Regulon engineering for rapid growth of Saccharomyces cerevisiae on non-native sugars

A dissertation submitted by

Venkatesh Endalur Gopinarayanan

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biotechnology Engineering

Tufts University

May 2018

Adviser: Nikhil U. Nair, Ph.D

Abstract

The rapid development of industries and decreasing fossil fuel reserves appeals for cleaner and sustainable methods for eco-friendly, industrial scale production of chemicals using biochemical methods with cheap, abundant and readily available feedstocks. However, not all industrially relevant microbes can metabolize these carbon sources, thereby requiring heterologous incorporation of substrate assimilation pathways. One such model organism, *Saccharomyces cerevisiae* (baker's yeast), has been widely used in diverse applications, from bread and wine making, to production of industrially relevant chemicals and high-value pharmaceuticals. However, it cannot metabolize pentoses, which make up a significant portion of lignocellulose.

Substantial research years have been expended on engineering pentose metabolism in *S. cerevisiae*. To tackle the problem, researchers have taken a direct approach of constitutively overexpressing necessary catabolic enzymes to direct flux towards glycolysis. However, in stark contrast, native sugar metabolism is usually carefully regulated using sensing, signaling and metabolic components using regulatory systems referred to as regulons. In this work, I analyzed a well characterized natural sugar detection and assimilation system in yeast, the galactose (GAL) regulon, and compared it with engineering methods used for pentose metabolism. From literature review, we hypothesized that downstream genes of the GAL regulon might enhance growth on pentose. As the role of the downstream GAL regulon genes are uncharacterized, we uncoupled regulation from metabolism and demonstrated that these genes are essential for rapid growth of yeast on galactose. To make use of these genes for growth on xylose, we systematically re-engineered every component of GAL regulon resulting in high aerobic growth rates and cell

densities on xylose. Transcriptomics analyses re-affirmed our hypothesis that downstream genes of GAL regulon required for growth, also get upregulated in the xylose regulon.

We extended this approach for a second substrate, arabinose, and demonstrate the general applicability of this strategy. Finally, we re-designed the regulon engineering technique to construct a platform strain that obviates the need to re-engineer multiple components of the regulon for metabolizing a non-native substrate. Using this approach, we show enhanced growth in sugars, irrespective of whether they are detected by the regulon. Overall, this thesis deals with the need for new strategies to engineer non-native substrate utilization and provides a powerful and easy-to-implement 'Regulon Engineering' strategy in this yeast as a potential paradigm.

Acknowledgements

The experience and knowledge I have gained as a graduate researcher has been enjoyable, primarily because of the help, motivation and guidance provided by the people around me, all of whom have immensely helped me in completing this thesis work.

First, I would like to thank my parents, my sisters and friends who have been around me during the course of my Ph.D. They have given their support, encouragement, motivation, and believed in me, even when I had self-doubts. Without their help, none of this would have been possible.

I would like to express my sincere thanks to my adviser, Dr. Nikhil U. Nair for taking me into his lab, supporting me, teaching me the skills required and honing my scientific and critical thinking abilities. He has been very patient with me, especially in the initial years of PhD, when he made himself easily approachable and has cleared even the basic doubts I had in research. He was receptive to a lot of my ideas and encouraged me to incorporate them into this thesis, while making sure I do not diverge away from the goal.

I would like to thank Dr. Kyongbum Lee for taking me into his lab during my first year, when Prof Nair hadn't joined Tufts and made me get the feel of working in a lab. He along with my other committee members, Dr. Joshua Kritzer and Dr. Lee Lynd provided me with valuable suggestions in research and helped me present scientific data clearly to an audience, for which I am deeply grateful. I would also like to thank many of the professors who have taught me several courses. They made learning at Tufts a thoroughly enjoyable experience and taught me several concepts that have been directly applied in this thesis.

During my years as a Ph.D student, I have worked with Dr. Soha Hassoun and Sara Amin from the Dept. of Computer Science on multiple projects. I am indebted to them for diversifying my research experience and they have influenced me to look at organisms from a systems-level.

Finally, I would like to thank my present and previous lab members, Dr. Entela Nako, Todd Chappell, Josef Bober, Zachary Mays, Bruno Nascimento, Dr. Vikas Trivedi, Sean Sullivan, Trevor Nicks, Brett Irwin, and Aaron Love. They have helped me with multiple experiments, provided valuable suggestions on research and provided feedbacks after sitting through hours of my presentations during lab meetings. Overall, they have made these years fly by and made the research experience enjoyable. In particular, Dr. Entela Nako taught me multiple molecular biology techniques during the first year of my lab and helped me kick-start my thesis work, Todd Chappell, Josef Bober and Dr. Vikas Trivedi helped me troubleshoot problems, helped with growth experiments and provided valuable suggestions and feedbacks on multiple occasions.

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Introduction

Since the industrial revolution, there has been a constant and increasing need to generate energy and industrial chemicals. In the past few decades, depleting fossil fuel reserves and need for clean energy have pushed the production of energy and chemicals from the traditional chemical engineering sector towards environmental-friendly microbe-based production. Moreover, unlike chemical industries that are sensitive to price volatility of oil, microbe-based chemical production relies on feedstocks as raw materials and microbial cell factories as catalysts. Although domestication of microbes and culturing techniques for metabolite production such as beer, wine etc. has been practiced for thousands of years, non-native chemical production in a microbial host was first carried out thirty-five years ago, when Ensley et al., transferred necessary genes for production of Indigo from *Pseudomonas putida,* and introduced them in *E. coli* (Ensley et al., 1983). This was followed by reports of production of 2-Keto-L-Gulonate, an intermediate in L-Ascorbate synthesis (Anderson et al., 1985), as well as 7-Aminocephalosporanic acid (7ACA) production (Isogai et al., 1991) in non-native hosts. These studies cemented the possibility of producing industrially relevant chemicals in heterologous hosts through genetic manipulation. In 1991, Bailey introduced the discipline of metabolic engineering and defined it as (Bailey, 1991),

"Metabolic engineering is the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology. The opportunity to introduce heterologous genes and regulatory elements distinguishes metabolic engineering from traditional genetic approaches to improve the strain. This capability enables construction of metabolic configurations with novel and often beneficial characteristics. Cell function can also be modified through precisely targeted alterations in normal cellular activities."

Encouraged by these initial successes, more research in molecular biology, recombinant DNA (rDNA) technology and analytical techniques ensued, leading to significant growth in the field of metabolic engineering and industrial biotechnology. With funding in global industrial biotechnology consistently increasing, the impact of metabolic engineering is forecasted to surge in the coming years.

Metabolic Engineering

Metabolic reactions can be classified into catabolic reactions, reactions of the central carbon metabolism and anabolic reactions. Catabolic reactions are those that convert sugars and carbon sources into central carbon metabolites. The central carbon metabolism acts as a metabolite freeway to funnel in various carbon sources, interconvert precursor metabolites (pyruvate, acetyl-CoA etc.), as well as provide energy and reducing power. Anabolic reactions are involved in using the precursor metabolites, energy and reducing power to produce components for cellular maintenance and growth (cell wall, DNA, proteins etc.) (Jullesson, David, Pfleger, & Nielsen, 2015). Thus, in the overall metabolic infrastructure, flux from various carbon sources converge into a single metabolite freeway, through which various branch points for anabolic reactions emerge. Research in metabolic engineering has primarily focused on regulating or re-routing this flow of fluxes through precursor metabolites to either increase native metabolite production or introduce heterologous reactions to produce non-native industrially relevant chemicals **(Fig. 1)**.

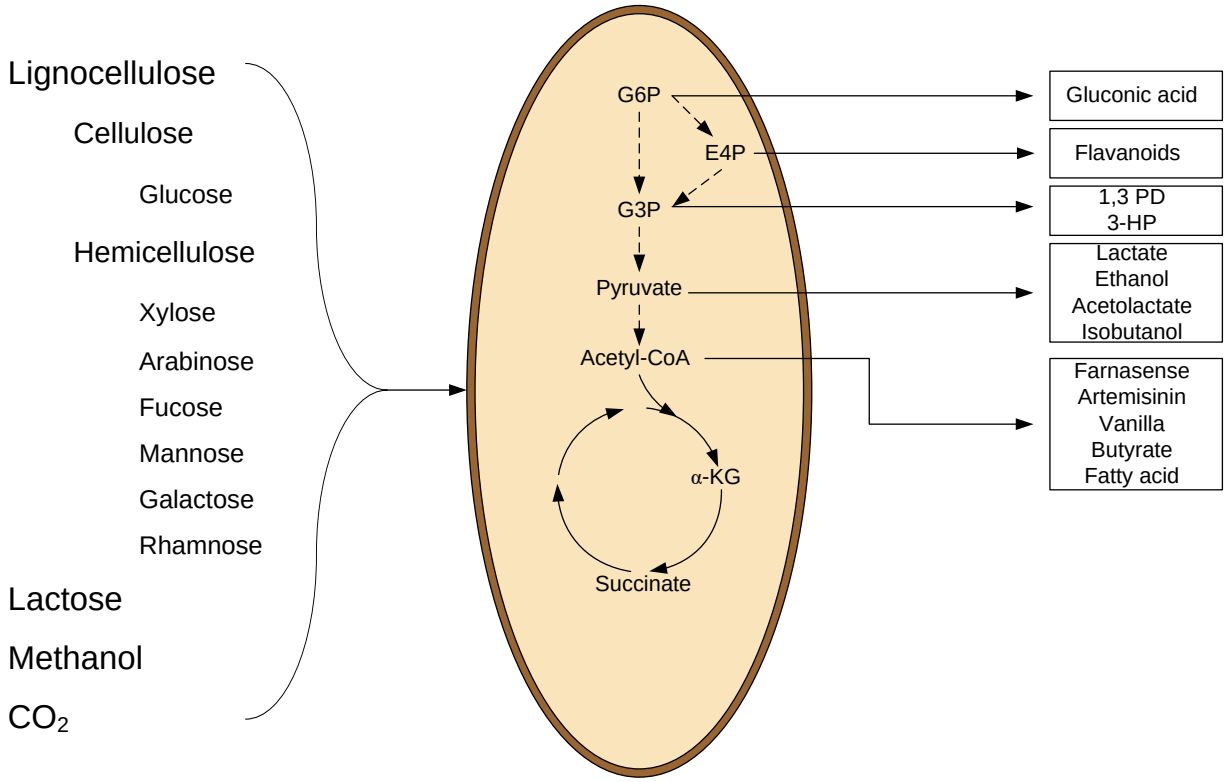


Figure 1: Overview of metabolic reactions and metabolic engineering. *Carbon sources enter the metabolic network through central carbon metabolism. Biochemicals are synthesized by re-routing the flux from central nodes or branch points. The complex polysaccharide lignocellulose, which is composed of cellulose and hemicellulose faction, is made of simple sugar monomers and are represented as a nested column.*

Initially, metabolic engineering was simply applied molecular biology. However, molecular biology tools are not sufficient to increase the product yield as the genetic modifications introduced using molecular biology has to be characterized, the strain has to be analyzed for its performance and based on that, the next set of genetic interventions have to be designed and implemented (design – build – test cycle **(Fig. 2)**). Hence tools for analysis and design are also equally important. In recent years, several tools developed in the fields of synthetic biology, systems biology and metabolic engineering have enabled faster transition through this Design-Build-Test cycle. In the

domain of metabolic pathway engineering, Genome-Scale Metabolic (GSM) models along with various pathway prediction tools such as *ProPath* (Yousofshahi, Lee, & Hassoun, 2011), BNICE (Hatzimanikatis et al., 2005), RetroPath (Carbonell, Planson, Fichera, & Faulon, 2011), OptStrain (Pharkya, Burgard, & Maranas, 2004), DESHARKY (Rodrigo, Carrera, Prather, & Jaramillo, 2008), etc., have partially automated the process of designing pathways, and identification of gene sequences for non-native metabolite production (Design step). Tools such as CRISPR- AID (Lian, HamediRad, Hu, & Zhao, 2017), MAGE (H. H. Wang et al., 2009), TALENS (Joung & Sander, 2013), Zinc finger nucleases (Carroll, 2011), programmable artificial restriction enzymes (Enghiad & Zhao, 2017) etc., along with lowered cost of DNA synthesis, have accelerated the processes of combinatorial library generation, genome engineering, and directed evolution to simultaneously generate variants in the order of $10^{9\text{-}10}$ (Build step). Finally, the multitude of omics tools as well as Next Generation Sequencing (NGS) techniques have greatly reduced the cost and time required to analyze the performance of the strains obtained from the 'build' step. Moreover, the advent of automated bio-foundries such as the MIT-Broad foundry (http://web.mit.edu/foundry/), Edinburgh Genome Foundry (Fletcher, Rosser, & Elfick, 2016), iBioFAB (Si et al., 2017) etc., that integrate some or all of the steps in the cycle, have introduced the possibility of automating the entire process with minimal or no human intervention. The wealth of tools, information, and techniques we have for metabolic engineering have all focused, rightly so, on the optimization and streamlining the production pipeline.

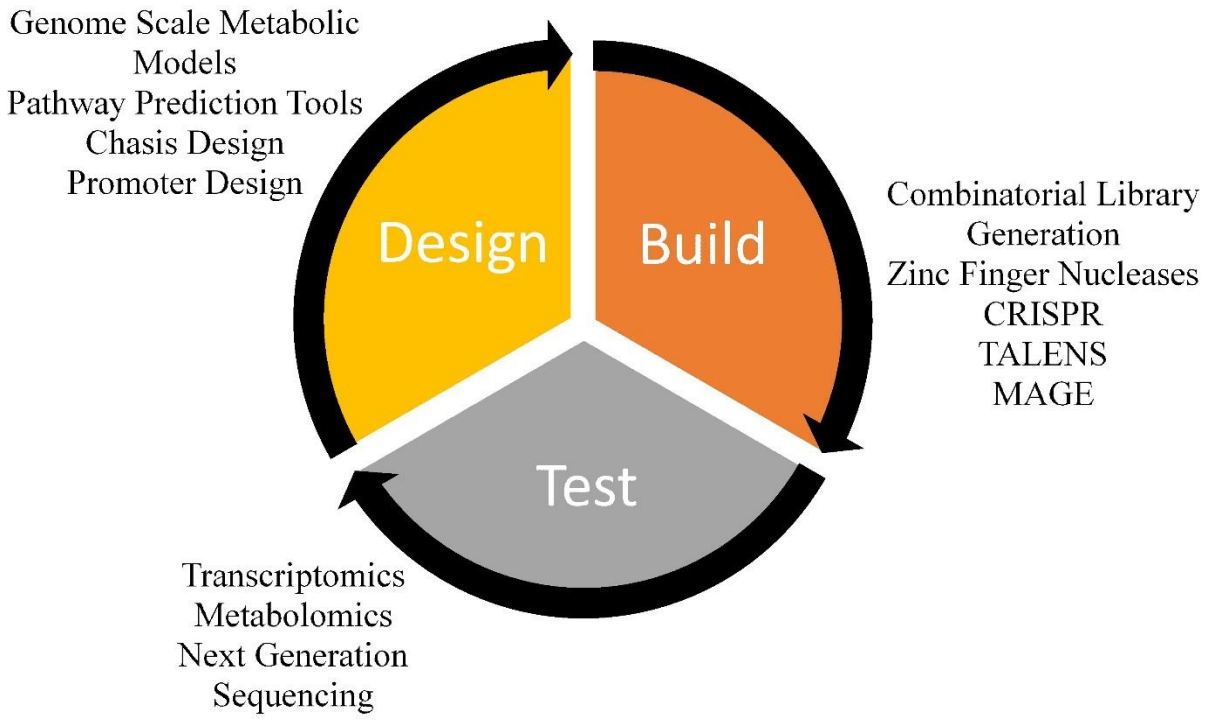


Figure 4: The design-build-test cycle. *Some of the strategies and tools available to fasten each of the steps in the cycle.*

However, the success of metabolic engineering at industrial scales relies largely on using carbon feedstocks that are cheap and readily available. The extension of substrate range of microbes by introducing non-native catabolic reaction pathway is another important research area of metabolic engineering. Researchers have tried incorporating multiple substrate utilization pathways in numerous microbial hosts, such as sucrose utilization in *E. coli K-12* (Sabri, Nielsen, & Vickers, 2013; Tsunekawa, Azuma, Okabe, Okamoto, & Aiba, 1992), lactose utilization in *Corynebacterium glutamicum* (Brabetz, Liebl, & Schleifer, 1991), *Xanthomonas campestris* (Fu & Tseng, 1990), and *Saccharomyces cerevisiae* (Domingues, Dantas, Lima, & Teixeira, 1999), pentose utilization in *S. cerevisiae* (Ren Amore et al., 1991; Becker & Boles, 2003; Moes,

Pretorius, & H Van Zyl, 1996) etc. In stark contrast to the tools, strategies and guidelines laid out for metabolic engineering for product synthesis, there is a lack of tools and strategies for streamlining the process of non-native substrate assimilation. Only at the start of this decade, have researchers started to identify the need to develop tools and techniques to fill in the missing gap (E. Young, Lee, & Alper, 2010). Using pentose utilization in *S. cerevisiae* as a model system, this thesis work aims to address the missing research gap by introducing a faster engineering strategy, which we refer to as 'Regulon Engineering'.

Thesis scope and outline

One of the most studied catabolic engineering topics is the utilization of xylose and arabinose in *S. cerevisiae*. Significant efforts have been expended over the past few decades in addressing this issue. The general strategy has been to overexpress necessary genes using strong constitutive promoters, identify rate-limiting reaction followed by de-bottlenecking downstream pathways. Ideally, this cycle has to be repeated several times to obtain fast growing, efficient sugar consuming strains. However, the strategy is laborious and slow. Along with this, Adaptive Laboratory Evolution (ALE) based strain engineering has been employed to increase the growth rate. But, it can only be employed after several rounds of strain optimization (Garcia Sanchez et al., 2010; Karhumaa, Garcia Sanchez, Hahn-Hägerdal, & Gorwa-Grauslund, 2007; Kuyper, Toirkens, et al., 2005; S.-M. Lee, Jellison, & Alper, 2014; Pitkänen, Rintala, Aristidou, Ruohonen, & Penttilä, 2005; Scalcinati et al., 2012; Marco Sonderegger & Sauer, 2003; Wisselink et al., 2007; H. Zhou, Cheng, Wang, Fink, & Stephanopoulos, 2012). Metabolomics and transcriptomics analysis of the ALE engineered strains reveal flux limitation in the lower half of glycolysis, uncoordinated control of central carbon metabolism along with increased expression levels of

genes involved in stress response and nutrient starvation, highlighting the problems associated with this approach (Y. Jin, Laplaza, & Jeffries, 2004; Salusjärvi et al., 2008; Salusjärvi, Pitkänen, Aristidou, Ruohonen, & Penttilä, 2006; Wahlbom, Otero, Zyl, & Jo, 2003; Wasylenko & Stephanopoulos, 2015). Further, this approach does not take into consideration how the heterologous genes and enzymes integrate into the larger systems level regulatory infrastructure of the host, which probably results in uncoordinated metabolism as well as expression of stress and nutrient starvation response genes. Interestingly, this kind of constitutive substrate assimilation system markedly varies from natural systems that often employ genome-scale regulatory infrastructures, called regulons, to coordinate substrate catabolism with other cellular functions. In such natural regulons, protein-based sensors detect a specific signal (input), which causes signal transduction and amplification (computation), finally resulting in global genetic regulation (output), such as activation of genes necessary for growth, metabolite secretion etc.

The overall objective of this thesis is to analyze the design of substrate assimilation systems in a model organism from a systems level and to use the information to engineer efficient and elegant synthetic catabolic systems in yeast (*S. cerevisiae*). By analyzing the natural regulons and dissecting its regulatory and metabolic components, we gain insight into the advantages conferred by downstream genes of a regulon. With this information, we reverse engineer every aspect of a regulon for non-native sugar utilization, demonstrate significant improvements in growth rate and biomass production while consuming non-native sugars. In the process, we also construct a platform strain aimed at future engineering of strains for non-native sugar consumption. Overall, this thesis work stresses the need to re-think the principles of catabolic engineering by taking into account how heterologous pathways integrate into the native regulatory networks of the host organism.

The thesis is organized into the following chapters.

Chapter 1: Native and non-native sugar utilization in yeast (Literature review)

We performed an extensive review of literature on one of the well-characterized natural regulons, the galactose (GAL) regulon in *S. cerevisiae* to better understand how native sugar metabolism and regulation operate. We also carried out an in-depth literature review on non-native sugar metabolism in yeast, especially xylose and arabinose. These two sugars have been the focus of catabolic engineering for past few decades in *S. cerevisiae,* and thus would help us identify state-of-the-art intervention strategies, as well as genetic, and protein engineering techniques that have been used to address this problem. Finally, we compare how native galactose metabolism differs from xylose and arabinose metabolism. This provided us with valuable insights to engineer better xylose and arabinose catabolic systems.

Chapter 2: A semi-synthetic GAL-type xylose regulon enables rapid growth of yeast on xylose

The review on galactose and pentose metabolism carried out in the previous chapter, raised questions on the role of downstream genes of the GAL regulon and if they can assist growth in other non-native sugars, which are addressed in this chapter. To study the role of downstream genes of GAL regulon on the growth phenotype, we decoupled its metabolism and regulation to create a strain devoid of GAL regulon, but constitutively expresses GAL metabolic genes. We compared its growth with the native GAL regulon and observed the benefits conferred by the regulon towards growth. With this knowledge, we re-engineered the GAL regulon for sensing, signal transduction, and metabolism of xylose. When compared to conventionally engineered strain with similar metabolic interventions for xylose metabolism, the regulon-controlled strain exhibited higher growth rates, near-complete sugar consumption as well as higher final cell

density. Finally, we used RNA-seq to analyze the transcriptomics of strains grown on galactose or xylose, to determine what contributes to the phenotypic changes observed. By systematic manipulation of the GAL regulon, we show that hundreds of genes controlled by GAL regulon get activated, which probably assists growth not only on galactose, a native sugar, but also on xylose, a non-native sugar.

Chapter 3: Extensions of regulon engineering

In chapter 2, we established the advantages of a sugar regulon both in native sugar, galactose and non-native sugar, xylose. In this chapter, we extend this approach to a second non-native sugar, arabinose and show regulon-based sensing as well as metabolism of the sugar. Similar to the xylose (XYL) regulon, the arabinose (ARA) regulon also exhibited significant improvement in growth rate and biomass accumulation when compared to a strain constitutively expressing arabinose metabolic genes. Finally, to circumvent the need to engineer the galactose sensor to interact with every non-native sugar that we plan on using as a carbon source for yeast, we developed a versatile mutant of Gal3p that can activate the GAL regulon in the absence of galactose or other sugars, but under the repression of glucose. We used this as a platform strain for non-native substrate assimilation, by testing for growth in native sugar galactose, as well as non-native sugars, xylose and arabinose.

Chapter 1

Native and non-native sugar utilization in yeast (Literature review)

1.1 Abstract

The rapid development of industries and decreasing fossil fuel reserves appeals for cleaner and sustainable sources of energy and biochemicals. One of the attractive options is to use the abundant lignocellulosic feedstock as a raw material using baker's yeast (*S. cerevisiae*) for the synthesis of value-added chemicals, drugs, biomolecules, etc. However, inefficient assimilation of non-native, lignocellulosic sugars xylose and arabinose by yeast is a major limitation for renewable biofuels and biochemicals production. Three decades of research have focused on expressing exogenous pentose metabolizing enzymes as well as other endogenous enzymes for effective pentose assimilation, growth and biofuel production. In this chapter, we performed an extensive review of literature to study the engineering strategies and techniques that have been carried out to incorporate pentose metabolism in yeast. We also carried out an extensive review of native sugar, galactose metabolism, and regulation and compared it with pentose metabolism in yeast, to identify missing links in pentose metabolism research. In the case of galactose metabolism, multiple signals regulate and aid growth in the presence of the sugar. However, for pentoses that are non-native, it is not clear if similar growth and regulatory signals are activated. While research on pentose metabolism have mostly concentrated on pathway level optimization, recent transcriptomics

analyses highlight the need to take into account regulatory, structural and signaling components of the organism.

1.2 Introduction

Biotechnological production of fuels and chemicals, instead of conventional chemical processes is an attractive eco-friendly option. This provides us with the possibility of using agricultural and dairy wastes as raw materials such as lignocellulose and whey for sustainable chemical and fuel production. Lignocellulose is an attractive and feasible source of biomass as it is the most abundant and cheap material in the biosphere. It is made up of cellulose, hemicellulose, and lignin. Of these, cellulose and hemicellulose are made up of sugar monomers and constitute 60 – 90 % of the total biomass composition (Malherbe & Cloete, 2002). Cellulose is made up of repeating units of glucose linked by α-1,4-linkages (Malherbe & Cloete, 2002) and hemicellulose is a heteropolymer composed of hexoses (glucose, galactose, mannose and glucuronic acid), de-oxy hexoses (fucose and rhamnose) and pentoses (arabinose and xylose) (Scheller & Ulvskov, 2010).

Lignocellulose has a tertiary structure that is stabilized by a number of covalent, and hydrogen bonds, both inter- and intra-molecular (Malherbe & Cloete, 2002). Thermo-chemical and biochemical processes have been in use to break down the complex lignocellulose structure into simpler residues (Limayem & Ricke, 2012). The thermo-chemical route involves gasification of the substrate to syngas (CO and H_2) at 800°C followed by conversion to other industrially relevant chemicals, in the presence of catalysts. The biochemical route involves hydrolysis of cellulose and hemicellulose into monomers which are then used as a carbon source by microorganisms. The thermochemical process is more prevalent of the two and is used more widely in industries than the biochemical process (Lynd, 2017). Cellulose degraded into glucose monomers by cellulases can be utilized by most microorganisms. Unlike cellulose, hemicellulose is a heteropolymer with both hexoses and pentoses whose individual composition varies depending on the plant type.

Irrespective of the plant, xylose seems to be the major sugar component of hemicellulose followed by galactose and arabinose (Table 1). In order to efficiently utilize lignocellulose, any industrial microbe must be capable of incorporating these sugars at a high uptake rate and convert it to the product of choice.

To utilize these monomers of lignocellulose simultaneously, research is underway in several microbes, including model organisms like *E. coli* and *S. cerevisiae* (Clomburg & Gonzalez, 2010; Kuhad, Gupta, Khasa, Singh, & Zhang, 2011). *S. cerevisiae* is a Generally Regarded As Safe (GRAS) organism and has been traditionally used for alcoholic beverages and bread making. With the advent of metabolic engineering, the model eukaryote is being explored as a potential host for producing a wide array of biochemicals, medicinal drugs, antibodies. Success stories include production of bulk commodity chemicals like lactic acid, highvalue compounds such as anti-malarial precursor amorphadiene, biofuels like ethanol, antioxidants like resveratrol etc., (Borodina & Nielsen, 2014). Further, extensive knowledge on the organism's genetics, physiology, and biochemistry, with an array of genetic manipulation and synthetic biology tools, makes it one of the attractive industrial workhorses for biological production of chemicals and biofuels. However, it is unable to utilize pentose sugars that are abundant in hemicellulose. Since the 1990s, there have been numerous attempts to integrate xylose metabolism effectively into *S. cerevisiae*. Though, initial attempts focused primarily on ethanol production, engineering the yeast for the synthesis of chemicals and biomass from pentoses has also attracted much attention in the recent years (W. Guo, Sheng, Zhao, & Feng, 2016; S. J. Kim et al., 2017; S. J. Kim, Seo, Park, Jin, & Seo, 2014; Scalcinati et al., 2012).

Table 1.1: Percentage of hemicellulose monomers in different plant types (Schädel, Blöchl, Richter, & Hoch, 2010)

Functional plant types	xylose (%)	arabinose (%)	Galactose (%)	Glucose (%)
Broad-leaved trees	40 – 80%	10 -30%	5 – 20%	5%
Conifers	17 – 30%	20 – 50 %	15 – 25%	5 – 10%
Grasses	60%	20%	5 – 10%	5%
Herbs	40%	20%	10 – 25%	5 – 15%

In this chapter, to understand the issues in pentose metabolism, we carried out an extensive review of the engineering strategies that has been carried out for pentose metabolism in *S. cerevisiae*. We also carried out an extensive review of literature on the utilization of native sugar galactose, its metabolic and regulatory infrastructure to compare how native sugar metabolism differs from engineering strategies used for utilizing non-native sugars. Understanding native sugar consumption, regulation and growth mechanisms in *S. cerevisiae*, might provide us with valuable cues, that can be used to engineer better catabolic systems for xylose and arabinose metabolism.

1.3 Galactose regulon

In living systems, any change in the external environment triggers a signal response which regulates multiple genes and pathways to adapt to the modified environment through operons and regulons (Orphanides, 2002). Operons exert an elementary level of control in prokaryotes in a single pathway through polycistronic transcripts. However, for regulating genes across multiple pathways, control is established by specific transcription factor controlled global regulatory systems, also known as regulons. Regulons are ubiquitous in nature, such as the heat shock response regulon in *E. coli* (Nonaka, Blankschien, Herman, Gross, & Rhodius, 2006; Yamamori & Yura, 1980), the galactose regulon in *S. cerevisiae* (Douglas & Hawthorne, 1966) and cold response regulons in *Arabidopsis thaliana* (Fowler & Thomashow, 2002). Of these regulons, the galactose regulon in *S. cerevisiae* is probably the most well-characterized. The galactose regulon can be broken down into three sets of genes, depending on their role. The metabolic genes that enable yeast to assimilate galactose into central carbon metabolism, the regulatory genes that enable activation of genes under the galactose regulon, and the downstream genes that have been observed to get activated by the regulon, but their exact role in galactose regulation and metabolism is unknown.

1.3.1 Galactose metabolism

The galactose metabolic pathway genes consist of *GAL1*, *GAL7*, *GAL10*, along with a transporter gene *GAL2* (Lohr, Venkov, Zlatanova, Program, & Academy, 1995). The transporter Gal2p is a high affinity, low flux galactose transporter, with an equal affinity towards glucose (Maier, Völker, Boles, & Fuhrmann, 2002) and severely lowered affinity towards pentose sugars xylose and arabinose (Cirillo, 1968; Farwick, Bruder, Schadeweg, Oreb, & Boles, 2014). Although, Gal2p in theory can transport glucose, the expression of the gene is tightly repressed in the presence of

glucose making it a galactose-specific transporter. Intracellular galactose flows through the Leloir pathway that begins with phosphorylation of galactose to galactose-1-phosphate by galactokinase encoded by the gene *GAL1*. This is followed by uridylyl transfer from UDP-glucose to galactose-1-phosphate by *GAL7* that encodes for galactose-1-phosphate uridyl transferase to generate glucose-1-phosphate and UDP-galactose. The UDP-galactose is isomerized to UDP-glucose by UDP-galactose-4-epimerase, encoded by *GAL10* (Timson, 2007). Gal10p is also bifunctional, and can anomerize alpha-D-glucose and alpha-D-galactose to their beta conformations (Majumdar, Ghatak, Mukherji, Bhattacharjee, & Bhaduri, 2004). Glucose-1-phosphate finally gets isomerized to glucose-6-P, an intermediate of central carbon metabolism, by *PGM1* and *PGM2* genes that code for phosphoglucomutase. Of the two isoforms, Pgm2p is responsible for 80% of the activity (Tsoi & Douglas, 1964).

1.3.2 Galactose regulation

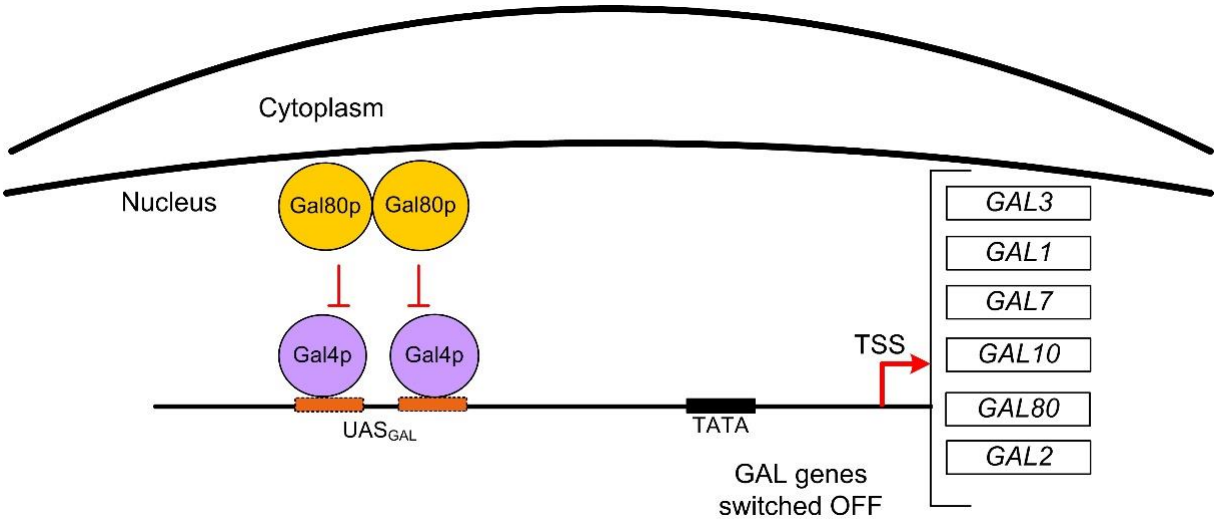
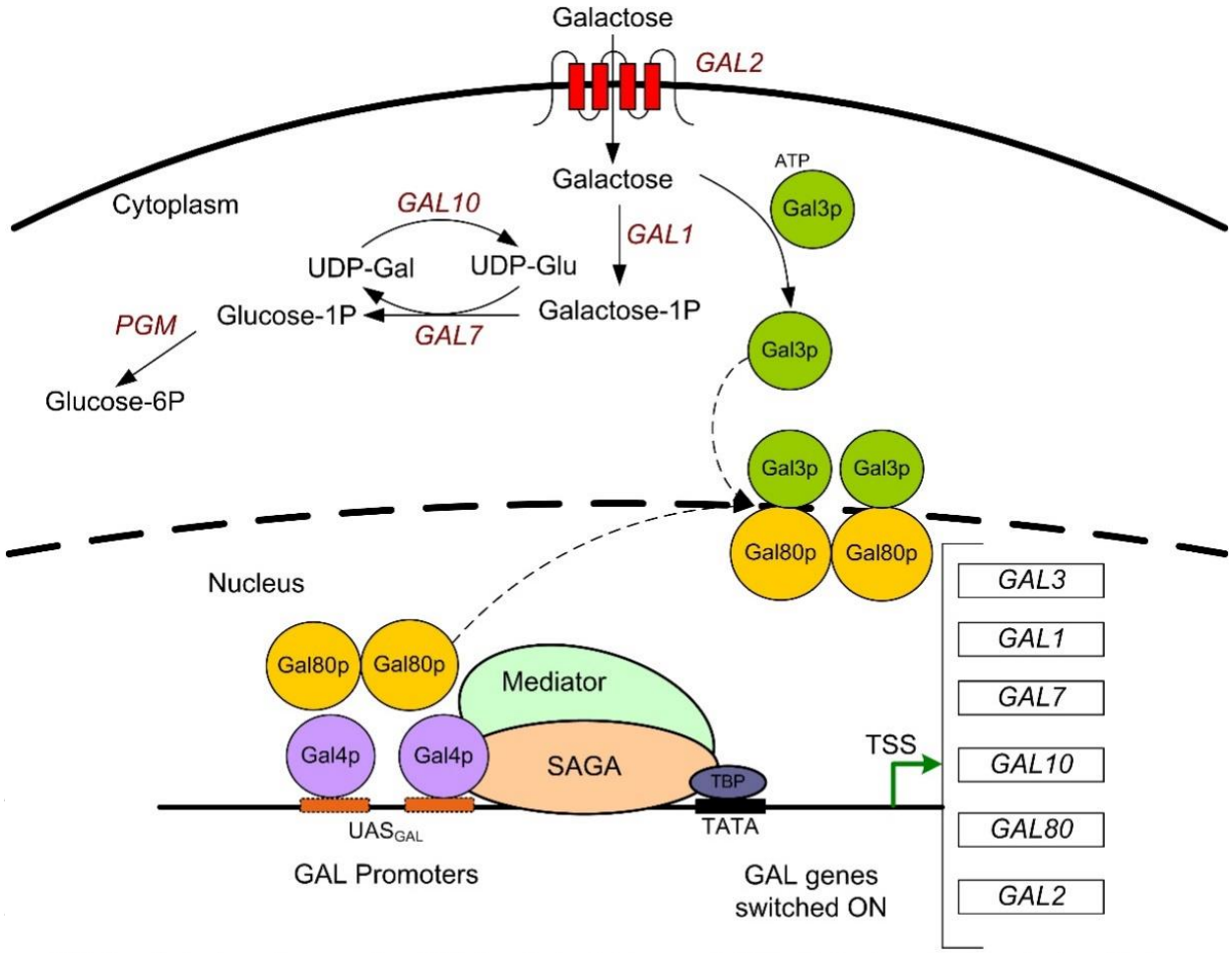


Figure 1.1: GAL regulon is turned OFF in the absence of galactose. *Gal4p is poised for induction at the GAL promoters. Gal80p represses Gal4p by binding to it and preventing activation of the GAL regulon.*

The regulation and activation of the GAL regulon is primarily due to three regulatory proteins, Gal3p, Gal80p, and Gal4p. The GAL regulatory switch is tightly controlled through two different mechanisms and is turned ON or OFF depending on the type of carbon source present (Johnston, 1987; Lohr, 1993; Lohr et al., 1995; Sellick, Campbell, & Reece, 2008). In the presence of glucose, even when galactose is present, none of the structural enzymes are expressed due to catabolite repression by glucose. This OFF state is the result of repression of the transcriptional activator Gal4p by glucose (Griggs & Johnston, 1991). In the presence of other carbon sources like glycerol, ethanol, raffinose etc., the carbon catabolite repression is turned OFF, but the GAL regulon is still not turned ON. The GAL regulon is said to be "poised" for induction and Gal4p, the transcription factor binds to the UAS (Upstream Activation Sequence). But transcription is still repressed by the binding of Gal80p, a transcriptional repressor to Gal4p (Platt & Reece, 1998) **(Fig 1.1)**. In the presence of galactose, the sugar enters the cytoplasm through minimally expressed Gal2p transporter and interacts with Gal3p present in cytoplasm along with the cofactors Mg^{2+} and ATP (Bhat & Murthy, 2001). Gal3p is loosely regulated and has basal levels of expression in the absence of glucose, even without galactose. Galactose bound Gal3p recruits Gal80p, forms a dimer and leads to de-repression of Gal4p. Two different hypotheses for the mechanism of activation have been suggested. One hypothesis proposes that a tripartite complex of Gal3p-Gal80p-Gal4p is formed which enables transcription (Lavy, Kumar, He, & Joshua-Tor, 2012; Thoden, Sellick, Timson, Reece, & Holden, 2005). The other hypothesis suggests that binding of galactose facilitates recruitment of Gal80p, which usually equilibrates between cytoplasm and nucleus. Binding of Gal80p with Gal3p shifts the equilibrium towards cytoplasm resulting in decrease of



GAL2 – Galactose transporter; GAL1 - Galactokinase; GAL7 - Galactose-1-phosphate uridyl transferase; GAL10 - UDP-glucose-4-epimerase; PGM – Phosphoglucomutase; GAL3 – Galactose sensor; GAL80 – Transcriptional repressor; GAL4 – Transcriptional activator

Figure 1.2 ***: GAL regulon activation in the presence of galactose.*** *Galactose is transported through GAL2. Gal3p interacts with galactose and forms Gal3p-Gal80p dimers, which relieves repression of Gal4p by Gal80p. Gal4p recruits necessary transcription factors for activation of the genes under the GAL regulon. GAL1, GAL7 and GAL10 genes are expressed for galactose metabolism and growth.*

Gal80p concentration in nucleus, thus freeing Gal4p for transcription of galactose genes (Campbell, Leverentz, Ryan, & Reece, 2008; Sellick et al., 2008). Another theory, combines both the hypothesis and suggests that during early induction, a Gal3p-Gal80p complex is formed in cytoplasm as well as Gal3p-Gal80p-Gal4p complex in the nucleus. The saturation of Gal80p sites

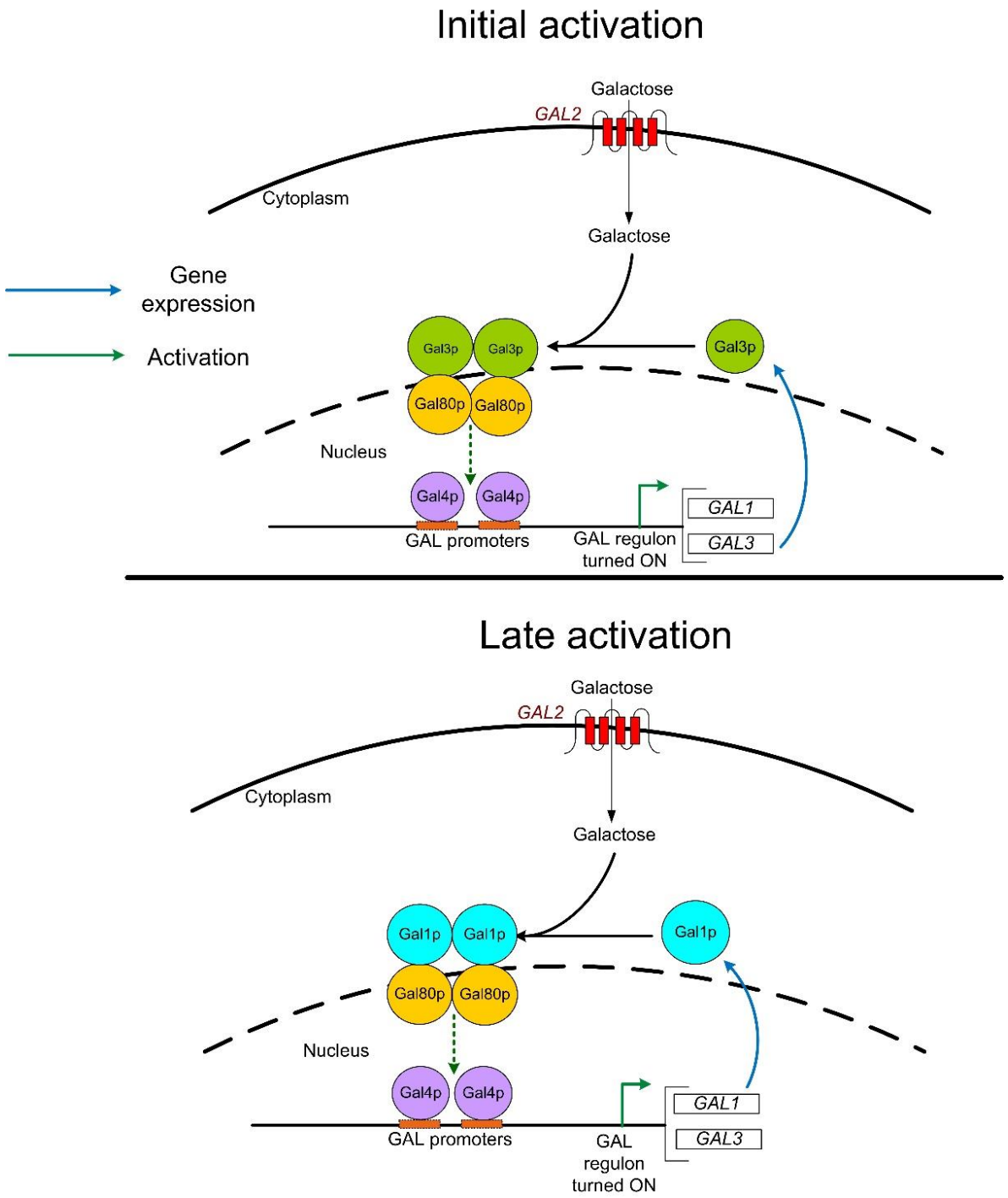


Figure 1.3: Dynamics of GAL activation. *In the initial stages of activation, Gal3p acts as the major transcriptional regulator. However, once Gal1p is transcribed and translated, Gal1p replaces Gal3p and is responsible for GAL regulon activation. Gene expression is shown in blue and GAL regulon activation is shown in green.*

with Gal3p enables transcription and prevents repression by Gal80p (Abramczyk, Holden, Page,

& Reece, 2012). Contributing to this theory, it has been observed that Gal80p dimers are dissipated and Gal3p-Gal80p dimers are formed once the system is induced with galactose (Egriboz et al., 2013). Irrespective of the pathway, it is clear that Gal3p binding with galactose activates Gal4p resulting in recruitment of transcriptional machinery composed of SAGA and mediator complex (Traven, Jelicic, & Sopta, 2006). This switches ON a regulatory cascade enabling transcription of GAL regulon genes and a 1000-fold increase in the expression levels of GAL metabolic enzymes (Johnston, 1987; Johnston, Flick, & Pextont, 1994) **(Figure 1.2)**.

