Effects of Ultrasound on Ethanol Fermentation
by *Saccharomyces cerevisiae*

THESIS

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By

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Abstract

Ethanol is the primary transportation biofuel used in the world. In the United States, more than 15 billion gallons of ethanol per year are made by fermentation of corn starch and lignocellulosic biomass. Ultrasound pretreatment of biomass has been shown to improve starch release and ethanol yield; however, less attention has been given to the ability of ultrasound to improve the mass transfer of substrates and products to and from yeast cells during fermentation. Previous studies have shown that certain levels and methods of ultrasound can improve or inhibit fermentation. Improvements have been attributed to reductions in mass transfer limitations; however, calculations of the external and intraparticle observable modulus indicate that no external or intraparticle mass transfer limitations should exist for yeast fermentation from glucose. The purpose of this study was to investigate the effects of ultrasound at different levels of intensity on fermentation of glucose to ethanol by yeast *Saccharomyces cerevisiae*. The effects of ultrasound on glucose uptake rate, ethanol and carbon dioxide production rates, and yeast cells population and viability were measured. Ultrasound was applied to a fermentation medium containing nutrients and glucose at 200 g/L. Four treatments were compared: direct probe ultrasound at 23 and 32 W/L, indirect ultrasound, and no-ultrasound. Both direct and indirect ultrasound had negative effects on yeast performance; they reduced the rates of glucose uptake and ethanol production, and negatively affected yeast viability.
Results indicate that ultrasound during fermentation, at the levels applied, is ineffective in improving the rate of ethanol production.
Dedicated to my parents and my sister.
Acknowledgments

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Chapter 1: Literature Review

1.1. Environment and energy

Energy is the capacity to do work. Society uses energy to perform many activities. The world total primary energy production for 2015 was $1.62 \times 10^8$ kWh, the energy consumption was $1.60 \times 10^8$ kWh (Enerdata, 2016). Primary energy is the energy stored in raw fuels or energy received as an input to a system (Gustavsson & Joelsson, 2010). It can be non-renewable or renewable, and it ignores conversion efficiency. Fossil fuels (oil, coal, or natural gas), solar, wind, falling water, tidal, biomass, and geothermal are examples of primary energy. Secondary energy is defined as the energy made from a primary energy sources, such as electricity, biofuel or gasoline, also called energy carriers; it is energy that can directly be used (Falk, Herrmann, & Schmid, 1983).

As shown in Figure 1, the country with the highest energy consumption is China, but the highest consumption per capita occurs in United States. Energy consumption growth is larger in developing countries than in developed countries. Most of the energy consumed is derived from fossil fuels.
The extraction and burning of fossil fuels in the form of oil, shale, carbon, and gas emits large quantities of carbon dioxide and other greenhouse gases into the atmosphere. This contributes to climate change due and leads to air quality deterioration, loss in biodiversity, oil spills, melting glaciers and ice caps, acid rain, acidification of the ocean (Kump, Bralower, & Ridgwell, 2009), and sea level rise. These issues and the fact that economically recoverable fossil fuels will be depleted within the next 30 to 100 years (Shafiee & Topal, 2009), are severe problems limiting human growth and development.
By the end of 2015 the carbon dioxide concentration in the atmosphere reached 398 ppm (National Oceanic & Atmospheric Administration, 2016). In order to relieve some of the problems caused by climate change, an atmospheric concentration of carbon dioxide of around 350 ppm (Hansen et al., 2008) needs to be achieved. The use of renewable energy technologies can reduce and mitigate some of these problems. It can also help to alleviate some of the socioeconomic and environmental issues associated with fossil fuels (Haines et al., 2007).

1.2. Renewable Energy and Ethanol

Renewable energy refers to a form of energy that is clean; does not deplete over time; generate less greenhouse gas emissions during its production and use, than fossil fuels; and can be restored by natural processes (IPCC, 2012). Renewable energy technologies are needed to mitigate climate change because they can help meet energy needs, and are more environmentally friendly than using fossil fuels. Currently, renewable energy technologies are at different stages of development and cost (IPCC, 2012). Renewable energy technologies are the backbone of a green economy (Gasparatos, Doll, Esteban, Ahmed, & Olang, 2017).

The adoption of renewable energy improves the environment (UNEP, 2011) and increases energy independence from fossil fuels (Aronoff, Sitty, & Taft, 2013). According to the UNEP (2011), renewable energy has the potential to reduce poverty by providing energy to people that do not have access to it and through the creation of jobs.
in the production of renewable energy. The UNEP claims that allocating 1% of the world GDP to renewable energy and energy efficiency will help mitigate climate change, save fossil energy, reduce dependence on oil, reduce carbon emissions (Sims, Rogner, & Gregory, 2003), and generate jobs, especially in rural areas, where they are most needed (U.S. Department of Energy, 2016b). Renewable energy also poses various challenges such as high capital cost, and intermittency. Social, economic, and technical problems are also present in the development, implementation, and use of renewable energy technologies (Krauter & Kissel, 2004). Renewable energy technologies also provide a buffer to fossil fuel price volatility (UNEP, 2011).

Renewable energy technologies use different approaches to collect and store energy. Solar technology converts sunlight into electricity using the photovoltaic effects, or indirectly into heat used to run generators using concentrated solar power (Parida, Iniyan, & Goic, 2011). Wind energy is generated by turbines rotated by large blades which are moved by air currents (Joselin Herbert, Iniyan, Sreevalsan, & Rajapandian, 2007). Hydropower is generated by turbines rotated by fresh flowing water (Yang & Jackson, 2011). Geothermal technology harvests heat from the earth’s crust (Lund & Boyd, 2016). Finally, biomass or bioenergy is produced by organic matter, which contains water and carbon structures, such as sugars.

In the United States nearly all of the renewable energy produced is consumed domestically, including hydroelectric, geothermal, solar, wind, wood, waste, and biofuels (Figure 2). In 2015, 9,466 BTU were produced, and 9,450 BTU were consumed (U.S. Energy Information Administration, 2016c), which indicates that future consumption will increase as production increases.
1.2.1. Bioenergy

Bioenergy refers to the energy produced from organic matter such as sludge or plant material; rather than from fossil fuels. Some types of bioenergy are:

- Methane (CH4), gas produced by anaerobic digestion of organic matter. The process produces biogas that contains methane and carbon dioxide; liquid called digestate; and solids. Methane is a greenhouse gas with a higher global warming potential than carbon dioxide. It is also produced naturally by wetlands and cows (Bitton, 2005) and is the main component of natural gas, a fossil fuel.
• Biodiesel, is produced from oils by transesterification. It consists of fatty acid methyl or ethyl esters (FAMEs). Common feedstocks are animal fats, vegetable oils, soybean, jatropha, palm oil, and algae. It is non-toxic and biodegradable. It is the most common biofuel in Europe (European Biodiesel Board, 2016).

• Butanol (C₄H₉OH) is produced via acetone-butanol-ethanol fermentation (ABE). Butanol can be used directly in a car engine without blending.

• Methanol (CH₃OH) or methyl alcohol is the simplest alcohol, it can be produced from wood or by industrial processes from coal or methane. Methanol can be used as fuel and is also used as denaturing agent.

• Ethanol (CH₃CH₂OH) is produced by fermentation of corn or sugar cane, and is used as fuel.

Depending on the feedstock and conversion used, biofuels can be classified as first, second, and third generation:

• First generation: biofuels produced from crops, such as corn in United States; sugar cane, soybean, and canola oils in Brazil; oilseeds and sugar cane in Sub-Saharan Africa (Gasparatos et al., 2013), and cassava in Nigeria and Ghana.

• Second generation, also known as advanced biofuels, come from non-food and lignocellulosic sources, such as agricultural waste based on lignocellulosic biomass like straw or wood. Dupont has the largest cellulosic ethanol facility in the world (Dupont, 2016), located in Nevada, Iowa. Common feedstock for the second generation of biofuels are wood, grasses and inedible parts of plants.
Lignocellulosic biomass is more difficult to process and requires thermal and/or chemical treatments to be converted into sugars.

- Third generation biofuels include lipids from algae, animal fats, used oil, and garbage.

The development of lignocellulosic ethanol, second generation of biofuel, is driven in part by the debate on the first-generation ethanol about food versus fuel, which was in competition with food production, supplies, and carbon emission (Inderwildi, O., and King, 2009). The second generation of biofuels are produced from cheaper sources (Lin & Tanaka, 2006), crops that are not traditionally used as food source. Although they are still grown in land that could be used to produce food. Third generation fuels are produced from wastes or sources grown in water.

1.2.2. Ethanol

Figure 3 shows the world’s ethanol production; for the year 2015 it was 26,668 million gallons, from which United States produced 14,807; Brazil 7,093; and the rest of the world 4,768 (Renewable Fuel Association, 2016b). United States is the largest corn ethanol producer, followed by sugar cane ethanol from Brazil (Crago, Khanna, Barton, Giuliani, & Amaral, 2010). Brazilian ethanol from sugar cane produces waste called bagasse, is used to produce heat and power (Crago et al., 2010; Soybean & Corn Advisor, 2016). In tropical countries like Brazil, the main feedstock is sugar cane, while in higher latitudes such as United States, corn is a better option. United States produced $3.45 \times 10^{11}$ kg of corn in 2015, form which 38% was used for ethanol production, some of that corn
is returned as dry distilled grain with solubles, to be used as animal feed (National Corn Growers Association, 2016).

Figure 3. Ethanol world production. United States, Brazil, and rest the world (ROW) (Renewable Fuel Association, 2016b).

Figure 4 shows ethanol production, consumption, and net exports and imports from the year 2000 to 2015. Ethanol production in 2015 was 14,807 million gallons, and the consumption was 13,947 million gallons. From 2000 to 2015, U.S. has had a net ethanol export of 1,920 million gallons of ethanol.
In an ethanol production plant, the typical process after receiving the feedstock is milling. Around 90% of corn ethanol is produced by dry milling and the rest (10%) by the wet milling process (U.S. Department of Energy, 2016a). After dry milling, the following steps are cooking, liquefaction, fermentation, distillation, molecular sieving, denaturation, storage, and distribution. Ethanol production by wet milling and from cellulose use different processes.

The rise in ethanol production after 2000 was due to renewable fuels mandates implemented by the US government. Ethanol also rose because it can be used as fuel oxygenate to replace methyl tert-butyl ether (U.S. Department of Energy, 2016a).
Today there are 231 ethanol production plants in the United States, 15 of which also use cellulose as a feedstock (Ethanol Producer Magazine, 2016). According to the Renewable Fuel Association, by January 2016, there were 199 operating ethanol plants (Figure 5).

Figure 5. Operating ethanol production facilities in United States (Renewable Fuel Association, 2016a).

Gasoline is a fossil-derived fuel used in internal combustion engines throughout the world. In 2015 the U.S. consumed 140.3 billion gallons of gasoline (U.S. Energy
Information Administration, 2016b). Gasoline in the U.S. is blended with ethanol for economic and environmental reasons. Ethanol is miscible with gasoline and is used as an additive at different proportions. Gasoline blends differ depending on the producer, use and the season. In order to stimulate ethanol production in U.S., the Renewable Fuel Standard (RFS) program requires increasing amounts of renewable fuels to be used each year (U.S. Department of Energy, 2016c).

The most common blend in United States is E10, which means 10% ethanol and 90% gasoline; almost 97% of the gasoline in United State is E10 (U.S. Energy Information Administration, 2016d). The letter “E” represents the percentage of ethanol in the mixture; ethanol percentages, range from E5 to E85. Each of the blends has their own regulations, all of the blends used in U.S. increase the octane, improve air quality compared to gasoline, and are used to meet the Renewable Fuel Standards (U.S. Department of Energy, 2016b).

The energy contained in fuels is represented per unit of volume as energy density (J/L) or mass (J/kg) as specific energy. Ethanol has a gasoline gallon equivalent (GGE) around 1.5, but a higher octane. Octane refers to the resistance a fuel has to ignition, the rate value represents the performance of the given fuel in an engine. The higher the value, the more compression the fuel can resist before igniting. Higher octane ratings are used for engines with higher efficiency, high compression engines. Research Octane Number (RON) is a way to measure the octane rate. Ethanol and gasoline are compared in Table 1. Energy density (U.S. Energy Information Administration, 2016a), specific energy (IOR Energy, 2016), carbon dioxide emissions (do not take into account CO, NOx,
sulfates and particulates), and RON (Eyidogan, Ozsezen, Canakci, & Turkcan, 2010) are compared.

Table 1. Comparing ethanol and gasoline.

