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OPTIMIZATION OF ETHANOL PRODUCTION BY YEASTS FROM
LIGNOCELLULOSIC FEEDSTOCKS

By

STEPHANIE LEE GROVES

A Thesis

Submitted in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

(Biological Sciences)

MICHIGAN TECHNOLOGICAL UNIVERSITY

2009

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This thesis, "Optimization of ethanol production by yeasts from lignocellulosic feedstocks," is hereby approved in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in BIOLOGICAL SCIENCES.

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List of Abbreviations

AP – Acid Pretreatment

CSL– Corn Steep Liquor

DAD – Diode Array Detector

DAP – Dilute Acid Pretreatment

ER – Ethanol Red (*Saccharomyces cerevisiae*)

HMF – 5-Hydroxymethylfurfural

HPLC – High Performance Liquid Chromatography

k – growth rate

RID- Refractive Index Detector

SD – Standard Deviation

SDB - Sabouraud Dextrose Broth

YNB – Yeast Nitrogen Base

YP – Yeast Peptone Broth

YPG – Yeast Extract-Peptone-Glucose

YPX- Yeast Extract-Peptone-Xylose

YP-Hexose – Yeast Extract-Peptone-Hexose

YP-Pentose – Yeast Extract-Peptone-Pentose

Abstract

Ethanol from lignocellulosic feedstocks is not currently competitive with corn-based ethanol in terms of yields and commercial feasibility. Through optimization of the pretreatment and fermentation steps this could change. The overall goal of this study was to evaluate, characterize, and optimize ethanol production from lignocellulosic feedstocks by the yeasts *Saccharomyces cerevisiae* (strain Ethanol Red, ER) and *Pichia stipitis* CBS 6054.

Through a series of fermentations and growth studies, *P. stipitis* CBS 6054 and *S. cerevisiae* (ER) were evaluated on their ability to produce ethanol from both single substrate (xylose and glucose) and mixed substrate (five sugars present in hemicellulose) fermentations. The yeasts were also evaluated on their ability to produce ethanol from dilute acid pretreated hydrolysate and enzymatic hydrolysate. Hardwood (aspen), softwood (balsam), and herbaceous (switchgrass) hydrolysates were also tested to determine the effect of the source of the feedstock. *P. stipitis* produced ethanol from 66-98% of the theoretical yield throughout the fermentation studies completed over the course of this work. *S. cerevisiae* (ER) was determined to not be ideal for dilute acid pretreated lignocellulose because it was not able to utilize all the sugars found in hemicellulose. *S. cerevisiae* (ER) was instead used to optimize enzymatic pretreated lignocellulose that contained only glucose monomers. It was able to produce ethanol from enzymatically pretreated hydrolysate but the sugar level was so low (>3 g/L) that it would not be commercially feasible.

Two lignocellulosic degradation products, furfural and acetic acid, were evaluated for whether or not they had an inhibitory effect on biomass production, substrate utilization, and ethanol production by *P. stipitis* and *S. cerevisiae* (ER). It was determined that inhibition is directly related to the concentration of the inhibitor and the organism.

The final phase for this thesis focused on adapting *P. stipitis* CBS 6054 to toxic compounds present in dilute acid pretreated hydrolysate through directed evolution. Cultures were transferred to increasing concentrations of dilute acid pretreated hydrolysate in the fermentation media. The adapted strains' fermentation capabilities were tested against the unadapted parent strain at each hydrolysate concentration. The fermentation capabilities of the adapted strain were significantly improved over the unadapted parent strain. On media containing 60% hydrolysate the adapted strain yielded $0.30 \text{ g_ethanol/g_sugar} \pm 0.033 \text{ (g/g)}$ and the unadapted parent strain yielded $0.11 \text{ g/g} \pm 0.028$. The culture has been successfully adapted to growth on media containing 65%, 70%, 75%, and 80% hydrolysate but with below optimal ethanol yields (0.14-0.19 g/g). Cell recycle could be a viable option for improving ethanol yields in these cases.

A study was conducted to determine the optimal media for production of ethanol from xylose and mixed substrate fermentations by *P. stipitis*. Growth, substrate utilization, and ethanol production were the three factors used to evaluate the media. The three media tested were Yeast Peptone (YP), Yeast Nitrogen Base (YNB), and Corn Steep Liquor (CSL). The ethanol yields (g/g) for each medium are as follows: YP - 0.40-0.42, YNB - 0.28-0.30, and CSL - 0.44-0.51. The results show that media containing CSL result in slightly higher ethanol yields than other fermentation media.

P. stipitis was successfully adapted to dilute acid pretreated aspen hydrolysate in increasing concentrations in order to produce higher ethanol yields compared to the unadapted parent strain. *S. cerevisiae* (ER) produced ethanol from enzymatic pretreated cellulose containing low concentrations of glucose (1-3g/L). These results show that fermentations of lignocellulosic feedstocks can be optimized based on the substrate and organism for increased ethanol yields.

Introduction

As the push for ethanol to reduce the reliance on petroleum-based fuels increases, so does research in the area of optimizing ethanol production through the bioconversion of lignocellulose. Lignocellulose is the most abundant renewable resource on the planet (Zaldivar et al., 2001). It is made up of three components: cellulose; hemicellulose; and lignin. Cellulose and hemicellulose can be converted to ethanol through a multistage process that typically begins with a chemical and/or mechanical pre-treatment. The pretreatment makes the material more available to hydrolysis by increasing the surface area (Karimi et al., 2006). The pretreatment is followed by a hydrolysis step, which liberates sugars locked in the complex structure. This either can be carried out chemically or by enzymes. The hydrolysate then provides the substrate for the bioconversion of monosaccharides to ethanol through microbiological fermentation (van Maris et al., 2006). Current research in the area of fermentation is focused on the optimization of the bioconversion of monosaccharides and disaccharides to ethanol. The majority of it is centered on finding an optimum organism for a given substrate. Coupled with finding the organism is developing the optimal fermentation conditions.

Pichia stipitis CBS 6054 is a strain of yeast that has been shown to produce ethanol from many of the sugars found in lignocellulosic material (Agbogbo et al., 2006). It is a native xylose-fermenting (the most abundant sugar in hemicelluloses) yeast that can yield up to 0.42-0.47 gram of ethanol per gram of substrate used (Agbogbo and Coward-Kelly, 2008). Numerous studies have shown its ability to be adapted to dilute acid pretreated hydrolysate for optimum ethanol yields (Nigam, 2001; Parekh et al., 1988; Sreenath and

Jeffries, 2000). For this reason, *P. stipitis* CBS 6054 has the potential to be an excellent candidate for optimizing ethanol production in a lignocellulosic feedstock system. *Saccharomyces cerevisiae* (ER) has not been reported in the literature as being used in lignocellulosic fermentations. It has the ability to ferment three of the five sugars found in lignocellulosic feedstocks to high ethanol yields. The yeast is ideal for using in fermentation involving enzymatically pretreated cellulose, which consists solely of glucose monomers.

Thesis Objectives

Lignocellulosic-based ethanol is a viable alternative to corn-based ethanol. However, the system is more complex than a simple starch to ethanol fermentation. Research was conducted in order to optimize each stage of biofuel production from lignocellulosic feedstocks (Figure 1). The optimization of the fermentation step was divided into two stages: organism evaluation and condition optimization (Figure 2). Two organisms, *Pichia stipitis* CBS 6054 and *Saccharomyces cerevisiae* (ER) that have shown to utilize one or more sugars present in lignocellulose were evaluated.

The specific objectives were:

1. Characterize the ability of *S. cerevisiae* (ER) and *P. stipitis* CBS 6054 to produce ethanol from the sugars present in lignocellulosic material from aspen, balsam, and switchgrass.
2. Characterize the toxic effect of dilute acid-pretreated hemicellulose and enzymatic hydrolysate for each yeast species in terms of growth, substrate utilization, and ethanol production.
3. Determine the effectiveness of adaptation of *P. stipitis* CBS6054 to the conditions present in dilute acid pretreated hemicellulose for improved ethanol production and substrate utilization.
4. Optimize the fermentation system for the maximum ethanol yields from aspen hydrolysate through fermentation media and cell recycle.

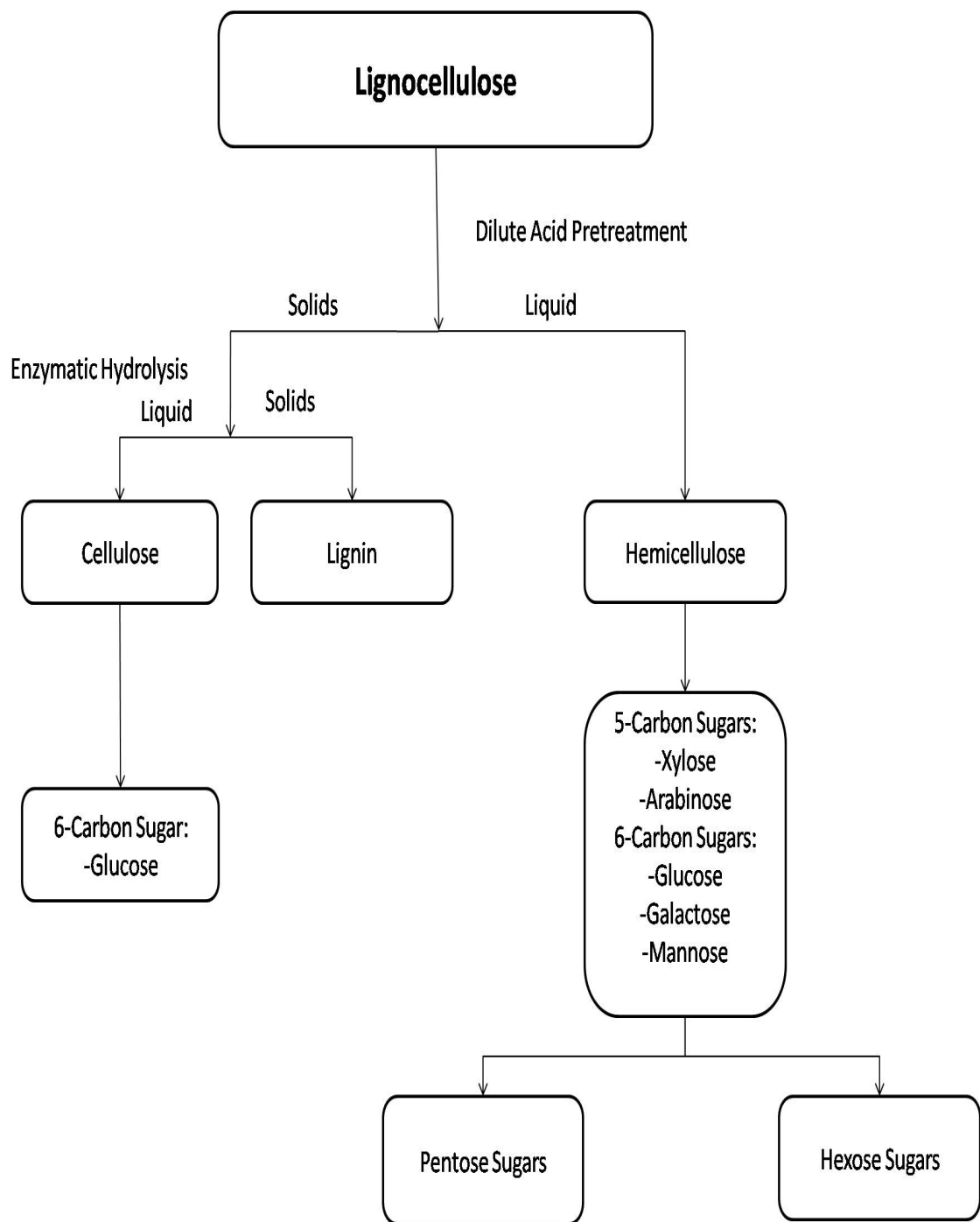


Figure 1. Diagram of carbohydrate components of lignocellulose after dilute acid pretreatment and enzymatic hydrolysis.

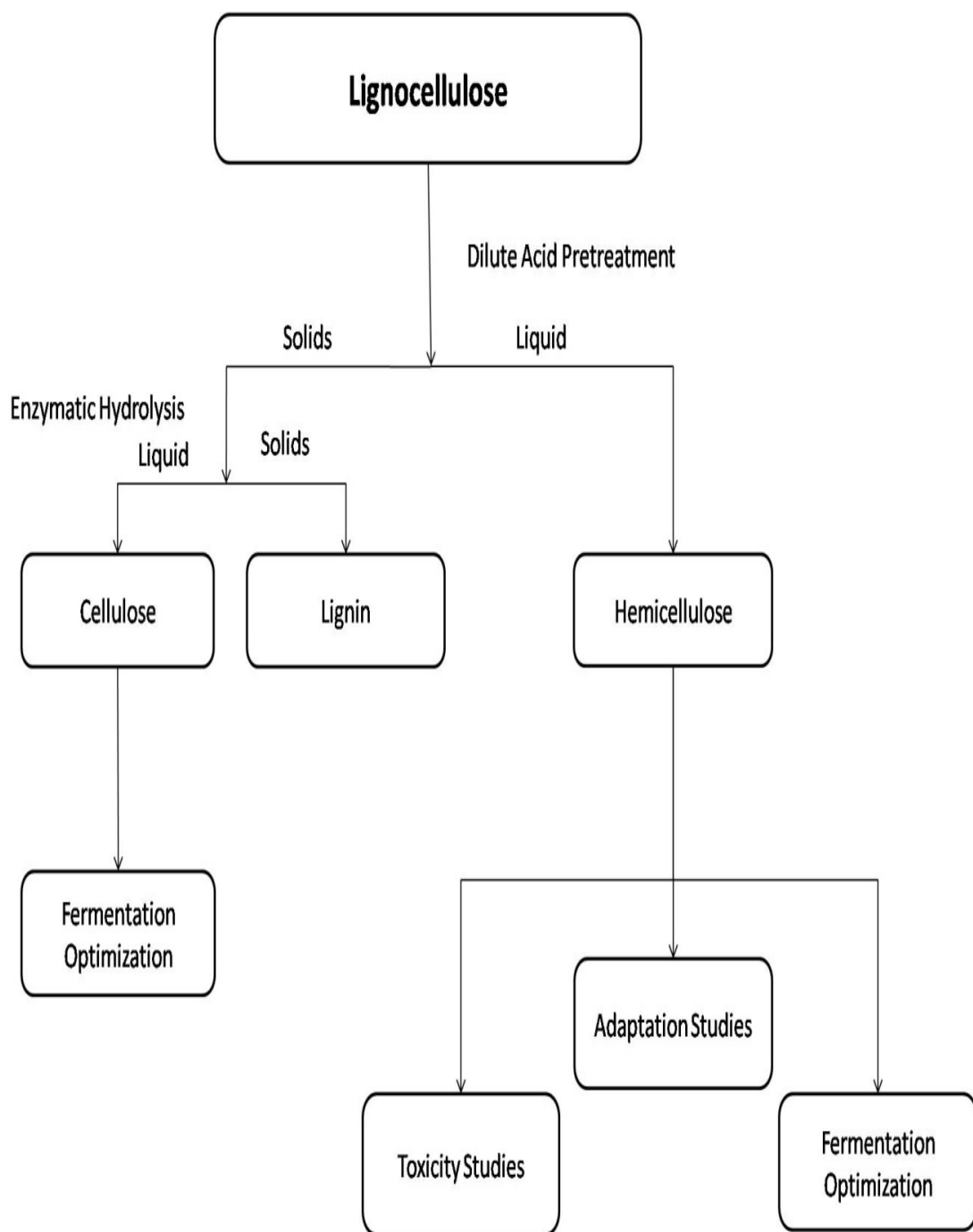


Figure 2. Diagram of the evaluations that were performed on the hemicellulose and cellulose feedstocks after hydrolysis.

Chapter 1. Literature Review

1.1 Lignocellulosic Material and Ethanol producing Microorganisms

When using lignocellulosic feed stock there are three possible substrates that can be used in fermentations. They are cellulose, hemicellulose and a mixture of the two. Each type of substrate contains fermentable sugars (Figure 3). Cellulose is composed of the hexose (6-carbon sugar) glucose. Hemicellulose is composed primarily of the hexoses: glucose, galactose, and mannose and the pentoses (5-carbon sugar) xylose and arabinose (Zaldivar et al., 2000). The fermentation processes for each of the three substrates will be discussed in detail, as well as the ability of many different organisms to ferment the available substrates to ethanol (Table 1).

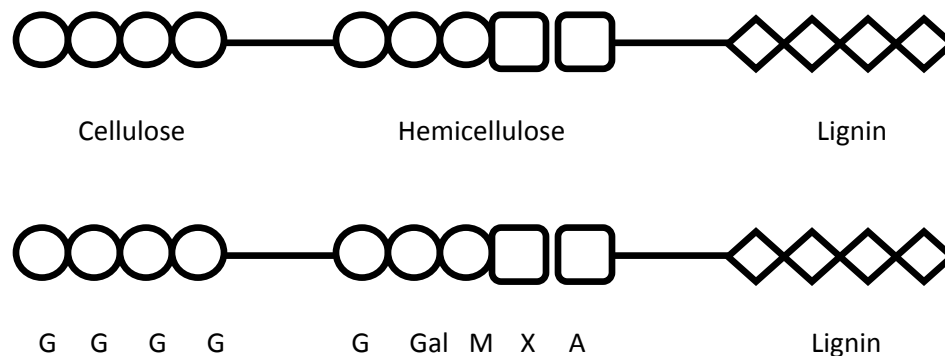


Figure 3. The composition of lignocellulose and the breakdown of the sugars present in cellulose and hemicellulose. The letters represent a different sugar: G-glucose; Gal-galactose; M-mannose; X-xylose; and A-arabinose (Modified from Zaldivar et al., 2000)

Table 1. Organisms that can use sugars in lignocellulosic material for the production of ethanol.

Organism	O ₂ ^a	Optimum		Substrate ^b		Pros for Use	Cons for Use	References
	Fermentation conditions	Temp (°C)	pH	Cellulose	Hemicellulose			
Yeast								
<i>Saccharomyces cerevisiae</i> (Wild-type)	Anaerobic ^d Aerobic	30	5	Yes	Yes – M, Gal	-High Ethanol Tolerance -Native Metabolism (glucose)	-No cellulase activity -Complex Growth Factors	Van Maris et al., 2006
<i>S. cerevisiae</i> (Ethanol Red – high temp. and EtOH tolerant strain)	Anaerobic ^d Aerobic	30-40	5	Yes	Yes- M and Gal	-High Ethanol Tolerance -Native Metabolism -SSF	-No cellulase activity -Complex Growth Factors	Karimi et al., 2006
<i>Kluyveromyces marxianus</i>	Anaerobic ^d Aerobic	40	5	Yes	No	-Ideal Temp for SSF -Native Metabolism	-Low Ethanol Yields -Complex Growth Factors	Ballesteros et al., 2004
<i>Pichia stipitis</i> CBS 6054	Microaerophilic ^d Aerobic	30-40	5	Yes	Yes-X, G, Gal, and M	Native Metabolism	-Low Ethanol tolerance -Uses only Xylose	Jefferies and Jin, 2000
Mold								
<i>Fusarium oxysporum</i>	Microaerophilic	30	4.5	Yes	Yes-all	-Native Metabolism -Cellulase Activity	-Low Ethanol yields	Ruiz et al., 2006
Bacteria								
<i>Zymomonas mobilis</i>	Anaerobic	30-40	5	Yes	Yes -all	-Ideal Temp for SSF -Native Metabolism	Not Hardy	Lin and Tanaka, 2005
<i>Escherichia coli</i> KO11	Anaerobic Microaerophilic ^c	30-35	6-8	Yes	Yes - all	-Easy to culture - Minimal growth requirements	-Narrow pH Range -Genetic Modification	Underwood et al.,2002

^a Oxygen conditions under which the organism produces ethanol^b Refers to whether the organism can ferment the sugars to ethanol. Cellulose contains: glucose. Hemicellulose contains: glucose; galactose (Gal); mannose (M); xylose (X); and arabinose (A)^c *E. coli* KO11 was shown to have higher xylose utilization ratios with microaeration (Okuda et al., 2006)^d Optimum ethanol yield

Cellulose

Cellulose is a polymer of glucose connected by β -(1, 4)-glycosidic bonds (Ohgen et al., 2006). Due to its close association with the other material in plant biomass and its conformation, it is highly resistant to hydrolysis (Zaldivar et al., 2000). The hydrolysis of cellulose is made possible by chemicals or enzymes. Enzymatic hydrolysis is the preferred means because it does not produce inhibitory compounds that chemical hydrolysis produces (van Maris et al., 2006). However, there are two antibiotics added during this hydrolysis, tetracycline and cycloheximide. These compounds are added to prevent microbial growth during the enzymatic pretreatment. Cycloheximide, which inhibits ribosomal function in eukaryotes, has the potential to negatively impact growth and subsequent ethanol production by the yeast cultures (de Kleot, 1966).

The benefit of using cellulose to produce ethanol is that glucose is easily and naturally metabolized by many organisms, such as wild-type strains of *S. cerevisiae* (Figure 4). Its high yields and high ethanol tolerance make it the ideal organism for glucose fermentations. *S. cerevisiae* does not have any natural cellulolytic activity and the fact that cellulose is resistant to hydrolysis does pose some problems. By default, the added step of enzymatic hydrolysis makes cellulose more expensive to work with than hemicellulose. Simultaneous Saccharification and Fermentation (SSF), however, can be done using cellulase enzymes coupled with *S. cerevisiae*. The fermentations can be carried out aerobically (lower yields) and anaerobically, which is another added benefit to using cellulose (Karimi et al., 2006). The efficiency of *S. cerevisiae* at glucose metabolism is

unmatched especially under anaerobic conditions; it converts 1 mol of glucose to 2 mol of ethanol (Zaldivar et al., 2000).

There are a few reasons to use or even consider using other organisms to carry out this conversion. One is apparent when conducting SSF. The cellulase enzymes require a higher temperature for complete hydrolysis of the cellulose. The optimum temperature is around 40-45°C for cellulytic enzymes and the optimal growth temperature for most yeast is 30°C (Ballesteros et al., 2004).

