Continuous Production of Ethanol *via* a Magnetically Stabilized Bioreactor

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Abstract

The goal of this project was to produce ethanol using a magnetically stabilized, continuous, bioreactor. This was achieved by the entrapment of *Saccharomyces Cerevisiae* yeast in calcium alginate beads, doped with magnetite. The bioreactor was constructed in-house and consisted of a flow through chamber in which the beads were stabilized with an array of magnets. Ethanol production was monitored over a period of up to 8 days using gas chromatography analysis of headspace vapor. Immobilization of yeast cells allowed for the optimization of ethanol production. With a working volume of 250mL, a 13% ethanol concentration was achieved within 72 hours. The system continued to produce ethanol at this level for an additional 5 days with the daily addition of glucose and new media. In the future this system would be designed in a manner that varying feed stocks and organisms could be used to optimize the production of ethanol while reducing the time and costs associated with current methods.

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Introduction

The world energy demands are projected to increase 49 percent from 495 quadrillion British thermal units (BTUs) in 2007 to 739 quadrillion BTUs in 2035 [1]. In 2008, the United States consumed 99.2 quadrillion BTUs, approximately 20 percent of the total energy used in the world [2]. Roughly 85 percent of that energy was generated through the use of conventional fossil fuels such as oil, coal and natural gas [3]. Also in 2008, 71% of the 37.1 quadrillion BTUs of the energy used from petroleum went towards the transportation sector which consumed in total 27.8 quadrillion BTUs [4]. While the United States used 26.4 quadrillion BTUs of energy from petroleum for transportation, only 3% of the total transportation energy consumed came from a renewable source [4]. Considering transportation was the second largest sector for US fuel consumption, only 11% of the total renewable energy produced went towards transportation [4]. With such an enormous energy quota to fill, the need for alternative renewable energy is of concern.

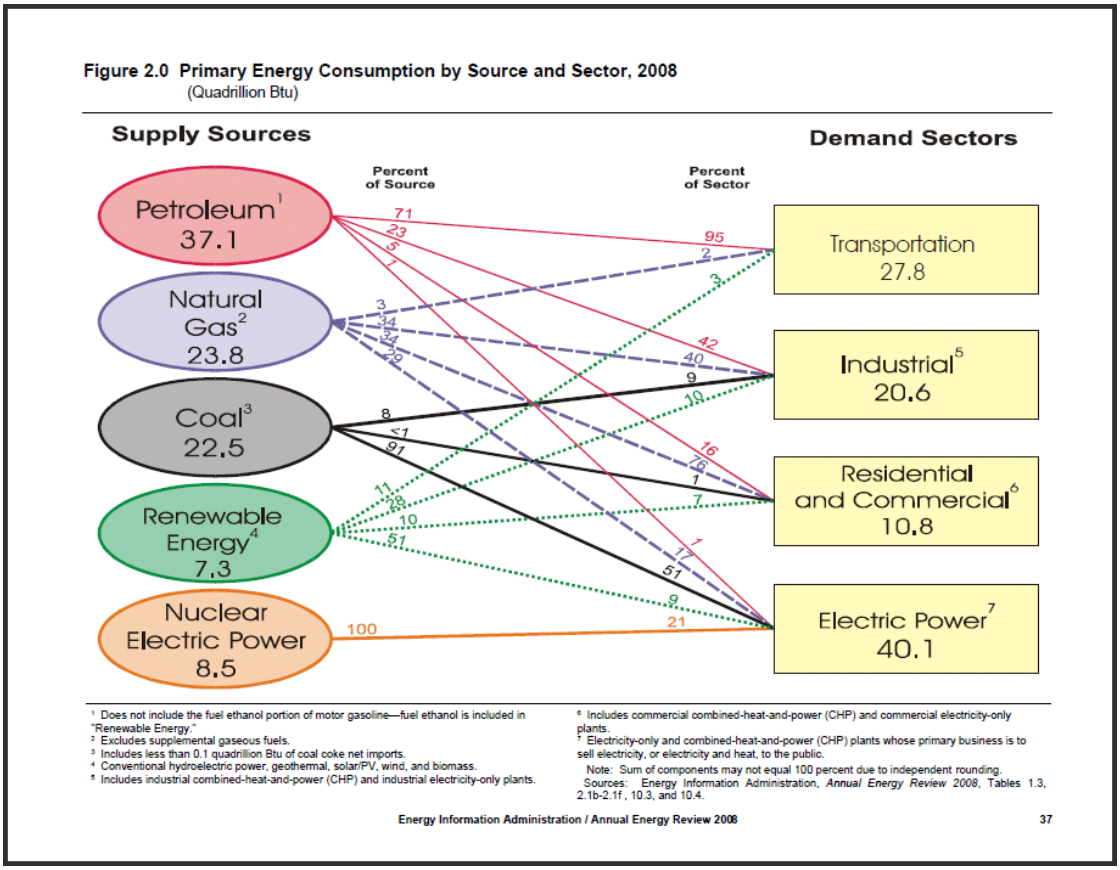


Figure 1: Primary energy consumption by source and sector [4]

In the past, renewable energy sources have generally been more expensive to produce and use compared to fossil fuels. Currently, renewable energy sources make up 8 percent of the total energy consumption in the United States [4]. The most common sources are hydropower, wind, solar and biofuels such as ethanol [5]. These systems can be beneficial when implemented in the right location. As seen with biofuels, the majority of production comes from the Midwest. This is where there is an abundance of corn which is the current feed stock for ethanol production. Another concern is the environment. Due to varying climate conditions in each area, it is hard to maintain a consistent energy supply for many of these sources. For example, during droughts, the reduced amount of water hinders hydroelectric power production; during cloudy days, solar power production is reduced; during calm days wind power cannot be used and if the supply of the carbon source is interrupted, biofuels cannot be manufactured. Currently there is not one source that will solve all of the energy demand problems which is why many companies are doing research to find new sources and methods of energy production.

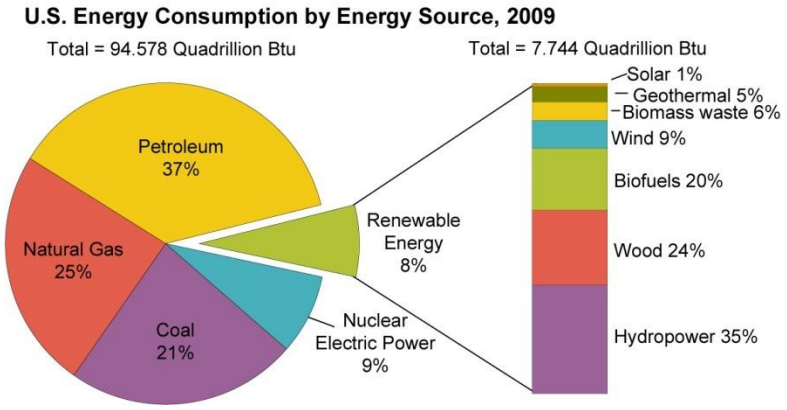


Figure 2: U.S. Energy Consumption by Energy Source [5]

Ethanol production from the fermentation of corn is one of the major energy sources that have become more prominent over the past thirty years. From 1981 to 2010 the consumption of commercially produced ethanol for fuels increased from 7 trillion BTUs to 1,017 trillion BTUs per year [6]. In 2008, of the 833 trillion BTUs of renewable transportation fuel consumed, 793 trillion BTUs came from ethanol consumption [4]. About 99 percent of all fuel ethanol produced is added to gasoline in varying concentrations. All gasoline powered vehicles can run on a 90 percent gas and 10 percent ethanol mixture. Starting in 2007, it was mandated that all engines

must be designed to run on 85 percent gas and 15 percent ethanol. A flex-fuel vehicle has also been designed which can run on 85 percent ethanol and 15 percent gasoline [7].

The current method of producing ethanol has been found to be too expensive, requires too much energy and is linked to environmental problems. David Pimentel, leading professor of ecology and agriculture at Cornell University, concluded that "abusing our precious croplands to grow corn for an energy-inefficient process that yields low-grade automobile fuels amounts to unsustainable subsidized food burning" [8]. Currently large scale production of ethanol is only possible due to the $1 billion a year in current federal and state subsidies. Without these subsidies to large corporations ethanol production for biofuel use would be much less due to the inefficiency in the process [8].

The inefficiencies of corn based ethanol production starts with the growing of corn. An acre of corn contains roughly 7,110 pounds of corn which can be converted into about 328 gallons of ethanol. In order to grow and harvest the crops however an investment of about 140 gallons of fossil fuels are necessary, costing about $350 per acre. This means that even before ethanol is produced, it costs $1.05 per gallon of ethanol [8].

Once the corn reaches the processing plant, a series of energy dependent steps are required for crushing the grain, fermenting the mixture, distillation and further separating of ethanol from water. The combined processes use 131,000 BTUs of energy to make one gallon of ethanol. One gallon of ethanol however only provides 77,000 BTUs which means it requires about 70 percent more energy to make a gallon of ethanol than the ethanol contains [8].

After processing, the cost to make one gallon of ethanol is around $1.74 while the cost to produce one gallon of gasoline is only $0.95. Since it is cheaper to produce and use fossil fuels, ethanol is not used to produce more ethanol [8]. Pimentel says, "The growers and processors can’t afford to burn ethanol to make ethanol. U.S. drivers couldn’t afford it, either, if it weren’t for government subsidies to artificially lower the price" [8].

Another problem that is overlooked is the environmental damages associated with producing ethanol from corn. Currently growing corn in the same spot in repeating years, also known as monocropping, depletes the soil of nutrients faster compared to growing multiple crops [8]. Due to this, corn growing erodes soil 12 times faster than the soil can reform. Reforming soil involves a natural process where no crops are grown in that area, allowing for the replenishment

of nutrients. Also, the amount of water needed for irrigation can deplete groundwater sources 25 times faster than the natural recharge rate of the ground. The cost of these problems adds another $0.23 per gallon bringing it to $1.97 to produce one gallon of ethanol [8].

However, despite the hidden costs associated with ethanol production, some people would rather see their vehicles fueled by ethanol that was produced in the United States instead of using imported fossil fuels. Pimentel calculated the amount of corn necessary to power an average car from the United States driving 10,000 miles a year solely on 100 percent ethanol. It was determined that it would require 11 acres of land to produce 852 gallons of ethanol. This same amount of land can feed seven Americans. Theoretically, if every automobile was fueled by 100 percent ethanol, 97 percent of the entire United States would be necessary for growing corn crops [8]. Comparatively, a company investing in algae biodiesel states that it would require 7 million acres of land to replace 15% of the current fuel usage. If ethanol was used it would require 90 million acres of land to replace the same 15%. Currently Sapphire is able to achieve an efficiency of 3,000 gallons/acre/year [31]. Due to the production of ethanol from corn being inefficient, researchers are looking into alternative crops which take less time to grow, produce more ethanol and are cheaper [8].

Table 1 is a chart comparing different sources which could be used for biofuel production. As can be seen, corn produces the least amount of fuel per hectare; however it is being implemented the most due to federal and state subsides.

Table 1: Comparison of some sources of Biofuels [9]

Crop	Oil yield (L/ha)	Land area needed (M ha) [a]
Corn	172	1540
Soybean	446	594
Canola	1190	223
Jatropha	1892	140
Coconut	2689	99
Oil palm	5950	45
Microalgae [b]	136,900	2
Microalgae [c]	58,700	4.5

a For meeting 50% of all transport fuel needs of the United States.
b 70% oil (by wt.) in biomass.
c 30% oil (by wt.) in biomass.

The goal of this project was to be able to design a small scale system which could continuously produce ethanol. Theoretically this technology could be used in the future for large scale ethanol production. The system would be designed in a manner that varying feed stocks and organisms could be used to optimize the production of ethanol and reduce the time and costs associated with current methods.

Background

Alcoholic fermentation is the process of converting sugar to energy with ethanol and carbon dioxide as waste products. Yeast and some types of bacteria are examples of micro-organisms that can carry out this process. Yeast are unicellular eukaryotes classified in the fungi kingdom. The most common strain of yeast, Saccharomyces Cerevisiae, has been used for alcoholic fermentation and baking for thousands of years [10].