<table>
<thead>
<tr>
<th>Fuel type</th>
<th>MJ/L (Gross)</th>
<th>MJ/kg</th>
<th>CO2 emission (kg/kg)</th>
<th>RON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>18.4 - 21.2</td>
<td>23.4 - 26.8</td>
<td>1.91</td>
<td>108.6</td>
</tr>
<tr>
<td>Gasoline</td>
<td>32.0 - 34.8</td>
<td>44.4 - 48.3</td>
<td>~3.30</td>
<td>91-92</td>
</tr>
</tbody>
</table>

Ethanol has less energy density than gasoline, which means that it takes more ethanol, to get the same energy as gasoline. It is also hygroscopic, which means it absorbs water from the atmosphere which can cause ethanol to separate from blends with gasoline. Ethanol has higher octane rating than gasoline, which increases thermodynamic efficiency. Combustion of gasoline and ethanol produce a different ratio of carbon dioxide and water. During combustion the gasoline or ethanol reacts with oxygen and produce carbon dioxide, water, and heat.

- Ethanol: \( \text{CH}_3\text{CH}_2\text{OH} + 3\text{O}_2 \rightarrow 2\text{CO}_2 + 3\text{H}_2\text{O} \)
- Gasoline: \( 2\text{C}_8\text{H}_{18} + 25\text{O}_2 \rightarrow 16\text{CO}_2 + 18\text{H}_2\text{O} \)

Bioethanol is produced from biomass, which refers to organic matter, usually plants and plant based materials. Almost any plant can be converted to ethanol, because almost all contain sugars that can be fermented.

Ethanol has many uses in addition to being used as a biofuel it is used in medicine, solvents, cleaning products, and alcoholic beverages. Ethanol is also called
ethyl alcohol, pure alcohol, grain alcohol; its chemical formula can be written as EtOH, CH₃CH₂OH, C₂H₆O, and C₂H₅OH. It is composed of an ethyl (methyl group, methylene group) and hydroxyl group. Ethanol production from biomass through fermentation plays an important role in energy and chemicals production.
1.3. Fermentation

Fermentation is a microbial anaerobic process in which an organic molecule is used as an electron acceptor for metabolism. Common substrates are sugar, starch, or cellulose. Products include alcohols, acids, and gases. Common products made using fermentation are bread, beer, wine, yogurt, pharmaceutical compounds, and fuels.

Fermentation starts with glycolysis, which is an 11 step metabolic pathway where a molecule of glucose is processed into two molecules of pyruvate (Berg, J., Tymoczko, J., and Stryer, 2002). Glycolysis occurs completely within the cytoplasm of microbial cells (Zamora, 2009). Different microorganisms process pyruvate in different ways, which gives rise to different products (Figure 6).
1.3.1. Ethanol fermentation

During alcoholic fermentation by yeast *Saccharomyces cerevisiae*, one molecule of glucose is transformed into 2 molecules of ethanol and 2 molecules of carbon dioxide. The molecular weight for glucose is 180 g, ethanol 46 g, and carbon dioxide 88 g. The theoretical yield for ethanol is 0.511 g of ethanol per g of glucose and for carbon dioxide is 0.488 g of CO$_2$ per g glucose (Table 2). Biomass (yeast growth), is also produced from glucose reducing the actual yield.
Table 2. Glucose, ethanol, and CO₂: molecular structure, weight, and yield.

<table>
<thead>
<tr>
<th>1 mole of glucose</th>
<th>2 ethanol</th>
<th>2 carbon dioxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₁₂O₆</td>
<td>2 CH₃CH₂OH</td>
<td>2 CO₂</td>
</tr>
<tr>
<td>180 × 1 = 180</td>
<td>46 g × 2 = 92 g</td>
<td>44 g × 2 = 88 g</td>
</tr>
<tr>
<td>Yield</td>
<td>92 g / 180 g = 0.511</td>
<td>88 g / 180 g = 0.488</td>
</tr>
</tbody>
</table>

Ethanol fermentation is shown in more detail in Figure 7, where pyruvate is decarboxylated in to ethanol (acetaldehyde) and carbon dioxide by the enzyme pyruvate decarboxylase, which uses magnesium and thiamine pyrophosphate as cofactor (Spencer, Spencer, & Figueroa, 1997). Ethanal is reduced to ethanol by the enzyme alcohol dehydrogenase, which uses zinc as cofactor (Zamora, 2009). For the yeast cell, the main purpose of fermentation is to produce ATP from pyruvate, to produce an organic molecule (Keeton, 1967) and to regenerate NADH into NAD⁺ when there is not a final electron acceptor, such as oxygen.
1.3.2. Yeast

Yeast are eukaryotic chemoorganotrophs. *Saccharomyces cerevisiae* is one species within the yeasts (Kurtzman, 1994). It is the most effective microorganism in fermenting sugars to ethanol (Aristidou, A., Baghaei, N., Javed, M., and Hartley, 2012), due to its great resistance to high ethanol concentrations (Fleet, 1993). Yeast plays a central role in the biological production of fuels and chemicals. The efficiency of the
ethanol fermentation requires that yeast remain in a viable state so that they can consume all the fermentable sugars (Bisson, 1999)

*S. cerevisiae* cells can grow to a diameter of 5 to 10 µm. It has been used as a model organism for research on eukaryotic cells, human biology, and microbial fuel cells to generate electricity (Schaetzle, O., Barriere, F., and Baronian, 2008). As shown in Figure 8, yeast can metabolize sugars with and without oxygen (Boulton, B., Singleton, V., Bisson, L., and Kunkee, 1996), using respiration or fermentation. However, yeast prefers fermentation rather than aerobic respiration. If oxygen is present, and the concentration of sugars is high and available, yeast will prefer to ferment alcohol, an effect known as the Crabtree effect (De-Deken, 1966).
Yeast has sexual and asexual reproductive cycles, the most common is asexual reproduction by budding (Balasubramanian & Bi, 2004) and fission. Budding refers to a small offspring cell that grows on the side of the original mother cell. The offspring separate from its mother when it is mature, leaving a scar (Yeong, 2005); however, this does not always happen, and clusters of yeast cells may form. Fission refers to the division of the cell into two new cells (Bailey, J. and Ollis, 1986). Under optimal
conditions a yeast can double their population every 100 min (Herskowitz & Of, 1988), and can go through up to 26 cell divisions (Kaeberlein et al., 2005).

In batch fermentation, yeast follow a typical growth curve, where different phases can be identified. These include the lag, exponential, stationary, and death phases. During the lag phase, after inoculation, yeast adapts to a new environment and to the metabolism of a carbon source, such as glucose (El-Mansi, E., Ward, B., and Chopra, 2012). The outcome of the lag phase is an adapted cell able to convert sugars into chemicals and biomass in an optimal rate. During the exponential phase, the cells increase in size and reproduce, the population increases. During the stationary phase, the growth limiting nutrient has been exhausted, or the concentration of rate limiting inhibitors such as ethanol or carbon dioxide, reach its inhibitory levels. During this phase the total number of cells remains stable. In the death phase energy for cell metabolism has been exhausted and a general decrease in the population is observed. Yeast can also be inhibited by high concentrations of glucose, 380 g/L (Maiorella, Blanch, & Wilke, 1983); ethanol, 70 g/L according to Zang et al (2015), and carbon dioxide.

1.3.3. Mass transfer

Mass transfer in yeast fermentations occurs due to the movement of substrates and products to and from the cell from the fermentation broth. As shown in Figure 9, glucose must move from the bulk liquid to the boundary layer, where the concentration begins to decrease due to cell uptake, to the cell surface, through the cell wall, and then inside the cell. Ethanol and carbon dioxide produced within the cell must move from the inside of
the cell through the boundary layer to the bulk liquid. (Doran, 2013). Intraparticle and external mass transfer resistances related to porous catalysts, immobilized cell systems, or fungal pellets (F. C. Michel & Grulke, 1992) are widely studied and shown to limit the rates of fermentation and bioconversion. External mass transfer limitation can be minimized by properly managing the reactor design or the mixing system (Hussain, Kangwa, Abo-Elwafa, & Fernandez-Lahore, 2015).

![Figure 9. Yeast cell and mass transfer dynamics.](image)

During ethanol fermentation, glucose molecules are transferred from the bulk media to the surface of the yeast cell and into the yeast cell, while ethanol and carbon dioxide are transferred in the opposite direction. Glucose enters the cell by facilitated diffusion; ethanol and carbon dioxide diffuse out of the cell by simple diffusion (Zamora, 2009). Facilitated diffusion refers to the passive transport of molecules or ions across the
cell’s membrane through specific proteins. Simple diffusion refers to the transport of molecules or ions across the cell’s membrane without any intermediary. Both types of diffusion occur simultaneously through the yeast cell.

1.3.3.1. Glucose

Yeasts transfer sugars (glucose, fructose, and mannose) into the cell across the membrane by facilitated diffusion, where sugars bind to carrier proteins called hexose transporters on the outer side of the membrane and are released into the cytoplasm (Barnett, 1976; Z. Liu & Li, 2014; Luyten, Riou, & Blondin, 2002). During the facilitated diffusion of glucose, no metabolic energy is required and the glucose concentration gradient over the membrane is the main driver of the process (Weusthuis, Pronk, Broek, & Dijkenl, 1994). The rate of glucose uptake in yeast is dictated by the activity and concentration of glucose transporters in the plasma membrane (Panalver, Lucero, Moreno, & Lagunas, 1998) and the glucose concentration at the cell surface and within the cell. Since ethanol denatures proteins by interacting with their non-polar parts (Z. Liu & Li, 2014), not all the transporter proteins may be free or ready for the glucose to bind, .

1.3.3.2. Ethanol release

Ethanol increases fluidity of the yeast plasma membrane (Curran, M. and Seeman, 1979), which negatively affects yeast performance. Continuous exposure to ethanol may cause cell lysis (R P Jones & Greenfield, 1987). Removing ethanol from the fermentation broth continuously can increase productivity due to an increase in the number of viable yeast cells, (Liu, H. and Hsu, 1990).
1.3.3.3. Carbon dioxide

Carbon dioxide is another product of glycolysis. It is highly soluble and is present in various gas and dissolved forms such as carbon dioxide, carbonic acid, bicarbonate, and carbonate (CO$_2$, H$_2$CO$_3$, HCO$_3^-$, CO$_3^{2-}$). The total dissolved carbon concentration is pH dependent (Bailey, J. and Ollis, 1986). The pH of the media is influenced by the balance of dissolved carbon dioxide and bicarbonate (Moss, 1973). Figure 10 shows the pH and dissolved carbon dioxide behavior.

The yeast membrane is impermeable to ionic and polar compounds, but is permeable to neutral or highly hydrophobic molecules; therefore the concentration and net flux of CO$_2$ across the yeast membrane is regulated by the concentration of CO$_2$ in the intracellular and extracellular fluids (Rodney P Jones & Greenfield, 1982). Nucleation is one of the early steps in the development of a new thermodynamic phase of structure, in
the case of CO₂ from aqueous CO₂ to gas. Yeast is not able to nucleate CO₂, so concentrations increase in the fermentation medium until bubble formation occurs (Delente, Akin, Krabbe, & Ladenburg, 1969). Nucleation improves with agitation, resulting in a decrease in CO₂ concentration in the fermentation medium, and an enhancement in the yeast fermentation rate (Ough & Groat, 1978). Fermentation rates improve thanks to the CO₂ getting out of the cell into the fermentation medium and out of the fermentation medium.

1.3.3.4. External and intraparticle mass transfer

The external and intraparticle observable moduli are factors used to evaluate the magnitude of external or intraparticle mass transfer resistances. These factors are a function of the concentrations in the bulk liquid (C_b) and the cell surface (C_s) as well as the diffusivity, viscosity, density, yeast cell size and density.

The external mass transfer modulus is the ratio of the concentration of a component at the surface of a particle and the concentration in the bulk liquid. If the concentration in the bulk liquid is the same as the concentration at the surface of the particle, then no boundary layer exist when C_s/C_b ≈1. This would indicate that the external mass transfer limitation is not significant, because the concentration at the surface is almost the same as the concentration in the bulk. However, if C_{As}/C_{Ab} << 1, it indicates that the external mass transfer limitation is significant and that a boundary layer exists. An observable modulus for external mass transfer is calculated using equation 1.

\[
\Omega = \frac{R}{3} \times \left( \frac{r_{A,bs}}{k_s \times C_{Ab}} \right)
\]

Equations 1
Where:
$\Omega =$ dimensionless observable modulus for external mass transfer
$R =$ radius of the yeast, m
$r_{A,\text{obs}} =$ observed reaction rate per unit volume of catalyst
$k_s =$ liquid-solid mass transfer coefficient
$C_{Ab} =$ substrate concentration in the bulk liquid

The intraparticle mass transfer is calculated by the Thiele modulus, also called Weisz’s modulus. It is a dimensionless number defined as the ratio of the reaction rate to the diffusion rate (Kraakman, Rocha-Rios, & Van Loosdrecht, 2011). It can be used to estimate any intraparticle mass transfer limitations of substrate, without previous knowledge of the kinetics (F. C. Michel & Grulke, 1992). If the Thiele observable modulus ($\Phi$) has a value greater than 0.3, it indicates that internal mass transfer limitations are significant. If the value is lower than 0.3, it indicates that there is no internal mass transfer limitation in the system. Expressions for spheres and flat plates are different (Doran, 1995); equation 2 is the expression for spheres.

$$\Phi = \left(\frac{R}{3}\right)^2 \times \left(\frac{r_{A,\text{obs}}}{D_{Ae} \times C_{As}}\right) \quad \text{Equation 2}$$

Where:
$\Phi =$ dimensionless observable number Thiele modulus
$R =$ radius of the yeast, m
$r_{A,\text{obs}} =$ observed reaction rate per unit volume of catalyst
$D_{Ae} =$ effective diffusivity of substrate
$C_{As} =$ substrate concentration at the external surface
1.4. Ultrasound

Ultrasound is defined as sound waves with frequencies above 20 kHz. Human hearing range is between 20 to 20,000 Hertz (20 kHz) and is called acoustic. If the frequency is below that range, it is called infrasound (Figure 11). Bats, porpoises, and some insects are able to emit and detect ultrasound waves (Kastelein, Bunskoek, Hagedoorn, Au, & de Haan, 2002; Spangler, 1984; Werner, 1981).

