Assessment of Stress Tolerance and Fermentation Performance in Traditional Norwegian Landrace (Kveik) Yeast

Thesis Submitted to the University of Nottingham for the degree of Master of Research

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Table of Contents

Abstract

Kveik yeast are believed to constitute a subspecies of *Saccharomyces cerevisiae* used by rural Norwegian brewers to brew traditional ales for personal and ceremonial consumption. These yeasts have remained largely unexplored until recent times, where their potential for use in mainstream beer production has been realised. Due to the nature of their origins and use, kveik cultures often comprise multiple strains, although single-strain isolates have been identified. Irrespective of their composition, the advantage of kveik yeast is that they ferment rapidly at high temperatures and, under these conditions, produce a relatively neutral flavour contribution when compared to contemporary brewers yeasts. However, a full understanding of the properties and potential for kveik strains has yet to be achieved.

This thesis aimed to investigate several aspects of kveik yeast. Firstly, the nature and strain composition of kveik slurries was explored to determine the extent of variation within a defined culture. Subsequently, the capacity of individual kveik strains to tolerate stressors present during fermentation was investigated. Based on this analysis, the fermentation performance of kveik strains was compared to contemporary brewers yeast at a range of temperatures. This was achieved through assessment of fermentation progression, as well as the development of flavour compounds. As part of this study, the contribution of individual isolates to a fermentation was assessed using various blends of kveik yeast, derived from the same 'mother' culture.

The data obtained demonstrates that kveik yeast are able to ferment successfully at high temperatures, due to their capacity to endure a range of stress factors, including a high degree of innate thermal tolerance. Furthermore, although kveik yeasts were shown to exhibit a similar capacity to attenuate wort as their contemporary brewing yeast counterparts, volatile analysis indicated that kveik strains are unique and distinct when compared to other brewing yeast strains.

Individual strain analysis also suggested the possibility that specific strains may be dominant within a collective whole, potentially imparting a greater impact on the resulting beer from the perspective of both alcohol yield and flavour production.

List of Figures

Page

List of Figures (cont.)

Page

List of Figures (cont.)

Page

List of Tables

Page

List of Abbreviations

ABV	Alcohol by Volume
ADF	Apparent Degree of Fermentation
APCI	Atmospheric Pressure Chemical Ionization Mass Spectrometry
CoA	Coenzyme A
CTA	Catalase Gene
DMS	Dimethyl Sulphide
FAN	Free Amino Nitrogen
GC-MS	Gas Chromatography-Mass Spectroscopy
HOG	High Osmolarity Glycerol
HSP	Heat Stress Protein
NCYC	National Collection of Yeast Cultures
POF	Phenolic Off-flavour
psi	Pounds per square inch
RO	Reverse Osmosis
ROS	Reactive Oxygen Species
RPM	Revolutions per Minute
SG	Specific Gravity
SOD	Superoxide Dismutase
STRE	Stress Response Element Gene
VDK	Vicinal Diketone
WLN	Wallerstein Laboratory Nutrient
YPD	Yeast Peptone Dextrose

Chapter 1

1. Introduction

1.1. The History of Brewing

Beer is a beverage that is derived from four main ingredients: water, malted cereal grains, hops, and yeast. The earliest known creation of a beer-type product dates to approximately 13,000 years ago from a Natufian burial site at Raqefet Cave, Israel (Liu et al., 2018). Beer brewing in ancient times was small in operation as early humans were predominantly nomadic, but as civilization progressed, settled collectives began to develop specialized roles within the community. Different positions within the commune would participate in the different individual processes, such as water collection, foraging, hunting, construction, and grain cultivation for both beer and bread (Meussdoerffer, 2009). As time continued, beer brewing became more advanced; the construction of permanent breweries (and bakeries), along with improved farming techniques are believed to have been critical to the advancement of the population. A key stage in the evolution of the process of brewing came during the industrial revolution, after which beer brewing became specialized and highly advanced (Unger, 2013). Major innovations such as steam generation, temperature control, refrigeration, and understanding of the microbiological nature of fermentation were critical to beer progressing to the beverage we know today (Hornsey, 2003).

1.2. Brewing Raw Materials

1.2.1. Water

Water is the most abundant ingredient in beer and is often referred to as ‘liquor’ in the brewing lexicon. The sourcing of fresh water is arguably a key step in

selecting a location for a brewery. Although in current times water is mostly derived from a municipal source, historically deep wells were constructed or surface water sources were utilized. Irrespective of source, the water should be potable and free of any local, regional, or nationally-declared contaminants. It should also be free of odour, sediment, and microbes (including bacteria), suspended solids, metals (such as iron), and high ion contents (such as chlorine residues from public water works treatment) (Palmer & Kaminski, 2013).

1.2.2. Cereal Grains

The types of cereal grains used in brewing greatly influence the flavours and aromas of the final product (Mallett, 2014). Barley and wheat are used predominantly (Lewis & Young, 2012), although depending on the type of beer being made, the geographical location, agricultural limitations or cultural preferences, other grains such as rye, millet, sorghum, corn, or rice can also be used (Briggs et al., 2004). Although raw grains can be used directly in brewing (for example to promote head retention), their use is relatively minimal (Stewart, 2016a). Instead, cereal grains are usually malted over a 7-10 day during which biochemical and physical changes occur resulting in product that is much more suitable for brewing. The procedure involves three primary steps: steeping, germination, and kilning. These stages instigate enzyme production, break down cell walls and proteins, as well as releasing sugars and starches that can ultimately be utilized by yeast during fermentation (Mallett, 2014).

Once in the brewery, malt undergoes a process called mashing and the main product of this procedure is wort: a nutrient-laden liquid substrate that yeast utilizes to produce beer (Kunze, 2019). Malt provides the bulk of nutrients for yeast including sugars, carbohydrates, minerals, and nitrogen in the form of proteins and amino acids. Malted barley also contributes directly to colour and allows for the creation of beers ranging from very pale gold to an opaque black.

1.2.3. Hops

Although hops (*Humulus lupulus*) are used in the majority of beers consumed across the world, their introduction to the brewing process is relatively recent. The earliest recorded date indicates that they began to be used in central Europe around 800 AD (Edwardson, 1952). Hops provide bitterness and aromatic qualities to beer, but also possess antimicrobial attributes. Lupulin glands within the hop cone contain resins with alpha acids, beta acids, and essential oils (Briggs et al., 2004) and bitterness is a result of a physiochemical transformation (isomerization) of the alpha acids via heat which produces isomerized alpha acids. This happens during the kettle boil; the longer the hops are present in the boil, the higher the potential bitterness (Kunze, 2019). This attribute is also 'breed specific'; each variety of hop has its own alpha acid value, albeit this can change from year to year. The essential oils in hops (between 0.5% and 5% by weight) provide a wide array of aromatic compounds that range from citrus, stone fruit, herbal, various spices, woodsy, or grassy flavours. The antimicrobial capacity of the hop is also derived from the presence of alpha and beta acids and their respective isomers (Hrnčič et al., 2019).

1.2.4. Yeast and Fermentation

While there are some beer styles that can be produced using bacteria, as well as ‘wild’ yeasts, most products are created using domesticated *Saccharomyces* yeast strains (White & Zainasheff, 2010). These yeast strains have often been selected over centuries by humans for their specific properties and the spectrum of products appropriate for particular beer styles. Key attributes associated with yeast domestication include temperature or alcohol tolerance, speed of fermentation, attenuation (the ability to ferment wort sugars), flocculation (the capacity of yeast cells to aggregate and settle from the media), and flavour production (Boulton & Quain, 2008). While ethanol, CO_2, and biomass (new cells)

are the primary products of fermentation, yeast metabolism also produces a range of flavour and aroma compounds that are integral to the overall impression of the beer. These products include esters, higher alcohols, carbonyl compounds (such as acetaldehyde and vicinal diketones or VDKs), sulphur compounds, and phenols (Kunze, 2019).

The type of beer produced can be broadly defined as either ale or lager, determined by the species of yeast used in fermentation. Ale products are fermented using *Saccharomyces cerevisiae* and lagers with *Saccharomyces pastorianus*. Both types of yeasts assimilate sugars and other nutrients from brewers wort but are manipulated to produce different flavours and aromas (Briggs et al., 2004). Whereas ale type products have a longer lineage in history, pale lager style beers are a more recent concept and dominate in terms of sales volume and popularity at the present time (Gorter De Vries et al., 2019).

1.3. The Brewing Process

The first step in the brewing process is the milling of malt, to create a 'grist' that is then mixed with hot liquor to create a mash (Mallett, 2014). This process releases proteins, sugars, and precursors for a multitude of flavour compounds, as well as colour to the wort. Once the process of enzymatically degrading starch into simpler sugars is complete, the separation of solids from the liquid fraction is achieved through a process called lautering, which allows the collection of sweet wort which is then pumped to a kettle where it is boiled (Briggs et al., 2004).

Arguably, the primary reason for boiling wort is for sanitation, but this process also serves to arrest enzymatic activity, allow hop isomerization to increase bitterness, and precipitation of protein-polyphenol compounds for clarification. In addition, the boiling process allows concentration of the wort and volatilization of aromatic compounds such as DMS, as well as pH reduction and colour development (Kunze, 2019). At the end of the boil, the wort is clarified using a

whirlpool and cooled using a heat exchanger as it is sent to the fermenter. At this stage, the addition of oxygen is also usually performed inline, a process critical for yeast metabolism at the onset fermentation (White & Zainasheff, 2010).

Yeast is introduced, or pitched, to the cooled wort within the vessel and this allows fermentation to begin. Yeast cells assimilate sugars and other nutrients extracted from the wort and produce ethanol, carbon dioxide, and flavour and aroma compounds (Boulton & Quain, 2008). The length of fermentation is dependent on the yeast strain, the temperature, as well as other parameters, such as the amount of yeast used (pitch rate), aeration/oxygenation levels, and the viability/vitality of the yeast culture (Briggs et al., 2004). Once fermentation is complete the beer undergoes a maturation phase that can take from several days up to many weeks. This step allows for flavour development and additional clarification due to the settling of particulate matter.

The beer can be clarified further if desired (usually by centrifugation and/or filtration) and is then sent to a final ‘bright beer’ tank, where carbonation levels are adjusted to desired specification and final sensory analysis takes place. Upon established acceptability, the beer is ready for packaging into can, keg, cask, or bottle and is ready for delivery to the point of sale.

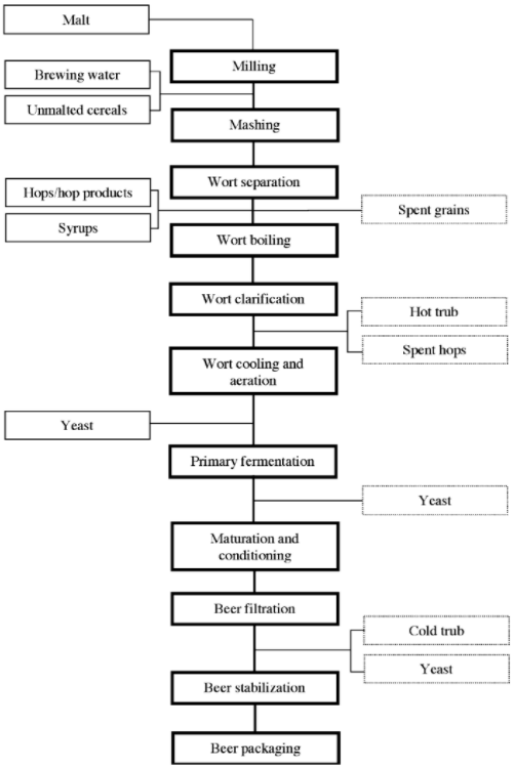


Figure 1.1. Overview of the brewing process. Input flows are indicated on the left of the image and outputs on the right-hand side (Willaert, 2007).

1.4. Yeast

Yeasts are members of the Fungi Kingdom and can be used in a variety of biotechnical applications. These include beer, wine, sake, ciders, and spirit production, as well as for other foodstuffs such as baking and chocolate. Other industrial uses include for the manufacture of bioethanol as a fuel source, enzyme production for pharmaceutical and medical practices, and for organic degradation of materials in bioremediation (Walker, 1998). As described briefly above (Section 1.2.4), within the brewing industry yeasts belonging to the *Saccharomyces* genus are predominantly used. The main species *S. cerevisiae* and *S. pastorianus* are used to create ales and lagers, respectively (White & Zainasheff, 2010). The derivation of the nomenclature is thus: *Saccharo-* meaning

sugar, and -*myces* meaning fungus. In the case of ale type yeasts, the term *cerevisiae* is derived from the Latin word for beer (Pires & Brányik, 2015), while for *S. pastorianus*, the species name recognises the famed 19th century microbiologist Louis Pasteur. It should be noted that lager strains were formerly classified at different times as *S. cerevisiae*, *S. carlsbergensis*, and *S. uvarum*, and this terminology can sometimes still be encountered today (Boulton & Quain, 2008).

Ales ferment at warmer temperatures than lagers and, because of this, their metabolism is faster and overall fermentation time is shorter (Boulton & Quain, 2008). Due to the elevated temperature of fermentation, ales have a pronounced fruity character, notably derived from an increase in ester, aldehyde, and higher alcohol production during fermentation (Briggs et al., 2004). Although there are variations according to individual yeast strains and the beers being produced, ales are typically used for fermentation at approximately 18˚C - 22˚C, and lagers at 8˚C - 15˚C (Table 1.1). Although not present in brewer's wort, lager strains can metabolize the sugar melibiose, whereas ale strains cannot (Stewart & Russell, 2009). Since melibiose does not exist in brewers wort, it is specifically used in a laboratory environment for identification purposes (Table 1.1).

Differences Between Ale and Lager Yeast	
Ales	**Lagers**
Can grow up to 37˚C	Cannot grow above 34˚C
Typically used in fermentations at 18˚C - 22˚C	Typically used in fermentations at 8˚C - 15˚C
Faster fermentation at several days	Slower fermentations at 1 -3 weeks
Higher ester and higher alcohol production	Lower ester and higher alcohol production
Lower sulphur production	Higher sulphur production
Cannot ferment melibiose	Able to ferment melibiose
Genome sequence length = 1	Genome sequence length = 1.5

Table 1.1. Metabolic and phenotypic differences between ale and lager yeast. Adapted from (Stewart, 2016)

1.4.1. Cell Morphology

Brewing yeast cells are approximately 5-10 µm in diameter and ovoid to ellipsoidal in structure (Figure 1.2). Since they are eukaryotic organisms, they possess defined organelles and a nucleus within the cell (Stewart & Russell, 2009). The key organelles include the nucleus, mitochondria, vacuole, golgi, and endoplasmic reticulum, as well as the cell envelope which acts as a semi-permeable barrier separating the cell from its environment (Figure 1.3)

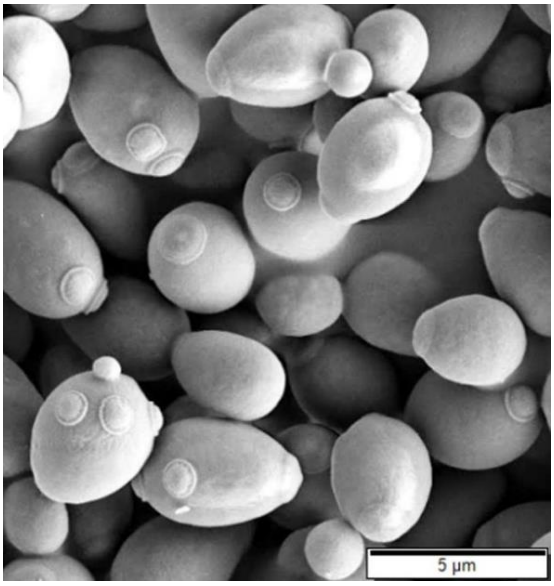


Figure 1.2. Scanning electron microscope image illustrating the size and shape of *Saccharomyces cerevisiae* yeast cells (Murtey, 2016).

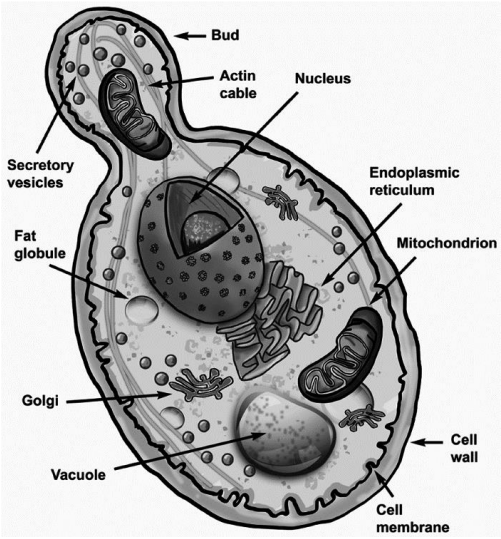


Figure 1.3. Key organelles found within the yeast cell (Stewart, 2017).

1.4.2. Classification and Identification

Contemporary yeast identification techniques utilize genetic sequencing, which allows classification based on total or specific sequences. Development of such methods has been facilitated by an understanding of the yeast genetic make-up; *S. cerevisiae* was the first eukaryotic organism to have its full genome sequenced (Goffeau et al., 1996). More traditional identification methods were based on physiological and/or phenotypic characteristics, such as cell and colony morphology, the capacity to produce spores, and the ability to assimilate certain sugars (Morris & Hough, 1956; Gallone et al., 2018).

1.5. Role of Yeast in Brewing

1.5.1. Fermentation and Ethanol Production

During the initial phase of fermentation, yeast cells rapidly utilize oxygen to produce fatty acids and sterols for cell and organelle membrane synthesis. This facilitates yeast growth, ensures that membrane structures are healthy, and assists with regulating overall membrane functionality (Boulton & Quain, 2008). During fermentation, nutrients enter the cell; these include, but are not limited to, glucose, fructose, sucrose, maltose, and maltotriose, nitrogenous compounds, lipids, vitamins, and minerals. In addition to key metabolic end products (biomass, CO_2, and ethanol), several by-products are formed including esters, higher alcohols, sulphur compounds, carbonyls (including acetaldehyde and VDKs), and, depending on the yeast strain, phenolic compounds (Kunze, 2019).

The process of alcoholic fermentation begins with sugar breakdown the glycolytic pathway. The main objective of this pathway is to create adenosine triphosphate (ATP), which is the primary energy source for the cell. The end product of this pathway is pyruvate which can be converted by yeast to acetaldehyde and then

to ethanol. The first step yields CO_2 which is released from the cell and provides carbonation originates of the beer. As the alcohol content rises, so does the toxicity of the surrounding environment of the yeast cell (Ding et al., 2009). This can act to reduce the activity of yeast cells, slowing fermentation and causing issues related to viability and vitality, such as reduced alcohol yield, higher desired final gravity, higher ester content, as well as an increase in VDKs, sulphur, and acetaldehyde (White & Zainasheff, 2010). Stress tolerance is therefore a key requirement for industrial yeast strains (Section 1.6.1).

Overall activity begins to slow as sugars and nutrients are consumed for cell metabolism and reproduction and become limited. At this point, yeast prepares for 'dormancy,' and cells build up energy reserves in the form of glycogen and trehalose. At the same time, flocculation typically occurs which describes the aggregation of yeast cells which then separates from the medium. The mechanism and extent of flocculation is both species and strain dependent (Stewart, 2016), but in general, brewing strains tend to be highly flocculant when compared to their wild counterparts. Upon completion of fermentation, the yeast is harvested and stored prior to reuse in a practice known as serial repitching.

1.5.2. Flavour and Aroma Production

1.5.2.1. Higher Alcohols

Higher alcohols, sometimes referred to a fusel alcohols, are primarily by-products of nitrogen and lipid metabolism (Hough et al., 2012). Lower levels of higher alcohols contribute to the overall flavour and aroma of beer, but excessive levels can bring forth 'hot' or solvent notes which can be considered a flaw (White & Zainasheff, 2010). Levels of higher alcohols are dictated by yeasts strain and environmental conditions (Lewis & Bamforth, 2006). Higher temperatures will increase higher alcohol production. An increase in wort gravity can also increase

concentrations as essentially more fermentation takes place. Similarly, aspects that favour cell growth, such as higher wort aeration and higher FAN, will also increase levels of these compounds due to the corresponding increase in amino acids within the cell. Underpitching can also increase higher alcohols, as the need for cell growth is high, so there is a need for increased amino acid synthesis for cellular protein production (Boulton & Quain, 2008).

1.5.2.2. Esters

Esters are arguably the most important flavour and aroma compounds produced by yeast (Kunze, 2019). There are over 100 known esters in beer and these are responsible for fruity characters. They are predominantly created by the reaction between a fatty acid (or acetyl-CoA) and an alcohol (Verstrepen et al., 2003). The amount and type of esters produced in beer are directly related to the variety of yeast used and its environmental conditions (Briggs et al., 2004). Each yeast strain has its own ideal temperature range and certain strains will produce a high quantity of a specific ester (Pires et al., 2014).

Ester content is largely correlated to fermentation temperature, and this acts as a key major sensory difference between ales and lager beers due to their typical temperature ranges (Hough & Stevens, 1961). It should be noted that when temperature becomes too high, regardless of strain, the esters produced can become excessive and produce a solvent-like character in the beer, which is undesirable (Pires et al., 2014). As with higher alcohols, a higher wort gravity can also increase ester content as yeast activity is enhanced. Esters can have a synergistic effect on the flavour profile of a beer, meaning that although individual esters may be below sensory perception, collectively they can contribute towards the overall flavour and aroma profile of a beer (Janish, 2016).

1.5.2.3. Vicinal Diketones (VDKs)

Arguably the most important 'green' or non-matured beer characteristic stems from the carbonyl groups consisting of vicinal diketones, or VDKs. The most important VDK, called diacetyl, provides a buttery-like flavour profile to the beer. While low levels of diacetyl can be acceptable in certain beer styles, excessive quantities are regarded as a flaw (Krogerus & Gibson, 2013). Once fermentation is complete, an additional period of time is often required to remove the compound, known as a 'diacetyl rest.' This can be 1-2 days for an ale and a week or more for lager beers, and the precise time can also depend on temperature, yeast variety, vitality, and the extent of flocculation (Inoue, 2008).

1.5.2.4. Sulphur Compounds

Small amounts of sulphur complexes arrive from all raw ingredients as well as processing aids. Yeast cell metabolism drives many sulphur compounds as yeast require sulphur for synthesis of amino acids, proteins and enzymes, such as CoA (Ferreira & Guido, 2018). Requirements and production will be yeast species and strain specific and will be present in the beer in various quantities dependent on fermentation temperature, vigour of fermentation, and time of conditioning. Typical sensory descriptors of sulphur compounds include rotten egg, struck match, skunk, rubber, garlic/onion, or canned corn.

1.5.2.5. Phenols

Phenolic compounds are responsible for medicinal, plastic, smoke, or spicy characters (Lentz, 2018). These are yeast strain dependent and their flavour threshold can be extremely low, making small quantities very apparent to the consumer. Although common among many Belgian ales and Bavarian hefeweizens, most brewers yeast strains have been selected (via yeast domestication) for the absence of this characteristic (Mukai et al., 2010). As such,

phenolic presence in a beer outside of those styles may indicate a wild yeast infection.

1.5.3. Attenuation

Attenuation describes the degree to which available sugars (extract) are consumed by yeast during fermentation and is expressed as a percentage (Kunze, 2019). Some ale yeast strains are referred to as super-attenuators and they are often characterised as belonging to the subspecies known as *S. cerevisiae var. diastaticus.* These yeasts can metabolise carbohydrates/dextrins which are not able to be assimilated by *S. pastorianus* or standard *S. cerevisiae* strains (Boulton & Quain, 2008). This trait is derived from the presence of three genes (STA1-3) that allow the yeast to produce a glucoamylase enzyme that breaks down carbohydrate into fermentable sugars (Suiker & Wösten, 2022). The high degree of attenuation results in beers with higher alcohol, thinner body, and higher levels of carbonation (Briggs et al., 2004). If a beer is packaged with this yeast present, the added carbonation due to extended fermentation can cause gushing in the final package or even dangerous bottle explosions (Štulíková et al., 2021). Circumstantially, the STA gene is adjacent to the gene labelled as the POF (Phenolic Off Flavour) gene, causing the traits of high attenuation and phenolic presence to often be found together (Boulton & Quain, 2008).

1.5.4. Flocculation

Flocculation is the property of yeast cells to aggregate together and is dictated by a number of specific flocculation genes (Vidgren & Londesborough, 2011). The aggregation of cells promotes either yeast sedimentation to the bottom of the vessel, or alternatively a rise to the surface due to entrapment of CO_2 in cell aggregates (Speers, 2016). Either of these mechanisms enable the harvesting of

yeast and are the derivation of the terms 'bottom cropping' and 'top cropping', respectively.

Highly flocculant strains settle early and can produce brighter, clearer beers which can be beneficial, but the fast sedimentation reduces the surface area interaction of the yeast with the surrounding beer causing for some potential issues such as increased diacetyl, underattenuation, and can produce a sweeter product. Non-flocculant yeasts do not settle as readily, so they tend to have a faster rate of green beer flavour reduction and can result in beer with a drier character due to the high surface area contact with the maturing beer (Stewart & Russell, 2009). These yeasts may also cause hazier beers, which can result in clarification and filtration issues and require the use of additional fining/clarifying agents.

1.5.5. Serial Repitching

Brewing is the only sector within the fermentation industry where yeast is harvested and reused for subsequent fermentations (Stewart, 2016b). This process is known as 'serial repitching' and is cost-effective compared to purchasing fresh yeast for every fermentation. After fermentation, the yeast can be directly transferred to another fermenter and used immediately. Another option is to draw the yeast from the fermenter into a smaller collection vessel, sometimes called a 'brink', and placed into cold storage before being used (Stewart & Russell, 2009). After harvest and storage, the slurry is added (pitched) into a batch of for the subsequent wort for another fermentation. Each time this occurs, another 'generation' is added to the age of the yeast. As the number of generations increases, the likelihood of genetic drift or infection by bacteria or wild yeast also increases. Further repetition of multiple stress factors on yeast cells as they age, including osmotic, oxidative, alcoholic, thermal, pH shifts, and nutrient starvation, can exacerbate adverse physical health effects of the cell also

induce negative fermentation performance parameters (Gibson et al., 2007). These include differences in flocculation, time of fermentation, attenuation, ethanol production, viability, vitality, and overall flavour profile (Jenkins et al., 2003).

1.6. Brewing Yeast Quality

1.6.1. Stress Factors Associated with Fermentation and Yeast Handling

Yeast cells experience different stresses during propagation, fermentation, and storage. These challenges, both individually and collectively, can negatively impact fermentation performance and yeast cell health. Of particular importance are stresses related to oxidative reactions, membrane osmotic pressures, ethanol production, and thermal stress (Bleoanca & Bahrim, 2013). These exposures in relation to a yeast's process timeline are depicted in Figure 1.4.

