTGATGATGTTTCTTGGAGAGTT
	TACCGTTC
PDR15F2	AATATTAAGCTTAACAATGTCCTCAGATATCAGAGACGTAGAGGAAC
PDR15R2	CGGCCCTCTAGACTAATGGTGATGGTGATGATGCTTCTTGGGTTTTT
	CGGAAATC

4.2.5 Plasmid construction and analyses of yeast strains overexpressing transporter genes

The gene specific primers and iProof High-Fidelity DNA Polymerase (Bio-Rad) were employed to amplify target genes by PCR with *S. cerevisiae* BY4741 genomic DNA as a template. DNA fragments were purified and cloned into an expression vector pYES2 (Table 4-2). The recombinant plasmids were prepared using QIAprep Miniprep Kit (Qiagen), transformed into *S. cerevisiae* mutants by electroporation [150], and followed by selection on Geneticin-containing minimal medium plates. To investigate effects of mutants expressing transporters on cell growth and alkane accumulation, cells harboring a recombinant plasmid were grown in an induction medium plus alkanes. Subsequently, optical density of cells at 600 nm (OD600) and intracellular alkanes were measured.

Table 4-2 Yeast strains and plasmids used in this study

Strains/plasmids	Description	Source
<u><i>S. cerevisiae</i> strains</u>		
BY4741	MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0	ATCC
BYL2K	snq2::kanMX derived from BY4741	This study
BYL25K	pdr5::kanMX derived from BYL2K	This study
BYL251K	yor1::kanMX derived from BYL25K	This study
BYL2515K	pdr15::kanMX derived from BYL25K	This study
<u>Plasmids</u>		
pYLYOR1	Ampr, YOR1 gene cloned into pYES2 at SacI	This study

	and XhoI sites	
pYLSNQ2	Ampr, SNQ2 gene cloned into pYES2 at SacI and XhoI sites	This study
pYLPDR5	Ampr, PDR5 gene cloned into pYES2 at SacI and XhoI sites	This study
pYLPDR15	Ampr, PDR15 gene cloned into pYES2 at HindIII and XbaI sites	This study

4.3 Results and discussion

4.3.1 Overexpression of transporters leads to improved tolerance to biofuel alkanes

Among the aforementioned mechanisms, we were interested in further identifying roles of plasma membrane efflux pumps in alkane tolerance and export because (i) plasma membrane efflux pumps are the first line of defense against drugs or organics, and (ii) microbial efflux pumps reportedly serve as a direct mechanism to improve biofuel tolerance [4,34-37] as well as productivity [4]. In fungi, efflux pumps, especially ATP-binding cassette (ABC) efflux pumps (e.g. Pdr5p and Snq2p in *S. cerevisiae*, Cdr1p and Cdr2p in *Candida albicans*), which contribute to multidrug efflux resistance, have been most extensively studied [25-28,38]. In our study, all the four efflux pumps induced by alkanes are ABC transporters, and considered as major pleiotropic drug transporters that comprise a PDR network in yeast [151]. Among them, Snq2p, Pdr5p, and

Pdr15p belong to a pleiotropic drug resistance (PDR) protein subfamily, and Yor1p belongs to a multidrug resistance-associated protein (MRP) subfamily. Their protein architecture contains multiple transmembrane domains (TMDs) that bind chemical compound and provide a translocation channel, and nucleotide-binding domains (NBDs) that couple ATP hydrolysis to substrate transport. Given that the induced ABC transporters play roles in reducing intracellular alkane accumulation, tolerance of *S. cerevisiae* cells against alkanes could be improved. Hence, we formed a hypothesis that the significantly induced transporters might also help cells to reduce the intracellular alkane accumulation and thus, provide protection to cells against alkanes.

To identify roles of each of the induced transporters in reducing intracellular alkane levels and improving alkane tolerance levels, we constructed triple knock-out mutants of transporters, BYL251K (*yor1* Δ *snq2* Δ *pdr5* Δ) and BYL2515K (*snq2* Δ *pdr5* Δ *pdr15* Δ). Subsequently, we cloned the four transporter genes (YOR1, SNQ2, PDR5, and PDR15) into plasmid pYES2 respectively, and transformed recombinant plasmids into the triple mutants. Under control of the galactose-inducible promoter PGAL1 and exposure to alkanes, the aforementioned transporter genes (YOR1, SNQ2, and PDR5) were expressed in the triple mutant BYL251K respectively, and PDR15 was expressed in the triple mutant BYL2515K. Cell tolerance and intracellular alkane levels were then analyzed (Figure 4-1). Figure 4-1A shows that there was similar growth among the BYL251K cells with pYES2 (control) and with SNQ2 or PDR5 in an alkane-free induction medium. Upon exposure to C10, cell growth of the triple mutant BYL251K

expressing each of Snq2p and Pdr5p was significantly enhanced, compared to BYL251K with the plasmid pYES2 (Figure 4-1B). This result suggested that expression of Snq2p and Pdr5p, respectively, increased cellular tolerance to C10. Furthermore, we tested growth of cells expressing Pdr5p and Snq2, respectively, under 5% C11. In support of our hypothesis, the triple knock-out mutant BYL251K expressing the transporter Snq2p or Pdr5p showed much higher cell density than the control cells, even though C11 did not induce expression of those transporters according to our microarray data. This result suggested that tolerance to C11 was significantly improved by the transporters Snq2p and Pdr5p. Note that the Resistance-Nodulation-Division (RND) efflux pumps such as AcrAB-TolC pump, Mex pumps, Ttg pumps from Gram-negative bacteria have been previously studied as an effort to improve tolerance to biofuels in bacteria [52-56]. However, the RND pumps utilize proton or sodium gradients as a source of energy [49] instead of ATP by ABC transporters. This suggests that yeast and gram-negative bacteria employ different types of efflux pumps for improving the biofuel tolerance.

Figure 4-1 Growth of triple knock-out mutants expressing transporters under alkane exposure. The triple mutant BYL251K (*yor1* Δ *snq2* Δ *pdr5* Δ) expressing Snq2p and Pdr5p, respectively, were grown in induction media without alkanes (A) or with 2% C10 (dashed lines) or 5% C11 (solid lines) (B). OD600 values represent cell density.

4.3.2 Transporter SNQ2 and PDR5 contribute to alkane efflux pumping

As shown in Figure 4-1B, expression of Snq2p and Pdr5p, respectively, increased cellular tolerance to C10 and C11. To look into the possible reasons for higher alkane tolerance, intracellular alkane level was determined. Figure 4-2 shows that upon 24 h exposure to C10, intracellular C10 amount was lowered by 25% and 33% in the triple mutant BYL251K expressing either Snq2p or Pdr5p. This result indicated a crucial role of Pdr5p and Snq2p in reducing the intracellular C10 levels, consistent with the hypothesis on the role of transporters in improving alkane tolerance as mentioned above. Furthermore, we tested intracellular alkane of cells expressing Pdr5p and Snq2, respectively, under 5% C11. Figure 4-2 shows that intracellular C11 levels were lowered by 87.4% and 94.4% in the triple mutant BYL251K expressing Snq2p and Pdr5p, respectively. This result indicated a crucial role of Pdr5p and Snq2p in reducing the intracellular C11 levels, consistent with the aforementioned hypothesis on the role of transporters in improving the tolerance to C11. GC peaks were shown in Figure 4-3.

However, even though the transporter genes (*YOR1*, *SNQ2*, *PDR5*, and *PDR15*) were all induced by both C9 and C10 according to our microarray

data, there was no improvement in tolerance against C9 by complementation of each of the four transporters. In addition, there was no improvement in tolerance against C10 and C11 by complementation of Yor1p or Pdr15p. This outcome was probably due to variance in substrate binding efficacy of various transporters, and/or the involvement of such mechanisms as stress response and membrane modification, in addition to transporters, in cellular tolerance to C9. Engineering approaches such as site-mutation, evolutionary engineering, and gene shuffling can be employed to improve substrate binding efficacy [51, 132, 152]. In addition, future studies could also include use of other cellular mechanisms besides transporters as an effort to improve alkane tolerance.

Figure 4-2 Intracellular alkane levels of triple knock-out mutants expressing transporters under alkane exposure. The triple mutant BYL251K (*yor1Δ snq2Δ pdr5Δ*) with empty plasmid (control), expressing Snq2p or Pdr5p, respectively, were grown in induction media with 2% C10 or 5% C11. After 24 h exposure, intracellular C10 and C11 levels were measured by GC and normalized to the total protein amount

and the internal standard C12. Alkane amount in control cells was set as 1. Error bars represent standard derivations of at least three biological replicates. *, statistically significant difference (Student t-test, $p < 0.05$) compared to cells with pYES2.

Figure 4-3 GC chromatograms of C10 and C11 from *S. cerevisiae* BYL251K cells. IS, internal standard. Peaks were indicated by arrows.

4.4 Conclusions

To our knowledge, this is the first report providing the evidence of the direct linkage between alkane tolerance and indigenous transporters, and of being able to develop alkane-tolerant *S. cerevisiae* by directly harnessing indigenous transporters. Since transporters could possibly aid in alkane export, thereby reducing the cytotoxicity, we formed the hypothesis that those induced transporters could contribute to alkane tolerance. In support of this hypothesis, we demonstrated the roles of the transporters Snq2p and Pdr5p in reducing the intracellular levels of C10 and C11, and enhancing the tolerance levels against C10 and C11. This result provided the evidence that Snq2p and Pdr5p were associated with alkane export and tolerance in *S. cerevisiae*. We envision that the membrane transporter engineering strategy established in this study can readily be extended to develop robust microbial hosts for alkane biofuels production. At the same time, other than the indigenous transporters in *S. cerevisiae*, transporters from other microbes could also be introduced to improve tolerance against biofuel alkanes, which was described in **Chapter 5**.

CHAPTER 5

IDENTIFICATION OF TRANSPORTERS FROM

***YARROWIA LIPOLYTICA* INVOLVED IN**

ALKANE TOLERANCE

5.1 Introduction

In **Chapters 3**, we hypothesized multiple molecular mechanisms underlying interactions between alkanes and *S. cerevisiae* through transcriptome analyses. Based on the hypothesized mechanisms, in **Chapter 4**, we demonstrated the involvement of indigenous ATP-binding cassette (ABC) transporters in alkane tolerance via transporter efflux pumping. These findings inspired us that in addition to indigenous transporters, heterologous ABC transporters from other strains may have the potential to endorse higher alkane tolerance in *S. cerevisiae*. Hence, here we were interested in identification and engineering heterologous transporters for improved alkane tolerance.

