STRAIN ENGINEERING AND BIOSENSOR DEVELOPMENT FOR EFFICIENT BIOFUEL PRODUCTION BY *SACCHAROMYCES CEREVISIAE*

BY

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DISSERTATION

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Abstract

Metabolic engineering of *Saccharomyces cerevisiae* is an attractive approach to enhance the production of cellulosic ethanol, fatty alcohols and other advanced biofuels. Production of cellulosic ethanol from lignocelluloses has attracted a lot of interest and significant improvement has been made to construct and optimize the recombinant *S. cerevisiae* strains capable of converting glucose or pentose sugars into ethanol. Unfortunately, pentose sugars, which constitute up to 30% of biomass hydrolysate, cannot be co-utilized simultaneously with glucose by recombinant *S. cerevisiae* strains. Great efforts have been made to improve the co-utilization efficiency of sugars derived from lignocellulose hydrolysates. A lot of research has been carried out to lower the effect of glucose repression that leads to inefficient pentose sugars utilization in the presence of glucose, but it remains challenging to overcome this issue by depletion of genes involved in transcriptional regulation or optimization of pentose sugar transportation and utilization.

To overcome the glucose repression problem in *S. cerevisiae*, we designed a strategy to construct a *S. cerevisiae* strain capable of simultaneously utilizing cellobiose and xylose derived from lignocellulose. The high efficiency pathway containing a cellobiose transporter and a β-glucosidase enables fast cellobiose utilization and ethanol production, and glucose repression is avoided by the intracellular utilization of cellobiose. Distinguished from existing glucose derepression methods, glucose utilization is not impaired, while xylose utilization is

improved because of the synergistic effects.

To optimize the cellobiose utilization efficiency, the functional role of an important enzyme in glucose conversion, aldose 1-epimerase (AEP), was investigated. AEP is supposed to maintain the intracellular equilibrium of α-glucose and β-glucose when the spontaneous conversion between the two glucose anomers is not sufficient. However, the heterologous cellobiose utilization pathway results in excess β-glucose accumulation and lowers the rate of glucose glycolysis, which limits efficient utilization of cellobiose in engineered *S. cerevisiae* strains. We found three AEP candidates (Gal10, Yhr210c and Ynr071c) in *S. cerevisiae* and investigated their function in cellobiose utilization. Deletion of Gal10 led to complete loss of both AEP activity and cell growth on cellobiose, while complementation restored the AEP activity and cell growth. In addition, deletion of YHR210C or YNR071C resulted in improved cellobiose utilization. These results suggest that the intracellular mutarotation of β-glucose to α-glucose might be a rate controlling step and Gal10 plays a crucial role in cellobiose fermentation by engineered S. cerevisiae.,

The production of advanced biofuels, such as higher alcohols, fatty acid derived fuels, and hydrocarbons, is considered to be a better fuel alternative solution. Because their physiochemical properties are more compatible with the current gasoline-based infrastructure than ethanol. However, compared to current progress in ethanol production, a lot more efforts are needed to make these advanced biofuels commercially available. Recent efforts in

advanced biofuels synthesis have been focused on the design, construction and optimization of pathways and strains, but detection becomes the bottleneck step that hinders high-throughput screening. Genetic biosensors convert chemical concentrations into detectable fluorescence signal via transcriptional regulation, and may serve as an important tool for screening and cell sorting. We have constructed a genomic sensor that correlates intracellular malonyl-CoA concentration to a fluorescence signal by transcriptional regulation. Malonyl-CoA is the building block for the biosynthesis of fatty acids, 3-hydroxypropionic acid, polyketides, and flavonoids, which can either be used directly or be used as a precursor for the production of biofuels and value-added chemicals. The sensor was combined with a genome wide mutant library in *S. cerevisiae*, and used to screen for mutants with higher productivity of malonyl-CoA, thus improving the downstream production of the reporter chemical, 3-hydroxypropionic acid. The constructed malonyl-CoA sensors can also be employed as control elements in order to modulate gene expression of biosynthetic pathways of important compounds that are of particular interest to the pharmaceutical and biofuel industries.

The development of transcriptional-regulation based sensors relies on the discovery and identification of transcription factors and operators, which are usually heterologous to the platform microorganism. We explored a novel strategy to discover multiple sensors by transcriptional profiling. The strategy utilizes the native regulation mechanisms in *S.*

cerevisiae, minimizes extrinsic manipulation and screens for multiple metabolite-responsive promoters with various transcription activities in a short time. A proof-of-concept sensor targeting acetyl-CoA was established and validated and the development of more sensors is in progress. This strategy provides an innovative approach for metabolite monitoring and pathway control.

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Chapter 1 Introduction

1.1. Biofuels

1.1.1.An Alternative Fuel Source

Biofuels derived from biomass has attracted great attentions as a promising alternative energy source compared to fossil fuels due to environmental, economic and energy security considerations (1). Firstly, there are much less greenhouse gas emission derived from biofuels than from fossil fuels: on a life cycle basis, the greenhouse gas emission from biofuel producing process is only 20% to 86% of that from gasoline producing process (2). Bioethanol is also “cleaner” than gasoline as it is a safe and fully-biodegradable fuel additive (3). Secondly, the supply of raw materials for biofuel production is adequate. Various feedstocks including crops, perennial plants, agricultural residues as corn stovers and wheat straws, forest residues, urban waste or even manures are utilized to produce biofuel (4). In the United States, there are more than a billion tons of available biomass which can be converted to 80-100 billion gallons of biofuels per year (5), which can relieve the dependence on imported gasoline. Besides, evaluated by the standard of energy economics, the ratio of the amount of usable energy acquired from biofuels to the amount of energy expended to obtain them, or the term called energy returned on energy invested (EROEI) can be as high as 36, which is much higher than that of oil or natural gas (2). The high amount of net energy gained from cheap feedstock makes biofuels an affordable energy source. Thus the supply of

biofuels is abundant, the production process is domestic and the price is affordable. Resultantly, researchers, government and companies are committed to advancing technological solutions to promote and increase the use of clean, abundant, affordable, and domestically- and sustainably-produced biofuels, not only to reduce greenhouse gas emissions, but also to diversify the energy sources in market and to reduce the dependence on fossil fuels.

In 2007, federal policy played a key role in the emergence of the biofuels industry. The Energy Independence and Security Act (EISA) of 2007 was passed with the intention of moving the United States toward greater energy security. The Renewable Fuels Standards (RFS) was extended from the one set at 2005 and the minimum volume of biofuels used in the national transportation fuel supply each year was increased. The mandate of biofuels required to be used each year rises from 9 billion gallons in 2008 to 36 billion gallons in 2022. Policy support from the government stimulates the development of biofuel production technologies. Corn ethanol, cellulosic ethanol and biodiesels and other advanced biofuels are all involved and improvements and innovations are needed for the high demand of renewable biofuels (6).

1.1.2.First Generation Bioethanol

Among various sorts of biofuels, bioethanol is the most widely used. Ethanol produced by microbial fermentation has been used by mankind since 9000 years ago (7) and

the utilization of ethanol as a transportation fuel has been reported many times in 19th century. After 1940, bioethanol production slowed down due to the competition of gasoline with a much cheaper price and it had been three decades before the production of bioethanol was resumed in 1970s due to the first energy crisis. The increasing price of fossil fuels becomes one of the dominant driving force supporting bioethanol production: counted on a time range of five years, the average annual real oil prices for 2007-11 were 220% above the average for 1997-2001, while for coal the increase was 141% and for gas 95%. Responsively, the annual global bioethanol production increased from 5.4 billion gallons in 1997 to 22.7 billion gallons in 2011 (8). It is predicted that renewable energy supply which is mainly composed of bioethanol will grow by a factor of 2 from 2011 to 2030, accounting for 17% of the increase in global energy supply (9).

Beginning three decades ago in the Midwest, bioethanol production in the U.S. boomed: the annual bioethanol production increased from 0.2 billion gallons in 1980 to 13.3 billion gallons in 2012, which composed 61% of the global production (10). To be noticed, the U.S. has surpassed Brazil to be the largest ethanol producer in the world since 2005 (10). 2013 saw the expansion of bioethanol industry in the U.S.: there were nearly 200 plants operating in 29 states, with annual capacity of 13.3 billion gallons (11).

Bioethanol production is the largest scale microbial process by far. After simple distillation and purification, ethanol can be used directly as a transportation fuel. Current

industrial ethanol production uses either starch or sugar cane molasses. Corn ethanol produced from enzymatically digested starch dominates the U.S. market, while in Brazil ethanol is produced mostly from sugar cane. These two kinds of bioethanol produced from food crops are defined as the 1st generation bioethanol. 1st generation bioethanol is the dominant biofuel on market and is widely used for transportation purpose. However, the sustainability and economics of 1st generation bioethanol is problematic. First, crops grown for fuel usage compete for land and water against food crops, which is well known as the "Food vs. Fuel" dilemma. It is reported that in the 2010/11 agricultural marketing year, 40% of corn and 14% of soybean oil production was used to produce biofuels (8). Second, the cost of production highly relies on the cost of substrate and processing: depending on different processes, the cost of substrate consists about 50% to 70% of the total cost of production. Due to the lack of competitive advantage in price, 1st generation bioethanol usually require government subsidies in the competition with fossil fuels (12). Third, though bioethanol production is coupled with lower greenhouse gas emissions, the consumption of fertilizers and energy weakens the reduction of greenhouse gas emission from 1st generation bioethanol production. The total reduction is around 20% compared to petroleum. For the last, because of the energy input in feedstock growth, distillation and transportation, the ERORI of 1st generation bioethanol is usually between 1.38 and 2.51 (13). Take all these into consideration, it remains a challenge to develop an advanced biofuel derived from biomass.

1.1.3.Second Generation Bioethanol and Other Advanced Biofuels

There is an increasing interest in developing 2nd generation bioethanol produced from non-food biomass such as lignocellulosic feedstock materials including agricultural and forest residues (corn stover, wheat straw, sugar cane bagasse and wood chips), wastes (organic components of municipal solid wastes) and energy crops. The feedstock materials have much less competition with food crops and the cost is much lower than corn or sugar cane. Besides, instead of reducing the Greenhouse gas (GHG) emission, the production of 2nd generation bioethanol fixes extra GHG into soil, leading to a negative carbon balance. Lastly, the ERORI of 2nd generation bioethanol can be as high as 36, which is the 2nd highest in current fuels, only lower than the 80 of coal (2). Hence the 2nd generation bioethanol is a more sustainable and cleaner energy than 1st generation bioethanol.

However, bioethanol is not the ideal alternative fuel molecule currently. It contains only 70% of the energy content of gasoline, which is a significant disadvantage for the use of transportation. Compared to ethanol, long chain alcohols such as isopropanol and n-butanol have higher energy intensity and lower hygroscopicity, which make them better alternative energy molecules than bioethanol (14). Natural microbes as *Clostridium* species can produce isopropanol and n-butanol, but their slow growth rate and anaerobic growth condition limit the large scale application. Engineered *Escherichii coli* and *Saccharomyces cerevisiae* were reported as host strains for isopropanol and n-butanol production and small amount of long

chain alcohols was observed (14-17). Strategies include the introduction of CoA-dependent pathway (17,18) or the construction of non-fermentative keto acid pathway through which multiple long chain alcohols can be produced from 2-keto acids to aldehydes and then reduced to alcohols including isobutanol, 1-butanol, 2-methy1-butanol, 3-methy1-butanol and 2-phenylethanol (16,19,20). Details will be discussed in 1.3.2.

Advanced biofuels also include biodiesel, which contains a group of mono-alkyl esters of long chain fatty acids, for example, fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE). The energy intensity of biodiesel is about 1.5-fold of that of bioethanol. The chemical characteristics of biodiesel are quite similar to those of petroleum diesel, which makes it a direct substitute. It can also be blended with petroleum diesel in any percentage in virtue of the compatibility with current existing distribution, storage and transportation conditions. Such advantages make biodiesel a preferable biofuel molecule to bioethanol.

Biodiesel available on market now is usually derived from oils from soybeans, rapeseed, sunflowers or animal tallow by trans-esterification with methanol (21). Commercial production of biodiesels in the U.S. started from around 5 million gallons in 2001 to 1.1 billion gallons in 2012, which grows by a factor of 213 times in 11 years (8). Due to the fast expansion of biodiesel industry, the United States has been the first biodiesel producing nation in the world, followed by Argentina, Germany, Brazil, France, and Indonesia (8). However, due to the cost on feedstock and energy input in planting, biodiesel is still less

affordable than that fossil based diesel and the low ERORI makes the cost of production too high. As a result, approaches bypassing vegetable oils or animal oils have gained a lot of interests. Algae are used as a platform to produce biodiesel with high oil content. This approach does not compete with food production as it requires neither farmland nor fresh water. Though the commercial production does not exist at present, many companies are focusing on algae fermentation and scaling up processes (22). Microbial fermentation is another approach winning lots of interests. Strategies have been developed such as engineering *E. coli* strains to overproduce free fatty acid and fatty acid ethyl esters (FAEEs) via the introduction of ethanol production genes from *Z. mobilis* and overexpression of endogenous wax-ester synthase. Biodiesels can further be produced from lignocelluloses by the expression of hemicellulases in recombinant fatty acid derivative producers and secreted into the medium to realize consolidated bioprocessing of hemicellulose biomass directly into biodiesels (23). More approaches will be included in 1.3.2.

Long chain hydrocarbon molecules as alkanes and alkenes are another two major fuel molecules among advanced biofuels (24). They have high energy intensity and low hygroscopicity. They are also suitable jet fuel candidates, which require a low freezing point, a high energy density and comparable net heat combustion. Alkanes can be produced from fatty acid metabolites in microbes, insects and plants. Alkane pathways from plant or cyanobacteria have been constructed in non-native hosts. Long-chain alkene production

through the expression of a three-gene cluster from *M. luteus* in a fatty acid overproducing *E. coli* strain was achieved. After a series of biochemical characterizations of the strain, a metabolic pathway for alkene biosynthesis was proposed involving acyl CoA thioester and decarboxylative Claisen condensation catalyzed by OleA (25). In another publication, intermediates of fatty acid metabolism are converted to alkanes and alkenes by an acyl carrier protein reductase and an aldehyde decarbonylase. Heterologous production of C13 to C17 mixtures of alkanes and alkenes was achieved in *E. coli* by the expression of this pathway (26). Alike other advanced biofuels, extremely more efforts are required for host and pathway optimization in order to make them compatible for industrial fermentation.

1.2.*Saccharomyces cerevisiae*

1.2.1.*Saccharomyces cerevisiae*: Pros and Cons

Saccharomyces cerevisiae, also known as Baker's yeast, has been used for bread, wine and beer production for thousands of years. The Latinized Greek word "Saccharo-myces" means "sugar-fungus" and the word "cerevisiae" means "beer", which directly emphasizes its important role in fermentation. Wild type *S. cerevisiae* strains are able to ferment a series of sugars including glucose, maltose, galactose and fructose to produce ethanol anaerobically or even aerobically by Crabtree effect (27). Besides the broad range of substrates it can utilize, there are also other advantages making *S. cerevisiae* a popular model microorganism for fermentation study. As a popular eukaryotic microorganism in research,

there are well-developed genetic tools to produce multiple products. Its complete genome sequence has been obtained in 1996 and multiple genome databases and strain databases such as SGD (Saccharomyces Genome Database) and EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis) have been established, providing not only biological materials but also sequence databases to all researchers in the world. Besides well-studied genetic background and well-developed tools, *S. cerevisiae* is suitable for not only laboratory study but also for industrial fermentation due to its high tolerance of acids, inhibitors derived from upstream feedstock pretreatment and multiple alcohols, which enables high productivity and stable producing process. It also has high osmotolerance to sugars and salts, which is preferable for concentrated industrial fermentation. The capability of anaerobic fermentation also eliminates the chance to get contamination from other microorganisms that cannot survive without oxygen. Compared to other eukaryotic microorganisms, the short lag phase and low nutrition requirement guarantee high production efficiency and low cost. The capability of catabolizing multiple different substrates also provides *S. cerevisiae* a competitive advantage to outgrow other microorganisms. Last but not least, besides producing various products, *S. cerevisiae* is also a valuable byproduct widely used as animal feed or protein supplement, which increases its competiveness especially in industrial production.

Unfortunately, *S. cerevisiae* also has its disadvantages: it cannot utilize pentose to

produce ethanol due to the lack of key enzymes to introduce pentose into the cellular metabolism (28). Second, the functional temperature is limited to 30-38 ºC but not a higher temperature which may avoid contamination and enhance productivity. Third, there is little genetic work on industrial strains as the genetic characteristics are poorly studied and genetic engineering tools are limited. Regarding lignocelluloses utilization, it is not cellulolytic and thus has limited compatibility with cellulosic hydrolysis, which is a significant drawback especially in 2nd generation bioethanol production.

1.2.2.Lignocelluloses

Lignocellulose is the dominant and most abundant feedstock for cellulosic ethanol production. It is composed of cellulose, hemicellulose and lignin (29). The former two carbohydrate polymers can be converted to fermentable sugars and the last aromatic polymer cannot be utilized as a fermentable substrate. Cellulose is a polysaccharide composed of a linear chain of several hundred to over ten thousand D-glucose units linked by β-(1,4) bonds. Hemicellulose is a polysaccharide with much more complex structure. It is composed of several matrix polysaccharides such as xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan that consist of glucose, xylose, arabinose and other molecules (30). Lignin is a polymer of aromatic alcohols. It covalently binds to cellulose and hemicellulose and provides the major mechanical strength to support the plant (31).

Lignocellulose has to be processed before fermentation. There are two major

operations in lignocellulose processing: pretreatment and hydrolysis. Pretreatment loosens the rigid lignocellulose structure and prepares the substrates ready for enzyme catalyzed hydrolysis, and hydrolysis converts the carbohydrate polymers to sugar monomers. Enzymatic hydrolysis of cellulose and hemicellulose is one of the key steps affecting the cost of production. During hydrolysis, cellulose can be hydrolytically broken down by exocellulases and endocellulases to a disaccharide named cellobiose, and cellobiose can be further converted to monomeric glucose by β-glucosidase. Hemicellulose can be hydrolyzed by hemicellulases and the matric polymers can be converted to a mixture of xylo-oligosaccharides and other oligosaccharides, and then the mixture can be converted to glucose, xylose, arabinose, mannose and other sugars. Lignin cannot be hydrolyzed in this hydrolysis step, but can be burnt for electricity generation. Resulted from the processing of lignocelluloses, a mixture of sugar hydrolysates including six carbon sugars (hexoses) such as glucose, galactose and mannose and five carbon sugars (pentoses) such as xylose and arabinose is produced. Hexoses can be utilized easily by most natural microorganisms but pentose utilization is quite limited with lower efficiency and reaction rates. Thus an engineered *S. cerevisiae* strain capable of co-fermenting both hexoses and pentoses efficiently is useful for production of biofuels and other value-added chemicals.

1.2.3.Glucose Repression

Glucose repression, also called carbon catabolite repression, is one of the major

limitations in mixed sugar fermentation for ethanol production (32). It exists in almost all microorganisms and presents a significant negative effect on bioethanol production in *S. cerevisiae* (33). In the presence of glucose, utilization of other sugars is inhibited, which lowers sugar utilization efficiency and also ethanol production rate. The preference for glucose results in a sequential utilization of xylose after glucose depletion, which greatly limits fermentation efficiency using sugar hydrolysates from lignocelluloses (32,34-36).

Glucose represses other sugars' utilization on the transcriptional level, and a large number of genes are involved (32). Glucose repression either interferes transcription activators, or activates expression of proteins that have a negative effect on transcription (37). Elements in the glucose repression pathway include (a) activators such as the Hap2/3/4/5 complex, Gal4, Mal63 and Adr1, which are capable of activating the transcription of key genes involved in the catabolism of pentose and other sugars; (b) repressors such as Mig1/2/3, which play a key role in glucose repression and are capable of binding to a variety of promoters that are repressed by glucose; (c) intermediary elements such as Snf1 and Snf4, which encode protein kinases associating with other proteins; (d) glucose sensors such as Snf3 and Rtg2, which are located on yeast membranes. Under the condition of high concentration of glucose, Snf3 expression is repressed while under the condition of low concentration of glucose, Rtg2 expression is repressed. Thus, Snf3 is considered as a sensor activated on low levels of glucose while Rtg2 is activated on high levels of glucose (34,35).

However, the complicated glucose repression pathways have not been fully understood and only two pathways were studied, which covers only a small part of the regulation system (32-35).

In order to overcome glucose repression, great efforts have been made in the past decades. Two routes of glucose derepression are developed either by establishing a genetic model to study the mechanisms using systems biology tools (38-41), or by constructing glucose derepressed strains by gene modulation (37).

In the mechanistic studies, two glucose sensors, Snf3 and Rgt2, were investigated (42). They are involved in the regulation of sugar transporters including Hxt transporters. At high concentrations of glucose, Grr1 deactivates the repression of Rgt1 on Hxt transcription to avoid excess glucose transportation inside cells (42). Another pathway involves Hxk2, which is a glycolytic enzyme transducing the intracellular glucose concentration signal to Snf1 and inactivates Snf1 by a protein phosphatase Glc7-Reg1. Additionally, Snf1 is capable of phosphorylating the Mig1 protein which can translocate from the nucleus to the cytosol when it is phosphorylated. Then Mig1 is able to regulate the sugar assimilation by binding to promoters or by inducing the repression of relative genes in the assimilation of pentose sugars. Therefore, with high concentrations of glucose, Snf1 is repressed and then the dephosphorylated Mig1 represses the utilization of xylose and other sugars. On the other hand, at low concentrations of glucose, Snf is activated, which phosphorylates Mig1 to

translocate into the cytosol, thus avoiding glucose repression (34,35).

With ^{13}C-labeled glucose, phenotypic characterization of *S. cerevisiae* strains was obtained by metabolic flux analysis (43-45). The Mig1 family including Mig1, Mig2 and Mig3 was characterized (46), and the correlation between Mig1 and Mig1-dependent Hxk2 was analyzed (39). All these studies aim at clarifying Mig1-related glucose repression mechanisms (39,46-48). Based on these studies, Mig1 disrupted *S. cerevisiae* strains were constructed. However, the engineered strains (Δ*mig1*, Δ*mig2* or Δ*mig1*Δ*mig2*) did not show any significant improvement in glucose derepression as expected (37).

1.3. Advanced Biofuels Production

The development of biologically-derived ethanol has achieved significant success in the past few decades (49,50). However, ethanol exhibits some intrinsic limitations, such as low energy content and corrosiveness, which hampers its large-scale application as a fuel alternative. In contrast, advanced biofuels, such as higher alcohols, fatty acid derived fuels, and hydrocarbons, are considered to be better fuel alternatives as their physiochemical properties are more compatible with the current gasoline-based infrastructure (51).

