ABSTRACT

SVENDSEN, CLAIRE INGRID. Investigation of *Lachancea thermotolerans* as a Novel, Single Culture Brewing Yeast. (Under the direction of Dr. John Sheppard).

Isolated and propagated at North Carolina State University, a novel strain of yeast, *Lachancea thermotolerans* NCSU*,* has been investigated for its application as a single-strain brewing yeast. Beer is primarily brewed with yeast of the genus *Saccharomyces*. Generally, other yeasts are considered spoilage organisms in beer; however, a few have been noted for their usefulness in brewing. These other accepted brewing yeasts are typically co-fermented with a *Saccharomyces* yeast, due to their inability to produce sufficient ethanol or their tending to produce off-flavors (e.g. *Brettanomyces*). *L. thermotolerans* NCSU has proven to be a viable brewing yeast in laboratory and pilot-scale fermentations, as it fermented the principal wort sugars (i.e. maltose and maltotriose) while producing CO_2, ethanol, glycerol, and lactic acid. In a pilot-plant scale fermentation of Lambic-style wort (malted barley and wheat, original gravity (OG) 1.057), *L. thermotolerans* NCSU was able to produce 6.8% alcohol by volume (ABV) and 7.3 g/L of lactic acid, reducing the pH to 3.60 (final gravity (FG) 1.005). Furthermore, *L. thermotolerans* NCSU improved fermentation ability in comparison to type strain NRRL Y-8284 (ATCC® 56472™) in an all-barley malt (OG 1.053); differences included FG (1.016 vs. 1.041), increased maltose utilization (Δ 61.4 g/L vs. Δ 3.5 g/L), ethanol production (4.15% vs. 1.06% ABV) and pH reduction (3.65 vs. 4.87). This demonstrated *L. thermotolerans* NCSU as a unique strain, and more fit as a brewing yeast compared to NRRL Y-8284. The capability to produce beer with *L. thermotolerans* NCSU will provide brewers with an alternative to *Saccharomyces* for creating innovative beer styles and flavors using a single-strain of yeast. With its ability to produce lactic acid, novel sour beers can be created without bringing bacteria or other ‘contaminant’ yeasts into the brewhouse.

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Investigation of *Lachancea thermotolerans* as a Novel, Single Culture Brewing Yeast.

by
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DEDICATION

I would like to dedicate my work to my parents, Hugh Blake and Sarah Sowers Svendsen. Thank you for your unwavering encouragement, support, and for inspiring my lifelong love for the NC State Wolfpack.

BIOGRAPHY

Claire Ingrid Svendsen was born in San Jose, California and now resides in Raleigh, North Carolina. Ms. Svendsen first joined North Carolina State University (NCSU) and the Department of Food, Bioprocessing and Nutrition Sciences as an undergraduate student. She graduated in December of 2012, with a B.S. in Food Science and a minor in Agricultural Business Management. After graduation, Ms. Svendsen interned at NCSU in the Biomanufacturing Training and Education Center (BTEC) analytical laboratory under the direction of Dr. Nathaniel Hentz. Ms. Svendsen began her Masters degree in the Fall of 2013, majoring in Food Science and minoring in Biomanufacturing, under the guidance of Dr. John Sheppard. During that time, she served as a teaching assistant, research assistant, and also worked in the NC State brewery. She was active in the Food Science Club and served on the executive board from 2014-2015. At the Institute of Food Technologist (IFT) national conference in 2015, Ms. Svendsen, as part of a three person development team, was awarded first place in the Dairy Research Institute® Product Development Competition for creating a ‘Shake and Go-Kefir’, a fruit-on-the-bottom fermented dairy beverage.

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TABLE OF CONTENTS

LIST OF TABLES vii
LIST OF FIGURES viii

CHAPTER 1: LITERATURE REVIEW 1
1. Introduction 2
2. Beer Ingredients 3
3. Brewing Process 5
3.1. Milling & Mashing 6
3.2. Wort Boiling 7
3.3. Fermentation and Maturation 8
4. *Saccharomyces* Yeast 8
5. Non-*Saccharomyces* Microorganisms Found in Beer 11
6. Non-*Saccharomyces* Microorganisms Found in Wine 12
7. *Lachancea thermotolerans* 13
8. Beer Flavor 15
8.1. Alcohols 17
8.2. Esters 17
8.3. Carbonyls 18
8.4. Sulfur Compounds & Fatty Acids 19
9. Wine Flavor 19
10. Analytical Techniques for Beer Flavor 21
11. Sample Preparation Techniques 22
12. Chromatographic Analysis 25
REFERENCES 27

CHAPTER 2: INVESTIGATION OF *LACHANCEA THERMOTOLERANS* AS A NOVEL, SINGLE CULTURE BREWING YEAST 34
1. Introduction 35
2. Materials and Methods 37
2.1. Beer Sample Production 37
2.1.1. Yeast Management 37
2.1.2. Wort Production 37
2.1.3. Propagation and Fermentation 38
2.2. Beer Sample Analysis 39
2.2.1. Ultra Performance Liquid Chromatography (UPLC®): Amino Acids 39
2.2.2. High Performance Liquid Chromatography (HPLC): Sugars and Alcohols 40
2.2.3. Cellomter: Cell Counts 40
2.2.4. Density Meter: Gravity Readings 41
2.2.5. pH: Acid Production 41
2.2.6. Gas Chromatography-Mass Spectrometry (GC-MS): Aromatic Flavor 41

3. Results 43
3.1 Preliminary Fermentation Data of *L. thermotolerans* NCSU (Phase 1) 43
3.2 Fermentation Performance of *L. thermotolerans* NCSU Compared to the Type Strain in Pilsner Wort (Phase 2) 44
3.3. Fermentation Performance of *L. thermotolerans* NCSU in Regular Pilsner Wort Compared to High-Gravity Pilsner Wort (Phase 3) 54
3.4. Fermentation Performance of *L. thermotolerans* NCSU in Lambic Wort at the Pilot Plant Scale (Phase 4) 63
4. Discussion 68
4.1. Preliminary Fermentations of *L. thermotolerans* NCSU (Phase 1) 68
4.2. Comparison of *L. thermotolerans* NCSU to the Type Strain (Phase 2) 69
4.3. *L. thermotolerans* NCSU in High Gravity and Regular Wort (Phase 3) 71
4.4. Pilot Scale Fermentation of *L. thermotolerans* NCSU (Phase 4) 73
4.5. Suggested Future Work..75
5. Conclusion 75
REFERENCES 77

APPENDICES 79
Appendix A (Phase 2 Data) 80
Appendix B (Phase 3 Data)...84
Appendix C (Phase 4 Data)...88

LIST OF TABLES

Table 1. Composition of sugars utilized by brewing yeasts during fermentation..................10

Table 2. Amino acids grouped by rate of assimilation (Adapted from He, 2014).................11

Table 3. Comparing pure culture fermentations of *L. thermotolerans* and *S. cerevisiae* (Adapted from Kapsopoulou, 2005) 14

Table 4. Flavor thresholds, concentration range and aroma impression of important flavor compounds in lager beer (Adapted from Pires, 2015) 16

Table 5. Outline of parameters of experimental phases conducted.......................................36

Table 6. Preliminary data of *L. thermotolerans* NCSU fermentations at 18 °C in wort (n=2) 43

Table 7. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotoerans* NCSU and NRRL Y-8284 (n=3) 51

Table 8. Aromatic analysis of *L. thermotolerans* NCSU compared to the type strain at the beginning of maturation (Week 0) (n=3 with one standard deviation reported) 52

Table 9. Quantitative volatile comparison of *L. thermtotolerans* NCSU (n=3) to NRRL Y-8284 (n=1)..53

Table 10. Aromatic analysis of *L. thermotolerans* NCSU start of maturation (Week 0) and the end of maturation (Week 3) (n=3 with one standard deviation reported).........................54

Table 11. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotolerans* NCSU in regular and HG Pilsner wort (n=3) 61

Table 12. Aromatic analysis of *L. thermotolerans* NCSU at the start of maturation in Pils and HG wort (n=3 with one standard deviation reported 62

Table 13. Aromatic analysis of *L. thermotolerans* NCSU at Week 3 of maturation in Pils and HG wort (n=3 with one standard deviation reported 63

Table 14. Sugar utilization of *L. thermotolerans* NCSU in Lambic wort (n=1).....................64

Table 15. Production of acid, glycerol and ethanol by *L. thermotolerans* NCSU in Lambic wort (n=1) 64

Table 16. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotolerans* NCSU in Lambic wort (n=1) 66

LIST OF FIGURES

Figure 1. Overview of the beer production process (Adapted from Hutkins, 2006) 6

Figure 2. Basic schematic of a gas chromatography (GC) system 22

Figure 3. Schematic of HS-SPME extraction procedure (Adapted from Qian, 2010)...........24

Figure 4. Change in specific gravity of wort by *L. thermotolerans* strains NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$) 44

Figure 5. Comparison of *L. thermotolerans* strains NCSU (A) and NRRL Y-8284 (B) maltose utilization and ethanol production at 18 °C (n=3 with ±1 standard deviation) 46

Figure 6. Comparison of *L. thermotolerans* strains NCSU (A) and NRRL Y-8284 (B) sugar utilization and glycerol production at 18 °C (n=3 with ±1 standard deviation) 48

Figure 7. pH of *L. thermotolerans* NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$)..49

Figure 8. Comparison of cell concentration of *L. thermotolerans* NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$)................................50

Figure 9. Specific gravity of *L. thermotolerans* NCSU fermentations in high gravity (HG) and Pilsner (Pils) wort at 22 °C (n=3 with ±1 standard deviation, $p < .05$)............................55

Figure 10. Comparison of *L. thermotolerans* NCSU average maltose utilization and ethanol production at 22 °C in Pilsner (A) and HG (B) wort (n=3 with ±1 standard deviation) 56

Figure 11. Comparison of *L. thermotolerans* NCSU sugar utilization and glycerol production in Pilsner (A) and HG (B) Pilsner wort at 22 °C (n=3 with ±1 standard deviation)................58

Figure 12. Change in pH by *L. thermotolerans* NCSU in HG and Pils fermentations at 22 °C (n=3 with ±1 standard deviation, $p < .05$)..59

Figure 13. Cell concentration of regular Pils and HG worts during fermentation at 22 °C (n=3 with ±1 standard deviation, $p < .05$)..60

Figure 14. Change in pH during fermentation of *L. thermotolerans* NCSU in Lambic-style wort (n=1) 65

Figure 15. Chromatogram of aromatic analysis of Lambic-style fermentation at pilot-scale; peaks are labeled with a peak number and correspond to the table showing the identified compound, and peak ratio average (n=1)...67

CHAPTER 1: LITERATURE REVIEW

1. Introduction

While originally all beer was inoculated spontaneously, most modern brewing fermentations utilize a single-strain starter culture of yeast (Barnett, 2001; Steensels, 2014). Alcoholic fermentations for beer and wine commonly use *Saccharomyces cerevisiae* (or a close relative) as the starter culture (Steensels, 2015). Generally, other yeasts are considered spoilage organisms in beer; however, a few have been noted for their usefulness in brewing. These other accepted brewing yeasts typically must be co-fermented with a *Saccharomyces* yeast, due to the inability to produce sufficient ethanol and the production of off-flavors (e.g. *Brettanomyces*). However, there is growing interest in beer fermentations with non-*Saccharomyces* yeasts due to the unique flavor compounds these microorganisms can produce. Isolated from a bumble bee in Dr. Rob Dunn's laboratory at North Carolina State University, a novel strain of yeast, *Lachancea thermotolerans,* has been investigated for its application as a single-strain brewing yeast.

Grape must contains naturally existing organisms from the environment, which means wine can never be fermented with a pure culture, the way beer can. These naturally occurring microbiota introduce yeasts to wine fermentations that are not typically encountered in the brewhouse. Though no scientific literature has been reported on *L. thermotolerans* in beer, *L. thermotolerans* has been reported in the wine industry and noted for its alcohol resistance, low production of volatile acidity, high production of fixed acidity in the form of L(+)lactic acid and the absence of off-flavor production (Ribéreau-Gayon, 1976).

Primary experimental objectives were to determine whether *L. thermotolerans* NCSU was metabolically different than the type strain, *L. thermotolerans* NRRL Y-8284 (ATCC® 56472™), and capable of producing positive metabolites (e.g., ethanol, CO_2, higher alcohols, esters, etc.) from brewer's wort. Fermentations with *L. thermotolerans* were conducted on the laboratory and pilot plant scales; liquid and gas chromatographic methods were employed to analyze the yeast's metabolic profile (sugar and amino acid assimilation, ethanol and glycerol production, aromatic flavor production).

2. Beer Ingredients

There are just four necessary ingredients needed to make beer: water, malted barley, hops, and yeast. Beer has one of the oldest food regulatory laws; in 1516 the "German Purity Law" or the *Reinheitsgebot* limited the ingredients in beer to water, barley, and hops (Pires, 2015). The fourth ingredient, yeasts, would not be identified as the microorganisms required for alcoholic fermentation until Louis Pasteur studied beer in France in the 19th century. The modern beer purity law *Vorläufiges Biergesetz* introduced in 1993 has been updated to include yeasts; the law also includes changes like the addition of adjuncts, such as corn and rice (for sugar sources), as allowable in top-fermenting brews (Pires, 2015). Within the four traditional ingredients, wild, novel brewing yeasts are being isolated and used for developing new styles of beer.

Not only is water the principal constituent of beer, but it is also required for daily operations in the brew house (e.g. cleaning, rinsing, etc.). Often breweries will choose their manufacturing locations with special consideration on the area's natural quality and abundance of water. Water must always be potable and free of pathogens, and may need to be adjusted for the brewing process (Pires, 2015).

Common adjustments to water include removing microbial contamination, removal of suspended solids, and reduction of any unwanted minerals (Pires, 2015). Mineral ions play a large role in influencing the brewing process, and the most pertinent mineral for brewers to monitor is calcium; by lowering the pH for optimum enzymatic activity, calcium ions help guard α-amylase from early inactivation (Pires, 2015). Furthermore, calcium helps with the precipitation of excess nitrogen compounds during boiling, prevents hop components from being over-extracted, and is a mandatory component for yeast flocculation (Stratford, 1989). There are other minerals that influence brewing; in high concentrations manganese and iron may change a beers taste and color (Pires, 2015). The ability for yeast growth and fermentation can be hindered by nitrites, but increased by zinc ions (Narziss, 1992; Wunderlich, 2009).

Malted barley is the traditional starch source of beer; prepared by germination and kilning (or drying), these grains provide key enzymes and nutrients for the brewing process (Hutkins, 2006). Today, many breweries use different starch sources either to reduce cost or alter the color, flavor or aroma of the beer. Common adjuncts used include unmalted barley, wheat, rice, corn, or sugars/sugar syrups (Pires, 2015). Though these adjuncts bring additional nutrients, they contain no natural enzymes and thus may only be used in light malts, like Pilsen malt, which contain enough enzymes to break down more than twice their weight. US commercial breweries are allowed to use unmalted cereals up to 34% (w/w) of the total malt. Though adjuncts can be present in high amounts, malted barley remains the most commonly used (Pires, 2015).

Barley is a member of the grass family, with seeds (known as grains or kernels) that grow on the ears of the plant. The barley can grow one or many grains per node of the ear, and two species of barley are frequently used in brewing: two-row barley, with one grain per node, and six-row barley, with three grains per node. Two-row barley has fewer grains per node, so the grains will be richer in starch and larger. Due to smaller grains, six-row barley contains less starch, but yields a higher protein content (Pires, 2015).

Unmalted barley is simply the dormant seeds of the plant, however, these seeds must be malted, or germinated controllably to produce malt. Germination begins as the embryo grows by using reserve nutrients stored in the kernel. For malting, germination is accomplished by steeping barley for two to three days at 10 to 20 °C (Hutkins, 2006). When grains reach the optimum conditions, they will will release enzymes (amylitic, proteolytic, etc.) to break down the remaining nutrients in the grain and form a new plant. Once adequate degradation of the endosperm occurs, germination is halted by a process called kilning. Kilning involves drying the grains with cool air so that enzymes remain undamaged. This produces pale-malted barley, also known as Pilsen malt (Pires, 2015). From this point, malt can be used or can be further kilned at higher temperature to roast the malt and produce styles like caramel malt. Additional kilning can produce richer, darker colors and flavors through the Maillard reaction. However, exposing the grains to higher temperatures can

reduce the enzymatic activity. Thus, Pilsen malt will have the highest enzymatic activity while chocolate malt has no enzymatic activity (Pires, 2015).

Hops are female flowers (cones) from the plant *Humulus lupulus*. In beer, hops are used to affect the flavor of the beer and are commonly categorized as either "aroma hops" and "bittering hops" (Hutkins, 2006; Pires, 2015). The bitter flavor of hops is due to alpha-acids, while aromatics are attributed to the essential oils within the cone; thus, bittering hops contain more alpha-acids and while aroma hops are higher in essential oils (Hutkins, 2006; Pires, 2015). Modern brewers rarely uses hop cones and instead rely on pellets and hop extracts for their beer. Extracts performed with ethanol or carbon dioxide and result in a highly concentrated, sticky, resin-like substance. Although hop extracts and pellets have different chemical compositions than hop cones, they are valued for their ease of storage and long shelf life (Pires, 2015).

Since ancient times yeasts have been involved in brewing; DNA from *Saccharomyces cerevisiae* was discovered in ancient Chinese pots, dating all the way back to 7,000-5,500 BC (McGovern, 2004). However, for the majority of history people were oblivious to the fact that microorganisms were responsible for these fermentations. Antonie van Leeuwenhoek was the first person to see yeasts through a microscope in 1680, but it was Louis Pasteur who discovered these living cells were transforming wort to beer and he published his results in "Etudes sur la biere", or "Studies about beer" in 1876 (Hutkins, 2006; Pires, 2015). Brewing yeasts are eukaryotic, unicellular, heterotropic, facultative anerobic microorganisms and the primary genera of brewing yeast is *Saccharomyces* (Pires, 2015). Yeasts for alcoholic fermentation are discussed more thoroughly in the next section.

3. The Brewing Process

Although the ingredient list for beer is short, beer production requires many steps and processes which can be difficult to control, as shown in Figure 1. First, malting and mashing are enzymatic activities key in breaking down the barely and extracting sugars to make wort. During fermentation and maturation, yeast use those sugars from the wort to produce by-

products like ethanol, CO_2 and flavor compounds. At this point the beer is ready to drink, but further processing and packaging steps may be included.

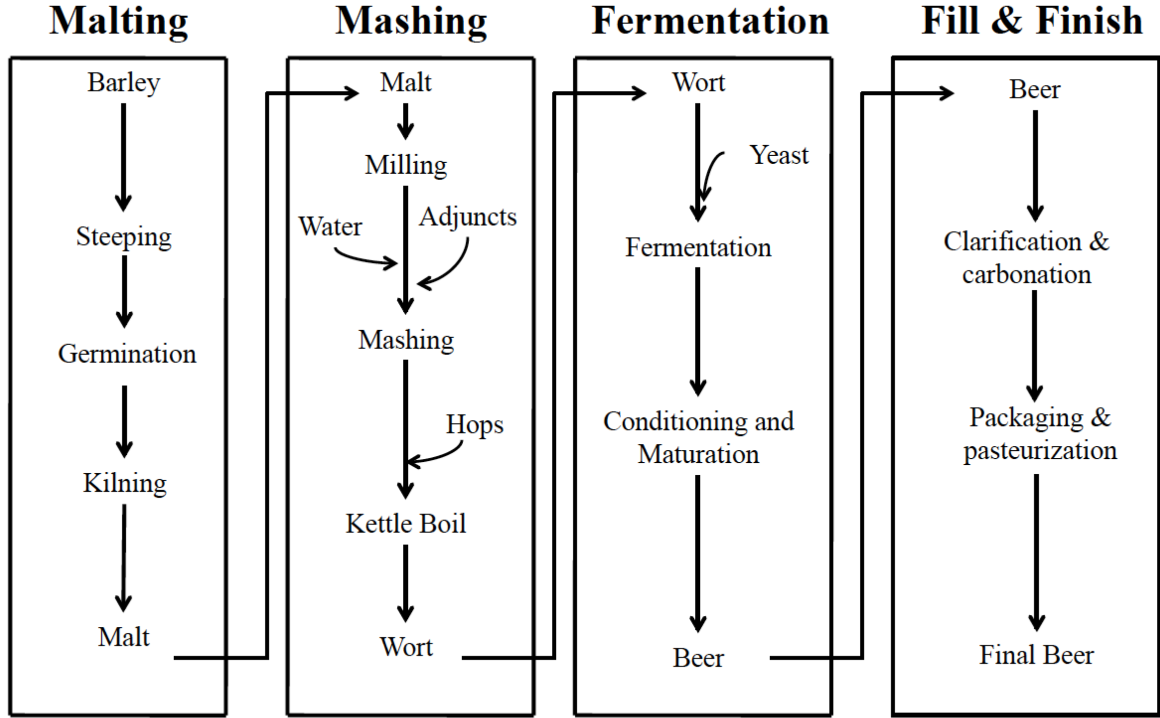

Figure 1. Overview of the beer production process (Adapted from Hutkins, 2006).

