CONSTRUCTION AND ANALYSIS OF A MODIFIED YEAST STRAIN FOR NEXT GENERATION BIOFUEL PRODUCTION

by

Jeffrey Ross Swana

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APPROVED:

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Dr. Reeta Prusty Rao, Thesis Advisor

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Dr. Pamela J. Weathers, Committee Member

_____

Dr. Luis Vidali, Committee Member

Abstract

Current research efforts are focused on 'second generation biofuels', which includes biofuels produced from lignocellulosic material. Lignocellulosic material is primarily composed of cellulose, a glucose polymer, xylose rich hemicellulose and non-fermentable lignin. *Saccharomyces cerevisiae* is widely used on an industrial scale for the production of ethanol from glucose; however, native *S. cerevisiae* does not contain the genes required for fermentation of xylose into ethanol. Others have sequentially expressed trans-genes from xylose fermenting organisms to engineer strains of *S. cerevisiae* capable of fermenting this pentose. The goal of this thesis was to generate a single cassette of 9 genes which have been shown to ferment xylose and arabinose. The 17 kb DNA fragment harboring all the genes necessary was introduced into the yeast genome using one-step homologous recombination based transformation. Expression of this cassette was verified by demonstrating that the first and last genes on this cassette were transcribed. The modified strain exhibited xylose utilization under microaerobic fermentation conditions. Further genetic and process engineering methods may be employed to improve the yield. The experiments described here demonstrate that generating a functional cassette of pentose fermenting genes is still achievable.

Acknowledgements

Upon entering into post-graduate study, I had two goals in mind, to learn as much as I could about the biofuel field and contribute to it. Unfortunately, I cannot claim that both goals were achieved with this thesis as I do not see these results as contributing to the field, as they are generally negative. Fortunately, I believe I have learned great deal about the biofuel field through my experiences as a student. I have also received a greater appreciation for scientific design, an inherent byproduct of thesis based research. With this experience, I will undoubtedly look carefully at the design of my future experiments. I find a quote from Thomas Edison to be quite fitting, “Negative results are just what I want. They’re just as valuable to me as positive results. I can never find the thing that does the job best until I find the ones that don’t.” It is a sentiment that has been stated over and over, but it has a resonance with my work and, I think, for scientific research as a whole.

Upon the culmination of this thesis, I think it is important to thank all those who have helped me through this process. First and foremost, is my girlfriend Kathleen as she has been there to support me through some of the most trying moments of my life. I also need to thank all of my family and friends, especially my parents for allowing me to choose my own path but always directing me down the right one. To my lab-mates, Luca, Alisha, and Charu, thank you all for helping in my experiments and being just downright awesome people. I also need to thank everyone else that I’ve had the pleasure of meeting at WPI for the unforgettable experiences and kindness. It is also important to thank the professors in the Biology department for their time and dedication to science; as, if it weren’t for all of them, none of this would have been possible. Specifically, Reeta Prusty Rao, Pam Weathers, and Luis Vidali deserve thanks for their help in the progression of this work.

I also need to thank the EdenIQ for funding the initiation of this project. An MQP group including Stephanie Carcieri, Elizabeth Clardy, and Neda Seher Zahid were integral to the assessment of the engineered strain. This group has a published MQP from 2010 describing the ethanol production by the modified yeast strain.

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Chapter 1: Introduction

Significance

Clean, renewable energy has become a research priority amid increasing global energy demands, reliance on petroleum, and concerns over global warming. Among renewable energy technologies, biofuels, fuels derived from biomass (Giampietro, Ulgiati, and Pimentel 1997; Lin and Tanaka 2006; Cardona and Sánchez 2007) have come to the forefront. One such biofuel under active research is biologically derived ethanol. Ethanol is a simple two-carbon alcohol and is advantageous because it is a product of microbial fermentation.

There are a number of obstacles that must be overcome before any serious move to an ethanol fuel economy can be accomplished. Among the greatest of these is obtaining adequate substrate for microbial conversion to ethanol. Current industrial production relies on crop-based materials, like sugarcane and corn starch, which are also used for food and fodder. This competition for substrate drives up the price of both food and fuel; in 2008, 23% of the total United States corn crop was used to produce ethanol, yet this resulted in supplying only 2% of the total transportation requirement (Demain 2009).

These crop-based materials contain glucose, which is readily usable by microorganisms and humans, making agricultural crops attractive as food and for biofuel production. Plants also contain polymers, composed primarily of sugars, which are discarded as wastes in agricultural processes because of the structural rigidity and complexity. These polymers, cellulose, hemicellulose, and lignin are collectively referred to as lignocellulose. Cellulose is composed almost entirely of glucose and is the most common natural polymer, and makes up more than half of global biomass (Peters 2006). Hemicellulose is amorphous and contains a variety of sugars, including glucose and the pentoses, xylose, and arabinose. Lignin, like hemicellulose, is an amorphous polymer but contains no readily fermentable sugars

(Ragauskas et al. 2006). Lignocellulosic material is often discarded as waste despite being composed of sugars because the polymerized sugars are energetically unfavorable for humans, and livestock must rely on microbes to digest them. Since agricultural by-products that contain lignocellulose are otherwise discarded, it is an attractive potential feedstock for industrial biofuels.

Lignocellulose is abundant in common agricultural and municipal waste products (Table 1). Economic calculations further bolster the case for using lignocellulose as the substrate of industrial ethanol production. A study by Hinman et al. (1989) determined that it is necessary to improve upon the industrial processing of lignocellulose for production of bioethanol to viably compete with fossil fuels.

Table 1. Lignocellulosic content of common biological wastes

(Adapted from Sun and Cheng (2002)

Agricultural waste	**Lignocellulosic content (%)**
Wheat straw	95
Discarded newspapers	~100
Leaves and lawn refuse	90
Swine waste	>30
Cattle manure	15

Microbes generally do not readily use xylose or arabinose anaerobically but can be engineered to do so (Shi et al. 1999). While some bacteria and fungi can naturally use such sugars anaerobically, their primary means of growth is nonfermentative (Sonderegger and Sauer 2003). Nevertheless, identifying organisms naturally capable of efficiently converting lignocellulose into alcohol is an area of active research (Jeffries and Shi 1999; Jeffries 2006; Rao, Bhadra, and Shivaji 2008). Similarly, much work is focused genetically modifying an organism to produce ethanol from lignocellulosic feedstock (Koskinen et al. 2008; Menon et al. 2010).

One organism in particular, which has received much attention in producing biofuels from lignocellulosic materials, is *Saccharomyces cerevisiae*. The reasons behind this are numerous, and include ease of use and safety in working with an FDA approved, GRAS (generally recognized as safe) organism (Nevoigt 2008). Consequently, many groups are working to modify the yeast to produce biofuels on an industrial scale. Our approach, however, is novel because we generated a genetic construct that harbored all the enzymatic machinery required to reduce pentose sugars to ethanol. This lignocellulosic transgenic cassette (LTC) includes 9 genes that individually have been shown to enhance conversion of pentose sugars into ethanol (Träff et al. 2001; Jin and Jeffries 2004; Chu and Lee 2007; Kuyper et al. 2005; Walfridsson et al. 1997; Wisselink et al. 2007; B Hahn-Hägerdal et al. 2001). The aim of the project was to improve the utilization of xylose and arabinose by a lab strain of *S. cerevisiae,* BY4741 (Lee, Jellison, and Alper 2012; Alper et al. 2006). The use of the lab strain allowed for easy manipulation and genetic tractability during the integration of the LTC. Furthermore, BY4741 only marginally metabolizes these sugars (Matsushika et al. 2009; Batt et al. 1986), so even a modest increase in fermentation would be measurable.

Background

The process of generating ethanol from simple sugars is what is generally termed 'first generation biofuels,' a process which is well understood. First generation biofuels are considered as necessary for the rapid implementation of biofuels into the transportation sector in order to reduce a reliance on petroleum imports and fossil fuels as a whole. Currently, biofuel production within the United States relies heavily on the conversion of corn starch and sugarcane into ethanol; a process that is not trivial, yet sets up a scenario for competition between foods versus fuel production. Though the conversion of corn starch to ethanol is energetically favorable, the conversion of lignocellulosic material to ethanol has a greater net energy return (Hammerschlag 2006). Any process of converting non-food based material to liquid fuels is generally referred to as 'second generation biofuels'.

The Energy Independence and Security Act of 2007 (EISA 2007) issued by the United States government contains a mandate known as the Renewable Fuel Standard or RFS which calls for 36 billion gallons per year (BG/Y) of biofuel to be produced by the year 2022. Considering the maturity and efficiency of first generation bioethanol production, a considerable portion of this mandate, 15 BG/Y, is expected to come from corn starch derived ethanol. The remaining 21 BG/Y is to be made up of second generation biofuels (Figure 1).

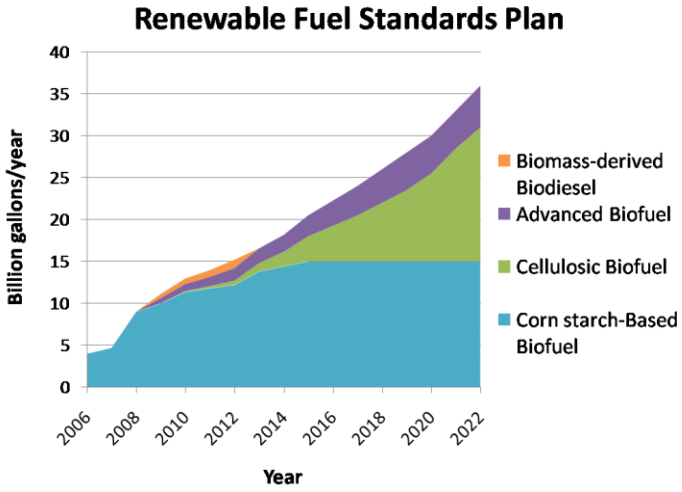

Figure 1: Renewable Fuel Standard from the Energy Independence and Security Act of 2007 (EISA 2007). The bill calls for the eventual production of 36 billion gallons per year of biofuels. (Adapted from EISA 2007)

In the EISA of 2007, the term cellulosic biofuel is defined as any fuel derived from cellulose, hemicellulose, or lignin that has lifecycle greenhouse gas emissions at least 60% lower than the baseline lifecycle emissions of gasoline. The term advance biofuel is written more broadly and includes any fuel, other than ethanol derived from corn starch, which has lifecycle greenhouse gas emissions at least 50% lower than the baseline lifecycle emissions of gasoline.

The RFS mandate was based primarily on a significant research effort jointly produced by the United States Department of Energy (DOE) and the United States Department of Agriculture (USDA) where

multiple scenarios were modeled based on production of lignocellulosic material within the United States (Perlack et al. 2005). Though the report provides an inventory of the potential tonnage of lignocellulosic material, there was no direct assessment of how much biofuel could potentially be produced based on the biomass availability in the report.

Through calculations of available agricultural wastes, corn stover and wheat straw, as well as energy crops, poplar and switchgrass, it was determined that sustainable harvesting practices would only supply 10.5 BG/Y of ethanol derived from the glucose fraction of this lignocellulosic material (Swana et al. 2011), substantially less than the 21 BG/Y mandated by the RFS. However, the 10.5 BG/Y estimate does not include all waste streams of lignocellulosic material nor does it include conversion of all fermentable carbohydrates (Swana et al. 2011). Specifically, the pentose sugars, xylose and arabinose, make up a substantial portion of lignocellulose and numerous research groups are currently developing efficient fermentation processes for these sugars (Bera et al. 2011; Van Vleet and Jeffries 2009).

The RFS mandate is indicative of the importance of lignocellulosic biofuels within the United States, and efficient use of available pentose sugars is an integral part of the process. In order to better understand the technical details of fermentation of the pentose fraction of lignocellulose, it is important to describe the general process with which lignocellulose is converted into its monomeric sugar components.