![Frequency ranges corresponding to infrasound, acoustic, and ultrasound](image)

Figure 11. Frequency ranges corresponding to infrasound, acoustic, and ultrasound

A wave is a form of disturbance that travels through a medium or without a medium. If the waves do not need a medium to travel through, they are called electromagnetic, and if the waves do need a medium to travel through, they are called mechanical. Waves are classified as transverse and longitudinal, depending on their direction and the direction of the particles in the medium. In transverse waves, the particles of the medium move perpendicular to the direction of the wave. In longitudinal waves, the particles of the medium travel in the same direction that the wave is traveling. Waves are also classified as standing or progressive. Progressive waves transfer energy
from one place to another without transferring the particles in the medium, while standing waves store their energy in one place. A standing wave is a pattern resulting from the interference of two progressive waves of the same frequency, similar in amplitude, and traveling opposite directions within the same medium. Sound waves are mechanical, longitudinal, and progressive (Figure 12).

![Standing Waves Image](http://avstop.com/ac/apgeneral/sound.html)

**Figure 12.** Waves being generating by the vibrations of a tuning fork. (http://avstop.com/ac/apgeneral/sound.html)

Amplitude is defined as the maximum displacement of a medium particle from its rest position; from rest to antinode (positive or negative) in standing waves, or from rest to crest and rest to trough in progressive waves. The rest position is between two antinodes in standing waves, and between a crest and a trough in progressive waves. Nodes and antinodes occur in standing waves; a node is the point where amplitude is 0.
and do not move from its rest position, an antinode moves up and down as crests and troughs in progressive waves, with the difference that it does not move forward. Crests and troughs occur in transverse waves; longitudinal waves have compressions and rarefactions (Figure 12). Compressions represent regions where the particles are closer together, and rarefactions represent regions where the particles are further apart. In longitudinal waves, the distance between two consecutive points that are in phase, two compressions or two rarefactions, is equal to a wavelength. Frequency of a wave is the number of wavelengths per second and is measured in Hertz (Hz).

1.4.1. Cavitation and ultrasound

Cavitation is a physical phenomenon that occurs from a sudden drop of pressure in a liquid, which creates vacuum bubbles or cavities (Figure 13). This is followed by an immediate return to the original pressure, which causes those cavities to collapse, releasing energy, which is then transferred into the medium (F. Michel & Kozyuk, 2015). Cavitation creates powerful local temperatures and pressures for a short period of time (Suslick et al., 1999). The incidence of cavitation depends on the acceleration at which the change in pressure occurs. Higher acceleration results in higher differential pressures, resulting in a higher probability of cavities.

In ultrasound, the acceleration depends on the frequency and the amplitude of oscillation. Ultrasound generates alternating high and low pressure areas, causing compressions and rarefactions cycles in the liquid. Rarefactions lead to the formation of cavities, which implode during compression. During the implosion of the cavities, high temperatures (4,700°C), high pressures ($2 \times 10^5$ kPa), and high rates of cooling
(1×10^{11}^\circ\text{C}/\text{second}) are reached (Suslick et al., 1999) (Figure 13). The intensity of ultrasound is then subjected to the energy input (W) divided by the surface area of the ultrasound probe (cm^2), also called sonotrode. Watts (W) divided by volume of the sample to treat is equal to energy density.

![Figure 13. Creation of cavitation bubbles.](image)

(a) Displacement from rest position, (b) transient cavitation, (c) stable cavitation, (d) pressure due to compressions and rarefactions. Adapted from (Santos, H., Lodeiro, C., Capelo, 2009)

There has been an increased use of ultrasound in liquids and slurries of all kinds (Figure 14). Some of the common uses of ultrasound are enzyme activation, particle separation (Groschl, 1998) emulsification, water treatment, wastewater treatment (Altmetric, Articles, Review, & Samir Kumar Khanal, David Grewell, 2007), degassing,
inactivation of microbes in the food industry (Mason, Paniwnyk, & Lorimer, 1996), disrupting cell membranes to harvest intracellular content (D. Liu, Zeng, Sun, & Han, 2013), wet-milling, de-agglomeration, particle size reduction, and dispersing and biomass pretreatment for biofuels production (Bussemaker, M., and Zhang, 2013; Sulaiman, Ajit, Yunus, & Chisti, 2011). Due to such a variety of applications, ultrasound devices have also evolved.

Figure 14. Classification of the effects of ultrasound
Modified from (Suslick et al., 1999).
1.4.2. Ultrasound equipment

The application of ultrasound into a sample can be direct or indirect. Direct application of ultrasound refers to the one applied straight into the sample medium; indirect application, refers to the application of ultrasound into a different medium before reaching the target sample, as shown in Figure 15. Although both techniques apply ultrasound to the sample, there are differences in their effectiveness, efficiency, and process capabilities. In laboratory scale processes, ultrasound is usually applied with an ultrasonic bath (indirect) or an ultrasonic probe (direct), and performance will be different when comparing them.

Figure 15. Diagram of an ultrasonic bath.
Kwiatkowska, et al. (2011)
One example of indirect application of ultrasound is a liquid within a bottle placed in an ultrasonic bath (Figure 15). In this system, the ultrasound waves cross two barriers to reach the sample to be treated. The first barrier is the liquid in the ultrasonic bath, which is heated due to the waves, and the second is the wall of the sample container before finally reaching the sample medium. The energy received by the sample is less than that originally emitted by the transducer. The heat of the liquid in the ultrasonic bath can be transmitted to the sample, therefore during experimentation, it may not be clear if the observed effects are due to heat transmitted to the sample or the ultrasonic effects (Patricio, C., Fernandez, A., Mota, A., and Capelo, 2006). Cooling systems such as water baths are usually used to keep a stable temperature in the system.

In bath ultrasound, there is an uneven distribution of the waves due to non-uniformity in power outputs from the sonic transducer and the interference of incident and reflective waves inside the tank (Kiani, Zhang, Delgado, & Sun, 2011). Reproducibility and scalability of bath ultrasound is difficult to achieve due to variations in the wave distribution; therefore, a common application for ultrasonic baths is cleaning, since it does not require strict parameters.

The differences between bath ultrasound and probe ultrasound make each system suitable for different kinds of applications. Probe ultrasound is known to have high localized intensity compared to bath ultrasound; however, bath ultrasound can process larger volumes than probe ultrasound (Dhanalakshmi & Nagarajan, 2011).

Direct ultrasound is usually applied with an ultrasonic probe that is submerged into the sample medium. It has been found to be more intense and localized compared to bath ultrasound (Dhanalakshmi & Nagarajan, 2011); however, under experimental
conditions, the sample may be more prone to contamination, when taking the probe in and out while taking samples. When ultrasound is applied with an ultrasonic probe, cavitation beneath the probe occurs (Figure 16).

![Figure 16. Ultrasonic probe transmitting sound waves into liquid. The disturbed area beneath the probe indicates cavitation hot spot area](www.hielscher.com)

A number of different parts are used in an ultrasonic device (Figure 17). The ultrasonic processor converts the AC supply voltage to a high frequency alternating current above 20 kHz, and also works as a control panel. The ultrasonic converter
transforms the electrical energy into mechanical vibrations at a fixed frequency. The standard and booster probes increase the amplitude generated by the converter and transmit the energy into the sample. When the probes resonate, they expand and contract longitudinally on its center. The greater the mass ratio between the upper and lower section of the probe, the greater the amplification (“Sonics & Materials, Inc.,” 2016). There are different types of probes for different types of applications and volumes.

Figure 17. Parts of an ultrasonic probe.
a) Ultrasonic processor or control panel, converts main voltage into high frequency electrical energy; b) Ultrasonic converter, converts electrical energy into vibrations; c) Probes, standard and boosters, increase the ultrasound amplitude and transmit the energy into the sample; d) Probes or extenders, screw into threaded end probes, transmit ultrasound to the sample.

Direct ultrasound leads to an increase in the medium temperature, due to higher amplitude. High temperatures can cause problems such as degradation of the compound
of interest or volatilization. Strategies to control the temperature include using an ice bath, special reactors, pulse mode ultrasound, or secondary temperature controlled water baths (Santos, H., Lodeiro, C., Capelo, 2009). The application of ultrasound in a pulse mode, also called duty cycle, refers to the time that the sonicator is ON; therefore, a duty cycle of 40% means that the ultrasonicator was ON 4 seconds and off OFF 6 seconds.

An important factor to consider when applying direct ultrasound, is the shape and size of the reactor. Reactor shape influences the reflection of the ultrasound waves (Thangavadivel, Owens, Lesniewski, & Okitsu, 2013). Choosing the right reactor prevents death zones, which are zones where cavitation is not achieved and usually occurs when large volumes of sample are subjected to ultrasound, regardless the medium; dead zones does not refer to dead microorganisms. Cavitation phenomena decreases axially and radially very fast, so, low volumes increase the area of exposure (Santos, H., Lodeiro, C., Capelo, 2009). This is why it is important to keep a short distance between the wall of the reactor and the tip of the probe (Capelo, J., Galesio, M., Felisberto, G., Vaz, C., and Costa, 2005).

As seen before, ultrasound has been used for a variety of purposes, which has led to the development of different ultrasound devices. Even, ultrasound devices that are identical can be used for different purposes, depending on the method of application and the energy provided.
1.5. Ultrasound in bioprocesses

The application of low power ultrasound has proved to promote the growth of cell cultures and their products (Choi, Ahn, Kim, Han, & Kim, 2015; Chuanyun, Bochu, Chuanren, & Sakanishi, 2003; Jomdecha & Prateepsen, 2006; Lanchun, Bochu, Zhiming, et al., 2003; Kazuo Matsuura, Hirotsune, Nunokawa, Satoh, & Honda, 1994; Neel, Gedanken, Schwarz, & Sendersky, 2012; Schläfer, Sievers, Klotzbücher, & Onyeche, 2000; Sulaiman et al., 2011; Wood, Aldrich, & Ingram, 1997), however, high power ultrasound has also proved to disrupt cells, inactivating them, or affecting their productivity (Anderson, 1953; Guerrero, Tognon, & Alzamora, 2005; Ma, Chen, Liu, & Ye, 2008; Radel, McLoughlin, Gherardini, Doblhoff-Dier, & Benes, 2000; Tsukamoto, Yim, et al., 2004a; Tsukamoto, Constantinou, Furuta, Nishimura, & Maeda, 2004; Vivek, Subbarao, & Srivastava, 2016). Ultrasound has been used with enzymatic treatments (Ali Kashkooli, Rooney, & Roxby, 1980), for environmental remediation (Entezari & Petrier, 2003), during fermentation (Radel et al., 2000), and for the hydrolysis of biopolymers (Montalbo-lomboy, 2008).

Anderson (1953) and Jomdecha (2006) investigated the effects of direct ultrasound treatment on fermentations using *Saccharomyces cerevisiae*. Anderson applied ultrasound at 0.6 kHz, power of 10 W, and an intensity of 10.5 W/cm² at an intermittent and continuous modes, the duty cycles were 25% for 40 min, 50% for 20 min, 75%, and 100% for 10 min to a volume of 5 mL. Anderson found that carbon dioxide generation decreased after the fermentation medium was subjected to ultrasound, especially during fermentation medium containing younger cells. Fermentation rate was
also decreased in all the cases, even with the same energy provided, but different duty cycles, a shorter but more intense ultrasound caused more inhibition in the rate of fermentation. Ultrasound appeared to have a greater effect on younger than older cells, and extended the lag phase.