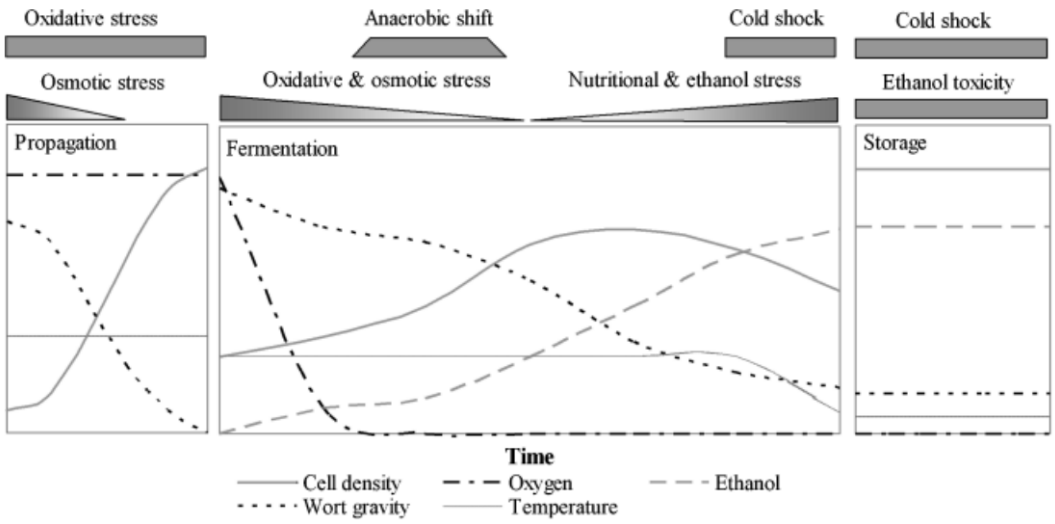


Figure 1.4. Stress factors associated with brewery yeast handling (Gibson et al., 2007).

Organelles and cell structures such as membranes are of particular importance since they are affected by stresses and can be readily damaged (Figure 1.5). Furthermore, since brewers yeast is generally repitched (Section 1.5.5), stress is cyclical and can cause a decline in yeast vitality (health). In extreme conditions, stress can also cause 'necrosis' or cell death (Farrugia & Balzan, 2012). This can invariably have a direct impact on the quality of the yeast, and subsequently the beer created.

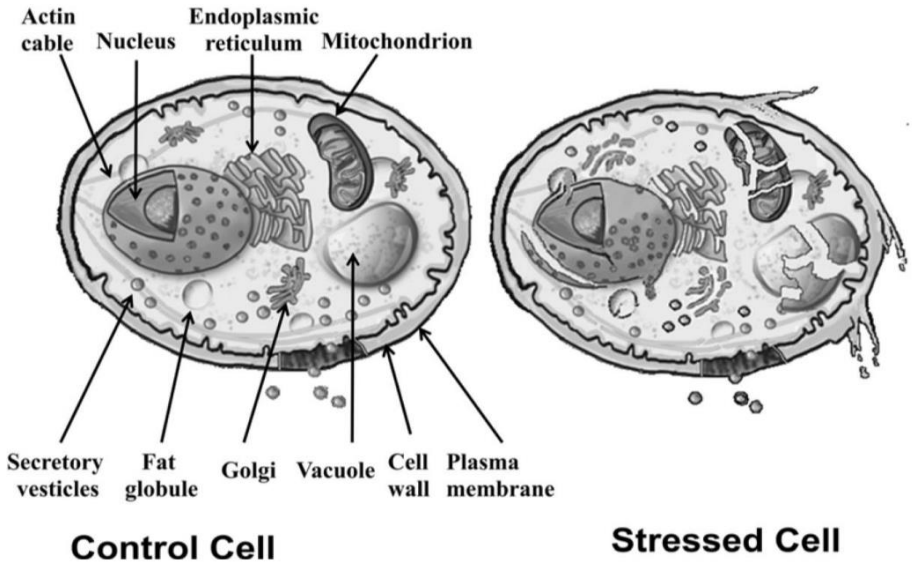


Figure 1.5. Artistic impression of the effect of stress factors on yeast organelles (Stewart, 2020).

1.6.1.1. Oxidative Stress

During propagation and the early stage of fermentation, yeast cells reside in a highly aerobic environment. Oxygen is necessary for yeast cells to create sterols and unsaturated fatty acids which are critical for cell and organelle membrane synthesis (Gibson et al., 2007). While aeration of wort is vital to yeast health, oxygen derivatives, collectively called Reactive Oxygen Species (ROS) can have detrimental effects on cell health; the extent to which this occurs is correlated to

time and intensity (Briggs et al., 2004). Oxidative stress can occur due to molecular ground-state oxygen (O_2), but more commonly from other forms, including superoxide (O_2^-), peroxide anions (O_2^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and perhydroxyl radicals (OOH•) (Vanderhaegen et al., 2006; Bamforth, 2017).

Each of these species act on the yeast in a variety of ways, but their presence typically imposes cellular damage on proteins, lipids and even DNA. Lipid damage degrades membrane fluidity and can cause inactivity of cellular enzymes and receptors on the membrane (Martin et al., 2003). Proteins and enzymes can be damaged due to breakage in peptide bonds and DNA degradation can create petite depleted mitochondria, and cause for complications regarding genetic insertions, deletions, or mutations to the cell. These problems can be exacerbated on subsequent generations considering serial repitching due to genetic mutation and/or viability (Gibson et al., 2008; Bleoanca & Bahrim, 2013).

1.6.1.2. Osmotic Stress

When yeast is taken from a storage vessel and pitched into wort, it transitions from a nutrient-deficient to a nutrient-rich environment. Since the main component of wort is sugar, this creates an osmotic stress, derived from the formation of a concentration gradient across the cell membrane (Bleoanca & Bahrim, 2013) (Figure 1.4). When the concentration of solutes outside the cell exceeds the concentration within, it becomes hyper-osmotic and there is a tendency for solutes to diffuse into, and water to leak out of, the cell (Estruch, 2000; Meledina et al., 2021). This pressure is directly proportional to solute concentration (osmolality) and can directly affect metabolism and cell performance with a decrease in viability (Heggart et al., 1999; Zhuang et al., 2017). Increases in osmotic pressure have shown to hinder cell growth and stall fermentation, potentially due to high levels of ethanol localised with the cell

(D'Amore et al., 1988). To counteract osmotic stress, yeast cells upregulate the HOG pathway (O'Rourke et al., 2002), which results in production of compatible solutes, such as glycerol, which act to stabilise the cell. The reverse scenario (hypo-osmotic shock) is less common but can also exist, for example if a yeast slurry is exposed to deionized water, extracellular water will enter the cell leading to enhanced cell turgor (Csonka & Hanson, 1991).

Irrespective of the nature of osmotic stress, this is of particular relevance to many breweries that carry out high-gravity brewing, a process where higher wort concentrations are used in the fermenter with the final product being diluted with deaerated water prior to being packaged (Stewart, 2010). The use of high gravity worts therefore exacerbates osmotic stress across the process.

1.6.1.3. Ethanol Stress

Ethanol is a key product of brewing and its presence exerts a stress to yeast cells. This is especially significant for high gravity products where concentrations of 10% ethanol (Briggs et al., 2004) can be exceeded, but is also true for standard beers of 3% - 6% ABV (alcohol by volume). Although desirable in beer production, ethanol is toxic to yeast at elevated levels and can negatively affects cell viability (Bleoanca & Bahrim, 2013). Alcohol acts to harm membranes and cellular proteins (denaturing) and adversely impacts overall membrane fluidity, meaning that the selective exchange of materials through the membrane can be compromised (Gibson et al., 2007). This directly affects nutrient transportation and consequently cell metabolism and division (Meledina et al., 2021). Elevated levels of ethanol can also instigate mutation of mitochondrial DNA causing for the creation of mitochondrially-depleted 'petites' which cannot undergo respiration (Jenkins et al., 2009).

Ethanol related damage can also occur during storage if not controlled; a high ethanol environment during storage negatively affects cell viability, producing

cells that are not appropriate for repitching (Figure 1.6). These damaging effects may also be exacerbated with increasing temperature (Boulton & Quain, 2008). Incidentally, although damage to yeast has direct implications on beer quality, there are also indirect links. For example, deficient or damaged membrane structures can lead to excretion of protease which impact beer quality by reducing head retention in the final product (Lentini et al., 2003).

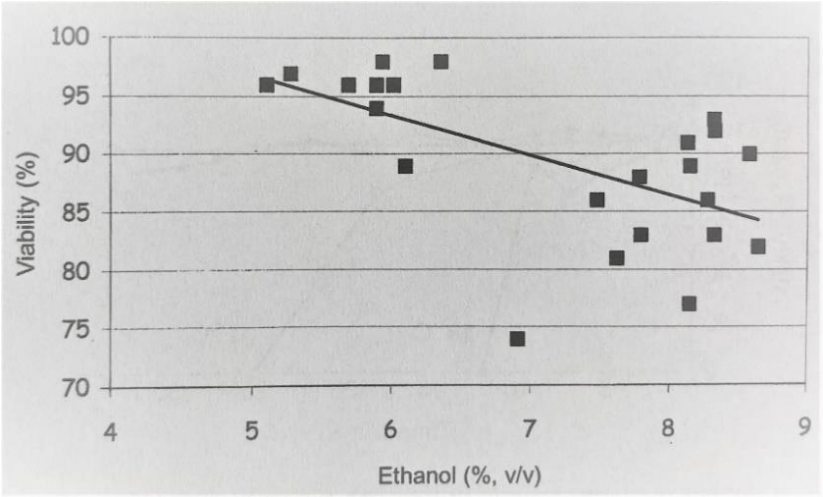


Figure 1.6. Relationship between exogenous ethanol concentration and viability of yeast in storage tank (Boulton & Quain, 2008).

1.6.1.4. Thermal Stress

From the perspective of yeast cells, which are usually able to survive well at temperatures between 25°C and 35°C, the brewing process is 'cold.' Production yeast strains have operating temperature ranging from 18°C-25°C and 8°C-15°C for ale and lager strains, respectively (Walker & Stewart, 2016). Temperature influences metabolic processes that affect cellular health, directly impacting the flavour and aroma profile of the beer produced. Fluctuations in temperature (even inside normal functional range) can trigger a thermal shock response in order to maintain cellular regularity (Bleoanca & Bahrim, 2013). While yeast cells

experience stresses directly due to temperature, it must be noted that, as alluded above (Section 1.6.1.3), increases in temperature can exacerbate other stresses.

Thermal stress due to very high temperatures causes the denaturing of proteins and negatively impacts hydrogen bonds and hydrophobic interactions within the cell. These can eventually lead to cell death (Walker, 1998). Yeast also exhibit cold shock sensitivity. Upon completion of fermentation, yeast is harvested for storage and repitching. To facilitate this process, the fermenter is rapidly cooled (crashed cooled) to induce rapid settling and yeast is further chilled to <4°C either inline or within the collection vessel after transfer. This can induce cold shock, especially if the temperature shift is sudden (Aguilera et al., 2007). Under such conditions, the cell membrane fatty acid arrangements are altered and the lipid layer transitions from a liquid to a gel state. Both these actions increase the fluidity/permeability of the membrane as a whole (Suutari et al., 1990; Gibson et al., 2007).

During storage, yeast should be kept in a sanitary environment, as cold as possible without freezing (1°C - 3°C), free from oxygen, under little pressure, gently agitated for uniformity, and for the shortest period of time as possible (White & Zainasheff, 2010) An increase in time and/or temperature will deplete glycogen reserves, which is in high demand at the onset of the subsequent fermentation and can induce stuck or longer fermentations, lower attenuation, lower alcohol production, potentially produce off flavours, and create yeast that is not suitable for repitching (Stewart, 2020) (Figure 1.7).

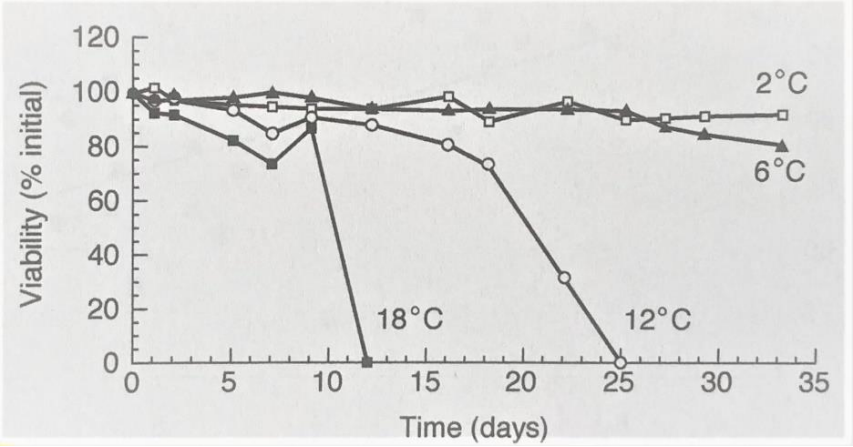


Figure 1.7. Effect of time and temperature on the viability of lager yeast during extended storage (Boulton & Quain, 2008).

1.6.2. General Yeast Stress Response

As described in Section 1.6.1, environmental stresses such as ethanol, high osmotic pressure, temperature, and reactive oxygen species (oxidative stress) are prevalent in brewing environments, and these can all have an impact on yeast health and beer quality. Fortunately, *Saccharomyces* species have defence mechanisms to combat stresses that are critical to evolutionary survival, allowing them to compete with other microorganisms (Rodrigues-Pousada et al., 2005). Primarily, *Saccharomyces* species possess a stress response element (STRE) system that proves to resolve the negative effects of a wide variety of environmental stresses factors. Upon the onset of a particular stress, the yeast cell manages the potential harmful effects by utilizing a rapid molecular response to restore the damage and shield against further exposure to the stress in question. These are predominantly in the form of a range of stress proteins (Estruch, 2000). In addition, a key product of the STRE pathway is the production of trehalose, an important carbohydrate reserve that is known to aid yeast cells under periods of stress by maintaining the cell membrane and internal membrane structures (Majara et al., 1996).

1.6.2.1. Oxidative Stress Response

Yeast possess both non-enzymatic and enzymatic antioxidant defence mechanisms (Jamieson, 1998). Non-enzymatic constituents include trehalose and glutathione (Martin et al., 2003). Glutathione is the most abundant radical scavenging molecule in cell that reacts with oxidants and reduces hydrogen peroxide to water and oxygen (Jamieson, 1998). Trehalose assists in protecting the plasma membrane and enzymes as well as enabling the repair of proteins (Ding et al., 2009). Additionally, it acts as a carbon source during nutrient-deprived storage periods (Gibson et al., 2007)

Enzymatic sources include peroxidases and reductases, as well as catalase (Kwon et al., 2003). The catalase response is STRE-triggered (Section 1.6.2) and is caused by the presence of oxygen and ROS (Swan et al., 2003). Two catalase genes, CTA1 and CTA2, facilitate the removal of hydrogen peroxide (H_2O_2) by hydrolysis to water and oxygen (Martin et al., 2003). Superoxide dismutase, SOD, working in tandem with copper, zinc, or manganese, has shown to be one of the principal enzymes in eliminating superoxide anions from the cytosol (Bleoanca & Bahrim, 2013). In addition, glutathione reductase and glutathione peroxidase enzymes both assist in preserving a high reduction-oxidation (redox) ratio within the cell (Auesukaree, 2017). The reductase enzyme reduces oxidized glutathione and peroxidase acts as an electron donor to glutathione, enabling glutathione to act as a reductant towards hydroperoxides (Jamieson, 1998).

1.6.2.2. Osmotic Stress Response

Yeast respond to osmotic stress by accumulating osmoprotectants such as glycerol, which acts to stabilize cellular components including membranes and proteins (White et al., 2003). Glycerol is accumulated rapidly when yeast is subjected to a highly osmotic environment and this increase has been shown to resist high osmotic pressure gradient (Gibson et al., 2007).

The reaction to osmotic stress can depend on initial cell health, wort sugar and carbohydrate spectrum, and the stage of fermentation. It has been suggested that the yeast cell is more susceptible to damage during the exponential stage of fermentation because glycogen and trehalose, two reserve carbohydrates, are reduced at this point (Meledina et al., 2021). The disaccharide trehalose, which also assists in oxidative, alcohol, and thermal stability, acts to protect the cell membrane. It has also been shown that cells high intracellular trehalose levels experience a lower rate of death under stressful environments (Hounsa et al., 1998).

1.6.2.3. Ethanol Stress Response

During the aerobic phase of fermentation, yeast utilize oxygen to develop unsaturated fatty acids and sterols in the cell membrane. This assures efficient membrane transport and assists the cell in combating multiple stresses including ethanol. While this trait is exhibited by all yeasts, it has been shown that higher ethanol-tolerant strains possess higher quantities of long-chain fatty acids than less ethanol-tolerant strains (Chi & Arneborg, 1999). Correspondingly, alcohol tolerance is generally believed to be linked to the genetic backgrounds of the strain (Briggs et al., 2004; Boulton & Quain, 2008). As previously mentioned, trehalose also plays a role in ethanol tolerance due to its protective effects on the cell membrane (Majara et al., 1996) (Section 1.6.2).

Enzymatically, oxidative stress-induced manganese superoxide dismutase has been shown to lessen the toxicity of ethanol, but this enzyme is only active at the onset of fermentation when oxidative stress is high and declines as fermentation progresses (Costa et al., 1997). Metal cations such as calcium and magnesium, sourced from raw ingredients and exogenous salts, may also provide a degree of protection (Dombek & Ingram, 1986).

1.6.2.4. Thermal Stress Response

The response that yeast have towards thermal stress is related to expression of specific heat shock genes. The genetic reaction to thermal stress is called Heat Shock Response (HSR). When non-lethal temperature increases damage proteins, gene expression (directed by STRE; Section 1.6.2) stimulates the production of Heat Shock Proteins (HSP). These provide homeostasis and act as 'chaperones' to replace the proteins and enzymes lost to heat-induced denaturation (Mühlhofer et al., 2019). Circumstantially, HSPs also assist in other stressing parameters such as the presence of ethanol or ROS (Heggart et al., 1999) (Table 1.2). Approximately 70 HSPs have been identified that are produced in high quantities under stress conditions. These function to maintain cellular homeostasis, allowing cell growth to continue. Other HSPs act in restorative function by repairing denatured proteins (Briggs et al., 2004). In relation to temperature shock, trehalose safeguards yeast from higher temperature by strengthening the cell membrane (Bolat, 2008).

Cold shock also induces a response by yeast, leading to accumulation of trehalose, glycerol, and heat-shock proteins in order to stabilize the cell. This is a critical survival mechanism especially relevant to brewery yeast cultures, since they are stored cold to prevent cellular activity during serial repitching (Somani et al., 2019). During this storage phase, STRE-induced accumulation of both glycogen and trehalose stabilise both protein and membrane structures as a survival mechanism (Aguilera et al., 2007).

Heat Shock Protein	Physiological function
Hsp104	Essential for thermotolerance acquisition. It is expressed constitutively in respiring cells, that do not ferment, entering stationary phase.
Hsp 100	Involvement in solubilization of protein aggregates and degradation of proteins
Hsp 90	Similar function to chaperonin and Hsp 70
Hsp83	Chaperone function
Hsp70 family	Interact with denatured proteins, helps with their solubilization and simultaneous refolding, having a chaperone function Implication in post- translational import
Hsp60	Functions similar to Hsp70, facilitates post-translational protein assembly
Reduced size Hsp	
Hsp30 Hsp26 Hsp12	Cellular role is not entirely known; it seems they are involved in the initiation of stationary phase and in induction of sporulation Hsp30 may regulate plasma membrane ATP– ase
Other proteins	
Ubiquitin	Implicated in the turnover of stress- degraded proteins
Part of glycolitic enzymes	Enolase (Hsp48), glyceraldehide 3 – phosphate dehydrogenase (Hsp35) and phosphoglycerate kinase
Catalase	Antioxidant defence
GP400 and P150	Implicated in HSP secretion.

Table 1.2. Heat Shock proteins involved in thermal stress response in *Saccharomyces cerevisiae* (Bleoanca & Bahrim, 2013).

1.7. Origins of Brewing Yeast

1.7.1. Origins of Ale and Lager Strains

Yeast are believed to have originated more than 300 million years ago (Lin & Li, 2014), and all strains prior to domestication would have been considered to be 'wild' yeasts by today's standard. Yeast can operate in both aerobic and anaerobic environments, which give them competitive advantage over other microorganisms in a defined environment (Piškur et al., 2006). The domestication of strains is believed to have coincided with the advent of civilization, specifically the baking of bread (Hardwick, 1995). Since this time, yeast strains have been using have been selected for their various attributes, for example properties

related to their suitability for baking, winemaking, or for brewing (Katz et al., 1991). Consequently, it has been argued that *Saccharomyces cerevisiae* yeast is the oldest domesticated microorganism (Donalies et al., 2008). There are many etymological examples of this, for example the English word 'ale' stems from the Nordic 'öl,' itself related to the old Saxon word 'alu' (Hoad, 1996).

Original fermentations used to make beer-type products would invariably have comprised a collection of different yeast and bacteria, and the fermentation results would have been varied with little consistency (Sparrow, 2005; Tyakht et al., 2021). It is widely accepted that selection of yeast strains by performance coupled with positive-outcome mutations has given rise to the industrial yeasts used today. Domestication in this way has provided yeasts with desirable characteristics, including the ability to ferment wort sugars (specifically maltose and maltotriose), alcohol tolerance, appropriate flocculation properties, and the suppression of non-desirable flavours such as phenolic notes (Gibson et al., 2020). It has been shown that due to domestication, the multitude of industrial yeasts that humans utilize today are derived from five groups, or clades of 'original' yeast (Figure 1.8). Each of these clades are genetically distinct from one another (Figure 1.9) and from 'wild' strains found in nature (Gallone et al., 2016). Two of these clades include the vast majority of brewing yeasts; one group includes various strains of Asian origin (such as sake yeasts); another houses the wine yeasts; and a final clade, which is relatively diverse, containing baking and other yeasts.

The two beer clades, simply referred to as Beer 1 and Beer 2, collectively comprise more than 85% of all known brewing strains. Beer 1 includes those from three geographically distinct locations: Belgium/Germany, Britain, and the United States; while Beer 2 lacks a defined set of geographical locations (Figure 1.8). However, Beer 2 yeasts are more closely related to the wine clade and comprises roughly 1/5 of all brewing strains (Gallone et al., 2016). Incidentally, in general Beer 1 yeasts displayed more traits of domestication; these strains produce less

off-flavours (POF-), increased maltotriose utilization, and do not sporulate (Figure 1.9).

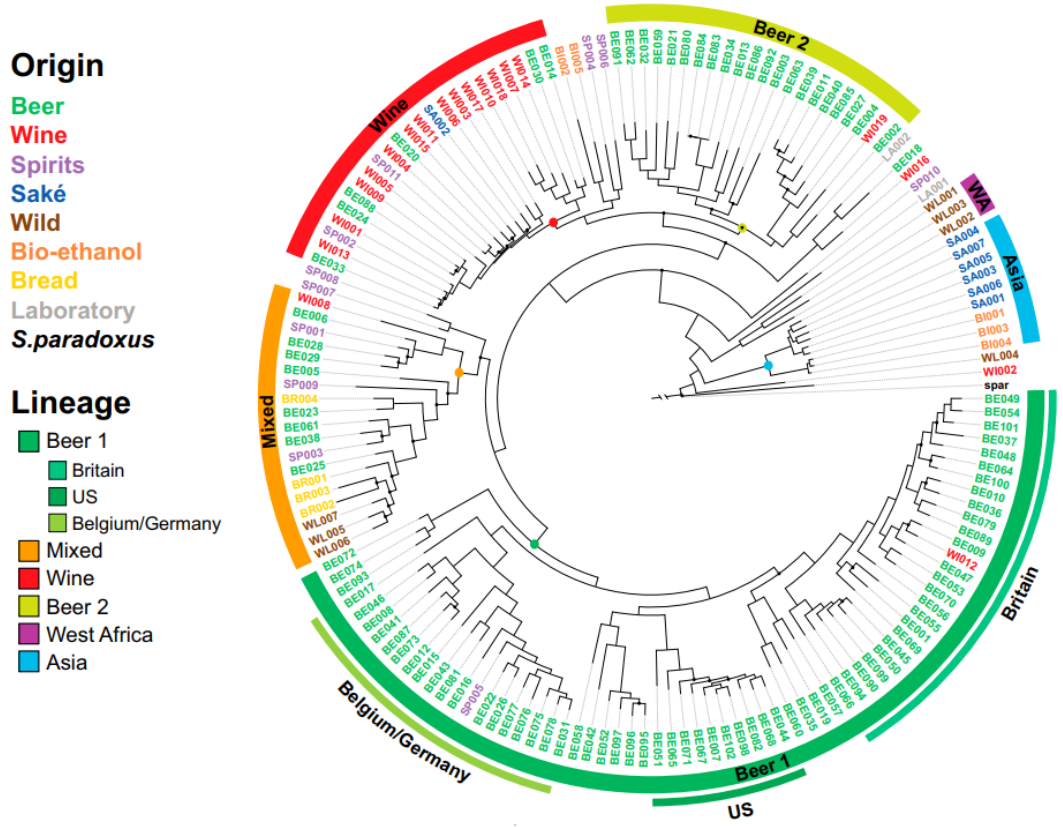


Figure 1.8. Maximum likelihood phylogenetic tree of *S. cerevisiae* strains (Gallone et al., 2016).

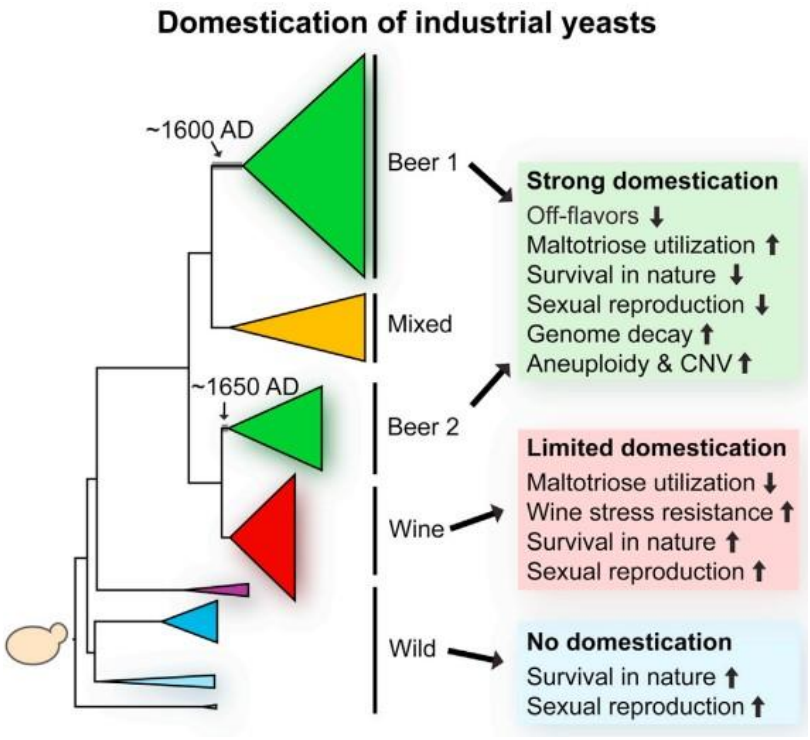


Figure 1.9. Representation of domesticated yeast based on clade separation (Gallone et al., 2016).

The use of lager yeast dates from the 15th century in central Europe and is commonly linked to modern-day Bavaria (Bing et al., 2014; Gonçalves et al., 2016). Indeed, the English word 'lager' originates from the German 'lagern,' which means 'to store.' It was only recently that the industry discovered that lager yeasts are hybrid organisms, derived from a cross between two parental strains most likely to belong to the species *S. cerevisiae* and *S. eubayanus*. The latter is a yeast strain first isolated from tree bark in Patagonia (Libkind et al., 2011) and the specifics of how an Argentinean yeast journeyed to Europe, mixed with an existing ale strain, was allowed to hybridize, and subsequently disappeared is not fully understood. However, *S. eubayanus* strains are increasingly found in alternative locations, such as Tibet, providing a theory that the yeast may have arrived in Europe via the Silk Road through trading patterns dating 2000 years ago (Bing et al., 2014). More recently, *S. eubayanus* has been

discovered in North America and Australasia (but so far not in Europe), further complicating the genetic mystery (Monerawela & Bond, 2017).