3.1. Milling & Mashing

Malt (and other grains, if used) must be milled, or ground up, prior to mashing to increase the surface area of grain to water (Pires, 2015). Milling is usually done by a roller or hammer mill, and how fine the malt is milled depends on the filtration method (Pires, 2015). Traditional breweries use a lauter tun with a false bottom for wort filtration, which demands that the grain's husks are not too damaged as they act as a filter to separate clear wort. Conversely, brewers may choose to use mash filters which do not require course grits (Pires, 2015). During mashing, smaller milled grain particles make it easier to extract fermentable material, but slows down rate of separation of the wort from the grain bed (Pires, 2015).

Mashing involves mixing milled barley with water ("mashing-in") at a set temperature to create a slurry. The temperature of the slurry may be increased in order to

activate enzymes to break down sugars and proteins. Mashing may have a defined pathway of heating that is followed (infusion mashing), or done by removing and boiling parts of the slurry and mixing them back in (decoction mashing) (Pires, 2015).

Enzyme activity increases with temperature, however, so does the rate of enzyme degradation (Pires, 2015). Enzyme activity is affected by the pH and wort composition, in addition to temperature (Rajesh et al., 2013). Barley malts have four starch-degrading enzymes: α-amylase, β-amylase, α-glucosidase, and limit dextrinase. These enzymes, particularly α-amylase and β-amylase, are responsible for breaking down starch to fermentable sugars and non-fermentable dextrins; the degradation of α-amylase is optimum between a temperature of 72-75 °C and pH of 5.6-5.8, while β-amylase is optimum at 60-65 °C and pH 5.4-5.5 (Pires, 2015).

After mashing is complete, the aqueous solution (wort) must be separated from the spent grains by filtration, or lautering. Lautering can be done by vorlaufing and sparging water over grains with a lauter tun and false bottom system (Pires, 2015).

3.2. Wort Boiling

After mashing is complete, the separated wort is boiled for approximately 90 to 120 minutes (Pires, 2015). During this time hops are added for either bittering (earlier addition time) or aroma (later addition time). Additionally, other seasoning (e.g. cinnamon, cloves, orange peel) or sugar adjuncts (e.g. sucrose, sugarcane, malt syrup) may be added in the boil (Pires, 2015). After the boil, the wort is separated from hop and protein solids, aerated, cooled, and ready to transfer to the fermenter.

The boiling step accomplishes seven key purposes: heats and kills most microorganisms for near sterility, inactivates majority of enzymes from mashing, extracts oils and resins from hops while catalyzing isomerization of alpha-acids, “hot break” or precipitation of proteins for enhanced clarity, enhanced color development from catalyzing Maillard browning, undesirable volatiles (like sulfur compounds) are boiled off, and wort concentration by water evaporation (Hutkins, 2006).

3.3. Fermentation and Maturation

After the wort is transferred to the fermentation tank, it is time for yeast to be pitched (i.e. inoculated with a slurry of suspended yeast cells). To avoid contamination, pitching should occur soon after the wort is prepared and closed fermentation tanks should be used. Yeasts are typically pitched at a concentration of 15-20 x 10^6 cells mL^{-1} (Pires, 2015); depending on the activity of the inoculum there may be a lag period of six to eighteen hours before the yeast begin fermentation (Hutkins, 2006). During fermentation, yeasts start assimilating fermentable sugars, amino acids, and minerals while producing metabolites like ethanol, CO_2, higher alcohols, and esters (Pires, 2015).

Attenuation is the amount of fermentable extract (sugars) of wort and is the primary parameter for the progression of fermentation (Pires, 2015). Typically, regular wort will start with 80% fermentable extract and have around 10% remaining when the beer is transferred prior to maturation (for sufficient formation of dissolved CO_2); some breweries, however, allow all extracts to ferment and add in more of the original wort (or sugar adjuncts) (Pires, 2015). Fermentation time is dependent on the wort's attenuation, fermentation temperature, and the yeast physiology (Pires, 2015).

Maturation consists of a secondary fermentation, where residual sugars are utilized and CO_2 is formed. Also during this time, more flavors are produced and off flavors diminish (e.g. aldehydes, sulfur compounds, diacetyl) (Pires, 2015). During maturation the temperature is dropped (-2 to 3 °C for lager beers) and beer is clarified due to the precipitation of cold break particles and yeast sedimentation (Pires, 2015). After beer is finished maturing, it may go through some or all of the following processes: filtration, colloidal stabilization, packaging, and pasteurization (Pires, 2015).

4. Saccharomyces Yeasts

Saccharomyces species are ideal yeasts for alcoholic beverage fermentations due to their ability to produce and survive high levels of ethanol, produce desirable flavor compounds, all without producing health-threatening toxins (Steensels, 2015). Literally

meaning “sugar fungus” (in Greek, Saccharo = sugar and myces = fungus), *Saccharomyces* species are commonly found in sugary environments, i.e. the surface of ripe fruits (Pires, 2015). Although *Saccharomyces* is commonly cultivated in man made environments like wine, bread and beer, it can persist even in harsh winters and travel the world by surviving in the guts of social insects, like wasps (Stefanini, 2012). Within the genus *Saccharomyces*, there are two major species used in brewing: ale yeast (*Saccharomyces cerevisiae*) and lager yeast (*Saccharomyces pastorianus*).

Also known as “ale” or “top fermenting” yeast, *S. cerevisiae* acquired the name from rising to the top of the fermenter in the foam. *S. cerevisiae* works best at 18 to 25 °C (ales are historically made in warmer climates) and has short fermentations producing notably fruity aromas (Hutkins 2006, Pires 2015). Lager yeast, *S. pastorianus* (previously known as *S. carlbergensis* and *S. uvarum*), is “bottom fermenting” as it sinks to the bottom of fermenters. These yeast work best at from 7 to 15 °C and have crisp flavor (Hutkins, 2006; Stewart, 2014). *S. pastorianus* is an aneuploid hybrid of *S. cerevisiae* and *S. eubayanus*, a cryotolerant yeast (Libkind, 2011).

It is important for brewing yeasts be able to utilize the constituents in wort (i.e. sugars and amino acids) to prevent growth of spoilage organisms and to produce adequate amount of ethanol. Brewery wort contains approximately 90% carbohydrates (He, 2014). The primary sugars found in brewer’s wort are maltose and maltotriose, with maltose making up 50-60% (Stewart, 2014) and maltotriose making up 10-14% of the total sugar (Lodolo, 2008). In general, brewing strains are able to utilize the sugars in wort in this approximate sequence: sucrose, fructose, glucose, maltose, and maltotriose (Stewart, 2014). The structure of these sugars is displayed in Table 1. Lager strains are distinguished from ale strains due to their ability to ferment melibiose (Lodolo, 2008).

Sugars may pass across the cell membrane intact (e.g. maltose and maltotriose) or be hydrolyzed outside the cell by enzymes (e.g. sucrose is broken down to glucose and fructose) (Stewart, 2014). Though maltose and maltotriose are most abundant, they will not be utilized until the monosaccharides have been depleted; this is due to ‘the carbon catabolite repression

of the metabolic pathways involved in the uptake and utilization of alternative sugars' (Lagunas, 1993). It is imperative that brewing yeasts be able to ferment these two sugars quickly to help prevent other unwanted organisms from growing.

Table 1. Composition of sugars utilized by brewing yeasts during fermentation.

	Sugar	Chemical formula	Structure	
Monosaccharides	Glucose	$C_6H_{12}O_6$		
	Fructose	$C_6H_{12}O_6$		
	Sugar	Unit 1	Unit 2	Unit 3
Disaccharides & Trisaccharides	Sucrose	glucose	fructose	-
	Maltose	glucose	glucose	-
	Maltotriose	glucose	glucose	glucose

Growing yeast use nitrogen to synthesize proteins and other nitrogenous compounds, with nitrogen uptake slowing as yeast growth halts (Stewart, 2014). Yeast use nitrogen in the form of amino acids (available form the proteolysis of barley), and there are 19 amino acids present in wort. Similar to sugars, there is a general order to which amino acids are assimilated as shown in Table 2. Group A are utilized immediately after pitching, with Group B assimilating slower. Amino acids from Group C are only utilized after Group A is depleted. Group D consists only of Proline, which is utilized poorly or not at all, despite being the most abundant amino acid in wort (Stewart, 2014).

Table 2. Amino acids grouped by rate of assimilation (Adapted from He, 2014).

Group	A	B	C	D
Absorption	Fast	Intermediate	Slow	Littler or none
Amino Acid	Threonine Serine Asparagine Methionine Lysine	Glutamine Leucine Isoleucine Aspartate Histidine Arginine Valine Phenylalanine	Glutamate Tyrosine Glycine Alanine Tryptophan	Proline

5. Non-*Saccharomyces* Microorganisms Found in Beer

Typically beer fermentations aim to use a single-strain, pure culture starter for brewing beer and avoid any contaminant organisms. Beer is a very stable beverage in part to the high concentration of ethanol (0.5-10% w/w), bitter hop compounds (ca. 17-55 ppm of iso-α-acids), carbon dioxide gas (approximately 0.5% w/v), a low pH (3.8-4.7) and decreased oxygen concentration (less than 0.3 ppm) (Suzuki, 2006). Furthermore, available nutrients are rapidly depleted by the yeasts fermentation process (Suzuki, 2011).

Some microorganisms are able to survive in beer's harsh environment, and may be unintentionally grown and contaminate the final beer. Common bacteria that can contaminate beer consist primarily of four genera: *Pectinatus*, *Megasphaera*, *Pediococcus*, and *Lactobacillus* (Back, 1994; Back, 2005). *Pectinatus* and *Megasphaera* are strict anaerobes that have the ability to contaminate packaged beer (Suzuki, 2011). *Pediococcus*, and *Lactobacillus* are lactic acid bacteria (LAB), a group of Gram-positive bacteria containing many genera (Suzuki, 2011). Some common issues with these bacterial contaminations include acidification, off-flavors, haze, and sedimentation.

Though most beers utilize just one yeast, some beer styles, notably Lambic beers from Belgium, utilize wild yeasts and bacteria for complex, uncontrollable spontaneous fermentations. Lambic style beer traditionally comes from the Payottenland region of Belgium and have long fermentations, lasting one to three years. After wort is produced it is

left open, so airborne microorganisms can inoculate the beer before being stored in casks for fermentation. This may serve as a base for making a fruit Lambic, by simply adding fruit, or Gueuze, made from mixing young Lambic (~1 year) with an old Lambic (~2 to 3 years) followed by bottle conditioning (Thompson-Witrick, 2015). In a study monitoring two Lambic fermentations over two years, over 2,000 bacterial and yeast isolates were identified (Spitaels, 2014).

According to Spitaels et al., "sour beers are currently attracting interest outside Belgium, especially in the USA" (Spitaels, 2014). Attempting to re-create sour, Lambic style ales, the American coolship ale (ACA) is a spontaneously fermented beer that mimics the traditional three year Lambic process (Bokulich, 2012). A study of ACA microbial succession showed an initial dominance of *Enterobacteriaceae* in the first month, followed by *Saccharomyces* spp. and *Lactobacillales* for the following year; *Brettanomyces bruxellensis* was the dominant yeast after one year, along with persisting *Lactobacillales* (Bokulich, 2012).

In contrast to spontaneous fermentations, brewers can intentionally add non-*Saccharomyces* yeasts or bacteria to brew unique beer styles. Non-*Saccharomyces*, or non-conventional yeasts, are becoming increasingly popular in the fermentation industry (Ciani, 2011; Cordero-Buseo, 2013; Gonzalez, 2013; Johnson, 2013). For example, *Brettanomyces* has been explored for alcoholic beverage production because of its amylase activity and unique flavor profile (Daenen, 2009); it also has the ability to produce acetic acid in addition to ethanol (Steensels, 2015). However, *Brettanomyces* flavor is very characteristic, and has been described with many terms including mousy, barnyard, medical, "band-aid" metallic, sweaty, goat-like and tropical (Heresztyn, 1986).

6. Non-Saccharomyces Microorganisms Found in Wine

Similar to the beer industry, in the wine industry *Saccharomyces cerevisiae* is the primary yeast genera used for fermentation. Winemakers also utilize 'wild' non-*Saccharomyes* yeasts in their processing (*S. cerevisiae* is still the dominant fermenter, either

inoculated or indigenous) due to the natural microbiota of grape musts (Fleet, 1990). Of the 1500 yeast species identified today, over 40 have been isolated from grape musts (Jolly, 2006; Ciani, 2010). Yeasts can be considered spoilage organisms if they produce off-flavors commonly considered defects in wine (i.e. excessive hydrogen sulfide and other sulphur volatiles, acetic acid, various esters, and volatile phenols) (Sponholz, 1993; Fleet, 1992, 1998; Fugelsang, 1997; Du Toit, 2000). Winemakers have developed strategies to deter 'spoilers' (e.g. *Dekkera bruxellensis*) and allow 'compatbile' wild yeasts to persist (e.g. *Torulaspora delbrueckii, Pichia kluyveri, Candida/Metschnikowia pulcherrima* and *Lachancea thermotolerans*) (Jolly, 2013).

These yeasts are generally thought to be active early on in the fermentation, though they do not all persist due to high levels of ethanol, low pH, or oxygen deficiency (Jolly, 2013). Inoculation of non-*Saccharomyces* yeasts for wine production has shown that all of the following yeasts were poor fermenters and need to be co-fermented with *Saccharomyces*: *Torulaspora, Candida, Hanseniaspora, Zygosaccharomyces, Schizosaccharomyces, Lachancea* (Jolly, 2013).

7. *Lachancea thermotolerans*

Lachancea thermotolerans (previously known as *Kluyveromyces thermotolerans;* Lachance & Kurtzman, 2011) is a yeast found in wine fermentations cited as being commonly found in many wine producing regions (Mora, 1988). This yeast has been investigated for wine fermentations due to its moderate alcohol resistance, low volatile acidity production, high production of fixed (non-volatile) acidity by L(+)lactic acid, and lack of off-flavor production (Ribéreau-Gayon, 1976). It is noted as being a useful yeast in wine for the purposes of bioacidification and aroma enhancement (Moreno-Arribas, 2009).

L. thermotolerans has been reported of producing wine with higher levels of lactic acid, glycerol and 2-phenylethanol in mixed fermentations (Kapsopoulou, 2007; Comitini, 2011; Gobbi, 2013). Wines scored higher in 'spicy' and 'acidity' attributes for co-fermentation of *L. thermotolerans* and *S. cerevisiae* (in Sangiovese must, commerecial-scale

10000 L fermentation) when compared to pure-culture *S. cerevisiae* wine (Gobbi, 2013). In a study where *L. thermotolerans* was innoculated to the natural microbiota of grape must, the fermentation produced 7.5 g/L lactic acid and reduced the pH to 3.10 (Mora, 1990).

The time of innoculation with *S. cerevisiae* is important, and the later that *L. thermotolerans* ferment is innoculated with *S. cerevisiae*, the more lactic acid and glycerol the final wine contains (Kapsopoulou, 2007; Gobbi, 2013).

Previous results (Kapsopoulou, 2005) investigated a pure culture fermentation of *L. thermotolerans* and found it produced 9.6 g/L of L-lactic acid and 7.58% v/v of ethanol in 1 L flask fermentations containing 163 g/L of fermentable sugars (Table 3). It also demonstrated *L. thermotolerans* can be grown in the presence of 3% v/v and 6% v/v ethanol at pH 3.5 at 20 °C. When tested at 9% v/v ethanol, *L. thermotoerans* did not grow but also did not lose any viability for 10 days (Kapsopoulou, 2005). *L. thermotolerans* is known to have a moderately high ethanol tolerance (<13.5 vol.%) when compared to *Saccharomyces* species (Moreno-Arribas, 2009). Additionally, when inoculated at $5x10^5$ cfu/mL, *L. thermotolerans* was found to have a rapid increase in cell concentration reaching $1x10^8$ cfu/mL in four days (Kapsopoulou, 2005).

Table 3. Comparing pure culture wine fermentations of *L. thermotolerans* and *S. cerevisiae* (Adapted from Kapsopoulou, 2005).

Chemical Analysis of Wine	**Grape Must**	***Lachancea thermotolerans***	***Saccharomyces cerevisiae***
Sugars (g/L)	163	-	-
Ethanol (% v/v)	-	7.58	9.6
Residual sugar (g/L)	-	38.8	1.66
Titratable acidity (g tartaric acid/L)	7.3	16.9	7.5
Volatile acidity (g acetic acid/L)	-	0.18	0.41
pH	3.15	2.9	3.06
L-lactic acid (g/L)	0.03	9.6	0.03
L-malic acid (g/L)	1.57	1.15	1.48
Glycerol (g/L)	-	3.33	4.82
Acetaldehyde (g/L)	-	0.57	0.04

8. Beer Flavor

When food is consumed, the experience we call "flavor" is a combination of the organoleptic sensations of gustation, olfaction, and chemesthesis. With gustation, there are five basic tastes (bitter, salty, sour, sweet, and umami) that contribute to flavor. These components are usually non-volatile at room temperatures and are perceived by taste buds on the tongue (Belitz, 2009). Chemesthesis is detected by nerve endings in mucosal membranes and stimulates the trigeminal nerve; it is described as perceiving "the burn of hot peppers and mustards, the tingle and pricking of carbonation and the sharp coolness of peppermint" (Green, 1996).

Although chemesthesis and basic tastes contribute to flavor, the majority of flavor is perceived through olfaction. Humans are only able to distinguish five basic tastes, but can identify thousands of aromas, each made up of potentially hundreds of volatile compounds (Parker, 2015). These compounds responsible for aroma are highly volatile, low molecular weight compounds that are found in foods at low levels (Parker, 2015). Aroma active compounds are detected by olfactory tissue in the nasal cavity and reach the receptors by traveling either through the nose in the orthonasal passage, or in the throat after being released through chewing in the retronasal passage (Belitz, 2009).

While beer receives flavor from many sources, such as malt and hops, by far yeast play the biggest role in creating unique flavors in beer. Malt flavor is especially important in darker beers (e.g. porter and stout), where the Maillard reaction between amino acids and sugars produce not only deeper colored malt, but highly flavored compounds like furaneol and maltol; Maillard produces a nut-like, toasted or maltly flavor (Barth, 2013). Hops contribute flavor compounds like terpenoids, polyphenols and resins which give a characteristic flavor and bitterness to beers; of these, beer researchers have regarded terpenoids (particularly hydrophilic terpene alcohols) as important to flavor (Takoi, 2010).

Carbon dioxide and ethanol are the primary products produced by yeast, but they have a small influence on the final flavor of beer (Stewart, 2014). Beer flavor can mostly be attributed to hundreds of flavor-active compounds produced through the brewing process.

Yeast metabolism plays a role in the formation and excretion of these compounds, and metabolism can be affected by many parameters such as yeast strain, fermenter design, wort pH, buffering capacity, and wort gravity (Stewart, 2014). Many of these compounds are metabolic intermediates and are formed by yeast during fermentation, either through catabolism of wort constituents (i.e., sugars, nitrogenous compounds and sulphur compounds) or synthesis of components required for yeast growth (i.e., amino acids, proteins, nucleic acids, lipids, etc.) (Lodolo, 2008). The following compound groups have been identified in beer: organic and fatty acids, alcohols, esters, carbonyls, sulfur compounds, amines, phenols, and other various compounds (Stewart, 2014). For any aroma active volatile compound to be detected by humans, it must occur above its odor threshold. An odor threshold is the concentration at which an individual perceives the stimulus (Parker, 2015). Common aroma compounds and their threshold values in beer are listed in Table 4.

Table 4. Flavor thresholds, concentration range and aroma impression of important flavor compounds in lager beer (Adapted from Pires, 2015).

Compound	Threshold (mg/L)	Concentration range (mg/L)	Aroma impression
Acetate esters			
Ethyl acetate	25-30	8-32	Fruity, solvent
Isoamyl acetate	1.2-2.0	0.3-3.8	Banana
Phenylethyl acetate	0.2-3.8	0.1-0.73	Roses, honey
MCFA ethyl esters			
Ethyl hexanoate	0.2-0.23	0.05-0.21	Apple, fruity
Ethyl octanoate	0.9-1.0	0.04-0.53	Apple, aniseed
Higher alcohols			
n-Propanol	600	4-17	Alcohol, sweet
Isobutanol	100	4-57	Solvent
Isoamyl alcohol	50-65	25-123	Alcoholic, banana
Amy alcohol	50-70	7-34	Alcoholic, solvent
2-phenylethanol	40	5-102	Roses
Vicinal Diketones			
Diacetyl	0.1-0.15	0.02-0.07	Sweet, buttery
2,3-Pentanedione	0.9-1.0	0.01-0.02	Buttery, toffee-like

8.1. Alcohols

Higher alcohols, or fusel alcohols, in beer are called so because they contain a higher number of carbons compared to ethanol (C_2H_6O). Higher alcohols are the most abundant organoleptic compounds present in alcoholic beverages (Nykänen, 1986; Pires 2014). Although over 40 alcohols have been identified, the most common higher alcohols in beer and spirits include: *n*-propanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol (Stewart, 2014). Higher alcohols are noted for having a strong and pungent smell and taste (Nykänen, 1986).

Higher alcohols can be formed through amino acid catabolism or via pyruvate from carbohydrate metabolism (Stewart, 2014). Brewing yeasts absorb amino acids so they may use their amino groups in their own structures, leaving behind an α-keto acid. The α-keto acid then enters the irreversible Ehrlich pathway to ultimately become a higher alcohol (Pires, 2014).

