METHODS AND PROCEDURES FOR THE QUANTIFICATION OF ETHANOL PRODUCTION FROM SWEET SORGHUM

By

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Abstract

This paper describes the protocols developed to facilitate the fermentation of ethanol from sweet sorghum juice. Sweet sorghum is being investigated as an ethanol feedstock because of its high sugar content and its ability to grow quickly in warm and dry climates. Methods were developed for storing yeast and creating an inoculant, pre-treating the sorghum juice to improve ethanol yield, performing fermentations and quantifying the ethanol produced. Before fermentation, juice should be centrifuged to remove any dense particulate matter and pH adjusted to 5.5. Fermentations should be performed using a 1:10 inoculant to total reaction volume ratio, and carried out for 72 hours to maximize ethanol yield. Ethanol should be measured using a colorimetric assay kit or with more accurate methods such as HPLC and GC.

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Introduction

The high demand for energy in America currently causes several economic, political and environmental challenges. Much of the energy supply, especially in the transportation sector, comes from petroleum. The combustion of petroleum, a mixture of hydrocarbons, can be used to power an engine, make steam for electricity or as heat directly. The property of petroleum that makes it controversial is that its use is unsustainable—it must be removed from the ground to be useful, yet is released into the atmosphere as carbon dioxide where it remains until reabsorbed by plants for photosynthesis. Because we are removing it from the ground at a rate greater than plants can be transformed into it, it is considered a non-renewable source of energy.

Over the years, an overwhelming ratio of the import of crude oil to domestic production[1] has led to unpredictable oil price fluctuations. These fluctuations affect several areas of the American lifestyle, from driving habits to having a warm home during the winter months. High gas prices affect every American, and are often an additional financial strain during tough economic times. Furthermore, an increasing demand coupled with the constantly decreasing global oil supply can only cause the price of oil to increase over time.

The dependence on oil is not only an inconvenience for the average person, but also a threat to national security. A large part of the world's oil supply is controlled either by nations that have a history of adversity with the United States or by nations led by dictators. In neither of these cases is it in America's best interest to be transferring billions of dollars for oil.

The concern over the environmental impact of fossil fuel usage is growing rapidly among many citizens and politicians. These factors have served as the driving force for the research and development of new and renewable energy sources.

Biofuels are a renewable energy source that has significant potential to supplement petroleum use. Unlike fossil fuels, biofuels are made from recently grown biological material that is processed into solids, liquids or gases, and can be used for transportation fuel, heating and cooking.

Biodiesel and ethanol are two biofuels that can be used for transportation. These fuels have significant commercial potential because they are somewhat comparable to diesel #2 and gasoline, respectively. They are compatible with most vehicles already in use and require little modification to the vehicles in order to be used effectively. There are also several vehicles in production that have the capability of running entirely on the high ethanol fuel E85. Ethanol is already being used in several states as an oxidizing agent and replacement for methyl tert-butyl ether, or MTBE.

[1] Energy Information Administration, 2009

Ethanol is a byproduct of fermentation, an anaerobic process where yeast convert sugar into ethanol and carbon dioxide. The sugars that yeast consume are glucose, sucrose and fructose. Certain yeast cultures will switch from their normal carbon dioxide producing metabolism to an ethanol producing metabolism when oxygen becomes unavailable. This allows the yeast to continue living until it can find more oxygen. Ethanol, however, can be toxic to yeast at too high of a level.

The fermentation process typically occurs in a large bioreactor filled with a sugar solution and a yeast culture. The mixture is allowed to ferment for a certain period of time, and when finished, the ethanol can be distilled from the remaining solution.

The main source of sugars for ethanol fermentation in the United States is corn. The United States produces around 80 million acres of corn every year.[2] Corn is used primarily as a feed grain but is also used in many areas of the food industry, including as a sweetener and oil source.

Corn faces several limitations when being considered as a source of sugars for ethanol production. The majority of the corn grown in the United States is grown in the Midwest. Production of corn in other regions is limited because corn requires good soil and a relatively large amount of water to be grown. A key strategy behind the development and implementation of renewable energy is to use the local resources of a region to their highest potential while having diverse technologies to accommodate different areas. This simply means that windmills should be used in areas where there is wind, and solar collectors in areas where there is sunshine. However, many regions of the country are currently importing corn in order to produce ethanol. This is why it has been the focus of much research to find other crops that can efficiently produce sugars for ethanol fermentation.

There are still other problems with using corn. Because it is used in so many areas of the human food chain, any corn diverted to ethanol production only serves as an additional stress on the food industry. Furthermore, corn based ethanol requires that sugars be extracted from the starches in a process known as saccharification. This process involves the use of special enzymes and may take several days depending on the amount of corn being used. This delay ultimately adds to the production cost of the ethanol.

Other major sources of sugars for ethanol production include sugarcane and cellulose. Sugarcane is an ideal crop for ethanol production because the juice extracted from it has a high sugar content and can be readily fermented by yeast. Brazil, for example, uses the ethanol produced from sugarcane to power the vast majority of its automobiles. Sugarcane, however, does not grow well in many areas of the United States and requires two years to mature. Cellulose, on the other hand, is found in every plant but is difficult to ferment. Cellulose is similar to starch in that it is a long chain of sugars, but the sugars in cellulose are more tightly

[2] Economic Research Service, 2009

held together than those in starch. This gives plants their rigid properties, but presents problems for fermentation purposes. Cellulose must undergo special chemical and biological treatments before its sugars can be converted into ethanol, adding to production costs.

Sweet sorghum is a sugarcane-like crop that has been grown successfully in the United States since the early seventeenth century[3]. It is a tall grass that produces stalks with high sugar content. It can be grown in warm climates and only takes a few months to cultivate. Because it is not a major feed crop, sweet sorghum can be grown in lower quality soil with reused irrigation water.