To this end, we considered ABC transporters from *Y. lipolytica*, an oleaginous yeast that efficiently assimilates and utilizes hydrophobic substrates such as alkanes, fatty acids and lipids [153-155]. Further, the mutants lacking part of ABC transporters show defective phenotypes for alkane utilization as a carbon source [156, 157]. Consequently, in this study, we attempted to improve the tolerance of *S. cerevisiae* against alkanes using *Y. lipolytica* ABC transporters. Notably, we demonstrated that ABC2 and ABC3 transporters maintained 5- and 30-fold lower intracellular decane and undecane levels respectively, and significantly improved tolerance in *S. cerevisiae*, evidenced by over 80-fold increase in the tolerance limit against decane.

5.2 Materials and methods

5.2.1 Strains and media

All cells involved in cloning experiments were *E. coli* TOP10 (Invitrogen) unless otherwise stated. Luria-Bertani (BD) was used as the medium for cloning studies unless otherwise stated. Ampicillin (100 µg/mL) was added to the culture media for antibiotic selection where appropriate.

The yeast strains *S. cerevisiae* BY4741 (ATCC 201388) and *Y. lipolytica* CLIB122 (CIRM) were used for function characterization. *S. cerevisiae* BY4741 was cultured in rich medium (YPD), synthetic minimal medium lacking uracil (SC-U) or induction medium. YPD medium (1% yeast extract, 2% peptone and 2% D-glucose) was used to routinely maintain wild type strain. SC-U medium (0.67% yeast nitrogen base, 0.192% uracil dropout and 2% raffinose) was used for growing pYES2 transformants. Induction medium (0.67% yeast nitrogen base, 0.192% uracil dropout, 1% raffinose and 2% galactose) was used for protein induction in *S. cerevisiae* cells. Medium containing 0.67% yeast nitrogen base supplemented with 0.5% casein hydrolysate and 2% glucose was used for growth of *Y. lipolytica* for qRT-PCR sample preparation. Solid media were supplemented with 2% agar. Yeast growth media components were purchased from Sigma-Aldrich.

Alkanes (octane (C8), nonane (C9), decane (C10), undecane (C11) and dodecane (C12)) purchased from Sigma-Aldrich were added to culture media for protein function analysis where appropriate.

5.2.2 Plasmid construction

A list of the oligonucleotides used is shown in Table 5-1. Plasmid pYES2 (Invitrogen) with the GAL1 promoter was used as an expression vector.

To clone 6x His-tagged ABC2, genomic DNA of *Y. lipolytica* CLIB122 was used as a PCR template with two pairs of primers ABC2-F1/ABC2-R1, and ABC2-F2/ABC2-R2. The two PCR products were combined through the Splicing Overlap Extension (SOE) method [158] using flanking primers ABC2-F1 and ABC2-R2. The resulting DNA fragment was digested with HindIII and NotI and cloned into pYES2 cut with the same restriction enzymes, creating pYES2ABC2. Plasmid pYES2ABC3 was constructed as for pYES2ABC2. Site-directed mutagenesis of the transporters ABC2-E988Q, ABC2-H1020A, ABC3-E989Q and ABC3-H1021A were constructed by mutating glutamate to glutamine and histidine to alanine respectively.

Plasmid pYES2ABC2-EGFP, which encodes yeast enhanced green fluorescent protein (EGFP) at the C-terminus of the ABC2 open reading frame, was constructed as follows. We used pYES2ABC2 as a PCR template with primer set ABC2-F1 and ABC2-EGFP-R2. The resulting DNA fragment was digested with HindIII and NotI and cloned into pYES2 cut with the same restriction enzymes, creating pYES2ABC2-1. EGFP was amplified from pKT127 (Euroscarf) [159] using primer sets EGFP-F and EGFP-R, digested with NotI and SphI and inserted into the same restriction sites of pYES2ABC2-1 to create pYES2ABC2-EGFP. Plasmid pYES2ABC3-EGFP was constructed as for pYES2ABC2-EGFP. For construction of pYES2EGFP, EGFP was amplified by PCR from pKT127 using primers EGFP-control-F and EGFP-R, digested with NotI and SphI

and cloned into pYES2. All restriction and ligation enzymes were purchased from New England Biolabs (NEB).

Table 5-1 Primers used in this study. Restriction sites are bold.

Primer name	Primer sequence from 5' to 3'
ABC2-F1	ACCCAAGCTTTAAAAAATGTCTCAAACACCTCCAGACTACACTGGTCTGG
ABC2-R1	CATCTCGGGGGACAC/ CCTGATAATTCCATT
ABC2-F2	AATGGAATTATCAGG/ GTGTCCCCCGAGATG
ABC2-R2	ATAAGAATGCGGCCGCCTAATGGTGATGGTGATGATGAGCCTTCTGAGCCTTGACTTCTTG
ABC3-F1	ACGGGGTACCTAAAAAATGTCTGATCCTGTACCGATCACGCAGG
ABC3-R1	GATTTTTCGGGGAATGC/ CCTGATAATTCCATTCA
ABC3-F2	TGAATGGAATTATCAGG/ GCATTCCCCGAAAAATC
ABC3-R2	ACATGCATGCCTAATGGTGATGGTGATGATGGGCCTTCTGCTTTTCGAGACTTTTTTG
ABC2-E988Q-R1	GACCGGAAGTAGGTTGATCCAGGAACAGC
ABC2-E988Q-F2	GCTGTTCTGGATCAACCTACTTCCGGTC
ABC2-H1020A-R1	GCAGAGGGCTGAGCAATGGTACACAGAATAGC
ABC2-H1020A-F2	GCTATTCTGTGTACCATTGCTCAGCCCTCTGC
ABC3-	GGAAGTGGGTTGATCAAGGAACAGC

E989Q-R1	
ABC3- E989Q-F2	GCTG TTCCTTGATCAACCCACTTCC
ABC3- H1021A- R1	GCAGAGGGCTGAGCAATAGTACACAG
ABC3- H1021A-F2	CTGTGTACTATTGCTCAGCCCTCTGC
ABC2- EGFP-R2	ATAAGAATGCGGCCGCGAGCCTTCTGAGCCTTGGACTTCTTG
ABC3- EGFP-R2	ACATGCATGCAACATGGATGCGGCCGCGGCCTTCTGCTTTTCGAGA CTTTTTGG
EGFP-F	ATAAGAATGCGGCCGCGATCGGTGACGGTGCTGGTTTAA
EGFP-R	ACATGCATGCTTAATGGTGATGGTGATGATGTTTGTACAATTCATC CATAACCATGGGTA
EGFP- control-F	ATAAGAATGCGGCCGCTAAAAAATGTCTAAAGGTGAAGAATTATTC ACTGGTGTTG
ABC1- qPCR-F	CGACGACAACAGCGTGAGGAACT
ABC1- qPCR-R	CCACTGAGACACGGCGAAATCAGT
ABC2- qPCR-F	CCTGTTGTGTGGGCTGAGTCTCCT
ABC2- qPCR-R	TCGCTCGGTAGTGTTCTGTCTGCAC
ABC3- qPCR-F	CGTGGCAGGCTTTCATGGGCT
ABC3-	AAGAGGGCTCCTCGTTCGCCA

qPCR-R	
ABC4- qPCR-F	GGCCACCAAAGAGCAGATGCCTG
ABC4- qPCR-R	ACGTTACCGGGGCTCGTCCAA
Actin-F	TCCAGGCCGTCCTCTCCC
Actin-R	GGCCAGCCATATCGAGTCGCA

5.2.3 Quantitative RT-PCR

Total RNA samples from *Y. lipolytica* CLIB122 cells, which were treated and untreated with alkane for 24 h, were prepared using RNeasy Mini Kit (Qiagen), followed by cDNA synthesis using H minus Reverse transcriptase kit (Fermentas). Quantitative RT-PCR analysis was performed on the Bio-Rad iQ5 real-time PCR detection system using SsoFast EvaGreen Supermix kit (Bio-Rad). The actin gene (YALI0D08272g) [160] was used as reference gene for *Y. lipolytica*. Relative mRNA levels were derived using comparative CT method. PCR primer sequences are listed in Table 5-1.

5.2.4 Western Blot Analysis

S. cerevisiae cells carrying the plasmids encoding the 6x His-tagged ABC2 and ABC3 were cultured in induction medium and harvested at OD₆₀₀=1-2 (early exponential phase). The protein extraction method here is based on

alkaline lysis [161] and glass bead lysis [162] methods. The following handling process was carried out in the cold room (~4°C). Cell pellets (around 14mg) were re-suspended in 300µl cold lysis buffer (0.1M NaOH, 2% β-mercaptoethanol, and protease inhibitor mixture (Roche Applied Science)). After 5 min, glass beads (425-600µm, Sigma) were added to the suspension. The cells were lysed by vortexing for 2 min. The lysate obtained was clarified by transferring supernatant into a new tube. Proteins in the lysate were fully dissolved by adding SDS (final concentration around 2%) and gently stirring for 10min. After centrifugation, the supernatant was mixed equally with Laemmli sample buffer (Bio-Rad) and separated on a SDS-polyacrylamide gel. The sample gels were used for blotting. Proteins were blotted onto a 0.2 µm nitrocellulose membrane (Bio-Rad) through Trans-Blot Turbo Blotting System (Bio-Rad). 6x His-tagged ABC2 and ABC3 were detected using anti-6x His-tag antibody (HRP) (ab1187, Abcam) and 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system (Sigma).

5.2.5 Fluorescence Microscopy

S. cerevisiae BY4741 cells carrying plasmid pYES2ABC2-EGFP or pYES2ABC3-EGFP were grown to the early exponential phase in induction medium, harvested and mounted on the poly-L-lysine-coated slide glass. EGFP fluorescence was analyzed with a fluorescent microscope (Zeiss Axio Scope A1).

5.2.6 Alkane susceptibility assays

Alkane susceptibility test on agar plate: Alkane susceptibility test on plates was performed according to the methods of Mauersberger et al. [155, 156]. Exponentially growing cells in induction medium were centrifuged and re-suspended until OD600 reaches 1. Ten microliter aliquots of successive 10-fold dilutions (non diluted, 10⁻¹, 10⁻², 10⁻³) of the cells were spotted onto the induction medium plate. Alkanes were supplied as vapour phase by placing 200µl alkane on a sterile filter paper in the lid of the petri dish. Plates were incubated at 28°C for 2 days.

Alkane susceptibility test in liquid culture: Overnight cultures were diluted into 5ml induction medium in 50ml glass bottle (Sigma) at an initial OD600 of 0.4. Alkanes were added at different final concentrations. Bottles were sealed tightly with butyl rubber stopper (Sigma) and silver aluminum seal (Sigma). Liquid culture was performed at 28°C with shaking. Growth was monitored by measuring the OD600 at different time points. Cell cultures used for time point OD600 checking were collected from the glass bottle using needles and syringes.

