THE EFFECTS OF WORT OXYGENATION SCENARIOS ON FERMENTATION PERFORMANCE, VOLATILE FLAVOR COMPOUND DEVELOPMENT, AND FLAVOR STABILITY IN HIGH GRAVITY BREWING

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ABSTRACT

The Effects of Wort Oxygenation Scenarios on Fermentation Performance, Volatile Flavor Compound Development, and Flavor Stability in High Gravity Brewing

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High gravity (HG) brewing has become the most used strategy for maximizing fermenter productivity in commercial brewing. While HG brewing has many benefits, the additional stress placed on the yeast due to the higher concentration of fermentable sugars in the wort can negatively impact fermentation performance and flavor compound formation. A proper dissolved oxygen (DO) level is vital to guarantee adequate yeast performance during HG fermentations. Dissolved oxygen is vital to yeast viability throughout the fermentation process, as yeast requires oxygen to synthesize vital cell membrane components needed for continued anaerobic growth and cell division. Previous research has demonstrated the importance of DO in wort for regular gravity fermentation and flavor compound production. However, the impact of dissolved oxygen during HG brewing on fermentation performance and how this will impact the production of flavor compounds have not been fully researched.

The objectives of this research were to analyze the impact of wort aeration timing and concentration on fermentation performance, flavor stability, and the formation of volatile flavor compounds, determined using gas chromatography. Gas chromatography analysis was modeled after the ASBC Method Beer-48. Flavor stability and staling was analyzed during aging under normal and accelerated conditions utilizing TBA analysis.

Pre-pitch oxygen treatments at levels greater than 8 ppm dissolved oxygen significantly increased attenuation when compared to the unoxygenated controls. Post-pitch oxygenation significantly increased attenuation, with DO treatments at levels of 8 ppm showed the most significant decrease in wort specific gravity. Aldehyde, ester, and higher alcohol production were all significantly affected by DO concentration. Aldehyde production decreased with increased DO concentration. Ester production increased from 0 to 8 ppm DO treatment and decreased at DO treatments greater than 8 ppm. Higher alcohol production increased from 0 to 10 ppm and decreased with DO treatments greater than 10 ppm. Greater concentrations of DO resulted in greater TBA index values after normal and accelerated aging, with accelerated aging producing greater TBA index values than normal aging.

Keywords: High gravity, fermentation, flavor, oxygenation, GC-MS

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Chapter 1

INTRODUCTION

Beer is defined as a yeast fermented beverage, having half a percent or more of alcohol by volume, brewed from malt, certain malt substitutes, and typically hops (27 C.F.R § 25.11). Beer is composed of water, ethanol, and 4% carbohydrates. In addition, it contains small amounts of proteins, vitamins, and a variety of flavor compounds (Ramirez et al. 2003). These compounds make beer a very complex food system.

The beer industry in the United States contributes over $328 billion to the economy, which represents roughly 1.6% of the United States GDP, and directly employs nearly 70,000 Americans (John Dunham & Associates 2019). From 2014 to 2018, over 3,500 breweries have opened in the US and more continue to open each year ("National Beer Sales", n.d.). Beer consumption is on the rise in the United States. In 2019, alcohol consumers in the United States alone consumed 25.9 gallons of beer and cider per capita ("Industry Fast Facts", n.d.). Due to ever increasing consumer demand and growth in the beer industry, breweries are being challenged to produce more beer each year. One method of increasing brewhouse capacity is through utilization of high gravity brewing techniques. (Casey et al. 1984)

Specific gravity is a measure of the density of a liquid in relation to water, which correlates directly to the concentration of sugar in an unfermented beer. Normal gravities for beer fermentation are typically in the range of 11-12 °P, resulting in roughly 5% ethanol (v/v) concentrations after fermentation, whereas high gravity (HG) fermentations typically contain 18 °P or higher and result in ethanol concentrations of 7.5% (v/v) or higher (Piddocke et al. 2009; Puligundla et al. 2011). This technique is used to make high alcohol by volume (ABV) beers. Also, the concentrated beer resulting from high gravity fermentation can be diluted with water to the desired ethanol concentration to produce a larger volume of beer than produced by traditional brewing methods. High gravity (HG) brewing therefore represents an attractive option to many breweries that wish to improve brewhouse capacity and efficiency without the purchase of new equipment.

High gravity brewing is not without its trade-offs (McCaig et al. 1992). The increased concentration of fermentable sugars introduces yeast stressors such as increased hyper-osmotic stress and ethanol concentrations that can reach toxic levels (Pratt et al. 2003). These stressors can cause off-flavors,

slow fermentations, and an overall decrease in the viability of healthy yeast (Brányik et al. 2008; Lei et al. 2012). Previous research on high gravity fermentations has focused on osmotic pressure, nitrogen concentration, and the use of adjunct syrups (Sigler et al. 2009; Piddocke et al. 2009; Lei et al. 2012)

One of the major factors that affect fermentation is oxygen. Oxygen is essential to the growth of healthy yeast as it is used to create vital cell components at the beginning of fermentation (Kucharczyk and Tuszyński 2017). Oxygen's role in regular fermentation and staling is well documented, but it's effect on fermentative performance and flavor development during high gravity brewing requires further investigation (Depraetere et al. 2008; Kucharczyk and Tuszyński 2017). The objective of this research is to investigate the effect of wort oxygenation on fermentative performance, flavor development, and staling in high gravity brewing conditions.

Chapter 2

LITERATURE REVIEW

2.1 A Brief History of Beer

Beer has been present with humankind for as long as agriculture and the domestication of grains has existed. It is theorized that during the Neolithic period (10,000 BCE), when humans began forming communities around agriculture, the first fermented beverages were produced (Meussdoerffer 2009). This is supported by Archaeological evidence of fermented beverages, which has been unearthed in the Nile river valley near the regions where agriculture first began (Rose 1959). The first form of beer was very likely a porridge that had been fermented by natural yeast, adding a warm and intoxicating sensation to the meal. It is believed that this accidental discovery was one incentive for the beginning of agriculture and the end of humankind's nomadic lifestyle (Mosher 2017).

In the Sumerian empire, which existed between 3300 and 3100 BCE, beer was commonly consumed and played a key role in society. In the Sumerian Epic of Gilgamesh, savages are civilized by introducing them to the eating of bread and the drinking of beer (Meussdoerffer 2009). Evidence has also been uncovered that beer had an important role in the religious ceremonies and offerings of the Sumerian people. Unearthed stone tablets indicate that Sumerians prayed to Ninkasi, the goddess of beer, often including the beverage in their ceremonies (Rose 1959).

Sumerians were not the only ancient society to place a high value on beer. Ancient Egyptians credited the creation of beer to their god Osiris, master of fertility, death, and resurrection (Meussdoerffer 2009). Beer was often used in religious ceremonies and is commonly discovered in Egyptian burial chambers. Though the Egyptians' beer was low in alcohol with a very short shelf-life, it was consumed daily in all sectors of the population as a reliable source of potable drinking water. (Hornsey 2003).

Not all ancient societies respected beer. Amongst these were the Greeks and Romans. Culturally, these empires were wine drinkers. Many of the people conquered by the Greeks and Romans, both to the north and south, were beer drinkers. This includes the Egyptians, conquered in 331 BC under the rule of Alexander the Great, as well as the Germanic peoples to the north of Italy that were encountered in the late second century AD (Nelson 2005; Meussdoerffer 2009).

When the Romans first encountered the Germanic peoples of central Europe, they found that the Bavarian and Bohemian regions they inhabited were rich in cereals. These bountiful grains were used for both the production of bread and beer. Archaeological evidence of germinated barley in modern Denmark and Sweden around the first century AD seem to indicate that these harvested grains were used for the intentional manufacture of beer (Nelson 2005). In these cold Bohemian regions, the air-drying of grains, as was common in southern beer drinking regions, often resulted in mold. In order to combat mold spoilage while malting. central European brewing practices introduced a step after malting, the kilning of grains (Meussdoerffer 2009).

Brewing traditions in the British Isles likely began when the Germanic Saxons took over following the failed Roman rule, bringing with them a beer and mead drinking culture. However, Irish Christians of Celtic descent, never quite influenced by the beer prejudice of the Romans, developed their own brewing culture that was heavily linked to the church. During this time, the development of monastic brewing took place (Meussdoerffer 2009). The ale brewed by these Irish monasteries was red in color, and produced mainly from barley, rye, wheat, and oats (Hornsey 2003). In the fifth century AD, Irish missionaries began to spread their monastic brewing culture to the rest of previously Roman-occupied Europe, from there monastic brewing practices spread throughout central Europe, and in fact, we see the first mention of hops by Abbot Adalhardus of the monastery of Corvey in Germany around AD 822 (Hornsey 2003; Meussdoerffer 2009).

Before the introduction of hops, many monastic and homebrewers in Europe used a blend of herbs and vegetables to flavor their ales, commonly known as gruit (Hornsey 2003). However, archaeobotanical evidence has revealed that hops were prevalent in Europe by the ninth and tenth centuries AD (Meussdoerffer 2009). By the end of the 14th century, hops are regularly mentioned in English brewing (Hornsey 2003). Although they were popular with many brewers, some disliked hops and preferred ales, which then referred to an unhopped beer. In 1530, Henry VIII outlawed hops in brewing, allowing only ales to be brewed until 1552 when Edward VI repealed the act (Hornsey 2003).

Although beer had been brewed since ancient times, little was known about the process of creating alcohol from sugars until the late 18th century. Antonie Lavoisier was a French chemist that first studied the chemical balance of alcoholic fermentation between 1784 and 1789 (Meussdoerffer 2009). Lavoisier's

experiments showed that the chemical products of fermentation, then known to be ethanol, CO_2, and acetic acid, had similar compositions of carbon, hydrogen, and oxygen as the sugar used to fuel the fermentation. This understanding set down the foundation for the study of alcoholic fermentation (Hornsey 2003).

Partway through the 19th century, another French chemist named Louis Pasteur was working with local alcohol manufacturers to determine the root cause of spoilage in alcohol beverages (Bramen 2009). By studying the "diseases" of beer, Pasteur discovered the relation between microorganisms and fermentation ("Brewing Mysteries", 2016). At the time, fermentation was thought to be a purely chemical process. Louis Pasteur's 1876 Études sur la Bière (Pasteur 1876) was instrumental in showing the roles that microorganisms play in the fermentation of beer, dispelling the widely believed theory of spontaneous generation of life (Eßlinger 2009).

Today, the science and industry behind the brewing of beer has advanced considerably. Beer has spread to all corners of the globe and contributes a considerable amount to the worldwide economy. Beer has become the world's leader in the alcoholic drinks market, comprising over 74.58% of the market share (Mordor Intelligence 2018). In 2018, the value of the US beer market reached $328 billion USD and the global beer market is projected to reach $758 billion in 2023 (Mordor Intelligence 2018). Beer contributed $111.4 billion to the economy in the US alone during 2017, selling over 207.4 million barrels ("Industry Fast Facts", 2018). In today's world, beer is a major economic force in all parts of the globe.

2.2 Beer Ingredients

Beer is typically made from a combination of four basic ingredients: water, malted barley, hops, and yeast. Each ingredient is used in the brewing process for a specific function. In addition, each of beer's principal ingredients are measured for a variety of parameters that are of importance to the brewer.

2.2.1 Water

By volume, water is the most abundant raw material used in the production of beer and represents well over ninety percent of a finished beer's composition. Although treated water is used in almost every step of beer production, untreated water is also used in the brewhouse as a cleaning and sanitizing agent. For this reason, water is a costly resource whose usage should be monitored closely. For breweries

attempting to minimize their water usage, only five to six times the volume of water found in their final beer should be used (Bamforth 2006, 2009).

The presence of mineral salts, such as calcium or magnesium chlorides, sulfates, and bicarbonates, has a direct effect on the hardness and alkalinity of brewing water (Lewis and Young 2004). Hardness describes the total sum of alkaline-earth ions in water (Eßlinger 2009). Hardness prevents water from lathering with soap, while soft water can easily lather. Hardness can either be temporary or permanent, depending on the composition of mineral salts in the water. Temporary hardness is caused by the presence of bicarbonates of calcium and magnesium, which can be removed by boiling. Boiling of these bicarbonate salts decomposes the bicarbonates, precipitating carbonates and releasing carbon dioxide. Permanent hardness is caused by sulfate, nitrate, and chloride salts, which cannot be removed by boiling (Bamforth 2006). Instead, permeant hardness is typically treated using a water softening machine which uses ion exchange to remove magnesium and calcium ions and replace them with sodium or potassium ions, reducing mineral hardness in the water (Tofighy and Mohammadi 2011).

Different minerals can cause different effects during the brewing of beer. Magnesium ions for example play a role in minimizing the effect of environmental stressors, like high osmotic pressure, temperature, or ethanol concentration (Pironcheva 2000). Sulfates often are implicated with the astringency and dryness of a finished beer (Bamforth 2006). Calcium promotes the activity of certain enzymes, like α-amylase, and can help to reduce the pH of wort, and aid in the flocculation of yeast (Bamforth 2006).

Certain styles are often brewed with specific levels of calcium salts. For example, top-fermenting ales will typically use water reminiscent of conditions in Burton-on-Trent in England, an area with waters high in calcium salts (350 ppm). While, in contrast, bottom-fermenting Pilsner style beers often use water similar to that in the area of Plzeň in the Czech Republic, where the water contains less than 10 ppm calcium (Bamforth 2009).

2.2.2 Barley

Malted barley serves as the primary fermentable in the production of beer. Malt for the use of brewing is produced by the germination and kilning of barley (*Hordeum vulgare*), in order to make fermentable sugars available. These barley grains contain mostly starch (62 to 65%), between 11 and 13%

moisture, protein, polysaccharides, a variety of enzymes, and a small fraction of fat, vitamins, and minerals (Bamforth 2006; Eßlinger 2009). Barley that is plump, low in nitrogen (protein), and viable for germination is preferred for malting, to achieve the highest conversion of starches to fermentable sugars. Levels of protein above 13% are undesirable in barley, as less starch will be available for conversion and protein can often produce haze in a finished beer (Lewis and Young 2004).