When it is cultivated, the stalks are juiced and the juice is fermented. However, the leftover material (the bagasse) may still be of value. The current trend is to burn it in order to generate electricity, but it may be possible to extract even more sugars from the cellulosic bagasse. These factors all serve to lower the production cost of ethanol from sweet sorghum and make it a viable ethanol crop for many areas of the country.

Two research groups at the University of Arizona, led by Dr. Mike Ottman and Dr. Dennis Ray are researching sweet sorghum to determine how it can best be grown in Arizona. Dr. Ray's research focuses on studying the combinations of planting time, harvest time and different treatments among different lines of sorghum to produce the optimal conditions in which sorghum can be grown as an ethanol feed.

The fermentation of sweet sorghum presents several challenges. There are many variables that needed to be addressed, and each had unique complications that needed to be overcome. These included how to keep the process sterile, what type of reaction vessel should be used, how much inoculant to use, how to create an anaerobic environment and how to accurately quantify ethanol. The research presented in this paper focused on overcoming these challenges in order to develop a fermentation process with a high ethanol yield.

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Materials and Methods

Yeast Culture Preparation and Storage

The Ethanol Red variety of the yeast *Saccharomyces cerevisiae* was selected for the fermentation procedure. This type of yeast is common in industrial ethanol production because it is tolerant to high levels of ethanol and produces a high yield.

The yeast was obtained in an active dry form and rehydrated in distilled water at 30°C for 30 minutes. The yeast was then transferred onto a plate of Yeast-Mold Agar and incubated at 30°C overnight. Initially, the yeast was stored on the YM plates in the incubator, being transferred to a new plate every two to three days. However, this method has been replaced with cryopreservation of the yeast in a YPD Broth/Glycerol mixture at -20°C.

Yeast Growth Characterization

The first goal was to determine if the yeast could grow at all in the sweet sorghum juice. A sample of yeast was transferred from one of the YM plates to the juice and placed into the incubator overnight. A standard inoculation loop was used to transfer a sample of the juice back to a YM plate and incubated overnight. A sample from that plate was examined under a microscope and compared to a sample of yeast to ensure that the yeast had grown in the juice.

The next goal was to characterize the growth constant for the yeast and determine the doubling time in both aerobic and anaerobic environments. An inoculation loop of yeast was transferred from an overnight culture in YPD broth to two flasks of juice. One flask was kept open (loosely covered with foil) and the other was closed with a rubber stopper. Cell density measurements were taken every two hours for twelve hours.

The density of yeast was initially determined by performing a plate titer. This method has since been replaced by measuring the UV/Visible spectrum absorbance at 570 nm. Cell counts can be approximated using Beer's Law, shown in Equation 1 below.

$$A = \varepsilon bc$$

Equation 1

In this equation, A is the absorbance, b is the path length through which the light travels, c is the concentration and ε is the extinction coefficient, a constant that depends on the solution and the wavelength of light.

Yeast growth in solution is typically divided into three phases: a lag phase where the yeast's metabolism is changing because of the new environment, an exponential growth phase and a plateau phase where yeast have reached their maximum density.

Yeast growth in its exponential phase can be modeled by the differential equation:

$$\frac{dx}{dt} = \mu X$$

Equation 2

Solving this equation leads to an equation for exponential growth:

$$X(t) = X_0 e^{\mu t}$$

Equation 3

X_0 is considered the initial population size and μ is a growth constant.

Yeast Inoculant Preparation

In order to inoculate the sorghum juice for fermentation, cryopreserved yeast aliquots were reconstituted in a 30°C water bath. After centrifugation, the broth/glycerol cryopreservation mixture was replaced with pure broth and the yeast was transferred into an incubator to grow for 24 hours.

Juice Characterization

The sweet sorghum juice used in these experiments was grown by the research groups of Dr. Mike Ottman and Dr. Dennis Ray at the University of Arizona. The characteristic of juice that is of most concern is its sugar content. Over the course of these experiments, sugar content was measured or approximated in three different ways, each with its own degree of accuracy. The least accurate method used a refractometer to measure the refractive index of the juice. This is scaled in units called degrees Brix, which is the weight to weight percent of sugar to solution. A more accurate method used was a colorimetric glucose assay kit. This kit contained reagents that react with glucose to produce a color change that could be measured with a UV/visible spectrophotometer. This method, however, was only useful for determining glucose content and did not provide any information about sucrose or fructose. The best method for determining sugar content was High Performance Liquid Chromatography, or HPLC. HPLC is a semi-automated process that, once set up, can measure the sugar levels in multiples samples quickly and accurately using a small sample volume. All HPLC measurements used in these experiments were performed by Dr. Ray's laboratory.

Juice Pretreatment

Several methods of pretreatment were researched to determine their effect on the fermentation procedure and its results. All juice samples were pH adjusted to 5.5 and centrifuged at 4000 rpm for 20 minutes before being used.

Another step in the juice pretreatment required the addition of salts to juice samples. Ammonium sulfate was added at 0.2% and magnesium sulfate was added at 0.05%, in accordance with values obtained from literature.

The initial protocol required juice samples to be sterilized before inoculated. In order to sterilize the samples, two methods were tested. The first method was to filter the juice using a 1.2 micron filter. This pore size was chosen because it would have filtered out any unwanted microorganisms. The second sterilization method was to autoclave the juice. Autoclaving involves exposing the juice to high pressure steam for several minutes. All autoclaving was performed on the Liquids setting. The effect on final ethanol production of autoclaving versus not autoclaving was analyzed in an experiment using the fermentation procedure described below.

