12-2015

# Production of Ethanol from Cereal Food Fines using Dilute-Acid Hydrolysis

Andrew Edward Arnett
*University of Arkansas, Fayetteville*

Production of Ethanol from Cereal Food Fines using Dilute-Acid Hydrolysis

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Chemical Engineering

by

Andrew Edward Arnett
University of Arkansas
Bachelor of Arts in Psychology, 2013

December 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Dr. Robert R. Beitle
Thesis Director

_____
Dr. Edgar C. Clausen
Committee Member

_____
Dr. Christa Hestekin
Committee Member

_____
Dr. Jin-Woo Kim
Committee Member

**Acknowledgements**

There are more people who helped get me to where I am right now than I could ever be able to thank, but I would like to extend my gratitude to a few people who deserve mention. First are my parents, who gave me the freedom to choose any path in life that I wanted while always extending their unconditional support. I've probably pushed my own personal boundaries in ways that they would never have been comfortable doing, but they let me make my mistakes and learn my own way while always trusting that I would figure things out. I would like to thank my research advisor, Dr. Robert Beitle, who, aside from all the obvious things an advisor does for their graduate students, was always the voice of reason every time I felt I was on the brink of disaster, which was a regular occurrence. I would also like to thank all the graduate students I spent time with on a daily basis, as our common struggle bonded us together and I know a made a lot of great friends that I will have for life.

# Abstract

Cereal food fines are a leftover by-product from breakfast cereal processing that is typically sold as animal feed or used as a pet food ingredient; however this product could be of greater value as a feedstock for the production of fuel ethanol via fermentation. In order for this material to be fermented it has to be broken down in to simple sugars using hydrolysis. One method of hydrolysis is called dilute-acid hydrolysis, whereby low concentrations of acid are added to the feedstock to facilitate the breaking of chemical bonds. This study investigates the effect of different concentrations of acid to determine optimal conditions for the production of ethanol. It was found that higher concentrations of acid yielded greater production of ethanol and, overall, this particular feedstock showed promise as a future source of fuel ethanol

## Table of Contents

## List of Figures

## List of Tables

# Chapter 1

## 1.0 Introduction

Cereal food fines are the leftover byproduct of breakfast cereal food processing. This leftover material is generally used as an ingredient in pet food or animal feed. This material, however, could be of much greater value if used in the production of bio-fuels such as bioethanol. An estimated 300 tons of this material is produced per year, so finding an efficient method for converting this leftover material into useable bioethanol could be of significant interest to industries needing to find uses for this common food processing byproduct. Conversion of food biomaterials into ethanol is a process that has been widely developed worldwide and fuel-grade ethanol is currently the largest competitor to the petroleum market with 24.57 billion gallons being produced in 2014 (1). The process generally consists of three distinct steps: 1) breakdown of biomass, most commonly corn, rice, starch, or lignocellulosic materials, into fermentable sugars, 2) fermentation using microorganisms, most commonly the yeast *Saccharomyces cerevisiae*, and 3) recovery of the ethanol. The first step is achieved via hydrolysis, where water is used to break the more complex sugars down in to glucose. There are a number of different chemical, physical and biological methods of hydrolysis currently available (2), but the subject of this research is a method called dilute-acid hydrolysis, where low volume percentage acid solutions, in the range of 0.1% – 10% acid, are mixed with the biomass and heated to high temperatures. The resulting mixture is then made suitable to host the specific microorganism, such as by pH modification, aeration, etc. The bacterial strain will naturally consume the broken down sugars and create ethanol as a by-product. There are many methods of recovery, but only the hydrolysis and fermentation steps are the subject of this research project.

The primary focus of investigation is the effect of acid concentration on ethanol production from cereal food fines. A dilute sulfuric acid hydrolysis process was used with several sample groups of different acid concentrations in the above-mentioned range as the independent variable. The hydrolyzate mixtures were inoculated with *Saccharomyces cerevisiae* and allowed to incubate for a period of at least two days. Gas chromatography is an effective technique for separating and comparing relative amounts of a compound from a mixture and was used as the primary means of measuring and comparing ethanol production between the different groups as well as determining concentrations of ethanol produced within the samples. A larger-scale batch reactor experiment was also conducted to measure ethanol levels during the growth phase for the purpose of obtaining a growth curve.

# Chapter 2

## 2.0 Bio-Ethanol Production

Bio-ethanol is ethanol produced via fermentation of biological materials for use as a transportation fuel. This type of technology has existed since the turn of the 20th century in the United States and corn-based ethanol was commonly used in automobiles until the 1930s. After World War II, however, the availability and abundance of petroleum sources led to fossil fuel-derived gasoline becoming the primary fuel source for the country (3). The 1970s Oil Embargo led to renewed interest in domestically-produced alternative fuels, chief among these being corn-based ethanol. As a result, fuel ethanol production facilities have steadily increased since this time and research in to optimization and expansion of this domestically produced energy supply has as well. In the United States, the vast majority of ethanol produced is from corn; however, many different biological sources are now readily converted in to ethanol using a variety of different methods. This chapter will be a review of the science and chemistry behind the conversion of biological materials in to fuel-grade ethanol, with a particular emphasis on the materials and methods used in this study.

## 2.1 Conversion of Cereal Grains into Ethanol

The feedstock used in this research project is a product known as cereal food fines. A commodity profile obtained from McNess company (a rural commodities exporter) defines cereal food fines as consisting "of ground and fine particles of breakfast cereals that are obtained as a byproduct of the processing of them" (4). The profile further states that cereal food fines represent "many varieties of breakfast food consisting of a mixture of cereal grains or processed products thereof, specific amino acid composition specifications in general can be expected to reflect that of corn, oats, rice and wheat combinations." Thus, this particular feedstock is to be

considered a combination of different starch-based sources. As mentioned earlier there are a number of different sources that can be and are currently used for the production of bioethanol, but there are typically considered to be three main categories: starches, sugars and lignocellulosic materials. Starches include sources such as corn, wheat, potato, rice and cassava; sugars include molasses, sugar cane and sugar beet while lignocelluloses, in the context of bio-ethanol production, generally refer to agricultural residues such as sugar cane bagasse, corn stover or switchgrass (5, 6). Conversion of biomass into ethanol generally involves the same fundamental process for all types of feedstocks. For starch and lignocellulose, a saccharification step is required to break down the more complex sugars that make up the materials into simple sugars that can be digested by particular microorganisms, most typically yeast (*Saccharomyces cerevisiae*), although the use of other strains such as *Zymomonas mobilis* has become more common (7). The resulting sugars are then fermented by the microorganisms and ethanol is produced as a by-product. What generally separates the three different groups of feedstocks is the type of pre-treatment required prior to fermentation. For starches, a process called hydrolysis is required.

### 2.1.1 Hydrolysis of Starch

Starch is a polymer of glucose that consists of two main structural components, amylose and amylopectin. Amylose is a straight chain polymer of D-glucose molecules linked together via α-1, 4 glycosidic linkages while amylopectin is a larger branched molecule composed of both α-1, 4 and β-1, 6 linkages.

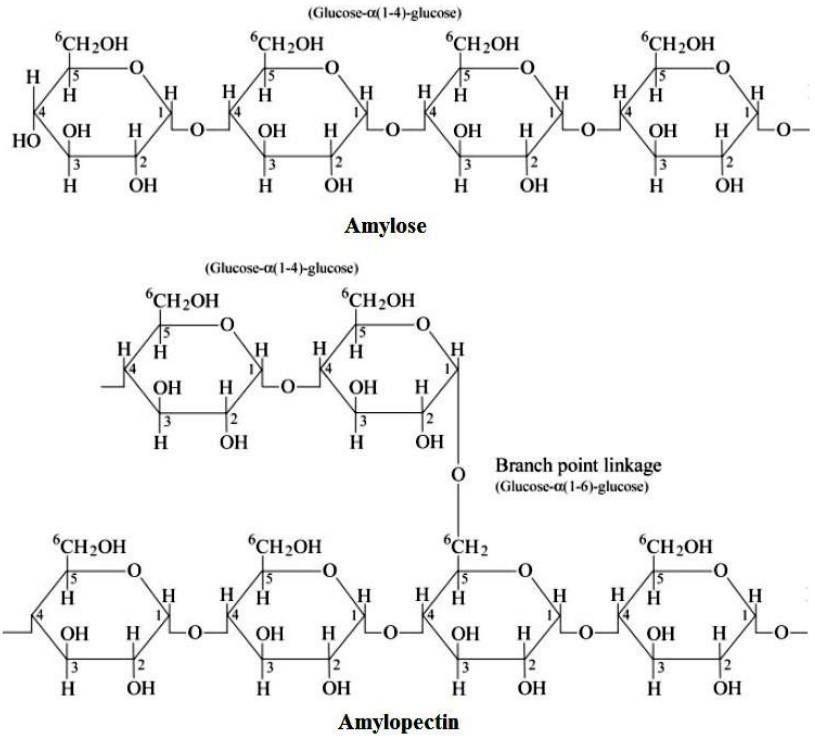


Figure 1: Amylose and Amylopectin (8)

In order for the bacteria to convert the starch into ethanol, the polymeric amylose and amylopectin must be broken down into monomeric glucose. This is achieved, both biologically and chemically, through a process called hydrolysis. Hydrolysis is the breaking of a chemical bond through the addition of water. In polysaccharides specifically, this occurs through the breaking of the glycosidic bonds linking the monomeric sugar units together.

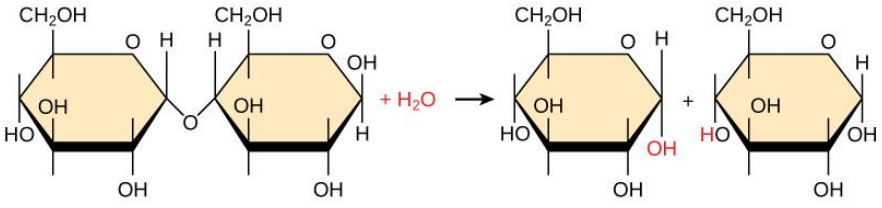


Figure 2: Polysaccharide Hydrolysis (9)

The vast majority of industrially-produced ethanol from starch uses an enzymatic hydrolysis process, where the endogenous enzyme α-amylase is used to liquefy the starch and the enzyme glucoamylase hydrolyzes the liquefied starch into glucose. There are also, however, two chemical hydrolysis methods available, dilute and concentrated acid hydrolysis. Dilute-acid hydrolysis typically uses acid solutions between 1 and 10 percent acid while concentrated-acid hydrolysis uses solutions between 10 and 30 percent (10). Generally, dilute-acid processes require higher temperatures and minimal to no acid recovery, as well as shorter reaction times compared to concentrated processes, but dilute-acid hydrolysis also results in the formation of undesired by-products, while concentrated-acid hydrolysis gives higher sugar yields compared to dilute-acid hydrolysis (10). The chosen method for this research project was the dilute-acid hydrolysis method, so there will be a further discussion on factors affecting this method.