8.2. Esters

Although esters are present in trace amounts in beer, they provide a large impact on the final flavor due to their low odor threshold in beer (Meilgaard, 1975; Saison, 2009). Esters are vital to the aroma of most fruits, and they make up the majority of volatile compounds in fruits like melons, apples, pineapple, and strawberries (Parker, 2015). At low levels in beer, esters are known to have pleasant fruity and floral aromas, though if overproduced, they can negatively affect the beer by imparting a bitter, overly-fruity taste (Pires, 2014).

Esters are formed during primary fermentation by the enzymatic chemical condensation of alcohols with organic acids (Pires, 2014). There are two primary groups of esters found in beer: acetate esters and medium-chain fatty acid (MCFA) ethyl esters. Acetate esters are synthesized from ethanol (or another higher alcohol) and acetic acid (acetate). Ethyl esters are synthesized when ethanol forms the alcohol radical (-OH) and the acid side is a MCFA (Pires, 2014). Numerically, ethyl esters are the largest group of flavor constituents

in alcoholic beverages (Nykänen, 1986). Dozens of esters have been identified, but there are six key esters in beer: ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), phenyl ethyl acetate (roses and honey aroma), ethyl hexanoate (sweet apple aroma) and ethyl octanoate (sour apple aroma) (Pires 2014).

8.3. Carbonyls

Over 200 carbonyl compounds have been reported in alcoholic beverages, but the most important groups in beer flavor include aldehydes and vicinal diketones (Stewart, 2014). An intermediate in ethanol formation, acetaldehyde is produced by the decarboxylation of pyruvate (Pires, 2014). Acetaldehyde may have an undesirable 'grassy' or 'green apple' flavor if present above its flavor threshold (~10 mg/L) (Stewart, 2014). The amount of acetaldehyde produced, like esters and higher alcohols, is determined by yeast strain and fermentation environment. Parameters like increased wort oxygen concentration, temperature, and pitching rate can favor acetaldehyde buildup (Stewart, 2014).

Diacetyl (2,3-butanedione) and 2,3 pentanedione are both flavor active vicinal diketones found in beer. These compounds are considered an off-flavor due to the 'butterscotch' or 'stale milk' aroma they release (Stewart, 2014). The flavor threshold of Diacetyl (~0.1 ppm) is ten fold lower than 2,3 pentanedione (~1.0 ppm), and thus is a higher concern for brewers (Stewart, 2014; Krogerus, 2013). In lighter beers, it is easier to detect vicinal diketones because they are not covered up by the flavor of malt and hops. Although usually considered an off-flavor, diacetyl is detectable and acceptable in some beer styles such as the Bohemian Pilsner and select English ales (Krogerus, 2013).

Diacetyl and 2,3 pentanedione are the intermediates of amino acid (valine and isoleucine, respectively) formation in yeast, and diacetyl production peaks at the end of the yeasts active growth (Stewart 2014). Through spontaneous oxidative decarboxylation, α-acetohydroxy acids (intermediates in the biosynthesis) excreted in the wort form vicinal diketones. During maturation, these vicinal diketones may be further metabolized by yeast dehydrogenases; diacetyl can be reduced to acetoin, then 2,3-butanediol (and 2,3

pentanedione to it's equivalent diol). These diols have a moderately low flavor threshold, and thus reduction of vicinal diketones is imperative to create palatable beer (Stewart, 2014).

8.4. Sulfur compounds & Fatty acids

Sulfur compounds can be acceptable and desirable at low levels; however, if excessive sulfur compounds are produced they can impart unpleasant off-flavors (Stewart, 2014). Sulfur compounds and off-flavors created include sulfide (rotten egg aroma) and sulfur dioxide (burnt match aroma). These sulfur compounds occur as by-products of yeast synthesis of the two sulfur containing amino acids, cysteine and methionine, from sulfate (Stewart, 2014).

Acetic, propionic, butanoic and lactic acids are short-chain fatty acids that can result as a product of fermentation. These acids are formed in the early stages of fermentation. Some are volatile and affect aroma, including: acetic (vinegar), propionic (goaty), and butanoic (spoiled butter) (Thompson-Witrick, 2015).

9. Wine Flavor

Although beer and wine fermentations are quite different, they both utilize *Saccharomyces* yeasts; this means similar flavors compounds are produced by yeast metabolism in wine as they are in beer. Vinification and wine flavor are complex processes; there are numerous different microorganisms interacting to transform sweet, acidic, low flavored grape must into a flavorful alcoholic beverage (Moreno-Arribas, 2009). The composition of wine is influenced by many parameters including: grape variety, geographical conditions of the grape cultivation, the microbial ecology of the grape and fermentation process, and winemaking practices (Cole, 1995). Grape quality is greatly affected by microorganisms prior to harvest, and through fermentation where they emit ethanol, CO_2, and hundreds of secondary end-products. These naturally occurring yeasts, bacteria and filamentous fungi (fungus) influence wine production, although due to fermentation, yeasts

have the largest impact because they conduct alcoholic fermentation (Fleet, 1993; Fugelsang, 1997).

There are several ways yeasts contribute to wine flavor during fermentation: (i) utilizing grape juice constituents, (ii) producing ethanol and other solvents that help to extract flavor components from grape solids, (iii) producing enzymes that transform neutral grape compounds into flavor active compounds, (iv) producing many hundreds of flavor active, secondary metabolites (e.g., acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulfur compounds), (v) autolytic degradation of dead yeast cells (Cole, 1995; Lambrechts, 2000).

In wine, byproducts of glycolysis are found in the highest concentration: ethanol, glycerol, acetic acid (Styger, 2011). Ethanol concentration can vary between 8 to 16 % v/v in red and dry white wines and impacts the perceived (alcohol) hotness, body and perceived viscosity, as well as sweetness, acidity, aroma and flavor intensity of wines (Moreno-Arribas, 2009; Gawel, 2007). Glycerol is desirable in wine for the complexity and contribution to mouthfeel it brings (Styger, 2011). Acetic acid is the most important volatile fatty acid produced during alcoholic fermentation, both quantitatively and sensorially. Acetic acid plays the most important role in wine quality and accounts for more than 90% of total wine volatile acidity (Eglinton, 1999).

Like in beer, anaerobic fermentation by *Saccharomyces* yeasts produce a variety of volatile metabolites in wine; thus, the same groups of esters, higher alcohols, volatile fatty acids, carbonyls, and volatile sulfur compounds are present. Esters contribute largely to the fruity flavor of wines, and are prominent in young red and white wines. Higher alcohols are quantitatively the largest group of volatile compounds in wine. Imparting a pleasant rose-like aroma, 2-phenylethanol is a higher alcohol that has been noted as a positive contributor to wine flavor (Swiegers, 2005). Acetaldehyde accounts for more than 90% of the aldehyde content in wine (Nykänin, 1986). Acetaldehyde is said to contribute a ‘bruised apple’ and ‘nutty’ characteristic when present above it’s odor threshold of 100 mg/L in wine (Schreier, 1979).

There are other ways wine flavor can be influenced in steps post-fermentation. Malolactic fermentation is a secondary fermentation some wines can undergo after alcoholic fermentation. Malolactic fermentation if the deacidification of wine by converting malic acid to L-lactic acid and carbon dioxide (Styger, 2011). Diacetyl is formed by lactic acid bacteria during malolactic fermentation and by yeast during alcoholic fermentation, but the majority of it is reduced to acetoin and 2,3-butanediol (Bartowsky, 2004). Furthermore, wine undergoes a long aging period either in the bottle or in oak barrels. Interactions can occur between the fermenting grape juice and the wood barrels. These reactions are mainly reductive, like the conversion of carbonyl compounds to their equivalent alcohols (Moreno-Arribas, 2009).

10. Analytical Techniques for Beer Flavor

Chromatography is a separation technique which is based on the partitioning of a sample between two phases; the most common is between a mobile phase and a stationary phase. Stationary phases are commonly solid, while mobile phases may be liquid (liquid chromatography, LC) or gas (gas chromatography, GC). A detector must be coupled with the chromatographic system for qualitative and quantitative analysis; many detectors exist that may be selected based on sensitivity or selectivity (Qian, 2010).

First introduced in the 1950's, GC is a well known analytical technique that is suitable for the analysis of thermally stable volatile materials and is the most commonly used technique for analyzing flavor compounds due to their volatility. Analytical instrumentation for the detection of volatile flavor components has been continually optimized in order to achieve better sensitivity and specificity by utilizing new instrumentation and sample extraction/preparation techniques (Andrés-Iglesias, 2014). Typical constituents of a GC system include a gas supply system, injection port, column, oven, detector and a recorder/integrator (Figure 2). The air-tight injection port is crucial for introduction of sample without introducing air from the surrounding environment. The column and column oven are the key components involved in the separation of analyte.

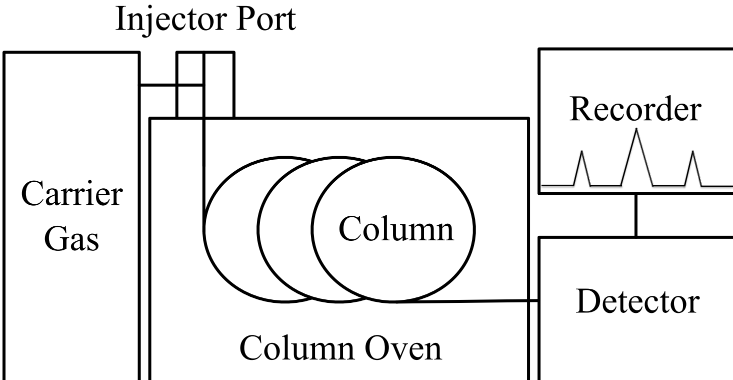

Figure 2. Basic schematic of a gas chromatography (GC) system.

GC samples are introduced at the injection port, vaporized, and carried through the system by a high-purity, inert gas (typically helium or nitrogen). The stationary phase is either a packed or capillary column that is stored in an oven with either a isothermal or gradient temperature (Qian, 2010). Chromatographic separation is affected by both the interaction of the stationary phase and the analyte, as well as the boiling point of the compounds. A higher oven temperature will cause compounds to elute faster, but will result in decreased resolution. Isothermal methods are rare, and most methods are temperature-programmed and start at a lower temperature and follow a gradient (e.g. 10 °C/min) to a higher temperature (Qian, 2010).

11. Sample Preparation Techniques

As there are a large quantity of non-volatile components present in beer that may damage the column, direct injection of beer samples is not suitable for GC analysis (Kobayashi, 2008). With the high temperature of the injection port, non-volatile constituents will be degraded and cause unwanted peaks on the GC (Qian, 2010). Additionally, water can cause back flash 'where the expansion volume of the solvent will exceed the volume of the injection port' (Restek, 2015) if it is injected in large volumes. Therefore, a sample extraction must be utilized to detect aromatics present in low concentrations in beer.

Selection of an extraction method is crucial for the success of analyzing flavor. The method ‘should allow the extraction of all compounds that contribute to the flavor of the food product but not alter the profile of characteristic volatiles, and in particular it should not form artifacts’ (Majcher, 2009). Several sample extraction techniques exist for the detection of aromatic compounds including headspace techniques, solvent extraction, solid-phase extraction, and solid-phase microextraction.

Headspace sampling (HS-GC) is one of the simplest methods and involves injecting only the gas phase of a sample for analysis. HS-GC has been commonly used to analyze numerous aromatic compounds in beer (Šmogrovičová, 1999). HS-GC is a straightforward technique that is advantageous over to direct injection because it does not inject any solid or liquid material, however, the sensitivity is relatively low (Pinho, 2006). Furthermore, injection volumes for direct HS are restricted to 5 mL or less, limiting the amount of sample that can be introduced to the system (Qian, 2010).

Solid-phase microextraction (SPME) is an extraction method that has grown in popularity due to its sensitivity and ease of use compared to other extraction techniques, like solvent assisted flavor evaporation (SAFE). Arthur and Pawliszyn developed SPME and it has been found to be a fast, solvent free extraction suitable for volatile analysis, particularly when combined with headspace sampling (HS-SPME) (Horák, 2009). The size of a syringe needle, SPME has an absorbant or adsorbant phase bound to the outside of a fused silica filament that is either immersed in the sample or used to sample the headspace for a period of extraction time. Figure 3 outlines the steps in SPME sampling: (1) pierce the septum with SPME fiber holder, (2) expose the SPME fiber and extract volatiles (3) retract the fiber into the holder and remove. After the extraction time, the filament is retracted into a protective metal sheath, pushed through the GC septum, and the volatiles are thermally desorbed (Qian, 2010).

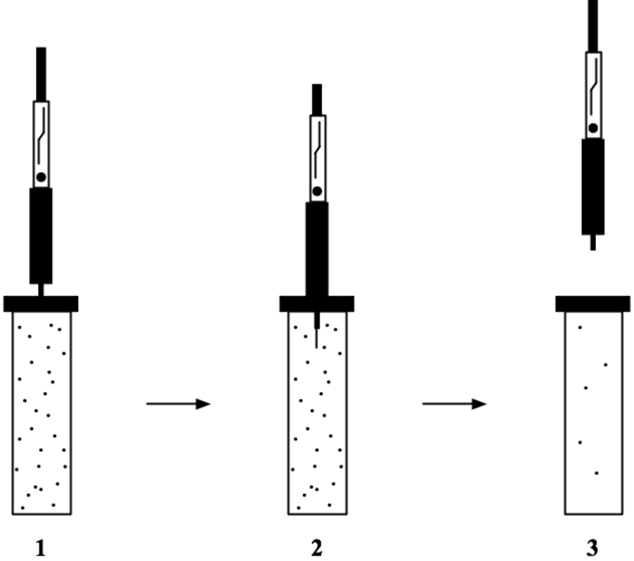

Figure 3. Schematic of SPME extraction procedure; (1) pierce sample septum, (2) expose fiber and extract volatiles, (3) retract fiber and remove (Adapted from Quin, 2010).

There are several different phases and thicknesses of SPME fibers available, which the analyst must consider in order to extract all potential volatiles in the sample. Fiber coatings may be relatively thick (100 μm) to better attract volatiles or thinner (7 to 30 μm) which is better for large molecules. Typical phases include polydimethylsiloxane (PDMS), a nonpolar phase coating used to extract nonpolar compounds, and Carbowax (CW) or polyacrylate which are polar phase coatings used for polar compound extraction. Also, Carboxen (CAR) and divinylbenzene (DVB) are porous fibers which are suitable for the extraction of highly volatile compounds (Qian, 2010). Multiphase fibers may be used to encompass a larger range of polarity volatiles.

Beer volatile analysis has been successfully conducted with single and multiphase fibers such as PDMS, CAR-PDMS (Charry-Parra, 2011) and DVB-CAR-PDMS (Gonçalves, 2014). There are many published examples that compare fibers and other analytical parameters for the optimization of beer flavor analysis. Pinho et al., compared 100 μm PDMS, 85 μm PA, and 75 μm CAR-PDMS for the analysis of beer with HS-GC and concluded CAR-PDMS proved to extract the most volatiles (Pinho, 2006). Saison et al.,

compared 100 μm PDMS, 65 μm PDMS-DVB, 85 μm CAR-PDMS, 70 μm CW-DVB and 50/30 μm DVB-CAR-PDMS and found the optimal extraction with the CAR-PDMS-DVB fiber (Saison, 2008).

The degassing of beer and addition of NaCl are critical to improving the sensitivity of HS-SPME. Degassing beer is critical as carbon dioxide (CO_2) bubbles are in suspension in the beer matrix and will overwhelm the headspace if not removed prior to analysis. Beer is commonly degassed by exposure to an ultrasonic bath. Sometimes referred to as "salting out", NaCl is added to samples to help drive volatiles into the headspace. Saison et al., states 'the solubility of numerous organic compounds will decrease with increasing salt concentration, especially the more hydrophobic ones' (Saison, 2008). Varying levels of salt have been reported for addition to beer for HS-SPME: 3.5 g NaCl/10 mL beer (Saison, 2008), 2 g NaCl/5 g beer (Pinho, 2006), and 2.7 g/10 mL beer (Silva, 2008).

When SPME was compared to continuous liquid-liquid extraction/solvent assisted flavor evaporation (CLLE-SAFE) for the analysis of Gueuze beer flavor analysis, neither technique proved superior with SPME detected 40 volatiles and CLLE-SAFE detecting 36. However, the SPME technique takes one hour compared to the 24 hours it took to continuously extract the beer for CLLE-SAFE (Thompson-Witrick, 2015). Overall, SPME has become a popular sample preparation technique due to the reproducibility, low cost, and high sensitivity. It has particularly shown its suitability for solvent-free analysis of volatiles.

12. Chromatographic Analysis

In chromatography, qualitative data is produced by matching the elution time of a peak to a chemical compounds. Mass spectrometry (MS) detectors utilize 'hard ionization' and are able to fragment the compounds to produce unique mass fingerprints. These fingerprints are then able to be matched in a compound library and tentatively identified. To confirm the identity of a peak, analytical standards should be injected individually, and retention times matched for identification.

Furthermore, quantitative data may be obtained in chromatography by utilizing either an internal or external standard. For external standards, the peak area of a sample may be compared to the peak area of standards injected known concentration injected separately. By comparing an analytes peak area to this external standard curve, the concentration of they analyte in the sample can determined (Ismail, 2010).

Internal standards are used to help reduce the variability that may occur during analysis, either with operator technique, sample preparation, or the analysis apparatus. This technique involves the addition of a structurally similar compound, not naturally occurring in the sample, which elutes at a different time than analytes of interest (Ismail, 2010). Then, the area of detected peaks is compared to that of the internal standard peak. Values may be reported as ratios, or the internal standard can be added to an external calibration curve to quantify the results (Ismail, 2010).

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CHAPTER 2: INVESTIGATION OF LACHANCEA THERMOTOLERANS AS A NOVEL, SINGLE CULTURE BREWING YEAST

1. Introduction

New yeasts are being investigated for their application in brewing. In particular, a novel strain of *Lachancea thermotolerans* has been isolated and has demonstrated qualities that are desirable in a single-strain brewing yeast; this yeast will be referred to as *L. thermotolerans* NCSU (Sheppard and Dunn, NCSU, unpublished).

The yeast *L. thermotolerans* (previously known as *Kluyveromyces thermotolerans*; Lachance & Kurtzman, 2011) has been cited in beverage fermentations, being noted as a non-*Saccharomyces* yeast found in wine producing regions (Mora, 1988). As a wine yeast, *L. thermotolerans* has received mixed evaluations. It is noted as being a useful yeast in wine for the purposes of bioacidification and aroma enhancement (Moreno-Arribas, 2009). The ability of *L. thermotolerans* to bring pleasant acidity to wine has been credited to its production of L(+) lactic acid (Ribéreau-Gayon, 1976). However, *L. thermotolerans* has also been cited as a ‘poor fermenter’ that needs to be co-fermented with a *Saccharomyces* yeast to produce wine (Jolly, 2013).

Despite appearing in wine literature, *L. thermotolerans* has yet to be reported for brewing beer. A desirable yeast for beer fermentation has considerably different requirements than a yeast for wine fermentation. First, beer wort and grape must comprise different substrates which are available to the yeasts to assimilate and metabolize. The main sugars in grape must are glucose and fructose, both monosaccharides. Other sugars available include mannose, galactose, arabinose, ribose, xylose, and rhamnose (Moreno-Arribas, 2009). For beer wort, the main sugars available are maltose and maltotriose, di- and tri-saccharides of glucose (Stewart, 2014; Lodolo, 2008). Thus, selecting a brewing yeast with the ability to metabolize the select sugars available in wort is critical. Furthermore, the concentration of alcohol that yeast is expected to produce and tolerate is different for the beer and wine industries. Wines typically range from 8 to 14% alcohol by volume (ABV) while beers contain from 4 to 8% ABV, approximately.

Saccharomyces species are ideal for brewing due to their ability to rapidly metabolize wort sugars, produce and withstand high levels of ethanol, and producing pleasant flavor

profiles. Most commonly, a *Saccharomyces cerevisiae* (or a close relative) is used as the starter culture for alcoholic fermentations (Steensels, 2015). Thus, utilizing unique yeasts, like *L. thermotolerans*, for fermentations may allow for the creation of new, sour beer styles that have never been tasted before. Traditional sour beers (e.g. Lambic beers) are time consuming and can take from one to three years to produce. Furthermore, Lambic beers are spontaneously fermented and can have over 2,000 bacterial and yeast isolates making fermentations difficult to control (Spitaels, 2014).

There were two primary experimental objectives of this study. The first objective was to demonstrate that *L. thermotolerans* NCSU was metabolically different than the type strain, *L. thermotolerans* NRRL Y-8284 (ATCC® #56472). The second objective was to demonstrate that *L. thermotolerans* NCSU can be used as brewing strain. These objectives were obtained through four Phases, outlined in Table 5. In Phase 1, preliminary data was collected to observe how quickly *L. thermotolerans* NCSU could ferment wort at 18 °C. Next, to establish that *L. thermotolerans* NCSU was metabolically distinctive compared to the type strain, the two yeasts were compared in laboratory-scale fermentations at 18 °C in Pilsner wort (Phase 2). Following, in Phase 3 the experiment compared the ability of *L. thermotolerans* NCSU to ferment in Pilsner wort and high gravity (HG) Pilsner wort in laboratory-scale fermentations at 22 °C. Finally, a 2.5 BBL pilot-scale fermentation of Lambic wort with *L. thermotolerans* NCSU was conducted in the NC State Brewery; this fermentation was monitored to collect additional data and demonstrate the ability of *L. thermotolerans* NCSU to produce beer in a stainless-steel fermenter (Phase 4).

