Spring 2014

Developing an unstructured model to investigate the effect of ethanol on product yields for glucose and xylose cofermentation in Saccharomyces cerevisiae 424A (LNH-ST)

Shane D. Clingenpeel

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By Shane Clingenpeel

Entitled
Developing an Unstructured Model to Investigate the Effect of Ethanol on Product Yields for Glucose and Xylose Cofermentation in Saccharomyces cerevisiae 424A (LNH-ST)

For the degree of Master of Science in Agricultural and Biological Engineering

Is approved by the final examining committee:
Nathan Mosier
Osvaldo Campanella
Martin Okos

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Nathan Mosier

Approved by Major Professor(s): ___________________________________________________________________

Approved by: Bernard Engel 03/18/2014

Head of the Department Graduate Program  Date
DEVELOPING AN UNSTRUCTURED MODEL TO INVESTIGATE THE EFFECT
OF ETHANOL ON PRODUCT YIELDS FOR GLUCOSE AND XYLOSE
COFERMENTATION IN SACCHAROMYCES CEREVISIAE 424A (LNH-ST)

A Thesis
Submitted to the Faculty
of
Purdue University
by
Shane Clingenpeel

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science in Agricultural and Biological Engineering

May 2014
Purdue University
West Lafayette, Indiana
To my wife for always being a rock for me. I only hope I can return the favor. - CLSB

To anyone who thinks they are not capable of greatness, this is proof otherwise.
ACKNOWLEDGEMENTS

I need to first thank my advisor, Dr. Nathan Mosier. Without his help and encouragement none of this would have been possible. I would also like to thank my other committee members Drs. Martin Okos and Osvaldo Campanella for their suggestions of this work. I would like to thank the Purdue Graduate School for awarding me with the Ross Fellowship and Purdue Agricultural Research for allowing this research to have funding.

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ABSTRACT

Clingenpeel, Shane D. M.S.A.B.E., Purdue University, May 2014. Developing an Unstructured Model to Investigate the Effect of Ethanol on the Glucose and Xylose Cofermentation in *Saccharomyces cerevisiae* 424A (LNH-ST). Major Professor: Nathan Mosier.

Production of bio-ethanol from lignocellulose requires the efficient fermentation of glucose and xylose, even in the presence of inhibitors. The desired product, ethanol itself, will inhibit the fermentation. A further understanding of how ethanol affects the organism is critical to overcoming its inhibition.

This thesis evaluated the effect of ethanol on the cofermentation of glucose and xylose in two different cases. The first case had an unstructured model created for *Saccharomyces cerevisiae* 424A (LNH-ST), a genetically modified strain of yeast capable of cofermenting glucose and xylose. The differential equations were based around sugar consumption, and the product yields were investigated to see how each were affected by ethanol. Results show that ethanol has a significant impact on all xylose product yields except for cell growth and xylitol.

The second case compared the specific xylose consumption rates at increasing ethanol concentrations of *S. cerevisiae* 424A (LNH-ST) and two adapted strains. The ethanol adapted strain performed better, and it produced ethanol at a higher yield.
CHAPTER 1. INTRODUCTION

1.1 Research Motivation

The dependence on fossil fuels has resulted in research toward alternative means of fuel production. One promising biofuel is ethanol. The production of ethanol via fermentation from glucose is a well-known process that has been used for years in industry. The main source of glucose is corn starch or sugar cane, with corn being the main source in the United States, however use of these plants for ethanol production does not seem sustainable (IEA 2007).

It would be preferable to be able to use a non-food plant source for ethanol production. Agricultural waste and dedicated energy crops (both herbaceous and woody) also called lignocellulose, has arisen as a promising feedstock for biofuel production. Lignocellulose consists of lignin, cellulose, and hemicellulose. Each of these are structurally complicated, and must be broken down through pre-treatment in order to access the fermentable sugars. Cellulose is a large polymer of glucose, and these bonds will be broken during the pre-treatment process allowing the glucose to be utilized. Similarly, hemicellulose will break down and release mainly xylose, and lignin will break down to release phenolics. The pre-treatment process will result in roughly 44% glucose, 30% xylose, and 26% phenolics by weight (Galbe and Zacchi 2012; Saha 2003).
Use of lignocellulosic biomass as a fuel source does have its drawbacks. First, *Saccharomyces cerevisiae* is unable to ferment xylose naturally, and effective fermentation of both glucose and xylose is necessary for lignocellulosic biomass to be a viable source for ethanol production. Fortunately, strains of *S. cerevisiae* have been genetically altered in order to utilize xylose in the fermentation process (Demeke et al. 2013; Ho et al. 2000; Ho et al. 1998; Karhuma et al. 2007). Second, lignin cannot be utilized in the fermentation process, so the cellulose and hemicellulose must be separated from the lignin. Lastly, the pretreatment process will result in production of acetic acid, furfural, and hydroxymethylfurfural (HMF) (Klinke et al. 2004). Each of these byproducts are inhibitory to the fermentation of the sugars and production of ethanol, especially from xylose (Casey et al. 2010; Warner 2006), and their removal would increase the cost of the overall process.

Along with these inhibitors from pretreatment, relatively low concentrations of ethanol will inhibit the production of more ethanol from xylose (Athmanathan et al. 2011). As ethanol is the key product of the fermentation, high ethanol concentrations are desired. Knowledge of how each inhibitor affects the fermentation can give insight on how best to combat their inhibitive properties, however this work will focus on ethanol inhibition.

### 1.2 Organization of Thesis

The remainder of this thesis is divided into four chapters. Chapter 2 reviews the current literature on the development of genetically modified microorganisms for glucose/xylose cofermentation and a more detailed review of the development of the organism examined in this thesis, *S. cerevisiae* 424A (LNH-ST). Also reviewed is what has been proposed in
terms of modeling kinetic processes, mainly of yeast, including what has been found for the strain used in this thesis. Chapter 3 presents the development of an unstructured kinetic model for the cofermentation of glucose and xylose in *S. cerevisiae* 424A (LNH-ST). This model is applied to fermentation data to see the effect of ethanol on the product yields. Chapter 4 uses a previously developed model to compare xylose consumption rates of inhibitor resistant strains and the parent strain, *S. cerevisiae* 424A (LNH-ST). Chapter 5 concludes the thesis with a summary of the major findings, and it gives recommendations for future work.
1.3 References


Casey E, Sedlak M, Ho NWY, Mosier NS. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae*. Fems Yeast Research 10(4):385-393.


Warner RE. 2006. Effects of Furfural and HMF on Co-Fermentation of Glucose and Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae*: Purdue University. 104 p.
CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

The main challenge in the production of ethanol from cellulosic sugars is the mixture of 5 and 6 carbon sugars which result from the breakdown of cellulosic biomass, namely glucose and xylose. No microorganism can naturally and efficiently use both sugars to produce ethanol. There are species, such as *Escherichia coli* and *Pichia Stipitis*, that can utilize both sugars, but they are poor ethanol producers. On the other hand *Saccharomyces cerevisiae* and *Zymomonas mobilis* are great at producing ethanol, but lack the ability to uptake xylose. Genetic alteration of these strains in order to make up for what they lack has been investigated.

2.2 Previous Work with Potential Microorganisms

*E. coli* is a bacteria that is capable of using multiple sugars, but it lacks the ethanol fermentation pathway. The enzymes pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* were placed into *E. coli* in order to facilitate the conversion of pyruvate to ethanol, and the resulting strain produced ethanol from glucose as 95% of its fermentative products (Ingram et al. 1987). This strain is also able to ferment xylose to ethanol, as well as arabinose, galactose, and mannose (Beall et al. 1991)
*P. stipitis* is a yeast that is capable of fermenting glucose and xylose under oxygen deprived conditions. However, it will naturally only grow in aerobic conditions which will only allow limited ethanol production. *S. cerevisiae* is able to grow anaerobically, and it has been shown that dihydroorotate dehydrogenase in the cytosol is the key for this (Nagy et al. 1992). This enzyme was inserted into *P. stipitis* in order to facilitate anaerobic growth, which allowed the *P. stipitis* strain to grow on glucose after a significant lag phase and it did not grow well on xylose (Shi and Jeffries 1998).

*Zymomonas mobilis* is able to produce ethanol rapidly and efficiently from glucose, but it is unable to ferment xylose naturally. Operons encoding enzymes for xylose assimilation and the pentose phosphate pathway were constructed and inserted into *Z. mobilis* (Zhang et al. 1995). This recombinant strain was capable of producing ethanol at a rate of 3.45 g/L/hr (Lawford and Rousseau 2002).