1.3.3 Dynamics of galactose regulation

During early induction of *GAL* genes, Gal3p plays the role of transcriptional activator, but in later stages, Gal1p undertakes this function. In later stages, the concentration of galactokinase increases to a much higher concentration than Gal3p and outcompetes for binding sites with Gal3p **(Fig 1.3)** (Diep et al., 2006; Thoden et al., 2005). This kind of shift occurs due to the difference in *GAL3* and *GAL1* expression. *GAL3* is loosely regulated but also has lower expression level. On the other hand, *GAL1* is tightly regulated but has very high expression once activated. In the absence of Gal3p, activation of GAL regulon by Gal1p takes days rather than hours even though Gal1p is upregulated 1000-fold and Gal3p is upregulated only by 3-fold (Hsu et al., 2012; Lohr et al., 1995). This kind of loose regulation for a sensor and tight regulation for the enzyme prevents over-expression of galactokinase which could result in the buildup of toxic galactose-1-phosphate while enabling faster activation of the regulon by Gal3p. Also, loose regulation of *Gal3p* is required for a basal level expression of the sensor, to detect galactose (Conrad et al., 2014). It has also been observed that *GAL3* activity can be replaced by expressing *GAL1* from a plasmid. But, over-expressing either of them has been shown to result in galactose independent activation of the GAL regulatory system (Bhat & Hopper, 1992). Further, as a result of increased concentration of *GAL1*,

a transcriptional memory of galactose is observed. When cells grown on galactose were shifted to glucose and then back to galactose, the lag phase was much shorter than cells previously unexposed to galactose. Such a memory effect has been attributed to lingering galactokinase which ensures faster re-activation of the GAL regulon. However, after a few generations, galactokinase gets diluted or degraded, leading to delayed activation (Zacharioudakis, Gligoris, & Tzamarias, 2007).

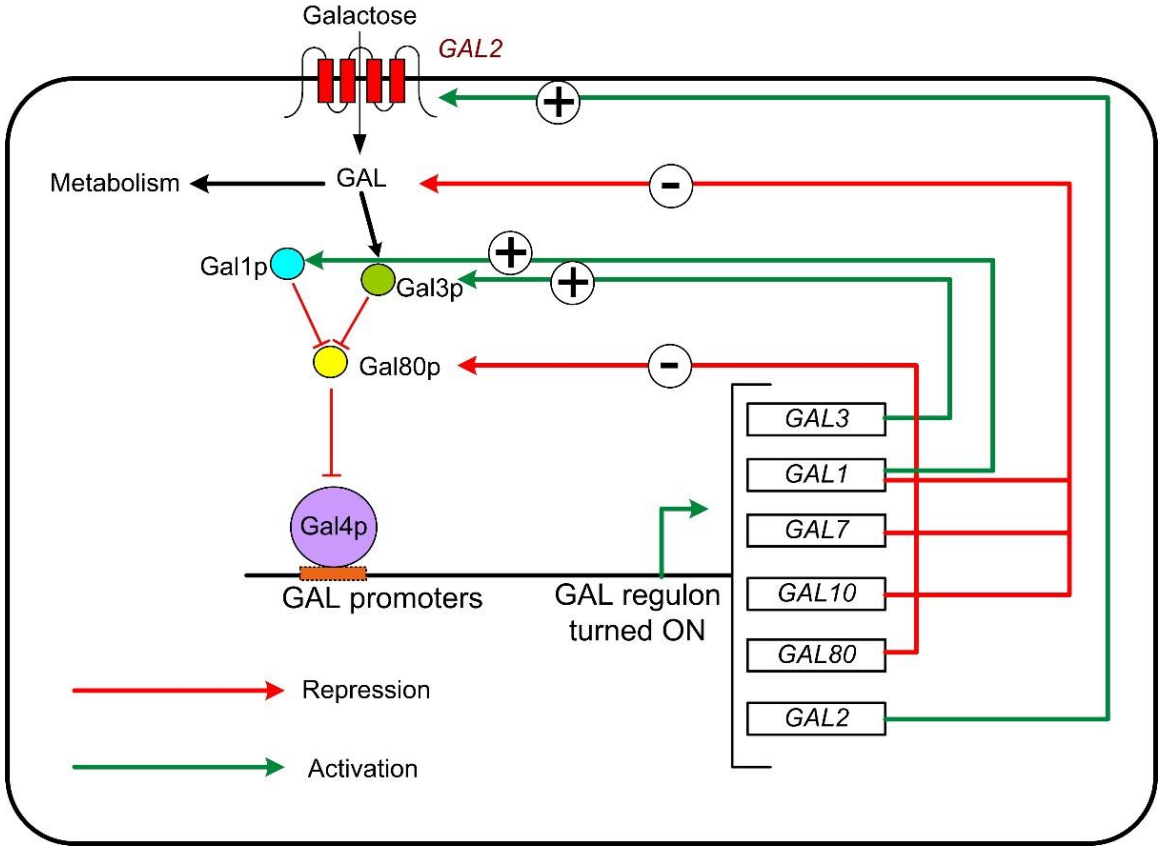


Figure 1.4: A system of interlocking positive and negative feedback loops of the GAL regulon. *Three positive feedback loops are created by Gal3p, Gal1p and Gal2p. Two negative feedback loops are created by GAL metabolism and Gal80p. Positive feedback loops that activate the regulon are represented using green lines with a '+' symbol. Negative feedback loops that repress the regulon are shown in red lines with a '-' symbol.*

The galactose regulatory system can be thought of as five feedback circuits acting in parallel, resulting in GAL induction **(Figure 1.4)**. Gal2p, the galactose transporter, and Gal3p, the galactose

sensor forms two positive feedback loops, by enabling regulon activation, which leads to induction of *GAL3* and *GAL2*. Gal80p, the transcriptional repressor is a negative feedback loop. Gal1p, Gal7p, and Gal10p together metabolize galactose and decrease galactose concentration, thereby acting as a second negative feedback loop. Finally, Gal1p, also interacts with Gal80p, thereby acting as a secondary galactose sensor and results in a third positive feedback loop (Hittinger & Carroll, 2007). Thus, Gal1p in addition to Gal3p creates two interlocking positive feedback loops (Acar, Mettetal, & Van Oudenaarden, 2008; Kundu & Peterson, 2010; Venturelli, El-samad, & Murray, 2012).

1.3.2.2 Yeast galactose sensor, Gal3p

The sensor Gal3p belongs to the GHMP (Galactokinase, Homoserine kinase and mevalonate kinase) kinase family of proteins but does not possess any enzymatic activity. It is made up of 520 amino acid and shares 90% similarity and 72% sequence identity with yeast galactokinase (*GAL1*). Moreover, the galactose binding residues of Gal3p and Gal1p, Lys-258, Glu-53, His-54, Asp-56, Asn-205, Asp-206, and Tyr-266 are conserved (Lavy et al., 2012). *GAL1* and *GAL3* are paralogs that emerged from a single enzyme/activator gene, from which coding sequences and promoter regions for the two genes diverged (Hittinger & Carroll, 2007). Gal3p exists in open and closed conformations depending on whether it is bound to its ligands. The open conformation or the apo-Gal3p is when it doesn't interact with ligands and doesn't have any interactions with Gal80p. Binding of Gal3p to ATP and galactose results in a closed conformation which open sites for binding of Gal80p (Lavy et al., 2012). The Gal3p-Gal80p dimer that is formed is believed to play a role in transcriptional activation.

1.3.2.3 Genes activated by the galactose regulon

Gal4p, the master regulator of the galactose regulon belongs to a family of $Zn(II)_2Cys_6$ proteins. The DNA binding site for the members from the family contains a trinucleotide, mostly 5'-CGG-3' either in single or in repeats (Traven et al., 2006). In the case of Gal4p, it binds to inverted repeats of 5'-CGG-3' and has a consensus sequence, 5'-$CGG(N)_{11}$CCG-3'. This knowledge has been used to predict Gal4p binding sites. Around 300 Gal4p-binding regions have been identified in the promoter regions (Traven et al., 2006). Of these, 41 genes have increased transcript levels when the GAL regulatory network is perturbed (Ideker et al., 2001) and upstream regions of seven of these genes have been shown to directly interact with Gal4p using ChIP analysis (Reimand, Vaquerizas, Todd, Vilo, & Luscombe, 2010; Ren et al., 2000). It has also been found that mutations in *RAS2* can increase growth rate on galactose, suggesting that Gal4p is connected to other regulatory and signaling pathways (Conrad et al., 2014; K.-K. Hong, Vongsangnak, Vemuri, & Nielsen, 2011; K. K. Hong & Nielsen, 2012). Very little is known about the downstream genes of the GAL regulon including the complete set of genes that Gal4p activates, whether they are activated directly by Gal4p binding, or indirectly through activation of other transcription factors. The role played by the downstream genes of the regulon is also not known.

1.3.3 Studies on engineering galactose regulon

1.3.3.1 Engineering GAL promoters based on UAS_{GAL} sites

Expression levels and regulation of GAL genes depend on the number of UAS_{GAL} elements present in the promoter region of the gene (Lopez & Lohr, 1995). *GAL2* has 5, *GAL1* and *GAL10* share 4, *GAL3* and *GAL80* have one each. More UAS_{GAL} elements in the promoter results in recruiting additional Gal4p, as well as increased binding of Gal80p to Gal4p. This results in more controlled activation or repression of the GAL enzymes along with an increase in expression level (Traven

et al., 2006). While for engineering applications, it is tempting to re-use the strong GAL promoters present, there is also the possibility of genetic instability as *S. cerevisiae* can efficiently recombine homologous sequences. Hence, synthetic GAL promoters were created by either varying the number of UAS_{GAL} elements or by placing them in tandem. This also allows modulating the expression levels to create a panel of GAL promoters of varying strengths (Blazeck, Garg, Reed, & Alper, 2012). Research from the Alper lab have also shown that synthetic minimal GAL promoters can be created by introducing UAS_{GAL} elements to minimal core elements of a promoter (Redden & Alper, 2015). More recently, GAL promoters from other species of *Saccharomyces* genus was incorporated in *S. cerevisiae*, and their expression strengths were characterized. It was found that several of these promoters had expression strengths higher than the commonly used *GAL1/10P* promoters (Peng, Wood, Nielsen, & Vickers, 2018). The modularity of the UAS_{GAL} elements along with the ease with which promoter strengths of GAL promoters can be altered have made the GAL system an attractive inducible system for metabolic engineering and synthetic biology applications.

1.3.3.2 Increasing galactose consumption and uptake rates

Since galactose is one of the more commonly used substrates when *S. cerevisiae* is used in metabolic engineering, there has been attempts to increase the assimilation rate of galactose. Ostergaard et al., increased galactose consumption by either removing the negative feedback loop of Gal80p, along with Gal6p and Mig1p (involved in carbon catabolite repression) or by overexpressing Gal4p. Interestingly, the increase in flux through Leloir pathway was not followed by a concomitant increase in growth rate or biomass production. Instead, the flux was rerouted towards ethanol production and a lowered biomass yield (Ostergaard, Olsson, Johnston, & Nielsen, 2000). Analysis of the two mutant strains from the study showed increased transcript levels of

PGM2. A reverse-engineered strain that over-expressed *PGM2* was later constructed that showed similar galactose consumption profiles (Bro, Knudsen, Regenberg, Olsson, & Nielsen, 2005). The study also demonstrated that even minor increase in transcript levels like the one they observed for *PGM2* is sufficient to change the glycolytic flux and cause flux re-allocation. To increase growth rates of galactose, adaptive evolution of yeast grown on galactose have been carried out. Sequencing of the strains revealed accumulation of mutations in *RAS2*, proteins involved cAMP-pkA signaling pathway or in ergosterol metabolism, but no mutations in galactose metabolic genes were observed (K.-K. Hong et al., 2011; K. K. Hong & Nielsen, 2012). However, when the strains carrying the mutations were grown in glucose, the growth rate decreased from the wildtype, suggesting that there is an inherent trade-off between growth on galactose and glucose that has been hard-wired into the yeast regulatory network. Similar techniques along with expression of *S. cerevisiae* genomic library on multi-copy plasmid have been employed to increase galactose consumption (K.-S. Lee et al., 2011). Mutations in *TUP1*, a regulatory gene as well as overexpression of *SEC53*, phosphomannomutase with limited phosphoglucomutase activity were observed. The mutations obtained by Lee et al. (2011) agree with the previously observed mutations from Hong et al. and suggest that galactose regulon is connected to other regulatory pathways through mechanisms that is yet to be deciphered (K.-K. Hong et al., 2011; K.-S. Lee et al., 2011).

1.3.3.3 Re-wiring GAL regulon

Very few engineering attempts have been made so far in re-engineering the galactose regulon. Constitutive *GAL3* mutants have been expressed by pheromone responsive *FIG1p* promoters to activate the GAL regulon. This resulted in coupled GPCR based signaling with galactose regulon and enabled activation of regulon in response to the pheromone, α-factor (Ryo, Ishii, Iguchi,

Fukuda, & Kondo, 2012). Similarly, constitutive Gal3p mutants, along with temperature-sensitive Gal4p mutants were employed to activate the galactose regulon under low-temperature conditions (P. Zhou et al., 2018). These attempts demonstrate that it is possible to activate the regulon using inputs other than galactose.

1.4 Xylose metabolism

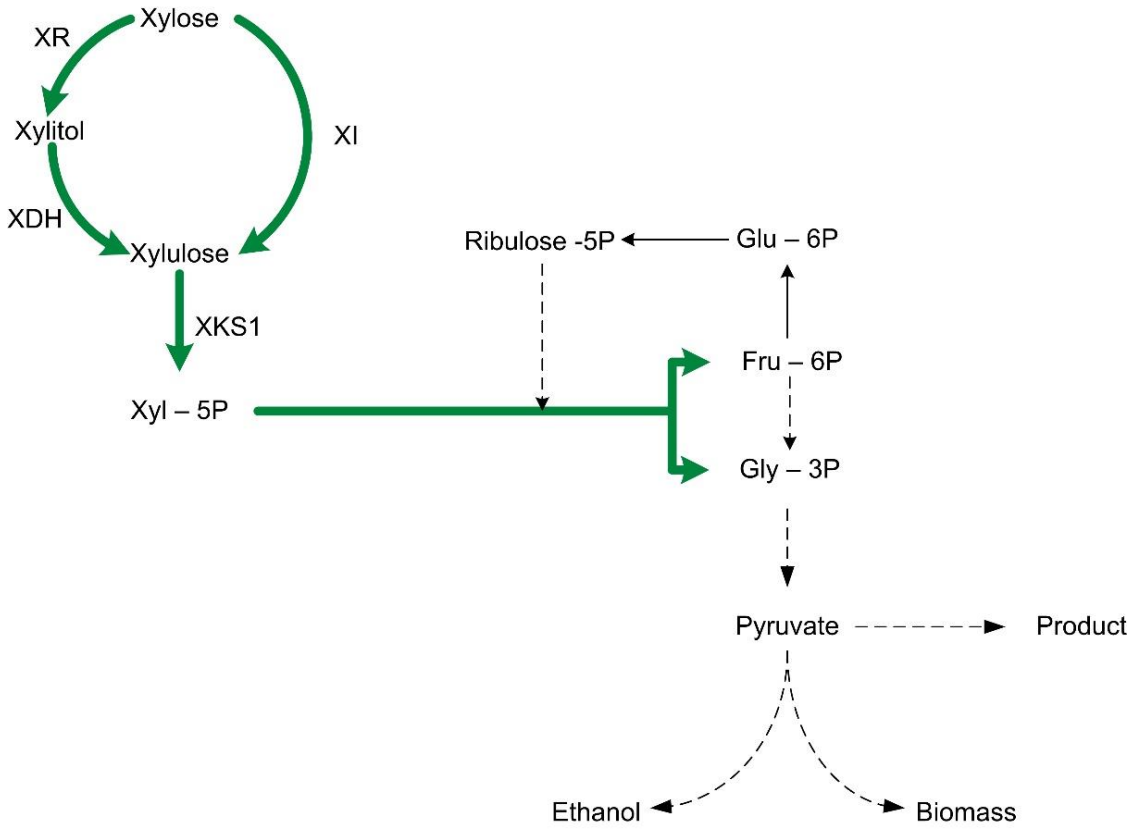


Figure 1.5: Xylose metabolic pathway. *A summary of xylose metabolic pathways that involves either the oxidoreductive route or the isomerase route. Irrespective of the pathway, xylose gets converted to xylulose, which is phosphorylated to xylulose-5-phosphate, a pentose phosphate pathway (PPP) intermediate. Pathways that are usually upregulated (xylose metabolic pathways and PPP) are shown in green.*

Xylose is a five-carbon pentose sugar and is usually the second most abundant component of lignocellulosic feedstock after glucose (Schädel, Blöchl, Richter, & Hoch, 2010). While glucose is packed as cellulose, xylose along with arabinose, galactose and fucose forms the heteropolymer, hemicellulose (Scheller & Ulvskov, 2010). Unlike glucose, which can be broken down by most organisms, xylose cannot be metabolized efficiently by some microbes including *S. cerevisiae*. Two different metabolic engineering pathways have been employed to incorporate it, either the oxidoreductive pathway or the isomerase pathway. Both routes differ in the path used to transform xylose to xylulose, which is then phosphorylated to xylulose-5-phosphate and enters carbon metabolism through pentose phosphate pathway **(Fig 1.5)**. While oxidoreductive pathway involves reduction to xylitol followed by oxidation to form xylulose, the isomerase involves direct isomerization of xylose to xylulose (P Kotter, Amore, Hollenberg, & Ciriacy, 1990; Walfridsson, Bao, Anderlund, & Lilius, 1996). Since most initial studies focused on ethanol production from xylose, a bulk of literature discussed below is concentrated on ethanol production from xylose. However, it is to be noted that efficient consumption of xylose is a necessary trait irrespective of whether the flux is routed towards growth, ethanol, or other products.

1.4.1 Oxidoreductive pathway

Most of the fungi and yeasts with innate xylose-utilizing ability have a two-step oxidoreduction mechanism where xylose is reduced by NAD(P)H dependent xylose reductase (XR) to form xylitol which is further oxidized by NAD^+ dependent xylitol dehydrogenase (XDH) to xylulose (Chakravorty, Veiga, Bacila, & Horecker, 1962; Chang Ching & Knight, 1960; Suikho, Suomalinen, & T.M, 1983; Vandeska, Kuzmanova, & Jeffries, 1995). Finally, xylulose enters the

pentose phosphate pathway (PPP) shunt as xylulose-5-phosphate by the action of xylulokinase (XK).

1.4.1.1 Engineering oxidoreductive pathway in *S. cerevisiae*

Since most fungi and yeasts that have an innate xylose metabolic pathway use the oxidoreductive pathway, initial studies expressed XR, XDH, and XK for xylose metabolism. Interestingly, *S. cerevisiae* has been shown to contain endogenous xylose-utilizing enzymes that consist of xylose reductase (*GRE3*), xylitol dehydrogenase (*XYL2*) and xylulokinase (*XKS1*), all of which together complete the oxidoreductase pathway. Studies have attempted to use *GRE3* and *XYL2* together or along with enzymes from other organisms for xylose utilization (S. R. Kim, Kwee, Kim, & Jin, 2013; M H Toivari, Penttilä, & Richard, 1999, 2000; Mervi H Toivari, Salusjärvi, Ruohonen, Salusja, & Penttila, 2004; Träff, Jönsson, & Hahn-Hägerdal, 2002). However, slow growth and poor xylose consumption were observed.

Of the natural xylose consumers, *S. stipitis* has been known to have the fastest growth rate on xylose with a high ethanol yield. Attempts at expressing xylose reductase gene *(XYL1*) from *S. stipitis* resulted in xylose uptake but with a concomitant xylitol secretion, since xylitol dehydrogenase required for uptake of xylitol was not present (Ren Amore et al., 1991; P Kotter et al., 1990; Takuma et al., 1991). With the idea of emulating xylose metabolism of *S. stipitis*, recombinant *S. cerevisiae* expressing *XYL1* and *XYL2* genes was constructed, but it had very low ethanol yield and secreted large amounts of xylitol (P Kotter et al., 1990; Tantirungkij, Nakashima, Seki, & Yoshida, 1993).

1.4.1.2 Overcoming limitations of the oxidoreductive pathway

1.4.1.2.1 Cofactor engineering in the oxidoreductive pathway

One of the major shortcomings of the approach is secretion of xylitol in large amounts. It was suggested that cofactor imbalance might be one of the causes for increased xylitol secretion (Hahn-Hägerdal et al., 1996). XR has a dual cofactor specificity with higher affinity for NADPH but can also utilize NADH (Km = 3.2 µmol/l for NADPH and Km = 40 µmol/l for NADH), whereas XDH is NAD^+ specific (E. Young et al., 2010). Since the two enzymes XR and XDH have different cofactor specificity, NADPH and NAD^+ must be regenerated to maintain cofactor balance. NAD^+ that is minimally formed during xylitol production could be readily regenerated during xylulose production by XDH, but regeneration of NADPH solely depends on NADPH utilizing enzymes like G6PDH and IDP2 (C. Guo & Jiang, 2013). Failure to regenerate NADPH results in increased xylitol secretion and stunted growth rates (Walfridsson, M, Hallborn J, Penttila M, Keranen S, 1995).

Natural xylose assimilating microbes including *S. stipitis* efficiently utilize xylose despite the dual cofactor specificity of the XR and can maintain redox balance. Comparison of NADPH regenerating enzymes G6PDH and IDP2 in wild-type *S. stipitis* and recombinant *S. cerevisiae* has shown that though the specific activity of IDP2 is low in both cases, the activity of G6PDH is ten times higher in *S. stipitis* than in *S. cerevisiae* (C. Guo & Jiang, 2013). Hence *S. stipitis* can regenerate NADPH and NAD^+ through G6PDH and XDH respectively while *S. cerevisiae* cannot. Further, increase in NADPH regeneration also supports growth as evident from the high growth rate of *S. stipitis* on xylose. Predictions from genome-scale model suggested that cofactor balancing would increase ethanol production by almost 25% (Ghosh, Zhao, & Price, 2011).

To establish a cofactor balance within the xylose-utilizing enzymes, attempts have been made at redesigning the cofactor specificity of both reductase and dehydrogenase. Since XR has lower Km for NADPH, studies on modifying its cofactor preference have focused on reversing its affinity from NADPH to NADH, resulting in increased xylose consumption and decreased xylitol production (Bengtsson, Hahn-Hägerdal, & Gorwa-Grauslund, 2009; Jeppsson et al., 2006; S.-H. Lee, Kodaki, Park, & Seo, 2012; David Runquist, Hahn-Hägerdal, & Bettiga, 2010; Watanabe, Abu Saleh, et al., 2007; Watanabe, Pil Pack, et al., 2007; Xiong et al., 2013). In the case of XDH, a complete reversal of cofactor specificity has been achieved by site-directed mutagenesis but with no significant increase in ethanol yield or decrease in xylitol secretion (Hou, Shen, Li, & Bao, 2007; Khattab, Watanabe, Saimura, & Kodaki, 2011; Krahulec, Klimacek, & Nidetzky, 2009; Watanabe, Kodaki, & Makino, 2005).

Strategies that involve overexpression of NADH-dependent enzymes or deletion of other NADPH-dependent enzymes have also been carried out to reduce xylitol yield and improve redox imbalance. Roca et al (2003), was able to decrease xylitol yield by deleting native *GDH1* (NADPH-dependent glutamate dehydrogenase) and overexpressing *GDH2* (NADH-dependent glutamate dehydrogenase) (Roca, Nielsen, & Olsson, 2003). Another strategy was overexpressing water producing NADH oxidase (*noxE* gene) from *Lactococcus lactis* to generate a regenerative cycle between XDH and NADH oxidase (G.-C. Zhang, Liu, & Ding, 2012). An alternative approach is the use of transhydrogenases that could convert NADPH to NADH. Since such enzyme have not been discovered in nature, genes that code for malic enzyme (*MAE1*), malate dehydrogenase (*MDH2*) and pyruvate carboxylase (*PYC2*) were overexpressed to create a transhydrogenase-like shunt that resulted in partial relief of the redox imbalance (Suga, Matsuda, Hasunuma, Ishii, & Kondo, 2013). In summary, studies have focused on altering the cofactor

preference, engineering synthetic transhydrogenase-like shunt, redox cycles, or innocuous NADH consuming enzymes to bring about a redox balance.

1.4.1.2.2 Relative activities of XR, XDH, and XK - interplay in the expression levels between enzymes

In any linear metabolic pathway, under a particular physiological condition, the reaction with the slowest rate (rate-limiting step) controls the rate of the entire pathway. In the case of xylose utilization, XR, XDH, and XK form a linear metabolic pathway from xylose to xylulose-5-phosphate. Hence along with cofactor balance discussed earlier, relative activities of the enzyme might also contribute to productivity. A kinetic model on the three enzymes predicted that XR: XDH: XK activity ratio of 1: ≥10: ≥4 would be ideal for decreasing xylitol and enhancing ethanol yield. This was also confirmed experimentally (Eliasson, Hofmeyr, Pedler, & Hahn-Hägerdal, 2001). Further studies also confirmed that increased XDH activity leads to decrease in xylitol secretion and better xylose uptake (Y.-S. Jin & Jeffries, 2003; S. R. Kim, Ha, Kong, & Jin, 2012). Unlike XDH, very high expression levels of XK have been found to inhibit xylose growth and uptake (Y.-S. Jin & Jeffries, 2003; R. N. Johansson, Christensson, Hobley, & Hahn-Hägerdal, 2001; T.-H. Lee et al., 2003; Ni, Laplaza, & Jeffries, 2007). Such inhibition can be explained by the turbo design of organisms where ATP is required during the first step of substrate uptake. It has been suggested that substrate uptake through phosphorylation at a very high rate may overcome the net ATP production rate leading to ATP depletion (S. R. Kim, Park, Jin, & Seo, 2013; Teusink, Walsh, van Dam, & Westerhoff, 1998). Hence an optimum level of XK is necessary for increased xylose consumption and growth. Another study that compared the activities of XR, XDH, and XK in wild-type *S. stipitis* and recombinant *S. cerevisiae* (EP490) found the ratios were 1:1.2:0.22 and 1:13:0.8 respectively. While the recombinant strain secreted more xylitol and less

ethanol, *S. stipitis* showed high ethanol and zero xylitol secretion (C. Guo & Jiang, 2013). Though the general consensus regarding the three enzymes is that the activity of XK should be higher or relatively same as that of XR and XDH, other studies have described else wise (Jeppsson, Johansson, Jensen, Hahn-Hägerdal, & Gorwa-Grauslund, 2003; Karhumaa, Fromanger, Hahn-Hägerdal, & Gorwa-Grauslund, 2007; Matsushika & Sawayama, 2011; Parachin, Bergdahl, van Niel, & Gorwa-Grauslund, 2011).

1.4.2 Isomerase pathway

The xylose isomerase pathway found mostly in bacteria and rarely in fungi requires no cofactors making it an attractive pathway to incorporate in *S. cerevisiae*. In this pathway, the enzyme xylose isomerase (XI) isomerizes xylose to xylulose, which is phosphorylated by xylulokinase to xylulose-5-phosphate (Rolf.M & R.watson, 1953). Initial attempts at heterologous expression of xylose isomerase from *E. coli*, *Bacillus*, *Actinoplanes* and *Clostridium thermosulforegenes* all failed probably due to improper protein folding of bacterial enzymes in yeast (Rene Amore, Wilhelm, & Hollenberg, 1989; Moes et al., 1996; Sarthy et al., 1987). Finally, xylose isomerase gene (*XYLA*) from *Thermus thermophilus* was functionally expressed in *S. cerevisiae*. But being a thermophile, the enzyme reached optimal activity at 85°C and was only 4% active at 30°C (Walfridsson, Bao, Anderlund, Lilius, & Hahn-Hägerdal, 1996). Cold adaptation of the enzyme from *T. thermophilus* via random mutagenesis generated variants capable of ethanol yields as high as 0.43 g/g, but had very low productivity and growth rates due to extremely poor specific activity of xylose isomerase (A. Lönn, Gárdonyi, van Zyl, Hahn-Hägerdal, & Otero, 2002; a Lönn, Träff-Bjerre, Cordero Otero, van Zyl, & Hahn-Hägerdal, 2003). Extensive search to identify xylose isomerase from fungi led to successful expression of *XYLA* gene from *Piromyces* & *Orpinomyces*

sp in *S. cerevisiae.* These strains exhibited negligible xylitol secretion and high ethanol yield but low growth rates (Kuyper, Hartog, et al., 2005; Madhavan, Tamalampudi, Srivastava, et al., 2009; Madhavan, Tamalampudi, Ushida, et al., 2009). Later, functional expression from bacteria such as *Clostridium phytofermentans* (Brat, Boles, & Wiedemann, 2009), *Prevotella ruminicola* (Hector, Dien, Cotta, & Mertens, 2013), *Burkholderia cenocepacia* (Vilela et al., 2013) and gut Bacteroidetes cluster (Peng, Huang, Liu, & Geng, 2015) were also shown. To counteract the problem of lower growth rate, debottlenecking of endogenous rate-limiting steps, and evolution engineering have been employed to increase growth rates and xylose consumption, which is discussed in later sections.

1.4.2.1 Overcoming limitations of isomerase pathway

Xylitol secretion, a problem usually associated with XR-XDH pathway due to cofactor imbalance was also observed in the isomerase pathway due to the expression of the native *GRE3* gene coding for aldose reductase. Deletion of the gene prevented xylitol secretion and increased ethanol yield (Kuyper, Hartog, et al., 2005; Madhavan, Tamalampudi, Srivastava, et al., 2009; Träff, Cordero, & Zyl, 2001). The issue of improper folding of bacterial enzymes was solved recently by expressing chaperonins. This enabled functional expression of *XYLA* from *E. coli*, (Xia et al., 2016). The apparent lower growth rates of XI pathway when compared to native and XR-XDH pathway expressing *S. cerevisiae* has been attributed to poor specific activity of xylose isomerase enzyme. Engineering the enzyme XI isolated from *Piromyces sp* to improve its activity has been performed resulting in aerobic growth rate increase from 0.001 h^{-1} to 0.061h^{-1} and a concomitant increase in xylose uptake and ethanol production (S.-M. Lee, Jellison, & Alper, 2012a). In strains expressing bacterial isomerases, codon usage optimization for *S. cerevisiae* is another popular strategy. Depending on the bacterium, modest increase in growth rates and xylose consumption

have been achieved (Brat et al., 2009; Hector et al., 2013). Similar to oxidoreductase pathway, altering expression of XK has been found to be beneficial increasing the xylose consumption rate (Madhavan, Tamalampudi, Srivastava, et al., 2009).

1.4.3 Endogenous pathway and transport limitations

1.4.3.1 Limitations in central carbon metabolism

Initial research in identifying the bottlenecking steps involved in xylose utilization found non-oxidative PPP enzymes to be rate limiting since sedoheptulose-7-phosphate (S7P) was found to be accumulating in xylose metabolizing recombinant *S. cerevisiae* (Koetter & Ciriacy, 1993). Hence, transaldolase (*TAL1*), the enzyme that utilizes S7P as a substrate was overexpressed, increasing growth rate on xylose (S.-M. Lee et al., 2012a; Matsushika et al., 2012; Walfridsson, M, Hallborn J, Penttila M, Keranen S, 1995). Overexpression of all of the enzymes involved in non-oxidative PPP enzymes (*RPE1, RKI1, TAL1, TKL1*) increased xylose growth rates and ethanol yield even further (Karhumaa, Hahn-Hägerdal, & Gorwa-Grauslund, 2005; Kuyper, Hartog, et al., 2005). However, over expression of non-oxidative PPP failed to increase the growth rate on xylose when xylose metabolic pathway enzymes were only moderately expressed (Aloke K Bera, Ho, Khan, & Sedlak, 2011; B. Johansson & Hahn-Hägerdal, 2002), showing that significant control of the pathway still lies in the initial steps of xylose metabolism. Apart from overexpression of enzymes of the non-oxidative pentose phosphate pathway, another strategy involved deletion of G6PDH (glucose-6-phosphate dehydrogenase), thereby decreasing unwanted CO_2 formation and forcing XR to act on NADH alone. This approach has been shown to increase ethanol yield, and decrease byproduct xylitol secretion. (Jeppsson, Johansson, Hahn-hägerdal, & Gorwa-grauslund, 2002; Verho, Londesborough, Penttila, & Richard, 2003). However, decrease in NADPH depended XR

activity and a total decrease in NADPH pool needed for growth and anabolism resulted in stunted growth and slow xylose consumption. So, in order to maintain sufficient levels of NADPH pool, $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (GDP1) was over expressed leading to increased xylose uptake rate and ethanol yield (Verho et al., 2003). Metabolomics on a fast-growing strain of xylose carrying many of these genetic interventions revealed flux limitations on lower part of glycolysis (Wasylenko & Stephanopoulos, 2015). Thus, as a community, through iterative de-bottlenecking, flux limitation was shifted from the initial steps of the xylose metabolic pathway to the tail-end of glycolysis.

1.4.3.2 Transport limitations

S. cerevisiae doesn't have dedicated pentose transporters as xylose is not a naturally metabolized sugar. It is dependent on hexose transporter system, mainly Hxt4p, Hxt5p, Hxt7p and Gal2p for pentose transport (Hamacher, Becker, Gárdonyi, Hahn-Hägerdal, & Boles, 2002). Hence, Gardonyi et al., (2003) analyzed the possibility of transport as a rate controlling step on xylose metabolism and that increase in flux through XR would shift the control from XR to transport (Gárdonyi, Jeppsson, Lidén, Gorwa-Grauslund, & Hahn-Hägerdal, 2003). Although initial research suggested that xylose transport is not rate controlling, further studies have confirmed that xylose transport exercises significant control of the xylose metabolic pathway (Gárdonyi et al., 2003; Kuyper, Hartog, et al., 2005). In a *S. cerevisiae* strain expressing XR and XDH, the flux control coefficient (FCC) for xylose transport was only around 0.15. But when xylose concentration was decreased below 0.6 g/l, the FC coefficient increased to more than 0.5 (Gárdonyi et al., 2003). Further, xylose transport becomes rate-limiting in the presence of other sugars, such as glucose or galactose. The native sugars have higher preference for hexoses, resulting in diauxic growth pattern (Katahira, Mizuike, Fukuda, & Kondo, 2006).