1.3.1.Biosynthetic Pathways for Advanced Biofuels

Propanol and butanol are two higher alcohols than ethanol. Native *Clostridia* strains are able to produce isopropanol and n-butanol to low levels (~2 g/L). Isopropanol production pathways have been constructed in engineered *E. coli* strains by introducing enzymes

converting acetyl-CoA to acetone and enzymes converting acetone to isopropanol. Genes for the former function were from either *Clostridium acetobutylicum* (*thl*, *ctfAB*, and *adc*) encoding acetoacetate decarboxylase or *E. coli* (*atoAD*) encoding acetyl-CoA acyltransferase and the gene encoding alcohol dehydrogenase (*adh*) was from *Clostridium beijerinckii* (52). Similar to ethanol, isopropanol is also toxic to *E. coli*. The titer was enhanced to 143 g/L by immediate removal of isopropanol in medium by gas trapping. The CoA-dependent *Clostridia* pathway was introduced into *E. coli* for n-butanol production and the titer was enhanced to 30 g/L by introduction of a transenoyl-CoA reductase (*pdaA*) from *Ralstonia eutrophus*, the overexpression of a pyruvate dehydrogenase complex complex (*aceEF–lpd*) from *E. coli*, and the deletion of NADH-competing enzymes as *frd*, *ldhA*, and *adhE* encoding fumarate reductase, lactate dehydrogenase and alcohol dehydrogenase (19). Use of non-fermentative pathway such as the 2-keto-acid pathway is another strategy for higher alcohols production in engineered *E. coli*, in which 2-keto acids were converted to aldehydes by 2-keto acid decarboxylases and aldehydes were converted to alcohols by alcohol dehydrogenases, which resulted in 0.85 g/L n-butanol production (52). The titer of higher alcohols was further enhanced to 22 g/L isobutanol after the flux towards the keto acid was increased by the overexpression of enzymes as *alsS* and *ilvCD* encoding acetohydroxy acid isomeroreductase and dihydroxy acid dehydratase (52).

Fatty acids is an important precursor of fatty alcohols, FAEEs, alkanes and alkenes

and is also a major native product in wild type cells. Thus the synthesis, engineering and regulation of fatty acids attract great interests in advanced biofuel production. The native fatty acid pathway in bacteria starts from acetyl-CoA which is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) and then converted to malonyl-ACP by malonyl-CoA:ACP transacylase (FabD). Fatty acyl is elongated by FabH which condenses malonyl-ACP and acetyl-CoA to generate a β-ketone type acetoacetyl-ACP, The acetoacetyl-ACP is then reduced to an alcohol. The resulting alcohol is dehydrated to generate a trans double bond, which is further reduced by NADPH to generate a saturated chain of a fatty acid catalyzed by a series of enzymes including FabG, FabZ, and FabI (53). This cycle can be repeated several times by adding malonyl-ACP to elongate the acyl chain (54).

FAEEs and fatty alcohols production in engineered *E. coli* strains were reported. The *fadD* gene was overexpressed to convert fatty acids to acyl-CoAs and either a wax-ester synthase (AtfA) to esterify acyl-CoAs to FAEEs or an acyl-CoA reductase (Acr1) to reduce acyl-CoAs to alcohols was expressed respectively (55). FAEE production in *S. cerevisiae* includes the deletion of the genes involved in storage lipids synthesis including *dga1, lro1, are1* and *are2,* and the overexpression of a wax-ester synthase WS/DGAT (56).

Alkanes can be produced in by expressing cyanobacteria genes in engineered *E. coli*. An acyl-ACP reductase (AAR) was expressed together with an aldehyde decarboxylase (ADC) to convert acyl-ACPs to aldehydes and aldehydes to alkanes, respectively (26). An

alkene synthetic pathway was constructed by expressing a three-gene cluster from *Micrococcus luteus* to condense two acyl-CoAs head to head and then to reduce and dehydrate the intermediates for alkene production in *E. coli* (25). Besides, alkenes can also be produced from fatty acid decarboxylation by expressing a cytochrome P450 enzyme OleTJE from *Jeotgalicoccus spp.* in *E. coli* (57).

1.3.2.Pathway Optimization

To enhance the productivity of advanced biofuels for commercialization, optimization of their biosynthetic pathways and hosts are needed. To this end, many strategies have been developed to control the flux of the biosynthetic pathways. For example, the copy number of a gene can be manipulated by using plasmids with varying copy numbers (58) or by integrating the pathway into genome at single or multiple locations (59,60). Transcriptional levels can be manipulated and balanced by the utilization and combination of constitutive or inducible promoters with different strengths (61-64) or by the control of transcriptional termination efficiency using synthetic terminators. Translational level manipulations can be realized by artificial ribosome binding site (RBS) with different strengths (65) or by inserting functional RNA segments into intergenic regions of operons (66) to regulate the processing and stability of mRNAs. Multiple enzymes can be chosen and combined from a library of enzyme candidates from various microorganisms (67) or directed evolution (68) while enzyme stability can be controlled by programmable degradation rate using peptide tags (69).

However, the productivity is fixed whenever the pathway construction process is completed and no more monitoring or dynamic controlling can be done, which limits further optimization during fermentation. To address this issue, researchers have developed dynamic controlling approaches by constructing a biosensor detecting the concentration of acyl-CoAs in an FAEE producing *E. coli*. The transcriptional regulation based biosensor can reflect the intracellular concentration of acyl-CoAs by fluorescence signal and control the expression of genes in FAEE biosynthetic pathways (70). This strategy provides a novel way of pathway control and real-time monitoring, which serves as an important tool in synthetic biology. Utilization of transcriptional regulation as an approach to regulate the metabolic flux and report intracellular metabolite concentrations has attracted increasing interests (71-73).

1.4.Biosensors in Microorganisms

The development of synthetic biology promotes the construction of pathways with multiple genes and corresponding genetic elements for the production of various compounds. Automation technology also improves large-scale gene library construction and microbial strain library construction (74). Although building a biosynthetic pathway becomes easier, high throughput screening for higher productivity and yield is extremely important and critical in applications. Thus it is essential to develop sensors for monitoring productivity and for controlling pathways dynamically. Recently, many biosensors have been developed in

microorganisms to detect environmental signals, extracellular and intracellular chemicals based on different mechanisms.

1.4.1.FRET-based Biosensors

Fluorescence resonance energy transfer (FRET) transfers the energy from one donor fluoromophore to the receptor fluoromophore in living cells (75). A FRET biosensor consists of a recognition module binding to the target ligand and two fluorescence proteins with different emission wavelengths. The efficiency of fluorescence energy transfer between the two fluorophores is highly dependent on their distance and orientation. A conformational change in the binding domain leads to a FRET efficiency change, thus the sensor is able to detect target chemicals with trace concentrations. FRET sensors have been reported in the quantification of key metabolites including ATP, NADH, cAMP, cGMP, ribose, glucose, maltose, sucrose and glutamate as well as ions such as calcium or phosphate (76-81). An arabinose sensor using FRET mechanism was established in *E. coli* by the utilization and optimization of the binding domain derived from a high-affinity L-arabinose binding protein AraF (82). When the domain is bound to arabinose, energy is transferred from eCFP to Venus and a Venus/eCFP emission ratio of ~2 was achieved. This sensor was used for monitoring intracellular arabinose levels in *E. coli*. Maltose sensors were also established following the same strategy, making it possible to calculate the accumulation rates after the addition of maltose (82,83).

1.4.2.Transcriptional-Regulation-Based Biosensors

Biosensors based on transcriptional regulation have been constructed and utilized in microorganisms to detect a series of key metabolites and products including alcohols (70), S-adenosylmethionine (SAM) (84), farnesyl pyrophosphate (FPP) (85) and acyl-CoAs (70). The key elements in these sensors include a metabolite-responsive transcription factor (TF) which is either an activator or a repressor, an operator which the transcription factor binds to and a reporter which is usually a fluorescence protein. The affinity of TF to the operator changes by the conformational change due to the binding of the target metabolite and the signal intensity changes because of the regulated transcription of reporter genes. Moreover, a gene circuit responding to the metabolite concentration can be constructed to regulate downstream and upstream gene expression.

A dynamic sensor-regulator system was constructed for the detection and control of acyl-CoA concentration in *E. coli*, in which transcription of several heterologous genes were controlled by a fatty acid/acyl-CoA responsive protein FadR. An n-butanol sensor using a putative σ54-transcriptional activator (BmoR) and a σ54-dependent, alcohol-regulated promoter (PBMO) derived from *Pseudomonas butanovora* was also constructed and utilized in the screening of high productivity *E. coli* strains (86).

Recently, Xu *et al.* reported the construction of a malonyl-CoA sensor in *E. coli* by incorporating the *B. subtilis* trans-regulatory protein FapR and the cis-regulatory element

fapO. The engineered hybrid promoter-regulator system could respond to a range of intracellular malonyl-CoA from 0.1 nmol/mg DW to 1.1 nmol/mg DW (87). Liu *et al.* also constructed a malonyl-CoA sensor and gene circuit using the same TF. The reported gene circuit in *E. coli* could respond to the intracellular concentration of malonyl-CoA and regulate the transcription of acetyl-CoA carboxylase (ACC) which converts acetyl-CoA to malonyl-CoA (88).

Due to the complex regulation system and the existence of a nucleus, it is much more difficult to construct a transcriptional regulation based biosensor in yeast. Moreover, TFs were usually identified in bacteria, which limits the efficient expression. A successful example of sensor in *S. cerevisiae* is the construction a gene circuit of S-adenosylmethionine (SAM). Umeyama *et al.* (84) utilized the met operator and MetJ repressor of *E. coli* to construct a synthetic gene circuit to report intracellular SAM concentrations and to screen for target genes for enhancing SAM productivity from a genomic library. Though the need of sensors for efficient biofuel production in yeast is urgent, there is limited research on advanced biofuel or corresponding metabolite sensors.

1.4.3.Riboswitch-based Biosensors

There are many regulatory RNA molecules serving as biosensors for intracellular metabolites. A typical riboswitch is composed of two parts: an aptamer which is single-stranded nucleic acids possessing unique binding characteristics to the target, and an

expression platform with structural changes in response to the changes in the aptamer. Riboswitches detecting riboflavin or thiamin utilizing mRNA aptamer responsive to riboflavin or thiamin in *B. subtilis* (89) or *E. coli* (90)were developed.

Aptamers can be selected against a wide variety of target molecules including small organics, peptides and proteins (91,92) with a great range of binding affinities from the picomolar scale to the nanomolar scale, and aptamers can differentiate closely related compounds. By virtue of these features, artificial aptamers specific to target metabolites can be potentially selected using systematic evolution of ligands (93). A hammerhead Sm1 ribozyme from *Schistosoma mansoni* was modified and inserted into the coding region of a mammalian cell gene, and a target toyocamycin was obtained from a library of small molecules screened for their abilities to regulate Sm1 activity (94). In another study, an antisense RNA sequence was added to a well-characterized theophylline responsive aptamer (95). The antisense sequence was able to interact with a target mRNA to affect translation in the presence of theophylline. Extending this strategy to other aptamers requires rescreening of compatible secondary structures to create functional riboswitches, which can be difficult.

1.4.4.Byproduct-based Biosensors

There are various approaches for biosensor construction. An example of auxotroph-based strategy is a sensor detecting the concentration of mevalonate which is a key intermediate in the isoprenoid biosynthetic pathway (96). The engineered *E. coli* strain

expressing GFP is mevalonate auxotroph, so the mevalonate concentration was reflected by the fluorescence intensity. The sensor could be used in high throughput screening but practical application of this strategy is limited because it cannot be used to regulate corresponding pathways and the target metabolite has to be membrane permeable. Santos and Stephanopoulos (97) also described a tyrosine biosensor in *E. coli* by converting tyrosine to melanin as a reporter of tyrosine productivity. Melanin is a black pigment and can be easily screened in solid culture. By virtue of the conversion to colored pigment, the concentration of tyrosine was converted to a visible signal ready to be measured by a spectrophotometer.

1.5. Project Overview

This thesis focuses on metabolic engineering of *S. cerevisiae* for efficient cellulosic ethanol production and improving advanced biofuel production based on monitoring and dynamic control of intracellular metabolites. *S. cerevisiae* cannot utilize the sugar mixture from biomass simultaneously mainly because of glucose repression. Most studies focused on the improvement of xylose utilization, while the utilization of glucose is either ignored or weakened. Here we designed a novel strategy enabling co-utilization of cellobiose and xylose derived from lignocelluloses for cellulosic ethanol production. Engineering of *S. cerevisiae* for advanced biofuel production such as fatty alcohol production has attracted a lot of interests, but the productivity is limited by inefficient screening tools and insufficient

methods for dynamic control of metabolic flux. Here we developed *in vivo* biosensors capable of detecting the concentration of precursors in the production of advanced biofuels and proposed the strategy to construct advanced biofuel producing pathways dynamically regulated by transcriptional regulation.

In Chapter 2, I established a novel approach to improve the efficiency of mixed sugar fermentation. Cellobiose is the main source of glucose during hydrolysis and the cellobiose-xylose mixture is easy to get with lower cost on hydrolases compared to what?. Native *Neurospora crassa* is able to assimilate cellobiose by cellobiose transporters and β-glucosidase. Identification of the corresponding proteins in *N. crassa* facilitated the construction of a recombinant *S. cerevisiae* strain capable of utilizing cellobiose. Together with the xylose utilization pathway constructed in the strain, the resultant strain is able to co-utilize cellobiose and xylose, bypassing glucose repression without compromising the glucose utilization efficiency. A library of cellobiose utilization pathways were established and compared and the co-fermentation of cellobiose and xylose or cellobiose, glucose and xylose was investigated. The resultant strain showed high capability of sugar co-utilization and ethanol production. The novel sugar co-utilization strategy may reduce the production cost of all fuels and chemicals from biomass.

Chapter 3 further investigated the cellobiose utilization process in *S. cerevisiae*. Though the cellobiose-xylose co-utilization performance surpasses the glucose-xylose

utilization performance, the utilization rate of cellobiose is still lower than that of glucose. To further improve cellobiose utilization, the role of glucose anomers in sugar co-utilization was investigated for the first time. Due to different cellobiose hydrolysis environments, the major product of intracellular cellobiose hydrolysis is β-glucose, which limits rapid glycolysis reactions. A crucial enzyme Gal10, an aldose 1-epimerase (AEP), was studied by genetic modification and the deletion of Gal10 was found to decrease the cellobiose utilization rate in mixed cellobiose and xylose fermentation. Another two putative AEPs were identified and compared and a complicated regulation system was discovered in cellobiose utilization.

In Chapter 4, a malonyl-CoA sensor based on transcriptional regulation was established in *S. cerevisiae*. The sensor responds to cytosolic malonyl-CoA concentration and provides an efficient tool for high throughput screening. Malonyl-CoA is the key intermediate in native fatty acid synthesis and can be used as an indicator for production of multiple chemicals including FAEEs, fatty alcohols and value-added chemicals such as 3-hydroxypropionic acid. The sensor monitors intracellular malonyl-CoA concentration and converts chemical concentration hard to detect to fluorescence signals that can be detected in a short time. The sensor was combined with an RNAi based genome wide mutant library in *S. cerevisiae*, and used to screen for mutants with higher productivity of malonyl-CoA, thus improving the downstream production of 3-hydroxypropionic acid.

In Chapter 5, I explored a novel strategy to discover *in vivo* biosensors for native

metabolites. By transcriptional profiling, a series of promoters responsive to metabolites can be found. The Plug and Play strategy utilizes the native regulation mechanisms in *S. cerevisiae*, minimizes extrinsic manipulation and screens for multiple “Plug-in”s with various transcription activities in a short time. A series of sensors for the detection of sugar phosphates and acetyl-CoA were established and evaluated. The acetyl-CoA sensor was further optimized and was able to screen for constructs with higher productivity of n-butanol. This strategy provides an innovative approach for metabolite monitoring and pathway control.

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Chapter 2. Construction of a *S. cerevisiae* Strain Capable of Simultaneously Utilizing Cellobiose and Xylose

2.1.Introduction

Cellobiose is one of the intermediate products from cellulose hydrolysis. Catalyzed by a cellulose cocktail composed of exocellulases, endocellulases and β-glucosidases, cellulose is degraded to cellobiose and cellobiose is further converted to glucose (1-5) . In the conventional methods for mixed sugar fermentation in *S. cerevisiae*, a mixture of glucose and pentose sugars derived from lignocellulose are used, where cellobiose inhibits endoglucanases and cellohydrolysases in hydrolysis. To relieve the inhibitory effect, simultaneous saccharification and fermentation (SSF) process is utilized by combining lignocellulose hydrolysis and fermentation of glucose and pentose sugars (1,2,5).

Compared to the study on the SSF process, research on intracellular cellobiose utilization was rare. The very few publications about ethanol production from cellobiose either lacked an efficient sugar uptake pathway, or used a low efficiency pathway that cannot be further improved (6,7). Gurgu and coworkers reported the construction of a cellobiose utilizing *S. cerevisiae* strain by heterologous expression of a *Saccharomycopsis fibuligera* β-glucosidase gene (BGL1) under the control of a constitutive promoter and observed ethanol production from the recombinant strain (6). β-glucosidase was secreted extracellularly where cellobiose was hydrolyzed to glucose. Limited by the expression level of β-glucosidase, only

small amount of glucose was produced, resulting in marginal concentration of ethanol produced. Other transporters and enzymes with relatively low activities were also expressed in *S. cerevisiae* in order to construct cellobiose assimilating strains. For example, a cellobiose phosphorylase and a lactose permease were co-expressed in a recombinant *S. cerevisiae* strain to enable intracellular cellobiose hydrolysis and utilization in *S. cerevisiae* (7). The expression of lactose permease derived from *Kluyberomyces lactis* facilitated cellobiose transportation and the expression of a cellobiose phosphorylase derived from *Clostridium stercorarium* converted intracellular cellobiose to glucose-phosphate, which entered the glycolysis pathway after catalysis. However, the cellobiose utilization efficiency was limited by the low activity of cellobiose phosphorylase and the low transportation efficiency of the nonspecific lactose permease.

A natural cellulolytic fungi *Neurospora crassa* was studied for the identification and characterization of cellodextrin (glucose polymers including cellobiose, cellotriose, cellotetraose, etc) transporters (8). Two celledextrin transporters, CDT1 and CDT2, were discovered. CDT1 is a symporter with higher cellobiose uptake activity and CDT2 is a facilitator with lower activity. These two transporters were re-constituted in *S. cerevisiae* and were proven to promote efficient cell growth on cellodextrins.

After the expression of cellodextrin transporters in *S.* cerevisiae, it is reasonable to co-express both the transporters and enzymes converting cellobiose to fermentable glucose or

glucose-derived intermediates in S. cerevisiae to improve cellobiose utilization together with other sugars in the hydrolysates. At the same time as Professor Yong-su Jin's group was evaluating the feasibility of co-fermenting cellobiose and xylose by co-expressing a cellobiose transporter and a β-glucosidase together with xylose utilization enzymes in *S. cerevisiae* (9), we also designed a similar strategy to enhance the utilization efficiency of cellobiose (Figure 2.1). The high efficiency pathway enables fast cellobiose utilization and ethanol production, which makes it an attractive platform for mixed sugar fermentation. Besides, this strategy represents a novel approach to address the problem called glucose repression. Cellobiose rather than glucose is used as the main carbon source in our new strategy. A mixture of cellobiose and xylose is used for ethanol production. Cellobiose is transported inside yeast cells via the heterologous cellobiose transporters while xylose is transported by endogenous hexose transporters, thus preventing direct competition between glucose and pentose sugars in the transport process. Once inside yeast cells, cellobiose is converted to glucose by β-glucosidase and immediately consumed by yeast cells, which results in a low intracellular glucose concentration, thereby further alleviating glucose repression. Distinguished from existing glucose derepression methods, there is no gene deletion in the yeast strain, and glucose utilization is not impaired, while xylose utilization is improved because of synergistic effects. This strategy avoids the almost inevitable glucose repression in lignocelluloses fermentation for the first time, and improves both sugars' utilization at the same time. Based on this engineered cellobiose-xylose co-utilization strain,

evolutionary engineering and metabolic flux modification can be carried out to obtain more efficient ethanol producing strains.

2.2.Results

2.2.1.Comparison of Various Cellobiose Utilization Pathways in a Laboratory *S. cerevisiae* Strain

As proof of concept, the mixed sugar fermentation consisting of xylose and cellobiose was used as a model system. Specifically, an engineered xylose-utilizing yeast strain HZ3001 was used as a host to co-express a cellobiose transporter gene and a β-glucosidase gene. In this strain, the xylose utilization pathway consisting of xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis* was integrated into the chromosome. Three cellodextrin transporter genes from *N. crassa*, including *cdt-1*, *NCU00809*, and *cdt-2* and two β-glucosidase genes, one from *N. crassa* (*gh1-1*) and the other from *Aspergillus aculeatus* (*BGL1*), were evaluated. A total of six different strains, referred to as SL01 through SL06, were constructed by introducing a pRS425 plasmid harboring one of the cellobiose transporter genes and one of the β-glucosidase genes into the HZ3001 strain (Figure 2.2). In each plasmid, the cellobiose transporter gene and the β-glucosidase gene were assembled into the multi-copy plasmid pRS425 by the DNA assembler method (10). The empty pRS425 plasmid was introduced to the HZ3001 strain to yield the SL00 strain, which was used as a negative control. All strains were cultivated in the YPA medium supplemented with 40 g/L

cellobiose and 50 g/L xylose in shake-flasks, and their sugar consumption rates, cell growth rates, and ethanol titers were determined (Figure 2.3).

Among all strains, the SL01 strain expressing *gh1-1* and *cdt-1* showed the highest sugar consumption rate and ethanol productivity. Thus, this strain was selected for further characterization.

2.2.2. Co-fermentation of Cellobiose and Xylose in an Engineered Laboratory *S. cerevisiae* Strain

Both SL01 and SL00 were cultivated using the YPA medium supplemented with 40 g/L cellobiose and 50 g/L xylose in both shake-flasks and bioreactors (Figure 2.4). In the shake-flask cultivation (Figure 2.4 a and b), 83% of the cellobiose was consumed in 96 hours by SL01, with a 41.2% higher overall xylose consumption rate (from 0.33 g/L h to 0.46 g/L h) compared to SL00. Consistent with the enhanced sugar consumption rate, 2.3-fold higher overall dry cell weight growth rate was observed (from 0.031 g dry cell weight/L h to 0.072 g dry cell weight/L h). The ethanol productivity was increased by more than 3.1-fold, from0.07 g/L h to 0.23 g/L h. The highest ethanol yield of 0.31 g per g sugar was reached in 48 hours, and the overall ethanol yield was 0.28 g per g sugar, representing a 23% increase compared to the SL00 strain. In the SL01 cultivation, a faster xylose consumption rate was observed, without the lag phase that is the hallmark of glucose repression in co-fermentation of glucose and xylose. Moreover, improved cell growth and ethanol production were also observed. In

the bioreactor cultivation (Figure 2.4c and d), almost all of the cellobiose and 66% of the xylose were consumed in 48 hours by SL01, representing 42% increased xylose consumption rate (from 0.48 g/L h to 0.68 g/L h) and 1.02-fold increased dry cell weight growth rate (from 0.08 g dry cell weight/L h to 0.17 g dry cell weight /L h) compared to SL00. The ethanol productivity was increased by more than 4.4-fold (from 0.09 g/L h to 0.49 g/L h) and the ethanol yield was 0.39 g per g sugar. Compared to shake-flask cultivations, sugar consumption rates in the first 24 hours were lower due to the low cell density used in the beginning of batch cultivation.