The process of alcoholic fermentation occurs when there is a lack of oxygen and the yeast cells cannot fully carry out the remaining steps of cellular respiration. Cellular respiration is the metabolism of nutrients into energy with the release of waste products. The first series of steps in cellular respiration is called glycolysis. Further metabolism is carried out by the Krebs citric acid cycle (TCA cycle). Glycolysis is a ten step process that converts one glucose molecule into two pyruvate molecules. The ten steps can be divided into two separate stages. Stage I (steps 1-5) require an initial energy investment of two Adenosine triphosphate (ATP) molecules. During stage II (steps 6-10) energy is recovered from four ATP molecules. The total process allows for a net production of two ATP molecules from Adenosine diphosphate (ADP) and inorganic Phosphate (P_i). ATP, ADP and P_i are essential for energy production and transfer in all eukaryotic cells. Figure 3 shows the structures of ATP and ADP [10].

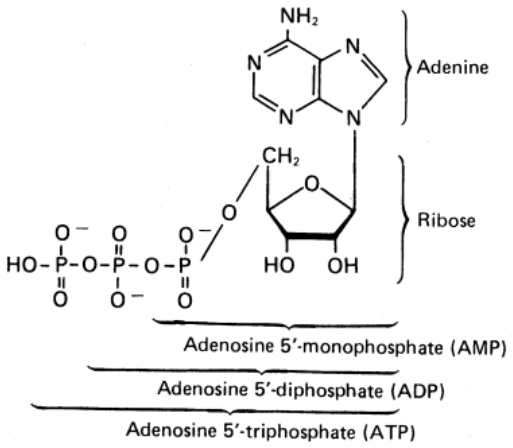


Figure 3: Structure of ATP and ADP [10]

Steps of Glycolysis

Stage I – Energy Investment

During steps 1-5 of glycolysis, two high energy ATP molecules are converted to two lower energy ADP molecules. The first step of glycolysis involves the phosphorylation of a glucose molecule. A phosphate group from ATP binds to the C-6 carbon of glucose. This results in ATP being converted to ADP and glucose becomes glucose-6-phosphate. This higher energy form of glucose is more unstable which drives the following steps. Glucose-6-phosphate is converted to fructose-6-phosphate to make a more symmetrical molecule during the second step. The third step phosphorylates the C-1 carbon of fructose-6-phosphate. The second ATP molecule is converted to ADP and fructose-1,6-bisphosphate is formed. During the fourth step, cleavage of fructose-1,6-bisphosphate yields glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). Both GAP and DHAP are three carbon monosaccharides. During glycolysis however, only GAP can be used for further reactions. The fifth step of stage I involves converting DHAP into GAP to be used in the stage II reactions. Figure 4 shows a summary and detailed outline of these steps [10].

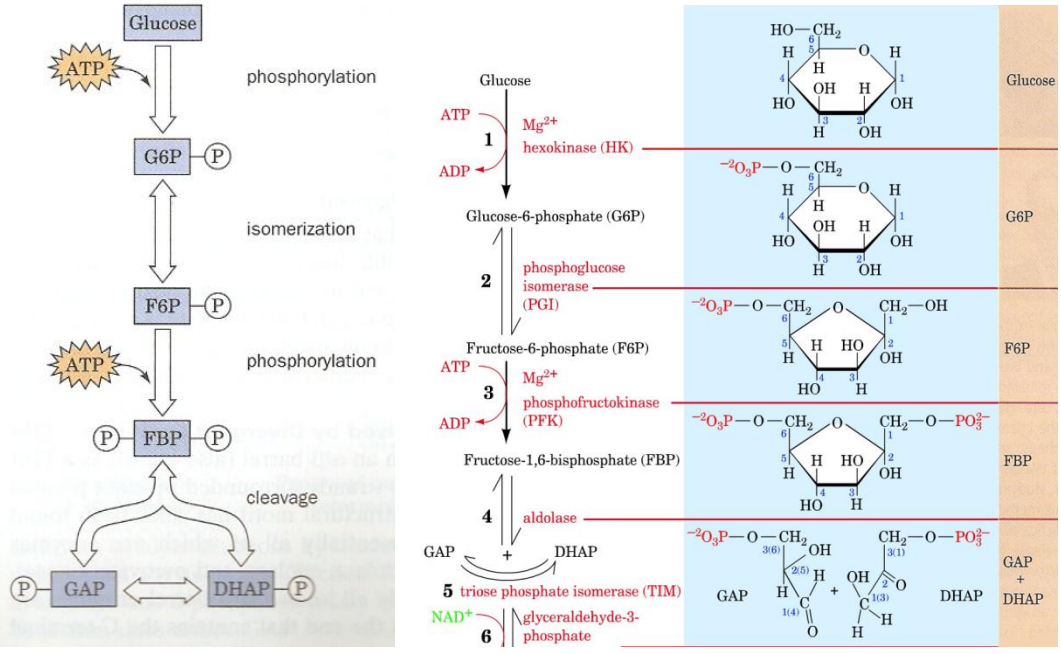


Figure 4: Summary and Detailed Steps of Stage I of Glycolysis [10]

Stage II – Energy Recovery

During steps 6-10 of glycolysis, two GAP molecules are oxidized yielding four ATP molecules and two pyruvate molecules. The first step of stage II or the sixth step of glycolysis is the oxidation and phosphorylation of GAP by P_i and NAD^+. This reaction yields 1, 3-bisphosphoglycerate (1,3-BPG). The seventh step is the first step where energy is recovered in the form of two ATP molecules. 1,3-BPG and ADP react to form 3-phosphoglycerate and ATP. To carry out the ninth step of glycolysis an energetically neutral reaction must occur first. During the eighth step, 3-phosphoglycerate is converted to 2-phosphoglycerate. In the ninth step a high energy phosphorylated compound is made by the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The last step of glycolysis yields pyruvate and the final two ATP molecules by the reaction of phosphoenolpyruvate and ADP. Figure 5 shows a summary and detailed outline of these steps [10].

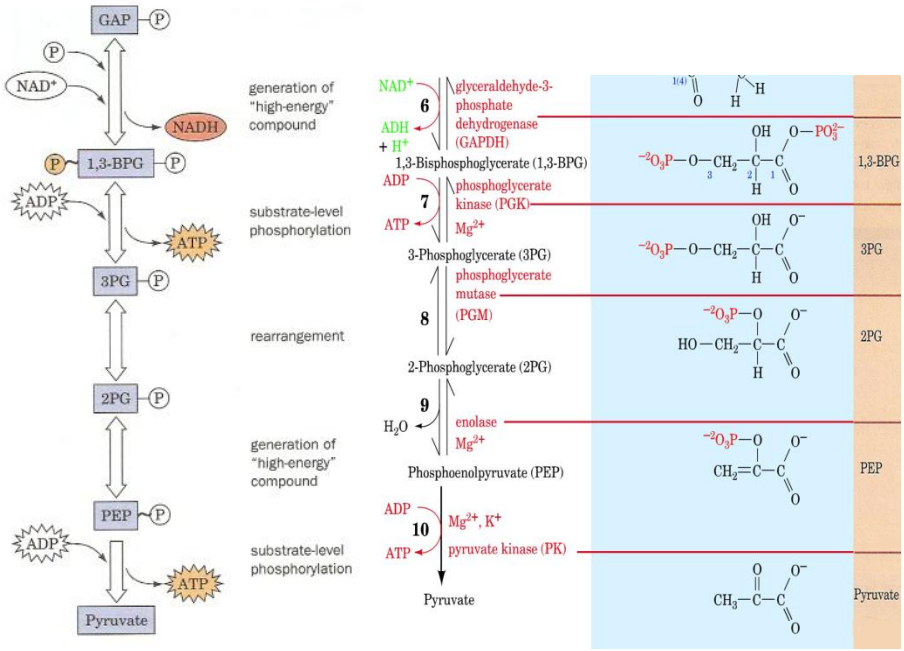


Figure 5: Summary and Detailed Steps of Stage II of Glycolysis [10]

Fates of Pyruvate

After glucose is converted to pyruvate *via* glycolysis, there are three fates for pyruvate depending on the amount of oxygen present. Aerobic environments have an excess of oxygen while anaerobic environments are in short supply of oxygen. In an aerobic environment

pyruvate is completely oxidized through a series of reactions called the citric acid cycle. All eukaryotic cells can carry out this reaction. In an anaerobic environment, ATP can be synthesized rapidly through homolactic fermentation or alcoholic fermentation. However, unlike the citric acid cycle, either homolactic fermentation or alcoholic fermentation will occur depending on the organism. Figure 6 shows the different pathways pyruvate can take [10].

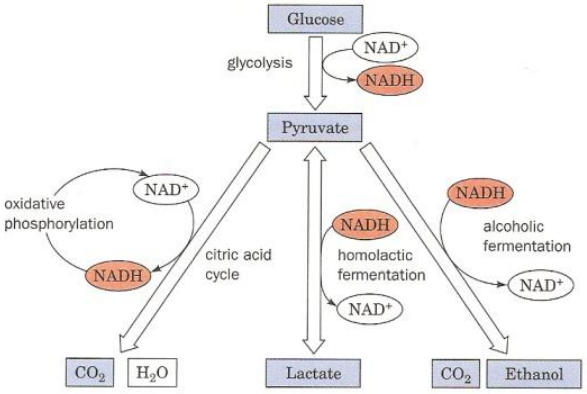


Figure 6: Fates of Pyruvate [10]

The Citric Acid Cycle

The citric acid cycle is usually thought of as a supplemental series of reactions to glycolysis; however, the citric acid cycle is also responsible for recovering energy from macromolecules such as carbohydrates, fats and proteins. The citric acid cycle is a series of eight reactions that oxidize macromolecules and break them down into carbon dioxide and water. This generates different forms of usable energy and precursors for many compounds including certain amino acids. This process is essential for all living cells in aerobic and anaerobic conditions. In an aerobic environment the citric acid cycle will oxidize the macromolecules and pyruvate. In an anaerobic environment, the citric acid cycle will only oxidize the macromolecules [10].

To initiate the citric acid cycle a preliminary step of converting pyruvate to acetyl-Coenzyme A (acetyl-CoA) is necessary. The first step of the citric acid cycle is a condensation reaction between oxaloacetate and Acetyl-CoA. This process is named after the product of the

second reaction with yields a citrate molecule. The citrate molecule is then converted into isocitrate via a reversible isomerization reaction. The third step produces the first carbon dioxide and NADH molecules of the citric acid cycle. Isocitrate is oxidized by being decarboxylated into α-ketoglutarate.α-ketoglutarate is then further decarboxylated into succinyl-CoA producing the second carbon dioxide and NADH molecules. The fifth step of the reaction cleaves the high energy succinyl-CoA molecule and synthesizes a high energy triphosphate molecule and succinate. In animals guanosine triphosphate (GTP) is usually synthesized while in plants and bacteria adenosine triphosphate (ATP) is synthesized. Succinate is dehydrogenated into fumarate reducing a flavin adenine dinucleotide (FAD), into $FADH_2$ to be used as an oxidizing agent. The seventh step converts fumarate into malate by hydrogenating the double bond. The last and most important step involves regenerating oxaloacetate. The hydroxyl group on malate is oxidized to form oxaloacetate which means the citric acid cycle acts as a multistep catalyst which can oxidize an unlimited number of acetyl groups. Figure 7 shows a summary and detailed outline of these steps [10].