To complicate further, two genetically distinct subspecies of *S. pastorianus* exist known as Saaz-type (often called Group 1) and Frohberg-type (Group 2); both are cryotolerant due to their *S. eubayanus* lineage and exhibit reduced floral and fruitier production compared to their *S. cerevisiae* counterparts (Gibson et al., 2013). The Saaz-type strains have been shown to have experienced more genetic loss from its *S. cerevisiae* ancestor when compared to the Frohberg-type strains, meaning that the Saaz-type more emulate its *S. eubayanus* ancestor while Frohberg-types are closer in relation to *S. cerevisiae* (Dunn & Sherlock, 2008). Saaz-type yeasts are believed to have gained use in breweries located in the modern-day Czech Republic and the Danish Carlsberg Brewery. The Frohberg strains appear to stem from breweries located in the Netherlands, non-Carlsberg Danish breweries, and some North American breweries. These two groups of lager yeast have some major physiological differences. Studies have shown that Saaz-type strains exhibit better performance at 10°C than Frohberg-type strains, while Frohberg strains operate better at an elevated temperature of 22°C. Frohberg-type yeasts also yield higher attenuation and higher alcohol contents from the same types of all-malt wort, due to inefficient maltotriose metabolism in Saaz-type yeasts (Gibson et al., 2013). The same experiment demonstrated that Saaz-types produced less esters and higher alcohols than Frohberg-types regardless of fermentation temperature.

1.7.2. Mixed Fermentations and Monocultures

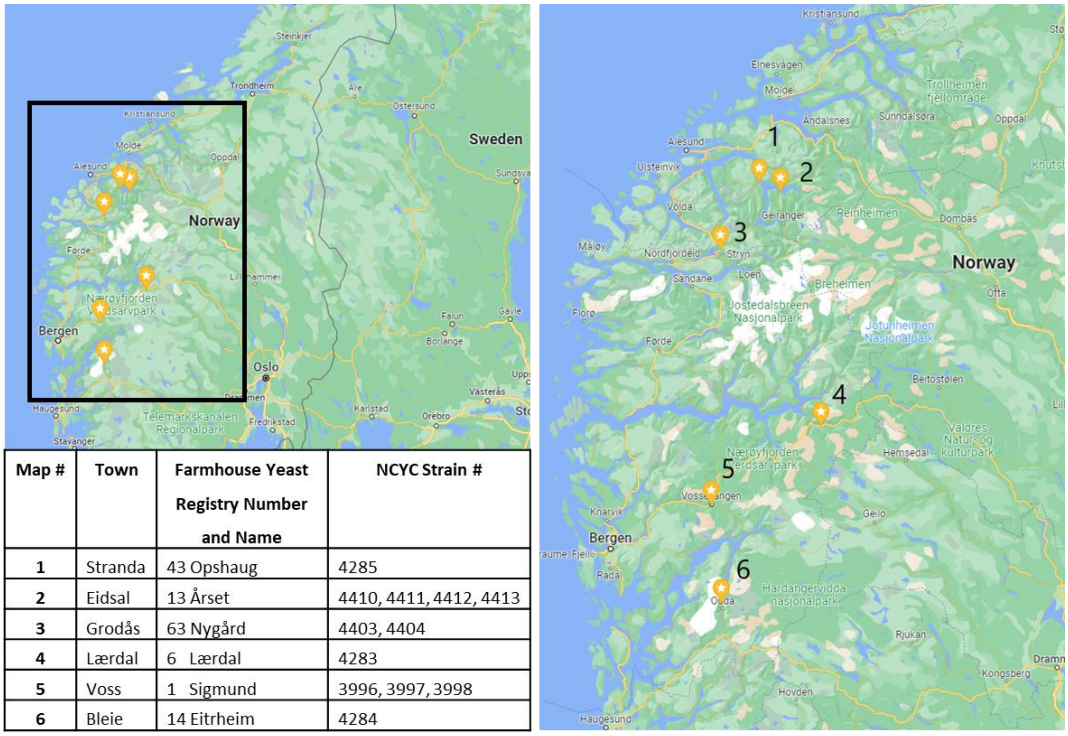
During the early stages of brewing domestication (and by today's standards), fermentations would have been considered 'mixed' as they inevitably included multiple yeast strains as well as bacteria. The inoculation of wort was exclusively 'spontaneous' and derived from the microflora found in the local environment

(Boulton & Quain, 2008). These types of 'wild' fermentations are rare today, but can still be found in the production of traditional Belgian lambics, German Berliner weisse, or American sour ales, for example (Sparrow, 2005; Tonsmeire, 2014). Mixed fermentations, either via multiple yeast strains, or with a combination of yeast(s) and bacteria can also be found in the making of many traditional Nordic and Baltic beers (Garshol, 2020) as described in Section 1.7.3.

The work of Louis Pasteur in the mid to late 19th century proved to be invaluable to the brewing industry as it demonstrated that yeast were responsible for fermentations and that beer spoiling bacteria could be reduced or destroyed (Briggs et al., 2004; White & Zainasheff, 2010). Emil Hansen, of the Carlsberg Brewery in Copenhagen, Denmark, further demonstrated that pure cultures could be effectively isolated, maintained, and used (Gallone et al., 2016). Based on this research, most breweries now utilize pure cultures to perform fermentations and the presence of wild yeast or bacteria is undesirable leading to the production of fresh cultures from original stocks or reserves.

1.7.3. Origins and Applications of Kveik

Kveik are a unique subspecies of *Saccharomyces cerevisiae* which originate from isolated farms, towns, or valleys in Norway. These yeast cultures were traditionally passed down between generations within a family a farming region for decades (perhaps centuries). These strains were often used indiscriminately for both brewing and the baking of bread (Garshol, 2020), and in Norwegian, the word kveik means 'to bring a spark to' or 'bring life to' (Geithung, 2019). When referring to the topic of brewing, 'kveik' is used as a term for 'yeast,' and the world has adopted this word as a catch-all for the collective of these yeasts, even though individual strains have their own differences. It must be noted that kveik is not a beer style *per se* and the term refers to beers made with these particular yeast cultures (Garshol, 2020).



Map #	Town	Farmhouse Yeast Registry Number and Name	NCYC Strain #
1	Stranda	43 Opshaug	4285
2	Eidsal	13 Årset	4410, 4411, 4412, 4413
3	Grodås	63 Nygård	4403, 4404
4	Lærdal	6 Lærdal	4283
5	Voss	1 Sigmund	3996, 3997, 3998
6	Bleie	14 Eitrheim	4284

Figure 1.10. Source locations of kveik strains used in study. The 'name' of each strain refers to designation based on Farmhouse Yeast Registry Number, while the NCYC number(s) reflect individual isolates from each culture.

Until recently, kveik strains were only used within local communities and their application in commercial brewing was largely unexplored. Slurries from a specific location frequently contain multiple yeast strains, sometimes including bacteria, but these additional microorganisms often impart little in undesirable flavours (Preiss et al., 2018; Garshol, 2020). This is rather unique as most modern breweries utilize pure yeast cultures.

It should be noted that it is difficult to discern precisely where each yeast originated, as their lineage is often described only by word-of-mouth, with little to no documentation (Garshol, 2020). However, recent genetic studies have shown that kveik cultures originate from the Beer 1 clade and are closely related to three German wheat beer (hefeweizen) yeasts (Preiss et al., 2018). This indicates that kveik cultures share a common ancestor with British, American,

and some Belgian and German ale strains (Figure 1.11). During the same study, sections of kveik DNA were compared to strains analysed in the study by Gallone et al., (2016). This indicated that the yeasts possessed genetic material outside the Beer 1 clade, providing an additional theory that the kveik strains may have genetic links to both Beer 1 and an unknown wild yeast strain (Figure 1.12).

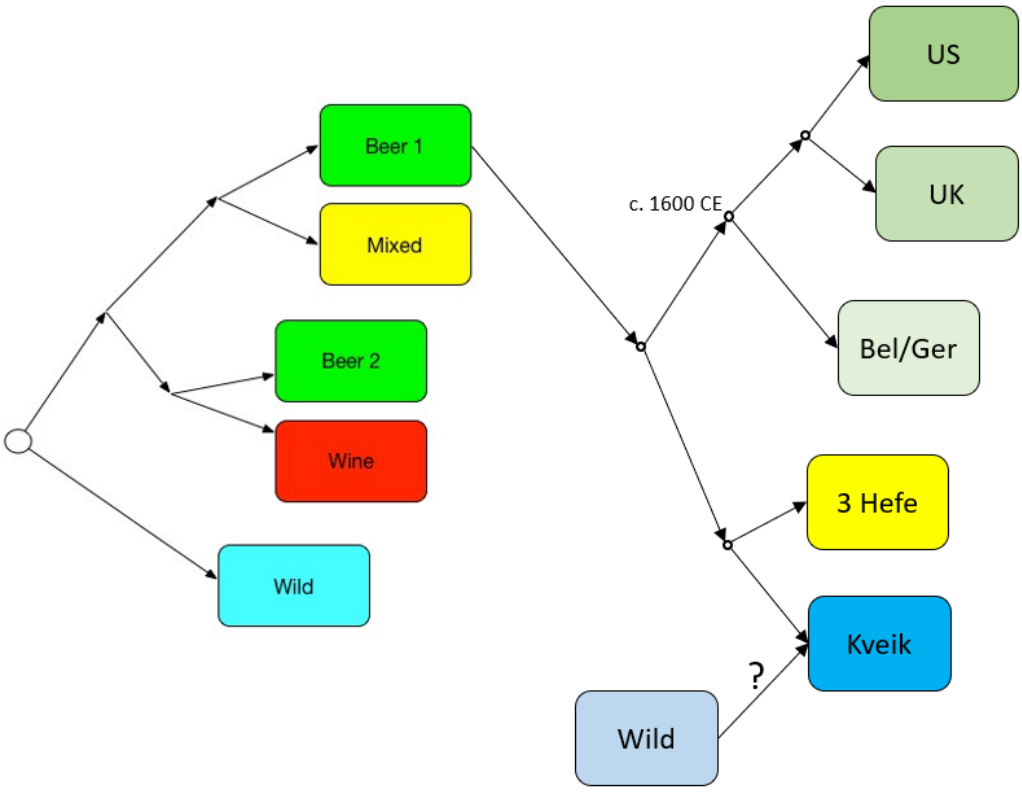


Figure 1.11. Kveik lineage adapted from (Garshol, 2017; Preiss et al., 2018).

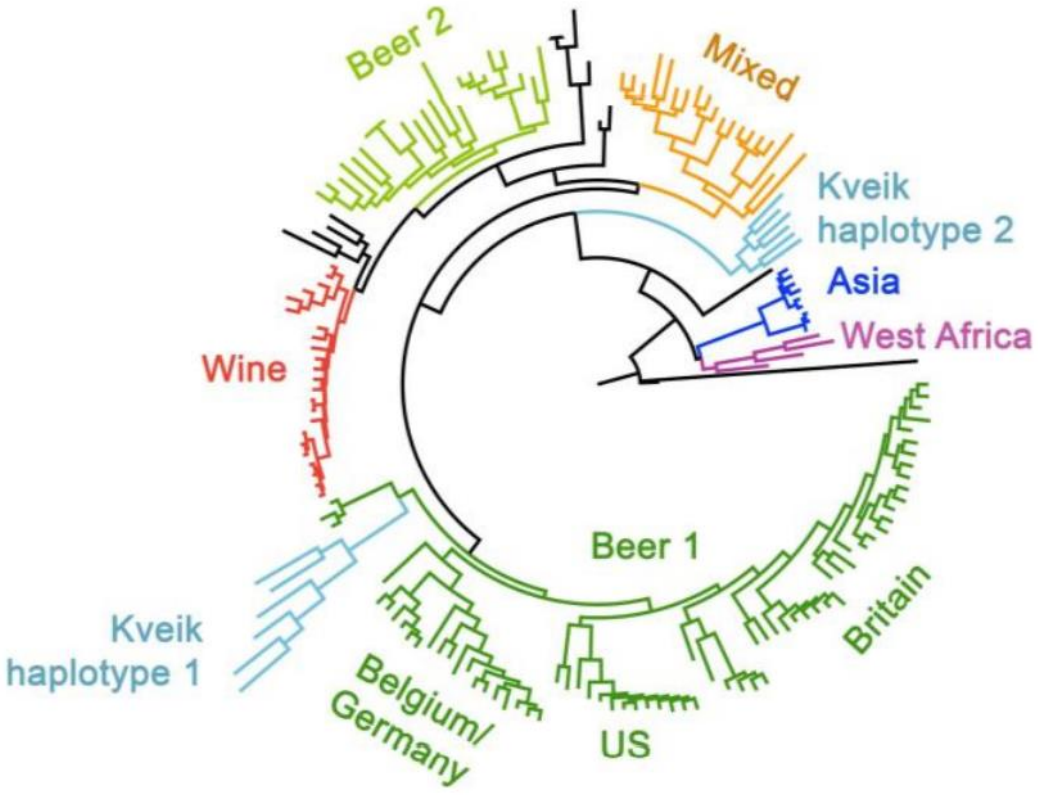


Figure 1.12. Simplified dendrogram indicating the relationship between kveik and other domesticated yeasts, obtained via whole genome sequencing (Garshol & Preiss, 2018; Preiss et al., 2018).

Previous analysis has shown that kveik strains, like most other brewing yeast, can ferment malt-based sugars, do not produce phenolic flavours, tolerate high alcohol levels, and exhibit flocculation (Preiss et al., 2018). Furthermore, these yeasts do not have diastatic power and are not super attenuators (Garshol & Preiss, 2018). Since these traits are not typical attributes of wild yeast, it can be surmised that these characteristics are indicative of domestication (Gallone et al., 2016). Aside from these characteristics, one of the interesting hallmarks of kveik strains is the ability to thrive at exceedingly high temperatures. Kveiks are often used for fermentations in excess of 30˚C and some can even exceed 40˚C which is indicative of thermotolerance (Garshol, 2021). Because of these elevated

temperatures, rates of fermentation are exceedingly fast, enabling kveiks to reach attenuation in 1-2 days (Garshol & Preiss, 2018). The reason for this deviation from ‘standard’ brewing properties is likely derived from the fact that, historically, brewers did not have efficient cooling equipment. The brewer would therefore need to either wait for the wort to cool from boiling to pitching temperature before retiring to bed (Garshol, 2021), or pitch the yeast ‘warm’ (Figure 1.13). Since a longer period of cooling could also increase the likelihood of wild yeast or bacterial infection (Hornsey, 2003), the latter was likely considered best practice and, repeating this during extended serial repitching, could have led to development of thermotolerance over multiple generations.

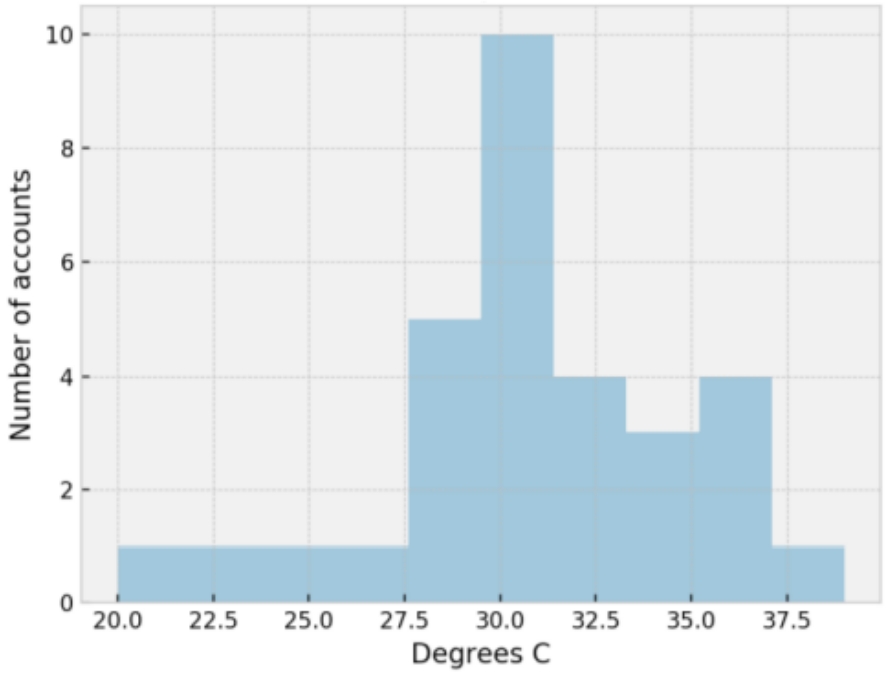


Figure 1.13. Pitch temperatures reported by the owners of collected kveiks (Garshol, 2021).

An intriguing aspect of kveik production is how the yeast culture is handled and stored. Complex rings of wood are traditionally used to store yeast, as shown in Figure 1.14. These rings, called ‘gjærkans,’ are coated with the yeast after fermentation and hung up to dry. Once dried, they can be stored until the next brew, at which point they are lowered into the cooled wort to activate the yeast and allow fermentation to ensue. A similar but alternative design is a block of wood called a ‘kveikstokk,’ which has many holes drilled within the structure (Figure 1.15). These two designs create a large surface area to which the yeast can adhere (Garshol, 2020).

Figure 1.14. Kveik ring belonging to Sigmund Gjernes from Voss, Norway (Garshol, 2020).

Figure 1.15. An old yeast log displayed at Voss Folkemuseum in western Norway (Laitinen, 2020).

An alternative traditional practice for yeast harvesting was to skim the foam during active fermentation (high kräusen) or from the bottom of the fermenter after transferring the beer to the final container. This yeast would then spread out onto a sheet to dry. After the advent of refrigeration in the late 19th century, the flaked dry yeast would then be stored in a household freezer or refrigerator until the next use, which may be several months later (Garshol & Preiss, 2018).

1.8. Aims and Objectives

The yeast strain employed in fermentation plays a vital role in generating the final flavour and aroma profile of beer and its performance must be reliable and consistent. The choice of yeast for a particular beer style may be primarily dictated by flavour generation, but other parameters are also considered,

including flocculation capacity, attenuation limits, rate of fermentation, and general robustness in an industrial setting. In particular, the ability of yeast to reliably withstand the multitude of stress factors experienced during successive fermentations, is important. This is because yeast health over the course of serial repitching has a direct and immediate effect on product quality.

Recent investigations into the use of non-conventional strains for brewing has led to interest in kveik cultures originating in Norway. These yeasts are able to ferment rapidly at exceedingly high temperatures when compared to standard brewing strains, thus potentially reducing fermentation time by days. In addition, anecdotal evidence suggests that the balance of flavours generated at these high temperatures is acceptable when compared to those produced using standard brewing yeasts. Despite this, little is known about the physiology of kveik strains, and their potential for use in mainstream brewing for mass-market products.

In this study, kveik yeast cultures were investigated for their tolerance to industrially relevant stress factors. To achieve this, the capacity of strains to resist stress factors derived from ethanol, osmotic and oxidative challenges, as well as temperature, were compared to a selection of ale and lager yeasts currently used within the brewing industry. In addition, the relationship between fermentation temperature, sugar utilisation and flavour development were investigated. Finally, in order to determine the relative impact of individual isolates within mixed populations, the effect of varying proportions of kveik strain ‘mixtures’ on flavour production was examined. It was anticipated that the information generated could have potential applications in commercial brewing. For example, the possibility of creating a standard beer of desired specifications in a shorter period of time than normal could be a lucrative proposition for small and large brewing companies. Furthermore, by exploring the physiology and fermentation characteristics of kveik yeasts, their potential both for the production of novel beverages and for the generation of novel strains through traditional breeding techniques could be investigated.

Chapter 2

2. Materials and Methods

2.1. Yeast Strains Used in this Study

In total, 12 kveik yeast cultures were obtained from the National Collection of Yeast Cultures (NCYC), Norwich, United Kingdom (Table 2.1). These yeasts were derived from 6 yeast cultures, as indicated by the 'groupings' provided by the Yeast Farmhouse Registry. Hence, the three NCYC strains designated 3996-3998 were isolated from a single culture. Similarly, the groups 4403-4404, and 4410-4413 were comprised of two and four strains respectively, while the remaining strains designated NCYC 4283, 4284 and 4285 were 'pure' cultures derived from single strains. In the case of these three cultures, only a single isolate was discovered in the original slurries by the NCYC.

Yeast Source Identifier and Farmhouse Registry Number	Origination of Strain Depositor	Additional Notes
NCYC 3996 Registry Number: 1	Voss, Norway Sigmund Gjernes c/o Lars Marius Garshol	Isolate from 3-strain NCYC 3995 (Strain A)
NCYC 3997 Registry Number: 1	Voss, Norway Sigmund Gjernes c/o Lars Marius Garshol	Isolate from 3-strain NCYC 3995 (Strain B)
NCYC 3998 Registry Number: 1	Voss, Norway Sigmund Gjernes c/o Lars Marius Garshol	Isolate from 3-strain NCYC 3995 (Strain C)
NCYC 4283 Registry Number: 6	Lærdal, Norway Dagfinn Wendelbo and Arvid Solheim	Single strain isolate
NCYC 4284 Registry Number: 63	Bleie, Norway Reidar Eitrheim c/o Lars Marius Garshol	Single strain isolate *For Research Only*
NCYC 4285 Registry Number: 43	Stranda Valley, Norway Harald Opshaug c/o Lars Marius Garshol	Single strain isolate
NCYC 4403 Registry Number: 63	Grodås, Norway Idar Nygård c/o Lars Marius Garshol	Isolate from 2-strain NCYC 4394 (Strain A)
NCYC 4404 Registry Number: 63	Grodås, Norway Idar Nygård c/o Lars Marius Garshol	Isolate from 2-strain NCYC 4394 (Strain B)
NCYC 4410 Registry Number: 13	Eidsdal, Norway Jakob Torp Årset c/o Lars Marius Garshol	Isolate from 4-strain NCYC 4409 (Strain A)
NCYC 4411 Registry Number: 13	Eidsdal, Norway Jakob Torp Årset c/o Lars Marius Garshol	Isolate from 4-strain NCYC 4409 (Strain B)
NCYC 4412 Registry Number: 13	Eidsdal, Norway Jakob Torp Årset c/o Lars Marius Garshol	Isolate from 4-strain NCYC 4409 (Strain C)
NCYC 4413 Registry Number: 13	Eidsdal, Norway Jakob Torp Årset c/o Lars Marius Garshol	Isolate from 4-strain NCYC 4409 (Strain D)

Table 2.1. Kveik strains used in this study. All strains were obtained from NCYC.

Yeast Identifier and Source	**Origination of Strain Depositor**	**Additional Notes**
NCYC 1332 National Collection of Yeast Cultures, Norwich, United Kingdom	Unknown British Brewery	*Saccharomyces cerevisiae* (Ale)
M2 Oxford Brookes University Brewing Yeast Culture Collection	Morrells Brewery, Oxford, UK c/o Steve Whisker	*Saccharomyces cerevisiae* (Ale)
CBS 1174 Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands	Unknown	*Saccharomyces pastorianus* Lager (Saaz-type)
CBS 1260 Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands	Unknown	*Saccharomyces pastorianus* Lager (Frohberg-type)
W34/70 Hefebank Weihenstephan Hallertau, Germany	Weihenstephan Brauerei Weihenstephan, Germany	*Saccharomyces pastorianus* Lager (Frohberg-type)

Table 2.2. Traditional brewing yeast strains used in this study. All yeasts were obtained from the University of Nottingham culture collection, derived from various sources as indicated.

2.2. Preparation of Media: Wort

All worts used in this study were sourced from the AB InBev Research Brewery, located at the University of Nottingham, Sutton Bonington Campus, UK. Wort was derived from all-malt raw materials and stored frozen in 10-litre bulk containers at -20°C prior to use. Wort was thawed prior to fermentation and diluted with sterile reverse osmosis (RO) water to a specific gravity of 1.060. Water was sterilised via autoclave at 121 °C and 15 psi for 15 minutes in 1-litre increments. Fermentations were 1-litre in final volume and conducted within 2-litre bottles that were sterilised at 121 °C and 15 psi for 15 minutes. Airlocks used during fermentations were sterilised using 70% ethanol spray.

2.3. Preparation of Media: Yeast Peptone Dextrose (YPD)

YPD media consisted of 1% (w/v) yeast extract (Oxoid, UK), 2% (w/v) bacteriological peptone (Oxoid, UK) and 2% (w/v) β-D-glucose (VWR Chemicals, Germany). Sterile reverse osmosis (RO) water was used for all dilutions and preparations unless otherwise specified. All media was sterilised by autoclaving at 121°C and 15 psi for 15 minutes after preparation stored cold at 4°C prior to use. If solid YPD media was desired, agar (Oxoid, UK) was added to YPD media at 1.2% (w/v) prior to autoclaving.

2.4. Preparation of Media: Wallerstein Laboratory Nutrient (WLN)

WLN media consisted of 6% (w/v) WLN powder (Sigma-Aldrich, UK) and 1.2% (w/v) agar (Oxoid, UK). Reverse osmosis (RO) water was used for all dilutions and preparations unless otherwise specified. All media was sterilised by autoclaving at 121°C and 15 psi for 15 minutes after preparation. Prior to cooling, the media was poured into sterile 92 mm petri dishes (Sarstedt, Germany) and allowed to set prior to use or cold storage at 4°C.

2.5. Yeast storage

2.5.1. Long-Term Cryogenic Storage of Yeast Cultures

Stock cultures of yeast were cryogenically preserved in sterile 1.5 ml cryovials at -80°C for long term storage. In each instance, a liquid yeast culture was grown to stationary phase in sterile YPD media. 500µl of this culture was added to the cryovial along with 500µl of 40% (v/v) sterile glycerol (Fisher Scientific, UK). The latter was added as a cryoprotectant to maintain cell viability during storage. All sterilisations were performed at 121°C and 15 psi for 15 minutes.

2.5.2. Short-Term Storage of Yeast Cultures

YPD agar slopes (slants) and plates were used to store short term stocks of each yeast strain at 4°C. Agar slopes and plates were produced by adding molten YPD agar media to the appropriate container (universal bottles or sterile 92 mm petri dishes (Sarstedt, Germany) respectively) after autoclaving. Once media had set, a sterile loop-full of a pre-prepared liquid yeast culture was streaked onto the media aseptically. Slopes and plates were then incubated at 25°C in a static incubator (Sanyo, Japan) for 48 – 72 hours to allow for growth before being transferred to 4°C for storage. Cultures on plates and slopes were stored at 4°C for a maximum of three weeks and three months, respectively, prior to being discarded and another culture propagated from frozen stock.

2.6. Enumeration of Cells (Cell Count)

An estimation of cell number was obtained using an Aber Countstar (Aber Instruments, UK). In each instance, yeast slurries grown from stock were diluted to an appropriate concentration using DO water, and a 15 µl aliquot was

transferred to an Aber Countstar slide (Aber Instruments, UK). Cell count was determined automatically based on analysis of discrete cells and the total number of cells per ml within the original culture was determined.

2.7. Giant Colony Morphology Assay (according to growth on WLN Media)

The morphological characteristics of yeast colonies were determined by visible characterisation of colonies following cultivation on WLN agar media (Section 2.4) for an extended time period. WLN agar (Sigma-Aldrich, Germany) was prepared and autoclaved at 121°C and 15 psi for 15 minutes before being poured into petri dishes to cool and set, as described above (Section 2.4). For analysis, each yeast strain was propagated in YPD media (Section 2.3) for 24 hours in an orbital shaker at 25°C and 150 rpm (Sartorius Certomat BS-1, Germany) until they had reached stationary phase of growth. Yeast cells were harvested by centrifugation, washed twice with sterile RO water, and enumerated using an Aber Countstar (Section 2.6) before being diluted to a concentration of approximately 500 cells/ml. A 100µl aliquot of this dilution was then spread onto WLN agar and incubated aerobically at 25°C for 15 days to yield approximately 10-50 colony forming units. These colonies were examined visually and characterised based on differences in size, shape, appearance, and the distribution of colour.

