Engineering synthetic promoters as dynamic controllers in *saccharomyces cerevisiae*

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

ENGINEERING SYNTHETIC PROMOTERS
AS DYNAMIC CONTROLLERS
IN *SACCHAROMYCES CEREVISIAE*

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2014

ENGINEERING SYNTHETIC PROMOTERS
AS DYNAMIC CONTROLLERS
IN SACCHAROMYCES CEREVISIAE

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To God be the glory, for in Him we live and move and have our being.

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EXECUTIVE SUMMARY

Towards the discovery and production of biofuels as a solution to energy security and environmental concerns, metabolic engineering of microbes is an attractive method that has great potential. *Saccharomyces cerevisiae*, which is also known as Baker's yeast, has been used industrially for bioethanol production, winemaking and brewing. Furthermore, *S. cerevisiae* is a well-researched model organism for biotechnology applications, resulting in an abundance of tools and knowledge for metabolic engineers to build upon. While bioethanol production has been industrialized, the challenge now is to engineer yeast for production of advanced biofuels, such as fatty acid derived biofuels, which can be used directly in the existing transportation infrastructure, and biofuel production from cheaper carbon sources, such as lignocellulosic biomass.

For high productivity biofuel production using microbes, it is crucial that the cells are able to grow well. This will enable large cell amounts to be accumulated which can in turn quickly convert substrates into products. In this thesis, six strategies for improving engineered cell viability were examined; first, the engineering of global transcription factors aided by systems biology, second, engineering of efflux pumps, third, spatial organization and optimization of pathway enzymes, fourth, engineering of dynamic sensing-regulation of protein expression, fifth, the balancing of co-factor usage and consumption in bio-catalytic pathways, and sixth, the use of computational tools to aid engineering. While all the

strategies expounded are recognised and important ways to boost cell viability and productivity, dynamic sensing-regulation, which is to develop synthetic promoters that function as dynamic controllers was selected for research and development.

Firstly, synthetic promoters systems that can sense and regulate protein expression upon detection of fatty acids/fatty acyl-CoA were constructed and characterized. As fatty acid derivatives have ideal properties for use as drop-in biofuels to existing transportation infrastructure, dynamic control of protein production in response to concentrations of fatty acids is a good strategy to optimize the engineered strain. In yeast, native transcription factors responsive to fatty acids have repressed activities in glucose rich conditions. Hence, in order to create a fatty acid sensor-regulator in yeast, bacterial FadR transcriptional repressors and yeast synthetic promoters that contained DNA binding operators were used. The functionality of FadR repressors was demonstrated. Tuning of the sensing system was carried out by changing promoter strength upstream to FadR coding sequence, by varying the number of operator sites in the synthetic promoter, and by using transcriptional repressors from two bacterial sources (*Escherichia coli* and *Vibrio cholerae*) that possess different ligand sensitivities.

Secondly, AND-gate dynamic controllers were created using synthetic promoters combining various upstream enhancer elements and core promoter allowing for response to fatty acids/fatty acyl-CoA. As the expression of heterologous proteins in metabolic engineering efforts can

be harmful to cells due to increased cellular resources consumption, dynamic controls, which enable on-demand protein expression, are effective for the engineering and optimization of microbes towards improved cell viability and enhanced biochemical productivity. AND-gate dynamic controllers which combine two dynamic control strategies, inducible promoters and sensing-regulation were created and characterized. These dynamic controllers were constructed using synthetic hybrid promoters. Yeast promoter upstream enhancer elements were combined with a synthetic GAL1 core promoter that contained DNA binding sites for the binding of FadR. Results demonstrated that the synthetic GAL1 core promoter can be used as a modular promoter part for constructing synthetic hybrid promoters and enabling fatty acid inducibility. Further, the performance of the AND-gate dynamic controllers, where two inputs (fatty acid and copper presence / phosphate starvation) were required to switch the AND-gate ON was shown.

Thirdly, xylose biosensors were created and characterized. For economically sustainable biofuel production, cheap and affordable starting material such as lignocellulosic biomass is needed. However, lignocellulosic hydrolysates contain various sugars, one major component of which is xylose that cannot be metabolized by yeast. Hence, engineering and optimization of xylose assimilating yeast strains has been the focus of much research. Genetically encoded xylose biosensors that can regulate protein expression upon detection of xylose sugars are a possible optimization strategy. Xylose biosensors were

created with constitutive expression of XylR repressors, which function as protein sensors, and synthetic promoters with XylR operator sites, which are bound by XylR in the absence of xylose to repress transcription. Upon xylose detection, XylR DNA binding activity is antagonized, and the downstream yEGFP reporter expression increases. Three XylR variants and the matching synthetic promoters were used, and the expression levels of the XylR proteins were changed to optimize the biosensor. XylR was shown to function in yeast to repress yEGFP expression from the synthetic promoters. Addition of exogenous xylose sugars to regulate yEGFP expression was also demonstrated.

In conclusion, synthetic promoters have been shown to be effective tools for dynamic regulation of protein expression in yeast. Fatty acid/fatty acyl-CoA and xylose can be detected by FadR and XylR repressors respectively, and these repressors control the output from the constructed synthetic promoters. Replacement of promoter upstream enhancer elements allowed AND-gate dynamic controllers to be made, which enabled both inducible control and dynamic sensing-regulation. While these successes were shown, challenges remain. This includes the ability to fully remove repressor activity upon inducer detection, and growth inhibition by repressor overexpression. The cost and benefit of expressing the heterologous repressors needs to be evaluated on a case-by-case basis to determine its true utility in optimizing engineered yeast strains.

CHAPTER 1 : INTRODUCTION

1.1 BACKGROUND

1.1.1. Energy and biofuels

Energy is crucial in our daily lives. Electricity powers our homes, offices and factories whereas oil and gas enables transportation and heating. The production or harvesting of energy sources at a sufficient and sustainable level is critical for our well-being. Sources of energy include non-renewable energy of petroleum, natural gas, coal and nuclear, which will deplete in the foreseeable future, and renewable energy of biomass, hydropower, geothermal, wind and solar, which are naturally replenished (Figure 1.1). Besides ensuring energy security and supply for us and the future generations, environmental concern is also an important factor that must be considered in the pursuit of energy production. The drilling for petroleum and natural gas and mining for coal in addition to their combustion can significantly change the landscape and release large amounts of greenhouse gases that lead to climate change.

Towards the discovery and production of renewable energy sources, much effort has been put into biofuels to make large quantities at competitive prices. However, the biofuels of bioethanol and biodiesel are being used as blends with petrol or diesel respectively as a result of incompatibility with existing transportation infrastructure. In addition, bioethanol is corrosive towards engines and transportation pipes, and the distillation of bioethanol is a costly process. On the other hand, biodiesel will degrade if stored for a long time and can even freeze when

temperatures drop. Furthermore, both bioethanol and biodiesel have poorer energy content relative to the natural petrol and diesel (Balat and Balat 2008; Peralta-Yahya et al. 2012) (Figure 1.2).

Figure 1.1 Non-renewable and renewable energy sources

Figure 1.2 Drawbacks of bioethanol and biodiesel

As a result of the non-ideal bioethanol and biodiesel properties, the discovery and production of advanced biofuels with ideal properties suitable for direct use in existing transportation infrastructure is important. To this end, scientists and engineers set out to discover new molecules and molecules similar to petroleum with high energy content and excellent fuel properties.

Fatty acid derived biofuels, including alkane, alkene and fatty alcohol are suitable for drop-in to existing transportation systems (Figure 1.3). They have high energy density, are hydrophobic to ease separation and are chemically similar to the petroleum analogs (Tai and Stephanopoulos 2012). However, as opposed to yeast fermentation to produce bioethanol, fatty acid derived biofuels are found only at small quantities in microbes. Hence, the ability to boost the production to economically sustainable levels is needed.

Figure 1.3 Fatty acid derived biofuels

1.1.2 Metabolic engineering of Baker's yeast

Metabolic engineering offers the potential for the creation of synthetically engineered microbes that can produce high levels of desired products (Figure 1.4). By manipulating the genetic landscape in a selected host microorganism, which is by introducing foreign genes, disrupting existing genes or changing the existing expression levels of various proteins, researchers can equip the engineered microorganisms to produce desired molecules.

Baker's yeast, *Saccharomyces cerevisiae* has long been used for baking, brewing and wine making. As a result, much biotechnology research has been carried out to understand and improve yeast's

Figure 1.4 Production of biochemicals from biomass derived carbon sources via metabolic engineering

properties for various purposes. Knowledge about yeast's genetics, physiology, biochemistry, methods for its engineering as well as infrastructure and technology have amassed over time. Furthermore, yeast is classified as GRAS (generally regarded as safe) by the US Food and Drug Administration (Nevoigt 2008). These properties make yeast an attractive microbe for engineering for production of desired biochemical molecules. In fact, while yeast has mainly been used for bioethanol production, recent metabolic engineering endeavours have reported the production of many more important and valuable molecules. Some examples include pharmaceuticals (Resveratrol, Artemisinin, and Aureothin), fine and bulk chemicals (vanillin, glycerol, propanediol, and muconic acid) and fuels (butanol, farnesane, and biodiesel) (Figure 1.5) (Keasling 2010; Liu et al. ; Nevoigt 2008).

Figure 1.5 Some examples of biochemical production from engineered yeast reported in literature

1.1.3 Towards improved production levels

In order to successfully engineer yeast metabolism with high productivity and titers, various strategies are required. This includes selection and screening of enzymes with high activities (Schirmer et al. 2010; Shi et al. 2012; Steen et al. 2010), expression of the enzymes at appropriate levels using promoters (Dahl et al. 2013; Zhang et al. 2012b), knocking out competing pathways to redirect reaction flux (Runguphan and Keasling 2014; Tang et al. 2013), engineering the accumulation of important pathway intermediates (Chen et al. 2014a; Chen et al. 2014b; Tang and Chen 2014) and pumping out products that could be toxic to

the host cell (Chen et al. 2013; Ling et al. 2013). However, even with extensive work on engineering yeast for industrial production of drop-in advanced biofuels, it remains a key challenge to obtain high yields of advanced biofuels (Peralta-Yahya et al. 2012; Rabinovitch-Deere et al. 2013).

All the strategies mentioned are crucial towards achieving the final engineered yeast strain. Promoters, which control the transcriptional level of genes, and ultimately protein expression levels, were chosen for research and development. In yeast, the promoters consist of the upstream enhancer element, which contains DNA sequence for binding by a DNA binding transcriptional activator, and the core promoter region, which includes the TATA box for binding of the TATA binding protein (Figure 1.6) (Barberis and Petrascheck 2001).

Figure 1.6 Transcription initiation in eukaryotic promoters. DBD, DNA binding domain; AD, activation domain.

1.2 OBJECTIVES

As fatty acid derivatives have ideal properties to be used as drop-in biofuel in today's transportation infrastructure, metabolic engineering of microbes capable of producing such molecules with high yields and titers is paramount. Dynamic controllers with promoters enable protein expression to be triggered on-demand. Dynamic control using inducible promoters allows cells to proliferate quickly before directing resources toward formation of desired chemicals (Holtz and Keasling 2010; Lo et al. 2013). In addition, dynamic control via the sensing of key intermediates to control gene expression enables real-time modifications to a cell's metabolic flux when conditions change, in effect facilitating efficient use of cellular resources (Farmer and Liao 2000; Zhang et al. 2012b).

Moreover, towards affordable biofuels, the use of lignocellulosic biomass as a starting material could prove to be crucial. However, yeast does not naturally assimilate xylose, which is a major component in lignocellulosic hydrolysates. Hence, strategies towards developing xylose utilizing yeast strains and their optimization are also of great interest. The development of dynamic controllers based on xylose can empower engineered yeast cells to regulate the expression of xylose utilizing enzymes to achieve optimal cellular resource usage. Also, a xylose based dynamic controller can assist the screening of xylose transporters in yeast, which is a possible bottleneck in xylose consumption pathways.

The objectives of this thesis are as follow:

- 1) Review strategies to engineer microbes with high cell viability for production of biochemicals
- 2) Design and characterize a fatty acid/fatty acyl-CoA sensing system in *S. cerevisiae*, and tune the sensing system using different sources of protein regulator, different number of operators in synthetic hybrid promoters and different expression levels of the protein regulator
- 3) Construct and characterize AND-gate dynamic controllers, where the function of the inducible promoter and sensing of fatty acid to regulate protein expression are combined in a promoter system
- 4) Develop and characterize xylose biosensors, which enable xylose dependant control of protein expression
- 5) Discuss challenges in dynamic controller development, evaluate the importance of the dynamic controllers and recommend future opportunities for further promoter development

1.3 THESIS OUTLINE

This present introduction **Chapter 1** lays down the background describing the importance and motive of engineering synthetic promoters in yeast. In **Chapter 2**, I will review strategies available for improving cell viability. The improvement of cell viability in engineered microbes is especially important given the extra metabolic load given to the cell, and that high biochemical productivity is dependent on good cell growth.

While all the strategies detailed in Chapter 2 are proven and important methods to boost cell viability and productivity, the approach of developing synthetic promoters that function as dynamic controllers for experimental research was chosen for this study. In **Chapter 3**, the development and characterization of synthetic promoters systems that can sense and regulate protein expression in response to fatty acids/fatty acyl-CoA are described. **Chapter 4** continues on the creation of dynamic controllers, where AND-gates are made using synthetic promoters combining various upstream enhancer elements and core promoter that are responsive to fatty acids/fatty acyl-CoA. **Chapter 5** describes the development and characterization of xylose biosensors, which empowers cells to control protein expression based on the concentrations of xylose sugars detected.

Chapter 6 concludes this thesis by evaluating the importance of dynamic controllers, the challenges faced, as well as recommending future opportunities for synthetic promoter development.

CHAPTER 2 : MICROBIAL ENGINEERING STRATEGIES TO IMPROVE CELL VIABILITY FOR BIOCHEMICAL PRODUCTION

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2.1 INTRODUCTION

During the microbial production of chemicals such as biofuels, pharmaceutical drugs, bio-polymers and other value-added compounds, bio-catalytic cells are often subjected to a variety of biotic and abiotic stresses that may threaten cell viability (Attfield 1997). Diminished cell viability usually results in negative consequences, for example, lower productivity and sluggish fermentation (Mannazzu et al. 2008). In industrial production plants, process interruption due to sluggish fermentation or constant cell replacement may result in considerable economic losses (Souza et al. 2007). To ensure continuous bio-conversion with cell recycle for minimal process interruption and maximized bio-catalytic productivity, improving cell viability is crucial.

Cell viability is defined by various factors, namely stress tolerance towards toxic chemical compounds (substrates, intermediates, products), harsh external environments (pH, temperature, osmolarity, starvation, oxidation), and growth rates. Improving cell stress tolerance has been the main focus for many prior studies (Atsumi et al. 2010; Borden and Papoutsakis 2007b; de Bont 1998; Nicolaou et al. 2010; Ruhl et al. 2009) because host cells with robust stress tolerance can grow well and accumulate biomass quickly. However, in the context of bio-catalysis, other factors such as heterologous protein expression (Scott et al. 2010; Scott and Hwa 2011) and metabolic flux balance can also greatly influence growth rates.

Towards the goal of enhancing bio-catalytic productivity, this review discusses various engineering strategies that can be harnessed for improvement of cell viability (Figure 2.1). First, the engineering of global transcription factors aided by systems biology approaches can improve the host cell's stress tolerance. Second, through engineered efflux pumps, the accumulation of intracellular toxic products can be reduced in bio-catalytic cells, leading to increased cell viability. Third, spatial organization and optimization of pathway enzymes can potentially improve cell viability via reduction of toxic intermediates accumulated. Fourth, the engineering of dynamic sensing-regulation of protein expression enables improved metabolic balance and efficient usage of cellular resources, resulting in improved growth rates. Fifth, the balancing of co-factor usage and consumption in bio-catalytic pathways

Figure 2.1 An overview of the key approaches towards developing a microorganism platform for improved viability.

helps to maintain the cellular redox state for viable growth. Finally, to facilitate this multi-faceted scheme and balance conflicting objectives (e.g. enzyme expression vs. growth rates), this review also emphasizes the use of computational tools that assist in engineering optimized bio-catalysts.

2.2 TRANSCRIPTION FACTOR ENGINEERING FOR IMPROVED STRESS RESPONSE

The development of bio-catalytic cells often requires genetic manipulation of microbial cells to carry heterogeneous genetic regulatory parts and metabolic pathways. For maximized productivity of the bio-catalysts, further optimization by engineering other physiological functions, such as growth rates, ancillary morphological changes (e.g. flagella or pilus development, biofilm formation) and tolerance to environment, is necessary (Lanza and Alper 2011). These physiological functions are complex phenotypes which are determined by complicated interactions of multiple genetic components and environmental factors. As a result, it has been difficult to obtain a desired phenotype by a gene-by-gene modification approach (Patnaik 2008). Rather, strains that show desired phenotypes have been found by chance or by artificially inducing genetic mutations through a vast number of successive cultures. However, this approach has been limited to inflexible host choices, long time-frame and lack of modular host optimization processes. Therefore, there has been increasing interest in rationally

engineering complex phenotypes while overcoming the above-mentioned limitations.

One approach to engineer desired complex phenotypes was to engineer global transcription machineries. Alper and co-workers have attempted to alter ethanol tolerance and lycopene production by combinatorial mutation of global transcription machineries such as sigma factors in *E. coli* (Alper 2006) and a TATA-binding protein and other associated factors in the yeast *S. cerevisiae* (Alper et al. 2006; Alper and Stephanopoulos 2007). A similar approach has also been employed in *S. cerevisiae* to improve xylose fermentation (Liu et al. 2010). In addition to global transcription machineries, a few known transcription factors have been engineered to improve resistance to stress conditions. For example, cyclic AMP receptor protein (CRP) was fused with various types of zinc finger DNA-binding proteins in *E. coli* (Lee et al. 2008), and a randomly mutated CRP was expressed in *E. coli* (Zhang et al. 2012c). In other studies, IrrE, a global regulator in *Deinococcus radiodurans*, was shown to confer osmotic pressure and ethanol tolerance in *E. coli* upon expression (Ma et al. 2011). Further, it has been shown that an introduced exogenous regulator indeed significantly reconstituted transcriptome and proteome profiles (Chen et al. 2012).