2.2.3.Co-fermentation of Cellobiose and Glucose in an Engineered Laboratory *S. cerevisiae* Strain

To determine whether a small concentration of glucose will repress cellobiose utilization significantly, a mixture of 10 g/L glucose and 40 g/L cellobiose was tested using the SL01 strain. For SL01, with solely 10 g/L glucose, 91.3% glucose was consumed and the maximum ethanol productivity and yield reached 0.40 g/L h and 0.32 g per g sugar, respectively, at 9 hours. After that, ethanol was gradually consumed (Figure 2.5 b). In comparison, with 10 g/L glucose and 40 g/L cellobiose, the ethanol productivity and yield were 0.38 g/L h and 0.28 g per g sugar, respectively, at 9 hours, and reached the maximum level (0.44 g/L h and 0.30 g per g sugar, respectively) at 24 hours (Figure 2.5 a). Thus, the effect of cellobiose on the maximum ethanol yield and productivity was insignificant. For

SL00, with 10 g/L glucose and 40 g/L cellobiose, no cellobiose consumption was observed (Figure 2.5 c), while with solely 10 g/L glucose, the profile of glucose consumption and ethanol production was almost identical to that of SL01. It was found that the presence of cellobiose increased the overall ethanol productivity, but its effect on the maximal ethanol yield and productivity seems to be insignificant.

2.2.4.Co-fermentation of Cellobiose, Xylose, and Glucose in an Engineered Laboratory *S. cerevisiae* Strain

A small amount of glucose (less than 10% of total sugars) is typically present in lignocellulosic hydrolysates when cellulose cocktails deficient in β-glucosidase were used to catalyze the hydrolysis of lignocellulosic materials. Thus, the fermentation performance of the engineered SL01 strain was also investigated using a mixture of cellobiose, xylose and glucose. Two concentrations of glucose, 5 g/L or 10 g/L, were combined with 40 g/L cellobiose and 50 g/L xylose as a mixed carbon source in bioreactors.

In the batch cultivation with 5 g/L glucose (Figure 2.6a and b), 81.5% cellobiose and 69.3% xylose were consumed, respectively, by SL01 at 48 hours. Compared to SL00, the xylose consumption rate was increased by 89%, from 0.38 g/L h to 0.73 g/L h. The ethanol productivity was increased by 2.2-fold (from 0.13 g/L h to 0.43 g/L h) while the ethanol yield was increased from 0.24 g per g sugar to 0.30 g per g sugar. In the batch cultivation with 10 g/L glucose (Figure 2.6 c and d), 74.3% cellobiose and 74.4% xylose were consumed

respectively, by SL01 at 48 hours. Compared to SL00, the xylose consumption rate was increased by 52%, from 0.51 g/L h to 0.77 g/L h. The ethanol productivity was increased by 1.1-fold (from 0.21 g/L h to 0.45 g/L h) and the ethanol yield was increased from 0.27 g per g sugar to 0.31 g per g sugar at 72 hours.

2.2.5.Cellobiose Utilization in an Industrial *S. cerevisiae* Strain

Compared to laboratory *S. cerevisiae* strains, industrial *S. cerevisiae* strains have much higher ethanol production capability and robustness. Despite of the advantages of industrial strains in fermentation, it is difficult to make gene modification based on very limited information of the multi-ploid industrial strains.

To test the performance of cellobiose assimilating system on the platform close to large scale fermentation, construction of an industrial strain capable of utilizing cellobiose is necessary. Based on the cellobiose assimilating system utilized in a laboratory strain, we introduced a multi-copy plasmid harboring the cellobiose pathway (*cdt1-gh1-1*) into an industrial strain, which resulted in the SLI01 strain capable of utilizing cellobiose efficiently. We compared its fermentation performance with that of the wild type industrial strain SLI00.

In shake-flask cultivation with 90 g/L cellobiose supplemented (Figure 2.7 a and b), 94.2% cellobiose was consumed by SLI01 in 48 hours, while the wild type strain hardly showed any consumption. The ethanol productivity was 0.64 g/L h while the ethanol yield was 0.42 g per g sugar, close to theoretical yield. Biomass production was quite high due to

the characteristics of the parent strain.

Based on the cellobiose cultivation result, we asserted that an industrial strain with an engineered cellobiose pathway was capable of efficiently producing ethanol using cellobiose and xylose as carbon sources.

2.3.Discussions

To create an efficient cellobiose utilizing pathway in *S. cerevisiae*, the performance of different combinations of cellobiose transporter and β-glucosidase was evaluated because the balance between the cellobiose uptake rate and the cellobiose conversion rate plays an important role in efficient sugar consumption. Three cellobiose transporters from *N. crassa* and two β-glucosidases were used to create six different cellobiose utilization pathways in a multi-copy plasmid for further overexpression in a target yeast strain. In order to obtain a *S. cerevisiae* strain capable of co-utilizing xylose and cellobiose, a mixture of cellobiose and xylose was used to select the most efficient cellobiose utilization pathway for further analysis. By comparing sugar consumption rate, ethanol productivity and yield, and biomass production in shake-flask fermentation, the combination with *N. crassa* cellobiose transporter *cdt1* and *N. crassa* β-glucosidase *gh1-1* was selected.

There are two types of *S. cerevisiae* strains, modified laboratory strains and real “wild type” industrial strains. The former was derived from naturally existing *S. cerevisiae* strains,

but then modified to make a simple model for genetic studies. As a result, laboratory strains are often used as benchmark strains because of their advantages such as well-studied gene background, available auxotrophic or antibiotic resistant markers, and haploid genotype which enables simple gene modification. The wild type industrial strains were discovered in long term fermentation adaptation and chosen from industrial fermentation process. Usually industrial strains have fast sugar utilization, ethanol production and biomass production. Industrial strains are usually diploid or multi-ploid, non-auxotrophic, and antibiotic resistant markers are not available. Although it is difficult to modify or engineer, industrial strains are robust and efficient ethanol production hosts. Here we tested the cellobiose utilization pathway in both a laboratory yeast strain and an industrial yeast strain.

In the laboratory yeast strain, we tested different combinations of sugar mixtures, including cellobiose and xylose, cellobiose and glucose, and cellobiose, xylose and glucose. The combination of cellobiose and xylose aimed at co-fermentation ability of these two sugars from lignocelluloses, while additive glucose was used to test whether glucose repression exists in cellobiose based co-fermentation process. Both small-scale fermentation in shake-flasks and bioreactor fermentation were tested. Shake-flask cultivation was used as a simple and easy method and batch cultivation in a bioreactor exhibited better productivity due to the precise oxygen supply and pH control. We found out that in the cellobiose and xylose co-fermentation system, our engineered laboratory strain showed significantly

improved sugar utilization, and the synergistic effect made it even better than the single cellobiose or xylose fermentation system. The ethanol productivity and yield shown here were much higher than what was obtained from single xylose fermentation. The cellobiose-xylose co-fermentation system represents a high-efficiency system with no glucose repression. Besides, from the results with added glucose to single cellobiose, or to the cellobiose-xylose mixture, we still found greatly improved sugar consumption and limited glucose repression, which suggests even with a small amount of glucose derived from sugar hydrolysates, the utilization of cellobiose and xylose is still efficient enough.

To construct the cellobiose-xylose co-utilizing pathway in an industrial strain, we introduced a cellobiose pathway into an industrial strain containing an integrated xylose utilization pathway. The resultant strain showed high ethanol production and sugar utilization, which were much higher than the laboratory strain: ethanol productivity was enhanced from 0.23 g/L h to 0.64 g/L h and cellobiose utilization rate was enhanced from 0.35 g/L h to 1.77 g/L h. The robust and efficient industrial yeast strain enables further establishment of the cellobiose-xylose utilizing system. The industrial strain also could serve as a model for glucose derepression study.

2.4.Conclusions and Outlook

Glucose repression is a well-studied regulatory mechanism in *S. cerevisiae*. Various approaches have been attempted to overcome glucose repression, such as evolutionary

engineering and deletion of key genes involved in glucose repression. However, these approaches met with only limited success. In our new strategy, the cellobiose will be transported into yeast cells via a heterologous cellobiose transporter, while pentose sugars will be transported into yeast cells by endogenous hexose transporters, thus mitigating the direct competition between glucose and pentose sugars for the same transporters that partly causes glucose repression. Once inside yeast cells, cellobiose will be converted to glucose by β-glucosidase and consumed, which should result in a low intracellular glucose concentration, thereby further alleviating glucose repression.

By co-expressing a cellobiose transporter gene and a β-glucosidase gene either in an engineered xylose-utilizing *S. cerevisiae* laboratory strain or in a high-productivity industrial strain, and using sugars including xylose and cellobiose or xylose, cellobiose, and a small amount of glucose, or single cellobiose as carbon sources, we demonstrated that these sugars can be consumed simultaneously to produce ethanol with high yields.

Overcoming glucose repression in mixed sugar fermentation in *S. cerevisiae* improved the overall sugar utilization efficiency and ethanol productivity, which is highly desirable in biofuels production. Varied pathways have been established in multiple microorganisms for cellobiose utilization (11-14). Recent progress in combinatorial pathway optimization and directed evolution significantly improves the efficiency of cellobiose utilization by both protein evolution and transcriptional optimization (15,16). Studies on the discovery and

engineering of more efficient transporters and enzymes catalyzing hydrolysis or phosphorylation coupled with engineering of a more efficient xylose-utilizing pathway may further enhance the co-utilization efficiency of lignocelluloses for biofuel production.

2.5.Materials and Methods

2.5.1.Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae L2612 (*MATα leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal*+) was a gift kindly provided by Professor Yong-su Jin (17). *Escherichia coli* DH5α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5%ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in un-baffled shake-flasks at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition .*E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific unless noted otherwise.

2.5.2.Strain and Plasmid Construction

To integrate the xylose utilization pathway consisting of xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis*, the genes and corresponding promoters

and terminators (ADH1 promoter-xylose reductase-ADH1 terminator, pGK1 promoter-xylitoldehydrogenase-CYC1 terminator, pYK1 promoter-xylulokinase-ADH2terminator) were PCR-amplified and cloned into the pRS416 plasmid using the DNA assembler method. BamHI and HindIII were used to remove the DNA fragment encoding the xylose utilization pathway and then ligated to the pRS406 plasmid digested by the same two restriction enzymes. The resulting plasmid was then linearized by ApaI and integrated into the URA3 locus on the chromosome of L2612, resulting in a recombinant xylose-utilizing yeast strain HZ3001. The pRS425 plasmid (New England Biolabs, Ipwich, MA) was used to co-express a cellobiose transporter gene and a β-glucosidase gene. As shown in Figure 2.2, the pRS425 plasmid was digested by BamHI and ApaI. The PYK1 promoter and the ADH1 terminator were added to the N-terminus and C-terminus of the β-glucosidase respectively, while the TEF1 promoter and the PGK1 terminator were added to the N-terminus and C-terminus of the cellobiose transporter respectively (Table 2.2). These DNA fragments were assembled into the linearized pRS425 shuttle vector using the DNA assembler method (10). Three cellobiose transporter genes *cdt-1* (GenBank Accession number XM_958708), NCU00809 (GenBank Accession number XM_959259) and *cdt-2* (GenBank Accession number XM_958780) from *N.crassa* and two β-glucosidase genes *gh1-1*(GenBank Accession numberXM_951090) from *N. crassa* and *BGL1* (GenBank Accession number D64088) from *Aspergillus aculeatus* were used. There are six combinations in total, each with one cellobiose transporter gene and one β-glucosidase gene (Table 2.1).

Yeast plasmids were then transferred into *E. coli* DH5α, which were plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Miniprep Kit (QIAGEN). These plasmids were transformed into the L2612 strain individually to yield the following strains: SL01 (containing the plasmid harboring the *cdt-1* cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL02(containing the plasmid harboring the NCU00809 cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL03 (containing the plasmid harboring the NCU08114cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL04(containing the plasmid harboring the *cdt-1*cellobiose transporter gene from *N. crassa* and the BGL1 gene from *A. aculeatus*), SL05 (containing the plasmid harboring the NCU00809 cellobiose transporter gene and the BGL1 gene from *A. aculeatus*), and SL06 (containing the plasmid harboring the *cdt-2*cellobiose transporter gene from *N. crassa* and the *BGL1* gene from *A. aculeatus*). The empty pRS425 plasmid was transformed into the HZ3001 strain to yield the SL00 strain as a negative control. Yeast transformation was carried out using the standard lithium acetate method (18). The resulting transformation mixtures were plated on SC-Ura-Leu medium supplemented with 2% glucose. To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Orange, CA) and then transformed into *E. coli* DH5α cells. The resulting cells were spread on LB plates

containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion using ClaI and HindIII. All restriction enzymes were obtained from New England Biolabs (Ipwich,MA).

2.5.3.Mixed Sugar Fermentation in Shake-Flasks

For each yeast strain, a single colony was first grown up in 2 mL SC-Ura-Leu medium plus20 g/L glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake-flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 40 g/L cellobiose and 50 g/L xylose; 40 g/L cellobiose, 50 g/L xylose, and 5 g/L glucose; or 40 g/L cellobiose, 50 g/L xylose, and 10 g/L glucose in a 250 mL un-baffled shake-flask. YPA media supplemented with 10 g/L glucose and 40 g/L cellobiose or solely 10 g/L glucose were also used to determine the ethanol productivity in the presence of cellobiose. Starting from an initial OD600 ~ 1, cell cultures were grown at 30 °C at 100 rpm for fermentation under oxygen limited conditions. OD600 readings and cell culture samples were taken at various time points. Dry cell weight was measured gravimetrically using an aluminum foil weighing dish after evaporating under 65 °C for approximate 72 hours. Sugars and ethanol concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and Shimadzu RID-10A refractive index

detector following the manufacturer's protocol. The HPX-87H column was kept at 65 °C using a Shimadzu CTO-20AC column oven. 0.5mM sulfuric acid solution was used as a mobile phase at a constant flow rate of 0.6 mL/min. 10 μL of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 25 minutes after the injection. The concentration of the sugars and ethanol were determined using a standard curve generated using a series of external standards. Each data point represented the mean of triplicate samples. The mixed sugar fermentation data for the strains ranging from SL00 to SL06 are shown in Figure 2.3. The best strain SL01 was selected for further characterization. In addition, both SL00 and SL01 were cultivated using the YPA media supplemented with a mixture of 10 g/L glucose and 40 g/L cellobiose or solely 1% glucose (Figure 2.5).

2.5.4.Mixed Sugar Fermentation in Bioreactors

The Multifors system (Infors-HT, Bottmingen, Switzerland) was used for mixed sugar fermentation. Each vessel has a total capacity volume of 750 mL. For each vessel, there was one set of a pO2 sensor, air sparger, exit gas cooler, temperature sensor, inoculation port, spare port, dip tube, antifoam sensor, pH sensor, drive shaft, heater block, rotameter, and peristaltic pump system. The whole bioreactor system was equipped with a ThermoFlex900 cooling system (Thermo Scientific, Waltham, MA). Single colonies of each constructs were first grown up in 2 mL SC-Ura-Leu medium plus 20g/L glucose, and then inoculated into 50

mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, 10 mL saturated culture were inoculated in 500 mL YPA medium supplemented with 40 g/L cellobiose and 50 g/L xylose; 40 g/L cellobiose, 50 g/L xylose, and 5 g/L glucose; or 40 g/L cellobiose, 50 g/L xylose, and 10 g/L glucose. The temperature was maintained at 30 °C and the pH was maintained at 5.5, adjusted by addition of either $2NH_2SO_4$ or 4N NaOH. In the first 48 hours, the air flow rate was maintained at 0.5 L/min, with the impeller speed at 250 rpm. Afterwards, the air flow rate was adjusted to 0.2 L/min to achieve high ethanol production under oxygen limited conditions. Triplicate samples were taken at various time points and the OD600, sugar concentration, and ethanol concentration were determined as described above.

2.6.Tables

Table 2.1 Constructed cellobiose assimilating strains

Strain Name	Cellobiose Transporter	β-Glucosidase
SL01	cdt1	gh1-1
SL02	NCU00809	gh1-1
SL03	cdt2	gh1-1
SL04	cdt1	BGL1
SL05	NCU00809	BGL1
SL06	cdt2	BGL1
SL00	-	-

Table 2.2 (to be continued) List of primers used in pathway construction

	SL01	SL02	SL03
PYK 1promoter-for	5’-TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGCAATGCTACTATTTTGG-3’		
PYK 1 promoter-rev	TCCTTAGGAA GAGACATTGT GATGATGTTT TATTTGTTTT GATTGGTGTC TTGTAAATAG		
gluc osidase-for	5’-TTACAAGACACCAATCAAAACAAATAAAACATCATCACAATGTCTCTTCCTAAGGATTTC-3’		
gluc osidase-rev	5'-TGGAGACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTTAGTCCTTCTTGATCAAAG-3'		
adh1 terminator-for	5’-TCTTTGATCAAGAAGGACTAAAGCTTTGGACTTCTTCGCCAGAGGTTTGGTCAAGTCTCC-3’		
adh1 terminator-rev	5'-TGGAAGAGTAAAAAAGGAGTAGAAACATTTTGAAGCTATCATGCCGGTAGAGGTGTGGTC-3'		
TEF 1 promoter-for	5'-TAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGATAGCTTCAAAATGTTTC-3'		
TEF 1 promoter-rev	5'-CTTCTCGGTGCTGGCCCCGTCATGGGAGCCGTGAGACGACATTTTGTAATTAAAACTTAG-3'	5'-ATGGGCACCCATGGCCTCCTTTTCGTTTATGCTGTGAGCCATTTTGTAATTAAAACTTAG-3'	5'-GTCGACGGCCTGAGCCACGGGCTTCTTGTTGAAGATGCCCATTTTGTAATTAAAACTTAG-3'
trans porter-for	5’-AGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGTCGTCTCACGGCTCCCATG-3’	5’-AAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCTCACAGCATAAACGAAAAG-3’	5'-AGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGGCATCTTCAACAAGAAGC-3'
trans porter-rev	5'-AAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATCTAAGCAACGATAGCTTCGG-3'	5'-AAAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATCTAAATTGTAACTTTCTCG-3'	5'-AAAAAATTGATCTATCGATTTCAATTCAATTCAATTCAAGCAACAGACTTGCCCTCATGC-3'
PGK 1 terminator-for	5’-CCAGGCCGACGGCCATGTGTCCGAAGCTATCGTTGCTTAGATTGAATTGAATTGAAATCG-3’	5’-CACCATGGGAGCGCCGGATGACGAGAAAGTTACAATTTAGATTGAATTGAATTGAAATCG-3’	5'-GAGATTCACGAGCATGAGGGCAAGTCTGTTGCTTGAATTGAATTGAATTGAAATCGATAG-3'
PGK 1 terminator-rev	5'-TCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGCAGGAAGAATACACTATACTGG-3'		

Table 2.2 (continued) List of primers used in pathway construction

	SL04	SL05	SL06
PYK1 promoter-for	5’-TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGCAATGCTACTATTTTGG-3’		
PYK1 promoter-rev	5’-GAGGGATAGAATGGAGGAGAGAACGCCAGTTCATCCATTGTGATGATGTTTTATTTG-3’		
glucosidase-for	5'-CAAGACACCAATCAAAACAAATAAAACATCATCACAATGGATGAACTGGCGTTC-3'		
glucosidase-rev	5'-ATTGGAGACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTCTATTGCACCTTCGGGAG-3'		
adh1 terminator-for	5'-AGCTGCCCCTTCACGCAGCGCTCCCGAAGGTGCAATAGAGCTTTGGACTTCTTCGCCAG-3'		
adh1 terminator-rev	5'-TGGAAGAGTAAAAAAGGAGTAGAAACATTTTGAAGCTATCATGCCGGTAGAGGTGTGGTC-3'		
TEF1 promoter-for	5'-TAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGATAGCTTCAAAATGTTTC-3'		
TEF1 promoter-rev	5'-CTTCTCGGTGCTGGCCCCGTCATGGGAGCCGTGAGACGACATTTTGTAATTAAAACTTAG-3'	5'-ATGGGCACCCATGGCCTCCTTTTCGTTTATGCTGTGAGCCATTTTGTAATTAAAACTTAG-3'	5'-GTCGACGGCCTGAGCCACGGGCTTCTTGTTGAAGATGCCCATTTTGTAATTAAAACTTAG-3'
transporter-for	5’-AGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGTCGTCTCACGGCTCCCATG-3’	5’-AAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCTCACAGCATAAACGAAAAG-3’	5'-AGAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGGCATCTTCAACAAGAAGC-3'
transporter-rev	5'-AAAGAAAAAAATTGATCTATCGATTTCAATTCAATTCAATCTAAGCAACGATAGCTTCGG-3'	5'-AAAAGAAAAAAATTGATCTATCGATTTCAATTCAATCTAAATTGTAACTTTCTCG-3'	5'-AAAAAATTGATCTATCGATTTCAATTCAATTCAATTCAAGCAACAGACTTGCCCTCATGC-3'
PGK1 terminator-for	5’-CCAGGCCGACGGCCATGTGTCCGAAGCTATCGTTGCTTAGATTGAATTGAATTGAAATCG-3’	5’-CACCATGGGAGCGCCGGATGACGAGAAAGTTACAATTTAGATTGAATTGAATTGAAATCG-3’	5'-GAGATTCACGAGCATGAGGGCAAGTCTGTTGCTTGAATTGAATTGAATTGAAATCGATAG-3'
PGK1 terminator-rev	5'-TCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGCAGGAAGAATACACTATACTGG-3'		

2.7.Figures

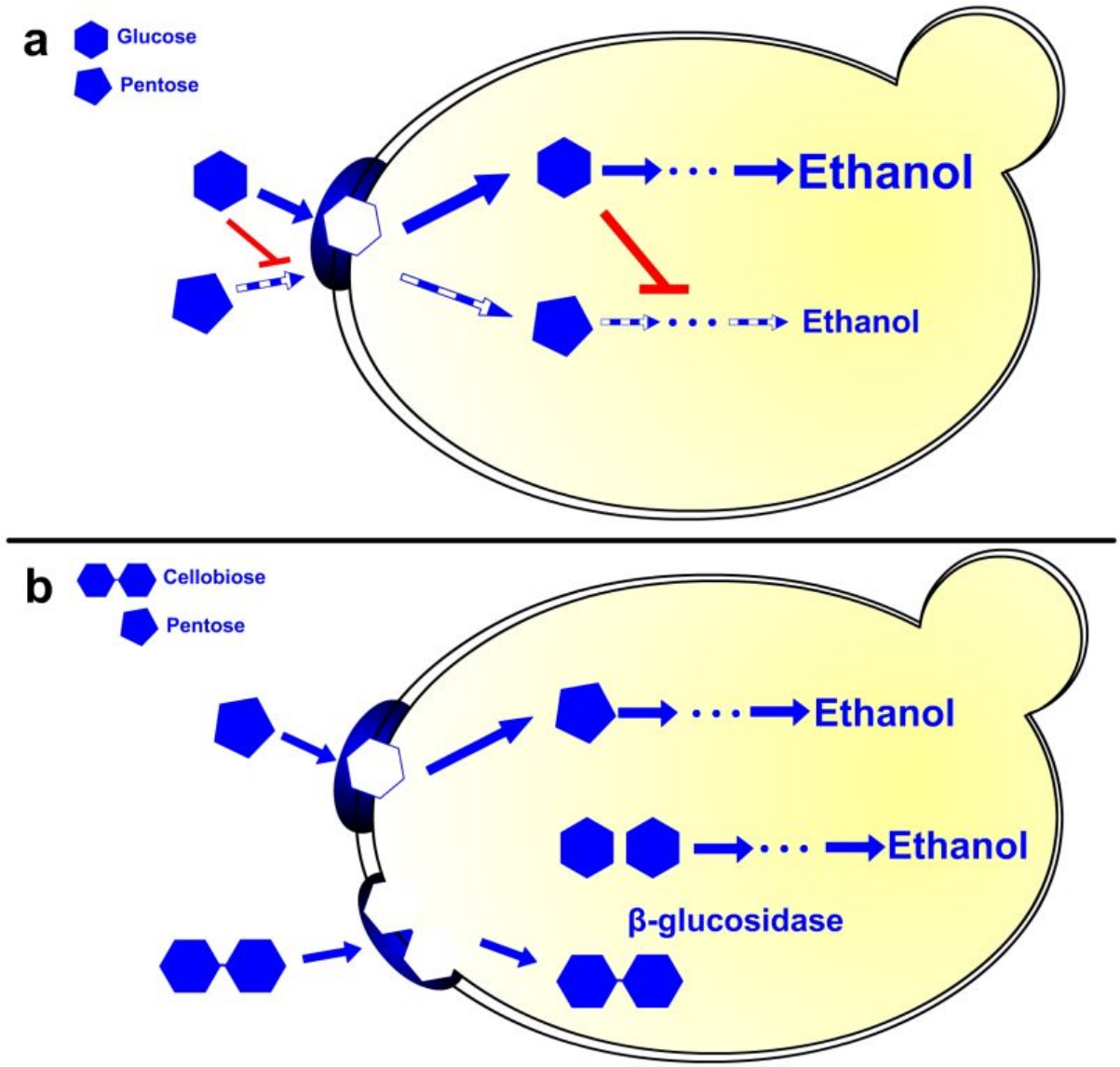

Figure 2.1 Schematic of glucose repression mechanism in co-fermentation of glucose and pentose sugars (a); proposed glucose de-repression mechanism of the strain co-expressing a cellobiose transporter and a β-glucosidase (b).