Jomdecha (2006) applied direct ultrasound at 20 kHz, at an intensity of 0.2, 0.4, and 0.8 W/cm² on 200 mL of yeast extract and peptone (YP) medium. Ultrasound was applied to reactors under non-shaking and shaking at 100 rpm. The samples were subjected to ultrasound at the levels described for 10 and 15 min at a 10% duty cycle. Yeast population growth was reported to be faster in the ultrasound treatments than the control. Jomdecha states that the mechanisms are still unclear and more research has to be done in the topic.

Neel (2012) reported an acceleration of ethanol fermentation from glucose by yeast *S. cerevisiae* at 20 and 30°C and 20 and 40% glucose solution in a 100 mL flask and 50 mL working volume, where 1 g of yeast was added. Indirect ultrasound was applied at 40 kHz and 120 W, in an ultrasonic bath with a 4 L volume, where the flasks were located, resulting in continuous mild ultrasound at 30 W/L. The ultrasound treatments were not stirred, while the control treatments were stirred at 1,000 rpm. Fermentation under stirring and ultrasound were faster than unstirred fermentation. Indirect ultrasound fermentation at 30°C was completed at 11 h, compared to the control treatment 18 h. At 20°C, fermentation was completed in 19 h in the ultrasound treatment, and in 36 h in the control treatment. Yeast agglomerate more in the stirring treatment than in the ultrasound treatment. Neel (2012) found that ultrasound provided better mixing and hypothesized that it may have helped to remove ethanol from the yeast surface and CO₂.
from the fermentation medium, promoted changes in membrane permeability, and enhanced mass transfer. Neel (2012) also did an experiment with a horn-type ultrasound where after 30 min, the yeast cells were ruptured and no ethanol was obtained, no energy input level was described.

Ultrasound in the ethanol industry is not still widely used, however, its effect, cavitation, has been applied as a pretreatment in order to release sugars and improve ethanol fermentation (Ramirez, Kozyuk, Lyle, & Michel, 2016; Ramirez, Kozyuk, & Michel, 2013). Ramirez (2013) applied cavitation to a corn slurry, by hydrodynamic cavitation. This type of cavitation happens when bubbles form in a liquid flow, due to fast changes in pressure, with the objective to release more starch and make it available to the enzymes for converting to simple sugars and then fermentation.

Table 3 summarizes several studies where ultrasound has been applied during fermentation, as a pretreatment, and to inactivate cells. The table also includes the method in which ultrasound was applied, the microorganism used, frequency of ultrasound, volume treated, energy provided, intensity, energy density, and results of the experiment. The method of ultrasound applied was either direct, by using a probe, or indirect, using an ultrasound bath, or a probe but not to the sample medium directly. Not all the studies completely describe the experimental conditions, but when conditions are described, they vary between studies. Method of application, energy applied, duty cycle, volume, substrate, and microorganism are some of the variables that differ in the systems. High ultrasound densities and the use of a probe in direct ultrasound seem to inhibit and inactivate the microorganisms, compared to applying indirect ultrasound.
Table 3. Previous studies.
Ultrasound has been applied directly or indirectly in fermentation, as a pretreatment, and to inactivate cells. The table shows organism, frequency of ultrasound, volume treated, intensity of ultrasound, power output, ultrasound density, and results.

<table>
<thead>
<tr>
<th>Source</th>
<th>Ultrasound</th>
<th>Microorganism</th>
<th>Frequency (kHz)</th>
<th>Volume (mL)</th>
<th>Intensity (W/cm²)</th>
<th>Power (W)</th>
<th>Ultrasonic density (W/L)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Huezo, 2017)</td>
<td>Direct</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>20</td>
<td>125</td>
<td>2.14, 2.91</td>
<td>22.76, 30.86</td>
<td></td>
<td>Inhibited fermentation compare to control. Improved ethanol production</td>
</tr>
<tr>
<td>(Sulaiman et al., 2011)</td>
<td>Direct</td>
<td><em>K. marxianus</em></td>
<td>20</td>
<td>3150</td>
<td>11.8</td>
<td>15</td>
<td>5</td>
<td>Improved ethanol production yield Improved ethanol fermentation</td>
</tr>
<tr>
<td>(Wood et al., 1997)</td>
<td>Direct</td>
<td><em>Klebsiella oxytoca</em></td>
<td>36</td>
<td>10,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Anderson, 1953)</td>
<td>Direct</td>
<td><em>S. cerevisiae</em></td>
<td>0.6</td>
<td>5</td>
<td>10.5</td>
<td>10</td>
<td>2,000</td>
<td>Inhibited fermentation Accelerated growth</td>
</tr>
<tr>
<td>(Jomdecha &amp; Prateepsen, 2006)</td>
<td>Direct</td>
<td><em>S. cerevisiae</em></td>
<td>20</td>
<td>200</td>
<td>0.2, 0.4, 0.8</td>
<td>0.001 - 0.004</td>
<td></td>
<td>Accelerated growth</td>
</tr>
<tr>
<td>(Radel et al., 2000)</td>
<td>Indirect</td>
<td><em>S. cerevisiae</em></td>
<td>2200</td>
<td>64</td>
<td>14</td>
<td>219</td>
<td></td>
<td>Killed 25%</td>
</tr>
<tr>
<td>(K. Matsuura, Hirotsune, Nunokawa, Satoh, &amp; Honda, 1994)</td>
<td>Indirect</td>
<td><em>S. cerevisiae</em></td>
<td>43</td>
<td>50 to 1,000</td>
<td>0.03</td>
<td>0.59</td>
<td>12 to 0.6</td>
<td>Improved ethanol productivity</td>
</tr>
<tr>
<td>(Schläfer et al., 2000)</td>
<td>Indirect</td>
<td><em>S. cerevisiae</em></td>
<td>25</td>
<td>4000</td>
<td></td>
<td>0.3, 12</td>
<td></td>
<td>Improved ethanol productivity</td>
</tr>
<tr>
<td>(Lanchun, Bochu, Liancai, et al., 2003)</td>
<td>Indirect</td>
<td><em>S. cerevisiae</em></td>
<td>24</td>
<td>300</td>
<td>2</td>
<td>7</td>
<td></td>
<td>Enhanced permeability to proteases Improved fermentation</td>
</tr>
<tr>
<td>(Neel et al., 2012)</td>
<td>Indirect</td>
<td><em>S. cerevisiae</em></td>
<td>40</td>
<td>100 in 4,000</td>
<td>120</td>
<td>30</td>
<td></td>
<td>Improved fermentation Improved ethanol production</td>
</tr>
<tr>
<td>(Choi et al., 2015)</td>
<td>Indirect</td>
<td></td>
<td>40</td>
<td>~450</td>
<td>120-200</td>
<td>267, 356, 444</td>
<td></td>
<td>Improved ethanol production</td>
</tr>
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</table>

Continued
Table 3 continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Organism</th>
<th>Time</th>
<th>Temperature</th>
<th>Concentration</th>
<th>Effect</th>
</tr>
</thead>
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<tr>
<td>(Lanchun, Bochu, Zhiming, et al., 2003)</td>
<td>S. cerevisiae</td>
<td>18 to30</td>
<td>300</td>
<td>2 -8</td>
<td>Promoted growth</td>
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<tr>
<td>(Bochu, et. al., 2003)</td>
<td>S. cerevisiae</td>
<td>24</td>
<td></td>
<td>2</td>
<td>Enhanced permeability to Ca2+</td>
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<tr>
<td>Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Nikoli, Mojovi, Rakin, Pejin, &amp; Pejin, 2010)</td>
<td>Saccharomyces cerevisiae</td>
<td>40</td>
<td></td>
<td></td>
<td>Increased [glucose] and [ethanol]</td>
</tr>
<tr>
<td>(Khanal, Montalbo, Leeuwen, Srinivasan, &amp; Grewell, 2007)</td>
<td>Direct</td>
<td>20</td>
<td>35</td>
<td></td>
<td>Reduced particle size</td>
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<tr>
<td>(Nitayavardhana et al., 2010)</td>
<td>Direct</td>
<td>20</td>
<td>35</td>
<td>2,000, 4,000, 8,500</td>
<td>Increased [starch] and [ethanol]</td>
</tr>
<tr>
<td>Inactivation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Vivek et al., 2016)</td>
<td>Direct</td>
<td>30</td>
<td>184–368</td>
<td></td>
<td>Inactivated microorganisms</td>
</tr>
<tr>
<td>(Guerrero et al., 2005)</td>
<td>Direct</td>
<td>S. cerevisiae</td>
<td>20</td>
<td>95</td>
<td>600</td>
</tr>
<tr>
<td>(Tsukamoto, Constantinou, et al., 2004)</td>
<td>Direct</td>
<td>S. cerevisiae</td>
<td>27.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(Tsukamoto, Yim, et al., 2004b)</td>
<td>Direct</td>
<td>S. cerevisiae</td>
<td>27.5</td>
<td></td>
<td>42,000</td>
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<tr>
<td>(D. Liu et al., 2013)</td>
<td>Direct</td>
<td>S. cerevisiae</td>
<td>20</td>
<td>200</td>
<td>120 to 600</td>
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2.1. Abstract

Ethanol is the primary transportation biofuel used in the world. In United States, around 15 billion gallons of ethanol per year are made by fermentation of corn starch and lignocellulosic biomass. Ultrasound pretreatment of biomass has been shown to improve starch release and ethanol yield; however, less attention has been given to the ability of ultrasound to improve the mass transfer of substrates and products to and from yeast cells during fermentation. Previous studies have shown that certain levels and methods of ultrasound can improve or inhibit fermentation. Improvements have been attributed to reductions in mass transfer limitations; however, calculations of the external and intraparticle observable modulus indicated that no external or intraparticle mass transfer limitations should exist for yeast fermentation from glucose. The purpose of this study was to investigate the effects of ultrasound at different levels of intensity during fermentation of glucose to ethanol, by yeast *Saccharomyces cerevisiae*. The effects of ultrasound on glucose uptake rate, ethanol and carbon dioxide production rates, and yeast cells population and viability were measured. Ultrasound was applied to a fermentation medium containing nutrients and glucose at 200 g/L. Four treatments were compared:
direct probe ultrasound at 23 and 32 W/L, indirect ultrasound, and no-ultrasound. Both
direct and indirect ultrasound had negative effects on yeast performance; they decreased
rates of glucose uptake and ethanol production, and yeast viability. Results indicate that
ultrasound during fermentation, at levels applied, is not effective at improving the rate of
ethanol production. Different methods of application, energies, and experimental set ups
must be tested to get a broad understanding of the effect of ultrasound and its potential,
when applied during fermentation.

2.2. Introduction

The world total primary energy production for 2015 was $1.62 \times 10^8$ kWh, and its
energy consumption was $1.60 \times 10^8$ kWh (Enerdata, 2016). United States, in 2015,
consumed $2.55 \times 10^7$ kWh of energy, which represents 16% of the world consumption,
despite having just 4% of the world population (Worldometers, 2016). The U.S.
consumed $2.14 \times 10^6$ kWh more than what the country itself produced. In world and the
U.S., most of the energy consumed is derived from fossil fuels.

The extraction and burning of fossil fuels (oil, coal and gas emit large quantities
of carbon dioxide and other greenhouse gases to the atmosphere, causing climate change.
Their use also causes problems with the health of the environment and living things.
They are non-renewable and will be depleted within the next 30 to 100 years at current
use rates (Shafiee & Topal, 2009). To help reduce and mitigate some of these problems,
renewable energy technologies are being rapidly developed and used. They will help mitigate some of the socioeconomic and environmental issues of fossil fuel use.

Renewable energy technologies are needed to reduce greenhouse gas emissions (Sims et al., 2003), meet energy needs, achieve energy independence from fossil fuels and from other countries (Aronoff et al., 2013), mitigate climate change, and generate green jobs (U.S. Department of Energy, 2016b). In 2015, 9,450 BTU of renewable energy including hydroelectric, geothermal, solar, wind, wood, waste, and biofuels were consumed (U.S. Energy Information Administration, 2016c).

One source of renewable energy is biomass, from which biofuels are derived. Ethanol is the principal biofuel produced in United States. In 2015, United States produced 14,807 million gallons of ethanol, which represents 55% of the total ethanol production in the world (Renewable Fuel Association, 2016b). United States has 231 ethanol production plants, and 15 of them use cellulose as a feedstock (Ethanol Producer Magazine, 2016).

In a corn ethanol processing plant, the process begins with dry milling; cooking, liquefaction, fermentation, distillation, molecular sieve, denaturant, storage, and distribution. Corn ethanol processing plants differ from lignocellulosic ethanol plants in delignification, release of hemicellulose and cellulose, and de-polymerization of hemicellulose and cellulose into hexose and pentose sugars. It is then similar to the corn ethanol plant in the fermentation, distillation, molecular sieve, denaturant, storage, and distribution. All the ethanol from biomass is produced through fermentation.