The above-mentioned studies chose to modify genetic components which could lead to desired phenotypes by inducing global changes in a biological system. While these targets were not derived from systems-level understanding of physiological functions, there have been studies

to identify targets by characterizing complex phenotypes and reconstituting genetic regulatory networks towards systems engineering. As mentioned earlier in this section, complex phenotypes are outcome of interactions between multiple genetic components and environmental factors. In other words, it is genetic regulatory networks that determine transcriptomic profiles that lead to desired phenotypes under a specific condition. It is worth to note that genetic regulatory networks consist of hierarchical, but highly inter-connected interactions of genetic components. In addition, genetic regulatory networks can be dynamically rewired due to activity of transcriptional regulators and degeneracy of transcription factors and transcription factor binding sites under different conditions (Balleza et al. 2009). These features of genetic regulations imply that different combinations of transcription factors can simultaneously bind to their regulon members in a condition-dependent way. Therefore, it is important to identify global regulators that change their activities for a particular physiological function.

To this end, Brynildsen and co-workers identified 16 transcription factors of which activities were changed in response to isobutanol stress in *E. coli*. Network component analysis was used to predict activity changes of transcription factors from gene expression data and the essentiality of a few identified transcription factors was verified by gene deletion (Brynildsen and Liao 2009). While this study was limited to inferring the activity changes of known transcription factors, a new algorithm was developed to reconstruct regulatory networks using the same transcriptome data used in the above study and predicted 7 new

transcription factor activity changes (Fu et al. 2011). Although these studies identified transcriptional regulators and reconstructed regulatory networks for rational engineering of complex phenotypes, it was not addressed whether the engineering of the identified targets could rewire regulatory networks leading to desired phenotypes such as improved tolerance to stress conditions. Toward this goal, we have shown that rewiring of genetic regulatory networks can be achieved by combinatorial deletion of transcription factors identified from transcriptomic data (Kang and Chang 2012). We demonstrated that the rewired genetic regulatory networks resulted in increased tolerance to isooctane in *E. coli*. While iterative characterization and identification of regulatory networks can further improve isooctane tolerance, we established that modification of transcription factors could be a promising strategy to rationally engineer complex phenotypes towards improved bio-catalytic performance (Figure 2.2).

Figure 2.2 Rational engineering of complex phenotypes by three approaches introduced in the text. A schematic diagram presents steps of identification and reconstitution of regulatory networks towards improved cellular responses.

2.3 HARNESSING EFFLUX PUMPS AGAINST PRODUCT TOXICITY

Product toxicity is often a major limitation to bio-catalytic conversion especially at industrial scales which require high titers and yield of end-products. The engineering of host cells with improved cell tolerance has led to significant boosts in productivity. For example, ethanol production in an engineered strain of *S. cerevisiae* was improved by 15% when its ethanol and glucose tolerance was improved through global transcriptional machinery engineering (Alper et al. 2006). Commonly used engineering strategies for improving cell tolerance towards toxic end-products include mutagenesis, adaptive evolutionary engineering, genome shuffling and creation of genomic libraries (Alper et al. 2006; Borden and Papoutsakis 2007a; Hou 2010; Stanley et al. 2010). However, these classical tolerance-improving strategies are often time-consuming and laborious. To illustrate, more than 6 months was needed to generate ethanol-tolerant mutants via the adaptive evolutionary engineering method. If the genomic library approach is to be used, huge libraries (>>10,000 transformants) have to be obtained and screened. In contrast to the above approaches, harnessing efflux pumps to bio-catalytic cells provides a direct strategy for efficiently improving tolerance by expelling toxic compounds from cells.

Efflux pumps are membrane transporters involved in expulsion of toxic compounds from cells. There are two major classes of efflux pumps, ATP-binding cassette (ABC) transporters and secondary efflux pumps. ABC transporters are primary transporters that utilize ATP as energy

source to catalyse the translocation of substrates. ABC transporters consist of two trans-membrane domains that bind the chemical compound and provide a translocation channel, and two cytoplasmic nucleotide-binding domains that hydrolyze ATP to drive transport (Rees et al. 2009). On the other hand, secondary efflux pumps utilize proton or sodium gradients as a source of energy. They consist of three protein subunits: a cytoplasmic membrane export protein responsible for chemical compound recognition and proton exchange, a periplasmic linker, and an outer membrane channel (Koronakis et al. 2000).

The use of efflux pumps for active excretion of toxic compounds from cells has received much attention for its roles in alleviating toxicity and increasing productivity (Higgins 2007) (Huffer et al. 2012). Examples of characterized efflux pumps for reducing toxicity include *Pseudomonas aeruginosa* Mex pumps (MexA-MexB-oprM, MexC-MexD-OprJ, and MexE-MexF-OprN) for organic solvents such as n-hexane, and *p*-xylene (Li et al. 1998; Ramos et al. 2002), *P. putida* Ttg pumps (TtgABC, TtgDEF, and TtgGHI) for toluene (Rojas et al. 2001), and *E. coli* AcrAB-TolC pump for hexane, heptane, octane and nonane (Tsukagoshi and Aono 2000). Recently, efflux pumps from the hydrophobe / amphiphile efflux (HAE1) family were heterogeneously expressed in *E. coli* to improve tolerance towards five representative biofuels (geranyl acetate, geraniol, α -pinene, limonene and farnesyl hexanoate) (Dunlop et al. 2011a). In addition, we discovered that the expression of heterologous ABC transporters conferred significant tolerance to hydrocarbons in yeast cells (unpublished data). Besides tolerance improvement, bio-

catalyst productivity can also be enhanced by efflux pump-mediated export of toxic compounds. The yeast multidrug resistance ABC transporter Pdr18p plays an indirect role in ethanol efflux through decreasing ethanol-induced plasma membrane permeabilization and reducing the intracellular ethanol concentration, thereafter leading to 17% higher ethanol production yield at a toxic concentration (Teixeira et al. 2012). In addition, the limonene-producing strain expressing an efflux pump demonstrates a 1.6 fold improvement in limonene yield (Dunlop 2011). However, while limonene is toxic to the *E. coli* host, the production yield is not yet at a toxic level. Hence, the efflux pump may have increased the productivity of the limonene bio-catalyst by relieving product inhibition of metabolic pathway enzymes. In another example of efflux pumps serving to relieve product inhibition of metabolic pathway enzymes for improved productivity, by overexpression of the ABC transporter AvtAB, avermectin productivity was improved by around 1.5 fold (from 3.3 to 4.8 g/l) in *Streptomyces avermitilis*. Moreover, the ratio of intracellular to extracellular accumulation of avermectin dropped from 6:1 to 4.5:1. It is highly likely that the AvtAB transporter exports avermectin, and thus reduces the feedback inhibition of avermectin biosynthesis inside the cell (Qiu et al. 2011).

While efflux pumps provide a direct mechanism for improving tolerance and productivity of bio-catalysts, there are a few barriers on harnessing efflux pumps. Firstly, pump overexpression can be toxic to host cells because excessive efflux pumps may overload membrane insertion machinery, and hamper cell division and protein homeostasis (Wagner

et al. 2007; Wagner et al. 2008). To avoid toxicity from pump overexpression, inducible expression by exogenous inducers is more preferred than constitutive expression (Figure 2.3A). Further, several studies have contributed to resolving membrane protein toxicity by synthetic biology design (Figure 2.3B). For example, the expression of a family of ion pumps was increased 5- to 25-fold in *E. coli* by changing the frequency of transcription initiation under a “restrained expression” mode (Narayanan et al. 2011). This suggests a mechanism of balancing the rates of protein production to the cell’s capacity for membrane protein biogenesis. Furthermore, a model was developed for improving

Figure 2.3 Improvement of cell viability and pathway productivity by harnessing efflux pumps.

(A) Efflux pump expression is induced by an exogenous inducer. (B) Efflux pump expression is regulated by a transcription factor (TF), a synthetic promoter and end-product (Dunlop et al., 2010). (C) Efflux pumps are engineered with desirable substrate specificity. P, exogenous inducer-responsive promoter; Ps, synthetic promoter responsive to TFs and end-product; purple ovals, efflux pumps; red stars, end-product synthesized in biocatalysts; blue stars, exogenous inducer; blue diamonds, chemicals or metabolites; circles, transcription factors. Blue and red arrows, transcriptional activation; end line, transcriptional repression.

microbial productivity using a synthetic feedback loop that controls efflux pump levels to balance toxicity due to end-products and pump overexpression. As a result, final product yields were significantly improved (Dunlop et al. 2010). Secondly, availability of efflux pumps with desirable substrate specificity is still limited. As shown in Figure 2.3C, directed evolution provides a strategy on altering the specificity of efflux pumps. According to Bokma et al., the shuffled *E. coli* TolC variants adapted to the non-cognate *Pseudomonas* MexAB translocase, and conferred resistance to the efflux substrate novobiocin (Bokma et al. 2006). As described by Huffer et al., directed evolution of *E. coli* AcrB enhanced the growth of *E. coli* by about 20% when short to medium chain alcohols were supplied exogenously in the medium (Huffer et al. 2012). However, it is still challenging to develop desirable efflux pumps specific to biofuels especially to short chain alcohols such as ethanol, n-butanol, and isopentanol (Dunlop et al. 2011a). Thirdly, pump evaluation is limited due to shortage of producing strains. The functionality of efflux pumps towards chemicals added in growth medium might leave a gap between promise and practice.

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

specificity towards improving tolerance and productivity; and (ii) evaluating novel efflux pumps in producing strains.

2.4 SPATIAL ENGINEERING AND COMPARTMENTALIZATION AGAINST INTERMEDIATE TOXICITY

In the microbial biosynthesis of chemicals, intermediates generated in the pathway may be toxic to the host cell (Agapakis et al. 2012; Chen and Silver 2012; Conrado et al. 2008; Lee et al. 2012; Zhu et al. 2012). Reducing intermediate concentration for improved cell viability is undesirable as low intermediate concentrations limit the reaction rate. High intermediate concentrations without exposing the host cell to toxic compounds can be achieved by localizing the intermediates into compartments. In nature, organelles (Kerfeld et al. 2010; Martin 2010) and large enzyme complexes (Conrado et al. 2008) are commonly used as compartments to segregate pathways and their metabolites. Metabolic engineers can take advantage of available compartments by tagging proteins with localization signals (Martin 2010), or create synthetic compartments to spatially organize and optimize heterologous pathways. To this end, researchers have developed synthetic scaffolds using proteins (Dueber et al. 2009), DNA (Conrado et al. 2012) and RNA (Delebecque et al. 2011) as well as protein based shells (Chen and Silver 2012; Lee et al. 2012).

One demonstration of the effectiveness of synthetic scaffolds to reduce metabolic load and improve productivity involves the mevalonate biosynthetic pathway in *E. coli* (Dueber et al. 2009). This pathway

involves three enzymes, one being acetoacetyl-CoA thiolase (AtoB), which is naturally present in the host, and two others, hydroxyl-methylglutaryl-CoA synthase (HMGS) and hydroxyl-methylglutaryl-CoA reductase (HMGR), which are heterologous to *E. coli*. The lower enzyme activity of HMGS relative to HMGR results in flux imbalance and accumulation of the toxic intermediate HMG-CoA. To overcome these challenges, the authors created protein scaffolds using three protein-protein interaction domains derived from metazoans. By varying a number of interaction domains on glycine-serine linkers, different ratios of pathway enzymes were co-localized on the scaffold. This enabled the engineering of a mevalonate production strain with a 77-fold increased product titer in addition to faster growth rates. At this point, the mechanism by which synthetic scaffolds enhance growth and productivity is unclear. It has been hypothesized that scaffold compartmentalization reduces accumulation of toxic intermediates outside the scaffold (Lee et al. 2012). By co-localizing pathway enzymes at optimal stoichiometries, intermediates can be quickly converted into products.

Another potential strategy in compartmentalizing pathways involves protein based shells (Chen and Silver 2012; Lee et al. 2012). By encapsulating pathway enzymes into protein shells, the shells act as physical barriers which can selectively regulate metabolite entry and exit. In this way, pathways which generate toxic intermediates can be sequestered to protect the host from the toxicity from the intermediates. Protein shells are available in nature, such as bacterial

microcompartments (Choudhary et al. 2012), lumazine synthase complex (Wörsdörfer et al. 2011) and encapsulins (Sutter et al. 2008). These shells have been successfully assembled in heterologous hosts and tagged proteins were localized into the shells. However, the sequestration of synthetic metabolic pathways has yet to be demonstrated. Much work remains to be done, including finding a balance between shell complexity and the number of proteins that can be localized, and engineering metabolite selectivity of protein pores (Lee et al. 2012).

2.5 DYNAMIC SENSING-REGULATION OF PROTEIN EXPRESSION

The engineering of synthetic pathways in bio-catalytic cells requires the heterologous expression of proteins. This creates a competition for cellular resources, resulting in an increased metabolic burden that adversely affects cell growth (Görgens et al. 2001). Furthermore, some proteins or metabolites could be toxic, resulting in lower cell viability and growth rates. In order to minimize undesired proteins and metabolites from being produced at the “wrong time” and hence maximize the cell’s ability to proliferate, inducible promoters are often used. Inducible promoters enable the separation of growth phase from production phase; at low cell densities, cells proliferate and accumulate biomass without the additional metabolic load of heterologous protein expression, whereas at higher cell densities, the inducible promoters are added, so that the cells produce enzymes necessary for production of desired chemicals (Figure 2.4A). Even though inducible promoter systems are

widely used in metabolic engineering for production of useful chemicals, the cost of inducers may prohibit their usage at industrial scales (Keasling 2010). On the other hand, inducible promoters regulated by native signals, such as nutrient starvation (Price et al. 1990; Shin and Seo 1990; Vogel and Hinnen 1990) and cell density (Miller and Bassler 2001; Saeidi et al. 2011; Tsao et al. 2010), enable temporal control and inducer-free protein expression.

In the construction of synthetic pathways for production of chemicals, often multiple genes are required. However, enzymes at different steps in the pathway may have varying expression levels (or activities). This results in bottlenecks, leading to accumulation of intermediates that may be toxic to the host. This also indicates a waste of cellular resources in the overproduction of enzymes for non-rate limiting steps. Hence, coordination of protein expression levels would greatly enhance growth and maximize productivity (Keasling 2010). To this end, researchers have developed strategies for tuning gene expression, including promoter strength (Blazeck and Alper 2012), ribosome binding sites (Salis et al. 2009), copy number (Jones et al. 2000) and enzyme degradation (Mateus and Avery 2000). Indeed, these strategies have been found to be effective in improving pathway productivity and cell growth. However, when disturbances in bioreactor conditions result in sub-optimal conditions, for which these static controllers have not been designed, the system may not respond accordingly, and productivity is not maximized. A more stable system can be created by the use of

Figure 2.4 Dynamic sensing-regulation of protein expression.

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

feedback mechanisms, i.e. dynamic regulation of protein levels (Holtz and Keasling 2010). Dynamic regulation enables real-time adjustments of metabolic flux upon variation in the host or environmental conditions. It also facilitates efficient utilization of cellular resources, where

expression of pathway enzymes is controlled to an optimal level for conversion of substrates into products, and intermediates are converted into downstream products immediately after synthesis. These advantageous properties of dynamic regulation lead to robust cell growth and enhanced pathway productivity.

Dynamic regulation is commonly used in cells, with one example being catabolite repression. The cell's ability to sense glucose enables metabolism of preferred carbon sources, minimizing expression of enzymes for utilization of alternative carbon sources (Gancedo 1998). To synthetically engineer such a feedback mechanism, a sensor-regulator system for the metabolite of interest needs to be identified (Figure 2.4B). Most basic sensor-regulator systems involve endogenous sensing systems, where one would only need to identify and use promoters or native riboswitches (Montange and Batey 2008) involved in the regulation mechanism. However, many metabolites and desired products in metabolic engineering endeavours are not naturally found in the host cell. Therefore, introduction of heterologous proteins or synthetic riboswitches is often necessary. A recent review by Michener et al. has described the possible protein and RNA sensors that can potentially be applied in the construction of sensor-regulator systems (Michener et al. 2012).

The first demonstration of dynamic sensing-regulation of a heterologous pathway for improved growth and productivity was by Liao and co-workers (Figure 2.4C) (Farmer and Liao 2000). In this study, a global

regulatory system in *E. coli*, the Ntr regulon was engineered to regulate a synthetic lycopene biosynthesis pathway. The deletion of the transcription factor nitrogen regulator II (NRII) allowed NRI to become a sensor for acetyl phosphate (ACP). The sensing of intracellular ACP levels, which were suggested to be a glucose availability indicator (Nyström 1994), together with the *glnAp2* promoter, enables regulation of two rate limiting enzymes in lycopene biosynthesis. Hence, excess glucose flux can be efficiently utilized for the desired lycopene biosynthesis pathway and at the same time, reduce formation of the toxic product acetate. This dynamic regulation control loop was found to significantly enhance lycopene production and reduce growth retarding metabolic imbalance.

Recently, a dynamic sensor-regulator system (DSRS) was developed by Keasling and co-workers for the production of the biodiesel fatty acid ethyl ester (FAEE) (Figure 2.4D) (Zhang et al. 2012b). Instead of employing indirect indicators (as used in the lycopene biosynthesis example), the direct sensing of critical pathway intermediates would serve to create a better regulation system, where production and consumption of intermediates can be controlled to maintain optimal concentrations and maximize pathway productivity. In this work, fatty acyl-CoA was identified to be a key intermediate for regulation. Since *E. coli*'s native FadR transcription factor is capable of responding to fatty acyl-CoA, the DNA-binding sequence for FadR was integrated into phage promoters along with DNA-binding sequence for LacI, a repressor that can be antagonised by isopropyl β -D-1-

thiogalactopyranoside (IPTG). The creation and adoption of this hybrid promoter responsive to both IPTG, which eliminates leaky expression and enables synthetic exogenous induction, and fatty acyl-CoA, which controls expression levels of key enzymes led to a superior FAEE producing strain. FAEE yield increased three-fold, and metabolic balance was improved, while the engineered strain had greater genetic stability.