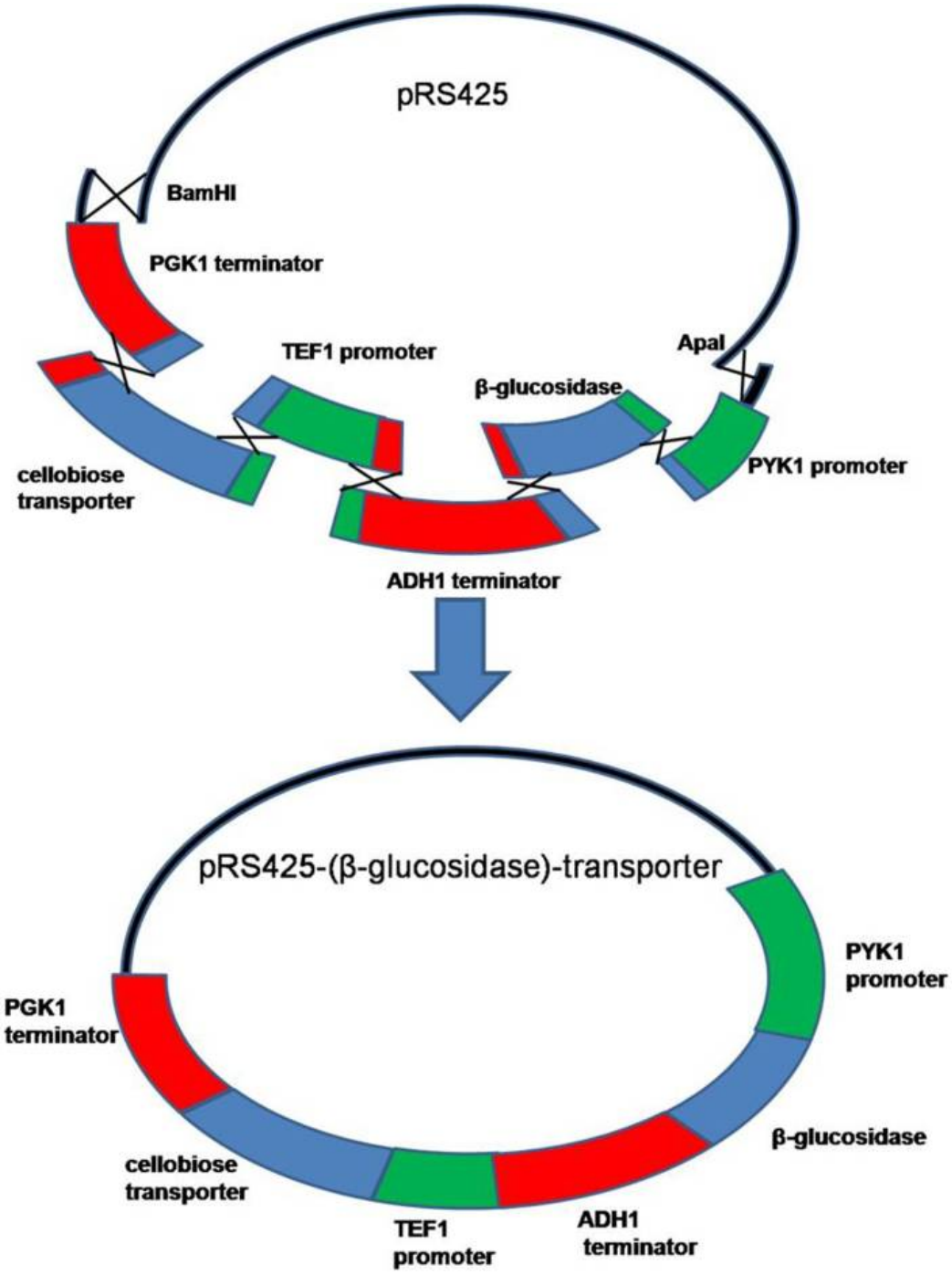

Figure 2.2 Scheme of plasmid construction

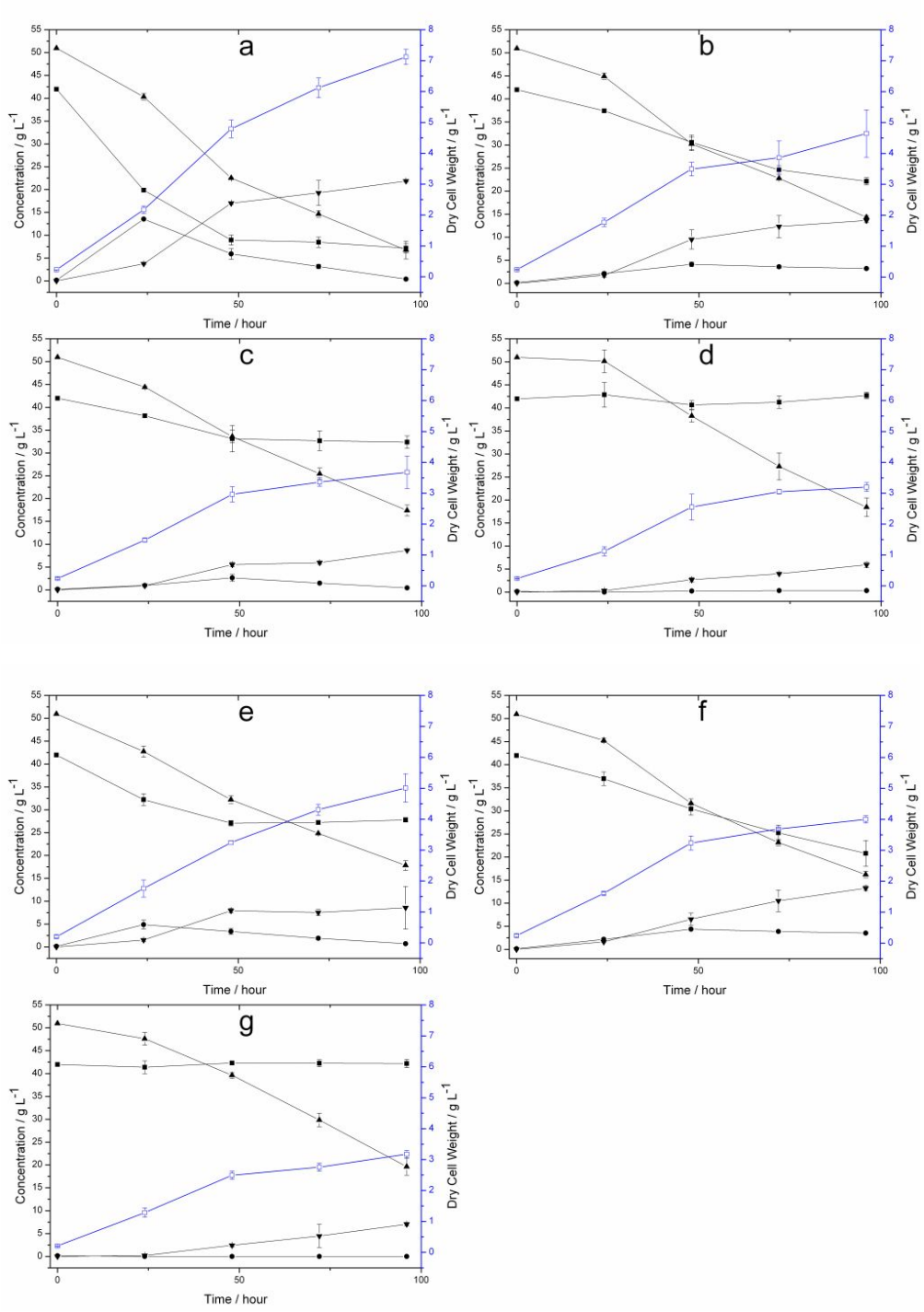

Figure 2.3 Concentrations of cellobiose (■), glucose (●), xylose (▲), ethanol (▼), and dry cell weight (□) in the co-fermentation of 40 g/L cellobiose and 50 g/L xylose of SL01(a), SL02 (c), SL03(e), SL04 (b), SL05 (d), SL06 (f), and SL00 (g), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

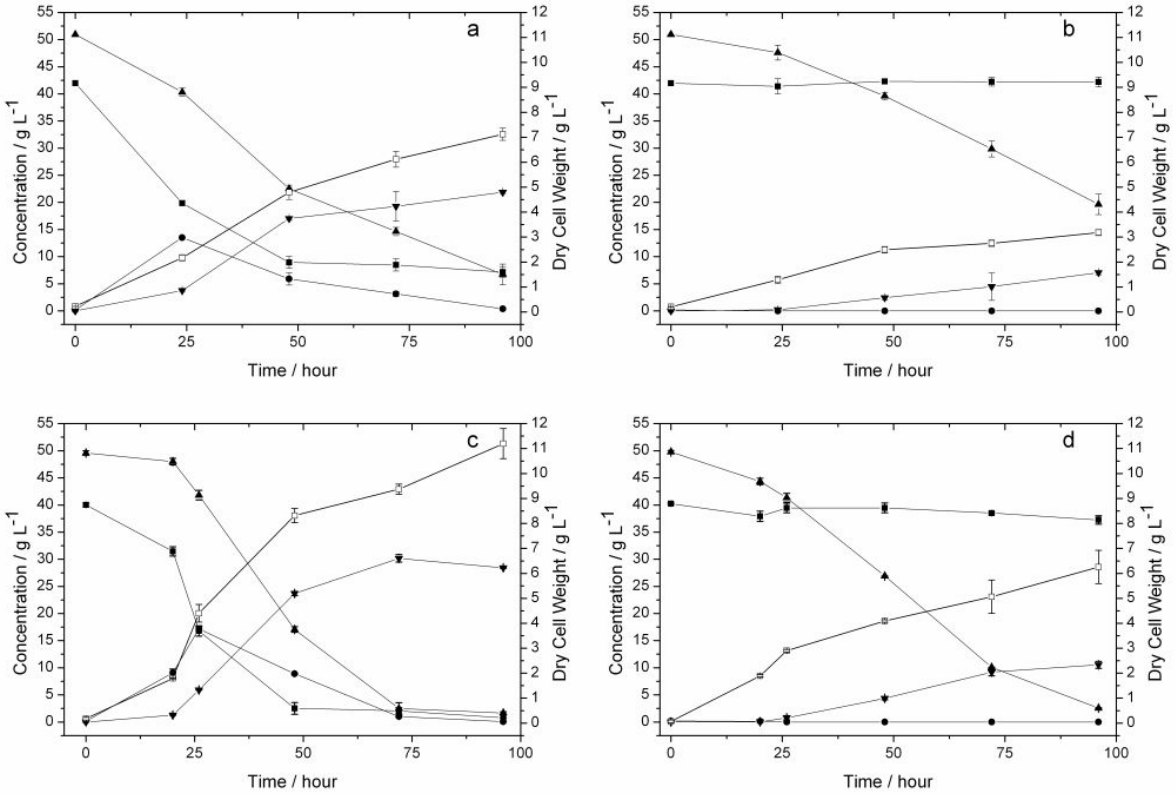

Figure 2.4 Concentrations of cellobiose (■), glucose (●), xylose (▲), ethanol (▼), and dry cell weight (□) of strains SL01 (a, c) and SL00 (b, d) in YPA medium supplemented with 40 g/L cellobiose and 50 g/L xylose in shake-flasks (a, b) and bioreactors (c, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

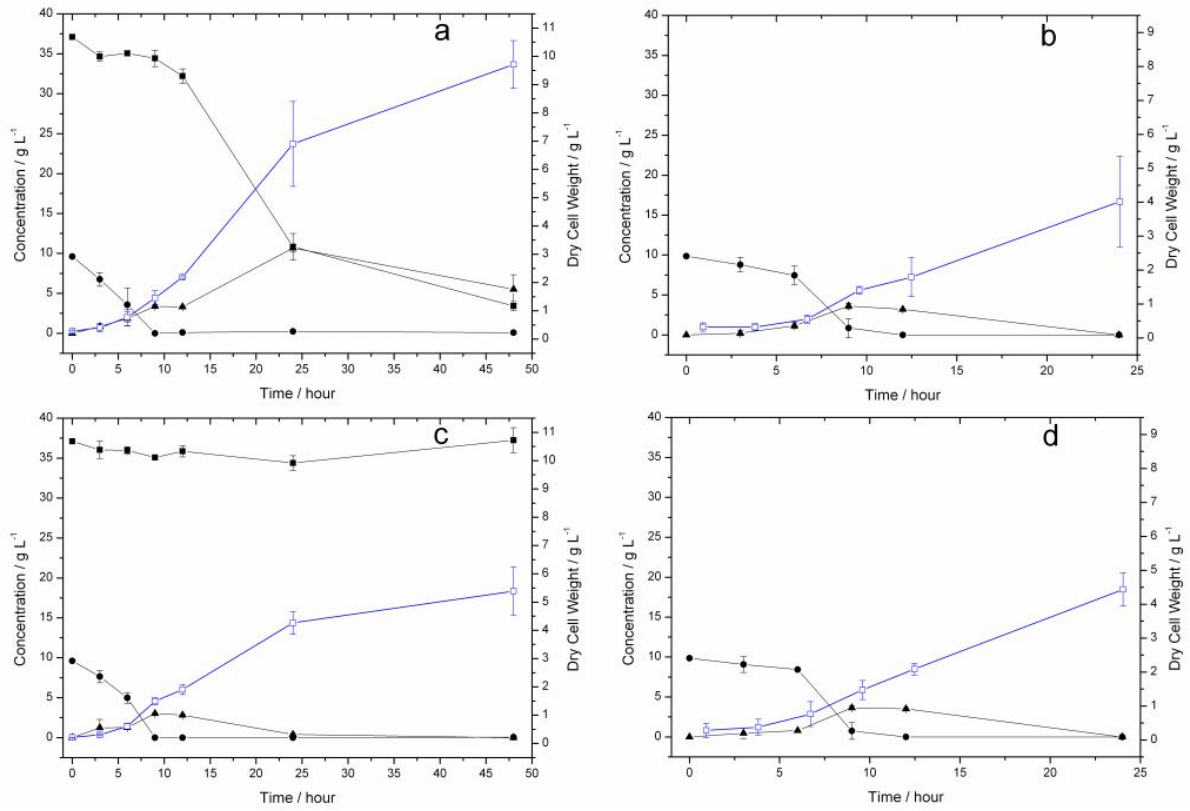

Figure 2.5 Concentrations of cellobiose (■), glucose (●), ethanol (▲), and dry cell weight (□) of SL01 (a, b) and SL00 (c,d) in the co-fermentation of 40 g/L cellobiose and 10 g/L glucose (a, c), or 10 g/L glucose (b, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

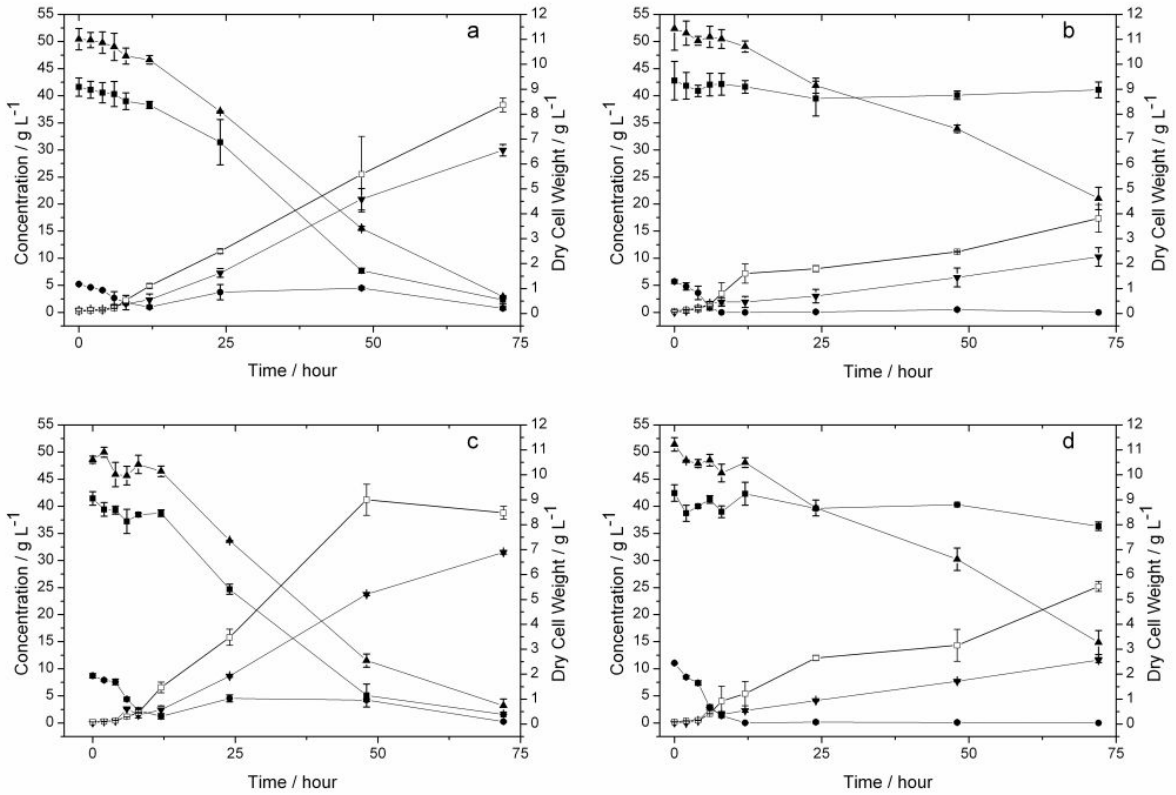

Figure 2.6 Concentrations of cellobiose (■), glucose (●), xylose (▲), ethanol (▼), and dry cell weight (□) of strains SL01 (a, c) and SL00 (b, d) in YPA medium supplemented with 5 g/L glucose–40 g/L cellobiose–50 /L xylose (a, b) or 10 g/L glucose– 40 g/L cellobiose–50 g/L xylose (c, d) in bioreactors, plotted as a function of time. Error-bars indicate standard deviations of duplicate samples.

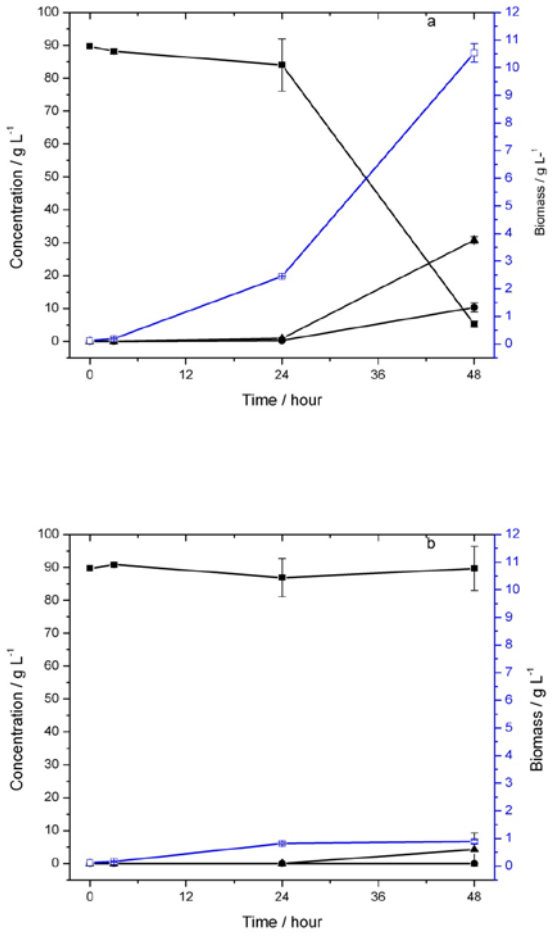

Figure 2.7 Concentrations of cellobiose (■), glucose (●), ethanol (▲),and dry cell weight (□) of strains SLI01 (a) and SLI00 (b) in YPA medium supplemented with 80 g/L cellobiosein shake-flasks, plotted as a function of time. Error-bars indicate standard deviations of duplicate samples

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Chapter 3 Investigation of the Functional Role of Aldose 1-Epimerases in Cellobiose Utilization

3.1. Introduction

It was observed that there was a small percentage of glucose existing in cellobiose fermentation. Although no glucose was supplemented at the beginning of fermentation, a glucose peak was detected by HPLC analysis of the culture broth. In shake-flask fermentation of the laboratory strain SL01, with 40 g/L cellobiose and 50 g/L xylose, glucose concentration reached a maximum of 12 g/L in the middle of fermentation, and in the bioreactor study, this value achieved as high as 17 g/L. Despite of such high glucose concentration, no obvious glucose repression was observed in all cultivations, which was opposite from those glucose repression studies reported in the literature (1-4). The uncommon production and fermentation of glucose led us to propose a mechanism of glucose inter-conversion in cellobiose utilization.