2.8. Stress Tolerance Analysis

2.8.1. Oxidative Stress Test

Spot plate analysis was used to determine the susceptibility and tolerance of yeast strains to oxidative stress, based on the growth of colonies derived from within each yeast culture. To achieve this, YPD agar (Section 2.3) was supplemented with hydrogen peroxide (Fisher Scientific, UK) to final

concentrations of 0% (control), 0.01%, 0.02%, and 0.03%. Plates were inoculated with yeast that had been pre-cultivated from agar slopes into 10 ml YPD and incubated for 48 hrs at 25°C in an orbital shaker (Sartorius Certomat BS-1, Germany) at 150 rpm. Viable cell counts were performed as described previously (Section 2.6) and suspensions of yeast were created at a standard $1x10^8$ cells/ml. These were then used to produce sequential dilutions at 10^2, 10^3, 10^4 and 10^5 using sterile RO water. 10µl of each diluted cell aliquot was then 'spotted' in a grid format onto the supplemented YPD agar plates and incubated for 3 days in a static incubator (Sanyo, Japan) to allow growth at 30°C. In each instance, tests were performed in triplicate.

2.8.2. Osmotic Stress Test

Spot plate analysis was used to determine the susceptibility and tolerance of yeast strains to osmotic stress, based on the growth of colonies derived from within each yeast culture. To achieve this, YPD agar (Section 2.3) was supplemented with sorbitol (Merck, Germany) to final concentrations ranging from of 0-30%. Plates were inoculated with yeast that had been pre-cultivated from agar slopes into 10 ml YPD and incubated for 48 hrs at 25°C in an orbital shaker (Sartorius Certomat BS-1, Germany) at 150 rpm. Viable cell counts were performed as described previously (Section 2.6) and suspensions of yeast were created at a standard $1x10^8$ cells/ml. These were then used to produce sequential dilutions at 10^3, 10^4, 10^5, and 10^6 using sterile RO water. 10 µl of each diluted cell aliquot was then 'spotted' in a grid format onto the supplemented YPD agar plates and incubated for 2 days in a static incubator (Sanyo, Japan) to allow growth at 30°C. In each instance, tests were performed in triplicate.

2.8.3. Ethanol Stress Test

Spot plate analysis was used to determine the susceptibility and tolerance of yeast strains to ethanol stress, based on the growth of colonies derived from within each yeast culture. To achieve this, YPD agar (Section 2.3) was supplemented with ethanol (Merck, Germany) at final concentrations ranging from 0-20%. Plates were inoculated with yeast that had been pre-cultivated from agar slopes into 10 ml YPD and incubated for 48 hrs at 25°C in an orbital shaker (Sartorius Certomat BS-1, Germany) at 150 rpm. Viable cell counts were performed as described previously (Section 2.6) and suspensions of yeast were created at a standard $1x10^8$ cells/ml. These were then used to produce sequential dilutions at 10^2, 10^3, 10^4 and 10^5 using sterile RO water. 10 µl of each diluted cell aliquot was then 'spotted' in a grid format onto the supplemented YPD agar plates and incubated for 2 days in a static incubator (Sanyo, Japan) to allow growth at 30°C. In each instance, tests were performed in triplicate.

2.8.4. Thermal Tolerance

Spot plate analysis was used to determine the susceptibility and tolerance of yeast strains to osmotic stress, based on the growth of colonies derived from within each yeast culture. To achieve this, yeast were incubated on YPD agar plates (Section 2.3) at 25°C, 30°C, 37°C, and 42°C. Plates were pre-inoculated with yeast cultivated from agar slopes into 10 ml YPD and incubated for 48 hrs at 25°C in an orbital shaker (Sartorius Certomat BS-1, Germany) at 150 rpm. Viable cell counts were performed as described previously (Section 2.6) and suspensions of yeast were created at a standard $1x10^8$ cells/ml. These were then used to produce sequential dilutions of 10^2, 10^3, 10^4 and 10^5 using sterile RO water. 10 µl of each diluted cell aliquot was then 'spotted' in a grid format onto the supplemented YPD agar plates and incubated for 5 days in static incubators

(Sanyo, Japan) at the respective temperature to determine cell growth. In each instance, tests were performed in triplicate.

2.9. Fermentation Analysis

2.9.1. Fermentation Preparation and Monitoring

Viable cell counts were obtained for yeast cultures grown for 3 days in YPD broth using the Aber Countstar (Section 2.6). Based on this assessment, calculation of desired total slurry volume needed for a final cell count of 1 x 10^6 cells/ml wort/°P was achieved. The appropriate volume of yeast slurry was obtained via centrifugation (Eppendorf 5810 R, Germany) and pitched into 1000 ml wort (1.060 specific gravity) contained within a 2-litre glass fermenter. As described above (Section 2.2), the wort used was a hopped all-malt wort (AB InBev Research Brewery, UK), diluted to the desired gravity with sterile RO water.

After inoculation and mixing, fermentations were monitored daily via gravity analysis. Samples (50 ml) were taken aseptically and yeast was removed via centrifugation at 4,000 rpm for 10 minutes (Eppendorf 5810 R, Germany). The gravity of the supernatant was then determined using an Anton Paar DM-35 (Anton Paar, UK). Gravity readings were taken at least daily and measured with a handheld densitometer after centrifugation. The Apparent Degree of Fermentation (ADF) was measured with the following equation (values in specific gravity with result as a percentage): ADF% = [(starting gravity – final gravity)/(starting gravity – 1)]*100%. All fermentations were performed in triplicate and data represents the mean ± the standard deviation (S.D.) at each time point. Final beers were also sampled for yeast characteristics (Section 2.6) and flavours as below (Sections 2.9.2 and 2.9.3). For the latter, samples were immediately frozen and stored at -20°C prior to analysis.

2.9.2. Flavour Characterisation by Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS) *in vitro* Analysis

Flavour distribution was determined using an MS Nose interface (Micromass, UK) fitted to a Quattro Ultima mass spectrometer (Waters, USA). Each sample (Section 2.9.1) was thawed at room temperature (20°C) and 40 ml aliquots transferred to a 100 ml Duran bottles for static headspace in vitro analysis. Bottles were sealed with a screw cap to minimise the loss of volatile compounds and the headspace was evaluated by APCI-MS by passing nitrogen through the headspace volatiles at a rate of 30 ml/min and into the transfer line, before proceeding to the ionization region. The 'Selected Ion' mode was used, with a cone voltage of 50 V; the source temperature was 30 °C and the transfer line temperature was 120°C. The silica capillary tube used inside the transfer line was 50 m in length with a 0.32 mm internal diameter Zebron deactivated tubing (Phenomenex, USA). A dwell time of 0.1 second was used for all acquisitions. The MS mode was set to ‘Full Scan’ mode with the mass to charge ratio of m/z 20–200. All analyses were performed in triplicate.

2.9.3. Gas Chromatography and Mass Spectrometer (GC-MS) Analysis

A trace 1300 series Gas Chromatograph coupled with a Single-Quadrupole Mass Spectrometer (Thermo Fisher Scientific, UK) was used for analysis of volatile aroma compounds. Samples were incubated under constant shaking at 50°C for 5 min. A 50/30 µm DVB/CAR/PDMS SPME Fibre (Supelco, Sigma Aldrich, UK) was used to extract volatile aroma compounds from the sample headspace (extraction for 5 min then desorption for 15 min). The inlet temperature was set at 200°C and a split mode was used due to the connection of GC-Olfactory, and the constant carrier pressure was at 124.11 kPa.

Separation was carried out using a ZB-WAX Capillary GC Column (length 30 m, inner diameter 0.25 mm, and film thickness 1 µm; Phenomenex Inc., UK). Column temperature was held initially at 40°C for 2 min, increased by 6°C/min to 240°C and held for 5 min. 'Full scan' mode was used to detect the volatile compounds (mass range from 20 to 300 AMU). Volatiles were identified by comparison of each mass spectrum against either the spectra from authentic compounds or spectra derived from reference libraries (NIST/EPA/NIH Mass Spectral Library, version 2.0, Faircom Corporation, USA). The relative abundance of each volatile compound present in the headspace was determined from the GC peak area found in the beer sample (average of 3 replicates) and quantified based on relative concentration against the internal standard, 3-heptanone.

Based on the raw data, TraceFinder 5.1 software (Thermo Fisher Scientific, UK) was utilised to obtain concentrations of specific volatile constituents. The 11 compounds present in the highest quantity with matching scores from the MS database were used in the comparative study. For comparison and presentation, results were logarithmically normalised.

2.9.4. Statistical Analysis

The intensity of MS ion peaks obtained using in vitro static headspace analysis were recorded for triplicate replicates and analysed by Principal Component Analysis via the online software MetaboAnalyst5.0 (https://www.metaboanalyst.ca/). To achieve this, two principal components, 173 (PC2) and 177 (PC1) were identified by the software algorithm and used to map flavour profiles for each strain. The relative abundance of each aroma compound was filtered by matching score first, then normalized by $\log_{10}$ ratio for comparison.

Chapter 3

3. Results and Discussion

3.1. Differentiation of Kveik Isolates based on Giant Colony Analysis

The twelve kveik yeast strains used in this study were derived from 6 individual farmhouse cultures, as described in Section 2.1. Three of these comprised multiple strains (NCYC 3996-3998; 4403-4404; and 4410-4413), while the remainder were designated as 'pure' cultures comprising single strains (NCYC 4283, 4284 and 4285). To perform a simplistic characterisation of each isolate, individual cells from each strain were analysed for their capacity to form 'giant colonies' on WLN media. These colonies were compared firstly to each other, and also to their more 'traditional' brewing counterparts (lager and ale), as described in Section 2.7. Characterising yeast strains in this way is a long-recognized procedure (Richards, 1967), allowing differentiation based on colony topography and the capacity of individual strains to assimilate and/or reduce bromocresol green dye contained within the media (Hall, 1971). It should be noted that while the overall appearance of giant colonies is unique to each yeast strain it is accepted some strains may be more distinct than others, while the description of colony type can also be subjective. Despite these limitations, analysis was performed primarily to determine whether the cultures obtained (both single strain and mixed cultures) were phenotypically distinct.

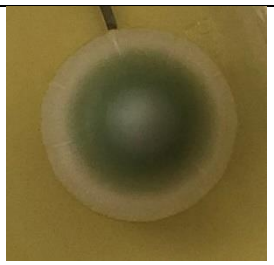
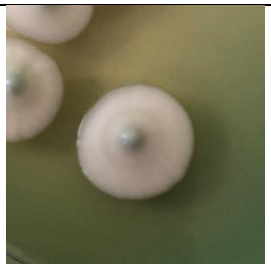
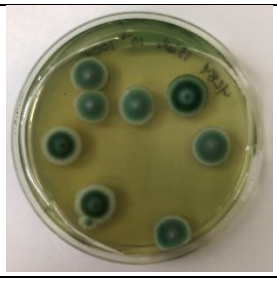
The individual yeast strains 4283, 4284, and 4285 represent isolates from different sources and as such were anticipated to possess different morphologies. This was confirmed following growth on WLN agar, with each strain producing distinctive structures with different patterns of colouration (Table 3.1). The yeast 4284 was significantly different in appearance compared to the other strains analysed in this study as it possessed multiple rings alternating between cream, teal and darker green, and had no striations. For comparative purposes, strain

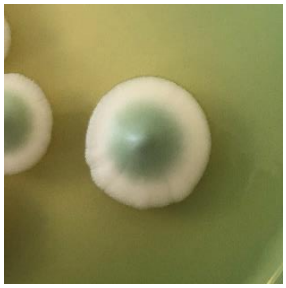
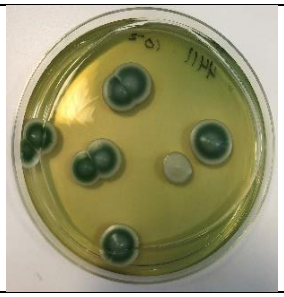
4285 can be seen to be striated with a gradual colour change from cream exterior to a light green centre. A similar pattern of results was seen when the standard brewing strains were analysed (Table 3.2), where each strain was able to be differentiated based on colony type. In this instance, the ale yeast strains M2 and 1332 both had a striated appearance with an uneven/rugged periphery, but could be differentiated based on colouration. All the other brewing strains were generally circular in pattern; the lager strain 1174 was dark in colour a shiny colony type. The other lager yeasts, 1260 and W34/70, exhibited a lighter exterior with darker green interiors. However, strain 1260 could be identified based on the more gradual colour transition observed, and lighter exterior when compared to W34/70.

Once the 'single' strain isolates had been analysed, the same approach was taken to investigate the mixed strain cultures. This was performed to determine if the methodology would provide a mechanism for identification of individual strains within each mixture. It can be seen from Table 3.1 that the three yeast strains designated NCYC 3996, 3997 and 3998 (derived from the same original slurry) showed some similarities in morphology type, but each yeast was distinct. All colonies exhibited a whitish cream exterior, with teal green centres. Strain 3996 had a pronounced border between the colours with a larger green centre, while 3997 was more gradual in this regard and possessed a smaller green centre. Strain 3998 had dominant striations, with stripes radiating from the centre to the exterior of the colony.

Similarly, when comparing the two-strain mixture (NCYC 4403 and 4404), each yeast was markedly different in morphology. Strain 4403 possessed minor striations with a whitish exterior and teal interior, while 4404 was a darker green, with little colour variation. Given that there were major differences in morphology, this may be an indication that these yeasts were not related and may have originated from different sources. Although further analysis of these yeast based on genetic homology would be required to support this hypothesis, it

is interesting to note that a different pattern of results was observed for the four-strain culture. The yeast strains 4410, 4411, 4412, and 4413 were all derived from the same slurry, all had a small lighter coloured exterior ring surrounded by a darker green interior and were similar in appearance with only minor differences observed (Table 3.1). This may suggest that these yeast strains were derivatives of a common parental source, or represent variants of the dominant strain within the culture, but this finding would be speculative and would require additional genetic investigation. In either scenario, it suggests that these yeasts may have been together during domestication over a prolonged timeframe. Based on this analysis, it can be concluded that the methodology applied could provide a convenient mechanism to analyse mixed kveik population dynamics, particularly for those cultures where colony morphology was distinct (NCYC 3996-3998 and NCYC 4403-4404). Furthermore, it is interesting to note that the strain mixtures analysed were not consistent in relation to their individual strain-components, with some isolates appearing to be more diverse than others. This conclusion is conjecture, but is somewhat supported by the fact that the number of yeast isolates within each culture was also variable, suggesting that the evolution of kveik cultures is complex. Additional analysis could prove to be insightful. This is likely to have arisen due to a combination of random mixing events in conjunction with selection of isolates through yeast handling, as well as individual strain evolution over time.

Kveik Strains			
NCYC Code	**Single Colony**	**Entire Plate**	**Notes**
3996			Abrupt color change Larger green centre
3997			Minor striations Smaller green centre
3998			Pronounced striations Gradual colour change
4283			Smooth centre Very minor striations
4284			Multiple colour rings on all colonies

4285			Mostly cream exterior with light green centre. Striated
4403			Minor exterior striations. Pronounced colour variation. Cream exterior with matte teal centre
4404			Green striations from center to exterior. Light green exterior with matte green interior.
4410			Pronounced colour variation. White exterior with green ‘ring’ and light green centre
4411			Pronounced white outer exterior with darker ring and light green center. Glossy

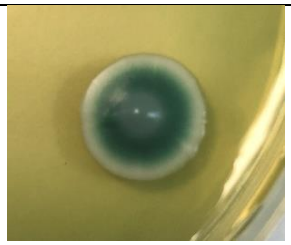
4412			Pronounced white outer exterior with darker ring and light green center. Glossy
4413			Pronounced white outer exterior with darker ring and light green center. Glossy

Table 3.1. Morphology of giant colonies produced from the growth of individual kveik yeast cells on Wallerstein Laboratory Nutrient (WLN) agar. Serially diluted slurry were prepared to yield 10-50 colonies on each plate and incubated aerobically at 25°C for 15 days. Images reflect representative examples of each colony type per strain.

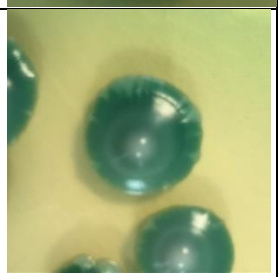
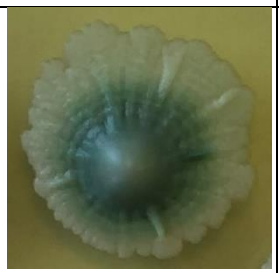
Non-Kveik Brewing Yeast Strains			
W34/70 (lager)			Light green color with very minor dark 'ring'
M2 (ale)			Striated, All green with multiple minor 'rings.' Rough exterior
CBS 1174 (lager)			Lighter exterior striations. Almost all darker green. Glossy
CBS 1260 (lager)			Smooth mostly white/cream coloured exterior with small green centre.
1332 (ale)			Light green exterior with darker centre. Striated with very rough appearance

Table 3.2. Morphology of giant colonies produced from the growth of individual traditional brewing yeast cells on Wallerstein Laboratory Nutrient (WLN) agar. Serially diluted slurry were prepared to yield 10-50 colonies on each plate and incubated aerobically at 25°C for at least 15 days. Images reflect representative examples of each colony type per strain.

3.2. The Capacity of Kveik Strains to Resist Industrially Relevant Stress Factors

Kveik cultures are known to be able to ferment well at high temperatures and have been reported to show stress resistance properties which exceed those of standard brewing strains with those stressors including oxidative, osmotic, alcoholic, and thermal (Section 1.6.1) (Garshol, 2020). In order to determine if this is a general and widespread characteristic of all kveik strains, 12 kveik and 5 brewing yeast strains were compared for stress tolerance. Spot plate assays were performed using YPD agar media supplemented with stressors to determine tolerance based on growth potential (Xu et al., 2014). For this study, sorbitol was used as an osmotic stressor, hydrogen peroxide as an oxidant, ethanol as an alcoholic stress, and elevated temperature for thermal stress.

3.2.1. Tolerance of Yeast Strains to Oxidative Stress

Oxidative stress occurs due to the cellular production of oxidative species collectively called ROS (Reactive Oxygen Species) (Section 1.6.1.1). ROS induce cellular damage on proteins and lipids, causing a degradation in cell fluidity (Martin et al., 2003). This has a detrimental effect on yeast viability and vitality, especially at elevated temperatures, and can also negatively affect the flavour profile of the beer directly, as well as indirectly (Gibson et al., 2007). To investigate the effect of oxidative stress on kveik and conventional brewing strains, YPD agar plates were supplemented with hydrogen peroxide (H_2O_2) at percentages of 0%, 0.01%, 0.02%, 0.03%, and 0.04% v/v prior to 10 µl 'spot' inoculation with progressively diluted yeast slurries. Dilution of slurries were derived from an initial culture standardised to $1x10^8$ cells/ml in each instance. Relative tolerance was determined by visual comparison to a range of typical brewing strains, as described in Section 2.8.1.

It can be seen from Table 3.3 that all the yeast strains analysed showed a progressive decrease in growth capacity (spot size) with increasing

concentrations of hydrogen peroxide. As anticipated, a concentration of 0% H_2O_2 (control) yielded a consistent reduction in cell growth with progressive dilutions in each instance. At 0.01% hydrogen peroxide, all strains (kveik and conventional) exhibited a similar decrease in growth as dilution factor increased. However, all strains produced visible biomass even when diluted, indicating that growth under these conditions could occur without hinderance across all the strains.

In comparison, cell growth in the presence of 0.02% hydrogen peroxide was noticeably reduced for all strains. This was especially apparent as samples were diluted, with some dilutions leading to visibly reduced growth or the complete absence of biomass (Figure 3.1). For all the kveik strains analysed, growth capacity exceeded that observed for the conventional strains (Figure 3.1). The conventional non-kveik brewing strains 1174 (lager), 1260 (lager), and 1332 (ale) did not show any growth at all at this concentration, while strains W34/70 (lager) and M2 (ale) showed only limited growth at the lowest dilution factor (10^2). In contrast, all the kveik strains showed some tolerance to 0.02% hydrogen peroxide, with most strains able to grow well. The exception to this were the strains 4412 and 4413, which showed similar growth patterns to W34/70 and M2. However, all the other strains showed enhanced tolerance, as demonstrated by good growth at higher dilutions rates (10^3 and greater). This was also true for the yeast strains 4403 and 4283, which showed the best growth at high dilution rates. The yeast 4283 was also the only strain able to produce growth at 0.03% hydrogen peroxide, indicating that this yeast exhibited the highest tolerance to oxidative stress, based on permissive growth capacity, when compared to the other yeast strains in the study.

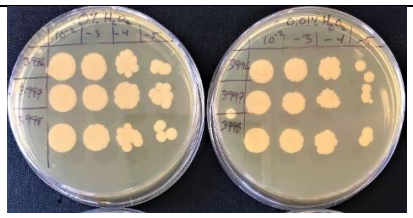
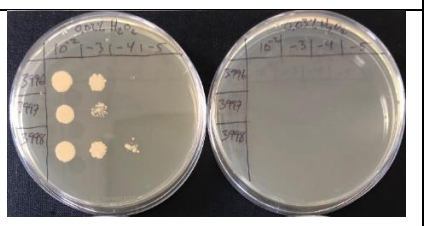
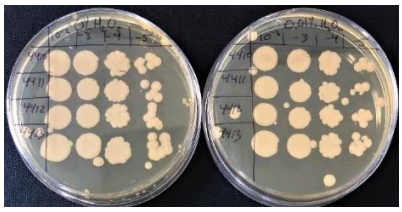
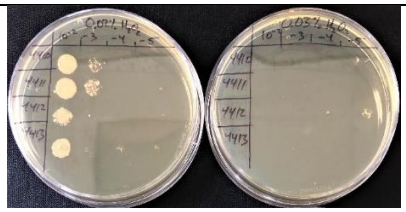
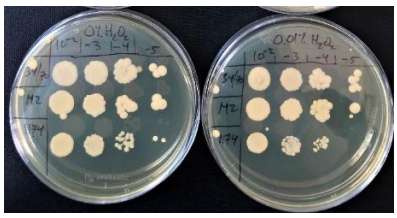
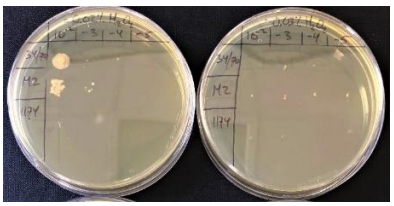
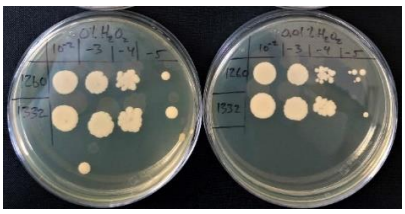
Yeast Strains	0% (L) and 0.01% (R) Hydrogen Peroxide	0.02% (L) and 0.03% (R) Hydrogen Peroxide
Kveik 3996 3997 3998		
Kveik 4283 4284 4285		
Kveik 4403 4404		
Kveik 4410 4411 4412 4413		
Non-Kveik W34/70 (lager) M2 (ale) 1174 (lager)		
Non-Kveik 1260 (lager) 1332 (ale)		

Table 3.3. Tolerance of yeast strains to hydrogen peroxide-induced oxidative stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of H_2O_2 as indicated. Serial dilutions (10^2 – 10^5) from an initial cell concentration of 1 x 10^8 cells/ml for each strain were

spotted on plates, from left to right, and incubated aerobically for 3 days at 30°C. Concentrations of H_2O_2 used were 0% (control), 0.01%, 0.02%, and 0.03%. Data obtained using a concentration of 0.04% is not shown since initial experiments revealed that yeast growth did not occur, regardless of yeast strain or dilution factor.

3.2.2. Tolerance of Yeast Strains to Osmotic Stress

Osmotic stress occurs during propagation and fermentation, and can which negatively affect cell membrane fluidity, potentially reducing cell growth and population viability (Section 1.6.1.2) (Zhuang et al., 2017). Furthermore, continually submitting yeasts to highly osmotic environments can also reduce the performance of yeast and negatively affect flavour (Stewart, 2020). While all fermentations may give rise to osmotic stress, the effects may be exacerbated in breweries conducting high gravity brewing operations. Hence, the capacity of kveik strains to tolerate osmotic stress could indicate suitability for high gravity brewing, especially if the flavour profile is appealing to the consumer.

To determine the capacity of yeast strains to tolerate osmotic stress, populations of cells were analysed by spot plate testing, utilizing YPD agar media supplemented with sorbitol (Section 2.8.2). Sorbitol is a sugar-alcohol that cannot be assimilated by yeast and its presence in the media simply provides an osmotically charged environment for cells. The concentrations of sorbitol used (0% (control), 20%, 30%, and 40%) were designed to simulate those typically encountered in the brewing industry (Zhuang et al., 2017). To determine tolerance to osmotic stress, all twelve kveik strains and two non-kveik strains, W34/70 (lager) and 1332 (ale) were analysed.

It can be seen from Figure 3.3 that all strains used (both kveik and conventional brewing strains) exhibited a decline in growth capacity as the percentage of

sorbitol increased and with an increase in dilution rate. Importantly, it can be seen that each of the kveik strains showed higher tolerance to osmotic stress when compared to the two ale and lager conventional non-kveik strains. At 20%, a similar sensitivity towards sorbitol was observed with the kveik strains when compared to the 0% control. However, less cell growth was noted for both non-kveik strains at 20% sorbitol when compared to the 0% control. This trend continued for cells subjected to 30% sorbitol, where all strains demonstrated decreased growth with smaller and fewer colonies than at 20% sorbitol. At this higher concentration, a 10^4 dilution factor reflected the limit for effective growth. There was extremely low growth at a dilution rate of 10^5 for the kveik strains and no growth at all for the two non-kveik strains analysed here (Table 3.4). At 40% sorbitol, all strains showed growth at 10^3, and 10^4, but with a continued decrease in overall size and with fewer colonies. Cell growth for the kveik strains at 10^5 was significantly reduced, but some biomass could be observed. There was no cell growth for strain W34/70 regardless of dilution at 40% sorbitol. Similarly, strain 1332 exhibited only minimal growth at 40% sorbitol up to a dilution of 10^4, showing that for this study, these two yeasts strains had a higher level of sensitivity to osmotic stress relative to the other yeasts tested. This represents typical tolerances exhibited by yeasts in past studies (Zhuang et al., 2017).

