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Comparison of *Saccharomyces cerevisiae* and the novel wild yeast used in beer fermentation and their future in industrial biotechnology.

By

Patience Zviuya

2090726

DISSERTATION

Submitted in fulfilment of the requirements for the degree.

Master of Science by Research

In

Molecular and Cell biology

In the faculty of science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Professor Karl Rumbold

Co-Supervisor: Dr Sanchia Moodley

DECLARATION

I declare that the entirety of the work contained in this thesis for the master's in science degree, except where otherwise indicated, therein is my original work and has not in its entirety or in part submitted by me for obtaining any qualification at another university. I furthermore cede a copy of the thesis in favor of the University of the Witwatersrand.

Signature: 

Date: 06 June 2024

ABSTRACT

The alcohol industry has grown over centuries due to the increase in alcohol demand by customers. Beer consumers now understand the brewing process and they well understand the role of yeast in fermentation. The alcohol industry now has a lot of customers because of the growth that this sector has made, and this has resulted in an increased demand for new beer styles and high flavor profile beers. Due to this demand, research has been conducted on unconventional wild yeasts that can be employed in making beer, demonstrating the variety of fermentation yeasts that are available and capable of enhancing beer quality and producing a wide range of new beer varieties. The commercial *S. cerevisiae* yeast and eight wild yeasts (*Samson's Saison, Ragnarok, Dark knight, B. brusc, Neipa, The Proletariat, La Trappist* and *B. clauss*) were used to ferment pale ale in different fermentation vessels. The commercial yeast was the control of this research because this yeast has been used for generations in the brewing industry. The wild yeast strains used were identified using sanger sequencing and seven of these yeasts were *S. cerevisiae* wild species with similarity index of more than 80% and one was *B. bruxellensis* strain similarity index more than 95% before and after fermentation. The research outcomes demonstrated that most of the wild yeast performed the same as the commercial yeast in terms of physical and chemical parameters however most of the wild yeast produced more volatiles and esters as compared to the commercial brewing yeast. Commercial *S. cerevisiae* produced the highest alcohol content 4.5% and the average alcohol content for wild yeast was 3.4% because they are challenging to regulate during fermentation and have low alcohol tolerance unlike the commercial yeast that has been harvested and used for generations.

Overall, the utilization of unconventional wild yeast to make beer was identified as a promising alternative to produce beers with exotic flavours and alcohol-free beers. Future work identifying specific yeast that suit different fermentation processes and beer types are recommended.

DEDICATION

This thesis is dedicated to my daughter who means the entire world to me. Thank you for being the source of determination and motivation to do great things in my life and I will keep fighting to give you the life that you deserve. This thesis is also dedicated to my mother, who serves as both a mentor and a source of strength for me. I appreciate your financial assistance, encouraging words, and belief in me. You have helped me become a strong, self-assured lady who believes she can accomplish anything she sets her mind to, and you never cease to amaze me. You have taught me that prayer conquers everything, and I prayed every step of the way whilst writing my thesis. You are incredibly fortunate to be my mother; you have never given up on me. Lastly, I dedicate this thesis to Leonard Zviuya, my late father, I did this project to fulfil the promise I made to him before his passing. Thank you for being the best father a girl can ever ask for and may you continue to rest in peace until we meet again, forever in heart.

To God, be the glory.

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List of symbols and abbreviations

°C: Degrees Celsius

mg/l: milligram per litre

ppm: parts per million

%: percent

et al: and others

g: grams

kg: kilograms

ml: millilitre

L: litre

10x: ten times

$: Dollar

μm: micrometre

kb: kilobases

DO: dissolved oxygen

CO2: carbon dioxide

SO2: Sulphur dioxide

YPDA: yeast peptone dextrose agar

S. cerevisiae: Saccharomyces cerevisiae

B. bruxellensis: Brettanomyces bruxellensis

CCVs: Cylindroconical vessels

ITS: internal transcribed spacer

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

Sec: Second

Min: Minute

EBC: European Brewery Convention units

HPLC: High-Pressure Liquid Chromatography

GC: Gas Chromatography

rpm: revolutions per minute

bp: base pairs

CHAPTER 1

1.1 Introduction

Microorganisms such as fungi, yeasts, bacteria, and algae play an important part of food and beverage manufacturing processes (Capece et al., 2018). Amongst these microorganisms' yeasts are the most notable for industrial fermentation, producing a variety of products in commercial food operations, including fermented beverages, chocolate, coffee, bread, and countless other food components (Avallone et al., 2001). A few properties which make yeast formidable microorganisms in the food and beverage industry is their ability to withstand harsh fermentation stresses, have short replication times and are simple to cultivate (Liszkowska & Berlowska, 2021).

Humans have benefited greatly from the widespread use of microbial fermentation as a metabolic process. Due to their significance in both the economy and culture, fermented drinks like lagers and ales have played a major role since the discovery of yeasts and their capacity for fermentation (Bruner et al., 2021). Microbial fermentation dates back centuries from the time of the agricultural revolution (Gibbons and Rinker, 2015). Globally, most of the manufactured food is fermented and this has a huge impact on the world's economy because fermentation has allowed to produce storable food products in early times when storage technologies were limited, and spoilage was a major problem, (Zilberman and Kim, 2011). By turning ordinarily perishable items like grapes into wine and pickling different food items, fermentation improves welfare during the winter or other less fruitful seasons.

The most frequently used yeasts in industrial processes are *Saccharomyces cerevisiae* (Walker & Stewart, 2016). *S. cerevisiae* is traditionally used to make alcoholic beverages and to ferment bread (Jespersen, 2003). More recently, new techniques in biotechnology have led to *S. cerevisiae* being used in the bioethanol industry as well as in the medical sector to produce medicines like hepatitis vaccines, insulin, and human papilloma virus serums (Nielsen, 2013). The brewing sector has benefited a lot from yeasts research because their manufacturing process solely depends on this fermentation organism. Brewing laboratories have made important developments in the identification of yeasts and the preparation of single cell cultures (Barnett, 1998, 2003, 2007). New technologies in industrial biotechnology have led to an evolution in the small- and large-scale brewing sector which was mainly triggered by customer demand for diversity in beers which are available on the market (B. Gibson et al., 2017). There are various modern brewing techniques used in alcohol production which evolved from ancient

brewing based on the type of yeasts and ingredients that early brewers used (Iorizzo et al., 2021a). Yeasts used in beer production are very important due to the different flavors produced by the various yeasts' strains (Maicas, 2020). While beer makers commonly use yeasts strains like *S. cerevisiae* known as the brewer's yeasts, the demand for beer with a better flavor profile has led to the use of wild yeasts strains like non-*Saccharomyces* yeasts which possess important characteristics for industrial fermentations. Non-*Saccharomyces* wild yeasts possess unique traits such as being able to breakdown complex sugars in wort during fermentation as well as producing exotic flavor compounds (Nyhan et al., 2023).

Beer quality is determined by type of yeasts strain used, its activity on the sugars in the wort as well as the aroma they produce due to the production of volatiles and esters as secondary metabolites (Capece et al., 2018). Beer fermentation is a technique which relies on yeasts such as *S. cerevisiae* and any other microbes are considered as foreign organisms (Callejo et al., 2017). However, the brewing sector now has a lot of consumers and an increased demand for innovative beer products. This demand has led to the use of novel wild yeasts and uncharacterized starter cultures which produce unique and exotic beer flavors. Efforts have been made to produce beer with high sensory profiles (Humia et al., 2019) and this has resulted in brewers opting to use wild yeasts like non-saccharomyces (Ubeda et al., 2014). Wild yeasts are generally more sensitive to fermentation stresses caused by the by-products of fermentation like ethanol, carbon dioxide, lactic acid, and hydrogen gas (Maicas, 2020). Wild yeasts produce low yields of byproducts; however, they provide unique aromatic compounds and high flavor profiles (Samoticha et al., 2019). Wild yeasts such as non-*Saccharomyces cerevisiae* yeasts strains can be used to manufacture light beers with a lower alcohol content and zero percent alcohol beverages (Capece et al., 2018).

1.2 Aim of the study

The aim of the study is to compare the brewing capability of numerous unconventional wild yeast strains to commercial yeasts, *S. cerevisiae* which is the control in improving beer quality through comparison of the esters, volatiles, aroma, and taste of beer made with different yeasts strains.

Specific objectives:

- To sample and isolate wild yeasts using enrichment culturing methods.
- To evaluate the potential of different yeast strains on their potential for brewing and fermentation of beer.
- To evaluate flavor profiles produced by the wild yeast and *S. cerevisiae* after fermentation.
- To compare fermentation metabolites produced by commercial and wild yeasts fermented beer.

CHAPTER 2

LITERATURE REVIEW

2.1 General yeast cell biology

Yeasts are eukaryotic microorganisms found in wide ecological niches, mainly water, soils, air, on plants and fruit surfaces (Maicas, 2020). Classified as fungi, yeasts perform a crucial role in various ecological functions such as metabolism, decomposition, and fermentation in natural environments. Typically yeasts cell sizes are 5×10µm (Figure 2.0), with similar organelles of mature eukaryotic cells such as the golgi apparatus, mitochondria, endoplasmic reticulum, vacuoles, and cytoskeleton (Botstein et al., 1997; Sherman, 2002; Tofalo & Suzzi, 2016). Generally, considered as single-celled organisms, yeasts reproduce by budding by single cells or divide by fission and vary in shapes i.e., globose, oval, elongated and rectangular (Tofalo & Suzzi, 2016). For example, species in the genus *Saccharomyces* reproduce primarily by budding, while yeast in *Schizosaccharomyces* through fission, a direct division process (Vyas et al., 2021).

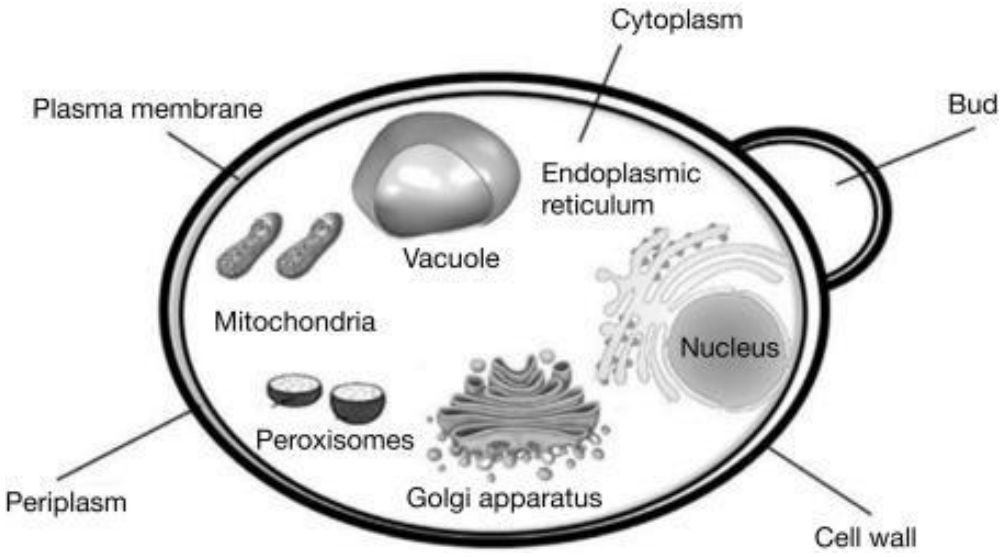


Figure 2.0: Structure and morphology of a typical yeast cell (Source: Tofalo & Suzzi, 2016)

The optimal growth conditions for yeasts reproduction include temperatures between 20- 28°C, while pH ranges between 3.5 to 4 (J. Ferreira & Du Toit, 2004). In some instances, yeasts can reproduce rapidly under very strict anaerobic conditions, especially in liquid environments (Parapouli et al., 2020). To date, reports have identified approximately 90 genera containing 1000 yeasts species. These include those classified as Ascomycetes (57), Basidiomycetes (36),

and 2 unclassified. Unlike other fungi such as molds and mushrooms, yeasts do not produce secondary toxic metabolites.

2.2 Use of Yeasts in Industrial Processes

Considered as man's oldest industrial microorganism, yeasts are used in the process of fermentation, food preservation and biotechnology (Mannaa et al., 2021). Yeast have been used in beverage and food manufacturing for more than 5000 years (Samuel, 1996). However, the work by Louis Pasteur in the late 1860's expanded on the use of yeasts in industrial processes and alcohol fermentation (Manchester, 2007). With advancements in molecular and microbiology techniques, it has become possible to isolate yeasts in pure culture forms for industrial use for example in food (bread) and beverage (beer) manufacturing (Tamang et al., 2016), and with the human society. Notably, yeasts in the genus *Saccharomyces* are the most used in food and beverage manufacturing (Walker & Stewart, 2016). These microorganisms have been employed to ferment sugars from grain crops such as maize, *hordeum vulgare* (barley) and sorghum to make alcohol and raising dough during baking (Parapouli et al., 2020). In addition, yeasts are often supplemented or added as an additive in commercial products such as soy sauce, canned meats, cheese, yoghurt, and bakery items because yeasts are a taste booster in processed foods due to the peptides and amino acids produced during autolysis of the yeast's protein (Tao et al., 2023). Yeasts are often taken as vitamin supplements to improve human health and productivity because scientific research in food technology has proved that yeasts are rich in folic acid, proteins, vitamin B and niacin (Kennedy, 2016). Other commercial products such as animal feed contain varying proportions of live and dead yeasts cells for animal nutrition, (Pang et al., 2022). Yeasts is also used in animal feed as a probiotic because it offers health benefits to animals when administered in the right quantities. The genera *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, and *Candida* contain the yeast strains that are primarily utilized as probiotics in animal feed (Elghandour et al., 2020). Within these genera, there are species such as *K. fragilis, S. boulardii, C. pintolopesii, K. lactis, C. saitoana,* and *S. cerevisiae* (Kumura et al., 2004).

2.3 Yeast in Alcoholic Beverage Fermentation

Alcoholic fermentation is the most imperative stage during the manufacturing of alcoholic beverages such as wine and beer ("Metabolomics: Wine-Omics.," 2008) The process incorporates yeast in multifaceted biochemical steps which change sugars to ethanol, carbon dioxide, volatiles, and esters (Maicas, 2020). These byproducts make up the body and taste of

alcoholic beverages (Maicas, 2020). In addition, some alcoholic beverages such as red and white wines are further subjected to an additional fermentation step, known as malolactic fermentation (Paramithiotis et al., 2022). This secondary fermentation involves the use of lactic acid bacteria which carry out deacidification and contribute to flavor and microbial stability of the wine (Virdis et al., 2021) however lactic acid is only used in some red and white wines during malolactic fermentation. Most wines use yeast in secondary fermentation. For example secondary fermentation is achieved by *Schizosaccharormyces pombe* which is the most used yeasts in wine making because other yeasts like *S. cerevisiae* are not able to efficiently degrade l-malate hence it cannot be used in wine making.

Selection of yeasts for alcoholic beverages play a pivotal role in maximizing alcohol yield, at the same time maintaining beverage sensory qualities (Walker & Stewart, 2016). Different strains of yeasts, when used in the fermentation process, have a prodigious effect on final product, influencing beer sensory. Various yeasts are used commercially to make several types of alcoholic beverages (Table 2). In the brewing industry pure yeasts strains are selected for fermentations such as winemaking, distilled spirit production, and beer brewing (Budroni et al., 2017). It is customary in brewing to refer to lager yeasts as bottom strains of *Saccharomyces carlsbergensis* and to ale yeasts as top strains, which are typically utilized in top fermentation (Stewart et al., 2013). Top fermentation is a harsh fermentation that is performed at very high temperatures which forces the yeasts cells to go to the top of fermentation vessels. These type of yeasts ferments at higher temperatures and bottom fermentation uses yeasts that ferments at lower temperatures for a long period of time. However, all brewing strains are now classified as *S. cerevisiae* by modern yeasts systematics, and many ales are now prepared by bottom fermentation using what were once top strains (Dzialo et al., 2017).

Table 2.0: Types of yeasts used in commercial alcoholic beverages production.

Yeasts strain (s)	Alcoholic Beverage	References
Saccharomyces pastorianus	Lager beers	(Gyurchev et al., 2022)
Saccharomyces cerevisiae	Ale	Stewart, 2016
Brettanomyces bruxellensis	Lambic beer	Serra Colomer et al., 2019
Saccharomyces bayanus	Wine	Januszek et al., 2020
Schizosaccharomyces pombei	Rum	Stewart, 2022
Kluyveromyces marxianus	Cheese whey derived beverage	(Rivera Flores et al., 2023)
Zygosaccharomyces bailii	Kombucha	VillarReal-Soto et al., 2018

2.4 Beer production

Beer production is one of the ancient techniques in food production which has continued to grow over the years and has had a positive impact on the economic status of the world (Raihofer et al., 2022). The fermentation of beer using yeasts entails making wort from malted cereal, water, and hops (Humia et al., 2019). Beer is produced in stages which include malting (the conversion of cereal to malt), mashing (grain starches are converted into sugars by malt enzymes.), fermentation, maturation, and packaging (Figure 2.1) (Rachwał et al., 2020). Although the full biochemical processes of ancient brewing were not understood, ancient brewers selected the most promising yeasts which produced the best fermentation results. *S. cerevisiae* is now called brewer's yeasts because it outperformed other yeasts during fermentation and it has been cultured for centuries by brewers (Legras et al., 2007).

With an estimated 360 million hector litres of beer produced annually, China ranks first in the world's beer production rankings. United States comes in second with an estimated volume of 195 million hector litres per annum. Today, the brewing industry is a global enterprise. Over

$107.6 billion was spent on beer by Americans in 2016, surpassing the amount spent on wine, spirits, and water combined. In Africa the leading beer producing country is South Africa which produces an estimated 31 million hector litres of beer annually and ranked as the 12th biggest beer consuming country in the world. South African Breweries company produces the highest beer volume in South Africa with 87% market share in beer industry. The craft beer sector saw the biggest gains in sales in recent years, rising 10% between 2015 and 2016, significantly outpacing the general beer category, which saw rises of only 1.3% to 3.5%. In terms of nutrition, moderate beer drinking has been linked to possible changes in lipid profiles and a lower risk of osteoporosis. For instance, it has been stated that beer includes soluble fibre, antioxidants, and trace levels of B vitamins, calcium, copper, potassium, manganese, selenium, and iron, all of which are good for human health. The alcohol percentage of beer ranges from 3% to 4%, with the majority of pale lagers having 4% to 6% alcohol (Sherrell 2023).

2.5 Brewing process

2.5.1 Malting and Milling

Rough oats, wheat, barley, or rye are germinated in a malt house before being used in brewing. It is customary to "steep" or soak fully matured grains in cold water until they become saturated (Kabirullah & Rahman, 2001). The grains are put into shallow tanks and agitated and aerated after the water is changed once a day for 40 to 70 hours (Eaton, 2017). Enzymes that transform starch into sugar are released during germination, including malt diastase (Lekjing & Venkatachalam, 2020). The grain is put into a kiln to be dried and/or roasted following germination, which can take up to six days. Over several hours, the kiln's temperature is progressively raised (Holt et al., 2019). Beer color and flavor are significantly influenced by germination circumstances, roasting temperatures, and timeframes.

After the product has finished kilning, it is called malt. The majority of the carbohydrates and sugars are found in the endosperm, which is exposed after the material is milled to split apart the kernels (Neylon et al., 2020). Hammer mills or roller mills (sometimes called grind mills) are commonly used for dry milling of grains. The ideal grinding parameters break up the grains just enough to reveal the starchy core of the seed without rupturing the protective hulls (Hourston et al., 2017). In contemporary plants, hydrating grain prior to milling increases the husk's pliability and decreases breakage. If the crush is too coarse, not enough starch will be available for fermentation. During the leaching process, the husks act as a bed for filtering the brew; if the crush is too fine, the husks will be destroyed, and the beverage will turn sticky and

unusable. Hammer mills are often used to produce a finer mash when mash filters are going to be employed during lautering (van Donkelaar et al., 2016).