Cellobiose is a disaccharide composed of two molecules of glucose linked by β-1,4-bond. As a result, the main product of cellobiose catalyzed by β-glucosidase is β-glucose. In aqueous solution, there are two anomers of glucose, α-glucose and β-glucose, which maintain a swift equilibrium between these two compounds (5-8). α-Glucose and β-glucose are two predominant pyranose structures, which differ from each other in the configuration of the hydroxyl group at carbon-1 of the ring (6). It was hypothesized that β-glucose is not preferred

in glycolysis reactions whereby it cannot activate glucose repression. The hypothesis about the preference between α-sugar and β-sugar is supported by a galactose utilization study: although α-glucose is phosphorylated by glucokinase in glycolysis, β-galactose has to be transformed to α-galactose before phosphorylation by galactokinase (5). In contrast, glucose and galactose dehydrogenases exhibit specificity for the β-form of their respective sugars (5,9,10).

In conventional bioethanol production process, cellobiose is converted to β-glucose, which is converted to α-glucose swiftly to maintain the equilibrium between two anomers of glucose. Both α-glucose and β-glucose are transported into *S. cerevisiae* to enter the glycolysis pathway and finally to be converted to ethanol. In contrast, in our strategy, cellobiose is transported into *S. cerevisiae* directly by a cellobiose transporter and mainly β-glucose is produced by β-glucosidase *in vivo*. Though the anomers will interconvert in water, the rate of inter-conversion in the cytoplasm does not seem to be sufficient enough. As a result, it takes longer time to convert β-glucose to α-glucose inside yeast cells. Thus we hypothesized that the accumulated glucose was from excess β-glucose, which showed a limited effect on glucose repression. Excess β-glucose not only limits the sugar consumption rate thus limiting ethanol productivity, but at the same time extracellular β-glucose released from yeast cells to culture medium may induce contamination from other glucose-assimilating microorganisms and inhibits cellobiose utilization. So the excessive β-glucose

accumulation due to inefficient conversion between β- and α- forms of glucose is a limiting factor for efficient utilization of cellobiose in engineered *S. cerevisiae* strains.

To improve the conversion from β-glucose to α-glucose, aldose 1-epimerase (AEP), *a.k.a* mutarotase, was investigated to enhance sugar utilization efficiency in engineered yeast strains. AEP is an enzyme catalyzing the inter-conversion between α-anomers and β-anomers of hexose sugars, such as glucose or galactose. It has been found in a wide range of organisms including bacteria, fungi, plants, and mammals such as human beings. In the literature, AEP functional studies were mainly focused on lactose utilization to convert β-galactose to α-galactose (insert citations). There are also epimerases existing in wild type *S. cerevisiae*. One typical aldose 1-epimerase is GAL10, which is a fusion protein to another enzyme of the Leloir pathway, named UDP-glucose-4-epimerase. Investigation of the function of AEP in *S. cerevisiae* will be beneficial to the engineering effort to enhance cellobiose utilization efficiency.

Here we investigate the functional role of aldose 1-epimerase (AEP) in engineered cellobiose utilization. One AEP (Gal10) and two putative AEPs (Yhr210c and Ynr071c sharing 50.6% and 51.0% amino acid identity with Gal10, respectively) were selected. Deletion of Gal10 led to complete loss of both AEP activity and cell growth on cellobiose, while complementation restored the AEP activity and cell growth. In addition, deletion of YHR210C or YNR071C resulted in improved cellobiose utilization. These results suggest

that the intracellular mutarotation of β-glucose to α-glucose might be a rate controlling step and Gal10 plays a crucial role in cellobiose fermentation by engineered *S. cerevisiae.*

3.2. Results

3.2.1.Comparison of Cellobiose Fermentation and Glucose Fermentation

Introduction of a cellobiose transporter (CDT-1) and an intracellular β-glucosidase (GH1-1) from *N. crassa* into *S. cerevisiae* enables fermentation of cellobiose as a carbon source (11,12). However, the fermentation rate of cellobiose by engineered *S. cerevisiae* is much lower than that of glucose. The engineered *S. cerevisiae* BY4741 strain consumes 81.0 g/L of cellobiose within 72 h, and consumes 80.0 g/L of glucose within 20 h (Figure 3.1), indicating a four-fold lower consumption rate of cellobiose than that of glucose. This result suggests that there might be some unknown steps hindering efficient cellobiose fermentation.

3.2.2.Identification of One Aldose 1-Epimerase Gene and Two Putative Genes in *S. cerevisiae*

The *gal10* gene in *S. cerevisiae* codes for a bifunctional enzyme with AEP and UDP galactose 4-epimerase activities. Three dimensional structure analysis revealed that Gal10 possesses both a galactose 4-epimerase domain (N-terminal region) and an aldose 1-epimerase domain (C-terminal region) (13). Using the *AEP* gene from *N. crassa* (*aep-Nc*) as a probe sequence for BLAST search, we identified two more putative AEP genes (*yhr210c* and *ynr071c*) in *S. cerevisiae*. GAL10, YHR210c and YNR071c have 24.7%, 24.2%, and

26.6% amino acid sequence similarity with AEP-Nc respectively. The YHR210c was annotated as a putative protein of unknown function. However, its sequence similarity to GAL10 was reported (5). For the YNR071c, its function is unknown. YHR210c and YNR071c also have 50.6% and 51.0% amino acid sequence similarity with GAL10 respectively.

3.2.3.Gal10 Plays an Important Role in Cellobiose Utilization

To determine the function of putative AEPs in the cellobiose utilization process, a plasmid harboring the cellobiose utilization pathway was introduced into three BY4741 strains which had the *yhr210c, ynr071c,* or *gal10* genes deleted respectively. Cellobiose was used as the sole carbon source and cultivations were tested on both an agar plate and liquid YP medium. The ΔGAL strain showed no growth on the cellobiose plate. After complementing the ΔGAL strain with a plasmid overexpressing *gal10* gene, the ability of cellobiose utilization was restored in the complementation strain (Figure 3.2).

When the resultant ΔYHR, ΔYNR, ΔGAL and control strains with the cellobiose utilization pathway were grown in YP medium supplemented with 8% cellobiose, the ΔGAL strain also showed almost complete loss of growth on cellobiose: At the end of fermentation, only 12.0 g/L cellobiose consumption was observed. As a result, there was no glucose accumulation or ethanol production tested in the ΔGAL strain (Figure 3.3).

3.2.4.Deletion of YHR210c or YNR071c Led to Improved Cellobiose Utilization

In contrast to the loss of cellobiose utilization ability in the ΔGAL strain, ΔYHR and ΔYNR strains showed interestingly improved cellobiose utilization abilities (Figure 3.3). The cellobiose consumption rates were enhanced from 0.78 g/L h to either 1.26 g/L h or 1.25 g/L h in ΔYHR strain and ΔYNR strain, which represented either 60.3% or 59.9% improvement over the wild type strain respectively. The ΔYHR and ΔYNR strains showed higher glucose accumulation than the wild type strain, which was proportional to the improved cellobiose consumption rate of the ΔYHR and ΔYNR strains. Considering the higher cellobiose consumption rates by the ΔYHR and ΔYNR strains, ethanol production was correlated to cellobiose consumption.

3.2.5.AEP-deletion Strains Exhibited Distinct Mutarotase Activities

To further probe the function of AEPs in yeast, we determined the mutarotase activity of the AEP deletion strains (Figure 3.4). The AEP deletion strains were grown on either cellobiose or glucose as a sole carbon source and the specific mutarotase activity was measured after 48 hour of cultivation. Using cellobiose as the sole carbon source, the specific mutarotase activity of the ΔYHR strain was 45.7% of that of the control strain, while the specific mutarotase activity of the ΔYNR strain was 89.5% of that of the control strain. No mutarotase activity was detected in the ΔGAL strain due to the poor growth rate (Figure 3.4A). AEP activities of the same strains tested were quite different using glucose as a sole

carbon source. The specific mutarotase activities of ΔYHR strain and ΔYNR strain were approximately 3-fold higher than the activities of ΔGAL strain and control strain (Figure 3.4B).

3.2.6.Overexpression of AEP did not Improve Cellobiose Utilization

In order to determine the effect of AEP overexpression, we introduced plasmids overexpressing AEP into the engineered cellobiose-utilizing BY4741 strain and cellobiose utilization was measured. However, no improvement in cellobiose consumption was observed and only marginal improvement in ethanol production was found (data not shown). Additionally, another cellobiose-utilizing strain D452-BT overexpressing AEP did not show improvement either (Figure 3.5). The results indicate that simple overexpression of AEP cannot facilitate cellobiose utilization, suggesting allosteric regulation of AEP might play a role in controlling cellobiose utilization.

3.3.Discussions

For cellulosic biofuel production, efficient utilization of glucose and xylose is necessary. However, the sequential utilization of glucose and xylose has several limitations such as low ethanol productivity and low ethanol yield from xylose (14,15). To address these issues, we developed a new strategy for co-fermentation of cellobiose and xylose, which

drastically improved the ethanol yield and productivity in mixed sugar fermentation (11,12,16). However, the cellobiose fermentation rate was four fold slower than that of glucose alone albeit they had similar ethanol yields. This result suggested that there might be unknown limiting steps for efficient cellobiose utilization. Glucose induction is known to be initiated by signaling mechanisms from cell membranes (1). However, in the case of cellobiose fermentation, since glucose is produced inside of the cell from cellobiose by intracellular β-glucosidase, the normal glucose signaling mechanisms may not be efficient, resulting in slow cellobiose utilization.

N. crassa is known to utilize cellobiose, and both the cellobiose transporter (CDT1) and the intracellular β-glucosidase (GH1-1) from *N. crassa* have been cloned and characterized (17). Therefore, we examined the transcriptomic analysis data from *N. crassa* to figure out the limiting steps in cellobiose utilization by an engineered *S. cerevisiae*. Interestingly, we found that the expression level of AEP was 160 times higher in the minimal medium containing *Miscanthus* hydrolysate compared to that in the sucrose containing medium (18). This result suggested that the high expression level of AEP may facilitate cellobiose utilization by *N. crassa*. In the engineered cellobiose utilization, β-glucose is produced by β-glucosidase. However, the interconversion of β-glucose to α-glucose may not be high enough *in vivo* even though the interconversion of the glucose anomers occurs spontaneously *in vitro* (19,20). Because yeast is known to prefer α-glucose, the activity of AEP could be rate-

limiting for efficient cellobiose fermentation (21).

Based on our studies on the AEP knock-out strains, a complex AEP regulation mechanism might exist in the BY4741 strain. Although the GAL10 has higher activity than the other two epimerases, its expression might be repressed by YHR210c and YNR071c whenever high AEP activity is not required. We proposed that under the cellobiose fermentation condition where high AEP activity is required, GAL10 is expressed efficiently, and the deletion led to limited AEP enzyme activity and no cell growth (Figure 3.3). However, because of the fast cellobiose depletion, the AEP activities of the ΔYHR and ΔYNR strains were relatively lower than that of the control strain (Figure 3.4A). Under the glucose fermentation condition, since the GAL10 expression is repressed by abundant α-glucose, the ΔGAL strain and the control strain showed almost identical AEP enzymatic activities, where GAL10 expression is limited either by gene deletion or by repression. Due to the deletion of YHR210C or YNR071C in ΔYHR or ΔYNR strains, the expression of GAL10 was not repressed, which is consistent with the high AEP activities observed in the ΔYHR and ΔYNR strains (Figure 4B).

Further investigation of AEP overexpression was tested in both BY4741 and D452-BT strains. However, we found little differences between the overexpression strains and control strain, which may be due to unknown regulation system of glucose inter-conversion. Therefore, we conclude the role AEP plays in cellobiose metabolism is important but also

complicated. GAL10 may play an indispensable role in cellobiose utilization, whereas other regulatory mechanisms also exist, leading to the poor performance of AEP overexpressing strains. Although the cellobiose fermentation rate is still lower than that of glucose, this study represents a step towards solving the limitations of cellobiose fermentation by an engineered *S. cerevisiae*. There might be several rate-limiting steps for efficient cellobiose fermentation. For example, one possible rate-limiting step is the hexokinase catalyzing the phosphorylation of hexose immediately after glucose is uptaken. Because glucose is now produced inside of the cell from cellobiose by an intracellular β-glucosidase, it may result in a too high glucose concentration that overwhelms the hexokinase. Further investigation is needed to identify additional rate-limiting steps through transcriptomics and metabolomics studies.

3.4.Conclusions and Outlook

Extracellular glucose accumulation was observed in the cellobiose fermentation, which may be due to inefficient conversion between β-glucose and α-glucose. It was also proved that α-sugar is preferred in metabolic reactions to some extent. Although interconversion between two anomers can be executed swiftly in aqueous environment, it is more difficult to complete this reaction inside *S. cerevisiae* cells. To facilitate cellobiose consumption and eliminate possible contamination, glucose interconversion should be

accelerated. One reasonable approach is to introduce aldose 1-epimerase which can catalyze the interconversion into cellobiose assimilating *S. cerevisiae*. Overexpression of related aldose 1-epimerase genes has been proved to show some benefits on the cellobiose consumption rate. However, to study the AEP function, analysis of the AEP disrupted strains showed much more complicated performance than expected. From the results we obtained so far, we can conclude that GAL10 is the dominant aldose 1-epimerase which can regulate sugar utilization in a cellobiose assimilating strain. The totally different trend in cellobiose fermentation and glucose fermentation shows that cellobiose-glucose utilization is a system more complicated than we expected, and the aldose 1-epimerases working together with other proteins regulates cellobiose and glucose utilization by a delicate mechanism. The study about aldose 1-epimerases may lead to significant improvement in sugar metabolism, which will facilitate bioethanol production.

3.5. Materials and Methods

3.5.1.Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae L2612 (*MATα leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal+*) was a gift from Professor Yong-su Jin(22). BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), BY4742 (*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*), and corresponding YKO strains (their names are listed in Table 3.3) were purchased from Open Biosystems Products

(Huntsville, AL). *Saccharomyces cerevisiae* Classic Turbo was purchased from Homebrew (Everett, WA). *Escherichia coli* DH5α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in un-baffled shake-flasks at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

3.5.2.Strain and Plasmid Construction

To integrate the AEP genes into a multi-copy plasmid, corresponding AEP genes were PCR-amplified and cloned together with the HXT7 promoter and the HXT7 terminator into pRS424 plasmid using the DNA assembler method (23). The resulting plasmid was then transferred into *E. coli* DH5α, which was plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). These plasmids were transformed into the HZ3001 strain with pRS425-*cdt-1-gh1-1* plasmid individually to yield AEP1 (containing the plasmid harboring the YHR210c

gene from *S. cerevisiae*) and AEP2 (containing the plasmid harboring the NCU09705 gene from *N. crassa*). The empty pRS424 plasmid was transformed into the HZ3001 strain together with the cellobiose-assimilating pRS425-*cdt-1-gh1-1* plasmid to yield the AEP0 strain as a negative control. Yeast transformation was carried out using the standard lithium acetate method (24). The resulting transformation mixtures were plated on SC-Trp-Leu medium supplemented with 2% glucose. To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (ZymoResearch, Orange County, CA) and then transformed into *E. coli* DH5α cells. The resulting cells were spread on LB plates containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion using *Eco*RI and *Hind*III. All restriction enzymes were obtained from New England Biolabs (Ipwich, MA).

To construct single AEP knockout strains in SL01, corresponding ORFs were removed by the *loxP-kanMX-loxP* disruption cassette using the DNA assembler method. The kanamycin resistance marker was then rescued with *cre*-bearing pSH47 plasmid and the double AEP knockout strain was constructed following the same protocol. Both SL01 AEP-disrupted strains and the YKO strains were transformed with the pRS425--*cdt-1-gh1-1* plasmid individually to enable the cellobiose assimilating ability.

3.5.3.Cellobiose Fermentation in Shake-Flasks

For each AEP overexpression strain, a single colony was first grown up in 2 mL SC-Ura-Leu medium plus 20 gL^{-1} glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. For each AEP disrupted strain, single colony was first grown up in 2 mL SC-Leu medium plus 20 gL^{-1} glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 80 g/L cellobiose. Starting from an initial OD600 $\approx$ 1, cell cultures were grown at 30 °C at 100 rpm for fermentation under oxygen limited conditions. OD600 readings and cell culture samples were taken at various time points. Sugars and ethanol concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and Shimadzu RID-10A refractive index detector (Shimadzu Scientific Instruments, Columbia, MD) following the manufacturer's protocol. The HPX-87H column was kept at 65°C using a Shimadzu CTO-20AC column oven. 0.5 mM sulfuric acid solution was used as mobile phase at a constant flow rate of 0.6 mL/min. 10 μL of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 25 minutes after the injection. The concentrations of the sugars and ethanol were determined using a standard curve generated using a series of external standards. Each

data point represented the mean of duplicate samples.

3.5.4.Mutarotase Activity Assay

The mutarotase activities in cellobiose assimilating *S. cerevisiae* strains were determined using the BY4742 strains. Both AEP-disrupted strains and AEP-overexpressed strains with a cellobiose assimilating pathway were investigated. In the mutarotase activity assay for the AEP-disrupted strains, cell cultures were grown in tubes filled with 5 mL YPA medium supplemented with 20 gL^{-1} glucose or 80 gL^{-1}cellobiose. The culture tubes were grown at 30°C at 250 rpm for 48 hours. And in AEP-overexpressed strains, cell cultures were grown in tubes filled with 5 mL SC medium supplemented with 20 gL^{-1} glucose. The culture tubes were grown at 30°C at 250 rpm for 48 hours. Cells were resuspended in Y-PER Extraction Reagent (ThermoFisher Scientific, Rockford, IL) following the manufacturer's instructions. Supernatants were then collected for measurement of protein concentration and mutarotase activity.

To determine the total protein concentration, BCA Protein Assay Reagent (ThermoFisher Scientific, Rockford, IL) was used following the manufacturer's instructions. A Synergy 2 Multi-Mode Microplate Reader (BioTeck, Winooski, VT) was used to measure the change of absorbance. Total protein concentration was calculated following the manufacturer's instructions.

To determine the mutarotase activity, a mixture containing 0.34 mM NAD^+, 0.05U of

glucose dehydrogenase and 50 mM Tris hydrochloride (pH 7.2) was made. 820 μL of the mixture was pipetted into a UV cuvette and then 130 μL mutarotase containing solution was added. Then, 50 μL of a 166 μM freshly prepared α–glucose was added to the cuvette and the increase in absorption at 340 nm was recorded for 3 minutes.

3.6.Tables

Table 3.1 AEP genes overexpressed in the SL01 strain

Strain Name	Gene	Source
AEP1	*YHR210c*	*S. cerevisiae*
AEP2	*NCU09705*	*N. crassa*
AEP0	-	

Table 3.2 List of primers in AEP overexpression

YHR-for	5’-CAAAAAGTTTTTTTAATTTTAATCAAAAAATGTCAAATAATAAGGCTGGCGGTGAATAT-3’
YHR-rev	5’-GATCATGAATTAATAAAAGTGTTCGCAAACTACACCGCAAAACGATATCGAGTCTTAGAA-3’
NCU-for	5’-CAAAAAGTTTTTTTAATTTTAATCAAAAAATGTCTGACGCAATCGCCTCCTTCATCCCC-3’
NCU-for	5’-CTACTCCTTCCACGCCCTGTACAGGATCTTTTTGCGAACACTTTTATTAATTCATGATC-3’

Table 3.3 List of YKO strains

Strain	Genotype
BY4741 ΔYHR	*Mat a; his31; leu20; met150; ura30; YHR210c::kanMX4*
BY4741 ΔYNR	*Mat a; his31; leu20; met150; ura30; YNR071c::kanMX4*
BY4741 ΔGAL	*Mat a; his31; leu20; met150; ura30; YBR019c::kanMX4*
BY4742 ΔYHR	*Mat α; his31; leu20; met150; ura30;YHR210c::kanMX4*
BY4742 ΔYNR	*Mat α; his31; leu20; met150; ura30; YNR071c::kanMX4*
BY4742 ΔGAL	*Mat α; his31; leu20; met150; ura30; YBR019c::kanMX4*

3.7.Figures

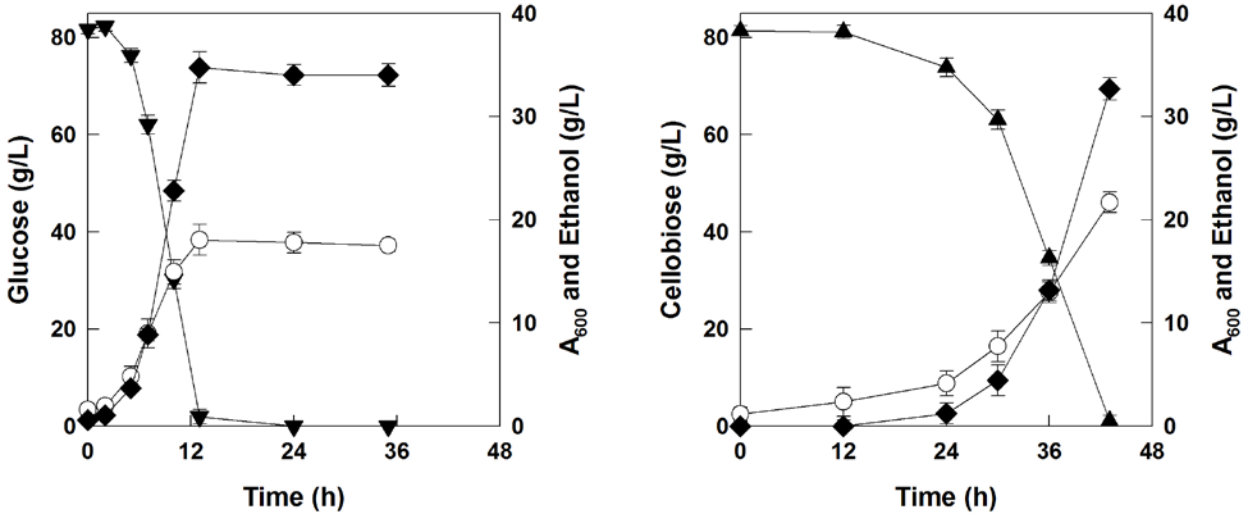

Figure 3.1 Comparison of glucose fermentation (left) and cellobiose fermentation (right). Symbols: OD (○), glucose (▼), cellobiose (▲), and ethanol (□). In all fermentation results, values are the mean of two independent fermentations, and error bars represent the standard deviations.