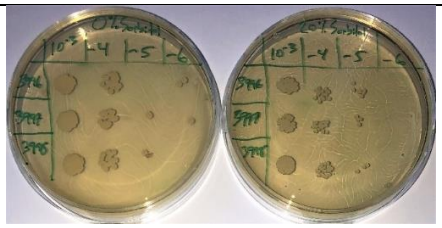
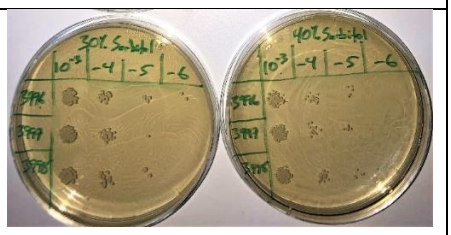
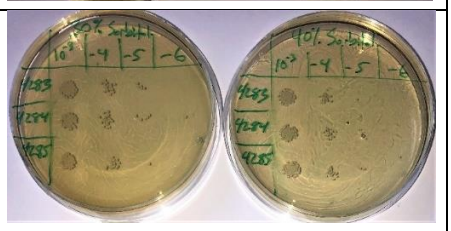
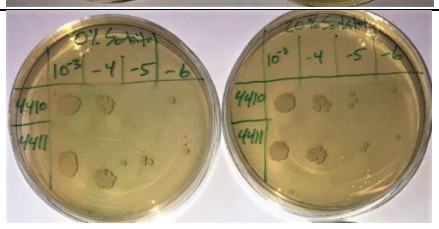
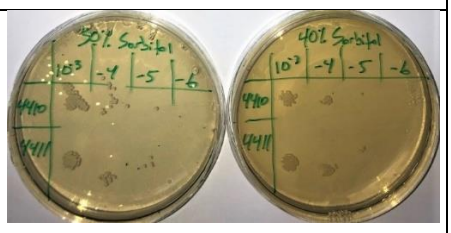
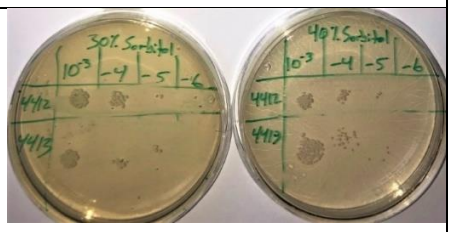
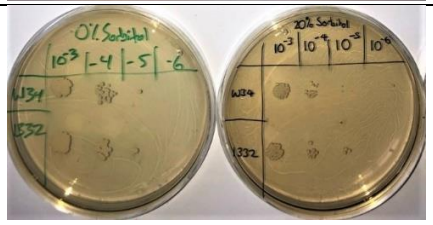
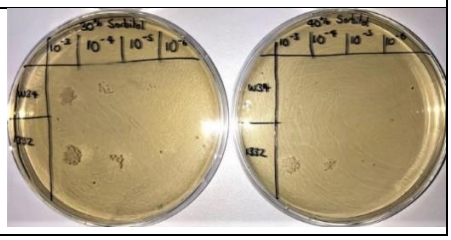
Yeast Strains	0% (L) and 20% (R) Sorbitol	30% (L) and 40% (R) Sorbitol
Kveik 3996 3997 3998		
Kveik 4283 4284 4285		
Kveik 4403 4404		
Kveik 4410 4411		
Kveik 4412 4413		
Non-Kveik W34/70 (lager) 1332 (ale)		

Table 3.4. Tolerance of yeast strains to sorbitol-induced osmotic stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of sorbitol as indicated. Serial dilutions (10^3 - 10^6) from an initial cell concentration of 1 x 10^8 cells/ml for each strain were spotted on plates from left to right and incubated aerobically for 2 days at 30°C.

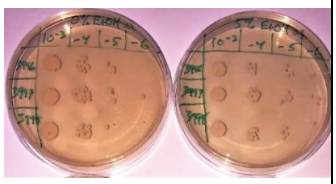
3.2.3. Tolerance of Yeast Strains to Ethanol Stress

Since ethanol is arguably the most valuable end-product of fermentation, the ability of yeast to tolerate higher concentrations of alcohol is of great relevance when determining the suitability of a strain for brewing. In order to evaluate the capability of each yeast to grow under alcoholic conditions (Section 1.6.1.3), populations of cells were analysed by spot plate testing, utilizing YPD agar media supplemented with varying concentrations of ethanol (0% (control), 5%, 10%, 15%, and 20% as described in Section 2.8.3). Ethanol stress tests were conducted using twelve kveik strains and five conventional brewing yeast strains.

As expected, all yeast strains in the experiment exhibited diminishing cell growth (spot size) as the percentage of alcohol increased, and as cultures were diluted (Table 3.5). All kveik strains in the study showed a negligible difference in growth when subjected to alcohol levels of 0% and 5% ethanol. Unsurprisingly, all strains demonstrated growth reduction as both the concentration of ethanol increased and as the dilution of slurry increased. All conventional brewing yeast strains in the study appeared to be more sensitive to ethanol when compared to the kveik strains. These yeasts produced less growth when progressing from 0% to 5% ethanol, with strains 1174 (lager) and 1260 (lager) being the most sensitive. This trend continued at 10% ethanol, where all the kveik strains exhibited greater growth than the other yeasts analysed. Strains W34/70 (lager), 1332 (ale), and M2 (ale) displayed minimal growth at a dilution of 10^3, and no growth at subsequent dilutions. Neither 1174 (lager) nor 1260 (lager) showed growth at 10% ethanol, irrespective of dilution rate, indicating that they were the most sensitive to ethanol stress in the experiment.

It is interesting to note that the kveik group of isolates consisting of 3996, 3997, 3998 (all from the same original source), demonstrated the highest capacity of growth under ethanolic conditions when compared to all other yeasts, including kveik strains (Table 3.5). At 15% and 20% ethanol, only these kveik strains

showed any biomass growth. The same strains also displayed some growth at 20% ethanol, albeit only at low dilution rates (10^3 to 10^4), with no growth thereafter.

Yeast Strains	0% (L) and 5% (R) Ethanol	10% (L) and 15% (R) Ethanol	20% Ethanol
Kveik 3996 3997 3998			

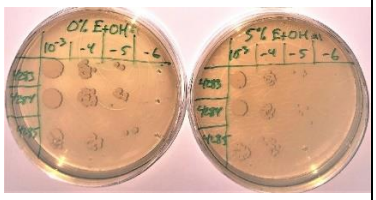
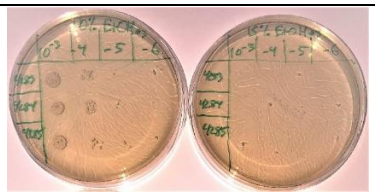
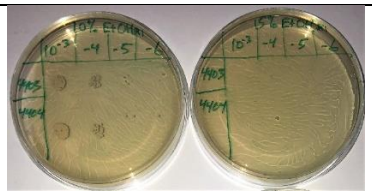
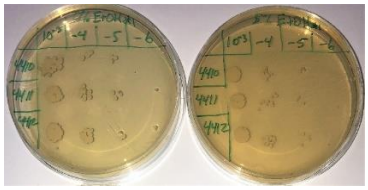
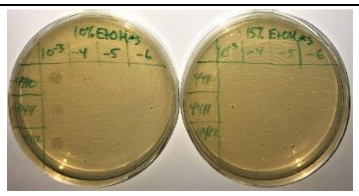
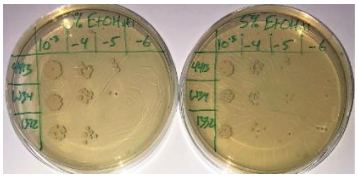
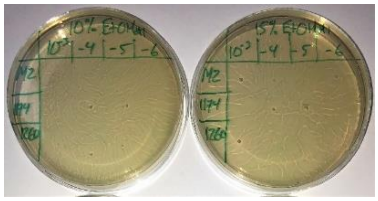
Yeast Strains	0% (L) and 5% (R) Ethanol	10% (L) and 15% (R) Ethanol
Kveik 4283 4284 4285		
Kveik 4403 4404		
Kveik 4410 4411 4412		
Kveik 4413 **Non-Kveik** W34/70 (lager) 1332 (ale)		
Non-Kveik M2 (ale) 1174 (lager) 1260 (lager)		

Table 3.5. Tolerance of yeast strains to ethanol stress. Growth of brewing yeast strains was assessed on YPD agar plates containing different concentrations of ethanol as indicated. Serial dilutions (10^3 – 10^6) from an initial cell concentration

of 1 x 10^8 cells/ml for each strain were spotted on each plate from left to right and incubated aerobically for 2 days at 30°C. The three kveik strains 3996, 3997, and 3998 were the only strains that exhibited growth at 20% ethanol. Since the other strains analysed did not yield growth at 15% ethanol, the data for 20% is omitted here.

3.2.4. Tolerance of Yeast Strains to Thermal Stress

Yeast strains are typically employed in fermentation at pre-defined and specific temperatures, selected to control both the extent of yeast growth and flavour production. The standard temperatures applied in brewing (often between 12-18°C, but influenced by beer style) are 'cold' from the perspective of the individual yeast cell. However, in contrast, kveik strains are typically used at much warmer temperatures, without creating the flavour imbalance that would normally be expected (Section 1.5.2.2). This suggests that the relationship between kveik yeast strain activity and temperature may be fundamentally unique and may extend to tolerance limits. While high temperatures are usually linked to cell damage, strains that can successfully ferment under such conditions are of interest since inevitably the time taken for fermentation is reduced accordingly.

In order to assess the capacity of yeast to grow at elevated temperatures, strains were analysed using a thermal stress test as described previously (Section 2.8.4). Yeast were cultivated on YPD agar plates and incubated aerobically at predetermined temperatures (25°C, 30°C, 37°C, and 42°C) for 5 days. Note that 37°C was selected since this temperature reflects the typical limit of growth for lager (*S. pastorianus*) yeasts. Hence, it was anticipated that the lager yeasts should not be able to exhibit growth, whilst the ale and kveik (all *S. cerevisiae*) strains would still be able to produce visible colonies (Stewart, 2016). It can be seen from Table 3.6 that at 25°C, 30°C, and 37°C, all the kveik strains exhibited

nearly identical patterns of growth, with an expected reduction based on dilutions at 10^4 and 10^5. Although at 42°C, this growth decline was more prominent, all strains successfully demonstrated cell growth at this elevated temperature. In contrast, the conventional brewing yeasts all showed weaker growth than the kveik strains at 25°C and 30°C in this study. The three *S. pastorianus* strains, W34/70, 1174, and 1260 all exhibited reduced growth at 30°C, with 1174 appearing to be the most affected. Also, as expected, none of the lager yeasts showed growth at 37°C and 40°C, while the ale strains, M2 and 1332, both displayed growth at 37°C, but a significant decline in biomass production at 42°C. In comparison, all the kveik yeast strains exhibited cell growth at 37°C and 42°C. Irrespective of dilution rate, which largely matched growth at lower temperatures, biomass production was not inhibited by temperature for any of the kveik yeasts analysed here. This clearly demonstrated that the kveik yeast were exclusively more tolerant to high temperatures than any of the conventional brewing strains analysed. This phenomenon can warrant subsequent investigations regarding kveik use in an industrial setting as fermentations would potentially be completed in less time when compared to non-kveik counterparts.

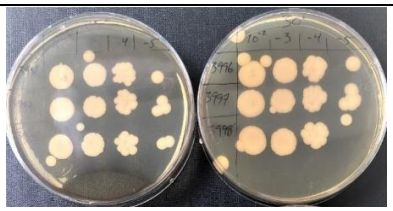
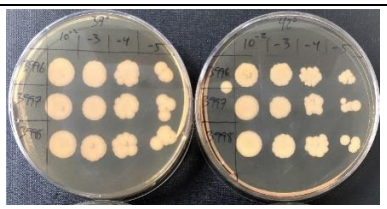
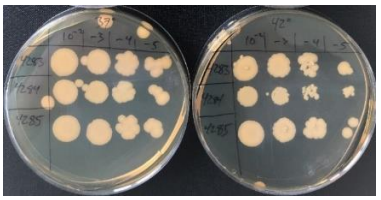
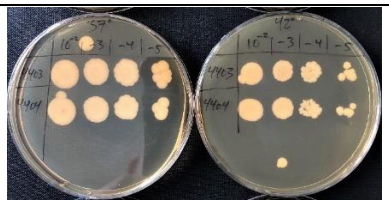
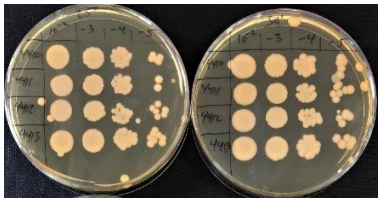
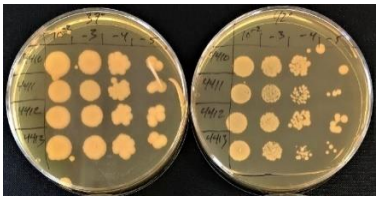
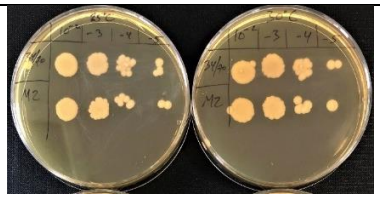
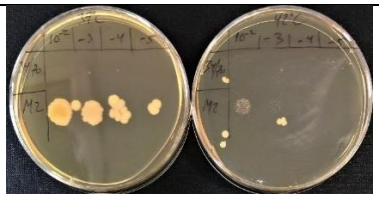
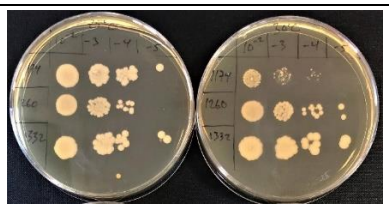
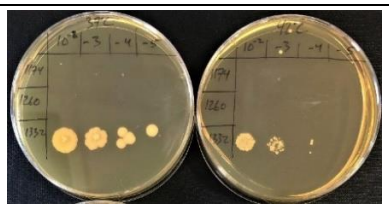
Yeast Strains	25°C (L) and 30°C (R)	37°C (L) and 42°C (R)
Kveik 3996 3997 3998		
Kveik 4283 4284 4285		
Kveik 4403 4404		
Kveik 4410 4411 4412 4413		
Non-Kveik W34/70 (lager) M2 (ale)		
Non-Kveik 1174 (lager) 1260 (lager) 1332 (ale)		

Table 3.6. Tolerance of yeast strains to thermal stress. Growth of brewing yeast strains was assessed on YPD agar plates subjected to the temperature indicated. Serial dilutions (10^2 – 10^5) from an initial cell concentration of 1 x 10^8 cells/ml for each strain were spotted on each plate from left to right and incubated aerobically for 5 days. Temperatures utilised were 25°C, 30°C, 37°C, and 42°C.

3.2.5. Summary of Yeast Tolerances

All kveik and non-kveik brewing yeast isolates varied in their tolerance capacity when subjected to the various stressors used in the experiments. When compared to the conventional non-kveik brewing strains, the kveik strains exhibited the capacity to tolerate higher degrees of oxidative, osmotic, alcoholic, and thermal stressors. It is worth noting that the kveik isolates derived from multiple-strain slurries were all distinct and exhibited varying levels of visual growth when exposed to the same concentration of a given stressor. This suggests that each isolate is sufficiently different in that they possess a unique stress response, even though they derive from the same original slurry. Further investigations would be needed to determine the nature and extent of genetic variation between strains to fully understand this phenomenon in similarity to past studies (Gibson et al., 2007; Foster et al., 2022), yet specify towards individual isolates within original yeast slurries.

When considering data from all of the stressors, it appeared that all of the kveik strains tested had a higher capacity for cell growth when subjected to stress-inducing environments than the conventional non-kveik brewing yeasts. Collectively, it appeared that the kveik strains 3996, 3997, and 3998, which all originate from one three-strain culture, were the most tolerant to the various stressors employed. Conversely, the strains 4410, 4411, 4412, and 4413, which all derive from a four-strain slurry, were the most sensitive of the kveik strains to the stressors, even though they did display higher levels of growth when compared to the non-kveik strains.

3.3. Fermentation Performance of Kveik Yeast Cultures

3.3.1. Fermentation Dynamics of Individual Kveik Yeast Isolates

To compare the fermentation characteristics of each yeast, twelve kveik strains and five conventional non-kveik yeasts were pitched into wort at 1.060 SG within 2000 ml fermenters (1000 ml working volume) using a standard pitching rate ($1x10^6$ cells/ml wort/°P). Fermenters were subsequently incubated statically at 20°C according to Section 2.9.1 and each fermentation was monitored for gravity until attenuation had been reached. Final beer was then analysed for volatile flavour compounds as described in Section 2.9.2. The fermentation profiles generated by each yeast are shown in Figure 3.1 and the associated data is tabulated in Table 3.7. In addition, each strain was ranked by ADF% as shown in Figure 3.2. Finally, fermentation dynamics were assessed by determining the time taken for each strain to achieve 50% attenuation (Figure 3.3), and to reach complete attenuation (terminal gravity) (Figure 3.4). A summary of this data can be found in Table 3.7.

When fermentations were conducted at 20°C, it was shown that all yeast isolates produced typical but distinct fermentation profiles (Figure 3.1), with varying rates of attenuation and apparent degrees of fermentation (ADF) in each instance (Figure 3.2 and Table 3.7). Overall, the lager strain 1174 exhibited the lowest ADF at 79.53%, while the ale strain M2 achieved the highest at 94.48%. Within the kveik strains, 4284 attenuated to 79.57% for the lowest in the study, while strain 4404 attenuated to 91.88% for the highest ADF of the kveik strains. This data was not surprising since it is well documented that individual strains vary in terms of attenuative performance (Lewis & Young, 2012). However, it was interesting to note that the kveik strains analysed here produced data typical of commercial brewing yeast strains. Within the multi-strain kveik groups, the two-strain culture consisting of 4403 and 4404 had the highest difference in apparent degree of

attenuation at 79.62% and 91.88%, respectively (Figure 3.2). This variation between strains belonging to the same culture was also observed for the other mixed-strain groups, albeit to a lesser extent. The three-strain kveik group (3996, 3997, 3998) and the four-strain group (4410, 4411, 4412, 4413) showed variation ranging from 86-91% and 84-88% respectively (Figure 3.2).

In order to provide greater insight into the fermentation dynamics of each strain, the time required to reach both 50% attenuation and terminal gravity were also determined (Figures 3.3 and 3.4). Evaluating 50% ADF was achieved by estimating on Figure 3.1. The ale strain 1332 had the highest rate of fermentation, reaching 50% ADF at 13 hours, while the kveik strain 4411 exhibited the slowest, reaching 50% ADF in 42 hours. Overall, the traditional brewing strains reached 50% ADF at between 13-40.5 hours and the kveik strains from 16-42 hours. Based on this analysis it can be concluded that the kveik yeasts again showed properties that were typical of more conventional industrial strains. Interestingly, there were noticeable differences in the time to complete attenuation when comparing kveik strains within each distinct group. For example, kveik strain 3996 was considerably slower to ferment relative to the other two isolates (3997 and 3998) in the three-strain group (Figures 3.3 and 3.4). As a collective, the kveik yeast belonging to the four-strain culture (4410, 441, 4412, and 4413), showed the lowest overall rate of fermentation, taking the longest to reach terminal gravity (Figures 3.3 and 3.4). The time to obtain complete attenuation was significantly longer than the other yeasts in the study and the 240-hour ADF time was estimated due to laboratory closure nearing the completion of fermentation. They did however, attenuate to levels ranging from 84.08% to 88.19%, which was typical of the yeast strains tested.

An important observation was that the rate at which 50% or complete attenuation was achieved was not related to the relative degree of fermentation. For example, the ale strain M2, which was the second slowest to obtain 50% ADF (40.5 hours), had the highest level of attenuation (94.48%) (Figures 3.2 and 3.3).

In contrast, yeasts such as 4283 or 4284 that were able to rapidly assimilate wort sugars (time to 50% ADF) were not as attenuative as some of the other strains examined. This may indicate that although these yeasts were healthy and active, they were not able to assimilate some of the larger more complex sugars (such as maltotriose), as efficiently as other strains in the experiment. Further analysis related to individual sugar metabolism would be needed to investigate this hypothesis. A study by (Foster et al., 2022) showed that kveik strains have varying degrees by which they can metabolise malt sugars. Irrespective, there was a wide variety in the levels of attenuation and rate of fermentation for the kveik strains analysed. However, as alluded to above, the kveik strains analysed were similar to conventional yeasts in this regard and therefore it can be concluded that they have broad fermentation potential which may be of commercial value.

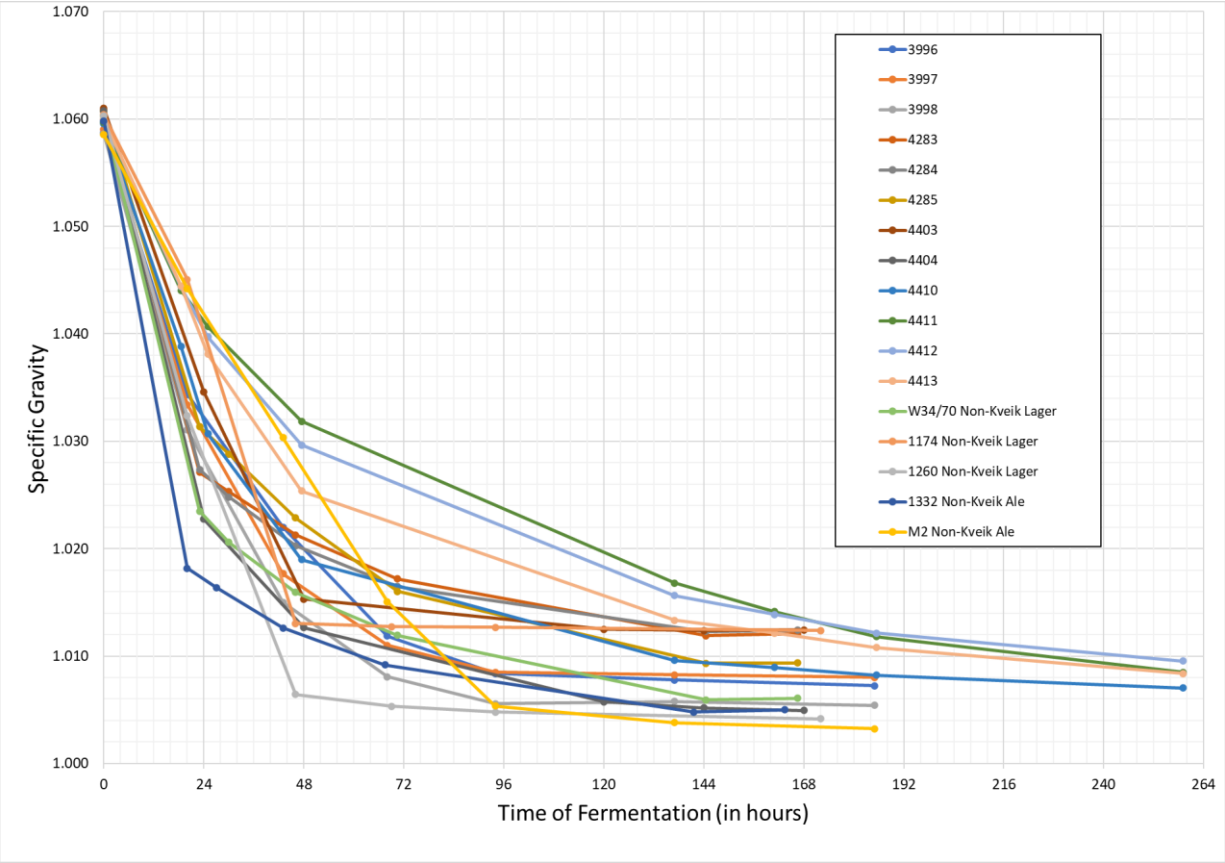


Figure 3.1. Fermentation profiles for kveik and conventional brewing yeast isolates. Values shown represent average of triplicate fermentations performed at 20°C with a pitch rate of $1x10^6$ cells/ml wort/°P. Note that this figure is for illustrative purposes only and error bars are not included to aid presentation.

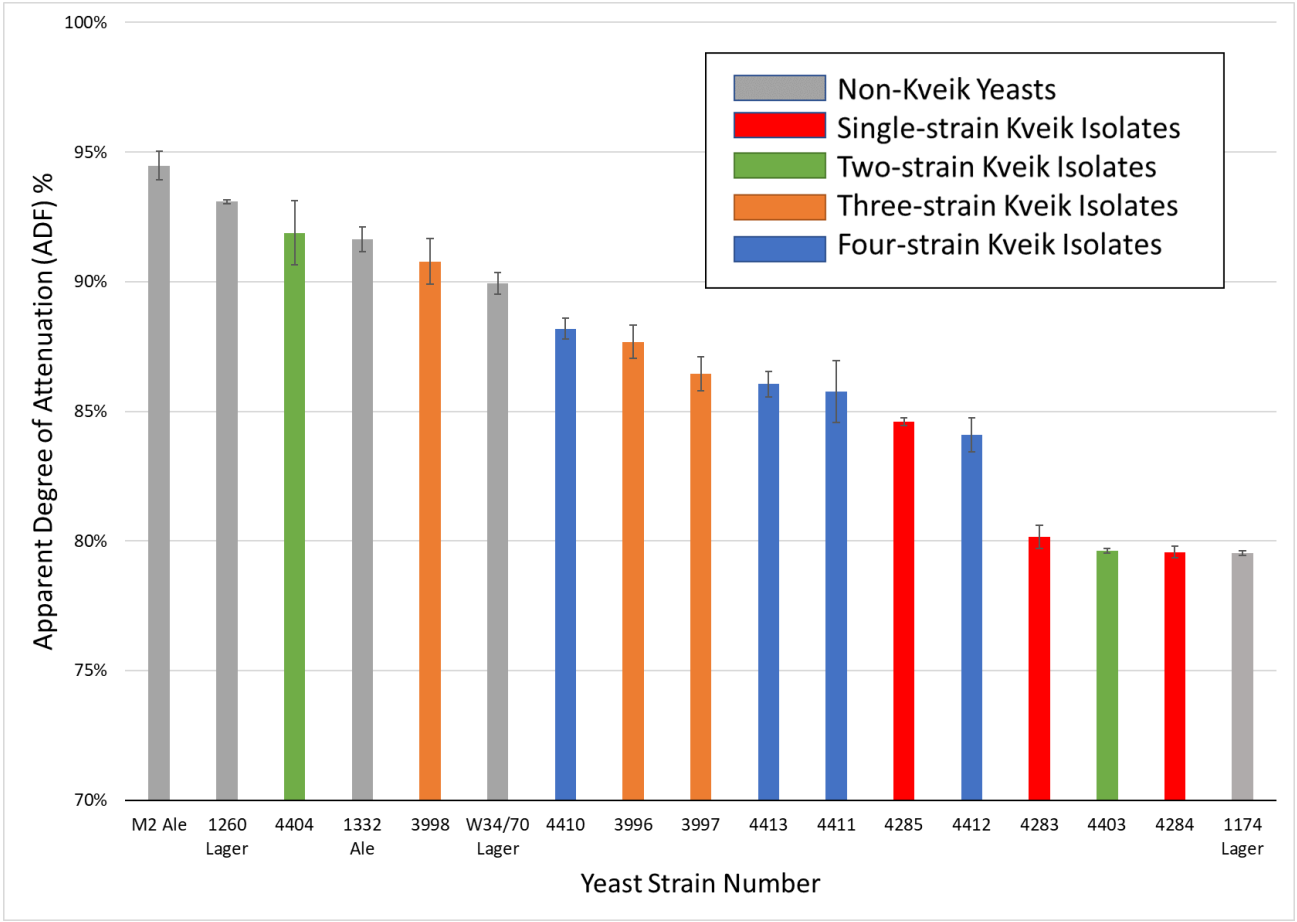


Figure 3.2. Apparent Degree of Fermentation (ADF) of yeast strains by percentage, sorted from highest to lowest. Data represents the average of triplicate fermentations performed at 20°C, with error bars indicating the standard deviation at each time point

Yeast	Starting Gravity	Estimated 50% ADF Gravity	Final Gravity	Estimated time to 50% ADF (in hrs)	Estimated Time to Attenuation (in hrs)	Average ADF %
M2 (non-kveik ale)	1.0585	1.0309	1.0032	40.5	160	**94.48%**
1260 (non-kveik lager)	1.0603	1.0322	1.0042	20.5	105	**93.09%**
4404	1.0608	1.0329	1.0049	17.5	130	**91.88%**
1332 (non-kveik ale)	1.0598	1.0324	1.0050	13	120	**91.64%**
3998	1.0586	1.0320	1.0054	19	95	**90.78%**
W34/70 (non-kveik lager)	1.0603	1.0332	1.0061	17	140	**89.93%**
4410	1.0596	1.0333	1.0070	23	240	**88.19%**
3996	1.0587	1.0330	1.0072	23	160	**87.68%**
3997	1.0590	1.0335	1.0080	21	100	**86.44%**
4413	1.0600	1.0342	1.0084	32	240	**86.05%**
4411	1.0597	1.0341	1.0085	42	240	**85.76%**
4285	1.0608	1.0351	1.0094	20	140	**84.59%**
4412	1.0599	1.0347	1.0095	36	240	**84.08%**
4283	1.0608	1.0365	1.0121	16	140	**80.16%**
4403	1.0610	1.0367	1.0124	22	120	**79.62%**
4284	1.0607	1.0366	1.0124	16.5	140	**79.57%**
1174 (non-kveik lager)	1.0604	1.0364	1.0124	27	50	**79.53%**

Table 3.7. Performance data for kveik and non-kveik fermentations, sorted by Apparent Degree of Fermentation (ADF %) of yeast strains by percentage from highest to lowest. Values shown are averages of triplicate fermentations performed at 20°C at a pitch rate of $1x10^6$ cells/ml wort/°P. Estimation of time based on data derived from Figure 3.1.