Table 1.2: Aerobic growth rate of previously engineered S. cerevisiae strains on xylose

Strain	Description	Growth condition	μ_{max} (h^{-1})	Reference
RWB 217	*XYLA, XKS1, TAL1, TKL1, RPE1, RKI1, Δ gre3*	Anaerobic, synthetic minimal medium	0.09	(Kuyper et al., 2005a)
RWB 218	*Evolved strain of RWB 217*	Anaerobic, synthetic minimal medium	0.12	(Kuyper et al., 2005b)
ADAP8	*xylA/XKS/SUT1, evolved*	Aerobic, synthetic minimal medium	0.133	(Madhavan et al., 2009)
H131-A3^{SB-1}	*XYLA, PsXYL3, PsTAL1, TKL1, RPE1, RKI1; evolved*	Aerobic, synthetic minimal medium	0.2	(Zhou et al., 2012)
CMB.GS010	*MATa URA3 HIS3 LEU2 TRP1 SUC2 MAL2-8C pTDH3-PsXYL1 pTDH3-PsXYL2 pTDH3-PsXYL3; evolved*	Aerobic, synthetic minimal medium	0.18	(Scalcinati et al., 2012)
ATCC –F2	*pgXR, pcXDH, aoXKS; combinatorial library*	Aerobic, synthetic minimal medium	0.15	(Kim et al., 2013)
SXA-R2PE	*xylA*3, tal1, XKS1, Δgre3, Δpho13; evolved*	Aerobic, synthetic minimal medium	0.128	(Lee et al., 2014)
SyBE005	*MATalpha, leu2, ura3, trp1: PGK1p-XYL1-PGK1t, PGKp-mXYL2-PGKt, PGKp-XKS1-XKS1t: LEU2: AUR1:: pAUR101–RPE1–TAL1, trp1:: pRS304–RKI1–TKL1 ; evolved*	Aerobic, YP medium	0.165	(Zha et al., 2014)
Ll110A	*SsXR, SsXDH*, SsXK, SsRPE, SsRKI, SsTKL, SsTAL; combinatorial library; evolved*	Aerobic, Synthetic minimal medium	0.25	(Latimer et al., 2014)
UUU	*UbXR1, UbXDH, UbXK*	Aerobic, synthetic minimal medium	0.15	(Lee et al., 2016)

Different heterologous transporters have been expressed in *S. cerevisiae* with the idea of relaxing the limitation on xylose transport (Du, Li, & Zhao, 2010; Hector, Qureshi, Hughes, & Cotta, 2008; Katahira et al., 2006; D Runquist, Fonseca, Rådström, Spencer-Martins, & Hahn-Hägerdal, 2009; Saloheimo et al., 2007; E. M. Young, Comer, Huang, & Alper, 2012). Some of these transporters, Gxf1p from *Clostridium intermedia* and Xut3p from *S. stipitis* have also been engineered through directed evolution for increased xylose uptake. Heterologous expression of these transporters in *S. cerevisiae* not only increased the growth rate but also changed the dynamics of diauxic shift during glucose and xylose co-cultures (E. M. Young et al., 2012). An alternative approach has been to engineer native sugar transporters, especially Gal2p and Hxt7p to be better xylose transporters. Most of such studies have concentrated on directed evolution to increase transport of xylose in a strain devoid of hexose transporters (Apel, Ouellet, Szmidt-Middleton, Keasling, & Mukhopadhyay, 2016; Nijland et al., 2014; O. Reznicek et al., 2015; Shin et al., 2015; M. Wang, Li, & Zhao, 2015). Some of these have also focused on altering the preference of hexose transporters, primarily *GAL2* from hexoses to pentoses, to aid in simultaneous co-consumption of xylose and hexoses, glucose or galactose (Farwick et al., 2014; E. M. Young, Tong, Bui, Spofford, & Alper, 2014).

1.4.4 Evolutionary engineering and other approaches

Adapting organisms to suit our needs has been an age-old approach that was historically practiced through selective breeding between species that carried the required traits. In the recent years, adaptive laboratory evolution (ALE) or evolutionary engineering has been exploited for human needs and is practiced extensively to generate industrial microorganisms. Evolutionary engineering is the process of evolving strains by applying a selective pressure that selects for beneficial mutations. Strains are usually grown in continuous cultivation, either using a chemostat

or diluting periodically in shake flasks, in a medium with xylose as a sole carbon source. Strains that accumulate beneficial mutations, which increase growth rate, are enriched in the population until further beneficial mutations accrue in a subpopulation of these cells. Carrying out this process over several generations results in strains that have accumulated mutations that enable it to grow better on xylose. Initially, Sondregger et al. used a xylose-utilizing strain expressing the oxidoreductase pathway enzymes to increase growth and ethanol production under anaerobic conditions by cultivating them under oxygen-limited conditions followed by a complete anaerobic condition for over a period of 460 generations (M Sonderegger, Jeppsson, Hahn-Hägerdal, & Sauer, 2004). Following this, adaptive evolution was carried out to increase the product yield as well as growth rates on xylose in a strain carrying isomerase pathway, with *XYLA* from *Piromyces sp* (Kuyper, Hartog, et al., 2005). Similar evolutionary engineering strategies involving adaptive evolution along with chemical mutagenesis, genome shuffling, and combinatorial library designs have been employed to increase xylose consumption rates, growth rates and ethanol yield (Demeke et al., 2013; Hawkins & Doran-Peterson, 2011; Latimer et al., 2014; Peng et al., 2012; Shen et al., 2012; Tang et al., 2012; Tomás-Pejó, Ballesteros, Oliva, & Olsson, 2010; Xia et al., 2016). Global transcription profiles of these evolved strains from both the pathways showed overexpression of non-ox PPP enzymes like *TAL1* and *TKL1*. (Scalcinati et al., 2012; H. Zhou et al., 2012). Whole genome sequencing of several strains obtained from adaptive evolution, identified mutations in *PHO13*, a phosphatase (S. R. Kim, Skerker, et al., 2013; Ni et al., 2007). Follow-up studies confirmed that *PHO13* deletion increased growth and *PHO13* was thought to play a role in downregulating PPP genes or phosphorylating xylulose-5-phosphate, both of which would lead to decreased flux and growth rates. Later, it was identified to non-specifically dephosphorylate all

PPP shunt intermediates, especially sedoheptulose-7-phosphate leading to accumulation of a dead-end metabolite, sedoheptulose (Xu et al., 2016).

Transcriptome analysis is another often used tool to assess the set of gene expression changes encountered in adaptively evolved strains. Transcriptome analysis and differential gene expression analysis provides us with all the genes that have varied expression levels with respect to control conditions. In most studies, the expression level under xylose is analyzed using strains grown in glucose as a control. An interesting observation from several such transcriptome analysis is the consistent upregulation of genes involved in galactose metabolism and regulation (Y. Jin et al., 2004; Scalcinati et al., 2012; M Sonderegger et al., 2004; Wahlbom et al., 2003). Similarly, upregulation of genes involved in starvation and stress responses have also been observed by several studies (Matsushika, Nagashima, Goshima, & Hoshino, 2013; Salusjärvi et al., 2008, 2006).

Transcriptome analysis provides us with hundreds or sometimes even thousands of genes that have expression level differences, thus making it harder point out the causal genes that play a role in the modified expression profile. Further, it is hard to dissect causation from effect. That is, we cannot differentiate gene expression changes that resulted in the observed change, from gene expression variations that are a result of the observed change. Hence, further analysis of the probable causal genes such as transcription factors is necessary. Alternatively, transcriptomics can be used in combination with other omics techniques such as whole genome sequencing, metabolomics or fluxomics to analyze the causal genes. Recently, Sato et al., using a combination of these approaches were able to identify causal genes as well as epistatic interactions between them. Genes such as *HOG1*, *ISU1*, and *IRA2* that are not directly involved in metabolism but are signaling pathway genes were identified (Sato et al., 2016). Similarly, other studies have identified mutations

in non-metabolic genes such as *CYC8* (Nijland et al., 2017), MAPK pathway genes and genes involved in cAMP-pKA signaling (Dos Santos et al., 2016; Sato et al., 2016).

Other approaches such as transcription factor-based engineering have also been explored. In this strategy, information of genome-wide regulatory networks is captured using an algorithm, which is used to identify transcription factors for deletion or overexpression that are necessary to transcriptionally reprogram yeast metabolic network. This method yielded high xylose uptake rates and ethanol production. Transcription factors such as *CAT8* and *HAP4* deletion were found to assist in shifting the transcriptional state from a respiratory to a fermentative state (Michael et al., 2016). Recently, overexpression and downregulation of endogenous genes for increasing xylose consumption and growth were carried out. Overexpression of *MDH1*, *VPS13,* and *COX5a,* along with downregulation of *CDC11* were found to be beneficial (HamediRad, Lian, Li, & Zhao, 2018).

Taken together, studies on identifying bottlenecking steps suggest limitations not only in metabolic enzymes such as *TAL1*, *PHO13,* etc. but also in transcription factors, signaling pathways as well as regulatory and structural proteins. These studies also underscore the need to analyze the problem of catabolic engineering from a systems-level, rather than analyzing it from a pathway level, and the need for transcriptional reprogramming. Although strategies like transcriptome engineering (Michael et al., 2016) have taken initial steps in that direction, more research on systems-level understanding of catabolism, regulatory mechanisms involved and how metabolic, regulatory, structural and phenotypic genes are interlinked is warranted.

1.4.5 Xylose sensing in *S. cerevisiae*

Xylose is a sugar that cannot be naturally metabolized by *S. cerevisiae*. Even though endogenous xylose metabolic pathway have been discovered (Mervi H Toivari et al., 2004), the growth is

insignificant and the signaling response xylose elucidates in yeast is unknown. It has been known that sugars like glucose and galactose exert repressive effects on certain pathways to varying extents. For example, both sugars have been shown to repress glyoxylate cycle genes such as *ICL1* (Polakis & Bartley, 1965; Schöler & Schüller, 1993). But how does yeast respond to xylose that is not naturally metabolized? To test this, Belinchón et al., studied the effect of xylose and found catabolite repression of xylose on genes involved in glycolysis, TCA cycle, glyoxylate cycle, galactose and sucrose metabolism (Belinchón & Gancedo, 2003). However, this repressive effect might not be the result of xylose acting as a glucose analog to exert repression as hexokinase 2, a protein involved in carbon catabolite repression was unable to interact with Mig1p in the presence of xylose (Moreno, Vega, & Herrero, 2016). Further, xylose induces a conformation state in Hxk2p that is usually observed under de-repressed conditions (Shoham & A. Steitz, 1982). These results confirm that xylose-based catabolite repression is not analogous to glucose-based catabolite repression.

Recently, Gorwa-Grauslund lab from Lund University conducted several studies on xylose sensing. They assessed the effect of xylose when GFP was expressed under various promoters in strains that can or cannot metabolize xylose (Brink, Borgström, Tueros, & Gorwa-Grauslund, 2016; Osiro et al., 2018). Their results demonstrated that extracellular xylose was not detected by *S. cerevisiae*. However, intracellular xylose exerted a partial de-repressive effect. Further, when strains consuming xylose was used, a carbon starvation response was observed. Similar observations were also made when transcript profiles of xylose consuming yeast strains were analyzed (Matsushika et al., 2013; Salusjärvi et al., 2008, 2006). Taken together, the mechanism by which xylose triggers signaling response in *S. cerevisiae* is still not clear, and further analyses are needed.

Alternatively, the idea of heterologous xylose sensors in *S. cerevisiae* have been explored by incorporating bacterial XylR based transcriptional sensors from various prokaryotes and having their respective DNA recognition sites incorporated at strategic transcriptional machinery binding regions of a promoter, upstream of a reporter gene (Teo & Chang, 2014; M. Wang et al., 2015).

1.5 Metabolic engineering for arabinose utilization in *S. cerevisiae*

Arabinose is the second most abundant pentose sugar present in hemicellulose after xylose **(Table 1.1)**. *S. cerevisiae* cannot metabolize arabinose, like xylose, necessitating the need to engineer arabinose utilization pathway. Like xylose metabolism, arabinose metabolic pathway occurs either through the predominantly fungal oxidoreductive pathway or the bacterial isomerase pathway. The fungal arabinose utilization pathway utilizes oxidoreduction reactions to convert arabinose to D-xylulose. An NADP-dependent aldose reductase (mostly a xylose reductase, XR) reduces L-arabinose to L-arabinitol which is oxidized to L-xylulose by NAD^+ dependent arabinitol dehydrogenase (LAD). L-xylulose is then isomerized to D- xylulose by the action of two enzymes with xylitol as the intermediate: NADPH dependent xylulose reductase (LXR) and NAD^+ dependent xylitol dehydrogenase (XDH) (Gene, Richard, Putkonen, & Va, 2002; Richard, Londesborough, Putkonen, Kalkkinen, & Penttilä, 2001; Richard, Verho, Putkonen, Londesborough, & Penttila, 2003) **(Fig 1.5)**.

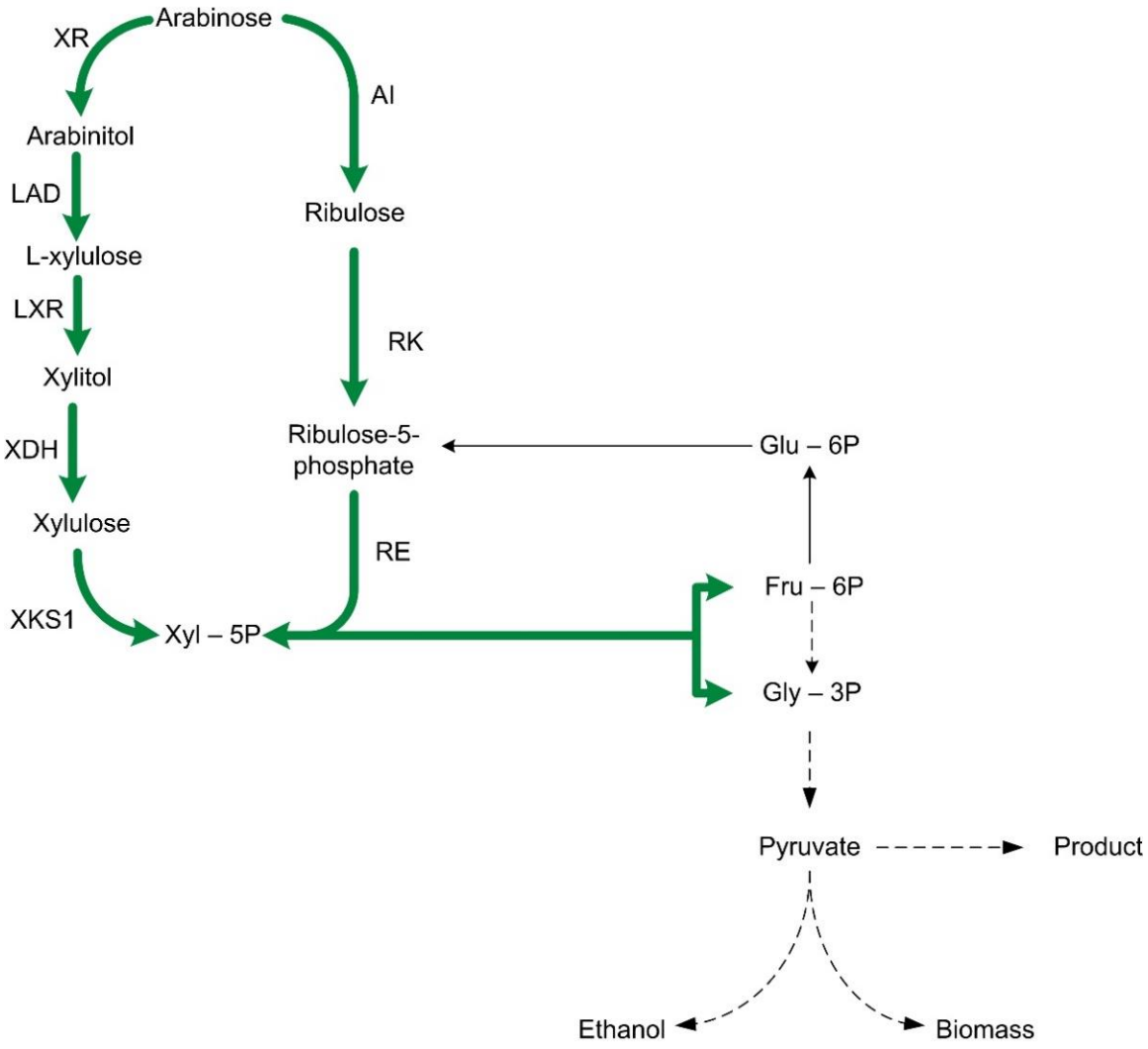


Figure 1.6: Arabinose metabolic pathway. *A summary of arabinose metabolic pathways that involves either the oxidoreductive route or the isomerase route. Pathways that are usually upregulated (arabinose metabolic pathways and PPP) are shown in green.*

The oxidoreductase pathway from fungi has been employed for arabinose utilization using *xyl1-xyl2* genes from *S. stipitis*, in addition to *lad1* (L-arabinitol dehydrogenase) & *lxr1* (L-xylulose reductase) genes from *Trichoderma reesei*. Low ethanol production was observed and growth or arabinose consumption was not studied (Richard et al., 2003). Similar to the oxidoreductive pathway engineering for xylose, the hampered flux through arabinose consuming pathway has

been attributed to the cofactor imbalance created by the differential preference of the enzymes to use NADPH and NAD^+ (van Maris et al., 2006). A strain optimized for xylose metabolism (PPP enzymes overexpressed, non-specific aldose reductase, GRE3 deleted) was further engineered to express a NADH specific mutated aldose reductase and NADH dependent L-xylulose reductase (ALX1) from *Ambrosiozyma monospora*, which resulted in better growth and arabinose consumption (Bettiga, Bengtsson, Hahn-Hägerdal, & Gorwa-Grauslund, 2009). A similar study aimed at expressing fungal oxidoreductase pathway of xylose and arabinose has demonstrated the ability of the strain to ferment a mixture of five sugars – glucose, mannose, galactose, xylose, and arabinose anaerobically (Aloke Kumar Bera, Sedlak, Khan, & Ho, 2010). Recently, bioprospecting for efficient arabinose consumers revealed a fungal strain, *Ustilago bevomyces*. Recombinant *S. cerevisiae* strains carrying genes from *U. bevomyces* showed better growth rates and arabinose consumption. Surprisingly, the arabinose reductase also moonlighted as a xylulose reductase, thereby requiring only three enzymes to convert arabinose to xylulose (S. M. Lee, Jellison, & Alper, 2016). In spite of these improvements, the oxidoreductase pathway has a high arabinitol yield of ≈ 0.35 - 0.48 g of arabinitol per g of arabinose consumed probably due to cofactor imbalance. Moreover, low growth rates have been observed while using this pathway, with the maximum growth rate of only $0.05h^{-1}$ (S. M. Lee et al., 2016).

Table 1.3: Aerobic growth rate of previously engineered S. cerevisiae strains on arabinose

Strain	**Description**	**Growth condition**	**μ_{max} (h^{-1})**	**Reference**
TMB 3130	*HIS3::ADH1p- XYL1-ADH1t, PGKp-XYL2-PGKt, pPGK-XKS1-tPGK TRP1:: YlpAraB KanMX, NTS2::HXT7p-AraA-CYC1t and NTS2:: HXT7p-AraD-CYC1t, KanMX additional copies*	Aerobic, synthetic minimal medium	0.033	(Karhumaa et al., 2006)

	of NTS2::HXT7p-AraA-CYC1t			
IMS0002	*MATa ura3-52 HIS3 leu2-3,112 TRP1 MAL2-8c Wisselink SUC2 loxP-PTPI::(-266, -1)TAL1 gre3::hphMX et al. (2007) pUGPTPI-TKL1 pUGPTPI-RPE1 loxP-PTPI:: (-40, -1) RKI1 {pRW231, pRW243} Strain constructed for growth on arabinose; promoters of TKL1, TAL1, RPE1 and RKI1 replaced by strong TPI promoter; transformed with plasmids pRW231 and pRW243, containing Lactobacillus plantarum AraA, AraB and AraD selected for anaerobic growth in L-arabinose*	Aerobic, synthetic minimal medium	0.15	(Wisselink et al., 2007)
TMB3075	*CEN.PK 2-1C, Δ gre3, his3:: p PGK1-XKS1- t PGK1, TAL1:: p PGK1-TAL1- t PGK1, TKL1:: p PGK1-TKL1- t PGK1, RKI1:: p PGK1-RKI1- t PGK1, RPE1:: p PGK1-RPE1- t PGK1, leu2, trp1, ura3 KanMX, pHXT7tr-AraB (E. coli)-tCYC1, TRP1 pHXT7tr-AraA (B. subtilis)-tCYC1, NTS2::pHXT7tr-AraD (E. coli)- tCYC1 LEU2 XYL1 (P. stipitis), XYL2 (P. stipitis), URA3*	Aerobic, synthetic minimal medium	0.03	(Bettiga et al., 2008)
TMB3076	*CEN.PK 2-1C, Δ gre3, his3:: p PGK1-XKS1- t PGK1, TAL1:: p PGK1-TAL1- t*	Aerobic, synthetic minimal medium	0.03	(Bettiga et al., 2008)

	PGK1, TKL1:: p PGK1-TKL1- t PGK1, RKI1:: p PGK1-RKI1- t PGK1, RPE1:: p PGK1-RPE1- t PGK1, leu2, trp1, ura3 KanMX, pHXT7tr-AraB (E. coli)-tCYC1, TRP1 pHXT7tr-AraA (B. subtilis)-tCYC1, NTS2::pHXT7tr-AraD (E. coli)- tCYC1 LEU2 pHXT7tr-XI (Pyromyces sp.)-tCYC1, URA3			
BWY1-SI	*YEparaAsynth, YEparaBG361A, YEparaD, YEpGAL2 Evolved for aerobic growth in arabinose*	Aerobic, synthetic minimal medium	0.112	(Wiedemann and Boles, 2008)
TMB3664	*CEN.PK 2-1C, MATa, leu2-3 112, ura3-52, Δ gre3, his3::HIS3 PGK1p-XKS1-PGK1t, TAL1::PGK1p-TAL1-PGK1t, TKL1::PGK1p-TKL1-PGK1t, RKI1::PGK1p-RKI1-PGK1t, RPE1::PGK1p-RPE1-PGK1t TDH3p-XYL1(K270R)-ADH1t, PGK1p-XYL2-PGK1t, URA3*	Aerobic, synthetic minimal medium	0.05	(Bettiga et al., 2009)
XR3* Δpho13	*p425GPD_sLAD1_sALX1 UbXR3*, UbLAD, UbXK, pho13Δ*	Aerobic, synthetic minimal medium	0.05	(Lee et al., 2016)

In the isomerase pathway mostly prevalent in bacteria, arabinose is isomerized to ribulose by arabinose isomerase (AI), which is then phosphorylated by ribulokinase (RK) to ribulose-5-phosphate. Finally, ribulose-5-P-4-epimerase (RE) acts on ribulose-5-P and converts it to xylulose-

5-phosphate (Sedlak & Ho, 2001) **(Fig 1.5)**. The *E. coli* araBAD operon containing the arabinose utilization genes has been expressed in *S. cerevisiae* by Sedlak & Ho, (2001) but no ethanol was detected (Sedlak & Ho, 2001). Growth rate increase and ethanol production was achieved only after replacing arabinose isomerase (*AraA*) of *E. coli* with that of *Bacillus subtilis*, overexpressing *GAL2* and adapting the strain through evolutionary engineering over hundreds of generations. Analysis of the evolved strain revealed a mutation in ribulokinase (*AraB*) gene that decreased its activity(Sedlak & Ho, 2001). Codon-optimized araBAD genes from *B. subtilis* have also been used. But it resulted only in a modest increase in growth rate and ethanol yield (Wiedemann & Boles, 2008). Isomerase pathway genes of arabinose metabolism from *L. plantarum*, along with *XYLA* from *Piromyces* were expressed in *S. cerevisiae*. Evolutionary engineering in this strain led to high uptake rates of glucose, arabinose and xylose mixtures. (Wisselink et al., 2007; Wisselink, Toirkens, Wu, Pronk, & van Maris, 2009). Characterization of the evolved strain revealed increased expression of the genes of GAL regulon, transketolase isoenzyme (TKL2) and transaldolase isoenzyme (YGR043c) (Wisselink et al., 2010), similar to evolutionary engineering studies carried out for xylose assimilation (Scalcinati et al., 2012; M Sonderegger et al., 2004). Few studies have also attempted xylose-arabinose co-consumption since many of the reactions between the two metabolic pathways are conserved. In both studies, extensive adaptive evolution was required to assimilate the sugars simultaneously (Karhumaa, Wiedemann, Hahn-Hägerdal, Boles, & Gorwa-Grauslund, 2006; van Maris et al., 2006). In one such study, arabinose could not be oxidized to ethanol and increased secretion of arabinitol was observed (Karhumaa et al., 2006). Bioprospecting for arabinose transporters has also been explored recently, resulting in arabinose specific transporters (P. Knoshaug et al., 2015; Subtil & Boles, 2011). Compared to studies on xylose assimilation, less work has been carried out for arabinose assimilation, probably due to the

increased number of genes in the pathway, or that further increase in growth rates, or arabinose utilization, have proven to be recalcitrant.

1.6 Industrial second-generation biofuel production strains

Table 4: Overview of commercial plants for second-generation biofuels. Adapted from Jansen et al., (Jansen et al., 2017) and UNCTAD report (UNCTAD, 2016)

Company	***Country***	***Country***	***Feedstock***	***Capacity (Million Liter/ year)***
Aemtis	USA	Bioethanol	NA	283.9
DuPont	USA	Bioethanol	Corn stover	113.6
Poet-DSM	USA	Bioethanol	Corn cobs/stover	75.7
Quad County	USA	Bioethanol	Corn fiber	7.6
Fiberight	USA	Bioethanol	Waste stream	1.9
ICM Inc.	USA	Bioethanol	Biomass crops	1.2
American Process Inc.	USA	Bioethanol	Other	1.1
Abengoa	USA	Bioethanol	NA	1.0
LS9	USA	Bioethanol	NA	94.6
ZeaChem Inc.	USA	Bioethanol	Biomass crops	37.8
Enerkem Alberta Biofuels	Canada	Bioethanol	Municipal waste	38.0
Enerkem Inc.	Canada	Bioethanol	Woody biomass	5.0
Iogen Corporation	Canada	Bioethanol	Crop residue	2.0
Woodlands Biofuels Inc.	Canada	Bioethanol	Woody biomass	2.0
GranBio	Brazil	Bioethanol	Bagasse	82.4
Raizen	Brazil	Bioethanol	Sugarcane bagasse/ straw	40.3
Longlive Bio-technology Co.	China	Bioethanol	Corn cobs	63.4
Mussi Chemtex	Italy	Bioethanol	*Arundo donax*, rice straw, wheat straw	75
Borregaard Industries AS	Norway	Bioethanol	Wood pulping residues	20
Dynamic Fuels (REG)	USA	Biodiesel	NA	284
NatureWorks	USA	Renewable Chemicals	NA	140.1
EcoSynthetix	USA	Renewable Chemicals	NA	126.2

Gevo	USA	Biobutanol	NA	60.6
Metabolix	USA	Renewable Chemicals	NA	56.8

The first-generation industrial bioethanol plants used starch as the raw material. However, this creates a competition, between food and fuel, and is not sustainable in a world where the human population is projected to increase for the foreseeable future. Second generation biofuels have been in use for the past few years that uses cheap lignocellulosic feedstocks as raw materials and several of them use *S. cerevisiae* as biocatalyst. While detailed information on the technology behind several of these plants are not available, some of the plants employ engineered *S. cerevisiae* strains capable of utilizing both hexoses and pentoses. Some of the noteworthy examples include, Poet-DSM's collaborative 'Project Liberty' that uses *S. cerevisiae* strains capable of fermenting hexoses and pentoses to ethanol (http://www.poetdsm.com/liberty). Similar collaborative projects have been established between Novozymes and Brazilian ethanol producing companies such as GranBio and Raizen (O.G. Silva, P. Vaz, & X.F.Filho, 2018). Both utilize enzymes and yeast engineered by Novozymes that can use hexoses and pentoses. More recently, Quad County Corn Processors started evaluating a strain of *S. cerevisiae* from Taurus Energy AB of Sweden that can produce ethanol from hexoses and xylose (Zimmerman, 2017). Of these plants, several of them have been shut down including those of DuPont and Abengoa (Hirtzer & Renshaw, 2017), showing that some of these technologies currently in use are unsustainable.

1.7 Conclusion

In this chapter, I presented an overview of the galactose metabolism and regulation, as well as engineering strategies for xylose and arabinose utilization. The review on galactose metabolism

reveals Gal4p to be the primary driver of galactose metabolism that activates the initial pathway genes, as well as other regulatory genes. However, Gal4p activation is dependent on Gal80p, a transcriptional repressor, and Gal3p, a transcriptional activator cum ligand sensor. The dynamics of the galactose regulon, though well-characterized still has certain missing links and new unforeseen mechanisms are still being discovered. One part of the missing puzzle with respect to galactose regulation is the set of genes that are controlled by the regulon. Although initial ChIP-based analysis narrowed down the number of genes under the GAL regulon (Ren et al., 2000), recent studies seem to question this finding. The GAL regulon has been shown to be influenced and regulated by signaling genes such as *RAS2*, amino acid pathway genes etc., suggesting that previously unknown interactions of the GAL regulon play a role in sugar metabolism (K.-K. Hong et al., 2011; K. K. Hong & Nielsen, 2013; Ideker et al., 2001; Reimand et al., 2010). Several of the genes identified are not connected to central carbon metabolism or Leloir pathway. But the mechanism involved, and the total number of genes activated by the regulon, either directly, or indirectly remains to be elucidated.

In the case of non-native sugars, this chapter focused mainly on the metabolism of xylose and to a certain extent on arabinose. The engineering interventions introduced to increase growth rates, sugar consumption and ethanol production in *S. cerevisiae* have also been discussed. Of the two pathways used for xylose and arabinose metabolism, the isomerase pathway seems to be more attractive as it obviates the need for cofactors and is a shorter pathway. While initial engineering attempts identified flux limitations in xylose isomerase and PPP shunt, further directed evolution and metabolic engineering have iteratively removed rate limitations in several of these metabolic reactions. Adaptive evolution studies on extensively engineered strains, have been identifying mutations in proteins that are involved in regulation, signaling, etc., insinuating the need to look

at limitations beyond cellular metabolism (Dos Santos et al., 2016; HamediRad et al., 2018; Nijland et al., 2017; Sato et al., 2016).

In yeast, depending on the sugar, the transcriptional response has been shown to differ drastically (Syriopoulos, Panayotarou, Lai, & Klapa, 2008), which is a result of evolutionary adaptation over the years. When yeast is grown in glucose, which is an abundant substrate, the cellular objective is fast consumption of the sugar. This results in Crabtree effect where even under aerobic conditions, the glycolytic flux increases by more than 10-fold resulting in ethanol production (respiro-fermentative growth). Yeast can tolerate high concentrations of ethanol giving it an advantage over other organisms in its surroundings. Finally, once glucose is consumed, the cell shifts to a strict respiratory mode to consume ethanol and produce biomass. However, when grown on galactose, which is present only in minute quantities, the cell upregulates the genes necessary for metabolism using multiple feedback loops. Further, the cellular objective is optimized for growth, while preventing the metabolic burden of expressing the required metabolic enzymes (Malakar & Venkatesh, 2014). Since the end objective for *S. cerevisiae* when grown in both of these sugars is very different, we can expect different transcriptional responses, as had been observed previously (Syriopoulos et al., 2008). This is clearly demonstrated by the work of Hong et al., whereby evolving yeast for increased growth on galactose, that resulted in a *RAS2* mutant, growth on glucose was compromised. Thus, the cell seems to act with a defined set of constraints for growth and metabolism. Disrupting or modifying one of the constraints automatically results in a trade-off. Hence, it is not surprising that once metabolic limitations are removed on xylose metabolism, the inherent regulatory and transcriptional limitations surfaced. Moreover, although recent studies have tried to assess the signal response elucidated by intracellular xylose, there is no conclusive evidence for any interaction that xylose exhibits. Since xylose is not a natively

metabolizable sugar, it is possible that the required substrate signaling pathways are not triggered leading to carbon, and amino acid starvation signals (Matsushika et al., 2013; Osiro et al., 2018; Salusjärvi et al., 2008, 2006). Alternatively, xylose could also result in partial de-repression of certain pathways, as observed previously (Salusjärvi et al., 2006). Any, or all, of these phenomena, might explain why growth rates and pentose consumption is poor until adaptive evolution is carried out. It is possible that signaling pathways are re-oriented during adaptive evolution leading to better performance of the evolved strains. For example, multiple strains of yeast engineered through adaptive evolution to grow better on xylose, upregulate regulatory and metabolic pathways for galactose (Y. Jin et al., 2004; Scalcinati et al., 2012; M Sonderegger et al., 2004; Wahlbom et al., 2003). This could easily occur as a result of mutations in Gal80p, Gal4p or Gal3p leading to activation of the regulon. Since the galactose regulon gets activated, substrate signaling pathways would rewire for galactose leading to increased growth rates. Taken together, these data suggest that certain uncharacterized downstream genes of the GAL regulon, could assist growth on galactose and maybe even in non-native sugars, xylose and arabinose. In the next chapter, these questions will be addressed through studies on galactose metabolism, combined with protein engineering, metabolic engineering, and transcriptome analysis.

Chapter 2

A semi-synthetic GAL-type xylose regulon enables rapid growth of yeast on xylose[1]

2.1 Abstract

Substrate assimilation is a key first step that allows biological systems to proliferate and produce value-added products. Yet, implementation of heterologous catabolic pathways has so far relied on constitutive gene expression without consideration for global regulatory systems that may enhance substrate assimilation and cell growth. In contrast, natural systems generally prefer substrate-responsive gene regulation (called regulons) that controls multiple cellular functions necessary for cell survival and growth. In *Saccharomyces cerevisiae*, by partially- and fully-uncoupling galactose (GAL)-responsive regulation and metabolism, we demonstrate the significant growth benefits conferred by the GAL regulon. Next, by adapting the various aspects of the GAL regulon, such as sensing, signal transduction, and metabolism for a non-native substrate, xylose, we built a semi-synthetic XYL regulon that exhibits higher growth rate, better substrate consumption, and improved growth fitness compared to the traditional and ubiquitous constitutive expression strategy. This work provides a new, elegant paradigm to integrate non-native substrate catabolism with native, global cellular responses to support fast growth.

[1] Endalur Gopinarayanan, V., & Nair, N. U. (2018). A semi-synthetic regulon enables rapid growth of yeast on xylose. *Nature Communications*, *9*(2018), 1233.

2.2 Introduction

Efforts in synthetic biology and metabolic engineering have largely focused on rationally-designing regulatory infrastructures around biosynthetic/anabolic pathways. In recent years, dynamic pathway regulation has yielded significant improvements in product titers using either native (Dahl et al., 2013; F. Zhang, Carothers, & Keasling, 2012) or heterologous transcription factors (Skjoedt et al., 2016; Teo & Chang, 2014; M. Wang et al., 2015). Conversely, rationally-designed regulatory controls for efficient and complete utilization of exogenously available substrates in synthetic biological systems are underdeveloped. Current efforts to engineer substrate assimilation pathways take a straightforward approach of over expressing catabolic pathway enzymes without regard for how that integrates into the larger cellular infrastructure that encompasses central metabolism, stress-responses, cell doubling, etc. Examples include engineering pentose catabolism in *S. cerevisiae* (Bettiga et al., 2009; S.-M. Lee et al., 2012a, 2014; Moes et al., 1996; Scalcinati et al., 2012; Sedlak & Ho, 2001; Walfridsson, Bao, Anderlund, Lilius, et al., 1996; H. Zhou et al., 2012), C1 (*viz.* CO_2 or methanol) feedstock usage in *E. coli* (Müller et al., 2015; William B. Whitaker, Sandoval, Bennett, Fast, & Papoutsakis, 2015), or even amorphous cellulose utilization by various yeasts and bacteria (den Haan, van Rensburg, Rose, Görgens, & van Zyl, 2015; Fan, Zhang, Yu, Xue, & Tan, 2012; Yamada, Hasunuma, & Kondo, 2013). In stark contrast, natural systems often use genome-scale regulatory infrastructures, called regulons, to coordinate substrate catabolism with other cellular functions (Caldara, Charlier, & Cunin, 2006; Gross, Kelleher, Iyer, Brown, & Winge, 2000; Jayadeva Bhat, 2008; Jenkins, Barton, Robertson, & Williams, 2013; Lyons et al., 2000; Ravcheev, Gelfand, Mironov, & Rakhmaninova, 2002; Santos-Beneit, 2015; Weickert & Adhya, 1993). Such systems include sensors to detect a specific nutrient or substrate (input), signal transduction and integration (computation), and global genetic

regulation (output). One of the best studied native regulons is the galactose (GAL) system in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), where Gal3p-mediated sugar detection initiates a genome-wide response effected by Gal4p and Gal4p-responsive transcription factors (TFs) (Jayadeva Bhat, 2008).

In this study, we first assess the role of the GAL regulon in enabling efficient galactose utilization for cell growth by decoupling its regulatory responses from sugar catabolism. We provide evidence that regulon-controlled galactose assimilation is better than constitutive expression of the catabolic genes in supporting fast growth rates to higher cell densities. Next, we assessed whether a regulon could enable more complete and efficient utilization of a substrate that is non-native to this yeast – xylose. We first adapted the GAL regulon to respond to xylose through directed evolution of Gal3p, coupling nutrient stimulus with sensing, computation, and regulatory actuation. Next, by using a rational, model-guided approach, we test two different positive feedback signal transduction loop designs for the regulon and demonstrate their individual merits and weaknesses. We also show that implementation of a GAL-type xylose-responsive regulon can regulate multiple genes across the yeast genome and enable more homogeneous population-wide gene expression. By integrating a minimal set of heterologous catabolic genes into the synthetic regulon we demonstrate high cellular growth rates and high final cell densities on xylose as well as better growth in non-inducing carbon sources. Finally, we compare the genome-wide expression profiles of strains grown with regulon assistance and conventionally engineered strains to identify mechanistic reasons that account for the different phenotypes observed. We posit that this study strongly supports the need to re-evaluate how substrate assimilation systems are currently implemented and introduces a new and unexplored paradigm of adapting a native regulon for efficient non-native sugar assimilation.