2.5.2 Mashing and Lautering

After the grain is ground, it is combined with hot water in a sizable container known as a mash tun to create mash (Devnani et al., 2023). A good beer's production depends heavily on the quality of the water used for mashing. To get the highest level of enzyme activity during the mashing, make sure the acid and calcium contents are appropriate. Saccharification, a process that can be used for fermentation, is the result of mashing starches into sugars (Vuong Mai & Vuong Mai Procédé, 2022). Additionally, during mashing, complex proteins are broken down, which lessens haze in beer. Mashing typically takes an hour or two, during which time temperatures are carefully regulated to maximize the breakdown of proteins and carbohydrates. Lower temperatures are used to make dry beers because they produce more highly fermentable sugars. Higher temperatures result in stronger, sweeter beers. These days, mash processes can include the addition of enzymes. Raising the temperature to approximately 75°C after mashing encourages the release of more starch and reduces the viscosity of the mash before lautering.

After mashing, the procedure known as "lautering" involves removing the liquid wort from the solid grains' material (Russey Russey, 2016). To begin the lautering process, the mash is placed into a lauter tun, which is a vessel with a false bottom. The next step is sparging, which removes the clear wort from the spent grains. At the end of the mash, sparging is the process of adding extra water to rinse the leftover grains and extract more sugars (Ledley et al., 2021). In order to prevent disturbing the spent grain bed that filters the wort, this is done gradually. To prevent the extraction of unwanted bitter tannins, sparging should not be done for an extended period of time. Some lauter tuns have knives or rakes which slice through the layer of wasted grains to keep the flow nice and steady. Another option is while lautering with a mash filter, grain can be used as a filtration medium in place of grains. Plate-in-frame filters, or mash filters, have plates inside of them to support filter cloths. Additionally, bladders found in more recent mash filters force liquid out while sparging.

2.5.3 Boiling

Hops are added to the boiling wort in a big copper brew kettle (Chrisfield et al., 2020). Because of its excellent heat transmission qualities, copper has historically been the metal chosen for the brew kettle. In addition to sterilizing the beer, boiling releases the bitterness from the hops by isomerizing their acids (Thesseling et al., 2019). Hops get more bitter as they simmer longer

because more alpha acids are isomerized during this process. You can add hops to the boiling process at any point. (Klimczak & Cioch-Skoneczny, 2023). Through their oils, hops incorporated at the end of the boiling process give the beer taste and aroma instead of bitterness. After the boil is finished, the mixture is thrown into a whirlpool to get rid of coagulated proteins and hop matter (Aroh, 2019). The wort is taken from the tank by being forced into the center of the bottom by the centripetal force of the whirlpool. The beer is then rapidly chilled to stop oxidation and the development of bad flavor. In contemporary breweries, plate heat exchangers are used for cooling.

2.5.4 Fermenting

The fermenter, which is typically a sizable steel and stainless tank is where the wort is transferred after it has cooled and aerated (Pires & Bányik, 2015). After adding yeast, the wort starts to ferment, producing carbon dioxide and alcohol from the carbohydrates. Warm, cool, and spontaneous fermentation are the three basic techniques. Saccharomyces cerevisiae is one type of yeast used in warm fermentation, which occurs at temperatures between 15°C and 20°C (Liszkowska & Berlowska, 2021). Warm fermenting beers are often ales that can be consumed three weeks after fermentation starts. Around 10°C is the starting point for cool fermentation, which continues at nearly freezing temperatures for several weeks to months during storage (lagering) (Aroh, 2019). Lagers are supposed to have a cleaner flavor due to cool fermentation. Yeast inoculation is not a part of spontaneous fermentation. Malt liquors can ferment for more than a year in wooden barrels utilizing indigenous bacteria found in the wood (Vermote et al., 2023).

2.5.5 Biochemistry of beer Fermentation

The goal of wort fermentation is to continuously break down the components of wort into ethanol, carbon dioxide, and other fermentation products in order to produce beer that is stable and of acceptable quality. (Stewart 2006). Both strains of *S. cerevisiae* and non-*S. cerevisiae* are capable of absorbing and fermenting a variety of sugars, such as glucose, fructose, galactose, mannose, maltose, and maltotriose. Furthermore, the release of glucoamylase enables *Saccharomyces diastaticus*, a subspecies of *S. cerevisiae*, to utilize dextrin material. Any sugar's first use usually involves either its intact passage across the cell membrane or its hydrolysis outside the membrane, with part or all of the hydrolysis products then entering the cell (Carbo and Rodriguez 2023). While sucrose and dextrins are digested by extracellular enzymes (glucoamylase, or amyloglucosidase in the case of sucrose) and the products of the

breakdown are taken up by the cell, maltose and maltotriose are examples of sugars that pass through the cell membrane intact. The aglucosidase system hydrolyzes both sugars to glucose units once they are within the cell. By fermenting the sugars in cereals, yeast cells produce CO_2 and ethanol. At the same time, hundreds of secondary metabolites are produced that influence the flavor and aroma of beer. Variations in these metabolites amongst yeast strains account for the unique way that yeast affects beer flavor (Dzialo et al., 2017). The VDKs 2,3-pentanedione and diacetyl (2,3-butanedione) give beer an unpleasant smell similar to old milk or butterscotch. According to Stewart et al. (2013), both VDKs are produced in beer as byproducts of the processes that produce valine and isoleucine. Part of the a-acetohydroxy acids, which are biosynthetic intermediates, is eliminated into the fermenting medium. Here, they go through an oxidative decarboxylation process that spontaneously produces VDKs.

2.5.6 Conditioning and Filtering

Upon the yeast consuming all accessible carbohydrates, primary fermentation comes to a stop (Maicas, 2020). Throughout conditioning, the live yeast continues to function, absorbing undesirable flavors created throughout fermentation. For ales, this procedure can take a week, while for lagers, it may take many months. Some lager brewers speed up the process by injecting still-fermenting wort to resume fermentation, a procedure called as krausening. The dormant yeast is then removed from the fermentation tank by mechanical filtration, filtering agents, or both (Bušić et al., 2018). Dormant yeast settles to the bottom of the tank after that. Filtration stabilizes and adds a clean sheen to the flavor of beer. Filters might be sheets or a fine powder such as diatomaceous earth.

2.5.7 Pasteurization and Packaging

To eradicate any leftover yeast and stop more ethanol synthesis, beer can be pasteurized (Milani & Silva, 2022). Real draft beers are not pasteurized; instead, they need to be refrigerated to retain flavor and reduce any residual yeast activity. Common packaging for beer includes kegs, cans, or bottles. Extra carbon dioxide gas may occasionally be added during bottling (Speers & MacIntosh, 2013). The final aroma of the beer can be enhanced by adding back carbon dioxide from fermentation tanks. Bottle conditioning is a technique used by certain brewers to extend the shelf life of their beer and exclude any oxygen from the bottle. After packaging, it entails adding wort, sugar, or yeast to start the second fermenting process in the container that was sealed. This results in an increase in flavor depth and natural carbonation.

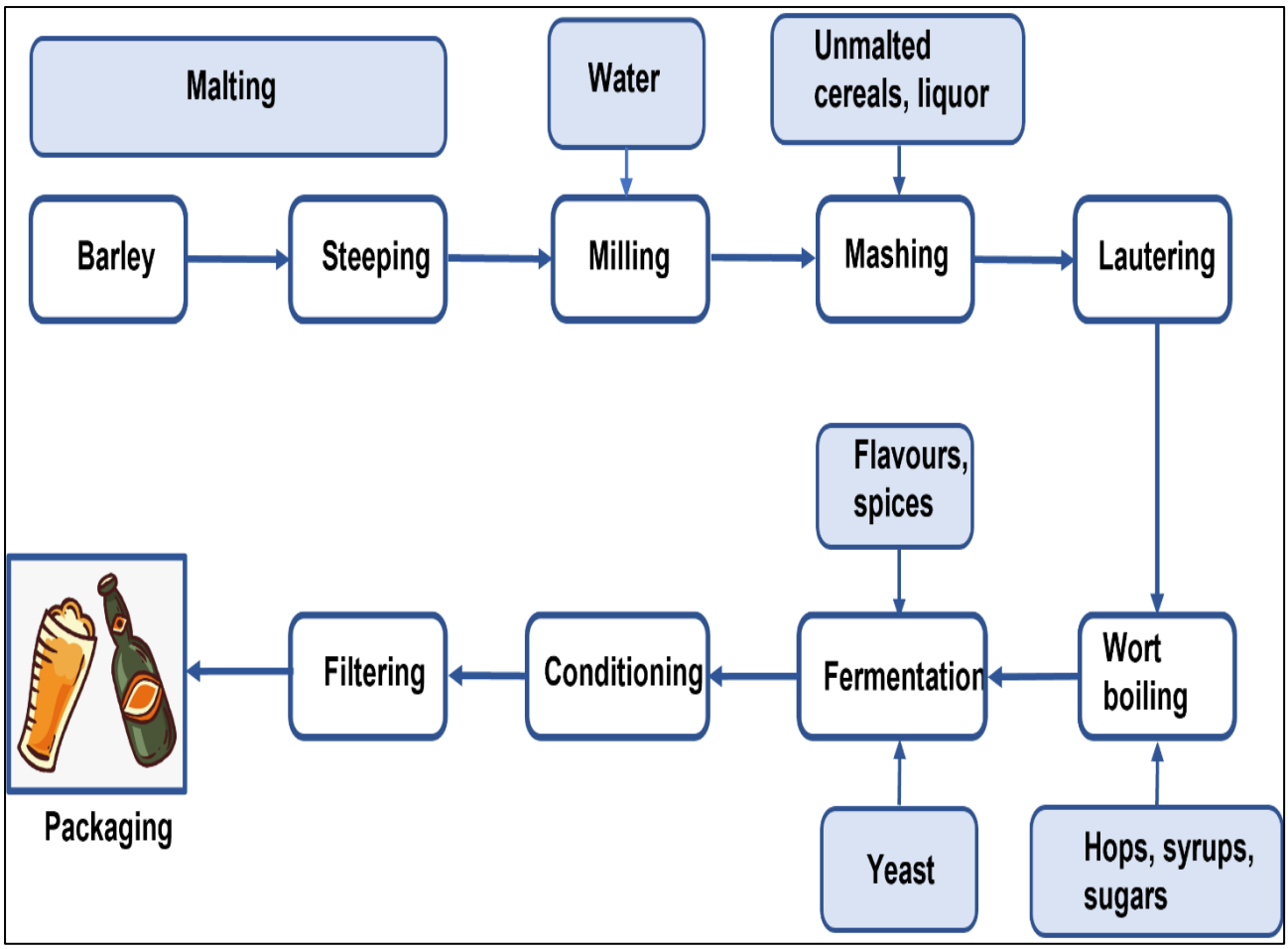


Figure 2.1: Flow diagram showing the process of beer production. Adapted from Application in Wine and Brewing industry (Chakraborty et al., 2016).

2.6 Fermentation systems in Beer Production

Over time, fermentation systems have developed. Fermentation systems were initially based on the characteristics of the yeasts, beer type, and fermentation vessel material availability (Boulton & Quain, 2006). Fermenters can be open or closed squares, horizontal or vertical cylindrical tanks, or any combination of these. Cylindroconical vessels (CCVs) are commonly used in batch fermentation, and they have a cylindrical shape and cone base (Sajid Jan et al., 2018). CCVs have become the favored technology in modern breweries because they are cheap to buy and maintain, they have high vessel usage, CIP efficient and CO2 collection. Stainless steel is commonly used in the construction of CCVs. Strength and rigidity are desired attributes, as are inertness and corrosion resistance, cleanability, malleability, and heat conductivity. Application of continuous fermentation methods in brewing is limited because of increased risk of bacterial contamination and genetic drift of the culture (Boulton & Quain, 2006). Pilot-scale research on a 20 Litre reactor system demonstrated that producing lagers using a

continuous, immobilized system was viable. For over 14 months, in terms of fermentation effectiveness and flavour compound production, the primary fermentation remained constant (Virkajarvi, 2001).

Commercial immobilized reactor development has been very beneficial when used for rapid beer maturation (Strobl & Strobl, 2019). However, separate studies to optimize different continuous fermentation systems yielded inconsistent results, particularly in terms of reaching the desired taste characteristics (Dragone et al., 2007). Continuous systems will require much more study and development before replacing present fermentation techniques in beer making sector. The rate and extent of yeasts development can be controlled using process control criteria like an adequate feed supply, the right inoculation (pitching) rate, the right amount of dissolved oxygen (DO), and temperature management (Lodolo et al., 2008). The fermenter design is determined by yeasts flocculation properties. Ale yeasts (top yeasts) may float and can capture CO2 bubbles to produce a yeasts "head" that can be removed from the top of fermentation vessels. Yeasts cells group together and form flocs which settle at the bottom of CCV cones hence lager strains (bottom yeasts) are most suited to CCVs (Lodolo et al., 2008).

2.7 Wort Fermentation

Wort fermentation involves a consistent process of metabolization of wort ingredients to produce fermentation byproducts with suitable quality and consistency (Stewart, 2016). In beer production the yeasts culture can be cropped and used in the next fermentation vessel, but in other fermentation processes such as distilled alcohol manufacturing and cider production, the yeasts culture is only used once (Maicas, 2020). It is critical to zealously guard harvested yeasts because only pure starter cultures can be pitched in future wort fermentation to acquire beer that does not have off flavors which are normally caused by spoilage microorganisms when contaminated yeasts is used. The study of the molecular biology of the brewer’s yeasts has become a subject of interest in industrial biotechnology throughout the years (Stewart, 2016). The properties of the brewer’s yeasts strains used, as well as the fermentation temperatures, are key variations in the manufacture of top and bottom fermented beers. Many breweries and universities have performed extensive study on this issue (Gallone et al., 2016), and the usual variances between lager and ale yeasts strains have been documented. Advanced technology in molecular biology such as gene sequencing has shown that some brewing yeasts strains such as *Saccharomyces pastorianus* is an interspecies hybrid of *S. cerevisiae* and *S. eubayanus* (Monerawela et al., 2015).

2.8 Brewing yeast

2.8.1 *Saccharomyces cerevisiae*

S. cerevisiae possesses desirable characteristics and is hence called the ideal eukaryote and a unique organism for scientific research (Parapouli et al., 2020). The major reason why *s. cerevisiae* is the ideal organism for industrial processes is because of one of its features known as 'make-accumulate-consume' (Pfeiffer & Morley, 2014). This part of its lifestyle is based on the Crabtree effect, whereby *S. cerevisiae* yeasts strains under normal respiratory conditions does not use respiratory machinery to break down sugars and increase biomass growth but rather produces ethanol and other by-products (Hagman et al., 2014). *S. cerevisiae*, well known as the brewer's yeast is a unicellular eukaryotic organism which is made up of genetic material of 12068 kilobases (kb) of DNA which are arranged in 16 chromosomes (Avramia & Amariei, 2021). *S. cerevisiae* was the first eukaryote to go through genome sequencing, it is the first unicellular organism to have its genome modified using bio techniques and used in the production of food (Hanson, 2018), and it became the first unicellular organism with a synthetic chromosome (Annaluru et al., 2014). *S. cerevisiae* is not only being used in beverage and food production, but it is also being used to manufacture industrial bio chemicals and for heterologous expression of proteins (Mattanovich et al., 2014).

S. cerevisiae is easy to isolate, and this has resulted in these yeasts becoming the most studied eukaryotic organism and it has become an essential tool for scientific studies in fermentation research. *S. cerevisiae* are the most important organisms in biotechnology processes because of their characteristics which enable them to perform fermentation and produce by-products like ethanol and CO_2 while withstanding the temperatures and no oxygen conditions in the wort (Azhar et al., 2017). *S. cerevisiae* has been used in the food sector for thousands of years and it has become the model organism in beer production, bread making and biofuel synthesis (Parapouli et al., 2020). *S. cerevisiae* is the most frequently used yeasts strain in beer fermentation. This model organism benefits from its unique characteristics such as rapid replication, ease of culturing, effectiveness of sporulation, and uncommon pathogenicity, and its ability to tolerate various environmental stresses. Fundamental studies, such as the de novo synthesis of an entire chromosome (Annaluru et al., 2014), and application-focused engineering of intricate processes, which includes the production of vanillin and amorpha diene, have successfully demonstrated yeast's potential as a powerful host for synthetic biology.

Brewers have done a lot of research on yeasts, and they do not use genetically modified yeasts for fermentation (Astola et al., 2023). Brewers crop yeasts after each fermentation and they keep these yeasts refrigerated and keep using these yeasts for centuries in fermentation and they term this procedure domestication (Kucharczyk et al., 2020). Most of the yeasts they use has developed unique characteristics over the years due to this domestication process (Onyema et al., 2023). Biotechnology of yeasts has not been exploited much because beer has been produced using organic raw materials for centuries hence customers are not so keen on having beer produced using genetically modified yeasts. However genetically modified yeasts have become popular in other food manufacturing industries as compared to the beer industry (Tenbült et al., 2008). There are reports that prove that yeasts that have had their genome manipulated have potential to produce beer with better quality and they can also improve fermentation efficiency, nevertheless they are not quite popular in commercial brewing sector, but they have been used as a fermentation instrument in wine production in America after approval by the FDA (Volschenk et al., 2004). A lot of research has been done on *S. cerevisiae* strains and they have been isolated from different locations. Ale yeasts are *S. cerevisiae* that are very diverse and have been used in the brewing sector as top-fermenting yeasts for centuries of years. After fermentation ale yeasts are collected from the top of the fermentation vessel and used again in the next fermentation vessel (Bokulich & Bamforth, 2013).

2.8.2 *Non-Saccharomyces cerevisiae*

In brewing, the non-*saccharomyces cerevisiae* is classified in the wild yeasts category because they are not frequently used in industrial beer fermentation and their existence in this process means it is a foreign organism (Miguel et al., 2022). Hence, beer is normally spoiled by rogue strains of non-*Saccharomyces cerevisiae* such as *Brettanomyces, Torulaspora delbrueckii* and *Zygosaccharomyces* (Michel, 2017). Recent studies on fermentation have proved that non-conventional wild yeasts, which were previously considered as foreign microbes in fermentation, can boost the aroma profile and chemical composition of alcoholic beverages like wine (Padilla et al., 2016). Demand in the alcoholic beverage industry has led to an increase on research on the uses of *non-Saccharomyces* yeasts in fermentation. Brewers have suggested that the increase in *non-Saccharomyces* research could have been attributed to varying consumer tastes and choices as well as having different customers with varying preferences for beer styles. In recent years, *non-Saccharomyces* cerevisiae species have been used in the brewing sector in search for exotic flavors and higher profile beer with unique secondary metabolites that produce beautiful aromas (Bruner and Fox 2020). Furthermore, an

aspiration for the introduction of new technical skills for fermenting lagers and other high-profile beer has led to the use of different and non-conventional yeasts species. Amongst them, *S. pastorianus* is the most used non-*Saccharomyces cerevisiae* yeasts strain, which was used in the production of lager beer for ages in the brewing sector (Budroni et al., 2017).

Using yeast starters other than *Saccharomyces* is becoming more and more common in the winemaking sector. They are advised to boost aromatic complexity in order to minimize the likelihood of microbial deterioration and restore some of the qualities of spontaneous fermentation (Ciani and Comitini, 2011). Some yeast species have been linked to potential benefits; commercial strains of *Torulaspora delbrueckii* have been identified, among others. Non-*Saccharomyces* yeast strains have demonstrated to improve aromatic character, enhance colour stability, volatile acidity and minimising alcohol concentration (Ciani et al., 2016). When non-*Saccharomyces* starters are used in co-inoculation, two different species are represented by equivalent cell counts for a reasonably long period of time (Curiel et al., 2017). On the other hand, in wine production that is traditionally inoculated, *Saccharomyces cere*visiae is predominant nearly from the beginning of fermentation. Therefore, through interactions with *S. cerevisiae*, the inoculation of non-*Saccharomyces* strains may have a direct or indirect biological impact on winemaking. *S. cerevisiae* and *T. delbrueckii* have been shown to collaborate in a few recent situations to produce higher concentrations of 3-sulfanylhexan-1-ol (Renault et al., 2016); similarly, *S. cerevisiae* and *Debaryomyces vanrijiae*, which is commonly referred to as *Candida sake*, work synergistically to produce an increased aroma profile (Taillandier et al., 2014).

Non-Saccharomyces cells almost invariably vanish or lose viability when co-inoculated with other commercial yeast used in beer or wine making (Wang et al., 2016). Although the majority of this dominance is due to the indirect impacts of sugar consumption rates, nutritional depletion, and ethanol generation, several direct mechanisms causing animosity between yeast species have also been documented. For instance, *S. cerevisiae* killer factors have long been known. Only a few yeast species are impacted by these released peptides that are encoded by extrachromosomal elements (Parapouli et al., 2020). Similar toxins have been discovered to be present in a few other yeast species (Velázquez et al., 2015). Furthermore, it was recently demonstrated that a peptide fragment of the glycolytic enzyme GAPDH from *S. cerevisiae* inhibits the growth of multiple species of wine yeasts and bacteria (Branco et al., 2021).