5.2.7 Determination of intracellular alkane levels

After induction for 48 h with or without addition of alkanes, *S. cerevisiae* cells transformed with pYES2, pYES2ABC2 and pYES2ABC3 were harvested at 6000 g for 5 min at 4 °C. After washing with 50 mM Tris.Cl, the cells were equally divided into two parts, one part for intracellular

alkane extraction and the other for determination of total protein content. For alkane extraction, cell pellets were re-suspended in freshly prepared HPLC grade Chloroform/Methanol (v/v, 2:1). Dodecane was added into cell suspension as an internal standard. Acid-washed glass beads were added until the suspension was covered. Cells were then lysed by mechanical agitation using FastPrep-24 (MPBio) for 6 min at 6 m/s. The crude extract was obtained by pipettes. After addition of autoclaved ddH₂O, the crude extract was emulsified for 10 min by inversion. After centrifugation, the crude extract was separated into two phases. The bottom phase containing alkane was transferred into a new 1.5 ml microcentrifuge tube and purified as above with chloroform and ddH₂O until particulate matter was no longer observed. The purified solution was transferred into a clear GC vial for GC analysis. To check the total protein content, the cell pellets were re-suspended into 50 mM Tris.Cl and lysed via mechanical agitation with acid-washed glass beads. Protein content of obtained crude extract was determined using the Bradford protein assay (Bio-Rad). Intracellular alkane levels were normalized to internal standard and cell lysate protein content [163, 164].

5.3 Results and discussion

5.3.1 Transcription activity of ABC transporters with alkanes

To assess whether ABC1, ABC2, ABC3 and/or ABC4 transporters were involved in alkane transport in *Y. lipolytica*, we analysed the effects of alkanes with different chain lengths (C8-C12) on the transcription levels of

these four ABC transporter genes using quantitative RT-PCR. The reason that C8 to C12 alkanes were tested in this study was that longer alkanes showed no toxicity toward *S. cerevisiae* (data not shown). Compared with control samples without alkane treatment, the transcription levels of ABC1 and ABC4 showed no change when the cells were treated with different alkanes (C8-C12) (Figure 5-1). However, the mRNA levels of ABC2 were significantly increased when *Y. lipolytica* was treated with octane (C8), nonane (C9), decane (C10) and undecane (C11) ($p < 0.05$), while the mRNA levels of ABC3 were significantly increased toward nonane (C9) and decane (C10) ($p < 0.05$) (Figure 5-1). These results strongly suggested that two of the ABC transporters, ABC2 and ABC3, might play a critical role in the transport of alkanes for C8, C9, C10 and C11 alkanes. Thus, based on the qRT-PCR results, ABC2 and ABC3 were chosen for further analysis of their alkane transport capability.

Our alkane susceptibility assays revealed that C12 or longer alkanes exhibited little or no toxicity toward *S. cerevisiae* (data not shown), while C8-C11 alkanes were toxic. This result suggests that longer chain length alkanes have lower cell toxicity toward *S. cerevisiae*, in agreement with the previous study by Gill and Ratledge (1972) [141] demonstrating that the toxicity of alkanes is related to their chain length. The proposed mechanisms for this phenomenon are as follows. First, higher chain lengths lead to increasing molecular weight. The higher the molecular weight is, the harder it is for the compound to penetrate yeast cell membrane. Second, a previous study [140] suggested that a compound whose log Pow is between 1.5 and 6.0 is considered extremely toxic to

microorganisms and other living cells, such as nonane (log Pow = 5.5) and decane (log Pow = 6.0). Higher chain length alkanes have higher log Pow value. For example, C12 alkane has log Pow of 7.0 and alkanes with chain length above 12 have higher log Pow. This reported correlation between toxicity and log Pow is in line with our observation that alkanes with longer chain length (C12 or longer) show insignificant toxicity toward *S. cerevisiae*. Since longer alkanes showed insignificant toxicity toward *S. cerevisiae*, this study was focused on improving the tolerance of *S. cerevisiae* against alkanes with C12 and below.

Figure 5-1 mRNA transcript levels of ABC1–4 genes in *Y. lipolytica*. Quantitative RT-PCR analyses of ABC1, ABC2, ABC3 and ABC4 in *Y. lipolytica* cells upon treatment with octane, nonane, decane, undecane and dodecane (C8-C12). Each value of qRT-PCR was normalized to β -actin expression levels and expressed as the fold change relative to the levels detected in control samples, which were cells without alkane treatment and set equal to 1. Error bars represent the standard deviation (SD) of three biological replicates. Primers for qRT-PCR are listed in Table 5-1.

5.3.2 Expression and subcellular localization of ABC2 and ABC3

To confirm the expression of these two transporters in *S. cerevisiae*, a 6x His tag was attached to the C terminus of ABC2 and ABC3. Through immunodetection of 6x His-tagged proteins, specific bands were assigned to ABC2 (165 kDa) and ABC3 (167 kDa) (Figure 5-2A). This western blot result confirmed the expression of ABC2 and ABC3 in *S. cerevisiae*. Next, to further analyse the localization of ABC2 and ABC3, each of them was tagged with EGFP at its C terminus. As shown in Figure 5-2B, strong fluorescence was observed on the plasma membrane of cells containing ABC2-EGFP and ABC3-EGFP fusion proteins, respectively. These results suggest that ABC2 and ABC3 were expressed and located on the plasma membrane of *S. cerevisiae*.

To investigate whether EGFP would affect the localization of the transporters, the functionality of ABC2- and ABC3-EGFP was compared with that of ABC2 and ABC3. This functionality was determined by the viability of the cells in the presence of alkanes. The growth patterns of strains expressing ABC2/3-EGFP fusion protein and ABC2/3 were similar (data not shown). These data indicate that EGFP fusion proteins function similarly as the untagged proteins. Thus, EGFP tag does not affect the localization of the transporter proteins.

Figure 5-2 Expression and subcellular localization of ABC2 and ABC3. (A) Expression of ABC2 and ABC3. The expression of ABC2 and ABC3 carrying 6xHis tag was confirmed by western blot analysis. The positions of molecular mass markers are indicated at left. Western blot analyses were performed as described under “Methods”. (B) Subcellular localization of ABC2 and ABC3 determined by fluorescence microscopy. Yeast cells carrying plasmids encoding ABC2+EGFP or ABC3+EGFP fusion proteins were cultured and harvested. Phase contrast figures and fluorescence figures are shown. The scale bar represents 5 microns.

5.3.3 ABC2 and ABC3 contribute to increasing alkane tolerance

After confirming that ABC2 and ABC3 were expressed on the cell membrane, we conducted susceptibility assays to study the effect of ABC2 and ABC3 on the tolerance of the cells toward alkanes. The toxic effects of alkanes on *S. cerevisiae* with ABC2 and ABC3 were measured through alkane susceptibility assays on agar plates. Figure 5-3A shows that octane, nonane, decane and undecane were toxic to *S. cerevisiae* cells. However, in cells expressing ABC2 and ABC3, cellular tolerance toward decane and undecane was considerably improved. It was observed that the expression of ABC2 led to higher tolerance toward decane than ABC3. Note that ABC1 and ABC4 expression led to no tolerance improvement (Figure 5-4).

Therefore, the results above suggest that ABC2 and ABC3 successfully improved alkane tolerance in *S. cerevisiae*.

Figure 5-3 Alkane susceptibility assay for *S. cerevisiae*. Alkane susceptibility assays were performed in *S. cerevisiae* cells expressing ABC2, ABC3 or with an empty plasmid. (A) Alkane susceptibility assay on agar plates. Serial 10-fold dilutions

(from left to right: non diluted, 10⁻¹, 10⁻², 10⁻³) of cells were spotted on agar plates with alkanes (octane, nonane, decane, undecane and dodecane) as vapor phase. Plates were incubated at 28°C for 2 days. (B) Alkane susceptibility assay in liquid culture. Overnight cell culture was diluted into induction medium (final OD₆₀₀=0.4) with alkanes (decane or undecane). The cell culture was incubated for 48 h at 28°C. The OD₆₀₀ value of each sample was determined and plotted against its corresponding alkane concentration (0.1%, 0.25%, 0.5%, 0.75%, 1%, 2%, 5%, 10% and 20% vol/vol). Each point represents the mean of three biological replicates; standard deviations are presented. Arrows indicate increased cell tolerance towards alkanes. (C) Growth file of cells in alkane-inhibiting conditions (0.5% (vol/vol) decane or 20% (vol/vol) undecane). Symbols for strains are: control sample with empty plasmid (open circle), cells expressing ABC2 (filled circle), cell expressing ABC3 (filled triangle).

Figure 5-4 Alkane susceptibility assay on agar plates. Serial 10-fold dilutions (from left to right: non diluted, 10⁻¹, 10⁻², 10⁻³) of cells were spotted on agar plates with alkanes (octane, nonane, decane, undecane and dodecane) as vapor phase.

To further examine quantitative effects of ABC2 and ABC3 toward decane and undecane, alkane susceptibility tests in liquid culture were conducted. As shown in Figure 5-3, the tolerance limit of *S. cerevisiae* was below 0.25% and 10% for decane and undecane, respectively. With ABC2 transporter,

the tolerance limit was increased to over 20% for both decane and undecane. With ABC3 transporter, the tolerance limit of *S. cerevisiae* was improved to over 0.75% and 20% for decane and undecane, respectively. This result suggests that the expression of ABC2 and ABC3 transporters significantly improved the tolerance toward decane and undecane.

To study the time-course behavior of ABC2- and ABC3-expressing *S. cerevisiae* cells in the presence of 0.5% decane and 20% undecane, which caused no growth in the wild-type cells, the growth patterns were studied. As shown in Figure 5-3C, while *S. cerevisiae* cells without the transporters exhibited no growth, the cells with ABC2 and ABC3 transporters grew normally after initial growth delays. ABC3 expressing cells underwent a longer growth delay under both alkane treatments, but exhibited a slightly higher growth rate with decane treatment. Note that *S. cerevisiae* cells expressing ABC2 and ABC3 transporters exhibited similar growth rates in the absence of alkanes (Figure 5-3C, Table 5-2).