2.3 Results

2.3.1 Downstream GAL regulon genes support fast growth (Book no. 7, page no. 32, -38, 41-42)

The GAL regulon exerts control over the initial galactose metabolic genes (Leloir pathway) as well as several downstream genes not directly involved in assimilating galactose (Hu, Killion, & Iyer, 2007; Ideker et al., 2001; Reimand et al., 2010; Ren et al., 2000). While the roles of initial galactose catabolic and regulatory genes have been established, the control exerted by the regulon on downstream genes and their phenotypic effects are not well elucidated. We decided to assess the role played by downstream genes of the GAL regulon in growth on galactose by fully- or partially-uncoupling their communication. To decouple growth on galactose from the regulon's function, we knocked out *GAL4*, the gene that encodes for the master GAL regulon transcription factor and placed the initial galactose metabolic genes (*GAL1*, *GAL7*, and *GAL10*) under the control of strong constitutive promoters, *TEF1p*, *GPM1p*, and *TPI1p*, respectively, to create a constitutive galactose metabolic gene expression strain, GAL-CONS **(Fig. 2.1A)**. We compared the growth rates and final cell densities of GAL-CONS with GAL-REG (wild-type) and observed that the former had a > 5-fold lower growth rate and took three-times longer to reach stationary phase **(Fig. 2.1B)**. The decrease in growth rate can either be attributed to inability of the strain GAL-CONS to activate the downstream genes of GAL regulon that are required for growth, or the difference in promoter strengths between the GAL and constitutive promoters that transcribe the Leloir pathway genes, or both. To determine true cause of growth rate decrease, we re-introduced Gal4p in GAL-CONS but deleted genomic Leloir pathway genes (and placed the genes under constitutive expression) as well as *GAL3* and *GRE3* (which encodes for non-specific aldose reductase) to create the strain GAL-

CONS-*GAL4* **(Fig. 2.1A)**. This re-factored, partially-coupled, system should enable activation of downstream genes through Gal1p-Gal80p-Gal4p pathway (Abramczyk et al., 2012), but keeps the Leloir pathway genes out of the GAL regulon control. Thus, if the downstream genes of the GAL regulon assist in growth, the partially coupled strain should have growth rates higher than the GAL-CONS strain. On the other hand, if the observed decrease is a result of the difference in promoter strengths between native GAL promoters and constitutive promoters, the GAL-CONS-*GAL4* strain should have the same growth rate as that of the GAL-CONS strain. We tested the GAL-CONS-*GAL4* strain for growth on galactose and observed that the strain recovers a significant portion of its growth fitness relative to GAL-CONS **(Fig. 2.1B)** suggesting that the downstream genes under the control of the regulon *trans*-activated by Gal4p positively affect the ability of yeast to grow on galactose. It should be noted that constitutive promoters (expressed on multicopy plasmids) have higher expression strength than GAL promoters **(Appendix Fig. 2.1 & Appendix Note 2.1).** Moreover, comparing growth of wild-type and GAL-CONS-*GAL4* strains **(Fig. 2.1B)** suggests that the difference in promoter strengths do not play a major role in dictating the growth rate of the strains tested. Here, the benefits of using GAL promoters are demonstrated by the slightly higher growth rate. However, most of growth benefit is gained by activation of downstream (non-Leloir) genes. Next, we wanted to test whether this observation can be extended to alternative substrates, particularly to those that are non-native to yeast.

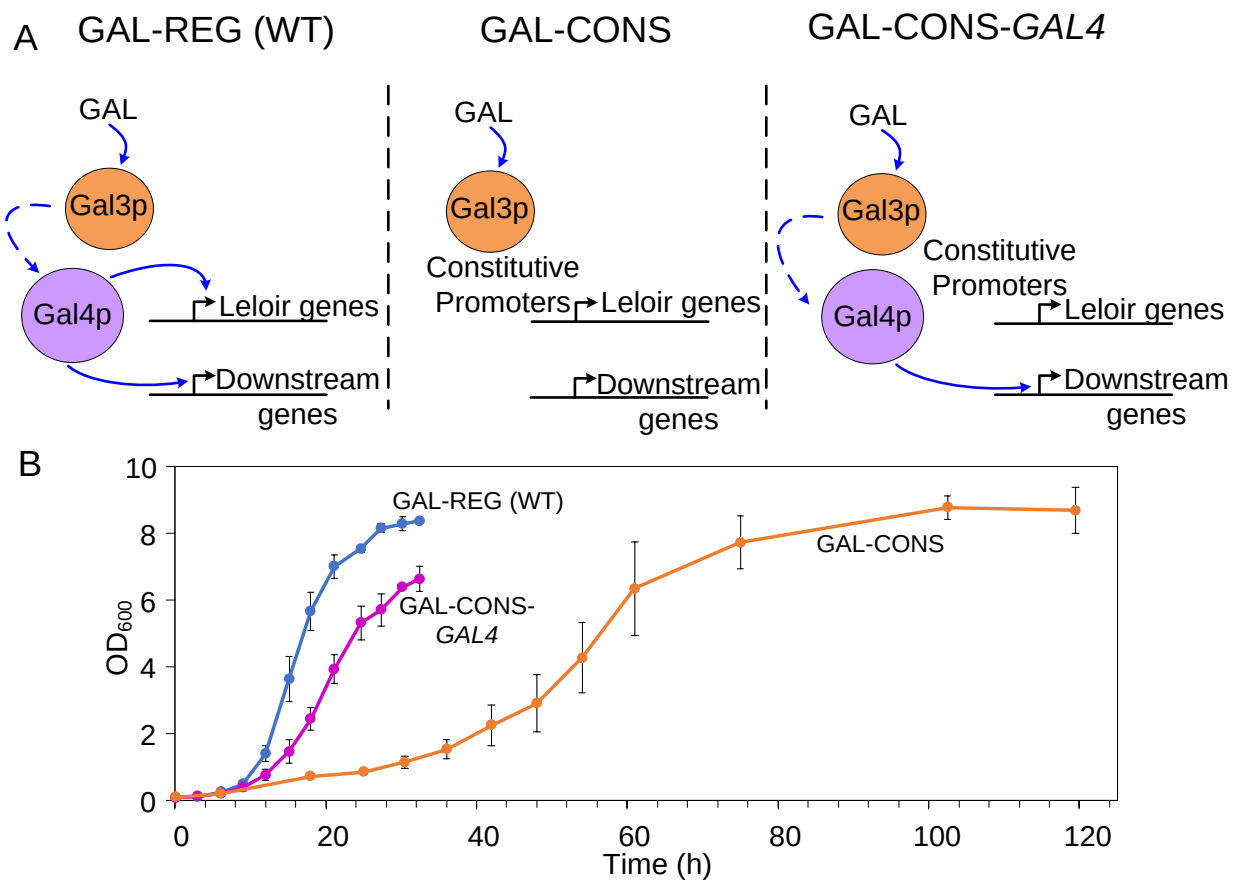


Figure 2.1: Gal4p-mediated activation of genes other than the Leloir pathway enzymes significantly contribute to the ability of yeast to grow well on galactose. *(A) Schematic of regulon design used to test effects of regulon on growth in galactose, where the left panel denotes the wild-type with intact regulon with Leloir pathway genes and downstream genes controlled by Gal4p. Middle panel represents the GAL-CONS mutant with Gal4p knocked out and Leloir pathway genes under strong constitutive promoters. Right panel denotes the GAL-CONS-GAL4 mutant with downstream genes under Gal4p, but Leloir pathway genes under constitutive promoters. (B) Growth of the three strains in galactose. Each of the data points denotes the average of three individual replicates, error bars are ±sd.*

2.3.2 Design of a synthetic GAL-type xylose regulon

So far, implementation of heterologous sugar assimilation systems in yeast and bacteria have relied on constitutive overexpression of metabolic genes (Bettiga et al., 2009; Peter Kotter, Amore,

Hollenberg, & Ciriacy, 1990; Müller et al., 2015; Sedlak & Ho, 2001; Walfridsson, Bao, Anderlund, Lilius, et al., 1996; William B. Whitaker et al., 2015; M. Zhang & et al., 1995) rather than regulon-controlled sugar assimilation. This is not only because there is no evidence to demonstrate the advantages of regulon-assisted growth, but also due to lack of tools and guidelines available to build a synthetic regulatory network that can coordinate substrate detection with metabolism and cell growth. Hence, rather than building a synthetic regulatory network from the bottom-up where each of the genes to be activated are chosen rationally to build an elaborate regulatory and metabolic network, we decided on a more efficient approach by reverse-engineering the GAL regulon into a xylose-responsive regulon. We hypothesized that since many of the downstream genes required for growth are relatively conserved irrespective of carbon source (Ideker et al., 2001; Reimand et al., 2010), most of the genes activated by the GAL regulon would also be beneficial for growth on xylose. To realize this regulatory system, we decided to engineer the three different components of the regulon – substrate detection, signal transduction, and substrate metabolism **(Figure 2.2)**. First, the galactose sensor Gal3p must be adapted to detect xylose and bind Gal80p to activate the regulon. Second, the signal transduction loop present in the galactose regulon must be re-designed for the xylose regulon, and finally, the genes required for xylose metabolism must be placed under the control of the regulon.

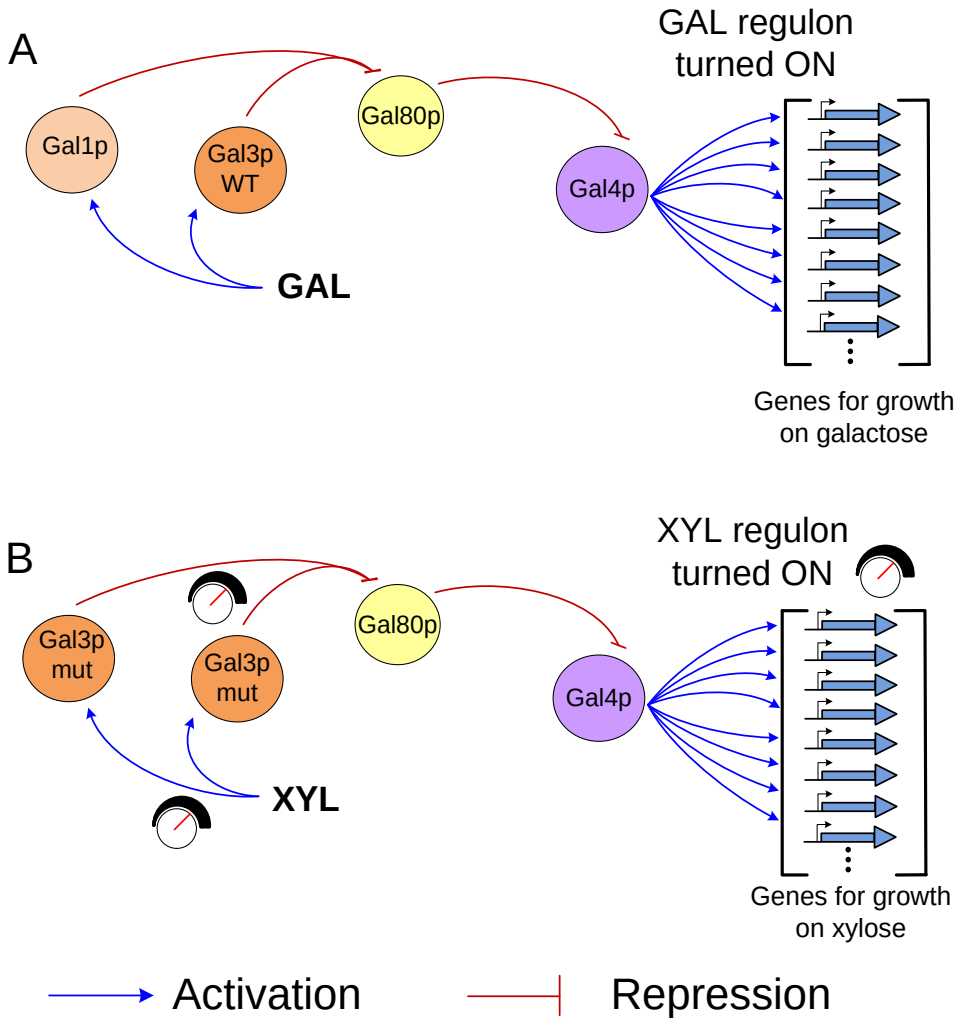


Figure 2.2: Design of a synthetic GAL-type xylose regulon. *(A) Schematic of galactose-based activation of the GAL regulon, where galactose-bound Gal3p relieves repression of Gal4p by binding with Gal80p, thereby turning ON the regulon, including the genes required for growth on galactose. Gal1p, one of the GAL regulon genes also interacts with Gal80p resulting in a dual positive feedback loop. (B) Engineering of xylose regulon. First stage of engineering involves protein engineering of Gal3p, such that Gal3p-xylose interaction relieves repression of Gal4p. Second stage involves capturing the dual positive feedback loop created by Gal3p and Gal1p by using a single gene under different promoters. Final stage involves, integrating genes required for growth on xylose under GAL activated promoters to create a xylose sensing and metabolizing synthetic regulon. Every stage of the regulation where interventions are necessary is marked with a symbol for tuning valve.*

2.3.3 Engineering Gal3p for improved response to xylose

2.3.3.1 Selection and screening system for Gal3p mutagenesis (Book no.2, Book no.3, Book no.5, page no. 1-23, 56-64)

To identify mutations within Gal3p that enhance its responsiveness to xylose, we developed a reporter strain and a robust selection and screening system. We deleted the genes of galactose metabolism (*GAL1*, *GAL7*, and *GAL10*) so that galactose acts only as an inducer of GAL regulon and like xylose, is not metabolized. We also knocked out *GAL3*, to be expressed through a plasmid for mutagenesis and *GRE3* (non-specific aldose reductase) to prevent reduction of sugars to polyols, to create the reporter strain, VEG16. Thus, the reporter strain lacks both the sensory proteins as well as metabolic enzymes required for galactose metabolism. Next, we developed a selection and screen based on G418 antibiotic resistance and Enhanced Green Fluorescent Protein (EGFP), respectively, by placing the two marker genes *KANMX* and *EGFP* under bidirectional *GAL1p* and *GAL10p* promoters. We placed *GAL3* under its own native promoter, *GAL3p,* along with the selection and screening construct into a multi-copy plasmid (pVEG8). Thus, any Gal3p-sugar interaction would activate the GAL regulon, resulting in expression of the *KANMX* gene for high throughput antibiotic selection and *EGFP* for quantitative fluorescence screening **(Fig. 2.3A)**.

2.3.3.2 Engineering xylose-responsive Gal3p to switch on the GAL regulon (Book no.4, page no. 25-55, 65-87,91-116)

Our initial fluorescence screening assays with Gal3p-WT showed a typical dose-response sigmoidal curve with galactose, as expected. When tested with xylose, we observed a linear increase in fluorescence at xylose concentrations above 2 % **(Fig. 2.3B)**. When compared to its native substrate galactose, the fluorescence exhibited in the presence of xylose was several-fold

lower and observable only at high sugar concentrations where transport is not expected to be an issue (Gárdonyi et al., 2003; Peter Kotter & Ciriacy, 1993). But the presence of fluorescence at high concentrations also indicates weak Gal3p-xylose interaction, suggesting that Gal3p active site is sufficiently flexible to accommodate xylose. Upon binding to its ligand, Gal3p undergoes a conformational change that enables it to dimerize with, and sequester, Gal80p and activate the GAL regulon. Since Gal3p-Gal80p interaction is the key step involved in activation of the GAL regulatory system, we decided to strengthen its interaction by mutagenizing Gal3p (Yano & Fukasawa, 1997). Analysis of the Gal3p-Gal80p (Lavy et al., 2012) co-crystal structure revealed two loops on Gal3p that interacts with Gal80p **(Appendix Fig. 2.2A)**. Residues from 93-115 (referred as loop 1) form a dynamic loop that remains closed when Gal3p is in *apo*-form but opens up during Gal3p-galactose interaction. A second stationary loop (loop 2) from 345-381 also interacts with Gal80p. We carried out random mutagenesis using error-prone PCR (epPCR) on the two loops, to obtain a mutant library of 10^4 variants with loops 1 and 2 having an average of 1.2 ± 0.8 and 1.1 ± 0.5 amino acid mutations respectively. We transformed the VEG16 strain with Gal3p loop mutant library and carried out selection and screening with 2 % xylose to obtain a mutant, Gal3p-1.1, which had a significantly higher fluorescence on xylose than Gal3p-WT **(Appendix Fig. 2.2B)**. Next, we characterized the fluorescence profile of the mutant by varying the concentration of xylose from 4 % to 0.002 % and observed more than 7-fold increase in fluorescence above 2 % xylose concentration **(Fig. 2.3C)**. Fluorescence dropped down significantly at xylose concentrations below 0.2 % and was indistinguishable from the control, confirming that the mutation did not result in constitutive activation of the GAL regulon. We sequenced the mutant and found it to be an A109T mutation. Analysis of the Gal3p crystal structure shows A109 residue present on the dynamic loop of Gal3p facing Gal80p **(Appendix Fig. 2A).**

When Gal3p-1.1 was titrated with different concentrations of galactose, we observed a 100-fold increase in sensitivity to galactose as well **(Fig 2.3D)**. Taken together, results indicate that the mutation seems to increase Gal3p-Gal80p interaction efficiency resulting in increased sensitivity and fluorescence upon induction with xylose and galactose.

To explore other residues at position 109 that could improve fluorescence, we carried out single site saturation mutagenesis with NNK codons to obtain a diversity of 32 codons and screened 3,000 variants (~100-fold coverage). The fluorescence profile of the best mutant, Gal3p-2.1 (A109V mutation), indicated a marked increase in signal strength and sensitivity at 2 % and 0.2 % xylose. In fact, there was an almost twelve-fold change in fluorescence at 2 % xylose (**Fig 2.3C**). Simultaneously, we also explored mutations that could further increase the GAL regulon induction strength through epPCR based random mutagenesis on the entire protein with Gal3p-1.1 as the template. From a library of $10^{\wedge 5}$ variants with an amino acid mutation rate of 1.9 ± 0.8, through selection and screening, we obtained three variants with better fluorescence profiles **(Appendix Fig 2.2B)**. All three variants showed increased signal strengths. Gal3p-3.1 and Gal3p-3.3 exhibited ten-fold increase in signal at 2 % xylose. Gal3p-3.2 had much lower fold induction values due to higher background fluorescence **(Fig 2.3C)**. Although the fold change varied between the mutants, the fluorescence at 2 % xylose concentration were relatively similar and comparable to Gal3p-2.1 mutant. However, the fold increase in fluorescence at 0.2 % xylose was less than five-fold in these mutants. Previous studies on xylose metabolism in *S. cerevisiae* have suggested transport as a rate limiting step at low xylose concentrations (Gárdonyi et al., 2003). To alleviate any possible xylose transport limitations, we knocked-in an engineered xylose transporter *GAL2-2.1* (O. Reznicek et al., 2015; Ondrej Reznicek, Sandra, Kassandra, Bernhad, & De Waal, 2014) at the *LEU* locus of VEG16 to create VEG20, which was used for the next round of mutagenesis.

Sequencing of the Gal3p-3.1, Gal3p-3.2, and Gal3p-3.3 mutants revealed that some of the mutations introduced during random mutagenesis arose close to one another **(Table 2.1)**. We employed a primer-based synthetic shuffling strategy (Ness et al., 2002) to combine mutations using multiple primers containing degenerate codons that coded for wild-type or the mutated nucleotides. We amplified and spliced together six fragments with *GAL3-2.1* as the template to cover the eight mutations from variants 3.1, 3.2, and 3.3 and transformed VEG20 with the library for functional selection. Since the motivation to use synthetic shuffling was to obtain mutants with better sensitivity, we carried out selection and screening at 0.2 % xylose. The best mutant, Gal3p-Syn4.1 showed a fluorescence profile with lower background, higher sensitivity, and a ten-fold increase in fluorescence at 0.2 % xylose **(Fig 2.3C)**. Comparing fold changes at 2 % xylose of Gal3p-Syn4.1 with fluorescence of Gal3p-WT at 2 % galactose, we observed that the mutant exhibits a similar fold-change, suggesting that Gal3p-Syn4.1 has sufficient transcriptional activation strength.

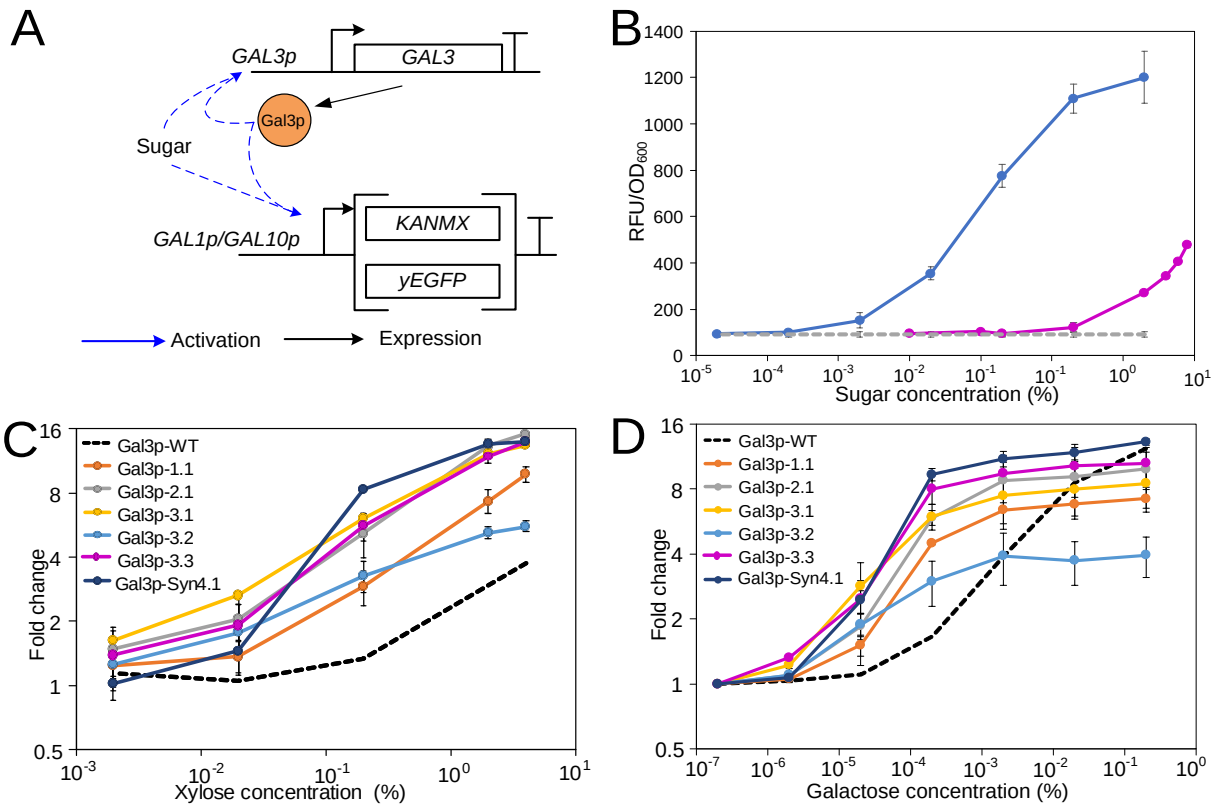


Figure 2.3: Engineering a Gal3p to respond to xylose: *(A) Design of the screening and selection system. Gal3p expressed under its native promoter, in the presence of activation sugar (galactose/xylose), would activate the GAL regulon, and switch ON GAL1p and GAL10p that drive expression of genes KANMX and EGFP, resulting in G418 sulfate resistance (antibiotic selection) and fluorescence (fluorescence screening). (B) Gal3p-WT interaction with galactose and xylose measured using the fluorescence assay. VEG16 transformed with selection and screening construct was grown in sucrose with varying concentrations of galactose (2 % - 2 ×10^{-6} %) or xylose (8% - 2 ×10^{-3} %). (C) Fold change in fluorescence of the best Gal3p mutants when incubated with varying concentrations of xylose. (D) Fold change in fluorescence of the best Gal3p mutants when incubated with varying concentrations of galactose. Each data point represents average of three individual replicates ± sd.*

2.3.3.3 Gal3p mutants still retain their galactose binding ability (Book no.3, 65-116)

We also checked the fluorescence profile of the best mutants from every step, in the presence of galactose. While Gal3p-1.1 had lower fluorescence and fold change compared to Gal3p-WT at 0.2 % galactose, we observed a 100-fold increase in sensitivity and reached saturation levels of fluorescence at 0.002 % concentrations of galactose. The mutants Gal3p-2.1, Gal3p-3.1, and Gal3p-3.3 showed increased fluorescence and sensitivity to galactose when compared to the wild-type, but had a lower fold change at 0.2 % galactose, due to increased background fluorescence. The final mutant, Gal3p-Syn4.1 also exhibited a similar profile, but with a marginal increase in fold-change when compared to Gal3p-WT **(Appendix Fig 2.2B)**. Thus, by targeting mutations at the protein-protein interaction sites and carrying out random mutagenesis on the entire protein, we could obtain a multi-sugar sensor with more than 15-fold induction in the presence of xylose **(Fig 2.3C),** while still retaining its native galactose binding function **(Fig 2.3D)**.

Table 2.1: List of mutations on the Gal3p variants during each round of mutagenesis.

Mutant name	Mutations	Round
Gal3p-1.1	A109T	Interaction loop mutagenesis
Gal3p-2.1	A109V	Site Saturation Mutagenesis
Gal3p-3.1	K22R, D68N, I77T, C123G, N141S, L394I, A109T	Error prone PCR
Gal3p-3.2	I271L, L394I, A109T	Error prone PCR
Gal3p-3.3	V69M, L394I, A109T	Error prone PCR
Gal3p-Syn4.1	D68N, V69M, I271L, A109V	Synthetic shuffling

2.3.4 Model-assisted tuning of XYL regulon activation and control (Book no. 7, page no. 15-28,34-47, MATLAB file "Xyl_regulon_run.m")

The GAL regulon has been described mathematically as a bi-stable system with negative- and positive-feedback loops (Venturelli et al., 2012). The negative feedback is mediated by the repressive function of Gal80p on Gal4p, whereas the positive feedbacks are mediated through

Gal3p and Gal1p-based de-repression of Gal4p (Abramczyk et al., 2012; Thoden et al., 2005). Further, the high-basal, weak, *GAL3p* promoter-driven *GAL3* expression along with a low-basal, strong, *GAL1p* promoter-driven *GAL1* expression creates a dual positive feedback loop, which has been shown to increase sensitivity and decrease noise (Abramczyk et al., 2012; Hsu et al., 2012; Venturelli et al., 2012). Such a dual positive feedback loop enables rapid, strong, and homogeneous expression during induction and low basal expression in the absence of inducer. To recapitulate this, we decided to build a dual positive feedback loop. However, since Gal1p is a galactokinase with affinity towards galactose rather than xylose, we could not use Gal1p to create the dual positive feedback loop. Instead, we hypothesized that placing *GAL3-Syn4.1* under both, *GAL3p* and *GAL1p*, promoters should be sufficient to create a dual positive feedback loop. To test this, we used the ODE model from Venturelli et al., that captures the interplay between Gal3p, Gal80p, Gal4p, and Gal1p (Venturelli et al., 2012) and modified it by modeling GFP expression under *GAL1/10p* promoter so that it can be compared with our experimental results **(Appendix Note 3)**. We assessed the cooperativity of Gal4p binding on *GAL10p, GAL3p* and *GAL80p* promoters used in the model expreimentally by expressing EGFP under the three promoters. We measured fluorescence output at varying concentrations of xylose, which was then used to fit a Hill curve to determine cooperativity **(Appendix Fig 2.3)**.

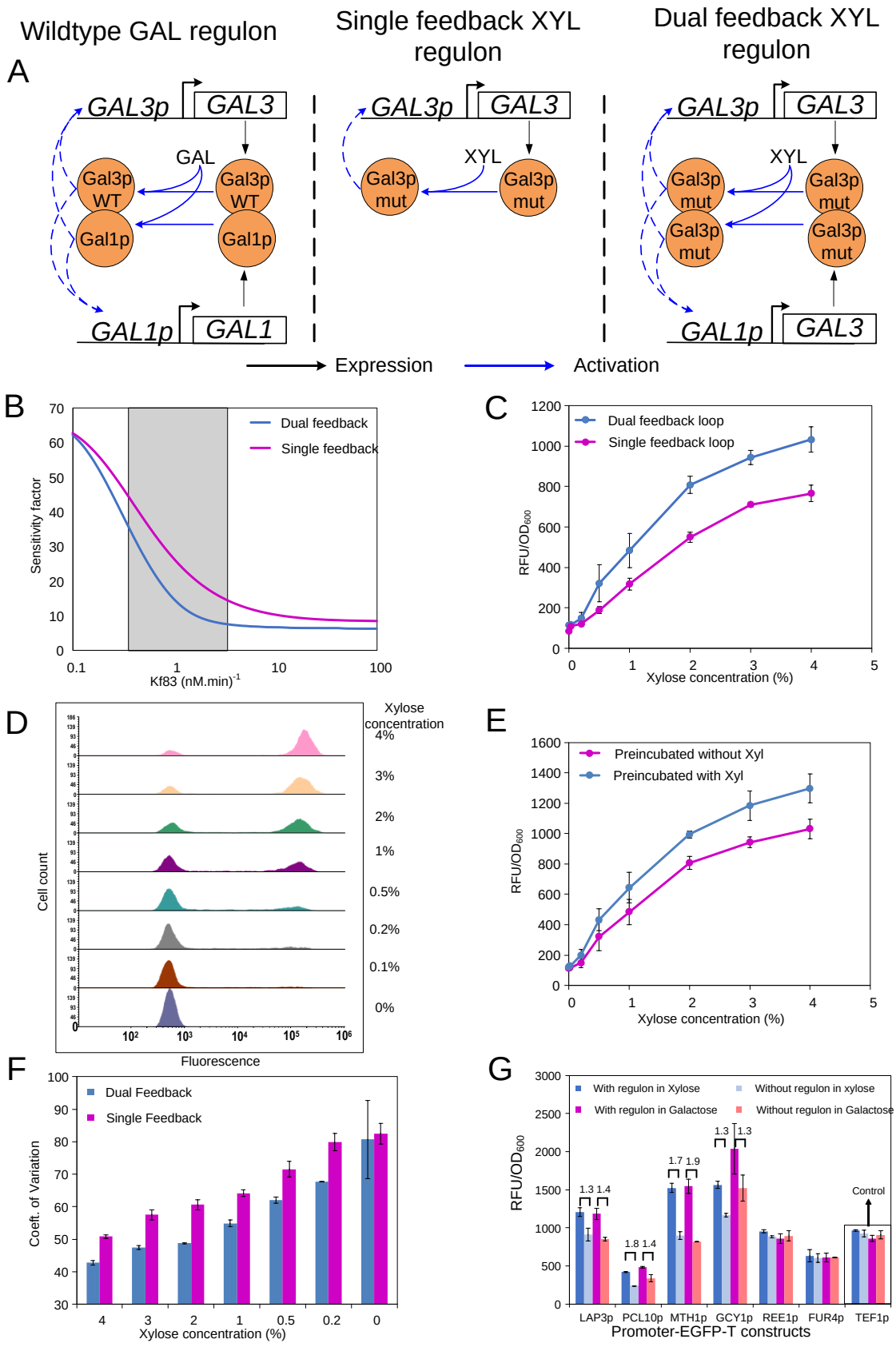

A
Wildtype GAL regulon
Single feedback XYL regulon
Dual feedback XYL regulon
GAL3p
GAL3
GAL
XYL
Gal3p WT
Gal3p mut
Gal1p
GAL1p
GAL1
Expression
Activation
B
Dual feedback
Single feedback
Sensitivity factor
Kf83 (nM.min)-1
C
Dual feedback loop
Single feedback loop
RFU/OD600
Xylose concentration (%)
D
Xylose concentration
4%
3%
2%
1%
0.5%
0.2%
0.1%
0%
Cell count
Fluorescence
E
Preincubated without Xyl
Preincubated with Xyl
RFU/OD600
Xylose concentration (%)
F
Dual Feedback
Single Feedback
Coeff. of Variation
Xylose concentration (%)
G
With regulon in Xylose
Without regulon in xylose
With regulon in Galactose
Without regulon in Galactose
RFU/OD600
Control
LAP3p
PCL10p
MTH1p
GCY1p
REE1p
FUR4p
TEF1p
Promoter-EGFP-T constructs

Figure 2.4: Tuning of xylose regulon activation. *(A) Schematic of dual positive feedback loop in the galactose regulon (left panel), single (middle panel), and dual positive feedback loop (right panel) in the xylose regulon. While Gal3p and Gal1p act in concert to create the dual feedback in the galactose regulon, Gal3p-Syn4.1 driven by two different promoters is used to create the dual positive feedback loop in the xylose regulon. (B) Model simulation of Sensitivity factor (concentration of inducer required to attain half of the maximum fluorescence) versus forward binding rate constant of Gal3p-Gal80p (kf83). The shaded region represents the range of kf83 where the difference between the two feedback models is prominent. (C) Fluorescence under GAL10p promoter in single and dual feedback systems at different concentrations of xylose (4 %, 3 %, 2 %, 1 %, 0.5 %, 0.2 %, 0.1 %, 0 %). (D) Flow cytometry histograms of cell population from dual feedback system that are either ON or OFF at different xylose concentrations, showing bimodal distribution of cells when induced with xylose. (E) Fluorescence of EGFP gene under GAL10p promoter in dual feedback system at different concentration of xylose either preincubated in 4 % xylose or 0 % xylose, showing hysteresis. (F) Coefficient of variation obtained from flow cytometry measurements of single and dual feedback system as a measure of cellular homogeneity when induced with different concentrations of xylose. (G) Promoters that drive expression the downstream genes of the galactose regulon were used to express EGFP. Fluorescence was measured for cells grown on ethanol/glycerol in the presence or absence of xylose and galactose regulon. The fold change obtained in xylose and galactose regulon is shown on top of the bars for comparison. Each data point represents average of three individual replicates ± sd.*

By varying the rate of galactose input for the two feedback models, we show that the sensitivity factor (concentration of inducer required to attain half the maximum fluorescence) is lower for the dual feedback than for the single feedback system **(Appendix Fig 2.4A)**. We carried out the experiment and observed that the trend of the simulation matched with experimental data **(Appendix Fig. 4A & Appendix Fig. 4B)**. While it has been known that the dual feedback system created by Gal3p and Gal1p increases sensitivity when compared to single feedback system

without Gal1p, we show that simply by having *GAL3* under *GAL3p* and *GAL1p* promoters, similar sensitivity can be achieved.

In the case of xylose regulon, Gal3p-Syn4.1-xylose-Gal80p interaction is much weaker than Gal3p-WT-galactose-Gal80p interactions **(Fig 2.3C & 2.3D)**. To take that into consideration, we varied the forward binding constant (kf83) of Gal3p binding to Ga80p in the presence of inducer (galactose/xylose) over five orders of magnitude from 0.1 – 10000 $nM^{-1}min^{-1}$ and tracked the sensitivity factor. We show that at very low kf83 values, single and dual feedback systems display similar sensitivity factor, probably due to poor association of Gal3p-Gal80p **(Appendix Fig 2.4C & Fig 2.4B)**. As we increased kf83, at intermediate strengths of Gal3p-Gal80p binding, sensitivity factor is significantly lowered for the dual feedback loop **(Appendix Fig 2.4E & Fig 2.4B)**. Finally, when Gal3p-Gal80p interaction is strong, the sensitivity factor saturates in both the feedback models with the dual feedback having higher sensitivity than the single feedback system **(Appendix Fig 2.4A & Fig 2.4B)**. Thus, the model predicts that dual feedback loop is more effective when Gal3p has relatively weaker interactions with the inducer, as is the case with xylose regulon. To test the model prediction that increased sensitivity could be observed under xylose induction, we placed *GAL10p* upstream of *EGFP* and compared fluorescence for strains with single and dual positive feedback. We incubated them with different concentrations of xylose or galactose with either $Gal3p^{WT}$ or $Gal3p^{mut}$ **(Appendix Fig 2.4B, Appendix Fig 2.4D & Appendix Fig 2.4F)** and observed that simulation and experiments have similar trends **(Fig 2.4C & Appendix Fig 2.4)**. In **Fig 2.4C,** we can see that the dual positive feedback loop shows increased fluorescence than a single feedback loop. We observed a similar increase in sensitivity with other GAL promoters – *GAL1p* and *GAL7p* **(Appendix Fig 2.5)**.

The GAL regulon has been known to exhibit bimodality (results in heterogeneous population in suboptimal environment, thus increasing fitness) and hysteresis (a history dependent response to galactose), which are characteristic features of a stochastic bistable system (Venturelli et al., 2012). To test if the xylose activated regulon still retains bistability observed in the parent regulon, we decided to test bimodality and hysteresis in the XYL regulon. To demonstrate bimodality, we integrated *GAL10p-EGFP-T* cassette into the chromosome and compared fluorescence of the two feedback systems at a cellular level under different concentrations of xylose using flow cytometry. Over the concentration range tested, we observed distinct populations of cells that were either turned ON or OFF confirming that the xylose regulon still retains bimodality **(Fig 2.4D)**. Next, we pre-incubated the yeast strain carrying dual feedback system in media with and without the inducer (xylose) for 24 hours, and later shifted the cells to media with varying concentrations of xylose. We observed a pre-incubation-dependent response (hysteresis) at all concentrations of xylose tested. The cells that were pre-incubated with xylose showed a higher fluorescence than strains that were not incubated with xylose **(Fig 2.4E)**. Together, these data show that the GAL-type xylose regulon retains bistability observed in the galactose regulon (Venturelli et al., 2012). In 2005, Brandman et al. hypothesized using mathematical simulations that interlinked dual positive feedback loops with fast and slow feedback responses, results in a faster response as well as a more stable signal output with low noise when compared to using either of the feedback loops in isolation. The fast response was attributed to the fast feedback loop, and the low noise with a stable response was attributed to the slow feedback loop. Since *GAL3p* and *GAL1p* promoters provide fast and slow feedback loops, respectively, we decided to test if the absence of slow feedback loop (*GAL1p*) would result in increased noise in the system. To test that, we calculated coefficient of variation (CV), a measure of cellular heterogeneity and noise, for dual feedback and single

feedback systems. The dual feedback loop had lower CV than the single feedback loop across different concentrations of xylose tested **(Fig 2.4F),** consistent with observations of Brandman et al. (Brandman, Ferrell, Li, & Meyer, 2005)

2.3.4.1 Activation of downstream genes under the synthetic xylose regulon (Book no. 7, page no. 15-27)

It has been shown previously that the GAL regulon differentially regulates hundreds of genes which include not only genes of galactose metabolism (*GAL1*, *GAL7*, *GAL10*) and regulation (*GAL3, GAL80, GAL4*) but also genes responsible for other cellular functions, such as *GCY1, FUR4, LAP3, MTH1, PCL10, REE1,* etc (Hu et al., 2007; Reimand et al., 2010; Ren et al., 2000). By amplifying the upstream regions of genes *GCY1, LAP3*, *MTH1*, *PCL10, FUR4,* and *REE1,* and placing them upstream of *EGFP* we created *Promoter-EGFP-TEFt* constructs. As control, we also included the constitutive promoter *TEF1p* promoter upstream of *EGFP-TEFt*. The constructs were grown on sucrose and tested for fluorescence with or without the regulon. As positive control, the strains were also incubated on galactose, to check the maximum possible induction for these promoters. **Appendix Fig.6** shows the fluorescence obtained under the conditions tested. Only three of the six promoters, *GCY1p*, *MTH1p,* and *PCL10p* had increased fluorescence with the xylose regulon consistent with fluorescence increase observed when the same genes were induced with galactose. Under these assay conditions, we did not observe activation of the other three promoters even though it has been shown to be upregulated by galactose (Choi, Jeong, Ham, Sung, & Yun, 2008; Ren et al., 2000). To check if there is sucrose induced regulation of these genes, we switched the growth medium from sucrose to a mixture of ethanol (3 %) and glycerol (2 %). With the new growth medium, we observed activation of *LAP3p* by both xylose and galactose, suggesting possible sucrose-mediated regulation of *LAP3p*. The other two promoters, *FUR4p* and

REE1p, probably have weak up-regulation that couldn't be detected by the fluorescence assay. Overall, we show that in the presence of xylose, Gal3p-Syn4.1 controls genes of the GAL regulon that are both strongly and weakly *trans*-activated, similar to galactose-based activation **(Fig 2.4G)**.