Standard brewing malts are characterized by their low nitrogen content (1.5 - 1.85%), a high degree of modification, and lighter color resulting from a lower temperature kilning process (Bamforth 2006). Examples of standard malts include Pilsner, Vienna, Munich, and various other pale malts. Other specialty malts are often used to impart flavor or color to wort during mashing, such as smoked malts, which are kilned in the presence of wood or peat to impart a smoky flavor to the malt (Eßlinger 2009).

2.2.3 Hops

Hops are the cone-shaped flowers of the hop vine (*Humulus lupulus*) and a member of the Cannabaceae family of plants native to Europe, North America, and some parts of west Asia (Bamforth 2006; Eßlinger 2009). Hops are a unique agricultural product as nearly all hops grown contribute to the production of beer alone (Bamforth 2009). Hops have two major applications during brewing - flavor formation and preservation.

In terms of flavor, hops lend an array of aromas via hop oils and bitterness from iso-alpha acids. Alpha acids are the precursor of the main bittering compounds in beer, iso-alpha acids, and exist in hops, on average, 9 - 10% by weight although this concentration is highly strain dependent (Steenackers et al. 2015). Sharpe and Laws (1981) classified hop oil components and found that 50-80% of the oil is comprised of hydrocarbons, while the rest includes sulfur-containing and oxygenated compounds (Sharpe and Laws 1981). Hop oil aroma compounds are numerous. Previous studies have determined hop derived aroma compounds to comprise 42.7% percent of beer aroma compounds identified (Tressel et al. 1978).

Hops also impart preservative properties on a finished beer, improving flavor stability and acting as a mild antibacterial agent. Antioxidant compounds, like polyphenols, derived from hops are one of the main sources of flavor stability. Hop polyphenols can prevent lipid oxidation, a major cause of beer staling (Schonberger and Kostelecky 2011). Other studies have suggested that alpha and beta acids found in hop

cones can form stable radicals that can also act as antioxidants, improving flavor stability (Ting et al. 2008). In terms of bacterial stability, iso-alpha acids have been shown to have higher effectiveness than alpha or beta acids, despite their lesser solubility (Sakamoto and Konings 2003). In order to induce an antimicrobial effect, iso-alpha acids act as protonophores, transporting protons across the cell membrane and inhibiting nutrient uptake leading to damage to the cellular metabolism and possible cell death (Schurr et al. 2015).

2.2.4 Yeast

Brewer's yeast of the genus *Saccharomyces* are unicellular fungi that are the driving force behind fermentation of beer, wine, and bread. In the case of beer, two main species *cerevisiae* and *pastorianus* are used (Eßlinger 2009). The main role of yeast during fermentation is to utilize wort carbohydrates to produce mainly ethanol and carbon dioxide, using carbohydrates in sequence starting with sucrose, glucose, and fructose, and ending with maltose and maltotriose (Bamforth 2006). In addition to the production of ethanol, yeast also produce small amounts of other chemical compounds during fermentation, these compounds include higher alcohols, vicinal diketones (VDKs), and esters that affect the final flavor profile of beer (Bamforth 2009).

2.3 Brewing Process

To produce beer, the four principal ingredients (water, malted barley, hops, and yeast) must be utilized and transformed to enable fermentation. This is done during a stepwise process with several steps, including malting of the barley, mashing to extract fermentable sugars, boiling of the unfermented wort, fermentation by yeast, conditioning the final beer, and packaging.

2.3.1 Malting

The first step in the brewing process is the malting of barley, by which germination of the barley kernel produces natural enzymes for the conversion of starch into fermentable sugars that are digestible by the yeast (Fox et al. 2003). To germinate, the grains are first steeped in water. Water enters the micropyle of the barley kernels, which makes its way to the embryo and stimulates hormones that begin the production of enzymes, such as α-amylase, b-amylase, b-glucanase, limit dextrinase, and proteases (Lewis and Young 2004).

The barley is then heated to halt the malting process and introduce darker colors and flavors, by way of caramelization and the Maillard reaction (Coghe et. al 2012). This process of heating the malted barley is called kilning and serves to dry the malt, ending the germination process (Bamforth 2009). The kilning process needs to be done at a relatively low temperature to ensure that the heat-sensitive enzymes produced during germination survive the process (Bamforth 2006). After the barley is prepared by malting and kilning, it is ready to be transformed by the brewer during the mashing process.

2.3.2 Mashing

During this stage, the malt is heated in water to gelatinize starch granules, releasing amylose, amylopectin, and enzymes that convert the starches into fermentable sugars. This is often done in a dedicated mashing vessel called the "mash tun". The first step in the mashing process is the soaking of barley grains in warm water to initiate gelatinization of starch granules within the malted barley, releasing the polysaccharides amylose and amylopectin (Bamforth 2006). A variety of enzymes, including α-amylase, β-amylase, β-glucanase, limit dextrinase, and proteases, are activated at certain temperatures (Table 1) and convert the natural starches within the malt into fermentable sugars (Sammartino 2015).

Table 1 Function, conversion, and deactivation temperatures of mash enzymes

Enzyme	Function	Ideal Conversion Temperatures	Deactivation Temperature
α-amylase	Cleaves any 1 ⟶ 4 glycosidic bonds of starch	162 - 172°F	176°F
β-amylase	Cleaves only linear 1⟶4 glycosidic bonds of starch; produces maltose	140 - 149°F	158°F
Limit dextrinase	Cleaves 1 ⟶ 6 bonds of amylopectin	131-140°F	145°F
β-glucanase	Break down β-glucans of barley cell wall	113 - 122°F	140°F
Proteases	Cleaves bonds between amino acids; increases FAN content	113 - 130°F	158 - 167°F

Common enzymes produced in malted barley and used in the production of beer. Enzymes are activated during heating during the mash. Adapted from Sammartino 2015.

As the temperature of water in the mash tun slowly increases, different enzymes become active. First, a small amount of β-glucanase and proteases begin to break cell walls and barley protein, respectively (Sammartino 2015). Next, starch digesting enzymes like α-amylase, β-amylase, and a small amount of limit dextrinase begin to break down the glycosidic bonds of starch molecules to produce dextrins and fermentable mono-, di-, and tri-saccharides (Muller 2000). These biochemical reactions increase the amount of soluble sugars in the solution, which is called wort, and convert the carbohydrates from the malt into sugars fermentable by yeast.

After all the extractable substances are dissolved into the wort, the mash is lautered to separate the liquid from the solids (e.g. barley husks). This is often performed using a vessel, called a lauter tun, with a perforated false-bottom (Eßlinger 2009). Once the mash is added to the lauter tun, mechanical rakes agitate the bed of grain while hot water (called sparge water) is used to "rinse" the grain of sugars and dissolved solids to be collected by runoff pipes under the false bottom (Lewis and Young 2004). Once the mash has been lautered to separate the sweet wort from the solids, the wort can be transferred to the boil kettle.

2.3.3 Boiling

The wort is at this point transferred to a large kettle for the boil. Boiling performs several functions, including sterilization, clarification, and hop bitterness and aroma additions. During this time, the wort will be heated to boiling for typically 30 - 60 minutes and hops can be added. This boiling process extracts several substances from the hops, including aroma compounds like terpenes, bittering alpha-acids, and various antimicrobials (Schönberger and Kostelecky 2012; Oladokun et. al 2016).

Hop bitterness is mainly a result of the isomerization of hop derived alpha acids during boiling. These alpha acids are composed of three major compounds (humulone, cohumulone, and adhumulone) which were first identified in the early 1950's (Rigby and Bethune 1953). During boiling, the insoluble α-acids (humulone, cohumulone, adhumulone) present in hops are readily isomerized to form the very soluble cis and trans iso-α-acids that give beer its characteristic bitterness (Bamforth 2006). After boiling, the ratio of cis-isohumulones to trans-isohumulones is roughly 70:30. Previous studies have shown that this ratio is

an important marker of quality, as trans-isohumulones are less stable and tend to degrade over time (Schonberger and Kostelecky 2011).

Hops also contain anywhere from 0.5% - 3.0% volatile, flavorful essential oils, which oftentimes are evaporated by the boiling process (Lewis and Young 2004). To minimize this volatilization of flavor compounds, hops can be added later during the boiling process, known as late-hopping, or after boiling, known as dry hopping (Forster and Gahr 2013). After boiling the hopped wort is filtered and cooled to roughly room temperature to prepare for the addition of yeast, the final ingredient in beer.

2.3.4 Fermentation

Addition of yeast starts the fermentation process, which converts sugars into ethanol, carbon dioxide, and other trace volatile compounds. First, the prepared wort must be cooled and transferred to the fermentation vessel. Before fermentation can begin, the wort must be oxygenated to ensure yeast viability during the initial stages of fermentation. Wort oxygenation allows for the yeast to produce unsaturated lipids and sterols, which are important yeast cell membrane components (Kucharczyk and Tuszyński 2017). Previous studies have suggested that the ideal oxygen concentration exists in the range of 10 - 12 ppm (Rees and Stewart 2012).

Brewer's yeast strains are broadly classified into ale and lager yeasts. *Saccharomyces cerevisiae* is the main yeast associated with ale brewing, whereas *S. uvarum* and *S. pastorianus* species are associated with lager brewing. Although many differences exist, these categories of yeast are mainly differentiated by their optimal temperature range, maximum growth temperature, and flocculation tendencies (Table 2). Typical ale yeasts can maintain growth up to 37°C, with an optimal range of fermentation temperature between 18 – 22°C In contrast, lager yeast strains show a maximum growth temperature of 34°C and ferment optimally from 8 – 15°C (Stewart et al. 2013).

Table 2 Traditional differences between ale and lager yeast strains

	Associated species	Fermentation temperature	Maximum growth temperature	Flocculation behavior
Ale yeast	*S. cerevisiae*	18 - 22°C	37°C	'Top' fermenting
Lager yeast	*S. uvarum* *S. pastorianus*	8 - 15°C	34°C	'Bottom' fermenting

Common characteristics of typically used ale and lager yeast species. Adapted from Stewart et al. 2013.

Within the brewery, yeast cultures are managed and propagated in order to increase viability and vitality for fermentation. Yeast management includes storage, acid washing, and recycling. Optimal storage temperatures are low, but above freezing (Stewart et al. 2013). Other storage methods have been evaluated, with emphasis on cryogenic storage. Wellman and Stewart investigated performance of *S. cerevisiae* and *S. uvarum* strains following cryogenic liquid nitrogen storage, showing that long term storage (3 years) can yield high rates of viability (Wellman and Stewart 1973).

In order for a successful fermentation to be carried out, yeast conditions such as pitch rate and fermentation temperature must be closely monitored. For a healthy fermentation, pitch rate should be at least 1 x 10^6 cells/mL/°Plato and temperatures should be maintained between 15 - 20°C for ale yeasts or 6 - 13°C for lager yeasts (Lewis and Young 2004). If these conditions are not met, issues may arise including stuck-fermentations and off-flavors, especially in high-gravity brewing (Stewart 2017). Once fermentation has been completed, the beer is known as "green" beer and is ready for further conditioning before bottling (Stewart 2004).

2.3.5 Conditioning

Conditioning of a green beer serves several purposes, including clarification and carbonation, as well as allowing time for final quality assurance checks. During this stage, the beer is made clear or "bright" (Lewis and Young 2004). After the fermentation stage beer is made bright by removing suspended materials from fermentation by one of several methods, including membrane filtration (via dead-end filtration or cross-flow filtration), Kieselguhr filtration (via plate and frame filtration or a leaf design), and centrifugation (Bamforth 2006; Eßlinger 2009). Stabilization is also done on this bright beer, to ensure the

removal of haze forming compounds like tannins and proteins via enzymatic, adsorptive, or chemical fining methods (Eßlinger 2009).

2.4 Fermentation

Fermentation is possibly the most important brewery operation. During fermentation yeast convert fermentable sugars in wort into carbon dioxide, ethanol, and other trace compounds including higher alcohols and esters. This process requires specific inputs, like oxygen, sugar, and nitrogen, and requires specific parameters to be monitored for the fermentation's duration, such as temperature, sugar, and ethanol concentrations (Gibson et al. 2007). Variables such as osmotic pressure, mineral concentrations, carbon, and nitrogen sources greatly affect the viability and fermentation performance of brewer's yeast. A deficit or excess in any of these factors can lead to stuck fermentations, decreased yeast growth, or negatively affected final flavor profiles (Bisson 1999). Factors affecting yeast performance are constantly shifting during fermentation, leading to various stress responses (Gibson et al. 2007). These factors are of utmost importance to the brewer and must be monitored as fermentation progresses.

2.4.1 Osmotic Pressure

Osmotic stress occurs when an imbalance of intracellular solutes and wort solutes exist. This can either be hypo-osmotic causing an influx of water into the cell, or hyper-osmotic leading to the loss of intracellular water to the environment. Increased osmotic pressure can cause cell shrinkage and cavitation of yeast cells, leading to decreased viability and vitality which indicates cell death (Pratt et al. 2003). In addition, D'Amore and collaborators showed that higher osmotic pressure can increase the amount of intracellular ethanol present during fermentation (D'Amore et al. 1988). The researchers observed that this increase in intracellular ethanol was correlated to a decrease in fermentation and yeast growth. These results were confirmed by Sigler et al. (2009) whose research showed a decrease in cellular reproduction and an increase in fermentation time necessary to produce a 4% alcohol solution (Sigler et al. 2009).

In order to adapt to increased osmotic pressure, brewer's yeast tend to synthesize one or more compounds, known as osmoticum, which increases internal osmotic potential (Gibson et al. 2007). This increased osmotic potential prevents the negative effects of hyper-osmotic pressure by minimizing cellular

water loss. Adaptability to hyper-osmotic stress is strain dependent, with strains able to produce glycerol more likely to adapt to hyper-osmotic conditions (Attfield and Kletsas 2001). Adaption of yeast cells to hyper-osmotic conditions has been well studied, but adaption to hypo-osmotic conditions is not well understood. However, studies investigating yeast cell stress response during hypo-osmotic conditions have revealed that cells vary rapidly export osmoticum, such as glycerol (Tamas et al. 2002).