### 2.1.2 Factors Affecting Efficiency of Dilute-Acid Hydrolysis

As already mentioned, both in industry and research, the enzymatic hydrolysis method for starches is a very well refined and mature practice and because of this is essentially the accepted method for the hydrolysis of starches for the production of glucose. This means, however, there is very little published research that investigates the efficiency of acid hydrolysis of starches for the production of glucose. However, dilute-acid hydrolysis is still a common practice as a pre-treatment of lignocelluloses, specifically, for the conversion of the cellulose components to glucose and the conversion of the hemicellulose fraction of lignocelluloses to xylose (11). The process and parameters investigated are similar enough that the studies on dilute-acid hydrolysis of lignocelluloses could help inform optimal process conditions for the hydrolysis of starch material.

The primary variables open to manipulation in an acid hydrolysis are temperature, pressure, residence time, and concentration of acid. It is also of interest to measure the formation of by-products, as the presence of these can affect both the efficiency of the hydrolysis reaction as well as subsequent fermentation of the sugars in to ethanol.

Studies on the effects of the mentioned variables are often aimed at developing kinetic parameters to measure the combined effect of changes of these variables on the production of sugars from various lignocellulosic feedstocks. Attempts to develop these parameters go back to Saeman's (12) work in 1945 measuring the effect of temperature and concentration of sulfuric acid on glucose production from Douglas fir wood. For models of glucose production from cellulose, there are typically two first-order rate constants, one for the degradation of cellulose to glucose and the other for the degradation of glucose to by-products (13). Numerous studies on various lignocellulosic materials, such as sugar cane bagasse, rice straw, corn stover and corn fiber (14-18) all show that glucose yield increases with temperature, residence time, pressure or acid concentration. However, these more extreme conditions also generally lead to greater production of by-products, most notably furfural and 5-hydroxymethylfurfural (HMF), which have also been shown to be by-products of starch hydrolysis (19,20). Finding balance between a maximum yield of glucose while minimizing formation of by-products is typically the goal in developing kinetic parameters, and this can help inform optimal conditions for a hydrolysis of cellulose, starch or other biomaterial.

### 2.1.3 Yeast Fermentation of Glucose for the Production of Ethanol

Once a biomaterial is broken down to the constituent sugars (most typically glucose) a biological fermentation process takes place where baker's yeast (*Saccharomyces cerevisiae*) uses the glucose for the production of energy while creating ethanol as a by-product. This is the same

fermentation process used in baking or by which alcoholic beverages are made. This chemical process occurs when there is a lack of oxygen available and is known as anaerobic glycolysis, and is the same energy production process responsible for the production of lactic acid in the human body during short periods of intense physical exercise.

Glycolysis is a metabolic process whereby glucose is used for the production of ATP and NADH for cellular energy in the absence of oxygen. This occurs through a series of enzyme-catalyzed reactions where glucose is converted to pyruvate and eventually ethanol and carbon dioxide. Optimally, for every molecule of glucose, two moles of ATP, NADH, carbon dioxide and ethanol are created. The glycolysis pathway used by yeasts is known as the Embden-Meyerhoff-Parnas pathway (EMP pathway) and is the most common pathway used by cells. However, ethanol fermentation can occur via other glycolytic pathways as well, such as the Entner-Doudoroff pathway (ED pathway) used by *Zymomonas mobilis.*

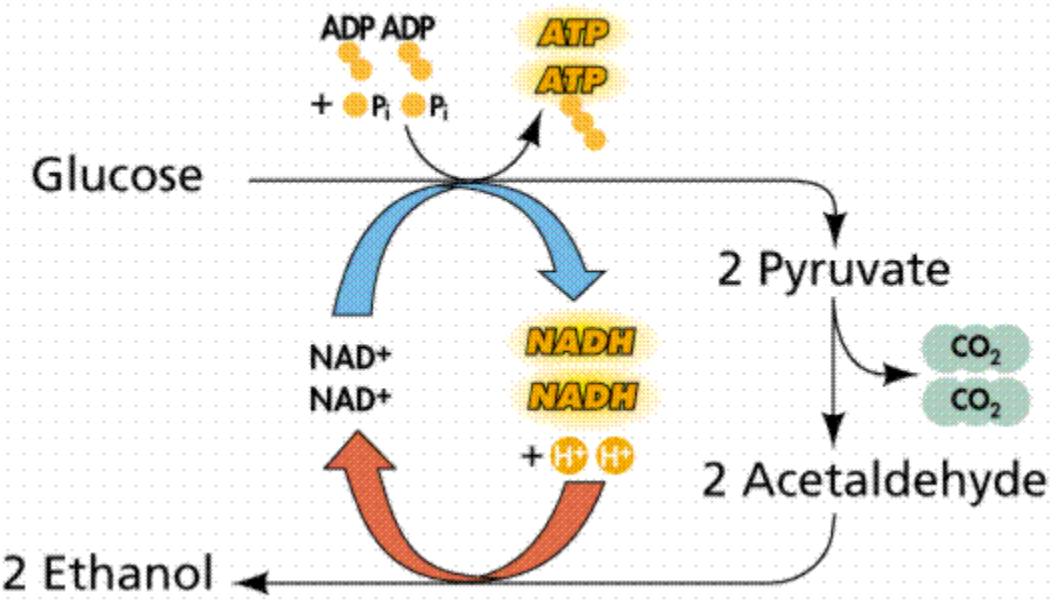


Figure 3: Glycolysis Summary (19)

### 2.1.4 Conditions for Yeast Fermentation

Several factors can help contribute to a successful fermentation, whether it is maintenance of the requisite conditions to promote growth or the inclusion of additional measures to promote either overall yield or rate of production. Since there are many different strains of microorganisms as well as many different ways to manipulate a fermentation, only the factors most relevant to the current study will be elaborated upon.

In order for the yeast to grow, it has to be suspended in a sufficiently nutritive aqueous medium. For yeast fermentation for the production of ethanol produced from a dilute-acid hydrolysis, this is going to be the hydrolyzate itself, which has the necessary glucose to provide nutrition for the cells. In general, however, a medium needs to contain some kind of carbon source that can be used by the cells for energy, most typically some kind of carbohydrate broth. Often pH buffering is required for optimal growth conditions. Most yeast thrive best under slightly acidic conditions (pH 5.0 -5.5) but this can vary depending on the strain used and other growth conditions. As has already been mentioned, yeast fermentation into ethanol is an anaerobic process, so in order to have a viable growth environment, there needs to minimal to no oxygen present. A method called nitrogen sparging was used for this study, where nitrogen ($N_2$) gas was circulated throughout the medium to dissolve the oxygen. Temperature and time are also important factors in fermentation. The optimal temperature range for yeast fermentation is 32–35 °C and complete growth typically takes 48-72 hours. The by-products formed from the hydrolysis stage have been shown to have inhibitory effects on fermentation, most notably in the case of starches, furfural and 5-hydroxymethylfurfural. Numerous detoxification methods, however, have been developed to help mitigate this problem (20).

# Chapter 3

## 3.0 Experimental

### 3.1 Materials

Pre-ground cereal food fines were obtained from ConAgra Corporation and stored at room temperature. Sodium Hydroxide pellets and Dibasic Potassium Phosphate were obtained from Sigma-Aldrich in St. Louis, MO. Compressed nitrogen gas was obtained from VWR Scientific. Lyophilized *Saccharomyces cerevisiae* cells in MRS media were obtained from American Type Culture Collection and stored at 37° C in an incubator.

### 3.2 Equipment

#### 3.2.1 G.C.

A Shimadzu GC-2014 with attached AOC-20i Autoinjector was used to measure ethanol content of samples. Samples were measured using flame ionization detection (FID) with helium as a carrier gas. Further gas chromatography specifications can be found in Appendix I.

#### 3.2.2 Reactor

A 1 L Applikon Bioreactor was used for the batch reactor experiment. BioExpert supervisory software was used to control pH, agitation and temperature.

Figure 4: Shimadzu GC-2014 and AOC-20i AutoInjector

Figure 5: 100mL Batch Reactor and Set-up

### 3.3 Procedure

#### 3.3.1 Small Batch (Culture Bottle) Experiments

The first part of this investigation studied the effect of acid concentration on ethanol production by creating four sample groups with varying concentrations of sulfuric acid. The four groups were 0.2%, 3%, 6% and 10% sulfuric acid by volume. The procedure was as follows: 10 g cereal fines were mixed with water and concentrated sulfuric acid (18 M) in a 100 mL solution. For an example 10% acid solution, 10 mL sulfuric acid would be added to 90 mL water. The mixture was then steam autoclaved at 120° C for 30 minutes. After being allowed to cool to room temperature, the mixture was vacuum filtered using a Whatman no. 3 filter to remove undissolved solids. To help maintain buffering, 1.0 M potassium phosphate was added to give a final concentration of 25 mM. Next, 3 M sodium hydroxide solution was added until a pH between 7.0 and 8.0 was obtained. At this point, the mixture was separated in to culture bottles with clamp-sealable lids; five bottles with 20 mL each for each sample group. To remove oxygen from the sealed bottles, nitrogen gas was bubbled in for approximately 20 minutes for each bottle.

Completion of preceding procedure meant that the media was suitable for fermentation. 1.0 mL of *S. cerevisiae* suspended in MRS media was injected in to each bottle and allowed to incubate at 37° C for a period of at least two days. Once ready, 1.0 mL samples from each bottle were taken and centrifuged at 14,100 rpm for seven minutes. The liquid was then separated from the formed pellet and the sample was stored and frozen for later analysis.

#### 3.3.2 Batch Reactor Experiment

A larger-scale batch reactor experiment was conducted to obtain a growth-curve vs. time. The hydrolyzate media was prepared in the same method as in the previous section with a 10%

sulfuric acid concentration hydrolysis. A 1 L reactor was used, so between 400 and 500 mL of hydrolyzate was desired. This was accomplished by originally making 200 mL of hydrolyzate prior to pH balancing with sodium hydroxide, as the previous experiment with 10% acid indicated that about an equal volume of sodium hydroxide solution was needed to balance the pH, and this resulted in a final volume of 437 mL hydrolyzate media. After being transferred to the reactor, the media was bubbled with nitrogen gas for 30 minutes. 10 mL of *S. cerevisiae* in the same hydrolyzate media was added and conditions for growth were set; the reactor was maintained at 37° C, a pH of 7.0 and stirred at a constant rate of 150 rpm. Sample taking began approximately 24 hours after inoculation and were taken every six hours after that for two days and every twelve hours for three days after that to give a five day growth period. All samples were 1.0 mL samples that were centrifuged, transferred in to new storage tubes to remove pellets and frozen directly after being taken.

### 3.3.3 Gas Chromatography Experiments

All experimental data was obtained from gas chromatography. Frozen samples were thawed out and filtered using 0.45 μm syringe filters. In addition to the experimental groups mentioned in the previous sections, an ethanol standard was prepared for determining ethanol peak locations and magnitude. This standard consisted of pure ethanol and water mixtures of 0.1%, 1%, 3% and 5% ethanol by volume samples. For the standard, two repetitions for each sample were analyzed for a total of eight data files. For the culture bottles, five samples were taken from each variable group and three repetitions were conducted for a total of fifteen data files per variable group. A common method file was used for all samples and its specifications are in Appendix I.

# Chapter 4

## 4.0 Results

This chapter summarizes results obtained from the small batch and batch reactor experiments using gas chromatography peak data to detect ethanol formation and calculate concentrations. For the culture bottle experiments, five samples from each sample group were analyzed, however all of these were theoretically equal samples and, for the large majority, the data supported this, so mean data will be presented here. However, if any significant disparities were apparent between any of the individual samples within the same sample group, then this will be noted. The complete data set is given in Appendix II.