Table 5-2 Specific growth rates under C10 and C11 alkane treatments

		$\mu(h^{-1})$
w/o alkane treatment	empty plasmid	0.189±0.008
	ABC2 expressing	0.173±0.019
	ABC3 expressing	0.164±0.011
with 0.5% C10 treatment	empty plasmid	0
	ABC2 expressing	0.143±0.002
	ABC3 expressing	0.168±0.009
with 20% C11 treatment	empty plasmid	0

	ABC2 expressing	0.204±0.021
	ABC3 expressing	0.178±0.015

ABC2 and ABC3 were selected as candidates based on their potentials toward alkane efflux pumping. Although both ABC2 and ABC3 expressing cells showed increased C10 and C11 alkane tolerance, ABC2 exhibited better performance than ABC3, suggesting that ABC2 is a more effective pump for alkane transport. Thus, we looked into the possible reasons for the difference in efficiency between these two transporters. One plausible reason is the difference in kinetics of substrate–protein interactions and/or nucleotide–protein interactions between ABC2 and ABC3. For example, Low et al. (2010) [165] found that difference in TMD residues can lead to differences in ABC transporter efficiency. It was hypothesized that the TMD residues might affect substrate affinity, resulting in differences in substrate–protein kinetics. Another reason for the different efflux efficiencies may be post-translational modification patterns. Protein phosphorylation was found to be essential for the stability of the yeast multidrug transporter Pdr5p [166]. As hypothesized before, transporters, such as Pdh1p that requires more phosphorylations than Cdr1p, may take longer time to reach its active, substrate-effluxing form [167]. Thus, similar to Pdh1p, it is possible that ABC3 requires longer phosphorylation time to arrive at its active alkane-effluxing form.

Besides the difference in efflux pumping efficiencies of ABC2 and ABC3, we also observed that ABC2 and ABC3 did not increase tolerance toward shorter chain alkanes such as C8 and C9. Future work therefore may

include directed evolution of these transporters to broaden their substrate specificity, mainly toward shorter chain alkanes. For example, random mutagenesis was shown to affect substrate specificity of ABC transporters [168]. Also, protein structure studies may be needed for better understanding of the exact substrate binding and transport mechanism.

5.3.4 ABC2 and ABC3-mediated efflux pumping of alkanes

The results on agar plates and in liquid medium showed that both ABC2 and ABC3 transporters greatly enhanced tolerance toward decane and undecane, with ABC2 leading to stronger tolerance. We therefore hypothesized that the tolerance generated by ABC2 and ABC3 was linked to the efflux pumping of decane and undecane. Alkane efflux pumping might help to maintain an intracellular alkane concentration under a toxic threshold.

To verify this hypothesis, intracellular alkane accumulation was analysed after 48 h incubation with 0.5% decane or 20% undecane. As shown in Figure 5-5, cells expressing ABC2 and ABC3 had ~5-fold lower intracellular decane level relative to the control. Further, ABC2 and ABC3 transporters were shown to reduce the intracellular undecane level approximately 30-fold compared to the control. The sharp decrease in intracellular alkane levels strongly suggests that ABC2 and ABC3 function as decane and undecane efflux pumps.

Figure 5-5 Intracellular alkane levels of ABC2 and ABC3. *S. cerevisiae* BY4741 with and without ABC2/3 were cultured under exposure to 0.5% (vol/vol) alkane or 20% (vol/vol) undecane. After 48 h incubation, intracellular alkane levels were measured as described under “Methods”. Intracellular alkane levels were normalized to that of control cells carrying an empty plasmid. Data shown are the mean $\pm$ SD of four biological replicates.

5.3.5 Glutamate is required for energy-dependent efflux pumping of ABC2 and ABC3

The main characteristic of ABC transporters, which differs from that of other transporters is that ABC transporters utilize the energy of ATP hydrolysis to carry out biological processes. The ATP hydrolysis model is essential for ABC transporters. Thus, to better understand ABC2 and ABC3’s mechanisms in alkane export, we looked into the possible ATP hydrolysis models for both transporters. Two different models of ATP hydrolysis mechanisms were proposed for ABC transporters before: the “catalytic carboxylate” model [169] and the “catalytic dyad” model [170].

According to the “catalytic carboxylate” model, the highly conserved glutamate residue at the C terminus of the Walker B motif is essential for ATP hydrolysis. However, in the “catalytic dyad” model, interactions between glutamate of the Walker B motif and the histidine of the H-loop are a prerequisite for ATP hydrolysis.

Protein analyses revealed four possible locations of characteristic domains in ABC transporters, namely two nucleotide-binding domains (NBD1 & 2) and two transmembrane domains (TMD1 & 2) [171, 172]. Sequence alignment of ABC2, ABC3, PDR5 and PDR15 showed that these proteins have high similarities in NBD domains which include Walker A motif, Walker B motif, C-loop and H-loop (Figure 5-6A). Similar to the widely studied PDR5 model [171, 172], critical amino acids such as glutamate in C-terminus of Walker B motif and histidine of H-loop are only present in NBD2 but not in NBD1 for ABC2 and ABC3 [173].

Hence, to determine the ATP hydrolysis mechanism of ABC2 and ABC3, the glutamate (E988 for ABC2 and E989 for ABC3) and the histidine (H1020 for ABC2 and H1021 for ABC3) in NBD2 of ABC2 and ABC3 were mutated to glutamine and alanine, respectively (Figure 5-7). As shown in Figure 5-6B, ABC2-E988Q and ABC3-E989Q mutants were highly sensitive against both decane and undecane, while ABC2-H1020A and ABC3-H1021A mutants still showed increased tolerance against undecane and decreased resistance against decane. Therefore, histidine is deemed not as essential as glutamate for these transporters' activity, and ATP is most likely to be hydrolyzed by the catalytic carboxylate mechanism.

Figure 5-6 Transporter sequence comparison and alkane susceptibility assay of ABC2 and ABC3 mutants. (A) Multiple sequence alignment of NBD2 of ABC transporters. ABC2 [GenBank: CAG82364]; ABC3 [GenBank: CAG82646]; PDR5 [GenBank: CAA99359]; and PDR15 [GenBank: AAB64846]. Starting amino acid numbers are shown in each line. Sequences were aligned in ClustalW, and the shading was created by Mobyle Pasteur Boxshade. Single letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (B) Alkane susceptibility assay with ABC2 and ABC3 mutants. Susceptibility assays were performed as described under “Methods”.

Figure 5-7 Topology model of ABC2 and ABC3. To generate a topology model of ABC2 (A) and ABC3 (B), the TMHMMfix algorithm was used (www.sbc.su.se/melen/TMHMMfix). The resulting model was visualized by the TOPO2 transmembrane protein display software (www.sacs.ecsf.edi/TOPO2). The Walker A motifs are shown in blue, the Walker B motifs are shown in green, and the C-loop are shown in orange. Residues that were mutated in this study are shown in red.

5.4 Conclusions

In this study, we demonstrated that transporter engineering through expression of heterologous transporters led to improved tolerance against alkanes in *S. cerevisiae*, a key industrial microbial host. In particular, we showed that the tolerance limit of *S. cerevisiae* was increased over 80-fold against decane. Further, we provided the evidence that the improved

tolerance was primarily due to the lowering of the intracellular alkane level wherein ABC2 and ABC3 function as an efflux pump. To our knowledge, this is the first study to harness a heterologous transporter engineering strategy for alkane tolerance improvement in eukaryotic host cells, which is readily applicable to alkane biofuel-producing microbes. We believe that our work here laid the groundwork for developing robust alkane-producing yeast cells through transporter engineering, which will greatly aid in next-generation alkane biofuel production and recovery.

CHAPTER 6

**CONCLUSIONS AND FUTURE
CONSIDERATIONS**

6.1 Conclusions

The development of renewable biofuels such as bio-ethanol [174], butanol [13], bio-diesel [175-177] and jetfuels [178] helps to address energy security and climate change concerns. Recently, the biological production of hydrocarbon alkanes, components of major fuels, has drawn much attention because alkanes have high energy content and are compatible with existing transportation infrastructure.

Efficient production of biochemicals, including biofuels, using engineered microbes as whole-cell biocatalysts requires robust cell viability. High viability leads to high productivity and improved bioprocesses by allowing repeated cell recycling. However, our results indicated that medium chain alkanes (C8-C11) affected cell viability, which can be a main bottleneck for eventual high alkane production yields. Thus, to uncover the alkane toxicity mechanisms, in **Chapter 3**, we performed transcriptome analyses. Based on our transcriptome analyses and the functions and physiological roles of the regulated genes, we hypothesized that *S. cerevisiae* responded to alkanes stresses through integrative mechanisms. First, efflux pump genes (ABC transporter in yeast cells) - YOR1, SNQ2, PDR5, and PDR1, are induced to export intracellular alkanes to reduce alkane toxicity. Second, alkanes exposure generates radicals and induces stress-responsive genes towards radical detoxification. Alkane molecules penetrate cell wall, disperse into cytoplasmic membrane, and generate radicals, while stress-responsive genes (GRE1, GRE2, CTT1, HSP12, TSA2, and YHB1) are induced to detoxify radicals including ROS. Third, more energy sources are generated through acceleration of glucose and fatty acid metabolism.

Hexose transport genes (HXT5, HXT6, and HXT7) are induced to increase glucose import. Thereafter, energy sources are enriched through enhanced glucose metabolism involving pentose phosphate pathway (GND2), β -oxidation of fatty acids (FAA4, and POT1), and ALD3-involved pathways. ATP may be used for alkane export by transporters. Fourth, the increase in glucose import and consumption aids in modifying membrane components, thereby helping cells to adapt to alkanes exposure. Enriched glucose inside the cells may lead to the synthesis of more fatty acids, lipids, and derivatives, contributed to by such induced genes as FAS1, FAS2, ACC1, ICT1, OLE1, CHO1, PSD1, INO1, ITR1, and OPI3. Further, ergosterol biosynthesis is enhanced by the four induced genes HMG1, EGR3, ERG6, and ERG25. Such a change in the expression of genes involved in lipid and ergosterols metabolism may lead to membrane modification, possibly facilitating adaptation to alkane exposure.

Among the aforementioned mechanisms, in **Chapter 4**, we further identified roles of plasma membrane transporters in alkane tolerance because microbial transporters reportedly serve as a direct mechanism to improve biofuel tolerance. According to the microarray data, four potential transporters - YOR1, SNQ2, PDR5, and PDR15 were tested for alkane tolerance. Among them, the expression of Snq2p and Pdr5p respectively increased cellular tolerance to both C10 and C11 alkanes. Further, upon exposure to C10 alkanes, intracellular alkane amount was lowered by 25% and 33% in cells expressing Snq2p and Pdr5p respectively, while under exposure to 5% C11, intracellular C11 levels were lowered by 87.4% and 94.4% in cells expressing Snq2p and Pdr5p, respectively. We

demonstrated the roles of the transporters Snq2p and Pdr5p in reducing intracellular levels of C10 and C11 alkanes, as well as enhancing tolerance levels against C10 and C11 alkanes. In summary of **Chapter 3** and **Chapter 4**, we investigated the cellular mechanisms of *S. cerevisiae* response to alkane biofuels at a systems level through transcriptome analyses and based on these mechanisms, we identified transporters involved in alkane tolerance in *S. cerevisiae*.