Fermentation Procedure

The general procedure for ethanol fermentation was as follows:

1. Inoculate a sample with the yeast inoculant previously described
2. Allow the inoculated juice to ferment for a specified period of time
3. Collect and store samples at -20°C until measurements could be taken

Fermentations were generally run in triplicate in 125 ml Erlenmeyer flasks. The specific aspects of each of step were varied depending on what experiment was being run.

In order to determine the appropriate ratio of inoculant to juice, the inoculate density was varied while holding other reaction conditions constant. Densities of 1:10 and 1:100 were compared. The juice was allowed to ferment for 12 hours before samples were collected for ethanol quantification.

Another set of experiments was designed to determine the best way to achieve anaerobic conditions for fermentation. Three methods were compared: An open flask (as a control), a flask sealed with Parafilm and a flask both sealed with Parafilm and purged with N_2 gas for 30 seconds. Each condition was inoculated with a 1:10 density of inoculant, and samples were taken at 0, 10, 11 and 12 hours.

In order to determine an appropriate amount of time to carry out fermentations, an experiment was run with the following conditions: inoculant density of 1:10, reaction vessels purged with N_2 gas and samples taken at 24, 48 and 72 hours.

Further experiments were done in order to determine if samples could be fermented in a high-throughput capacity. Samples were fermented using a 1:10 inoculant density for 72 hours in a N_2 purged vessel. Fermentations were carried out on 96 well plates, 12 well plates, standard Petri dishes and 125 ml Erlenmeyer flasks.

Ethanol Quantification

Two methods were employed in order to quantify the ethanol production of fermentation trials. The first method was to measure the specific gravity of the solution using a hydrometer. Specific gravity is the ratio of the density of the liquid to the density of water as shown in Equation 4.

$$S.G. = \frac{\rho}{\rho_{H_2O}}$$

Equation 4

Because the density of ethanol is less than that of water, the specific gravity of a solution will decrease as the amount of ethanol in that solution increases. These measurements allowed for a relative estimation of the amount of ethanol produced. However, sugar content of a solution also affects the specific gravity. Because both sugar levels were decreasing and ethanol levels were increasing, another quantification method was necessary to determine the absolute ethanol production.

The EnzyChrom Ethanol Assay Kit from BioAssay Systems[4] was chosen as a method of accurately quantifying ethanol in samples. This kit used ethanol dehydrogenase, an ethanol specific enzyme, to produce a color change over time. The color change was measured as the change in optical density at 570 nm over 5 minutes. Ethanol standards were prepared as a calibration, from which unknown ethanol quantities could be determined.

The small amount of reagents provided with the kit required that new reagents be made. As each reagent was reproduced, calibrations were recorded in order to make sure that the kit was still functioning as intended.

Ethanol Yield

Ethanol yield is the mass-to-mass ratio of ethanol output to sugar input. Yield is an important benchmark for measuring the efficiency of the fermentation process. The maximum theoretical yield can be obtained from the stoichiometrically balanced equation for glucose decomposing into ethanol and carbon dioxide:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH$$

Equation 5

The theoretical maximum yield is 0.511g ethanol per gram glucose. The realistic maximum yield is always less than the theoretical yield because some carbon from the sugar must be used

[4] (BioAssay Systems, 2008)

for growth. The degree to which this happens depends on the yeast strain. According to data from an Ethanol Red supplier[5] the maximum practical yield is 0.48g ethanol per gram glucose.

[5] (Fermentis, 2009)

Results and Discussion

Yeast Growth Characterization

It is useful to take the logarithm of the growth curve and check for linearity. Doing this allows the detection of a lag phase, which often is hard to detect on the standard growth curve.

The logarithmic form of Equation 3 is linear, as shown in Equation 6.

$$\ln X = \ln X_0 + \mu t$$

Equation 6

Figure 1 and Figure 2, below, show the growth curve of yeast in sweet sorghum on a normal and logarithmic scale, respectively. The R^2 values close to 1 for the linear fit trend lines indicate that the data is exponential. This means that the variety of yeast used has a very short lag phase of less than 2 hours, when the first sample was measured.

Having a short lag phase is important for industrial applications. Longer lag phases require longer fermentation periods and will often lead to decreased ethanol yields at higher costs.

The doubling time can also be determined from the growth constant, as shown in Equation 7,

$$T_{double} = \frac{\ln 2}{\mu}$$

Equation 7

where μ is the slope of the logarithmic graph. This makes the doubling times for closed and open growth 3.25 hrs and 3 hrs, respectively.