Non-*Saccharomyces* yeast such as *Kluyveromyces lacti*, *Scheffersomyces stipitis*, *Dekkera bruxellensis* and *Yarrowia lipolytica* have been used in alcoholic beverage production by commercial brewers. Despite the unlimited work that has been done concerning usage of some wild yeasts strains on a lab scale, especially for wine fermentation, more research still needs to be done for the industrial fermentation using non-*Saccharomyces* yeasts strains and their efficacy in the production of beer (Bruner et al., 2021). The utilization of innovative wild yeasts in the brewing process has fresh prospects for enhancing and updating the sensory characteristics of beers, along with offering innovative technological benefits and maturation methods. The increased diversity in non-*Saccharomyces* yeasts strains that have been studied in biotechnology articles were inspired by new experimental techniques that have made it easier to detect non-*Saccharomyces* yeasts strains (Gibson et al., 2007). The growing demand for advanced beer products, new flavors and alcohol-free beer resulted in exploitation of different yeasts strains including non-*Saccharomyces* strains and phylogenetics is already being used to identify different yeasts strains that are used for fermentation (Miguel et al., 2022). Wild yeasts isolated from nature can produce top quality beer with different aroma and flavor characteristics because of their fermentation technique, which results in innovative beer profiles and styles (Postigo et al., 2021). Non-*Saccharomyces* represent yeasts strains that are not domesticated but have ability to breakdown complex sugars which results in formation of different higher secondary metabolites, and this affects the flavor profiles and style of beer formed (Budroni et al., 2017). There are not enough studies that have been done on non-*Saccharomyces* yeasts hence not much is known about their characteristics but these yeasts strains have already showed that capability to improve the brewing sector through production of new beer styles with completely different flavor profiles that can increase demand for new innovative beers. The alcoholic beverage sector has expanded over the years and brewers have implemented a lot of new brewing techniques such as using different hops or malts and preparation of specialized blends to meet customer demands and satisfaction (Faganel & Rižnar, 2023). When a business grows it is very important to improve the products hence in brewing sector using non-*Saccharomyces* yeasts strains can produce beer with increased flavor profiles. Non-*Saccharomyces* yeast species have been shown to be capable of producing beer with a high flavor profile, however their usage as replacement yeasts in brewing still demonstrates little to no use of these organisms as fermentation instruments (Capece et al., 2018).

2.9 Brewing potential of wild yeast species

In recent years, wild yeasts have garnered a lot of interest in brewing yeasts research. Yeasts that exist naturally in the environment, water and air are called wild yeasts (Basso et al., 2016; Molinet & Cubillos, 2020). Wild yeasts clings to surfaces in the vineyard, winery ad on equipment. Wild yeasts are any fermentation organisms that were not intentionally introduced to a brewing process and are considered spoilage organisms. The discovery of *S. eubayanus* which is a wild yeast (Libkind et al., 2011) has led to its commercial use in the brewing sector (Hittinger et al., 2018). Its characteristics which result in production of sour beers in bacterial free methods (Osburn et al., 2018) and promising results in making beers with zero to low alcohol content (Bellut et al., 2019) are only a few of the explanations for the increased curiosity about wild yeasts. The genetic and phenotypic diversity of commercial *S. cerevisiae* strains is restricted, according to studies comparing commercial and wild yeast strains carried out over the past 20 years (Molinet & Cubillos, 2020). This lack of variety restricts the availability of alternate wines and beers, emphasizing the importance of exploring novel genetic resources to make every fermentation product unique. In this way, wild strains have greater genetic and phenotypic variety than previously imagined, making them a viable choice for developing novel fermentative drinks. For a long time, researchers believed that *S. cerevisiae* evolved in fruit or in environments connected to fermentation. It has been isolated from wasps, flies, oak trees, and their related substrates like bark, soil, and flowers, but bio prospecting studies have shown that it is far more abundant (Molinet & Cubillos, 2020). Other yeasts species can be considered as wild strains despite the discovery of *S. paradoxus, S. kudriavzevii*, and *S. eubayanus* in domesticated hybrids with *S. cerevisiae* (Nikulin et al., 2020). Wild yeasts species have a tendency of producing off flavors in beer such as vinyl phenols (Nikulin et al., 2018), which limits their usage in fermentation. However, these phenolic qualities are thought to be an integral component of the flavor profile in several specialty beer varieties, such as saisons and wheat beers (Lentz 2018).

2.10 Identification of yeast

For the end product, as well as the quality and purity of the yeast strain, yeast identification and characterization are crucial (Hutzler et al., 2021). The identification of yeast species using conventional methods heavily depends on the physiological traits studied, such as the isolates' capacity to ferment or assimilate various carbon sources (Posteraro et al., 2015). As may be imagined, a small number of traits that set closely related species apart from one another may be regulated by a single changeable gene in certain cases (Martinsen et al., 2008). This is true

for species that are closely linked to *Saccharomyces cerevisiae*, which are categorized according to their varied ability to use less than 10 distinct carbon sources, as per Bai et al.'s 2022 classification (Onyema et al., 2023). This also applies to the *Saccharomyces Meyen ex Reess* genus. Since the development of molecular technology, the accepted method for classifying yeasts has been the sequencing of conserved DNA sections. The internal transcribed spacer (ITS) region, which includes the ITS1–5.8S nrDNA–ITS2 gene cluster, and the 28S nuclear ribosomal DNA (nrDNA) are the most often utilized and recognized molecular targets for yeast identification (Leaw et al., 2006). The ITS sequence from *S. cerevisiae* has been modeled in its secondary folding structure. This sequence is composed of multiple structural domains, each with one or two loops. The sequence of *S. cerevisiae* is an excellent option for studying the molecular phylogeny of yeast species that are closely related because, interestingly, the sequences of ITS sections from related yeast species could only partially match to the *S. cerevisiae* ITS sequence (Ramazzotti et.al., 2016). When *Saccharomyces cerevisiae*, a model eukaryote, genome sequencing was first done in 1996, it gave researchers the first indication of a substantially conserved eukaryotic gene set (Gibney et al., 2013). Ortholog comparisons are important for comparing the evolutionary and functional links between genes from various species. Genes that most closely match a common ancestor's sequence but have diverged due to speciation are known as orthologs (Studer 2009).

Based on the genetic information obtained from DNA and RNA sequencing, phylogenetic trees may be built to show the evolutionary links between creatures after yeast identification (Dylus et al., 2023). Nearly all genera will be redefined as a result of a significant revision in yeast systematics brought about by phylogenetic analysis of gene sequences (Kurtzman et al., 2015). This increased understanding of species linkages has led to the development of new guidelines for the naming and classification of yeasts and other fungi, which are presented in the recently adopted Melbourne Code, the International Code of Nomenclature for Algae, Fungi, and Plants. Previously, phylogenetic analyses were limited to morphological areas and relied on external features and internal architecture. On data at the whole genome level, scholars have been focusing on computer methods for ancestral reconstructions and phylogeny. The majority of current methods are limited to processing simulated datasets with similar genome content and distinct genome markers, or simplified actual genome datasets (Feng et al., 2017). Furthermore, their ability to handle intricate evolutionary events such whole genome duplication, insertion, deletion, and duplication is limited (Farhat et al., 2023). A crucial basis for research on microbes, phylogenetic analysis examines the evolutionary links among

organisms (Shakya et al., 2020). The creation of trustworthy phylogenetic trees is a crucial first step in the biomedical field's characterization of novel diseases and treatment approaches. Because the goal of this project is to ascertain the role that various yeast will play in industrial biotechnology, phylogeny plays a crucial role in comprehending the evolution of the yeast that is currently utilized in brewing.

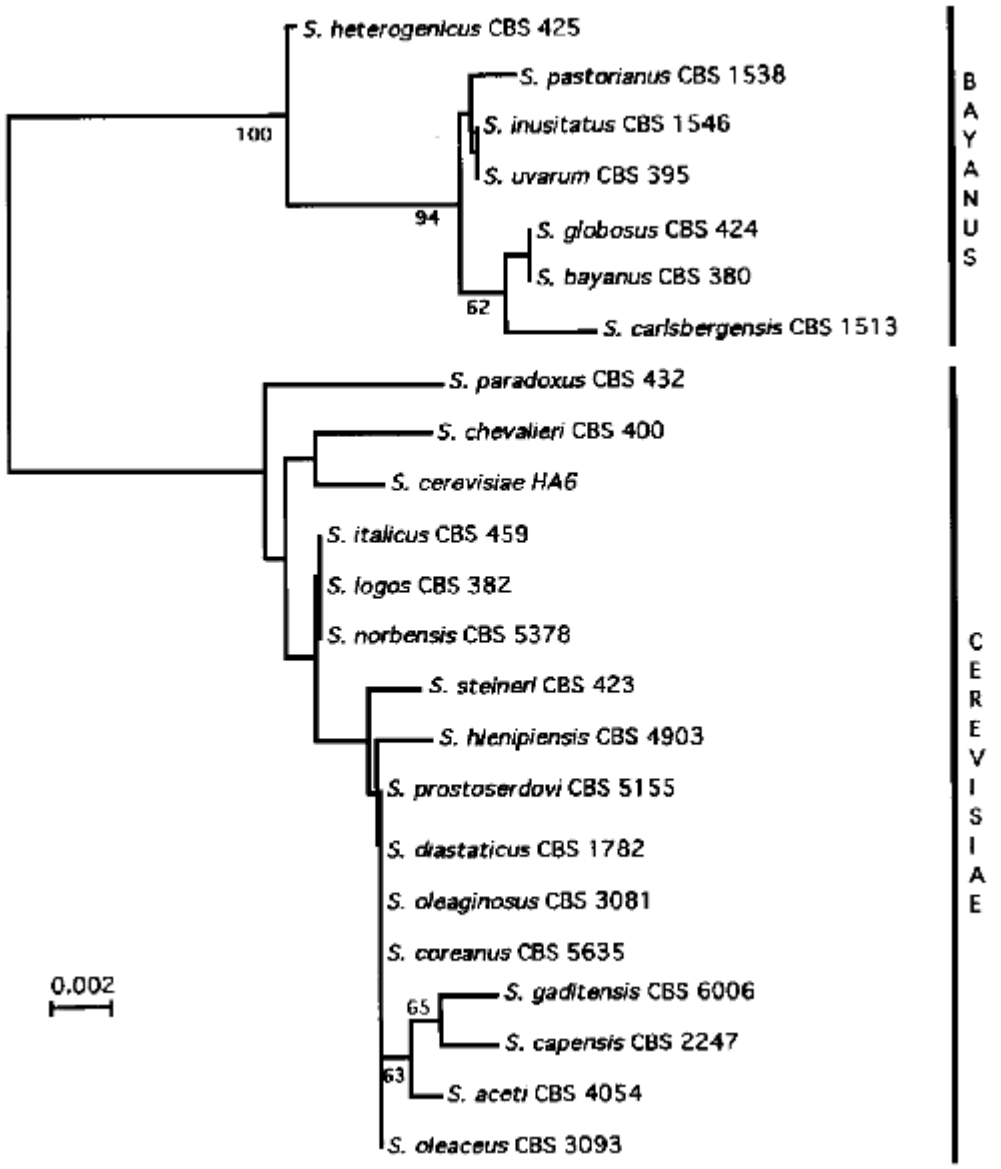


Figure 2.2: Based on the PCR-RFLP analysis of the ITS and IGS rDNA spacers, an unrooted phylogenetic dendrogram is presented that illustrates the taxonomic placements of *Saccharomyces* type strains and wine yeast strains. Taken from Montrocher and colleagues (1998), (Montrocher et.al, *Ijs-48-1-295 (2)*, n.d.1998)

2.11 Brewing Yeast Niche

Saccharomyces cerevisiae, the common budding yeast, can be found in a range of environments, including fruit trees and oak trees as well as human-related businesses like baking and fermentation (Pontes Ciências Da Vida, n.d. et al., 2022). The current endeavors to describe the variety of *S. cerevisiae* on a broad whole-genome sequencing has been applied to *cerevisiae*. The findings have revealed a relatively distinct population structure: strains that have been isolated from a particular niche or malt liquor have a closer relationship with other yeasts that are present there. (Gallone et al., 2016). Yeast's genomes are believed to have been influenced by their place of origin as well as past human migrations because of their close relationship with humans. Three large clades of beer-brewing yeasts exist within the yeast family tree, and these are some of the best-characterized examples of this adaptation or domestication associated with humans (Large et al., 2020). A species barrier is the primary distinction between *S. cerevisiae* ale yeast and lager yeasts, which are hybrids of *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011). The ale yeasts are divided into two main categories, Beer 1 and Beer 2, as well as a few smaller categories of mixed origin that contain yeast from wineries, distilled alcoholic beverages and baking industries. The Beer 1 yeasts, often known as ale yeasts, are a broad group of largely tetraploid strains from United States of America and other European countries, whereas the Beer 2 group is primarily made up of diploid individuals employed in classic Belgian styles (L Large et al., 2020). Multiple *S. cerevisiae* subpopulations that shared genomic characteristics with current populations of European and Asian wine strains, as well as certain extinct beer brewing strains, are considered to have been mixed historically to produce ale yeasts (Fay et al., 2019). Because of their diversity and structure, these populations are excellent tools for studying the genetic underpinnings of domestication. Specifically, their structure has allowed for a detailed analysis of the distinct molecular adaptations that human-established brewing yeasts have made to their environment.

In previous articles, genotype association and phenotyping have been used to link a multitude of unique genetic variations to traits beneficial for the flavor of a certain beer or for growth in a beer brewing environment. First, by inactivating two genes, PAD1 and FDC1, the ale brewing yeasts are unable to create 4-vinylguaiacol (4-VG), a taste molecule that is undesirable in some beer types, compared to wild strains (P. Chen et al., 2015). Curiously, functional alleles are retained by a lineage of ale yeasts that are exclusively utilized to produce wheat beers where 4-VG is a desired trait, underscoring the diversity and specialization of the domesticated strains (Gonçalves et al., 2016). Furthermore, according to Gallone et al. (2016), Two very important

sugar sources for beer brewing, maltose and maltotriose, are absorbed and broken down by an increase of genes that ale yeasts encode. Lastly, Will et al. (2010) found that loss of function alleles in AQY1 and AQY2 in wine and ale brewing yeasts result in greater Osmo tolerance in settings with high sugar content. Nonetheless, some of the ale beer strains contain a functioning allele of AQY2, which may suggest that this allele is not selected for or that there is a selective advantage that varies with the environment. Majority of brewing ale yeast are genetically stable but more research needs to be done to determine the genetic diversity of these yeast which results from adaptive evolution or domestication (Beer 1) (Large et al., 2020).

Additionally, unlike other mutations that are common in the lineage which are more challenging to understand, these single gene occurrences have made it easier to link them to possible symptoms. Tracts of homozygosity have been widely found in ale brewing yeasts, probably due to their decreased capacity to complete meiosis and their higher rate of mutation for both aneuploidy and mitotic recombination in tetraploids (Mayer and Aguilera, 1990). Due to significant aneuploidy and mitotic recombination across and between these two genomes, similar tracts of homozygosity favouring particular *S. cerevisiae* or *S. eubayanus* alleles have been identified in lager-brewing yeasts (Monerawela et al., 2015). While the implications of these intragenomic events in ale and lager yeasts remain uncertain, prior research conducted by our group and others has demonstrated that, on the short-term experimental evolution time scale, loss of heterozygosity (LOH) resulting from mitotic recombination in a previously heterozygous strain can have drastic fitness consequences (Rodrigues-prause et al., 2018). Additionally, widespread aneuploidy and LOH can result in a variety of phenotypic consequences in other yeasts, including competitive growth and greater drug resistance in Candida albicans and Cryptococcus neoformans (Ni et al., 2013). Lastly, LOH of a non-functioning tumor suppressor allele in human cells undergoing mitosis can raise the risk of cancer development, particularly BRCA1-mediated ovarian and breast cancer (Ryland et al., 2015). Although LOH has been commonly detected in ale yeasts and has been observed to have phenotypic implications in other populations of mitotic cells, it has not yet been definitively related to characteristics in ale brewing yeasts (Garge et al., 2024).

Furthermore, the diminished capacity of ale yeasts to undergo meiosis hampers the application of conventional quantitative trait mapping techniques for the interpretation of genetic variation. Genetic screens and other non-meiotic methods, including experimental evolution, have yielded important insights into adaptation in general. These discoveries include the significance of particular mutations, copy number variation, and ploidy (Bergero et al., 2021). Professional

brewers typically employ a technique called repitching, or back slopping, in which they serially reuse populations of yeast to brew batches of beer. This approach lowers the cost of purchasing yeast on a regular basis and allows the yeast to get physiologically acclimated to the brewery. The process begins when a brewery (population size of around 2 x 1013) purchases a batch of a certain yeast strain from a propagation company. Usually, a patch of yeast taken from a clonal glycerol stock maintained at -80°C is used to culture these yeasts. In order to meet the demand for a particular strain, a propagation company would often ship out these yeasts, expanding the stock beyond what one brewery could use. This implies that before the yeasts reach the brewery, a number of generations of yeast development (at least 50 yeast generations) must occur. Once at the brewery, the yeasts are "pitched" or inoculated into wort, which is essentially a grain and cereal-based beer medium. Yeasts are recovered from the bottom of the fermenter once the fermentation process, which typically takes 10 to 14 days, is complete. The brewer would typically gather around one-third of the yeast and re-pitch it into a fresh fermentation vessel filled with wort after removing the trub, which is made up of hop and protein particles. The precise quantity of yeast cells injected varies from one brewery to another and is frequently adjusted in accordance with the media's initial sugar level and the yeast's viability at that moment (Powell et al., 2003).

Brewers avoid using re-pitched yeast more than eight times because there is high risk of contamination, mutation and reduction in viability after reuse. (Large et al., n.d.). Phenotyping clones derived from populations of repurposed yeasts in a continuous use fermenter allowed researchers to determine early on how genetic changes affect brewing-relevant features in yeasts (Duina et al., 2014). A second study by a separate team found that clone isolates from yeasts that were repeatedly utilized over a period of years varied in their flocculation activity, maybe due to a deletion mutation in a flocculation-related gene (Sato. M et al., 2001). Furthermore, utilizing gel-electrophoresis based approaches, a study looking at population samples from serial reuse of yeasts was unable to demonstrate any genetic change during 135 serial fermentations (Powell & Diacetis, 2007). On the other hand, new research on buckwheat and quinoa beer lager fermentations has discovered possible changes in chromosomal length after serial repitching (Deželak et al., 2014). Despite new studies showing that genetic variation is probably certainly the cause of the significant phenotypic differences amongst brewing yeasts, genetic variation-based changes in beer qualities rarely occur over short-term repitching. In line with these findings, professional and amateur brewers frequently cultivate a yeast strain over a long period of time in order to produce a so-called "house strain" with

modified brewing characteristics. This suggests that genetic alterations are likely to take place in the brewery over a brief period of time. However, current high-throughput whole genome sequencing has not provided a complete record of the mutational foundation, timing, and impact of these modifications (Raphael et al., 2014).

CHAPTER 3

METHODS AND MATERIALS

3.1 Yeasts strains media

Different species of wild yeast were used in this research. The different yeast was isolated from different ecological niches. *B. clauss* was isolated from fermented kombucha tea, *B. brusc* from tree bark, *Ragnarok* from wasp, *proletariat* from fermenting Sauvignon wine, *Neipa* from red grapes peel, *dark knight* from fermented milk, *La Trappist* and *Samson's saison* from vineyard soil in the Durbanville Hill wine farms in the Western Cape Province of South Africa. The control for this research was the commercial yeasts *S. cerevisiae* which was acquired from a local brewing store called the Beer Lab in Cape town.

Table 3.0: Summary of wild yeast used in the research and their sample codes before identification.