These examples have experimentally proven the importance and effectiveness of dynamic regulation for the engineering of microbial cell factories with improved productivity and growth. Besides the direct sensing and regulation via critical pathway intermediates, further improvement of the system would exclude the need for any exogenous inducer, and at the same time, maintain minimal basal expression at early growth phases. Our group has recently developed an engineered *E. coli* strain for production of lignin-derived chemicals. In this work, a sensor-regulator for the substrates lignin constituents was engineered to enable dynamic regulation of producing enzymes. This was coupled with a time-delay element, allowing the cells to auto-induce pathway enzymes at higher cell densities. We found the growth rate and productivity of the engineered strain to be significantly improved (unpublished data). Other research groups have established the importance and utility of dynamic regulation *in silico* (Anesiadis et al. 2008; Dunlop et al. 2010; Gadkar et al. 2005). The role of modelling in the engineering of production strains with robust growth and improved productivity will be addressed in Section 2.7.

Even though sensor-regulator systems were found to be effective in improving host growth and productivity, suitable sensor-regulator systems for metabolic engineering purposes are limited. Metabolite biosensors that use auxotrophic strains, or protein and RNA sensors with small dynamic ranges can only be used as screening tools instead of pathway regulation (Michener and Smolke 2012; Zhang and Keasling 2011). Besides identifying sensors for a metabolic pathway of interest, much engineering effort is required to create regulators (e.g. hybrid promoters) that possess appropriate strength and dynamic range (Zhang et al. 2012b). Signal to noise ratios (Dietrich et al. 2010) as well as tightness of regulation are also crucial factors that determine the utility of a sensor-regulator system in pathway regulation applications.

2.6 BALANCING CO-FACTORS IN PATHWAYS AND KEY METABOLISMS

Redirecting metabolic fluxes by altering the level of co-factors such as NADH, NADPH and CoA is one of the promising metabolic engineering approaches to boost productivity in bio-catalysts. Co-factor manipulation allows overcoming bottleneck of co-factor availability in co-factor dependent bio-synthesis pathways. Furthermore, co-factors influence a broad range of host cellular functions such as cellular respiration, metabolite synthesis and membrane transport (Futai 1974). As such, utilization of co-factors during bio-chemical synthesis may re-shape the metabolic landscape of the host, resulting in diminished host viability. One example is metabolic engineering for succinate production in *E. coli*

(Bunch et al. 1997), where gene knockouts ($\Delta ldhA$; $\Delta pfkB$) were performed to redirect the metabolic flux towards succinate production, but resulted in a 2:1 NADH/NAD⁺ imbalance. The unusually high intracellular NADH/NAD⁺ ratio led to the growth impairment of the engineered strain. Hence, in recognition of the importance of maintaining the intracellular co-factor balance for host viability, this section is devoted towards strategies for limiting co-factor imbalances, which often result from metabolic engineering and novel pathway introduction.

One key co-factor imbalance that needs to be addressed promptly is the NADH/NAD⁺ ratio (Figure 2.5). NADH/NAD⁺ ratio may be regarded as the indicator of the intracellular redox state (Singh et al. 2009), and it plays a major role in microbial metabolism by overseeing over 300 redox reactions (San et al. 2002), notably, in glycolysis. During glycolysis, glucose is oxidized using NAD⁺ and the reducing equivalent in the form of NADH is produced. For continued cell growth, the reduced NADH needs to be re-oxidized to NAD⁺ to support glucose oxidation. One approach to regenerate NAD⁺ is to recycle intracellular NAD breakdown products and scavenge pyridine compounds such as nicotinic acid from the environment. To do so, the enzyme nicotinic acid phosphoribosyltransferase (NAPRTase) that converts pyridine compounds such as nicotinic acid into a direct precursor of NAD⁺ can be expressed. NARTases such as PncB from *Salmonella typhimurium* were expressed in *E. coli* (Berrios-Rivera et al. 2002b; San et al. 2002), and PncB1 and PncB2 from *Mycobacterium tuberculosis* were studied for

Figure 2.5 Approaches towards balancing co-factors for boosting anaerobic growth under batch condition, supplying reducing power for reducing substrate to product and restoring NADPH / NADH balance.

their role in NAD synthesis (Boshoff et al. 2008). It was observed that overexpression of PncB in *E. coli* had greater effects on the final distribution of metabolites during anaerobic batch cultivation than in anaerobic chemostat conditions (Berrios-Rivera et al. 2002b). Under anaerobic batch cultivation, increase in total level of NAD increases the rate of NADH-dependent pathways. As such, cultivation conditions became one of the key considerations when the NAD⁺ level is increased. In addition to NAPRTase expression, alternative approaches to boost NAD⁺ availability include expression of water-forming NADH oxidase, which was successfully attempted in *Lactococcus lactis* NZ9800 (Lopez de Felipe et al. 1998) by using the *nox-2* gene from *Streptococcus mutans*, and in *S. cerevisiae* (Heux et al. 2006) by using the *noxE* gene from *L. lactis*, which reduced cytosolic NADH accumulation.

While increased NAD⁺ availability aids in maintaining NADH/NAD⁺ intracellular pool, in situations where NADH is needed to reduce metabolic intermediates for bio-chemical synthesis, NADH availability becomes the primary focus (Figure 2.5). To regenerate NADH from NAD⁺, Berrios-Rivera and co-workers (Berrios-Rivera et al. 2002a) expressed NAD⁺-dependent formate dehydrogenase (FDH) from *Candida boidinii* in *E. coli*, which doubled the yield of NADH from 2 to 4 mol NADH per mol glucose consumed at the end of anaerobic fermentation. The increase in availability of NADH even induced a shift to fermentation in *E. coli* during aerobic growth (Berrios-Rivera et al. 2002a). Other FDHs from organisms such as *Mycobacterium vaccae*

N10 and *S. cerevisiae* were expressed to improve D-mannitol productivity (Heuser et al. 2009) and to increase glycerol yield on glucose in *S. cerevisiae* (Geertman et al. 2006), respectively. Another approach for improving NADH regeneration is the use of methanol oxidation pathways. Methanol, rather than commonly used glucose, serves as an auxiliary substrate for NADH regeneration in terms of 2 molecules of NADH produced per molecule of methanol. The oxidation of methanol to CO₂ is spontaneous as it is driven by a favorable thermodynamic force (Schroer et al. 2010). Additionally, this pathway exists naturally in methylotrophic yeasts such as *Pichia pastoris*. Schroer and co-workers (Schroer et al. 2010) demonstrated that by overexpressing formaldehyde dehydrogenase (FLD), one of the key enzymes in the methanol utilization pathway, endogenously in *Pichia pastoris*, higher theoretical NADH formation rate was achieved, and butanediol production rate was significantly improved for whole-cell bio-transformation.

Though the NADH/NAD⁺ ratio can be maintained through enzymatic expression that oversupplies NADH, compartmentalization of NADH production in eukaryotic hosts was shown to have important metabolic consequences. For example, Hou and co-workers (Hou et al. 2010) evaluated the effect of increasing NADH in *S. cerevisiae*'s mitochondria and cytosol. Increasing mitochondrial NADH in *S. cerevisiae* triggers ethanol production while increasing NADH in cytosol results in glycerol production. This finding suggests possible manipulation of carbon flux in

the central carbon metabolism for rational engineering of eukaryotic hosts through compartmentalizing NADH production.

In addition to the NADH/NAD⁺ ratio, the NADH/ NADPH ratio is also of importance (Figure 2.5). While NADH is the chief redox product of catabolism, NADPH plays a major role in anabolism where it serves as a reducing agent for amino acid, lipid and nucleotide biosynthesis (Hou et al. 2009). Introduction of bio-synthesis pathways such as α -santalene formation pathway (Scalcinati et al. 2012) leads to a net production of NADH and consumption of NADPH. To restore NADH / NADPH balance, Scalcinati and co-workers reshaped the host co-factor landscape by deleting the NADPH consuming reaction of glutamate dehydrogenase (GDH1) and coupling it with overexpression of NAD⁺-dependent glutamate dehydrogenase (GDH2) that resulted in NADH consumption. Such a strategy may require the use of genome-scale modelling of hosts to predict the global network effects of cofactor balancing, as demonstrated by Ghosh and co-workers (Ghosh et al. 2011) in their attempt to engineer *S. cerevisiae* towards effective utilization of pentose sugars. In addition to metabolic engineering for co-factor balancing, enzymatic specificity towards NADH versus NADPH can be adjusted for desired outcome. Hasegawa and co-workers (Hasegawa et al. 2012) changed co-factor requirements for L-valine synthesis from NADPH to NADH by modifying the co-factor specificity of acetohydroxy acid isomeroreductase (AHAIR) through site-directed mutagenesis, where NADP-specific positively charged arginine was replaced with NAD-specific negatively charged glutamate.

Another important co-factor is CoA and its derivative acetyl-CoA. Acetyl-CoA is a key intermediate in various metabolic pathways for energy production, and is involved in the synthesis of esters and lipid molecules (San et al. 2002). In the work by Lee and co-workers (Lee et al. 2009) where *E. coli* was engineered to convert ferulic acid into vanillin, the introduced bio-synthesis pathway led to the entrapment of CoA as acetyl-CoA, a by-product of vanillin synthesis. This entrapment caused a shortage of CoA for the first step in the vanillin production pathway which requires ferulic acid to be converted to feruloyl-CoA. To overcome this problem, Lee and co-workers overexpressed the citrate synthase *gltA* which catalyzes the first reaction of the TCA cycle so that acetyl-CoA (by-product) could be utilized to synthesize citrate for CoA release. The *gltA* amplification led to a 2-fold increase in vanillin production.

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

2.7 COMPUTATIONAL TOOLS FOR DESIGN AND PREDICTION

Towards balancing multi-faceted objectives such as cell growth, flux optimization, thermodynamics, economic costs and other factors in bio-catalytic cells, computational methods are extremely useful in handling these enormous data and providing some very handy predictions for the *in silico* design. Current computational methods on designing new enzymatic pathways have been developed based on characterized pathways and theoretical thermodynamic feasibility, and re-adjustment of metabolic routes (gene knock-out or amplification), which are useful for tuning the host cell and constructing genetic parts required for the assembly of genetic circuits. In this section, we relate the use of these tools to optimizing host viability (Figure 2.6).

2.7.1 Host engineering

The first stage of engineering bio-catalysts is the construction of synthetic pathways that lead to the synthesis of target compounds. To aid in designing bio-synthetic routes, useful databases such as the Kyoto Encyclopedia of Genes and Genome (KEGG) (Kanehisa et al. 2006) and MetaCyc (Caspi et al. 2012) can be referred to. However, information on biochemical pathways and metabolic networks from those databases is frequently insufficient because energetic costs of producing more enzymes should also be taken into consideration when new pathways are introduced. Since the metabolic load of heterologous enzyme expression directly affects cell viability (Scott and Hwa 2011),

Figure 2.6 An *in silico* approach for designing the cell for bio-chemical production, but with host viability as key consideration. After determining bio-synthesis pathways, both the top-down and the bottom-up approaches can be carried out concurrently. The top-down approach refers to host cell engineering which requires the prediction of the impact on the global flux distributions by metabolic engineering and novel pathway introduction. The bottom-up approach involves optimizing DNA sequences and adjusting genetic circuit layout for minimal metabolic requirements, but yet achieving maximal bio-catalytic performances.

pathway distance should be considered carefully. However, pathway distance can only be an applicable criterion if a host is selected *a priori*, since a host cell may contain one or more enzymes from the designed pathway in its inherent metabolic network. This issue can be alleviated by using computational strategies such as the one employed in the Biochemical Network Integrated Computational Explorer (BNICE) framework (Finley et al. 2009; Henry et al. 2010), which can be useful for prioritizing novel biosynthetic routes based on pathway length, thermodynamic feasibility, maximum achievable yield and maximum achievable enzymatic activity. Henry and co-workers (Henry et al. 2010) applied the BNICE framework to generate novel shortest pathways for synthesis of 3-hydroxypropanoate from glucose. In addition, another prediction system DESHARKY (Rodrigo et al. 2008) included transcription, translation and metabolic load into its calculation, which can be particularly useful for optimizing cell viability because it provides information on the impact of designed metabolic pathways together in a cellular context.

Once an enzymatic pathway has been selected for introduction into a host organism, its impact on host cells can be predicted using genome-scale modelling. Genome-scale modelling utilizes the reconstructed map of genome-scale metabolic bio-chemical reactions which are defined by their reaction stoichiometry. In this manner, model predictions can be made based on the constraints imposed on the reaction fluxes and a defined objective function, such as cell biomass growth, for which the fluxes are optimized. Users can refer to the Biochemically, Genetically

and Genomically structured (BiGG) (Schellenberger et al. 2010) knowledgebase for reconstructed genome-scale metabolic maps. These maps are designed to work with analytic tools such as the Constraint Based Reconstruction and Analysis (COBRA) (Becker et al. 2007) Toolbox for quantitative prediction of cellular metabolism. COBRA Toolbox has been frequently used for constraint-based analyses on genome-scale models (Medema et al. 2012). Our group utilized COBRA Toolbox on BiGG maps such as *E. coli* iAF1260 for predicting the impact of the designed bio-synthesis pathways on steady-state metabolic flux distribution and growth rate. This was done by adding stoichiometric coefficients and novel compounds from the designed reactions into the BiGG map, and then, new steady-state flux distribution was predicted using the constraint-based flux balance analysis (FBA) (Orth et al. 2010), a function available in COBRA Toolbox. Besides FBA, COBRA Toolbox v2.0 (Schellenberger et al. 2011) can also be used to direct metabolic flux towards the production of targeted compounds. This may frequently require gene deletions which can have a considerable impact on host viability. Therefore, to examine whether the suggested gene deletion set would have minimal impact on host growth, the OptKnock (Burgard et al. 2003) function in COBRA Toolbox v2.0 can be used. The OptKnock algorithm identifies gene deletion strategies that allow production of targeted compounds to become an obligatory by-product of cellular growth. Further, the OptKnock-suggested gene-deletion sets can be completed by the minimization of metabolic adjustment (MOMA) (Segrè et al. 2002) function in COBRA Toolbox. MOMA works based on

the hypothesis that the knock-out metabolic fluxes would undergo a minimal redistribution with respect to the flux configuration of the wild-type. Brochado and co-workers (Brochado et al. 2010) improved vanillin production by 2-fold by using MOMA as the biological objective function for their *in silico* simulations.

2.7.2 Genetic circuit construction

After the COBRA Toolbox has been used for *in silico* prediction and thus, the whole genome-scale metabolic landscape has been reshaped, genetic parts construction frequently starts. Genetic parts such as promoters, ribosome binding sites (RBS) and coding sequences (CDS) play a significant role in allocation of metabolic resources, which in turn affects host growth. For example, strong constitutive promoters and RBS initiate high level of gene expression which may consume considerable amount of metabolic resources. The diversion of cellular resources from essential metabolic activities may subsequently lead to growth retardation (Scott and Hwa 2011) and deviation from engineered behaviors (Canton et al. 2008; You et al. 2004), both of which can decrease yield and productivity. To avoid such situations, the minimalist approach of moderate expression of enzymes can be employed in biocatalytic cells. The first step towards this approach is the codon optimization of CDS. Codon optimization circumvents rate-limiting translations, a consequence due to codon bias in host organisms. By identifying rate-limiting codon sets and replacing them with synonymous codons that are more recognizable by abundant tRNA species in the

host, codon elongation rate can be higher (Racle et al. 2012). One handy web-based tool Optimizer (Puigbò et al. 2007) can be utilized for codon optimization process for over 150 prokaryotic species. For advanced users who prefer visual computer-aided design (CAD) tool, Gene Designer 2.0 (Villalobos et al. 2006) can be helpful. In addition to codon optimization, the intrinsic properties of mRNA sequence should also be examined, including mRNA stability and secondary structure. Having a loop structure at the 5'-end of the mRNA may impede translation. To both minimize the 5' structure and match codon bias, the backtranslation in Gene Designer 2.0 can be used, which first minimizes the 5' structure, and then designs remaining gene sequences for desired codon usage (Welch et al. 2011). An optimized CDS enables a higher level of translation per mRNA transcript, therefore could allow the use of weaker synthetic promoters to reduce over-transcription and save on precious metabolic resources.

Further, the optimized CDS can be coupled with a suitable RBS for tuning expression towards optimality. Enzymes with different kinetic efficiencies should be expressed according to their levels of activity. For instance, highly active enzymes can be expressed on a weaker RBS to prevent overexpression and results in a more efficient use of metabolic resources. To tune the expression towards the desired level, synthetic RBSs designed using RBS Calculator (Salis et al. 2009) can be used. Users can decide on the level of translation by simply inputting the CDS and the preferred translation initiation rate which ranges from 0 to 100,000-fold into the RBS Calculator, and the synthetic RBS sequences

to deliver the desired outcome would be generated. While this approach enables greater control of protein expression levels, the optimal translation rate has to be obtained through trial-and-error.

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

2.8 CONCLUSION

Biological production of chemicals is gaining importance and could possibly define the future of chemical synthesis. However, commercially feasible bioprocesses for the production of biofuels, pharmaceuticals, chemicals, and other valuable compounds demand high yields and titers, and at the same time, low costs. Given that bio-catalytic performances are frequently affected by toxicity arising from chemical production and stresses resulting from metabolic imbalances, engineering host cells to sustain robust viability during bioconversion processes, which can increase biomass accumulation and stability in bio-catalytic performances, is certainly one of the most critical steps for the successful development of commercially feasible bio-catalytic cells. Consequently, it is imperative to design engineering strategies to improve microorganisms' viability against the aforementioned stresses and limit their impact.

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

**CHAPTER 3 : BACTERIAL FADR AND SYNTHETIC
PROMOTERS FUNCTION AS MODULAR FATTY ACID
SENSOR-REGULATORS IN SACCHAROMYCES
CEREVISIAE**

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3.1 INTRODUCTION

High energy prices as a result of limited oil and gas supply and climate concerns have led to the search and development of renewable energy sources. The naturally produced ethanol biofuel from yeast fermentation can be used as a transportation fuel, and significant efforts are being made towards engineering the use of biomass as feedstock (Lin and Tanaka 2006; Weber et al. 2010). However, in comparison to petrol fuels, ethanol possesses only 70% of the energy content, in addition to corroding engines and transportation pipes, and requiring expensive distillation to be separated from the fermentation broth. As a result, ethanol is often used as blends with petrol (Stephanopoulos 2007; Tai and Stephanopoulos 2012). Another petrol alternative is biodiesel. This too has its downside, where the energy content is lower, and biodiesel degrades in prolonged storage and even freezes at lower temperatures (Balat and Balat 2008). The production of biofuels with more ideal properties is desirable as they would serve to be drop-in fuels to existing transportation infrastructure.