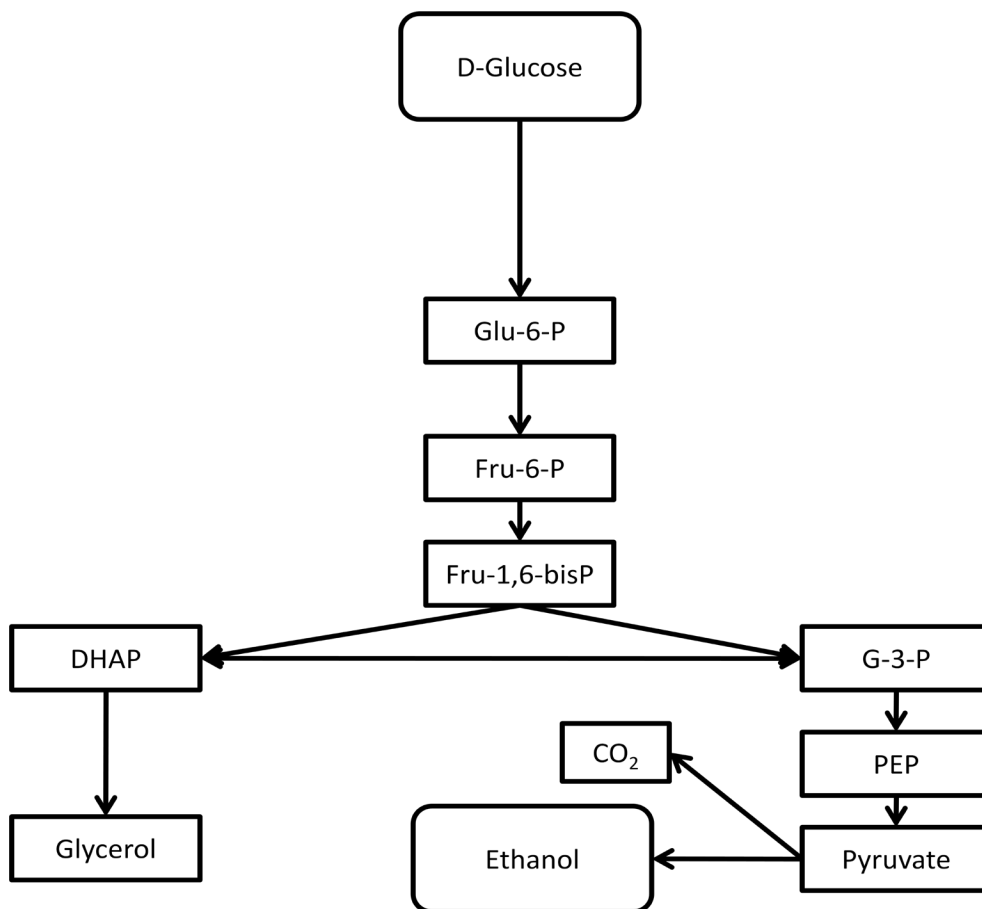


Figure 4. The catabolism of glucose by *Saccharomyces cerevisiae* using the Embden-Meyerhof glycolytic pathway (Modified from van Marin, 2006).

In addition to *S. cerevisiae* and its respective strains, there are some bacteria that will convert glucose to ethanol at levels that rival yeast. The bacterium *Zymomonas mobilis* produces ethanol at roughly the same theoretical yields as *S. cerevisiae*, 90-95% (Yomano et al., 1998). Thermophilic bacteria and other species of thermotolerant yeasts have also been used for SSF but they have shown reduced levels of ethanol production compared to *Z. mobilis* and *S. cerevisiae* (Ballesteros et al., 2004) (Karimi et al., 2006). *S. cerevisiae* is typically chosen over ethanologenic bacteria, like *Z. mobilis*, because it is a hardy culture that is easy to maintain (Lin and Tanaka, 2005).

Hemicellulose

Unlike cellulose that is a polymer of one sugar, hemicellulose is a polymer of several sugars. Hemicellulose may be converted directly to monomeric sugars from the pretreatment of the material with dilute acid hydrolysis (Jefferies and Jin, 2000). This direct hydrolysis may result in the presence of compounds that will inhibit bioconversion of the sugars. The most abundant of the sugars in hemicellulose is xylose. Xylose is a pentose that is not naturally fermented by many organisms. For the most part, genetically modified organisms need to be utilized for xylose fermentations on an industrial scale. A small number of yeast will ferment the sugar but they are not industrially feasible because they have low yields and low ethanol tolerance (Jeppenson et al., 1996). However, one promising yeast is *Pichia stipitis*, which naturally ferments xylose to ethanol at high yields (Jeffries et al., 2007) (Table 1). *P. stipitis* converts xylose to xylulose using the pentose-phosphate pathway and two enzymes, xylose reductase and xylitol

dehydrogenase (Almeida et al., 2008). Xylose must be converted to xylulose in order to be utilized by *Pichia*'s fermentation pathway without genetic modification (Figure 5).

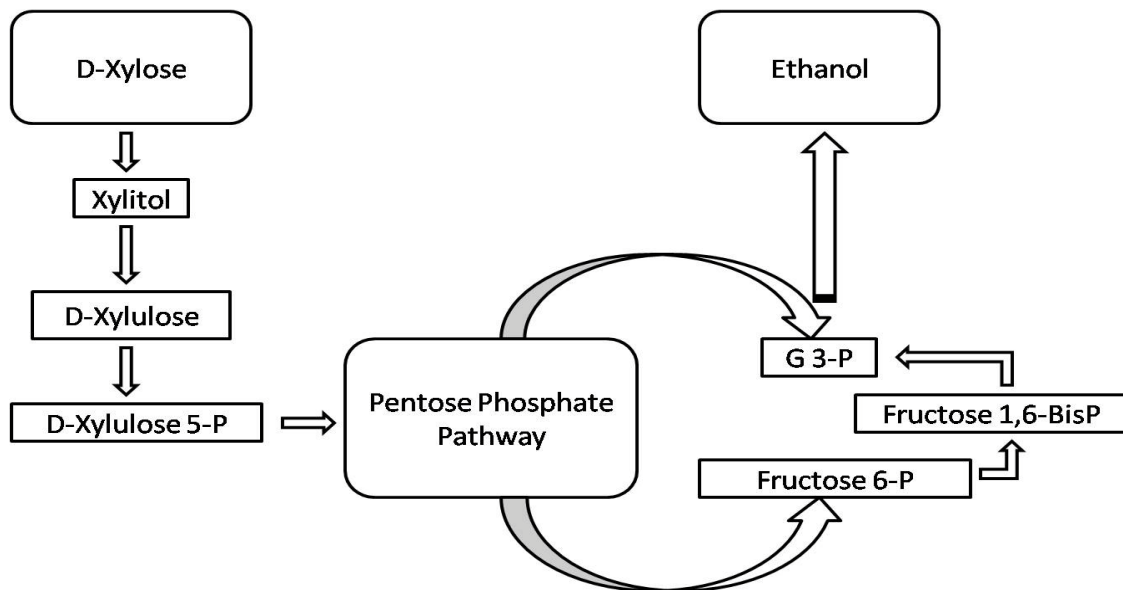


Figure 5. The native metabolic pathway of wild-type *Pichia stipitis* for ethanol production from xylose. The products utilize the pentose phosphate pathway (PPP) once xylose has been converted to xylitol and then to xylulose (Zalidivar *et al.*, 2001) (Modified from van Maris *et al.*, 2006).

S. cerevisiae has shown an ability to ferment xylulose at slow rates but it lacks the pathway to convert xylose to xylulose and therefore cannot produce ethanol from it (Kuyper *et al.*, 2005). The two genes encoding for xylose reductase and xylitol dehydrogenase from *P. stipitis* have been integrated into *S. cerevisiae*'s genetic material to make the conversion occur. The over-production of xylitol, a by-product, must be overcome and the bacterial enzyme xylose isomerase must be expressed in order for there to be an optimization of xylose metabolism (Kuyper *et al.*, 2005). Though *S. cerevisiae* is not able to utilize xylose without genetic modification, it can use other hemicellulose

sugars. It readily ferments the glucose isomers mannose and fructose. Using the Leloir pathway, it will also convert galactose and mannose to ethanol (Figure 6). This pathway however is regulated by the presence of glucose (van Maris *et al.*, 2006).

Bacteria that naturally produce ethanol from xylose seem to suffer the same problems as the yeasts that utilize xylose: they are not suitable for commercial production of ethanol. The most promising xylose-fermenting bacteria are strains of genetically modified *Escherichia coli*. One strain, KO11, using the native glycolytic pathway, has the ability to produce ethanol at high yields from xylose and other hemicellulose sugars (Underwood *et al.*, 2002). KO11 contains genes from *Z. mobilis*. These genes encode for pyruvate decarboxylase and alcohol dehydrogenase (*pdc* and *adhB*) and are integrated in to its chromosome (Underwood *et al.*, 2002). In addition to utilizing xylose, KO11 will simultaneously use glucose, galactose, arabinose, and mannose (Moniruzzaman and Ingram, 1998). This makes it an excellent candidate for mixed substrate fermentations. Unfortunately, KO11 can only operate in a narrow pH range and is less hardy than yeast (Lin and Tanaka, 2005). Another *Escherichia coli*, a mutant of strain K12, has been developed to convert glucose or xylose to ethanol at 82% yields using its native metabolism (Kim *et al.*, 2007).

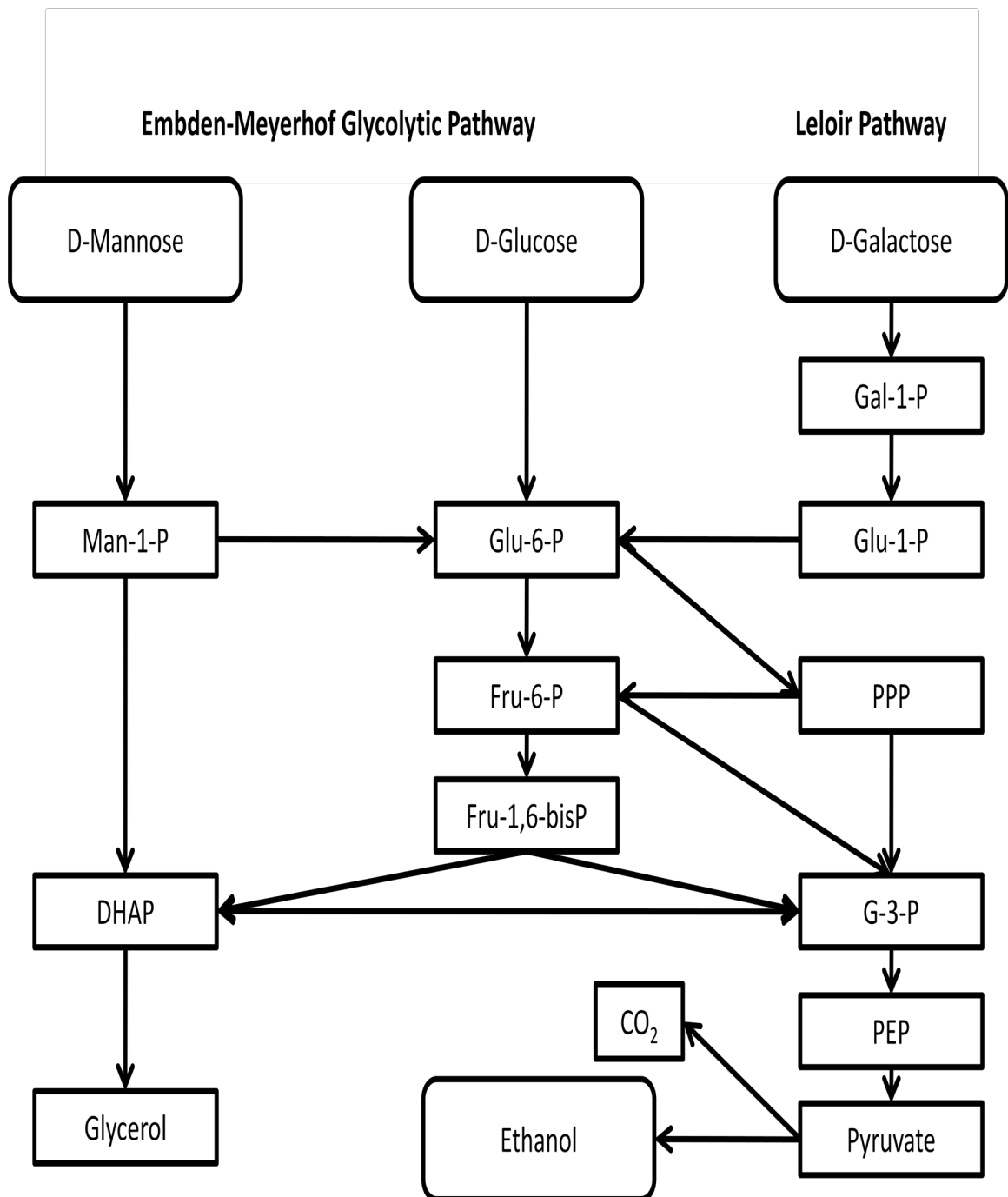


Figure 6. The catabolism of glucose and its isomers by *Saccharomyces cerevisiae* (Modified from van Marin, 2006).

Mixed Substrate

Mixed substrate fermentations for lignocellulose would require a bacterium or yeast that could utilize a mixture of hexoses and pentoses (Table 1). *P. stipitis* CBS 6054 (CBS refers to the Netherlands' Culture Collection and 6054 is a strain designation) is a strain of yeast that has been shown to produce ethanol from both xylose and glucose (Agbogbo et al., 2006). *P. stipitis* CBS 6054 was first isolated from insect larvae and is considered to have the best native xylose to ethanol fermentation capacity of any known organism (Jeffries et al., 2007). *Fusarium oxysporum* is a direct cellulose to ethanol converter. It produces cellulases, xylanases, and β -glucosidases and is able to metabolize both hexoses and pentoses into ethanol (Ohgen et al., 2006). A study performed by Ruiz et al., found that *F. oxysporum* will produce ethanol from xylose/glucose mixes (Ohgren et al., 2006). A genetically modified *S. cerevisiae* has also been shown to carry out mixed substrate (glucose/xylose) fermentations (Jeppenson et al., 1996; Ohgren et al., 2006).

Mixed substrate fermentations have one downside. In the presence of glucose the utilization of the other main sugars (xylose and arabinose) is often delayed due to catabolite repression (Nichols et al., 2001). A mutation of the glucose phosphotransferase gene, *ptsG*, in *E. coli* was shown to eliminate the catabolite repression, resulting in simultaneous utilization of all sugars (Nichols et al., 2001). Another method for eliminating the effect of catabolite repression is controlling the ratio of glucose to xylose. Lawford and Rousseau (1994) stated that xylose uptake is inhibited when glucose exceeds 40% of the total sugar present in the fermentation media. Controlling the ratio of sugars is an easy way to get around genetic modification. Catabolite repression is a

problem that can arise with all mixed substrate fermentations regardless of the organism used. Yeast will use other sugars in the presence of glucose only after all the glucose has been removed (Roca et al., 2003). Genetic modification has to occur to avoid this. Epimerase enzymatic hydrolysis could open the door for a broad range of sugar utilization by *S. cerevisiae*. A mixed culture, multiple organisms in one fermentation, is also a possibility.

1.2 Pretreatment and Toxicity

Before fermentation can occur the sugars must be liberated from cellulose and hemicellulose. In the case of hemicellulose the pretreatment will result in near complete chemical hydrolysis (van Maris et al., 2006). This means the monomer sugars are ready for fermentation without further hydrolysis, unlike with cellulose. Cellulose must undergo chemical or enzymatic hydrolysis. This can occur in one of two ways. The hydrolysis can be either carried out before the fermentation (separate hydrolysis and fermentation, SHF) or during the fermentation (SSF) (Karimi et al., 2006). SSF is the preferred method because it can be carried out in the same fermentor, thus reducing equipment costs. It is also beneficial in the case of enzyme hydrolysis. The enzyme is not inhibited by the build-up of sugars because they are removed by the microorganism as the fermentation proceeds (Ballesteros et al., 2004). The problem with SSF is that the optimal temperature of enzymatic hydrolysis is not the optimal temperature for common ethanologenic organism growth. For example, the typical optimum temperature for enzyme hydrolysis is 10 – 20°C higher than the optimal temperature for *Saccharomyces cerevisiae*, the main ethanol producing yeast (Karimi et al., 2006). The use of thermotolerant organisms,

mixed cultures, and the addition of a pre-hydrolysis step in which the organism is added after a period of high temperature, are all ways around this problem (Karimi et al., 2006).

Through dilute acid pretreatment the hemicellulose portion of lignocellulose feedstock can be broken down into fermentable sugars for ethanol production (Figure 7). The Michigan Tech Dept. of Chemical Engineering is currently optimizing a dilute sulfuric acid pretreatment of aspen, balsam, and switchgrass hydrolysate for the highest monomeric sugar yields and subsequent enzymatic hydrolysis. In addition to the sugars (xylose, glucose, galactose, mannose, and arabinose), several degradation products are produced (Taherzadeh and Karmimi, 2007) (Figure 8). These compounds can potentially negatively affect growth, substrate utilization, and ethanol production (Liu et al., 2004). The key compounds of concern in the pretreated hydrolysate are acetic acid, furfural and 5-hydroxymethylfurfural (HMF) (Almeida et al., 2008). Acetic acid is produced as the structure of hemicelluloses degrades. Furfural and HMF are sugar degradation products. Furfural results from the degradation of pentose sugars and HMF as a result hexose degradation (Almeida *et al.*, 2008). Each compound has its own mechanism of action, for fermentation inhibition. Acetic acid, in its dissociated form, acts to decrease the cells internal pH, thus inhibiting growth (Taherzadeh and Karmimi 2007). Furfural initially increases the lag time but has little effect on final ethanol yield (Liu et al., 2004). HMF has a very limited inhibitory effect on growth and subsequent ethanol production (Liu et al., 2004). Though the individual effects are apparent, the synergistic effects of these compounds must be examined when dealing with the hydrolysate. This effect must be dealt with in order to optimize ethanol production.

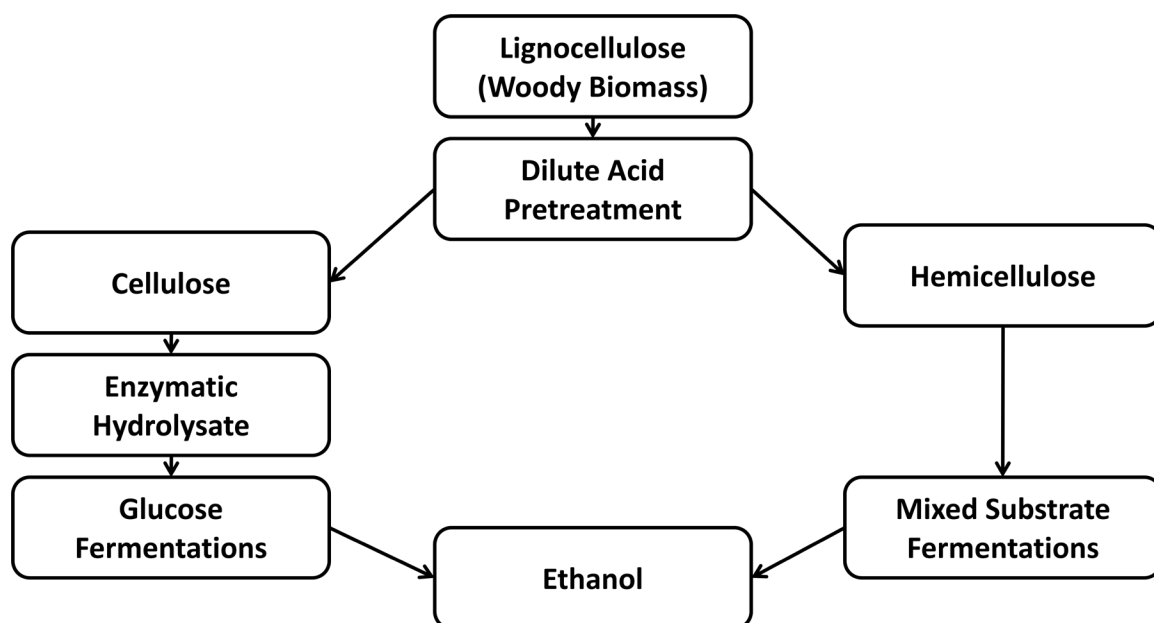


Figure 7. A summary of lignocellulosic feedstreams that can be used to produce ethanol (Modified from Karakashev *et al.*, 2007).

Several means of detoxifying the hydrolysate have been developed: treating with activated charcoal, extraction with organic solvents, ion exchange, ion exclusion, molecular sieves, over liming, and steam stripping (Olsson and Hahn-Hagerdal, 1996). Though extremely effective, detoxification of the hydrolysate can be costly. Another time-saving and cost-effective method is to adapt a natural ethanol-producing organism to the growth conditions present in the hydrolysate. *P. stipitis* CBS 6054 has the potential of overcoming the toxic effect of the hydrolysate over the ethanol-producing powerhouse *S. cerevisiae*. *P. stipitis* has a xylose reductase/xylitol dehydrogenase pathway which has been shown to reduce HMF and furfural (Almeida et al., 2008). This pathway reduces the toxic effect of the two furfuraldehydes. In the past, this *Pichia* strain has shown adaptability to dilute acid hydrolysate resulting in increased ethanol production (Nigam,

2001a; Amartey and Jefferies, 1996). Adaptation has shown to increase ethanol yields in several studies using dilute acid pretreated hydrolysate substrates. Nigam (2001a) saw a 1.6 fold increase in ethanol yield after adaptation to dilute acid pretreated red oak. Ethanol yields increased almost 13% on aspen hydrolysate after adaptation (Parekh et al., 1986). *S. cerevisiae* has limited ability to produce ethanol from pentoses and does not possess the xylose reductase pathway (Almeida et al., 2008) making it a less desirable choice for adaptation.