The key for ethanol production from xylose for both *Z. mobilis* and *S. cerevisiae* is the production of glycolysis intermediates from xylose via the pentose phosphate pathway. However, *Z. mobilis* uses a different glycolytic pathway than *S. cerevisiae*. *Z. mobilis* utilizes the Entner-Doudoroff mechanism (Swings and Deley 1977) whereas *S. cerevisiae* uses the, more common, Embden-Meyerhof-Parnas pathway. The Enter-Doudoroff pathway (figure 2.1) results in a net production of 1 ATP, 1 NADPH, and 1 NADH from 1 molecule of glucose. This is in contrast to the Embden-Meyerhof-Parnas pathway which has a net production of 2 ATP and 2 NADH from 1 glucose molecule.
2.3 Genetically altering *Saccharomyces cerevisiae*

This thesis is based on a genetically altered strain of *S. cerevisiae*, so the following will be a more detailed account of how *S. cerevisiae* works and what was changed. *S. cerevisiae* is like *Z. mobilis* in the fact that it can quickly and efficiently produce ethanol from glucose, but it cannot naturally utilize xylose. A major problem is that glucose is a six-carbon sugar, whereas xylose is a five-carbon sugar. Therefore xylose is unable to take the same path as glucose. However, five-carbon sugars are metabolized through the pentose phosphate pathway of the cell. An isomer of xylose is xyulose, and *S. cerevisiae* is able to use xyulose-5-phosphate in the pentose phosphate pathway in the presence of ribose-5-phosphate. This calls for a closer look at the workings of the pentose phosphate pathway.
The pentose phosphate pathway can be categorized into the oxidative and non-oxidative sections. The oxidative section is not very pertinent to xylose metabolism, but this is where glucose will be converted to ribulose-5-phosphate (Kruger and von Schaewen 2003). The non-oxidative portion of the pathway picks up directly from the end of the oxidative section. Ribulose-5-phosphate will be converted into either ribose-5-phosphate or xyulose-5-phosphate. Ribose-5-phosphate is made via D-ribulose-5-phosphate-3-epimerase, and xyulose-5-phosphate is made via ribose-5-phosphate-ketol-isomerase. Xyulose-5-phosphate and ribose-5-phosphate combine and react with the enzyme, transketolase to form glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. Glyceraldehyde-3-phosphate can combine with sedoheptulose-7-phosphate via transaldolase and create fructose-6-phosphate and erythrose-4-phosphate. Finally, erythrose-4-phosphate can combine with another xyulose-5-phosphate and transketolase will produce glyceraldehyde-3-phosphate and fructose-6-phosphate (Williams et al. 1978). The production of these products indicates the end of the pentose phosphate pathway.

The pentose phosphate pathway produces glyceraldehyde-3-phosphate and fructose-6-phosphate. Both of these products are intermediates in glycolysis. Therefore if *S. cerevisiae* could be modified to convert xylose into xyulose-5-phosphate, xylose could be fermented to ethanol.

As stated before, wild *P. stipitis* are able to ferment xylose to ethanol, and they do this by converting xylose to xyulose-5-phosphate. Unfortunately, these yeasts have a low ethanol tolerance compared to *S. cerevisiae* and therefore would not be economically
efficient to use in industry. Bacterial species, such as *Bacillus subtilis* and *Actinoplanes missouriensis*, can also convert xylose into xyulose. However, the bacterial species accomplish the conversion in a different way than the yeasts. If one of these methods could be transferred into *S. cerevisiae*, a species could be created that potentially could ferment glucose as well as xylose efficiently.

The wild yeasts, such as *P. stipitis* convert xylose to xylitol via xylose reductase (XR). Xylitol can then be converted to xyulose by the enzyme, xylitol dehydrogenase (XDH). Bacterial species convert xylose directly to xyulose via xylose isomerase (XI). Both the yeast and bacteria then use xylulokinase (XK) to create xyulose-5-phosphate from xyulose. Strains of *S. cerevisiae* have successfully been planted with the XI gene (Amore et al. 1989; Sarthy et al. 1987; Walfridsson et al. 1996), but rapid fermentation and growth were not seen without additional metabolic engineering (Kuyper et al. 2005). However they do not perform as well as the strains utilizing the XR-XDH pathway that will be explained below (Karhumaa et al. 2007).

The XR and XDH enzymes were cloned from *P. stipitis*, which had been determined as the most efficient xylose fermenting yeast of the non-*Saccharomyces* strains, however there are some drawbacks. One major roadblock is found at the reaction from xylitol to xyulose. XDH catalyzes the reversible reaction, but it favors the formation of xylitol rather than xyulose. Also, XR requires NADP as its cofactor, but yeasts do not have an efficient system to convert NAD to NADP. Lastly, many *Saccharomyces* yeast have low XK levels (Xue and Ho 1990). These three signs all point to the fact that even if the
genes were properly cloned into *S. cerevisiae*, the yeast would produce xylitol instead of ethanol. An overview of the last two paragraphs is shown in Figure 2.2.

Dr. Ho et al. (1998), were able to produce a viable strain of *S. cerevisiae*. This was accomplished by cloning and inserting all three genes (XR, XDH, and XK) from *P. stipitis* even though *S. cerevisiae* already contained XK. The expressions of the genes were changed to mimic the expression in the glycolytic genes in the yeast by replacing the regulatory sequences for gene expression (Ho et al. 2000). This allows xylose and glucose to be co-fermented because the XR, XDH, and XK genes are no longer suppressed by the presence of glucose. This is why XK had to be cloned even when *S. cerevisiae* already contained that enzyme. If this had not been done, the genes for xylose
conversion would not be expressed until glucose was completely consumed. This would result in a lag phase when the yeast is switching from glucose to xylose as its carbon source known as a diauxic shift.

In the viable strain, XK was cloned and overexpressed (Ho et al. 1998). *S. cerevisiae* naturally expresses XK in the presence of xylose, but it is inefficient. By overexpressing the XK gene, this yeast strain can effectively ferment xylose to ethanol or use xylose for growth. This overexpression allows the reversible XDH catalyzed reaction to be pulled toward xyulose production. This strain of yeast after some additional modifications, known as *S. cerevisiae* 424A (LNH-ST), is now stable in terms of its genetic changes. Therefore, even after multiple generations of growth without xylose, the strain will still keep its xylose fermenting genes.

Now that a stable xylose fermenting strain of *S. cerevisiae* has been created, it would be ideal to be able to model the fermentation process. In order to do this, previous models need to be looked at beginning with single substrate models of *S. cerevisiae* and then cofermentations in other species.

### 2.4 Previous modeling approaches

Lee et al. (1983) looked at product production from one substrate in *S. cerevisiae*, and they based their model on cell growth. The cell growth equation started with a Monod growth term (Monod 1949), and inhibition terms were added to try and properly model the growth. These inhibition terms were based on the findings that at a certain product concentration the cell growth will cease (Levenspiel 1980).
\[
\frac{dC}{dt} = \left( \frac{\mu_0 \cdot S}{K_m + S} \right) \left( 1 - \frac{P}{P_{max}} \right)^n \left( 1 - \frac{C}{C_{max}} \right)^m
\]

Here \( C \) is cell concentration (g/L), \( S \) is substrate concentration (g/L), \( P \) is ethanol concentration (g/L), \( \mu_0 \) is the maximum specific growth rate (1/hr), \( K_m \) is the substrate saturation constant (g/L), \( P_{max} \) is the ethanol concentration where cell growth stops (g/L), \( C_{max} \) is the cell concentration where cell growth stops (g/L), and \( m \) and \( n \) are the inhibition powers for cells and ethanol, respectively (Lee et al. 1983).

From the cell growth equation, the equations for substrate consumption and ethanol production can be related with yield coefficients

\[
\frac{dS}{dt} = -Y_{S/C} \frac{dC}{dt}
\]
\[
\frac{dP}{dt} = Y_{P/C} \frac{dC}{dt}
\]

where \( Y_{S/C} \) is the yield coefficient for substrate consumption (g substrate/g cell) and \( Y_{P/C} \) is the yield coefficient for product production (g product/g cell). This approach based product production on cell growth which will only work if the substrate will only form one product (Lee et al. 1983) which is not the case in my work.

Another approach by Maiorella et al. (1984) also looks at \( S.\) cerevisiae creating product (ethanol) from one substrate (glucose), however their model is based on ethanol production given as:
\[
\frac{dP}{dt} = \left(\frac{v_{max} \cdot S}{K_m + S}\right) \cdot \left(1 - \frac{P}{P_{max}}\right)^n \cdot C
\]

where \( v_{max} \) is the maximum specific ethanol production rate (\( g/g_{dcw}/h \)) and in this case \( P_{max} \) is the concentration at which ethanol production ceases (\( g/L \)). The cell growth and glucose consumption were then related in the same manner that the Lee et al. (1983) model was:

\[
\frac{dS}{dt} = -Y_{s/p} \frac{dP}{dt}
\]

\[
\frac{dC}{dt} = Y_{c/p} \frac{dP}{dt}
\]

This is a more robust model, as it does not have the constraint of only one product being formed in order to be valid. There is an over-simplification in the fact that they claim that the yield coefficients are constant values throughout the experiment and they don’t model the secondary products, but this is a sufficient model for consumption of only glucose in \( S. cerevisiae \).

Working with a genetically modified strain of \( S. cerevisiae \) capable of fermenting xylose will require the knowledge of how this strain utilizes xylose in order to effectively model the cofermentation. Athmanathan et al. (2011) investigated how the 424A (LNH-ST) strain consumes xylose and how it is affected by ethanol in both the cofermentation with glucose as well as when ethanol is added externally. The xylose consumption was based on the following equation:
\[
- \frac{dX}{dt} = v_{max,\text{specific}} \frac{X}{X + K_S} \left(1 - \frac{P}{P_{max}}\right)^n \cdot C
\]

where \(X\) is the xylose concentration (g/L) and \(v_{max,\text{specific}}\) is the maximum specific xylose consumption rate (g/g_{dcw}/h). Through this investigation it was found that \(v_{max,\text{specific}}\) is 0.621 g/g_{dcw}/h, \(K_S\) is 16.7 g/L, \(n\) is 1.0, and \(P_{max}\) is 121 g/L during cofermentation (Athmanathan et al. 2011).