Fatty acids derivatives, including fatty alcohols, fatty acid ethyl esters, fatty acid methyl esters, alkenes and alkanes, have ideal properties that enable their use as drop-in biofuels; they have high energy density, are hydrophobic and are chemically similar to their petroleum analogs (Peralta-Yahya et al. 2012; Tai and Stephanopoulos 2012). In recent years, various groups have engineered microbes to produce fatty acids derived biofuels (Peralta-Yahya et al. 2012; Schirmer et al. 2010; Steen

et al. 2010). For economically sustainable biofuel production, the yield, titers and productivity must be sufficiently high. Several strategies for strain optimization are available, such as tuning of promoter strength (Alper et al. 2005) and ribosome binding sites (Salis et al. 2009) to regulate gene expression, improving of tolerance to toxic biofuels concentration (Dunlop et al. 2011b), and metabolic engineering of fatty acids overproducing strains (Cho and Cronan 1995; Steen et al. 2010) to increase intermediate concentrations.

Another effective strategy to improve engineered strains involves dynamic regulation of enzyme levels via sensing of intermediates concentration. This was recently demonstrated, where researchers created a dynamic sensor-regulator system in *E. coli* that dynamically controls biodiesel producing enzyme levels in response to fatty acids/fatty acyl-CoA concentrations (Zhang et al. 2012b). In that study, *E. coli*'s native fatty acyl-CoA/fatty acid responsive transcription factor, FadR was used in partnership with various synthetic hybrid phage promoters to tune the transcription level of various key enzymes. It was shown that such dynamic regulation increased the yield three-fold, in addition to improved genetic stability and metabolic balance.

We sought to design a dynamic fatty acid sensor-regulator system in the yeast *S. cerevisiae*. In this eukaryotic organism, the induction of fatty acid responsive genes is dependent on two transcription factors, Oaf1 and Pip2. When Oaf1 is activated by fatty acids, it forms a heterodimer with Pip2, and subsequently localizes to promoters containing Oleate

Responsive Elements (OREs) to trigger transcription. However, in the presence of glucose, the activities of both Oaf1 and Pip2 are directly repressed (Karpichev and Small 1998; Rottensteiner et al. 1997). Since glucose is an affordable and important carbon source for yeast, the use of ORE containing promoters to regulate the levels of fatty acid derivative-producing enzyme is unsuitable.

Hence, in this chapter, we describe for the first time the development of a modular fatty acid regulation system in *S. cerevisiae* using heterogeneous bacterial FadR transcriptional repressors and synthetic yeast promoters. FadR represses transcription by binding to specific operator sites and is de-repressed by the ligands fatty acids/fatty acyl-CoAs (DiRusso et al. 1992). Further, in contrast to Oaf1/Pip2, FadR is unaffected by catabolite repression (Feng and Cronan 2012). Strategies to tune the system included promoter strength, the number of operator sites and bacterial origin of FadR. The functionality of the FadR repressors towards the construction of fatty acids/fatty acyl-CoAs responsive system in the eukaryotic organism *S. cerevisiae* was demonstrated.

3.2 MATERIALS AND METHODS

3.2.1 Plasmids, strains and media

Plasmids, *E. coli* and *S. cerevisiae* strains used are listed in Table 3.1. Primers used for PCR amplification are listed in Table 3.2. *E. coli* strain Top10 was used for transformation and amplification of plasmids, cultured at 37 °C in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin. *S. cerevisiae* strain BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) was obtained from ATCC (Manassas, VA). Yeast extract and peptone were obtained from BD (Franklin Lakes, NJ), while other chemicals were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise stated. Yeast strains were grown in YPD medium (yeast extract 10 g/l, peptone 20 g/l, D-glucose 20 g/l), minimal medium (yeast nitrogen base 6.7 g/l, yeast synthetic drop-out medium supplements – Ura- 1.92 g/l, D-glucose 20 g/l), or induction medium (yeast nitrogen base 6.7 g/l, yeast synthetic drop-out medium supplements – Ura- 1.92 g/l, galactose 20 g/l). Fatty acids (oleic acid, palmitic acid, myristic acid and lauric acid) were solubilized with Tween 80 and added to a final concentration of 0.5% Tween 80, at the stated fatty acid concentrations (medium pH 5.5). 2% agar was added, where necessary, for solid medium. Plasmid pESC-URA (Agilent Technologies, Santa Clara, CA) was used for reporter protein expression whereas plasmid pIS112 (EUROSCARF, Frankfurt, Germany) (Sadowski et al. 2007) was used for promoter characterization as well as FadR expression.

Table 3.1 Yeast strains and plasmids used in this study

Strain or plasmid	Description	Source
<u>S. cerevisiae strains</u>		
BY4741	MATa; <i>his3</i> Δ1; <i>leu2</i> Δ0; <i>met15</i> Δ0; <i>ura3</i> Δ0	ATCC
BY-CON	<i>ADE8::null</i> derived from BY4741	This study
BY-TEF1-E	<i>ADE8::TEF1p-yEGFP-Adh1t</i> derived from BY4741	This study
BY-CYC1-E	<i>ADE8::CYC1p- yEGFP-Adh1t</i> derived from BY4741	This study
BY-TEF1-EC	<i>ADE8::TEF1p-FadR_E.coli</i> derived from BY4741	This study
BY-CYC1-EC	<i>ADE8::CYC1p-FadR_E.coli</i> derived from BY4741	This study
BY-TEF1-VC	<i>ADE8::TEF1p-FadR_V. cholerae</i> derived from BY4741	This study
BY-CYC1-VC	<i>ADE8::CYC1p-FadR_V. cholerae</i> derived from BY4741	This study
<u>Plasmids</u>		
pESC-URA	2μ plasmid vector; Used for expression of reporter construct (synthetic GAL1 promoter- <i>yEGFP</i>)	Agilent
pIS112	Integrative plasmid to ADE8 locus; Used for expression of <i>yEGFP</i> for promoter characterization, and for expression of FadR	EUROSCARF
pESC-1XEC	GAL1-1XFadBA_EC- <i>yEGFP</i> cloned into pESC-URA at XhoI and NotI	This study
pESC-3XEC	GAL1-3XFadBA_EC- <i>yEGFP</i> cloned into pESC-URA at XhoI and NotI	This study
pESC-1XVC	GAL1-1XFadBA_VC- <i>yEGFP</i> cloned into pESC-URA at XhoI and NotI	This study
pESC-3XVC	GAL1-3XFadBA_VC- <i>yEGFP</i> cloned into pESC-URA at XhoI and NotI	This study

Table 3.2 Primers used in this study

Name	Sequence	Site*
GAL1-f	CCAAC <u>CTCGAG</u> ACATGGCATTACCACCATA TACATATCCATATC	XhoI
GAL1tata-r	TGGTAGGATCCATTTATATATCTGTTAATAG ATCAAAAATCATCGCTTC	BamHI
GAL1end-r	CGAGGGAATTCTATAGTTTTTCTCCTTGA CGTTAAAGTATAGAGG	EcoRI
GAL1VC-r	GTTGAAAGTATTTTCTGGTTTGACCAGTTA GCTTTTCCATTTATATATCTGTTAATAGATC AAAAATCATCG	-
GAL1VC-f	GGAAAAGCTAACTGGTCAAACCAGAAAATA CTTCAACATTTTCAGTTTGTATTACTTCTT ATTCAAATG	-
GAL1EC-r	GTTGAAAGTATTATCTGGTCGTACCAGATA GCTTTTCCATTTATATATCTGTTAATAGATC AAAAATCATCG	-
GAL1EC-f	GGAAAAGCTATCTGGTACGACCAGATAATA CTTCAACATTTTCAGTTTGTATTACTTCTT ATTCAAATG	-
FadR_EC-f	CCACAGAATTCATGGTCATTAAGGCGCAAA GCC	EcoRI
FadR_EC- NLS-r	CGGTTGCGGCCGCTTATCTAGTTACCTTGC GCTTTTTCTTGGGAGATCCTCGCCCCTGAA TGGCTAAATCAC	NotI
TEF1-f	CCATAGGATCCGCCGTACCACTTCAAAACA CCC	BamHI
TEF1-r	CTTGAATTCCTTTGTAATTA AAACTTAGATTA GATTGCTATGCTTTCTTTC	EcoRI
CYC1-f	CCATAGGATCCGGTAACAGTATTGATGTAA TCAATTTCTACCTG	BamHI
CYC1-r	CCTTGAATTCATTAATTTAGTGTGTGTATT TGTGTTTGTGTGTCTATAG	EcoRI
Egfp-f	CCTCGAATTCATGTCTAAAGGTGAAGAATT ATCACTGGTGTGTGCCC	EcoRI
Egfp-r	CGGTTGCGGCCGCTTATTTGTACAATTCAT CCATACCATGGGTAATACC	NotI
Adh1T-r	GGTGGAAGCTTGAGCGACCTCATGCTATA CCTGAG	HindIII

*Restriction sites in the primers are indicated with underlines

3.2.2 Cloning procedures

Restriction enzymes used were obtained from New England Biolabs (NEB, Ipswich, MA) and digestions were carried out according to the recommended protocols. PCR reactions were performed with Iproof High Fidelity DNA Polymerase (Biorad, Hercules, CA) at recommended conditions. Ligations were done with T4 DNA Ligase (NEB) at 16 °C. Gel extractions and PCR purifications were carried out using QIAquick gel extraction kit and QIAquick PCR purification kit respectively (Qiagen, Venlo, The Netherlands). Minipreps of plasmids from *E. coli* were performed using QIAprep Spin Miniprep kit (Qiagen). Standard chemical transformation methods for *E. coli* were used for transformation of ligation products.

3.2.3 Plasmid construction

To construct plasmids for characterization of promoters, yEGFP was PCR amplified from pKT127 using primers Egfp-f and Egfp-r, digested with EcoRI and NotI and ligated to pESC-URA. Promoters TEF1 and CYC1 were cloned from BY4741 genomic DNA using primers TEF1-f and TEF1-r, and CYC1-f and CYC1-r respectively, digested with EcoRI and ligated to pESC-URA containing yEGFP, also digested with EcoRI. This is followed by amplification with TEF1-f (and CYC1-f) and Adh1-T-r to obtain the promoter-yEGFP-terminator cassette, digested with BamHI and HindIII, and ligated to pIS112.

To construct plasmids for expression of FadR from *E. coli*, primers FadR_EC-f and FadR_EC-NLS-r were used to amplify FadR from *E. coli*

MG1655 genomic DNA, and to add the nuclear localization signal for nuclear targeting of FadR. The PCR product was then digested with EcoRI and NotI and ligated to pESC-URA. Addition of promoters TEF1 and CYC1, and Adh1 terminator was carried out as described above. To construct plasmids for expression of FadR from *V. cholerae*, FadR sequence from *V. cholerae* was codon optimized and synthesized (GeneArt Carlsbad, CA), digested with EcoRI and NotI and ligated to pESC-URA. The above cloning procedure is then repeated for addition of promoters and terminator, followed by ligation to pIS112.

The construction of reporter plasmids containing operator sites for FadR protein binding was as follows: For pESC-1XEC, primers GAL1-f and GAL1EC-r were used to amplify the front portion of the GAL1 promoter of BY4741, whereas GAL1EC-f and GAL1end-r were used to amplify the back portion of the GAL1 promoter. The synthetic promoter containing a single repeat of operator site was obtained by splicing overlap extension (SOE), where the PCR products from the front and back portion of GAL1 promoter was combined by amplifying using primers GAL1-f and GAL1end-r. For pESC-1XVC, primers GAL1-f and GAL1VC-r were used for the front portion, GAL1VC-f and GAL1end-r was used for the back portion, and the portions combined with primers GAL1-f and GAL1end-r. The SOE products were then digested with XhoI and EcoRI and ligated to pESC-URA containing yEGFP to obtain pESC-1XEC and pESC-1XVC. For pESC-3XEC and pESC-3XVC, primers GAL1-f and GAL1tata-r were used to amplify the front portion of the GAL1 promoter and then digested with XhoI and BamHI. The back portion of GAL1

promoter, which contains three repeats of FadR binding operators, was synthesized (GeneArt) and digested with BglII and EcoRI. The front and back portions of the GAL1 promoter were combined by three-way ligation into pESC-URA containing yEGFP.

3.2.4 Transformation and marker rescue of *S. cerevisiae*

Transformation of *S. cerevisiae* and URA3 marker rescue were carried out according to (Sadowski et al. 2007). For characterization of promoters, constructs carrying promoter-yEGFP were first transformed into yeast, followed by marker rescue and then an empty pESC-URA plasmid was introduced. For fatty acid/fatty acyl-CoA sensor strains, constructs carrying promoter-FadR were first transformed into yeast, followed by marker rescue and then reporter plasmids were introduced.

3.2.5 Biosensor characterization with a microplate reader

For characterization of the fatty acid biosensor, *S. cerevisiae* strains, integrated with various constructs and transformed with different plasmids, were inoculated overnight in 1 mL of induction medium. The strains were then diluted to OD₆₀₀ of 0.2 in 1 mL fresh induction medium. At 24 hours incubation at 30 °C with shaking, 200 µl of cells were loaded to 96-well microplates and read for fluorescence and optical density (600 nm) using a Synergy HT Microplate reader (Biotek Instruments, Winooski, VT). Excitation and emission filters used were 485/20 and 528/20 respectively. Minimal medium was used as blank followed by background subtraction of negative control strains containing no yEGFP

constructs. For characterization of the biosensor with exogenously added fatty acids, the strains were inoculated overnight in 2 mL induction medium, re-inoculated into 40 mL induction medium overnight, centrifuged and re-suspended in 2 mL induction medium. The strains were then diluted to OD₆₀₀ of 2 in 2 mL fresh induction medium or medium containing 2% galactose and fatty acids. At 2, 4, 8 and 24 hour incubation at 30 °C with shaking, 200 µl of cells were loaded to 96-well microplates and read for fluorescence and optical density.

3.3 RESULTS

3.3.1 Design of synthetic fatty acid/fatty acyl-CoA biosensor constructs.

In the absence of fatty acids/fatty acyl-CoAs, the repressor FadR binds to specific operator sites, whereas when fatty acids/fatty acyl-CoAs ligands are present, the DNA binding activity is antagonized. As fatty acids and fatty acyl-CoAs are naturally present in yeast, these ligands may be present in significant amounts to antagonize FadR DNA binding activity, hence resulting in an 'always on' reporter construct. Since the amount of repressors present in the cell can enable tuning of sensitivity towards the ligands (Murphy et al. 2010), here, FadR was expressed under promoters of different strengths (strong, TEF1p; weak, CYC1p)(Figure 3.1A). Further, to tune the sensor's sensitivity towards fatty acids/fatty acyl-CoAs, FadR from two different bacterial sources, *E. coli* and *V. cholerae* was cloned. It was previously reported that *V. cholerae* FadR had ~2.5-fold higher affinity towards oleoyl-CoA in comparison with *E. coli* FadR (dissociation constant ~25 nM and ~70 nM for *V. cholerae* and *E. coli* FadR respectively) (Iram and Cronan 2005). As ligand binding and DNA binding are interdependent, higher ligand affinities indicate increased sensitivity of the sensor. The higher ligand sensitivity of *V. cholerae* FadR is further supported by *in vivo* experiments, where the β -galactosidase activity of engineered *E. coli* carrying *V. cholerae* FadR was elevated 14-fold relative to un-induced levels, compared to only a 6-fold increase in *E. coli* expressing its own FadR.

Figure 3.1 Diagram for synthetic fatty acid/fatty acyl-CoA biosensor constructs.

(A) FadR transcription factors from both *E. coli* and *V. cholerae* were cloned downstream of the TEF1p (strong) or CYC1p (weak) promoters and integrated into the ADE8 locus of BY4741 yeast strain. FadR binds to the DNA binding site (operator) in synthetic GAL1 promoters to inhibit transcription of a reporter gene, yEGFP, cloned into multicopy vector pESC-URA. In the presence of fatty acids/fatty acyl CoA, the DNA binding activity of FadR is antagonized, allowing transcription of yEGFP and detection of a fluorescent signal. (B) Diagram of synthetic GAL1 promoters containing the 17 bp operator sequences of FadR from both *E. coli* and *V. cholerae*. The TATA box and FadBA operator sites are shown by base position relative to the transcription start site. 1X denotes a single operator site, whereas 3X denotes triple operator-containing promoters. (C) Characterization of TEF1 and CYC1 promoters.

Another engineering strategy to tune gene expression involves the construction of synthetic promoters. By placing a different number of operators at various positions in the promoter, the dose response curve and gene expression noise from anhydrotetracycline induction of TetR repressed promoters could be controlled (Murphy et al. 2007). Here, synthetic promoters containing one and three operators downstream of the GAL1 TATA box to tune fatty acid/fatty acyl-CoA response were created (Figure 3.1B). Figure 3.1C shows the expression levels of yEGFP from TEF1p and CYC1p in BY4741 strain.

3.3.2 Selection of suitable biosensor constructs in BY4741 strain.

The gene expression levels of the constructed biosensors were measured to compare the basal expression levels in galactose medium without exogenous addition of fatty acids. The Relative Fluorescence Units (RFU) was normalized with the OD₆₀₀ to account for any variance in cell density. TEF1p driven expression of *E. coli* FadR, in combination with synthetic 1X operator GAL1 promoter, repressed expression of the promoter by almost 50%, while use of synthetic 3X operator enabled even stronger repression, limiting expression to about 13% of the maximal levels. On the other hand, CYC1p driven expression of *E. coli* FadR did not significantly repress the synthetic promoters (Figure 3.2A).

The DNA binding activity of FadR from *V. cholerae*, which has a lower dissociation constant towards acyl-CoAs, was found to be highly antagonized. Strong expression of *V. cholerae* FadR repressed expression of yEGFP in a similar trend with *E. coli* FadR, but the basal

expression was much higher, whereas weak *V. cholerae* FadR expression did not repress yEGFP expression (Figure 3.2B).