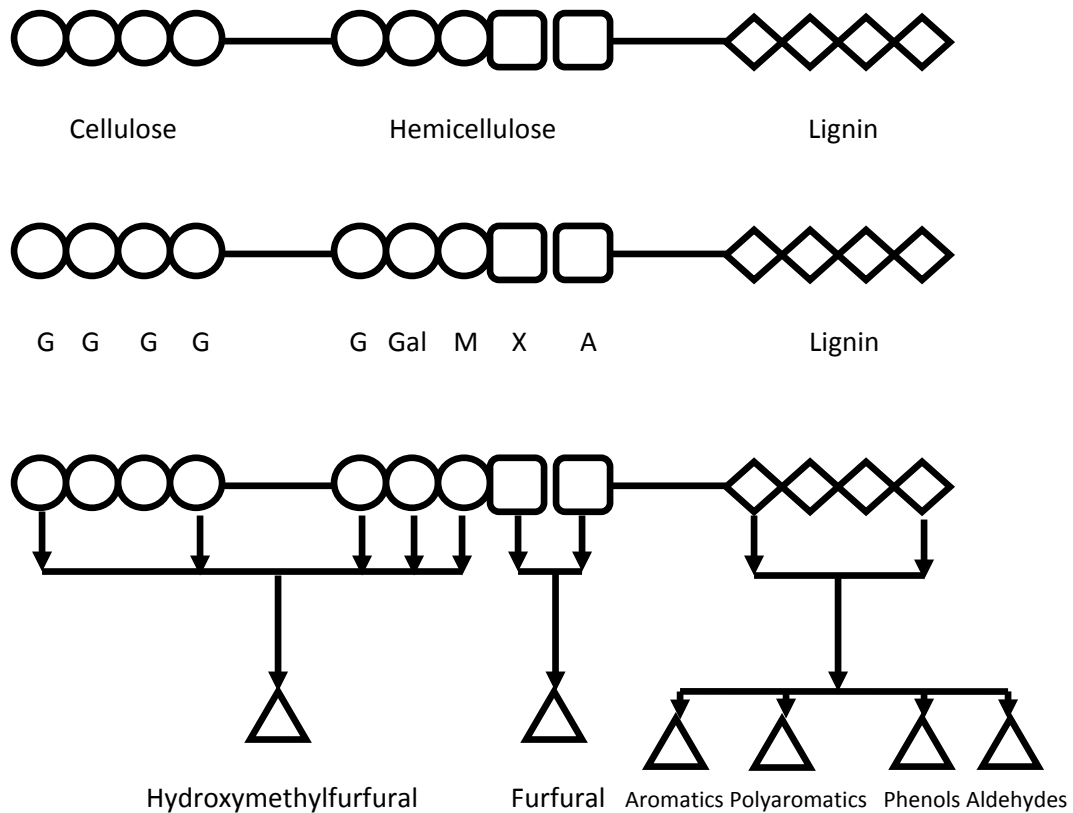


Figure 8. The composition of lignocellulose and the breakdown of the sugars present in cellulose and hemicellulose. The dilute acid pretreatment degradation products and their sources are shown. The letters represent a different sugar: G-glucose; Gal-galactose; M-mannose; X-xylose; and A-arabinose (Modified from Zaldivar et al., 2001).

1.3 Optimization of Fermentation Conditions

The optimization of fermentation conditions involves a series of organism-specific factors. The hydrolysate characteristics also affect these factors. These factors include: cultivation method, cultivation/inoculation media, and cultivation conditions. Each factor can be optimized separately or as a unit depending on the desired result.

Cultivation methods fall into two categories: continuous and batch fermentations. Continuous fermentations are where media and nutrients are fed through the system at a continuous rate and effluent (spent media) and products are removed at the same rate. There is typically a cell recycle system set up in these types of fermentations. Batch culture fermentations are closed-system fermentations. Media and conditions are set at the beginning of the fermentation and once inoculated, the fermentation proceeds until a set stop point. There are considered to be three phases in industrial batch fermentations: lag, growth, and harvesting (Shuler and Kargi, 2002). A type of batch fermentation where substrate and other nutrients are fed throughout the fermentation but nothing is removed is referred to as a fed-batch fermentation. They are done in an effort to overcome limitations such as substrate inhibition and toxicity (Shuler and Kargi, 2002; Taherzadeh et al., 1998). Both continuous and fed-batch have shown to reduce the toxic effect of the dilute acid hydrolysate for furfural and HMF in *S. cerevisiae* fermentations (Bandberg et al., 2005; Taherzadeh et al., 1998).

Cultivation media are directly dependent on the substrate type and are organism-specific (Hahn-Hagerdal et al., 2005). Cultivation conditions refer to pH, temperature, and

agitation. They are fairly well defined for *P. stipitis* (Bandberg et al., 2005; Nigam, 2001). *P. stipitis*' optimum temperature is 30°C and the optimum pH range is 4.0-5.0 (du Preez et al., 1985). Media play important roles in the ethanol production by *Pichia stipitis* CBS 6054. Several nutrients have shown to play a role in increasing both biomass and ethanol yield (Agbogbo and Coward-Kelly, 2008). Three common media used in the literature are Yeast-Peptone medium (YP), Yeast Nitrogen Base medium (YNB), and Corn Steep Liquor medium (CSL) (Table 2) (Agbogbo et al., 2006; Amartey and Jefferies, 1994). YNB has been shown to increase ethanol concentrations but causes a decrease in biomass production, whereas CSL has been shown to increase both biomass and ethanol concentration (Amartey and Jefferies, 1994). YP media are typically used as a cultivation and storage media for *P. stipitis*. It does, however, function as a supplement for fermentation media (Rodrigues et al., 2008). A number of other media can be used to produce ethanol using *P. stipitis*; it is a matter of choosing what is optimal for the application. *S. cerevisiae* (ER) can also be grown on all three media types. The effect of each on ethanol production has yet to be reported. Wild-type *S. cerevisiae* is capable of growing on YP and YP is used as the fermentation medium (Wahlbom et al., 2003)

Changing the cultivation method, media, and conditions are key to determining the optimum conditions for ethanol production by *P. stipitis* CBS 6054 and *S. cerevisiae* (ER). Choosing whether to separate the enzymatic and dilute acid pretreated portions of the feed-streams into a two fermentations or combined them for a single fermentation also needs to be considered.

Table 2. Media composition .^{a, b}

Components (g/L)	YP	YNB	CSL
Yeast Extract	10	-	-
Peptone	20	6.56	-
Urea	-	2.26	-
Yeast Nitrogen Base	-	1.17	-
Corn Steep Liquor	-	-	30

^aYP- Yeast Peptone, YNB- Yeast Nitrogen Base, and CSL- Corn Steep Liquor

^bpH of all media is 5.5 ± 0.1 .

Chapter 2. Methods

2.1 Organism Cultivation

Organism Source

The focus of this research was on *Pichia stipitis* CBS 6054 (provided courtesy of Dr. Thomas Jeffries, Institute for Microbial and Biochemical Technology, USDA, Forest Service, Forest Products Laboratory, Madison, Wisconsin) and *Saccharomyces cerevisiae* (Ethanol Red) provided by *Fermentis* (Marcq-en-Baroeul, France). These two yeast species were chosen as a result of their adaptability and ethanol producing capabilities (Table 1). They were used in a series of studies in order to determine if they were suitable for ethanol production from lignocellulosic material. Their capabilities will be described in respect to the different types of substrates present in the lignocellulosic material.

Culture Maintenance

P. stipitis was maintained on Yeast-Peptone-Xylose (YPX) plates (xylose 20 g/L, and agar 15 g/L), with incubation at 30°C for 24 hours. All *P. stipitis* inocula are grown in YPX broth at 30°C for 24 hours. *S. cerevisiae* (ER) was maintained and grown on YPG (glucose 20 g/L, and agar 15 g/L). All studies are carried out in YP supplemented with sugar solutions. Early *S. cerevisiae* (ER) experiments were performed in Sabouraud Dextrose Broth (SDB). The inoculum was prepared by initially growing the yeast in YPX or YPG medium at 30°C for 24 hours on a gyratory shaker in 125 mL Erlenmeyer flasks with 25 mL of media. The well-mixed culture was divided into 1 mL aliquots and

centrifuged (3000 rpm for 5 minutes); the supernatant was discarded. The pellet was resuspended in sterile distilled water (Agbogbo et al., 2006).

Specific media requirements or modifications are listed in Table 2 for the composition of all media used over the course of this investigation. The sugar requirements for each individual study are described as well as any other media additives with the methods for that study. All media and supplements were filter-sterilized using vacuum filtration and 0.2 μm filters (47mm diameter), except for CSL that was autoclaved for 20 minutes at 121°C.

The properties of each yeast have been described extensively in the literature. An initial series of growth, sugar utilization, and ethanol production screening experiments were designed to verify these properties. Due to time constraints and the extensive background work already done with these organisms replicates were not performed on some studies.

2.2 Growth Studies

All of the growth studies were carried out in 250 mL baffled-bottom sidearm flasks with a working volume of 25 mL. A 5% inoculum of actively growing culture (grown in the same medium as the fermentation) was used. The media composition of the growth studies varied with what was actually being tested but all treatments were supplemented with YP. The growth rate and generation time were determined over a 24 hour period using absorbance as an estimate of dry weight [equations 1, *P. stipitis*, and 2, *S. cerevisiae* (ER), (Govindaswamy and Vane, 2006)]. All absorbance measurements were taken using UV/VIS spectroscopy at 600nm (*P. stipitis*) and 660nm (*S. cerevisiae*) using

a Thermo Genesys 20 spectrometer (Model 4001/4). Total biomass yield was determined by filtering a sample onto a pre-weighed 0.2 µm filter (47 mm diameter), then drying the filter for 24 hours at 80°C in a drying oven. The filter was then reweighed and the total biomass (g/L) was determined.

Equation 1. Estimated dry weight (W, g/L) of *Pichia stipitis* CBS 6054

$$W = 2.04 + 1.80A_{600}$$

Equation 2. Estimated dry weight (W, g/L) of *Saccharomyces cerevisiae* (Ethanol Red)

$$W = 1.06 + 4.76 A_{660}$$

2.3 Fermentations

All fermentations were carried out in 125 ml Erlenmeyer flasks with a 50 ml working volume. The media varied for each treatment depending on what was being tested. Each substrate that was to be fermented was supplemented with YP. An inoculum was grown for 18-24 hours in the same media as the fermentation media. Fermentations were carried out for 24 hours for *S. cerevisiae* (ER) and 72 hours for *P. stipitis*. These flasks were covered with aluminum foil and sealed with parafilm incubated on a gyratory shaker at 30°C. Samples were taken aseptically by using a sterile pipette and removing 0.5 mL from the flask.

2.4 Organism Characterization for Lignocellulosic Substrate Utilization

Single Substrate Utilization

The growth characteristics of *S. cerevisiae* (ER) were determined using concentrations of 20, 50, and 100 g/l glucose in SDB. The sugar solutions were filter-sterilized and added to 30 ml of SDB without a carbon/energy source (Brandberg et al., 2005). The broth was inoculated with 100 µl of yeast inoculum.

P. stipitis was tested for growth characteristics using YPX (20 g/L) under the same conditions. In a study of xylose utilization and ethanol production by *Pichia stipitis* the yeast was grown in YPX for 72 hours. That culture was then used to inoculate two flasks containing 30 ml of YPX. These flasks were incubated for 24 hrs.

Both strains were tested for their ability to produce biomass from each of the five sugars known to be present in aspen hydrolysate. These five sugars are xylose, glucose, galactose, mannose, and arabinose. 100 ml YP was supplemented with either 20 g/l xylose, glucose, arabinose, galactose, or mannose in 250 ml Erlenmeyer flasks. The flasks were inoculated with 1 ml of either *P. stipitis* or *S. cerevisiae* (ER). They were sealed and incubated for 24 hours. The absorbance was taken at 24 hours to determine the maximum biomass production in that time period for the specific substrate. Growth was monitored over time. A study was performed to test whether or not this setup would result in ethanol production by *S. cerevisiae* (ER). Four flasks were filled with YPG. Three of the flasks were inoculated with culture and the fourth was used as the control. All were tightly sealed in an incubator for 24 hours. The samples were then analyzed

using high performance liquid chromatography (HPLC) to determine the presence of ethanol and the disappearance of glucose. Replicates were not performed.

Mixed Substrate Utilization

S. cerevisiae (ER) was tested for its ability to produce ethanol from glucose: xylose mixtures. 30 ml of YP was inoculated with 0.13 g of dried *S. cerevisiae* (ER). (0.13 g was the average dry weight of a 1 ml aliquot from the inoculation media.) Each flask contained a different sugar mix concentration for a total of five treatments and one control: (% glucose/% xylose) and (YP – Sugar Free). Each treatment had a total sugar concentration of 4 g/L. The five treatments were: 100% glucose, 75%:25% glucose: xylose, 50%:50% glucose: xylose, 25%:75% glucose: xylose, and 100% xylose. Samples were taken at 6 hour intervals and analyzed using HPLC (in a later section) to test for ethanol production and sugar disappearance. Growth was monitored every two hours for a 48 hour period using the absorbance values at 660 nm. The treatments and sampling methods were designed based on those of Agbogbo et al. (2006).

P. stipitis was grown in YPX and YPG (glucose 50 g/L) and grown overnight. These cultures were then used to inoculate YPX, YPG (glucose 50 g/L), YP-Hexose mix (glucose 25 g/L, galactose 12.5 g/L, and mannose 12.5 g/L), and YP-Pentose mix (xylose 25 g/L and arabinose 25 g/L) for 24 hours. Cultures grown in YPX were used to inoculate YPX and the YP-Pentose mix and cultures grown in YPG were used to inoculate YPG and the YP-Hexose mix. These flasks were sealed with aluminum foil and parafilm and incubated for 72 hrs. All fermentations were carried out in either 125 ml or 250 ml Erlenmeyer Flasks with a 50 ml and 100 ml working volume, respectively. Each sugar

solution was supplemented with YP broth. The flasks were then inoculated with 1 ml of yeast suspended in YP broth. All media and supplements were filter-sterilized. Samples were taken every 24 hours until there was complete sugar disappearance or the ethanol concentration dropped. The samples were analyzed using HPLC and sugar and ethanol concentrations were determined. Replicates were not performed

2.5 Lignocellulose Hydrolysate Studies

Enzymatic Hydrolysate

The enzymatic hydrolysate was made according to previous literature (Jensen et al., 2009.)

Enzymatic Hydrolysate-Toxicity

The toxic effect of cycloheximide was tested at the concentration seen in the enzymatic hydrolysate (0.03g/L) (NREL LAP009). Flasks containing 50 ml YPG [for *S. cerevisiae* (ER)] and YPX (for *P. stipitis*) were supplemented with 1.5 mg of cycloheximide. The flasks were inoculated with 1 ml of its respective yeast suspended in YP. Another set of flasks with YPG and YPX without cycloheximide were also inoculated and served as the control. All four flasks were sealed and incubated for a 16 hour period. The dry weight was estimated through absorbance values.

Enzymatic Hydrolysate-Fermentation

Both *P. stipitis* and *S. cerevisiae* (ER) were used to determine ethanol production from three types of enzymatic hydrolysate: aspen, balsam, and switchgrass. 250 mL Erlenmeyer flasks were filled with 25 mL of enzymatic hydrolysate and 75 mL of YP

broth. The flasks were then inoculated with 1 mL of yeast suspended in YP broth. The flasks were sealed and incubated. Samples were taken every 24 hours for a period of 120 hours. The samples were analyzed using HPLC and ethanol and glucose concentrations were determined.

Synthetic Acid Hydrolysate

P. stipitis CBS 6054 ability to produce ethanol was evaluated on synthetic media containing five sugars (xylose, glucose, mannose, galactose, and arabinose) in a ratio reflective of that seen in dilute acid pretreated aspen, balsam, and switchgrass hydrolysate (Table 3). These data are the mean concentrations and were obtained from HPLC analysis of dilute acid-pretreated lignocellulosic material by Michigan Tech's Dept. of Chemical Engineering. The concentrations reflect the mean of nine pretreatments for each type of hydrolysate. The time at which the maximum xylose concentration was achieved was used to obtain the concentrations for the other sugars. This is representative of the ratio of sugars that would be observed if the pretreatment was optimized for obtaining maximum xylose monomers. The yeast was grown in YP broth supplemented with the sugars for 72 hours. Replicates were not performed

Table 3. Sugar concentrations (g/L) for synthetic dilute acid pretreated hydrolysate fermentations by *Pichia stipitis* CBS 6054. Taken from data obtained by Michigan Tech

Treatment ID	Xylose	Glucose	Galactose	Mannose	Arabinose
Aspen^a	18.5±1.0	4±0.9	1±0.1	2.5±0.9	1±0.8
Balsam^a	6.5±0.4	4.5±0.3	3±0.2	10±0.7	3.5±0.6
Switchgrass^a	22±0.9	5±0.08	3±0.06	1±0.2	3±0.1

^aMean ±2SD, n=9

2.6 Toxicity Studies

Furfural Toxicity Assays for Saccharomyces cerevisiae (Ethanol Red)

S. cerevisiae (ER) was grown aerobically in SDB at 30°C on a gyratory shaker for 24 hours. This culture served as the inoculum for the experiment. Each experiment was carried out in 250 ml Nephelo flasks containing 30ml of SDB. Different concentrations of furfural were then added to each flask to achieve specific experimental conditions or treatments, as follows:

1. 0 g/L furfural Control
2. 1 g/L furfural
3. 2 g/L furfural
4. 4 g/L Furfural

The levels of furfural were chosen as a result of HPLC analysis performed by Michigan Tech's Dept. of Chemical Engineering. Replicates were not performed.

Furfural and Acetic Acid Toxicity Assays for Pichia stipitis CBS 6054

Initial toxicity studies were carried out in YPX media supplemented with a given concentration of either acetic acid or furfural. Acetic acid was tested at 2, 4, 6, 8, and 10 g/L and furfural was tested at 1, 2, and 4 g/L. Toxicity assays were also performed in synthetic aspen hydrolysate media (Table 4) supplemented with furfural, acetic acid, or combinations of the two. The synthetic hydrolysate was made by combining 1 L of YP with the five sugars. The mixture was then filter-sterilized. The toxic effect of furfural and acetic acid were tested on the growth of *P. stipitis*. The toxicity studies were carried out in 125 mL Erlenmeyer flasks containing 50 mL of media. Dry weight was determined

using absorbance. Furfural was tested at 2 and 4 g/l and acetic acid at 8 and 10 g/L. Two combinations of the two compounds were also tested. There was a 2 g/L furfural with 8 g/L acetic acid in synthetic aspen hydrolysate treatment and a 4 g/L furfural and 10 g/L acetic acid treatment. To determine furfural's and acetic acid's effect on ethanol production and substrate utilization increased levels of inhibitory compounds were used: 1.5, 3, and 4.5 g/L furfural and 4, 8, and 12 g/L acetic acid. Combinations of the two were tested using 1.5:4, 3:8, and 4.5:12 g/L of furfural and acetic acid respectively. These concentrations mimic what would be seen in a 1X, 2X, and 3X aspen hydrolysate. Replicates were not performed

Table 4. Medium composition for synthetic hydrolysate.

Compound	Concentration (g/L)
Xylose	18.5
Glucose	4
Galactose	1
Mannose	2.5
Arabinose	1
Yeast Extract	10
Peptone	20

Sugar Level Toxicity

Synthetic concentrated dilute acid pretreated aspen media was made by taking the sugar ratio seen in the hydrolysate and making 1X, 2X, and 3X sugar media. This was supplemented with YP. The cultures were incubated for 120 hours. Samples were taken

every 24 hours to determine growth, substrate utilization, and ethanol production. Replicates were not performed

2.7 Adaptation

Dilute Acid Pretreatment

The conditions used for the dilute acid pretreated aspen used to produce the hydrolysate were reported by Jensen et al. (2008). The dilute acid pretreated aspen (5% solids) was neutralized with KOH (pH 5.5 ± 0.1) and filter-sterilized to remove the precipitate and particulates.

*Developing an Adapted Strain of *Pichia stipitis* CBS 6054*

The average dilute acid pretreated aspen hydrolysate composition was determined using HPLC. A synthetic aspen hydrolysate was supplemented with 10, 20, 30, 40, 50, 60, 65, 70, 75%, or 80% (v/v) dilute acid pretreated aspen hydrolysate (Figure 9). The adaptation was carried out over a period of three weeks or until the cells could tolerate up to 80% of the hydrolysate (Amarthey and Jefferies, 1994).

The original stock culture was grown in synthetic aspen media for 24 hours. The cells were then harvested and used to inoculate 2 125 mL Erlenmeyer flasks. Each flask had 50ml of 10% adaptation medium (X% of hydrolysate, YP, and synthetic aspen media). The cultures were incubated for 24 hours. Then, the contents of one of the flasks were transferred to two new flasks containing the next higher level of hydrolysate (20%) and the process was repeated until an evolved population that could withstand growth at 80%

hydrolysate was reached. The second flask went to storage after a sample for HPLC analysis had been removed.

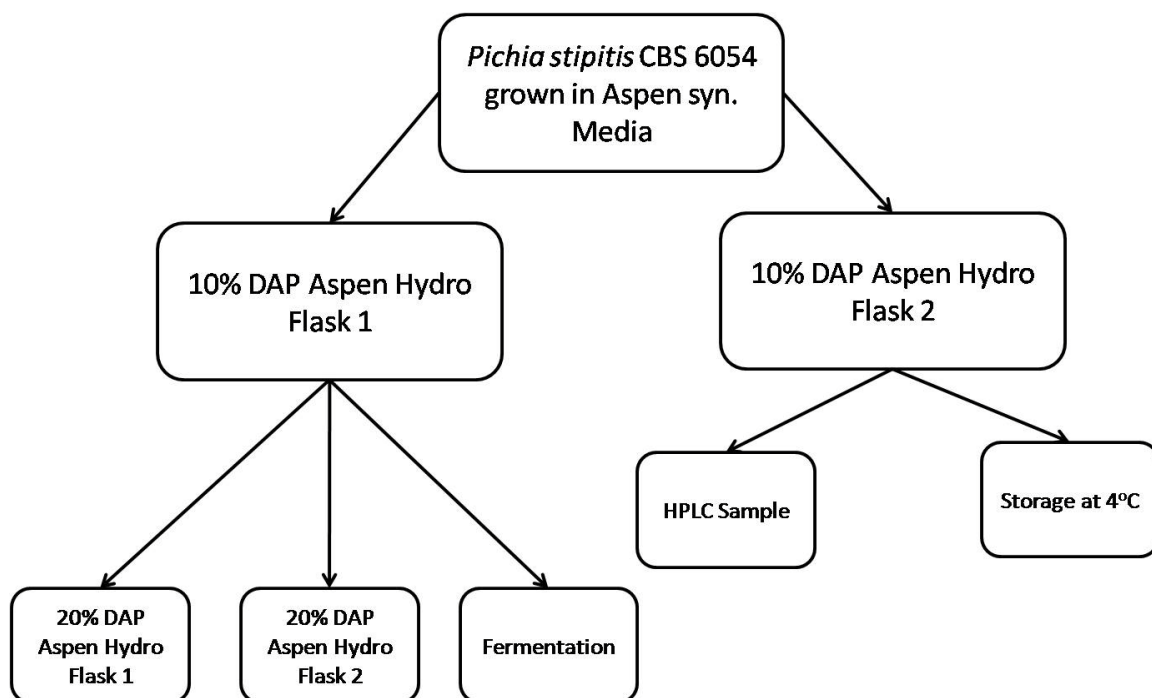


Figure 9. Sample flow chart diagramming the adaptation study of *Pichia stipitis* CBS6054 for greater ethanol production from aspen hydrolysate (DAP-Dilute Acid Pretreated, Syn.-synthetic aspen media, Hydro – hydrolysate).