## 4.1 Ethanol Standard Curve

The following table gives the ethanol standard data with percent (%) ethanol being the volume percentage of the ethanol/water sample and peak being the chromatography elution peak height for ethanol. The slope of the curve on the plot generated from this data was used as the reference for calculating ethanol concentrations in the experimental groups.

Table 1: EtOH Standard Curve Data

| % EtOH | Peak |
|---|---|
| 0.1 | 1,214,071 |
| 1 | 1,918,711 |
| 3 | 4,861,232 |
| 5 | 6,525,398 |

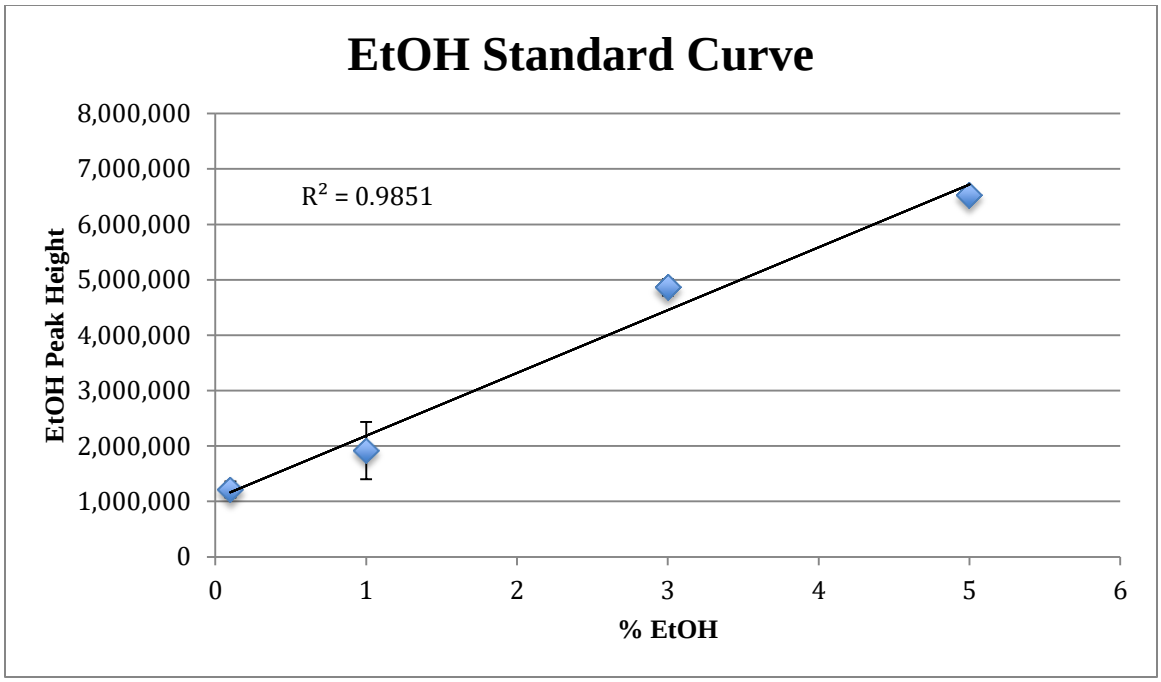


Fig. 6: EtOH Standard Curve Slope

Using the point-slope equation:

$$y = mx + b \text{ (eq. 1)}$$

a satisfactorily linear fit ($R^2$ = .9378) is found for the standard data where *y* is the peak, *x* is the concentration of ethanol, *x* is the slope and *b* is the y-intercept, also, error bars are presented using standard error of the mean. The value for the slope, *m*, is found to be 1,133,611.17 and this value will be used to calculate ethanol concentrations of the experimental groups.

Figures 2 – 5 are the peaks for the different ethanol standard samples. The presence of ethanol is indicated by a peak at a retention time between 2.7 and 3.0 minutes.