Yeast	**Sample code**
B. brusc	*B. brusc*
B. clauss	*B. clauss*
Ragnarok	*LC018*
The Proletariat	*LC082*
Neipa	*LC035*
Dark Knight	*LC011*
La Trappist	*LC009*
Samson's saison	*LC006*

3.1.1 Media

Yeast Peptone Dextrose agar (YPD) media was used to cultivate wild yeast strains. A liter of distilled water was used to dissolve 10 grams of yeast extract, 20 grams of peptone, 20 grams of dextrose, and 15 grams of agar to create the YPD medium (Li et al., 2016). A pH meter from

Lasec South Africa was used to measure the pH, which was then adjusted to 6.16. The medium was autoclaved at 120^0C for 20 minutes. The media was autoclaved, allowed to cool to room temperature, and then transferred into petri dishes or culture plates.

3.1.2 Yeast culturing

Propagation of the wild yeasts on YDA was started from stored cultures (Durcanska et.al, 2019). For each wild yeast three plates were prepared. The isolates were streaked onto YDA using a disposable inoculation loop and incubated for 48 hours at 30^0C, with three replicate plates for each wild yeasts strain. After 48 hours of growth, each plate was checked for growth and subcultures were made from the replicate plates for each strain, (Marrs et al., 2021). Thereafter, subsequent subcultures were performed for several weeks on YDA to obtain yeasts cultures that were going to be used for beer fermentation. The commercial yeasts bought was ready to use so it was not cultured.

3.2 DNA Extraction and Yeast Identification

Using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005), genomic DNA was recovered from the cultures. The ITS and 16S target regions were amplified as presented in Table 3.1 and Table 3.2.

Table 3.1: ITS Primers sequences.

NAME OF PRIMER	TARGET	SEQUENCE (5' TO 3')
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC

Table 3.2: 16S Primers sequences

NAME OF PRIMER	TARGET	SEQUENCE (5' TO 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

Inqaba Biotechnical Industries supplied the NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S), genomic DNA (10–30ng/µl), forward and reverse primers (10µM), and nuclease-free water (Catalogue No. E476) for the PCR. The DNA Thermal Cycler was programmed to run at 94°C for five minutes, then 35 cycles at 94°C for thirty seconds, 50°C for thirty seconds, and 68°C for one minute. Finally, it ran for ten minutes at 68°C for one extension. Conditions were the same for both primers.

Agarose Gel Analysis:

A 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd.) stained with EZ-vision® Bluelight DNA Dye was used to visualize the integrity of the PCR amplicons (Mitchell & Ladarola, 2010). As a size standard, the NEB Fast Ladder was applied to all gels (N3238) (Yu et al., 2022).

PCR Amplicon Purification:

The ExoSAP method (NEB M0293L; NEB M0371) was used to enzymatically purify segmentation.

Sanger Sequencing:

Using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific), the amplicons were purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM, Catalogue No. D4050) and sequenced in both forward and reverse directions (Nimagen, BrilliantDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000).

Data Analysis:

Finch TV (https://finchtv.software.informer.com/1.4/) can be used to see the raw chromatogram files (.abi). In this study the forward and reverse sequencing data was combined using CLC Bio Main Workbench to get a consensus sequence for each sample. BLASTn analysis (with default parameters) (Altschul et al., 1997) was carried out to ascertain whether a sequence in the database matches the query sequence over a specified threshold (99% query coverage; 99% identity).

3.2 Brewing preparation

American pale ale beer was prepared using a warm fermentation method (Thesseling et al., 2019) with five buckets for each yeasts strain. To kill off beer spoiling bacteria and other yeasts, fermentation equipment i.e., buckets (20 L) were sterilized and cleaned before the brewing was started. The solution used to clean the equipment was an acid-based star sign sanitizer and hot water. The buckets were left to soak in the sterilizing solution for 3 hours and air dried afterwards.

3.2.1 Beer wort preparation

To make the wort (unfermented beer), a thermometer was attached to the side of a large stock pot (Krenzke, 2014) and Five liters of water was heated up to 170º C (Figure 3). Once the water reached the required temperature (170º C), the heat was turned off. One kilogram of malt was measured and placed in a cheesecloth bag which was tied into a knot to tightly secure the malt. Thereafter the sack of malt was placed in the heated water, stirred until all the grains were completely soaked and steeping in the water (Turner et al., 2019). The malt was left to steep in the water for 30 minutes. After 30 minutes, the sack of grains was removed from the pot and the remaining wort was brought back up to a boil. Once the wort was boiling, the heat was shut off. Twenty grams of malt extract syrup was added, mixed with the wort, and left to boil for 30 minutes. Twenty-eight grams of hop pellets were added, and the mixture was boiled for an additional 40 minutes, another portion of hop pellets 28 grams was added and boiled for 20 minutes. Right before taking the wort off the heat, 56 grams of hop pellets were added to the mixture. The pot was removed from the heat and covered loosely with foil and placed in a sink with ice cold water to take down the temperature to 70 ºC. The temperature of the wort was monitored with a thermometer.

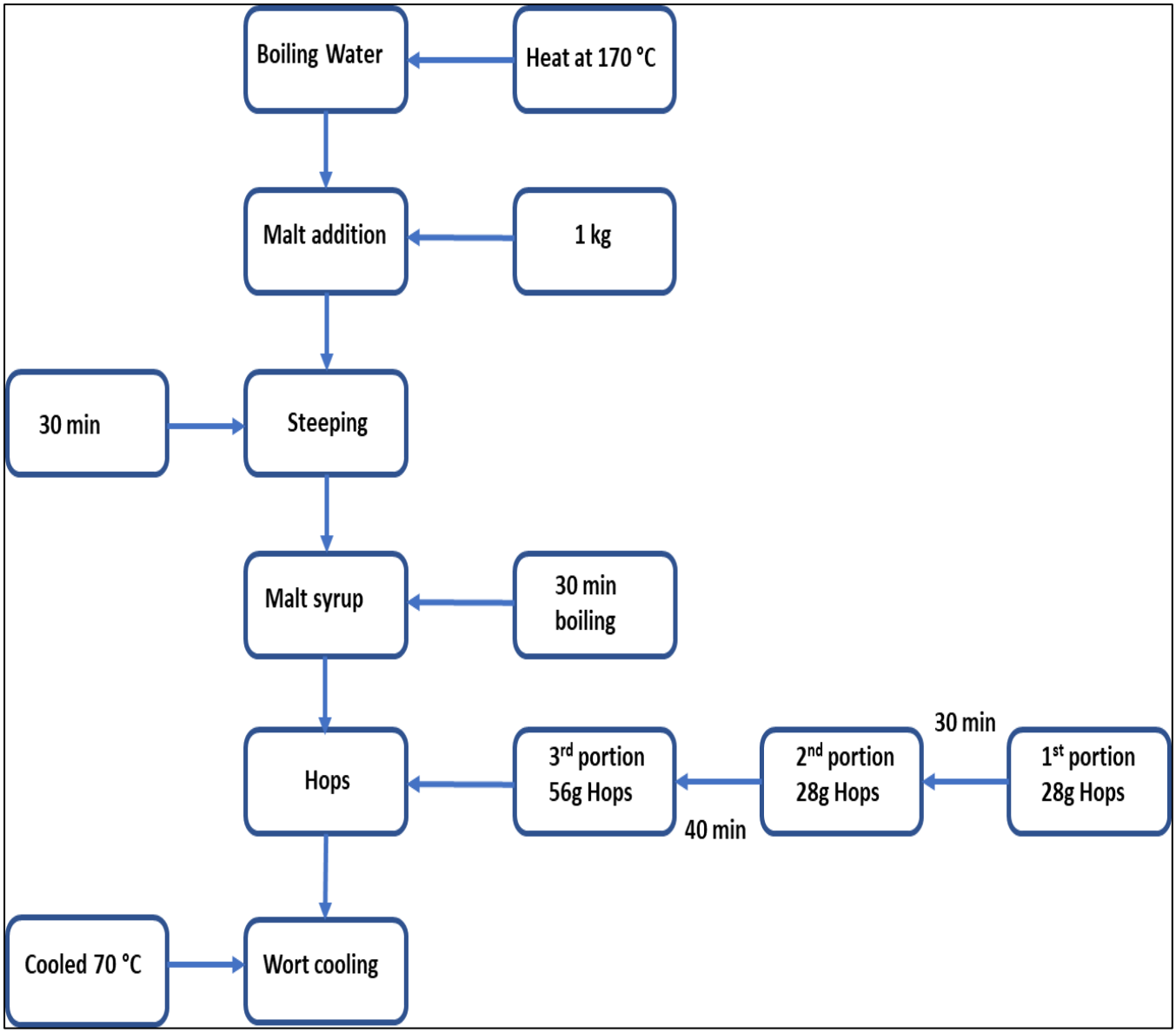


Figure 3.0: Process flow for beer wort preparation adapted from Micet group 2021, Brewing Process Step-By-Step

3.2.2 Yeasts pitching

Yeasts pitching was done using the yeasts cultures obtained in section 3.1.2. Starter tubes containing 15ml of sterile wort were used to increase culture size (Harrison & Curtin, 2021). A sterile inoculation loop was used to transfer the yeast's culture into the starter tube two times. The Starter tube was loosely capped and left at room temperature for 24 hours. 200ml of the wort was poured into a 500ml Erlenmeyer flask and the mixture in the starter tube was pitched into the flask with the wort. After being wrapped in aluminum foil, the flask was let to sit at room temperature for a full day. Aluminum foil was used to cover the flask because yeast development depends on both oxygen and agitation, and oxygen can enter through the foil cap

(Josey & Food Science, 2018). The yeast was ready to pitch into a fermentation vessel after 24 hours. The yeast pitching was done in different starter tubes and Erlenmeyer flasks for the different wild yeasts and the control which was the commercial *S. cerevisiae*.

3.2.3 Fermentations in beer wort

Fermentation buckets were filled up with 5 liters of spring water. Before adding the yeast, the cooled wort was added to the buckets of water and vigorously agitated for 10 to 25 minutes to maximize oxygenation (Wachter & Barta, 2022). The pitched wild yeasts were shaken and poured on top of the wort in the fermentation buckets. This was done the same for the commercial yeasts. Tight lids were placed over the fermentation buckets, and an airlock was placed on top of each to let carbon dioxide escape throughout the fermentation process without letting anything inside (Potter, 2015). After shaking the bucket for five minutes to evenly spread the yeast, it was left to ferment at room temperature for two weeks at 22 degrees Celsius.

3.2.4 Bottling

Equipment for siphoning and bottling buckets were disinfected by immersing them in an acid-based sanitizer for ten minutes. The bottling buckets were set on the floor beneath the fermenting buckets after the priming sugar had been dissolved in hot water. A racking cane was attached to a siphon tube (Murray et al., 2019). The racking cane was put inside the fermentation bucket and the siphon line was held upright to stop any liquid sanitizer from spilling into the bucket. We disposed of the remaining sanitizing solution after transferring it into a cup. The siphon tube was inserted into the bucket below to allow gravity to carry the wort from the fermentation bucket into the bottling bucket. A blend of priming sugar was added to the beer during the pouring process to increase its flavor and carbonation. The bottling bucket was filled with wort, set on a table, and let to sit for twenty minutes. The bottling tubing was connected to the bottling wand and spigot of the bottling bucket. The wand was put into the beer bottle, and the spring tip was pressed down to fill it. The beer was loaded to a small overflow in order to extend its shelf life and drive out any excess oxygen that would deteriorate it. A sterile cap, often known as a crown, was placed over the bottle and fastened with a bottle capper. The procedure was repeated until all of the beer was bottled. To allow the yeast to eat the sugar and make carbon dioxide, which carbonates beer, the bottles were held at 22ºC for two weeks (Janssens et al., 2015). Prior to the tasting, eight bottles were chilled in the refrigerator, and additional samples were collected for physicochemical examination in the lab.

3.2.5 Yeast harvesting

After fermentation the yeast left in the fermentation buckets for each strain was harvested, (Rezaei et al., 2014). The sediment left in the fermentation buckets was mixed with sterile water and transferred to a jar and covered with a lid. The jars were placed in a refrigerator for a week so that the yeast can settle at the bottom of the jar. The liquid on top of the yeast was poured out and the yeast was inoculated on YPDA plates and incubated for 48 hours at 30 degrees. The yeast cultures that grew were used for yeast identification using the method in 3.1.3.

3.3 Physicochemical and volatile compound determination

3.3.1 Alcohol content

Alcohol content was measured using Alcolyzer (Golston, 2023) from Anton Paar company in Gauteng Province South Africa. The beer samples were placed in a warm water bath and monitored with a thermometer until the temperature reached 22^0C and then filtered using Whatman Qualitative filter paper (South Africa). After filtering, the samples were poured into separate conical flasks to remove foam from the beer. Thereafter, samples we loaded in the Alcolyzer for analysis. The procedure was repeated seven times for beer made from each yeast strain.

3.3.2 Sulphur dioxide

Sulphur dioxide was measured using a Skalar (Merck, South Africa) (Kunz & Methner, 2009). The samples were filtered using Whatman filter paper and 10 ml of each beer sample made with the different yeasts was measured using a measuring cylinder. The samples were analyzed on the skalar where the beer sample is diluted in a sulfuric acid solution and then heated to a high temperature of 90^0C. The heating forces the beer samples to release the bound Sulphur dioxide molecules, and this results in the formation of gaseous SO_2. The formed SO_2 is dialyzed into a phosphate buffer solution and goes on to react with 2,2'-dinitro-5'-dinitrobenzoic acid (Ellman's reagent) which is reduced to 2-nitro-5-thiobenzoate. The absorption will give us the reading for the actual SO_2 in the beer sample at 430nm absorbance.

3.3.3 Color

The color of each beer produced during fermentation with different yeasts, was measured using a Shimadzu spectrophotometer (Eureka Scientific, South Africa) (Molitor et al., 2022). Beer samples were placed in a warm water bath using a thermometer to measure the beer temperature

until it reached to 22^{0}C and filtered using a filter paper. Thereafter, each beer sample (15 ml) from the different yeasts strains was placed in small cuvets, placed inside the spectrophotometer for analysis. Color is measured in European Brewery Convention units (EBC) (Koren et al., 2020). Figure 3.1 shows a color chart used for identification of different beers, while the colors in the chart are identified in the range shown in the Table (3.3).

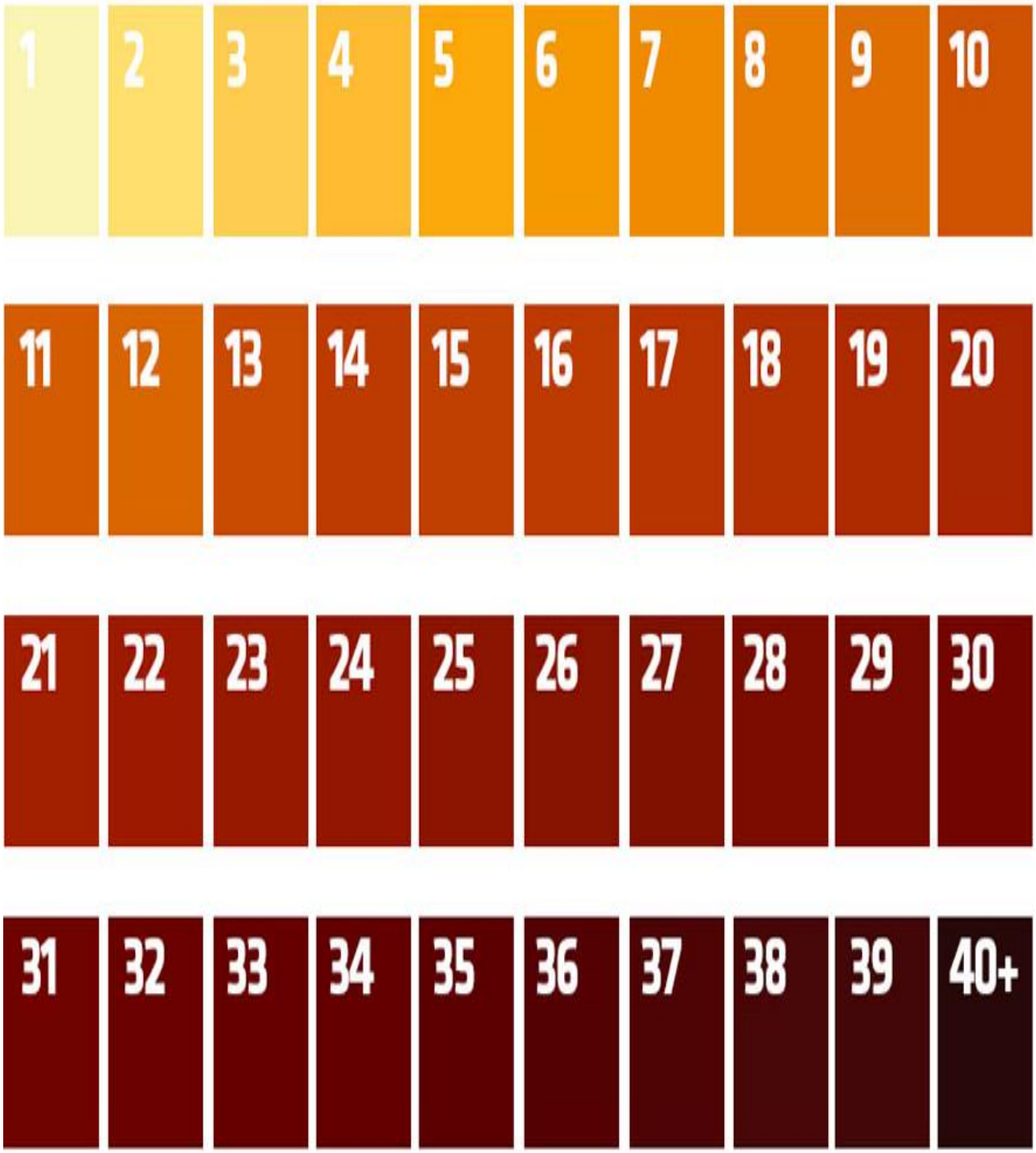


Figure 3.1: Color chart adapted from just beer showing the different colors of beer ranging from 1 EBC to 40+ EBC (European Brewery Convention).

Table 3.3: beer color ranges used in the brewing industry (430nm wavelength).

Color Scale	Range
1-2	Beige
3-4	Yellow
5-6	Gold
7-14	Light copper
15-17	Copper
18-20	Light brown
21-23	Brown
24-30	Light amber
31-36	Amber
37-39	Dark amber

3.3.4 Volatiles and Esters quantification

Volatiles such as acetaldehyde, ethyl acetate, ethyl hexanoate and isoamyl acetate were measured using the Agilent Gas Chromatography (GC) (Ogawa et al., 2022). This GC has a 3 layer sandwich injections and 16 vials. The needle valve flow is set at 20 mL/min and pressure of up to 200bar. The fermented beer samples were filtered using kieselguhr which is a filtering powder and Whatman Qualitative filter paper. Five milliliters of each sample were poured into different 20 ml vial and closed with a septum cap. The samples were placed inside the GC and baked at 60^0C for 30 minutes. Septum was injected to release the volatile from the vial headspace (Maurya et al., 2015). Samples were run through the column for 4 minutes to obtain the result. Esters which are diacetyl and pentane, the samples were filtered with kieselghur and centrifuged at 3000 rpm, poured 10ml of each sample into the vial, bake and run through the GC.

3.3.5 Isohumalone/ bitterness

A 1260 Infinity II LC System High-Pressure Liquid Chromatography (HPLC) from Chemetrix was used to measure isohumalone, ad-humalone and co-humalone which are the compounds that determine bitterness in beer. This HPLC has a pressure range of up to 600 bar and flow rates up to 10 mL/min. 1260 Infinity II LC HPLC contains a thermostat that can support up to four columns. Each column may be accessed directly through a quick-change switching valve which does not need to be setup every time it moves to the next column. The depth of each column is 468mm and injection range is 0.1-100 µL. Samples were prepared and run on the HPLC (Tofana et al., 2009). The samples were centrifuged at 3000 rpm. 10ml of each sample were extracted after centrifugation using a syringe and filtered using Millipore filter. The sample was poured into a 1ml vial and run on the HPLC. The solvent used during analysis to solubilize the analytes was methanol.

3.4 Sensory analysis

A descriptive sensory analysis was performed with 3 certified beer tasters. Beer samples were poured into glasses and each taster had to smell and sip the beer to determine the aroma and flavor of the beer. A scale which ranged between 1-9 (Table 3.4), was used to determine the brand criteria of pale ale and how it is supposed to taste and smell (Higgins et al., 2020).