Table 5. Outline of parameters of experimental phases conducted.

Phase #	Temperature (°C)	# of Replications	Fermentation Vessel	Comparison
1	18	Duplicate	Flask	n/a
2	18	Triplicate	Flask	Yeast Strains
3	22	Triplicate	Flask	Wort Gravity
4	18	Single	Stainless Steel Fermenter	n/a

2. Materials and Methods

2.1 Beer Sample Production

2.1.1. Yeast Management

In this study two strains of *Lachancea thermotolerans* were utilized. The first was a novel strain obtained from a bumble bee and isolated at North Carolina State University, denoted as *L. thermotolerans* NCSU. The second, *L. thermotolerans* NRRL Y-8284, was obtained from the American Type Culture Collection (ATCC® #56472). Master glycerol stocks (250 µL of 50% glycerol in water solution with 1000 µL of yeast slurry) of both yeast were prepared and stored at -80 °C. Yeast potato dextrose agar (YPDA) containing 1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar (Sigma Aldrich, MO, U.S.A.) media was used to grow yeast cultures and was stored at 4 °C until use.

2.1.2. Wort Production

Wort was produced at the pilot scale, using a 2.5 BBL (1 BBL = 117 L) mash tun and boil kettle (Diversified Metal Engineering Ltd., P.E.I., Canada). The barley malt was milled using a roller mill (Appolo Machine & Products Ltd., Saskatoon, Canada). For fermentations, two worts were created: a Pilsner wort for laboratory scale experiments and a Lambic wort for pilot scale experiments. The Pilsner wort was collected in a 10-L carboy and stored at -4 °C until needed. Wort was used as media for propagation of yeasts and used in subsequent fermentations. To produce high gravity wort, Pilsner wort was supplemented with maltose prior to autoclaving. All wort was autoclaved prior to analysis to maintain an aseptic environment.

To create the Pilsner wort, milled 2-row superior Pilsen malt and 2-row premium pale malt (Canada Malting, Calgary, Canada) were added to 1.6 hL of filtered water at 52 °C containing 50 g of $CaSO_4$. The grain and water mixture was manually stirred and held at 50 °C for 30 min. The temperature was raised to 65 °C over the next 30 min while stirring continuously, held at 65 °C for 15 min, raised to 72 °C while stirring, covered and held at 72 °C for 10 minutes, and then mashed out at 76 °C. The wort was then vorlaufed to remove any

solids and drained to the boil kettle. Sparge water (1 hL at 80 °C) was sprayed into the mash tun and allowed to completely run off. The original gravity (OG) of the wort was adjusted to 1.046 g/cm^3 and the wort was boiled for 90 min.

To create the Lambic wort, milled 2-row pale malt, caramel malt, and wheat malt (Canada Malting, Calgary, Canada) were added to 1.6 hL of fitered water at 54 °C with 120 g of $CaSO_4$. After a 20 minute protein rest, the temperature was raised to 72 °C over the next 45 minutes, then held at 72 °C for 10 minutes. The temperature was then raised to 76 °C and maintained for 10 minutes, and then mashed out at 76 °C. The wort was vorloufed and 1.5 hL of 80 °C sparge water was added. The boil was 90 minutes and the OG was adjusted to 1.057 g/cm^3.

2.1.3. Propagation and Fermentation

To propagate yeast cultures, t-streaks were performed on YPDA using the master glycerol stocks and stored at 30 °C for 48 hours. To create a yeast slurry, two 25-mL flasks (Pyrex, NY, U.S.A.) containing 10 mL of autoclaved wort were inoculated with 1 CFU each and agitated in a shake incubator (New Brunswick Scientific-I26, NJ, U.S.A.) at 22 °C for 48 hours. The two 10-mL portions of yeast slurry were then added to a single portion of 300 mL autoclaved wort in a 1-L baffled flask (Pyrex, NY, U.S.A.) and agitated at 22 °C for 48 hours. Yeast slurry was then pitched to autoclaved wort to create a total of 500 mL volume in a 2-L flask (Pyrex, NY, U.S.A.) with yeast at starting concentration of 2×10^7 cfu/mL. The samples were capped and then fermented at ale temperature (18 or 22 °C). During fermentation samples were taken daily in order to monitor pH, gravity, cell counts, sugar utilization and alcohol production. After the samples reached a gravity reading of approximately 1.015 g/cm^3, they were matured for 3 weeks at 14 °C and sampled weekly for flavor compound analysis. Samples were collected aseptically using a 15-mL serological pipette and stored in 15-mL Falcon tubes (Corning Inc., NY, U.S.A.). Samples were stored at -18 °C.

2.2. Beer Sample Analysis

2.2.1 Ultra Performance Liquid Chromatography (UPLC®): Amino Acids

To characterize the profile of amino acids available to yeasts during fermentation, a reverse phase UPLC® method was employed. The Acquity UPLC including the sample manager, column manager, and photodiode array (PDA) detector was coupled with Empower software for instrument control and data analysis (Waters Corporation, MA, U.S.A.). Separation occurred through an ACCQ-TAG Ultra C18 1.7 µm 2.1x100 mm column with ACCQ-TAG Eluent A and B as mobile phases (Waters Corporation, MA, U.S.A.). The flow rate was 0.7 mL/min with UV absorbance detection at 260 nm.

Samples were degassed in 15-mL conical tubes in the Digital Ultrasonic Cleaner (Fisher Scientific, GA, U.S.A) for 10 min and centrifuged in the Sorvall Legend RT+ (Thermo Scientific™, MA, U.S.A.) for 5 min at 1000xg. Samples were derivatized using the AccQ Tag™ Ultra Reagents (Waters Corporation, MA, U.S.A.). Samples were first diluted 1:3 with deionized H_2O and vortexed in 0.5-mL Eppendorf tubes (Fisher Scientific, GA, U.S.A). In 12x32 mm glass total recovery screw vials with LectraBond™ caps (Waters Corporation, MA, U.S.A.), 70 µL of borate buffer (pH 8.0), 10 µL of diluted sample and 20 µL of derivatization reagent were added and thoroughly mixed with a pipette tip after each addition. The samples were capped, vortexed, and heated at 55 °C in the Multi-Blok Barnstead Lab-Line Heater (Thermo Scientific™, MA, U.S.A.) for 10 min. The vials were then cooled to ambient temperature and lightly agitated by hand to remove any air bubbles prior to analysis.

Standards were prepared the day of analysis and derivatized with the same method as samples. L-Amino acid analytical standards (Sigma Aldrich, MO, U.S.A.) were removed from -20 °C freezer storage and combined with borate buffer to create a 50 pmol/µL standard. Borate buffer was used as the diluent to create six standards, ranging from 0.78 to 50 pmol/µL.

2.2.2 High Performance Liquid Chromatography (HPLC): Sugars and Alcohols

HPLC was employed using a anion exchange method to analyze maltose, fructose, glucose, glycerol, ethanol and lactic acid. A Prominence UFLC system (Shimadzu, Kyoto, Japan) was used and included the DGU-20A$_3$ degasser, LC-20AD pumps, SIL-20AC HT autosampler, CBM-20A communications bus model, CTO-20A column oven, RID-10A refractive index (RI) detector and Labsolutions Software for control and analysis.

The autosampler was kept at 4 °C with an injection volume of 10 µL. The column was a 300 x 7.8 mm Rezex RHM-Monosaccharide H+ (Phenomenex, CA, U.S.A.) stored in the column oven at 60 °C. The mobile phase was 100% DI Milli-Q (Millipore, MA, U.S.A) water flowing at 0.6 mL/min, degassed and filtered. The RI detector was kept at 40 °C with positive polarity.

Samples were degassed and centrifuged, as described in Section 2.2.2. Supernatant was collected and 150 µL was pipetted into 1.5-mL clear glass screw thread vials with PTFE/Silicon caps for analysis (Thermo Scientific™, MA, U.S.A.).

USP grade maltose, glucose, fructose, glycerol (Sigma Aldrich, MO, U.S.A.), and denatured anhydrous ethanol (Fisher Scientific, GA, U.S.A) were used to prepare standards. Standards were created by making 30 g/L (w/v) stocks in 10-mL graduated cylinders (Fisher Scientific, GA, U.S.A) and homogenized by stirring. Stocks were then filtered with a 10-mL Luer-Lok™ syringe (BD, NJ, U.S.A.) and 0.45-µm syringe filter (Millipore, MA, U.S.A) to 15-mL Falcon tubes (Corning Inc., NY, U.S.A.) and stored at 4 °C until needed for further use.

2.2.3 Cellometer: Cell Counts

Cell counts were obtained each day using the Cellometer® Vision (Nexcelom Bioscience, MA, U.S.A.) and included methylene blue, which stains dead eukaryotes blue while "live" cells remain colorless, allowing yeast viability to be determined. Yeasts were diluted 1:10 by mixing 10 µL of yeast sampled, 10 µL 0.1% methylene blue, and 80 µL of DI water. After thorough mixing, 15 µL of diluted sample was pipetted on to the cellometer slide

and inserted to the system to perform the cell count; readings were done in triplicate and averaged for each sample.

2.2.4. Density Meter: Gravity Readings

Throughout fermentation the gravity of the wort was recorded using a density meter, DMA 4500M (Anton-Paar, Graz, A.U.T.). Sample was collected in a 5-mL Injekt™ syringe (B.Braun, Melsungen, D.E.U.), degassed by manual agitation, and inserted into the density meter until the U-tube was visibly filled and no air bubbles remained. Once the temperature reaches 20 °C, the U-tube shakes and the resulting oscillation of the liquid is determined. The gravity (g/cm^3) and sugar concentration (g/L) were recorded and used to determine when sugar utilization had ended and fermentation was complete.

2.2.5. pH Meter: Acid Production

The pH of the beer samples was monitored during fermentation using a SevenGo Duo pH Meter and InLab® 413 SG electrode (Mettler Toledo, Greifensee, C.H.E.). The pH meter was calibrated with buffer solutions of pH 4.0, 7.0, and 10.0 (Fisher Scientific, GA, U.S.A) prior to analysis.

2.2.6. Gas Chromatography-Mass Spectrometry (GC-MS): Aromatic Compounds

GC-MS coupled with head space solid-phase micro extraction (HS-SPME) was used to analyze the aromatic flavor compounds in beer samples throughout maturation. The GC-MS QP2010 SE (Shimadzu, Kyoto, Japan) was paired with GCMS LabSolutions software. A Stabilwax® 30 m x 0.32 mm ID x 0.25 µm column (Restek, PA, U.S.A.) was stored in the column oven and held at 40 °C for 2 min, then raised to 150 °C at a rate of 3 °C/min (38.67 min total run time). Ultra-high purity helium was used as the mobile phase with the flow control mode set to “Linear Velocity”, a total flow of 16.6 mL/min, and a column flow of 1.51 mL/min. The injector port was equipped with a low-volume liner for SPME (SGE, Melbourne, A.U.S.) and gas tight septum Thermogreen® LB-2 Septa (Sigma Aldrich, MO,

U.S.A.) The injector port was held at 200 °C and operated in splitless mode. The mass spectrometer operated with the ion source temperature and interface temperature held at 200 °C and with 10000 scan speed.

Samples were degassed in 50-mL Falcon tubes (Corning Inc., NY, U.S.A.) in the Digital Ultrasonic Cleaner (Fisher Scientific, GA, U.S.A) for 20 min. Then, 2.7 g of sodium chloride (Fisher Scientific, GA, U.S.A) and an 8-mm stir bar (Fisher Scientific, GA, U.S.A) were added to a clear 15-mL vial with 22-mm hole caps and 22-mm PTFE/silicone septa (Sigma Aldrich, MO, U.S.A.). Then 9.99 mL of degassed beer along with 10 µL of 2-heptanol solution was added to the vials. The 2-heptanol was added as an internal standard, and all vials contained a final concentration of 0.1 mg/L of 2-heptanol.

Samples were vortexed until all salt was in solution, then placed on a Cimarec Stirring Hot Plate (Thermo Scientific™, MA, U.S.A.) inside a 22-mm heating block insert to maintain a temperature of 50 °C. The temperature was monitored with an Ertco™ Exact-Temp™ mercury thermometer (Fisher Scientific, GA, U.S.A). After incubating for 5 minutes, a 50/20 µm DVB/CAR/PDMS, Stableflex SPME fiber in a manual SPME holder (Sigma Aldrich, MO, U.S.A.) was inserted into the septum of the sample vial, the fiber was exposed to the headspace, and incubated for 30 min.

The mass spectrometer utilized electron ionization (EI), a quadrupole mass analyzer and the detector was operated in scan mode for analysis. In addition to the chromatographic data obtained, mass spectra data was available for each eluting compound. For unknown compounds detected, a tentative identification was determined by comparing the resulting mass spectra fragmentation pattern with the mass spectra library available with the GCMSsolution Workstation Software. The following analytical grade standards were obtained and injected to confirm compound identity: isobutyl alcohol, isoamyl alcohol, ethyl acetate, phenylethyl acetate, phenyl acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate, diacetyl, and acetylacetone (Sigma Aldrich, MO, U.S.A.).

Additionally, samples from Phase 2 were sent for external quantitative volatile analysis for comparison of *L. thermotolerans* NCSU to the type strain NRRL Y-8284. This

external laboratory conducted volatile analysis according to the standard method Beer-29: Lower boiling volatiles in beer or ale (ASBC). This method utilizes a flame-ionization detector (GC-FID), coupled with a 15 ft x 1/8 in stainless steel packed column with 20% Carbowax 20 M on Chromosorb W (HDMS), 60/80 mesh held at 80 °C. Helium (30 mL/min) was the mobile phase, with an injector temperature of 170 °C and a detector temperature of 180 °C. Sample was introduced to the system via direct injection of 10 µL with an internal standard, and quantitation was accomplished with an external calibration curve using the internal standard.

3. Results

3.1. Preliminary Fermentation Data of L. thermotolerans NCSU (Phase 1)

In Phase 1, *Lachancea thermotolerans* NCSU was fermented at 18 °C in wort to determine whether it could be used as a brewing strain. As shown in Table 6, *L. thermotolerans* NCSU utilized 97.3% of the maltose by Day 6, only reducing a further 1.7 g/L between Day 6 and Day 10 (94.5% total maltose utilized). Similarly, 97.0% of the ethanol was produced by Day 6, with an addition of only 1.2 g/L of ethanol produced between Day 6 and Day 10.

Table 6. Preliminary data of *L. thermotolerans* NCSU fermentations at 18 °C in wort (n=2).

Day	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	ABV%
1	66.8	5.1	6.7	1.4	5.9	0.8%
2	51.0	0.1	1.0	2.0	18.6	2.4%
3	33.2	0.1	0.9	2.3	27.2	3.5%
4	17.6	0.3	1.3	2.8	34.3	4.4%
5	9.3	0.2	1.2	2.9	38.4	4.9%
6	5.4	0.5	1.4	3.0	40.2	5.1%
7	4.6	0.5	1.4	3.1	40.5	5.1%
8	3.9	0.5	1.3	3.2	41.4	5.3%
9	3.7	0.5	1.3	3.2	41.5	5.3%
10	3.7	0.5	1.3	3.2	41.4	5.3%

3.2. Fermentation Performance of L. thermotolerans NCSU Compared to the Type Strain in Pilsner Wort (Phase 2)

In Phase 2, *L. thermotolerans* NCSU was compared to the type strain *L. thermotolerans* NRRL Y-8284 (ATCC® 56472™) in triplicate in laboratory scale fermentations at 18 °C. All statistical analysis was conducted using t-tests with $p < .05$. Figure 4 shows the NCSU strain reduced the specific gravity faster and further than the NRRL Y-8284 strain in wort; the original gravity (OG) was 1.053 (13 °P). Sampling of both fermentations was stopped at Day 7 when the gravity of the *L. thermotolerans* NCSU fermentation slowed; the final gravity was 1.016 (45.9 g/L residual sugar). The specific gravity of the *L. thermotolerans* NRRL Y-8284 fermentation slowed after reaching 1.041 on Day 2; the FG was 1.040 (108.9 g/L residual sugar).

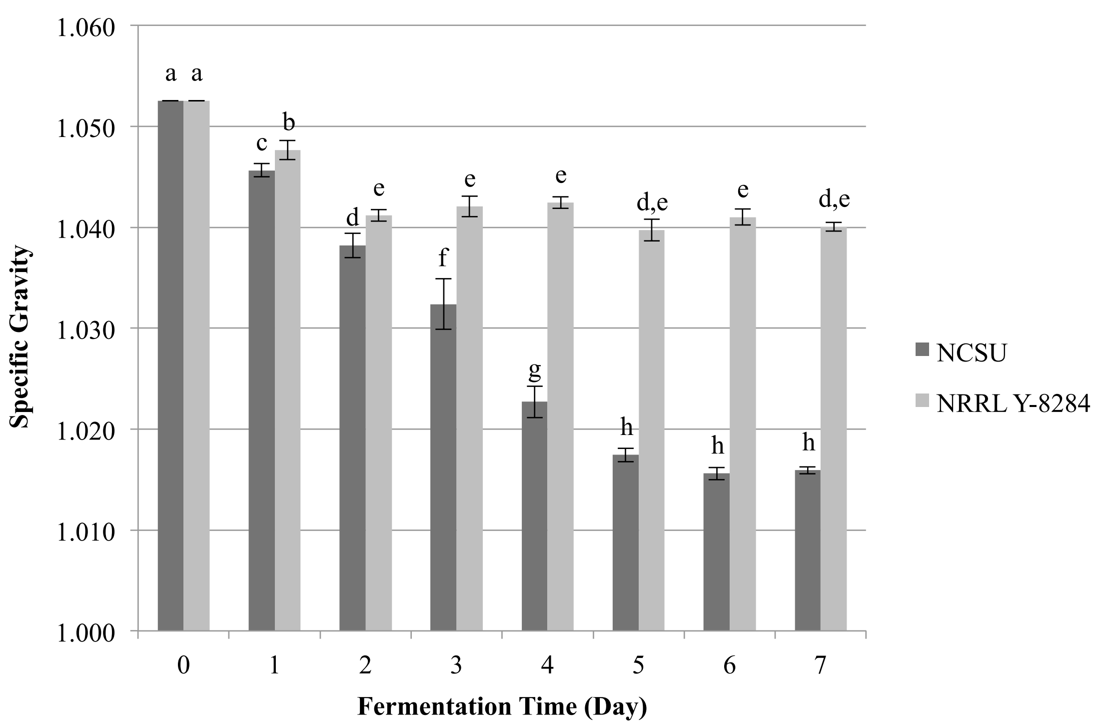
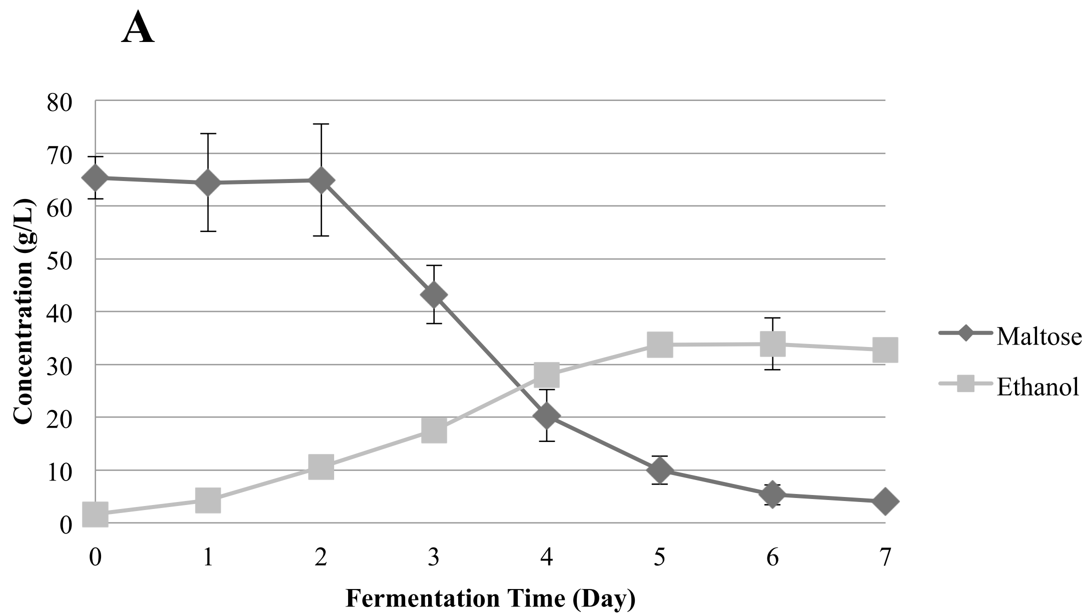


Figure 4. Change in specific gravity of wort by *L. thermotolerans* strains NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$).

L. thermotolerans NRRL Y-8284 did not utilize maltose or produce ethanol as efficiently as *L. thermotolerans* NCSU, as displayed in Figure 5. *L. thermotolerans* NRRL Y-8284 started at 64.9 g/L maltose and ended at 61.4 g/L (5.4% total maltose utilized). *L. thermotolerans* NCSU started at 65.4 g/L maltose and ended at 4.0 g/L (93.8% total maltose utilized). More ethanol was produced by *L. thermotolerans* NCSU, which had a final ABV of 4.15% (32.7 g/L of ethanol). *L. thermotolerans* NRRL Y-8284 did not produce any additional ethanol from Day 2 to Day 7 and had a final ABV of 1.06% (8.4 g/L of ethanol).