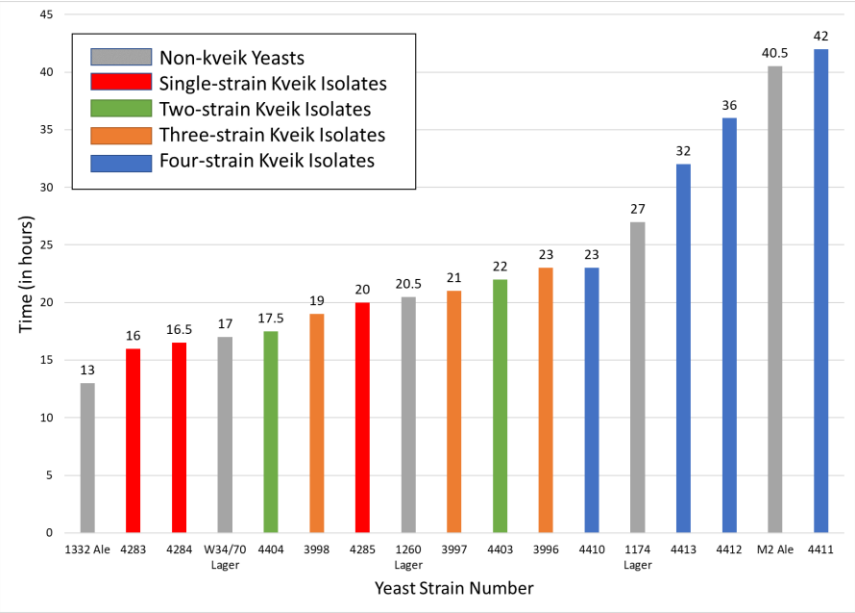


Figure 3.3. Rate of fermentation of yeast strains by approximate time to obtain 50% Apparent Degree of Fermentation (ADF), in hours, sorted from lowest to highest. Values shown are averages of triplicate fermentations performed at 20°C with a pitch rate of $1x10^6$ cells/ml wort/°P. Estimation of time based on data derived from Figure 3.1.

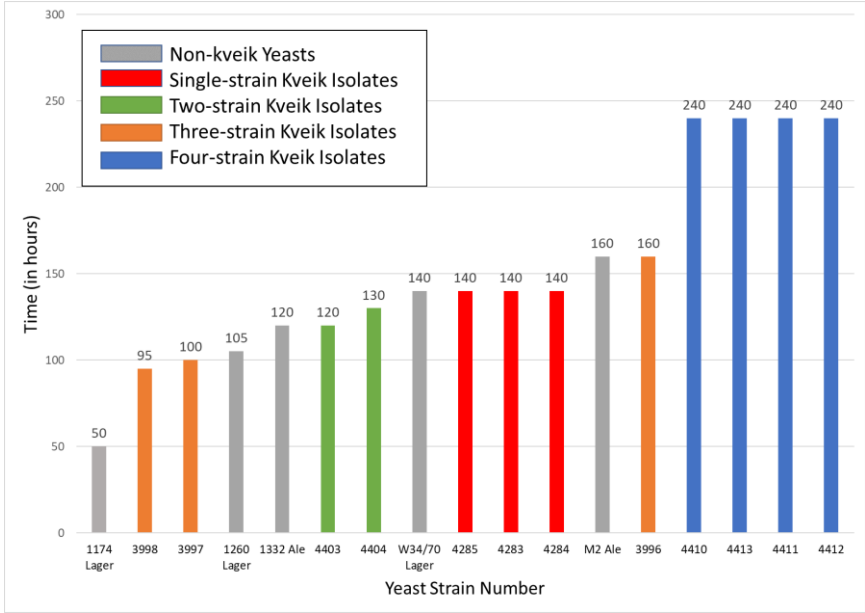


Figure 3.4. Rate of fermentation of yeast strains sorted by approximate time to obtain complete attenuation, in hours, sorted from lowest to highest. Values shown are averages of triplicate fermentations performed at 20°C with a pitch rate of $1x10^6$ cells/ml wort/°P. Estimation of time based on data derived from Figure 3.1.

3.3.2. Flavour Production of Individual Kveik Yeast Cultures

Once the fermentations described above (Section 3.3.1) had reached terminal gravity, samples of beer were taken and analysed for volatile spectrum via APCI-MS as detailed in Section 2.9.2. It should be noted that although this method does not denote specific compounds, it provides a characteristic 'fingerprint' representation of volatile compounds produced by each yeast during fermentation (Ashraf et al., 2010). This technique is therefore useful in distinguishing between yeast strains, allowing the extent of variation between groups of yeast to be rapidly characterised. Based on this analysis, Principal Component Analysis was performed (177:173) (where each number is molecular weight minus one) and used to produce a score plot displaying the volatile expression of each triplicate fermentation performed for the twelve kveik isolates and five conventional non-kveik yeasts (Figure 3.5). In addition, a heat map displaying volatile expression patterns for each of these seventeen yeast isolates is shown in Figure 3.6.

The scores plot shown in Figure 3.5 demonstrate that within each biological triplicate analysis, profiles were remarkably similar, indicating a high degree of repeatability. However, as expected, each of the strains analysed showed a unique flavour profile. Interestingly, the extent to which a strain differed was not related to its brewing specification. For example, the lager strain W34/70 produced a similar profile to the kveik strains 3998 and 4404. Another interesting observation was that some of the kveik isolates, for example 3996, 3997, and 3998 (originating from the same three-yeast culture), all exhibited a very similar heat map signature. A similar pattern was observed for 4403 and 4404, isolated from the same two-strain culture. However, when analysing yeast from the four-strain culture (4410, 4411, 4412, and 4413), it can be seen that isolate 4413 appeared to be distinct from the others in regard to volatile production (Figure 3.5).

The data above was confirmed based on APCI-MS score plot (heat map), as shown in Figure 3.9. This indicated that the lager yeast W34/70 was distinct to the other brewing yeasts analysed, however the kveik yeast strains from the two-strain group (4403 and 4404) and 3 strain group (3996, 3997 and 3998) showed similar flavour profiles. In addition, this data also showed that strain 4413 was unique when compared to the other three strains within the same original culture (4410, 4411 and 4412), supporting the previous results above. This suggests that it is likely that isolate 4413 has a different heritage than the other strains in the system and entered the culture at an unknown time point. An extension of this hypothesis is that this strain may be a potential 'contaminant' yeast, since brewers would borrow yeast from local farms or villages if fermentations didn't proceed as planned (Garshol, 2020). Potentially, this 'anomalous' yeast allowed desirable flavour impact on the beers to be produced, or yielded improved fermentation efficiency, such that the culture was not discarded and was reused, eventually developing into its own own unique blend.

An intriguing observation when viewing the volatile analysis of the individual kveik isolates 4283, 4284, and 4285, was the close proximity of their flavour profiles (Figure 3.5), supported by the visual differentiation of compound groups (Figure 3.6). It was expected that these yeasts may be different, especially considering that there is a major mountain range, a large inland fjord and almost 500 km separating these areas in the country from which they were isolated (Figure 1.10 map locations 1 (4285), 4 (4283), and 6 (4284)). Based on this, it is perhaps suprising that they have such similarities in their volatile expression, since distribution of strains would have been limited. The chance that they share a common ancestor, however plausible, would be speculative and additional analysis of the genetic composition of these strains would be required to investigate this further.

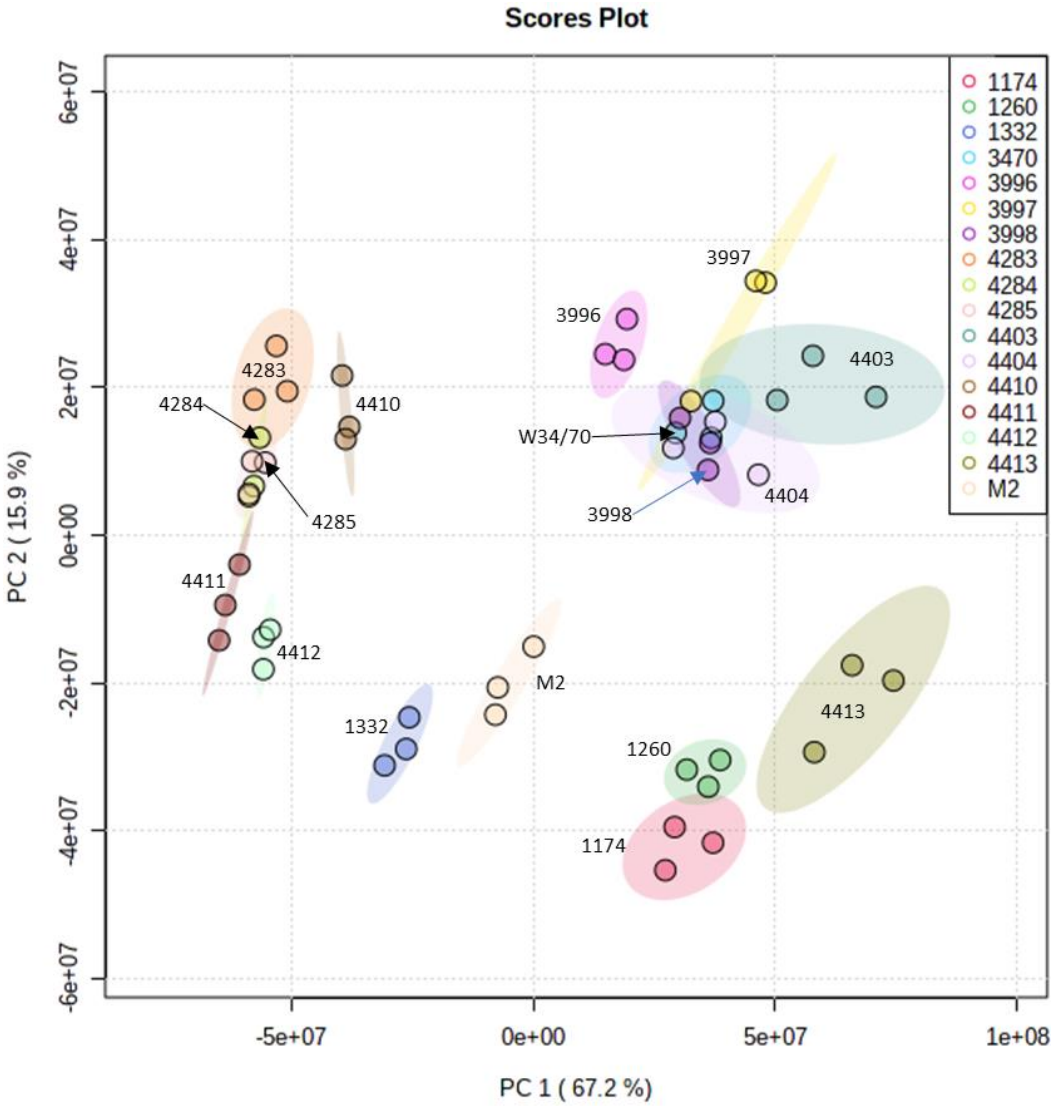


Figure 3.5. Principal component projection of flavour compounds produced by kveik and traditional non-kveik isolates. Colours represent individual yeast strains as noted. All fermentations were performed at = a pitch rate of $1x10^6$ cells/ml wort/°P. Data is displayed here, with gates indicating similarity/variation within each sample group. Data was evaluated using principal component analysis: PC1: 177 and PC2: 173 as described in Section 2.9.4.

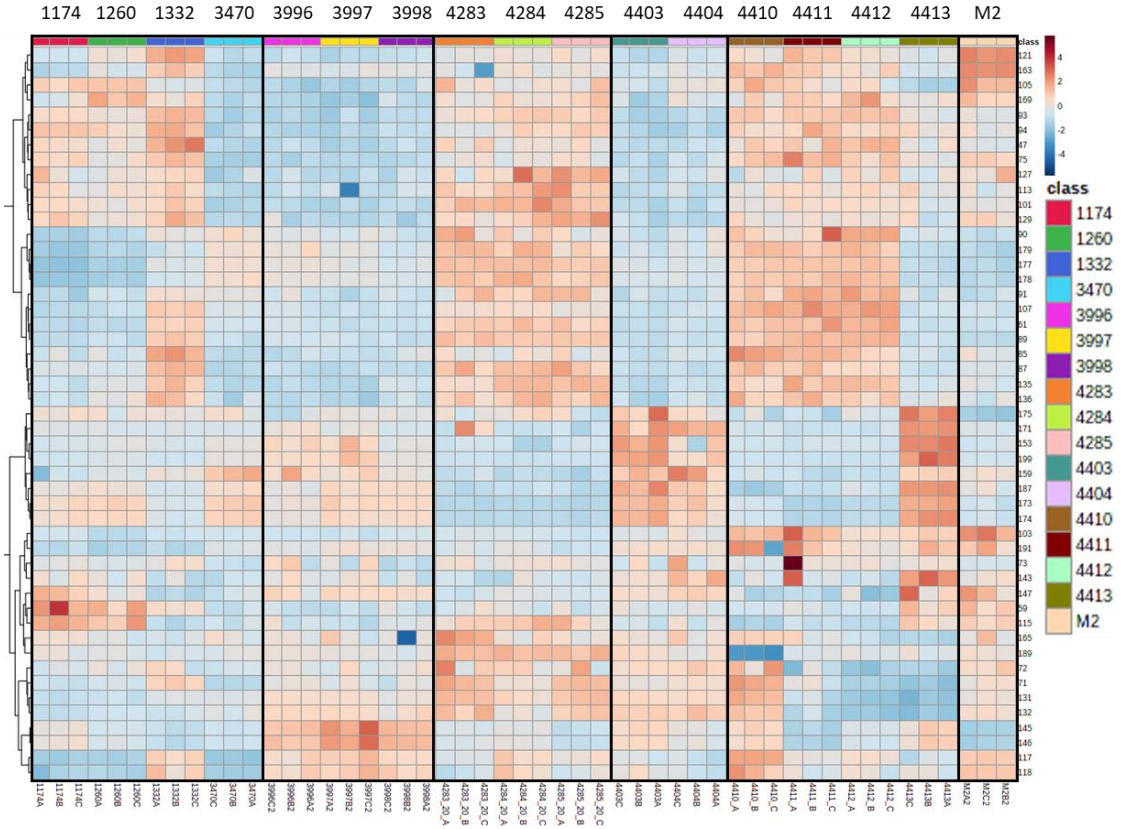


Figure 3.6. Distribution of volatile compounds for each fermentation, as determined by APCI-MS (Section 2.9.2). Each yeast strain is coded by colour as indicated, and in each instance, data indicates those obtained from triplicate samples. The Y-axis displays the respective molecular weight of volatiles, determined algorithmically for generation of this heat map (by molecular weight minus 1). Each 'cell' represents the relative ion signal concentration and is accompanied by a colour intensity, where red represents relatively higher normalised signal intensity and blue represents a lower relative signal intensity.

3.3.3. The Relationship Between Fermentation Temperature and Flavour Production in Kveik Yeast Strains.

Kveik fermentations are typically conducted 'hot' when compared to mainstream products, without yielding the flavour imbalance usually associated with such regimes (Section 1.5.2). To fully investigate the impact of temperature on the fermentation performance and flavour production of kveik yeast, two single strain isolates (strains 4283 and 4285) were selected along with two traditional brewing yeasts (1332 (ale) and W34/70 (lager)). The chosen kveik isolates were selected since they were monocultures, and were sufficiently different to both contemporary strains in terms of flavour production based on previous analysis (Section 3.3.2). Each strain was used for fermentation of all-malt wort (1.060 specific gravity) at 10°C, 15°C, 20°C, 30°C and 40°C. The temperatures of 10°C, 15°C, and 20°C were selected to reflect the typical ranges applied in standard beer production (Section 1.4), while temperatures above 20°C reflect practices more closely associated with traditional kveik fermentations. Each fermentation was monitored for gravity (Section 2.9.1), speed of attenuation and apparent degree of fermentation, as well as flavour characteristics of the final products via GC-MS evaluation (Section 2.9.2 - 2.9.4). The effect of temperature on the performance of each yeast strain can be found in Figure 3.7. Related to this, the differences between strains at each temperature can be seen in Figure 3.8. Data from each of the fermentations performed can also be found in Table 3.8, where starting and final gravity, apparent degree of fermentation (ADF), and the estimated time to reach both 50% ADF and final gravity in hours are displayed.

It can be seen that as temperature increased, the rates of fermentation also increased for each strain (Figure 3.7). This included both the time required to reach 50% attenuation, as well as the time to obtain terminal gravity (Table 3.8). In addition, each strain performed differently according to the experimental parameters applied. To illustrate this, at 10°C, all yeasts fermented wort at varying rates, with different degrees of attenuation (Figure 3.8; Table 3.8). The

lager strain W34/70 exhibited a rapid rate of fermentation, both in time to achieve 50% ADF and time to terminal gravity, but also in total degree of attenuation. This was broadly anticipated, as 10°C reflects the normal operating range (10°C – 14˚C) for lager yeast and for this strain specifically (Hefebank, 2022), and is below the fermentation temperatures normally associated with ales (Stewart, 2016). Both W34/70 and NCYC 1332 (ale strain) exhibited a higher degree of attenuation than the kveik strains at this temperature, perhaps indicating that 10°C is below the normal operating range of these kveik yeast. This was somewhat corroborated by a study by (Foster et al., 2022), where kveik strains and non-kveik strains exhibited varying degrees of attenuation at temperatures lower than typical kveik use, albeit with different yeast strains. It should be noted, however, that the speed of fermentation and the time to reach attenuation was similar to the conventional brewing yeasts.

When the temperature was increased to 15°C, all rates of fermentation increased, as expected (Figures 3.7 and 3.8). An interesting outlier was the ale strain 1332, which exhibited a slower rate of fermentation relative to the other three yeasts (Figure 3.8B). This strain eventually attenuated to a level higher than the two kveik strains, however, and data obtained at other temperatures indicates that this profile may not be a true reflection of the performance of this strain at 15°C. When comparing the two kveik yeasts, strain 4283 was again less attenuative than strain 4285, reaching a final gravity of 1.013 compared to 1.007 respectively (Table 3.8).

As seen in Figures 3.7 and 3.8, the fermentations at 20°C showed a progressive increase in the rate of fermentation for all strains. At this temperature, both W34/70 and NCYC 1332 exhibited a faster rate of fermentation and a lower terminal gravities than the two kveik strains (Table 3.8). This perhaps suggests that 20°C remains below the optimal fermentation temperature for kveik strains. This was supported by analysis of performance at 30°C, where all of the yeasts showed an increased rate of fermentation, but in this instance the kveik strains

had comparable fermentation rates to the traditional brewing strains, while the ADF for all strains finished at similar levels (Figure 3.8; Table 3.8).

At 40°C, it was expected that the lager yeast W34/70 would perform poorly, as this exceeds the threshold for efficient growth of this strain (Section 3.2.4) and *S. pastorianus* yeast in general (Walker & Stewart, 2016). This was indeed the case, with the decrease in gravity observed attributed to the period of time between the pitching of yeast on the laboratory benchtop and reaching incubation temperature (Figure 3.7A). Similarly, the non-kveik 1332 ale yeast also did not function efficiently at this temperature, with fermentations stalling at around 24 hours (Figure 3.7B). However, the two kveik strains both performed well, and exhibited more rapid rates of fermentation when compared to other temperatures (Figure 3.7). However, of these strains, strain 4285 performed the best with an ADF of 85.89% compared to 77.86% for strain 4283 (Table 3.8). The data obtained for strain 4283 may indicate that the optimum temperature for fermentation may be lower than 4285, likely falling between 30˚C and 40˚C. Nonetheless, the data obtained here further provides support to previous observations that kveik strains can successfully ferment at temperatures where conventional lagers and some ale strains cannot (Foster et al., 2022).

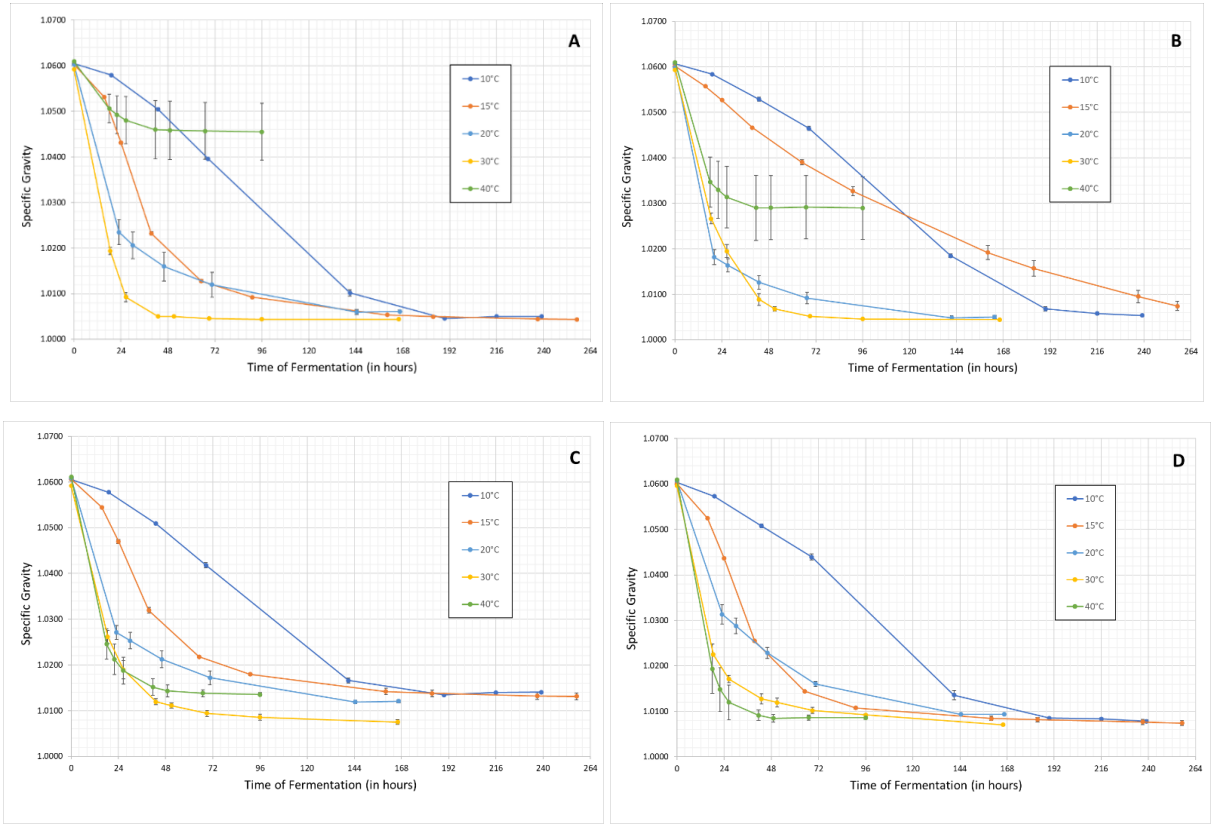


Figure 3.7. The relationship between temperature and fermentation performance of W34/70 (A), 1332 (B), and kveik strains 4283 (C) and 4285 (D). Fermentations were performed at 10°C, 15°C, 20°C, 30°C, and 40°C. Data represents the average of triplicate fermentations, with error bars indicating the standard deviation at each time point.

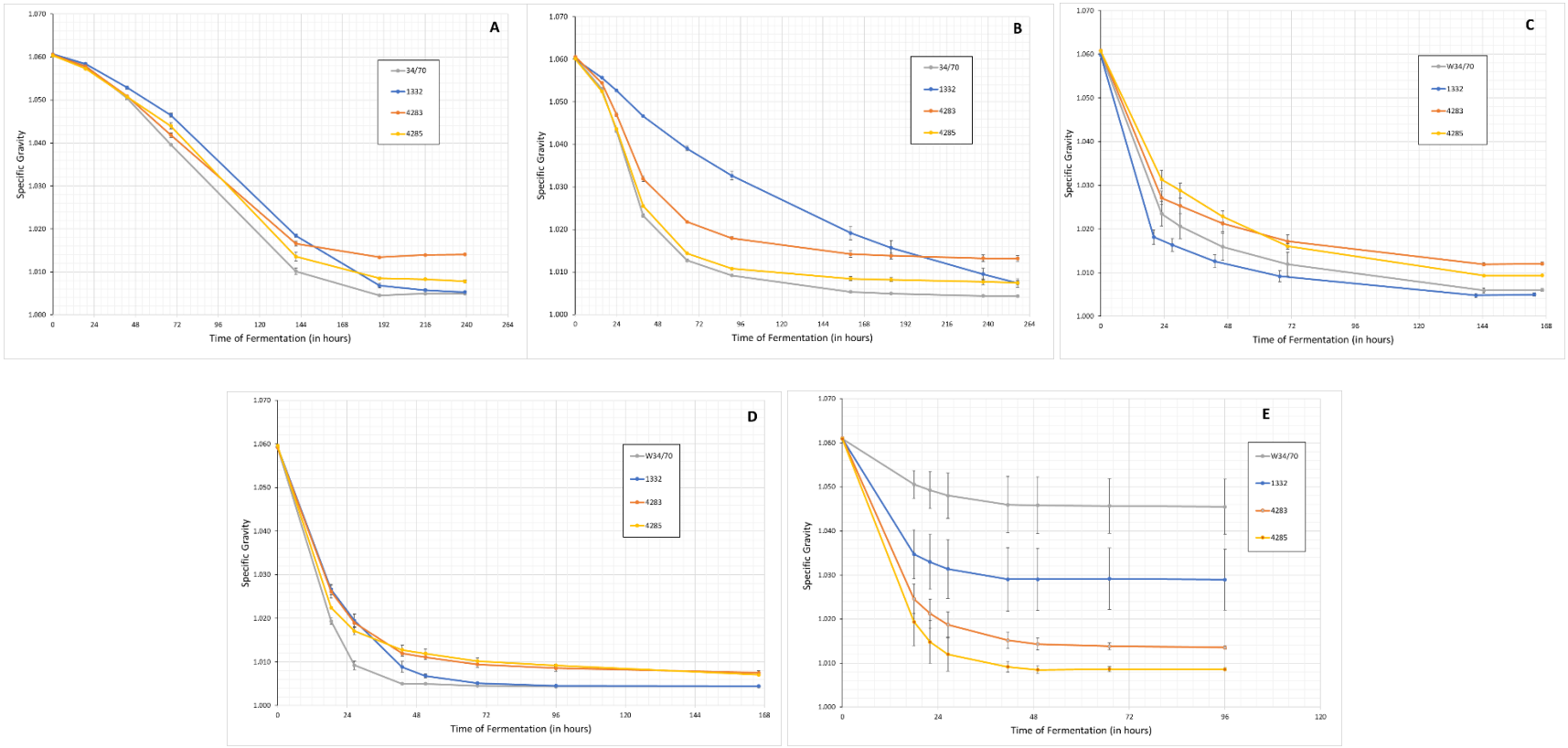


Figure 3.8. The effect of temperature on fermentation progression for yeast strains 4283 and 4285 (kveik), W34/70 (lager) and 1332 (Ale). Fermentations were conducted at 10°C (A), 15°C (B), 20°C (C), 30°C (D), and 40°C (E). Data represents the average of triplicate fermentations, with error bars indicating the standard deviation at each time point.