Pretreatment and enzymatic saccharification of corn starch and lignocellulosic biomass, yields monomeric sugars, which are the substrates upon which *Saccharomyces*
S. cerevisiae grows. *S. cerevisiae* is the most effective microorganism in fermenting sugars to ethanol (Aristidou, A., Baghaei, N., Javed, M., and Hartley, 2012), due to its great resistance to high ethanol concentrations (Fleet, 1993). Yeast plays a central role in the biological production of fuels and chemicals. The success of the ethanol fermentation is based on maintaining yeast in a viable state so that they can consume all the fermentable sugars (Bisson, 1999). Genetic improvements to yeast have been made to increase the range of substrates it can utilize (Dequin, 2001). Another possible method used to improve its conversion efficiency is to stimulate yeast with ultrasound (Anderson, 1953; Bochu et al., 2003; Jomdecha & Prateepsen, 2006; Lanchun, Bochu, Liancai, et al., 2003; Lanchun, Bochu, Zhiming, et al., 2003; K. Matsuura et al., 1994; Neel et al., 2012; Radel et al., 2000; Schläfer et al., 2000).

Ultrasound is sound waves with frequencies above 20 kHz. Sound is a longitudinal wave, a form of disturbance, which travels through a medium. Ultrasound generates alternating high and low pressure areas, causing compressions and rarefactions cycles in the liquid. Rarefactions leads to the formation of cavities, which implode during compression, the process of cavity formation is called cavitation (F. Michel & Kozyuk, 2015; Suslick et al., 1999). During the implosion of the cavities, high temperatures, high pressures, and high rates of cooling are reached. The incidence of cavitation depends on the acceleration at which the change in pressure occurs. Higher acceleration results in higher differential pressures, resulting in a higher probability of cavities.

Ultrasound can be applied directly or indirectly. Direct application of ultrasound refers to application straight into the sample medium; in comparison to indirect application, which refers to the application of ultrasound into a different medium before
reaching the target sample, referred to as bath ultrasound. The differences between bath ultrasound (indirect) and probe ultrasound (direct) make each system suitable for different kinds of applications. Probe ultrasound is known to have high localized intensity compared to bath ultrasound; however, bath ultrasound can process larger volumes of solution than probe ultrasound, (Dhanalakshmi & Nagarajan, 2011). Direct ultrasound is usually applied with an ultrasonic probe submerged into the sample medium, which is why it has been found to be more intense and localized compared to bath ultrasound (Dhanalakshmi & Nagarajan, 2011). When ultrasound is applied directly or indirectly to a medium, it usually generates heat, which is transmitted to the sample. The heat generated to the medium may or may not affect the sample. If it does have an effect, it will confound the effect of ultrasound (Patricio, C., Fernandez, A., Mota, A., and Capelo, 2006).

Little research has been done to study the effect of ultrasound on the yeast as opposed to the substrate. Ultrasound has been used in three different ways, as a pretreatment (Khanal et al., 2007; Nikoli et al., 2010; Nitayavardhana et al., 2010), to deactivate cells (Vivek et al., 2016), and as an enhancer during fermentation.

During ethanol fermentation, glucose molecules are transferred from the bulk media to the surface of the yeast cell and into the yeast cell, while ethanol and carbon dioxide are transferred in the opposite direction. Glucose enters the cell by facilitated diffusion; ethanol and carbon dioxide diffuse out of the cell by simple diffusion (Zamora, 2009). Both types of diffusion occur simultaneously.

Yeast transfers sugars (glucose, fructose, and mannose) into the cell across the membrane by facilitated diffusion, where sugars bind to carrier proteins called hexose
transporters on the outer side of the membrane and are released into the cytoplasm (Barnett, 1976; Z. Liu & Li, 2014; Luyten et al., 2002). During the facilitated diffusion of glucose, no metabolic energy is required and the glucose concentration gradient over the membrane is the main driver of the process (Weusthuis et al., 1994). The rate of glucose uptake in yeast is dictated by the activity and concentration of glucose transporters in the plasma membrane (Panalver et al., 1998) and the glucose concentration outside and inside the yeast cell.

Ethanol increases fluidity of the yeast plasma membrane (Curran, M. and Seeman, 1979), which negatively affects yeast performance. Continuous exposure to ethanol may cause cell lysis (R P Jones & Greenfield, 1987). Carbon dioxide is another product of fermentation, and it is highly soluble and present in various gas and dissolved forms. Therefore, the concentration and net flux of CO$_2$ across the yeast membrane is regulated by the concentration of aqueous CO$_2$ in the intracellular and extracellular fluids (Rodney P Jones & Greenfield, 1982). Ethanol and CO$_2$ at high concentration can inhibit yeast metabolism.

One mechanism by which ultrasound may improve ethanol fermentation is by reducing mass transfer limitations for glucose, ethanol, and carbon dioxide in the vicinity of yeast cells. If mass transfer limits the movement of these products away from yeast cells, they may adversely affect the rate of fermentation by limiting glucose concentration at the cell surface.

By implementing ultrasound, we expect a greater rate of ethanol production due to a decrease in the concentration of ethanol at the cell surface, greater CO$_2$ release from the cell surface to the medium and from the medium to the air, and better glucose uptake.
Since ultrasound and hydrodynamic cavitation (Ramirez, 2012) have been used as a pretreatment of biomass, the purpose of this study is to evaluate the effects of ultrasound on improving mass transfer limitations during ethanol fermentation from glucose.

The hypothesis of this study is that ultrasound may enhance the performance of the yeast \textit{Saccharomyces cerevisiae} by interfering with the boundary layer at the cell surface and reduce mass transfer limitations for glucose, ethanol, and carbon dioxide.

The objective of this study was to evaluate the effect of ultrasound on yeast performance by measuring glucose uptake, ethanol and CO\textsubscript{2} production, and yeast cell population and viability.
2.3 Materials and Methods

2.3.1. Preliminary experiments

Preliminary experiments (Appendix A) were performed to develop a system to test the effects of ultrasound cavitation on the rate of glucose uptake, ethanol and CO₂ production, and yeast viability during fermentation. These experiments indicated the time required to harvest the inoculum and the way to inoculate the fermentation broth. An adequate reactor, probe, stopper, agitation system, temperature control, duty cycle, and a second sonicator were required to conduct the experiments. The details and system developed is described in the following sections.

2.3.2. Inoculum preparation

Inoculum for fermentation was prepared according to Ramirez (2013), Dowe and McMillan (2008), and Montalbo-Lomboy (2008). Yeast extract and peptone (YP) 10X was prepared by mixing 100 g of yeast extract and 200 g of peptone in double-deionized water (DDIW) to a total volume of 1,000 mL. This solution was autoclaved at 121°C for 30 minutes and used as a stock for future media preparation. Glucose solution at a concentration of 50% was prepared by diluting 500 g of glucose in DDIW to a total volume of 1,000 mL. YP-glucose 5% was prepared by mixing 100 mL of YP 10X and 100 mL of glucose solution 50% in 800 mL of DDIW. Citrate buffer (pH 4.5, 1M) was prepared by diluting 192 g of anhydrous citric acid into DDIW for a total volume of 1,000 mL; the solution was titrated to a pH of 4.3 with a solution of sodium hydroxide.
10M (NaOH); the base was prepared by diluting 400 g of NaOH in DDIW for a total volume of 1,000 mL.

The yeast pre-culture media was then prepared by mixing 1,000 mL of YPD-glucose 5%, 100 mL of citrate buffer, and adding 3.07 g of magnesium sulfate heptahydrate (MgSO₄·7H₂O), 1.80 g of potassium phosphate monobasic (KH₂PO₄), 4.87 g of sodium phosphate monohydrate (Na₂HPO₄·H₂O), 0.32 g of zinc sulfate heptahydrate (ZnSO₄·7H₂O), and DDIW to a total volume of 1,500 mL. Finally, 1 g of dried yeast, *Saccharomyces cerevisiae* (Bio-Ferm XR, North American Bioproducts Corporation) was added to the pre-culture media. The yeast inoculum was incubated at 32°C, 180 rpm for 19 hours in a shaker (Innova™ 2300, Platform shaker, New Brunswick) to get a viable yeast population of ~1×10⁸ cells/mL.

2.3.3. Fermentation

Glucose was used as the only substrate to avoid the effects of ultrasound on other solid suspended material, and allow the examination of direct and indirect ultrasound on the performance of *Saccharomyces cerevisiae*.

Fermentation was conducted in autoclaved 125 mL flasks with a working volume of 125 mL. Fermentation medium consisting of glucose 200 g/L and 6.25 mL YP 10X medium, was inoculated with 6.25 mL of yeast inoculum (see above), for a total volume of 125 mL. Flasks were sealed with rubber stoppers to maintain anaerobic conditions. A needle was inserted into each rubber stopper to allow the CO₂ gas to come out of the system. All the treatments were kept at 32°C by placing them in a warm-room incubator.
The fermentation progress was measured every 12 hours for 36 hours. Fermentation was stopped at 36 hours when more than 75% of the glucose was consumed, and the peak of production of ethanol and carbon dioxide already had passed. The reactors were located in a water-bath-shaker (Model G76D, Gyrotory ® Water Bath Shaker, New Brunswick Scientific Co. INC.) and a shaker (Innova ™ 2300, Platform shaker, New Brunswick), both shaking at 180 rpm to prevent dead zones in the flasks. Dead zones refer to the areas of the reactor where the effects of ultrasound does not reach, not to dead microorganisms. The flask reactors were located in a warm room at 32°C. The water-bath-shaker was used to maintain temperature at 32°C.

2.3.4. Ultrasound

Ultrasound was applied directly and indirectly. Two treatments received direct ultrasound in which a probe was inserted 1 cm into the fermentation medium. A rubber ring around the probe served as a stopper to maintain anaerobic conditions, and a needle was inserted for the CO₂ to be released. A picture of the ultrasound unit is shown in Figure 18.

Probe ultrasound was applied to the flasks using an ultrasound probe produced by Sonics® & Materials, Inc. It includes a vibracell control panel that operates at 750 W, converter (Model CV33), and probe (13 mm diameter, threaded end). Ultrasound was provided to the fermentation broth at a frequency of 20 kHz.

In initial experiments yeast were completely killed by the heat in the reactors, because it did not have any medium to dissipate the heat. In order to prevent this rise in
temperature due to ultrasound, reactors were placed in a water bath, to dissipate the heat and maintain the temperature at 32°C.

The ultrasound equipment power was managed by changing the amplitude setting; the ultrasound dose by changing the duty cycle. The ultrasound duty cycle used was 15 seconds with 1 second ON and 14 seconds OFF, which is 4 s/min. Duty cycle expressed in percentage is equal to the proportion of the pulse time to the cycle time, in this case \((1\text{ s}/15\text{ s}) \times 100\% = 6.67\%\) duty cycle. Two ultrasound energies were applied to the two direct ultrasound treatments D-1 and D-2, the energy densities were 173 and 237 J/min or 23 and 32 W/L respectively.

Figure 18. Ultrasound equipment.
A) Ultrasound equipment including control panel VC 750 W, converter (Model CV33), and probe (13 mm diameter, threaded end). B) Ultrasound probe inside the flask reactor, laboratory set up.
2.3.5. Sampling and measurements

Sampling from each treatment and measurements were done every 12 hours. Carbon dioxide generation was obtained by weight loss. Centrifuged and filtered samples were used to measure concentrations of glucose and ethanol by high performance liquid chromatography (HPLC, Agilent Technologies 1200 series). Yeast active and dead cells were manually counted after staining the sample with Trypan Blue solution, put on a hemocytometer and observed through a microscope. Hydrated yeast cells are considered active. Hydrated cells, in comparison with inactive cells, do not remain blue after staining with Trypan Blue solution, while inactive and dead cells remain blue.

2.3.6. Experimental design

Four different ultrasound intensities were investigated: direct ultrasound at 23 W/L (D-1); direct ultrasound at 32 W/L (D-2); indirect ultrasound (I), and no-ultrasound (N) in a complete randomized design. All ultrasound treatments were inside a water-bath-shaker (direct and indirect). For treatments D-1 and D-2 the ultrasound probe was directly into the reactor. The indirect ultrasound (I) treatment was also inside the water-bath-shaker and received the waves emitted from the other two direct ultrasound reactors, water was the sound transfer medium. The no-ultrasound treatment (N) was located in a shaker, outside the water-bath. Treatments D-1 and D-2 had 3 replicates each; I, 6 replicates; and N, 6 replicates. Fermentation conditions remain the same in all the reactors. Treatments and responses analyzed are in Table 4.
Table 4. Treatments studied and response variables analyzed.