2.4.2 Ethanol Toxicity

Ethanol can also have a negative effect on brewer's yeast. Previous research has shown that high ethanol concentrations can be detrimental to yeast health, causing reduced cell size, slower fermentation rates, lowered viability, reduced respiration, enzymatic inactivation, and increased membrane permeability (Birch and Walker 2000; Pratt et al. 2003; Gibson et al. 2007).

The main site of ethanol inhibition in yeast are the cell membrane components, causing cavitation and cell shrinkage (Pratt et al. 2003). Therefore, the ethanol tolerance of a yeast cell is highly dependent on the composition of the plasma membrane. An influx of magnesium ions (Mg^{2+}) can help maintain membrane integrity, increasing viability of the cell. When investigated by Birch and Walker (2000), magnesium ions had a protective effect on ethanol and heat stressed yeast cells, specifically by reducing cell mortality, preventing damage and increased permeability of the cell surface, and repression of stress protein synthesis (Birch and Walker 2000). Magnesium ions can be added prior to or during ethanol shock to increase viability and overall cell growth (Gibson et al. 2007).

Since heat stress and ethanol toxicity both hurt membrane permeability, cells respond to both by producing heat shock proteins (Birch and Walker 2000; Gibson et al. 2007). In addition to magnesium, substances produced by yeast have been implicated in the ethanol stress response of yeast. Several studies of the relation between ethanol concentration and concentration of intracellular solutes have been performed. Some of these solutes, like glycerol and trehalose, have shown that an increase in ethanol will increase synthesis of glycerol and treahalose (Ogawa et al. 2000; Jung and Park 2005). Both glycerol and trehalose have been previously investigated for their positive effect on cell viability and performance and have been linked to maintenance of the cell membrane during ethanol stress responses (D'Amore et al. 1990; Pratt et al. 2003; Vriesekoop et al. 2009; Bandara et al. 2009).

2.4.3 Oxygen Concentration

Oxygen is an essential component of a healthy yeast proliferation. Oxygen is introduced early in the fermentation to aid the yeast in synthesis of vital cell components such as lipids and sterols, as well as increasing biomass (Kucharczyk and Tuszyński 2017). Visser et al. compared the growth of various species of fermentative yeasts in media with different oxygen concentrations, finding that *S. cerevisiae* grows best (and better than other yeasts) under low to zero oxygen conditions (Visser et al. 1990). Kirsop (1973) explored the effects that oxygen deficiency has on fermentation, reporting slowed yeast reproduction. Kirsop (1973) also reported this decreased growth during fermentation was further correlated with lowered production of higher alcohols and a decrease in ester production, affecting beer flavor.

Oxygen at later stages of fermentation can be detrimental to yeast health. Oxygen in the wort can form reactive oxygen species (ROS) such as hydrogen peroxide, radical oxygen, and hydroxyl radicals. These ROS may be the reason why cells age and cannot be re-pitched indefinitely, according to the radical theory of aging (Beckman and Ames 1998). ROS are highly damaging towards DNA, lipids, and proteins. Yeast cells produce antioxidant molecules as a rapid response to oxidative stress, in order to protect vital cell components from oxidation (Jamieson 1998). This response to oxidative stress is reported to be strain dependent, with some strains being more adaptable than others (Gibson et al. 2007). The importance maintaining and monitoring oxygen concentration cannot be overlooked during brewery operations, with oxygen early in the fermentation promoting yeast health while oxygen at later stages being detrimental to fermentation progress.

2.4.4 Temperature

S. cerevisiae achieves optimal growth between the temperatures of 25 - 30°C (Morano et al. 2012). Beyond or below this temperature range, brewer's yeast activates stress responses that change yeast physiology. In *S. cerevisiae* a cold-shock response is experienced when temperatures fall below 20°C activating the TIP1 (temperature shock-inducible protein) gene (Kondo and Inouye 1991). One common cause of this cold-shock response during brewery operations is yeast handling, such as storage or acid washing before pitching, which typically happens in a reduced temperature environment (Gibson et al. 2007). During this period, membrane fluidity is decreased, this is due to fatty acid chains in the membrane

converting from a liquid crystalline arrangement to a gel state (Thieringer et al. 1998). This hurts yeast health by decreasing cell membrane fluidity and affecting the transport of metabolites across the membrane, disrupting cell metabolism and leading to decreased viability (Gibson et al. 2007).

When *s. cerevisiae* experiences temperatures above 30°C, cells activate a heat shock response. At these higher temperatures, cellular membranes are damaged by denaturation and aggregation of proteins. The heat shock response mediates this damage by the synthesis of the disaccharide trehalose and the release of a family of heat-shock proteins, preventing the denaturation and stabilizing cell membrane proteins (Hohmann et al. 2010). Heat shock proteins act as yeast "chaperones", refolding and repairing partially denatured proteins (Morano et al. 2012).

Increased fermentation temperature can also increase fermentation rate. In an investigation by Fernandez et al. (1985), an increase in temperature directly resulted in a decreased fermentation time to reach maximum attenuation (Fernandez et al. 1985). As shown by Olaniran et al. (2011), fermentation temperature can also affect the viability and density of spent yeast after fermentation with higher temperatures resulting in both lower viability and density (Olaniran et al. 2011).

2.4.5 Mineral Concentration

The mineral composition of the water used for brewing can greatly affect yeast metabolism during the fermentation process. Minerals like magnesium, calcium, and sodium are commonly found in municipal water sources and can affect fermentation performance in different ways. Previous studies have tested the effects of magnesium and calcium on fermentation performance (Saltukoglu and Slaughter 1983; Rees and Stewart 1997; Rees and Stewart 1999). Typically, increased levels of magnesium in brewing water lead to increased ethanol production, percentage of viable yeast, total cell number, and initial rate of fermentation. In contrast, an increased concentration of calcium in brewing water decreased fermentation rates, ethanol production, viability, and total cell number. Therefore, the ratio of magnesium to calcium ions in wort should favor higher magnesium content and lower calcium content to enhance fermentative performance. These results were displayed during both normal and high gravity conditions (Rees and Stewart 1999).

2.4.6 Carbon and Nitrogen Sources

Yeast need both nitrogen sources and carbon sources (primarily carbohydrates) for metabolism. Without these, yeast growth can be stunted, and proliferation can cease. Nitrogen sources can be categorized as assimilable and non-assimilable for brewer's yeast, with the non-assimilable nitrogen sources being polypeptides and proteins and more assimilable sources including ammonium salts, short peptides, and amino acids. These assimilable amino acids are separated into four lettered groups (Table 3) and are taken up sequentially, beginning with group A (Lei et al. 2012). When nitrogen resources in the wort are reaching depletion yeast cells change from selective amino acid uptake to non-selective amino uptake, forgoing the sequential pattern (Gibson et al. 2007).

Table 3 Assimilable Amino Acid Groups and their Member Amino Acids

Assimilable Amino Acid Group	Members
A	Aspartic acid, glutamic acid, serine, threonine, lysine, arginine
B	Valine, methionine, leucine, isoleucine, histidine
C	Glycine, phenylalanine, tyrosine, tryptophan, alanine
D	Proline

Previous studies have explored the relationship between free amino nitrogen (FAN) concentration, fermentation performance, and volatile compound formation. Using high gravity worts with various levels of maltose syrup adjunct addition, it was observed that utilization rate of amino acids decreased with increasing wort gravity (Lei et al. 2012). The study also showed that worts having higher overall FAN concentrations decreased the overall production of higher alcohols and esters. Higher FAN consumption was shown to correlate with higher cell growth speed, flocculation, and sugar uptake. These results helped to explain that diluting FAN in wort with low-amino syrup adjuncts can cause issues with yeast physiology and beer flavor profile. Worts with a low FAN content can lead to elevated diacetyl concentrations, an undesirable flavor compound and a common marker of beer flavor (Brányik et al. 2008).

Carbon sources such as fermentable carbohydrates are also an important nutrient to yeast. Wort solids consist of roughly 90% carbohydrates. Of these carbohydrates 20 - 30% are unfermentable dextrins, 15% maltotriose, 45 - 65% maltose, 5% sucrose, and 10% fructose (Gibson et al. 2007). Other trace carbohydrates are present such as arabinose, ribose, isomaltose, panose, isopanose, and xylose. These carbohydrates are not all equally fermentable and are taken up sequentially in terms of fermentability. Sucrose is the first to be metabolized, followed by fructose and glucose, and finally maltose and maltotriose. For this reason, high amounts of maltose and maltotriose residuals often exist in fermented beer (Patel and Ingledew 1973).

2.4.7 pH

During fermentation, wort pH is typically reduced by between 1 and 1.5 units in lager fermentations while intracellular pH keeps a narrow range between 5.9 - 6.4 (Rowe et al. 1994). This decrease in pH can affect the gene expression of yeasts. This is an effect of the creation of carbonic acid by CO_2, secretion of organic acids by the yeast, and the consumption of buffering components such as amino acids and primary phosphates (Coote and Kirsop 1975). A reduction in pH can also change what flavor compounds are produced. A decrease in pH of 5.75 to 5.46 can increase dimethyl sulfide production up to 50% and a pH decrease from 5.5 to 4.0 can increase diacetyl production four times (Anness and Bamforth 1982; Gibson et al. 2007). In addition to changing the flavor profile produced by yeasts, pH also can affect yeast growth. As initial pH of the medium trends towards 3.0, rates of growth and fermentation are decreased (Charoenchai et al. 1998). This is consistent with results found by Pampulha and Loureiro-Dias (1989), where decreasing intracellular pH was strongly correlated with the inhibition of fermentation. In addition, the same study also found that complete inhibition of fermentation by intracellular pH is dependent on external ethanol concentration (Pampulha and Loureiro-Dias 1989). In a of 8% (w/v) ethanol medium fermentation was inhibited at pH 5.5, while in the absence of ethanol fermentation was inhibited at pH 4.8.

2.5 High Gravity Fermentation

High gravity worts are high in fermentable sugars and yield a higher ABV beer at the end of fermentation when compared to regular gravity worts. This higher ABV beer is often diluted with distilled water to create a larger volume of beer at a lower ethanol concentration. For example, a brewer may dilute 100 gallons of an 8% ethanol beer with distilled water to reduce the ethanol concentration to 4% and increase the volume to 200 gallons. High gravity (HG) brewing therefore represents an attractive option to many breweries that wish to improve brewhouse capacity and efficiency without the purchase of new equipment.

Implementing the use of high gravity wort can provide many benefits. One such benefit is a decrease in water requirements, by using a high-solids mashing technique that utilizes a lower water to starch ratio (Puligundla et al. 2011). Previous studies have predicted that a water savings of 58.5% potentially exists by increasing wort gravities from 16 to 31 °P (Thomas et al. 1996). This decrease in water

usage decreases energy costs and increases overall productivity of the process without investment in additional infrastructure. Additionally, studies have shown that dilution of high gravity beers can increase volume by greater than 100% to achieve a 5.5% ethanol beer (Lima et al. 2011). For these reasons, high gravity brewing remains an attractive option for brewer's trying to increase productivity with minimal investment.

Despite the potential water and processing cost savings, high gravity brewing can also pose several challenges when compared to normal wort. The high level of fermentable sugars in a HG wort introduces increased osmotic stress, and later during fermentation, high ethanol concentrations that can lead to yeast cell damage (Pratt et al. 2003). Increased yeast stress during high gravity brewing can also cause off-flavors, stuck fermentations, and decrease viability of fermenting yeast (Brányik et al. 2008; Lei et al. 2012).

2.5.1 Fermentation Performance and Yeast Viability

High gravity brewing is an intense form of brewing, with higher than normal concentrations of solutes and ethanol. Because of the intensified conditions of high gravity brewing, yeast cells can often become stressed or damaged during fermentation. This can affect yeast viability and overall fermentative performance. Ability of *S. cerevisiae* to adapt to the elevated stresses of high gravity fermentation is highly strain dependent. Blieck et al. (2007) attempted to isolate and characterize yeast variants that showed improved fermentation ability under the stress of high gravity conditions, making note of their ability to produce glycogen and trehalose, part of yeast stress response. Strains of yeast with improved high gravity fermentation abilities showed faster utilization of glycogen and lower concentration of trehalose at the end of fermentation (Blieck et al. 2007).

Lima et al. (2011) investigated the effect of dissolved oxygen, fermentation temperature, wort gravity, and pitch rate of yeast, it was found that wort concentration, dissolved oxygen concentration, and temperature had the greatest effect on fermentation time. By raising initial dissolved oxygen concentration and keeping fermentation temperatures below 18°C, high gravity fermentation time was reduced to that of the control gravity fermentation (Lima et al. 2011).

Some studies have focused on acceleration of the high gravity fermentation process. Barber et al. (2002) attempted to increase high gravity fermentation rate by daily additions of acetaldehyde (an electron acceptor) during fermentation. The study found that additions of acetaldehyde decreased the length of the lag phase of fermentation, but increased production of some undesirable volatile compounds such as 2-3-butanediol, acetate, and 2-methylpropanol (Barber et al. 2002).

High gravity fermentation conditions can also affect the viability of yeast cells after fermentation. Many brewery operations reuse yeast harvested from fermentation vessels several times in subsequent batches, sometimes as many as 20 times (Kordialik-Bogacka and Diowksz 2013). Therefore, the health of yeast cells after fermentation is of great importance to brewers. Zhimin et al. (2012) compared the physiological activities of lager and ale yeasts in normal, high, and very high gravity worts ranging from 10.93 to 23.66 °P. This study indicated that the viability of yeasts collected after fermentation decreased with increasing wort gravity (Zhimin et al. 2012).

Increased levels of osmotic pressure and ethanol present during high gravity brewing affect the viability of cropped yeast, as indicated in previous research (Pratt et al. 2003). Pratt et al. (2003) also reported increased osmotic pressure and ethanol concentration significantly decreased yeast viability of lager strains studied, whilst ale strains viability remained relatively constant.

2.5.2 Role of Oxygen in High Gravity Fermentation

As previously discussed, oxygen is required at the beginning of fermentation for yeast cells to produce unsaturated lipids and sterols, which are important yeast cell membrane components (Kucharczyk and Tuszyński 2017). O'Connor-Cox et al. (1993) previously determined other usages of oxygen by the yeast cell during high gravity fermentation in the absence of unsaturated lipid synthesis. The study found that oxygen also plays a role in mitochondrial functionality and development, a critical component of cellular energy production (O'Connor-Cox et al. 1993).