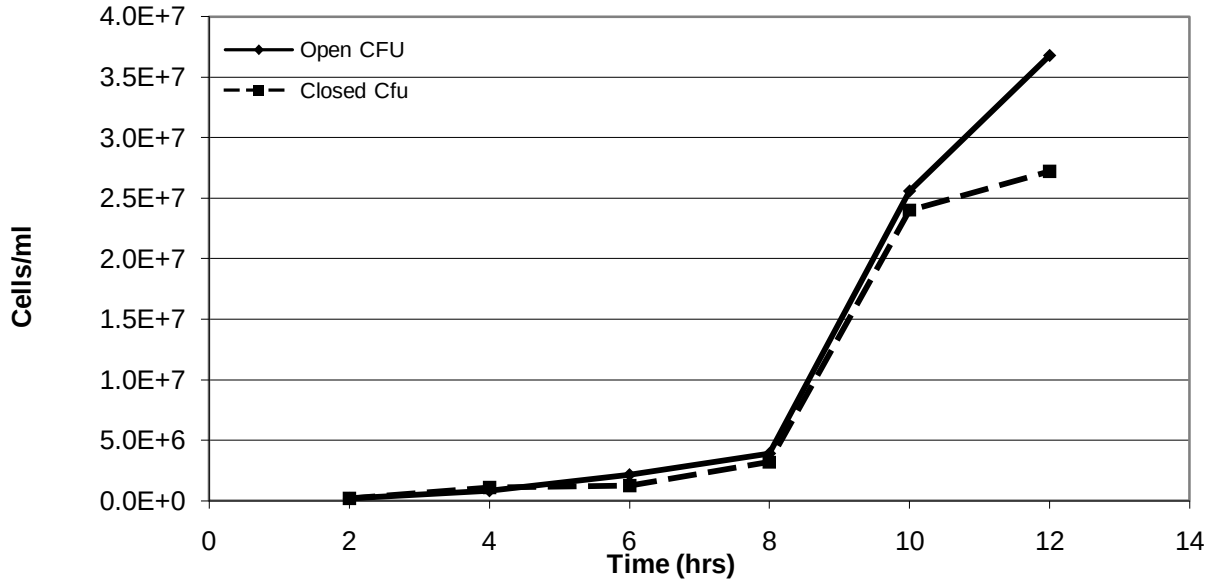

Figure 1. Yeast Growth in Sorghum

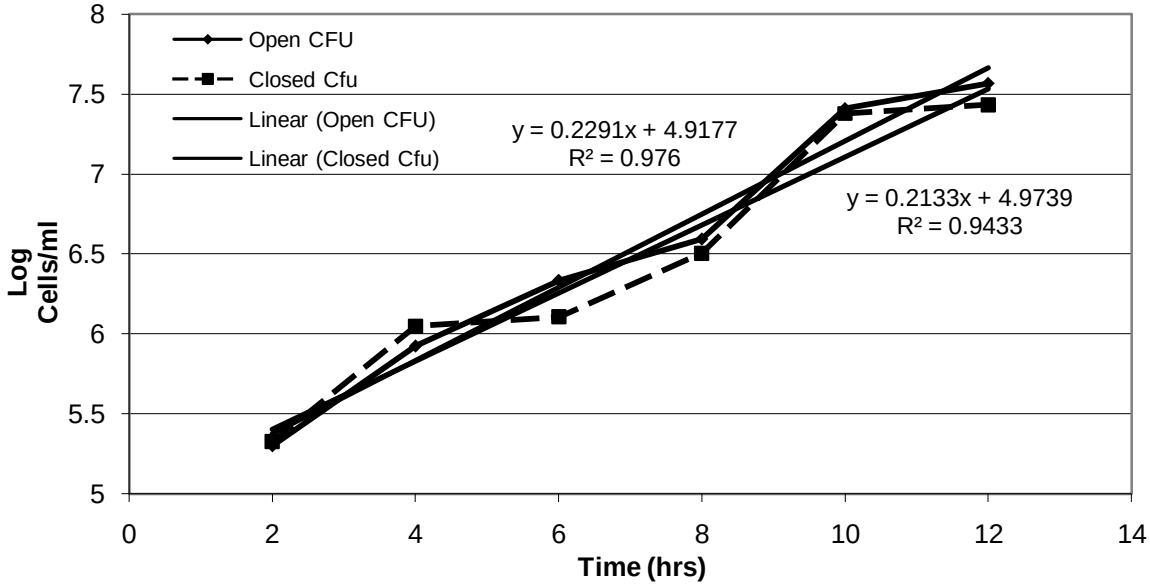

Figure 2. Yeast Growth in Sorghum (Logarithmic Scale).

Juice Characterization

The data on sugar content has for the most part been researched by Dr. Ray's Lab. Appendix A contains data from the 2007 season harvest and Appendix B Contains data on the 2008 season's harvest.

Juice Pretreatment

The pretreatment step was an important factor on the overall ethanol production. If the juice contained too many microorganisms before the inoculant was added, there would be a competition for resources and the yeast would have less sugar for ethanol production. Out of the two sterilization methods described, only autoclaving was successful. It was found that the juice was too viscous to be filtered through micropore filters with diameters small enough to remove bacteria. Autoclaving also presented challenges. An unidentifiable residue forms when the juice is heated for too long. It is possible this is created through the Maillard reaction, in which sugars combine with amino acids at high temperatures. Any loss of sugar should be avoided, so other sterilization methods have been evaluated. Presently, antibiotics are added to the juice right after it is being harvest in order to decrease the amount of unwanted organisms present.

Fermentation Procedures

In order to gain repeatable ethanol production results, it was first necessary to determine an appropriate amount of yeast for inoculating samples. The chart presented in Figure 3 shows the results of this experiment. The specific gravity is expected to decrease over time as sugar is converted to ethanol because ethanol is less dense than water and sugar solutions. The chart shows that, while all three samples started with the same specific gravity, after 12 hours there were noticeable differences. The 1:100 condition showed the least amount of change, which was expected because there were less microorganisms to utilize the sugars in biological processes. The 1:10 nitrogen purged flask showed considerable decrease in 12 hours. This was also expected because the high density of yeast combined with the lack of oxygen.