Current practices for Lignocellulosic alcohol production

Conventionally, there are three main steps in the conversion of lignocellulosic material into biofuel: pretreatment, hydrolysis, and fermentation (Figure 2). Typical pretreatment involves a thermo-mechano-chemical step to remove the structural rigidity of the plant material and the indigestible lignin from the sugars. Microbes are being considered for pretreatment, but the biological degradation of lignin is slow and inefficient as compared to the other options (Sun and Cheng 2002). Common treatments for this step involve acid hydrolysis, steam explosion, ammonia fiber expansion, and sulfite

pretreatment to overcome recalcitrance of lignocellulose (Himmel et al. 2007). This step is a crucial one, especially if subsequent steps involve microbial populations. The choice of the method ultimately depends on the feed material and type of downstream processing. A thorough evaluation on the different pretreatment options and their advantages and disadvantages was published by Hendriks and Zeeman (2009).

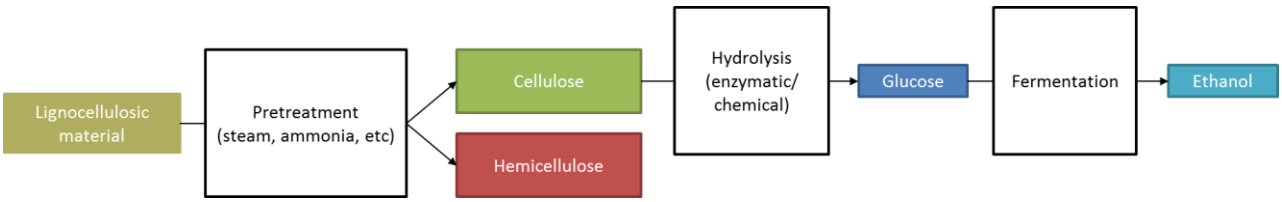

Figure 2: Representation of conversion of lignocellulose to ethanol. Three major steps are required for conversion of lignocellulosic material to ethanol, pretreatment, hydrolysis, and fermentation. This process assumes use of *S. cerevisiae* so the hemicellulose fraction is not included in fermentation. (Adapted from Margeot et al. (2009))

The second and third steps, hydrolysis and fermentation, result in the depolymerization of cellulose and hemicellulose into fermentable sugar products (i.e., cellulose depolymerizes to glucose monomers) and conversion of those sugars into ethanol. Hydrolysis of the pretreated material can be performed either chemically or enzymatically, both have their drawbacks however as the cost of purified enzymes can be substantial (Fischer, Klein-Marcuschamer, and Stephanopoulos 2008), while the use of chemical hydrolysis results in the formation of toxic compounds such as aliphatic acids, furan derivatives, and phenolic compounds (Chandel, Singh, and Rao 2010). The microorganism for fermentation is then added later to convert the monomeric sugars into ethanol. Commonly, *S. cerevisiae* is used in this fermentation process due to its ability to tolerate high sugar concentrations as well as the toxic inhibitors generated during the hydrolysis step. Unfortunately, native *S. cerevisiae* is incapable of effectively fermenting pentose sugars into ethanol (Batt et al. 1986), so a separate fermentation step is required to achieve fermentation of all available sugars .

Pentose Fermentations Using Yeasts

Historically, *Saccharomyces cerevisiae* has been widely used for alcohol production from glucose (Weber et al. 2010). On the other hand, a number of other yeasts have the ability to ferment pentose sugars into ethanol; *Scheffersomyces stipitis, Pichia segobiensis, Candida shehatae, Pachysolen tannophilus*,and *Kluyveromyces marxianus* can directly ferment xylose into ethanol (Jeffries 2006; Prusty Rao, Dufour, and Swana 2011). These are of particular interest to the biofuel industry for the efficient conversion of xylose and arabinose to alcohols. Since lignocellulosic material contains glucose, xylose and arabinose, it is conceivable to use a co-culture of *S. cerevisiae* and *S. stipitis* to convert all available sugars (Taniguchi et al. 1997). However, to achieve high ethanol productivities pentose fermenting yeasts require oxygen while *S. cerevisiae* requires anaerobic conditions during glucose fermentation, resulting in low yield (Chu and Lee 2007). Clearly there is an unmet need to engineer yeasts to express xylose metabolizing enzymes for efficient anaerobic xylose fermentations.

Engineering yeasts for Xylose Fermentation

Anaerobic fermentation of pentose sugars has been widely studied in *S. cerevisiae* (Prusty Rao, Dufour, and Swana 2011; Weber et al. 2010). In particular, these studies focus on first understanding the rate limiting steps for xylose metabolism (Chu and Lee 2007; Jin and Jeffries 2004) and next incorporating exogenous or modifying endogenous genes for generating yeasts capable of efficient xylose fermentation (Liu et al. 2010; Brat, Boles, and Wiedemann 2009; Sonderegger and Sauer 2003; Walfridsson et al. 1997).

The pentose metabolizing pathway of *S. stipitis* (formerly *Pichia stipitis*) includes xylose reductase (EC 1.1.1.21; XR) and xylitol dehydrogenase (EC 1.1.1.9; XDH). *S cerevisiae* has these enzymes but shows minimal metabolism of xylose, primarily producing xylitol (Batt et al. 1986; Van Vleet and Jeffries 2009).

In the case *of S. cerevisiae,* it has been shown that the endogenous xylose reductase enzyme (XR) and xylitol dehydrogenase enzyme (XDH) show preference for producing xylitol from xylose due to cofactor specificity of the enzymes (for a recent review see Jeffries 2006). Briefly, the endogenous *S. cerevisiae* XR gene, *GRE3,* has a strong preference for NADPH as a cofactor for the conversion of xylose to xylitol while the *S. cerevisiae* XDH gene, *XYL2*, has strong preference for NAD+ as a cofactor (Jeffries 2006). This process results in a gradual depletion of the NADPH pool resulting in xylitol accumulation. Conversely, the *S. stipitis* XR gene, *XYL1*, demonstrates dual-cofactor specificity and can use either NADH or NADPH allowing for the continual replenishment of NADH. Efforts have been made to evolve the *S. cerevisiae* XR as well as the *S. stipitis* XR to show increased specificity for NADH with some success through traditional mutagenesis, as well as adaptive evolution techniques (Chu and Lee 2007; Jeffries 2006; Sonderegger and Sauer 2003).

While increasing ethanol productivities using native xylose fermenting yeasts is one approach, another area of intense research for xylose fermentation is towards generating a microbe capable of fermenting both hexose and pentose sugars in a single fermentation step. Specifically, much work has been invested in engineering *S. cerevisiae* through insertion of exogenous genes (Weber et al. 2010). To achieve this, integration of two exogenous pathways into *S. cerevisiae*, the XR/XDH pathway and the xylose isomerase (EC 5.3.1.5; XI) pathway have been widely studied (Jeffries 2006; Chu and Lee 2007). *S. cerevisiae* strains have been generated displaying either the *S. stipitis* XR/XDH pathway (Kotter and Ciriacy 1993; Walfridsson et al. 1997; Jin and Jeffries 2004; Jin and Jeffries 2003; Sonderegger and Sauer 2003) or the XI pathway (Kuyper et al. 2005; Kuyper et al. 2004; Karhumaa, Hahn-Hägerdal, and Gorwa-Grauslund 2005).

In terms of the fungal XR/XDH pathway, Jin and Jeffries (2003) described transgenic strains of *S. cerevisiae* with the XR and XDH from *S. stipitis* either integrated on the chromosome, or on a multiple

copy plasmid under control of a strong constitutive promoter. Optimal conversion of xylose to xylulose was attained in a strain where the *S. stipitis* XR was integrated in a single site on the chromosome, and the *S. stipitis* XDH was maintained on a multicopy vector. Thus, lower expression of XR compared to XDH was demonstrated as important for efficient xylose metabolism.

After the expression of the exogenous XR/XDH pathway, ethanol production in *S. cerevisiae* is negligible (Kotter and Ciriacy 1993), unless there is an abundance of the enzyme responsible for phosphorylation of xylulose to xylulose-5-phosphate, xylulokinase (EC 2.7.1.17; XK) (Moniruzzaman et al. 1997). When overexpressed, the endogenous *S. cerevisiae* XK gene, *XKS1*, demonstrated increased flux from xylose to ethanol, but only at a yield ~60% of the theoretical maximum. Similarly, in *S.cereviasiae* strains containing the engineered XR/XDH pathway the *S. stipitis* XK enzyme coded by the *XYL3* gene displayed a titer which was increased twofold compared to an isogenic strain lacking *XYL3* (Jin et al. 2003).

Other attempts to increase yield from xylose to ethanol in *S. cerevisiae* identified the non-oxidative pentose phosphate pathway (PPP) as a bottleneck (Gancedo and Lagunas 1973; Fiaux et al. 2003). In yeast, the non-oxidative PPP is responsible for the conversion of xylulose into glycolytic intermediates, but in *S. cerevisiae* this pathway is not efficient (Matsushika et al. 2009). Two endogenous genes coding for enzymes within the non-oxidative PPP, *TAL1*, a transaldolase (EC 2.2.1.2; TAL), and *TKL1*, a transketolase (EC 2.2.1.1; TKL), were overexpressed in isogenic strains containing the *S. stipitis* XR/XDH pathway and analyzed for growth on xylose (Walfridsson et al. 1995). In comparison to the base strain, the *TKL1* overexpressing strain showed decreased growth, while the *TAL1* overexpressing strain showed improved growth. Further studies have overexpressed the entire non-oxidative PPP with varying improvement in ethanol yield (Bera et al. 2011; Chu and Lee 2007; Matsushika et al. 2009), nonetheless increasing flux through this pathway has been shown to be important for efficient xylose utilization.

Compared to the two-step XR/XDH pathway, the xylose isomerase (XI) enzyme catalyzes a one-step conversion of xylose to xylulose eliminating the cofactor imbalance issues observed with the fungal XR/XDH pathway (Matsushika et al. 2009). A gene coding for XI from the fungus *Piromyces sp.* E2 (ATCC 76762), *XYLA*, has been demonstrated as expressed and active in *S. cerevisiae* strains (Kuyper et al. 2004). The resulting transgenic strain underwent a lengthy adaptation protocol involving constant cultivation in xylose, but was able to grow anaerobically on xylose at a specific growth rate of 0.03 h^{-1} and a yield of 0.42 g/g (ethanol to xylose) which equates to 85% of the theoretical maximum (0.51 g/g ethanol to sugar) (Kuyper et al. 2004). It is important to note that the genetic background of this strain did not include overexpression of any other exogenous or endogenous genes; rather, the group relied on sequential outgrowths of the strain in media containing only xylose, aerobically then anaerobically. Conversely, directed engineering approaches expressing bacterial and fungal XI have demonstrated xylose fermentation to ethanol by overexpressing XK, and non-oxidative PPP enzymes (Bettiga, Hahn-Hägerdal, and Gorwa-Grauslund 2008; Kuyper et al. 2005; Karhumaa, Hahn-Hägerdal, and Gorwa-Grauslund 2005).

Isogenic strains of *S. cerevisiae* harboring either the *S. stipitis* XR/XDH pathway or the *Piromyces* XI pathway were compared directly for the ability to anaerobically ferment xylose to ethanol (Bettiga, Hahn-Hägerdal, and Gorwa-Grauslund 2008). The strain containing the exogenous XR/XDH pathway generated a higher overall ethanol titer, 14.7 g/L, compared to the XI strain, 11.8 g/L. However, the ethanol yield from consumed xylose was higher in the XI strain, 0.41 g/g (84% the theoretical maximum), than the XR/XDH strain, 0.32 g/g (63% the theoretical maximum). This conflicting data is lacking comparison to a strain harboring both pathways, an approach which had yet to be published until our study.