3.5 Statistical analysis

Physicochemical parameter and volatile compound data was checked for normal distribution before being subjected to statistical analysis (Ghasemi & Zahediasl, 2012). The Shapiro Wilk test was used to see if data was normally distributed or not. Data which was normally distributed was subjected to a one-way ANOVA test to compare differences between physicochemical parameters and volatile compounds (Jia et al., 2021). Physicochemical and volatile compounds data which was not normally distributed was compared between strains using Dunn (1964) Kruskal-Wallis multiple comparison, where p-values were adjusted with the Benjamini-Hochberg method. All analyses were performed using RStudio version 4.2.2 (Rstudio Team, 2020). Probabilities less than 0.05 were considered as statistically significant.

Table 3.4: Pale ale brand profile scale

9.0-7.5	**7.0-5.0**	**4.5-4.0**	**3.5-1.0**
Attributes that define Pale ALE	**Deviations from a perfect ALE**	**Emerging defects**	**Beer taste completely off/ maximum defects**
Medium hop aroma	**Moderate malty**	**Slight cheesy**	**Hazy**
Malty profile	**Strong astringent**	**Sweaty**	**Musty**
Slight sweet	**Slight after bitter**	**Cooked vegetables**	**Chlorine**
Slight sulphurs	**Slight-clear apple (acetalydehyde)**	**Rotten eggs**	**Oil**
Fruity and floral taste		**Dairy/worty/brothy**	**Vinegar/ buttery**
Crisp finish		**Meaty, burnt rubber, winery**	

Table 3.5: Definitions of beer sensory descriptors (Lexicon)

Descriptor	**definition**
Floral	Sweet scents with a hint of perfume that make you think of a blooming garden.
Citrusy	hints of lemon, orange, or grapefruit, adding a zesty, invigorating fruitiness.
Fruity esters	Yeast can produce flavors ranging from apple, pear, and banana to more exotic fruits, adding a lively, fruity character to the beer.
Malt complexity	The flavors that yeast may produce range from banana, apple, and pear to more exotic fruits, giving the beer a vibrant, fruity flavor.
Aroma	Turn your attention to the symphony of smells. The rules include figuring out where fragrances originate, whether it's from the hops, grain, or special flavors brought on by fermentation. Determining these aromas is like figuring out the beer's genetic makeup.
Spicy phenols:	These could give a spicy element to the beer's flavor profile, such as warmth akin to cloves or peppery zest.
Wild yeast characters:	Barnyard, horse blanket, and cheese Flavors—all representative of wild or sour beers—introduce an interesting, frequently learned flavor.
Flat	Little or no carbonation, which can result in a smoother, occasionally syrupy mouthfeel; nonetheless, this is frequently seen negatively unless it fits the style.

CHAPTER 4

RESULTS

4.1 Culturing and Sanger sequencing

4.1.1 Culturing

Wild yeast samples were cultured on yeast dextrose agar and incubated. The yeast showed different growth patterns on the media and growth was observed after 48 hours (Figure 4.0). Wild Yeast isolates that were cultured formed shiny butyrous and smooth white raised colonies on YPD agar media they were inoculated on. Smooth colonies are associated with performing well in fermentation as opposed to rough colonies that are known for disrupting fermentation.

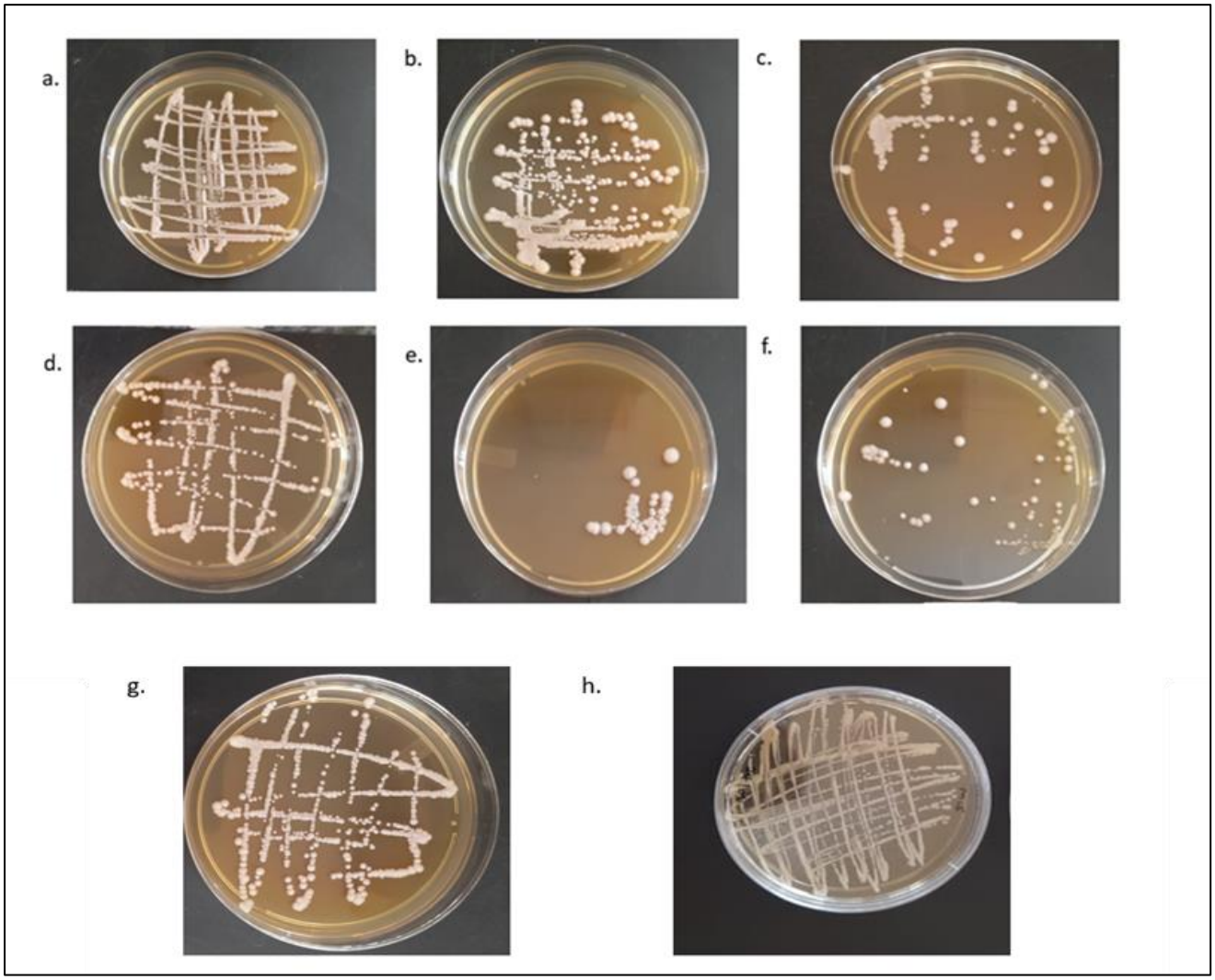


Figure 4.0: Images of the cultured wild yeast samples after 48hrs of growth (a) Samson's Saison, (b) Ragnarok, (c) Dark knight, (d) B. brusc, (e) Neipa, (f) The Proletariat, (g) La Trappist and (h) B. clauss.

4.1.2 Sanger Sequencing

After sequencing, an agarose gel (Figure 4.1) was performed to show the amplification of the ITS target region (~600bp). The agarose gel showed that DNA samples from Samson's Saison (LC006) amplified at 1008bp, La Trappist (LC009) amplified at 412bp, Dark Knight (LC011) amplified at 947bp, Neipa (LC035) amplified at 168bp, The Proletariat (LC082) amplified at 420bp, Ragnarok (LC018) amplified at 593bp, B. brusc amplified at 1015bp, while B. clauss separated at 425bp.

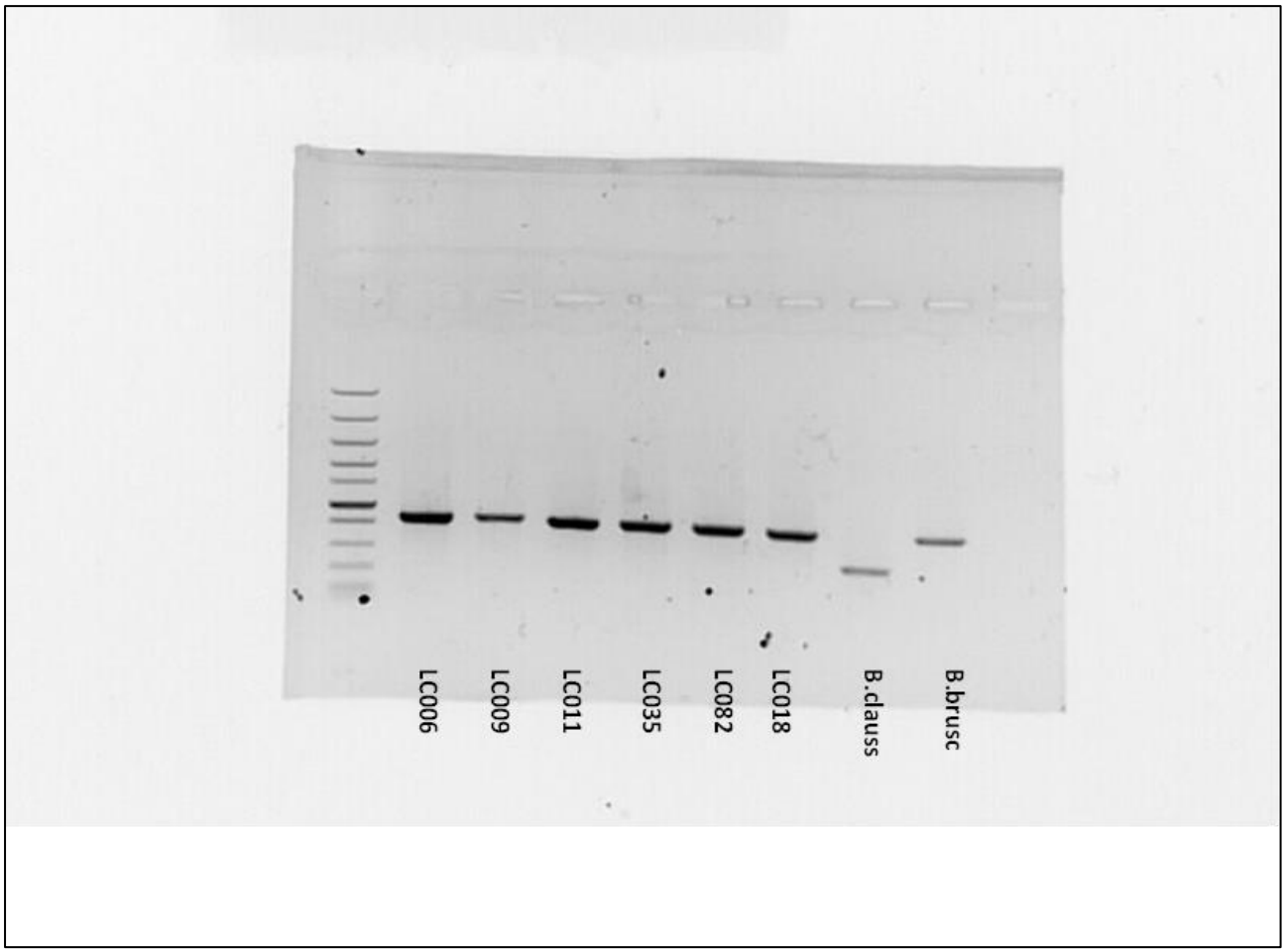


Figure 4.1: A photographic image of an agarose gel indicating the amplification of the ITS target region (~600bp).

For every sample, a consensus sequence was created by assembling the forward and reverse sequences. Following assembly, the NCBI website was used for a BLASTn examination. The

analysis revealed that majority of the yeast samples were *S. cerevisiae*, except for the wild yeast B_brusc which was identified as *Brettanomyces bruxellensis* (Table 4.0)

Table 4.1: BLAST results: Similarity between the sequence queried and the biological sequences within the NCBI database.

Sample name	Predicted organism	GenBank Accession	% identity
LC006 *Samson's Saison*	*Saccharomyces cerevisiae*	KM982975.1	92.1%
LC009 *La Trappist*	*Saccharomyces cerevisiae*	KX887500.1	95.89%
LC011 *Dark Knight*	*Saccharomyces cerevisiae*	KM982975.1	94.49%
LC035 *NEIPA*	*Saccharomyces cerevisiae*	MK267784.1	83.33%
LC082 *The Proletariat*	*Saccharomyces cerevisiae*	KF953892.1	94.05%
LC018 *Ragnarok*	*Saccharomyces cerevisiae*	KX023222.1	95.12%
B. clauss	*Brettanomyces bruxellensis*	MT734873.1	99.53%
B. brusc	*Saccharomyces cerevisiae*	KM982975.1	92.62%

4.2 Brewing potential of yeast after fermentation

To investigate the influence of various yeast on fermentation, eight wild yeast and a commercial yeast were used to produce beer. After two weeks of fermentation, alcohol content, sulfur dioxide, pH, color, and volatiles were measured to quantify differences in brewing performance between the yeast. After fermentation the yeast at the bottom of the fermentation vessels was harvested, washed and cultured. DNA extraction was performed, and sanger sequencing was used to identify the samples. The sequencing results showed that the same yeast identified before fermentation were the same species identified after brewing (Figure and Table 4.2). Hence there was no mutation in the yeast samples after fermentation.

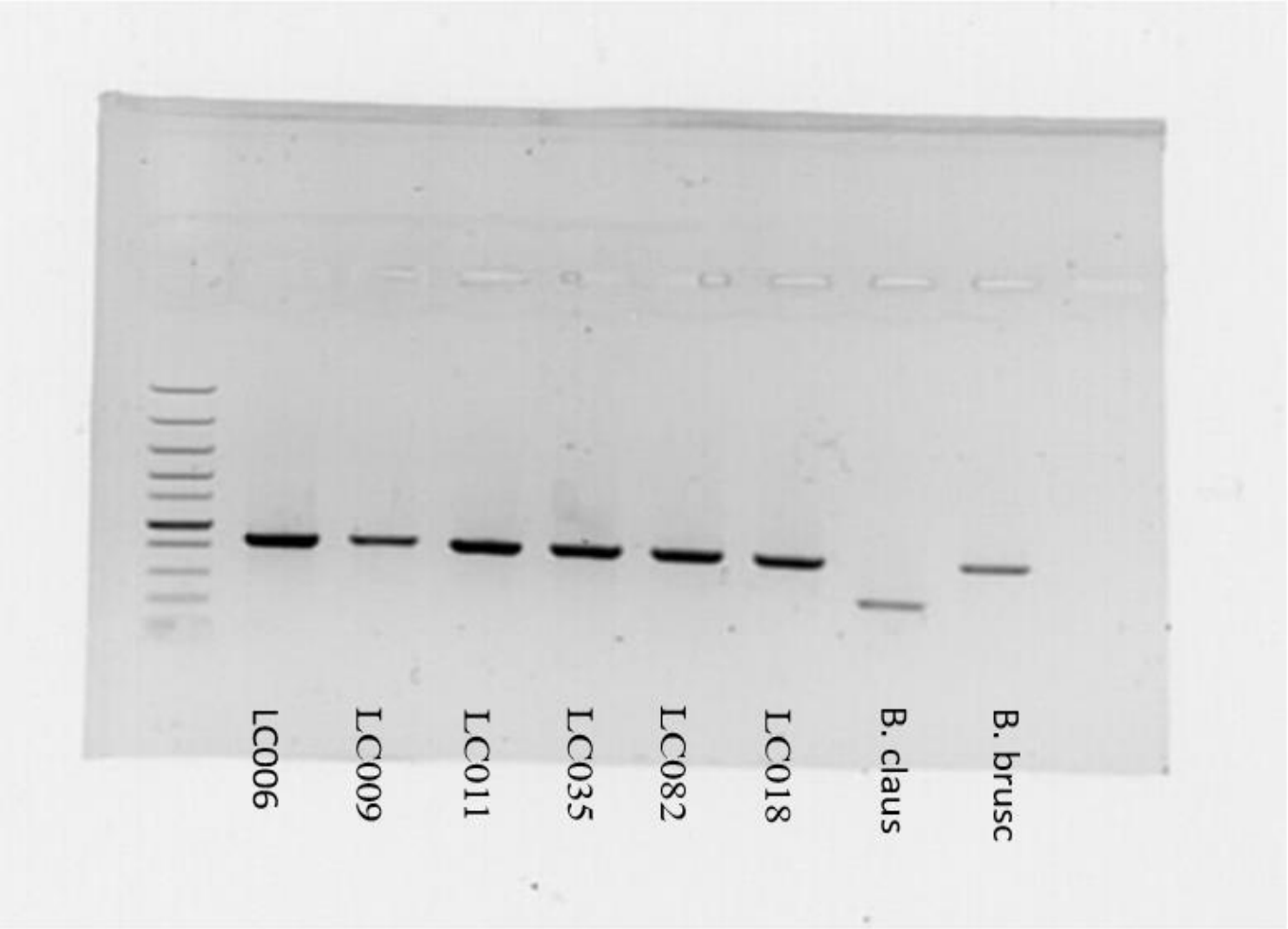


Figure 4.2: A photographic image of an agarose gel indicating the amplification of the ITS target region (~600bp) of the yeast samples after fermentation.

Table 4.2: Yeast samples identification after fermentation

Yeast sample used for fermentation	**Predicted organism**	**GenBank Accession**	**% identity**
LC006 Samson's Saison	*Saccharomyces cerevisiae*	KM982975.1	89.43%
LC009 La Trappist	*Saccharomyces cerevisiae*	KX887500.1	92.67%
LC011 Dark Knight	*Saccharomyces cerevisiae*	KM982975.1	93.60%
LC035 NEIPA	*Saccharomyces cerevisiae*	MK267784.1	86.12%
LC082 The Proletariat	*Saccharomyces cerevisiae*	KF953892.1	92.20%
LC018 Ragnarok	*Saccharomyces cerevisiae*	KX023222.1	94.99%
B. clauss	*Brettanomyces bruxellensis*	MT734873.1	96.12%
B. brusc	*Saccharomyces cerevisiae*	KM982975.1	89.04%

A principal coordinate analysis (PCA) was plotted to show possible groupings between yeast cultures and understanding the relationship between the variables measured during the brewing process (Figure 4.2). The resulting analysis showed that all treatments, besides B_clauss grouped closely together to the commercial yeast *S. cerevisiae* (Figure 4.2). Overall, the PCA shows that all treatments besides, *B_clauss* have little variation between them with regards to brewing parameters.

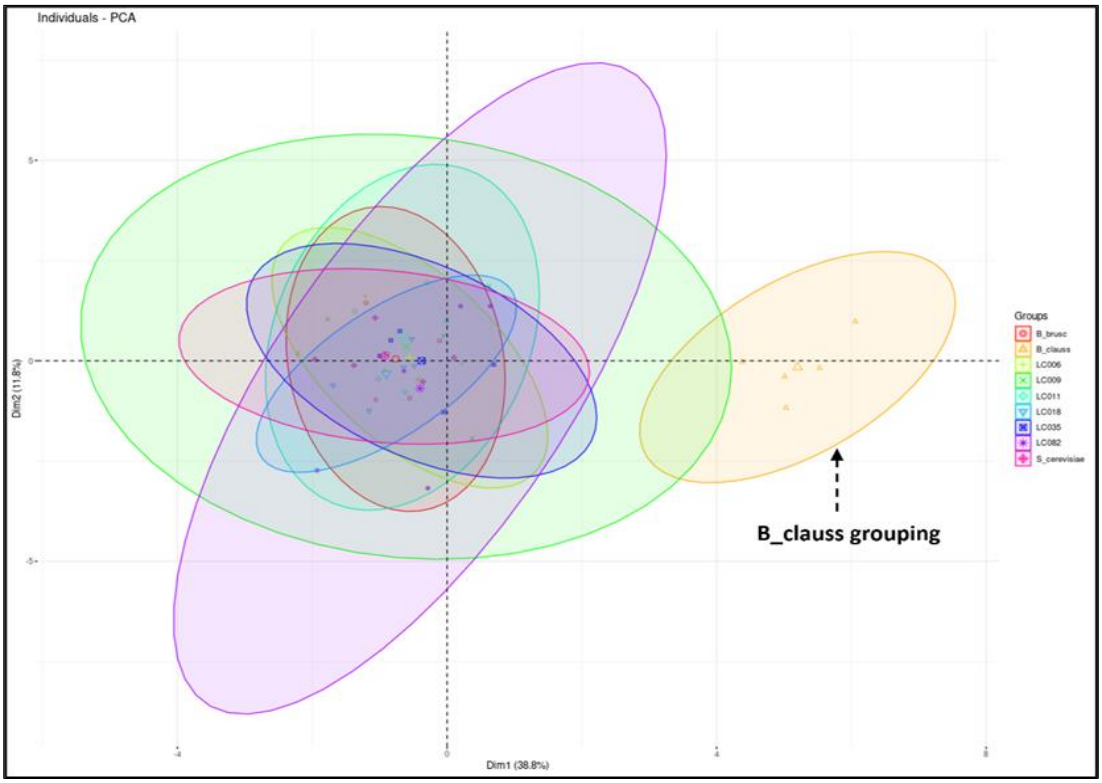


Figure 4.3: Principal coordinate analysis of a commercial yeast and wild yeast cultures for brewing characteristics

4.2.1. Physicochemical parameters

Physicochemical parameters of the brewed samples were tested for quality of the brewing process with the different yeast samples (Supplementary Table 1). The wild and commercial yeast exhibited differences in alcohol production during the fermentation process (Figure 4.4). There were significant differences ($p < 0.005$) observed between the yeast with regards to alcohol content. The highest alcohol percentage was observed with the commercial yeast *S. cerevisiae* (4.5%), which was significantly different to wild yeast LC018 (3.6%), LC082 (3.6%), LC011 (3.6%), and *B_clauss* (1.4%). Statistically, the wild yeast *B_brusc* (3.7%), LC009 (3.8%), LC006 (3.6%), LC035 (3.8%) produced similar alcohol content after brewing

when compared to the commercial yeast. The wild yeast *B_clauss* produced the lowest alcohol content.