Due to the high concentration of dissolved solids in high gravity wort, the solubility of oxygen is greatly reduced. Therefore, supplying the correct amount of oxygen during high gravity fermentation becomes a challenge. Jones et al. (2007) investigated the timing and concentration of dissolved oxygen during very high gravity beer fermentation at bench-top and pilot scales. At the bench-top scale, worts

provided with 25 ppm dissolved oxygen after twelve hours of fermentation showed the fastest rates of fermentation. Worts provided with 25 ppm dissolved oxygen at both initial fermentation and after 12 hours showed the second fastest rate of attenuation (Jones et al. 2007). Lima et al. (2011) demonstrated the effect of dissolved oxygen in wort on fermentation rates in high gravity brewing. Using statistical methods to optimize for low fermentation time, high ethanol production, and low final apparent extract this study's data suggests an optimal concentration of dissolved oxygen at 22 ppm for a 22 °P wort, resulting in a total fermentation time of 11.9 ± 0.2 days (Lima et al. 2011).

Rees and Stewart investigated the combined effects of magnesium, calcium, and wort oxygenation on fermentation of high gravity worts. This study's data suggests that elevating the level of magnesium in oxygenated worts increased initial fermentation rates and overall ethanol production compared to unoxygenated samples high gravity samples (Rees and Stewart 1998).

Anderson and Kirsop previously investigated the use of oxygen as a regulator of ester formation during high gravity wort fermentation. It was reported that treatment with oxygen significantly reduced levels of isoamyl acetate and ethyl acetate in the final fermentation, in addition to increasing overall yeast mass and fermentation rates; concentrations of higher alcohols were not affected significantly by oxygen treatment (Anderson and Kirsop 1974).

2.5.3 Volatile Flavor Compound Formation in High Gravity Worts

During fermentation, *S. cerevisiae* synthesize many flavor- and aroma-active compounds that affect the final flavor profile of beer. Several studies have attempted to monitor the effects of environmental factors like fermentation temperature, yeast pitching rate, and dissolved oxygen concentration on the production of flavor compounds in beer. Saerens et al. (2008) studied the effect of initial gravity and fermentation temperature on formation of flavor. This study found that increased wort gravity (16 to 18 °P) increased the formation of acetate esters and ethyl hexanoate (associated with sweet, fruity aroma) when compared to less dense worts (12 and 14 °P). When a higher fermentation temperature (15℃ for lager strains, 24℃ for ale strains) was used, the concentration of phenyl ethyl acetate (responsible for a floral aroma) was increased when compared to lower fermentation temperatures (12℃ for lager strains, 20℃ for ale strains) (Saerens et al. 2008).

Lei et al. (2012) previously researched the combined effects of wort gravity and nitrogen level on fermentation performance, suggesting that increasing nitrogen concentration in high gravity worts resulted in increased higher alcohol and ester formation during fermentation (Lei et al. 2012). While investigating the effect that pitching rate had on formation of flavor-active volatile compounds in high gravity wort, Erten et al. (2007) found that higher pitch rates increased production of certain higher alcohols, but decreased production of others. Increasing pitch rate led to higher concentrations of iso-butanol, but decreased concentrations of isoamyl alcohol and 3-methyl-1-butanol. In terms of ester production, pitching rate was reported to have no effect except in the case of isoamyl acetate, where lower pitch rates increased concentration (Erten et al. 2007).

2.6 Beer Flavor

Beer flavor is complex and unique, with flavor notes ranging from bitter, toasty, and even fruity. Beer flavors come from various sources, including different varieties of malt and hop. During fermentation yeast also synthesize various flavor compounds as a part of their normal metabolism, including desirable compounds like esters and higher alcohols and undesirable compounds like vicinal diketones (Bamforth 2014). These flavor compounds are perceived in part because of their volatile nature, evaporating and contributing to both taste and aroma. As with most chemicals that we taste, beer flavor compounds have a detection threshold, a minimum concentration where the taste is detected 50% of the time (Meilgaard 1982). Beer flavor may also be synergistically or antagonistically affected by flavor compounds present in concentrations below their detection threshold (Sterckx et al. 2011). Because of the effect of individual flavor compounds on the overall flavor profile of a beer, much research has been done to identify and quantify volatile flavor compounds in beer.

2.6.1 Volatile Flavor Compounds in Beer

Beer flavor is a combined result of interactions between hundreds of chemical compounds and their perception (Blanco et al. 2014). Many research efforts have been devoted to the identification and quantification of these compounds in various beer styles produced in a large amount of ways (Palamad et al. 1971; Chen 1981; Sakuma et al. 1999; Huimin et al. 2012). The main flavor contributing compounds

can be classified by structure and include esters, alcohols, vicinal diketones, and sulfur-containing compounds (Bamforth 2014).

Esters in beer are a result of yeast metabolism, derived from the combination of an alcohol and an acid (Bamforth 2014). Ester synthesis is highly dependent on yeast strain and fermentation conditions such as sugar profile and temperature, with each strain producing a different combination of esters in different concentrations (Verstrepen et al. 2003). Generally, esters provide fruity aromas to a beer's flavor profile (Table 4). Notable esters typically found in beer include isoamyl acetate (banana, bubble gum), ethyl acetate (pear drops, nail polish), and ethyl hexanoate (apple, aniseed) (Bamforth 2014).

Table 4 Esters and Their Beer Flavor Contributions

Ester	Flavor contribution
Isoamyl acetate	Banana, bubble gum
Ethyl acetate	Pear drops, nail polish
Ethyl octanoate	Apple, fruity
Ethyl butyrate	Papaya, mango, canned pineapple
Ethyl hexanoate	Apple, aniseed (at high concentrations)
Phenethyl acetate	Rose, honey
Ethyl caprylate	Apple, sweet, fruity

Table adapted from Bamforth 2014.

Another major contributor to beer flavor are higher alcohols resulting from yeast metabolism, produced from a combination of hydrogen atoms with carbonyl compounds like aldehydes and ketones (Pires et al. 2014). Like esters, the production of higher alcohols by yeast is highly dependent on the fermentation parameters such as oxygen level and temperature (Blanco et al. 2014). Higher alcohols (also known as fusel alcohols) are mostly associated with alcoholic flavors in beer but can have other floral and fruity aromas (Table 5). Notable higher alcohols associated with beer flavor include propanol (alcoholic), isoamyl alcohol (alcoholic, banana, wine-like), and phenyl ethanol (roses, perfume) (Bamforth 2014).

Table 5 Alcohols and Their Beer Flavor Contributions

Alcohol	Flavor contribution
Ethanol	Alcoholic
Propanol	Alcoholic
Isobutanol	Alcoholic
Isoamyl alcohol	Alcohol, banana, wine-like
Tyrosol	Bitter
Phenylethanol	Roses, perfume

Table adapted from Bamforth 2014.

Some flavor compounds that contribute to beer flavor in undesirable ways, producing off flavors and aromas. Most common are vicinal diketones (VDKs), the most common being diacetyl and pentanedione (Bamforth 2014). Diacetyl and pentanedione are formed during fermentation as a result of amino acid synthesis by the yeast and add a buttery or butterscotch off flavor to a beer that is undesirable (Krogerus and Gibson 2013).

2.6.2 Gas Chromatography Techniques for Volatile Analysis

In order to identify and quantify the concentration of volatile flavor compounds in beer, gas chromatography (GC) is the most typical method used. GC is a type of chromatography that utilizes an inert gas as the moving phase to separate compounds contained within a volatilized sample as it moves through a column. These separated compounds are analyzed using various sensors, including mass spectrophotometers (MS) or flame ion detectors (FID), which output an electrical signal. The area under the curve of this electrical signal can be further analyzed to determine the concentration of analytes in the original sample (Mcnair and Miller 2009). Several methods of sample delivery are available including needle injection and headspace analysis.

One of the most common sample delivery methods for GC is injection, which can occur in various forms. The most common form is isothermal split or splitless injection, where a small volume of sample (usually only 1-2 µL) is injected and instantly volatilized by the hot inlet and either split, where a large amount of the sample is diverted before the entering the column, or not split where the full sample is injected into the column (Hoh and Mastovska 2008). On-column injection involves injection of sample components directly into the GC column, but usage is limited to simple sample matrices as separation of analytes is made more difficult (Zrostlikova et al. 2001).

Headspace solid phase microextraction (HS-SPME) is a quick, simple, and solvent less method of extracting volatile flavor compounds from a beer sample's headspace (Pinho et al. 2006). Many research efforts have been dedicated to the optimization of this technique, both in general and as used in specific applications for specific compounds (Roberts et al. 2000; Huimin et al. 2012; Filipowska et al. 2020). HS-SPME is generally performed using a porous fiber that absorbs volatile flavor compounds from a sample's headspace before being inserted into the gas chromatography system. Pinho et al. (2006) attempted to optimize the HS-SPME technique using different SPME fibers and found that a 75 µm Carboxen-polydimethylsiloxane (CAR-PDMS) enabled the extraction for the widest array of compounds including ethers, esters, acids, aldehydes, ketones, alcohols, and sulfur-containing compounds (Pinho et al. 2006).

2.6.3 Staling and Shelf Life

Over time, the flavor of a beer in storage will be altered by a variety of chemical reactions. This change in flavor can present itself by the decrease of fresh aromas and bitterness and an increase in off-flavors such as cardboard (Dityrch et al. 2019). Many of these flavors are contributed by aldehyde compounds. During storage the concentration of aldehydes in a beer increases, especially for furfural and Strecker degradation aldehyde products like 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal (Malfliet et al. 2008). As indicated by research performed by Saison et al. (2010), the presence of oxygen during storage accelerates the production of aged flavors typical of trans-2-nonenal, acetaldehyde, Strecker degradation aldehydes, and diacetyl (Saison et al. 2010).

Baert et al. (2012) reviewed the origin of staling aldehydes in beer, providing several recommendations for the decrease of aldehyde production during beer storage including achieving lowest

in-package dissolved oxygen content, addition of antioxidants, and the addition of yeast during bottling (referred to as “bottle conditioning”) in low concentrations for their oxygen scavenging capabilities (Baert et al. 2012).

In order to analyze the effect of storage on the flavor stability of beer, a forced or accelerated aging technique is often used. Barnette and Shellhammer (2019) previously investigated the impact of dissolved oxygen and aging on dry-hopped aroma stability utilizing an accelerated aging protocol (Barnette and Shellhammer 2019). Beer samples were stored at either an elevated temperature (30°C) as a surrogate for aging, or a cold storage (3°C) condition as a control. Results showed that accelerated storage conditions at higher temperatures had a greater effect on the hoppy characteristics of dry-hopped beers than beers in cold storage. Li et al. (2015) evaluated the effects of storage temperature on beer staling and kinetics, observing that increasing temperature increased the rate of beer ageing (Li et al. 2015). Results indicated the development of stale flavors during the storage of beer for 1 day 50°C was equivalent to storage for 4 weeks at room temperature and storage for 1 day at 60°C was equivalent to 8 weeks of room temperature storage.

2.6.4 Thiobarbituric Acid (TBA) Analysis

Thiobarbituric acid (TBA) is a cyclic, organic acid commonly used in assays that detect products of lipid oxidation. TBA reacts, when heated, with products of oxidation to form a pink complex that is measured using a spectrophotometric method (Botsoglou et al. 1994). Using the measured absorbance at 448 nm wavelengths, a unitless TBA index (TBI) value is calculated and indicates the level of staling. TBA analysis for the presence of lipid oxidation is commonly performed on many foodstuffs such as poultry, frozen meats, dried milk products, and countless other products (Sidwell et al. 1955; Salih et al. 1987; Tomas and Funes 1987).

TBA analysis has been evaluated previously as a determination of beer quality and staling. Grigsby and Palamand (1975) were the first to apply TBA analysis in measurement of beer oxidation and determined the correlation between extent of beer staling and TBA values (Grigsby and Palamand 1975). Lynch and Seo (1987) previously used TBA analysis to correlate the production of ethylene during beer storage with TBA values indicating staling. TBA values determined for beer samples stored at 5°C were

much lower than TBA values for samples stored at 40°C during the same period, indicating an increase in staleness (Lynch and Seo 1987). A number of similar studies have used TBA values to indicate staleness in beer for various purposes including the optimization of flavor stability, comparison to and evaluation of novel staling indicators, and in the use of mathematically modeling canned beer quality (Bamforth and Parsons 1985; Kaneda et al. 1991; Corzo and Bracho 2006). As shown, TBA analysis has long since been used as a quick and effective indicator of staling and beer quality in previous research.

Chapter 3

THE EFFECTS OF WORT OXYGENATION SCENARIOS ON FERMENTATION PERFORMANCE, VOLATILE FLAVOR COMPOUND DEVELOPMENT, AND FLAVOR STABILITY IN HIGH GRAVITY BREWING

3.1 Materials and Methods

3.1.1 Chromatography Compounds

All chemicals used for volatile analysis were food grade and purchased from Sigma-Aldrich (St. Louis, MO) in the highest available purity: benzaldehyde (>99%), ethyl hexanoate (>99%), isoamyl alcohol (>98.5%), diacetyl (>98%), hexanal (>97%), acetaldehyde (>99.5%), ethyl acetate (>99.5%), isoamyl acetate (>97%), isobutanol (>99%), propanol (>99.5%), phenethyl alcohol (>99%). Reagents for TBA analysis were purchased from Sigma-Aldrich (St. Louis, MO) in the highest available purity: glacial acetic (>99%) and thiobarbituric acid (>98%).

3.1.2 Wort Preparation

Malt extract was used in this study to brew wort in 5 gallon batches. In order to brew, approximately 9 lbs. of Briess Pilsen Light dry malt extract (BSG Handcraft, Shakopee MN) was dissolved in 5 gallons of water and was brought to boil. Once malt extract was dissolved and the wort was boiling, a quantity of 10 g of US Centennial hops (Doc's Cellar, San Luis Obispo CA) was added and boiled for one hour. After boiling, wort was cooled to room temperature. Sterile water was added to account for evaporation and ensure a final batch volume of 5 gallons.