### 2.5 Casey Model

With the kinetics of glucose and xylose now investigated, a comprehensive model should be put together that models the consumption of both sugars, cell growth, and creation of all products. Dr. Casey (2013) has modeled batch co-fermentations with glucose and xylose to ethanol with the genetically engineered \(S.\ cervisiae\) 424A (LNH-ST). The modeling equations will be shown below with an explanation of each equation (Casey 2013).

#### 2.5.1 Cell Growth

The cell growth has been modeled by Monod kinetics (Monod 1949) and the addition of two inhibitory terms, where one of them is pseudo-inhibitory. The pseudo-inhibitory term is \(1 - \frac{C}{C_{max}}\), which represents the slowing of cell growth as the actual cell growth approaches the carrying capacity (or \(C_{max}\)) of the experiment. The other inhibition term represents the slowing of cell growth due to ethanol concentration. It is well known the
ethanol is inhibitory to *S. cerevisiae* cell growth. Both inhibitory terms used are represented by Levenspiel inhibition terms (Levenspiel 1980).

\[
\frac{dC}{dt} = \mu \cdot C \cdot \left(1 - \frac{C}{C_{\text{max}}} \right) \cdot \left(1 - \frac{P}{P_{\text{max, grow}}} \right)^n
\]

Where \(C\) is cell density (g dry cell/L), \(C_{\text{max}}\) is the maximum cell density (g dry cell/L), \(P\) is ethanol concentration (g/L), \(P_{\text{max, grow}}\) is the lowest ethanol concentration that halts cell growth (g/L), \(\mu\) is the cell growth rate (1/hr), and \(n\) is an exponential inhibition factor. In the case of cell growth, \(n\) is estimated at 1 and \(P_{\text{max, grow}}\) is estimated at 87 g/L (Ghose and Tyagi 1979).

### 2.5.2 Substrate Consumption

In most other glucose or xylose fermentation models, the ethanol production is modeled first and the substrate consumption is then modeled with yield coefficients. The model constructed by Casey took the opposite approach and modeled substrate consumption first. This is allowable because fermentation to ethanol is considered a Type I process (Mosier and Ladisch 2009). Therefore, substrate consumption and product formation are directly related. A model of general substrate consumption with a Levenspiel inhibition term was started with.

\[
\frac{dS}{dt} = -\frac{v_{\text{max}} \cdot S}{K_m + S} \cdot \left(1 - \frac{P}{P_{\text{max}}} \right)^n \cdot C
\]

Where \(S\) is substrate concentration, \(v_{\text{max}}\) is the maximum specific substrate consumption rate, and \(K_m\) is the substrate concentration when \(v_{\text{max}}\) is at half its value.
Now, this model can be applied to glucose and xylose. When applying the model to glucose it can be simplified. The $K_m$ value for glucose is 0.315 g/L (Maiorella et al. 1984) which is very small compared to the concentrations of glucose seen over the batch fermentation process. This means that the denominator of the first term is dictated by the concentration of glucose only. Therefore the glucose concentration terms will then nullify each other and allow the glucose consumption model to be simplified further (steps shown below).

\[
\frac{dG}{dt} = -\frac{v_{max,g} \times G}{K_{m,g} + G} \times \left(1 - \frac{P}{P_{max,g}}\right)^n \times C
\]

\[
\frac{dG}{dt} = -\frac{v_{max,g} \times G}{G} \times \left(1 - \frac{P}{P_{max,g}}\right)^n \times C
\]

\[
\frac{dG}{dt} = -v_{max,g} \times C \times \left(1 - \frac{P}{P_{max,g}}\right)^n
\]

Where $G$ is concentration of glucose (g/L), $v_{max,g}$ is the maximum consumption rate of glucose (g glucose/g cells/hr), and $P_{max,g}$ is the maximum ethanol concentration allowable for glucose consumption (g/L). $P_{max,g}$ has been estimated at 140 g/L for $S.\ cerevisiae$ 424A (LNH-ST) (data unpublished). It should be noted that $P_{max,g}$ is larger than $P_{max,grow}$. This indicates that ethanol has greater inhibition towards cell growth than glucose fermentation. Therefore glucose will continue to be fermented even after cell growth ceases.
For xylose consumption, it has been found that glucose is a competitive inhibitor of xylose uptake. This is believed to be caused by the transport proteins having a higher affinity for glucose over xylose. Reifenberger et al. (1997) determined that the glucose transporters have glucose binding constants of approximately 1mM, whereas the binding constant for xylose is approximately 130 mM (Saloheimo et al. 2007). Therefore, a competitive inhibition term has been added in the xylose consumption model.

\[
\frac{dX}{dt} = -\frac{v_{max,x} \cdot X \cdot C}{K_{m,x} \cdot (1 + \frac{G}{G_{max}}) + X} \cdot \left(1 - \frac{P}{P_{max,x}}\right)^n
\]

Where \(X\) is xylose concentration (g/L), \(v_{max,x}\) is the maximum consumption rate of xylose (g xylose/g cells/hr), \(K_{m,x}\) is the xylose concentration when \(v_{max,x}\) is half its value, \(G_{max}\) is the glucose inhibition constant (g/L), and \(P_{max,x}\) is maximum concentration of ethanol allowable for xylose consumption. \(G_{max}\) was determined to be 10 g/L by observing the glucose and xylose concentration profiles and finding the glucose concentration at which significant xylose consumption is first found (Casey 2013). \(P_{max,x}\) has been estimated at 121 g/L and \(n\) determined as 1 for this yeast strain as stated earlier (Athmanathan et al. 2011). It should be noted that \(P_{max,x}\) is less than \(P_{max,g}\). Therefore xylose consumption is more inhibited by ethanol than glucose consumption. An acetic acid inhibition term was also in the xylose consumption equation (Casey 2013), however that term was not included here as this thesis work does not deal with any initial acetic acid concentration.
2.5.3 Ethanol Formation

As mentioned before, ethanol fermentation has a Type I relationship. This allows the ethanol formation to be expressed via yield coefficients. Ethanol will have different yields depending on the substrate, so two yield coefficients are used in this model.

\[
\frac{dP}{dt} = -\frac{Y_{P/G}}{G} \frac{dG}{dt} - \frac{Y_{P/X}}{X} \frac{dX}{dt}
\]

Where \( Y_{P/G} \) is the yield coefficient for ethanol formed during consumption of glucose (g ethanol/g glucose) and \( Y_{P/X} \) is the yield coefficient for ethanol formed during consumption of xylose (g ethanol/g xylose).

2.5.4 Byproduct Formation

No system results in 100% yield, and this cofermentation is no exception. Ideally, all of the substrate should be consumed and used towards cell growth or production of ethanol. Some xylitol is produced through the conversion of xylose to xyulose, as mentioned previously, and some glycerol is produced through glycolysis. Since both glucose and xylose eventually go to glycolysis the glycerol model will need to account for both substrates, however xylitol is only formed from xylose. Acetic acid will occasionally be produced by the hydrolysis of acetaldehyde which is produced in the step before ethanol is produced. Therefore, acetic acid can be produced by both sugars, and the will need to appear in the acetic acid model. The Type I relationship of this fermentation allows for the use of yield coefficients again.
\[
\frac{dGly}{dt} = -Y_{Gly/G} \frac{dG}{dt} - Y_{Gly/X} \frac{dX}{dt}
\]

\[
\frac{dXyl}{dt} = -Y_{Xyl/X} \frac{dX}{dt}
\]

\[
\frac{dAA}{dt} = -Y_{AA/G} \frac{dG}{dt} - Y_{AA/X} \frac{dX}{dt}
\]

Where Gly is the concentration of glycerol (g/L), Xyl is the xylitol concentration (g/L), AA is the concentration of acetic acid (g/L), \(Y_{Gly/G}\) is the yield coefficient for glycerol formed via glucose consumption (g glycerol/g glucose), \(Y_{Gly/X}\) is the yield coefficient of glycerol formed via xylose consumption (g glycerol/g xylose), \(Y_{Xyl/X}\) is the yield coefficient of xylitol formed via xylose consumption (g xylitol/g xylose), \(Y_{AA/G}\) is the yield coefficient of acetic acid formed via glucose consumption (g acetic acid/g glucose), and \(Y_{AA/X}\) is the yield coefficient of acetic acid formed via xylose consumption (g acetic acid/g xylose). This model is the most ideal so far, as it models the consumption of both glucose and xylose as well as modeling of all possible products.
2.6 References


CHAPTER 3. DEVELOPING AN UNSTRUCTURED MODEL TO EVALUATE ETHANOL EFFECT ON PRODUCT YIELDS FOR THE GLUCOSE/XYLOSE COFERMENTATION OF S. CEREVISIAE 424A (LNH-ST)

3.1 Introduction

The efficient fermentation of xylose at high ethanol concentrations is essential for the use of lignocellulosic biomass as means of bio-ethanol production. Pretreatment of the lignocellulosic biomass releases acetic acid, furfural, and hydroxymethylfurfural (HMF) (Klinke et al. 2004). All three of these have been shown to inhibit the fermentation through decreased sugar consumption and ethanol production rate, especially with regards to xylose (Casey et al. 2010; Lu et al. 2009; Warner 2006).