Results from Figure 3.2A and 3.2B show that both FadRs are functional in yeast, being able to bind its operator and respond to naturally occurring fatty acids/fatty acyl-CoAs in the cell. The higher sensitivity of *V. cholerae* FadR, however, is unfavourable in yeasts for constructing fatty acid/fatty acyl-CoA sensors with low basal expressions.

Figure 3.2 Gene expression from the set of synthetic GAL1 promoters in BY4741 biosensor strains.

(A) Strains expressing *E. coli* FadR. **(B)** Strains expressing *V. cholerae* FadR. Bars represent the RFU per OD₆₀₀. Diamond markers represent expression relative to the controls. Error bars represent standard deviation from 3 replicates.

3.3.3 Induction of the fatty acid/fatty acyl-CoA sensor system.

From the results above, the combination of TEF1p driven expression of FadR from *E. coli* and synthetic 3X operator GAL1 promoter was identified to be most suitable for construction of a modular fatty acid/fatty acyl-CoA biosensor system. In the engineered BY4741 strain, the basal expression was low (or repression efficiency was high). To test the system for responsiveness towards exogenously added fatty acids, the strains were grown in induction medium with 1 mM of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) or oleic acid (C18:1). Since in microbial cell factories the production phase should be separated from the growth phase, the cell number was normalized to a starting OD₆₀₀ of 2, and measured the RFU and OD₆₀₀ at various time-points. Figure 3.3A shows a dynamic response of the sensor strain toward different chain length fatty acids. Myristic acid was found to be most effective in antagonizing DNA binding of FadR to increase yEGFP expression. Oleic acid and palmitic acid could also induce a response in the sensors, albeit at lower efficiencies. For lauric acid, yEGFP expression was found to decrease initially before rising to give a small induction ratio.

As myristic acid was most effective in the induction of the sensor, the sensor strain was further characterized using different concentrations of myristic acid (Figure 3.3B). The sensor system was shown to be able to provide higher levels of induction in response to higher myristic acid concentrations. While even higher concentrations of fatty acids may

trigger greater sensor responses, 1 mM fatty acid was found to be the solubility limit under our experimental conditions.

A growth profile of the sensor strain showing TEF1 driven expression of *E. coli* FadR and the Null strain without FadR (Figure 3.3C) shows that FadR expression did not affect the growth rate of the cells at the exponential phase, though the OD₆₀₀ attained after 24 hours culture was found to be slightly lower. As expression of FadR would consume metabolic resources, the impact on cellular growth was expected. This highlights the importance of the regulation of protein expression levels for optimized usage of cellular resources to maximize growth and productivity.

Figure 3.3 Induction of the fatty acid/fatty acyl-CoA sensor system consisting TEF1 driven expression of *E. coli* FadR and synthetic 3X operator GAL1 promoter in BY4741 strain.

(A) Sensor strain (TEF1-EC +3XEC) was grown in induction medium with 1 mM of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) or oleic acid (C18:1). Cells were also grown in induction medium without fatty acid addition (galactose) or with 0.5% Tween 80 as controls. The RFU and OD₆₀₀ were measured using a microplate reader after 2, 4, 8 and 24 hours incubation. Shown are the induction ratios normalized to galactose medium control. **(B)** Sensor strain (TEF1-EC +3XEC) was grown in induction medium with 0 (galactose), 0.25, 0.5 or 1 mM myristic acid (C14:0). The RFU and OD₆₀₀ were measured using a microplate reader after 2, 4, 8 and 24 hours incubation. Shown are the induction ratios normalized to galactose medium control. **(C)** A growth profile of the sensor strain with TEF1 driven expression of *E. coli* FadR and the Null strain without FadR expression as a control. Error bars represent standard deviation from 3 replicates.

3.4 DISCUSSION

Exogenous addition of oleic acid to sensor strains was found to not give a big induction ratio. This is likely due to the low bioavailability of fatty acids/fatty acyl-CoAs in the cell nucleus. For efficient antagonization of FadR, oleic acid must (1) diffuse across the cell membrane, (2) be activated to its acyl-CoA form, and (3) make its way into the nucleus for interaction with FadR. The compartmentalization and spatial optimization of the eukaryotic yeast may become a barrier for induction of the sensing system. On the other hand, the highest induction ratio was obtained from use of myristic acid. This result was unexpected as oleoyl-CoA is 50 times more potent than myristoyl-CoA to dissociate the FadR-DNA complex (DiRusso et al. 1992). Possible explanations include differing diffusion and transportation rates, differing fatty acid activation activities, myristic acid significantly altering gene expression profiles and enabling fatty acid/fatty acyl-CoA accumulation, or even a combination of these possibilities.

We have shown that the bacterial FadR transcription factors from both *E. coli* and *V. cholerae* are functional in *S. cerevisiae*. In the absence of fatty acids/fatty acyl-CoAs, the repressor binds to its operator to block transcription whereas in the presence of the ligands, its DNA binding activity is antagonized, triggering an increase in yEGFP expression. In addition, our results from the application of weak and strong promoters to drive expression of FadR, and the number of operator sites in the reporter construct, as well as the use of two FadR sources with different

ligand sensitivities (Figure 3.2) illustrate the natural levels of fatty acids/fatty acyl-CoAs present in the cell. As can be explained using a dose response curve (Figure 3.4A), weak expression of FadR results in an 'always on' biosensor, due to high ligand sensitivities. This is seen in strains expressing FadR under the weak CYC1 promoter. Medium level of FadR expression or sensitivity of the system would include strains expressing FadR from *V. cholerae* under the strong TEF1 promoter and strains expressing FadR from *E. coli* under the strong TEF1 promoter for reporter constructs with only one operator site. At this level, the biosensor is 'partially on'. Strong expression of FadR from *E. coli* coupled with reporter constructs with three operator sites reduced fatty acids/fatty acyl-CoAs sensitivities and hence the lowest basal expressions were observed. To trigger sensor response, a significant increase in inducer concentrations is necessary. However, exogenous addition of saturating levels of fatty acid to the culture medium was found to only provide a small increase in signal. This suggests that the intracellular concentration of inducers is only marginally increased in *S. cerevisiae* cells with a complex network of fatty acid pathways that are strictly regulated to maintain lipid homeostasis (Shin et al. 2012). Deregulation of the network would enable *S. cerevisiae* strains to be capable of accumulating greater amounts of fatty acids in the cell, leading to bigger sensor responses. Besides showing the functionality of FadR in *S. cerevisiae*, our results also illustrate three methods of tuning the sensitivity of the fatty acids/fatty acyl-CoAs biosensor, including adjusting the strength of the promoter upstream to FadR coding

sequence, using FadR with different ligand sensitivities, and changing the number of operator sites in the reporter construct (Figure 3.4B).

Towards the construction of regulation systems capable of responding to fatty acids concentrations in eukaryotic cells, here, we report for the first time that the bacterial transcription factor FadR can function as a modular sensor-regulator in the yeast *S. cerevisiae*. In particular, using FadR and yeast synthetic promoters containing DNA binding operators, novel fatty acid/fatty acyl-CoA biosensors were constructed in *S. cerevisiae*. The sensing system was tuned by varying promoter strength upstream to FadR coding sequence, by varying the number of operator sites in the synthetic promoter, and by using FadR from *E. coli* and *V. cholerae* with different ligand sensitivities. We envision that our fatty acid/fatty acyl-CoA biosensors can be used for regulation of protein expression based on the availability of fatty acid intermediates, which will potentially assist in balancing of cellular metabolism and improvement of the genetic stability of engineered strains during fatty acid derivatives production in yeast.

Figure 3.4 Dose-response curves for different repressor expression levels and strategies for tuning biosensor availability.

(A) Dose-response curves for different repressor expression levels. Weaker expression of repressors results in increased inducer sensitivity, whereas strong expression of repressors requires higher concentrations of the inducer to trigger a response. Figure adapted from (Murphy et al. 2010). Dashed lines represent nominal intracellular fatty acids/fatty acyl-CoAs concentrations. **(B)** Strategies for tuning biosensor sensitivity. An increase in sensitivity shifts the dose-response curve to the left, whereas a reduced sensitivity shifts the dose-response curve to the right. This tuning of sensitivity can be achieved via adjustment of promoter strength upstream to FadR coding sequence, usage of FadR with different ligand sensitivities, as well as manipulation of the number of operator sites in the reporter construct.

CHAPTER 4 : DEVELOPMENT AND CHARACTERIZATION OF AND-GATE DYNAMIC CONTROLLERS WITH A MODULAR SYNTHETIC GAL1 CORE PROMOTER IN SACCHAROMYCS CEREVISIAE

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4.1 INTRODUCTION

Metabolic engineering of bio-catalytic cells for biochemical production requires expression of one or more heterologous proteins. The introduction of new proteins increases the demand for cellular resources which can be detrimental to bio-catalytic cells. Constitutive expression or overexpression of the pathway proteins can impede cell growth rates, which in turn reduce biochemical productivity. On the other hand, expression of pathway enzymes at too low a level results in bottlenecks in the reaction pathway, limiting product yield and titers. Strategies for optimization of engineered pathways include static controls such as tuning of promoter strength (Blazeck and Alper 2013), ribosome binding sites (Salis et al. 2009) or copy number of the vector (Jones et al. 2000) to enable balanced pathway reaction flux and remove bottlenecks, resulting in higher product yield. More advanced optimization strategies would involve dynamic controls where protein expression can be triggered on-demand. Dynamic control using inducible promoters enable the separation of growth phase from production phase, allowing cells to accumulate biomass before channelling resources for formation of desired chemicals (Holtz and Keasling 2010; Lo et al. 2013). Furthermore, dynamic control via the sensing of key intermediates to regulate protein expression levels empowers the cell to make real-time adjustments to its metabolic flux when host or environmental conditions change (Farmer and Liao 2000; Zhang et al. 2012b). This facilitates efficient consumption of cellular resources, with optimal protein expression levels at all times.

Baker's yeast *S. cerevisiae* is an important laboratory and industrial microorganism for biotechnological applications. In addition to the wide array of tools available for genetic manipulation, *S. cerevisiae* is also easily cultured and classified as a generally recognized as safe (GRAS) microorganism (Da Silva and Srikrishnan 2012). *S. cerevisiae* has long been used for wine making, brewing and bioethanol production at industrial scales and metabolic engineers have more recently used it to produce valuable biochemicals, such as alcohols, ergosterol and isoprenoids (Nevoigt 2008). However, for sustainable industrial scale production of biochemicals, the yields and titers obtained from engineered yeast cells need to be further increased. Hence, the development of optimization strategies and tools is crucial. Towards the engineering and optimization of the yeast *S. cerevisiae*, strategies employed have included gene knockout and overexpression to accumulate intermediate metabolites (Tang et al. 2013), expression of efflux transporters to pump out toxic chemicals (Chen et al. 2013) and creation of libraries of constitutive and regulated promoters (Blazeck et al. 2012).

Genetic AND-gates enable the integration of multiple environmental and cellular signals to generate a programmable response (Anderson et al. 2007; Auslander et al. 2012; Bonnet et al. 2013; Kramer et al. 2004; Moon et al. 2012; Wang et al. 2011). Here, we describe the development and characterization of AND-gate dynamic controllers that can be used to engineer yeast towards robust growth and enhanced pathway productivity. These AND-gate dynamic controllers combine the

two dynamic control strategies described above, where both inducible promoters and sensing-regulation are employed in a dynamic controller. In an AND gate, two input signals are needed in order to turn the output ON. The absence of any one input will result in the output being OFF. At low cell densities, the inducer of the inducible promoter is absent, resulting in the output being OFF, hence allowing the cells to quickly proliferate without the additional burden of heterologous protein expression. Upon reaching higher cell densities, the inducing conditions can be added to satisfy the first input condition. For the second input signal, a key intermediate can be sensed real-time, where varying concentrations of the key intermediate can tune the strength of the input signal, and ultimately regulate protein expression for biochemical production (Figure 4.1).

Various approaches have been employed towards the construction of genetic AND-gates, such as the use of RNAs, RNA binding proteins, transcription factors, chaperones and co-activating proteins (Anderson et al. 2007; Auslander et al. 2012; Moon et al. 2012; Wang et al. 2011),

Figure 4.1 AND-gate dynamic controller for dynamic control of protein expression.

where *E. coli* is often chosen for development and validation of the AND-logic gates. Our strategy of creating the AND-gate dynamic controllers in yeast involved the construction of synthetic hybrid promoters. As eukaryotic promoters contain two separate regions, the upstream enhancer elements (UEEs), which control transcriptional frequency, and core elements, which determine the transcriptional direction and start site (Blazeck and Alper 2013), various UEEs were fused with a synthetic GAL1 core promoter containing DNA binding sites for protein repressors that reduced DNA affinity upon interaction with key intermediates in biochemical pathways. Since fatty acids are key intermediates for production of fatty alcohols, fatty acid esters, alkenes and alkanes, which are advanced biofuels, *E. coli*'s fatty acid responsive FadR repressor and its operator sequence were used to demonstrate the functionality of the dynamic controllers. The synthetic GAL1 core promoter was established to function as a modular promoter part for constructing synthetic hybrid promoters and conferring fatty acid inducibility. We further showed the performance of the AND-gate dynamic controllers, where two inputs (fatty acid and copper presence / phosphate starvation) were required to switch the AND-gate ON. This work provides a convenient platform for constructing AND-gate dynamic controllers, i.e. promoters that combine inducible functionality with regulation of protein expression levels upon detection of key intermediates towards the engineering and optimization of bio-catalytic yeast cells.

4.2 MATERIALS AND METHODS

4.2.1 Plasmids, strains and media

Plasmids and *S. cerevisiae* strains used are listed in Table 4.1. Primers used for PCR amplification are listed in Table 4.2. *E. coli* strain Top10 was used for transformation and amplification of plasmids, cultured at 37 °C in LB broth supplemented with 100 µg/ml ampicillin. Yeast extract and peptone were obtained from BD, while other chemicals were purchased from Sigma Aldrich, unless otherwise stated. Yeast strains were grown in minimal medium (yeast nitrogen base 6.7 g/l, yeast synthetic drop-out medium supplements – Ura- 1.92 g/l, D-glucose 20 g/l). For copper induction, 1 mM copper sulphate was added; to trigger phosphate starvation for induction, yeast nitrogen base without phosphates (MP Biomedicals, Santa Ana, CA) was used; for induction with fatty acids, oleic acid, palmitic acid, myristic acid and lauric acid were solubilized with Tween 80 and added to a final concentration of 0.5% Tween 80, at the stated fatty acid concentrations.

Table 4.1 Yeast strains and plasmids used in this study

Strain or plasmid	Description	Source
<u>S. cerevisiae strains</u>		
BY-CON	<i>ADE8::null</i> derived from BY4741	(Chapter 3) (Teo et al. 2013)
BY-TEF1-EC	<i>ADE8::TEF1p-FadR_E.coli</i> derived from BY4741	(Chapter 3) (Teo et al. 2013)
<u>Plasmids</u>		
pESC-3XEC	GAL1-3X _{Fad} BA_EC-yEGFP cloned into pESC-URA at XhoI and NotI	(Chapter 3) (Teo et al. 2013)
pESC-GAL1m*	GAL1m* cloned into pESC-URA- yEGFP at XhoI and EcoRI	This study
pESC-TEF1-GAL1m*	UEE(TEF1)-GAL1m* cloned into pESC-URA-yEGFP at XhoI and EcoRI	This study
pESC-PGK1-GAL1m*	UEE(PGK1)-GAL1m* cloned into pESC-URA-yEGFP at XhoI and EcoRI	This study
pESC-HXT7-GAL1m*	UEE(HXT7)-GAL1m* cloned into pESC-URA-yEGFP at XhoI and EcoRI	This study
pESC-CUP1-GAL1m*	UEE(CUP1)-GAL1m* cloned into pESC-URA-yEGFP at XhoI and EcoRI	This study
pESC-PHO5-GAL1m*	UEE(PHO5)-GAL1m* cloned into pESC-URA-yEGFP at XhoI and EcoRI	This study

Table 4.2 Primers used in this study

Name	Sequence	Site
GAL1m* for	CCAACCTCGAGTATATAAATGGATCTGCT ATCTGGTACG	XhoI
GAL1 rev	CGAGGGAATTCTATAGTTTTTCTCCTTG ACGTTAAAGTATAGAGG	EcoRI
TEF1 for	CCAAACTCGAGATGTTTCTACTCCTTTTT TACTCTTCCAGATTTTCTC	XhoI
TEF1 rev	TGGTAGGATCCATTTATATAAAAATTTTTAT TGCCTTTTTTCGACGAAGAA	BamHI
PGK1 for	CCAAACTCGAGGAAGTACCTTCAAAGAA TGGGGTCTC	XhoI
PGK1 rev	TGGTAGGATCCATTTATATATATGTAAATGT AAGTTTCACGAGGTTCTAC	BamHI
HXT7 for	CCAAACTCGAGCCGTGGAAATGAGGGGT ATGCAG	XhoI
HXT7 rev	TGGTAGGATCCATTTATATACTTTTTTTTTT TCAGTTCTAGTATATTTTGTATTTTCCTCC	BamHI
CUP1 for	CCTTTCTCGAGTCCCATTACCGACATTTG GGC	XhoI
CUP1 rev	TGGTAGGATCCATTTATATATGATTCTGAC AATCCATATTGCGTTGG	BamHI
PHO5 for	CCTTTCTCGAGTTTTCTTTGTCTGCACAA AGAAATATATATTAAATTAGC	XhoI
PHO5 rev	GGTAGGATCCATTTATATACGTTTCATTTC GACAATTCAAAGATGTTTAC	BamHI

*Restriction sites in the primers are indicated with underlines

4.2.2 Cloning procedures

Restriction enzymes used were obtained from NEB and digestions were carried out according to the recommended protocols. PCR reactions were performed with Iproof High Fidelity DNA Polymerase at recommended conditions. Ligations were done with T4 DNA Ligase (NEB) at 16 °C. Gel extractions and PCR purifications were carried out using QIAquick gel extraction kit and QIAquick PCR purification kit respectively (Qiagen). Minipreps of plasmids from *E. coli* were performed using QIAprep Spin Miniprep kit (Qiagen). Standard chemical transformation methods for *E. coli* were used for transformation of ligation products. The LiAc/PEG method was used for transformation of yeast cells.