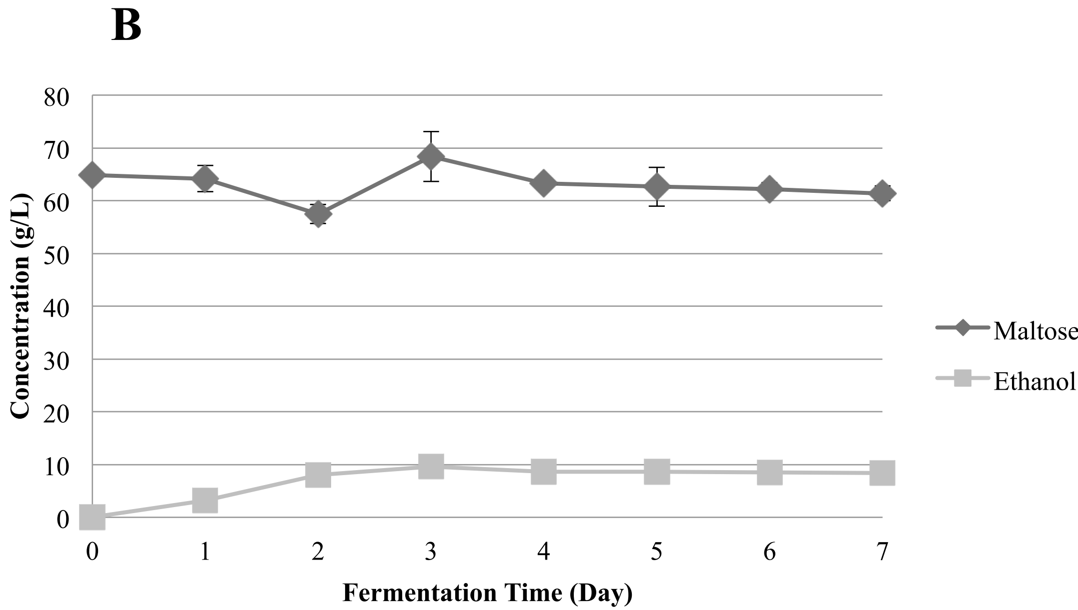
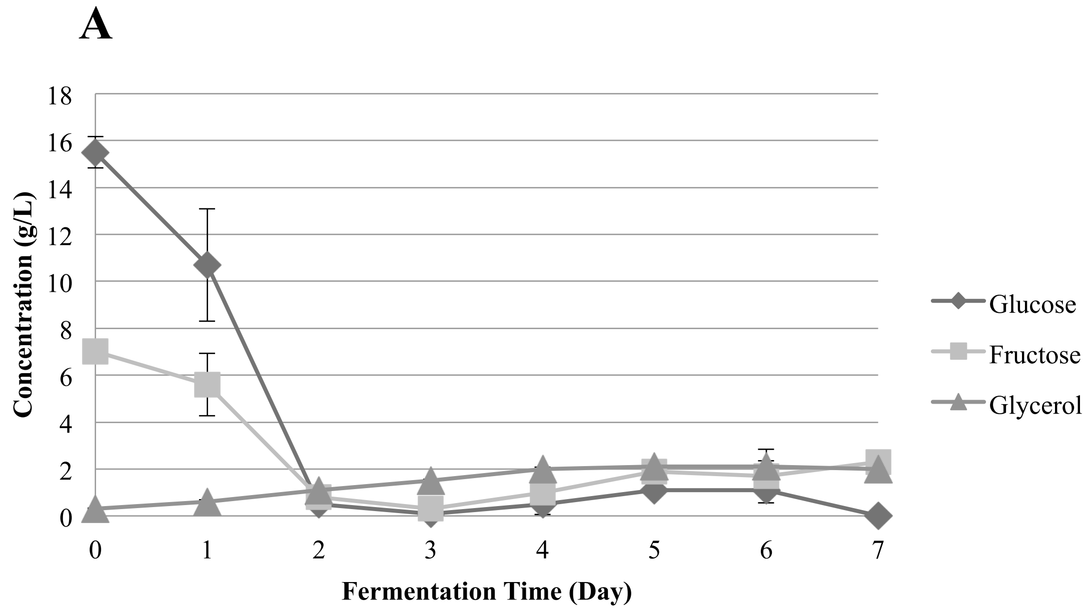


Figure 5. Comparison of *L. thermotolerans* strains NCSU (A) and NRRL Y-8284 (B) maltose utilization and ethanol production at 18 °C (n=3 with ±1 standard deviation).

Both *L. thermotolerans* NRRL Y-8284 and NCSU utilized the available glucose and fructose. As seen in Figure 6, *L. thermotolerans* NRRL Y-8284 reduced both sugars to completeness by approximately Day 2. *L. thermotolerans* NCSU reduced both sugars by Day 3, but on Day 4 both monosaccharides had increased in concentration. Both strains produced glycerol, but the *L. thermotolerans* NCSU produced more glycerol than *L. thermotolerans* NRRL Y-8284; during fermentation, the NCSU strain produced 1.7 g/L of glycerol compared to NRRL Y-8284 strain, which produced 0.8 g/L.



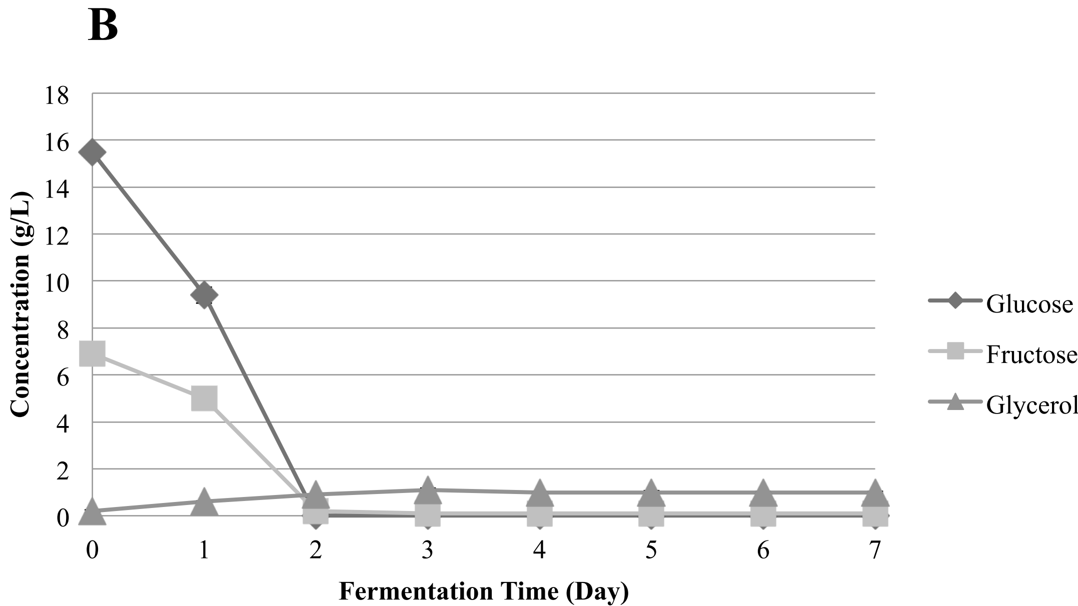


Figure 6. Comparison of *L. thermotolerans* strains NCSU (A) and NRRL Y-8284 (B) sugar utilization and glycerol production at 18 °C (n=3 with ±1 standard deviation).

The pH was monitored during fermentation from an initial wort pH of 5.56 (Figure 7). *L. thermotolerans* NCSU strain dropped the pH by 1.01 after Day 1 and finished acid production around Day 3 for a final pH of 3.65 (Δ 1.91). *L. thermotolerans* NRRL Y-8284 did not produce as much acid as the NCSU strain, dropping the pH by only 0.60 after Day 1 and finishing acid production around Day 2 for a final pH of 4.87 (Δ 0.77).

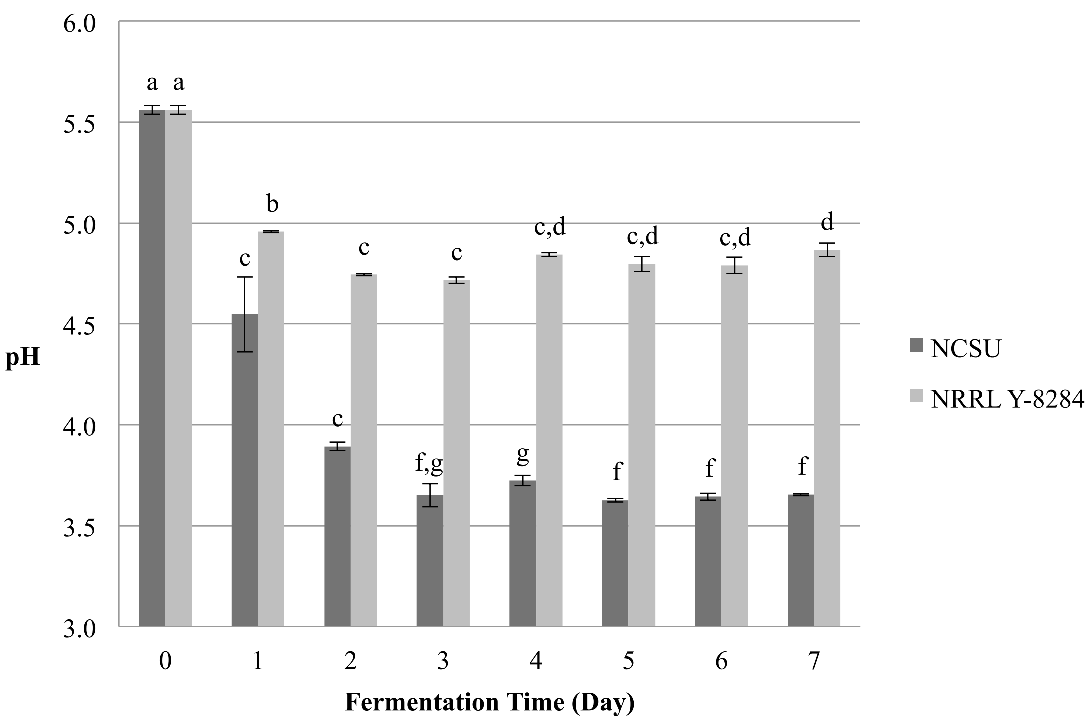


Figure 7. pH of *L. thermotolerans* NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$).

Cell counts were also monitored via a cellometer; *L. thermotolerans* NCSU and NRRL Y-8284 strains were inoculated at $1x10^7$ cfu/mL and remained viable throughout fermentation, though the type strain did have a higher average concentration and reached concentrations up to $1x10^8$ cfu/mL during fermentation (Figure 8).

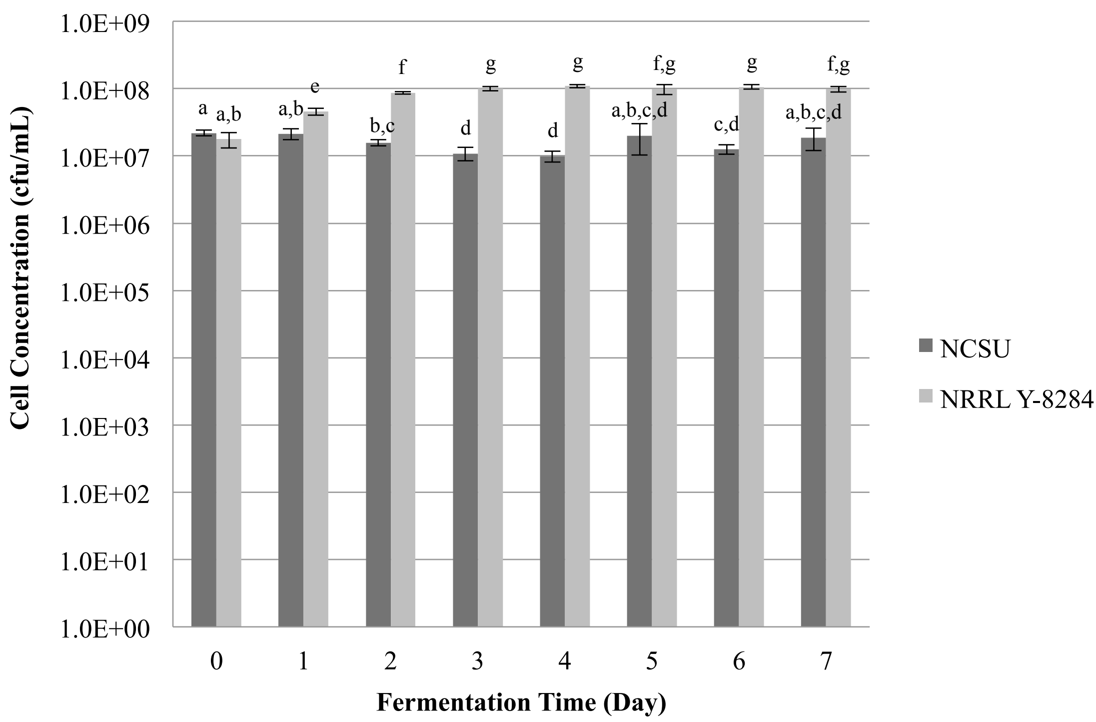


Figure 8. Comparison of cell concentration of *L. thermotolerans* NCSU and NRRL Y-8284 during fermentation at 18 °C (n=3 with ±1 standard deviation, $p < .05$).

Amino acids were analyzed at the beginning (Day 0) and end (Day 7) of fermentations for both *L. thermotolerans* NCSU and NRRL Y-8284 strains; note that wort contains 19 amino acids, as it excludes cysteine. Ammonia (NH_3) utilization was analyzed for comparison to the type strain. Amino acid utilization is displayed in four groups: Group A (Thr, Ser, Asn, Met, Lys), Group B (Gln, Leu, Ile, Asp, His, Arg, Val, Phe), Group C (Glu, Tyr, Gly, Ala, Trp), and Group D (Proline) (Table 7). Based on the average remaining amino acids, *L. thermotolerans* NCSU utilized Group A more than *L. thermotolerans* NRRL Y-8284. In Group B, *L. thermotolerans* NCSU appeared to produce glutamine compared to *L. thermotolerans* NRRL Y-8284 which utilized all but 24% of the available glutamine. Strain NRRL Y-8284 utilized Group D further than strain NCSU. For both *L. thermotolerans*

NCSU and NRRL Y-8284, Group D had the highest percentage of amino acids remaining, followed by Group C, Group B, and finally Group A.

Table 7. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotoerans* NCSU and NRRL Y-8284 (n=3).

	NCSU			NRRL Y-8284		
Amino Acid	Starting Concentration (mg/L)	Remaining (%)	Group Average Remaining (%)	Starting Concentration (mg/L)	Remaining (%)	Group Average Remaining (%)
GROUP A						
Thr	80.2	22%	29%	89.8	10%	34%
Ser	102.9	30%		114.6	44%	
Asn	129.4	34%		148.8	28%	
Met	48.5	30%		47.3	48%	
Lys	109.5	27%		121.1	41%	
GROUP B						
Gln	10.4	233%	69%	12.2	24%	51%
Leu	198.2	29%		221.5	44%	
Ile	80.2	35%		102.0	57%	
Asp	92.5	48%		99.5	43%	
His	60.2	63%		67.0	66%	
Arg	131.7	9%		153.5	36%	
Val	124.9	82%		167.5	79%	
Phe	130.5	50%		175.9	59%	
GROUP C						
Glu	104.0	69%	78%	113.0	69%	81%
NH3	75.1	82%		81.8	94%	
Tyr	147.2	84%		163.6	81%	
Gly	52.0	76%		59.0	76%	
Ala	163.3	69%		180.3	93%	
Trp	46.7	86%		61.4	73%	
GROUP D						
Pro	498.4	98%	98%	560.7	90%	90%

Aromatic analysis was conducted at the beginning of maturation (Week 0) to compare *L. thermotolerans* NCSU to NRRL Y-8284 (Table 8). These 14 compounds were selected for

comparison based upon their high frequency of occurrence among samples analyzed. Aromatics were also identified by their mass spectra. There compounds were analyzed and compared to the internal standard (0.1 mg/L of 2-heptanol) and reported as peak ratio (PR), or the peak area of the analyte divided by the peak area of the internal standard. The 14 compounds included alcohols (5), esters (7), aldehydes (1), and terpenes (1). Of the 14 compounds detected, *L. thermotolerans* NCSU had a higher peak ratio for 13 compounds when compared to NRLL Y-8284. *L. thermotolerans* NRRL Y-8284 had a higher ratio of 1-hexanol detected.

Table 8. Aromatic analysis of *L. thermotolerans* NCSU compared to the type strain at the beginning of maturation (Week 0) at 18 °C (n=3 with one standard deviation reported).

		NCSU (Peak Ratio)			NRRL Y-8284 (Peak Ratio)		
Elution #	Compound	Average	Standard Deviation	CV (%)	Average	Standard Deviation	CV (%)
1	Acetaldehyde	0.21	0.02	0.12	0.06	0.01	0.11
2	Ethyl acetate	4.16	0.81	0.19	0.46	0.12	0.27
3	Ethyl butyrate	0.01	0.00	0.12	n.d.	n.d.	n.d.
4	Isobutanol	0.45	0.09	0.20	0.15	0.00	0.02
5	Isoamyl acetate	0.35	0.02	0.06	0.07	0.02	0.32
6	Isoamyl alcohol	9.17	1.46	0.16	3.92	0.16	0.04
7	Ethyl hexanoate	0.14	0.10	0.71	0.01	0.01	0.55
8	1-Hexanol	0.01	0.01	1.00	0.04	0.02	0.52
9	Ethyl octanoate	0.07	0.01	0.10	0.01	0.00	0.22
10	2,3-Butanediol	0.02	0.02	1.00	n.d.	n.d.	n.d.
11	Linalool	0.12	0.02	0.19	0.08	0.01	0.12
12	Ethyl decanoate	0.06	0.04	0.71	n.d.	n.d.	n.d.
13	Phenylethyl acetate	0.01	0.00	0.17	n.d.	n.d.	n.d.
14	Phenylethyl alcohol	3.09	0.06	0.02	0.99	0.09	0.09

Quantitative volatile analysis was conducted and the results are displayed in Table 9. Of the compounds detected, NCSU produced acetaldehyde in the highest concentration; NRRL Y-8284 produced ethyl acetate in the highest concentration, an order of magnitude more than NCSU.

Table 9. Quantitative volatile comparison of *L. thermtotolerans* NCSU (n=3) to NRRL Y-8284 (n=1).

Compound	NRRL Y-8284 (ppm)	NCSU (ppm)
Acetaldehyde	9.2	176.0
Ethyl Acetate	1018.7	21.2
Methanol	5.6	5.8
n-Propanol	6.0	14.7
Isobutanol	9.2	14.1
1-Butanol	0.7	0.1
Amyl alcohol	32.6	53.8

Aromatic analysis was conducted at the beginning (Week 0) and end (Week 3) of maturation for *L. thermotolerans* NCSU (Table 10). Of the 14 compounds analyzed, four increased from Week 0 to Week 3 while the rest decreased the four compounds that increased were acetaldehyde, 1-hexanol, 2,3-butanediol, and ethyl decanoate. The compound with the highest peak ratio at Week 3 is phenylethyl alcohol.

Table 10. Aromatic analysis of *L. thermotolerans* NCSU start of maturation (Week 0) and the end of maturation (Week 3) (n=3 with one standard deviation reported).

		Week 0 (Peak Ratio)			Week 3 (Peak Ratio)		
Elution #	Compound	Average	Standard Deviation	CV (%)	Average	Standard Deviation	CV (%)
1	Acetaldehyde	0.21	0.02	0.12	0.62	0.06	0.10
2	Ethyl acetate	4.16	0.81	0.19	2.22	1.06	0.48
3	Ethyl butyrate	0.01	0.00	0.12	n.d.	n.d.	n.d.
4	Isobutanol	0.45	0.09	0.20	0.36	0.09	0.24
5	Isoamyl acetate	0.35	0.02	0.06	0.11	0.07	0.64
6	Isoamyl alcohol	9.17	1.46	0.16	1.75	1.48	0.84
7	Ethyl hexanoate	0.14	0.10	0.71	0.05	0.06	1.24
8	1-Hexanol	0.01	0.01	1.00	0.02	0.00	0.19
9	Ethyl octanoate	0.07	0.01	0.10	0.05	0.01	0.12
10	2,3-Butanediol	0.02	0.02	1.00	0.06	0.02	0.30
11	Linalool	0.12	0.02	0.19	0.10	0.01	0.13
12	Ethyl decanoate	0.06	0.04	0.71	0.10	0.02	0.22
13	Phenylethyl acetate	0.01	0.00	0.17	n.d.	n.d.	n.d.
14	Phenylethyl alcohol	3.09	0.06	0.02	3.08	0.55	0.18

3.3. Fermentation Performance of L. thermotolerans NCSU in Regular Pilsner Wort Compared to High-Gravity Pilsner Wort (Phase 3)

In Phase 3, *L. thermotolerans* NCSU was fermented in regular Pilsner (Pils) wort and high gravity Pilsner (HG) wort, or Pilsner wort supplemented with 61.2 g/L maltose; both fermentations were conducted in triplicate. All statistical analysis was conducted using t-tests with $p < .05$. The single-strain, lab-scale fermentations were conducted at 22 °C, with the Pils wort density starting at 1.043 (10.5° Plato) and the HG starting at 1.064 (15.5° Plato). The Pils and HG fermentations lasted four and seven days, respectively, and were stopped when the gravity reached approximately 1.015 (Figure 9). The final gravity for the regular Pils was 1.015 (43.9 g/L residual sugars) and the HG was 1.013 (39.5 g/L residual sugars).