An ongoing area of focus is increasing the transport and fermentation of xylose when glucose is present in the media (Bärbel Hahn-Hägerdal et al. 2007; Wisselink et al. 2009). Briefly, xylose transport has been identified as a potential bottleneck for ethanol production in xylose metabolizing strains (Gárdonyi et al. 2003). *S. cerevisiae* has endogenous hexose transporters which are capable of xylose transport when xylose is at high enough concentrations, but are severely inhibited by glucose (Boles and Hollenberg 1997). Constitutive expression of individual Hxt transporters from *S. cerevisiae* has been shown to improve xylose fermentation (Sedlak and Ho 2004). Specifically, Hxt7 and Hxt5 showed the highest xylose assimilation and metabolism rates when overexpressed in a transporter null strain including XR/XDH from *S. stipitis*. Hxt7 belongs to the 'high-affinity' glucose transporters ($K_{m\ glucose}$ ~1.5mM) while Hxt5 belongs to the 'moderate-affinity' glucose transporters ($K_{m\ glucose}$ 10mM). The distinction between high, moderate, and low affinity glucose transporters has been reviewed in the context of dual specificity for pentose and hexose sugars (Matsushika et al. 2009). Generally, the high-affinity Hxt transporters are viewed of as useful for xylose uptake, though still not ideal as they are repressed in the presence of glucose. The low-affinity Hxt transporters are constitutively expressed, but are poor at xylose uptake (Sedlak and Ho 2004; Chu and Lee 2007). Looking at exogenous sugar transporters, *SUT1,* from *S. stipitis* transformed into a *S. cerevisiae* strain containing the XR/XDH pathway from *S. stipitis* increased the yield from xylose to ethanol from 0.39 g/g to 0.44 g/g during a glucose and xylose co-fermentation process using 5% glucose and 5% xylose (w/v) (Katahira et al. 2008).

Further work on fermentation of mixed sugars has led to the development of a *S. cerevisiae* strain capable of simultaneously fermenting xylose and cellobiose, a glucose dimer (Ha et al. 2011). The strain contained the XR/XDH pathway and XSK from *S. stipitis* as well as a cellodextrin transporter (*cdt-1*) and an intracellular β-glucosidase (*gh1-1*) from *Neurospora crassa*. For xylose transport, endogenous hexose transporters were relied on as no glucose was present in the media. This experiment demonstrated a much improved co-fermentation rate of pentose and hexose than previous studies have demonstrated.

Arabinose Fermentation

Arabinose is approximately 5% of the total sugars in lignocellulosic material compared to xylose, which is ~30% (Prusty Rao, Dufour, and Swana 2011). Therefore, methods to improve arabinose fermentations are less common; though, when considering the disparity between the RFS mandate and current production levels, it is important to take advantage of all available resources.

Engineered strains of *S. cerevisiae* harboring an arabinose fermenting pathway from bacterial sources have been successful (Becker and Boles 2003; Wisselink et al. 2007); specifically Wisselink et al. (2007) demonstrated anaerobic fermentation of arabinose by expressing three genes from *Lactobacillus plantarum*, *araA*, *araB*, and *araD*, coding for an arabinose isomerase, ribulokinase, and ribulose-5-phosphate 4-epimerase respectively. The resulting strain provided an ethanol yield of ~85% of the theoretical under anaerobic conditions. Additional work in this area is focused on improving the co-fermentation of mixed sugars bottleneck observed in xylose metabolism engineering (Weber et al. 2010).

Advancing pentose fermentation by yeasts is an important area of research for lignocellulosic biofuel production, but due to current limitations with ethanol as a fuel, it is important to consider alternative products as well.

Biologically derived butanol

Considering the current limitation that ethanol can only be blended to relatively minor percentages for use in current combustion engines (10-15%), it is clear that other options should be investigated. In the wording of the EISA 2007 the definition of both 'cellulosic biofuels' and 'advanced biofuels' include any fuel other than corn-starch derived ethanol, indicating a role for fuel products other than ethanol.

One promising alternative to biologically derived ethanol is butanol; butanol holds a variety of characteristics that make it attractive as a fuel when compared with ethanol, including the ability to be pumped through our existing pipeline infrastructure, increased hydrophobicity, and lower corrosiveness (Keasling and Chou 2008). The energy content of butanol is higher than that of ethanol (Wu et al. 2007), and butanol can be blended at higher concentrations than ethanol with gasoline for use in current combustion engines (Dürre 2008). Additionally, butanol can serve to replace a percentage of our chemical feedstocks that are currently generated from petroleum processing. N-Butanol has been listed as one of the top 30 industrial organic chemicals (Nikolau et al. 2008).

The current standard for producing butanol from sugar sources involves the bacteria *Clostridium acetobutylicum* (Ezeji, Qureshi, and Blaschek 2007). The disadvantages to using this bacterium for fusel alcohol production include its obligate-anaerobic growth, which makes it a slow and difficult organism to cultivate in a laboratory setting. Furthermore, the microbe is recalcitrant to modern genetic and molecular biological applications, and is not tolerant to high concentrations of alcohols (Papoutsakis 2008). These considerations have led researchers to investigate other microbial systems (Keasling and Chou 2008). *S. cerevisiae* has been considered for production of butanol (Steen et al. 2008) due in part to its ease of manipulation, rapid growth rate and resistance to alcohol concentrations (Fischer, Klein-Marcuschamer, and Stephanopoulos 2008).

As this country continues to make strides towards renewable energy sources, ethanol will continue to be a major contributor, but it is clear that we need to develop new fuels that will help solve our long term needs.

Butanol pathway engineering

Introduction of 2 transgenes, *kviD (Lactococcus lactis)* and *ADH1 (S. cerevisiae)* into *Escherichia coli* allowed the biosynthetic pathways for valine to be shunted towards butanol (Atsumi, Hanai, and Liao 2008, Figure 3).

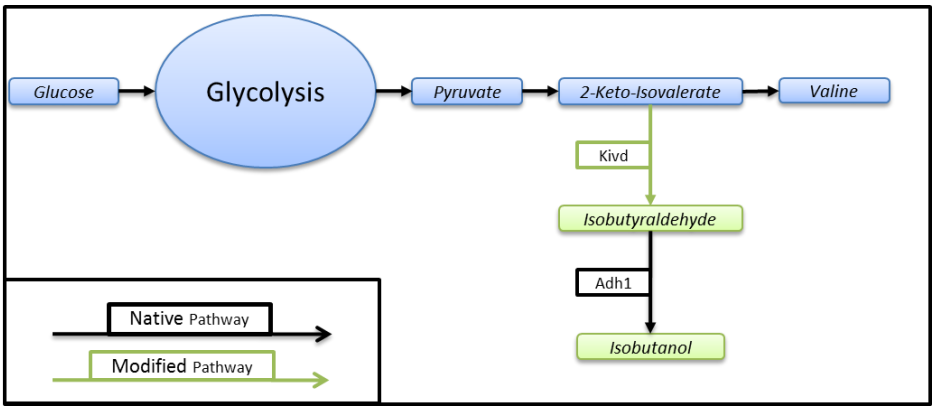

Figure 3.The amino acid shunt pathway to generate fusel alcohols, such as isobutanol. The modification needed in *S. cerevisiae* is marked in green.

In *S. cerevisiae* the Ehrlich pathway (J Richard Dickinson, Salgado, and Hewlins 2003; J R Dickinson, Harrison, and Hewlins 1998; Hazelwood et al. 2008) has been shown to shunt amino acid biosynthesis into alcohol by enzymatic activity on 2-keto acids, a byproduct of fermentation (Bigelis et al. 1983). This provides an important proof-of concept and manipulation of this pathway should yield fusel alcohols, such as isobutanol. Since we are using *S. cerevisiae* for our studies, the only modification required would be to insert the same *kivD* gene from *L. lactis* into our yeast strain of choice (Figure 3, marked by a green arrow).

Project Goals

Wild-type *S. cerevisiae* is unable to ferment xylose/arabinose. Previous studies have described genes that individually enabled *S. cerevisiae* to ferment alternate sugars (Kotter and Ciriacy 1993; Tanaka et al. 2002; Kuyper et al. 2005; Karhumaa, Hahn-Hägerdal, and Gorwa-Grauslund 2005; Bettiga, Hahn-Hägerdal, and Gorwa-Grauslund 2008; Becker and Boles 2003). The goal of this research project was to

introduce 9 genes contained in a cassette, the lignocellulosic transgenic cassette (LTC), using homologous recombination (Figure 4, Table 2). The LTC alone should enable *S. cerevisiae* to ferment xylose and arabinose into ethanol; however, the endogenous xylose reductase (*GRE3*) has been shown to produce xylitol, which inhibits xylose fermentation. Deletion of *GRE3* from *S. cerevisiae* should further bolster metabolic flux from pentose sugars to ethanol (Träff et al. 2001). We hypothesized that introduction of the LTC along with *gre3*Δ would together enable efficient fermentation of xylose and arabinose.

Metabolic engineering of *E. coli* to produce butanol has been demonstrated. Considering *S. cerevisiae* is known for producing high amounts of ethanol it was hypothesized that *S. cerevisiae* would be more tolerant to butanol than *E. coli*. If correct, engineering a strain of yeast for butanol production could be beneficial considering the potential impact of producing high amounts of butanol from lignocellulose.

Chapter 2: Integration of a putative lignocellulosic transgenic cassette (LTC) for the metabolism of pentose sugars by *S. cerevisiae*

Structure of the lignocellulosic transgenic cassette (LTC)

The lignocellulosic transgenic cassette (LTC) was synthesized by DNA 2.0 (Menlo Park, CA) and contains 9 genes and an antibiotic resistance marker, each under the control of a constitutive promoter and each with a terminator sequence (Figure 4). For the promoter sequences, two constitutive promoters were used, one from *ADH1* which is moderately active and the other from *TEF1* which is one of the strongest promoters in *S. cerevisiae* (Da Silva and Srikrishnan 2012).The promoters are not all the same, but the terminator sequences are all code for the terminator sequence of *ADH1*

The cassette of 9 genes is flanked on either end by homology regions to the coding region of the *SPT15* gene of *S. cerevisiae*; the entire integration cassette is flanked on either end by *Pac*I sites for cloning purposes. The genes of the LTC are designed to provide xylose and arabinose fermentation in a single transformation step.

Table 2: 9 genes that constitute the lignocellulosic transgenic cassette, LTC.

Gene	Source Organism	Protein/Enzyme	Purpose
XYL1	*Scheffersomyces stipitis*	Xylose Reductase (XR)	Reduces D-xylose to xylitol
XYL2	*Scheffersomyces stipitis*	Xylulose reductase/Xylitol dehydrogenase	Oxidizes xylitol to D-xylulose
XYL3	*Scheffersomyces stipitis*	Xylulokinase	Converts D-xylulose to D-xylulose-5-P
xylA	*Streptomyces diastaticus*	Xylose Isomerase	Converts D-xylose to D-xylulose
TAL1	*Saccharomyces cerevisiae*	Transaldolase	Increase flux through the pentose phosphate pathway
STL1	*Scheffersomyces stipitis*	Sugar Transporter Like protein	Xylose uptake
araA	*Salmonella typhimurium*	L-arabinose isomerase	Converts L-arabinose to L-ribulose
araB	*Salmonella typhimurium*	L-ribulokinase	Converts L-ribulose to L-ribulose-5-P
araD	*Bacillus subtilis*	L-ribulose-5-phosphate 4-epimerase	Converts L-ribulose-5-P to D-xylulose-5-P

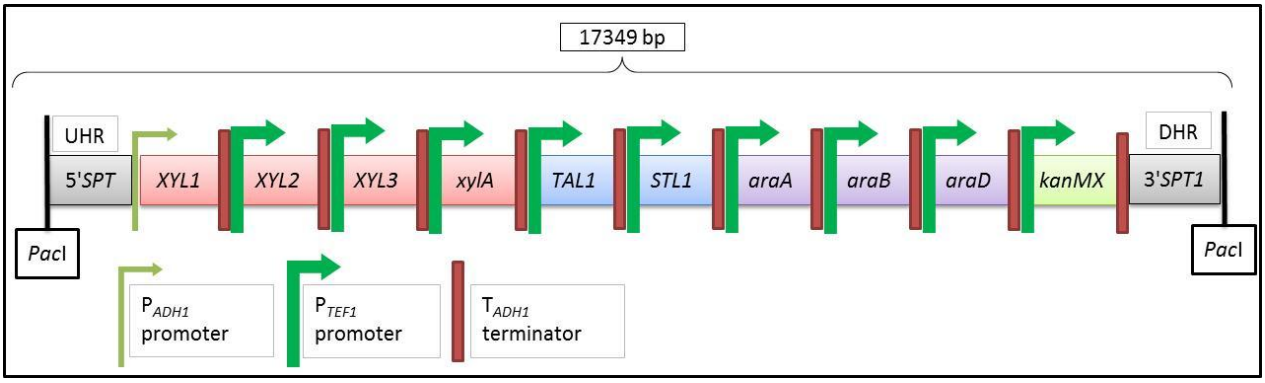

Figure 4: Structure of lignocellulosic transgenic cassette (LTC). The heavy green arrow represents a strong constitutive promoter, P_{TEF1}, while the lighter green arrow represents a weaker constitutive promoter, P_{ADH1}. Genes involved in xylose metabolism (red) and arabinose metabolism (purple) flank *TAL1* and *STL1* (blue) which are transaldolase and transporter genes respectively. *kanMX* (green) is used as a drug resistance marker. The upstream homology and downstream homology regions (UHR/DHR) are homologous to the end of the *SPT15* gene (grey). The entire construct is flanked on either end by *Pac*I restriction sites for cloning purposes.