To improve alkane tolerance by harnessing heterologous transporters, in **Chapter 5**, we attempted to exploit ABC transporters from *Y. lipolytica* based on the observation that *Y. lipolytica* utilizes alkanes as a carbon source. We first confirmed the increased transcription of ABC2 and ABC3 transporters upon exposure to a range of alkanes in *Y. lipolytica*. Similarly to the native transporter characterization, we then showed that the heterologous expression of ABC2 and ABC3 transporters significantly increased tolerance against C10 and C11 alkanes in *S. cerevisiae* through maintaining lower intracellular level. In particular, ABC2 transporter increased the tolerance limit of *S. cerevisiae* over 80-fold against C10. We demonstrated that transporter engineering through expression of heterologous transporters led to significantly improved tolerance against alkane biofuels in *S. cerevisiae*.

Overall, in this study, we elucidated genetic mechanisms underlying cellular response to alkane, and based on these mechanisms, we improved alkane tolerance by harnessing either indigenous or heterologous ABC transporters. We believe that our results here provide valuable insights into designing microbial engineering strategies (transcriptome analysis and

transporters engineering) for developing robust alkane-producing yeast cells, which will greatly aid in next-generation alkane biofuel production and recovery.

6.2 Future considerations

Considering the importance of C8 to C11 alkanes as drop-in biofuels and the direct effect of transporters to improve cell tolerance toward biofuels, in this study, we mainly focused on the characterization of potential transporters. As presented in **Chapter 4** and **Chapter 5**, four identified transporters, PDR5 and SNQ2 from *S. cerevisiae*, and ABC2 and ABC3 from *Y. lipolytica*, effectively reduced intracellular C10 and C11 alkanes, resulting in increased cell viability. However, these identified transporters showed no effects towards C8 and C9 alkanes which are regarded as important gasoline alternatives but pose high cellular toxicity. Therefore, the following future works may be considered:

- 1) Directed evolution can be performed for the characterized transporters in order to broaden their substrate specificity to shorter chain alkanes such as C8 and C9 alkanes. For example, random mutagenesis was shown to change substrate specificity of ABC transporters [168]. Also, protein structure studies may be needed for better understanding of the substrate binding and transport mechanism.
- 2) Toward the aim of improving alkane tolerance in yeast, other than heterologous transporter engineering, native strain engineering strategies

including mutagenesis, adaptive evolutionary engineering, genome shuffling and genomic library [29] can also be utilized. These native engineering strategies can further be combined with heterologous transporter engineering to achieve a synergistic effect on alkane tolerance improvement.

BIBLIOGRAPHY

1. Harger M, Zheng L, Moon A, Ager C, An JH, Choe C, Lai Y-L, Mo B, Zong D, Smith MD *et al*: **Expanding the Product Profile of a Microbial Alkane Biosynthetic Pathway**. *ACS Synthetic Biology* 2012;120926103747002.
2. Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB: **Microbial biosynthesis of alkanes**. *Science* 2010, **329**(5991):559-562.
3. Bernard A, Domergue F, Pascal S, Jetter R, Renne C, Faure J-D, Haslam RP, Napier JA, Lessire R, Joubès J: **Reconstitution of Plant Alkane Biosynthesis in Yeast Demonstrates That Arabidopsis ECERIFERUM1 and ECERIFERUM3 Are Core Components of a Very-Long-Chain Alkane Synthesis Complex**. *The Plant Cell Online* 2012.
4. Gong P-F, Xu J-H: **Bio-resolution of a chiral epoxide using whole cells of *Bacillus megaterium* ECU1001 in a biphasic system**. *Enzyme and Microbial Technology* 2005, **36**(2-3):252-257.
5. Klibanov AM: **Improving enzymes by using them in organic solvents**. *Nature* 2001, **409**(6817):241-246.
6. Inoue A, Horikoshi K: **A *Pseudomonas* thrives in high concentrations of toluene**. *Nature* 1989, **338**(6212):264-266.
7. Sun X, Dennis JJ: **A Novel Insertion Sequence Derepresses Efflux Pump Expression and Preadapts *Pseudomonas putida* S12 for Extreme Solvent Stress**. *Journal of Bacteriology* 2009, **191**(21):6773-6777.
8. Tatham MH, Matic I, Mann M, Hay RT: **Comparative Proteomic Analysis Identifies a Role for SUMO in Protein Quality Control**. *Sci Signal* 2011, **4**(178):rs4-.
9. Ding C, Cantor CR: **A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS**. *Proceedings of the National Academy of Sciences* 2003, **100**(6):3059-3064.
10. Ooi SL, Shoemaker DD, Boeke JD: **A DNA Microarray-Based Genetic Screen for Nonhomologous End-Joining Mutants in *Saccharomyces cerevisiae***. *Science* 2001, **294**(5551):2552-2556.
11. Lee SK, Chou H, Ham TS, Lee TS, Keasling JD: **Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels**. *Curr Opin Biotechnol* 2008, **19**(6):556-563.
12. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD: **Microbial engineering for the production of advanced biofuels**. *Nature* 2012, **488**(7411):320-328.
13. Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD: **Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol**. *Microb Cell Fact* 2008, **7**:36.
14. Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC: **Metabolic engineering of *Escherichia coli* for 1-butanol production**. *Metabolic Engineering* 2008, **10**(6):305-311.
15. Bond-Watts BB, Bellerose RJ, Chang MCY: **Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways**. *Nat Chem Biol* 2011, **7**(4):222-227.
16. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **451**(7174):86-89.
17. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS: **Identification and microbial production of a terpene-based advanced biofuel**. *Nat Commun* 2011, **2**:483.

18. Zhang F, Rodriguez S, Keasling JD: **Metabolic engineering of microbial pathways for advanced biofuels production.** *Curr Opin Biotechnol* 2011, **22**(6):775-783.
19. Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD: **Microbial production of fatty-acid-derived fuels and chemicals from plant biomass.** *Nature* 2010, **463**(7280):559-562.
20. Akhtar MK, Turner NJ, Jones PR: **Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities.** *Proceedings of the National Academy of Sciences* 2012.
21. Rude MA, Baron TS, Brubaker S, Alibhai M, Del Cardayre SB, Schirmer A: **Terminal Olefin (1-Alkene) Biosynthesis by a Novel P450 Fatty Acid Decarboxylase from *Jeotgalicoccus* Species.** *Applied and Environmental Microbiology* 2011, **77**(5):1718-1727.
22. Mendez-Perez D, Begemann MB, Pfleger BF: **Modular Synthase-Encoding Gene Involved in α -Olefin Biosynthesis in *Synechococcus* sp. Strain PCC 7002.** *Applied and Environmental Microbiology* 2011, **77**(12):4264-4267.
23. Beller HR, Goh E-B, Keasling JD: **Genes Involved in Long-Chain Alkene Biosynthesis in *Micrococcus luteus*.** *Applied and Environmental Microbiology* 2010, **76**(4):1212-1223.
24. Sukovich DJ, Seffernick JL, Richman JE, Gralnick JA, Wackett LP: **Widespread Head-to-Head Hydrocarbon Biosynthesis in Bacteria and Role of OleA.** *Applied and Environmental Microbiology* 2010, **76**(12):3850-3862.
25. Sukovich DJ, Seffernick JL, Richman JE, Hunt KA, Gralnick JA, Wackett LP: **Structure, Function, and Insights into the Biosynthesis of a Head-to-Head Hydrocarbon in *Shewanella oneidensis* Strain MR-1.** *Applied and Environmental Microbiology* 2010, **76**(12):3842-3849.
26. Attfield PV: **Stress tolerance: The key to effective strains of industrial baker's yeast.** *Nat Biotech*, **15**(13):1351-1357.
27. Mannazzu I, Angelozzi D, Belviso S, Budroni M, Farris GA, Goffrini P, Lodi T, Marzona M, Bardi L: **Behaviour of *Saccharomyces cerevisiae* wine strains during adaptation to unfavourable conditions of fermentation on synthetic medium: Cell lipid composition, membrane integrity, viability and fermentative activity.** *International Journal of Food Microbiology* 2008, **121**(1):84-91.
28. Atsumi S, Wu T-Y, Machado IMP, Huang W-C, Chen P-Y, Pellegrini M, Liao JC: **Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*.** *Mol Syst Biol* 2010, **6**.
29. Borden JR, Papoutsakis ET: **Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*.** *Applied and Environmental Microbiology* 2007, **73**(9):3061-3068.
30. de Bont JAM: **Solvent-tolerant bacteria in biocatalysis.** *Trends in biotechnology* 1998, **16**(12):493-499.
31. Nicolaou SA, Gaida SM, Papoutsakis ET: **A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation.** *Metabolic Engineering* 2010, **12**(4):307-331.
32. Rühl J, Schmid A, Blank LM: **Selected *Pseudomonas putida* Strains Able To Grow in the Presence of High Butanol Concentrations.** *Applied and Environmental Microbiology* 2009, **75**(13):4653-4656.
33. Scott M, Gunderson CW, Mateescu EM, Zhang Z, Hwa T: **Interdependence of Cell Growth and Gene Expression: Origins and Consequences.** *Science* 2010, **330**(6007):1099-1102.