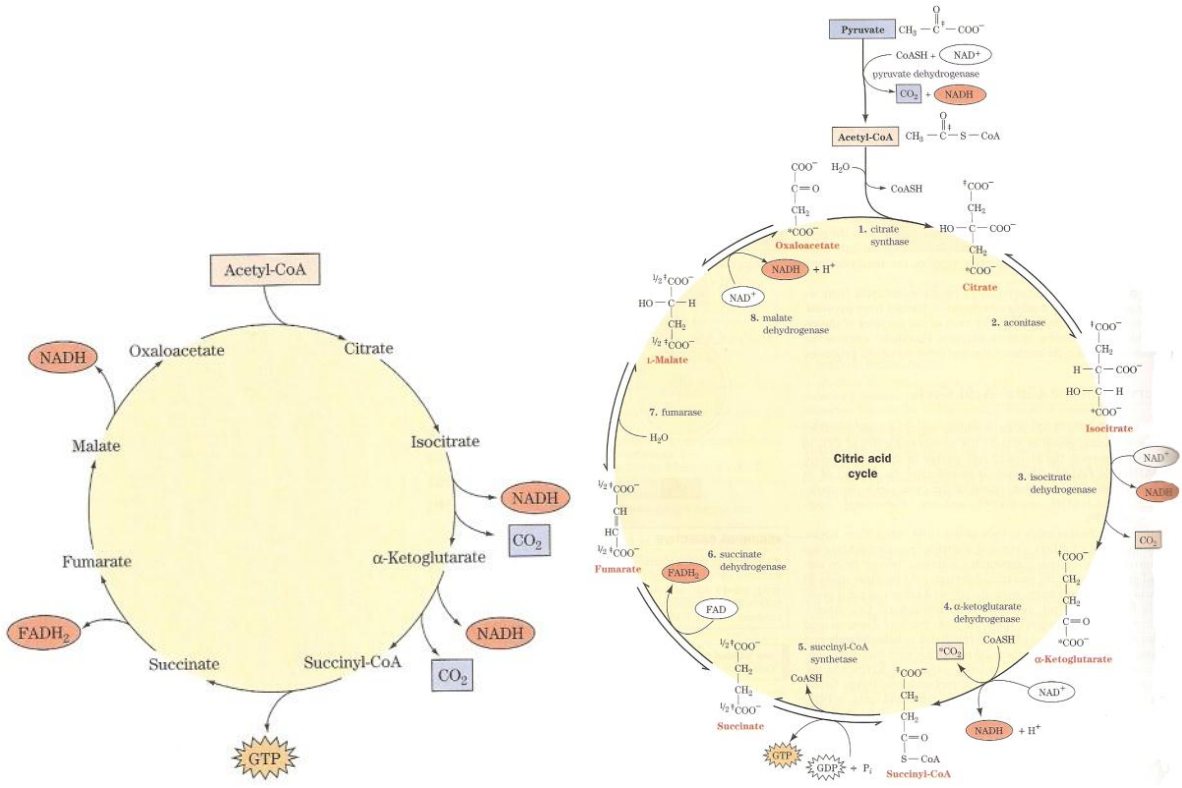


Figure 7: Summary and Detailed Steps of the Citric Acid Cycle [10]

Homolactic Fermentation

In humans, under strenuous activity, muscle cells will carry out homolactic fermentation when the oxygen supply is low. Pyruvate is reduced to lactate which quickly synthesizes ATP molecules to be used for energy. Homolactic fermentation is often referred to as step 11 of glycolysis because it is readily reversible and lactate and pyruvate are in equilibrium. Unfortunately the formation of lactate is essentially a "dead end" for glucose metabolism. The lactate is either converted back into pyruvate or it is exported from the cell and converted back into glucose in the liver [10].

Alcoholic Fermentation

Unlike mammalian cells, yeast cells carry out alcoholic fermentation when in anaerobic conditions. Alcoholic fermentation is the conversion of pyruvate into ethanol and carbon dioxide, producing NAD^+. This two-step process has been used for thousands of years in beer and wine making as well as providing the CO_2 to leaven bread. The first step involves the decarboxylation of pyruvate into acetaldehyde producing CO_2. The second step reduces acetaldehyde to ethanol by a NADH catalyzed reaction. The NAD^+ produced can be used for the sixth step of glycolysis [10]. This process can be seen in Figure 8.

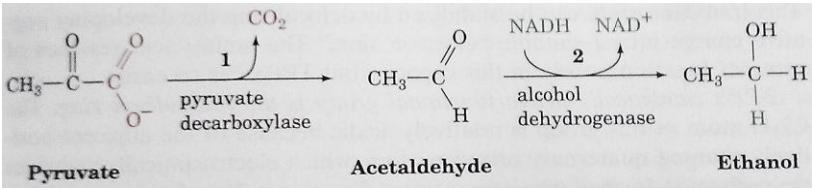


Figure 8: Conversion of Pyruvate to Ethanol [10]

Since ethanol is a waste product, at a certain point the yeast cells will not be able to survive any longer. Each strain of yeast is capable of surviving in varying ethanol concentrations. Yeast used for brewing beer can survive in 6-10% ethanol while champagne yeast can survive in 16-18% ethanol concentrations. In a study conducted by Kyung Man You, Claire-LiseRosenfield and Douglas C. Knipple from the Department of Entomology at Cornell University, it was determined that ethanol tolerance in Saccharomyces Cerevisiae is dependent on the lipid membrane fluidity [11]. The lipid membranes in Saccharomyces Cerevisiae are composed mostly of the two unsaturated fatty acids palmitoleic acid and oleic acid as seen in Figure 9. Palmitoleic acid is an omega-7 monounsaturated fatty acid and oleic acid is an omega-

9 monounsaturated fatty acid. When in an absence of ethanol, the lipid membrane is composed mostly of palmitoleic acid. When exposed to a 5 percent ethanol concentration, stearic acid is converted to oleic acid by an oxygen and NADH dependent desaturation reaction. This results in a fourfold increase of the ratio of oleic acid to palmitoleic acid. The increase of oleic acid results in a decrease in the membrane fluidity that counteracts the fluidizing effects of ethanol [11].

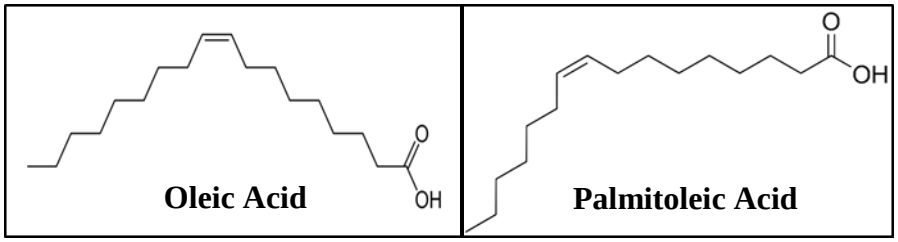


Figure 9: Comparison of Oleic Acid and Palmitoleic Acid Structures [10]

Stages of Fermentation

Fermentation occurs in two different stages. There is a primary fermentation stage and a secondary fermentation stage. During the primary fermentation stage, which lasts about three to five days, 70 percent of the fermentation activity will occur. Rapid fermentation and growth occurs resulting in a considerable amount of foaming. To remove the excess carbon dioxide produced, a fermentation lock is implemented allowing for a flow of gas out of the system while preventing an inward flow. Usually during this stage, the container is in an aerobic environment that allows for the growth of the yeast cells. Although most of the fermentation activity occurs, a lot of the yeasts energy is put into reproduction. Yeast cells are capable of multiplying by 100 to 200 times during the first few days alone [12] [13].

The secondary stage of fermentation can last anywhere from one to two weeks depending on the availability of nutrients. During this time the remaining 30 percent of fermentation activity occurs. This stage takes place in an anaerobic environment that results in a slower reaction. The lack of oxygen forces the yeast to use its energy for alcohol production instead of reproduction [12] [13].

Bioreactors

A bioreactor can be any device or system that sustains a biologically active environment [14]. The most common forms of bioreactors involve chemical reactions occurring in living

organisms such as fermentation and cell growth. These systems can range from small scale setups on a lab bench to commercially used setups requiring whole buildings. Bioreactors can be designed to control factors such as temperature, pH, pressure, light exposure and aerobic or anaerobic conditions [15].

There are two major types of bioreactors: batch and continuous. A batch bioreactor is a general name for a single vessel where a biological process occurs. Batch bioreactors are currently used for alcoholic fermentation processes. The reactor consists of a sealable tank, which can vary in size depending on what is needed, an agitator to stir the mixture and a heating and cooling system. Senor probes are used to monitor and thus maintain optimal reaction conditions. Reactants and vapor products are removed through connections at the top and any liquids and solids can be discharged out of the bottom of the tank. A schematic of this design can be seen in Figure 10. A batch bioreactor is a versatile piece of equipment because it can carry out various types of operations in the same system. This is important when containment cannot be compromised. It is also capable of operating in semi-batch mode. This entails priming the tank with one reactant and then slowly adding a second [15] [16].

The benefits of a batch bioreactor are:

- Common place in current industries and technology has been optimized
- An effective economic solution for many types of reactions
- Can also be used for distillation, crystallization, liquid-liquid extraction and even something as simple as storage
- Excellent at handling difficult materials

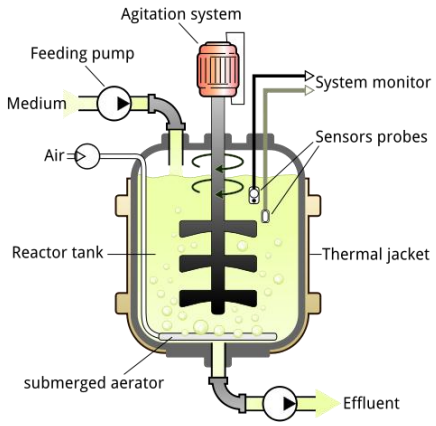


Figure 10: Schematic of Batch Bioreactor [15]

The second major type is a continuous bioreactor. Continuous bioreactors refer to a system which uses a flow of reactants to induce a biological process to occur producing products. This flow can be designed in a "looped" system or can flow from point A (reactants) to point B (products). Unlike batch reactors, continuous reactors are generally smaller and products can be removed upon formation further driving the reaction. Continuous reactors may be designed as pipes or a series of interconnected stages. Continuous bioreactors are currently used in many pharmaceutical, chemical and food industries [15] [16].

The benefits of a continuous bioreactor are:

- Products and waste materials can be removed while new media is introduced
- Greater separation between reactants and products giving a better concentration profile
- Smaller size allows for increased mixing rates
- Amount of product produced can be controlled by varying the run time, thus increasing operating flexibility for manufacturers

A promising continuous bioreactor design for 2010 comes from Renewed World Energies located in Georgetown, South Carolina [17]. A similar design can be used for varying micro-organisms and feed stocks. These bioreactors can also be paired with local industrial plants to use effluent sources. This particular bioreactor was designed with algae in mind, however with simple modifications to the bioreactor and the automation behind it, other organisms could be used. Every aspect of the system was designed to decrease cost and increase

yield of algal mass (Figure 11). This bioreactor contains vertical "ponds" that are spaced out to allow maximal sunlight absorption by each unit (Figure 11). The technology facilitates algae farm expansion by adding units through quick-connect piping headers. The system is fully automated which makes the cultivating process hands-free, and the design is also wireless which avoids wiring from each unit to the main controls which is very cost efficient. Back-flushing systems are used to remove algae that adhere to the sides of the ponds to promote highest yield during the algal mass harvest as well as avoiding the cost of expensive mechanical wipers to serve the same purpose. Furthermore, the liquid medium is mixed by impeller pumps to avoid shear that could damage cell structure. The design allows for a commercial scale production of algae biomass for a third of the cost of some smaller scale models [17].