Chromosomal site for integration of the LTC

Mutations in the transcription factor, Spt15, increase ethanol tolerance and production (Alper et al. 2006). Thus, this site was chosen for integration of the LTC. The LTC was engineered with homology regions to introduce these specific mutations in the *SPT15* gene. The 3 individual point mutations within the *SPT15* gene that improved the ethanol tolerance are $Phe^{177}Ser$, $Tyr^{195}His$, and $Lys^{218}Arg$ (Alper et al. 2006). The modified strain showed a 15% improvement in ethanol yield compared to the control strain. We included these mutations in the strain construction by designing the upstream homology region (UHR) of the LTC against the 3' end of the coding region of *SPT15* (Figures 4 and 6). This allowed us to use a recombination mediated one-step integration protocol (Da Silva and Srikrishnan 2012). The LTC construct was engineered to contain a $G418^R$ marker (*kanMX*) flanked by *loxP* sites which allow efficient Cre recombinase mediated marker removal (Gueldener 2002). Strain confirmation was achieved by purifying genomic DNA (Hoffman and Winston 1987) and performing PCR with primer pairs flanking the insert (Figure 6).

The genes included in the LTC

The alcohol dehydrogenases (ADH) in *S. cerevisiae* that control pyruvate conversion to ethanol is highly active (Piskur et al. 2006), so focus was given to reactions upstream to increase the flux from importing of the sugars to entering glycolysis. We synthesized a ~17 kb transgenic cassette containing 9 genes for lignocellulosic alcohol production (Table 2). An overview of the carbon flux facilitated by the LTC genes is shown in Figure 5.

The primary step of carbohydrate metabolism involves transport of the sugars from the extracellular milieu to the cytosol. Wild-type *S. cerevisiae* is known to have a preferential hexose transport system encoded by the Hxt family of proteins (Hamacher et al. 2002). Hxt2, Hxt6, and Hxt7 are high affinity glucose transporters that are induced at low concentrations of glucose while Hxt1, Hxt3, and Hxt4 are

constitutively expressed low affinity transporters (Chu and Lee 2007). At high concentrations of xylose, the Hxt transporters are capable of xylose uptake ($K_{m\ glucose}$ = 1.5mM, $K_{m\ xylose}$ = 137-190mM for the high affinity group; $K_{m\ glucose}$ = ~20-35mM, $K_{m\ xylose}$ = ~ 1.5M for low affinity group) (Chu and Lee 2007). We designed an active transport system into the cell for the pentose sugars in question. Slt1p is a member of the Major Facilitator Superfamily (MFS) and is a transporter from *S. stipitis* (Altschul et al. 2005; Altschul et al. 1997). This transporter was selected due to its sequence homology to other pentose sugar transporters. Xylose can also enter the cell by simple diffusion. At high enough concentrations of xylose ($\geq$20 g/L xylose), the rate of xylose uptake is not the limiting factor to xylose metabolism as it is 30-fold higher than the xylose utilization rate by *XYL1* (Kotter and Ciriacy 1993).

The xylose reductase (XR) and xylulose dehydrogenase (XDH), encoded by *XYL1* and *XYL2,* respectively must be maintained at proper relative concentrations for efficient conversion from xylose to xylulose (Walfridsson et al. 1997). To prevent the build-up of xylitol (a preferred product of xylose metabolism by *S. cerevisiae*) the intracellular concentration of XDH should be higher than XR (Walfridsson et al. 1997). Therefore we used the constitutive *ADH1* promoter (P_{ADH1}) to drive expression of *XYL1* while a stronger *TEF1* promoter (P_{TEF1}) was used for *XYL2* expression. The expression of the remaining genes was also driven by the P_{TEF1} promoter system (Mumberg, Müller, and Funk 1995).

Of the genes in the LTC, 8 are exogenous to *S. cerevisiae* and have been shown to allow for metabolic flux of xylose or arabinose through the pentose phosphate pathway to pyruvate and finally to ethanol. The one endogenous gene included in the cassette results in an overexpression of *TAL1* which is responsible for a transaldolase reaction in the pentose phosphate pathway. Inclusion of this gene in combination with *XYL1* and *XYL2* has been shown to increase flux to ethanol in *S. cerevisiae* (Walfridsson et al. 1997).

In addition to the XR/XDH pathway from *Scheffersomyces stipitis*, it has also been shown that inclusion of a bacterial xylose isomerase (*xylA*) allows for direct conversion of xylose to xylulose while avoiding formation of xylitol (Brat, Boles, and Wiedemann 2009). Xylose isomerase is known to be inhibited by xylitol formation, therefore xylitol production must be minimized (Chu and Lee 2007).

While not included in the LTC, Gre3p, a putative XR, has been demonstrated to inhibit xylose metabolism (Jin and Jeffries 2004). Specificity for the NADPH cofactor by Gre3 during xylose to xylitol conversion results in a redox imbalance as the downstream XDH enzyme uses NAD+ as a cofactor and does not regenerate the NADPH pool. Therefore, deletion of *GRE3* should increase flux from pentose to ethanol (Träff et al. 2001).

After glucose, xylose is the second most abundant carbohydrate content of lignocellulosic material making up ~30% of the total fermentable sugars(Prusty Rao, Dufour, and Swana 2011). Next, arabinose comprises ~5% of the total fermentable sugars in lignocellulosic material (Tkác et al. 2000). Therefore, we designed the LTC to integrate bacterial genes shown to improve flux from arabinose to ethanol (Wisselink et al. 2007). Specifically, we used 3 genes, *araA*, *araB*, and *araD*, that convert arabinose to xylulose-5-phosphate and subsequently through the non-oxidative pentose phosphate pathway (Figure 5).

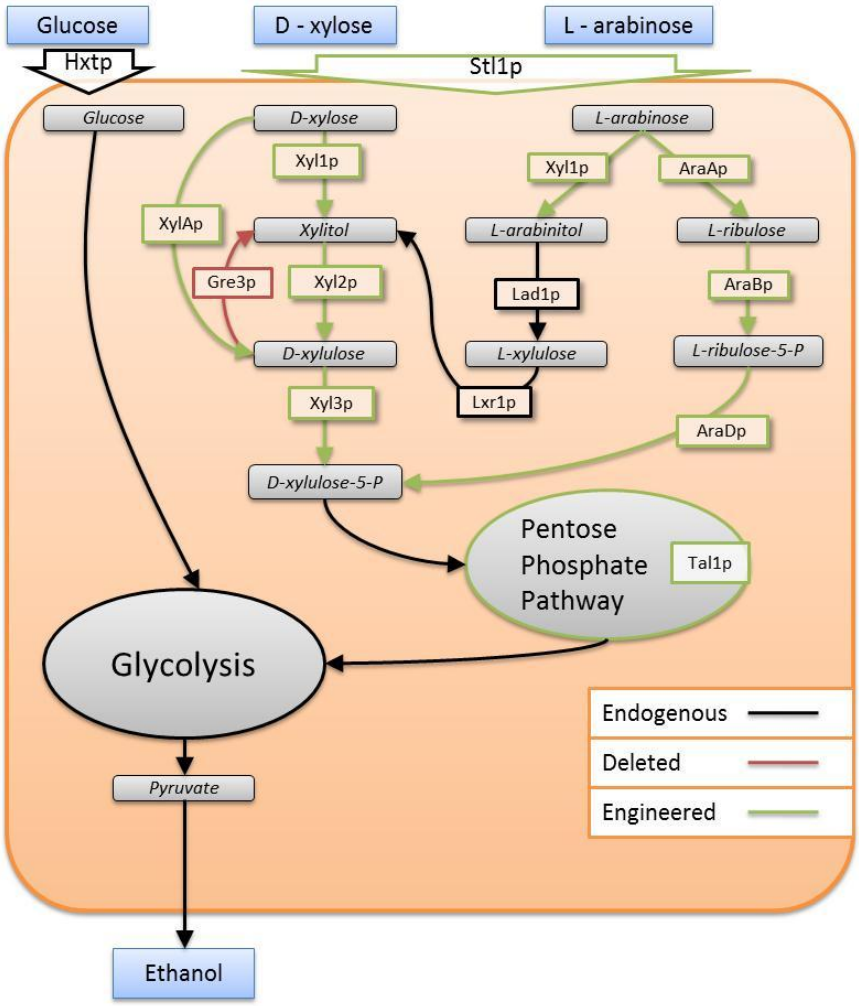

Figure 5: Schematic representation of *Saccharomyces cerevisiae* harboring the LTC. The yeast is genetically modified for improved xylose and arabinose metabolism for the production of alcohol. Black lines represent pathways endogenous to *S. cerevisiae*, green lines represent engineered pathways, and red lines represent steps that have been removed.

Chapter 3: Materials and Methods

Media and Growth Conditions

Yeast cultures were grown in standard yeast peptone dextrose(YPD) (Sherman 2002) media overnight at 30°C unless indicated otherwise. Absorbance of cultures at 600nm (A_{600}) were used to measure culture density. Permanent cultures were stored in glycerol stocks.

Strains

We used the *S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) as the parent strain.

Table 3: Strains used in study.

Strain designation	**Genotype**	**Source**
BY4741	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	ATCC
BY4741 *gre3Δ*	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 gre3Δ*	This study
YLTC LTC integrated into BY4741	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 XYL1 XYL2 XYL3 xylA TAL1 STL1 araA araB araD*	This study
YLDG *gre3*Δ in YLTC	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*BY4741 *XYL1 XYL2 XYL3 xylA TAL1 STL1 araA araB araD gre3*Δ	This study

Purification and amplification of DNA/RNA

BY4741 PCR reactions were completed using standard Taq polymerase (NEB Inc., Ipswich, MA) according to manufacturer instructions using a Bio-rad (Hercules, CA) DNA Engine multi-bay thermal cycler. Lists of primers used are included in the appendix.

Plasmid extractions were completed using Qiagen (Venlo, Netherlands) mini-prep kits. Genomic DNA was purified by standard methods (Hoffman and Winston 1987). RNA was purified via phenol

chloroform precipitation (Schmitt, Brown, and Trumpower 1990). cDNA generation from purified RNA was completed using the SuperScript master mix provided by Life Technologies (Carlsbad, CA).