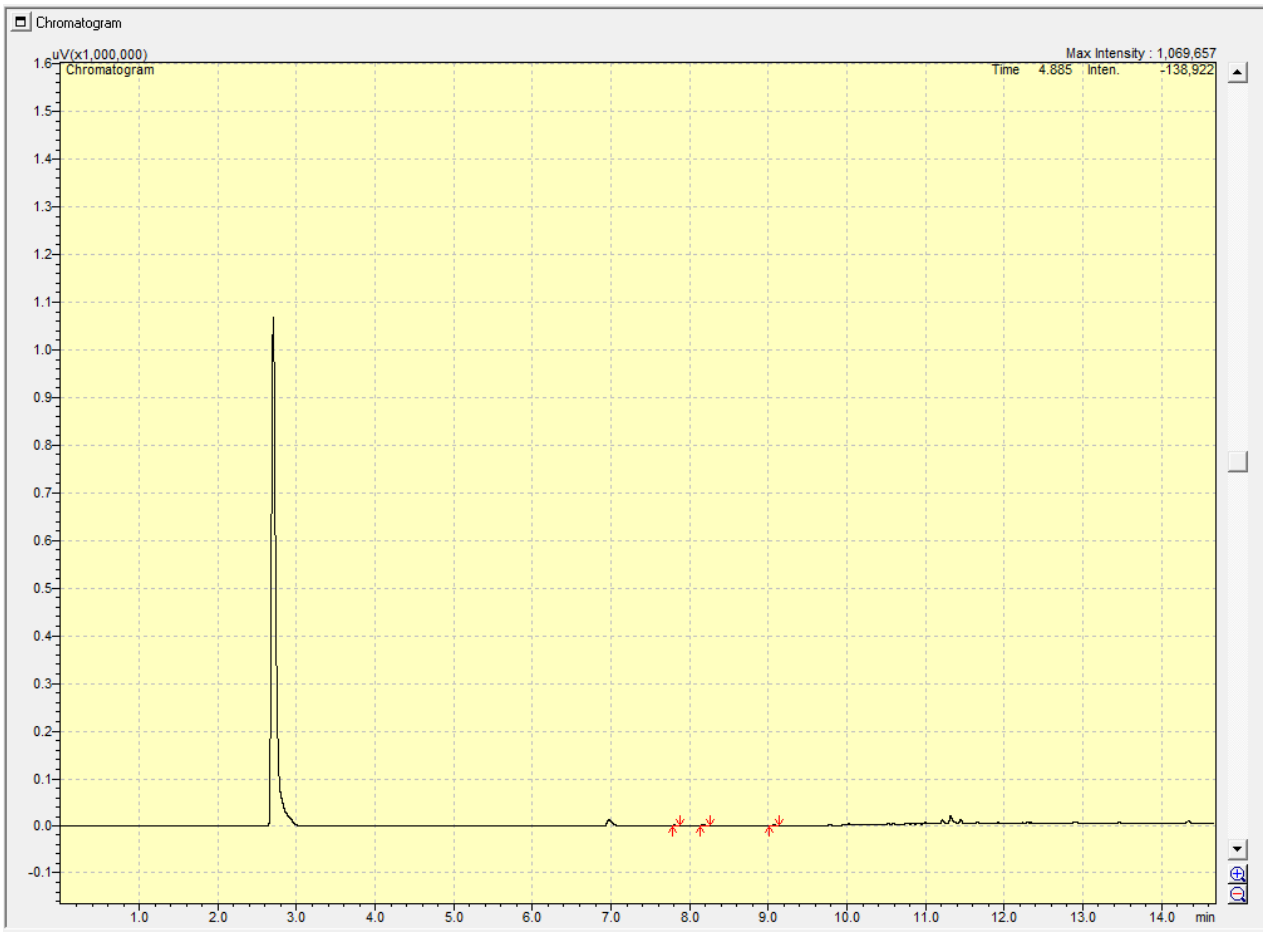


Figure 7: 0.1% EtOH Standard Chromatogram

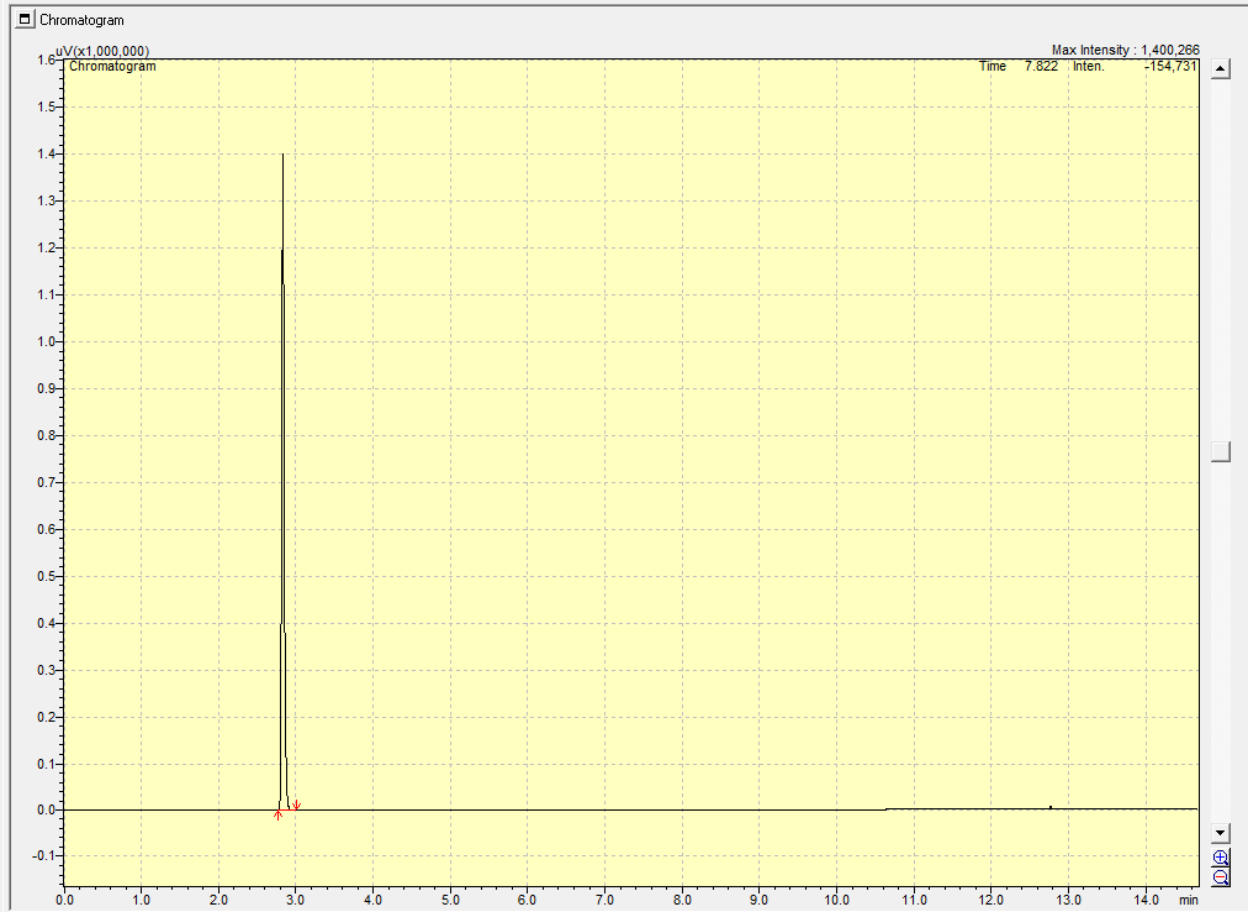


Figure 8: 1% EtOH Standard Chromatogram


Figure 9: 3% EtOH Standard Chromatogram

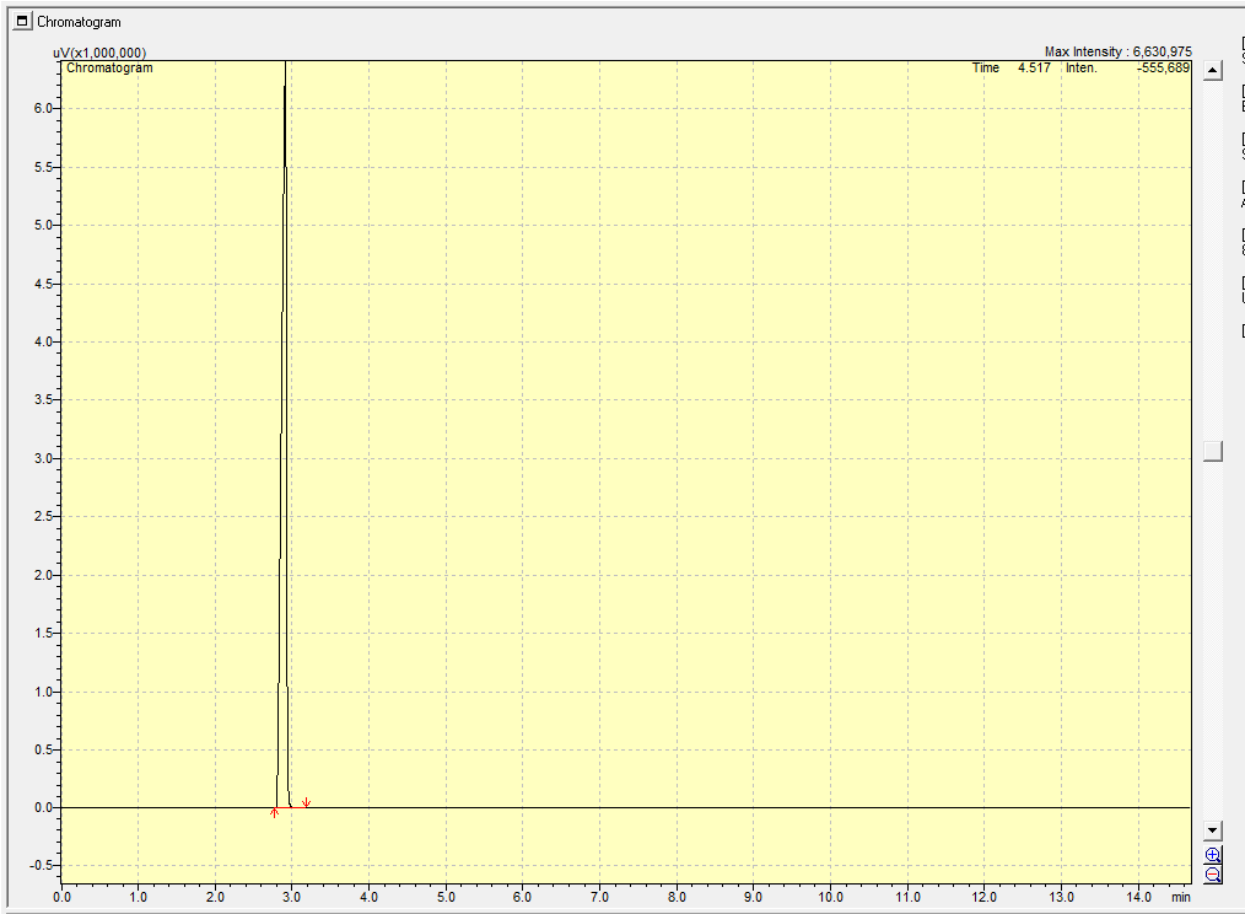


Figure 10: 5% EtOH Standard Chromatogram

### 4.2 Culture Bottle Experiments

The four sample groups analyzed were 0.2%, 3%, 6% and 10% sulfuric acid by volume. Five samples from each group were analyzed with three repetitions each. Ethanol formation was indicated by the retention time peak and a mean peak height value was found to determine concentration using the slope formula determined in the previous section. Groups are presented in the order the experiments were performed.

#### 4.2.1 3% Sulfuric Acid

Disappointingly, the 3% sulfuric acid sample group did not present a significant ethanol peak, as shown in figure 11. The mean peak height for this sample group was 81,284; which is not significant compared to even the lowest standard concentration which yielded peak heights on the order of 10^6. The concentration given from this value is 0.071% ethanol by volume with a standard deviation of 0.0264%. The retention time data does however present multiple other significant peaks, suggesting the presence of inhibitory by-products. This possibility is supported by the literature (2) and data in this study, as multiple non-ethanol peaks were common to all the samples that yielded little to no ethanol. These peaks were, however, present in samples that did yield significant amounts of ethanol, though in not as large amounts. Studies indicate that by-product formation from hydrolysis increases with increased ethanol formation (13), so the idea that these by-products are causing there to be no ethanol formation seems unlikely, however, it may be possible that more of the glucose was converted to degradation products, leaving less available for fermentation. It may be valuable to determine what substances are responsible for these other peaks, but retention times and peak heights vary across chromatography studies and it would be inaccurate to try to make any judgements on the nature of these compounds from other chromatography studies without developing our own standards of whatever compounds are

expected.  Results of this sample group could also be attributed to incomplete sample mixing, measurement error from the equipment or just growth failure in general.

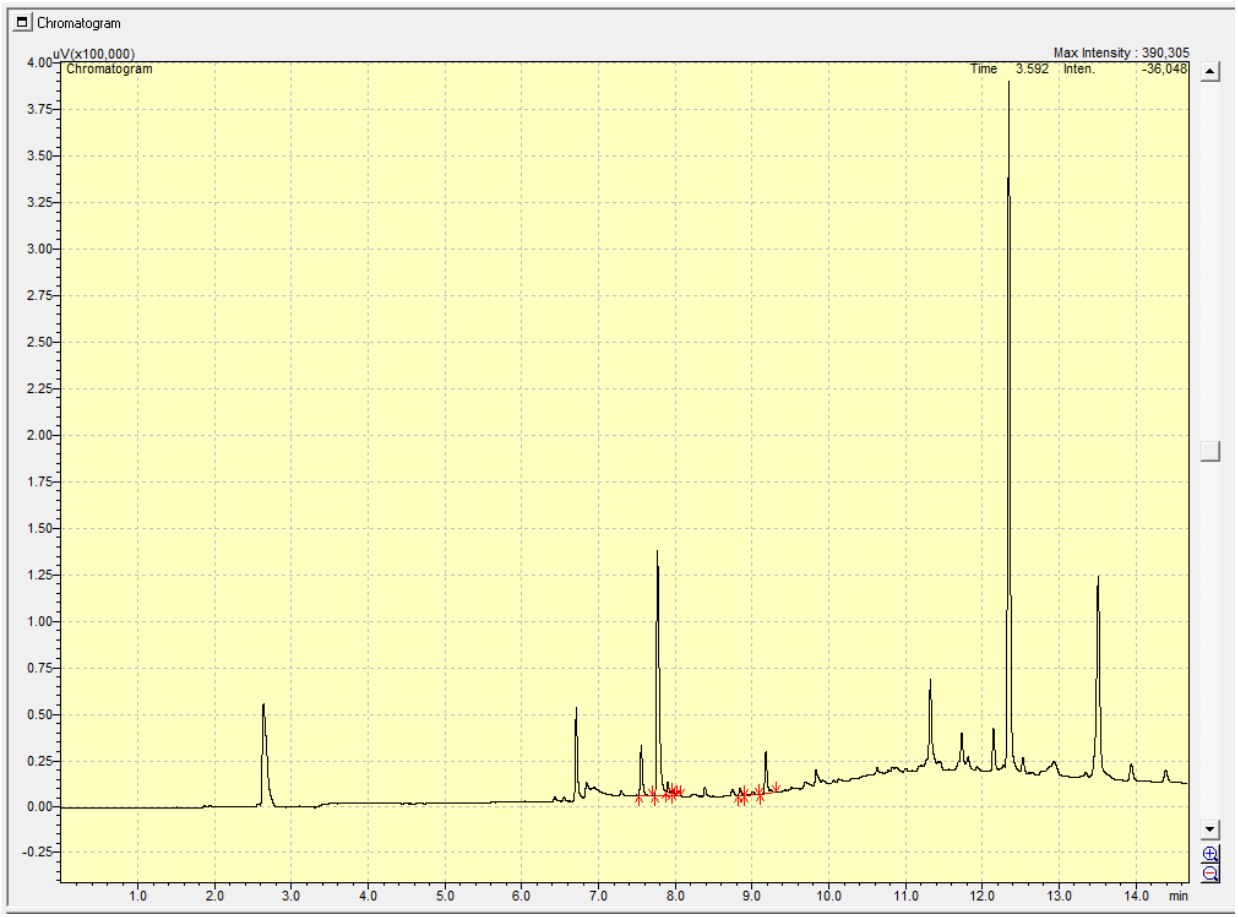


Figure 11: Chromatogram of 3% Hydrolysis Fermentation Product

### 4.2.2 6% Sulfuric Acid

The 6% sulfuric acid group showed good and consistent production of ethanol (figure 12). The mean peak height obtained was 6,279,157, yielding a mean concentration of 5.54% (SD = .5084%). Also, there were no significant peaks aside from the ethanol, suggesting hydrolysis by-products could in fact be a significant factor in determining the difference between successful and unsuccessful batches.