The presence of ethanol affects the production of additional ethanol, and this strain is no exception. This work focuses on ethanol inhibition as it is the desired product so it will be seen in high concentrations, whereas the pretreatment could be optimized to not produce the previously mentioned inhibitors. The specific xylose consumption rate drops as ethanol concentration increases. This strain has been shown to have a higher specific xylose consumption rate when the ethanol is produced in situ from glucose fermentation rather than adding the ethanol in a single dose (Athmanathan et al. 2011).

Current literature tends to only report how inhibitors affect sugar consumption, ethanol production, and cell growth, however they typically do not report how the byproducts are
affected. They also report these as overall or metabolic yields, which are constant values rather than reporting how the yields change throughout the experiment. In this study an unstructured model was developed in order to see how ethanol affects instantaneous product and byproduct yields over a wide range of ethanol concentrations including concentrations that would typically be seen in industrial applications. An unstructured model was previously created for *S. cerevisiae* 424A (LNH-ST) by Dr. Casey, and that will used as the basis for this model with adaptations (Casey 2013).

3.2 Materials

Yeast extract and Bacto Peptone were obtained from Becton, Dickinson and Co. (Sparks, MD). Reagent-grade glucose and xylose were obtained from Sigma Aldrich (St. Louis, MO). Ethanol (UPS Grade, 200 proof) was obtained from AAPER Alcohol and Chemical Company (Pharmco-AAPER, Shelbyville, KY).

3.3 Methods

3.3.1 Inoculum Preparation

Prior to each experiment, two 2 L flasks each containing 600 mL of a solution consisting of 2% w/v Bacto Peptone, 1% w/v Yeast extract, and 2% w/w glucose were inoculated with *S. cerevisiae* 424A (LNH-ST) stock culture (2% v/v per flask). The glucose was added after sterilization of the YEP (yeast extract and peptone) solution. Sterilization of the flasks occurred in an autoclave at 121°C for 30 minutes. The flasks were incubated aerobically for 24 hours at 29°C and 200 rpm. Following the 24 hour growth period, the
solutions were centrifuged at 3100xg for 5 minutes. The precipitate was resuspended in YEP (24 mL total), and this cell suspension was used to inoculate for the experiment.

3.3.2 Medium Preparation

Each fermentation took place in 300 mL sidearm flasks. 90 g of xylose were added to each flask and an appropriate amount of glucose or ethanol was added. Enough YEP was then added to each flask in order to dissolve the sugar(s). Once dissolved, YEP was added to get 95 mL of solution. All flasks and YEP were sterilized using the previously stated method before use. The amount of glucose added to a cofermentation flask ranged from 0% w/v to 25.3% w/v, and the ethanol added to a xylose only flask ranged from 0% w/v to 11% w/v.

3.3.3 Fermentations

All fermentations were performed micro-aerobically in the 300 mL sidearm flasks that were prepared earlier and in an incubator-shaker at 29°C and 200 rpm. In addition, all experiments were performed in duplicate. The flask that contained no glucose/ethanol had the cell suspension added until the optical density reached 400 Klett units (~ 4 mL). This same volume of cell suspension was added to the remaining flasks beginning with the flask with the highest concentration of glucose/ethanol. This was done because the cells will not start their fermentation in the extreme conditions as quickly as the more dilute solutions, which allows for a more accurate “time 0” reading.

Throughout the fermentation, 1 mL samples were taken for analysis. These samples were then centrifuged at 9300xg for at least 10 minutes. The supernatant of each sample was
removed and kept frozen at -20°C until needed for HPLC analysis. While the samples were spinning down, the optical density of each flask was determined using a Klett unit meter. The Klett unit meter readings were converted to biomass concentrations based on the calibration curve given by (Bera et al. 2010).

3.3.4 HPLC Analysis

Samples taken during the course of fermentation were analyzed by HPLC. The HPLC system consisted of a Waters 2414 refractive index detector (Waters Corp., Milford, Mass.), an Aminex HPX-87H 300 × 7.8 mm column (Bio-Rad Laboratories, Hercules Cal.), and an Alliance Waters 2695 separations module (Waters Corp., Milford, Mass.). Column temperature was maintained at 65°C. The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6 mL/min. Samples were analyzed for glucose, xylose, xylitol, glycerol, ethanol, and acetic acid concentrations (g/L) using peak-area based calibration curves of standards of pure compounds.

3.3.5 Sugar Consumption Models

When the sugars are consumed they are either going to be seen in cell growth, ethanol production, or production of a byproduct. In fact, the products are a direct result of the central carbohydrate catabolic pathways of the cell, making this a type I fermentation (Mosier and Ladisch 2009). Therefore, if the sugar consumption is correctly modeled, all of the products can be modeled directly from those equations. The equations for the consumption of glucose and xylose are taken from Casey (2013) and are below:
\[
\frac{dG}{dt} = -v_{max,G} \cdot C \cdot (1 - \frac{P}{P_{max,G}})^n
\]

\[
\frac{dX}{dt} = -\frac{v_{max,X} \cdot X \cdot C}{K_{m,X} \cdot (1 + \frac{G}{G_{max}}) + X} \cdot (1 - \frac{P}{P_{max,X}})^m
\]

where \( G \) is glucose concentration (g/L), \( X \) is xylose concentration (g/L), \( P \) is ethanol concentration (g/L), \( C \) is cell mass concentration (g/L), \( v_{max,G} \) is the maximum specific consumption rate of glucose (g glucose/g cell/hr), \( P_{max,G} \) is the maximum tolerable ethanol concentration for glucose consumption (g/L), and \( n \) is the ethanol inhibition factor for glucose consumption. \( P_{max,G} \) has been determined to be 140 g/L for this strain (Casey 2013) and \( n \) was assumed to be 1 for this experiment. \( v_{max,X} \) is the maximum specific consumption rate of xylose (g xylose/g cell/hr), \( K_{m,X} \) is the xylose concentration when the consumption rate is at half its maximum (g/L), \( G_{max} \) is the glucose inhibition on xylose consumption (g/L), \( P_{max,X} \) is the maximum tolerable ethanol concentration for xylose consumption (g/L), and \( m \) is the ethanol inhibition factor for xylose consumption. For this strain, \( v_{max,X}, K_{m,X}, P_{max,X}, \) and \( m \) have been determined to be 0.621 g xylose/g cell/hr, 16.7 g/L, 121 g/L, and 1 respectively (Athmanathan et al. 2011). \( G_{max} \) has also been determined to be 10 g/L (Casey 2013).

### 3.3.6 Ethanol Production Model

As stated before, the products are a direct result from the catabolic pathways. Therefore, a certain ratio will go toward ethanol production, and that production can be modeled using yield coefficients. Ethanol can be produced by either glucose or xylose in this
strain, so there must be a different yield coefficient for each sugar. Ethanol production is represented by

\[
\frac{dP}{dt} = -Y_{P/G} \frac{dG}{dt} - Y_{P/X} \frac{dX}{dt}
\]

where \(Y_{P/G}\) is yield coefficient of ethanol formed per glucose consumed (g ethanol/g glucose), and \(Y_{P/X}\) is the yield coefficient of ethanol formed per xylose consumed (g ethanol/g xylose).

### 3.3.7 Byproduct Production Models

The byproducts that we will see in this fermentation are glycerol, xylitol, and acetic acid. These three can all be modeled with the same strategy used in the ethanol equation. Glycerol and acetic acid can be produced by both sugars, but xylitol will only be formed from xylose. This is because xylitol is an intermediate when converting xylose to xyulose-5-phosphate for use in the pentose phosphate pathway (Ho et al. 2000). Therefore, the xylitol model will only depend on xylose consumption.

\[
\frac{dGly}{dt} = -Y_{Gly/G} \frac{dG}{dt} - Y_{Gly/X} \frac{dX}{dt}
\]

\[
\frac{dXyl}{dt} = -Y_{Xyl/X} \frac{dX}{dt}
\]

\[
\frac{dAA}{dt} = -Y_{AA/G} \frac{dG}{dt} - Y_{AA/X} \frac{dX}{dt}
\]
Where $Gly$ is the concentration of glycerol (g/L), $Xyl$ is the concentration of xylitol (g/L), $AA$ is the acetic acid concentration (g/L), $Y_{Gly/G}$ is the yield coefficient for glycerol formed per glucose consumed (g glycerol/g glucose), $Y_{Gly/X}$ is the yield coefficient for glycerol formed per xylose consumed (g glycerol/g xylose), $Y_{Xyl/X}$ is the yield coefficient for xylitol formed per xylose consumed (g xylitol/g xylose), $Y_{AA/G}$ is the yield coefficient for acetic acid formed per glucose consumed (g acetic acid/g glucose), and $Y_{AA/X}$ is the yield coefficient for acetic acid formed per xylose consumed (g acetic acid/g xylose).