Chromosomal integration of the LTCBY4741

The DNA fragment harboring the LTC was synthesized by DNA2.0 (Menlo Park, CA) and cloned into *E. coli*. A permanent culture of this clone was maintained. When needed the plasmid was digested with *Pac*I (Figure 4) to release the LTC and gel purified. The linear DNA fragment was then transformed into BY4741 using a lithium acetate protocol described previously (Gietz and Woods 2002). Transformants were selected using the $G418^R$ marker that renders *S. crevisiae* resistance to gentamycin, or G418, an analog of kanamycin. Positive colonies were isolated and maintained on G418 containing media. Since the LTC is ~17 kb we confirmed positive colonies using PCR (Hoffman and Winston 1987) at the insertion junctions to generate YLTC. The G418 marker was then removed from the YLTC by *loxP* mediated recombination by expression of the Cre recombinase protein, a technique commonly used during genetic modification of *S. cerevisiae* (Gueldener 2002). Briefly, expression of Cre recombinase excises the $G418^R$ selection marker between the 34 bp *loxP* recombination sites by 'looping out' of the DNA between these sites, and leaves behind a single *loxP* sequence (Gueldener 2002).

Deletion of GRE3 from YLTC

To delete *GRE3* from YLTC a PCR product was generated with the $G418^R$ marker flanked by homologous sequences upstream and downstream of *GRE3*. This DNA fragment was transformed using the lithium acetate protocol described previously (Gietz and Woods 2002) and transformants were selected using the $G418^R$ marker. Positive colonies were isolated and maintained on G418-containing media. Cre recombinase mediated recombination was used to remove the $G418^R$ marker from confirmed transformants. The resulting strain was YLDG. The same protocol was used on BY4741 to generate BY4741 *gre3*Δ.

Generation of growth curves

The YLTC, BY4741 *gre3*Δ and isogenic control BY4741 strains were inoculated in triplicate into 5 mL of YPD media containing either 5% w/v glucose, 5% w/v xylose + 1% w/v arabinose, or 5% w/v glucose + 1% w/v arabinose + 5% w/v xylose at an initial A_{600} of 0.001. Cultures were grown aerobically at 30°C, rotating; A_{600} readings were taken at 0, 3, 6, 9, 12, and 24 hours.

Calculation of specific growth rate (μ) between for each strain was determined using linear regression of the ln(A_{600}) versus time during the cultures' linear growth phase, between 6 and 12 hrs (Palmqvist et al. 1999). The equation is displayed:

$\mu = ((\text{Ln}\ (t_f\ A_{600})) - (\text{Ln}\ (t_0\ A_{600}))\ /\ \Delta t$

$t_f\ A_{600} = A_{600}$ during the final time point

$t_0\ A_{600} = A_{600}$during the initial time point

Δt = the change in time between t0 and tf

Xylose quantification by HPLC

The YLDG and isogenic control BY4741 strains were grown for 48 hours in 5 mL of YPD media, 10 μL of these cultures were used to inoculate 15 mL conical tubes with 5 mL of yeast nitrogen base (YNB), which included 100 mM glucose and either 100 mM or 200 mM xylose. An identical set up was used for both aerobic and microaerobic conditions. For the aerobic condition the caps were left loose for free gas exchange and for the microaerobic condition the caps were sealed tight. After 3 days of fermentation the cultures were centrifuged at high speed (13,000 xg for 5 minutes), the supernatant was transferred to a fresh tube and centrifuged again to remove all cell debris.

Glucose and xylose concentrations were measure in undiluted samples using HPLC. A Microsorb amino column (5 μm, 250 x 4.6 mm, 100Å, Varian Analytical Instruments, Walnut Creek, CA) with a R401 refractive index detector (Waters, Milford, MA) was used with 85:15::Acetonitrile:H_2O (Vol:Vol) as the

mobile phase at a flow rate of 1.0 mL/min. Each condition was tested in triplicate and compared to a standard curve between 100 mM and 400 mM xylose. Typically, the xylose peak appeared at 7.1-7.2 minutes and the glucose peak at 10.6 minutes depending on the concentration of sugar loaded (Towler, Wyslouzil, and Weathers 2007)

Statistical analysis

To determine the significance of xylose concentration values between strains in separate experimental conditions univariate analysis of variance (ANOVA) methods were used. SPSS software (IBM inc. Armonk, N.Y.) was used to conduct statistical analysis.

Chapter 4: Results

To generate a strain capable of efficiently fermenting pentose sugars to ethanol, the LTC (Figure 4, Table 2) was integrated at a chromosomal location in *S. cerevisiae* (Figure 6). The LTC presumably includes all the genetic elements necessary to improve xylose and arabinose metabolism (Träff et al. 2001; Jin and Jeffries 2004; Chu and Lee 2007; Kuyper et al. 2005; Walfridsson et al. 1997; Wisselink et al. 2007; B Hahn-Hägerdal et al. 2001). Furthermore, it was shown previously that deletion of the endogenous aldose dehydrogenase, *GRE3*, improves the efficiency of fermentation (Träff et al. 2001). Therefore the strategy included integrating the LTC followed by deletion of the *GRE3*. Next, it was necessary to ensure that the LTC was transcribed and perform preliminary assessments for xylose metabolism in the modified strain.

Integration of the LTC

Our group sought to express a set of 8 exogenous genes and 1 endogenous gene which independently demonstrate improvement in the fermentative capacity of *S. cerevisiae* for pentose sugars (Träff et al. 2001; Jin and Jeffries 2004; Chu and Lee 2007; Kuyper et al. 2005; Walfridsson et al. 1997; Wisselink et al. 2007; B Hahn-Hägerdal et al. 2001). This set of 9 genes was included in a cassette referred to as the lignocellulosic transgenic cassette (LTC). We hypothesized that insertion of these genes would collectively increase the efficiency of pentose fermentation. The integration site for the LTC was chosen to be immediately downstream of the wild-type *SPT15* locus as this would allow us to simultaneously introduce 3 point mutations in the *SPT15* gene ($Phe^{177}Ser$, $Tyr^{195}His$, and $Lys^{218}Arg$) which have been previously shown to increase ethanol tolerance in *S. cerevisiae* (Alper et al. 2006). The LTC was synthesized (by DNA 2.0, Inc. Menlo Park, CA), including a 380bp homology region that overlaps with the 3' coding region of *SPT15* and a 138bp homology region immediately downstream of the *SPT15* ORF (Figure 4, Figure 6).

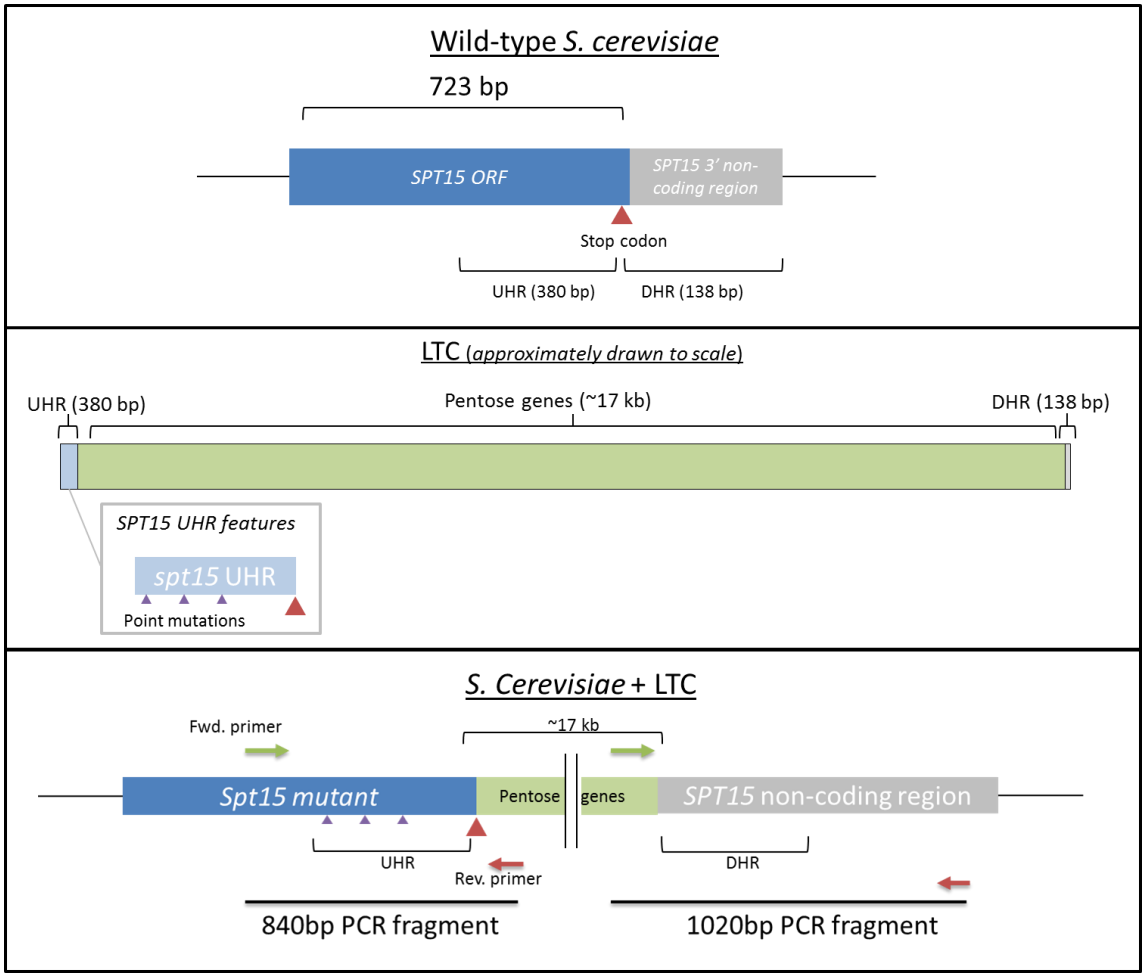

Figure 6: Graphical depiction of the integration of the LTC into the *SPT15* locus. The LTC was designed with overlapping homology regions of the SPT15 locus. The upstream homology region (UHR) of the LTC includes approximately half (380bp) of the coding sequence of the *SPT15* open reading frame (ORF) and includes 3 point mutations (purple triangles). The UHR ends with the 3' stop codon 'TGA' of the *SPT15* gene (red triangle), but is immediately followed by the 9 promoter – gene – terminator sequences coding for pentose fermenting genes of the LTC and the $G418^R$ marker. Following the string of 9 genes is the downstream homology region (DHR) of the LTC which is homologous to the non-coding region of *SPT15* immediately following the stop codon. Green and red arrows at the bottom of the diagram represent the forward and reverse primers, respectively, which were used in PCR reactions to determine proper integration of the LTC.

The LTC was integrated into the chromosome by targeted homologous recombination (Figure 6). G418 resistant colonies were selected (Figure 4). Proper integration of the LTC was confirmed by PCR on both the upstream and downstream regions of the cassette (Figure 7).

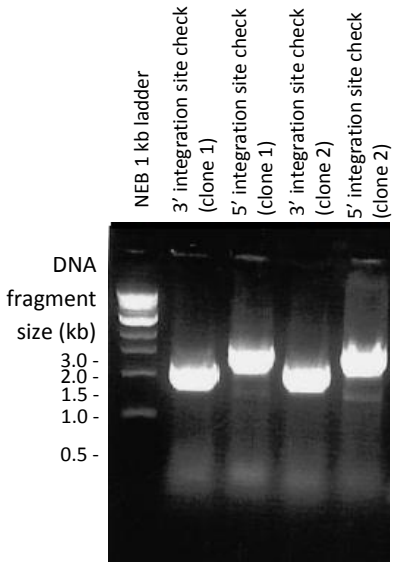

Figure 7: Confirmation of LTC integration. Spanning PCR for two transformants confirmed proper integration of LTC sequence. Proper 5' and 3' junctions were confirmed by the presence of the 1020 bp and 840bp PCR product respectively.