<table>
<thead>
<tr>
<th>Treatments/ hours</th>
<th>Resp.</th>
<th>Glucose uptake</th>
<th>Ethanol production</th>
<th>CO₂ generation</th>
<th>Yeast,</th>
</tr>
</thead>
<tbody>
<tr>
<td>No-ultrasound</td>
<td>N</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Indirect ultrasound</td>
<td>I</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Direct ultrasound 1</td>
<td>D-1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Direct ultrasound 2</td>
<td>D-2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

2.3.7. Mass transfer limitation

To test the hypothesis that ultrasound reduces external and intraparticle mass transfer limitations for glucose, ethanol, and carbon dioxide, the external and intraparticle observable modulus for those molecules were calculated under worst case conditions (high ethanol concentration, saturating CO₂ concentration, and low glucose concentration).

The external mass transfer modulus is the ratio between the concentration of the component in the surface of the particle and the concentration of the component in the bulk liquid. If the concentration in the bulk liquid is the same as the concentration at the surface of the particle, then no boundary layer exist when. If \( C_{As}/C_{Ab} \approx 1 \), it indicates the external mass transfer limitation is not significant, because the concentration at the surface is almost the same as the concentration in the bulk. However, if \( C_{As}/C_{Ab} << 1 \), it indicates that the external mass transfer limitation is significant due to a well-defined boundary layer. An observable modulus for external mass transfer is represented as follow:
\[ \Omega = \frac{R}{3} \times \left( \frac{r_{A,\text{obs}}}{k_s \times C_{Ab}} \right) \]

Where:
\( \Omega \) = dimensionless observable modulus for external mass transfer  
\( R \) = radius of the yeast, m  
\( r_{A,\text{obs}} \) = observed reaction rate  
\( k_s \) = liquid-solid mass transfer coefficient  
\( C_{Ab} \) = substrate concentration in the bulk liquid

The intraparticle mass transfer is calculated by the Thiele modulus, also called Weisz’s modulus. It is a dimensionless number defined as a ratio of the reaction rate to the diffusion rate (Kraakman et al., 2011). It can be used to estimate any intraparticle mass transfer limitations of substrate, without previous knowledge of the kinetics (F. C. Michel & Grulke, 1992). If the Thiele observable modulus (\( \Phi \)) has a value greater than 0.3, it indicates that internal mass transfer limitations are significant. If the value is lower than 0.3, it indicates that there is no internal mass transfer limitation in the system. Expressions for spheres and flat plates are different (Doran, 1995); equation 1 is the expression for spheres.

\[ \Phi = \left( \frac{R}{3} \right)^2 \times \left( \frac{r_{A,\text{obs}}}{D_{Ae} \times C_{As}} \right) \]
Where:
\[ \Phi = \text{dimensionless observable number Thiele modulus} \]
\[ R = \text{radius of the yeast, m} \]
\[ r_{A, \text{obs}} = \text{observed reaction rate per unit volume of catalyst} \]
\[ D_{Ae} = \text{effective diffusivity of substrate} \]
\[ C_{As} = \text{substrate concentration at the external surface} \]

In order to calculate the external and intraparticle modulus, values for yeast radius, diffusivity, concentration, and rate of uptake or production were calculated for glucose, ethanol, and carbon dioxide, shown in Table 5. Values for concentration and rate are based on experimental results.

### Table 5. Values to calculate mass transfer

<table>
<thead>
<tr>
<th>Yeast radius (µm)</th>
<th>Diffusivity ( m^2/s )</th>
<th>Concentration (g/L)</th>
<th>Rate ((g/L)/12 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 1 and 5</td>
<td>7.3×10^{-10}</td>
<td>200 and 20</td>
<td>25 and 75</td>
</tr>
<tr>
<td>Ethanol 1.4×10^{-9}</td>
<td>10 and 100</td>
<td>15 and 50</td>
<td></td>
</tr>
<tr>
<td>CO₂ 2.1×10^{-9}</td>
<td></td>
<td>1.6×10^{-2} M</td>
<td></td>
</tr>
</tbody>
</table>

Values for diffusivity of glucose and ethanol are based on Gutenwik, Nilsson, and Axelsson (2002), diffusivity of carbon dioxide is based on Chaix, Guillaume, and Guillard (2014).

2.3.8. Statistical analysis

The effects of direct ultrasound (D-1 and D-2), indirect ultrasound (I), and no-ultrasound (N) on glucose uptake, ethanol production, carbon dioxide generation, and active and dead yeast cells were statistically analyzed using JMP Pro 12.2.0. Data was
collected at 0, 12, 24, and 36 hours after the fermentation was started. ANOVA or Welch ANOVA were performed within each response variable; p-values < 0.05 were considered significant and then the response variable means were analyzed by Tukey-Kramer’s HSD test.
2.4. Results and Discussion

2.4.1. Mass transfer limitation

In order to calculate the external and intraparticle modulus, values for yeast radius, diffusivity, concentration, and rate of uptake or production were calculated for glucose, ethanol, and carbon dioxide. The value for yeast radius used was 3.5 µm. Diffusivity value for glucose at 32°C is $7.3 \times 10^{-10}$ m$^2$/s (Gutenwik et al., 2002); ethanol, $1.4 \times 10^{-9}$ m$^2$/s (Gutenwik et al., 2002); and carbon dioxide, $2.1 \times 10^{-9}$ m$^2$/s (Chaix et al., 2014). The concentrations used for glucose and ethanol came from the experimental results, glucose 200 and 20 g/L, corresponding to different times during fermentation; ethanol, 10 and 100 g/L; and carbon dioxide, $1.6 \times 10^{-2}$ M (Chaix et al., 2014). The rate of glucose, ethanol, and carbon dioxide rates used are from the experimental results, glucose 25 and 75 (g/L)/12 h; ethanol and carbon dioxide 15 and 50 (g/L)/12 h.

The external mass transfer modulus calculated were below the limit value of 1. If the value is bigger than 1, the external mass transfer limitation is significant. The external mass transfer showed that the concentration of substrate in the bulk liquid is the same as in the surface of the yeast cell.

The intraparticle Thiele observable modulus calculated from different concentrations, rates, and yeast radius were all well below 0.3, where the intraparticle diffusion resistant is found to limit the rate. This value indicates that there is no limitation for glucose to enter the yeast cell. Similar low values were found for ethanol and carbon dioxide.
The rate of glucose uptake in yeast is dictated by the activity and concentration of glucose transporters in the yeast cell plasma membrane (Panalver et al., 1998). The concentration of this transporter is affected by the effects of increasing ethanol concentration (W. Liu, Bratko, Prausnitz, & Blanch, 2004). Ethanol denatures proteins by interacting with the non-polar parts of proteins (Z. Liu & Li, 2014).

Since no limitation exists, we based this experiment on previous studies (Neel et al., 2012; Sulaiman et al., 2011) that used ultrasound during fermentation in order to better understand the mechanism behind the use of ultrasound. Previous studies that concluded that improvements were due to reducing mass transfer limitations provided no explanation of how that occurs and the calculations above indicate that there is no intraparticle mass transfer limitation to be improved.

2.4.2. Fermentation

Experiments were conducted to study the effects of direct and indirect ultrasound on glucose uptake, ethanol and carbon dioxide production, and active and dead yeast cells during fermentation. Direct ultrasound at 2 different levels was applied D-1, 23 W/L (173 J/min); D-2, 32 W/L (237 J/min); a third treatment was subjected to indirect ultrasound (I); and a fourth treatment had no-ultrasound (N). The means of glucose uptake, ethanol, and CO2 production during fermentation for the 4 treatments are shown in Figure 19.
2.4.3. Glucose uptake

Glucose is a sugar used as a carbon source for energy and growth by yeast *Saccharomyces cerevisiae*. In this experiment, glucose was used as the only substrate for fermentation to study the effects of ultrasound on glucose mass transfer, and not on other substrates. Biomass substrates, such as corn slurry, have already shown to release...
additional starch under ultrasound or cavitation conditions (Khanal et al., 2007; Ramirez et al., 2016). Furthermore, there is no evidence that ultrasound degrades the glucose molecule, the wavelength is too long compared to the size of the glucose molecule (Bang & Suslick, 2010).

The concentration of glucose was measured among treatments during fermentation to compare the effects of ultrasound on glucose uptake by yeast. Initial concentration of glucose was 200 g/L. There was no risk of shocking the yeast due to high concentration of sugars (Moulin, Boze, & Galzy, 2016). Shocking due to high concentration of sugars occurs at levels around 380 g/L, when it will inhibit the yeast by osmotic pressure effects (Maiorella et al., 1983).

During the first 12 hours of fermentation, glucose uptake in the no-ultrasound treatment (N) was significantly faster than in the 2 direct ultrasound treatments, D-1 and D-2, by 64 and 38% respectively (Figure 20). This findings indicate that the negative effect of ultrasound at the levels previously described were manifested since the beginning of the fermentation.

Between 12 and 24 hours the highest glucose uptake rate was observed for all the treatments, no significant differences were found in this time range. Between 24 and 36 hours, the glucose consumed by the two direct ultrasound treatments was greater than the no-ultrasound treatment (N), this because they have more glucose available at the end, compare to treatment N, which consumed more sugar at the beginning of the fermentation and had less glucose available by the end. Treatments I, D-1, and D-2 were catching up with the sugars they had left. D-1 and D-2 consumed glucose at a slower rate
initially, which is why they had more glucose available at the end of the fermentation (Table 6).

Table 6. Glucose uptake per treatment.

<table>
<thead>
<tr>
<th>Glucose uptake rate (g/L)</th>
<th>00-12 h</th>
<th>12-24 h</th>
<th>24-36 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>68.43 ±3.41 a</td>
<td>80.36 ±6.40 a</td>
<td>44.88 ±1.42 b</td>
</tr>
<tr>
<td>I</td>
<td>57.39 ±10.23 ab</td>
<td>73.85 ±1.98 a</td>
<td>33.12 ±2.27 c</td>
</tr>
<tr>
<td>D-1</td>
<td>24.52 ±10.37 c</td>
<td>80.78 ±7.92 a</td>
<td>52.37 ±5.66 a</td>
</tr>
<tr>
<td>D-2</td>
<td>42.24 ±6.43 bc</td>
<td>82.19 ±6.64 a</td>
<td>46.14 ±1.58 ab</td>
</tr>
</tbody>
</table>

Means are followed by ± standard deviation.

The glucose consumed after 36 hours in treatment N (no-ultrasound) was 19, 12, and 15% higher than the two direct (D-1 and D-2) and one indirect ultrasound treatments respectively (Figure 20). The high glucose uptake rate by the no-ultrasound treatment indicates that there was a negative impact of ultrasound on glucose uptake. According to Radel (2000), sub lethal levels of ultrasound may not directly kill yeast, but may affect the morphology of some structures, such as the vacuole, which may negatively impact yeast metabolism, and subsequently die.
Figure 20. Glucose uptake (g/L) as affected by ultrasound treatments. Each bar indicates the amount of glucose consumed in a 12 hour period from 0 to 12, 12 to 24, and 24 to 36 hours. Each texture pattern and treatment contains the result of the Tukey-Kramer HSD test, $\alpha = 0.05$; lower case letters indicate differences among treatments at the same time range; capital letters indicate differences among the total glucose uptake between treatments. Same letters indicate no significant difference between treatments, different letters indicate a significance differences. Each treatment has an error bar indicating the standard deviation.

2.4.4. Ethanol production

Ethanol is the product of interest from fermentation in the biofuel and energy industry. Ethanol is an inhibitor of yeast and its accumulation near the yeast cell wall could potentially lead to higher internal concentrations and inhibit the rate of fermentation. The effect of ultrasound on ethanol production from glucose was studied by applying ultrasound to the fermentation medium at different levels and measuring the ethanol concentration over time.
During the first 12 hours of fermentation, the ethanol production in the no-ultrasound treatment (N) and indirect ultrasound (I) were significantly higher than D-1 and D-2 (Table 7), which indicates that direct ultrasound may have a negative effect in the production of ethanol. Lanchun (2003) stated that yeast cells are generally most sensitive during the lag phase because they are preparing for reproduction, which make them even more sensitive if subjected to ultrasound. Between the 12 and 24 hour, treatment N had the highest ethanol production, but was not significantly higher than all the other treatments. In the last 12 hours, treatments D-1 and D-2 produced more ethanol than treatments N and I, the reason is similar to the one for glucose uptake. Treatments D-1 and D-2 produce ethanol at a lower rate than N and I, which allows them more sugar to convert to ethanol at the end. If we left the fermentation to run until completion, all treatments would likely have produced the same amount of ethanol (Table 7).

Table 7. Ethanol production per trt.