2.3.5 A semi-synthetic XYL regulon enables better growth on xylose (Book no. 3 page no. 88,-89, book no. 6, page no. 3-19)

We placed genes necessary for xylose metabolism (*XYLA*3* (S.-M. Lee, Jellison, & Alper, 2012b), *XKS1*, *GAL2-2.1* (O. Reznicek et al., 2015), and *TAL1*) under the control of *GAL1p* and *GAL10p* promoters and transformed them along with the dual feedback loop system to create the strain, XYL-REG that has a complete xylose regulon capable of xylose detection and metabolism **(Fig 2.5A).** We built a metabolic control strain, XYL-CONS, where the four xylose catabolic genes were placed under the control of strong constitutive *TEF1p* and *TPI1p* promoters **(Fig 2.5B)**. Initial growth studies in synthetic complete (SC) xylose medium resulted in a growth rate of 0.12 h^{-1} for with XYL-REG and 0.07 h^{-1} for XYL-CONS **(Appendix Fig 2.8A)**. However, xylose was not fully consumed possibly due to substrate limitation in the minimal SC medium. Hence, we tested growth in complex YP medium supplemented with 2 % xylose and observed growth rates of 0.15 h^{-1} for XYL-REG and 0.06 h^{-1} for XYL-CONS. While XYL-REG reached an OD_{600} of 11, the XYL-CONS attained an OD_{600} of only 7. In concurrence with the OD values, most of xylose was consumed by XYL-REG and less than 0.15 % residual xylose was observed at the end of 60 hours. But, in the case of XYL-CONS, almost one-third (> 0.7 %) of xylose remained unused in the spent medium **(Fig 2.5C)**. These data show that implementation of a synthetic xylose regulon resulted in a higher growth rate, complete xylose consumption, as well as increased biomass density with minimal metabolic engineering.

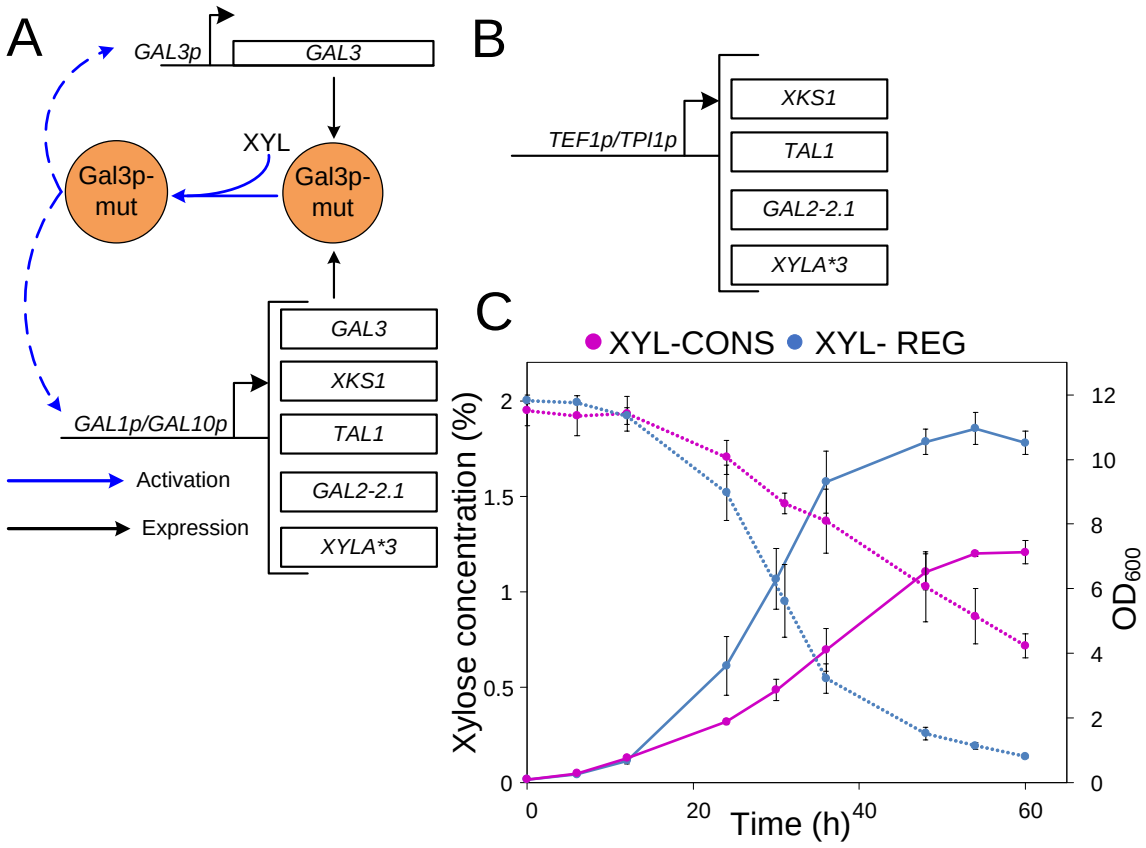
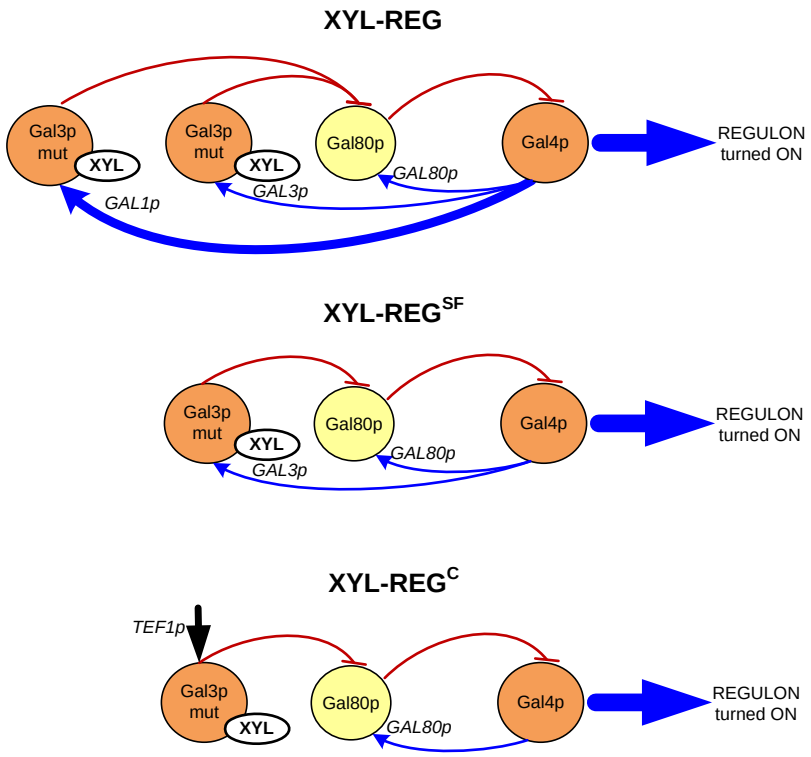


Figure 2.5: Growth on xylose facilitated by the xylose regulon. *(A) Design of the genes placed under the synthetic regulon and mode of activation of the xylose regulon. Apart from GAL3-Syn4.1 placed under GAL3p and GAL1p promoters, other metabolic genes XYLA*3, XKS1, TAL1, and xylose transporter GAL2-2.1 were expressed under GAL1p and GAL10p promoters. (B) Design of the metabolic control strain (XYL-CONS) built by placing the genes XYLA*3, XKS1, TAL1, and GAL2-2.1 under strong TEF1p and TPI1p promoters. (C) Growth (bold lines) and xylose consumption profile (dotted lines) of the engineered strain, XYL-REG, and metabolic control, XYL-CONS under aerobic conditions. Each data point represents average of three individual replicates ± sd.*

We also compared the effect of other GAL regulon designs on growth on xylose. We have shown that dual positive feedback exhibits better sensitivity and lower noise when compared to single feedback design. Next, we tested if these characteristics would also translate to improved growth.

We transformed plasmids carrying necessary genes for xylose metabolism under the control of *GAL1p* and *GAL10p* promoters along with Gal3p-Syn4.1 downstream of *GAL3p* promoter to create a single feedback strain, XYL-REG^{SF}. In this strain, the plasmid carrying Gal3p-Syn4.1 downstream of *GAL1p* promoter necessary for dual feedback was excluded. We also tested the effect of constitutively expressing Gal3p-Syn4.1 by placing the gene downstream of *TEF1p* promoter (XYL-REG^{C}). It is to be noted that the regulatory architectures tested in this study differ only in the dynamics and strength of activation of the regulon and should in theory activate the same set of genes, both the xylose metabolic genes and the downstream genes **(Fig 2.6A)**. The strains XYL-REG^{SF} and XYL-REG^{C} exhibited a growth rate of 0.12 h^{-1} and a final OD of ~8, a 20% decrease in growth rate, and a 27% decrease in final biomass compared to the dual feedback design (**Fig. 2.6B**). This clearly showcases the growth benefits of the wild-type-like dual positive feedback system in XYL-REG.

A


XYL-REG
Gal3p mut
XYL
Gal3p mut
XYL
Gal80p
Gal4p
REGULON turned ON
GAL80p
GAL3p
GAL1p
XYL-REG^SF
Gal3p mut
XYL
Gal80p
Gal4p
REGULON turned ON
GAL80p
GAL3p
XYL-REG^C
TEF1p
Gal3p mut
XYL
Gal80p
Gal4p
REGULON turned ON
GAL80p

B

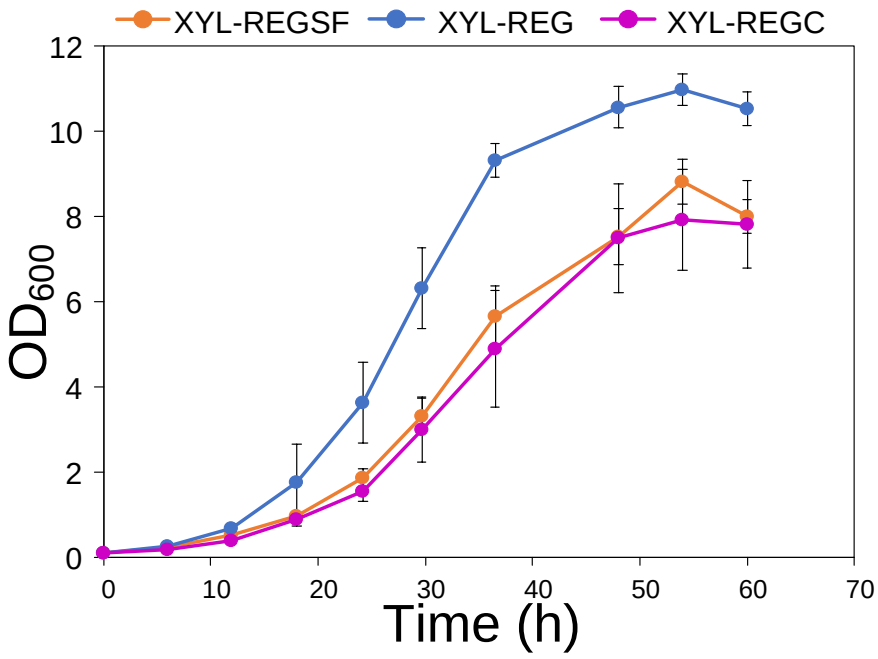

XYL-REGSF
XYL-REG
XYL-REGC
OD600
Time (h)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70

Figure 2.6: Effect of different GAL regulatory networks on xylose growth. *(A) Schematic of the regulon designs used. The XYL-REG and XYL-REG^{SF} are dual and single positive feedback networks analyzed previously. In XYL-REG^{C}, the Gal3p-Syn4.1 is expressed constitutively by TEF1p promoter, resulting in constant activation of the regulon in the absence of catabolite repression by glucose. (B) Comparison of growth curves (right) in complex medium (YPA + 2% xylose) of dual feedback strain XYL-REG (μ = 0.15 h^{-1}, $OD_{max} \approx 11$), with single feedback, XYL-REG^{SF} (μ = 0.12 h^{-1}, $OD_{max} \approx 8$), and constitutively active XYL regulon XYL-REG^{C} (μ = 0.12 h^{-1}, $OD_{max} \approx 8$). Each data point represents average of biological triplicates ± sd.*

2.3.6 XYL regulon enables better growth on native sugars (Book no. 5, page no. 132-137, book no. 6, page no. 11-13)

Since mutations on Gal3p-Syn4.1 are not in the sugar binding pocket, it retains its galactose binding function **(Fig. 2.3D)**. To test if Gal3p-Syn4.1 can still function as a galactose transcriptional regulator, we transformed the plasmid containing Gal3p-Syn4.1 in a *GAL3Δ* strain to create GAL-Gal3p-Syn4.1. The strain and the unmodified parent strain, W303-1a, when grown in SC medium containing 2 % galactose showed similar growth rates of 0.3 h^{-1} and reached similar cell densities **(Fig. 2.7A)**. Finally, since strains containing the regulon switch ON gene expression only when xylose is present, we hypothesized that XYL-REG would have better growth fitness than XYL-CONS under conditions where the regulon is either uninduced or repressed. We tested growth of these strains on sucrose and glucose, to assess if regulated expression of xylose catabolic genes provides improved fitness even under non-inducing conditions, compared to constitutive expression. As expected, the growth rate of XYL-REG was higher than that of XYL-CONS in both sugars. The strains having the regulon grew at ~0.3 h^{-1} while the XYL-CONS had a growth rate of 0.24 h^{-1} in both sugars **(Fig. 2.7B & Fig. 2.7C)**. Comparing the growth of strains with and

without regulon in multiple sugars, it is clear that presence of regulon upregulates necessary genes for sugar metabolism only when the sugar is detected. This induction system leads to high growth rates and also prevents metabolic burden in the presence of non-inducing sugars **(Table 2.2)**. Thus, a regulon-based strategy not only results in faster growth rates to higher final cell densities and more complete sugar consumption in the non-native sugar it is designed to respond to, but also exhibits lower metabolic burden and improved growth in alternate sugars.

Table 2.2: *Comparison of growth rates (μ) for different strains cultured in different sugars, xylose, galactose, sucrose, and glucose.*

	Growth rate on sugar, μ (h^{-1})			
Strain	**Xylose**	**Galactose**	**Sucrose**	**Glucose**
WT (GAL-REG)	-	0.30±0.02	0.30±0.01	0.31±0.01
XYL-CONS	0.06±0.01	-	0.24±0.01	0.25±0.01
XYL-REG	0.15±0.01	-	0.31±0.01	0.30±0.002
GAL-CONS	-	0.056±0.002	-	-
GAL-GAL-CONS*4*	-	0.22±0.03	-	-

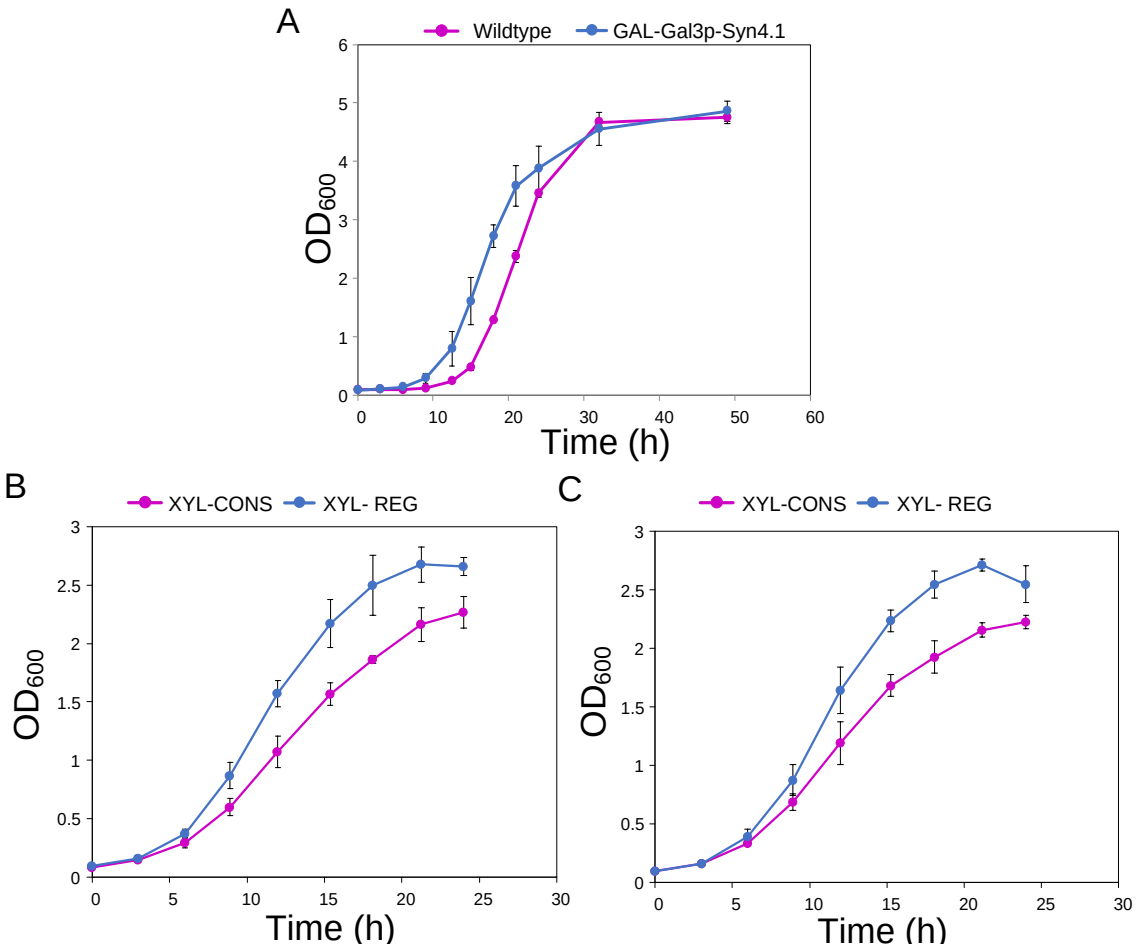


Figure 2.7: Growth of strains in inducing and non-inducing sugars. *(A) Growth of wildtype and a strain expressing Gal3p-Syn4.1 in a GAL3Δ background on 2 % galactose (μ = 0.3 h^{-1} for both). Growth of strains XYL-REG and XYL-CONS in minimal media supplemented with (B) non-repressing sugar sucrose and (C) repressing sugar glucose. Each data point represents average of biological triplicates ± sd.*

2.3.7 Transcriptomic analysis of strains (Book no. 7, page no. 50 - 55, R code files are "regulon_vs_cons.R", "TA_regulon_vs_cons.R", and "heatmaps.R")

To provide insight into the genes differentially expressed in REG strains (GAL-REG (WT) and XYL-REG) when compared to CONS strains (GAL-CONS and XYL-CONS) that result in vastly different growth phenotypes, we carried out RNA-seq to profile their transcriptome. We used triplicates of these strains grown in their respective carbon sources, harvested them during mid-exponential phase of growth and used them for RNA-seq. We found that the transcriptome profiles of the REG and CONS strains varied drastically **(Appendix Fig. 10)**. Next, we carried out a differential gene expression analysis between XYL-REG and XYL-CONS as well as between GAL-REG (WT) and GAL-CONS strains using the limma (Ritchie et al., 2015) and edgeR(Robinson, McCarthy, & Smyth, 2009) packages. Genes with a statistical *p*-value less than 0.05 after Benjamini–Hochberg correction were considered as differentially regulated. Total of 4202 genes were differentially regulated between GAL-REG and GAL-CONS strains and 3314 genes between XYL-REG and XYL-CONS strains **(Fig. 2.8A & Fig 2.8B)**. We reasoned that if there are genes either directly or indirectly regulated by Gal4p, they would be differentially expressed in not just the strains grown on galactose (GAL-REG versus GAL-CONS) but also in strains grown on xylose (XYL-REG versus XYL-CONS). Further, we also hypothesized that since both the CONS strains lack regulation for sugar detection, those strains should exhibit a starvation-like response. To test both, we decided to select for genes that are up- and down- regulated in both the differential gene expression analyses. We found 452 genes that were upregulated and 507 genes that were downregulated in both the regulon strains **(Fig. 2.8C)**.

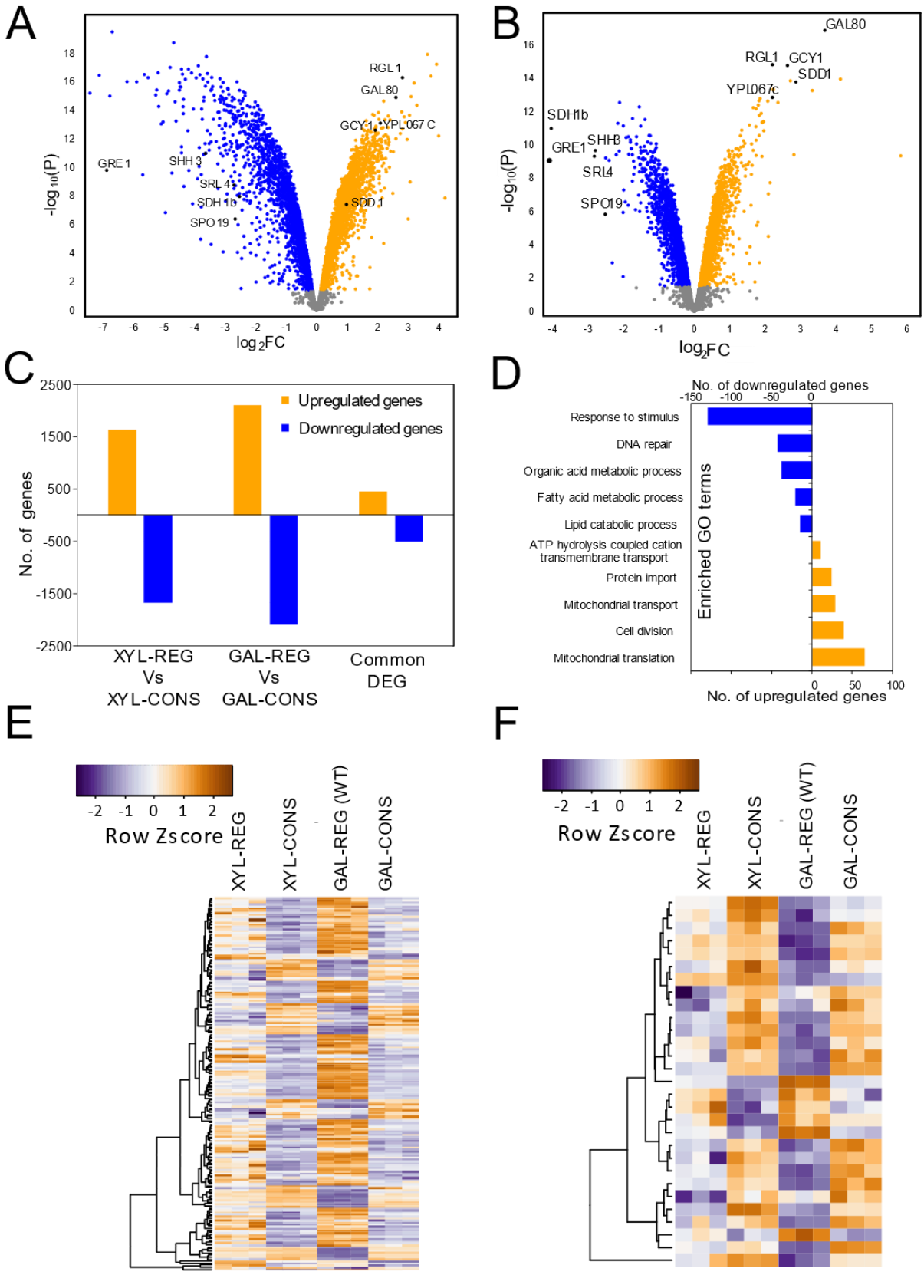

A
RGL1
GAL80
GCY1
YPL067C
GRE1
SHH3
SRL4
SDH1b
SDD1
SPO19
-log10(P)
log2FC
B
GAL80
RGL1
GCY1
SDD1
YPL067c
SDH1b
GRE1
SHH3
SRL4
SPO19
-log10(P)
log2FC
C
Upregulated genes
Downregulated genes
No. of genes
XYL-REG Vs XYL-CONS
GAL-REG Vs GAL-CONS
Common DEG
D
No. of downregulated genes
Response to stimulus
DNA repair
Organic acid metabolic process
Fatty acid metabolic process
Lipid catabolic process
ATP hydrolysis coupled cation transmembrane transport
Protein import
Mitochondrial transport
Cell division
Mitochondrial translation
Enriched GO terms
No. of upregulated genes
E
Row Zscore
XYL-REG
XYL-CONS
GAL-REG (WT)
GAL-CONS
F
Row Zscore
XYL-REG
XYL-CONS
GAL-REG (WT)
GAL-CONS

Figure 2.8: RNA-seq analysis of REG and CONS strains. *Differential Expressed Genes (DEG) between (A) GAL-REG (WT) versus GAL-CONS grown on galactose and (B) XYL-REG versus XYL-CONS grown on xylose. DEGs that are upregulated are shown in red while downregulated genes are shown in blue. Common DEGs with high fold change values are labeled and shown in black. (C) Number of genes that are differentially expressed between REG and CONS strains as well as genes common between the two pairs. (D) Relevant GO biological process terms of common DEGs. Negative numbers represent downregulated genes and positive ones represent upregulated genes in the REG stains. Heatmap of normalized log counts of DEG (E) controlled by Gal4p, and (F) that are transcription factors (TFs).*

Next, we evaluated the functional relation between these genes by examining the Gene Ontology (GO) biological process terms that are enriched in up- and down- regulated genes. Of the genes that are upregulated in the REG strains, we found 36 enriched GO terms, including those relating to mitochondrial translation and transport, cell division, ATP production, protein import, etc. We also found 11 GO biological process terms in the case of genes that were downregulated, which were involved in processes such as fatty acid and lipid metabolism, DNA repair, response to stimulus, etc. **(Fig. 2.8D and Appendix Fig. 11)**. Out of the genes enriched under the GO term for response to stimulus, 58 genes were associated with stress response. Since, we hypothesized many of these genes are regulated by Gal4p, we extracted genes that are known to be regulated by Gal4p from YEASTRACT(M. C. Teixeira, 2006; Miguel C. Teixeira et al., 2017) and compared them with differentially regulated genes from our analysis. We identified 181 genes as hits from the overlap of the two sets **(Fig. 2.8E & Appendix Data 1)**. GO term analysis of the genes show that many of the processes previously enriched in upregulated genes such as cell division, cell wall organization, mitochondrial translation, and membrane transport were enriched, suggesting that upregulation of these vital cellular pathways are a direct consequence of activating the GAL regulon. Interestingly, none of the previously enriched GO process terms from the downregulated genes were enriched

in this analysis indicating that GAL regulon is probably not involved in downregulation of genes observed in REG strains. Between the REG and CONS strains, we also analyzed for differentially expressed genes that code for transcription factors (TFs) **(Fig. 2.8F & Appendix Data 1)**. We identified 29 TFs that were upregulated in the CONS strains, of which several are involved in response to stress, nutrient starvation, and DNA replication stress (e.g. *CBF1* (Tkach et al., 2012), *RPH1* (Bernard et al., 2015; Jang, Wang, & Sancar, 1999), *GSM1* (Ho, Jona, Chen, Johnston, & Snyder, 2006), *HAA1* (Tkach et al., 2012), *WAR1* (Kren et al., 2003), *YOX1* (Loll-Krippleber & Brown, 2017; Tkach et al., 2012), *SUT2* (Rützler, Reissaus, Budzowska, & Bandlow, 2004), *MIG3* (Dubacq, Chevalier, & Mann, 2004; Lewis & Gasch, 2012), and *GCN4* (Hinnebusch & Natarajan, 2002; Natarajan et al., 2001)). TFs involved in gluconeogenesis and glyoxylate cycle (*RDS2* (Turcotte, Liang, Robert, & Soontorngun, 2010)) as well as drug resistance (*RDR1* (Hellauer, Akache, MacPherson, Sirard, & Turcotte, 2002) and *STB5* (Kasten & Stillman, 1997)) were also upregulated in CONS strains. Upregulation of the above-mentioned TFs along with GO term enrichment of processes involved in DNA repair as well as upregulation of several stress response genes in the CONS strains reaffirms our hypothesis that CONS strains exhibit a starvation- and stress-like response when grown in carbon sources for which substrate sensing systems are absent. In the case of REG strains, TFs responsible for cell wall production (*INO4* (Santiago & Mamoun, 2003; Schwank, Ebbert, Rautenstrauß, Schweizer, & Schüller, 1995)), cell cycle progression (*SWI5* (Cosma, Tanaka, & Nasmyth, 1999; Moll, Tebb, Surana, Robitsch, & Nasmyth, 1991) and *HCM1* (Pramila, Wu, Miles, Noble, & Breeden, 2006; Zhu & Davis, 1998)), and flocculation suppression (*SFL1* (Robertson & Fink, 1998)) were upregulated **(Fig. 2.8E)**. Taken together with growth studies, the data suggests GAL regulon-controlled upregulation of pathways involved in growth such as cell wall maintenance, cell division, mitochondrial biogenesis, and cell cycle

progression support the observed growth phenotype. On the other hand, unregulated constitutive expression of sugar metabolizing genes seems to trigger stress, starvation, and DNA damage responses.

2.4 Discussion

In this study, we compared regulon-assisted control that is prevalent in nature to constitutive expression strategy, which is widely used in synthetic biology and metabolic engineering communities for non-native sugar assimilation. By first assessing the growth of *S. cerevisiae* on a native sugar, galactose, using the two strategies, we provide evidence that GAL regulon offers significant advantages for promoting growth compared to constitutive expression. We attribute this to the fact that apart from dynamically regulating the (upstream) Leloir pathway genes, the GAL regulon also regulates hundreds of other downstream metabolic and regulatory genes (Ideker et al., 2001; Reimand et al., 2010; Ren et al., 2000). We show that activation of these pathways acts synergistically and provide growth benefits to yeast on galactose. With the knowledge that GAL regulon can enhance growth and that the downstream genes were not galactose metabolism-specific, we adapted it for heterologous xylose metabolism in *S. cerevisiae* and circumvented the need for extensive genome-scale engineering that would otherwise be needed for synthetic regulon construction. We engineered the GAL regulon into a xylose (XYL) regulon and with minimal metabolic engineering, obtained better growth and final cell density compared to constitutive expression of upstream xylose metabolic genes. It should also be noted that this is in stark contrast to most published studies where growth rate on xylose prior to adaptive evolution is low. The growth rate of XYL-CONS, 0.06 h^{-1} is obtained due to superior *XYLA*3*, which was engineered by Lee et al., 2012, who report similar growth rate in their work (S.-M. Lee et al., 2012b). While

a number of studies have obtained higher growth rates than what we report for XYL-REG, those strains have all been engineered extensively with overexpression of all of the non-oxidative pentose pathways as well as adaptive evolution (Y. Jin, Alper, Yang, & Stephanopoulos, 2005; S.-M. Lee et al., 2014; Scalcinati et al., 2012; H. Zhou et al., 2012). We would also like to point out that mRNA expression levels of *XYLA*3* and *XKS1* from XYL-REG strain is several folds higher than expression in XYL-CONS strain and can, to some extent, contribute to the observed increase in growth rate **(Appendix Note 3, Appendix Fig. 7)**. But, transcriptomic analysis by RNA-seq revealed that genes responsible for cell wall biogenesis, mitochondrial biogenesis, and ATP biosynthesis were upregulated in the REG strains, suggesting that GAL regulon mediated activation of downstream genes also plays a major role in promoting fast growth of the REG strains. On the other hand, genes involved in response to stress, starvation, DNA damage, and lipid metabolism were upregulated in the CONS strains as a consequence of being forced to metabolize unrecognized substrates. Thus, the GAL regulon seems to aid in growth by upregulating several growth-related pathways and transcription factors while suppressing stress and starvation responses, which are upregulated in strains with unregulated substrate catabolic pathways. While metabolite sensing is increasingly employed in metabolic engineering, they are used to *trans*-activate only a small set of genes (Mahr & Frunzke, 2016; Rogers & Church, 2016; Siedler, Stahlhut, Malla, Maury, & Neves, 2014; Silva-Rocha & de Lorenzo, 2012; Skjoedt et al., 2016; Slomovic & Collins, 2015; Teo & Chang, 2014; M. Wang et al., 2015). Other approaches such as transcription factor-based engineering, which involves deletion or overexpression of specific transcription factors have also been carried out for non-native sugar metabolism such as xylose or cellobiose (Lin et al., 2014; Michael et al., 2016). However, as far as we know, this is the first known engineering effort that rationally couples substrate sensing to direct global cellular state for fast-growth while also repressing stress and starvation responses that is generally observed when *S. cerevisiae* is grown

in a non-native sugar (Lin et al., 2014; Matsushika & Hoshino, 2015; Salusjärvi et al., 2006). We also demonstrate that this cellular state is congruent with observed transcriptional and phenotypic responses on native and non-native substrates.

One of the initial steps for protein engineering of Gal3p was to study the binding affinity of Gal3pWT for xylose. Fluorescence was observed at xylose concentrations above 2% suggesting weak affinity of Gal3p towards xylose. Previous transcriptomics analyses of *S. cerevisiae* grown on xylose have also observed an increase in transcript levels of GAL metabolic and regulatory genes (Y. Jin et al., 2004; Scalcinati et al., 2012; M Sonderegger et al., 2004; Wahlbom et al., 2003). More importantly, this observation further adds credence to our hypothesis that downstream genes of the galactose regulon can get activated in the presence of xylose. Based on this data although Gal3p seems promiscuous, the sensitivity and response of Gal3p towards galactose is more than 3 magnitudes higher than that of xylose. Hence it is possible that other mutations in GAL regulatory genes synergize with this promiscuity, leading to increased GAL regulated transcripts and also better growth.

Sellick and Reece, (2006), have previously shown promiscuity of Gal3p towards glucose which shares a similar ring structure with xylose (Xylose differs from glucose by the absence of C6 carbon) (Sellick and Reece, 2006). However, previous mutation analysis on the active site of galactokinase (Gal1p), a structural homolog of Gal3p either resulted in abolishing the activity of Gal1p or didn't have any effect (Sellick and Reece, 2006; Timson and Reece, 2003). Further, mutations away from the active site of Gal1p have been shown to increase the promiscuity of the enzyme, suggesting us to resort to random mutagenesis instead of targeted saturation mutagenesis in the sugar binding pocket. Hence, the first round of random mutagenesis, targeted at the Gal3p-Gal80p interaction loop aimed to increase the interaction strength and thereby increase Gal3p-

sugar binding. The advantageous mutation A109T, is present on the dynamic loop of Gal3p, which opens up only when Gal3p interacts with a sugar. By not targeting the sugar binding pocket of the protein, we were able to retain the native galactose binding ability. Hence, when Gal3p-WT was replaced with Gal3p-SYN4.1, no difference was observed in growth rates. We also show that a required property can be obtained by mutating residues far away from the active site of the protein.

Maintaining tight regulatory control along with rapid and robust response to a nutrient is an essential characteristic for nutrient-induced regulon activation. For growth fitness in a competitive environment, yeast evolved mechanisms for initiating rapid and robust response to assimilate available galactose through mutiple feedback loops while maintaining tight regulatory control. In this chapter, I show that a single protein (Gal3p-Syn4.1) involved in repressor (Gal80p) sequestration when driven by promoters of different expression strengths can create a dual positive feedback loop that has increased response to the inducer, exhibits bistability, and is resistant to noise. Further, this dual positive feedback regulatory structure supports higher growth rate and cell density when compared to single positive feedback or constitutive GAL activation systems. The dual positive feedback loop conceived in this study is a synthetic implementation of the native GAL system. Thus, the high growth rate obtained by the XYL-REG strain is a synergistic effect of engineering the native regulatory and metabolic architecture of the GAL regulon at various levels such as sensing, transduction, and metabolism.

Altogether, this work provides a new and unexplored paradigm of engineering semi-synthetic regulons for substrate assimilation and highlights not only the importance of sensing substrates, but also how they integrate into other cellular functions to ensure activation of growth responses and repression of starvation/stress responses. This approach can be easily extended for other

abundant, but non-native substrates. We suggest that the regulon engineering strategy is potentially a rational, faster, and possibly more elegant approach than prevailing strategies.

2.5 Methods:

2.5.1 Strains and plasmids used

List of plasmids and strains used are listed in Appendix table 2.1 and Appendix table 2.2**,** respectively. Abbreviations used are SC (Synthetic Complete), YP (Yeast extract Peptone), S (Sucrose), G (Galactose), X (xylose), Leu (Leucine), Ura (Uracil), His (Histidine), Trp (Tryptophan) and A (Adenine).

2.5.2 Materials

Strain W303-1a *(MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15}),* and plasmids pkT209 and pBK415 were obtained from Euroscarf (Frankfrut, Germany). All enzymes for cloning were purchased from NEB (Beverly, MA). DNA primers were ordered from Operon Inc (Huntsville, AL). Sequencing of plasmid DNA was outsourced to Operon Inc. Growth media and chemicals were purchased from Amresco (Solon, OH) or RPI Corp (Mount Prospect, IL). Ampicillin was obtained from RPI and G418 sulfate from Life Technologies (Grand Island, NY). 5-Fluoroorotic Acid (FOA) was purchased from Zymo Research (Irvine, CA). E.Z.N.A.® Plasmid Mini Kit I, PCR Purification and Gel Extraction Kits were obtained from Omegabiotek (Norcross, GA). *XYLA*3* DNA sequence was provided by Prof. Hal S. Alper (University of Texas at Austin) and was synthesized from Twist Biosciences (San Francisco, CA). *GAL2-2.1* DNA sequence was provided by Prof. Bernard Hauer (University of Stuttgart, Stuttgart, Germany). Complete

Supplement mixture without His, Leu, Ura and Trp mixture was obtained from Sunrise Science Products, Inc (San Diego, CA).

2.5.3 Strain construction

2.5.3.1 Gene knock-outs

The yeast strain W303-1a, purchased from Euroscarf was used for constructing all the strains used in the study. The knock out protocol "Delitto perfetto"(Storici & Resnick, 2006) was used with modifications. Instead of using CORE (Counterselectable Reporter) cassette containing *URA3* and *KANMX* markers, only *URA3* was used. Selection was performed in SC (Synthetic Complete) medium without uracil and counterselection using SC medium with 1 g/l of 5-FOA. *URA3* gene was PCR amplified from pkT209 plasmid using primers with 40 bp flanking ends which are homologous to the gene ends to be knocked out. The deletion cassette was inserted through homologous recombination by lithium acetate transformation protocol of Gietz (2014)(Gietz, 2014). The transformed cells were selected for *URA3* cassette insertion by selecting in SC-Ura medium and confirmed using colony PCR. To remove the cassette, two colony PCRs were performed to amplify the flanking ends of gene using primers with overlapping ends. The two fragments were spliced using Overlap-Extension PCR (OE-PCR), transformed and selected in SC+FOA plates and confirmed using colony PCR.

2.5.3.2 Gene knock-ins

GAL2 along with its native promoter was amplified from yeast genome and single point mutations at different sites were introduced through OE-PCR. *GAL2p-GAL2-2.1-TEF1t* construct with XhoI and NotI restriction sites were restriction digested and ligated with pRS405 to create pRS405-*GAL2p-GAL2-2.1-TEF1t*. The plasmid was linearized by making a single cut with EcoRI at the

LEU locus, transformed in VEG16 strain and selected for colonies in SC-Leu medium supplemented with 2 % glucose and confirmed through colony PCR. The *GAL10p-GFP-TEFt* construct from pRS426 was restriction digested with BamHI and SalI and cloned into pRS406. The integrative vector was then linearized with NdeI and used to transform VEG16 strain.