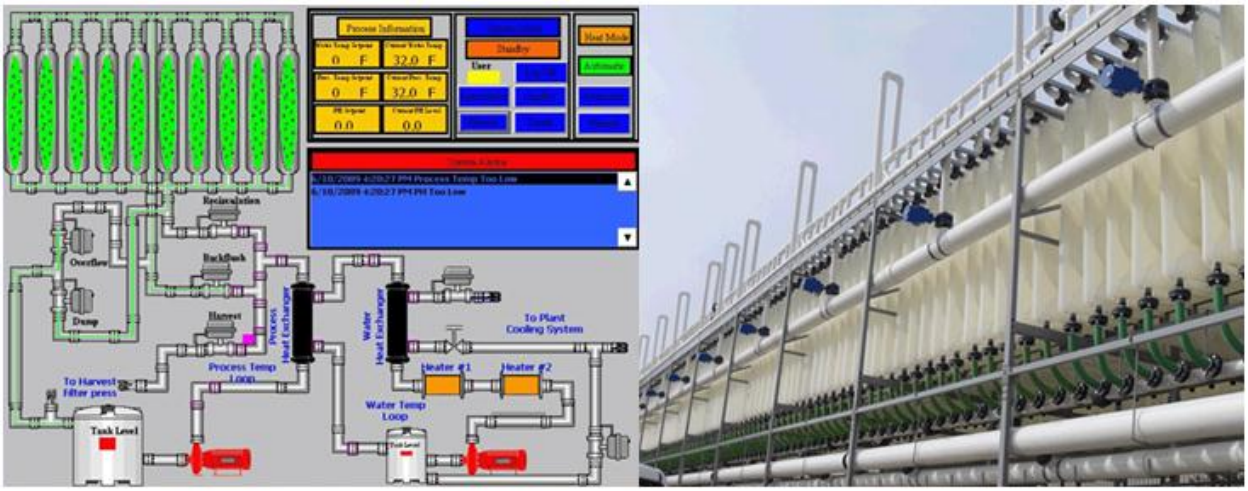


Figure 11: Blueprint and real-life view of Renewed World Energies bioreactor design [17]

Current Methods of Large Scale Batch Ethanol Production

Most ethanol is currently produced by yeast fermenting corn in large batch reactors. Currently the dry grind and wet mill processes are the two major ways of industrially producing ethanol [18]. In each of these cases, the reaction can be limited by three ways:

- If the ethanol concentration becomes too high, the yeast cells will die
- If the concentration of sugar becomes too low, the yeast will not be able to ferment
- Addition of enzymes to stop the reaction

Dry Grind Process

The dry grind process is the cheaper of the two and accounts for 67 percent of total ethanol production. The focus of a dry grind plant is solely to maximize the capital return per gallon of ethanol. The process involves grinding the corn and mixing it with water to form a corn slurry. The slurry is heated and allowed to cook. Enzymes are added which help aid in the conversion of the starches to sugars. The slurry is cooled and yeast is added. The yeast converts the sugars into ethanol resulting in a mixture of ethanol and solids. The mixture is first distilled to obtain roughly a 95 percent ethanol solution. It is then dehydrated using a molecular sieve to achieve a 100 percent pure ethanol solution. The remaining solids are dried and sold to be used as farm feed [18]. Figure 12 shows the overall dry grind process.

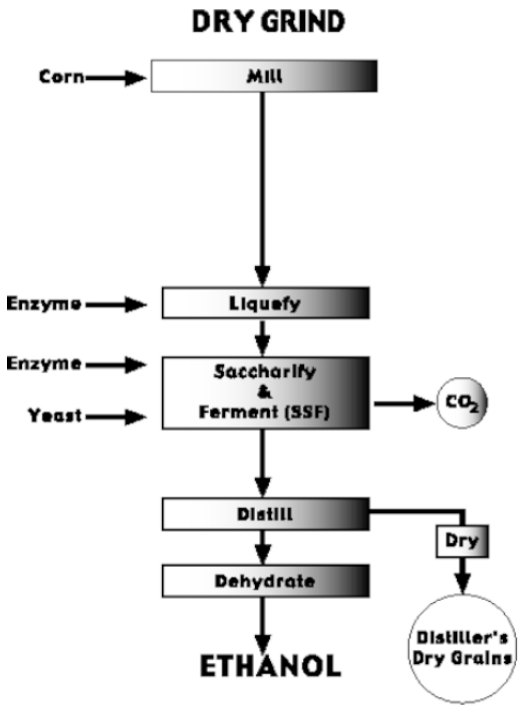


Figure 12: Overview of Dry Grind Ethanol Production [18]

Wet Mill Process

The wet mill process is more expensive and accounts for 33 percent of total ethanol production. This process requires more capital and energy however it can be considered a true biorefinery because it produces numerous high value products. Initially, the corn grain is separated into its core components of starch, fiber, gluten and germ. Separating the germ from the kernel allows for the extraction of corn oil. The left over germ meal is mixed with the fiber

and the hull of the corn. This produces a high protein corn gluten animal feed. The starch and sugar solution is separated from the solids and yeast is added. This solution produces ethanol which is then distilled and further dehydrated via a molecular sieve [18]. Figure 13 shows the overall wet mill process.

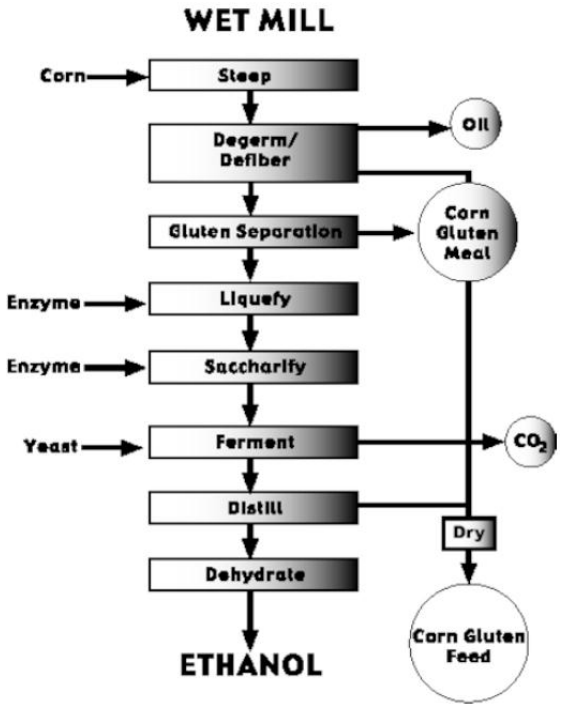


Figure 13: Overview of Wet Mill Ethanol Production [18]

Current Methods of Continuous Ethanol Production

The continuous production of ethanol is not a new concept. This entails simultaneously producing ethanol as well as extracting ethanol in the same system at the same time. Theoretically, this would be able to reduce costs and increase ethanol production as opposed to conventional batch methods. An innovative continuous two-stage bioreactor (Figure 14) was designed with a working volume of 13.5L and was able to produce ethanol at a rate of 41 g L^{-1} hr^{-1}[19]. The system contained a first reactor which focused on cell growth, and a second reactor dedicated to ethanol production. After fermentation, the media was filtered and permeate was collected. A feedback loop was implemented between reactor 2 and 1 as well as between the filter and reactor 2. This allowed for recycling of unused media and potential increase of cell activity [19].

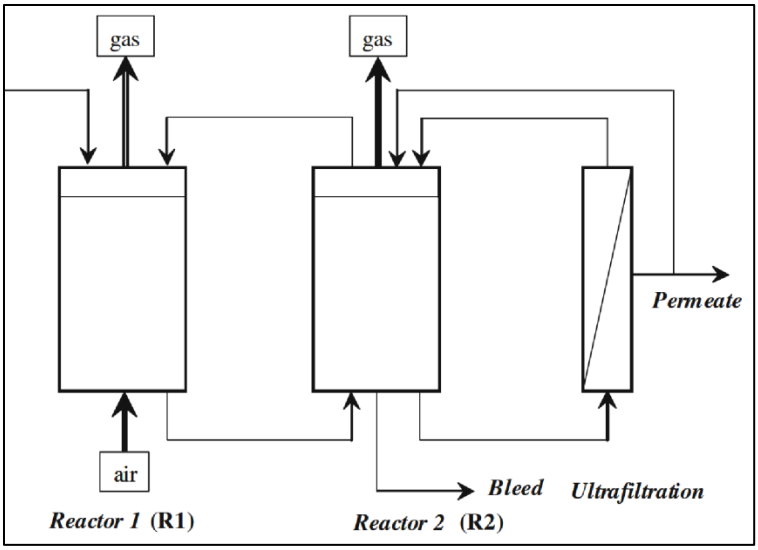


Figure 14: Simplified Schematic of Two-Stage Bioreactor[19]

Currently for small scale continuous bioreactors, distillation is not required to separate ethanol from solution (Figure 14). In large scale batch reactors, fossil fuels are needed to heat the mixture to a point where the ethanol will evaporate out of the solution. The distillation process uses more energy than the produced ethanol will be able to provide. For example, if gasoline is used to heat the solution, upon combustion gasoline provides 47.0 kJ/g of heat energy. Alternatively, when ethanol is combusted only 29.7 kJ/g of heat energy is generated. This means gasoline produces 1.6 times as much energy per gram compared to ethanol so there is a negative energy gain in the system [8]. In a continuous system the ethanol is immediately removed usually by a filter or molecular sieve that requires no outside energy resulting in a positive energy gain.

In a continuous system the ethanol is removed shortly after it is produced, which drives the fermentation reaction forward producing more ethanol. An additional benefit is that the yeast cells remain alive throughout the process because the total ethanol concentration of the system never becomes deadly. Different strains of yeast can survive in varying ethanol concentrations. Typical brewing yeast can withstand about 6% to 10% ethanol concentration.

Immobilization of Yeast Cells

An immobilized cell is defined as a living cell that, by natural or artificial means, is prevented from moving independently from its original location to all parts of an aqueous phase of a system [20]. Immobilization can reduce damaging shear forces caused from the liquid flow in a bioreactor, allow for the reduction of cell growth, contamination and a potential increase in product yield [20]. Immobilization of yeast can be achieved by entrapment of cells in a variety of different substrate matrices. Three different substrates that have been used before are pectin gel [21], carrageenan gel [22] and calcium alginate gel [23] [24].

Through literature research, calcium alginate was found to be the most beneficial. Calcium alginate is a water-insoluble gel. It is formed by aqueous sodium alginate reacting with aqueous calcium chloride. The gel matrix forms instantly when sodium is replaced with calcium ions. The alginate structure has a higher affinity towards calcium which induces a conformational change (Figure 15). Due to this property, if the sodium alginate is added to a calcium chloride bath drop wise, the resulting structure will maintain this shape. This process is not limited to only the entrapment of yeast cells. Any substrate or microorganism which can dissolve in the aqueous sodium alginate will be contained in the calcium alginate gel matrix [23] [24].

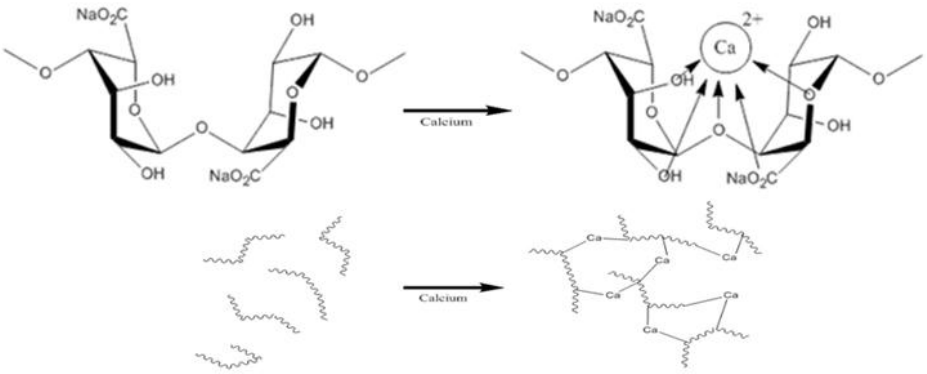


Figure 15: Formation of Calcium Alginate Matrix

Materials and Methods

Red Star Pasteur Champagne (Foxboro, MA) brewing yeast (*Saccharomyces Cerevisiae*) was obtained at a local brewing store and was used throughout all the experiments. Glucose and calcium chloride was obtained from Sigma Aldrich (St. Louis, MO).