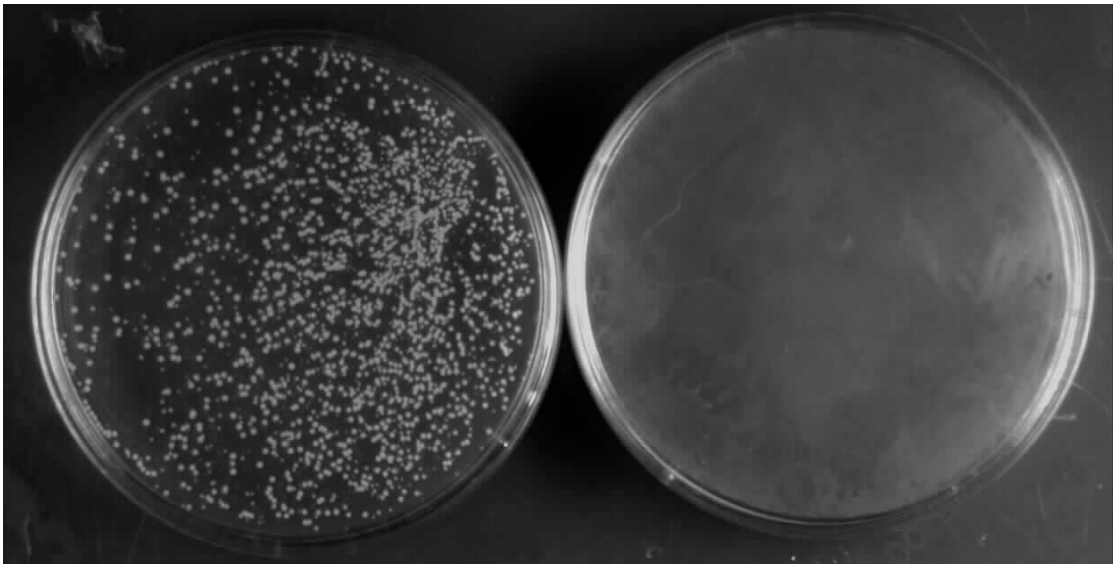

Figure 3.2 Growth performance on plate with cellobiose as sole carbon source: ΔGAL strain with pRS423-GAL10 overexpressing plasmid (left) and ΔGAL strain with pRS423 blank plasmid (right).

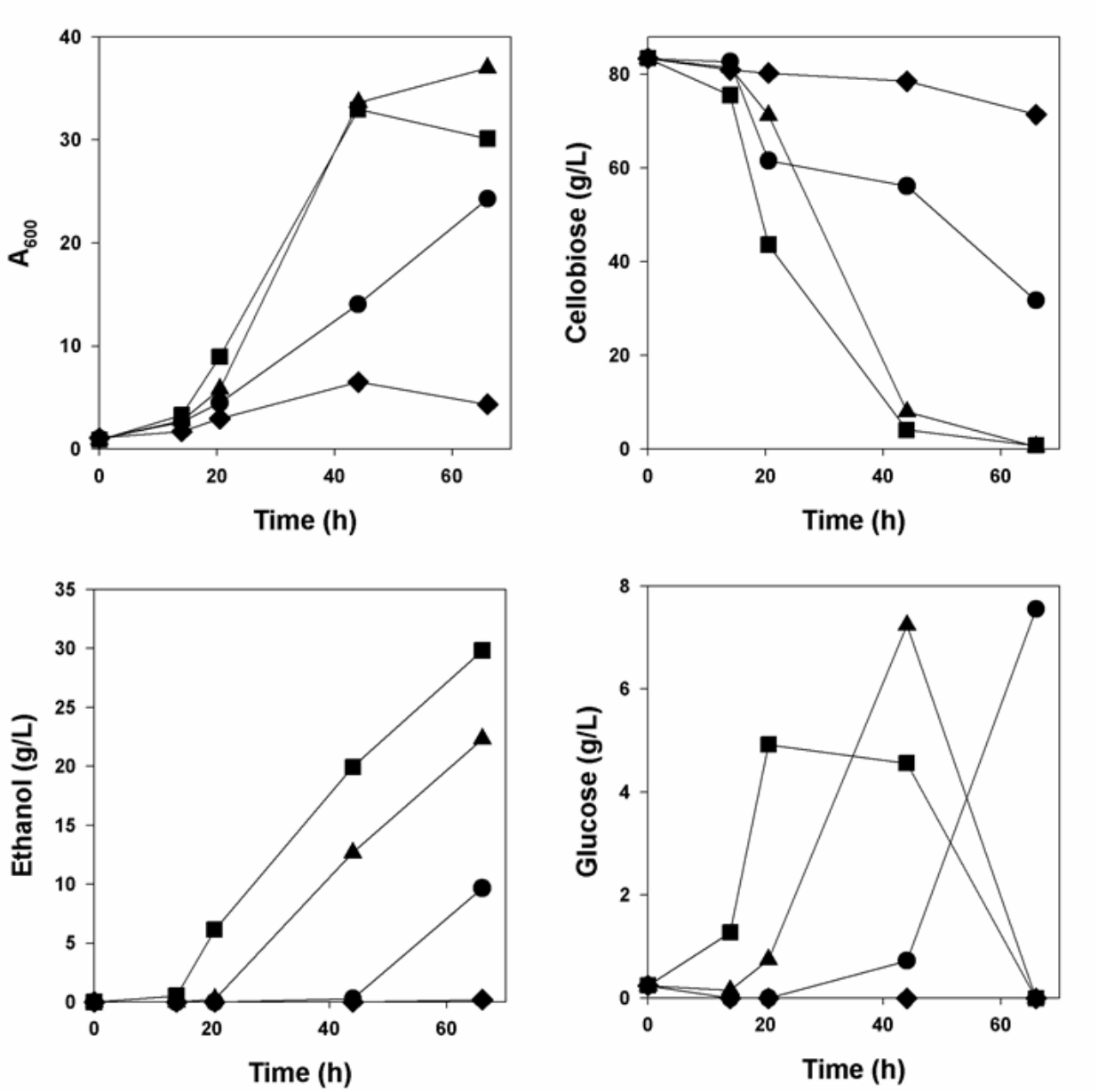

Figure 3.3 Comparison of cellobiose fermentation by BY4741 ΔYHR, ΔYNR and ΔGAL strains with a cellobiose fermentation pathway. Values are the mean of two independent fermentations and standard deviations are within 15%. Symbols: control (●), ΔYHR (▲), ΔYNR (■), and ΔGAL (♦).

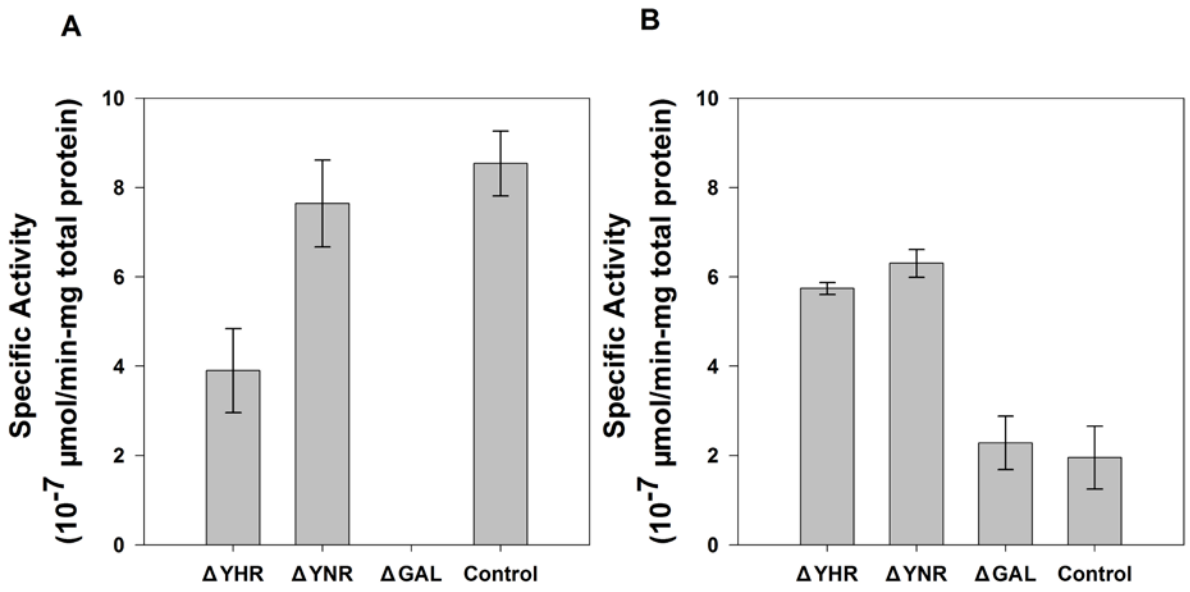

Figure 3.4 Specific AEP activity of the BY4741 AEP knock-out strains grown on cellobiose (A) or glucose (B). One unit of AEP activity is defined as the amount of enzyme converting 1 µmol of α-glucose to β-glucose in 1 min in addition to the non-enzymatic rate at 22 °C. Values are the mean result of two activity assays, and error bars represent the standard deviations.

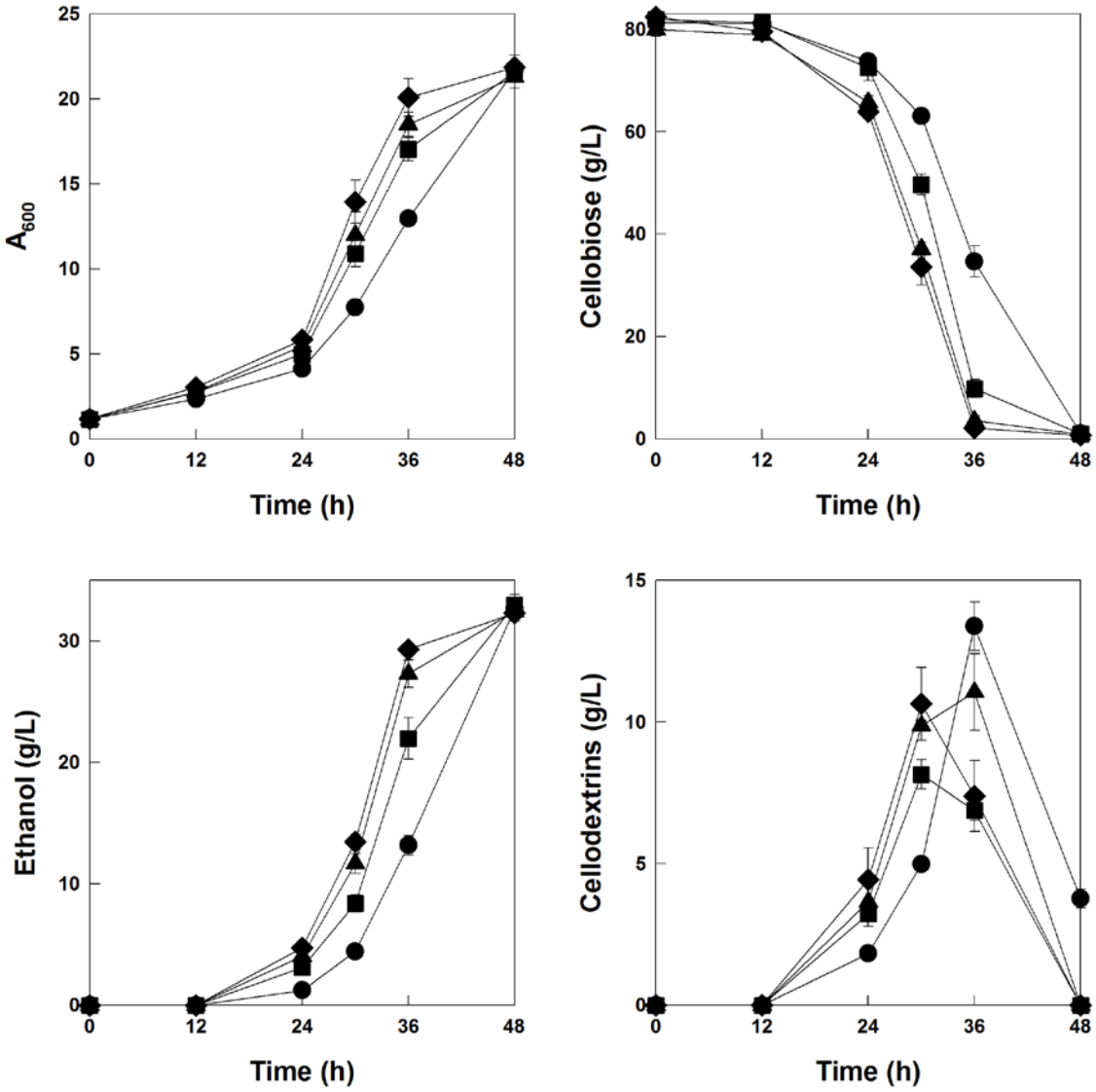

Figure 3.5 Comparison of cellobiose fermentation by three *S. cerevisiae* D452-BT strains overexpressing an AEP gene (GAL10-Sc, YHR210C, and YNR071C) into the engineered *S. cerevisiae* D452-BT with a cellobiose fermentation pathway. Symbols: control (□), YHR210C (▲), YNR071C (□), and GAL10-Sc (□). In all fermentation results, values are the mean of two independent fermentations, and error bars represent the standard deviations.

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Chapter 4 Construction and Utilization of a FapR-based Malonyl-CoA Sensor

4.1. Introduction

Recent efforts in biosynthesis have been focused on the design, construction and optimization of pathways and strains (1). Progress in controlling gene expression levels involve approaches of designing pathways with enzymes derived from various microorganisms (2) and construction of promoter (3), ribosome binding site (RBS) (4) or intergenetic region libraries (5) to optimize and balance transcriptional and translational efficiencies. The development of automated workstations accelerates the construction process of large libraries, which enables genome-wide coverage of gene mutations or adequate coverage of regulation libraries (6,7). Compared to the fast development of library generation technologies, the development of high throughput screening or selection technologies have lagged behind and the existing methods typically cannot meet the requirement for high-throughput mutant selection or screening, mainly due to the need for developing independent analysis methods towards different target compounds. Chromatography-based quantification methods provide accurate measurement of target compounds despite of the low throughput and strict requirement of sample pretreatment (8). Fluorescence-activated cell sorting (FACS) provides a throughput as high as 10^9 variants per experiment (9) and could act as an efficient tool for large scale screening (10). However, the approach linking intracellular chemical concentrations to steady fluorescence signals is the bottleneck that has the biggest impact on the screening step. Genetic biosensors convert chemical concentrations into detectable fluorescence signal via transcriptional regulation, which may serve

as an important tool for screening and sorting (8,11).

Malonyl-CoA is a key metabolite in cell growth and a basic building block for the biosynthesis of fatty acids, 3-hydroxypropionic acid, polyketides, and flavonoids (12,13), which can either be used directly or be used as a precursor for the production of biofuels and value-added chemicals (14). It is involved in fatty acid elongation as an elementary unit and also inhibits the consumption of fatty acid by regulating the rate-limiting step in beta-oxidation. The conventional analytical approaches as HPLC-MS or immunoassays require large amounts of cells, complicated sample preparation and long analysis time, which hinders rapid screening or fermentation monitoring (15) (16). As a result, the development of malonyl-CoA responsive sensor is highly desired to study the intracellular regulation and the synthesis of valuable compounds in the biofuel and pharmaceutical industries.

To develop a malonyl-CoA responsive sensor, a regulator capable of binding to malonyl-CoA and regulating gene transcription is needed. FapR is a bacterial transcription factor from *Bacillus subtilis* (17). It represses the expression of many genes involved in fatty acid metabolism (18). FapR undergoes a conformational change when it specifically binds to malonyl-CoA and dissociates from its 34 bp operator *fapO,* allowing the access of the RNA polymerase for transcription (18). It has been reported that FapR can be utilized as a specific repressor together with a fluorescent protein to generate a malonyl-CoA sensor in mammalian cells (19) or *E. coli* (20,21). However, there is no report about the construction of a FapR-based malonyl-CoA sensor in *S. cerevisiae* to date. Here we firstly report the development of a malonyl-CoA sensor in *S. cerevisiae*. The sensor is transcriptionally regulated by malonyl-CoA and links the fluorescence signal to the concentration of cytosol malonyl-CoA. We used the

malonyl-CoA sensor for the screening of a genome-wide RNA interference and cDNA overexpression library in *S. cerevisiae* to discover strains with enhanced production of malonyl-CoA. The resultant mutant strains with a co-expressed plasmid containing a 3-hydroxypropionic acid (3HPA) biosynthetic pathway showed improved 3HPA production, and an over 50% higher titer than the wild type strain was achieved in one of the mutant strains. This sensor can be used for the screening of enzymes involved in fatty acid and other compound synthesis and for the enhancement of desired compound productions.

4.2. Results

4.2.1.Design of the Malonyl-CoA Sensor

To create the malonyl-CoA sensors in *S. cerevisiae*, we constructed a FapR-regulated fluorescent protein expression plasmid by inserting the *fapO* operator into a Gpm1 promoter in front of a gene encoding for a fluorescent protein (tdTomato). We codon-optimized the *fapR* gene to increase its expression in *S. cerevisiae* and attached a Nuclear Localization Sequence (NLS) to the C-terminus of the FapR protein to enable the import of FapR into nucleus for transcriptional regulation. We also constructed another FapR expressing plasmid without the NLS for comparison. The fluorescence signal of the engineered strains were measured by a Tecan plate reader and normalized by the cell density. Strains expressing the tdTomato protein showed at least 5-fold higher fluorescence than the blank CEN.PK2 strain (data not shown).

The engineered strain with the intact malonyl-CoA sensor exhibited 30.3% of the fluorescence intensity of the strain without FapR expression, and the strain lacked the NLS showed a fluorescence intensity similar to that without the repression of FapR on transcription

(Figure 4.1).

4.2.2.Characterization of the Malonyl-CoA Sensor

To validate that the sensor is responsive to intracellular malonyl-CoA levels, we altered the malonyl-CoA level by co-expressing enzymes affecting the concentrations of malonyl-CoA together with the sensor. Expression of acetyl-CoA carboxylase (Acc) synthesizing malonyl-CoA from acetyl-CoA resulted in over 2-fold increased fluorescence and the expression of [acyl-carrier-protein] S-malonyltransferase (Mct) resulted in decreased reporter fluorescence that was 89.5% of that of the wild type construct (Figure 4.2).

To validate further that the malonyl-CoA sensor is able to report relatively quantitative change of intracellular malonyl-CoA levels, we altered the concentrations of malonyl-CoA in gradient by adding an inhibitor cerulenin into the medium which will block fatty acid elongation and build up malonyl-CoA. The response of the sensor to malonyl-CoA is reflected by the ratio of fluorescence intensity of the culture with or without cerulenin added. The ratio of fluorescence intensity increased from 1 to 11.3, which was positively correlated with the level of cerulenin added in the culture medium ranging from 0.5 mg/L to 5 mg/L, indicating the accumulation of malonyl-CoA in the cell. The control construct co-expressing an empty pRS424 and the pRS425-tdTomato showed slightly decreased fluorescence in the presence of cerulenin, which was due to the inevitable cell lysis resulted from the effect of cerulenin (Figure 4.3).

4.2.3.Screening for Malonyl-CoA Overproducing Mutants from Genome-Wide RNA Interference/Overexpression libraries

We recently developed a highly efficient method called RNA interference (RNAi)-

Assisted Genome Evolution (RAGE) (22) for genome-scale engineering in *S. cerevisiae*. A functional RNAi machinery was constructed in *S. cerevisiae* by the expression of Dicer and Argonaute proteins from a related species *Saccharomyces castellii*, while small interference RNAs (siRNA) were converted from double strand RNAs (dsRNA) synthesized by two convergent promoters to mediate the knockdown of homologous genes. Here we used this method together with the malonyl-CoA sensor to screen for mutant strains over-producing malonyl-CoA intracellularly. In addition, we prepared a plasmid-based cDNA overexpression library to achieve gene overexpression on a genome-scale, which was also combined with the malonyl-CoA sensor in the library screening. In each round of fluorescence-activated cell sorting (FACS), the modifications conferring enhanced fluorescence intensity were identified and the best cassettes from both overexpression and knockdown libraries were cloned into a plasmid to facilitate malonyl-CoA overproduction. Repeated cycles of FACS accumulated the beneficial genetic modifications continuously. As proof-of-concept, two rounds of FACS were applied to improve malonyl-CoA production in *S. cerevisiae*. After first round, one overexpression construct and two knockdown constructs showed over 3-fold increased fluorescence intensity compared to the wild type construct and were selected for further malonyl-CoA overproduction assays.

4.2.4.Improvement of 3-Hydroxypropionic Acid Production

3-Hydroxypropionic acid is an attractive value-added chemical as the precursor of a series of chemicals such as acrylates (23). 3-HPA fermentation in bacteria or yeast has been reported by the introduction of the *mcr* gene from *Chloroflexus aurantiacus*, encoding a bi-functional enzyme acting as both an NADPH-dependent malonyl-CoA reductase (Mcr) and a 3-

hydroxypropionate dehydrogenase (Hpdh) or by the expression of a 3-hydroxypropionate dehydrogenase from *Metallosphaera sedula* and a malonyl-CoA reductase from *Sulfolobus tokodaii* (24,25). However, 3-HPA production in yeast has not been optimized and the titer is relatively low. Therefore we co-expressed the Mcr enzyme from *C. aurantiacus* in the malonyl-CoA overproducing strain screened by the sensor and improved 3-HPA production ranging from 24.5% to 58.4% (Figure 4.4).

4.3.Discussions

We designed the malonyl-CoA sensor based on a naturally-occurring malonyl-CoA-responsive transcription factor, FapR, from the Gram-positive bacteria *B. subtilis*. FapR was codon-optimized for the expression in eukaryotic cells and SV40 was assembled to the C-terminus of FapR as a nuclear localization sequence to enable nuclear transport. It was proven that FapR with a NLS was able to regulate transcription while FapR only cannot repress the fluorescent protein expression (Figure 4.1) because it lacks the capability to be transported into nucleus. The 34 bp *fapO* sequence was inserted at the TATA box region in the Gpm1 promoter to enable the blocking of tdTomato transcription from the binding of FapR. As a result, when malonyl-CoA is adequate, FapR binds with malonyl-CoA and dissociates from *fapO*, allowing the expression of tdTomato. When the concentration of malonyl-CoA is low, FapR blocks the transcription of tdTomato and results in a low fluorescence intensity. The concentration of intracellular malonyl-CoA is positively correlated with the fluorescence intensity. To our knowledge, it is the first report of a malonyl-CoA sensor based on transcriptional regulation in *S. cerevisiae*.