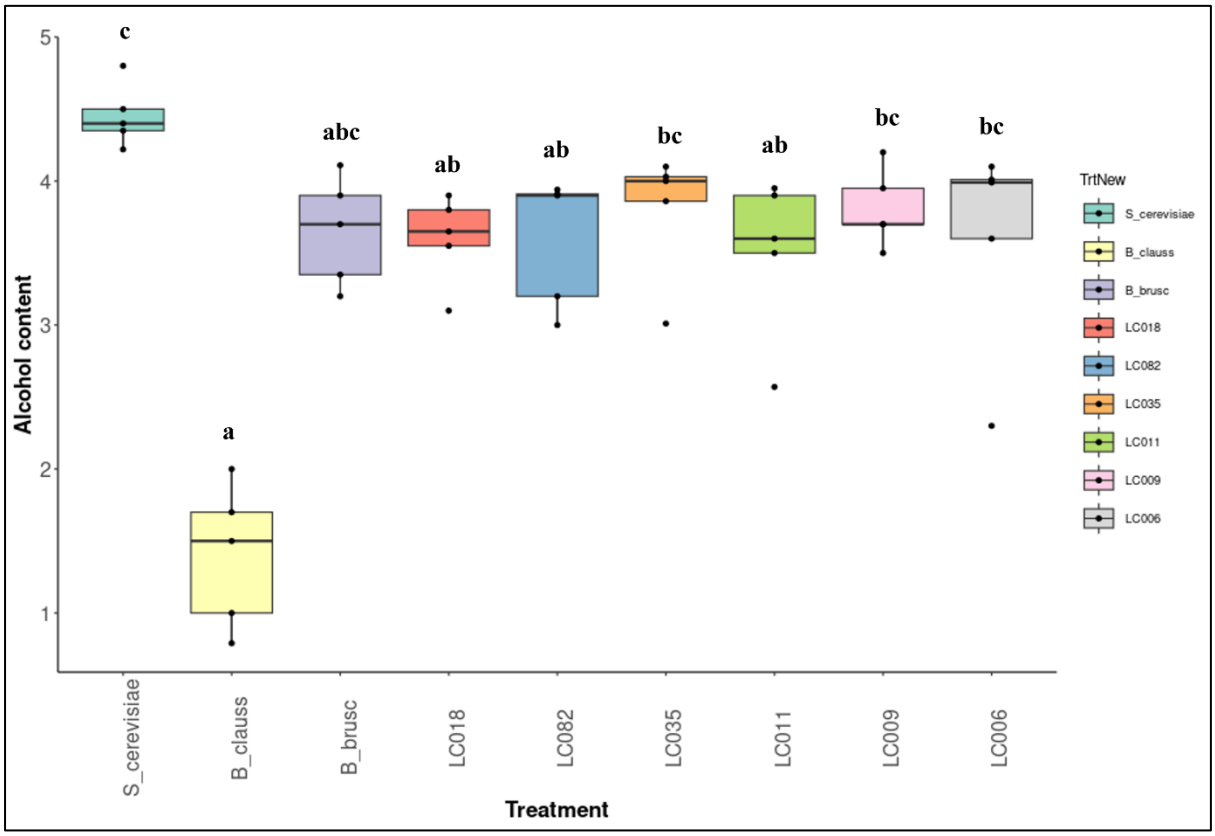


Figure 4.4: Alcohol content (%) produced in fermented wort by the different yeast strains.

With regards to the sulfur dioxide content (SO_2), there were no significant differences ($p > 0.05$) between the wild strains and *S. cerevisiae* (Figure 4.5). Numerically, LC018 had the highest sulfur dioxide content (0.97 mg/l) two weeks after brewing. The pH of the fermented wort showed a similar trend between samples, with no significant differences ($p > 0.1$) observed two weeks after brewing (Figure 4.6). The pH ranged between 3.6 to 4.3, with *B_clauss* numerically having the highest pH.

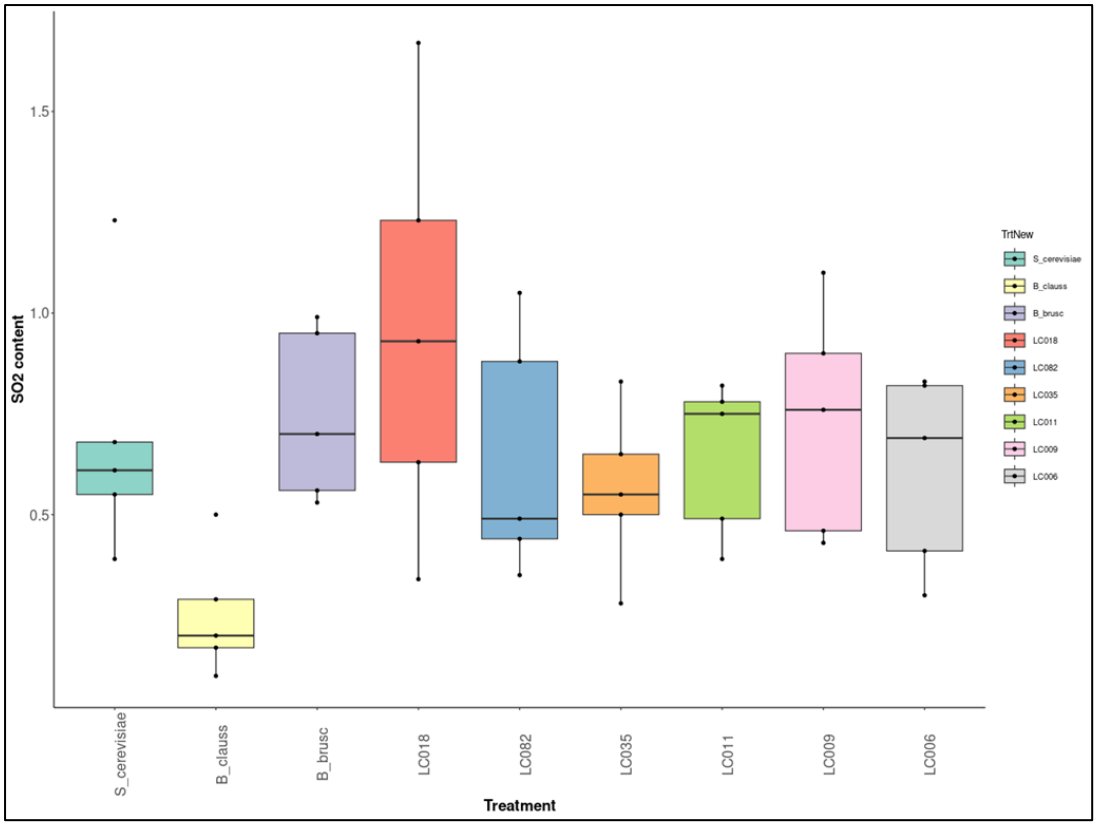


Figure 4.5: Sulfur dioxide content (mg/l) produced in fermented wort by the different yeast strains.

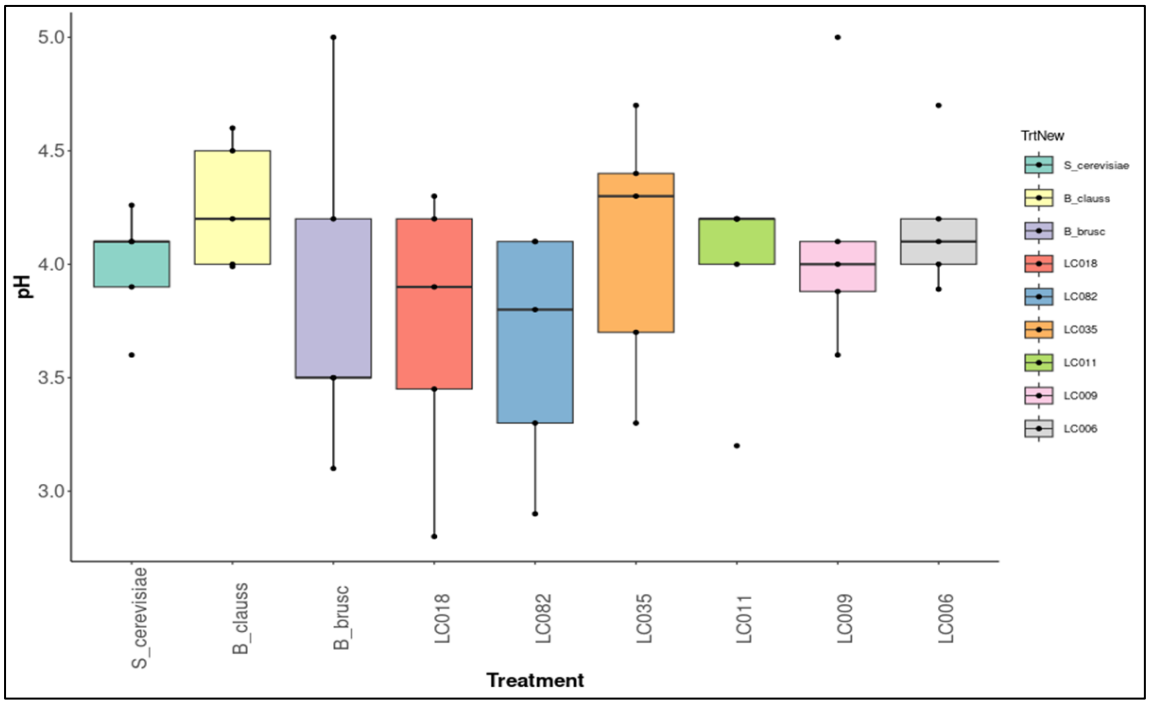


Figure 4.6: pH (scale 0-14) of samples produced in fermented fermented with wild yeast strains and *S. cerevisiae*.

The color of pale ale produced after fermentation was checked and scored according to Australian Pale ale color prolife which ranges from golden to light amber. Although the colors varied between the pale ale samples, there were no significant differences in beer color two weeks after fermentation (Figure 4.7).

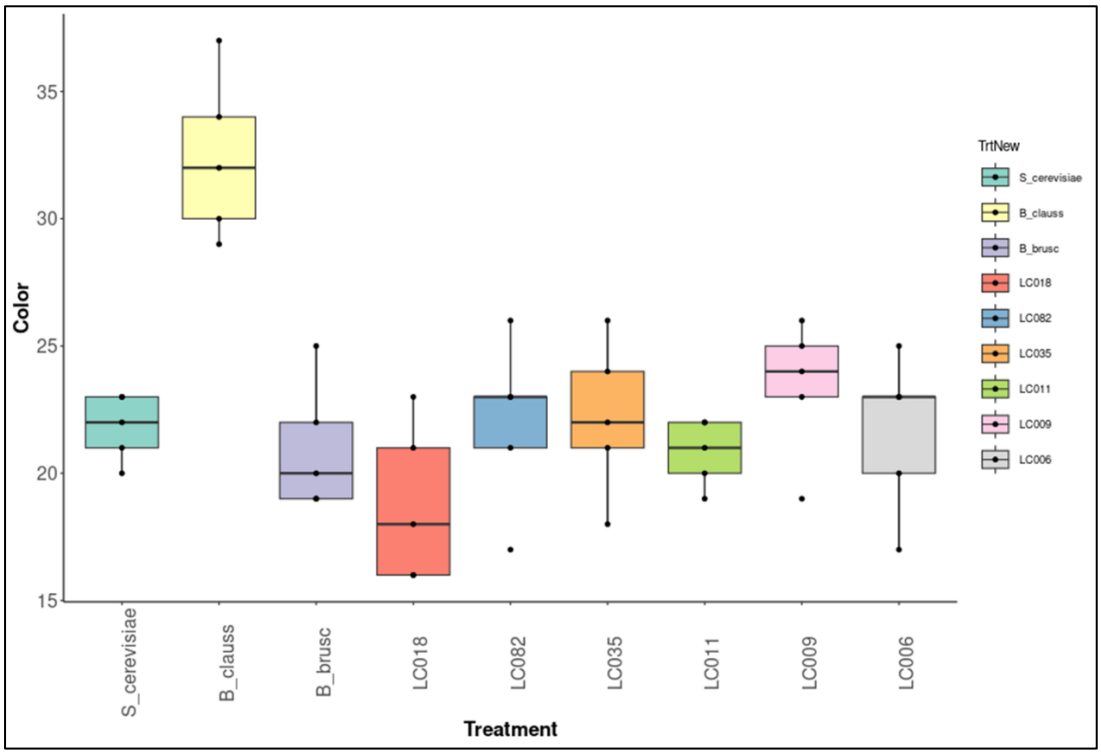


Figure 4.7: Color (EBC) of pale ale produced by wild yeast strains and *S. cerevisiae* after fermentation @430nm wavelength.

4.2.2. Volatile compounds and Ester determination

Diacetyl was detected in all beer samples fermented with the different yeast strains (Figure 4.8). There were no significant differences ($p > 0.1$) observed in the diacetyl content produced by *S. cerevisiae* and wild yeast. Numerically, the wild yeast strains produced the highest content of diacetyl after fermentation, with the highest observed in beers brewed with *B_brusc* (29.4 ppm).

Similarly, no significant differences ($p > 0.05$) were found in the volatile, Pentane (Figure 4.9) and Isoamyl acetate (Figure 4.10). With Pentane, numerically the concentration was higher in the wild yeast strain *B_clauss* (3.62 ppm) two weeks after fermentation. This was also the same for isoamyl acetate, with the yeast *B_clauss* (1.1 ppm) numerically producing the highest concentration of the volatile after brewing.

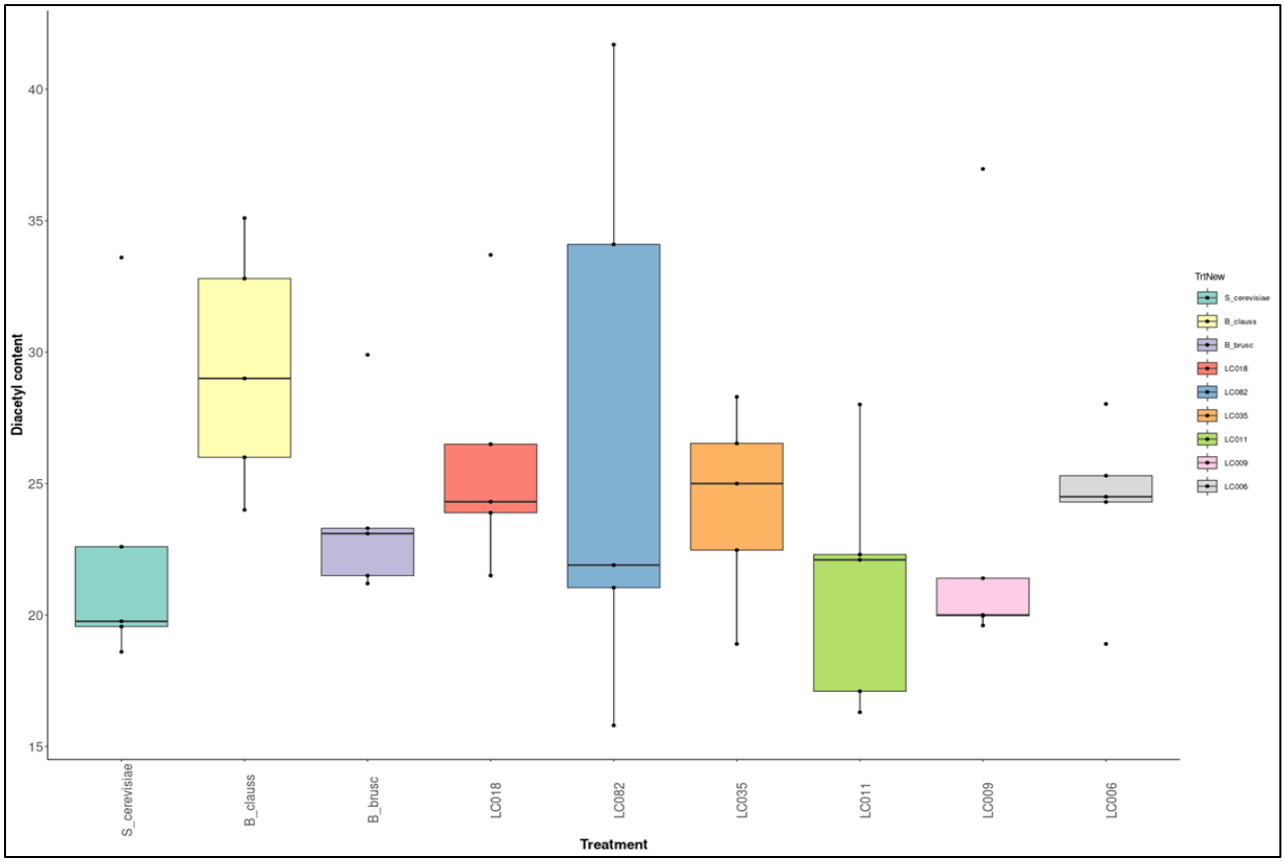


Figure 4.8: Diacetyl concentration (ppb) produced by wild yeast and *S. cerevisiae*.

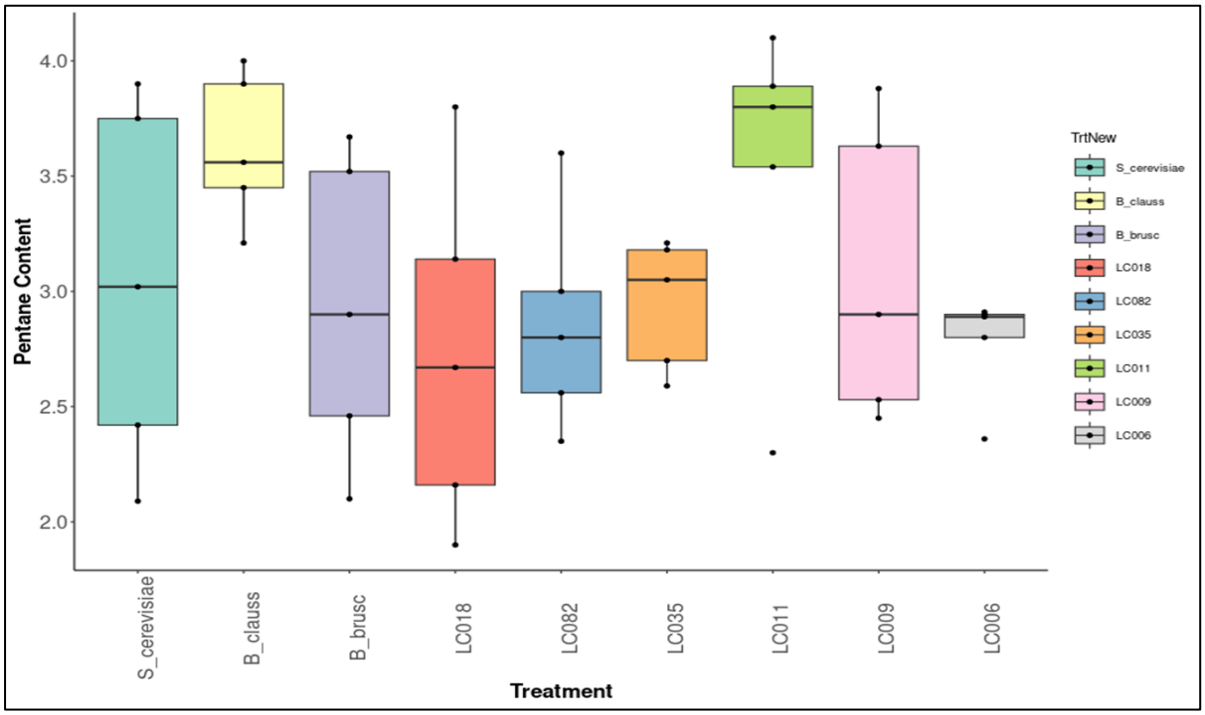


Figure 4.9: The volatile metabolite pentane (ppm) concentration produced by wild yeast strains and *S. cerevisiae* during fermentation.