Magnetic Calcium Alginate Beads with Yeast

A $CaCl_2$ (0.05M) bath was prepared in a glass dish with $CaCl_2$ (0.985g) and H_2O (175mL) and was stirred until completely dissolved. To proof the yeast, H_2O (50mL) was heated to 40°C and Red Star Pasteur Champagne yeast (4.903g) was added. Proofing yeast is a process of testing the viability of the yeast cells. The temperature is important for this step because if it is too hot or too cold the yeast can be destroyed. The mixture was removed from the heat source and was left to sit for 10 minutes, swirling occasionally. Sodium alginate (0.9855g) was added to a beaker of H_2O (25mL) and heated to 50°C to dissolve completely. Magnetite powder (0.795g) was then added to the sodium alginate solution and was swirled periodically to prevent settling of magnetite. The yeast mixture was then combined with the magnetite and sodium alginate solution along with H_2O (5mL). This solution was pumped drop wise through a hypodermic needle (gauge 20) at a flow rate of 12 mL/min. The individual calcium alginate beads (2mm diameter) containing yeast and magnetite were formed upon immediate contact with the $CaCl_2$ bath. The beads were allowed to cure at room temperature for fifteen minutes before using. This procedure was modified from the procedure found in *Alcohol Production by Magnetic Immobilized Yeast* [23].

Characterization of Calcium Alginate Beads with Stained Yeast

To show the distribution of yeast throughout the beads, yeast cells were stained with DAPI, a fluorescent stain that binds to the A-T areas of DNA. Beads were made with the stained yeast and were characterized using a confocal microscope. Confocal microscopy is an optical imaging technique that eliminates out-of-focus light to increase resolution and contrast. It uses point illumination and a spatial pinhole to better detect fluorescence very close to the sample compared to wide-field microscope. Due to only one point in the sample being illuminated at a time, imaging requires scanning over a rectangular pattern of parallel lines to create an image. Depending on the wavelength of light and lens used determines the potential scanning depth. All

of these parameters allow for confocal microscopy to be well suited for 3D imaging and surface profiling [25]. Figure 16 shows the components of a confocal microscope.

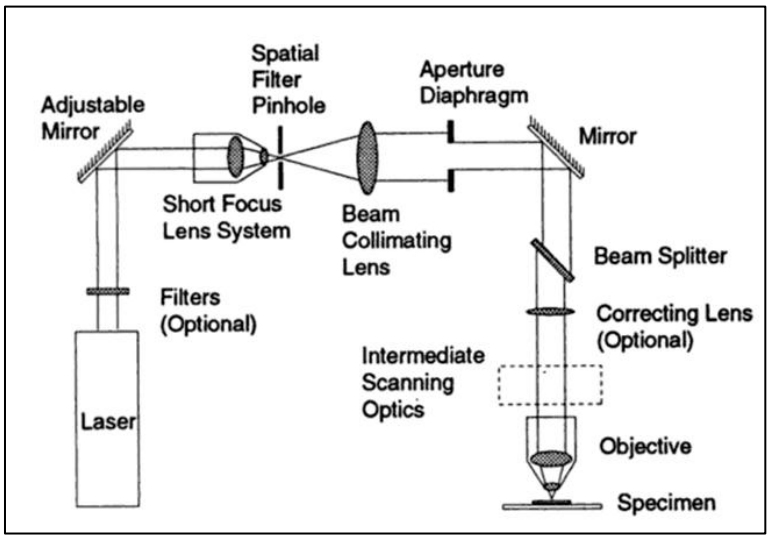


Figure 16: Components of a Confocal Microscope [25]

Yeast Staining

H_2O (15mL) was heated to 40°C. Yeast (0.1120 grams) and glucose (0.222 grams) were added and left to proof for 10 minutes. The mixture (15 mL) was placed into a centrifuge tube (15 mL volume) and was centrifuged for 5 minutes to allow for complete separation of the yeast/media. The media was extracted and DAPI (0.001 grams) dissolved in H_2O (2mL) was added to the centrifuge tube containing the yeast. The tube was covered with aluminum foil to avoid light exposure and was agitated via the vortex genie. The tube was allowed to sit for 10 minutes undisturbed. The mixture was placed into the centrifuge for 5 minutes to allow for separation of the yeast/DAPI solution. The solution was extracted and phosphate buffer solution (PBS) (3mL) was added. The mixture was agitated, centrifuged and the solution was extracted. This process was repeated a second time to insure complete rinsing of the excess DAPI. PBS (3mL) was added to the mixture and was left covered until used for bead making. The procedure for staining the yeast cells was modified from the procedure found in *Detection of Active Yeast Cells in Frozen Dough Sections* [26].

Calcium Alginate Beads with Stained Yeast

PBS/stained yeast mixture (1mL) was removed and diluted 10x via addition of PBS. This solution was added to 5 mL of a 4% (w/v) sodium alginate solution and was mixed

thoroughly. The mixture was pumped through a 20 gauge needle at a flow rate of 12 mL/min and beads were formed upon landing in a 0.05M $CaCl_2$ bath. The beads formed were allowed to cure in a dark drawer to avoid light exposure for 10 minutes.

Characterization of Calcium Alginate Beads with Stained Yeast via Confocal Microscopy

A bead containing stained yeast cells was placed into a dish where the bottom was the thickness of a slide cover. The confocal microscope took 75 pictures over a 22 micron range starting from the surface of the bead and moving inwards. The data was compiled into a z-stack and two different side view videos showing the total distribution of yeast in the observed region.

Generation of a Standardized Curve via Headspace Gas Chromatography

To be able to calculate the percent ethanol the bioreactor is producing over time, a standardized curve was generated. Prior to testing, the retention times of ethanol and 1-butanol were determined. By using an internal standard of 1-butanol, peak areas of the two alcohols were used to establish a ratio depending on ethanol concentration. A standardized curve was plotted to obtain a quadratic equation that would allow for the determination of an unknown ethanol concentration.

Samples with known concentrations of Ethanol

Seventeen, 10mL volumetric flasks were filled halfway with H_2O. To each flask, ethanol was added ranging from 0mL to 1.6mL increasing by 0.1mL per flask. The remainder of each flask was filled with H_2O to give a total volume of 10mL per flask. This provided 17 samples with ethanol concentrations ranging from 0% to 16% increasing by 1% per flask.

Addition of 1-Butanol as an Internal Standard

Seventeen, Gas Chromatography vials (2mL) were labeled 0 to 16. Each sample (1mL) was removed from the volumetric flask and put into its respective vial. 1-Butanol (30 µL) was then added to each vial. Each vial was covered with a septum cap and was allowed to sit over night to equilibrate at room temperature.

Gas Chromatography

A Perkin Elmer Gas Chromatograph with a Flame Ionization Detector (FID) was used. The FID temperature was set to 200^{o}C, the injector temperature was set to 200^{o}C and the oven

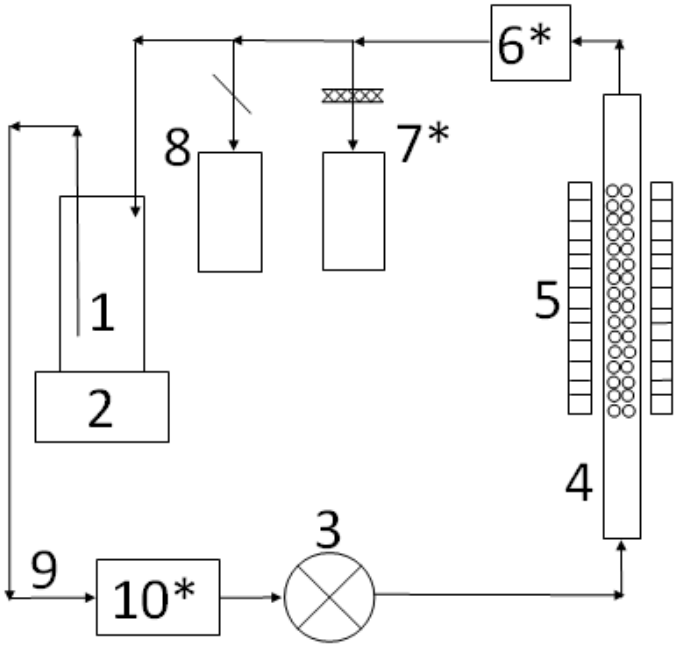
temperature was set to 40^{o}C. The air flow rate was set to 450mL/min and the H_2 flow rate was set to 45mL/min. Due to the inability of the column to handle aqueous solutions, gas chromatography of head space vapor was necessary. A vapor sample (2µL) was removed from each vial before being injected and tested for 2.5 minutes. Each sample was tested three times to obtain an average value. The areas of the peaks were integrated and the ratios of the ethanol to 1-butanol peaks were plotted vs. percent ethanol. A trend line was added giving a quadratic equation and a R^2 value. This equation allowed for the calculation of unknown ethanol percentages of bioreactor samples.

Bioreactor

Components of Bioreactor

The labeled diagram below shows the major components of the bioreactor. Objects with a * designates future implementation.

1: Initial medium flask (250mL)
2: Stirrer plate
3: Pump
4: Bioreactor with magnetic beads
5: Magnet holder
6*: Filter (<5 µm)
7*: Molecular sieve and collection flask
8: Sampling valve
9: Plastic tubing (2mm inside diameter)
10*: Dialysis unit



Magnet holder

In order to hold the beads in place a magnetic field surrounding the bioreactor was necessary. To do this a magnet holder was designed in SolidWorks, a 3-D computer aided design (CAD) software. The model was constructed using laser cut acrylic pieces which were glued together. Figure 17 below shows one side of the magnet holder. Each square represents where a magnet was placed. The magnets used were 0.5 inch cube neodymium magnets. To aid in

holding the magnets in place, set screws were added. To form the final product, two of these holders were glued together with a gap large enough to house the reactor part of the system.

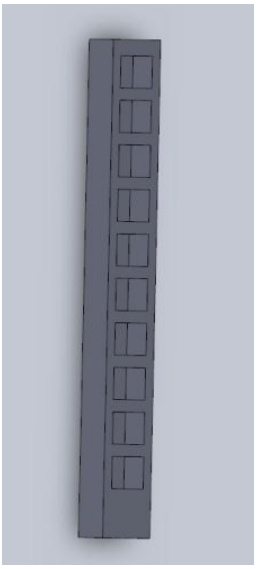


Figure 17: 3-D CAD of Magnet Holder

Fermentation

A medium solution was made consisting of H_2O (350mL), KH_2PO_4 (1.708g), NH_4SO_4 (0.748g), $MgSO_4$ (0.165g) [27]. Calcium alginate beads (15g) were removed from the $CaCl_2$ bath. To load the beads into the bioreactor, medium (50mL) was added to help correctly position the beads between the magnets. Medium (200mL) was added to the Erlenmeyer flask to reach a total working volume of 250mL. An initial amount of glucose (12.167g) was added to obtain a 0.26 molar solution. A magnetic stir bar was placed into the solution to help dissolve the glucose and maintain a uniform concentration throughout the system. The system was sealed to be air tight and the pump was set to a flow rate of 20mL/min. The reactor was left to ferment with samples being taken every 24 hours to test for ethanol concentrations. An additional amount of glucose (11g) was added after each sample was obtained. New media was also added at this point to make up for the lost volume of removing samples.

Headspace Gas Chromatography for Bioreactor Samples

The reactor was allowed to run for nine days. Seven samples were taken over this period of time to be analyzed. This allowed for the plotting of percent ethanol vs. time after being

compared to the standardized curve. Samples were taken from the system via a three-way valve. Upon turning the flow would be redirected to our collection flask as opposed to flowing through the system. The first 2mL were discarded to "clean" out the valve. An extra 3mL was collected as the sample and the valve was turned back allowing the system to flow normally. The sample was filtered through a 0.2 micron syringe filter and put into a labeled vial. The sample number, date, time and total hours passed since the reactor started were recorded.

Sample Preparation/ Adding 1-Butanol as an Internal Standard

Seven Gas Chromatography vials (2mL) were labeled 1 to 7. Each sample (1mL) was removed from the vial and put into its respective GC vial. 1-Butanol (30 µL) was then added to each GC vial. Each vial was covered with a septum cap and was allowed to sit over night to equilibrate at room temperature.