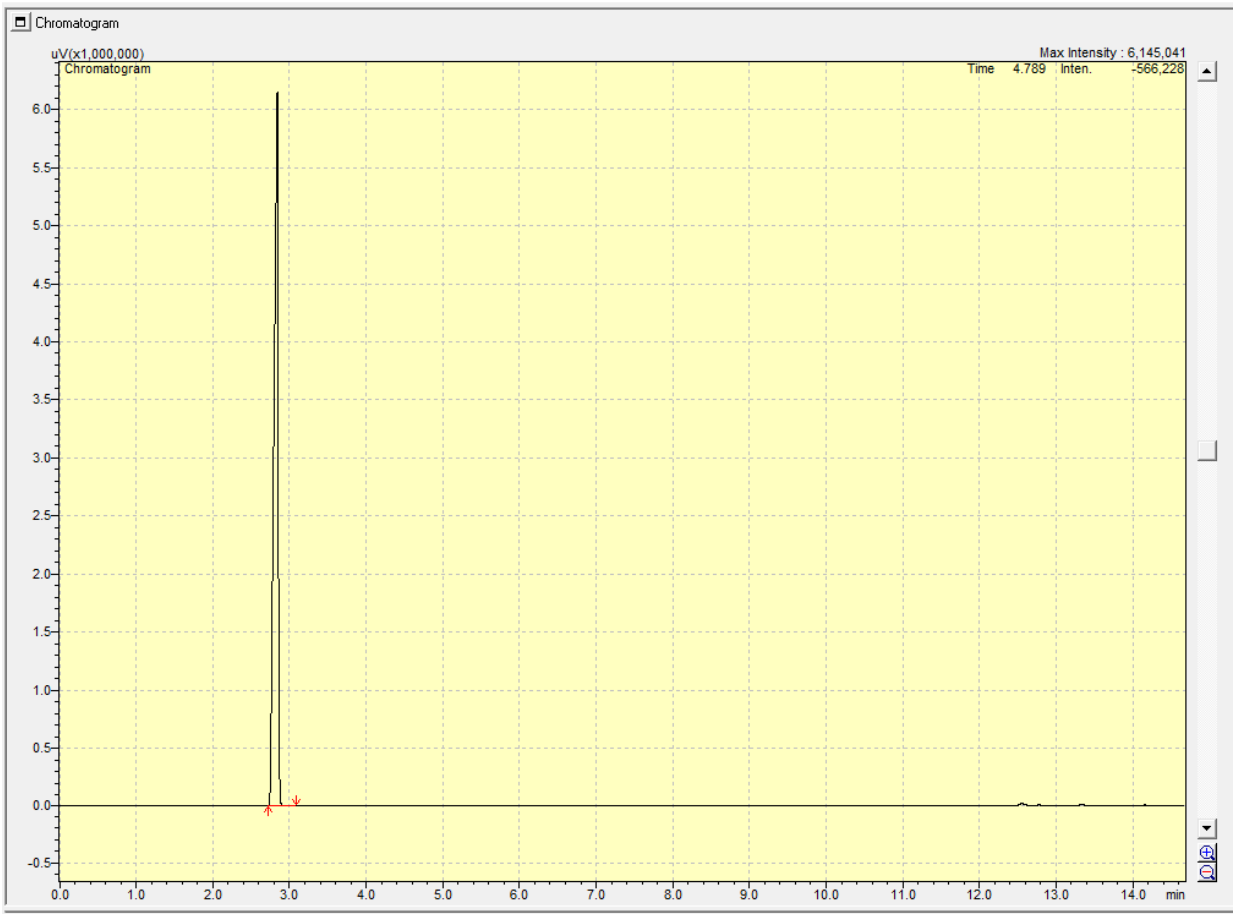


Figure 12: Chromatogram of 6% Hydrolysis Fermentation Product

### 4.2.3 10% Sulfuric Acid

The 10% sulfuric acid group showed good and consistent production of ethanol as well (figure 13). The mean peak height for this sample group was 11,626,940, giving a mean concentration of 10.26% (SD = 1.175%). Once again ethanol was the only significant peak, suggesting the ethanol production process was dominant. The increase of this batch from the 6% batch seems to indicate that, when done successfully, ethanol production from dilute-acid hydrolysis increases with acid concentration. This result would be consistent with the wide body of literature (14-18). This, however, is premature to conclude with only two data points. However, it does appear that two successful ethanol production experiments indicated that the highest percentage of acid yielded the most ethanol, and it is for this reason that 10% acid was used for the batch reactor experiment. Also, the hypothesis that byproduct formation was the cause behind the lower ethanol yields in the 3% acid group seems even more unlikely, as the higher concentrations of the 6% and 10% groups would lead to more byproduct formation according to this idea, yet this is clearly not the case. There still might be a relationship between the presence of byproducts and lower ethanol yields, but a causative relationship appears unlikely.

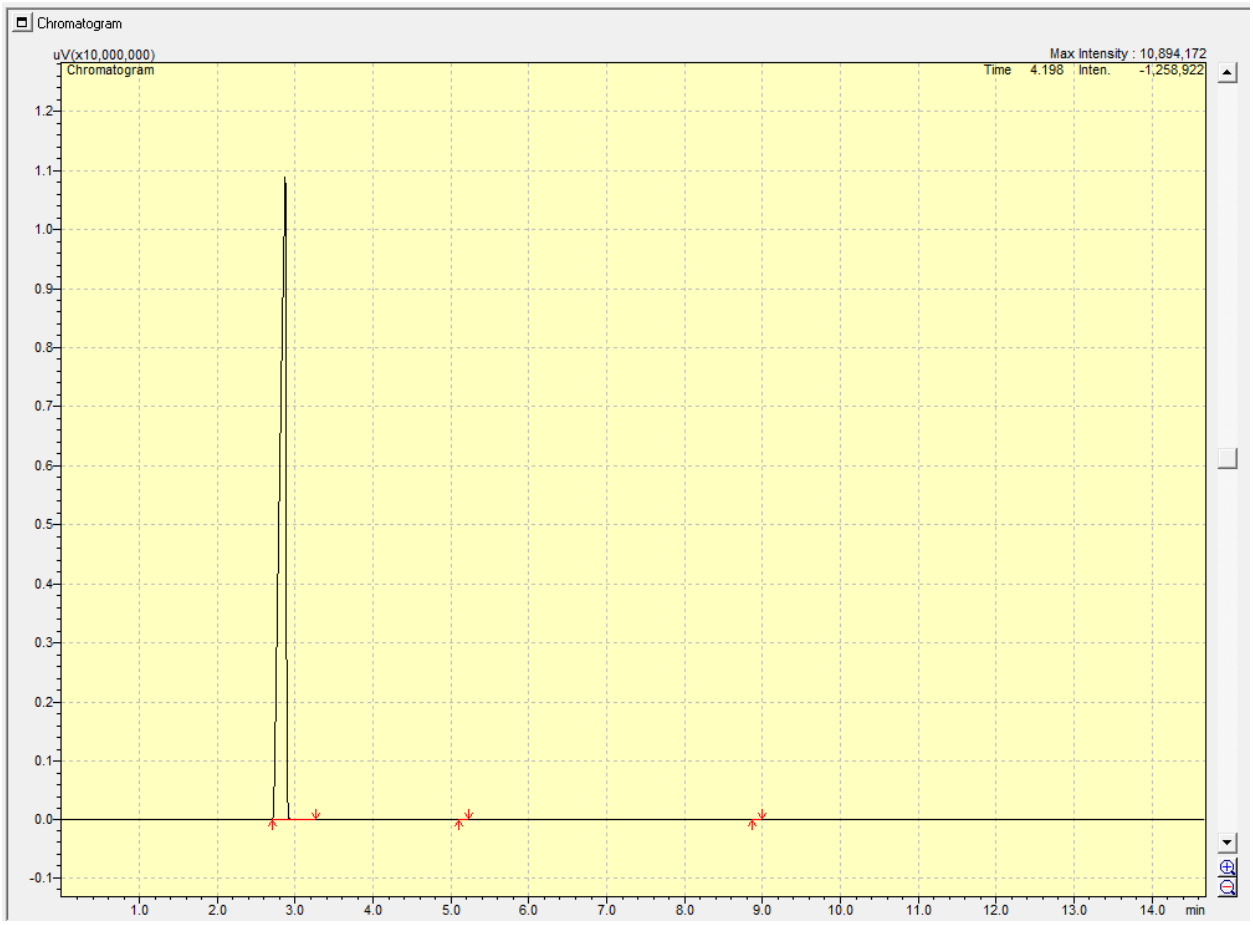


Figure 13: Chromatogram of 10% Hydrolysis Fermentation Product

### 4.2.4 0.2% Sulfuric Acid

The 0.2% sulfuric acid batch yielded interesting results. One of the samples showed no ethanol content and had the familiar non-ethanol peaks seen for the 3% batch. However, the remaining four samples successfully produced ethanol and showed no significant by-products. Since these samples were only separated after hydrolysis and prior to inoculation, this suggests that whatever led to the inhibition of ethanol production occurred in the fermentation phase, such as a failure of growth, but also could simply be an issue of incomplete mixing when the sample was taken. There was also an instance of a repetition measurement of the same sample showing no significant ethanol content while the other repetitions on the sample showed ethanol content consistent with the remaining samples. This would most likely be attributed to a measurement error from the equipment. For the sake of presenting meaningful data, the points that indicated no ethanol content (no peak at a retention time of 2.8 minutes) were not included in the average presented here. The complete data set, however, is given in Appendix II along with the average and standard deviation. The samples that displayed significant growth had surprisingly high peak values, with a mean peak of 11,230,446 and a mean concentration of 9.91% (SD = .8226%). This is comparable to the 10% acid batch and seems to contradict the trend from the previous two data groups that indicated that acid concentration and ethanol production were positively correlated. Two factors are likely responsible for this result, and both have to do with the amount of sodium hydroxide added in the pH balancing stage. For the 10% acid batch, a nearly equal volume of NaOH solution needed to be added to the hydrolyzate mixture in order to create a neutral pH solution; however, less than 5 mL of NaOH solution was necessary for the 0.2% acid batch. This meant that a total of 200 mL of hydrolyzate media was made for the 10% batch and a total of a little more than 100 mL was created for the 0.2% acid batch, meaning that only half of the

mixture from the 10% batch, and subsequently, half of the sugars from the original 10 g of fines were separated in to the culture bottle samples and available for fermentation, while nearly all of the hydrolyzate mixture from the 0.2% batch was used for fermentation. This possible dilution effect was not considered at the time of the experiments and exact concentrations of NaOH solution were not recorded, so this factor remains a confounding variable in interpreting the results of these experiments. Aside from dilution of samples, however, it is possible that large salt concentration itself could have been a factor in growth inhibition in the groups that required more buffering. There is some precedent for this in the literature, as well (23).