3.1.3 Wort Oxygenation

Two oxygenation scenarios, pre-pitch and post-pitch, were evaluated. For pre-pitch oxygenation scenarios, wort was split into 3 L samples and dosed with pure oxygen (Benzomatic 1.4 oz oxygen tank, Worthington Industries, Pomona, CA) through a 0.5 µm diffusion stone to achieve three levels of dissolved oxygen: 8 ppm, 14 ppm, and an unoxygenated control. Pre-pitch oxygenation samples were oxygenated immediately before pitching yeast.

For post-pitch oxygenation scenarios, wort was split into 3 L samples and dosed to achieve 8 ppm of dissolved oxygen immediately before pitching yeast. After 12 hours of fermentation had elapsed, samples were again dosed with oxygen to achieve five dissolved oxygen levels: 8 ppm, 10 ppm, 12 ppm, and 14 ppm with an unoxygenated control. All samples were measured for dissolved oxygen concentration using a MW600 DO Meter (Milwaukee Instruments, Rocky Mount, NC).

3.1.4 Yeast Propagation

A liquid starter culture was prepared using liquid yeast (WLP001 California Ale Yeast, White Labs, San Diego, CA). One packet of liquid yeast was pitched in a 1 L flask containing 1 L of sterile wort. To prepare the wort, 1 L of DI water was brought to boil and 100 g of light dry malt extract (Briess Pilsen Light DME 2.0° L, BSG Handcraft, Shakopee MN) was dissolved. Starter culture was allowed to sit at room temperature for 48h or until cell density was appropriate for pitching. The target pitch rate was 1.0 x 10^6 cells/mL•°P for each wort inoculated.

3.1.5 Fermentation

Aliquots of 900 mL treated wort were split 1 L glass bottles with airlock (ThermoFisher Scientific, Waltham, MA) for fermentation, with three glass bottles per oxygen treatment. Propagated yeasts were pitched in the 1 L glass bottles containing 900 mL of wort at a rate of 1.0 x 10^6 cells/mL•°P and thoroughly mixed. For post-pitch oxygenation trials, fermentation was allowed to begin at room temperature before being dosed with oxygen after 12 hours. Fermentation was completed at a constant 20°C over 7 days. Daily samples were taken to analyze for specific gravity using 4500M Density Meter (Anton Paar). Samples for GC analysis were taken at the end of fermentation for post-pitch oxygenation samples. All analyses were performed in triplicate.

Viable cells were counted with a microscope and hemocytometer after staining with 0.1% methylene blue dye solution. The viability was determined by dividing the viable cells by the total number of cells, with results obtained as percentages.

3.1.6 Gas Chromatography Analysis

Volatile analysis of beer samples was performed following a modified Beer-48 Method by the American Society of Beer Chemists (ASBC 2012) and performed in triplicate. For analysis, 5 mL of beer and 100 µL of n-butanol internal standard was added to a 10 mL vial and mixed. Aliquots (1.75 mL) of mixed beer sample and internal standard were put into 2 mL autosampler vials before injection into the GC injector. To create standard curves for each volatile compound, stock solutions serially diluted with ethanol (Sigma-Aldrich) for each compound were analyzed to create a calibration curve. These ranges can be found in Table 6.

Table 6 Concentration Ranges of Volatile Compounds in Calibration Curves

Compound	Concentration Range in Calibration Curve	
	Minimum (ppm)	Maximum (ppm)
Benzaldehyde	0.07	29.12
Acetaldehyde	0.79	31.52
Ethyl acetate	0.92	36.08
Iso-amyl acetate	0.18	7.01
Iso-butyl alcohol	0.80	32.08
Iso-amyl alcohol	3.24	129.60
Phenethyl alcohol	6.12	244.80

Volatile analyses were performed using an Aligent 7890B GC (Aligent Scientific, Santa Clara, CA, CN10847018). The column used was an HP-5ms (length = 30 m, internal diameter = 0.25 mm, film thickness = 0.25 µm). Helium was used as the carrier gas with a flow rate of 3.5 mL/min. The oven temperature was set to 70°C for 8 minutes, increased by 10°C/min to 90°C, held at that temperature for 4 minutes, increased by 25°C to 165°C, and increased to 210°C by 75°C/min, and held at that temperature for

10 minutes (ASBC Method Beer-48). Compounds were identified by comparing retention times with reference standards of each compound analyzed previously using the same procedure.

3.1.7 Thiobarbituric Acid (TBA) Index Analysis

Thiobarbituric acid (TBA) index analysis was used as an indicator of beer flavor stability during storage. Beer samples were prepared and analyzed according to American Society of Brewing Chemists method Wort 21 (ASBC 2009). Beer samples for TBA analysis were pretreated by first degassing and performing a 10-fold dilution with deionized water. Samples were prepared by combining 10 mL of diluted beer sample with 5 mL of TBA in a test tube before mixing. Beer samples for analysis were prepared in triplicate. All test tubes (calibration blanks and beer samples) were placed in a 70°C water bath and heated for one hour, after which the tubes were rapidly cooled to 20°C by placing test tubes in a cool water bath.

During analysis, the spectrophotometer was set to measure absorbance at 448 nm wavelengths. Before analyzing blanks and samples, a sample of deionized water was used to zero the spectrophotometer. Once zeroed, blanks and beer samples were analyzed for absorbance. These absorbances were used to calculate the unitless TBA Index (TBI) using the equation below (1). All analyses were performed in triplicate.

Equation 1 Calculation of TBA Index (TBI)

$$TBI = (Sample\ absorbance - Blank\ absorbance) \times 10 \times Dilution\ factor$$

3.1.8 Statistical Analysis

Specific gravity and volatile concentration were analyzed using one-way ANOVA and Tukey's pairwise comparisons were used to assess significant differences. Minitab 19 statistical software was used for all analysis (Version 19.1, Minitab LLC, State College, PA). Significance was defined as $P \leq 0.05$.

3.2 Results and Discussion

The purpose of this research was to determine the impact of wort aeration timing and concentration on fermentation performance, development of volatile flavor compounds during fermentation, and staling behavior of high gravity brewing. While previous research has demonstrated the importance of dissolved oxygen (DO) for fermentation performance, flavor compound production, and staling in standard gravity beers, few studies have focused on the impact of oxygenation timing and concentration on these factors during high gravity brewing (Visser and Scheffers 1990; Rees and Stewart 1999; Kucharczyk and Tuszynski 2017).

To assess the effect of wort oxygenation prior to the pitching of yeast, high gravity worts were oxygenated to DO concentrations of 8 and 14 ppm, along with an unoxygenated control sample, prior to addition of yeast. Results of the pre-pitch oxygenation trials are shown in Table 7. Wort specific gravity was monitored daily to evaluate fermentation performance. The initial specific gravity for all high gravity worts was 1.085, and final specific gravity was significantly ($p \leq 0.05$) lower for all oxygen treatments after seven days of fermentation. The lowest final specific gravity after fermentation was observed in the 14 ppm treatment, while the highest final specific gravity was recorded for the control. No significant difference ($p \leq 0.05$) in specific gravity was found between the 8 and 14 ppm oxygen treatments during fermentation. However, the specific gravity for both the 8 ppm and 14 ppm oxygen treatments were significantly different ($p \leq 0.05$) from the control treatment after two days of fermentation, indicating an increased degree of fermentation for the treatment samples.

Table 7 Mean Specific Gravity for Pre-Pitch Oxygen Treatment During Fermentation

Day	Control	8 ppm	14 ppm
0	$1.085^{a} \pm 0.000$	$1.085^{a} \pm 0.000$	$1.085^{a} \pm 0.000$
1	$1.068^{a} \pm 0.004$	$1.071^{a} \pm 0.008$	$1.066^{a} \pm 0.001$
2	$1.072^{a} \pm 0.002$	$1.069^{b} \pm 0.002$	$1.067^{b} \pm 0.006$
3	$1.065^{a} \pm 0.002$	$1.060^{b} \pm 0.006$	$1.061^{b} \pm 0.005$
4	$1.067^{a} \pm 0.001$	$1.064^{b} \pm 0.002$	$1.065^{b} \pm 0.005$
5	$1.065^{a} \pm 0.001$	$1.060^{b} \pm 0.001$	$1.060^{b} \pm 0.005$
6	$1.067^{a} \pm 0.004$	$1.063^{b} \pm 0.004$	$1.061^{b} \pm 0.006$
7	$1.065^{a} \pm 0.004$	$1.059^{b} \pm 0.005$	$1.056^{b} \pm 0.009$

*Statistical significance is notated by letter pairs. Mean concentrations in the same row and different columns with different letters are significantly different ($p \leq 0.05$).

During pre-pitch oxygenation trials, the alcohol level in the fermenting worts were calculated and recorded throughout the fermentation (Figure 1). As can be seen from Figure 1, after seven days of fermentation, all samples showed an alcohol by volume (ABV) above 2.5%, with the highest ABV found in 14 ppm treated wort at 3.63%. The lowest recorded ABV was found in the control at 2.67%.

Results for the pre-pitch oxygenation trials showed that the addition of dissolved oxygen enhanced the fermentation performance of the high gravity fermentation. A previous study by Rees and Stewart (1999) also demonstrated that the addition of oxygen, either via simple mixing or bubbling of O2 at a level of 1 ppm per °Plato, to worts of both normal (12 °P) and high (20 °P) gravities enhanced fermentation performance in both ale and lager yeast strains. This increase in fermentation performance was attributed to greater yeast viability, where oxygenation increased viability by 13 - 15% in lager strains and 18 - 16% in ale strains. Additionally, a study by Moonjai et al. (2002) also observed the positive effect of oxygenation on fermentation performance. In this study, the researchers observed that an oxygenated synthetic media showed rates of attenuation that were much higher than those of de-aerated media. Under the experimental conditions, media aerated to 8 ppm of DO showed higher yeast viability and increased biomass throughout fermentation leading to increased rates of fermentation compared to de-aerated media.

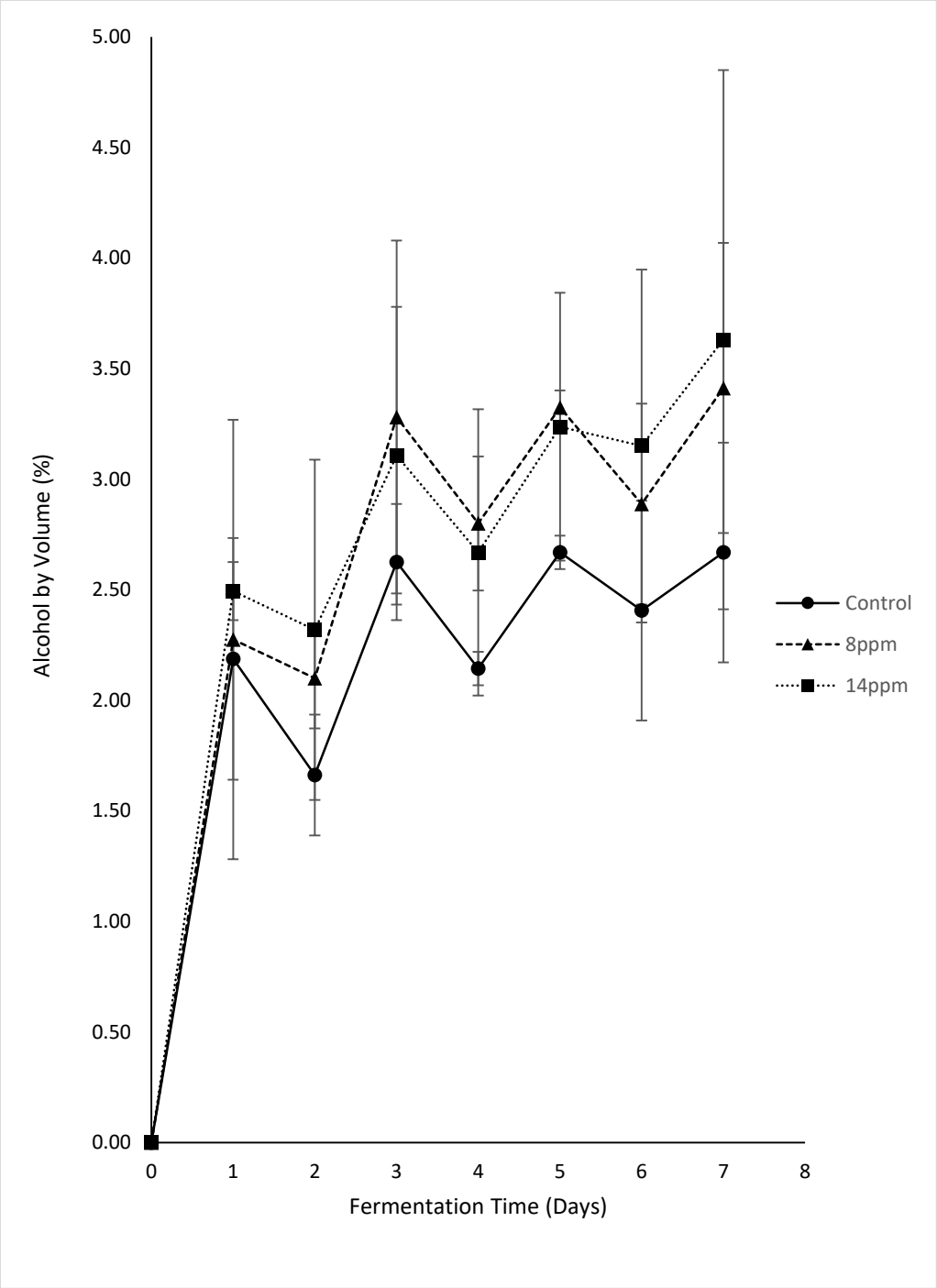

Figure 1 Alcohol by Volume (ABV) in Pre-Pitch Oxygenation Trials

Previous research has also concluded that the level of DO is an important factor in the improvements of fermentation performance. A study by Kucharczyk and Tuszynski (2017) demonstrated

that worts oxygenated to 7 and 10 ppm DO showed no significant difference in daily attenuation, while concentrations of DO at 12 ppm resulted in an increase in daily attenuation at laboratory scales, and decreased fermentation time by 10% at industrial scales. As observed in previously discussed research by Rees and Stewart (1999) and Moonjai et al. (2002), worts aerated with 12 ppm DO showed the greatest increases in cell biomass and yeast vitality. This study by Kucharczyk and Tuszynski (2017), noted that higher degrees of aeration have beneficial effects on yeast cell metabolism, due to increased permeability of the cell wall leading to ease of metabolite exchange.