Gas Chromatography

A Perkin Elmer Gas Chromatograph with a Flame Ionization Detector (FID) was used. The FID temperature was set to 200^{o}C, the injector temperature was set to 200^{o}C and the oven temperature was set to 40^{o}C. The air flow rate was set to 450mL/min and the H_2 flow rate was set to 45mL/min. Due to the inability of the column to handle aqueous solutions, gas chromatography of head space vapor was necessary. A vapor sample (2µL) was removed from each vial before being injected and tested for 2.5 minutes. Each sample was tested three times to obtain an average value. The areas of the peaks were integrated and the ratios of the ethanol to 1-butanol peaks were calculated. The quadratic formula, generated from the standardized curve, was used to calculate the percent ethanol in each sample from the ratios of the peak areas. The percent ethanol was plotted vs. time (hours) to obtain the ethanol production rate of the bioreactor.

Results

Magnetic Calcium Alginate Beads with Yeast

The individual calcium alginate beads had an average diameter of 2mm (Figure 18) and an average mass of 0.02 grams. Figure 19 shows the total amount of beads used in the bioreactor with a mass of 14 grams. A penny was used as a scale next to approximately ten beads.

Figure 18: Average Diameter of Beads

Figure 19: Calcium Alginate Beads compared to a penny

The calcium alginate beads with yeast and magnetite were placed in a flask to demonstrate the magnetic properties of the beads. Figure 20 shows the beads under no magnetic influence (left) and the beads under magnetic influence (right). Neodymium magnets were used to induce the magnetic field.

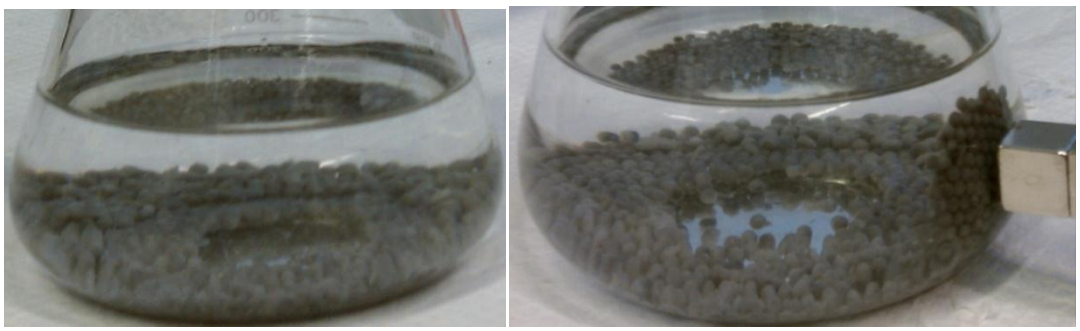


Figure 20: Beads with no Magnetic Influence and Beads with Magnetic Influence

Characterization of Calcium Alginate Beads with Stained Yeast

Yeast cells were stained with DAPI, a fluorescent dye that stains DNA, and a confocal microscope was used to obtain a z-stack of 75 pictures over a 22 µm range. Each blue dot represents the nucleus of an individual yeast cell. Figure 21 (a) and (b) show the distribution of all the yeast cells from (a) a top view and (b) a side view of the entire z-stack. Figure (c) shows the distribution of yeast at 5 µm and figure (d) shows the distribution of yeast at 10 µm.

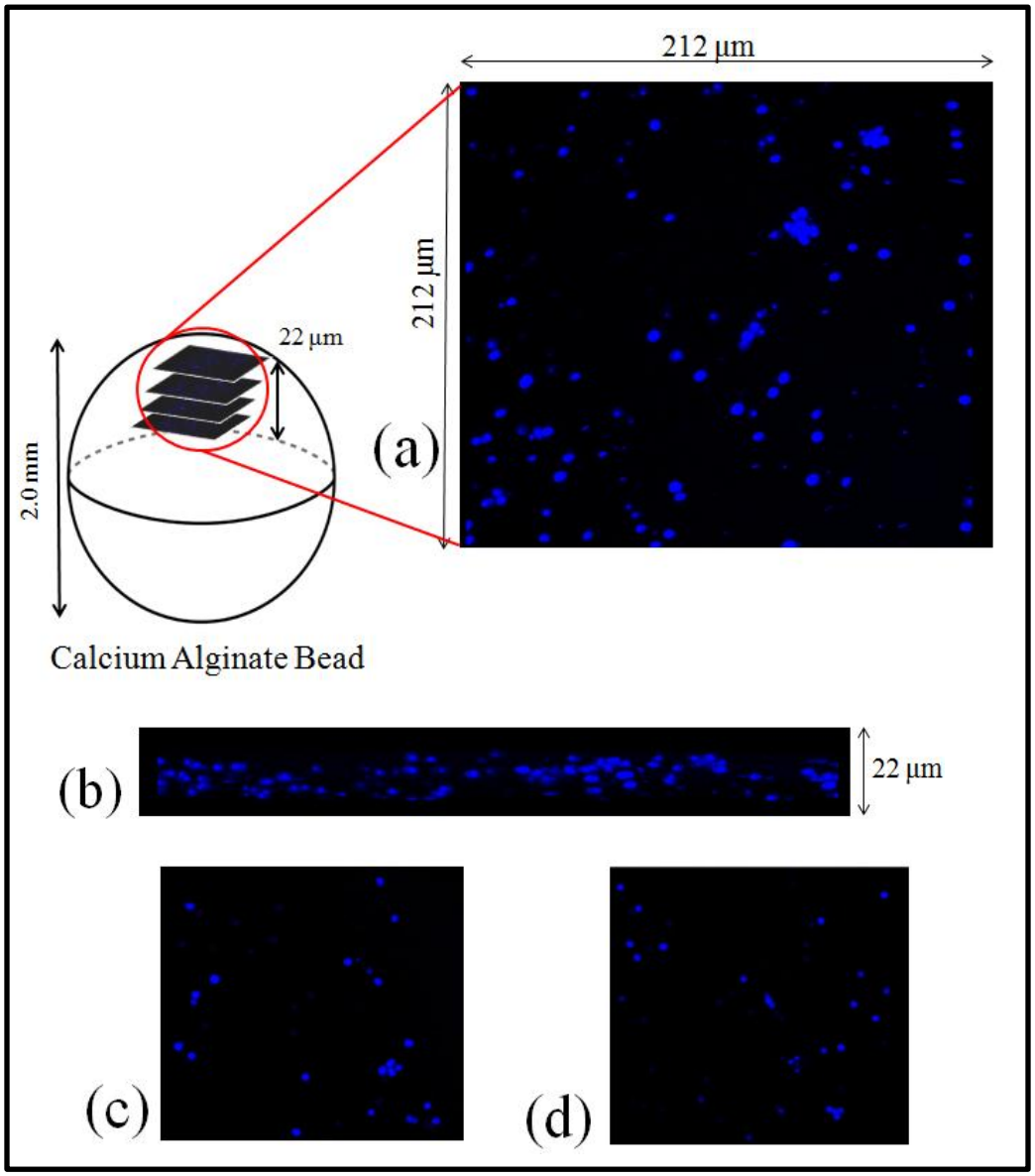


Figure 21: Confocal Microscope Images

Generation of a Standardized Curve via Headspace Gas Chromatography

The plotting of the ratios of the areas of ethanol to 1-butanol vs. percent ethanol yielded the following data which can be seen in Figure 22. The trend line was a 2nd order polynomial with an equation of $y = 0.0049x^2 + 0.0488x$ with a R^2 value of 0.997. Figure 23 is the chromatograph acquired from the 3 percent ethanol sample. The first peak shown is for ethanol while the second peak is showing 1-butanol. The initial parameters of the GC were changed in order to increase separation between the two alcohols. This was done because the peaks were too close to integrate separately. The temperatures used were: oven temp 40°C, FID temp 200°C, injector temp 200°C. With these conditions the retention time for ethanol is at 50 seconds and 1-butanol is at 120 seconds.

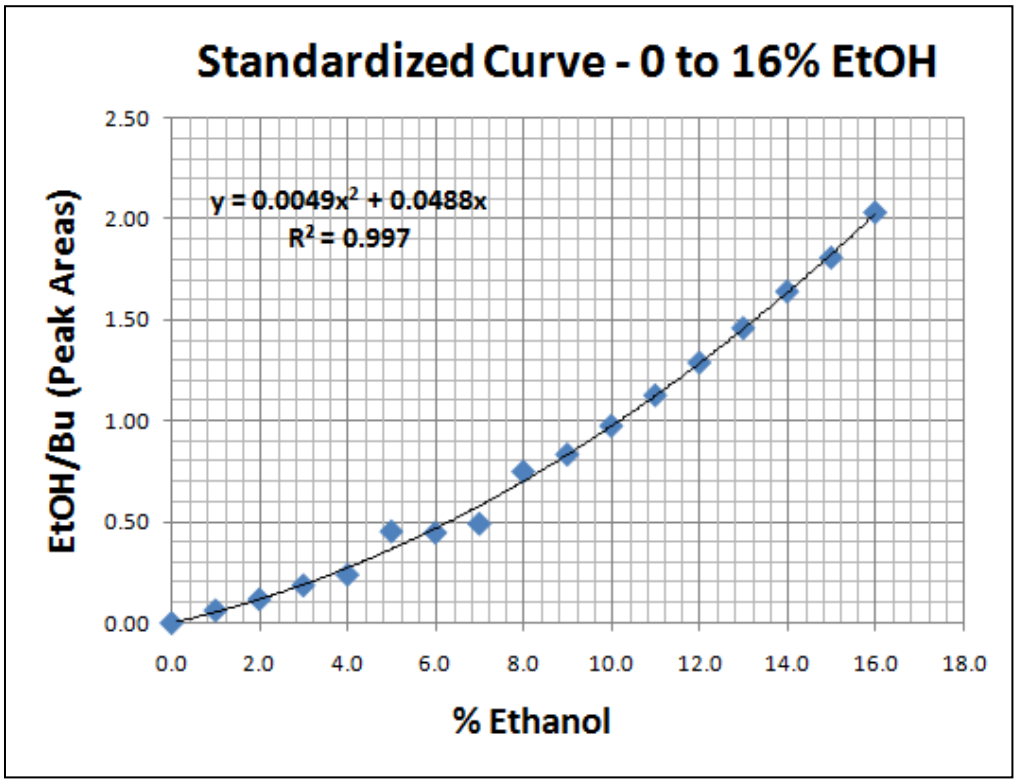


Figure 22: Standardized Curve - 0% to 16% Ethanol

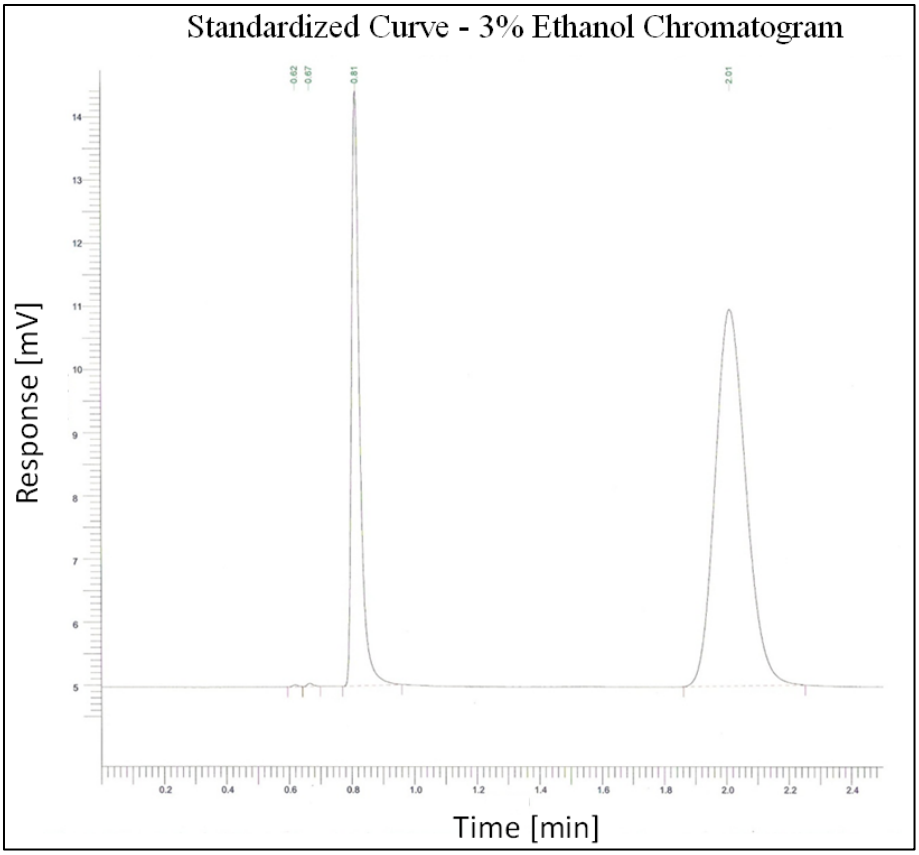


Figure 23: Standardized Curve - 3% Ethanol Chromatogram

Bioreactor

Components of Bioreactor

Observations of bioreactor over the working period:

- Yeast built up underneath beads and spread throughout system
- To allow CO_2 to escape, bioreactor was tilted to a 45 degree angle
- Limited amount of beads were used to allow movement within the magnetic field
- Added screens to each side of bioreactor column to avoid beads clogging plastic tubing
- A pre-sample was taken to flush out built up residue in sampling valve and was discarded before acquiring samples for testing

Figure 24 shows the actual bioreactor setup. Components are numbered and labeled below.