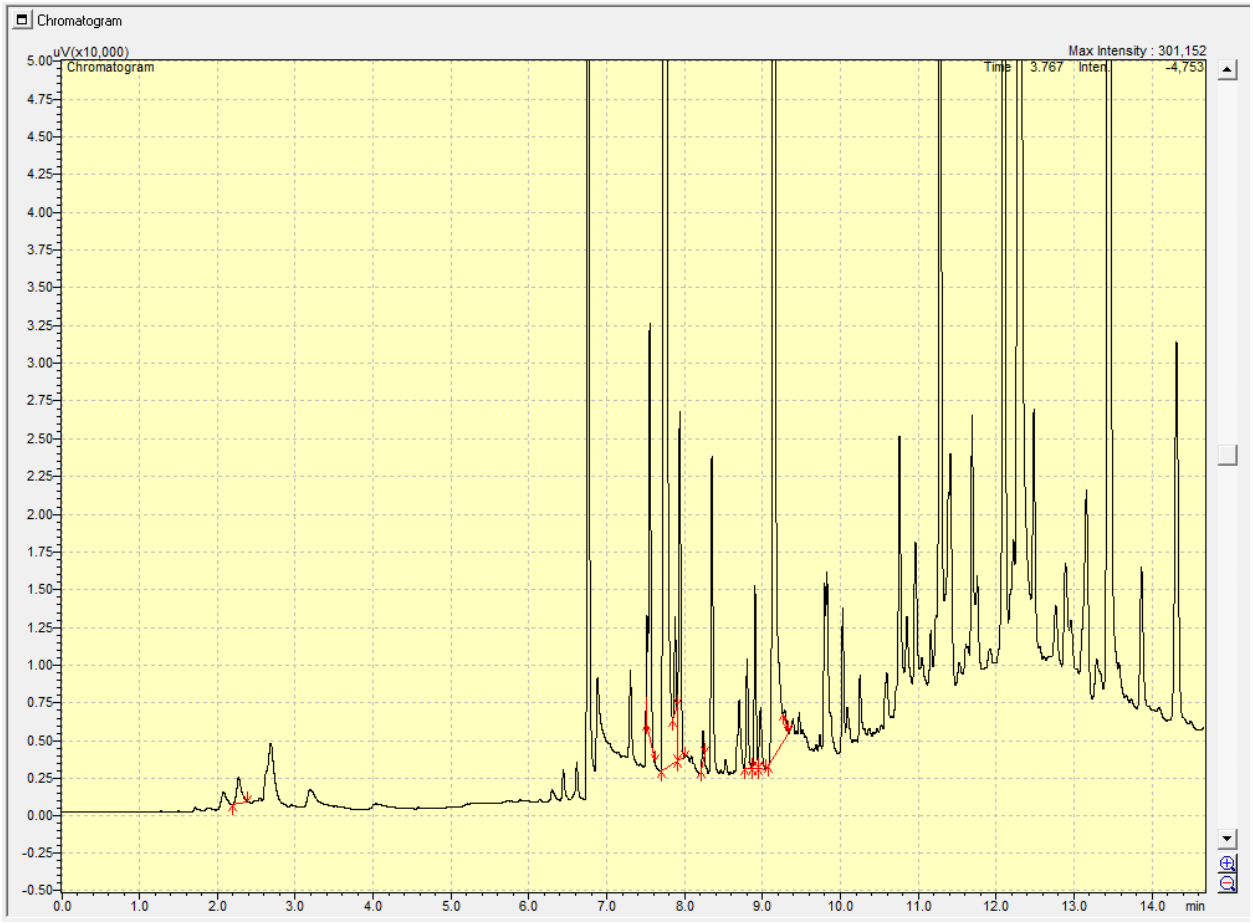

Figure 14: Chromatogram of 0.2% Hydrolysis Fermentation Product; 1st Sample (No EtOH Production)

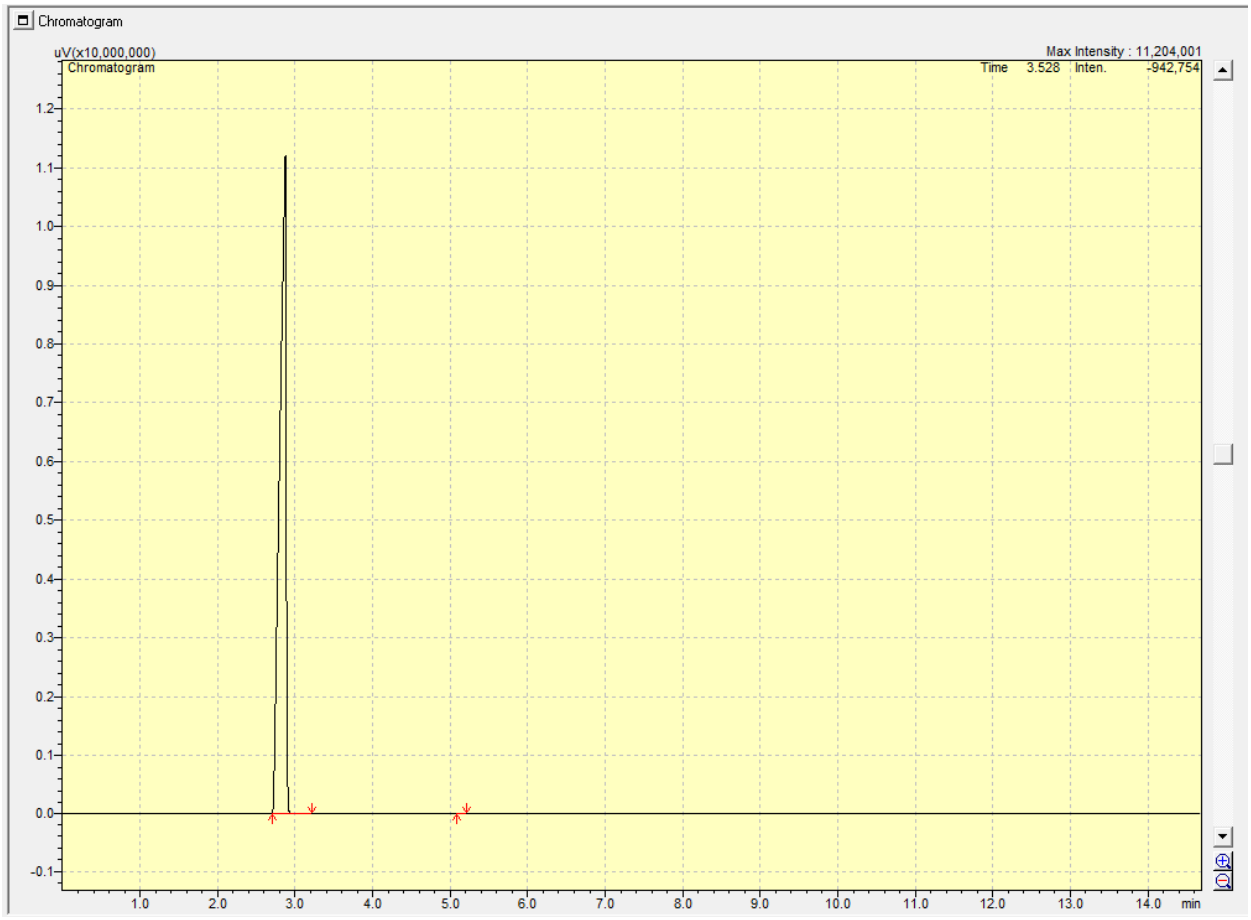


Figure 15: Chromatogram of 0.2% Hydrolysis Fermentation Product; 4$^{th}$ Sample (Significant EtOH Production, Characteristic of 2$^{nd}$ through 5$^{th}$ Samples)

### 4.3 Batch Reactor Experiment

A batch reactor experiment was conducted to develop a curve for cell growth/ethanol production versus time. Samples were taken starting one day after inoculation and were taken every six hours for two days and every twelve hours for three days after that for a total of five days analysis. Three repetitions of each sample were recorded. The data from this experiment is presented in table 2.

Table 2: Batch Reactor Data

| Sample # | Time (min.) | Peak | Avg. | Peak Present Avg. |
|---|---|---|---|---|
| 1 | 1352 | 6,997,845 | | |
| | | 7,944,837 | 7,368,040 | 7,368,040 |
| | | 7,161,438 | | |
| 2 | 1612 | 143,772 | | |
| | | 145,090 | 139,241 | N/A |
| | | 128,862 | | |
| 3 | 2039 | 2,575 | | |
| | | 8,225,697 | 5,161,210 | 7,740,527 |
| | | 7,255,358 | | |
| 4 | 2457 | 7,086,444 | | |
| | | 8,000 | 5,335,387 | 7,999,081 |
| | | 8,911,717 | | |
| 5 | 2817 | 32,710 | | |
| | | 134,101 | 3,522,724 | 10,401,360 |

Table 2: Batch Reactor Data (Cont.)

| Sample # | Time (min.) | Peak | Avg. | Peak Present Avg. |
|---|---|---|---|---|
| | | 10,401,360 | | |
| 6 | 3192 | 52,904 | | |
| | | 62,994 | 60.724 | N/A |
| | | 64,925 | | |
| 7 | 3814 | 68,078 | | |
| | | 62,126 | 114,894 | N/A |
| | | 216,299 | | |
| 8 | 4167 | 28,016 | | |
| | | 7,927 | 15,282 | N/A |
| | | 9,903 | | |
| 9 | 4612 | 6,280 | | |
| | | 17,807 | 21,732 | N/A |
| | | 41,108 | | |
| 10 | 5352 | 9,941,177 | | |
| | | 10,128,277 | 10,063,092 | 10,063,092 |
| | | 10,179,822 | | |
| 11 | 6152 | 92,305 | | |
| | | 32,710 | 3,833,546 | 11,375,624 |
| | | 11,375,624 | | |
| 12 | 6912 | 91,602 | | |
| | | 17,807 | 39,661 | N/A |

Table 2: Batch Reactor Data (Cont.)

| Sample # | Time (min.) | Peak | Avg. | Peak Present Avg. |
|---|---|---|---|---|
| | | 9,573 | | |
| 13 | 7542 | 93,413 | | |
| | | 103,394 | 3,337,331 | 9,815,186 |
| | | 9,815,186 | | |
| 14 | 8382 | 13,608 | | |
| | | 10,808 | 3,446,961 | 10,316,466 |
| | | 10,316,466 | | |

It's clear from this data that the results of this experiment are largely inconsistent. Many samples did not indicate any significant ethanol content, while multiple samples showed growth for some measurements but not for others. Possible explanations for this have been mentioned in previous sections; however, these aberrations make taking an average of the repetitions and also developing a curve based on every sample not meaningful toward deducing a growth trend. One can see, though, that significant ethanol peaks were present for samples at the beginning, end, and throughout the growth period indicating that there was in fact ethanol produced by the experiment and it should not be considered a complete failure. So for the sake of meaningful interpretation of the data, the fifth column in table 2 was included, which only takes in to account the data representing significant ethanol content (i.e. peak heights on the order of 10^6) and this data is presented graphically as a plot of peak height vs. time is figure 13.

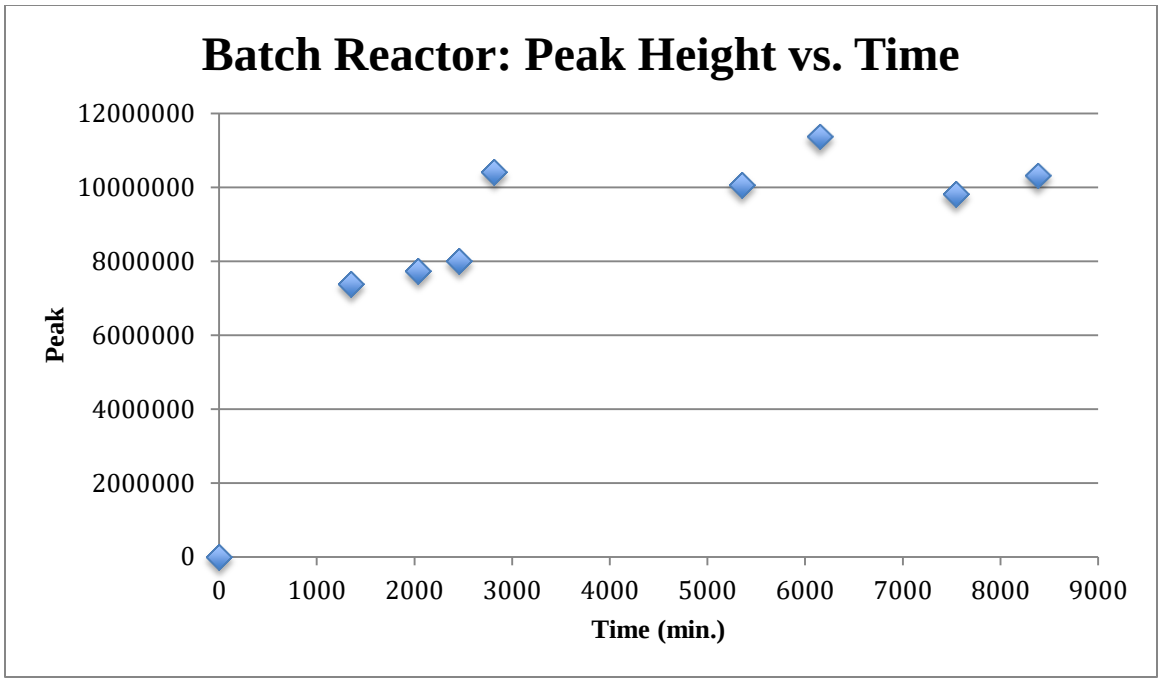


Figure 16: Peak Height vs. Time for Batch Reactor Experiment

When only using the data indicating significant ethanol content, the results of this experiment seem to be closer to what would be expected. At the end of the first day of growth the ethanol content is 6.5% and rises during the second day to 7.1% and by the start of the third day of measurement is near 10% and moves between 8 and 10 percent for the rest of the growth period, which is near enough to the average determined from the 10% culture bottle experiment to suggest consistent growth and accurate readings. However, the fact that only three data points are in the growth phase and that there is a sharp jump to an equilibrium value suggests this data is not representative enough to give an accurate growth curve. If this experiment were to be conducted again, sampling should begin earlier than 24 hours after inoculation, should be taken more often (perhaps every 3-4 hours) and should not need to be necessary after 3 days.