Fermentation Studies on Adapted Strain of Pichia stipitis CBS6054

Fermentation studies were carried out using dilute acid pretreated aspen hydrolysate. The hydrolysate was supplemented with YP. Depending on the concentration of hydrolysate used it was also supplemented with xylose, glucose, galactose, arabinose, and mannose at the concentration in synthetic aspen media (Table 4). The unadapted strain was used in a series of fermentations (30 - 80% hydrolysate media) to determine the baseline data for each adaptation level. The adapted cells were also evaluated at each level. In the case of the adapted strain the flasks were inoculated directly from one of the adaptation flasks.

The inoculum for the unadapted strains came from culture grown overnight in their respective percentage of hydrolysate. The fermentations were allowed to run for 72 hours. Samples were taken at 0 and 72 hours and analyze on the HPLC. Each mutant was graded in the areas of growth, substrate utilization, and ethanol production/yield. The final adapted strain is maintained on yeast peptone xylose plates and in 80% aspen hydrolysate media.

2.8 Media Optimization

Three media were evaluated: YP, YNB, and CSL (Table 2) (Agbogbo and Coward-Kelly 2008). The media were supplemented with either 50 g/L xylose as well as aspen synthetic media. For the treatments using aspen synthetic media the YP component was replaced with YNB or CSL. The fermentation was allowed to proceed for 72 hours.

2.7 Analytical Methods

High-performance liquid chromatography was used to determine ethanol, carbohydrate, and toxin levels in all samples following the methods described by Jensen et al. (2008). An Agilent 1200 Series HPLC with a Bio-Rad aminex HPX-87P column was used. Carbohydrates and ethanol was measured on a refractive-index detector (RID). The furfuraldehydes were measured on a diode array detector (DAD).

Chapter 3. Results and Discussion

3.1 Single Substrate Utilization by *P. stipitis* and *S. cerevisiae* (ER)

Glucose utilization by S. cerevisiae

S. cerevisiae (ER) was tested at varying concentrations of glucose. The results of these aerobic 250 ml Nephelo flask cultures show that glucose at these levels produces similar growth rates (Table 5), i.e., no apparent osmotic stress impacts. The typical growth rate for *S. cerevisiae* in glucose fermentations can range anywhere from 0.3-0.65 h⁻¹ (Papagianni et al., 2007). Similar growth rates were found for *S. cerevisiae* at the 20 g/L (0.41 h⁻¹); the growth rates reported in this work for 50 and 100 g/L are slightly higher than those reported in the literature (Papagianni et al., 2007). The lack in difference in growth rates reported in this work for the three concentrations shows that glucose does not have an inhibitory effect at these concentrations. This is expected because a typical glucose fermentation has a concentration above 100 g/L (Lee et al., 1999).

Table 5. Growth rate of *S. cerevisiae* (ER) at varying glucose concentrations in YP media (30⁰C, pH 5.5 for 24 hours)

Treatment (g/L)	k (hr-1)
20	0.41
50	0.45
100	0.42

Pichia stipitis Single Substrate Utilization

A series of fermentations was setup to test substrate utilization patterns and subsequent ethanol production. *Pichia stipitis* CBS 6054 was evaluated for its ability to ferment

xylose, glucose, glucose and xylose mixtures, hexose mixtures (glucose, galactose, and mannose), and pentose mixtures (arabinose and xylose). In single substrate xylose fermentations (50 g/L), 45.9% of sugar was utilized after 72 hours. Ethanol yields for xylose were 80% of theoretical and 84% of theoretical yields for glucose (Table 6). Biomass production was similar for both glucose and xylose. This is in agreement with studies showing higher ethanol yields from media containing glucose compared to ones containing xylose as the sole substrate (Agbogbo et al., 2006; du Preez et al., 1985).

A parallel study was conducted in baffled flasks to see if the added aeration had an effect on ethanol production. In the baffled flasks ethanol yields dropped to 0.32 g/g and 0.27 g/g in xylose and glucose, respectively. An increase in sugar consumption was observed in flasks containing xylose. There was no change in sugar consumption in the flask containing glucose. This observation is consistent with the literature. In the case of *P. stipitis*, oxygenation results in an increase in xylose uptake (Skoog and Hahn-Hagerdal, 1990). The sugar utilization in the xylose went to 99.7% with no change in utilization of glucose. Biomass increased in the baffled flasks for both glucose and xylose (34 g/L and 25 g/L, respectively). The baffled flasks thus have a negative effect on ethanol production for both substrate types and were not used for further fermentations. The flasks drastically decreased the ethanol yields. The decrease in ethanol production can be attributed to an increase in aeration (Agbogbo and Coward-Kelly, 2008).

Table 6. Summary of single substrate fermentation data for xylose and glucose fermentations by *Pichia stipitis* CBS 6054 for 72 hours (30⁰C, pH 5.5).^a

Sugar	Total Sugars (g/L)	% Sugar Utilized	Ethanol Conc. (g/L)	Ethanol Yield g_EtOH/g_sugar	Dry Weight (g/L)
Xylose	49.0±0.12	45.7	9.25 ± 1.5	0.41±0.01	13.5±0.01
Glucose	53.8±0.01	100	22.9 ± 0.5	0.43±0.01	12.2±0.01

^aMean values (n=2, ±2SD)

Biomass Concentration from Five Sugars for P. stipitis and S. cerevisiae (ER)

S. cerevisiae (ER) and *P. stipitis* were used to determine the maximum biomass that each strain could produce on the five sugars found in hemicellulose (xylose, glucose, arabinose, galactose, and mannose). *S. cerevisiae* (ER) yielded the highest biomass on the hexose sugars (Table 7). There was no net growth on either pentose sugar; *S. cerevisiae* is not able to utilize pentose sugars (Jeppsson et al., 1996). The growth that is observed in those treatments can be attributed to the fact that YP supports very low-level growth of *S. cerevisiae* in the absence of an additional carbon source (Hahn-Hagerdal et al., 2005). In a separate study, *S. cerevisiae* was grown just in xylose and then just in YP without a carbon source (Figure 10). There is similar biomass production for the two treatments, i.e., 3.09 g/L and 3.01 g/L, respectively. The higher biomass yields between the two studies are due to a difference between a 2% inoculum and 10% inoculum. It is important to note that YP and xylose produce similar biomass concentrations. *Pichia* produced the most biomass on glucose and mannose and the other three sugars yield similar results

(Table 7). This is in agreement with other studies that show glucose yields a greater biomass than other substrates (Agbogbo et al., 2006). Background growth on YP media was performed for *P. stipitis*. When tested just on YP without a carbon source *P. stipitis* produced no net increase in biomass over a 24 hour period (Appendix B).

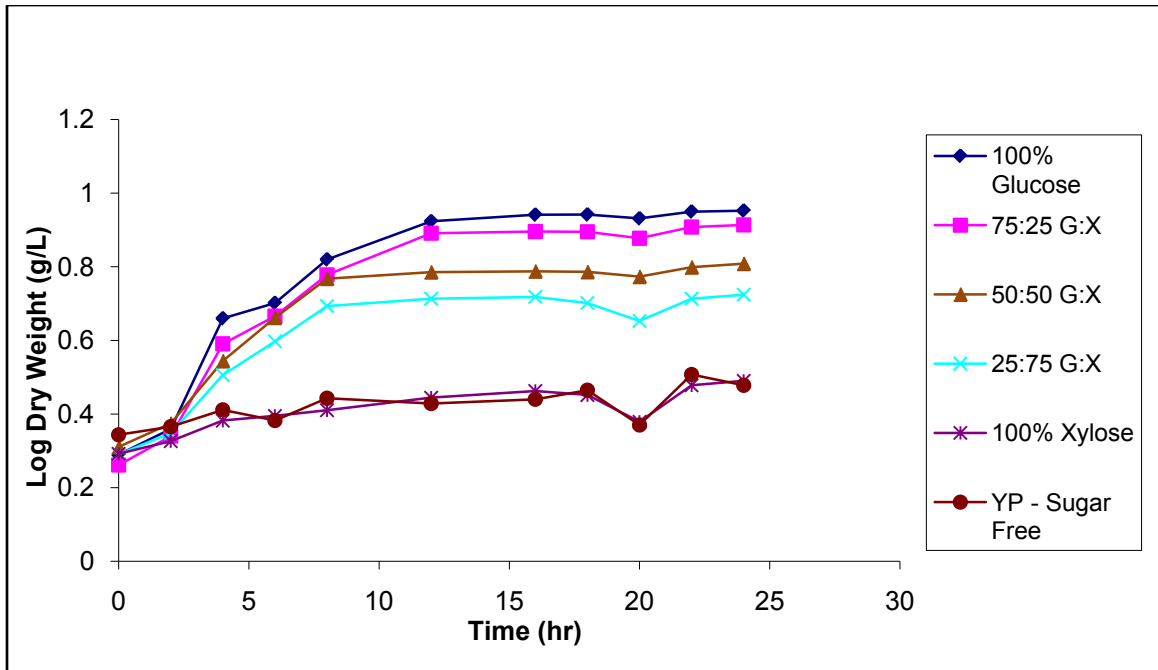


Figure 10. The effect of glucose/xylose concentrations (5 g/L) (w/v) on *S. cerevisiae* (ER) growth. ER was grown in YP with the varying levels of glucose/xylose mixtures (35°C for 24 hours, pH 5.5).

Table 7. The maximum biomass produced by *S. cerevisiae* (ER) (1) and *Pichia stipitis* (2) on glucose, xylose, galactose, mannose, and arabinose in YP media at 30°C, pH 5.5.

Sample ID	Biomass at T=0 (g/L)	Biomass at T=24 (g/L)	% Increase
Glucose 1	2.08	8.85	325
Xylose 1	2.90	4.18	44.1
Arabinose 1	2.78	4.46	60.8
Galactose 1	3.24	9.24	185
Mannose 1	3.24	9.01	178
Glucose 2	1.96	9.43	381
Xylose 2	2.15	8.13	279
Arabinose 2	3.39	9.04	167
Galactose 2	2.67	7.66	187
Mannose 2	2.29	8.86	287

3.2 Mixed Substrate Utilization and Fermentations

Saccharomyces cerevisiae (ER) fermentation and biomass production on glucose and xylose mixtures

S. cerevisiae (ER) was used to determine the effect of pentose/hexose mixed substrate fermentations on ethanol production and growth. The fermentation setup was first tested to see if ethanol could be produced. Results of that investigation can be seen in Appendix A, section 1. The treatments were set up to be 100% glucose, 75:25% glucose to xylose, 50:50% glucose to xylose, 25:75% glucose to xylose, and 100% xylose. The HPLC data showed that the mixtures were 100%, 85%, 38%, 27%, and 0% glucose with xylose

making up the other fraction. This is either due to a sampling error or an error in the HPLC standards. The presence of xylose resulted in a reduction in biomass production (Figure 10) and reduced growth rates (Table 8). As noted earlier, this is not actually due to xylose utilization but rather background growth on YP or growth on residual materials and utilization of the glucose in the mixtures (Hahn-Hagerdal et al., 2005). The different growth rates observed can only be attributed to the presence of glucose in the media. *S. cerevisiae* biomass production was directly proportional to the amount of glucose in the media (Figure 10). The results of the HPLC analysis showed ethanol production only in treatments containing glucose. The results of the HPLC analysis also yielded glucose concentrations lower than what should have been in the treatments. This could be due to an HPLC sample preparation error. A shift in the retention time of glucose was also observed. The xylose levels remained, for the most part, constant throughout the experiment. This was the expected result because *S. cerevisiae* cannot metabolize xylose. The results, in terms of biomass of the 100% xylose and the YP-sugar free treatment, are relatively similar. It has been established that *S. cerevisiae* does not utilize xylose and it can grow on YP without a carbon source (Jeppsson et al., 1996; Hahn-Hagerdal et al., 2005). From this it can be inferred whatever growth is observed is due to background growth on YP. The mean biomass produced in 24 hours from YP media was determined to be $0.97 \text{ g/L} \pm 0.24$. Results of this fermentation can be found in Appendix A.

Mixed Substrate Fermentations by Pichia stipitis

In a glucose: xylose mixed substrate fermentation by *P. stipitis*, glucose was completely used in the first 24 hours while 53.2 % of the xylose was consumed within this same time

period. The xylose was completely gone within 48 hours. The total ethanol yield was 0.17 g/g_sugar (Table 8). The yield was 34% of theoretical yield. This is an extremely low level according to the literature. Agbogbo et al., (2006) reports an ethanol yield of 0.44 g/g on a 50:50 mix of glucose on xylose at 72 hours. In this work the fermentation was stopped at 48 hours. If it was allowed to precede to 72 hours higher ethanol levels might be observed. In the case of *P. stipitis*, xylose is consumed only after glucose is completely utilized (Agbogbo et al., 2008). Xylose consumption was reduced in the presence of glucose, which is due to the fact that glucose inhibits xylose uptake (Kilian and Uden 1988).

Table 8. The growth rate (k) of *S. cerevisiae* (ER) during mixed substrate fermentations. (ER was grown in YP with the varying levels of glucose/xylose mixtures at 35°C for 24 hours, pH 5.5.)

Treatment	k (hr⁻¹)
100% Glucose	0.24
75:25 G:X	0.23
50:50 G:X	0.22
25:75 G:X	0.19
100% Xylose	0.04
YP – Sugar Free	0.03

It is well established in the literature that glucose consumption proceeds xylose consumption so the experiment was not designed to observe the order of sugar utilization (Agbogbo et al., 2008). If the study were to be repeated the order of sugar utilization would be taken in account. If glucose composes more than 40% of the media it has an inhibitory effect on other substrate utilizations (Lawford and Rousseau, 1994). Based on this information it can be assumed that mannose and galactose were not utilized until after the glucose was. If the rate of glucose consumption can be increased, the length of the fermentation will decrease.

Agbogbo et al. (2006) reported that, in 50:50 mixtures of glucose and xylose, the sugar consumption rate is 0.54 g/L h and 0.37 g/L h, respectively. Applying those rates to this work's initial sugar concentration in the same mixture implies that the sugars should not be completely consumed at the 24-hour mark. From this it can be inferred that, if calculated, the rate of glucose and xylose consumption would be faster than those reported in the literature.

The pentose mixture had the lowest of ethanol yield (0.14 g/g) of all the mixed substrate fermentations (Table 8). This can be attributed to the fact that *Pichia* does not ferment arabinose to ethanol (Agobogbo and Coward-Kelly, 2008). The hexose mixture had the highest ethanol yield (0.21 g/g) (Table 9). Galactose and mannose are isomers of glucose and are fermented by the Leloir and the EM glycolytic pathways, respectively (Figure 6) (VanMaris et al., 2006). The ethanol concentration showed that same pattern that the g/g yield did (Table 9). In terms of biomass production the hexose mix produced the most, 26.5 g/L, followed by glucose xylose mix, 20 g/L, and then the pentose mix 17.8 g/L. Graphical results for each mixture can be found in Appendix B Section 1.

Table 9. Ethanol production by *P. stipitis* CBS 6054 from glucose:xylose, pentose (arabinose and xylose), and hexose (galactose, glucose, and mannose) sugar mixtures in a 48 hour period.

Sugar Mixture	Total Sugars	Ethanol Conc. (g/L)	Ethanol Yield (g/g_sugar)
Glucose:Xylose^a	102.6	17.5	0.17
Pentose^b	55.76	7.86	0.14
Hexose^c	54.64	11.6	0.21

^a50:50 mixture

^b75:25 arabinose to xylose

^c50:25:25 glucose to galactose to mannose

3.3 Enzymatic Hydrolysate Fermentations

Enzymatic Hydrolysate- Cycloheximide Toxicity for P. stipitis and S. cerevisiae (ER)

The main component of the liquor is glucose. As a result, *S. cerevisiae* (ER) and *P. stipitis* were used in these investigations. There are two antibiotics added during the hydrolysis, tetracycline and cycloheximide. Cycloheximide, which inhibits ribosomal function in eukaryotes, has the potential to negatively impact growth and subsequent ethanol production by the yeast cultures (de Kleot, 1966). The toxic effect of cycloheximide was first tested at the concentration seen in the enzymatic hydrolysate (0.03g/L) (NREL LAP009, 2008). *P. stipitis* and *S. cerevisiae* (ER) were tested to

determine if cycloheximide had an inhibitory effect on growth in either organism. The results show that the compound, at this level, did not have a effect on growth (Table 10). After 48 hours, an unidentified pink yeast was found to have contaminated the enzymatic hydrolysate. This yeast was able to utilize both xylose and glucose but did not produce ethanol in subsequent tests. Some pink yeasts are known for their ethanol utilization (Belloch et al., 2007) properties. The decrease in ethanol concentration after 48 hours could possibly be a result of this contamination. As a result the enzymatic pretreatment was filter-sterilized right after hydrolysis.

Table 10. Biomass of *P. stipitis* (PS) and *S. cerevisiae* (ER) produced in 16 hours in media, (YPX or YPG) containing 0.03g/L cycloheximide

Treatment	Biomass (g/L) at T=0	Biomass (g/L) at T=16	% Biomass increase
PS YPX	3.2	8.9	183%
PS YPX -Cycloheximide	3.5	8.6	146%
SC YPG	2.7	6.6	145%
SC YPG-Cycloheximide	2.7	7.4	171%

Enzymatic Hydrolysate-Fermentation

A mixture of 25% YP to 75% hydrolysate was chosen as the medium, because it provided the highest glucose concentration with the optimal supplement level. Both organisms, *P. stipitis* and *S. cerevisiae*, were tested for their ability to produce ethanol on this mixture. *P. stipitis* did not produce any ethanol over a 120 hour period. This could be

due to the minimal sugar levels available. *P. stipitis* produces ethanol optimally at sugar levels above 50 g/L (Agbogbo and Coward-Kelly, 2008). The enzymatic hydrolysate had between 0.25-2.14 g/L of glucose, depending on the source (Table 11). This is low compared to reported values (Jensen et al., 2009). The low level is most likely due to a contaminant, a pink-pigmented yeast, that was found in the enzymatic hydrolysate. *S. cerevisiae* (ER) produced ethanol in the greatest quantity between 24-48 hours (Figure 11). It also produced the most ethanol from switchgrass enzymatic hydrolysate and the least from balsam. All treatments show the presence of ethanol at T=0. It is unknown why this may have occurred. As a result, the data were corrected to determine the ethanol yield (Table 11). The yields were higher than theoretical yields for glucose fermentations (0.51 g/g) (>100%). This could be to an analytical error or a sampling error. Another possibility is that the glucose levels were in fact higher than what was calculated from the HPLC data. When the samples were initially run on the HPLC, right after the hydrolysis was complete, the concentration of glucose was 3.97 g/L (aspen), 0.40 g/L (balsam), and 2.39 g/L (switchgrass). Using those values as the initial sugar concentration rather than the calculated values the ethanol yields were 57% and 73% of the theoretical yield for aspen and switchgrass respectively. Despite the low sugar levels and contamination, this study demonstrates that enzymatic hydrolysate can be converted to ethanol by *S. cerevisiae*. Further steps to optimize this process were not performed due to the low sugar levels seen in the hydrolysate. Results for substrate utilization and ethanol production can be found in Appendix B section 2.

Table 11. Glucose levels in enzymatic pretreated cellulose from aspen, balsam, and switchgrass and ethanol yield by *S. cerevisiae* (ER) for 24 hours (30°C, pH 5.5).

Cellulose Source	Glucose (g/L)	Ethanol Yields (g/g)
Aspen	2.14	0.64
Balsam	.25	0
Switchgrass	1.87	0.97*

*48 hour value

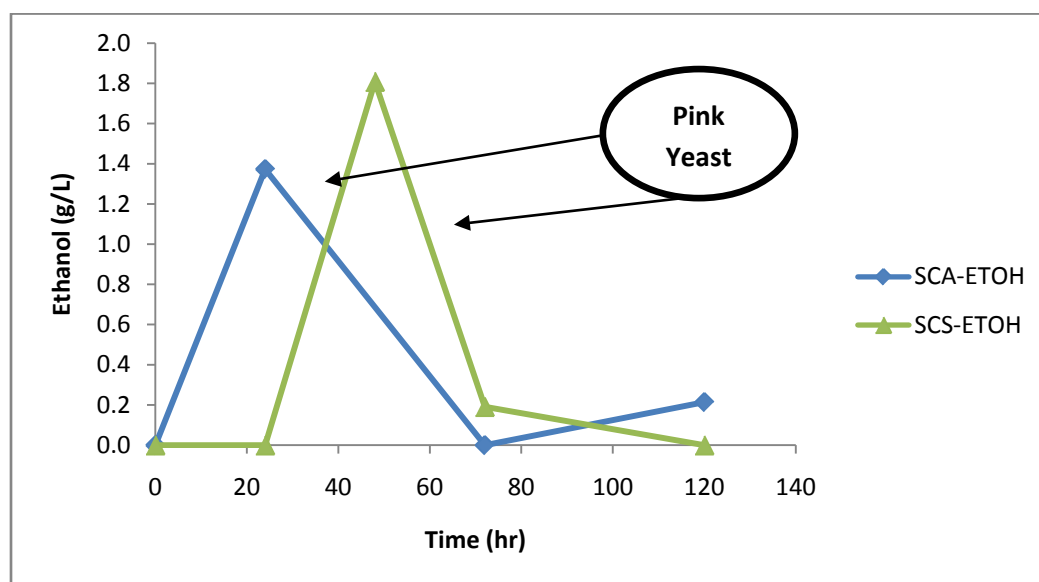


Figure 11. Ethanol yield from enzymatic pretreated hydrolysate (Aspen –SCA, Balsam – SCB, and Switchgrass- SCS) fermented by *S. cerevisiae* (ER) over 120 hours (30°C, pH 5.5).