Under the conditions of the current experiment, it was observed that high gravity fermentations utilizing a pre-pitch wort oxygenation strategy had a significant reduction ($p \leq 0.05$) in wort specific gravity over the course of fermentation with 8 ppm or greater dissolved oxygen treatments when compared to the control. The results observed in the current study are consistent with those of previous research, where increased DO concentrations lead to increased fermentation rates when compared to lower degrees of wort pre-oxygenation, likely due to greater yeast biomass and increased cell viability. Additionally, under the conditions of this experiment it was observed that high gravity fermentations with 8 ppm or greater dissolved oxygen treatments produced more ethanol than the control. Higher final volumes of ethanol are indicative of greater ethanol productivity by the yeast during the course of fermentation. As shown in previous work performed by Jones et al. (2007), increased oxygen supply can lead to significantly increased ethanol productivity and overall reduction in fermentation time indicative of increased fermentation performance.

The effect of wort oxygenation post-pitching was also assessed for high gravity worts. To assess the effect of wort oxygenation post-pitching of yeast, high gravity worts were oxygenated to DO concentrations of 0, 8, 10, and 14 ppm after 12 hours of pitching the yeast. The specific gravity for the post-pitch oxygenation trials was monitored daily throughout fermentation (Table 8). Based on the results from the pre-pitch oxygenation trial, worts were oxygenated at a base level of 8 ppm before pitching the yeast. Initial specific gravity of the worts used for the post-pitch oxygenation trials was 1.0884.

After seven days of fermentation, wort gravity was significantly ($p \leq 0.05$) lower in all samples. The lowest final specific gravity was observed in the 8 ppm treatment, while the highest specific final gravity was observed in the control. Significant difference ($p \leq 0.05$) in specific gravity was found between

treatments, with the highest specific gravity observed in the control treatment, followed by the 14 ppm, 10 ppm, 12 ppm and 8 ppm treatments respectively. The specific gravity for the 12 ppm treatment was significantly lower than the control and 14 ppm treatments after seven days. Overall, the 8 ppm treatment's specific gravity was significantly lower ($p \leq 0.05$) than all other treatments except the 12 ppm treatment after seven days of fermentation.

Attenuation rates as a percentage reduction in wort specific gravities were calculated after seven days of fermentation (Table 9). Control samples showed the lowest rate of attenuation after seven days of fermentation at 53.39%. All samples treated with post-pitch oxygenation showed a greater rate of attenuation than the control. Samples treated with 8 ppm of dissolved oxygen post-pitch were observed to have the highest rate of attenuation at 73.30%.

Table 8 Mean Specific Gravity by Post-pitch Oxygen Treatment During Fermentation

Day	Control	8 ppm	10 ppm	12 ppm	14 ppm
0	$1.0884^{a} \pm 0.0$	$1.0884^{a} \pm 0.0$	$1.0884^{a} \pm 0.0$	$1.0884^{a} \pm 0.0$	$1.0884^{a} \pm 0.0$
1	$1.0704^{a} \pm 0.002$	$1.0571^{b} \pm 0.002$	$1.0606^{b} \pm 0.001$	$1.0584^{b} \pm 0.002$	$1.0666^{a} \pm 0.002$
2	$1.0699^{a} \pm 0.001$	$1.0498^{e} \pm 0.002$	$1.0571^{c} \pm 0.001$	$1.0532^{d} \pm 0.002$	$1.0638^{b} \pm 0.0004$
3	$1.0637^{a} \pm 0.0004$	$1.0410^{c} \pm 0.002$	$1.0513^{b} \pm 0.001$	$1.0471^{b\,c} \pm 0.001$	$1.0521^{b} \pm 0.001$
4	$1.0572^{a} \pm 0.001$	$1.0337^{e} \pm 0.002$	$1.0438^{c} \pm 0.001$	$1.0392^{d} \pm 0.002$	$1.0497^{b} \pm 0.0001$
5	$1.0519^{a} \pm 0.002$	$1.0283^{d} \pm 0.003$	$1.0382^{c} \pm 0.003$	$1.0342^{c} \pm 0.002$	$1.0442^{b} \pm 0.0001$
6	$1.0479^{a} \pm 0.002$	$1.0264^{d} \pm 0.002$	$1.0343^{c} \pm 0.003$	$1.0306^{c\,d} \pm 0.001$	$1.0404^{b} \pm 0.0001$
7	$1.0412^{a} \pm 0.003$	$1.0236^{d} \pm 0.001$	$1.0317^{b\,c} \pm 0.001$	$1.0277^{c\,d} \pm 0.001$	$1.0360^{b} \pm 0.001$

*Statistical significance across columns is notated by letter pairs. Mean concentrations in the same row and different columns with different letters are significantly different ($p \leq 0.05$).

Table 9 Overall Attenuation Rate After Seven Days of Fermentation

	Control	8 ppm	10 ppm	12 ppm	14 ppm
Attenuation (%)	53.39	73.30	64.14	68.67	59.28

During post-pitch oxygenation trials, the alcohol level in the fermenting worts were calculated and recorded throughout the fermentation (Figure 2). As shown in Figure 2, all samples showed an alcohol by volume (ABV) above 6%, with the highest ABV found in 8 ppm treated wort at 8.59%. The lowest recorded ABV was observed in the control at 6.27%. Significant differences ($p \leq 0.05$) in ABV content were found between treatments. The control treatment produced significantly less ($p \leq 0.05$) alcohol than all other treatments. Treatment with 8 ppm DO produced significantly more ($p \leq 0.05$) alcohol than the 10 and 14 ppm treatments. No significant differences ($p \leq 0.05$) were observed between the 10 and 12 ppm treatments. No significant differences were ($p \leq 0.05$) observed between the 10 and 14 ppm treatments. These pairwise comparisons are shown in Table 10.

Table 10 Final ABV % of Post-Pitch Oxygen Treatments

Treatment	Mean
Control	6.27^{d}
8 ppm	8.59^{a}
10 ppm	$7.52^{b\,c}$
12 ppm	$8.04^{a\,b}$
14 ppm	6.95^{c}

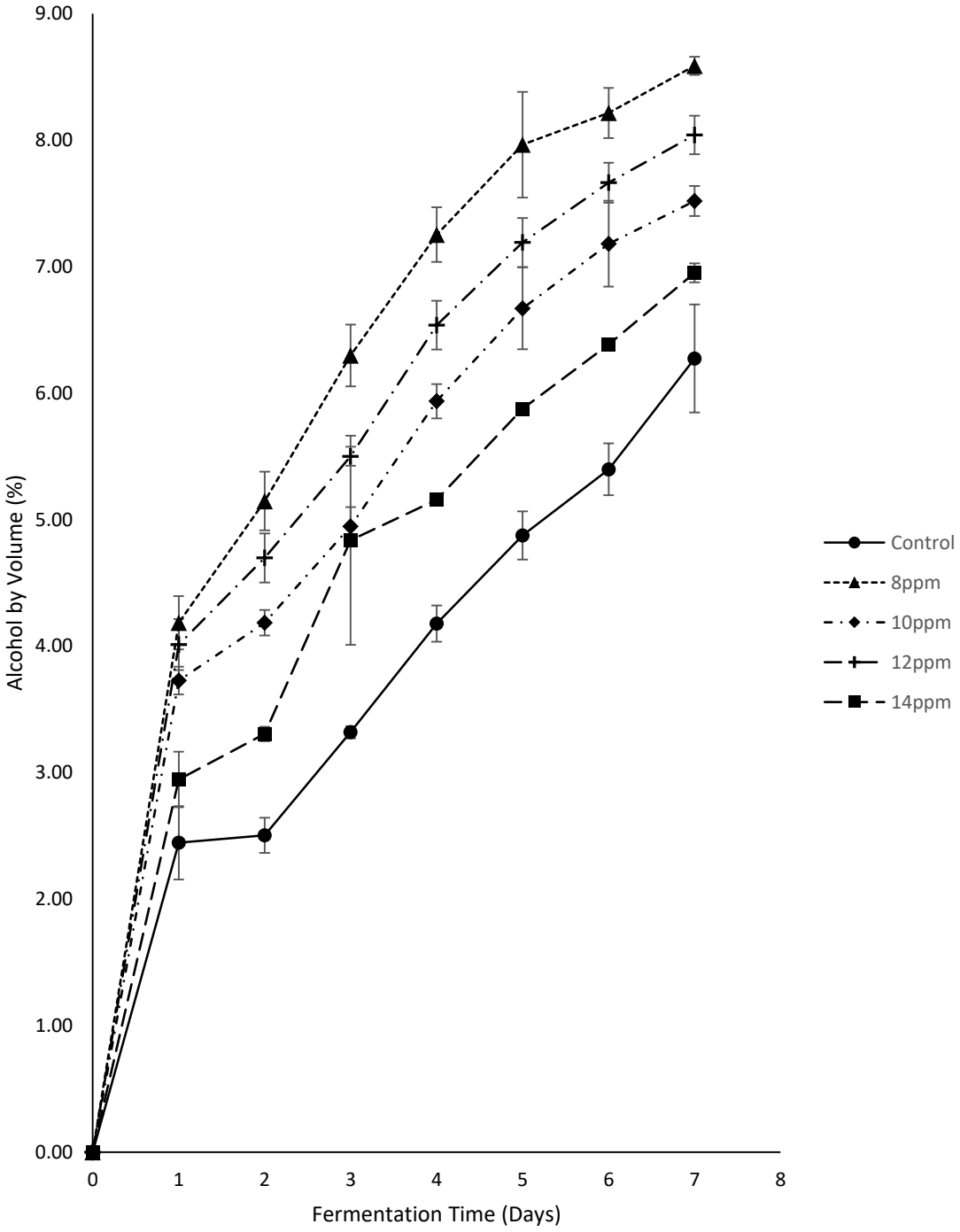

Figure 2 Alcohol by Volume (ABV) in Post-Pitch Oxygenation Trials

Results for the post-pitch oxygenation trials showed that the addition of dissolved oxygen after 12 hours enhanced fermentation performance of high gravity fermentations. Previous research also observed that standard gravity worts oxygenated with 25 ppm DO at 12 hours post-pitch showed increased fermentation performance. This increase in fermentation performance was measured as a significant (33%) reduction in fermentation time when compared to control fermentations oxygenated at 0 hours post-pitch (Jones et al. 2007). Additionally, in a study conducted by O'Connor-Cox and Ingledew (1990), oxygen supplied between 10 and 14 hours post-pitch also resulted in increased fermentation performance. Post pitch oxygenation resulted in a higher rate of ethanol formation and high gravity wort attenuation, indicating an increase in fermentation performance. When oxygen was delivered at 2, 4, or 6 hours post-pitch, increases in ethanol formation and wort attenuation was not as effective as oxygen supplied 12 hours post-pitch (O'Connor-Cox and Ingledew 1990).

In the current study, it was observed all worts that were treated with at least 8 ppm of dissolved oxygen twelve hours post-pitch were observed to have significantly ($p \leq 0.05$) lower specific gravities after 7 days of fermentation when compared to the control. Results observed in this study are consistent with those of previous research, where post-pitch oxygenation of high gravity worts led to increases in attenuation rates and ethanol formation. Results for the post-pitch oxygenation trials showed that post-pitch oxygen dosages of greater than 8 ppm resulted in the most significant decrease in specific gravity and showed the highest attenuation compared to all other treatments. Additionally, treatment with 8 ppm DO at 12 hours post-pitch produced the highest degrees of ethanol production and the highest rates of attenuation when compared to other treatments.

Oxygen is an essential component of healthy yeast growth. Oxygen introduced in the early stages of fermentation aid in synthesis of vital cell components such as unsaturated fatty acids (UFA) and sterols that increase membrane fluidity and permeability, as well as contributing to an increase in overall yeast biomass (Verbelen et al. 2009; Kucharczyk and Tuszynski 2017). Increased permeability of the cell wall, due to the synthesis of UFA and sterols, increases the rate of carbohydrate metabolism and other metabolic processes (Kucharczyk and Tuszynski 2017). This increased rate of metabolism can result in greater ethanol productivity and rates of attenuation over the course of fermentation (Jones et al. 2007). Oxygen

deficiency has also previously been observed to slow overall yeast production and growth during fermentation (Kirsop 1973). Therefore, it is clear that optimization of wort oxygenation leads to increases in yeast growth, viability, and rates of metabolism. The effects of oxygen on yeast growth and viability can explain the results observed in the current study, where samples provided with post-pitch oxygenation showed the most significant rates of wort attenuation when compared to the control.

Previous studies have estimated that the oxygen requirement for yeast growth is between 5 and 10 ppm DO (Salmon 2006), however oxygen requirements are highly strain dependent. In a previous study by Jakobsen and Thorne (1980) investigating the oxygen requirements of bottom fermenting yeast strains (*S. uvarum*), more than half of strains investigated had oxygen requirements exceeding 20 ppm while other strains showed no benefits between wort attenuation for when oxygenated above 5 ppm. As shown in previous research by Kirsop (1974), the variation of oxygen requirement in yeast strains reflect differences in sterol metabolism, as oxygen is primarily utilized in for the synthesis of essential sterols and unsaturated fatty acids.

High gravity fermentations under the conditions of this experiment utilizing an 8 ppm pre-pitch oxygenation showed the most significant ($p \leq 0.05$) decrease in wort specific gravity (1.024) , greatest rates of attenuation, and highest amount of ethanol produced when compared to all other oxygenation levels. This observation is consistent with recommendations and estimations that the ideal DO for brewing yeasts exists between 5 and 10 ppm dissolved oxygen. However, these results are strain dependent and vary between yeast strains. Additionally, increases in wort oxygenation can have detrimental effects on flavor stability due to the production of reactive oxygen species (ROS) that can form aldehydes and other compounds responsible for off-flavors (Bamforth and Parsons 1985). For this reason, increasing oxygen concentration past 8 ppm, where the greatest reduction in wort gravity are found, can be detrimental to the long-term flavor stability of bottled beer.