### 4.4 Overall Process Efficiency and Analysis

The primary trend from the experiments is that increasing acid concentration led to more ethanol production. Even though there was a large of amount confounding and unsuccessful data, there was enough meaningful data to give a relevant and consistent trend. Table 3 gives mean volume of ethanol produced per gram cereal fine input for the three samples groups that gave meaningful results. Total volume is included as well because of the varied final volumes due to the different pH buffering requirements; these totals were not recorded during the experiments and are estimates.

Table 3: Mean Production Efficiencies

| | 0.2% | 6% | 10% |
|---|---|---|---|
| Total volume (mL) | 100 | 180 | 200 |
| mean EtOH conc. (%) | 9.91 | 5.54 | 10.25 |
| mL EtOH / g fines (mL/g) | .991 | .997 | 2.05 |

First of note here is that the 0.2% batch produced an overall efficiency equal to that of the 6% batch. This reinforces the idea that the significantly lower salt concentration necessary for buffering the lower volume acid solution gave it an advantage by having a less toxic growth environment than higher concentration solutions, which theoretically yielded higher glucose concentration hydrolyzates. However, the 10% batch showed that, overall, the effectiveness of higher acid concentrations eventually outweighs the detrimental effect of increased pH buffering and yielded the most productive results. Table 4 compares our optimal production rate to theoretical ethanol yields for different cereal grain feedstocks found in the literature.

Table 4: Theoretical Ethanol Yields from Various Grain Feedstocks (24) and Experimental Yield

| Feed Grain | Theoretical Yield (mL/g) |
|---|---|
| Rice | .57 |
| Corn | .52 |
| Wheat | .55 |
| Barley | .41 |
| Sorghum | .52 |
| Oat | .42 |
| Fines (10% Experimental) | 2.05 |

It is unlikely that the yield from this experiment led to a 2- to 4-fold increase from even the best theoretical yields on ethanol production from this type of feedstock, especially considering that actual yields are generally lower than these estimates (24) and employ more efficient and optimized methods of production than those used in this experiment. The most logical source of error for this would be from the standard curve development. The fact that an order of 10 reduction in the experimental yield data would give very reasonable results in comparison to the values from the literature lends more credibility to such a possibility.

If this possibility were to be the case, and a yield of .21 mL of ethanol were produced per gram of feedstock, then 300 tons of annual material would be able to produce 15,098 gallons of ethanol. If the standard curve isn't the source of error, and we were to assume yields within a reasonable range for this type of materials, say between .35 and .5 mL per gram feedstock, this would give between 25,163 and 35,948 gallons of ethanol annually.

# Chapter 5

## 5.0 Conclusions and Recommendations

Overall, the experiments led to predicted results given what was indicated by the body research in this field. It has been well established that higher acid concentrations from dilute or even concentrated acid hydrolyses lead to greater glucose production and, subsequently, higher ethanol yields. These studies, however, measure glucose production directly following hydrolysis and do not indirectly measure ethanol formation after fermentation. The issue of higher salt concentrations required for the higher acid concentration groups seemingly leading to inhibition of fermentation even more reinforces that measuring glucose concentration immediately following hydrolysis would have led to greater clarity in determining hydrolysis efficiency as well as helping to isolate which steps in the experimental process might have been responsible for creating confounding results. Chromatography can and has been used to measure glucose levels in a similar manner (25) as for ethanol in this experiment and would not be an unreasonable experimental manipulation for future studies. Worth consideration in future chromatography work might be the use of an internal standard, where a standard amount of an unrelated analyte is used in all sample and concentrations are measure relative to this separate analyte. The consistency of the absolute values in this study as well as the lack of intermediate steps between drawing the sample and the GC measurement suggests that the external standard used in this study was the better choice.

The most fundamental issue to be addressed in any future studies would be to obtain a precise characterization of the feedstock. The nature of this product lends to there likely being a combination of many different materials at different concentrations within the mixture. This could have wide-ranging effects on what sort of treatments would be necessary and/or optimal

and also creates a highly variable and uncertain economic cost/benefit profile. The product profile given in chapter 2 describes fines as largely consisting of starch material, but it is also likely that the by-product of breakfast cereal contains a large amount of free sugar as well as undesired waste that becomes intermixed with the fines during its collection. So based on the exact composition of the feedstock a number of alternative routes would be available. If it were determined that a significant portion of the fines were simply free sugar, then this material could be directly fermented, since yeast is able to directly ferment sucrose. This of course would eliminate the cost and time necessary for a hydrolysis procedure. It might also be possible to separate the sugar from the starch and only need to hydrolyze the starch material. Under this circumstance, a hot water treatment of the material would suffice in isolating free sugar from the insoluble material. Such a wash would provide two sources of carbohydrates – one capable of fermentation without hydrolysis, and one requiring additional treatment, respectively. It is also possible that a portion of the material is either lignin or waste material, neither of which is readily fermentable and would skew efficiency measurements (ethanol produced / gram cereal fines). So any economic assessment of the viability of this project going forward would be dependent on determining the exact composition of the fines to give an accurate forecast of how to optimally treat the material. Several assays are available to for the detection of sugars such as α-amylase or sucrose, such as enzymatic assays and the 3,5-dinitrosalicylic acid (DNS) assay. Relative compositions could then be determined using either high performance liquid chromatography (HPLC) or GC. A meaningful economic assessment of the possible process efficiency of producing ethanol from this material could then be performed.

As far as new experimental manipulations to the current method are concerned, a few potentially beneficial alterations would be worth considering. First, Qureshi et al. (23) not only

helped to confirm that high salt concentrations inhibited fermentation, but also successfully showed that removal of salts using electrodialysis prior to fermentation significantly increased fermentation product yield. So looking in to the costs and benefits of adding a salt removal step prior to fermentation would very likely increase process efficiency. It is important to note that the acid hydrolysis conditions used were standards set for lignocellulosic materials, which require more extreme treatments that starches. It is likely the conditions that were chosen for this work were too harsh, as there is minimal guiding literature useful for the determination of acid hydrolysis conditions for starch. Less severe conditions for hydrolysis might have led to similar, if not better, yields. A logical alternative to either of these recommendations is, however, to consider using an enzymatic hydrolysis process, as this is the industry standard for hydrolysis of this type of feedstock, and has been for some time (21). This practice has been shown to eliminate many of the disadvantages associated with acid hydrolyses. It would however, be factored in to a cost estimate because such an addition is not without cost.

Finally, a consideration of the ethanol yield must be discussed in terms of recommended work. A large impediment to the project lay in the timing of sample analysis due to multiple users vying for the instrument used to measure the ethanol produced, which eventually led to calculated yields that are theoretically impossible. To this end, immediately analyzing the product and standard curves would be prudent.

**Bibliography**


1. http://www.afdc.energy.gov/data/10331

2. Sun, Ye, and Jiayang Cheng. "Hydrolysis of lignocellulosic materials for ethanol production: a review." *Bioresource technology* 83.1 (2002): 1-11.

3. Bothast, R. J., and M. A. Schlicher. "Biotechnological processes for conversion of corn into ethanol." *Applied Microbiology and Biotechnology* 67.1 (2005): 19-25.

4. http://www.mcness.com/sites/default/files/file_attach/CEREALFOODFINESCDNH03.pdf

5. Mojović, Ljiljana, et al. "Progress in the production of bioethanol on starch-based feedstocks." *Chemical Industry and Chemical Engineering Quarterly* 15.4 (2009): 211-226.

6. Balat, Mustafa, Havva Balat, and Cahide Öz. "Progress in bioethanol processing." *Progress in energy and combustion science* 34.5 (2008): 551-573.

7. Sanchez, Oscar J., and Carlos A. Cardona. "Trends in biotechnological production of fuel ethanol from different feedstocks." *Bioresource technology* 99.13 (2008): 5270-5295.

8. Babak Ghanbarzadeh and Hadi Almasi (2013). Biodegradable Polymers, Biodegradation - Life of Science, Dr. Rolando Chamy (Ed.), ISBN: 978-953-51-1154-2, InTech, DOI: 10.5772/56230. Available from: http://www.intechopen.com/books/biodegradation-life-of-science/biodegradable-polymers

9. Boundless. “Hydrolysis.” Boundless Biology. Boundless, 21 Jul. 2015. Retrieved 20 Oct. 2015 from https://www.boundless.com/biology/textbooks/boundless-biology-textbook/biological-macromolecules-3/synthesis-of-biological-macromolecules-53/hydrolysis-295-11428/

10. Broder, J. D., and J. W. Barrier. "Producing ethanol and coproducts from multiple feedstocks." American Society of Agricultural Engineers (Microfiche collection)(USA) (1988).

11. Taherzadeh, Mohammad J., and Keikhosro Karimi. "Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review." *BioResources* 2.3 (2007): 472-499.

12. Saeman, Jerome F. "Kinetics of wood saccharification-hydrolysis of cellulose and decomposition of sugars in dilute acid at high temperature." *Industrial & Engineering Chemistry* 37.1 (1945): 43-52.

13. Wyman, Charles E., et al. "Hydrolysis of cellulose and hemicellulose." *Polysaccharides: Structural diversity and functional versatility* 1 (2005): 1023-1062.

14. Gámez, Sara, et al. "Study of the hydrolysis of sugar cane bagasse using phosphoric acid." *Journal of food engineering* 74.1 (2006): 78-88.

15. Saha, Badal C., et al. "Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol." *Process Biochemistry* 40.12 (2005): 3693-3700.

16. Bhandari, Nandan, Douglas G. Macdonald, and Narendra N. Bakhshi. "Kinetic studies of corn stover saccharification using sulphuric acid." *Biotechnology and bioengineering* 26.4 (1984): 320-327.

17. Noureddini, H., and J. Byun. "Dilute-acid pretreatment of distillers' grains and corn fiber." Bioresource technology 101.3 (2010): 1060-1067.

18. Aguilar, R., et al. "Kinetic study of the acid hydrolysis of sugar cane bagasse." *Journal of Food Engineering* 55.4 (2002): 309-318.