To evaluate the malonyl-CoA sensor, we varied the intracellular malonyl-CoA concentrations by overexpressing a malonyl-CoA-accumulating enzyme Acc or a malonyl-CoA-consuming enzyme Mct into the sensor strain. As expected, the fluorescence intensity of the cell culture was increased by over 2-fold with Acc overexpressed, and the fluorescence intensity decreased to 89.5% with the Mct overexpressed, which may be due to the relatively low activity of Mct. In addition, we varied the intracellular malonyl-CoA concentrations by using cerulenin that blocks the native fatty acids pathway and as a result forces the cell to build up malonyl-CoA. Cells cultured in the same tube were aliquoted and added with different amounts of cerulenin (0, 0.5, 1, 2, 4 mg/L). The concentration of cerulenin used is much lower than the functional concentration of cerulenin used as an antibiotic, because of the lack of cerulenin resistance in the sensor construct. We observed that tdTomato expression linearly increased with the concentration of cerulenin ranging from 0 to 2 mg/L. However, when the concentration of cerulenin reached 4 mg/L, no more increase of fluorescence was observed, which was mainly because of the saturation of intracellular malonyl-CoA or the saturation of FapR. Interestingly, the fluorescence intensity of the control construct dropped to half of the original value in the presence of cerulenin, which may be due to cell lysis from the toxicity of cerulenin.

The malonyl-CoA sensor enables fast and high-throughput detection of intracellular metabolites, thus acts as an efficient tool for large-scale library screening. Here we reported the combination of the sensor together with a newly developed genome evolution strategy to increase the production of malonyl-CoA intracellularly. Through iterative cycles of creating a library of RNAi induced reduction-of-function mutants and a library of cDNA overexpression induced mutants, the modified RAGE method can continuously improve target trait(s) by

accumulating multiplex beneficial genetic modifications in an evolving yeast genome. Coupled with the fluorescence sensor and high throughput screening techniques such as FACS, desired target traits will not be limited to growth deficient phenotypes, but can be broadened to intracellular metabolite concentrations that are not directly correlated to selection or conventional screening methods. Three genetic modifications enhancing malonyl-CoA production were discovered and more cycles of RAGE will lead to accumulation of the beneficial modification for even higher malonyl-CoA production. 3-HPA produced from malonyl-CoA was used as another reporter indicating the intracellular malonyl-CoA levels and the overproduction of malonyl-CoA has been proven by the overproduction of 3-HPA detected by HPLC.

4.4.Conclusions and Outlook

In conclusion, we have constructed a genomic sensor that correlates intracellular malonyl-CoA concentration to a fluorescence signal. The constructed malonyl-CoA sensors can be employed as control elements in order to modulate gene expression of biosynthetic pathways of important compounds that are of particular interest to the pharmaceutical and biofuel industries. The negative feedback of malonyl-CoA derived from FapR can also be used to alleviated growth inhibition caused by malonyl-CoA, improving fatty acid titers and productivities.

4.5.Materials and Methods

4.5.1.Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae CEN.PK2-1C (*MATa; ura3-52; trp1-289; leu2-3,112; his3Δ1;*

MAL2-8C; SUC2) was purchased from EUROSCARF (Frankfurt, Germany) and used as a background strain. *Escherichia coli* DH5α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in baffled shake-flasks at 30 °C and 250 rpm for aerobic growth. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

4.5.2.Strain and Plasmid Construction

The gene encoding for FapR was codon-optimized and synthesized by Genscript (Piscataway, NJ). The *fapR* gene with or without an SV40 sequence at 3' end was assembled with the Tef1 promoter and the Hxt7 terminator into the pRS424 plasmid to yield pRS424-fapR-NLS or pRS424-fapR using the DNA assembler method (26). The tdTomato gene was cloned from ptdTomato plasmid purchased from Clontech (Clontech Laboratories, Inc, Mountain View, CA) and assembled with the Gpm1p-fapO promoter and the Adh1 terminator into pRS425 to yield pRS425-tdTomato using the DNA assembler method (26). Gene encoding malonyl-CoA reductase from *Chloroflexus aurantiacus* (*caMcr*) was cloned from *C. aurantiacus* cDNA into a pRS423 plasmid with the Tef1 promoter and the Hxt7 terminator using the DNA assembler method (26). Genes encoding acetyl-CoA carboxylase or [acyl-carrier-protein] S-malonyltransferase were cloned into pRS416 plasmid with the Pyk1 promoter and the Eno1 terminator. The resulting plasmids were then transferred into *E. coli* DH5α, which was plated on

LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion. All restriction enzymes were obtained from New England Biolabs (Ipwich, MA). These plasmids were transformed into the CEN.PK2-1C strain using the standard lithium acetate method (27). The resulting transformation mixtures were plated on SC dropout medium supplemented with 2% glucose.

4.5.3.Assay for Sensor Activity

The fluorescence signal intensity was used to characterize the promoter activity among the engineered sensors. Host cells transformed with different sensor plasmids were grown overnight in SC dropout media supplemented with 2% glucose at 30 °C and 250 rpm agitation. The overnight culture was inoculated into 2 ml fresh SC media with 2% glucose in 14 mL culture tubes at 30 °C, 250 rpm for approximately 24 hour. Different amounts of cerulenin were added into the media when necessary. Subsequently, 20 µl cell culture was transferred to 180 µL water in a Corning 96-well, clear bottom fluorescence plate (ThermoFisher Scientific, Rockford, IL). Cell density and expression of the tdTomato fluorescence protein were simultaneously detected using a Tecan Infinite M1000 PRO microplate reader (Tecan US, Inc, Morrisville, NC). Cell density was read at 600 nm and the excitation and emission wavelengths for tdTomato were set at 559 ± 20 nm and 581 ± 20 nm, respectively. Fluorescence intensity was normalized to cell density. All experiments were performed in duplicates.

4.5.4.Quantification of 3-HPA

3-HPA concentration was determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, CA), a Shimadzu RID-10A refractive index detector (Shimadzu Scientific Instruments, Columbia, MD) and a Shimadzu SPD-10A UV/Vis detector (Shimadzu Scientific Instruments, Columbia, MD) following the manufacturer's protocol. The HPX-87H column was kept at 25°C using a Shimadzu CTO-20AC column oven. 0.5 mM sulfuric acid solution was used as a mobile phase at a constant flow rate of 0.4 mL/min. 10 μL of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 60 minutes after the injection. The concentrations were determined using a standard curve generated using a series of external standards of 3-HPA purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

4.5.5.High-throughput screening

The RAGE library plasmids were transformed into the sensor strain the standard lithium acetate method (27). After transformation, the yeast cells were recovered at 30 °C with shaking for 1 h in YPA medium (1% yeast extract, 2% peptone and 0.01% adenine hemisulphate) with 2% glucose. The cells were then centrifuged and resuspended in SC-Leu-Trp-Ura medium with 2% glucose for growing at 30 °C with shaking overnight. On the next day, cells were analyzed on a BD FACS Aria III cell sorting system (BD Biosciences, San Jose, CA) and 4000-10000 tdTomato-positive cells with high fluorescence intensity were collected. After growth at 30 °C in both liquid and solid SC-Leu-Trp-Ura medium with 2% glucose for 2 days, colonies were separated and plasmids were extracted using the Zymoprep Yeast Plasmid Miniprep II Kit (Zymo

Research, Orange, CA). The plasmids were then electroporated into DH5α competent cells for amplification. The miniprepped plasmids were then subjected to the next round of screening and overexpression.

4.6.Figures

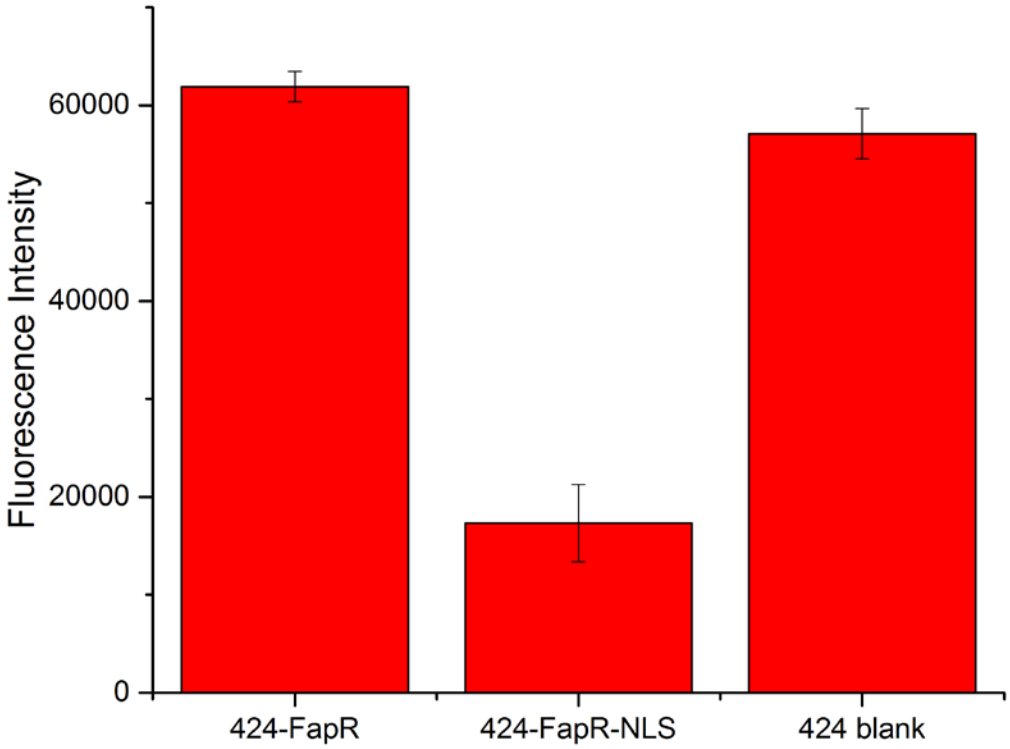

Figure 4.1 Fluorescence intensity of constructs expressing pRS425-tdTomato together with pRS424-FapR (no NLS), pRS424-FapR-NLS and blank pRS424. Error bars indicate standard deviations from duplicates.

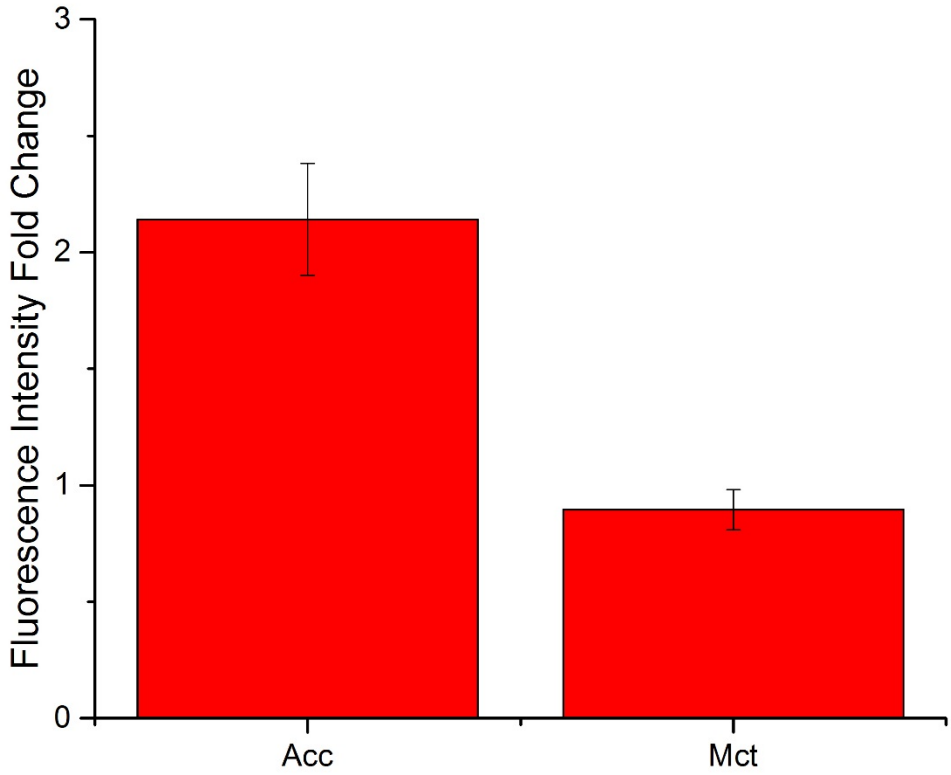

Figure 4.2 Ratio of fluorescence intensity in constructs overexpressing Acc or Mct to that of the wild type sensor construct. Error bars indicate standard deviations from duplicates.

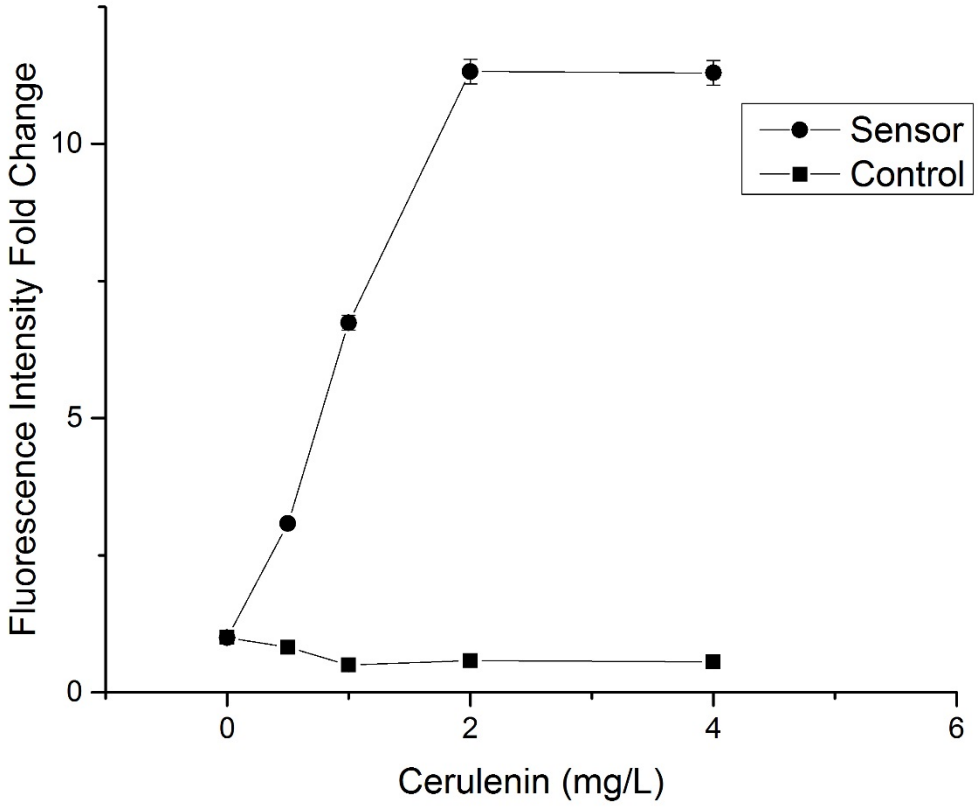

Figure 4.3 Fold change of fluorescence intensity when different levels of cerulenin were added into the culture medium. Sensor: ●, Control: ■. Error bars indicate standard deviation from duplicates.

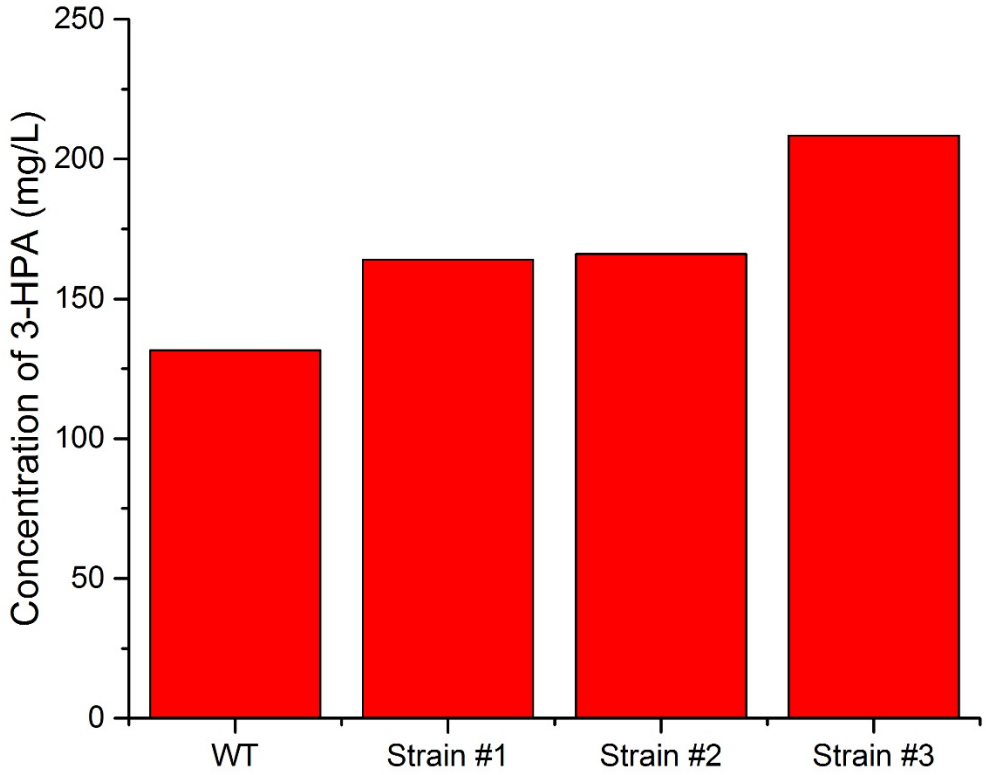

Figure 4.4 Concentration of 3-HPA produced from RAGE-improved malonyl-CoA overproducing strains.

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Chapter 5 Development of Sensors Based on Transcriptome Analysis

5.1. Introduction

Current efforts in microbial production of chemicals are mainly focused on pathway construction (1). Thus high throughput screening approaches based on sensors are extremely important for monitoring productivity and yield of the final products (2). Besides, engineering regulatory components to improve product titers and conversion yields of heterologous pathways dynamically is also reported as an efficient tool for chemical biosynthesis (3). A dynamic sensor-regulator system (DSRS) responsive to special target metabolites was developed to regulate the production of fatty acid–based molecules in *E. coli* (4). The DSRS utilized a transcription factor sensing the target key metabolite dynamically regulates the expression of genes involved in biodiesel production, thus increase the titer of biodiesel to 1.5 g/L (4). This transcriptional regulation based strategy can be extended to other pathways to increase product titers and yields and to maintain the host cell growth rate as well.

However, the development of such sensor reflecting key metabolite concentrations or such dynamic regulation system relies on the discovery and identification of transcription factors and operators, which are usually heterologous to the platform microorganism such as *E. coli* or *S. cerevisiae* (5). Significant efforts are needed to identify and characterize the two elements for the successful construction of sensors in these hosts (6-8).

Fortunately, multiple endogenous transcriptional regulation systems for a variety of

intracellular molecules have been evolved in nature, which could be used to detect the biosynthetic intermediate inside the host cells directly (9-11). Transcriptional factors (TFs) widely exist in wild type *S. cerevisiae* to regulate gene expression (11), which can be utilized directly without extra cloning. They bind to specific DNA sequences in a promoter region to either repress or activate transcription of upstream/downstream genes. The DNA-binding activity of many transcriptional factors can be affected by binding to metabolites, while many of these metabolites are involved in metabolic pathways responsible for synthesizing valuable products and act as target molecules of sensors. Building of such sensor endogenously can be easier: a metabolite-responsive transcription factor would have exist in the host cell already, the cognate DNA sequence (a promoter with a putative operator region) could be screened by transcriptome analysis under target metabolite deficient or abundant conditions, and then used in a designed reporter system that converts the target metabolite concentration to the fluorescence signal. The promoter-reporter plasmid is expressed within the host *in situ*, thus the expression of transcriptional factors can be bypassed. The cognate promoters screened with various responses to target molecules can also be used to regulate downstream or upstream gene expression to construct the dynamic regulation system that allows an organism to adapt its metabolic flux to changes within the host in real time and to enhance final product titer by controlling the key metabolite levels. Compared to conventional transcriptional regulation based sensor construction strategy, this key metabolite responsive promoter screening strategy utilizes the endogenous transcriptional factors existing within the host, thus avoids the complicated TF identification process. The utilization of endogenous TFs also lowers the expression burden derived from conventional heterologous protein expression. Moreover, through this screening strategy,

multiple promoters responsive to a target metabolite can be discovered in a short time, thus enables simultaneous regulation of multiple genes in a pathway.

Here we report the strategy for developing of multiple sensors targeting various key metabolites including acetyl-CoA, long chain acyl-CoAs, acetate, oxaloacetate, pyruvate and sugar-phosphates in *S. cerevisiae*. So far we have developed an effective fluorescent sensor responsive to cytosolic acetyl-CoA levels. The sensors can be used for real time fermentation monitoring and be combined with large-scale libraries to enhance the titer of products. The development of a dynamic regulation system as well as sensors responsive to other key metabolites are in progress.

5.2. Results

5.2.1. Construction of Key-Metabolite-Responsive-Promoters

Genome wide transcriptome analysis enables the profiling of transcriptional events for a given condition (12-14), and provides an efficient tool for the screening of key metabolite responsive promoters. Taking advantage of the transcriptional profiling study of the changes occurring in response to cellular depletion of the yeast acyl-CoA-binding protein (Acb1p) (12), we obtained a list of genes whose expressions were significantly changed by the depletion of Acb1p, which were usually involved in fatty acid and phospholipid synthesis. Both genome cDNA microarray and quantitative real-time PCR (Q-RT-PCR) data were investigated in order to identify transcriptional changes of putative genes in response to the cellular depletion of Acb1p (12). Though a total of 134 genes were identified with more than 1.6-fold changes of expression

levels, of which 44 genes were down-regulated and 90 genes were up-regulated, and 22 genes were examined by Q-RT-PCR. 12 of the genes with significant fold change in either the microarray analysis or the Q-RT-PCR assay were selected for promoter cloning and the entire DNA sequence between the open reading frame (ORF) of the target gene and the ORF of its upstream gene was cloned to ensure the maximal coverage of the functional region of the promoter. The 12 promoters of the genes responsive to metabolite change were assembled into a multi-copy pRS425 plasmid expressing eGFP as the fluorescent reporter to make the sensor plasmid candidates (listed in Table 5.1).