34. Lanza A, Alper H: **Global Strain Engineering by Mutant Transcription Factors**. In: *Strain Engineering*. Edited by Williams JA, vol. 765: Humana Press; 2011: 253-274.
35. Patnaik R: **Engineering Complex Phenotypes in Industrial Strains**. *Biotechnology Progress* 2008, **24**(1):38-47.
36. Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G: **Engineering yeast transcription machinery for improved ethanol tolerance and production**. *Science* 2006, **314**(5805):1565-1568.
37. Alper H, Stephanopoulos G: **Global transcription machinery engineering: A new approach for improving cellular phenotype**. *Metabolic Engineering* 2007, **9**(3):258-267.
38. Liu H, Yan M, Lai C, Xu L, Ouyang P: **gTME for Improved Xylose Fermentation of *Saccharomyces cerevisiae***. *Appl Biochem Biotechnol* 2010, **160**(2):574-582.
39. Lee JY, Sung BH, Yu BJ, Lee JH, Lee SH, Kim MS, Koob MD, Kim SC: **Phenotypic engineering by reprogramming gene transcription using novel artificial transcription factors in *Escherichia coli***. *Nucleic Acids Research* 2008, **36**(16):e102.
40. Zhang H, Chong H, Ching CB, Jiang R: **Random mutagenesis of global transcription factor cAMP receptor protein for improved osmotolerance**. *Biotechnol Bioeng* 2012, **109**(5):1165-1172.
41. Ma R, Zhang Y, Hong H, Lu W, Lin M, Chen M, Zhang W: **Improved Osmotic Tolerance and Ethanol Production of Ethanologenic *Escherichia coli* by IrrE, a Global Regulator of Radiation-Resistance of *Deinococcus radiodurans***. *Curr Microbiol* 2011, **62**(2):659-664.
42. Chen AH, Silver PA: **Designing biological compartmentalization**. *Trends in Cell Biology* 2012, **22**(12):662-670.
43. Balleza E, López-Bojorquez LN, Martínez-Antonio A, Resendis-Antonio O, Lozada-Chávez I, Balderas-Martínez YI, Encarnación S, Collado-Vides J: **Regulation by transcription factors in bacteria: beyond description**. *FEMS Microbiology Reviews* 2009, **33**(1):133-151.
44. Brynildsen MP, Liao JC: **An integrated network approach identifies the isobutanol response network of *Escherichia coli***. *Mol Syst Biol* 2009, **5**.
45. Kang A, Chang MW: **Identification and reconstitution of genetic regulatory networks for improved microbial tolerance to isooctane**. *Mol Biosyst* 2012, **8**(4):1350-1358.
46. Hou L: **Improved production of ethanol by novel genome shuffling in *Saccharomyces cerevisiae***. *Appl Biochem Biotechnol* 2010, **160**(4):1084-1093.
47. Stanley D, Fraser S, Chambers PJ, Rogers P, Stanley GA: **Generation and characterisation of stable ethanol-tolerant mutants of *Saccharomyces cerevisiae***. *J Ind Microbiol Biotechnol* 2010, **37**(2):139-149.
48. Rees DC, Johnson E, Lewinson O: **ABC transporters: the power to change**. *Nat Rev Mol Cell Biol* 2009, **10**(3):218-227.
49. Koronakis V, Sharff A, Koronakis E, Luisi B, Hughes C: **Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export**. *Nature* 2000, **405**(6789):914-919.
50. Higgins CF: **Multiple molecular mechanisms for multidrug resistance transporters**. *Nat Genet* 2007, **446**(7137):749-757.
51. Huffer S, Roche CM, Blanch HW, Clark DS: ***Escherichia coli* for biofuel production: bridging the gap from promise to practice**. *Trends in biotechnology* 2012, **30**(10):538-545.

52. Li X-Z, Zhang L, Poole K: **Role of the Multidrug Efflux Systems of *Pseudomonas aeruginosa* in Organic Solvent Tolerance.** *Journal of Bacteriology* 1998, **180**(11):2987-2991.
53. Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A: **Mechanisms of solvent tolerance in gram-negative bacteria.** *Annu Rev Microbiol* 2002, **56**:743-768.
54. Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL, Segura A: **Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E.** *Journal of Bacteriology* 2001, **183**(13):3967-3973.
55. Tsukagoshi N, Aono R: **Entry into and Release of Solvents by *Escherichia coli* in an Organic-Aqueous Two-Liquid-Phase System and Substrate Specificity of the AcrAB-TolC Solvent-Extruding Pump.** *Journal of Bacteriology* 2000, **182**(17):4803-4810.
56. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A: **Engineering microbial biofuel tolerance and export using efflux pumps.** *Mol Syst Biol* 2011, **7**:487.
57. Teixeira M, Godinho C, Cabrito T, Mira N, Sá-Correia I: **Increased expression of the yeast multidrug resistance ABC transporter Pdr18 leads to increased ethanol tolerance and ethanol production in high gravity alcoholic fermentation.** *Microb Cell Fact* 2012, **11**(1):1-9.
58. Qiu J, Zhuo Y, Zhu D, Zhou X, Zhang L, Bai L, Deng Z: **Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*.** *Appl Microbiol Biotechnol* 2011, **92**(2):337-345.
59. Wagner S, Baars L, Ytterberg AJ, Klussmeier A, Wagner CS, Nord O, Nygren P-Å, van Wijk KJ, de Gier J-W: **Consequences of Membrane Protein Overexpression in *Escherichia coli*.** *Molecular & Cellular Proteomics* 2007, **6**(9):1527-1550.
60. Wagner S, Klepsch MM, Schlegel S, Appel A, Draheim R, Tarry M, Hogbom M, van Wijk KJ, Slotboom DJ, Persson JO *et al*: **Tuning *Escherichia coli* for membrane protein overexpression.** *Proc Natl Acad Sci U S A* 2008, **105**(38):14371-14376.
61. Narayanan A, Ridilla M, Yernool DA: **Restrained expression, a method to overproduce toxic membrane proteins by exploiting operator-repressor interactions.** *Protein Sci* 2011, **20**(1):51-61.
62. Dunlop MJ, Keasling JD, Mukhopadhyay A: **A model for improving microbial biofuel production using a synthetic feedback loop.** *Syst Synth Biol* 2010, **4**(2):95-104.
63. Bokma E, Koronakis E, Lobedanz S, Hughes C, Koronakis V: **Directed evolution of a bacterial efflux pump: Adaptation of the *E. coli* TolC exit duct to the *Pseudomonas* MexAB translocase.** *FEBS Letters* 2006, **580**(22):5339-5343.
64. Agapakis CM, Boyle PM, Silver PA: **Natural strategies for the spatial optimization of metabolism in synthetic biology.** *Nat Chem Biol* 2012, **8**(9):527-535.
65. Conrado RJ, Varner JD, DeLisa MP: **Engineering the spatial organization of metabolic enzymes: mimicking nature's synergy.** *Curr Opin Biotechnol* 2008, **19**(5):492-499.
66. Lee H, DeLoache WC, Dueber JE: **Spatial organization of enzymes for metabolic engineering.** *Metabolic Engineering* 2012, **14**(3):242-251.
67. Zhu L, Zhu Y, Zhang Y, Li Y: **Engineering the robustness of industrial microbes through synthetic biology.** *Trends in Microbiology* 2012, **20**(2):94-101.
68. Kerfeld CA, Heinhorst S, Cannon GC: **Bacterial Microcompartments.** *Annu Rev Microbiol* 2010, **64**(1):391-408.

69. Martin W: **Evolutionary origins of metabolic compartmentalization in eukaryotes.** *Philosophical Transactions of the Royal Society B: Biological Sciences* 2010, **365**(1541):847-855.
70. Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KLJ, Keasling JD: **Synthetic protein scaffolds provide modular control over metabolic flux.** *Nat Biotech* 2009, **27**(8):753-759.
71. Conrado RJ, Wu GC, Boock JT, Xu H, Chen SY, Lebar T, Turnšek J, Tomšič N, Avbelj M, Gaber R *et al*: **DNA-guided assembly of biosynthetic pathways promotes improved catalytic efficiency.** *Nucleic Acids Research* 2012, **40**(4):1879-1889.
72. Delebecque CJ, Lindner AB, Silver PA, Aldaye FA: **Organization of Intracellular Reactions with Rationally Designed RNA Assemblies.** *Science* 2011, **333**(6041):470-474.
73. Choudhary S QM, Sanders MA, Johnson ET, Schmidt-Dannert C: **Engineered Protein Nano-Compartments for Targeted Enzyme Localization.** *PLoS ONE* 2012, **7**(3).
74. Wörsdörfer B, Woycechowsky KJ, Hilvert D: **Directed Evolution of a Protein Container.** *Science* 2011, **331**(6017):589-592.
75. Görgens JF, van Zyl WH, Knoetze JH, Hahn-Hägerdal B: **The metabolic burden of the PGK1 and ADH2 promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium.** *Biotechnol Bioeng* 2001, **73**(3):238-245.
76. Price VL, Taylor WE, Clevenger W, Worthington M, Young ET: **[25] Expression of heterologous proteins in *Saccharomyces cerevisiae* using the ADH2 promoter.** In: *Methods in Enzymology*. Edited by David VG, vol. Volume 185: Academic Press; 1990: 308-318.
77. Shin PK, Seo J-H: **Analysis of *E. coli* *phoA-lacZ* fusion gene expression inserted into a multicopy plasmid and host cell's chromosome.** *Biotechnol Bioeng* 1990, **36**(11):1097-1104.
78. Vogel K, Hinnen A: **The yeast phosphatase system.** *Molecular Microbiology* 1990, **4**(12):2013-2017.
79. Miller MB, Bassler BL: **QUORUM SENSING IN BACTERIA.** *Annu Rev Microbiol* 2001, **55**(1):165-199.
80. Saeidi N, Wong CK, Lo T-M, Nguyen HX, Ling H, Leong SSJ, Poh CL, Chang MW: **Engineering microbes to sense and eradicate *Pseudomonas aeruginosa*, a human pathogen.** *Mol Syst Biol* 2011, **7**.
81. Tsao C-Y, Hooshangi S, Wu H-C, Valdes JJ, Bentley WE: **Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of *E. coli*.** *Metabolic Engineering* 2010, **12**(3):291-297.
82. Blazeck J, Alper HS: **Promoter engineering: Recent advances in controlling transcription at the most fundamental level.** *Biotechnol J* 2013, **8**(1):46-58.
83. Salis HM, Mirsky EA, Voigt CA: **Automated design of synthetic ribosome binding sites to control protein expression.** *Nat Biotech* 2009, **27**(10):946-950.
84. Jones KL, Kim S-W, Keasling JD: **Low-Copy Plasmids can Perform as Well as or Better Than High-Copy Plasmids for Metabolic Engineering of Bacteria.** *Metabolic Engineering* 2000, **2**(4):328-338.
85. Mateus C, Avery SV: **Destabilized green fluorescent protein for monitoring dynamic changes in yeast gene expression with flow cytometry.** *Yeast* 2000, **16**(14):1313-1323.
86. Holtz WJ, Keasling JD: **Engineering Static and Dynamic Control of Synthetic Pathways.** *Cell* 2010, **140**(1):19-23.