Fermentation of Synthetic Dilute Acid Pretreated Hydrolysate by Pichia stipitis

P. stipitis was used to test whether or not it could produce ethanol from a synthetic media containing a mixture of sugars at the concentrations seen in the aspen, balsam, and switchgrass dilute acid pretreated hydrolysates. After HPLC analysis it was concluded that *Pichia* could in fact produce ethanol from the mixture of sugars found in all three synthetic hydrolysates types (Table 12). In all three hydrolysate types, the hexose sugars were completely utilized in the 72 hour period (Appendix B). Arabinose was not utilized

in any of the treatments. Xylose was utilized in all three hydrolysate types, but only the balsam showed its complete disappearance within 72 hours. Although, synthetic media were used, the sugar concentrations mimicked those seen in the real dilute acid pretreated hemicellulose. Any differences in sugar consumption related directly to the concentration (Table 12). Dilute acid pretreated balsam only has 6.5 g/L xylose compared to aspen and switchgrass which have 18.5 and 22 g/L, respectively. This study was performed as a screening measure for the adaptation study to determine if *P. stipitis* would grow and produce ethanol from the sugars present in the hydrolysate. These initial results matched that of the literature for synthetic hydrolysate media and therefore were not repeated (Nigam, 2001).

Table 12. Ethanol yield by *Pichia stipitis* on synthetic non-toxic hydrolysate after 72 hrs. of growth (30°C, pH 5.5).

Hydrolysate Type	Total Sugars	% Sugars Utilized	Ethanol Conc. (g/L)	Ethanol Yield (g/g_substrate)
Aspen	27	83.9	8.25	0.38
Balsam	27.5	92.1	8.79	0.36
Switchgrass	34	70.1	8.52	0.37

3.4 Toxicity

Furfural Toxicity –Saccharomyces cerevisiae (ER)

Several growth experiments were performed to determine if furfural had any inhibitory effects on the growth of *S. cerevisiae* (ER). It has been determined that different strains of *S. cerevisiae* have different tolerance levels to furfurals (Martin et al., 2003). The concentrations of furfural used in this experiment were determined by what is typically seen in a wood hydrolysate, (1.0-4.0 g/L) (Jensen et al., 2008). Concentrations of furfural at 1 and 2 g/L resulted in an increase in lag phase. Eventually those cultures reached the similar concentrations of biomass as the control (Figure 16). This result was expected because furfural causes an increase in the duration of lag phase in batch growth (Liu et al., 2004). Delgenes et al. (1996) reported a 90% reduction in biomass production compared to the control on 2 g/L furfural for *S. cerevisiae*. That drastic of an inhibition was not observed in this work, but it was not stated at which time the growths were compared in that study. However, both Delgenes et al. (1996) and this work contradict the finding of Taherzadeh et al. (1999), who found that increases in the lag phase of batch growth occur at concentrations above 2 g/L. The 4 g/l concentration of furfural did have a negative impact on culture growth. No growth was observed during the normal time period (2 – 8 hours). This culture was left in the incubator for 24 hrs, after which time similar biomass concentrations were seen as in the control and the other furfural concentrations. Although furfural has been shown to be inhibitory to biomass production by *S. cerevisiae*, this inhibition can be overcome through adaption to increased concentrations of furfural (Banerjee et al., 1981). It is also able to reduce furfural to a less toxic furfuryl alcohol (Almeida et al., 2008). These characteristics are especially important for *S. cerevisiae* when used in dilute acid pretreated fermentations.

Pichia stipitis Toxicity Assays

Three separate studies evaluating the effect of furfural, acetic acid, and combinations of the two on the biomass production of *P. stipitis* were conducted. The first study evaluated the effect of furfural and acetic acid in YPX media. The second evaluated furfural, acetic acid, and combinations of the two in synthetic dilute acid pretreated hydrolysate. The third was done in concentrated synthetic dilute acid pretreated aspen hydrolysate (1X, 2X, and 3X) with comparable levels of furfural and acetic acid.

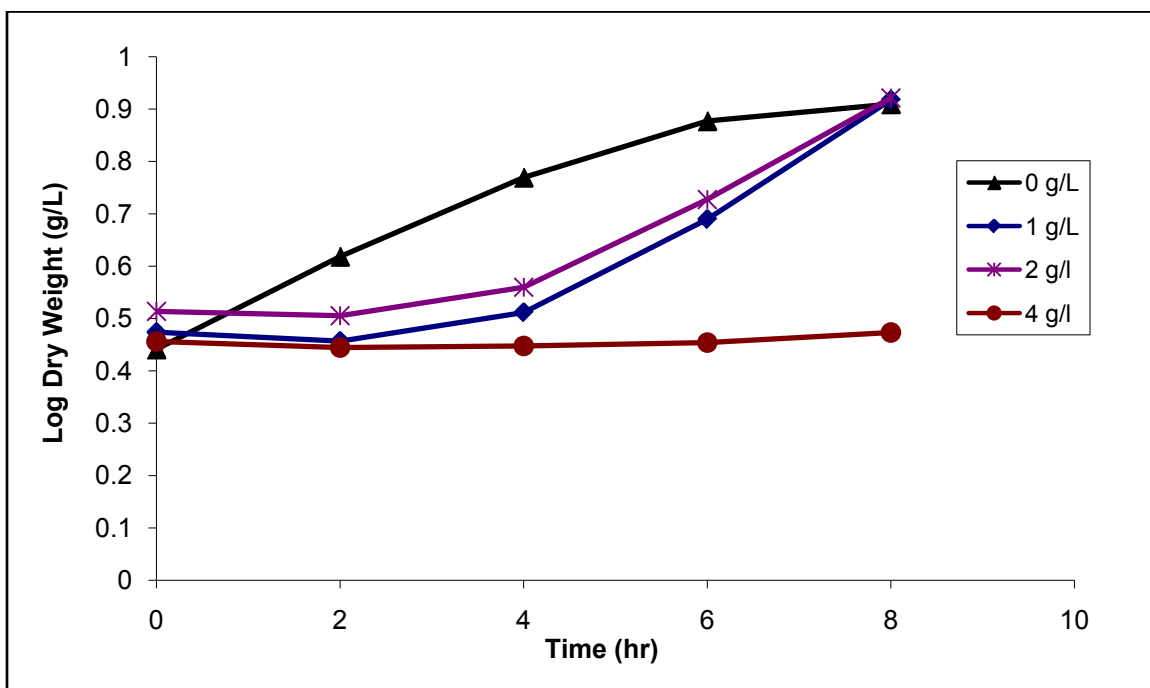


Figure 12. The effects of furfural concentrations on *S. cerevisiae* (ER) (biomass production in YPG media for 24 hours (30°C, pH 5.5).

Pichia stipitis Furfural and Acetic Acid Toxicity Assays in YPX

In YPX (20 g/L) media, furfural was not inhibitory at 1 g/L but at 2 g/L and 4 g/L showed substantial growth inhibition, as seen by a decrease in the biomass production (Table 14). Under aerobic conditions furfural is inhibitory to *P. stipitis* above 2 g/L (Palmqvist and Hahn-Hagerdal, 2000). Previous studies have shown that furfural concentration of 0.5 and 2 g/L cause a 25% and 99% decrease in growth, respectively (Delgenes et al., 1996). Ethanol production, at the same concentrations, is reduced 29% and 95% (Delgenes et al., 1996). Similar results were achieved in this study (Table 13). At 1 g/L, furfural causes a 43.6% reduction in biomass production and 2 g/L furfural resulted in a 98.2% reduction in biomass.

Acetic acid was inhibitory at concentrations above 2 g/L (Table 14). Van zyl et al., (1991) reported a 17% reduction in the growth rate at 2.1 g/L. In this work a 18.75% reduction in the growth rate at 2.0 g/L was observed. Previous work has shown that at a concentrations of 5 g/L a 37% reduction in growth and at 10 g/L a 36% reduction in growth compared to the control is observed (Delgenes et al., 1996). At these same concentrations there is a 70% and 75% reduction in ethanol production (Delgenes et al., 1996). These results could not be confirmed through this study. At 10 g/l a 98% decrease in biomass was observed. Acetic acid inhibits cell growth by decreasing the internal pH of the cell (Palmqvist and Hahn-Hagerdal, 2000). Neutralization of the media to pH of 6.5 tends to negate the toxic effect of acetic acid. However, xylose is converted to ethanol optimally by *P. stipitis* by a pH between 4-5.5 (Van Zyl et al., 1991).

Any compound that results in a decrease in biomass production or a declined growth rate affects the production of ethanol. Ethanol is a primary metabolite that is made only

during exponential growth phase in batch growth. Overall furfural and acetic acid have negative impacts on biomass production. Both compounds, however, have been used to improve ethanol yields at concentrations below the inhibitory levels (Klinke et al., 2004). Like *S. cerevisiae* (ER), the inhibitory effect of these compounds on growth and ethanol production by *P. stipitis* can be overcome through adaptation (Nigam 2001). Growth rates for this investigation can be found in Appendix C.

Table 13. Biomass production (g/L) by *Pichia stipitis* with different concentrations of furfural in YPX medium after 24 hours(30°C, pH 5.5).

Furfural (g/L)	Initial Biomass (g/L)	Final Biomass (g/L)	Total Biomass (g/L)	% Biomass Increase	% of Control
0	2.1	5.2	3.1	148.2%	
1	2.1	4.2	2.1	98.9%	66.4%
2	2.1	2.2	0.1	2.7%	1.8%
4	2.1	2.2	0.1	3.6%	2.4%

Table 14. Biomass production (g/L) by *Pichia stipitis* with different concentrations of acetic acid in YPX medium after 24 hours(30°C, pH 5.5).

Acetic Acid (g/L)	Initial Biomass	Final Biomass	Total Biomass	% Biomass Increase	% of Control
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	(g/L)	(g/L)	(g/L)		
0	2.2	4.6	2.4	106.8%	
2	2.1	2.9	0.77	35.9%	32.3%
4	2.2	2.1	0	0%	0%
6	2.2	2.1	0	0%	0%
8	2.1	2.1	0.08	3.9%	3.4%
10	2.1	2.2	0.05	2.2%	2.0%

Pichia stipitis Furfural and Acetic Acid Toxicity Assays in Synthetic Aspen Media

The inhibitory effects of furfural and acetic acid were tested on *P. stipitis* in synthetic aspen hydrolysate media (Table 15). This study was performed to show that, at the levels typically seen in dilute acid pretreated hydrolysate, there is inhibitory effect caused by furfural and acetic acid. This study was not performed to determine at which levels the compound was inhibitory or the degree of inhibition. This study was used as the justification for performing the adaptation study because it showed that the compounds found in the hydrolysate at these levels are inhibitory and need to be dealt with. No net growth was observed in these treatments over an 18-hr period compared to the control (Table 16). Results for the individual toxins in synthetic aspen hydrolysate media (furfural 2 and 4 g/L and acetic acid 8 and 10 g/L) were similar to those generated from the same concentrations in media containing just xylose. The synergistic effect of the toxins could not be determined from these data because without replicates any growth

can be attributed to a number of factors. Graphical representation of this data and growth rates can be found in Appendix C.

Table 15. Biomass production (g/L) by *Pichia stipitis* with different concentrations of furfural(ff), acetic acid(aa), and combinations of the two in synthetic aspen media.

Treatment (g/L)	Initial Biomass (g/L)	Final Biomass (g/L)	Total Biomass (g/L)	% Biomass Increase	% of Control
Aspen–control (0)	2.15	5.25	3.10	144.0	
ff2	2.11	2.15	0.04	2.0	1.39
ff4	2.11	2.14	0.03	1.5	0.99
aa8	2.14	2.27	0.13	6.1	4.18
aa10	2.15	2.20	0.05	2.3	1.63
ff2:aa8	2.22	2.27	0.05	2.3	1.63
ff4:aa10	2.23	2.34	0.11	4.8	3.48

Concentrated Sugar and Furfural and Acetic Acid Toxicity-Pichia stipitis

Typical lignocellulosic pretreatment will yield anywhere from 10 - 60 g/L fermentable sugars (Robinson et al., 2003). This concentration is low compared to a typical starch fermentation (100 - 300 g/L) (Robinson et al., 2003). Methods exist for concentrating the sugars in the hydrolysate and are focused on the removal of the water from the feedstream. Evaporation has yielded glucose concentrations of 170 g/L (Lee et al., 1999). The main problem with concentrating of the sugars in hydrolysate is that toxins present could also be concentrated making fermentation that much more difficult. However, there

are several methods used to detoxify the hydrolysate that fall in the categories of biological, chemical, and physical (Mussatto and Roberto, 2004). Evaporation, in fact, is also a method of detoxification. It removes acetic acid, vanillin, furfural, and other volatiles (Mussatto and Roberto, 2004). Treating the hydrolysate with activated charcoal and overliming are other effective methods that have been employed. The very cheap and effective means of detoxification is culture adaptation.

The inhibitory compounds, furfural and acetic acid were tested at levels that mimic what would be seen in a 1X, 2X, and 3X aspen hydrolysates. The initial experiment resulted in minimal biomass production in all treatments except for acetic acid, 4g/L and furfural, 1.5 g/L; growth was seen at 3 g/L furfural after 96 hours (See Appendix C). The synergistic effect of the inhibitory compounds was apparent in that no growth was seen at 72 hours in any combined treatment (Table 16). These samples were not tested for ethanol production. Acetic acid has been shown to not be toxic at levels below 4 g/L (Van Zyl et al., 1991). Also, most concentrating methods result in its removal. As a result the samples were not tested for ethanol production. The furfural samples were tested for ethanol production. The 1.5 g/L furfural concentration had an ethanol yield of 0.27 g/g (48 hrs.) compared to the control's 0.40 g/g (72 hrs) yield. Furfural concentrations of 3.0 g/L and 4.5 g/L yielded no ethanol (see Appendix C).

Table 16. Biomass production by *Pichia stipitis* CBS 6054 in the presence of: acetic acid (aa; 4, 8, 12 g/L), 5B. furfural (ff; 1.5, 3, 4.5 g/L); and combinations of the two (4:1.5, 8:3, 12:4.5 g/L) in synthetic aspen hydrolysate in synthetic aspen media after 24 hours (30°C, pH 5.5).

Treatment	Initial Biomass (g/L)	Final Biomass (g/L)	Total Biomass (g/L)	% Biomass Increase	% of Control
Control	2.2	3.9	1.8	81.0	
ff 1.5	2.1	4.1	2.0	91.9	111.7
ff 3	2.3	2.2	-0.1	-4.9	-6.3
ff 4.5	2.5	2.2	-0.3	-13.8	-19.7
aa 4	2.4	2.3	-0.1	-2.8	-3.7
aa 8	2.2	2.3	0.0	1.9	2.3
aa 12	2.0	2.2	0.1	7.1	8.2
ffaa4:1.5	2.4	2.4	0.0	-0.5	-0.7
ffaa8:3	2.2	2.3	0.1	3.8	4.7
ffaa12:4.5	2.3	2.2	-0.1	-4.0	-5.2

Sugar Level Toxicity on P. stipitis

Another factor to consider when concentrating the hydrolysate is the inhibitory effect of the sugars. High sugar concentrations can cause osmotic stress. Xylose has been shown to be inhibitory to growth at levels between 76 - 99 g/L (du Preez et al. , 1986). Maximum ethanol production occurs at 50 g/L (Agbogbo and Coward-Kelly, 2008). The goal of this study was to determine the effect, if any, concentrated sugars had on fermentations by *P.*

stipitis. In each treatment, 100% of the sugars were used within 48 hours. Aspen 2X had the highest ethanol yield (0.51 g/g). Ethanol productivity was the lowest in aspen 3X, which could be due to osmotic stress (Table 17). The ethanol yield on aspen 3X was 73% of the theoretical yield (0.51 g/g), which is still considered a sufficient yield (du Preez et al., 1986). *P. stipitis* is more sensitive to low substrate concentrations than high substrate concentrations in terms of ethanol productivity (du Preez et al., 1986). The biomass yields were highest on aspen 1X. They were slightly less on aspen 3X and lowest on aspen 2X. Without replicates these data cannot be verified.

Table 17. Maximum ethanol yields from concentrated sugar mixtures modeled after aspen hydrolysate by *P. stipitis* CBS 6054 over 120 hours in YP (30°C, pH 5.5).

Treatment	Total Sugars (g/L)	% Sugar Utilized	Ethanol Conc. (g/L)	Ethanol Yield (g_EtOH/g_sugar)	Dry Weight (g/L)
1X	17.30	100%	7.30 ^a	0.42	22.4
2X	36.95	100%	18.97 ^a	0.51	16.6
3X	65.03	100%	23.96 ^b	0.37	18.6

^a 48 hour value

^b 64 hour value

3.5 Adaptation

Adaptation to Dilute Acid Pretreated Hydrolysate

P. stipitis was adapted to dilute acid pretreated aspen hydrolysate. The hydrolysate differs between batches but the overall characteristics remain the same (Table 18). Using dilute acid-pretreated aspen hydrolysate made by the Chemical Engineering Dept. and synthetically made hydrolysate, *P. stipitis* has been adapted to media containing up to 80% hydrolysate. The 65%-80% adapted strains have not yet reached optimal production

ethanol yields (0.06-0.19 g/g) and are not reported in this work. At 60% (v/v) of hydrolysate, ethanol yields are between 0.28-0.33 g/g (Table 19). Compared to the unadapted strain, there was a 2.7 ± 0.31 -fold increase in g/g ethanol yield. This is comparable with 2.4 ± 0.10 -fold increase in ethanol yields from an adapted strain of *P. stipitis* on 60% dilute acid pretreated wheat straw (Nigam, 2001c). It has been reported that a 1.6-fold increase in ethanol yields has been achieved through adaptation to dilute acid pretreated hardwood (60%) (Nigam, 2001a). Nigam (2001a) reported data for an adapted strain of *P. stipitis* CBS 6054 for up to 60% (v/v) dilute acid pretreated red oak hydrolysate. Ethanol yields of 0.4 g/g_{sugar} were reported for that adapted strain. Several other adapted cultures have reported similar values (Table 20). Michigan Tech's adapted strain of *Pichia* is performing as well as other adapted and wild type strains grown in similar studies in terms of ethanol yields (Agobogbo and Wenger, 2007). In terms of time to maximum ethanol concentrations, it is performing better. *P. stipitis* CBS 6054 typically produces 0.37 - 0.44 g/g within 48-72 hours; the adapted strain in this work is producing ethanol at those levels (Figure 13).

The adapted strain at Michigan Tech was compared to its parent strain through a series of fermentations in order to verify a successful adaptation. The ethanol yields are from the yeast's first exposure to the hydrolysate after adaptation. If the cells are recycled (actively growing cultures repeatedly transferred to media of the same composition), higher ethanol yields, shorter fermentation times, and greater substrate utilization are likely to be observed (Amartey and Jefferies, 1996). Every batch of hydrolysate is different (Table 18). The synthetic media were made from an average pretreatments. Comparisons between the adapted and unadapted parent strain can be found in Appendix D section 1.

Table 18. Composition of two batches of dilute acid pretreated aspen hydrolysate.

Compound	Aspen Hydrolysate Batch 1 (g/L)	Aspen Hydrolysate Batch 2 (g/L)
Glucose	1.72	1.65
Xylose	6.92	7.00
Galactose	0.56	0.56
Mannose + Arabinose	1.62	1.27
Furfural	0.96	1.14
Total sugars	10.82	11.61

Table 19. Ethanol yields from an adapted strain of *Pichia stipitis* CBS 6054 on real dilute acid pretreated aspen hydrolysate after 72 hours (30°C, pH 5.5).^a

Percent Hydrolysate	Total Sugars (g/L)	% Sugars Utilized	Ethanol Conc. (g/L)	Ethanol Yield (g_EtOH/g_sugar)	Biomass (g/L)^b
30%	28.62±0.16	100	12.47 ± 0.9	0.44 ± .032	8.43
40%	24.63±0.22	100	11.07 ± 0.6	0.45 ± .024	6.98
50%	13.24±0.11	100	4.10 ± 0.03	0.31 ± .0018	10.1
60%	11.72±0.13	100	3.56± 0.4	0.30 ± .033	6.19

^aMean (n=2) ±2 SD

^bThe biomass was determined on one sample only

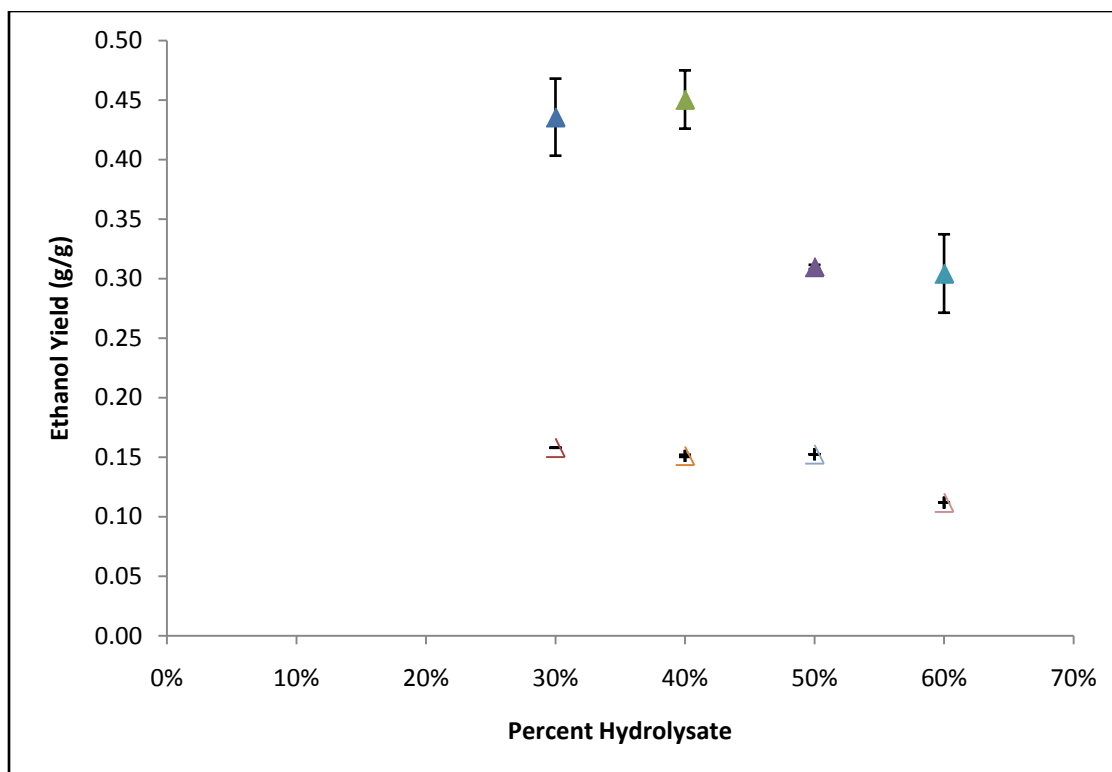


Figure 13. Comparison of 72 hour ethanol yields produced by an adapted *Pichia stipitis* CBS 6054 (solid markers) and the parent strain (hollow markers) on varying percentages of dilute acid pretreated aspen hydrolysate supplemented with a sugar mix. Data presented as mean ($n=2$) ± 2 SD.