Figure 25 shows a close up of the bioreactor column (4) and magnet holder (5).

1: Initial medium flask (250mL)
2: Stirrer plate
3: Pump
4: Bioreactor with magnetic beads
5: Magnet holder
6: Plastic tubing (2mm inside diameter)
7: Sampling valve

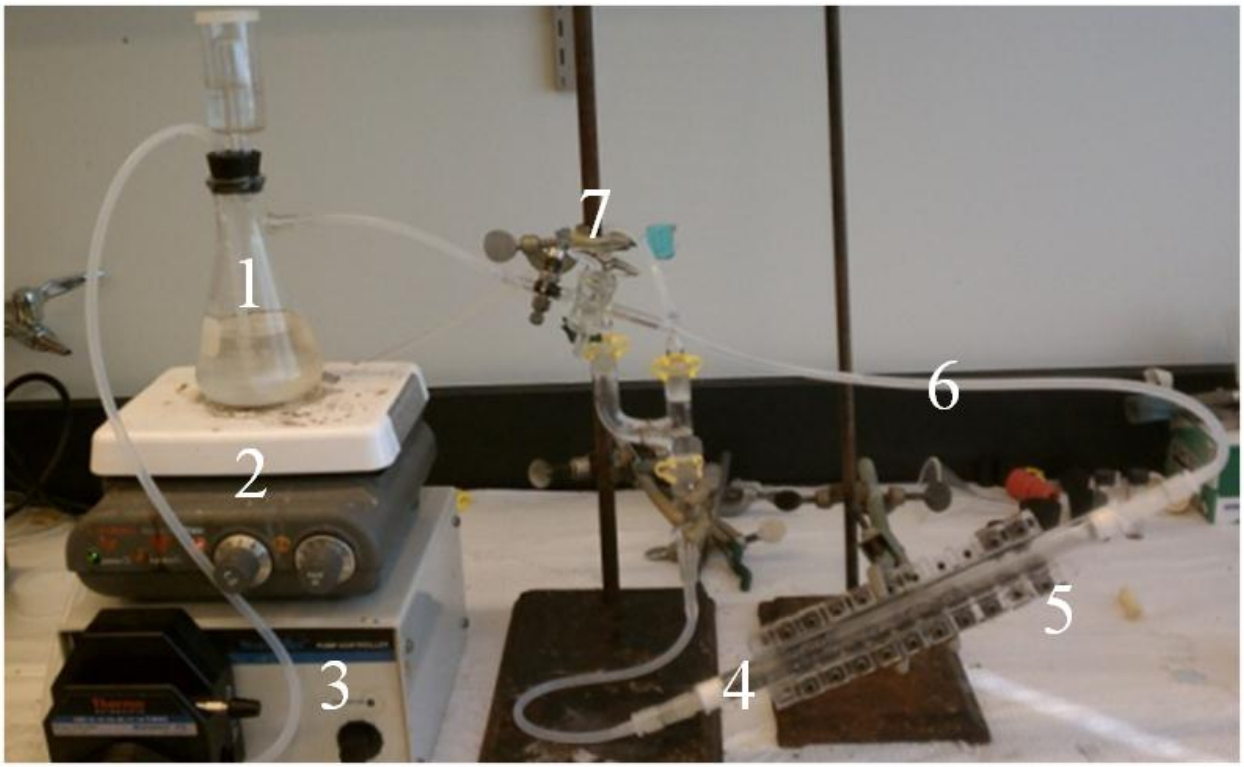


Figure 24: Working Bioreactor set up

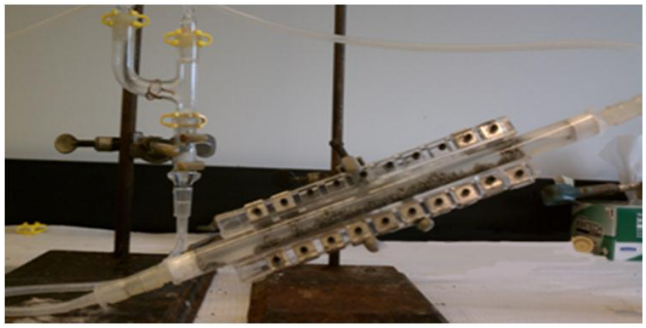


Figure 25: Bioreactor column and Magnet holder

Magnet holder

Initially, the magnet holder was completely full with magnets. However, after testing it was determined that carbon dioxide being produced was not able to easily escape the bioreactor column. After numerous trials the configuration seen in Figure 26 was used for the remaining bioreactor runs. Magnets were placed in every slot along the bottom while removing the middle of the top row. With this setup the beads were held against the bottom of the column thus allowing CO_2 to escape upwards and flow out of the column.

Figure 26: Beads held in place by magnetic field

Fermentation

To maintain proper levels of nutrients, an excess of medium solution was made consisting of H_2O (350mL), KH_2PO_4 (1.708g), NH_4SO_4 (0.748g), $MgSO_4$ (0.165g). The working volume of the bioreactor was 250mL. The excess 100mL of media was then able to be used to replace the volume lost after a sample was removed. The initial glucose concentration was 0.26 mol/L. Each day an additional amount of glucose (11g) was added after each sample was obtained.

During the first four days of fermentation, rapid carbon dioxide formation was noticeable. This can be characterized by large amounts of foaming and visible gas release through the fermentation lock. Even with the addition of glucose, over the course of the next five days it was obvious that the reaction was slowing down because of the reduced amount of carbon dioxide being formed. After testing the samples it was determined that the ethanol concentration increased rapidly during the first four days and slowly plateaued to a final concentration of 14.14%.

Headspace Gas Chromatography for Bioreactor Samples

To plot the percent ethanol produced vs. time (hours), the ratios of the areas of ethanol to 1-butanol were calculated. These ratios were then put into the equation given by the trend line to find the percent ethanol in each sample. Table 2 shows the data for each sample tested. Figure 27 is a plot of all the data points acquired from the bioreactor. Figure 28 is the chromatograph acquired from the first bioreactor sample. The first peak is from ethanol and the second peak is from 1-butanol. As show in Table 2, the ratio of peak areas for sample 1 is 0.269 which means there is 3.96 percent ethanol after 24 hours. There was an unexpected result in that the bioreactor samples were of increased purity compared to samples made with stock alcohols

acquired from Sigma Aldrich. This can be seen by comparing Figure 23 to Figure 28 and the absence of additional peaks.

Table 2: Bioreactor Sample Data

Sample	Time (hours)	EtOH/Bu (areas)	% Ethanol
0	0	0.000	0.000
1	24	0.269	3.96
2	48	0.646	7.55
3	72	1.460	13.00
4	94	1.522	13.35
5	168	1.562	13.57
6	190	1.666	14.14