A Cre-recombinase/*loxP* system was employed to remove the G418^R resistance marker that is flanked on either end by *loxP* recombination sites (Gueldener 2002). Briefly, expression of cre-recombinase excises the G418^R selection marker between the *loxP* recombination sites by 'looping out' of the DNA between these sites. This resulted in colonies that no longer displayed resistance to G418; DNA from these colonies was subjected to PCR analysis to confirm the genotype. In this case, primers spanning from the *araD* gene to a region downstream of the *SPT15* DHR was expected to yield a fragment of 800 bp. Though the resulting PCR product is faint, there is a positive amplification product for one of the colonies at this position (Figure 8).

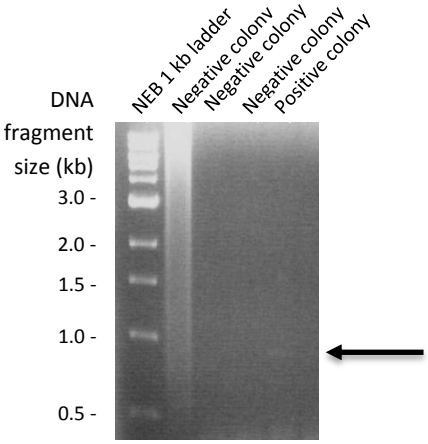

Figure 8: Confirmation of G418^R marker removal. Removal of G418^R marker was confirmed by PCR resulting in an 800 bp product (lane 5, black arrow) instead of a ~2500 bp fragment if the marker was still present.

Deletion of GRE3

The endogenous aldose reductase gene *GRE3* catalyzes the conversion of xylose to xylulose using NADPH. This creates an cofactor imbalance for the XR/XDH pathway, therefore deleting this gene may improve ethanol production from xylose (Träff et al. 2001). The removal of *GRE3* was performed using homologous recombination replacing the entire ORF with the G418^R marker. Complete deletion of the ORF was confirmed by PCR (Figure 9). The resulting strain is referred to as YLDG. The same process of deleting *GRE3* from BY4741 was performed (data not shown) as a control for growth curve comparisons.

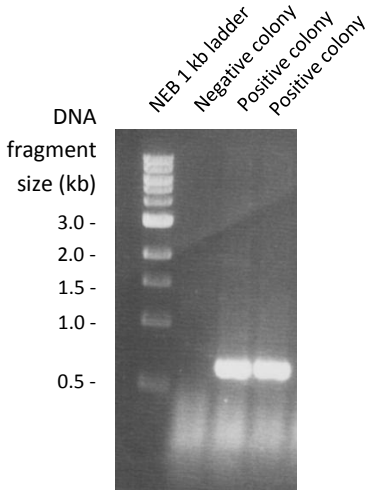

Figure 9: Confirmation of *GRE3* deletion. Deletion of the aldose reductase gene *GRE3* was confirmed by PCR, a product of 650 base pairs shows proper integration of the $G418^R$ marker into the *GRE3* locus.

Expression of the LTC

To test the expression of the genes in the LTC, cDNA products were analyzed for the genes at either end of the LTC (Figure 10). To obtain the cDNA, RNA was purified from the YLDG strain using a phenol chloroform extraction method (Schmitt, Brown, and Trumpower 1990) and cDNA generation was performed using the SuperScript reverse-transcriptase kit from Life Technologies (Carlsbad, CA). Integrity and quality of the RNA prep was determined by verifying the presence of distinct bands for the 18S and 26S rRNA subunits. The presence of a 100 bp amplicon indicates that *XYL1* and *araD* genes at either end of the LTC, were transcribed (Figure 10). The gene for actin (*ACT1)* was used as a positive control and an amplicon of 100 bp was observed as well (appendix).

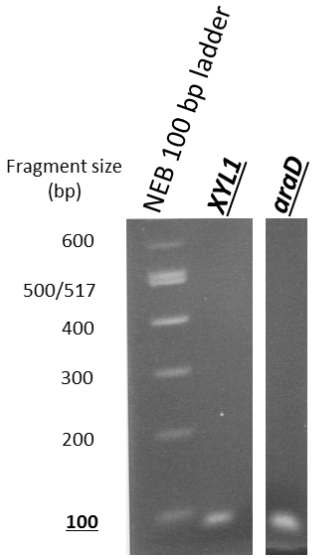

Figure 10: Transcription of the genes on either end of the LTC. The presence of 100 bp RT-PCR amplicon in lanes marked *XYL1* and *araD* indicate that the genes at either end of the LTC were being expressed.

Strain characterization

To evaluate the ability of YLDG to utilize xylose, we compared the growth profile of YLDG to the isogenic control BY4741 and analyzed their respective xylose content after fermentation.

Growth curves

A variety of sugar combinations were tested to measure the ability of YLDG to metabolize pentose sugars. Media containing 5% xylose + 1% arabinose, 5% glucose only, or a combination of 5% xylose + 5% glucose + 1% arabinose were inoculated with various strains (percentages are all based on weight to volume of sugar to media). Absorbance at 600 nm (A_{600}) was measured every 3 hours to generate the growth curves (Figure 11) of 3 strains of *S. cerevisiae* BY4741, YLTC, and BY4741 *gre3*Δ. Equal number of cells were used to inoculate 5 mL cultures in triplicate and grown in standard aerobic growth conditions. The isogenic BY4741 *gre3Δ* strain was generated as a control for the experiment below and does not show the same impact on growth as the LTC.

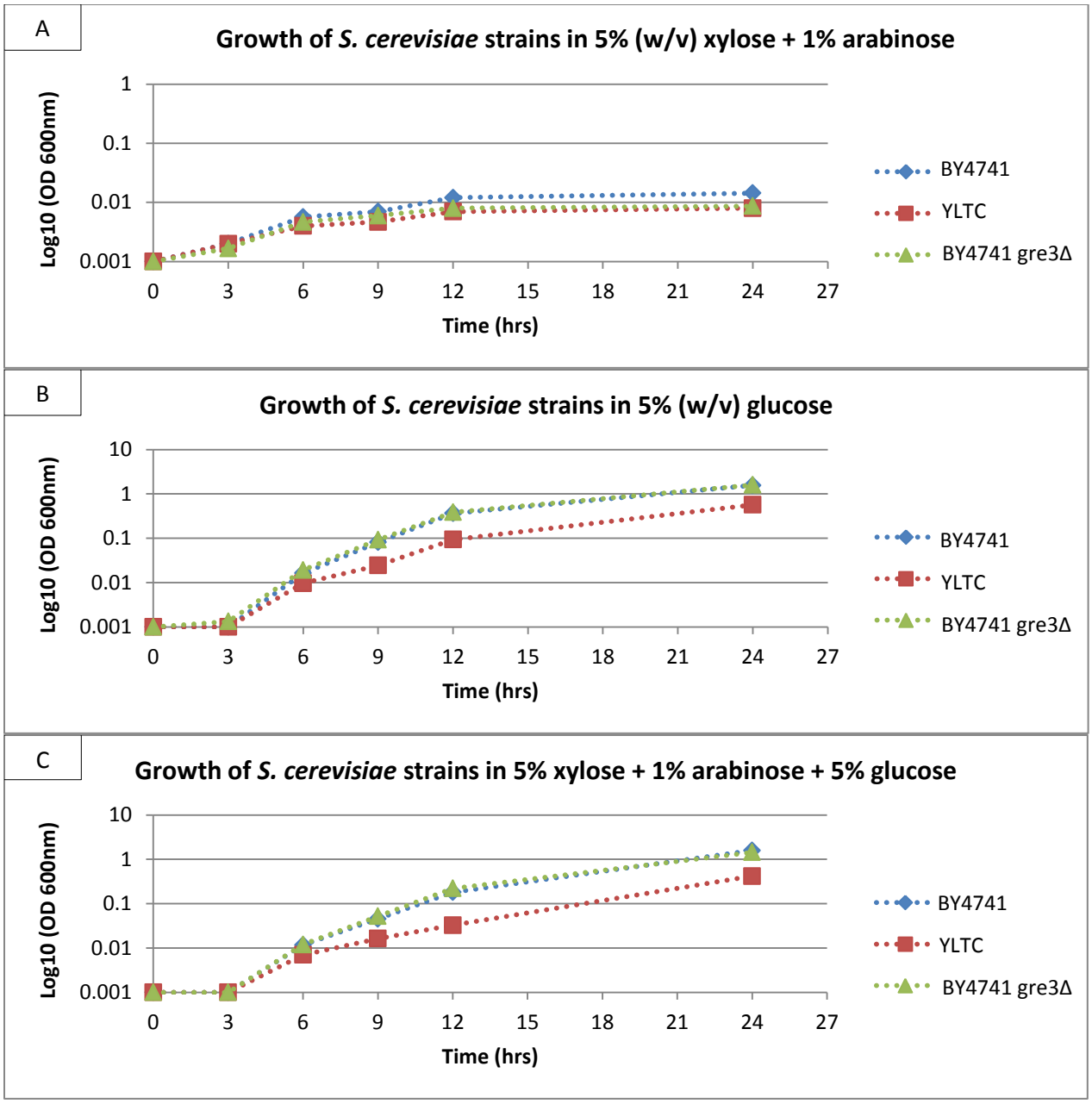

Figure 11: Growth comparison of modified *S. cerevisiae*. The modified strain YLTC harboring the LTC construct was compared to the base-strain (BY4741), and a *gre3*Δ strain. No growth from any strain was detected in media containing xylose and arabinose (A). The strain containing the LTC shows a decreased growth rate in media containing glucose (B and C). Samples were tested in triplicate.

In the presence of glucose, strain YLTC showed a decrease in growth rate as compared to the BY4741 *gre3Δ* strain and isogenic wild type strain (Table 4). In the presence of xylose, the growth curves for YLTC and BY4741 *gre3Δ* strains were indistinguishable from their wild type counterpart.

Table 4: Growth rates with pentoses versus glucose

Specific growth rate (ΔOD/hr)			
	5% xylose + 1% arabinose	5% glucose	5% xylose + 1 % arabinose + 5% glucose
BY4741	0.10	0.52	0.46
BY4741 *gre3*Δ	0.06	0.50	0.49
YLTC	0.02	0.38	0.26

Xylose consumption analysis

To evaluate sugars consumption during the fermentation, we measured sugar concentrations after fermentation using HPLC analysis. The YLDG strain and isogenic control BY4741 strain were inoculated into YNB minimal media with 100 mM glucose (1.8% w/v) and either 100 or 200 mM xylose (1.5% or 3.0% w/v). After 3 days the cells were removed by centrifugation and HPLC was used to determine the residual concentration of xylose in the conditioned media (Figure 12).

Under microaerobic conditions, the YLDG strain showed a statistically significant increase in xylose utilization compared to BY4741 but no significant difference was observed in aerobic conditions (Figure 12).