87. Gancedo JM: **Yeast Carbon Catabolite Repression**. *Microbiology and Molecular Biology Reviews* 1998, **62**(2):334-361.
88. Montange RK, Batey RT: **Riboswitches: Emerging Themes in RNA Structure and Function**. *Annual Review of Biophysics* 2008, **37**(1):117-133.
89. Michener JK, Thodey K, Liang JC, Smolke CD: **Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways**. *Metabolic Engineering* 2012, **14**(3):212-222.
90. Farmer WR, Liao JC: **Improving lycopene production in Escherichia coli by engineering metabolic control**. *Nat Biotech* 2000, **18**(5):533-537.
91. Nyström T: **The glucose-starvation stimulon of Escherichia coli: induced and repressed synthesis of enzymes of central metabolic pathways and role of acetyl phosphate in gene expression and starvation survival**. *Molecular Microbiology* 1994, **12**(5):833-843.
92. Anesiadis N, Cluett WR, Mahadevan R: **Dynamic metabolic engineering for increasing bioprocess productivity**. *Metabolic Engineering* 2008, **10**(5):255-266.
93. Gadkar KG, Doyle IJ, Edwards JS, Mahadevan R: **Estimating optimal profiles of genetic alterations using constraint-based models**. *Biotechnol Bioeng* 2005, **89**(2):243-251.
94. Zhang F, Keasling J: **Biosensors and their applications in microbial metabolic engineering**. *Trends in Microbiology* 2011, **19**(7):323-329.
95. Dietrich JA, McKee AE, Keasling JD: **High-Throughput Metabolic Engineering: Advances in Small-Molecule Screening and Selection**. *Annual Review of Biochemistry* 2010, **79**(1):563-590.
96. Futai M: **Stimulation of Transport into Escherichia coli Membrane Vesicles by Internally Generated Reduced Nicotinamide Adenine Dinucleotide**. *Journal of Bacteriology* 1974, **120**(2):861-865.
97. Bunch PK, Mat-Jan F, Lee N, Clark DP: **The IdhA Gene Encoding the Fermentative Lactate Dehydrogenase of Escherichia Coli**. *Microbiology* 1997, **143**(1):187-195.
98. Singh A, Lynch MD, Gill RT: **Genes restoring redox balance in fermentation-deficient E. coli NZN111**. *Metabolic Engineering* 2009, **11**(6):347-354.
99. San K-Y, Bennett GN, Berríos-Rivera SJ, Vadali RV, Yang Y-T, Horton E, Rudolph FB, Sariyar B, Blackwood K: **Metabolic Engineering through Cofactor Manipulation and Its Effects on Metabolic Flux Redistribution in Escherichia coli**. *Metabolic Engineering* 2002, **4**(2):182-192.
100. Berri, amp, x, os-Rivera SJ, Bennett GN, San K-Y: **Metabolic Engineering of Escherichia coli: Increase of NADH Availability by Overexpressing an NAD⁺-Dependent Formate Dehydrogenase**. *Metabolic Engineering* 2002, **4**(3):217-229.
101. Boshoff HIM, Xu X, Tahlán K, Dowd CS, Pethe K, Camacho LR, Park T-H, Yun C-S, Schnappinger D, Ehrt S *et al*: **Biosynthesis and Recycling of Nicotinamide Cofactors in Mycobacterium tuberculosis: AN ESSENTIAL ROLE FOR NAD IN NONREPLICATING BACILLI**. *Journal of Biological Chemistry* 2008, **283**(28):19329-19341.
102. Lopez de Felipe F, Kleerebezem M, de Vos WM, Hugenholtz J: **Cofactor Engineering: a Novel Approach to Metabolic Engineering in Lactococcus lactis by Controlled Expression of NADH Oxidase**. *Journal of Bacteriology* 1998, **180**(15):3804-3808.
103. Heux S, Cachon R, Dequin S: **Cofactor engineering in Saccharomyces cerevisiae: Expression of a H₂O-forming NADH oxidase and impact on redox metabolism**. *Metabolic Engineering* 2006, **8**(4):303-314.
104. Heuser F, Marin K, Kaup B, Bringer S, Sahm H: **Improving d-mannitol productivity of Escherichia coli: Impact of NAD, CO₂ and expression of a putative sugar**

- permease from *Leuconostoc pseudomesenteroides*. *Metabolic Engineering* 2009, **11**(3):178-183.
105. Geertman J-MA, van Maris AJA, van Dijken JP, Pronk JT: **Physiological and genetic engineering of cytosolic redox metabolism in *Saccharomyces cerevisiae* for improved glycerol production**. *Metabolic Engineering* 2006, **8**(6):532-542.
 106. Schroer K, Peter Luef K, Stefan Hartner F, Glieder A, Pscheidt B: **Engineering the *Pichia pastoris* methanol oxidation pathway for improved NADH regeneration during whole-cell biotransformation**. *Metabolic Engineering* 2010, **12**(1):8-17.
 107. Hou J, Scalcinati G, Oldiges M, Vemuri GN: **Metabolic Impact of Increased NADH Availability in *Saccharomyces cerevisiae***. *Applied and Environmental Microbiology* 2010, **76**(3):851-859.
 108. Hou J, Lages NF, Oldiges M, Vemuri GN: **Metabolic impact of redox cofactor perturbations in *Saccharomyces cerevisiae***. *Metabolic Engineering* 2009, **11**(4-5):253-261.
 109. Hasegawa S, Uematsu K, Natsuma Y, Suda M, Hiraga K, Jojima T, Inui M, Yukawa H: **Improvement of the Redox Balance Increases L-Valine Production by *Corynebacterium glutamicum* under Oxygen Deprivation Conditions**. *Applied and Environmental Microbiology* 2012, **78**(3):865-875.
 110. Lee E-G, Yoon S-H, Das A, Lee S-H, Li C, Kim J-Y, Choi M-S, Oh D-K, Kim S-W: **Directing vanillin production from ferulic acid by increased acetyl-CoA consumption in recombinant *Escherichia coli***. *Biotechnol Bioeng* 2009, **102**(1):200-208.
 111. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M: **From genomics to chemical genomics: new developments in KEGG**. *Nucleic Acids Research* 2006, **34**(suppl 1):D354-D357.
 112. Caspi R, Altman T, Dreher K, Fulcher CA, Subhraveti P, Keseler IM, Kothari A, Krummenacker M, Latendresse M, Mueller LA *et al*: **The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases**. *Nucleic Acids Research* 2012, **40**(D1):D742-D753.
 113. Bar-Even A, Noor E, Lewis NE, Milo R: **Design and analysis of synthetic carbon fixation pathways**. *Proceedings of the National Academy of Sciences* 2010, **107**(19):8889-8894.
 114. Erb TJ: **Carboxylases in Natural and Synthetic Microbial Pathways**. *Applied and Environmental Microbiology* 2011, **77**(24):8466-8477.
 115. Finley SD, Broadbelt LJ, Hatzimanikatis V: **Computational framework for predictive biodegradation**. *Biotechnol Bioeng* 2009, **104**(6):1086-1097.
 116. Rodrigo G, Carrera J, Prather KJ, Jaramillo A: **DESHARKY: automatic design of metabolic pathways for optimal cell growth**. *Bioinformatics* 2008, **24**(21):2554-2556.
 117. Becker SA, Feist AM, Mo ML, Hannum G, Palsson BO, Herrgard MJ: **Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox**. *Nat Protocols* 2007, **2**(3):727-738.
 118. Medema MH, van Raaphorst R, Takano E, Breitling R: **Computational tools for the synthetic design of biochemical pathways**. *Nat Rev Micro* 2012, **10**(3):191-202.
 119. Orth JD, Thiele I, Palsson BO: **What is flux balance analysis?** *Nat Biotech* 2010, **28**(3):245-248.
 120. Schellenberger J, Que R, Fleming RMT, Thiele I, Orth JD, Feist AM, Zielinski DC, Bordbar A, Lewis NE, Rahmanian S *et al*: **Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0**. *Nat Protocols* 2011, **6**(9):1290-1307.

121. Burgard AP, Pharkya P, Maranas CD: **Optknock: A bilevel programming framework for identifying gene knockout strategies for microbial strain optimization.** *Biotechnol Bioeng* 2003, **84**(6):647-657.
122. Segrè D, Vitkup D, Church GM: **Analysis of optimality in natural and perturbed metabolic networks.** *Proceedings of the National Academy of Sciences* 2002, **99**(23):15112-15117.
123. Brochado A, Matos C, Moller B, Hansen J, Mortensen U, Patil K: **Improved vanillin production in baker's yeast through in silico design.** *Microb Cell Fact* 2010, **9**(1):84.
124. Canton B, Labno A, Endy D: **Refinement and standardization of synthetic biological parts and devices.** *Nat Biotech* 2008, **26**(7):787-793.
125. Racle J, Overney J, Hatzimanikatis V: **A computational framework for the design of optimal protein synthesis.** *Biotechnol Bioeng* 2012, **109**(8):2127-2133.
126. Puigbò P, Guzmán E, Romeu A, Garcia-Vallvé S: **OPTIMIZER: a web server for optimizing the codon usage of DNA sequences.** *Nucleic Acids Research* 2007, **35**(suppl 2):W126-W131.
127. Villalobos A, Ness J, Gustafsson C, Minshull J, Govindarajan S: **Gene Designer: a synthetic biology tool for constructing artificial DNA segments.** *BMC bioinformatics* 2006, **7**(1):285.
128. Fischer CR, Klein-Marcuschamer D, Stephanopoulos G: **Selection and optimization of microbial hosts for biofuels production.** *Metabolic Engineering* 2008, **10**(6):295-304.
129. Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD: **Biofuel alternatives to ethanol: pumping the microbial well.** *Trends in biotechnology* 2008, **26**(7):375-381.
130. Pinkart HC, White DC: **Phospholipid biosynthesis and solvent tolerance in *Pseudomonas putida* strains.** *Journal of Bacteriology* 1997, **179**(13):4219-4226.
131. Nuwaysir EF, Bittner M, Trent J, Barrett JC, Afshari CA: **Microarrays and toxicology: The advent of toxicogenomics.** *Molecular Carcinogenesis* 1999, **24**(3):153-159.
132. Afshari CA, Nuwaysir EF, Barrett JC: **Application of Complementary DNA Microarray Technology to Carcinogen Identification, Toxicology, and Drug Safety Evaluation.** *Cancer Research* 1999, **59**(19):4759-4760.
133. Chang W, Toghrol F, Bentley WE: **Toxicogenomic Response of *Staphylococcus aureus* to Peracetic Acid.** *Environmental Science & Technology* 2006, **40**(16):5124-5131.
134. Matsui K, Hirayama T, Kuroda K, Shirahige K, Ashikari T, Ueda M: **Screening for candidate genes involved in tolerance to organic solvents in yeast.** *Appl Microbiol Biotechnol* 2006, **71**(1):75-79.
135. Ghosh AK, Ramakrishnan G, Rajasekharan R: **YLR099C (ICT1) Encodes a Soluble Acyl-CoA-dependent Lysophosphatidic Acid Acyltransferase Responsible for Enhanced Phospholipid Synthesis on Organic Solvent Stress in *Saccharomyces cerevisiae*.** *Journal of Biological Chemistry* 2008, **283**(15):9768-9775.
136. Yasokawa D, Murata S, Kitagawa E, Iwahashi Y, Nakagawa R, Hashido T, Iwahashi H: **Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis.** *Environmental Toxicology* 2008, **23**(5):599-606.
137. Yasokawa D, Iwahashi H: **Toxicogenomics using yeast DNA microarrays.** *J Biosci Bioeng* 2010, **110**(5):511-522.
138. Maeng JH, Sakai Y, Tani Y, Kato N: **Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. strain M-1.** *Journal of Bacteriology* 1996, **178**(13):3695-3700.