In addition to fermentation performance, oxygenation also has an effect on the formation of volatile compounds. Previous research has shown that oxygen has a significant effect on the production of esters, aldehydes, and higher alcohols during fermentation and storage (Anderson and Kirsop 1974; Depraetere et al. 2008; Verbelen et al. 2009; Wietstock et al. 2016). These volatile compounds have a strong effect on overall beer flavor, and thus the consumer acceptability of the final beer. While it is

important to monitor factors affecting fermentation performance to ensure a healthy and complete fermentation, it is equally important to monitor the development of flavor compounds to ensure a consistent and quality product is produced.

In order to assess the impact of wort oxygenation on beer flavor production, volatile compounds were analyzed via gas chromatography. Gas chromatography (GC) has often been applied to the analysis of beer flavor due to its sensitivity and ease of analysis, utilizing small volumes of sample to sensitively detect small concentrations of volatile flavor compounds (Roberts et al. 2000; McNair and Miller 2009). Researchers commonly use the technique to determine the volatile compound profile of different beer styles under different processing conditions. Pinho et al. (2006) previously investigated the use of headspace solid-phase microextraction (SPME) and GC to detect alcohols, ethers, aldehydes, acids, esters, and other aromatic compounds in beer. Li et al. (2012) previously analyzed volatile flavor compounds present in wheat beer using headspace gas chromatography to quantify amounts of aldehydes like acetaldehyde, alcohols such as N-propanol and isobutyl alcohol, and esters such as isoamyl acetate and ethyl hexanoate. Filipowska et al. (2020) also optimized a method of headspace SPME and GC-MS to extract and quantify aldehydes present in pale malts.

Volatile compound concentrations in the various dissolved oxygen treatments were analyzed by gas chromatography using a modified ASBC Method Beer-48, "Headspace Gas Chromatography – Flame Ionization Detection Analysis of Beer Volatiles" (ASBC 2012). Before analysis, the GC analysis method was optimized for adequate separation and sensitivity for the compounds to be analyzed. In order to optimize chromatographic separation of flavor volatiles, a mixed standard solution of selected flavor volatiles was analyzed and retention times for each compound recorded. Once the observed separation was determined to be suitable for quantitative analysis, calibration curves for each compound were constructed using five calibration points, utilizing n-butanol as an internal standard. Each curve was linear over the concentration range analyzed (Table 11).

Eight volatile flavor compounds – two esters (ethyl acetate, isoamyl acetate), two aldehydes (acetaldehyde, benzaldehyde), and four higher alcohols (isoamyl alcohol, isobutyl alcohol, phenethyl alcohol, propanol) – were chosen for this analysis. These volatile compounds were chosen as they are representative of the major classifications of beer's volatile flavor compounds (esters, higher alcohols, and

aldehydes) and most noticeably affect the flavor of beer (Verstrepen et al. 2003; Lei et al. 2012). Descriptions of the selected compounds' flavor notes are shown in Table 12.

Table 11 Concentration Ranges and Linearity of Calibration of Volatile Compounds

Compound	Concentration Range (mg/L)		Linearity (R^2)
	Minimum	Maximum	
Ethyl acetate	0.92	36.08	0.9594
Iso-amyl acetate	0.18	7.01	0.9506
Acetaldehyde	0.79	31.52	0.9900
Benzaldehyde	0.07	29.12	0.9968
Iso-amyl alcohol	3.24	129.60	0.9984
Iso-butyl alcohol	0.80	32.08	0.9979
Phenethyl alcohol	6.12	244.80	0.9979
Propanol	0.96	38.54	0.9995

Table 12 Flavor Notes Contributed by Volatile Compounds

Compound	Flavor notes	Typical Concentrations (mg/L)
Ethyl acetate	Sweet, fruity	12.1 - 26.7[1]
Iso-amyl acetate	Banana, fruity	1.1 – 3.1[1]
Acetaldehyde	Tart, green apple	2.85 – 9.0[1]
Benzaldehyde	Bitter almond, cherry	< 0.1[2]
Iso-amyl alcohol	Banana, alcoholic	39.5 – 70[1]
Iso-butyl alcohol	Alcoholic	10.2 – 14[1]
Phenethyl alcohol	Rose, alcoholic	5 – 102[3]
Propanol	Alcoholic	6.8 – 18[1]

Typical concentrations adapted from [1]Branyik et al. 2012, [2]Meilgaard 1993, and [3]Pires et al. 2014

The concentration of the ester compounds ethyl acetate and isoamyl acetate was measured after post-pitch oxygenation trials were completed and results are shown in Table 13. Ethyl acetate and iso-amyl acetate both typically represent positive flavor notes, depending on the style of beer being produced. The concentration of iso-amyl acetate ranged between 2.17 and 3.33 ppm, with the highest concentration observed in the 8 ppm treatment. The lowest concentration of iso-amyl acetate was observed in the 14 ppm treatment. The iso-amyl concentration in the 8 ppm treatment was observed to be significantly different ($p \leq 0.05$) from all other treatments. No significant differences were observed between 0, 10, 12, and 14 ppm treatments.

The concentration of ethyl acetate ranged between 17.23 and 81.11 ppm, with the highest concentration in the 8 ppm treatment. The lowest concentration of ethyl acetate was observed in the 14 ppm treatment. Significantly lower ($p \leq 0.05$) concentrations of ethyl acetate were observed in the 12 and 14 ppm oxygen treatments compared to the 0 – 10 ppm oxygen concentration treatments. The general trend observed was an increase in ester concentration from the 0 to the 8 ppm treatment, followed by a decrease in ester concentration for the higher dissolved oxygen treatments.

These results are consistent with previous studies. Anderson and Kirsop (1974) observed the effects of oxygenation on high gravity fermentation, where oxygenation with 76 ppm oxygen resulted in a significant reduction in the production of both ethyl and iso-amyl acetate when compared to oxygenation at only 38 ppm oxygen (Anderson and Kirsop 1974). Plata et al. (2005) analyzed the influence of glucose and oxygen on ethyl and isoamyl acetate esters during alcoholic fermentation, where anaerobic and semi-anaerobic conditions produced greater concentrations of acetate esters than semi-aerobic fermentations (Plata et al. 2005). Verbelen et al. (2009) previously observed a significant decrease in ester production during high-cell density fermentations when high levels of oxygen were added to fermenting wort, citing a repression of the acetyl transferase gene ATF1 as responsible for these observed decreases in ester level (Verbelen et al. 2009).

Acetate esters are synthesized during fermentation, combining the acetic acid group of Acetyl-CoA and a higher alcohol utilizing a variety of enzymes known as alcohol acetyltransferase (AATases) (Pires et al. 2014). Therefore, the biosynthesis of volatile ester compounds in beer is significantly affected by the availability of higher alcohols and free acetyl-CoA. Wort oxygenation stimulates yeast growth and

the utilization of Acetyl-CoA in the citric acid cycle (which provides energy to the yeast cell through oxidation of Acetyl-CoA), reducing the amount of free Acetyl-CoA available for ester synthesis (Verstrepen et al. 2003). It is very likely that the addition of high levels of oxygen lead to a decrease in the free Acetyl-CoA concentration in the high gravity worts, thereby causing the reduced acetate ester formation observed in our study with the higher dissolved oxygen concentrations. As seen in our results, the addition DO at concentrations between 0 and 8 ppm increases the production of esters, but higher concentrations of DO lead to decreased ester production.

Table 13 Final Concentration of Ester Compounds After Fermentation

	Average Final Compound Concentration (ppm)	
Treatment	Ethyl acetate	Iso-amyl acetate
0 ppm	$52.10^{a} \pm 5.78$	$2.26^{a} \pm 0.14$
8 ppm	$81.11^{a} \pm 14.76$	$3.33^{b} \pm 0.11$
10 ppm	$74.67^{a} \pm 4.75$	$2.23^{a} \pm 0.20$
12 ppm	$20.83^{b} \pm 0.74$	$2.44^{a} \pm 0.10$
14 ppm	$17.23^{b} \pm 0.41$	$2.17^{a} \pm 0.01$

Statistical significance between treatments is notated by letter pairs. Mean concentrations in the same column with different letters are significantly different ($p \leq 0.05$).

The concentration of the aldehydes acetaldehyde and benzaldehyde analyzed in the post-pitch oxygenation trials are shown in Table 14. Acetaldehyde flavor can be desirable in low concentrations. The concentration of acetaldehyde ranged between 2.58 and 5.99 ppm, with the highest concentration observed in control treatments. The lowest concentration of acetaldehyde was observed in 14 ppm treatments. Significant differences ($p \leq 0.05$) were found between the control and higher oxygen treatments, excluding the 10 ppm treatment. The 10 ppm treatment was not significantly different ($p \leq 0.05$) from either group.

Table 14 Final Concentration of Aldehyde Compounds After Fermentation

Treatment	Average Final Compound Concentration (ppm)	
	Acetaldehyde	Benzaldehyde
0 ppm	$5.99^{a} \pm 0.94$	$79.39^{c} \pm 6.90$
8 ppm	$3.81^{b} \pm 0.02$	$117.73^{b} \pm 4.62$
10 ppm	$4.17^{a\,b} \pm 0.04$	$155.14^{a} \pm 3.23$
12 ppm	$3.22^{b} \pm 0.35$	$83.73^{c} \pm 9.04$
14 ppm	$2.58^{b} \pm 0.54$	$54.72^{d} \pm 0.76$

Statistical significance between treatments is notated by letter pairs. Mean concentrations in the same column with different letters are significantly different ($p \leq 0.05$).

Benzaldehyde flavor can be desirable in certain fruit forward beer styles, such as kriek (cherry) lambic and other cherry-flavored beers, at a low concentration (Gonzalez et al. 2019). The concentration of benzaldehyde ranged between 54.72 and 155.14 ppm, with the highest concentration observed in the 10 ppm treatment and the lowest concentration observed in the 14 ppm treatment. Significant differences ($p \leq 0.05$) in benzaldehyde concentration were observed between the 8, 10, and 14 ppm treatments. While no significant difference ($p \leq 0.05$) was reported between 0 and 12 ppm treatments.

Acetaldehyde concentration decreased between 0 and 8 ppm treatments, with a small increase at 10 ppm. Acetaldehyde concentration again decreased between 10 and 14 ppm treatments. Benzaldehyde concentrations increased between 0 and 10 ppm treatments but decreased between 10 and 14 ppm treatments. The lowest concentration of both aldehydes was observed in 14 ppm treatments. In general, aldehyde concentrations increased from the control to the 10 ppm treatment and decreased from 10 to 14 ppm treatments.

For both aldehyde compounds analyzed in this study, a significant difference in concentration was found between dissolved oxygen treatments. Acetaldehyde concentrations decreased from the control onwards, whereas benzaldehyde concentrations increased from the control to 10 ppm treatments and decreased from 10 ppm onwards. These results are in agreement with previous studies which demonstrated that increased oxygen concentrations decreased the production of aldehyde. A study by Wietstock et al. (2016) showed that production and storage of beer under an oxygen atmosphere increases the degree of

oxidative degradation of amino acids into Strecker aldehydes, such as benzaldehyde and acetaldehyde, when compared to production and storage under a nitrogen atmosphere. Additional research by Depraetere et al. (2008) has also observed that unaerated wort fermented by yeast pre-exposed to oxygen at levels of 8 ppm had no significant effect on flavor stability indicators, including many aldehydes, both during fermentation and 1-year of beer storage at 20°C.

Several pathways for the synthesis of different aldehydes in beer exist. Aldehydes found in beer can be produced through fatty acid oxidation, enzymatic oxidation, photo-oxidation, maillard reactions, and strecker degradation (Baert et al. 2012). For example, aldehydes like trans-2-nonenal, which produce a "papery" or "cardboard" flavor, result from the oxidation of unsaturated fatty acids (Svoboda et al. 2011). Furfural and 5-hydroxymethylfurfural (HMF) on the other hand, are produced through the Maillard reaction (Rico-Yuste et al. 2016), and aldehydes like acetaldehyde and benzaldehyde can be produced through the Strecker degradation (Baert et al. 2012). The presence of oxygen and reactive oxygen species can affect a variety of these aldehyde synthesis pathways, changing the concentrations of aldehydes produced during fermentation and storage (Wietstock et al. 2016).

Our results show that for both aldehyde compounds analyzed, a significant difference in concentration was found between dissolved oxygen treatments, with increasing oxygen treatments decreasing aldehyde formation. This decrease in aldehyde formation can likely be explained by a decrease in Strecker degradation. During Strecker degradation, amino acids react with dicarbonyls formed during the Maillard reaction to form Strecker aldehydes (Ferreira and Guido 2018). Acetaldehyde and benzaldehyde are both aldehydes that can be formed through the Strecker degradation of FAN (Depraetere et al. 2008; Baert et al. 2012). Greater degrees of oxygenation stimulate yeast growth and increase overall yeast biomass. Increased yeast growth leads to greater utilization of FAN by the yeast, leaving less FAN in the wort for the production aldehydes from Strecker degradation, although further investigation is required to confirm the exact mechanism (Lei et al. 2012). The findings of this study are consistent with previous research showing increasing dosages of DO from 7 to 12 ppm decreased production of acetaldehyde (Kucharczyk and Tuszynski 2016).

The concentration of higher alcohol compounds (isoamyl alcohol, isobutyl alcohol, phenethyl alcohol, and propanol) measured after post-pitch oxygenation trials are shown in Table 15. Isoamyl alcohol

flavor can be undesirable in high concentrations. The final concentrations of iso-amyl alcohol were in the range of 23.14 and 32.75 ppm, with the highest concentration observed in 8 ppm dissolved oxygen treatment while the lowest concentration was observed in the control treatment. Concentration of iso-amyl alcohol in the control was significantly lower ($p \leq 0.05$) from the 8 and 10 ppm treatments. The 12 and 14 ppm treatments were not significantly different ($p \leq 0.05$) from any other treatments.