Table 20. MTU adapted strain of *Pichia stipitis* CBS 6054 compared to other adapted strains of *Pichia* strains in the literature.

Hydrolysate Type	% Hydrolysate	% Sugar Utilized	Ethanol Yield (g/g)	% T^c Yield	Fermentation Time (hr)	Ref.
DAP^a Aspen	60	99.9	0.30-0.33	61-65	72	This Study, (2009)
DAP Red Oak	30	66.94	0.31	62	140	Nigam, (2001a)
AP^b Aspen woodchips	Not reported	95	0.41-0.47	82-94	96	Parekh et al., (1988)
DAP Poplar	Not reported	Not reported	0.31-0.38	62-76	50	Fenske et al., (1998)
DAP hard and softwood mixes	80% ^d	Not reported	0.44	88	72	Sreenath and Jeffries (2000)

^aDAP – Dilute sulfuric acid pretreatment

^bAP – Sulfuric Acid Pretreatment

^cT- Theoretical yield

^dPartially detoxified

3.6 Fermentation Optimization

Media composition

The effects of different fermentation media on ethanol production by *P. stipitis* was determined. Three media were evaluated: YNB, YPX, and CSL (Table 21) (Agbogbo and Coward-Kelly, 2008). Growth and biomass production for YNB and YPX have been assessed. The growth rate was slower for YPX than YNB, 1.32 and 0.72 hr⁻¹, respectively (Appendix E). In preliminary studies, YNB produced a mean ethanol yield of 0.52 g/g, whereas YPX produced 0.23 g/g. Another fermentation study was conducted with all three media types (Table 18). CSL and YNB were supplemented with 50:50 glucose:xylose. YPX consisted just of xylose (50 g/L). CSL media gave the highest ethanol yields but utilized the least amount of sugar. YPX utilized the greatest percentage of sugar (and had relatively high ethanol yields).

Table 21. Summary of fermentation results of three different media types: (Yeast Nitrogen Base (YNB), Yeast Peptone Broth (YPX), and Corn Steep Liquor Media (CSL) using *Pichia stipitis* on glucose and/or xylose (30°C, pH 5.5)^a.

Medium	Total Sugars (g/L)	% Sugar Utilized	Ethanol Conc. (g/L)	Ethanol Yield (g_EtOH/g_sugar)	Dry Weight (g/L)
YPX	49.0±0.1	49.9	10.3±1.46	0.42±0.01	13.6
YNB	77.6±0.7	35.5	8.25±0.49	0.30±0.07	13.4
CSL	75.0±1.3	24.9	9.44 ±1.44	0.51±0.01	15.4

^aMean (n=2) ±2 SD

Conclusions

- *S. cerevisiae* (ER) could not utilize the pentose sugars present in the dilute acid pretreated hydrolysate and was not considered for fermentations where pentose sugars were present. *Pichia stipitis* was able to grow on all five sugars present in the hydrolysate. It also could produce ethanol from four out of the five.
- Some compounds (furfural and acetic acid) that are produced as a result of dilute acid pretreatment are inhibitory to both types of yeast.
- *P. stipitis* was successfully adapted to dilute acid pretreated hydrolysate for improved ethanol yields. The final adaptation resulted in 2.6 fold increase in ethanol production compared to the unadapted parent strain.
- Adaptation is an effective and inexpensive means of improving ethanol yields from lignocellulosic feedstocks.
- Three media were tested to determine the optimum for ethanol production by *P. stipitis* CBS 6054. The three tested media all had comparable yields. CSL had higher ethanol yields than the YPX and YNB.

Recommendations

The adapted strain of *P. stipitis* needs to be adapted to higher concentrations of dilute acid pretreated hydrolysate. Cell recycle also needs to be looked at as a means of improving ethanol yields. Scale-up needs to be done with the adapted strain of *P. stipitis* in order to determine if the procedures that worked in the bench scale studies can be applied to large scale fermentations. It also needs to be determined that this is in fact adaptation and not just acclimation. In terms of media, given these results more factors, such as cost and availability, need to be examined in order to optimize the fermentation on the basis of media.

More work needs to be done with concentrated hydrolysate and mixed hydrolysate fermentations should be examined. Each biomass type has its own characteristic and properties that can lead to increased ethanol yields. Fermentation involving combinations of the enzymatic and dilute acid hydrolysate and co-culture fermentations should be performed.

The fermentation of lignocellulosic feedstocks have the potential to become as commercially and economically feasible as corn based ethanol. It hinges on producing comparable ethanol yields. This can be done by selecting the right organism and then adapting it to the feedstock. The condition must be optimized for that organism. A small scale example is presented in this thesis but it can and should be applied to scale-up methods in similar studies.

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Appendix A. Supplementary Data for *S. cerevisiae*

A.1 Ethanol production from glucose by *S. cerevisiae* (ER)

S. cerevisiae (ER) was tested in the bench scale flask system to obtain baseline ethanol production data for the system. YPG (20 g/L) was used. A mathematical equation was developed to predict ethanol yields using the bench scale system.

Table A.1. Production of ethanol from glucose by *S. cerevisiae* in 24 hours (30°C, pH 5.5).

Glucose (g/L)	EtOH (g/L)	Ethanol g/g Yield	% Theoretical
20	3.27	0.17	33.17%
20	3.87	0.20	39.47%
20	5.69	0.29	57.87%

Preliminary ethanol production data for Ethanol Red (*S. cerevisiae*) led to the creation of an equation for predicting ethanol yield within this experimental design (equation A.1.1).

Equation A 1

$$\frac{\text{Initial Sugar Conc. (g/L)} - \text{Final Sugar Conc. (g/L)}}{4.9} = \text{EtOH Conc. (g/L)}$$

Table A.2. Example of the ethanol yield model using results of HPLC data for a 100% glucose (5 g/L) fermentation using *S. cerevisiae* (ER) after 24 hours in YP media (30°C, pH 5.5).

Treatment	Initial Glucose (g/L)	Final Glucose (g/L)	Difference (g/L)	Expected EtOH (g/l)	Actual EtOH (g/l)	% of expected
100% Glucose	4.17	0.46	3.70	0.75	0.73	96

A.2 Fermentation results for *S. cerevisiae* (ER) in xylose and glucose mixtures

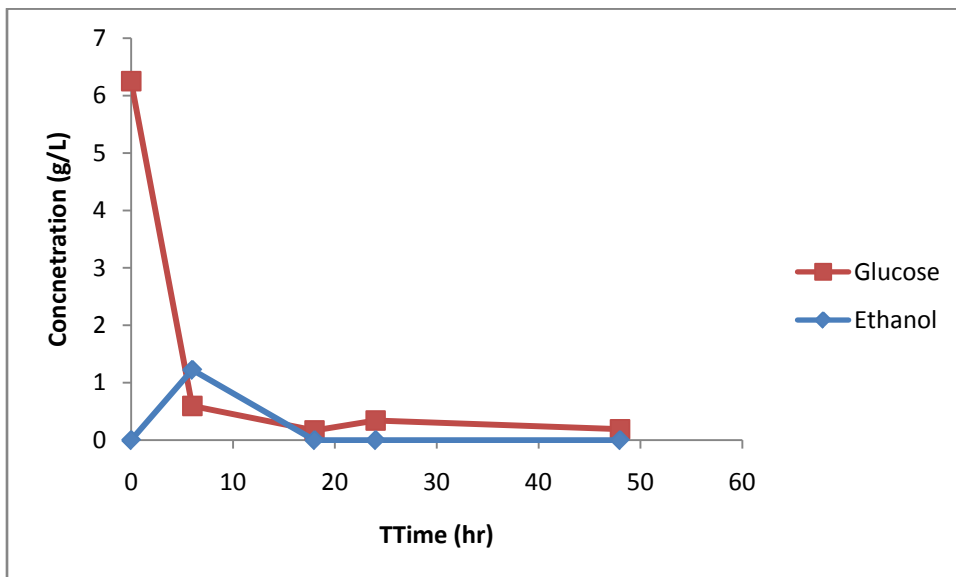


Figure A.1. Ethanol production and substrate utilization by *S. cerevisiae* on 100% glucose (6.25 g/L) after 24 hours in YP media (30°C, pH 5.5).

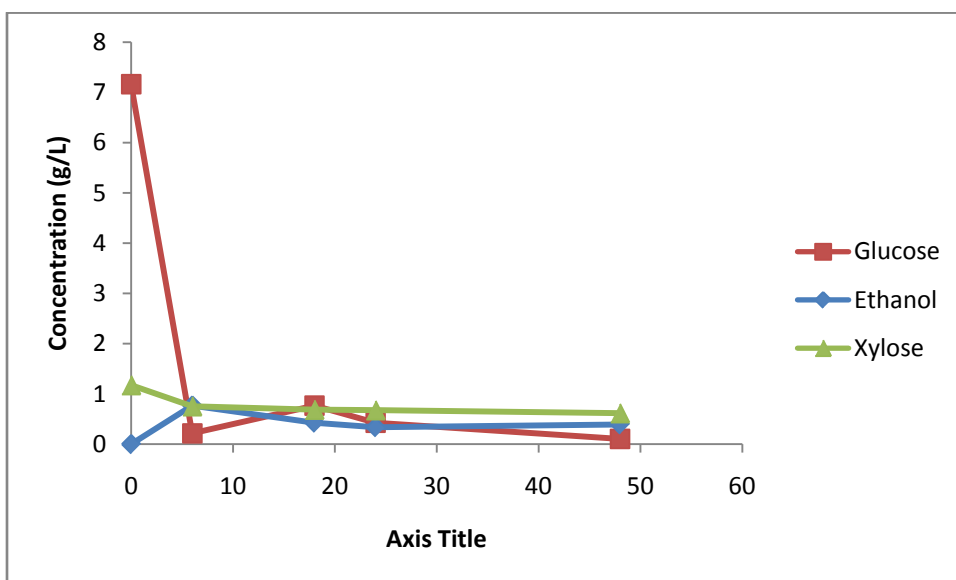


Figure A.2. Ethanol production and substrate utilization by *S. cerevisiae* on 75% glucose:25% xylose mixtures (8.2 g/L) after 24 hours in YP media (30°C, pH 5.5).

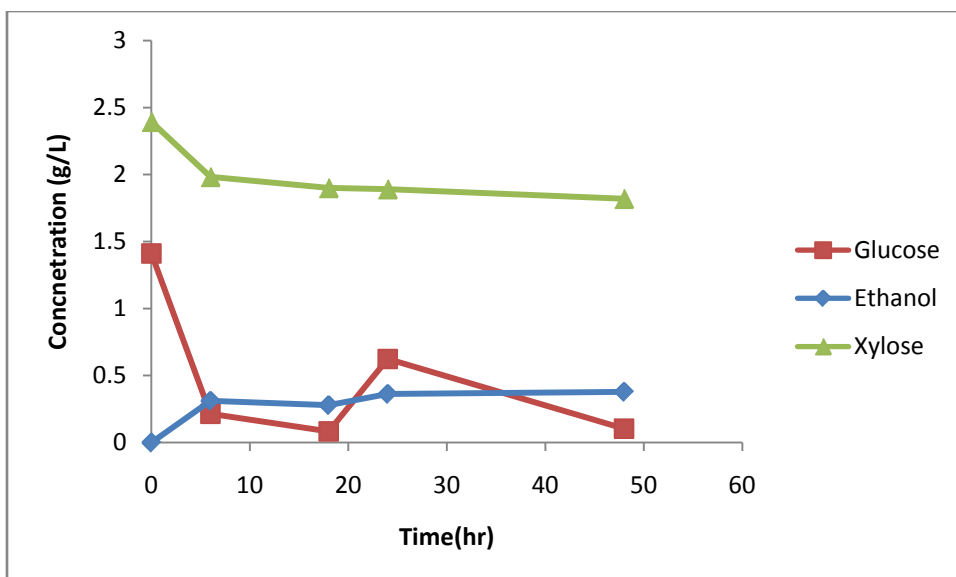


Figure A.3. Ethanol production and substrate utilization by *S. cerevisiae* on 60%:40% glucose : xylose (3.8 g/L) after 24 hours in YP media (30°C, pH 5.5).

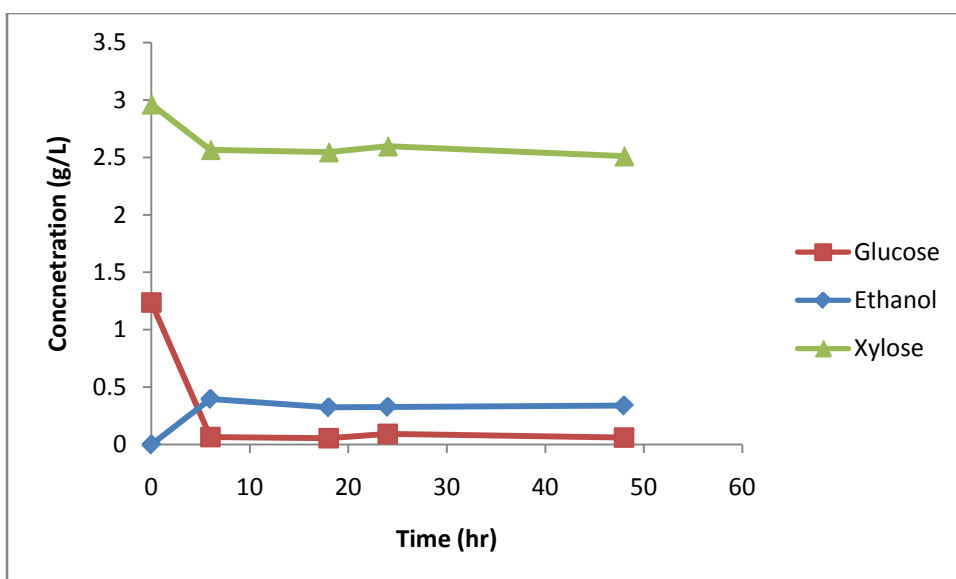


Figure A.4. Ethanol production and substrate utilization by *S. cerevisiae* on 25%:75% glucose : xylose (4.1g/L) after 24 hours in YP media (30°C, pH 5.5).

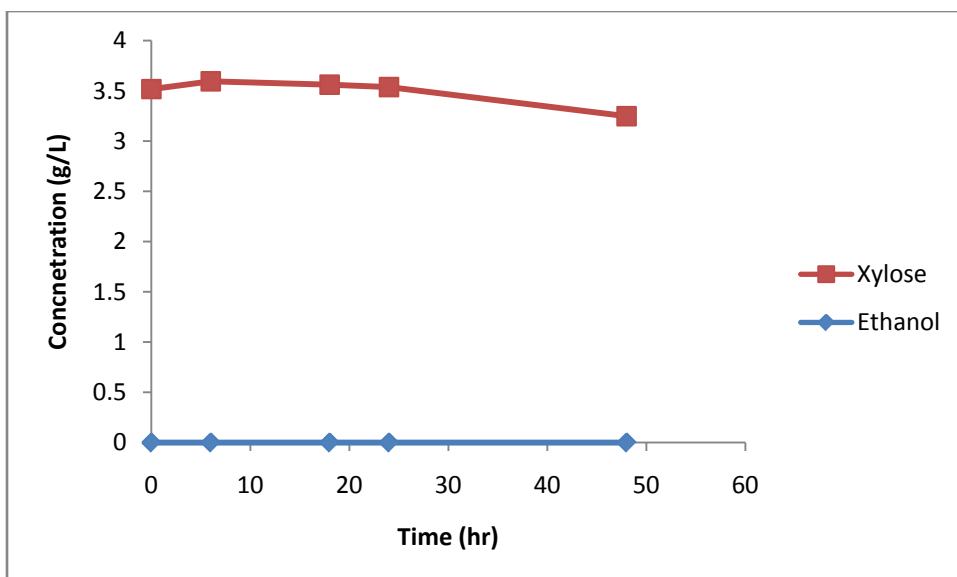


Figure A.5. Ethanol production and susbstrate utilization by *S. cerevisiae* on 25%:75% glucose : xylose (3.5 g/L) after 24 hours in YP media (30°C, pH 5.5).

Appendix B. Supplementary Data for Fermentations by *P.stipitis* and Enzymatic Hydrolysate Fermentations by *S. cerevisiae* (ER)

B.1 Growth of *P. stipitis* on YP-sugar free vs. YPX

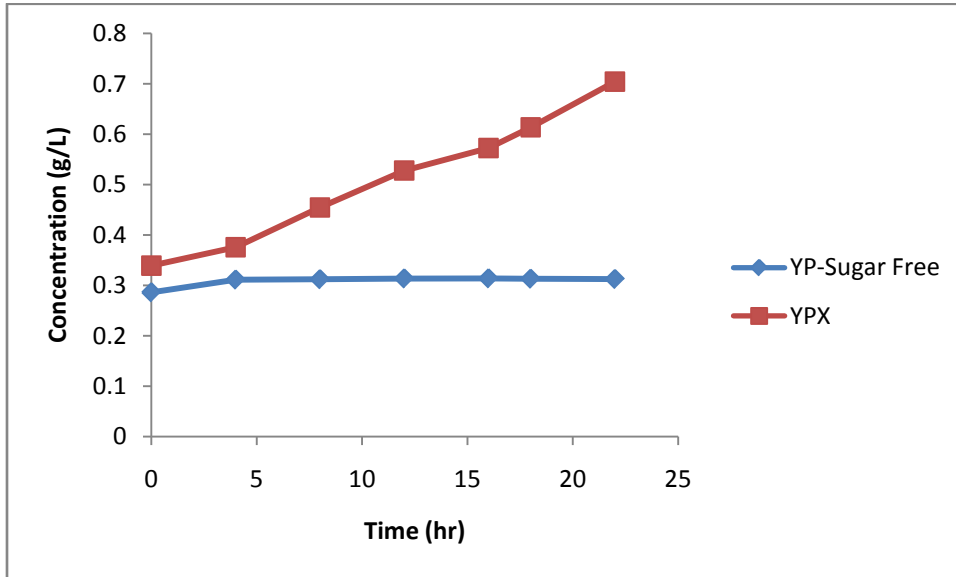


Figure B.1. Growth of *P. stipitis* on YP media without a carbon source compared to YPX (20g/L) media after 24 hours (30⁰C, pH 5.5).

B.2 mixed substrate fermentations by *P. stipitis*

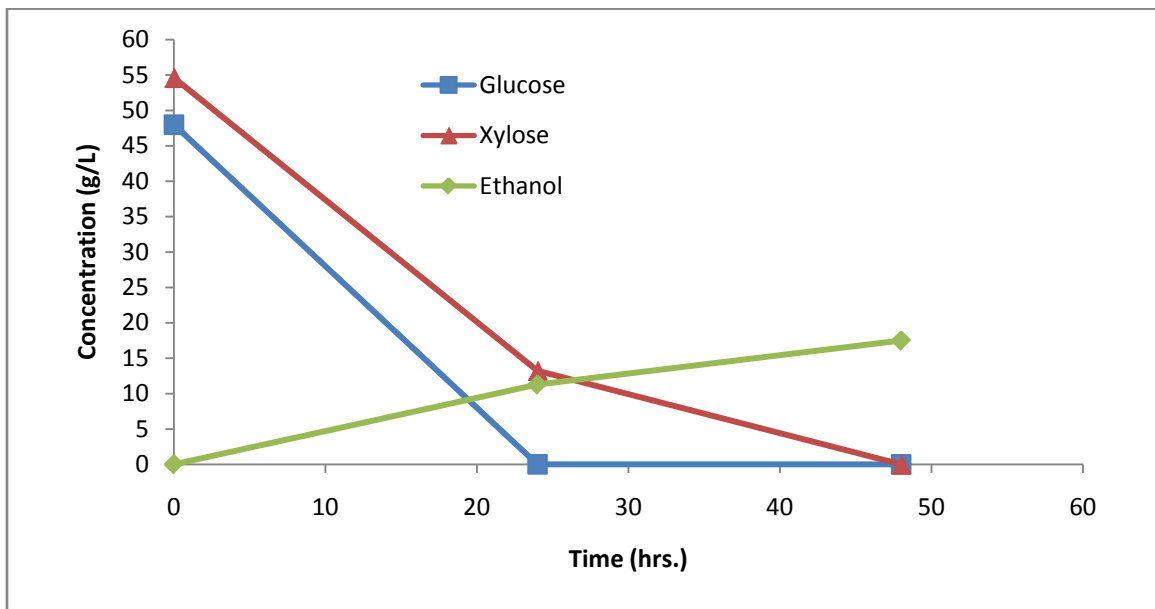


Figure B.2. Fermentation of a 50:50 mixture xylose (50g/L) and glucose (50g/L) in YP media by *Pichia stipitis* CBS 6054 after 72 hours (30°C, pH 5.5).