19. Purves, William K., et al. *Life: The Science of Biology: Volume III: Plants and Animals*. Vol. 3. Macmillan, 2003.

20. Whistler, Roy L., and James R. Daniel. *Starch*. John Wiley & Sons, Inc., 1978.

21. Lorenz, K., and J. A. Johnson. "Starch hydrolysis under high temperatures and pressures." *Cereal chemistry* (1972).

22. Palmqvist, Eva, and Bärbel Hahn-Hägerdal. "Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification." Bioresource technology 74.1 (2000): 17-24.

23. Qureshi, Nasib, et al. "Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw: production of butanol from hydrolysate using Clostridium beijerinckii in batch reactors." *Biomass and bioenergy* 32.12 (2008): 1353-1358.

24. Gawande, Sheetal B., and I. D. Patil. "Utilization of Cereal Grains for Bioethanol Production: A Critical Review." 60.

25. Scholz, Matthew J., Mark R. Riley, and Joel L. Cuello. "Acid hydrolysis and fermentation of microalgal starches to ethanol by the yeast Saccharomyces cerevisiae." *Biomass and Bioenergy* 48 (2013): 59-65.

# Appendix I

## Gas Chromatography Method File

## Gas Chromatography Method File

Method

<Analytical Line 1>

[Auto Sampler AOC-20i+s]

| | | |
|---|---|---|
| Injection Volume | : 1.0 uL | |
| # of Rinses with Solvent(Pre-run) | | : 3 |
| # of Rinses with Solvent(Post-run) | | : 9 |
| # of Rinses with Sample | : 5 | |
| Plunger Speed(Suction) | : High | |
| Viscosity Comp. Time | : 0.2 sec | |
| Plunger Speed(Injection) | : High | |
| Syringe Insertion Speed | : High | |
| Injection Mode | : Normal | |
| Multi Injection Count | : 1 | |
| Pumping Times | : 5 times | |
| Injection Port Dwell Time | : 2.0 sec | |
| Terminal Air Gap | : No | |
| Plunger Washing Speed | : High | |
| Washing Volume | : 6 uL | |
| Syringe Suction Position | : 0 mm | |
| Syringe Injection Position | : 0 mm | |
| Solvent Selection | : All A,B,C | |

[Injection Port SPL1]

| | |
|---|---|
| Injection Mode | : Split |
| Temperature | : 250.0 C |
| Carrier Gas | : He |
| Flow Control Mode | : Velocity |
| Pressure | : 58.3 kPa |
| Total Flow | : 22.8 mL/min |
| Column Flow | : 1.80 mL/min |
| Linear Velocity | : 30.0 cm/sec |
| Purge Flow | : 3.0 mL/min |
| Split Ratio | : 10.0 |
| High Pressure Injection | : OFF |
| Carrier Gas Saver | : OFF |
| Splitter Hold | : OFF |

[Column Oven]

| | |
|---|---|
| Initial Temperature | : 40.0 C |
| Equilibration Time | : 0.5 min |

=Column Oven Temperature Program=

Total Program Time : 14.67 min

| | Rate(C/min) | Temperature(C) | Hold Time(min) |
|---|---|---|---|
| | ------ | 40.0 | 0.00 |
| 1 | 6.0 | 60.0 | 0.00 |
| 2 | 20.0 | 160.0 | 0.00 |
| 3 | 30.0 | 230.0 | 4.00 |

[Column Information]

| | |
|---|---|
| Column Name | : ZB-FFAP |
| Serial Number | : 277075 |
| Film Thickness | : 0.25 um |
| Column Length | : 30.0 m |
| Inner Diameter | : 0.32 mm ID |
| Column Max Temp | : 250 C |
| Installation Date | : 2013/08/16 |

[Detector Channel 1 SFID1]

| | |
|---|---|
| Temperature | : 250.0 C |
| Signal Acquire | : Yes |
| Sampling Rate | : 40 msec |
| Stop Time | : 14.67 min |
| Delay Time | : 0.00 min |
| Subtract Detector | : None |

[General]
< Ready Check Heat Unit >
Column Oven : Yes
SPL1 : Yes
SFID1 : Yes
DINJ1 : Yes
< Ready Check Detector (FTD) >
< Ready Check Baseline Drift >
SFID1 : No
< Ready Check Injection Flow >
SPL1 Carrier : Yes
SPL1 Purge : Yes
DINJ1 Carrier : No
< Ready Check Add. Flow >
< Ready Check Detector APC Flow >
External Wait : No
Auto Flame On : Yes
Auto Flame Off : Yes
Reignite : No
Auto Zero After Ready : Yes

[Peak Integration Parameters - Channel 1]

| | | | |
|---|---|---|---|
| Width | : 5 sec | Slope | : 20000 uV/min |
| Drift | : 0 uV/min | T.DBL | : 1000 min |
| Min.Area/Height | : 1000 counts | | |

=Integration Time Program=

| Enable | Time(min) | Event | Value |
|---|---|---|---|
| 1 [Yes] | 0.010 | Integration Off | ***** |
| 2 [Yes] | 2.000 | Integration On | ***** |
| 3 [Yes] | 2.600 | Integration Off | ***** |
| 4 [Yes] | 2.700 | Integration On | ***** |
| 5 [Yes] | 3.500 | Integration Off | ***** |
| 6 [Yes] | 5.000 | Integration On | ***** |
| 7 [Yes] | 5.600 | Integration Off | ***** |
| 8 [Yes] | 7.500 | Integration On | ***** |
| 9 [Yes] | 8.250 | Integration Off | ***** |
| 10 [Yes] | 8.750 | Integration On | ***** |
| 11 [Yes] | 9.500 | Integration Off | ***** |

[Quantitative Parameters - Channel 1]
=Quantitative Parameters=

| | | | |
|---|---|---|---|
| Quantitative Method | : External Standard | | |
| Calculated by | : Area | Calibration Level# | : 3 |
| Calibration Curve | : Linear | Through Origin | : Not through |
| Weight Regression | : None | Unit | : % |

=Identification Parameters=

| | | | |
|---|---|---|---|
| Window/Band | : Window | Window | : 5 % |
| Default Band Time | : --- | Identification Method | : Absolute |
| Peak Select | : All Peaks | Grouping | : None |
| Correction RT | : No Change | | |

[Compound Table - Channel 1]

| ID# | Name | Type | Ret.Time | Conc.1 | Conc.2 | Conc.3 | Unit |
|---|---|---|---|---|---|---|---|
| 1 | RT2.815 | Target | 2.815 | 0.260 | 0.540 | 1.120 | |
| 2 | RT8.858 | Target | 8.858 | 0.250 | 0.500 | 1.000 | |

[Column Performance Parameters - Channel 1]

| | | | |
|---|---|---|---|
| Calc. Method | : JP | Column Length | : 0 mm |
| Time of Unretained Peak | : Time of 1st Peak | Set Time | : --- |
| Calculated for Identified Peak | : OFF | | |

## Appendix II

## Complete Ethanol Peak Height Data Set

| Sample Group | Sample | Repetition | Ethanol Peak Height |
|---|---|---|---|
| Standard | 1 (0.1%) | 1 | 1,069,657 |
| | | 2 | 1,358,485 |
| | 2 (1%) | 1 | 2,437,156 |
| | | 2 | 1,400,266 |
| | 3 (3%) | 1 | 5,007,054 |
| | | 2 | 4,715,410 |
| | 4 (5%) | 1 | 6,630,975 |
| | | 2 | 6,419,821 |
| 3% | 1 | 1 | 55,749 |
| | | 2 | 43,001 |
| | 2 | 1 | 93,743 |
| | | 2 | 43,089 |
| | 3 | 1 | 88,967 |
| | | 2 | 95,988 |
| | 4 | 1 | 111,078 |
| | | 2 | 118,653 |
| 6% | 1 | 1 | 4,459,748 |
| | | 2 | 6,154,041 |
| | | 3 | 6,066,087 |
| | 2 | 1 | 6,290,143 |
| | | 2 | 6,258,520 |
| | | 3 | 6,270,327 |
| | 3 | 1 | 5,832,156 |
| | | 2 | 6,726,706 |
| | | 3 | 6,869,421 |
| | 4 | 1 | 6,574,819 |
| | | 2 | 6,293,583 |
| | | 3 | 6,453,695 |
| | 5 | 1 | 6,662,088 |
| | | 2 | 6,701,270 |
| | | 3 | 6,574,752 |
| 10% | 1 | 1 | 10,314,756 |
| | | 2 | 10,396,001 |
| | | 3 | 10,612,394 |
| | 2 | 1 | 10,894,172 |
| | | 2 | 13,228,022 |
| | | 3 | 10,491,375 |
| | 3 | 1 | 11,068,884 |
| | | 2 | 10,662,988 |
| | | 3 | 10,528,444 |
| | 4 | 1 | 12,158,941 |
| | | 2 | 14,033,775 |
| | | 3 | 13,546,340 |
| | 5 | 1 | 12,530,607 |

| Sample Group | Sample | Repetition | Ethanol Peak Height |
|---|---|---|---|
| | | 2 | 10,760,093 |
| | | 3 | 13,177,312 |
| 0.2% | 1 | 1 | 0 |
| | | 2 | 0 |
| | | 3 | 0 |
| | 2 | 1 | 11,388,355 |
| | | 2 | 10,417,304 |
| | | 3 | 11,299,260 |
| | 3 | 1 | 11,866,451 |
| | | 2 | 9,317,542 |
| | | 3 | 11,108,312 |
| | 4 | 1 | 5,800 |
| | | 2 | 11,204,001 |
| | | 3 | 10,842,053 |
| | 5 | 1 | 12,543,194 |
| | | 2 | 12,317,983 |
| | | 3 | 0 |
| Batch | 1 | 1 | 6,997,845 |
| | | 2 | 7,944,837 |
| | | 3 | 7,161,438 |
| | 2 | 1 | 143,772 |
| | | 2 | 145,090 |
| | | 3 | 128,862 |
| | 3 | 1 | 2,575 |
| | | 2 | 8,225,697 |
| | | 3 | 7,255,358 |
| | 4 | 1 | 7,086,444 |
| | | 2 | 8,000 |
| | | 3 | 8,911,717 |
| | 5 | 1 | 32,710 |
| | | 2 | 134,101 |
| | | 3 | 10,401,360 |
| | 6 | 1 | 52,904 |
| | | 2 | 62,994 |
| | | 3 | 64,925 |
| | 7 | 1 | 68,078 |
| | | 2 | 62,126 |
| | | 3 | 216,299 |
| | 8 | 1 | 28,016 |
| | | 2 | 7,927 |
| | | 3 | 9,903 |
| | 9 | 1 | 6,280 |
| | | 2 | 17,807 |
| | | 3 | 41,108 |

| Sample Group | Sample | Repetition | Ethanol Peak Height |
|---|---|---|---|
| | 10 | 1 | 9,941,177 |
| | | 2 | 10,128,277 |
| | | 3 | 10,179,822 |
| | 11 | 1 | 92,305 |
| | | 2 | 32,710 |
| | | 3 | 11,375,624 |
| | 12 | 1 | 91,602 |
| | | 2 | 17,807 |
| | | 3 | 9,573 |
| | 13 | 1 | 93,413 |
| | | 2 | 103,394 |
| | | 3 | 9,815,186 |
| | 14 | 1 | 13,608 |
| | | 2 | 10,808 |
| | | 3 | 10,316,466 |

0.2 % Batch: Average = 7487350.333; Standard Deviation = 5530112.073