Table 15 Final Concentration of Higher Alcohol Compounds After Fermentation

Treatment	Average Final Compound Concentration (ppm)			
	Iso-amyl alcohol	Iso-butyl alcohol	Phenethyl alcohol	Propanol
0 ppm	$23.14^{b} \pm 0.88$	$7.66^{a} \pm 0.53$	$6.87^{a} \pm 0.43$	$4.66^{b} \pm 0.31$
8 ppm	$32.75^{a} \pm 1.06$	$9.84^{a} \pm 0.73$	$8.75^{a} \pm 0.24$	$6.67^{b} \pm 0.10$
10 ppm	$31.35^{a} \pm 1.85$	$9.54^{a} \pm 0.29$	$8.99^{a} \pm 0.45$	$9.53^{a} \pm 1.04$
12 ppm	$28.46^{a\,b} \pm 0.55$	$8.77^{a} \pm 0.47$	$7.74^{a} \pm 0.53$	$6.25^{b} \pm 0.42$
14 ppm	$25.89^{a\,b} \pm 3.08$	$6.03^{a} \pm 2.19$	$7.48^{a} \pm 0.89$	$1.79^{c} \pm 0.18$

Statistical significance between treatments is notated by letter pairs. Mean concentrations in the same column with different letters are significantly different ($p \leq 0.05$).

Both isobutyl and phenethyl alcohol flavor is undesirable in high concentrations. The final concentrations of iso-butyl alcohol ranged between 6.03 and 9.84 ppm, with the highest concentration found in the 8 ppm treatment while the lowest concentration was observed in the 14 ppm treatment. The final concentrations of phenethyl alcohol ranged between 6.87 and 8.99 ppm, with the highest concentration observed in the 10 ppm treatment and the lowest concentration found in the control. No significant difference ($p \leq 0.05$) was found between treatments for both isobutyl and phenethyl alcohols.

Propanol flavor is undesirable in high concentrations. The final concentrations of propanol ranged between 1.79 and 9.53 ppm, with the highest concentration found in the 10 ppm treatment and the lowest concentration found in the 14 ppm treatment. Significant differences ($p \leq 0.05$) in propanol concentration between several treatments were observed. No significant difference ($p \leq 0.05$) was found between 0, 8,

and 12 ppm treatments. Treatments of 10 and 14 ppm were significantly different ($p \leq 0.05$) from all other treatments.

Concentrations of iso-butyl and phenethyl alcohol were not significantly affected by oxygen treatments. For both iso-amyl alcohol and propanol, concentrations increased between 0 and 10 ppm treatments and decreased between 10 and 14 ppm treatments. In general, higher alcohol concentrations increased from the control to the 10 ppm treatment and decreased from 10 to 14 ppm treatments.

Out of the higher alcohols analyzed in this study, only iso-amyl alcohol and propanol showed significant differences in concentration between oxygenation treatments. For both iso-amyl alcohol and propanol, a peak in concentration was observed in the 10 ppm oxygen treatment with lower concentrations observed at higher dissolved oxygen treatments. Both iso-butyl alcohol and phenethyl alcohol showed no significant differences between dissolved oxygen treatments, but follow the same general trend with higher alcohol concentration increasing slightly from 0 to 10 ppm treatments and decreasing at concentrations greater than 10 ppm.

Higher alcohols are synthesized during fermentation by means of the catabolic Ehrlich pathway or by anabolic amino acid metabolism (Ferreira and Guido 2018). First reported by Felix Ehrlich at the beginning of the 20th century, the Ehrlich pathway enzymatically transaminates amino acids to α-keto amino acids, which are then decarboxylated into fusel aldehydes, and are finally reduced to higher alcohols (Hazelwood et al. 2008; He et al. 2014; Pires et al. 2014). This process requires free amino acids and increasing concentrations of FAN have been observed to increase the concentration of higher alcohols produced (Lei et al. 2012; Hill and Stewart 2019).

Our results show that, in general, higher alcohol concentration increased from 0 to 10 ppm DO treatments and decreased at DO treatments greater than 10 ppm. As seen previously with aldehyde production in this study, greater degrees of oxygenation stimulate yeast growth and increase overall yeast biomass. Increased yeast growth leads to greater utilization of FAN by the yeast, leaving less FAN in the wort for use in the Ehrlich pathway and the production of higher alcohols.

In order to assess the effect of dissolved oxygen during high gravity brewing on the degree of staling and beer flavor stability during storage, a thiobarbituric acid (TBA) index analysis was utilized. TBA index analysis has been previously applied to determine the degree of beer oxidation and sensory

quality during storage due to its ease of sample preparation and quick reaction time (Grigsby and Palamand 1976; Bamforth and Parsons 1985). Saison et al. (2009) previously utilized TBA index analysis to determine the effects of yeast strain on the production of staling precursors. Corzo and Bracho (2004) previously used TBA index analysis to create a predictive model for the sensory quality of canned beer during storage conditions.

TBA index analysis is a method used to assess the concentration of lipid oxidation products in both beer and worts. When heated under acidic conditions, TBA reacts with products of oxidation to form a pink complex that is quantified by measuring absorbance at wavelengths of 448 nm (Botsoglou et al. 1994). Absorbance at 448 nm is used to calculate a unitless TBA index value, with higher index values indicating a greater degree of staling.

The TBA index value for each of the dissolved oxygen treatments was determined for refrigerated and accelerated aging beer samples, results are shown in Table 16. The TBA index values for the refrigerated samples ranged between 44.900 and 49.067, with the largest value observed in the 12 ppm DO treated sample. Significant differences ($p \leq 0.05$) in TBA index between the 0 and 12 ppm treatments were observed, with the 12 ppm treatment having a significantly higher TBA index. For refrigerated samples, TBA index increased between 0 and 12 ppm treatments, but decreased between the 12 and 14 ppm treatments.

TBA index values for samples subjected to accelerated aging at 30°C ranged between 47.600 and 58.600, with the largest value observed in the 14 ppm DO treated sample. The 14 ppm DO treatment was significantly different ($p \leq 0.05$) from the other DO treatments, with the 14 ppm treatment having a significantly higher TBA index. For samples that underwent accelerated aging at 30°C, TBA index increased between 0 and 8 ppm treatments and decreased between 8 and 12 ppm treatments. An increase in TBA index was observed between the 12 and 14 ppm treatments.

Table 16 TBA Index of Beer Samples After Storage at Refrigeration and Elevated Temperatures

Treatment	Control - No Aging	Accelerated Aging at 30°C
0 ppm	$44.900^{b} \pm 0.900$	$47.600^{b} \pm 2.227$
8 ppm	$46.333^{a\ b} \pm 1.069$	$50.167^{b} \pm 1.815$
10 ppm	$46.967^{a\ b} \pm 0.551$	$49.567^{b} \pm 3.782$
12 ppm	$49.067^{a} \pm 1.922$	$47.633^{b} \pm 0.603$
14 ppm	$46.400^{a\ b} \pm 1.473$	$58.600^{a} \pm 3.195$

Statistical significance between dissolved oxygen treatments is notated by letter pairs. Mean TBA values in the same column with different letters are significantly different ($p \leq 0.05$).

Under the conditions of this experiment, both refrigerated samples and samples that underwent aging at 30°C were observed to have significant differences in TBA index value between oxygen treatments. For refrigerated samples, the control was observed to have a significantly lower TBA index than the 12 ppm treatment. For samples that underwent aging at 30°C, the 14 ppm treatment showed a significantly higher TBA index than all other treatments.

This observed increase in TBA value for higher oxygenation treatments is likely due to the greater production of reactive oxygen species (ROS) (Bamforth and Parsons 1985). ROS are formed from molecular oxygen via a one-electron reduction through many methods, including mineral-induced and photochemical formation, creating singlet oxygen, superoxides, peroxides, and hydroxyl radicals (Schoonen et al. 2005; Zhang et al. 2014). Greater degrees of oxygenation leave more dissolved molecular oxygen available for the production of ROS. ROS in beer can react with all matter of organic molecules present, changing the sensory profile of a beer (Vanderhaegen et al. 2006). In this case, increased concentrations of ROS can result in lipid oxidation and the creation of staling aldehydes.

Samples that were aged at 30°C were generally observed to have higher TBA index values. These findings are consistent with previous research indicating that storage at higher temperatures resulted in greater TBA index values when compared to storage at lower temperatures (Kaneda et al. 1991; He et al. 2013; Li et al. 2014). Kaneda et al. (1991) found that storage of beer at 60°C resulted in significantly increased TBA values compared to beer stored at 37°C. Li et al. (2014) observed that the rate of beer aging

increased with increased storage temperature. He et al. (2013) showed that TBA values in beers stored at 20°C were highest when compared to beers stored at 5, 10, and 15°C.

The results observed in this study lead to practical implications for beer brewing. Using the correct oxygen concentration during fermentation, without addition of excess oxygen, will reduce oxidative staling during storage. Additionally, staling during storage is accelerated at higher temperatures, which confirms that refrigerated storage is one of the best ways to slow the beer staling process.

Some limitations to the generalizability of these results exist. While TBA analysis is appropriate for measuring the quantity of lipid peroxides in beer, other staling and undesirable flavor compounds are developed during storage, particularly aldehydes. Staling aldehydes, like furfural, 5-hydroxymethyl-furfural, and trans-2-nonenal, can increase in concentration during beer storage, often correlating with an increase in "papery" flavor of a beer (McDougall et al. 1963; Barker et al. 1983). Additionally, while chemical analysis like TBA or gas chromatography provide information about how beer flavor changes over time, sensory tests are better suited for determining how the desirability and acceptability of beer flavor changes. Future research should focus on adding sensory analysis on desirability and acceptability in addition to chemical analysis, in order to correlate perceived flavor and staling reactions.

Chapter 4

FUTURE RESEARCH

Overall, the results of this analysis of volatile beer flavor compounds have some limitations. Although applicable at smaller scales, the bench-top scale conditions of this experiment may not perfectly align with pilot or commercial scale fermentations with industry-standard equipment and materials. Future research should focus on increasing the scale of fermentations performed, with special attention payed to commercial scale brewing to obtain results directly applicable to real brewing industry scenarios. Additionally, subsequent studies should broaden the scope of analysis to additional volatile flavor compounds, including hop oil compounds, to gain a greater understanding of the effect of oxygenation on the overall flavor of beer. Additional studies including sensory analysis would also aid in correlating changes in flavor compound concentrations and consumer acceptability, as quantitative instrumental data alone is not equivalent to qualitative sensory data.

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APPENDICIES

APPENDIX A. RAW DATA FOR PRE-PITCH OYXGENATION TRIAL

	Control Specific Gravity			8 ppm Specific Gravity			14 ppm Specific Gravity		
Day	A	B	C	A	B	C	A	B	C
0	1.085	1.085	1.085	1.085	1.085	1.085	1.085	1.085	1.085
1	1.067	1.073	1.065	1.073	1.071	1.059	1.065	1.067	1.066
2	1.074	1.073	1.070	1.067	1.070	1.070	1.065	1.063	1.074
3	1.065	1.063	1.067	1.063	1.053	1.064	1.060	1.067	1.057
4	1.068	1.069	1.069	1.065	1.065	1.061	1.059	1.068	1.067
5	1.065	1.064	1.065	1.060	1.060	1.059	1.055	1.063	1.063
6	1.064	1.071	1.065	1.061	1.061	1.067	1.054	1.064	1.065
7	1.063	1.069	1.062	1.059	1.054	1.064	1.047	1.060	1.065

APPENDIX B. RAW DATA FOR POST-PITCH OXYGENATION TRIAL

	Control Specific Gravity			8 ppm Specific Gravity			10 ppm Specific Gravity		
Day	A	B	C	A	B	C	A	B	C
0	1.0727	1.0701	1.0683	1.0574	1.0585	1.0554	1.0603	1.0600	1.0616
1	1.0704	1.0687	1.0706	1.0508	1.0509	1.0478	1.0566	1.0568	1.0580
2	1.0637	1.0641	1.0634	1.0413	1.0428	1.0391	1.0510	1.0503	1.0526
3	1.0559	1.0579	1.0577	1.0323	1.0355	1.0334	1.0432	1.0431	1.0450
4	1.0502	1.0528	1.0526	1.0297	1.0306	1.0247	1.0358	1.0381	1.0407
5	1.0463	1.0494	1.0480	1.0269	1.0276	1.0247	1.0318	1.0340	1.0370
6	1.0431	1.0431	1.0375	1.0231	1.0242	1.0235	1.0317	1.0308	1.0326
7	1.0385	1.0420	1.0397	1.0226	1.0221	1.0211	1.0287	1.0275	1.0297

	12 ppm Specific Gravity			14 ppm Specific Gravity		
Day	A	B	C	A	B	C
0	1.0599	1.0568	1.0586	1.0685	1.0657	1.0655
1	1.0549	1.0523	1.0525	1.0643	1.0634	1.0637
2	1.0478	1.0468	1.0468	1.0449	1.0559	1.0557
3	1.0407	1.0377	1.0392	1.0496	1.0498	1.0498
4	1.0359	1.0335	1.0333	1.0441	1.0444	1.0442
5	1.0320	1.0298	1.0301	1.0403	1.0405	1.0404
6	1.0291	1.0271	1.0270	1.0354	1.0364	1.0364
7	1.0258	1.0242	1.0250	1.0328	1.0330	1.0404

APPENDIX C. RAW DATA FOR TBA ANALYSIS

Control

Sample	Blank	A	B	C	Mean TBI
0 ppm	0.073	0.513	0.522	0.531	44.900
8 ppm	0.074	0.549	0.535	0.528	46.333
10 ppm	0.094	0.558	0.569	0.564	46.967
12 ppm	0.068	0.538	0.576	0.562	49.067
14 ppm	0.064	0.519	0.545	0.520	46.400

Accelerated Aging at 30°C

Sample	Blank	A	B	C	Mean TBI
0 ppm	0.086	0.586	0.558	0.542	47.600
8 ppm	0.081	0.562	0.590	0.596	50.167
10 ppm	0.083	0.535	0.601	0.600	49.567
12 ppm	0.087	0.557	0.569	0.564	47.633
14 ppm	0.077	0.699	0.638	0.652	58.600