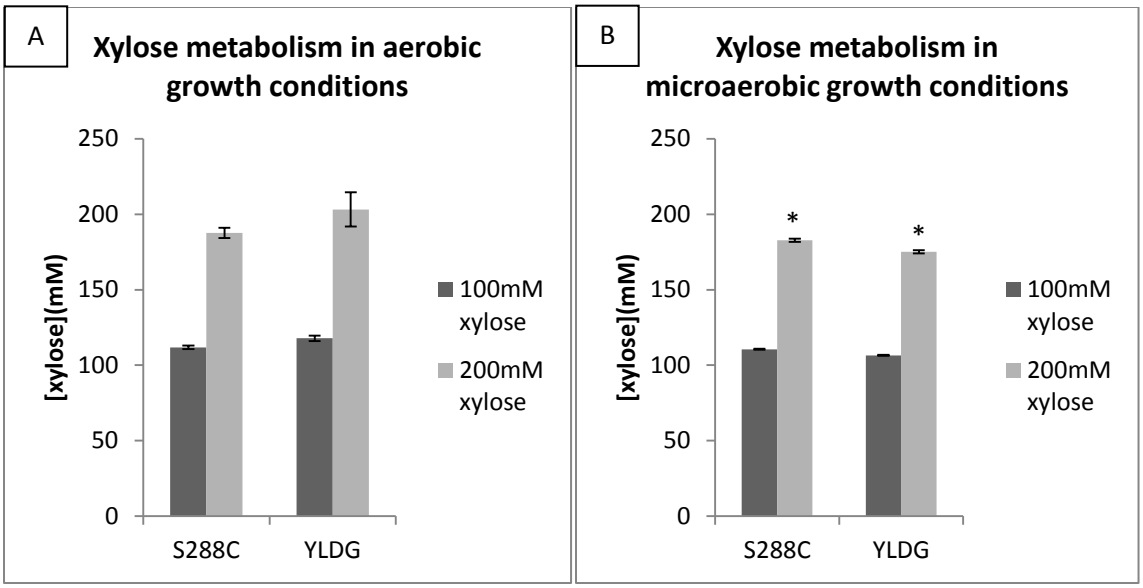

Figure 12: Xylose metabolism analysis. The figure above displays the concentration of xylose remaining after 3 days of incubation at 30°C with minimal media including 100mM glucose and either 100 mM xylose or 200 mM xylose in both aerobic (A), and microaerobic (B) conditions. Samples were tested in triplicate. Asterisks above the 200mM xylose microaerobic condtion indicate a significant difference in xylose concentrations. YLDG: BY4741, LTC, *gre3*Δ (Note: xylose concentrations are based on a standard curve)

Butanol tolerance

We tested BY4741 along with a strain of *E. coli* (J96) for growth tolerance in increasing concentrations of isobutanol. The assay was run in triplicate in 96-well plates in isobutanol concentration from 0.5 % - 5.0 % v/v isobutanol/media. Growth was measured by absorbance at 600 nm and compared to J96 and BY4741 in media lacking isobutanol. As was anticipated, *S. cerevisiae* outperformed *E. coli* in its tolerance to high concentrations of isobutanol (Figure 13). We compared the relative MIC_{50} (50% Minimum Inhibitory Concentration) of both plots and determined the average MIC_{50} for BY4741 and J96 to be ~3.9 % and ~3.4 % isobutanol respectively.

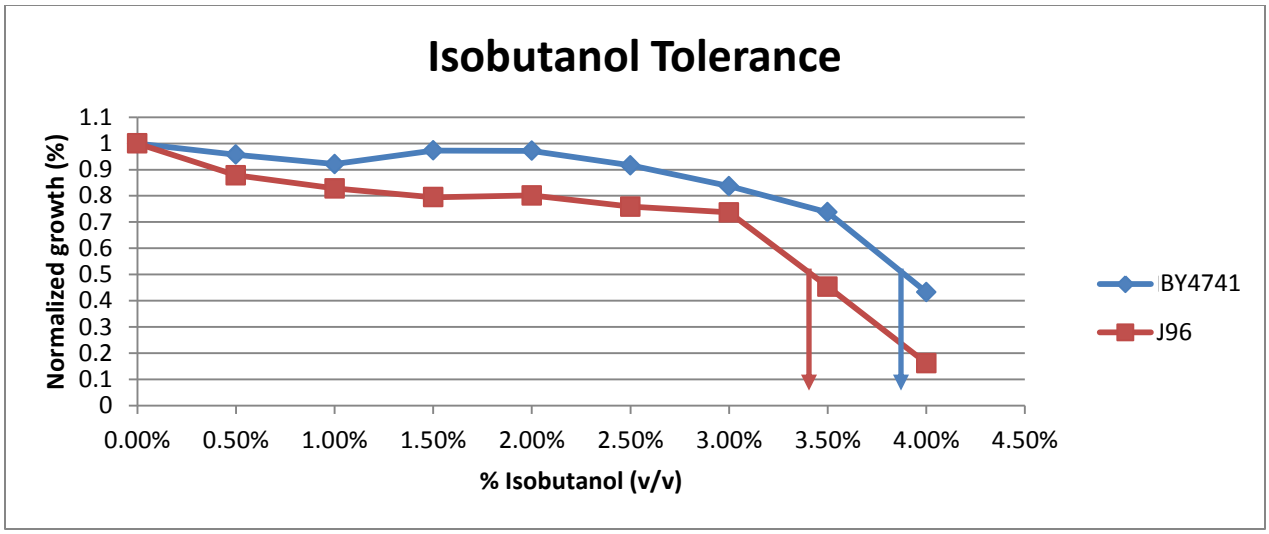

Figure 13: Tolerance to isobutanol. Comparison of the minimum inhibitory concentration of isobutanol for *E. coli* (J96) and *S. cerevisiae* (BY4741)

Chapter 5: Discussion

Traditional metabolic engineering approaches are demonstrated at a modular level (Dueber et al. 2009). Our goal was to use the previously demonstrated successes of multiple other groups to generate a cassette (LTC) harboring all the required elements for pentose fermentation. The goal of these experiments was to introduce the LTC into *S. cerevisiae* for pentose fermentation using a single genetic manipulation. We were successful in integrating the LTC into BY4741. This result in itself was a significant achievement because introducing a large number of heterologous genes (Krivoruchko, Siewers, and Nielsen 2011; Da Silva and Srikrishnan 2012) is not trivial.

Transcriptional analysis of the genes at the ends of the LTC (*XYL1* and *araD*) confirmed that these genes were expressed. This result implied proper transcription of the entire LTC but expression of the 7 other genes was not confirmed.

Analysis of the growth kinetics of the LTC harboring strain in glucose containing media demonstrated a decrease in growth rate (Table 4). This result was not necessarily surprising as many modified strains show a similar growth defect, yet are more metabolically productive than the control strains. Unfortunately, the LTC harboring strain did not exhibit the robust growth in xylose (Figure 11A) which is typically observed in *S. cerevisiae* strains harboring either the XR/XDH or XI pathways (Chu and Lee 2007; Kotter and Ciriacy 1993; Matsushika et al. 2009; Karhumaa, Hahn-Hägerdal, and Gorwa-Grauslund 2005; Bettiga, Hahn-Hägerdal, and Gorwa-Grauslund 2008).

It was hypothesized that the addition of the LTC should have provided the genetic pathways required for fermentation of xylose into ethanol. The xylose consumption assay does show a slight (10 mM) decrease in xylose concentrations after microaerobic fermentation with the modified strain (YLDG) as compared to the wild-type (Figure 12); though this result is statistically significant, the aerobic fermentation results are not significant (Figure 12). Considering the role of aeration for mixed sugar fermentations using

exogenous xylose metabolizing enzymes (Kuyper et al. 2005; Chu and Lee 2007; Kotter and Ciriacy 1993), it was hypothesized that any observed difference would have been in the aerobic fermentation condition. This was not the case, nor was the observed difference in xylose utilization substantial. As a point of comparison, one of the first ever xylose metabolizing strains produced by Kotter and Ciracy (1993) consumed ~150 mM xylose in 72 hours, substantially greater than the result above; though in the fermentation run by Kotter and Ciracy, a high cell density was used for inoculum while our fementation conditions were not ideal (Lee, Jellison, and Alper 2012). Optimizing fermentation conditions could potentially display improved xylose metabolism in the LTC strain.

A study by Carcieri, Clardy, and Zahid (2010) confirmed the decrease in growth kinetics of a strain harboring the LTC as compared to an isogenic wild-type strain. In the same study, the group evaluated production of ethanol using various glucose/xylose ratios using a high density inoculum into media containing 1% w/v total sugars. Growth and ethanol titer in media containing only xylose was negligible by either the wild-type or modified strains. Similarly, in every media combination tested, ethanol production of both strains was comparable. In one experiment where the xylose and glucose content were 0.4% w/v and 0.6% w/v respectively, the modified strain did produce more ethanol (0.042%) than theoretically possible from the glucose fraction which was 0.03%. Unfortunately, the wild-type strain showed a similar titer at 0.041% which is also higher than the theoretical maximum (Carcieri, Clardy, and Zahid 2010). While it is known that wild-type *S. cerevisiae* does contain enzymes capable of xylose metabolism, ethanol formation from xylose is negligible (Batt et al. 1986). Sampling errors were mentioned throughout the report so it is likely that this is an over-estimation of the actual ethanol titer. Nonetheless an ideal xylose to glucose ratio of 4 g/L xylose to 6 g/L glucose for mixed sugar fermentation was identified and could be used in future experiments.

The primary factor that accounts for minimal pentose fermentation is that the expression of 7 internal transgenes of the LTC was not confirmed, though expression was confirmed for the ends of the LTC (Firgure 10). Expression of the internal transgenes can be tested by RT-PCR, primer pairs for each of the 9 genes on the LTC have been designed to produce ~100 bp amplicons. Proper RT-PCR could easily be completed by purifying RNA, generating cDNA and comparing PCR products from both the RNA and cDNA samples, while using LTC plasmid DNA as a positive control. Detection of amplicons from the cDNA sample and no detection in the RNA sample would indicate transcription of the genes as opposed to amplification from residual genomic DNA in RNA samples. In addition to the lack of a complete transcriptional profile, protein levels for the genes on the LTC were not tested. To test whether the proteins are being translated, SDS-PAGE and western blot analysis could be used on wild-type and LTC harboring strains. Enrichment of proteins using protein purification techniques such as ion-exchange and size exclusion chromatography are also possible (Verduyn et al. 1985). Similarly, enzymatic assays are commonly used and could detect the activity of xylose metabolizing genes (Jin and Jeffries 2003). Another factor which could account for low pentose metabolism is the genetic background. The parent strain, BY4741, was chosen because of the tools it provides; however, its fermentative capacity is low. The conscious decision to use this strain enabled us to demonstrate that the one-step genomic insertion is achievable. This technique is easily replicated in strains with more fermentative competency as needed.

Depending on the results of these experiments, it would be possible to determine the effectiveness of 1) the fermentative capacity of the LTC harboring strains and 2) an accurate genetic depiction of the LTC within the strains. Experiments could be performed quickly as they require little planning or research in order to obtain results and provide information for the future direction of the project.

Although the engineered strain did not metabolize substantial amounts of xylose, the assay conditions were not ideal for determining the true fermentative capacity of the strain (Lee, Jellison, and Alper 2012). What has been presented in this report is a summary of the experiments which have been performed, but also a path forward for the eventual goal of a transgenic cassette which may impart a one-step solution to pentose metabolism in *Saccharomyces cerevisiae*.

Aside from ethanol, *S. cerevisiae* has been widely adopted for industrial production of biologically derived products (Ro et al. 2006; Huang et al. 2008). In light of the increased tolerance to butanol of *S. cerevisiae* versus *E. coli* (Figure 13) and the recent work done by Atsumi et al. (2008), it seems feasible to introduce a single gene and produce a strain of *S. cerevisiae* capable of butanol fermentation from carbohydrates. While integration of *kivD* into the *S. cerevisiae* genome has yet to be attempted, an engineered strain of *S. cerevisiae* could be produced capable of fermenting butanol from pentose sugars.

Chapter 4: Conclusion

Engineering of *S. cerevisiae* for the production of biofuels has provided incremental steps in the progress of second generation biofuels, yet much work remains to be done. Unfortunately, the design and analysis of a 'super cassette' for the fermentation of pentose sugars to ethanol, described here as the LTC, provided little in these efforts to understand the process or improve the field. What should be taken away from this work is, while it is technically feasible to design, construct, and integrate a cassette containing 9 constitutively active genes, it should not be attempted in the manner described. Step-wise construction and verification of the individual components of the xylose and arabinose metabolism pathways should be proven; then assembly of larger cassettes could be attempted.

Despite the ineffectiveness of the LTC for the intended purpose of a one-step solution to pentose fermentation by *S. cerevisiae*, the concept is still novel and valid, and a functional LTC could be generated with proper planning and execution of experiments. On the same note, assuming production of isobutanol by *S. cerevisiae* could be demonstrated, a pathway moving from xylose to butanol using *S. cerevisiae* would be a remarkable achievement; though, again, substantial planning and execution of experiments is essential.

Chapter 5: References

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Appendix

Table A1: Raw data from growth curve comparison. It should be noted that during this experiment the strain designated 'BY4741 + LTC, GRE3(Δ)' became contaminated and these values should not be considered valid.