2.5.4 Plasmid construction

2.5.4.1 Selection and screening plasmid construction

The plasmid for screening and selection, pVEG8 was built through two sequential cloning steps. The bidirectional promoters *GAL1p/GAL10p* and *HXT7t* terminator were amplified from the yeast genome. *KANMX* gene was amplified from plasmid pBK415; *EGFP* and *ADH1t* terminator were amplified from pkT209. All of them were spliced using OE-PCR, restriction digested with BamHI and SalI, ligated and cloned into pRS426 to create pVEG7. *GAL3* gene along with its native promoter was amplified from the yeast genome, spliced with *TEF1t* terminator amplified from plasmid pkT209 and cloned into pVEG7 backbone using BamHI and NotI restriction sites to create pVEG8.

2.5.4.2 Plasmid construction for galac2.5.4.tose metabolism

Three constitutive promoters, *TEF1p, TPI1p,* and *GPM1p,* three terminators, *TEF1t, ADH1t,* and *HXT7t* along with genes *GAL1, GAL7,* and *GAL10* were amplified from the genome of *S. cerevisiae* using colony PCR with primers containing appropriate flanking regions for extension. Three constructs *TEF1p-GAL1-Hxt7t, TPI1p-GAL10-ADH1t,* and *GPM1p-GAL7-TEF1t* were built using OE-PCR and assembled onto pRS426 backbone using DNA assembler(B. Kim, Du, Eriksen, & Zhao, 2013).

2.5.4.3 Plasmid construction for xylose metabolism

Two plasmids for xylose metabolism were built using genes *XYLA*3* from *Piromyces sp* and *XKS1*, *TAL1* and *GAL2-2.1* from *S. cerevisiae. KANMX* and *EGFP* were replaced by *XYLA*3* and *XKS1* in pVEG7 to create pVEG11. Similarly, *TAL1* and *GAL2-2.1* were cloned into the promoter-gene-terminator construct of pVEG7, but in pRS423 backbone to create pVEG12. The *GAL1p/GAL10p* promoters of pVEG11 and pVEG12 were replaced by divergent *TEF1p* and *TPI1p* promoters to create pVEG10 and pVEG13 respectively. The wild-type or the Syn4.1 mutant of *GAL3* was sub-cloned into pRS414 from pVEG8 to create $pVEG16^{WT}$ and $pVEG16^{*}$ respectively. Finally, wild-type and Syn4.1 mutant of *GAL3* was also placed under *GAL1p* promoter and cloned into pRS415 backbone to create $pVEG17^{WT}$ and $pVEG17^{*}$ respectively.

2.5.4.4 Plasmids for Gal4p based activation of genes

Promoters of genes *GAL1*, *GAL7*, *GAL10*, *FUR4*, *TEF1*, *PCL10*, *REE1*, *LAP3*, and *MTH1* were amplified from yeast genome, spliced with *EGFP-ADH1t* construct using appropriate primers from pVEG8, cloned onto pRS426 backbone to create the respective *pRS426-Promoter-EGFP-ADH1t* constructs.

2.5.5 Antibiotic selection and fluorescence screening

S. cerevisiae strain, VEG16 or VEG20 were transformed with mutant libraries of pVEG8 using established protocols(Gietz, 2014) and recovered for six hours in 1.2 ml of YP supplemented with 2 % of sucrose and xylose before plating on the agar plates with same medium, supplemented with 100 µg/ml of G418 sulfate. The plates were incubated at 30 °C for 2-3 days and colonies were streaked in SC-Ura medium with glucose. Grown colonies were then inoculated in both 2 % sucrose and 2 % sucrose supplemented with xylose (2 % or 0.2 % as mentioned) in 96-well plates

and incubated for 18 hours in a shaker. Fluorescence (excitation at 488 nm and emission at 525 nm) and OD_{600} were measured in a Spectramax M3 spectrophotometer to obtain RFU/OD_{600}. Only strains that exhibited low basal fluorescence on sucrose and higher fluorescence than wild-type on xylose were taken for further screening. Characterization of the fluorescence profile of mutants were carried out by inoculating the strains on medium with SC-Ura with sucrose supplemented with different concentrations of xylose or galactose and RFU/OD_{600} was measured after 18 hours of incubation in a plate shaker.

2.5.6 Dose response curve for fluorescence:

For all dose response curve experiments, the cells were grown in 2 % sucrose unless specified. The cells were first pre-grown for 24 hours, except for hysteresis experiments where the cells where grown in media supplemented with 4 % xylose for full induction. The cells were diluted 1:100 fold in SC medium containing sucrose and specified concentrations of galactose or xylose. They were incubated at 30 °C in a microplate shaker and RFU/OD_{600} was measured in a spectrophotometer with excitation at 488 nm and emission at 509 nm. For comparing dual and single feedback cell populations, fluorescence was measured using Attune Nxt5 flow cytometer. Blue laser (488 nm) was used for excitation. At least 10,000 cells were measured for each of the flow cytometry experiments.

2.5.7 Growth studies on xylose, galactose, sucrose, and glucose

The strains were grown overnight in appropriate dropout SC medium supplemented with sucrose. They were washed thrice in the growth medium to be inoculated and then diluted to an initial OD_{600} of 0.1 in the same medium with appropriate sugar (2 %), incubated in 5 ml test tubes, and OD_{600} was measured every couple of hours. Growth studies with xylose or galactose were carried

out in 250 ml shake flasks containing 20 ml of media. Growth studies with sucrose or glucose were carried out in 15 ml test tubes containing 5 ml of media. For measuring concentration of extracellular xylose, samples collected during OD_{600} measurement were centrifuged at 10,000 × g for 1 minute and the supernatant was stored at -20 °C.

2.5.8 Media and Transformation

Yeast strains were grown in YPA medium or SC medium (Yeast nitrogen base (1.67 g/l), ammonium sulfate (5 g/l), complete supplement mixture without His, Leu, Ura and Trp (0.6 g/l)) with appropriate nutrient. Luria Bertani (LB) broth and LB agar plates with 100 mg/l of ampicillin (when required) were used for all *E. coli* propagation and transformation experiments. *E. coli* NEB5α was used to transform the ligated mixture to create all of the plasmids described using MES transformation except for mutant libraries which were created by electroporating the ligation mixture. The plasmids were sequenced and transformed into the appropriate yeast strain using the protocol of Gietz (2014)(Gietz, 2014)

2.5.9 Mutagenesis

2.5.9.1 Error-prone PCR

Random mutagenesis libraries were created by error prone PCR with 0.3 ng μl^{-1} of template plasmid, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 5 mM $MgCl_2$, $MnCl_2$ (0.05 mM for mutagenesis on the entire protein and 0.3 mM for mutations on the loops), 0.05 U ml^{-1} *Taq* DNA polymerase, and 0.4 mM of the forward and reverse primers. The reaction was amplified using the following PCR cycle conditions: 95 °C denaturation, 5 min; 16 cycles of 95 °C denaturation, 30 s; 46 °C annealing, 45 s; and 68 °C extension, 3 min, 16 cycles for mutagenesis

on the entire protein and 25 cycles for mutations on the loops, followed by 68 °C extension for 5 min. The mutated gene was spliced with *GAL3p* promoter and *TEF1t* terminator using OE-PCR, restriction digested, cloned into pVEG7 plasmid and electroporated to *E. coli* NEB5α cells. Five transformants were randomly chosen and their gene sequenced to determine the error rate of the library.

2.5.9.2 Single site saturation mutagenesis

For saturation mutagenesis, forward and reverse primers with NNK degenerate nucleotides at position 109 were used to create fragments that were spliced using OE-PCR, restriction digested with BamHI and NotI enzymes, ligated, and cloned into pVEG7 background.

2.5.9.3 Synthetic shuffling

Five primers containing degenerate nucleotides at sites that has to be mutated were used. The degenerate nucleotides either code for wild-type or mutated nucleotide covering all of the eight mutations obtained from random mutagenesis were used to amplify fragments from *GAL3-2.1* variant, spliced with OE-PCR, restriction digested, and cloned into pVEG7 background.

2.5.10 Extracellular xylose measurement

Xylose concentration was measured using an Agilent HPLC system equipped with a Hi-Plex H-column and detected using 1260 Agilent ELSD detector. Mobile phase was 0.1 % Trifluoroacetic acid (TFA) with a flow rate of 0.6 ml/min. The ELSD detector's nebulizer and evaporation temperature were set at 30 °C and nitrogen flow rate at 1.6 SLM (standard liter per minute).

2.5.11 RNA extraction, sequencing, and analysis

Triplicates of strains WT (GAL-REG), XYL-REG, GAL-CONS and XYL-CONS were grown in their respective carbon source (galactose or xylose) till mid exponential phase and approximately 2×10^7 cells were washed twice in water, pelleted and stored at -80 °C. RNA extraction as well as library preparation and sequencing were outsourced to Genewiz, Inc. (South Plainfield, NJ). RNA-seq was performed on Illumina HiSeq. Possible adapter sequences and nucleotides with poor quality were trimmed, sequence reads shorter than 50 bp were excluded and the remaining were aligned to the reference genome W303 obtained from Saccharomyces Genome Database (http://www.yeastgenome.org) along with *XYLA*3* sequence. The obtained gene count data was normalized based on library size, converted to cpm (counts per million) using edgeR package (Robinson et al., 2009). To prevent skewing of data, genes that were either deleted or overexpressed were removed from the dataset for further analysis. For differential gene expression analysis, limma package (Ritchie et al., 2015) with voom transformation (Law, Chen, Shi, & Smyth, 2014) was applied to the samples. Finally, a linear model was fit to each gene using limma (lmfit function) and differential gene expression was analyzed. Genes with Benjamini–Hochberg corrected *p*-values less than 0.05 were considered as differentially expressed.

2.5.12 Gene Ontology (GO) term enrichment analysis

GO term enrichment analysis was carried out using the GO term tool from Princeton University (http://go.princeton.edu/) by querying for commonly up- or down-regulated genes separately, after applying Bonferroni correction and with a cut-off *p*-value of 0.05. The obtained GO terms were summarized using REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) toolbox with default settings.

2.5.13 Computational modeling

The ODE model from Venturelli et al.(Venturelli et al., 2012) was used with minor modifications. Since the regulatory network remains the same, but the mode of activation is either galactose or xylose, we retained all the parameters used by the previous study. In our case, Gal3p-Syn4.1 induction by xylose is weaker than Gal3p-WT activation by galactose. Since the model doesn't differentiate free Gal3p with sugar bound Gal3p, we assume Gal3p-sugar-Gal80 interaction (kf83 and kr83) to be a lumped parameter that represents Gal3p based Gal4 activation. In our model, Gal1p was not considered since it has been deleted in our strains and represents the single feedback model. For dual feedback, the expression of Gal3p under *GAL1p* promoter was modeled by adding a second Gal3p production term under *GAL1p* promoter. In both the models, EGFP expression under *GAL1p* promoter was modeled. Since EGFP has low degradation rate, decay of EGFP was ignored. Both the models are provided in the supplemental section. Further details on parameters as well as ODE equations for both models are provided in the supplemental section.

2.6 Appendix

Appendix Notes:

Appendix Note 2.1: Characterization of expression strengths of GAL-activated versus constitutive promoters

To assess which Gal4p-mediated genes assist in growth on galactose, we compared growth between strains that have Leloir genes under GAL-activated promoters (WT strain, GAL-REG) and constitutive promoters (GAL-CONS). First, we compared the expression strengths of GAL-activated (*GAL1p*, *GAL7p*, and *GAL10p*) and constitutive promoters (*TEF1p*, *TPI1p*, and *GPM1p*) by placing EGFP gene downstream of these promoters. Since the WT strain has a single copy of Leloir pathway genes, we placed the $GAL_{promoter}$ – EGFP constructs in pRS406 integration plasmid and knocked it into the *URA3* locus of the chromosome. To account for locus-based expression differences, we also cloned the constructs in low copy plasmids (2 – 5 copy number(Karim, Curran, & Alper S. Hal, 2012)) and considered them to be the maximum possible expression of GAL promoters in the WT strain. Next, we cloned EGFP under constitutive promoters in multicopy pRS426 plasmid and compared their fluorescence on galactose **(Appendix Fig.1a).** Comparing the results, we observe that the constitutive promoters in high copy plasmids have stronger expression strength than single copy GAL promoters integrated in the chromosome or similar expression strength with low-copy GAL promoters. This observation is consistent with mRNA expression data obained from RNA-seq analysis **(Appendix Fig.1b)**.

Appendix Note 2.2: ODE models for single and dual feedback systems:

The feedback models were adapted from Venturelli et al.(Venturelli et al., 2012) with modifications as described in the methods section, including all of the parameters used **(Appendix Table 2.3)**. Fluorescence assay for measuring Gal3p-WT-galactose-Gal80p interaction was compared with the model prediction. While dual and single feedback showed a difference as predicted by the model, the trends were different. To match them, the forward binding rate constant, kf83, was decreased to 2.5 $nM^{-1}min^{-1}$. Further, it is known that Gal4p binds cooperatively to UAS_{GAL} sites present on GAL activated promoters(Giniger & Ptashne, 1988; Kang, Martins, & Sadowski, 1993). We checked the cooperativity of *GAL10p, GAL3p* and *GAL80p* promoters used in the model experimentally by using the promoters to drive EGFP and measured fluorescence output after inducing them with varying concentrations of xylose **(Appendix Fig.3)**. We fit the data to Hill equation and obtained cooperativity values of 2, 1, and 1 for *GAL10p, GAL3p,* and *GAL80p* respectively, which were used for both the feedback models. It is to be noted that the parameter kf83 has been chosen to better reflect the experimental data and that the conclusion drawn from the experiments does not change when kf83 is modified in the model, provided the order of binding interaction strength for the three cases are preserved: Gal3pWT-xylose-Gal80p < Gal3p-Syn4.1-xylose-Gal80p < Gal3p-WT-galactose-Gal80p. The simulations along with experimental data confirm that while at low interaction strengths Gal3p-sugar interaction is inefficient, as the interaction gets stronger, the difference between the two models becomes more prominent with the dual feedback showing increased signal.

Single feedback model:

$$\frac{dG3}{dt} = vgal + \alpha G3 * \frac{[G4]^{n3}}{KG3^{n3} + [G4]^{n3}} - kf83\,[G3][G80] + kr83[C83] - \gamma_{G3}\,[G3]$$

$$\frac{dG4}{dt} = \alpha G4 - kf84\,[G4][G80] - \gamma_{G4}[G4]$$

$$\frac{dG80}{dt} = \alpha_o G80 + \alpha G80 * \frac{[G4]^{n80}}{KG80^{n80} + [G4]^{n80}} - kf83\,[G3][G80] + kr83[C83] - \gamma_{G80}\,[G80] - kf84\,[G3][G80] + kr83[C84] - \gamma_{G80}\,[G80]$$

$$\frac{dGFP}{dt} = \alpha G1 * \frac{[G4]^{n1}}{KG1^{n1} + [G4]^{n1}}$$

$$\frac{dC83}{dt} = kf83\,[G3][G80] - kr83[C83] - \gamma_{C83}[C83]$$

$$\frac{dC84}{dt} = kf84\,[G4][G80] - kr84[C84] - \gamma_{C84}[C84]$$

Dual feedback model:

$$\frac{dG3}{dt} = vgal + \alpha G3 * \frac{[G4]^{n3}}{KG3^{n3} + [G4]^{n3}} + \alpha G1 * \frac{[G4]^{n1}}{KG3^{n1} + [G4]^{n1}} - kf83\,[G3][G80] + kr83[C83] - \gamma_{G3}\,[G3]$$

$$\frac{dG4}{dt} = \alpha G4 - kf84\,[G4][G80] - \gamma_{G4}[G4]$$

$$\frac{dG80}{dt} = \alpha_o G80 + \alpha G80 * \frac{[G4]^{n80}}{KG80^{n80} + [G4]^{n80}} - kf83\,[G3][G80] + kr83[C83] - \gamma_{G80}\,[G80] - kf84\,[G3][G80] + kr83[C84] - \gamma_{G80}\,[G80]$$

$$\frac{dGFP}{dt} = \alpha G1 * \frac{[G4]^{n1}}{KG1^{n1} + [G4]^{n1}}$$

$$\frac{dC83}{dt} = kf83\,[G3][G80] - kr83[C83] - \gamma_{C83}[C83]$$

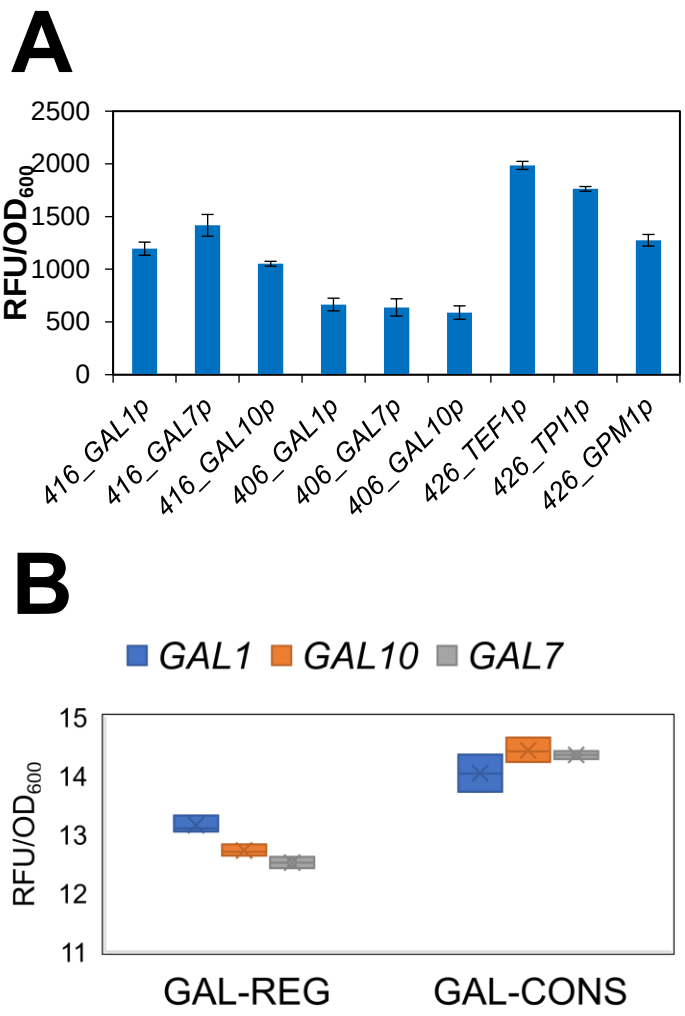
$$\frac{dC84}{dt} = kf84\,[G4][G80] - kr84[C84] - \gamma_{C84}[C84]$$

Appendix Note 2.3: Characterization of expression strengths of *GAL1p/10p* and constitutive promoters on xylose

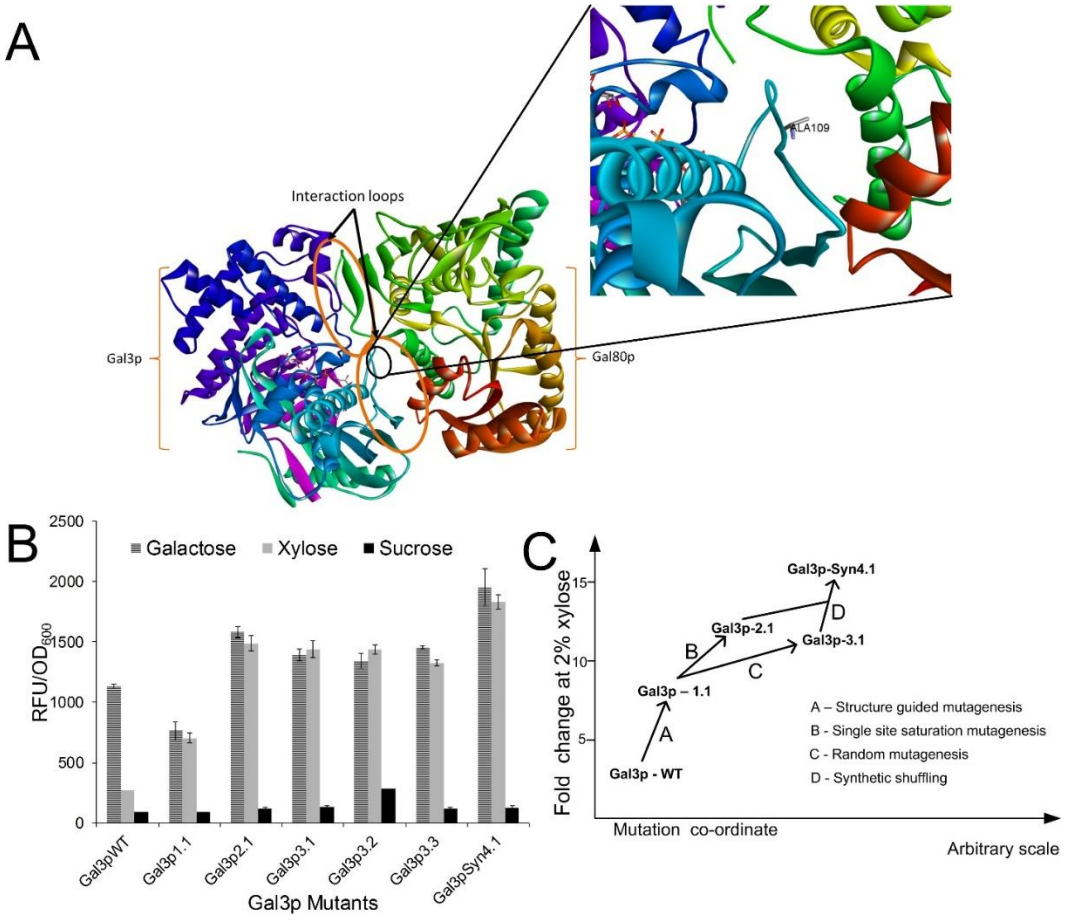
To check if there is a difference in expression strengths between *GAL1p/10p* and *TEF1p/TPI1p* results in observed difference in growth rates of XYL-REG and XYL-CONS strain on xylose, we transformed VEG16 strain with either pRS426-GAL1p, pRS426-GAL10p, pRS426-TEF1p or pRS426-TPI1p plasmid along with XYL regulon plasmids (pVEG16* and pVEG17*) and compared the fluorescence levels after growing the strains on sucrose along with xylose. To replicate the conditions of growth on xylose, we used high copy plasmids. Since XYL-REG and XYL-CONS strains were grown in 2 % xylose, we checked for fluorescence in 2 % xylose, as well at maximum xylose concentration of 4 %. From **Appendix Fig.7a**, it can be seen that the fluorescence at 2 % xylose concentration, constitutive promoters have 1.6-fold higher fluorescence than the GAL promoters and at 4 % xylose concentration, the fluorescence levels remain the same. On the contrary, mRNA expression data from RNA-seq analysis reveal mRNA levels of *XYLA*3* and *XKS1* from XYL-REG to be several folds higher than XYL-CONS **(Appendix Fig.7b)**. It is possible that either increased levels of mRNA doesn't translate completely into protein or presence of sucrose as the growth substrate suppresses GAL regulon resulting in lowered fluorescence of strains carrying pRS426-GAL1p and pRS426-GAL10p plasmids. Either way, the difference in expression strengths of GAL regulated promoters and constitutive promoters alone does not explain the difference in growth rates as increased expression need not directly translate to

increased growth rate. In the case of GAL-CONS, though expression of *GAL1,7,10* genes were several folds higher than in GAL-REG, growth rate was lower. Finally, as seen from RNA-seq analysis **(Fig. 6)**, the increased expression of XYLA*3 and XKS1 in XYL-REG is not sufficient to explain the observed upregulation of several growth-related pathways.

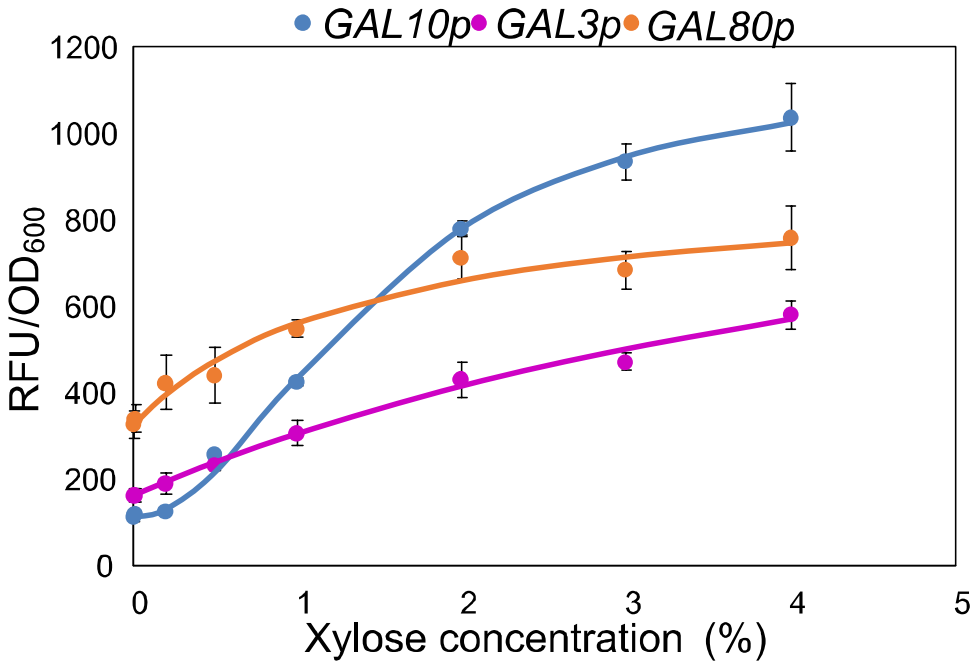
Appendix Figures



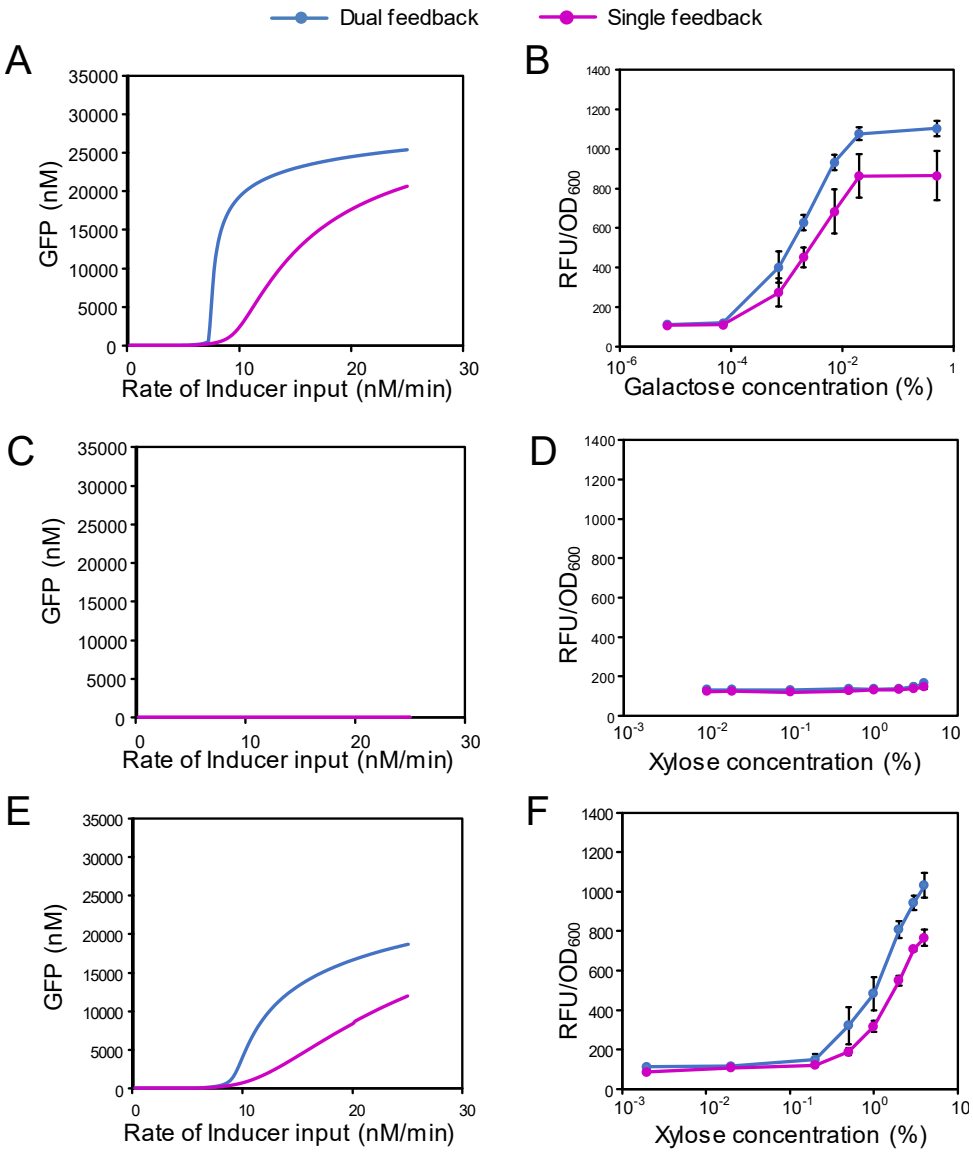
Appendix Figure 2.1: *Comparison of expression strengths of GAL regulated and constitutive promoters when grown on galactose. (A) GAL and constitutitve promoters expressing EGFP in single chromosomal copy (406), low copy plasmid (416), and multicopy plasmid (426). (B) Box and whisker plot of normalized log counts per million mapped reads of GAL1, GAL7, and GAL10 mRNA in GAL-REG (WT) and GAL-CONS strains. Each data point represents average of three biological replicates ± sd.*



Appendix Figure 2.2: *Gal3p-mutagenesis. (A) Crystal structure of Gal3p with the two interaction loops circled in orange (figure adapted from Lavy T, et al., 2012*(Lavy et al., 2012)*). The position of A109 residue is zoomed in to show that A109 faces the Gal80p suggesting that mutation to Thr or Val probably increases this interaction and thus increases the strength of GAL regulon activation by xylose. (B) Fluorescence of Gal3p-WT and the best mutants from successive rounds of mutagenesis were incubated with sucrose alone, 2 % xylose or 2 % galactose. (C) Roadmap to the final mutant Gal3p-Syn4.1 and increase in fold change at 2 % xylose with every successive round of mutation. Each data point represents average of biological triplicates ± sd.*

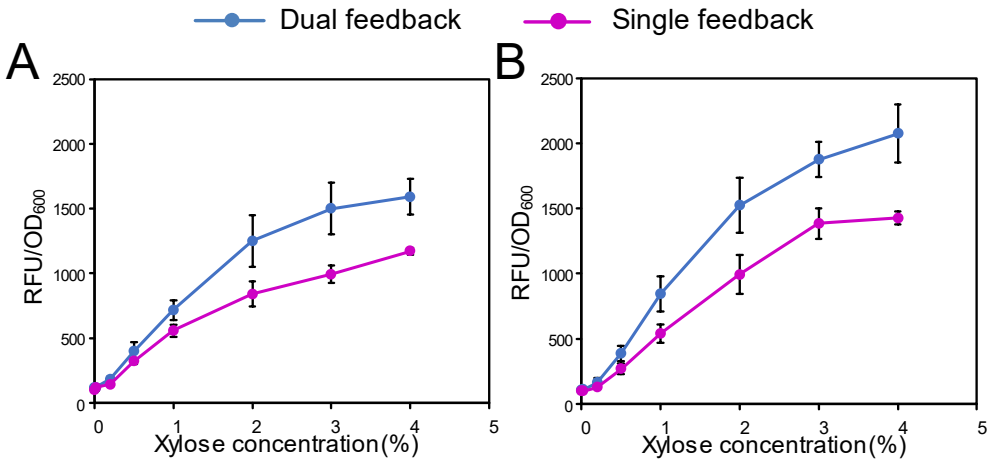


Appendix Figure 2.3: *Fluorescence assay of constructs with GAL10p, GAL3p, and GAL80p promoters driving EGFP expression. Data points represent actual experiments and the lines represent hill curve fits for the data. Each data point represents average of biological triplicates ± sd.*

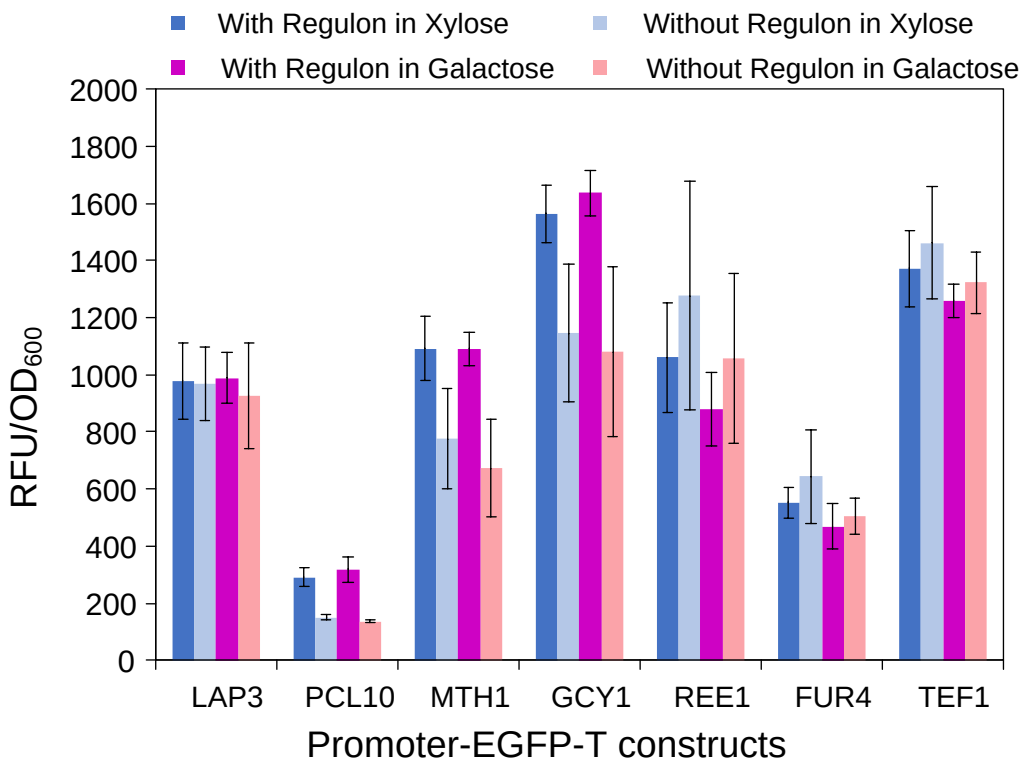


Appendix Figure 2.4: *Comparison of steady state ODE simulation for single and dual feedback models with varying levels of kf83 with experimental data measuring varying interaction strengths of Gal3p-sugar-Gal80p binding. (A & B) Comparison of simulation and experiment for Gal3p-WT-galactose-Gal80p interaction with the model having kf83 of 2.5. (C & D) Comparison of simulation and experiment for Gal3p-WT-xylose-Gal80p interaction with the model having kf83 of 0.1. (E & F) Comparison of simulation and experiment for Gal3p-Syn4.1-xylose-Gal80p*

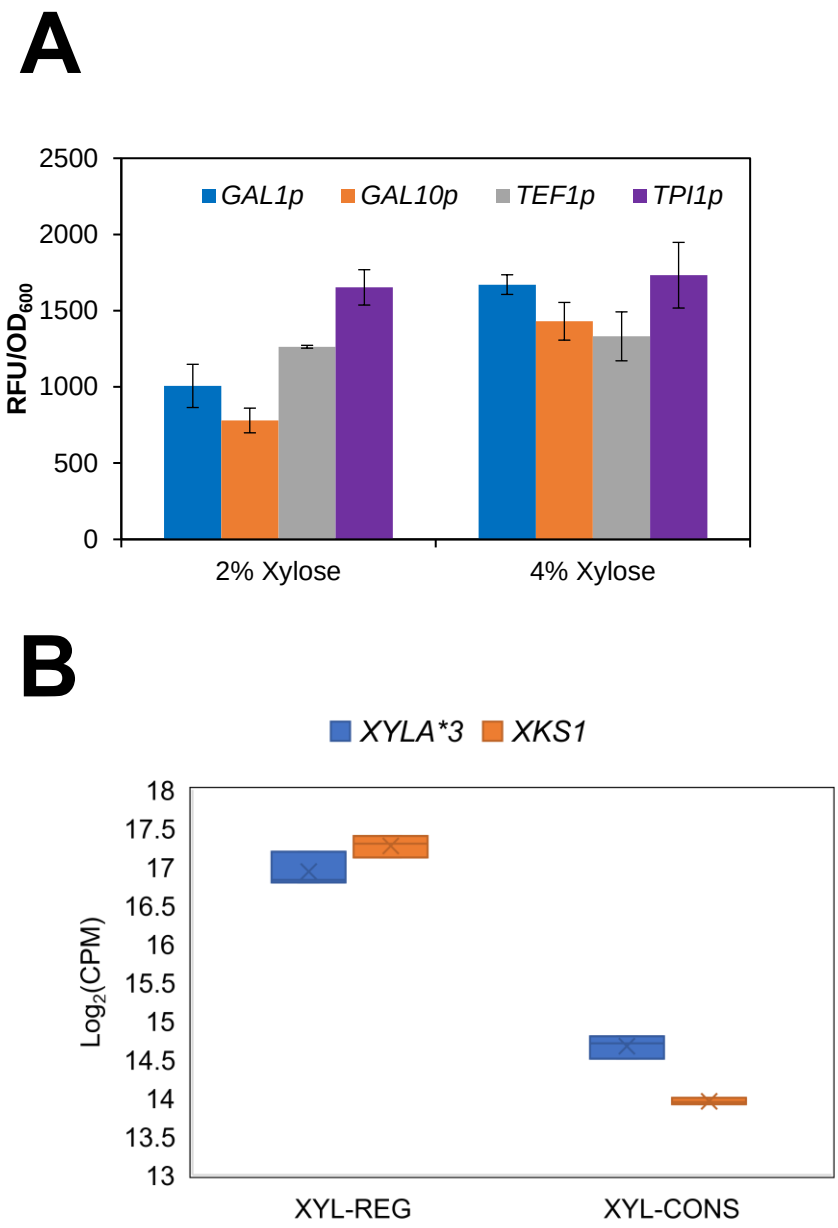
interaction with the model having kf83 of 1. Each data point represents average of biological triplicates ± sd.