4.2.3 Plasmid construction

Construction of plasmids containing synthetic hybrid promoters was carried out as follows: Primer pairs (TEF1 for / rev, PGK1 for / rev, HXT7 for / rev, CUP1 for / rev, PHO5 for / rev) were used to PCR amplify the respective UEE sequences using BY4741 genomic DNA as a template. The DNA fragments obtained were then digested with BamHI. The synthetic GAL1 core promoter part containing 3 DNA operator sites for FadR binding, here named GAL1m* was synthesized (GeneArt) and digested with BglII and EcoRI. The UEEs were ligated with GAL1m* followed by PCR using primer pairs (TEF1 for, PGK1 for, HXT7 for, CUP1 for or PHO5 for / GAL1 rev) to obtain DNA fragments of synthetic hybrid promoters with UEE fused to the GAL1m* part. The PCR

products were digested with XhoI and EcoRI and ligated to plasmid pESC-3XEC (Chapter 3)(Teo et al. 2013) containing reporter protein yEGFP, also digested with XhoI and EcoRI. To construct the promoter without any UEE fused to the GAL1m*, primer pair GAL1m* for and GAL1 rev were used for PCR amplification, followed by digestion with XhoI and EcoRI and ligation with the digested pESC-3XEC plasmid. All plasmids constructed were then transformed into yeast strains BY-CON and BY-TEF1-EC (Chapter 3) (Teo et al. 2013), where the former does not express FadR repressors whereas the latter expresses FadR repressors under the strong TEF1 promoter.

4.2.4 Synthetic hybrid promoter characterization with a microplate reader

For characterization of the synthetic hybrid promoters, *S. cerevisiae* transformants with different plasmids were inoculated overnight in 2 mL of minimal medium, re-inoculated into 40 ml minimal medium overnight, centrifuged and re-suspended in 2 mL minimal medium. The strains were then diluted to OD₆₀₀ of 2 in 2 mL fresh minimal medium and incubated at 30 °C with shaking. At the stated time points, 200 µl of cells were loaded to 96-well microplates and read for fluorescence and optical density (600 nm) using a Synergy HT Microplate reader (Biotek Instruments). Excitation and emission filters used were 485/20 and 528/20 respectively. Minimal medium was used as blank. For induction of the copper inducible synthetic hybrid promoter, copper sulphate was added to a final concentration of 1 mM. For induction of the phosphate

starvation inducible synthetic hybrid promoter, yeast nitrogen base without phosphates was used. For alleviation of FadR operator binding, fatty acids were added at the stated concentrations.

4.3 RESULTS

4.3.1 Construction of the synthetic hybrid promoters

A synthetic GAL1 core promoter containing DNA binding sites for protein repressors that have reduced DNA affinity upon interaction with key intermediates in biochemical pathways is required for demonstration of the sensing-regulation functionality of the AND-gate dynamic controllers. As *E. coli*'s FadR repressor protein binds to specific operator sites in the absence of fatty acids / fatty acyl-CoAs ligands and releases the DNA binding upon ligand detection, the use of FadR and the corresponding operator sites can demonstrate sensing of fatty acids for regulation of fatty acid derivatives production (Chapter 3) (Teo et al. 2013). Here, a synthetic GAL1 core promoter containing 3 tandem repeat of FadR operator sequences, named GAL1m* was used for fusion with various UEEs to construct synthetic hybrid promoters. UEEs used include constitutive enhancers (TEF1, PGK1, HXT7) – which created synthetic hybrid promoters that are regulated by the levels of fatty acids, and inducible enhancers (CUP1, PHO5) – which created AND-gate synthetic hybrid promoters that are regulated by both the levels of fatty acids and the inducing conditions of the enhancer element (presence of copper for CUP1; absence of phosphates for PHO5) (Figure 4.2). To select the UEE region, promoter sequences from literature were used (Blazeck et al. 2012; Koller et al. 2000; Partow et al. 2010; Zhu and Zhang 1999), but removed sequences from the TATA box to the -1 of the start codon. Note that the GAL1m* core promoter included the GAL1 TATA box.

Details of the DNA sequences of the hybrid promoter parts used are listed in Table 4.3.

Figure 4.2 Schematic illustration of the synthetic hybrid promoters
(A) Schematic illustration of the synthetic hybrid promoters with upstream enhancer elements (UEEs) fused to the synthetic GAL1m* core promoter containing 3 tandem FadR operator sites. FadR repressors bind to the DNA operator sites to block transcription. Upon detection of fatty acids / fatty acyl-CoAs (FAs), the DNA binding activity of FadR is antagonized, triggering transcription of the downstream gene.
(B) Synthetic hybrid promoters constructed include UEEs from constitutive (TEF1, PGK1 and HXT7), copper inducible (CUP1) and phosphate starvation inducible (PHO5) promoters. For hybrid promoters with constitutive UEEs, the transcription of yEGFP will be triggered by the detection of FAs. For CUP1-GAL1m*, both inducers Cu^{2+} and FAs are needed to turn on yEGFP transcription. For PHO5-GAL1m*, yEGFP transcription will only trigger upon phosphate starvation and detection of FAs.

Table 4.3. List of synthetic hybrid promoter parts used in this study. GAL1m* is the engineered synthetic GAL1 core promoter containing FadR operators while UEE stands for upstream enhancer elements.

DNA Part	Inclusive basepairs (5' of start codon)	Length (bp)	Reference
GAL1m*	-147 to -1	147	This work
UEE (TEF1)	-401 to -198	204	(Blazeck et al. 2012)
UEE (PGK1)	-982 to -152	831	This work
UEE (HXT7)	-587 to -168	420	This work
UEE (CUP1)	-456 to -143	314	This work
UEE (PHO5)	-400 to -102	299	This work

4.3.2 Functionality of GAL1m* in synthetic hybrid promoters

To confirm the ability of the synthetic hybrid promoters for gene expression, the reporter protein, yEGFP was fused downstream of the promoters and measured the relative fluorescence units (RFU) / OD₆₀₀ in the presence and absence of FadR repressors. RFU was normalized to the optical density to account for any difference in cell number. Figure 4.3A shows the kinetic expression of the synthetic hybrid promoters with constitutive UEEs. In the absence of FadR, yEGFP expression was strong whereas in the presence of FadR, yEGFP expression was strongly repressed. For the synthetic hybrid promoters with inducible UEEs, inducing conditions were triggered at time 0 and observed the kinetic response of the promoter. Figure 4.3B shows the hybrid CUP1-GAL1m* performance, where in the absence of FadR, strong yEGFP expression levels was quickly triggered upon the addition of 1 mM Cu²⁺. In the presence of FadR, yEGFP expression was also triggered upon addition of 1 mM Cu²⁺, but was significantly repressed by FadR. In the

Figure 4.3 Kinetic expression of the synthetic hybrid promoters in the presence (FadR) and absence (Null) of FadR. **(A)** Hybrid promoters with constitutive UEEs (TEF1-GAL1m*, PGK1-GAL1m* and HXT7-GAL1m*). GAL1m* is shown as a control containing no fused enhancers. **(B)** CUP1-GAL1m*. **(C)** PHO5-GAL1m*. Error bars represent standard deviation from 3 replicates.

absence of Cu^{2+} , basal expression was observed from the CUP1-GAL1m* hybrid promoter in the strain without FadR expression. In the FadR expressing strain, basal expression was minimal. Figure 4.3C shows the hybrid PHO5-GAL1m* promoter's kinetic expression. Here, two inducing conditions were used, first, the use of medium without any phosphates, and second, the use of medium with a limited amount of phosphates (0.22 mM). In the strain containing no FadR repressors, strong yEGFP expression levels was observed 4 hours after the transfer of cells to growth medium containing no phosphates whereas in the presence of a low phosphates concentration (0.22 mM), yEGFP expression was delayed to 8 hours post transfer. This indicates temporal control of the promoter via growth medium nutrient adjustments. In the strain with FadR expressed, yEGFP expression was also triggered upon phosphate starvation, but was strongly repressed by FadR. In phosphate rich medium, basal expression from the PHO5-GAL1m* hybrid promoter was marginal. Comparison between CUP1-GAL1m* and PHO5-GAL1m* hybrid promoters in strains without FadR expression under non-inducing conditions, i.e. no exogenous copper and phosphate rich, indicate that significant basal expression was present in CUP1-GAL1m* but absent in PHO5-GAL1m*. This can be explained in that the CUP1 promoter is induced by the transcriptional activator Cup2p (Thiele 1988) and that trace amounts of copper ions are present in the culture medium, resulting in leaky expression. On the other hand, the PHO5 promoter is protected by 4 positioned nucleosomes under phosphate rich conditions, enabling a tight repression of the promoter (Svaren and

Hörz 1997). These results demonstrate the functionality of the hybrid promoters, where TEF1, PGK1, HXT7, CUP5 and PHO5 UEEs can enhance and regulate gene expression while the GAL1m* synthetic core promoter is effective as a modular core promoter to impart inducible fatty acid regulation.

4.3.3 Fatty acid induction of TEF1-GAL1m* synthetic hybrid promoter

Upon confirmation of good hybrid promoter capacity for protein expression, we proceeded to test the ability of fatty acids to trigger an increase in yEGFP expression. The TEF1-GAL1m* hybrid promoter was chosen to represent the constitutive UEEs and added various chain lengths fatty acids to the culture medium containing glucose as carbon source (Figure 4.4A). Myristic acid (C14:0) was found to be the most effective inducer for triggering an increase in fluorescence signal. The effect of lauric acid (C12:0) and palmitic acid (C16:0) were small whereas oleic acid (C18:1) did not give higher yEGFP expression levels. In addition, the fatty acid sensor system was found to provide graded responses according to the concentrations of myristic acid added (Figure 4.4B).

4.3.4 Induction of AND-gate dynamic controllers

The constructed AND-gate synthetic hybrid promoters were also characterized with a range of myristic acid inducers in the presence and absence of the UEE inducing conditions. For CUP1-GAL1m* (Figure 4.5A), upon the addition of 1 mM Cu²⁺, yEGFP expression was quickly triggered. Further addition of myristic acid resulted in increased RFU /

OD600, where higher myristic concentrations gave higher fluorescence levels. In the absence of exogenous Cu^{2+} , a basal expression was observed, and this basal level was increased slightly when myristic acid was added. For PHO5-GAL1m* (Figure 4.5B), yEGFP expression was observed 4 hours after transfer of the cells to minimal medium without phosphates. The addition of a concentration range of myristic acids also resulted in graded RFU / OD600 levels. In non-inducing conditions (phosphate-rich), basal expression was very low, and addition of myristic acids did not trigger increase in basal levels.

Figure 4.4 Induction of the TEF1-GAL1m* synthetic hybrid promoter sensor system with **(A)** various chain length fatty acids. Cells were grown in minimal medium with 1 mM of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) or oleic acid (C18:1). Cells were also grown in minimal medium without fatty acid addition (glucose) or with 0.5% Tween 80 as controls. **(B)** different concentrations of myristic acid (C14:0). Cells were grown in minimal medium with 0 (glucose), 0.25, 0.5 or 1 mM myristic acid. Error bars represent standard deviation from 3 replicates.

Figure 4.5 Induction of the AND-gate dynamic controllers.

(A) CUP1-GAL1m*. Cells were grown in minimal medium with 1 mM Cu^{2+} and 0 (glucose), 0.25, 0.5 or 1 mM myristic acid. Cells were also grown in the absence of exogenously added Cu^{2+} with 0 or 1 mM myristic acid. **(B)** PHO5-GAL1m*. Cells were grown in minimal medium without phosphates and 0 (glucose), 0.25, 0.5 or 1 mM myristic acid. Cells were also grown in phosphate rich medium with 0 or 1 mM myristic acid.

4.4 DISCUSSION

In yeast, the galactose promoters which exhibit tight control and 1000 fold induction ratios are commonly employed as inducible promoters (Da Silva and Srikrishnan 2012). However, the use of an affordable carbon source, such as lignocellulosic biomass is crucial for lowered biochemical production costs. Since lignocellulose contains glucose (Rubin 2008), the galactose promoters are catabolite repressed, rendering them unsuitable for constructing the AND-gate dynamic controller. To overcome the glucose repression barrier, synthetic hybrid promoters with UEEs from the copper inducible CUP1 promoter and phosphate starvation inducible PHO5 promoter were constructed. Recently, the use of hormone based gene expression system was characterized (Liang et al. 2013; McIsaac et al. 2013). This will enable the use of inducers with very low costs and even single gene-specificity induction. We can capitalize on this novel hormone based gene expression system to construct AND-gate dynamic controllers by fusing the UEE of the hormone promoter to the GAL1m* core promoter in addition to expressing the artificial transcription factor.

In the construction of synthetic hybrid promoters, truncated CYC1 and LEU2 promoters have been used as core promoters (Blazeck et al. 2012). However, these core promoters contain multiple TATA binding sites (Zhu and Zhang 1999) and are unsuitable for synthetically introducing operator sites for repressor binding. Operators for TetR and LacI have previously been inserted downstream of the TATA boxes of

GAL1, GAL10 and ADH1 promoters (Blake et al. 2003), where introduction of operator sites need to be carried out for each core promoter. By showing that the synthetic GAL1 core promoter can be used as a modular part with other UEEs, this permits flexible selection and cloning of UEEs with desired regulation mechanisms. Furthermore, the identification and use of a modular core promoter can have its operator locations optimized for good and reliable performance. Thus, a synthetic GAL1 core promoter can be used as a modular part for constructing synthetic regulatory networks in yeast.

Synthetic hybrid promoters with various UEEs fused to a synthetic GAL1 core promoter was established to be able to provide inducible and AND-gate regulation properties. However, basal levels of yEGFP expression without the exogenous addition of fatty acids were significant, resulting in small induction ratios when comparing samples with controls. This is due to naturally present fatty acids / fatty acyl-CoAs, which are able to antagonize FadR DNA binding activity (DiRusso et al. 1992; Tang et al. 2013). It is likely that differing amounts of various chain length fatty acids present in yeast cells and the chain –length dependent potency of fatty acid/fatty acyl-CoA to antagonize FadR DNA binding both contribute to the basal levels of yEGFP expression without exogenous fatty acid addition. While the use of other repressors such as TetR and LacI would give more contrasting induction levels upon the addition of ligands tetracycline and IPTG respectively, these ligands are not key intermediates in bio-catalytic reactions for demonstration of a promoter capable of dynamic sensing-regulation. In fact, for the AND-gate

dynamic controllers, basal expression at low cell densities due to naturally present fatty acid ligands is minimal, as the upstream enhancer is inactive. Only upon reaching a higher cell density that calls for triggering of production phase, is the upstream enhancer activated and the fatty acid sensor-regulator required.

To demonstrate the modularity of GAL1m*, various UEEs have been used for conferring promoter strength and regulation. Further engineering of the synthetic hybrid promoters is possible, where upstream activating sequences (UAS), which are minimal regions of UEEs that contain essential sequences for transcriptional and inducible control, can be used. By identifying and fusing tandem or combinations of UAS elements to core promoters and endogenous promoters, the dynamic range of promoters can be tuned (Blazeck et al. 2012). Spacer-region diversification can also enable tuning of promoters, though this is likely to result in weaker promoters (Blount et al. 2012; Ellis et al. 2009).

In this work, the performance of *E. coli* FadR in yeast under glucose repressing conditions have also been characterized. In the presence of glucose, the beta-oxidation pathway in yeast is blocked as fatty acyl-CoA oxidase (POX1) expression is repressed (Luo et al. 1996). Hence, the catabolism of fatty acids is prevented. This may allow for more accumulation of fatty acids in the cell to antagonize FadR DNA binding. However, similar to growth on galactose medium (Chapter 3) (Teo et al. 2013), myristic acid is found to be the most potent inducer while other fatty acids have minimal effect. Explanations on factors affecting the

ability of fatty acids to trigger induction of the fatty acid sensor in yeast remain unclear.

In conclusion, we have established that the synthetic GAL1 core promoter can be used as a modular promoter part for constructing synthetic hybrid promoters and conferring fatty acid inducibility. The performance of the AND-gate dynamic controllers was shown, where two inputs (fatty acid and copper presence / phosphate starvation) were required to switch the AND-gate ON. While the fatty acid metabolism regulator protein, FadR have been used to exhibit the utility of a modular synthetic GAL1 core promoter, the identification and application of other DNA binders that respond to chemical ligands or to other signals (Ramos et al. 2005) can enable the construction of more synthetic regulatory networks. This approach of combining synthetic GAL1 core promoters with desired upstream enhancers provides a simple and convenient platform for the construction of non-catabolite repressed, AND-gate dynamic controllers, i.e. promoters that combine inducible functionality with regulation of protein expression levels upon detection of key intermediates towards the engineering and optimization of biocatalytic yeast cells.

**CHAPTER 5 : BACTERIAL XYLRS AND SYNTHETIC
PROMOTERS FUNCTION AS GENETICALLY ENCODED
XYLOSE BIOSENSORS IN SACCHAROMYCES
CEREVISIAE**

5.1 INTRODUCTION

The development and production of biofuels is a solution towards addressing concerns with regards to energy security and climate change. Biofuels, including bioethanol, due to their incompatibility with existing transportation infrastructures, are frequently used as blends with gasoline. The discovery and production of novel and advanced biofuel molecules, including longer chain alcohols, fatty acid derivatives, polyketide derivatives and isoprenoid derivatives, have properties more suitable for direct use in current transportation infrastructures (Peralta-Yahya et al. 2012; Tippmann et al. 2013). In any case, for economical biofuel production, the minimization of costs is crucial. One major cost contributor is the carbon source used, where the use of lignocellulosic biomass, such as sugarcane bagasse, corn stover, wheat straw, grasses and wood chips, can provide an economically viable means towards biofuel production. In addition, lignocellulosic biomass is a plentiful and renewable source of sugar (Buschke et al. 2013; Hasunuma and Kondo 2012). However, hydrolysis of lignocellulosic biomass releases not only glucose, but also galactose, arabinose and a large proportion of xylose.