T=3Hrs	BY4741	BY4741 + LTC	BY4741 + LTC, GRE3(Δ)	BY4741, GRE3(Δ)
Xyl				
1	0.002	0.002	0.024	0.002
2	0.002	0.002	0.024	0.002
3	0.002	0.002	0.022	0.001
Avg	0.002	0.002	0.023333	0.001667
StDv	0	0	0.001155	0.000577
Xyl+Ara				
1	0.002	0.002	0.017	0.002
2	0.002	0.002	0.017	0.002
3	0	0.002	0.017	0.002
Avg	0.001333	0.002	0.017	0.002
StDv	0.001155	0	0	0
Xyl+Ara+Glu				
1	0	0.001	0.015	0.001
2	0	0	0.019	0
3	0	0	0.017	0
Avg	0	0.000333	0.017	0.000333
StDv	0	0.000577	0.002	0.000577
Glu				
1	0.001	0.003	0.048	0.001
2	0.001	0.003	0.055	0.001
3	0.001	0.002	0.052	0.002
Avg	0.001	0.002667	0.051667	0.001333
StDv	0	0.000577	0.003512	0.000577
T=6Hrs	BY4741	BY4741 + LTC	BY4741 + LTC, GRE3(Δ)	BY4741, GRE3(Δ)
Xyl				
1	0.006	0.004	0.29	0.005
2	0.006	0.004	0.299	0.005
3	0.005	0.004	0.29	0.004
Avg	0.005667	0.004	0.293	0.004667
StDv	0.000577	0	0.005196	0.000577

Xyl+Ara				
1	0.006	0.005	0.2	0.005
2	0.006	0.008	0.213	0.005
3	0.007	0.005	0.211	0.005
Avg	0.006333	0.006	0.208	0.005
StDv	0.000577	0.001732	0.007	0
Xyl+Ara+Glu				
1	0.011	0.008	0.208	0.011
2	0.012	0.005	0.225	0.013
3	0.012	0.008	0.198	0.012
Avg	0.011667	0.007	0.210333	0.012
StDv	0.000577	0.001732	0.01365	0.001
Glu				
1	0.017	0.01	0.55	0.02
2	0.016	0.009	0.579	0.02
3	0.016	0.01	0.567	0.018
Avg	0.016333	0.009667	0.565333	0.019333
StDv	0.000577	0.000577	0.014572	0.001155
T=9Hrs	BY4741	BY4741 + LTC	BY4741 + LTC, GRE3(Δ)	BY4741, GRE3(Δ)
Xyl				
1	0.007	0.005	1.97	0.006
2	0.007	0.005	1.95	0.006
3	0.007	0.004	1.97	0.006
Avg	0.007	0.004667	1.963333	0.006
StDv	0	0.000577	0.011547	1.06E-18
Xyl+Ara				
1	0.008	0.005	1.55	0.006
2	0.008	0.005	1.65	0.006
3	0.008	0.006	1.53	0.006
Avg	0.008	0.005333	1.576667	0.006
StDv	0	0.000577	0.064291	1.06E-18
Xyl+Ara+Glu				
1	0.048	0.016	1.58	0.047
2	0.047	0.017	1.69	0.06
3	0.041	0.016	1.58	0.049
Avg	0.045333	0.016333	1.616667	0.052
StDv	0.003786	0.000577	0.063509	0.007
Glu				
1	0.082	0.024	2.61	0.098
2	0.079	0.024	2.54	0.092

3	0.082	0.025	2.53	0.088
Avg	0.081	0.024333	2.56	0.092667
StDv	0.001732	0.000577	0.043589	0.005033
T=12Hrs	BY4741	BY4741 + LTC	BY4741 + LTC, GRE3(Δ)	BY4741 GRE3(Δ)
Xyl				
1	0.012	0.007	4.5	0.008
2	0.012	0.007	4.37	0.008
3	0.012	0.007	4.27	0.008
Avg	0.012	0.007	4.38	0.008
StDv	2.12E-18	0	0.115326	0
Xyl+Ara				
1	0.012	0.006	4.11	0.008
2	0.011	0.007	4.22	0.007
3	0.011	0.007	4.33	0.007
Avg	0.011333	0.006667	4.22	0.007333
StDv	0.000577	0.000577	0.11	0.000577
Xyl+Ara+Glu				
1	0.172	0.034	3.27	0.208
2	0.198	0.032	3.21	0.253
3	0.172	0.032	3.2	0.202
Avg	0.180667	0.032667	3.226667	0.221
StDv	0.015011	0.001155	0.037859	0.027875
Glu				
1	0.367	0.091	3.31	0.406
2	0.356	0.097	3.13	0.393
3	0.37	0.094	2.86	0.365
Avg	0.364333	0.094	3.1	0.388
StDv	0.007371	0.003	0.226495	0.020952
T=24Hrs	BY4741	BY4741 + LTC	BY4741 + LTC, GRE3(Δ)	BY4741, GRE3(Δ)
Xyl				
1	0.013	0.009	4.58	0.008
2	0.014	0.008	4.65	0.009
3	0.016	0.007	4.43	0.009
Avg	0.014333	0.008	4.553333	0.008667
StDv	0.001528	0.001	0.112398	0.000577
Xyl+Ara				
1	0.016	0.008	4.57	0.007
2	0.014	0.008	4.58	0.01

3	0.012	0.007	4.49	0.009
Avg	0.014	0.007667	4.546667	0.008667
StDv	0.002	0.000577	0.049329	0.001528
Xyl+Ara+Glu				
1	1.55	0.45	2.69	1.48
2	1.64	0.41	2.34	1.5
3	1.57	0.4	2.92	1.35
Avg	1.586667	0.42	2.65	1.443333
StDv	0.047258	0.026458	0.292062	0.081445
Glu				
1	1.6	0.54	2.72	1.62
2	1.53	0.59	2.65	1.6
3	1.56	0.58	2.62	1.58
Avg	1.563333	0.57	2.663333	1.6
StDv	0.035119	0.026458	0.051316	0.02

Table A2: Peak area integrations from HPLC analysis of xylose standard curve. Used to calculate effective xylose concentrations.

blank media [xylose] (M)	Peak area
0.1	513027
0.1	496356
0.1	480605
0.1	483910
0.1	480693
0.2	1158360
0.2	1130947
0.2	1099999
0.2	1102967
0.2	1086111
0.4	3091668
0.4	2935754
0.4	2732836

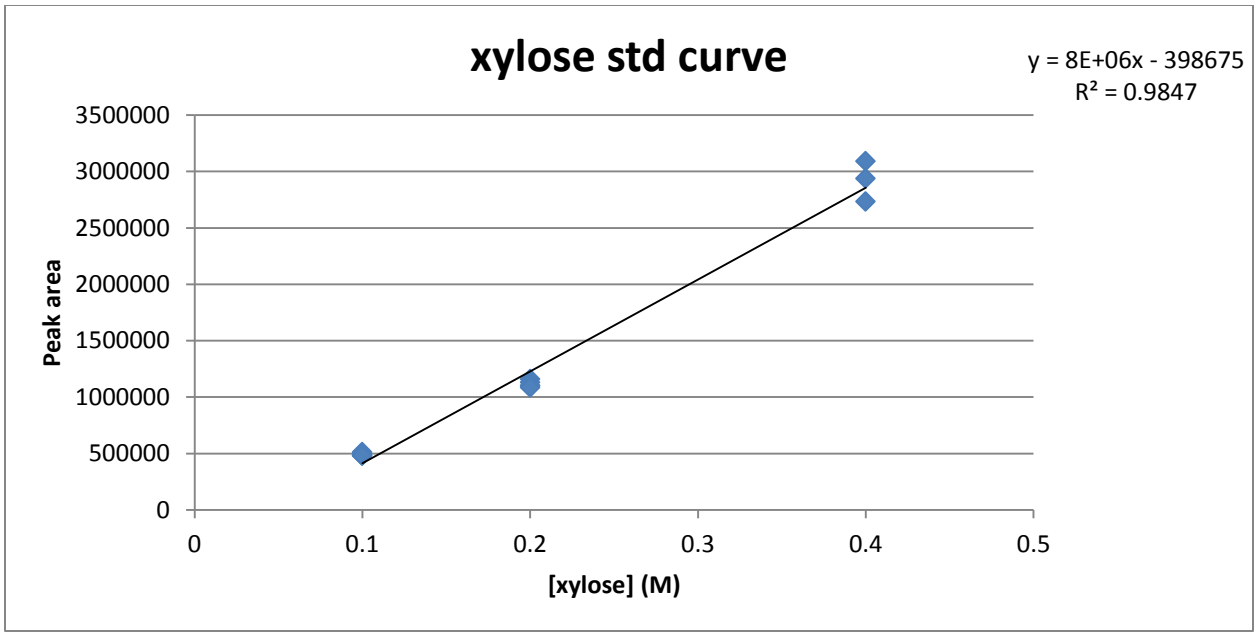

Figure A1: Standard curve used for calculating xylose concentrations.

Table A3: Peak area integrations from HPLC analysis of YLDG and BY4741. In either 100mM xylose (designated #2) or 200mM xylose (designated #3). A versus AN represent aerobic versus microaerobic growth conditions respectively.

Peak area	Strain-media #- respiration
489343	BY4741-2-A
487617	BY4741-2-A
473344	BY4741-2-A
1067637	BY4741-3-A
1119887	BY4741-3-A
1080627	BY4741-3-A
471038	BY4741-2-AN
472671	BY4741-2-AN
476037	BY4741-2-AN
1042654	BY4741-3-AN
1055787	BY4741-3-AN
1053941	BY4741-3-AN
523799	YLDG-2-A
523021	YLDG-2-A
548181	YLDG-2-A
1159354	YLDG-3-A
1318121	YLDG-3-A
1164266	YLDG-3-A
440373	YLDG-2-AN

439379	YLDG-2-AN
443105	YLDG-2-AN
983668	YLDG-3-AN
993802	YLDG-3-AN
991936	YLDG-3-AN

Table A4: Primers used for confirmation of genotypes

Primer #	Primer name	Sequence	Tm
373	5' *SPT15*	GAGCTGCCCCAGAATCTG	62.2
381	3' *XYL1*	CAGCAGGCATATCGTAGCCCG	66.5
380	5' *G418*	GTCGGGCAATCAGGTGCGACA	66.4
377	3' *SPT15*	GCTGGAAAGGGAGCTTGCTT	62.4
383	5' *araD*	GGACGGAGAGGTGGTTGAAGGC	66.5
359	5' *GRE3D*	TAATATAAATCGTAAAGGAAAATTGGA-AATTTTTTAAAGCAGGTCGACAACCCTTAAT	-
360	3' *GRE3D*	TGTTCATATCGTCGTTGAGTATGGTTTT-ACTGGCTGGAGTGGCTGGAGTGGATCTGATATCACCTA	-
361	5' *GRE3*	AGACGCAGATACTGTAAATG	43
371	3' *GRE3*	CACTCACTCATCGCCCCC	50

Table A5: Primers used for RT-PCR analysis

Primer #	Name	Sequence	Tm (°C)
384	5' XYL1	5 - AACTTCCCAGGCGCATTACT - 3	56.9
385	3' XYL1	5 - TATGGGTGGTGTTCGACCTGA - 3	58
401	5' ARAD	5 - TCGCAATGGGCTACTAGTTGGGCAC - 3	63
402	3' ARAD	5 - AAACTCCCGGCACCTGTTCGTA - 3	60.8
403	5' ACT1	5 - CTCCACCACTGCTGAAAGAGAA - 3	56.9
404	3' ACT1	5 - CCAAGGCGACGTAACATAGTTTT - 3	55.5

FigureA2: RT-PCR of *ACT1* control. cDNA was generated from S. cerevisiae strains harboring the LTC. Primers were designed against *ACT1* as a control. An amplicon of 100 bp indicates transcription of *ACT1*.**Δ**