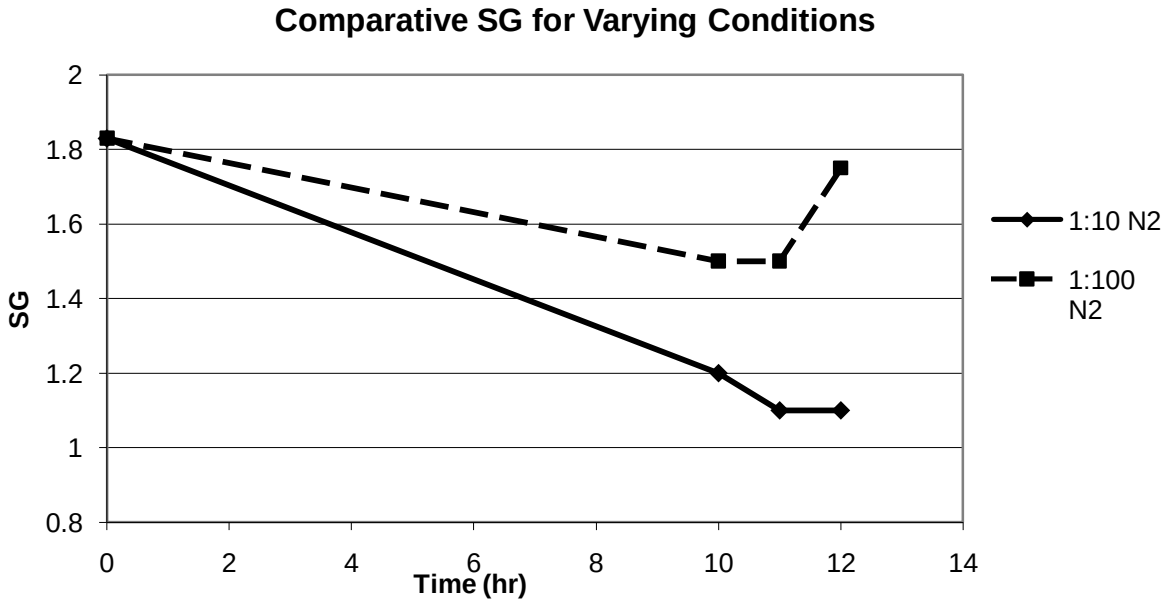

Figure 3. Inoculant density experiment results

Yeast requires an anaerobic environment in order to produce ethanol. Two different conditions and a control were examined. The first experimental condition used a closed flask that was purged with nitrogen. This, in theory, would cause the yeast to switch immediately to their ethanol producing metabolism. The other condition was to close the flask without purging. The yeast would be allowed to grow and use the oxygen in the flask before switching to their ethanol producing metabolism. Figure 4 shows the results of these tests.

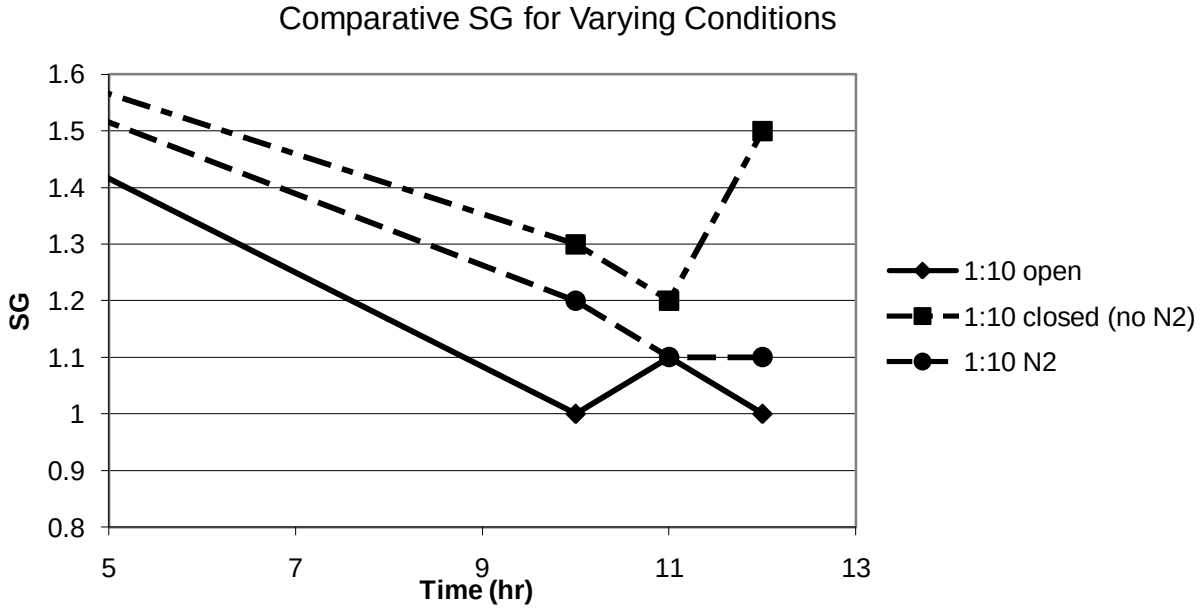

Figure 4. Anaerobic Conditions experiment results.

This chart shows that the nitrogen purged flask resulted in a lower final specific gravity reading than the closed flask. It is reasonable that the closed flask had less ethanol produced, because some of the sugar present in the juice would have been used for growth and therefore unavailable for ethanol production.

Two anomalies are present in this chart. The high reading of the closed flask at 12 hrs is likely to be a misread error. This is possible due to the hydrometer used lacking precision. The other anomaly is that the open flask shows the lowest specific gravity. This could be because the change in specific gravity is dominated more by sugar decrease than ethanol increase. Because of these inaccuracies and uncertainties, it is not recommended that ethanol be quantified using a hydrometer, but rather using a more precise and repeatable method.

The last important fermentation protocol concerned the overall time the juice should be allowed to ferment. Figure 5 shows the results of a fermentation run for 72 hours.