Baker's yeast *S. cerevisiae*, which is used industrially for bioethanol production, brewing and winemaking, is an important host microorganism for biotechnological applications (Nevoigt 2008). The robust growth properties and abundant genetic tools available for its manipulation make yeast an ideal target for engineering biofuel

production. However, *S. cerevisiae* is unable to ferment xylose, the second most abundant sugar present in lignocellulosic hydrolysates after glucose (Cai et al. 2012). As a result, yeast has been metabolically engineered to allow xylose utilization via the introduction of heterologous xylose assimilating pathways (Kuyper et al. 2003; Tanino et al. 2010; Zha et al. 2012). To optimize yeast strains capable of xylose utilization, strategies employed included identification of active xylose assimilating pathways and enzymes, engineering of cofactor balance in the heterologous pathway, and engineering of simultaneous co-fermentation of mixed sugars such as by cellobiose utilization or enhanced xylose transport (Farwick et al. 2014; Ha et al. 2011; Kim et al. 2012; Kim et al. 2013; Wei et al. 2013; Young et al. 2013).

Genetically encoded biosensors, including transcription factor-based and RNA-based biosensors, can specifically sense and translate the intracellular concentration of desired biochemicals into detectable and graded outputs (Michener et al. 2012; Schallmey et al. 2014). Biosensors can aid high-throughput screening of enzymes for pathway optimization and also be used to enable dynamic regulation of pathway enzymes, which helps maximize use of cellular resources and minimize cellular stress (Abatemarco et al. 2013; Seo et al. 2012; Seo et al. 2013; Zhang et al. 2012b). In this work, we created genetically encoded transcription factor-based xylose biosensors that can regulate expression of a fluorescent reporter upon detection of xylose sugars. Three XylR variants and the corresponding synthetic promoters were cloned and characterized: XylR from *Tetragenococcus halophile*,

Clostridium difficile and *Lactobacillus pentosus* (Chaillou et al. 1998; Nariya et al. 2011; Takeda et al. 1998). To optimize the biosensor, two promoters with different strengths were used to express the XylR proteins. The ability of XylR to repress reporter protein expression from the synthetic promoters was demonstrated. Xylose sugars added exogenously to the engineered yeast cells was also shown to trigger an increase in reporter protein expression.

5.2 MATERIALS AND METHODS

5.2.1 Plasmids, strains and media

Plasmids used are recorded in Table 5.1. *S. cerevisiae* strain BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0) (ATCC) was used in this study. DNA primers used for PCR are listed in Table 5.2. *E. coli* strain Top10 was used for transformation and amplification of plasmids, grown at 37 °C in LB broth supplemented with 100 μ g/ml ampicillin. Yeast extract and peptone were obtained from BD, whereas other chemicals were purchased from Sigma Aldrich, unless otherwise stated. Yeast strains were grown in minimal medium (yeast nitrogen base 6.7 g/l, yeast synthetic drop-out medium supplements – Ura- 1.92 g/l, D-glucose 20 g/l). D-xylose was used at 4 g/l, 20 g/l and 100 g/l to induce sensor response.

5.2.2 Cloning procedures

Restriction enzymes used were purchased from NEB and digestions were done according to the suggested protocols. PCR amplifications were performed with Iproof High Fidelity DNA Polymerase (Biorad) at recommended conditions. Ligations were carried out with T4 DNA Ligase (NEB) at 16 °C. Gel extractions and PCR purifications were carried out using QIAquick gel extraction kit and QIAquick PCR purification kit respectively (Qiagen). Qiagen MinElute PCR

Table 5.1 Yeast plasmids used in this study

Plasmid	Description	Source
pESC-URA	2 μ plasmid vector	Agilent
pESC-3XEC	pESC-URA plasmid, with yEGFP cloned at EcoRI and NotI, and a synthetic promoter cloned at XhoI and EcoRI.	(Chapter 3) (Teo et. al. 2013)
pESC-LP	XylR (<i>L. pentosus</i>) cloned into pESC-3XEC at XhoI and NheI	This study
pESC-CD	XylR (<i>C. difficile</i>) cloned into pESC-3XEC at XhoI and NheI	This study
pESC-TH	XylR (<i>T. halophila</i>) cloned into pESC-3XEC at XhoI and NheI	This study
pESC-LP-PGK1-TEF1-GAL1mLP	PGK1p-TEF1-GAL1mLP cloned into pESC-LP at XhoI and EcoRI	This study
pESC-CD-PGK1-TEF1-GAL1mCD	PGK1p-TEF1-GAL1mCD cloned into pESC-CD at XhoI and EcoRI	This study
pESC-TH-PGK1-TEF1-GAL1mTH	PGK1p-TEF1-GAL1mTH cloned into pESC-TH at XhoI and EcoRI	This study
pESC-LP-PFY1-TEF1-GAL1mLP	PFY1p-TEF1-GAL1mLP cloned into pESC-LP at XhoI and EcoRI	This study
pESC-CD-PFY1-TEF1-GAL1mCD	PFY1p-TEF1-GAL1mCD cloned into pESC-CD at XhoI and EcoRI	This study
pESC-TH-PFY1-TEF1-GAL1mTH	PFY1p-TEF1-GAL1mTH cloned into pESC-TH at XhoI and EcoRI	This study
pESC-PGK1-TEF1-GAL1mLP	PGK1p-TEF1-GAL1mLP cloned into pESC-3XEC at XhoI and EcoRI	This study
pESC-PGK1-TEF1-GAL1mCD	PGK1p-TEF1-GAL1mCD cloned into pESC-3XEC at XhoI and EcoRI	This study
pESC-PGK1-TEF1-GAL1mTH	PGK1p-TEF1-GAL1mTH cloned into pESC-3XEC at XhoI and EcoRI	This study
pESC-yEGFP	yEGFP cloned into pESC-URA at XhoI and NheI	This study
pESC-yEGFP-PGK1	PGK1p-TEF1-GAL1mCD cloned into pESC-yEGFP at XhoI and EcoRI	This study
pESC-yEGFP-PFY1	PFY1p-TEF1-GAL1mCD cloned into pESC-yEGFP at XhoI and EcoRI	This study

Table 5.2. List of primers used in this study

Name	Sequence (5'-3')	Site*
LP for	ACCTT <u>CTCGAG</u> ATGGAAAACAGATCCATCTCCAGAA	XhoI
LP rev	TGGTTGCTAGCTTATCTGGTAACCTTTCTCTTTTTC	NheI
TH for	ACCTA <u>CTCGAG</u> ATGCAAACCTTACAACCCAAC	XhoI
TH rev	TGGTTGCTAGCTTATCTGGTGACCTTTCTCTTTTTC	NheI
CD for	ACCTA <u>CTCGAG</u> ATGCAAACAACCTTGTTGT	XhoI
CD rev	TGGTTGCTAGCTCATCTGGTAACCTTTCTCTTCTTC	NheI
TEF1 for	AACGCGGATCCATGTTTCTACTCCTTTTTTACTCTTCC AGATTTTCTC	BamHI
TEF1U EE rev LP	GTTGAAAGTATTAGTTGGTTGGTTGTTGAATACAAC ATTTATATAAAAATTTTATTGCCTTTTTCGACGAAGAA AAAG-3'	-
GAL1m for LP	ATTAGTTGTATTCAACAACCAACCAACTAATACTTTCA ACATTTTCAGTTTGTATTACTTCTTATTCAAATG	-
TEF1U EE rev TH	GAAAATGTTGAAAGTATTAAGTTAGTACGAAATTCGTA CTAACTATTTATATAAAAATTTTATTGCCTTTTTCGAC GAAGAAAAAG-3'	-
GAL1m for TH	TAGTTAGTACGAATTTTCGTACTAACTTAATACTTTCAA CATTTTCAGTTTGTATTACTTCTTATTCAAATG	-
TEF1U EE rev CD	CAAACGAAAATGTTGAAAGTATTAGTTTGTAGTATA TTAACTAACTTATTTATATAAAAATTTTATTGCCTTTT TCGACGAAGAAAAAG-3'	-
GAL1m for CD	TAAGTTAGTTTAATATACTAACAACTAATACTTTCAAC ATTTTCAGTTTGTATTACTTCTTATTCAAATG	-
GAL1 end rev	CGAGGGAATTCTATAGTTTTTCTCCTTGACGTTAAAG TATAGAGG	EcoRI
PGK1 for	AACGAGGATCCGAAGTACCTTCAAAGAATGGGGTCTC	BamHI
PGK1 rev	CCTTTCTCGAGTGTTTTATATTTGTTGTAAAAAGTAGA TAATTACTTCCTTGATGATC	XhoI
PFY1 for	CCATAGGATCCCTGTGTGGGAGGTTTTACCATGATT	BamHI
PFY1 rev	CCGTTCTCGAGAATTTGCGATCGTAGTTGGGTTTATG TTGTG	XhoI
EGFP for	GGAA <u>ACTCGAG</u> ATGTCTAAAGGTGAAGAATTATTCAC TGGTGTTG	XhoI
EGFP rev	GGTTTGCTAGCTTATTTGTACAATTCATCCATACCATG GTAATACC	NheI

*Restriction sites in the primers are indicated with underlines

purification kit was used where concentrations were low. Minipreps of plasmids from *E. coli* were executed using QIAprep Spin Miniprep kit. Standard chemical transformation methods for *E. coli* were used for transformation of ligation mixtures. The LiAc/PEG technique was used for transformation of yeast cells.

5.2.3 Xylose biosensor plasmid construction

To obtain plasmids with XylR repressors, the following steps were carried out. DNA encoding XylR repressors from *T. halophile*, *C. difficile* and *L. pentosus* were codon optimized and the SV40 nuclear localization signal was added at the C-terminus. The DNA sequences were split into 2 parts and synthesized using GeneArt Strings or gBlocks gene fragments (Integrated Device Technology, San Jose, CA). The coding sequences were combined by splicing overlap extension (SOE) PCR using primers LP for/rev, TH for/rev and CD for/rev. The SOE-PCR products were then digested with XhoI and NheI and ligated with an expression vector containing yEGFP, pESC-3XEC (Chapter 3) (Teo et al. 2013), also digested with the same enzymes.

To create synthetic promoters containing operator sites for XylR binding, the following PCR steps were carried out. Primer pairs TEF1 for and TEF1UEE rev LP/TH/CD were used to amplify the TEF1 promoter upstream region, primer pairs GAL1m for LP/TH/CD and GAL1 end rev were used to amplify the GAL1 core promoter region, using BY4741 genomic DNA as a template. Upstream and core promoters were combined by SOE-PCR to obtain TEF1-GAL1m promoters with a XylR

operator sequence immediately after the TATA box. At the same time, the promoters controlling XylR expression were inserted. Primer pairs PGK1 for/rev and PFY1 for/rev were used to amplify the yeast native promoters PGK1 and PFY1 from BY4741 genomic DNA. The PCR fragments of promoters PGK1/PFY1 and TEF1-GAL1m LP/CD/TH were digested with BamHI, ligated together, and PCR was carried out to obtain PGK1-TEF1-GAL1m LP/CD/TH and PFY1-TEF1-GAL1m LP/CD/TH. These fragments were then digested with EcoRI and XhoI and ligated to vectors pESC-LP/CD/TH also digested with the same enzymes. To obtain the promoter strength without XylR repression, DNA fragment of PGK1-TEF1-GAL1m LP/CD/TH were digested with XhoI and EcoRI and ligated to pESC-3XEC, also digested with the same enzymes.

To construct plasmids to characterize the strength of the promoters controlling XylR expression, yEGFP was amplified from pESC-3XEC using primers EGFP for/rev, digested with XhoI and NheI, and ligated to pESC-URA that was also digested with the same enzymes. Promoters PGK1 and PFY1 were digested out from pESC-PGK1-TEF1-GAL1mCD and pESC-PFY1-TEF1-GAL1mCD to obtain PGK1-TEF1-GAL1mCD and PFY1-TEF1-GAL1mCD using EcoRI and XhoI and ligated to pESC-yEGFP, also digested with EcoRI and XhoI.

5.2.4 Characterization with a microplate reader

To obtain growth curves, cells were inoculated overnight in 1 mL minimal medium, followed by re-dilution in 2 mL fresh minimal medium

at an initial OD₆₀₀ of 0.2, and incubated at 30 °C with shaking. OD₆₀₀ was measured using a Synergy HT Microplate reader (Biotek Instruments) at 0, 6, 12, 24 and 48 hours. To determine promoter strength of PGK1 and PFY1, and similarly to determine the repression efficiency of the various XylRs, cells were inoculated overnight in 1 mL minimal medium, followed by re-dilution in 2 mL fresh minimal medium at an initial OD₆₀₀ of 0.2. At 24 hours incubation at 30 °C with shaking, 200 µL of cells were loaded to 96-well microplates for fluorescence measurement. Excitation and emission filters used were 485/20 and 528/20 respectively. Minimal medium was used as blank. Cells carrying empty pESC-URA were also used for background subtraction. For induction of the xylose biosensor systems, D-xylose was added to the minimal medium at varying concentrations of 4 g/L, 20 g/L and 100 g/L and fluorescence measured at 8 and 24 hours.

5.3 RESULTS

5.3.1 Design of the xylose biosensors

Three variants of XylR repressors from three bacterial species, namely *T. halophila*, *C. difficile* and *L. pentosus* were used as protein sensors to sense and respond to the presence of xylose inducers. Their corresponding DNA binding sites for XylR recognition was cloned into synthetic hybrid promoters composed of a constitutive upstream enhancer element from the TEF1 promoter, and the core promoters from GAL1p that were previously demonstrated to be modular and suitable for modification and addition of operator sequences (Figure 5.1) (Chapter 4)(Teo and Chang 2013). yEGFP was cloned downstream of the synthetic hybrid promoter as a reporter gene. To control the level of XylR expression, native yeast constitutive promoters of PGK1 and PFY1 were cloned upstream of the XylR coding sequences. In these systems, constitutively expressed XylR repressors bind to the operator sites in the synthetic hybrid promoter in the absence of xylose inducers to repress yEGFP transcription. When xylose molecules are detected, XylR affinity to the operator will be reduced, resulting in an increase of yEGFP expression.

Figure 5.1 Schematic illustration of the xylose biosensor systems

(A) Schematic illustration of the xylose biosensor systems. XylR repressors from 3 different bacteria strains were constitutively expressed. XylR repressors bind to synthetic hybrid promoters containing the respective XylR operator sequence to block transcription. Detection of xylose sugars by XylR results in antagonization of XylR DNA binding, triggering transcription of the downstream reporter, yEGFP. **(B)** Design of the synthetic hybrid promoter. A constitutive upstream enhancer element (UEE) from yeast's TEF1 promoter was fused upstream of a yeast modified GAL1 core promoter. Individual XylR operator sequences were inserted immediately after the TATA box, generating 3 synthetic promoters for the 3 versions of XylR.

5.3.2 Growth of engineered strains

Yeast strains were transformed with the biosensor plasmids and a growth curve was constructed (Figure 5.2A). Compared to the control, all engineered biosensor strains had reduced growth rates. Strains with PGK1p-expressed XylRs grew the slowest, while strains with PFY1p-expressed XylRs grew quicker. Also, strains with PGK1p-expressed XylRs and PFY1p-expressed XylR (*L. pentosus*) were observed to have longer lag times as well as attained lower cell densities after 48 hours cell growth. Yeast transformation with PGK1p-expressed XylR (*L. pentosus*) did not yield any transformants on agar plates, even after multiple attempts, indicating severe growth deficiency as a result of XylR (*L. pentosus*) expression. Figure 5.2B shows the relative promoter strength of PGK1p and PFY1p. Hence, too high a level of XylR overexpression is unfavorable for good cell growth.

Figure 5.2 Growth curves and promoter strength characterization.

(A) Growth curves of the engineered yeast strains. pESC is the empty vector control. PGK1p TH and PGK1p CD are strains carrying PGK1p-expressed XylRs from TH and CD sources respectively. PFY1p LP, PFY1p TH and PFY1 CD are strains carrying PFY1p-expressed XylRs from LP, TH and CD sources respectively. (B) Promoter strength of PGK1p and PFY1p. Error bars represent the standard deviation from 3 replicates. RFU, Relative fluorescence units; TH, *Tetragenococcus halophila*; CD, *Clostridium difficile*; LP, *Lactobacillus pentosus*.

5.3.3 Determination of XylR repression efficiency in the absence of xylose

To determine the ability of XylR to bind its operator in synthetic hybrid promoters, engineered yeast strains were grown in minimal medium containing glucose, without adding xylose inducers. Measurement of the relative fluorescence units (RFU) / OD₆₀₀ was carried out for engineered strains encoding or not encoding XylR repressors. RFU was normalized to the OD₆₀₀ to account for any variance in cell number. The expression levels of the synthetic hybrid promoters in strains without the encoding of XylR repressors were comparable (Figure 5.3A). Figure 5.3B showed that strains carrying XylR under PGK1p expression had lower basal expression whereas strains carrying PFY1p-expressed XylR gave higher basal expression, which is in line with a stronger expression of XylR due to PGK1p. Further, XylR repressors from *C. difficile* and *L. pentosus* were more effective in repressing the synthetic hybrid promoters compared to XylR from *T. halophila*. Combination of the data from Figure 5.3A and 5.3B yields Figure 5.3C, which indicates excellent repression in all systems constructed, except PFY1p-expressed XylR (*T. halophila*). For characterization with xylose inducers, one from each XylR variant was chosen: PGK1p-expressed XylR (*T. halophila*), which showed good repression efficiency, PFY1p-expressed XylR (*C. difficile*), which exhibited good repression efficiency and better growth, and PFY1p-expressed XylR (*L. pentosus*).

Figure 5.3 XylR represses yEGFP reporter expression.

(A) yEGFP expression levels of the synthetic hybrid promoters in the absence of XylR repressors. (B) yEGFP expression levels from the xylose promoter systems under xylose-free conditions. * No growth on transformation plates. (C) Repression efficiency of XylR under xylose-free conditions. * No growth on transformation plates. Error bars represent the standard deviation from 3 replicates. RFU, Relative fluorescence units; TH, *Tetragenococcus halophila*; CD, *Clostridium difficile*; LP, *Lactobacillus pentosus*.