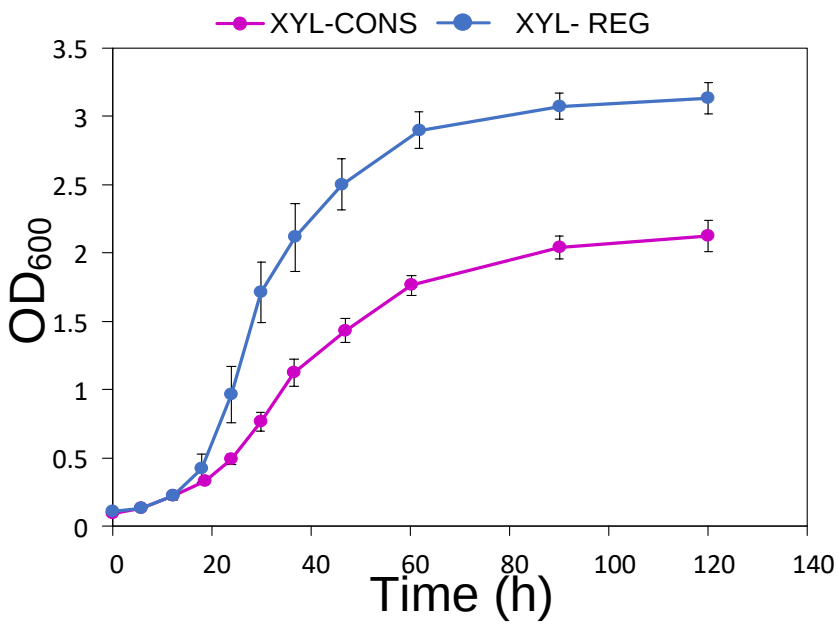
Appendix Figure 2.5: *Increase in sensitivity of dual positive feedback system over single feedback system using (A) GAL1P-EGFP-T and (B) GAL7P-EGFP-T as reporter constructs. Each data point represents average of biological triplicates ± sd.*



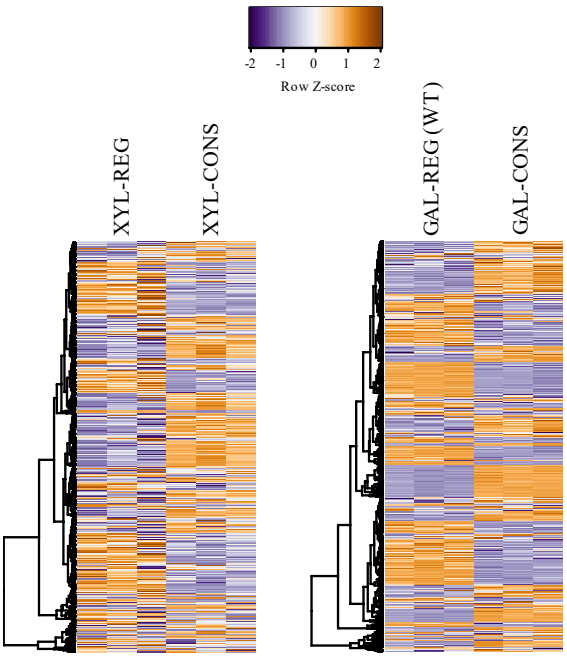
Appendix Figure 2.6: *Some of the promoters that drive the downstream genes of the galactose regulon were used to drive EGFP. Fluorescence was measured in the presence or absence of xylose and galactose regulon when grown on sucrose. Each data point represents average of biological triplicates ± sd.*



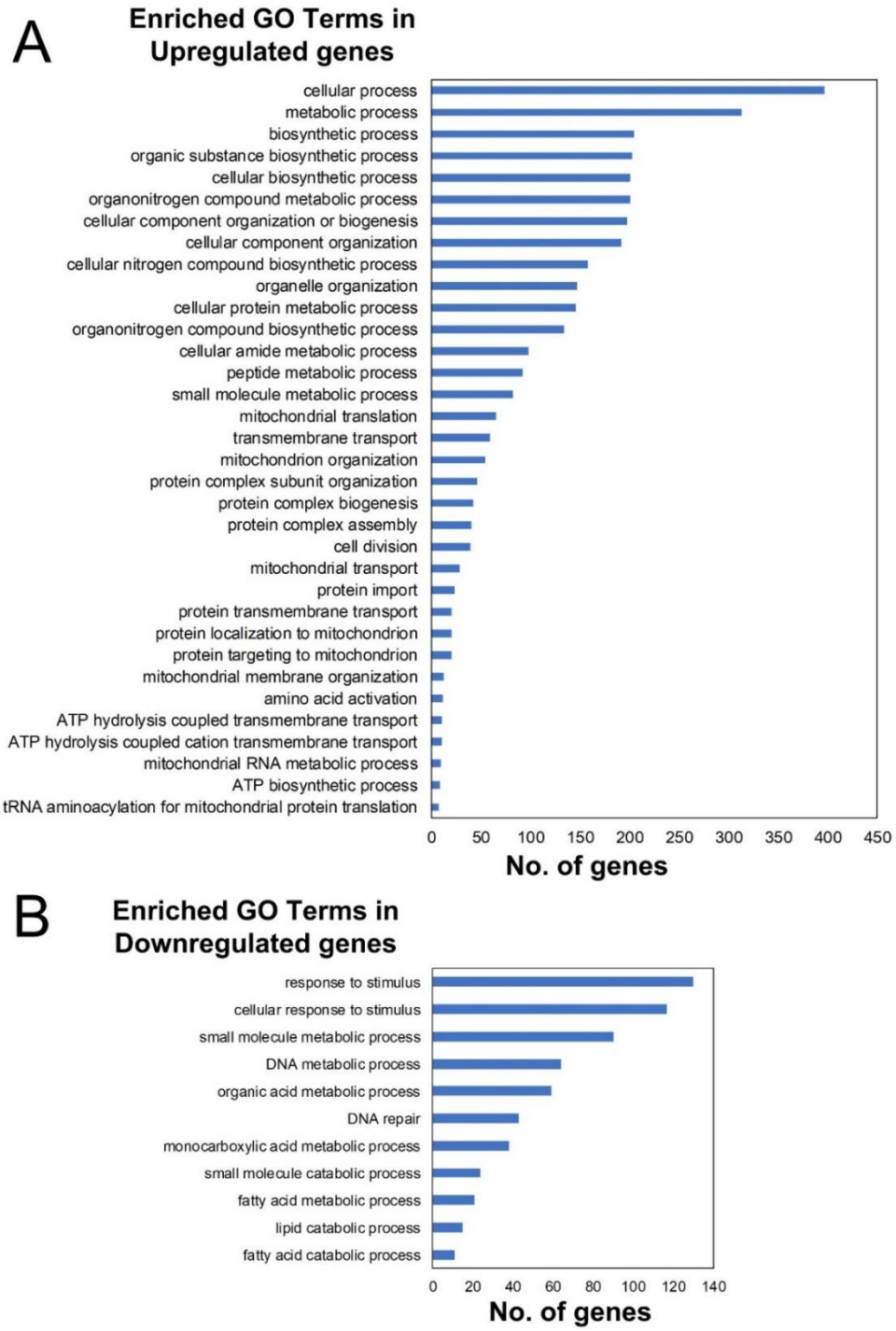
Appendix Figure 2.7: *Comparison of expression strengths of GAL regulated and constitutive promoters when grown on xylose. (A) GAL and constitutitve promoters expressing EGFP when grown on sucrose with 2 % or 4 % xylose. (B) Box and whisker plot of normalized log counts per million mapped reads of XYLA* and XKS1 mRNA in XYL-REG and XYL-CONS strains. Each data point represents average of biological triplicates ± sd.*



Appendix Figure 2.8: *Growth of engineered strains in xylose. (A) Growth of XYL-REG (μ = 0.12 h^{-1}, $OD_{max} \approx 3.2$) and XYL-CONS (μ = 0.07 h^{-1}, $OD_{max} \approx 2.1$) strains in minimal medium supplemented with 2 % xylose*



Appendix Figure 2.9: *Hierarchically clustered heatmap of transcriptome profiles of REG vs CONS strains.*



Appendix Figure 2.10: *GO term enrichment analysis of genes that are (A) upregulated in REG strains (B) downregulated in REG strains, both compared to CONS strains.*

Appendix Tables

Appendix Table 2.1: List of plasmids used.

Plasmids	Description
pCONS-GAL	*pRS426, 2µ ori, URA3, TEF1t-GAL7-GPM1p-ADH1t- GAL10- TPI1p-TEF1p –GAL1-HXT7t*
pVEG7	*pRS426, 2µ ori, URA3, ADH1t-EGFP- GAL1p/GAL10p-KANMX-Hxt7t*
pVEG8	*pRS426, 2µ ori, URA3, GAL3p-GAL3-TEF1t, ADH1t-EGFP-GAL1p/GAL10p-KANMX-HXT7t*
pVEG11	*pRS426, 2µ ori, URA3, ADH1t- Piromyces_XYLA*3- GAL1p/GAL10p -XKS1-HXT7t*
pVEG12	*pRS423, 2µ ori, HIS, ADH1t- TAL1- GAL1p/GAL10p–GAL2-2.1-HXT7t*
pVEG13	*pRS423, 2µ ori, HIS, ADH1t- TAL1- TEF1p-TPI1p –GAL2-2.1-HXT7t*
pVEG15	*pRS426, 2µ ori, URA3, ADH1t- Piromyces_XYLA*3- TEF1p-TPI1p -XKS1-HXT7t*
pVEG16	*pRS414, CEN ori, TRP, GAL3p-GAL3-TEF1t*
pVEG17	*pRS415, CEN ori, LEU, GAL1p-GAL3-TEF1t*
pVEG16*	*pRS414, CEN ori, TRP, GAL3p-GAL3-Syn4.1-TEF1t*
pVEG17*	*pRS415, CEN ori, LEU, GAL1p-GAL3-Syn4.1-TEF1t*
pVEG16^{C}	*pRS414, CEN ori, TRP, TEF1p-GAL3-Syn4.1-TEF1t*
pRS405-GAL2-2.1	*pRS405, no ori, LEU, GAL2p-GAL2-2.1-TEF1t*
pRS426-GAL1p	*pRS426, 2µ ori, URA3, GAL1p-EGFP-ADH1t*
pRS426-GAL3p	*pRS426, 2µ ori, URA3, GAL3p-EGFP-ADH1t*
pRS426-GAL80p	*pRS426, 2µ ori, URA3, GAL80p-EGFP-ADH1t*
pRS426-GAL7p	*pRS426, 2µ ori, URA3, GAL7p-EGFP-ADH1t*
pRS426-GAL10p	*pRS426, 2µ ori, URA3, GAL10p-EGFP-ADH1t*

pRS416-GAL1p	*pRS426, CEN ori, URA3, GAL1p-EGFP-ADH1t*
pRS416-GAL7p	*pRS426, CEN ori, URA3, GAL7p-EGFP-ADH1t*
pRS416-GAL10p	*pRS426, CEN ori, URA3, GAL10p-EGFP-ADH1t*
pRS406-GAL1p	*pRS406, URA3, GAL1p-EGFP-ADH1t*
pRS406-GAL7p	*pRS406, URA3, GAL7p-EGFP-ADH1t*
pRS406-GAL10p	*pRS406, URA3, GAL10p-EGFP-ADH1t*
pRS426-REE1p	*pRS426, 2μ ori, URA3, REE1p-EGFP-ADH1t*
pRS426-LAP3p	*pRS426, 2μ ori, URA3, LAP3p-EGFP-ADH1t*
pRS426-PCL10p	*pRS426, 2μ ori, URA3, PCL10p-EGFP-ADH1t*
pRS426-FUR4p	*pRS426, 2μ ori, URA3, FUR4p-EGFP-ADH1t*
pRS426-MTH1p	*pRS426, 2μ ori, URA3, MTH1p-EGFP-ADH1t*
pRS426-GCY1p	*pRS426, 2μ ori, URA3, GCY1p-EGFP-ADH1t*
pRS426-TEF1p	*pRS426, 2μ ori, URA3, TEF1p-EGFP-ADH1t*
pRS426-TPI1p	*pRS426, 2μ ori, URA3, TPI1p-EGFP-ADH1t*
pRS426-GPM1p	*pRS426, 2μ ori, URA3, GPM1p-EGFP-ADH1t*

Appendix Table 2.2: List of strains used.

Strains	**Description**
W303-1a (GAL-REG)	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*
VEG6	*W303-1a, ΔGAL3*
VEG16	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10*

VEG20	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10; LEU::GAL2p-GAL2-2.1-TEFt*
CONS-GAL	*W303-1a, ΔGAL4;* transformed with pCONS-GAL
CONS-GAL-*GAL4*	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10;* transformed with pCONS-GAL
XYL-CONS	VEG16 transformed with pVEG15, pVE13, pRS41416 and pRS415
XYL-REG	VEG16 transformed with pVEG11, pVE12, pVEG16* and pVEG17*
XYL-REG^{SF}	VEG16 transformed with pVEG11, pVE12, pVEG16* and pRS415
XYL-REG^{C}	VEG16 transformed with pVEG11, pVE12, pVEG16^{C} and pRS415
GAL-Gal3p-Syn4.1	VEG6 transformed with pVEG16$^{Syn4.1}$
VEG21	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10; URA3::GAL10p-EGFP-TEFt*
VEG22	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10; URA3::GAL1p-EGFP-TEFt*
VEG23	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10; URA3::GAL7p-EGFP-TEFt*

Appendix Table 2.3: List of constants used in single and dual feedback models

Parameter	**Description**	**Units**	**Values**
kf83	Forward binding rate constant of Gal3p and Gal80p	$(nM.min)^{-1}$	2.5
kr83	Unbinding rate constant of Gal3p and Gal80p	$(min)^{-1}$	462
kf84	Forward binding rate constant of Gal4p and Gal80p	$(nM.min)^{-1}$	100
kr84	Unbinding rate constant of Gal4p and Gal80p	$(min)^{-1}$	1300
$\alpha G1$	Production rate of Gal3p from *GAL1p* promoter	$nM.min^{-1}$	35

$\alpha G3$	Production rate of Gal3p	nM.min^{-1}	8
$\alpha G4$	Production rate of Gal4p	nM.min^{-1}	3.6
$\alpha G80$	Production rate of Gal80p	nM.min^{-1}	9
$\alpha_o G80$	Basal production rate of Gal80p	nM.min^{-1}	5.9
KG3	Transcriptional feedback threshold of Gal3p	nM	64.9
KG80	Transcriptional feedback threshold of Gal80p	nM	1.5
n1	Hill Coefficient for Gal4p binding to *GAL1p* promoter	Dimensionless	2
n3	Hill Coefficient for Gal4p binding to *GAL3p* promoter	Dimensionless	1
n80	Hill Coefficient for Gal4p binding to *GAL80p* promoter	Dimensionless	1
γ_{G3}	Gal3p decay rate	min^{-1}	0.004
γ_{G4}	Gal4p decay rate	min^{-1}	0.0119
γ_{G80}	Gal80p decay rate	min^{-1}	0.0073
γ_{C83}	Gal3p-Gal80p complex decay rate	min^{-1}	0.0527
γ_{C84}	Gal4p-Gal80p complex decay rate	min^{-1}	0.0177

Chapter 3

Extensions of regulon engineering

3.1 Abstract

Regulon Engineering can be used to integrate non-native heterologous substrate catabolic pathways into yeast metabolic network while taking into consideration the existing regulatory infrastructure used by the host for cell growth and metabolism. Although regulon engineering is faster and aids growth better than conventional approaches, it suffers from certain limitations, which are addressed in this chapter. In the previous chapter, I showed that the downstream genes of the GAL regulon aid growth in D-galactose and D-xylose. However, it is not clear if these genes can assist growth in other non-native substrates. To shed light on this issue, we employed regulon engineering for catabolic engineering of L-arabinose in *S. cerevisiae*. We achieved higher growth rates as well as final cell densities when compared to *S. cerevisiae* strain engineered using conventional constitutive overexpression strategy. Another limitation with regulon engineering is the need to engineer the sensory protein (Gal3p) to interact with the non-native sugar that has to be metabolized. While the sensor can be engineered easily to interact with galactose-like substrates, engineering to detect and interact with structurally different substrates would involve the extensive reshaping of the binding site as well as the entire protein. To avoid this, we constructed self-activating Gal3p mutants that need not interact with a sugar to activate the regulon. We used this approach to efficiently metabolize xylose and arabinose with high growth rates. Overall, this chapter extends the possibilities of regulon engineering while addressing some of the limitations present in this approach, thereby enabling rapid design and construction of strains

capable of growing in non-native substrates at high-growth rates without the need for extensive engineering and other time-consuming approaches.

3.2 Introduction

In the previous chapter, I demonstrated the advantages of a regulon in promoting growth and the benefits offered by engineering each aspect of the regulon- sensing, signal amplification, and metabolism, for a non-native substrate xylose. There are many unanswered questions from the previous chapter. First, it is not clear if the technique can be extended to other non-native substrates. Secondly, while transcriptomics analyses showed hundreds of genes differentially regulated in the REG strains, the exact set of genes that are activated by the regulon is unknown. Further, if those genes are either directly or indirectly regulated and how they aid in cell growth remains to be elucidated. Finally, one of the limitations with extending this technique for every non-native carbon source is the need to incorporate dynamic GAL regulon activation for every one of those substrates that need to be metabolized by yeast. Answering these could further expand our understanding of the regulatory infrastructure, how extensive they are, and also provide us with cues for fine-tuning the regulon depending on the substrate that needs to be utilized. This chapter addresses some of these questions.

To assess if regulon engineering can be extended for other non-native substrates, we developed a synthetic arabinose regulon for efficient metabolism and cell growth. Arabinose is the second most abundant pentose in the hemicellulosic fraction of lignocellulose and usually makes up for ≈ 2% of the total biomass (van Maris et al., 2006). In the case of other sources such as pectin hydrolysates, corn fiber, etc., arabinose content of up to 20% is present (Grohmann & Bothast, 1994; Lynd, van Zyl, McBride, & Laser, 2005; Saha, Dien, & Bothast, 1998). Since corn fibers has the highest arabinose content among biomass feedstocks, the lignocellulosic biofuels industry is focused on research in developing effective arabinose-utilizing strains (Jansen et al., 2017).

Significant research years have been expended in trying to engineer arabinose metabolism in *S. cerevisiae* either through the oxidoreductive or isomerase pathways. However, oxidoreductive pathways, in general, have resulted in low growth rates, increased arabinitol secretion and slow arabinose consumption, due to increased cofactor imbalance (S. M. Lee et al., 2016; Wisselink et al., 2009). In the case of isomerase pathway, *L. plantarum* and *B. subtilis* genes of arabinose metabolism, *AraA, AraB,* and *AraD* have been expressed in yeast for growth (Becker & Boles, 2003; Sedlak & Ho, 2001; Wiedemann & Boles, 2008; Wisselink et al., 2007). The highest growth rate of 0.15 h^{-1} was achieved by expressing genes of non-oxidative pentose phosphate pathway along with *AraA, AraB* and *AraD* genes from *L. plantarum*, followed by adaptive evolution for a period of five months (Wisselink et al., 2007). While research in recent years have focused on ethanol production, as well as co-utilization of glucose, xylose, and arabinose with moderate levels of success, the underlying cause for decreased growth rates and the need to carry out extensive evolutionary engineering has never been addressed.

In this chapter, by using an arabinose sensitive Gal3p, I activated the GAL regulon in the presence of arabinose. Further, by placing genes of arabinose metabolism under the control of GAL promoters, we created a regulon capable of arabinose-sensing, signal transduction, and metabolism. When compared to conventionally engineered arabinose metabolic strain where metabolic genes are expressed constitutively, the regulon-controlled strain exhibited superior growth rate as well as higher biomass accumulation, confirming that the approach can be generalized for other non-native substrates.

However, for regulon engineering to be applied for any non-native carbon source, Gal3p must be engineered to bind to the substrate, and activate the regulon. Gal3p mutations to accommodate xylose and arabinose was achieved relatively easily, partly owing to the structural similarity of

these sugars to galactose. To accommodate substrates such as cellobiose, a disaccharide or single carbon substrate such as methanol or methane, radical alterations in the active site would be required. One way to get around the issue and activate galactose regulon is to delete the transcriptional repressor Gal80p (Ryo et al., 2012; P. Zhou et al., 2018). Although this modification results in increased galactose consumption, an attractive trait, the flux is re-routed primarily towards ethanol production and does not contribute to growth rate increase (Ostergaard et al., 2000). Overexpressing Gal4p also results in a similar flux re-allocation (Ostergaard et al., 2000). To produce value-added products from galactose, flux re-routing towards ethanol should be avoided. Hence, as an alternative, we used a Gal3p mutant that interacts with Gal80p even in the absence of a sugar. Previous studies on Gal3p have shown two mutants, F237Y and S509P to be self-activating (Blank, Woods, Lebo, Xin, & Hopper, 1997). Using these mutants in combination with our previous Gal3p mutant, we achieved regulon activation without the need for any interaction with a sugar ligand. Further, we tested the applicability of this technique, by assessing the growth of strains carrying these mutants on xylose and arabinose. The growth rates for both xylose and arabinose increased significantly, holding promise for developing a generalized platform strain for catabolic engineering.

3.3 Results

3.3.1 Regulon Engineering extension: Semi-synthetic regulons for non-native sugar, arabinose (Book no. 6, page 10 -93)

As discussed in the previous chapter, for regulon engineering, interventions at three different domains is required. First, Gal3p must be engineered to interact with the substrate and elicit the

regulon response. Next, the signal transduction architecture of the GAL regulon must be engineered, and finally, the genes for substrate metabolism must be expressed under GAL promoters. However, since we have demonstrated the benefits and characteristics of having a dual feedback architecture for the regulon, the same architecture can be retained for arabinose metabolism. Hence interventions only on the sensory and metabolic components are required **(Fig 3.1)**.

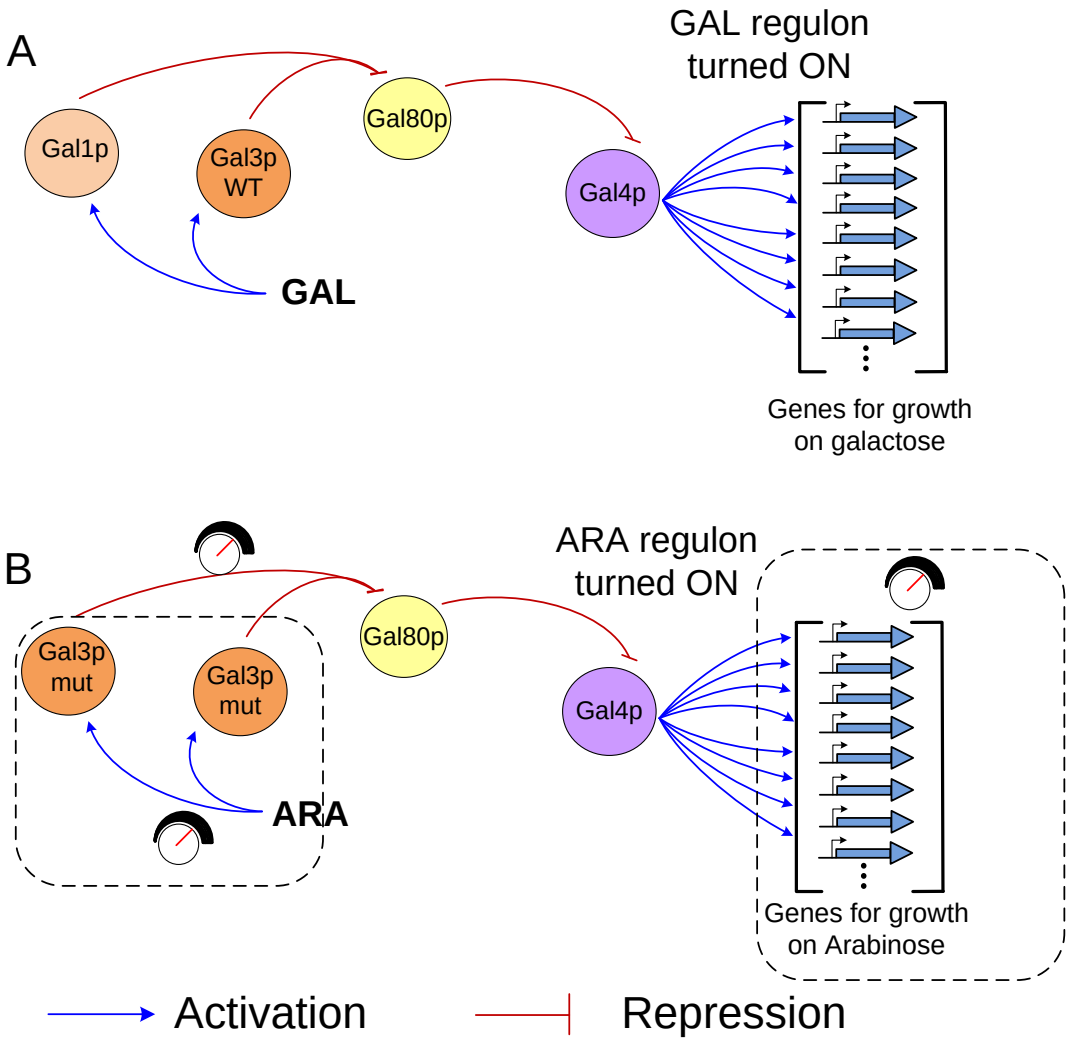


Figure 3.1: Design of a synthetic GAL-type arabinose regulon. *(A) Schematic of galactose-based activation of the GAL regulon, which was discussed in the previous chapters. (B) Engineering of arabinose regulon. Every stage of the regulon where interventions are necessary is marked with a tuning valve. Of the three interventions, we use the same dual feedback architecture previously used for xylose regulon. The engineering modifications that must be introduced for arabinose regulon are represented by black dotted boxes.*

3.3.1.1 Engineered Gal3p variant that interacts with arabinose

To develop an arabinose-sensitive Gal3p, we employed the same reporter strains (VEG16 and VEG20) as well as the selection strategy that was used for engineering the xylose-sensitive Gal3p. Thus, the interaction of Gal3p with arabinose will activate GAL regulon and will be measured as fluorescence output **(Fig 3.2A)**. First, we tested the arabinose-Gal3p-WT interaction strength. The fluorescence profile revealed a stronger interaction than was previously observed with xylose **(Fig 3.2B)**. This can partly be explained by the fact that arabinose is more similar to galactose than xylose and differs only in the absence of hydroxymethyl group (-CH_2OH) at C6 position. However, fluorescence is observed only at high concentrations of arabinose, which is not ideal to be used as a transcriptional activator. Arabinose has been previously shown to activate *GAL2*, corroborating the results obtained (Cirillo, 1968; Kou, Christensen, & Cirillo, 1970). Since xylose and galactose with sufficient interaction strengths, we hypothesized that Gal3p-Syn4.1 would be able to also interact with arabinose with a reasonable affinity. We tested the affinity of Gal3p-Syn4.1 towards arabinose by titrating with different concentrations of the activation sugar (0.002%, 0.02%, 0.2%, 2% and 4% arabinose) **(Fig 3.2C)**. Gal3p-WT interacts with arabinose better than xylose and

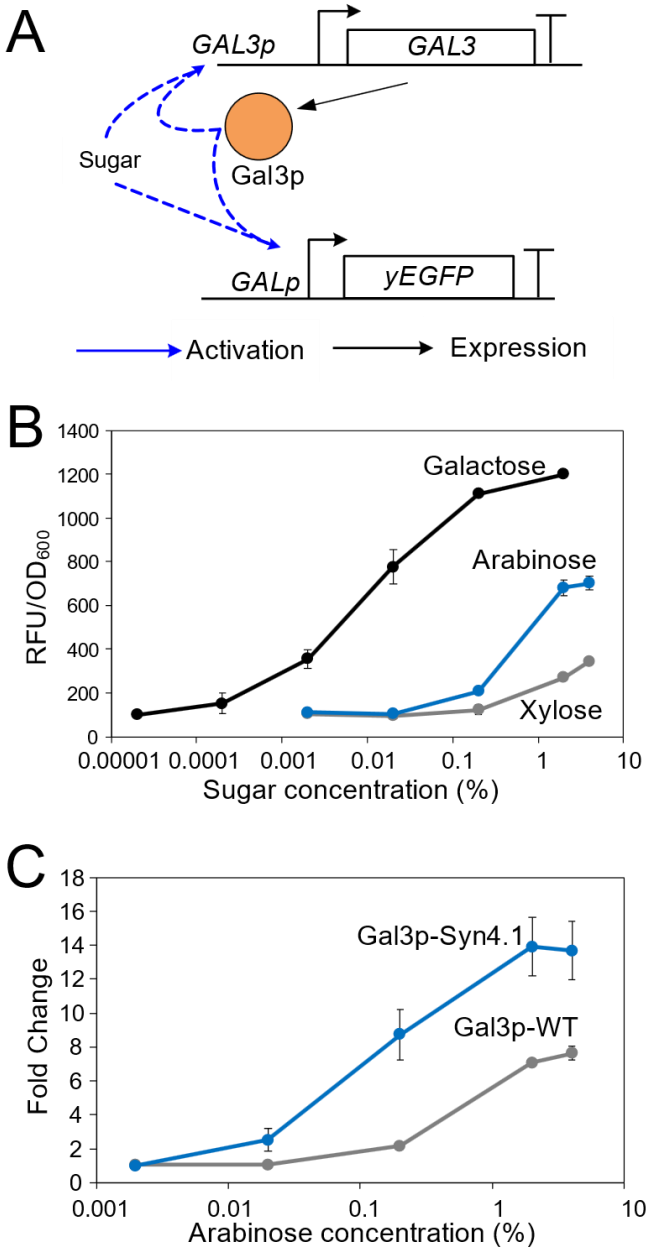


Figure 3.2: Gal3p interaction with arabinose*. A) Selection strategy used for assessing the interaction strength of Gal3p and sugars. Presence of a sugar that can activate the regulon, would result in expression of EGFP that is placed under GAL controlled promoter. B) Fluorescence profile of Gal3p-WT with sugars, galactose, xylose and arabinose. C) Fold change of fluorescence in the presence over absence of arabinose for Gal3p-WT and Gal3p-Syn4.1. Each data point represents the average of two biological replicates ± sd.*

Gal3p-Syn4.1 mutant interacts with both Gal3p-Syn4.1 had a 14-fold change in fluorescence in

the presence of arabinose at 2% when compared to fluorescence without the activation sugar. More importantly, the fluorescence profile of Gal3p-Syn4.1 saturated at 2% and exhibited higher sensitivity than Gal3p-WT at 0.2% arabinose. Together, the data suggest that the mutant, Gal3p-Syn4.1 is sufficiently flexible to accommodate multiple sugars, galactose, xylose, and arabinose.

3.3.1.2 Semi-synthetic ARA regulon enables better growth on arabinose

To create an arabinose-sensing and -metabolizing regulon, we decided to retain the regulatory architecture that was designed for xylose metabolism and incorporate arabinose metabolizing genes under the regulon. The highest growth rate achieved on arabinose in *S. cerevisiae* used bacterial arabinose isomerase pathway genes extracted from *L. plantarum* (Wisselink et al., 2007). Hence, we amplified the protein-coding regions of *AraA* (arabinose isomerase), *AraB* (ribulokinase) and *AraD* (ribulose-5-phosphate-4-epimerase) from *L. plantarum*, placed them downstream of *GAL1p*, *GAL10p* and *GAL7p* promoters respectively, and cloned them in multicopy plasmids, pRS426 and pVEG12, to construct pVEG37 and pVEG38 respectively. Finally, we transformed the two plasmids along with plasmids, pVEG16* and pVEG17* required for dual feedback in VEG16 to create a regulon-assisted arabinose growing strain, ARA-REG. As a metabolic control, we placed the genes *AraA*, *AraB* and *AraD* under the control of strong constitutive promoters, *TEF1p*, *TPIp*, and *GPM1p*, cloned them in multicopy plasmids, pRS426 and pVEG13, to construct pVEG39 and pVEG40 respectively. We transformed the plasmids along with empty vectors, pRS414 and pRS415 in VEG16 to create the ARA- CONS strain.

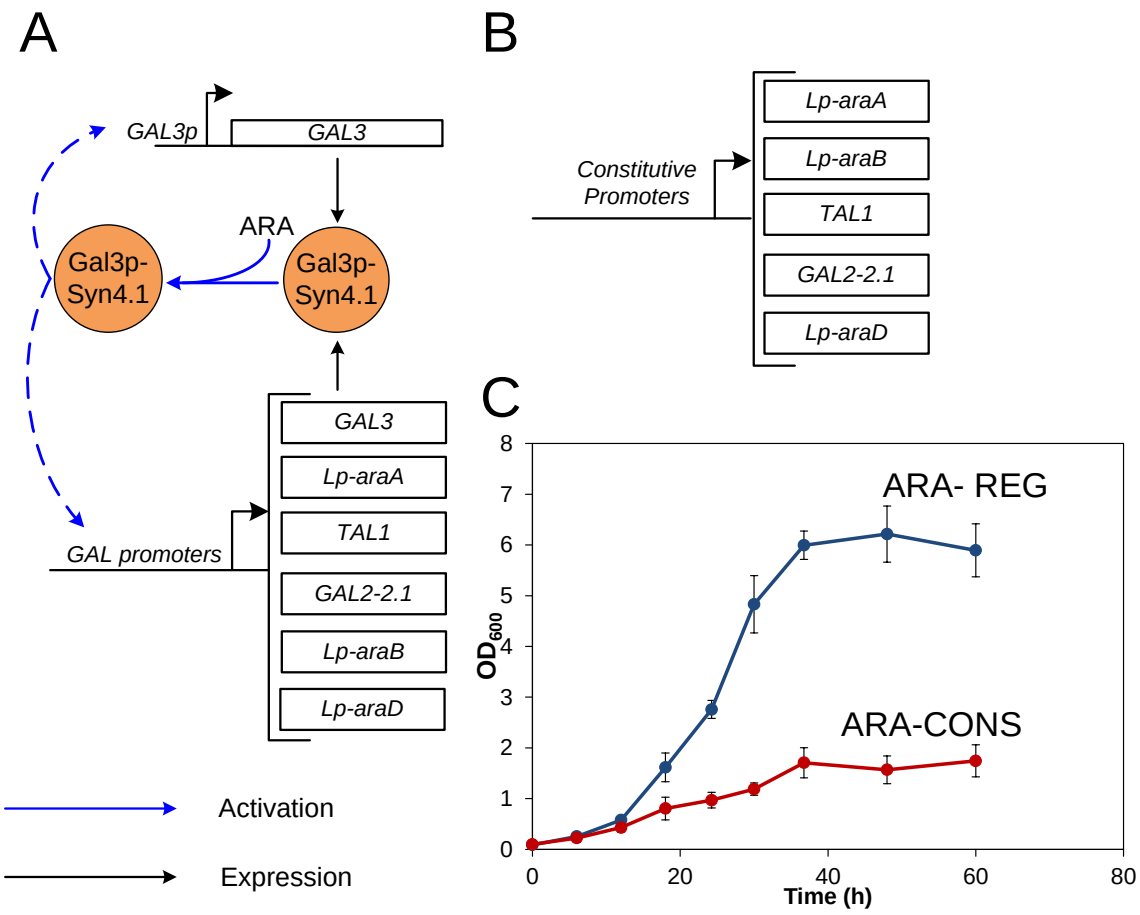


Figure 3.3: Growth on arabinose facilitated by the arabinose regulon. *(A) Design of the genes placed under the synthetic regulon and mode of activation of the arabinose regulon. Apart from GAL3-Syn4.1 placed under GAL3p and GAL1p promoters, other metabolic genes araA, araB, araD, TAL1, and pentose transporter GAL2-2.1 were expressed under GAL promoters. (B) Design of the metabolic control strain (XYL-CONS) built by placing the genes araA, araB, araD, TAL1, and GAL2-2.1 under strong constitutive promoters. (C) Growth of the engineered strains, ARA-REG, and metabolic control, ARA-CONS under aerobic conditions. Each data point represents average of two individual biological replicates ± sd.*

Next, we tested the growth profile and biomass accumulation of both these strains by growing them in YP media supplemented with 2% arabinose **(Fig 3.3C)**. ARA-CONS exhibited a growth rate of 0.05 h^{-1} and a maximum OD_{600} of 1.7. In contrast, we observed a growth rate of 0.13 h^{-1}

and a maximum OD_{600} of 6.2 with ARA-REG. This represents a 2.5-fold increase in growth rate, a 3.5-fold increase in cell density and the highest growth rate achieved on arabinose by *S. cerevisiae* without any evolutionary adaptation. It seems that activating GAL regulon assists growth on not just the native sugar, galactose but also other non-native sugars, xylose, and arabinose. Thus, regulon engineering holds promise to be used as a general catabolic engineering strategy for incorporating any non-native catabolic pathway into *S. cerevisiae* metabolism.

3.3.2 Regulon Engineering extension: Self-activating Gal3p for rapid metabolic engineering (Book no. 7, page 64-79)

In order to engineer xylose and arabinose regulons, we re-engineered Gal3p to bind to the non-native sugar and activate the native GAL regulon. This strategy worked for xylose and arabinose which are pentoses and are structurally similar to galactose. However, in the case of structurally different substrates such as cellobiose or methanol, extensive modifications to Gal3p might be necessary. To resolve this issue, a mutant of Gal3p that retains the closed conformation required for GAL regulon activation, even in the absence of an activation sugar needs to be designed and constructed.