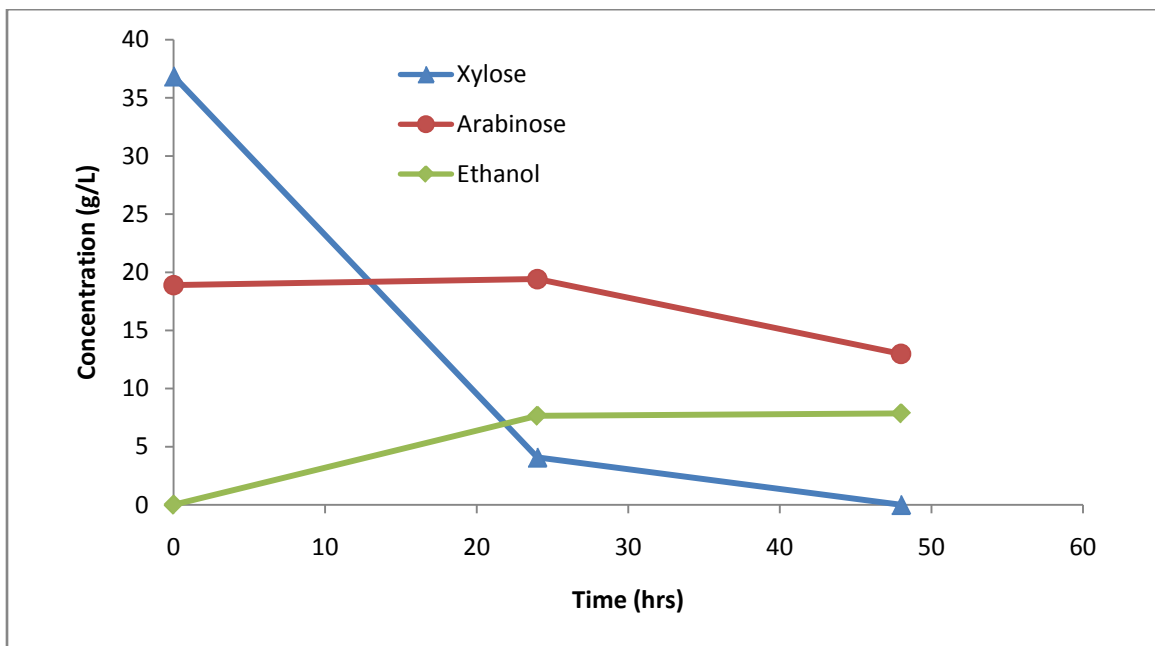


Figure B.3. Fermentation of a mixture of 1:2 mixture of arabinose and xylose in YP Media by *Pichia stipitis* CBS 6054 after 72 hours (30°C, pH 5.5).

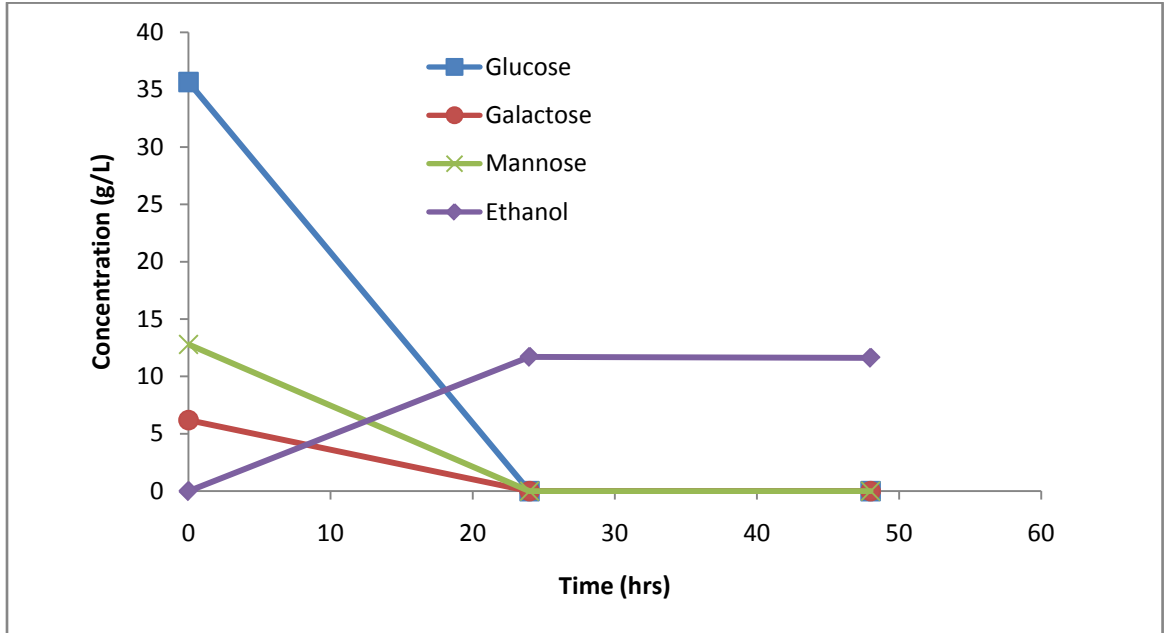


Figure B.4. Fermentation of a mixture of 1:1:2 mixture of galactose, mannose, and glucose in YP Media by *Pichia stipitis* CBS 6054 after 72 hours (30°C, pH 5.5).

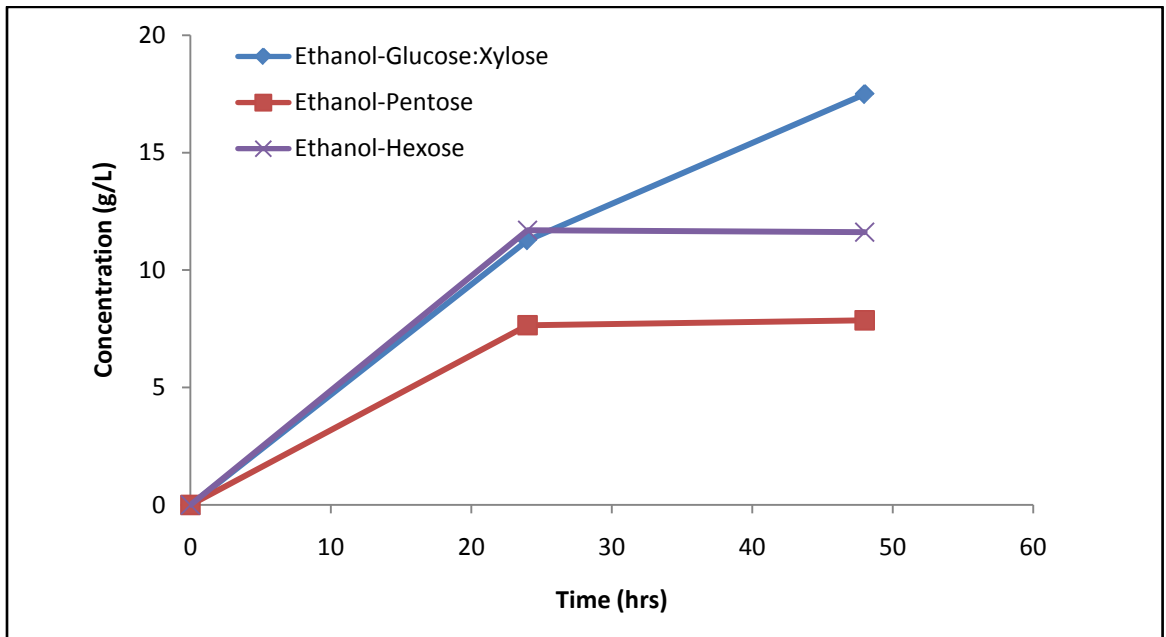


Figure B.5. Comparison of ethanol concentrations in a glucose: xylose, pentose, and hexose fermentation by *Pichia stipitis* CBS 6054 after 72 hours in YP media (30°C, pH 5.5).

B.2 Ethanol production by *P. stipitis* in synthetic hydrolysate media

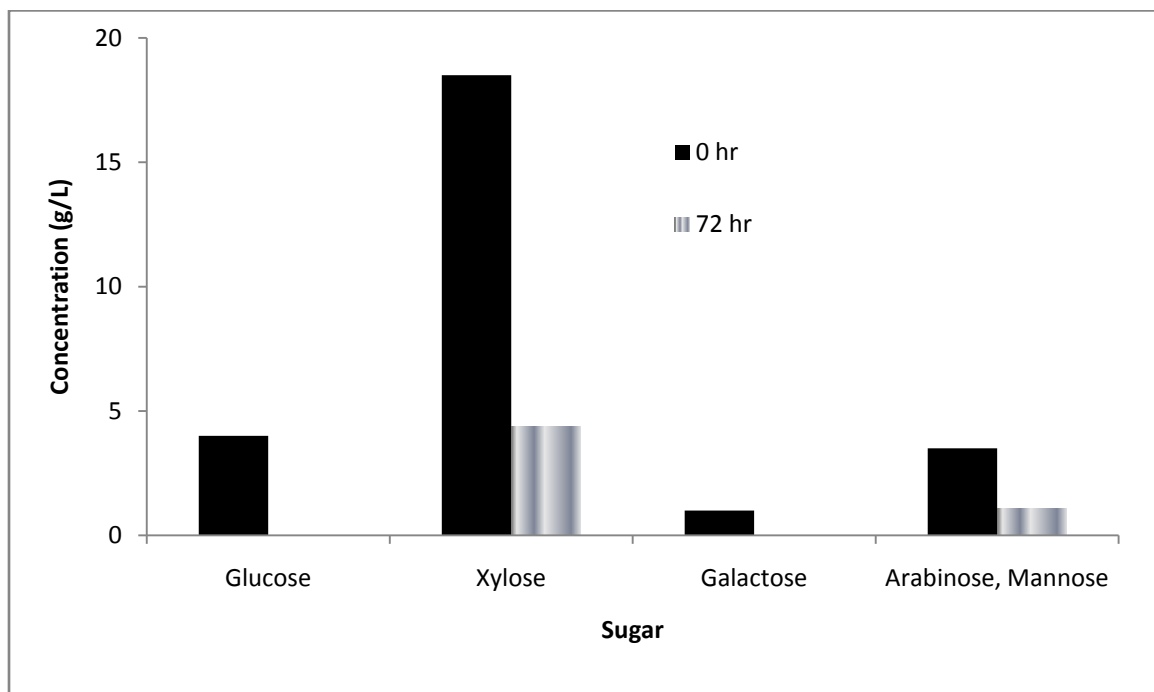


Figure B.6. Sugar concentrations in synthetic dilute acid pretreated aspen hydrolysate at 0 and 72 hours during a fermentation by *P. stipitis* (30°C, pH 5.5).

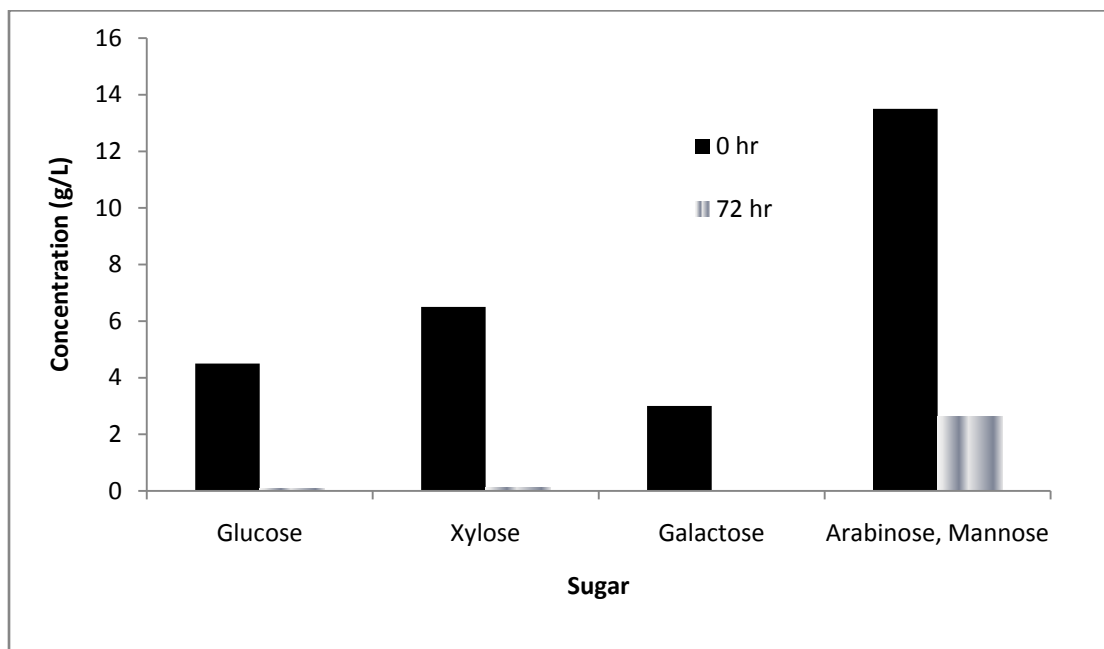


Figure B.7. Sugar concentrations in synthetic dilute acid pretreated balsam hydrolysate at 0 and 72 hours during a fermentation by *P. stipitis* (30°C, pH 5.5).

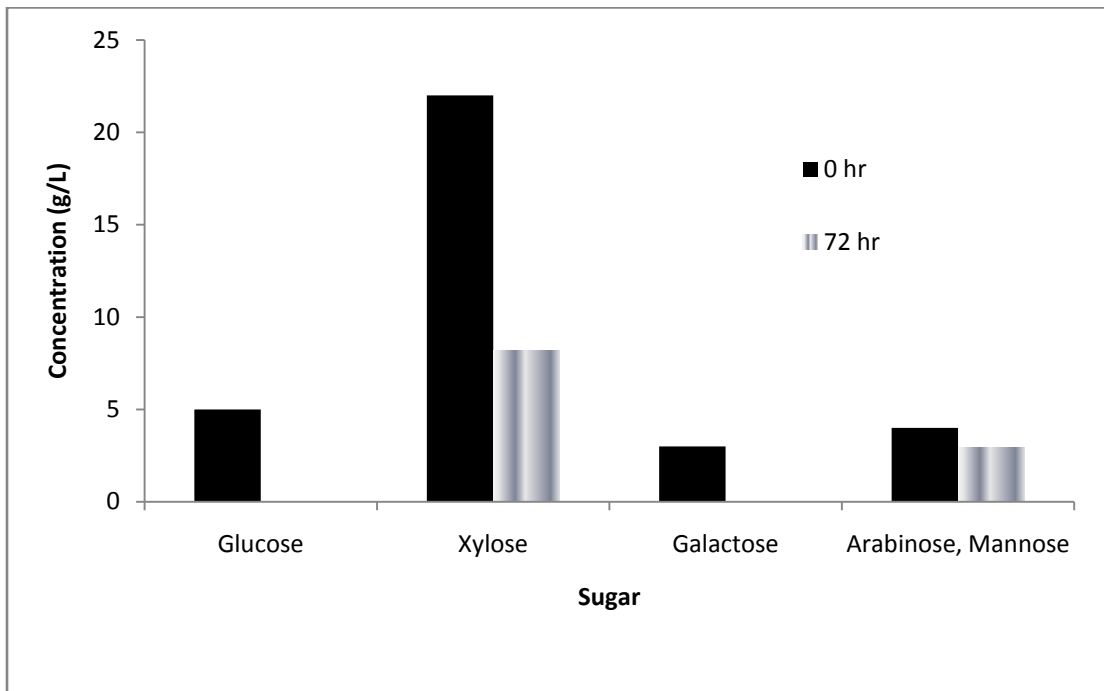


Figure B.8. Sugar concentrations in synthetic dilute acid pretreated switchgrass hydrolysate at 0 and 72 hours during a fermentation by *P. stipitis* (30°C, pH 5.5).

B.3 Fermentation of aspen, balsam, and switchgrass enzymatic hydrolysate by *S. cerevisiae* (ER)

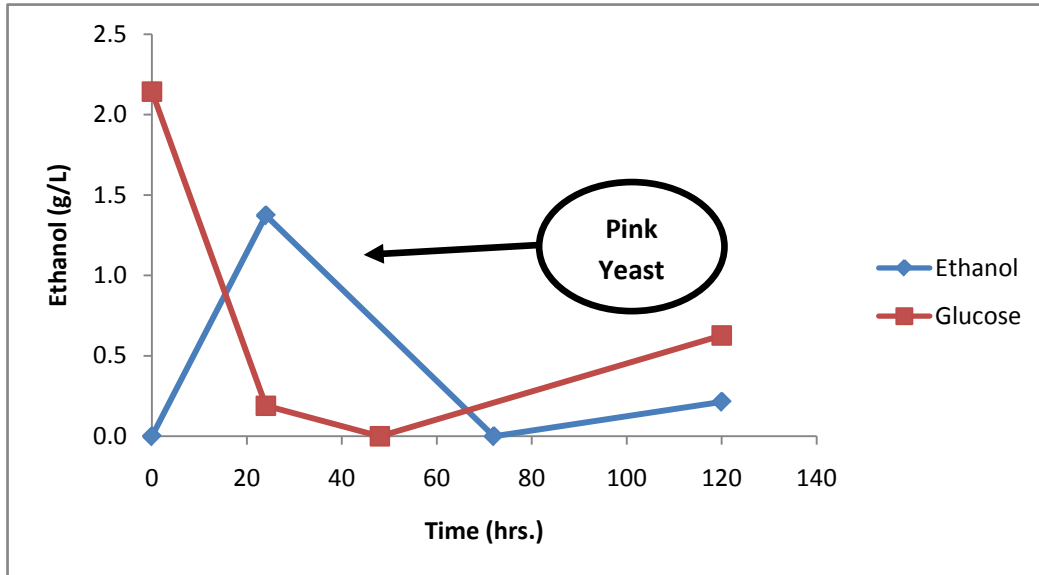


Figure B.9. Fermentation results of ethanol production from enzymatic pretreated aspen by *S. cerevisiae* after 24 hours in Enzymatic hydrolysate supplemented with YP media (30°C, pH 5.5).

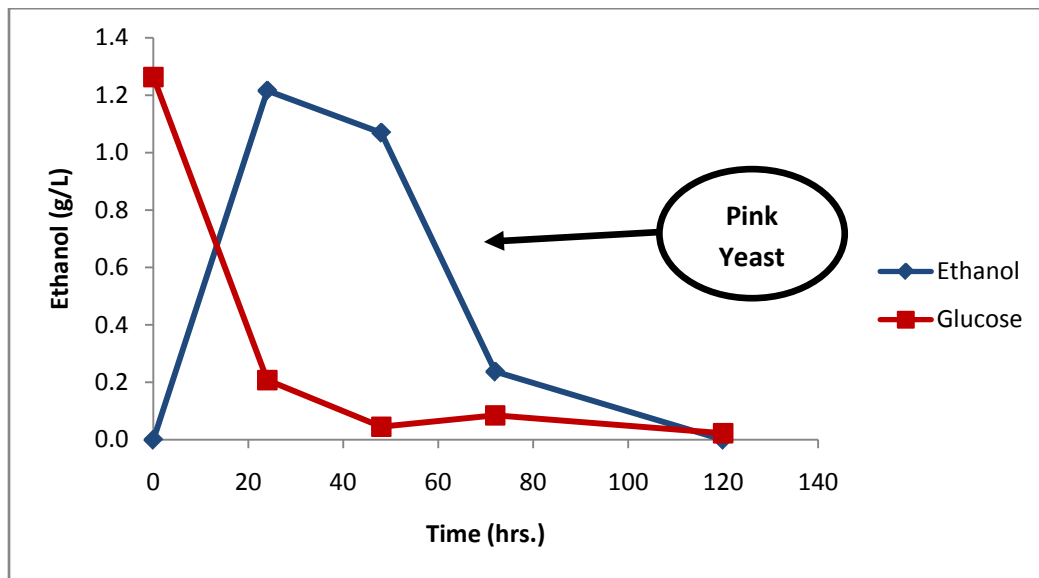


Figure B.10. Fermentation results of ethanol production from enzymatic pretreated balsam by *S. cerevisiae* after 24 hours in Enzymatic hydrolysate supplemented with YP media (30°C, pH 5.5).

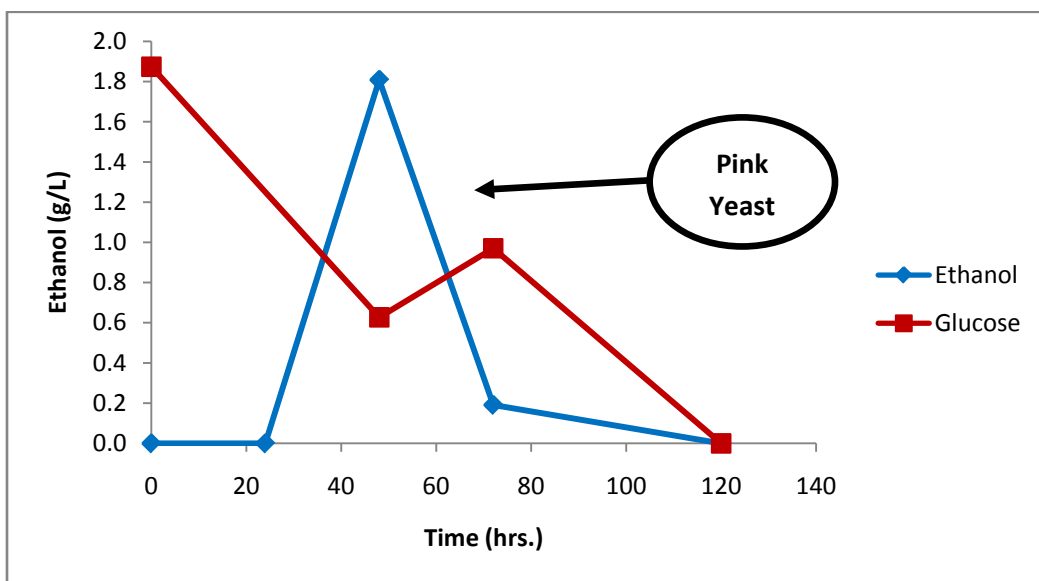


Figure B.11. Fermentation results of ethanol production from enzymatic pretreated switchgrass by *S.cerevisiae* after 24 hours in Enzymatic hydrolysate supplemented with YP media (30°C, pH 5.5).

Appendix C. Toxicity Assay Supplementary Information

C.1 Effect of furfural and acetic acid in YPX media on biomass production by *P. stipitis*

Table C.1. The growth rate (k) of *Pichia stipitis* with different concentrations of furfural in YPX after 24 hours (30°C, pH 5.5).

Furfural (g/L)	k (hr ⁻¹)
0	0.23
1	0.21
2	≤0.05
4	≤0.05

Table C.2. The growth rate (k) and generation time (t) of *Pichia stipitis* with different concentrations of acetic acid in YPX after 24 hours (30°C, pH 5.5).