Yeast Strain	Temperature	Starting Gravity	Final Gravity	Time to Reach 50% ADF (hours)	Time to Reach Final Gravity (hours)	Apparent Degree of Fermentation (ADF)
Non-kveik W34/70	10˚C	1.0605	1.0045	85	180	91.51%
	15˚C	1.0603	1.0043	32	180	92.82%
	20˚C	1.0603	1.0059	16	144	90.15%
	30˚C	1.0592	1.0044	12	70	92.63%
	40˚C	1.0609	1.0455	14	40	25.33%
Non-kveik 1332	10˚C	1.0607	1.0043	104	240	91.21%
	15˚C	1.0601	1.0074	86	260	87.69%
	20˚C	1.0598	1.0048	17	140	91.98%
	30˚C	1.0594	1.0045	14	100	92.50%
	40˚C	1.0610	1.0290	11	40	52.51%
Kveik 4283	10˚C	1.0606	1.0140	82	190	76.94%
	15˚C	1.0606	1.0131	34	230	78.34%
	20˚C	1.0608	1.0119	16	144	80.44%
	30˚C	1.0592	1.0075	14	100	87.32%
	40˚C	1.0611	1.0135	12	72	77.86%
Kveik 4285	10˚C	1.0604	1.0078	92	230	87.08%
	15˚C	1.0601	1.0074	32	240	87.64%
	20˚C	1.0608	1.0093	20	144	84.64%
	30˚C	1.0596	1.0071	13	100	88.14%
	40˚C	1.0609	1.0086	11	48	85.89%

Table 3.8. Fermentation data for brewing yeast W34/70 (lager) and 1332 (ale) and the kveik strains 4283 and 4285. Fermentations conducted at temperatures indicated with a pitch rate of $1x10^6$ cells/ml wort/°P. All values are presented as averages of triplicate fermentation.

Once the performance (sugar utilisation) of yeast strains at different temperatures had been determined, the volatile compounds produced during fermentation were characterised by GC-MS analysis of the finished beers (Section 2.9.3). This was conducted in order to explore the relationship between fermentation temperature and flavour development in traditional brewing and kveik yeast strains. In each instance, the top 11 volatile compounds (identified based on matching to the MS database; Section 2.9.3) were normalised logarithmically and used to prepare spider diagrams. These enable a comparison of flavours produced by each strain based on fermentation temperature, as shown is Figure 3.9. Furthermore, the differences between each strain at a given temperature could also be investigated (Figure 3.10). It should be noted that normalisation of the data was conducted to make visual presentation easier, but an artefact of this is that numerical changes based on logarithmic scaling could vary in actual significance, especially when regarding flavour profiling and the relative thresholds of different flavour compounds.

It can be seen from Figure 3.9 that all yeasts examined experienced changes in volatile expression as fermentation temperature increased. It was noted that, to varying degrees, all of the yeasts produced the lowest concentrations of esters when fermentations were performed at 10˚C and at 40˚C (Figure 3.9). This was perhaps unsurprising given that esters typically increase with increasing temperature (Section 1.5.2.2), and that cell growth may have been impacted at 40˚C. However, a surprising finding was that the ester spectrum remained similar between the temperatures 15˚C, 20˚C, and 30˚C for all the strains examined. A noted point of this could be that while visually similar on the graph, the subtle difference in sensory perception may be noticeable as the scale is logarithmic to aid presentation. This would require additional analysis. This trend could be seen more clearly when comparing flavour production by each yeast according to temperature (Figure 3.10). However, it should be noted that at 10˚C, the two kveik isolates show less volatile production when compared to the traditional

brewing yeasts, especially regarding isobutyl acetate, phenylethyl alcohol, acetic acid, as well as the octanoic and decanoic esters. This could potentially be attributed to the fact that 10˚C is considerably lower than standard kveik operating temperature, as described above. At 40˚C, the ratios of volatile compounds produced were generally similar, but the two traditional brewing yeasts W34/70 and 1332 produced lower levels of phenylethyl alcohol and decanoic acids, and a much more pronounced decrease in acetic acid ester (Figure 3.10). This is likely to reflect the failure to complete fermentation, although the similarity in other flavour products is perhaps surprising here.

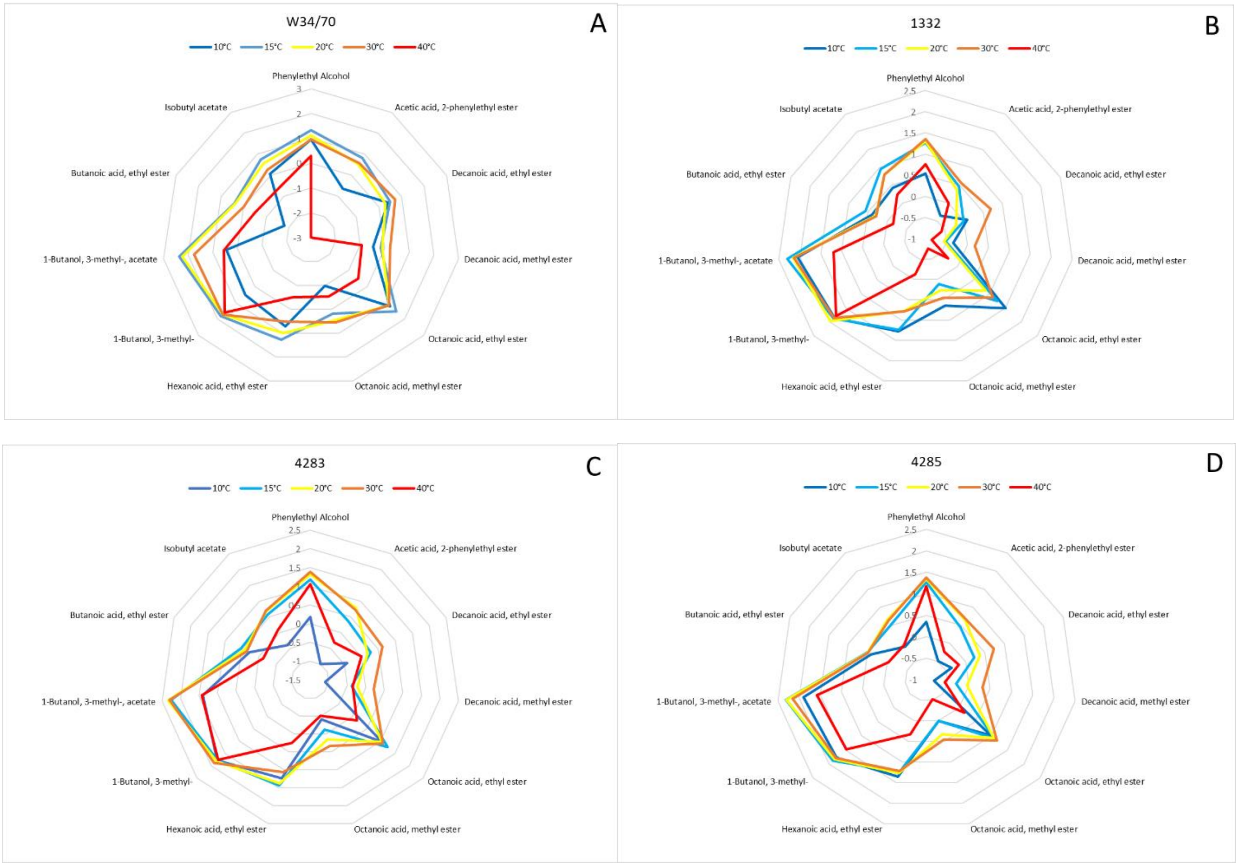


Figure 3.9. The effect of temperature on the volatile production of brewing strains W34/70 (A) and 1332 (B), and kveik yeast 4283 (C) and 4285 (D). Fermentations were performed at 10°C, 15°C, 20°C, 30°C and 40°C and results reflect the average of triplicate samples and data is expressed in $\log_{10}$ scale with 1 = 10 mg/l.

Figure 3.10. Comparison of flavour production of the brewing yeasts W34/70 and 1332, and the kveik strains 4283 and 4285 following fermentations conducted at 10˚C (A), 15°C (B), 20°C (C), 30°C (D), and 40°C (E). Results reflect the average of triplicate samples and data is expressed in $\log_{10}$ scale with 1 = 10 mg/l.

3.3.4. Fermentation Analysis Using Blends of Culture Isolates

Many kveik cultures utilised in traditional Norwegian brewing are mixtures of multiple yeast isolates (Section 1.7.3). However, this occurrence is largely due to circumstance rather than design, and the precise impact of each component isolate on overall fermentation performance and product character remains unknown. To investigate the relative influence of individual isolates on fermentation, predetermined proportions of yeasts were pitched into wort (1.060 specific gravity) and fermented at 20˚C (Section 2.9.1). In each instance, isolates were propagated from stocks and added to wort in various percentages in order to achieve a combined total pitch rate of $1x10^6$ cells/ml/°P. For this set of experiments, the two-culture slurry (consisting of strains 4403 and 4404) and the three-culture slurry (consisting of strains 3396, 3997, and 3998) were examined. As previously described (Section 2.9.1), fermentations were analysed for gravity until attenuation, and flavours were determined using GC-MS (Section 2.9.3).

3.3.4.1. Fermentation of Two-Isolate Culture Blends

To determine the relative contribution of each strain to fermentation, various mixtures (blends) of strains 4403 and 4404 were pitched into fermenters as described above. The blends comprised: 1) 75% 4403 cells and 25% 4404 cells; 2) 50% of each isolate; and 3) 75% 4403 cells and 25% 4404 cells. These blends were also compared to the pure isolate fermentations performed previously (Section 3.3.1). It can be seen from Figure 3.11 that each mixture produced a distinct fermentation curve. For example, the pure isolate 4403 fermented more slowly and yielded a higher final attenuation than the pure culture of 4404, which was more rapid and reached a lower final attenuation. However, the rates of fermentation (based on analysis of time to 50% ADF and to reach terminal gravity) were varied and did not follow a specific trend (Table 3.9). The key difference observed was related to final attenuation and ADF%. The general trend

observed was that as the percentage of 4403 cells decreased (and therefore 4404 increased), the ADF% increased (from 79.62% to 91.88%; Table 3.9). This suggests that strain 4404 is more capable of assimilating malt-based sugars than 4403, even though they were both derived from the same original yeast slurry. As a side observation, it was noted from visual analysis of the pure isolates, that strain 4403 was more flocculant in appearance than strain 4404. Similarly, in the blending experiment, it was noted that the degree of yeast sediment was greatest for those fermentations conducted with strain 4403. This difference in flocculation may have reinforced the attenuative nature of 4404 versus 4403, as the former remained in suspension for longer potentially allowing fermentations to be completed more efficiently. The study by (Foster et al., 2022) showed that varied strains of kveik and non-kveik consume the malt sugars glucose, maltose, and maltotriose at different rates, however that study did not compare these parameters compared to flocculation. Additional analysis of the flocculation properties and/or the capacity of cells to assimilate specific wort sugars would be required to further investigate this phenomenon.

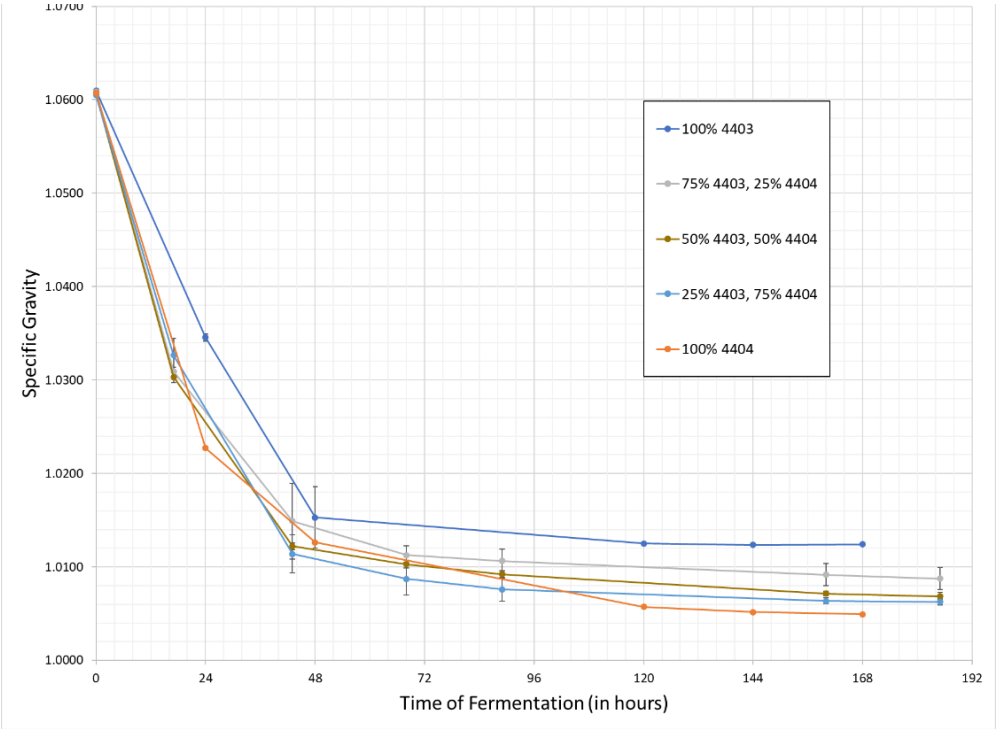


Figure 3.11. The relationship between starting cell inoculum rate and fermentation progression for kveik blends using mixtures of isolates 4403 and 4404 as indicated. All fermentations were conducted in triplicate. Data represents the average of triplicate fermentations, with error bars indicating the standard deviation at each time point.

Yeast Blend Percentage	Final Gravity	Time to Reach 50% ADF, in hours	Time to Reach Final Gravity, in hours	Apparent Degree of Fermentation (ADF)
100% 4403	1.0124	22	120	79.62%
75% 4403, 25% 4404	1.0088	14.5	170	85.59%
50% 4403, 50% 4404	1.0069	15	160	88.68%
75% 4404, 25% 4403	1.0063	16.5	160	89.64%
100% 4404	1.0049	17.5	140	91.88%

Table 3.9. Quantification of fermentation dynamics for kveik blends produced using strains 4403 and 4404.

Once fermentation differences had been confirmed, the relative contribution of each yeast blend to flavour production was determined by analysis of the final beer. Quantification of beer volatiles revealed considerable differences in ester production for each of the blends investigated (Figure 3.26). It can be seen that the yeast blend consisting predominantly of strain 4404 (75% 4404 and 25% 4403) produced the highest level of volatiles overall when compared to all of the other blends. Conversely, the blend consisting predominantly of 4403 produced the least concentration of volatiles. As might be expected based on this, the 50% blend yielded a final flavour profile between these two extremes. Coupled with the rate of fermentation and attenuation levels exhibited, it appears that kveik strain 4404 was the more dominant in terms of flavour contribution and sugar utilisation. It is possible that this may be related to individual strain growth dynamics and further investigation into growth rates and biomass yield under the conditions applied would be insightful.

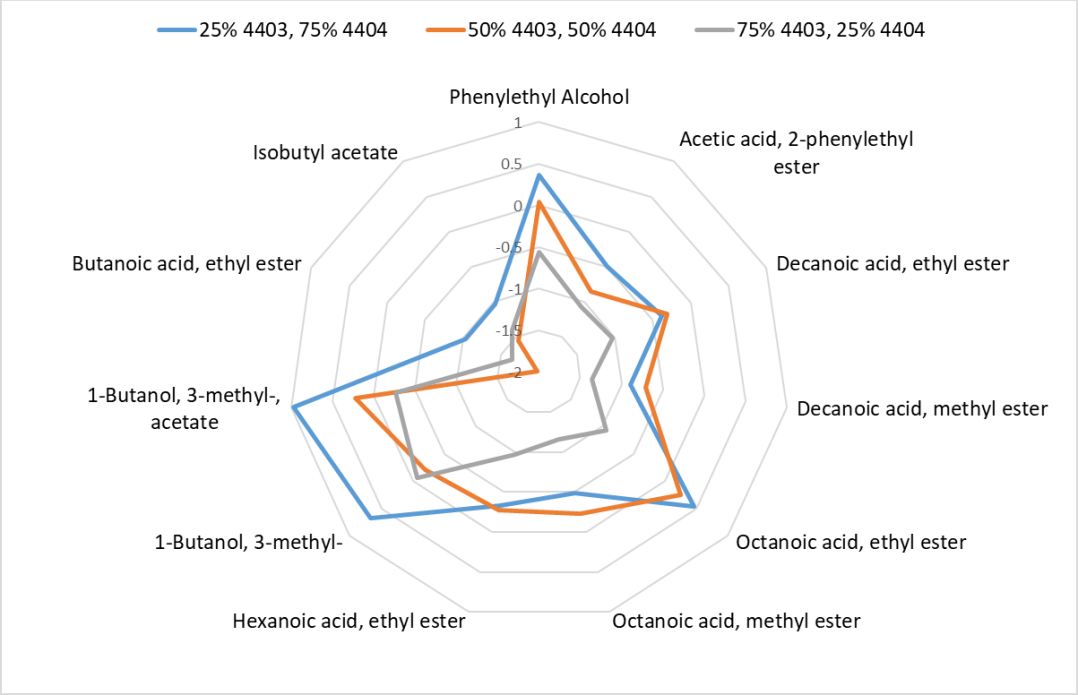


Figure 3.12. The effect of population dynamics on flavour production in kveik strains 4403 and 4404. Fermentations were conducted using 3 blends of yeast (75% 4403 cells and 25% 4404; 50% of each isolate; 75% 4403 cells and 25% 4404 cells) as indicated. Results reflect the average of triplicate analyses and data is expressed in $\log_{10}$ scale with 1 = 10 mg/l.

3.3.3.2. Fermentation of Three-Isolate Culture Blends

Similar to the experiments described above (Section 3.3.3.1), a second experiment was performed using blends of the kveik isolates 3396, 3997, and 3998, all of which were derived from the same original yeast slurry. In this instance, each strain was used in blends of 70%, 33% and 15%, such that all variations of strain combinations were achieved. This data was also compared to the pure isolate fermentations for each strain reported previously (Section 3.3.1). As before, each set of fermentations yielded a unique profile with distinctive attenuation curves (Figure 3.13). Analysis of this data (Table 3.10) indicated that strain 3998 was the most attenuative (90.78% ADF), followed by strain 3996 (87.68% ADF), and finally 3997 being the least attenuative (86.44% ADF). As might be expected, when viewed as mixed cultures, the fermentations consisting predominantly of 3998 were also the most attenuative (91.08% ADF) (Table 3.10), the blends consisting of mainly 3996 cells yielded the second highest ADF (90.82%), while the least attenuative blend possessed mainly 3997 cells (89.56% ADF). However, beyond this comparison, the data was less clear cut, with the rate of fermentation for blends of yeast not always representative of the dominant strain. For example, the most rapid fermentations (time to 50% ADF) were those comprising 70% 3996, 70% 3997 and an equal mixture (33%) of all strains (Table 3.10. Consequently, this data is perhaps inconclusive and may require further investigation, including a more frequent analysis of gravity over time.

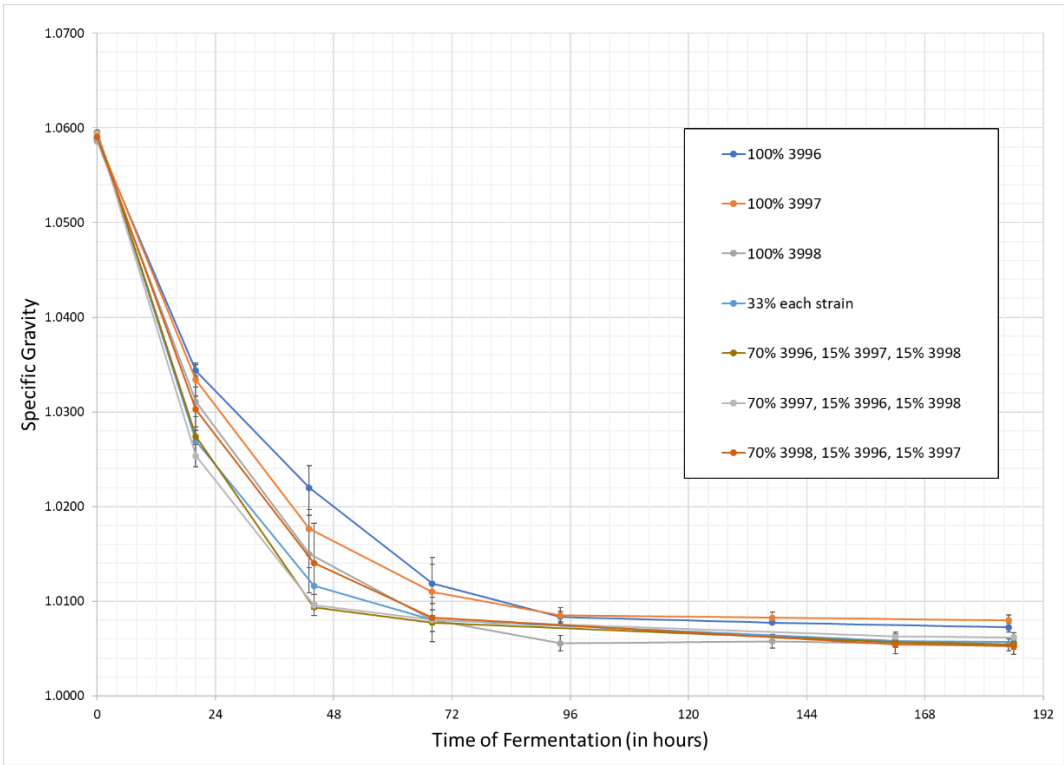


Figure 3.13. The relationship between starting cell inoculum rate and fermentation progression for kveik blends using mixtures of isolates 3996, 3997, and 3998 as indicated. All fermentations were conducted in triplicate. Data represents the average of triplicate fermentations, with error bars indicating the standard deviation at each time point.

Yeast Blend Percentage	Final Gravity	Time to Reach 50% ADF, in hours	Time to Reach Final Gravity, in hours	Apparent Degree of Fermentation (ADF)
100% 3996	1.0072	23	150	87.68%
100% 3997	1.0080	21	150	86.44%
100% 3998	1.0054	19	150	90.78%
33% each 3396, 3997, 3998	1.0057	16	170	90.47%
70% 3996, 15% 3997, 15% 3998	1.0055	16	170	90.82%
15% 3996, 70% 3997, 15% 3998	1.0062	15	170	89.56%
15% 3996, 15% 3997, 70% 3998	1.0053	19	170	91.08%

Table 3.10. Quantification of fermentation dynamics for kveik blends produced using strains 3996, 3997, and 3998.

Irrespective of the lack of trends observed for the fermentations shown above, flavour analysis was performed on each of the final beers produced using GC-MS. Analysis of all four yeast strain blends exhibited negligible differences in volatile expression, except for one blend (70% 3398). This strain combination yielded a reduction in decanoic acid, ethyl ester and decanoic acid, methyl ester (Figure 3.14). Considering the high similarities otherwise observed within this group of strains (based on heat map analysis; Section 3.3.2), it is possible that the organoleptic effect of this difference on final beer character was negligible.

However, it should equally be noted that other constituents not shown in Figure 3.14 (i.e. those omitted by the software analysis based on selection of the top 11 volatile compounds matched to the MS database) may also have a significant impact on the final product. Additional insight involving a trained sensory panel would be required.

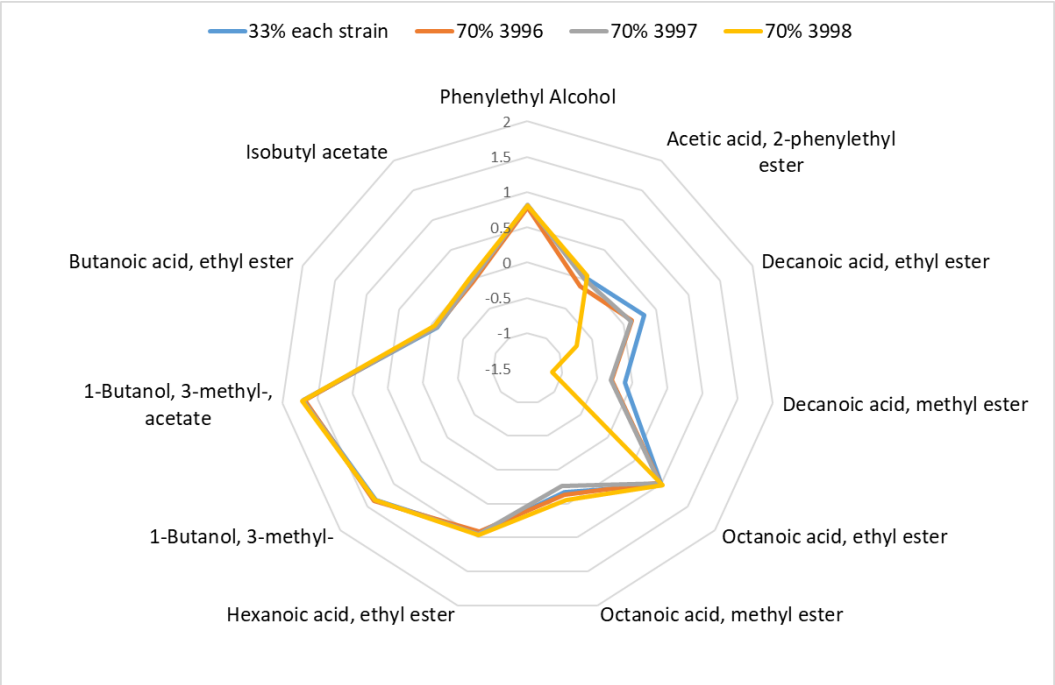


Figure 3.14. The effect of population dynamics on flavour production in kveik strains 3996, 3997, and 3998. Fermentations were conducted using 4 blends (3 of which comprised 70% of a 'dominant' isolate, with an equal 15% of the other two strains; and the final culture comprising 33% of each isolate). Results reflect the average of triplicate analyses and data is expressed in $\log_{10}$ scale with 1 = 10 mg/l.

Chapter 4

4. Conclusions

The purpose of this thesis was to investigate the physiological and fermentative properties of traditional Norwegian landrace yeast strains. This group of 'kveik' yeast are known to ferment well at high temperatures and at a rapid rate when compared to contemporary strains, however their full potential for mainstream brewing, and as potential substitutes for current yeasts, has not been fully investigated. One of the main objectives of this work was to test the capacity for kveik yeasts to tolerate stressors typically encountered during brewing operations, including those induced by oxidative, osmotic, alcoholic, and elevated thermal environments. The capacity for kveik strains to ferment wort thoroughly and in a timely manner (a highly desirable property for industrial use), was also examined, along with a characterisation of the flavour profiles of final beers. Lastly, since kveik cultures often consist of multiple strains, the impact of individual isolates on fermentation performance and flavour production were assessed.

The data obtained indicated that kveik strains can tolerate equivalent or higher levels of the various stressors typically encountered during fermentation than contemporary strains. These results support the observations of (Preiss et al., 2018; Foster et al., 2022) who also showed a similar pattern of results. However, it was interesting to note that individual isolates of kveik yeast from the same culture all demonstrated similar levels of resistance, indicating that this was not an artefact of the presence of a 'dominant' strain, but instead perhaps simply a reflection of their close heritage. Although stress tolerance was a characteristic trait, there did not appear to be a trade off with performance; each kveik strain reached levels of attenuation and rates of fermentation comparable to contemporary brewers yeast strains. Similarly, they were shown to produce distinct flavour and aroma compounds when compared to the other brewing

yeasts, albeit within acceptable limits. Interestingly, some of the kveik isolates analysed produced flavour profiles that were more similar to lager yeast than those resulting from ale strains, suggesting potential for a range of product types. Within each kveik culture, there was some strain diversity, and altering the proportion of constituent strains was shown to affect the flavour profile of the beer being made.