3.3.8 Cell Growth Yield

Dr. Casey assumed that the cells should be modeled by logistic growth with inhibition terms. This is a simplified approach as it tends to model the cell growth well, however it does not relate the cell growth to the sugar consumption. When either sugar is consumed, a certain ratio will be allotted to cell growth. Even though cell growth is not a result of the central catabolic pathways, yield coefficients will be used.

$$\frac{dC}{dt} = -Y_{C/G} \frac{dG}{dt} - Y_{C/X} \frac{dX}{dt}$$

Where $Y_{C/G}$ is the yield coefficient for cell growth per glucose consumed (g cell/g glucose) and $Y_{C/X}$ is the yield coefficient for cell growth per xylose consumed (g cell/g xylose).
3.3.9 Parameter Determination

Microsoft Excel was used to numerically evaluate the above equations using the Euler method with a time step of 0.5 hours. The Euler method was used for its simplicity in order to arrive at these initial results. Unknown parameters were estimated using the Solver add-in in Microsoft Excel by minimizing the total sum of square errors (SSE) between the experimental values and predicted values from the model.

Independent datasets were used to determine glucose and xylose dependent parameters. The unknown glucose dependent parameters were investigated using glucose and xylose cofermentations provided by Dr. Wu. These data were provided to me for analysis by Dr. Wu from experiments that she conducted independently prior to this work. Her fermentation procedure was the same as described above with the following differences. The fermentations only lasted 16 hours with samples taken every hour for the first 6 hours and samples taken every two hours after that. Also, the experiments started with no initial ethanol concentration.

The unknown xylose dependent parameters were estimated using the xylose fermentation with externally added ethanol experiments described earlier. The yield coefficients were assumed to be constant over each experimental condition and then later investigated for ethanol dependence. The Solver feature in Microsoft Excel was used as described above.

Since the sugar consumption is most affected by ethanol, each parameter was investigated to see if it was a function of ethanol concentration. As previously mentioned, acetic acid is also inhibitory, but its inhibitory properties were not
investigated. This is because the datasets used for parameter determination had no initial acetic acid concentration. Once the parameters were determined, the predictive model was tested against the cofermentation experiments described earlier and carbon mass balance was used in order to validate the model.

3.4 Results and Discussion

3.4.1 Glucose Parameters

The yield coefficient for cell growth due to glucose consumption was looked at first. The plot of this data did not show a substantial trend (Figure 3.1). When a linear fit was tried, the slope of the line was essentially zero. Each experiment showed that cell growth yield would begin in the upper range (~0.009-0.014) and as the experiment progressed the yield would drop until cell growth ceased. This pattern was seen regardless of the initial ethanol concentration, indicating there is a time dependence on cell growth yield not caused by ethanol. We believe because the cells are grown aerobically before the inoculation, the yeast grows rapidly by using the oxygen that is present initially. Once the oxygen has been depleted the yeast can no longer produce lipids and sterols needed for cell membrane production, and the yeast can no longer grow.

Therefore the cell growth yield coefficient from glucose was taken to have no dependence on ethanol and the average value was used. No significant trend is seen with any yield coefficients based on glucose consumption (data not pictured). All glucose yield coefficients were found using their respective averages (table 3.1).
As a comparison, Casey (2013) estimated her glucose parameters, from the same strain, to be the following: \(1.646 \text{ g cell/g glucose/hr}\) for \(v_{max,G}\), \(0.353 \text{ g ethanol/g glucose}\) for \(Y_{P/G}\), and \(0.111 \text{ g glycerol/g glucose}\) for \(Y_{Gly/G}\). It should be noted that these values were taken from experiments performed in sealed 1L anaerobic fermenters, whereas our experiments were in a micro-aerobic environment. Our flasks had to be opened briefly (less than 10 seconds per sample) in order to retrieve a sample, so the yeast occasionally had access to oxygen. This would explain why \(v_{max,G}\) is larger in this study, and this could also be the reason for the difference in \(Y_{P/G}\) and \(Y_{Gly/G}\) as well.

![Figure 3.1. Cell growth yield per glucose consumed vs. ethanol concentration](image)

### 3.4.2 Xylose Parameters

#### 3.4.2.1 Cell Growth Yield

Figure 3.2 shows the data of cell growth due to xylose consumption. The same trend is seen here as was seen for cell growth due to glucose consumption. There does seem to be a time dependence, however it is not with ethanol. This yield coefficient was taken to have a constant value of \(0.022 \pm 0.019 \text{ g cell/g xylose}\).
Table 3.1. Summary of glucose derived parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v_{\text{max},\text{G}}$</td>
<td>3.305 ± 0.899</td>
<td>g glucose/g cell/hr</td>
</tr>
<tr>
<td>$Y_{C/G}$</td>
<td>0.0087 ± 0.0042</td>
<td>g cell/g glucose</td>
</tr>
<tr>
<td>$Y_{P/G}$</td>
<td>0.425 ± 0.011</td>
<td>g ethanol/g glucose</td>
</tr>
<tr>
<td>$Y_{\text{Gly}/G}$</td>
<td>0.070 ± 0.005</td>
<td>g glycerol/g glucose</td>
</tr>
<tr>
<td>$Y_{\text{AA}/G}$</td>
<td>0</td>
<td>g acetic acid/g glucose</td>
</tr>
</tbody>
</table>

Figure 3.2. Cell growth per xylose consumed vs. ethanol concentration.

3.4.2.2 Ethanol Yield

In order to fit the ethanol yield data (figure 3.3), a piecewise function was used. The yield was essentially constant, and therefore kept as 0.4 when the initial ethanol concentration was less than 70 g/L. After 70 g/L, the data was fit using a Levenspiel inhibition function (Levenspiel 1980) where 115 g/L was the concentration at which
ethanol production would cease, and the inhibition factor being 0.6. In order to have the Levenspiel inhibition function begin at 70 g/L ethanol, the function needed to be shifted. Therefore the $P$ and $P_{max}$ terms in the function had 70 subtracted from them in order to shift the function.

$$Y_{P/X} = \begin{cases} 
0.4 & \text{when } P < 70 \\ 
0.4 \left(1 - \frac{P - 70}{45}\right)^{0.6} & \text{when } 70 \leq P \leq 115 \\ 
0 & \text{when } P > 115 
\end{cases}$$

![Ethanol yield due to xylose consumption vs. initial ethanol concentration](image)

Figure 3.3. Ethanol yield due to xylose consumption vs. initial ethanol concentration

Casey (2013) found that the ethanol yield from xylose consumption is 0.405 g ethanol/g xylose. This value is almost identical to the value we have predicted when the yield coefficient is not inhibited by ethanol. *S. cerevisiae* strains that use the xylose isomerase (XI) pathway have reported ethanol yields of 0.43 ± 0.03 (Karhumaa et al. 2007) and 0.46 ± 0.00 g ethanol/g xylose (Demeke et al. 2013).
3.4.2.3 Glycerol Yield

The data for the glycerol yield from xylose consumption was also modeled with a piecewise function (figure 3.4). There is a sharp drop in the 0-20 g/L ethanol range, and that section is modeled with a linear function. It should be noted that no data were obtained between 0-20 g/L ethanol, but this region needed to be modeled. Therefore a linear function may not accurately describe the glycerol yield from xylose consumption in that range. When the ethanol concentration is greater than 20 g/L, the data seemed to show a similar drop as seen in the ethanol yield. Therefore, this portion of the data was modeled with a Levenspiel inhibition function with glycerol production ceasing at 103 g/L ethanol, and the inhibition factor being 0.2. The same strategy used in shifting the Levenspiel inhibition function for ethanol yield was used here as well.

\[ Y_{\text{Gly}}/X = \begin{cases} 
-9.28 \times 10^{-4}P + 0.0615 & \text{when } P < 20 \\
0.0442 \left(1 - \frac{P - 20}{83}\right)^{0.2} & \text{when } 20 \leq P \leq 103 \\
0 & \text{when } P > 103 
\end{cases} \]

Casey (2013) estimated that \( Y_{\text{Gly}}/X \) was 0.030 g glycerol/g xylose. According to our glycerol yield equation, that production wouldn’t be seen until 91 g/L ethanol. This difference could again be from the difference of the micro-anaerobic environment in which our experiments were performed. The XI strains have reported glycerol yields of 0.07 ± 0.02 (Karhumaa et al. 2007) and 0.06 ± 0.00 g glycerol/g xylose (Demeke et al. 2013). These values were reported from experiments where the highest the ethanol concentration ever became was 16.1 g/L. These values are similar for the 424A (LNH-ST) strain under the same conditions.
Figure 3.4. Glycerol yield from xylose consumption vs. initial ethanol concentration.

### 3.4.2.4 Xylitol Yield

Xylitol yield from xylose consumption is seen to be essentially constant, but the yield rises after 100 g/L ethanol (figure 3.5). There is not enough data after 100 g/L to accurately determine how that portion would be modeled, and the cells are struggling to function as they have already stopped growing at 87 g/L ethanol (Ghose and Tyagi 1979). Therefore, the xylitol yield will be assumed constant and the average value will be used which is 0.0874 ± 0.0085 g xylitol/g xylose.