<table>
<thead>
<tr>
<th></th>
<th>00 - 12 h</th>
<th>12 - 24 h</th>
<th>24 - 36 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>27.24 ±1.16 a</td>
<td>42.52 ±1.33 a</td>
<td>25.08 ±2.00 a</td>
</tr>
<tr>
<td>I</td>
<td>28.14 ±0.19 a</td>
<td>37.43 ±1.47 b</td>
<td>18.22 ±0.94 b</td>
</tr>
<tr>
<td>D-1</td>
<td>12.55 ±2.74 c</td>
<td>38.38 ±2.19 ab</td>
<td>25.58 ±5.21 a</td>
</tr>
<tr>
<td>D-2</td>
<td>20.69 ±2.06 b</td>
<td>40.14 ±0.38 ab</td>
<td>26.64 ±0.66 a</td>
</tr>
</tbody>
</table>

Means are followed by ± standard deviation.

After 36 hours, the no-ultrasound treatment (N) produced 11, 19, and 9% more ethanol than treatments I, D-1, and D-2 respectively. Although the maximum production
of ethanol was found in the no-ultrasound treatment (N) and ultrasound had a negative impact on the production of ethanol in the other treatments, no statistical differences were found among the treatments for the cumulative production (Figure 21).

![Figure 21. Ethanol production (g/L) per treatment. Each treatment bar contains the amount of ethanol produced every 12 hours. Each texture pattern (12 h period) and treatment contains the result of the Tukey-Kramer HSD test indicated with letters. Each treatment have an error bar indicating the standard deviation.](image)

2.4.5. Carbon dioxide generation

Carbon dioxide is a by-products of fermentation. In order to investigate the effect of ultrasound on CO₂, weight loss was recorded during fermentation by weighing the flasks every 12 hours. As shown in Figure 22, direct ultrasound treatments (D-1 and D-2) seem to release more CO₂ than indirect ultrasound (I) and no-ultrasound (N). All 4
treatments are significantly different to each other, D-1 and D-2 show the highest weight loss.

Figure 22. Carbon dioxide production (g/L) per treatment. Each treatment bar represent the weight lost every 12 hours. Each texture pattern and treatment contains the result of the Tukey-Kramer HSD test indicated with letters. Each bar have an error bar indicating the standard deviation.

Similar to glucose uptake and ethanol production, the fastest rate of CO₂ generation occurred between 12 and 24 hours; D-1 and D-2 generated significantly more CO₂ than the indirect (I) and no-ultrasound (N) treatments. During the first 12 hours, the amount of CO₂ generated by D-2 was significantly higher than the other 3 treatments, 38, 45, and 56%, for N, I, and D-1 respectively. D-2 also had the greatest standard deviation.
High concentrations of dissolved CO₂ have been shown to inhibit yeast performance in fermenting ethanol (Chen & Gutmains, 1976). Ultrasound has been found to decrease the concentration of dissolved CO₂ (K. Matsuura et al., 1994), and then to enhance fermentation. A rapid release of CO₂ can cause the formation of foam in the surface of the fermentation medium, which increases the risk of losing liquid when opening the flasks. It is possible that in the direct ultrasound treatments (D-1 and D-2), some fermentation medium was lost due to foaming, and this was mistakenly attributed to CO₂ loss by weight. Fermentation medium may have also been lost when taking the ultrasound probes out of the flasks. This may have affected the weight loss values for the two direct ultrasound treatments giving erroneous CO₂ loss, which contradict the glucose and ethanol results.

Rapid release of CO₂ occurs when the fermentation medium is stimulated after being stagnant for a period of time, yeast settle down, and dissolved CO₂ concentration increase in the fermentation medium, caused by the lack of energy input to the system. This period of rest happens while sampling, around 10 min. After sampling, installing the ultrasound probe, and setting up the shaker, the reactors receive energy, shaking, and the ultrasound raise the yeast from the bottom of the flask and agitate the fermentation medium enough to release a greater amount of CO₂ during the first couple of minutes. The fermentation medium was observed to foam within seconds of starting ultrasound as shown in Figure 23, the foam decreased minutes later.
2.4.6. Yeast viability

In this study, *Saccharomyces cerevisiae* was used to ferment ethanol from glucose. Ultrasound was applied during fermentation, not as a pretreatment for biomass as previous studies (Luo, Fang, & Smith, 2014), but to enhance yeast performance. To isolate the effects of ultrasound on the yeast, glucose was used as feedstock, avoiding effects of ultrasound on other particles other than yeast. Most of the studies in the field have used ultrasound as pretreatment for different types of biomass such as corn meal (Nikoli et al., 2010), cassava slurry (Nitayavardhana et al., 2010), wastewater (Pilli et al., 2011); or to deactivate microbes (Drakopoulou, Terzakis, Fountoulakis, Mantzavinos, & Manios, 2009); just few of them have applied ultrasound during fermentation (Neel et al., 2012; Sulaiman et al., 2011).
In order to investigate the effects of ultrasound on yeast cells viability, manual counting was done at 0, 12, 24, and 36 hours after the fermentation started, using a microscope, hemocytometer, and a hand counter. Active yeast and dead cells (Figure 24) quantities were distinguished by staining with Trypan Blue solution. All fermentation treatments started with $\sim6.92\times10^6$ yeast cells/mL.
Figure 24. Concentrations of active and dead yeast cells during fermentation. Error bars indicate the standard deviation for each treatment (N, n=7; I, n=6; D-1 and D-2, n=3).

The no-ultrasound treatment (N) contained higher concentrations of active yeast cells than all the other treatments during the course of the fermentation (Figure 24). Even
though it was higher than all the other treatments, there were not significant differences.
The fastest growth of yeast occurred during the first 12 hours of the fermentation. This
period was the only one where a significant difference occurred, it was between the no-
ultrasound treatment (N) and direct ultrasound (D-1), as shown in Table 8. After 12 hours
of fermentation, treatment D-1 had significantly higher quantity of dead cells than the
other 3 treatments. Treatment N at 36 hours of fermentation had significantly less dead
cells than treatments I and D-1 (Figure 24). Any level of ultrasound affected the quantity
of dead cells, the no-ultrasound treatments had less dead cells along the fermentation.
Negative values are found in Table 8, which indicate that the yeast cells population
started to decline, which means that there were more cells dying that reproducing.

Table 8. Active yeast cell rate ± standard deviations.

<table>
<thead>
<tr>
<th></th>
<th>00 - 12 h</th>
<th>12 - 24 h</th>
<th>24 - 36 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2.3E+8 ±3.7E+7 a</td>
<td>2.6E+6 ±2.6E+7 a</td>
<td>-1.9E+7 ±3.4E+7 a</td>
</tr>
<tr>
<td>I</td>
<td>1.7E+8 ±2.0E+7 ab</td>
<td>2.3E+7 ±2.2E+7 a</td>
<td>-2.1E+7 ±2.4E+7 a</td>
</tr>
<tr>
<td>D-1</td>
<td>1.2E+8 ±2.1E+7 b</td>
<td>2.6E+7 ±1.5E+7 a</td>
<td>-5.6E+6 ±2.4E+7 a</td>
</tr>
<tr>
<td>D-2</td>
<td>1.6E+8 ±1.3E+7 ab</td>
<td>3.3E+7 ±2.2E+7 a</td>
<td>-1.3E+7 ±4.2E+7 a</td>
</tr>
</tbody>
</table>

As shown in Figure 25, ultrasound had a negative effect on yeast viability.
Ultrasound can cause cytolytic effects, but this effect depends on the experimental
conditions and type of microbes. In the direct ultrasound treatments, smaller cells and
particulate matter was scattered in the extracellular medium, similar to Radel. (2000)
found. Yeast viability for the no-ultrasound (N) was above 90% during the fermentation, while the indirect and direct ultrasound treatments a steady decrease in viability over time was observed reaching 78, 74, and 84% for I, D-1, and D-2 respectively. At 12 h of fermentation, the fraction of viable yeast cells in D-1 was significantly lower than in the other treatments. At 24 hours, treatment D-1 was still significantly lower than I and N; and at 36 hours, N was significantly higher than the ultrasound treatments, which showed 16, 21, and 10% non-viable cells in I, D-1, and D-2 respectively.

Figure 25. Percentage of viable cells.

The decrease in viability in the control treatment (N) was likely caused by the progressively increasing concentration of ethanol, which is an inhibitor of yeast (Lucero,
Penalver, Moreno, & Lagunas, 2000; Rosa & Sá-Correia, 1996), since no-ultrasound was applied to it. During fermentation, the concentration of ethanol reached ~80 g/L, and it has been found that ethanol concentrations of 70 g/L begin to inhibit yeast cells (Maiorella et al., 1983). In biological membranes, water penetrates the lipid bilayer and forms hydrogen bonds with the polar groups of phospholipids. At increasing ethanol concentrations, this water is replace by ethanol, altering the structure and function of the membrane (Crowe, Mouradian, Crowe, Jackson, & Womersley, 1984). Another possible explanation provided by Liu and Li (2014) is that ethanol denatures proteins by interacting with to non-polar parts of proteins.

The effect of ethanol on the cell membrane, plus the effect of ultrasound seem to have contributed to a faster decline in yeast cell viability. The beginning for the decline of viability for treatment N coincided with higher ethanol concentrations. The higher ethanol concentration plus ultrasound was also a cause of the viability decline of the other treatments (I, D-1, D-2). Other studies have not found negative effects in cell physiology by using standing waves in retention systems (Doblhoff-Dier et al., 1994), but had found morphological changes such as the cell vacuole, intracellular material leakage, increased intracellular material in the extracellular space and dead cells (Radel et al., 2000).

To better understand the qualitative effects of ultrasound on the yeast morphology, pictures of the yeast on a hemocytometer and under a microscope were taken for each treatment at 0, 12, 24, and 36 hours of fermentation (Figure 26). The effect of ultrasound on yeast cells (Figure 26 D and F) is visually negative compared to the no-ultrasound treatment (Figure 26 C and E). There are less blue dots in the no-ultrasound treatment, blue dots indicate dead yeast cells, and more and bigger cells are observed
(Figure 26 C and E). The background of the image (Figure 26 C and E) looks cleaner; small particles in the background may indicate intracellular material that got out from the cell due to cell lysis, intracellular material were not measured.

Radel (2000) says that agglomeration of yeast cells within the pressure nodal planes appears to minimize damaging effects due to ultrasonic. However compared to Radel, in this experiment there were no nodal points in the reactors because they shake at 180 rpm, which disrupt and prevent standing patterns to occur.
Figure 26. Microscopy images of yeast, different treatments and fermentation stages. A) yeast in the fermentation medium just after inoculation, 0 hours; B) indirect ultrasound, 12 hours after inoculation; C) and D), yeast cells after 24 h of inoculation for no-ultrasound and direct ultrasound respectively; and E) and F); 36 h after inoculation for no-ultrasound and direct ultrasound respectively. The square with a triple line border represent 1 mm.
2.4.7. Ultrasound in fermentation

The external and intraparticle observable modulus results showed that there is no external nor intraparticle mass transfer limitation for glucose, ethanol, or carbon dioxide; none of the values are greater than 1 in the case of external mass transfer, and none is greater than 0.3 in the case of intraparticle mass transfer. The yield for ethanol and glucose were also very close or equal to the theoretical yield, which indicates a normal fermentation. The absence of mass transfer limitations and values that are very close to the theoretical yield are possibly because glucose was used as a substrate, which is the simplest form of sugar that yeast can take, and the fermentation medium was close to ideal with nutrients, sugar, water, and yeast. This is different for fermentation mediums where slurries are present, which contains more non-fermentable solids.

Stirring has proved to enhance process capabilities (G. Zhang, Wan, Gao, & Dong, 2014). Stirring together with ultrasound increase the cavitation effect (Saad & Williams, 1985) and efficiency of the probe. High localized effects of ultrasound happens in a small region beneath the probe, the effect of the ultrasound probe may not reach the whole reactor, and this may cause death zones, which lower its efficiency (Capelo, J., Galesio, M., Felisberto, G., Vaz, C., and Costa, 2005); dead zones are define as areas of the reactor where ultrasound cannot reach; not to areas were yeast is dead. Shaking at 180 rpm was used to prevent dead zones and give a uniform distribution of ultrasound.

Ultrasound waves are longitudinal and progressive, and depending of the characteristics of the reactor, turbulence and standing waves can be formed. In this experiment, the waves created by the ultrasound probe traveled first in the medium contained in the direct ultrasound treatment reactor (D1 and D2); these waves then passed
through the wall of the reactor; the waves then travelled through the water in the bath, reaching the indirect ultrasound treatment reactor (I). The effect of indirect ultrasound was minimized due to the movement in the water caused by the shaking, but the reactor still received the impacts of the waves as exhibited by a reduction in yeast cell viability and slower rates of fermentation.

Ultrasound has been used more as a pretreatment than as an enhancer during fermentation. Most of the studies using ultrasound as a pretreatment aimed to reduce particle size of the biomass or to extract more sugars to make them more available. More available sugars and smaller particle sizes increase surface area and promote yeast growth (Nitayavardhana et al., 2010; Nitayavardhana, Rakshit, Grewell, Leeuwen, & Khanal, 2008).