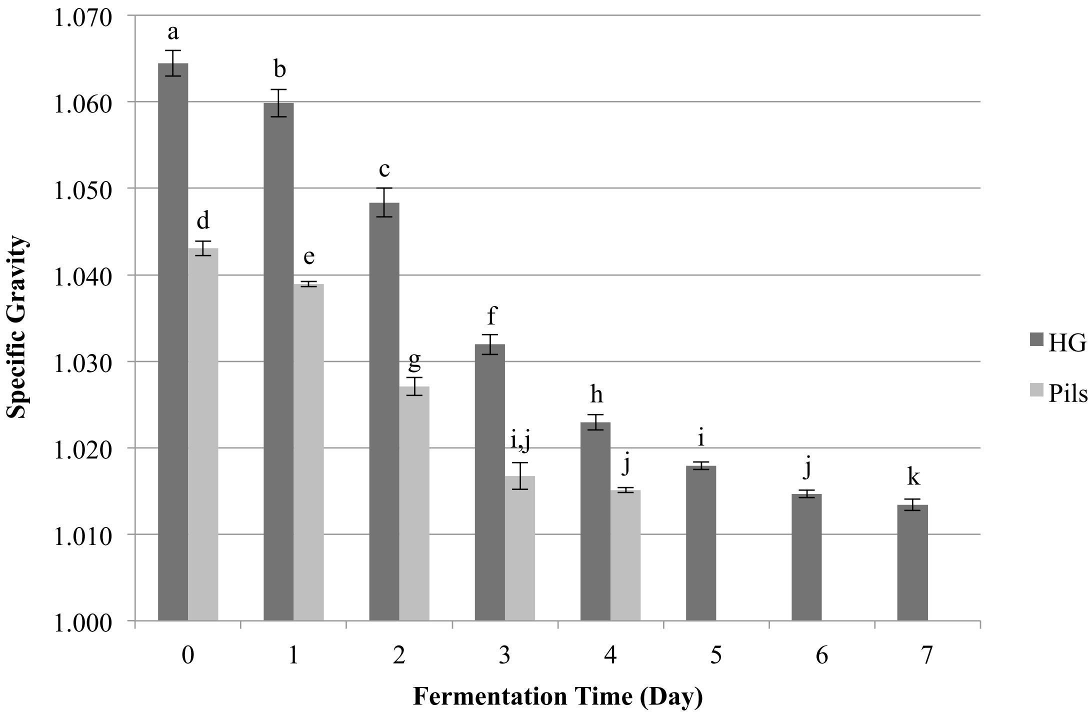
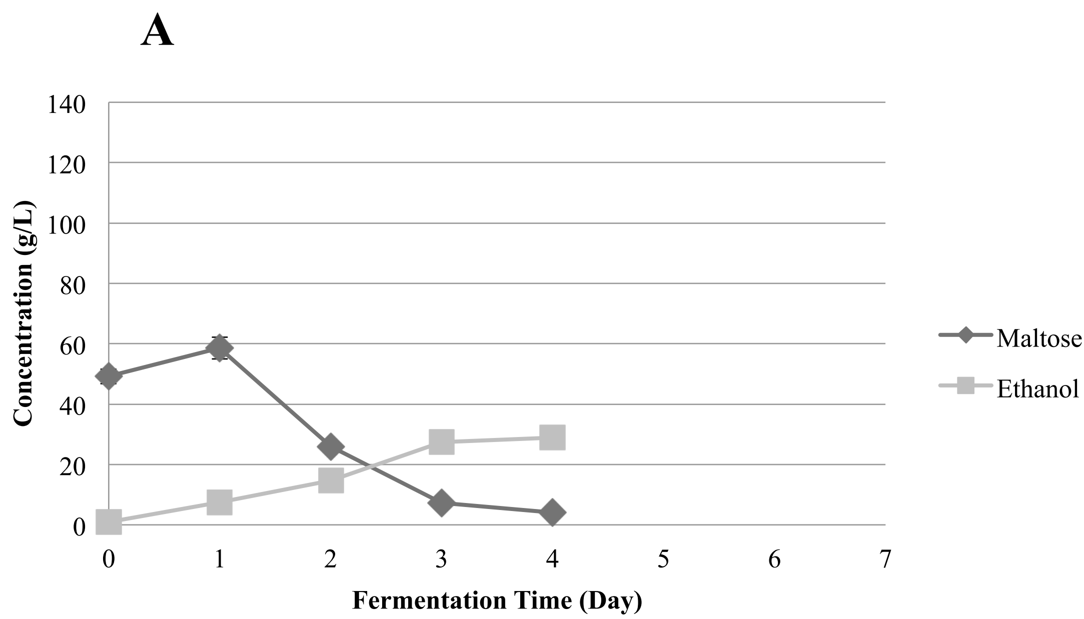


Figure 9. Specific gravity of *L. thermotolerans* NCSU fermentations in high gravity (HG) and Pilsner (Pils) wort at 22 °C (n=3 with ±1 standard deviation, $p < .05$).

L. thermotolerans NCSU was efficient at utilizing the available maltose and producing ethanol in both fermentations (Figure 10). In both fermentations, *L. thermotolerans* utilized 92% of the available maltose; the regular Pilsner wort started at 49.2 g/L maltose and the HG wort starting at 110.3 g/L maltose. The HG fermentation generated more ethanol than the regular Pilsner fermentation, yielding 56.0 g/L ethanol (7.10% ABV), versus 28.9 g/L ethanol (3.66% ABV), respectively.



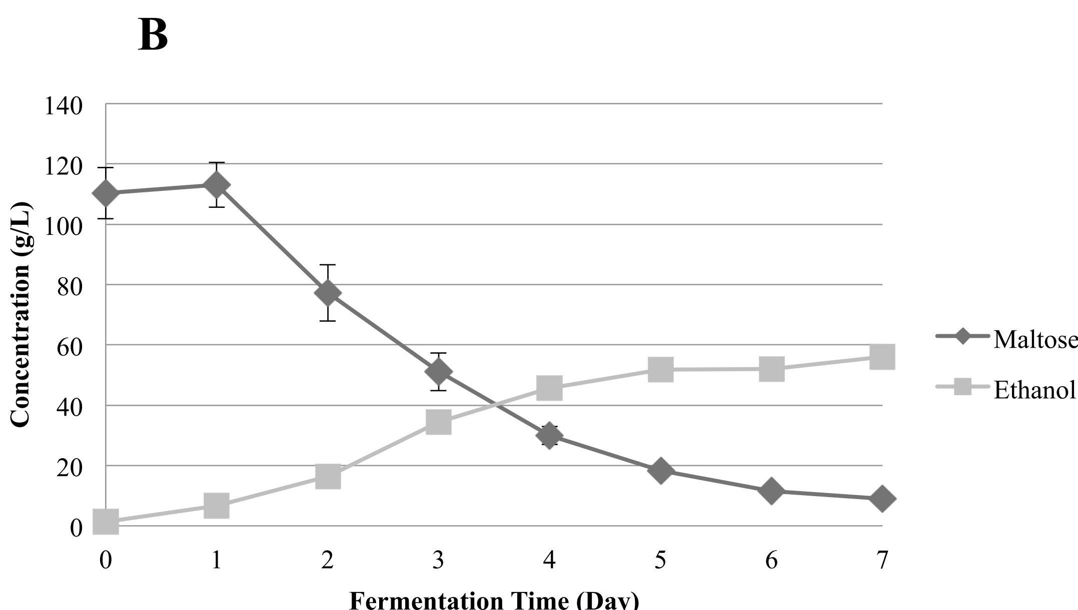
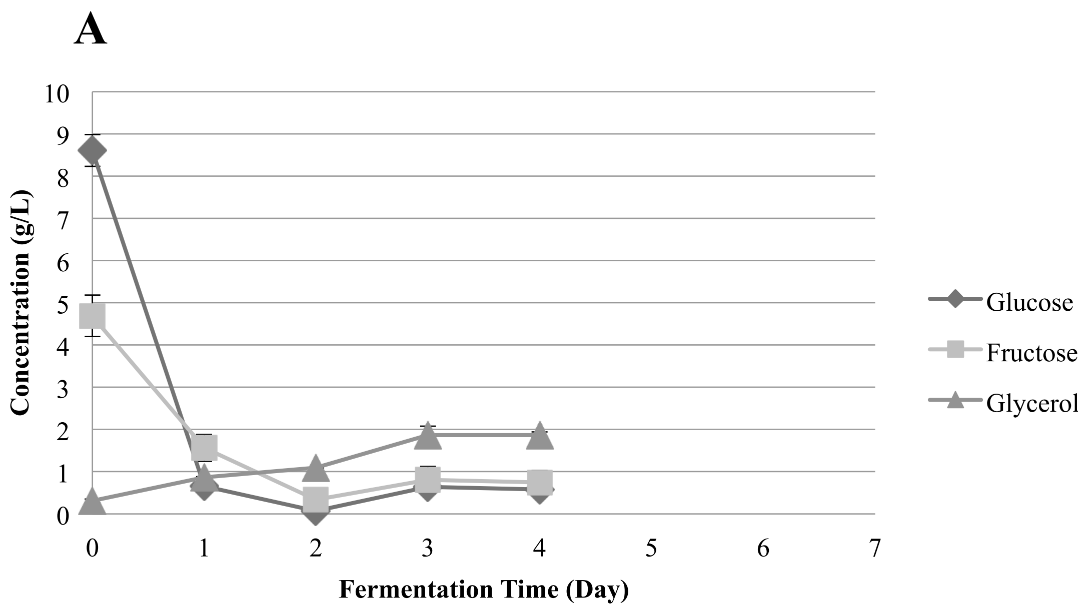


Figure 10. Comparison of *L. thermotolerans* NCSU average maltose utilization and ethanol production at 22 °C in Pilsner (A) and HG (B) wort (n=3 with ±1 standard deviation).

In HG wort, *L. thermotolerans* NCSU utilized glucose and fructose quickly, depleting glucose by Day 2 and reducing the fructose to under 0.5 g/L by Day 3 (Figure 11). However, on Day 6 the concentration of both glucose and fructose increased. In the regular Pils wort, NCSU utilized the majority of the available glucose and fructose by Day 2, but monosaccharide concentrations increased on Day 3. Glycerol was produced during fermentation in both HG and regular Pils wort, producing 2.9 g/L and 1.6 g/L of glycerol, respectively.



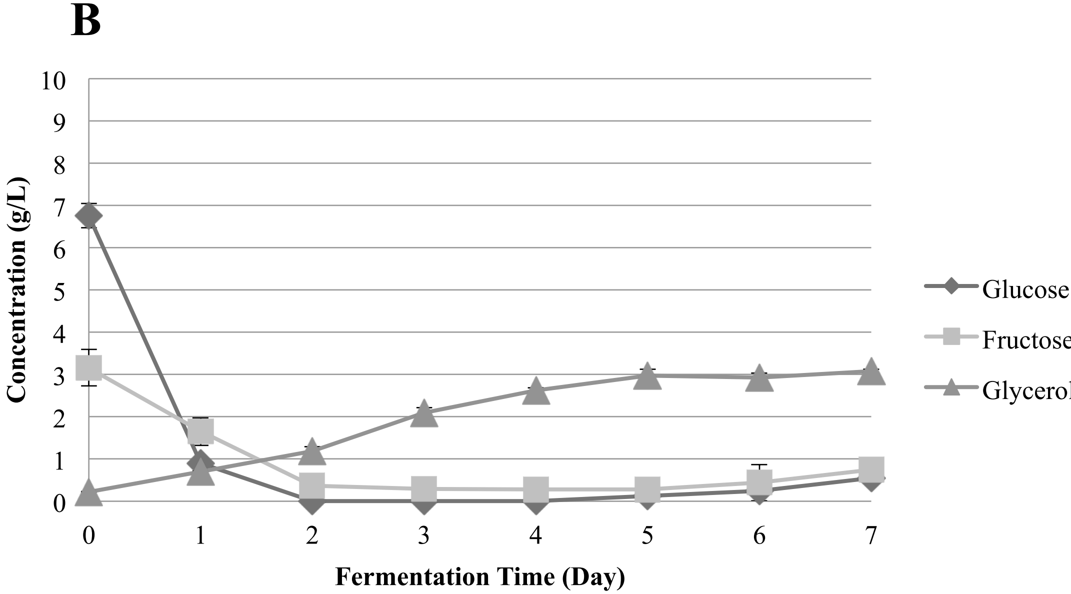


Figure 11. Comparison of *L. thermotolerans* NCSU sugar utilization and glycerol production in Pilsner (A) and HG (B) Pilsner wort at 22 °C (n=3 with ±1 standard deviation).

The pH of both fermentations decreased rapidly, with the regular Pils fermentation starting at 5.28 and HG starting at 5.26 (Figure 12). The Pils decreased in pH by 1.37 by Day 1, and had a final pH of 3.54 (Δ 1.74). The pH of the HG fermentation was reduced by 1.30 by Day 1, and also had a final pH of 3.54 (Δ 1.72).

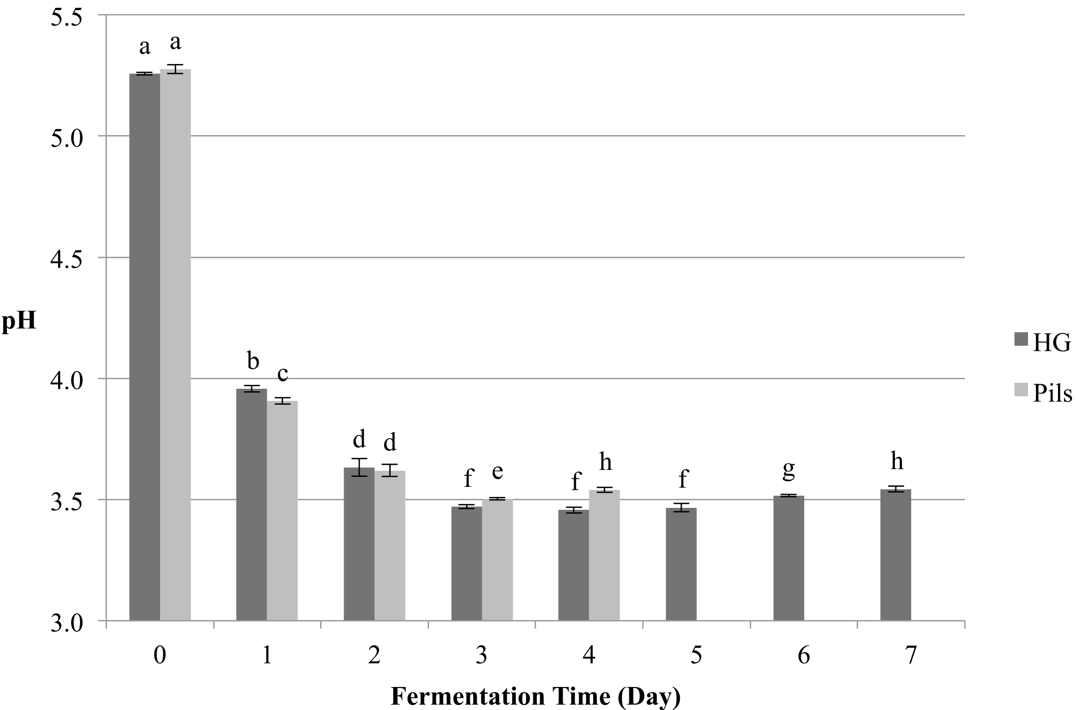


Figure 12. Change in pH by *L. thermotolerans* NCSU in HG and Pils fermentations at 22 °C (n=3 with ±1 standard deviation, $p < .05$).

Cell counts were also monitored for both fermentations; both high gravity and regular Pils fermentations were inoculated at $1x10^7$ cfu/mL and remained stable and viable throughout fermentation. (Figure 13).

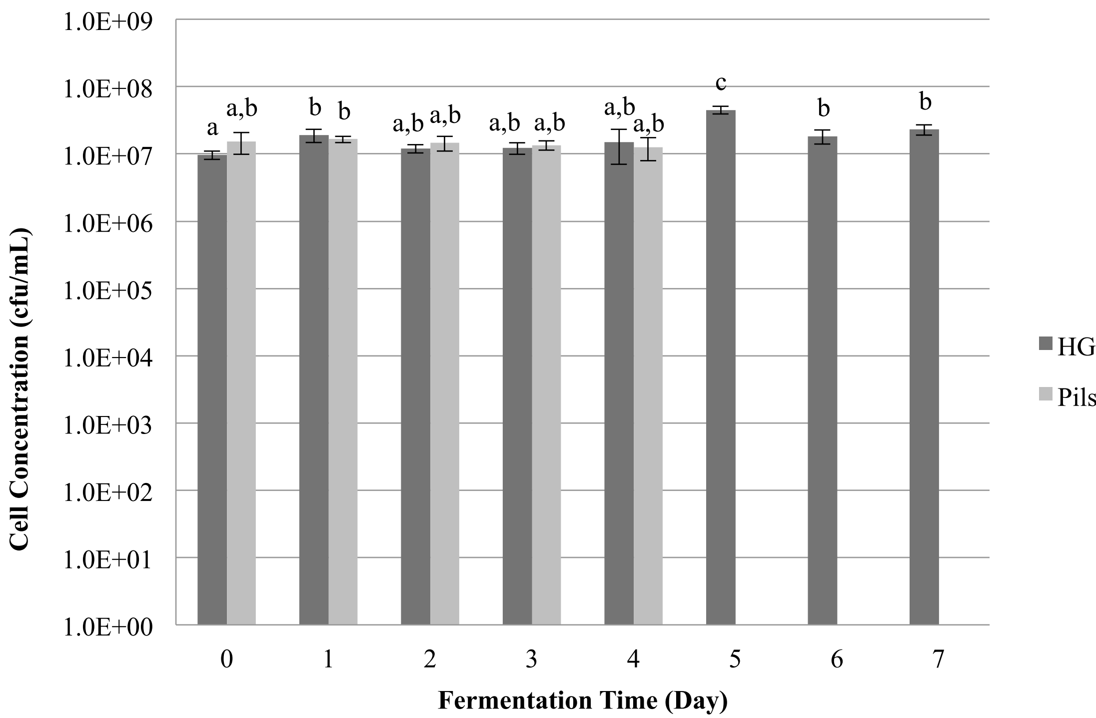


Figure 13. Cell concentration of regular Pils and HG worts during fermentation at 22 °C (n=3 with ±1 standard deviation, $p < .05$).

Amino acids were analyzed at the beginning and end of fermentation and analyses are grouped as reported in Section 3.1. While in the high gravity wort Group D was actually utilized, in the regular wort, the average available amino acid for Group D had increased at Day 4. In the regular fermentation, Group C had two amino acids increase in concentration; tyrosine and tryptophan. In the high gravity fermentation, Group B amino acid, glutamine, had not increased or decreased. In both fermentations (Table 11), Group D had the highest percentage of amino acids remaining, followed by Group C, Group B, and Group A. Additionally, the high gravity fermentation had lower percentage of remaining amino acids for each group compared to the fermentation in regular wort.

Table 11. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotolerans* NCSU in regular and HG Pilsner wort (n=3).

	Regular Pilsner			High Gravity Pilsner		
Amino Acid	Starting Concentration (mg/L)	Remaining (%)	Average Remaining (%)	Starting Concentration (mg/L)	Remaining (%)	Average Remaining (%)
GROUP A						
Thr	70.2	18%		71.0	7%	
Ser	90.3	29%		102.0	11%	
Asn	121.2	28%	29%	124.0	10%	9%
Met	23.9	25%		24.0	13%	
Lys	89.1	0%		87.0	2%	
GROUP B						
Gln	25.5	77%		28.0	100%	
Leu	135.0	26%		171.0	3%	
Ile	100.3	18%		81.0	7%	
Asp	77.0	45%	43%	78.0	15%	26%
His	57.1	67%		58.0	47%	
Arg	123.2	8%		125.0	5%	
Val	131.0	65%		134.0	24%	
Phe	112.6	38%		142.0	4%	
GROUP C						
Glu	90.1	72%		91.0	36%	
Tyr	133.1	108%		134.0	63%	
Gly	48.0	78%	86%	49.0	71%	57%
Ala	146.1	66%		149.0	45%	
Trp	39.5	106%		53.0	68%	
GROUP D						
Pro	449.7	104%	104%	464.0	93%	93%

Aromatic analysis was conducted at the beginning (Week 0) of maturation for *L. thermotolerans* NCSU in regular and HG Pilsner wort (Table 12). Of the 14 compounds compared, one remained constant (1-hexanol) and all other compounds were present in higher amounts in the HG Pilsner fermentation.

Table 12. Aromatic analysis of *L. thermotolerans* NCSU at the start of maturation in Pils and HG wort (n=3 with one standard deviation reported).