A value of 0.118 g xylitol/g xylose was reported from Casey (2013). The anaerobic condition of their experiment may explain the discrepancy between the values, as the brief exposure to oxygen had a large effect on $v_{max,G}$. The XI strains report xylitol yields of 0.04 ± 0.02 (Karhumaa et al. 2007) and 0.04 ± 0.00 (Demeke et al. 2013). The lower values for the XI strain are expected given that XI provides the yeast with an alternative metabolic pathway for converting xylose to xyulose than the 424A (LNH-ST) strain uses.
Figure 3.5. Xylitol yield from xylose consumption vs. initial ethanol concentration.

3.4.2.5 Acetic Acid Yield

Lastly, the data for acetic acid yield from xylose consumption shows no acetic acid production until around 40 g/L ethanol (figure 3.6). The non-zero data was modeled with a linear function, and all yield values are zero when the ethanol concentration is less than the line’s x-intercept.

\[
Y_{AA/X} = \begin{cases} 
0 & \text{when } P < 38 \\
0.0022P - 0.083 & \text{when } P \geq 38
\end{cases}
\]

3.4.3 Model Validation

A carbon mass balance for xylose consumption was used to check if the model was reasonable. This was done by adding all of the yield coefficients from xylose consumption at various ethanol concentrations and theoretically their sum should be 1. A yield coefficient for carbon dioxide was added to the mass balance as one mole of carbon dioxide is produced for every mole of ethanol or acetic acid. It should be noted, there
was no mass conservation constraint for the model, but the mass balance is able to account for > 90% of the carbon from xylose when the ethanol is no greater than 85 g/L (table 3.2). This result gives us more confidence in the proposed yield coefficients.

Figure 3.6. Acetic acid yield from xylose consumption vs. initial ethanol concentration.

With all the parameters known, the cofermentation experiments were tested against the proposed model. Figure 3.7 shows the actual data and the model prediction from a cofermentation experiment with approximate initial concentrations of 138 g/L glucose, 90 g/L xylose, and 4.22 g/L cells. The model predicts that glucose is consumed too quickly, but it has the xylose being consumed too slowly. With the model predicting slower consumption of xylose, this causes the model to predict the ethanol concentration too low.

The crutch of this model was to accurately predict sugar consumption. Without an accurate prediction of sugar consumption all of the products will also be modeled incorrectly. As can be seen in figure 3.7, the model does not match up with the actual
data for sugar consumption. Similarly, the sugar consumption was not properly modeled for the other cofermentation conditions.

Table 3.2. Carbon mass balance from xylose to products.

<table>
<thead>
<tr>
<th>[Ethanol] g/L</th>
<th>Y_{CX}</th>
<th>Y_{P/X}</th>
<th>Y_{Gly/X}</th>
<th>Y_{Xyl/X}</th>
<th>Y_{AA/X}</th>
<th>Y_{CO2/X}</th>
<th>Total Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.022</td>
<td>0.4</td>
<td>0.0615</td>
<td>0.0874</td>
<td>0</td>
<td>0.3843</td>
<td>0.9552</td>
</tr>
<tr>
<td>20</td>
<td>0.022</td>
<td>0.4</td>
<td>0.0442</td>
<td>0.0874</td>
<td>0</td>
<td>0.3843</td>
<td>0.9379</td>
</tr>
<tr>
<td>40</td>
<td>0.022</td>
<td>0.4</td>
<td>0.0413</td>
<td>0.0874</td>
<td>0.005</td>
<td>0.3876</td>
<td>0.9433</td>
</tr>
<tr>
<td>60</td>
<td>0.022</td>
<td>0.4</td>
<td>0.0375</td>
<td>0.0874</td>
<td>0.049</td>
<td>0.4170</td>
<td>1.0129</td>
</tr>
<tr>
<td>80</td>
<td>0.022</td>
<td>0.3440</td>
<td>0.0321</td>
<td>0.0874</td>
<td>0.093</td>
<td>0.3925</td>
<td>0.9710</td>
</tr>
<tr>
<td>100</td>
<td>0.022</td>
<td>0.2069</td>
<td>0.0193</td>
<td>0.0874</td>
<td>0.137</td>
<td>0.2901</td>
<td>0.7626</td>
</tr>
<tr>
<td>120</td>
<td>0.022</td>
<td>0.0</td>
<td>0</td>
<td>0.0874</td>
<td>0.181</td>
<td>0.1206</td>
<td>0.4110</td>
</tr>
</tbody>
</table>

In order to see if the predicted product yields were reliable the cell growth, ethanol production, and byproduct production models needed to be evaluated when the sugar consumption was modeled correctly. Using the Solver add-in tool, the two sugar consumption equations were optimized for this experiment, but the predicted product yields were not changed. This allowed for the sugars to be accurately fit, and therefore we could evaluate if the product equations fit better.

The SSE for each function was evaluated with the proposed model and after the sugar consumption parameters had been optimized (table 3.3). The fit of each product was better once the sugars were modeled properly except for acetic acid. This result shows that the approach used was successful, however the yield coefficient for acetic acid from xylose consumption needs reevaluated or acetic acid production is unable to be modeled by this equation.
Figure 3.7. Model prediction of a cofermentation experiment compared to actual data. (Top) Sugars and Ethanol and (bottom) Cell mass and byproducts.
Table 3.3. Comparing individual model SSE before and after optimal sugar consumption fit.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Xylose</th>
<th>Cell Growth</th>
<th>Ethanol</th>
<th>Glycerol</th>
<th>Xylitol</th>
<th>Acetic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SSE_{before}</strong></td>
<td>572.4</td>
<td>681.7</td>
<td>1.76</td>
<td>455.9</td>
<td>2.94</td>
<td>3.71</td>
<td>24.31</td>
</tr>
<tr>
<td><strong>SSE_{after}</strong></td>
<td>380.4</td>
<td>8.78</td>
<td>1.61</td>
<td>205.8</td>
<td>1.30</td>
<td>0.70</td>
<td>45.41</td>
</tr>
</tbody>
</table>

### 3.5 Conclusions

An unstructured model was developed in order to observe how ethanol affects all product yields and cell growth of *S. cerevisiae* 424A (LNH-ST) for glucose and xylose consumption. The model was able to show the ethanol dependence for each product yield, and it showed that ethanol is not the key factor for cell growth.

The sugar consumption models do not accurately match the experimental data over all concentrations of ethanol. This causes issues as each product equation is directly related to sugar consumption. When the sugar consumption was optimally fit the product models fit better (except for acetic acid), letting us know that most of our product yields are reliable.

### 3.6 Recommendations

Ideally, the yield parameters from glucose consumption should be estimated from fermentations that contain only glucose. We did not have access to such data, nor did we perform these experiments. In addition, Dr. Wu’s data only contained four fermentations
with the highest ethanol concentration reaching 60 g/L, so the data we were able to use was sparse.

The sugar consumption equations need to be evaluated in order to accurately model consumption over the entire range of ethanol conditions. All of the yields should also be looked at for inhibition by any other factors (acetic acid, glycerol, xylitol, etc.). This may give some insight to why the acetic acid model is poor, and what the cell growth is dependent upon.
3.7 References


Casey E, Sedlak M, Ho NWY, Mosier NS. 2010. Effect of acetic acid and pH on the co-fermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae. Fems Yeast Research 10(4):385-393.


Warner RE. 2006. Effects of Furfural and HMF on Co-Fermentation of Glucose and Xylose to Ethanol by Recombinant Saccharomyces cerevisiae: Purdue University. 104 p.
CHAPTER 4. COMPARISON OF XYLOSE CONSUMPTION RATES IN ADAPTED STRAINS AND PARENT STRAIN SACCHAROMYCES CEREVISIAE 424A (LNH-ST).

4.1 Introduction

Ethanol production from the fermentation of cellulosic biomass is becoming well understood. Xylose makes up 30-40% dry weight of the fermentable sugars in cellulosic biomass (Galbe and Zacchi 2012; Saha 2003), making its fermentation an important factor in the ethanol production. Many strains of Saccharomyces cerevisiae have been developed that are able to co-ferment glucose and xylose effectively (Demeke et al. 2013; Ho et al. 1998; Karhumaa et al. 2007), however they are susceptible to the inhibitory effects of ethanol and acetic acid.

Although ethanol is the byproduct that is desired, it has an inhibitory effect on the production of more ethanol via xylose fermentation (Athmanathan et al. 2011). In a starch-based process, ethanol is not cost effective to distill if the concentration is less than 8% w/v (Galbe and Zacchi 2012), therefore it is necessary to have a microorganism capable of surpassing this concentration.

In order to make the sugars fermentable, the biomass must be pre-treated (Galbe and Zacchi 2012). This pre-treatment will result in the creation of acetic acid at the concentration of 3-5% dry weight (Lu et al. 2009). Acetic acid has also been shown to
have an inhibitory effect upon xylose fermentation (Casey et al. 2010), and it also becomes a byproduct of the xylose fermentation of the 424A (LNH-ST) strain at higher ethanol concentrations, specifically greater than 3.8% w/v (as shown in the previous chapter). The removal of acetic acid would increase the cost of the overall process, so it would be desirable to have a strain of microorganism that is adapted to acetic acid.