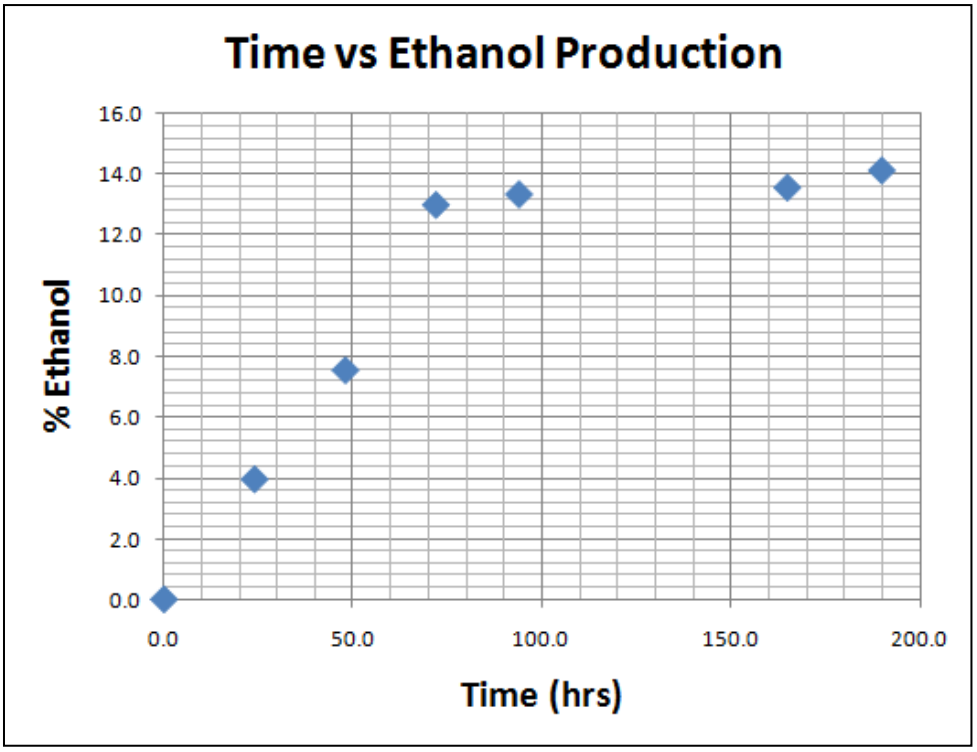


Figure 27: Bioreactor - Time vs. Percent Ethanol

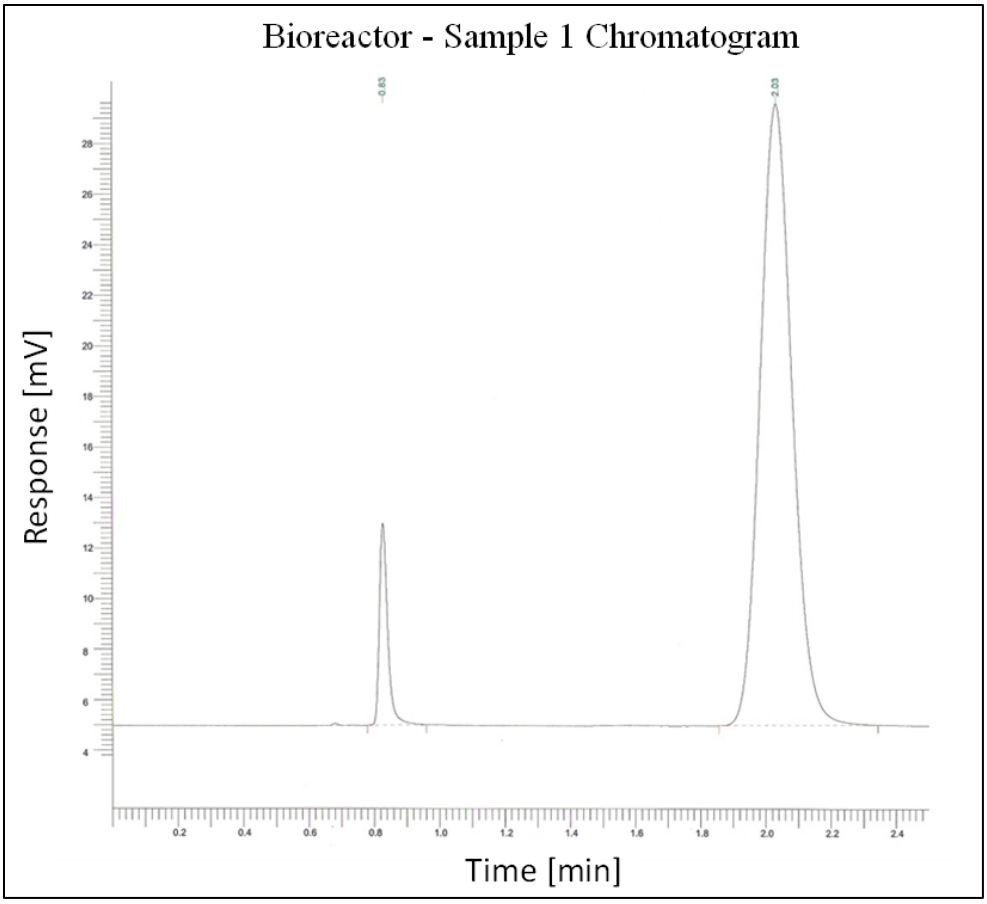


Figure 28: Bioreactor - Sample 1 Chromatogram

Conclusions and Future Work

It was determined that a 13% ethanol concentration could be achieved after 72 hours during the primary fermentation stage when 70% of fermentation activity occurs. After that time the production of ethanol drastically slowed down as the yeast entered the secondary fermentation stage; which requires an average of one to two weeks to reach its maximum ethanol concentration. A potential benefit of the bioreactor in combination with a molecular sieve or membrane would allow for continuous production and removal of ethanol. This would keep the ethanol concentration low enough where the yeast would remain in the primary fermentation stage allowing for maximum ethanol production as long as proper nutrient levels are maintained.

A potential problem of keeping the yeast in the primary fermentation stage is that while maximum ethanol production is achieved, reproduction rates are also at their highest. Immobilization of yeast in calcium alginate beads helps to reduce the contamination however; some yeast cells are still able to escape the beads. The potential addition of a filter would prevent escaping yeast cells from contaminating a dialysis unit and fresh media.

A method that has been used in the past to remove ethanol from solution is pervaporation through a sodium alginate membrane or pervaporation through a zeolite membrane [28] [29]. In either case, separation of ethanol from solution was achieved. However, as the water concentration increased, performance of both membranes decreased. Each of these systems worked best at 96 or higher percent ethanol. This means that distillation was necessary before membrane pervaporation. Another method to separate ethanol from water is through membrane distillation [30]. Membrane distillation (MD) is a hydrophobic porous membrane that separates two aqueous solutions at different temperatures. The process induces a vapor pressure gradient, at temperatures lower than the boiling points of the products, which causes a selective mass transfer across the membrane. As a control the MD unit was not attached to the bioreactor. Over the course of 22 hours the system and was able to convert 60% of the total glucose to produce 24.4gL^{-1} of ethanol. Comparatively, in a subsequent trial a MD unit was implemented. Over the course of 18 hours the system converted 100% of the total glucose producing 32.0gL^{-1} of ethanol. The ethanol productivity increased 87% from 0.99$gL^{-1}h^{-1}$ in the absence of MD unit to 1.85$gl^{-1}h^{-1}$ when coupled with a MD unit [30]. If a MD unit was implemented into our reactor system, a similar increase in ethanol production would be expected. Figure 29 shows the overall

schematic of the bioreactor and MD unit. Figure 30 shows the comparison of ethanol production when a MD unit is implemented. In both cases, as glucose concentration decreased, ethanol and biomass concentration increased. With no MD unit, this process was not as efficient and yielded a lower ethanol concentration. With the addition of a MD unit, this process became more efficient and yielded a higher ethanol concentration in a shorter period of time.

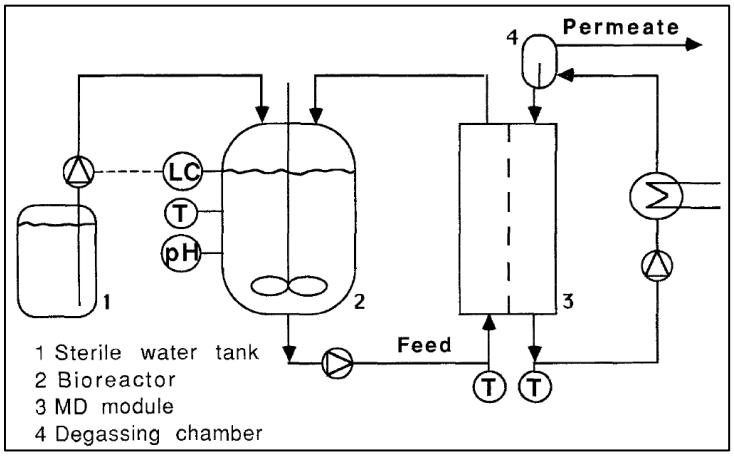


Figure 29: Schematic of bioreactor with MD unit [30]

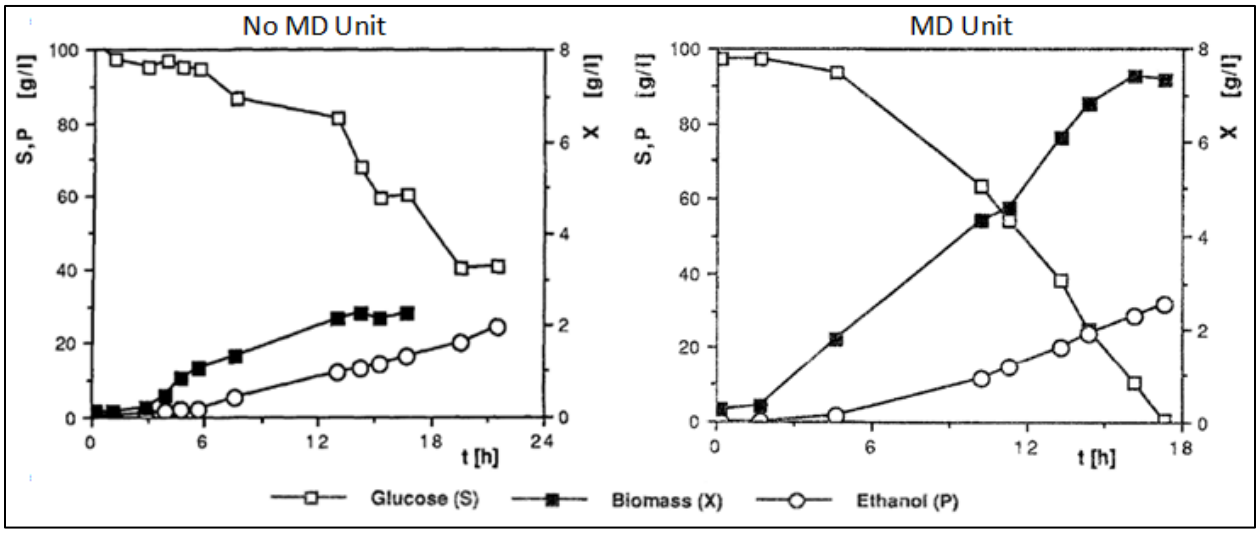


Figure 30: Comparison of Ethanol Production [30]

Another aspect to be considered is the potential contamination of the entire system by yeast. To limit the spread yeast throughout the system, a filter could be used. Most common strains of yeast are 5-10μm in diameter. By implementing a filter, smaller than 5μm, after the bioreactor column would theoretically trap all the yeast within the column. A potential problem is that over time the filter will clog and will need to be replaced. Depending on the design of the bioreactor, this could mean that the system would have to be stopped and opened up in order to replace the filter. An addition of a 'Y' shaped valve at the end of the column would allow for two filters to be set up while only one is used at a time. Once one filter clogs, a simultaneous opening and closing of the valves would redirect the flow to the unused filter. This would allow for time to change the clogged filter without contaminating and stopping the system. If extraction of escaped yeast was possible, this yeast could potentially be used in making more beads to fuel another bioreactor.

In addition to implementing a filter, our system could contain a dialysis unit in order to introduce a clean source of new media. A dialysis unit works on the principle of diffusion. As yeast consume glucose and other nutrients, the concentration of reactants within the system decreases. The dialysis unit would contain a high concentration of these nutrients and thus constantly replenish the system. Currently this technology is used extensively in the medical field. Due to the sterility needed in medical environments, a dialysis unit would limit the amount of contamination in our bioreactor. This would allow for the system to remain sealed while receiving fresh media.

To bring the system from the lab bench to a commercial scale would be feasible if a more efficient method of immobilizing yeast was developed. Currently, calcium alginate beads are viable on small scale but it is a labor intensive process. If the system were to be scaled up, too many beads would be required for it to be practical unless an automated process was designed and utilized. However, even if this process was automated and enough beads were made, they would still not be able to completely contain the yeast. In a scaled up bioreactor due to the increase in initial amount of yeast used, the amount of escaping yeast would much larger than seen on a small scale.

Another problem with scaling up the bioreactor would be the need to find a consistent source of feedstock. Glucose was used on a small scale; however this would not be practical on a

commercial scale. As seen with current designs for algae plants, waste effluent sources such as CO_2 emissions from power plants can be used to grow the algae. Another example is the use of brackish water compared to fresh water that could be used for drinking. For ethanol production from yeast, corn is currently used as the feedstock. This is not practical but is the current method only because of the amount of subsidizes given out by the government.

Approximately one billion dollars a year from tax payer's money is given out in federal and state subsidies, usually to large corporations. Currently 70% of corn grain is used as food for livestock. Using a portion of this for fuel can have dramatic consequences such as increased prices for meat, milk, and eggs. This means that not only are people paying tax dollars for ethanol subsidies, consumers would be paying significantly higher food prices in the marketplace as well [8]. Using corn also raises the food versus fuel debate. With a limited amount of usable land to grow the nation's crops, by setting aside a portion of this just for fuel production can be seen as a huge problem. However, if a waste product from already existing process could be utilized, this would increase the viability of continuously producing ethanol. An example of this is in Iran and the use of cane molasses as a waste feedstock [24]. The sugar cane industry in Iran generates three million kilograms of molasses every year. This effluent can create major environmental problems if not disposed of properly such as ground water contamination and increased insect population. Using molasses as feedstock for ethanol production solves these problems as well as create an alternative renewable fuel [24].

With the benefits of bioreactors, the United States could implement a similar process if the correct feedstock and organism are used. With current ethanol production methods it would only be feasible as an additive to gasoline and not a standalone fuel due to the energy capabilities of ethanol. During combustion ethanol only produces 60 percent of the total energy that is contained in fossil fuels. Ethanol production requires more energy input to produce a less amount of energy meaning the process is energy negative. Therefore, the major benefit of using ethanol is for environmental purposes and not economic [8]. It would require 90 million acres of corn in order to replace only 15 percent of the current United States fuel demands [31]. As stated previously, other organisms such as algae are able to produce biofuel on a much larger scale more efficiently. Sapphire Energy, an algae oil company, is currently able to produce 3000 gallons/acre/year of green crude oil. Using conventional oil refinery techniques a light sweet

category of oil comparable to current fossil fuels is produced. Sapphire would require 7 million acres of open ponds to replace 15 percent of United States fuel demands [31]. However, optimization of bioreactors could potentially reduce the amount of land needed to grow algae. This technology could be applied to several micro-organisms and different feed stocks to produce a variety of biofuels.

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