		Pils (Peak Ratio)			HG (Peak Ratio)		
Elution #	Compound	Average	Standard Deviation	CV (%)	Average	Standard Deviation	CV (%)
1	Acetaldehyde	0.09	0.02	0.21	0.24	0.03	0.14
2	Ethyl acetate	2.09	0.34	0.16	6.17	0.62	0.10
3	Ethyl butyrate	0.01	0.00	0.32	0.06	0.01	0.12
4	Isobutanol	0.38	0.02	0.05	1.06	0.14	0.13
5	Isoamyl acetate	0.27	0.06	0.22	0.70	0.04	0.06
6	Isoamyl alcohol	7.10	1.04	0.15	16.40	1.51	0.09
7	Ethyl hexanoate	0.11	0.02	0.14	0.23	0.13	0.56
8	1-Hexanol	0.01	0.00	0.35	0.01	0.00	0.10
9	Ethyl octanoate	0.03	0.01	0.41	0.13	0.03	0.24
10	2,3-Butanediol	n.d.	n.d.	n.d.	0.20	0.05	0.24
11	Linalool	0.02	0.00	0.06	0.03	0.01	0.16
12	Ethyl decanoate	0.02	0.00	0.10	0.08	0.01	0.12
13	Phenylethyl acetate	0.27	0.15	0.55	0.43	0.20	0.46
14	Phenylethyl alcohol	2.41	0.33	0.14	4.41	0.28	0.06

Aromatic analysis was conducted at the end (Week 3) of maturation for *L. thermotolerans* NCSU in Pils and HG Pilsner wort (Table 13). While at the beginning of maturation, both fermentations had the same peak ratio of 1-hexanol, at the end of maturation, the average peak ratio of 1-hexanol was higher in the HG fermentation. Conversely, linalool had a higher peak ratio in the HG fermentation at the beginning of maturation, while at the end of maturation both fermentations linalool had the same peak ratio. Additionally, at the end of maturation the average peak ratio of phenylethyl acetate was lower in the HG than the Pils wort, even though the HG had a higher peak ratio of phenylethyl acetate than the Pils at the beginning of maturation.

Table 13. Aromatic analysis of *L. thermotolerans* NCSU at Week 3 of maturation in Pils and HG wort (n=3 with one standard deviation reported).

		Pils (Peak Ratio)			HG (Peak Ratio)		
Elution #	Compound	Average	Standard Deviation	CV (%)	Average	Standard Deviation	CV (%)
1	Acetaldehyde	0.13	0.02	0.12	0.24	0.02	0.07
2	Ethyl acetate	0.19	0.01	0.05	3.27	0.90	0.27
3	Ethyl butyrate	n.d.	n.d.	n.d.	0.11	0.08	0.69
4	Isobutanol	0.18	0.01	0.05	0.92	0.12	0.13
5	Isoamyl acetate	0.01	0.00	0.19	0.53	0.27	0.51
6	Isoamyl alcohol	3.56	0.19	0.05	16.47	2.36	0.14
7	Ethyl hexanoate	0.01	0.00	0.36	0.39	0.15	0.38
8	1-Hexanol	0.01	0.00	0.13	0.02	0.01	0.35
9	Ethyl octanoate	0.27	0.36	1.34	0.32	0.12	0.38
10	2,3-Butanediol	0.01	0.00	0.31	0.11	0.03	0.28
11	Linalool	0.03	0.00	0.15	0.03	0.00	0.06
12	Ethyl decanoate	n.d.	n.d.	n.d.	0.01	0.01	0.90
13	Phenylethyl acetate	0.35	0.46	1.32	0.16	0.05	0.32
14	Phenylethyl alcohol	1.80	0.16	0.09	4.12	0.86	0.21

3.4. Fermentation Performance of L. thermotolerans NCSU in Lambic Wort at the Pilot Plant Scale (Phase 4)

In Phase 4, at the pilot plant scale (in a 300-L fermenter) *L. thermotolerans* NCSU was tested for the production of a Lambic-style ale; while the Pilsner fermentations were an all barley malt, the Lambic-style recipe includes wheat. The fermentation was conducted at 18 °C. The pitching rate of 4 L of active NCSU yeast was halved due to excessive flocculation, for a total of 2 L aerobic active yeast. The gravity was monitored throughout fermentation; the OG was 1.057 (14 °P) and fermentation was stopped at Day 25, the FG was 1.011 (33.0 g/L residual sugar).

For the pilot-scale fermentation, maltotriose was analyzed to confirm *L. thermotolerans* NCSU could utilize the second most abundant sugar in wort; these comprise the total sugars reported (Table 14). After Day 25, there was no fructose detectable in the sample and the residual glucose was less than 1 g/L. Maltose was the most abundant sugar

analyzed and was the most reduced (Δ 64.0 g/L). Maltotriose was the second most abundant, and had the second highest quantity utilized (Δ 24.4 g/L), but had the lowest percent utilization (84% utilized).

Table 14. Sugar utilization of *L. thermotolerans* NCSU in Lambic wort (n=1).

Day	Maltotriose (g/L)	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Total Sugars (g/L)
0	29.0	67.0	18.7	6.8	121.5
25	4.8	3.5	0.8	n.d.	9.0
% Utilized	84%	96%	97%	100%	93%

n.d. = not detected

Table 15 displays the byproducts of the Lambic-style fermentation. In addition to ethanol and glycerol production, propionic, acetic and lactic acid were analyzed. Of these acids, only lactic acid was detected and was found to have reached 7.3 g/L by Day 25. The Lambic-style fermentation also produced 2.4 g/L of glycerol and had a final ABV of 6.84%.

Table 15. Production of acid, glycerol and ethanol by *L. thermotolerans* NCSU in Lambic wort (n=1).

Day	Lactic Acid (g/L)	Glycerol (g/L)	Ethanol (g/L)	ABV (%)
0	n.d.	n.d.	n.d.	n.d.
25	7.33	2.37	53.96	6.84%

n.d. = not detected

The pH was recorded throughout fermentation. The pH started at 5.35 and reached a final pH of 3.60 (Δ 1.75). The majority of acid production was completed by Day 4 (Figure 14) and remained fairly constant until Day 25; the final pH was 3.63.

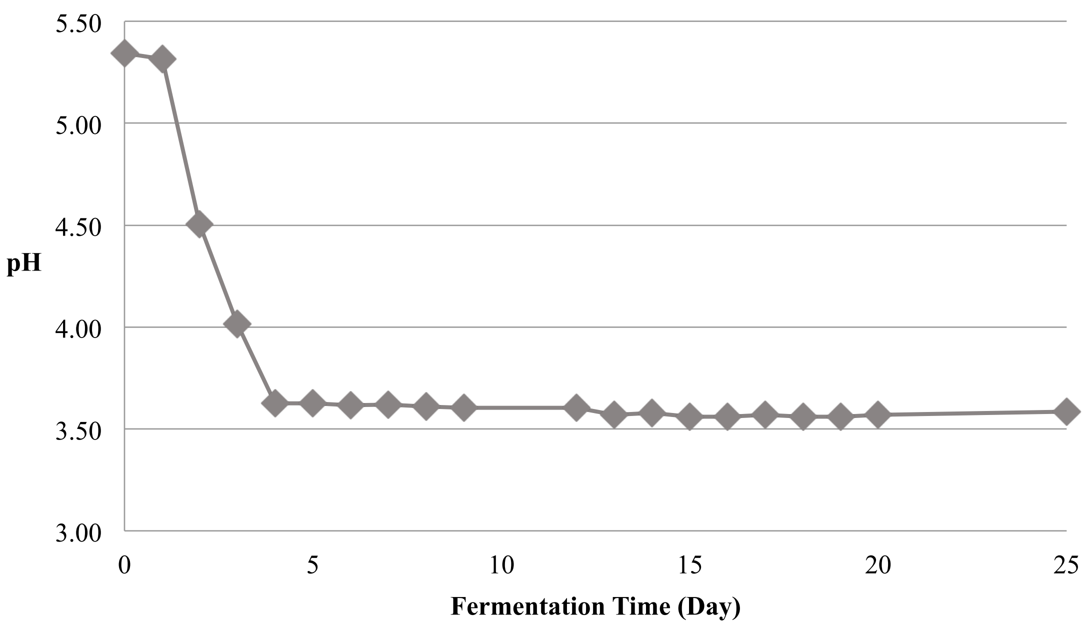


Figure 14. Change in pH during fermentation of *L. thermotolerans* NCSU in Lambic-style wort (n=1).

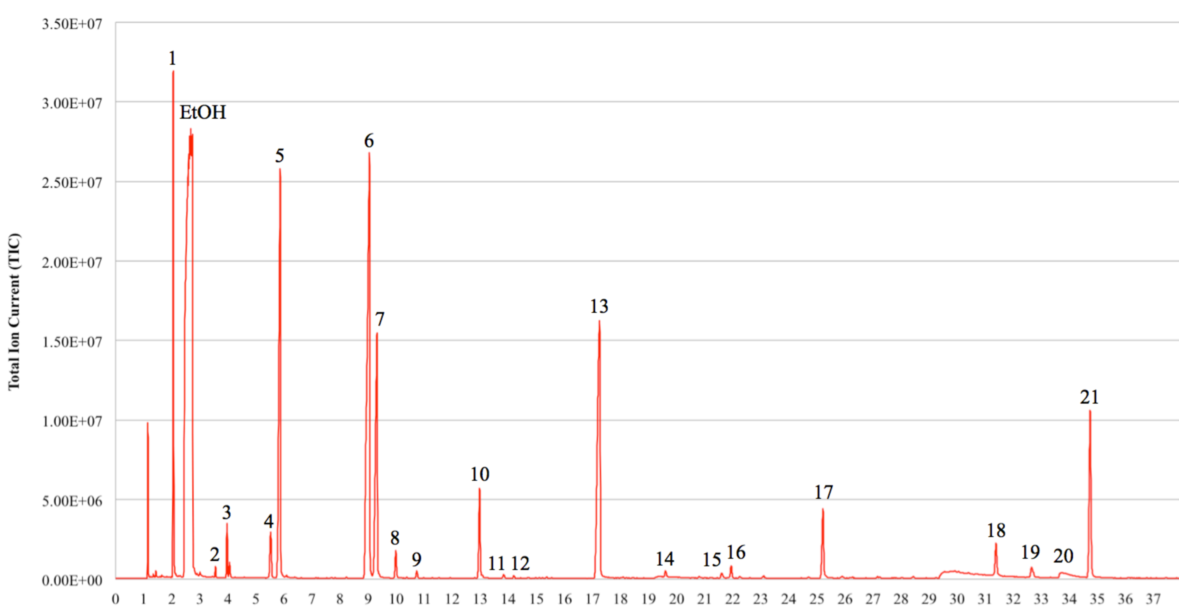
Amino acids were analyzed at the beginning and end of fermentation and analyses are grouped as reported in Section 3.1. Group A utilized all amino acids to a concentration below 25 g/L. Group B utilized amino acids to concentrations ranging between 25 g/L to 100 g/L. Group C utilized amino acids to concentrations ranging between 40 g/L to 150 g/L. For the Lambic-style fermentation, Group D had the highest average remaining amino acid percentage (106%), followed by Group C (88%), Group B (50%), and Group A (29%), as shown in Table 16. Group D, or proline, increased in concentration. In Group C, glycine also increased in concentration during fermentation.

Table 16. Starting concentration of amino acids and percentage remaining after fermentation of *L. thermotolerans* NCSU in Lambic wort (n=1).

	Lambic Fermentation		
Amino Acid	Starting Concentration (mg/L)	Remaining (%)	Group Average Remaining (%)
GROUP A			
Thr	77.2	8%	
Ser	104.7	15%	
Asn	122.8	19%	29%
Met	43.1	25%	
Lys	105.5	12%	
GROUP B			
Gln	49.4	86%	
Leu	200.3	23%	
Ile	90.9	33%	
Asp	85.9	38%	50%
His	60.8	66%	
Arg	155.9	42%	
Val	149.7	60%	
Phe	163.1	49%	
GROUP C			
Glu	103.7	75%	
Tyr	148.8	84%	
Gly	48.2	114%	88%
Ala	160.7	96%	
Trp	66.3	69%	
GROUP D			
Pro	581.3	106%	106%

Aromatic analysis was conducted at the end of maturation for *L. thermotolerans* NCSU in Lambic-style wort; the resulting chromatogram is displayed in Figure 15. From resulting mass spectra, 19 compounds were tentatively identified; these compounds include 11 esters, 6 alcohols, 1 aldehyde and 1 acid. The five compounds with the highest average peak ratios were isoamyl alcohol, ethyl octanoate, isoamyl acetate, ethyl hexanoate, ethyl

acetate, phenylethyl alcohol. Note that acetaldehyde is not detected in the Lambic fermentation.



Peak #	Compound	Peak Ratio	Peak #	Compound	Peak Ratio
1	Ethyl acetate	3.56	12	1-Hexanol	0.04
2	Isobutyl acetate	0.08	13	Ethyl octanoate	6.67
3	Ethyl butyrate	0.41	14	2-Ethylhexanol	0.07
4	Isobutanol	0.58	15	2,3-Butanediol	0.06
5	Isoamyl acetate	6.57	16	Linalool	0.15
6	Isoamyl alcohol	9.77	17	Ethyl decanoate	1.04
7	Ethyl hexanoate	4.28	18	Phenylethyl acetate	0.47
8	Styrene	0.35	19	Ethyl laurate	0.17
9	Acetic acid, hexyl ester	0.08	20	Caproic acid	0.38
10	2-Heptanol	1.00	21	Phenylethyl Alcohol	2.46
11	Ethyl lactate	0.04			

Figure 15. Chromatogram of aromatic analysis of Lambic-style fermentation at pilot-scale; peaks are labeled with a peak number and correspond to the table showing the identified compound, and peak ratio average (n=1).

4. Discussion

4.1. Preliminary Fermentations of L. thermotolerans NCSU (Phase 1)

In Phase 1, preliminary data demonstrated that *L. thermotolerans* NCSU could perform the two most critical functions required of a brewing strain: the assimilation of the primary sugar, maltose, and production of the primary desired product, ethanol. By Day 4, 74% of the available maltose was utilized and 80% of the total ethanol was produced.

Brewing yeast (i.e. *S. cerevisiae* and *S. pastorianus*) can only assimilate and metabolize glucose, fructose, maltose and maltotriose (sucrose is broken down extracellularly); the sugars are assimilated in a preferential order with glucose and fructose being metabolized first, followed by maltose and maltotriose (Pires, 2015). Similar results were seen in fermentations with *L. thermotolerans* NCSU, which utilized glucose and fructose first, followed by maltose and maltotriose. Results showed that while glucose and fructose were quickly utilized, they increased in concentration starting on Day 4. Brewer's yeasts have invertases that hydrolyze sucrose (to fructose and glucose) outside of the yeast cell, where as the other sugars are able to be transported into the cytoplasm (Pires, 2015); this suggests that *L. thermotolerans* NCSU also has invertases to hydrolyze sucrose, resulting in the increased concentrations of glucose and fructose over time. The metabolism of glucose, fructose and sucrose is important for brewers yeast as these sugars are readily available in wort.

Glycerol was also produced during fermentation; 1.8 g/L of glycerol was produced and 78% of the glycerol was produced by Day 4. All brewing strains produce glycerol; glycerol is a viscous sugar alcohol that has a slightly sweet taste, and mostly contributes to body in fermented beverages (Scanes, 1998). In beer, glycerol ranges from 1 to 3 g/L and has a taste threshold of 10 g/L (Briggs, 2004; Nykänen and Suomaleinen, 1983; Parker and Richardson, 1970); based on these values, *L. thermotolerans* NCSU produces glycerol within the desirable range. This means glycerol can contribute to the mouthfeel of beer made by *L. thermotolerans* NCSU without occurring over the flavor threshold, and disturbing the balance of the beer.

4.2. Comparison of L. thermotolerans NCSU to the Type Strain (Phase 2)

In Phase 2, *L. thermotolerans* NCSU distinguished itself metabolically and proved to be a superior brewing strain when compared to the type strain, *L. thermotolerans* NRRL Y-8284. *L. thermotolerans* NCSU was able to utilize more sugars than *L. thermotolerans* NRRL Y-8284. The NRRL Y-8284 strain demonstrated the ability to utilize glucose and fructose to completion by Day 2; however, these values did not increase like they did in fermentations with the NCSU strain. This suggest that *L. thermotolerans* NRRL Y-8284 does not contain the same invertases, or levels of invertases, that *L. thermotolerans* NCSU does. A major difference between the two yeasts was the assimilation of maltose; by Day 4, *L. thermotolerans* NCSU had utilized 69% of the available maltose, while *L. thermotolerans* NRRL Y-8284 had only reduced the maltose by 2%.

Likely due to this inability to metabolize maltose, *L. thermotolerans* NRRL Y-8284 produced less ethanol and glycerol than *L. thermotolerans* NCSU. *L. thermotolerans* NRRL Y-8284 had reached an ABV of 1.02% by Day 2, and did not significantly increase after that time; the glycerol produced totaled 0.8 g/L, which is lower than the average glycerol expected in beer. Conversely, *L. thermotolerans* NCSU produced ethanol throughout fermentation and reached a final ABV of 4.15%; a total of 1.7 g/L of glycerol was produced, which falls in line with the average glycerol range expected.

Cell counts and pH were monitored throughout fermentation and, again, differed between the two *L. thermotolerans* yeast strains. Both strains were pitched at $1x10^7$ cells/mL; while *L. thermotolerans* NCSU remained viable throughout fermentation at $1x10^7$ cells/mL, *L. thermotolerans* NRRL Y-8284 remained viable and actually increased to $1x10^8$ cells/mL during the fermentation. Both strains reduced the pH of the wort, but *L. thermotolerans* NCSU was able to reduce the pH (Δ 1.91) more than *L. thermotolerans* NRRL Y-8284 (Δ 0.69). It is likely that *L. thermotolerans* NCSU was able to produce more acid because of its ability to metabolize more of the available wort sugars, and utilize them as a carbon sources to produce more acids.

Differences between the two strains were also observed comparing their utilization of amino acids. When comparing the average percent remaining of the amino acid groups, *L. thermotolerans* NCSU utilized Group A and Group C more than *L. thermotolerans* NRRL Y-8284. However, in the *L. thermotolerans* NCSU fermentation, glutamine (Group B) was produced during fermentation. This data point skews the average percent remaining of Group B for *L. thermotolerans* NCSU. However, it is in line with literature on typical brewer's yeast, which has determined *Saccharomyces* can synthesize glutamine from glutamate and ammonia (catalyzed by the glutamine synthase encoded by GLN1) (Pires, 2015). Adjustments could be made in Group B, as arginine was the most utilized amino acid by *L. thermotolerans* NCSU, it could be moved to Group A for future analysis.

Aromatic comparisons were conducted at NC State and in an external laboratory for the comparison of *L. thermotolerans* NCSU and NRRL Y-8284. Our data showed strain NCSU had a higher peak ratio for all compounds, except 1-hexanol. According to the ASBC Flavor Database (2014) 1-hexanol has aroma descriptors of "coconut, green leaves and unpleasant". Data from the external laboratory showed that strain NRRL Y-8284 had an extremely high concentration of ethyl acetate (1018.72 ppm) compared to NCSU (21.21 ppm); ethyl acetate has a "solvent, fruity, sweet, estery" aroma and a flavor threshold ranging from 25-45 ppm in beer (ASBC, 2014). It should be noted that results from NC State show that ethyl acetate is higher in *L. thermotolerans* NCSU fermentations; these differences in results may be attributed to the fact that the external laboratory utilized a direct injection method (ASBC, Beer-29), while NC State results were obtained using solid-phase microextraction (SPME) as a sample preparation technique. Further results from the external laboratory showed that strain NCSU was well above the flavor threshold (10-20 ppm) for acetaldehyde (175.96 ppm) and within the threshold range (50-70 ppm) of amyl alcohol (53.75 ppm). All other compounds for NCSU and NRRL Y-8284 were below their flavor thresholds.

For *L. thermotolerans* NSCU, aromatic evaluation was done (at NC State) for Week 0 of maturation (end of fermentation) and Week 3 of maturation. While most compounds either

stayed consistent or decreased in peak ratio, acetaldehyde increased from Week 0 (0.21 peak ratio) to Week 3 (0.62). The purpose of maturation is to allow flavors to mellow and produce a more balanced beer (e.g., diacetyl is an off flavor that is reduced by yeasts during maturation). This indicates that *L. thermotolerans* NCSU may not be able to reduce acetaldehyde during maturation, and special care should be taken to reduce the amount of acetaldehyde produced during fermentation. This could be a potential issue, as acetaldehyde has a 'grassy' off-flavor when present above its flavor threshold (Meilgaard, 1975). Additional discussion on acetaldehyde formation by *L. thermotolerans* NCSU is discussed in Section 4.4.

4.3. L. thermotolerans NCSU in High Gravity and Regular Wort (Phase 3)

In Phase 3, *L. thermotolerans* NCSU was fermented in regular Pilsner (10.5 °P) and high gravity Pilsner (15.5 °P) worts. Like in previous fermentations with *L. thermotolerans* NCSU, levels of glucose and fructose were rapidly depleted and then slightly increased in concentration. *L. thermotolerans* NCSU was able to utilize 92% of the available maltose in both fermentations. Brewers utilize high gravity brewing (typically 16-18 °P) to increase their volumetric productivity, saving labor and energy costs (He, 2014); thus, it is desirable for brewing yeasts to be able to utilize a high concentration of sugars for economical reasons. Our data demonstrates *L. thermotolerans* NCSU was able to ferment the increased sugar concentration and can be used for high gravity brewing.