In order to address these inhibitors, *Saccharomyces cerevisiae* 424A (LNH-ST), capable of glucose/xylose cofermentation to ethanol, were continuously cultured under selective pressure in order to develop derivative strains adapted to ethanol and acetic acid (Wu 2013). One strain was adapted to ethanol while fed only xylose, and one strain was adapted to acetic acid while fed only xylose.

This work investigates the performance of the two adapted strains under increasing ethanol concentrations compared to the parent strain without any initial acetic acid present. The performance will be measured by calculating the apparent maximum specific xylose consumption rate and the specific xylose consumption rate at the point in which sugar consumption has reached 50% completion for that experiment. The 424A (LNH-ST) strain has been shown to produce more ethanol from xylose when the ethanol is first produced by the glucose in the cofermentation compared to if the equivalent amount of ethanol is added in externally at the beginning of the xylose fermentation as can be seen in figure 4.1 (Athmanathan et al. 2011). Both fermentation conditions were investigated to see if the same trend was followed in the adapted strains.
Figure 4.1. Sample fermentation profiles for S. cerevisiae 424A (LNH-ST) for (top) externally added ethanol of 70 g/L and (bottom) equivalent amount of ethanol generated from glucose.
4.2 Materials

Yeast extract and Bacto Peptone were obtained from Becton, Dickinson and Co. (Sparks, MD). Reagent-grade glucose and xylose were obtained from Sigma Aldrich (St. Louis, MO). Ethanol (UPS Grade, 200 proof) was obtained from AAPER Alcohol and Chemical Company (Pharmco-AAPER, Shelbyville, KY).

4.3 Methods

4.3.1 Inoculum Preparation

Prior to each experiment, two 2 L flasks each containing 600 mL of a solution consisting of 2% w/v Bacto Peptone, 1% w/v Yeast extract, and 2% w/w glucose were inoculated with the appropriate strain stock culture (2% v/v per flask). The glucose was added after sterilization of the blank YEP (yeast extract and peptone) solution. Sterilization of the flasks occurred in an autoclave at 121°C for 30 minutes. The flasks were incubated aerobically for 24 hours at 29°C and 200 rpm. Following the 24 hour growth period, the solutions were centrifuged at 3100xg for 5 minutes. The precipitate was resuspended in blank YEP (24 mL total), and this cell suspension was used to inoculate for the experiment.

4.3.2 Medium Preparation

Each fermentation took place in 300 mL sidearm flasks. 90 g of xylose were added to each flask and an appropriate amount of glucose or ethanol was added. Enough YEP was then added to each flask in order to dissolve the sugar(s). Once dissolved, YEP was
added to get 95 mL of solution. All flasks and YEP were sterilized using the previously stated method before use. The amount of glucose added to a cofermentation flask ranged from 0% w/v to 25.3% w/v, and the ethanol added to a xylose only flask ranged from 0% w/v to 11% w/v.

4.3.3 Fermentations

All fermentations were performed micro-aerobically in the 300 mL sidearm flasks that were prepared earlier and in an incubator-shaker at 29°C and 200 rpm. In addition, all experiments were performed in duplicate. The flask that contained no glucose/ethanol had the cell suspension added until the optical density reached 400 Klett units (~ 4 mL). This same volume of cell suspension was added to the remaining flasks beginning with the flask with the highest concentration of glucose/ethanol. This was done because the cells will not start their fermentation in the extreme conditions as quickly as the more dilute solutions, which allows for a more accurate “time 0” reading.

Throughout the fermentation, 1 mL samples were taken for analysis. These samples were then centrifuged at 9300xg for at least 10 minutes. The supernatant of each sample was removed and kept frozen at -20°C until needed for HPLC analysis. While the samples were spinning down, the optical density of each flask was determined using a Klett unit meter. The Klett unit meter readings were converted to biomass concentrations based on the calibration curve given by (Bera et al. 2010).
4.3.4 HPLC Analysis

Samples taken during the course of fermentation were analyzed by HPLC. The HPLC system consisted of a Waters 2414 refractive index detector (Waters Corp., Milford, Mass.), an Aminex HPX-87H 300 × 7.8 mm column (Bio-Rad Laboratories, Hercules Cal.), and an Alliance Waters 2695 separations module (Waters Corp., Milford, Mass.). Column temperature was maintained at 65°C. The mobile phase was 5 mM H$_2$SO$_4$ at a flow rate of 0.6 mL/min. Samples were analyzed for glucose, xylose, xylitol, glycerol, ethanol, and acetic acid concentrations (g/L) using peak-area based calibration curves of standards of pure compounds.

4.3.5 Parameter Determination

Apparent maximum xylose consumption rates ($v_{\text{max,specific,app}}$) and xylose consumption rates at 50% of consumed xylose ($v_{50}$) were found by using the kinetic model proposed by Casey (2013). We removed the ethanol inhibition term from the xylose differential equation. This allowed for a much better fit at larger ethanol concentrations which will allow for more accurate findings. The model was used to generate data and a total SSE was calculated based on the experimental data. The Solver Add-In tool in Microsoft Excel was then used to minimize the total SSE by optimizing all model parameters, in which $v_{\text{max,specific,app}}$ was included. The data generated from the optimized model was used to find $v_{50}$. 
4.4 Results

4.4.1 Fermentations with Xylose and Externally Added Ethanol

The results of these fermentations can be seen in figure 4.2. Both adapted strains consume xylose at a higher rate over the entire range of initial ethanol concentrations (table 4.1). The ethanol adapted strain consumed xylose at the highest rate when the ethanol concentration was no greater than 80 g/L. Once the ethanol concentration surpassed that, the acetic acid adapted strain consumed xylose the quickest. The parent strain always consumed xylose at the slowest rate. The same pattern is seen for the xylose consumption rates when 50% of the xylose had been consumed.

4.4.2 Fermentation with Xylose and Glucose

The same trend is seen here where both adapted strains perform better than the parent strain, with the ethanol adapted strain performing best for all conditions. Both the ethanol adapted strain and the acetic acid adapted strain show a significant improvement in $v_{max,specific,app}$ and $v_{50}$ throughout all ethanol conditions from fermented glucose (figure 4.3) except for the most extreme conditions (> 90 g/L). Under those conditions, all strains were heavily inhibited and the adapted strains showed a less improved $v_{max,specific,app}$ and $v_{50}$ (table 4.2).

4.5 Discussion

The 424A (LNH-ST) and acetic acid adapted strain both have lower consumption rates compared to the cofermentation results. This is to be expected as the cells do not have
time to adjust to the growing concentration of ethanol (Athmanathan et al. 2011; Damore et al. 1990; Damore and Stewart 1987; Monteiro and Sa-Correia 1998; Rosa and Sacorreia 1992). The ethanol adapted strain shows an increase in $v_{\text{max, specific, app}}$ and $v_{50}$ compared to the cofermentation experiment. This boost in xylose consumption rate in conjunction with the drop of the acetic acid adapted strain, makes the ethanol adapted strain significantly better when initial ethanol is <70 g/L. However, the acetic acid adapted strain outperforms the ethanol adapted strain when the initial ethanol concentration is >80 g/L.

During the fermentations with externally added ethanol, the acetic acid adapted strain saw an increase in $v_{\text{max, specific, app}}$ and $v_{50}$ over the range of 70-90 g/L initial ethanol. This phenomenon could be caused by two things. First, acetic acid becomes the major byproduct of xylose consumption once the ethanol concentrations are greater than 80 g/L. The selective growth of that strain could allow it to dispose of some acetic acid over that range, lessening its inhibitive qualities. Second, the kinetic model begins to show a poor fit when the initial ethanol concentration is 80 g/L or greater which could result in incorrect $v_{\text{max, specific, app}}$ values. This could explain the odd uptrend that is seen.

Along with xylose consumption rates, the product yields for each strain were evaluated when no ethanol/glucose was initially present (table 4.3). The acetic acid adapted strain produces less xylitol, but it produces more glycerol making the ethanol yield essentially the same as the parent strain. However, the ethanol resistant strain produces less xylitol and glycerol, but it produces more ethanol compared to the parent. These findings
indicate that the ethanol adapted strain became more efficient at producing ethanol rather than just having increased xylose uptake.

Table 4.1. Comparison of the apparent maximum specific xylose consumption rates of all three strains in the externally added ethanol fermentations.