There were several aspects of analysis, however, that may have impacted some results obtained. Wort oxygen is known to affect yeast growth and fermentation progression; although all worts were processed in the same fashion (via aeration prior to inoculation), it was difficult to determine whether oxygen levels were below those used in 'standard' brewing practices. There were also several instances during stress testing where inconsistencies may have been present. For example, when preparing media for oxidative stress tests, there was potential for degradation of H_2O_2 which may have introduced inconsistencies. Similarly, when creating YPD agar media for the ethanol stress test, pure ethanol was added to the media while still warm (prior to setting), a proportion of which may have been lost to ethanol volatisation. Finally, during fermentation testing, and due to the speed of fermentations, it was not always possible to determine the precise time point at which attenuation was reached. Further investigation into key strains with optimised fermentation parameters using an automated system for gravity analysis would provide insightful data.

While the data produced here provides evidence of the potential for the use of kveik yeasts strains in brewing, future studies could also be conducted to investigate this further. It should be noted that this study only utilised twelve isolates from six original sources, but there are many other kveik strains available. Expanding this study to include more kveik isolates and culture blends, as well as additional traditional farmhouse yeasts from other areas of Scandinavia and the Baltics may prove to deliver further discoveries. Additionally, although the capability of yeasts to tolerate stress factors associated with brewery operations

were investigated, these stressors were applied individually. Future experiments could involve the application of multiple stress factors simultaneously, to more readily emulate existing brewing operations and to provide additional insight into overall stress resistance. Additionally, utilising sugar sources other than malt-based substrates (such as wine or cider must, honey, or other simple sugar mixtures), could provide useful information for the fermentation industry outside of brewing, or for identifying suitability for the production of alcoholic bases used in the manufacture of flavoured alcoholic beverages (FAB's), Ready-To-Drinks (RTD's), hard seltzers, or radlers.

This study has shown that kveik strains have the potential to be appealing to brewers that produce a range of products. While suitable for those interested in producing novel beverages in a short period of time, they may also find use in stressful high gravity brewing procedures, and in fermentations that are conducted 'warm', potentially increasing production throughput. This versatility of traditional Norwegian landrace yeasts is likely a consequence of their history and usage, reflecting the tangible link between a brewer and his or her ancestors. Since kveik strains have historically been passed from one generation to the next, they remain celebrated by their communities and are an enduring part of local culture. Formerly isolated, their entry into the modern brewing scene could open new doors for brewers looking to experience a piece of culture that was once unknown outside the small towns and hidden farms in Norway.

Chapter 5

5. References

Aguilera, J., Randez-Gil, F., & Prieto, J. A. (2007). Cold response in Saccharomyces cerevisiae: new functions for old mechanisms. *Federation of European Microbiological Societies*, *31*(3), 327–341. https://doi.org/https://doi.org/10.1111/j.1574-6976.2007.00066.x

Ashraf, N., Linforth, R., Bealin-Kelly, F., Smart, K., Taylor, A. (2010). Rapid analysis of selected beer volatiles by atmospheric pressure chemical ionisation-mass spectrometry. *International Journal of Mass Spectrometry*, 294(1), 47-53 https://doi.org/10.1016/j.ijms.2010.05.007.

Auesukaree, C. (2017). Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. *Journal of Bioscience and Bioengineering*, *124*(2), 133–142. https://doi.org/10.1016/J.JBIOSC.2017.03.009

Bing, J., Han, P.-J., Liu, W.-Q., Wang, Q.-M., & Bai, F.-Y. (2014). Evidence For a Far East Asian Origin of Lager Beer Yeast. *Current Biology*, *24*(10), R380–R381. https://doi.org/https://doi.org/10.1016/j.cub.2014.04.031

Bleoanca, I., & Bahrim, G. (2013). Overview on brewing yeast stress factors. *Romanian Biotechnological Letters*, *18*(5), 8559–8572. https://doi.org/http://www.rombio.eu/vol18nr5/1%20Bleoanca.pdf

Bolat, I. (2008). The importance of trehalose in brewing yeast survival. *Innovative Romanian Food Biotechnology*, *2*, 1–10. https://doi.org/http://www.bioaliment.ugal.ro/ejournal.htm

Boulton, C., & Quain, D. (2008). *Brewing yeast and fermentation*. John Wiley & Sons.

Briggs, D. E., Brookes, P. A., Stevens, R., & Boulton, C. A. (2004). *Brewing: science and practice* (Vol. 108). Woodhead Publishing.

Chi, Z., & Arneborg, N. (1999). Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in Saccharomyces cerevisiae. *Journal of Applied Microbiology*, *86*(6), 1047–1052. https://doi.org/https://doi.org/10.1046/j.1365-2672.1999.00793.x

Costa, V., Amorim, M. A., Reis, E., Quintanilha, A., & Moradas-Ferreira, P. (1997). Mitochondrial superoxide dismutase is essential for ethanol tolerance of Saccharomyces cerevisiae in the post-diauxic phase. *Microbiology*, *143*(5), 1649–1656. https://doi.org/https://doi.org/10.1099/00221287-143-5-1649

Csonka, L. N., & Hanson, A. D. (1991). Prokaryotic Osmoregulation: Genetics and Physiology. *Annual Review of Microbiology*, *45*(1), 569–606. https://doi.org/https://doi.org/10.1146/annurev.mi.45.100191.003033

D'Amore, T., Panchal, C. J., Russell, I., & Stewart, G. G. (1988). Osmotic pressure effects and intracellular accumulation of ethanol in yeast during fermentation. In *Journal of Industrial Microbiology* (Vol. 2). https://academic.oup.com/jimb/article/2/6/365/5987269

Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D., & Zhang, K. (2009). Tolerance and stress response to ethanol in the yeast Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology*, *85*(2), 253–263. https://doi.org/10.1007/s00253-009-2223-1

Dombek, K. M., & Ingram, L. O. (1986). Magnesium Limitation and Its Role in Apparent Toxicity of Ethanol During Yeast Fermentation. *Applied and Environmental Microbiology*, *52*(5), 975–981. https://doi.org/https://doi.org/10.1128/aem.52.5.975-981.1986

Donalies, U. E. B., Nguyen, H. T. T., Stahl, U., & Nevoigt, E. (2008). Improvement of *Saccharomyces* Yeast Strains Used in Brewing, Wine Making and Baking. In U. Stahl, U. E. B. Donalies, & E. Nevoigt (Eds.), *Food Biotechnology* (pp. 67–98). Springer Berlin Heidelberg. https://doi.org/10.1007/10_2008_099

Dunn, B., & Sherlock, G. (2008). Reconstruction of the genome origins and evolution of the hybrid lager yeast Saccharomyces pastorianus. *Genome Research*, *18*(10), 1610–1623. https://doi.org/https://genome.cshlp.org/content/18/10/1610

Edwardson, J. R. (1952). Hops—Their botany, history, production and utilization. *Economic Botany*, *6*(2), 160–175. https://doi.org/https://doi.org/10.1007/BF02984875

Estruch, F. (2000). Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *Federation of European Microbiological Societies*, *24*(4), 469–486. https://doi.org/https://doi.org/10.1111/j.1574-6976.2000.tb00551.x

Farrugia, G., & Balzan, R. (2012). Oxidative Stress and Programmed Cell Death in Yeast. *Frontiers in Oncology*, *2*. https://doi.org/https://doi.org/10.3389/fonc.2012.00064

Ferreira, I. M., & Guido, L. F. (2018). Impact of Wort Amino Acids on Beer Flavour: A Review. *Fermentation*, *4*(2), 23. https://doi.org/https://doi.org/10.3390/fermentation4020023

Foster, B., Tyrawa, C., Ozsahin, E., Lubberts, M., Krogerus, K., Preiss, R., & van der Merwe, G. (2022). Kveik Brewing Yeasts Demonstrate Wide Flexibility in Beer Fermentation Temperature Tolerance and Exhibit Enhanced Trehalose Accumulation. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.747546

Gallone, B., Mertens, S., Gordon, J. L., Maere, S., Verstrepen, K. J., & Steensels, J. (2018). Origins, evolution, domestication and diversity of Saccharomyces beer yeasts. In *Current Opinion in Biotechnology* (Vol. 49, pp. 148–155). Elsevier Ltd. https://doi.org/10.1016/j.copbio.2017.08.005

Gallone, B., Steensels, J., Prahl, T., Soriaga, L., Saels, V., Herrera-Malaver, B., Merlevede, A., Roncoroni, M., Voordeckers, K., & Miraglia, L. (2016). Domestication and Divergence of Saccharomyces cerevisiae Beer Yeasts. *Cell*, *166*(6), 1397-1410. e16. https://doi.org/https://doi.org/10.1016/j.cell.2016.08.020

Garshol, L. M. (2020). *Historical Brewing Techniques: The Lost Art of Farmhouse Brewing*. Brewers Publications.

Garshol, L. M. (2021). Pitch Temperatures in Traditional Farmhouse Brewing. *Journal of the American Society of Brewing Chemists*, *79*(2), 181–186. https://doi.org/https://doi.org/10.1080/03610470.2020.1805699

Garshol, L. M., & Preiss, R. (2018). How to Brew with Kveik. *MBAA Technical Quarterly*, *55*(4), 76–83. https://doi.org/https://doi.org/10.1094/TQ-55-4-1211-01

Geithung, I. (2019). *In Norway, kveik means more than just yeast*. https://doi.org/https://www.youtube.com/watch?v=MCC3tBS2j_Q

Gibson, B., Dahabieh, M., Krogerus, K., Jouhten, P., Magalhães, F., Pereira, R., Siewers, V., & Vidgren, V. (2020). Adaptive Laboratory Evolution of Ale and Lager Yeasts for Improved Brewing Efficiency and Beer Quality. *Annual Review of Food Science and Technology*, *11*, 23–44. https://doi.org/https://doi.org/10.1146/annurev-food-032519-051715

Gibson, B. R., Lawrence, S. J., Leclaire, J. P. R., Powell, C. D., & Smart, K. A. (2007). Yeast responses to stresses associated with industrial brewery handling. *Federation of European Microbiological Societies*, *31*(5), 535–569. https://doi.org/https://doi.org/10.1111/j.1574-6976.2007.00076.x

Gibson, B. R., Storgårds, E., Krogerus, K., & Vidgren, V. (2013). Comparative physiology and fermentation performance of Saaz and Frohberg lager yeast strains and the parental species Saccharomyces eubayanus. *Yeast*, *30*(7), 255–266. https://doi.org/https://doi.org/10.1002/yea.2960

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., & Johnston, M. (1996). Life with 6000 genes. *Science*, *274*(5287), 546–567. https://doi.org/https://doi.org/10.1126/science.274.5287.546

Gorter De Vries, A. R., Pronk, J. T., & Daran, J. M. G. (2019). Lager-brewing yeasts in the era of modern genetics. *FEMS Yeast Research*, *19*(7), 63. https://doi.org/10.1093/FEMSYR/FOZ063

Hall, J. F. (1971). DETECTION OF WILD YEASTS IN THE BREWERY. *Journal of the Institute of Brewing*, *77*(6), 513–516. https://doi.org/https://doi.org/10.1002/j.2050-0416.1971.tb03413.x

Hardwick, W. A. (1995). *Handbook of Brewing*. Marcel Dekker, Inc.

Hefebank Weihenstephan. (n.d.). *https://www.hefebank-weihenstephan.de/en/products/yeast/bierhefen-untergaerig-bruchhefe/w-34-70/*.

Heggart, H. M., Margaritis, A., Pilkington, H., Stewart, R. J., Dowhanick, T. M., & Russell, I. (1999). Factors Affecting Yeast Viability and Vitality Characteristics: A Review. *MBAA Technical Quarterly*, *36*(4), 383–406. https://doi.org/https://www.mbaa.com/publications/tq/tqPastIssues/1999/Documents/36_383.pdf

Hoad, T. F. (Ed.). (1996). *The Concise Oxford Dictionary of English Etymology*. Oxford University Press. https://doi.org/10.1093/acref/9780192830982.001.0001

Hornsey, I. S. (2003). *A History of Beer and Brewing*. Royal Society of Chemistry. https://doi.org/https://doi.org/10.1039/9781847550026

Hough, J. S., Briggs, D. E., Stevens, R., & Young, T. W. (2012). *Malting and Brewing Science: Hopped Wort and Beer* (2nd ed., Vol. 2). Springer.

Hough, J. S., & Stevens, R. (1961). Beer Flavour: Factors Affecting the Production of Fusel Oil. *Journal of the Institute of Brewing*, *67*(6), 488–494. https://doi.org/https://doi.org/10.1002/j.2050-0416.1961.tb01828.x

Hounsa, C.-G., Brandt, E. V., Thevelein, J., Hohmann, S., & Prior, B. A. (1998). Role of trehalose in survival of Saccharomyces cerevisiae under osmotic stress. *Microbiology*, *144*(3), 671–680. https://doi.org/https://doi.org/10.1099/00221287-144-3-671

Hrnčič, M., Španinger, E., Košir, I. J., Knez, Ž., & Bren, U. (2019). Hop Compounds: Extraction Techniques, Chemical Analyses, Antioxidative, Antimicrobial, and Anticarcinogenic Effects. *Nutrients*, *11*(2), 257. https://doi.org/https://doi.org/10.3390/nu11020257

Inoue, T. (2008). *Diacetyl in Fermented Foods and Beverages*. American Society of Brewing Chemists.

Jamieson, D. J. (1998). Oxidative Stress Responses of the Yeast Saccharomyces cerevisiae. *Yeast*, *14*(16), 1511–1527. https://doi.org/https://doi.org/10.1002/(SICI)1097-0061(199812)14:16%3C1511::AID-YEA356%3E3.0.CO;2-S

Janish, S. (2016). *Esters and Fusel Alcohols: A Look at Studies and Experimental Brew*. https://doi.org/http://scottjanish.com/esters-and-fusel-alcohols/

Jenkins, C. L., Lawrence, S. J., Kennedy, A. I., Thurston, P., Hodgson, J. A., & Smart, K. A. (2009). Incidence and Formation of Petite Mutants in Lager Brewing Yeast *Saccharomyces Cerevisiae* (Syn. *S. Pastorianus*) Populations. *Journal of the American Society of Brewing Chemists*, *67*(2), 72–80. https://doi.org/10.1094/ASBCJ-2009-0212-01

Katz, S. H., Maytag, F., & Civil, M. (1991). Brewing an Ancient Beer. *Archaeology*, *44*(4), 24–33. https://doi.org/https://www.jstor.org/stable/pdf/41765984.pdf

Krogerus, K., & Gibson, B. R. (2013). 125th Anniversary Review: Diacetyl and its control during brewery fermentation. *Journal of the Institute of Brewing*, *119*(3), 86–97. https://doi.org/https://doi.org/10.1002/jib.84

Kunze, W. (2019). *Technology Brewing & Malting* (6th ed.). VLB Berlin.

Kwon, M., Chong, S., Han, S., & Kim, K. (2003). Oxidative stresses elevate the expression of cytochrome c peroxidase in Saccharomyces cerevisiae. *Biochimica et Biophysica Acta - General Subjects*, *1623*(1), 1–5. https://doi.org/10.1016/S0304-4165(03)00151-X

Laitinen, M. (2020). *Brewing Nordic*. https://doi.org/https://www.brewingnordic.com/farmhouse-ales/practical-guide-to-kveik-and-other-farmhouse-yeast-reusing-and-maintaining-yeast/

Lentini, A., Rogers, P., Higgins, V., Dawes, I., Chandler, M., Stanley, G., & Chambers, P. (2003). The Impact of Ethanol Stress on Yeast Physiology. In *Brewing Yeast Fermentation Performance* (2nd ed., pp. 23–38). Blackwell Publishing. https://doi.org/https://doi.org/10.1002/9780470696040.ch3

Lentz, M. (2018). The Impact of Simple Phenolic Compounds on Beer Aroma and Flavor. *Fermentation*, *4*(1), 20. https://doi.org/https://doi.org/10.3390/fermentation4010020

Lewis, M. J., & Bamforth, C. W. (2006). *Essays in Brewing Science*. Springer Science & Business Media.

Lewis, M. J., & Young, T. W. (2012). *Brewing* (2nd ed.). Springer Science & Business Media.

Libkind, D., Hittinger, C. T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P., & Sampaio, J. P. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences*, *108*(35), 14539–14544. https://doi.org/https://doi.org/10.1073/pnas.1105430108

Lin, Z., & Li, W.-H. (2014). Comparative Genomics and Evolutionary Genetics of Yeast Carbon Metabolism. In *Molecular Mechanisms in Yeast Carbon Metabolism* (pp. 97–120). Springer. https://doi.org/https://doi.org/10.1007/978-3-642-55013-3_5

Liu, L., Wang, J., Rosenberg, D., Zhao, H., Lengyel, G., & Nadel, D. (2018). Fermented beverage and food storage in 13,000 y-old stone mortars at Raqefet Cave, Israel: Investigating Natufian ritual feasting. *Journal of Archaeological Science: Reports*, *21*, 783–793. https://doi.org/https://doi.org/10.1016/j.jasrep.2018.08.008

Majara, M., O'Connor-Cox, E. S. C., & Axcell, B. C. (1996). Trehalose—An Osmoprotectant and Stress Indicator Compound in High and Very High Gravity Brewing. *Journal of the American Society of Brewing Chemists*, *54*(3), 149–154. https://doi.org/10.1094/ASBCJ-54-0149

Mallett, J. (2014). Malt: a practical guide from field to brewhouse. In *Brewing Elements Series* (Vol. 4). Brewers Publications.

Martin, V., Quain, D. E., & Smart, K. A. (2003). Brewing Yeast Oxidative Stress Responses: Impact of Brewery Handling. In B. Publishing (Ed.), *Brewing Yeast Fermentation Performance* (2nd ed., pp. 61–73). Blackwell Publishing. https://doi.org/https://doi.org/10.1002/9780470696040.ch6

Meledina, T. v, Ivanova, V. A., Golovinskaia, O. v, & Harba, R. (2021). YEAST. MORPHOLOGY AND PHYSIOLOGY. *ITMO University*. https://doi.org/https://books.ifmo.ru/file/pdf/2760.pdf

Meussdoerffer, F. G. (2009). A Comprehensive History of Beer Brewing. In Dr. H. M. Eßlinger (Ed.), *Handbook of Brewing: Processes, Technology, Markets* (pp. 1–42). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/https://doi.org/10.1002/9783527623488.ch1

Monerawela, C., & Bond, U. (2017). Brewing up a storm: The genomes of lager yeasts and how they evolved. *Biotechnology Advances*, *35*(4), 512–519. https://doi.org/https://doi.org/10.1016/j.biotechadv.2017.03.003

Morris, E. O., & Hough, J. S. (1956). SOME ASPECTS OF THE GIANT COLONY CHARACTERS OF *SACCHAROMYCES CEREVISIAE*. *Journal of the Institute of Brewing*, *62*(6), 466–468. https://doi.org/10.1002/j.2050-0416.1956.tb02890.x

Mühlhofer, M., Berchtold, E., Stratil, C. G., Csaba, G., Kunold, E., Bach, N. C., Sieber, S. A., Haslbeck, M., Zimmer, R., & Buchner, J. (2019). The Heat Shock Response in Yeast Maintains Protein Homeostasis by Chaperoning and Replenishing Proteins. *Cell Reports*, *29*(13), 4593-4607. e8. https://doi.org/https://doi.org/10.1016/j.celrep.2019.11.109

Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., & Iefuji, H. (2010). PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in Saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering*, *109*(6), 564–569. https://doi.org/https://doi.org/10.1016/j.jbiosc.2009.11.011

Murtey, M. das. (2016). Sample Preparations for Scanning Electron Microscopy – Life Sciences. In P. R. E.-M. J. E.-R. Kral (Ed.), *Modern Electron Microscopy in Physical and Life Sciences* (p. Ch. 8). IntechOpen. https://doi.org/10.5772/61720

O'Rourke, S. M., Herskowitz, I., & O'Shea, E. K. (2002). Yeast go the whole HOG for the hyperosmotic response. *Trends in Genetics*, *18*(8), 405–412. https://doi.org/10.1016/S0168-9525(02)02723-3

Palmer, J. J., & Kaminski, C. (2013). Water: A Comprehensive Guide for Brewers. In *Brewing Elements Series*. Brewers Publications.

Pires, E., & Brányik, T. (2015). *Biochemistry of Beer Fermentation*. Springer. https://doi.org/https://doi.org/10.1007/978-3-319-15189-2

Pires, E., Teixeira, J., Brányik, T., & Vicente, A. A. (2014). Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology*, *98*(5), 1937–1949. https://doi.org/https://doi.org/10.1007/s00253-013-5470-0

Piškur, J., Rozpędowska, E., Polakova, S., Merico, A., & Compagno, C. (2006). How did Saccharomyces evolve to become a good brewer? *TRENDS in Genetics*, *22*(4), 183–186. https://doi.org/https://doi.org/10.1016/j.tig.2006.02.002

Preiss, R., Tyrawa, C., Krogerus, K., Garshol, L. M., & van der Merwe, G. (2018). Traditional Norwegian Kveik are a Genetically Distinct Group of Domesticated Saccharomyces cerevisiae Brewing Yeasts. *Frontiers in Microbiology*, *9*, 2137. https://doi.org/https://doi.org/10.3389/fmicb.2018.02137

Richards, M. (1967). THE USE OF GIANT-COLONY MORPHOLOGY FOR THE DIFFERENTIATION OF BREWING YEASTS. *Journal of the Institute of Brewing*, *73*(2), 162–166. https://doi.org/https://doi.org/10.1002/j.2050-0416.1967.tb03028.x

Rodrigues-Pousada, C., Nevitt, T., & Menezes, R. (2005). The Yeast Stress Response: Role of the Yap family of b-ZIP transcription factors The PABMB Lecture delivered on 30 June 2004 at the 29th FEBS Congress in Warsaw. *Federation of European Microbiological Societies*, *272*(11), 2639–2647. https://doi.org/https://doi.org/10.1111/j.1742-4658.2005.04695.x

Somani, A., Box, W. G., Smart, K. A., & Powell, C. D. (2019). Physiological and transcriptomic response of Saccharomyces pastorianus to cold storage. *FEMS Yeast Research*, *19*(4). https://doi.org/10.1093/femsyr/foz025

Sparrow, J. (2005). *Wild Brews: Beer Beyond the Influence of Brewer's Yeast*. Brewers Publications.

Speers, A. (2016). Brewing Fundamentals, Part 3: Yeast Settling–Flocculation. *MBAA Technical Quarterly*, *53*(1), 17–22. https://doi.org/http://dx.doi.org/10.1094/TQ-53-1-0302-04

Stewart, G. (2016). Adjuncts. In C. W. Bamforth (Ed.), *Brewing Materials and Processes* (pp. 27–46). Elsevier.

Stewart, G. (2020). Stresses Imposed on Yeast During Brewing Wort Fermentations and Their Effects on Cellular Activity. *2019 Master Brewers Conference*, *57*(1), 1–8. https://doi.org/https://doi.org/10.1094/TQ-57-1-0131-01

Stewart, G. G. (2010). High-Gravity Brewing and Distilling—Past Experiences and Future Prospects. *Journal of the American Society of Brewing Chemists*, *68*(1), 1–9. https://doi.org/https://doi.org/10.1094/ASBCJ-2009-1214-01

Stewart, G. G. (2017). Introduction. In G. G. Stewart (Ed.), *Brewing and Distilling Yeasts* (pp. 1–9). Springer International Publishing. https://doi.org/10.1007/978-3-319-69126-8_1

Stewart, G., & Russell, I. (2009). *The IBD Blue Book on Yeast: Vol. III*. The Institute of Brewing and Distilling.

Štulíková, K., Vrzal, T., Kubizniaková, P., Enge, J., Matoulková, D., & Brányik, T. (2021). Spoilage of bottled lager beer contaminated with Saccharomyces cerevisiae var. diastaticus. *Journal of the Institute of Brewing*, *127*(3), 256–261. https://doi.org/https://doi.org/10.1002/jib.653

Suiker, I. M., & Wösten, H. A. B. (2022). Spoilage yeasts in beer and beer products. *Current Opinion in Food Science*, *44*, 100815. https://doi.org/https://doi.org/10.1016/j.cofs.2022.100815

Swan, T., Baldwin, B., Clarke, F., Tonissen, K., Anderson, M., & Rogers, P. (2003). The development and application of natural yeast reductants to the stabilisation of beer. *Proceedings of the Congress: European Brewing Convention*, *29*, 707–722.

Tyakht, A., Kopeliovich, A., Klimenko, N., Efimova, D., Dovidchenko, N., Odintsova, V., Kleimenov, M., Toshchakov, S., Popova, A., & Khomyakova, M. (2021). Characteristics of bacterial and yeast microbiomes in spontaneous and mixed-fermentation beer and cider. *Food Microbiology*, *94*(103658). https://doi.org/https://doi.org/10.1016/j.fm.2020.103658

Unger, R. W. (2013). *Beer in the Middle Ages and the Renaissance*. University of Pennsylvania Press.

Vanderhaegen, B., Neven, H., Verachtert, H., & Derdelinckx, G. (2006). The chemistry of beer aging–a critical review. *Food Chemistry*, *95*(3), 357–381. https://doi.org/https://doi.org/10.1016/j.foodchem.2005.01.006

Verstrepen, K. J., Derdelinckx, G., Dufour, J.-P., Winderickx, J., Thevelein, J. M., Pretorius, I. S., & Delvaux, F. R. (2003). Flavor-Active Esters: Adding Fruitiness to Beer. *Journal of Bioscience and Bioengineering*, *96*(2), 110–118. https://doi.org/https://doi.org/10.1016/S1389-1723(03)90112-5

Vidgren, V., & Londesborough, J. (2011). 125th Anniversary Review: Yeast Flocculation and Sedimentation in Brewing. *Journal of the Institute of Brewing*, *117*(4), 475–487. https://doi.org/https://doi.org/10.1002/j.2050-0416.2011.tb00495.x

Walker, G. (1998). *Yeast Physiology and Biotechnology*. John Wiley & Sons.

Walker, G. M., & Stewart, G. G. (2016). Saccharomyces cerevisiae in the Production of Fermented Beverages. *Beverages*, *2*(4), 30. https://doi.org/https://doi.org/10.3390/beverages2040030

White, C., & Zainasheff, J. (2010). Yeast: The Practical Guide to Beer Fermentation. In *Brewing Elements Series*. Brewers Publications.

White, P. A., Kennedy, A. I., & Smart, K. A. (2003). The Osmotic Stress Response of Ale and Lager Brewing Yeast Strains. In K. A. Smart (Ed.), *Brewing Yeast Fermentation Performance* (2nd ed., pp. 46–60). Blackwell Publishing.

Willaert, R. (2007). The Beer Brewing Process: Wort Production and Beer Fermentation. In Y. H. Hui (Ed.), *Handbook of food products manufacturing*. John Wiley & Sons.

Xu, X., Lambrecht, A. D., & Xiao, W. (2014). Yeast Survival and Growth Assays. In W. Xiao (Ed.), *Yeast Protocols* (3rd ed., Vol. 1163, pp. 183–191). Springer. https://doi.org/10.1007/978-1-4939-0799-1_13

Zhuang, S., Smart, K., & Powell, C. (2017). Impact of Extracellular Osmolality on Saccharomyces Yeast Populations During Brewing Fermentations. *Journal of the American Society of Brewing Chemists*, *75*(3), 244–254. https://doi.org/https://doi.org/10.1094/ASBCJ-2017-3505-01