L. thermotolerans NCSU was able to produce more ethanol and glycerol in the high gravity wort than the regular wort; this increased production is due to the increased maltose concentration (i.e., carbon source). Again, the glycerol produced fell within the average range of 1 to 3 g/L; the regular fermentation produced 1.55 g/L and high gravity produced 2.86 g/L. With ethanol production, the regular fermentation had a final ABV of 3.66%, while the high gravity fermentation reached 7.10%. The approximate concentration of ethanol in beer is 4.44% ABV (Hutkins, 2006) and ABV typically ranges from 4-6%; however, certain styles may reach as high as 12% ABV.

Saccharomyces species are able to produce and tolerate high ethanol concentrations (e.g., some wine fermentations may reach up to 16% ABV). Relative to other non-*Saccharomyces* yeast in wine, *L. thermotolerans* is known to have a moderately high ethanol tolerance of <13.5 v/v% (Moreno-Arribas, 2009). A study of pure culture fermentations in wine found from 163.00 g/L sugars, *L. thermotolerans* TH942 made 7.58 v/v% ethanol compared to *S. cerevisiae* SCM952 9.60 v/v% (Kapsopoulou, 2005).

Our data demonstrated that the NCSU strain can produce sufficient ethanol to cover the typical range of beers; furthermore, the NCSU strain can produce concentrations of ethanol higher than in average beer. It is important that *L. thermotolerans* NCSU be able to produce and withstand a large quantity of ethanol to be comparable to *Saccharomyces* as a brewing yeast. Yet, it is also important for brewers to balance the flavors of a beer, and high concentrations of ethanol can yield a strong, solvent aroma and taste.

Comparing the average percent of amino acids remaining, the high gravity fermentation reduced Groups A, B, C and D further than the regular fermentation. For brewing, the carbon to nitrogen (C:N) ratio is low in all-malt wort fermentations, and after fermentation, when yeast growth has ceased, nitrogenous compounds are abundant (He, 2014). However, by supplementing adjuncts like sugar syrups, the C:N ratio increases and the available nitrogen is diluted (He, 2014). Proline is the least preferred amino acid by yeasts, and is not typically utilized during fermentation. Piddocke et al. (2009) found that with an increase in gravity, some proline was assimilated by *Saccharomyces* under microaerobic conditions. Our results show that *L. thermotolerans* NCSU also began to assimilate proline by the end of fermentation in high gravity wort (proline was not utilized in the regular fermentation). It is important for amino acids to be supplemented with high gravity brewing as a lack of nitrogen affects metabolic processes (i.e., higher alcohol production).

Different sugar adjuncts can affect the flavor of beer. Piddocke et al. (2009) determined that more balanced beer was achieved when the gravity was increased by adding maltose syrup as opposed to glucose syrup; additionally, they determined an increased

gravity resulted in an increase in the concentration of ethyl acetate and isoamyl acetate in the final beer. Negative effects associated with high gravity brewing include increased acetate esters and higher alcohol concentrations; Anderson and Kirsop (1974) observed an 8-fold increase in acetate ester production occurred when specific gravity was doubled. Additionally, elevated concentrations of higher alcohols and volatile esters were found in high gravity brewing (16 °P) compared to normal gravity (13 °P) (He, 2014). Aromatic analysis of *L. thermotolerans* NCSU fermentations displayed increased aromatic production, as 13 of the aromatics observed were found in higher quantities in the high gravity beer. It is important for brewers to manage ester and higher alcohol production, or resulting beers can have overly fruity or solvent-like aromas (He, 2014). However, no true conclusions can be made about the flavor imparted by these detected compounds until their exact concentrations are determined and compared to the flavor thresholds of those compounds in acidic beer.

4.4. Pilot Scale Fermentation of L. thermotolerans NCSU (Phase 4)

As previous fermentations demonstrated, *L. thermotolerans* NCSU assimilated glucose, fructose, and maltose. As maltotriose had not been analyzed in previous phases, it was analyzed and confirmed to be assimilated by *L. thermotolerans* NCSU (84% utilized). Again, the glycerol produced fell in the desirable range (2.37 g/L) and the ethanol production exceeded the average range of beer (6.84% ABV).

The pH of wort is approximately 5.6, with the pH of beer being approximately 4.2 (Hutkins, 2006). Comparatively, beer produced from fermentations with *L. thermotolerans* NCSU is more acidic, and beer from all phases ranged in pH from 3.54-3.65. From analyzing the Lambic-style beer (starting pH 5.35), it was determined that *L. thermotolerans* NCSU was producing lactic acid during fermentation (7.33 g/L, final pH 3.60). Comparatively, *Saccharomyces* can produce acetic acid during growth in a glucose medium (Woo, 2014) but has not been found to produce lactic acid.

Our research is in line with results found from wine fermentations; Kapsopoulou et al., (2005) found *L. thermotolerans* TH941 in grape must was able to produce 9.57 g/L of

lactic acid while *S. cerevisiae* SCM952 did not produce any; the starting pH of the grape must was 3.15 and *L. thermotolerans* and *S. cerevisiae* finished the 30 day fermentation with a pH of 2.90 and 3.06, respectively. In beer, lactic acid is typically only encountered when a contamination of lactic acid bacteria (LAB) occurs, as they are able to persist beers harsh environment.

Despite initial aerobic conditions of wort, it is known that brewer's yeast sugar metabolism occurs via the Embden-Meyerhoff-Parnas glycolythic pathway; in this pathway wort sugars are metabolized to pyruvate (Hutkins, 2006). From there, pyruvate is decarboyxlated (via pyruvate decarboxylase) and forms CO_2 and acetaldehyde. Acetaldehyde is then reduced (via alcohol dehydrogenase) to form ethanol (Hutkins, 2006). However, pyruvate also can also be reduced to lactic acid via lactate dehydrogenase (using NADH) when oxygen is limited or absent (Holzer, 2001); thus, it is possible that *L. thermotolerans* NCSU is using lactate dehydrogenase for the formation of lactic acid. This production of lactic acid makes *L. thermotolerans* NCSU unique as brewing yeast and gives the potential to create novel single-culture sour beers that do not rely on LABs and mixed culture fermentations.

Aromatics were also determined for the Lambic-style beer. A noticeable difference from previous results was that the compound acetaldehyde was not detected. Acetaldehyde is the major aldehyde present in beer, as it is an intermediate of ethanol and acetate (Lodolo, 2008); however, acetaldehyde has a flavor threshold of 10-20 mg/L and above its threshold results in a 'grassy' flavor (Meilgaard, 1975). High pitching rate, high fermentation temperature and high oxygen levels result in a high acetaldehyde concentration in beer (ASBC, 2014). In Phase 2 and 3 (laboratory scale), fermentations were conducted in flasks that were uncapped each day and gently swirled with the goal of achieving a homogenous sample; this practice would have increased the oxygen level of laboratory fermentations. Additionally, Phase 2 and 3 were both overpitched at $1x10^7$ cfu/mL (the desired pitching rate is $1x10^6$ cfu/mL). Both these practices likely attributed to the overproduction of acetaldehyde by *L. thermotolerans* NCSU in Phase 2 and 3. Meanwhile, Phase 4 was conducted in a gas-

tight stainless steel fermenter, yeast was not overpitched and aromatic analysis results showed *L. thermotolerans* NCSU did not produce acetaldehyde.

While Phase 1, 2, and 3 demonstrated *L. thermotolerans* NCSU can grow in flasks at laboratory scale, Phase 4 showed that strain NCSU can grow in a stainless steel fermenter, which is the typical beer production method. This successful fermentation is invaluable for the justification that *L. thermotolerans* NCSU can be used by industry to produce beer in the brewhouse environment.

4.5. Suggested Future Work

Future studies that could be conducted for brewing with *L. thermotolerans* NCSU would include flocculation properties, co-fermentations with *Saccharomyces* yeasts, and determination of how much ethanol the yeast can produce while remaining viable. Further, quantitative determination of aromatics, particularly acetaldehyde, should be investigated and the threshold of these compounds should be determined for acidic beer. Finally, sensory panels could be conducted to determine consumer liking of this new style of beer.

5. Conclusion

From the research conducted, both of the primary experimental objectives were achieved. First, *Lachancea thermotolerans* NCSU proved to be metabolically unique in comparison to the type strain, *L. thermotolerans* NRRL Y-8284 (ATCC® #56472); metabolic differences observed included that strain NCSU had overall gravity reduction, increased maltose utilization, further pH reduction, and increased production of ethanol and glycerol compared to strain NRRL Y-8284. Additionally, dissimilarities in amino acid utilization and flavor production were observed. These distinctions demonstrated that *L. thermotolerans* NCSU is a unique yeast.

L. thermotolerans NCSU also demonstrated that it can be used a brewing strain, utilizing primary wort sugars and producing sufficient ethanol concentrations to cover the average range of beer. Furthermore, *L. thermotolerans* NCSU could potentially be utilized

for high gravity brewing. The NCSU strain also established the ability to produce beer in a brewhouse environment. Compared to *Saccharomyces* species, *L. thermotolerans* NCSU has the unique ability to rapidly produce lactic acid and can be utilized to make pure-culture sour beers. To conclude, *L. thermotolerans* NCSU can be used at industrial scales for alcoholic fermentations as a single culture brewing yeast.

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APPENDICES

Appendix A (Phase 2 Data)

Table I. Utilization of Sugars and Production of Alcohol and Sugar Alcohol Throughout Fermentation of BB101 at 18°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	65.4	15.5	7.0	0.3	1.7	0.2%
1	64.4	10.7	5.6	0.6	4.2	0.5%
2	64.9	0.5	0.8	1.1	10.5	1.3%
3	43.2	0.1	0.3	1.5	17.4	2.2%
4	20.3	0.5	1.0	2.0	28.1	3.6%
5	10.0	1.1	1.9	2.1	33.8	4.3%
6	5.3	1.1	1.7	2.1	33.9	4.3%
7	4.0	n.d.	2.3	2.0	32.7	4.2%

Table II. Standard Deviations of Sugars and Alcohols Throughout Fermentation of BB101 at 18°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	4.0	0.7	0.5	0.0	0.2	0.0%
1	9.3	2.4	1.3	0.1	1.1	0.1%
2	10.6	0.2	0.4	0.1	1.3	0.2%
3	5.5	0.0	0.1	0.1	1.5	0.2%
4	4.9	0.4	0.5	0.1	1.5	0.2%
5	2.6	0.0	0.1	0.0	0.5	0.1%
6	1.9	0.0	1.1	0.3	4.9	0.6%
7	0.0	n.d.	0.1	0.1	0.6	0.1%

Table III. Utilization of Sugars and Production of Alcohol and Sugar Alcohol Throughout Fermentation of ATCC #56472 at 18°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	64.9	15.5	6.9	0.2	n.d.	n.d.
1	64.2	9.4	5.0	0.6	3.1	0.4%
2	57.5	n.d.	0.2	0.9	8.1	1.0%
3	68.3	n.d.	0.1	1.1	9.6	1.2%
4	63.3	n.d.	0.1	1.0	8.7	1.1%
5	62.6	n.d.	0.1	1.0	8.6	1.1%
6	62.2	n.d.	0.1	1.0	8.5	1.1%
7	61.4	n.d.	0.1	1.0	8.4	1.1%

Table IV. Standard Deviations of Sugars and Alcohols Throughout Fermentation of ATCC #56472 at 18°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	0.7	0.2	0.1	0.1	n.d.	n.d.
1	2.5	0.4	0.2	0.0	0.1	0.0
2	1.8	n.d.	0.1	0.0	0.2	0.0
3	4.7	n.d.	0.1	0.1	0.8	0.0
4	0.9	n.d.	0.1	0.0	0.1	0.0
5	3.7	n.d.	0.1	0.1	0.5	0.0
6	1.2	n.d.	0.1	0.0	0.2	0.0
7	1.4	n.d.	0.1	0.0	0.1	0.0

Table V. Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 18°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	5.6	1.1	141.6	$2.19x10^7$
1	4.6	1.0	123.5	$2.13x10^7$
2	3.9	1.0	104.0	$1.57x10^7$
3	3.7	1.0	88.8	$1.09x10^7$
4	3.7	1.0	63.8	$9.92x10^7$
5	3.6	1.0	49.9	$2.01x10^7$
6	3.6	1.0	46.0	$1.26x10^7$
7	3.7	1.0	45.9	$1.88x10^7$

Table VI. Standard Deviations of Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 18°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	0.0	n.d.	n.d.	$2.09x10^6$
1	0.2	0.0	1.7	$3.95x10^6$
2	0.0	0.0	3.2	$1.60x10^6$
3	0.1	0.0	6.5	$2.47x10^6$
4	0.0	0.0	3.9	$1.90x10^6$
5	0.0	0.0	1.8	$9.84x10^6$
6	0.0	0.0	0.5	$1.94x10^6$
7	0.0	0.0	0.9	$6.89x10^6$

Table VII. Acid Production, Density Depletion and Cell Counts Throughout Fermentation of ATCC #56472 at 18°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	5.6	1.1	141.6	$1.77x10^7$
1	5.0	1.0	128.7	$4.59x10^7$
2	4.7	1.0	111.8	$8.68x10^7$
3	4.7	1.0	114.1	$1.01x10^8$
4	4.8	1.0	115.1	$1.09x10^8$
5	4.8	1.0	108.0	$9.84x10^7$
6	4.8	1.0	111.4	$1.06x10^8$
7	4.9	1.0	108.9	$9.77x10^7$

Table VIII. Standard Deviations of Acid Production, Density Depletion and Cell Counts Throughout Fermentation of ATCC #56472 at 18°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	0.0	n.d.	n.d.	$4.58x10^6$
1	0.0	0.0	2.5	$5.52x10^6$
2	0.0	0.0	1.5	$3.80x10^6$
3	0.0	0.0	2.7	$7.60x10^6$
4	0.0	0.0	1.6	$5.54x10^6$
5	0.0	0.0	2.8	$1.67x10^7$
6	0.0	0.0	2.1	$8.16x10^6$
7	0.0	0.0	1.2	$8.68x10^6$

Appendix B (Phase 3 Data)

Table IX. Utilization of Sugars and Production of Alcohol and Sugar Alcohol Throughout Fermentation of BB101 at 22°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	49.2	8.6	4.7	0.3	0.9	0.1%
1	58.6	0.7	1.6	0.9	7.4	0.9%
2	25.9	0.1	0.3	1.1	14.8	1.9%
3	7.2	0.6	0.8	1.9	27.5	3.5%
4	4.1	0.6	0.7	1.9	28.9	3.7%

Table X. Standard Deviations of Sugars and Alcohols Throughout Fermentation of BB101 at 22°C.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	2.4	0.4	0.5	0.1	0.1	0.1%
1	3.7	0.0	0.3	0.0	0.1	0.0%
2	1.5	-0.1	0.3	0.0	0.4	0.1%
3	1.6	0.0	0.3	0.2	2.8	0.4%
4	0.6	0.0	0.3	0.1	1.0	0.1%

Table XI. Utilization of Sugars and Production of Alcohol and Sugar Alcohol Throughout Fermentation of BB101 at 22°C in High Gravity Wort.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	110.3	6.8	3.2	0.2	1.3	0.2%
1	113.1	0.9	1.7	0.7	6.7	0.8%
2	77.2	n.d.	0.4	1.2	16.4	2.1%
3	51.1	n.d.	0.3	2.1	34.4	4.4%
4	30.0	n.d.	0.3	2.6	45.7	5.8%
5	18.2	0.1	0.3	3.0	51.8	6.6%
6	11.5	0.2	0.4	2.9	52.0	6.6%
7	8.9	0.6	0.7	3.1	56.0	7.1%

Table XII. Standard Deviations of Sugars and Alcohols Throughout Fermentation of BB101 at 22°C in High Gravity Wort.

Day of Fermentation	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (g/L)	Alcohol by Volume (%)
0	8.5	0.3	0.4	0.0	0.3	0.0%
1	7.5	0.0	0.3	0.0	0.1	0.0%
2	9.3	n.d.	0.3	0.1	1.5	0.2%
3	6.2	n.d.	0.3	0.1	1.7	0.2%
4	3.0	n.d.	0.3	0.1	1.0	0.1%
5	1.5	0.0	0.3	0.2	1.3	0.2%
6	1.0	0.1	0.4	0.1	1.4	0.2%
7	1.1	0.1	0.3	0.0	1.1	0.1%

Table XIII. Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 22°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	5.28	1.043	116.7	$1.54x10^7$
1	3.91	1.039	106.0	$1.65x10^7$
2	3.62	1.027	75.1	$1.46x10^7$
3	3.50	1.017	48.1	$1.35x10^7$
4	3.54	1.015	43.9	$1.26x10^7$

Table XIV. Standard Deviations of Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 22°C.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	0.02	0.001	2.1	$5.55x10^6$
1	0.01	0.000	0.8	$1.78x10^6$
2	0.02	0.001	2.7	$3.62x10^6$
3	0.00	0.002	3.9	$2.16x10^6$
4	0.01	0.000	0.7	$4.73x10^6$

Table XV. Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 22°C in High Gravity Wort.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	5.26	1.064	172.8	$9.67x10^6$
1	3.96	1.060	160.8	$1.91x10^7$
2	3.63	1.048	130.6	$1.20x10^7$
3	3.47	1.032	87.8	$1.23x10^7$
4	3.46	1.023	64.3	$1.50x10^7$
5	3.47	1.018	51.2	$4.50x10^7$
6	3.52	1.015	42.8	$1.84x10^7$
7	3.54	1.013	39.5	$2.31E+0^7$

Table XVI. Standard Deviations of Acid Production, Density Depletion and Cell Counts Throughout Fermentation of BB101 at 22°C in High Gravity Wort.

Day of Fermentation	pH	Density (g/cm^3)	Sugar (g/L)	Cell Count (cfu/mL)
0	0.00	0.001	3.9	$1.41x10^6$
1	0.01	0.002	4.1	$4.28x10^6$
2	0.04	0.002	4.3	$1.68x10^6$
3	0.01	0.001	3.1	$2.49x10^6$
4	0.01	0.001	2.3	$7.97x10^6$
5	0.02	0.000	1.2	$5.77x10^6$
6	0.00	0.000	1.1	$4.47x10^6$
7	0.01	0.001	1.7	$4.09x10^6$

Appendix C (Phase 4 Data)

Table XVII. HPLC analysis of Lambic sugar utilization.

Day	Maltotriose (g/L)	Maltose (g/L)	Glucose (g/L)	Fructose (g/L)	Total Sugars (g/L)
0	29.0	67.0	18.7	6.8	121.5
1	25.5	62.7	15.0	6.6	109.8
2	27.5	66.5	11.6	6.1	111.7
3	27.5	66.2	5.1	4.3	103.1
4	24.5	59.4	0.6	1.7	86.2
5	27.4	65.7	n.d.	1.4	94.4
6	25.2	60.6	n.d.	0.7	86.4
7	27.9	65.9	n.d.	0.7	94.5
8	23.0	55.1	n.d.	0.5	78.6
9	25.0	59.5	n.d.	0.6	85.0
12	23.9	56.5	n.d.	0.5	81.0
13	22.1	50.3	0.7	0.4	73.6
14	13.4	29.8	1.1	n.d.	44.4
15	13.4	25.9	1.1	n.d.	40.4
16	13.4	22.9	1.3	n.d.	37.6
17	9.6	17.5	1.7	n.d.	28.7
18	8.1	15.2	1.5	n.d.	24.8
19	6.9	12.7	1.4	n.d.	20.9
20	5.1	9.7	1.0	n.d.	15.8
25	4.8	3.5	0.8	n.d.	9.0
33	4.7	3.1	0.5	n.d.	8.3
41	4.6	3.0	0.6	n.d.	8.2
Δ Utilized	24.4	64.0	18.1	6.8	113.3
% Utilized	84%	96%	97%	100%	93%

Table XVIII. Lactic acid, pH, glycerol and ethanol analysis of Lambic fermentation.

Day	pH	Lactic Acid (g/L)	Glycerol (g/L)	Ethanol (g/L)	ABV (%)
0	5.4	n.d.	n.d.	n.d.	n.d.
1	5.3	1.0	n.d.	n.d.	n.d.
2	4.5	2.4	n.d.	1.6	0.2%
3	4.0	3.8	0.6	5.6	0.7%
4	3.6	6.1	1.1	7.5	1.0%
5	3.6	6.9	1.3	9.0	1.1%
6	3.6	6.3	1.2	8.6	1.1%
7	3.6	7.0	1.4	9.5	1.2%
8	3.6	5.7	1.1	7.8	1.0%
9	3.6	6.4	1.2	8.8	1.1%
12	3.6	6.9	1.3	11.6	1.5%
13	3.6	7.4	1.6	17.2	2.2%
14	3.6	5.3	1.1	18.0	2.3%
15	3.6	6.1	1.4	24.7	3.1%
16	3.6	7.6	1.9	36.6	4.6%
17	3.6	7.6	2.1	42.5	5.4%
18	3.6	7.4	2.1	43.5	5.5%
19	3.6	7.5	2.2	47.1	6.0%
20	3.6	6.9	2.0	45.0	5.7%
25	3.6	7.4	2.4	54.0	6.8%
33	3.6	7.5	2.4	55.3	7.0%
41	3.6	7.3	2.4	53.8	6.8%