Furfural (g/L)	k (hr ⁻¹)
0	0.16
2	0.13
4	≤0.05
6	≤0.05
8	0.09
10	≤0.05

C.2. Effect of furfural, acetic acid and combinations of the two compounds in synthetic aspen media on biomass production by *P. stipitis*

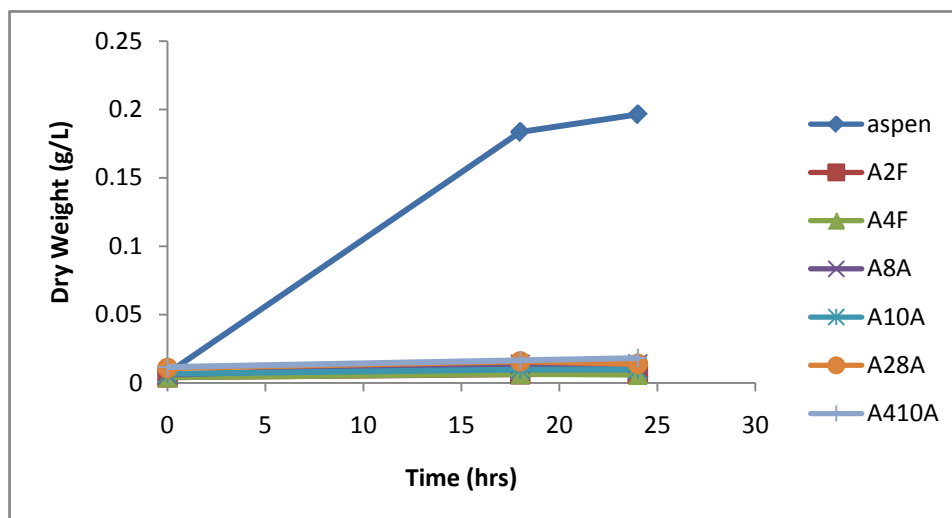


Figure C.1 Biomass production of *Pichia stipitis* in synthetic aspen hydrolysate supplemented with acetic acid [8 g/L (A8A) and 10 g/L (A10A)], furfural (2 g/L (A2F) and 4 g/L (A4F)], and combinations of the two toxins [2 g/L furfural with 8 g/L acetic acid (A28A) and 4 g/L furfural with 10 g/L acetic acid (A410A)] in synthetic aspen media (30°C, pH 5.5).

Table C.3 Growth rate (k) of *Pichia stipitis* in synthetic aspen hydrolysate supplemented with acetic acid [8 g/L (A8A) and 10 g/L (A10A)], furfural (2 g/L (A2F) and 4 g/L (A4F)], and combinations of the two toxins [2 g/L furfural with 8 g/L acetic acid (A28A) and 4 g/L furfural with 10 g/L acetic acid (A410A)] in synthetic aspen media (30°C, pH 5.5).

Growth rate	k (hr ⁻¹)
Aspen	0.20
A2F	≤0.05
A4F	≤0.05
A8A	≤0.05
A10A	≤0.05
A28A	≤0.05
A410A	≤0.05

C.3. Effect of concentrated furfural levels on biomass and ethanol production

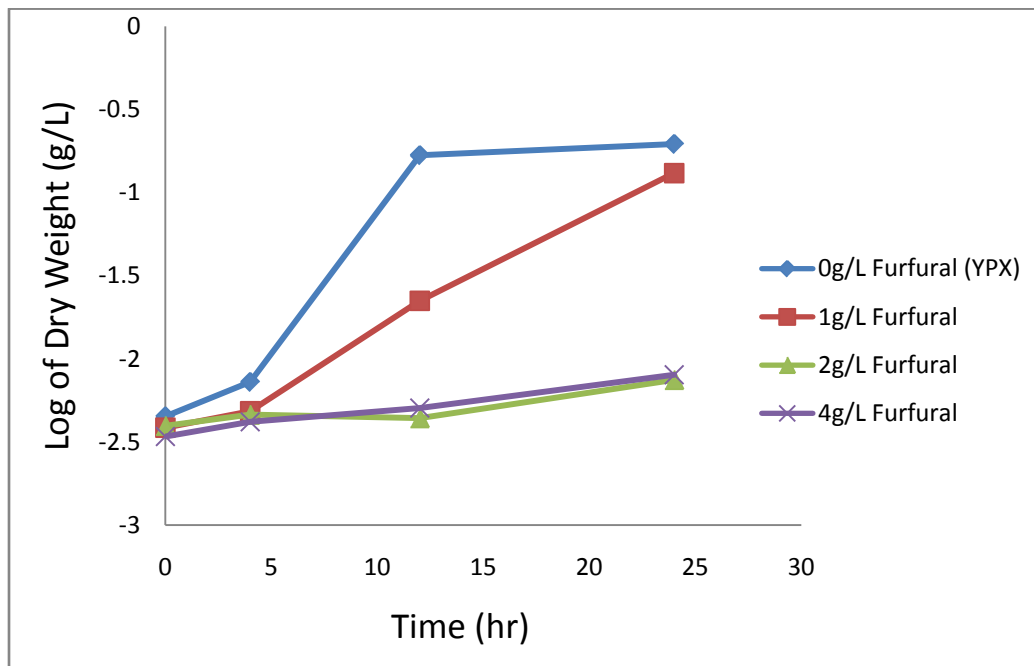


Figure C.2. Biomass production by *P. stipitis* in the presence of furfural after 24 hours in YPX (30°C, pH 5.5).

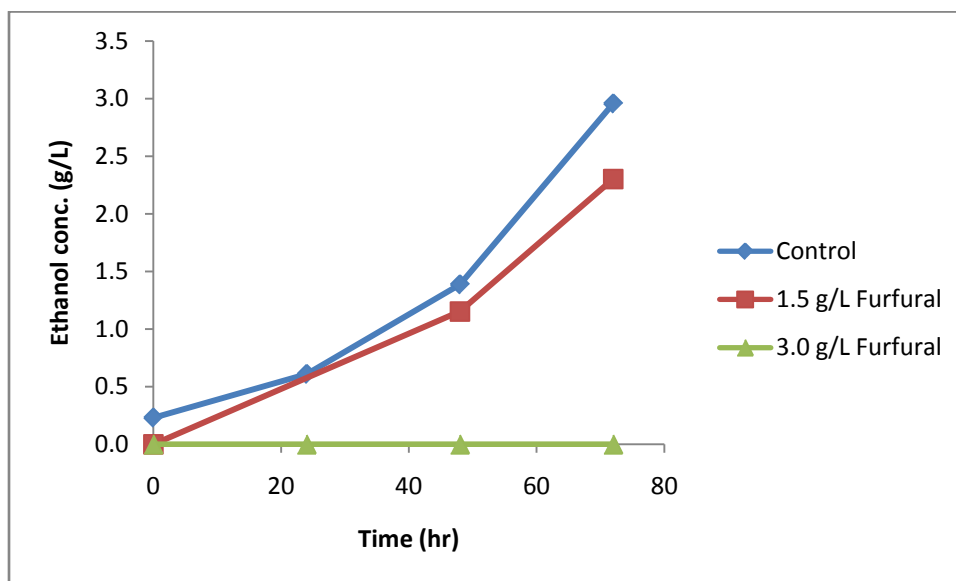


Figure C.3. Ethanol production by *P. stipitis* in the presence of furfural in after 72 hours synthetic aspen media (30°C, pH 5.5).

Appendix D. Supplementary Adaptation Data

D.1 Adaptation studies comparison data

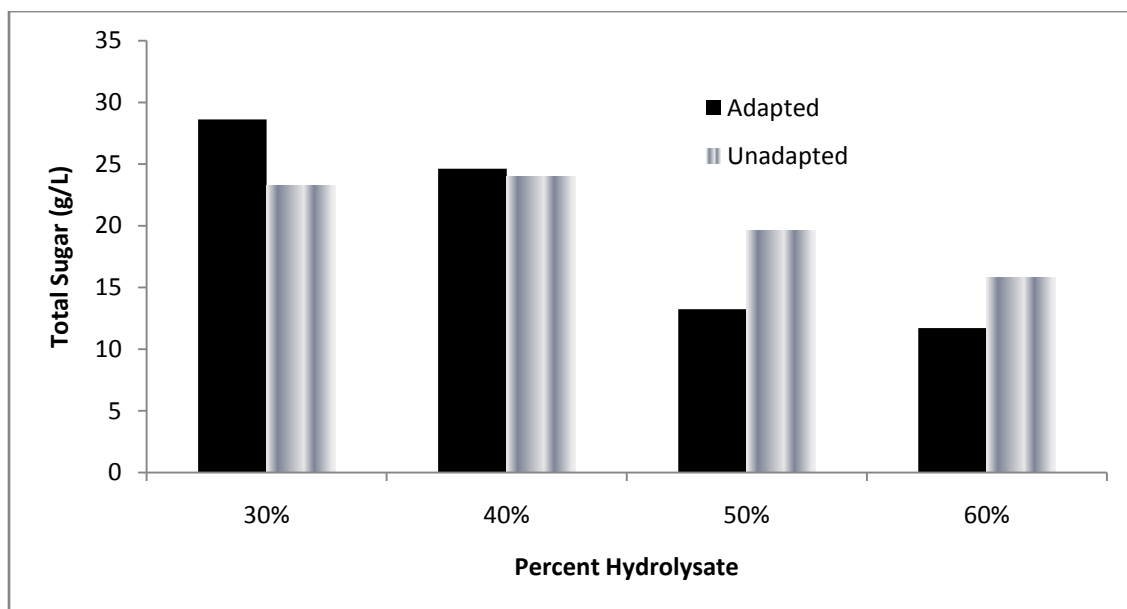


Figure D.1. Comparison of total starting sugars used by an adapted *Pichia stipitis* CBS 6054 and the parent strain on varying percentages of dilute acid pretreated aspen hydrolysate supplemented with a sugar mix (n=2) (30°C, pH 5.5).

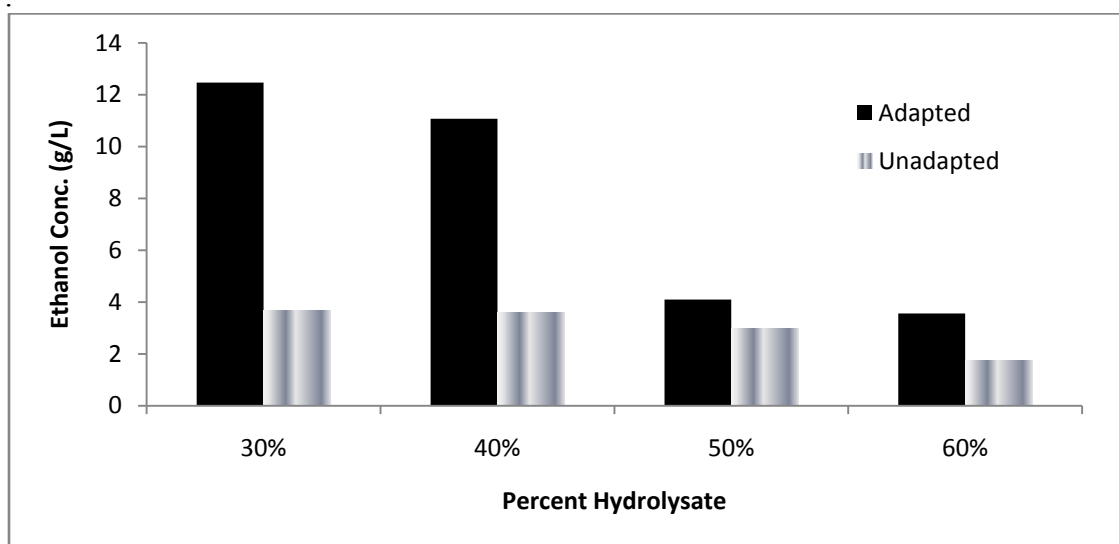


Figure D.2. Comparison of 72 hour ethanol concentrations produced by an adapted *Pichia stipitis* CBS 6054 and the parent strain on varying percentages of dilute acid pretreated aspen hydrolysate supplemented with a sugar mix (n=2) (30°C, pH 5.5).

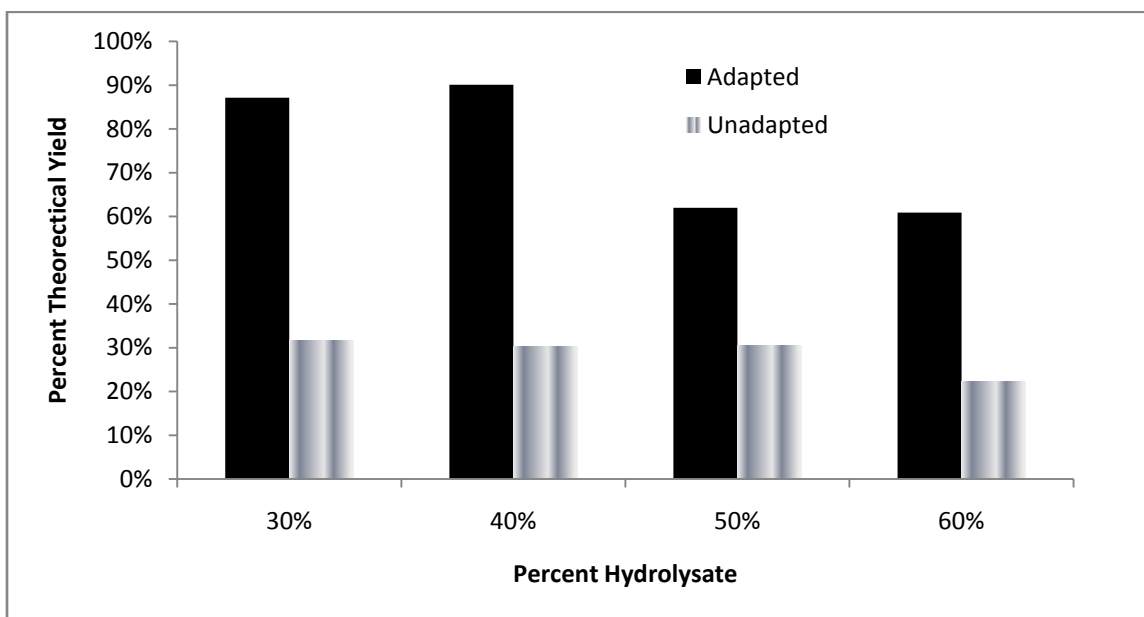


Figure D.3. Comparison of 72 hour percent theoretical yield produced by an adapted *Pichia stipitis* CBS 6054 and the parent strain on varying percentages of dilute acid pretreated aspen hydrolysate supplemented with a sugar mix (n=2) (30°C, pH 5.5).

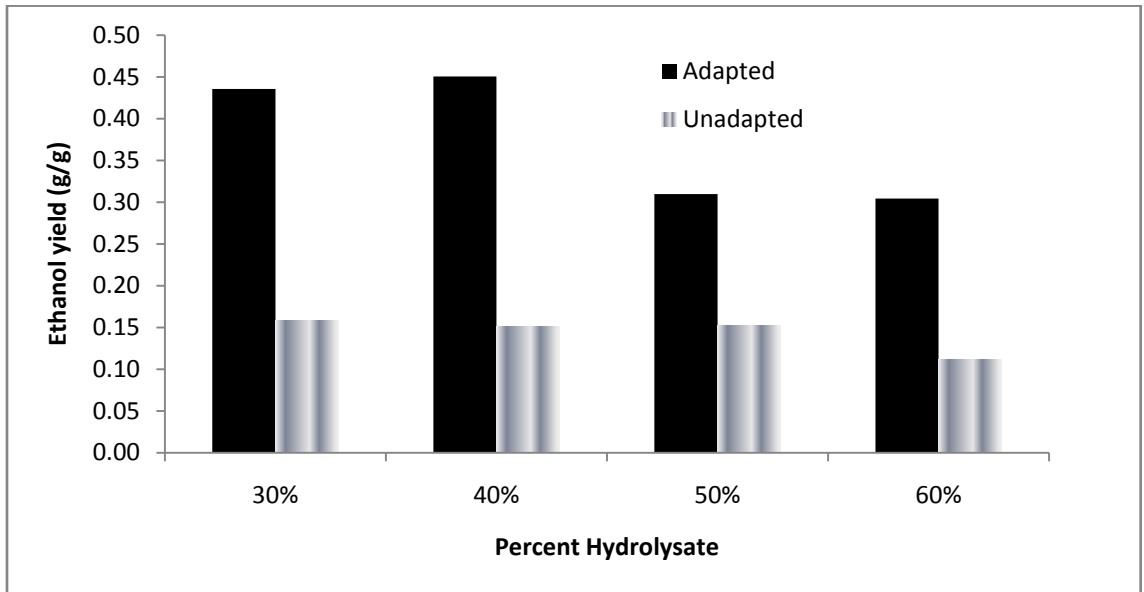


Figure D.4. Comparison of 72 hour ethanol yields produced by an adapted *Pichia stipitis* CBS 6054 and the parent strain on varying percentages of dilute acid pretreated aspen hydrolysate supplemented with a sugar mix (n=2) (30°C, pH 5.5).

Table D.1. Summary of adapted *Pichia stipitis* CBS 6054 adapted to dilute acid pretreated hydrolysate (30°C, pH 5.5). ^a

Unadapted Strain				
Percent Hydrolysate	% Sugars Utilized	Ethanol (g/L)	g_EtOH/g_Substrate yield	% Theoretical yield
30	99.6	3.7 ± 0.4	0.16 ± 0.30	31.4
40	99.6	3.6 ± 0.5	0.15 ± 0.11	29.4
50	99.7	3.0 ± 0.1	0.15 ± 0.03	29.2
60	99.8	1.8 ± 0.1	0.11 ± 0.0	21.6
Adapted Strain				
30	100.0	12.5 ± 0.9	0.44 ± 0.03	99.7
40	99.8	11.1 ± 0.6	0.45 ± 0.02	90.1
50	99.9	4.1 ± 0.03	0.31 ± 0.00	61.9
60	99.9	3.6 ± 0.4	0.30 ± 0.03	60.9

^aMean (n=2) ± 2SD

Appendix E. Growth of *P. stipitis* in YPX and YNB

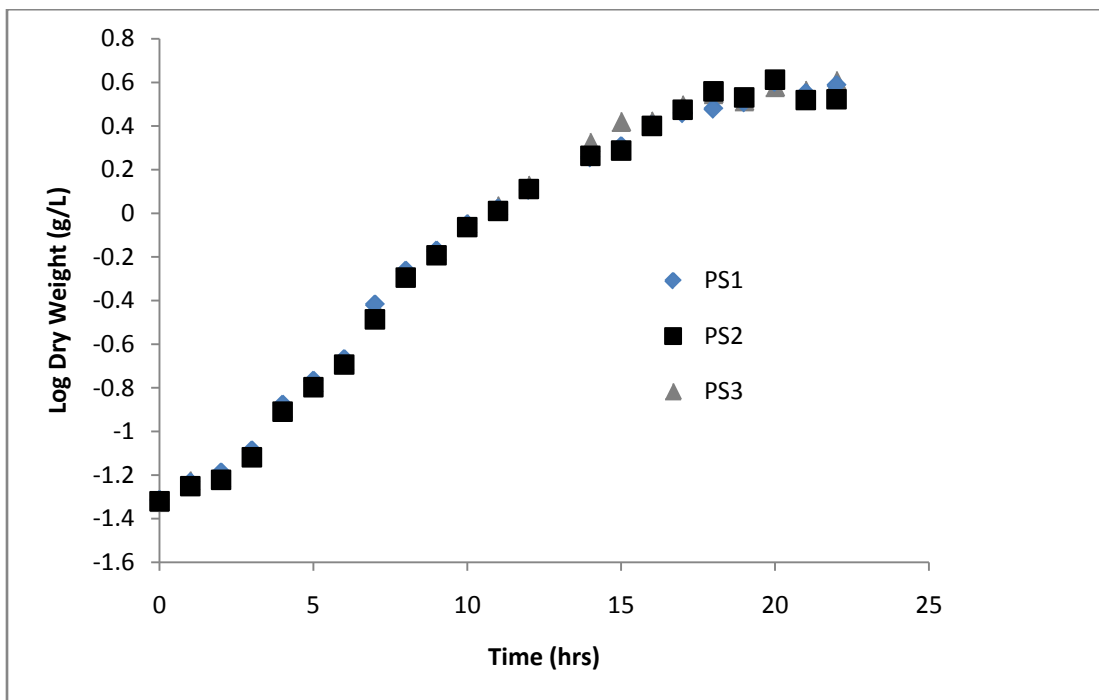


Figure E. 1. Growth of *P. stipitis* in YPX media (50g/L) during 24 hours (30°C, pH 5.5).

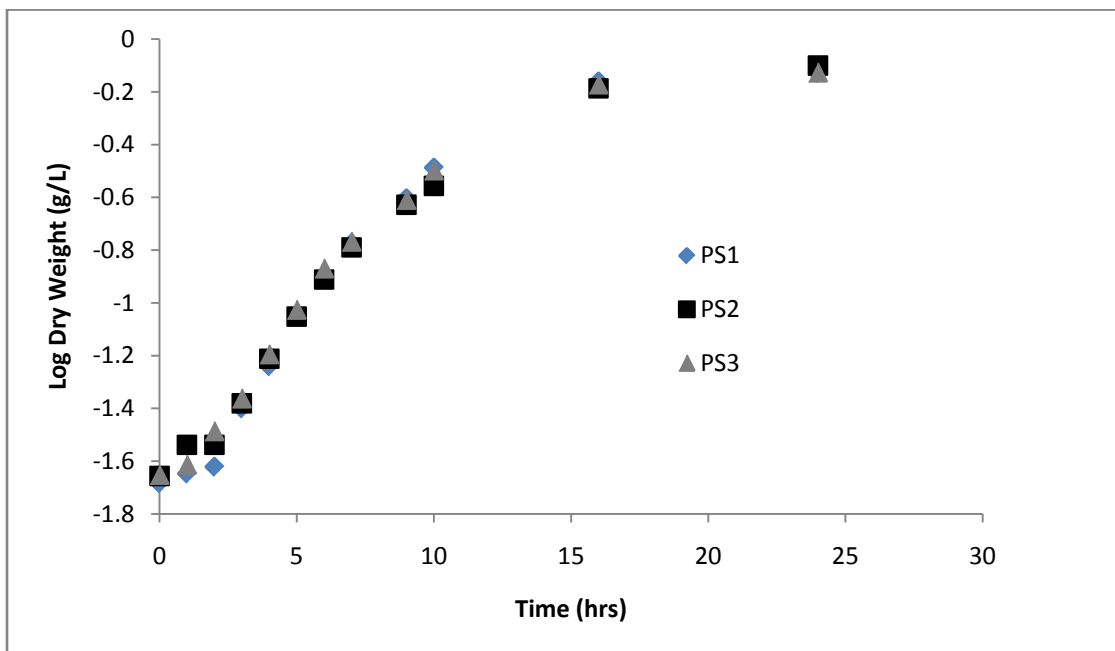


Figure E. 2. Growth of *P. stipitis* in YNB media (50g/L) over 24 hours (30°C, pH 5.5).