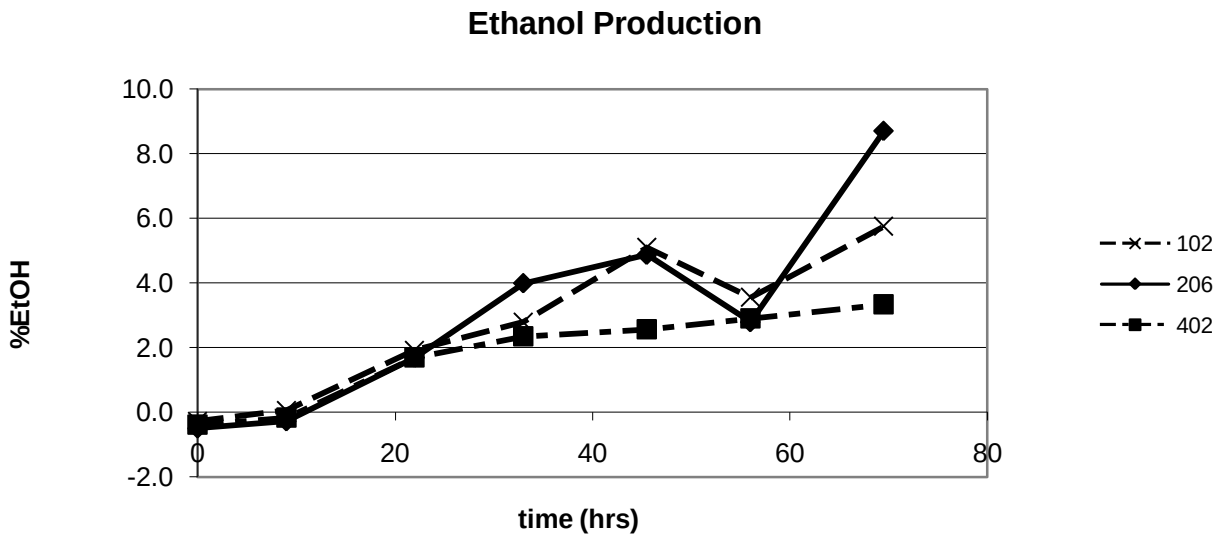

Figure 5. Ethanol production over a 72 hour period.

Ethanol production continues until at least 72 hours. The different series correspond to different sorghum samples, and each set was fermented in triplicate. The data points shown are the average values.

Several fermentations were attempted in a high-throughput capacity. Wells on both 96- and 12-well plates were filled with juice and inoculated with a 1:10 inoculant density. After fermenting for 72 hours, little ethanol could be detected. It was determined that the small radii of the wells discouraged the mixing needed to keep the yeast in suspension where they would produce ethanol.

Ethanol Quantification

While measuring the specific gravity with a hydrometer gave relative ethanol levels, absolute quantification of ethanol required the use of a colorimetric ethanol assay. The kit from BioAssay Systems was chosen because it provided both accuracy and a good detection limit. In order to use the kit, the change in optical density (color) of a sample was compared to the change in color of standards with known ethanol content. The standard readings were used to create a calibration curve such as the one in Figure 6. Ethanol percentages in unknown samples could be determined by interpolating the curve. Samples were typically diluted into the range of the calibration and the resulting percent would be multiplied by the dilution ratio to obtain the final ethanol percentage.

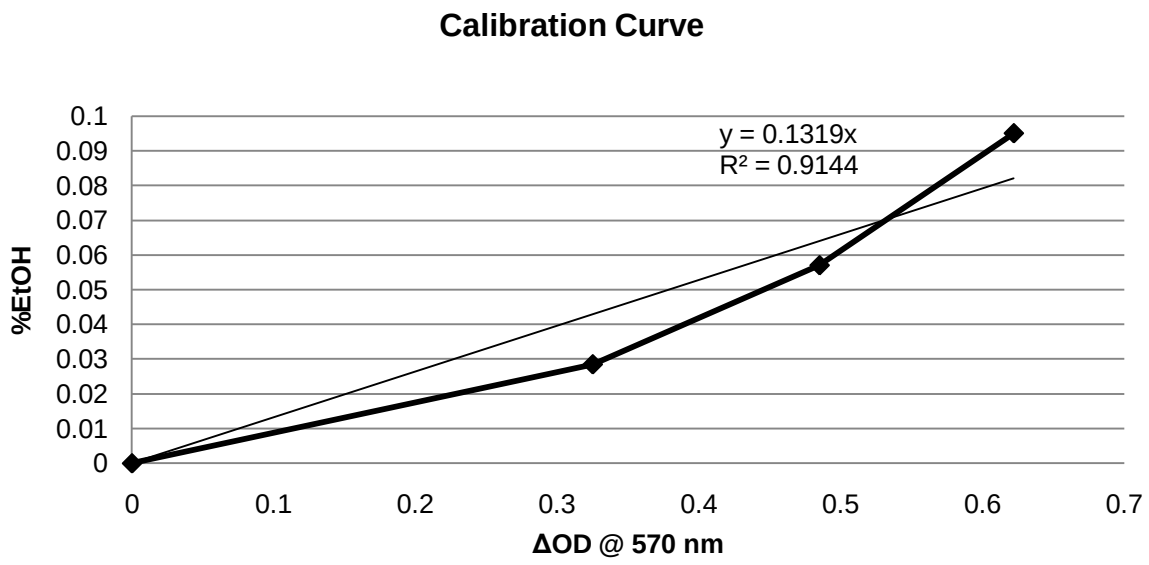

Figure 6. A calibration curve.

There were a few problems encountered while using the ethanol assay kit. The biggest problem was not actually the kit itself, but the UV/Visible spectrum plate reader used to detect optical densities. The Opsys MR plate reader has an accuracy of ±0.01 OD units[6]. When the range of the calibration curve was from 0-0.8 OD units, this produced an accuracy of the kit of 0.001 % ethanol. However, as the reagents degraded over time, the range of the OD change decreased significantly. There would often only be a maximum OD change of 0.08 OD units or less. This produced an accuracy of ±0.01% ethanol. Because samples were typically diluted at a ratio of 1:100 with water to be in the correct range of the calibration curve, the overall accuracy of using the ethanol assay was ±1% ethanol. This also limited the upper bound of ethanol detection to 10% because any samples with higher ethanol content would require a 1:1000 dilution and would have an accuracy of ±10%.