5.2.2. Screening for Key-Metabolite-Responsive-Promoters responsive to target molecules

To validate whether the sensors are responsive to long chain acyl-CoAs, long chain fatty acids with the length from C12 to C18 (dodecanoic acid, myristic acid, palmitic acid and stearic acid) were supplemented into the culture medium to increase the intracellular acyl-CoA concentrations. However, little growth or fluorescence changes were observed (data not shown) because of the toxicity of long chain fatty acids and low transportation activity of fatty acids into the cell (15,16). A plasmid constructed by my labmate Jiazhang Lian which harbored an acetyl-CoA overproducing pathway including cytosolic pyruvate dehydrogenase (PDH), ATP-dependent citrate lyase (ACL), and acetyl-CoA synthetase (ACS) (17) was co-expressed with the putative sensors to increase the intracellular acetyl-CoA/acyl-CoA concentration. Sensors with *ole, fas, erg* and *ino* promoters exhibited altered fluorescence signal (Figure 5.1) with the overproduced acetyl-CoA. As expected, the utilization of *ole* and *fas* promoters induced fluorescence negatively correlated to the level of acetyl-CoA, while *erg* and *ino* promoters induced a positive correlation between the fluorescence intensity and the acetyl-CoA level. At the

same time, the constitutive promoter *tef1* was used as a control and the fluorescence level remained at the same level when acetyl-CoA was overproduced.

5.2.3. Validation of the Selected Acetyl-CoA Sensor

To further validate whether the acetyl-CoA sensor could respond to different levels of cytosolic acetyl-CoA, two acetyl-CoA overproducing plasmids (17) with different levels of activities were co-expressed together with the *erg* promoter based sensor and the fluorescence intensity was measured. The two plasmids, ACS* and HZ1983, contain either an acetyl-CoA synthetase or a three-gene pathway composed of a pyruvate dehydrogenase, an ATP-dependent citrate lyase and an acetyl-CoA synthetase, and the later exhibited higher levels of acetyl-CoA production and resulted in over 4-fold higher n-butanol production than the former (17). A blank pRS424 plasmid was used as the wild type control in this analysis. The *erg* sensor responded to different pathways and showed either 55.0% or 3.47-fold higher fluorescence intensity in these two acetyl-CoA overproducing strains compared to the wild type control strain (Figure 5.2).

5.2.4. Erg-Promoter-Based Sensor Responded to Cytosolic Acetyl-CoA Rather than Long Chain Acyl-CoAs

To further investigate the target molecules of the *erg* sensor, the sensor was expressed in strains producing n-butanol via a reversed β-oxidation pathway respectively. A new biofuel production platform based on the reversed β-oxidation pathway enables the synthesis of fatty alcohols via acyl-CoAs in *S. cerevisiae* (18), coupled with simultaneous acetyl-CoA consumption and acyl-CoA production in the cytoplasm. The opposite trend enables the comparison of the response from the *erg* sensor to different CoAs.

Two plasmids, rP32 and rP35, containing reversed β-oxidation pathways were used. Both plasmids contain the entire pathway consisting of 4 enzymes converting acetyl-CoA to butyryl-CoA and higher acyl-CoAs, and the *trans*-2-enoyl-CoA reductases (TERs) derived from different microorganisms determined the varied activities. rP35 was reported with higher n-butanol, octanoic acid and hexanoic acid production than those of rP32, indicating higher levels of acyl-CoAs and lower level of acetyl-CoA. The fluorescence intensities from the strains co-expressing rP32 or rP35 with the *erg* sensor were only 71.6% or 58.7% of that of the wild type strain (Figure 5.3), indicating the responsive effect of the *erg* sensor to acetyl-CoA consumption is stronger than to the long chain acyl-CoAs production.

5.3. Discussions

Conventional sensor construction strategies are limited by the identification and characterization of transcription factors. Here we described a new strategy for the development of sensors in *S. cerevisiae* by screening for metabolite-responsive promoters from transcriptome analysis. This strategy utilizes natural regulation within *S. cerevisiae*, avoids expression and optimization of heterologous transcription factors and screens for multiple promoters in a short time, enabling both sensor construction and dynamic regulation of pathways composed of multiple genes. This strategy can be utilized for the development of sensors for various key metabolites in yeast metabolism including acetyl-CoA, malonyl-CoA, long chain acyl-CoAs, acetate, oxaloacetate, pyruvate and sugar-phosphates. As proof of concept, we utilized the transcriptional profiling data of an acyl-CoA-binding protein deletion strain to construct sensors responsive to acyl-CoAs. As proof of concept, we screened for a series of promoters responsive to acetyl/acyl-CoA and constructed a sensor using the *erg* promoter. The *erg* sensor showed a

positive correlation between the fluorescence intensity and the target molecule concentration.

There are two problems to solve in the sensor development process: a) the availability of promoter candidates: although there might be many promoters responsive to metabolite changes, lots of them might not be functional to regulate the fluorescent reporter expression directly. Instead, due to the complexity of the transcriptional regulation system within yeast, the number of promoters containing operator sequences can be limited. Among over 20 promoters, only 4 of them (*ole, fas, erg* and *ino*) showed altered fluorescence signal responsive to regulation. b) Specificity of the sensors: the transcriptional factor determines the specificity of the sensor system. However, more efforts on transcriptional factor characterization and identification are needed to investigate whether the sensor is specific to the target molecule or not. In the proof-of-concept *erg* sensor study, depletion of the Acb1p was used to induce metabolite changes. *In vitro* experiments have shown that Acb1p attenuates the inhibitory effect of long chain acyl-CoAs on mitochondrial adenine nucleotide translocase, stimulates the mitochondrial long-chain acyl-CoA synthetase, extracts membrane-imbedded acyl-CoAs and donates them to utilizing systems such as glycerolipid synthesis and β-oxidation (19,20). Thus the depletion of Acb1p that both transport acyl-CoA and facilitate acyl-CoA (21) is coupled with altered levels of both long chain acyl-CoAs and acetyl-CoA. To distinguish the responses to acetyl-CoA and long chain acyl-CoAs, we utilized the reversed β-oxidation pathway inducing opposite metabolic directions of the two putative target molecules: decreased acetyl-CoA concentration and increased acyl-CoAs concentration due to the biosynthesis converting acetyl-CoA to acyl-CoAs. Because of the specificity of the terminal enzymes such as the thioesterases and acyltransferases, the products were mainly limited to n-butanol. Thus the comparison is mainly between acetyl-CoA and

butyryl-CoA. Various pathway activities have been reported in the former study of our group, determined by different *trans*-2-enoyl-CoA reductases. Interestingly, we observed a lowered signal in the acyl-CoA overproducing strains, indicating the *erg* sensor reflects acetyl-CoA consumption rather than acyl-CoA. Thus we conclude the *erg* sensor has a higher responsive effect to cytosolic acetyl-CoA than to acyl-CoAs. Therefore, the strategy for the development of sensors based on transcriptome analysis could act as an efficient tool for the detection of cytosolic metabolites in yeast.

The transcriptome analysis based sensor development strategy can be utilized to construct sensors responsive to various cytosolic chemicals. For prokaryotic cells such as *E. coli*, this strategy can be widely expanded for the construction of intracellular chemicals (22). However, for eukaryotic cells such as *S. cerevisiae*, this strategy is limited by the cellular compartmentalization problem (23). The transcriptional factors are only functional when the target molecules binding to them exist in cytosol. As a result, sensors of metabolites mainly located in the enclosed cellular compartments such as mitochondria, chloroplasts, peroxisomes, lysosomes, the endoplasmic reticulum, or the Golgi apparatus (24) are hard to develop. Fortunately, abundant metabolic communications between cytoplasm and cellular compartments such as mitochondrion have be evolved in nature and many intermediates playing important roles in both cytoplasm and mitochondrion can be transported by shuttles and transporters, allowing the detection by sensors located in cytoplasm (25). For example, acetyl-CoA is the substrate for fatty acid synthesis, but besides that acetyl-CoA is converted from acetate in cytoplasm catalyzed by the acetyl-CoA synthetase (26), the production of acetyl-CoA occurs predominantly in the mitochondria matrix derived from the catalysis of a pyruvate dehydrogenase (27), the breakdown

of fatty acids (28) and the catabolism of ketogenic amino acids (29). Though the mitochondrial membrane is impermeable to acetyl-CoA molecules, acetyl-CoA can be transported depending on the activity of the citrate/malate exchange transporter, and formation of citrate from oxaloacetate and acetyl CoA inside mitochondrion using the condensing enzyme, and production of acetyl CoA and oxaloacetate in the cytoplasm using ATP-dependent citrate lyase (30), which enables the development of cytosolic sensors. By virtue of such transport systems, we can obtain the cytosolic concentration of the key metabolites that is able to reflect the global level of the target molecules in various cellular compartments.

To validate the acetyl-CoA sensor, two strains co-expressing an acetyl-CoA synthetase or a three-gene pathway composed of a pyruvate dehydrogenase, an ATP-dependent citrate lyase and an acetyl-CoA synthetase were used to induce altered fluorescence intensity. To enhance the cytosolic acetyl-CoA production, a heterologous acetyl-CoA biosynthetic pathway containing Acs with enhanced activity was reported to be functional. The overexpression of the entire PDH-bypassing pathway including three genes was reported with an even higher acetyl-CoA production (17). An acetyl-CoA dependent n-butanol pathway was constructed by Lian *et al.* as a model to report the acetyl-CoA level in the cytosol of yeast. From this work, the n-butanol titer in the acetyl-CoA synthetase overexpression strain was 2.5 mg/L while that in the three-gene pathway was over 13 mg/L (17). Consistent with the n-butanol reporter, the *erg* sensor also showed higher fluorescence in the three-gene pathway than the single acetyl-CoA synthetase overexpression strain, exhibiting a ~2-fold higher fluorescence signal. As a result, the *erg* promoter based acetyl-CoA sensor can work as effectively as the n-butanol reporter system. The trend is consistent, the time of analysis is much shorter, and the introduction of the heterologous

pathway can be bypassed. All these advantages prove the sensor constructed can be used as an efficient tool for the detection of CoAs in biological systems, which is much more convenient than the tedious and labor intensive extractions and analysis protocols (31,32).

5.4. Conclusions and Outlook

Here we developed a new strategy to screen for key-metabolite-responsive promoters for sensor construction and dynamic transcriptional regulation of biosynthesis pathways. The strategy utilizes promoters responsive to target molecules evolved in nature and the natural transcriptional regulation machinery existing in yeast, thus avoiding protein purification and characterization to identify the regulators. Large numbers of promoters can be discovered in a short time and be combined to regulate biosynthetic pathways dynamically. In a proof-of-concept study, 4 promoters responsive to cytosolic acetyl-CoA levels were screened and validated, and advanced investigation was carried out based on the e*rg* sensor, which exhibits obvious positive correlation along with the acetyl-CoA concentration. The erg sensor succeeded in differentiating two n-butanol producing pathways with different activities. We are continuing to develop more sensors based on this strategy to detect the concentrations of cytosolic metabolites such as malonyl-CoA, long chain acyl-CoAs, acetate, oxaloacetate, pyruvate and sugar-phosphates.

5.5. Materials and Methods

5.5.1. Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae CEN.PK2-1C (*MATa; ura3-52; trp1-289; leu2-3,112; his3Δ1; MAL2-8C; SUC2*) was purchased from EUROSCARF (EUROSCARF, Frankfurt, Germany) and used as a background strain. *Escherichia coli* DH5α was used for recombinant DNA

manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in baffled shake-flasks at 30 °C and 250 rpm for aerobic growth. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

5.5.2.Strain and Plasmid Construction

The putative promoters responsive to metabolite changes were cloned from the genomic DNA of *S. cereviaie* into a pRS425 plasmid together with an *eGFP* gene and a *Tef1* terminator using the DNA assembler method (33). The resultant plasmids were then transferred into *E. coli* DH5α, which was plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion. All restriction enzymes were obtained from New England Biolabs (Ipwich, MA). These plasmids were transformed into the CEN.PK2-1C strain using the standard lithium acetate method (34). The resulting transformation mixtures were plated on SC dropout medium supplemented with 2% glucose.

5.5.3.Assay of Sensor Activity

The fluorescence signal intensity was used to characterize the promoter activity among the engineered sensors. Host cells transformed with different sensor plasmids were grown

overnight in SC dropout media supplemented with 2% glucose at 30 °C and 250 rpm agitation. The overnight culture was inoculated into 2 ml fresh SC media was inoculated with 2% glucose in 14 mL culture tubes at 30 °C, 250 rpm for approximately 24 hour. Subsequently, 20 μL cell culture was transferred to 180 μL water in a Corning 96-well, clear bottom fluorescence plate (ThermoFisher Scientific, Rockford, IL). Cell density and expression of tdTomato fluorescent protein were simultaneously detected using a Tecan Infinite M1000 PRO microplate reader (Tecan US, Inc, Morrisville, NC). Cell density was read at 600 nm and the excitation and emission wavelengths for eGFP were set at 488 ± 20 nm and 509 ± 20 nm, respectively. Fluorescence intensity was normalized to cell density. All experiments were performed in triplicates.

5.5.4.Assay of Sensor Activity with additive fatty acids

Host cells transformed with different sensor plasmids were grown overnight in SC dropout media supplemented with 2% glucose at 30 °C and 250 rpm agitation. The overnight culture was inoculated into 2 ml fresh SC media was inoculated with 2% glucose in 14 mL culture tubes at 30 °C, 250 rpm for approximately 12 hour. Long chain fatty acids (dodecanoic acid, myristic acid, palmitic acid and stearic acid) were then supplemented into the culture medium with the final concentration of 20 mM.

5.6. Tables

Table 5.1 List of putative promoters

Promoters	Size (bp)	Transcriptional change responsive to Abp1
Ole1	1010	1.9
elo1	557	1.2
fas1	1029	1.6
fas2	504	1.6
erg11	868	-1.9
ino1	440	151
psd2	476	1.3
cho2	616	1.6
opi3	161	6.5
psd1	360	1.3
cho1	504	1.7
gpd1	1373	5.5

Table 5.2 List of plasmids involved in this study.

Plasmids	Constructs
p424	pRS424, multi-copy plasmid with *TRP1* marker
p425	pRS425, multi-copy plasmid with *LEU2* marker
p426	pRS426, multi-copy plasmid with *URA3* marker
rP32	p426-cytoFOX3-cytoYlKR-cytoYlHTD-TdTer-EcEutE-CaBdhB
rP35	p426-cytoFOX3-cytoYlKR-cytoYlHTD-cytoETR1-EcEutE-CaBdhB
pOle	p425-ole1p-eGFP-tef1t
pFas	p425-fas2p-eGFP-tef1t
pErg	p425-erg11p-eGFP-tef1t
pIno	p425-ino1p-eGFP-tef1t
pTef	p425-tef1p-eGFP-tef1t
ACS*	p424-Se*Acs*^{L641P}
HZ1983	pRS424-*PDC1-ALD6*-Se*Acs*^{L641P}

5.7. Figures

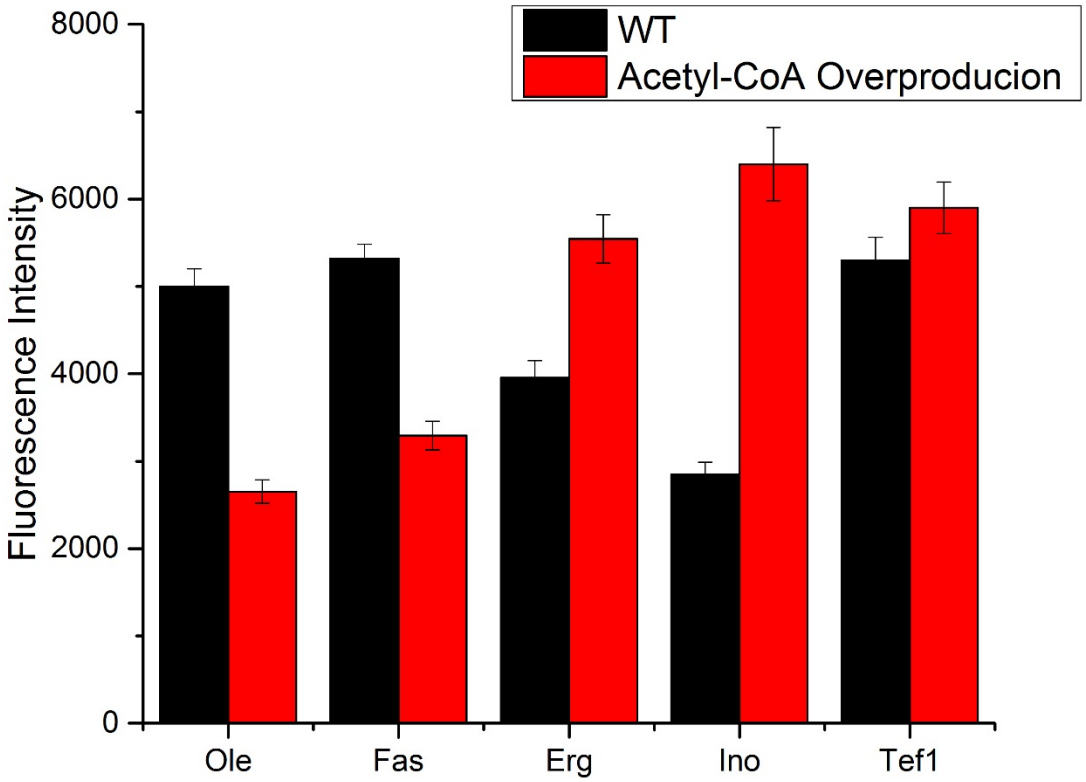

Figure 5.1 Sensors with altered fluorescence intensity responsive to overproduced acetyl-CoA. Constitutive promoter *tef1* was used as the control. Error bars indicate standard deviation from duplicates.

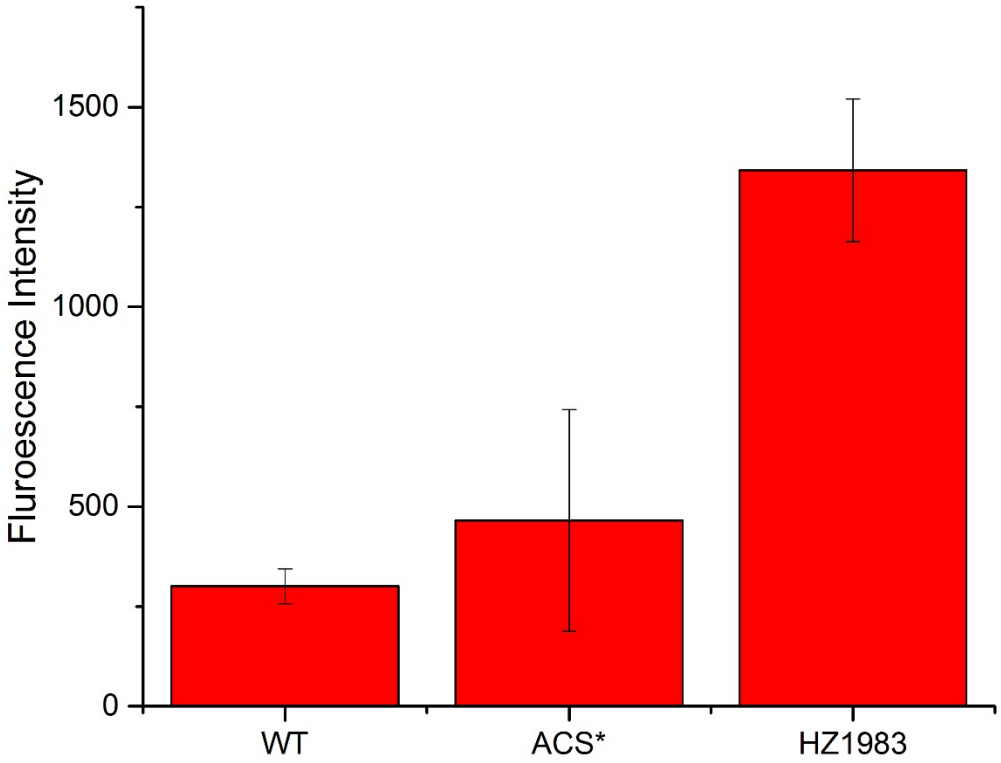

Figure 5.2 Altered fluorescence intensity in *erg* sensor strain responsive to different acetyl-CoA overproducing pathways. Error bars indicate standard deviation from duplicates.

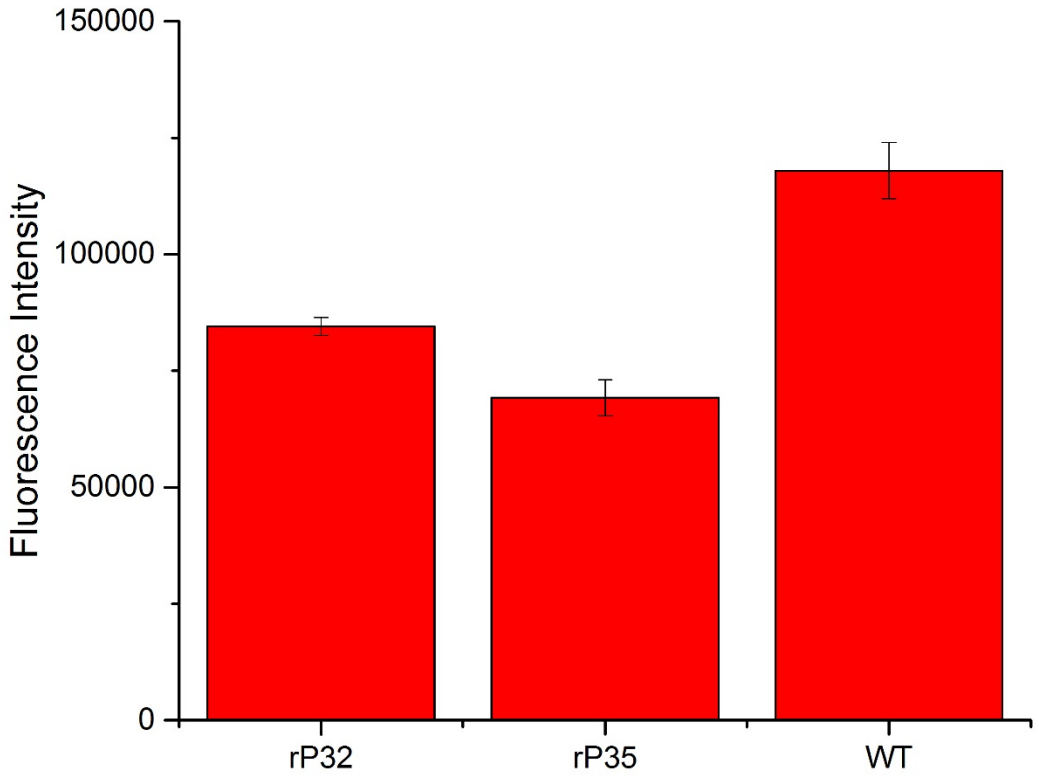

Figure 5.3 Altered fluorescence intensity in the *erg* sensor strain responsive to different reversed β-oxidation pathways. Error bars indicate standard deviation from duplicates.

5.8. References

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