<table>
<thead>
<tr>
<th>Initial Ethanol Concentration (g/L)</th>
<th>Vmax,spec,app of 424A (LNH-ST) (g/g_dcw/hr)</th>
<th>Percent Increase of the Ethanol Adapted Strain</th>
<th>Percent Increase of the Acetic Acid Adapted Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.730</td>
<td>122.9</td>
<td>51.4</td>
</tr>
<tr>
<td>20</td>
<td>0.554</td>
<td>137.5</td>
<td>56.9</td>
</tr>
<tr>
<td>40</td>
<td>0.318</td>
<td>230.3</td>
<td>97.4</td>
</tr>
<tr>
<td>50</td>
<td>0.251</td>
<td>208.7</td>
<td>60.4</td>
</tr>
<tr>
<td>60</td>
<td>0.155</td>
<td>240.6</td>
<td>91.4</td>
</tr>
<tr>
<td>70</td>
<td>0.087</td>
<td>197.5</td>
<td>102.3</td>
</tr>
<tr>
<td>80</td>
<td>0.088</td>
<td>138.2</td>
<td>128.6</td>
</tr>
<tr>
<td>90</td>
<td>0.060</td>
<td>160.5</td>
<td>232.1</td>
</tr>
<tr>
<td>100</td>
<td>0.044</td>
<td>163.5</td>
<td>267.1</td>
</tr>
<tr>
<td>110</td>
<td>0.014</td>
<td>10.7</td>
<td>564.4</td>
</tr>
</tbody>
</table>

Table 4.2. Comparison of the apparent maximum specific xylose consumption rates of all three strains in the cofermentations.

<table>
<thead>
<tr>
<th>Ethanol Produced from Glucose (g/L)</th>
<th>Vmax,spec,app of 424A (LNH-ST) (g/g_dcw/hr)</th>
<th>Percent Increase of the Ethanol Adapted Strain</th>
<th>Percent Increase of the Acetic Acid Adapted Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.736</td>
<td>94.5</td>
<td>46.5</td>
</tr>
<tr>
<td>20</td>
<td>0.612</td>
<td>76.5</td>
<td>51.3</td>
</tr>
<tr>
<td>40</td>
<td>0.500</td>
<td>67.8</td>
<td>46.4</td>
</tr>
<tr>
<td>50</td>
<td>0.438</td>
<td>59.6</td>
<td>55.7</td>
</tr>
<tr>
<td>60</td>
<td>0.373</td>
<td>49.3</td>
<td>27.7</td>
</tr>
<tr>
<td>70</td>
<td>0.211</td>
<td>107.3</td>
<td>74.1</td>
</tr>
<tr>
<td>80</td>
<td>0.148</td>
<td>52.3</td>
<td>30.8</td>
</tr>
<tr>
<td>90</td>
<td>0.113</td>
<td>65.3</td>
<td>20.0</td>
</tr>
<tr>
<td>100</td>
<td>0.105</td>
<td>24.0</td>
<td>9.7</td>
</tr>
<tr>
<td>110</td>
<td>0.083</td>
<td>15.1</td>
<td>9.3</td>
</tr>
</tbody>
</table>
Figure 4.2. (top) Apparent maximum specific xylose consumption rate and (bottom) specific xylose consumption rate after 50% xylose had been consumed for a range of initial added ethanol concentrations.
Figure 4.3. (Top) Apparent maximum specific xylose consumption rates and (bottom) specific xylose consumption rates when 50% xylose has been consumed over a range of ethanol produced from glucose fermentation.
Table 4.3. Metabolic yields (g/g xylose) for each strain with no initial ethanol/glucose.

<table>
<thead>
<tr>
<th></th>
<th>Ethanol</th>
<th>Xylitol</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>0.398 ± 0.004</td>
<td>0.081 ± 0.009</td>
<td>0.055 ± 0.002</td>
</tr>
<tr>
<td>AA Adapted</td>
<td>0.398 ± 0.004</td>
<td>0.065 ± 0.004</td>
<td>0.079 ± 0.006</td>
</tr>
<tr>
<td>EtOH Adapted</td>
<td>0.437 ± 0.035</td>
<td>0.044 ± 0.009</td>
<td>0.039 ± 0.004</td>
</tr>
</tbody>
</table>

Demeke et al. (2013) have developed an industrial strain of *S. cerevisiae* that utilizes xylose isomerase to allow the yeast to be able to ferment xylose. In an experiment with only xylose and no initial ethanol, that strain has a maximum xylose consumption rate of 1.10 g/g<sub>dcw</sub>/hr and an ethanol yield of 0.46 g xylose/g ethanol (Demeke et al. 2013). Under similar conditions, the ethanol resistant strain we are working with shows a maximum xylose consumption rate of 1.53 ± 0.14 g/g<sub>dcw</sub>/hr and an ethanol yield of 0.44 ± 0.03 g xylose/g ethanol.

### 4.6 Conclusions

The two adapted strains consumed xylose at a higher rate over the entire range of the externally added ethanol fermentations except for the ethanol adapted strain at 110 g/L ethanol. They also consumed xylose at a significantly higher rate during the xylose-glucose cofermentation when the ethanol produced from glucose fermentation was no greater than 70 g/L. The ethanol adapted strain performed better during the externally added ethanol fermentation, while the other two strains preferred the cofermentation conditions. Testing should now be done under increasing conditions of acetic acid to see how it affects all three strains.
4.7 References


Casey E, Sedlak M, Ho NWY, Mosier NS. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae. Fems Yeast Research 10(4):385-393.


Wu C-L. 2013. Systems Biology Approaches to Determine the Factors for Acetic Acid Resistance by Comparing *S. cerevisiae* 424A (LNH-ST) and 424A (LNH-ST)-AAR during Glucose/Xylose Co-Fermentation: Purdue University. 146 p.
CHAPTER 5. CONCLUSION

5.1 Summary of Chapters

Chapter 1 explained why production of ethanol from lignocellulose is a viable alternative to typical methods of bioethanol production. It also laid out the main hindrances to the fermentation of the sugars in lignocellulose. Ethanol was identified as the inhibitor that would be the focus of this thesis.

Chapter 2 discussed the previous attempts on creating a microorganism capable of efficiently fermenting both glucose and xylose to ethanol, with the major focus on the creation of *S. cerevisiae* 424A (LNH-ST). Chapter 2 also reviews models that have been developed for glucose fermentation in yeast, as well as the cofermentation of glucose and xylose.

Chapter 3 presents the development of an unstructured kinetic model in order to evaluate the effect that ethanol has on product yields during the cofermentation of glucose and xylose in *S. cerevisiae* 424A (LNH-ST). The resulting model was validated with a carbon mass balance along with testing against real fermentation data. The data revealed that the sugar consumption equations do not fit as well as we desire. By optimizing the sugar consumption fit, the product models all fit better except for acetic acid. This allows
us to conclude that the proposed yield parameters (not acetic acid) are a good estimate of how ethanol affects them throughout the cofermentation.

Chapter 4 compared the xylose consumption rates of *S. cerevisiae* 424A (LNH-ST) and two adapted strains and how they were affected by ethanol. The ethanol resistant strain showed the highest xylose consumption at almost all conditions. Further analysis showed that the ethanol resistant strain has a greater ethanol yield from xylose, as well as a lower yield of glycerol and xylitol.

### 5.2 Conclusions and Recommendations for Future Work

The modeling approach allowed me to quantify how ethanol affects coproduct yields in the 424A (LNH-ST) strain during xylose fermentation. The validation of this model through the mass balance and cofermentation data show that these yields are reliable. The model used in chapter 4 allowed us to see quantify how ethanol affects xylose consumption rates in the 424A (LNH-ST) and two adapted strains. This model was also able to show us that the coproduct yields of each strain are different, indicating that the ethanol adapted strain is also better at producing ethanol.

Through the model validation it was shown that the sugar consumption equations did not fit well to cofermentation data. All of the product modeling equations rely directly on the sugar consumption equations, therefore it is key that they are modeled correctly. The approach I would take to rectify this involves getting more data to determine the Michaelis-Menten kinetics values for both sugar consumption equations ($v_{max}$, $K_m$, and $G_{max}$). The approach that I used in the modeling chapter involved relying on previous
findings for these values, but the way they determined their values was different than what I did. This could be an indication to why the sugar consumption was modeled poorly. If that did not solve the issue, I would then suggest discarding those modeling equations and creating new ones.

Once the sugars are modeled properly attention should be directed toward the acetic acid equation. As shown in chapter 3, it had a worse fit when the fit of sugar consumption was optimized. I would suggest gathering more data to see if that would change the yield coefficients for acetic acid and result in a better fit. If the production of acetic acid is still fit poorly, I would look into using something other than just yield coefficients to see if those were better able to describe the production.

The results from chapter 4 show that not only do the adapted strains consume xylose faster than the parent strain under increasing ethanol concentrations, but the product yields of the adapted strains are different from each other and the parent strain, even with no ethanol present (table 4.3). This indicates a genetic change in each of the adapted strains. As an example, xylitol yield is lower in both adapted strains which would seem to indicate a greater expression of the enzyme xyulokinase (XK), as a greater XK expression would drive more xylitol into xyulose-5-phosphate. The expression of this gene, and any other genes in the biochemical pathway of converting xylose to xyulose should be evaluated. This could give insight to the genetic changes that occurred during selective growth of the adapted strains.
VITA
VITA

Shane Clingenpeel was born in 1989 in the small town of Burlington, IN. He graduated from Carroll Jr./Sr. High School in Flora, IN in 2007. In 2012, he received his B.S. in Agricultural and Biological Engineering with a focus in Biological and Food Process Engineering with a mathematics minor from Purdue University. He was the first one in his family to attend and receive a degree from a four year university. In order to combine his passion for mathematics and biological systems, he continued with graduate school at Purdue University. In April of 2014 he moved to Rochester, MN to join Kerry Flavours & Ingredients as a Production Supervisor with aspirations of moving to the research and development team.