Figure 7 shows a one series from the same ethanol production chart in Figure 5. The calibration associated with this chart only produced an optical density change of 0.03 OD units, resulting in a final measurement accuracy of ±3.33%. This is indicated by the error bars.

[6] (Dynex)

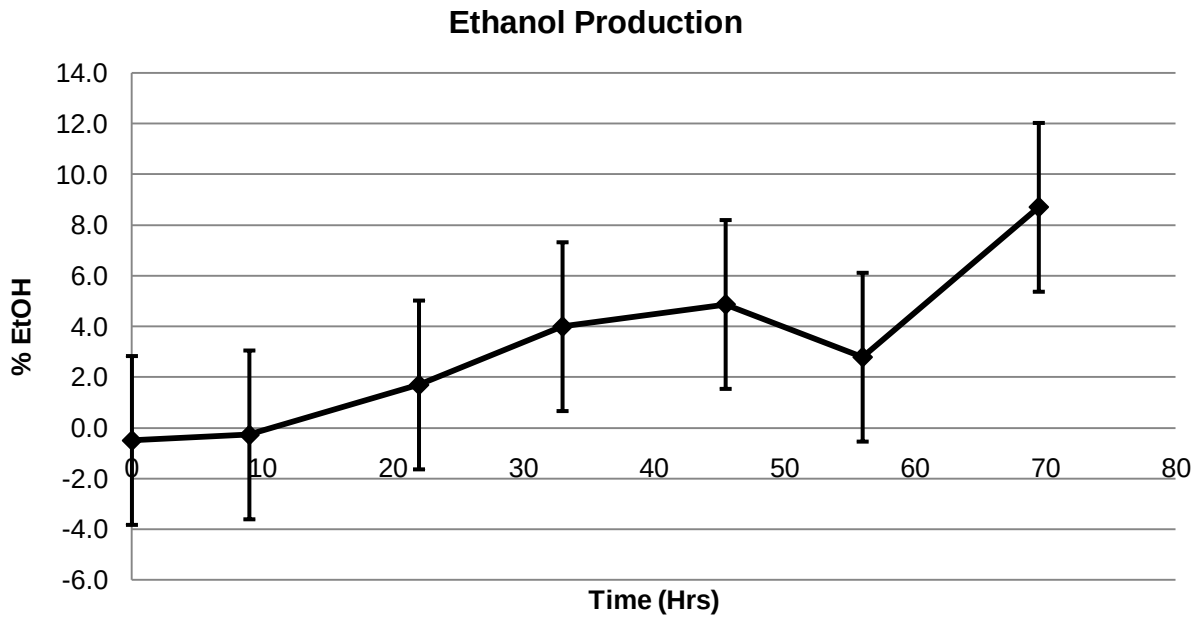

Figure 7. Ethanol production with measurement error.

The relatively large margin of error makes results difficult to analyze. From this chart it is impossible to tell if there is 0 or 4% ethanol at 24 hours and the margin of error at the 72 hour point is over half of the measured value.

Another problem using the ethanol assay was that the amounts of reagents in the kit were limited, and the kit was relatively expensive. In order to work around this, reagents had to be recreated from raw chemicals. This required a stoichiometric analysis of the chemical process involved in producing the color change.

Ethanol Yield

Yield calculations have shown interesting data trends. Yield is determined by converting ethanol percentages into grams of ethanol per liter and dividing by the corresponding grams of sugar per liter for the sample. In general, ethanol yield decreases as sugar concentrations increase. This means that, despite more total ethanol production, the fermentation process becomes less efficient when more sugar is present. Figure 8 shows this trend.

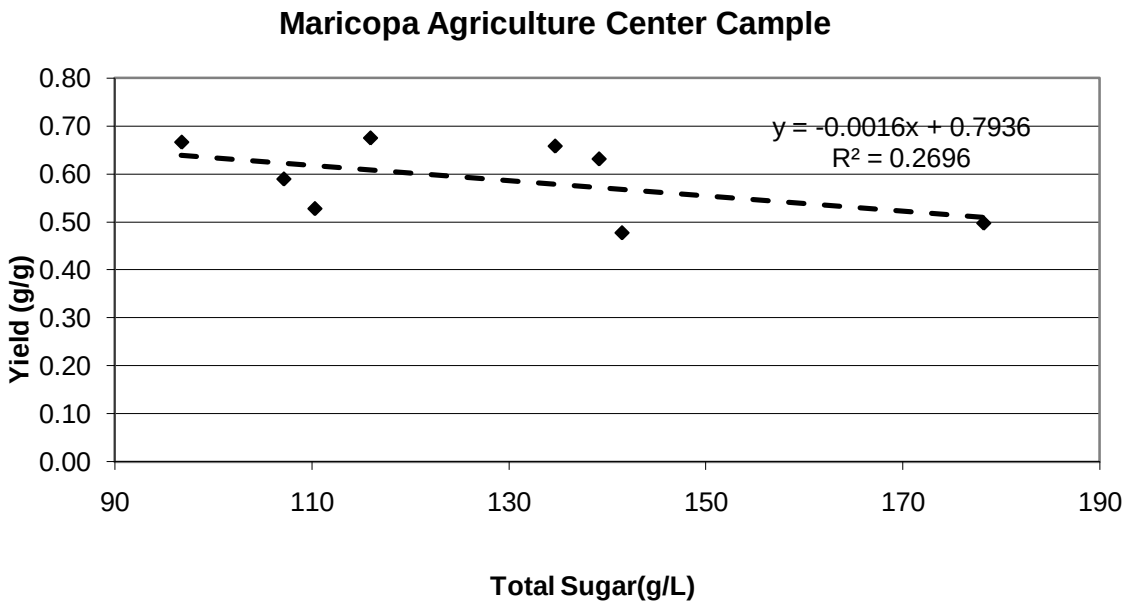

Figure 8. Yield correlation to total sugar.