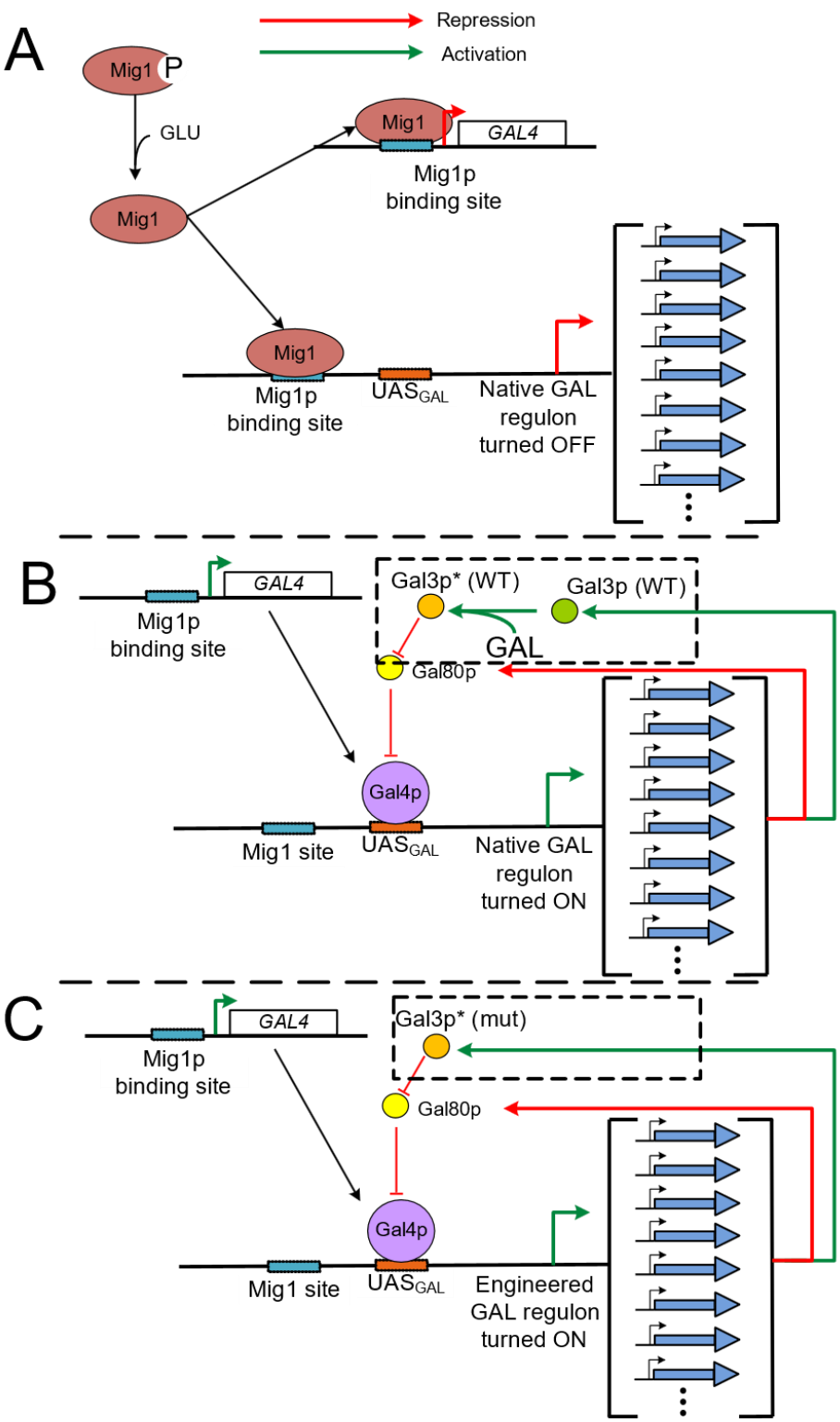

A
Repression
Activation
Mig1
P
GLU
Mig1
Mig1
GAL4
Mig1p
binding site
Mig1
Mig1p
binding site
UAS$_{GAL}$
Native GAL
regulon
turned OFF
B
GAL4
Mig1p
binding site
Gal3p* (WT)
Gal3p (WT)
GAL
Gal80p
Gal4p
Mig1 site
UAS$_{GAL}$
Native GAL
regulon
turned ON
C
GAL4
Mig1p
binding site
Gal3p* (mut)
Gal80p
Gal4p
Mig1 site
UAS$_{GAL}$
Engineered
GAL regulon
turned ON

***Figure 3.4**: **Design of the self-activating Gal3p.** A) GAL regulon in turned OFF in the presence of glucose. Mig1p is dephosphorylated in the presence of glucose, allowing it to enter the nucleus, resulting in repression of the GAL regulon by binding to Mig1p binding sites at upstream region of GAL4 as well as other GAL activated promoters. B) GAL regulon is turned ON in the presence of galactose by Gal3p. Gal3p changes conformation after binding with galactose and de-represses Gal4p by forming a Gal3p-Gal80 complex, leading to activation of the GAL regulon. C) Design of a self-activating Gal3p mutant that de-represses Gal4p and forms Gal3p-Gal80p complex even in the absence of galactose. Difference in mechanism of GAL regulon activation between Gal3p(WT) and Gal3p(mut) are highlighted using black dotted rectangles. Activated Gal3p mutants are represented as Gal3p*.*

3.3.2.1 Design and engineering of a self-activating Gal3p mutant

The activation of Gal3p, normally occurs in the presence of ATP and an activation sugar, usually galactose. However, studies have demonstrated constitutive overexpression of Gal3p to result in GAL regulon activation, suggesting that a subpopulation of Gal3p can interact with Gal80p even in the absence of galactose (Bhat & Hopper, 1992). Other reports have identified mutants of Gal3p that favor the Gal3p-Gal80p interaction even in the absence of a sugar (Blank et al., 1997). A number of these mutants have been analyzed in the context of Gal3p structure (Lavy, Yanagida, & Tawfik, 2016). Some of the mutations such as V69E/D70V, F237Y, D368V and S509P lie near the pivot region of Gal3p, region involved in the formation of the closed conformation when Gal3p interacts with a sugar. Such a conformational change exposes the residues that interact with Gal80p, thereby favoring Gal3p-Gal80p interaction. For example, the amino acid phenylalanine at 237 position lies in a hydrophobic pocket made of F247, M403, L65, F414, F418, and I245, in the open conformation. However, in the closed conformation, the phenylalanine residue moves to a more hydrophilic location. So, it was reasoned that F237Y mutation facilitates a hydrophilic interaction with S509, similar to the closed conformation. Such an alteration in the local position

would achieve the same output as binding to a sugar, leading to Gal3p-Gal80p interaction and activation of the GAL regulon (Lavy et al., 2016). Careful analysis of the constitutive Gal3p mutants from the work of Blank et al., revealed mutations F237Y and S509P to have the highest Gal3p activation response in the absence of galactose. By expressing alpha-galactosidase under GAL promoters, they assessed the activity of the mutants with or without galactose. The two mutants not only showed increased activity without galactose but in the presence of the sugar, the wildtype activity was restored. The results show that the Gal3p mutants favor Gal3p-Gal80p interaction and only a fraction of the population remains unbound to Gal80p. Using such self-activating mutants of Gal3p would obviate the need for an external activation sugar. However, regulation can still be preserved, as glucose represses GAL regulon through Mig1p. In the presence of glucose, Mig1p gets dephosphorylated by Reg1p-Glc7p phosphatase, enabling Mig1p to translocate into the nucleus and repress *GAL4* as well as other genes of the GAL regulon (Schüller, 2003). In the absence of glucose, Mig1p is phosphorylated by Snf1p kinase complex, resulting in export of the protein from the nucleus, thereby relieving glucose based repression (Santangelo, 2006; Schüller, 2003) **(Fig 3.4A)**. Thus, with self-activating mutants of Gal3p, the control of GAL regulon activation would shift from the presence of an activation sugar and absence of glucose, to solely on the absence of glucose **(Fig 3.4 B, C)**.

As a first step, we mutated Gal3p-WT to constitutive Gal3p variants, F237Y, S509P, and a third variant that contained both these mutations, using OE-PCR. We studied these mutants for their ability to express GFP in the reporter strain VEG16 carrying the fluorescence selection system **(Fig 3.5A)**. For the fluorescence assay, we pre-grew the Gal3p variants (wildtype as well as the mutants) on glucose for 24 hours, followed by sub-culturing either in the same medium or medium supplemented with sucrose, along with different activation sugars, galactose, xylose, or arabinose

for 18 hours. We observed increased fluorescence among all of the conditions tested for the F237Y mutant when compared to Gal3p-WT **(Fig 3.5B)**. In the presence of glucose, we observed a 3-fold increase in fluorescence and in the absence of the sugar, the fluorescence increased 7-fold. Additionally, when galactose or xylose was added, the fluorescence increased even further, reaching levels observed for Gal3p-Syn4.1. However, in the presence of arabinose, fluorescence did not increase, suggesting that the mutant is incapable of binding with arabinose. In the case of S509P mutant, we did not observe any fluorescence increase compared to Gal3p-WT, irrespective of the conditions tested, suggesting that the mutation failed to activate the GAL regulon in the absence of glucose. Moreover, the mutant strain carrying both F237Y & S509P mutations exhibited similar fluorescence profile as that of F237Y mutation, further confirming that S509P mutation does not aid in creating a self-activating Gal3p.

Next, to test if better transport of sugars or a Gal3p mutant with increased sugar sensitivity would have an impact, we introduced F237Y mutation on Gal3p-Syn4.1 mutant, to create Gal3p-5.1, and tested the effect in VEG20. We assessed the fluorescence levels in different sugars **(Fig 3.5C)**. Gal3p-5.1 mutant showed clear fluorescence increase when compared to WT-F237Y, especially when incubated with xylose or galactose. Although it has been shown that Gal3p-Syn4.1 mutant can interact with arabinose **(Fig 3.2)**, the Gal3p-5.1 mutant showed no increase in fluorescence when incubated with arabinose. The fluorescence levels of Gal3p-5.1 mutant incubated with either sucrose or sucrose and arabinose were similar, suggesting arabinose doesn't interact with Gal3p-5.1. Taken together, it is clear that F237Y mutation prevents arabinose-binding while allowing interactions with galactose and xylose.

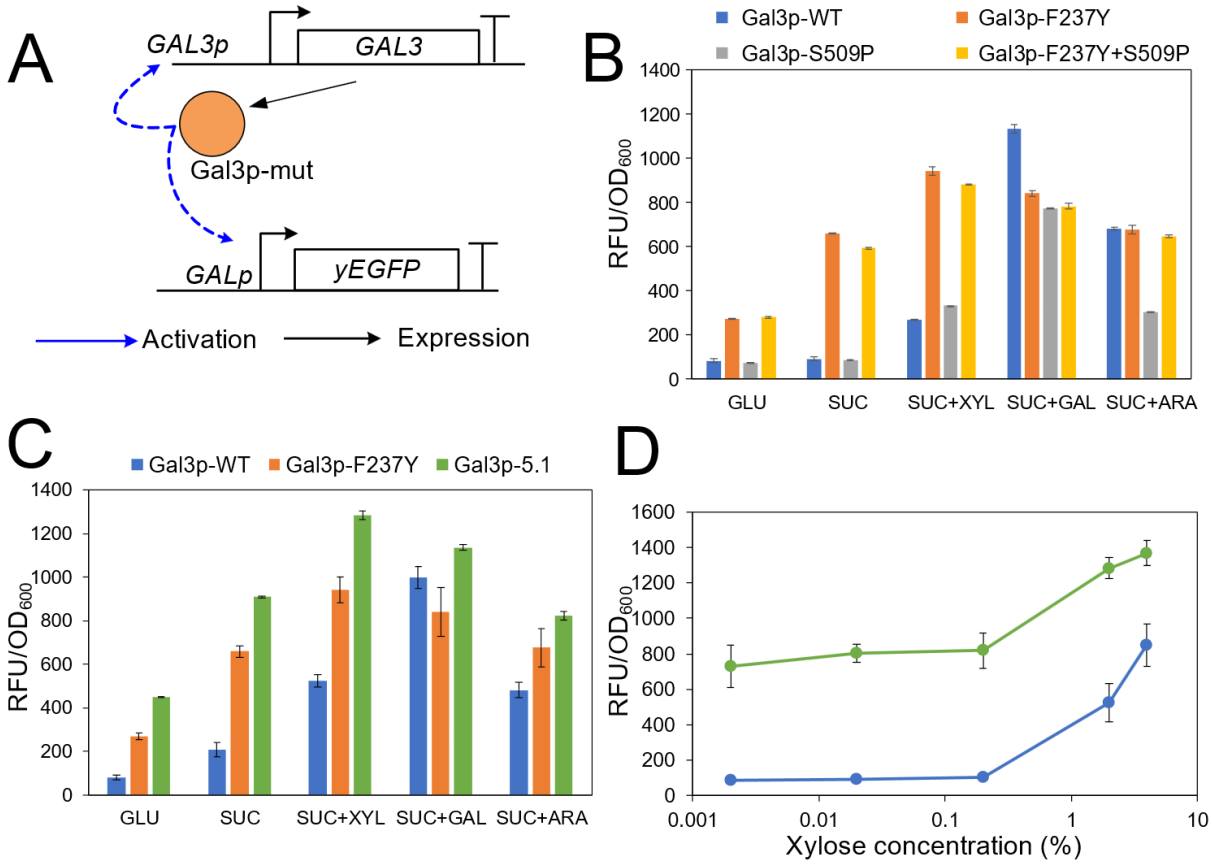


***Figure 3.5**: **Engineering a self-activating Gal3p.** A) Design of the fluorescence-based screening system as described in the previous chapter. B) Fluorescence normalized to OD_{600} value of either Gal3p-WT or Gal3p mutants in VEG16 strain. All Gal3p variants were incubated in 2% of sugar concentrations. C) Normalized fluorescence values of Gal3p-WT or Gal3p-5.1 mutant in VEG20 strain compared to Gal3p-F237Y in VEG16 strain. D) Fluorescence profiles of Gal3p-WT and Gal3p-5.1 variants at different xylose concentrations (4%, 2%, 0.2%, 0.02% and 0.002%). Each data point represents average of biological triplicates repeated on two different days ± sd.*

Next, we studied the fluorescence profile of the mutant, Gal3p-5.1 by titrating it with different concentrations of xylose and compared with the fluorescence profile of Gal3p-WT. As expected, at lower concentrations, fluorescence was similar to the basal level, and only at concentrations above 0.2%, fluorescence increased. The data confirms that on glucose, GAL regulon is partly activated at basal levels, but is kept in check by glucose-based carbon catabolite repression. In the absence of glucose, when sucrose is present, the regulon is activated at high levels. The regulon activation is further increases in the presence of galactose or xylose, which can interact with the final mutant Gal3p-5.1. However, the F237Y mutation prevents interaction with arabinose.

3.3.2.2 Self-activating Gal3p mutant assists growth in non-native sugars

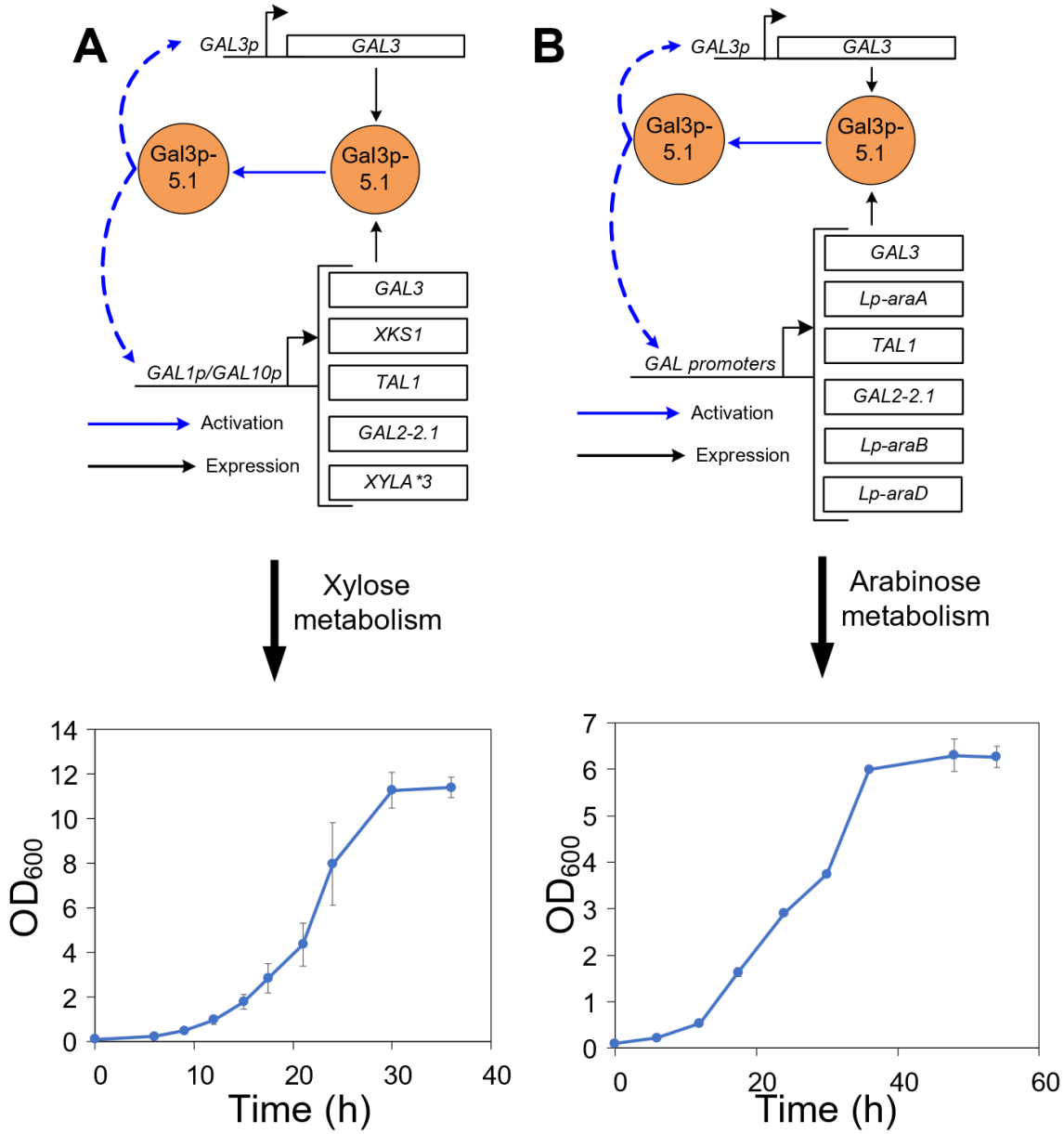


***Figure 3.6**: Self-activating Gal3p activates regulon for growth on xylose and arabinose. A) Design of the genes expressed for xylose metabolism to create the strain, XYL-REG^{SA} (top panel) and growth of XYL-REG^{SA} in 2% xylose (bottom panel) B) Design of the genes expressed for arabinose metabolism to create, ARA-REG^{SA} (top panel) and growth of the strain in 2% arabinose (bottom panel).*

For a Gal3p mutant to be used as a general regulon activator, it must aid growth in not just non-native substrates that it can interact with, but also other non-native carbon sources that Gal3p cannot detect. In the case of Gal3p-5.1 mutant, while it can interact with xylose, it cannot interact with arabinose. So, using xylose and arabinose as test cases for the two scenarios, we evaluated the ability of Gal3p-5.1 to act as a transcriptional activator for growth on the two sugars. As a first step, we constructed two plasmids required for dual feedback by placing GAL3-5.1 gene under *GAL3p* and *GAL1p* promoters (pVEG16-5.1 and pVEG17-5.1). Next, we transformed the necessary plasmids for xylose and arabinose metabolism, (pVEG11 and pVEG12 for xylose metabolism, pVEG37 and pVEG38 for arabinose metabolism) along with dual feedback plasmids in VEG16 strain to create XYL-REG^{SA} and ARA-REG^{SA} strains. Next, we evaluated the two strains for growth on xylose and arabinose. In the case of xylose metabolism, the strain achieved a growth rate of 0.19 h^{-1} and reached stationary phase by 30 hours. When compared to the XYL-REG strain, this represents a 26% increase in growth rate. In the case of arabinose metabolism, we observed a growth rate of 0.154 h^{-1} and the strain attained stationary phase by 36 hours, a 15% growth rate increase when compared to ARA-REG strain. Thus, the self-activating Gal3p mutant can successfully transactivate the GAL regulon even if the sugar to be metabolized cannot interact with Gal3p.

3.4 Discussion

In this chapter, I explored the possible extensions of regulon engineering, while addressing some of the limitations associated with the strategy. In particular, we assessed two of the concerns, namely, the ability of regulon engineering to aid the growth of other non-native substrates, and, circumventing the need to engineer Gal3p for Gal3p-sugar interaction for regulon activation. First,

to evaluate the ability of regulon engineering to assist growth in other non-native sugars, we chose arabinose, the second most abundant pentose in lignocellulose, which cannot be metabolized by *S. cerevisiae*. Since arabinose is structurally similar to galactose and xylose, and Gal3p-Syn4.1, was a flexible sugar sensor, we hypothesized that Gal3p-Syn4.1 can interact with arabinose. Using the fluorescence assay discussed previously, we captured Gal3p (WT and mutant) interactions with arabinose, which revealed high-affinity of Gal3p-Syn4.1 for arabinose. Next, we expressed the genes for arabinose metabolism under the control of regulon by placing the genes under GAL controlled promoters. Compared to a conventionally engineered strain that constitutively expresses the genes for arabinose metabolism (ARA-CONS), the regulon strain, ARA-REG exhibited superior growth rates and biomass accumulation. The slow growth rate as well as poor biomass accumulation of the control strain is corroborated by other reports who have observed similar poor growth characteristics in strains that weren't evolutionarily engineered, irrespective of whether the oxidoreductive or isomerase pathway is being expressed (Bettiga et al., 2009; Bettiga, Hahn-Hägerdal, & Gorwa-Grauslund, 2008; Karhumaa et al., 2006; S. M. Lee et al., 2016). Compared to the fastest aerobically growing strain, which was engineered by adaptive evolution for almost four months (Wisselink et al., 2007), the ARA-REG strain grows at a similar, but slightly lower growth rate of $0.13h^{-1}$, without the need for evolutionary engineering, by employing the regulon engineering strategy.

The regulon engineered strain, however, grew to an OD_{600} of 6.6 on arabinose. While it is higher than the ARA-CONS strain, when compared to the strain XYL-REG engineered in the previous chapter that grew to an OD_{600} of 11, the final cell density is significantly low. This lowered cell density could be a result of inherent transport or metabolic pathway limitations. It is to be noted that the ARA-REG strain consists of support catabolic genes, primarily optimized for xylose

metabolism. For example, the transporter employed GAL2-2.1 is a high-affinity xylose and galactose transporter (O. Reznicek et al., 2015). Although the transport of arabinose was observed during our fluorescence assay, it is possible to increase transport further by using a better arabinose transporter. Similarly, the genes of arabinose metabolism, *AraA, AraB* and *AraD* are all bacterial genes. In general, bacterial genes have been shown to have folding issues in *S. cerevisiae*, which could be resolved either by codon optimizing the genes (Wiedemann & Boles, 2008), expressing specific bacterial chaperones (Xia et al., 2016) or by simply mutating the bacterial genes. Directed evolution of bacterial arabinose metabolic genes followed by selection for mutants that exhibit better growth properties, indirectly selects for enzymes with higher specific activity in the yeast cytosolic environment. Lending more credibility to this theory, Wisselink et al., observed no significant enzyme activity of *AraD* and very low activity of *AraB* genes from *L. planatarum* when expressed in *S. cerevisiae* (Wisselink et al., 2007). Although in this study, we overexpressed the genes using strong GAL promoters, it is possible that a significant portion of the expressed enzymes might not fold accurately or would have poor enzyme activity. Another potential issue is the use of four auxotrophic marker plasmids for growth. Growth rate decrease due to auxotrophic markers is a well-documented phenomenon and is observed irrespective of whether 2μ (high-copy) or centromere (low-copy) plasmids are expressed. Moreover, the growth rate, as well as copy number, have been shown to vary depending on the promoters present (Karim et al., 2012). While plasmids are easy to work with, these limitations could restrict yeast from attaining high growth rates. Stable chromosomally integrated copies of the genetic constructs could be another way to resolve the issue and would also serve as a platform strain for future metabolic engineering applications.

In the previous chapter, for re-engineering GAL regulon to a XYL regulon, we carried out interventions at three points. Protein engineering of Gal3p for sugar sensing, fine-tuning the regulon architecture for signal transduction, and finally, metabolic engineering for xylose assimilation. Of the three interventions, the dual feedback architecture using Gal3p can be retained irrespective of the type of carbon source. Hence for arabinose regulon, only two interventions were originally needed, Gal3p engineering, and incorporating genes for arabinose metabolism under GAL promoters. However, since Gal3p-Syn4.1 was capable of interacting with arabinose, only one of the engineering steps had to be carried out. On the other hand, if regulon engineering for structurally different sugars such as cellobiose, methanol etc., need to be carried out, extensive modifications in the active site of Gal3p would be needed. This would make the strategy cumbersome and time-consuming. To circumvent the issue, we decided to create self-activating Gal3p mutants. Previously, several such mutants have been characterized (Blank et al., 1997). Of those, we tested two of the mutants (F237Y and S509P) that exhibited the highest Gal80p binding ability, for regulon activation in the absence of glucose, using our fluorescence assay. While F237Y showed satisfactory activation of the regulon, S509P did result in fluorescence increase. The contrasting results obtained for S509P could be explained by the difference fluorescence assay used in this chapter and the one used by Blank et al.(Blank et al., 1997). While in our assay, activation is dependent on positive feedback loop created by Gal3p, in the previous study Gal3p was expressed constitutively from the *ADH2p* promoter. Thus, it is possible that S509P mutant can activate the regulon independent of an activation sugar, but only when present at high enough concentrations.

By using the self-activating mutant Gal3p-5.1, we observed regulon activation when grown on sucrose, without a second activation sugar. In the presence of xylose or galactose, fluorescence

increased further, indicating that only a subpopulation of Gal3p-5.1 gets self-activated without the activation of sugar. But, when xylose or galactose is present, the subpopulation of Gal3p-5.1 that is not activated, also gets turned ON, and a further increase in fluorescence is observed. Interestingly, Gal3p-5.1 did not interact with arabinose. Using xylose and arabinose as a proof of principle, it was demonstrated that Gal3p-5.1 assisted growth in the two sugars, irrespective of whether the sugar interacts with Gal3p or not. Moreover, when using the regulon, the strains attained higher growth rates in both xylose and arabinose when compared to the previous regulon engineered strains, XYL-REG and ARA-REG. In the case of arabinose, the strain exhibited highest growth rate reported in published literature.

In the self-activating Gal3p mutants, higher basal expression of GAL regulon genes was observed on glucose, resulting in slower growth than wildtype strains even on glucose. Engineering yeast for better non-native sugar growth seems to have resulted in a trade-off leading to slow growth in native sugars. This phenomenon can be attributed to ineffective Mig1p based repression. Evolutionarily it is not desirable to have strict control by Mig1p since other negative feedback loops help keep the regulation in check. However, in this scenario, regulation by other negative feedback loops such as Gal80p gets overridden by the self-activating Gal3p. This makes Mig1p the lone controller of GAL regulon, thereby causing leaky and higher basal expression on glucose. This can be mitigated by introducing Mig1p repression sites at *GAL3p* promoter to bring down the basal level expression. Overall, this chapter established that regulon engineering can be used as a general catabolic engineering strategy irrespective of the carbon source that needs to be assimilated. Further, by employing self-activating Gal3p, the need for extensive Gal3p engineering can be obviated, thereby enabling faster and easier employment of the regulon Engineering technique.

3.5 Methods

3.5.1 Strains and plasmids used

List of plasmids and strains used are listed in Table 3.1 and Table 3.2 respectively.

3.5.2 Materials

Strain W303-1a *(MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15}),* and plasmids pkT209 and pBK415 were obtained from Euroscarf (Frankfrut, Germany). All enzymes for cloning were purchased from NEB (Beverly, MA). DNA primers were ordered from Operon Inc (Huntsville, AL). Sequencing of plasmid DNA was outsourced to Operon Inc. Growth media and chemicals were purchased from Amresco (Solon, OH) or RPI Corp (Mount Prospect, IL). Ampicillin was obtained from RPI. E.Z.N.A.® Plasmid Mini Kit I, PCR Purification and Gel Extraction Kits were obtained from Omegabiotek (Norcross, GA). Complete Supplement mixture without Histidine (His), Leucine (Leu), Uracil (Ura) and Tryptophan (Trp) mixture was obtained from Sunrise Science Products, Inc (San Diego, CA).

3.5.3 Plasmid construction

3.5.3.1 Plasmids for arabinose metabolism

The genes *AraA* and *AraB* were spliced with *GAL1p* /*GAL10p* bidirectional promoters and *HXT7t* and *ADH1t* terminators using OE-PCR carrying restriction sites, SalI and NotI to create the plasmid pVEG37. The insert was cloned in pRS426 vector through restriction digestion cloning using the SalI and NotI sites. AraD was spliced with *GAL7p* promoter and *TEFt* terminator carrying NotI and SacI restriction sites and cloned into pVEG12 plasmid to create pVEG38.

Control metabolic plasmids were created using the same genes, terminators and restriction sites, but with a few modifications. Instead of *GAL1p*, *GAL7p*, and *GAL10p* promoters, *TEF1p*, *GPM1p* and *TPI1p* promoters were used. *AraA* and *AraB* expressed under *TEF1p* and *TPI1p* promoters were cloned in pRS426 to create pVEG39. *AraD* expressed under *GPM1p* promoter was cloned in pVEG13 to create pVEG40.

3.5.3.2 Plasmids for self-activating Gal3p mutants

GAL3 mutants, *GAL3-F237Y*, *GAL3-S509P*, *GAL3-F237Y+S509P*, and *GAL3-5.1* were created as described below and was cloned into pVEG7 construct to create the plasmid pVEG8-F237Y, pVEG8-S509P, pVEG8-F237Y+S509P and pVEG8-5.1 respectively. pVEG16-5.1 plasmid was constructed by cloning the *GAL3p-GAL3-5.1-TEFt* insert from pVEG8-5.1 in pRS414 vector using BamHI and NotI restriction sites. Plasmid pVEG17-5.1 was constructed by first splicing GAL3-5.1-TEFt construct with GAL1p promoter using OE-PCR. The insert as well as pRS415 vector were restriction digested with BamHI and SalI, ligated and transformed in *E. coli* NEB5α strain.

3.5.4 Strain construction

VEG16 was used for constructing all the strains used in this chapter. VEG16 was transformed with pVEG16*, pVEG17*, pVEG37 and pVEG38 to create ARA-REG. For construction of ARA-CONS, pVEG39, pVEG40, pR414 and pRS415 were transformed in VEG16. For construction of strains carrying self-activating Gal3p mutant that grows on xylose or arabinose, pVEG16-5.1 and pVEG17-5.1 was transformed in VEG16. Along with the two plasmids for dual feedback regulon, either pVEG11 and pVEG12 were transformed to create XYL-REG^{SA} or pVEG37 and pVEG38 were transformed to create ARA-REG^{SA}. Transformation was carried out using the Lithium

Acetate (LiAc) method of Gietz (Gietz, 2014) and grown in SC dropout media without Leu, His, Ura and Trp along with appropriate sugar as the carbon source.

3.5.5 Dose-response curve for fluorescence

For dose-response curve experiments, the cells were pre-grown in 2 % sucrose for studies involving measuring interactions between Gal3p-Syn4.1 and arabinose. When self-activating mutants of Gal3p were tested, the cells were pre-grown in 2% glucose, to prevent premature expression of Gal3p and activation of GAL regulon on sucrose. The cells were pre-grown for 24 hours, diluted 80-fold in SC medium containing 2% sucrose along with specified concentrations of galactose, xylose or arabinose. The cells were also diluted in medium containing 2% glucose, incubated at 30 °C in a microplate shaker and RFU/OD_{600} was measured in a spectrophotometer with excitation at 488 nm and emission at 525 nm.

3.5.6 Growth studies on xylose and arabinose

The strains were grown overnight in appropriate dropout SC medium supplemented with sucrose. They were washed thrice in the growth medium to be inoculated and then diluted to an initial OD_{600} of 0.1 in the same medium with 2% of the appropriate sugar, and OD_{600} was measured every couple of hours. All growth studies with were carried out in 250 ml shake flasks containing 20 ml of YPA media.

3.5.7 Media and Transformation

Yeast strains were grown in YPA medium or SC medium (Yeast nitrogen base (1.67 g/l), ammonium sulfate (5 g/l), complete supplement mixture without His, Leu, Ura and Trp (0.6 g/l))

with appropriate nutrient. Luria Bertani (LB) broth and LB agar plates with 100 mg/l of ampicillin (when required) were used for all *E. coli* propagation and transformation experiments. *E. coli* NEB5α was used to transform the ligated mixture to create all of the plasmids described using MES transformation except for mutant libraries which were created by electroporating the ligation mixture. The plasmids were sequenced and transformed into the appropriate yeast strain using the protocol of Gietz, 2014 (Gietz, 2014)

3.5.8 Mutagenesis

For targeted *GAL3* mutations, forward and reverse primers with specific mutations at either F237Y, S509P or at both positions were used to amplify two fragments of the *GAL3p-GAL3-TEFt* construct using *GAL3-WT*, or *GAL3-Syn4.1* as a template. The two fragments obtained were spliced with OE-PCR and cloned in pVEG7 to create the *GAL3* mutants.

3.6 Appendix

Table 3.1: List of plasmids used in the study

Plasmids	Description
pVEG7	*pRS426, 2µ ori, URA3, ADH1t-EGFP- GAL1p/GAL10p-KANMX-Hxt7t*
pVEG8	*pRS426, 2µ ori, URA3, GAL3p-GAL3-TEF1t, ADH1t-EGFP-GAL1p/GAL10p-KANMX-HXT7t*
pVEG11	*pRS426, 2µ ori, URA3, ADH1t- Piromyces_XYLA*3-GAL1p/GAL10p -XKS1-HXT7t*
pVEG12	*pRS423, 2µ ori, HIS, ADH1t- TAL1- GAL1p/GAL10p–GAL2-2.1-HXT7t*
pVEG13	*pRS423, 2µ ori, HIS, ADH1t- TAL1- TEF1p-TPI1p –GAL2-2.1-HXT7t*
pVEG16*	*pRS414, CEN ori, TRP, GAL3p-GAL3-Syn4.1-TEF1t*
pVEG17*	*pRS415, CEN ori, LEU, GAL1p-GAL3-Syn4.1-TEF1t*
pVEG8-F237Y	*pRS426, 2µ ori, URA3, GAL3p-GAL3^{F237Y}-TEF1t, ADH1t-EGFP- GAL1p/GAL10p-KANMX-HXT7t*
pVEG8-S509P	*pRS426, 2µ ori, URA3, GAL3p-GAL3^{S509P}-TEF1t, ADH1t-EGFP- GAL1p/GAL10p-KANMX-HXT7t*
pVEG8-F237Y+S509P	*pRS426, 2µ ori, URA3, GAL3p-GAL3^{F237Y,S509P}-TEF1t, ADH1t-EGFP- GAL1p/GAL10p-KANMX-HXT7t*
pVEG8-5.1	*pRS426, 2µ ori, URA3, GAL3p-GAL3-Syn4.1^{F237Y}-TEF1t, ADH1t-EGFP- GAL1p/GAL10p-KANMX-HXT7t*
pVEG16-5.1	*pRS414, CEN ori, TRP, GAL3p-GAL3-5.1-TEF1t*
pVEG17-5.1	*pRS415, CEN ori, LEU, GAL1p-GAL3-5.1-TEF1t*
pVEG37	*pRS426, 2µ ori, URA3, ADH1t- AraA- GAL1p/GAL10p -AraB-HXT7t*

pVEG38	*pRS423, 2µ ori, HIS, ADH1t- TAL1- GAL1p/GAL10p–GAL2-2.1-HXT7t-GAL7p-AraD-TEF1t*
pVEG39	*pRS426, 2µ ori, URA3, ADH1t- AraA- TEF1p-TPI1p –AraB-HXT7t*
pVEG40	*pRS423, 2µ ori, HIS, ADH1t- TAL1- TEF1p-TPI1p –GAL2-2.1-HXT7t-GPM1p-AraD-TEF1t*

Table 3.2: List of strains used in the study

Strains	**Description**
W303-1a	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*
VEG16	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10*
VEG20	*W303-1a, ΔGAL3; ΔGRE3; ΔGAL1; ΔGAL7; ΔGAL10; LEU::GAL2p-GAL2-2.1-TEFt*
ARA-CONS	VEG16 transformed with pVEG39, pVEG40, pRS414 and pRS415
ARA-REG	VEG16 transformed with pVEG37, pVEG38, pVEG16* and pVEG17*
XYL-REG^{SA}	VEG16 transformed with pVEG11, pVE12, pVEG16-5.1 and pVEG17-5.1
ARA-REG^{SA}	VEG16 transformed with pVEG37, pVEG38, pVEG16-5.1 and pVEG17-5.1

Conclusion

In this thesis work, I aimed to address the lack of tools and strategies in the field of catabolic engineering, for integrating non-native substrates into yeast cellular machinery for growth and biomass accumulation. In chapter one, by carrying out a comprehensive survey of literature, I analyzed the mechanism involved in detection, signaling and metabolism of a native substrate, galactose, and compared it with engineering strategies employed for non-native substrate metabolism (xylose and arabinose). We gained valuable insights into the lack of knowledge regarding the genes required for growth in both native and non-native substrates and discovered a missing gap between substrate metabolism and cellular growth. The literature review further raised questions about the role played by downstream genes of the GAL regulon and if they assist growth in yeast, irrespective of the carbon source. In chapter 2, by untangling galactose metabolism from regulation, I assessed the role played by downstream genes of the GAL regulon and demonstrated that downstream genes of the GAL regulon aids growth. Next, we systematically re-engineered the substrate detection, signal transduction and metabolic components of the regulon for xylose, using protein and metabolic engineering tools. For substrate detection, we targeted the galactose sensor, Gal3p, and using four rounds of targeted and random mutagenesis, we obtained a Gal3p variant that can detect xylose and activate the regulon. To enable rapid signal transduction with minimal noise and increased sensitivity, we developed a dual positive feedback loop and coupled it with substrate detection. When xylose metabolic genes were expressed under the re-programmed regulon, high growth rates were observed when compared to conventionally engineered strains. Finally, transcriptome analyses of the regulon assisted versus conventionally engineered strains, grown either on galactose or xylose revealed regulon-based upregulation of transcripts involved in cell growth, cell wall and mitochondrial biogenesis. Moreover, unregulated (conventionally

engineered strains), upregulated transcripts responsible for substrate starvation as well as stress and DNA damage response. After conclusively demonstrating the benefits of regulon in aiding growth on galactose and xylose, in the next chapter, I tested if the regulon can aid growth in another economically important non-native pentose sugar arabinose. Like xylose regulon, arabinose regulon greatly improved growth rate and increased cell densities. Together, these data re-affirm the theory that downstream genes of GAL regulon can support growth on a sugar, irrespective of whether the sugar can be metabolized by yeast naturally. Finally, to carry out regulon engineering strategy with relative ease, we compressed the multi-step engineering required, into a single step by employing a self-activating GAL regulon activator, Gal3p, that can switch ON the regulon and activate genes for substrate metabolism, irrespective of whether the substrate is detected by yeast. Overall, this thesis work stresses the need to re-consider how non-native substrate catabolism integrates with the host cellular machinery, regulation, signaling etc. and provides a new strategy, regulon engineering for elegant, faster and efficient incorporation of heterologous catabolic pathways into yeast. Further, this technology can be directly applied to industrial yeast strains reviewed in Table 3, that are currently being used for metabolizing lignocellulosic feedstocks. Depending on the final product requirement, minor optimization might be needed.

Future work recommendations

In this thesis work, I established a new strategy for catabolic engineering and demonstrated the benefits of employing a natural regulon for non-native sugar assimilation. While two of the possible extensions of regulon engineering were explored in the final chapter of this thesis, there are several areas that still need to be studied but is out of the scope of this thesis. Some of the possible areas for future work is described below.

Systematic adaptive laboratory evolution

To engineer fast growth of yeast on xylose and arabinose, most studies employ adaptive evolution. As described in the first chapter of this thesis, most of these studies use strains with poor growth rates on pentose and after adaptive evolution, have identified transcriptional changes in signaling pathways, galactose regulon genes, phenotypic genes etc. However, in the regulon strain that is developed in this work, yeast is already programmed for activating galactose regulon with many of the phenotypic growth-related genes getting upregulated. So, evolving regulon engineered strains in parallel with strains constitutively expressing genes for xylose growth might shed light on the evolutionary trajectory both of these strains would take for a common end objective of better growth on xylose. To analyze how re-wiring of transcriptional networks changes evolution, other differently regulated XYL-REG strains, such as a strain carrying a single feedback for regulon activation, or a strain with constitutively active xylose regulon can also be evolved in parallel. These studies would provide us with a deeper understanding on whether evolution converges or diverges if strains with different regulatory architectures and different evolutionary start points aim to attain the same end objective.

Regulon Engineering for growth on novel substrates

The catabolic engineering platform strain developed in the final chapter can be used for assimilating other non-native, but economically important substrates by expressing the necessary catabolic genes under the control of GAL promoters. For cellobiose metabolism, a cellodextrin transporter and beta-glucosidase can be expressed under regulon control. Cellodextrin transporter transports cellobiose into yeast and is cleaved to glucose molecules by beta-glucosidase. Although glucose is a native substrate, recognized by yeast, absence of extracellular glucose as well as minute concentrations of intracellular glucose results in absence of substrate detection, leading to carbon starvation-like response (Chomvong, Benjamin, Nomura, & Cate, 2017), similar to what is observed on xylose and arabinose metabolism. Thus, employing GAL regulon for cellobiose regulation would resolve this issue and has the potential to greatly improve growth in this sugar.

Another attractive source of substrate is methanol, owing to the increase in production worldwide and a decrease in the price of the C1 compound. Although, a methanol-based economy is an attractive option, most methylotrophs are not genetically tractable and do not produce small molecules at sufficient quantities. All of these have nudged the metabolic engineering community to engineer methylotrophy in model microbes. Research has been underway in production of methylotrophic *E. coli*, but success has been limited (Müller et al., 2015; W. Brian Whitaker et al., 2017; William B. Whitaker et al., 2015; W. Zhang et al., 2017). The strategy involves usage of Ribulose Mono Phosphate pathway (RuMP pathway) to assimilate formaldehyde and ribulose-5-phosphate to fructose-6-phosphate. The same approach can be used in *S. cerevisiae* but would use galactose regulon for expression of genes required for RuMP pathway. Since, *S. cerevisiae* has a higher tolerance to methanol than prokaryotes like *E. coli*, with specialized vesicles such as

peroxisomes to handle toxic metabolites, methylotrophic *S. cerevisiae* might be a viable and easier engineering alternative to methylotrophic *E. coli*.

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