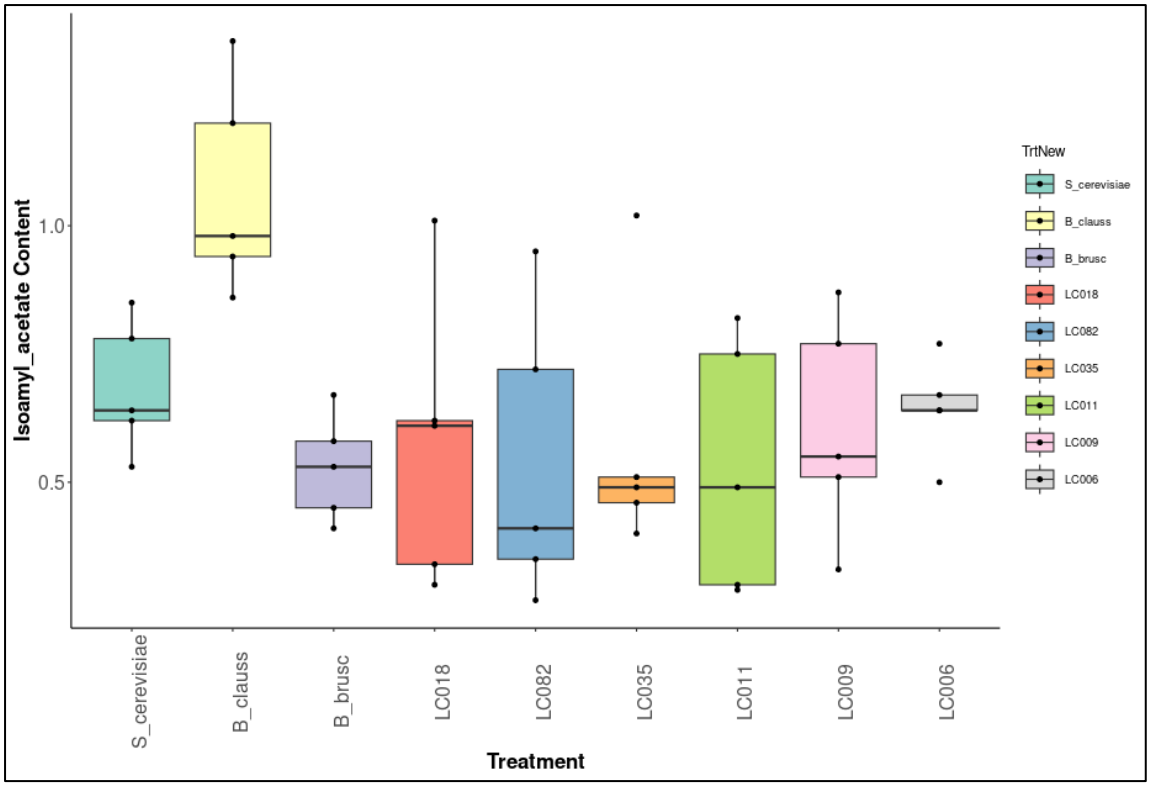


Figure 4.10: The volatile metabolite isoamyl acetate (ppm) concentration produced by wild yeast strains and *S. cerevisiae* during fermentation.

With regards to the volatile acetaldehyde, significant differences ($p < 0.005$) were observed two weeks after fermentation (Figure 4.11). The concentration of the volatile ranged between 4.86-6.86 ppm. The highest concentration was observed in the yeast *B_clauss*. Overall, the yeast *B_clauss* significantly had a higher concentration of acetaldehyde compared to the commercial yeast (*S. cerevisiae*), *B_brusc*, LC018, LC035, LC011, and LC009.

Overall, the concentration of the volatile ethyl-acetate in the samples ranged between 7.33-11.9 ppm, after brewing (Figure 4.12). The results showed that acetaldehyde was significantly ($p < 0.001$) higher in the beer samples brewed with the yeast *B_clauss* (11.9 ppm) compared to the other yeasts, including the commercial yeast *S. cerevisiae*.

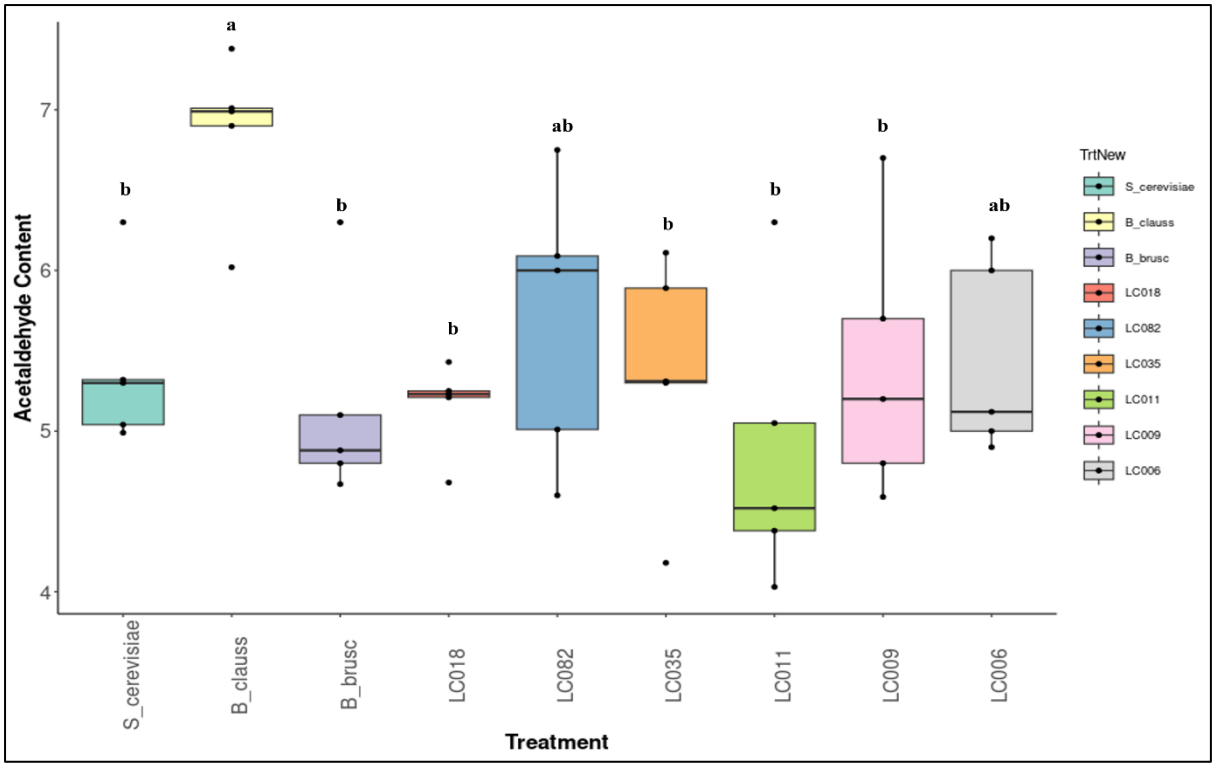


Figure 4.11: The volatile metabolite acetaldehyde (ppm) concentration produced by wild yeast strains and *S. cerevisiae* during fermentation.

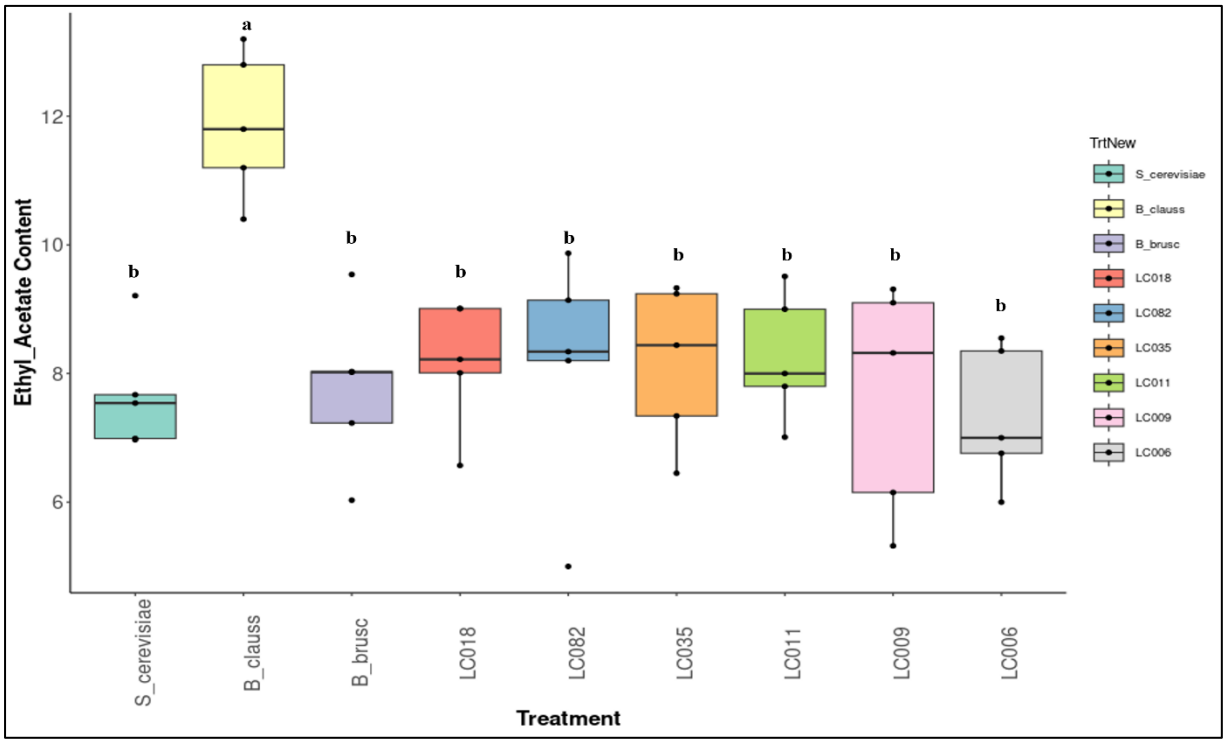


Figure 4.12: The volatile metabolite ethyl-acetate (ppm) concentration produced by wild yeast strains and *S. cerevisiae* during fermentation.

The results showed that there were significant differences ($p < 0.001$) in the concentrations of the volatile ethyl hexanoate in the beer samples two weeks after fermentation (Figure 4.13). The highest concentration of the volatile was observed in beer samples brewed with *B_clauss* (1.6 ppm), with the lowest concentration in the LC009 (0.82 ppm). Overall, the concentration of ethyl hexanoate was significantly greater in beer samples fermented with *B_clauss* compared the commercial yeast *S. cerevisiae*, LC082, LC035, LC011, LC009, and LC006.

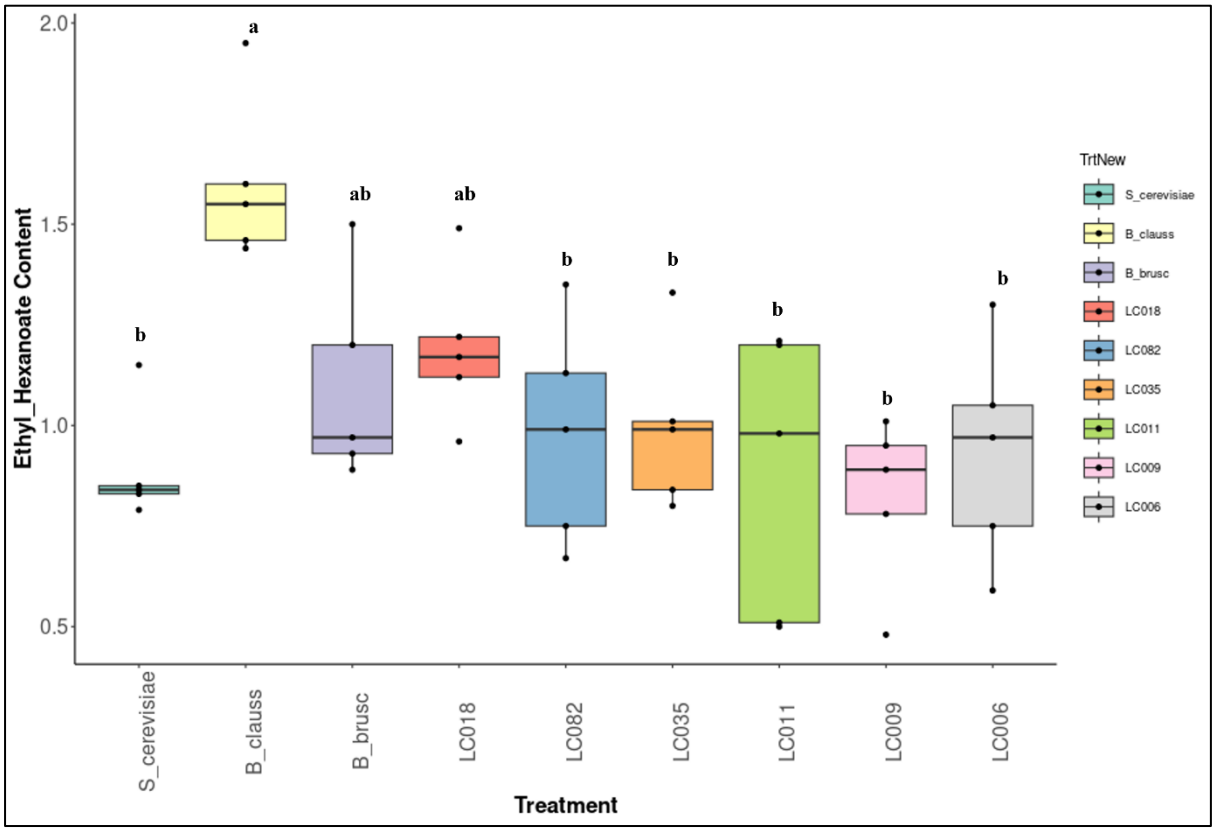


Figure 4.13: The volatile metabolite ethyl-hexanoate (ppm) concentration produced by wild yeast strains and *S. cerevisiae* during fermentation.

Supplementary information

Supplementary Table 4.3: Mean values of physicochemical parameters produced by wild yeast strains and *S. cerevisiae* during fermentation.

	Physicochemical parameter			
Treatment	**Alcohol (%)**	**SO_2 (mg/l)**	**pH (0-14)**	**Color**
B_brusec	3.65 ^{abc}	0.74 ^{ab}	3.86 ^{a}	21.00 ^{b}
B_clauss	1.39 ^{a}	0.25 ^{b}	4.26 ^{a}	32.40 ^{a}
LC006	3.60 ^{bc}	0.61 ^{ab}	4.18 ^{a}	21.60 ^{b}
LC009	3.81 ^{bc}	0.73 ^{ab}	4.12 ^{a}	23.40 ^{b}
LC011	3.50 ^{ab}	0.64 ^{ab}	3.96 ^{a}	20.80 ^{b}
LC018	3.60 ^{ab}	0.96 ^{a}	3.73 ^{a}	18.80 ^{b}
LC035	3.80 ^{bc}	0.56 ^{ab}	4.08 ^{a}	22.20 ^{b}
LC082	3.59 ^{ab}	0.64 ^{ab}	3.64 ^{a}	22.00 ^{b}
S. cerevisiae	4.54 ^{c}	0.69 ^{ab}	3.99 ^{a}	21.80 ^{b}

***Mean values with the different letters were significantly different ($p < 0.05$), while those with the same letters were not significantly different ($p > 0.05$).**

Supplementary Table 4.4: Mean values of volatiles compounds and Esters produced by wild yeast strains and *S. cerevisiae* during fermentation.

	Volatiles and Esters					
Treatment	**Diacetyl (ppb)**	**Pentane (ppm)**	**Ethyl-hexanoate (ppm)**	**Acetaldehyde (ppm)**	**Ethyl-acetate**	**Isoamyl-acetate(ppm)**
B_brusc	23.08[a]	2.93[a]	1.10[ab]	5.15[b]	7.77[b]	0.528 [a]
B_clauss	29.38[a]	3.62[a]	1.60[a]	6.86[a]	11.9[a]	1.068 [a]
LC006	24.20[a]	2.77[a]	0.93[b]	5.44[ab]	7.33[b]	0.644 [a]
LC009	23.58[a]	3.08[a]	0.82[b]	5.40[b]	7.64[b]	0.606 [a]
LC011	21.16[a]	3.53[a]	0.88 [b]	4.86[b]	8.26[b]	0.530 [a]
LC018	25.98[a]	2.73[a]	1.19[ab]	5.16[b]	8.16[b]	0.576 [a]
LC035	24.24[a]	2.95[a]	0.99[b]	5.36[b]	8.16[b]	0.576 [a]
LC082	34.10[a]	2.86[a]	0.97[b]	5.69[ab]	8.11[b]	0.540 [a]
S. cerevisiae	22.82[a]	3.04[a]	0.89[b]	5.39[b]	7.68[b]	0.684 [a]

***Mean values with the different letters were significantly different ($p < 0.05$), while those with the same letters were not significantly different ($p > 0.05$).**

CHAPTER FIVE

DISCUSSION

Demand for novel and enhanced beer quality and aroma characteristics has increased recently (Canonico et al., 2016; Estela-Escalante et al., 2016; Michel, 2017). New fermenting technologies have been developed in this area with the goal of maximizing beer production. Among these advances are the use of wild yeasts strains in brewing (Coulibaly et al., 2022; Molinet & Cubillos, 2020). Some wild yeasts have had successful industrial scale application, with improving fermentation processes, physicochemical parameters and producing desirable concentrations of fermentation metabolites (volatiles) of brewing importance Hittinger et al., 2018; Nikulin et al., 2020). This research looked at the differences in flavor and aroma that can be produced by commercial yeasts, *S. cerevisiae*, versus wild yeasts in pale ale brewing.

Due mainly to human selection, there is genetic variation between commercial brewing yeast and wild yeast strains (Gallone et al., 2016). Commercial yeast strains produce small amounts of organic aromatic compounds, do not tolerate high levels of PH and withstand high alcohol content concentration (Molinet & Cubillos, 2020). The TCA cycle is accelerated in wild strains, fatty acid synthesis is elevated, and extracellular concentrations of several secondary metabolites (including aspropyl acetate, isoamyl acetate, and ethyl acetate) are relatively high (Carpena et al., 2021). These characteristics immediately result in increased fatty acid synthesis, which is linked to a more resilient reaction to several stressors, including elevated concentrations of ethanol and salt, high temperatures, and oxidative stress (Sun et al., 2018). Wild strains therefore have a tendency to show wider ranges of resistance to various environments and, as a result, present a substantial opportunity for use in a variety of industrial applications (Krogerus & Gibson, 2020). Moreover, industrial strains exhibit self-centered behavior due to their big cell size, high rate of glucose consumption, and internal nutrient storage. Wild strains, on the other hand, behave cooperatively and release secondary metabolites to ensnare or eat rivals (Filteau et al., 2017). It's possible that this behavior will help during fermentation. As per Stanzer et al. (2023), wild isolates derived from sugar-rich sources and flowers have the potential to yield a distinct range of volatile compounds, hence broadening the range of fragrances and flavors that can be produced during alcoholic fermentation. Accordingly, wine fermentations employing native wild strains derived from oak

trees yield significant concentrations of citrus and floral characteristics in addition to earthy and sulfurous organoleptic characteristics (Chandra et al., 2020). While wild strains obtained from oak barks show stuck fermentation characteristics, native strains isolated from vineyards, grapes, and soil show outstanding fermentation properties (Freel et al., 2015). As a result, methods for improving wild strains are required, and their direct usage may be restricted.