It should be noted that some of the yields are higher than both the practical and theoretical values. This could potentially be attributed to several things. The previously discussed accuracy of the ethanol assay kit affects the accuracy of the yield numbers. Also, the total sugar values only include glucose, fructose and sucrose even though other unknown sugar polymers have been detected in the juice. These unknown sugars are potentially being digested and fermented while not being taken into account in the yield calculations.

Conclusions

The ethanol fermentation process involves many factors other than yeast and sugar. Many parameters must be carefully considered before the process begins in order to obtain accurate, repeatable results. A strain of yeast should be chosen that efficiently converts sugar to ethanol. Juice should be pH adjusted to suit the needs of the yeast and contamination should be avoided to improve yields. In order to jump start the yeast's fermentation metabolism, it might be beneficial to eliminate any oxygen from the reaction vessel. Fermentation should be continued for at least 72 hours because ethanol is still being produced. Lastly, an accurate way to measure ethanol production should be used. The colorimetric assay, while itself accurate, was ineffective because the absolute amount of ethanol the assay was able to detect was much smaller than the amount of ethanol in the samples. This required dilutions to be performed that amplified measurement error.

Future Work

In order for this project to continue developing in both potential and merit, a new method of ethanol quantification must be employed. Colorimetric assay kits that worked with higher percentages of ethanol are available and might be useful. It might also be possible to recalibrate the reagents used presently to work with higher percentages of ethanol. Gas chromatography and high-performance liquid chromatography would ultimately be the best choices for ethanol quantification.

For the fermentations, it would be beneficial to look at what sugars are being consumed at different points in the fermentation process. This would help the plant science aspect of the project by providing information about what sugars are most beneficial for producing ethanol quickly.

Lastly, it would be beneficial to run scaled-up fermentation models to test if the same results are achievable in larger scale operations. The challenges here would be ensuring appropriate mixing and keeping the mixture sterile.

Acknowledgements

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Appendix A – 2007 Sugar Analysis Data

Plot #	Brix (Ray Lab)
101	12.4
102	12.7
103	11.5
104	11
105	10.7
106	12
107	12.5
201	12.6
202	11.5
203	10.5
204	11
205	10.8
206	12.5
207	12.3
301	11.47
302	10.5
303	9.5
304	11.4
305	10.3
306	10.9
307	12.2
401	11.5
402	10
403	11
404	11.9
405	9.9
406	11
407	12.7

Appendix B – Sample 2008 Sugar Analysis Data

2008 HPLC DATA

plot	planting date	harvest date	total sugar (g/L)
A1	4/25/2008	6/5/2008	11.47071
A2	4/25/2008	6/5/2008	0
A3	4/25/2008	6/5/2008	
B1	4/25/2008	6/5/2008	0
B2	4/25/2008	6/5/2008	0
B3	4/25/2008	6/5/2008	6.069903
C1	4/25/2008	6/5/2008	0
C2	4/25/2008	6/5/2008	9.29277
C3	4/25/2008	6/5/2008	0
D1	4/25/2008	6/5/2008	
D2	4/25/2008	6/5/2008	0
D3	4/25/2008	6/5/2008	0
A1	4/25/2008	6/23/2008	15.55383
A2	4/25/2008	6/23/2008	27.98568
B1	4/25/2008	6/23/2008	9.44802
B2	4/25/2008	6/23/2008	19.88736
C1	4/25/2008	6/23/2008	19.32582
C2	4/25/2008	6/23/2008	22.75842
D1	4/25/2008	6/23/2008	10.46942
D2	4/25/2008	6/23/2008	15.77544
A1	4/25/2008	7/29/2008	51.58533
A2	4/25/2008	7/29/2008	31.5168
A3	4/25/2008	7/29/2008	56.45721
B1	4/25/2008	7/29/2008	26.85543
B2	4/25/2008	7/29/2008	24.80364
B3	4/25/2008	7/29/2008	26.07546
C1	4/25/2008	7/29/2008	95.90241
C2	4/25/2008	7/29/2008	102.5172
C3	4/25/2008	7/29/2008	99.5526
D1	4/25/2008	7/29/2008	32.75088
D2	4/25/2008	7/29/2008	29.56023
D3	4/25/2008	7/29/2008	28.13496
A1	5/20/2008	7/29/2008	29.74107
A2	5/20/2008	7/29/2008	25.55508
A3	5/20/2008	7/29/2008	22.02228
B1	5/20/2008	7/29/2008	21.70506
B2	5/20/2008	7/29/2008	23.82093
B3	5/20/2008	7/29/2008	12.69951
C1	5/20/2008	7/29/2008	49.24323
C2	5/20/2008	7/29/2008	43.9713
C3	5/20/2008	7/29/2008	31.31658
D1	5/20/2008	7/29/2008	16.16823
D2	5/20/2008	7/29/2008	15.92274
D3	5/20/2008	7/29/2008	19.1286

C1	4/25/2008	8/26/2008	124.612
C2	4/25/2008	8/26/2008	137.7442
C3	4/25/2008	8/26/2008	155.369
A1	4/25/2008	9/16/2008	148.5958
A2	4/25/2008	9/16/2008	141.2232
A3	4/25/2008	9/16/2008	151.5672
C1	5/20/2008	9/16/2008	150.3534
C2	5/20/2008	9/16/2008	150.5265
C3	5/20/2008	9/16/2008	