5.3.4 Xylose induction of selected xylose biosensors

Three biosensor systems were shown to have excellent repression efficiency in the absence of xylose inducers, giving low basal expression to the promoters. To test the ability of xylose to trigger an increase in yEGFP expression, the cells were grown in minimal medium with 2% glucose, and xylose was added at 0% (control), 0.4%, 2% and 10%. Fluorescence and optical density readings were made at 8 hours (exponential phase, glucose rich), and 24 hours (stationary phase, glucose poor). Figure 5.4 presents the response of the biosensor strains to xylose. Strain with PFY1p-expressed XylR (*C. difficile*) showed graded response to increasing xylose concentrations at both 8 hour and 24 hour measurements. For strain with PFY1p-expressed XylR (*L. pentosus*), while graded response was also observed, however, the signal increase was much smaller (compare y-axis). For strain with PGK1p-expressed XylR (*T. halophila*), basal expression was higher, resulting in a smaller signal to basal expression ratio, and at 24 hours, the graded response to increased xylose concentrations was absent.

Figure 5.4 Induction of the xylose promoter systems (A) PFY1p-CD; (B) PFY1p-LP; (C) PGK1p-TH. Cells were grown in minimal media with 2% glucose in addition to 0% (control), 0.4%, 2% and 10% xylose. Error bars represent the standard deviation from 3 replicates. RFU, Relative fluorescence units; TH, *Tetragenococcus halophila*; CD, *Clostridium difficile*; LP, *Lactobacillus pentosus*.

5.4 DISCUSSION

Synthetic biology aims to forward-engineer cellular behavior towards applications in biotechnology and medicine. This requires the availability of well characterized and reliable molecular parts, such as promoters, terminators and transcriptional regulators (Cameron et al. 2014; Keren et al. 2013; Yamanishi et al. 2013). In this work, the characterization of xylose biosensors, which involve synthetic hybrid promoters and XylR transcriptional regulators, were carried out in the model microbial species *S. cerevisiae*. Three XylRs that were found to be independent of glucose were used (Chaillou et al. 1998; Nariya et al. 2011; Takeda et al. 1998). The independence of the biosensor system from glucose is crucial as this enables its use in growth media that contain glucose, which is most abundant in lignocellulosic hydrolysates. More XylR repressors are available in nature; however, they may interact with glucose and glucose-6-phosphate, which can be anti-inducers or inactivators of XylR (Dahl et al. 1995; Gu et al. 2010).

For the promoters used in this work, literature available constitutive promoters have been selected to ensure that the biosensor constructs are constitutively functional. The PGK1 promoter, which controls expression of 3-phosphoglycerate kinase, a key enzyme in glycolysis and gluconeogenesis, and the TEF1 promoter, which regulates expression of translational elongation factor 1 alpha, a protein for the binding reaction of aminoacyl-tRNA to ribosomes, are well characterized strong constitutive promoters in yeast (Partow et al. 2010; Sun et al.

2012; Vickers et al. 2013). In addition, the PFY1 promoter, which drives expression of the yeast profilin gene, a housekeeping component of the actin cytoskeleton, is constitutively transcribed at a moderate level with stable constitutive output (Angermayr et al. 2003; Blount et al. 2012). Besides, the hybrid promoter used in this work utilized the upstream activating sequence of the constitutive TEF1 promoter, which was shown to be a modular amplifier of transcription with constitutive output (Chapter 4)(Blazeck et al. 2012; Teo and Chang 2013).

All the engineered biosensor strains had reduced growth rates compared to the control. This will not pose an issue if the biosensor is used as a tool for high throughput screening for xylose transporters with superior activities. However, for enabling on-demand protein synthesis to help optimize cellular resource consumption and minimize cellular stress via flux balance in synthetic xylose utilization pathways, growth defects as a result of XylR expression will limit its utility. Indeed, efficient production of biochemicals using engineered microbes requires robust cell viability (Ling et al. 2014; Lo et al. 2013). Hence, any benefit from use of the biosensor for on-demand protein synthesis must exceed that of the growth defect sustained. In addition, the growth retardation of the XylR proteins used in this work towards yeast differed across variants. This could be attributed to different protein levels even though the coding sequences were codon optimized, or XylR proteins bound to other parts of the genome causing off-target effects, affecting the genetic landscape of the cell and ultimately fitness. The discovery or engineering of XylR proteins with minimal or no off-target effects will be

important for dynamic control of single gene expression (McIsaac et al. 2014; McIsaac et al. 2013).

While the addition of xylose to the engineered biosensor strains can trigger an increase in yEGFP expression, the synthetic promoters are found to fall short of maximum levels. Relating the xylose responsive promoters to normally used promoters, such as the strong constitutive PGK1 promoter, the medium strength constitutive PFY1 promoter, and strong constitutive TEF1 upstream activating sequence driven hybrid promoters (Figure 2B and 3A), it can be seen that the xylose responsive promoters are significantly weaker. Compared to the synthetic repression-induction systems using TetR and LacI (Ellis et al. 2009; Murphy et al. 2007), which can attain very high induction ratios, it is likely that xylose is unable to diffuse freely in the cell to interact with XylRs in the nucleus. Another biosensor system that faced similar challenge is the fatty acid sensor using FadR repressors, where maximum induction levels were also limited (Chapter 3 and 4)(Teo and Chang 2013; Teo et al. 2013).

Further improvement of the xylose biosensor may be possible with screening of a XylR library. XylR will need to be selected based on good repression efficiencies in the absence of xylose sugars, and excellent dynamic response range. Additionally, the expression of XylR in yeast cells should have minimal effect on the host cell growth. This could be made possible with bioinformatic tools and comparison of XylR operator

sequences to genomic targets to select XylRs with minimal binding sites in yeast's genome.

The expression of transcription factors and insertion of their DNA operator site into synthetic promoters enabled construction of the xylose biosensors. The use of such rational promoter design strategy can help boost metabolic engineering, where distinct applications need different expression range and inducibility. Also, better understanding of transcription factors and their DNA operator site will allow rational and predictive control of gene expression (Blazeck and Alper 2013). In this work, the DNA operator site for XylR binding was placed immediately adjacent to GAL1 core promoter's TATA box. This is in contrast with the 9 or 10 basepairs spacer included between operator site and the GAL1p TATA box used in previous synthetic promoter works (Chapter 3) (Murphy et al. 2007; Teo et al. 2013). As the constitutive strength of the promoters containing XylR operators remained strong, future synthetic promoter studies may insert the DNA operator sites beside the TATA box without spacer.

In conclusion, we have created genetically encoded xylose biosensors that can control protein expression based on the xylose concentration detected. This biosensor can permit cells to regulate the expression levels of xylose assimilating proteins towards the construction of a xylose-assimilating yeast strain that expresses optimal levels of xylose consuming genes. Moreover, the xylose biosensor will be a useful tool toward screening and characterization of xylose transporters, which is a

potential bottleneck for xylose assimilation in yeast. Recently, a new method for xylose transporters screening in wild-type yeast cells was demonstrated (Wang et al. 2013). A xylosidase gene expressed in yeast can quickly hydrolyze a xylose analog, p-nitrophenyl- α -D-xylopyranoside to p-nitrophenol, which has a yellow color. This enables xylose transporter activities to be calculated using the p-nitrophenol production rate. This method is based on yellow signals detected outside the cell at a population level. However, the use of a genetically encoded xylose biosensor will enable detection of yEGFP signals inside cells on a single cell basis. Hence, the biosensor system makes possible the mutation and screening of xylose transporters using flow cytometry and cell sorting.

To make the xylose biosensor, heterologous XylR repressors were constitutively expressed, and synthetic promoters with XylR operator sites were constructed. Three XylR variants and the matching synthetic promoters were cloned and characterized, including XylR from *T. halophile*, *C. difficile* and *L. pentosus*. To optimize the biosensor, two promoters with different strengths were used to express the XylR proteins. The ability of XylR to repress the reporter protein expression yEGFP from the synthetic promoters was shown. Xylose sugars added exogenously to the cells was also demonstrated to result in increased reporter protein expression.

CHAPTER 6 : CONCLUSIONS AND FUTURE CONSIDERATIONS

6.1 SUMMARY

In **Chapter 1**, the importance of metabolic engineering towards discovery and production of biofuels was described. Also, the advantages of yeast as a host cell were explained.

In **Chapter 2**, various microbial strategies for improving cell viability, which is crucial for high productivity in synthetic microbes, were reviewed. Six strategies were examined, including the engineering of global transcription factors aided by systems biology approaches, engineering of efflux pumps, spatial organization and optimization of pathway enzymes, engineering of dynamic sensing-regulation of protein expression, the balancing of co-factor usage and consumption in biocatalytic pathways, and the use of computational tools to aid engineering.

While all the strategies explained are proven and important ways to boost cell viability and productivity, synthetic promoters that function as dynamic controllers was selected for experimental research. **Chapter 3** described the development and characterization of synthetic promoters systems that can sense and regulate protein expression in response to fatty acids/fatty acyl-CoA. As fatty acid derivatives have ideal properties for use as drop-in biofuels to existing transportation infrastructure, an effective way to engineer microbes to maximize productivity and yield employs dynamic control of protein production in response to concentrations of key intermediates. In yeast, the natural transcription factors responsive to fatty acids have repressed activities in glucose rich

conditions. To create a fatty acid dynamic controller system in yeast, fatty acid/fatty acyl-CoA biosensors with bacterial FadR transcriptional repressors and yeast synthetic promoters that contained DNA binding operators were constructed. The functionality of FadR repressors was demonstrated in yeast. Furthermore, the tuning of the sensing system was carried out by varying promoter strength upstream to FadR coding sequence, by changing the number of operator sites in the synthetic promoter, and by using transcriptional repressors from two bacterial sources (*E. coli* and *V. cholerae*) that possess different ligand sensitivities.

Chapter 4 presented AND-gates created using synthetic promoters combining various upstream enhancer elements and core promoter allowing for response to fatty acids/fatty acyl-CoA. The expression of heterologous proteins in metabolic engineering efforts can be harmful to cells due to increased cellular resources consumption. Dynamic controls, which enable on-demand protein expression, are effective for the engineering and optimization of microbes towards improved cell viability and enhanced biochemical productivity. The development and characterization of AND-gate dynamic controllers in yeast which combine two dynamic control strategies, inducible promoters and sensing-regulation were described in this chapter. These dynamic controllers were built using synthetic hybrid promoters. Promoter upstream enhancer elements were fused to a synthetic GAL1 core promoter that contained DNA binding sites for the binding of FadR, a repressor that reduced DNA affinity upon interaction with fatty acids/fatty

acyl-CoAs, which are key intermediates in production of fatty alcohols, fatty acid esters, alkenes and alkanes, which are advanced biofuels. Results showed that the synthetic GAL1 core promoter can be used as a modular promoter part for making synthetic hybrid promoters and enabling fatty acid inducibility. The performance of the AND-gate dynamic controllers, where two inputs (fatty acid and copper presence / phosphate starvation) were required to switch the AND-gate ON was also revealed.

Chapter 5 described the development and characterization of xylose biosensors in yeast. Towards economical and sustainable biofuel production, cheap and affordable starting material like lignocellulosic biomass is needed. However, lignocellulosic hydrolysates contain various sugars, one major component of which is xylose that cannot be metabolized by the industrial workhorse *S. cerevisiae*. As a result, engineering of *S. cerevisiae* to assimilate xylose has been the focus of much research to develop and optimize xylose-assimilating yeast strains. The construction of genetically encoded xylose biosensors that can regulate protein expression upon detection of xylose sugars is a possible tool for this purpose. Metabolite biosensors can enable the regulation of protein expression to minimize cellular stress via flux balance in synthetic pathways, allow on-demand protein synthesis at each pathway step, and help screening for proteins with higher activities. Xylose biosensors were constructed with constitutively expressed XylR repressors, which function as protein sensors, and synthetic promoters with XylR operator sites, which are bound by XylR

in the absence of xylose to repress transcription. When xylose sugars are detected, XylR DNA binding activity is antagonized, resulting in an increase in the downstream yEGFP reporter expression. Three XylR variants and the matching synthetic promoters were used, including XylR from *T. halophile*, *C. difficile* and *L. pentosus*. The expression levels of the XylR proteins were modulated to optimize the biosensor. XylRs were shown to function in yeast to repress yEGFP expression from the synthetic promoters. Also, xylose sugars added exogenously in the culture media can be detected by the biosensor to control yEGFP expression.

6.2 CONCLUSION AND FUTURE CONSIDERATIONS

In this thesis, I have shown that synthetic promoters are useful genetic elements for creating dynamic controllers in yeast. The identification of DNA binding protein that responds to desired ligands, and the DNA operator site for the protein binding are the basic pieces of information required for development of a promoter system that senses the ligand to regulate protein expression. Many transcription factors are found in nature, but their dynamic behavior has not been characterized in yeast. This presents many opportunities for researchers to explore and develop synthetic promoters in yeast.

A general strategy to create the dynamic controller would be to constitutively express a heterologous transcriptional repressor which senses the desired ligand, and construct synthetic promoters with one or more DNA operator sites in the core promoter region. Various upstream enhancer elements can be used, depending on the researcher's aim. If the transcription factor is an activator in prokaryotic systems, an activation domain such as VP16 or Gal4 (Barberis and Petrascheck 2001) will need to be fused to the transcription factor, and the DNA operator be inserted in the upstream enhancer element instead of the core promoter.

The cost and benefit expressing the heterologous transcription factor needs to be evaluated on a case-by-case basis. Overexpression of proteins in a host cell may burden the transcription and translation machinery, resulting in reduced growth rates. However, this could be

more beneficial, should the dynamic controller be able to control production of toxic proteins, membrane proteins or proteins that produce toxic molecules. Expression of the heterologous transcription factor hence needs to be at an optimal level, that is the minimal level of expression for good promoter regulation with little metabolic burden, to provide a favorable outcome of improved cell viability and pathway productivity.

While the microbial production of alkanes has been demonstrated by a few groups, the use of yeast has not shown the same promise (Choi and Lee 2013; Harger et al. 2012; Schirmer et al. 2010). At my research institution, the production of alkenes has been attained in yeast at a low titer (unpublished). Hence, it is crucial to determine the true bottleneck or barriers towards fatty acid derivatives production in yeast in order to achieve higher titers and productivity. Further efforts in developing synthetic promoters for dynamic regulation should focus on other functions that would have better application opportunities.

One challenge for adapting non-native transcription factors to eukaryotic cells such as yeast is sensitivity obtained. Desired ligands first need to diffuse or be transported into the cells. Further, as eukaryotes possess a nucleus to house genetic information, as opposed to prokaryotes which do not have nuclei, desired ligands need to make its way into the nucleus to interact with the transcription factor and induce a desired response. From the data presented in Chapter 3 and 4, I found that saturating levels of fatty acids could not trigger a complete de-

repression by FadR repressors. This could be a result of fatty acids being unable to reach the intended target, since the nucleus and cell membrane may pose to be significant barriers. Data in Chapter 5, too, showed that high levels of xylose could not totally turn on the XylR repressed synthetic promoters. In addition, I have also attempted to create synthetic sensor systems for the detection of hydrocarbon alkanes, to no avail (results not shown). Transcription factors attempted included YAS1, 2, and 3 from *Yarrowia lipolytica*, AlkS from *Pseudomonas Oleovorans* and AlkR from *Acinetobacter Baylyi ADP1* (Hirakawa et al. 2009; Yuste et al. 1998; Zhang et al. 2012a). Excellent dynamic response was attainable for synthetic promoter systems responsive towards tetracyclines (TetR repressor) and IPTG (LacI repressor) molecules but not so for hydrophobic alkanes or fatty acids (Ellis et al. 2009). Hence, future works should take into consideration the solubility of ligands, transport of ligands into the cell, and dynamic response required in a synthetic dynamic controller system.

Another aspect to consider would be the choice of reporter gene. In this thesis, the work was carried out using the fluorescent protein, yEGFP. This protein requires oxygen for maturation. Thus, ligands chosen should be non-volatile, as opposed to volatile alkanes. The aeration of the cell culture may result in much volatile ligands being evaporated and hence unable to trigger biosensor response. Other non-oxygen requiring reporter genes would function better to detect volatile ligands.

Finally, I present a synthetic dynamic controller idea for yeast that employs glucose starvation to regulate gene expression, which is an excellent tool for engineering microbes for biochemical production. In order to enable cells to grow quickly when cell density is low, the metabolic burden of heterologous protein production should be shut off at low cell density, and turned on at higher cell densities (Holtz and Keasling 2010; Michener et al. 2012). The use of inducible promoter will incur costs of inducers. The creation of dynamic controllers responsive to glucose concentration will enable exogenous inducer-free trigger of production phase, that is the phase where heterologous proteins are produced for biochemical production.

In yeast the native ADH2 promoter is strongly repressed in the presence of glucose, and de-repressed when glucose is exhausted and ethanol consumption begins (Price et al. 1990). However, the promoter regulation and behavior is complex; it is found to be dependent on starting glucose concentration, is more strongly expressed in rich non-selective media, and is also repressed by galactose and raffinose (Michael Lee and DaSilva 2005; Shah 2007). One possible strategy to create glucose starvation inducible promoter is to make use of MIG1 and its binding site. In yeast, MIG1 binds to operators in the presence of glucose to shut off many genes, including galactose and sucrose consumption genes (Schüller 2003). When glucose concentration drops, MIG1 no longer represses the promoters, enabling transcription. The use of multiple MIG1 binding sites in synthetic promoters may enable

the creation of synthetic promoters regulated by glucose concentrations in the culture medium (Gertz et al. 2009).

Synthetic promoters were cloned by PCR or gene synthesis and located upstream of the yEGFP reporter in the multicopy pESC-URA vector backbone. Results shown in Figure 6.1 demonstrates that MIG1 operator indeed can enable the promoter to be glucose dependent, where more copies of MIG1 operator sequences can improve the induction ratio between glucose rich and glucose poor conditions. However, the synthetic promoters were found to be much weaker when multiple copies of MIG1 operator sites were added to the core promoter region. In addition, repression by MIG1 protein did not give very low basal expression. More engineering work and/or other approaches will be necessary towards improving the current setup of a glucose starvation inducible dynamic controller in yeast. The development of a glucose starvation inducible promoter system will empower engineers to control gene expression in engineered microbes in an exogenous inducer-free manner towards affordable and sustainable microbial biochemical production.

Figure 6.1 Glucose starvation synthetic promoter system.

(A) Schematic diagram **(B)** Expression from the promoter at glucose rich (exponential phase) conditions at 8 hours, and at glucose limited (stationary phase) conditions at 24 hours. Grey squares represent MIG1 operators sequence from the GAL1 promoter. Black squares represent strong MIG1 operators taken from (Gertz et al. 2009)

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