There are a few studies where ultrasound was used directly to enhance fermentation. These studies include Sulaiman (2011), Neel (2012), and Lanchun (2003). Sulaiman (2011) used ultrasound during fermentation of lactose by *Kluyveromyces marxianus* (Sonicator® 3000 20 kHz, Misonix, Inc., standard tapped probe). The intensity used by Sulaiman was 11.8 W/cm² at different duty cycles (10, 20, and 40%) in a volume of 3.5 L, which gives an ultrasonic energy density of 5 W/L. Sulaiman reported a decrease in cell growth at the highest duty cycle. The fermentation medium was continually recirculated to an external chamber where the bioreactor was also stirred.

Neel (2012) reported an acceleration of ethanol fermentation from glucose by yeast *S. cerevisiae* at 20 and 30°C, 20 and 40% glucose solution in a 100 mL flask and 50 mL working volume, where 1 g of yeast was added. Indirect ultrasound was applied at 40 kHz and 120 W, in an ultrasonic bath with a 4 L volume, where the flasks were located,
applying continuous mild ultrasound at 30 W/L. The ultrasound treatments were not stirred, while the control treatments were stirred at 1,000 rpm. Fermentation under stirring and ultrasound was faster than unstirred fermentation. Indirect ultrasound fermentation at 30°C was completed at 11 h, compared to the control treatment, which took 18 h. At 20°C, fermentation was completed in 19 h in the ultrasound treatment, and in 36 h in the control treatment. Yeast agglomerate more in the stirring treatment than in the ultrasound treatment. Neel also harvested the yeast after fermentation to be used in subsequent fermentation, stating that yeast is reusable. Neel found that ultrasound provided better mixing and that may have helped to remove ethanol from the yeast surface and CO₂ from the fermentation medium, promoted changes in membrane permeability, and enhanced mass transfer, though, no method for measuring is described. Neel also did an experiment with an ultrasound prove, where after 30 min, the yeast cells were ruptured and no ethanol was obtained, no energy was described.

Lanchun (2003) found that ultrasound can accelerate the growth and improve mass transfer of *S. cerevisiae* in the lag and log phase, where ultrasound optima were different for each one; even though Lanchun does not provide an explanation how ultrasound can do so. Fermentation was done in 300 mL YPD medium and ultrasound from 18 to 40 kHz, with ultrasound times from 1 to 30 seconds.

Probably the widest use of ultrasound on living things is to inactivate pathogenic microorganism or to harvest intracellular compounds, such as proteins (Iida, Tuziuti, Yasui, Kozuka, & Towata, 2008). Ultrasound is a technique used for cell disruption (Chisti & Moo-Young, 1986). Tsukamoto (2004) studied the inactivation of *S. cerevisiae*
by probe ultrasound at 27.5 kHz at 42 W/mL and found that the lower the initial cell concentration, the more effective the ultrasound is in inactivating the yeast.

Anderson (1953) and Jomdecha (2006) applied direct ultrasound during fermentation with *Saccharomyces cerevisiae*. Anderson applied ultrasound at 0.6 kHz, power of 10 W, and an intensity of 10.5 W/cm² at an intermittent and continuous modes, the duty cycles were 25% for 40 min, 50% for 20 min, 75%, and 100% for 10 min. Anderson found that the carbon dioxide generation decrease after the fermentation medium was subjected to ultrasound, especially from fermentation medium with younger cells. Fermentation rate was also decreased in all the cases, even with the same energy provided, but different duty cycles, a shorter but more intense ultrasound cause more inhibition. Ultrasound has more effect in younger cells than in older cells provoking longer lag phases.

Jomdecha applied direct ultrasound at 20 kHz, intensity of 0.2, 0.4, and 0.8 W/cm² on 200 mL of YP medium. Ultrasound was applied to non-shaking and shaking at 100 rpm reactors. The samples were subjected to ultrasound at the levels described for 10 and 15 min at a 10% duty cycle. Yeast growth was reported higher in the ultrasound treatments than the control. Jomdecha state that the mechanism are still unclear and more research has to be done in the topic.

The 2 energy densities used in this experiments were 23 W/L for D-1 and 32 W/L for D-2, and negative effects to yeast performance were observed. Neel (2012) found an acceleration of fermentation applying 30 W/L; Anderson (1953) found that ultrasound applied at 2,000 W/L inhibited fermentation; Jomdecha and Prateepsen (2006) on the other hand found an acceleration in growth applying 0.004 W/L.
In summary, results from the ultrasound experiment conducted in this study, show that ultrasound had a negative effect on fermentation. The ultrasound conditions used in this study were unique to this experiment. An ultrasound probe was applied during batch fermentation without recirculation while shaking in a water bath. The ultrasound intensity provided was around 23 and 32 W/L which was well below some of the previous studies. All the previous experiments, described in the introduction have different set ups, which make comparison difficult.
2.5. Conclusions

In this study, direct and indirect ultrasound was applied to a medium during ethanol fermentation and its effect on glucose uptake, and ethanol and carbon dioxide production were investigated. The hypotheses was that ultrasound may enhance the performance of the yeast *Saccharomyces cerevisiae* by interfering with the boundary layer at the cell surface and reduce mass transfer limitations for glucose, ethanol, and carbon dioxide.

However, the external and intraparticle moduli results showed there is no mass transfer limitation for glucose, ethanol, or carbon dioxide; none of the values are greater than 1 in the case of external mass transfer, and none is greater than 0.3 in the case of intraparticle mass transfer. Previous studies have proposed several mechanisms for how ultrasound improves fermentation, but these conclusions were not based on an analysis or calculation of the magnitude of the actual mass transfer limitations in the system.

Direct ultrasound negatively affected the performance of yeast during fermentation. Indirect ultrasound also negatively affected the performance during fermentation, but not as much as the direct ultrasound. Negative effects of ultrasound have been also found by other researchers. The three ultrasound treatments had significantly lower rates of glucose uptake, ethanol production, and yeast viability. Treatment N had the highest ethanol production. Yeast population and viability were also affected by ultrasound. Direct ultrasound affected the yeast population and viability more than indirect and no-ultrasound. The quantity of dead cells was significantly lower in the
no-ultrasound treatment. The effect of ultrasound on yeast depends on the energy provided.

The negative effect of ultrasound was mostly found at the beginning of the fermentation process, the first 12 hours. Direct ultrasound treatments were most of the time lower in terms of glucose uptake, ethanol production, and cell viability than indirect and no-ultrasound treatments throughout the study. The results from the ultrasound experiment, show that ultrasound did not kill all the yeast, but had a negative effect on its performance.

Findings from this study compared with previous studies suggest that there are different ways to apply ultrasound, and these lead to inconsistent effects on the fermentation process.
References


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Appendix A: Preliminary experiments

This appendix describes preliminary experiments conducted to determine the necessary conditions for investigating the ability of ultrasound during ethanol fermentation from glucose, by the yeast *Saccharomyces cerevisiae*.

Experiment 1: Inoculum Preparation

Different incubation times for yeast pre-cultures are reported in the literature ranging from 4 to 20 hours (Nikoli et al., 2010; Ramirez et al., 2013; Sulaiman et al., 2011). The objective of this experiment was to understand the kinetics of the yeast pre-culture growth, in order to determine the length of time needed to grow dry yeast to viable cells with a cell density of $1 \times 10^8$ viable cells/mL for subsequent inoculation.

Yeast were grown in a propagation medium containing 100 mL of YP medium with 5% glucose, 10 mL of citrate buffer (pH 4.3), trace elements, and water for a total volume of 150 mL. Samples were taken every 3 hours to measure the concentration of viable (hydrated and active) yeast by cell counting after blue methylene staining using a microscope and a hemocytometer (Fig.1). Glucose and ethanol concentrations (mg/mL) were measured using HPLC (Fig. 2 and 3).
Results of this experiment showed that there was a long lag phase (~15 h) during which little growth occurred, followed by rapid growth from 15 to 21 hours after inoculation. After approximately 19 hours, the pre-culture reached the target cell density of ~1×10^8 viable cells/mL (Figure 27); this indicates that pre-cultures should be harvested approximately 19 hours after inoculation with the dry yeast.

![Figure 27. Live yeast cell density. Values are averages (n=4). Standard deviation are shown in error bars.](image)

Experiment 2: Fermentation

The second experiment was conducted to investigate various ways of inoculating the culture and to determine the behavior of yeast during fermentation under different
media conditions. The objective was to generate a stable rate of glucose uptake and ethanol production so that the effects of ultrasound could be clearly seen.

Three different treatments were tested (Table 9). All the treatments were inoculated with yeast resuspended in 7.5 mL of pre-culture medium (treatment 2) or water (treatment 1 and 3) with ~10⁷ cells/mL; 7.5 mL of YP medium (10X), needed for growth and metabolism, were added to treatment 2 and 3, 7.5 mL of water were added to treatment 1 instead of YP medium. The treatments started fermentation with an initial glucose concentration of 200 g/L and a volume of 150 mL. In treatment 1, the yeast pre-culture was centrifuged to remove the spent medium, then resuspended in water, and added to the fermentation medium containing the glucose solution. In treatment 2 the pre-culture was used without centrifugation and added to the fermentation medium containing both glucose and YP medium. In Treatment 3, the pre-culture was centrifuged, resuspended in water, and inoculated into the fermentation medium containing glucose and YP medium.

Table 9. Treatments tested during fermentation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yeast pre-culture (7.5 mL)</th>
<th>Nutrients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>centrifuge and resuspended in water</td>
<td>Water</td>
</tr>
<tr>
<td>2</td>
<td>original medium</td>
<td>YP medium</td>
</tr>
<tr>
<td>3</td>
<td>centrifuge and resuspended in water</td>
<td>YP medium</td>
</tr>
</tbody>
</table>
The progress of the fermentation was measured by weighing the flasks every 6 hours to determine CO₂ generation. Yeast density, glucose, and ethanol concentrations were measured every 12 hours.

The performance of the different fermentation is shown in Figure 28 and Figure 29, including CO₂ generation, yeast density, and glucose and ethanol concentrations. Treatment 1 did not show any activity and therefore was not included in the graphs and subsequent experiments. Treatment 2 showed the fastest rate of fermentation and growth, and was similar to commercial scale yeast fermentations. Treatment 2 was used for the future ultrasound experiments.

Figure 28. CO₂ generation and live yeast cell density during fermentation. Standard deviation are shown in error bars (n=4).
Experiment 3: Ultrasound

The objective of this experiment was to develop a system for conducting ultrasound experiments. Due to the size of the ultrasound probe, and the requirement that nothing attach to the ultrasound tip during operation, a 1,000 mL volume flask was needed to accommodate the probe. The fermentation medium was inoculated with 35 mL of yeast *Saccharomyces cerevisiae* culture with ~$4 \times 10^7$ cells/mL to a total volume of 750 mL.

Two set-ups were tested; in one, the ultrasound equipment was installed inside a shaker incubator (Figure 30 A); this approach did not work well due to the lack of space and problems caused by shaking the ultrasound probe; the second set up improved on this
by using stir plates in a 32°C warm room (Figure 30 B) Ultrasound was applied using a tapered microtip attached to a standard probe (Figure 30 C). This set up allowed the ultrasound equipment to remain stationary and worked for 60 hours, after that, the ultrasound stopped, it is expected it was due to the concentration of alcohol in the solution.

An ultrasound treatment and 2 controls (no-ultrasound) were tested. Carbon dioxide generation was measured every 6 hours; yeast cells, glucose and ethanol concentrations were measured every 12 hours. Low intensity ultrasound conditions were tested first (22% amplitude during cycles of 1 second ON, followed by 10 seconds OFF) using one of the fermentation conditions developed from a previous experiment. The energy provided was around 11,500 J (0.59 W), an energy intensity of 4.66 W/cm2, and an energy density of 0.78 W/L.

Figure 30. Preliminary experiment set ups.
A) Standard probe and micro tapered tip, in a shaker incubator; B) Standard probe with a tapered microtip, using magnets for stirring; C) Tapered microtip.
Results from this preliminary experiment show a slightly increase in the glucose uptake, ethanol production, and carbon dioxide generation (Figure 31 and Figure 32) from the ultrasound treatment compared to the control treatment. Yeast population was also slightly higher than the no-ultrasound treatment.

Even with apparent positive results of this preliminary experiment, this set up was not used due to inconsistency in the energy provided by the sonicator. Since the microtip is not designed for systems containing low surface tension liquids, such as ethanol, because these liquids may penetrate into the tip and overload the system. This problem was observed in the experiment where ultrasound stopped suddenly without any warning. In the case of this fermentation, ultrasound stop at 60 hours and could not be resumed.

![Figure 31. Carbon dioxide generation and live yeast density during fermentation. Standard deviations are shown in error bars (n=2)](image-url)
Figure 32. Glucose and ethanol concentrations during fermentation. Standard deviations are shown in error bars (n=2)