5.1 Identified Wild yeast used for brewing.

The wild yeast used in this research were identified using PCR and Sanger sequencing. Sanger sequences of certain molecular markers, primarily from the ribosomal DNA operon, are currently used to identify the species of yeasts and other fungi. These sequences are characterized by hundreds of tandem repeats of the 18S, ITS1, 5.8S, ITS2, and LSU loci (Colabella et al., 2018). B. brusc, Ragnarok, The Proletariat, Neipa, Dark Night, La Trappist and Samson's saison are *saccharomyces cerevisiae* yeast strains. *S. cerevisiae* is known as the brewing yeast and this genus has a lot of species that have been identified using old and new molecular techniques (Onyema et al., 2023). Because they belong to the same genus, the yeast strains that were recognized as S. cerevisiae species share traits with the commercial yeast employed in this study. *S. cerevisiae* wild strains are not domesticated hence they tend to perform differently from commercial/ domesticated *s. cerevisiae*. *B. clauss* was identified as *Brettanomyces bruxellensis* yeast strain. According to Varela et al. (2019), B. bruxellensis is generally the most active fermenter in the Brettanomyces family, with some strains having the ability to completely attenuate wort. Compared to their Saccharomyces cousins, some strains are forty times more concentrated in particular esters, indicating their extreme aromaticity (Tocci et al., 2023). Additionally, compared to other species, B. bruxellensis often yields a higher number of funky phenols, making it the preferred choice for funk bombs (Branco et al., 2021). Since *B. bruxellensis* can thrive in anaerobic environments with low pH, high alcohol content, low levels of fermentable sugars, and other adverse conditions, regulating its development has proven to be a major difficulty (Schifferdecker et al., 2014). This yeast can go into a viable but not culturable (VBNC) state under stress, which is a physiological situation in which cells cannot divide on culture media even when they are still alive and exhibiting metabolic activity (Salma et al., 2013). Yeasts other than *Saccharomyces* have a lower ability for fermentation and a greater sensitivity to ethanol stress. They may also result in cloudiness,

filtration issues, and viscosity issues with beer. However, the flavors and aromas that these microbes produce can impart unique qualities to beer.

5.2 Physicochemical parameters

In the fermentation process, there was variation between *S. cerevisiae* and some wild yeasts with respect to physicochemical parameters, highlighting the importance of the type of yeasts selection for industrial processes. Alcohol is produced as a byproduct during fermentation when yeasts convert the sugars (glucose) in wort to ethyl alcohol and carbon dioxide. Furthermore, different yeasts determine the amount of alcohol produced during fermentation (Maicas, 2020). In turn, the alcohol content in beer also adds to the flavor and body of the beer. The alcohol percentage after fermentation ranged between 1.4% - 4.5%. Overall, the highest alcohol content was observed with commercial *S. cerevisiae*, while the lowest alcohol content was in beer fermented with the *Brettanomyces bruxellensis* yeast. This shows that the commercial yeasts had a higher tolerance to alcohol compared to wild yeasts. Similar studies showed that wild yeast had a lower alcohol tolerance compared to commercial yeast (Benito et al., 2019), hence more research on alcohol tolerance needs to be done because there are several factors that affect byproducts of fermentation like yeast strain, fermentation period, raw materials, and environmental conditions. Wild yeasts have a range of alcohol tolerance ratings because they are challenging to regulate during fermentation. The alcohol amount of the brew is limited since the majority of wild yeast species cease fermentation processes when the alcohol concentration reaches 6% (Foster et al., 2022). *S. cerevisiae* have been known to dominate the food and beverage industries worldwide (Maicas, 2020; Parapouli et al., 2020). Considered as the model yeasts, particular strains of *S. cerevisiae* employed in fermentation, have been reported to have a profound influence on several physicochemical parameters, including alcohol percentage (Walker & Stewart, 2016). However, alcohol percentage in beer normally ranges from 1% to 8%. This study showed that all the wild yeasts used during fermentation produced alcohol above the lower threshold of alcoholic beverages. This confirms the use of wild yeasts in beer fermentation to create different beer styles with different alcohol concentrations (Hittinger et al., 2018; Libkind et al., 2011). For example, the wild yeasts *Saccharomyces eubayanus* has been successfully used in fermentation for different beer flavors and aromas (B. Gibson et al., 2017; Libkind et al., 2011; Mardones et al., 2020; Osburn et al., 2018; Sampaio, 2018). Furthermore, this gives consumers an option to choose a beer that

suits their palate and mouth feel. In this research *B. bruxellensis* yeast produced the lowest alcohol content and this proves the facts in literature which states that production of fermentation metabolites in *B. bruxellensis* peaks after 14 days hence this yeast is best used in beers that are fermented for longer periods of time or can be used in production of low alcohol content beers.

Sulphur dioxide (SO_2) occurs naturally in beverages because of fermentation (Dvořak et al., 2006; Guido, 2015). In beer, SO_2 works as an antioxidant and is important for shelf life and flavor stability (Carneiro et al., 2006; Chen et al., 2012). In this study the levels of SO_2 were different between the yeasts, ranging between 0.25 – 0.96 mg/L, with the highest in the isolate *Ragnarok* (LC018). B. claus produced the lowest levels of Sulphur dioxide with an average of 0.25mg/L. When used in the right concentration SO_2 content produces high quality beer because of its antioxidant properties (Nardini & Foddai, 2020). However, there are thresholds for SO_2 concentration as this compound is considered an allergen (Chen et al., 2012; Guido, 2015). Therefore, SO_2 levels in beer need to be strictly monitored. In the EU, for example, according to Commission Regulation (EU) 2011/1129 the sulfur dioxide content may not exceed the limit of 20 mg/L (Guido, 2016). In South Africa the legal limit for SO_2 is 10 mg/l. In industry concentrations exceeding 10 mg/L SO_2 must be indicated on the label "Contains sulfites" (Position EU, 2011). In Africa, most brewing companies indicate SO_2 as an allergen on the label as a cautionary statement for consumers who might be allergic (Chang et al., 2023). SO_2 concentration in beer determines whether the beer will be consumed immediately if the levels are not high and beer with high SO_2 will only be consumed a few days or weeks when the SO_2 is within acceptable limits because SO2 levels drops once the beer is packaged. There are numerous methods that are used to check SO_2 levels in beer and some of these methods like p-rosaniline method includes use of toxic carcinogenic substances such as p-rosaniline, formaldehyde, and mercury chloride, (Martinez-Periñan et al., 2011). hence most commercial brewers use Skalar to check SO_2 levels compliance in packaged beer products which is reliable method and does not involve use of harmful substances. Sulphur dioxide smells and tastes like burnt matches (Beukers & Blijleven,2023) hence brewers and tasters can easily pick up high levels of SO_2. Almost all beers contain Sulphur dioxide, and this compound occurs because of yeast fermentation in some beers, or it can be added to the beer to preserve the final beer product and its raw materials like hops. The wild yeast and commercial yeast used in this research produced SO_2 that was below the upper

threshold for SO_2 compliance in South Africa. This indicated that the wild yeast used in this research performed the same as the commercial yeast and produced desirable levels of SO_2.

Other parameters, such as pH levels in the beer were tested in this study. Due to their impact on sensory qualities, biological stability, and chemical stability, pH and total acidity are crucial parameters for brewers (Silva et al., 2022). The brewing industry typically prefers pH levels between 3.90 and 4.20 for light lager and ale beers (Pai et al., 2015). The beer used in this study had a pH ranging from 3.64 to 4.26. *B. claus*, which is *B. bruxellensis* had the highest pH and The Proletariat, which is *S. cerevisiae* species had the lowest pH. Past research that has been done has shown that most beers have pH that range from 4.0 to 4.6 (Senila et al., 2023) and some of the yeast used in this study had pH in the same range as the findings from similar research. Research done by Campolongo et al. (2014) showed that when the external pH drops, *B. bruxellensis* can continue to be viable and even increase its pH gradient; but, when the external pH rises to a high level (pH 8), it cannot sustain a pH gradient and perishes. When beer becomes too acidic it can be a sign of bacterial contamination or spoilage (Ciont et al., 2022). PH monitoring is very important because it helps to keep in control the final product and the pH of the water used for brewing can be adjusted with calcium sulphate. The pH during various stages of fermentation and brewing processes affects parameters such as beer color, flavor development by yeasts, foam stability, hop bitterness and alterability of the beer (Silva et al., 2022). pH is monitored during all stages of beer production from brewing, fermentation and packaging before beer goes to customers and consumers and this alone shows how important this parameter is in beer making. In addition, during storage lower pH help in inhibiting the growth of bacteria improving beer quality (Vriesekoop et al., 2012). Ale beers are acidic because during the brewing process, natural acids are released, and the pH of the wort and beer becomes more acidic. As a result of yeasts using buffering resources (free amino nitrogen) and the production of organic acids, the pH decreases during fermentation (Bamforth, 2001).

Beer color is one of the qualities that consumers see before they taste the beer. One important aspect of a product's quality is its look, which includes its repeatable foam and color (Koren et al., 2020). Malts are a major contributor influencing beer color. Simply put, the more the malts are roasted, the darker the wort will be (Castro et al., 2021). The pale ale produced after fermentation had colors that ranged from light brown to amber and there was no significant difference between

commercial *S. cerevisiae* and the wild yeasts, and the color ranged from 18.8 to 32.4 EBC. Furthermore, the wild yeasts formed clumps during fermentation and did not cause haze in beer. When yeasts do not flocculate or form clumps and settle at the bottom of the fermentation vessel it causes haze in the beer which affects the beer color (Stewart, 2018). This shows the wild yeasts had the same flocculation characteristics as the commercial yeast used. Research done by Govender (2009) showed that saccharomyces cerevisiae yeast flocculate and settle at the bottom of the fermentation vessel after primary fermentation and the yeast used in this research exhibited the same properties. Another factor that contributes to beer color is the pH of the water during steeping of the grains and mashing (https://www.lowercasebrewing.com/beer-blog/what-determines-beer-color). This agrees with similar pH levels observed between *S. cerevisiae* and the wild yeasts contributing to similar color of the pale ale beer. The beer fermented with B. claus had the highest color and looked more darker than the other pale ales. In the wine industry, B. bruxellensis are known as spoilage organisms (Branco et al., 2021), however in beer making industry *B. bruxellensis* are becoming popular as they produce exotic flavors and they require longer fermentation period to produce desirable flavors.

5.3 Volatile compounds and Esters

Vicinal Diketones (VDKs) are a group of compounds which influence flavor and aroma in beer (Boulton & Quain, 2006). In this study the VDKs that were tested were diacetyl and pentane (pentanedione). Diacetyl and pentane are the most noteworthy VDKs when considering beer flavor. With regards to diacetyl, there were no significant differences between the wild yeasts and commercial *S. cerevisiae*, with most of the wild yeasts showing a higher capacity of forming the compound compared to commercial *S. cerevisiae*. Diacetyls are characterized by a sweet butter, caramel, or butterscotch flavors and aromas (I. M. Ferreira & Guido, 2018). However, if concentrations are too high, diacetyls can cause off flavors in beer. Molinet & Cubillos, (2020) had similar findings of wild yeasts producing more secondary metabolites during fermentation compared to commercial yeasts.

Pentane contributes honey-like notes to fermented beverages which enables beer to be paired with different foods (Boulton & Quain, 2006). Although the *B.claus*, *Dark Knight* and *La Trappist* wild yeasts had a higher capacity for forming pentane compared to commercial *S. cerevisiae*, the levels were similar among all the yeasts and there was no significant difference. *B. claus* produced the

highest levels of pentane. When VDKs are added to lagers in amounts above flavor thresholds, the beer tastes bad. Because diacetyl (2,3-butanedione) has a flavor threshold in lager that is about ten times lower than pentanedione's, it has a greater effect on beer flavor than 2,3-pentanedione (I. M. Ferreira & Guido, 2018). Even though VDKs are produced during fermentation, a long-chain reaction results in their creation rather than yeasts producing them directly. Alpha-acetolactate and alpha-ketobutyrate are released by yeast cells during the synthesis of the amino acids valine and isoleucine, respectively (Brányik et al., 2008). They are the ancestors of VDKs, and at about the midway point of fermentation, their concentration peaks. After that, they naturally break down into VDKs, whose concentration peaks at the end of fermentation.

Generally, most of the wild yeasts had a higher capacity for forming esters in beer compared to the commercial yeasts *S. cerevisiae* showing that there is diversity of competitive yeast that can be explored in the brewing industry to produce different beer styles instead of relying only on domesticated yeast that have been used for decades. The ability of wild yeast to produce high levels of esters indeed explains the different and supplementary flavor aromas that are found in beers fermented with wild yeasts (Nikulin et al., 2020). There are different strains of yeast that can be used for brewing. However, in this research the wild yeast strains that were used were B. bruxellensis species and *S. cerevisiae* and the yeast were identified using sanger sequencing. The *B. bruxellensis* species, which was B. claus produced the highest levels of esters compared to the other strains of *S. cerevisiae* that were used for brewing in this study. Past research has proved that *B. bruxellensis* used in brewing has unique profile (Budroni et al., 2017). In fermented drinks, volatile esters fall into two primary categories. (Jespersen & Jakobsen, 1996). Small quantities of esters can give the aroma of beer a nice, full-bodied quality. When esters are overdone, the aroma of beer becomes overly fruity, which is not to everyone's taste. The esters that were investigated in this study were ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), ethyl hexanoate (anise seed, apple-like odor), and acetaldehyde (unripe green apple aroma). About one-third of the esters in beer, ethyl acetate, was found to be produced in the highest concentration (11.9 ppm) in beer fermented with *B. claus* yeast (*B. bruxellensis* strain), and in the lowest concentration (7.33 ppm) in beer fermented with *Samson's saison* yeast (*S. cerevisiae* strain). The amount of ethyl acetate that the yeast utilized in this study produced varied significantly. Nonetheless, the majority of wild yeast generated this ester at levels comparable to those of *S. cerevisiae* sold commercially. The type of yeast used will determine how the cells and fermenting

media are distributed, however the intracellular fermentation of yeast cells produces longer-chain fatty acids such ethyl acetate esters (which are C13–C22 long). Ethyl esters can permeate past cellular membranes into the fermenting phase because they are lipid soluble, which accounts for their higher amounts in beer (Saerens et al., 2008). The taste results showed that there were no off flavors in wild yeasts fermented beer meaning that the volatile esters produced where within the brewing threshold. This opens doors for breweries to new fermentation that includes using wild yeasts from all over the world.

There was a significant difference in the amount of acetaldehyde produced by the wild yeast and commercial yeast used in the research. *B. claus* produced the highest concentration of acetaldehyde and Dark Knight had the lowest concentration of acetaldehyde. The commercial yeast produced similar levels of acetaldehyde as the other wild yeast (*La Trappist, Samson saison, Dark knight, Neipa, B. brusc, Ragnarok and the Proletariat*). This shows that other wild yeast can be studied further and used in brewing to produce lagers, pale ales, pilsners, and wines. Acetaldehyde is normally present in beer in very low concentrations because high levels of this ester can cause off flavors in the beer. During fermentation acetaldehyde is produced before ethanol however traces of ester are found in the final liquor because it isn't always completely decomposed (Eram & Ma, 2013). Ethyl hexanoate was produced in all the beers that were brewed with both the wild yeast and commercial yeast. There was a significant difference in the amount of ethyl hexanoate produced by the wild yeast and commercial yeast. *S. cerevisiae* wild and commercial yeast produced lower levels of ethyl hexanoate compared to *B. claus* which is *B. bruxellensis* species that produced the highest level of ethyl hexanoate. Isoamyl acetate was tested in all beer samples, and it was present. There was no significant difference in the levels of isoamyl acetate that were produced by the wild yeast and commercial yeast. Isoamyl acetate adds aroma and flavor to beer however very high concentrations of this ester can cause banana like off flavors in beer (Holt et al., 2019). *B. claus* was the only yeast that produced isoamyl acetate that was above 1ppm and the other yeast had similarly low levels of this ester. The ability of the wild yeast used in this research to produce same levels of esters as the commercial yeast shows that there is room for growth in the brewing industry as more yeast can be used to brew beer which can result in new beer styles and creating new flavor profiles. The brewing industry has been limited to use of domesticated yeast samples for centuries however biotechnology is showing that wild yeast has a future in

brewing industry as more yeast are now being studied and craft beers are now increasing on the market because of wild yeast that are now being used to create new beer styles.

5.4 Beer flavor and aroma

Beer sensory indices are the most important quality check before beer goes to a customer and sensory speaks to the flavor profile. According to Habschied et al. (2022), production of various craft beers is increasing offering many distinct sensory characteristics of beer. Pale ale beer is well known for its intense flavor profile and the findings from this study are consistent with reports from other research in literature (Kawa-Rygielska et al., 2022). The pale ale made with commercial yeasts had a malty and citrusy flavor, while the pale ale brewed with wild yeast showed an interesting pattern of exotic flavors which varied from batch to batch. Overall, the study showed that the pale ale brewed with wild yeasts were more aromatic than the beer brewed with commercial yeast. This shows the ability of wild yeast to produce exotic flavors and create new beer styles. Recent studies on wild yeast strains have identified alleles that may favor favorable traits for use in brewing, such as nitrogen intake, tolerance to high or low temperatures, and the synthesis of metabolites like glycerol and aroma compounds (Molinet & Cubillos, 2020). Wort's sugars are fermented into ethanol and volatile substances like esters and higher alcohols, which are concurrently produced as byproducts of yeast metabolism. The unique flavor characteristics of beer are mostly caused by these metabolic processes that occur within the yeast cell (Olaniran et al., 2017). These compounds have a significant impact on the flavor and aroma of beer.

5.5 Yeast identified after brewing.

The yeast strains identified were *S. cerevisiae* and *B. bruxellensis*. The wild yeast samples used at the beginning of the experiment and the samples identified after the experiment were the same strains hence there was no mutation or any phenotypic changes that occurred during the experiment. Similar research was performed by (Iorizzo et al., 2021) using saccharomyces and non-saccharomyces yeast. Yeast that mutates during fermentation can impair beer quality because they are uncontrollable resulting in poor fermentation which affects the final product quality (Capece et al., 2018). Although they are frequently found in breweries, yeast alterations may go undetected (Astola et al., 2023). Since the mutant can't compete with regular yeast and usually vanishes quickly, it usually has no negative effects. However, occasionally mutant yeast will outcompete regular brewing yeast and exhibit a wide range of expressions. For instance, the

fermentation of maltotriose may be impacted by a mutation, or the rate of fermentation may fluctuate continuously. Previous research have used genotype association and phenotyping to link a number of distinct genetic variations to features that are advantageous for the flavor of a particular beer (Chen et al., 2015).

CHAPTER 6

CONCLUSION

Numerous advancements are made concerning the yeast strains used in fermentations during the creation of alcoholic beverages (Walker & Stewart, 2016). In addition to increasing the efficiency of converting sugar to alcohol, research is being done to find new yeasts that can impart desired tastes and use diverse carbohydrate source materials (Walker & Stewart, 2016). The brewing sector is changing as a result of consumer demand, the choice of quality raw ingredients, and the development of novel brewing techniques. Since they have been cultivated and utilized for hundreds of generations, yeasts are the most essential raw material in fermentation and are a dependable tool in the brewing business.

The results clearly show that wild yeasts have a potential to play a huge role in industrial biotechnology because of their ability to produce different VDKs and alcohol content which can be used to produce different beer styles to suit different palates of consumers. This research showed that there are more yeasts in the environment that can be exploited and used in fermentation as they possess almost the same characteristics as the commercial brewing yeasts.

Yeasts produce vital by-products which can be used in other processes hence research on this organism contributes positively to the economy of the world and shows yeasts can play a big role in industrial biotechnology. The brewing industry is one of the biggest contributors to world economy and growth in this sector is desperately needed to produce new fermented beverages to suit the different needs of consumers and this research clearly shows the possibility of reaching the goals of brewing through exploitation of novel yeasts to produce more flavors and new beer styles. More research is still needed in identifying the strains of the wild yeasts that perform better than the known brewing yeasts.

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