139. De Smet MJ, Kingma J, Witholt B: **The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of escherichia coli.** *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1978, **506**(1):64-80.
140. Kawamoto T, Kanda T, Tanaka A: **Preparation of an organic solvent-tolerant strain from baker's yeast.** *Appl Microbiol Biotechnol* 2001, **55**(4):476-479.
141. GILL CO, RATLEDGE C: **Toxicity of n-Alkanes, n-Alk-1-enes, n-Alkan-1-ols and n-Alkyl-1-bromides towards Yeasts.** *Journal of General Microbiology* 1972, **72**(1):165-172.
142. Bissinger PH, Kuchler K: **Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. A yeast ABC transporter conferring mycotoxin resistance.** *Journal of Biological Chemistry* 1994, **269**(6):4180-4186.
143. Grigoras I, Lazard M, Plateau P, Blanquet S: **Functional characterization of the *Saccharomyces cerevisiae* ABC-transporter Yor1p overexpressed in plasma membranes.** *Biochimica et Biophysica Acta (BBA) - Biomembranes* 2008, **1778**(1):68-78.
144. Mahé Y, Parle-McDermott A, Nourani A, Delahodde A, Lamprecht A, Kuchler K: **The ATP-binding cassette multidrug transporter Snq2 of *Saccharomyces cerevisiae*: a novel target for the transcription factors Pdr1 and Pdr3.** *Molecular Microbiology* 1996, **20**(1):109-117.
145. Ernst R, Klemm R, Schmitt L, Kuchler K: **Yeast ATP - Binding Cassette Transporters: Cellular Cleaning Pumps.** In: *Methods in Enzymology*. Edited by Helmut S, Lester P, vol. Volume 400: Academic Press; 2005: 460-484.
146. Schneiter R, Guerra CE, Lampl M, Tatzer V, Zellnig G, Klein HL, Kohlwein SD: **A Novel Cold-Sensitive Allele of the Rate-Limiting Enzyme of Fatty Acid Synthesis, Acetyl Coenzyme A Carboxylase, Affects the Morphology of the Yeast Vacuole through Acylation of Vac8p.** *Molecular and Cellular Biology* 2000, **20**(9):2984-2995.
147. Hong M-E, Lee K-S, Yu BJ, Sung Y-J, Park SM, Koo HM, Kweon D-H, Park JC, Jin Y-S: **Identification of gene targets eliciting improved alcohol tolerance in *Saccharomyces cerevisiae* through inverse metabolic engineering.** *J Biotechnol* 2010, **149**(1-2):52-59.
148. Monteiro PT, Mendes ND, Teixeira MC, d'Orey S, Tenreiro S, Mira NP, Pais H, Francisco AP, Carvalho AM, Lourenço AB *et al*: **YEASTRACT-DISCOVERER: new tools to improve the analysis of transcriptional regulatory associations in *Saccharomyces cerevisiae*.** *Nucleic Acids Research* 2008, **36**(suppl 1):D132-D136.
149. Guedener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH: **A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast.** *Nucleic Acids Research* 2002, **30**(6):e23.
150. Manivasakam P, Schiestl RH: **High Efficiency transformation of *Saccharomyces cerevisiae* by electroporation.** *Nucleic Acids Research* 1993, **21**(18):4414-4415.
151. Paumi CM, Chuk M, Snider J, Stagljär I, Michaelis S: **ABC Transporters in *Saccharomyces cerevisiae* and Their Interactors: New Technology Advances the Biology of the ABCC (MRP) Subfamily.** *Microbiology and Molecular Biology Reviews* 2009, **73**(4):577-593.
152. Lo T-M, Teo WS, Ling H, Chen B, Kang A, Chang MW: **Microbial engineering strategies to improve cell viability for biochemical production.** *Biotechnology Advances* 2013, **31**(6):903-914.
153. Beopoulos A, Chardot T, Nicaud JM: ***Yarrowia lipolytica*: A model and a tool to understand the mechanisms implicated in lipid accumulation.** *Biochimie* 2009, **91**(6):692-696.

154. Barth G, Gaillardin C: **Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica***. *FEMS Microbiology Reviews* 1997, **19**(4):219-237.
155. Mauersberger S, Wang HJ, Gaillardin C, Barth G, Nicaud JM: **Insertional Mutagenesis in the n-Alkane-Assimilating Yeast *Yarrowia lipolytica*: Generation of Tagged Mutations in Genes Involved in Hydrophobic Substrate Utilization**. *Journal of Bacteriology* 2001, **183**(17):5102-5109.
156. Thevenieau F, Le Dall MT, Nthangeni B, Mauersberger S, Marchal R, Nicaud JM: **Characterization of *Yarrowia lipolytica* mutants affected in hydrophobic substrate utilization**. *Fungal Genet Biol* 2007, **44**(6):531-542.
157. Thevenieau F, Beopoulos A, Desfougeres T, Sabirova J, Albertin K, Zinjarde S, Nicaud JM: **Uptake and Assimilation of Hydrophobic Substrates by the Oleaginous Yeast *Yarrowia lipolytica***. 2010:1513-1527.
158. Heckman KL, Pease LR: **Gene splicing and mutagenesis by PCR-driven overlap extension**. *Nat Protoc* 2007, **2**(4):924-932.
159. Sheff MA, Thorn KS: **Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae***. *Yeast* 2004, **21**(8):661-670.
160. Blanchin-Roland S, Da Costa G, Gaillardin C: **ESCRT-I components of the endocytic machinery are required for Rim101-dependent ambient pH regulation in the yeast *Yarrowia lipolytica***. *Microbiology* 2005, **151**(Pt 11):3627-3637.
161. Riezmann H, Hase T, Loon APGMv, Grivell LA, Suda K, Schatz G: **Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane**. *the The European Molecular Biology Organization Journal* 1983, **2**(12):2161-2168.
162. Conzelmann A, Riezmann H, Desponds C, Bron C: **A major 125-kd membrane glycoprotein of *Saccharomyces cerevisiae* is attached to the lipid bilayer through an inositol-containing phospholipid**. *the The European Molecular Biology Organization Journal* 1988, **7**(7):2233-2240.
163. Ramaswamy G, Karim MA, Murti KG, Jackowski S: **PPAR α controls the intracellular coenzyme A concentration via regulation of PANK1 α gene expression**. *Journal of Lipid Research* 2004, **45**(1):17-31.
164. Oda T, Hirota K, Nishi K, Takabuchi S, Oda S, Yamada H, Arai T, Fukuda K, Kita T, Adachi T *et al*: **Activation of hypoxia-inducible factor 1 during macrophage differentiation**. *American Journal of Physiology - Cell Physiology* 2006, **291**(1):C104-C113.
165. Low KO, Mahadi NM, Abdul Rahim R, Rabu A, Abu Bakar FD, Abdul Murad AM, Illias RM: **Enhanced secretory production of hemolysin-mediated cyclodextrin glucanotransferase in *Escherichia coli* by random mutagenesis of the ABC transporter system**. *J Biotechnol* 2010, **150**(4):453-459.
166. Decottignies A, Owsianik G, Ghislain M: **Casein Kinase I-dependent Phosphorylation and Stability of the Yeast Multidrug Transporter Pdr5p**. *Journal of Biological Chemistry* 1999, **274**(52):37139-37146.
167. Wada S, Niimi M, Niimi K, Holmes AR, Monk BC, Cannon RD, Uehara Y: ***Candida glabrata* ATP-binding cassette transporters Cdr1p and Pdh1p expressed in a *Saccharomyces cerevisiae* strain deficient in membrane transporters show phosphorylation-dependent pumping properties**. *J Biol Chem* 2002, **277**(48):46809-46821.
168. Egner R, Rosenthal FE, Kralli A, Sanglard D, Kuchler K: **Genetic Separation of FK506 Susceptibility and Drug Transport in the Yeast Pdr5 ATP-binding Cassette Multidrug Resistance Transporter**. *Molecular Biology of the Cell* 1998, **9**(2):523-543.

169. Moody JE, Millen L, Binns D, Hunt JF, Thomas PJ: **Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters.** *J Biol Chem* 2002, **277**(24):21111-21114.
170. Zaitseva J, Jenewein S, Jumpertz T, Holland IB, Schmitt L: **H662 is the linchpin of ATP hydrolysis in the nucleotide-binding domain of the ABC transporter HlyB.** *EMBO J* 2005, **24**(11):1901-1910.
171. Schmitt L, Tampé R: **Structure and mechanism of ABC transporters.** *Current Opinion in Structural Biology* 2002, **12**(6):754-760.
172. Rutledge RM, Esser L, Ma J, Xia D: **Toward understanding the mechanism of action of the yeast multidrug resistance transporter Pdr5p: a molecular modeling study.** *J Struct Biol* 2011, **173**(2):333-344.
173. Ernst R, Kueppers P, Klein CM, Schwarzmüller T, Kuchler K, Schmitt L: **A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5.** *Proc Natl Acad Sci U S A* 2008, **105**(13):5069-5074.
174. Matsushika A, Watanabe S, Kodaki T, Makino K, Sawayama S: **Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, NADP(+)-dependent xylitol dehydrogenase, and xylulokinase.** *J Biosci Bioeng* 2008, **105**(3):296-299.
175. Kalscheuer R, Luftmann H, Steinbüchel A: **Synthesis of Novel Lipids in *Saccharomyces cerevisiae* by Heterologous Expression of an Unspecific Bacterial Acyltransferase.** *Applied and Environmental Microbiology* 2004, **70**(12):7119-7125.
176. Yu KO, Jung J, Kim SW, Park CH, Han SO: **Synthesis of FAEEs from glycerol in engineered *Saccharomyces cerevisiae* using endogenously produced ethanol by heterologous expression of an unspecific bacterial acyltransferase.** *Biotechnol Bioeng* 2012, **109**(1):110-115.
177. Shi S, Octavio Valle-Rodriguez J, Khoomrung S, Siewers V, Nielsen J: **Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production.** *Biotechnology for Biofuels* 2012, **5**(1):7.
178. de Jong B, Siewers V, Nielsen J: **Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels.** *Curr Opin Biotechnol* 2011.